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ORIGINAL ARTICLE – CANCER RESEARCH

Characterization of soluble PD-L1 in pleural effusions of mesothelioma patients: potential implications in the immune response and prognosis

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Abstract

Purpose Programmed death-ligand 1 (PD-L1) protein plays a central role in the antitumor immune response, and appears to be a predictor of prognosis and efficacy for PD-L1 and programmed death 1 (PD-1) blockade therapy. The immunoregulatory role and prognostic impact of PD-L1 soluble form (sPD-L1) have been investigated in biological fluids of patients with different tumors. In malignant pleural mesothelioma (MPM), circulating sPD-L1 has been recently reported in patients' sera, but no data are available in pleural effusions (PE). In our study, we evaluated the baseline expression levels of sPD-L1 in PE from 84 MPM patients and correlated them with PD-L1-status in matched tumors and patients' overall survival (OS).

Methods sPD-L1 in PE was determined by ELISA and tumor PD-L1 by immunohistochemistry. Association of sPD-L1 with OS was estimated using the Cox regression model.

Results We observed that sPD-L1 was variably expressed in all the PE and tended to be higher (by 30%) in patients with PD-L1-positive tumors (cut-off ≥ 1% stained cells) as compared to patients with PD-L1-negative tumors (geometric mean ratio = 1.28, *P* value = 0.288). sPD-L1 levels were significantly higher than those of sPD-1 (*P* value = 0.001) regardless of the MPM histotypes and they were positively correlated ($r = 0.50$, *P* value < 0.001). Moreover, high PE sPD-L1 concentrations were associated with a trend towards increased OS (hazard ratio 0.79, 95% CL 0.62–1.01, *P* value = 0.062).

Conclusions Our study documents the presence of sPD-L1 in PE of MPM patients, and suggests its possible biological and prognostic role in MPM.

Keywords Soluble PD-L1 · Pleural effusion · Mesothelioma · Immune response · Overall survival

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Introduction

Programmed cell death-ligand 1 (PD-L1/B7-H1/CD274) is one of two ligands interacting with programmed cell death protein 1 (PD-1), a major immune checkpoint receptor down-regulating the function of activated T and B lymphocytes, natural killer cells, and myeloid cells (Pardoll 2012; Riella et al. 2012). PD-L1 is expressed on a variety of human cancers (Dong et al. 2018) including malignant pleural mesothelioma (MPM) (Cedrés et al. 2015; Patil et al. 2018; Minnema-Luiting et al. 2018). Also, it is present at the cell surface of immune cells, such as T and B cells, dendritic cells (DC), and macrophages (Mφ), as well as stromal/stem cells and vascular endothelial cells (Pardoll 2012; Zou and Chen 2008).

The PD-1/PD-L1 signaling pathway negatively regulates T-cell-mediated immune response involved in peripheral T-cell tolerance, autoimmunity (Keir et al. 2008), and tumor immunity (Wu et al. 2019). Indeed, PD-1/PD-L1 interaction results in T-cell inactivation leading both to inhibition of effector functions such as cytotoxicity and cytokine release (Keir et al. 2008) and promotion of T-cell apoptosis (Dong et al. 2002) or exhaustion (Blank and Mackensen 2007).

Engagement of PD-1 on effector T lymphocytes by PD-L1 expressed on tumor cells is a mechanism to evade the host immune response through inhibition of tumor-reactive T lymphocytes (Iwai et al. 2002; Jiang et al. 2019). In this context, monoclonal antibodies blocking the PD-1/-PD-L1 axis have proven effective in the treatment of some human cancers, although only a proportion of patients benefit from this treatment (Topalian et al. 2012; Brahmer et al. 2012; Herbst et al. 2014).

PD-L1 molecule does not exist only as a membrane-bound form (mPD-L1) but also as a soluble form (sPD-L1) that can originate from both immune and tumor cells predominantly by proteolytic cleavage of mPD-L1 (Chen et al. 2011), spliced transcripts lacking the exon encoding the PD-L1 transmembrane domain (Zhu and Lang 2017), or release of exosome-associated PD-L1 (Chen et al. 2018).

It is still controversial whether sPD-L1 can affect T lymphocyte activation. Some in vitro studies reported the ability of recombinant sPD-L1 to inhibit CD4 + and CD8 + T-cell activation and proliferation (Zhou et al. 2017), while the tumor-derived sPD-L1 can induce T-cell apoptosis (Frigola et al. 2011). In contrast, it has been reported that sPD-L1 splice variants can act as "decoy receptors" of anti-PD-L1 antibody (Gong et al. 2019) or as PD-1 receptor antagonists (Ng et al. 2019). These sPD-L1 molecules retain PD-1 binding activity, but they do not inhibit T-cell activation (Gong et al. 2019; Ng et al. 2019).

sPD-L1 has emerged as a prognostic biomarker in cancer patients prior to treatment with immune checkpoint inhibitors (ICI). Its biological significance is, however, still controversial. On one hand, high levels of serum/plasma sPD-L1 have been associated with poor prognosis in different solid tumors including melanoma (Zhou et al. 2017), renal cell carcinoma (Frigola et al. 2011), lung cancer (Okuma et al. 2017), and hepatocellular carcinoma (Finkelmeier et al. 2016) suggesting its possible role in antitumor immune suppression. On the other hand, high sPD-L1 levels have been found in gastric cancers characterized by a better outcome (Zheng et al. 2014) and sPD-L1 did not influence advanced pancreatic cancer prognosis (Kruger et al. 2017). It is noteworthy that increasing levels of sPD-L1 during treatment with PD-1/PD-L1 blockade improved clinical response in melanoma (Zhou et al. 2017), lung and gastric cancer (Ando et al. 2019), as well as in malignant pleural mesotelioma (Chiarucci et al. 2020).

Based on our previous finding that MPM cell lines expressing mPD-L1 can release sPD-L1 in the culture supernatant (Pistillo et al. 2020), we investigated whether sPD-L1 can be found in pleural effusions (PE) derived from patients affected by MPM. Indeed, sPD-L1 in PE may be a biomarker of MPM prognosis. To date, to our knowledge, no data are available on this issue.

In the present study, we show that sPD-L1 is detectable in PE from MPM patients with different histotypes at diagnosis and its abundance correlates with that of PD-1 soluble isoform (sPD-1), density of PD-L1-positive tumor cells in matched MPM tissues and patients' overall survival.

Material and methods

Patients and specimens

This study included 84 patients who had been diagnosed with MPM at the Pneumology Division of Azienda Sanitaria Locale 5, La Spezia, Italy, from April 2008 to February 2017. The follow-up period ended by December 31st, 2019.

Forty-one out of eighty-four MPM patients (48.8%) were treated with standard care and 18 patients (21.4%) received supportive care only. Among the patients treated with first-line therapy, 20 (23.8%) underwent pemetrexed plus cisplatin or carboplatin and, alternatively, gemcitabine plus carboplatin or cisplatin. Twenty patients (23.8%) received also second-line therapy (pemetrexed plus cisplatin) and one patient (1.2%) received third-line therapy (vinorelbine). Fourteen out of eighty-four patients (16.7%) reveived surgical treatment with pleurectomy and also radiotherapy. For 25 out of 84 patients (29.8%), the therapeutic regimen was unknown, since they moved to other hospitals.

PE specimens were collected by thoracentesis before any treatment and centrifuged at 1500 × *g* for 10 min at 4 °C, and

the supernatant was frozen at − 20 °C until ELISA determinations were performed.

ELISA of sPD-L1 and sPD-1

Soluble PD-L1 and PD-1 levels in PE were measured using enzyme-linked immunosorbent assays (Human PD-L1 (28–8) SimpleStep ELISA® Kit ab214565, Abcam, Cambridge, UK, and human PD-1 ELISA Kit BMS2214, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The minimum detectable doses of sPD-L1 and sPD-1 were 2.91 pg/ml and 1.14 pg/ml, respectively. Each sample was analyzed in duplicates. The intra-assay and inter-assay coefficients of variation were below 15%.

Immunohistochemistry for tissue PD-L1 expression

Four micron-thick sections were cut from formalin-fixed paraffin-embedded tissue blocks. Immunohistochemistry (IHC) was performed using the Ventana BenchMark ULTRA (Ventana Roche Diagnostics, Basel, Switzerland) automated immunostainer as per the manufacturer's instructions. The PD-L1 antibody (Clone 22C3-Dako Agilent, Santa Clara, CA, United States) was used undiluted and incubated for 40 min at 37 °C, after heat-based antigen retrieval for 64 min. Signal amplification was obtained using the UltraView Universal AP red immunohistochemistry detection kit (Ventana, Tucson, Arizona, USA) and OptiView Amplification kit (Ventana, Tucson, Arizona, USA). Tonsil squamous epithelium was used as a positive on-slide control (Bragoni et al. 2017), while liver tissue was used as a negative control.

The slides were simultaneously analyzed by two pathologists (L.M. and F.G.) and discrepancies were resolved by consensus. The extent of PD-L1 expression in tumor cells was defined as the proportion of tumor cells showing positive PD-L1 staining with a distinct membranous, and possible cytoplasmic pattern, of any intensity, in the tumor area on the whole slide. A cut-off of ≥ 1% of tumor cells expressing PD-L1 was used to define PD-L1 positivity (Cedrés et al. 2015; Chapel et al. 2019; Ghanim et al. 2020). Tumors with < 1% stained cells were considered PD-L1 negative (Cedrés et al. 2015; Chapel et al. 2019; Ghanim et al. 2020).

Tumor cells were easily discriminated from tumor-infiltrating lymphocytes by their morphology.

Statistical analysis

Patients and disease characteristics were explored using descriptive statistics. Discrete variables (e.g., gender, histotype, ECOG-PS, etc.) were expressed as relative frequencies (percentages) and continuous variables (e.g., age at diagnosis, N–L ratio, platelet count, etc.) were summarized using median and range values. Analysis of contingency tables and related Chi-square test were used to assess the associations between categorical variables, while Student's *t* test was applied to compare distributions of continuous variables (sPD-L1 and sPD-1) among subgroups of patients.

The first analytical step was to evaluate the association between sPD-L1 and sPD-1 in PE according to histotype subgroups using a multiple normal regression model in which the geometric mean (GM) of sPD-L1 and sPD-1 levels were log-transformed to fulfill the normality assumption and/or to reduce the influence that outlying measurements can unduly exert on regression results. In this context, the geometric mean ratio (GMR) was used as an index of association.

The second step involved the analysis of PE sPD-L1 levels according to the tumor PD-L1 status (positive vs negative). Also in this case, a multiple normal regression model was applied to log-transformed sPD-L1 levels and GMR was estimated as an index of association.

The last step consisted in evaluating the joint effect of PE sPD-L1 levels and tumor PD-L1 status on patients' life expectancy using the Cox regression model. In this analysis, the prognostic role of both variables was expressed as mortality rate (hazard) ratio (HR).

In all regression settings, results were adjusted for age at diagnosis, gender, N–L ratio, disease stage, histotype, and therapy, while the statistical precision of regression estimates was quantified using 95% confidence limits (95% CL).

A two-tailed *P* value less than or equal to 0.05 was considered as statistically significant.

All analyses were performed using Stata (StataCorp. Stata Statistical Software. Release 13.1. College Station, TX, USA, 2013).

Results

Patients characteristics

We studied 84 MPM patients at diagnosis, before any treatment. The mean age of patients was 72 years (range 45–98) and most of them were male (85.7%). The MPM histotypes were 57 (67.9%) epithelioid, 27 (32.1%) non-epithelioid including 15 (17.8%) sarcomatoid, 9 (10.7%) biphasic, and 3 (3.6%) desmoplastic.

Forty-six (54.8%) patients had MPM stage 1, 23 (27.4%) stage 2, and 15 (17.9%) stage 3 or 4. Thirty patients (35.7%) had ECOG-PS = 0, 34 (40.5%) = 1, and 20 (23.8%) > 1. Other patients' characteristics are shown in Table 1. At the end of the study period, 83 patients (98.8%) died, the median follow-up time was 13.5 months (95% CL 11.3–16.1), and the median survival time was 13.4 months (95% CL 11.0–15.8).

Table 1 Characteristics of patients analyzed for sPD-L1 in pleural effusion

Characteristics	*N*	%
Age (years) at diagnosis (median, range)	(72, 45–98)	
Gender		
Male	72	85.7
Female	12	14.3
Smoking habit		
Never	32	38.1
Current	13	15.5
Former	37	44.1
Unknown	2	2.3
Asbestos exposure		
Non-exposed	29	34.5
Exposed	53	63.1
Unknown	2	2.3
Disease stage		
1	46	54.8
2	23	27.4
3–4	15	17.9
Histotype		
Epithelioid	57	67.9
Non-epithelioid	27	32.1
Therapy		
No	18	21.4
Yes	41	48.8
Unknown	25	29.8
ECOG-PS		
0	30	35.7
1	34	40.5
2–3	20	23.8
N–L ratio (median, range)	(3.68, 0.12-40.0)	
Platelet count $\times 10^3$/μl (median, range)	(284, 117–678)	
Whole sample	84	100.0

Non-epithelioid included 15 sarcomatoid, 9 biphasic, and 3 desmoplastic histotypes

ECOG-PS Eastern Cooperative Oncology Group-Performance Status, *N–L ratio* neutrophil-to-lymphocyte ratio

Determination of sPD-L1 levels in PE and comparison with sPD-1 levels

sPD-L1 levels were evaluated by ELISA in PE specimens from all 84 MPM patients and compared in two subgroups of epithelioid (57 Epi) and non-epithelioid (27 non-Epi, including 15 sarcomatoid, 3 desmoplastic, and 9 biphasic) histotypes.

sPD-L1 was detectable in all the PE samples analyzed showing a wide range of variation (23.04–1139.9 pg/ml) with a GM value of 130.2 pg/ml that was slightly higher (1.2-fold) in the non-Epi subgroup (GM 148.0 vs 122.6) (Table 2).

Furthermore, we compared the baseline expression levels of sPD-L1 with those of sPD-1 in the same PE. sPD-1 levels in the whole sample resulted to have a wide variability (range 12.3–708.7 pg/ml) with a GM value of 97.6 pg/ml (Table 2). sPD-L1 levels were found significantly higher than those of sPD-1 both in the whole samples (GMR = 1.34, 95% CL 1.13–1.58, *P* value = 0.001) and in each histotype subgroup (Epi: GMR = 1.22, 95% CL 1.00–1.48, *P* value = 0.050; non-Epi: GMR = 1.62, 95% CL 1.16–2.28 *P* value = 0.007) (Table 2).

Relationship between sPD-L1 and s-PD-1 levels in PE

Next, we investigated the relationship between the expression levels of sPD-L1 and sPD-1 in the same PE, using a multiple normal regression model in which base 2 log-transformed sPD-L1 (log-2-sPD-L1) was used as a predictor variable and natural log-transformed sPD-1 (log-e-sPD-1) as the dependent variable. After adjusting for age at diagnosis, gender, N–L ratio, disease stage, histotype and therapy, we observed a positive association between sPD-L1 and sPD-1 levels. In particular, when the sPD-L1 level doubled (i.e., increased by 100%), also the sPD-1 level increased, although at a lower extent, of about 40% (GMR = 1.39, 95% CL 1.23–1.57; *P* value < 0.001, Fig. 1a).

Table 2 Determination of sPD-L1 levels and comparison with sPD-1 levels in PE from all MPM patients and in two histotype subgroups

MPM histotype	sPD-L1 GM (95% CL)	sPD-1 GM (95% CL)	sPD-L1 vs sPD-1 GMR (95% CL)	*P* value
Whole sample (*n* = 84)	130.2 (108.6–156.2)	97.6 (83.4–114.2)	1.34 (1.13–1.58)	0.001
Non-epithelioid (*n* = 27)	148.0 (107.3–204.1)	91.1 (65.4–126.9)	1.62 (1.16–2.28)	0.007
Epithelioid (*n* = 57)	122.6 (97.9–153.6)	100.8 (84.4–120.4)	1.22 (1.00–1.48)	0.050

Detection of soluble PD-L1 (sPD-L1) and soluble PD-1 (sPD-1) in pleural effusion (PE) of MPM patients was performed by ELISA. All samples were tested in duplicate with intra- and inter-assay coefficient of variation below 15%

Results are expressed as geometric means (GM) and the geometric mean ratio (GMR), i.e., the ratio between the GMs of sPD-L1 and sPD-1 levels in the whole sample and in the two MPM histotype (non-epithelioid and epithelioid) subgroups

95% CL 95% confidence limits for GMR, *P value* probability level associated with Student's *t* test

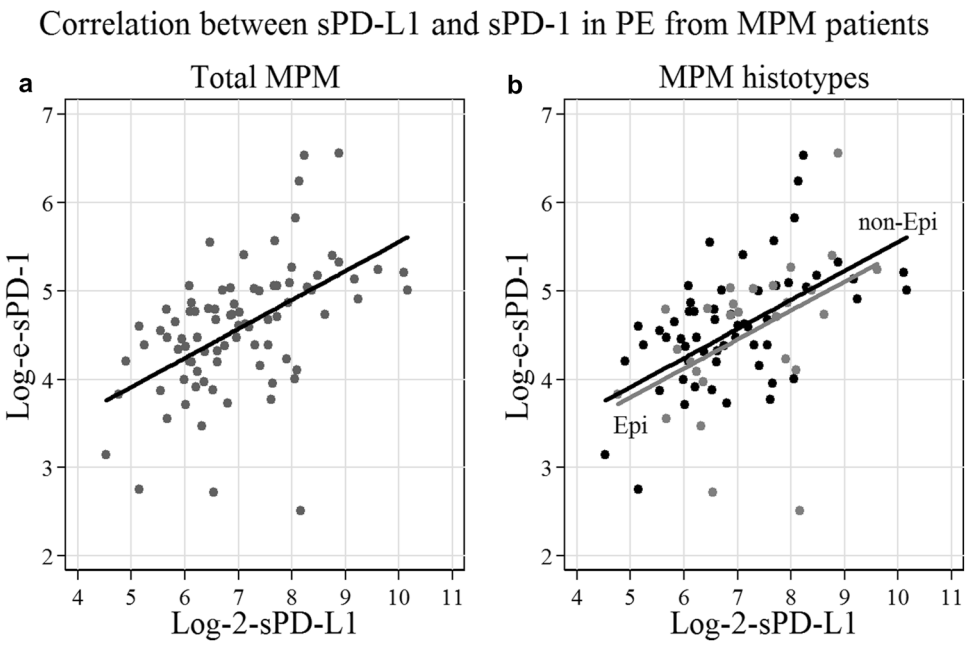

Fig. 1 Correlation between sPD-L1 and sPD-1 levels in pleural effusion samples from all MPM patients and two histotype subgroups. The correlation analysis was performed using a multiple normal regression modeling in which base 2 log-transformed sPD-L1 (log-2-sPD-L1) was assumed as a predictor variable and natural log-transformed sPD-1 (log-e-sPD-1) as the dependent variable. For an explanation of log-transformations, see text. Results are adjusted for age at diagnosis, gender, N–L ratio, disease stage, histotype, and therapy. Lines and dots represent fitted and observed log-e-sPD-1 values, respectively. Black and gray lines/dots indicate non-Epi and Epi patients, respectively. **a**, **b** Indicate the positive linear correlation between sPD-L1 and sPD-1 levels in the same PE. In particular, **a** refers to the analysis of total PE samples in which, for each sPD-L1 doubling, sPD-1 level increased, in a linear manner, by about 40% ($r = 0.50$; GMR = 1.38, 95% CL 1.23–1.57; P value < 0.001); **b** refers to the analysis of PE samples in the epithelioid (Epi) and non-epithelioid (non-Epi) MPM histotypes. In this analysis, sPD-1 levels showed the same correlation with sPD-L1 as in **a**, but its levels were lower (by 16%) in the Epi subgroup (gray line) as compared to the non-Epi (black line) subgroup (GMR = 0.84, 95% CL 0.62–1.13; P value = 0.250)

When the sPD-L1/sPD-1 relationship was evaluated in the two histologic subgroups (Epi vs non-Epi) using the same statistical model, we found that the type of relationship between sPD-L1 and sPD-1 was identical to that observed in the whole cohort (i.e., about 40% sPD-1 increase for each sPD-L1 doubling) (Fig. 1a), although sPD-1 levels in the Epi patients were on average 16% lower (GMR = 0.84, 95% LC 0.62–1.13; P value = 0.250) than those in the non-Epi patients (Fig. 1b).

Correlation of sPD-L1 levels in PE with density of PD-L1-positive tumor cells in matched tissues

Moreover, we evaluated the correlation between PE sPD-L1 levels and density (percentage) of PD-L1-positive tumor cells analyzed by IHC. A patient was defined as PD-L1 positive if her/his tumor cells expressing PD-L1 were greater than or equal to 1% (Cedrés et al. 2015; Chapel et al. 2019; Ghanim et al. 2020).

Among the 74 patients for whom we could evaluate both PD-L1 biomarkers in PE and matched MPM tissues, we observed that 19 (25.7%) patients were PD-L1-positive and 55 (74.3%) patients were PD-L1-negative.

Using a normal regression model, we found that PD-L1 positive patients had PE sPD-L1 levels that were about 30% higher than those in patients with a PD-L1 negative status (GMR = 1.28, 95% CL 0.81–2.04, P value = 0.288), although such a difference was not statistically remarkable (Table 3).

Images of PD-L1 positive and negative IHC stainings in MPM tissues are shown in representative cases (Fig. 2b, d respectively).

Association between sPD-L1 levels and overall survival

Finally, we analyzed the joint effect of log-2-sPD-L1 and tumor PD-L1 expression on patients' OS through the Cox regression analysis (Table 4). In particular, after adjusting for age at diagnosis, gender, N–L ratio, disease stage, histotype and therapy, we estimated that for each doubling in sPD-L1 levels, the mortality rate was subject to a reduction of about 20% (HR 0.79; 95% CL 0.62–1.01; P value = 0.062). On the contrary, patients with a positive tumor PD-L1 expression

Table 3 Association of sPD-L1 levels in PE with tumor PDL-1 status in matched MPM tissues estimated through a normal regression analysis

Tumor PD-L1 status	GMR	95% CL	*P* value
Negative (< 1%)	1.00	(Ref.)	0.288
Positive (≥ 1%)	1.28	0.81–2.04	

Detection of soluble PD-L1 (sPD-L1) in pleural effusion (PE) of MPM patients was performed by ELISA. All samples were tested in duplicate with intra- and inter-assay coefficient of variation below 15%. Tumor PD-L1 expression was evaluated by immunohistochemical (IHC) staining of formalin-fixed, paraffin-embedded MPM tissue sections and expressed as percentage of PD-L1 positive tumor cells in the whole tumor area using a cut-off of ≥ 1% stained cells, as described in "Materials and methods"

GMR geometric mean ratio adjusted for age at diagnosis, gender, N–L ratio, disease stage, histotype and therapy, *95% CL* 95% confidence limits for GMR, *Ref.* reference category, *P value* probability level associated with Student's *t* test

(≥ 1%) showed an increased death rate of about 70% (HR 1.73; 95% CL 0.93–3.22; *P* value = 0.086) in comparison to those with a PD-L1-negative status (Table 4).

No correlation between levels of sPD-L1with other clinical parameters was found.

Discussion

PD-L1 is a glycoprotein expressed mainly by tumor cells, Mφ and DC that, upon ligation with PD-1 receptor, expressed primarily by T cells, inhibits T-cell activation and effector functions (Pardoll 2012; Riella et al. 2012), thus allowing tumor cell survival and proliferation. Recently, it has been shown that also the soluble form of PD-L1 may have an immunosuppressive activity (Zhou et al. 2017; Frigola et al. 2011) and represents a prognostic factor in a variety of tumors (Zhou et al. 2017; Frigola et al. 2011; Gong et al. 2019; Okuma et al. 2017; Finkelmeier et al. 2016; Zheng et al. 2014; Kruger et al. 2017).

In the present study, we investigated whether sPD-L1 was detectable in PE derived from MPM patients at diagnosis and explored its correlation with PE sPD-1, tumor PD-L1, as well as its clinical relevance as a possible prognostic marker.

Levels of sPD-L1 in PE were found to be heterogeneous among MPM patients and slightly higher in the non-epithelioid subgroup suggesting a possible involvement of sPD-L1 levels in the worse prognosis that commonly characterizes this MPM histotype (Musk et al. 2011).

Because PD-L1 plays a prominent role in the PD-1/PD-L1 axis, we analyzed the relationship between sPD-L1 and sPD-1 levels in the same PE. Interestingly, we observed that the levels of sPD-L1 were higher than those of sPD-1 in all the PE samples, regardless of the tumor histotype. In addition, sPD-L1 levels positively correlated with the levels of sPD-1 ($r = 0.50$, P value < 0.001). Yet, for each twofold increase of sPD-L1, sPD-1 showed a lower increase of about 40% (1.4-fold). A positive correlation between levels of sPD-L1 and sPD-1 has also been reported in serum samples from advanced pancreatic patients (Kruger et al. 2017), and they suggested that it was most likely due to their common origin from immune cells (Kruger et al. 2017).

A possible explanation for the different levels of sPD-L1 and sPD-1 in PE of our patients may be their different expression levels released by immune cells and/or source. Both soluble isoforms most likely reflect the activation status of immune cells, either in the tumor microenvironment or circulating in the PE, and they may parallel the different expression levels of the membrane-bound isoforms. In particular, sPD-1 could be induced and released after activation of T cells, whereas sPD-L1 could be constitutively expressed and further upregulated upon activation of T cells or other immune cells (Chen et al. 2019). In addition, the release of sPD-L1 by MPM tumor cells (Pistillo et al. 2020) can contribute to its higher levels in PE with respect to sPD-1.

In this context, we found that indeed sPD-L1 levels in PE tended to be higher in MPM patients with PD-L1-positive tumors (≥ 1% stained cells) with respect to patients with PD-L1-negative tumors (< 1% stained cells), suggesting that sPD-L1 might derive, at least in part, from MPM tumor cells. This finding is in agreement with our previous in vitro observation that MPM tumor cells can produce and release the sPD-L1 isoform (Pistillo et al. 2020) and would confirm this occurrence also in vivo.

Another possibility for the presence of sPD-L1 in PE is the diffusion from the blood to the pleural fluid which is consistent with the increased capillary permeability that characterizes the exudate nature (Valdés et al. 2010) of the PE derived from our patients.

In our study, we found a proportion (25.7%) of MPM patients with PD-L1-positive tumors that is in line with data reported in the literature (Minnema-Luiting et al. 2018). As expected, patients with a higher percent of PD-L1-positive tumor cells showed a tendency to a worse OS (data not shown) as previously reported in MPM (Cedrés et al. 2015; Chapel et al. 2019; Inaguma et al. 2018; Mansfield et al. 2014) and other tumors (Nomi et al. 2007; Ohigashi et al. 2005; Mu et al. 2011; Hamanishi et al. 2007).

Our findings provided preliminary evidence of a possible association of high sPD-L1 levels in PE at diagnosis with a better OS, suggesting that the prognostic potential of sPD-L1 in PE may be independent of that shown by PD-L1 expression on tumors. The favorable role of high sPD-L1 levels would reflect a pre-existing stimulation and activation of the immune system.

Our results extend our previous finding on soluble CTLA-4 (sCTLA-4) that we detected in PE from the same

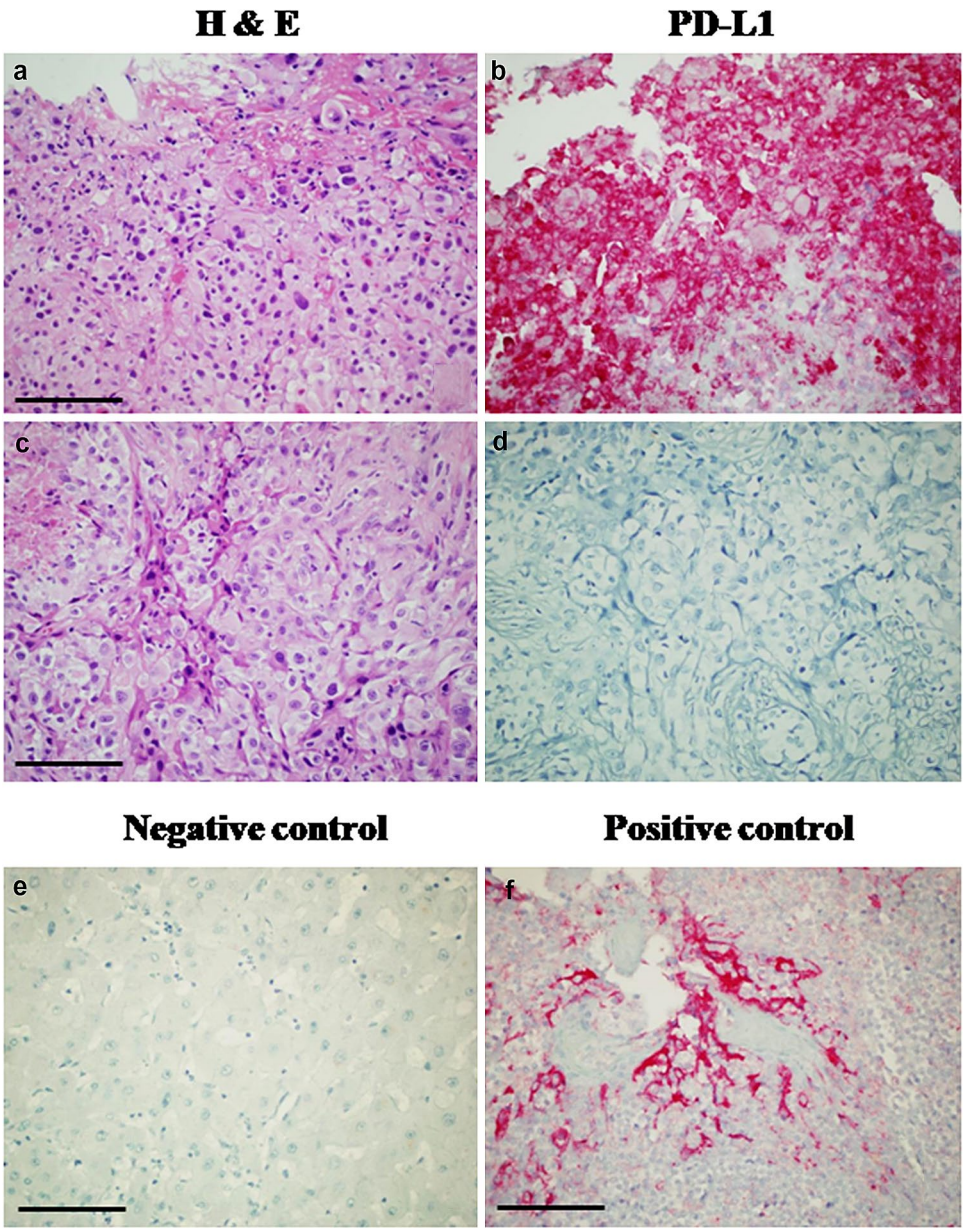

Fig. 2 Analysis of PD-L1 expression in MPM tissues. PD-L1 expression was evaluated by immunohistochemical (IHC) staining of formalin-fixed, paraffin-embedded MPM tissue sections and expressed as percentage of PD-L1-positive tumor cells in the whole tumor area using a cut-off of ≥ 1% of stained cells, as described in "Materials and methods". Representative IHC stainings for PD-L1 (antibody clone 22C3-Dako) in epithelioid mesothelioma are shown. **a** Hematoxylin and eosin-stained section and **b** paired serial section in the same field stained for PDL-1 with intense membrane and cytoplasmic staining. **c** Hematoxylin and eosin-stained section and **d** paired serial section in the same field stained for PD-L1 with negative staining. **e** Sample of liver tissue used as a negative control for PD-L1 staining. **f** Sample of tonsil tissue used as a positive control for PD-L1 staining. *H&E* hematoxylin and eosin. Original magnification: × 40 for all figures. Scale bar 100 µm

MPM patients (Roncella et al. 2016) to other soluble immune checkpoint molecules, namely sPD-L1 and sPD-1. Indeed, patients with a higher sCTLA-4 expression in PE had a better OS than patients with a lower expression. Therefore, soluble forms of different IC molecules, such as CTLA-4, PD-L1, and PD-1, can be present in the PE of MPM patients and a delicate balance likely exists between their free and membrane-bound isoforms. Minimal alterations of this functional balance may determine the final effect of IC regulators on the immune response (activation versus downregulation).

We hypothesize that both PE sPD-L1 and sPD-1 can modulate the function of mPD-L1 and mPD-1 expressed by immune cells and, consequently, the antitumor immune response. In particular, sPD-L1, by binding to mPD-1, could avoid its interaction with the membrane-bound forms of both ligands PD-L1 and PD-L2, thereby preventing PD-1 negative signaling. In addition, sPD-L1 could bind to CD80 expressed by the antigen-presenting cells (Butte et al. 2007) thus competing for binding to CTLA-4 as well as CD28 on T cells and avoiding T-cell downregulation (due to CTLA-4) or costimulation (due to CD28).

On the other hand, sPD-1, as it has been proposed by some authors (Amancha et al. 2013), could prevent the PD-L1/2:mPD-1 interaction, by binding to PD-L1/PD-L2 ligands expressed on the antigen-presenting cells, thereby blocking the negative signal transduced by the membrane form of PD-1.

Table 4 Joint effect of PE sPD-L1 levels and tumor PD-L1 status on patients' overall survival estimated through a Cox regression analysis

Variable	HR	95% CL	*P* value
Tumor PD-L1% status			0.086
Negative (< 1%)	1.00	(Ref.)	
Positive (≥ 1%)	1.73	0.93–3.22	
PE log-2-sPD-L1			0.062
Linear trend	0.79	0.62–1.01	

Life expectancy results according to PE sPD-L1 levels and tumor PD-L1 status estimated through the Cox regression model

HR mortality rate (hazard) ratio adjusted for age at diagnosis, gender, N–L ratio, disease stage, histotype and therapy, *95% CL* 95% confidence limits for HR, *P value* probability level associate with the likelihood ratio test, *Ref.* reference category, *PE* pleural effusion, *Log-2-sPD-L1* base 2 log-transformed sPD-L1 levels, *sPD-L1* soluble PD-L1

It is noteworthy that, in our patients, high basal PE sPD-L1 levels seem to favor a better OS at variance with findings referred to serum sPD-L1 in patients with other tumors (Zhou et al. 2017; Frigola et al. 2011; Okuma et al. 2017; Finkelmeier et al. 2016) including MPM (Chiarucci et al. 2020). One explanation for this difference could reside in the fact that the biological significance of sPD-L1 in PE may be different from that generally reported in serum. In this context, it has been shown that PE of mesothelioma patients are characterized by a high percentage of PD-L1-positive immune cells and contain a higher proportion of CD3 + PD-L1 + T cells compared to their paired peripheral blood (54.8% vs 2.9%) (Khanna et al. 2016). These PE samples also contain PD-L1 + tumor cells suggesting a possible interaction of T lymphocytes and tumor cells in PE besides at the tumor site (Khanna et al. 2016).

Regarding the difference with results on serum sPD-L1 from a previous study by other authors in MPM (Chiarucci et al. 2020), their patients analyzed underwent therapy with ICI, whereas our patients underwent chemotherapy only. Thus, the PE sPD-L1 levels at diagnosis, by reflecting an ongoing antitumor immune response, may influence chemotherapy outcomes, as previously reported for the effect of PD-L1 gene polymorphisms in non-small cell lung cancer patients (Lee et al. 2016).

We are aware of the limitations of the present study, such as the relatively small sample size and the lack of functional studies regarding the biological role of sPD-L1 in PE. However, it is conceivable that this role can be elusive due to the presence of several membrane-linked and soluble immune receptors and ligands present in PE, peripheral blood and at the tumor site. Nevertheless, we deduce from our findings that high levels of sPD-L1 in PE may have a key role in the biology of the MPM tumor microenvironment. Additional investigations are needed to clarify the immunological functions and clinical implications of sPD-L1 in MPM PE.

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Author contributions MPP and SR: conceived and designed the study; MPP, SR, VF, and AP: evaluated results and wrote the manuscript; RC: carried out ELISA of pleural effusion; VF: performed statistical analyses; LM and FG: performed immunohistochemical stainings; PF collected MPM pleural effusions and created the database. PAC: recruited MPM patients, PD performed MPM diagnosis and AV treated MPM patients; AM and BB: contributed to the final manuscript; AP and UP: critically revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to disclose.

Ethics approval The study protocol was approved by the Ethics Committee of the Liguria Region (P.R. 207REG2014) after obtaining written informed consent from all participating patients. All procedures performed in this study were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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