Monocyte subsets associated with the efficacy of anti-PD-1 antibody monotherapy

RYOTARO OHKUMA[1,2], YUKI FUJIMOTO[1-3], KATSUAKI IEGUCHI[2,3], NOBUYUKI ONISHI[2,3], MAKOTO WATANABE[2-5], DAISUKE TAKAYANAGI[1-3], TSUBASA GOSHIMA[1-3], ATSUSHI HORIIKE[1], KAZUYUKI HAMADA[1], HIROTSUGU ARIIZUMI[1], YUYA HIRASAWA[1], TOMOYUKI ISHIGURO[1], RISAKO SUZUKI[1], NANA IRIGUCHI[1], TOSHIAKI TSURUI[1], YOSUKE SASAKI[6], MAYUMI HOMMA[6], TOSHIKO YAMOCHI[6], KIYOSHI YOSHIMURA[1,3,7], MAYUMI TSUJI[4,5], YUJI KIUCHI[4,5], SHINICHI KOBAYASHI[3], TAKUYA TSUNODA[1] and SATOSHI WADA[1-3]

[1]Division of Medical Oncology, Department of Medicine, School of Medicine, Showa University, Tokyo 142-8555; [2]Department of Clinical Diagnostic Oncology, Clinical Research Institute for Clinical Pharmacology and Therapeutics, Showa University; [3]Clinical Research Institute for Clinical Pharmacology and Therapeutics, Showa University, Tokyo 157-8577; [4]Division of Medical Pharmacology, Department of Pharmacology, School of Medicine; [5]Pharmacological Research Center, Showa University, Tokyo 142-8555; [6]Department of Pathology, Showa University School of Medicine; [7]Department of Clinical Immuno-oncology, Clinical Research Institute for Clinical Pharmacology and Therapeutics, Showa University, Tokyo 157-8577, Japan

Received February 20, 2023; Accepted June 26, 2023

DOI: 10.3892/ol.2023.13967

Abstract. Immune checkpoint inhibitors (ICIs) are among the most notable advances in cancer immunotherapy; however, reliable biomarkers for the efficacy of ICIs are yet to be reported. Programmed death (PD)-ligand 1 (L1)-expressing $CD14^+$ monocytes are associated with shorter overall survival (OS) time in patients with cancer treated with anti-PD-1 antibodies. The present study focused on the classification of monocytes into three subsets: Classical, intermediate and non-classical. A total of 44 patients with different types of cancer treated with anti-PD-1 monotherapy (pembrolizumab or nivolumab) were enrolled in the present study. The percentage of each monocyte subset was investigated, and the percentage of cells expressing PD-L1 or PD-1 within each of the three subsets was further analyzed. Higher pretreatment classical monocyte percentages were correlated with shorter OS (r=-0.32; P=0.032), whereas higher non-classical monocyte percentages were correlated with a favorable OS (r=0.39; P=0.0083). PD-L1-expressing classical monocytes accounted for a higher percentage of the total monocytes than non-classical monocytes with PD-L1 expression. In patients with non-small cell lung cancer (NSCLC), a higher percentage of PD-L1-expressing classical monocytes was correlated with shorter OS (r=-0.60; P=0.012), which is similar to the observation for the whole patient cohort. Comparatively, higher percentages of non-classical monocytes expressing PD-L1 were significantly associated with better OS, especially in patients with NSCLC (r=0.60; P=0.010). Moreover, a higher percentage of non-classical monocytes contributed to prolonged progression-free survival in patients with NSCLC (r=0.50; P=0.042), with similar results for PD-L1-expressing non-classical monocytes. The results suggested that the percentage of monocyte subsets in patients with cancer before anti-PD-1 monotherapy may predict the treatment efficacy and prognosis. Furthermore, more classical monocytes and fewer non-classical monocytes, especially those expressing PD-L1, are involved in shortening OS time, which may indicate the poor efficiency of anti-PD-1 treatment approaches.

Correspondence to: Professor Satoshi Wada, Department of Clinical Diagnostic Oncology, Clinical Research Institute for Clinical Pharmacology and Therapeutics, Showa University, 6-11-11 Kitakarasuyama, Setagaya, Tokyo 157-8577, Japan
E-mail: st-wada@med.showa-u.ac.jp

Key words: cancer immunotherapy, immune checkpoint inhibitors, anti-programmed death-1 antibody, programmed death-ligand 1, monocyte subsets, classical monocytes, non-classical monocytes, biomarker

Introduction

Programmed death protein-1 (PD-1) and PD-ligand 1 (PD-L1) are the central factors of immune checkpoints that intervene in immune escape. These immune checkpoints are actuated by ligand and receptor binding, blocking their signaling pathways (1). Previous studies have reported that the PD-1/PD-L1 pathway has a role in resistance to antitumor immunity in numerous types of cancer (1,2). Immune checkpoint inhibitors (ICIs) are among the biggest breakthroughs in immunotherapy, showing notable efficacy against various cancer

types by suppressing immune checkpoint-mediated immune escape (3,4).

PD-L1 expression in tumor cells suppresses activation of tumor-infiltrating lymphocytes and promotes tumor progression (5,6). This suggests that the level of PD-L1 expression may have a greater than expected effect on tumor dynamics. Moreover, cumulative evidence has indicated that not only tumor cells with PD-L1 expression, as quantified by the tumor proportion score (TPS), but also PD-L1-expressing immune cells, as quantified by the combined positive score, play a crucial role in predicting the response to ICIs (4,7,8). An association has previously been reported between the therapeutic efficiency of anti-PD-1 antibodies and PD-L1 expression in both tumor and tumor-infiltrating immune cells in lung cancer (8), breast cancer (9) and malignant melanoma (10). Therefore, the expression levels of PD-L1 molecules in either tumor cells or tumor-infiltrating immune cells may serve as biomarkers to predict the therapeutic response to ICIs. However, there are no ideal biomarkers for treatment prediction (11), and PD-L1 expression, detected using immunohistochemistry (IHC), has several limitations as a predictive biomarker. Tumor tissue collection can be difficult in some patients; it is invasive and it cannot be frequently monitored. Moreover, PD-L1 expression in either tumor or immune cells is determined using immunostaining and through visual inspection of tumor tissue by pathologists, which limits objectivity in determining PD-L1 expression levels. In addition, different IHC assays for PD-L1 have been developed for each ICI drug, and different antibodies have been used to predict efficacy, thus raising the question of how different these IHC assays are, whether they need to be performed separately, and whether harmonization is possible (12). To validate this issue, an international comparative study was conducted: The Blueprint study sought to differentiate between the following four antibodies: 28-8, 22C3, SP142 and SP263 (13). The three antibodies 28-8, 22C3 and SP263 showed similar positivity rates, whereas SP142 showed a lower positivity rate for PD-L1. Although the three aforementioned assays showed similar analytical performance for PD-L1 expression, switching assays and cut-off values could lead to misclassification of PD-L1 status. In addition, the cut-off values used in the aforementioned clinical trials varied from 1 to 50% (13), creating bias in defining positive PD-L1 expression. Furthermore, tissue-to-tissue and temporal heterogeneity of PD-L1 expression limits accurate and precise assessment, and there are a number of challenges associated with assessing PD-L1 expression by IHC (12).

To overcome the aforementioned limitations, we have focused on the immune checkpoint expression in circulating peripheral blood. We have previously measured soluble forms of PD-L1 (sPD-L1) and PD-1 (sPD-1) in the peripheral blood of patients with non-small cell lung cancer (NSCLC), gastric cancer and bladder cancer before treatment, as well as their relationship with treatment response and prognosis in patients treated with PD-1 blockade monotherapy. The results indicated that the increase in sPD-1 and sPD-L1 levels was associated with tumor size in patients with various types of cancer who received anti-PD-1 antibody monotherapy (14,15). Therefore, sPD-L1 and sPD-1 may be used as predictive and prognostic biomarkers to identify primary responders to anti-PD-1 antibody treatment. Furthermore, we investigated the clinical significance of pretreatment PD-L1 expression levels in peripheral blood mononuclear cell (PBMC) subsets such as $CD3^+$, $CD4^+$, $CD8^+$ and $CD14^+$ in patients with cancer treated with anti-PD-1 antibody monotherapy, as in a previous study (16). In the study, it was found that increased $CD14^+$ monocytes, which express PD-L1, were significantly correlated with shorter overall survival (OS) time, suggesting that PD-L1-expressing monocytes may contribute to poor prognosis for patients with cancer treated with anti-PD-1 antibody (16).

Monocytes are classified into three subsets according to the expression levels of CD14 and CD16: Classical (CD14 high and $CD16^-$), intermediate (CD14 high and $CD16^+$), and non-classical (CD14 low and $CD16^+$) (17,18). Monocytes play key roles in tumor metastasis, invasion, angiogenesis and immune regulation (19), and are thus associated with tumor progression. Nevertheless, the role of each monocyte subset in cancer prognosis remains unclear. Therefore, the present study focused on the three subsets of monocytes, with PD-L1 and PD-1 expression analyzed in each subset, and their clinical significance in patients treated with anti-PD-1 antibody monotherapy was investigated. The current study assessed the immune response in patients treated with anti-PD-1 antibody, to identify biomarkers for the prediction of patient survival. The cell population and immune-associated phenotypic markers in peripheral blood samples collected from patients with various types of cancer before anti-PD-1 antibody monotherapy were investigated.

Materials and methods

Patient population and treatment schedule. The current study included patients with unresectable or metastatic NSCLC, gastric cancer and esophageal cancer treated with anti-PD-1 antibody monotherapy at the Division of Medical Oncology, Department of Medicine, School of Medicine, Showa University (Tokyo, Japan). All patients were treated between January 2017 and January 2021 with an anti-PD-1 antibody; either 200 mg pembrolizumab given intravenously every 3 weeks or 240 mg nivolumab given intravenously every 2 weeks. Regimens were administered according to the clinical routine and preference of the physician. A schematic diagram of the schedule is shown in Fig. S1.

Assessment of treatment response. Radiologists and physicians performed imaging assessments using computed tomography. The responses to anti-PD-1 antibody were evaluated according to the Response Evaluation Criteria in Solid Tumors version 1.1 (20). OS was defined as the time from the start of anti-PD-1 antibody treatment to either patient death from any cause or last follow-up. Progression-free survival (PFS) was defined as the time from the start of anti-PD-1 antibody treatment to the first documented progressive disease, death from any cause or the last follow-up, whichever occurred first. The follow-up cutoff was August 2021.

Peripheral blood samples and PBMC stock preparation. Peripheral blood samples were obtained from each patient before the first administration of pembrolizumab or nivolumab, and stored in BD Vacutainer CPT cell preparation tubes containing sodium heparin (Becton, Dickinson and Company).

The supernatant was separated by centrifugation at 1,600 x g for 20 min at 20˚C, and the pellet was first resuspended and then washed in PBS. The separated PBMCs were stored in BAMBANKER® (Lymphotec Inc.) at -80˚C in liquid nitrogen.

Flow cytometry. PBMCs were stained immediately as previously described (14). For individual evaluation, a total of $1x10^7$ PBMCs were resuspended in PBS containing 2% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), incubated in human BD FC Block (cat. no. 564220; Becton, Dickinson and Company) at 25˚C for 10 min, stained with 7-AAD (cat. no. 559925; Becton, Dickinson and Company) at 4˚C for 30 min to remove dead cells, PD-L1 phycoerythrin (PE)-labeled antibody (cat. no. 557924; PE mouse anti-human CD274) and PD-1 Brilliant Violet 480 (BV480)-labeled antibody (cat. no. 566112; BV480 mouse anti-human CD279), and stored on ice for 30 min with anti-CD14 Brilliant Violet 650 (BV650)-labeled antibody (cat. no. 563419; mouse anti-human CD14) and anti-CD16 FITC-conjugated antibody (cat. no. 555406; mouse anti-human CD16), which centered on staining $CD14^+$ and $CD16^+$ monocytes (all antibodies were used as provided by Becton, Dickinson and Company). Thereafter, the cell suspension was washed twice in PBS containing 2% FBS, and the absorbance was detected at each wavelength [FITC, 515-545 nm; PE (Blue Laser), 562-588 nm; 7-AAD, 685-735 nm; BV650, 655-685 nm; BV480, 425-475 nm; PE-Cy7, 750-810 nm]. Flow cytometry was performed using a BD LSRFortessa™ Cell Analyzer (Becton, Dickinson and Company). The negative threshold strategies for gating were set according to single-stained samples and isotype controls. The data were analyzed using FlowJo (version 10.5.3; Becton, Dickinson, and Company). The levels of CD14 were expressed using '++' as a higher positive than '+'.

IHC evaluation of PD-L1 expression on tumor cells. Briefly, formalin-fixed paraffin-embedded tissue samples with a thickness of 5 μm were obtained from the biopsy specimens of the patients. The specimens were fixed in 10% neutral buffered formalin for 12-72 h. For companion diagnostics, the PD-L1 IHC 28-8 PharmaDX kit (cat. no. SK005; Dako; Agilent Technologies, Inc.) for nivolumab and PD-L1 IHC 22C3 PharmaDX kit (cat. no. SK006; Dako; Agilent Technologies, Inc.) for pembrolizumab were used, according to the manufacturer's instructions on the appropriate automated staining devices [Dako Link AS-48 (Dako; Agilent Technologies, Inc.)]. PD-L1 expression was assessed quantitatively as the TPS with reference to previously conducted clinical trials (21,22).

IHC evaluation of CD68-stained area in tumor tissue. To evaluate the infiltration of macrophages into the tumor tissue, the tissue specimens were immunostained for CD68 using the IHC-DAB method for the 31 patients whose tissue specimens were available for analysis. Immunohistological analysis of CD68 expression in cancer tissues was performed using an automated immunostainer (Bond III; Leica Microsystems, Ltd.) according to the manufacturer's protocol. Briefly, 3-μm formalin-fixed paraffin-embedded tissue sections underwent heat-mediated antigen retrieval using Bond epitope retrieval solution 1 (Leica Microsystems, Ltd.) at 98˚C for 10 min. The sections were incubated with an anti-CD68 antibody (clone E3O7V; 1:500 dilution; cat. no. 97778; Cell Signaling Technology, Inc.) for 15 min at room temperature, and the signal was detected using a horseradish peroxidase-conjugated compact polymer system (BOND Polymer Refine Detection kit; cat. no. DS9800; Leica Microsystems, Ltd.) and DAB as the chromogen. The slides were incubated with Post Primary reagent (adjusted by manufacturer) at room temperature for 8 min, following which, the slides were incubated with Polymer reagent (adjusted by manufacturer) as a secondary antibody at room temperature for an additional 8 min. Visualization with DAB was performed at room temperature for 10 min. The sections were counterstained with hematoxylin and viewed under a bright-field fluorescence microscope. Pathological reviewing of the CD68-stained tissue specimens was conducted by two independent pathologists. The BZ-X800 microscope (Keyence Corporation) was used to semi-quantify $CD68^+$ cells. Tumor tissues were observed with 4x magnification and three representative fields of view were selected. The mean value of the three areas occupied by CD68-stained cells was calculated using the hybrid cell count function of the all-in-one fluorescence microscope (BZ-X800; Keyence Corporation), which can objectively quantify the stained area in the immunostained sections (23).

Statistical analysis. Pearson correlation coefficients were calculated to analyze the association among the variables. Patient survival duration was assessed using the Kaplan-Meier analysis and compared using the log-rank test. A cut-off value was defined using the receiver operating characteristic (ROC) curve for the Kaplan-Meier analysis. The comparison of values between the two groups was conducted using the Mann-Whitney U test. JMP® Pro (version 15.0; SAS Institute, Inc.) and GraphPad Prism (version 9.4.1; Dotmatics) were used for analyses. All tests performed on comparisons between the two groups were two-sided. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The present study included 44 patients with either gastric cancer, NSCLC or esophageal cancer, and their characteristics are summarized in Tables I and II. The histopathological types identified were adenocarcinoma for gastric cancer and squamous cell carcinoma for esophageal cancer. Regarding NSCLC, 12 patients had adenocarcinoma, 4 had squamous cell carcinoma and 1 had not otherwise specified lung cancer. A total of 2/17 patients with NSCLC were positive for an EGFR mutation (Table SI). The Mann-Whitney U test was performed to compare gene mutation status and monocyte percentages. No association between gene mutation status and monocyte percentages was observed (Fig. S2).

Flow cytometric analysis of each subset of peripheral monocytes and their respective percentages. Monocytes in human peripheral blood are heterogeneous and categorized into three subsets based on their CD14 and CD16 expression levels. In the present study, monocytes were gated according to their size and granularity in forward and side scatter plots using flow cytometry (Fig. 1). Classical monocytes expressed high CD14 but no CD16 ($CD14^{++}$ $CD16^-$), intermediate

Table I. Clinicopathological features of all patients (n=44).

Case no.	Sex	Age, years	Cancer type	Stage	Performance status	ICI regimen	ICI administration cycles	Number of prior systemic therapy regimens	Radiation therapy history	Progression-free survival, months	Overall survival, months
1	M	78	NSCLC	IV	1	Nivolumab	98	1	-	48.4	53.4
2	M	64	NSCLC	IV	1	Nivolumab	3	3	+	1	4.5
3	M	67	NSCLC	IV	0	Nivolumab	28	1	+	14.6	30.3
4	M	67	NSCLC	IV	1	Nivolumab	1	1	+	4.5	8.2
5	M	84	NSCLC	IV	0	Nivolumab	17	1	+	9	32.3
6	F	73	NSCLC	IV	1	Nivolumab	14	1	-	8.7	23.0
7	F	81	NSCLC	IV	1	Nivolumab	9	1	-	4.1	20.2
8	M	72	NSCLC	IV	1	Pembrolizumab	7	0	+	4.9	7.2
9	F	76	NSCLC	IV	2	Pembrolizumab	1	1	+	1	1.0
10	M	71	NSCLC	IV	1	Pembrolizumab	16	0	+	29	34.1
11	M	59	NSCLC	IV	1	Pembrolizumab	1	0	-	1	1.5
12	M	64	NSCLC	IV	3	Pembrolizumab	9	0	+	7.2	21.0
13	M	70	NSCLC	IV	1	Pembrolizumab	2	0	+	1.6	1.6
14	M	71	NSCLC	IV	2	Pembrolizumab	4	0	+	2.5	4.4
15	M	68	NSCLC	IV	1	Pembrolizumab	37	1	+	36.3	36.3
16	M	65	NSCLC	IV	1	Pembrolizumab	3	0	-	21.5	24.0
17	M	57	NSCLC	IV	1	Pembrolizumab	24	0	-	22.9	22.9
18	M	63	GC	IV	2	Nivolumab	10	4	-	3.4	4.2
19	M	74	GC	IV	1	Nivolumab	5	3	-	2.4	4.8
20	F	68	GC	IV	1	Nivolumab	5	2	-	2.3	2.3
21	M	60	GC	IV	2	Nivolumab	2	2	+	1.4	1.4
22	F	49	GC	IV	1	Nivolumab	1	2	-	2.4	2.4
23	F	75	GC	IV	1	Nivolumab	8	2	-	5.6	9.4
24	F	57	GC	IV	2	Nivolumab	2	2	-	1.1	1.5
25	M	62	GC	IV	2	Nivolumab	3	2	-	2.1	2.1
26	M	67	GC	IV	1	Nivolumab	4	2	-	2.3	4.2
27	M	77	GC	IV	1	Nivolumab	2	2	-	0.9	1.4
28	M	72	GC	IV	2	Nivolumab	4	2	-	1.6	2.7
29	M	73	GC	IV	1	Nivolumab	4	1	+	2.5	7.4
30	F	71	GC	IV	1	Nivolumab	5	2	-	2.5	4.9
31	M	60	GC	IV	2	Nivolumab	3	2	-	2.1	2.8
32	M	75	GC	IV	1	Nivolumab	31	2	-	13.8	13.8
33	M	82	GC	IV	1	Nivolumab	9	1	-	4	6.1
34	M	58	GC	IV	2	Pembrolizumab	2	1	+	1.3	7.8

Table I. Continued.

Case no.	Sex	Age, years	Cancer type	Stage	Performance status	ICI regimen	ICI administration cycles	Number of prior systemic therapy regimens	Radiation therapy history	Progression-free survival, months	Overall survival, months
35	F	73	GC	IV	2	Nivolumab	14	2	-	7.3	7.9
36	M	71	GC	IV	1	Nivolumab	4	2	-	4.7	6.7
37	M	75	GC	IV	1	Nivolumab	3	1	-	1.9	1.9
38	M	73	EC	IV	2	Nivolumab	4	1	-	2.3	4.2
39	M	64	EC	IV	1	Nivolumab	6	0	+	4.7	10.2
40	F	67	EC	IV	2	Nivolumab	4	1	+	2.1	3.2
41	M	74	EC	IV	1	Nivolumab	4	1	-	1.8	2.2
42	M	74	EC	IV	1	Nivolumab	4	0	-	1.4	9.0
43	M	66	EC	IV	1	Nivolumab	3	1	+	1.3	9.1
44	M	72	EC	IV	1	Nivolumab	24	0	-	10.3	10.3

M, male; F, female; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung carcinoma; GC, gastric cancer; EC, esophageal cancer.

monocytes expressed CD16 and high CD14 ($CD14^{++}$ $CD16^{+}$), and non-classical monocytes expressed CD16 but lower CD14 than intermediate monocytes ($CD14^{+}$ $CD16^{+}$; Fig. 1). The percentage of monocytes in each of the three subsets is shown in Table SII. The percentages of classical, intermediate and non-classical monocytes were 73.5, 20.3 and 6.1%, respectively (Fig. 2A), which is similar to previously reported results (18).

Correlation between each monocyte subset and survival outcomes in patients with cancer receiving anti-PD-1 antibody monotherapy. In the whole patient cohort (n=44), higher percentages of non-classical monocytes were statistically correlated with longer OS (r=0.39; P=0.0083; Fig. 3A). By contrast, a negative correlation was simultaneously obtained for classical monocytes; higher percentages of classical monocytes were significantly correlated with shorter OS (r=-0.32; P=0.032; Fig. 3C). Additional analyses were performed separately for each type of cancer. In patients with gastric cancer (n=20) and esophageal cancer (n=7), no correlation was shown between each monocyte subset and OS (Fig. 3D-F and J-L). Focusing on patients with NSCLC (n=17), results demonstrated the same trend in the monocyte populations as for the whole patient cohort. However, a stronger correlation was observed between classical monocytes of patients with NSCLC and shorter OS (r=-0.53; P=0.028; Fig. 3I), whereas a higher percentage of non-classical monocytes was significantly related to longer OS (r=0.57; P=0.017; Fig. 3G). There was no correlation between the intermediate monocyte subset and OS (Fig. 3B, E, H and K). Statistical analysis was performed using the same strategy for PFS. There were positive correlations between non-classical monocyte subsets and PFS in the whole patient cohort and patients with NSCLC (r=0.36; P=0.015; Fig. 4A, and r=0.50; P=0.042; Fig. 4G, respectively). No statistical correlation was observed between the other monocyte subsets and PFS for any of the cancer types investigated (Fig. 4B-F and H-L).

Flow cytometric analysis of each subset of peripheral monocytes expressing the immune checkpoints (PD-L1 and PD-1) and their respective percentages. The expression of PD-L1 and PD-1 in each monocyte subset was analyzed by flow cytometry. The percentage of PD-L1-expressing cells in each monocyte subset was investigated (Table SIII). In terms of the percentage of monocytes that expressed PD-L1, classical monocytes were the most common (26.6% of all monocytes), followed by intermediate (10.0% of all monocytes) and non-classical monocytes (2.1% of all monocytes; Fig. 2B). For those monocytes expressing PD-L1, the classical monocyte subset expressing PD-L1 accounted for a larger percentage compared with the others. The percentage of $PD\text{-}L1^{+}$ intermediate or non-classical monocytes was lower than that of $PD\text{-}L1^{+}$ classical monocytes. Similarly, the percentage of monocytes expressing PD-1 within each monocyte subset is listed in Table SIV. A total of <2% of the sum of the three monocyte subsets expressed PD-1 (Fig. 2C), indicating that the percentage of monocytes expressing PD-1 was lower than that of the monocytes expressing PD-L1.

Correlation between PD-L1-expressing monocytes and survival outcomes in patients with cancer receiving anti-PD-1 antibody monotherapy. Within the whole

Table II. Summary of the clinicopathological features of all patients (n=44).

Clinicopathological characteristics	Total
Mean age ± SD, years	69.1±7.22
Sex, n	
Male	34
Female	10
Cancer type, n	
Non-small cell lung cancer	17
Gastric cancer	20
Esophageal cancer	7
Performance status, n	
0	2
1	29
2	12
3	1
Type of ICIs, n	
Nivolumab	33
Pembrolizumab	11
Median no. of ICI administration cycles (minimum-maximum)	10 (1-98)
Median progression-free survival time, months (minimum-maximum)	3.0 (0.9-51.0)
Median overall survival time, months (minimum-maximum)	6.4 (1.4-53.4)

ICI, immune checkpoint inhibitor.

patient cohort (n=44) and the NSCLC cohort (n=17), positive correlations were observed between PD-L1-expressing non-classical monocytes and OS (r=0.47; P=0.0012; Fig. 5A, and r=0.60; P=0.010; Fig. 5G, respectively). By contrast, higher percentages of PD-L1-expressing classical monocytes in the NSCLC cohort were statistically correlated with shorter OS (r=-0.60; P=0.012; Fig. 5I). No significant correlation was observed between each subset of PD-L1-expressing monocytes and OS in the gastric cancer group (n=20; Fig. 5D-F) and esophageal cancer group (n=7; Fig. 5J-L). No correlation was observed between the PD-L1$^+$ intermediate monocyte subset and OS (Fig. 5B, E, H and K). Moreover, statistical analyses were performed using the same strategy for PFS, and similar trends were obtained as in the relationship between PD-L1-expressing monocytes and OS. Within the whole patient cohort (n=44), and particularly patients with NSCLC (n=17), a positive correlation was observed between PD-L1-expressing non-classical monocytes and PFS (r=0.48; P=0.0011; Fig. 6A, and r=0.58; P=0.014; Fig. 6G, respectively). No significant correlation between PD-L1-expressing intermediate and classical monocyte subsets and PFS was identified (Fig. 6B, C, E, F, H, I, K and L). Regarding PD-L1-expressing classical monocytes, higher PD-L1-expressing classical monocytes tended to be correlated with shorter PFS times only in patients with NSCLC (r=-0.48; P=0.051; Fig. 6I).

Correlation between PD-1-expressing monocytes and survival outcomes in patients with cancer receiving anti-PD-1 antibody monotherapy. In the whole patient cohort (n=44), no correlation was observed between any of the subsets of PD-1-expressing monocytes and OS (Fig. S3A-C). For the NSCLC cohort (n=17), both PD-1-expressing intermediate and classical monocytes showed significant results; higher percentages of PD-1-expressing monocytes were correlated with a shorter OS (r=-0.53; P=0.029; Fig. S3H, and r=-0.41; P=0.022; Fig. S3I, respectively). No statistically significant correlations were found for the cohort of patients with either gastric or esophageal cancer (Fig. S3D-F and J-L) or for the PD-1$^+$ classical monocytes of the NSCLC cohort (Fig. S3G). Similar analyses were also performed for PFS. There were no correlations between any of the subsets of PD-1-expressing monocytes and PFS (Fig. S4A-L).

Survival time analysis using the Kaplan-Meier method. Survival analyses using the Kaplan-Meier method were additionally performed to determine the association between the percentage of each monocyte subset and OS. A cut-off value was defined using the ROC curve, and patients were divided into two groups, namely 'high' and 'low', for each subset of monocytes (Fig. S5A-I). The results of the survival time analysis showed that high percentages of classical monocytes were associated with a shorter OS in the whole patient cohort and in the cohort of patients with NSCLC (P=0.0015; Fig. S6C, and P=0.037; Fig. S6I, respectively), but no significant results were obtained for patients with either gastric or esophageal cancer (Fig. S6F and L). In addition, no significant findings were observed for either the non-classical or intermediate monocytes and OS (Fig. S6A, B, D, E, G, H, J and K). Survival analysis was performed using the same methods for PD-L1- and PD-1-expressing monocytes. Similarly, for the whole patient cohort and patients with NSCLC, high percentages of classical monocytes expressing PD-L1 were associated with shorter OS compared with the low group (P=0.046; Fig. S7C, and P=0.0073; Fig. S7I), but no statistically significant difference was obtained for the PD-L1 expressing non-classical and intermediate monocytes (Fig. S7A, B, D, E, G, H, J and K). In addition, no significant difference in OS was observed among the other cancer types compared with that of NSCLC (Fig. S7F and L). Regarding PD-1-expressing monocytes, high percentages of classical monocytes expressing PD-1 were associated with shorter OS compared with the low group for patients with NSCLC (P=0.027; Fig. S8I). There were no other statistically significant differences between the percentages of PD-1-expressing monocytes and OS (Fig. S8A-H and J-L).

Correlation between PD-L1 expression on tumor cells and circulating monocytes. The present study aimed to determine whether there was a significant association between PD-L1 expression on tumor cells and the percentage of each PD-L1-expressing monocyte subset in the tissues of 22 patients with either gastric cancer (n=6) or NSCLC (n=16), in which PD-L1 expression of tumor cells was investigated in routine clinical settings. PD-L1 expression was investigated on tumor cell membranes using IHC, in addition to detecting the percentages of PD-L1-expressing monocyte subsets by flow cytometry (Table SIII). A representative image of PD-L1

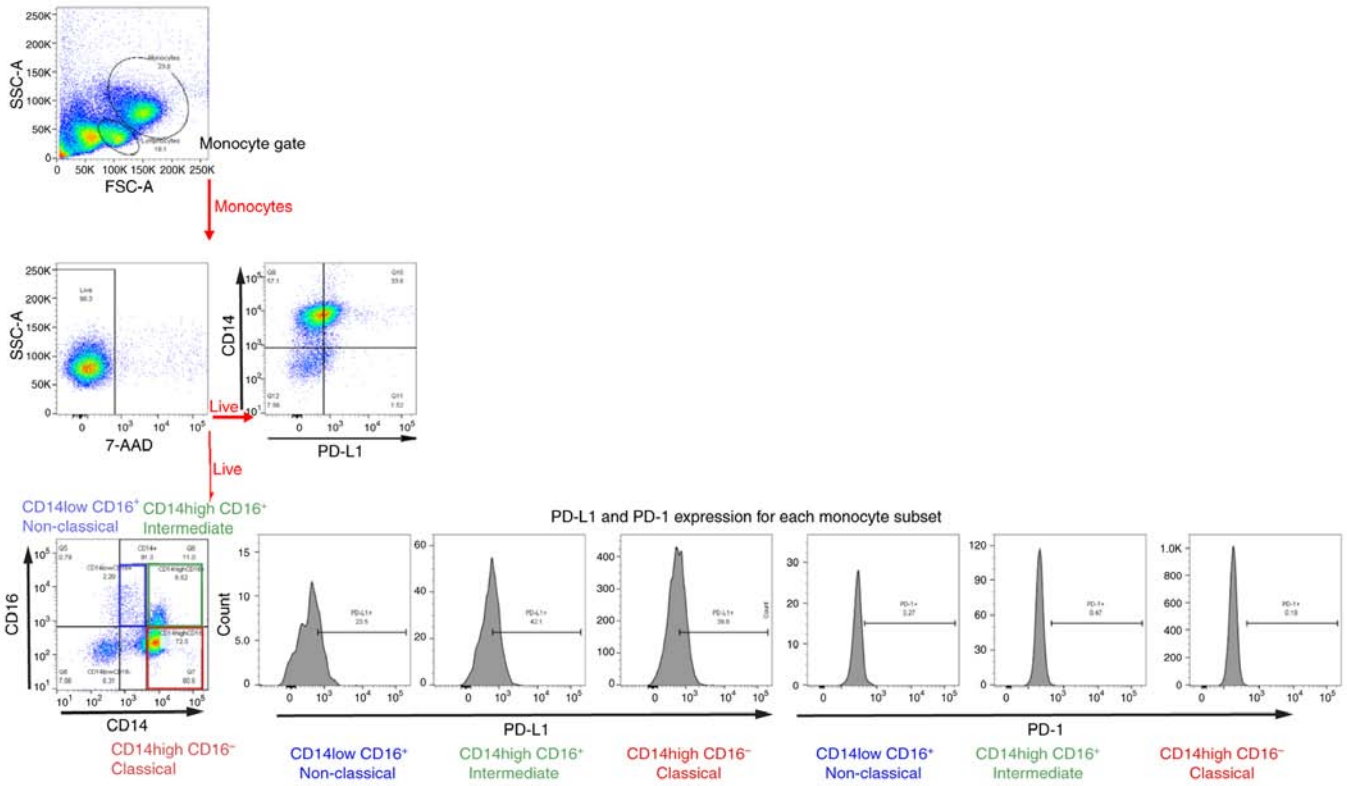

Figure 1. Schematic diagram of the gating strategy for each monocyte subset, and PD-L1- and PD-1-expressing monocyte subpopulations. The schematic shows the gating techniques for circulating PD-1/PD-L1-expressing CD14+ monocytes, CD14high CD16- classical monocytes, CD14high CD16+ intermediate monocytes and CD14low CD16+ non-classical monocytes. PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

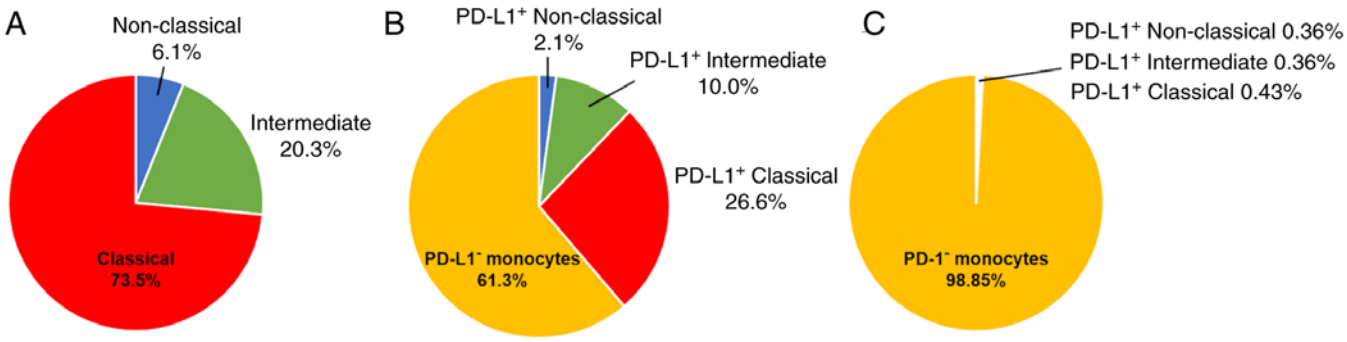

Figure 2. Pie charts showing the percentages of each monocyte subset. (A) Percentage of each monocyte subset: Classical, intermediate and non-classical. Percentages of (B) PD-L1- and (C) PD-1-expressing monocyte subsets in the total number of monocytes from all three subsets. PD-1, programmed death 1; PD-L1, programmed death-ligand 1.

expression analyzed using IHC is shown in Fig. S9. The results showed no significant correlation between immunohistochemical PD-L1 expression on tumor cells and the percentage of either circulating, PD-L1-expressing non-classical or intermediate monocyte subsets (Fig. 7A and B). By contrast, a positive correlation was observed between PD-L1 expression on tumor cells and the percentage of PD-L1-expressing classical monocytes in 22 patients (r=0.54; P=0.009; Fig. 7C).

Correlation between PD-L1-expressing monocytes and survival outcome in patients with NSCLC with high PD-L1 expression on tumor cells. NSCLC is known as a cancer type in which high PD-L1 expression on tumor cells is used as a predictive biomarker for therapeutic response (21,22); therefore the present study focused on patients with NSCLC (n=16). The Kaplan-Meier method was used to assess the relationship between PD-L1 expression on tumor cells (TPS), and OS and PFS in the high and low PD-L1 expression groups with PD-L1 cutoffs of 50% (Fig. 8A and B) and 1% (Fig. 8I and J). These cut-off values are generally applied in clinical settings for predicting the efficacy of anti-PD-1 antibodies in treating patients with NSCLC (21,22). The patients with high PD-L1 (TPS) ≥50% had a more favorable OS than the low PD-L1 expression group (P=0.0286; Fig. 8A); however, no significant results were obtained using a cut-off value of 1% for PD-L1 expression (P=0.870; Fig. 8I) by Kaplan-Meier survival analysis. No significant association between PD-L1 (TPS) and PFS was found in either group (P=0.139; Fig. 8B, and P=0.682;

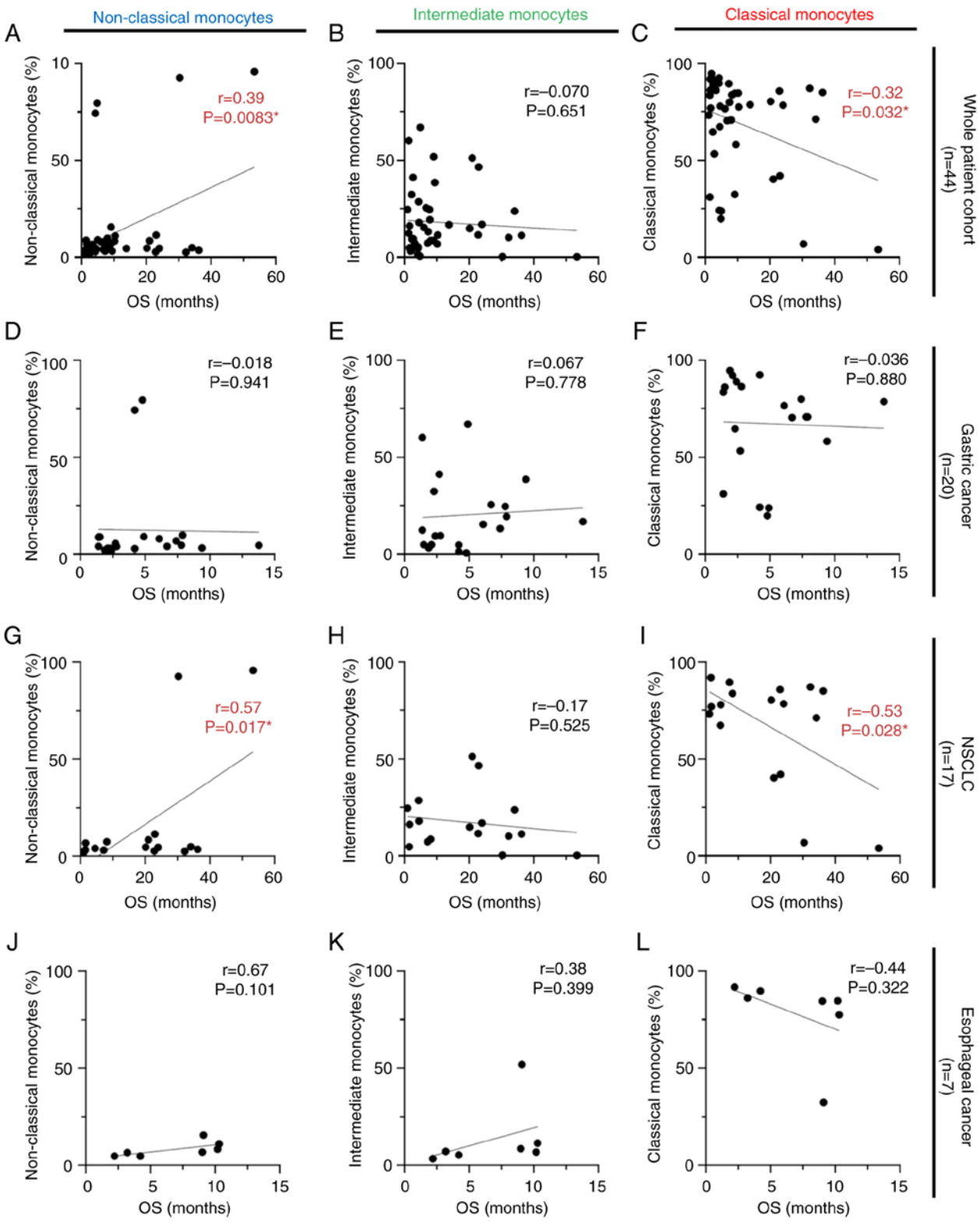

Figure 3. Correlation between each monocyte subset and OS. Graphs show the association between OS and the percentage of (A) non-classical, (B) intermediate and (C) classical monocytes in the whole patient cohort (n=44); OS and the percentage of (D) non-classical, (E) intermediate and (F) classical monocytes in the gastric cancer cohort (n=20); OS and the percentage of (G) non-classical, (H) intermediate and (I) classical monocytes in the NSCLC cohort (n=17); and OS and the percentage of (J) non-classical, (K) intermediate and (L) classical monocytes in the esophageal cancer cohort (n=7). Each dot represents a sample from each patient cohort. *P<0.05. OS, overall survival; NSCLC, non-small cell lung cancer.

Fig. 8J). Furthermore, patients with high PD-L1 expression, including those with PD-L1 (TPS) ≥50% (n=10; Fig. 8C-H) or PD-L1 (TPS) ≥1% (n=13; Fig. 8K-P) were selected. The focus was on the high PD-L1 group, and the association between the percentage of each PD-L1-expressing monocyte subset and OS and PFS was investigated. In patients with NSCLC whose PD-L1 (TPS) was ≥50%, there was an inverse correlation between PD-L1-expressing classical monocytes and OS

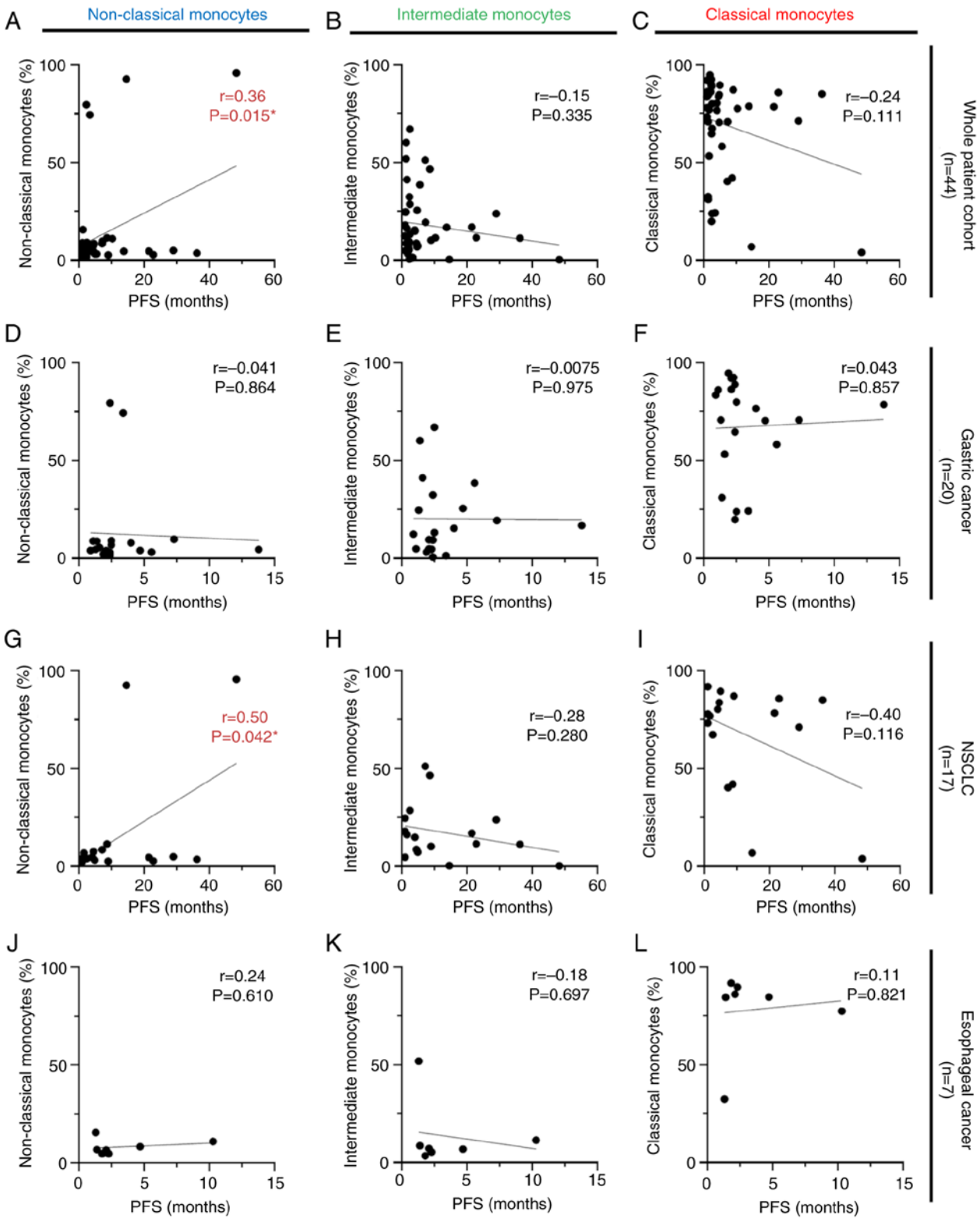

Figure 4. Correlation between each monocyte subset and PFS. Graphs show the association between PFS and the percentage of (A) non-classical, (B) intermediate and (C) classical in the whole patient cohort (n=44); PFS and the percentage of (D) non-classical, (E) intermediate and (F) classical monocytes in the gastric cancer cohort (n=20); PFS and the percentage of (G) non-classical, (H) intermediate and (I) classical monocytes in the NSCLC cohort (n=17); and PFS and the percentage of (J) non-classical, (K) intermediate and (L) classical monocytes in the esophageal cancer cohort (n=7). Each dot represents a sample from each patient cohort. *P<0.05. PFS, progression-free survival; NSCLC, non-small cell lung cancer.

(r=-0.68; P=0.030; Fig. 8E), but not between non-classical and intermediate monocyte subsets (Fig. 8C and D). Meanwhile, regarding PD-L1 (TPS) ≥1%, PD-L1-expressing non-classical monocytes showed a clear positive correlation with both OS and PFS (r=0.63; P=0.022; Fig. 8K, and r=0.60; P=0.029; Fig. 8N). Conversely, higher PD-L1-expressing classical monocytes were significantly correlated with poorer OS and PFS (r=-0.68; P=0.010; Fig. 8M, and r=-0.58; P=0.036;

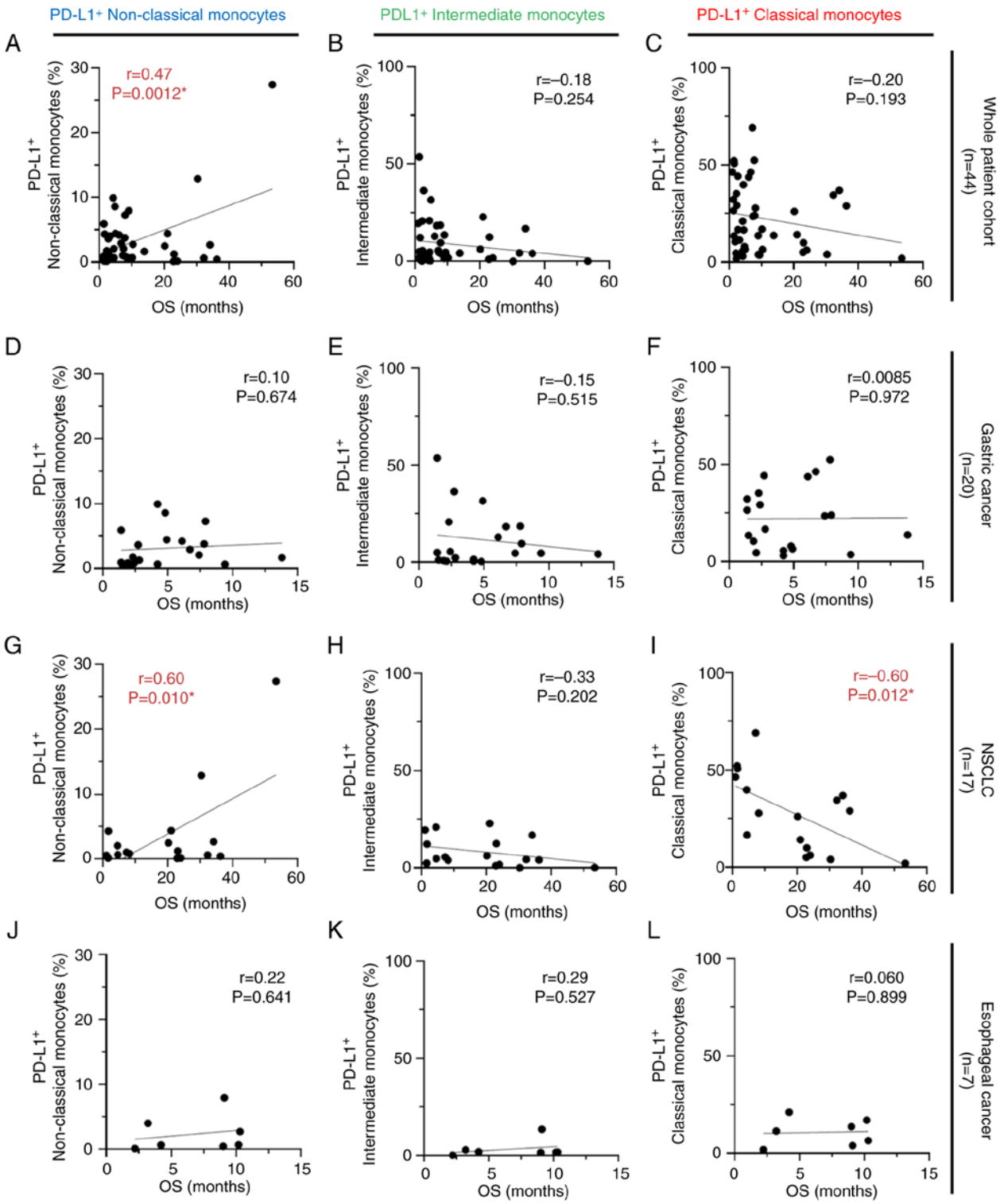

Figure 5. Correlation between each monocyte subset expressing PD-L1 and OS. Graphs showing the association between OS and the percentage of (A) PD-L1+ non-classical, (B) PD-L1+ intermediate and (C) PD-L1+ classical monocytes in the whole patient cohort (n=44); OS and the percentage of (D) PD-L1+ non-classical, (E) PD-L1+ intermediate, (F) PD-L1+ classical monocytes in the gastric cancer cohort (n=20); OS and the percentage of (G) PD-L1+ non-classical, (H) PD-L1+ intermediate and (I) PD-L1+ classical monocytes in the NSCLC cohort (n=17); and OS and the (J) percentage of PD-L1+ non-classical, (K) intermediate and (L) classical monocytes in the esophageal cancer cohort (n=7). Each dot represents a sample from each patient cohort. *P<0.05. OS, overall survival; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer.

Fig. 8P). There was no correlation between PD-L1-expressing intermediate monocytes and either PFS or OS (Fig. 8L and O). PD-L1 expression on tumor cells (TPS) alone is likely inadequate as a biomarker for predicting therapeutic efficacy, especially when the PD-L1 cut-off value is 1% (TPS). In light of these results, it was hypothesized that focusing on the PD-L1-expressing circulating monocytes in peripheral blood, in addition to PD-L1 expression on tumor cells (TPS), might improve its usefulness as a predictive and prognostic biomarker.

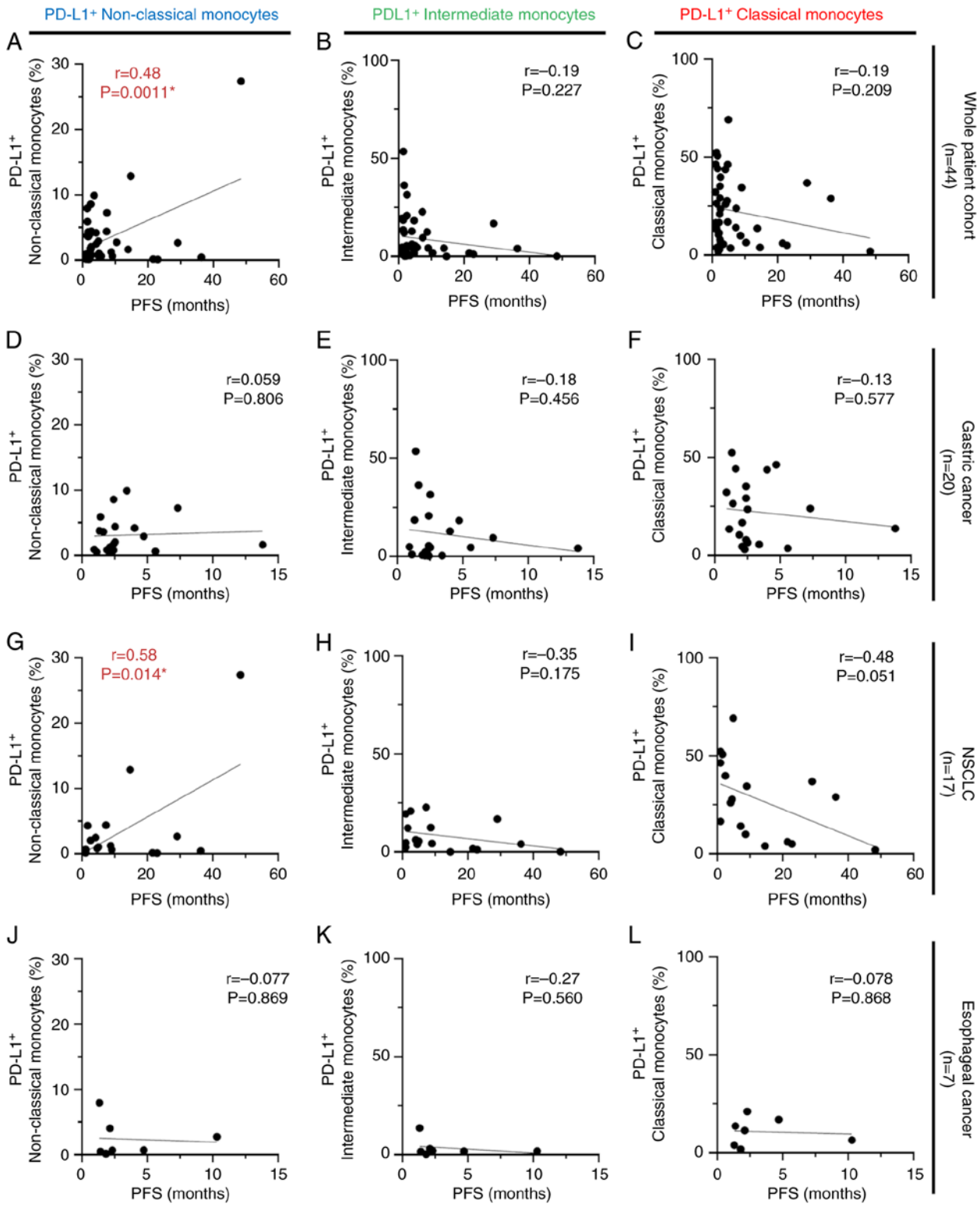

Figure 6. Correlation between each monocyte subset expressing PD-L1 and PFS. Graphs showing the association between PFS and the percentage of (A) PD-L1+ non-classical, (B) PD-L1+ intermediate and (C) PD-L1+ classical monocytes in the whole patient cohort (n=44); PFS and the percentage of (D) PD-L1+ non-classical, (E) PD-L1+ intermediate and (F) PD-L1+ classical monocytes in the gastric cancer cohort (n=20); PFS and the percentage of (G) PD-L1+ non-classical, (H) PD-L1+ intermediate and (I) PD-L1+ classical monocytes in the NSCLC cohort (n=17); and PFS and the percentage of (J) PD-L1+ non-classical, (K) intermediate and (L) classical monocytes in the esophageal cancer cohort (n=7). Each dot represents a sample from each patient cohort. *P<0.05. PFS, progression-free survival; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer.

Correlation between CD68+ cells and survival outcome in patients with cancer. The CD68-stained area was calculated using the hybrid cell count software function (Table SV), and the correlation with survival duration was investigated. Representative images of CD68 immunostaining and CD68-stained areas assessed using the hybrid cell count function are shown in Fig. 9A-D. The stained area of CD68+ cells was statistically correlated with longer PFS and OS in the

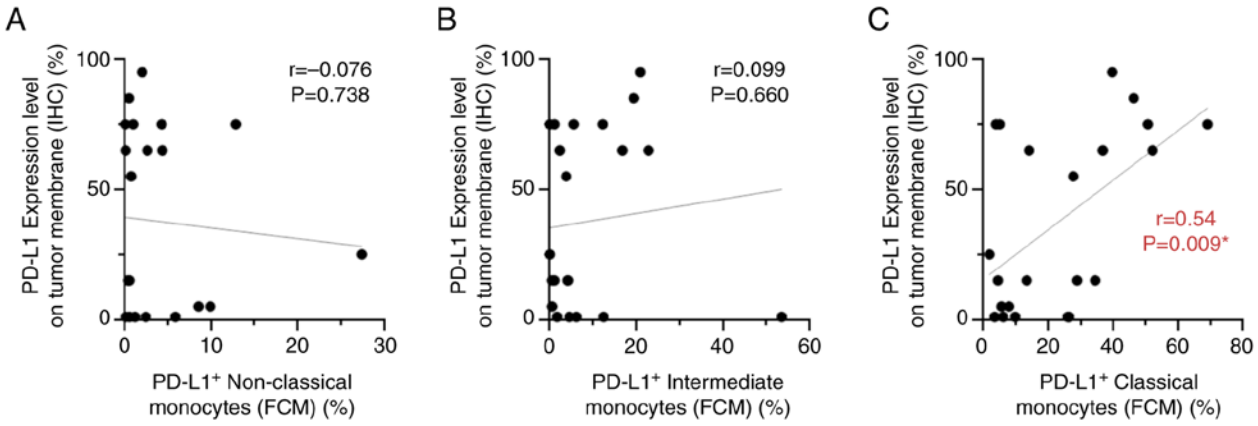

Figure 7. Correlation between PD-L1 expression levels on the tumor membrane and the percentage of PD-L1-expressing monocytes in each subset. Investigation of the relationship between PD-L1 expression on tumor cells and the percentage of monocytes expressing PD-L1 for 22 patients, some with gastric cancer (n=6) and others with non-small cell lung cancer (n=16), using IHC. The PD-L1 expression levels on tumor cells were measured using IHC and were compared with the percentage of PD-L1-expressing (A) non-classical, (B) intermediate and (C) classical monocytes detected using flow cytometry. *P<0.05. IHC, immunohistochemistry; FCM, flow cytometry; PD-L1, programmed death-ligand 1.

whole patient cohort (n=31; r=0.4557; P=0.010; Fig. 9E, and r=0.3857; P=0.032; Fig. 9F). Therefore, macrophages in tumor tissues may have a favorable effect on therapeutic efficacy and prognosis. Furthermore, the association between CD68-stained cells and percentages of non-classical, intermediate and classical monocytes was analyzed. No statistical correlations were observed between the $CD68^+$ area and monocyte percentage of each subset with or without expression of PD-L1 and PD-1 (Fig. S10).

Discussion

The present study demonstrated that higher percentages of classical monocytes were associated with shorter OS. By contrast, higher percentages of non-classical monocytes were significantly associated with longer OS, particularly in the cohort of patients with NSCLC. For non-classical monocytes, similar statistically significant results were also obtained for PFS. Namely, the percentage of each subset of monocytes, especially the relative balance between non-classical and classical monocytes, was suggested to be an essential factor for predicting prognosis and therapeutic efficacy of anti-PD-1 antibodies.

Monocytes in human peripheral circulating blood play a key role in cancer pathophysiology and progression, including tumor angiogenesis, invasion, metastasis and immune regulation (24,25). Moreover, each subset of monocytes plays a specific role in tumor growth (26). This is thought to be due to monocytes presenting pro- and antitumor immunity, such as the secretion of mediators, promotion of angiogenesis, recruitment of lymphocytes and differentiation into macrophages (25). Monocytes are heterologous and are classified into three different subsets primarily based on the expression levels of markers within the cluster of differentiation, CD14 and CD16. The percentage of each subset of monocytes has been reported as follows: Classical (~85%), intermediate (~5%) and non-classical (~10% of the monocyte population) (17,18). The current study showed a similar trend in terms of the highest percentages of the classical monocyte subset. The study showed that the percentages of intermediate monocytes were higher than those of non-classical monocytes. Most monocytes that express CD14, but not CD16, on their surface correspond to classical monocytes that mediate inflammatory responses and differentiate into various macrophages that can inhibit immune responses (27,28). Moreover, classical monocytes are collected at tumor sites that contribute to macrophage capacity, and promote tumor development and metastasis (29). Human circulating monocytes in peripheral blood migrate into the tumor tissue and are differentiated into tumor-associated macrophages, which are polarized into classically activated macrophages (M1) and alternatively activated macrophages (M2) (30,31). M1 macrophages have an antitumor role, whereas M2 macrophages have a tumor-promoting role (32). It remains unclear whether the main monocytes that differentiate into M2 macrophages with tumor-promoting functions are classical or non-classical monocytes (33). It is possible that the classical monocytes present in the plasma prior to anti-PD-1 antibody administration mobilize to the tumor site as M2 macrophages and function in a tumor-promoting manner, thereby explaining the positive correlation between the percentage of classical monocytes and shorter OS observed in the present study. By contrast, non-classical monocytes are involved in complement and Fc γ-mediated phagocytosis. They also have functions such as tumor cytotoxicity, natural killer cell recruitment, adhesion and inhibition of regulatory T cells (25), which may be involved in favorable cancer prognosis, as suggested by the results of the present study. In addition, IHC of CD68-stained cells was performed to evaluate the infiltration of macrophages into tumor tissues. As monocyte subsets in the peripheral blood were not associated with macrophages in the tumor tissue analyzed by IHC, the results suggested the possibility that circulating monocytes and macrophages in the tumor tissue could be independent factors. Macrophages that are present in tumor tissues do not originate only from monocytes in the peripheral blood, and the existence of tissue-resident macrophages originating from the yolk sac during the fetal period has been reported (34). Macrophages in tumor tissues are generally considered to have a tumor-promoting function.

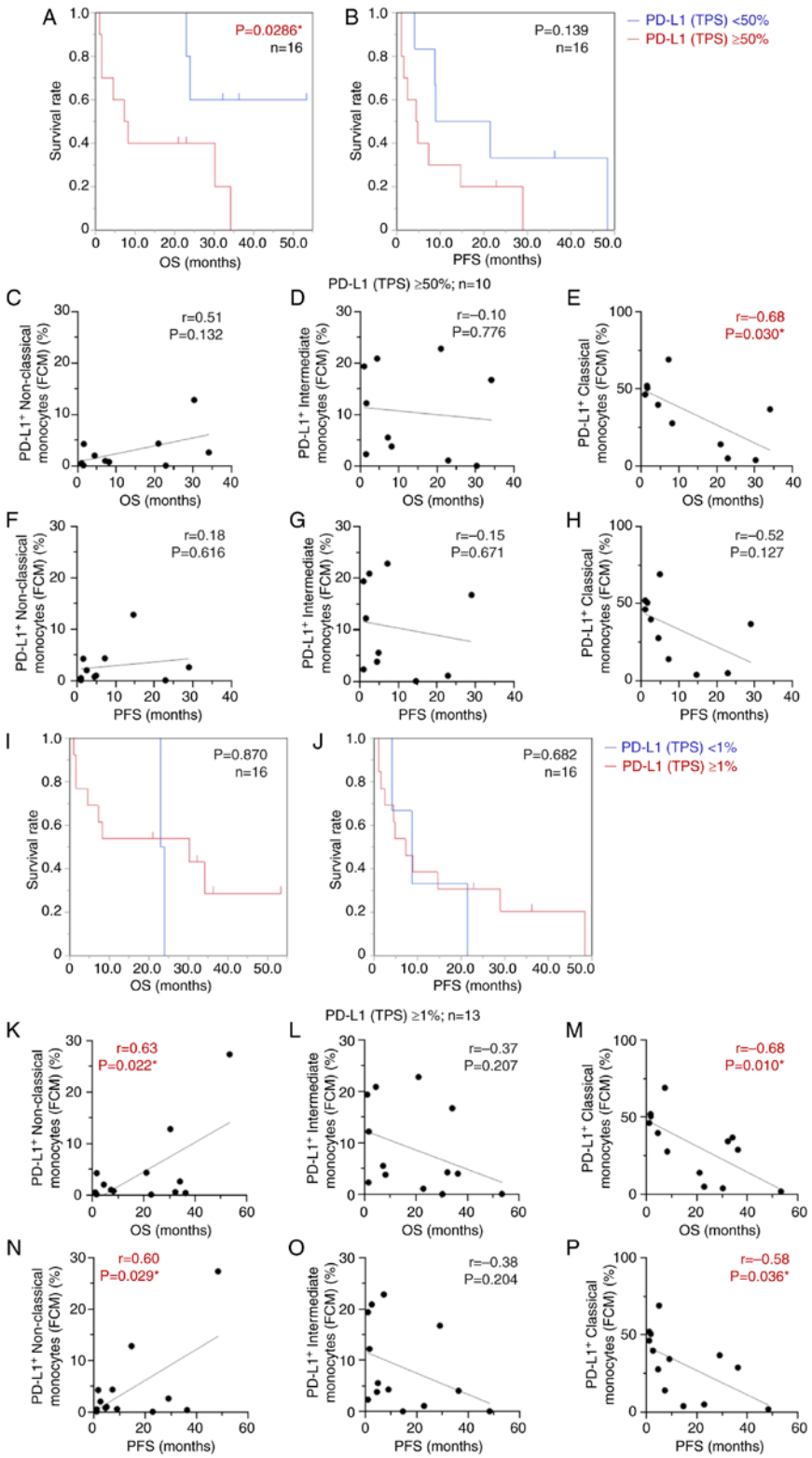

Figure 8. Kaplan-Meier curve and correlation analysis to evaluate the association between PD-L1-expressing monocyte subsets, PD-L1 expression on tumor cells (TPS) and survival duration. Kaplan-Meier analysis of the relationship between PD-L1 expression on tumor cells (TPS) and (A and I) OS and (B and J) PFS. Patients with non-small cell lung carcinoma (n=16) were separated into high and low PD-L1 expression groups with PD-L1 cutoffs of either (A and B) <50% or ≥50%, or (I and J) <1% or ≥1%. Patients with NSCLC were split into high and low PD-L1 expression groups with PD-L1 cutoffs of either ≥50 or ≥1%. Patients with high PD-L1 (TPS) ≥50% (n=10 in C-H) and PD-L1 (TPS) ≥1% (n=13 in K-P) were selected to analyze the association between the percentage of each monocyte subset expressing PD-L1 and OS and PFS. Correlation between (C) PD-L1$^+$ non-classical monocytes, (D) PD-L1$^+$ intermediate monocytes and (E) PD-L1$^+$ classical monocytes, and OS was analyzed in patients with PD-L1 expression ≥50%. Correlation between (F) PD-L1$^+$ non-classical monocytes, (G) PD-L1$^+$ intermediate monocytes and (H) PD-L1$^+$ classical monocytes, and PFS was analyzed in patients with PD-L1 expression ≥50%. A similar analysis was conducted with a cutoff value of 1% of PD-L1 expression. Correlation between (K) PD-L1$^+$ non-classical monocytes, (L) PD-L1$^+$ intermediate monocytes and (M) PD-L1$^+$ classical monocytes, and OS were analyzed in patients with PD-L1 expression ≥1%. Correlation between (N) PD-L1$^+$ non-classical monocytes, (O) PD-L1$^+$ intermediate monocytes and (P) PD-L1$^+$ classical monocytes, and PFS was analyzed in patients with PD-L1 expression ≥1%. Red line, high level; blue line, low level. *P<0.05. PD-L1, programmed death-ligand 1; OS, overall survival; PFS, progression-free survival; TPS, tumor proportion score; NSCLC, non-small cell lung cancer.

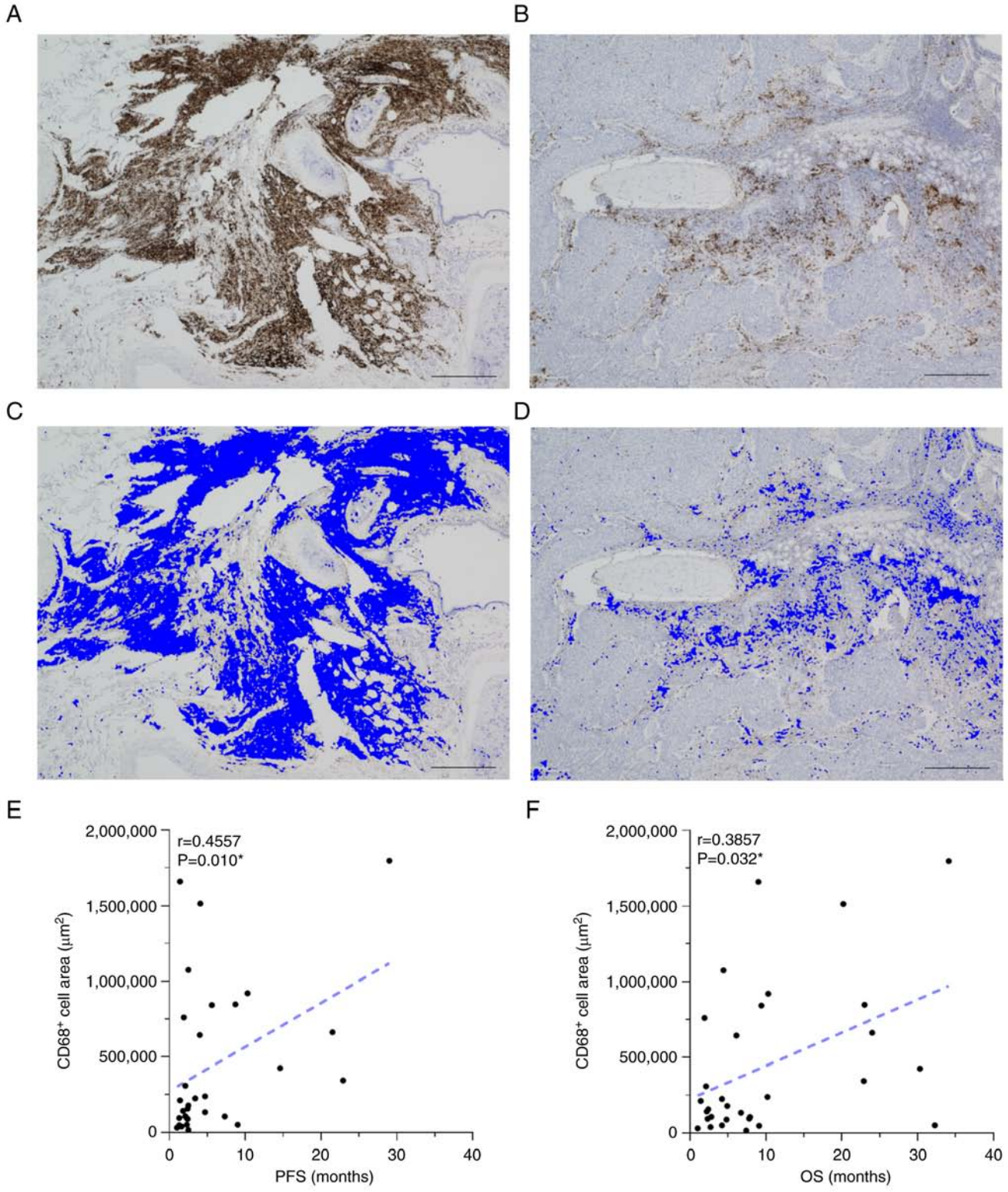

Figure 9. Immunohistochemistry analysis of CD68$^+$ cells and correlation between CD68-stained area and survival outcome in patients with cancer. To evaluate the infiltration of macrophages into the tumor tissue, tissue specimens were immunostained for CD68 in 31 patients from the patient cohort. (A and B) Representative images of CD68 immunostaining of (A) case no. 7 and (B) case no. 3, both of which are cases of patients with NSCLC. (C and D) Representative images of (C) case no. 7 and (D) case no. 3 showing the CD68-stained areas using the hybrid cell count function. Pearson correlation coefficient was performed to analyze the correlation between the CD68-stained area, and (E) OS and (F) PFS. *P<0.05. Scale bar; 500 μm. PFS, progression-free survival; OS, overall survival.

However, the present study indicated that macrophages in the tumor tissue may have a favorable effect on therapeutic efficacy and prognosis. Tumor-associated macrophages are thought to change between antitumor and tumor-promoting roles during tumor development and proliferation (35). In future studies, subsets of macrophages will be analyzed and their relationship to ICI treatment and monocyte subsets in the peripheral blood will be further investigated.

To date, numerous studies have focused on bone marrow-derived suppressor-like cells (MDSCs) (36-38). MDSCs are myeloid immune cells that are more immature than monocytes and are highly immunosuppressive, exerting negative effects not only on the tumor microenvironment but also on the tumor immune system of the patient as a whole (39). The immunosuppressive function of MDSCs has been implicated in resistance to ICI treatment. Monocytic MDSCs (M-MDSCs) have been reported to be involved in the resistance to ICI treatment via TGF-β and IL-6 (40,41). This finding supports the role of some immunosuppressive cytokines in mediating the negative effects of M-MDSCs on the antitumor immune response. M-MDSCs are abundant in the tumor microenvironment, where they rapidly differentiate into tumor-associated macrophages. Broute *et al* (42) showed that high M-MDSC levels are strongly associated with primary resistance to immunotherapy. However, a previous study reported controversial results (43), in which patients who responded to anti-PD-1 antibodies had higher pretreatment MDSC levels. There is no robust evidence that M-MDSCs are associated with resistance to ICI treatment. The current exploratory study focused on monocytes. The mechanism of tolerance to ICI through a series of immune cells, including MDSCs, monocytes and macrophages, should be systematically investigated in future studies.

Previous studies have shown that high circulating monocyte levels are associated with worse prognosis in several cancer types, such as prostate, hepatocellular cervical, pancreatic and gastric cancer (27,44-48). Additionally, it has been shown that higher pretreatment absolute monocyte counts are associated with a shorter response time in patients with NSCLC who responded to ICI therapies, underscoring the impact of monocytes on predicting the efficacy of ICI (49). Further studies investigating the association between the efficacy of ICIs and monocytes with PD-L1 expression have reported that higher pretreatment PD-L1-expressing monocytes are associated with worse clinical outcomes for ICI treatment (16,50-52). However, data on the relationship between the efficacy of ICI, focusing on each monocyte subset expressing immune checkpoints such as PD-1 and PD-L1, remain limited (46). The current study is one of the few valuable reports (53,54), examining the relationship between each monocyte subset and immune checkpoints expressed on monocytes. It was demonstrated that higher percentages of pretreatment classical monocytes expressing PD-L1 were correlated with shorter OS, whereas non-classical monocytes were correlated with favorable survival, especially in patients with NSCLC. In patients with hepatocellular carcinoma, Jeon *et al* (53) reported that PD-L1$^+$ classical monocyte percentages were elevated after 1 week of anti-PD-1 antibody therapy and were associated with a non-durable clinical benefit. Meanwhile, it is not clear why high PD-L1 expression on monocytes, especially on classical monocytes, is associated with poor prognosis. It has been suggested that either IL-6 or IL-10 can contribute to a numerical increase in PD-L1$^+$ classical monocytes early after anti-PD-1 therapy (53). Moreover, hypoxia has been reported to increase surface PD-L1 expression on a variety of immune cells, namely MDSCs, macrophages and antigen-presenting dendritic cells, and tumor cells (55). Hypoxia has also been shown to play a role in tumor aggressiveness (56); therefore, hypoxia may influence PD-L1 expression on monocytes in aggressive tumors. The origin and role of PD-L1-expressing monocytes and their association with prognosis or treatment efficacy remain to be elucidated. Furthermore, the association between PD-1 molecules and therapeutic efficacy of anti-PD-1 antibody remains to be determined. The present study demonstrated that PD-1 expression on monocytes was much lower than that of PD-L1. It was hypothesized that PD-L1, but not PD-1, may have a more essential role in the expression of immune checkpoints in monocytes.

In some cancer types, high PD-L1 expression on tumor cells has been used as a predictive biomarker for the therapeutic efficacy of PD-1 blockade therapy, especially in lung cancer (21,22), and PD-L1 expression levels in tumor cells have been used as a companion diagnosis to determine the indication of anti-PD-1 antibodies. However, PD-L1 expression in tumor cells is not a perfect biomarker as there are some cases in which anti-PD-1 antibody therapy does not respond even when PD-L1 expression in tumor cells is high as shown by IHC (57). In the present study, high or low PD-L1 expression in tumor cells did not have a significant association with PFS in patients with NSCLC. Moreover, patients with NSCLC whose PD-L1 (TPS) was ≥1%, had notably positive correlations between the PD-L1-expressing non-classical monocytes and both OS and PFS. Contrary to this, higher PD-L1-expressing classical monocytes were significantly correlated with poorer OS and PFS. PD-L1 expression on tumor cells (TPS) alone is possibly insufficient as a biomarker for predicting complete therapeutic efficacy, especially in positive cases with a PD-L1 cut-off value of ≥1%. In light of these results, it was hypothesized that focusing on the circulating monocyte subsets in peripheral blood that express PD-L1, in addition to the PD-L1 expression on tumor cells (TPS), may improve its usefulness as a predictive and prognostic biomarker.

No association was observed between genetic alterations (EGFR, ALK, ROS1 and BRAF mutations) and monocyte percentages in the present study. To date, to the best of our knowledge, no studies have focused on the relationship between genetic mutations and monocyte levels in lung cancer, and this will be one of the subjects of future research.

Regarding statistical methodology, two methods were used for analysis: The Pearson correlation coefficient was used to evaluate linear correlation, and the Kaplan-Meier method with cut-off values defined by a ROC curve analysis to compare survival time at high and low levels for each monocyte subset. The results were similar between the two types of methods. Although similar trends were shown in part of the analysis, there were some results that were not consistent. Comparison of two different statistical methods for the overall population of the monocyte subset significantly showed that high percentages of classical monocytes were associated with shorter OS in the whole patient cohort and patients with NSCLC by both methods of statistical analysis. On the other hand, the Kaplan-Meier analysis did not show that non-classical monocytes were associated with a better prognosis, as indicated by the correlation analysis. Similarly, in the analysis of PD-L1-expressing monocyte subsets, classical monocytes expressing PD-L1 were similarly significant in both analyses, but not PD-L1$^+$ non-classical monocytes. For the whole patient cohort, high percentages of classical

monocytes expressing PD-L1 were associated with favorable OS only using Kaplan-Meier analysis. Furthermore, both analysis methods for PD-1$^+$ monocytes showed that classical monocytes expressing PD-1 in patients with NSCLC were associated with a shorter OS time, but the other results regarding intermediate and non-classical monocytes expressing PD-1 were not significantly different in patients with NSCLC. Briefly, the two statistical methods showed similar results with statistical significance for particularly classical monocyte subsets. Since no clear standards to define the cut-off values of high and low monocytes have previously been reported, there are limitations in interpreting the analysis results using the Kaplan-Meier method. In the present study, it was hypothesized that the correlation analysis using the Pearson correlation method is the more principal result. Regarding Kaplan-Meier analyses for patients with esophageal cancer, when the patients were divided into two groups, 'high' and 'low' groups, exactly the same number of patients were classified into both groups. Therefore, in some cases, the Kaplan-Meier curves and the analysis results completely matched. These issues were due to the small number of patients with esophageal cancer.

There are several notable limitations in the present study. The patient population was heterogeneous and small, including three cancer types: Lung, gastric and esophageal cancer. Also, there was no single histological type for patients with lung cancer. Future studies should unify the line of ICI administration. In the current study, the focus was on patients who were treated with anti-PD-1 antibody monotherapy. Selecting patients treated with monotherapy enabled the direct examination of the relationship between anti-PD-1 antibodies and monocytes, and excluded the influence of concomitant agents. Of note, the number of patients who met the eligibility criteria could not be increased due to the increasing use of combination therapy. Additionally, the analysis of gastric and esophageal cancer did not yield results similar to those of NSCLC. Individual cancer types were analyzed and cross-sectional data across all cancer types were compiled because ICIs are used across different cancer types. Due to limited blood samples from patients in the current study, the dynamic changes in each monocyte subset before and after anti-PD-1 treatment could not be analyzed. In addition, experiments could not be carried out to explore molecular mechanisms. Prospective trials and elucidation of molecular mechanisms are required to resolve these limitations.

In conclusion, despite the shortcomings associated with the heterogeneity of the patient cohort, higher percentages of classical monocytes were associated with shorter OS. In comparison, higher percentages of non-classical monocytes were significantly associated with longer OS, particularly in patients with NSCLC. For non-classical monocytes, similar significant results were also obtained for PFS. Moreover, focusing on PD-L1 expression in monocytes, a higher percentage of classical monocytes expressed PD-L1 than intermediate and non-classical monocytes. The analysis of immune checkpoints on monocytes, especially in NSCLC, revealed that classical monocytes expressing PD-L1 at a high level were correlated with a shorter OS. By contrast, a positive correlation between PD-L1-expressing non-classical monocytes, and longer OS and PFS was observed. Classical and non-classical monocytes, especially PD-L1-expressing monocytes, are potential candidate biomarkers for predicting prognosis in patients treated with ICIs and treatment response. PD-L1 expression on monocytes is superior because it can be measured in circulating peripheral blood. Further studies are warranted to resolve some of the limitations of the present study.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RO, TaT and SW conceptualized the study. RO, YF, KI, NO, MW, DT, TG, YS, MH, TY and SW designed the methodology used. RO, YF and MW used software. AH, KH, HA, YH, TI, RS, NI, ToT, KY, MT, YK, SK and TaT analyzed the data, and validated the reproducibility of the data involved in the flow cytometry analysis and clinical data of patients. YS, MH, and TY conducted IHC experiments and performed data verification for data collection and analysis. YS, MH, and TY validated the reproducibility of the IHC data, then confirmed all the data involved in the pathological experiment. RO and SW confirm the authenticity of all raw data. RO carried out the formal analysis. RO, YF, KI, NO, MW, DT, TG, YS, MH and TY carried out the investigation. RO, AH, KH, HA, YH, TI, RS, NI, ToT, KY, TaT and SW provided resources. RO, YF and RS carried out data curation; RO wrote and prepared the original draft. TaT and SW reviewed and edited the manuscript. RO completed data visualization. KY, MT, YK, SK, TaT and SW carried out project supervision. SW carried out project administration. TaT and SW acquired funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of The Declaration of Helsinki and was approved by the Ethics Committee of Showa University School of Medicine, Tokyo, Japan (approval nos. 2165, 2253, 283 and 285). All patients provided written informed consent for their participation in the current study.

Patient consent for publication

All patients provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

References

1. Dosset M, Vargas TR, Lagrange A, Boidot R, Végran F, Roussey A, Chalmin F, Dondaine L, Paul C, Lauret Marie-Joseph E, *et al*: PD-1/PD-L1 pathway: An adaptive immune resistance mechanism to immunogenic chemotherapy in colorectal cancer. Oncoimmunology 7: e1433981, 2018.
2. Han Y, Liu D and Li L: PD-1/PD-L1 pathway: Current researches in cancer. Am J Cancer Res 10: 727-742, 2020.
3. Vaddepally RK, Kharel P, Pandey R, Garje R and Chandra AB: Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. Cancers (Basel) 12: 738, 2020.
4. Gong J, Chehrazi-Raffle A, Reddi S and Salgia R: Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: A comprehensive review of registration trials and future considerations. J Immunother Cancer 6: 8, 2018.
5. Yi M, Niu M, Xu L, Luo S and Wu K: Regulation of PD-L1 expression in the tumor microenvironment. J Hematol Oncol 14: 10, 2021.
6. Bardhan K, Anagnostou T and Boussiotis VA: The PD1:PD-L1/2 pathway from discovery to clinical implementation. Front Immunol 7: 550, 2016.
7. De Marchi P, Leal LF, Duval da Silva V, da Silva ECA, Cordeiro de Lima VC and Reis RM: PD-L1 expression by tumor proportion score (TPS) and combined positive score (CPS) are similar in non-small cell lung cancer (NSCLC). J Clin Pathol 74: 735-740, 2021.
8. Kulangara K, Zhang N, Corigliano E, Guerrero L, Waldroup S, Jaiswal D, Ms MJ, Shah S, Hanks D, Wang J, *et al*: Clinical utility of the combined positive score for programmed death ligand-1 expression and the approval of pembrolizumab for treatment of gastric cancer. Arch Pathol Lab Med 143: 330-337, 2019.
9. Noske A, Wagner DC, Schwamborn K, Foersch S, Steiger K, Kiechle M, Oettler D, Karapetyan S, Hapfelmeier A, Roth W and Weichert W: Interassay and interobserver comparability study of four programmed death-ligand 1 (PD-L1) immunohistochemistry assays in triple-negative breast cancer. Breast 60: 238-244, 2021.
10. Daud AI, Wolchok JD, Robert C, Hwu WJ, Weber JS, Ribas A, Hodi FS, Joshua AM, Kefford R, Hersey P, *et al*: Programmed death-ligand 1 expression and response to the anti-programmed death 1 antibody pembrolizumab in melanoma. J Clin Oncol 34: 4102-4109, 2016.
11. Zeng Z, Yang B and Liao Z: Biomarkers in immunotherapy-based precision treatments of digestive system tumors. Front Oncol 11: 650481, 2021.
12. Hutarew G: PD-L1 testing, fit for routine evaluation? From a pathologist's point of view. Memo 9: 201-206, 2016.
13. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, Jansson M, Kulangara K, Richardson W, Towne P, Hanks D, Vennapusa B, *et al*: PD-L1 immunohistochemistry assays for lung cancer: Results from phase 1 of the blueprint PD-L1 IHC assay comparison project. J Thorac Oncol 12: 208-222, 2017.
14. Ohkuma R, Ieguchi K, Watanabe M, Takayanagi D, Goshima T, Onoue R, Hamada K, Kubota Y, Horiike A, Ishiguro T, *et al*: Increased plasma soluble PD-1 concentration correlates with disease progression in patients with cancer treated with anti-PD-1 antibodies. Biomedicines 9: 1929, 2021.
15. Ando K, Hamada K, Watanabe M, Ohkuma R, Shida M, Onoue R, Kubota Y, Matsui H, Ishiguro T, Hirasawa Y, *et al*: Plasma levels of soluble PD-L1 correlate with tumor regression in patients with lung and gastric cancer treated with immune checkpoint inhibitors. Anticancer Res 39: 5195-5201, 2019.
16. Ando K, Hamada K, Shida M, Ohkuma R, Kubota Y, Horiike A, Matsui H, Ishiguro T, Hirasawa Y, Ariizumi H, *et al*: A high number of PD-L1^{+} CD14^{+} monocytes in peripheral blood is correlated with shorter survival in patients receiving immune checkpoint inhibitors. Cancer Immunol Immunother 70: 337-348, 2021.
17. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, *et al*: Nomenclature of monocytes and dendritic cells in blood. Blood 116: e74-e80, 2010.
18. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM and Wong SC: The three human monocyte subsets: Implications for health and disease. Immunol Res 53: 41-57, 2012.
19. Sidibe A, Ropraz P, Jemelin S, Emre Y, Poittevin M, Pocard M, Bradfield PF and Imhof BA: Angiogenic factor-driven inflammation promotes extravasation of human proangiogenic monocytes to tumours. Nat Commun 9: 355, 2018.
20. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer 45: 228-247, 2009.
21. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, *et al*: Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 373: 1627-1639, 2015.
22. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, Gottfried M, Peled N, Tafreshi A, Cuffe S, *et al*: Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med 375: 1823-1833, 2016.
23. Iida Y, Tanaka H, Sano H, Suzuki Y, Shimizu H and Urano T: Ectopic expression of PCSK9 by smooth muscle cells contributes to aortic dissection. Ann Vasc Surg 48: 195-203, 2018.
24. Prat M, Le Naour A, Coulson K, Lemée F, Leray H, Jacquemin G, Rahabi MC, Lemaitre L, Authier H, Ferron G, *et al*: Circulating CD14^{high} CD16^{low} intermediate blood monocytes as a biomarker of ascites immune status and ovarian cancer progression. J Immunother Cancer 8: e000472, 2020.
25. Olingy CE, Dinh HQ and Hedrick CC: Monocyte heterogeneity and functions in cancer. J Leukoc Biol 106: 309-322, 2019.
26. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA and Pollard JW: CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 475: 222-225, 2011.
27. Shigeta K, Kosaka T, Kitano S, Yasumizu Y, Miyazaki Y, Mizuno R, Shinojima T, Kikuchi E, Miyajima A, Tanoguchi H, *et al*: High absolute monocyte count predicts poor clinical outcome in patients with castration-resistant prostate cancer treated with docetaxel chemotherapy. Ann Surg Oncol 23: 4115-4122, 2016.
28. Xu H, Manivannan A, Crane I, Dawson R and Liversidge J: Critical but divergent roles for CD62L and CD44 in directing blood monocyte trafficking in vivo during inflammation. Blood 112: 1166-1174, 2008.
29. Movahedi K, Laoui D, Gysemans C, Baeten M, Stangé G, Van den Bossche J, Mack M, Pipeleers D, In't Veld P, De Baetselier P and Van Ginderachter JA: Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. Cancer Res 70: 5728-5739, 2010.
30. Robinson A, Han CZ, Glass CK and Pollard JW: Monocyte regulation in homeostasis and malignancy. Trends Immunol 42: 104-119, 2021.
31. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD and Fantino E: Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. Am J Respir Cell Mol Biol 53: 676-688, 2015.
32. Richards DM, Hettinger J and Feuerer M: Monocytes and macrophages in cancer: Development and functions. Cancer Microenviron 6: 179-191, 2013.
33. Italiani P and Boraschi D: From monocytes to M1/M2 macrophages: Phenotypical vs functional differentiation. Front Immunol 5: 514, 2014.
34. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F and Rodewald HR: Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. Nature 518: 547-551, 2015.
35. Allavena P, Sica A, Garlanda C and Mantovani A: The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 222: 155-161, 2008.
36. Xu Q, Liu H, Qile M and Wuren T: Dynamic changes in myeloid-derived suppressor cells during the menstrual cycle: A pilot study. Front Med (Lausanne) 9: 940554, 2022.
37. Nonaka K, Saio M, Umemura N, Kikuchi A, Takahashi T, Osada S and Yoshida K: Th1 polarization in the tumor microenvironment upregulates the myeloid-derived suppressor-like function of macrophages. Cell Immunol 369: 104437, 2021.
38. Trikha P and Carson WE III: Signaling pathways involved in MDSC regulation. Biochim Biophys Acta 1846: 55-65, 2014.
39. Marvel D and Gabrilovich DI: Myeloid-derived suppressor cells in the tumor microenvironment: Expect the unexpected. Clin Invest 125: 3356-3364, 2015.
40. Pogoda K, Pyszniak M, Rybojad P and Tabarkiewicz J: Monocytic myeloid-derived suppressor cells as a potent suppressor of tumor immunity in non-small cell lung cancer. Oncol Lett 12: 4785-4794, 2016.

41. Koh J, Kim Y, Lee KY, Hur JY, Kim MS, Kim B, Cho HJ, Lee YC, Bae YH, Ku BM, *et al*: MDSC subtypes and CD39 expression on CD8+ T cells predict the efficacy of anti-PD-1 immunotherapy in patients with advanced NSCLC. Eur J Immunol 50: 1810-1819, 2020.
42. Broute G, Petracci E, De Matteis S, Canale M, Zampiva I, Priano I, Cravero P, Andrikou K, Burgio MA, Ulivi P, *et al*: High levels of circulating monocytic myeloid-derived suppressive-like cells are associated with the primary resistance to immune checkpoint inhibitors in advanced non-small cell lung cancer: An exploratory analysis. Front Immunol 13: 866561, 2022.
43. Feng J, Chen S, Li S, Wu B, Lu J, Tan L, Li J, Song Y, Shi G, Shi YG and Jiang J: The association between monocytic myeloid-derived suppressor cells levels and the anti-tumor efficacy of anti-PD-1 therapy in NSCLC patients. Transl Oncol 13: 100865, 2020.
44. Sasaki A, Iwashita Y, Shibata K, Matsumoto T, Ohta M and Kitano S: Prognostic value of preoperative peripheral blood monocyte count in patients with hepatocellular carcinoma. Surgery 139: 755-764, 2006.
45. Lee YY, Choi CH, Sung CO, Do IG, Huh S, Song T, Kim MK, Kim HJ, Kim TJ, Lee JW, *et al*: Prognostic value of pre-treatment circulating monocyte count in patients with cervical cancer: Comparison with SCC-Ag level. Gynecol Oncol 124: 92-97, 2012.
46. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, Mitchem JB, Plambeck-Suess SM, Worley LA, Goetz BD, *et al*: Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: A role for targeting the CCL2/CCR2 axis. Clin Cancer Res 19: 3404-3415, 2013.
47. Hayashi T, Fujita K, Nojima S, Hayashi Y, Nakano K, Ishizuya Y, Wang C, Yamamoto Y, Kinouchi T, Matsuzaki K, *et al*: Peripheral blood monocyte count reflecting tumor-infiltrating macrophages is a predictive factor of adverse pathology in radical prostatectomy specimens. Prostate 77: 1383-1388, 2017.
48. Feng F, Zheng G, Wang Q, Liu S, Liu Z, Xu G, Wang F, Guo M, Lian X and Zhang H: Low lymphocyte count and high monocyte count predicts poor prognosis of gastric cancer. BMC Gastroenterol 18: 148, 2018.
49. Parikh K, Kumar A, Ahmed J, Anwar A, Puccio C, Chun H, Fanucchi M and Lim SH: Peripheral monocytes and neutrophils predict response to immune checkpoint inhibitors in patients with metastatic non-small cell lung cancer. Cancer Immunol Immunother 67: 1365-1370, 2018.
50. Yasuoka H, Asai A, Ohama H, Tsuchimoto Y, Fukunishi S and Higuchi K: Increased both PD-L1 and PD-L2 expressions on monocytes of patients with hepatocellular carcinoma was associated with a poor prognosis. Sci Rep 10: 10377, 2020.
51. Riemann D, Schütte W, Turzer S, Seliger B and Möller M: High PD-L1/CD274 expression of monocytes and blood dendritic cells is a risk factor in lung cancer patients undergoing treatment with PD1 inhibitor therapy. Cancers (Basel) 12: 2966, 2020.
52. Zhang W, Liu Y, Yan Z, Yang H, Sun W, Yao Y, Chen Y and Jiang R: IL-6 promotes PD-L1 expression in monocytes and macrophages by decreasing protein tyrosine phosphatase receptor type O expression in human hepatocellular carcinoma. J Immunother Cancer 8: e000285, 2020.
53. Jeon SH, Lee YJ, Kim HD, Nam H, Ryoo BY, Park SH, Yoo C and Shin EC: Dynamic changes in peripheral blood monocytes early after anti-PD-1 therapy predict clinical outcomes in hepatocellular carcinoma. Cancer Immunol Immunother 28: 371-384, 2023.
54. Bianchini M, Duchêne J, Santovito D, Schloss MJ, Evrard M, Winkels H, Aslani M, Mohanta SK, Horckmans M, Blanchet X, *et al*: PD-L1 expression on nonclassical monocytes reveals their origin and immunoregulatory function. Sci Immunol 4: eaar3054, 2019.
55. Noman MZ and Chouaib S: Targeting hypoxia at the forefront of anticancer immune responses. Oncoimmunology 3: e954463, 2015.
56. Reck M, Kerr KM, Grohé C, Manegold C, Pavlakis N, Paz-Ares L, Huber RM, Popat S, Thatcher N, Park K, *et al*: Defining aggressive or early progressing nononcogene-addicted non-small-cell lung cancer: A separate disease entity? Future Oncol 15: 1363-1383, 2019.
57. Shen X and Zhao B: Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: Meta-analysis. BMJ 362: k3529, 2018.