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ORIGINAL ARTICLE

Programmed death-ligand 1 and its soluble form are highly expressed in nasal natural killer/T-cell lymphoma: a potential rationale for immunotherapy

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Abstract Nasal natural killer/T-cell lymphoma (NNKTL) is an aggressive neoplasm with poor therapeutic responses and prognosis. The programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) pathway plays an important role in immune evasion of tumor cells through T-cell exhaustion. The aim of the present study was to examine the expression of PD-L1 and PD-1 molecules in NNKTL. We detected the expression of PD-L1 in biopsy samples from all of the NNKTL patients studied. PD-L1 was found on both malignant cells and tumor-infiltrating macrophages, while PD-1-positive mononuclear cells infiltrated the tumor tissues in 36% of patients. Most significantly, soluble PD-L1 (sPD-L1) was present in sera of NNKTL patients at higher levels as compared to healthy individuals and the levels of serum sPD-L1 in patients positively correlated with the expression of PD-L1 in lymphoma cells of tumor tissues. In addition, the high-sPD-L1 group of patients showed significantly worse prognosis than the low-sPD-L1 group. Furthermore, we confirmed that membrane and soluble PD-L1 was expressed on the surface and in the culture supernatant, respectively, of NNKTL cell lines. The expression of PD-L1 was observed in tumor tissues and sera from a murine xenograft model inoculated with an NNKTL cell line. Our results suggest that sPD-L1 could be a prognostic predictor for NNKTL and open up the possibility of immunotherapy of this lymphoma using PD-1/PD-L1 axis inhibitors.

Keywords Nasal NK/T-cell lymphoma · PD-L1 · PD-1 · Soluble form · Immunotherapy

Abbreviations

CAEBV	Chronic active Epstein–Barr virus infection
CTLs	Cytotoxic T lymphocytes
EBER	Epstein–Barr virus encoded small RNA
EBV	Epstein–Barr virus
FBS	Fetal bovine serum
FFPE	Formalin-fixed, paraffin-embedded
IFN	Interferon
IL	Interleukin
IPI	International Prognostic Index
ISH	In situ hybridization
LDH	Lactate dehydrogenase
LMP	Latent membrane protein
mAbs	Monoclonal antibodies
NK	Natural killer
NNKTL	Nasal natural killer/T-cell lymphoma
OS	Overall survival
PD-1	Programmed death-1

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PD-L1	Programmed death-ligand 1
PINK	Prognostic index of natural killer lymphoma
ROC	Receiver-operating characteristic
RT	Room temperature
sPD-L1	Soluble programmed death-ligand 1

Introduction

Nasal natural killer (NK)/T-cell lymphoma (NNKTL) is characterized by progressive necrotic lesions in the nasal cavity, palate, and/or nasopharynx [1]. NNKTL cells are derived from two CD56-positive lineages, NK- and γδT-cell, although the NK-cell lineage is the predominant phenotype [2–4]. Our previous studies further showed the presence of Epstein-Barr virus (EBV) DNA, EBV oncogenic proteins, and the clonotypic EBV genome in this lymphoma, indicating that NNKTL is an EBV-related malignancy [2, 3, 5].

It has been established histologically that many inflammatory cells such as lymphocytes, monocytes, and macrophages infiltrate NNKTL tissue [1]. This finding gives rise to the speculation that tumor-reactive cytotoxic T lymphocytes (CTLs) might also exist in NNKTL tissue to attack the lymphoma cells. It is especially possible that EBV-specific CTLs could recognize and destroy NNKTL cells infected by EBV, because EBV-associated proteins are exogenous antigens that induce immune responses [6]. However, NNKTL shows poor prognosis caused by its rapid progression, suggesting that this lymphoma might be able to evade the antitumor immune response using several mechanisms. For example, several amino-acid changes are seen in the sequence encoding the major HLA-A2 restricted CTL epitopes of latent membrane protein (LMP)-1 and LMP-2A, which are EBV oncogenic proteins, resulting in the reduction of CTL recognition for lymphoma cells [7, 8]. In addition, NNKTL cells secrete immune suppressive factors such as interleukin (IL)-10 [9, 10] and transforming growth factor-β [11] that suppress the activity of CTLs, inhibit the effect of tumor-specific helper T cells, and skew naïve helper T cells to regulatory T cells. Although numerous groups including our group are involved in clarifying the immune regulation of NNKTL, details regarding immune evasion are unknown.

Many research groups have recently focused on the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis, which is an immune checkpoint pathway. In general, the PD-1 receptor is expressed on activated T cells, while the PD-L1 ligand is expressed on tumor cells or antigen-presenting cells [12, 13]. The interaction between PD-1 and PD-L1 attenuates T-cell function and proliferation, leading to antitumor immunosuppression through CTL exhaustion [14]. Thus, blockade of the PD-1/PD-L1 pathway with monoclonal antibodies (mAbs) can restore and enhance the CTL response, promoting T-cell survival and proliferation [15]. Furthermore, a number of investigators have reported that a soluble form of PD-L1 can be detected in the blood of patients with some malignant diseases and might be a potent predictive biomarker [16–19]. To further our understanding of the mechanism of immune evasion by NNKTL, it is, therefore, of great interest to examine the expression of PD-L1 and PD-1 in this lymphoma.

In the present study, we aimed to assess the expression of membrane and soluble PD-L1 (sPD-L1) as well as of PD-1 in NNKTL. Using biopsy tissues, cell lines, and a murine xenograft model, we clearly show that PD-L1 is expressed on tumor cells and tumor-infiltrating macrophages. Furthermore, we show that increased sPD-L1 levels are detectable in the blood of patients and correlate with poor prognosis. These results suggest that the PD-1/PD-L1 pathway may play an important role in evasion of the antitumor immune response and that sPD-L1 may be a valuable predictor for prognosis in NNKTL.

Materials and methods

Patients

Seventeen patients with NNKTL were analyzed in this study, all of whom were newly diagnosed according to the World Health Organization classification of hematological malignancies at Asahikawa Medical University (Asahikawa, Japan) between 2000 and 2014. Patient clinicopathological characteristics and follow-up data are summarized in Tables 1 and 2. Serum lactate dehydrogenase (LDH) was considered elevated if >240 IU/mL. The International Prognostic Index (IPI) and the prognostic index of natural killer lymphoma (PINK) were determined based on published criteria [20, 21]. As the primary treatment, all patients received concurrent chemoradiotherapy. Patients were treated with MPVIC-P (ifosfamide, carboplatin, methotrexate, peplomycin, etoposide, and prednisolone) (12 patients) [22], DeVIC (dexamethasone, etoposide, ifosfamide, and carboplatin) (two patients) [23], MTCOP-P (pirarubicin, cyclophosphamide, vincristine, methotrexate, peplomycin, and prednisolone) (one patient) [24], or THP-COP (pirarubicin, cyclophosphamide, vincristine, and prednisolone) (one patient) [25] chemotherapy regimens. One patient received etoposide alone. We also analyzed 23 healthy volunteers as controls. All patients and volunteers signed informed consent forms. This study was conducted with the approval of the Institutional Review Board at Asahikawa Medical University.

Table 1 Characteristics of the 17 patients with NNKTL

Case no.	Age	Sex	B symptoms	ECOG PS	Ann Arbor stage	Lymph-node involvement	No. of extranodal sites	Bone marrow involvement	Serum LDH (IU/L)[a]	IPI	PINK	Primary treatment	Chemotherapy regimen	Course (months)
1	52	F	−	0	I	None	1	−	163	0	0	CCRT	MTCOP-P	Alive (110)
2	52	M	+	3	IV	Regional	2	+	219	3	1	CCRT	THP-COP	Died (1)
3	48	F	−	0	I	None	1	−	205	0	0	CCRT	MPVIC-P	Alive (104)
4	60	M	−	0	I	None	1	−	236	0	0	CCRT	MPVIC-P	Alive (109)
5	64	M	−	0	I	None	1	−	162	1	1	CCRT	MPVIC-P	Alive (104)
6	48	M	+	1	I	None	1	−	176	0	0	CCRT	MPVIC-P	Alive (51)
7	40	F	+	1	I	None	1	−	144	0	0	CCRT	MPVIC-P	Alive (116)
8	45	M	+	2	IV	Regional	2	+	782	4	1	CCRT	DeVIC	Died (1)
9	67	F	−	0	IV	Regional	2	−	171	3	2	CCRT	DeVIC	Died (7)
10	63	M	−	0	I	None	1	−	219	1	1	CCRT	MPVIC-P	Alive (90)
11	20	M	+	1	I	None	1	−	177	0	0	CCRT	MPVIC-P	Alive (89)
12	63	M	−	0	I	None	1	−	151	1	1	CCRT	MPVIC-P	Alive (84)
13	58	M	−	0	II	Regional	1	−	765	1	0	CCRT	MPVIC-P	Alive (86)
14	67	M	+	2	I	None	1	−	626	3	1	CCRT	MPVIC-P	Alive (48)
15	21	M	+	2	I	None	1	−	205	1	0	CCRT	MPVIC-P	Alive (42)
16	85	M	+	1	I	None	1	−	398	2	1	CCRT	Etoposide	Died (27)
17	67	M	+	1	I	None	1	−	293	2	1	CCRT	MPVIC-P	Alive (9)

ECOG PS Eastern Cooperative Oncology Group Performance Status, *LDH* lactate dehydrogenase, *IPI* International Prognostic Index, *PINK* prognostic index of natural killer lymphoma, *CCRT* Concurrent chemoradiotherapy, *MTCOP-P* pirarubicin, cyclophosphamide, vincristine, methotrexate, peplomycin, and predonisolone, *THP-COP* pirarubicin, cyclophosphamide, vincristine, and predonisolone, *MPVIC-P* ifosfamide, carboplatin, methotrexate, peplomycin, etoposide, and predonisolone, *DeVIC* dexamethasone, etoposide, ifosfamide, and carboplatin

[a]LDH was considered as elevated when it was >240 lU/mL

Table 2 Pathological characteristics and PD-L1/PD-1 expression in NNKTL patients

Case no.	EBER ISH	CD56 expression on malignant cells	% of PD-L1+ cells in CD56+ cells	% of CD68+ cells	% of PD-L1+ cells in CD68+ cells	Infiltration of PD-1+ cells	Serum sPD-L1 (pg/mL)
1	+	+	40	30	30	+	3041
2	+	+	70	80	40	−	1387
3	+	+	10	40	20	−	307
4	+	+	50	50	30	−	622
5	+	+	10	40	40	−	300
6	+	+	50	NE	NE	NE	1796
7	+	+	40	50	30	−	1066
8	+	+	50	40	40	−	851
9	+	+	50	50	40	+	1059
10	+	+	50	50	40	−	1230
11	+	+	40	50	30	−	658
12	+	+	NE	NE	NE	NE	257
13	+	+	NE	NE	NE	NE	330
14	+	+	30	60	20	−	307
15	+	+	40	50	20	+	<13
16	+	+	40	70	30	+	NE
17	+	+	40	60	30	+	400

ISH in situ hybridization, *sPD-L1* soluble PD-L1, *NE* not examined

Cell lines

The characteristics of the cell lines are summarized in Supplemental Table S1. SNK-6 [4], SNK-1 [26], and SNK-10 [27] were provided by Dr. Norio Shimizu (Tokyo Medical and Dental University, Tokyo, Japan). Raji was established from Burkitt lymphoma [28]. HDLM-2 [29] and Molt-4 [30] were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Culture Collection (Manassas, VA), respectively. SNK-6, SNK-1, and SNK-10 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 700 units/mL recombinant human IL-2. Raji, HDLM-2, and Molt-4 were cultured in RPMI 1640 supplemented with 10% FBS. All cell lines were used within ten passages after thawing of frozen cells.

Immunohistochemical staining and in situ hybridization

Formalin-fixed, paraffin-embedded (FFPE) samples were prepared from pretreatment biopsy tissues of NNKTL patients or the subcutaneous tumors of mice transplanted with SNK-6 cells and were cut into 4-μm-thick sections. We used anti-PD-L1 (E1L3N, 1:200, Cell Signaling Technology), anti-PD-1 (NAT105, 1:100, dianova), anti-CD56 (1:50, Novocastra), anti-CD68 (PG-M1, 1:50, DAKO), and anti-CD8 (C8/144B, 1:100, DAKO) mAbs as the primary Ab. The Envision HRP System (DAKO) was used for visualization of the signal. For antigen retrieval, slides were treated with Target Retrieval Solution pH 9 (DAKO) for 15 min in a microwave oven. Serial sections were used for PD-L1 and CD56, PD-L1 and CD68, PD-L1 and PD-1, CD8 and PD-1, or CD56 and CD68 staining. We considered a case CD68-positive if >5% of the cells in tumor cell regions were positive for CD68 staining in three representative high-power (400×) fields [31]. A case was considered as PD-L1 positive if >10% of the CD56-positive or CD68-positive cells were also PD-L1 positive, as previously described by other groups [32, 33]. When >5% of tumor-infiltrating mononuclear immune cells were PD-1 positive, the specimen was defined as PD-1 positive [34, 35]. EBV encoded small RNA (EBER) in FFPE tissue sections was detected by in situ hybridization (ISH) as previously described [3].

Immunofluorescent staining

Double immunofluorescent staining was performed on 2-μm-thick FFPE sections. After antigen retrieval using Target Retrieval Solution High pH (DAKO), the sections were incubated with anti-PD-L1 mAb (E1L3N, 1:500, Cell Signaling Technology) at room temperature (RT) for 60 min. The sections were treated with secondary Abs, Histofine Simple Stain MAX PO (Nichirei Biosciences Inc.), at RT for 30 min, and were then treated with the TSA

Cyanine 3 system (PerkinElmer Inc.) at RT for 10 min. A second antigen retrieval was performed using Target Retrieval Solution Citrate pH 6 (DAKO). The sections were incubated with anti-CD56 mAb (1:150, Novocastra) or anti-CD68 mAb (PG-M1, 1:150, DAKO) at 4 °C overnight, and then with anti-mouse AlexaFluor 488 (Abcam) at RT for 120 min. After DAPI (Invitrogen) staining, the sections were embedded in mounting medium.

Measurement of sPD-L1 using ELISA

sPD-L1 in blood from patients or mice was measured using the PDCD1LG1 ELISA Kit (USCN Life Science Inc.). All sera from patients were taken at diagnosis and were frozen at −80 °C. sPD-L1 in cell culture supernatants was measured using the PathScan Total PD-L1 Sandwich ELISA Kit (Cell Signaling Technology). Recombinant human PD-L1 (R&D Systems) was used as the standard. Cell lines (5×10^5/mL) were cultured in 96-well round-bottomed plates, and supernatants of the cell cultures were collected after 24 h. The minimum detectable concentration was 13 pg/mL and the minimum quantitative range was 49 pg/mL. Measurements for serum samples and cell culture supernatants were done in duplicate and triplicate, respectively. The intra-assay and inter-assay variations were below 20%. The results correspond to means ± SEM.

Flow cytometric analysis

Flow cytometric analysis was done using anti-PD-L1 (MIH1) or PD-1 (MIH4) mAb conjugated with PE (eBioscience) as previously described [36]. PE-conjugated mouse IgG1 kappa (eBioscience) was used as an isotype control. The FACS Accuri flow cytometer (BD Biosciences) was used to measure fluorescence and analyze the data.

Western blot analysis

Western blot analysis was performed using anti-PD-L1 (E1L3N, 1:1000, Cell Signaling Technology), anti-PD-1 (D4W2J, 1:1000, Cell Signaling Technology), or control anti-β-Actin (C4, 1:1000, Santa Cruz Biotechnology) mAb as previously described [6]. ImageJ software (National Institute of Health) was used to quantify the amount of PD-L1 protein against β-Actin.

Transplantation of SNK-6 cells into NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice

Six-week old female NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained under specific pathogen free conditions in the animal laboratory of the Center for Advanced Research and Education, Asahikawa Medical University (Asahikawa, Japan). Twelve mice were injected subcutaneously with 2×10^6 SNK-6 cells in a shaved flank. Thirty days later, the mice were sacrificed, and the subcutaneous tumors or blood samples were collected. All animal experiments were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University.

Statistical analysis

The Mann–Whitney *U* test was used to compare sPD-L1 values between NNKTL patients and healthy controls. The relationship between the serum sPD-L1 values and the expression of molecules including PD-L1 in tissues was evaluated by the Spearman rank correlation. The optimal cut-off value of serum sPD-L1 was determined based on receiver-operating characteristic (ROC) curve analysis for survival (death due to NNKTL or censored). The Kaplan–Meier method was used to estimate survival curves and the statistical significance of differences in survival curves was examined using the log-rank test. Correlations between the sPD-L1 level and the main clinical features were determined using Fisher's exact test. $P<0.05$ was considered statistically significant. All graphics and analyses were done using GraphPad Prism 5 (GraphPad Software) and JMP version 12 (SAS Institute).

Results

Lymphoma cells and tumor-infiltrating macrophages in tissues from NNKTL patients express PD-L1

We initially assessed whether the expression of PD-L1 was detected in the tissues from 15 of the 17 NNKTL patients using immunohistological staining (Table 2). Atypical lymphoid cells infiltrated the nasal mucosa in all samples (Fig. 1a). In addition, most tumor cells were positive for EBER (Fig. 1b). Staining using serial sections showed that both CD56 and PD-L1 were expressed in all 15 patients and that CD56-positive lymphoma cells were preferentially localized at PD-L1-expressing areas (Fig. 1c, d). To confirm that tumor cells expressed PD-L1, double immunofluorescent staining for PD-L1 and CD56 was performed. CD56-positive cells coexpressed PD-L1 (Fig. 1e). This result indicated that the malignant cells of all 15 patients showed positive PD-L1 staining.

It is known that tumor-infiltrating CD68-positive macrophages in several lymphomas such as Hodgkin's lymphoma and diffuse large B-cell lymphoma strongly express PD-L1 [32, 33]. We, therefore, investigated whether CD68-positive macrophages infiltrated

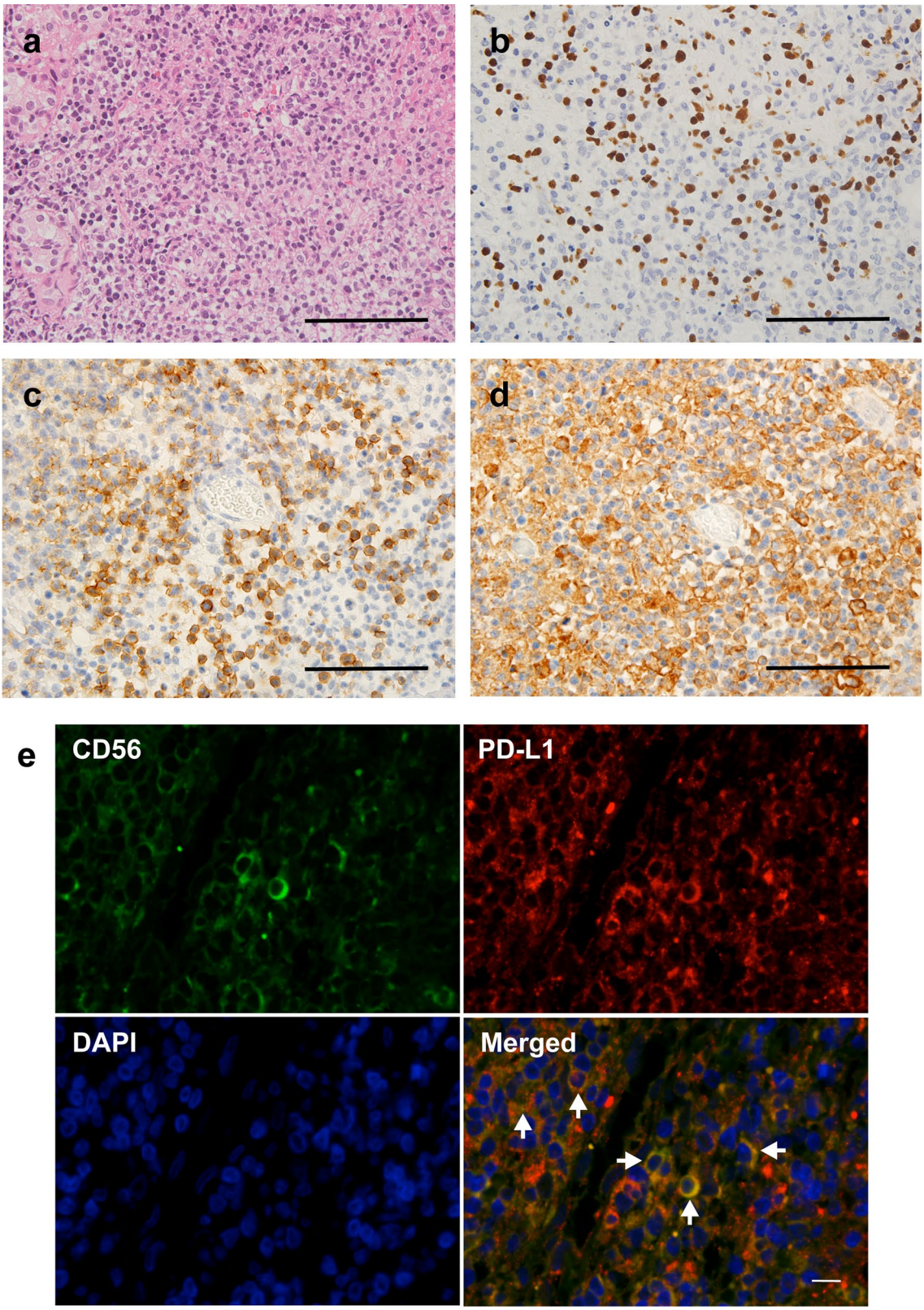


Fig. 1 Expression of PD-L1 on lymphoma cells in biopsy tissues from NNKTL patients. **a–d** Representative immunohistological features of FFPE samples (Patient 17). **a** Nasal mucosa is infiltrated by atypical lymphoid cells (H&E staining). **b** ISH for EBER. Nuclei of EBER-positive cells are stained (*brown*). **c** Staining for CD56. **d** Staining for PD-L1. Serial sections were used for **c** and **d**. *Scale bar* in **a–d** is 100 µm. **e** Double immunofluorescent staining of CD56 (*green*) and PD-L1 (*red*), counterstained with DAPI (*blue*). *Arrows* indicate colocalization of CD56 and PD-L1. *Scale bar* is 50 µm

NNKTL tissues. CD68-positive macrophages (Fig. 2a) were observed in all 14 of the patients tested, as shown in Table 2. Furthermore, immunohistochemical staining using serial sections indicated that CD68-positive macrophages were localized at regions corresponding to PD-L1-expressing areas (Fig. 2a, b). Double immunofluorescent staining for PD-L1 and CD68 confirmed that CD68-positive cells coexpressed PD-L1 (Fig. 2c), indicating that tumor-infiltrating CD68-positive macrophages clearly expressed PD-L1 in NNKTL.

PD-1-positive mononuclear immune cells infiltrate the tissues of patients with NNKTL

We next investigated the expression of PD-1 in NNKTL tissues. Immunohistological staining showed that 5 of 14 samples (36%) were positive for PD-1 (Table 2). Representative immunohistological staining of PD-1-positive mononuclear cells is shown in Fig. 3a. We further examined the infiltration pattern of PD-1-positive cells in NNKTL tissues with PD-L1-positive cells. Staining using consecutive slides showed that PD-1-positive cells

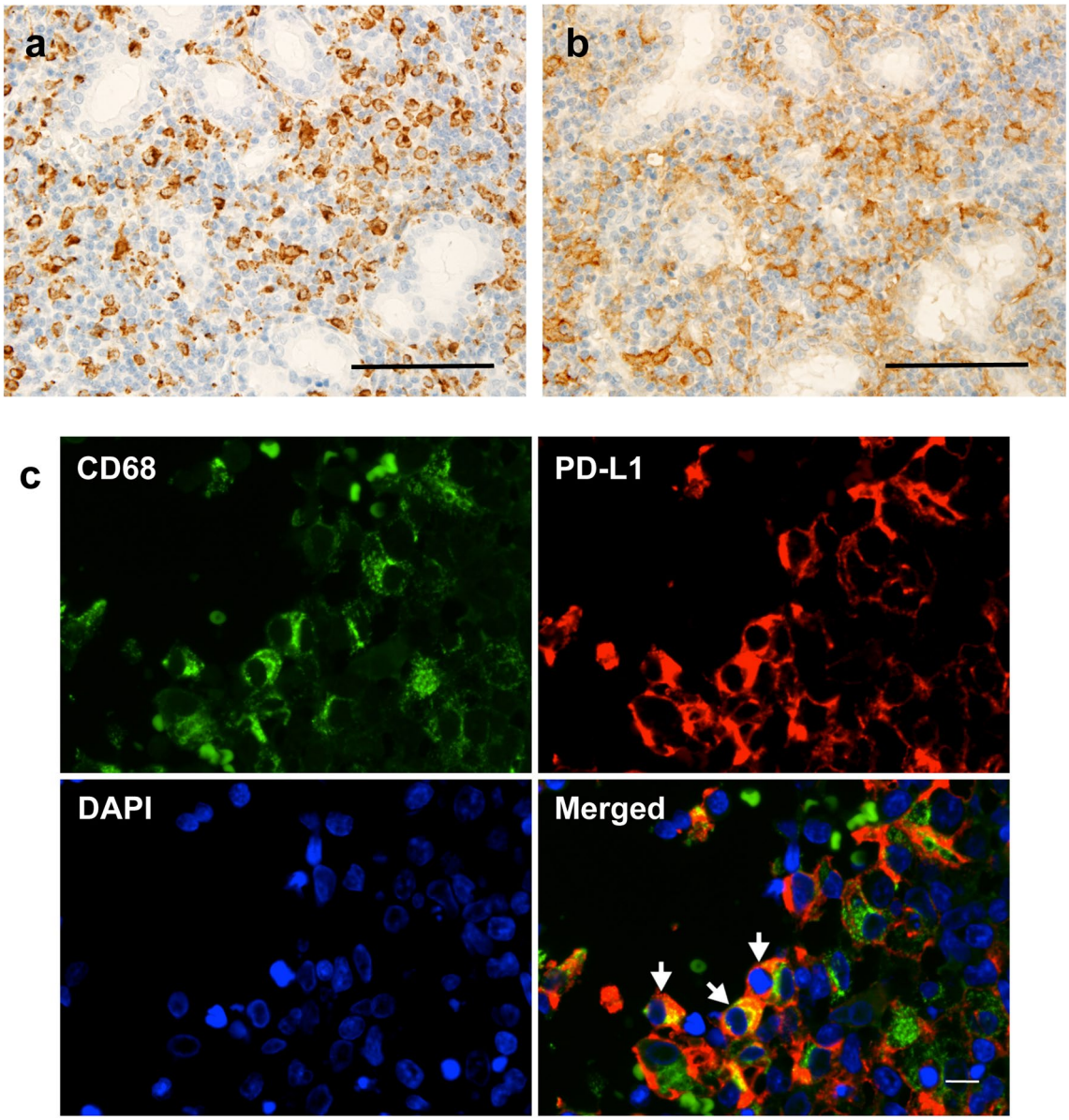


Fig. 2 Expression of PD-L1 on tumor-infiltrating macrophages in biopsy tissues from NNKTL patients. Representative immunohistological features of FFPE samples (Patient 16) stained for CD68 (**a**) and PD-L1 (**b**). Serial sections were used for **a** and **b**. *Scale bar* in **a** and **b** is 100 μm. **c** Double immunofluorescent staining of CD68 (*green*) and PD-L1 (*red*), counterstained with DAPI (*blue*). *Arrows* indicate colocalization of CD68 and PD-L1. *Scale bar* is 50 μm

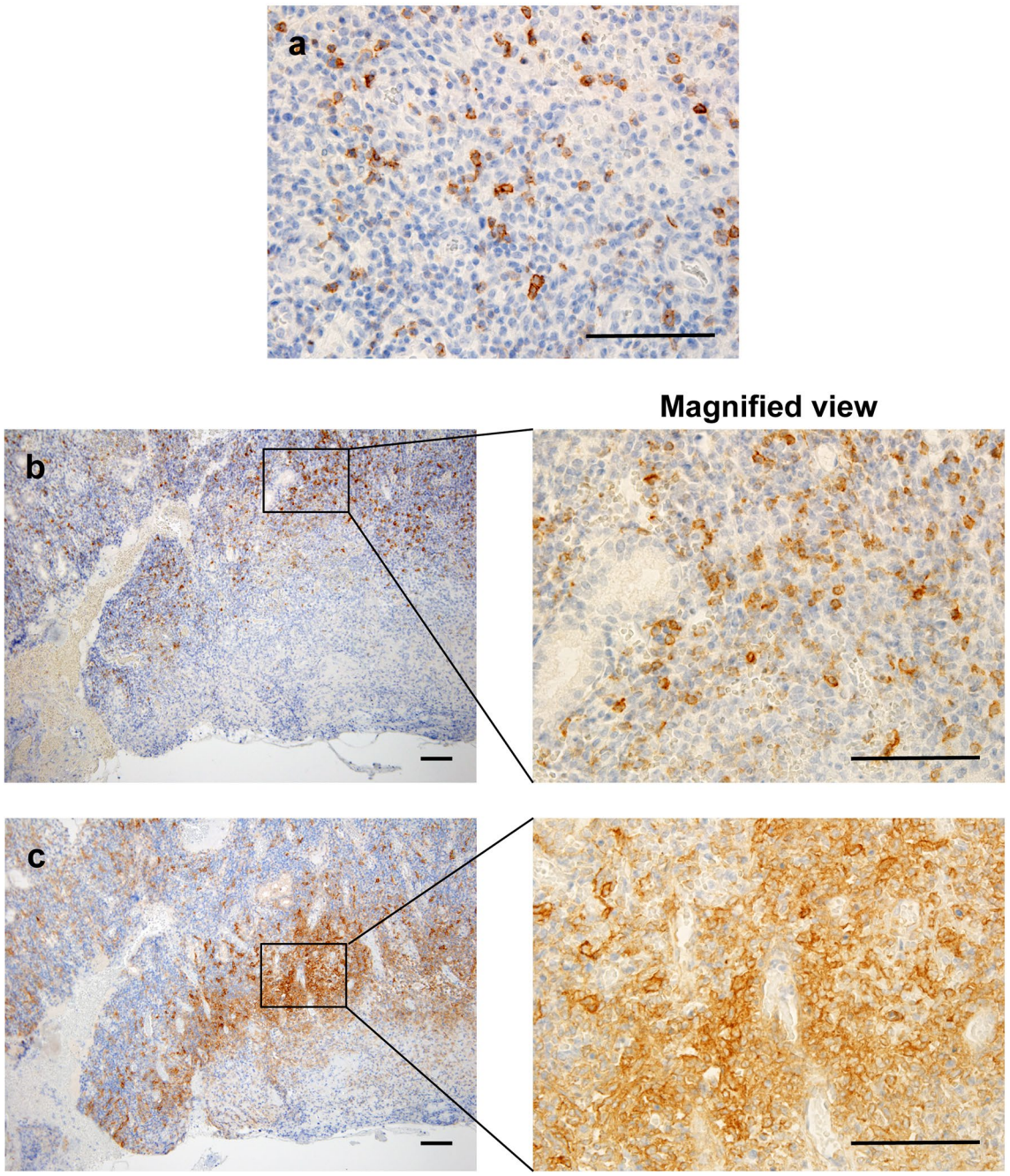


Fig. 3 Localization of PD-1 and PD-L1-positive cells in NNKTL tissues. Representative immunohistological features of NNKTL FFPE samples. **a** Staining for PD-1 (*brown*) (Patient 15). *Scale bar* is 100 μm. Original magnification, ×400. **b, c** Staining for PD-1 (**b**) and PD-L1 (**c**) was performed using serial slides (Patient 17). *Right panels* show enlargement of the *boxed areas* that are positive for PD-1 or PD-L1 staining. PD-1 or PD-L1-positive cells are stained *brown*. *Scale bar* is 100 μm. Original magnification, ×100 (*left*) and ×400 (*right*)

mainly infiltrated around PD-L1-positive cells (Fig. 3b, c, and Supplemental Fig. S1) including both CD56-positive lymphoma cells and CD68-positive macrophages, since these cells were present in the same regions of tumor tissue (Supplemental Fig. S2). To assess whether CD8-positive CTLs expressed PD-1, we performed immunohistochemistry for CD8 and PD-1 using serial slides from PD-1-positive patients. As shown in Supplemental Fig. S3, some of the CD8-positive cells were positive for PD-1, suggesting that PD-1-positive CD8 T cells were present in NNKTL tissues.

Elevated sPD-L1 levels are detectable in the sera of NNKTL patients and correlate with poor prognosis

We measured the concentration of sPD-L1 in the sera of NNKTL patients and healthy volunteers. As shown in Fig. 4a, the sPD-L1 serum level was significantly increased in NNKTL patients (mean ± SD = 850 ± 738 pg/mL; median = 639 pg/mL; range = 13-3041 pg/mL) as compared to healthy individuals (mean ± SD = 324 ± 296 pg/mL; median = 221 pg/mL; range = 13–872 pg/mL; $p = 0.0074$). The serum sPD-L1 levels in patients significantly correlated with the expression of PD-L1 in CD56-positive lymphoma cells ($p = 0.0141$, Fig. 4b). The correlation between the serum sPD-L1 level and the expression of PD-L1 in CD68-positive cells showed a trend toward statistical significance ($p = 0.0746$, Supplemental Fig. S4b), while statistical significance was not observed for correlations between the serum sPD-L1 level and the percentage of CD68-positive cells ($p = 0.9962$, Supplemental Fig. S4a). We further investigated whether the sPD-L1 level correlated with prognosis in NNKTL patients. Based on ROC curve analysis for survival, the optimal sPD-L1 cut-off value was 851 pg/mL, which was almost the same as the mean value of 850 pg/mL (Supplemental Fig. S5). We, therefore, separated the patients into two groups, sPD-L1 low and sPD-L1 high, using a cut-off value of 850 pg/mL. Patients in the low-sPD-L1 concentration group (<850 pg/mL) demonstrated a favorable clinical course with

a 5-year overall survival (OS) rate of 100%, whereas patients in the high concentration group (≥850 pg/mL) showed an unfavorable course with a 5-year OS rate of 57% (Fig. 4c). These results indicate a significant difference between survival rates of the two groups ($p = 0.0332$). OS according to the main clinical features was also investigated

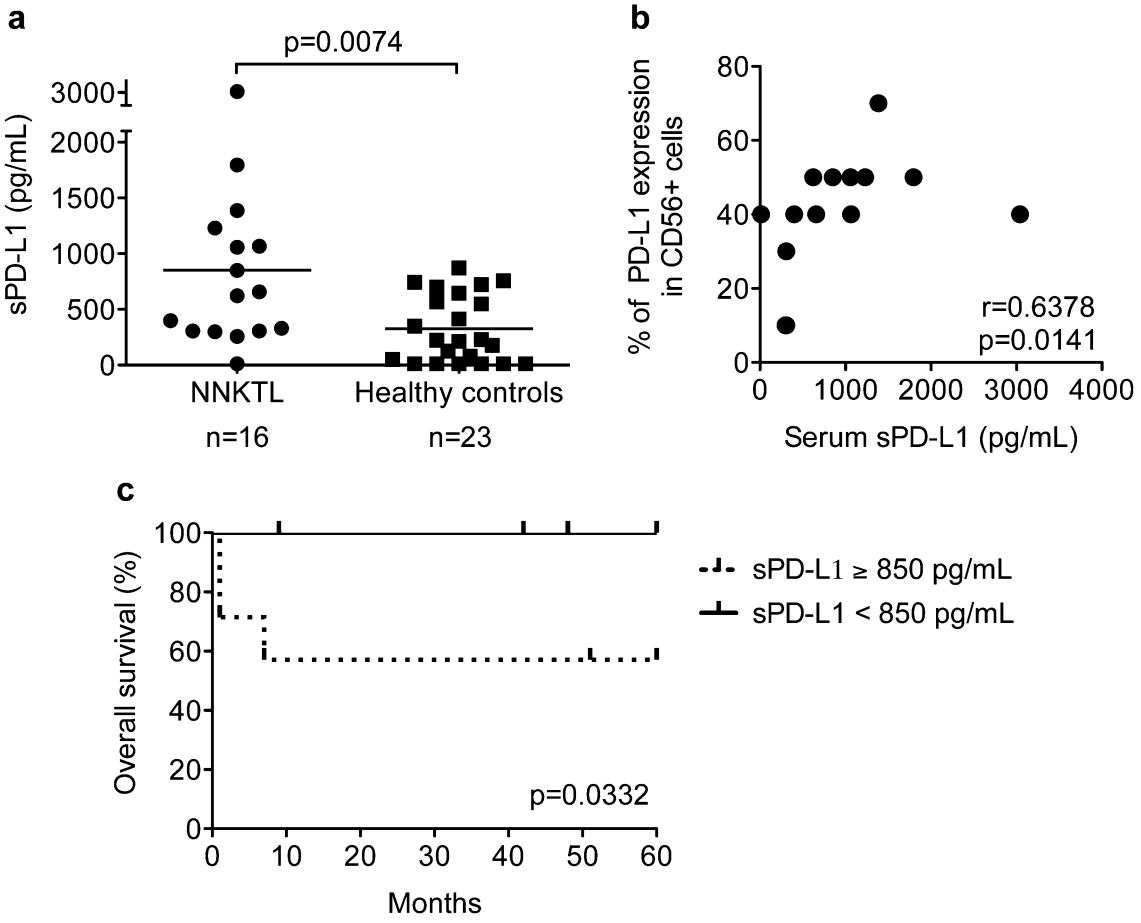
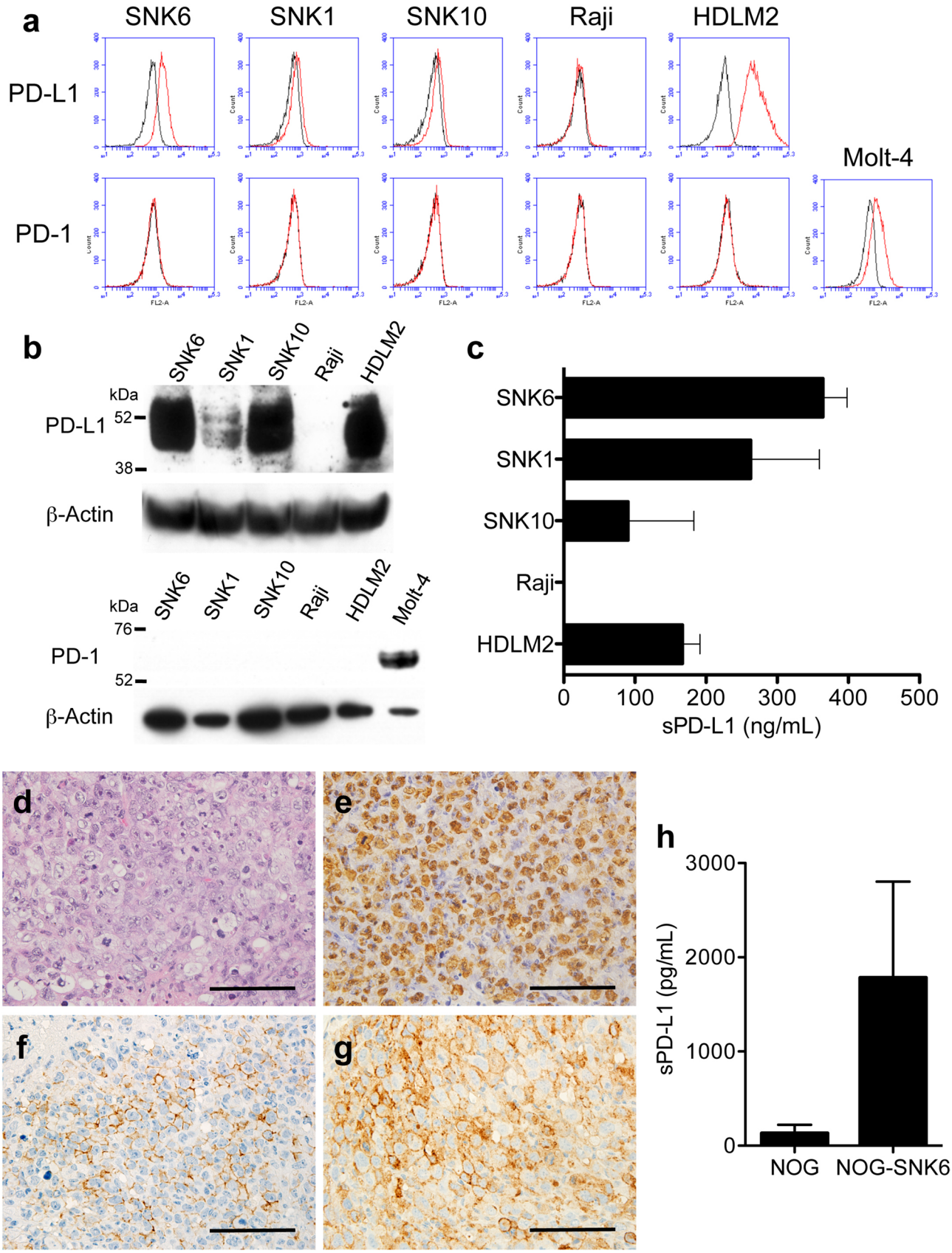


Fig. 4 Levels of sPD-L1 in sera and correlation with overall survival. **a** sPD-L1 levels in the sera from 16 NNKTL patients and 23 healthy controls were measured using ELISA. The *horizontal lines* indicate mean values. Statistical significance was determined using the Mann–Whitney *U* test. **b** Correlation between the levels of serum sPD-L1 and the expression levels of PD-L1 in CD56-positive cells of tumor tissues in NNKTL patients. Statistical significance was determined using the Spearman rank correlation. **c** Kaplan–Meier curves for overall survival of the 16 NNKTL patients. The high-sPD-L1 group ($n = 7$) showed significantly worse prognosis than the low-sPD-L1 group ($n = 9$). Statistical significance was determined using the log-rank test


a
SNK6
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b
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Raji
HDLM2
kDa
52
38
PD-L1
β-Actin
SNK6
SNK1
SNK10
Raji
HDLM2
Molt-4
kDa
76
52
PD-1
β-Actin
c
SNK6
SNK1
SNK10
Raji
HDLM2
0
100
200
300
400
500
sPD-L1 (ng/mL)
d
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2000
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sPD-L1 (pg/mL)
NOG
NOG-SNK6

◂**Fig. 5** Expression of PD-L1 in NNKTL cell lines and in tumor tissue and sera from a murine xenograft model inoculated with SNK-6 cells. **a** Flow cytometric analysis of the surface expression of PD-L1 or PD-1 in the indicated cell lines. Cells were stained with a PE-conjugated anti-PD-L1 or anti-PD-1 mAb (*red lines*). *Black lines*, cells stained with isotype control Ab. **b** PD-L1 or PD-1 expression in cell lines as assessed by western blotting. β-Actin was used to verify the amount of loading. **c** Supernatants from the indicated cell cultures (5×10^5/mL) in 96-well round-bottomed plates were collected after 24 h, and sPD-L1 production was assessed using ELISA. *Columns* means of triplicate determinations; *bars* SEM. In **a–c**, HDLM-2 and Raji cells were used as positive and negative controls, respectively, for the expression of PD-L1. Molt-4 cells were used as a positive control for the expression of PD-1. **d–g** Representative immunohistological features of FFPE tumor samples from a murine xenograft model. **d** H&E-stained section showing tumor tissue formed by highly atypical lymphoid cells. **e** ISH for EBER. Nuclei of EBER-positive cells are stained *brown*. **f** Staining for CD56. **g** Staining for PD-L1. *Scale bar* is 100 μm. **h** sPD-L1 levels in the sera from NOG mice inoculated with SNK-6 cells (NOG-SNK-6, $n=5$) and normal NOG mice (NOG, $n=4$) were measured using ELISA. *Columns* means; *bars* SEM

and patients in the late stage (IV), with a higher IPI, and with a higher PINK showed significantly shorter OS (Supplemental Fig. S6). We examined the correlation between the sPD-L1 level and the main clinical features of NNKTL patients; however, no significant correlation was observed (Supplemental Table S2).

NNKTL cell lines express membrane PD-L1 and produce sPD-L1

To confirm the above findings that were obtained using NNKTL patient materials, we next examined the expression of PD-L1 on the surface of the NNKTL cell lines, SNK-6 and SNK-1, using flow cytometric analysis. We also assayed the SNK-10 cell line, which was established from a patient with chronic active EBV infection (CAEBV) and is of EBV-positive NK-cell lineage, because some CAEBV patients develop from CAEBV to NNKTL with CAEBV. PD-L1 was clearly expressed on the surface of SNK-6, while a low level of expression of membrane PD-L1 was observed on the surface of SNK-1 and SNK-10 (Fig. 5a; Supplemental Fig. S7a). As reported previously [32, 37], HDLM-2 and Raji were positive and negative for the expression of PD-L1, respectively. In addition, we used flow cytometric analysis to investigate whether NNKTL and CAEBV cell lines also expressed PD-1; however, PD-1 was not detected on the surface of the cell lines except for Molt-4, which was used as a positive control for the expression of PD-1 (Fig. 5a). The expression of PD-L1 and PD-1 in cell lines was also assessed by western blot analysis. The PD-L1 protein was expressed in SNK-6, SNK-1, and SNK-10 cells, as well as in the positive control HDLM-2, but it was barely detected in the negative control Raji (Fig. 5b; Supplemental Fig. S7b). On the other hand, the PD-1 protein was not detected in SNK-6, SNK-1, SNK-10, Raji, or HDLM-2 cells, although it was clearly expressed in the positive control Molt-4 (Fig. 5b), confirming that PD-1 is not expressed in these NNKTL cell lines. We further measured the level of sPD-L1 in the supernatants from these cell lines using ELISA (Fig. 5c). sPD-L1 was detectable in the supernatants of SNK-6, SNK-1, SNK-10, and HDLM-2 cells, but was not detectable in the supernatant of the PD-L1 negative cell line Raji.

PD-L1 is detectable in tumor tissue and blood from a murine xenograft model inoculated with an NNKTL cell line

We then extended our investigation to a murine xenograft in vivo model, using NOG mice inoculated subcutaneously with SNK-6, to ascertain the importance of our in vitro findings. Tumor formation at the inoculated site was detectable in all of the NOG mice. Immunohistological staining was performed to analyze PD-L1 expression in FFPE tissue sections prepared from the subcutaneous tumor. Histologically, the tumor was formed by atypical lymphoid cells (Fig. 5d) that were positive for EBER and CD56 (Fig. 5e, f). These characteristics were similar to those of the malignant cells that form the tumor tissue of NNKTL patients. These neoplastic cells clearly expressed PD-L1 (Fig. 5g). Most significantly, high levels of sPD-L1 were detected in the sera from NOG mice inoculated with SNK-6 as compared with the levels in normal NOG mice (Fig. 5h). These results suggest that not only cultured SNK-6 cells but also the SNK-6 tumor in the in vivo xenograft model could express membrane PD-L1 and secrete sPD-L1.

Discussion

In the present study, using serial tissue sections and double immunofluorescent staining, we clearly demonstrated that PD-L1 was expressed by lymphoma cells in the tissue samples from all of the 15 NNKTL patients examined. Chen and colleagues previously reported that the malignant cells of 4 of 6 NNKTL cases (66.7%) were immunohistochemically positive for PD-L1 expression [32]. In recent reports, Kim et al. [38] and Jo et al. [39] further indicated that PD-L1 expression in lymphoma cells was positive in 56.2 and 79.7% of cases, respectively, using immunohistochemistry. Our results and the data of other researchers, therefore, suggest that the majority of NNKTL cases highly express PD-L1 and that the PD-1/PD-L1 pathway might play an important role in immune escape of NNKTL from CTL recognition. However, it is controversial whether PD-L1 expression of lymphoma cells in NNKTL tissues associates with prognosis. In a study of 79 NNKTL biopsy

samples, Jo et al. [39] found that PD-L1 positivity in lymphoma cells was not significantly associated with OS. On the other hand, in a study of 73 NNKTL patients, Kim and colleagues [38] found that patients with PD-L1-positive tumors exhibited significantly better OS than patients with PD-L1-negative tumors, although PD-L1 positivity was not significantly correlated with clinicopathological variables. Thus, further studies of tissue samples from NNKTL are needed to clarify the correlation between tissue PD-L1 expression and prognosis. We further found that CD68-positive macrophages abundantly infiltrated NNKTL tissues and that these cells also expressed PD-L1. Other studies have indicated that tumor-infiltrating immune cells expressed PD-L1 in 62–79% of NNKTL cases [38, 39]. Kim et al. reported that these cells morphologically resembled macrophages, although they did not confirm the expression of macrophage markers using serial sections or double-staining methods [38]. These results including ours are similar to those of recent studies on Hodgkin's lymphoma and diffuse large B-cell lymphoma [32, 33]. Because interferon (IFN)-γ is secreted by NNKTL cells [9, 10] and is known to induce PD-L1 expression on macrophages [40], NNKTL might utilize the PD-L1/PD-1 pathway to inhibit immune suppression via two distinct mechanisms: direct inhibition by PD-L1 expression on tumor cells and indirect inhibition by induction of PD-L1 expression on macrophages via IFN-γ production.

To confirm PD-L1 expression on NNKTL tumor cells, we investigated PD-L1 expression in NNKTL cell lines. We found that PD-L1 is highly expressed in the SNK-6 cell line, which was established from a primary NNKTL lesion, and this result is consistent with that of a recent report [41]. We further demonstrated that not only SNK-6 cells cultured in vitro but also SNK-6 cells inoculated into NOG mice could produce PD-L1, suggesting that SNK-6 cells could maintain the expression of PD-L1 in an in vivo mouse model. We also detected the expression of PD-L1 in SNK-1 and SNK-10 cells, which were isolated from the peripheral blood of NNKTL and CAEBV patients, respectively, although these cell lines weakly expressed cell surface PD-L1 compared with SNK-6 cells. Other studies have indicated that loss of PD-L1 expression could occur during the establishment or the serial passage of human cell lines [37]. Future studies will be required to clarify the reason why there is a difference in the PD-L1 expression level between the cell lines cultured in vitro.

Several recent studies have reported the detection of high concentration levels of sPD-L1 in blood and their association with poor prognosis in some malignancies [17–19]. Because we observed a high number of PD-L1-expressing cells in NNKTL tissues, we speculated that sPD-L1 might be secreted by these cells and, therefore, that higher levels of sPD-L1 might be detected in the blood of NNKTL patients compared to healthy controls. As expected, elevated sPD-L1 levels were detected in the sera of NNKTL patients. Furthermore, the correlation of serum sPD-L1 levels with PD-L1 expression in tissue lymphoma cells and in tumor-infiltrating macrophages was statistically significant and showed a trend towards statistical significance, respectively, and higher sPD-L1 levels were correlated with poor prognosis. In general, our findings correspond with the results reported by Bi et al. [41]. Those authors reported that the concentration of serum sPD-L1 in NNKTL patients with stage I and II was significantly higher than that in healthy controls and positively correlated with the PD-L1 expression level in tumor tissues. They also showed that patients in the high-sPD-L1 group exhibited a significantly worse OS compared to patients in the low-sPD-L1 group. These results presented by Bi et al. and by us suggest that sPD-L1 may be a valuable predictor for prognosis in NNKTL. However, in this study, it should be taken into consideration that all 3 patients who died in the high-sPD-L1 group were late-stage cases that showed significantly shorter OS than early stage cases (Supplemental Fig. S6). In addition, the correlation between the sPD-L1 level and stage showed a trend towards statistical significance ($p = 0.0625$, Supplemental Table S2). Thus, we cannot rule out the possibility that late-stage might have affected the unfavorable course in the high-sPD-L1 group. We intend to investigate further NNKTL samples including late-stage samples as they become available in the future to address the association between the sPD-L1 level and clinical stage. Although the sources of sPD-L1 remain unknown, it is possible that either tumor cells and/or tumor-infiltrating immune cells could produce sPD-L1. We demonstrated that sPD-L1 levels detected in the sera from SNK-6-bearing NOG mice were elevated compared to those of normal mice, which means that the main source of sPD-L1 in the blood of this mouse model was the inoculated tumor cells. This result suggests that tumor cells are one of the sources of circulating sPD-L1 in the blood of patients with NNKTL, although CD68-positive macrophages might also be able to produce sPD-L1. It has been reported that sPD-L1 that is secreted into the supernatant by tumor cell lines or that is circulating in the sera maintains the ability to bind to the PD-1 receptor [42] and that sPD-L1 from culture supernatants can deliver immunosuppressive signals to T cells [16]. Because high-sPD-L1 levels correlate with poor prognosis in malignant diseases, sPD-L1, similar to membrane PD-L1, might induce immunosuppressive activity through binding to PD-1.

Immunohistochemical analysis using serial sections of NNKTL tissues detected PD-1-positive cells in the tissues of 5 of 14 patients (36%) and these cells were mainly localized around PD-L1-positive lymphoma cells and macrophages. Furthermore, PD-1 was not expressed in

NNKTL cell lines. These results suggest that it is infiltrating immune cells including lymphocytes and not lymphoma cells that express PD-1 in NNKTL. We also demonstrated that some of the tumor-infiltrating CD8-positive cells expressed PD-1, indicating that PD-1-positive CD8 T cells are present in NNKTL tissues, although it is possible that other immune cells including CD4 T cells also express PD-1. The association between PD-1 expression on infiltrating lymphocytes and prognosis is controversial. It has been reported that PD-1 expression on infiltrating lymphocytes was correlated with poor prognosis in renal cell carcinoma [34], Hodgkin's lymphoma [43], and nasopharyngeal carcinoma [44]. On the other hand, other studies have shown that the expression of PD-1 on infiltrating T cells was associated with better survival in follicular lymphoma [35] and human papillomavirus-associated head and neck cancer [45]. Therefore, further study with a greater sample size is required to identify the origin of the PD-1-expressing cells and to evaluate the clinical relevance of PD-1 expression in NNKTL.

In conclusion, our results show that PD-L1 is expressed on malignant cells and tumor-infiltrating macrophages in NNKTL tissues. More importantly, increased sPD-L1 levels were detectable in the sera of patients and correlated with poor prognosis. The expression of PD-L1 was observed in tumor tissues and sera from a murine xenograft model inoculated with an NNKTL cell line, suggesting that tumor cells are one of the sources of circulating sPD-L1 in blood. These findings indicate that a high-sPD-L1 level is a possible valuable predictor of poor prognosis and that the PD-1/PD-L1 pathway may play a crucial role in the escape of NNKTL from the antitumor immune response. We believe that immunotherapy with mAbs targeting PD-1 and PD-L1 could benefit NNKTL patients.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflict of interest.

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