Prognostic impact of the tumor immune microenvironment in synovial sarcoma

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The association between the immune status within the tumor microenvironment and prognosis in synovial sarcoma is not well understood. We aimed to investigate the tumor immune microenvironment and analyze its prognostic impact for patients with synovial sarcoma. A total of 36 primary patients who were treated in our institution were retrospectively evaluated. Infiltration of lymphocytes (CD4+, CD8+, and FOXP3+), CD163+ macrophages, and expression of human leukocyte antigen (HLA) class I and programmed death ligand 1 (PD-L1) were evaluated by immunohistochemistry. Moreover, we investigated PD-L1 and programmed death ligand 2 (PD-L2) mRNA expression in 19 of the 36 cases, using real-time PCR. The Kaplan-Meier method was used to estimate overall survival and progression-free survival. Infiltration of lymphocytes and macrophages varied among the patients. Furthermore, the expression of HLA class I was negative or downregulated in 11 specimens. No PD-L1 expression was observed using immunohistochemistry. Moreover, although PD-L1 mRNA expression was observed in 18 of 19 specimens, the expression level was low. A higher infiltration of CD8+ or FOXP3+ lymphocytes in patients was associated with a favorable overall survival. In addition, a higher infiltration of CD163+ macrophages indicated a significantly worse overall and progression-free survival. Infiltration of CD4+ lymphocytes, HLA class I, PD-L1, and PD-L2 expression were not associated with patient prognosis. This represents the first report investigating the tumor immune microenvironment as a prognostic factor in synovial sarcoma, indicating that CD163+ macrophages are associated with tumor progression. Our results underscore the clinical significance of the tumor immune microenvironment in synovial sarcoma.

KEYWORDS
CD163+ macrophage, prognosis, programmed death ligand 1, synovial sarcoma, tumor immune microenvironment

1 INTRODUCTION

Synovial sarcoma is a mesenchymal tumor that comprises 5%-10% of soft tissue sarcomas. The 5-year OS rate of synovial sarcoma is 64%-
76%. Standard treatment for primary localized SS consists of surgery, radiotherapy, and chemotherapy. However, local or metastatic recurrences occur in approximately 50% of patients at 5 years, and the benefit of adjuvant chemotherapy in localized primary SS cases is still controversial. In addition, the prognosis of metastatic or relapsed SS remains poor. Thus, to improve the survival rate of patients with SS, the development of novel therapies is needed.

Synovial sarcoma presents a specific chromosomal translocation t(X;18) (p11.2;q11.2), resulting in SS18-SSX1 or SS18-SSX2, and rarely into the SS18-SSX4 fusion proteins. Moreover, the NY-ESO-1 cancer testis antigen is expressed in approximately 80% of SS cases. Recently, immunotherapies consisting of SYT-SSX or NY-ESO-1-delivered peptide vaccines or autologous T cells transduced with a T-cell receptor directed against NY-ESO-1 were attempted for patients with SS. Importantly, clinical trials using an adoptive cell transfer of autologous T cells transduced with anti-NY-ESO-1 have shown promise in triggering objective responses in patients with metastatic or refractory disease. These data suggest that immunotherapies may be effective in the treatment of SS.

Programmed cell death 1 and its ligands, PD-L1 and PD-L2, play a key role in the immune escape of tumor cells by inactivating T-cell function. Several authors have shown that antibodies blocking PD-1 or PD-L1 lead to improved clinical outcomes in a variety of malignancies. However, previous reports have indicated that expression of PD-L1/ PD-L2 has different prognostic value in various cancers. Association between PD-L1 expression and the prognosis of soft tissue sarcoma, including SS, was also examined. However, because these studies comprised a small number of patients with SS, the expression of PD-L1 in SS and its clinical significance remain controversial.

Understanding how the tumor immune microenvironment relates to the prognosis of patients with SS may clarify the potential for effective immunotherapy for the treatment of SS. However, to the best of our knowledge, the tumor immune microenvironment including TILs, macrophages, and the expression of HLA class I and PD-L1 are not well understood in SS. The purpose of the present study was to investigate the tumor immune microenvironment and evaluate the prognostic impact of various immunological factors in patients with SS. In addition, we are the first to investigate the tumor immune environment of primary non-treated patients with SS and analyze its clinical significance in SS.

2 MATERIALS AND METHODS

2.1 Patient tissue samples

A total of 39 primary patients with SS, who were diagnosed and treated in Niigata University Hospital and Niigata Cancer Center Hospital between 1983 and 2017, were enrolled. To evaluate the natural tumor immune microenvironment, three patients were excluded because they had already received some treatment in other hospitals or for which specimens without neoadjuvant chemotherapy were not available. Thus, a total of 36 patients were retrospectively examined in this study. We used biopsy specimens for the immunohistochemical examination if the patient had received neoadjuvant chemotherapy. In any other case, we used surgical specimens for the examination. In all cases, H&E staining was prepared from the archives. The SS diagnosis was confirmed and representative samples, including viable tumor cells, were selected by an experienced pathologist. The patient’s clinical information and follow-up data were collected from the medical records for statistical analysis. Clinical characteristics of the patients are summarized in Table 1. There were 23 male and 13 female patients with a mean age of 36 years (ranging from 10 to 76 years). Three of these patients showed lung metastasis at diagnosis. Ten tumors were located in the upper extremities, 23 in the lower extremities, and three in the trunk. The American Joint Committee on Cancer (AJCC) 7th edition staging system was used to classify disease staging. 14 patients were stage I, 19 were stage II, and three were stage IV.

This study was approved by the Institutional Review Board of the Niigata University Hospital (No. 2016-0024) and was conducted in line with the Declaration of Helsinki. Written informed consent was obtained from all patients and/or their families prior to their participation in this study.

2.2 Immunohistochemistry

To evaluate the tumor immune microenvironment in patients with SS, immunohistochemical staining for TILs (CD4, CD8, FOXP3),

| Characteristic                  | n (%) |
|--------------------------------|-------|
| Total number of patients       | 36 (100) |
| Age at diagnosis, y            |       |
| <20                            | 6 (16.7) |
| ≥20                            | 30 (83.3) |
| Gender                         |       |
| Male                           | 23 (63.9) |
| Female                         | 13 (36.1) |
| Location                       |       |
| Upper extremities              | 10 (27.8) |
| Lower extremities              | 23 (63.9) |
| Trunk                          | 3 (8.3) |
| Tumor size, cm                 |       |
| >5                             | 22 (61.1) |
| ≤5                             | 14 (38.9) |
| Translocation                   |       |
| SYT-SSX 1                       | 24 (66.7) |
| SYT-SSX 2                       | 6 (16.7) |
| NA                             | 6 (16.7) |
| Stage (AJCC system)            |       |
| II                             | 14 (38.9) |
| III                            | 19 (52.8) |
| IV                             | 3 (8.3) |

AJCC, American Joint Committee on Cancer systems 7th edition staging manual; NA, not available.
CD163+ macrophages, HLA class I, and PD-L1 was conducted. For each specimen, the immunohistochemical analysis was carried out on 4-μm sections from representative blocks of formalin-fixed, paraffin-embedded tumor tissues. The sections were deparaffinized with xylene and rehydrated with graduated ethanol. Heated antigen retrieval was carried out in citrate pH 6.0 (for CD163, HLA class I) or in Tris-EDTA pH 9.0 (Nichirei Bioscience, Tokyo, Japan, for CD4, CD8, FOXP3), using an autoclave (at 121°C) for 5 minutes. For PD-L1, the sections were boiled for 40 minutes in Tris-EDTA pH 9.0 (Nichirei Bioscience) for antigen retrieval. Endogenous peroxidase activity in tissues was blocked by incubation in 0.3% hydrogen peroxide, and the sections were incubated with primary antibodies. The following monoclonal antibodies were used: CD4 (1F6, 1:50; Nichirei Bioscience), CD8 (C8/144B, 1:200; Nichirei Bioscience), FOXP3 (236A/E7, 1:200; Abcam, Cambridge, UK), CD163 (10D6, 1:500; Leica, Wetzlar, Germany), HLA class I (EMR8-5, 1:500; Hokudo, Sapporo, Japan), PD-L1 (28-8, 1:200; Abcam). The sections were incubated with the primary antibodies at 4°C overnight (CD4, CD8, CD163, FOXP3, and HLA class I), or at room temperature for 1 hour (PD-L1), followed by incubation with Histofine Simple Stain MAX PO MULTI (Nichirei Bioscience) at room temperature for 30 minutes. Peroxidase activity was detected with 3'-diaminobenzidine tetrahydrochloride (Simple Stain DAB; Nichirei Bioscience). Sections were counterstained with hematoxylin (Vector Laboratories Inc., Burlingame, CA, USA). Appropriate positive and negative controls were used for CD4, CD8, FOXP3, CD163, and PD-L1. Expression of HLA class I within endothelial cells served as an internal positive control. To validate our immunohistochemistry technique for PD-L1, immunohistochemical staining for PD-L1 MDA-MB231 was conducted.

2.3 Evaluation of immunohistochemistry

For TILs and macrophages, tumor sections were evaluated at low magnification, and the areas with the most abundant TILs or macrophages were selected. A maximum of five high-power fields (×200) was then digitally photographed in a size of 0.093 mm² with an Olympus DP73 digital camera (Olympus, Tokyo, Japan) and counted manually. The count was conducted two times by an experienced pathologist who was blinded to previously available results and clinical information. Average value in an area of 1 mm² was used for the statistical analysis. For each immunohistochemical marker, we divided the patients into subgroups. The patients were classified into “high” and “low” infiltration groups except for HLA class I, where the “high” group displayed a number of TILs or macrophages that was above the median. For HLA class I, we classified the 36 cases into high (number of positive cells >50%), low (number of positive cells <50%), and negative (the samples were negative for these cells). For PD-L1, the patients were assigned to the “high” group when the number of positive cells >1%, as previously reported.

2.4 Cell culture

HS-SYII and SYO-1 SS cells lines were obtained from Dr H. Sonobe (Department of Pathology, Kochi Medical School, Kochi, Japan) and Dr A. Kawai (Department of Orthopedic Surgery, Okayama University, Okayama, Japan), respectively. MDA-MB-231 breast cancer cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). All cell lines were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS and 1% Antibiotic-Antimycotic (Invitrogen Life Technologies). Cell lines were then incubated in a humidified atmosphere containing 5% CO2 at 37°C.

2.5 Quantitative real-time PCR

Quantitative real-time PCR for PD-L1 and PD-L2 was carried out in two SS cell lines and freshly frozen specimens from 19 non-treated patients with SS. Total RNA was isolated from cell lines or frozen specimens using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA was converted to cyclic DNA using the PrimeScript RT reagent kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using the SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara, Shiga, Japan), and the results were analyzed using the Thermal Cycler Dice Real Time System TP800 (Takara, Shiga, Japan). The GAPDH housekeeping gene was used as a reference gene. The primer sequences used were as follows: primer pairs used for human PD-L1 5’-CAATGTGACCGACACTGAGA-3’, (forward) and 5’-GGCATATAAGATGGCTCCCAGA-3’ (reverse); PD-L2 5’-A TCAACCTTGCTTGCAC-3’ (forward) and 5’-CTCCCAAGACCACAGGTTCA-3’ (reverse); and GAPDH, 5’-GCACCGTCAAGGCTGA GAAC-3’ (forward) and 5’-TGGTGAAGCCACGTGGA-3’ (reverse). mRNA expression levels in each sample were normalized by the GAPDH levels. Gene copy number for PD-L1 was calculated using a standard curve that was constructed using the MDA-MB-231 cell line. MDA-MB-231 was previously reported as a positive control and was used as a calibrator. Nineteen patients were divided into two subgroups for the statistical analysis. The patients were considered as belonging to the “high” group when the PD-L1 or PD-L2 mRNA level was above the median, and the levels for the rest of the patients were low.

2.6 Western blot analysis

HS-SYII and SYO-1 were subjected to western blot analysis to assess endogenous expression of the PD-L1 protein as previously reported. Briefly, the cells were suspended in a sample buffer containing 62.5 mmol/L Tris, pH 6.8; 2% SDS; 5% glycerol; and 6 mol/L urea. Then, dithiothreitol (1 mol/L; Sigma-Aldrich, St Louis, MO, USA) and bromophenol blue were added to the lysates, and the lysates were boiled for 5 minutes and stored at −80°C. Equal amounts of proteins (50 μg/lane) were run on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA, USA) and were transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were incubated overnight at 4°C with primary antibody against PD-L1(28-8, 1:2000; Abcam) and actin (AC-74, 1:3000; Sigma-Aldrich), and with HRP-labeled anti-rabbit IgG (GE Healthcare).
at room temperature. Bands were visualized by ECL detection reagents (GE Healthcare).

2.7 | Statistical analysis

Statistical analyses were conducted using IBM SPSS version 21.0 (IBM, New York, NY, USA) or GraphPad Prism v6.0 software (La Jolla, CA, USA). Spearman’s rank correlation coefficient test was used to analyze the relationship between groups. OS and PFS were measured from the date of the initial biopsy. A terminal point for OS was determined as the time of death or the time the patient was last seen. A terminal point for PFS was defined as the time of local recurrence, appearance of distant metastasis, disease progression, or the last follow up. Kaplan-Meier method was used to estimate OS and PFS probabilities and survival differences were analyzed using the log-rank test. Multivariate analysis was conducted using the COX proportional hazard model. P-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical course of the patients

Mean follow-up times were 112.7 months (6-366 months). OS probabilities of the 36 patients with SS at 5 and 10 years were 82.7% and 75.2%, respectively. The 5 and 10-year PFS probabilities were both 67.5%. Local recurrence was observed in one case and 12 patients developed distant metastasis. Sites of distant metastasis were the lung in 10 patients, and the lymph nodes, kidney, and vertebra in one patient.

3.2 | Infiltration of lymphocytes and macrophages

Representative images displaying high or low levels of infiltration for CD8+, FOXP3+ lymphocytes, or CD163+ macrophages are shown in Figure 1. Figure 2 shows the number of CD4+, CD8+, and FOXP3+ TILs, and CD163+ macrophages that infiltrated into the tumor per mm². Although CD163+ macrophages were seen in all patients, 10 patients were negative for CD4, one patient was negative for CD8, and nine patients were negative for FOXP3. Median number of infiltrating TILs and CD163+ macrophages was as follows: CD4+ lymphocytes 45.2 (0-697.3), CD8+ lymphocytes 188.3 (0-1358.0), FOXP3+ lymphocytes 18.3 (0-639.2), and CD163+ macrophages 444.4 (139.9-1628.1). Next, the correlation between infiltration of each immune cell was examined, and there was a correlation between the density of CD8+ and FOXP3+ lymphocytes and that of CD163+ macrophages. In addition, there was a strong correlation between CD8+ and FOXP3+ lymphocytes (Table 2).

3.3 | Expression of HLA class I and PD-L1

Immunohistochemical staining for HLA class I was conducted and representative images for the high or low expression of HLA class I are shown in Figure 1. Twenty-five (69.4%) of the 36 specimens were graded as high expression, five (13.9%) were classified as low expression, and the remaining six cases (16.7%) were considered negative for HLA class I. Patients with high expression of HLA class I showed a trend toward a higher number of infiltrated CD8+ lymphocytes compared with low or negative expression of HLA class I, but this was not statistically significant (data not shown). Although no case was positive for PD-L1 following the immunohistochemical analysis, intense staining for PD-L1 in MDA-MB231 cells was observed (Figure 3A).

3.4 | Programmed death ligand 1/PD-L2 mRNA and protein expression

Programmed death ligand 1 and PD-L2 mRNA expression were quantified in patients with SS and two SS cell lines by real-time PCR. Both synovial sarcoma cell lines and all 19 specimens expressed PD-L1 mRNA to a certain extent. However, no cases or cell lines showed a higher expression of PD-L1 mRNA than MDA-MB-231 (Figure 3B). Western blot analysis showed no protein expression of PD-L1 in both synovial sarcoma cell lines (Figure 3C). Therefore, PD-
3.5 Prognostic significance of the immune microenvironment

Patient survival curves are shown in Figure 5. Patients with higher levels of CD8+ or FOXP3+ lymphocyte infiltration or lower levels of CD163+ macrophage infiltration were associated with a favorable OS. High expression of HLA class I showed a tendency toward longer OS compared with negative expression of HLA class I, but did not achieve statistical significance ($P = 0.052$). Next, to examine whether the tumor immune microenvironment was associated with the progression of SS, we evaluated PFS. Although no significant association was found for CD8+ lymphocytes or FOXP3+ lymphocytes, lower CD163+ macrophage infiltration levels in patients were also significantly associated with a longer PFS. These results suggest that CD163+ macrophages are associated with tumor progression in patients with SS. No prognostic impact on OS and PFS was found for CD4+ lymphocyte infiltration, or HLA class I PD-L1, and PD-L2 expression. Univariate analysis of clinicopathological factors showed that advanced AJCC stage (III vs IV) was significantly associated with unfavorable prognosis (Table 3). Multivariate analysis was also carried out to evaluate the tumor immune microenvironment and clinicopathological factors. We excluded tumor size because AJCC staging system includes tumor size. We also excluded infiltration of FOXP3+ lymphocytes, because there was a strong correlation between the number of CD4+ and FOXP3+ lymphocytes. Multivariate analysis indicated that AJCC stage, number of CD8+ lymphocytes, and number of CD163+ macrophages were significantly associated with OS, respectively (Table 4).

4 DISCUSSION

Some studies have examined the tumor microenvironment, including infiltration of CD8+ lymphocytes and expression of PD-L1 in soft tissue sarcomas. However, those studies often lump together heterogeneous types of soft tissue sarcomas and include limited numbers of patients with SS. To the best of our knowledge, the present study is the first report to focus on the tumor immune environment in patients with SS. We investigated the tumor immune microenvironment including infiltration of CD4+, CD8+, and FOXP3+ TILs, that of CD163+ macrophages, expression of HLA class I and PD-L1, and their association with the prognosis of SS.

Since CD8+ lymphocytes can kill tumor cells presenting tumor-associated antigen, CD8+ lymphocytes are essential for adaptive tumor immunity. As a result, a high infiltration of CD8+ TILs has a positive impact on the clinical course of many types of cancer. However, studies investigating the associations between TILs and the prognosis of soft tissue sarcomas, including SS, are limited. Fujii et al. reported that a higher infiltration of CD8+ lymphocytes was associated with a favorable prognosis and correlated with distant metastasis-free survival in patients with angiosarcoma. In contrast, van Erp et al. suggested that a high infiltration of CD8+ lymphocytes in SS tumors was associated with a worse metastasis-free

**TABLE 2** Correlation of each immune parameter

|            | CD4 density | CD8 density | FOXP3 density | CD163 density |
|------------|-------------|-------------|---------------|---------------|
| CD4 density | $r = 0.343$ | $r = 0.457$ | $r = 0.110$   |               |
|            | $P = 0.040$ | $P = 0.005$ | $P = 0.525$   |               |
| CD8 density | $r = 0.883$ | $r = 0.393$ |               | $P = 0.018$   |
|            | $P < 0.001$ |             |               |               |
| FOXP3 density | $r = 0.401$ |             |               | $P = 0.015$   |
|            |             |             |               |               |
| CD163 density |             |             |               |               |

r, correlation coefficient. Bold values are statistically significant ($P < 0.05$).

L1 mRNA expression of both cell lines was considered negative. Regarding PD-L2, five of 19 specimens showed a higher expression of PD-L2 mRNA than MDA-MB231 (Figure 3D). The expression level of both PD-L1 and PD-L2 was not correlated with the density of each immune cell and correlation between number of infiltrated CD163+ macrophages and expression level of PD-L1 and PD-L2 is shown in Figure 4.
survival in patients with SS. However, the OS of patients with SS was not clarified in this study, and the prognostic value of CD8 for patients with SS remains unclear. In addition, Nowicki et al reported that the density of intratumor CD8$^+$ lymphocytes was not associated with OS or PFS in patients with SS. These results conflict with the data in our study. Our results indicate that although the infiltration of CD8$^+$ lymphocytes is not associated with PFS, a higher infiltration of CD8$^+$ lymphocytes is significantly associated with a favorable OS. Taken together, these findings suggest that although patients with a higher infiltration of CD8$^+$ lymphocytes may develop distant metastases, the prognosis of these patients may not be poor.

Downregulation of HLA class I was associated with poor CD8$^+$ infiltration and unfavorable prognosis. However, the present study has shown that HLA class I expression is not associated with prognosis. This discrepancy might arise from the small number of samples in this study because negative expression of HLA class I showed a trend toward lower CD8$^+$ infiltration and unfavorable prognosis.

Regulatory T cells represent a subset of CD4$^+$ lymphocytes characterized by coexpression of CD4 and CD25. FOXP3, a nuclear transcription factor, functions as a master regulator for the development of Treg and is considered the most reliable Treg marker. FOXP3$^+$ Treg promote the evasion of cancer cells from immune responses. Although CD4$^+$ lymphocytes did not show any association with the prognosis of patients with SS, in our study, a higher infiltration of FOXP3$^+$ lymphocytes was significantly associated with a better OS. However, Que et al evaluated the infiltration of FOXP3$^+$ lymphocytes in 163 cases of soft tissue sarcoma, including 21 cases of SS, and showed that a higher infiltration of FOXP3$^+$ lymphocytes was associated with a poor prognosis. This result is markedly different from ours. One possible explanation may be that FOXP3$^+$ T cells comprise functionally different subsets, including non-Treg. FOXP3 is not necessarily a specific marker of regulatory T cells. Considering that Que's study did not distinguish SS from other soft tissue sarcomas, FOXP3$^+$ lymphocytes that infiltrate into the tumor microenvironment in SS might represent a subset other than Treg. However, our data should be interpreted with care. As nine patients were negative for FOXP3, and the median value of infiltrated FOXP3$^+$ lymphocytes was very low, a favorable prognosis might arise as a result of our choice of cutoff value. Although present study used a median value as the cutoff, Que et al defined “high infiltration” as infiltration occurring in greater than 5%
expression was predominantly observed in metastatic specimens. In the present study, no expression of PD-L1 was observed in any patient using immunohistochemistry. Moreover, PD-L1 mRNA expression levels were extremely low in both the SS cell lines and in the clinical specimens. The data therefore suggest that SS escapes from the immune system by a mechanism other than the PD-1/PD-L1 axis. The role of PD-L2 in the tumor microenvironment is less understood compared to that of PD-L1 in sarcomas. Zheng et al first examined the expression of PD-L2 in 234 patients with musculoskeletal tumors including 127 cases of patients with SS, and 26 cases (20.5%) of SS were positive for PD-L2 by immunohistochemistry. However, the prognostic impact of PD-L1 and PD-L2 in SS remains unclear and further experiments are required to show the mechanism underlying the association among PD-L1, PD-L2, and prognosis.

Macrophages represent a major component of tumor immune cells, and may be classified into tumoricidal M1-like macrophages or pro-tumoral M2-like macrophages. The role of PD-L1 and PD-L2 in the tumor microenvironment is less understood compared to that of PD-L1 in sarcomas. Zheng et al first examined the expression of PD-L2 in 234 patients with musculoskeletal tumors including 127 cases of patients with SS, and 26 cases (20.5%) of SS were positive for PD-L2 by immunohistochemistry. However, the prognostic impact of PD-L1 and PD-L2 in SS remains unclear and further experiments are required to show the mechanism underlying the association among PD-L1, PD-L2, and prognosis.

Although some authors reported that PD-L1 expression was associated with the clinical prognosis of soft tissue sarcomas, including SS cases, expression of PD-L1 and the prognostic significance of PD-L1 in SS is still controversial. Kim C et al evaluated PD-L1 expression in 82 soft tissue sarcomas, including 19 SS, and Kim JR et al investigated 105 cases of soft tissue sarcoma, including 16 SS, by immunohistochemistry. They reported that 10 out of 19 (53%) and 12 out of 16 (75%) patients with SS were positive for PD-L1. In addition, Nowicki et al examined 29 SS specimens and showed that PD-L1 was expressed in SS. Conversely, D’Angelo et al investigated the expression of PD-L1 in 50 cases of soft tissue sarcoma, including five cases of patients with SS, whereas van Erp et al examined PD-L1 expression in a large cohort of primary sarcoma (n = 202), including 22 cases of patients with SS. Neither study identified any case that was positive for PD-L1 by immunohistochemistry. This discrepancy may possibly be as a result of the use of different antibodies or the definition of the cutoff value. Moreover, in the Nowicki et al study, greater than half of the specimens were metastatic specimens, and PD-L1 expression was predominantly observed in metastatic specimens. In the present study, no expression of PD-L1 was observed in any patient using immunohistochemistry. Moreover, PD-L1 mRNA expression levels were extremely low in both the SS cell lines and in the clinical specimens. The data therefore suggest that SS escapes from the immune system by a mechanism other than the PD-1/PD-L1 axis. The role of PD-L2 in the tumor microenvironment is less understood compared to that of PD-L1 in sarcomas. Zheng et al first examined the expression of PD-L2 in 234 patients with musculoskeletal tumors including 127 cases of patients with SS, and 26 cases (20.5%) of SS were positive for PD-L2 by immunohistochemistry. However, the prognostic impact of PD-L1 and PD-L2 in SS remains unclear and further experiments are required to show the mechanism underlying the association among PD-L1, PD-L2, and prognosis.

FIGURE 4 Correlation between CD163+ macrophages and programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) mRNA expression. There was no correlation between the number of infiltrated CD163+ macrophages and expression level of (A) PD-L1 and (B) PD-L2. NS, not significant.
Another mechanism for resistance to PD-1 targeted therapy was reported, showing that macrophages arrested CD8+ lymphocytes by shaping long-lasting interaction and inhibiting CD8+ lymphocyte migration into tumor nests and contact with tumor cells. Depletion of macrophages allowed CD8+ lymphocytes to migrate into tumor nests and scan tumor cells to improve the efficacy of PD-1 targeting immunotherapy. In this study, although the density of CD8+ lymphocytes was correlated with that of CD163+ macrophages, each
factor had an opposite impact on prognosis. This may have resulted from the fact that it is difficult to definitively distinguish tumor nests from stroma in SS, unlike other types of cancer. Further studies are needed to verify the interaction between CD8+ lymphocytes and CD163+ macrophages in patients with SS. Taken together, it is suggested that macrophage-targeting therapy or combining approaches targeting macrophages and PD-1 may represent a promising immunotherapy in patients with SS.

The present study has several limitations. First, as this was a retrospective study, the patient’s baseline characteristics varied. Thus, this study comprises patients with distant metastasis at diagnosis, and this might have influenced our results. To confirm these results, further studies are needed to evaluate a greater number of tumor specimens with a similar presentation status. Moreover, the evaluation of metastatic specimens was beyond the scope of our study, and we could not address the tumor immune microenvironment in metastatic tumors.
Finally, we evaluated the expression of PD-L1 using one antibody only. Therefore, our results could be influenced by the antibody used. To remedy this problem, a standardized method for testing the expression of PD-L1 using immunohistochemistry is required.

In conclusion, we showed that PD-L1 was not expressed in any of the patients, as assessed by immunohistochemistry, and that the PD-L1 mRNA expression level was low in the tumor microenvironment of patients with SS. In addition, our results showed that a lower infiltration of CD8+ and FOXP3+ lymphocytes and a higher infiltration of CD163+ macrophages were associated with an unfavorable prognosis in patients with SS. We also showed that the infiltration of CD163+ macrophages might play an important role in disease progression in patients with SS. We believe these data offer novel insights into the SS tumor immune microenvironment and will provide a foundation for developing immunotherapies for patients with SS.

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**CONFLICTS OF INTEREST**

Authors declare no conflicts of interest for this article.

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