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ORIGINAL ARTICLE

A high number of PD-L1$^+$ CD14$^+$ monocytes in peripheral blood is correlated with shorter survival in patients receiving immune checkpoint inhibitors

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Abstract

Purpose Targeting of anti-programmed cell death protein-1 (PD-1) and anti-programmed death-ligand 1 (PD-L1) is a standard therapeutic strategy for various cancers. The aim of the present study was to investigate the prognostic effect of pretreatment PD-L1 expression levels in peripheral blood mononuclear cell (PBMC) subsets for patients with several cancer types receiving anti-PD-1 blockade therapies.

Patients and methods Thirty-two patients undergoing anti-PD-L1 blockade therapy, including 15 with non-small cell lung cancer, 14 with gastric cancer, 1 with melanoma, 1 with parotid cancer, and 1 with bladder cancer, were recruited for the present study. PD-L1 expression levels in CD3$^+$, CD4$^+$, CD8$^+$, CD45RA$^+$ and CCR7$^+$ T cells; CD20$^+$ B cells; CD14$^+$ and CD16$^+$ monocytes were measured via flow cytometry before treatment. The percentages of PD-L1$^+$ cells in respective PBMC subsets were compared with respect to different clinicopathological conditions and the association with overall survival (OS) was assessed.

Results The percentages of PD-L1$^+$ with CD3$^+$, CD4$^+$ and CD8$^+$ T cells including naïve and memory T cell subsets, or CD20$^+$ B cells during pretreatment were not markedly correlated with the OS of patients ($p > 0.05$); however, the percentage of the PD-L1$^+$ CD14$^+$ monocyte subset was significantly correlated with OS ($p = 0.0426$).

Conclusion Increase in pretreatment expression levels of PD-L1 on CD14$^+$ monocytes is associated with the OS of patients treated with immune checkpoint inhibitors. Further evaluation of large sample size and each specific cancer type might clarify the predictive role of PBMC in patients.

Keywords Programmed death-ligand 1 · Programmed death-1 · CD14 · Prognosis · Nivolumab · Pembrolizumab

Introduction

Programmed death-1 (PD-1) and its ligand programmed death-ligand1 (PD-L1) play a pivotal role in immunosuppression [1–3]. The PD1/PD-L1 pathway is closely associated

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with resistance to antitumor immunity in the tumor microenvironment (TME) [4]. Therefore, targeting PD-1/PD-L1 signaling using immune checkpoint inhibitors (ICIs) such as anti-PD-1 antibodies (e.g., nivolumab and pembrolizumab) has provided remarkable therapeutic benefits in the treatment of various cancers [5–7]. PD-L1 expressed on cancer cells inhibits the activation of tumor-infiltrating lymphocytes, resulting in tumor progression, indicating that PD-L1 expression levels can potentially predict tumor dynamics [8–10]. Furthermore, accumulating evidence indicates that not only PD-L1-expressing tumor cells, as quantified by the tumor proportion score (TPS), but also PD-L1-expressing immune cells, referred to as the combined positive score (CPS), play an important role in predicting the response to ICIs [11–13]. The KEYNOTE-059 cohort 1 trial revealed that the CPS might improve the prediction of gastric cancer patients potentially benefiting from pembrolizumab, demonstrating its diagnostic utility [14]. Concurrently, the potential role of tumor-infiltrating PD-L1-expressing immune cells, especially lymphocytes and macrophages, has received increased research attention for improving ICI-based therapies. The recent most plausible explanation for this association may be that PD-L1-expressing immune cells maintain immunological self-tolerance by suppressing self-reactive lymphocytes such as regulatory T cells (Tregs) and M2 macrophages in the TME, suggesting potential therapeutic implications in various cancers [15, 16]. However, the prognostic significance and predictive role of circulating PD-L1-expressing immune cells in the peripheral blood for responses to ICIs remain unclear. Moreover, we previously reported that a reduction in sPD-L1 levels after four cycles of ICI treatment was significantly correlated with tumor regression in patients with non-small cell lung cancer (NSCLC) and gastric cancer, but not with overall survival (OS) [17].

To clarify these associations and suggest candidate biomarkers to improve patient selection and treatment outcome with ICIs, the aim of the present study was to identify prognostic factors in peripheral blood mononuclear cell (PBMC) subsets, including $CD3^+$, $CD4^+$, and $CD8^+$ T cells; $CD20^+$ B cells; and $CD14^+$ monocytes, and assessed the PD-L1-expressing subsets of each of these cells including naïve and memory T cell subsets as well as classical, intermediate and non-classical monocyte subsets in patients with various cancer types, along with their clinical implications in anti-PD-1 immunotherapy.

Patients and methods

Study subject recruitment

A total of 32 patients were recruited for this study. Fifteen patients had NSCLC, 14 had gastric cancer, 1 had melanoma, 1 had parotid cancer, and 1 had bladder cancer. All patients had received treatment with an ICI (240 mg nivolumab intravenously every 2 weeks, or 200 mg pembrolizumab intravenously every 3 weeks) at Showa University Hospital from January 2017 to August 2019. Patient characteristics, immunohistochemical analysis of PD-L1 in the tumor tissue during pathological diagnosis, and the number of $PD\text{-}L1^+$ cells in PBMC subsets during pretreatment were evaluated. OS was determined as the time from diagnosis to the final follow-up or death. Target lesions were assessed via computed tomographic imaging and the responses to ICIs were assessed in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 [18]. The percentage of $PD\text{-}L1^+$ cells in PBMC subsets was also determined in blood samples collected from two healthy volunteers (control samples) for comparison. Written informed consent was obtained from all participants prior to specimen collection, and the study was approved by the Ethics Committee of Showa University Hospital and adhered to the tenets of the 1975 Declaration of Helsinki.

Assessment of tumor PD-L1 levels

Immunohistochemical staining of tumor PD-L1 was performed as previously described [17]. In brief, formalin-fixed paraffin-embedded tissue samples were prepared from biopsy specimens of the patients for pathological diagnosis. As a companion diagnostic method, PD-L1 immunohistochemistry 28-8 PharmaDX and PD-L1 IHC 22C3 PharmaDX kits were used in accordance with the manufacturer instructions (Dako, Glostrup, Denmark). PD-L1 expression was quantitatively evaluated as the TPS.

PBMC preparation

Blood samples were obtained prior to ICI treatment and stored in BD Vacutainer CPT Cell Preparation Tubes containing sodium heparin (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The supernatant was separated via centrifugation at $1600 \times g$ for 20 min at 20 °C, and the pellet was resuspended in phosphate-buffered saline (PBS) and washed once with PBS. The separated PBMCs were stored in BAMBANKER (GC LYMPHOTEC, Tokyo, Japan) at − 80 °C and then in liquid nitrogen until further analysis.

Staining and flow cytometry analysis

$PD\text{-}L1^+$ cells in PBMC subsets were enumerated using a BD LSRFortessa X-20 Cell Flow Cytometer (Becton, Dickinson and Company). For individual assessments, 1×10^7 PBMCs were resuspended in PBS containing 2% fetal bovine serum (FBS), incubated with Human BD FC Block (Becton, Dickinson and Company) for 10 min at 25 °C, and stained with 7-AAD, PD-L1 phycoerythrin (PE)-conjugated

antibody (PE Mouse Anti-Human CD274), and with the following antibodies on ice for 30 min: anti-CD3 BD Horizon BV480-conjugated antibody (Mouse Anti-Human CD3), anti-CD8 allophycocyanin (APC)-conjugated antibody (Mouse Anti-Human CD8), anti-CD4 PE-Cy7-conjugated antibody (Mouse Anti-Human CD4), anti-CD45RA APC-conjugated antibody (Mouse Anti-Human CD45RA), anti-CCR7 BV421-conjugated antibody (Rat Anti-Human CCR7), anti-CD20 fluorescein isothiocyanate (FITC)-conjugated antibody (Mouse Anti-Human CD20), anti-CD14 PE-Cy7-conjugated antibody (Mouse Anti-Human CD14), and anti-CD16 FITC-conjugated antibody (Mouse Anti-Human CD16), which targeted $CD3^+$, $CD8^+$, $CD4^+$, $CD45RA^+$, $CCR7^+$ T cells, $CD20^+$ B cells, or $CD14^+$ and $CD16^+$ monocytes, respectively (all used as supplied and obtained from Becton, Dickinson and Company). Thereafter, the cell suspension was washed twice in PBS with 2% FBS and detected at the respective wavelengths. A minimum of 50,000 events was acquired. The gating strategies used for flow cytometry were based on single-stained samples and isotype controls, and outlined in Fig. 2, Supplementary Fig. 1a, as well as Supplementary Fig. 2a, b. The data were analyzed using FlowJo version 10.5.3 (Tree Star, Inc., Ashland, OR, USA) software.

Statistical analysis

An unpaired Student's *t* test was performed for between-group comparisons. Spearman's rank correlation analysis was performed for linear correlation analysis. Statistical analysis was performed using Microsoft Excel (Microsoft Co., Redmond, WA, USA) and JMP version 14.0 (SAS Institute, Cary, NC, USA). The results are presented as mean ± standard deviation values. All tests were two sided, and a p value of less than 0.05 was considered statistically significant.

Results

Association between PBMC subsets and patient outcomes

We assessed the potential prognostic predictors in different PBMC subsets. We enumerated $CD3^+$, $CD4^+$ and $CD8^+$ T cells; $CD20^+$ B cells; and $CD14^+$ monocytes or the percentage of PD-$L1^+$ cells of the respective subsets in 21 patients, including 11 with NSCLC, 9 with gastric cancer, and 1 with bladder cancer before ICI treatment, along with 2 healthy controls via flow cytometry (Fig. 1). Particularly, the configurations for initial gating were different and were set independently for lymphocytes and monocytes subsets. Therefore, the percentages of lymphocyte and monocyte subsets are represented as the proportion of cells within the independent lymphocyte and monocyte gates, respectively (Fig. 2). Four patients with NSCLC and all nine with gastric cancer were treated with nivolumab, and seven patients with NSCLC and the one patient with bladder cancer were treated with pembrolizumab, as previously described [17]. The clinicopathological characteristics of patients, including patient responses, and OS, the percentages of total or PD-$L1^+$ cells in each subset are summarized in Tables 1 and 2, respectively. As mentioned above, we set different gates independently for lymphocyte and monocyte subsets initially. The percentage of PD-$L1^+$ $CD14^+$ monocytes indicated the proportion of PD-$L1^+$ cells in total $CD14^+$ monocytes (Table 2). We initially analyzed the correlation between the number of respective PBMC subsets and OS. No association was observed between the number of $CD3^+$ T cells, $CD20^+$ B cells, and $CD14^+$ monocytes and OS (r = 0.1613, $p = 0.4850$; $r = -0.0175$, $p = 0.9398$; $r = -0.1070$, $p = 0.6443$; Fig. 3a, d, and e, respectively) among 21 patients before anti-PD-1 blockade therapy. An increased proportion of $CD8^+$ T cells tended to be positively associated with OS, although the correlation was not statistically significant ($r = 0.3901$, $p = 0.0804$, Fig. 3b). These results suggest that changes in the distribution of $CD8^+$ T cells may result from secondary factors influenced by unknown transitions in some PBMC subsets. Therefore, this finding prompted us to further investigate the percentages of $CD4^+$ T cells in 18 patients whose PBMC samples were available for additional experiments. Predictably, we found an inverse correlation between the percentage of $CD4^+$ T cells and OS ($r = -0.5224$, $p = 0.0261$, Fig. 3c).

PD-L1-expressing cells in PBMC subsets associated with different clinicopathological characteristics and clinical responses

As shown in Table 3, no significant correlation was observed between the median percentage of PD-$L1^+$ cells in respective PBMC subsets and the patients' sex, age, cancer type, pathological stage, and response to anti-PD-1 blockade therapy. Interestingly, an increased proportion of PD-$L1^+$ $CD14^+$ monocytes tended to be positively associated with progressive disease (PD) of the best overall response, although the association was not statistically significant ($p = 0.083$, Table 3). Therefore, a considerable improvement of guidelines for the assessment of response to immunotherapies, for instance, a modified RECIST 1.1 for immune-based therapeutics (iRECIST) [19], will be needed to open a window into the investigation of predictive markers for ICI response.

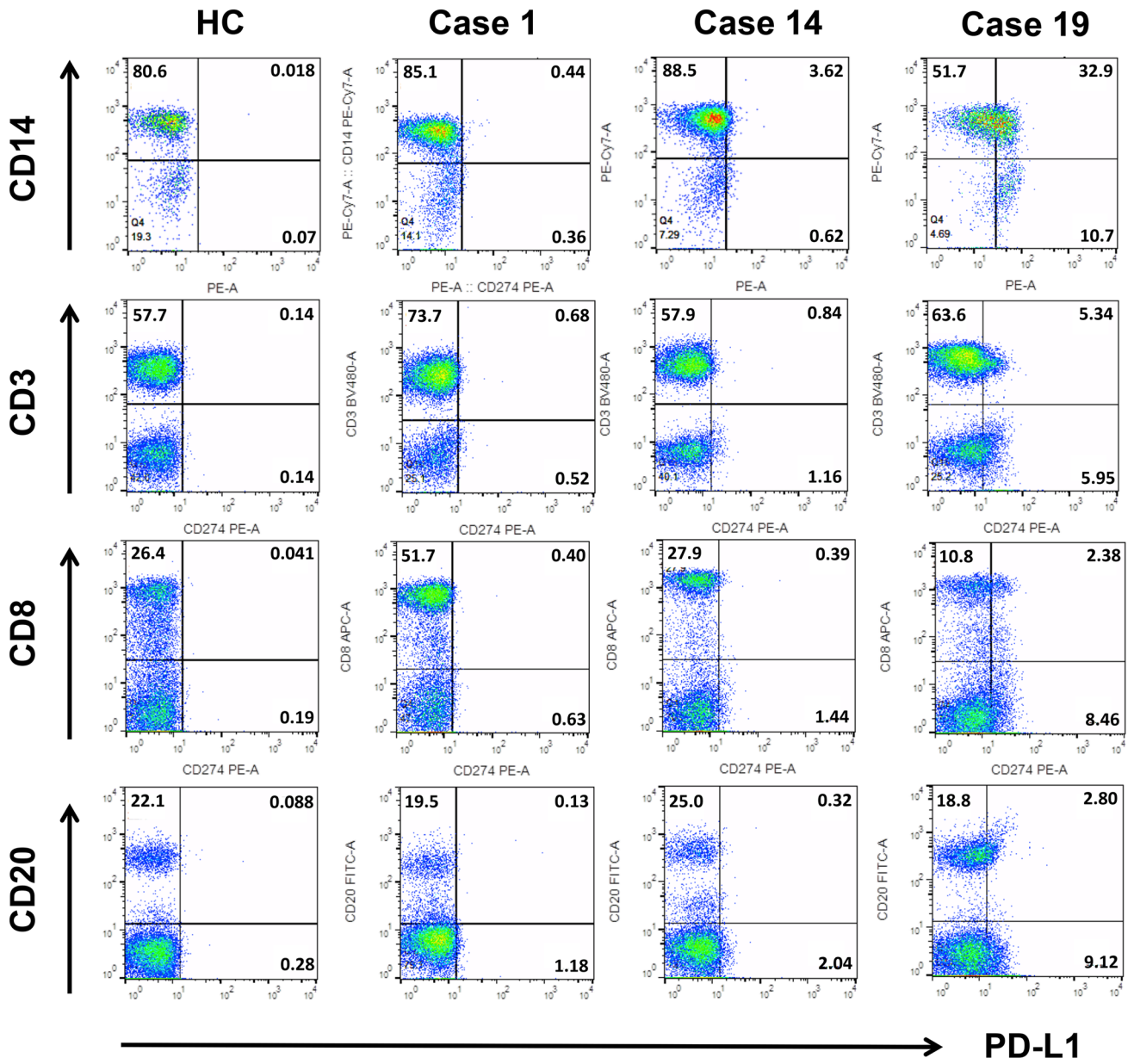

Fig. 1 Lymphocyte and monocyte subsets, and their PD-L1 expression levels in peripheral blood. Representative flow cytometry dot plots for peripheral $CD3^+$, $CD8^+$ T cells, $CD20^+$ B cells, and $CD14^+$ monocytes (vertical axis) and respective PD-$L1^+$ cells (horizontal axis) from healthy controls (HC) and patients (Case 1, 14, and 19). Lymphocyte and monocyte subpopulations were gated on the basis of forward and side scattering, and their subsets were determined through cell surface markers. The percentage of double-positive cells is shown in the upper right area. *PD-L1* programmed death-ligand 1; *CD* cluster of differentiation

Association between PD-$L1^+$ PBMC subsets and patient outcomes

Among the various subsets examined, an increase in the percentage of PD-$L1^+$ $CD14^+$ monocytes was significantly correlated with a shorter OS ($r = -0.4463$, $p = 0.0426$, Fig. 4d), whereas no significant association between OS and the number of PD-$L1^+$ $CD8^+$ T cells, PD-$L1^+$ $CD4^+$ T cells, or PD-$L1^+$ $CD20^+$ B cells ($r = -0.3412$, $p = 0.1302$; $r = 0.2332$, $p = 0.3518$; $r = -0.2051$, $p = 0.3724$, respectively) was observed among these 18 or 21 patients before anti-PD-1 blockade therapy (Fig. 4a, b, c).

To extend these findings to PD-$L1^+$ T cell subsets, we investigated the possible association between patient outcomes and the percentages of PD-$L1^+$ naïve or memory T cells. As shown in Supplementary Table 1, we analyzed the percentages of PD-$L1^+$ $CD4^+$ and PD-$L1^+$ $CD8^+$ T cell subsets in 18 patients based on expression

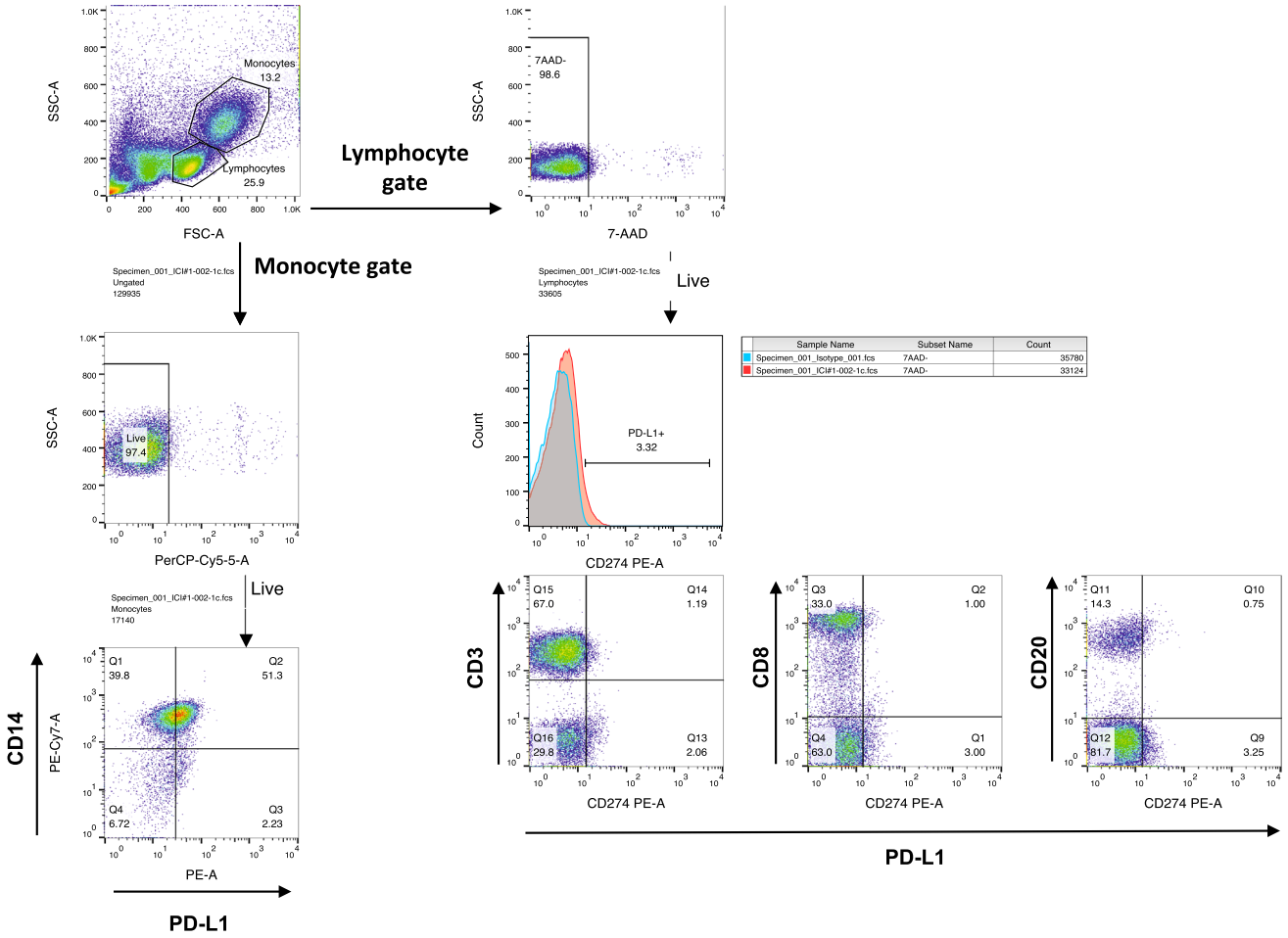

Fig. 2 A schematic representation of gating strategy for PD-L1 expressing lymphocyte and monocyte subpopulations. Gating strategies for peripheral PD-L1-expressing CD14$^+$ monocytes, CD3$^+$ T cells, CD8$^+$ T cells and CD20$^+$ B cells

of CCR7 in combination with CD45RA expression, which is a marker to classify naïve (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$), effector memory (CD45RA$^-$CCR7$^-$), and terminal differentiated effector memory (CD45RA$^+$CCR7$^-$) T cell subsets [20, 21]. No significant correlations were observed between patients' OS and PD-L1$^+$ CD4$^+$ naïve, central memory, effector memory, or terminally differentiated effector memory T cells (r = 0.0005, $p = 0.9984$, $r = 0.2793$, $p = 0.2617$; $r = 0.2774$, $p = 0.2651$; $r = 0.0001$, $p = 0.9997$, respectively; Supplementary Table 1 and Supplementary Fig. 2C a–d). Likewise, no significant correlations were detected between OS and PD-L1$^+$ CD8$^+$ T cell subsets regarding naïve, central memory, effector memory, or terminally differentiated effector memory T cells ($r = -0.3168$, $p = 0.2002$; $r = -0.2329$, $p = 0.3524$; $r = -0.2647$, $p = 0.2885$; $r = -0.1811$, $p = 0.4721$, respectively, Supplementary Table 1 and Supplementary Fig. 2C e–h).

In the past decade, CD16$^+$ monocytes have been subdivided into two subsets, intermediate (CD14^{high}) and non-classical (CD14^{low}), whereas CD16$^-$ monocytes are considered the classical subset [22]. The intermediate and non-classical monocytes are considered to differentiate into M2 macrophages, which have a tumor-promoting function [23]. Therefore, we next evaluated whether these monocyte subsets with PD-L1$^+$ expression could be correlated with patient outcomes. Although the PD-L1$^+$ intermediate monocytes showed a tendency to be associated with patient outcomes among these three subsets, there was no significant correlation observed between OS and the percentages of PD-L1$^+$ classical monocytes, PD-L1$^+$ intermediate monocytes, or non-classical monocytes ($r = -0.0873$, $p = 0.7303$; $r = -0.3148$, $p = 0.2032$; and $r = -0.1618$, $p = 0.5213$, respectively, Table 2 and Supplementary Fig. 2d).

To compensate for the relatively low sample size, which may have prevented detecting a significant effect, we recruited 11 additional patients to confirm whether the significant positive correlation between the percentages of PD-L1$^+$ CD14$^+$ monocytes and OS could be sustained in this setting. Including all 32 patients, we still found a

Table 1 Clinicopathological features, percentages of peripheral blood mononuclear cell subsets, and overall survival (OS) for all patients

Case no	Sex	Age,	Cancer type	Stage	ICI	PD-L1 IHC (%)	$CD3^+$ T cells (%)	$CD4^+$ T cells (%)	$CD8^+$ T cells (%)	$CD20^+$ B cells (%)	$CD14^+$ monocytes (%)	BOR	OS (days)
1	M	78	NSCLC	IV	NIVO	20–30	74.38	24.90	52.10	19.63	85.54	PR	952
2	F	70	NSCLC	IVB	NIVO	50–60	30.32	N/A	43.82	32.28	84.61	SD	167
3	M	67	NSCLC	$IIIA^R$	NIVO	70–80	72.37	31.80	39.39	12.12	81.65	PR	586
4	M	63	GC	$IIIA^R$	NIVO	N/A	73.89	17.70	14.57	5.55	85.63	PD	125
5	M	74	GC	IV	NIVO	N/A	82.02	60.10	37.51	5.88	84.61	PD	144
6	M	68	GC	IV	NIVO	N/A	51.61	N/A	17.48	16.56	70.50	SD	248
7	M	67	NSCLC	IIIA	NIVO	50–60	51.81	28.00	44.23	19.59	79.08	PD	246
8	F	68	GC	IV	NIVO	N/A	52.38	34.20	31.30	16.98	90.30	PD	68
9	M	66	GC	IIIB	NIVO	N/A	65.62	N/A	31.41	11.42	82.90	PD	220
10	M	60	GC	$IIIB^R$	NIVO	N/A	61.75	54.50	24.24	6.25	83.30	PD	43
11	F	49	GC	IIIB	NIVO	N/A	78.21	43.60	20.27	12.26	91.50	PD	74
12	F	75	GC	IV	NIVO	N/A	54.03	46.60	33.02	13.97	92.65	SD	281
13	F	57	GC	IV	NIVO	N/A	74.80	38.60	35.13	9.59	82.60	PD	45
14	M	72	NSCLC	IV	PEMBRO	70–80	58.74	32.80	28.29	25.32	92.12	PD	216
15	M	71	NSCLC	IV	PEMBRO	60–70	71.84	53.90	27.07	12.45	88.05	PR	803
16	M	59	NSCLC	IV	PEMBRO	60–70	73.75	39.20	35.44	20.04	91.90	PD	45
17	M	64	NSCLC	IV	PEMBRO	60–70	66.43	45.70	30.94	10.31	84.99	PR	630
18	M	70	NSCLC	IV	PEMBRO	70–80	52.33	32.50	33.76	10.26	91.60	SD	48
19	M	71	NSCLC	IV	PEMBRO	90	68.94	51.20	13.18	21.60	84.60	PD	133
20	F	70	BLDC	IV	PEMBRO	N/A	48.68	17.70	35.60	12.73	87.52	PR	527
21	M	68	NSCLC	IVB	PEMBRO	10–20	34.46	27.30	26.76	31.85	88.32	PR	161

BLDC bladder cancer; *BOR* best overall response; *F* female; *GC* gastric cancer; *ICI* immune checkpoint inhibitor; *IHC* immunohistochemistry; *M* male; *NIVO* Nivolumab; *NSCLC* non-small cell lung cancer; *PEMBRO* pembrolizumab; *R* recurrence

Table 2 Percentage of PD-L1-expressing peripheral blood mononuclear cell (PBMC) subsets and overall survival (OS) among all patients

Case no	PD-L1$^+$CD3$^+$ T cells (%)	PD-L1$^+$CD4$^+$ T cells (%)	PD-L1$^+$CD8$^+$ T cells (%)	PD-L1$^+$CD20$^+$ B cells (%)	PD-L1$^+$CD14$^+$ monocytes (%)	PD-L1$^+$CD14^{high} CD16$^-$ monocytes (%)	PD-L1$^+$CD14^{low} CD16$^+$ monocytes (%)	PD-L1$^+$CD14^{high} CD16$^+$ monocytes (%)	OS (days)
1	0.68	2.76	0.40	0.13	0.44	32.80	18.10	25.0	952
2	0.82	N/A	0.42	0.48	4.01	N/A	N/A	N/A	167
3	0.87	14.70	0.39	0.12	1.45	46.80	14.70	35.70	586
4	0.69	2.33	0.069	0.075	0.43	17.30	7.79	36.00	125
5	0.82	2.07	0.21	0.046	3.11	30.30	8.40	42.90	144
6	0.71	N/A	0.28	0.16	21.3	N/A	N/A	N/A	248
7	1.01	9.33	0.63	0.19	4.18	21.80	1.85	28.30	246
8	1.48	2.67	0.80	0.48	31.2	34.00	14.50	49.80	68
9	2.32	N/A	1.21	0.62	34.3	N/A	N/A	N/A	220
10	2.25	1.10	1.44	0.26	44.6	66.30	18.40	79.30	43
11	3.21	0.80	0.87	0.56	26.4	13.40	5.22	34.10	74
12	0.73	0.71	0.12	0.37	1.25	3.97	8.03	7.72	281
13	0.60	0.69	0.13	0.31	1.60	7.80	1.69	11.10	45
14	0.84	4.13	0.39	0.32	3.62	60.90	9.68	69.90	216
15	0.74	1.38	0.27	0.15	1.25	26.60	10.20	46.50	803
16	1.25	1.87	0.44	0.24	3.30	32.40	1.95	38.30	45
17	1.43	2.07	0.24	0.31	3.99	18.90	12.10	29.60	630
18	2.73	3.28	1.96	0.16	20.1	42.40	20.20	70.40	48
19	5.34	1.94	2.38	2.80	32.9	40.90	20.90	59.90	133
20	0.68	2.25	0.20	0.43	3.12	10.80	1.34	6.28	527
21	1.06	2.31	0.26	0.85	8.02	17.10	1.87	25.90	161

significant association between the percentage of PD-L1$^+$ CD14$^+$ monocytes and the patients' OS (higher percentage leads to shorter OS; $r = -0.3622$, $p = 0.04164$; Supplementary Table 2 and Supplementary Fig. 1B). However, this analysis per cancer type revealed no significant correlation between the percentage of CD14$^+$ PD-L1$^+$ monocytes and the overall survival of patients: 15 NSCLC, $p = 0.10459$; 14 GC, $p = 0.38005$ (Supplementary Fig. 1b). Therefore, we could not exclude the possibility of a selection bias. For instance, our data set revealed that GC patients showed a shorter OS than that of NSCLC patients ($p = 0.0307$). For validating the results, this study should be conducted on large number of patients.

Collectively, these findings suggest that an increase in the percentage of PD-L1$^+$ CD14$^+$ monocytes might help to predict a poor prognosis before anti-PD-1 blockade therapy in patients with various cancers.

Inverse correlation between the percentage of PD-L1$^+$CD14$^+$monocytes and CD8$^+$ T cells among PBMC subsets

Given accumulating evidence that the number of immune cells can effectively predict the response to anti-PD-1 blockade therapy, PD-L1-positive tumor-infiltrating immune cells have been implicated for ICI therapeutics [11]. This prompted us to investigate the potential correlation between the percentage of PD-L1$^+$ CD14$^+$ monocytes and PD-L1$^+$ CD3$^+$ or PD-L1$^+$ CD8$^+$ T cells among PBMC subsets of our patients. A significant positive correlation was observed between the percentages of PD-L1$^+$ CD14$^+$ monocytes and PD-L1$^+$ CD8$^+$ T cells ($r = 0.7584$, $p < 0.0001$, Fig. 5a) or PD-L1$^+$ CD3$^+$ T cells ($r = 0.7126$, $p = 0.0003$, data not shown). However, there was no correlation between PD-L1$^+$ CD14$^+$ monocytes and PD-L1$^+$ CD4$^+$ T cells ($r = -0.2286$, $p = 0.3615$, Fig. 5b). In contrast with PD-L1$^+$ CD8$^+$ T cells, the percentage of whole CD8$^+$ T cells among all PBMC subsets was negatively correlated with the percentage of PD-L1$^+$CD14$^+$ monocytes ($r = -0.4710$, $p = 0.0312$, Fig. 5c), supporting the tendency found for the reduction in the percentages of CD8$^+$ T cells with a poor OS (Fig. 3b). Notably, a weak but nonsignificant correlation was observed between the percentages of whole CD4$^+$ T cells and PD-L1$^+$CD14$^+$ monocytes ($r = 0.4336$, $p = 0.0722$, Fig. 5d). These results suggest that anti-PD-1 blockade therapy-related patient outcomes may be determined, at least in part, to changes in the relative proportions of CD4$^+$ T cell subsets rather than CD8$^+$ T cells.

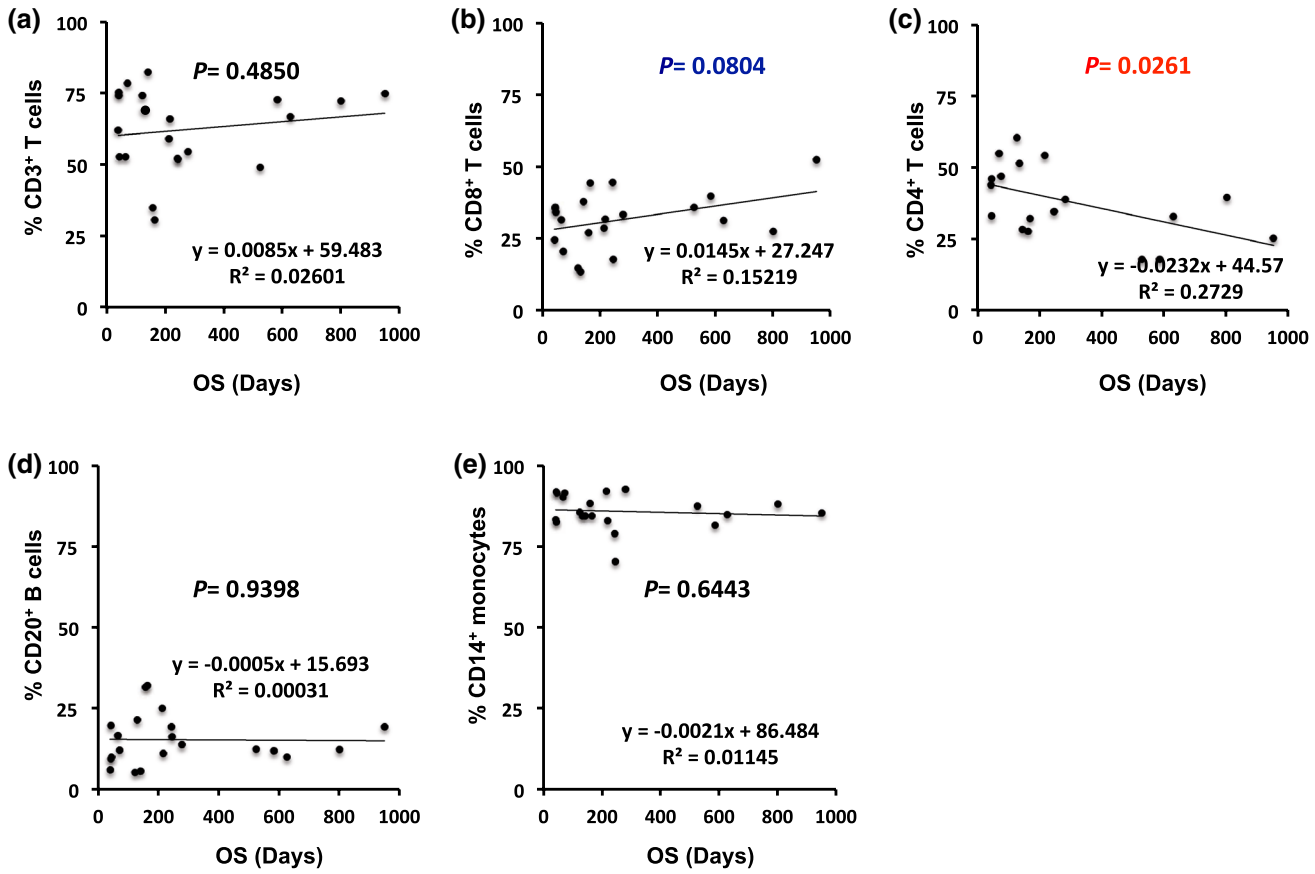

Fig. 3 Linear correlation between peripheral blood mononuclear cell subsets and overall survival (OS). Association between the percentage of peripheral **a** $CD3^+$ T cells, **b** $CD8^+$ T cells, **c** $CD4^+$ T cells, **d** $CD20^+$ B cells or **e** $CD14^+$ monocytes and OS. Each dot represents one specimen from a total of 21 or 18 patients

Discussion

Promotion of T cell-specific immune responses is a central feature of cancer immunotherapy. Effector cytotoxic T cells (CTLs) express the CD8 antigen and play an essential role in the direct elimination of cancer cells. Accumulating evidence has clarified the mechanism underlying cancer cell immune resistance through which the functions of CTLs are suppressed by tumor stromal cells, including cancer-associated fibroblasts, Tregs, and tumor-associated macrophages (TAMs) in the TME, in a phenomenon known as "immune exhaustion" [24, 25]. PD-1/PD-L1 blockade therapy is performed primarily to prevent CTL suppression, and the number of infiltrating $CD8^+$ T cells in the tumor, and PDL-1 expression in the TME have been proposed as candidate biomarkers of immunotherapy responses [12, 26]. Moreover, considering the recent success in using the CPS as a companion diagnostic tool for predicting responses to pembrolizumab, PD-L1 expression in either the tumor membrane or tumor-infiltrating lymphocytes and monocytes has emerged as a potential predictor of the treatment response [11]. However, the clinical implication of each mononuclear cell subset in the peripheral blood, including lymphocytes and monocytes, with respect to their predictive potential has remained unclear. This study shows the potential prognostic significance of patient PBMC subsets before ICI treatment by assessing the correlation between OS and different PBMC subsets or the $PD\text{-}L1^+$ PBMC subset in samples from patients with various cancer types. The most prominent feature of our results is that high rates of PD-L1-expressing $CD14^+$ monocytes detected in the peripheral blood before treatment of nivolumab/pembrolizumab displayed prognostic effects based on an association with poor patient survival.

Most peripheral blood $CD14^+$ monocytes differentiate into macrophages in the TME [27]. Macrophages are classified into at least two subsets as tumoricidal macrophages and tumor-promoting macrophages or TAMs, also referred to as M1 and M2 macrophages, respectively. There is a strong association between poor patient survival and high TAM infiltration in various cancers, including lung, gastric, and bladder cancers [28, 29]. Although it remains controversial whether TAM heterogeneity originates from independent

Table 3 Association between clinical factors and the number of PD-L1-expressing peripheral blood mononuclear cell (PBMC) subsets

Variable		All, n (%)	PD-L1$^+$CD3$^+$ Tcells (%)	*p* value	PD-L1$^+$CD8$^+$ Tcells (%)	*p* value	PD-L1$^+$CD20$^+$ Bcells (%)	*p* value	PD-L1$^+$CD14$^+$ monocytes (%)	*p* value
Sex	Male	15 (71.4)	1.01	0.549	0.39	0.213	0.19	0.847	3.99	0.863
	Female	6 (28.6)	0.78		0.31		0.46		3.57	
Age	< 70 Years	12 (57.1)	1.16	0.751	0.42	0.541	0.29	0.556	6.10	0.259
	≥ 70 Years	9 (42.9)	0.82		0.39		0.32		3.11	
Cancer type	NSCLC	11 (52.4)	1.01	0.63	0.40	0.449	0.24	0.471	3.99	0.179
	Other	10 (47.6)	0.78		0.25		0.34		12.21	
Clinical stage	III	6 (28.6)	0.82	0.603	0.28	0.649	0.31	0.394	3.62	0.319
	IV	15 (71.4)	1.63		0.75		0.23		15.29	
Observed Response	PD	10 (47.6)	1.25	0.150	0.63	0.226	0.31	0.396	4.18	0.083
	PR and SD	11 (52.4)	0.78		0.275		0.24		3.56	

Patients were divided on the basis of the median percentage of indicated subsets

NSCLC non-small cell lung cancer; *PD* progressive disease; *SD* stable Disease

lineages and/or environmental cues [30], the correlation between PD-L1-expressing CD14$^+$ monocytes in PBMCs and patient outcomes is of particular interest. Concurrent with the present results, a previous study reported that the number of PD-L1-expressing CD14$^+$ monocytes was increased in patients with cervical cancer and intraepithelial neoplasia in comparison with that of healthy controls, suggesting the potential involvement of these factors in tumorigenesis [31]. One plausible explanation for our findings is that a large number of PD-L1$^+$ TAMs might be mobilized at tumor sites when PD-L1$^+$ monocytes emerge in PBMC subsets.

Another remarkable finding of this study is that the percentage of PD-L1-expressing CD14$^+$ monocytes was inversely correlated with the percentage of CD8$^+$ T cells. In addition, a weak positive correlation was observed between percentages of PD-L1-expressing CD14$^+$ monocytes and CD4$^+$ T cells in PBMC subsets. A reduction in the percentage of CD8$^+$ T cells displayed a limited tendency to be positively associated with patient survival, whereas the percentage of CD4$^+$ T cells showed a significant negative correlation with patient survival. Thus, we presume that the relative increase in some CD4$^+$ T cell subsets, including Tregs, might influence patient survival. Tregs, as an immunosuppressive subset of CD4$^+$ T cells, are central players in cancer immunity; therefore, targeting Tregs has been an attractive method to potentiate immune therapy [32, 33]. Regarding infiltrating lymphocytes in the tumor tissue, an increasing number of FoxP3$^+$ Tregs has been significantly correlated with a poor patient prognosis in various tumors, whereas a high CD8$^+$/FoxP3$^+$ Tregs ratio was significantly associated with improved OS in certain cancers [34]. Similarly to the tumor tissue, an increase in the number of CD4$^+$ Tregs in PBMC subsets might result in a relative reduction in the number of CD8$^+$ subsets. However, in our experimental conditions, we observed no correlation between the percentage of CD4$^+$/FOXP3$^+$ Tregs and OS ($r = 0.0430$, $p = 0.9526$, Supplementary Fig. 3, Supplementary Table 3 and Supplementary Materials and Methods). Therefore, the predictive importance of the CD4$^+$/FOXP3$^+$ Tregs before ICI treatment might not lie on their presence in PBMC. Additionally, recent evidence suggests that the biological interaction between T cells and monocytes results in a significant proportion of cell doublets in flow cytometry [35]. Further improvements in evaluation focusing on CD4$^+$ and/or PD-L1$^+$ CD14$^+$ PBMC subsets will clarify their predictive importance for poor patient outcomes before ICI treatment.

In this study, we mainly focused on the clinical significance of PD-L1-expressing PBMC subsets for patients with several cancer types receiving treatment with anti-PD-1 antibodies. The following were the limitations of this study: the sample size was relatively small; the study relied on the evaluation of mixed samples from patients with several cancer

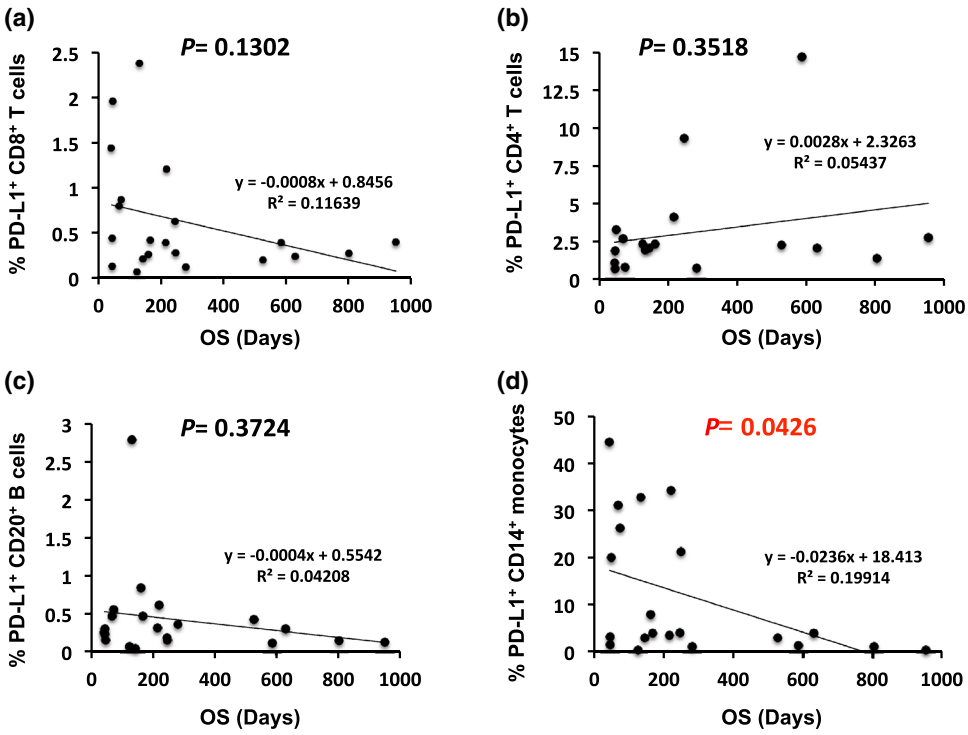

Fig. 4 Linear correlation between PD-L1-expressing peripheral blood mononuclear cell subsets and overall survival (OS). Association between the percentage of peripheral PD-L1-expressing **a** $CD8^+$ T cells, **b** $CD4^+$ T cells, **c** $CD20^+$ B cells, and **d** $CD14^+$ monocytes and OS. Each dot represents one specimen from a total of 18 or 21 patients

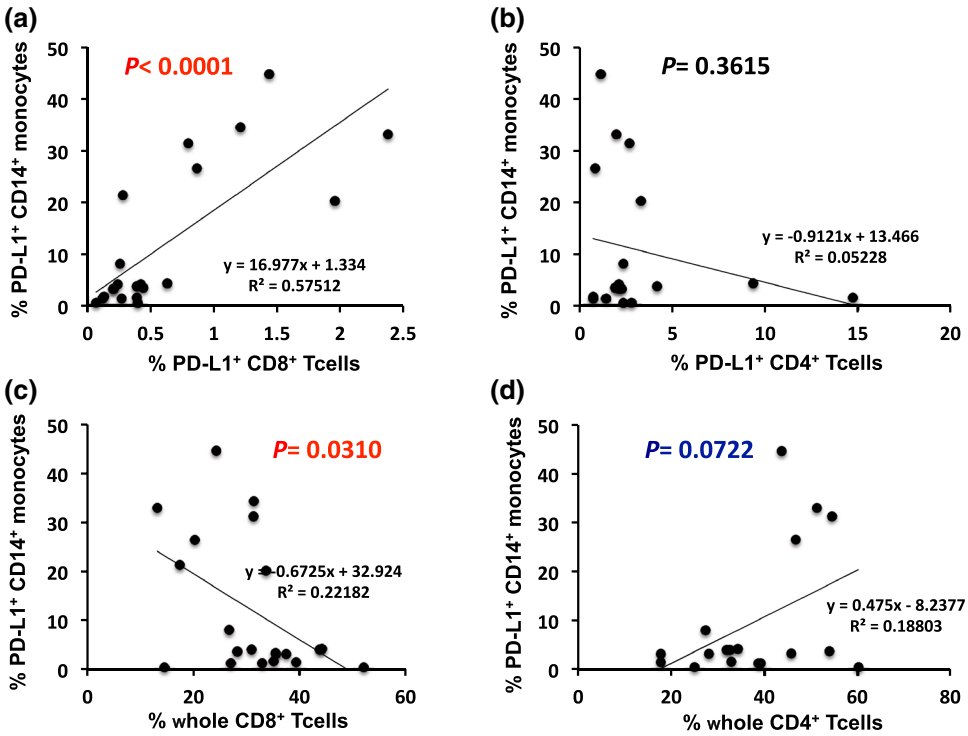

Fig. 5 Linear correlation between PD-L1-expressing $CD14^+$ monocytes and T cell subsets. Association between the percentage of peripheral PD-L1-expressing $CD14^+$ monocytes and PD-L1-expressing **a** $CD8^+$ T cells or **b** $CD4^+$ T cells, and peripheral PD-L1-expressing $CD14^+$ monocytes and whole **c** $CD8^+$ T cells or **d** $CD4^+$ T cells. Each dot represents one specimen from a total of 21 or 18 patients

types who were undergoing ICI treatment, implicating the focus was not on a single cancer. Nonetheless, to our knowledge, this is the first study to report the prognostic significance of the distribution of PD-L1-expressing subsets in circulating PBMCs, particularly $CD14^+$ monocytes. The main advantage in identifying circulating levels of these subsets is that the blood is more accessible than tumor samples in a clinical setting, thereby offering a non-invasive biomarker to facilitate repeated testing and monitoring of the treatment response. Further studies on improving cell surface markers

distinguishing PBMC subsets, including $CD4^+$ lymphocyte and $CD14^+$ monocyte subsets, are expected to reveal their prognostic impact and predictive factors of responses to ICI treatment.

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Author contributions KH and SW conceived and designed this study. KH, RO, YK, AH, HM, TI, YH, HA, TA, and SW collected samples and recorded the general data and patient indications. MS, MW, and RO prepared the samples. MS and RO performed all flow cytometry analyses. KA analyzed the data and wrote the first draft of the manuscript. JT, KY, TT, SK, and SW critically reviewed and revised the manuscript. All Authors reviewed and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest None of the Authors declares any conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The "Showa University Ethics Committee" approved the study with number 2165 and 2253.

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