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Involvement of M2-polarized macrophages in the ascites from advanced epithelial ovarian carcinoma in tumor progression via Stat3 activation

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**Summary**

Peritoneal macrophages are actively involved in the regulation of inflammation, immune response, and tumor growth in peritoneal cavity. Macrophages in the ascites from advanced epithelial ovarian cancer (EOC) are polarized to M2 immunosuppressive phenotype and involved in tumor progression by modulating the tumor microenvironment. However, the cell-to-cell interaction between peritoneal macrophages and ovarian cancer cells is still unclear. This study focused on the activation of signal transducer and activator of transcription 3 (Stat3) which is a critical signal transduction molecule at a point of convergence for numerous oncogenic signaling pathways as well as controlling the M2-polarization of macrophages. Immunohistochemistry using CD163 as a marker for M2 macrophages demonstrated that most macrophages in the ascites of EOC were polarized to the M2 phenotype. EOC ascites stimulated the proliferation of SKOV3 cells, a human ovarian cancer cell line, and immortalized human ovarian surface epithelial cells. It also induced the activation of Stat3 in THP-1 macrophages. In addition, the co-culture of M-CSF-primed M2 macrophages, but not GM-CSF-primed immature macrophages induced a strong Stat3 activation in SKOV3 cells. The expression of cyclin-D1 in SKOV3 cells was up-regulated by a co-culture with macrophages. The cyclin-D1 up-regulation in SKOV3 cells was significantly inhibited after blocking Stat3 by small interfering (si)RNA in the macrophages, thus indicating that Stat3-mediated M2 polarization of macrophages in the ascites is important for tumor cell survival. These results indicate that the cell-to-cell interactions between macrophages and EOC cells through Stat3 activation are positively involved in EOC progression.
Introduction

Epithelial ovarian carcinoma (EOC) is the forth or fifth most common cause of cancer death in females worldwide.\(^{(1-3)}\) Although current approaches, including surgery, combination chemotherapy, and hormonal therapy yield responses in 60-80% of patients with advanced disease, the majority of ovarian cancer patients eventually relapse and become refractory to additional treatment.\(^{(1-3)}\) Under the clinical situation of EOC, severe ascites is a hallmark of advanced EOC.

Peritoneal macrophages play an important role in the suppression of inflammation and the regulation of immune response. They are present in peritoneal cavity of healthy women, and the number of macrophages increases in the ascites of patients with pelvic endometriosis and other gynecological diseases.\(^{(4)}\) In advanced EOC ascites, there is also an increased number of macrophages and the macrophages are involved in cancer metastasis and progression by modifying the tumor microenvironment.\(^{(5-8)}\) Macrophage depletion in peritoneal ovarian cancer models suppresses cancer progression and accumulation of ascites.\(^{(6,9)}\)

Macrophages infiltrating cancer tissues in various malignant tumors, including EOC, are referred to as tumor-associated macrophages (TAMs), which modulate the tumor microenvironment by suppressing anti-tumor immune reactions and inducing angiogenesis.\(^{(10-12)}\) TAMs are generally considered to belong to the alternatively activated macrophage population (M2) because of their anti-inflammatory functions.\(^{(10-13)}\) Macrophages in the ascites of advanced EOC patients are polarized to M2 macrophages stimulated by cancer-derived factors such as interleukin 6 (IL-6), leukemia inhibitory factor (LIF), and macrophage colony stimulating factor (M-CSF).\(^{(14,15)}\) Recent studies have focused on the phenotypic polarization of TAMs and its involvement in tumor progression. In our observations, M2-polarized TAMs support tumor proliferation of human glioma and the density of M2-polarized macrophages is positively associated with a poor prognosis in patients with glioma.\(^{(16)}\) In addition, the number of M2-polarized TAMs and local production of M-CSF are positively correlated with the histological grade of malignancy in EOC.\(^{(17)}\)

On the basis of these findings, cancer-derived factors could play an indispensable role in macrophage differentiation and cell-to-cell interactions between M2 macrophages and cancer cells could be important in cancer progression. However, few studies have described the interaction of cancer cells and macrophages in human EOC. The present
study investigated the role of M2 macrophages in the proliferation and progression of ovarian cancer cells by focusing on the signal transducer and activator of transcription-3 (Stat3), since Stat3 is a critical signal transduction molecule at a point of convergence for numerous oncogenic signaling pathways as well as one of the major regulators of macrophage activation associated with M2 polarization.(18)

Materials and methods

Samples

Samples of ascites were collected from patients with ovarian carcinoma (International Federation of Gynecology and Obstetrics (FIGO) Stage I: 5 patients, Stage, III, IV; 15 patients) and myoma as a control (4 patients) undergoing surgery at Kumamoto University Hospital. Informed consent was obtained from all patients. Each sample was centrifuged prior to storage –80°C. Peripheral blood mononuclear cells (PBMC) were obtained from 3 healthy adult female volunteer donors, in accordance with the protocols approved by Kumamoto University Hospital Review Board.

Cell lines and cell culture

A human ovarian cancer cell line, SKOV3, was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in McCoy’s 5a Medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml sodium pyruvate. The cell culture supernatant was collected at 80% cell confluence. A human monocytic cell line, THP-1, was also purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml sodium pyruvate. The establishment and characterization of an immortalized human ovarian surface epithelial cell line (hOSE), H-OSE-E7/hTERT, has been described previously.(19) It was maintained in DMEM with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin. These cells were incubated at 37°C under a 5% CO₂ and harvested from subconfluent cultures.

Co-culture assay

CD14+ monocytes were purified from PBMCs by positive selection using magnetic-activated cell sorting technology (Miltenyi Biotec., Bergisch Gladbach,
Monocytes were cultured with GM-CSF (10 ng/ml, WAKO, Tokyo, Japan) or M-CSF (10 ng/ml, WAKO, Tokyo, Japan) for up to seven days to induce differentiation and maturation into macrophages. M-CSF differentiates human macrophages toward an M2 phenotype, whereas GM-CSF induces M1 differentiation in murine macrophages, but not in human macrophages. Following washing in PBS, monocyte-derived macrophages were co-cultured with SKOV3 cells for 5 days (1:1 ratio, each 1x10^6 per 10cm culture dish). To prepare the paraffin-embedded cell block specimens, cultured cells were detached by a cell-scraper (TPP, Trasadingen, Switzerland), and fixed in 10% neutral buffered formalin. Then cells were suspended in 1% sodium arginate and solidified by addition of 1M calcium chloride. Finally, gelatinous specimens containing SKOV3 cells and macrophages were embedded in paraffin in the routine manner.

For a separate culture, SKOV3 cells were plated onto BD Falcon™ cell culture inserts with 0.4 μm pores in a permeable membrane at a density of 5×10^5 cells per insert. Macrophages were seeded into wells of the Falcon companion plate (BD Biosciences, San Jose, CA) at a density of 1×10^6 cells per well. After 24 h, inserts containing SKOV3 cells were placed into wells containing macrophages and incubated for 5 days. After incubation, inserts containing SKOV3 were removed. Macrophages and SKOV3 cells were harvested and subjected to a Western blot assay.

**Cell growth assay**

SKOV3 and hOSE cells were seeded at a density of 2-5×10^3 cells per 96 well plates and maintained in DMEM medium with 10% FBS for 24 h before stimulation. Thereafter, the cells were cultured for 5 days in the same medium containing 20% ascites, or one of the following cytokines; IL-6, IL-10, or GRO-α (Pepro Thech, Rocky Hill, NJ) at corresponding concentrations observed in ascites of patients. Cell proliferation was assessed by the WST-1 cell proliferation assay (Dojindo Lab., Kumamoto, Japan). In cytokine blocking assay, neutralizing antibodies against IL-6 and IL-10 (Pepro Thech, Rocky Hill, NJ) were added with ascites at a final concentration 1 μg/ml.

**Immunostaining of ascites cells**

100 μl of ascites samples were attached to frosted micro slide glasses (Matsunami Glass Inc., Osaka, Japan) by centrifugation for 5 min. at 800 rpm at high acceleration in a
Cytospin 2 centrifuge (Shandon; Frankfurt, Germany) and dried for 10 min. Cytospin preparations were fixed in 4% paraformaldehyde for 10 min., washed twice in PBS, air dried and stored at −20°C. Samples were incubated with mouse anti-CD68 antibody (PG-M1; DAKO, Glostrup, Denmark) or mouse anti-CD163 antibody (AM-3K, Transgenic, Kumamoto, Japan). Horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Nichirei, Tokyo, Japan) was used as a secondary antibody.

**Immunohistochemistry**

Table 1 lists the primary antibodies and pretreatments used in this study. Deparaffinized sections were pretreated for antigen retrieval and then were sequentially incubated with 5% goat serum and reacted with one of the primary antibodies. After careful rinses, samples were incubated with HRP-labeled goat anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin (Nichirei, Tokyo, Japan). The immunoreaction was visualized using a diaminobenzidine substrate kit (Nichirei). All sections were counterstained with Mayer’s hematoxylin. Mouse or rabbit IgG was then used as a negative control. Evaluations of immunostaining were conducted by two pathologists (Y.K and K.T) who were blinded to information on the samples. Positive cells were counted in 20 high power fields (0.028 mm² per field) randomly and the data of Y.K and K.T were averaged.

**Double-immunostaining**

After the reaction with two antigen-specific antibodies of different animal origin, samples were incubated with HRP-labeled goat anti-mouse or alkaline phosphatase (ALP)-labeled goat anti-rabbit antibody (Nichirei). The streptavidin-biotin method was used for the immunostaining of pStat3. The reaction was visualized by the use of the diaminobenzidine substrate system (Vector, Burlingame, CA) and Fast Red/Fast Blue solutions as described previously.\(^{16}\)

**Cytokine array and cytokine quantification**

The cytokine array kits were purchased from RayBiotech (Norcross, CA) and then the assays were performed according to the manufacturer’s protocol. Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-8, IL-15, IL-10, IL-12, M-CSF, and MCP-1 were purchased from Biosource (Camarillo, CA), ELISA kit for LIF and GRO-\(\alpha\)
were purchased from R&D systems (Minneapolis, MN), TNF-α ELISA kit was purchased from BD Biosciences (San Jose, CA), and VEGF ELISA kit was purchased from Immuno-Biological Laboratories Co. (Takasaki, Japan).

Quantitative real-time polymerase chain reaction (Q-PCR)
Total RNA was extracted by using an RNA STAT-60 extraction kit (Tel-Test, Inc., Friendswood, TX). RNA was reverse-transcribed by means of the ExScript RT reagent kit (Takara, Shiga, Japan). Q-PCR was performed by using TaqMan polymerase, with the detection of Syber Green fluorescence (Takara) by an ABI PRISM 7300 Sequence Detector (Applied Biosystems, Foster City, CA). Primers were as follows:
CD163: 5'-CGAGTTAACGCCAGTAAGG-3’ (forward) and 5'-GAACATGTCACGCCAGC-3’ (reverse).
IL-6: 5'-CACACAGAGACAGCCACTCACC-3’ (forward) and 5'-GTGCCCTTTGCTGCTTTCAC-3’ (reverse).
IL-10: 5'-GGTTGCCAAGCCTTGTCTGA-3’ (forward) and 5'-AGGGAGTTCACATGCGCCT-3’ (reverse).
GAPDH: 5'-GCACCGTCAAGGCTGAGAAC-3’ (forward) and 5'-TGGTGAAGACGCCAGTGGA-3’ (reverse).
Relative quantitation of mRNA levels was normalized by using expression of GAPDH as a housekeeping gene.

Small interfering (siRNA) in human macrophages and SKOV3 cells
Primary monocyte-derived macrophages and SKOV3 cells were transfected with siRNA against human Stat3 (Santa Cruz, CA) using Lipofectamin™ RNAiMAX (Invitrogen, CA). Control siRNA (Santa Cruz, CA) was used as negative control.

Western blot analysis
Cellular proteins were solubilized in Tris buffer (TB) containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and Phosphatase Inhibitor Cocktail (Nacalai Tesq. Tokyo, Japan). The amount of protein was quantified by the bicinchoninic acid assay, and equal amounts of protein were separated by SDS-PAGE, and then were transferred to the polyvinylidene fluoride membrane. Following blocking in TB containing 2% BSA, membrane was stained with anti-Stat3
(Santa Cruz, CA) and anti-pStat3 (Cell Signaling Tec. Tokyo, Japan) antibody according to the manufacturer’s protocol.

**Statistical analysis**
The statistical analysis was performed using the Kruskal-Wallis test or Student’s *t*-test with the Mann-Whitney test and the level of significance was set at *p* < 0.05. All data was representative at least two independent experiments. Values are the mean ± SD of 3 independent experiments.

**Results**

*Macrophages in the ascites of EOC were polarized to the M2 phenotype*
Most peritoneal macrophages have functional characteristics of the M2 phenotype, however, it is unclear whether the peritoneal macrophages in ovarian cancer ascites are polarized to the M2 phenotype. The number of macrophages positive for CD68, a pan-macrophage marker, and CD163, a M2 macrophage marker, were examined using cytospin specimens of ascites to investigate the phenotype of macrophages in the ascites in advanced EOC patients. Since most macrophages in the ascites were positive for CD163 as well as CD68 (*Fig. 1A*), they were considered to have polarized to the M2 phenotype. No significant differences were found between the number of CD68*+* (*Fig. 1B*) or CD163*+* (*Fig. 1C*) cells in different clinical stages; however, their numbers in advanced EOC patients (FIGO stage III and IV) tended to be higher than those in benign or early EOC patients (FIGO stage I).

*EOC ascites contained soluble factors to activate cancer cells*
An ovarian cancer cell line (SKOV3) and hOSE (HOSE-E7/hTERT) were cultured in the presence of ascitic fluid to test whether the ascites of EOC patients could stimulate proliferation of tumor cells. Ascitic fluid from advanced EOC patients significantly enhanced the proliferation of SKOV3 cells and hOSE cells (*Fig. 2*). In contrast, the ascites from non-advanced EOC patients and non-cancer patients did not enhance the proliferation of these cells (*Fig. 2*). These results indicate that advanced EOC ascites contain some soluble factors to promote tumor cell proliferation.

*Ascites from advanced EOC patients contained high concentrations of IL-6, IL-10,*
**GRO-α, and VEGF**

Evaluations using a cytokine array disclosed that IL-6, IL-8, IL-10, IL-12, IL-15, M-CSF, MCP-1, VEGF and GRO-α were up-regulated in the ascites of advanced EOC patients (data not shown). Among these cytokines, IL-6, IL-10, GRO-α, and VEGF were found to have significantly increased in the ascites of advanced EOC patients and their concentration was strongly correlated with the clinical stages (Fig. 3A). It is noteworthy that the production of IL-6 was observed in both of cancer cells and macrophages in double immunostaining of cell-block specimens of advanced EOC ascites (Fig. 3B). The relevant concentrations of IL-6, IL-10 and GRO-α as in ascites significantly enhanced SKOV3 proliferation, and of these cytokines, dose dependency was seen in IL-6 and IL-10 (Fig. 3C). Although neutralizing antibody against each IL-6 or IL-10 did not influence the proliferation of SKOV3 cells, simultaneous inhibition of both these antibodies significantly suppressed the growth of SKOV3 cells (Fig. 3D). These data indicate that these cytokines play an important role for tumor growth.

**EOC ascites polarized macrophages toward the M2 phenotype via Stat3 activation**

Ascites from EOC patients (Stage I, III, and IV) induced significant activation of Stat3 in THP-1 cells while non-EOC ascites did not activate Stat3 (Fig. 4A, 4B). The macrophages stimulated by advanced EOC ascites produced higher levels of IL-10, a cytokine preferentially produced by M2 macrophages, than that of non-advanced EOC and non-malignant diseases (Fig. 4C). Similar results were obtained in human monocyte-derived macrophages (data not shown).

**Tumor cells were activated by cell-cell interaction with the macrophages**

EOC ascites contained many M2 macrophages and cancer cells. Direct cell-to-cell interaction between M2 macrophages and tumor cells is thought to play an important role in the tumor microenvironment. Therefore, the effect of such interaction between macrophages and tumor cells was investigated in a co-culture system. Although the discrimination of each cell is difficult when two different kinds of cells are mixed, this problem was overcome by making cell blocks to perform double immunostaining using cell-type specific antibodies (Fig. 5A). Little or no Stat3 activation was observed in a single culture of macrophages or SKOV3 cells (Fig. 5B). In contrast, when the macrophages and SKOV3 cells were co-cultured both cell types
showed strong pStat3 staining (Fig. 5C). M-CSF-primed macrophages induced stronger activation of Stat3 in SKOV3 cells than GM-CSF-primed macrophages (Fig. 5D). The expression of the cell cycle-related protein cyclin-D1 in SKOV3 cells was also significantly up-regulated by co-culture with macrophages (Fig. 5D). A separate culture was used to test whether direct cell-cell contact is necessary to activate Stat3 in both cell types. A transwell culture system demonstrated that Stat3 in macrophages and SKOV3 cells were apparently activated as in the mixed co-culture system (Fig. 5E). These results indicated that humoral factors were involved in Stat3 activation in macrophages and SKOV3 cells.

*Interaction between tumor cells and macrophages was mediated by Stat3 activation*

Stat3 was blocked in the macrophages by siRNA to confirm that Stat3 activation is actually involved in the interaction between tumor cells and macrophages (Fig. 6A, 6B). Stat3 activation in SKOV3 cells was significantly suppressed when the activation of macrophage Stat3 was suppressed by siRNA in co-culture (Fig. 6C). The expression of cyclin-D1 in SKOV3 cells was also suppressed by Stat3 deactivation in macrophages (Fig. 6C). Since production of IL-6 and IL-10, and Stat3 activation show intimate link (27), we first examined whether neutralization of IL-6 and/or IL-10 influences Stat3 activation in tumor cells or not. Neutralizing antibody against each cytokine did not influence Stat3 activation in SKOV3 cells, however, simultaneous inhibition of both cytokines significantly suppressed Stat3 activation in tumor cells (Fig. 6D). Then we examined whether the suppression of Stat3 in macrophages influence IL-6/IL-10 production. Deactivation of Stat3 by siRNA suppressed IL-6/IL-10 production as well as CD163 expression (Fig. 6E). These results indicate that the Stat3 activation is involved in cell-cell interactions between macrophages and cancer cells via soluble factors including IL-6 and IL-10.

*SKOV3 cell proliferation is suppressed by Stat3 silencing*

Stat3 activation in cancer cells are associated to the cell proliferation, survival, and resistance to chemotherapy, and the activation of Stat3 was found to be associated with a poor clinical prognosis in EOC.(28) We therefore tested the effect of Stat3 activation in SKOV3 cells and their proliferation. As shown in Fig. 7A and 7B, the proliferation of SKOV3 cells was suppressed significantly after siRNA treatment of Stat3.
Discussion

Most patients with EOC are diagnosed with advanced disease. Intraperitoneal metastases are already widespread, and the peritoneal cavity accumulates large amounts of ascites containing tumor cells and immune cells including macrophages. Peritoneal macrophages are thought to play an indispensable role in cancer progression.\(^{(6-9)}\) Generally, peritoneal macrophages express the characteristics of the M2 phenotype. Xu et al. demonstrated that peritoneal macrophages isolated from peritoneal dialysate from patients with kidney diseases display the M2 phenotype characterized by strong surface expression of CD163, lack of CD16, and production of IL-10 in response to LPS stimulation.\(^{(23)}\) The current study showed that most of the peritoneal macrophages in the ascites of EOC patients are positive for CD163, indicating that those of EOC patients are also polarized to the M2 phenotype.

The presence of M2 macrophages and tumor cells in ascites of advanced EOC patients, suggests that some soluble factors derived from M2 macrophages as well as tumor cells might stimulate the proliferation of tumor cells. Ascitic fluid from advanced EOC patients significantly enhanced the proliferation of SKOV3 cells and hOSE cells. IL-6 and IL-10 were highly increased in the ascites of advanced EOC patients, thus suggesting that these cytokines are possibly involved in tumor cell proliferation. Although it is well known that EOC cells produce IL-6,\(^{(17, 29-31)}\) we found the macrophages in ascites secret IL-6. In the present study, we did not investigate which cells produce IL-10, however, IL-10 is suggested to be mainly produced by macrophage because M2 macrophage produce highly amount of IL-10 and IL-10 production was not detected in culture supernatant of EOC cell lines. GRO-\(\alpha\) was also increased in the ascites of advanced EOC patients. GRO-\(\alpha\) is a CXC chemokine produced by cancer cells and induces the chemotaxis of neutrophil and dendritic cells.\(^{(32)}\) It is well known that has a function as autocrine growth factor and plays a major role in angiogenesis, tumor development, and metastasis. The silence of GRO-\(\alpha\) in melanoma cell line suppressed IL-6 and VEGF production from melanoma cell line.\(^{(33)}\) Although there is no report described the function of GRO-\(\alpha\) in ovarian cancer, GRO-\(\alpha\) suggested to be involved in ovarian cancer invasion and development.

The current study investigated the activation of Stat3, a signal transduction molecule that transmits M2-polarizing signals to macrophage nuclei\(^{(34)}\) to examine the effect of
ascites on the phenotypic change of macrophages. M2-polarizing cytokines such as IL-4, IL-10, and IL-13 activate Stat3 to induce macrophage differentiation toward the M2 phenotype.\(^{10,11}\) Though Stat3 is a constitutive activator in tumor cells\(^{35}\) and is involved in the initiation and progression of human EOC,\(^{28,36-38}\) the regulation and clinical significance of Stat3 signaling in macrophages in EOC ascites is not known. This study demonstrated that soluble factors of ascites induced Stat3 activation in macrophages and the Stat3 activation in macrophages was strikingly evoked by co-culture with cancer cells. Interestingly, Stat3 activation was also detected in cancer cells by co-culture with macrophages, and blockade of Stat3 activation in macrophages suppressed Stat3 activation in cancer cells. These results indicated that cancer cell survival and proliferation in the peritoneal microenvironment are significantly influenced by macrophage differentiation and activation toward the M2 phenotype via Stat3 signaling. Although the factor(s) involved in cell-cell interaction could not be determined, IL-6 is thought to be one of the candidates to activate Stat3 because IL-6 is a potent activator of Stat3\(^{37}\) and it is elevated in ascites of EOC patients and positive in ascites macrophages. M-CSF and VEGF are also candidate molecules, since these molecules are known to activate Stat3 signals,\(^{35,39}\) and their production was enhanced in the co-culture of macrophages and EOC cells.\(^{14}\)

In the present study, the inhibition of both IL-6 and IL-10 cytokines by neutralizing antibodies suppressed Stat3 activation of SKOV3 cells by cell-cell interaction, however, the suppression was statistically significant but the inhibition level was around 40%. This indicated that unknown molecules other than IL-6 and IL-10 are involved in cell-cell interaction between cancer cells and macrophages.

In summary, the present study indicates that interactions between macrophages and ovarian cancer cells through Stat3 activation are important for the development of the tumor microenvironment in the ascites of advanced EOC patients. Therefore, Stat3 inhibitors could be effective to prevent tumor progression in advanced EOC patients by regulating the tumor microenvironment.

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Culture, Sports, Science, and Technology of Japan. This study was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society.

Disclosure Statement
All authors have no conflict of interest.
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**Figure legends**

**Figure 1**: Macrophages in the ascites of EOC and control patients.

(A) Immunohistochemical staining of the cytopsin specimens of advanced EOC ascites. Many CD68$^+$ and CD163$^+$ macrophages are observed. (B, C) The numbers of CD68$^+$ and CD163$^+$ macrophages in the ascites. (Kruskal-Wallis test) [B]: benign disease, [I]: EOC patients of Stage I, [III, IV]: Stages III and IV.

**Figure 2**: Proliferation of SKOV3 cells and hOSE cells in the presence of ascitic fluids of EOC and control patients.

The ascites of advanced EOC induced strong proliferation of SKOV3 cells and hOSE cells. Cell numbers are expressed as the percentage of untreated controls. [B]: benign disease, [I]: EOC patients of Stage I, [III, IV]: Stages III and IV.

**Figure 3**: Cytokine levels in ascites of EOC and control patients.

(A) Increased levels of IL-6, IL-10, GRO-α, and VEGF-A were detected in advanced EOC ascites (Kruskal-Wallis test). [B]: benign disease, [I]: EOC patients of Stage I, [III, IV]: Stages III and IV. (B) Double immunostaining of cell-block of ascites revealed that CD163$^+$ macrophages as well as CD163$^-$ cancer cells produced IL-6 in advanced EOC ascites. Scale bar=50 μm. (C) SKOV3 cells were cultured with recombinant IL-6, IL-10, and GRO-α, and the cell proliferation was evaluated by a WST assay. SKOV3 cells proliferated significantly in the presence of these cytokines. (D) Neutralizing with both against IL-6 and IL-10 antibodies were added with ascites, and SKOV3 cells were cultured for 5 days. The proliferation of SKOV3 cells were significantly suppressed by blocking IL-6 and IL-10. The results are expressed as the percentage of untreated controls. (Mann-Whitney test, * $p < 0.01$, ** $p < 0.05$)

**Figure 4**: Stat3 activation and IL-10 production in THP-1 cells by ascitic fluids of EOC and control patients.

(A, B) Following the culturing of THP-1 cells in the presence of 20% ascites for 30 min, Stat3 activation was evaluated immunohistochemically using anti-pStat3 antibody. Distinct staining for pStat3 was observed in the nuclei of THP-1 cells treated with EOC ascites. Cont: no ascites added. SCS: SKOV3 cell-supernatant used as a positive control. (C) THP-1 macrophages were cultured in the presence of 20% ascites for 2 days, cells

- 18 -
were stimulated by lipopolysaccharide (100 ng/ml) for 24 h. IL-10 production was analyzed by means of ELISA. A significant increase in the IL-10 production was induced by the ascites of advanced EOC patients. [B]: benign disease, [I]: EOC patients of Stage I, [III, IV]: Stages III and IV. Statistical differences were evaluated by using Mann-Whitney test (B, C).

Figure 5: Stat3 activation by macrophages-to-cancer cells interactions.
(A) SKOV3 cells and primary monocyte-derived macrophages were mixed and cultured for 5 days. Cells were detached and prepared as cell block specimens. Stat3 activation and cyclin-D1, a molecule associated with cell proliferation, were analyzed via double immunostaining. (B) SKOV3 cells and monocyte-derived macrophages were cultured separately. Little or no Stat3 activation was detected in both experiments. Scale bar=50 μm. (C) Mixed culture of SKOV3 cells and macrophages (pretreated with M-CSF or GM-CSF from monocytes). Stat3 activation was analyzed using double immunostaining of cell-block specimens. Stat3 activation is seen in the nuclei of CD68+ macrophages and CD68− SKOV3 cells. The arrowheads indicate CD68+ macrophages with Stat3 activation. Scale bar=50 μm. (n=3 for each group) (D) Cyclin-D1 expression in mixed culture of SKOV3 cells and macrophages (pretreated with M-CSF or GM-CSF from monocytes). Cyclin-D1 was analyzed by double immunostaining. Many cyclin-D1+ cells were found in CD68− SKOV3 cells by double immunostaining. Scale bar=50 μm. The percentage of cyclin-D1+ SKOV3 cells was higher in co-culture than in single culture. (n=3 for each group) (E) SKOV3 cells and macrophages were co-cultured without actual cellular contact using a transwell co-culture system. Stat3 activation was evaluated by a Western blot assay. Stat3 activation in both cell types was observed in the transwell co-culture system (Mann-Whitney test, *p < 0.01, **p < 0.05).

Figure 6: Si-RNA mediated knockdown of endogenous Stat3 in macrophages
(A) Two days after suppression of Stat3 in macrophages, SKOV3 cells were added to the culture plate. After co-culture for 5 days, cells were prepared as cell block specimens. (B) The Stat3 expression was evaluated by a Western blot analysis. Stat3 expression in macrophages differentiated with GM-CSF was suppressed by siRNA. (C) Two days after suppression of Stat3 in macrophages, cells were washed in PBS to deplete the siRNA. After co-culture for 3 days with SKOV3 cells, cells were prepared
as cell-block specimens for immunostaining. Stat3 activation in SKOV3 was significantly suppressed by pretreatment of macrophages with siRNA. Scale bar=50 nm. Similarly, Cyclin-D1 expression in SKOV3 cells (CD68− cells) was also suppressed by siRNA pretreatment. (D) We evaluated cell-cell interaction by neutralizing IL-6 and IL-10. Neutralizing with both against IL-6 and IL-10 antibodies induced suppression of Stat3 activation in SKOV3 co-cultured with macrophages. (E) Cultured human macrophages were stimulated by SKOV3 culture supernatant for 2 days, and the mRNA expression of IL-6, IL-10, and CD163 was examined by quantitative PCR (Q-PCR). The expression of these molecules was suppressed by Stat3 siRNA. (Mann-Whitney test, *\( p < 0.01 \), **\( p < 0.05 \)). (n=3 for each group)

Figure 7: The involvement of Stat3 in growth of SKOV3 cells
(A) The Stat3 expression was evaluated by a Western blot analysis. Stat3 expression in SKOV3 cells was suppressed by siRNA. (B) Following siRNA treatment, SKOV3 was cultured for 5 days and the viability of SKOV3 cells was evaluated by WST-1 assay. The proliferation of SKOV3 was suppressed by down-regulation of Stat3 (n=3 for each group, Mann-Whitney test, *\( p < 0.01 \)).
Table 1 Monoclonal antibodies

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