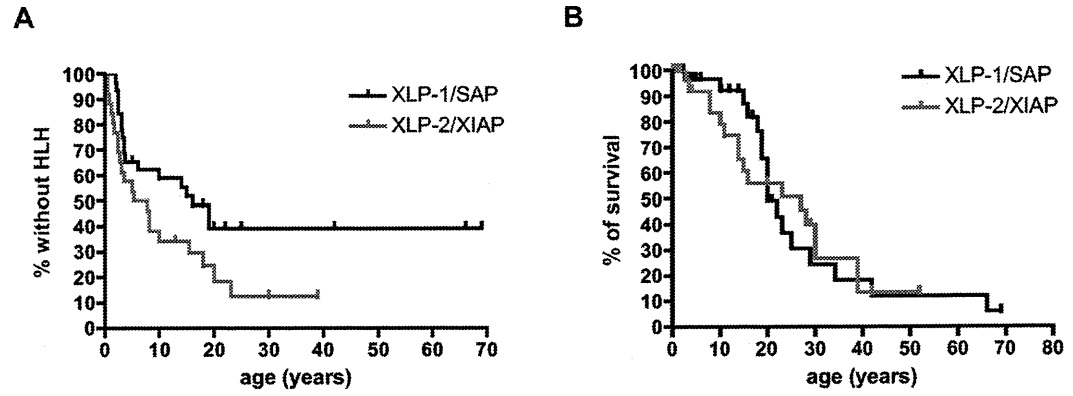

Figure 1. Comparison of HLH phenotypes and survival curves of SAP-deficient (XLP-1) and XIAP-deficient (XLP-2) patients. Kaplan-Meier survival curves were constructed on the basis of data presented in Table 1 and Table 2. Statistical analyses with log-rank tests. (A) Percentage of XLP-1/SAP and XLP-2/XIAP patients without HLH phenotype (P = .099). (B) Overall survival curves for XLP-1/SAP and XLP-2/XIAP patients (P = .948).

develop HLH, the others subsequently developed HLH within a period of time, varying from a few months to 19 years. In 2 XIAP-deficient patients, transient pancytopenia with splenomegaly was noticed after vaccinations against measles, mumps, and rubella or measles and rubella. Importantly, none of the XIAP-deficient patients developed B-cell lymphoproliferative disease.

PX4.1 underwent splenectomy at the age of 21 years, and histopathologic examination of the spleen showed reduced white pulp areas, and red pulp was extended with a mild fibrosis (supplemental Figure 2 top left). In the white pulp, most of the lymphocytes were $CD20^+$, whereas in the red pulp there was an accumulation of $CD3^+$ T cells that were mostly $CD8^+$ and cytotoxic (T-cell intracellular antigen-1^+; data not shown; supplemental Figure 2 bottom). Strikingly, features of hemophagocytosis were observed in the red pulp (supplemental Figure 2 upper right). Lymphocytes were negative for LMP-1 with very rare $EBER^+$ cells, suggesting that the infiltration was not related to EBV infection (data not shown). Altogether, these observations strongly suggest that these lymphoproliferative manifestations can be regarded as incomplete or attenuated forms of HLH.

In addition, 3 XIAP-deficient patients had liver disease (2 patients with cholangitis and 1 patient with chronic liver failure). In 2 of the patients, the cholangitis was associated with colitis, which are known to overlap.[18] For patient PX1.7, histopathologic examination of the liver showed granulomatous hepatitis in lobular areas with foci of macrophages around necrotic hepatocytes (supplemental Figure 3). Staining for LMP-1 was negative (data not shown). It is unclear whether these liver diseases should also be considered as an incomplete form of HLH.

Table 3. Comparison of XLP-1 and XLP-2 phenotypes

	SAP-/Y, n (%)	XIAP-/Y, n (%)	P*
HLH	18 of 33 (55)	22 of 29 (76)	NS
HLH relapses (/HLH-survivors)	2 of 7 (29)	11 of 14 (79)	NS
EBV at first HLH	11 of 12 (92)	15 of 18 (83)	NS
Fatal HLH	11 of 33 (33)	5 of 30 (17)	NS
Fatal HLH (/HLH patients)	11 of 18 (61)	5 of 22 (23)	.0230
Hypogammaglobulinemia	14 of 21 (67)	8 of 24 (33)	.0377
Lymphoma	10 of 33 (30)	0 of 30 (0)	.0010
Cytopenias (in the absence of full-blown HLH)	4 of 33 (12)	11 of 21 (52)	.0020
Splenomegaly (in the absence of full-blown HLH)	2 of 29 (7)	20 of 23 (87)	<.0001
Hemorrhagic colitis	0 of 33 (0)	5 of 30 (17)	.0203

*Calculated with Fisher exact tests.

Lymphoma

Ten of 33 SAP-deficient patients (30%) and none of the 30 XIAP-deficient patients developed lymphoma (Tables 1-3; supplemental Figure 1B; ***P = .001). Mean age at diagnosis of lymphoma was 15 years (range, 2-40 years). Diagnoses were non-Hodgkin lymphoma (n = 9), including EBV-positive Burkitt lymphoma (n = 6) and EBV-negative (n = 3). Lymphomas were localized in the ileocecal (n = 5), cerebral[19] (n = 1), cervical (n = 2), and spinal (n = 2) regions, and for one the origin was not known. One patient (PS1.3) had a second lymphoma at the age of 30 years, 23 years after the first one, and one patient (PS15.3) had myelodysplasia.

Dysgammaglobulinemia

Hypogammaglobulinemia was documented in 14 SAP-deficient patients (14 of 21, 67%) and in 8 XIAP-deficient patients (8 of 24, 33%) (*P = .0377) (Tables 1-3). Thirty percent (10 of 33) of SAP-deficient patients and 13% (4 of 30) of XIAP-deficient patients received intravenous immunoglobulin (IVIG) substitution (P = .1357) (supplemental Figure 1C). Interestingly, hypogammaglobulinemia was transient in 2 of the 8 XIAP-deficient patients. PX3.1 was substituted with IVIG between the age of 23 and 35 years, currently, 4 years after stopping IVIG, immunoglobulin levels remain within the normal range, and the patient does not experience recurrent respiratory infections. Two XIAP-deficient patients developed hypergammaglobulinemia, with higher than normal IgA and IgM levels in PX9.3 and elevated IgG and IgM levels in PX11.1, respectively.

Severe infections were noted in several SAP- and XIAP-deficient patients with hypogammaglobulinemia before initiation of the IVIG substitution when treated. Ten of the 14 SAP-deficient and 4 of the 8 XIAP-deficient patients had recurrent respiratory tract infections. Rare severe infections caused by *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Cryptococcus neoformans* were also observed in SAP- and XIAP-deficient patients (supplemental Table 1).

Colitis

Chronic colitis with hemorrhagic diarrheas or rectal bleeding or both evoking inflammatory bowel disease was observed in 5 of 30 XIAP-patients (17%) but in none of 33 SAP-deficient patients (*P = .0203; Tables 1-3). In PX1.4, colitis initially responded to immunosuppressive treatment with corticosteroids and cyclosporine A. However, corticosteroids could not be withdrawn, and the

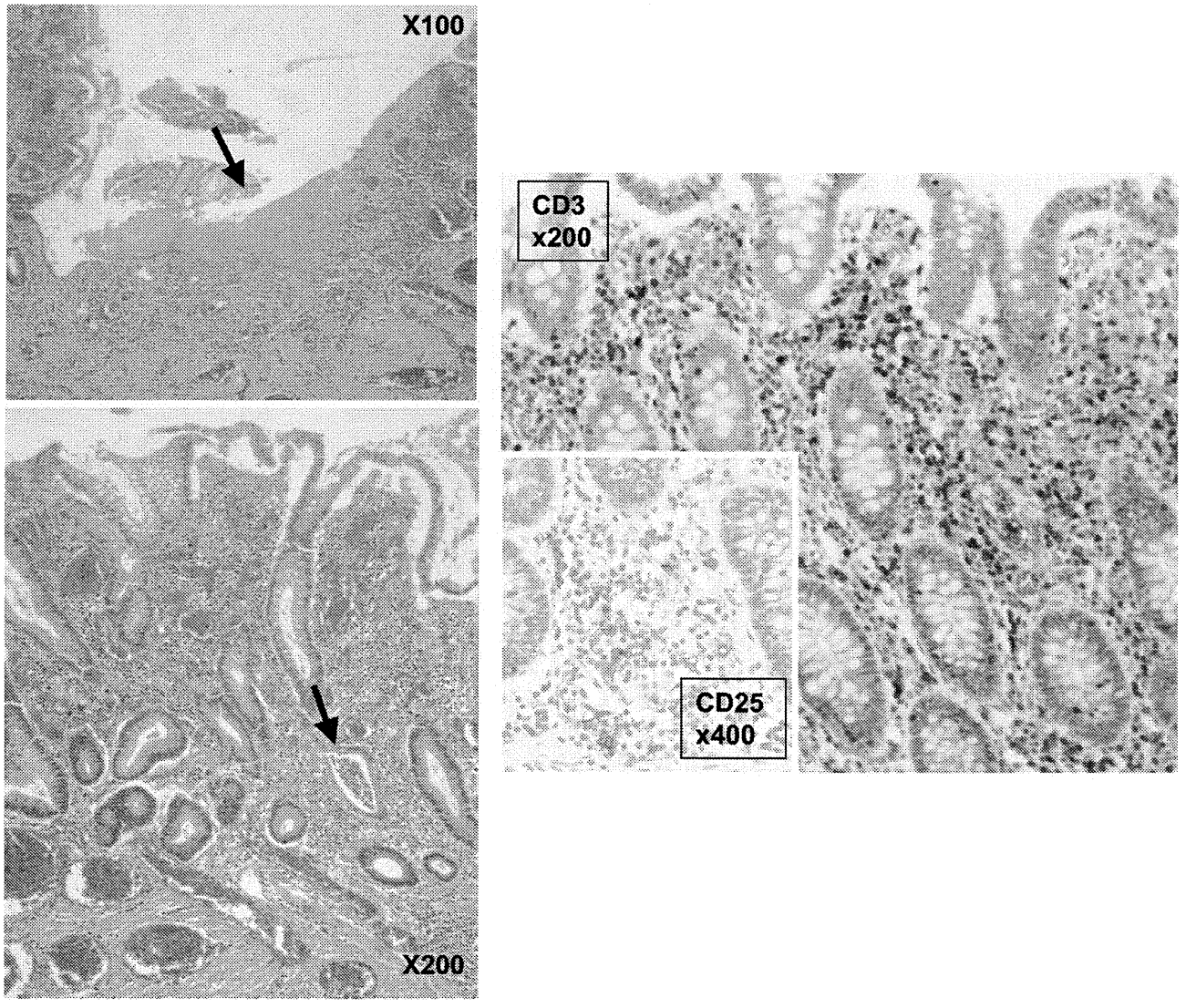

Figure 2. Histology of the large bowel of PX1.7 with XIAP deficiency. (Top left) On hematoxylin and eosin at low magnification (×100), a large ulceration is seen, indicated by an arrow. (Bottom left) Higher magnification (×200) shows a massive polymorphic inflammatory infiltrate associated with a crypt abscess (indicated by the arrow). (Central right) Immunostaining with anti-CD3 shows frequent lymphoid T cells (on the right, ×200), some of them express the activation marker CD25 (×400, inset).

addition of azathioprine could not prevent the recurrence of symptoms. Anti–tumor necrosis factor-α mAb treatment (infliximab) provided partial improvement. Recently, a colectomy was performed, but the patient now has terminal ileitis. In PX1.7, severe hemorrhagic colitis was associated with portal hypertension and massive gastroduodenal bleeding that lead to death of this patient. Patients PX9.6 and PX11.2 also suffered from chronic colitis and most probably died of intestinal hemorrhage.

Histopathologic examination of intestinal mucosa biopsy specimens was performed in 3 patients, PX1.4, PX1.7, and PX9.3. Representative images are shown in Figure 2. Hemorrhagic ulcerations of the colon associated with mononuclear infiltration consisting of lymphoid cells and plasma cells in the lamina propria were observed (Figure 2 left top). Crypt architecture was mostly preserved, except for rare crypt abscesses (Figure 2, left bottom), but frequent apoptotic crypt cells were seen (supplemental Figure 4). The lymphoid cells were mostly $CD3^+$ and $CD8^+$ with some lymphocytes expressing CD25 with numerous eosinophils (in PX1.4) (Figure 2; supplemental Figure 4). $CD20^+$ cells were rare, EBER staining was negative (not shown), and there was no granuloma formation. Microbiologic cultures were negative in all 3 cases.

Rare clinical manifestations

Rare clinical features (supplemental Table 1), each observed in 1 SAP-deficient patient, were hemolytic uremic syndrome associated with HLH, vasculitis, and arthritis. Clinical features, each observed in 1 XIAP-deficient patient, were Kawasaki syndrome and psoriasis. Additional infections in patients without hypogammaglobulinemia were caused by *Pseudomonas aeruginosa* (1 SAP-deficient patient), recurrent measles (1 XIAP-deficient patient), and HSV-(1 XIAP-deficient patient). Of note, 2 SAP-deficient patients (PS3.1 and PS3.2) had chronic gastritis.[20]

Survival and outcome

Sixteen of 33 SAP-deficient patients and 12 of 30 XIAP-deficient patients died at a mean age of 11 years (range, 2-69 years) and 16 years (range, 0.1-52 years), respectively. Survival rates did not differ between both patient groups ($P = .93$; Figure 1B), and the proportions of whom reached adulthood (age ≥ 16 years) were similar in both groups (17 of 33 SAP-deficient patients [52%] and

13 of 30 XIAP-deficient patients [43%]). Mortality was related to HLH (11 SAP- and 5 XIAP-deficient patients), lymphoma (2 SAP-deficient patients), myelodysplasia (1 SAP-deficient patient), colitis (3 XIAP-deficient patients), hepatitis (1 XIAP-deficient patient), complications of hematopoietic stem cell transplantation (2 SAP- and 4 XIAP-deficient patients), and pneumonia (1 XIAP-deficient patient). Mean age at last follow-up was 24.9 years (range, 10-66 years) for SAP-deficient patients and 17.5 years (range, 0.7- 39 years) for XIAP-deficient patients. Among the surviving 17 SAP-deficient patients, 4 are well without any treatment, 10 receive IVIG substitution, 2 are currently treated for a lymphoma, and 1 had successful hematopoietic stem cell transplantation. Among the surviving 17 XIAP-deficient patients, 10 are well without any treatment (among them 3 with splenomegaly), 2 received recently anti-CD20 antibody treatment because of EBV-related HLH, 2 are under IVIG substitution, 1 has terminal ileitis after colectomy, 1 has colitis treated with mesalazine and azathioprine, and 1 has recurrent HLH treated with cyclosporine A and dexamethasone. One XIAP-deficient and 2 SAP-deficient patients have never developed clinical signs and are considered to be asymptomatic.

Discussion

We report the first comparison of the clinical phenotypes of SAP- and XIAP-deficient patients. The present study was based on a retrospective analysis with data from medical records on 33 SAP- and 30 XIAP-deficient patients. The relatively small size of both cohorts obviously implies that data should be interpreted with caution.

The overall clinical phenotypes of the affected persons matched with the phenotypes previously reported.[2,7,9,21] In accordance to previous studies, we did not observe any genotype-phenotype correlation in the SAP-deficient patients. However, in our cohort of XIAP-deficient patients, we noticed that XIAP-deficient patients carrying non-null mutations had a tendency to be less prone to develop HLH by contrast to patients with null mutations. However, other genetic or environmental factors may contribute to the variety of phenotypes observed in XLP-1 and XLP-2.

HLH occurred both in SAP- and in XIAP-deficient patients but with more frequent neurologic involvement and fatal outcome in SAP-deficient patients than in XIAP-deficient patients. Splenomegaly often associated with cytopenia and fever was more frequent in XIAP-deficient patients than in SAP-deficient patients. Histologic analysis of one spleen showed accumulation of activated $CD8^+$ T cells and hemophagocytosis without EBV^+ cells. These symptoms probably represent incomplete forms of HLH. In addition, HLH relapses seemed to be more common in XIAP- than in SAP-deficient patients who survived HLH. Together, these findings suggest that HLH has a less severe disease course in XIAP- deficient patients than in SAP-deficient patients.

In most of the patients from both groups, the trigger of HLH was an EBV infection (> 80%); EBV may favor HLH by eliciting a potent CD8 T-cell response. It is also postulated that SAP and possibly XIAP are associated with activation pathways that are more important in triggering selective cytotoxicity toward B cells.[22-27] HLH in most hereditary conditions such as FHL, Griscelli syndrome type II, and Chediak-Higashi syndrome shares common pathophysiologic mechanisms, that is, global impaired cytotoxicity responses that lead to the inability of effector lymphocytes to kill infected cells and antigen-presenting cells.[28] In mice and humans, SAP-deficient $CD8^+$ T and NK cells exhibit defective cytotoxicity responses caused by abnormal functions of SLAM receptors.[29] This could explain the occurrence of HLH in SAP-deficient patients.[22-27] In contrast, NK-cell and T-cell cytotoxic responses appear to be preserved in XIAP-deficient patients[7,9] (C. Synaeve and S.L., unpublished data, 2009 and 2010). This might account for the lower severity of the HLH in the XIAP deficiency. Hence, the precise immune defects responsible for HLH in XIAP deficiency remain to be elucidated.

Only XIAP-deficient patients were at risk for chronic colitis with often a lethal outcome. This phenotype seems that is may be even worse than HLH, because the mortality in the group of patients with colitis (3 of 5) has a tendency to be higher than in the group with HLH (5 of 22). Histopathologic analysis of intestinal mucosal biopsy specimens showed an inflammatory process with an accumulation of activated T cells (and eosinophils in one patient) that could evoke inflammatory bowel disease. Interestingly, a recent report indicates that XIAP is involved in nucleotide-binding oligomerization domain containing 2 (NOD2) activation which is an intracellular pattern recognition receptor of the NOD-like receptor family.[30] Importantly, *NOD2* is a key susceptibility gene for Crohn disease.[31] Thus, defects in XIAP might lead to defective NOD2 responses as an additive risk factor for colitis in some of these patients. Of note, however, *NOD2* was sequenced in 2 XIAP-deficient patients with colitis, and none had the genotype shown to be a risk factor for Crohn disease (J.P. Hugot and S.L., unpublished data, June 2006).

One striking difference between XLP-1 and XLP-2 was that only SAP-deficient patients developed lymphoma, although it could not be formally excluded that XIAP-patients might develop lymphomas in the future. In SAP-deficiency, the occurrence of lymphomas may be explained by defective immunosurveillance of hematopoietic cells, resulting from alterations in SLAM receptor-mediated NK- and T-cell cytotoxicity responses,[22-24,26] but also by the proapoptotic functions that have been assigned to SAP.[32,33]

Another common finding shared by XLP-1 and XLP-2 is the hypogammaglobulinemia. Interestingly, 2 XLP-2 patients recovered from hypogammaglobulinemia, which so far seems not to be the case for XLP-1 patients. Numerous studies in mice and humans have documented that impaired antibody production found in XLP-1 resulted from a block in germinal center formation, leading to defects in the differentiation of Ig-isotype–switched memory B cells.[34-36] In most of the XIAP-deficient patients, Ig-isotype–switched memory B cells are not found to be decreased[7] (S. Siberil and S.L., unpublished data, 2008 and 2009). In XIAP deficiency, hypogammaglobunemia could be the consequence of increased activation-induced cell death of B cells, a hypothesis that needs to be tested.

In conclusion, the present comparison of the clinical features of SAP- and XIAP-deficient patients shows that SAP deficiency and XIAP deficiency share a main phenotype, that is, EBV-induced HLH. This similarity raises the possibility of a functional/molecular link between SAP and XIAP proteins. Alternatively, impairment of 2 independent pathways, both important in EBV immunity, could lead to a shared phenotype. Nevertheless, we also demonstrate that XLP-1 and XLP-2 can be distinguished on several clinical aspects, which could be helpful for diagnosis and therapeutic decisions.

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Authorship

Contribution: J.P.S. collected and analyzed the data and participated in study design, writing of the report, and patients' care; D.C. performed the immunohistochemistry experiments and analyzed histopathologic findings; F.H. participated in histopathologic analysis and writing of the report; C.L., N.L., and S.R. realized gene sequencing and protein expression tests; G.S.B. participated in data analysis; A.F. contributed to study design, data analysis, writing of the report, and patients' care; S.L. coordinated the study collected the data and contributed to sequencing, expression tests, data analysis, and wrote the report. The other authors provided and collected the clinical data on patients' status and contributed to the data analysis and patients' care.

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Clinical and Genetic Characteristics of XIAP Deficiency in Japan

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Abstract Deficiency of X-linked inhibitor of apoptosis (XIAP) caused by *XIAP/BIRC4* gene mutations is an inherited immune defect recognized as X-linked lymphoproliferative syndrome type 2. This disease is mainly observed in patients with hemophagocytic lymphohistiocytosis (HLH) often associated with Epstein–Barr virus infection. We described nine Japanese patients from six unrelated families with XIAP deficiency and studied XIAP protein expression, *XIAP* gene analysis, invariant natural killer T (iNKT) cell counts, and the cytotoxic activity of $CD8^+$ alloantigen-specific cytotoxic T lymphocytes. Of the nine patients, eight patients presented with symptoms in infancy or early childhood. Five patients presented with recurrent HLH, one of whom had severe HLH and died after cord blood transplantation. One patient presented with colitis, as did another patient's maternal uncle, who died of colitis at

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4 years of age prior to diagnosis with XIAP deficiency. Interestingly, a 17-year-old patient was asymptomatic, while his younger brother suffered from recurrent HLH and EBV infection. Seven out of eight patients showed decreased XIAP protein expression. iNKT cells from patients with XIAP deficiency were significantly decreased as compared with age-matched healthy controls. These results in our Japanese cohort are compatible with previous studies, confirming the clinical characteristics of XIAP deficiency.

Keywords X-linked lymphoproliferative syndrome · X-linked inhibitor of apoptosis · Epstein–Barr virus · hemophagocytic lymphohistiocytosis · invariant natural killer T cell

Abbreviations

BIR	Baculovirus IAP repeat
CTL	Cytotoxic T lymphocyte
HSCT	Hematopoietic stem cell transplantation
HLH	Hemophagocytic lymphohistiocytosis
IAP	Inhibitor of apoptosis
LCL	Lymphoblastoid cell line
MMC	Mitomycin C
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
iNKT	Invariant natural killer T
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
TCR	T cell receptor
XIAP	X-linked inhibitor of apoptosis
XLP	X-linked lymphoproliferative syndrome

Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency estimated to affect approximately one in one million males, although it may be underdiagnosed [1]. XLP is characterized by extreme vulnerability to Epstein–Barr virus (EBV) infection, and the major clinical phenotypes of XLP include fulminant infectious mononucleosis (60%), lymphoproliferative disorder (30%), and dysgammaglobulinemia (30%) [2]. In addition, XLP is associated with a variety of additional clinical phenotypes such as vasculitis, aplastic anemia, and pulmonary lymphoid granulomatosis. Patients with XLP often develop more than one of these phenotypes. The gene responsible for XLP was identified as *SH2D1A*, located on Xq25 and encoding the SLAM-associated protein (SAP) [3–5]. However, gene analysis revealed *SH2D1A* mutations in only 50–60% of presumed XLP patients [6]. Importantly, a mutation in the gene that encodes the X-linked inhibitor of apoptosis (XIAP) called *XIAP* or *BIRC4* was identified as a second causative gene for XLP [7]. *XIAP* is located close to the *SH2D1A* gene on the X chromosome and consists of six coding exons [8–10]. XIAP produces an anti-apoptotic molecule that belongs to the inhibitor of apoptosis (IAP) family proteins. It contains three baculovirus IAP repeat (BIR) domains that, together with flanking residues, bind to caspases 3, 7, and 9, thereby inhibiting their proteolytic activity [11].

The clinical presentations of XIAP-deficient patients have been frequently reported [7,12,13]. More than 90% of patients with XIAP deficiency develop hemophagocytic lymphohistiocytosis (HLH) which is often recurrent. Therefore, it was recently suggested that the phenotype of XIAP deficiency fits better with the definition of familial HLH than with XLP disease [12]. However, familial HLH is characterized by defects in $CD8^+$ T and NK cell cytotoxicity responses, while these responses are normal in XIAP deficiency [7,12]. Other symptoms of XLP, such as splenomegaly, hypogammaglobulinema, and hemorrhagic colitis, have been reported in patients with XIAP deficiency, but lymphoma has never been noted [7,12–15].

We searched for patients with XIAP deficiency in Japan by detection of *XIAP* gene mutations and flow cytometric assessment of lymphoid XIAP expression. We previously reported the first case of XIAP deficiency in Japan [14]. Thereafter, we identified eight additional cases from five families with XIAP deficiency in our country. In this study, we describe the clinical and laboratory findings from nine patients from six unrelated families with XIAP deficiency, including previous cases, to help further the understanding of the pathogenetic features of this disease.

Materials and Methods

Patient and Family Member Samples

Patients without indentified *SH2D1A* mutations but with presumed XLP phenotypes were screened for *XIAP* mutations. Their family members were also screened for the same mutation. Upon identification of *XIAP* mutations, the patients were enrolled in this study. Patient 2.2 passed away before a genetic diagnosis of XIAP deficiency was made, but he was the maternal uncle of patient 2.1 and had presented with a XLP phenotype (Table I). In the end, nine patients from six different families were found to have XIAP deficiencies, three of whom had been reported previously [13,14]. Upon the approval of the Ethics Committee of the University of Toyama and after obtaining informed consent, 5–10 mL heparinized venous blood was collected from the patients, their mothers, and 25 age-matched healthy children (1–13 years of age). All of the samples were

transferred to our laboratory at room temperature within 24 h for analysis.

Mutation Analysis of the *XIAP* Gene

DNA was extracted from peripheral blood using the QuickGene-Mini 80 nucleic acid extraction system (FUJIFILM Co., Tokyo, Japan). The coding regions and the exon–intron boundaries of the *XIAP* gene were amplified by polymerase chain reaction (PCR) using primers flanking each of the six exons by standard methods. PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. Sequencing analysis was performed on an Applied Biosystems Prism 310 Capillary Sequencer (Applied Biosystems).

Flow Cytometric Analysis of XIAP Protein Expression in Lymphocytes

XIAP protein expression was studied by flow cytometric techniques as previously described [16,17]. Peripheral blood mononuclear cells (PBMC) from patients 1, 2.1, 3.1, 3.2, 4, 5, 6.1, 6.2, and 25 age-matched healthy children were prepared by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA). The cells were first fixed in 1% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized in 0.5% saponin in washing buffer. The fixed and permeabilized cells were then incubated with an anti-XIAP monoclonal antibody (mAb) (clone 48 (BD Biosciences, Franklin Lakes, NJ, USA) or clone 2 F1 (Abcam, Cambridge, UK)) for 20 min on ice, washed, and then incubated with a FITC-labeled anti-mouse IgG1 antibody (SouthernBiotech, Birmingham, AL, USA) for 20 min on ice. The stained cells were analyzed on the FC500 flow cytometer (Beckman Coulter, Tokyo, Japan).

Western Blot Analysis of XIAP Protein Expression in Lymphocytes

PBMC from normal controls and patients 3.1, 5, and 6.2 were washed and pelleted. The cells were then lysed in 10 μL of lysing solution (1% Triton-X 100; 150 mmol/L NaCl; 10 mmol/L Tris–HCl, pH 7.6; 5 mmol/L EDTA-Na; 2 mmol/L phenylmethylsulfonyl fluoride) per 10^6 cells for 30 min on ice. The lysed cells were centrifuged for 10 min at 15,000*g* to remove nuclei, and the supernatants were diluted in the same volume of Laemmli's sample buffer. Samples were then electrophoresed in sodium dodecyl sulfate–polyarylamide 10% to 20% gradient gel and blotted on nitrocellulose filters. Blots were blocked in 5% skim milk in PBS for 1 h, treated with anti-XIAP mAb (clone 28 or clone 2F1) for 2 h, and then incubated with peroxidase-conjugated anti-mouse IgG antibody (Invitrogen, Grand Island, NY, USA) for 1 h. Immunoblots were developed by the ECL Western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, England).

Flow Cytometric Identification of Invariant Natural Killer T Cells

PBMC from eight patients (1, 2.1, 3.1, 3.2, 4, 5, 6.1, and 6.2) and 25 controls were incubated with fluorochrome-conjugated anti-CD3 (Dako Japan KK, Kyoto, Japan), anti-TCRVα24, and anti-TCRVβ11 mAbs (Beckman Coulter) to identify invariant natural killer T (iNKT) cells by flow cytometry. After the electronic gating of 100,000 $CD3^+$ T cells, iNKT cell populations were defined by the co-expression of TCRVα24 and TCRVβ11. The iNKT cell counts were evaluated at the diagnosis of XIAP deficiency.

Establishment of Alloantigen-Specific Cytotoxic T Lymphocyte Lines and Analysis of Cytotoxic T Lymphocyte-Mediated Cytotoxity

Alloantigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) lines were generated as described previously [18,19]. Briefly, PBMC were obtained from patients 1, 2.1, 3.1, and unrelated healthy individuals. These cells were co-cultured with a mitomycin C (MMC)-treated B lymphoblastoid cell line (LCL) established from an HLA-mismatched individual (KI-LCL). Using cell isolation immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA, USA), $CD8^+$ T lymphocytes were isolated from PBMC that had been stimulated with KI-LCL for 6 days. $CD8^+$ T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% human serum and 10 IU/mL interleukin-2 (Roche, Mannheim, Germany) and stimulated with MMC-treated KI-LCL three times at 1-week intervals. These lymphocytes were then used as $CD8^+$ alloantigen-specific CTL lines. The cytotoxic activity of CTLs was measured by a standard ^{51}Cr-release assay as described previously [20]. Briefly, alloantigen-specific CTLs were incubated with ^{51}Cr-labeled allogeneic KI-LCL or TA-LCL, which did not share HLA antigens with KI-LCL, for 5 h at effector/target cell ratios (E/T) of 2.5:1, 5:1, and 10:1. Target cells were also added to a well containing only medium and to a well containing 0.2% Triton X-100 to determine the spontaneous and maximum levels of ^{51}Cr release, respectively. After 5 h, 0.1 mL of supernatant was collected from each well. The percentage of specific ^{51}Cr release was calculated as follows: (cpm experimental release − cpm spontaneous release) / (cpm maximal release − cpm spontaneous release)×100, where cpm indicates counts per minute.

Table I Summary of our data

	Patient 1 [13]	Patient 2.1 [12]	Patient 2.2 [12]	Patient 3.1	Patient 3.2	Patient 4	Patient 5	Patient 6.1	Patient 6.2
Age at initial presentation	20 months	7 months	3 months	2 months	Asymptomatic	2 months	6 months	17 months	15 months
Current age	4 years	Deceased	Died of colitis	12 years	17 years	15 years	2 years	1 year	12 years
Family history	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes
HLH	+	+	−	+	−	−	+	+	+
Recurrent HLH	+	+	−	+	−	−	+	−	+
Fever	+	+	+	+	−	−	+	+	+
Splenomegaly	+	+	ND	−	−	−	−	+	+
Cytopenia	+	+	ND	+	−	−	+	+	+
EBV	+	−	ND	+	−	−	−	+	+
Hypogammaglobulinemia	−	+	ND	−	−	+	−	−	−
Colitis	−	−	+	−	−	−	+	−	−
Treatment	PSL	PSL		PSL			PSL, Dex		
	CsA	CsA	ND	CsA	−	IVIG	CsA, IVIG	IVIG, Dex	PSL
	Dex	Dex					Infliximab		
Allogeneic HSCT	−	+	−	−	−	−	−	−	−
Mutation	R238X	R381X	ND	W217CfsX27	W217CfsX27	E349del	Del of exons 1-2	N341YfsX7	N341YfsX7
XIAP protein expression	±	−	ND	−	−	+	±	±	±

HLH hemophagocytic lymphohistiocytosis, *ND* no data, *EBV* Epstein–Barr virus, *PSL* prednisolone, *CsA* cyclosporin A, *Dex* dexamethasone, *IVIG* intravenous immuglobulin, *HSCT* hematopoietic stem cell transplantation, + yes or positive, − no or negative, ± residual expression

Statistical Analysis

Student's *t*-test was used for statistics, with *P*-values <0.05 considered to be statistically significant.

Results

Clinical Manifestations of the Patients

Most of our patients presented with disease symptoms at very early ages; five patients presented in infancy and three patients presented in childhood (Table I). Three of the six families had family history records. Five of the nine patients had recurrent HLH, fever, splenomegaly, and cytopenia. EBV infection and hypogammaglobulinemia were also observed in multiple patients. Most patients with HLH were treated with corticosteroids with or without cyclosporin A to prevent an otherwise rapidly fatal disease course. Patients 2.2 and 5 presented with colitis, whereas patient 2.2 died; patient 5 improved with anti-TNF alpha mAb (infliximab®) treatment. Patient 2.1 underwent cord blood transplantation but died of complications. Patient 4 had a history of recurrent otitis media and pneumonia since 2 months of age, and he was found to have hypogammaglobulinemia. The patient was treated with intravenous immunoglobulin replacement therapy alone, and he is currently doing well. No patient developed lymphoma.

Detection of *XIAP* Mutations

We identified *XIAP* mutations in patients from all six unrelated families (Fig. 1) and analyzed all of the data using the US National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/SNP) to check for single-nucleotide polymorphism in the *XIAP* gene. As previously reported, patient 1 possessed a nonsense mutation, 712 C > T, resulting in an early stop codon R238X [14]. Patient 2.1 had a nonsense mutation in exon 5, 1141 C > T, resulting in R381X [13]. Patient 2.2 might have the same mutation as patient 2.1 because patient 2.2 was the maternal uncle of patient 2.1 [13]. Patients 3.1 and 3.2 were siblings and were found to have a one base pair deletion (650delG) in exon 1, resulting in a frameshift and premature stop codon (W217CfsX27). Patient 4 was found to have one amino acid deletion (1045_1047delGAG; E349del) in exon 3. Patient 5 has a large deletion, spanning exons 1 and 2. Patients 6.1 and 6.2 were brothers and had a two-nucleotide deletion (1021_1022delAA), which resulted in a frameshift and premature stop codon (N341YfsX7). All of the mothers of the patients from families 1–5 were heterozygote carriers of the mutations. Interestingly, we could not find any *XIAP* mutation in the mother of patients 6.1 and 6.2. We identified deleterious *XIAP* mutations in nine patients from six unrelated Japanese families that are likely to underlie their XLP phenotypes.

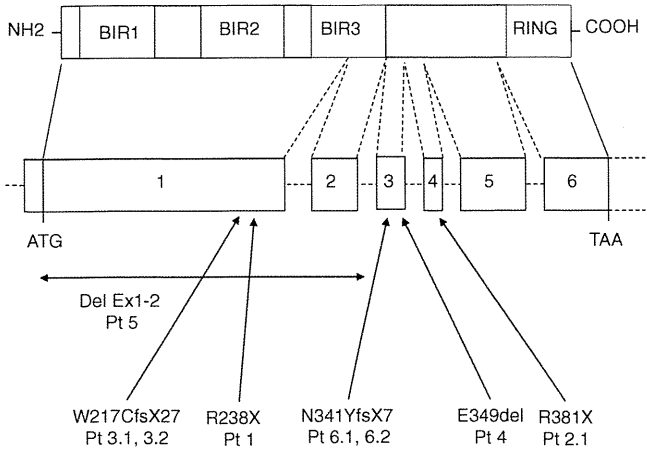

Fig. 1 *XIAP* gene mutations and their consequences for XIAP protein. *XIAP* comprises six exons and encodes the XIAP protein, which consists of 497 amino acids. XIAP contains three BIR domains and one RING domain. Mutations identified in our patients are indicated

XIAP Expression in Lymphocytes from the Patients and Carriers by Flow Cytometry

XIAP expression levels were analyzed in the lymphocytes of patients from all six families (Fig. 2). The lymphocytes of

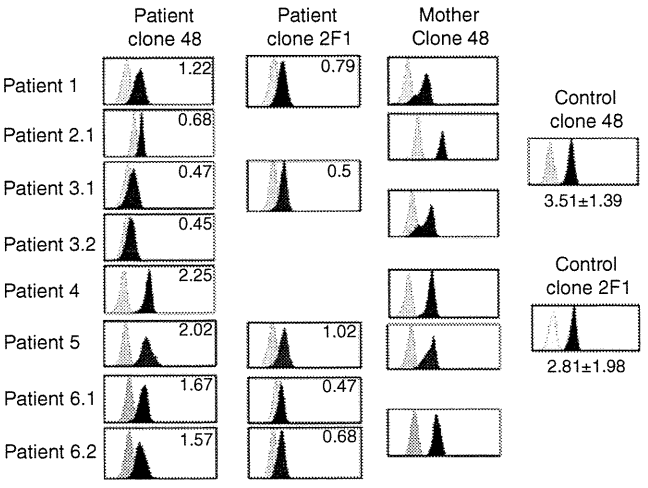

Fig. 2 XIAP protein expression in lymphocytes from the patients and their carriers. Flow cytometric detection of intracellular XIAP in lymphocytes from patients and their maternal carriers. The *gray* and *black areas* indicate the negative control and anti-XIAP staining, respectively. Anti-XIAP staining was performed using the clones 48 and 2 F1 antibodies where indicated. The *number in the box* indicates the log scale difference between the mean fluorescence intensity (ΔMFI) stained by the isotype antibody and that by the anti-XIAP antibodies. XIAP expression in 25 normal controls was also analyzed by the clone 48 and 2 F1 antibodies. The data of mean ± standard deviation of ΔMFI and each representative profile were shown

patients 1, 3.1, 5, 6.1, and 6.2 were examined by two different anti-XIAP mAbs. Using clone 48 antibody, patients 1, 2.1, 3.1, 3.2, 6.1, and 6.2 showed reduced XIAP expression, whereas XIAP was normally expressed in the lymphocytes of patients 4 and 5. In contrast to clone 48, clone 2F1 antibody showed reduced XIAP expression in patient 5. The effects of heterozygous *XIAP* mutations were studied in the lymphocytes of the patients' mothers by anti-XIAP mAb clone 48. The mothers of patients 1, 3.1, and 3.2 showed a bimodal pattern of XIAP protein (Fig. 2). The mothers of patients 2.1, 6.1, and 6.2 did not show a clear mosaic pattern, but all of these patients had reduced XIAP expression levels. Similarly to patients 4 and 5, the mothers of patients 4 and 5 demonstrated a normal XIAP expression pattern.

XIAP Expression in Lymphocytes from the Patients by Western Blot

Western blot analysis was used to evaluate the expression level of XIAP to determine the impact of patient *XIAP* mutations on protein expression and to compare this to the flow cytometric analysis. PBMCs from patients 3.1, 5.1, and 6.2 were available for Western blotting. All of these patients showed a reduction in XIAP protein expression (Fig. 3), fitting with the results obtained by flow cytometric analysis.

iNKT Cell Counts in the Patients

SAP-deficient patients had reduced numbers of NKT cells that expressed an invariantly rearranged T-cell receptor (TCR) consisting of TCRVα24 and TCRVβ11 chains [21,22]. The rare subset of iNKT cells was originally reported to be reduced in XIAP-deficient patients as well [7] but seemed to be present in normal numbers in a later study involving a larger patient cohort [23]. We analyzed the iNKT cell frequencies in 100,000 $CD3^+$ T cells in our XIAP-deficient patients and compared these with healthy controls (Fig. 4). The average frequency of iNKT cells within the $CD3^+$ T cell compartment of our XIAP patients was significantly reduced by twofold when compared with healthy controls (20 vs. 47 per 10^5 $CD3^+$ T cells). Therefore, we concluded that the number of iNKT cells was reduced in our patients with XIAP deficiency.

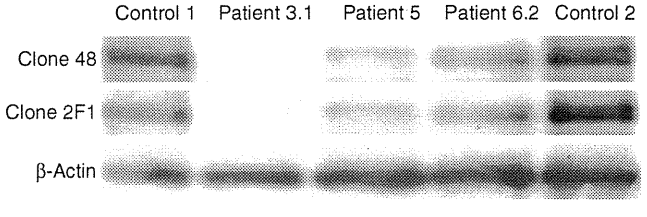

Fig. 3 XIAP expression in lymphocytes from the patients by Western blot. Analysis of XIAP expression in PBMC generated from patients with XIAP deficiency and normal controls using the antibody clone 48 (*upper panel*), the antibody clone 2 F1 (*middle panel*), and the β-actin antibody as an internal control (*lower panel*)

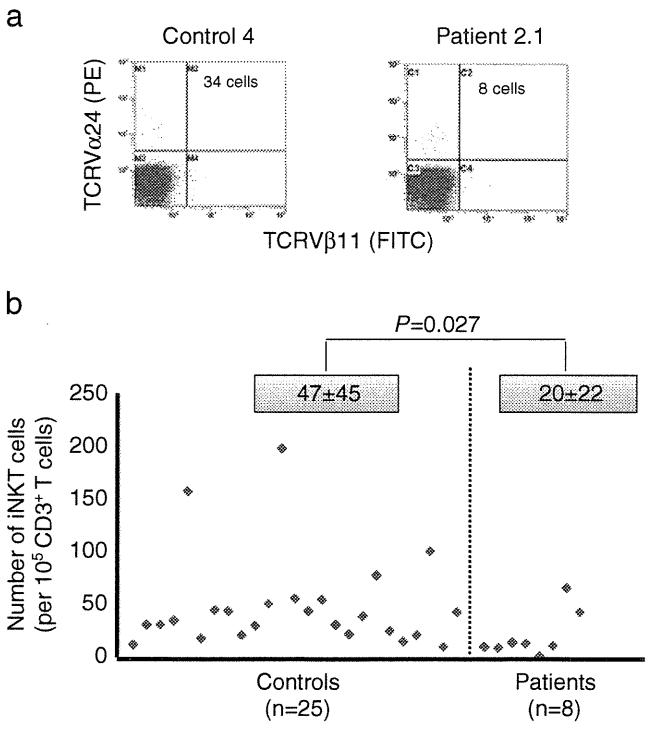

Fig. 4 iNKT cell counts in the patients and healthy controls. **a** Representative flow cytometric analysis of iNKT cells in $CD3^+$ lymphocytes from one XIAP-deficient patient and one healthy control. **b** Comparison of the number of iNKT cells in 100,000 $CD3^+$ lymphocytes between XIAP-deficient patients and control individuals. Statistical significance between patients and controls was determined with the Student's *t*-test (*p*-value=0.027)

Functional Analysis of CTL Lines Established from the Patients

To test whether our XIAP-deficient patients have similar defects in $CD8^+$ T cell cytotoxicity as described in other subtypes of familial HLH [20,38], we generated $CD8^+$ alloantigen-specific CTL from patients 1, 2.1, 3.1, and three healthy controls (Fig. 5). The cytotoxic activity of the CTL of these patients was similar to that of the healthy controls, indicating that XIAP patients clearly differ from other familial HLH patients in this aspect of the disease.

Discussion

XIAP deficiency is a rare but severe and life-threatening inherited immune deficiency [12,13]. Early diagnosis and life-saving treatment such as hematopoietic stem cell transplantation is especially important. The causative gene for

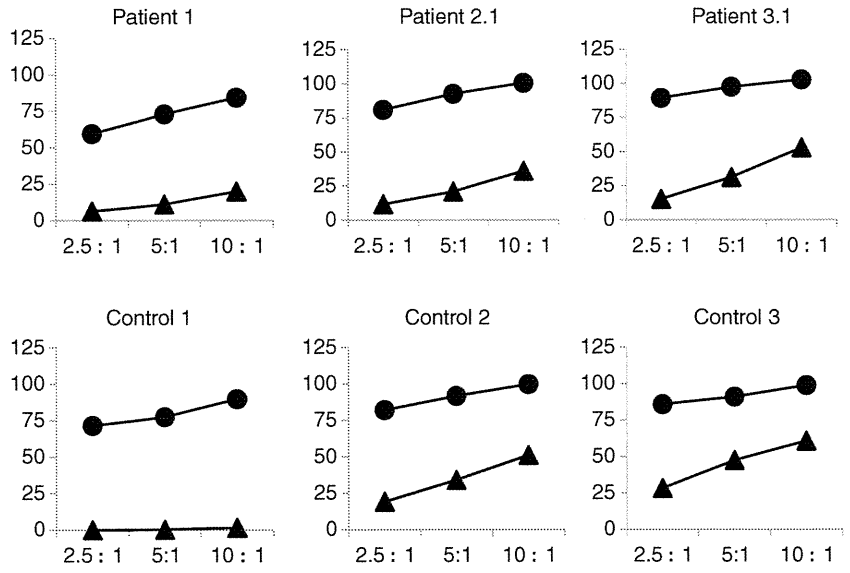

Fig. 5 Cytotoxicity of alloantigen-specific $CD8^+$ T cell lines. $CD8^+$ T cell lines were generated from PBMC of patients with XIAP deficiency and healthy controls by stimulation with allogeneic LCL (KI-LCL). Their cytotoxity was determined against allogeneic KI-LCL (*circles*) and against allogeneic TA-LCL (*triangles*), which does not share alloantigens with KI-LCL

XIAP deficiency was identified to be *XIAP/BIRC4*, and 25 mutations in the *XIAP* gene have been previously reported [7,12–14]. In the present study, we described four novel mutations (W217CfsX27, E349del, deletion of exons 1 and 2 and N341YfsX7) in the *XIAP* genes as well as previously described patients with R381X and R238X mutations [13,14]. The mother of patients 6.1 and 6.2 had no mutation in the *XIAP* gene. Because this is an X-linked inheritance, the failure to identify the same mutation in the mother suggests that the mother had a germline mosaicism for the mutation. Such mosaicism has not yet been described in XIAP deficiency, but it has been reported in Duchenne muscular dystrophy, X-linked severe combined immunodeficiency, X-linked agammaglobulinemia, and many other inherited diseases [24–26]. HLH is common in XIAP-deficient patients, and it is often recurrent [13,14]. In our study, six patients had HLH and five patients presented with recurrent HLH. Therefore, XIAP deficiency should be suspected in certain boys with HLH, especially in those with family history or recurrent HLH. The reason why XIAP deficiency increases susceptibility to HLH remains unclear. Murine studies have also failed to disclose a mechanism for the development of HLH [27]. Interestingly, Xiap-deficient mice possess normal lymphocyte apoptosis induced by a variety of means [28]. Three of our patients presented with EBV-associated HLH. EBV infection has been reported to be a trigger of the first HLH episode in patients with XIAP deficiency [13]. The excess of lymphocyte apoptosis in XIAP deficiency might account for the abnormal immune response to EBV [28]. Splenomegaly is not frequently observed in XLP type 1 or SAP deficiency but might be a common clinical feature in XIAP deficiency [12,13] as four (50%) of eight Japanese patients developed splenomegaly. Pachlopnik Schmid et al. [13] reported that recurrent splenomegaly occurring in the absence of systemic HLH was often associated with fever and cytopenia. XIAP-deficient patients are at risk for chronic colitis, which is possibly a more frequent cause of mortality than HLH [13]. Our study included two patients who developed colitis, and one of the patients died of colitis at 4 years of age. Although we did not have enough clinical information or samples from that patient because of his early death, his symptoms suggest that he had a XIAP deficiency complicated with colitis because he was the maternal uncle of patient 2.1. The other patient was 2 years old and also suffered from chronic hemorrhagic colitis.

In contrast to SAP deficiency, lymphoma has never been reported in XIAP deficiency, including our patients. Some studies indicate that the XIAP protein is a potential target for the treatment of cancer based on the anti-apoptotic function of XIAP [29]. Therefore, the absence of XIAP may protect patients from cancer, explaining why XIAP-deficient patients do not develop lymphoma. We generated a clinical summary to compare XIAP-deficient patients with the previous reports (Table II). Although our study included a relatively small number of patients, our results appear to be consistent with previous large studies [12,13] and confirm the clinical characteristics of XIAP deficiency.

Flow cytometry can be used for the rapid screening of several primary immunodeficiencies including XLP [30]. XIAP protein has been found to be expressed in various human tissues, including all hematopoietic cells [7,10]. Marsh et al. [16] described that XIAP was readily detectable in normal granulocytes, monocytes, and all lymphocyte subsets. Moreover, patients with *XIAP* mutations had decreased or absent expression of XIAP protein by flow cytometry [14,16]. We investigated XIAP expression in lymphocytes from eight patients by flow cytometry as previously described [16,17]. As demonstrated by Marsh et al. [16], clone 48 antibody provided brighter staining compared

Table II Comparison of patients with XIAP deficiency

	Marsh R et al. [12]	Pachlopnik Schmid J et al. [13]	Our study
Number of patients	10	30	9
HLH	9 (90%)	22/29 (76%)	6/9 (67%)
Recurrent HLH	6 (60%)	11/18 (61%))	5/6 (83%)
EBV-associated HLH	3 (30%)	16/19 (84%)	4/6 (67%)
Splenomegaly	9 (90%)	19/21 (90%)	4/8 (50%)
Hypogammaglobulinema	2 (20%)	8/24 (33%)	2/8 (25%)
Lymphoma	0	0	0
Colitis	0	5 (17%)	2 (22%)

to clone 2F1 antibody. In patients 5, 6.1, and 6.2, XIAP protein expression was normal when using clone 48 antibody but decreased when using clone 2F1 antibody. Western blot analysis showed XIAP expression in patients 3.1, ,5 and 6.2, and using clone 48 antibody, we found a discrepancy between flow cytometry and Western blot. Flow cytometric diagnosis may thus result in false positive results, and the gene sequencing of *XIAP* should be performed even when the patient shows normal XIAP expression levels.

All of the mothers examined in this study except for one were carriers of *XIAP* mutations. Analysis of XIAP expression in the mothers of patients 1, 3.1, and 3.2 revealed a bimodal expression pattern of XIAP in lymphocytes with cellular skewing towards expression of the wild-type XIAP allele as previously demonstrated [16]. However, the mother of patients 2.1, 6.1, and 6.2 demonstrated a normal expression pattern, possibly resulting from an extremely skewed pattern of X chromosome inactivation as shown in XIAP deficiency and other primary immunodeficiencies, and de novo mutations in *XIAP* are also observed [16,31]. The mother of patients 6.1 and 6.2 might have a germline mosaicism for the mutation, resulting in normal XIAP protein expression.

iNKT cells represent a specialized T lymphocyte subpopulation with unique features distinct from conventional T cells [32,33]. Human iNKT cells express an invariant TCR that recognizes self and microbacterial glycosphingolipid antigens presented by the major histocompatibility complex class I-like molecule CD1d [28]. The first series of XIAP-deficient patients showed decreased iNKT cell counts similar to SAP deficiency [7]. However, Xiap-deficient mice have normal numbers of iNKT cells and did not show an abnormal response to apoptotic stimuli [34]. Marsh et al. [23] reported a cohort of XIAP-deficient patients with normal numbers of iNKT cells, indicating that XIAP-deficient patients differ from SAP-deficient patients in this respect. In our cohort, we observed significantly decreased iNKT cell numbers in XIAP-deficient patients compared to healthy controls. However, we could not identify a correlation between the number of iNKT cells and the clinical disease features. Flow cytometric evaluation of iNKT cell counts can allow for the discrimination of XLP and other primary immunodeficiency diseases because patients may have normal XIAP protein expression in their lymphocytes.

CTLs kill their targets by one of two mechanisms: granule- or receptor-mediated apoptosis [35]. A recent study showed that the main pathway of cytotoxicity mediated by alloantigen-specific human $CD4^+$ and $CD8^+$ T cells is granule exocytosis and not the FAS/FAS ligand system [18]. Granzyme B is a major effector molecule of granule-mediated killing that rapidly induces cell death after entering the cytoplasm of the target cell [36]. The enzymatic activity of granzyme B is key to its ability to induce cell death. The executioner caspase-3 has been shown to be proteolytically processed and activated by granzyme B [37]. Although XIAP possesses an inhibitory effect for caspases, it is important to study the cytotoxic activities of CTLs in XIAP deficiency. Furthermore, many studies have indicated that some subtypes of patients with familial HLH show a deficiency in their cytotoxic activities [20,38]. To further investigate the function of antigen-specific CTLs, we studied $CD8^+$ alloantigen-specific CTL analysis among three XIAP-deficient patients. XIAP-deficient patients showed a normal level of cytotoxic activity, suggesting that XIAP might not play an important role in the cytotoxic responses of $CD8^+$ T cells as was previously suggested based on the normal NK cell-mediated cytotoxicity found in XIAP-deficient patients [7,12].

In this study, we have described nine Japanese patients with XIAP deficiency with clinical characteristics similar to those of patients in Europe and USA [12,13].

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