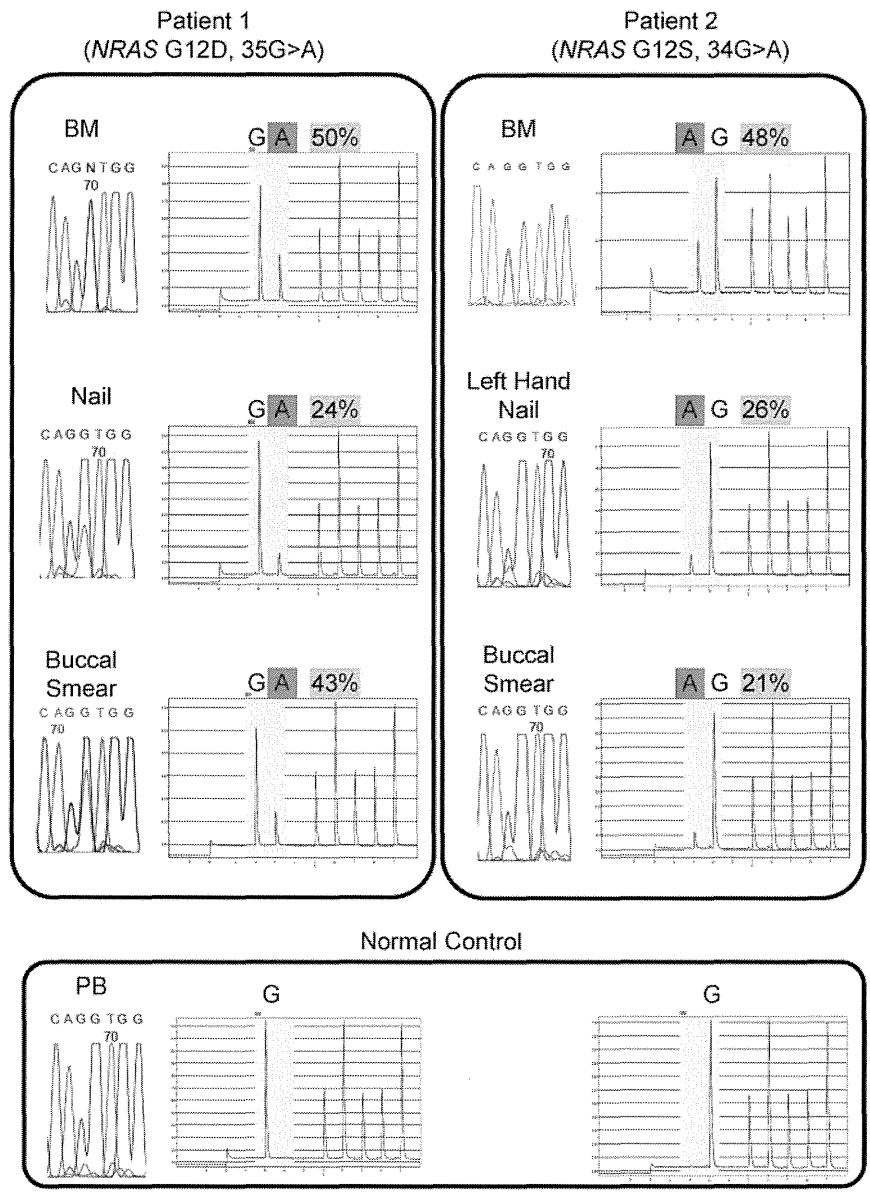

Figure 1. Direct sequencing and quantitative mutational analysis of *NRAS* in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs at diagnosis of JMML and in the nails and buccal smear cells. Quantification by pyrosequencing revealed that the fractions of mutated allele varied among different tissue types. For Patient 1: BM, 50%; nail, 24%; and buccal smear, 43%. For Patient 2: BM, 48%; left-hand nail, 26%; and buccal smear, 21%.

the first report of JMML patients with somatic mosaicism of mutations in RAS pathway genes.

Germline RAS pathway mutations are often associated with dysmorphic features similar to Noonan syndrome or its associated diseases. Correspondingly, JMML patients with germline *NRAS* or *CBL* mutations exhibit characteristic dysmorphic features.[3,10] Although our patients did not show any dysmorphic or developmental abnormalities, they should receive careful medical follow-up, especially for the occurrence of other cancers, because of the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if left untreated.[8] However, recent clinical genotype-phenotype analyses have revealed heterogeneity in their clinical course. We and other researchers have reported that patients with *PTPN11* mutations have a worse prognosis than patients with other gene mutations, including *NRAS* and *KRAS*.[15,16] Both of the JMML patients in the present study with somatic mosaicism of oncogenic *NRAS* mutations have had a mild and self-limiting clinical course. We analyzed nails of other 3 JMML patients with RAS mutations who experienced aggressive clinical course and none showed somatic mosaicism (data not shown). In analogy to the mild phenotype of JMML patients with germline mutations in *PTPN11*, we speculate that JMML patients with somatic mosaicism of RAS genes might have a mild clinical course. We are planning to confirm these observations in larger cohort.

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Authorship

Contribution: S.D. and H.M. designed and conducted the research, analyzed the data, and wrote the manuscript; A.S., M.M.-E., M. Sato, H.K., A.K., M. Sotomatsu, and Y.H. treated the patients; Y.T., Y.F.-H., K.Y., H.H., H.K., N.Y., H.S., A.N., X.W., O.I., Y.X.,

N.N., M.T., A.H., and K.K. conducted the research; and S.K. designed the research, analyzed the data, and wrote the manuscript.

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CASE REPORT

Two cases of partial dominant interferon-γ receptor 1 deficiency that presented with different clinical courses of bacille Calmette–Guérin multiple osteomyelitis

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Abstract We experienced two cases of unrelated Japanese children with bacille Calmette–Guérin (BCG) multiple osteomyelitis with partial interferon (IFN)-γ receptor 1 (IFNGR1) deficiency. Heterozygous small deletions with frame shift (811 del4 and 818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency. Case 1: a 2-year-old boy visited us because of limb and neck pain. He had been vaccinated with BCG at 17 months of age. Multiple destructive lesions were observed in the skull, ribs, femur, and vertebral bones. *Mycobacterium bovis* (BCG Tokyo 172 strain by RFLP technique) was detected in the bone specimen. The BCG multiple osteomyelitis was treated successfully without recurrence. Case 2: an 18-month-old girl developed multiple osteomyelitis 9 months after BCG inoculation. Radiologic images showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. *M. bovis* (BCG Tokyo 172 strain) was detected in the cultures from a bone biopsy. Her clinical course showed recurrent osteomyelitis and lymphadenitis with no pulmonary involvement. The effects of high-dose antimycobacterial drugs and IFN-γ administration were transient, and complete remission has since been achieved by combination antimycobacterial therapy, including levofloxacin. Partial dominant IFNGR1 deficiency is a rare disorder, but it should be considered when a patient presents with multiple osteomyelitis after BCG vaccination. The cases that are resistant to conventional regimens require additional second-line antituberculous drugs, such as levofloxacin.

Keywords Interferon-γ receptor 1 deficiency · Multiple osteomyelitis · Bacille Calmette–Guérin · Mycobacterial infection · Levofloxacin

Introduction

Interleukin-12 (IL-12)- and IFN-γ (IFNG)-mediated immunity plays an important role in host defense against intracellular pathogens [1]. Mendelian susceptibility to mycobacterial disease (MSMD) is a rare disorder and sometimes lethal disease that occurs in response to poorly virulent mycobacteria, such as bacille Calmette–Guérin (BCG) and environmental nontuberculous mycobacteria (NTM). In patients with MSMD, different types of mutations in six genes—IFNGR1, IFNGR2, IL12RB1, IL12B, STAT-1, and NEMO—have been revealed [2].

Sasaki et al. [3] previously reported a partial IFNGR1 mutation in three Japanese children with BCG osteomyelitis and in the father of one of the patients. We have followed the two unrelated cases over 10 years since their onset in the same department (Koshigaya Municipal Hospital). Based on our longitudinal experience, we intend to provide important clinical information for the diagnosis and treatment of IFN-γR1 deficiency in Japan.

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Case report

Case 1

A Japanese boy became spontaneously positive to a tuberculin purified protein derivative (PPD) skin test at the age of 11 months. There was no family history of tuberculosis. A chest X-ray film showed no abnormal findings. The PPD skin test turned negative after 6 months of prophylactic treatment with isoniazid (INH). He was inoculated with BCG (Tokyo 172 strain) by the multiple puncture technique at the age of 17 months. Nine months later (at 26 months of age), he started to limp and could not move his neck. He visited Koshigaya Municipal Hospital, and multiple osteolytic lesions were observed on his skull, vertebrae (cervical and lumbar), ribs, and femur by X-ray, bone scintigram, and magnetic resonance (MR) imaging. *Mycobacterium* was detected in the bone biopsy. *Mycobacterium bovis* was identified as the BCG Tokyo 172 strain by restriction fragment length polymorphism (RFLP). The BCG osteomyelitis was treated successfully with antimycobacterial therapy with isoniazid (INH), rifampicin (RFP), and streptomycin (SM) for 1.5 years without recurrence. He is now 17 years old and has not had a mycobacterial infection since the treatment.

Case 2 (Fig. 1)

An 18-month-old girl (13 years old at present) developed left axillary lymphadenitis 2 months after BCG inoculation at the age of 8 months. Multiple skin eruptions and abscesses appeared 9 months after the vaccination. At the BCG inoculation site, there were signs of hypertrophic scar and keloid. Granuloma was also observed below the inoculation site. X-ray, skeletal scintigram, and MR imaging showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. A bone biopsy specimen of the femur revealed granulomatous inflammation without central necrosis. *M. bovis* (BCG Tokyo 172 strain) was detected in cultures from the bone biopsy by RFLP. She was treated with INH, RFP, and SM, and showed slow improvement. Eighteen months after her initial presentation, she started to develop recurrent osteomyelitis. Additional administration of ethambutol (EB) and IFN-γ was effective but the effect was temporary. She exhibited osteomyelitis soon after discontinuation of EB and RFP. High-dose INH and EB, with the addition of clarithromycin (CAM) and IFN-γ, proved effective. Her osteomyelitis appeared to have subsided. However, later, at the age of 11 years, she experienced a third relapse of the osteomyelitis. Antimycobacterial therapy was started again, but lymphadenitis also developed on her right supraclavicle. The findings from the swollen lymph nodes were nonspecific. Additional administration of high-dose IFN-γ was partially effective against the osteomyelitis and the lymphadenitis. As the cervical lymphadenopathy appeared again, the CAM was changed to levofloxacin (LVFX). A three-drug regimen of INH, RFP, and LVFX for a period of 9 months was successful in achieving remission.

The clinical features of these two unrelated Japanese children with BCG multiple osteomyelitis are summarized in Table 1. Two-color flow cytometric analysis was performed [3] and showed significantly higher levels of IFNGR1 expression on monocytes in both cases. IL-12 and IFN-γ production was normal. Genomic DNA was obtained from peripheral blood mononuclear cells. cDNA sequences were analyzed by polymerase chain reaction. Heterozygous small deletions with frame shift (case 1, 811 del4; case 2,

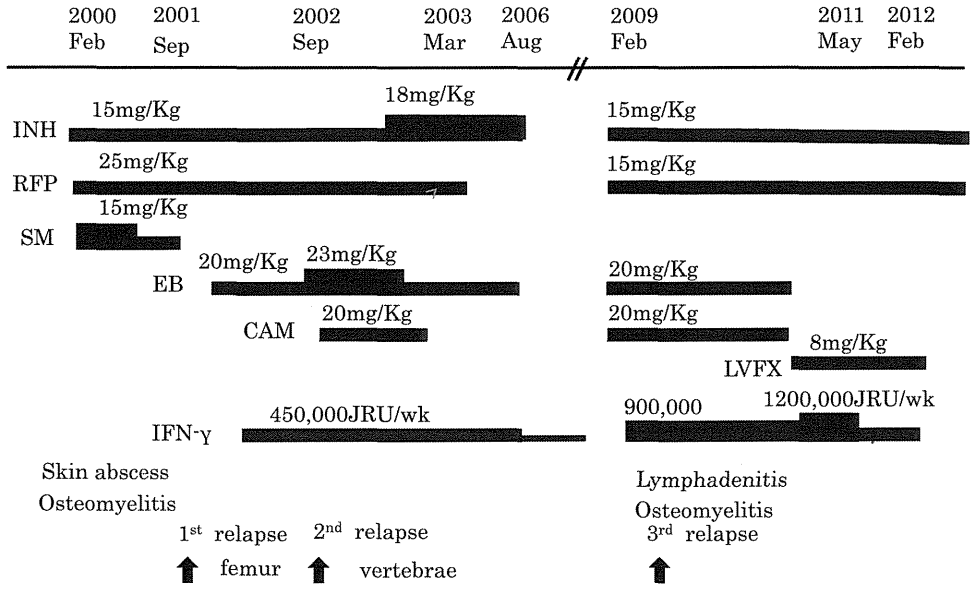

Fig. 1 Recurrent osteomyelitis and lymphadenitis in case 2. *INH* isoniazid, *RFP* rifampicin, *SM* streptomycin, *EB* ethambutol, *CAM* clarithromycin

Table 1 Immunological data at the onset of patients with bacille Calmette–Guérin (BCG) osteomyelitis

Case	1 (17 years/M)	2 (13 years/F)
BCG given at	1 year 5 months	8 months
Age at onset	2 years 2 months	1 year 5 months
Type	Multiple	Multiple, recurrent
Histology	Inflammation	Granuloma
Other organs	None	Skin, lymph node
WBCs/μl	5,300	29,600
Lymphocytes/μl	3,657	7,400
IgG, mg/dl	1,370	1,430
IgA, mg/dl	188	104
IgM, mg/dl	602	181
CD3 cells, %	40.7	56.6
CD4:CD8	3	3
CD19 cells, %	10.4	26.4
PHA response	Normal	Normal
Cytokine production IL-12/INF-γ	Normal	Normal

818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency (data not shown). Sequence analysis of six coding regions was performed and showed that none of the family members of the patients had any mutations. Furthermore, neither sets of parents were consanguineous. Thus, de novo mutation had occurred in both cases 1 and 2.

Discussion

Bacille Calmette–Guérin vaccines are safe in immunocompetent hosts, and Japanese BCG substrain Tokyo 172 is the safest BCG in the world [4]. Complications of BCG vaccination can be severe and life threatening in infants with immunodeficiency. Systemic adverse reactions to BCG vaccine, including osteomyelitis and disseminated BCG infection, are rare. Toida and Nakata [5] reviewed severe adverse reactions to BCG from 1951 to 2004 in Japan and identified 39 cases (incidence rate, 0.0182 cases per 100,000 vaccinations). Thirteen cases exhibited primary immunodeficiency; 5 of these exhibited chronic granulomatous diseases, 4 exhibited severe combined immunodeficiency, and 4 exhibited IFNGR1 deficiency. Unidentified defects in cellular immunity were observed in 6 cases. The 6 fatal cases had cellular immunodeficiencies. Bone and joint involvement was observed in 27 cases, 15 cases with multiple lesions and 12 cases with single site lesions.

Hoshina et al. [6] analyzed the clinical characteristics and the genetic background of 46 patients with MSMD in Japan from 1999 to 2009, and found that 6 had mutations in the IFN-γR1 gene. All the cases of IFN-γR1 deficiency exhibited multiple osteomyelitis, and disseminated mycobacterial infection recurred in 5 patients. All the patients exhibited the partial dominant type, and 4 of them had 818 del4. Two of the patients were from the same family, and therefore autosomal dominant inheritance was suspected. The 4 others were considered to have occurred spontaneously. In Taiwan, 3 patients from two unrelated families were identified with a hotspot IFNGR1 deletion mutation (818 del4) and exhibited chronic granulomatous disease-like features, presenting as cutaneous granuloma and multiple osteomyelitis infected with NTM [7]. Fewer patients of Asian origin have been reported with partial dominant IFNGR1 deficiency compared with those of Western countries [8]. The clinical phenotype of partial dominant IFNGR1 deficiency is milder than that of complete deficiency. In this type, BCG and NTM are the major pathogens. Complete IFN-γ receptor deficiency is associated with the early onset of severe disease caused by BCG or NTM, whereas the other genetic forms are associated with a milder course of mycobacterial infection [8].

Patients with partial IFGR1 deficiency usually respond well to antibiotic treatment, and for those who do not respond well, additional IFN-γ therapy has been shown to be effective [9]. There is no single standard regimen for the treatment of children with BCG osteomyelitis. *M. bovis* is resistant to pyrazinamides because of the expression of a pyrazinamidase. Case 1 was successfully treated with a long-term combination therapy of INH, RFP, and SM. However, in case 2, conventional therapy was inadequate to fight the infection. Additional administration of EB and relatively low dose IFN-γ was not able to control the intractable osteomyelitis. As NTM infection was also possible, high-dose EB, INH, and CAM were administered. The regimen was effective but temporary. Combination therapy, including LVFX and high-dose INF-γ, was the most successful strategy. Treatment with second-line antituberculous drugs, such as fluoroquinolone, and two first-line drugs (RFP and EB), may be more effective than RFP and EB alone against multidrug-resistant *M. bovis* [10]. LVFX plays an important role as a substitute agent for those patients who are intolerant of first-line antituberculous agents.

IFN-γ receptor deficiency is a rare disorder that should be considered when patients exhibit BCG lymphadenitis and disseminated osteomyelitis. Multifocal mycobacterial osteomyelitis without other organ involvement is only seen in dominant partial IFNGR1 deficiency [6, 8]. This type of immunodeficiency tends to exhibit recurrent mycobacterial infection and resistance to conventional antimycobacterial therapy. LVFX is likely an effective option for cases with the partial dominant type that are resistant.

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

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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

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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.

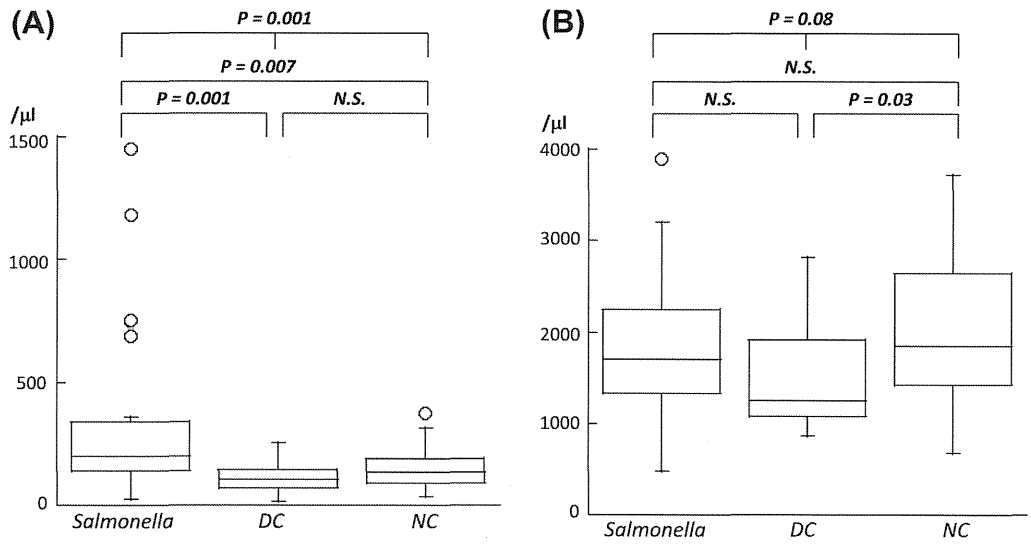

Fig. 1. The number of peripheral γδ T cells. (A) and αβ T cells (B) gated on lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls by flow cytometic analysis. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line 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

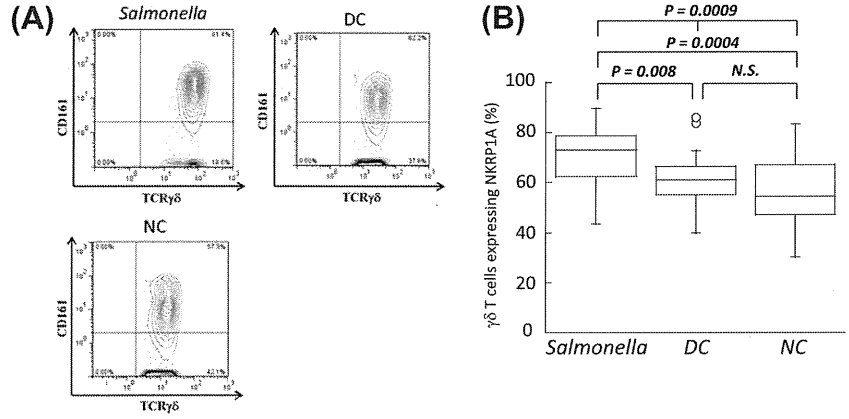

Fig. 2. Flow cytmetric analysis of NKRP1A$^+$ γδ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ γδ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence 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.

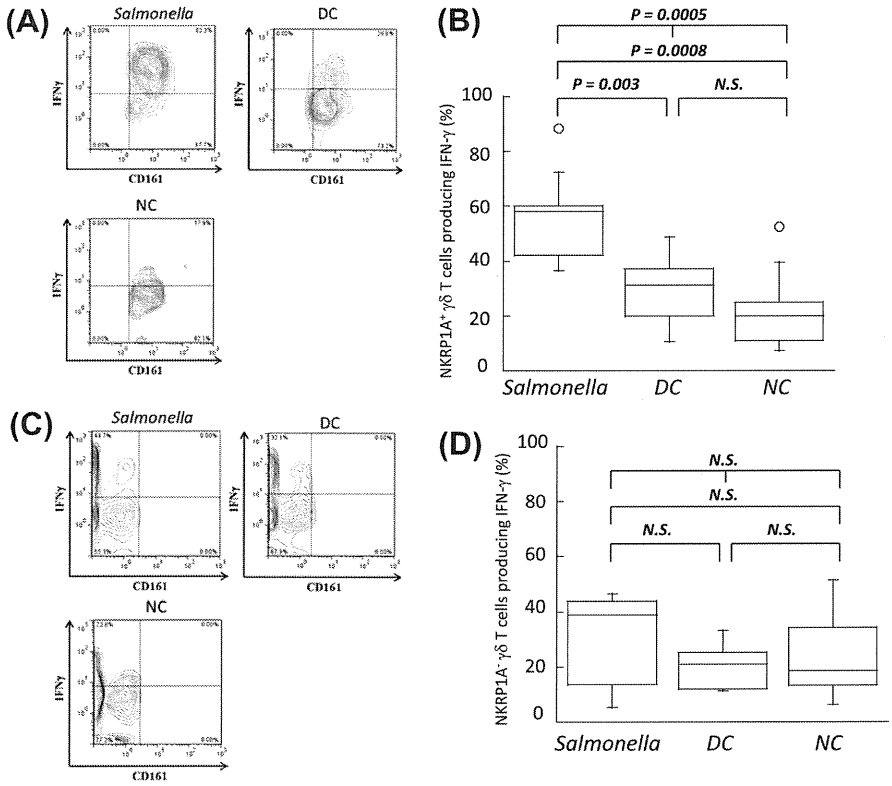

Fig. 3. Flow cytometric analysis of intracellular IFN-γ production of γδ T cells. (A and C) A representative dot plot of intracellular IFN-γ staining in CD161 (NKRP1A)$^+$ cells (A) and CD161$^-$ cells (C) gated on γδ T cells in the patients with *Salmonella* infection, disease controls and normal controls. 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.

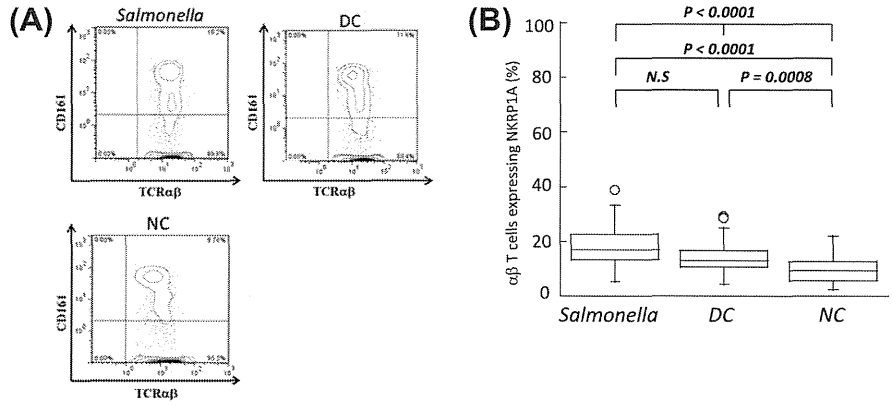

Fig. 4. Flow cytometric analysis of NKRP1A$^+$ αβ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ αβ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence 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.

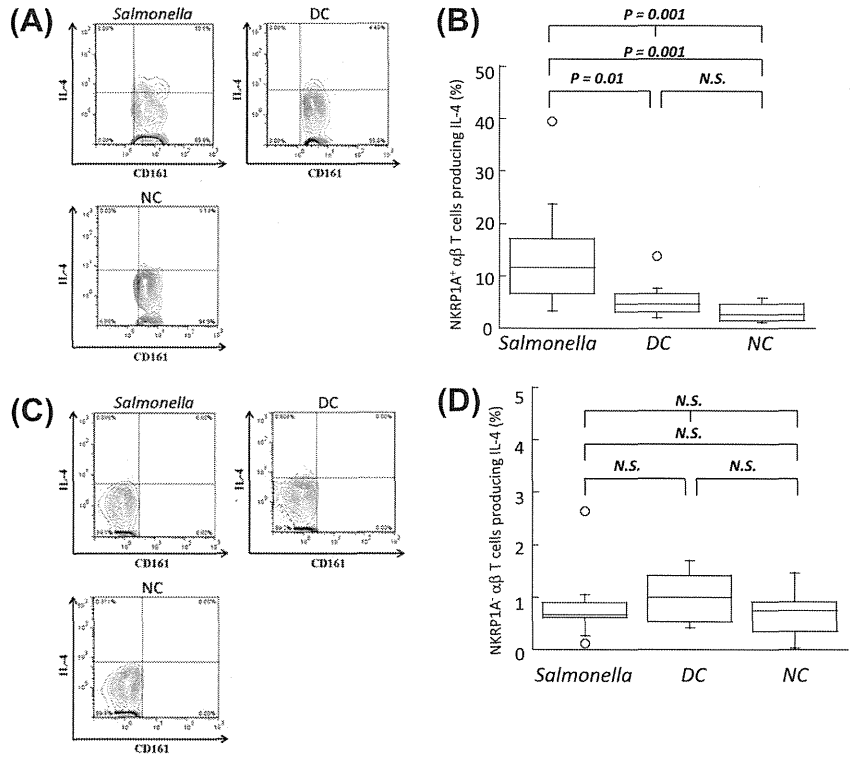

Fig. 5. Flow cytometric analysis of intracellular IL-4 production of αβ T cells. (A and C) A representative dot plot of intracellular IL-4 staining in CD161 (NKRP1A)$^+$ and CD161$^-$ cells (C) gated on αβ T cells in the patients with *Salmonella* infection, disease controls and normal controls. The 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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Pediatr Blood Cancer 2012;59:265–270

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

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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.

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Conflict of interest: Nothing to declare.

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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.

RESULTS

Profiles of EBV-HLH Patients

Clinical and laboratory findings of 22 patients at diagnosis are shown in Table I. The male/female ratio was 0.69. HLH occurred at median 5.5 years of age ranging from 11 months to 41 years. During the follow-up period (median: 2 years, range: 5 months–10 years), no survivors recurred HLH or developed immunodeficiency or lymphoma. T-cell infection was determined in 15 of 16 (94%) patients studied, 12 and 7 of whom showed EBV and TCR clonality, respectively (Fig. 1). Of 15 T-cell infections, $CD8^+$ subset was targeted in 9 patients examined. One B-cell infection was found in the series. Nineteen and three patients were determined as having primary infection and reactivation at the time of diagnosis, respectively. When the age of 16 patients (<15 years of age) was plotted according to the year, all but one had primary infection (Fig. 2). The primary infection occurred at higher age 2006–2010 than 1999–2005 ($P = 0.031$).

Treatment Outcomes of EBV-HLH Patients

Cohort diagram for the treatment outcome of 22 patients are shown in Figure 3. Two patients remitted spontaneously. A 14-month-old male (index case-1) showed high fever for 9 days and hepatosplenomegaly. Laboratory data showed WBC 2.19×10^9/L, hemoglobin 9.9 g/dl, platelet 48×10^9/L, CRP 0.23 mg/dl, LDH 517 U/L, and ferritin 738 ng/ml. EBV load (2.0×10^4 copies/ml) and negative EBNA indicated primary infection. At the day of admission, he had defervescence. A 21-month-old female (index-2) showed fever for 8 days and hepatosplenomegaly. Laboratory data showed WBC 2.52×10^9/L, hemoglobin 10.5 g/dl, platelet 25×10^9/L, LDH 3293 U/L, sIL-2R 4923 U/ml, and ferritin 8851 ng/ml. EBV load (1.0×10^5 copies/ml) and negative EBNA indicated primary infection. At the next day of admission, she had defervescence. A 10-year-old female was a representative ITx case showing high fever for 5 days and hepatosplenomegaly. Complete blood counts showed WBC 40.7×10^9/L, hemoglobin was 13.0 g/dl and platelet 68×10^9/L. PB smear revealed neutrophils 9.0%, lymphocytes 8.5%, atypical lymphocytes 1.5%, large granular lymphocytes 20% and unclassified cells 58.5%, mostly expressing CD3, CD8, and HLA-DR assessed by flow-cytometry. Laboratory data showed LDH 2,122 U/L, sIL-2R 8,684 ng/ml, and ferritin 5,436 ng/ml. EBV load (2.0×10^5 copies/ml), positive VCA-IgG and -IgM and negative EBNA indicated primary infection. On the next day after the start of γ-globulin (1 g/kg) and PSL (1.5 mg/kg/day), she had defervescence. Southern blotting revealed clonally proliferating EBV-positive $CD8^+$T-cells.

Of 20 patients with ITx, 16, 16, and 13 received γ-globulin, PSL, including 5 high dose methyl-PSL, and CSA, respectively. No HLH recurred in 11 patients who received CSA for 6 months after the remission. CTx was added to 8 patients; one, the other one and 3 patient(s) attained the remission after VP16 only, VP16 + CY, and CHOP-VP16, respectively. Of eight patients with CTx, a 41-year-old male died of HLH, and a 15-year-old male died after subsequent SCT. A 6-year-old female underwent successful allogeneic UCBT as reported elsewhere [27]. The remaining five patients attained remission with 100% Karnofsky score. As shown in Figure 4, 12 of 16 children <15 years of age (75%) required no CTx for the control of HLH, while 2 of 6 patients >16 years of age (33%) obtained remission without CTx.

Comparative Study on the Requirement of VP16-Based Chemotherapy

Clinical variables at diagnosis were studied in patients who underwent CTx or not (Table I). Febrile days until the treatment ($P = 0.011$), LDH ($P = 0.028$) and sIL-2R levels ($P = 0.018$) at the time of diagnosis were higher in CTx-group than seen in non-CTx group. The observation period was marginally longer in CTx- than non-CTx group ($P = 0.042$). To search the predictors requiring CTx, multiple logistic regression analysis was performed (Table II). As all three EBV-reactivated patients underwent CTx, EBNA was excluded for the analysis. When 22 patients were analyzed for 12 variables excluding sIL-2R, longer febrile days until the first ITx (odds ratio 1.10, $P = 0.017$) was associated with CTx. When 12 patients were analyzed for 13 variables

TABLE I. Comparison of Variables Between Etoposide-Required Patients and Non-Required Patients

	Total	Cytotoxic	Non-cytotoxic	*P*-value
Number of patients	22	8	14	
Sex—male:female	9:13	4:4	5:9	0.662*
Age at the onset of disease (yrs)	5.5 (0.9–41)	11 (1.6–41)	3.5 (0.9–19)	0.123**
Febrile days until the treatment (days)	8 (4–60)	19 (7–60)	7 (5–40)	0.011**
EBV DNA (copies/ml)	1×10^5 (2×10^2–5×10^7)	4×10^5 (1×10^4–3×10^6)	1×10^5 (2×10^2–5×10^7)	0.176***
ALT (IU/L)	130 (14–885)	372 (14–885)	118 (23–450)	0.204***
LDH (IU/L)	2,156 (291–14,743)	2,445 (1,016–14,743)	1,600 (124–3,576)	0.028***
Ferritin (ng/ml)	8,823 (265–99,280)	22,862 (599–99,280)	7,485 (265–44,760)	0.145***
Hemoglobin (g/dl)	10.6 (6.8–13.7)	10.6 (8.7–12.1)	10.2 (6.8–13.7)	0.283****
Platelet count ($\times 10^9$/L)	47 (20–112)	56 (20–112)	46 (25–75)	0.260**
Soluble IL-2 receptor (IU/L)	10,985 (1,008–28,100)	16,400 (6,000–28,100)	8,684 (1,008–11,600)	0.018****
Primary EBV infection (%)	86	62.5	100	0.036*
Target of infection, T/B/NK cells (%)	94/6/0	100/0/0	87/13/0	>0.99*
Observation period (yrs)	2 (0.4–10)	9 (4–10)	2 (0.4–6)	0.042**

EBV, Epstein–Barr virus; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; IL-2, interleukin-2; yrs, years. All variables were expressed as median (range). Each *P*-value was calculated by Fisher's exact test*, Wilcoxon-test**, $\log_{10}$ *t*-test*** and *t*-test****.

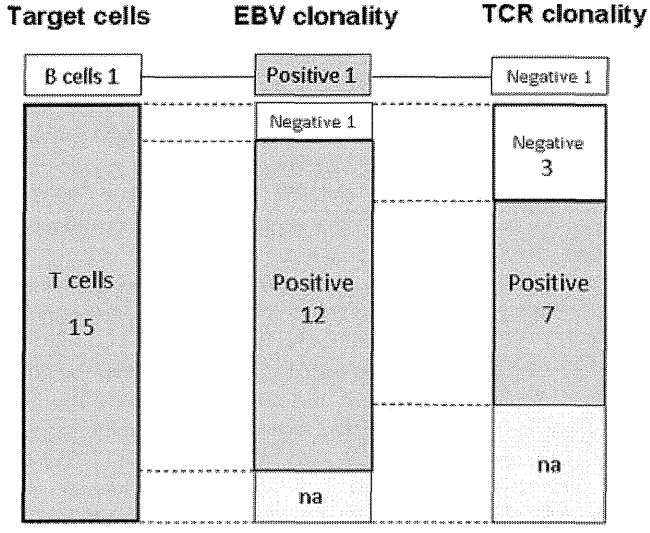

Fig. 1. Target cells of infection and clonal analyses in 16 patients with Epstein–Barr virus associated hemophagocytic lymphohistiocytosis (EBV-HLH). Major targeted cells were assessed by the copy number of EBV DNA in sorted cells. Clonal analyses were assessed by Southern blotting probed with EBV-terminal repeat sequences and T-cell receptor (TCR) genes. na: Not assessed.

including sIL-2R, sIL-2R levels (odds ratio 1.39, $P = 0.017$) was only selected in association with CTx.

DISCUSSION

We notably observed that EBV-HLH mostly occurred at primary infection, 64% of which remitted without VP16 therapy including spontaneous resolution. The target of infection was clonally proliferating $CD8^+$T-cells. Patients >15 years of age having positive EBNA at diagnosis had intractable course. Prolonged fever or high sIL-2R levels were indicated as the independent risk factors for requiring CTx. The early ITx could more effectively treat EBV-HLH than expected, although universal

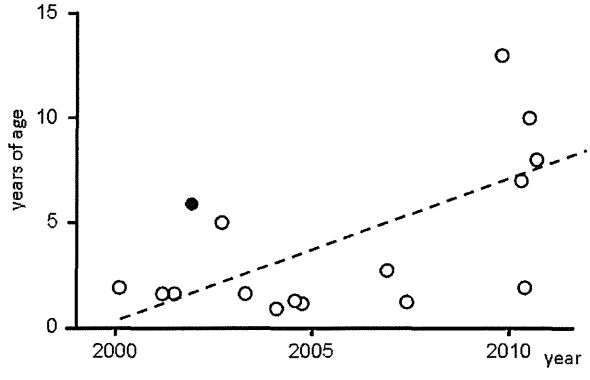

Fig. 2. Age and calendar year at the time of diagnosis in 16 patients <15 years of age. Open circle (○) means primary infection, and closed circle (•) represents reactivation of EBV. EBV status was serologically assessed by the positivity of EBNA at the time of diagnosis. Dashed line is the linear slope with correlation coefficient of 0.403; $P = 0.0101$.

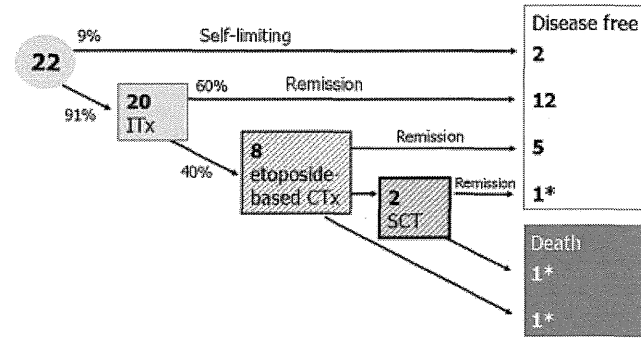

Fig. 3. Cohort diagram for the treatment course of 22 EBV-HLH patients. ITx: immunosuppresive/modulation therapy consisting of prednisolone, cyclosporine-A, and high dose therapy of γ-globulin. CTx: etoposide-based cytotoxic therapy including multidrug chemotherapy. SCT: allogeneic hematopoietic stem cell transplantation.

applicability of our strategy needs to be assessed. The treatment response suggested the modulating T-cell activation, as well as a progressive nature of EBV-driven T-lymphoproliferation.

The primary issue is the entity of EBV-HLH. In this study, EBV-HLH is defined as a non-inherited HLH having high EBV load but no evidence of malignancy or CAEBV [22]. Even using our criteria, acute IM, CAEBV and EBV-positive peripheral T-cell lymphoma might be incorporated into the subjects. Complete exclusion of inherited HLH is difficult. VP16 therapy improved the patient survival of FHL [26] and EBV-HLH [28] until 2000. Imashuku et al. [29] revealed a survival benefit if VP16 therapy was started within 4 weeks of diagnosis, while more than half of cases first underwent VP16 therapy in the multicenter study. HLH94/2004 originally aimed to control FHL patients until SCT. The feasibility of VP16 therapy for EBV-HLH has been an open question because several patients successfully remitted after ITx [7,15,30,31]. As reported here and by Belyea et al. [19], spontaneous remission occurred in patients <20 years of age on primary infection. In our study, one patient who had EBV-loaded B-cells and T-cells on primary infection was successfully treated with ITx, although it was unclear which lymphocytes carried clonal EBV. Both Japanese and non-Japanese studies revealed that T-cells [7–9,15] and rarely NK-cells [16] were the target of infection in EBV-HLH patients. More than 80% of Asian T-cell type CAEBV overlap the entity of "systemic EBV^+T-cell LPD of childhood" by the WHO2008 classification [10]. However, EBV-HLH is hardly defined pathologically. EBV-HLH patients on the virus reactivation might have a poor prognosis [31]. In our study, all three EBV-reactivated patients underwent CTx ± SCT, and two adult patients of them died. High EBV load indicated the poor treatment response [32]. However, irrespective of EBV load, 14 of 19 patients on primary infection (74%) attained resolution without CTx. None of 14 patients had late relapse of HLH. Advanced age at presentation of pediatric EBV-HLH in these 5 years (Fig. 2) reflected the increasing age of primary infection in Japan [33]. The ITx response in adolescent primary infection was favorable. Taken together, the clinical entity of "true EBV-HLH of childhood" could be defined as "systemic EBV-positive $CD8^+$T-cell LPD in immunocompetent subjects on the primary infection" and be treated with ITx during the early phase of disease.

Fig. 4. Sex, age at the time of diagnosis, EBV status, and treatment modality in 22 patients. Asterisk means the reactivation of EBV. Bold square represents death. Closed and open squares mean male and female, respectively. C: etoposide-based chemotherapy, S: chemotherapy and hematopoietic stem cell transplantation.

The prognosis of adult EBV-HLH is poor. Ahn et al. [18] reported that only 2 of 15 adult patients with EBV-HLH survived after ITx in Korea. Elazary et al. [17] reported a fatal case of a 27-year-old Jewish male with EBV-HLH at the primary infection. Sonke et al. [14] reported three fatal cases of adult onset CAEBV and HLH, one of whom showed positive EBV VCA-IgM and negative EBNA and systemic infiltration of EBV^+ polyclonal $CD8^+$T cells. Arai et al. [34] have reported five refractory cases of adult onset CAEBV, one of whom showed EBV^+ monoclonal $CD8^+$T cells and negative EBNA. Further study is needed for the pathogenesis of adult onset EBV-HLH.

Recurrent flare-up of disease activity is a critical issue in the treatment of EBV-HLH. There is no recommended therapy for patients who failed 8 weeks of HLH2004 protocol. EBV-infected T-cells evade the immune surveillance and could proliferate [6,24,35,36]. In our study, 8 of 22 EBV-HLH patients (36%) required CTx because of relapsing or persistent fever within 3 weeks after the initial ITx. Based on the subclinical activation represented by high IL-18 levels [37], CSA therapy was prolonged for refractory cases but failed in eight patients. Multivariate analyses indicated longer fever and higher sIL-2R levels as the risk of requiring CTx. It may raise the potential role of early ITx to avoid falling into the vicious cycle of hypercytokinemia as in the early VP16 therapy [29]. Alternately, high levels of sIL-2R and LDH might imply the nature of LPD. A snapshot of laboratory profiles hardly stratified the treatment arm of EBV-HLH, but our study raised a potential indicator of non-CTx. Clinical rationale of ITx, CTx and SCT for EBV-HLH is distinct from that for primary HLH, Asian type CAEBV (T-cell infection) [38,39], and Caucasian type CAEBV (B-cell infection) [40]. Early ITx, but not to be delayed VP16 therapy, may be recommended for EBV-HLH of childhood that meets all chosen criteria. The cytokine blockades of tumor necrosis factor-α or interferon-γ pathway are promising non-cytotoxic agents for the control of HLH [19]. Our strategy with limited number of cases may not be applicable to all patients beyond the racial backgrounds. Further prospective studies on the cellular tropism and EBV status are needed to confirm the preliminary results and establish the optimal treatment of EBV-HLH.

TABLE II. Variables Associated With the Requirement of Etoposide-Based Chemotherapy

Variables	Odds ratio	95% Confidence interval	*P*-value
Febrile days until the first ITx (1-day gain)[a]	1.10	1.01–1.23	0.017
sIL-2R (1×10^3 IU/L gain)[b]	1.39	1.04–2.52	0.017

ITx: immunotherapy with corticosteroid, cyclosporine-A, and/or γ globulin. sIL-2R: soluble IL-2 receptor. Multiple logistic regression analysis was performed to examine associations between variables at diagnosis and required chemotherapy, simultaneously adjusting for potential confounding factors by covariates. Likelihood ratio tests were used to assess statistical significance. [a]Twenty-two patients analyzed for 12 variables excluding sIL-2R; [b]Twelve patients for 13 variables including sIL-2R.

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Letter to the Editor

Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles

To the Editor:

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency associated with hypogammaglobulinemia and other various clinical manifestations. CVID was originally reported to be a disease primarily caused by defective B-cell function, with defective terminal B-cell differentiation rendering B cells unable to produce immunoglobulin. However, combined immunodeficiency (CID) involving both defective B and T cells is often misdiagnosed as CVID.[1] Indeed, one study reported that $CD4^+$ T-cell numbers were decreased in 29% of 473 patients with CVID[2]; similarly, another study found that naive T-cell numbers were markedly reduced in 44% (11/25) of patients with CVID.[3] These observations indicated that a subgroup of patients with clinically diagnosed CVID is T-cell deficient. Consistently, some patients with CVID have complications that might be related to T-cell deficiency, including opportunistic infections, autoimmune diseases, and malignancies, which is similar to that observed in patients with CID.[1,4] Therefore identifying novel markers to better classify CVID and distinguish CID from CVID will be required to best manage medical treatment for CVID.

We recently performed real-time PCR-based quantification of T-cell receptor excision circles (TREC) and signal joint immunoglobulin κ-deleting recombination excision circles (KREC) for mass screening of severe combined immunodeficiency (SCID)[5] and B-lymphocyte deficiency[6] in neonates. TREC and KREC are associated with T-cell and B-cell neogenesis, respectively.[7] Here we retrospectively report that TREC and KREC are useful for classifying patients with clinically diagnosed CVID.

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome [*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

Ages of patients with CVID ranged from 2 to 52 years (median age, 15.5 years). The sex ratio of the patients was 21 male/19 female patients. Serum IgG, IgA, and IgM levels were 370 ± 33 mg/dL (0-716 mg/dL), 30 ± 7 mg/dL (1-196 mg/dL), and 40 ± 6 mg/dL (2-213 mg/dL), respectively. TREC and KREC quantification was performed by using DNA samples extracted from peripheral blood, as reported previously.[5,6] Clinical symptoms were then assessed retrospectively. The study protocol was approved by the National Defense Medical College Institutional Review Board, and written informed consent was obtained from adult patients or parents of minor patients in accordance with the Declaration of Helsinki.

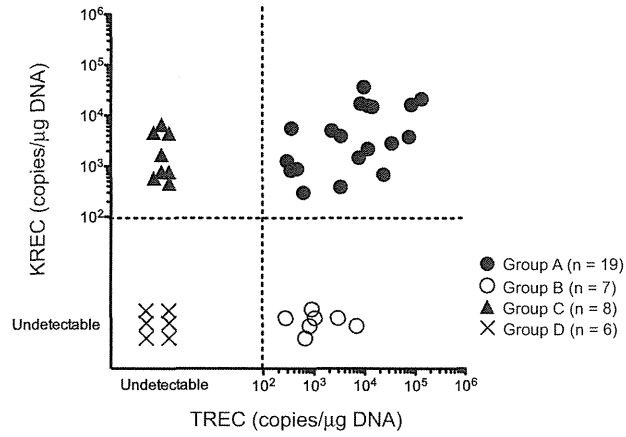

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable*, Less than 100 copies/μg DNA.

Based on TREC and KREC copy numbers, the 40 patients with CVID were classified into 4 groups (groups A, B, C, and D; Fig 1). Comparing lymphocyte subsets, $CD3^+$ T-cell numbers were similar among groups A, B, and D but were significantly lower in group C ($P < .05$; group A, 1806 ± 204 cells/μL; group B, 1665 ± 430 cells/μL; group C, 517 ± 124 cells/μL; and group D, 1425 ± 724 cells/μL; $P = .0019$, Tukey multiple comparison test based on 1-way ANOVA). $CD3^+CD4^+CD45RO^+$ memory T-lymphocyte percentages in groups B, C, and D were significantly higher than those in group A ($P < .0001$; group A, 37% ± 16%; group B, 67% ± 13% [$P = .0006$]; group C, 92% ± 8.2% [$P < .0001$]; and group D: 83% ± 14% [$P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org); additionally, the percentages of these cells in groups C and D were higher than in group B ($P = .0115$). These results indicate that group C and D patients have markedly decreased $CD4^+CD45RA^+$ naive T-cell counts than group A patients and that counts in group B are also significantly decreased, although less so than in groups C or D, which is consistent with a report showing lower TREC copy numbers in $CD4^+CD45RO^+$ cells. Some patients in groups B, C, and D exhibited normal $CD4^+CD45RO^+$ percentages, although TREC levels, KREC levels, or both decreased. This discrepancy indicates that TREC/KREC levels could be independent markers to determine the patient's immunologic status in addition to $CD4^+CD45RA^+$; the reasons underlying the discrepancy between $CD4^+CD45RA^+$ and TREC/KREC levels remain unsolved.

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and

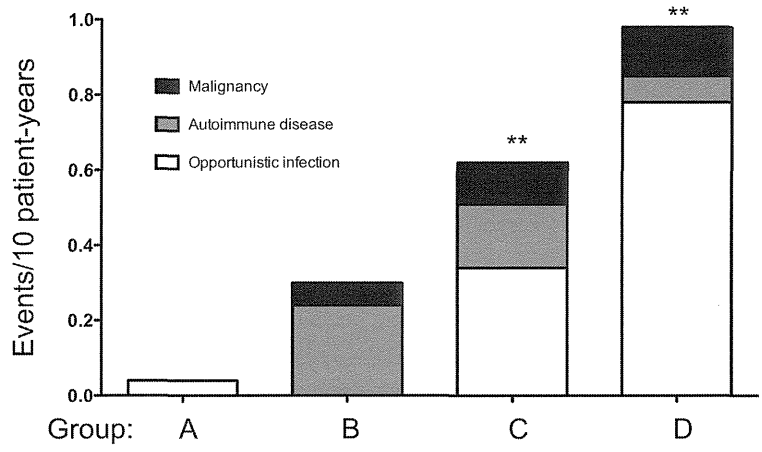

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years), and group B (0.30 events/10 patient-years) were higher than in group A (0.04 events/10 patient-years). Group A versus group D: $^{**}P = .0022$; group A versus C: $^{**}P = .0092$; group A vs group B: $P = .0692$.

IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

Because TREC and KREC levels decrease with age (see Fig E2 in this article's Online Repository at www.jacionline.org)[5,6] and age distribution was wide in this study, we compared patients' ages among groups at the time of analysis to determine whether classification was associated with age. TREC/KREC-based classification was independent of both age and sex because age distribution was not significantly different among groups ($P > .05$; group A, 12.7 ± 2.3 years [2-30 years]; group B, 23.4 ± 4.2 years [6-39 years]; group C, 21.5 ± 6.1 years [4-52 years]; and group D, 25.5 ± 4.4 years [15-46 years]; data not shown) nor was male/female sex ratio (overall, 21/19; group A, 10/9; group B, 2/5; group C, 5/3; and group D, 4/2; $P = .4916$, χ^2 test; data not shown).

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and 57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

Assessing these data, TREC/KREC-based classification matches clinical outcomes. Because group D patients exhibited the most frequent complications (opportunistic infections, autoimmune diseases, and malignancies), they could receive a diagnosis of CID based on these symptoms. If they are indeed determined to have CID, then TREC/KREC analysis is helpful to distinguish between CID and CVID. Their TREC(−)/KREC(−) phenotype might relate to defective V(D)J recombination in T- and B-cell development[8] because patients with B-negative SCID (*RAG1*, *RAG2*, *Artemis*, and *LIG4*), as well as patients with ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS; see Fig E5 in this article's Online Repository at www.jacionline.org),[5,6] were also negative for both TREC and KREC; it is intriguing to speculate that an unknown V(D)J recombination gene or genes is responsible. As for treatment, hematopoietic stem cell transplantation should be considered the preferred treatment to "cure" group D patients, as reported in patients with severe CVID/CID, because event-free survival is poor.[9]

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor