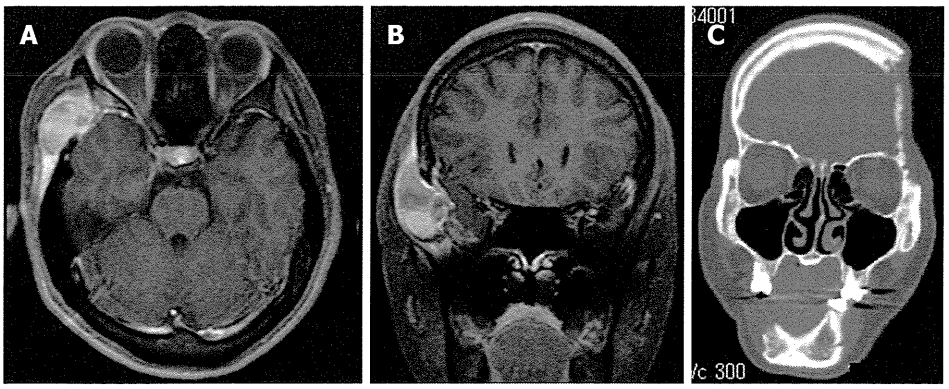

Figure 4 Langerhans cell histiocytosis skull mass (A, B) and lytic skull (C) lesion. A, B: Gadolinium-enhanced magnetic resonance imaging (T1W1) shows a mass with heterodensity at the right temporal area. axial view (A), coronal view (B); C: Computed tomography scan shows extensive lytic bones, at the left temporal bone and mandible. Defect of the right mandible is due to surgical resection.

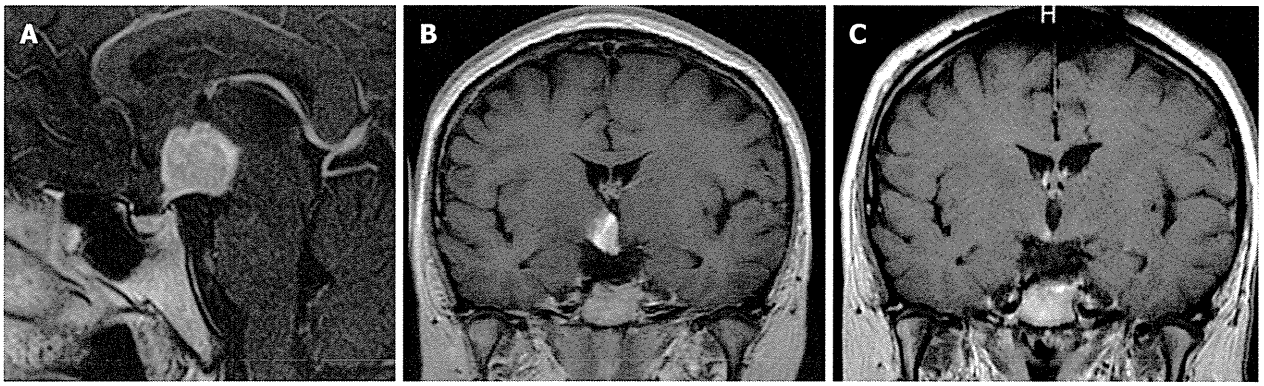

Figure 5 Langerhans cell histiocytosis mass at the hypothalamic area (A) and hypothalamic–pituitary area, comparison of pre and post chemotherapy (B, C). A: Gadolinium-enhanced magnetic resonance imaging (W1T1) shows a large mass at the hypothalamic area. Pituitary stalk is enlarged; B, C: Gadolinium-enhanced magnetic resonance imaging (T1W1) shows (B) a large mass before chemotherapy and (C) a residual mass post chemotherapy. The size of mass was significantly reduced after 2-deoxychloroadenosine treatment.

biopsy. Re-institution of systemic chemotherapy significantly (> 50%) reduced the size of hypothalamic mass. The patient has currently been in a state of ASAD.

Case 14

A 36-year-old female developed CDI in association with amenorrhea. However, the thickened pituitary stalk detected by MRI was put on under observation and not immediately treated. After progression of the thickened pituitary stalk into a significant Gd-enhanced mass at the HPR, a biopsy was performed to reveal typical LCH histology, when the patient had amenorrhea, fatty liver, reduced glucose tolerance. Systemic chemotherapy with 2CDA significantly (> 50%) reduced the mass size (Figure 5B and C). LCH lesions outside the CNS were not found. The patient remains markedly obese and diabetic, with residual active disease.

Case 15

A 38-year-old male was first diagnosed with primary hypothyroidism. Nineteen months later, he developed the symptoms of CDI, fatigue and disturbed consciousness along with disorientation and abnormal behaviors. A brain MRI revealed a Gd-enhanced mass at the HPR, which biopsy did not confirm the diagnosis of LCH. Two years later, osteolytic bone lesions appeared on the right femur and left clavicle, when the LCH was eventually diagnosed by a bone biopsy. The patient received systemic chemotherapy with 2CDA, but the mass size was reduced minimally (< 50%), and he remained significantly obese and diabetic. Four years from the initial CDI symptom, the patient developed retropharyngeal B-cell lymphoma. He has had active diseases of LCH as well as lymphoma.

Case 16

A 46-year-old male first presented with decreased libido and erectile dysfunction seven years after total gastrectomy for gastric adenocarcinoma. Four years later, a Gd-enhanced mass at the HPR was detected. Until then, he had ignored his polydipsia/polyuria symptoms, thus the diagnosis of CDI was delayed. Biopsy of the CNS mass led to a diagnosis of LCH. A systemic MRI survey also revealed multiple spinal involvements. The patient also had loss of concentration and short term memory deficits suggesting mild neurodegenerative disease, which findings were confirmed with brain MRI examination. Eventually, the patients received systemic chemotherapy

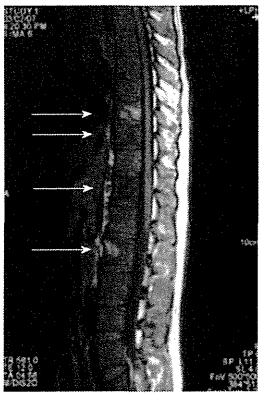

Figure 6 Langerhans cell histiocytosis spinal lesions. Non-enhanced magnetic resonance imaging (T1W1) shows high and low signals at multiple vertebral bodies (arrows).

with 2CDA, which markedly (> 50%) reduced the CNS mass size. However, currently, he has been treated for the regrowth of the CNS mass.

Case 17

A 23-year-old young adult was noted to have cerebellar ataxia and dysarthria. Past history revealed that at age of 16, he had been diagnosed as CDI; however, the exact cause was unidentified. At age 20, he was found to have polycystic lung disease with pneumothorax, followed by a mild ataxia. At age 23, he suffered a traffic accident when he was incidentally found to have a brain disease with a mass at the HPR as well as neurodegenerative disease on MRI performed at the emergency hospital. Eventually, LCH was diagnosed from the biopsy of lung tissues. He received monthly intravenous immunoglobulin therapy for neurodegenerative disease with dexamethasone[10]; however, the patient declined further treatment. Currently, he has remained with progressive neurological symptoms and with active lung disease.

Case 18

An 18-year-old man initially complained of low back pain and cervical mass. MRI revealed multiple spinal bone involvement (Figure 6). The initial diagnosis of LCH was made from the histology obtained by excisional iliac biopsy. A year later, he developed swelling of left cervical lymph nodes. CT scan of the chest also revealed a nodule in the right lung and the enlargement of left upper mediastinal lymph nodes. Histopathology of the biopsied cervical lymph node showed coexistence of two tumorous components; one was LCH and the other tissue of Hodgkin disease with Reed-Sternberg/Hodgkin cells being positive for CD30. The disease responded temporarily to irradiation (36 Gy) and systemic chemotherapy, but became refractory with relapses to the lungs and lymph nodes. Despite autologous followed by allogeneic HSCTs, he died of refractory Hodgkin disease at age of 23.

Summary of the cases

As summarized in Table 1, cases consisted of 3 SS-LCH (all CNS disease) and 15 MS-LCH. Regarding the initial symptoms, 7 (2 males and 5 females) of the 18 patients had CDI and other endocrine symptoms with thickened pituitary stalk or a mass at the HPR. Additional 2 patients initiated the disease with CDI with no immediate diagnosis. In the remaining patients, the disease begun with single (n = 3) or multiple (n = 1) spinal bone lesion(s) in 4 patients (all males), with multiple bone lesions in 3 patients (1 male and 2 females), with localized skull/muscle lesion in one female patient and with ambiguous symptoms including hypothyroidism in one male patient. Thyroid mass was noted in 2 patients. In terms of treatment, 9 patients received systemic immuno-chemotherapy alone, of whom 3 with CNS disease and 1 with multiple bone lesions received 2CDA. Five patients had a combination of immuno-chemotherapy with surgical resection or radiotherapy, 2 had immunotherapy alone, 2 had surgical resection followed by observation alone to date. Three patients received HSCT after extensive chemotherapy. In terms of outcome, 15 patients are alive (9 with active disease; AWAD, 6 without active disease; ASAD) with a median follow-up of 66 mo (range 17-166 mo) and 2 died of disease; 1 from sepsis-induced DIC and the other from progression of Hodgkin disease. The remaining 1 patient is lost to follow-up. As late sequelae, CDI (n = 9), neurodegenerative disease (n = 2) and obesity/diabetes mellitus (n = 3) are noted.

DISCUSSION

Clinical features

To date, adult LCH cases have mostly been reported as case series[14-18]. Here, we add another case series describing the clinical features and discussing the issues specifically relevant to adult LCH. Adult patients may have LCH as a recurrence of childhood LCH as well as *de novo* LCH developing first in adult life. Here described is all the latter type of LCH. None had a history of LCH in childhood.

Two major clinical features of non-pulmonary LCH in adults

It is apparent that there are two major groups; one is a CNS mass with endocrine problems (9/18) and the other is recalcitrant bone lesions (7/18). Both types of disease are histologically non-malignant, but extensive disease causes various impairments leading to the decreased quality of life, and limiting to achieve normal daily life activity.

Endocrine problems

Adult patients are often noted first with endocrine problems such as CDI, amenorrhea, loss of libido and obesity[9]. Particularly, hypothalamic-pituitary disease is the most common CNS manifestation of LCH, which leads to CDI and anterior pituitary hormone deficiencies. CDI is diagnosed from the finding that MRI scan shows absence of the bright spot of the posterior pituitary on

Table 1 Summary of 18 cases of non-pulmonary adult langerhans cell histiocytosis

Cases	Age (yr)/sex	Initial symptoms/signs	Subsequent symptoms/ disease progression	Treatment			Outcome	Follow-up (mo)
				Surgical resection	Radiation	Immune-chemo Rx/HSCT		
1	27/F	CDI/endocrine	None	Y	Y	Y[4]	LTF	-
2	25/F	CDI/endocrine	Nasal/thyroid/skin masses			Y	DOD[1]	216
3	35/F	MBL	MBL			Y/Y	ASAD	132+
4	37/M	CDI	MBL/skin lesions			Y/Y	ASAD	128+
5	40/M	MBL	MBL		Y	Y	ASAD	133+
6	46/F	Thyroid mass/spine (Th4)	MBL/LNs	Y		Y	AWAD	27+
7	40/M	Spine (C3)	Parietal bone	Y			ASAD	72+
8	40/M	Spine (C6)	Inguinal LNs			Y	AWAD	17+
9	27/F	Temporal bone	None	Y			ASAD	26+
10	31/M	Spine (Th1)	MBL			Y[4]	AWAD	108+
11	53/F	CDI	MBL	Y		Y	AWAD	166+
12	36/F	Endocrine	HPR mass			Y[3]	AWAD	28+
13	20/F	CDI/endocrine	Skin		Y	Y	ASAD	156+
14	36/F	CDI/endocrine	None			Y[3]	AWAD	52+
15	38/M	Hypothyroid	CDI/endocrine			Y[3]	AWAD	48+
16	46/M	Endocrine	CDI/MBL/ND-CNS			Y[3]	AWAD	60+
17	23/M	CDI	Lungs/ND-CNS			Y[4]	AWAD	72+
18	18/M	Spine (multiple)	LNs/Lungs		Y	Y/Y	DOD[2]	72

[1]From disseminated intravascular coagulation; [2]From Hodgkin disease; [3]Systemic chemotherapy with 2-deoxychloroadenosine; [4]Steroid alone with or without bisphosphonate. CDI: Central diabetes insipidus; MBL: Multiple bone lesions; HPR: Hypothalamic pituitary region; LNs: Lymph nodes; ND-CNS: Neurodegenerative central nervous system disease; Y: Yes; LTF: Lost to follow-up; ASAD: Alive without active disease; AWAD: Alive with active disease; DOD: Died of disease.

the T1-weighted sequences[9,19]; however, it is common that patients are taken care and followed up about these problems at the Endocrinology Unit until when Gd-enhanced MRI reveals a thickened pituitary stalk and/or a hypothalamic mass. Generally, it takes a year or longer for the mass to be biopsied and correct diagnosis be confirmed. Even when the diagnosis is confirmed, there are occasions that it takes time for the patient to be referred to hemato-oncologists for chemotherapy. Whenever the diagnosis and the introduction of treatment are delayed, the patient may develop not only endocrine problems but also cognitive impairment such as memory deficits as well as consciousness disturbances, as shown in our cases (Cases 12, 16, 17).

LCH in association with childbirth

The development of LCH in association with childbirth has not been well recognized. Regarding LCH occurring during pregnancy, only a few sporadic cases have been described previously[20,21]; however, no information is available how childbirth influenced on the development of LCH. In our series, the correlation between pregnancy/childbirth and LCH in adult female patients was noted in 4 cases (Cases 1-3, 12). Sex hormones are believed to participate in immune responses, as estrogens have been found to serve as enhancers in humoral immunity while androgens/progesterone appears to act as natural immune-suppressors[22]. For examples, postpartum thyroiditis/diabetes mellitus is speculated to be a consequence of the immunological flare that occurs after the lifting of the pregnancy-related immune suppression[23,24]. Moreover, pregnancy and the post-partum period are associated with increased breast cancer aggressiveness[25]. Thus, the hormonal imbalance in the postpartum period may trigger the development of LCH. Detailed examination of pregnancy and childbirth history in female LCH patients may clarify whether the associated hormonal changes influence the pathogenesis and the development of LCH.

LCH in association with various diseases/events

Two patients (Cases 16, 17) were noted to have cognitive disturbance due to LCH-related neurodegenerative CNS disease[10]. Additionally, two patients (Cases 15, 18) developed malignant lymphoma; one with concurrent LCH and Hodgkin disease and the other developed B-cell lymphoma after systemic chemotherapy for LCH. The association of LCH and other malignant lymphoid neoplasms has been well recognized[26-28]. In this series two patients (Cases 8, 9) with severe atopic dermatitis were found to develop LCH. This is an interesting topic considering the antigen-stimulation in the skin. It is also cautioned that recalcitrant or clinically atypical skin eruptions must be differentiated from LCH and other rare disorders[29] but no data is available that incidence of LCH is higher in patients with severe atopic dermatitis. Four patients (Cases 3, 5, 7, 8) were diagnosed to have LCH from spinal bone lesions. Particularly, of whom two had single spine (C3 or C6) involvement, not in the spinal body but in the arch. Spinal lesion should be searched for any adult who complained of cervical pain[30]. Intriguingly, discovery of LCH was triggered by road traffic accident in 2 patients (Cases 8, 17), although such reports are rarely found[31]. In Case 8, LCH lesion at the cervical spine was identified at the emergency hospital. In Case 17, traffic accident

incidentally led to the diagnosis of CNS- and pulmonary-LCH in the patient.

Importance of CT/MR/PET imaging for the diagnosis

To determine the precise biopsy/excision site of LCH, CT/MRI findings are inevitable. Particularly, bone scintigraphy for multiple bone lesions and Gd-enhanced MRI for CNS lesions are essential for the diagnosis of LCH[9,19]. However, more recently, ^{18}F-FDG PET is recommended. In one large study it was concluded that whole body FDG-PET scans can detect LCH activity and is useful to evaluate early response to therapy with greater accuracy than other imaging modalities (MRI, CT, plain films) in patients with LCH lesions in the bones and soft tissues[32]. Also, it is a useful tool for the monitoring of CNS disease activity in LCH[33,34]. It is said that ^{18}F-FDG PET might be useful to detect an early neurodegenerative lesions before MRI abnormalities appear, where bilateral hypometabolism is shown in the cerebellum and the basal ganglia (caudate nuclei) areas[34].

Therapeutic measures for adult LCH

In this case series, four patients received surgical resection of LCH mass without immuno-chemotherapy. Four patients received irradiation to the CNS-mass (n = 2), bone (n = 1) and lungs (n = 1), in association with immuno-chemotherapy. In the majority, systemic immuno-chemotherapy was given, mostly with a conventional combination of VBL/PSL or JCSL-96 protocol including VCR/cytosine arabinoside (AraC)/PSL[13] for induction. In 3 cases with CNS-LCH, 2CDA was employed. Previously proposed A1 protocol for adult LCH[6] was used only in one case in this series. With these measures, 6 ASAD cases were obtained, but necessity for further improvement of treatment for adult LCH seems apparent. As future trials, we have to scrutinize how efficiently we can employ AraC, 2CDA, clofarabine, and other novel agents for adult LCH patients. In the past, treatment reports on adult LCH cases were very limited[35,36]. In particular, the usefulness of intravenous 2CDA for CNS-LCH as well as for systemic MS-LCH was described in adult patients[37-40]. Windebank *et al*[41] also reported the usefulness of subcutaneous 2CDA treatment (5 mg/m^2 × 5 d, *sc*, q4 wk, for up to 6 cycles) in LCH. Effectiveness of the combination of 2CDA/AraC was described for extremely refractory cases[42]. More recently, effectiveness of clofarabine (25 mg/m^2 × 5 d, *iv*, q4 wk) has also been reported[43,44]. Particularly, Simko *et al*[44] demonstrated usefulness of clofarabine for multifocal skull lesions. On the other hand, Morimoto *et al*[11] reported the usefulness of Special C regimen of JLSG for treating adult LCH patients on ambulatory basis. Intriguingly, for the treatment of multiple bone LCH lesions in adults, Cantu *et al*[45] reported that AraC alone is an effective and minimally toxic, while VBL/PSL results in poor overall responses with excessive toxicity. Considering the fact that about 50% of LCH possess BRAF V600E mutation, molecular targetting treatment with vemurafenib has been proposed more recently[46]. As bone therapy regimen, Zoledronic acid as bisphosphonate is available, although its effectiveness on LCH bone lesions is still elusive[47]. Allogeneic HSCT for adult LCH is not within a scope of this article, although a few reports on pediatric LCH cases have been described[48,49]. As well recognized, in the recipients of allogeneic HSCT, care must be taken for the transplant related adverse events. In Table 2, a list of candidate systemic immuno-chemotherapy regimens is summarized, which we think is useful in choosing regimens for adult LCH patients. In practice, for an adult case of LCH with persistent minimal disease and systemic involvement, we prefer once a month or twice a month treatment, like Special C regimen of JLSG[11]. However, with these regimens, some adult patients may still show VBL neurotoxicity, MTX hepatotoxicity, or neutropenia due to mercatopurine hydrate (6MP); such events make it difficult to achieve the entire regimens as planned. Although we recognize that 2CDA is highly effective and could be useful in adult LCH, it is often difficult persuading the patients to stay in the hospital for the 5-d continuous treatment. If subcutaneous 2CDA is available at the outpatient care, this agent could be more employed in the treatment of adult LCH. In any case, it is important to make a most appropriate treatment plan for each patient individually. In summary, for adult patients with two major types of LCH, *i.e.*, recalcitrant multiple bone lesions and/or a mass at the HPR, early introduction of systemic immuno-chemotherapy using conventional regimens including AraC or alternative 2CDA or clofarabine regimens is recommended to overcome the disease-related impairment of qualify of life.

Table 2 Therapeutic options in the treatment of adult langerhans cell histiocytosis

Protocol	Drugs	Ref.
A1 protocol	VBL/PSL	[6]
JLSG-96	VCR/AraC/MTX/6MP/PSL	[13]
Cladribine-based	2CDA/PSL, 2CDA/AraC	[37-41]
Clofarabine-based	Clofarabine	[43,44]
JLSG-special C	VBL/MTX/6MP/PSL	[11]
Others	AraC alone	[45]
Molecular targetting	Vemurafenib	[46]
Bone therapy	Zoledronic acid	[47]

VBL: Vinblastine; PSL: Prednisolone; MTX: Methotrexate; 6MP: 6-mercatopurine hydrate; 2CDA: 2-deoxychloroadenosine.

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ORIGINAL ARTICLE

Therapeutic outcome of multifocal Langerhans cell histiocytosis in adults treated with the Special C regimen formulated by the Japan LCH Study Group

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Abstract Little information is available regarding effective systemic therapies for adult Langerhans cell histiocytosis (LCH). The Japan LCH Study Group has formulated an ambulatory treatment regimen for adult patients with LCH. In total, 14 patients (median age 43 years, range 20–70 years) with multifocal LCH with biopsy-confirmed histology were enrolled. None had received cytoreductive agents for LCH previously. Four had single system (SS) and ten had multi system (MS) disease. All were treated with the Special C regimen, which consists of vinblastine/prednisolone and methotrexate with daily 6-mercaptopurine for 36 weeks. At the end of the therapeutic regimen, all SS patients achieved no active disease (NAD), and six of the ten MS patients showed a response (NAD in two, partial response in four). At the last follow-up (median 34 months), 11 patients were alive (NAD in eight and active disease in three). Of the three deceased, one died of hemorrhage during the Special C treatment, and two of infections during subsequent therapy. Although this study is limited by the small sample size, this ambulatory regimen shows signs of efficacy for adult LCH. This was particularly evident for patients with multifocal SS disease, but half of those with MS disease also benefited.

Keywords Langerhans cell histiocytosis · Adult · Chemotherapy

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease that is characterized by the infiltration of clonal CD1a-positive

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dendritic cells. It mostly develops in infancy or early childhood with a childhood incidence of 2.2–8.9 cases per million; in adults, the incidence is one-third of the childhood incidence (1–2 cases per million) [1, 2]. LCH is categorized as a single system (SS) disease with multifocal or single/localized lesion(s) and as a multi system (MS) disease with or without risk organ (hematopoietic system, lung, liver, or spleen) involvement [3]. Children with multifocal SS or MS LCH are required to undergo systemic chemotherapy, but no such therapy is recommended for those with localized SS LCH [3]. Also in adults, systemic chemotherapy is required for multifocal SS or MS LCH lesions [1, 5], although adult-specific, smoking-related solitary pulmonary LCH lesions are treated differently [4]. While recent prospective, large-scale, multi-institutional trials have improved the therapeutic outcomes of multifocal childhood LCH [6, 7], only a few therapeutic trials involving a small number of cases have been performed for adult LCH [8–10].

A major obstacle in treating adult LCH patients is that they are often reluctant to take a leave of absence from their jobs for hospitalization, which can limit the provision of sufficient chemotherapy. Considering this adult-specific situation, the Japan LCH Study Group (JLSG) formulated Special C regimen for adult LCH patients in giving therapy safely at the outpatient clinic without hospitalization, which consisted of combinations of vinblastine (VBL)/prednisolone (PSL) and methotrexate (MTX) with daily 6-mercaptopurine (6-MP). These drugs were conventional agents and successfully employed as first-line chemotherapy for pediatric LCH patients [11]. The pilot study with the use of this regimen on adult patients with multifocal SS or MS LCH was performed. Results are reported here.

Patients and methods

This multicenter study was planned as a pilot study at the participating facilities of JLSG. The study was approved by the institutional review board (IRB). The study procedure was in accordance with the Helsinki Declaration. Eligible patients signed a detailed written informed consent statement meeting the requirements of the IRB. Patients were eligible for the study when having histologically diagnosed multifocal LCH who were at least 20 years of age. The diagnosis of LCH was confirmed by histopathology of biopsies of affected organs, which were positive for S-100 and/or CD1a antigen. Patients also needed to have adequate performance status and normal hepatic, renal, and cardiac functions. Exclusion criteria included the presence of serious infection and a history receiving cytoreductive chemotherapy for LCH. All patients were treated with the Special C regimen, which consisted of nine cycles of 6 mg/m^2 (max. 6 mg) of VBL on day 1, 2 mg/kg (max. 60 mg) of PSL on days 1–5, 20 mg/m^2 of MTX on day 15, and 1.5 mg/kg of 6-MP on days 1–28, over a period of 36 weeks. The dose of 6-MP was adjusted to white blood cell counts of 2,000–3,000 μ/L. Preventive medication of trimethoprim-sulfamethoxazole combination was recommended. At the end of treatment, the response was categorized as follows: no active disease (NAD) was defined as the disappearance of the signs or symptoms of disease, a partial response was defined as regression of >50 % of the signs or symptoms of disease without organ dysfunction and new lesions, no response was defined as regression of <50 % of the signs or symptoms of disease with or without organ dysfunction and the absence of new lesions, and progressive disease was defined as progression in the signs or symptoms of disease and/or the appearance of new lesions. Disease status at the last follow-up was defined as alive with NAD, alive with disease, or died. Common Terminology Criteria for Adverse Events v3.0 was used to grade adverse events.

Results

Fourteen adult patients with multifocal LCH (nine males and five females) were enrolled in this adult pilot study between 2002 and 2010 (Table 1). Four had a previous history of malignant disease (NK/T cell lymphoblastic lymphoma, renal cancer, diffuse large B cell lymphoma, and uterine cervical cancer). The median age at LCH onset was 34 years (range 16–69 years). In terms of prior medication other than cytoreductive agents for LCH, six patients were treated with PSL alone. Of the 14 patients with multifocal LCH, four had SS disease (skin, $n = 2$; multiple bones, $n = 2$) and ten had MS disease, of whom five had diabetes insipidus (DI) and one had central nervous system degeneration (CNSD) already at the time our treatment was initiated. The median time between disease onset to the initiation of our treatment was 2.4 years (range 0.1–32.7 years). The median age when our treatment was initiated was 40 years (range 20–70 years). Nine, three, and two of the patients were treated in the Departments of Internal Medicine, Dermatology, and Pediatrics, respectively. At the end of therapy, all SS patients attained NAD, while six of the ten MS patients had a response (NAD in two and a partial response in four) (Table 2). In terms of reactivation, two patients with SS disease in the skin had cutaneous reactivation and three patients with MS disease had reactivation in lymph node, bone, and mucosa (one in each patient). All reactivation sites were included in the primary lesions. Four of the five reactivations occurred approximately 1 year after therapy was initiated. In terms of treatment at reactivation, four patients underwent a

Table 1 Characteristics of 14 Adult LCH patients who participated in the JLSG-02 study

Disease type	UPN	Sex	Preceding malignancy	Onset age (years)	Preceding Tx. for LCH	Organ(s) involved	Interval between onset and regimen C treatment (years)	Age when treated with regimen C (years)
Single system	158	M	NK/T-LBL	29	None	Multi-B	0.3	29
	189	F	None	18	None	Multi-B	1.3	20
	202	M	Renal cancer	69	None	Sk	1.2	70
	E03	F	None	66	PSL	Sk	2.5	69
Multi system	36	M	None	38	PSL	Sk, B, LN, Pit	0.8	39
	95	M	None	40	PSL	Sk, B, ST, H	4.9	45
	120	M	None	19	PSL	Sk, LN, ST, Pit	1.1	20
	173	F	None	26	PSL	Muc, B, Pit	13.7	40
	208	M	None	16	None	B, L, Pit, CNSD	6.5	23
	249	M	DLBCL	62	None	Sk, ST	0.1	63
	295	M	None	18	None	Sk, B, L, Pit	32.7	50
	305	M	None	23	None	Sk, Muc, B, L	2.3	25
	E01	F	UCC	53	None	Sk, LN, Mus	5.0	58
	E02	F	None	54	PSL	B, Mus, Muc	2.8	56

NK/T-LBL NK/T cell lymphoblastic lymphoma, *DLBCL* diffuse large B cell lymphoma, *UCC* uterine cervical cancer, *PSL* prednisolone, *Tx* therapy, *Multi-B* multiple bone, *Sk* skin, *B* bone, *LN* lymph node, *Pit* pituitary, *ST* soft tissue, *H* hematopoietic system, *Muc* mucosa, *L* lung, *CNSD* central nervous system degeneration, *Mus* muscle

Table 2 Outcome of 14 adult LCH patients who were treated with the Special C regimen

Disease type	UPN	Response at the end of Tx.	Adverse effects more than Grade 2	Reactivation (time)	Second-line systemic Tx.	Permanent sequelae	Status at last follow-up (months)
Single system	158	NAD	No	None	No	None	AWND (68)
	189	NAD	Yes	None	No	None	AWND (57)
	202	NAD	No	Skin (28 months)	ND	None	Died (41)
	E03	NAD	No	Skin (14 months)	No	None	AWD (18)
Multi system	36	NR	No	NE	2CdA/HD-CA, HSCT	DI, cGVHD	AWND (107)
	95	NE	Yes	NE	No	NE	Died (0.1)
	120	PR	No	LN (9 months)	AraC/VCR/ PSL, AZP/ MTX	DI, skin scar	AWND (83)
	173	PR	Yes	Bone (14 months)	VBL/MTX/ 6MP	DI, hypothyroidism	AWND (47)
	208	NR	Yes	NE	ND	DI, CNSD	AWD (53)
	249	PD	No	NE	AraC/VCR/PSL	NE	Died (1.3)
	295	PR	No	None	No	DI, honeycomb lung	AWND (21)
	305	PR	No	Mucosa (12 months)	AraC/VCR/PSL	Loss of teeth, honeycomb lung	AWD (16)
	E01	NAD	Yes	None	No	None	AWND (24)
	E02	NAD	No	None	No	Loss of teeth	AWND (27)

NAD no active disease, *PR* partial response, *NR* no response, *PD* progressive disease, *NE* not evaluable, *ND* no data, *2CdA* cladribine, *HD-CA* high dose cytarabine, *HSCT* hematopoietic stem cell transplantation, *AraC* cytarabine, *VCR* vincristine, *PSL* prednisolone, *AZP* azathioprine, *MTX* methotrexate, *6MP* 6-mercaptopurine, *CNSD* CNS degeneration, *DI* diabetes insipidus, *cGVHD* chronic graft versus host disease, *AWND* alive with no active disease, *AWD* alive with disease

Grade 3 neutropenia in UPN E01, grade 4 neutropenia in UPN 189 and UPN 208, grade 3 infection (varicella-zoster virus reactivation) in UPN 173, grade 3 hepatic dysfunction (ALT 283 IU/L and total bilirubin 3.1 mg/dl) in UPN 208 and grade 5 bleeding in UPN 95)

cytarabine-containing regimen and one patient underwent hematopoietic stem cell transplantation. Treatment responses in the four patients (SS, $n = 2$; MS, $n = 2$) who had a history of malignant disease were NAD in three and PD in one. As a total, 3 patients died; one from hemorrhage and 2 from infections as commented later. In terms of eventual outcome, eleven patients were alive (NAD in eight and active disease in three) with a median follow-up duration of 34 months. Of whom, one patient (UPN 36) was a recipient of allogeneic bone marrow transplantation from unrelated donor, which was done because of disease progression, with the conditioning regimen of total body irradiation (8 Gy) and cyclophosphamide and with the graft versus host disease prophylaxis using a short-course MTX/tacrolimus. Eight patients had some sequelae of which association with LCH are well known, namely DI in five, CNSD in one, loss of teeth in two, and honeycomb lung in two. The overall survival and event-free survival rates at 3 years were 85.7 % (95 % confidence interval, 67.3–100 %) and 28.6 % (95 % confidence interval, 0–57.2 %), respectively (Fig. 1).

In terms of adverse events, Grade 3 or more adverse events were observed in 5 of the 14 patients (see footnotes in Table 2). Of the three deceased, two died of infections (pneumonia, sepsis) during subsequent therapy after stopping the Special C regimen (UPN 202 and UPN 249). UPN 95, which had a huge cystic lesion of LCH on his back and within the cyst occasional bleedings had been noted previously, died of fatal hemorrhagic shock due to massive bleeding in the cyst after 2 days of treatment with the Special C regimen.

Discussion

There are a number of issues that hamper the timely and effective treatment of adult LCH. First, it often takes time to correctly diagnose LCH in adults because the disease is not often seen by physicians who are taking care of adult patients, and the clinical features of LCH are quite heterogeneous. A report by the International Registry of the Histiocyte Society indicated that the median latency period between disease onset and diagnosis in adult patients with LCH was 4 months [5]. Second, the various symptoms of this rare disease cause patients to visit a variety of clinics including internal medicine, dermatology, orthopedics, dental surgery, otolaryngology, and neurology clinics. Indeed, more than one-third of our patients with multifocal LCH underwent our regimen at clinics other than the Department of Internal Medicine. Thus, patients with adult LCH are mostly treated in various clinics that apply various therapeutic regimens. Third, it is very common in adult LCH to adopt a "wait and see" strategy after diagnosis, even when it is multifocal LCH, because it is believed that most cases of adult LCH do not progress rapidly. Indeed, the International Registry of the Histiocyte Society report found that 30–40 % of adult patients with multifocal LCH did not receive chemotherapy when they are diagnosed [5]. Further supporting this is that, in our cohort, 50 % of the SS LCH cases and 40 % of the MS LCH cases did not receive any chemotherapy for more than 1 year after the onset of LCH. The reason of this treatment delay seems to be that LCH was not familiar to the treating physicians, and there were only a few evidences about how to treat adult LCH patients. It should be noted that LCH is a disease that causes late sequelae and that, during the "wait and see" period, patients often develop neurological sequelae such as DI, anterior pituitary hormone deficiencies, and CNSD [12]. Indeed, more than half of our patients had DI and one patient had CNSD already at the time our treatment was initiated. The incidence of DI in adult LCH is up to 30 % [5], which seems to be higher than in childhood LCH [13]. Fourth, a considerable proportion of adult LCH patients have a previous history of malignancy [5], which could cause LCH to become chemotherapy-resistant. In fact, four patients in our study had a history of malignant disease; however, numbers were too small to confirm the refractoriness in these cases. Anyway, all of these issues make it difficult to treat adult LCH patients.

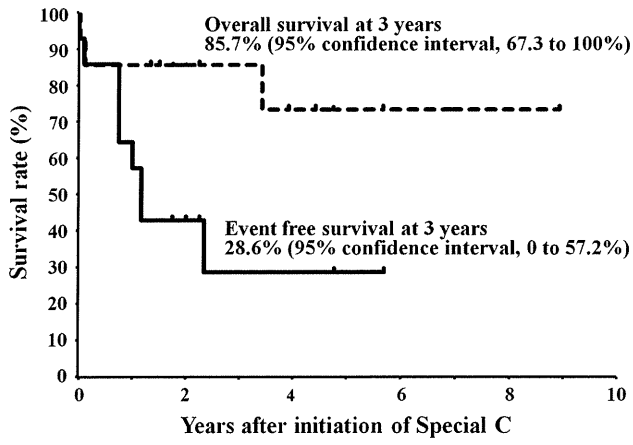

Fig. 1 Survival curve of adult patients treated with the Special C regimen

To date, only a few attempts have been made to establish an effective systemic therapy for adult patients with LCH [8–10]. Three case series involving a small number of patients have been reported (Table 3). In the case series of Saven et al., 12 patients underwent cladribine (2CdA) monotherapy [8]. Nine responded and six maintained a continuous response with a median follow-up of 3.6 years. Grade 3–4 neutropenia was observed in seven patients. Notably, the response rate in patients who were resistant to other cytoreductive chemotherapies was the same as the response rate of the other patients in their cohort. This

indicates that 2CdA is highly effective. 2CdA is a promising agent also in children with recurrent LCH, especially those with intracranial mass lesions [14]. 2CdA has also been found to be effective for adult patients with central nervous system LCH lesions [12]. However, this drug is not suitable as a first-line agent because of its high cost, with a risk of severe hematological toxicity [15] and secondary hematologic malignancies [8]. Another case series was that of McClain et al., who reported the responses of seven adult LCH patients in the LCH-A1 study of the International Registry of the Histiocyte Society, where patients were treated with a regimen derived from the pediatric LCH protocol that consists of VBL and PSL [9]. Three patients responded to the therapy, but five developed Grade 3–4 neuropathy, and only two were able to complete the treatment courses. Adult patients with LCH seem to be particularly sensitive to the neuropathic effects of VBL. The third case series of that of Derenzini et al., who recently reported the efficacy of MACOP-B regimen which is an intensive chemotherapy that was originally used for aggressive non-Hodgkin's lymphomas [10]. All seven patients responded and four have maintained a continuous

Table 3 Case series reports of the results of various treatments for adult LCH

Disease type (no. of pts.)	Age at Tx. years (range)	Regimen	Tx response	Adverse effects (≥Grade 3)	Eventual response	Median follow-up (years)	References
SS (3) MS (9)	44 (19–72)	2CdA 0.1 mg/kg, day 1–7, every 4 weeks Total duration: 2–6 months	SS: 2/3 MS: 7/9	Neutropenia 7/12 (58 %)	SS: 1/3 MS: 5/9	3.6	Saven et al. [8]
MS (7)	NA	Induction: VBL 6 mg/m^2, day 1, 8, 15, 22, 29, 36 PSL 1 mg/kg, day 1–28 Maintenance: VBL 6 mg/m^2, day 1 PSL 1 mg/kg, day 1–5 6-MP 30 mg/m^2, day 1–21 Total duration: 6 or 12 months	MS: 3/7	Neuropathy 5/7 (71 %)	MS: 3/7	0.5	McClain et al. [9]
SS (4) MS (3)	27 (18–62)	CY 350 mg/m^2, day 1, 15, 29, 43, 57, 71 ADR 50 mg/m^2, day 1, 15, 29, 43, 57, 71 MTX 400 mg/m^2, day 8, 36, 64 VCR 1.4 mg/m^2, day 8, 22, 36, 50, 64 Bleo 10 mg/m^2, day 22, 50, 78 PSL 40 mg/m^2, day 1–84 Total duration: 3 months	SS: 4/4 MS: 3/3	Neutropenia 2/7 (29 %)	SS: 3/4 MS: 1/3	6.5	Derenzini et al. [10]
SS (4) MS (10)	43 (20–70)	VBL 6 mg/m^2, day 1 PSL 2 mg/kg, day 1–5 MTX 2 mg/day, day 15 6-MP 1.5 mg/kg/day, day 1–28 Total duration: 9 months	SS: 4/4 MS: 6/10	Neutropenia 3/14 (21 %) Bleeding 1/14 (7 %) Infection 1/14 (7 %) Hepatic dysfunction 1/14 (7 %)	SS: 2/4 MS: 3/10	2.8	Present study

Tx treatment, *SS* single system, *MS* multisystem, *2CdA* cladribine, *VBL* vinblastine, *PSL* prednisolone, *6-MP* 6-mercaptopurine, *CY* cyclophosphamide, *ADR* adriamycin, *MTX* methotrexate, *VCR* vincristine, *Bleo* bleomycin

response with a median follow-up of 6.5 years. Despite the intensive therapy, Grade 3–4 neutropenia was observed only in two patients. This may reflect that fact that the patients in this cohort were relatively young (a median age of 27 years). Such intensive therapy may be best reserved for salvage in adult patients with resistant or progressive MS LCH [16].

In the present pilot study, 10 of the 14 patients responded and five patients maintained a continuous response with a median follow-up of 2.8 years. A response was obtained in all of the patients with SS disease and in half of the patients with MS disease. The Special C regimen was originally prepared to give it safely at the outpatient clinic. Therefore, it was a surprise that we observed a fairly high % of ≥grade 3 adverse events in our cohort, even including a fatal case. This may mean that, especially in the treatment of adult LCH, we have to be careful for various co-morbidities which could affect the patients' outcome associated with therapeutic procedures for LCH. In SS patients, the response rate is comparable to those achieved with the 2CdA and MACOP-B regimens, but the response rate of the MS patients may be slightly more inferior (Table 3). However, the Special C regimen is particularly significant because it is an ambulatory treatment. It may be necessary to intensify the treatment for patients with MS disease, but careful consideration should be given when the patient is elderly because two of the three patients over 60 years of age died from infection during subsequent treatments for refractoriness and reactivation.

A multicenter phase II study with a large number of patients with multifocal LCH that allows risk stratification is needed to establish a more efficient and less toxic regimen. To promote this, closer cooperation between pediatric and adult hematologists and experts in other fields (such as dermatology, orthopedics and neurology) is essential. Such a study may improve the quality of life of adult patients with LCH.

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ORIGINAL ARTICLE

IL-17A receptor expression differs between subclasses of Langerhans cell histiocytosis, which might settle the IL-17A controversy

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Abstract Langerhans cell histiocytosis (LCH) is a lymphoproliferative disorder consisting of abnormal Langerhans cell-like cells and other lymphoid cells. LCH presents as either a multisystem LCH (LCH-MS) or a single-system LCH (LCH-SS). Currently, neither the pathogeneses nor the factors that define these disease subclasses have been elucidated. The interleukin (IL)-17A autocrine LCH model and IL-17A-targeted therapies have been proposed and have engendered much controversy. Those authors showed high serum IL-17A levels in LCH and argued that serum IL-17A-dependent fusion activities in vitro, rather than serum IL-17A levels, correlated with LCH severity (i.e. *the IL-17A paradox*). In contrast, others could not confirm the IL-17A autocrine model. So began the controversy on IL-17A, which still continues. We approached *the IL-17A controversy* and *the IL-17A paradox* from a new perspective in considering the expression levels of IL-17A receptor (IL-17RA). We detected higher levels of IL-17RA protein expression in LCH-MS (n=10) as compared to LCH-SS (n=9) (P=0.041) by immunofluorescence. We reconfirmed these data by re-analyzing GSE16395 mRNA data. We found that serum levels of IL-17A were higher in LCH (n=38) as compared to controls (n=20) (P=0.005) with no significant difference between LCH subclasses. We propose an IL-17A endocrine model and stress that changes in IL-17RA expression levels are important for defining LCH subclasses. We hypothesize that these IL-17RA data could clarify the IL-17A controversy and the IL-17A paradox. As a potential treatment of LCH-MS, we indicate the possibility of an IL-17RA-targeted therapy.

Keywords Langerhans cell histiocytosis · Interleukin-17A · Interleukin-17A receptor · Photoshop-assisted image analysis · Staining intensity · Immunofluorescence

Abbreviations

AU	Arbitrary units
BH-FDR	False discovery rate controlled by the Benjamini–Hochberg procedure
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin-fixed paraffin-embedded
IL-17A	Interleukin-17A
IL-17RA	Interleukin-17A receptor
IS	Intensity score
LC	Langerhans cell

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LCH	Langerhans cell histiocytosis
LCH cell	Langerhans cell-like abnormal cell
LCH-MS	Multisystem LCH
LCH-SS	Single-system LCH
LC/MRM-MS	Liquid chromatography/multiple reaction monitoring-mass spectrometry
LC/MS	Liquid chromatography/mass spectrometry
MMP	Matrix metalloproteinase
S100	S100 protein

Introduction

Cicadas are hard of hearing like a very deaf man, concluded the French entomologist Fabre after observing that firing a cannon at them elicited no change in behavior [1]. Cicadas, however, can hear their own sound. If Fabre had been capable of observing the question from the viewpoint of specific receptors to sense particular sound, his conclusion might have been different.

Langerhans cell histiocytosis (LCH) is a lymphoproliferative disorder consisting of abnormal Langerhans cell (LC)-like cells (LCH cells) and other lymphoid cells [2, 3]. LCH presents as either an uncontrollable multisystem LCH (LCH-MS) or a single-system LCH (LCH-SS) with favorable prognosis. Currently, neither the pathogenesis nor the factors that define these disease subclasses have been elucidated.

Whether LCH is neoplastic or reactive has long been debated [2]. The presence of clonality [4, 5], cytogenetic aberrations [6], and BRAF mutations [7] suggests a neoplastic character, whereas the formation of granulomas [3] with spontaneous regression [8–10] and cytokine storms [2, 11–18] is more indicative of a reactive process. So LCH is regarded as a premalignant inflammatory process driven by aberrant BRAF signaling [7, 19], though BRAF mutation did not differ significantly between LCH-SS and LCH-MS [7].

As one such example, the incidence of BRAF mutation did not differ significantly [7] between pulmonary LCH that has been regarded as reactive to smoking [2, 20] and nonpulmonary LCH that has been regarded as a neoplastic process [2, 4–7]. Since smoking increases the number of LCs in chronic obstructive pulmonary disease [21], mutated precursor LCH cells may overreact to smoking. Similarly in cutaneous LCH, overreaction to stimuli such as a dermotropic viral infection might happen.

Coury et al. found IL-17A to be elevated in the serum of patients with LCH and suggested that it might be involved in LCH pathogenesis according to an IL-17A autocrine model [22]. The IL-17A autocrine model in LCH and the IL-17A-targeted therapies proposed by Coury et al. [22] have engendered much controversy. Those authors showed high serum IL-17A levels in LCH and argued that serum IL-17A-dependent healthy monocyte-derived dendritic cell fusion activities in vitro, rather than serum IL-17A levels, correlated with LCH severity (i.e., *the IL-17A paradox*) [22]. In contrast, Allen et al. [23–25] were unable to confirm the data presented in Coury et al. [22]. So began the controversy on IL-17A (Table 1), which still continues [19, 26].

IL-17A is a proinflammatory cytokine produced by various cells including T helper type 17 cells (Th17), γδT cells, CD8+ T cells, natural killer T cells, lymphoid tissue inducer-like cells, neutrophils, monocytes, and natural killer cells [27–29]. IL-17A acts in both innate and acquired immunity [29]. Innate lymphoid populations can rapidly produce IL-17A [29], which is maintained at low levels in the absence of stimulation [30]. For host defense, IL-17A/IL-17RA is important [31]. We approached *the IL-17A controversy* and *the IL-17A paradox* from a new perspective in considering the expression levels of IL-17A receptor (IL-17RA), based upon which we propose an endocrine model of LCH (Fig. 1).

Patients and methods

This study was approved by the Institutional Review Board of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan and Faculty of Medicine, Tottori University, Tottori, Japan.

Patients, tissue samples, and sera

We tested LCH cells in tissue and sera from LCH patients, all of which were taken before treatment. Formalin-fixed paraffin-embedded (FFPE) tissues were obtained from 19 patients (10 LCH-MS and 9 LCH-SS) (Table 2). Sera were also obtained from 23 LCH-MS and 15 LCH-SS patients and from 20 control individuals. Tissues or sera of LCH were obtained from patients registered with the Japan LCH Study Group between 2002 and 2009. Tissues or sera were taken from different patients with LCH. Twenty control sera were obtained from patients with non-LCH and under follow-up with inactive stable state. Some samples were from patients who visited Okayama University Hospital or Tottori University Hospital between 2002 and 2011.

Antibodies for immunofluorescence

Anti-CD1a antibody (monoclonal mouse anti-human CD1a antibody (IgG1, kappa, O10, Dako Japan, Kyoto, Japan) was used at 1:100 dilution. Anti-IL-17RA antibody (polyclonal rabbit anti-human IL17RA antibody, IgG, 200 μg/ml, IL17RA (H-168): sc-30175, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1:50 dilution. Anti-S100 protein (S100) antibody (polyclonal rabbit anti-S100 antibody, purified immunoglobulin fraction including IgG, Z0311 Dako Japan) was used at 1:1,000 dilution as substitute isotype control for the anti-IL-17RA antibody.

Table 1 Comparison among cellular and serum datasets concerning IL-17A in LCH

Authors [Ref.]	LCH cell				Serum IL-17A (protein)
	IL-17A		IL-17RA		
	mRNA	Protein	mRNA	Protein	Levels; detection method
Coury et al. [22]	N.E.	Detected[a]; immunofluorescence	N.E.[b]	N.E.	High levels; ELISA kit (Peprotech)
Allen et al. [23, 24]	Not detected; microarray	Not detected; Western blotting	Not mentioned[c]; microarray	N.E.	Low levels[d]; human IL17A Quantikine ELISA system (R&D)[d]; human IL17A ELISA system (eBioscience)
This paper	N.E.	0 of 1 LCH patient LC/MS and LC/MRM-MS[e]	N.E. detected[c]; re-analysis of GSE16395) [24]	Detected, (higher in LCH-MS than in LCH-SS); immunofluorescence	High levels, (no significant difference between LCH-MS and LCH-SS); Bio-Plex suspension array system (Bio-Rad)

DC dendritic cell, *ELISA* enzyme-linked immunosorbent assay, *LCH* Langerhans cell histiocytosis, *LCH cell* Langerhans cell-like abnormal cell, *LC/MRM-MS* liquid chromatography/multiple reaction monitoring-mass spectrometry, *LC/MS* liquid chromatography/mass spectrometry, *N.E.* not examined

[a] Allen et al. showed that immunofluorescence signals were due to nonspecific reactivity [23–25]

[b] Coury et al. assessed IL-17RA mRNA in healthy DCs (not LCH cells) and showed that IL-17A signaling alone did not change IL-17RA transcription levels

[c] We re-analyzed the GSE16395 microarray dataset submitted by Allen et al. [24], using the Subio Platform

[d] Makras et al. indicated high serum IL-17A levels using same ELISA kit [26]

[e] We used an LTQ Orbitrap XL (Thermo Fisher) and a QTRAP 5500 (AB SCIEX) with MRM for IL-17A. Samples were prepared by laser microdissection using a LMD7000 (Leica) and protein extraction using the Liquid Tissue™ MS Protein Prep kit (Expression Pathology)

Immunofluorescence for interleukin-17A receptor

The slides were incubated for 1 h in 5 % skim milk, then primary antibodies were applied against both IL-17RA and CD1a, and the slides were maintained for 3 h at 37 °C. After washing, incubation with the first antibodies, second antibodies, Alexa Fluor® 555 goat anti-mouse IgG (H +L), and Alexa Fluor® 488 goat anti-rabbit IgG (H + L) (Molecular Probes, Inc., Eugene, Oregon, USA) was done for 1 h at room temperature. Images were captured using a confocal laser microscope TCS SP2 (Leica Microsystems GmbH, Wetzler, Germany). Samples of dermatopathic lymphadenopathy, synovia of rheumatoid arthritis, or of normal skin were used throughout as controls as appropriate. Primary antibody against S100 was also applied instead of the primary antibody against IL-17RA in several similar slides.

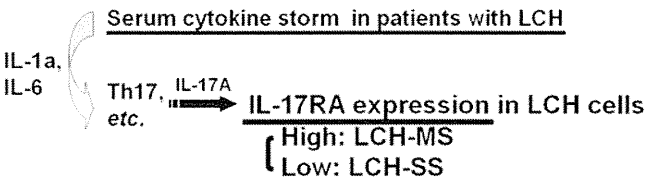

Fig. 1 The endocrine model of LCH is based on our own data: high IL-17A serum levels exist in both LCH-MS and LCH-SS; higher expression of IL-17RA is observed in LCH cells in LCH-MS as compared to LCH-SS. LCH tissues produce cytokines, including IL-1a and IL-6, leading to a cytokine storm that stimulates IL-17A-producing cells. Abbreviations: *IL-17A* interleukin-17A, *IL-17RA* IL-17A receptor, *LCH* Langerhans cell histiocytosis, *LCH cell* LC-like abnormal cell, *LCH-MS* multisystem LCH, *LCH-SS* single-system LCH, *Th17* T helper type 17 cells

Laser capture microdissection and protein extraction

An LCH lesion (FFPE tissue) was identified on serial sections stained with hematoxylin and eosin and for CD1a by immunohistochemistry. For proteomic analysis, a 10-μm-thick section prepared from the same tissue block was attached onto DIRECTOR™ Slides (Expression Pathology, Gaithersburg, MD, USA), de-paraffinized twice with xylene for 5 min, rehydrated with graded ethanol solutions and distilled water, and then stained by only hematoxylin. The stained uncovered slide was air-dried, and about 30,000 LCH cells were collected into 200 μl low-binding plastic tube using a Leica LMD7000 (Leica Microsystems). Protein extraction was performed using the Liquid Tissue MS Protein Prep kit ((Expression Pathology, http://www.expressionpathology.com/).

Liquid chromatography/mass spectrometry (LC/MS)

Peptide-mixture samples processed from microdissected FFPE LCH tissues were used for nanoflow reverse phase liquid chromatography followed by tandem MS, using an LTQ linear ion-trap mass spectrometer (Thermo Fischer, San Jose, CA, USA) [32]. All MS/MS spectral data were

Table 2 IL-17RA Intensity scores (IS) in patients with LCH

Patient	Age	Sex	Subtype	Biopsy site	IS (IL-17RA)
1000202	71 years	M	SS	Skin	22
1000219	2 years 11 months	M	SS	Bone	14
1000222	5 years 1 month	F	SS	Bone	44
1000227	1 year 5 months	M	SS	Bone	24
1000275	6 years 6 months	M	SS	Bone	80
130015	32 years	M	SS	LN	45
130016	61 years	M	SS	Lung	45
130019	86 years	F	SS	Bone	48
130025	5 months	F	SS	Skin	93
1000201	5 months	M	MS	Bone	35
1000216	1 year 1 month	F	MS	LN	25
1000230	4 months	M	MS	Skin	90
1000234	4 years 2 months	F	MS	Bone	36
1000277	0 month	M	MS	Skin	132
13009	8 years	F	MS	Bone	88
130014	7 years	F	MS	Skin, LN	137
130021	39 years	F	MS	Skin	100
100051	6 years	F	MS	Bone	113
130028	6 months	F	MS	Skin	110

The median age of the LCH-SS patients (n=9) was 6 years 6 months old (range, 5 months–86 years old). The median age of the LCH-MS patients (n=10) was 2 years 7 months old (range, 0–39 years old)

LN lymph node

searched against the SwissProt 55.6 Homo sapiens database (20,009 entries) using Mascot (version_2.1.04, Matrix Science, London, UK), in which the peptide and fragment mass tolerances were 2.0 and 0.8 Da, respectively. Reported results were obtained from triplicate LC/MS runs for each sample with all peptide hits included.

Liquid chromatography/multiple reaction monitoring-mass spectrometry (LC/MRM-MS)

Targeted analyses of human IL-17A (Research grade, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) by LC/MRM-MS were done. The LC/MRM-MS system used was DiNa nanoLC autoinjection system (KYA Technologies Corporation, Tokyo, Japan) coupled with a Q TRAP 5500 triple quadrupole linear ion trap mass spectrometer (ABI SCIEX, Carlsbad, CA, USA). Peptide-mixture samples processed from microdissected FFPE LCH tissues were analyzed using LC/MRM-MS system after targeted analyses of IL-17A by this system.

Digital image capture

Digital images for IL-17RA with the CD1a-positive area of the sections as representative of the overall immunostaining were acquired with a TCS SP2 confocal laser microscope (Leica Microsystems). All images were obtained using a× 40 objective of the DMIRE2 inverted microscope (Leica Microsystems). The software used was the Leica Confocal Software 2004 (Leica Microsystems). During the entire study, lighting conditions were maintained to keep the image intensity at an identical level. Files were saved in uncompressed TIFF format (size fixed to 289 kb).

Quantification of immunofluorescence

The immunofluorescence intensity was determined using Adobe Photoshop, version 5.0 J (Adobe Systems Incorporated, Japan office, Tokyo, Japan) [33, 34]. Using the magic wand tool in the select menu of Photoshop, the cursor was placed on IL-17RA-positive cytoplasm. The tolerance level of the magic wand tool was adjusted so that the entire positive cytoplasm was selected automatically. This selection was confirmed by checking both the selected figure and CD1a-positive cells. The mean staining intensity was calculated as follows: intensity score (IS) = mean of brightness of selected cells' green channel score (in arbitrary units, AU) using Adobe Photoshop, version 5.0 J (Fig. 2).

Re-analysis of GSE16395 data by Subio platform

In 2010, Allen et al. analyzed cell-specific gene expression in LCH lesions compared with epidermal LC and submitted the data to the Gene Expression Omnibus Web site under accession number GSE16395 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16395) [24]. We downloaded and re-analyzed using the Subio platform (http://www.subio.jp/products/platform). Before re-analysis, we re-categorized the cases into two subclasses of LCH-SS (n=8) and LCH-MS (n=5) from the four categories of LCH (group 1, multisystem risk patients (n=3); group 2, multisystem low-risk patients (n=2); group 3, patients with single-system "multifocal bone disease" or localized "special-site involvement" (n=1); and group 4, n=7) used by Allen et al. Among the four categories, three categories (groups 1–3) correspond to groups used in the Histiocyte Society LCH-III treatment protocol categories based on sites of LCH lesions [2, 35]. Group 4 included patients with single nonrisk lesions [24].

Measurement of serum humoral factors

Collected serum samples at diagnosis of LCH were stored until assay at −80 °C. Humoral factors of LCH patients were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA, USA) (2010 Histiocyte Society Meeting abstract: [56]). We chose the following 48 serum humoral factors: IL-1α, IL-1β, IL-1Ra, IL-2, IL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-

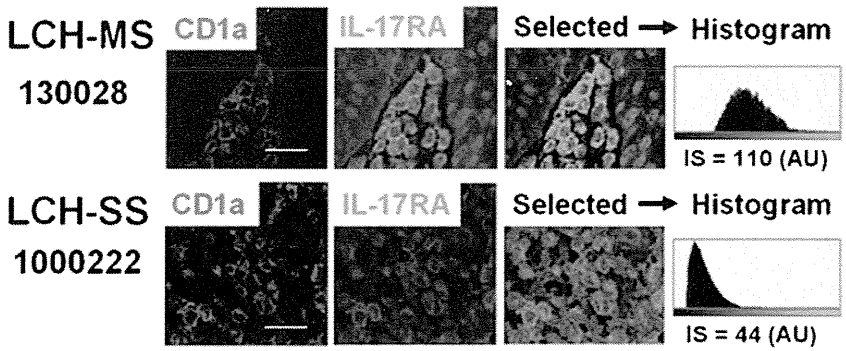

Fig. 2 Detection of IL-17RA signals from CD1a-positive LCH cells. Digital images for IL-17RA with the CD1a-positive area of the sections as representative of the overall immunostaining were acquired with the confocal laser microscope TCS SP2 (Leica Microsystems). The mean staining intensity was calculated as follows: intensity score (IS)=mean of brightness of selected cells' green channel score (in arbitrary units, AU) using Adobe Photoshop, version 5.0 J. *Scale bars*, 25 μm. Abbreviations: *IL-17RA* interleukin-17A receptor, *LCH* Langerhans cell histiocytosis, *LCH cell* LC-like abnormal cell, *LCH-MS* multisystem LCH, *LCH-SS* single-system LCH

12p70, IL-12p40, IL-13, IL-15, IL-16, IL-17, IL-18, G-CSF, M-CSF, GM-CSF, SCF, LIF, IFN-α2, IFN-γ, TNF-α, TNF-β, TRAIL, CXCL1, CXCL9, CXCL10, CXCL12, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL27, MIF, Basic FGF, HGF, β-NGF, PDGF-BB, SCGF-β, and VEGF.

Statistical analysis

In LC/MS analysis, a P value less than 0.05 was considered to indicate a statistically significant difference. Comparisons of immunofluorescence data of IL-17RA between LCH-SS and LCH-MS were performed using the Mann–Whitney U test. In the re-analysis of GSE16395, data were analyzed by the Mann–Whitney U test with the false discovery rate controlled by the Benjamini–Hochberg procedure (BH-FDR) at <0.10 or the Mann–Whitney U test. Comparisons of serum data were performed by the Mann–Whitney U test. Differences between values were considered statistically significant at $P<0.05$. Comparisons among sera of LCH-SS, LCH-MS, and controls were performed by the Mann–Whitney U test with Bonferroni adjustment. Differences between values were considered statistically significant at $P<0.0167=0.05/3$.

Results

Diagnosis of Langerhans cell histiocytosis

Histological sections and immunohistochemistry such as CD1a of all specimens were reviewed by pathologists to confirm the diagnosis [36].

Interleukin-17A in Langerhans cell histiocytosis tissue

Allen et al. showed that immunofluorescence signals for IL-17A in tissue analyses described by Coury et al. [22] were due to nonspecific reactivity [23–25]. We confirmed this result by LC/MS and LC/MRM-MS (0/1) (Table 1). The limit of detection for IL-17A by LC/MRM-MS was 20 pg (1.3 fmol (as monomer))/peptide mixtures of 740 cells.

Interleukin-17A receptor in Langerhans cell histiocytosis tissue

Next, we detected higher levels of IL-17RA protein expression in LCH-MS (n=10) as compared to LCH-SS (n=9) using a double immunofluorescence stain for both CD1a and IL-17RA (Table 2, Figs. 2 and 3). LCH cells in LCH

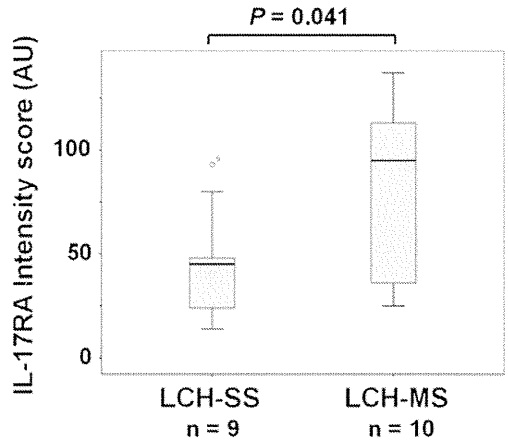

Fig. 3 Immunofluorescence data providing the IS of IL-17RA expression for LCH-MS and LCH-SS samples were plotted as box-whisker plots by the PASW Statics 18 program (Mann–Whitney U test, P=0.041; IBM Japan, Tokyo, Japan). The median IS data are 45 and 95, lower quartiles are 24 and 36, and upper quartiles are 48 and 113 in LCH-SS and LCH-MS, respectively. Abbreviations: *IL-17A* interleukin-17A, *IL-17RA* IL-17A receptor, *IS* intensity score, *LC* Langerhans cell, *LCH* Langerhans cell histiocytosis, *LCH cell* LC-like abnormal cell, *LCH-MS* multisystem LCH, *LCH-SS* single-system LCH, *Th17* T helper type 17 cells

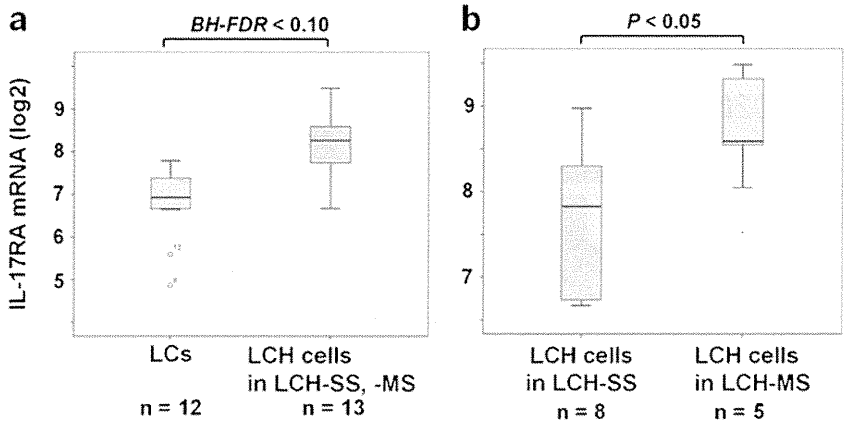

Fig. 4 Relationship between mRNA IL-17RA expression and LCH cells compared to LCs. LCH cells in LCH (both LCH-SS and LCH-MS) yielded 1,410 genes which were more than twofold higher in LCH cells (n=13) as compared to LCs (n=12) among 54,682 genes (Mann–Whitney U test, BH-FDR<0.10). LCH cells in LCH-MS yielded six genes which were more than twofold higher in LCH cells in LCH-SS among 1,410 genes (Mann–Whitney U test, P<0.05). IL-17RA mRNA expression was included among six genes. **a** IL-17RA mRNA expression in LCs and LCH cells from both LCH-SS and LCH-MS patients was plotted as box-whisker plots by the PASW Statics 18 program (IBM Japan). **b** IL-17RA mRNA expression in LCH cells from both LCH-SS and LCH-MS patients was plotted as box-whisker plots by the PASW Statics 18 program. The median IL-17RA mRNA (log2) data are 6.927, 8.261, 7.826, and 8.586; lower quartiles are 6.669, 7.740, 6.742, and 8.584; and upper quartiles are 7.310, 8.586, 8.281, and 9.316 in LCs; LCH cells in LCH (LCH-SS and LCH-MS); LCH cells in LCH-SS; and LCH cells in LCH-MS, respectively. Abbreviations: *BH-FDR* false discovery rate controlled by the Benjamini–Hochberg procedure, *IL-17RA* interleukin-17A receptor, *LC* Langerhans cell, *LCH* Langerhans cell histiocytosis, *LCH cell* LC-like abnormal cell, *LCH-MS* multisystem LCH, *LCH-SS* single-system LCH

(both LCH-SS and LCH-MS) yielded among 54,682 genes 1,410 genes with expression levels more than twofold higher in LCH cells (n=13) as compared to LCs (n=12) (Mann–Whitney U test, BH-FDR<0.10; Fig. 4). LCH cells in LCH-MS yielded six genes with expression levels more than twofold higher in LCH cells in LCH-SS among 1,410 genes (Mann–Whitney U test, P<0.05). IL-17RA mRNA expression was included among six genes. In addition among 1,410 genes, matrix metalloproteinase 3 (MMP3) and MMP12 were included.

Table 3 Serum IL-17A in patients with non-LCH as controls

Control	Age (years)	Condition	IL-17A (pg/ml)
C1	0.3	Non-LCH patient under follow-up with inactive state	0
C2	0.5	Non-LCH patient under follow-up with inactive state	0
C3	0.8	Non-LCH patient under follow-up with inactive state	0
C4	1	Non-LCH patient under follow-up with inactive state	0
C5	1	Non-LCH patient under follow-up with inactive state	117
C6	2	Non-LCH patient under follow-up with inactive state	0
C7	3	Non-LCH patient under follow-up with inactive state	0
C8	3	Non-LCH patient under follow-up with inactive state	0
C9	4	Non-LCH patient under follow-up with inactive state	0
C10	6	Non-LCH patient under follow-up with inactive state	544
C11	7	Non-LCH patient under follow-up with inactive state	0
C12	7	Non-LCH patient under follow-up with inactive state	0
C13	8	Non-LCH patient under follow-up with inactive state	252
C14	8	Non-LCH patient under follow-up with inactive state	243
C15	8	Non-LCH patient under follow-up with inactive state	0
C16	9	Non-LCH patient under follow-up with inactive state	0
C17	11	Non-LCH patient under follow-up with inactive state	365
C18	11	Non-LCH patient under follow-up with inactive state	0
C19	14	Non-LCH patient under follow-up with inactive state	0
C20	15	Non-LCH patient under follow-up with inactive state	171

The median age of the non-LCH patients (n=20) was 6.5 years old (range, 0.3–15 years old)

IL-17A interleukin-17A, *LCH* Langerhans cell histiocytosis

Serum interleukin-17A

We found that the serum levels of IL-17A were higher in LCH as compared to controls (P=0.005) with no significant differences among LCH subclasses (Tables 3, 4, and 5, Fig. 5).

Discussion

Our study resulted in three major findings. First, serum levels of IL-17A were higher in LCH as compared to controls with no significant differences among LCH subclasses. Second, higher levels of IL-17RA protein expression in LCH-MS were detected as compared to LCH-SS. Third, our results using LC/MS and LC/MRM-MS did not confirm the presence of IL-17A in LCH cells. An endocrine model reproduced our data: the IL-17A serum levels and expression levels of IL-17RA are higher in LCH tissue in patients with LCH. Accordingly, we postulate that IL-17RA expression defines the LCH subclass (Fig. 1).

We regard LCH as a reactive and neoplastic disorder that is influenced by environmental triggers such as pathogens or smoking. IL-17A is one of the proinflammatory cytokines acting against infection. A high serum IL-17A level might be taken to indicate the possibility of an infection in relation to LCH. Serum of patients with LCH might be related to up-regulation of IL-17RA in LCH cells as well as in healthy monocyte-derived dendritic cells. This hypothesis could clarify the IL-17A paradox presented by Coury et al. [22].

In general, cytokines work within autocrine or paracrine mechanisms; however, IL-3 and some proinflammatory cytokines exhibit endocrine mechanisms [30, 37, 38]. In our analysis of tissue, the results using LC/MS and LC/MRM-MS (Figs. 2 and 3) confirm the observation that the IL-17A reactivity in LCH cells observed by immunofluorescence [22] is due to nonspecific antibody binding as described by Allen et al. [23–25]. We solved this problem by adding data of IL-17RA mRNA expression (Fig. 4) uploaded by Allen et al. [24].

Generally, stimuli are recognized by receptors such as Toll-like receptors in LCs [29, 39]. LCH cells also express these receptors in the GSE16395 dataset [24]. LCH cells that are in an active state [12] can induce IL-17A producers in a similar manner as activated LCs promote Th17 polarization [29]. It would be useful to evaluate CD4/CD8 ratio

Table 4 Clinical characteristics and serum IL-17A in patients with LCH-SS

Patient	Age (years)	Site of involvement	Subtype	IL-17A (pg/ml)
X11	11	Bone	SS	0
X20	1	Bone	SS	333
X21	2	Bone	SS	653
X25	5	Bone	SS	364
X28	9	Bone	SS	99
X33	4	Bone	SS	163
X10	14	Bone	SS	989
X13	0.9	Bone	SS	50
X24	12	Bone	SS	647
X26	5	Bone	SS	0
X27	2	Bone	SS	397
X29	7	Bone	SS	163
X31	5	Bone	SS	0
X37	12	Bone	SS	0
X19	1	Skin	SS	381

The median age of the LCH-SS patients (n=15) was 5 years old (range, 0.9–14 years old)

IL-17A interleukin-17A, *LCH* Langerhans cell histiocytosis, *LCH-SS* single-system LCH

Table 5 Clinical characteristics and serum IL-17A in patients with LCH-MS

Patient	Age (years)	Site of involvement	Subtype	IL-17A (pg/ml)
X12	1	Bone, lung	MS	438
X8	1	Bone, LN, spleen	MS	101
X6	1	Skin, liver, spleen, lung	MS	0
X2	7	Skin, bone, pituitary gland	MS	193
X9	0.8	Skin, bone, liver, spleen	MS	21
X4	4	Skin, bone, pituitary gland, CNS	MS	0
X5	11	Bone, pituitary gland	MS	0
X14	0.5	Skin, liver, spleen, bone	MS	0
X3	0.4	Skin, bone, soft tissue	MS	372
X16	0.8	Skin, bone, thymus	MS	510
X17	0.9	Skin, bone, soft tissue	MS	319
X22	1	Skin, liver, spleen, lung, LN	MS	205
X18	1	Bone, LN	MS	803
X38	2	Bone, soft tissue, CNS	MS	0
X42	0.7	Skin, bone	MS	559
X39	0.5	Skin, soft tissue	MS	47
X34	0.9	Skin, bone, LN	MS	0
X36	0.4	Skin, bone, liver, lung, BM, etc.	MS	124
X32	6	Bone, hypothalamus	MS	409
X41	9	Bone, LN, pineal gland	MS	357
X35	4	Skin, bone, liver, spleen, CNS, etc.	MS	0
X40	12	Bone, esophagus, soft tissue	MS	33
X30	3	Skin, bone, pituitary gland	MS	634

The median age of the LCH-MS patients (n=23) was 1 year old (range, 0.4–12 years old)

CNS central nervous system, *IL-17A* interleukin-17A, *LCH* Langerhans cell histiocytosis, *LCH-MS* multisystem LCH, *LN* lymph node

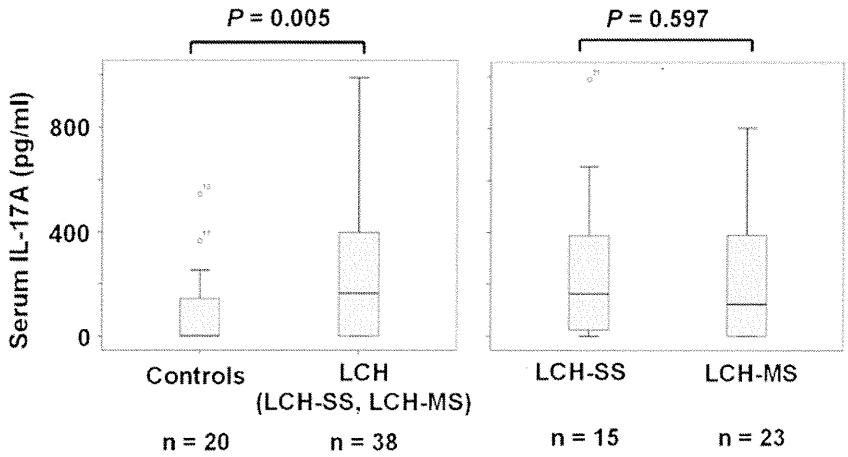

Fig. 5 Relationship between the serum IL-17A levels and LCH compared to controls. **a** Serum IL-17A in controls and LCH patients were plotted as box-whisker plots by the PASW Statics 18 program (Mann–Whitney *U* test, P=0.005; IBM Japan). **b** The serum levels of IL-17A were higher in LCH as compared to controls with no significant differences among LCH subclasses. The median age of the controls (n=20) was 7 years old (range, 0–15 years old). The median age of the LCH patients (n=38) was 2 years old (range, 0–14 years old). The median age of the LCH-SS patients (n=15) was 5 years old (range, 1–14 years old). The median age of the LCH-MS patients (n=23) was 1 year old (range, 0–12 years old). Serum levels of IL-17A were higher in each subclass of LCH as compared to controls (Mann–Whitney *U* test with Bonferroni adjustment: LCH-SS, P=0.012; LCH-MS, P=0.015). The median serum IL-17A level data are 0, 163, 163, and 124; lower quartiles are 0, 0, 25, and 0; and upper quartiles are 131, 392, 389, and 390 in controls; in patients with LCH (LCH-SS and LCH-MS); in patients with LCH-SS; and in patients with LCH-MS, respectively. Abbreviations: *IL-17A* interleukin-17A, *LCH* Langerhans cell histiocytosis, *LCH-MS* multisystem LCH, *LCH-SS* single-system LCH

and assess Th17 in the blood of LCH patients compared to healthy individuals. Though Allen et al. showed low serum levels of IL-17A [23, 25], Makras et al. showed high serum levels of IL-17A using the same enzyme-linked immunosorbent assay (ELISA) kit in both patients with LCH and controls without significant difference [26].

As IL-17RA is ubiquitously expressed [22, 29], it might be difficult to detect IL-17A in the blood as Delprat et al. replied to Allen et al. [23]. We analyzed sera using a Bio-Plex suspension array system (Bio-Rad), which is different from other ELISA systems [23, 25, 26]. We found that the serum levels of IL-17A were higher in LCH as compared to controls (P=0.005) with no significant differences among LCH subclasses (Fig. 5).

For host defense, IL-17A/IL-17RA is important [31]. IL-17A is commonly produced during viral infection [40]. In LCH, an overreaction by mutated LCH cells on stimuli such as viral infection might occur, including increased IL-17RA expression. In the context of infection, pathogens such as Epstein–Barr virus [41], human cytomegalovirus [42], and human herpes virus 6 [43, 44] were proven to exist in LCH cells. But they were regarded as bystander of the LCH lesion in a case-controlled sero-epidemiological study and in situ analysis [2, 45].

As reported in LCH tissue [2, 11, 13, 17, 46], serum levels of IL-1a and IL-6, which are known to stimulate Th17 [29], were also significantly higher as compared to controls (P< 0.05) (data not shown; Fig. 1). Our own analyses on LCH tissues using LC/MS and LC/MRM-MS could not confirm IL-17A positivity in LCH cells (i.e., the IL-17A autocrine model in LCH) [22]. Rather, we propose an IL-17A endocrine model and stress that changes in IL-17RA expression levels are important for defining LCH subclasses (Fig. 1). Low IL-17A levels in sera are maintained by γδT cells in emergencies such as infection [30]. Allen et al. also showed that CD3-positive cells in tonsils produced IL-17A (4/4) [23, 24].

IL-17A/IL-17RA signaling pathways include MMP3 or MMP12 [47–49]. These MMP3 and MMP12 belong to 1,410 genes, the levels of which were more than twofold higher in LCH cells as compared to LCs in the re-analysis of GSE16395 mRNA data. These higher expression levels of MMP3 and MMP12 not only confirm IL-17A/IL-17RA signaling roles in LCH cells but also explain the inflammatory process of LCH such as bone absorption and accumulation of eosinophils [50–52].

In summary, LCH is a neoplastic disorder driven by genetic abnormalities such as BRAF mutation [7] and the severity of LCH might be driven by an inflammatory process such as a cytokine storm, especially involving IL-17A/IL-17RA signaling pathways. In the future, stimuli that govern IL-17A or IL-17RA production might serve as therapeutic targets to stop LCH progression, similar to cessation of smoking which induces pulmonary LCH regression [2, 53]. In contrast, IL-17A-targeted treatments are now in preclinical development [19, 26, 54]. As a potential treatment of LCH-MS, we indicate the possibility of the use of an IL-17RA-targeted therapy such as brodalumab, which is currently in phase 2 clinical trials with known safety profiles [55].