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Immune checkpoint protein and cytokine expression by T lymphocytes in pleural effusion of cancer patients receiving anti–PD-1 therapy

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ABSTRACT

Objectives: Pleural effusion (PE) occasionally develops in cancer patients during treatment with antibodies to programmed cell death–1 (PD-1) or to its ligand PD-L1 (hereafter, αPD-1 therapy). Such effusion often contains infiltrated mononuclear cells, although the types of immune cell present as well as the outcome of such patients have remained unclear.

Materials and methods: We performed a multi-institutional, observational study to examine the clinical outcome of patients who develop PE after the onset of αPD-1 therapy. We compared the immune cell profiles and the immune status of lymphocytes in PE as determined by flow cytometry between nine patients who developed effusion during αPD-1 therapy (αPD-1 group) and 15 patients who developed PE during treatment with other anticancer agents (control group).

Results: Most mononuclear cells in PE were lymphocytes in both the αPD-1 and control groups. The frequency of both $CD4^+$ and $CD8^+$ T lymphocytes expressing the immune checkpoint proteins TIM-3 or TIGIT as well as that of $CD8^+$ T lymphocytes expressing PD-L1 were increased in the αPD-1 group compared with the control group. αPD-1 therapy continued for a substantial period after the emergence of PE in six of the nine patients in the αPD-1 group, and the frequency of $CD4^+$ T lymphocytes in PE expressing the immune checkpoint protein LAG-3 or the cytokine interkeukin-17 was lower for these patients than for those who did not receive a sustained treatment benefit.

Conclusion: Our results suggest a clinical benefit of continuing αPD-1 therapy in some patients who develop PE. We found that infiltrating T lymphocytes in PE manifest a more exhausted phenotype during αPD-1 therapy than during treatment with other cancer drugs, with subpopulations of these cells characterized by specific immune checkpoint protein and cytokine expression profiles possibly contributing to the antitumor immune response.

1. Introduction

The emergence of pleural effusion (PE) is usually a sign of progressive disease in individuals with cancer. In many such cases, the PE contains malignant cells and is referred to as malignant pleural effusion. It is treated with repeated thoracentesis or chemical pleurodesis, but it is associated with poor prognosis [1], with a median survival time after its diagnosis of ~6 months [2,3].

The development of antibodies to programmed cell death–1 (PD-1) and to its ligand PD-L1 (hereafter referred to as αPD-1 therapy) and of other immune checkpoint inhibitors has brought about a paradigm shift in the treatment of various cancers. αPD-1 therapy achieves a clinical response by interrupting the interaction of PD-L1 on tumor and other cells with PD-1 on $CD8^+$ T lymphocytes and thereby reactivating effector T cell function [4–9]. We recently experienced a case of PE containing abundant lymphocytes that developed in a kidney cancer patient after the onset of αPD-1 therapy and which was followed by pronounced shrinkage of a metastatic tumor in the chest wall [10]. The patient was able to continue αPD-1 therapy until a severe adverse event occurred, and no tumor recurrence was observed after the cessation of

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treatment. This case raised the question of whether emerging effusion under αPD-1 therapy has a distinct clinical meaning.

The pleural space of individuals with cancer and PE often contains abundant lymphocytes and can be considered a tumor microenvironment (TME) [11,12]. PD-1–PD-L1 signaling is a key mechanism for suppression of the activity of tumor-infiltrating lymphocytes in the TME, with the exhausted T cells being characterized by down-regulation of the production of effector cytokines such as interferon-γ (IFN-γ) and impaired cytotoxicity [13,14]. On the other hand, αPD-1 therapy does not always result in complete restoration of T cell function, and the outcome of such therapy remains limited [15,16]. This situation has promoted further study of the immune cells present in the TME for the identification of biomarkers that may allow prediction of the efficacy of αPD-1 therapy or serve as a target for enhancement of antitumor immunity.

Co-inhibitory receptors other than PD-1—such as T cell immunoglobulin mucin–3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), and lymphocyte activation gene–3 (LAG-3)—are up-regulated on exhausted T lymphocytes and regulate effector T cell function in the TME [17], although their precise roles have remained unclear. In addition to immune checkpoint molecules, cytokines produced by T cells in the TME are thought to contribute to the status of the antitumor response [18], but the mechanism by which tumor-infiltrating lymphocytes produce cytokines in PE after the initiation of αPD-1 therapy and the relation of such cytokine production to the outcome of treatment are unknown.

To elucidate how patients who develop PE might benefit from continuation of αPD-1 therapy and to provide insight into the characteristics of PE in such patients, we examined the clinical outcome of patients and analyzed infiltrating mononuclear cells by flow cytometry. In the present study, we analyzed not only immune cell profiles but also the expression of immune checkpoint proteins and cytokine production by T lymphocytes in PE from cancer patients undergoing αPD-1 therapy or other anticancer treatments in order to shed light on the immune response mediated by T lymphocytes during αPD-1 therapy and its possible association with clinical efficacy.

2. Materials and methods

2.1. Patients and sample collection

This multi-institutional, observational study was approved by the institutional review board of each institution and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent. PE was collected via thoracentesis at the time cancer patients developed PE during anticancer treatment. Patients who manifested clinical or laboratory signs of acute or chronic infection were excluded from the study. Those with transudative PE according to Light's criteria [19] were also excluded. The total cell count in PE was determined with an automated instrument at each hospital. The effusion samples were centrifuged at 400 × *g* for 5 min, and the cell pellets were washed twice with RPMI 1640 medium. If necessary, immune cells including lymphocytes were separated from red blood cells by centrifugation with Ficoll-Paque Plus (GE Healthcare Life Sciences) and were then suspended in RPMI 1640 medium.

2.2. Flow cytometric analysis

Antibodies used for flow cytometry were summarized in Supplementary Table 1 and surface staining of cells was performed as previously described [20]. For measurement of intracellular interleukin (IL)–17 and IFN-γ, mononuclear cells (1×10^6/ml) were stimulated for 6 h at 37 °C under 5% CO2 with Leukocyte Activation Cocktail with BD Golgiplug (2 μl/ml) (BD Biosciences). The cells were then stained with antibodies to CD3, CD4, and CD8 before fixation and permeabilization with the use of a Cytofix/Cytoperm-Plus Fixation/Permeabilization Kit (BD Biosciences). For measurement of intracellular perforin and granzyme B, cells were stained without stimulation, fixed and permeabilized in same ways as those of IL-17 and IFN-γ. Finally, intracellular staining was performed with antibodies to human IL-17, IFN-γ, perforin,-granzyme B and mouse isotype control, respectively.

2.3. Statistical analysis

Data were compared with Student's *t* test. All statistical analysis was performed with JMP software version 13 (SAS Institute). A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Clinical course of patients developing PE during αPD-1 therapy

Between February 2017 and October 2018, we identified nine patients who developed PE after the onset of αPD-1 therapy (αPD-1 group). The clinical course of these patients is summarized in Table 1. They included one patient with renal cell carcinoma, six with lung adenocarcinoma, and two with squamous cell lung carcinoma. Four of the nine patients received nivolumab, and the others received pembrolizumab. PE of six patients (67%) was found to contain malignant cells. The median time before development of PE after the onset of αPD-1 therapy was 31 days (range, 7–49 days).

To elucidate specific characteristics of PE emerging during αPD-1 therapy, we also examined patients who developed PE while receiving anticancer treatments other than αPD-1 therapy. During the same period, we identified 15 such patients (control group). The clinicopathologic characteristics of the αPD-1 group and the control group, including age, sex, type of cancer, histology, and therapy, are summarized in Table 2.

Table 1
Clinical course of patients in the anti–PD-1 therapy (αPD-1) group.

Patient	Diagnosis	TNM stage	PD-L1 TPS (%)	Malignant cells in PE	Therapy	Time to PE emergence after onset of αPD-1 therapy (days)	Continuation of αPD-1 therapy after PE	TTS (days)
1	Kidney cancer CCC	cTXN0M1b stage IV	UN	(–)	Nivo	26 (after 2 courses)	Yes	> 806 (NR)
2	Lung cancer ADC	cT2aN2M1a stage IV	80	(+)	Pembro	9 (after 1 course)	Yes	> 627 (NR)
3	Lung cancer ADC	cT1bN2M1c stage IV	≥90	(–)	Pembro	49 (after 3 courses)	Yes	> 217 (NR)
4	Lung cancer SCC	pT4N2M0 stage IIIB	65	(–)	Pembro	44 (after 2 courses)	Yes	148
5	Lung cancer ADC	cT4N2M1b stage IV	35	(+)	Pembro	31 (after 2 courses)	Yes	132
6	Lung cancer ADC	cT1bN2M0 stage IIIB	≥90	(+)	Pembro	33 (after 2 courses)	Yes	109
7	Lung cancer SCC	cT1cN3M1c stage IV	1–24	(+)	Nivo	7 (after 1 course)	Yes	46
8	Lung cancer ADC	cT1bNoM1a stage IV	15	(+)	Nivo	37 (after 3 courses)	No	37
9	Lung cancer ADC	cT2aN2M1a stage IV	0	(+)	Nivo	15 (after 1 course)	No	21

TPS, tumor proportion score; PE, pleural effusion; TTS, time before implementation of a change in treatment strategy; CCC, clear cell carcinoma; ADC, adenocarcinoma; SCC, squamous cell carcinoma; UN, unknown; Nivo, nivolumab; Pembro, pembrolizumab; NR, not reached.

Table 2
Clinicopathologic characteristics of patients in the anti–PD-1 therapy (αPD-1) group and the control group.

Characteristic			αPD-1 (n = 9)	Control (n = 15)
Age (years)				
		Median	67	70
		Range	64–75	48–79
Sex				
		Male	4	11
		Female	5	4
Type of cancer				
	Kidney cancer		1	2
		Clear cell carcinoma	1	2
	Lung cancer		8	13
		Adenocarcinoma	6	9
		Squamous cell carcinoma	2	3
		Small cell carcinoma	0	1
Malignant cells in PE				
		Positive	6	13
		Negative	3	2
Type of therapy				
		Nivolumab	4	0
		Pembrolizumab	5	0
		Cyototoxic drug	0	8
		Tyrosine kinase inhibitor	0	7

3.2. Up-regulation of immune checkpoint molecules on T cells in PE of the αPD-1 group

We analyzed mononuclear cells by flow cytometry in order to characterize the types of immune cell present in PE. The total cell number as well as the number of lymphocytes ($CD45^+CD3^+$ cells and $CD45^+CD19^+$ cells) in PE did not differ significantly between the αPD-1 group and the control group (Fig. 1A and B). The percentage of natural killer (NK) cells ($CD45^+CD3^-CD19^-CD56^+$ cells) or myeloid cells ($CD45^+CD3^-CD19^-CD11b^+$ cells) also did not differ between the two groups (Supplementary Fig. 1A and B). More than half of mononuclear cells in PE were found to be lymphocytes in both groups (Fig. 1C), showing that most infiltrating immune cells after the onset of αPD-1 therapy were lymphocytes. Further analysis of lymphocyte subsets revealed that the percentage of B lymphocytes ($CD45^+CD19^+$ cells) was significantly lower in the αPD-1 group ($P = 0.012$) (Fig. 1D), whereas neither the frequency of T lymphocytes ($CD45^+CD3^+$ cells), $CD4^+$ T lymphocytes ($CD3^+CD4^+$ cells), or $CD8^+$ T lymphocytes ($CD3^+CD8^+$ cells) nor the CD4/CD8 cell ratio differed significantly between the two groups (Fig. 1E and Supplementary Fig. 1C-E).

To examine whether αPD-1 therapy might affect the expression of immune checkpoint proteins on T lymphocytes in PE, we measured the surface expression of PD-1, TIM-3, TIGIT, LAG-3, and PD-L1 in both the αPD-1 group and the control group (Fig. 2). The frequency of both $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes expressing TIM-3 or TIGIT as well as that of $CD8^+$ T lymphocytes expressing PD-L1 were significantly higher in the αPD-1 group than in the control group (Fig. 3), suggesting that T lymphocytes show a more exhausted phenotype under αPD-1 treatment.

To investigate the specific functional characteristics of T lymphocytes in PE of the αPD-1 group, we also evaluated the expression profiles of the cytotoxicity mediators perforin and granzyme B as well as of the signature cytokines IL-17 and IFN-γ. The frequency of $CD4^+$ T lymphocytes or $CD8^+$ T lymphocytes producing each protein did not differ between the αPD-1 and control groups, however (Supplementary Fig. 2 and Supplementary Fig. 3).

3.3. Clinical benefit of continuing αPD-1 therapy after PE development

αPD-1 therapy for the study subjects continued after the development of PE if the investigator deemed the patients to be still receiving a clinical benefit. We defined a time before implementation of a change in treatment strategy (TTS) of ≥90 days as clinical benefit and a TTS of < 90 days as nonbenefit (Table 1). Six of the nine patients in the αPD-1 group were thus classified as having received a clinical benefit. All three of the nonbenefit patients manifested malignant cells in their PE, whereas three of the six patients in the benefit group did so. The median TTS of the clinical benefit group was 182.5 days, compared with 37 days for the nonbenefit group ($P = 0.132$).

3.4. Relation of immune checkpoint protein or cytokine profiles of T lymphocytes to the antitumor immune response during αPD-1 therapy

The fact that six of the nine patients in the αPD-1 group continued to show apparent disease control after the emergence of PE led us to perform an exploratory analysis of the relation between response to αPD-1 therapy and the characteristics of T lymphocytes in PE. We found that the number and frequency of lymphocytes did not differ significantly between the clinical benefit group and the nonbenefit group (data not shown). Examination of the expression of immune checkpoint proteins (Fig. 4A), cytotoxicity mediators (Supplementary Fig. 4), and cytokines (Fig. 4B and C) in T lymphocytes revealed that the frequency of $CD4^+$ T lymphocytes expressing LAG-3 or IL-17 was significantly higher in the nonbenefit group than in the clinical benefit group.

4. Discussion

Our observational study has shown that some patients are able to receive clinical benefit by continuing αPD-1 therapy after the emergence of PE, indicating that the clinical course of these patients differs from that of patients who develop PE during treatment with conventional anticancer drugs. We found that lymphocytes constituted the majority of immune cells in PE and that αPD-1 therapy did not significantly affect the number or percentage of lymphocytes, NK cells, or myeloid cells compared with other anticancer treatments, whereas αPD-1 therapy was associated with an increased proportion of T lymphocytes expressing TIM-3, TIGIT, or PD-L1. In addition, LAG-3 expression and IL-17 production by $CD4^+$ T lymphocytes were negatively associated with clinical outcome in patients receiving αPD-1 therapy. As far as we are aware, our study is the first to examine the expression of multiple checkpoint proteins and cytokine production by T lymphocytes in PE of cancer patients receiving αPD-1 therapy.

αPD-1 therapy has been associated with a pattern of immune-related responses [21] that differ from responses to cytotoxic agents. The clinical course and characteristics of PE for patients in the αPD-1 group of the present study suggest that the activation of T lymphocytes by such therapy might lead to an immune-related reaction that is occasionally followed by clinical tumor shrinkage. Case reports have described emergence of PE followed by clinical benefit during αPD-1 therapy [22–24]. Our observational study confirms the existence of such patients. At present, there is no definitive PE-based biomarker for prediction of subsequent clinical response to αPD-1 therapy. Patients who develop PE soon after the onset of αPD-1 therapy should thus be carefully evaluated so that some are able to receive clinical benefit by continuing such therapy.

In addition to PD-1 and cytotoxic T lymphocyte–associated protein–4 (CTLA-4), which serve as targets for immune checkpoint inhibitors in clinical practice, immune checkpoint proteins including TIM-3, TIGIT, and LAG-3 are expressed on exhausted or dysfunctional T lymphocytes [17,25]. The apparent functional overlap among these proteins in immune suppression might contribute to the maintenance of immune homeostasis in the event that one or more of them becomes

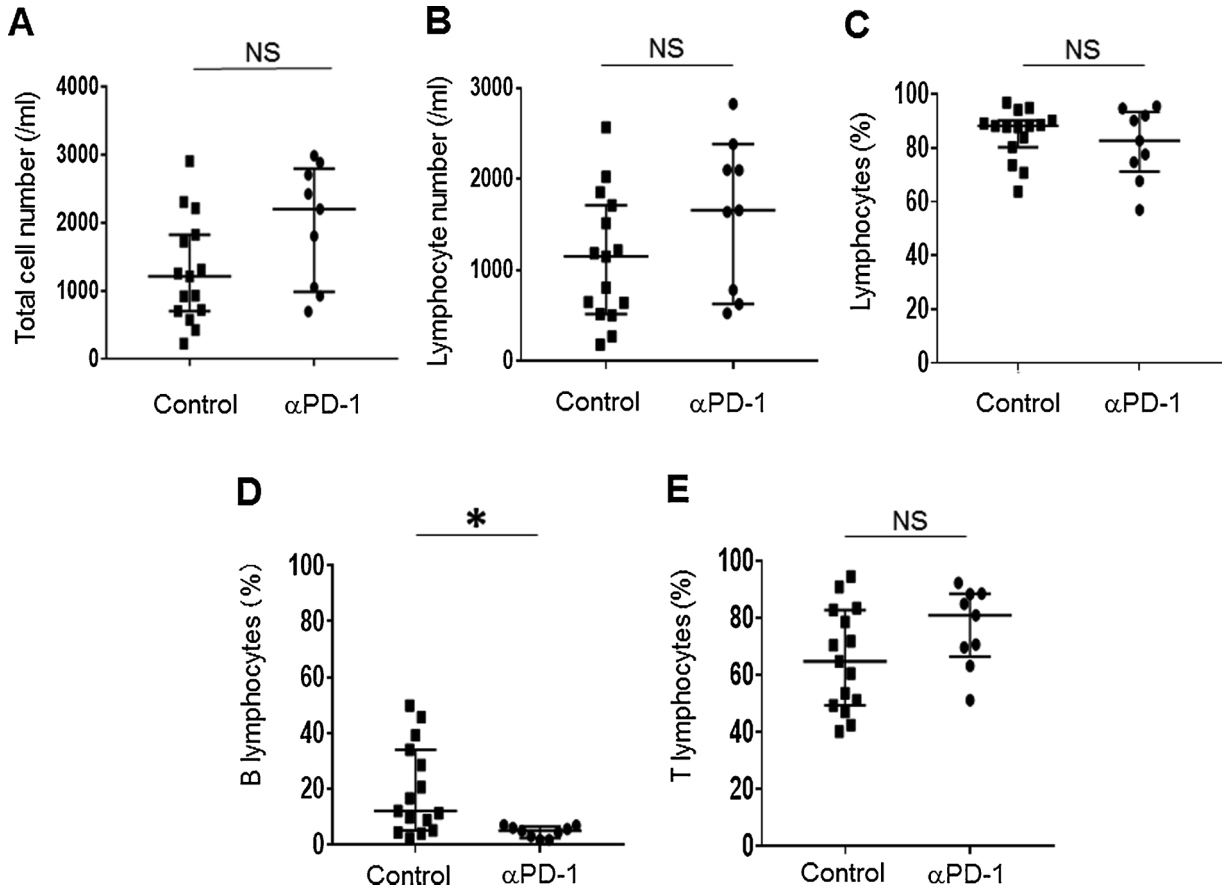

Fig. 1. Lymphocyte subsets in pleural effusion of patients in the anti–PD-1 therapy (αPD-1) and control groups as determined by flow cytometry. The total cell number (A); total number of lymphocytes ($CD45^+CD3^+$ cells and $CD45^+CD19^+$ cells) (B) and percentage of lymphocytes (C); frequency of B lymphocytes ($CD45^+CD19^+$ cells) (D) or T lymphocytes ($CD45^+CD3^+$ cells) (E) among all lymphocytes were determined for pleural effusion samples of patients in the αPD-1 (n = 9) and control (n = 15) groups. Bars indicate the median ± interquartile range. *$P < 0.05$; NS, not significant (Student's *t* test).

compromised. However, αPD-1 therapy was found to result in up-regulation of TIM-3 expression on $CD8^+$ T lymphocytes in association with the development of treatment resistance in a mouse model of lung cancer, and the addition of an antibody to TIM-3 to αPD-1 therapy overcame this resistance [26]. This previous study suggested to us that TIM-3, TIGIT, and LAG-3 expressed on the surface of T lymphocytes might all play specific roles in the TME. Our results now show that PD-1 blockade was associated with increased TIM-3 and TIGIT expression on both $CD4^+$ and $CD8^+$ T lymphocytes in PE as well as with increased PD-L1 expression on $CD8^+$ T lymphocytes. These findings might reflect the operation of an autoregulatory mechanism to prevent the overreaction of T lymphocytes in response to αPD-1 therapy. Among the checkpoint proteins examined, we found that an increased frequency of LAG-3 expression on $CD4^+$ T lymphocytes was associated with a shorter duration of response to αPD-1 therapy. LAG-3 has been found to be expressed on $CD4^+$ T lymphocytes with a regulatory function such as regulatory T cells [27]. Our findings thus suggest that regulatory T cells might contribute to the development of immune unresponsiveness to αPD-1 therapy [28].

Furthermore, the frequency of IL-17–producing $CD4^+$ T lymphocytes, which include T helper 17 (Th17) cells, was increased in PE of patients who did not benefit from αPD-1 therapy compared with those who did. In a mouse model of *KRAS* mutation–positive lung cancer, expression of an IL-17 transgene was shown to confer intrinsic resistance to αPD-1 therapy, suggesting that IL-17 contributes to tumor promotion [29]. On the other hand, pleural Th17 cells are associated with better survival in non–small cell lung cancer patients receiving cancer treatments other than αPD-1 therapy [30]. The functions of IL-17 and Th17 cells in the TME thus appear to be complex and might be dependent on treatment type. Our data suggest that an increased number of Th17 cells in PE might be a sign of early resistance to PD-1 blockade. We thus found that the response to αPD-1 therapy was associated not with the characteristics of $CD8^+$ T lymphocytes but with those of $CD4^+$ T cells, suggesting that $CD4^+$ T lymphocytes may play an essential role in orchestration of immune reactions in cancer patients receiving αPD-1 therapy [31–34].

Our study has several limitations. First, given that the number of cancer patients who develop PE after the onset of αPD-1 therapy is limited [6,35], the number of such patients enrolled in the study was small. Second, we did not compare immune checkpoint protein expression and cytokine production by T lymphocytes in PE with those in paired tumor specimens. However, sampling of tumor tissue during treatment is invasive and ethically not justifiable, especially in patients with lung cancer.

In conclusion, our study has revealed that there is a substantial number of patients who can benefit from continuation of αPD-1 therapy after the emergence of PE. Our findings indicate that lymphocytes account for most of the immune cells in PE and that αPD-1 therapy upregulates the expression of immune checkpoint proteins other than PD-1 on T lymphocytes in PE. Furthermore, increased expression of LAG-3 and IL-17 by $CD4^+$ T lymphocytes was apparent in emergent PE of patients refractory to αPD-1 therapy. Our results thus provide insight into immune responses in the TME and provide a basis for further development of biomarkers for prediction of the efficacy or expansion of the therapeutic reach of cancer immunotherapy.

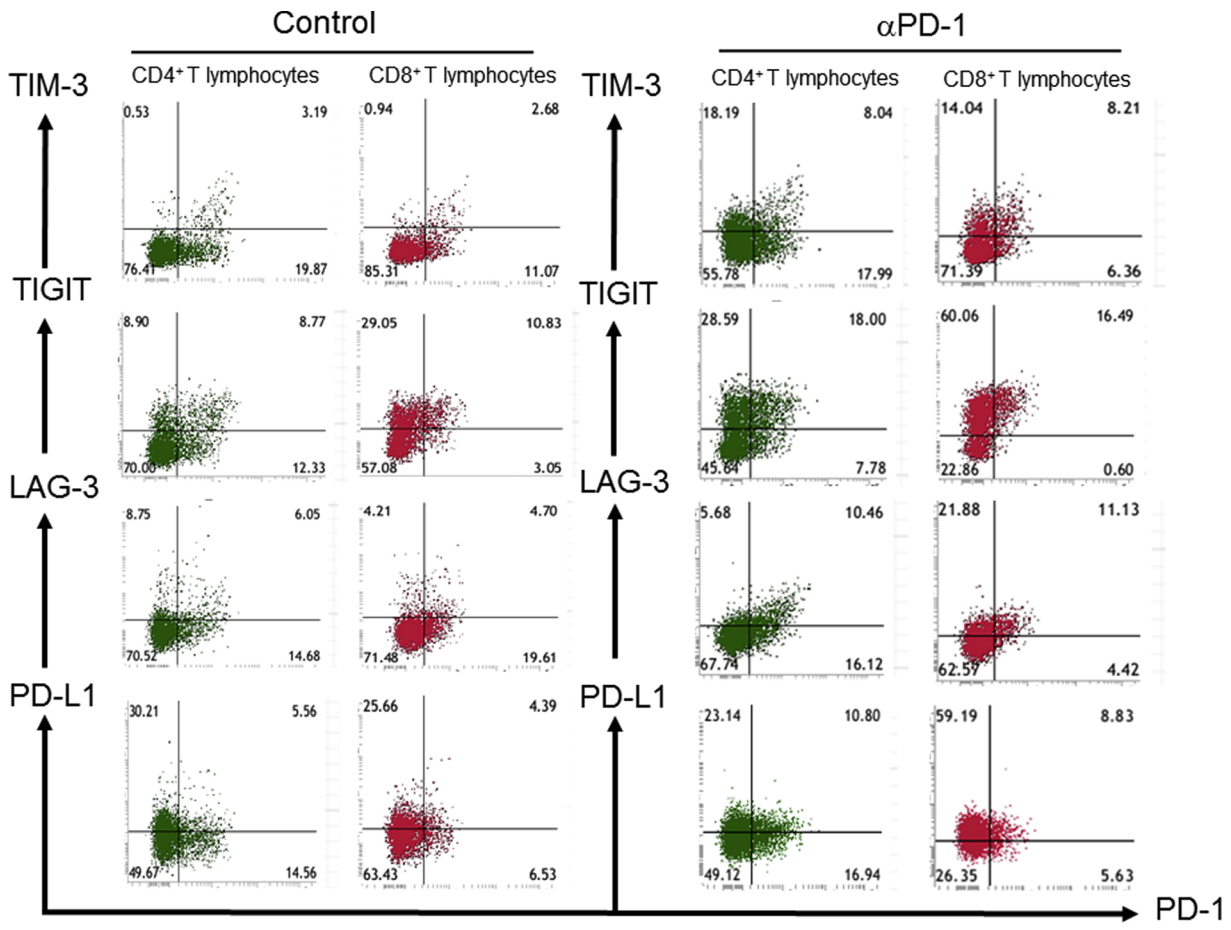

Fig. 2. Representative flow cytometric analysis of immune checkpoint protein expression on $CD4^+$ or $CD8^+$ T lymphocytes in pleural effusion of patients in the anti–PD-1 therapy (αPD-1) and control groups. The surface expression of PD-1 and either TIM-3, TIGIT, LAG-3, or PD-L1 is shown. The patient in the αPD-1 group is no. 2 in Table 1.

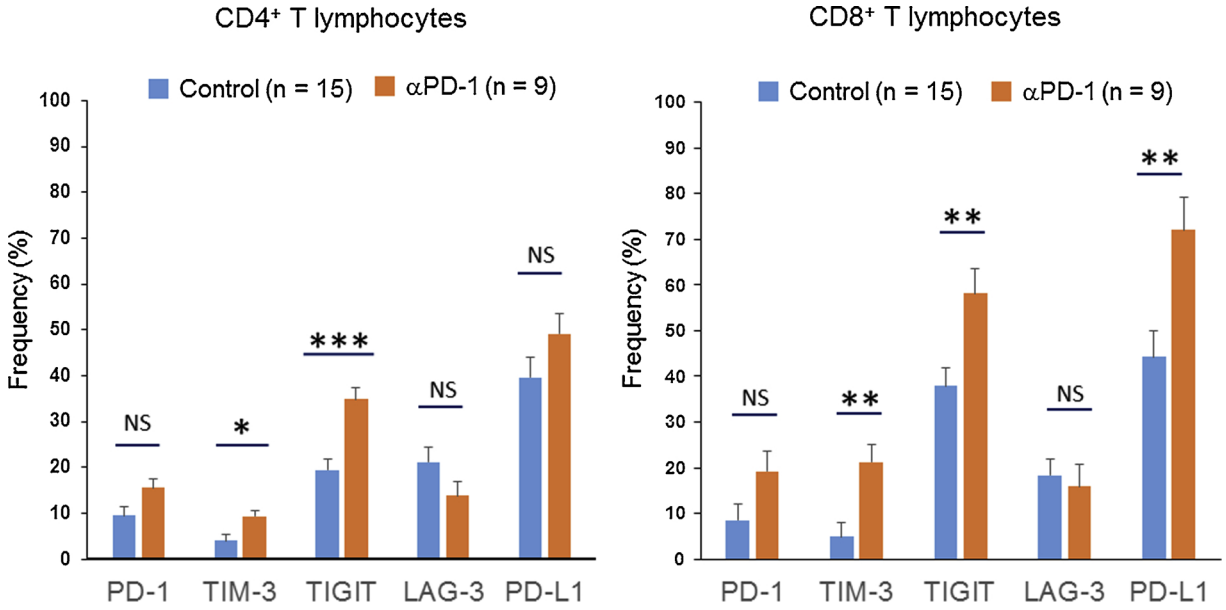

Fig. 3. Immune checkpoint protein expression by $CD4^+$ or $CD8^+$ T lymphocytes in pleural effusion of patients in the anti–PD-1 therapy (αPD-1) and control groups. Expression of PD-1, TIM-3, TIGIT, LAG-3, and PD-L1 for the αPD-1 group (n = 9) and the control group (n = 15) were determined by flow cytometry. Data in figure are means + SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; NS, not significant (Student's *t* test).

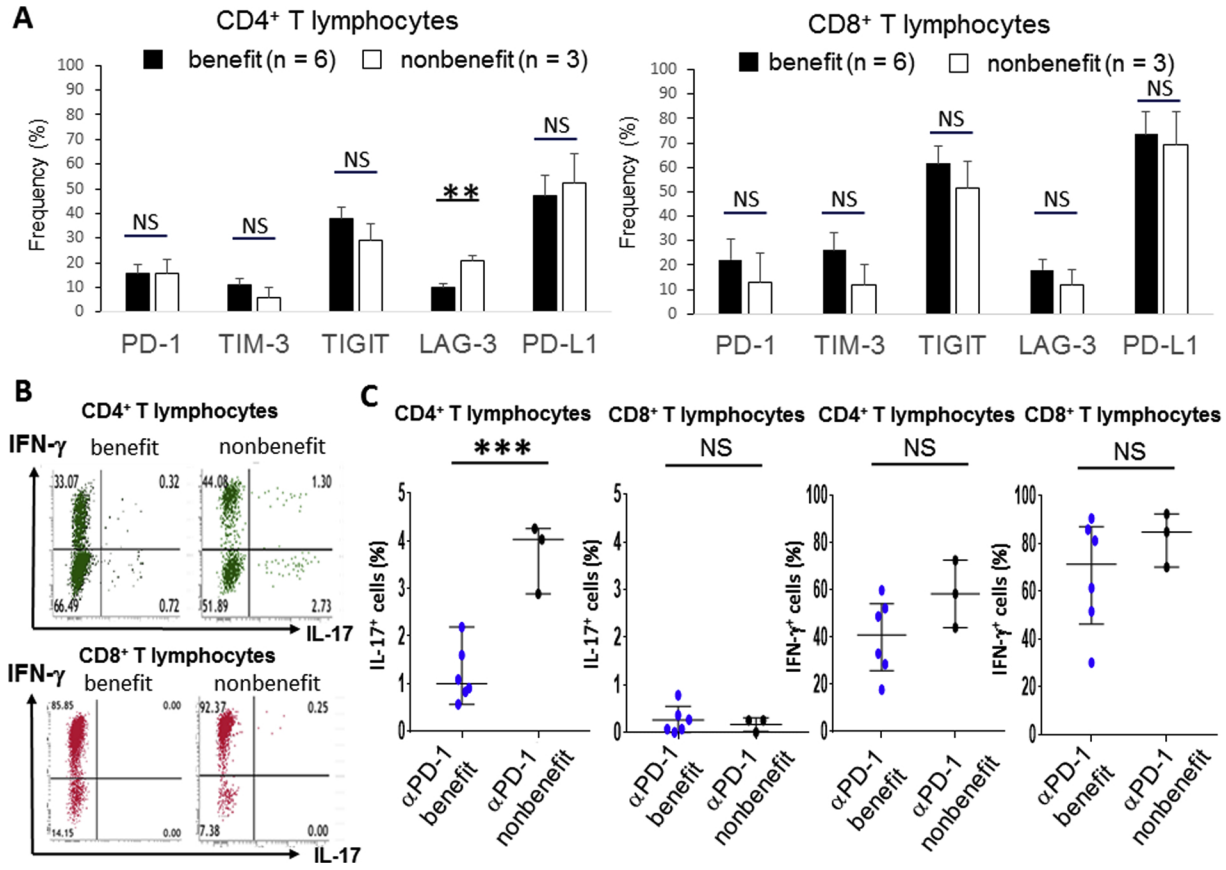

Fig. 4. Immune checkpoint protein expression and cytokine production by $CD4^+$ or $CD8^+$ T lymphocytes in pleural effusion of patients in the anti–PD-1 therapy (αPD-1) group who benefited or did not benefit from treatment. (A) Expression of PD-1, TIM-3, TIGIT, LAG-3, and PD-L1 for the benefit (n = 6) and nonbenefit (n = 3) subgroups of the αPD-1 group was determined by flow cytometry. (B and C) Representative flow cytometric analysis (B) and combined data (C) for IL-17 and IFN-γ production by $CD4^+$ or $CD8^+$ T lymphocytes for the benefit (n = 6) and nonbenefit (n = 3) subgroups of the αPD-1 group. Data in (A) are means + SEM, whereas bars in (C) indicate the median ± interquartile range. $^{**}P < 0.01$, $^{***}P < 0.001$; NS, not significant (Student's *t* test).

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.10.011.

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