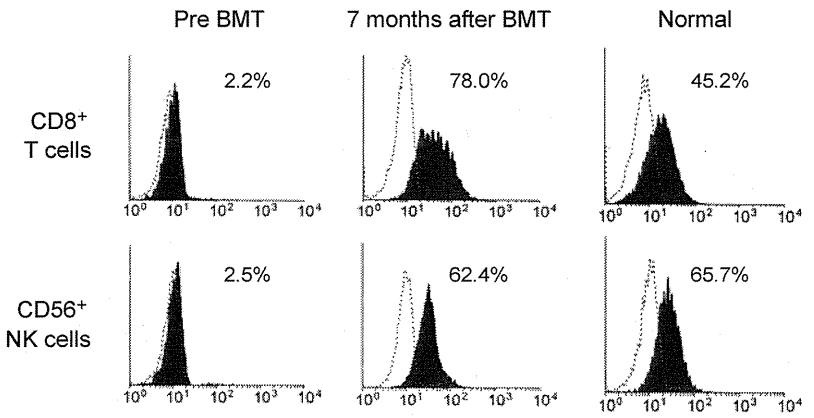

Figure 1 The SAP expression in $CD8^+$ T cells and NK cells from the patient (P16.1) and a normal adult donor. Dotted lines and shaded areas indicate staining by the control antibody and anti-SAP mAb (KST-3), respectively. A flow cytometric analysis demonstrated that deficient SAP expression in $CD8^+$ T cells and NK cells from the patient increased after he had undergone hematopoietic stem cell transplantation.

herpes virus 6 infection and P21.1 developed EBV encephalitis. Approximately 70% of the patients (23 of 33) were diagnosed by the time they were 5 yr of age, but two patients (P13.1 and P20.1) were diagnosed in adulthood. Eleven families (52%) had X-linked family histories. Ten patients (30%) presented with more than one clinical manifestation over time. Ten sibling cases were observed in this study, and seven families manifested different phenotypes. Fifteen patients (45%) were treated with intravenous immunoglobulin replacement therapy. In this study, the mortality rate was 21 of 32 patients (66%), and all the living patients were post-transplanted. Clinical characteristics of this study are summarized in comparison with those of previous study (Table 2).

Hematopoietic stem cell transplantation for patients with XLP-1

Twelve patients with XLP underwent HSCT in Japan (Table 3), and one patient (P9.2) died of *Pseudomonas* sepsis and multiple organ failure 14 days after HSCT. Two patients (P1.2 and P7.2) were transplanted from matched sibling donors, but the other patients were transplanted from matched or one-locus-mismatched unrelated donors, or mismatched familial donors. Various types of conditioning regimen were performed. Five patients (P1.2, P7.2, P9.1, P10.1, and P14.1) underwent HSCT following myeloablative conditioning, but the other patients did so following reduced intensity conditioning (RIC). Acute graft versus host disease (GVHD) was observed in 6 of 11 patients (Grade I, two patients; Grade II, three patients; Grade III, one patient). Chronic GVHD was observed in five patients, among whom 4 (P1.2, P7.2, P10.1, and P18.1) had extensive types and one (P14.1) had a limited type. Eleven patients (92%) have survived and had complete chimerism with a median follow-up of 7 yr and 9 months. A flow cytometric assay could be conducted to evaluate SAP expression in $CD8^+$ T cells and NK cells after HSCT in five patients (P7.2, P10.2, P16.1, P17.2, and P18.1). All the patients demonstrated an increase in SAP expression in $CD8^+$ T cells and NK cells after undergoing HSCT (Fig. 1).

Discussion

X-linked lymphoproliferative syndrome is a rare but life-threatening disease. A large cohort showed that most patients with XLP died by the age of 40 yr and more than 70% of the patients died before the age of 10 yr (2). Early diagnosis in non-familial cases may be difficult because XLP is heterogeneous in its clinical presentation. The ability to screen rapidly and make an accurate diagnosis of patients with XLP facilitates the initiation of life-saving treatment and preparation for HSCT. In a previous study, we generated an anti-SAP mAb, termed KST-3, which was applied to the flow cytometric evaluation of SAP deficiency (XLP-1) (11). All the patients evaluated in this study showed deficient SAP expression, although some patients with missense mutations might demonstrate normal expression of SAP, as shown in Western blotting (16).

Various types of *SH2D1A* mutation have been identified in Japan (11–15). The *SH2D1A*base (http://bioinf.uta.fi/SH2D1Abase) discloses that 133 unrelated patients were identified to have *SH2D1A* mutations. Missense and nonsense mutations appear in one-quarter each, and other types of mutation appear in half of the patients in this database. In the present study, Arg55stop mutations were most frequently found, in keeping with the *SH2D1A*base. No genotype and phenotype correlation was evident in this study, as well as in previous studies (1, 17).

Large cohort studies have shown that the major clinical phenotypes of XLP include FIM (60%), dysgammaglobulinemia (30%), and malignant lymphoma (30%) (1, 2). Aplastic anemia, lymphoid granulomatosis, and systemic vasculitis are minor clinical presentations at frequencies of approximately 3%. Although the present study included a limited number of patients with XLP-1, the distribution of the clinical manifestations seems to be similar to that in previous large studies

Table 1 Clinical and genetic data of patients with X-linked lymphoproliferative syndrome

Patient ID	Age at diagnosis	Family history	Clinical presentation	Epstein–Barr virus status	IVIG	Outcome	Cause of death	Age at death or presence	*SH2D1A* mutation	SAP expression
1.1	12 yr	+	Hypo-γ, LPD	+	+	Dead	GVHD	12 yr	NE	NE
1.2	7 yr	+	Hypo-γ, LPD	+	+	Alive*		21 yr	Asp33Tyr	NE
2.1	3 yr	−	FIM	+	−	Dead	FIM	3 yr	Arg55stop	NE
3.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	Arg55stop	NE
3.2	2 yr	+	FIM	+	−	Dead	FIM	2 yr	Arg55stop	NE
4.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	416C>T, fs	NE
4.2	4 yr	+	ML, vasculitis, HLH	−	−	Dead	HLH (MOF)	14 yr	416C>T, fs	Deficient
5.1	1 yr	+	FIM	+	+	Dead	FIM	1 yr	del of whole gene	NE
6.1	1 yr	−	FIM	+	−	Dead	FIM	1 yr	Gly27Ser	NE
7.1	1 yr	+	Hypo-γ, aplastic anemia	+	+	Dead	Sepsis	1 yr	NE	NE
7.2	3 yr	+	Hypo-γ, vasculitis	−	+	Alive*		30 yr	His8Asp	Deficient
8.1	1 yr	−	FIM	+	+	Dead	FIM	1 yr	584delA, fs	NE
9.1	6 yr	+	Hypo-γ	+	+	Alive*		18 yr	Arg55stop	Deficient
9.2	6 months	+	FIM	+	+	Dead*	Sepsis	6 yr	Arg55stop	Deficient
10.1	4 yr	+	ML	+	−	Alive*		15 yr	Gly49Val	Deficient
10.2	0 months	+	Healthy	−	−	Alive*		4 yr	Gly49Val	Deficient
11.1	1 yr	+	FIM	+	+	Dead	FIM (MOF)	1 yr	del of exons 3, 4	NE
11.2.	2 yr	+	FIM	+	+	Dead	FIM (MOF)	2 yr	del of exons 3, 4	Deficient
11.3	0 month	+	Healthy	−	+	Alive*		9 yr	del of exons 3, 4	Deficient
12.1	12 yr	+	Hypo-γ, ML	+	−	Dead	ML	12 yr	Ser34Gly	Deficient
12.2	10 yr	+	Hypo-γ	+	−	Unknown	Unknown	Unknown	Ser34Gly	Deficient
13.1	23 yr	−	FIM	+	−	Dead	FIM	23 yr	Tyr7Cys	Deficient
14.1	8 yr	−	Hypo-γ, ML	+	−	Alive*		16 yr	Arg55stop	Deficient
15.1	2 yr	−	FIM	+	−	Dead	FIM	2 yr	His8Asp	NE
16.1	10 yr	−	Hypo-γ, HLH	−	+	Alive*		17 yr	545insA, fs	Deficient
17.1	2 yr	+	FIM	+	−	Dead	FIM	2 yr	IVS2+1G>A	Deficient
17.2	2 yr	+	ADEM	−	−	Alive*		8 yr	IVS2+1G>A	Deficient
18.1	6 yr	−	Hypo-γ	+	+	Alive*		12 yr	312insG, fs	Deficient
19.1	10 months	+	Hypo-γ	+	+	Dead	DIC	10 months	NE	NE
19.2	1 yr	+	FIM	+	−	Dead		1 yr	NE	NE
19.3	3 yr	+	Hypo-γ, HLH, ML	+	+	Alive*		18 yr	del of exons 3, 4	Deficient
20.1	41 yr	−	FIM	+	−	Dead	FIM	42 yr	Ala3Ser	Deficient
21.1	3 yr	−	Encephalitis, LPD	+	−	Dead	Encephalitis	3 yr	538insA, fs	Deficient

Hypo-γ, hypogammaglobulinemia; LPD, lymphoproliferative disease; GVHD, graft versus host disease; FIM; fulminant infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis; MOF, multiple organ failure; ML, malignant lymphoma; ADEM, acute disseminated encephalomyelitis; DIC, disseminated intravascular coagulation; NE, not examined; fs, frameshift; del, deletion; ins, insertion.
P17.1 and 17.2 are monozygotic twins. Asterisk indicates the patients who underwent hematopoietic stem cell transplantation. P1.2, P2.1, P3.1, P3.2, P4.1, P5.1, P6.1, P7.2, P8.1, and P10.1 were described by Sumazaki et al. (14) P5.1 was described by Honda et al. (13) P9.1, P9.2, P11.1, P11.2, P11.3, P12.1, and P12.2 were described by Shinozaki et al. (11) P13.1 was described by Hoshino et al. (15) P16.1, P17.1, P17.2, P18.1, P19.3, and P20.1 were described by Zhao et al. (12). [Correction added on 10 April 2012, after first online publication: the *SH2D1A* mutation of P21.1 has been corrected.]

(Table 2) (2, 17). Lymphoid granulomatosis was not found in Japanese patients, but two patients have presented with systemic vasculitis (18). The vasculitis in these patients mainly affected the brain and was associated with encephalopathy. The mortality was different among clinical phenotypes, and the mortality of each phenotype in our study decreased from that in the XLP registry (2). However, in a recent worldwide study, the mortality associated with HLH decreased to 65%, lymphoproliferative disease to 8%, and dysgammaglobulinemia to 5% (16).

Hematopoietic stem cell transplantation is the only curative treatment for XLP-1. Twenty-one patients with XLP-1 did not undergo HSCT, and these patients died of the disease and complications. The outcome of one patient (P12.2) was unknown. Twelve patients underwent HSCT in Japan, and 11 patients survived. Most of the transplants were performed in different institutions, but the outcomes are similar to previously published data (9, 10, 17). This study revealed that unrelated donors could be used as donors as well as sibling donors. Although various types of conditioning regimen were performed, more than half included RIC regimen, and the result of RIC regimen is similar to that of myeloablative regimen. The RIC regimen should be performed for patients with XLP-1 to avoid regimen-related toxicity or morbidity (17). In

Table 2 Clinical phenotypes of patients with X-linked lymphoproliferative syndrome

	Present study (33 cases)		Seemayer (272 cases) (2)		Booth (91 cases) (17)	
Phenotype	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
FIM or HLH	18 (55%)	16/18 (89%)	157 (58%)	127/132 (96%)	35.2%	65.6%
ML or LPD	7 (21%)	3/7 (43%)	82 (30%)	46/71 (65%)	24.2%	9.0%
Hypogammaglobulinemia	12 (36%)	4/11 (36%)	84 (31%)	34/75 (45%)	50.5%	13.0%

FIM, fulminant infectious mononucleosis; HLH, hemophagocytic lymphohistiocytosis.

Table 3 Characteristics of HSCTs

Patient ID	Age at HSCT	Donor	Sources	Conditioning regimen	GVHD prophylaxis	Acute GVHD	Chronic GVHD	Outcome
1.2	7 yr	MSD (6/6)	PBSC	TBI/CY	CsA/sMTX	Grade I	Extensive	Alive (14 yr 8 months)
7.2	24 yr	MSD (6/6)	BM	BU/CY/ATG	CsA/sMTX	Grade II	Extensive	Alive (6 yr 6 months)
9.1	8 yr	MUD (6/6)	BM	BU/VP/CY	FK/sMTX	None	None	Alive (10 yr 6 months)
9.2	6 yr	mMFD (3/6)	BM	TBI 6Gy/BU 4 mg/kg	MMF/sMTX/mPSL	NE	NE	Dead (14 days)
10.1	4 yr	mMUD (5/6)	BM	BU/CY/AraC	FK/sMTX	Grade II	Extensive	Alive (11 yr 2 months)
10.2	1 yr	MUD (6/6)	BM	BU/TAI 3Gy/Flu/CY/ATG	FK/sMTX	None	None	Alive (3 yr 3 months)
11.3	8 months	mMUD (5/6)	PBSC	Flu/Mel/ATG/TAI 6Gy	FK/sMTX/mPSL	Grade II	None	Alive (9 yr 2 months)
14.1	10 yr	MUD (6/6)	BM	BU/CY	CsA/sMTX	Grade III	Limited	Alive (8 yr 2 months)
16.1	11 yr	mMUD (5/6)	BM	BU/TAI 3Gy/Flu/CY/ATG	FK/sMTX	None	None	Alive (5 yr 6 months)
17.2	3 yr	mMFD (4/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	Grade I	None	Alive (8 yr 10 months)
18.1	7 yr	MUD (6/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	None	Extensive	Alive (4 yr 7 months)
19.3	15 yr	MUD (6/6)	BM	Flu/Mel/TBI 3 Gy	FK/sMTX	None	None	Alive (3 yr 7 months)

MSD, matched sibling donor; MUD, matched unrelated donor; mMFD, mismatched familial donor; mMUD, mismatched unrelated donor; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; CY, cyclophosphamide; BU, busulfan; ATG, anti-thymoglobulin; VP, etoposide; Gy, gray; AraC, cytosine arabinoside; TAI, total abdominal irradiation; Flu, fludarabine; Mel, melphalan; GVHD, graft versus host disease; CsA, cyclosporine A; sMTX, short methotraxate; FK, tacrolimus; MMF, mycophenolate mofetil; mPSL, methylprednisolone; NE, not evaluated; HSCT, hematopoietic stem cell transplantation.

this study, two patients (P10.2 and P11.3) were diagnosed because of a family history and presented no clinical features of XLP. Their parents wanted them to undergo HSCT because of the poor prognosis of the disease. Although the decision to transplant a relatively well child has been more challenging, these patients underwent transplant and were free from chronic GVHD.

In conclusion, this study verified the clinical usefulness of a flow cytometric assessment of SAP to search for XLP-1 (SAP deficiency). Flow cytometric analysis of XIAP is also useful to detect patients with XLP-2 (7, 19, 20). A male with any of the clinical phenotypes of XLP with or without EBV infection should be initially examined with a flow cytometric assay to evaluate both SAP and XIAP (21). We also identified nine Japanese patients with XIAP deficiency with a combination of flow cytometry and genetic analysis (22). Needless to say, a mutation analysis is the gold standard for confirming a definite diagnosis. The outcome of patients with XLP-1 seemed to be poor in Japan, and HSCT is the only curative treatment for patients with XLP-1.

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Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes

Rebecca A. Marsh, Kanchan Rao, Prakash Satwani, Kai Lehmberg, Ingo Müller, Dandan Li, Mi-Ok Kim, Alain Fischer, Sylvain Latour, Petr Sedlacek, Vincent Barlogis, Kazuko Hamamoto, Hirokazu Kanegane, Sam Milanovich, David A. Margolis, David Dimmock, James Casper, Dorothea N. Douglas, Persis J. Amrolia, Paul Veys, Ashish R. Kumar, Michael B. Jordan, Jack J. Bleesing and Alexandra H. Filipovich

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Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes

Rebecca A. Marsh,[1] Kanchan Rao,[2] Prakash Satwani,[3] Kai Lehmberg,[4] Ingo Müller,[4] Dandan Li,[5] Mi-Ok Kim,[5] Alain Fischer,[6] Sylvain Latour,[7] Petr Sedlacek,[8] Vincent Barlogis,[9] Kazuko Hamamoto,[10] Hirokazu Kanegane,[11] Sam Milanovich,[12] David A. Margolis,[12] David Dimmock,[13] James Casper,[12] Dorothea N. Douglas,[14] Persis J. Amrolia,[2] Paul Veys,[2] Ashish R. Kumar,[1] Michael B. Jordan,[1] Jack J. Bleesing,[1] and Alexandra H. Filipovich[1]

[1]Division of Bone Marrow Transplantation and Immunodeficiency, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [2]Department of Haematology and Bone Marrow Transplantation, Great Ormond Street Hospital for Sick Children, London, United Kingdom; [3]Division of Pediatric Blood and Marrow Transplantation, Columbia University Medical Center, Morgan Stanley Children's Hospital of New York-Presbyterian, New York, NY; [4]Clinic for Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [5]Division of Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [6]Inserm U768, Unité d'Immunologie et d'hematologie, Hopital Necker, Assistance Publique-Hopitaux de Paris, Université Paris Descartes, Sorbonne Paris Cité, Institut Imagine, Paris, France; [7]Inserm U768, Hôpital Necker, Université Paris Descartes, Sorbonne Paris Cité, Institut Imagine, France; [8]Department of Pediatric Hematology and Oncology, University Hospital Motol, Charles University, Prague, Czech Republic; [9]Hôpital Timone Enfants, Marseille, France; [10]Department of Pediatrics, Hiroshima Red Cross Hospital, Hiroshima, Japan; [11]Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; [12]Division of Pediatric Hematology, Oncology and Bone Marrow Transplant, Medical College of Wisconsin, Children's Hospital of Wisconsin, Milwaukee, WI; [13]Division of Genetics, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI; and [14]University of Arizona College of Medicine, Phoenix Children's Hospital, Phoenix, AZ

Key Points

- High mortality rates are observed in patients with XIAP deficiency treated with myeloablative conditioning regimens for hematopoietic cell transplantation.

There have been no studies on patient outcome after allogeneic hematopoietic cell transplantation (HCT) in patients with X-linked inhibitor of apoptosis (XIAP) deficiency. To estimate the success of HCT, we conducted an international survey of transplantation outcomes. Data were reported for 19 patients. Seven patients received busulfan-containing myeloablative conditioning (MAC) regimens. Eleven patients underwent reduced intensity conditioning (RIC) regimens predominantly consisting of alemtuzumab, fludarabine, and melphalan. One patient received an intermediate-intensity regimen. Survival was poor in the MAC group, with only 1 patient surviving (14%). Most deaths were from transplantation-related toxicities, including venoocclusive disease and pulmonary hemorrhage. Of the 11 patients who received RIC, 6 are currently surviving at a median of 570 days after HCT (55%). Preparative regimen and HLH activity affected outcomes, and of RIC patients reported to be in remission from HLH, survival is 86% (*P* = .03). We conclude that MAC regimens should not be used for patients with XIAP deficiency. It is possible that the loss of XIAP and its antiapoptotic functions contributes to the high incidence of toxicities observed with MAC regimens. RIC regimens should be pursued with caution and, if possible, efforts should be made to ensure HLH remission before HCT in these patients. (*Blood*. 2013;121(6):877-883)

Introduction

Deficiency of X-linked inhibitor of apoptosis (XIAP) is associated with X-linked lymphoproliferative disease (XLP) and familial hemophagocytic lymphohistiocytosis (FHLH) phenotypes. Traditionally, patients with inherited immune deficiencies that cause HLH have been treated with allogeneic hematopoietic cell transplantation (HCT) because of the life-threatening nature of HLH. There is extensive experience with transplantation in patients with FHLH. Over the past 10 years, survival has generally approximated 60% with myeloablative conditioning (MAC) regimens.[1-7] More recently, improvements have been made with reduced-intensity conditioning (RIC) protocols, and current survival rates are as high as 80%.[8-11] There is less experience with transplantation in patients with XLP because of SLAM-associated protein (SAP) deficiency, but survival is generally accepted to be greater than 70% regardless of the intensity of the conditioning protocol.[12-14]

To date, little has been published concerning the outcomes of HCT for patients with XIAP deficiency. XIAP deficiency was first discovered in 2006,[15] and is associated with XLP, FHLH, and colitis phenotypes.[15-18] Patients with XIAP deficiency are unique compared with patients with the other genetic forms of HLH because, as the name suggests, XIAP is an inhibitor of apoptosis that is widely expressed outside of the immune system.[19] Thymocytes from XIAP-deficient mice have been shown to have normal apoptotic responses to a variety of apoptotic stimuli,[20] but hepatocytes are more sensitive to death induced by treatment with cross-linked Fas ligand.[21] XIAP-deficient mouse embryonic fibroblasts are also more sensitive to death after infection with MHV-68.[22] In addition, there is increasing experience with the use of XIAP inhibitors in conjunction with traditional cancer treatment. In this setting, XIAP inhibitors generally increase the susceptibility

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Table 1. Patient characteristics

Patient no	Age at initial presentation	EBV HLH before HCT	Non-EBV HLH before HCT	HLH not in full remission before HCT	Colitis before HCT	Other	XIAP mutation	Protein expression
1	3 mo	–	+	–	–		1443_1449 delins 24 (P482fsX508)	NE*
2	2 mo	–	+	+	–		1443_1449 delins 24 (P482fsX508)	NE
3	2 mo	–	+	–	–		563 G → A (G188E)	Reduced
4	Asymptomatic (symptomatic brother)	–	–	–	–		563 G → A (G188E)	Reduced
5	15 mo	–	–	–	+	Recurrent enterocutaneous fistulas; multiple episodes of polymicrobial sepsis	608G → A (C203Y)	Reduced
6	9 mo	–	+	–	–		E99KfsX129	Absent
7	9 y	+	–	–	–		497G → T, R166I	NE
8	7 mo	–	+	+	–		1141C → T (R381X)	Reduced
9	Infancy	–	+	–	–		1481 T → A (I494N)	NE
10	4 mo	–	+	+	–		1445 C → G (P482R)	Reduced
11	1 y	+	–	+	–	Repeated infections: pneumonia, otitis media, history of paracentesis, mastoidectomy	1189 delA (I397fsX414)	Absent
12	1 y	+	–	+	–		387_390del (D130fsX140)	Not reported
13	3 mo	–	+	–	–		Gross Deletion Exons 1-5	Absent
14	1 y	–	–	–	+	Recurrent fevers; pneumococcal sepsis.	758 C → G (S253X)	Absent
15	3 y	–	+	–	–	Ventricular septal defect	356_359del (N119fs384)	NE
16	7 y	+	+	–	–		1141C → T (R381X)	Reduced
17	8 y	+	–	+	–		310 C → T (Q104X)	Absent
18	Infancy	–	+	–	–	Liver failure in infancy required liver transplantation; nodular lung disease; positive CMV and fungal elements	Gross deletion exon 6	Truncated (robust detection of a smaller molecular weight protein by Western blot)
19	17 y	+	–	–	–		894_898 del 5 (K299fsX307)	Absent

*Not examined.

of cancer cells to undergo apoptosis.[23,24] Because of the importance of XIAP in preventing apoptosis, patients with XIAP deficiency may be at increased risk of treatment-related toxicities because of increased sensitivity to chemotherapeutic agents.

To investigate whether deficiency of XIAP adversely affects the survival of patients undergoing allogeneic HCT, we conducted an international survey to collect information regarding the transplantation outcomes of patients confirmed to have XIAP deficiency.

Methods

Data collection

Approval for this retrospective study was granted by the Cincinnati Children's Hospital Institutional Review Board. A spreadsheet questionnaire was sent to physicians who provided treatment for patients with XIAP deficiency who underwent allogeneic HCT. Physicians were identified through contact with our center, our review of the literature regarding XIAP deficiency, or a request made to all members of the Histiocyte Society.

Patients

Only patients with a confirmed *XIAP/BIRC4* (baculoviral inhibitor of apoptosis repeat containing protein 4) mutation or with a sibling with a confirmed mutation were included in this study (Table 1), which was conducted in accordance with the Declaration of Helsinki. Supplemental lymphocyte protein analysis was performed in some patients using either Western blot or intracellular flow cytometric analysis.[15-17,25]

Transplantation procedures

Patients received transplantation at centers in the United States (n = 12), Europe (n = 6), and Japan (n = 1) between the years 2001-2011. Transplantation procedures were carried out per institutional standard practices. Conditioning regimens and graft characteristics are listed in Table 2. Conditioning regimens were classified as MAC if they contained an alkylating agent (busulfan) or total body irradiation (TBI) at a dose that would not allow autologous BM recovery.[26] Conditioning regimens were classified as RIC if they did not meet the definition of MAC regimen.[26] If there was uncertainty regarding the intensity of the regimen (n = 1, patient 8), it was classified as an intermediate-intensity regimen. Neutrophil engraftment was considered to be the day the neutrophil count reached 0.5×10^9/L. Engraftment studies were done using either XY FISH for sex-mismatched donors or variable number of tandem repeat analysis for same-sex donors. Mixed chimerism was defined as having 5% or more host-derived cells in the whole blood on more than 1 occasion. Acute and chronic GVHD were assessed by standard criteria.[27,28] Patients received GVHD prophylaxis per institutional standard practices. Other routine transplantation care, such as antimicrobial prophylaxis, IV Ig replacement, and fluid and nutrition supplementation when needed, were also provided per institutional standard practices.

Table 2. Transplantation procedures

Patient no	Age at HCT, y	Type of conditioning	Conditioning regimen	Graft HLA match*	Graft source	Relationship
1	0.42	MAC	Bu, Mel, ATG	5/6	Cord	Unrelated
2	0.58	MAC	Bu, Cy, ATG, Etop	6/6	Cord	Unrelated
3	1	MAC	Bu, Cy, ATG	7/8	BM	Unrelated
4	4	MAC	Bu, Cy, ATG	10/10	BM	Unrelated
5	5	MAC	Bu, Flu, ATG	6/6	Cord	Unrelated
6	10	MAC	Bu, Cy, ATG	6/6	BM	Unrelated
7	14	MAC	Bu, Cy, ATG, Etop	7/8	PBSCs	Unrelated
8	1	Intermediate	TBI (6 Gy), Flu, Cy, Mel (80 mg/m^2)	7/8	Cord	Unrelated
9	0.40	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
10	0.98	RIC	Alem, Flu, Mel	9/10	BM	Unrelated
11	2	RIC	Alem, Flu, Mel	9/10	BM	Unrelated
12	3	RIC	Alem, Flu, Mel	9/10	Cord	Unrelated
13	3	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
14	3	RIC	Alem, Flu, Mel	10/10	BM	Unrelated
15	4	RIC	Alem, Flu, Mel	8/8	PBSCs	Maternal
16	7	RIC	Alem, Flu, Treo, Thio	10/10	PBSCs	Unrelated
17	9	RIC	Alem, Flu, Mel	7/8	BM	Unrelated
18	11	RIC	Alem, Flu, Mel	8/8	BM	Unrelated
19	19	RIC	Alem, Flu, Mel	10/10	BM	Sibling

Bu indicates busulfan; Mel, melphalan; ATG, antithymocyte globulin; Cy, cyclophosphamide; Etop, etoposide; Flu, fludarabine; Alem, alemtuzumab; Treo, treosulfan; Thio, thiotepa; and PBSCs, peripheral blood stem cells.

*Six to 10 alleles (HLA-A, HLA-B, HLA-C, HLA-DRB1, or HLA-DQB1).

Statistical analysis

Survival was analyzed using Kaplan-Meier curves created with XLSTAT 2011 software (Addinsoft). Comparison of survival curves was done using the log-rank test. For multivariate analysis of survival time and the impact of preparative regimen (MAC vs RIC), donor match, (full match vs mismatch), and HLH activity (remission vs nonremission), Cox proportional hazard regression model analysis was used. The patient who received the intermediate-intensity regimen was excluded from these analyses. Statistical significance was considered as $P < .05$.

Results

Patients

Nineteen patients with XIAP deficiency underwent allogeneic HCT between 2001 and 2011 at a median age of 3 years (range, 0.4-19). Patient characteristics before HCT and *XIAP/BIRC4* mutations are listed in Table 1. Approximately one-third of patients had developed EBV-related HLH before HCT, and approximately two-thirds of patients had developed non-EBV HLH before HCT. Six of these patients were reported to have either active HLH or HLH in partial remission just before HCT. Two patients with colitis were diagnosed and treated as having Crohn disease before the diagnosis of XIAP deficiency.

Transplantation procedures

Graft characteristics and conditioning regimens are shown in Table 2. Seven patients received a MAC protocol.[26] Most patients received busulfan, cyclophosphamide, and antithymocyte globulin with or without etoposide (n = 5). The remaining 2 patients received busulfan with either fludarabine or melphalan and antithymocyte globulin. Eleven patients received a RIC protocol.[26] Ten RIC patients received alemtuzumab, fludarabine, and melphalan, and 1 patient received alemtuzumab, fludarabine, treosulfan, and thiotepa. The remaining patient (patient 8) received an intermediate protocol consisting of TBI (6 Gy), fludarabine, cyclophosphamide, and melphalan (80 mg/m^2).

Eleven patients received fully matched related (n = 2) or unrelated (n = 9) grafts based on typing of 6-10 HLA antigens (HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1). Eight patients received a single allele mismatched graft. The stem cell source was BM in 11 patients, cord blood in 5 patients, and peripheral blood stem cells in 3 patients.

Engraftment

All patients engrafted with a median of 15 days (range, 8-22) except for patient 11, who died before engraftment on day +13.

Toxicities

There was a high incidence of conditioning-related toxicities among MAC patients (Table 3). There were 3 cases of hepatic venoocclusive disease (VOD), which contributed to deaths on days +17, +50, and +144 in patients 6, 2, and 1, respectively. Two of these patients also developed pulmonary hemorrhage. One patient (patient 3) developed pulmonary hypertension of uncertain etiology with pulmonary hemorrhage after transplantation and died on day +170. This patient had received MAC after having previously undergone HCT twice with RIC.

There were no cases of hepatic VOD or pulmonary hemorrhage in patients who received RIC. However, 1 patient (patient 11) developed multiorgan failure and cardiac toxicity with asystole and died at day +13. A second patient (patient 15) suffered an unexpected death related to idiopathic pneumonitis and respiratory failure at day +125.

Patient 8, who received the intermediate preparative regimen (consisting of TBI, fludarabine, cyclophosphamide, and melphalan), suffered posttransplantation cytokine storm syndrome with acute respiratory distress syndrome and died on day +22.

GVHD

Three patients developed acute GVHD of grade 2 or greater (Table 3). One additional patient developed acute GVHD after receiving a donor lymphocyte infusion that was administered as an intervention for declining donor contribution to hematopoiesis.

Table 3. Toxicities and complications

Patient no	VOD	Pulmonary hemorrhage	Acute VHD	Pneumonitis or ARDS	Confirmed bacteremia/sepsis	Fungal infection	Viremia with EBV, CMV, adenovirus, or HHV6	BK virus hemorrhagic cystitis
1	+	+	–	NR	NR	NR	NR	NR
2	+	–	–	NR	NR	NR	NR	NR
3	–	+ (shown by autopsy, not clinically)	II	–	+ (*S marcescens*)	–	+ (EBV, adenovirus)	–
4	–	+ (related to fungal septic thrombosis of the pulmonary veins and pulmonary artery)	III	–	–	+ (fungal septic thrombosis of the pulmonary veins and pulmonary artery)	+ (EBV, adenovirus)	+
5	–	–	I	–	+ (*K oxytoca*, *Enterococcus* sp, *P aeruginosa*)	–	+ (CMV, adenovirus, HHV6)	–
6	+	+	–	–	–	–	–	–
7	–	–	III	+	–	–	–	–
8	–	–	–	+	–	–	–	–
9	–	–	–	–	–	–	+ (adenovirus)	–
10	–	–	– (+ after DLI)	–	+ (*K oxytoca*, *S maltophilia*, *P aeruginosa*)	–	–	–
11	–	–	–	+	–	–	–	–
12	–	–	–	–	–	–	–	+
13	–	–	–	–	–	–	+ (adenovirus)	–
14	–	–	–	–	–	–	+ (EBV, CMV)	–
15	–	–	–	+	–	–	–	–
16	–	–	–	–	–	–	+ (adenovirus)	–
17	–	–	I	–	+ (*S aureus*)	–	+ (adenovirus)	–
18	–	–	–	–	+ (*S aureus*)	–	+ (CMV)	–
19	–	–	–	–	–	–	–	–

ARDS indicates acute respiratory distress syndrome; NR, not reported; and DLI, donor lymphocyte infusion.

Two patients developed chronic GVHD (limited, n = 1, and extensive, n = 1).

Infections

Most patients experienced an infectious complication of HCT (Table 3). Common viral complications included EBV viremia (n = 3, all patients received rituximab), CMV viremia (n = 3, all patients received CMV-directed therapy), and adenovirus viremia (n = 7, 4 patients received adenovirus-directed therapy). Other reported viral complications included human herpesvirus 6 (HHV6) viremia and encephalitis (n = 1), varicella zoster (n = 1), and BK virus hemorrhagic cystitis (n = 2).

Reported bacterial infections included pneumonias, bacteremias and episodes of sepsis (n = 5) related to *Serratia marcescens*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia*, *Enterococcus* sp, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. One patient developed fatal fungal septic thrombosis of the pulmonary veins and pulmonary artery.

Donor contribution to hematopoiesis

Six patients were reported to develop mixed donor and recipient chimerism (< 95% donor cells detected in peripheral blood) at a median of 37 days after HCT. All of these patients had received RIC. Patient 12 was reported to lose the graft by 35 days after HCT. For the remaining 5 patients (patients 9, 10, 13, 18, and 19), the lowest observed donor contributions to hematopoiesis ranged from 13.8%-92%. Three patients received a stem cell boost and/or donor lymphocyte infusion(s). At the time of last follow-up at a median of 867 days after HCT (range, 139-1706), all 5 patients possessed greater than 90% donor contribution to hematopoiesis and remained free of disease.

Survival and outcome

Only 1 of the 7 patients who received MAC is currently surviving, 414 days after HCT (Table 4). The other 6 patients died at a median of 97 days after HCT (range, 17-247) from toxicities and complications including VOD, pulmonary hemorrhage, pulmonary hypertension, GVHD, sepsis, multiorgan failure, and fungal septic thrombosis of pulmonary veins and pulmonary artery with pulmonary hemorrhagic necrosis.

Patient 8, who received an intermediate-conditioning regimen, also died, on day +22, of posttransplantation cytokine storm syndrome with acute respiratory distress syndrome.

Of the patients who received RIC, 6 of 11 are currently alive and well at a median of 570 days after HCT (55%). All but 1 survivor were given a Lansky or Karnofsky score of 100 at the time of last follow-up. Patients 10, 11, 12, 15, and 17 died at a median of 140 days after HCT (range, 13-416). Reported causes of death were heterogeneous and included pneumonitis with respiratory failure, cardiac toxicity with asystole and multiorgan failure, encephalitis, and ongoing CNS HLH (with loss of graft), sepsis, and pneumonia with respiratory failure (Table 4).

The 1-year probabilities of survival for MAC and RIC patients are 14% and 57%, respectively (Figure 1A), with long-term probabilities of survival of 14% and 43%, respectively (Figure 1B).

Influences on survival

We examined the significance of multiple factors known to influence transplantation outcomes including preparative regimen (MAC vs RIC),[11] donor match,[29] and HLH disease status at the time of transplantation.[2-4] HLH disease status at the time of transplantation was based on the judgment of the treating/contributing physician who reported HLH to be in remission, in partial

Table 4. Patient outcomes

Patient no	Follow-up, d	Outcome	Cause of death
1	144	Died	VOD and pulmonary hemorrhage
2	50	Died	VOD and MOF
3	170	Died	Pulmonary hypertension
4	247	Died	Fungal septic thrombosis of pulmonary veins and pulmonary artery with pulmonary hemorrhagic necrosis
5	414	Alive and well; limited skin GVHD	
6	17	Died	Pulmonary hemorrhage, VOD
7	50	Died	GVHD, MOF
8	22	Died	ARDS, posttransplantation cytokine storm syndrome
9	1765	Alive and well	
10	285	Died	Drug-resistant *P aeruginosa* sepsis
11	13	Died	Cardiac toxicity, MOF, asystole
12	140	Died	Encephalitis, HLH with CNS involvement
13	1057	Alive and well	
14	149	Alive and well	
15	125	Died	Pneumonitis and respiratory failure
16	273	Alive and well	
17	416	Died	Pneumonia and respiratory failure; chronic extensive GVHD
18	867	Alive and well	
19	139	Alive and well	

MOF indicates multiorgan failure; and ARDS, acute respiratory distress syndrome.

remission, or active. The patient who received the intermediate-intensity regimen (patient 8) was excluded from the analysis. Although there are a limited number of patients in our series, it is notable that of the surviving patients (n = 7), all were reported to be in remission of HLH at the time of HCT. Of the deceased patients (n = 12), half were reported to be in partial remission or have active disease at the time of HCT. It is also notable that of the 7 surviving patients, all but 1 received grafts from HLA-matched donors, whereas of the 12 deceased patients, only 3 received grafts from HLA-matched donors. Multivariate analysis suggested that MAC regimens and HLH that was not in remission conveyed statistically significant negative influences on survival (Figure 1C and Table 5). Match was significant in univariate analysis (data not shown), but was not significant once controlled for conditioning regimen and HLH remission status. Survival for patients receiving RIC who were reported to be in remission from HLH is 86% ($P = .03$; Figure 1C).

Because XIAP functions as an inhibitor of apoptosis and is widely expressed, we also sought to determine whether residual protein expression may offer some protective benefit for survival after allogeneic HCT. Twelve patients were reported to have had analysis of XIAP protein expression. Of 5 patients with no detectable XIAP, 2 are alive and well (40%). Of 7 patients with detectable decreased or truncated protein expression, 3 are alive and well (43%). We conclude that in this limited cohort, the presence of detectable XIAP does not appear to confer a survival advantage.

Discussion

Deficiency of XIAP is a newly recognized disorder, and the results of the present study survey reveal that transplantation outcomes overall appear poor compared with the outcomes typically expected of patients with XLP and FHLH. There was a high incidence of conditioning-related toxicity, which may be related to the lack of ubiquitously expressed XIAP and the resultant loss of its antiapoptotic and other functions. In particular, only 1 patient treated with MAC is currently surviving (14%). This is in sharp contrast to the typical survival rates in other forms of HLH, which are generally greater than 50%.[1-7,11-13] There was a preponderance of hepatic VOD and pulmonary hemorrhage in MAC patients. Although VOD has been reported in patients with HLH who undergo MAC regimens, it appears that the 50% incidence of VOD in this series is high compared with previous reports of 20%-30%.[3,4] However, because of the small number of patients included in the present study, it is difficult to conclude definitively that XIAP deficiency predisposes patients to an increased risk of liver and pulmonary toxicity. In addition, a high proportion of MAC patients received grafts from HLA-mismatched donors or had HLH that was not in remission at the time of transplantation, which may have contributed to the poor outcomes. Regardless, based on the poor survival outcomes, MAC protocols should be cautioned against and avoided in patients with XIAP deficiency.

With regard to the RIC cohort, the overall survival of just over half of patients appears to be decreased compared with the relatively high survival rates expected for HLH patients undergoing RIC HCT, which are typically greater than 80%.[10,11] However, the causes of death among the patients with XIAP deficiency were heterogeneous and we found no clear evidence to suggest that the deaths were related to deficiency of XIAP. The survival of RIC patients reported to be in remission from HLH was 86%, and the impact of HLH status was significant. This suggests that RIC transplantation outcomes for patients with XIAP deficiency who are in remission from HLH may be equivalent to that of other forms of XLP and FHLH. Infectious complications were common after HCT in both MAC and RIC patients. These complications do not appear to be increased compared with reports of transplantation outcomes for patients with HLH.[9,11]

Given our findings, the question of whether to pursue allogeneic RIC HCT is somewhat difficult to answer and is further complicated by the limited amount of information regarding outcomes of patients with XIAP deficiency not treated with transplantation. In the largest published series to date (N = 30), approximately 40% of patients with XIAP deficiency died at a mean age of 16 years predominantly because of HLH, colitis, or complications of allogeneic HCT.[30] Overall, the small numbers of patients make it difficult to draw a firm conclusion regarding recommendations for

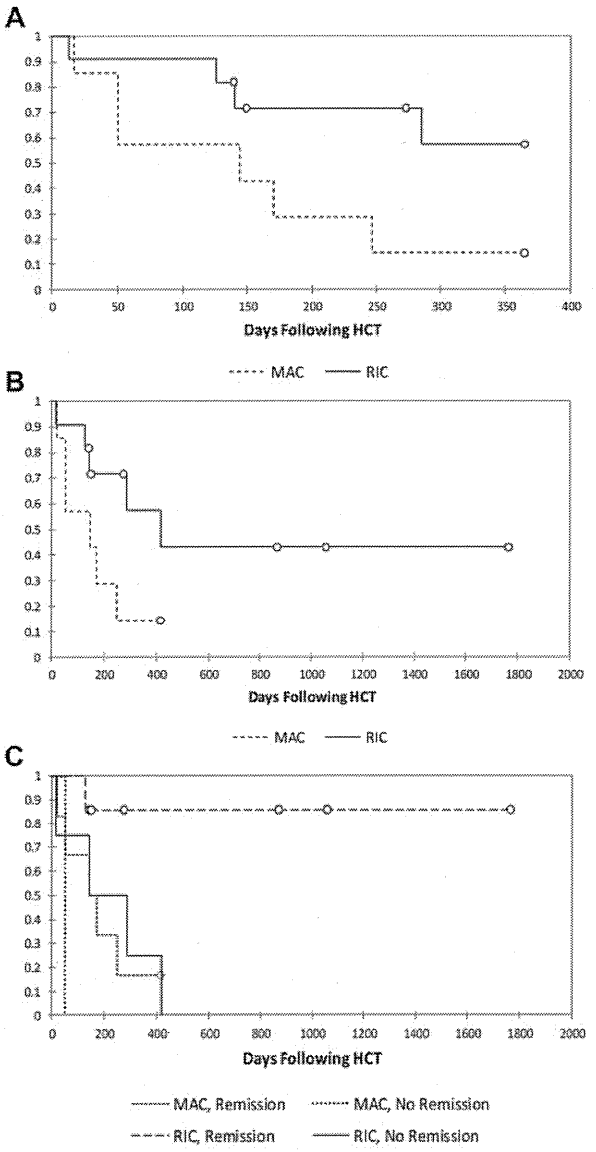

Figure 1. Kaplan-Meier survival analyses. Shown are analyses of 1-year survival (A), long-term survival (B), and survival stratified by reported HLH status at the time of transplantation (C; $P = .035$) in patients treated with MAC or RIC regimens.

RIC HCT for patients with XIAP deficiency. At this time, based on the available information, it is the our opinion that RIC protocols should be pursued with caution in young patients with XIAP deficiency who have a compelling clinical history and for whom a good stem cell donor is available. Preferably, patients should have no active lymphoproliferative disease or HLH and aggressive efforts should be made to ensure remission of HLH. The outcomes of all patients with XIAP deficiency should be monitored to further support evidence-based decisions regarding optimal treatment strategies.

Table 5. Cox proportional hazard regression model analysis

Variable	*P*	HR	HR 95% CI	
A				
Conditioning (MAC vs RIC)	.0251	7.524	1.287	44.000
Match (match vs mismatch)	.2744*	0.471	0.122	1.816
HLH activity (not in remission vs remission)	.0806	4.322	0.837	22.330
B				
Conditioning (MAC vs RIC)	.0181	6.348	1.371	29.394
HLH activity (not in remission vs remission)	.0218	5.301	1.275	22.046

In part A of the table, multivariate analysis included preparative regimen, match, and HLH activity; in part B, the effects of preparative regimen and HLH activity were analyzed with removal of the nonsignificant match effect.

HR indicates hazard ratio; and CI, confidence interval.

*The effect of match was statistically significant in univariate analysis.

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Authorship

Contribution: R.A.M. and K.R. designed the study, collected and analyzed the patient data, and wrote the manuscript; P.K., K.L., I.M., A.F., S.L., P.S., V.B., K.H., H.K., S.M., D.A.M., D.D., J.C., D.N.D., P.J.A., P.V., A.R.K., M.B.J., and J.J.B. collected the patient data and edited the manuscript; D.L. and M.K. performed the statistical analyses; and A.H.F. designed and oversaw the study and edited the manuscript.

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Correspondence: Rebecca A. Marsh, MD, Division of Bone Marrow Transplantation and Immune Deficiency, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229; e-mail: rebecca.marsh@cchmc.org.

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NKRP1A$^+$ γδ and αβ T cells are preferentially induced in patients with *Salmonella* infection

Takayuki Hoshina [a,*], Koichi Kusuhara [a,b], Mitsumasa Saito [a], Yumi Mizuno [c], Toshiro Hara [a]

[a] Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Pediatrics, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan
[c] Fukuoka Children's Hospital and Medical Center for Infectious Diseases, 2-5-1 Tojin-Machi, Chuo-ku, Fukuoka 810-0063, Japan

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ABSTRACT

NKRP1A$^+$ γδ and αβ T cells play an important role at the early phase of *Salmonella* infection in mice. Meanwhile, association between NKRP1A$^+$ T cells and human *Salmonella* infection has not been reported. The objective of this study was to investigate the role of the peripheral NKRP1A$^+$ T cells in immune response to *Salmonella* infection. Expression of NKRP1A in peripheral γδ and αβ T cells and production of interferon (IFN) γ and interleukin (IL)-4 in NKRP1A$^+$ γδ and αβ T cells were analyzed in 28 patients with acute phase *Salmonella* infection, 23 patients with acute bacterial enterocolitis other than *Salmonella* infection (disease controls) and 44 normal controls by flow cytometry. The proportion of γδ T cells expressing NKRP1A and that of IFNγ-producing cells in NKRP1A$^+$ γδ cells were significantly higher in *Salmonella* group than those in other two groups. Compared with normal controls, the proportion of αβ T cells expressing NKRP1A and that of IL-4-producing cells in NKRP1A$^+$ αβ cells were significantly higher in *Salmonella* group. These data suggested that NKRP1A$^+$ T cells might play an important role in the early defense mechanism against *Salmonella* infection.

1. Introduction

Salmonella species form a group of Gram-negative intracellular bacteria, and are common pathogens that cause enterocolitis in humans. The immune response to *Salmonella* infection includes innate immunity comprised of the intestinal epithelium, neutrophils, macrophages, dendritic cells, natural killer (NK) cells, NK T cells and γδ T cells, as well as adaptive immunity comprised of antigen-specific T cells and B cells [1–3]. Among the T cell populations, αβ T cells express CD4 and/or CD8 and recognize MHC-associated peptides, whereas the majority of γδ T cells lack CD4 and CD8 expression and recognize antigens independently of classical MHC class I- or class II-presenting molecules [4]. It was speculated that γδ T cells might bridge the innate and adaptive immunity and it was shown that these cells could act as antigen-presenting cells [5,6].

NKRP1A, an NK-cell receptor equivalent to the antigen NK1.1 in mice, is a type II transmembrane C-type lectin-like receptor expressed on the cell membrane as disulfide-linked homodimers [7]. It is expressed on almost all NK and NKT cells, and on a subset of ~25% of CD4$^+$ T cells [7,8]. Engagement of this receptor modulates several cell functions including cytokine release and transendothelial cell migration [8,9]. NKRP1A$^+$ T cells secrete several inflammatory cytokines such as IFNγ and tumor necrosis factor (TNF) α [8,10], and also play an immunoregulatory role in several diseases [11,12]. During the early phase of *Salmonella* infection in mice, MHC class II-dependent NK1.1$^+$ γδ T cells are induced to produce IFNγ, whereas NK1.1$^+$ αβ T cells are the main source of IL-4 production [13,14]. However, no association between NKRP1A$^+$ T cells and human *Salmonella* infection has so far been reported.

In the present study, to investigate the role of the peripheral NKRP1A$^+$ T cells in the human immune response to *Salmonella* infection, we compared the proportion of peripheral αβ T cells and γδ T cells expressing the NKRP1A molecule and then examined the IFNγ- or IL-4-producing cells within the NKRP1A$^+$ γδ and αβ T cell populations in patients with acute phase *Salmonella* infection, other non-*Salmonella* bacterial enterocolitis (disease controls) and healthy normal controls.

2. Materials and methods

2.1. Subjects

We studied 28 patients with *Salmonella* infection (14 males and 14 females; median age, 6.3 years; range, 1.0–33 years). Stool cultures were carried out for all patients, and blood cultures were carried out for the patients with a fever over 38 °C. The diagnosis of

* Corresponding author. Fax: +81 92 642 5435.
E-mail address: hoshina@pediatr.med.kyushu-u.ac.jp (T. Hoshina).

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Salmonella infection was made by positive stool culture and, in 5 cases, in combination with blood culture. Eleven patients were found to be infected with *Salmonella enterica* serovar Enteritidis, three with *S. enterica* serovar Typhimurium, three with *S. enterica* serovar Typhi, two with *S. enterica* serovar Paratyphi B, one with *S. enterica* serovar Thompson, one with *S. enterica* serovar Montevideo, one with *S. enterica* serovar Haifa, one with *S. enterica* serovar Infantis and five with other types (three with *Salmonella* O9, one with O4 and one with O7). All patients were immunocompetent and were successfully treated with antibiotics. All blood samples were obtained during the acute phase of *Salmonella* infection (median, 9th day of illness; range 4th–19th days).

Twenty-one patients with acute bacterial enterocolitis other than *Salmonella* infection (12 males and 9 females; median age, 10 years; range, 1.8–29 years) served as disease controls (*Campylobacter jejuni*, 14; pathogenic *Escherichia coli*, 6; *Shigella flexneri*, 1). All blood samples were obtained during the acute phase (median, 7th day of illness; range, 4th–12th day). Forty-four healthy subjects (24 males and 20 females; median age, 7.8 years; range, 1.0–38 years) served as normal controls. Informed consent was obtained from the subjects or their parents before the study. This study was approved by the Ethics Committee of Kyushu University.

2.2. Detection of cell-surface determinants by flow cytometry

Ethylenediaminetetraacetic acid blood samples were collected from both patients and controls. Fluorescein isothiocyannate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD161 (NKRP1A), and phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-TCRαβ and anti-TCRγδ antibodies (Beckman Coulter, Miami, FL, USA) were used as fluorochrome-conjugated monoclonal antibodies against the surface determinants. A three-color flow cytometric analysis was performed by using an EPICS XL instrument (Beckman Coulter). The analysis gate was set within the lymphocyte by using forward and side scatters as previously described [15]. Specificity of staining was assessed using fluorochrome-conjugated isotype-matched monoclonal antibodies. Each analysis was performed using at least 20,000 cells. Further analysis was conducted using FlowJo (version 7.6.5; Tree Star Inc.).

2.3. Intracellular cytokine detection by flow cytometory

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood samples via density-gradient centrifugation using LSM (Cappel-ICN Immunobiologicals, Costa Mesa, CA, USA) and were stimulated for 4 h at 37 °C and under 5% CO_2, with 25 ng/ml phorbol 12-myristate acetate (PMA; Sigma Chemical, St. Louis, MO, USA) plus 1 μg/ml ionomycin (Sigma Chemical) and 10 μg/ml brefeldin A in the culture medium (RPMI-1640 plus 10% fetal calf serum) containing gentamycin. After stimulation, 0.1 ml of the sample was stained with FITC-conjugated anti-CD161 (Medical & Biological laboratories, Nagoya, Japan) and PC5-conjugated anti-TCRαβ antibodies for IL-4 detection and PE-conjugated anti-CD161 and anti-TCRγδ antibodies for IFNγ detection, respectively. Erythrocytes were lysed for 10 min with 2 ml of 1X FACS lysing solution (Beckton Dickinson, San Jose, CA, USA). After washing, cells were treated with 500 μl of 1× FACS permeabilizing solution (Beckton Dickinson) for 10 min and washed again with PBS. Cells were incubated for 30 min with FITC-conjugated anti-IFNγ or PE-conjugated anti-IL-4 antibodies (Beckton Dickinson). Cells were washed and resuspended in PBS and analyzed by flow cytometry (EPICS XL, Beckman Coulter). Flow cytometric analysis was conducted by gating on lymphocytes and αβ T cells or γδ T cells. Specificity of staining was assessed using fluorochrome-matched isotype antibodies. Each analysis was performed using at least 20,000 cells. Further analysis was conducted using FlowJo (version 7.6.5; Tree Star Inc.).

2.4. Statistical analysis

The Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction was performed to analyze the differences in the proportion of αβ T cells or γδ T cells among patients with *Salmonella* infection, other bacterial infections and normal controls. The P values <0.017 (0.05/3) were considered to be statistically significant.

3. Result

The clinical features and laboratory data for the patients with *Salmonella* infections are shown in Table 1. Twenty-five patients had diarrhea, and 27 patients developed a fever over 38°C. Three patients had a fever without diarrhea. Two of them were infected with *S. enterica* serovar Typhi, and one with *S. enterica* serovar Thompson.

The number of peripheral γδ T cells in the patients with *Salmonella* infection (*Salmonella* group: median, 201 μl) was significantly higher than those in other two groups (disease controls: median, 104 μl, $P = 0.001$; normal controls: median, 133 μl, $P = 0.007$, Fig. 1A). On the other hand, no significant differences in the number of peripheral αβ T cells were seen among the three groups (*Salmonella* group: median, 1699 μl; disease controls: median, 1247 μl; normal controls: median, 1853 μl, Fig. 1B).

The proportion of $NKRP1A^+$ cells in the γδ T cell population (Fig. 2A) was significantly higher in the *Salmonella* group (median, 73.0%) than in the disease controls (median, 61.2%, $P = 0.008$) and normal controls (median, 57.3%, $P = 0.0004$) (Fig. 2B). The proportion of $NKRP1A^+$ γδ T cells producing IFNγ after stimulation with PMA plus ionomysin (Fig. 3A) was also significantly higher in the *Salmonella* group ($n = 10$, median, 58.3%) than in the disease controls ($n = 8$, median, 31.3%, P=0.003) and normal controls ($n = 10$, median, 20.1%, $P = 0.0008$) (Fig. 3B). On the other hand, no significant differences were seen in the proportion of $NKRP1A^-$ γδ T cells producing IFNγ after stimulation among the three groups (Salmonella group: median, 38.9%; disease controls: median, 21.1%; normal controls: median, 18.7%, Fig. 3C, D). Neither the proportion of IL-4-producing $NKRP1A^+$ nor $NKRP1A^-$ γδ T cells after stimulation was significantly different among the three groups (data not shown).

Within the αβ T cell population, the proportion of $NKRP1A^+$ cells (Fig. 4A) was significantly higher in the *Salmonella* group (median, 17.1 %) than in normal controls (median, 9.5%, $P < 0.0001$) but the difference between the *Salmonella* group and disease controls (median, 13.1%) was not significant (Fig. 4B). The proportion of $NKRP1A^+$ αβ T cells producing IL-4 (Fig. 5A) was significantly higher in the *Salmonella* group ($n = 10$, median, 11.7%) than in both the disease controls ($n = 8$, median, 4.6%, $P = 0.01$) and the normal controls ($n = 10$, median, 2.7%, $P = 0.001$) (Fig. 5B). Although the proportion of $NKRP1A^+$ cells in the αβ T cell population in the disease controls was also significantly higher than that in normal controls ($P = 0.0008$), the proportion of $NKRP1A^+$ αβ T cells producing IL-4 were not significantly different between the two groups. No significant differences were seen in the proportion of $NKRP1A^-$ αβ T cells producing IL-4 after stimulation among three groups (*Salmonella* group: median, 0.67%; disease controls: median, 0.99%; normal controls: median, 0.75%, Fig. 5C, D). Neither the proportion of IFNγ-producing $NKRP1A^+$ nor $NKRP1A^-$ αβ T cells after stimulation was significantly different among the three groups (data not shown).

Table 1
Clinical features and laboratory data of 28 patients with *Salmonella* infection.

Patient no.	Age (month)	Sex	Form of infection	Serotype	Fever	Diarrhea	WBC (/μl)	CRP (mg/L)
1	45	F	S	*S.* Typhi	(+)	(+)	6000	329.5
2	126	M	S	*S.* Typhi	(+)	(−)	2990	42
3	188	M	S	*Salmonella* O9	(+)	(+)	3800	284.2
4	283	F	S	*S.* Thompson	(+)	(−)	5800	259.8
5	398	F	S	*S.* Typhi	(+)	(−)	3400	50.8
6	12	F	G	*S.* Enteritidis	(+)	(+)	17300	44.6
7	13	M	G	*S.* Typhimurium	(+)	(+)	13800	126.4
8	21	F	G	*S.* Infantis	(+)	(+)	5910	77.6
9	35	M	G	*S.* Enteritidis	(+)	(+)	11310	119.1
10	35	M	G	*Salmonella* O7	(+)	(+)	10880	32.7
11	44	F	G	*S.* Haifa	(+)	(+)	7500	56.8
12	48	M	G	*S.* Enteritidis	(+)	(+)	6700	9.3
13	49	M	G	*S.* Enteritidis	(+)	(+)	3840	19.9
14	53	F	G	*S.* Paratyphi B	(+)	(+)	5950	60.8
15	64	F	G	*Salmonella* O9	(+)	(+)	5900	29
16	67	M	G	*S.* Enteritidis	(+)	(+)	3980	13.2
17	71	M	G	*Salmonella* O4	(+)	(+)	6850	41.9
18	74	M	G	*S.* Enteritidis	(+)	(+)	11420	21.6
19	78	F	G	*Salmonella* O9	(+)	(+)	10600	115.3
20	80	F	G	*S.* Typhimurium	(+)	(+)	6300	130.3
21	101	M	G	*S.* Enteritidis	(+)	(+)	6120	81.6
22	102	F	G	*S.* Typhimurium	(−)	(+)	6200	0.6
23	110	M	G	*S.* Enteritidis	(+)	(+)	7010	24
24	118	M	G	*S.* Montevideo	(+)	(+)	7720	35
25	132	F	G	*S.* Enteritidis	(+)	(+)	9520	270.6
26	138	F	G	*S.* Enteritidis	(+)	(+)	5300	6.7
27	153	F	G	*S.* Enteritidis	(+)	(+)	6660	54.5
28	182	M	G	*S.* Paratyphi B	(+)	(+)	3400	131.5

S: systemic infection, G: gastroenteritis. Systemic infection was defined that *Salmonella* species was isolated from blood culture.

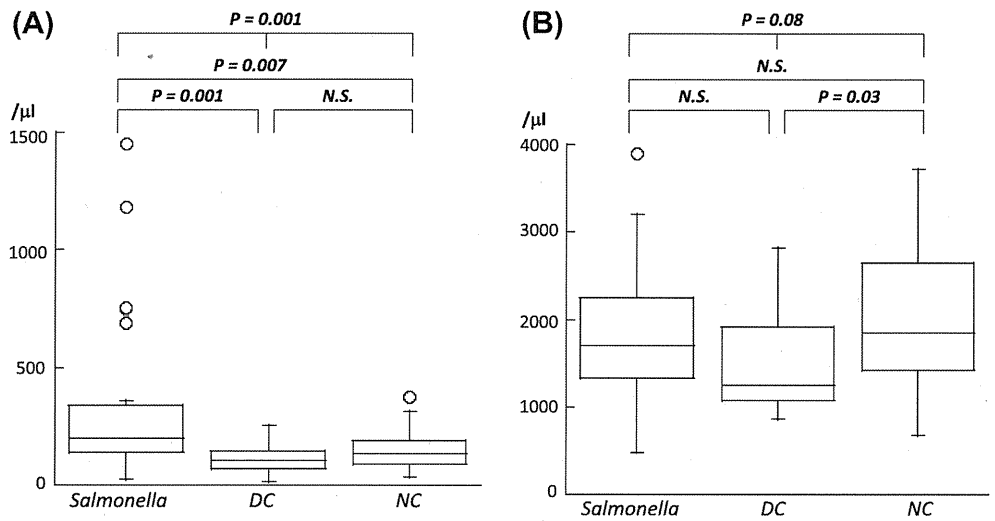

Fig. 1. The number of peripheral γδ T cells. (A) and αβ T cells (B) gated on lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls by flow cytometic analysis. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

4. Discussion

The present study showed that the proportion of NKRP1A$^+$ γδ T cells producing IFNγ was significantly higher in the patients with acute phase *Salmonella* infection than those of the patients with acute bacterial enterocolitis other than *Salmonella* infection and compared to healthy normal controls. In addition, the proportion of NKRP1A$^+$ αβ T cells producing IL-4 was significantly increased in the patients with an acute phase *Salmonella* infection. These data suggested that NKRP1A$^+$ T cells might play an important role during the early phase of *Salmonella* infection. To the best of our knowledge, the present study is the first to show that the proportion of NKRP1A$^+$ T cells was increased in human patients with *Salmonella* infection.

IFNγ plays an essential role in controlling bacterial replication in the course of a *Salmonella* infection [2,16]. In mice, neutralization of IFNγ with antibodies, or IFNγ knock-out results in increased bacterial numbers in the spleen and liver and decreases the survival of the host [17,18], whereas IFNγ treatment of infected mice results in the opposite outcome [19]. In humans, deficiencies of the IL-12/IL-23/IFNγ axis are associated with increased risks of recurrent *Salmonella* infection [20]. The main producers of IFNγ during

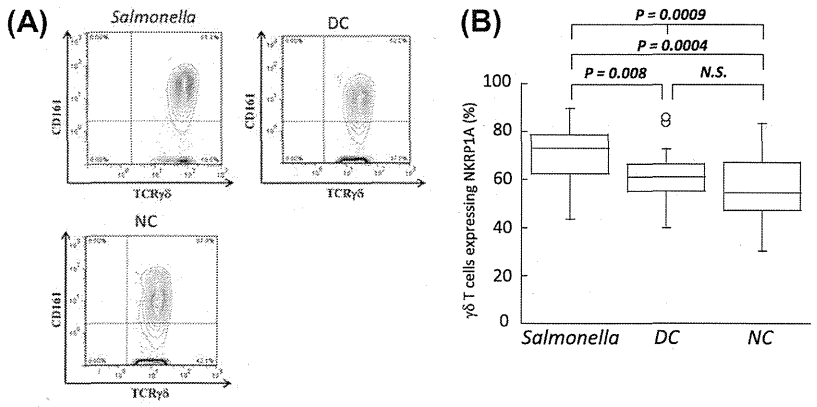

Fig. 2. Flow cytmetric analysis of NKRP1A$^+$ γδ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ γδ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of CD161-PE; the x-axis represents specific fluorescence of TCR-γδ-PC5. (B) The proportions of γδ T cells expressing NKRP1A in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

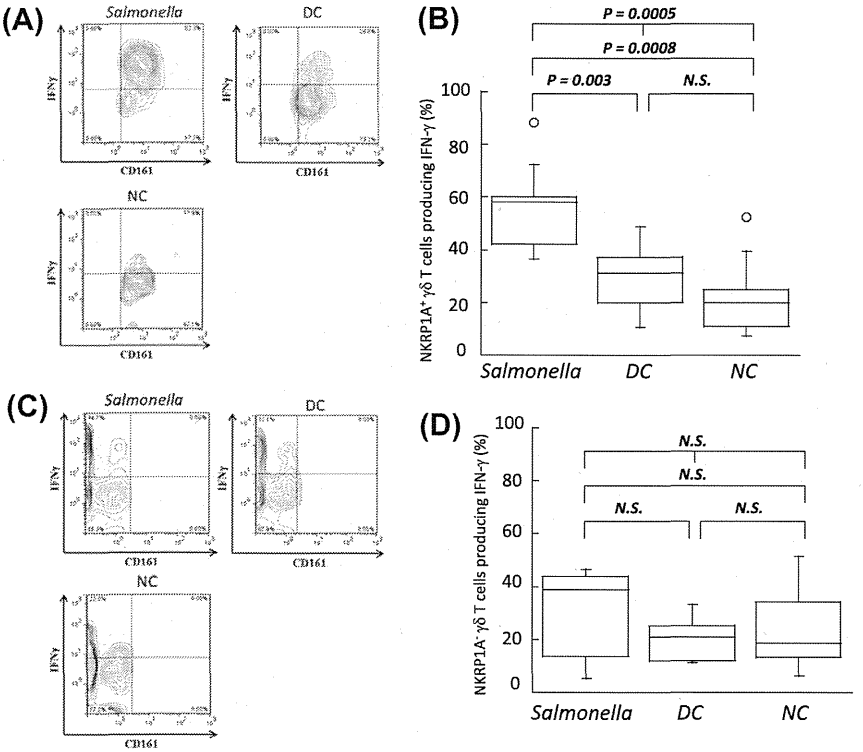

Fig. 3. Flow cytometric analysis of intracellular IFN-γ production of γδ T cells. (A and C) A representative dot plot of intracellular IFN-γ staining in CD161 (NKRP1A)$^+$ cells (A) and CD161$^-$ cells (C) gated on γδ T cells in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of IFNγ-FITC; the x-axis represents specific fluorescence of CD161-PE. (B and D) The proportions of NKRP1A$^+$ γδ T cells (B) and NKRP1A$^-$ γδ T cells (D) producing IFN-γ in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

the early phase of primary *Salmonella* infections appear to be macrophages, neutrophils, NK cells and NKT cells [1–3,21,22].

In our previous report, we found that γδ T cells were preferentially activated and expanded during a *Salmonella* infection, and this cell population expressed significantly higher level of IFNγ mRNA in patients during the acute phase of *Salmonella* infection than in healthy controls, suggesting that IFNγ -producing γδ T cells contribute to the early protection against *Salmonella* infection [23,24].

Recently, Pozo et al reported that NKRP1A crosslinking led to an enhanced IFNγ production through activation of acid sphingomyelinase and the resultant ceramide production [25]. In the present study, the proportion of NKRP1A$^+$ γδ T cells producing IFNγ was increased during the acute phase of *Salmonella* infection. It is possible that NKRP1A$^+$ γδ T cells might play an important role in the early defense against *Salmonella* infection as a main producer of IFNγ among the γδ T cells.

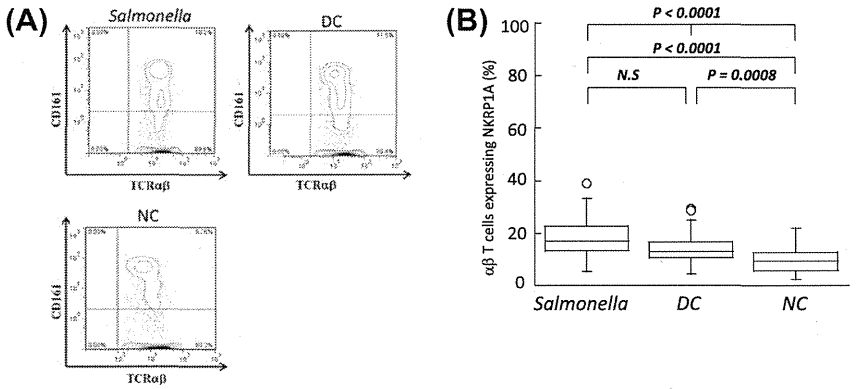

Fig. 4. Flow cytometric analysis of NKRP1A$^+$ αβ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ αβ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of CD161-PE; the x-axis represents specific fluorescence of TCR-αβ-PC5. (B) The proportions of αβ T cells expressing NKRP1A in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

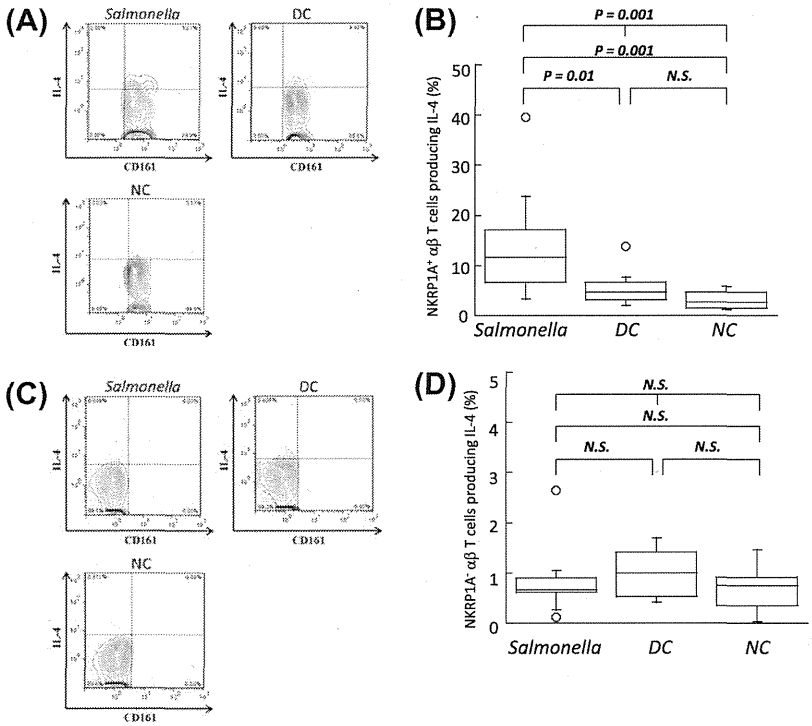

Fig. 5. Flow cytometric analysis of intracellular IL-4 production of αβ T cells. (A and C) A representative dot plot of intracellular IL-4 staining in CD161 (NKRP1A)$^+$ and CD161$^-$ cells (C) gated on αβ T cells in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of IL-4-PE; the x-axis represents specific fluorescence of CD161-FITC. (B and D) The proportions of NKRP1A$^+$ αβ T cells (B) and NKRP1A$^-$ αβ T cells (D) producing IL-4 in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

Similarly, Naiki et al. reported that NK1.1$^+$ αβ T cells were the main source of IL-4 production during the early phase of *Salmonella* infection in mice, and suggested that this cell population had an inhibitory function on the IL-12 production by macrophages and regulated the excessive inflammatory response [14]. As reduced IL-12-mediated signaling results in low IFNγ production, it was speculated that NKRP1A$^+$ αβ T cells and NKRP1A$^+$ γδ T cells, proportions of which were both increased during the acute phase of human *Salmonella* infection, might play a different role in supplementing IFNγ production.

In the present study, the proportion of NKRP1A$^+$ αβ T cells was also increased in the patients with acute bacterial enterocolitis other than *Salmonella* infection while the proportion of these cells producing IL-4 was not significantly increased, compared with those of healthy subjects. The NKRP1A molecule is also expressed on T-helper 17 cells which have been demonstrated to be responsible for the development of autoimmune diseases and allergic diseases [26]. Recent studies suggest that NKRP1A$^+$ αβ T cells can contribute to the mucosal response to pathogens by secreting a subset of cytokines such as IL-17 and IL-22

[27,28]. The increase in the proportion of NKRP1A$^+$ αβ T cells, which was commonly observed in *Salmonella* and non-*Salmonella* enterocolitis, may indicate that these cells play an important role in the gut mucosal barrier function.

In conclusion, the proportion of NKRP1A$^+$ T cells was elevated in the patients during the acute phase of a *Salmonella* infection, and it was suggested that this cell population might play an important role in the early defense against a *Salmonella* infection. Further investigations will be needed to elucidate the role and function of NKRP1A$^+$ T cells in humans during *Salmonella* and other infections.

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Treatment Choice of Immunotherapy or Further Chemotherapy for Epstein–Barr Virus-Associated Hemophagocytic Lymphohistiocytosis

Akira Shiraishi, MD,[1] Shouichi Ohga, MD, PhD,[1,2]* Takehiko Doi, MD, PhD,[1] Masataka Ishimura, MD,[1] Tomohito Takimoto, MD, PhD,[1] Hidetoshi Takada, MD, PhD,[1] Toshihiro Miyamoto, MD, PhD,[3] Yasunobu Abe, MD, PhD,[4] and Toshiro Hara, MD, PhD[1]

Background. Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) leads to an aggressive and often fatal course without appropriate treatment. Etoposide therapy is crucial for the better prognosis, although it remains unknown what patients need cytotoxic agents. Since we have complied with step-up strategy in a tertiary center, treatment outcomes were studied to search predictors for disease course. ***Methods.*** The study enrolled 22 EBV-HLH patients treated between 1999 and 2010 in Kyushu University. Immunotherapy, chemotherapy and stem cell transplantation (SCT) proceeded in stages unless patients attained a consecutive >21 days-afebrile remission. Clinical and laboratory data and outcomes were retrospectively analyzed. ***Results.*** Median age of 9 males and 13 females was 5 years (range: 9 months–41 years). Sixteen patients (73%) presented at age <15 years. Two patients remitted spontaneously, 12 attained remissions after immunotherapy, 5 after chemotherapy, and 1 after successful SCT. The remaining two patients died after chemotherapy and SCT, respectively. Median EBV load was 1×10^5 copies/ml of peripheral blood (range: 200–5×10^7). T-cells were exclusively targeted (94%; 15/16 examined) often with EBV/T-cell receptor clonality. EBV status indicated 19 primary infections and 3 reactivations. Either death occurred in EBV-reactivated patients who underwent chemotherapy ± SCT. Age at primary infection in pediatric patients increased in the last 5 years. Patients having prolonged fever ($P = 0.017$) or high soluble CD25 levels ($P = 0.017$) at diagnosis were at higher risk for requiring chemotherapy assessed by multivariate analyses. ***Conclusions.*** No cytotoxic agents were needed for >60% of EBV-HLH patients. Early immunotherapy may modulate T-cell activation and reduce the chance of unnecessary chemotherapy. Pediatr Blood Cancer 2012;59:265–270.

Key words: Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; etoposide; familial hemophagocytic lymphohistiocytosis; hematopoietic stem cell transplantation; immunochemotherapy

INTRODUCTION

Epstein–Barr virus (EBV)-associated hemophagocytic syndrome is one of the most common and serious forms of secondary hemophagocytic lymphohistiocytosis (HLH) [1,2]. The disease entity originates from the report of virus-associated hemophagocytic syndrome [3]. Affected patients present high fever, cytopenias, hepatosplenomegaly, hyperferritinemia and disseminated intravascular coagulopathy. Most patients may lead to a fatal course unless early etoposide (VP16) therapy is started. HLH is classified into primary and secondary forms, both of which arise from uncontrolled activation of lymphocytes and hemophagocytosing-macrophages, along with hypercytokinemia. The genetic basis of HLH is the defects in cytotoxic granule pathway and immune homeostasis; familial HLH (FHL) with *PRF1*, *UNC13D*, *STX11* or *STXBP2* mutations, Griscelli syndrome with *RAB27A* mutation, Hermansky–Pudlak syndrome with *AP3B1* mutation, Chediak–Higashi syndrome *LYST* mutation, along with X-linked lymphoproliferative disease (XLP) with *SH2D1A* or *BIRC4* mutations [4]. Although inherited HLH is rarely triggered by EBV infection, EBV-HLH occurs in children with no underlying diseases. The pivotal player in both types of HLH is interferon-γ producing $CD8^+$T-cells; defective cytotoxic T-cells governing immune homeostasis in FHL [5], and clonally proliferating EBV-infected T-cells in EBV-HLH [6,7]. However, optimal therapy has not been established for EBV-HLH patients.

In the last decade, molecular diagnosis has effectively distinguished FHL from EBV-HLH. Cellular target of EBV infection could make a distinction between B-cell and T-cell lymphoproliferative diseases (LPD). The broad spectrum of EBV-HLH may include infectious mononucleosis (IM), EBV^+B-cell LPD in immunodeficient patients, and chronic active EBV infection (CAEBV). In Japanese experiences, the major target of infection is $CD8^+$T-cells in EBV-HLH patients, and other T-cell subsets in CAEBV patients [8,9]. Because of aggressive infiltration of EBV^+T-cells, the Asian type CAEBV and EBV-HLH may overlap "systemic EBV-positive T-cell LPD of childhood" by the WHO classification 2008 [10]. VP16-based chemotherapy such as

Abbreviations: BM, bone marrow; CAEBV, chronic active Epstein–Barr virus infection; CB, cord blood; EBNA, Epstein–Barr virus nuclear antigen; EBV-HLH, Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; FHL, familial hemophagocytic lymphohistiocytosis; HLH, hemophagocytic lymphohistiocytosis; IM, infectious mononucleosis; LPD, lymphoproliferative disease; MNC, mononuclear cell; PB, peripheral blood; SCT, hematopoietic stem cell transplantation; sIL-2R, soluble interleukin-2 receptor; UCB, unrelated donor cord blood; VCA, viral capsid antigen; XLP, X-linked lymphoproliferative disease.

[1]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Department of Perinatal and Pediatric Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [3]Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [4]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

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*Correspondence to: Shouichi Ohga, MD, PhD, Department of Perinatal and Pediatric Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: ohgas@pediatr.med.kyushu-u.ac.jp

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HLH94/2004 leads to the cure of EBV-HLH, but not CAEBV or inherited HLH [11]. Long-term outcomes after stem cell transplantation (SCT) differ between EBV-HLH and FHL patients [12,13]. Recently, there have been an increasing number of adult patients with EBV-HLH in Eastern and Western countries [14–18]. VP16 therapy is crucial for the better prognosis of EBV-HLH, however the treatment strategy should be refined as long as self-limiting cases and secondary malignancies are of concern [19,20].

We analyzed predicting factors for the treatment of EBV-HLH in a tertiary institution, where the step-up strategy has long been employed. The clinical entity of EBV-HLH was discussed with reference to the viral status and treatment response.

PATIENTS AND METHODS

Diagnosis of EBV-HLH

Patients diagnosed as having EBV-HLH in Kyushu University between 1999 and 2010 were consecutively enrolled for the study. Inclusion criteria were the fulfillment of the diagnostic guideline for HLH [21] and high circulating EBV DNA (>200 copies/1 ml of peripheral blood [PB]). Excluded were patients having immunodeficiency, or histopathologically diagnosed malignancy. The exclusion criteria of CAEBV was based on the diagnostic guideline [22]: (1) During >6 months of HLH remission, no any infectious mononucleosis-like symptoms suggestive of CAEBV, including fever, lymphadenopathy, hepatosplenomegaly, uveitis, interstitial pneumonia, hydroa vacciniforme, and hematological, gastrointestinal, neurological, and cardiovascular disorders; (2) no abnormally high titers or unusual pattern of anti-EBV antibodies; and (3) no history of unexplained chronic illness or underlying diseases. EBV serostatus was determined by the Sumaya criteria; primary infection or reactivation was defined by positivity of EBV nuclear antigen (EBNA) at the onset of HLH [23]. Seroconversion of viral capsid antigen (VCA)-IgG and EBNA were confirmed during the disease course. Primary HLH consisting of FHL, XLP, and other secretary granule disorders were assessed by family history, pigmentary anomaly, natural killer (NK)-cell activity and flow-cytometry. The expression of perforin and Munc13.4 was screened for sequencing *PRF1*, *UNC13D*, and *STX11* approved by the ethics committee of Kyushu University. Clinical profiles, laboratory data, and treatment outcomes were collected from the hospital records.

Cell Sorting, EBV Load, and Clonality

Target cells of infection were determined by using real-time polymerase chain reaction (PCR) for EBV DNA in sorted cells [24]. Magnetic activated cell sorting (MACS) was performed on PB-mononuclear cells (MNCs) using Vario-MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) after staining with anti-CD3, CD4, CD8, and CD56 immunobeads (Miltenyi Biotec). Each cell fraction was collected using Lymphocyte Separation column (Miltenyi Biotec). The positive selection procedures yielded >92% purity. TaqMan real-time PCR for EBV DNA was performed as described previously. Gene dosages were analyzed by ABI PRISM 7700 (Applied Biosystems, Foster City, CA). DNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems), primers, and TaqMan probe. PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minutes. EBV-seropositive healthy persons show <200 copies EBV/ml and <40 copies EBV/μg DNA in PB and MNCs, respectively. Southern blotting for EBV-terminal repeat sequences was performed using PB-MNC by the established protocol [24].

Step-Up Treatment Strategy

Immunotherapy (ITx), chemotherapy (CTx), and SCT proceeded in stages unless the clinical remission continued for 3 weeks. ITx included high-dose γ-globulin (2 g/kg), conventional dose cyclosporine-A (CSA, oral 6 mg/kg/day) and/or prednisolone (PSL, oral 1–2 mg/kg/day), and additional methyl-PSL (i.v. 30 mg/kg/day for 3 days, n = 5). Antibiotic/antifungal therapy was performed with the control of coagulopathy. VP16-based CTx consisted of Fischer's protocol [25] or HLH94 [26], and subsequent CHOP-VP16 based regimen (VP16, vincristine, cyclophosphamide [CY], doxorubicin and PSL) [27]. Only one adult patient received additional high-dose cytarabin. Urgent SCT from unrelated donor cord blood (UCB) was performed in patients who were refractory to ITx + CTx. VP16 infusion (100–150 mg/m^2/day, consecutive 3 days in Fischer's protocol, 2 days per week in HLH 94) was added to the first line ITx if patients showed no sign of defervescence within 48 hours after the start of ITx, or had recurrent HLH within 21 days after all the above ITx. Unless patients had consecutive >21 afebrile days after the start of VP16, VP16 + ITx was switched to CHOP-VP16. The CTx was repeated up to six courses to patients whose afebrile period became prolonged by each course. When the interval of febrile bouts became shorter, SCT was conducted after myeloablative conditionings with VP16/busulfan/CY. The remission was defined as having no HLH findings of fever, detectable EBV DNA, cytopenias, DIC, levels of lactate dehydrogenase (LDH), transaminases, soluble CD25 (interleukin-2 receptor: sIL-2R) and ferritin. The step-up therapy was organized by two authors (S.O. and A.Y.).

Statistical Analysis

Computation was carried out by using JMP 8 Statistics and Graphics Guide, Second Edition. Logarithmic transformation was performed for continuous variables with skewed distribution. Difference in the mean values between CTx- and non-CTx groups was analyzed by Wilcoxon test and/or Student's *t*-test. Categorical difference between the two groups was assessed by Fisher's exact test. Multiple logistic regression analysis was performed to examine associations between parameters at diagnosis and requirement of CTx, simultaneously adjusting for potential confounding by covariates. For missing values, 12 (sex, age, EBV DNA, days until the first ITx, fibrinogen, leukocyte counts, hemoglobin concentration, platelet counts, alanine aminotransferase, LDH, triglyceride, and ferritin levels) and 13 variables (all the above and sIL2R) were selected in 22 and 12 patients, respectively. EBNA positivity was excluded from the analyses because of quasi-complete separation. The model was determined using forward stepwise regression, with odds ratios as estimates of relative risk. Likelihood ratio tests were used to assess statistical significance. P values <0.05 were considered to be significant.