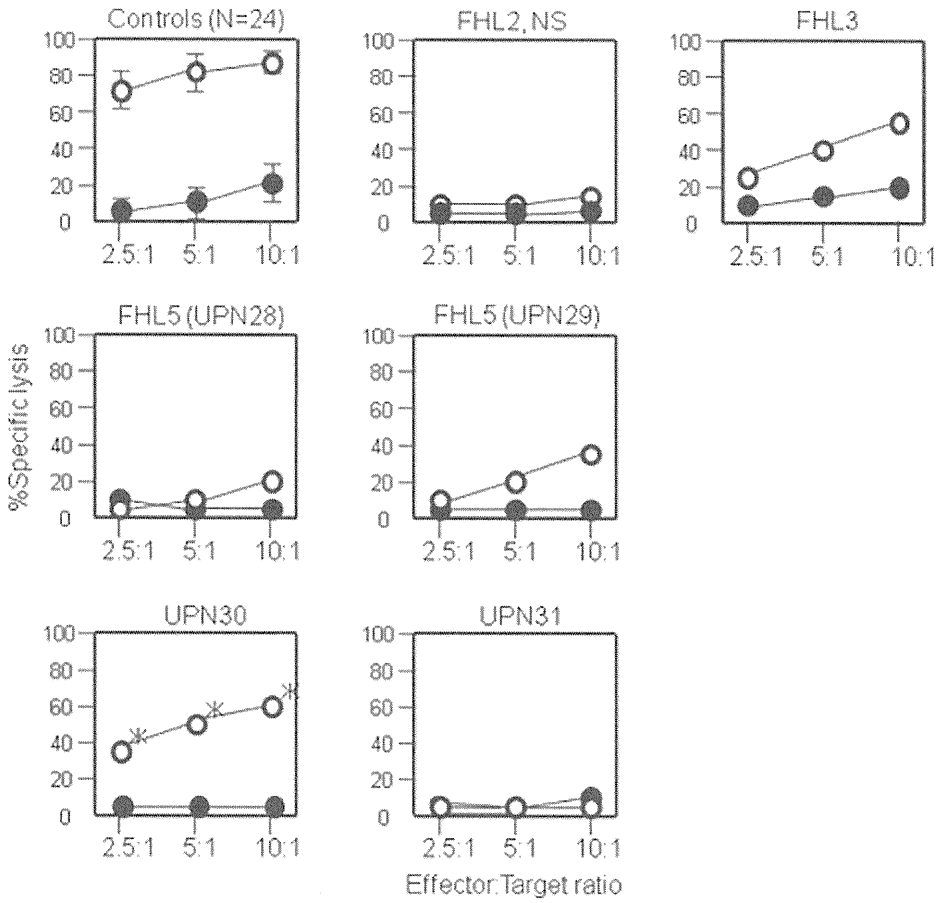

Figure 4. Cytotoxicity of alloantigen-specific CD8$^+$ T-cell lines. CD8$^+$ T-cell lines were generated from the PBMCs of the patients with FHL and 24 healthy individuals as controls by stimulation with allogeneic B-LCL (KI-LCL) cells. Their cytotoxicity was determined against allogeneic KI-LCL (clear circles) and against allogeneic TA-LCL (solid circles). All FHL patients showed various degrees of impairment of CTL-mediated cytotoxicity against allogeneic B-LCLs. NS indicates *PRF1* nonsense mutation.
doi:10.1371/journal.pone.0014173.g004

previously [14,21]. Cytotoxicity mediated by CTLs generated from 2 FHL5 patients also appeared to be low but still detectable. However, the cytotoxicity from 2 patients with unknown genetic mutations was variable; moderately impaired in one (UPN30), and deficient in the other (UPN31).

Degranulation analysis of CTL lines established from FHL patients

Degranulation activity mediated by CTLs established from healthy individuals and FHL patients are measured, and the representative data are shown in Fig. 5. The fluorescence intensities of CTLs cultured with and without alloantigen stimulation were compared by calculating MFI. Both control CTLs generated from healthy individuals and perforin-deficient (FHL2) CTLs showed a marked increase of fluorescence intensity following alloantigen stimulation, indicating that CTLs with perforin deficiency had no impairment of degranulation activity; MFI of CTLs generated from healthy individuals (n = 4) and the patient with perforin deficiency was 4.19±1.15 (mean ± SD) and 5.90, respectively. On the other hand, the increase of fluorescence intensity in Munc13-4-deficient (FHL3) CTLs following alloantigen stimulation was relatively slight; i.e. MFI was 1.81. In repeated experiments, similar degrees of degranulation were detected using CTLs established from other FHL2 or FHL3 patients. CTLs established from 2 FHL5 patients also showed a slight but significant change in fluorescence intensity (MFIs was 1.35). Notably, the increase of fluorescence intensity by CTLs established from 2 patients with unknown genetic mutations was also variable; a slight but significant change in UPN30 (MFI was 1.53), while completely undetectable even after alloantigen stimulation in UPN31 (MFI was 0.16).

Clinical and laboratory findings of 2 FHL patients with unknown genetic mutations

Clinical and laboratory findings of 2 FHL patients with unknown genetic mutations were analyzed. Both had splenomegaly, deficient NK cell activity and hemophagocytosis in bone marrow, and had shown onset of the disease at birth. One patient (UPN30) also showed hydrocephalus as CNS involvement. They had a positive family history of HLH, i.e. their sibling had shown severe hemophagocytosis and died in infancy. Both received immunochemotherapy with or without stem cell transplantation, but three subsequently died due to disease progression or complications related to the treatment.

Discussion

We have been performing a continuous nationwide survey of HLH in Japan [27]. Among 87 young patients with HLH registered so far, 31 were diagnosed as having FHL. Among these

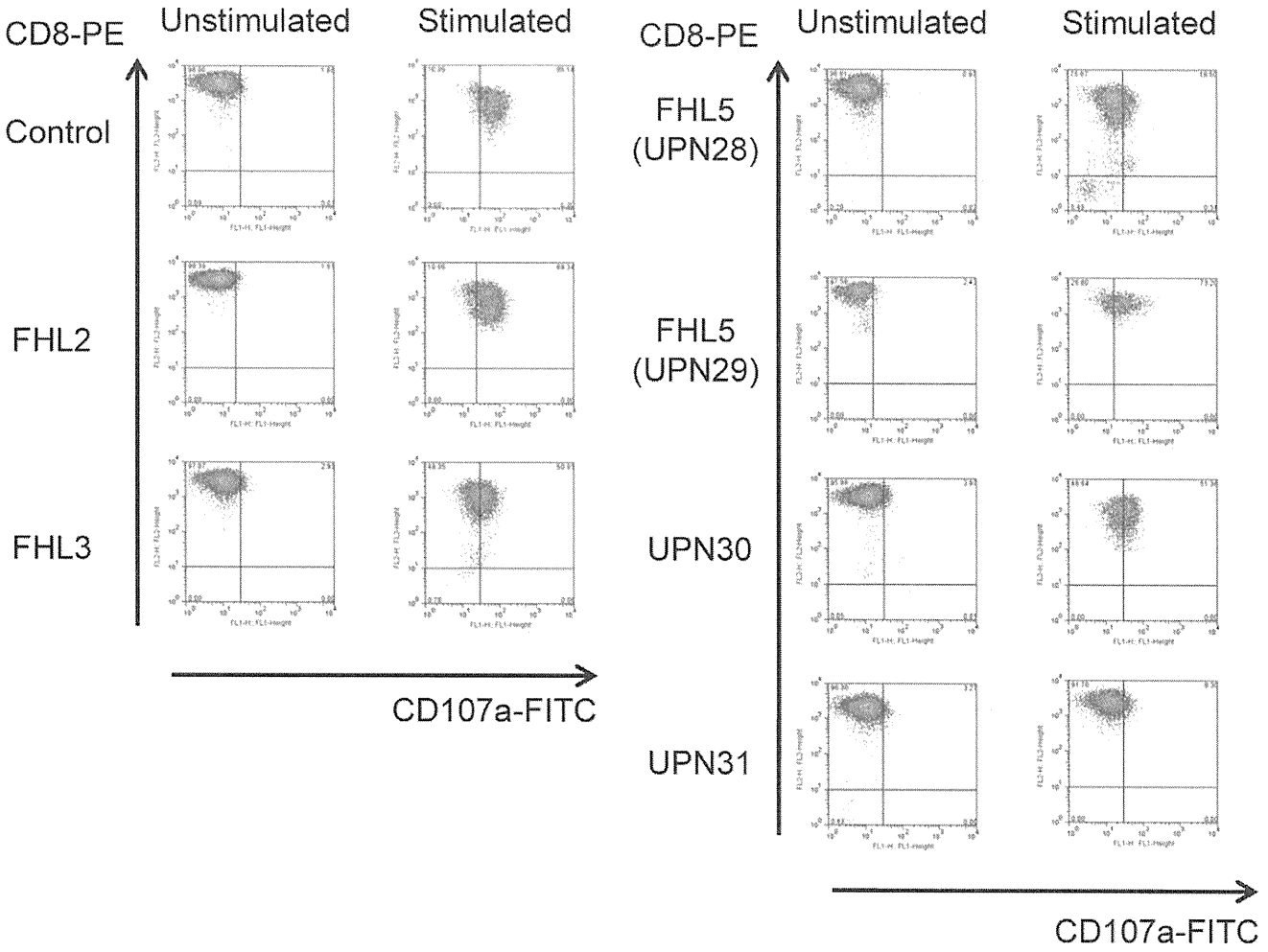

Figure 5. CD107a expression of alloantigen-specific CD8$^+$ T-cell lines. Flow cytometric analysis of CD107a expression was performed using CD8$^+$ T-cell lines generated from a healthy individual and FHL patients, as detailed in the text. Left panel of each column shows CD107a expression in CD8$^+$ T cells without any stimulation. Right panel of each column shows CD107a expression in CD8$^+$ T cells stimulated with KI-LCL cells.
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31 patients, 17 and 10 patients appeared to have FHL2 and FHL3, respectively, while no FHL4 patient was detected. In the present study, we carried out precise genetic characterizations of 4 non-FHL2/3/4 patients. Among these patients, 2 showed *STXBP2* mutations and were diagnosed as having FHL5. These findings demonstrate that the actual incidence of FHL2 and FHL3 in Japan is approximately 55% and 32%, respectively. FHL5 with *STXBP2* mutation accounts for only 6%, and no FHL4 patients have yet been found in Japan. Since more than 80% of FHL patients in Japan have been registered by our laboratory, these findings reflect the actual epidemiology of FHL in Japan. In a cohort study using samples from West Asian countries, mutations of 3 known genes (*PRF1*, *UNC13D*, *STX11*) were identified in 80% of FHL patients, while *STXPB2* mutation accounted for 10% and the causes remained unknown for the remaining 10% of FHL cases [17]. These data suggest the presence of other gene deficiencies responsible for FHL in various ethnic groups.

STXBP2 is a newly identified causative gene for FHL5. zur Stadt et al. reported 12 patients with 9 kinds of *STXBP2* mutations from Turkey, Saudi Arabia, and Central Europe [16]. Cote et al. also reported 9 patients from Turkey, Saudi Arabia and Palestine [17]. Among *STXBP2* mutations in FHL5, 1430C>T resulting in P477L and 1247-1g>c resulting in a splicing effect are the most frequent mutations in these countries [16,17]. The association between phenotype and genotype in FHL5 is still obscure. The former report described that patients with mildly impaired CD107 expression or residual CTL activity showed late onset [16]. The latter report mentioned that most of the FHL5 patients with 1430C>T showed very early onset and rapid death, whereas all of the patients with splice site mutation developed their symptoms several years later [17]. In the present study, 3 novel mutations of *STXBP2* were identified in 2 Japanese patients. Both of these patients showed onset in early infancy and the cytotoxic activities of their CTLs and NK cells were low. Further accumulation of FHL5 patients should make it possible to clarify the relationship between phenotype and genotype in this disease.

Bryceson et al. [28] demonstrated that syntaxin11 deficiency is predominantly manifested in the context of NK, rather than CD8$^+$ CTLs. Two recent studies [16,17] have shown that Munc18-2 deficiency is strongly manifested at the level of naive NK cells, whereas relatively milder defects are evident in CD8$^+$ CTLs. These studies suggest that NK deficiency is the likely trigger for at least two types of FHL (FHL4 and FHL5), while perforin and Munc13-4 deficiencies affect both cell types and thus the trigger cannot be discriminated. However, the number and cytotoxic function of NK cells vary depending on a number of factors,

including the nature of the disease, infections, and type of treatment, as indicated by Bryceson et al. [28]. Therefore measurements of NK cell activity using whole PBMCs may not accurately reflect the immune status of the patients [21]. We therefore established alloantigen-specific CTL lines from patients with the different subtypes of FHL and compared their cytotoxic activities. Consequently, CTL lines generated from 2 FHL5 patients showed markedly decreased but detectable cytotoxicity with a level similar to that in FHL3. In the SNARE systems, perforin is critical for granzyme delivery, and Munc13-4 is essential for priming of cytotoxic granules docked at the immunologic synapse, whereas syntaxin11 regulates membrane fusion events [29,30]. Via interaction with syntaxins, Munc18 proteins are required for secretory vesicle docking and fusion with the immunologic synapse [31,32]. A recent report has indicated that docked vesicles are primed for fusion by Munc13-4 when Munc18-2 clasps across the zippering 4-helix-assembled trans-SNARE complex [33]. These findings suggest that at the immunologic synapse of CTLs, the Munc18-2/syntaxin11 complex could play a role similar to that of Munc13-4 by regulating granule docking and the initiation of SNARE formation prior to the priming step. Our data indicating that the cytotoxic activities of CTLs and NK cells in FHL3 and FHL5 are impaired to a similar degree appear to support this hypothesis.

Interestingly, the degrees of cytotoxic activity mediated by CTL lines generated from 2 patients with unknown genetic mutations appeared to be significantly different, i.e. moderately decreased in one and undetectable in the other, as is the case for *PRF1* nonsense mutation [21]. A large amount of IFN-γ was produced by both of the CTL lines generated from these patients after stimulation with allogeneic LCL cells, and this cytokine production was abrogated by anti-HLA class I antibody, indicating that the antigen-recognition system mediated via the T-cell receptor/CD3 complex was intact in both cases. These data also indicate that immunological synapses are normally formed between CTLs from these FHL patients and target cells.

A recent study has indicated that CD107a expression mediated by antigen stimulation is a good candidate marker for the cytotoxic activity of CTLs and NK cells [34]. The lysosome-associated membrane protein-1, also known as CD107a, is usually located in cytotoxic granules in CTLs and NK cells. During the cytotoxic activity of CTLs and NK cells, these molecules are transported to the cell surface. Therefore, the level of CD107a expression is well correlated with degranulation activity in CTLs and NK cells. Indeed, activated NK cells derived from patients with FHL3 showed a sharply lower frequency and MFI of CD107a staining compared with healthy control subjects [35]. CD107a assay is effective tool for rapid identification of patients with FHL3 and other impaired degranulation. Furthermore, it has been reported previously that degranulation in Munc18-2-deficient CTLs is significantly impaired [16], and that transfection of these cells with the wild-type *STXBP2* gene results in recovery of the degranulation activity [17]. In our present study, determination of CD107a expression by flow cytometry indicated that Munc18-2-deficient CTLs also showed a significantly reduced level of degranulation activity. Similarly to cytotoxic activity, the degree of degranulation mediated by CTL lines generated from 2 patients with unknown genetic mutations appeared to differ significantly. That is, degranulation activity was moderately impaired in one patient and severely impaired in the other. These data also strongly suggest the presence of two types of FHL with unknown genetic mutation.

In summary, we have examined the genetic and immunological abnormalities in Japanese patients with different FHL subtypes, and our data have clarified the frequency of each FHL subtype in Japan, as well as strongly suggesting that unknown FHL subtypes are present. Further investigations to identify the molecular defects in these FHL patients will be required to clarify the pathogenesis of FHL. It is also expected that further progress in the study of FHL may clarify the detailed mechanisms of CTL- and NK cell-mediated cytotoxicity.

Supporting Information

Table S1 Primer sets for mutation screening of *STXBP2*.
Found at: doi:10.1371/journal.pone.0014173.s001 (0.06 MB DOC)

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Author Contributions

Conceived and designed the experiments: HF EI MY. Performed the experiments: KN KY HF JA TO KS TY MY. Analyzed the data: KN KY HF JA TO KS HT TH EI MY. Contributed reagents/materials/analysis tools: KN KY HT KK MS AM EI. Wrote the paper: KN KY EI MY.

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(1–3). In the first report by Barton et al (1), the IVIG infusion was followed by a decrease in IgG and IgM serum levels within 72 hours, and a typical biopsy-proved cryoglobulinemic membranoproliferative glomerulonephritis. In a case described by Odum et al (2), IVIG infusion was followed within 48 hours by diffuse purpura, a rise in plasma creatinine levels, a microscopic hematuria, and high-level proteinuria strongly suggestive of glomerulonephritis. Yebra et al reported a flare of hepatitis C virus–related cryoglobulinemic vasculitis 4 hours after the first IVIG infusion, an increase of cryoglobulin precipitation, and depletion of the monoclonal IgMκ after in vitro addition of IVIG, and suggested that this simple method could help to predict the risk of cryoglobulin–IVIG immune complex formation and should be performed before starting IVIG in patients with mixed cryoglobulinemia (3). As with infliximab and rituximab, we have reported in our article that polyvalent exogenous human immunoglobulins were also recognized in vitro by RF-positive IgMκ.

Taken together, these results strongly suggest that the recognition of monoclonal or polyclonal immunoglobulin by RF-positive IgMκ is not specific and that treating RF-positive IgMκ cryoglobulinemic vasculitis with either monoclonal immunoglobulins (e.g., rituximab or infliximab) or polyvalent immunoglobulins is associated with a risk of increased cryoprecipitation and vasculitis flare shortly after treatment initiation.

We believe that, in the presence of RF-positive IgMκ type II cryoglobulinemic vasculitis, any treatment with IVIG should be used with caution. IVIG does not have the clear benefit of rituximab in cryoglobulinemic vasculitis, and there is not a rationale for the use of monoclonal anti–tumor necrosis factor α antibodies. The use of rituximab, should, as well, be proposed cautiously in patients with a RF-positive IgMκ type II cryoglobulinemic vasculitis. We recommend that plasma exchanges should be performed to reduce high serum cryoglobulin levels, and that rituximab should be given in low doses. This precaution should also be recommended for other treatments that are based on B cell–depleting monoclonal antibodies, which have not yet been used in cryoglobulinemic vasculitis, such as veltuzumab (anti-CD20), inotuzumab ozogamicin, and epratuzumab (anti-CD22).

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Damien Sène, MD, PhD
Patrice Cacoub, MD, PhD
Hôpital Pitié-Salpêtrière
Assistance Publique Hôpitaux de Paris
CNRS UMR 7087 and Université Pierre et Marie Curie-Paris 6
Paris, France

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DOI 10.1002/art.27619

Enhanced NF-κB activation with an inflammasome activator correlates with activity of autoinflammatory disease associated with *NLRP3* mutations outside of exon 3: comment on the article by Jéru et al

To the Editor:

We wish to comment on a recent report regarding functional consequences of *NLRP3* mutations (1). Based on the genetic analysis of a family with atypical autoinflammatory symptoms, Jéru et al identified a mutation in the leucine-rich repeat (LRR) domain of *NLRP3*. *NLRP3* is a 9-exon gene comprising 3 major domains: an amino-terminal pyrin domain, a nucleotide-binding oligomerization domain (NOD), and carboxyl-terminal LRR. More than 50 disease-associated mutations have been described for *NLRP3*; most were found within the centrally located NOD encoded by exon 3.

Jéru et al reported that NLRP3 was an inhibitory molecule, although the function of NLRP3 in NF-κB signaling remains controversial. We previously reported that *NLRP3* mutations showed spontaneous ASC-dependent NF-κB activation, and this was clearly associated with disease severity in patients with cryopyrin-associated periodic syndrome (CAPS) (2). However, among 11 mutations identified from our recruited patients (3), G755R located in exon 4 did not show spontaneous NF-κB activation (Figure 1A), even though a patient carrying G755R had severe disease manifestations. To date, 2 other *NLRP3* mutations located outside of exon 3 have been reported: G755A (4) and Y859C (5). G755A was identified in a typical CAPS patient (4). In contrast, a patient with Y859C had only 1 episode of a transient rash and, of note, absence of fever (5). A family member with Y859C (1) also did not manifest skin eruptions and showed a relatively mild phenotype. As seen in Figure 1A, neither G755A in exon 4 nor Y859C in exon 6 exhibited NF-κB activation.

For *NOD2*, gain-of-function mutations associated with granulomatous disorders are recognized in the centrally located *NOD* and exhibit similar spontaneous activation of NF-κB without the *NOD2* ligand (6). Interestingly, the amino acids affected by an R260W mutation in *NLRP3* and an R334W mutation in *NOD2* are at analogous positions, suggesting a common molecular mechanism for development of autoinflammatory disease. In contrast, *NOD2* mutations at LRRs, related to Crohn's disease, show defective responses to the *NOD2* ligand. In the presence of R837, an NLRP3-inflammasome activator, a G755R mutation located outside exon 3 of *NLRP3* showed remarkably enhanced NF-κB activation with an activity level that was higher than that observed with the R260W mutation (Figure 1B). With regard to 2 other mutations located outside of exon 3, G755A showed slightly increased NF-κB activation with R837, whereasY859C, which was identified in the atypical CAPS family whose members had mild phenotypes, did not. Thus, the enhanced NF-κB activation after stimulation with R837 correlates with disease activity, including mutations outside of exon 3. However, we still do not know how R837 activates the NLRP3 inflammasome.

We agree that *NLRP3* mutations, especially those identified from de novo cases, should be carefully evaluated by functional analyses. We believe that, in addition to excess production of interleukin-1β (IL-1β), enhanced NF-κB activa-

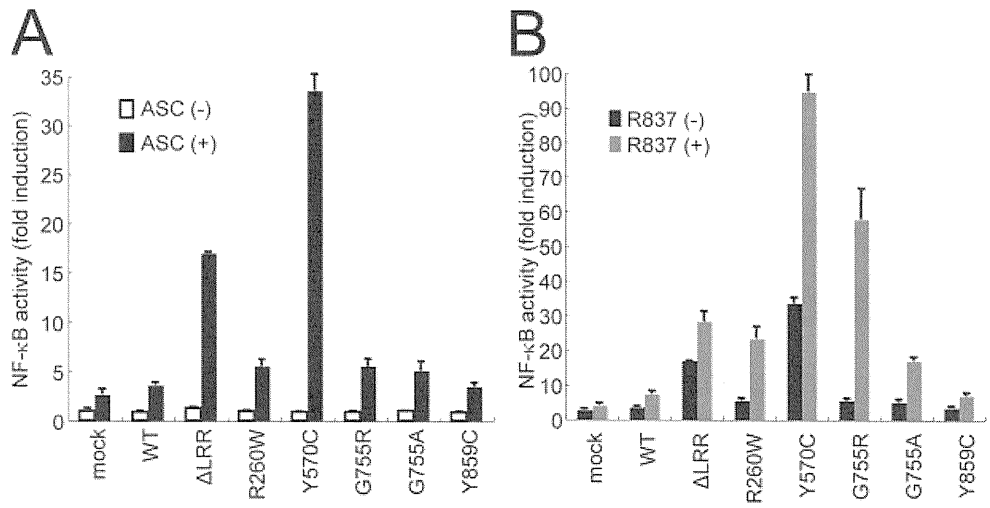

Figure 1. NF-κB activity in disease-associated mutations of *NLRP3*. **A,** Spontaneous NF-κB activation occurs in disease-associated mutations of *NLRP3*, but not in mutations located outside of exon 3. Expression plasmids for NLRP3, its deletion mutation lacking leucine-rich repeats (ΔLRRs), and ASC in the pEF-BOS vector background have been previously described (2). Mock is an empty vector. WT is wild-type NLRP3. R260W and Y570C are disease-associated mutations located within exon 3. G755R, G755A, and Y859C are the mutations outside of exon 3. **B,** Addition of R837 induces appreciable NF-κB activation in G755R mutations compared with WT *NLRP3*. ASC-dependent activation of NF-κB in the presence and absence of 10 μg/ml R837 was assessed. Values are the mean and SD of normalized data (in relation to mock with ASC [**A**] or R837 [**B**], set at 1), from triplicate cultures. Representative data from 3 independent analyses with similar results are shown.

tion may be associated with the accumulation of the IL-1β proform (7), which may also contribute to disease onset and the clinical manifestations of CAPS. In addition, careful observations of patients who bear *NLRP3* mutations outside of exon 3, who sometimes present with atypical symptoms of CAPS as in the cases described by Jéru et al, may provide an opportunity to better understand the physiologic and pathologic functions of NLRP3.

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Naotomo Kambe, MD, PhD
Takashi Satoh, MD
Chiba University
Chiba, Japan
Hideaki Tanizaki, MD
Akihiro Fujisawa, MD, PhD
Megumu K. Saito, MD, PhD
Ryuta Nishikomori, MD, PhD
Kyoto University
Kyoto, Japan

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Reply

To the Editor:

We thank Dr. Kambe and colleagues for their comments. As mentioned by Kambe et al and in our article, research on the function of NLRP3 in NF-κB signaling has led to many conflicting data. Several studies have shown an activating effect of NLRP3 in the presence of ASC (1–5), while

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REVIEW ARTICLE

The Inflammasome, an Innate Immunity Guardian, Participates in Skin Urticarial Reactions and Contact Hypersensitivity

Naotomo Kambe[1], Yuumi Nakamura[1,2], Megumu Saito[3,4] and Ryuta Nishikomori[3]

ABSTRACT

Urticarial rash, one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS), is caused by a mutation in the gene encoding for *NLRP3* (nucleotide-binding oligomerization domain, leucine-rich repeats containing family, pyrin domain containing 3). This intracellular pattern recognition receptor and its adaptor protein, called apoptosis associated speck-like protein containing a caspase-recruitment and activating domain (ASC), participate in the formation of a multi-protein complex termed the inflammasome. The inflammasome is responsible for activating caspase-1 in response to microbial and endogenous stimuli. From the analysis of cellular mechanisms of urticarial rash in CAPS, we have traced caspase-1 activated IL-1β in CAPS to a surprising source: mast cells. Recently, two groups have generated gene-targeted mice that harbored *Nlrp3* mutations. These mice had very severe phenotypes, with delayed growth and the development of dermatitis, but not urticaria. The reason for the differences in the skin manifestations observed with CAPS and these knock-in mice relates to the findings that the inflammasome also plays a role in contact hypersensitivity, and that IL-18, another cytokine involved with inflammasome-activation of caspase-1, may be a major player in dermatitis development.

KEY WORDS

contact hypersensitivity, IL-1β, inflammasome, NLRP3, urticaria

INTRODUCTION

The skin is the primary interface between the interior of the body and the external environment. The skin functions to retain water, prevent the permeation or loss of other molecules and maintain body temperature. The skin also physically protects us from microbial invasion. Furthermore, it has become established that the skin itself plays a major role in the immune system.[1]

Recognition of invading microorganisms is essential for inducing an effective immune response. This process is mediated by germ line-encoded pattern recognition receptors, which can also be found in plants that do not have circulating white blood cells. To date, the most extensively studied pattern recognition receptors have been the Toll-like receptors (TLRs). Using their leucine-rich repeats (LRRs), these transmembrane proteins recognize conserved bacterial constituents, such as lipopolysaccharide (LPS). More recently, another class of pattern recognition receptors, called nucleotide-binding oligomerization domain (NOD)-LRRs containing family (NLRs), have been identified.[2] While TLRs detect bacterial products at the outer cell surface or in endosomes, intracellular NLRs mediate cytoplasmic recognition of bacterial products.[3]

Several NLR members participate in the formation of a multi-protein complex termed the inflammasome that is responsible for activating caspase-1 in re-

[1]Department of Dermatology, Chiba University Graduate School of Medicine, Chiba, [3]Department of Pediatrics, Kyoto University Graduate School of Medicine, [4]Clinical Application Department, Center for iPS Cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan and [2]Department of Pathology and Comprehensive Cancer Center, The University of Michigan, Medical School, Ann Arbor, Michigan, USA.
Correspondence: Naotomo Kambe, MD, PhD, Department of Dermatology, Chiba University Graduate School of Medicine, 1–8–1 Inohana, Chuo-ku, Chiba 260–8670, Japan.
Email: nkambe@faculty.chiba-u.jp
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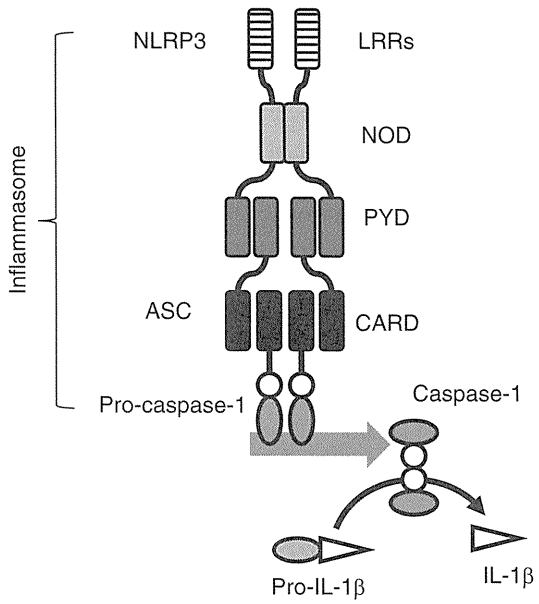

Fig. 1 The inflammasome is comprised of an NLR protein and an adaptor protein ASC, which can bridge the NLR to pro-caspase-1. Activation of the inflammasome results in self-cleavage and activation of pro-caspase-1 into the active protease. Then, activated caspase-1 cleaves its target molecules, including pro-IL-1β, into biologically active forms. LRRs, leucine-rich repeats; NOD, nucleotide-binding oligomerization domain; PYD, pyrin domain; CARD, caspase-recruitment and activating domain; NLRP3, NOD-LRRs containing PYD 3; ASC, apoptosis-associated speck-like protein containing a CARD.

sponse to microbial and endogenous stimuli.[4,5] The inflammasome is comprised of an NLR protein and an adaptor protein called apoptosis associated speck-like protein containing a caspase-recruitment and activating domain (ASC), which can bridge the NLR to pro-caspase-1 (Fig. 1). Activation of the inflammasome results in self-cleavage and activation of pro-caspase-1 into the active protease. Then, activated caspase-1 cleaves its target molecules, including pro-IL-1β, into biologically active forms.[6-9] IL-1β participates in systemic and local responses to infection, injury and immunological challenges by inducing fever, activating lymphocytes and by promoting leukocyte infiltrations at sites of injury or infection.[10,11]

In this review, we will discuss the functions of the inflammasome in the skin. In particular, we will focus on the inflammasome comprised of NLR family, pyrin domain containing 3 (NLRP3) in the regulation of IL-1β production and the implications for skin diseases.

URTICARIA

Urticaria, or hives, is a common disease that affects up to 20% of the general population at least once during their lifetimes.[12] This allergic disorder involving the skin is fleeting in nature, as it is characterized by the sudden appearance of wheals but the returning to its normal appearance without pigmentation, usually within 1-24 hours.[13] In its histological aspects, the wheal consistently exhibits localized edema of the dermis with dilatations of the post-capillary venules and lymphatic vessels. However, perivascular infiltrates show variable intensities comprised of neutrophils, and/or eosinophils, macrophages and T cells.[13] These inconsistent histological findings may underline the complex nature of the pathogenesis of urticaria, which has many sources including histamine release by activated mast cells.

Although patients with acute urticaria often complain of an upper airway infection, the eliciting cause is unclear in over 50% of patients. In particular, for chronic urticaria, defined as that persisting longer than 6 weeks, triggers remain unidentifiable in the majority of cases, despite extensive clinical and laboratory investigations.[12] In addition, urticaria is sometimes triggered by cold or heat contact, solar exposure, delayed pressure, mechanical stimuli and vibration. These subtypes of urticaria are classified as physical urticaria. Other types of urticaria are aquagenic, cholinergic, evoked by a contact irritant or exercise-induced.[13] Moreover, 2 or more different subtypes of urticaria can co-exist in any given patient.

Compared with the variety of proposed causes for urticaia, the strategy for treatment is relatively simple. Histamine H1-receptor antagonists are recommended as the first line of treatment because histamine release by cutaneous mast cells plays an important role in the development of urticaria. However, good responses using oral antihistamines have only been recorded for 40% of patients.[12] Antihistamines have been shown not to be effective for physical urticaria, suggesting that in a significant number of individuals, chronic urticaria is mediated via histamine-independent mechanisms.

CRYOPYRIN-ASSOCIATED PERIODIC SYNDROME

An urticarial rash (Fig. 2) that develops in the neonatal or early infant period is one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS). CAPS is caused by a mutation in the gene encoding for *NLRP3*, previously known as *NALP3/CIAS1*/cryopyrin.[14,15] CAPS includes a spectrum of hereditary periodic fever disorders that comprise 3 phenotypically overlapping, but relatively distinct syndromes: familial cold antoinflammatory syndrome [FCAS, Mendelian inheritance in men number (MIM) #120100], Muckle-Wells syndrome (MWS, MIM #191900) and chronic infantile neurological cutaneous and articular (CINCA) syndrome (MIM #607115), also known as neonatal-onset multisystem inflammatory disease. FCAS and MWS are characterized by periodic attacks of urticarial rash, fever and arthralgia; whereas patients with CINCA syndrome,

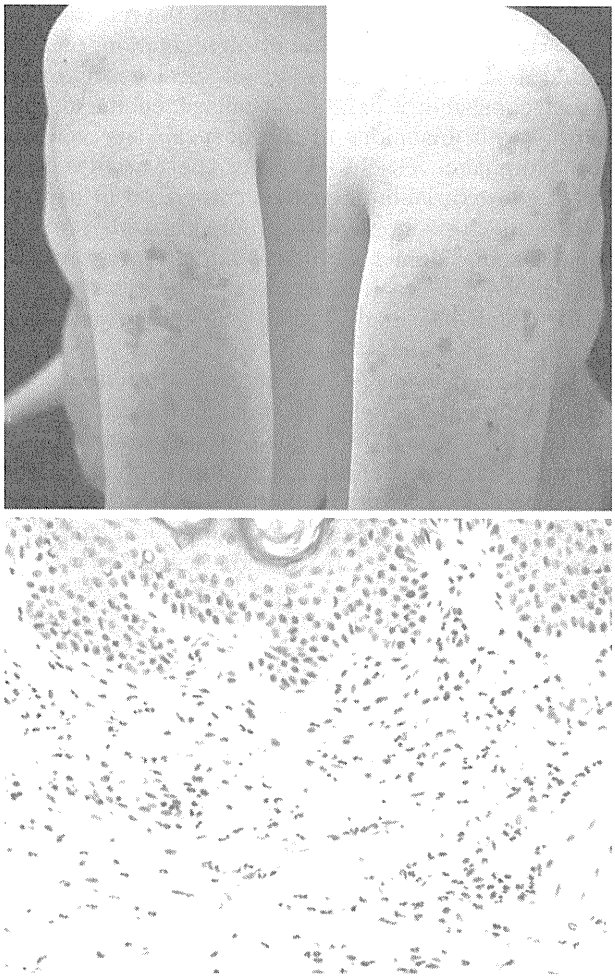

Fig. 2 Urticarial rash in a CAPS patient with an R260W mutation in *NLRP3*. The eruption is non-pruritic and returns to its normal appearance without pigmentation. Histopathological sample from a skin eruption shows edema in the dermis accompanied by the perivascular infiltration of inflammatory cells, predominantly neutrophils.

the most severe form of CAPS, exhibit chronic urticaria, as well as fever, arthropathy, chronic meningitis, papilledema, growth and mental retardation and hearing loss (Table 1).[16] The urticarial rash observed in CAPS is similar to that associated with common urticaria. However, unlike ordinary urticaria, the rash observed in most CAPS patients is not pruritic and responds to therapy with an IL-1 receptor antagonist rather than antihistamines.[17-19]

INFLAMMASOME ACTIVATION IN MAST CELLS

After development of an anti-IL-1β specific antibody (canakinumab), it was found that patients with CAPS have abnormally high levels of circulating IL-1β, approximately 5 times the normal amount.[20] In addition, treating patients with canakinumab relieved their

Table 1 Clinical manifestations of cryopyrin-associated periodic syndromes

	FCAS	MWS	CINCA
Skin rash	+	++	+++
Joint involvement	Arthritis?	Arthritis/ Arthralgia	Arthritis/ Arthralgia
Cartilage target	-	-	+++
Deafness	-	++	+++
Chronic meningitis	-	+?	+++
Eye involvement	-	+	+++
Amyloidosis	+	++	?

FCAS, familial cold auto-inflammatory syndrome; MWS, Muckle-Wells syndrome; CINCA, chronic infantile neurological, cutaneous and articular syndrome.

rashes within a day, suggesting that IL-1β was the sole cytokine responsible for the skin eruptions in CAPS. The disease-responsible gene, *NLRP3*, is predominantly expressed in monocytes, granulocytes and chondrocytes.[14] However, after performing immunohistochemical staining, we traced IL-1β in CAPS to a surprising source: mast cells.[21] Interestingly, mast cells in the skin samples from CAPS patients expressed active IL-1β without any treatment, whereas those from healthy donors only expressed active cytokine when appropriately stimulated.

Primary mast cells derived from mouse bone marrow and human cord blood expressed inflammasome components, including *NLRP3* and its adapter protein *ASC*. As was the case with macrophages, production of mature IL-1β via the NLRP3-inflammasome in mast cells required 2 signals. Microbial ligands, such as LPS, trigger the synthesis of the IL-1β precursor. A second ATP-triggered signal activates the inflammasome. Although a major function of LPS is to induce pro-IL-1β production, LPS also promotes the expression of *Nlrp3* in mast cells.

In macrophages, ATP-driven stimulation via the purinergic receptor P2X, ligand-gated ion channel 7 (P2RX7) is essential for caspase-1 proteolytic cleavage and IL-1β secretion by LPS-primed cells (Fig. 3).[22,23] P2RX7 forms a non-selective ion channel upon activation with ATP and, after stimulation, mediates K^+ efflux, which may be important for activating the inflammasome.[24] This ion channel mediated by P2RX7 rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore that allows passage of molecules as large as 900 Da.[22] It is possible, as has been proposed for macrophages,[25] that ATP promotes passage of microbial ligands, such as LPS, via pannexin-1 to trigger inflammasome activation in mast cells. Consistent with this conjecture, ATP alone did not induce IL-1β secretion by mast cells, even though ATP triggered large-pore formation. IL-1β secretion by mast cells was blocked for cells derived from *P2rx7*-deficient mice or by incubation in high K^+

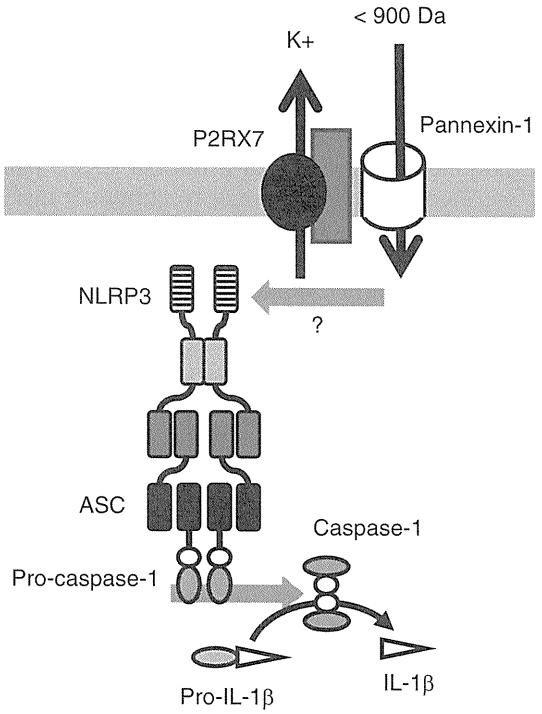

Fig. 3 P2RX7 forms a non-selective ion channel upon activation by ATP. Upon stimulation, P2R7X mediates K+ efflux and rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore, which allows passage of molecules as large as 900 Da.

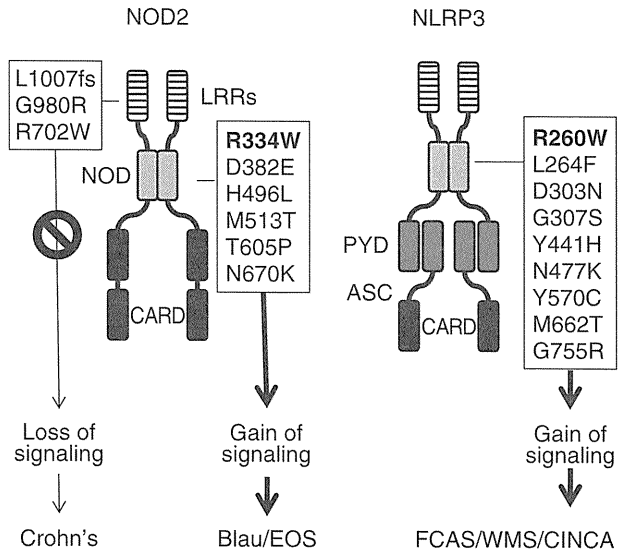

Fig. 4 Autoinflammatory disease-associated mutations in NOD2 and NLRP3. Crohn's disease-associated mutations found primarily in the LRRs of NOD2 result in a loss of activity, whereas mutations associated with Blau syndrome and early onset sarcoidosis (EOS) clustered in the NOD region of NOD2 cause constitutive activation of the molecule. Uncontrolled constitutive activation of NLRP3 in FCAS, MWS and CINCA are found in the NOD region of NLRP3. The mutations in NOD2 and NLRP3 associated with autoinflammatory diseases shown here are those found from our experience with cases and those mutations shown in bold are in analogous positions in the two proteins of NOD2 and NLRP3.

extracellular medium. Thus, ATP-driven P2RX7 and K^+ efflux are also required for effective IL-1β secretion by mast cells.[21]

CAPS-ASSOCIATED NLRP3 INDUCES URTICARIA

Disease-associated mutations associated with CAPS are localized to the centrally located NOD region in *NLRP3*. Of note, similar missense mutations in *NOD2* have been identified in patients with Blau syndrome, another autosomal-dominant autoinflammatory syndrome, and early-onset sarcoidosis, a set of sporadic granulomatous disorders that phenotypically resemble Blau syndrome.[26-28] Interestingly, the amino acids affected by an R260W mutation in *NLRP3* and an R334W mutation in *NOD2* are at analogous sequence positions, suggesting a common molecular mechanism for their roles in the development of autoinflammatory disease (Fig. 4). Via molecular interactions of their LRRs with their own NOD regions, NLRP3 and NOD2 are maintained in inactive conformations. This is relieved by ligand recognition via the LRRs.[29,30] Disease-associated mutations are thought to mimic active conformational changes that are induced by microbial ligands, and *in vitro* studies suggest that these mutations exert gain-of-function effects.[5,29] In the case of NLRP3, in addition of NF-κB activation, the mutation found in CAPS constitutively activates caspase-1 to produce active IL-1β.

Mouse *Nlrp3* mutants, corresponding to those observed in human CAPS, induced constitutive *Asc*-dependent NF-κB activation and IL-1β secretion.[21] Transfer of mast cells expressing an R258W mutant, corresponding to R260W of human CAPS-associated NLRP3, induced perivascular neutrophil-rich inflammation in mouse skin, a histological hallmark of the urticaria observed in CAPS patients. These findings are consistent with a previous report that showed enhanced production of IL-1β in the skin of CAPS patients.[18]

However, it remains unclear why mast cells with a constitutively activated NLRP3-inflammasome produce mature IL-1β, even in the absence of LPS. One possibility is that CAPS-associated NLRP3 mutants induce pro-IL-1β via constitutive activation of NF-κB induction.[31,32] Another possibility is that production of pro-IL-1β is induced by endogenous or environmental cues that operate in the skin independently of NLRP3. Consistent with this latter model, the characteristic skin rash observed in CAPS often develops within the first few weeks of life when the skin is first

exposed to environmental factors. This may include exposure to small amounts of LPS and/or other microbial stimuli. The observation that skin abnormalities in incontinentia pigmenti (MIM #308300) commence at birth[33] is also consistent with this possibility. This disorder is an X-linked dominant inherited disorder caused by a mutation of NEMO, a gene that encodes the regulatory component of the I-κB kinase complex responsible for activating the NF-κB signaling pathway.

Collectively, inflammasome activation in mast cells contributes to the pathogenesis of IL-1β-mediated diseases of the skin. Mast cells reside in numerous tissues and also participate in experimental models of arthritis[34] and encephalomyelitis.[35,36] Thus, it is possible that these cells play a role in disease pathogenesis not only in the skin, but also in the joints and central nervous system, which are also major diseased sites in CAPS patients.[16] Additional studies are needed in order to better understand the contributions of mast cells to IL-1β-mediated diseases associated with NLRP3.

Furthermore, urticaria that is associated with CAPS is usually non-pruritic and unresponsive to antihistamines. This clinical observation is in line with experimental results that NLRP3-inflammasome activation induces IL-1β secretion, but not degranulation, in mast cells. Nonetheless, mast cells that expressed a CAPS-associated NLRP3 mutant promoted vascular permeability, a cellular response critical for wheal formation *in vivo*. Because many cases of non-CAPS urticaria are unresponsive to histamine H1-receptor antagonists, it is possible that skin rash associated with histamine resistance is mediated via inflammasome activation in mast cells. Thus, understanding the pathophysiology of CAPS may provide critical insights into more common diseases, such as antihistamine-refractory urticaria.

CAPS AND KNOCK-IN MICE

Recently, two groups[37,38] have generated gene-targeted mice harboring *Nlrp3* mutations that mimic the amino acid substitutions in NLRP3 that were found to cause disease susceptibility in CAPS. The mice generated in both studies had very severe phenotypes. Newborn mice with an R258W mutation in *Nlrp3* exhibited delayed growth, decreased weight gain and increased mortality. Adult animals were infertile and developed dermatitis, but not urticaria, associated with increased sizes of lymphoid organs.[38] The mice with A350V in *Nlrp3* died between days 2 and 14 and exhibited profound growth delays, skin abscesses, and hair growth and pigmentation defects.[37]

At the cellular level, despite the observation that T cells in mutant mice displayed altered polarization profiles favorable to Th17, both groups found that this defect was due to the expression of the mutated *Nlrp3* in antigen-presenting cells (APCs), but not in T cells. They isolated bone marrow-derived macrophages or dendritic cells (DCs) and explored their responses to TLR ligands in the presence or absence of ATP. In contrast to a study of CD14-positive peripheral mononuclear cells from a human CAPS patient that secreted IL-1β without any treatment,[30] myeloid cells from mutant mice in both studies did not spontaneously secrete mature IL-1β. Rather, they displayed a considerably higher sensitivity to TLR ligands than cells from wild type (WT) mice.[37,38] Importantly, for optimal activation, addition of exogenous ATP was not required in order to release IL-1β, supporting the notion that the mutated Nlrp3 was constitutively active and, therefore, did not require additional stimuli for inflammasome activation.

An important outcome from these knock-in mice was that a common feature evoked by CAPS-associated *Nlrp3* was the development of severe skin lesions. These included erythema, abscesses, scaling and thickening of both the epidermis and dermis. However, these observations only recapitulated some, but not all, of the urticaria-like skin lesions reported in CAPS patients. Moreover, both studies still failed to address the critical question of whether or not TLR-dependent accumulation of pro-IL-1β was necessary for the burst of IL-1β secretion in CAPS.

CONTACT HYPERSENSITIVITY

The reasons for the discrepancies between the skin manifestations of CAPS and gene-targeted mice harboring the *Nlrp3* mutations could be directed to findings that the inflammasome also plays a role in contact hypersensitivity. Contact hypersensitivity involves the priming of naïve T cells after sensitizing chemicals penetrate the skin surface (sensitization) and primed T cells are activated upon re-exposure to the antigen (elicitation). Elicitation can be further subdivided into early and late phases.[39] The early phase, characterized by increased vessel permeability and local edema, peaks 8 hours after antigen re-exposure and is believed to be mediated by local release of mediators, including IL-1β and histamine.[40] The late phase develops 12-36 hours after antigen re-exposure and is due to cellular infiltration. During these processes, DC migration, antigen presentation, expansion of specific T cells and recruitment of T cells to the skin depend on the coordinated interactions of inflammatory cytokines, namely IL-1β and IL-18,[41] which are important cytokines for initiating specific T-cell-mediated immune responses.

A role for the inflammasome in contact hypersensitivity was analyzed by Watanabe *et al.*[42] who found that key components of the inflammasome were present in keratinocytes. Some contact sensitizers, such as trinitrochlorobenzene (TNCB), can induce caspase-1-mediated cleavage and activation of IL-1β and IL-18 in an ASC-dependent manner. Interestingly,

chemical irritants like SDS and physical agents like ultraviolet B could also trigger inflammasome activation in keratinocytes.[43,44] Subsequently, it was found that mice lacking *Nlrp3* and adaptor protein *Asc*-deficient mice had impaired early phase reactions during the challenges. Thus, the inflammasome can modulate the early effector phase of T cell-mediated immune responses.

These findings, however, are in contrast with a report by Sutterwala *et al.*[45] who found that impaired responses in *Nlrp3*- and *Asc*-deficient mice were seen in late-phase. Interestingly, in this report, *Nlrp3*-deficient mice that received cells from sensitized WT animals showed almost normal ear swelling, whereas WT mice that received cells from sensitized donors lacking *Nlrp3* failed to develop ear swelling. This suggests that Nlrp3 is necessary for inducing antigen-specific T-cell responses. Indeed, Langerhans cell migration and an optimal contact hypersensitivity response require functional caspase-1,[46] even though recent data suggest that important cross-presenting APCs in the skin are not Langerhans cells, but are langerin$^+$/CD103$^+$ DCs, most likely of dermal origin.[47,48]

NECROSIS AND NLRP3

How can we explain the discrepancies in these two reports on contact hypersensitivity in mice? One possibility is that TNCB is very potent when causing direct tissue damage, and that the signaling involved might be strong enough to overcome the requirement of inflammasome activation for T-cell priming.[49]

Of potential interest is a recent report showing that necrosis directly activates the NLRP3 inflammasome.[50,51] By treating human monocytic THP-1 cells with indirubin oxime derivative 7-bromoindirubin-3'-oxime, Li *et al.*[50] showed that the NLRP3 inflammasome was activated in cells undergoing necrosis, resulting in the production of mature IL-1β and IL-18. It is remarkable that inflammasome activation and the release of these caspase-1 targeting cytokines did not require LPS priming or other pro-inflammatory stimuli.

Along these lines, the activation of NLRP3 itself mediates a form of necrosis termed pyronecrosis.[52,53] We previously showed that the expression of a disease-associated mutation of *NLRP3* resulted in a caspase-1 independent, but cathepsin B-dependent form of cell death.[32] This characteristic cell death can also be observed in monocytes derived from CAPS patients. NLRP3-mutant monocytes rapidly and selectively underwent necrosis-like programmed cell death after treatment with LPS accompanied by the induction of *NLRP3* expression. This unique NLRP3 phenotype enabled us to differentiate NLRP3-mutated cells from WT cells in CAPS patients who had disease-associated mutant *NLRP3* as a latent mosaicism.[54]

Pyronecrosis is caspase-independent; neither the activating cleavage of effector caspase-3 nor its substrate poly-(adenosine diphosphate ribose) polymerase (PARP) occurs during cell death. Pyronecrosis proceeds in the presence of caspase-1-specific inhibitors, and even pan-caspase inhibitors. However, cell death is abrogated in the presence of CA074-Me, an inhibitor of the lysosomal protease cathepsin B, implicating the contribution of lysosome activity in the pathway. Pyronecrotic cells do not demonstrate DNA fragmentation or a loss of mitochondrial membrane potential. By electron microscopy, the morphological changes characteristic of pyronecrosis are consistent with necrosis and include membrane degradation and uncondensed chromatin. Similar to classic necrosis, pyronecrosis is accompanied by the release of the immune modulator high-mobility group box 1 (HMGB1). Pyronecrosis induced by NLRP3 activation suggests an exciting connection between cell death and inflammation in response to cellular insult to injury.

However, we should note that necrosis does not always induce inflammasome activation. Treatment with hydrogen peroxide or paclitaxel, which induce caspase-independent necrosis, fails to induce inflammasome activation. Similarly, induction of necroptosis through TNF-α in the presence of caspase-3 and caspase-9 inhibitors[55] did not result in inflammasome activation. If necrosis was induced too rapidly by repeated cycles of freeze-thaw or excessive osmotic shock, inflammasome activation was greatly reduced. Based on these findings, during activation of the NLRP3 inflammasome, the dissolution of the cellular architecture that occurs during some specific forms of necrosis may be required for lysosome destabilization.[56]

Another impressive point to remember from the study by Li *et al.*[50] is that it remains unclear how pro-IL-1β is induced in a sterile environment without bacterial components like LPS. Several endogenous danger signals released by necrotic cells have been shown to stimulate TLRs and other pattern recognition receptors and, therefore, have the potential to induce pro-IL-1β, although this has not been definitively demonstrated. Of note, endogenous molecules can serve a priming role for NLRP3-inflammasome activation.[51] Biglycan and hyaluronic acid, components of the extracellular matrix, were capable of priming macrophages for Nlrp3-inflammasome activation in response to pressure-disrupted necrotic cells. Hence, extracellular matrix components that accumulate in non-physiological sites or amounts can function as signal for the induction of pro-IL-1β accumulation, suggesting that inflammasome activation can occur *in vivo* in sterile settings without microbes.

Another possibility is that inflammasome activation during necrosis can lead to the release of mature IL-18, even in the absence of microbial stimuli, as IL-18

is constitutively expressed by several cell types. IL-18, in turn, via activation of the MyD88-dependent pathway, leads to the transcription of pro-inflammatory cytokine genes, including IL-1β,[57] and amplifies inflammation. Thus, IL-18 may be one of the earliest mediators of the sterile inflammatory response that is triggered by necrosis or tissue damage.

IL-18 AND DERMATITIS

In a report by Watanabe *et al.*,[42] IL-1 receptor deficiency resulted in a significant decrease in the intensity of ear swelling. However, it did not totally abrogate ear swelling after elicitation with TNCB, suggesting that other cytokines/signals are likely to modulate the early phases of contact hypersensitivity. This may be due, at least in part, to the presence of IL-18, which is also activated by caspase-1.[58,59]

Keratinocytes constitutively produce both pro-IL-1β[60] and pro-IL-18,[61] but lack endogenous caspase-1 activity under normal conditions.[60] Using established keratinocyte-specific caspase1-transgenic mice with a human keratin 14 promoter that specifically expressed the targeted gene at the basal layer of keratinocytes, Yamanaka *et al.*[62] showed that the mice spontaneously suffered from chronic dermatitis under specific pathogen-free (SPF) conditions, which was accompanied by abnormally elevated serum levels of IL-18 and IL-1β. Another transgenic mouse model in which epidermal cells over secreted IL-18 also spontaneously developed atopic dermatitis-like skin eruption under SPF conditions, and a deletion of *Il18* protected against the development of skin eruptions.[63] This finding suggests that excessive cutaneous IL-18 release is a causative factor for the development of dermatitis. Of potential interest is that the phenotypes in the skin of these caspase-1 and IL-18 transgenic mice closely resembled that in disease-associated *Nlrp3* knock-in mice.

Furthermore, Terada *et al.*[64] developed an intrinsic atopic dermatitis mouse model with daily applications of protein A, a surface molecule and virulence factor of *Staphylococcus aureus*, which resulted in destruction of the skin barrier with a subclinical dose of SDS. In this model, neutralizing anti-IL-18 antibodies and *Il18*-deficient mice could completely protect against SDS plus *S. aureus*-derived protein A-induced dermatitis, suggesting the importance of IL-18 for atopic dermatitis-like skin eruptions. This is also interesting when we consider the recent findings that hemolysins and bacterial lipoproteins in *S. aureus* can induce activation of the NLRP3 inflammasome.[65,66] Thus, another target inflammasome-activating cytokine, IL-18, may be a major player for dermatitis development.

LESSONS FROM CAPS

During the past decade, our understanding of the cellular and molecular mechanisms by which innate immune system molecules sense specific molecular patterns of components of invading organisms has increased tremendously. The NLRs, together with the TLRs, are now appreciated as parts of this important sensing system that allows the host to generate effective immune responses. NLRP3 also detects various endogenous, sterile danger signals in the absence of microbial infection. Progress in understanding the roles of NLRs will improve our knowledge to answer questions, such as how to transfer insights from mouse model systems to translational research focusing on human pathology, especially from the rare genetic disorder CAPS, associated with an *NLRP3* mutation, to more common diseases, such as ordinary urticaria and dermatitis. Interestingly, two NLRP3 single nucleotide polymorphisms (SNP, rs4612666 and rs10754558) were recently reported to be significantly associated with susceptibility to food-induced anaphylaxis as well as aspirin-induced asthma.[67] Functional analysis of the rs4612666 SNP located in intron 7 of *NLRP3* showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele, whereas rs10754558 in the 3'untranslated region affected the stability of the *NLRP3* mRNA. Thus, NLRP3 polymorphisms may increase the risk of the hypersensitive phenotype of allergy.

However, we still cannot answer all of the questions for the roles of the inflammasome in the skin. IL-18, as well as IL-33, are target molecules for inflammasome-activated caspase-1. Nevertheless, why does the urticarial rash in CAPS depend solely on IL-1β? Why can't we induce hives in mice, even if activated *Nlrp3* is induced? We should still look carefully at the clinical manifestations of CAPS in order to determine what happens in humans when the inflammasome is activated.

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Hematopoietic Stem Cell Transplantation for Familial Hemophagocytic Lymphohistiocytosis and Epstein–Barr Virus-Associated Hemophagocytic Lymphohistiocytosis in Japan

Shouichi Ohga, MD,[1,2]* Kazuko Kudo, MD,[2,3] Eiichi Ishii, MD,[2,4] Satoshi Honjo, MD,[1] Akira Morimoto, MD,[2,5] Yuko Osugi, MD,[6] Akihisa Sawada, MD,[7] Masami Inoue, MD,[7] Ken Tabuchi, MD,[8] Nobuhiro Suzuki, MD,[2,9] Yasushi Ishida, MD,[2,10] Shinsaku Imashuku, MD,[2] Shunichi Kato, MD,[2,11] and Toshiro Hara, MD[1]

Background. Post-transplant outcomes of hemophagocytic lymphohistiocytosis (HLH) patients were analyzed in Japan where Epstein–Barr virus (EBV)-associated severe forms are problematic. ***Methods.*** Fifty-seven patients (43 familial HLH [12 FHL2, 11 FHL3, 20 undefined], 14 EBV-HLH) who underwent stem cell transplantation (SCT) between 1995 and 2005 were enrolled based on the nationwide registration. ***Results.*** Fifty-seven patients underwent 61 SCTs, including 4 consecutive SCTs. SCTs were employed using allogeneic donors in 93% of cases (allo 53, twin 1, auto 3). Unrelated donor cord blood transplantation (UCBT) was employed in half of cases (21 FHL, 7 EBV-HLH). Reduced intensity conditioning was used in 26% of cases. The 10-year overall survival rates (median ± SE%) were 65.0 ± 7.9% in FHL and 85.7 ± 9.4% in EBV-HLH patients, respectively. The survival of UCBT recipients was >65% in both FHL and EBV-HLH patients. Three out of four patients were alive with successful engraftment after second UCBT. FHL patients showed a poorer outcome due to early treatment-related deaths (<100 days, seven patients) and a higher incidence of sequelae than EBV-HLH patients ($P = 0.02$). The risk of death for FHL patients having received an unrelated donor bone marrow transplant was marginally higher than that for a related donor SCT ($P = 0.05$) and that for UCBT ($P = 0.07$). ***Conclusions.*** EBV-HLH patients had a better prognosis after SCT than FHL patients. FHL patients showed either an equal or better outcome even after UCBT compared with the recent reports. UCB might therefore be acceptable as an alternate SCT source for HLH patients, although the optimal conditioning remains to be determined. Pediatr Blood Cancer 2010;54:299–306. © 2009 Wiley-Liss, Inc.

Key words: central nervous system disease; Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; familial hemophagocytic lymphohistiocytosis; hematopoietic stem cell transplantation; reduced intensity conditioning; umbilical cord blood transplantation

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is an immunohematologic emergency, characterized by fever, cytopenias, hepatosplenomegaly, hyperferritinemia, and disseminated intravascular coagulopathy (DIC) [1,2]. HLH comprises primary form of familial hemophagocytic lymphohistiocytosis (FHL) and secondary form occurring in association with infections, malignancies, and rheumatic diseases. FHL has currently been classified into FHL1 linked to chromosome 9, FHL2 with *PRF1* mutation, FHL3 with *UNC13D* mutation, and FHL4 with *STX11* mutation, although more than half of patients have no mutations of these genes [1]. HLH could also be a presenting symptom in patients with the other inherited disorders including X-linked lymphoproliferative disease (XLP), Griscelli syndrome, Hermansky–Pudlak syndrome, Chediak–Higashi syndrome and primary immunodeficiency diseases. HLH accounts for the common basis of hypercytokinemia arising from excessive immune activation, in which activated lymphocytes and hemophagocytosing-macrophages without malignant morphology infiltrate into systemic organs, including the bone

Additional Supporting Information may be found in the online version of this article.

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; CB, cord blood; CBT, cord blood transplantation; CNS, central nervous system; CT, computed tomography; EBV-HLH, Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; EEG, electroencephalography; FHL, familial hemophagocytic lymphohistiocytosis; HLH, hemophagocytic lymphohistiocytosis; PB, peripheral blood; SCT, hematopoietic stem cell transplantation; MRI, magnetic resonance imaging; OS, overall survival; SCT, hematopoietic stem cell transplantation; TRM, treatment-related mortality; RIC, reduced intensity conditioning; VOD, venoocclusive disease; XLP, X-linked lymphoproliferative disease/syndrome.

[1]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]The HLH/LCH and SCT Committees in the Japanese Society of Pediatric Hematology, Tokyo, Japan; [3]Division of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan; [4]Department of Pediatrics, Ehime University Graduate School of Medicine, Toon, Japan; [5]Department of Pediatrics, Jichi Medical University, Tochigi, Japan; [6]Division of Pediatrics, Osaka Municipal Medical Center, Osaka, Japan; [7]Department of Hematology/Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan; [8]Division of Hematology, Kanagawa Children's Medical Center, Yokohama, Japan; [9]Department of Pediatrics, Sapporo Medical University School of Medicine, Sapporo, Japan; [10]Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan; [11]Department of Cell transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

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*Correspondence to: Shouichi Ohga, Department of Pediatrics, 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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marrow (BM), liver, spleen, lymph nodes, skin, and central nervous system (CNS) [3,4]. FHL is a fatal disease if allogeneic hematopoietic stem cell transplantation (SCT) has not been successfully performed.

Epstein–Barr virus (EBV)-associated HLH (EBV-HLH) is a severe form of secondary HLH more frequently occurring in Asian children [5–7]. Activated EBV-infected $CD8^+$ T cells account for the disease process of EBV-HLH [8], however no predisposing factors have yet been clarified. EBV-HLH patients mostly respond to immunochemotherapy, but a small fraction of patients experience a fatal course without SCT. Therefore, although numbers were still small, SCT has been included in the salvage for refractory EBV-HLH cases [9–11]. The optimal timing of SCT, the source of donor cells and the conditioning are critical, particularly for young HLH patients. In this setting, the appropriate SCT for HLH patients needs to be established.

This study analyzed the outcomes of patients with FHL or EBV-HLH who underwent SCT in Japan over the past 10 years, in order to address the issues in the transplant-related problems including engraftment, late sequelae as well as to find out if there are distinct transplant strategies for FHL and EBV-HLH patients.

PATIENTS AND METHODS

Data Collection

The HLH/LCH Committee in the Japanese Society of Pediatric Hematology (JSPH) sent the first questionnaires to the hospitals administered by JSPH members based on the SCT registry in JSPH, asking if SCT was performed for any HLH patients between 1995 and 2005. The second questionnaires were sent to 57 hospitals with SCT cases, asking the patients' characteristics, treatment prior to SCT, donor sources, conditioning regimens, complications, and outcome. Of the 47 responses (recover rate 82%), 61 definite SCT cases from 33 hospitals were eligible for the study (mean 1.7 case/hospital, Supplemental Table). Forty-three FHL patients underwent 46 SCT, while 14 EBV-HLH patients underwent a total of 15 SCT. The majority of SCT (EBV-HLH 87%, FHL 89%) were performed between 2000 and 2005.

Diagnosis and Classification

All 57 patients fulfilled the diagnostic criteria of HLH [12]. FHL was diagnosed when the patient had a genetic abnormality, positive family history, and/or other evidence such as impaired natural killer cell activity [13]. The genetic study of FHL 2, 3, and 4, approved by the ethics committee of Kyushu University, Japan (No. 45), was partly completed postmortem according to our methods [14–17]. FHL2 and FHL3 determined by *PRF1* or *UNC13D* mutations accounted for 28% (n = 12), and 26% (n = 11), respectively, in this group. In addition, a total of eight patients were found with siblings diagnosed as having HLH. EBV infection might be associated with the development of HLH in four FHL patients (one FHL2, one FHL3, and two familial). These cases were classified as FHL, not as EBV-HLH. Other types of primary HLH such as XLP were excluded in this study.

EBV-HLH was diagnosed when a non-FHL patient had a primary infection or reactivation of EBV at the onset of HLH. EBV infection was assessed by the detection of EBV DNA and/or the pattern of serum EBV-specific antibody titers [18]. Cases with secondary HLH occurring in a chronic active EBV infection [19], and/or a histologically confirmed EBV-related lymphoma were excluded in this study. CNS involvement was determined when patients showed neurological manifestations, clinically as well as with any evidence of abnormality in the cerebrospinal fluids (CSF), neuroimagings (CT/MRI), and/or electroencephalography (EEG).

Prior Treatment to SCT

Treatment was based on the HLH-94 protocol using a combination of corticosteroid, cyclosporine-A (CSA), and etoposide (VP16) for both groups [20,21]. As the multidrug chemotherapy, CHOP-VP16-based regimen (VP16, vincristine, cyclophosphamide [CY], doxorubicin, and prednisolone) was chiefly employed. SCT was performed for all FHL patients, but limited for EBV-HLH patients who were resistant to any other treatments.

SCT

Allogeneic SCT was performed in 53 of the 57 patients (93%). Autologous SCT and identical-twin donor SCT were performed in three and one sporadic patients, respectively, because the molecular diagnosis was not available at the time of SCT. Donor sources, infused cell doses, conditioning regimens, and other SCT-related data are summarized in Table I. Allogeneic donor sources for EBV-HLH were HLA-matched sibling peripheral blood (PB) 1, haploidentical parent BM/PB 2, HLA-matched unrelated BM 1, HLA-matched unrelated cord blood (UCB) 2, and HLA-mismatched UCB 5, and those for FHL were HLA-matched related BM 7 (sibling 6), haploidentical parent BM/PB 2, HLA-matched unrelated BM 12, HLA-matched UCB 9, and HLA-mismatched UCB 12. All CBs were obtained from unrelated donors registered in the Japanese Cord Blood Bank Network. All unrelated donor BMs were obtained from the Japanese Marrow Donor Program. Myeloablative conditioning for EBV-HLH included VP16/busulfan (BU)/CY in 8 patients (4 in UCB transplantation [UCBT]) and other regimens in 3 patients, while those for FHL were VP16/BU/CY plus or minus anti-thymocyte globulin (ATG) in 23 patients (10 in UCBT) and others in 8 patients. Reduced intensity conditioning (RIC) for EBV-HLH included melphalan (MEL)/fludarabine (FLU) plus or minus thoracoabdominal irradiation in three patients (two in UCBT), and those for FHL were MEL/FLU plus or minus low-dose total body irradiation plus or minus ATG in eight patients (four in UCBT) and others in three patients. Donor chimerism was assessed by using short tandem repeats or sex chromosome analyses.

Evaluation of Late Sequelae

Long-term survivors were further questioned concerning their physical growth, endocrinological status, and neurological deficits. Neurological development including cognitive functions was assessed by Karnofsky score, developmental quotient and/or school performance.

Statistical Analysis

The 10-year overall survival (OS) rate with 95% confidence intervals were estimated by the Kaplan–Meier method. The OS was calculated for the period from the day of SCT until the death of any cause or the final observation. All results were updated to May 31,

TABLE I. Profiles of Patients Who Underwent Hematopoietic Stem Cell Transplantation

	EBV-HLH	FHL	*P*-value
Number, male:female	14, 4:10	43, 23:20	0.37
Age at onset (median, range)	5.5y, 6m–18y	0.5y, 6d–12y	<0.0001
Age at SCT (median, range)	5.9y, 1.4–18y	1.2y, 0.4–15y	0.0002
Observation period (median, range)	5.5y, 0.3–16y	4.8y, 0.2–19y	0.94
Manifestation at diagnosis (%)			
Fever	100	95	>0.99
Hepatosplenomegaly	86	86	>0.99
Lymphadenopathy	36	21	0.30
Skin eruption	7	14	0.67
Respiratory failure	36	14	0.12
DIC	50	33	0.26
Treatment prior to SCT (%)			
HLH94 only	36 (5/14)	60 (25/42)	0.14
Multidrug chemotherapy	57 (8/14)	19 (8/42)	0.017
Diagnosis to SCT (median, range)	5.8m, 1.8–24m	7.5m, 1.6–84m	0.18
SCT (n)			
Allogeneic	11	42	
Auto/Identical twin	3	1	
Nucleated cell doses ($\times 10^8$/kg)	1.3 (0.2–6.6)	2.5 (0.1–12.7)	0.14
Donor			
UCB	7	21	0.94
Others	7	22	
HLA disparity no	4	28	0.09
HLA disparity yes (>1 locus[a])	7	14	
Conditioning			
Myeloablative[b]	11	31	>0.99
RIC[c]	3	11	
Irradiation yes	4	11	0.73
Irradiation no	9	31	
ATG yes	0	8	0.18
ATG no	14	34	
CNS abnormality (%)			
At diagnosis	29[d] (4/14)	21[d] (9/42)	0.72
Before SCT	57 (8/14)	67 (28/42)	0.52
CSF pleocytosis	25 (2/8)	32 (7/22)	>0.99
MRI abnormality	36 (5/14)	51 (20/39)	0.36
Convulsion	43 (6/14)	41 (17/41)	0.93
Disturbed consciousness	36 (5/14)	24 (10/41)	0.49
Post-transplant state (n)			
Early death (<100 days)	2	7	0.48
Alive	12	29	0.31
Neurological deficit (%)	8[d] (1/12)	29[d] (7/24)	0.22
Late sequelae[e] (%)	8 (1/12)	52 (11/21)	0.022

ATG, anti-thymocyte globulin; BU, busulfan; CNS, central nervous system; CSF, cerebrospinal fluid; CY, cyclophosphamide; DIC, disseminated intravascular coagulopathy; EBV, Epstein–Barr virus; FHL, familial hemophagocytic lymphohistiocytosis; FLU, fludarabine; HLH, hemophagocytic lymphohistiocytosis; MEL, melphalan; MRI, magnetic resonance imaging; SCT, hematopoietic stem cell transplantation; TAI, thoracoabdominal irradiation; TBI, total body irradiation; UCBT, unrelated donor cord blood transplantation; VP16, etoposide. Parenthesis means the positive number of patients per the evaluable number of patients. The observation period means the time from the onset to the last visit or death. [a]Human leukocyte antigen (HLA) disparity was assessed by the serotyping data of HLA-A, -B, and -DR; [b]Myeloablative conditionings for EBV-HLH were VP16/BU/CY 8 (4 in UCBT) and others 3, and those for FHL were VP16/BU/CY + ATG 23 (10 in UCBT) and others 8; [c]Reduced intensity conditionings (RIC) for EBV-HLH were MEL/FLU + TAI 3 (2 in UCBT), and those for FHL were MEL/FLU + low dose TBI + ATG 8 (4 in UCBT) and others 3; [d]The proportion of patients having neurological abnormality was lower in survived patients with EBV-HLH ($P = 0.0015$). Survived patients were neurodevelopmentally assessed at the last visit to the hospital; [e]Late sequela(e) in EBV-HLH was hemiparesis (n = 1), and those in FHL were short stature (n = 5), endocrinological abnormality (n = 1), psychomotor retardation with or without seizure (n = 5), brain atrophy (n = 1), and hearing difficulty (n = 1).

2008. An analysis of the risk factors for SCT outcome was possible for FHL, but not for EBV-HLH because of the small number of subjects. Age at onset of HLH or at the SCT, duration from the onset to SCT, CNS disease before SCT, donor sources, and the type of conditioning were tested using the log-rank method. Cox proportional-hazard model was employed to examine the association between selected clinical variables and the risk for death. A logistic regression model was used to investigate factors associated with neurological sequelae. Chi-square test or Fisher's exact test were employed in other comparisons. P values less than 0.05 were considered to be significant.

RESULTS

Profiles of EBV-HLH and FHL Patients

A comparison of the clinical profiles (Table I) revealed that the ages at disease onset and at the time of SCT were each higher in EBV-HLH than in FHL patients ($P < 0.0001$, $P = 0.0002$, respectively). No clinical manifestations differed between the two groups during the disease course, including respiratory failure as well as CNS abnormalities at diagnosis. The proportion of patients who failed VP16 and CSA therapy including HLH94 protocol and needed combination chemotherapy such as CHOP-VP16 before planning SCT was higher in EBV-HLH patients than FHL patients (57% vs. 19%, $P = 0.0168$).

Outcomes of SCT

Engraftment and survival. Post-transplant outcomes of 43 FHL patients and 14 EBV-HLH patients are summarized in Figures 1 and 2. The 10-year OS rates (median ± SE%) of FHL and EBV-HLH patients were 65.0 ± 7.9% and 85.7 ± 9.4%, respectively ($P = 0.24$; Fig. 3). In the allogeneic SCT cases with FHL (Fig. 1), 29 attained engraftment, 6 had rejection or graft failure, and 7 were undetermined. On the other hand, in EBV-HLH (Fig. 2), seven were engrafted, three were rejected, and one was undetermined. Of all 29 FHL patients engrafted after the first SCT, 26 were alive with no HLH relapse, but 3 died of treatment-related mortality (TRM). Seven engrafted patients with EBV-HLH were alive and well at the final follow-up. Among the nine rejection/graft failure patients (six FHL, three EBV-HLH), a second UCBT was successful in three of the four patients (three FHL, one EBV-HLH). Twelve of the UCBT recipients for FHL that received a graft with the first UCBT and two that received a second UCBT were alive at the last follow-up; while seven died; six were due to TRM and one was due to active HLH disease. Six of the seven UCBT recipients for EBV-HLH were alive and well at the last follow-up, while only one died of active HLH disease on day 18 post-transplant. A total of 29 FHL survivors after allogeneic SCT(s) had 17 complete donor chimera (2 patients after second UCBTs), 3 mixed chimera (1 had 42% donor chimera in remission 18 months after SCT, 2 attained >90% donor chimera until 6 months after SCT), 8 undefined, and 1 graft failure with CNS disease. Ten EBV-HLH survivors after allogeneic SCT attained eight complete donor chimera (seven patients after the first SCT and one patient after second SCT [UCBT]), and two with autologous recovery. Two of three EBV-HLH patients who rejected allogeneic cells were alive and disease free more than 6 years post-transplant. One of two EBV-HLH patients who underwent autologous SCT was alive and well 13 years post-transplant [22]. One EBV-HLH patient was alive and well 10 years after the identical twin donor BMT.

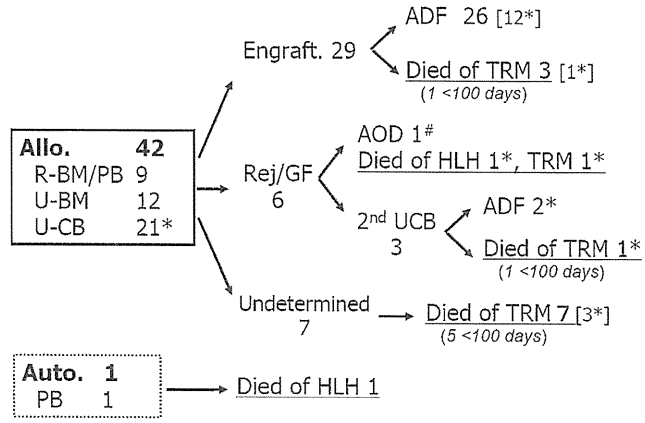

Fig. 1. Cohort diagram for the clinical outcome of 43 patients with familial hemophagocytic lymphohistiocytosis (FHL) who underwent stem cell transplantation (SCT). Of 42 patients after allogeneic SCT, 29 achieved engraftment (18 complete, 3 mixed) and 6 failed to engraft. One (#) with graft failure was alive with central nervous system disease 12 years after SCT. A total of 29 patients (67%) were alive after SCT. The underlined data indicate the number of deceased patients. Seven patients died within 100 days post-SCT (parenthesis). Asterisk (*) means UCB. R, related; U, unrelated; BM, bone marrow; PB, peripheral blood; CB, cord blood; ADF, alive with the disease free state; AOD, alive on disease; Rej/GF, rejection or graft failure; TRM, treatment-related mortality.

Causes of death. Of 14 deceased FHL patients, 12 died of TRM, including 3 chronic GVHD while 2 died of recurrent HLH. Seven patients experienced early death from TRM within 100 days after SCT (Fig. 1). One patient, later diagnosed with FHL2, died of CNS disease 5 years after autologous SCT [14]. Two EBV-HLH patients died of recurrent HLH within 50 days after SCT (Fig. 1). No TRM-related deaths were noted among the EBV-HLH patients.

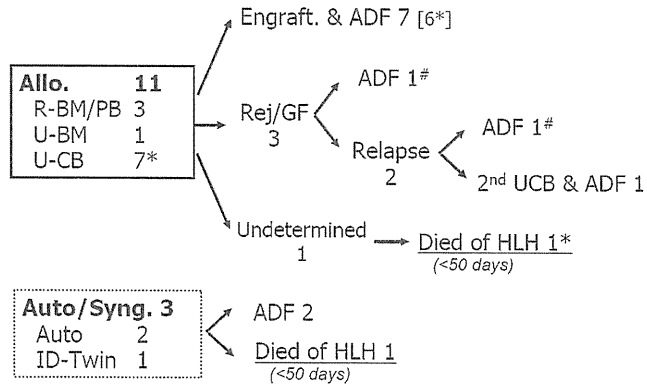

Fig. 2. Cohort diagram for the clinical outcome of 14 patients with Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) who underwent SCT. Among 11 patients after the first allogeneic SCT, 7 achieved successful engraftment and 3 failed to engraft. A total of 12 patients (86%) were alive after SCT. Two patients (#) were alive and well more than 6 years after SCT failure. The underlined data indicate the number of deceased patients. Two patients died within 50 days post-SCT (parenthesis). Asterisk (*) means UCB. Auto/Syng: autologous/syngeneic, ID: identical.

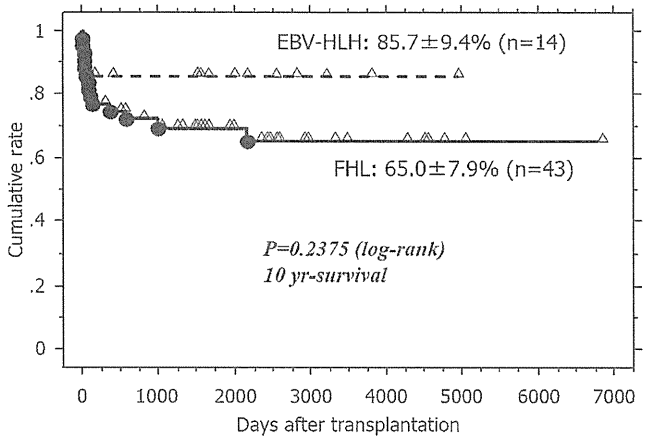

Fig. 3. Cumulative probability of post-transplant overall survival of FHL (*solid line*) and EBV-HLH patients (*dashed line*) who underwent SCT. Closed circle and open triangle represent deceased and alive patients, respectively. Each value indicates the 10-year overall survival rate plus or minus standard error assessed by the log-rank test.

Analysis of Prognostic Factors in FHL

A log-rank test on the OS rate did not show any significant difference in terms of age at SCT (<2 years vs. ≥2 years), time of SCT from HLH treatment (<6 months vs. ≥6 months), conditioning regimens (myeloablative vs. RIC) and various donor sources (R-PB/BM vs. UCBT vs. UBM; Table II). The Cox hazard model with adjustment for gender and age at engraftment indicated that the risk of death for UBM might be higher than that for R-PB/BM (adjusted hazard ratio = 0.07, 95% confidence interval [CI] = 0.01–1.02, $P = 0.05$) and that for UCB (0.27, 95% CI = 0.07–1.09, $P = 0.07$; Table II). No significant variables were found to predict the risk of early death within 100 days post-transplant, or the risk of neurological sequelae.

CNS Abnormalities and Late Sequelae

Table I shows that the frequency of CNS abnormalities at onset and the time of SCT did not differ between the EBV-HLH and FHL patients. Whereas, post-transplant CNS abnormalities were significantly higher in the FHL patients ($P = 0.0015$). Eleven FHL patients (52%) have had late sequelae including neurological as well as endocrinological problems, in comparison to only one EBV-HLH patient with left hemiparesis ($P = 0.022$). Late sequelae of FHL

TABLE II. Association Variables Influencing on the Risk of Mortality in FHL Patients

(A) Log-rank analysis Variables	No.	Survival (OS %)		*P*-value
Age				
<2 years	30	66.2 ± 8.7		0.56
≥2 years	12	75.0 ± 12.5		
Time from HLH treatment				
<6 months	14	62.9 ± 13.3		0.65
≥6 months	28	71.4 ± 8.5		
Conditioning				
Myeloablative	31	71.0 ± 8.2		0.50
RIC	11	60.6 ± 15.7		
Donor sources				
R-PB/BM, a	9	88.9 ± 10.5	a vs. b	0.22
UCB, b	21	65.6 ± 10.6	a vs c	0.15
UBM, c	12	58.3 ± 14.2	b vs c	0.61
(B) Cox's model analysis Variables	No.	Adjusted hazard ratio	95% CI lower–upper limit	*P*-value
Stem cell source				
Unrelated BM	12	1.00	Reference	
Unrelated CB	21	0.27	0.07–1.09	0.07
Related PB/BM	9	0.07	0.01–1.02	0.05
Conditioning				
Reduced intensity	11	1.00	Reference	
Myeloablative	31	0.48	0.09–2.47	0.38
Radiation				
No	31	1.00	Reference	
Yes	11	0.52	0.11–2.52	0.41
Use of ATG				
No	34	1.00	Reference	
Yes	8	0.91	0.18–4.70	0.91
HLA disparity				
No	28	1.00	Reference	
Yes (>1 locus)	14	2.79	0.75–10.38	0.13

Both analyses (A, B) were performed for 42 FHL patients who underwent the first allogeneic SCT. The Cox model analysis was performed with adjustment for selected variables including sex and age at engraftment.