Flow cytometry detection of cell type-specific expression of programmed death receptor ligand-1 (PD-L1) in colorectal cancer specimens

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ABSTRACT

Aim: PD-1/PD-L1 blockade therapy is now widely used for the treatment of advanced malignancies. Although PD-L1 is known to be expressed by various host cells as well as tumor cells, the role of PD-L1 on non-malignant cells and its clinical significance is unknown. We evaluated cell type-specific expression of PD-L1 in colorectal cancer (CRC) specimens using multicolor flow cytometry.

Methods: Single cell suspensions were made from 21 surgically resected CRC specimens, and immunostained with various mAbs conjugated with different fluorescent dyes. Tumor cells, stromal cells, and immune cells were identified as CD326(+) , CD90(+) and CD45(+) phenotype, respectively. CD11b(+) myeloid cells, CD19(+) B cells and CD4(+) or CD8(+) T cells were also stained in different samples, and their frequencies in the total cell population and the ratio of PD-L1(+) cells to each phenotype were determined.

Results: PD-L1 was expressed by all the cell types. The ratio of PD-L1(+) cells to CD326(+) tumor cells was 19.1% / 14.0%, lower than those for CD90(+) stromal cells (39.6% / 16.0%) and CD11b(+) myeloid cells (31.9% / 14.3%). The ratio of PD-L1(+) cells in tumor cells correlated strongly with the ratio in stromal cells, while only weakly with that in myeloid cells. Tumor cells were divided into two populations by CD326 expression levels, and the PD-L1 positive ratios were inversely correlated with the rate of CD326 highly expressing cells as well as mean fluorescence intensity of CD326 in tumor cells, while positively correlated with the frequencies of stromal cells or myeloid cells in CRC.

Conclusion: PD-L1 is differentially expressed on various cell types in CRC. PD-L1 on tumor cells may be upregulated together with CD326 downregulation in the process of epithelial mesenchymal transition. Quantification of cell type-specific expression of PD-L1 using multicolor flow cytometry may provide useful information for the immunotherapy of solid tumors.

1. Introduction

The tumor microenvironment (TME) contains not only cancer cells but also numerous cell types which include inflammatory leukocytes such as tumor infiltrating lymphocytes and tumor associated macrophages or neutrophils and stromal cells [1, 2]. The interaction between tumor cells and the host cells in the TME is critical to promote immune evasion as well as invasion and metastasis of tumor cells [3, 4]. Recently, it has become clear that immune checkpoint molecules play a crucial role in immune escape of tumor cells [5, 6].

The PD-1/PD-L1 pathway is a representative immune checkpoint system. Accumulating evidence indicates that activated T cells in the TME express PD-1, whereas tumor cells express PD-L1 and escape from T cell-mediated killing [5]. Numerous studies have shown that antibody blockade of the PD-1/PD-L1 pathway can elicit remarkable antitumor responses in a broad spectrum of cancers [7, 8]. Despite remarkable anti-tumor effects in some patients, a substantial number of patients are unresponsive to anti-PD-1/PD-L1 immunotherapy, suggesting the need to elucidate biomarkers for factors to predict a therapeutic effect [9, 10].

Based on the mechanism of action, the expression of PD-L1 protein in tumor tissue has been extensively examined using immunohistochemistry
performed multicolor staining using cell specific mAbs, and objectively evaluated the expression at the single cell level by flow cytometry (FCM). (IHC) as a possible biomarker. Although several studies have demonstrated that PD-L1 expression in tumor cells correlates with response to anti-PD-1/PD-L1 immunotherapy [11, 12], the association is not absolute, partly due to differences in processing of samples, staining, semi-quantitative cut-offs as well as the subjectivity of observers [13]. More importantly, PD-L1 is constitutively expressed on immune cells such as antigen-presenting cells and lymphocytes as well as stromal cells [14]. The role of PD-L1 on non-malignant cells has not been fully determined. However, multiple clinical trials indicate that patients with PD-L1-negative tumors also respond to checkpoint blockade therapy, suggesting the potential contribution of PD-L1 on host immune cells to tumor immune escape [14, 15]. Recently, the combined positive score which is the ratio of the number of all PD-L1 expressing cells (tumor cells, lymphocytes, macrophages) to the number of tumor cells, is used as a robust and reproducible PD-L1 scoring method that predicts responses in patients with gastric cancer [16, 17].

Taken together, previous studies suggest that it is necessary to quantitatively evaluate the expression of PD-L1 in various cell types and examine the correlation between the expression pattern and prognosis or therapeutic effects of anti-PD-1/PD-L1 immunotherapy. In this study, we isolated single cells from primary colorectal cancer (CRC) specimens, performed multicolor staining using cell specific mAbs, and objectively evaluated PD-L1 expression at the single cell level by flow cytometry (FCM).

2. Materials and methods

2.1. Patients and samples

CRC specimens were obtained from 21 patients who underwent colectomy in the Department of Gastrointestinal Surgery, Jichi Medical University Hospital between July and December 2019. Immediately after resection, specimens were dissociated into single cells by a semi-automated combined mechanical/ enzymatic process using gentleMACS Dissociator and a Tumor Dissociation Kit, human (Miltenyi Biotec). In brief, tumor specimens were cut into pieces of 5–6 mm pieces and transferred into C Tubes containing a mix of Enzymes H, R, and A included in the Kit. Mechanical dissociation was accomplished by performing on the step for three times. To allow for enzymatic digestion, the C Tube was rotated continuously for 30 min at 37 °C, after the first and second mechanical dissociation step. After dissociation, cells were filtered with EASY strainer™ 100 μm (Greiner Bio-One) to obtain a single cell suspension, which was used for immunostaining. Histologic status was evaluated by pathologists. This study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Jichi Medical University (approval no. clinic 18-133). Written informed consent was obtained from all the patients.

2.2. MAbs

Fc-blocker and FVS780 were purchased from Becton-Dickinson (San Jose, CA) and DAPI from ThermoFisher Scientific (Waltham, MA USA). PE-conjugated mAb to PD-L1, control mouse IgG2a and control mouse IgG2b were purchased from BioLegend (San Diego, CA). APC-conjugated mAb to CD326 (EpCAM) was purchased from Miltenyi Biotec (Auburn, CA). FITC-conjugated mAb to CD45, APC-conjugated mAb to CD19 and BV605-conjugated mAb to PD-L1, control mouse IgG2a and control mouse IgG2b were purchased from BioLegend (San Diego, CA). FITC-conjugated mAb to CD4 was purchased from BD Pharmingen (San Jose, CA). FITC-conjugated mAb to CD90, BV605-conjugated mAb to PD-L1, APC-conjugated mAb to CD19 and BV605-conjugated mAb to PD-L1, APC-conjugated mAb to CD11b, APC-conjugated mAb to CD8, BV605-conjugated mAb and PE-conjugated mAb to CD4 were purchased from BioLegend (San Diego, CA).

2.3. Cell processing and flow cytometry (FCM)

After centrifugation of single cell suspensions at 1500 rpm for 5 min, hemolysis was performed using the RBC Lysis Buffer (BioLegend). After washing with PBS +0.02% EDTA for 3 times, cells (1 × 10⁶) were resuspended in 100 μl of PBS +0.02% EDTA and incubated for 15 min in FVS780 to label dead cells. After washing with PBS, cells were incubated with 10 μl of Fc-blocker for 10 min and immunostained with sets of mAbs to CD4, APC-conjugated mAb to CD326 (EpCAM) was purchased from Miltenyi Biotec (Auburn, CA). FITC-conjugated mAb to CD4 was purchased from BD Pharmingen (San Jose, CA). FITC-conjugated mAb to CD90, BV605-conjugated mAb to CD4, APC-conjugated mAb to CD19 and BV605-conjugated mAb to PD-L1, APC-conjugated mAb to CD11b, APC-conjugated mAb to CD8, BV605-conjugated mAb and PE-conjugated mAb to CD4 were purchased from BioLegend (San Diego, CA).

Table 1. Pathological characteristics of 21 patients with colorectal cancer (CRC).

| No | Gender | Age | Tumor site | Histological type | pH | pH | PH | LY | v | pStage |
|----|--------|-----|------------|-------------------|----|----|----|----|---|--------|
| 1  | Male   | 91  | Sigmoid    | tub1              | 3  | 0  | 0  | 0  | 1 | II     |
| 2  | Female | 89  | Ascending  | tub2              | 2  | 0  | 0  | 0  | 0 | I      |
| 3  | Female | 75  | Rectum     | tub2              | 4  | 1  | 0  | 1  | 1 | III    |
| 4  | Female | 50  | Sigmoid    | tub1              | 2  | 1  | 0  | 1  | 1 | III    |
| 5  | Female | 63  | Sigmoid    | tub2              | 3  | 0  | 0  | 1  | 1 | II     |
| 6  | Female | 46  | Rectum     | tub2              | 3  | 1  | 0  | 1  | 1 | III    |
| 7  | Male   | 69  | Sigmoid    | tub1              | 2  | 0  | 0  | 0  | 0 | I      |
| 8  | Male   | 61  | Sigmoid    | tub2              | 4  | 0  | 0  | 0  | 1 | II     |
| 9  | Female | 81  | Cecum      | tub1              | 1  | 0  | 0  | 0  | 0 | I      |
| 10 | Male   | 74  | Sigmoid    | tub2              | 2  | 0  | 0  | 0  | 0 | I      |
| 11 | Male   | 67  | Transverse | tub2              | 3  | 1  | 0  | 1  | 1 | II     |
| 12 | Female | 71  | Sigmoid    | tub2              | 3  | 1  | 1  | 1  | 1 | II     |
| 13 | Male   | 48  | Rectum     | tub1              | 4  | 2  | 1  | 1  | 1 | II     |
| 14 | Male   | 70  | Sigmoid    | por               | 3  | 1  | 0  | 1  | 1 | III    |
| 15 | Female | 75  | Ascending  | tub1              | 3  | 1  | 0  | 1  | 0 | II     |
| 16 | Female | 87  | Cecum      | tub2              | 4  | 1  | 0  | 1  | 0 | II     |
| 17 | Male   | 32  | Descending | por               | 4  | 0  | 1  | 1  | 1 | III    |
| 18 | Male   | 65  | Cecum      | tub2              | 4  | 2  | 1  | 1  | 1 | III    |
| 19 | Male   | 67  | Sigmoid    | tub1              | 3  | 0  | 0  | 1  | 1 | II     |
| 20 | Male   | 68  | Sigmoid    | tub2              | 3  | 0  | 0  | 1  | 1 | II     |
| 21 | Male   | 83  | Rectum     | tub2              | 3  | 0  | 0  | 1  | 1 | II     |
Figure 1. Gating strategy of tumor cells and host cells in colorectal cancer (CRC) specimens and quantification of PD-L1 expression on each cell type using flow cytometry (FCM). Single cell suspensions were obtained and immunostained with mAbs as described in Materials and Method. Initially, DAPI (-) non-cellular components were excluded using FlowJo™ software (Becton-Dickinson). After doublet cell exclusions, FVS780(+) dead cells were excluded from all dots. In live nucleated cell population, tumor cells, stromal cells, and immune cells were identified as CD45(-) CD326(+) CD90(-), CD45(-) CD326(-) CD90(+), and CD45(+) CD326(-) CD90(-) phenotypes, respectively. The frequencies of PD-L1(+) cells on each cell population was quantified based on the threshold determined with the staining intensity with an isotype control (mouse-IgG2b). In different series, cells were stained with mAbs to CD19 and CD11b, or mAbs to CD4 and CD8, and expression of PD-L1 was similarly evaluated for each cell type.
CD45(-) CD326(-) CD90(+) cells, respectively, while infiltrating leukocytes were identified as CD45 (+) CD326(-) CD90(-) cells. The expressions of PD-L1 on each cell population was quantified in comparison to the staining levels with their isotype controls using FlowJo ™ software (Becton-Dickinson, San-Jose, CA). In parallel, cells were stained with anti-PD-L1 together with the combination of mAbs to CD4 and CD8 or the combination of mAbs to CD19 and CD11b, and the expressions of PD-L1 on CD4(+), CD8(+), T cells, CD19(+) B cells, and CD11b(+) cells were examined separately.

2.4. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8. Statistical differences in cell frequencies were evaluated with one-way ANOVA and correlation was examined with Pearson’s correlation coefficient analysis. In all tests, the standard of significance was set at p < 0.05.

3. Results

3.1. PD-L1 expression in various cell types in CRC specimens

The profile of 21 patients with CRC whose specimens were analyzed by FCM is shown in Table 1. A gating strategy for stained cells from a typical specimen is shown in Figure 1. At first, DAPI-negative small extracellular components which were mostly located in a low FSC area were excluded. Next, FVS780-positive dead cells were excluded in a single cell population which was gated in the FSC-A and FSC-H plot. In DAPI(+) FVS780(−) gated area, CD45(+), leukocytes were identified as infiltrating immune cells, while tumor cells and stromal cells were identified as CD90(−) CD326(+), and CD90(−) CD326(+) CD45(−) cell population, respectively. Then, the ratios of PD-L1 (+) cells was determined in comparison with isotype control on CD326(+) tumor cells and CD90(−) stromal cells, separately. In parallel, infiltrating lymphocytes and myeloid cells were identified by immunostaining with mAbs to CD4, CD8, CD19 and CD11b and ratios of PD-L1 (+) cells were examined in each cell population.

Figure 2A (upper panel) shows the percentage of cell types in single cell suspensions isolated from CRC specimens. More than half of the cells (64.0 ± 15.8%) were tumor cells with CD45(+) CD326(+), CD90(+) phenotype, while 27.5 ± 15.8% were CD45(+), CD326(+) infiltrating leukocytes. CD45(−) CD326(+) CD90(+) stromal cells accounts for only a small population (1.8 ± 1.4%). As shown in Figure 2B (lower panel), PD-L1 was significantly expressed in all the cell types. The ratio of PD-L1(+) cells in CD326(+) tumor cells was 19.1% ± 14.0%. However, the ratios in CD90(+) stromal cells and CD11b(+) myeloid cells were 39.6% ± 16.0% and 31.9% ± 14.3%, respectively, both of which were significantly higher than that in tumor cells. As shown in Figure 2B and C, PD-L1 positive ratios among tumor cells correlated strongly with ratios in stromal cells (r = 0.89, p < 0.001), while only weakly with ratios in myeloid cells (r = 0.39, p > 0.05). PD-L1 was also expressed in CD19(−) B as well as CD4(+) or CD8(+) T cells at similar levels as tumor cells.

We examined the correlation between PD-L1 expression in each cell type and age, gender, tumor location and pathological T, N and M stages. However, none of them showed significant impact on PD-L1 expression in any specific cell types (Table 2).

3.2. PD-L1 expression in tumor cells is correlated with expression levels of CD326 and cellular composition of CRC

As shown in Figures 1 and 3A, CD326 (+) tumor cells were divided into two cell populations according to their expression levels of CD326, and the ratios of tumor cells with high and low CD326 expressing cells were variable among the specimens. Interestingly, many tumor cells with low CD326 expression expressed PD-L1, while only a few cells with high CD326 expression were positive for PD-L1 (Figure 3A). Accordingly, the rates of PD-L1 expression tended to be high in specimens with low CD326 expression, and the ratios of PD-L1 (+) cells inversely correled with the rates of CD326 high expressing cells (r = -0.501, p < 0.021) as well as mean fluorescein intensity of CD326 in tumor cells (r = -0.413 p < 0.063) (Figure 3B). C). On the contrary, the percentages of stromal cells among the total number of live cells derived from CRC specimens positively correled with PD-L1 positive ratios in CD326(+) tumor cells (r = 0.630, p < 0.01) (Figure 3D). The rates of PD-L1 (+) cells in tumor cells also tended to be high in CRC specimens with high percentages of CD45(+) infiltrating leukocytes with various phenotypes (data not shown) and the correlation...
Table 2: PD-L1 expression in various cell types and clinical and pathological features of CRC.

| Age     | Gender | Location | pT category | pN category | pM category |
|----------|--------|----------|-------------|-------------|-------------|
| 69 < 95  | Male   | Right    | N(-)        | N(-)        | N(-)        |
| 69 < 95  | Female | Left     | N(-)        | N(-)        | N(-)        |

*Discussion*

PD-L1, also referred to as B7-H1, CD274, is well known to be expressed on various malignant cells and creates a local immune suppressive microenvironment leading to the poor outcomes of the patients [18, 19]. However, PD-L1 is also widely expressed also on non-tumor cells such as immune cells and stromal cells [14]. Recent studies have suggested that PD-L1 on these host cells also participates in immune regulation of tumors [5, 15, 16, 17]. In this study, we isolated single cells from surgically resected CRC specimens to examine the PD-L1 expression separately on tumor cells, stromal cells and tumor infiltrating immune cells using FCM.

In previous studies, the same methodology has been used to examined the PD-L1 expression on tumor cells and immune cells isolated from non-small lung cancer specimens [20, 21]. They showed that the results of flow cytometric detection of PD-L1 were highly concordant with the results using IHC and proposed that the differential quantification of PD-L1 on tumor cells and immune cells may allow for better prediction of response to anti-PD-1/PD-L1 antibody therapy. Generally, FCM has been considered to be unsuitable for the analysis of cells from tumor specimens because extracellular particles and debris are contaminants released during the dissociation process to create a single cell suspension, which hampers the widespread application of this strategy. In this study, we additionally stained the samples with DAPI which can clearly distinguish real cells from other extracellular components using a UV laser. This enables the acquisition of a much larger number of real cells gated in the FCM profile and gives more accurate and reproducible values of antigen expressions on specific cell types. Indeed, numerous DAPI-negative micro-particle components with small FSC values were initially excluded at acquisition and more than 10⁷ live cells could be analyzed from 200–300 mg of tissues from the tumor specimens.

In this study, we stained isolated cells with mAbs to CD45, CD326, and CD90 which are usually used as specific markers for hematopoietic, epithelial and mesenchymal cells, respectively, and determined the CD326(-)CD90(-) and CD326(-)CD90(+) cells as tumor and stromal cells in the CD45(-) cell population, respectively. CD45(+) immune cells were further classified using mAbs to CD11b, CD19 as well as CD4 and CD8, and PD-L1 expression was evaluated for each cell type. Using the gating strategy, we confirmed that PD-L1 was significantly expressed in all cell types in CRC specimens and the percentage of PD-L1(+) cells in CD90(-) stromal cells and CD11b(-) myeloid cells was significantly higher than that in CD326(-) tumor cells. In addition, PD-L1 positive ratio in tumor cells showed a strong correlation with that in CD90(+) stromal cells. CD326(-) CD90(+) cells in tumor tissues consist mainly of fibroblasts. Recent studies have demonstrated that PD-L1 is significantly expressed in some types of fibroblasts including cancer associated fibroblasts (CAF) and their expression levels are upregulated by IFN-γ, IL-1α, as well as TGF-β [22, 23, 24]. Since PD-L1 expression on tumor cells is strongly upregulated by inflammatory cytokines secreted by activated immune cells such as IFN-γ or IL-1α [25, 26], our data suggest that the expression level of PD-L1 in stromal fibroblasts is similarly regulated by those inflammatory cytokines resident in CRC tissue. In comparison, the correlation with that in CD11b(+) myeloid cells was not significant, suggesting that PD-L1 expression in leukocytes may be regulated partly by different mechanisms from tumor and stromal cells.

CD326 (EpCAM) is known to be widely expressed in a variety of human cancers and often used as a marker for circulating tumor cells in peripheral blood [27, 28]. In this study, we found that CD326(+) tumor cells in CRC are divided into two populations according to the expression levels of CD326 and the ratios of high and low CD326 expressing cells varied among the CRC specimens. Although CD326 is diffusely expressed on most tumor cells in CRC, it is known to be completely lost in Lynch syndrome-associated CRC [29]. Previous studies have shown that

with ratios of CD11b(+) myeloid cells was statistically significant (r = 0.498, p < 0.023) (Figure 3E).
CD326 expression is generally reduced in tumor cells at the invasive front of CRC tumors [30, 31, 32] and transient downregulation of CD326 is considered to be related to epithelial-mesenchymal transition (EMT) which is favorable for cell motility and metastasis [33]. From the results of this study, we suggest that CD326 high and low expressing cells in isolated cell populations are tumor cells with epitelial and mesenchymal phenotypes, respectively, and the ratios of two phenotypes may be related to the malignant potential of CRC.

In this series of 21 tumor specimens, no significant association was observed between the ratio of PD-L1(+) cells in tumor cells and the pT, pN and pM stages of CRC partly because of the small sample size. However, we found that the expression levels of PD-L1 and CD326 in CRC tumor cells showed inverse association, while PD-L1 expression positively correlated with the frequencies of CD90(+) stromal cells and CD11b(+) myeloid cells in CRC specimens. PD-L1 expression on tumor cells and antigen-presenting cells (APCs) is strongly upregulated by cytokines secreted by activated immune cells, such as IFN-γ or IL-1α [25, 26]. Several lines of evidence have indicated that stromal cells and immune infiltrates can efficiently promote the EMT process in a paracrine manner [34, 35, 36]. This suggests that those non-malignant cells in the TME may contribute to the induction of PD-L1 expression together with EMT on tumor cells. In fact, previous studies have shown that EMT is associated with upregulation of different immune checkpoint molecules, including PD-L1 [37, 38, 39]. The results of the present study are consistent with previous results and suggest that EMT promotes cancer progression partly through the immune escape with upregulation of PD-L1.

In summary, we present a novel FCM-based technology to evaluate PD-L1 expression on distinct cell types isolated from CRC specimens. In previous studies, evaluation of PD-L1 expression was mostly performed by IHC. However, many technical and biological issues are pointed out for the quantification of PD-L1 expression in IHC [12, 13]. In the FCM method used in this study, however, the cell separation is semi-automated, and the staining process is simple and rapid as compared with IHC. Moreover, evaluation is fully quantitative, less subjective and cell-type specific analysis is possible. Although further analysis with a larger sample size is necessary, unequivocal quantification of cell-type specific PD-L1 expression in tumor specimens using multicolor FCM analysis may provide useful information for cancer immunotherapy in the future.

Declarations

Author contribution statement

Joji Kitayama: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Akira Saito: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Mineyuki Tojo, Yuko Kumagai: Performed the experiments.
Hideyuki Ohzawa: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Hironori Yamaguchi: Performed the experiments.
Hideyo Miyato: Conceived and designed the experiments; Analyzed and interpreted the data.
Al Sadatomo, Daishi Naoi, Gaku Ota, Koji Koinuma, Hisanaga Horie: Contributed reagents, materials, analysis tools or data.
Alan Kawari Lefor, Naohiro Sata: Wrote the paper.

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Data availability statement
Data will be made available on request.

Declaration of interests statement
The authors declare no conflict of interest.

Additional information
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