

Characterization of PD-1/PD-L1 immune checkpoint expression in the pathogenesis of musculoskeletal Langerhans cell histiocytosis

A retrospective study

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Abstract

Recent data suggest that programmed cell death -1 (PD-1) and programmed cell death ligand-1 (PD-L1) are involved in the pathogenesis of Langerhans cell histiocytoma (LCH); however, their contributions are not well established. Also, the involvement of PD-1/PD-L1 molecules in musculoskeletal LCH remains particularly unclear. The current study aims to characterize the involvement of PD-1/PD-L1 immune checkpoint system in the pathogenesis of musculoskeletal LCH. PD-1/PD-L1 expression was evaluated in 6 patients, 3 men and 3 women with a mean age of 13.5 years, with musculoskeletal LCH who were treated at Kindai University Hospital and Osaka Women's and Children's Hospital between November 2005 and December 2020. The median follow-up period for all patients with musculoskeletal LCH was 41 months. We surveyed symptoms, number of lesions, treatment modality, and outcomes. Immunostaining for CD4, CD8, PD-1, and PD-L1 was also performed on pathological specimens obtained by biopsy. Multiple lesions were observed in 5 cases, and a single lesion was observed in 1 case. The chief complaint in 5 cases was pain. Four patients underwent spontaneous regression. The other 2 patients received chemotherapy. The outcomes included continuous disease-free (n=5) and alive with the disease (n=1). The CD4-, CD8-, PD-1-, and PD-L1-positive rates among all specimens were 100%, 100%, 16.6%, and 83.3%, respectively. The CD4/PD-L1, CD8/PD-L1, and PD-1/PD-L1 positive rates in all the specimens were 83.3%, 83.3%, and 16.6%, respectively. We believe that the PD-1/PD-L1 immune checkpoint molecules may play some role in the microenvironment of musculoskeletal LCH.

Abbreviations: LCH = Langerhans cell histiocytoma, PD-1 = programmed cell death -1, PD-L1 = programmed cell death ligand-1.

Keywords: immunohistochemistry, Langerhans cell histiocytoma, musculoskeletal system, programmed cell death -1, programmed cell death ligand-1

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This report complies with the Declaration of Helsinki. This study was also approved by the Ethics Committee of Kindai University Hospital (Approval number: 31-187, approved on January 16, 2020, Osaka, Japan).

Written informed consent has been obtained from the patient to publish this paper.

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The datasets generated during and/or analyzed during the current study are publicly available.

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1. Introduction

Langerhans cell histiocytosis (LCH) is a rare systemic disorder characterized by the accumulation of CD1a+/Langerin+ LCH cells.^[1] These LCH cells originate from myeloid dendritic cells rather than skin Langerhans cells.^[2] The annual incidence in children is about 5 cases per 1 million population.^[3] The male:female ratio is 1.2:1.^[3] The annual incidence in adults is 1 to 2 cases per 1 million population.^[3] The clinical manifestation of LCH varies from a single organ disease that can spontaneously undergo remission, to a systemic and aggressive disease that can lead to death.^[3] Thus, the pathogenesis of LCH is complex and remains unclear. A recent study has shown that immune checkpoint inhibitors are used to alleviate the immunosuppressive state of the tumor microenvironment in malignant neoplasms, such as melanoma, non-small cell lung cancer, and colon cancer by restoring the immune function of T cells and killing tumor cells.^[4] There are various genes related to the programmed cell death -1(PD-1)/ programmed cell death ligand-1 (PD-L1) immune checkpoint mechanism, but the main pathway is through T cells.^[5] In particular, the CD4/CD8-mediated pathway has been attracting attention in recent years, so we attempted to investigate CD4/CD8 in this study as well.^[6] Interestingly, a recent study showed PD-L1 to be expressed in histiocytic and dendritic cell disorders, however, there is insufficient evidence for the expression of PD-1/PD-L1 immune checkpoint molecule in

LCH.^[7] Moreover, the involvement of PD-1/PD-L1 in the pathogenesis of musculoskeletal LCH remains unclear. Herein, we investigated the expression of PD-1/PD-L1 immune checkpoint molecules including CD4/CD8 molecules in musculoskeletal LCH by using immunohistochemistry and characterized the involvement of the PD-1/PD-L1 immune checkpoint system in the pathogenesis of LCH. Additionally, this is the first report suggesting the involvement of PD-1/PD-L1 immune checkpoint system in the pathogenesis of musculoskeletal LCH.

2. Material and methods

We retrospectively reviewed 6 patients with musculoskeletal LCH treated at Kindai University Hospital and Osaka Women's and Children's Hospital between November 2005 and December 2020. The exclusion criterion was set as patients whose clinical course could not be followed. Informed consent was obtained from the study participants. The study was approved by the Ethics Committee of Kindai University Hospital (Approval number: 31-187; Approved on January 16, 2020).

The median follow-up period for all patients with musculoskeletal LCH was 41 (range, 4–73) months. Age, sex, site of origin, symptoms, treatment, and final clinical outcomes were studied. Antibodies against CD1a, S100, and langerin were used for immunohistochemical analyses to determine the immunophenotype of musculoskeletal LCH samples, as previously described.^[8]

Immunostaining was performed with specimens obtained using needle biopsies from patients with musculoskeletal LCH treated at Kindai University Hospital and Osaka Women's and Children's Hospital. Tissues were deparaffinized, rehydrated, and subjected to antigen retrieval using 3% hydrogen peroxide. The sections were incubated with the following primary antibodies: CD4 antibody (rabbit monoclonal, ab133616; Abcam) for 32 minutes at 37°C after 60 minutes of high pH (9.0) heat activation; CD8 antibody (rabbit monoclonal, Product No. 05298709001; Roche) for 32 minutes at 37°C after 60 minutes of high pH (9.0) heat activation; PD-1 (mouse monoclonal, ab52587; Abcam, Cambridge, UK) for 30 minutes

at 37°C after 30 minutes of low pH (6.0) heat activation; and PD-L1 antibody (rabbit monoclonal, ab205921; Abcam) for 32 minutes at 37°C after 60 minutes of high pH (9.0) heat activation. Sections were incubated with the corresponding secondary antibodies (for CD4, CD8, and PD-L1: Goat anti-rabbit IgG antibody (Cosmo Bio; SA00001–2) and for PD-1: Goat anti-mouse IgG antibody (Cosmo Bio; SA00001–1) for 30 minutes at 37°C. The reaction was visualized using 33'-diaminobenzidine (DAB; DAB Substrate Chromogen System; DAKO, Kyoto, Japan, Code K3467) and counterstained with hematoxylin. Slides were observed under a microscope (BIOREVO BZ-9000; KEYENCE, Osaka, Japan), and brown granules in the cytoplasm or nuclei indicated positive staining. Samples showing presence of CD4, CD8, and PD-1–positive tumor infiltrating lymphocytes were considered positive. We used just secondary antibodies as negative controls. The immune marker staining within the tumor was quantified in 4 representative high-power fields (40x magnification).^[9] The positivity rate was defined as the number of positive cells/total cell number. The positivity rate was quantified using the software provided with BIOREVO-BZ 9000 (Keyence),^[9] and staining >5% of total cells was considered positive. Positive staining rates for each immunostaining target (CD4, CD8, PD-1, and PD-L1) were calculated. Adjacent sections stained positive for CD4/PD-L1, CD8/PD-L1, and PD1/PD-L1 were considered co-positive. The co-staining rates for each immune molecule were also examined. The sample was evaluated by 3 observers (K.H., S.N., M.A) to exclude observation bias.

3. Results

The demographic and clinicopathological characteristics of the 6 patients with musculoskeletal LCH are summarized in Table 1. The cohort consisted of 3 men and 3 women with a mean age of 13.5 (range, 2–60) years. There were 5 cases with multiple lesions and 1 case with a single lesion. Lesions in the trunk such as a rib or lumbar vertebrae were observed in all 3 adults older than 20 years. In contrast, pediatric cases had lesions in the extremities. Five patients were symptomatic at the time of their first visit, while one was asymptomatic. Of the cases with symptoms, pain

Table 1
Characteristics of patients with musculoskeletal Langerhans cell histiocytoma.

Patient No.	Age	Sex	Site	Symptoms	Treatment	Outcome	Follow-up periods (Months)	CD4	CD8	PD-1	PD-L1
								+Lymphocytes positivity			
1	24	F	rt Tibia, left 7th rib	Pain and swelling	Spontaneous regression	CDF	13		+	–	+
								+	+	–	–
2	60	M	2nd, 3rd, 4th, 5th lumbar, Th10, rt 4th rib, sacrum, pelvic	without symptoms	Chemotherapy (6-mercaptopurine 110 mg, MTX 2.5 mg, PSL 5 mg)	CDF	63	+	+	–	+
								+	+	–	+
3	30	F	Rt 4th rib	Pain	Spontaneous regression	AWD	4	+	+	–	–
								+	+	–	–
4	2	F	Femur, 8th thoracic vertebra	Pain and claudication	Spontaneous regression	CDF	27	+	+	+	+
								+	+	–	+
5	2	M	Femur, Temporal bone, anterior mediastinum	Pain and claudication	Chemotherapy	CDF	55	+	+	–	+
								+	+	–	–
6	3	M	Elbow	Pain	Spontaneous regression	CDF	73	+	+	–	+
								–	+	–	–

CDF = continuous disease-free, AWD = alive with disease, rt = right, MTX = methotrexate, PSL = prednisolone.

was the most common complaint. Claudication was a common complaint in patients with limb-onset. Four patients underwent imaging follow-up, and 2 patients were treated with chemotherapy. The final clinical outcome was continuous disease-free in 5 patients and alive with disease in 1 patient. Tissue samples of all 6 cases stained positive for S100, CD1a, and langerin (Fig. 1A–C). Further, the overall immunohistochemistry positive staining rates were 6/6 (100%) for CD4, 6/6 (100%) for CD8, 1/6 (16.6%) for PD-1, and 5/6 (83.3%) for PD-L1. Representative positive histological findings are shown in (Fig. 2A–D). The CD4-positive and PD-L1-positive co-staining rates were 5/6 (83.3%). The CD8-positive and PD-L1-positive co-staining rates were 5/6 (83.3%). The PD-1 and PD-L1 concordance rates were 1/6 (16.6%). Interestingly, CD4 (5/6), CD8 (6/6), PD-1 (0/6), and PD-L1 (2/6) positivity were also observed in lymphocytes (Table 1, Fig. 3A and B).

4. Discussion

The involvement and mechanism of the PD-1/PD-L1 immune checkpoint system in the pathogenesis of LCH remains unclear. Here, we investigated the expression of PD-1/PD-L1 immune checkpoint molecules, including CD4/CD8 components, in musculoskeletal LCH using immunohistochemistry.

A previous study described PD-L1 positive rate of 5% and PD-1 positive rate of 5% to 20% in pulmonary LCH^[8]; and, another found a PD-L1 positive rate of 88% in LCH.^[10] In the current

study, 1/5 (16.6%) positive rate for PD-1 and 5/6 (83.3%) positive rate for PD-L1 were observed. Moreover, the positive rate of CD4/CD8 immune components was high. Therefore, these findings indicate that the PD-1/PD-L1 immune checkpoint system is involved in the pathogenesis of musculoskeletal LCH.

Previous studies have shown a predominance of M2 macrophages, T cells, and regulatory T cells, as well as increased expression of programmed death ligand 1 (PD-L1) in LCH cells.^[10–13] It has also been reported that there is a bias towards CD4+ T cells and regulatory T cells compared to CD8+ T cells. Both CD4+ and CD8+ T cells show increased expression of inhibitor receptors such as PD-1, TIM-3, and LAG-3; decreased cytokine production upon stimulation; and reduced effector function. It has been reported that T cells showed signs of exhaustion, such as decreased cytokine production upon stimulation and decreased effector function.^[10–14]

In the present study, tumor cells and lymphocytes were positive for PD-1/PD-L1 immune checkpoint mechanism molecules; there was no significant difference in the positivity of CD4 and CD8. These findings indicate that the same quantity of CD4 and CD8 are activated in the pathogenesis of musculoskeletal LCH.

Tumor infiltrating CD4+ or CD8+ T cells play an important role in regulating the tumor microenvironment in some cancer types.^[15,16] Some studies also showed that the tumor-infiltrating T cells may serve as indicators of prognosis or therapeutic targets.^[17–19] In the current study, we observed infiltration of CD4+ or CD8+ T cells in all cases. These findings suggest that

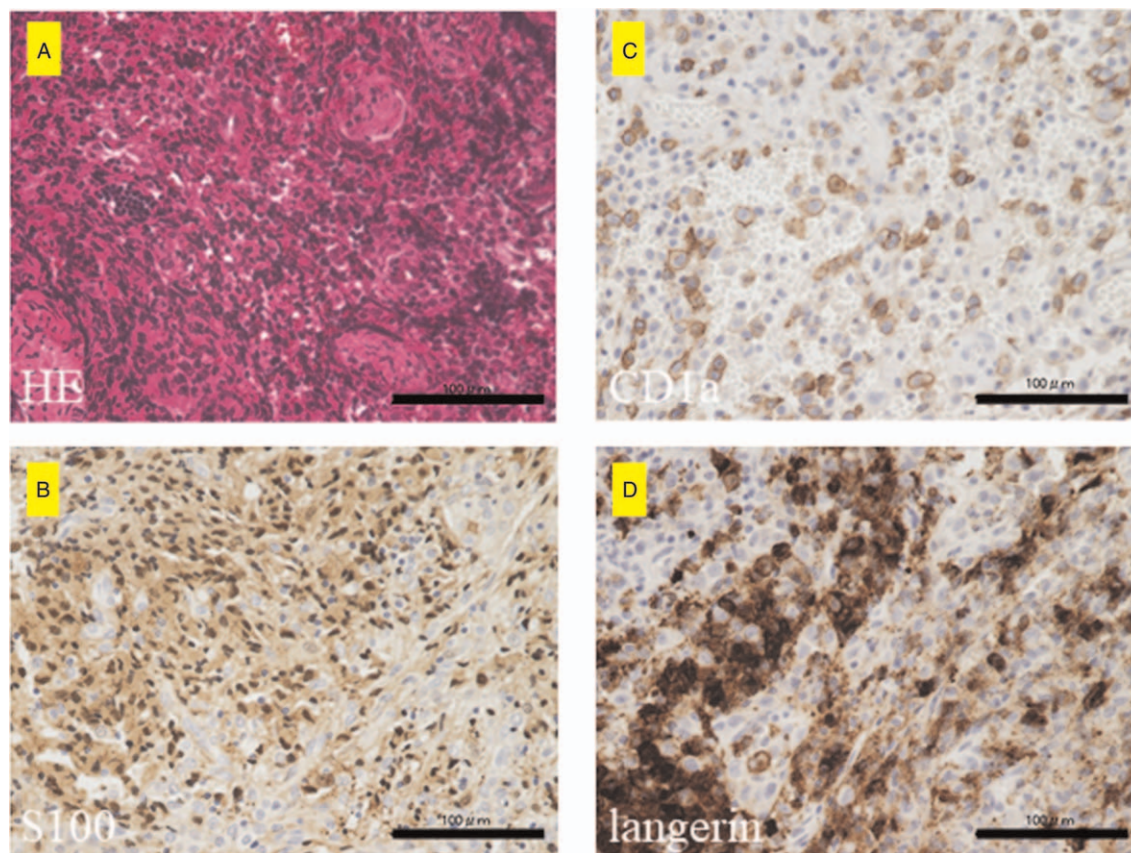


Figure 1. Immunohistochemical analyses of S100, CD1a, and langerin in Langerhans cell histiocytosis (LCH) samples. (A) Hematoxylin-Eosin (HE) staining of the representative LCH sample. Proliferation of Langerhans cells is observed. (B–D) Immunohistochemical staining for S100 (B), CD1a (C), and langerin (D). (B–D) indicate S100-, CD1a-, and langerin-positive cells, respectively. Scale bar = 100 μm.

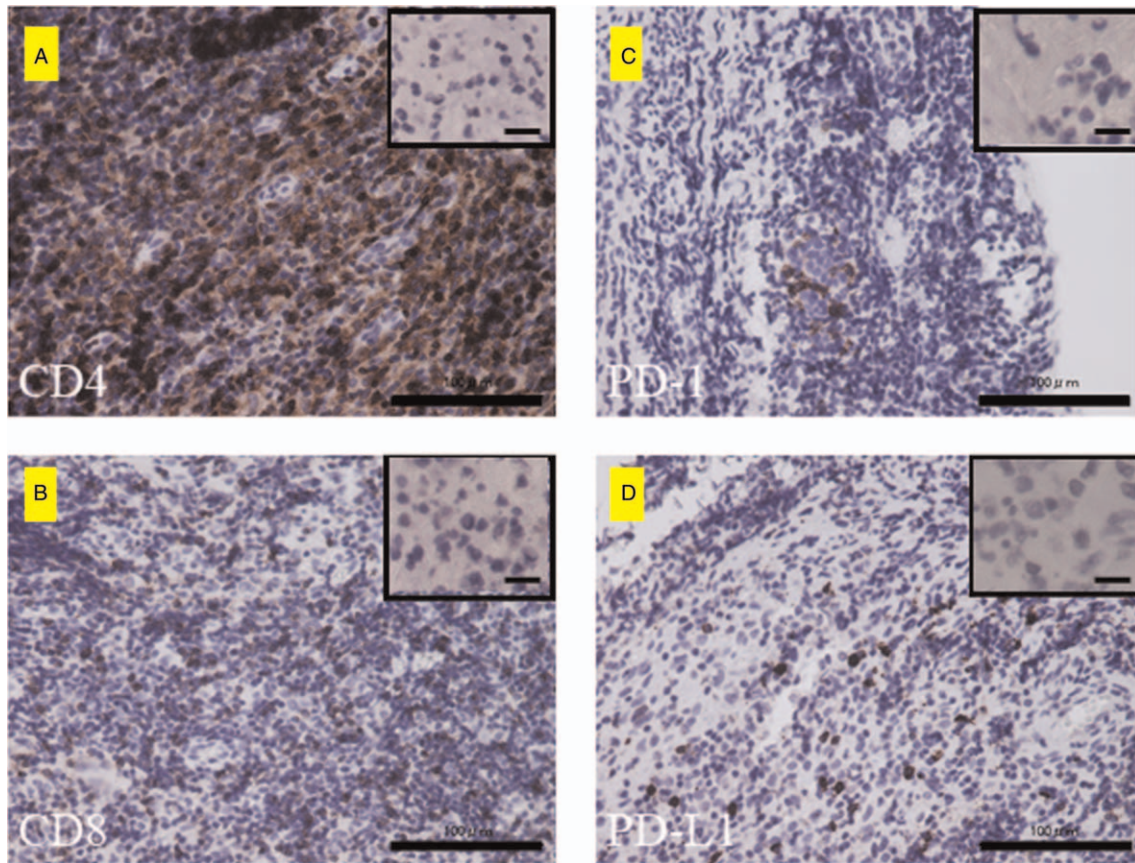


Figure 2. The histological findings of immunohistochemistry for CD4, CD8, PD-1, and PD-L1. (A–C) indicate CD4-, CD8-, and PD-1-positive lymphocytes, respectively. (D) PD-L1-positive tumor cells. Scale bar = 100 μm . The images in the upper right frame are the negative control in each (Scale bar = 10 μm). The CD4-positive and PD-L1-positive co-staining rates were 5/6 (83.3%). The CD8-positive and PD-L1-positive co-staining rates were 5/6 (83.3%). The PD-1 and PD-L1 concordance rates were 1/6 (16.6%). Interestingly, PD-L1 positivity was observed not only in the tumor cells but also in lymphocytes (Figure 3A and 3B).

infiltrating CD4+ or CD8+ T cells play an important role in the microenvironment of musculoskeletal LCH.

There is a significant debate for defining LCH either as immune disorder or neoplasm.^[20] A previous study showed that only one case of pulmonary LCH and 2 cases of extrapulmonary LCH expressed PD-1, suggesting PD-1 ligand-mediated immune escape to be an unlikely mechanism of disease progression.^[7] Here,

83.3% of the LCH specimens expressed PD-L1. Moreover, PD-L1 is typically expressed in tumor cells.^[21] Therefore, these findings support the classification of LCH as a neoplasm.

Major advances have been made in the characterization of the tumor microenvironment of soft tissue sarcoma, as “hot tumors” massively infiltrated by immune cells and “cold tumors” with no significant immune infiltration.^[22] Moreover, Petitprez et al

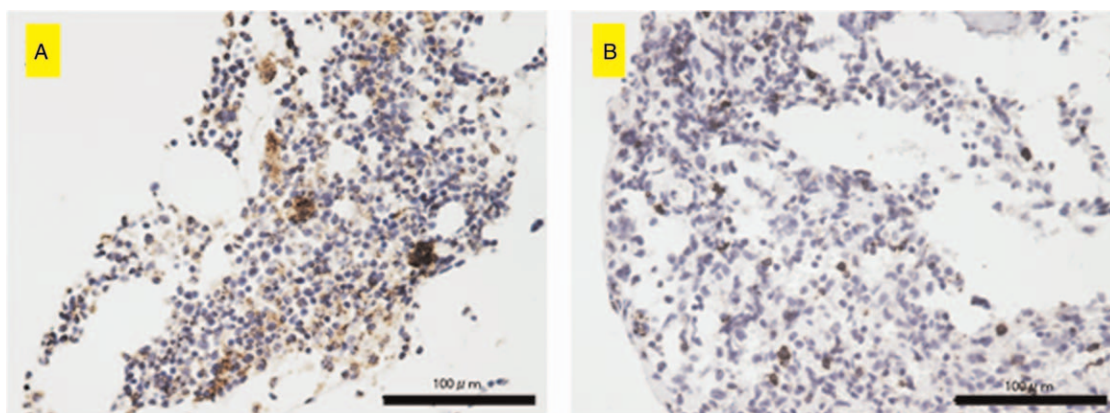


Figure 3. Immunohistochemical staining for PD-L1. The PD-L1-positive tumor cells are observed in (A) and positive lymphocytes are observed in (B). Scale bar = 100 μm .

established an immune-based classification system using the composition of the tumor microenvironment and identified 5 distinct phenotypes: immune-low (A and B), immune-high (D and E), and highly vascularized (C).^[22] The report also indicated that the class-E group demonstrated improved survival and high response rate to PD-1 blockade with pembrolizumab in a phase 2 clinical trial.^[22] In the present study, the LCH specimens expressed PD-1(1/6: 16.6%)/PD-L1 (5/6: 83.3%) immune checkpoint molecule. Therefore, musculoskeletal LCH can be classified as an immune-high (D or E) tumor.

In general, LCH is a benign tumor and shows favorable prognosis.^[2,3] However, some LCH cases have aggressive clinical features, as previously described.^[23] Here, 5/6 cases were continuous disease-free and 1/6 cases were alive with disease at the final follow-up. Although no case with an aggressive course was observed in this study, careful follow-up is necessary.

This study has some limitations. First, only 6 patients were enrolled in this study, which may have introduced bias into the analysis of the relationship between PD-L1 and musculoskeletal LCH. Second, this study is not a validation cohort. Therefore, we think it would be better to consider enrolling more patients into the study to reduce bias, or isolating cells from patients' tissues to confirm that blocking PD1/PD-L1 signaling is beneficial in the treatment of musculoskeletal LCH. Alternatively, a cohort of similar studies could be sought and analyzed to confirm the results. These will be the subjects of future research.

Third, BRAF mutations specific to LCH^[8,9] were not investigated. Fourth, we could not investigate the use of anti-PD-1 or anti-PD-L1 drugs in these patients because it was a retrospective study. However, despite these limitations, the study findings clearly indicate the involvement of PD-1/PD-L1 immune checkpoint molecules in the pathogenesis of musculoskeletal LCH. Nevertheless, we will attempt to overcome these limitations in future studies.

5. Conclusion

We suggest in this study that musculoskeletal LCH may be a highly immunocompetent neoplasm involving the immune checkpoint molecule, PD-1/PD-L1.

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