Serum levels of soluble programmed death protein 1 (sPD-1) and soluble programmed death ligand 1 (sPD-L1) in advanced pancreatic cancer

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ABSTRACT

Up to now, the efficacy of programmed death protein 1/programmed death ligand 1 (PD-1/PD-L1) blockade in pancreatic cancer (PC) remains uncertain. Serum levels of soluble PD-1 and PD-L1 (sPD-1/sPD-L1) have been reported to be independent prognostic factors in solid tumors susceptible to checkpoint blockade. Provenience, regulation and immunologic function of sPD-1 and sPD-L1 in cancer are poorly understood. To the best of our knowledge, sPD-1 and sPD-L1 have not been measured conjointly in any cancer type yet.

In contrast to other tumor entities, sPD-1/sPD-L1 levels did not indicate an adverse outcome in a cohort of 41 patients with advanced PC. We observed a close positive correlation of sPD-L1 levels with sPD-1 in patients with advanced PC, suggesting a common provenience and regulation of sPD-1 and sPD-L1 in cancer patients. Higher sPD-L1 levels were present in patients with elevated C-reactive protein or strong tumoral T cell infiltration, while no correlation of sPD-L1 levels with tumoral PD-L1 expression was found. Our findings indicate that sPD-1 and sPD-L1 are markers of systemic inflammation in (pancreatic) cancer. In a subset of PC patients, elevation in sPD-L1 levels might be caused by an inflammatory tumor type – independent of tumoral PD-L1 expression.

Abbreviations: CA 19–9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; ECOG, Eastern Cooperative Oncology Group; HCC, hepatocellular carcinoma; HR, hazard ratio; IHC, immunohistochemistry; OS, overall survival; PC, pancreatic cancer; PD-1, programmed death protein 1; PD-L1, programmed death receptor ligand 1; sPD-1, soluble programmed death protein 1; sPD-L1, soluble programmed death receptor ligand 1

Introduction

Pancreatic cancer (PC) is predicted to become the second leading cause of cancer-related death in the United States and Germany by 2030.1,2 The vast majority of patients are diagnosed with advanced disease and do not qualify for potentially curative resection. Despite minor advances using intensified chemotherapeutic regimens like FOLFIRINOX and gemcitabine plus nab-paclitaxel, prognosis in advanced PC remains dire with a median overall survival (OS) of less than 1 year.3,4 Accordingly, novel and innovative treatment strategies are urgently needed.

Inflammation is an important step in PC initiation and progression.5,6 Almost a decade ago, checkpoint blockade targeting the programmed death protein 1/programmed death ligand 1 (PD-1/PD-L1) pathway was described as a promising therapeutic target in PC and has been proven to be successful in a variety of other solid tumor entities.7–9 While the importance of PD-L1 in PC immune evasion was confirmed very recently, only preliminary – rather disappointing – data on the potential efficacy of PD-1/PD-L1 blockade is available in PC yet.10–12 High levels of soluble plasma PD-L1 (sPD-L1) were described as an adverse prognostic factor in several malignancies susceptible to PD-1/PD-L1 blockade, including renal cell cancer and hepatocellular carcinoma (HCC).13,14 To date, provenience, regulation and immunologic function of sPD-1 and sPD-L1 in cancer remain uncertain. Plasma levels of sPD-1 and sPD-L1 have not yet been determined conjointly in any cancer type. To our knowledge, neither sPD-1 nor sPD-L1 has previously been analyzed in patients with advanced PC.

Materials and methods

Patient characteristics

Serum samples of 41 patients with advanced PC (locally advanced: n = 6, metastatic: n = 35) diagnosed and/or treated at our high-volume comprehensive cancer center between 2011
and 2015 were prospectively collected before initiation of palliative chemotherapy. Clinical characteristics were obtained from a prospectively maintained database. All patients were followed up for survival status until September 2016. This translational research study was approved by the local ethics committee of Ludwig-Maximilians-University of Munich (approval number 284–10) and all patients gave written informed consent for the collection of blood and data analysis.

Blood sampling
Blood samples were obtained from each subject during routine venipuncture on day one of the first cycle of chemotherapy. To remove blood cells, serum tubes were centrifuged at 3,000 rounds per minute for 10 min at room temperature. Serum samples were aliquoted and stored at −80°C subsequently. Complete blood count, levels of C-reactive protein (CRP), carbohydrate antigen 19–9 (CA 19–9) and carcinoembryonic antigen (CEA) were measured at the central laboratory of LMU Munich University Hospital.

sPD-1 and sPD-L1 ELISAs
Soluble PD-1 (sPD-1) and PD-L1 (sPD-L1) were quantified by enzyme-linked immunosorbent assays (ELISA) using the human PD-1 antibody duosets for ELISA development (for PD-1 catalog number DY1086, for PD-L1 / B7-H1 DY156) from R&D Systems (Minneapolis, MN) that were applied on the MSD MesoScale Quickplex SQ120 platform (MSD MesoScale Diagnostics, Rockville, MD) enabling highly sensitive chemoluminescent detection. Antibody duosets contained streptavidin- and biotin-labeled capture and detection antibodies as well as appropriate standard material for PD-1 and PD-L1.

In brief, high-bind microtiter plates from MSD were incubated with 25 μL/well capture antibodies (concentrations 2 μg/mL for PD-1, and PD-L1, respectively), sealed and incubated overnight. On the next day, plates were washed (3 × 200 μL/well PBS with 0.05% Tween). Then, 150 μL/well BSA (5% in PBS) was added as blocking agent, the plate was sealed and shaken at 700 rpm for 1 h. After a washing step (3 × 200 μL/well PBS-T), 25 μL of calibrators or patient samples were added, sealed and incubated for 2 h under shaking conditions. Calibration curve consisted of 1:4 dilutions of the standard material ranging from 30 ng/mL to 7 pg/mL. After a further washing step (3 × 200 μL/well PBS-T), 25 μL/well unlabeled detection antibodies were added (concentrations 200 ng/mL for PD-1, and 100 ng/mL for PD-L1, respectively), sealed and incubated for 2 h under shaking conditions. Once again plates were washed (3 × 200 μL/well PBS-T) and 25 μL/well streptavidin-sulfo-tag antibodies were added and incubated for 2 h. After a further washing step (3 × 200 μL/well PBS-T), 150 μL/well reading buffer was added and chemiluminescent measurement was performed on the SQ120 QuickPlex reader. Absolute concentrations of sPD-1 and sPD-L1 (in ng/mL) in the patient samples were calculated by use of a four-point-fit calibration curve of the standard dilutions.

Prior to clinical testing, the assays were optimized regarding plate selection (high-bind vs. standard plate), buffers antibody concentrations (by titrating different combinations) and streptavidin-labeling. Best antibody combinations were identified by highest signal-to-noise ratio over several standard concentrations. In addition, intra- and inter-assay imprecision as well as dilution linearity were tested.

Defining a cohort with high sPD-1 and sPD-L1 serum levels
Up to now, there is no defined cut-off value for sPD-1 and sPD-L1 in patients with cancer. In other cancer entities, different approaches to determine prognostic cut-off values have been used: median sPD-L1 serum level in the analyzed cancer patient cohort, sPD-L1 serum levels in a corresponding cohort of healthy individuals or receiver operating characteristic (ROC) curve models to define an ideal prognostic cut-off value. In all aforementioned studies, the prognostic cut-off value was in the range of the 50th (i.e., median) and 75th percentile of the observed sPD-L1 levels in the whole analyzed patient cohort. When designing our study we therefore decided to use the median and the 75th percentile of sPD-1 and sPD-L1 levels observed in our study to define a cohort of patients with high versus low levels of the two molecules.

Tumor samples and immunohistochemistry
Formalin-fixed paraffin-embedded (FFPE) tissue containing histologically confirmed exocrine pancreatic cancer was retrieved from the archives of the Institute of Pathology of the Ludwig-Maximilians-University or from external pathologists. Tumor tissue of eight patients was included in a tissue microarray (TMA) consisting of three 1 mm diameter tissue cores of different tumor regions from resection or biopsy specimens of each patient. To examine tumoral CD3+ lymphocyte infiltration and PD-L1 expression using immunohistochemistry (IHC) on 4-μm thick whole mount tissue sections or TMA sections, a Ventana Benchmark Ultra autostainer was used (Ventana, Tucson, AZ, USA). Briefly, the slides were dewaxed and antigenicity was retrieved using the Ventana antigen retrieval solution CCI1 (pH 8.4, Ventana) for 64 min. The slides were then incubated with the rabbit monoclonal anti-PD-L1 antibody (1:100 dilution, clone E1L3N, Cell Signaling Technology, Danvers, MA, USA) for 32 min and after secondary antibody incubation (UltraView DAB Kit; Ventana) the staining was visualized using a dianamobenzidine system (Ventana). As a validation experiment, exemplary slides were also stained using a commercial Ventana PD-L1 assay kit. Briefly, slides were dewaxed and antigenicity was retrieved as described before. Slides were then incubated with the pre-diluted rabbit monoclonal anti-PD-L1 antibody (clone SP263, concentration 1.61 μg/mL, Ventana) and staining was visualized using a dianamobenzidine kit after secondary antibody incubation (OptiView DAB Kit; Ventana). Appropriate positive (human tonsil) and negative controls (human liver) if not already present on the slides were included in each staining run. Only membranous PD-L1 staining in tumor cells was scored using the percentage of positive cells in the whole tissue sample. Rarely occurring pure cytoplasmic staining was considered as artifact and not counted. The amount of CD3+ lymphocyte infiltration was scored semiquantitatively using a three-tier
score. Briefly, score 0 was considered very few (0–5) CD3+ per high power field (HPF) in 200× magnification. Score 1 was given when there were up to 50 CD3+ cells per HPF. Cases showing more than 50 CD3+ lymphocytes per HPF in 200-fold magnification were considered as score 2. All three tissue cores on TMA tissue as well as all the tumor tissue present on biopsy samples were read and CD3+ infiltrate was counted. Only true tumor infiltrating or tumor adjacent CD3+ cells were counted, excluding CD3+ cells in occasionally present lymph follicles or lymphatic aggregates.

Statistical analyses

OS was estimated using Cox-regression analysis and the Kaplan–Meier method. Median follow-up was calculated using the reversed Kaplan–Meier method. Differences in mean tumor marker levels were tested using an unpaired Student’s t-test. Differences in variance were tested for statistical significance using an F-test. Correlation analyses were performed using the Pearson coefficient analysis. SPSS PASW 23.0 (SPSS Inc., Chicago, IL, USA) software was used for survival analyses. Graphpad Prism 7.01 (GraphPad Software Inc., La Jolla, CA USA) was used for comparison and correlation analyses.

Table 1. Prognostic relevance of sPD-1 and sPD-L1.

| Parameter | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR | 95% CI | p value | HR | 95% CI | p value |
| Age > 65 y | 1.13 | [0.56–2.28] | 0.74 | | | |
| ECOG > 1 | 1.48 | [0.20–10.92] | 0.70 | | | |
| CA-19-9 > 1,000 U/mL | 1.96 | [0.98–3.9] | 0.06 | 2.29 | [1.07–4.9] | < 0.05 |
| CRP [mg/dL] | 1.06 | [1.01–1.11] | < 0.05 | 1.08 | [1.02–1.13] | < 0.01 |
| sPD-1 [ng/mL] | 0.74 | [0.44–1.24] | 0.25 | 0.74 | [0.01–489.78] | 0.93 |
| sPD-L1 [ng/mL] | 0.23 | [0.01–13.52] | 0.48 | 0.90 | [0.37–2.20] | 0.82 |

Abbreviations: CA 19–9: carbohydrate antigen 19–9; CEA: carcinoembryonic antigen; CI, confidence interval; CRP: C-reactive protein; ECOG: Eastern Cooperative Oncology Group performance status; HR, hazard ratio; sPD-1, soluble programmed death protein 1; sPD-L1, soluble programmed death-ligand 1.

Results

Patient characteristics

Between 2011 and 2015, serum samples of 41 consecutive patients with non-resectable PC (ductal adenocarcinoma: n = 40, acinar cell carcinoma n = 1) were prospectively collected before initiation of first-line chemotherapy. At the time of database lock in September 2016, 35 patients were deceased. Median OS for the whole study population was 10.8 mo (95% CI: 6.1 to 15.5 mo) with a median follow-up of 24.7 mo (95% CI: 19.6 to 30.0 mo). First-line palliative chemotherapy consisted of gemcitabine monotherapy (n = 11), different gemcitabine-based combination regimens (n = 11), 5-FU-based combination chemotherapy (n = 18; FOLFIRINOX = 10) or palliative chemoradiotherapy (n = 1).

Correlation of sPD-1 and sPD-L1 levels in patients with advanced pancreatic cancer

While sPD-1 was detectable in the serum of all patients analyzed, sPD-L1 was below the lower limit of detection in 15 cases. Median values for sPD-1 and sPD-L1 were 0.117 (range 0.038–25.93 ng/mL) and 0.012 ng/mL (range 0.007–0.632 ng/mL), respectively. We observed a close correlation between levels of sPD-1 and sPD-L1 suggesting a common provenience and a simultaneous release of these two soluble checkpoint molecules (Fig. 1).

Correlation of sPD-1 and sPD-L1 serum levels with overall survival using cox regression

To correlate levels of sPD-1 and sPD-L1 with OS, we applied a Cox regression model including established prognostic factors in advanced PC (Table 1). As expected, CRP levels and a high pre-treatment CA 19–9 value (> 1,000 U/mL) independently predicted an unfavorable prognosis in our patient cohort. Neither sPD-1 nor sPD-L1 was correlated with a shorter OS in advanced PC (HR for sPD-1: 0.74 [0.44–1.24]; HR for sPD-L1: 0.23 [0.01–13.52]).

Clinical characteristics of patients with high vs. low sPD-1 and sPD-L1 serum levels

Patients were grouped into sPD-1 / sPD-L1 high vs. low using the median sPD-1 or sPD-L1 concentrations, respectively.
Clinical characteristics such as age, Eastern Cooperative Oncology Group (ECOG) score and stage of disease were comparable in patients with sPD-1 and sPD-L1 high vs. low subgroups (Tables 2A and B).

To compare OS in patients with high vs. low sPD-1 and sPD-L1 serum levels, we used the Kaplan–Meier method. We observed no adverse outcome in the high sPD-1 or sPD-L1 group, respectively (11.93 vs. 9.53 mo for high sPD-1 vs. low sPD-1, p = 0.78; 11.92 vs. 9.53 mo for high sPD-L1 vs. low sPD-L1, p = 0.36) (see Table 2 and Fig. 2). Similar results were obtained when using the 75th percentile or very high levels of sPD-1 and sPD-L1 (as defined by sPD-1 > 1 ng/mL and sPD-L1 > 0.1 ng/mL) instead of the median sPD-1 and sPD-L1 levels (Fig. S1).

**Table 2A.** Clinical characteristics of patients with high vs. low levels of sPD-1.

|                  | Low sPD-1 (n = 20) | High sPD-1 (n = 21) |
|------------------|--------------------|---------------------|
| Age years (Median) | 66.05 [44–81]      | 67.10 [38–77]       |
| CRP mg/dL (Median, Range) | 1.6 [0.1–32.8]  | 1.4 [0.1–14.6]      |
| Leukocytes G/L (Median, Range) | 7.3 [4.5–18.0] | 7.2 [4.3–18.0]       |
| CA-19–9 U/mL (Median, Range) | 364 [2.6–122 000] | 1455 [2.6–422 000]   |
| CEA ng/mL (Median, Range) | 4.3 [0.7–122]  | 10.2 [0.2–432]      |
| Stage             |                    |                     |
| Locally advanced pancreatic cancer | 2                  | 4 |
| Metastatic pancreatic cancer | 18                 | 17              |
| Performance status |                    |                     |
| ECOG 0            | 10                 | 10                  |
| ECOG 1            | 9                  | 9                   |
| ECOG 2            | 0                  | 1                   |
| ECOG 3            | 1                  | 0                   |
| ECOG missing      | 0                  | 1                   |
| Median overall survival | 9.53 [95% CI: 5.06–11.93 [95% CI: 6.13–13.99] (months) | 17.72 |

**Abbreviations:** CA 19–9: carbohydrate antigen 19–9; CEA: carcinoembryonic antigen; CI: confidence interval; CRP: C-reactive protein; ECOG: Eastern Cooperative Oncology Group performance status; sPD-1, soluble programmed death protein 1; sPD-L1, soluble programmed death-ligand 1.

**Table 2B.** Clinical characteristics of patients with high vs. low levels of sPD-L1.

|                  | Low sPD-L1 (n = 20) | High sPD-L1 (n = 21) |
|------------------|--------------------|---------------------|
| Age years (Median, Range) | 66.05 [41–81]  | 67.10 [38–77]       |
| CRP mg/dL (Median, Range) | 0.9 [0.1–14.6]  | 1.6 [0.1–32.8]      |
| Leukocytes G/L (Median, Range) | 7.2 [4.5–18.0] | 7.4 [4.3–18.0]       |
| CA-19–9 U/mL (Median, Range) | 353 [2.6–16 257] | 1466 [2.6–422 000]   |
| CEA ng/mL (Median, Range) | 4.0 [0.7–59.8]  | 9.0 [0.2–432]       |
| Stage             |                    |                     |
| Locally advanced pancreatic cancer | 3                  | 3 |
| Metastatic pancreatic cancer | 17                 | 18              |
| Performance status |                    |                     |
| ECOG 0            | 10                 | 10                  |
| ECOG 1            | 9                  | 9                   |
| ECOG 2            | 0                  | 1                   |
| ECOG 3            | 1                  | 0                   |
| ECOG missing      | 0                  | 1                   |
| Median overall survival | 9.53 [95% CI: 5.06–11.92 [95% CI: 6.41–13.99] (months) | 17.44 |

**Abbreviations:** CA 19–9: carbohydrate antigen 19–9; CEA: carcinoembryonic antigen; CI: confidence interval; CRP: C-reactive protein; ECOG: Eastern Cooperative Oncology Group performance status; sPD-1, soluble programmed death protein 1; sPD-L1, soluble programmed death-ligand 1.

**Figure 2.** Overall survival for patients with high vs. low levels of sPD-1 or sPD-L1 (n = 41).

CA 19–9 and CEA in patients with high sPD-1 and sPD-L1 levels

CA 19–9 and CEA are established serum tumor markers in patients with advanced PC and indicative of prognosis and burden of disease. As expected, we found a significant positive correlation of pretreatment CA 19–9 and CEA levels in our patient cohort (Fig. S2A). Mean levels of CA 19–9 and CEA in patients with high vs. low sPD-L1 levels did not differ significantly (Fig. S2B). Further, we correlated levels of CA 19–9 and CEA to sPD-L1 levels in individual patients (Fig. S2C): No correlation between tumor marker levels and sPD-L1 serum levels was observed. Similar results were observed for sPD-1 (data not shown).

**Correlation of sPD-1 and sPD-L1 levels with levels of C-reactive protein, tumoral T cell infiltration and tumoral PD-L1 expression**

We hypothesized that sPD-1 and sPD-L1 might be upregulated in patients with elevated markers of systemic inflammation (e.g., CRP). Very high sPD-1 and sPD-L1 levels (as defined by sPD-1 > 1 ng/mL and sPD-L1 > 0.1 ng/mL) were almost exclusively present in patients with CRP elevation (p < 0.01 [sPD-1] and p < 0.0001 [sPD-L1] for variances between patients with normal vs. elevated CRP). Mean sPD-1 and sPD-L1 levels tended to be higher in patients with an elevated CRP (mean sPD-1: 490 pg/mL vs. 230 pg/mL, p = 0.36 and mean
sPD-L1 60 pg/mL vs. 18 pg/mL, \( p = 0.28 \) for patients with elevated vs. normal CRP values, respectively) (Fig. 3A). Archival tumor specimens to analyze tumoral infiltration by CD3+ T cells and tumoral PD-L1 expression were available from 30 of 41 patients. Reasons for missing tumor samples were diagnosis of PC at an external institution in eight cases and no remaining tumor tissue after diagnostic workup in three cases (Table 3). Tumor tissue was from metastatic sites in 17 cases and from the primary tumor in 13 cases. Two patients received neoadjuvant treatment before acquisition of the analyzed tumor sample. Median time from acquisition of tumor sample to blood draw was 12 d (range: −317 d [neoadjuvant treatment] to 1,205 d [relapse after surgery in curative intent]) (Table 3). A high infiltration by CD3+ T cells was found in 10/30 cases (33%) (Fig. S3 and Table 3). Interestingly – a subset of patients with a strong tumoral T cell infiltration had very high sPD-L1 levels (Fig. 3C). Of note in one patient with low sPD-L1 levels despite a strong T cell infiltration – neoadjuvant treatment was administered after acquisition of blood for sPD-L1 analysis (patient number 23, Table 3). Using adequate positive and negative controls, PD-L1 expression was found to be present in 9/30 cases (30%) (Fig. 3B and Table 3). When correlating levels of sPD-L1 with tumoral PD-L1 expression, no correlation was found (Fig. 3C).

**Discussion**

Clinical results of phase II/III trials investigating the efficacy of PD-1/PD-L1 blockade as monotherapy or in combination with other drugs in PC are still pending. Serum levels of sPD-1 and sPD-L1 have been reported to be independent prognostic factors in different solid tumors susceptible to immunotherapy targeting the PD-1 axis. Regulation, provenience and function of the soluble forms of PD-1 and PD-L1 in cancer are still under discussion. This is the first study to examine serum levels of sPD-1 and sPD-L1 in advanced PC.

The soluble forms of PD-1 and PD-L1 were initially described in autoimmune disorders where both sPD-1 and sPD-L1 are thought to be produced by immune cells upon stimulation with proinflammatory cytokines. To the best of our knowledge, sPD-1 and sPD-L1 have not yet been measured conjointly in any cancer type. As seen in autoimmune disorders, we observed a close positive correlation of sPD-1 and sPD-L1 levels in patients with advanced PC, suggesting a common provenience and regulation of sPD-1 and sPD-L1 in cancer patients.

While sPD-L1 has not been determined in PC before, conflicting findings on the prognostic relevance of membrane bound PD-L1 on PC tumor cells exist. Using a cox regression model including established prognostic factors for patients with advanced PC, we did not find an adverse influence of sPD-1 or sPD-L1 on OS. Likewise, we did not find a significant survival difference between patients with high vs. low sPD-1 or sPD-L1, respectively. This suggests, that neither sPD-1 nor sPD-L1 serum levels serve as adverse prognostic marker in advanced PC. This is in line with the recent report on the absent prognostic effect of PD-L1 expression on PC tumor cells and the rather disappointing early trial results of checkpoint blockade as monotherapy in advanced PC and could suggest that PD-L1 blockade alone might possibly not be sufficient to improve prognosis in advanced PC patients. Serum CA 19–9 and CEA are established tumor markers in PC. Higher CA 19–9 and CEA levels indicate higher tumor burden and poor outcome. In accordance with the observation that high sPD-1 or sPD-L1 values are not correlated with an adverse prognosis, no correlation between mean sPD-L1 levels and tumor marker levels was found in our cohort.

Levels of sPD-L1 have been reported to be elevated in patients with cancer and systemic inflammation (as defined by an elevated CRP or elevated sCD163) in HCC with a similar trend for CRP in gastric cancer. Accordingly, very high serum levels of sPD-1 and sPD-L1 were only observed in patients with elevated CRP levels in our study population (with a similar trend for...
higher mean sPD-1 and sPD-L1 levels in patients with elevated CRP). Importantly, serum levels of soluble PD-L1 were markedly elevated in a subset of patients with strong tumoral CD3+ T cell infiltration. This could indicate that systemic inflammation and subsequently elevated sPD-L1 levels are provoked by an inflammatory tumor type in a subset of advanced PC patients. Given the small number of patients included in our study, further studies are clearly necessary to confirm this observation. Importantly, such studies should aim to elucidate the reason for the missing upregulation of sPD-L1 in a subset of patients despite a high number of CD3+ T cells in immediate adjacency to malignant cells were counted.

Besides tumoral T cell infiltration, we analyzed tumoral PD-L1 expression in archival tissue samples: Using two different PD-L1 IHC antibodies, we were not able to confirm the high expression of PD-L1 reported very recently.27 In line with previous reports only a minority (30%) of all analyzed tumor specimens were classified as PD-L1 positive.27,28,30 However, PD-L1 expression might differ between primary tumor and metastases as described for renal cell carcinoma and lung adenocarcinoma.31-33 Therefore, it must be pointed out that a majority (57%) of tumor samples in our study was from metastatic sites, while previous studies in PC determined PD-L1 expression in the pancreatic primary.7,27-30 In lymphoma patients, levels of sPD-L1 have been reported to be independent of tumoral PD-L1 expression.15 Likewise, tumoral expression of PD-L1 did not correlate to serum levels of its soluble counterpart in our patient population. As reported very recently, PD-L1 expression in PC is not associated to an inflammatory tumor type as defined by an immunogenic gene signature.39 This could explain the correlation of high sPD-L1 levels with a strong CD3+ T cell infiltrate in the absence of a correlation between tumoral PD-L1 expression with sPD-L1 levels.

In conclusion, our study indicates that sPD-1 and sPD-L1 are markers of systemic inflammation in (pancreatic) cancer. In a subset of PC patients, elevated sPD-L1 levels might be caused by an inflammatory tumor type – independent of tumoral PD-L1 expression. Future studies should attempt to define the immune cell population(s) responsible for release of sPD-1 and sPD-L1 in cancer and possible functional consequences. A special focus should be on T cells and innate immunity.

### Table 3. Correlation of sPD-L1 levels with tumoral PD-L1 expression and tumoral CD3+ T cell infiltration in advanced pancreatic cancer.

| Patient number | sPD-L1 level [ng/mL] | pancreatic cancer cells | Tumor infiltration by CD3+ T cells | Type of tumor tissue | Δ [days] from acquisition of tissue sample to blood draw |
|----------------|----------------------|-------------------------|-----------------------------------|---------------------|--------------------------------------------------------|
| 1              | 0.013                | 0                       | 1                                 | liver metastasis    | 23                                                     |
| 2              | 0.007                | no remaining tumor tissue | —                                 | —                   | —                                                      |
| 3              | 0.012                | 5                       | 1                                 | peritoneal metastasis | 21                                                     |
| 4              | 0.138                | external pathology       | —                                 | —                   | —                                                      |
| 5              | 0.007                | 10                      | 1                                 | liver metastasis    | 7                                                      |
| 6              | 0.007                | external pathology       | —                                 | —                   | —                                                      |
| 7              | 0.007                | 0                       | 2                                 | peritoneal metastasis | 21                                                     |
| 8              | 0.007                | 30                      | 1                                 | liver metastasis    | 5                                                      |
| 9              | 0.007                | 0                       | 2                                 | primary tumor       | 28                                                     |
| 10             | 0.007                | external pathology       | —                                 | —                   | —                                                      |
| 11             | 0.029                | 0                       | 0                                 | liver metastasis    | 4                                                      |
| 12             | 0.007                | 0                       | 1                                 | liver metastasis    | 12                                                     |
| 13             | 0.007                | external pathology       | —                                 | —                   | —                                                      |
| 14             | 0.119                | 0                       | 1                                 | cutaneous metastasis| 16                                                     |
| 15             | 0.021                | 0                       | 1                                 | liver metastasis    | 5                                                      |
| 16             | 0.039                | 0                       | 1                                 | primary tumor after neoadjuvant treatment | -317          |
| 17             | 0.007                | 0                       | 2                                 | primary tumor       | 17                                                     |
| 18             | 0.007                | 20                      | 0                                 | liver metastasis    | 12                                                     |
| 19             | 0.043                | 30                      | 1                                 | liver metastasis    | 7                                                      |
| 20             | 0.009                | 0                       | 1                                 | primary tumor       | 12                                                     |
| 21             | 0.012                | 0                       | 1                                 | primary tumor       | -2                                                     |
| 22             | 0.007                | 0                       | 2                                 | liver metastasis    | 26                                                     |
| 23             | 0.632                | external pathology       | —                                 | —                   | —                                                      |
| 24             | 0.007                | 0                       | 1                                 | liver metastasis    | 0                                                      |
| 25             | 0.007                | external pathology       | —                                 | —                   | —                                                      |
| 26             | 0.078                | 30                      | 2                                 | primary tumor       | 77                                                     |
| 27             | 0.007                | 60                      | 2                                 | primary tumor after neoadjuvant treatment | -210         |
| 28             | 0.031                | external pathology       | —                                 | —                   | —                                                      |
| 29             | 0.047                | 0                       | 2                                 | liver metastasis    | 19                                                     |
| 30             | 0.007                | no remaining tumor tissue | —                                 | —                   | —                                                      |
| 31             | 0.222                | 0                       | 2                                 | primary tumor       | 5                                                      |
| 32             | 0.013                | 0                       | 1                                 | liver metastasis    | 6                                                      |
| 33             | 0.008                | no remaining tumor tissue | —                                 | —                   | —                                                      |
| 34             | 0.015                | 20                      | 1                                 | liver metastasis    | 11                                                     |
| 35             | 0.067                | 0                       | 2                                 | primary tumor       | 23                                                     |
| 36             | 0.008                | 20                      | 0                                 | liver metastasis    | -2                                                     |
| 37             | 0.026                | 0                       | 1                                 | primary tumor       | 14                                                     |
| 38             | 0.178                | external pathology       | —                                 | —                   | —                                                      |
| 39             | 0.012                | 0                       | 1                                 | primary tumor       | 49                                                     |
| 40             | 0.019                | 0                       | 1                                 | primary tumor       | 1,205                                                   |
| 41             | 0.093                | 0                       | 2                                 | primary tumor       | 38                                                     |

Scoring for tumor infiltration by CD3+ T cells: 2 = high number of peritumoral CD3+ T cells, 1 = intermediate number of peritumoral CD3+ T cells, 0 = low number CD3+ T cells. Only CD3+ T cells in immediate adjacency to malignant cells were counted.

**Abbreviations:** sPD-L1, soluble programmed death-ligand 1.
immune cells given the positive correlation of sPD-L1 with sCD163 (the soluble form of CD163, exclusively expressed on macrophages).  

Disclosure of potential conflicts of interest

Stefan Boeck has received honoraria for scientific presentations from Celgene and Roche, research funding from Celvios Oncology and Roche, travel grants from Celgene and Roche and acted as consultant for Celgene and Baxalta.

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Author contributions

Stephan Kruger and Stefan Boeck: analysis and interpretation of data; drafting of the manuscript. Stephan Kruger, Stefan Holdenrieder and Stefan Boeck: study concept and design. Marie-Louise Legenstein and Verena Roesgen: acquisition of data, collection of serum and tumor samples. Stef- fen Ormanns, Thomas Kirchner, Volker Heinemann, Stefan Holdenrieder and Stefan Boeck: critical revision of the final manuscript.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research commit- tee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local ethics committee (Approval number 284–10, ethics committee Ludwig-Maximilians-University Munich).

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