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Expression of multiple immune checkpoint molecules on T cells in malignant ascites from epithelial ovarian carcinoma

YUICHI IMAI[1-3], KOSEI HASEGAWA[1,2], HIROKAZU MATSUSHITA[4], NAO FUJIEDA[4], SHO SATO[1,2], ETSUKO MIYAGI[3], KAZUHIRO KAKIMI[4] and KEIICHI FUJIWARA[1,2]

[1]Department of Gynecologic Oncology, Saitama Medical University International Medical Center; [2]Gynecologic Oncology Translational Research Unit, Project Research Division, Research Center for Genomic Medicine, Saitama Medical University, Hidaka-shi, Saitama 350-1298; [3]Department of Obstetrics and Gynecology, Yokohama City University, Yokohama-shi, Kanagawa 236-0004; [4]Department of Immunotherapeutics, The University of Tokyo Hospital, Bunkyo-ku, Tokyo 113-8655, Japan

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Abstract. Expression of immune checkpoint molecules, including programmed cell death protein-1 (PD-1), has been reported on T cells in various types of cancer. However, the expression status of these molecules in the tumor microenvironment of epithelial ovarian cancer (EOC) has not yet been studied. A total of 54 cases of malignant ascites from patients with EOC were analyzed in the present study. The expression of PD-1, lymphocyte-activation gene-3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and B and T lymphocyte attenuator (BTLA) on cluster of differentiation (CD)4$^+$ and CD8$^+$ T cells in malignant EOC ascites were investigated using multicolor flow cytometric analysis. The expression of PD-L1 in tumor cells, PD-L2 in HLA-DR-positive cells and galectin-9 in ascitic fluid was also analyzed. In addition, cytokine profiling of ascitic fluid was performed to understand the immune microenvironment of EOC. PD-1, LAG-3 TIM-3, and BTLA were expressed on 65.8, 10.6, 4.3 and 37.6% of CD4$^+$ T cells, and on 57.7, 5.0, 4.9 and 15.7% of CD8$^+$ T cells, respectively. Programmed cell death protein-1 (PD-1), LAG-3 and BTLA were more frequently expressed on CD4$^+$ compared with CD8$^+$ T cells. The co-expression of immune checkpoints was further investigated and results indicated that 39 (72.2%) and 37 patients (68.5%) expressed multiple immune checkpoints on CD4$^+$ T cells and CD8$^+$ T cells, respectively. In addition, lower levels of TNF-α and interleukin-6 in ascitic fluid were significantly associated with multiple immune checkpoint expression on CD8$^+$ T cells. The present findings indicated that multiple immune checkpoint molecules were expressed on T cells in the EOC tumor microenvironment and the results may suggest the significance of simultaneous blockade of immune checkpoints to control EOC.

Correspondence to: Dr Kosei Hasegawa, Department of Gynecologic Oncology, Saitama Medical University International Medical Center, 1397-1 Yamane, Hidaka-shi, Saitama 350-1298, Japan
E-mail: koseih@saitama-med.ac.jp

Dr Hirokazu Matsushita, Department of Immunotherapeutics, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
E-mail: hmatsu924@m.u-tokyo.ac.jp

Abbreviations: PD-1, programmed cell death protein-1; LAG-3, lymphocyte-activation gene-3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; BTLA, B and T lymphocyte attenuator; EOC, epithelial ovarian cancer; PFS, progression free survival; OS, overall survival; PBMC, peripheral blood mononuclear cell

Key words: ovarian cancer, ascites, programmed cell death protein-1, lymphocyte-activation gene-3, T-cell immunoglobulin and mucin-domain containing-3, B and T lymphocyte attenuator

Introduction

Epithelial ovarian cancer (EOC) is the most lethal disease among gynecological malignancies. Unlike other carcinomas, peritoneal dissemination is the most common mechanism of disease progression in ovarian cancer, and up to 70% of cases present with massive malignant ascites with peritoneal implants (1). Among patients with advanced ovarian cancer who undergo primary debulking surgery, those with no residual disease have a much better survival than women with any residual disease. Therefore, control of dissemination seems to be the most important strategy in the treatment of ovarian cancer (2). Despite cytoreductive surgery and platinum and taxane combination chemotherapy, most patients with advanced ovarian cancer experience relapse. The peritoneal cavity is the most frequent site of recurrence, and most patients with intraperitoneal recurrence eventually become chemoresistant and die from the disease (3). Thus, development of new treatment strategies for EOC is required (4,5).

Recent studies have shown that tumor cells acquire escape mechanisms to evade host immunity in the tumor microenvironment (6,7). To circumvent these mechanisms, extensive studies have been undertaken for regulatory T cells, immune checkpoints, myeloid-derived suppressor cells and M2 type

macrophages (8-11). With the clinical success of immune checkpoint inhibitors such as ipilimumab and nivolumab for melanoma and lung cancer, immune checkpoints have received increased attention (12,13). Some of the early-phase clinical trials of immune checkpoint inhibitors for ovarian cancer, such as anti-programmed cell death protein 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) antibodies, have shown manageable safety profiles and demonstrated a durable anti-tumor response in a certain patient population (14). However, their response rates remain at 10 to 15% (15-17). Therefore, we need to explore predictive biomarkers for durable responders and to understand the underlying mechanism. Combination therapy with chemotherapy may be another way to enhance the value of immune checkpoint inhibitors for ovarian cancer (18). Since we observed relatively lower rates of clinical response in recurrent EOC patients in recent early-phase clinical trials for PD-1 blockade, we recently came to recognize not only PD-1 but also other immune checkpoint molecules, such as lymphocyte-activation gene-3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), B and T lymphocyte attenuator (BTLA), and VISTA, are expressed on T cells associated with cancer (19-21). A recent study showed that expression of PD-1 and LAG-3 on cluster of differentiation (CD)8^+ T cells derived from tumor-infiltrating or tumor-associated lymphocytes can result in impaired IFN-γ and TNF-α production compared with $CD8^+$ T cell subsets that express PD-1 alone (22). Dual blockade of PD-1 and LAG-3 pathways could potentially improve the therapeutic efficacy of cancer immunotherapy. Therefore, we sought to address the expression status of various immune checkpoints on T cells in the tumor microenvironment of EOC patients through the analysis of ascites cells.

Malignant ascites was thought to be an ideal source to assess the tumor immune microenvironment. Cells are basically in suspension in ascites, therefore it is easy to assess both immune and tumor cells by flow cytometric analysis. The expression of LAG-3, TIM-3, and BTLA on T cells in malignant ascites from EOC has not yet been assessed. Here, we evaluated the expression of immune checkpoint molecules on both $CD4^+$ and $CD8^+$ T cells in malignant ascites from EOC. In addition, expression of their potential ligands was addressed at the same time. Moreover, we measured levels of cytokines/chemokines in ascites fluid to understand the immunological background of the ovarian cancer tumor immune microenvironment.

Materials and methods

Patients and ascites. This study was reviewed and approved by the Institutional Review Board of Saitama Medical University International Medical Center (no. 13-092). Eighty-nine patients who were clinically suspected to have EOC before surgery at Saitama Medical University International Medical Center (Hidaka-shi, Japan) were enrolled in this study from December 2010 to November 2014. Eighty-two patients were pathologically diagnosed with malignant tumors, while two had borderline and five had benign ovarian tumors. Of 82 malignant ovarian tumors, 80 were diagnosed as EOC. One patient was diagnosed with ovarian metastasis of a primary colorectal cancer and one with a germ cell tumor. Twenty-six cases were excluded because of insufficient levels of ascites cells for analysis. Thus, ascites cells from the remaining 54 patients were analyzed. The median age of the patients was 63.5 years with a range of 30-80 years. The EOC cases consisted of 4 (7.4%) stage I, 4 (7.4%) stage II, 35 (64.8%) stage III and 11 (20.4%) stage IV according to the International Federation of Gynecology and Obstetrics (FIGO) system. There were 31 (57.4%) serous, 8 (14.8%) clear cell and 6 (11.1%) endometrioid carcinoma. Furthermore there were 13 (24.1%) type I and 41 (75.9%) type II. Informed written consent was obtained from all patients in this study.

Flow cytometry analysis. The following monoclonal antibodies (mAbs) were used for flow cytometry: FITC-labeled anti-human CD4 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), PE-labeled anti-human CD273 (B7-DC, PD-L2; BioLegend, Inc., San Diego, CA, USA), anti-human CD274 (PD-L1, B7-H1; BioLegend, Inc.), anti-human CD279 (PD-1; BioLegend, Inc.), anti-human CD366 (TIM-3; (BioLegend, Inc.), anti-human CD272 (BTLA; BioLegend, Inc.), anti-human LAG3 (R&D Systems Inc., Minneapolis, MN, USA) and mouse IgG_1 isotype (BioLegend, Inc.) antibodies, PC5-labeled anti-CD3 (BioLegend, Inc.) antibody, APC-labeled anti-CD326 (EpCAM), anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-HLA-DR (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse IgG_1 isotype (eBioscience, San Diego, CA, USA) antibodies, and Pacific Blue-labeled anti-CD45 (BioLegend, Inc.) and anti-CD8a (BioLegend, Inc.) antibodies. Fixable Viability Dye eFluor 780 (eBioscience) was used to exclude dead cells. Ascites cells were harvested by centrifugation, stained with the mAbs described above and analyzed on a Gallios (Beckman Coulter, San Diego, CA, USA). The data were processed using Kaluza software (Beckman Coulter).

Cytokine measurement. Cytokines, including interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, bFGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF in ascites fluid were measured using Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The assay was performed according to the manufacturer's instructions. Briefly, ascites was incubated with microbeads labeled with specific antibodies to one of the aforementioned cytokines for 60 min. Following a washing step, the beads were incubated with the detection antibody cocktail with each antibody specific to a single cytokine for 30 min. After another washing step, the beads were incubated with streptavidin-phycoerythrin for 10 min, washed again and then the concentration of each cytokine was determined using the array reader. Cytokines of which standard deviation values were larger than 20 were subsequently analyzed.

Measurements of galectin-9. Galectin-9 in ascites fluid was measured using a Human Galectin-9 DuoSet ELISA development kit (R&D Systems Inc.) according to the manufacturer's instructions.

Statistical analysis. Differences between the groups of patients were assessed by one-way ANOVA, Student's t-test and Chi-square test. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA,

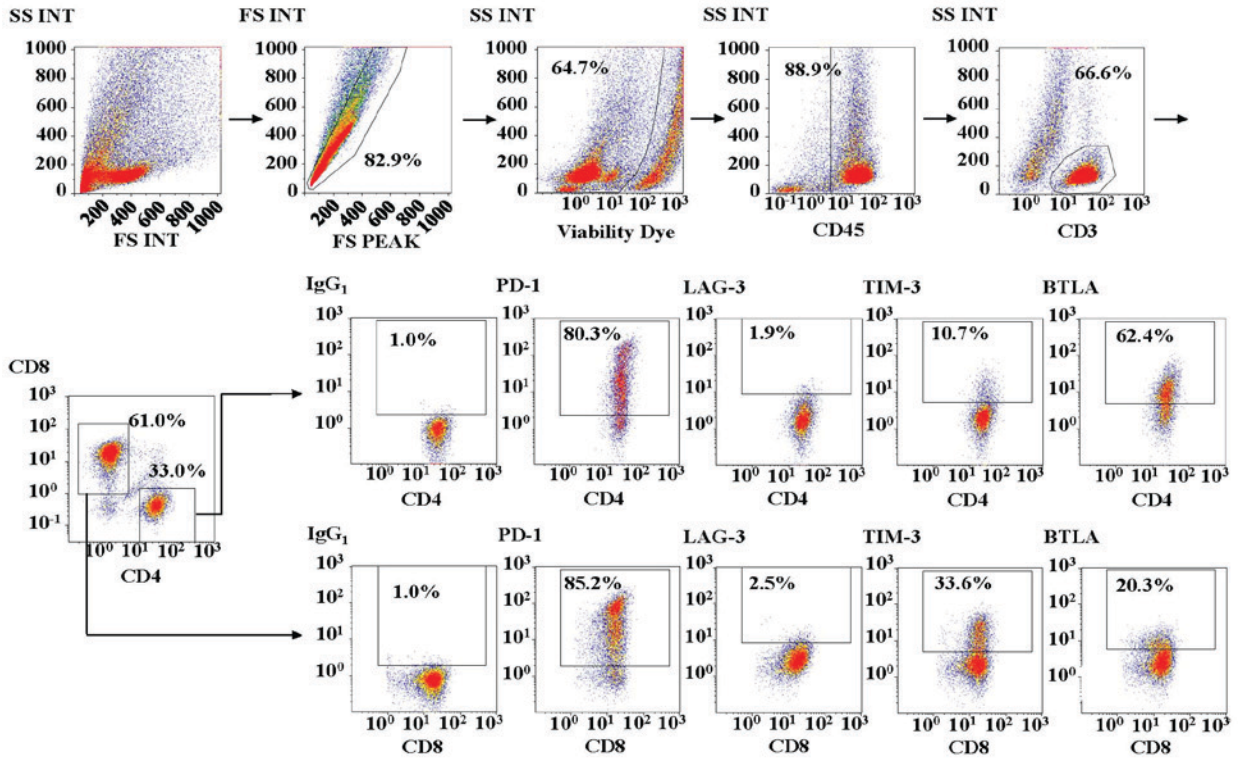

Figure 1. Analysis of immune checkpoint molecules PD-1, LAG-3, TIM-3, and BTLA on CD4+ and CD8+ T cells in malignant ascites from ovarian cancer by multicolor flow cytometry. Various immune checkpoint molecules were expressed on both CD4+ and CD8+ T cells in ascites from EOC. FS, forward scatter; SS, side scatter; INT, integral; PD-1, programmed cell death protein-1; LAG-3, lymphocyte-activation gene-3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; BTLA, B and T lymphocyte attenuator.

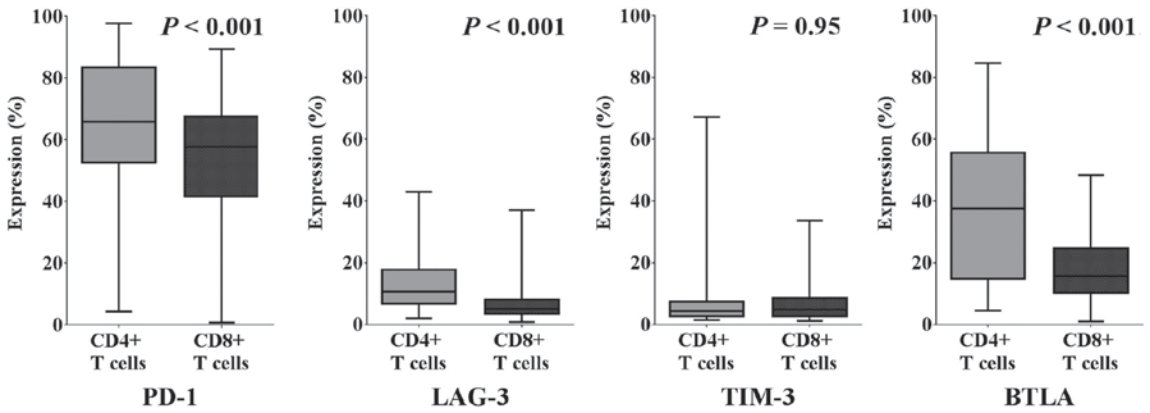

Figure 2. The median, quartile and range of expression rates of PD-1, LAG-3, TIM-3, and BTLA on CD4+ and CD8+ T cells in ovarian cancer ascites. PD-1, LAG-3, and BTLA exhibited higher expression levels on CD4+ T cells than on CD8+ T cells in ascites of EOC patients (P<0.001). PD-1, programmed cell death protein-1; LAG-3, lymphocyte-activation gene-3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; BTLA, B and T lymphocyte attenuator.

USA). All reported P-values were two-sided, and P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of immune checkpoint molecules on T cells in ascites from EOC patients. First, we investigated the expression of various immune checkpoint molecules on T cells in malignant ascites. Fig. 1 shows the representative analysis pipeline for the immune checkpoint molecules on T cells in malignant ascites. We observed that each immune checkpoint molecule was expressed at various levels on both CD4+ and CD8+ T cells in ascites from EOC. As shown in Fig. 2, 65.8% (range, 4.4-97.6%), 10.6% (1.9-43.0%), 4.3% (1.4-67.2%) and 37.6% (4.5-84.6%) of CD4+ T cells expressed PD-1, LAG-3, TIM-3, and BTLA, respectively. We also found that 57.7% (range, 0.7-89.4%), 5.0% (0.8-37.0%), 4.9% (1.2-33.6%) and 15.7% (1.0-48.4%) of CD8+ T cells expressed PD-1, LAG-3, TIM-3, and BTLA, respectively. We observed higher expression rates of PD-1, LAG-3, and BTLA on CD4+ T cells than on CD8+ T cells in ascites from EOC patients (P<0.001).

Clinicopathological features and immune checkpoint molecule expression in patients with EOC. Tables I and II summarize

Table I. Expression of immune checkpoint molecules on $CD4^+$ T cells in malignant ascites from ovarian carcinoma.

Characteristcs	High PD-1/total (%)	P-value	High LAG-3/total (%)	P-value	High TIM-3/total (%)	P-value	High BTLA/total (%)	P-value
Age (years)								
≥65	12/25 (48.0)	0.78	11/25 (44.0)	0.41	10/25 (40.0)	0.17	13/25 (52.0)	0.78
≤64	15/29 (51.7)		16/29 (55.2)		17/29 (58.6)		14/29 (48.3)	
FIGO stage								
I + II	5/8 (62.5)	0.44	5/8 (62.5)	0.44	3/8 (37.5)	0.44	3/8 (37.5)	0.44
III + IV	22/46 (47.8)		22/46 (47.8)		24/46 (52.2)		24/46 (52.2)	
Histology								
Serous	14/31 (45.2)	0.67	12/31 (38.7)	0.29	16/31 (51.6)	0.73	19/31 (61.3)	0.18
Clear cell	5/8 (62.5)		5/8 (62.5)		5/8 (62.5)		3/8 (37.5)	
Endometrioid	4/6 (66.7)		4/6 (66.7)		2/6 (33.3)		3/6 (50.0)	
Others	4/9 (44.4)		6/9 (66.7)		4/9 (44.4)		2/9 (22.2)	
Type								
I	10/13 (76.9)	0.03[a]	9/13 (69.2)	0.11	7/13 (53.8)	0.75	4/13 (30.8)	0.11
II	17/41 (41.5)		18/41 (43.9)		20/41 (48.8)		23/41 (56.1)	

Low grade serous carcinoma, low grade endometrioid carcinoma, clear cell carcinoma and mucinous carcinoma were included in type I EOC. High grade serous carcinoma, high grade endometrioid carcinoma and carcinosarcoma were included in type II EOC. PD-1, programmed cell death protein-1; LAG-3, lymphocyte-activation gene-3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; BTLA, B and T lymphocyte attenuator; FIGO, International Federation of Gynecology and Obstetrics.

Table II. Expression of immune checkpoint molecules on $CD8^+$ T cells in malignant ascites from ovarian carcinoma.

Characteristcs	High PD-1/total (%)	P-value	High LAG-3/total (%)	P-value	High TIM-3/total (%)	P-value	High BTLA/total (%)	P-value
Age (years)								
≥65	13/25 (52.0)	0.78	13/25 (52.0)	0.78	13/25 (52.0)	0.78	12/25 (48.0)	0.78
≤64	14/29 (48.3)		14/29 (48.3)		14/29 (48.3)		15/29 (51.7)	
FIGO stage								
I + II	6/8 (75.0)	0.13	5/8 (62.5)	0.44	3/8 (37.5)	0.44	3/ 8 (37.5)	0.44
III + IV	21/46 (45.7)		22/46 (47.8)		24/46 (52.2)		24/46 (52.2)	
Histology								
Serous	18/31 (58.1)	0.51	15/31 (48.4)	0.85	19/31 (61.3)	0.29	19/31 (61.3)	0.18
Clear cell	3/8 (37.5)		4/8 (50.0)		3/8 (37.5)		3/8 (37.5)	
Endometrioid	3/6 (50.0)		4/6 (66.7)		2/6 (33.3)		3/6 (50.0)	
Others	3/9 (33.3)		4/9 (44.4)		3/9 (33.3)		2/9 (22.2)	
Type								
I	7/13 (53.8)	0.75	7/13 (53.8)	0.75	3/13 (23.1)	0.03[a]	4/13 (30.8)	0.11
II	20/41 (48.8)		20/41 (48.8)		24/41 (58.5)		23/41 (56.1)	

PD-1, programmed cell death protein-1; LAG-3, lymphocyte-activation gene-3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; BTLA, B and T lymphocyte attenuator; FIGO, International Federation of Gynecology and Obstetrics.

Table III. Multiple immune checkpoint molecules expression on $CD4^+$ and $CD8^+$ T cells in malignant ascites from ovarian carcinoma.

	CD4+ T cells				CD8+ T cells			
Characteristcs	Multiple/total (%)	Single/total (%)	None/total (%)	P-value	Multiple/total (%)	Single/total (%)	None/total (%)	P-value
Age (years)								
≥65	16/25 (64.0)	7/25 (28.0)	2/25 (8.0)	0.21	16/25 (64.0)	5/25 (20.0)	4/25 (16.0)	0.51
≤64	23/29 (79.3)	4/29 (13.8)	2/29 (6.9)		21/29 (72.4)	5/29 (17.2)	3/29 (10.3)	
FIGO stage								
I + II	6/8 (75.0)	1/8 (12.5)	1/8 (12.5)	0.85	6/8 (75.0)	0	2/8 (25.0)	0.67
III + IV	33/46 (71.7)	10/46 (21.7)	3/46 (6.5)		31/46 (67.4)	10/46 (21.7)	5/46 (10.9)	
Histology								
Serous	22/31 (71.0)	7/31 (22.6)	2/31 (6.5)	0.46	24/31 (77.4)	5/31 (16.1)	2/31 (6.5)	0.07
Clear cell	7/8 (87.5)	0	1/8 (12.5)		5/8 (62.5)	1/8 (12.5)	2/8 (25.0)	
Endometrioid	5/6 (83.3)	1/6 (16.7)	0		5/6 (83.3)	0	1/6 (16.7)	
Others	5/9 (55.6)	3/9 (33.3)	1/9 (11.1)		3/9 (33.3)	4/9 (44.4)	2/9 (22.2)	
Type								
I	12/13 (92.3)	0	1/13 (7.7)	0.06	9/13 (69.2)	1/13 (7.7)	3/13 (23.1)	0.95
II	27/41 (65.9)	11/41 (26.8)	3/41 (7.3)		28/41 (68.3)	9/41 (22.0)	4/41 (9.8)	

CD, cluster of differentiation; FIGO, International Federation of Gynecology and Obstetrics.

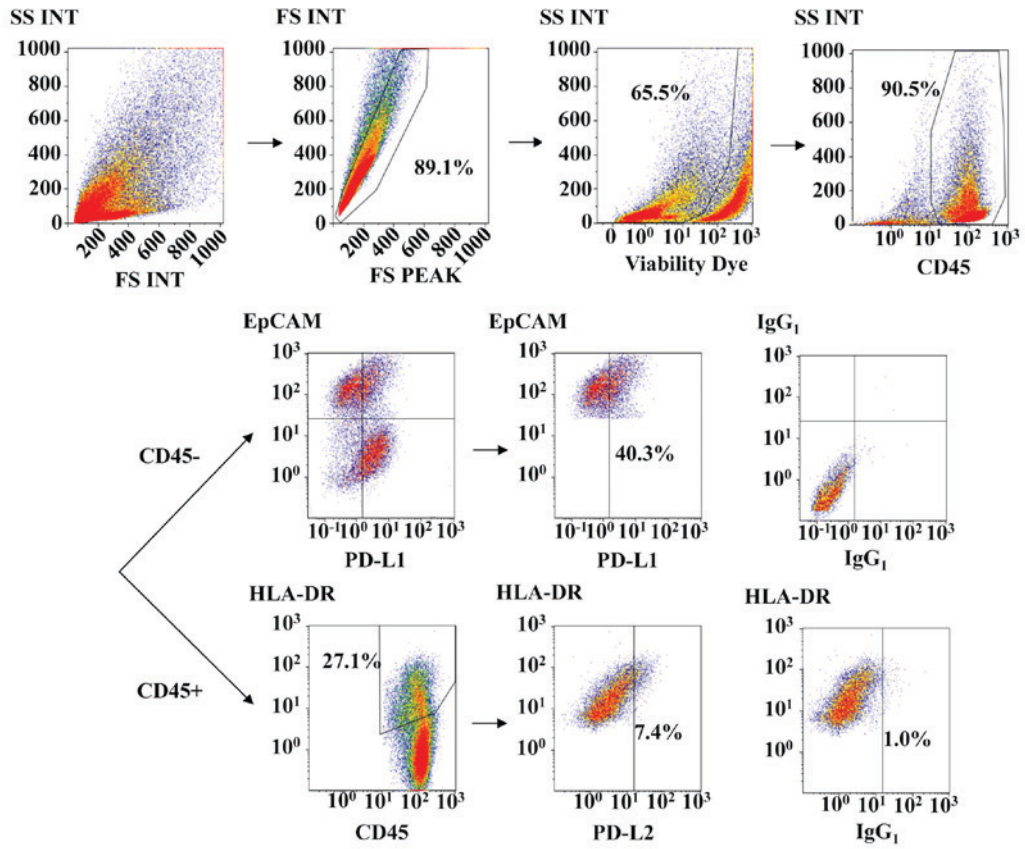

Figure 3. Analysis of PD-L1 and PD-L2 expression on EpCAM-positive cells and HLA class II-positive lymphocytes in malignant ascites by multicolor flow cytometry. PD-L1, programmed cell death-ligand 1; FS, forward scatter; SS, side scatter; INT, integral.

the relationship between clinicopathological features and the expression of immune checkpoint molecules on $CD4^+$ and $CD8^+$ T cells in malignant ascites from EOC. We found higher rates of PD-1 expression on $CD4^+$ T cells in ascites from type I EOC patients than that from type II EOC patients (76.9% vs. 41.5%, P=0.03). Likewise, high rates of TIM-3 expression were observed on CD8+ T cells in ascites from type II EOC than that from type I (58.5 vs. 23.1%, P=0.03). No correlation was found between the expression of immune checkpoint molecules on T cells and other clinical variables.

Multiple immune checkpoint molecule expression on T cells in ascites from EOC. Next, we asked whether there were any overlapping immune checkpoint inhibitory pathways on T cells from patients with malignant ascites. We therefore further investigated the multiple expression of immune checkpoint molecules on T cells in malignant ascites. We considered a higher percentage above the median values as higher immune checkpoint expression. We found that 39 (72.2%) patients and 37 (68.5%) patients exhibited expression of multiple immune checkpoint molecules on $CD4^+$ and $CD8^+$ T cells, respectively. We also examined the relationship between multiple immune checkpoint expression and clinicopathological factors but did not find any correlation (Table III).

PD-L1 and PD-L2 expression on ascites cells from EOC patients. We next assessed the expression of PD-1 ligands, such as PD-L1 and PD-L2, on tumor cells and antigen-presenting cells in malignant ascites. Of the 54 EOC patients, 30 cases could be analyzed for PD-L1 and PD-L2. Fig. 3 shows the representative analyses of PD-L1 and PD-L2 expression on EpCAM-positive cells and HLA class II-positive lymphocytes in malignant ascites, respectively. We investigated PD-L1 and PD-L2 expression based on the PD-1 expression status of T cells from the same patient. We defined above the median values of percent PD-1 expression as high PD-1 expression. As shown in Fig. 4A, PD-L1 expression was found in 43.9% (3.5-91.7%) of tumor cells in patients who had high PD-1-expressing $CD4^+$ T cells, but only 27.3% (8.5-60.0%) of tumor cells in patients who had low PD-1-expressing $CD4^+$ T cells (P=0.02). However, no difference in PD-L1 expression was observed between patients with high and low PD-1 expression on $CD8^+$ T cells, at 34.1% (3.5-91.7%) and 27.3% (8.5-68.0%), respectively. As shown in Fig. 4B, PD-L2 expression was 2.4% (0.8-8.7%) in patients who had high PD-1 on $CD4^+$ T cells and 3.4% (1.2-10.7%) in patients who had low PD-1 on $CD4^+$ T cells (P=0.63), and was 2.3% (0.8-10.7%) in patients who had high PD-1 on $CD8^+$ T cells and 3.2% (1.6-10.7%) in patients who had low PD-1 on $CD8^+$ T cells (P=0.99). No correlation was found between PD-L1/2 expression and clinical variables (Table IV). Moreover, we did not observe any association between PD-L1/2 expression and clinical outcomes (data not shown).

We also investigated the levels of galectin-9, a ligand of TIM-3, in ascites fluids from EOC patients. We observed higher levels of galectin-9 in patients who had high TIM-3 on $CD8^+$ T cells compared with those who had low TIM-3

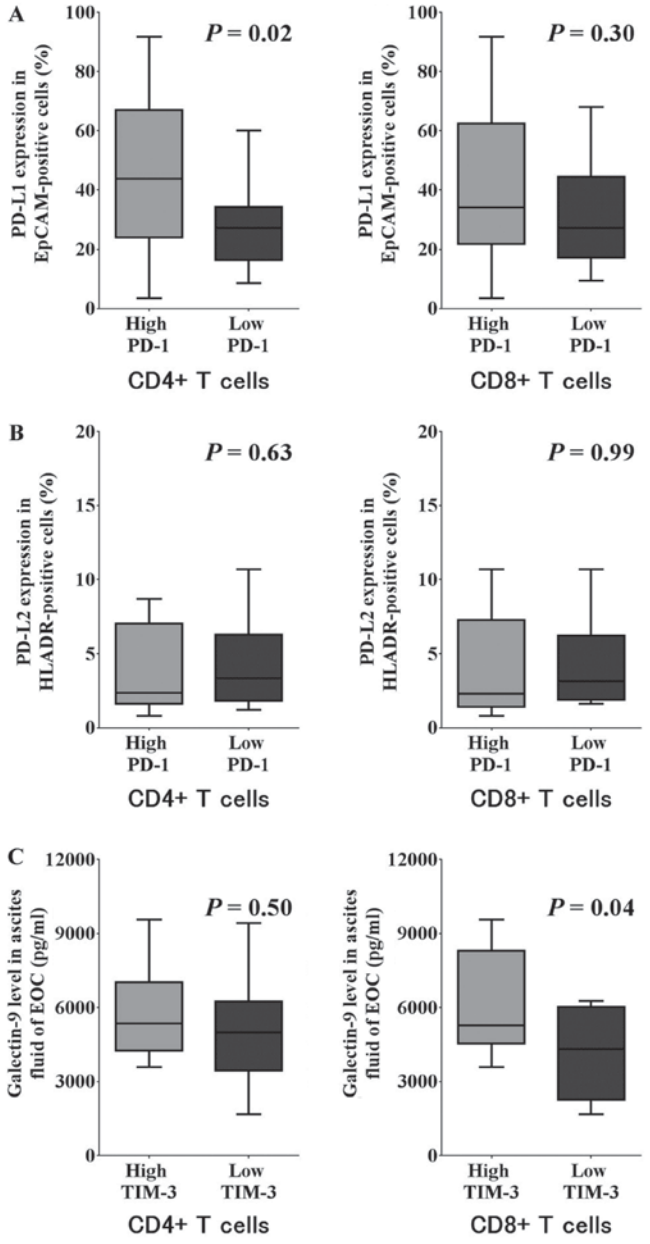

Figure 4. (A) Expression of PD-L1 on EpCAM-positive cells in high or low PD-1 expression groups of the $CD4^+$ and $CD8^+$ T cells in ascites from EOC. (B) Expression of PD-L2 on HLADR-positive cells in high or low PD-1 expression groups of the $CD4^+$ and $CD8^+$ T cells in ascites from EOC. No correlation between PD-L2 and PD-1 on $CD4^+$ and $CD8^+$ T cells was identified (*P*=N.S.). (C) Evaluation of galectin-9 in ascites fluid classified by high or low TIM-3 expression on $CD4^+$ and $CD8^+$ T cells.

(6,004 pg/ml [3,584.6-9,562.6 pg/ml]) vs. 4,067.0 pg/ml [667.5-9,428.6 pg/ml]) (P=0.04).

Relationship between immune checkpoint expression and ascites cytokine profile. To further investigate the local immune inhibitory environment, we determined the cytokine and chemokine profile of ascitic fluids by suspension arrays. We assessed the relationship between immune checkpoint molecule expression and ascites cytokine profile (Table V). We observed that lower TNF-α and IL-6 levels in ascitic fluids were significantly associated with multiple immune checkpoint expression on $CD8^+$ T cells (P=0.03 and P=0.02, respectively). Higher VEGF and lower G-CSF levels were also associated with multiple immune checkpoint expression with borderline significance (P=0.06).

Discussion

In this study, we focused on the expression of various immune checkpoint molecules on T cells in the tumor microenvironment of EOC through the analysis of ascites cells. PD-1 has been reported to be upregulated on T cells from patients with EOC. PD-1 expression on T cells isolated from peripheral blood mononuclear cells (PBMCs) and ascites from patients with malignant ovarian tumors was high compared with benign/borderline in ovarian tumors (23). However, the expression status of other immune checkpoint molecules such as LAG-3, TIM-3, or BTLA on T cells in EOC patients have not been addressed yet, with the exception of a report about TIM-3 on PBMCs of EOC patients (24). The co-expression status of immune checkpoint molecules on T cells in the tumor microenvironment of EOC is important to understand the complex immune inhibitory mechanism of EOC patients.

We investigated the expression of various immune checkpoint molecules on T cells in malignant ascites from EOC patients. Among them, PD-1 was the most frequently expressed, with median expression rates of 65.8 and 57.7% on $CD4^+$ T cells and $CD8^+$ T cells, respectively. Conversely, the median expression rates of LAG-3, TIM-3, and BTLA were 10.6, 4.3, and 37.6% of $CD4^+$ T cells; and 5.0, 4.9, and 15.7% of $CD8^+$ T cells, respectively. These data suggest the immune inhibitory environment caused by immune checkpoint molecules in ascites of EOC patients was PD-1/PD-L1-axis dominant, or might be because of varying sensitivity/specificity for each antibody to its molecule. This aspect should be carefully considered when comparing the expression levels and/or rates of different molecules by different antibodies. However, we at least found not only PD-1 but also LAG-3, TIM-3, and BTLA were expressed on T cells in the tumor microenvironment of EOC.

We did not observe a correlation between the expression of any of the immune checkpoint molecules examined and clinicopathological factors in our study. However, PD-1 expression was reported to be higher in advanced-stage breast (25), renal (26) and pancreatic cancers (27) than in the respective early-stage disease. Thus, the expression of immune checkpoint molecules in EOC seems to be independent from these factors, unlike in other cancer types. In other words, immune checkpoints were expressed even in early-stage EOC as well as in advanced-stage EOC. These results indicate that checkpoint blockade therapy can serve not only as second-line treatment for metastatic disease but as an adjuvant immunotherapy for early-stage EOC patients after initial surgery. With regard to patient survival, some of the previous studies reported that immune checkpoint molecule expression was associated with clinical outcomes (25,26,28-30). The presence of PD-1-expressing tumor-infiltrating lymphocytes correlates with poor prognosis in a number of cancer types, including lung (28), breast (25,29), renal (26), and nasopharyngeal cancer (30), and a low percentage of PD-1 expression on PBMCs was

Table IV. Expression of PD-L1 on EpCAM-positive cells and PD-L2 on HLA-DR-positive cells.

Characteristcs	High PD-L1/total (%)	P-value	High PD-L2/total (%)	P-value
Age (years)		0.46		0.46
≥65	8/14 (57.1)		8/14 (57.1)	
≤64	7/16 (43.8)		7/16 (43.8)	
FIGO stage		0.28		1
I + II	1/4 (25.0)		2/4 (50.0)	
III + IV	14/26 (53.8)		13/26 (50.0)	
Histology		0.29		0.25
Serous	10/21 (47.6)		9/21 (42.9)	
Clear cell	1/3 (33.3)		1/3 (33.3)	
Endometrioid	1/3 (33.3)		3/3 (100.0)	
Others	3/3 (100.0)		2/3 (66.7)	
Type		1		0.36
I	3/6 (50.0)		4/6 (66.7)	
II	12/24 (50.0)		11/24 (45.8)	

PD-L1, programmed cell death-ligand 1; PD-L2, programmed cell death-ligand 2; FIGO, International Federation of Gynecology and Obstetrics.

recently shown to be associated with improved progression free survival (PFS) and overall survival (OS) in ovarian cancer patients (31). We did not see a correlation between immune checkpoint expression and survival in EOC patients. This result might be because of a different source of T cells or the detection methods we used, or because of an insufficient number of events to determine it as a prognostic factor.

Since various immune checkpoint pathways have been reported in cancers (21), we further investigated the expression status of multiple immune checkpoint molecules on T cells in malignant ascites. We found that 72.2 and 68.5% patients had high multiple immune checkpoint molecule expression on $CD4^+$ and $CD8^+$ T cells, respectively. Data for multiple immune checkpoint molecules may be a reason for the relative low response rates of current PD-1/PD-L1 blockade therapy for recurrent EOC patients, which demonstrated response rates of 10 to 15% at most (15-17). Our findings may explain in part that single immune checkpoint inhibition alone may not be sufficient to control the growth of EOC. It is reasonable to consider combination therapy of immune checkpoint inhibitors for EOC patients. Several clinical trials for combination therapies of PD-1 inhibitor with other cancer immunotherapies are currently ongoing. In particular, a combination of nivolumab and ipilimumab for the treatment of melanoma increased PFS compared with either agent alone (32), and similar combination therapies are now being investigated in ovarian cancer (33). Double checkpoint blockade in which anti-PD-1 antibody is combined with immune modulators such as anti-LAG-3 antibody is currently under investigation for solid tumors as well (22). Based on our findings, combination therapy for the blockade of various immune checkpoint pathways would be effective as a multiple-targeting immunotherapy.

When we focused on the relationship between the expression of each immune checkpoint and its ligand, we observed expression of PD-1 on $CD4^+$ and TIM-3 on $CD8^+$ T cells was correlated with PD-L1 and galection-9 in ascites, respectively. We suggest that it may reflect an immune suppressive environment for EOC. Immune checkpoints and/or their ligand expression were considered as candidate biomarkers of EOC for immune checkpoint blockade therapy (34-37). Therefore, we postulate that EOC is a good target for blockade therapy of PD-1/PD-L1 and TIM-3/galectin-9 pathways. The relationship between PD-L1 and clinical outcomes is another issue because it remains controversial. Some reports have shown that PD-L1 expression is associated with poorer prognosis (34,35), but recent studies have shown better prognosis (36,37) in ovarian cancer. We demonstrated no correlation between PD-L1/L2 expression and clinical variables and outcome in this study, which might be because of the different antibodies, detection method, or different source of cancer cells (ascites or tumor) used.

To further evaluate the immune inhibitory environment in malignant ascites in patients with EOC, we assessed the relationship between immune checkpoint molecule expression and ascites cytokine/chemokine profiles. We observed lower TNF-α and IL-6 in ascitic fluids-indicative of impaired local inflammation-were significantly associated with multiple immune checkpoint expression on $CD8^+$ T cells. This result could reflect a strong immunosuppressive tumor microenvironment in patients who had multiple immune checkpoint expression on their T cells.

The limitations of our study need to be addressed. First, our study was not a prospective study and the number of cases we assessed was slightly limited. Second, our immune checkpoint expression data were not based on single T cells. We do not know whether individual T cells express multiple immune checkpoint molecules or not.

In conclusion, we report in this study that expression of various immune checkpoint molecules was observed on both $CD4^+$ and $CD8^+$ T cells in ascites from EOC patients, and

Table V. Cytokines in multiple immune checkpoint molecule expression on CD4+ and CD8+ T cells in malignant ascites from ovarian carcinoma.

	CD4+ T cells				CD8+ T cells			
Characteristcs	Multiple (n=39)	Single (n=11)	None (n=4)	P-value	Multiple (n=37)	Single (n=10)	None (n=7)	P-value
IFNγ (pg/ml)	378.2	372.8	430.2	0.85	369.4	394.3	420.6	0.48
TNFα (pg/ml)	165.0	174.4	281.2	0.33	150.2	202.0	267.7	0.03[a]
IL1Ra (pg/ml)	365.0	380.8	447.3	0.71	333.5	463.2	454.3	0.15
IL1b (pg/ml)	9.0	10.1	13.2	0.65	7.4	14.2	13.4	0.11
IL2 (pg/ml)	9.4	8.9	11.0	0.97	8.9	9.6	12.1	0.20
IL4 (pg/ml)	7.5	6.7	8.8	0.81	6.9	8.4	9.0	0.04[a]
IL5 (pg/ml)	6.4	6.2	7.6	0.94	6.2	7.0	7.3	0.63
IL6 (pg/ml)	5,315.4	4,859.1	6,607.5	0.99	4,411.6	5,742.1	9,246.3	0.02[a]
IL7 (pg/ml)	25.3	26.7	23.9	0.88	25.9	25.1	24.1	0.78
IL8 (pg/ml)	905.9	1,410.5	710.7	0.60	768.5	1,862.4	907.8	0.23
IL9 (pg/ml)	93.7	97.7	106.4	0.75	91.6	101.9	106.1	0.53
IL10 (pg/ml)	178.5	179.2	187.0	0.96	153.0	235.3	231.0	0.12
IL12 bp70 (pg/ml)	476.2	500.6	509.5	0.80	519.8	327.1	528.3	0.28
IL13 (pg/ml)	31.6	35.5	36.2	0.46	33.8	25.7	37.9	0.56
IL15 (pg/ml)	19.4	31.1	26.0	0.05[a]	21.4	24.9	23.8	0.56
IL17a (pg/ml)	90.2	104.0	124.3	0.42	88.7	99.4	124.9	0.36
CCL2 (MCP1) (pg/ml)	752.7	1179.0	1052.9	0.21	1020.1	605.1	468.1	0.12
CCL3 (MIP1a) (pg/ml)	20.2	18.0	15.7	0.85	15.6	37.7	11.9	0.41
CCL4 (MIP1b) (pg/ml)	825.1	676.4	787.5	0.78	746.7	1031.0	667.4	0.75
CCL5 (Rantes) (pg/ml)	122.4	221.1	107.1	0.36	127.9	255.5	50.9	0.55
CXCL10 (IP10) (pg/ml)	164,748.8	2,316,341.3	80,073.0	0.02[a]	813,639.9	72,861.6	312,573.7	0.28
CCL11 (Eotaxin) (pg/ml)	349.8	567.7	297.8	0.25	426.0	414.8	188.9	0.40
GMCSF (pg/ml)	95.4	68.6	89.4	0.15	84.7	84.3	116.2	0.38
bFGF (pg/ml)	71.9	79.7	73.9	0.64	77.4	61.1	74.9	0.41
VEGF (pg/ml)	7,117.9	11,619.7	5,046.1	0.56	10,638.1	1,843.0	2,943.0	0.06
PDGFbb (pg/ml)	153.8	145.1	82.8	0.76	178.0	90.0	69.9	0.22
GCSF (pg/ml)	105.5	91.5	110.2	0.73	87.8	107.1	171.9	0.06

[a]Statistical significance. CD, cluster of differentiation; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; GMCSF, granulocyte-macrophage colony-stimulating factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; PDGFbb, platelet derived growth factor-BB; GCSF, granulocyte colony stimulating factor; IL, interleukin; IL1Ra, interleukin-1 receptor antagonist; TNF, tumor necrosis factor; IFN, interferon.

that this expression was independent of clinicopathological factors. There seemed to be a partial correlation between immune checkpoint expression and their respective ligands. In addition, we observed approximately 70% of the EOC patients exhibited multiple immune checkpoint expression, and those patients had suppressive levels of inflammatory cytokines in their tumor microenvironment. These data suggest the potential application of combination therapy for immune checkpoint blockade in high-risk stage I/II EOC patients as well as advanced-stage EOC patients.

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