Immunological status of peripheral blood is associated with prognosis in patients with bone and soft-tissue sarcoma

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Abstract. Immune-checkpoint inhibitors have shown promising antitumor effects against certain types of cancer. However, specific immune-checkpoint inhibitors for patients with sarcoma have yet to be identified, whereas the immunological status of peripheral blood in patients with bone sarcoma and soft-tissue sarcoma (STS) remains unknown. In addition, it is unclear whether the immunological status from the peripheral blood could be used as a prognostic indicator. Therefore, the present study aimed to clarify the immunological status of peripheral blood samples derived from patients with bone sarcoma and STS. Immune monitoring was performed using the peripheral blood samples of 61 patients with no metastasis of high-grade sarcoma. A total of 25 patients with metastatic sarcoma were used for comparison. A total of 41 immune cell subsets were analyzed using multicolor-flow cytometry. The patients that did not have metastasis demonstrated higher quantities of monocytic myeloid-derived suppressor cells (M-MDSCs) and T cell immunoglobulin and mucin domain-3 (Tim-3)$^+$ CD8$^+$ T cells, which were significantly associated with poor disease-free survival (DFS) time, while higher quantities of NKG2D$^+$ CD8$^+$ T cells were significantly associated with improved DFS time. Multivariate Cox regression analysis demonstrated that the number of Tim-3$^+$ CD8$^+$ T cells was associated with lower DFS time. A significant association was also found between the number of M-MDSCs and progression-free survival (PFS) time in patients with metastasis. The results suggested the occurrence of immune surveillance, which indicated that the host immune reaction against cancer existed in patients with bone sarcoma and STS. Notably, a high number of M-MDSCs was associated with both DFS and PFS time, suggesting a strong prognostic value. The data suggested that the immune status of peripheral blood was associated with the prognosis in patients with sarcoma, as previously reported in patients with other cancer types. In summary, the results may assist with the development of novel strategies for sarcoma treatment, based on the use of biomarkers or immunotherapy.

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Introduction

Bone sarcomas and soft-tissue sarcomas (STSs) are very rare and biologically heterogeneous malignancies. There are 0.8 malignant bone tumors and ~2 malignant soft tissue sarcomas per 100,000. These are very few compared with that in other types of cancer in 2013. In addition, malignant bone tumors and soft tissue sarcomas are classified into ~20 and 40 types, respectively (1). The treatment option for patients with advanced STS is single systemic chemotherapy (2). In contrast to bone sarcomas and STSs, osteosarcoma (OS) and the Ewing sarcoma family of tumors (ESFT) are the most frequent primary malignant bone tumors found in adolescents and young adults, worldwide (3,4). The introduction of preoperative chemotherapy has significantly improved the overall survival time of these patients (5,6). Nevertheless, the prognosis of patients with OS and ESFT, who exhibit a poor response to chemotherapy remains unfavorable, due to their high risk of developing distant metastases. The mainstay of treatment for other bone sarcoma tumors, such as chondrosarcoma and

chordoma is surgical resection. The use of chemotherapy for these bone tumors is considered ineffective (7,8).

Immune surveillance against tumors has attracted considerable attention, due to the development of immune-checkpoint inhibitors, that have shown antitumor effects against certain types of cancer, such as breast, colorectal, gastric, lung, pancreatic and renal cancers (9,10). The balance between activation and inhibition of immune responses may determine whether cancers can avoid detection based on immune recognition. The simultaneous inhibition of more than one immune target may regulate the expression level of various molecules, including programmed death-1 (PD-1), cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), lymphocyte-activation gene-3 (LAG-3), T cell immunoglobulin and mucin domain-3 (Tim-3) and natural killer group 2 member A (NKG2A), which have also been recognized as immune-checkpoint molecules that are present on the surface of $CD4^+$ and $CD8^+$ T cells (11-14). Furthermore, co-stimulated activated molecules, such as CD28, CD134 (OX-40), CD137 (4-1BB), inducible co-stimulatory molecule (ICOS) and natural killer group 2 member D (NKG2D), are also known to be present on the surface of $CD4^+$ and $CD8^+$ T cells (15-19). Since the approval of immune checkpoint inhibitor therapies, such as the application of PD-1 and CTLA-4 antibodies, several clinical studies have been conducted worldwide on the effects of these treatments on various types of cancer, including colorectal and thyroid cancers and lymphoid malignancies (20-22). In addition, immune-suppressive cells, such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) do not trigger the activation and/or proliferation of effector T cells and thereby escape the immune response (23,24). Previous studies have shown higher quantities of MDSCs to be associated with poor outcomes in patients with certain solid tumors, such as colon cancer, melanoma, hepatocellular carcinoma and breast cancer (25-28). These studies were conducted using patient peripheral blood samples. Serial collection of tumor samples from patients with metastatic sarcoma is usually difficult. Therefore, the use of peripheral blood samples, which can be collected with minimally invasive methods, will be extremely valuable for the identification of potential biomarker candidates.

The immunological status (the number of immune cell subsets, such as T and B cells, NK cells and immuno-suppressive cells, as well as those corresponding to cells secreting immune checkpoint molecules) for patients with bone sarcoma and STS remain uncertain. In the present study, the immunological status of patients with bone sarcoma and STS was assessed in peripheral blood samples. The results provide more information on the host immune reaction against sarcoma based on analysis of T-cell expression in response to co-stimulation with activated molecules.

Materials and methods

Patients. Following institutional review board approval (approval no. 2014-287), the patients who were treated at the National Cancer Center Hospital (Tokyo, Japan) between April 2015 and March 2017 were prospectively enrolled. In total, 86 patients were enrolled, of which 61 patients had no metastasis of high-grade sarcoma and 25 patients presented with metastatic sarcoma. No metastasis was defined as stages I-III and metastatic sarcoma as stage IV. The characteristics of the patients are summarized in Table I. The patients provided written informed consent. The patients who met the following exclusion criteria were not enrolled in the study: i) Subjects under 15 years of age, ii) the presence of active viral infections, such as human immunodeficiency virus or hepatitis B and/or hepatitis C, and iii) ongoing treatment with steroids.

Clinicopathological factors and staining of peripheral blood mononuclear cells (PBMCs). The peripheral blood samples were collected prior to treatment, including surgery and chemotherapy. Levels of alkaline phosphatase (ALP), C-reactive protein (CRP) and lactate dehydrogenase (LDH), white blood count and differential count of leukocytes, including lymphocytes, neutrophils, monocytes and eosinocytes were analyzed. The blood samples were centrifuged at 425 x g for 10 min at room temperature, using density gradient centrifugation, and the separated plasma samples were cryopreserved at -80˚C in cryogenic tubes (Thermo Fisher Scientific, Inc.) using CELLBANKER (Nippon Zenyaku Kogyo). Fresh PBMC samples were stained for the myeloid cell subset, since the MDSC fraction was decreased by cryopreservation (29). Each sample was also stained for the subset of dendritic cells (DC) concomitantly with MDSC measurement, since a common flow cytometry panel was used for both of these cell subsets. The remaining PBMCs were cryopreserved and used for measurements of T, B and NK cell subsets.

PBMCs ($5x10^5$) were suspended in 100 ml staining buffer (PBS containing 2% FBS (Sigma-Aldrich; Merck KGaA). The antibodies for the surface markers were subsequently added followed by a 30-min incubation period at 4˚C. The staining of the intracellular proteins (LAG-3 and FOXP3) was performed using the Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent with a 30-min incubation at 4˚C (cat. no. 12-4777-42; eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The antibodies used were as follows: Lineage (Lin; CD3, CD16, CD19, CD20 and CD56) cocktail FITC (cat. no. 643397; BD Pharmingen; BD Biosciences), LAG-3 FITC (cat. no. ALX-804-806F-C100; Enzo Life Sciences, Inc.), OX-40 FITC (cat. no. 55837; BD Pharmingen; BD Biosciences), CD14 peridinin chlorophyll protein (PerCP)-Cy5.5 (cat. no. 561116; BD Pharmingen; BD Biosciences,), CD28 PerCP-cy5.5 (cat. no. 337181; BD Pharmingen; BD Biosciences), CD11b allophycocyanin (APC)-Cy7 (cat. no. 557754; BD Pharmingen; BD Biosciences), CD8 APC-Cy7 (cat. no. 557834; BD Pharmingen; BD Biosciences), CD33-phycoerythrin (PE)-Cy7 (cat. no. 333946; BD Pharmingen; BD Biosciences), ICOS PE-Cy7 (cat. no. 25-9948-42; eBioscience; Thermo Fisher Scientific, Inc.), NKG2D PE-Cy7 (cat. no. 320812; BioLegend, Inc.), CD11c Alexa Fluor700 (cat. no. 561352; BD Pharmingen; BD Biosciences), CD45RA Alexa Fluor700 (cat. no. 304120; BioLegend, Inc.), CD123 Brilliant Violet 421 (cat. no. 562517; BD Pharmingen; BD Biosciences), CD62-L (cat. no. 304828; BioLegend, Inc.), CD15 V500 (cat. no. 561585; BD Pharmingen; BD Biosciences), CD66b APC (cat. no. 561645; BD Pharmingen; BD Biosciences), PD-1 APC (cat. no. 558694; BD Pharmingen; BD Biosciences), NKG2A APC (cat. no. PN A60797; Beckman Coulter, Inc.),

Table I. Clinicopathological characteristics of the patients with bone and soft-tissue sarcoma, and with and without metastasis

Clinicopathological characteristic	Free of metastasis and high-grade sarcoma (n=61)		Metastatic sarcoma (n=25)	
	Value	Percentage	Value	Percentage
Median age (range), years	56 (19-83)		60.1 (24-77)	
Sex				
Male	39	65.0	14	56.0
Female	22	35.0	11	44.0
PS				
0	44	72.1	9	36.0
1	17	27.9	8	32.0
2			5	20.0
3			2	8.0
4			1	4.0
Location				
Bone	15		5	
Femur	7	46.6	4	16.0
Rib	3	20.0		
Tibia	2	13.3		
Scapula	1	6.7		
Radius	1	6.7		
Sacrum	1	6.7		
Toe			1	4.0
Soft tissue	46		20	
Femur	16	34.7	3	12.0
Retroperitoneum	8	17.3	4	16.0
Lower leg	6	13.1	3	12.0
Axilla	3	6.5	6	24.0
Neck	1	2.2	1	4.0
Back	2	4.4	1	4.0
Forearm	2	4.4		
Shoulder	2	4.4		
Foot	1	2.2		
Humerus	1	2.2		
Inguinal	1	2.2		
Knee	1	2.2		
Buttocks	2	4.4		
Chest wall			1	4.0
Pelvis			1	4.0
Sarcoma				
Liposarcoma	15	24.6	6	24.0
Myxoid LS	7		2	
Dedifferentiated LS	6		1	
Pleomorphic LS	2		3	
UPS	9	14.8	4	16.0
OS	9	14.8	4	16.0
Myxofibrosarcoma	8	13.1	1	4.0
MPNST	6	9.8	3	12.0
Chondrosarcoma	5	8.3	1	4.0
Synovial sarcoma	3	4.9	2	8.0
Epithelioidsarcoma	2	3.3		
Angiosarcoma	1	1.6		

Table I. Continued.

Clinicopathological characteristic	Free of metastasis and high-grade sarcoma (n=61)		Metastatic sarcoma (n=25)	
	Value	Percentage	Value	Percentage
Fibrosarcoma	1	1.6		
Leiomyosarcoma	1	1.6	2	8.0
Myxofibrosarcoma			1	4.0
Malignant rhabdoid tumor			1	4.0
Rhabdomyosarcoma			1	4.0
Malignant perineurolima	1	1.6		
TNM stage				
IIA	5	8.2		
IIB	9	14.8		
III	47	77.0		
IV			25	100

PS, performance stage; LS; liposarcoma; UPS, undifferentiated pleomorphic sarcoma; OS, osteosarcoma; MPNST, malignant peripheral nerve sheath tumor.

HLA-DR ECD (cat. no. PN IM3636; Beckman Coulter, Inc.), CD56 PE-CF 594 (cat. no. 562289; BD Pharmingen; BD Biosciences), FOXP3 PE (cat. no. 12-4777-42; eBioscience, Thermo Fisher Scientific, Inc.), CD16 BUV395 (cat. no. 563785; BD Pharmingen; BD Biosciences), CD3 BUV496 (cat. no. 564809; BD Pharmingen; BD Biosciences), CD4 Brilliant Violet 650 (cat. no. 317436; BioLegend, Inc.), CCR7 BV711 (cat. no. 353228; BioLegend, Inc.), 4-1BB BV711 (cat. no. 740798; BD Pharmingen; BD Biosciences), CTLA-4 BV786 (cat. no. 563931; BD Pharmingen; BD Biosciences), Tim-3 BV786 (cat. no. 345032; BioLegend, Inc.) and CD19 PE-Cy5.5 (cat. no. 35-0198-42; eBioscience, Thermo Fisher Scientific, Inc.). Isotype controls included the appropriate fluorochrome-conjugate as follows: Brilliant violet 421 mouse IgG1, κ isotype control (cat. no. 400158; BioLegend, Inc.), brilliant violet 711 mouse IgG2a, κ isotype control (cat. no. 400272; BioLegend, Inc.), BV786 mouse IgG2b-κ isotype control (cat. no. 563732; BD Pharmingen; BD Biosciences), FITC mouse IgG1, κ isotype control (cat. no. 400108; BioLegend, Inc.), PerCP-Cy5.5 mouse IgG1 κ isotype control (cat. no. 45-4714-82; eBioscience), PE mouse IgG1 κ isotype control (cat. no. 12-4714-82; eBioscience), PE-Cy7 mouse IgG1 κ isotype control (cat. no. 25-4714-42; eBioscience), APC mouse IgG1 κ isotype control (cat. no. 555751; eBioscience), brilliant violet 711 mouse IgG1, κ isotype control (cat. no. 400168; BioLegend, Inc.) and brilliant violet 785™ mouse IgG1, κ isotype control (cat. no. 400170; BioLegend, Inc.).

The stained cells were detected using an LSR Fortessa X-20 with the FACSDiva software (BD Biosciences). The analyses were performed using a FlowJo microplate reader (Tomy Digital Biology Co., Ltd.).

Definition and analysis of the immune cell subsets. A total of 41 immune cell subsets were analyzed in the present study. They were defined as follows: Monocytic-MDSCs (M-MDSCs): Lin-$CD14^+CD33^+CD11b^+HLA\text{-}DR^{low/-}$; granulocytic MDSCs (Gr-MDSCs): $CD33^{dim}CD15^+CD66^+CD11b^+$; Myeloid DCs (M-DCs): Lin-$CD14^-CD11c^+$ $HLADR^{high}$; plasmacytoid DCs (p-DCs): Lin-$CD14^-CD123^+HLA\text{-}DR^{high}$; naive T-regs: $CD3^+CD4^+CD45RA^-FOXP3^{high}$; and effector T-regs: $CD3^+CD4^+CD45RA^+FOXP3^{high}$. T cells were classified as naïve T cells ($CD45RA^+CCR7^+$), effector T cells ($CD45RA^+CCR7^-$), effector memory T cells ($CD45RA^-CCR7^-$) and central memory T cells ($CD45RA^-CCR7^+$), in populations of $CD4^+$ or $CD8^+$ cells (Fig. 1). NK cells were classified as $CD56^{bright}$ NK cells ($CD3^-CD19^-CD14^-CD16^+CD56^{bright}$) and $CD56^{dim}$ NK cells ($CD3^-CD19^-CD14^-CD16^+CD56^{dim}$). The quantities of CD28, 4-1BB, ICOS, OX-40, CTLA-4, PD-1, Tim-3, LAG-3, NKG2D, NKG2A and CD62-L were also assessed in $CD4^+$ and $CD8^+$ T cells and NK cells (Figs. 2 and 3). Isotype controls were used to determine the cut-off levels for distinguishing between positivity and negativity. The quantities of the lymphoid subsets were obtained by dividing the cell number of each subset by the cell number of the lymphocyte fraction, based on the results obtained from the flow cytometry analysis. The quantities of Gr-MDSCs were calculated by dividing the cell number of $CD33^{dim}CD15^+CD66^+CD11b^+$ cells by the number of PBMCs. The patients were divided, based on the median values, according to the proportion of each immune cell subset into elevated and non-elevated groups.

Statistical analysis. In the present study, DFS was defined follows: From the day of study registration and blood collection after the primary treatment for tumor ended until the day of confirmation of a new lesion or symptom of the tumor. PFS was defined follows: From the day of study registration and blood collection after the treatment of a disease until the day of confirmation of disease progression. Imaging studies, such as CT scans, were performed approximately every three months to check for disease progression and the appearance of new lesions, and was assessed according to the Response Evaluation Criteria in Solid Tumors guidelines (v1.1) (30). The

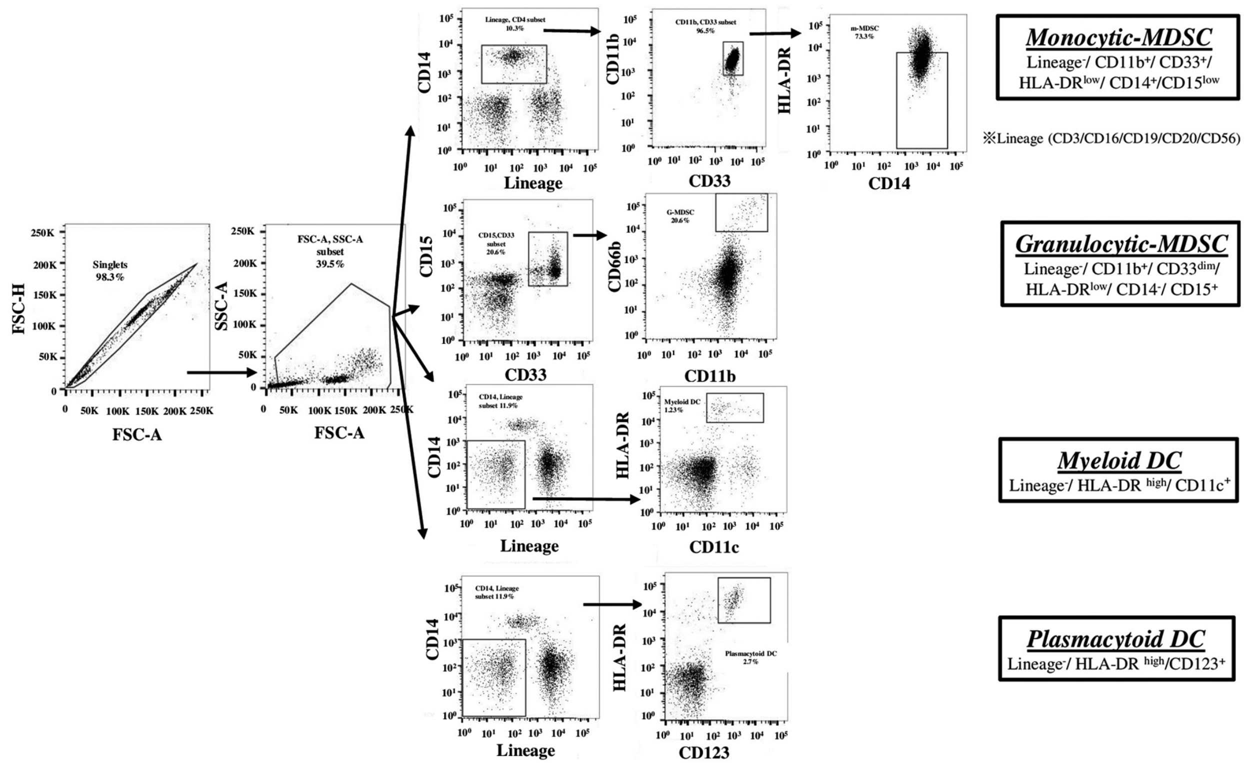

Figure 1. Gating strategy for the immune suppressor cells and antigen-presenting cells. Gating strategy and representative dot plots for M-MDSCs, Gr-MDSCs, M-DCs and p-DCs. The cells were defined as follows: M-MDSCs) Lin-$CD14^+CD33^+CD11b^+HLA\text{-}DR^{low/-}$; Gr-MDSCs: $CD33^{dim}CD15^+CD66^+CD11b^+$; M-DC: Lin-$CD14^-CD11c^+$ $HLA\text{-}DR^{high}$; p-DC: Lin-$CD14^-CD123^+HLA\text{-}DR^{high}$. MDSCs, myeloid-derived suppressor cells; M-MDSCs, monocytic-MDSCs; Gr-MDSCs, granulocytic MDSCs; DCs, dendritic cells; M-DCs, myeloid DCs; p-DCs, plasmacytoid DCs.

data are presented as the mean ± SD. The association between clinicopathological factors, and the different immune cell subsets with DFS and PFS were analyzed using univariate logistic analysis. The patients were divided into elevated and non-elevated groups based on the median values and according to the proportion of each immune cell subset. The DFS and PFS curves were calculated using the Kaplan-Meier method and compared with the log-rank test. Pearson's correlation was used to evaluate for correlations between each pair of immune cell subset. Multivariate cox regression analysis was used to investigate the association between clinicopathological factors and DFS. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using the GraphPad Prism software (v7; GraphPad Software, Inc.) and the SPSS statistical software (v21.0; IBM Corp.). The experiments were repeated three times.

Results

Quality of immune cell subsets in patients with bone sarcoma and STS. Each immune cell subset was detected using flow cytometry. The median values of the proportion and range in patients with high-grade non-metastatic are shown in Table SI. In addition, the median values of the proportion and range of the patients with metastatic sarcoma are shown in Table SII.

Associations between clinicopathological factors/number of suppressor cells and number of antigen-presenting cells/effector cells/cells secreting immune checkpoint proteins and DFS/PFS. A total of 61 patients with no metastasis and high-grade sarcoma, and 25 patients with metastatic sarcoma were examined. The associations between clinicopathological factors and DFS/PFS times are shown in Table II. The patients were divided into elevated and non-elevated groups based on the median value of each factor. No significant differences were noted between the DFS time in patients without metastasis and PFS time in patients with metastasis. The gating strategies for suppressor, antigen-presenting and effector cells are shown in Figs. 1 and 2. The associations between the quantities of these cell types and the DFS time in patients without metastasis and the PFS time in patients with metastasis are shown in Tables III and IV. The patients were divided into elevated and non-elevated groups based on the median values and according to the proportion of each immune cell subset. The DFS time in patients without metastasis and the PFS time in patients with metastasis were compared between each pair of immune cell subsets. High M-MDSC number was significantly associated with lower DFS time in patients without metastasis and PFS time in patients with metastasis. The gating strategies used to determine the expression levels of molecules, such as immune checkpoint proteins, in $CD4^+$ T, $CD8^+$ T and NK cells are shown in Figs. 2 and 3. There were 65.9 and 10.2% of the $NKG2D^+$ $CD8^+$ T cells, and 0.42 and 23.7% of the Tim-3^+ $CD8^+$ T cells in patient nos. 60 and 49. The associations between the expression levels of these molecules and DFS time in patients without metastasis and PFS time in patients with metastasis are shown in Tables III and IV. The patients were divided, based on the median values of the immune cell subsets, into elevated and non-elevated groups. DFS

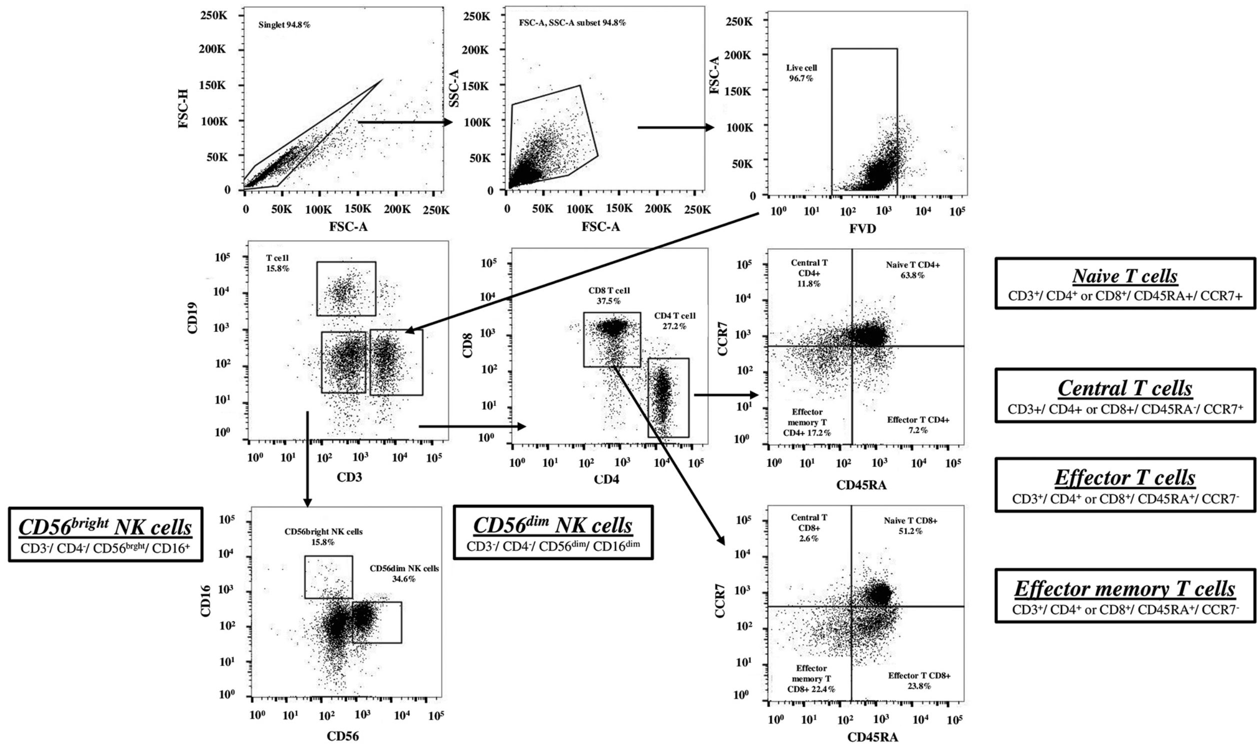

Figure 2. Gating strategy for T and NK cells. Gating strategy and representative dot plots for T and NK cells. The cells were defined as follows: T cells were classified as naïve T cells ($CD45RA^{+}CCR7^{+}$), central memory T cells ($CD45RA^{+}CCR7^{-}$), effector memory T cells ($CD45RA^{-}CCR7^{-}$) and central memory T cells ($CD45RA^{-}CCR7^{+}$), in populations of $CD4^{+}$ or $CD8^{+}$ cells. NK cells were classified as $CD56^{bright}$ NK cells ($CD3^{-}CD19^{-}CD14^{-}CD16^{+}CD56^{bright}$) and $CD56^{dim}$ NK cells ($CD3^{-}CD19^{-}CD14^{-}CD16^{+}CD56^{dim}$). NK, natural killer.

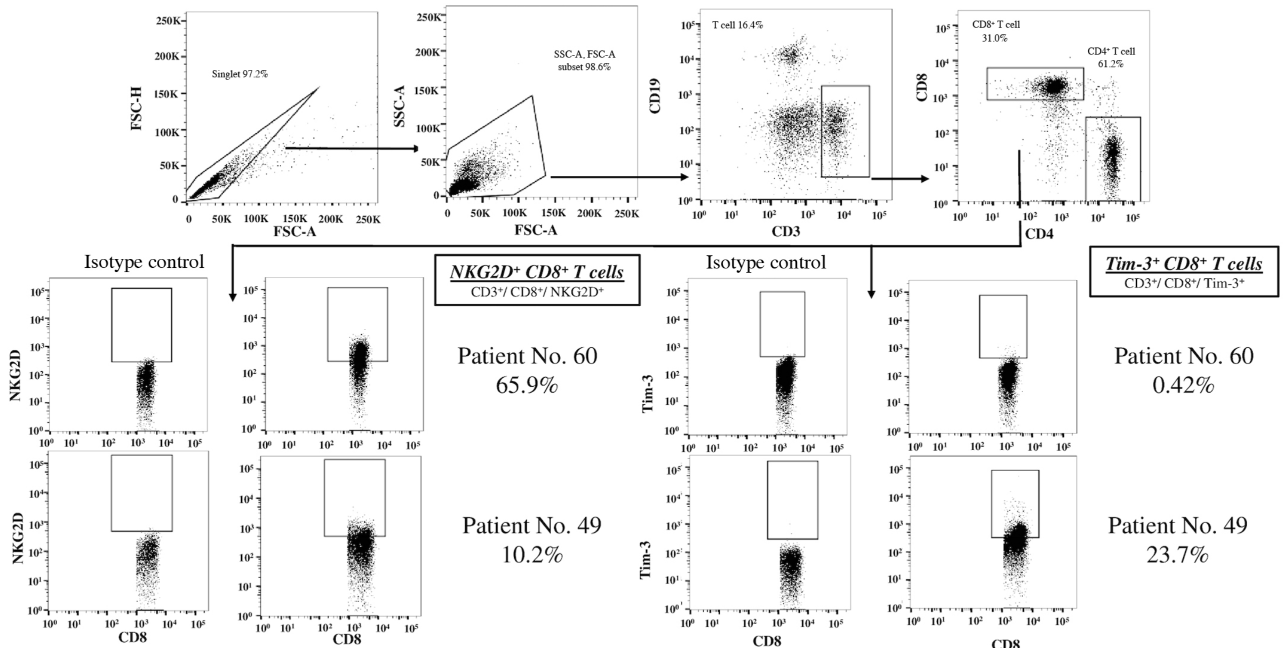

Figure 3. Gating strategy for $NKG2D^{+}$ $CD8^{+}$ T and Tim-3^{+} $CD8^{+}$ T cells. Gating strategy and representative dot plots for $NKG2D^{+}$ $CD8^{+}$ T cells and Tim-3^{+} $CD8^{+}$ T cells. A total of 2 independent dot plots are shown as high and low $NKG2D^{+}$ $CD8^{+}$ T cells, and high and low Tim-3^{+} $CD8^{+}$ T cells. Isotype controls were used to determine the cut-off levels for distinguishing between positivity and negativity. NKG2D, natural killer group 2 member A; Tim-3, T cell immunoglobulin and mucin domain-3.

time in patients without metastasis and PFS time in patients with metastasis were compared between molecules, such as immune checkpoint proteins on $CD4^{+}$ T, $CD8^{+}$ T and B cells. Low numbers of $NKG2D^{+}$ $CD8^{+}$ T cells and high numbers of Tim-3^{+} $CD8^{+}$ T cells were significantly associated with lower DFS time in patients without metastasis. However, no significant differences were noted in comparisons of PFS time in patients with metastasis between two groups.

Table II. Association between the clinicopathological factors and DFS and PFS.

	Free of metastasis and high-grade sarcoma (N=61)		Metastatic sarcoma (N=25)	
Clinicopathological factors	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years				
<56	Reference		Reference	
≥56	0.9 (0.3-2.6)	0.96	2.1 (0.8-3.4)	0.71
Sex				
Male	Reference		Reference	
Female	1.0 (0.3-3.0)	0.87	0.4 (0.4-3.7)	0.55
PS				
0	Reference			
1	1.1 (0.2-5.1)	0.88		
0-1			Reference	
2-4			2.2 (0.3-3.3)	0.43
ALP, U/l				
<322	Reference		Reference	
≥322	1.5 (0.4-5.7)	0.52	2.3 (0.7-5.5)	0.43
LDH, U/l				
<222	Reference		Reference	
≥222	3.3 (0.8-12.3)	0.08	4.3 (0.6-7.3)	0.66
WBC, μl				
<8600	Reference		Reference	
≥8600	2.1 (0.6-7.2)	0.25	2.3 (0.8-4.2)	0.39
Lymphocytes, %				
<38.9	Reference		Reference	
≥38.9	0.1 (0-2.3)	0.77	0.5 (0.5-2.6)	0.29
Neutrophils, %				
<72.7	Reference		Reference	
≥72.7	2.4 (0.9-7.0)	0.09	5.2 (0.4-6.1)	0.12
Monocyte, %				
<8.7	Reference		Reference	
≥8.7	1.0 (0.1-10.9)	0.95	3.6 (0.2-6.9)	0.55
Eosinocyte, %				
<5.0	Reference		Reference	
≥5.0	0.1 (0.1-2.4)	0.78	0.3 (0.3-2.9)	0.33
CRP, mg/dl				
<0.14	Reference		Reference	
≥0.14	1.9 (0.6-5.5)	0.23	7.9 (0.9-8.9)	0.41

CRP, C- reactive protein; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; WBC, white blood count; HR, hazard ratio; PS, performance stage; DFS, disease-free survival; PFS, progression-free survival.

Association of the number of M-MDSC, NKG2D⁺ CD8⁺ T cells and Tim-3⁺ CD8⁺ T cells with DFS. The association between the number of immune subset cells with DFS times in patients without metastasis was investigated. The patients were divided into 'elevated' and 'non-elevated' groups. The cut-off/threshold value was used based on the median value of immune subset cells. The data indicated that the number of M-MDSC, NKG2D$^+$ CD8$^+$ T cells and Tim-3$^+$ CD8$^+$ T cells was significantly associated with DFS based on the Kaplan-Meier method and the comparisons performed using the log-rank test (Fig. 4). High numbers of M-MDSC and Tim-3$^+$ CD8$^+$ T cells were significantly associated with poor DFS times (P=0.04 and 0.02, respectively), while high levels of NKG2D$^+$ CD8$^+$ T cells were significantly associated with longer DFS times (P=0.04). Multivariate Cox regression analysis revealed that the

Table III. Association between the quantity of each immune cell subset and disease-free survival in patients with high-grade sarcoma and without metastasis.

Immune cell subset	Median, %	HR (95% CI)	P-value
Suppressor cells			
M-MDSC	≤21.4	Reference	
	>21.4	2.9 (1.0-8.4)	0.04[a]
Gr-MDSC	≤0.04	Reference	
	>0.04	1.5 (0.5-4.3)	0.41
Naïve Tregs	≤0.31	Reference	
	>0.31	0.9 (0.3-2.7)	0.94
Effector Tregs	≤0.01	Reference	
	>0.01	0.7 (0.2-2.0)	0.50
Antigen-presenting cells			
Myeloid DC	≤0.31	Reference	
	>0.31	1.5 (0.5-4.4)	0.44
Plasmacytoid DC	≤0.22	Reference	
	>0.22	0.9 (0.3-2.6)	0.91
Effector cells			
$CD4^+$ T cells	≤35.5	Reference	
	>35.5	1.0 (0.4-3.1)	0.89
Naïve	≤15.4	Reference	
	>15.4	1.0 (0.4-3.0)	0.94
Effector	≤20.2	Reference	
	20.2	2.2 (0.7-6.8)	0.16
Effector memory	≤28.9	Reference	
	>28.9	0.4 (0.2-1.3)	0.13
Central memory	≤58.8	Reference	
	>58.8	0.7 (0.3-2.1)	0.58
$CD8^+$ T cells	≤42.8	Reference	
	>42.8	0.33 (0.4-3.5)	0.72
Naïve	≤27.0	Reference	
	>27.0	3.0 (0.9-7.8)	0.60
Effector	≤25.2	Reference	
	>25.2	2.9 (1.0-8.9)	0.12
Effector memory	≤64.8	Reference	
	>64.8	0.4 (0.1-1.1)	0.07
Central memory	≤30.3	Reference	
	>30.3	0.7 (0.2-1.9)	0.47
B cells	≤1.05	Reference	
	>1.05	0.9 (0.3-2.6)	0.89
$CD56^{bright}$ NK cells	≤11.5	Reference	
	>11.5	1.7 (0.6-4.9)	0.33
$CD56^{dim}$ NK cells	≤1.1	Reference	
	>1.1	0.6 (0.2-1.7)	0.33
Expression on $CD4^+$ T cells			
CD28	≤0.32	Reference	
	>0.32	0.6 (0.2-1.8)	0.41

Table III. Continued.

Immune cell subset	Median, %	HR (95% CI)	P-value
4-1BB	≤20.3	Reference	
	>20.3	2.6 (0.5-13.3)	0.24
ICOS	≤3.26	Reference	
	>3.26	0.8 (0.3-2.2)	0.65
OX-40	≤0.35	Reference	
	>0.35	1.4 (0.4-5.1)	0.59
CTLA-4	≤1.03	Reference	
	>1.03	0.4 (0.1-1.1)	0.07
PD-1	≤0.11	Reference	
	>0.11	0.7 (0.2-2.0)	0.50
LAG-3	≤0.08	Reference	
	>0.08	0.5 (0.17-1.4)	0.19
Tim-3	≤5.8	Reference	
	>5.8	1.3 (0.3-5.4)	0.72
NKG2D	≤10.2	Reference	
	>10.2	2.9 (0.8-11.9)	0.12
NKG2A	≤0.14	Reference	
	>0.14	2.2 (0.7-7.7)	0.19
CD62-L	≤16.7	Reference	
	>16.7	0.3 (0.1-1.0)	0.06
Expression on $CD8^+$ T cells			
CD28	≤0.11	Reference	
	>0.11	0.5 (0.17-1.5)	0.23
4-1BB	≤15.8	Reference	
	>15.8	15.9 (0.1-4.4)	0.75
ICOS	≤0.43	Reference	
	>0.43	1.3 (0.5-4.0)	0.54
OX-40	≤0.19	Reference	
	>0.19	1.6 (0.5-5.3)	0.38
CTLA-4	≤0.68	Reference	
	>0.68	0.4 (0.2-1.3)	0.15
PD-1	≤0.39	Reference	
	>0.39	0.3 (0.4-2.3)	0.21
LAG-3	≤7.6	Reference	
	>7.6	0.5 (0.2-1.5)	0.21
Tim-3	≤8.7	Reference	
	>8.7	3.4 (1.0-11.1)	0.04[a]
NKG2D	≤26.6	Reference	
	>26.6	0.3 (0.1-0.9)	0.05[a]
NKG2A	≤1.4	Reference	
	>1.4	1.6 (0.5-4.8)	0.41
CD62-L	≤24.7	Reference	
	>24.7	0.7 (0.2-2.0)	0.50

[a]P<0.05. M-MDSC, monocytic myeloid-derived suppressor cells; Gr, granulocytic; DC, dendritic cells; HR, hazard ratio; Treg, regulatory T cells.

Table IV. Association between the quantity of each immune cell subset and progression-free survival in patients with metastatic sarcoma.

Immune cell subset	Median, %	HR (95% CI)	P-value
Suppressor cells			
M-MDSC	≤44.2	Reference	
	>44.2	5.9 (1.3-26.7)	0.02[a]
Gr-MDSC	≤0.63	Reference	
	>0.63	0.3 (0.7-1.2)	0.09
Naïve Tregs	≤0.62	Reference	
	>0.62	3.2 (0.5-3.5)	0.34
Effector Tregs	≤0.03	Reference	
	>0.03	0.7 (0.2-7.9)	0.88
Antigen-presenting cells			
Myeloid DC	≤0.34	Reference	
	>0.34	1.5 (0.4-6.1)	0.52
Plasmacytoid DC	≤0.48	Reference	
	>0.48	2.1 (0.5-8.2)	0.30
Effector cells			
CD4$^+$ T cells	≤29.7	Reference	
	>29.7	0.5 (0.1-4.3)	0.53
Naïve	≤21.8	Reference	
	>21.8	0.2 (0.1-1.7)	0.14
Effector	≤3.42	Reference	
	>3.42	2.1 (0.3-16.5)	0.50
Effector memory	≤32.4	Reference	
	>32.4	0.1 (0.1-1.4)	0.09
Central memory	≤33.1	Reference	
	>33.1	0.3 (0.1-2.4)	0.23
CD8$^+$ T cells	≤26.8	Reference	
	>26.8	1.4 (0.2-11.8)	0.77
Naïve	≤16.7	Reference	
	>16.7	1.4 (0.1-15.7)	0.77
Effector	≤14.6	Reference	
	>14.6	5.1 (0.4-2.3)	0.33
Effector memory	≤46.7	Reference	
	>46.7	11.2 (0.6-21.7)	0.11
Central memory	≤9.28	Reference	
	>9.28	0.2 (0.1-3.0)	0.23
B cells	≤8.39	Reference	
	>8.39	0.3 (0.1-3.9)	0.36
CD56^{bright} NK cells	≤13.4	Reference	
	>13.4	1.2 (0.2-9.0)	0.83
CD56^{dim} NK cells	≤2.3	Reference	
	>2.3	7.4 (0.9-60.3)	0.62
Expression on CD4$^+$ T cells			
CD28	≤0.77	Reference	
	>0.77	0.3 (0.1-3.8)	0.38
4-1BB	≤10.2	Reference	
	>10.2	1.7 (0.2-12.1)	0.60
ICOS	≤4.98	Reference	
	>4.98	0.3 (0.1-2.1)	0.19
OX-40	≤0.53	Reference	
	>0.53	0.24 (0.1-2.9)	0.26
CTLA-4	≤1.31	Reference	
	>1.31	0.3 (0.1-2.4)	0.25
PD-1	≤0.52	Reference	
	>0.52	16.1 (0.6-4.1)	0.09
LAG-3	≤0.29	Reference	
	>0.29	0.4 (0.1-3.2)	0.39
Tim-3	≤3.58	Reference	
	>3.58	0.3 (0.1-2.6)	0.28
NKG2D	≤7.9	Reference	
	>7.9	0.1 (0.1-1.4)	0.09
NKG2A	≤0.82	Reference	
	>0.82	0.7 (0.1-4.1)	0.65
CD62-L	≤53.2	Reference	
	>53.2	6.0 (0.2-15.2)	0.28
Expression on CD8$^+$ T cells			
CD28	≤0.49	Reference	
	>0.49	6.8 (0.3-15.7)	0.23
4-1BB	≤8.07	Reference	
	>8.07	2.3 (0.4-2.8)	0.33
ICOS	≤0.45	Reference	
	>0.45	1.5 (0.1-24.1)	0.78
OX-40	≤1.44	Reference	
	>1.44	0.3 (0.3-1.8)	0.55
CTLA-4	≤0.57	Reference	
	>0.57	0.4 (0.4-4.1)	0.46
PD-1	≤0.23	Reference	
	>0.23	2.7 (0.2-32.2)	0.44
LAG-3	≤0.08	Reference	
	>0.08	0.2 (0.1-3.7)	0.30
Tim-3	≤0.88	Reference	
	>0.88	2.8 (0.4-9.2)	0.12
NKG2D	≤15.4	Reference	
	>15.4	1.5 (0.9-9.2)	0.87
NKG2A	≤3.68	Reference	
	>3.68	0.4 (0.9-12.5)	0.55
CD62-L	≤33.9	Reference	
	>33.9	0.1 (0.1-2.7)	0.18

[a]P<0.05. M-MDSC, monocytic myeloid-derived suppressor cells; Gr, granulocytic; DC, dendritic cells; HR, hazard ratio; Treg, regulatory T cells.

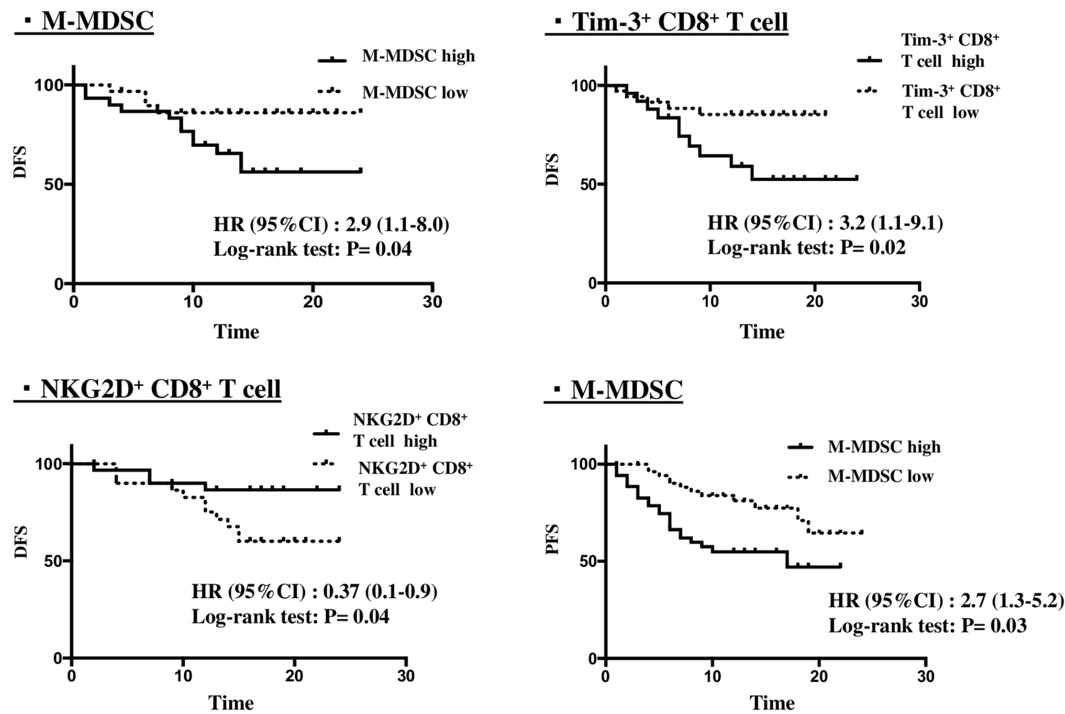

Figure 4. Association between the numbers of M-MDSCs, NKG2D+ CD8+ T cells and Tim-3+ CD8+ T cells with PFS. PFS curves were calculated using the Kaplan-Meier method and compared with the log-rank test. The Kaplan-Meier curves for PFS are shown according to the pretreatment quantities of M-MDSCs, NKG2D+ CD8+ T cells and Tim-3+ CD8+ T cells. M-MDSCs, monocytic- myeloid-derived suppressor cells; NKG2D, natural killer group 2 member A; Tim-3, T cell immunoglobulin and mucin domain-3, PFS, progression-free survival.

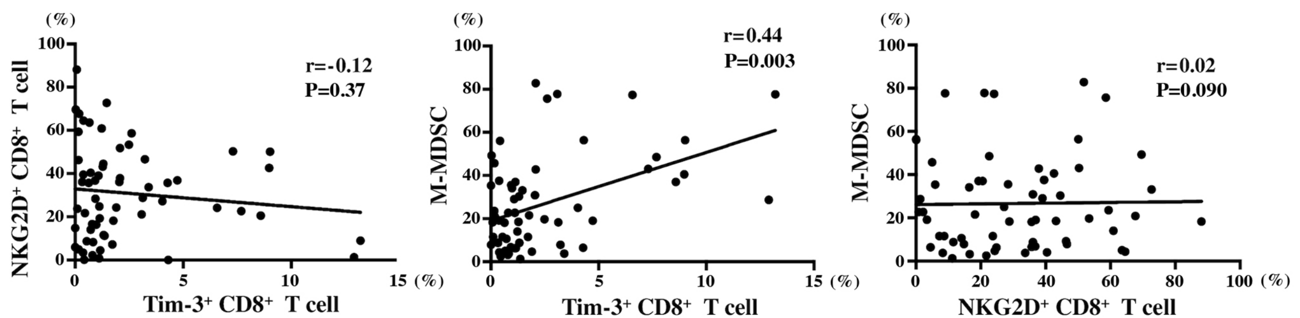

Figure 5. Correlations between the numbers of M-MDSCs, Tim-3+ CD8+ T cells and NKG2D+ CD8+ T cells, with each other. Pearson's correlation coefficient is indicated as 'r' M-MDSCs, monocytic-myeloid-derived suppressor cells; Tim-3, T cell immunoglobulin and mucin domain-3; NKG2D, natural killer group 2 member A.

quantities of Tim-3+ CD8+ T cells were associated with a lower DFS time (Table V).

Association between the number of M-MDSCs and PFS. The association between the number of immune subset cells and PFS time was examined. The patients with metastasis were divided into 'high' and 'low' groups. The cut-off/threshold value was used based on the median value of immune subset cells. The data indicated that the number of M-MDSCs was significantly associated with PFS time based on the Kaplan-Meier method and comparisons using the log-rank test (Fig. 4). A high number of M-MDSCs was significantly associated with poor PFS (P=0.03).

Pairwise correlation of Tim-3+ CD8+ T, M-MDSC and NKG2D+ CD8+ T cell number. The initial analysis demonstrated that the number of M-MDSCs, Tim-3+ CD8+ T cells and NKG2D+ CD8+ T cells was associated with DFS time. Subsequently, Pearson's correlation analysis was performed between each pair of these variables, corresponding to the three different cell types (Fig. 5). No correlation was found between the number of Tim-3+ CD8+ T and NKG2D+ CD8+ T cells (r=-0.12; P=0.37). Similarly, no correlation was noted between the number of NKG2D+ CD8+ T and M-MDSC cells (r=-0.02; P=0.90). However, a weak correlation was observed between the number of M-MDSCs and Tim-3+ CD8+ T cells (r=-0.44; P=0.003).

Discussion

In the present study, the immunological status of peripheral blood samples from patients with bone sarcoma and STS was investigated and also the association between the quantity of each immune cell subset and DFS/PFS times in patients with

Table V. Multivariate analysis for disease-free survival.

Clinicopathological characteristics	HR (95% CI)	P-value
M-MDSC, %		
<21.4	Reference	
≥21.4	2.3 (0.6-8.3)	0.17
NKG2D$^+$ CD8$^+$ T cell, %		
<26.6	Reference	
≥26.6	0.3 (0.1-1.3)	0.12
Tim-3$^+$ CD8$^+$ T cell, %		
<8.7	Reference	
≥8.7	3.7 (1.0-12.9)	0.04[a]

[a]P<0.05. HR, hazard ratio; M-MDSC, monocytic myeloid-derived suppressor cells.

sarcoma. A higher number of M-MDSCs and Tim-3$^+$ CD8$^+$ T cells was significantly associated with poor DFS times, while a higher number of NKG2D$^+$ CD8$^+$ T cells was significantly associated with longer DFS times. In addition, a higher number of M-MDSCs was significantly associated with poor PFS time and a weak positive correlation was found between the number of M-MDSCs and Tim-3$^+$ CD8$^+$ T cells.

In previous studies, high quantities of M-MDSCs in peripheral blood samples were identified as a poor prognostic factor for various types of cancer, such as melanoma, hepatocellular carcinoma, colorectal cancer and non-small cell lung cancer (25,26,28,31,32). In patients with gastric cancer, a higher number of granulocyte-MDSCs was found to be a significant adverse factor for PFS time (33). The present study revealed that higher quantities of M-MDSCs were associated with poor DFS and PFS times. However, the granulocyte-MDSC ratio was not associated with DFS and PFS. The findings suggested similar results with those obtained in other types of tumors.

MDSCs are a heterogeneous population of granulocyte and monocyte-like cells that inhibit T cell function (23). Significant accumulation of MDSCs has been observed in patients with certain conditions, including chronic infections, transplantation and multiple malignancies compared with that in healthy subjects (23). The functions of MDSCs, including production of arginase 1, release of reactive oxygen species and nitric oxide and secretion of immune-suppressive cytokines, leads to suppression of the immune responses (34). In addition, MDSCs are a potential therapeutic target. Clinical trials have examined the administration of multiple kinase inhibitors as inhibitors of MDSC proliferation (35,36). In addition, phosphodiesterase-5 inhibitors have been used to deactivate MDSCs and all-trans retinoic acid has been used to prevent the differentiation of MDSCs (34). These trials are ongoing. The present study demonstrated that the number of M-MDSCs in patients with metastatic sarcoma was higher compared with that in patients with no high-grade sarcoma metastasis. The quantities of M-MDSCs may depend on the potential tumor clinical stage. Therefore, the number of M-MDSCs in patients with metastatic sarcoma may cause suppression of immune responses and consequently the inhibition of M-MDSC proliferation may be a potential therapeutic strategy.

As a co-inhibitory receptor present on the surface of T cells, Tim-3 plays a role in immune regulation (14). Galectin-9 has been described as a binding receptor that mediates the T cell inhibitory effects of Tim-3 (37). Tim-3 has also been shown to be expressed on Th1 cells, as well as on CD4$^+$ and CD8$^+$ T cells, Treg, Th17, NK, DCs, monocytes and mast cells (38). Upregulation of TIM-3 expression has been observed on exhausted CD8$^+$ T cells (39,40). In certain tumors, such as lung cancer, lymphomas and breast cancer, higher quantities of Tim-3$^+$ CD8$^+$ T cells were associated with poor disease outcomes (41-45). Ge *et al* (42) reported that Tim-3$^+$ CD4$^+$ T and CD8$^+$ T cells may serve as novel diagnostic and prognostic biomarkers of OS. However, to the best of our knowledge, the number of Tim-3$^+$ CD8$^+$ T cells in peripheral blood specimens from patients with STS has not been previously investigated, with respect to disease progression and patient survival. The present study indicated that the higher number of Tim-3$^+$ CD8$^+$ T cells was associated with poor DFS time in peripheral blood specimens derived from patients with bone sarcoma and STS.

The results of the present study suggested that the host immune response to tumors occurred in some, but not all, patients with bone sarcomas and STSs. Feng and Guo (46) demonstrated that the Tim-3 protein was overexpressed and that its mRNA expression levels were increased in OS tissues *in vitro*, as demonstrated by immunohistochemistry and reverse transcription-quantitative PCR. These findings suggested that the anti-Tim-3 antibody may exert significant tumor-associated effects on STS cells, as well as on T cells expressing the Tim-3 protein on their surface.

In addition, the present study indicated that high levels of NKG2D$^+$ CD8$^+$ T cells were favorable factors for DFS time in patients with early stage bone sarcoma and STS. NKG2D is a stimulatory receptor expressed on the surface of NK cells and subsets of T cells (47). The function of NKG2D, as a co-stimulatory molecule on the surface of tumor infiltrating lymphocytes, involves its ligands, MICA/B and ULBPs, which are present in tumors, as well as the stimulation of the antitumor immunity (48,49). Several studies have shown that the protein expression levels of NKG2D were associated with optimal outcomes in patients with cancer, such as nasopharyngeal carcinoma, cervical cancer and pancreatic cancer (50-52). Similarly, the findings from the present study suggested that high levels of NKG2D$^+$ CD8$^+$ T cells were found to be favorable factors for DFS time in patients with early stage bone sarcoma and STS.

Furthermore, a high number of M-MDSCs was identified as a poor prognostic factor, indicating low PFS time in patients with metastasis. These observations suggested that immune surveillance, i.e. the host immune reaction against cancer, existed in patients with bone sarcomas and STSs. Notably, a high number of M-MDSCs was associated with DFS and PFS times, suggesting that it could be used as a prognostic factor.

In advanced cancer progression cases, the number of immunosuppressor cells, such as Tregs and MDSCs typically increases according to their tumor volume. Furthermore, T cell function is strongly suppressed. Therefore, the activation of the surface markers, such as Tim-3 and NKG2D and the associated fatigue caused on T cells may not correlate with prognosis in patients who are at the late disease stages (53-55).

The present study contains certain limitations. The cohort was small, since bone sarcomas and STSs are rare tumors. Furthermore, it is important to identify associations between the immunological status of peripheral blood samples and the findings of pathological specimens, and to compare the immune status between patients with non-metastatic sarcoma and with patients with subsequent metastatic to predict prognosis. However, in the current study, specimens were not collected in the same patient at subsequent metastases. These aspects will be investigated in future studies. In addition, the interference of other factors, such as chemotherapy could not be excluded. Therefore, patients with high-grade sarcoma and no metastasis or those with metastatic sarcoma were only included to minimize the impact of differences in disease background and interventions. This is a preliminary, exploratory study and the results will be subsequently validated in a larger number of patients in the future.

In conclusion, the present study demonstrated that the immune status of patients with high-grade sarcoma and no metastasis or those with metastatic sarcoma who were treated with non-immunotherapy methods was associated with PFS or DFS times. These results may indicate that patients with bone sarcoma and STS, who develop an antitumor immune response over the natural course of their disease and those who develop a strong antitumor immune response, may have improved disease outcomes. The results of the present study may aid the development of novel strategies for sarcoma treatment based on the use of specific biomarkers or immunotherapeutic targets.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EK, AK and SK supervised the research. YK, EK, YS, TN, KK and SK designed the study and performed the experiments. YK, EK, DK, YT, ME, FN, AK obtained patient consent and collected the samples. YK and AI analyzed and interpreted the data. All authors wrote the manuscript and approved the final version for publication.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board (approval no. 2014-287) from the National Cancer Center (Tokyo, Japan). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

Shigehisa Kitano reports personal fees from Astra Zeneca, Chugai Pharmaceutical Co., Ltd., Pfizer, Inc., Sanofi S.A., Nippon Kayaku Co., Ltd., Meiji Seika Kaisha, Ltd., Taiho Pharmaceutical Co., Ltd., Novartis International AG, Daiichi-Sankyo Co., Ltd., personal fees from Merck Sharp and Dohme Corp., Kyowa Kirin Co., Ltd., Celgene Corporation, Sumitomo Dainippon Pharma Co., Ltd., Astellas Pharma, Inc., Ono Pharmaceutical Co., Ltd., Bristol-Myers Squibb Company, AYUMI Pharmaceutical Corporation, Rakuten Medical, Inc., and Pharmaceuticals and Medical Devices Agency. In addition, grants and personal fees from Boehringer Ingelheim, Eisai Co., Ltd., and Regeneron Pharmaceuticals, Inc., and grants from Gilead Sciences, Inc., Japan Agency for Medical Research and Development, and Japan Society for the Promotion of Science, outside the submitted work.

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