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ORIGINAL ARTICLE

A comparative view on the expression patterns of PD-L1 and PD-1 in soft tissue sarcomas

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Abstract

Soft tissue sarcomas (STSs) are heterogeneous cancers associated with poor prognosis due to high rates of local recurrence and metastasis. The programmed death receptor ligand 1 (PD-L1) is expressed in several cancers. PD-L1 interacts with its receptor, PD-1, on the surface of tumor-infiltrating lymphocytes (TILs), thereby attenuating anti-cancer immune response. Immune checkpoint inhibitors targeting this interaction have been established as effective anti-cancer drugs. However, studies on the PD-L1 and PD-1 expression status in STS are commonly limited by small sample size, analysis of single STS subtypes, or lack of combinatorial marker assessment. To overcome these limitations, we evaluated the expression patterns of intratumoral PD-L1, the number of TILs, their PD-1 expression, and associations with clinicopathological parameters in a large and comprehensive cohort of 225 samples comprising six STS subtypes. We found that nearly all STS subtypes showed PD-L1 expression on the tumor cells, albeit with a broad range of positivity across subtypes (50% angiosarcomas to 3% synovial sarcomas). Co-expression and correlation analyses uncovered that PD-L1 expression was associated with more PD-1-positive TILs ($P < 0.001$), higher tumor grading ($P = 0.016$), and worse patients' 5-year overall survival ($P = 0.028$). The results were in line with several publications on single STS subtypes, especially when comparing findings for STS with low and high mutational burden. In sum, the substantial portion of PD-L1 positivity, the co-occurrence of PD-1-positive TILs, and the association of PD-L1 with unfavorable clinical outcome provide rationales for immune checkpoint inhibition in patients with PD-L1-positive STS.

Keywords PD-L1 · PD-1 · Tumor-infiltrating lymphocytes · Immune checkpoint inhibition · Soft tissue sarcoma

Abbreviations

GIST	Gastrointestinal stromal tumor
HPF	High-power field
IHC	Immunohistochemistry
MPNST	Malignant peripheral nerve sheath tumor

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STS	Soft tissue sarcoma
TIL	Tumor-infiltrating lymphocyte
TMA	Tissue microarray
UPS	Undifferentiated pleomorphic sarcoma

Introduction

Soft tissue sarcomas (STS) are heterogeneous and highly malignant tumors originating from the mesenchymal lineage [1] with more than 50 subtypes described to date [2]. Current therapy regimens for STS are limited mainly to surgery and radiation [3]. Benefits of neoadjuvant and adjuvant radio-chemotherapy are still under debate [3, 4]. In addition, established therapies appear not fully sufficient for long-term tumor control as many patients develop local relapse (up to 45%) and/or distant metastases (30%) [1, 5, 6], leading to fatal outcome.

Unfortunately, STS patients barely benefited from new and more sophisticated anti-cancer treatments like kinase inhibitors used in gastrointestinal stromal tumor (GIST) treatment [3, 7]. Innovative and more effective therapeutic alternatives are lacking, possibly due to the rarity and diversity of STS.

In the past two decades, immune checkpoint inhibitors revolutionized anti-cancer therapies. Immune checkpoint inhibitors like nivolumab interfere with an immunosuppressive mechanism by which cancer cells attenuate the anti-cancer activity of the patient's immune system [8]. Specifically, several cancer cells hijack a regulatory mechanism of the immune system by expression of programmed death receptor ligand 1 (PD-L1, also known as B7-H1 and CD274) [9]. PD-L1 is normally expressed on antigen presenting cells and binds to the programmed death receptor 1 (PD-1) on activated T cells, B cells, and macrophages, thereby blocking their activity and the recruitment of further immune cells [10]. By upregulation of PD-L1 on their surface, tumor cells can ligate the same way as antigen presenting cells the PD-1-positive cells, commonly CD8 + T cells, thereby inhibiting adaptive immune response against the tumor. By blockade of any of the interacting proteins, PD-L1 or PD-1, the down-regulation of the cancer patient's immune response, originating from the tumor, is removed, leading to a host versus tumor reaction [11]. For instance, in melanoma, squamous non-small cell lung cancer and urothelial carcinoma, inhibition of this immune checkpoint enhances anti-tumor immune activity, resulting in significantly improved clinical outcome [12–14].

However, to date, there is only limited knowledge on PD-L1 as a common feature in STS, its correlation with tumor-infiltrating lymphocytes (TILs), and the PD-1 status of TILs. Except for the work of Dancsok et al., previous studies focused mainly on single STS subtypes and based their analyses on small sample sizes, often neglecting TILs and PD-1, not addressing the PD-L1 expression at protein level, and not including survival data [10, 15–22] (Supplementary Table 1).

In the present study, we analyzed the PD-L1 and PD-1 expression patterns in a large and well-characterized tumor collective comprising 225 STS samples of six distinct subtypes with clinical annotation. Our results show that a substantial proportion of STS is positive for PD-L1 and that PD-L1 expression is associated with PD-1-positive TILs and poor patient outcome. In synopsis with previous studies, mainly on single STS subtypes, our results provide rationales for immune checkpoint inhibition in patients with PD-L1-positive STS.

Materials and methods

Patient cohort

The study was conducted in accordance with the Declaration of Helsinki. Formalin-fixed paraffin-embedded tumor material from STS patients was retrieved from the archive of the Institute of Pathology, LMU Munich, in agreement with the Ethics Committee of the LMU Munich University hospital (307-16 UE, 25.05.2016). To include as many samples of various STS subtypes as possible, all cases for the entire time range from 1989, from when on tumor material was still archived, to 2012, enabling 5-year follow-up, were investigated. Tumors were reclassified by TKn and EK according to the current WHO classification. Clinical data, including sex, age at diagnosis, tumor site, tumor size, metastasis, and grading were extracted from the archived pathological results and the database of the medical department of the LMU Munich. None of the patients received immune checkpoint inhibitors as treatment. Survival data were updated until 09/2017 in collaboration with clinicians performing follow-up and the patients' respective general physicians. The resulting cohort with tumor material and clinical data available comprised 225 STS cases (Table 1). As a second cohort without clinical data, 114 independent STS samples of six subtypes on tissue microarrays (TMAs) published in Baldauf et al. [23] and on an angiosarcoma TMA created by TKn were used (Supplementary Table 2).

Assembly of TMAs

For TMA assembly, all STS cases were considered for which material, defined diagnosis and clinical data were present. On hematoxylin and eosin (H&E) stained slides of the STS cases, such tumor areas were marked, which showed the most typical histology for the respective tumor entity with vital tumor cells and without regressive alterations or other

Table 1 Patients' characteristics

STS subtype	*n*	Fraction of cohort (%)	Sex (male/female)	Age at diagnosis (median, range) (years)	Follow-up (median, range) (months)
Angiosarcoma	6	2.7	3/3	48 (18–67)	14 (4–54)
Leiomyosarcoma	47	20.9	17/30	55 (19–79)	33 (6–287)
Dediff. liposarcoma	49	21.8	30/19	57 (24–79)	65 (0–189)
MPNST	11	4.9	7/4	37 (24–74)	20 (6–102)
Synovial sarcoma	29	12.9	11/18	42 (22–67)	54 (2–176)
UPS	83	36.9	56/37	56 (19–79)	32 (1–182)
All samples	225	100.0	114/111	54 (18–79)	36 (0–287)

artifacts. Based on the availability of such representative material on slides, formalin-fixed paraffin-embedded tumor blocks of each patient were chosen, and two cores with 0.6 mm diameter were taken from the marked area and integrated in a TMA precast as duplicates. Samples from tonsils were added to the TMAs as positive controls.

TMAs published by Baldauf et al. were additionally tested for PD-L1 and PD-1 positivity, as for those TMAs more representative material was available, enabling three cores per sample on the TMAs. The assembly of those TMAs has been published previously [23].

Immunohistochemistry and scoring of immunoreactivities

TMA sections of 5 µm were stained for H&E, PD-L1, PD-1, and Ki-67. For immunohistochemical PD-L1 staining, slides were pretreated with heat and the Epitope Retrieval Solution pH8 Novocastra (Leica Biosystems) and incubated with the monoclonal primary anti-PD-L1 antibody raised in rabbit (#E1L3N; 1:50; Cell Signaling Technology) for 60 min at room temperature. For detection, the SignalStain Boost IHC Detection Reagent (Cell Signaling Technology) and the chromogen DAB + (Agilent) were used. For immunohistochemical PD-1 staining, slides were pretreated with heat and Target Retrieval solution (S1699, Agilent) and incubated with the monoclonal primary anti-PD-1 antibody raised in mouse (315M; 1:80; Cell Marque) for 60 min at room temperature. Detection was performed with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories) and the chromogen DAB +. For both anti-PD-L1 and anti-PD-1 specificity in immunohistochemistry was validated with isotype and system controls (Supplementary Figure S1). Immunohistochemical stains for PD-L1 of the TMAs by Baldauf et al. plus the angiosarcoma TMA, and for Ki-67 were performed automatically on a Ventana Benchmark XT autostainer system with the XT ultra-View DAB Kit (Ventana Medical Systems, Roche) with monoclonal rabbit anti-PD-L1 antibody (#SP263; Ventana Medical Systems; currently used in diagnostics at the Institute of Pathology, LMU Munich) and monoclonal mouse anti-Ki-67 antibody (#MIB-1; 1:100; Agilent), respectively. Hematoxylin (Vector Laboratories) was used for counterstaining.

PD-L1 expression was scored by a sarcoma pathologist (TKn) and a physician experienced in immunohistology (EK) independently into PD-L1 negative and positive. If > 1% tumor cells exhibited membranous staining for PD-L1, the corresponding sample was considered as PD-L1 positive. TILs between tumor cells were count per high-power field (HPF) (400 × magnification, field of view 0.237mm^2) in H&E stained TMA slides, as routinely done by the pathologist. PD-1-positive TILs were also count per HPF, here using the PD-1 stained TMA slides. In case of discrepancies in the scoring results of both investigators, consent was built after individual reevaluation of each sample. The percentage of Ki-67-positive cancer cells was evaluated by three researchers (FCA, FW, LRP) independently, and the average percentage of Ki-67 positivity for each sample was taken as basis for further analysis. All researchers scoring the TMAs were blinded to the clinical data.

For five representative cases of the most prevalent entities (UPS, liposarcoma and leiomyosarcoma), consecutively cut open slides were stained for PD-L1 and PD-1 as described above, and additionally for CD4, CD8, CD19, CD56 and FOXP3. Stains for CD4, CD8, CD19 and CD56 were performed with a Ventana Benchmark XT autostainer system using the monoclonal anti-CD4 antibody raised in mouse (#4B12; 1:500; Leica Biosystems) and the OptiView detection kit (Ventana Medical Systems), the monoclonal anti-CD8 antibody raised in mouse (#C8/144B; 1:50; Cell Marque), the monoclonal anti-CD19 antibody raised in rabbit (#EP169; Cell Marque), or the monoclonal anti-CD56 antibody raised in mouse (#123C3; Ventana Medical Systems) and the UltraView detection kit. For FOXP3 stains, slides were pretreated with heat and Target Retrieval solution (S1699, Agilent) and then incubated with the

monoclonal anti-FOXP3 antibody (ab20034; 1:80; Abcam) raised in mouse for 60 min. Detection was performed with the ZytoChem Plus AP Polymer anti-Mouse kit (Zytomed Systems) and the chromogen Permanent AP Red (Zytomed Systems). Micrographs were taken from the identical areas of PD-L1 and PD-1 stained consecutive slides for each sample. Overlay images to interpret co-localization were generated with ImageJ (NIH). The number of CD4, CD8, CD19, CD56 and FOXP3 cells was count by a physician-scientist (MFO) in 17 HPF (corresponding to 4 mm^2 tumor area), and the average count per 1 mm^2 tumor area was used for further interpretations.

Statistical analyses

For statistical analyses, samples with ≥ 4 TILs per HPF (corresponding to > 12 TILs per mm^2, hence more than the mean PD-1-positive TILs count in PD-1 blockage non-responders reported in NSCLC [24]) were considered as positive for TILs, and if ≥ 4 TILs/HPF exhibited PD1 staining, as PD-1 positive. Statistical analyses were carried out and displayed using SPSS and GraphPad PRISM (v5). Associations between clinicopathological parameters and histological results were calculated with the Fisher's exact test and unpaired two-tailed Student's *t* test. Associations with survival were displayed with the Kaplan–Meier method, and significance was assessed with the log-rank test. *P* values < 0.05 were considered statistically significant.

Results

PD-L1 is expressed in several STS subtypes

To test whether PD-L1 expression at protein level is a common feature of STS, we stained TMAs with 225 STS samples represented as duplicates by immunohistochemistry (Table 1) using a well-established routine antibody for PD-L1 and considered all samples with > 1% membranous staining as PD-L1 positive, the remaining as negative (Fig. 1a). On average, 16% of the entire cohort exhibited PD-L1 expression. The fraction of positive samples comprised 50% of angiosarcomas, 23% of undifferentiated pleomorphic sarcomas (UPS), 13% of leiomyosarcomas, 12% of dedifferentiated liposarcomas, 3% of synovial sarcomas,

Table 2 PD-L1 expression in STS

STS subtype	PD-L1 positive
Angiosarcoma	50% (3/6)
Leiomyosarcoma	12.8% (6/47)
Dediff. liposarcoma	12.2% (6/49)
MPNST	0 (0/11)
Synovial sarcoma	3.4% (1/29)
UPS	22.9% (19/83)
All samples	15.6% (35/225)

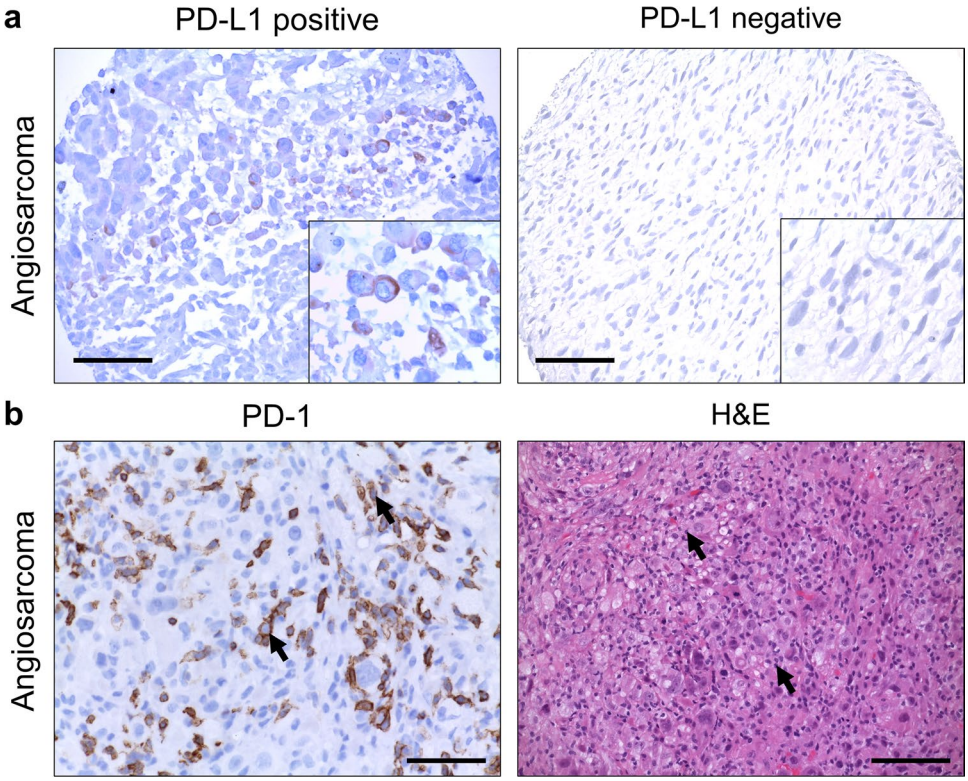

Fig. 1 PD-L1 is expressed in a fraction of STS, which is often positive for PD-1 and TILs. Representative micrographs of cores on a TMA representing angiosarcoma samples, immunohistochemically stained for **a** PD-L1 (brown, scale bar indicates 100 µm) and **b** PD-1 (brown) and H&E (scale bar indicates 50 µm), arrow-heads point to lymphocytes

and 0 malignant peripheral nerve sheath tumor (MPNST, Table 2). Likewise, the high fractions of PD-L1-positive angiosarcomas and UPS versus low fraction in synovial sarcomas were observed in a second independent TMA cohort of 114 samples (Supplementary Table 2). Hence, a substantial fraction of STS tumors expresses PD-L1 at protein level, albeit with variable proportions depending on the STS subtype.

PD-L1 expression in STS correlates with PD-1-positive TILs

Besides PD-L1 expression, another prerequisite for effective immune checkpoint inhibition in cancer is an actual interaction of PD-L1 with its receptor. Accordingly, we scored the total number of TILs and TILs positive for PD-1 for all samples tested for PD-L1 positivity considering samples with counts of ≥ 4 lymphocytes per HPF as positive (Fig. 1b).

From all 225 samples, sufficient material was available for 223 and 220 samples to evaluate the number of TILs and their PD-1 immunoreactivity, respectively. Across the entire cohort, 76.3% of samples were positive for TILs and 28.1% for PD-1. Interestingly, in the PD-L1-positive samples, the fractions for TIL and PD-1 positivity were 87.2% and 62.9% (Table 3). In fact, for all tested STS subtypes with more than three PD-L1-positive samples, the fraction of PD-1 positivity was higher in the PD-L1 expressing samples than in those negative for PD-L1. Similar results yielded the analyses of the second TMA cohort with 113 samples with sufficient material (23% positive for PD-1, Supplementary Table 2). Consistently, PD-L1 expression and PD-1 positivity were highly significantly correlated ($P < 0.001$, Table 3).

For better interpretation of the topologic localization of PD-1-positive TILs and PD-L1-positive tumor cells, FFPE material of five PD-1 and PD-L1 double-positive samples from the most prevalent STS subtypes (3 × UPS, 1 × leiomyosarcoma, 1 × liposarcoma) was consecutively cut and stained for both markers (Supplementary Figure S2a). PD-1 expressing TILs were found widely distributed throughout the vital tumor tissue, partly forming cell groups. PD-L1-positive tumor cells were found either directly next to PD-1-positive TILs or nearby, but never in areas of the tumor lacking PD-1-positive TILs. The same samples were additionally stained for CD8 (cytotoxic T cells), CD4 (T helper cells), FOXP3 (regulatory T cells), CD56 (natural killer cells) and CD19 (B cells) to further characterize the observed TILs. Across all five representative cases, CD8 + cytotoxic T cells constituted the largest fraction of TILs. FOXP3 staining was exclusively observed in UPS. The percentage of B cells among the TILs ranged from 0.5 to 7.9 (Supplementary Figure S2b).

Taken together, these results provide evidence that PD-L1-positive STSs are enriched for nearby located PD-1-positive TILs, pointing to an actual interaction of PD-L1 and PD-1-positive TILs in STS, which is for some cancer entities a prerequisite for patients' eligibility for immune checkpoint inhibitor therapy.

PD-L1 expression is associated with clinical outcome in STS

To test whether the PD-L1 status is associated with clinicopathological parameters, we correlated the PD-L1 scoring results obtained from assessors blinded to the clinical data with the most important prognostic parameters for STS patients.

While PD-L1 expression did not correlate with age and tumor size in any tested STS subtype, it was significantly enriched in males ($P = 0.027$) and significantly associated with the prognostically favorable tumor localization in the extremities ($P = 0.023$). In contrast, PD-L1 expression was significantly associated with the prognostically unfavorable parameters of high grading ($P = 0.016$) and metastasis at diagnosis ($P = 0.027$; Table 4). In accordance, intratumoral PD-L1 expression was associated with a significantly worse 5-year overall survival (40% vs. 59% 5-year overall survival

Table 3 PD-1-positive cells and TILs in STS

STS subtype	PD-1 positive	PD-1 in PD-L1 positive	*P* value association PD-L1–PD-1	TIL positive	TIL in PD-L1 positive	*P* value association PD-L1–TILs
Angiosarcoma	40% (2/5)	33.3% (1/3)	1.000 (FE)	83.3% (5/6)	66.7% (2/3)	1.000 (FE)
Leiomyosarcoma	17.4% (8/46)	66.7% (4/6)	0.006 (FE)	65.2% (30/46)	66.7% (4/6)	1.000 (FE)
Dediff. liposarcoma	18.8% (9/48)	66.7% (4/6)	0.008 (FE)	81.3% (39/48)	100% (6/6)	0.578 (FE)
MPNST	27.3% (3/11)	NA	NA	81.8% (9/11)	NA	NA
Synovial sarcoma	10.3% (3/29)	0% (0/1)	1.000 (FE)	48.3% (14/29)	0% (0/1)	1.000 (FE)
UPS	46.9% (38/81)	61.1% (11/18)	0.192 (FE)	85.5% (71/83)	94.7% (18/19)	0.280 (FE)
All samples	28.1% (63/220)	62.9% (20/31)	< 0.001 (FE)	76.3% (168/223)	87.2% (30/35)	0.139 (FE)

FE Fisher's exact test

Table 4 Association of PD-L1 expression and clinicopathological parameters in STS

STS subtype	Sex (M/F)	Age </≥ median	Extr./trunk	M status (M0/M1)	Grading (1–2/3)	Size </≥ 80 mm	Ki-67	Survival (5y-OS)
Angiosarcoma	1.000 (FE)	1.000 (FE)	NA	1.000 (FE)	1.000 (FE)	1.000 (FE)	0.180 (TT)	0.050 (LRT)
Leiomyosarcoma	0.653 (FE)	0.221 (FE)	0.614 (FE)	1.000 (FE)	0.161 (FE)	0.645 (FE)	0.365 (TT)	0.226 (LRT)
Dediff. liposarcoma	0.384 (FE)	0.098 (FE)	0.324 (FE)	0.068 (FE)	1.000 (FE)	0.565 (FE)	0.241 (TT)	0.001 (LRT)
MPNST	NA	NA	NA	NA	NA	NA	NA	NA
Synovial sarcoma	0.379 (FE)	1.000 (FE)	0.483 (FE)	1.000 (FE)	1.000 (FE)	1.000 (FE)	NA	0.010 (LRT)
UPS	0.113 (FE)	0.190 (FE)	0.001 (FE)	0.028 (FE)	0.587 (FE)	0.783 (FE)	0.441 (TT)	0.783 (LRT)
All samples	0.027 (FE)	0.271 (FE)	0.023 (FE)	0.027 (FE)	0.016 (FE)	0.325 (FE)	0.076 (TT)	0.028 (LRT)

FE Fisher's exact test, *TT* Student's *t* test, *LRT* log-rank test, *extr*. Extremities, *5y-OS* 5-year overall survival

probability, $P = 0.028$) (Table 4; Fig. 2). Interestingly, when separating the PD-L1-positive STS in two subgroups by their immunoreactivity for PD-L1 (low/high; 17 and 18 samples, respectively), not any statistical difference between both subgroups in the associations with clinical unfavorable markers was observed. Yet, both PD-L1-positive STS subgroups exhibited higher rates of unfavorable clinical markers than PD-L1-negative STS. Indeed, for lowly and highly PD-L1 expressing STS versus PD-L1-negative STS, rates of metastasis at diagnosis were 18% and 22% versus 7%, and rates of G3 grading were 82% and 56% versus 46%, respectively. Moreover, 5-year overall survival probability was 29 and 50 months versus 59 months for PD-L1 lowly and highly expressing STS versus PD-L1-negative STS. In synopsis, these data indicate that any PD-L1 expression is clinically relevant in STS patients.

Discussion

Immune checkpoint inhibitors interfering with the interaction of tumoral PD-L1 and PD-1 on TILs show convincingly, partly even impressive positive results as anti-cancer drugs in recent studies, e.g., for melanoma, non-small-cell lung carcinoma, cervical carcinoma, and bladder carcinoma [12–14, 25]. So far, the applicability of these inhibitors for STS patients has not been studied extensively (see also Supplementary Table 1) [19, 22, 26].

Since any targeted therapy is prone to fail in the absence of the target, we first assessed the PD-L1 expression in STS at protein level. To this end, we compiled a cohort of 225 tumor samples from STS patients with matched and well-curated clinical data, including median follow-up of 3 years (ranging from median 1.2–5.4 years depending on STS subtype). We did not observe any quality difference in the older

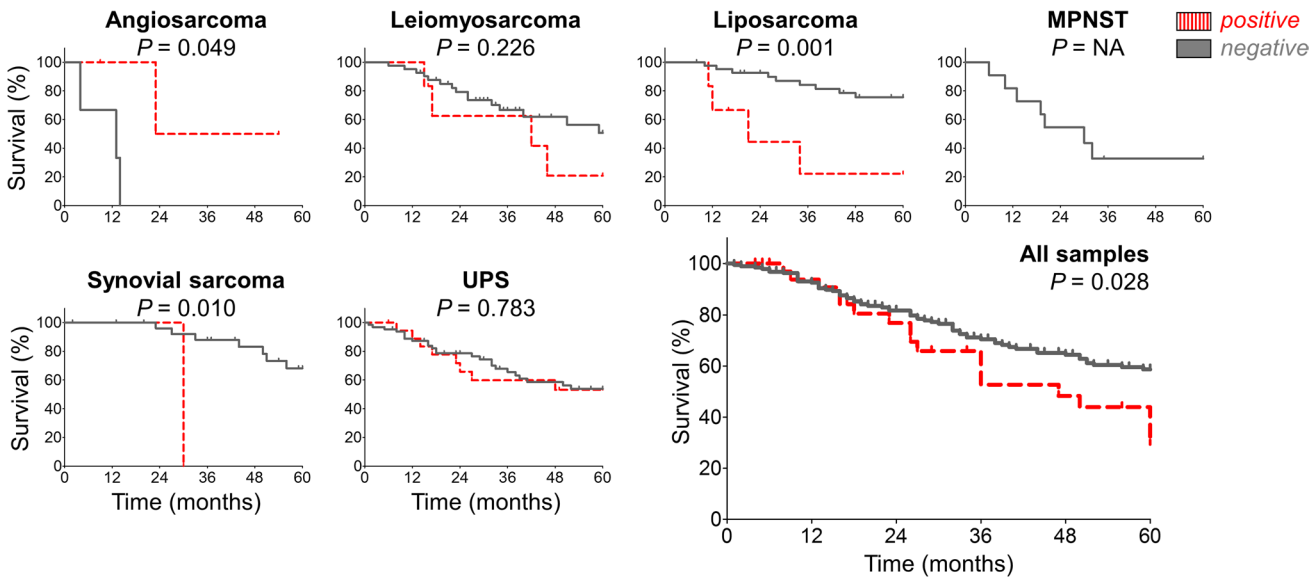

Fig. 2 PD-L1 is associated with overall survival in STS. Kaplan–Meier plots indicating the overall survival for the given STS subtypes and for all samples in the entire cohort. Dashed, red: PD-L1 positive; solid, gray: PD-L1 negative; significance was assessed by log-rank test

versus newer FFPE material (from 1989 to 2012), compatibly to a publication presenting even 40-year-old FFPE material suitable for IHC [27]. The cohort comprised six distinct and representative STS subtypes, including the three most common subtypes in adults (UPS, liposarcoma, leiomyosarcoma), which were represented by at least 47 samples, each. In contrast to several previous studies on single STS subtypes, this comprehensive sample set enables analyses across STS subtypes without inter-observer bias. Thus, our analyses were based on one of the largest and most comprehensive STS cohorts for IHC with associated survival data [20, 28, 29].

For MPNST not any sample was scored as positive for PD-L1, for angiosarcomas half of the six tested samples, which indicates a strong variability of PD-L1 expression depending on the STS subtype. However, it should be noted that for both subtypes showing extreme PD-L1 positivity rates only a limited number of samples (11 and 6, respectively) were available. Correspondingly, those extreme observations did not affect our further analyses across STS subtypes strongly. Nevertheless, 17 of 25 angiosarcoma samples in our second TMA cohort were positive for PD-L1 and high rates of PD-L1-positive cutaneous angiosarcomas have been reported previously [16]. In line, there is first evidence for the benefit of PD-L1-PD-1-axis blockage in angiosarcoma [30].

Interestingly, 23% of UPS cases (19/83) were positive for PD-L1, while only 13% of leiomyosarcomas (6/47) and 12% of dedifferentiated liposarcomas (6/49) showed PD-L1 expression. This is in line with prior studies in other cancers correlating mutational burden, which is much higher in UPS compared to leiomyosarcoma and liposarcoma [31], with enhanced neoantigen presentation [32, 33]. Hence, immune checkpoint inhibition may be in particular effective in UPS. Consistently, PD-L1 expression on protein level was relatively high for the TCGA sarcoma dataset, which comprises many UPS cases, compared to other tumor entities like bladder cancer or melanoma (The Cancer Proteome Atlas, [34]); high rates of PD-L1 positivity in UPS have been reported before [20, 21], and the majority of responders to pembrolizumab in the SARC028 trial are UPS patients [26].

As PD-L1 acts as ligand of PD-1, we next investigated the positivity for PD-1 expressing TILs in the same patients' specimens tested for PD-L1 positivity. Across all STS subtypes with > 11 samples, the rate of PD-1-positive samples in PD-L1 expressing ones was higher than in the negative ones. This result is supported by previous studies in smaller STS cohorts or single STS subtypes demonstrating a PD-L1 and PD-1 interaction [16, 18, 20, 28], which collectively provides a strong rationale that PD-L1-positive STS patients are eligible for immune checkpoint inhibitor therapy. Nevertheless, the actual co-localization of PD-L1-positive tumor cells and PD-1-positive TILs is a prerequisite for successful ligand/receptor interaction. To this end, five representative cases of our cohort (3 × UPS, 1 × leiomyosarcoma, 1 × liposarcoma) were investigated for both markers on consecutively cut and stained open slides. Indeed, not any PD-L1-positive tumor cell was observed without direct contact to a PD-1-positive TIL or at least location in close vicinity. Furthermore, this finding supports the hypothesis that cancer cells can dynamically increase PD-L1 expression to protect themselves in settings of increased numbers of TILs [35, 36], as an alternative mechanism of PD-L1 upregulation to PD-L1 driving mutations, which results in occurrence of PD-L1-positive tumor cells independent of TILs [37, 38]. When further characterizing these TILs, CD8-positive T cells were found to constitute the largest population of TILs, another marker correlating with immunotherapy response [26]. Additionally, we observed CD19-positive B cells in all samples, although to various extents. The presence of B cells might be another indicator of immune checkpoint therapy response, as B cells and especially the formation of tertiary lymphoid structures have been described very recently as strong predictors of immunotherapy response, even in sarcomas [39, 40].

The evaluations of PD-L1 expression and PD-1-positive TILs were performed on two independent cohorts. Strikingly, we found in our second TMA cohort, with usually three cores from different tumor areas per sample, that in 93.8% of samples the PD-L1 status was identical across all cores, and in 89.4% the PD-1 status. Thus, our results on TMAs are likely representative for the respective tumors.

Besides the role of PD-L1 as a biomarker to guide clinical decisions on the implementation of immunotherapy, we evaluated its prognostic relevance. In our large STS cohort, we found that intratumoral PD-L1 expression was associated with significantly worse 5-year overall survival ($P = 0.028$). Actually, PD-L1 was significantly positively associated with histological G3 grading, which further supports previous findings on superior response to checkpoint inhibitors in tumors with high mutational burden [32]. There was no statistically significant difference in the association of PD-L1 expressing STS with clinically unfavorable markers when subgrouping the samples by their degree of PD-L1 immunoreactivity. This may indicate that the quality of PD-L1/PD-1 interaction is more prognostically relevant than the quantity. As high histological tumor grading is a strong negative prognostic factor, and as PD-L1 and grading were correlated, we could not identify PD-L1 as independent prognostic factor in multivariate analysis. However, the association with survival

in our cohort of six STS subtypes, despite with different sample contributions, was in line with previous reports for single STS subtypes [16, 17, 21, 28], studies with smaller cohorts of various STS subtypes [15], and findings on a large cohort ($n = 758$) on the mRNA level [41].

This study focused on the potential eligibility of STS patients for immune checkpoint inhibition via the PD-L1/PD-1 axis. Notably, there are other attempts to inhibit negative immune regulation, like targeting CTLA4 [42, 43]. Future studies have to elucidate the expression patterns of CTLA4 ligands and abundance of CTLA4-positive lymphocytes in STS. However, a pilot study for synovial sarcoma did not find any benefit from CTLA4 blockage via ipilimumab [44].

Our findings on PD-L1 expression in STS, coincidence with PD-1-positive TILs and association with survival in a large and comprehensive STS cohort support previous studies on single STS subtypes and further confirm the comprehensive analyses by Dancsok et al. Thus, our study demands an extensive evaluation of the relevance of immune checkpoint blockage across various STS subtypes with increased sample sizes, and to also address the tumor microenvironment known to be relevant in the context of immune checkpoint blockage [45]. We further recommend evaluation beyond the subtypes included in this study. For instance, myxofibrosarcoma has been shown to be potentially drugable with nivolumab combined with ipilimumab [22], but was not included in our cohort as we found only four suitable samples in our archives, of which two were actually PD-L1 positive.

The observed prognostic relevance of the PD-L1/PD-1 axis indicates that especially PD-L1-positive STS patients should be considered for treatment with immune checkpoint inhibitors, as especially in these patients long-term tumor control may not be achieved with conventional treatment options.

We show for a large and comprehensive STS cohort the abundance of PD-L1, PD-1 and TILs across subtypes and provide evidence for the clinical relevance of PD-L1.

We conclude that immune checkpoint inhibitor treatment may constitute a promising approach for a substantial proportion of STS patients that show immunohistochemical evidence for intratumoral PD-L1 expression.

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Authors' contributions MFO analyzed the data, performed statistical analysis, and wrote the paper together with TGPG. EK and TKn scored the IHC staining of PD-L1 and PD-1. FCA, LRP, and FSW scored Ki-67 staining. VLB, MS, EN, LL, and RI supplied clinical data. AAH carried out statistical analyses. TGPG and TKi provided laboratory infrastructure. TKn conceived the project, drafted the paper, and supervised the analyses together with TGPG. All authors read and approved the final manuscript.

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committees of the LMU Munich University hospital (307-16 UE, 25.05.2016) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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