Original Article

T-Cell Infiltration and Clonality Correlate With Programmed Cell Death Protein 1 and Programmed Death-Ligand 1 Expression in Patients With Soft Tissue Sarcomas

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BACKGROUND: Patients with metastatic sarcomas have poor outcomes and although the disease may be amenable to immunotherapies, information regarding the immunologic profiles of soft tissue sarcoma (STS) subtypes is limited. **METHODS:** The authors identified patients with the common STS subtypes: leiomyosarcoma, undifferentiated pleomorphic sarcoma (UPS), synovial sarcoma (SS), well-differentiated/dedifferentiated liposarcoma, and myxoid/round cell liposarcoma. Gene expression, immunohistochemistry for programmed cell death protein (PD-1) and programmed death-ligand 1 (PD-L1), and T-cell receptor Vβ gene sequencing were performed on formalin-fixed, paraffin-embedded tumors from 81 patients. Differences in liposarcoma subsets also were evaluated. **RESULTS:** UPS and leiomyosarcoma had high expression levels of genes related to antigen presentation and T-cell infiltration. UPS were found to have higher levels of PD-L1 ($P \leq .001$) and PD-1 ($P \leq .05$) on immunohistochemistry and had the highest T-cell infiltration based on T-cell receptor sequencing, significantly more than SS, which had the lowest ($P \leq .05$). T-cell infiltrates in UPS also were more oligoclonal compared with SS and liposarcoma ($P \leq .05$). A model adjusted for STS histologic subtype found that for all sarcomas, T-cell infiltration and clonality were highly correlated with PD-1 and PD-L1 expression levels ($P \leq .01$). **CONCLUSIONS:** In the current study, the authors provide the most detailed overview of the immune microenvironment in sarcoma subtypes to date. UPS, which is a more highly mutated STS subtype, provokes a substantial immune response, suggesting that it may be well suited to treatment with immune checkpoint inhibitors. The SS and liposarcoma subsets are less mutated but do express immunogenic self-antigens, and therefore strategies to improve antigen presentation and T-cell infiltration may allow for successful immunotherapy in patients with these diagnoses. ***Cancer* 2017;123:3291-304.**

KEYWORDS: gene expression, immunotherapy, leiomyosarcoma, liposarcoma, pleomorphic, programmed cell death protein (PD-1), programmed death-ligand 1 (PD-L1), sarcoma, T-cell receptors.

INTRODUCTION

Cancer immunotherapies have induced durable responses for patients with many types of cancer.[1,2] T cells engineered with receptors that target tumor-associated antigens have antitumor activity and can induce complete and durable remissions in some patients with cancer.[3-7] Antibodies that block ligand engagement of T-cell checkpoint molecules such as CTLA-4 and programmed cell death protein (PD-1) have transformed the standard of care for melanoma and lung cancer,[8,9] and hold promise for many other tumor types.

Despite these successes, much work remains to make checkpoint blockade highly effective for the majority of patients with advanced cancers. How to best select patients, design combination strategies, and sequence regimens are areas of intense research that will hinge on an improved understanding of the tumor immune microenvironment. Approaches to interrogate the tumor microenvironment have included characterization of infiltrating immune cells, immunohistochemistry (IHC) to

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quantify the expression of inhibitory molecules including programmed death-ligand 1 (PD-L1), characterization of the lymphocyte infiltrates using T-cell receptor Vβ gene (TCR-Vβ) sequencing, and gene expression analysis.[10-12] However, few studies in any cancer type have used multiple modalities to understand how these different elements interact with one another.

Soft tissue sarcomas (STS) comprise < 1% of adult malignancies but are an ideal group of malignancies for the study of focused questions regarding the tumor immune microenvironment because many STS subtypes have unique and diverse biologic features. STS often are divided biologically into 2 categories: genetically "simple" and "complex" tumors. The simple tumors tend to have specific, reliably identifiable oncogenic alterations (such as translocations) and a limited number of mutated neoantigens.[13,14] Synovial sarcoma (SS) and myxoid/round cell liposarcoma (MRCL) both are translocation-driven malignancies and often express high levels of self-antigens, notably NY-ESO-1,[15,16] which has been targeted with promising signals of efficacy.[6,17]

The genetically "complex" STS tumors, such as undifferentiated pleomorphic sarcomas (UPS) and leiomyosarcoma (LMS), have numerous genetic mutations but no clear oncogenic driver. Although well-differentiated/dedifferentiated (WD/DD) liposarcomas may have a low mutation burden and are driven through overexpression of MDM2 and CDK4, DD liposarcomas can acquire additional mutations, ultimately becoming highly mutated.[18] Highly mutated tumors are more genetically heterogeneous and may provide multiple immunogenic mutated protein targets for T cells, which subsequently may be inhibited at checkpoints such as PD-1.[19,20] Indeed, recently presented provisional data have indicated that treatment with checkpoint inhibitors can result in a benefit in patients with certain sarcoma subtypes such as UPS.[21]

Overall, the outcome of patients with advanced STS remains poor, with a median overall survival in the region of 12 to 18 months. The objective of the current study was to identify key biologic patterns related to the immune response that could potentially serve as a biological rationale for future sarcoma immunotherapy trials as well as identify patterns that might be relevant to other solid tumors. Because of the rarity of some STS subtypes, the current study was focused on 4 of the more common subtypes (including tumors with both simple and complex karyotypes): 1) liposarcoma (both the WD/DD and MRCL liposarcoma subtypes); 2) SS; 3) UPS; and 4) LMS. We examined the expression of 760 genes, including a majority related to immune function, using a NanoString platform (NanoString Technologies, Seattle, Wash); PD-1 and PD-L1 expression were examined using IHC and TCR clonality was examined using TCR-Vβ sequencing.

MATERIALS AND METHODS

Patients

Institutional Review Board approval was obtained before commencing this study. A retrospective search of a prospectively maintained database was performed to identify consecutively listed patients with UPS, WD/DD liposarcoma, MRCL, LMS, or SS with available tumor tissue from the Institutional Review Board-approved, University of Washington/Fred Hutchinson Cancer Research Center tissue bank. All patients consented to participate in this tumor bank. Patients were treated at the University of Washington/Seattle Cancer Care Alliance with surgical resection between 2002 and 2012. Patient demographic data and histological characteristics were obtained from the database and chart review. In all cases, an experienced soft tissue pathologist confirmed the histological diagnosis and tumor grade. The French Federation of Comprehensive Cancer Centers (FNCLCC) grading system was used to grade tumors.[22] A hematoxylin-and-eosin-stained specimen was prepared from all tumor blocks to confirm the presence of viable tumor from each sample.

NanoString Gene Expression Analysis

nCounter gene expression assay: preparing the RNA lysate

Before making the cellular RNA lysate, tissue sections were deparaffinized in xylene 3 times at 5 minutes each and then rehydrated by immersing consecutively in 100% ethanol twice for 2 minutes each time, 95% ethanol for 2 minutes, and 70% ethanol for 2 minutes and then were immersed in distilled water until they were ready to be processed. Tissue was lysed on the slide by adding 10 to 50 μL of PKD buffer (Qiagen Inc, Gaithersburg, Md). Tissue was scraped from the slide and transferred to a 1.5-mL Eppendorf tube. Proteinase K (Roche Molecular Systems Inc, Branchburg, NJ) was added at $\leq$ 10% of the final volume and the RNA lysate was incubated for 15 minutes at 55°C and then for 15 minutes at 80°C. The Qubit Fluorometer (Thermo Fisher Scientific, Waltham, Mass) then was used for quantification. The RNA lysate was stored at -80°C until gene expression profiling was performed using the NanoString nCounter system (NanoString Technologies).

NanoString methodology

Per sample, 50 ng (RNA content) from the cellular lysate in a final volume of 5 μL was mixed with a 3' biotinylated

capture probe and a 5' reporter probe tagged with a fluorescent barcode from the desired gene expression code set. Probes and target transcripts were hybridized overnight at 65°C for 12 to 16 hours as per the manufacturer's recommendations. Hybridized samples were run on the NanoString nCounter preparation station using their high-sensitivity protocol in which excess capture and reporter probes were removed and transcript-specific ternary complexes were immobilized on a streptavidin-coated cartridge. The samples were scanned at maximum scan resolution capabilities using the nCounter Digital Analyzer (NanoString Technologies).[23,24]

Analysis of NanoString data

Data analysis was performed using quantile normalization, in which relative ranks of genes (across all genes on the NanoString code set) within each sample were replaced by values having the same relative rank from the pooled distribution (from all samples and genes in the data set). Normalization was performed using nSolver software (NanoString Technologies). All quantile normalized data underwent subsequent log10 transformation. Individual genes were compared using an analysis of variance (ANOVA) and if the P value was $<.05$ (Turkey multiple comparison test), these genes were included using an unsupervised hierarchical cluster analysis. The individual reduced gene lists for each region then were analyzed in ingenuity pathway analysis (IPA) (Qiagen) using CORE analysis to try and help to identify whether they had any significant relationships or associations with known functions or pathways. These predefined networks within the IPA (grouping genes by function, pathway, disease association, etc) are manually curated from a consortium of published articles and public data. Although this analysis was not used to demonstrate associations between specific genes and STS subtype, it was used to identify potential genes of interest.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were deparaffinized and rehydrated with serial passage through changes of xylene and graded ethanols for PD-L1 and PD-1 IHC. All slides were subjected to heat-induced epitope retrieval in Envision FLEX Target Retrieval Solution, High pH (Dako Corporation, Carpinteria, Calif). Endogenous peroxidase in tissues was blocked by incubation of slides in 3% hydrogen peroxide solution before incubation with the primary antibody (anti-PD-L1 clone 22C3 [Merck Research Laboratories, Palo Alto, Calif] or anti-PD-1 clone NAT105 [Cell Marque, Rocklin, Calif]) for 60 minutes. Antigen-antibody binding was visualized with the FLEX + polymer system (Dako) and application of 3,3' diaminobenzidine chromogen (Dako). Stained slides were counterstained with hematoxylin and coverslipped for review and scoring. Scoring was conducted by a pathologist (J.H.Y.) who was blinded to all patient clinical information using a semiquantitative scale of 0 to 5, in which positive cell frequency within the tumor tissue was grouped into the following categories: 0 indicates negative, 1 indicates rare, 2 indicates low, 3 indicates moderate, 4 indicates high, and 5 indicates very high. These patterns are illustrated for both PD-1 and PD-L1 in Supporting Information Figure 1.

Analysis of PD-1 and PD-L1 expression

Detectable PD-L1 and PD-1 expression was defined as an IHC score >0. A score >2 was used to delineate tumors with higher levels of expression. Binary variables were compared using a chi-square test, and continuous variables were compared using a Student t test by PD-L1/PD-1 status. The relationship between tumor size and PD-L1 expression was analyzed independent of histology using a logistical model, which included tumor size as a continuous variable. The Kaplan-Meier method was used to estimate overall survival (defined as the time from diagnosis to death), progression-free survival (PFS; defined as the time from diagnosis to disease progression or death), and recurrence-free survival (defined as the time from definitive surgery to disease recurrence or death) and compared between patient groups with the log-rank statistic. Cox proportional hazards models were used to estimate the hazard ratio and associated 95% confidence interval for defined groups of patients.

TCR-β Immunosequencing

DNA extraction of FFPE preserved solid tumor curls and TCR-β immunosequencing were performed at Adaptive Biotechnologies Corporation (Seattle, Wash) as previously described.[25-27] In brief, DNA was extracted and TCR-β complementarity determining region 3 regions were amplified using a multiplexed polymerase chain reaction method with 54 forward primers specific to TCR-Vβ gene segments and 13 reverse primers specific to TCR-Jβ gene segments. Next, the Illumina HiSeq platform (Illumina Inc, San Diego, Calif) was used to sequence the resulting amplicons. Housekeeping genes also were amplified, and their template counts quantitated to determine the amount of DNA usable for TCR-β sequencing. The number of total cells and T cells, the T-cell fraction, the number of unique rearrangements, and clonality were

calculated for each sample. A Mann-Whitney-Wilcoxon test was used to compare TCR sequencing metrics between histologic subtypes.

Integrative Analysis of Multiple Assays

NanoString gene expression data were normalized with the median- median absolute deviation normalization method for each sample in the log2 scale. A linear model was constructed to investigate the association between the T-cell fraction and TCR clonality and the normalized NanoString gene expression. For analysis of clonality and T-cell fraction with IHC staining, a model was constructed using only histology as an adjusting factor.

RESULTS

Clinical Demographics

Archival tumor samples were selected from 81 patients with a median age of 53 years (range, 18-57 years) who had provided written informed consent to the University of Washington/Fred Hutchinson Cancer Research Center tumor bank (Table 1). FNCLCC is the standard grading system used at the study institution, and we verified that this system was used in 77 of the 81 cases (>95%).[22] A total of 34 tumors (42%) were high grade (grade 3), 31 tumors (38%) were intermediate grade (grade 2), and 15 tumors (19%) were low grade (grade 1). The median tumor size was 9 cm (range, 1.2-40 cm).

Specimens from the patients who provided consent to the tumor bank included liposarcoma (27 specimens), LMS (19 specimens), UPS (20 specimens), and SS (15 specimens). Although the UPS generally were undifferentiated, 2 of these tumors had areas with a spindle cell appearance and 1 contained areas of tumor with myoid differentiation. Of the 27 liposarcomas, there were 15 WD/DD tumors, including 9 tumors with dedifferentiation and 6 aggressive WD liposarcomas (meaning either tumors measuring >10 cm or tumors that were recurrent/refractory). The other 12 liposarcoma specimens had MRCL histology. LMS tumors were not selected for having uterine (2 specimens) versus nonuterine (17 specimens) origin. Thirteen of the 15 SS tumors had monophasic histology. Patients had a mix of different clinical outcomes. Of the 81 patients, 38 (47%) developed metastatic disease, and 44 patients (54%) were alive at the time of last follow-up. Although the majority of specimens were from the resection of the primary tumor, 18 (22%) were from a recurrence of a metastatic lesion. A total of 36 patients (44%) with tumors examined in the current study had received no neoadjuvant chemotherapy or radiotherapy before undergoing surgical resection. A total of 21 patients (26%) received neoadjuvant chemotherapy alone and 17 patients (21%) had received neoadjuvant chemotherapy and radiotherapy. Prior chemotherapy ($P<.01$) but not radiotherapy ($P=.59$) was found to be associated with histology.

TABLE 1. Patient Characteristics

Characteristic	N = 81	
Age, y		
Median (range)	52	
Range	18-87	
Interquartile range	42-60	
Sarcoma type		
Liposarcoma	27	33%
WD/DD	15	56%
Myxoid/round cell	12	44%
LMS	19	23%
Nonuterine	17	89%
Uterine	2	11%
Pleomorphic	20	25%
SS	15	19%
Monophasic	13	87%
Biphasic	1	7%
Unknown	1	7%
Treatment prior to surgery		
None	36	44%
RT	7	9%
Chemotherapy	21	26%
Chemotherapy and RT	17	21%
Tumor size, cm		
Median (range)	9 (1.2-40)	
Interquartile range	5-12.6	
FNCLCC tumor grade		
1	15	19%
2	31	38%
3	34	42%
Unknown	1	1%
Local disease recurrence		
Yes	26	32%
No	54	67%
Unknown	1	1%
Developed metastasis		
Yes	38	47%
No	41	51%
Unknown	2	2%
Alive at time of last follow-up		
Yes	44	54%
No	37	46%

Abbreviations: FNCLCC, French Federation of Comprehensive Cancer Centers; LMS, leiomyosarcoma; RT, radiotherapy; SS, synovial sarcoma; WD/DD, well-differentiated/dedifferentiated liposarcoma.

More Highly Mutated STS Subtypes Express High Levels of Genes Related to Antigen Presentation and T-Cell Infiltration

RNA extraction and unsupervised cluster analysis

RNA was extracted from paraffin-embedded tumor samples and analyzed using a NanoString gene expression assay of 760 genes, a majority of which were related to the immune response but also related to many other

functions. Sufficient RNA for NanoString analysis was obtained in 18 of 19 LMS samples (95%), 24 of 27 liposarcoma samples (89%), 18 of 20 UPS samples (90%), and all 15 SS samples (100%), for a total of 75 of 81 samples (93%). An initial unsupervised clustering analysis including all samples and genes initially was performed to observe broad patterns in histologic subtype and gene expression clusters. This analysis generally separated out the individual histologic subtype with few exceptions (see Supporting Information Fig. 2).

Cluster analysis and IPA

To focus on the differential gene expression between STS subtypes, genes were analyzed in a subsequent unsupervised clustering analysis only if there was a P value $< .05$ confirming a difference in expression levels between at least 2 STS subtypes (Fig. 1A). This analysis identified 367 of the 760 genes as being significantly different between the subgroups (48%). Seven gene regions were defined within this analysis (Fig. 1A) using the dendrogram tree generated by the unsupervised clustering. The genes in each region are detailed in Supporting Information Table 1. The individual reduced genes in each region then were analyzed in IPA using CORE analysis to help identify whether they had any relationships or associations with known functions or pathways. These predefined networks within IPA (grouping genes by function, pathway, disease association, etc) previously had been curated manually from a consortium of published articles and public data.

Genes related to antigen presentation

One of the strongest associations identified using IPA was with the "antigen presentation pathway" and group 6, a group of genes whose calculated variance demonstrates higher differential expression in UPS and LMS and lower differential expression in liposarcomas (WD/DD) and SS. Human leukocyte antigen A (HLA-A), HLA-B, and HLA-C are critical genes for antigen presentation and are absolutely necessary for the recognition of tumor cells by conventional CD8-positive T cells. For each of these, UPS and LMS were found to have the highest expression, an expression level that was significantly higher than that for SS (HLA-A, $P<.0001$ [Fig. 1B]; others shown in Supporting Information Fig. 3A). A similar pattern was observed in the noncanonical HLA molecule HLA-G (Fig. 1B), as well as class II HLA genes such as *HLA-DPB1* (see Supporting Information Fig. 3A). *TAP1* (transporter-associated with antigen processing 1), a critical element of major histocompatibility complex (MHC)/peptide loading, also had significantly higher expression in UPS compared with all other subtypes with the exception of LMS ($P = .0052$) (Fig. 1B).

T-cell-related genes

Group 6 also had notable overlap with genes associated in the canonical pathways of "communication between adaptive and innate immune cells" as well as "cytotoxic T-lymphocyte-mediated apoptosis of target cells." *CD3D*, part of the CD3 complex and therefore a marker for T-cell infiltration, and *CD8A*, part of CD8 and therefore a marker of CD8-positive T cells, were included in this zone. *CD8A* is shown in Supporting Information Figure 3, as is interleukin 2 receptor subunit alpha (*IL-2RA*), which also may be associated with T-cell-mediated inflammation. *CD3D* expression was found to be highest in LMS and UPS specimens and was significantly higher for LMS compared with MRCL ($P = .0089$) (Fig. 1C). The IL-7 receptor (CD127, IL-7R) often is expressed on activated and antigen-experienced T cells and was found to be significantly higher for UPS and LMS ($P = .0021$) (Fig. 1C). *IDO* is a key regulator affecting these cells and also demonstrated a trend toward higher expression in UPS and LMS specimens (see Supporting Information Fig. 3B) as did the T-cell markers *CD4, CD27*, and CD28, as well as the chemokine receptor/ligands *CCL5* and *CCR5*. Genes in zones 1 and 4 had higher calculated variance in UPS and LMS specimens; this included *CD274* (PD-L1), in which a strong trend toward higher expression in UPS and LMS specimens was noted, suggesting that further analysis would be warranted. *PDCD1LG2* (PD-L2) was included in zone 5, in which genes had higher variance in UPS compared with any other subtype (Fig. 1D).

Other notable genes also are shown in Supporting Information Figure 3. A separate K-means clustering analysis of the same 367 genes also was performed (see Supporting Information Fig. 4) (genes are listed in Supporting Information Table 2). This analysis highlighted the importance of tumor-associated macrophages (TAM) in LMS, confirming observations made in prior studies (Fig. 1E).[28,29] A trend toward more regulatory T-cell gene signatures was observed in UPS and LMS specimens and some liposarcoma cases (see Supporting Information Figs. 5 and 6).

Immune-related gene expression in MRCL versus WD/DD

In our initial cluster analysis, all liposarcoma specimens were analyzed together. However, through our analysis of individual genes, we recognized that there were important

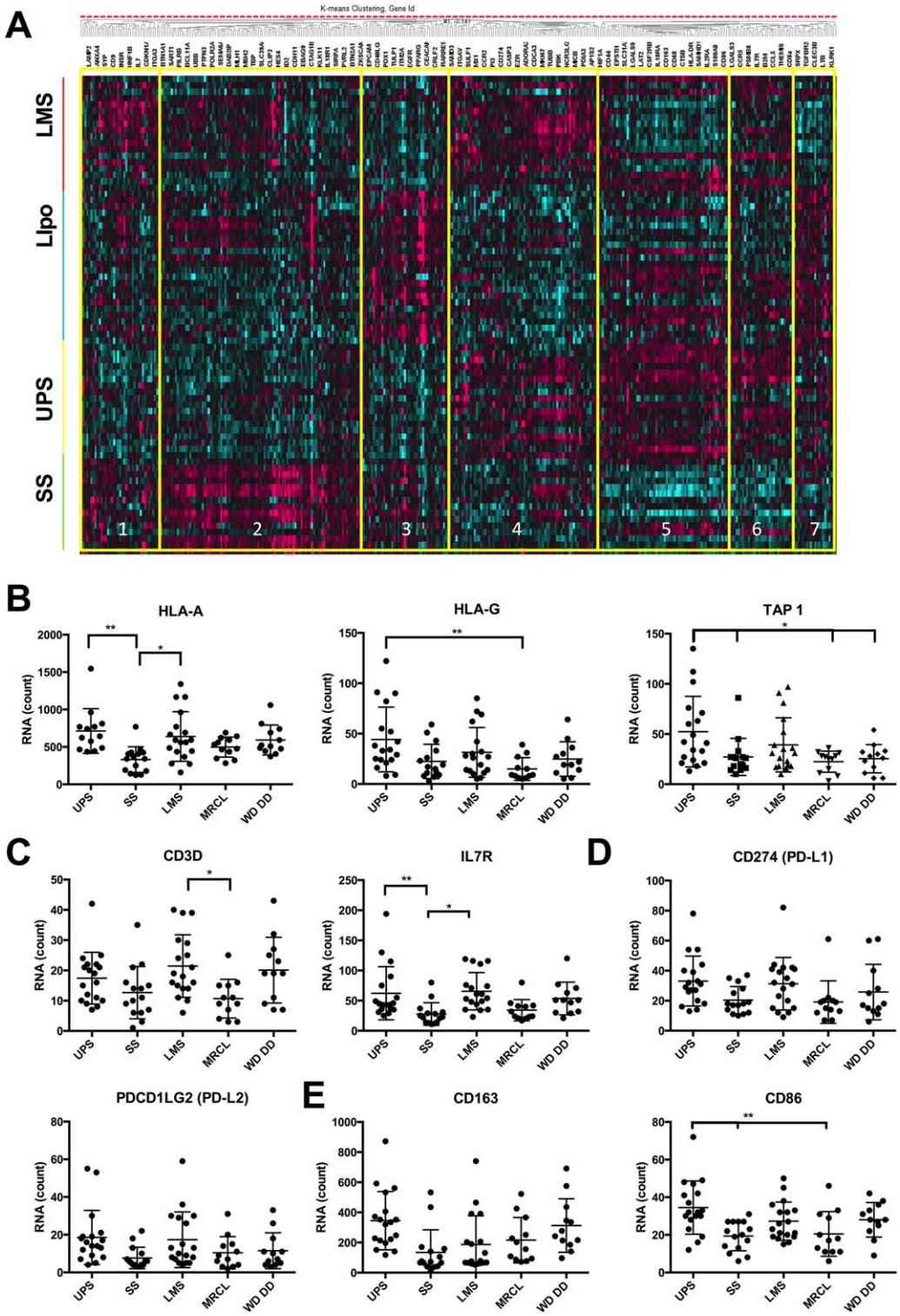

Figure 1. (A) Heat map of 367 genes found to be significantly different between at least 2 sarcoma subtypes ($P<.05$) after unsupervised clustering. (B) Gene expression by sarcoma subtype in selected genes related to antigen presentation. (C) Selected genes reflecting T-cell infiltration. (D) Gene expression for programmed death-ligand 1 (*PD-L1*) and *PD-L2*. (D) Selected markers found on infiltrating tumor-associated macrophages. * indicates $P\leq.05$; **, $P\leq.01$; ***, $P\leq.001$; ****, $P\leq.0001$; HLA, human leukocyte antigen; IL7R, interleukin 7 receptor; Lipo, liposarcoma; LMS, leiomyosarcoma; MRCL, myxoid/round cell liposarcoma; PDCD1LG2, programmed cell death 1 ligand 2; SS, synovial sarcoma; TAP1, transporter-associated with antigen processing 1; UPS, undifferentiated pleomorphic sarcoma; WD/DD, well-differentiated/dedifferentiated liposarcoma.

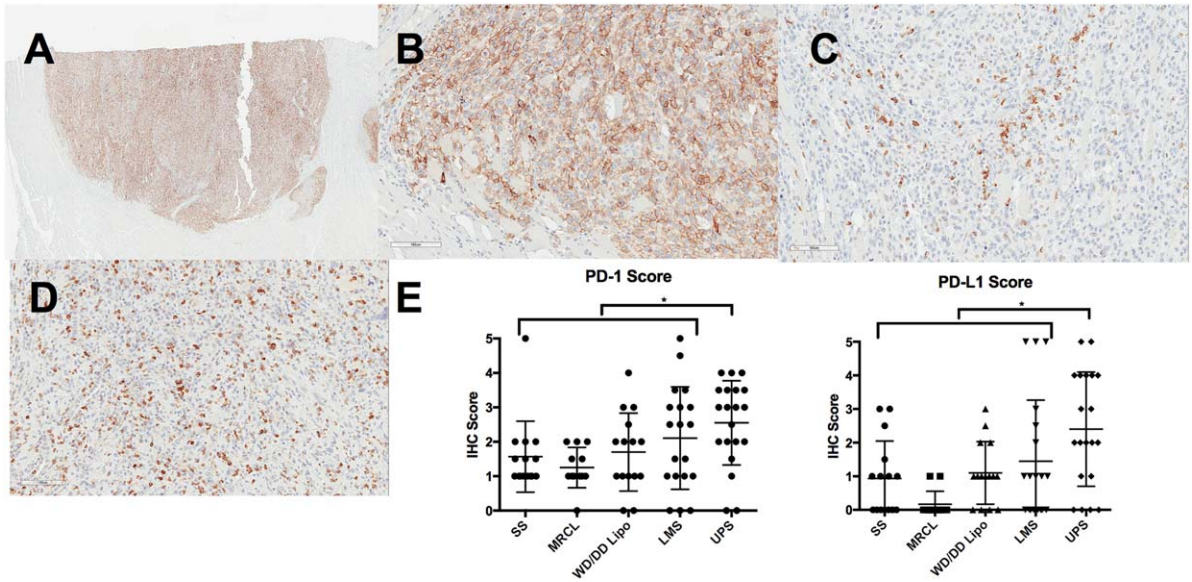

Figure 2. (A) Programmed death-ligand 1 (PD-L1) delineated undifferentiated pleomorphic sarcoma (UPS) tumor on low power; no staining was observed in adjacent normal tissue. (B) Very high tumor cell staining for PD-L1 in a UPS noted on high power. (C) High programmed cell death protein (PD-1) staining in a UPS. (D) Very high levels of PD-1-positive infiltrates in a leiomyosarcoma (LMS) tumor. (E) PD-1 and PD-L1 scores in sarcoma subtypes. * indicates $P<.05$; **, $P<.01$; ***, $P=.001$; ****, $P=.0001$; IHC, immunohistochemistry; MRCL, myxoid/round cell liposarcoma; SS, synovial sarcoma; WD/DD Lipo, well-differentiated/dedifferentiated liposarcoma.

differences in the immunobiology of these 2 biologically distinct entities. To parse the differences between the MRCL and WD/DD, a similar cluster analysis based on calculated variance was performed on only the liposarcoma tumors, which demonstrated markedly different expression patterns between these liposarcoma subsets (see Supporting Information Fig. 7) (individual genes are shown in Supporting Information Table 3). Again using IPA and CORE analysis, predefined interferon-related gene groups and TCR synapse-related groups were found to demonstrate higher variance in gene expression in the WD/DD tumors.[10,11] We suspected that DD tumors would have higher levels of inflammatory genes compared with WD tumors because they often acquire additional genetic mutations,[30] and we observed a nonsignificant trend in support of this (see Supporting Information Fig. 8).

Increased PD-L1 and PD-1 Expression Are Noted in UPS

The pattern that emerged from the above gene expression analysis was that UPS had high expression of genes that were related to both antigen presentation and T-cell-mediated immunity. LMS also generally had high expression of these genes, whereas SS and MRCL specimens were found to have lower expression. We were interested to learn whether this expression pattern would carry over to PD-L1 protein expression, which was suggested based on the RNA expression data and would have potential therapeutic implications (Fig. 1B). Several studies to date have examined the presence or absence of PD-1 and PD-L1 in STS samples, but to the best of our knowledge none has scored the level of protein expression based on IHC.[31,32] We stained FFPE sections by IHC and scored them using a scoring system of 0 to ≥ 5 as described above and illustrated in Supporting Information Figure 1.

Among all 81 patients, 48 (59%) expressed PD-L1, and 73 (90%) had detectable PD-1-expressing cells within the tumor. We expected to find PD-1-expressing lymphocytes in PD-L1-expressing tumors, and indeed all patients with PD-L1-expressing tumors were found to have at least some PD-1-expressing cells within the tumor (see Supporting Information Table 4A). Furthermore, only 2 tumors with PD-L1 expression of ≥ 2 were found to have PD-1 expression of < 2. A total of 28 tumors (35%) had levels of PD-L1 expression of ≥ 2 (see Supporting Information Table 4B), and 41 tumors (51%) had levels of PD-1 expression of ≥ 2.

Higher grade tumors were associated with higher expression levels of both PD-L1 ($P=.03$) and PD-1 ($P=.05$). Neither prior chemotherapy, radiotherapy, or the combination were associated with higher levels of

TABLE 2. Association Between PD-L1 and PD-1 With Tumor and Patient Characteristics

PD-1

	Negative[a]		Positive[a]		*P*	Low[a]		High[a]		*P*
Prior treatment					.96					.77
None	3	8%	33	92%		16	44%	20	56%	
RT	1	14%	6	86%		3	43%	4	57%	
Chemotherapy	2	10%	19	90%		8	38%	13	62%	
Both	2	12%	15	88%		5	29%	12	71%	
Mean age, y	49		52		.62	47		54		.02
Sarcoma type					.72					.06
Liposarcoma (WD/DD)	1	8%	11	92%		7	58%	5	42%	
Liposarcoma (MRCL)	2	13%	13	87%		7	47%	8	53%	
LMS (nonuterine)	2	12%	15	88%		5	29%	12	71%	
Pleomorphic	2	10%	18	90%		3	15%	17	85%	
SS	0	0%	15	100%		8	53%	7	47%	
FNCLCC tumor grade					.19					.05
1	3	20%	12	80%		10	67%	5	33%	
2	1	3%	30	97%		10	32%	21	68%	
3	4	12%	30	88%		11	32%	23	68%	

PD-L1

	Negative[a]		Positive[a]		*P*	Low[a]		High[a]		*P*
Prior treatment					.24					.65
None	5	29%	12	71%		9	53%	8	47%	
RT	9	43%	12	57%		15	71%	6	29%	
Chemotherapy	18	50%	18	50%		24	67%	12	33%	
Both	1	14%	6	86%		5	71%	2	29%	
Mean age, y	47		55		.01	49		56		.03
Sarcoma type					.007					.001
Liposarcoma (WD/DD)	10	83%	2	17%		12	100%	0	0%	
Liposarcoma (MRCL)	4	27%	11	73%		11	73%	4	27%	
LMS (nonuterine)	7	41%	10	59%		11	65%	6	35%	
Pleomorphic	4	20%	16	80%		6	30%	14	70%	
SS	7	47%	8	53%		11	73%	4	27%	
FNCLCC tumor grade					.07					.03
1	10	67%	5	33%		13	87%	2	13%	
2	12	39%	19	61%		22	71%	9	29%	
3	11	32%	23	68%		17	50%	17	50%	

Abbreviations: FNCLCC, French Federation of Comprehensive Cancer Centers; LMS, leiomyosarcoma, MRCL, myxoid/round cell liposarcoma; PD-1, programmed cell death protein; PD-L1, programmed death-ligand 1; RT, radiotherapy; SS, synovial sarcoma; WD/DD, well-differentiated/dedifferentiated liposarcoma.

[a] Negative indicates a score of 0; positive, score ≥ 1; low, score ≤ 2; high, score > 2.

PD-L1 expression or an increased frequency of PD-1-expressing lymphocytes. Although the current study was not designed to examine the impact of PD-L1 or PD-1 on PFS or overall survival, the analysis was performed and did not reveal a statistically significant impact for this relatively small cohort of patients.

Detectable PD-L1 was noted in each of the STS subtypes tested and PD-1-expressing lymphocytes were observed in a majority of tumors in each subtype (Figs. 2A-2D [LMS]). Although numerically fewer UPS were entirely negative for PD-L1, this was not statistically significant ($P = .16$). However, UPS had higher levels of PD-L1 expression ($P = .001$) and PD-1 expression ($P = .05$) compared with other sarcoma subtypes (Fig. 2E). UPS also had higher levels of expression compared with the WD/DD and MRCL subsets of liposarcoma, as well as the nonuterine subset of LMS for both PD-L1 ($P = .05$) and PD-1 ($P = .001$) (Table 2).

High (≥ 4) or very high (≥ 5) expression of PD-L1 was observed in 10 tumors, including 7 UPS and 3 LMS. Seven tumors had high or very high PD-1 expression, including 3 UPS and 2 LMS. In contrast, very few of the translocation-related tumors (SS or MRCL) were found to have high PD-1 or PD-L1 expression. For example, even though 100% of the SS tumors had some PD-1 expression, none had either ≥ 4 or ≥ 5 PD-1 expression.

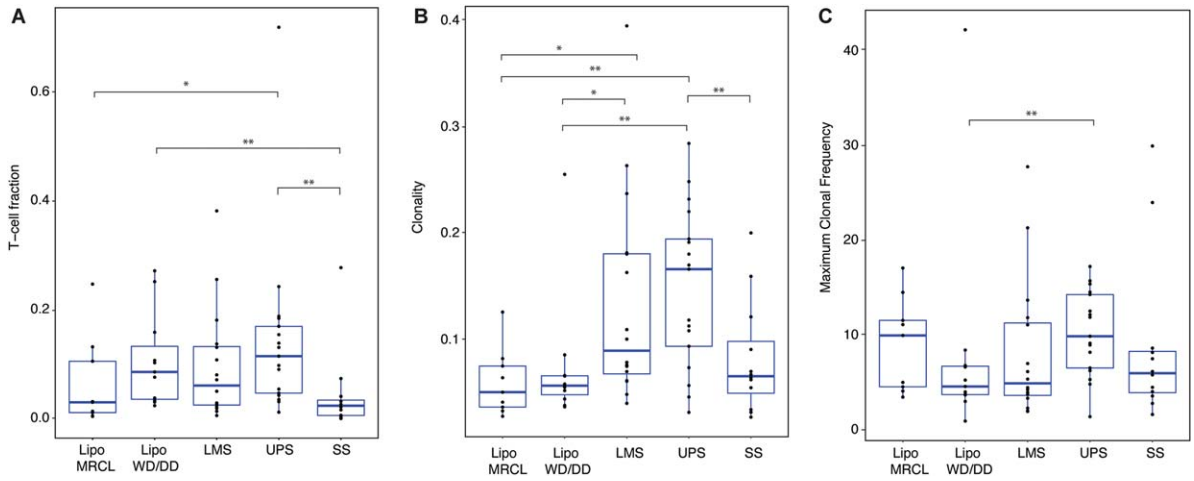

Figure 3. T-cell receptor sequencing in sarcoma subtypes examining (A) T-cell fraction, B) clonality, and C) maximum clonal frequency. * indicates $P<.05$; **, $P<.01$; ***, $P = .001$; ****, $P = .0001$; Lipo MRCL, myxoid/round cell liposarcoma; Lipo WD/DD, well-differentiated/dedifferentiated liposarcoma; LMS, leiomyosarcoma; SS, synovial sarcoma; UPS, undifferentiated pleomorphic sarcoma.

Only 4 SS tumors (27%) and none of the 12 MRCL tumors had > 2 + expression for PD-L1 (see Supporting Information Fig. 9).

UPS and LMS Have Higher TCR-Vβ Clonality Compared With SS and MRCL

Given that we noted higher expression of inflammatory-type genes in UPS and LMS as well as increased PD-1 and PD-L1 expression in UPS, we hypothesized that these tumors would have a more robust and focused T-cell response, and sought to confirm this through immunosequencing of the TCR-Vβ complementarity determining region 3 variable region of each tumor. FFPE tissue was available for immunosequencing for 72 of the 81 samples. Seven samples were filtered from further analysis because they yielded sequence data corresponding to <100 total cells or < 10 T cells. Among the 65 included samples, we observed an average of 18,545 total cells (range, 310-81,531 total cells), and an average of 1697 T cells (range, 11-28,449 T cells). The average number of unique TCR sequences observed was 664 (range, 9-4500 sequences). The maximum productive clonal frequency was 42.31%. The mean T-cell fraction, defined as the number of T cells in the sample divided by the total number of nucleated cells, was 0.097 (range, 0-0.720). Clonality, a metric characterizing the TCR distribution, in which more oligoclonal TCR repertoires are closer to 1 and more evenly (or randomly) distributed repertoires are closer to 0,[25-27] was 0.110 (range, 0.029-0.397) for the group. The distribution of clonality and TCR fractions for the group as a whole is depicted in Supporting Information Figures 10 and 11.

To have greater statistical power, liposarcomas were grouped together for further analysis. UPS (17 specimens) numerically had the highest T-cell fraction compared with all other sarcoma subtypes (mean T-cell fraction, 0.145) and were only statistically significantly different from SS (12 specimens; mean T-cell fraction, 0.044 [$P \leq .01$]) (Fig. 3A) and MRCL (11 specimens; mean T-cell fraction, 0.064 [$P \leq .05$]). WD/DD liposarcoma (9 specimens; mean T cell fraction, 0.107) also had a significantly higher T-cell fraction compared with SS ($P \leq .01$). The average T-cell fraction for LMS (16 specimens) was 0.096.

We analyzed the clonality of the different STS subtypes (Fig. 3B). We found that LMS (mean, 0.136) and UPS (mean, 0.151) specimens both had higher clonality scores than WD/DD liposarcoma (mean, 0.0759), MRCL (mean, 0.0615), and SS (mean, 0.084) specimens, reflecting a more oligoclonal T-cell repertoire (individual P value significance levels are indicated in Fig. 3B) (LMS and UPS as a group had significantly higher clonality [$P<.01$]). We also compared the maximum clonal frequency among the groups. UPS was found to have the highest average maximum clonal frequency (10.32%), although the only statistically significant difference was observed between UPS and WD/DD liposarcoma ($P \leq .05$) (Fig. 3C).

Neither prior radiotherapy nor chemotherapy were found to be associated with T-cell fraction or clonality even when adjusted for histology. Although these data

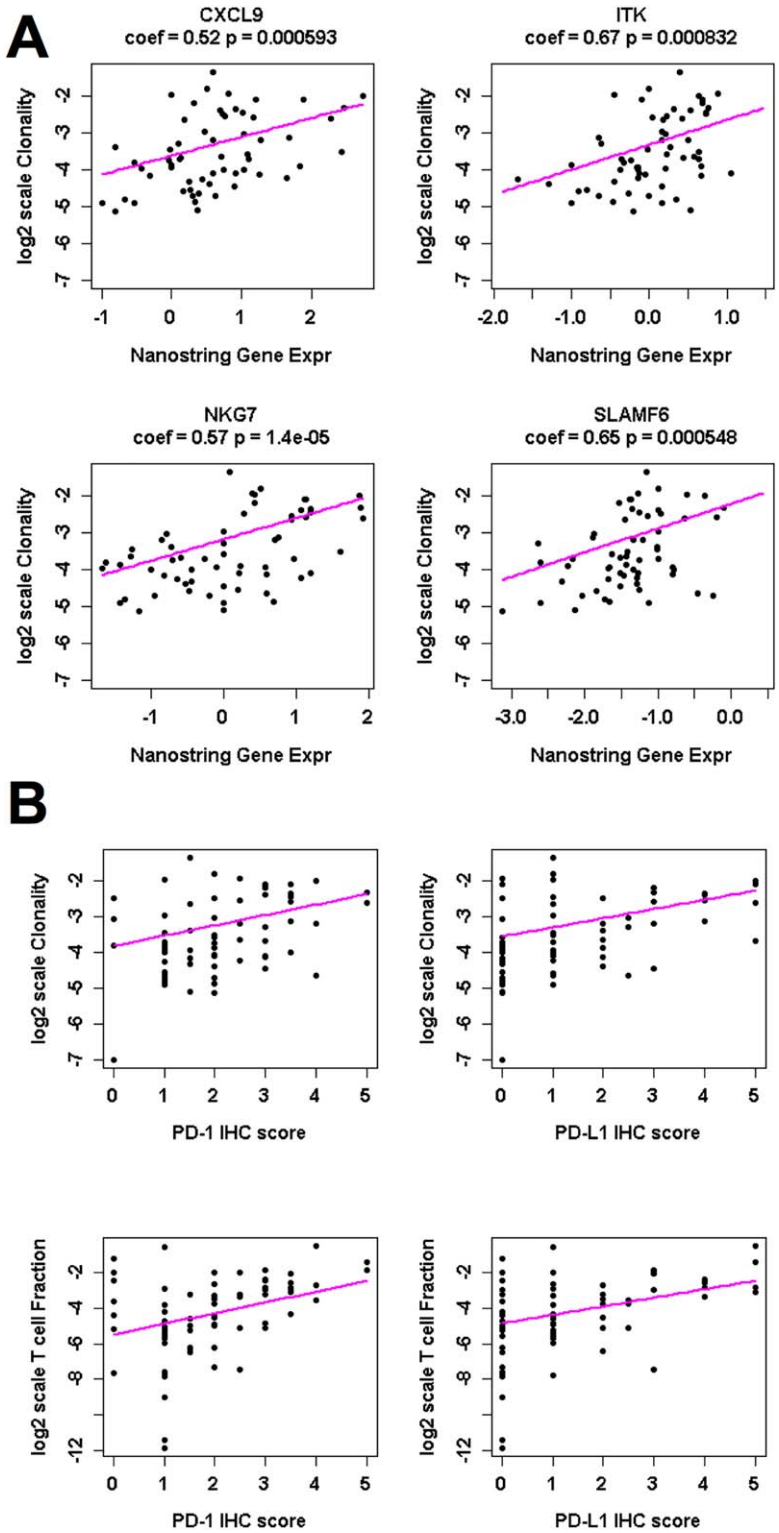

Figure 4. (A) Gene correlation with clonality. B) Correlation of T-cell fraction and clonality with programmed cell death protein (PD-1) and programmed death-ligand 1 (PD-L1) expression. Coef indicates coefficient; CXCL9, C-X-C motif chemokine ligand 9; Expr, expression; IHC, immunohistochemistry; ITK, IL-2 (interleukin-2)-inducible T-cell kinase; NKG7, natural killer cell granule protein 7; SLAMF6, SLAM family member 6.

were not powered to detect a relationship between clonality and survival, this was examined and no statistically significant correlation was observed. Neither was any association with prior chemotherapy or radiotherapy noted. Comparing the WD (4 specimens) with the DD (6 specimens) liposarcoma tumors, there were no significant differences noted with regard to clonality (0.062 vs 0.060, respectively) or maximum clonal frequency (0.057 vs 0.037; $P = .17$). In fact, the average T-cell fraction of WD (0.149) was actually higher than that of DD (0.55), as a result of one outlier WD tumor with a very high T-cell fraction (0.39).

PD-1 and PD-L1 Are Correlated With TCR Fraction and Clonality Adjusted For Histology

Our analysis indicated that UPS had a more inflammatory microenvironment than the other sarcoma subtypes. LMS and WD/DD liposarcoma tumors also appeared to share this inflammatory phenotype, although to a lesser degree and not by every metric. SS and MRCL generally had a noninflammatory phenotype. We then sought to integrate these data sets into an analysis that would look for a general correlation among all patients with sarcoma. No genes in this data set were found to have a statistically significant association with overall survival or recurrence-free survival in a Cox model adjusted for tumor grade. Gene expression was normalized with the median- median absolute deviation normalization method for each sample in the log2 scale. The clonality and T-cell fraction were log2 transformed to correct for the skewness of the data. A linear model was constructed to investigate the association between the clonality score of TCR sequencing and the normalized NanoString gene expression. Linear regression variable selection was conducted to search for possible confounding factors such as histology, tumor grade, tumor size, and marginal analysis. Histologic subtype was found to have a strong association with clonality ($P = .0168$). After adjusting for STS subtype, the expression levels of 4 genes were found to be significantly associated with clonality at a qvalue < 0.2 (see Supporting Information Table 5). These genes included *NKG7, SLAMF6, CXCL9*, and *ITK* (Fig. 4A).[23]

We also tested whether after adjusting for histology there would be correlation of genes with the T-cell fraction. The top 18 genes with a P value $< 10^{-5}$ are shown in Supporting Information Table 6. Although many of these genes were directly related to the T-cell infiltration, many also were related to antigen presentation. In fact, the 2 genes with the most significant correlation were class II MHC molecules HLA-DPB1 ($P = 1.38 \times 10^{-12}$) and HLA-DRA (3.3×10^{-12}). The third most significant association also was related to class II antigen presentation: CD74 ($P = 1.26 \times 10^{-9}$). None of these associations were explained in an analysis including prior chemotherapy or radiotherapy.

We then sought to test whether PD-1 and PD-L1 expression scores were correlated with T-cell fraction and clonality (Fig. 4B) (see Supporting Information Table 7) using a model that adjusted only for histology. T-cell fraction was found to be significantly associated with both PD-1 ($P = .00304$) and PD-L1 ($P = .00638$). To determine whether this association was due to a random influx of T cells into the tumor or a more organized tumor-specific infiltrate, we also tested whether clonality was associated with these staining patterns and clonality also was found to be significantly associated with both PD-1 ($P = .00711$) and PD-L1 ($P = .00369$).

DISCUSSION

Outcomes for patients with metastatic STS remain poor.[33] The standard first-line therapy for patients with metastatic STS is doxorubicin, with an overall response rate of 20% to 30% and a median PFS of approximately 4.6 months.[34-36] Although new drugs recently have been approved for metastatic STS that may add valuable additional months of survival for patients, there remains a clear need for therapies capable of inducing deep and durable responses.[37,38]

Immunotherapy has been relatively slow to be evaluated in sarcoma. To the best of our knowledge, the current study provides the most detailed characterization of the immune phenotype for the most common individual STS subtypes to date. This analysis included UPS, a highly mutated tumor type. Our analysis suggests these mutations may contribute to greater immunogenicity of this subtype, and result in the robust and more oligoclonal T-cell infiltrates observed herein. This tumor appears to fit the classic model of an inflammatory-type tumor, which is an ideal target for checkpoint inhibition, with high expression of genes related to antigen expression and T-cell-mediated apoptosis, high PD-1 and PD-L1 expression, and high TCR clonality. A caveat is that genes related to antigen presentation, including class I HLA molecules, are expressed on T cells and this could contribute to the increase in HLA-related genes observed in these tumors. Clearly, future work will need to examine MHC expression on tumor cells, and the function and specificity of the infiltrating T cells to determine whether they have the potential to recognize and lyse the tumors they inhabit.

Recent presentations have suggested disappointing activity of single-agent PD-1 inhibition in patients with LMS.[39,40] Conversely, the findings of the current study suggest that LMS is an inflammatory tumor type with high levels of T-cell-related gene expression, with several tumors demonstrating very strong expression of PD-L1 and containing PD-1-expressing cells. Nevertheless, we suspect that combination therapy may be necessary to treat these tumors because other investigators have suggested that immunosuppressive tumor-associated macrophages may be critical to immune evasion in these tumors.[29,41,42] Combinations of checkpoint inhibitors with drugs aimed at depleting or modulating these cells may prove successful in providing durable responses for these patients.

Likewise, WD/DD liposarcoma has many features of a highly inflammatory tumor such as a high T-cell fraction and relatively high HLA expression. However, unlike LMS and UPS, it had a low TCR clonality and few patients were found to have high PD-L1 expression. Given that these tumors have been observed to respond to checkpoint inhibition,[39] further study certainly is warranted. Additional study regarding the differences between WD and DD tumors also would be instructive. In the current study, we did not observe significant differences but this study was not designed or powered to examine these differences. However, because a given patient may have both WD and DD tumors, immunotherapy ideally will have activity against both histologies. It may be that the addition of a tyrosine kinase inhibitor (ClinicalTrials.gov identifier NCT023636725) or chemotherapeutic agent (ClinicalTrials.gov identifier NCT02888665) may increase the potency of checkpoint inhibition for patients with LMS and WD/DD liposarcoma.

SS and MRCL may pose a more complex challenge with regard to immunotherapy. These tumors expressed fewer inflammatory-type genes and, fittingly, had less staining for PD-L1 and had fewer PD-1 infiltrating cells. SS tumors had a significantly lower T-cell fraction, maximum clonal frequency, and clonality. Nearly all of the SS tumors in the current study were monophasic, and future studies should confirm these findings in biphasic tumors.

The data from the current study suggest that both SS and MRCL tumors evade immune recognition through mechanisms other than PD-L1, possibly via expression of lower levels of MHC, which is suggested by the gene expression data. Although these data suggest that checkpoint inhibition may not be an effective initial treatment of these tumors, both SS and MRCL often express the highly immunogenic protein NY-ESO-1.[43,44] Approaches using adoptive T-cell transfer and vaccination both currently are being investigated in these sarcomas, and analysis of tumors before and after therapy may reveal whether adaptive resistance mediated by upregulation of PD-L1 occurs.[45,46] Given that these patients have NY-ESO-1-specific T cells in their blood capable of recognizing and lysing tumor cells,[43] it may be that additional immune manipulation will be required to increase T-cell infiltration into the tumor, and tumor MHC expression. Further study of the SS and MRCL tumor microenvironment may alter currently ongoing investigations such as vaccine plus PD-L1 inhibitor trials (ClinicalTrials.gov identifier NCT02609984) or adoptive T-cell therapy (ClinicalTrials.gov identifier NCT01343043).

The impact of radiotherapy and chemotherapy on immune-related genes is an important direction for future study. Although none of the associations noted in the current analysis were impacted by prior radiation exposure, we know that radiation can have an important impact on the expression of immune-related genes. The current study was not designed to examine the impact of prior therapy on immune-related gene expression. Among all STS samples, we found a significant correlation between both the T-cell fraction and clonality with PD-1 and PD-L1 expression regardless of STS subtype. This association was true across a diverse set of tumors, including highly mutated UPS as well as the genetically simpler SS and MRCL tumors.[15,16] This observation supports the suggestion that inflamed tumors are better targets for checkpoint inhibition. Tumors with robust T-cell infiltration produce interferon γ and other cytokines, which in turn upregulate PD-L1 and other inhibitory ligands. Thus, these tumors are dependent on these checkpoints for immune evasion.

In the current study, we did not perform whole-exome sequencing to confirm the mutational load for each patient, but this clearly requires assessment. This analysis was not designed to examine the impact of radiotherapy on the sarcoma tumor micro-environment (TME) as well as the effect of prior chemotherapy/radiotherapy on the TME, but again these are areas that require further study and will be highly relevant to the SARC032 trial evaluating neoadjuvant pembrolizumab.

We believe that comprehensive immune profiling using multiple methodologies, in combination with the evaluation of clinical responses after immunotherapeutic intervention, will be important for establishing predictive algorithms. Dissection of the tumor immune microenvironment and precise understanding of the mechanisms of immune evasion for individual patients will be key to the

successful development of these promising therapies in STS.

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CONFLICT OF INTEREST DISCLOSURES

Jennifer H. Yearley has a patent pending (WO2015088930 A1) and is a full-time employee of Merck and Company. Ryan Emerson and Marissa Vignali are full-time employees of Adaptive Biotechnologies Corporation and Marissa Vignali owns stock in the company. Lee D. Cranmer has acted as a paid member of the Speakers' Bureau for Bristol-Myers Squibb for work performed outside of the current study. Venu G. Pillarisetty has received a grant from Merck and Company for work performed outside of the current study. Terrill K. McClanahan, Steven M. Townson, Erin Murphy, and Wendy M. Blumenschein are full-time employees of Merck and Company. Sharon Benzeno is an employee of and holds stock in Adaptive Biotechnologies Corporation.

AUTHOR CONTRIBUTIONS

Seth M. Pollack and **Robin L. Jones** conceptualized the project. **Seth M. Pollack, Qianchuan He, Jennifer H. Yearley, Terrill K. McClanahan**, and **Sharon Benzeno** contributed to the design and methodology of the experiments performed. **Qianchuan He, Ryan Emerson, Marissa Vignali, Yuzheng Zhang, Mary W. Redman**, and **Kelsey K. Baker** performed formal (statistical) analysis. **Jennifer H. Yearley, Ryan Emerson, Marissa Vignali, Erin Murphy**, and **Wendy M. Blumenschein** contributed to the experiments (investigation). **Jennifer H. Yearley, Ryan Emerson, Marissa Vignali, Terrill K. McClanahan, Erin Murphy, Wendy M. Blumenschein**, and **Sharon Benzeno** contributed key resources and reagents. **Sara Cooper, Bailey Donahue, Matthew B. Spraker**, and **Y. David Seo** contributed to data curation and management. **Seth M. Pollack** and **Robin L. Jones** wrote the original first draft of the article. **Elizabeth T. Loggers, Lee D. Cranmer, Matthew B. Spraker, Y. David Seo, Venu G. Pillarisetty, Robert W. Ricciotti, Benjamin L. Hoch**, and **Steven M. Townson** played key roles in writing with respect to review and editing (all authors contributed to the final article). **Seth M. Pollack, Jennifer H. Yearley, Marissa Vignali, Yuzheng Zhang, Mary W. Redman, Kelsey K. Baker, Robert W. Ricciotti, Benjamin L. Hoch, Erin Murphy**, and **Wendy M. Blumenschein** prepared the figures (visualization). **Seth M. Pollack, Stanley R. Riddell**, and **Robin L. Jones** provided oversight and leadership (supervision).

REFERENCES

1. June CH, Riddell SR, Schumacher TN. Adoptive cellular therapy: a race to the finish line. *Sci Transl Med*. 2015;7:280ps7.
2. Emens LA, Butterfield LH, Hodi FS Jr, Marincola FM, Kaufman HL. Cancer immunotherapy trials: leading a paradigm shift in drug development. *J Immunother Cancer*. 2016;4:42.
3. Dudley ME, Yang JC, Sherry R, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol*. 2008;26: 5233-5239.
4. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365:725-733.
5. Turtle CJ, Hanafi LA, Berger C, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J Clin Invest*. 2016;126:2123-2138.
6. Robbins PF, Kassim SH, Tran TL, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res*. 2015;21:1019-1027.
7. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol*. 2011;29:917-924.
8. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med*. 2014; 371:2189-2199.
9. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
10. Seiwert TY, Burtness B, Weiss J, et al. Inflamed-phenotype gene expression signatures to predict benefit from the anti-PD-1 antibody pembrolizumab in PD-L1+head and neck cancer patients [abstract]. *ASCO Meeting Abstracts*. 2015;33:6017.
11. Ribas A, Robert C, Hodi FS, et al. Association of response to programmed death receptor 1 (PD-1) blockade with pembrolizumab (MK-3475) with an interferon-inflammatory immune gene signature [abstract]. *ASCO Meeting Abstracts*. 2015;33:3001.
12. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014; 515:568-571.
13. Kawaguchi S, Tsukahara T, Ida K, et al. SYT-SSX breakpoint peptide vaccines in patients with synovial sarcoma: a study from the Japanese Musculoskeletal Oncology Group. *Cancer Sci*. 2012;103:1625-1630.
14. Bloom JE, McNeel DG, Olson BM. Vaccination using peptides spanning the SYT-SSX tumor-specific translocation. *Expert Rev Vaccines*. 2012;11:1401-1404.
15. Jungbluth AA, Antonescu CR, Busam KJ, et al. Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen

NY-ESO-1 but not MAGE-A1 or CT7. *Int J Cancer*. 2001;94:252-256.
16. Pollack SM, Jungbluth AA, Hoch BL, et al. NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. *Cancer*. 2012;118:4564-4570.
17. Somaiah N, Block MS, Kim JW, et al. Phase I, first-in-human trial of LV305 in patients with advanced or metastatic cancer expressing NY-ESO-1 [abstract]. *ASCO Meeting Abstracts*. 2015;33:3021.
18. Kanojia D, Nagata Y, Garg M, et al. Genomic landscape of liposarcoma. *Oncotarget*. 2015;6:42429-42444.
19. Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4 + T cells in a patient with epithelial cancer. *Science*. 2014;344:641-645.
20. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. Cancer genome landscapes. *Science*. 2013;339:1546-1558.
21. Tawbi HE. SARC 038: a phase II study of the anti-PD1 antibody pembrolizumab (P) in patients (Pts) with advanced sarcomas. Paper presented at: American Society of Clinical Oncology 2016 Annual Meeting; June 3-7, 2016; Chicago, IL.
22. Coindre JM, Terrier P, Bui NB, et al. Prognostic factors in adult patients with locally controlled soft tissue sarcoma. A study of 546 patients from the French Federation of Cancer Centers Sarcoma Group. *J Clin Oncol*. 1996;14:869-877.
23. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008;26:317-325.
24. Payton JE, Grieselhuber NR, Chang LW, et al. High throughput digital quantification of mRNA abundance in primary human acute myeloid leukemia samples. *J Clin Invest*. 2009;119:1714-1726.
25. Robins HS, Srivastava SK, Campregher PV, et al. Overlap and effective size of the human CD8 + T cell receptor repertoire. *Sci Transl Med*. 2010;2:47ra64.
26. Robins HS, Ericson NG, Guenthoer J, et al. Digital genomic quantification of tumor-infiltrating lymphocytes. *Sci Transl Med*. 2013;5:214ra169.
27. Robins HS, Campregher PV, Srivastava SK, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood*. 2009;114:4099-4107.
28. Lee CH, Espinosa I, Vrijaldenhoven S, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. *Clin Cancer Res*. 2008;14:1423-1430.
29. Ganjoo KN, Witten D, Patel M, et al. The prognostic value of tumor-associated macrophages in leiomyosarcoma: a single institution study. *Am J Clin Oncol*. 2011;34:82-86.
30. Seth D. Pollak: award for distinguished scientific early career contributions to psychology. *Am Psychol*. 2006;61:805-807.
31. Kim JR, Moon YJ, Kwon KS, et al. Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas. *PLoS One*. 2013;8:e82870.
32. D'Angelo SP, Shoushtari AN, Agaram NP, et al. Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment. *Hum Pathol*. 2015;46:357-365.
33. Constantinidou A, Miah A, Pollack S, Jones RL. New drugs and clinical trial design in advanced sarcoma: have we made any progress? *Future Oncol*. 2013;9:1409-1411.
34. Santoro A, Tursz T, Mouridsen H, et al. Doxorubicin versus CYVADIC versus doxorubicin plus ifosfamide in first-line treatment of advanced soft tissue sarcomas: a randomized study of the European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. *J Clin Oncol*. 1995;13:1537-1545.
35. Gelderblom H, Blay JY, Seddon BM, et al. Brostallicin versus doxorubicin as first-line chemotherapy in patients with advanced or metastatic soft tissue sarcoma: an European Organisation for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group randomised phase II and pharmacogenetic study. *Eur J Cancer*. 2014;50:388-396.
36. Judson IR, Verjeij J, Gelderblom H, et al. Result of a randomised phase III trial (EORTC 62012) of single agent doxorubicin versus plus ifosfamide as first line chemotherapy for patients with advanced, soft tissue sarcoma: a survival study by the EORTC Soft Tissue and Bone Sarcoma Group. Oral presentation at: 18th Annual Connective Tissue Oncology Society Meeting; October 30-November 2, 2013; New York, NY.
37. Rajendra R, Jones RL, Pollack SM. Targeted treatment for advanced soft tissue sarcoma: profile of pazopanib. *Onco Targets Ther*. 2013;6:217-222.
38. van der Graaf WT, Blay JY, Chawla SP, et al; EORTC Soft Tissue and Bone Sarcoma Group; PALETTE study group. Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. 2012;379:1879-1886.
39. Tawbi HA, Burgess MA, Crowley J, et al. Safety and efficacy of PD-1 blockade using pembrolizumab in patients with advanced soft tissue (STS) and bone sarcomas (BS): results of SARC028–a multicenter phase II study [abstract]. *ASCO Meeting Abstracts*. 2016;34:11006.
40. Miao D, Adeegbe D, Rodig SJ, et al. Response and oligoclonal resistance to pembrolizumab in uterine leiomyosarcoma: genomic, neoantigen, and immunohistochemical evaluation [abstract]. *ASCO Meeting Abstracts*. 2016;34:11043.
41. Espinosa I, Beck AH, Lee CH, et al. Coordinate expression of colony-stimulating factor-1 and colony-stimulating factor-1-related proteins is associated with poor prognosis in gynecological and non-gynecological leiomyosarcoma. *Am J Pathol*. 2009;174:2347-2356.
42. Edris B, Weiskopf K, Volkmer AK, et al. Antibody therapy targeting the CD47 protein is effective in a model of aggressive metastatic leiomyosarcoma. *Proc Natl Acad Sci U S A*. 2012;109:6656-6661.
43. Pollack SM, Jones RL, Farrar EA, et al. Tetramer guided, cell sorter assisted production of clinical grade autologous NY-ESO-1 specific CD8(+) T cells. *J Immunother Cancer*. 2014;2:36.
44. Pollack SM, Li Y, Blaisdell MJ, et al. NYESO-1/LAGE-1s and PRAME are targets for antigen specific T cells in chondrosarcoma following treatment with 5-Aza-2-deoxycitabine. *PLoS One*. 2012;7:e32165.
45. Somaiah N, Block MS, Kim JW, et al. Single-agent LV305 to induce anti-tumor immune and clinical responses in patients with advanced or metastatic sarcoma and other cancers expressing NY-ESO-1 [abstract]. *ASCO Meeting Abstracts*. 2016;34:3093.
46. Pollack S, Kim EY, Conrad EU, et al. Using G100 (glucopyranosyl lipid A) to transform the sarcoma tumor immune microenvironment [abstract]. *ASCO Meeting Abstracts*. 2016;34:11017.