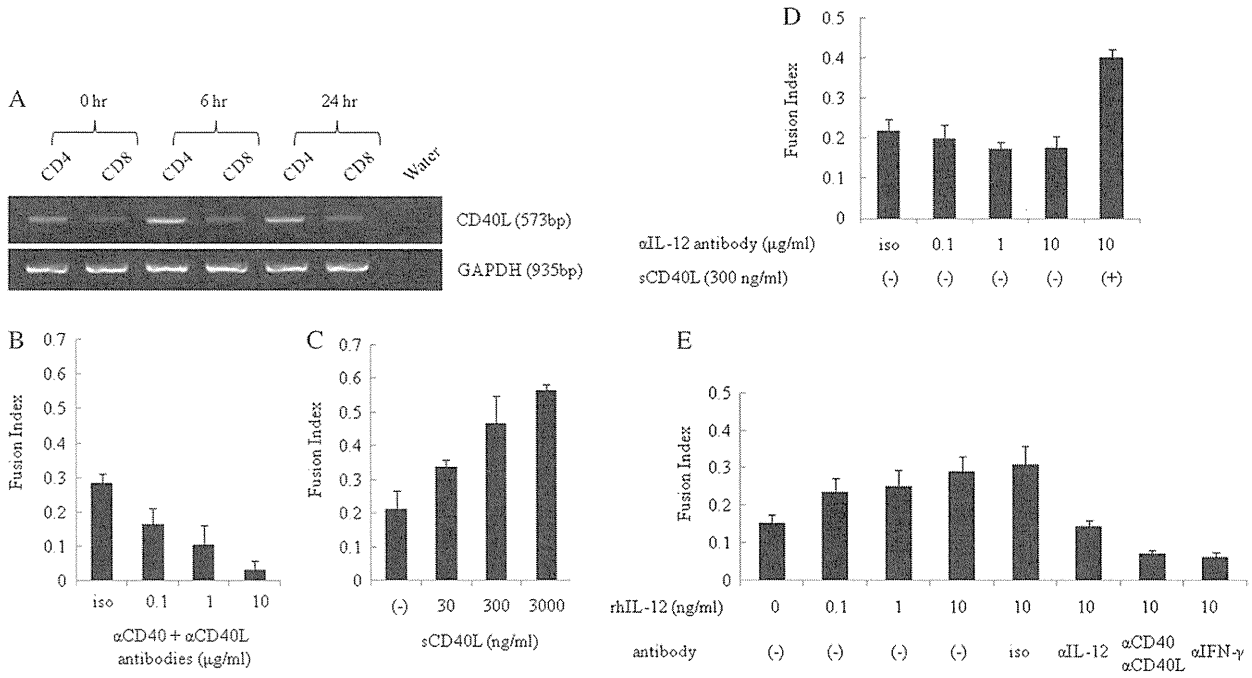

Fig. 4. The CD40-CD40L axis is necessary for LGC formation. (A) CD4+ T cells expressed more CD40L than CD8+ T cells when co-cultured with monocytes stimulated with ConA (5 μg ml^{-1}). RT–PCR analysis for the mRNA expression of CD40L from cells co-cultured for the indicated time periods is shown. (B) Addition of antibodies directed against CD40 and anti-CD40L abrogated LGC formation and (C) exogenous addition of sCD40L enhanced LGC formation. (D) An antibody against IL-12 had a minimal effect on LGC formation induced by the co-culture of monocytes and autologous T cells stimulated with ConA. 'Iso' refers to the isotype control. (E) Addition of rhIL-12 to the co-culture of monocytes and autologous T cells stimulated with ConA enhanced LGC formation. (B–E) Co-culture conditions were the same as in Fig. 2(A and B). Values represent the mean fusion index, and error bars indicate the SEM (n = 3 independent co-cultures).

(Fig. 5D). Taken together, these results demonstrate that T cells play a pivotal role by providing IFN-γ and CD40 stimulation to monocytes, but that a direct effect of ConA is also required for the induction of LGCs from human monocytes.

DC-STAMP is involved in LGC formation

Recently, it has been reported that several fusion-related molecules are involved in MGC formation, particularly in osteoclast differentiation and FGC formation (27–31). However, the involvement of these molecules in LGC formation has not been established. The mRNA expression of well-known fusion-related genes was therefore examined by RT–PCR. Notably, DC-STAMP, which is required for osteoclast formation and FGC formation, was up-regulated in monocytes stimulated with sCD40L, rhIFN-γ and ConA, whereas the expression of TREM-2, DOCK180, SIRPα, CD44, CD9, CD81 and MMP-9 was observed in the monocytes cultured with media alone (Fig. 6A).

To further explore the involvement of DC-STAMP in the LGC formation, siRNA-mediated knock-down of DC-STAMP was performed. Down-regulation of DC-STAMP mRNA was confirmed by RT–PCR (Fig. 6B). Transfection of monocytes with siRNAs against DC-STAMP decreased LGC formation in comparison with monocytes transfected with control siRNA, confirming the involvement of DC-STAMP in LGC formation by human monocytes stimulated with exogenous sCD40L, IFN-γ and ConA (Fig. 6C).

Inhibitors against nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases were used to delineate the signal transduction pathways involved in DC-STAMP up-regulation since CD40 stimulation induces the activation of these molecules (32–34). First, the activation of NF-κB and the MAP kinases (ERK kinase, JNK kinase, p38 kinase) during LGC formation was confirmed (Fig. 6D). The NF-κB inhibitors JSH-23 and BMS-345541, the ERK kinase inhibitor U0126, JNK kinase inhibitor II and the p38 kinase inhibitor SB-203580 reduced DC-STAMP mRNA expression and inhibited LGC formation (Fig. 6E and F). These data suggest that DC-STAMP up-regulation occurred through activation of NF-κB and MAP kinases and implied that this up-regulation is critical for LGC formation from monocytes stimulated with sCD40L, IFN-γ and ConA.

Discussion

Granulomas are generally formed as a consequence of the failure of the host to eliminate a specific pathogen such as *Mycobacterium* (1) or *Cryptococcus* (2). They also occur when the host is immunodeficient, as in the case of CGD (3). Non-infectious granulomas cause clinical symptoms in diseases such as systemic sarcoidosis and BS/EOS. In systemic sarcoidosis, idiopathic granuloma formation in the bronchus or pleura causes respiratory disorders (4) and in BS/EOS, granuloma formation in the uvea or synovium causes loss of vision or joint contractures, respectively

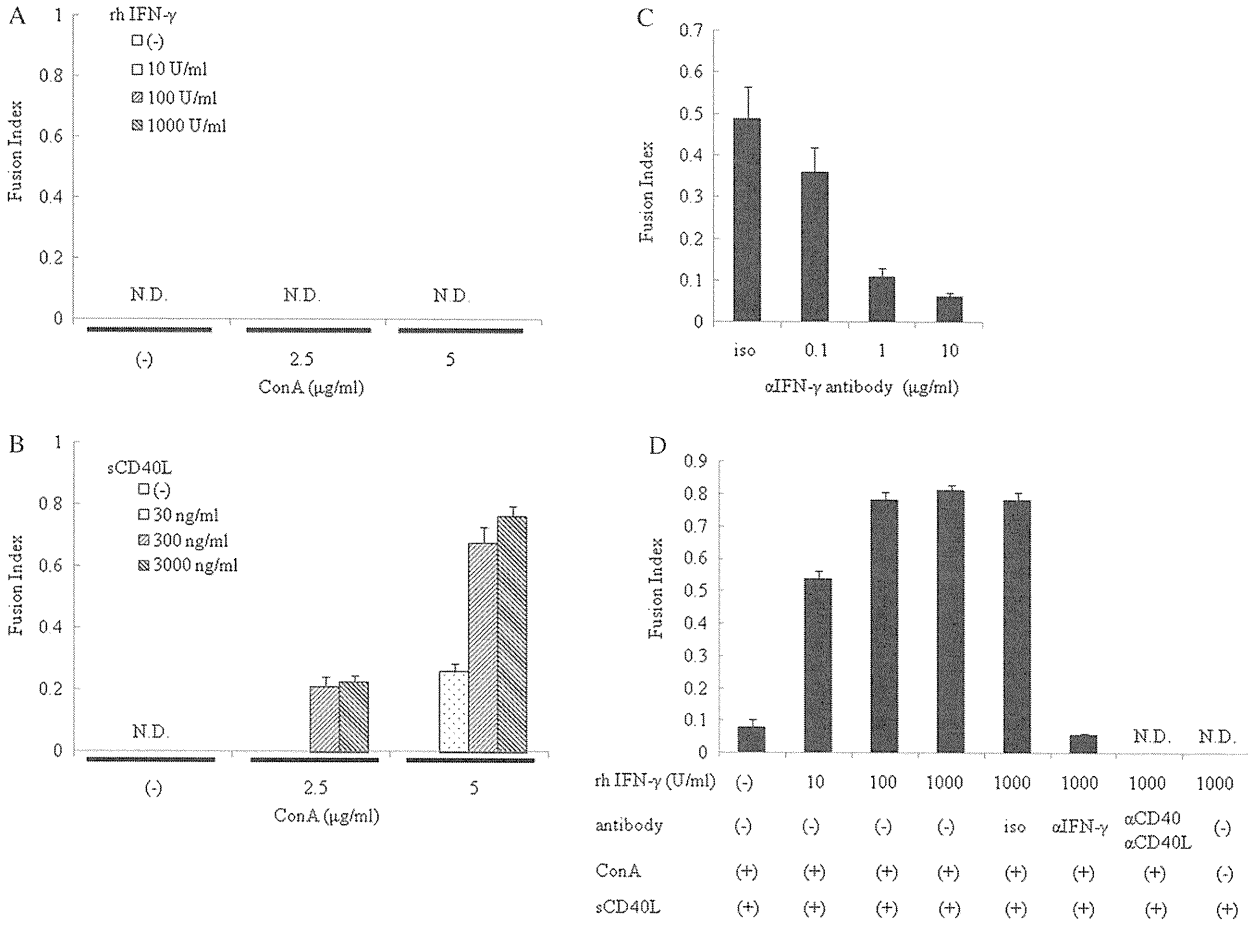

Fig. 5. T cells play a pivotal role in the induction of LGCs by providing IFN-γ and CD40 stimulation. (A) Monocytes stimulated with ConA did not differentiate into LGCs in the presence of exogenous rhIFN-γ. (B) Monocytes stimulated with ConA differentiated into LGCs in the presence of exogenous sCD40L. ND refers to 'not detected'. (C) Addition of an antibody against IFN-γ abrogated LGC formation induced by exogenous sCD40L (3 μg ml^{-1}) and ConA (5 μg ml^{-1}). 'Iso' refers to the isotype control. (D) The CD40-CD40L axis, IFN-γ and ConA were required for LGC formation. Highly purified monocytes isolated from T cell-depleted PBMC were cultured with the indicated concentration of rhIFN-γ. The indicated antibodies (10 μg ml^{-1}) were added to the culture medium, in addition to exogenous sCD40L (3 μg ml^{-1}) and ConA (5 μg ml^{-1}). Values represent the mean fusion index, and error bars indicate the standard mean of the error (n = 3 independent cultures).

(5–7). These granulomatous diseases are pathologically characterized by the presence of LGCs, which are considered to be closely related with the formation of granulomas. However, the pathophysiological mechanisms of LGC and granuloma formation are not well understood.

Based on the fact that granulomas usually contain LGCs and are surrounded by T cells (4, 35), a novel culture system was established whereby human monocytes were co-cultured with autologous T cells that were activated by ConA. This system resulted in efficient formation of LGCs. Although many systems that employ cytokines such as IFN-γ have been reported (15–19, 24), this is the first demonstration that a co-culture of monocytes and autologous T cells induces LGC-dominant MGC differentiation. Using this novel co-culture system, it was demonstrated that T cells play a pivotal role in LGC formation by stimulating monocyte fusion via IFN-γ and a CD40-mediated mechanism and that a direct effect of ConA on monocytes is also required for the induction of LGCs.

Since CD40 ligation on monocytes stimulates IL-12 production leading to IFN-γ production from activated T cells (26), it was possible that LGC formation did not require downstream signaling of CD40 in monocytes but required IFN-γ subsequently secreted from nearby T cells. However, LGC formation was not observed in monocytes stimulated with rhIFN-γ and ConA, and further addition of exogenous sCD40L was required to induce LGC formation (Fig. 5A and D). In addition, the contribution of IL-12 to LGC formation in the co-culture system of monocytes and T cells was minimal (Fig. 4D and E). Thus, CD40 signaling in monocytes contributes to LGC formation by a mechanism other than the induction of IL-12 production.

Although the molecular mechanism of macrophage fusion has been studied extensively in relation to osteoclast and FGC formation, little is known about the basis of LGC formation (27–31). In the present study, an *in vitro* human LGC formation system was established and the molecular mechanisms underlying the formation of LGCs were investigated.

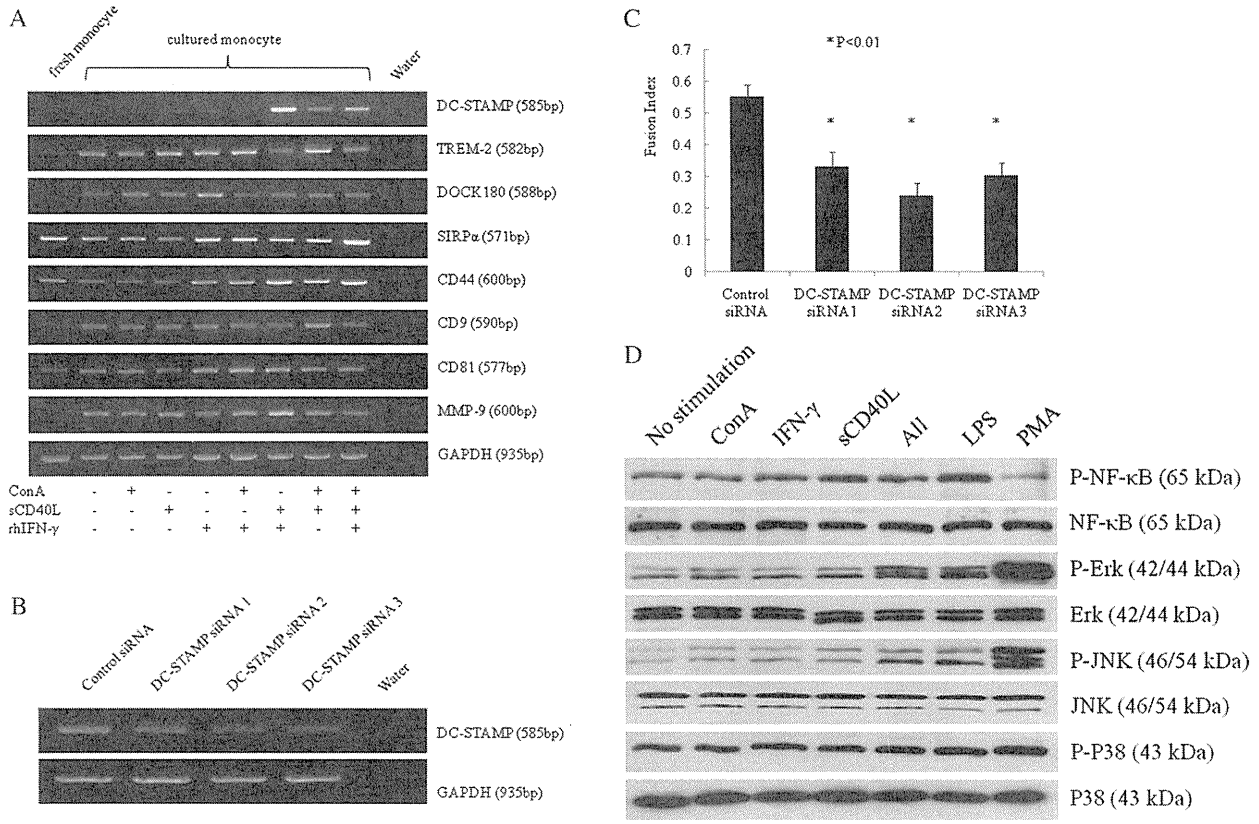

Fig. 6. DC-STAMP is involved in LGC formation. (A) DC-STAMP was up-regulated in LGC formation. RT–PCR analysis of several fusion-related genes in highly purified monocytes stimulated with the indicated reagents (exogenous sCD40L 3 μg ml^{-1}; rhIFN-γ 100 U ml^{-1}; ConA 5 μg ml^{-1}) is shown. (B) and (C) siRNA knock-down of DC-STAMP reduced LGC formation. (B) The down-regulation of DC-STAMP was confirmed by RT–PCR analysis in highly purified monocytes transfected with either control siRNA or siRNA against DC-STAMP, followed by stimulation with exogenous sCD40L (300 ng ml^{-1}), rhIFN-γ (100 U ml^{-1}) and ConA (5 μg ml^{-1}). Additional transfections were performed at 12 and 24 h of the culture. (C) LGC formation was reduced by siRNA against DC-STAMP. Highly purified monocytes transfected with either control or DC-STAMP siRNA were cultured with exogenous sCD40L, rhIFN-γ or ConA as above. Values represent the mean fusion index, and error bars indicate the standard mean of the error ($n = 3$ independent cultures). $*P < 0.01$ by Student's *t*-test. (D) NF-κB and MAP kinase signaling were involved in LGC formation in this system. Immunoblotting of whole cell lysates stimulated as indicated (LPS, 1 μg ml^{-1}; PMA, 4 μg ml^{-1}). (E) DC-STAMP up-regulation during LGC formation was abrogated in the presence of inhibitors of NF-κB or MAP kinases. RT–PCR analysis of DC-STAMP expression in the culture is shown. Each inhibitor was added 1 h before stimulation with ConA, sCD40L and rhIFN-γ (JSH-23, 20 μM; BMS-345541, 5 μM; U0126, 10 μM and JNK inhibitor II, 40 μM; SB203580, 10 μM). (F) LGC formation from highly purified monocytes stimulated with ConA, sCD40L and rhIFN-γ was prevented by the addition of inhibitors of NF-κB or the MAP kinases. (JSH-23, 10–20 μM; BMS-345541, 2.5–5 μM; U0126, 5–10 μM and JNK inhibitor II, 20–40 μM; SB203580, 5–10 μM).

Among the genes reported to be involved in macrophage fusion, DC-STAMP was involved in LGC formation in this co-culture system, similar to its involvement in osteoclast (36–39) and FGC formation (40). DC-STAMP was up-regulated by stimulating monocytes with exogenous sCD40L (Fig. 6A).

DC-STAMP is induced by the transcription factor nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) during osteoclast formation (40, 41) and by NF-κB and the transcription factor PU.1 during FGC formation (40). Because the CD40-CD40L axis signals through the NF-κB and the MAP kinases (32–34), up-regulation of DC-STAMP in our LGC formation system could have been caused by NF-κB and MAP kinases activation through the CD40-CD40L axis. Indeed, inhibition of NF-κB and MAP kinases reduced DC-STAMP mRNA up-regulation (Fig. 6E). Interestingly, BS/EOS, a rare Mendelian-inherited disease characterized by idiopathic granuloma formation in the skin, synovium and uvea, is caused by a gain-of-function mutation in nucleotide-binding oligomerization domain 2 (NOD2), which in turn causes the constitutive activation of NF-κB and the MAP kinases (42, 43). The precise mechanism mediating the up-regulation of DC-STAMP and the involvement of other fusion-related molecules during LGC formation remains to be elucidated.

Certain features of the LGCs formed by the present cell culture method were similar to those of osteoclasts, namely the up-regulation of DC-STAMP in pre-fusion monocyte-macrophage lineage cells. Activated T cells express receptor activator of NF-κB ligand (RANKL), which is necessary for osteoclast formation. This suggests the possibility that the co-culture of monocytes and T cells might have induced osteoclasts instead of LGCs. However, IFN-γ was required for LGC formation in our culture system (Fig. 2), whereas IFN-γ is a negative regulator of osteoclast formation. For example, IFN-γ induces rapid degradation of tumor necrosis factor receptor-associated factor 6, an adaptor protein of RANKL, which is a critical signaling pathway activated during osteoclast differentiation (44). On the other hand,

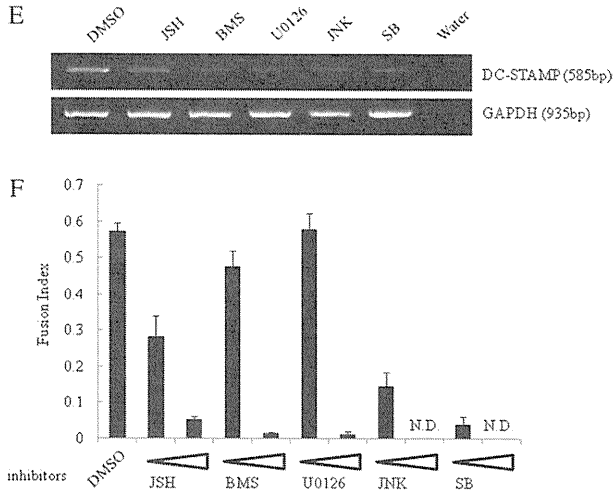

Fig. 6. Continued

RANKL does not appear to be involved in LGC formation in the present system, as a neutralizing antibody against RANKL failed to abrogate LGC formation (data not shown). These data imply that the differentiation of monocytes into LGCs versus osteoclasts occurs through different pathways.

In chronic inflammatory lesions of various etiologies, chemokines such as monocyte chemoattractant protein-1 produced by macrophages or vascular endothelial cells induce the chemotaxis of monocytes and T cells (45–48). The present study suggests that the interaction between monocytes and activated T cells caused LGC formation through pathways involving a CD40L and IFN-γ, both of which are provided by activated T cells. A potential scheme for LGC formation based on this data is shown in Fig. 7. ConA was a requisite for LGC formation in our system and acted directly on monocytes (Fig. 5D), and furthermore, the addition of methyl-α-D-mannopyranoside (Sigma), a ConA neutralizing agent, completely abrogated LGC formation (Supplementary Figure 1A is available at *International Immunology* Online). However, methyl-α-D-mannopyranoside did not inhibit the DC-STAMP up-regulation, the activation of NF-κB or the activation of MAP kinases (Supplementary Figure 1B and C is available at *International Immunology* Online). Although we speculate that ConA stimulation of monocytes yielded an activated phenotype, further study is required to delineate the mechanism through which ConA affects LGCs.

The present LGC formation system showed that the CD40-CD40L axis plays a critical role in LGC differentiation and might be a potential therapeutic target for pharmacologic treatment of granulomatous diseases. Although lethal thromboembolic events were initially reported for the humanized anti-CD40L antibody BG9588 (49), the humanized anti-CD40 monoclonal antibody dacetuzumab was well tolerated in a phase I study of patients with non-Hodgkin's lymphoma (50). The use of anti-CD40-CD40L axis agents for the treatment of diseases such as sarcoidosis and CGD colitis seems contradictory because these diseases are known to involve infectious agents and disrupting the CD40-CD40L axis weakens host defenses. However, these diseases are currently treated

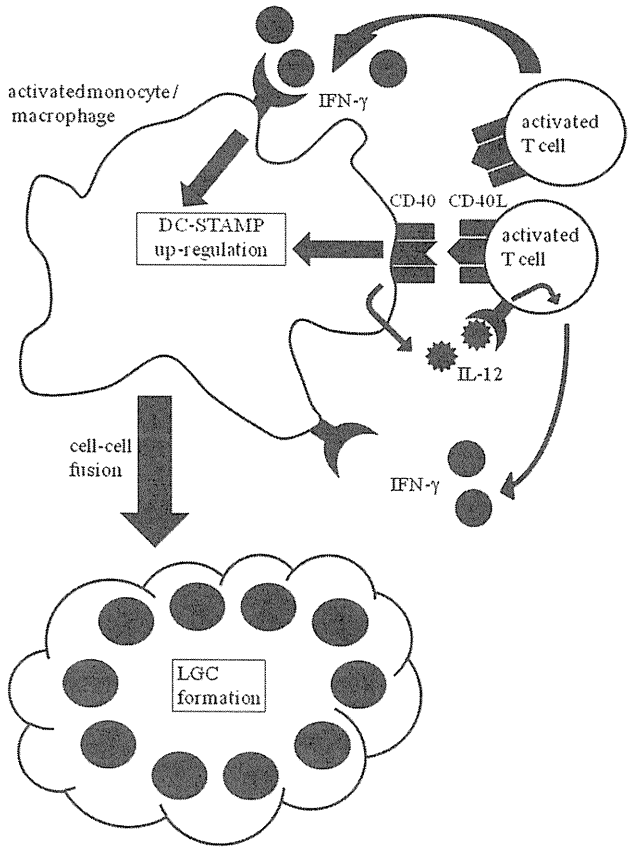

Fig. 7. Schematic presentation of the putative mechanism of human LGC formation. CD40L expressed on activated T cells binds to CD40 on monocytes/macrophages. The activated T cells produce IFN-γ, partially responding to IL-12 production from the activated monocytes/macrophages. Down-stream signaling from CD40 as well as IFN-γ and ConA is hypothesized to up-regulate the fusion-molecule DC-STAMP.

with corticosteroids, which also have both anti-inflammatory and immunosuppressive effects, which control the tissue damage caused by the granulomatous lesions. Thus, the treatment of granulomatous diseases with anti-CD40-CD40L axis agents as a targeted therapy could be a viable approach.

In conclusion, the present study demonstrates that the CD40–CD40L interaction as well as IFN-γ production was necessary for LGC formation in a new *in vitro* LGC formation system using human monocytes and autologous T cells. T cells were shown to initiate signaling to monocytes via CD40- and IFN-γ-mediated pathways, and DC-STAMP was involved in the fusion of monocytes into LGCs. These findings provide new insights into the molecular mechanism of LGC formation and have the potential to contribute to the establishment of novel therapeutics against corticosteroid-resistant or -dependent granulomatous diseases.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein

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Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein

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Familial hemophagocytic lymphohistiocytosis (FHL) is a potentially lethal genetic disorder of immune dysregulation that requires prompt and accurate diagnosis to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. In the present study, 85 patients with hemophagocytic lymphohistiocytosis were screened for FHL3 by Western blotting using platelets and by natural killer cell lysosomal exocytosis assay. Six of these patients were diagnosed with FHL3. In the acute disease phase requiring platelet transfusion, it was difficult to diagnose FHL3 by Western blot analysis or by lysosomal exocytosis assay. In contrast, the newly established flow cytometric analysis of intraplatelet Munc13-4 protein expression revealed bimodal populations of normal and Munc13-4–deficient platelets. These findings indicate that flow cytometric detection of intraplatelet Munc13-4 protein is a sensitive and reliable method to rapidly screen for FHL3 with a very small amount of whole blood, even in the acute phase of the disease. (*Blood.* 2011;118(5):1225-1230)

Introduction

The granule-dependent cytotoxic pathway is a major immune effector mechanism used by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[1] The pathway involves a series of steps, including cell activation, polarization of the lysosomal granules to the immunologic synapse, exocytosis of lytic proteins such as perforin and granzymes, and induction of apoptosis in the target cells.[2] In addition to its central role in the defense against intracellular infections and in tumor immunity, this pathway also plays an important role in the regulation of immune homeostasis. Defects in the granule-dependent cytotoxic pathway result in a catastrophic hyperinflammatory condition known as hemophagocytic lymphohistiocytosis (HLH).[1,3]

HLH is a life-threatening syndrome of immune dysregulation resulting from the uncontrolled activation and proliferation of CTLs, which leads to macrophage activation and the excessive release of inflammatory cytokines.[4,5] Clinical diagnosis of HLH is made on the basis of cardinal signs and symptoms including prolonged fever and hepatosplenomegaly, and by characteristic laboratory findings such as pancytopenia, hyperferritinemia, hypofibrinogenemia, increased levels of soluble IL-2 receptor, and low or absent NK cell activity.[5,6] HLH can be classified into primary (genetic) or secondary (acquired) forms according to the underlying etiology, although this distinction is difficult to make in clinical practice.[4,5]

Familial hemophagocytic lymphohistiocytosis (FHL) encompasses major forms of primary HLH for which mutations in the genes encoding perforin (*PRF1*; FHL2),[7] Munc13-4 (*UNC13D*; FHL3),[8] syntaxin-11 (*STX11*; FHL4),[9] and syntaxin-binding protein 2 (also known as Munc18-2) (*STXBP2*; FHL5)[10,11] have been identified to date. Perforin is a cytolytic effector that forms a pore-like structure in the target cell membrane. Munc13-4, syntaxin-11, and Munc18-2 are involved in intracellular trafficking or the fusion of cytolytic granules to the plasma membrane and the subsequent delivery of their contents into target cells.[1,12] Consequently, defective cytotoxic activity of CTLs and NK cells is one of the hallmark findings of FHL,[7,8,13-16] although NK cell activity is also decreased in some cases of secondary HLH.[15,17-20]

Prompt and accurate diagnosis of FHL is mandatory to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. Detection of perforin expression in NK cells with flow cytometry is a reliable method to screen for FHL2.[21] Another test analyzes the expression of CD107a on the surface of NK cells, which marks the release of cytolytic granules.[22] Reduced expression of CD107a implies impaired degranulation of NK cells and predicts a likelihood of FHL3.[23] However, this analysis is not available in some patients with extremely reduced NK cell numbers, such as during the acute phase of HLH.[19] In addition, NK-cell degranulation is also impaired in FHL4[24] and FHL5,[10,11] making it impossible to differentiate these disorders.

We reported previously that Munc13-4 protein is expressed in platelets and regulates the secretion of dense core granules.[25] Herein we report that Munc13-4 is expressed far more abundantly in platelets than in PBMCs. We also describe the development of a

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new method to screen for FHL3 rapidly by detecting intraplatelet Munc13-4 expression through flow cytometry.

Methods

Patients

Between January 2008 and March 2010, whole blood samples from 85 patients were screened for FHL3. The patients had been clinically diagnosed with HLH by their referring physicians and were suspected of possible FHL. Characteristics of the enrolled patients are summarized in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). As a control, blood obtained from healthy adults at the time of patient sampling was shipped for screening along with the patient samples. Before the laboratory studies were performed, informed consent was obtained from the patients and their parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Preparation of PBMCs and platelet samples

Whole blood samples treated with EDTA were centrifuged gently at 100*g* for 10 minutes, and platelets were collected from the supernatant plasma layer. Alternatively, platelets were prepared from small aliquots of blood samples by lysing red blood cells with ammonium chloride. PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation from the remaining sample. $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, and $CD45^+$ cells were separated from PBMCs using an AutoMACS Pro (Miltenyi Biotec) and magnetic bead–conjugated mAbs according to the manufacturer's instructions. Flow cytometric analysis revealed that each cell population contained > 95% $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, and $CD45^+$ cells (data not shown).

Mutation analysis

Genomic DNA was isolated from the PBMCs of patients with defective Munc13-4 expression using standard procedures. Primers were designed for the amplification and direct DNA sequencing of the *UNC13D*-coding exons, including the adjacent intronic sequences for the identification of splice-site variants. Primer sequences are available upon request. Products were sequenced directly with an ABI3130 genetic analyzer (Applied Biosystems).

Antibodies

Rabbit polyclonal antibodies raised against the N-terminal region (residues 1-262)[25] and full-length human Munc13-4 protein were used as primary antibodies for Western blot and flow cytometric analysis, respectively. Rabbit polyclonal anti-integrin αIIb (Santa Cruz Biotechnology) and mouse polyclonal anti–β-actin (Sigma-Aldrich) antibodies were used as primary antibodies for Western blotting. The mAbs used in the flow cytometric analysis were FITC-conjugated anti-CD3 (SK7; BD Pharmingen), phycoerythrin (PE)–conjugated anti-CD41a (HIP8; BD Pharmingen); allophycocyanin-conjugated anti-CD56 (N901; Beckman Coulter), and PE-conjugated anti-CD107a (H4A3; eBioscience).

Western blot analysis

Cell extracts were fractionated by SDS-PAGE, and the fractionated proteins were electrotransferred onto polyvinylidene fluoride membranes. The membranes were blocked overnight in blocking buffer (5% skim milk) and incubated for 1 hour at room temperature with the primary antibodies, followed by HRP-conjugated anti–rabbit or anti–mouse IgG polyclonal antibodies (Santa Cruz Biotechnology). Specific bands were visualized by the standard enhanced chemiluminescence method.

Flow cytometric analysis of Munc13-4 protein

After surface staining with anti-CD41a mAbs, platelets were fixed and permeabilized by Cytofix/Cytoperm (BD Biosciences) and washed 3 times with Perm/Wash buffer (BD Biosciences). After nonspecific reactions were blocked with Chrome-Pure human IgG (Jackson ImmunoResearch Laboratories), rabbit polyclonal antibody against the full-length human Munc13-4 protein was added, followed by FITC-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Platelets were gated on the basis of their appearance on forward- and side-scatter plots in log/log scale and by CD41a expression. The gated platelets were analyzed for Munc13-4 expression by flow cytometry (FACSCalibur; BD Biosciences).

Lysosomal degranulation assays

To quantify lysosome exocytosis by NK cells, 2×10^5 PBMCs were mixed with 2×10^5 human erythroleukemia cell line K562 cells and incubated for 2 hours in complete medium (RPMI 1640 medium supplemented with 2mM L-glutamine and 10% FCS) at 37°C in 5% CO_2. Cells were resuspended in PBS supplemented with 2% FCS and 2mM EDTA; stained with anti-CD3–FITC, anti-CD56–allophycocyanin, and anti-CD107a–PE mAbs; and analyzed by flow cytometry.

Platelet exocytosis of the lysosomal granules was analyzed as described previously[26] but with a minor modification. Briefly, platelets were suspended in PBS containing 2mM EDTA and PE-conjugated anti-CD107a mAb, stimulated with 5 U/mL of thrombin (Wako Pure Chemical Industries) for 10 minutes at 25°C, and immediately analyzed by flow cytometry. The degranulation index of platelets was calculated as: (mean fluorescence value of stimulated sample − mean fluorescence value of nonstimulated sample)/mean fluorescence value of nonstimulated sample.

Statistical analysis

Statistical analyses were performed with 1-way ANOVA followed by the Tukey post hoc test to compare multiple groups, with a $P < .05$ level considered to be significant.

Results

Diagnosis of FHL3 by Western blot analysis using platelets

Before screening for FHL3, the Munc13-4 expression level was compared between platelets and PBMCs. Munc13-4 expression in platelets was approximately 10 times higher than that in PBMCs (Figure 1A). $CD8^+$ cells expressed a similar level of Munc13-4 protein as other PBMC cell types (Figure 1B). Similar amounts of platelet- and PBMC-derived proteins could be obtained from a sample (data not shown). Therefore, platelets were used to perform Western blotting to screen for Munc13-4 deficiency. Of the 85 patients screened, 6 patients were diagnosed with FHL3 (Figure 1C). Munc13-4 protein was barely detected in the platelets of each FHL3 patient regardless of the gene mutation (Table 1). For each sample, no more than 1 mL of whole blood was required to perform the analysis.

Difficulty in diagnosing FHL3 in the acute phase of the disease

Patients in the acute phase of the disease who require screening for FHL often receive platelet transfusions because of thrombocytopenia.[4-6] To study the effect of transfused platelets on screening results, FHL3 screening was attempted in a patient receiving platelet transfusions. As expected, Western blotting using platelets could not detect Munc13-4 deficiency because of the normal expression of the protein in the transfused platelets (Figure 2A left column). Surprisingly, Western blotting using PBMCs also could not clearly identify Munc13-4 deficiency because a substantial number of platelets were present in the PBMCs obtained by the standard method (Figure 2A right column). By positively selecting $CD45^+$ cells and removing platelets, it was found that a considerable amount of the Munc13-4 protein detected in PBMC samples

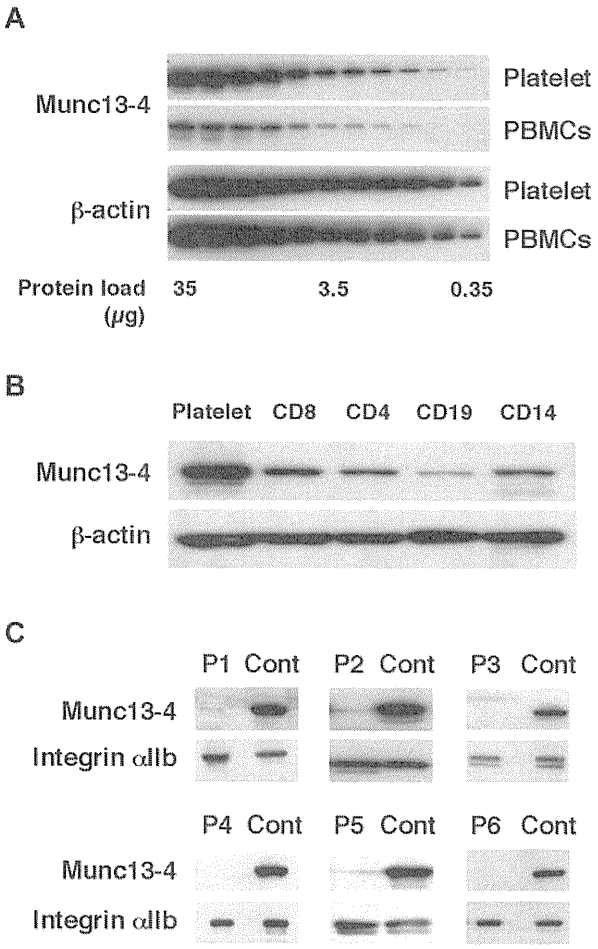

Figure 1. Diagnosing FHL3 by Western blotting using platelet protein. The amount of Munc13-4 protein expression was compared between platelets and PBMCs (A) and among platelets, $CD8^+$, $CD4^+$, $CD19^+$, and $CD14^+$ cells (B) by Western blotting. A representative result of 5 independent experiments is shown. (C) Six FHL3 patients were diagnosed by Western blotting for Munc13-4 protein using platelets.

obtained by standard density gradient centrifugation was actually derived from the contaminating platelets (Figure 2B).

We performed a NK-cell degranulation assay for every referred sample and found the assay to be defective for every FHL3 patient identified (data not shown). All of the other patients showed a normal release of lysosomal granules by NK cells; however, the analysis could not be performed in some patients because of the extremely low NK-cell number during the acute phase of the disease (data not shown).

We also examined the lysosomal granule release of platelets in 31 patients to determine whether this assay could be used as a screening method for FHL3. Lysosomal exocytosis of FHL3 platelets was partially impaired at steady state, but profound impairment was observed during the acute phase of the disease (Figure 3A-C). This profound impairment was also observed in platelets obtained from some secondary HLH patients during the acute phase (Figure 3B-C). These results indicate that it is difficult to diagnose FHL3 during the acute phase of HLH either by Western blot or by lysosomal degranulation assay.

Table 1. UNC13D gene mutations of FHL3 patients

Patient	Age at onset	Gender	Mutation	Genotype	Predicted effect
P1	14 days	Female	c.1596 + 1G → C	Homo	Splice error
P2	2 months	Male	c.322–1G → A	Hetero	Splice error
			c.990G → C	Hetero	p.Q330H
			c.3193C → T	Hetero	p.R1065X
P3	12 months	Female	c.754–1G → C	Hetero	Splice error
			c.2485delC	Hetero	p.L829fs
P4	4 months	Female	c.754–1G → C	Hetero	Splice error
			c.1799C → T	Hetero	p.T600M
			c.1803C → A	Hetero	p.Y601X
P5	2 months	Female	c.754–1G → C	Hetero	Splice error
			c.1596 + 1G → C	Hetero	Splice error
P6	5 months	Male	ND	ND	ND

Mutations were checked for single nucleotide polymorphisms using the dbSNP Build 132 database from the National Center for Biotechnology Information.

X indicates stop; fs, frame shift; and ND, not determined.

Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4

To overcome the difficulty in diagnosing FHL3 during the acute phase of HLH, antibodies were raised against the full-length human Munc13-4 protein (supplemental Figure 1) and a new method was developed to detect Munc13-4 protein in platelets by flow cytometry. A total of 35 patients, including 4 with FHL3 (P3-P6), were

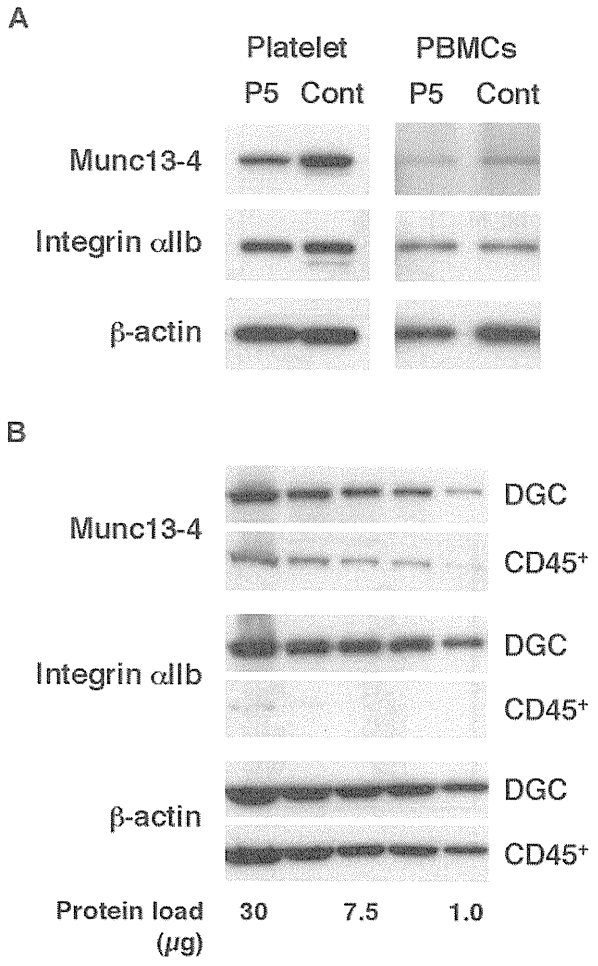

Figure 2. Effect of platelet transfusion on Western blot analysis. (A) Western blotting analysis for Munc13-4 expression using platelets and PBMCs from an FHL3 patient (P5) receiving platelet transfusions during the acute phase of the disease. (B) The expression of Munc13-4 was compared between PBMCs obtained by density gradient centrifugation (DGC) and $CD45^+$ cells obtained by magnetic sorting from healthy controls. A representative result of 3 independent experiments is shown.

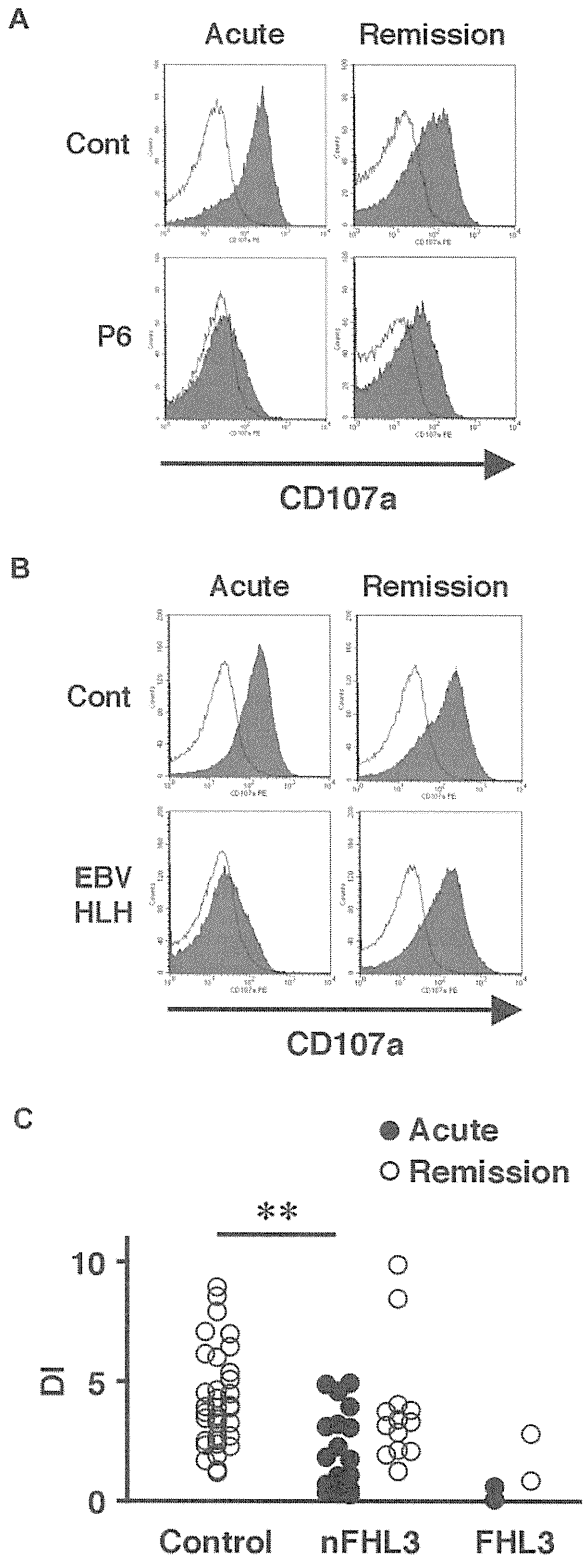

Figure 3. Analysis of lysosomal exocytosis using platelets from HLH patients. Platelets from an FHL3 patient (P6; A) and from a secondary (EBV-associated) HLH patient (B) along with healthy controls were left untreated (open histogram) or were stimulated with thrombin (closed histograms), and the surface expression of CD107a was analyzed by flow cytometry. Analysis was performed during the acute phase of the disease (left column) and after clinical remission (right column). (C) Degranulation index (DI) of platelets from HLH patients during the acute phase (●) and after clinical remission (○). HLH patients with normal NK-cell degranulation and Munc13-4 protein expression by Western blot analysis were defined as non-FHL3 (nFHL3). $^{**}P < .01$ by the Tukey post hoc test.

analyzed using this method. Munc13-4 deficiency was readily detected in all of the FHL3 patients, with a sample volume of < 100 μL of whole blood (Figure 4A-C). Munc13-4 protein was expressed at normal level in the platelets of parents and siblings of FHL3 patients carrying heterozygous *UNC13D* mutations (data not shown). In the FHL3 patient receiving platelet transfusions, flow cytometric analysis revealed bimodal populations of normal and Munc13-4–deficient platelets (P5 in Figure 4A). As shown in Figure 4B, the method was able to clearly identify Munc13-4–deficient platelets in whole blood samples stored at room temperature for 1 week.

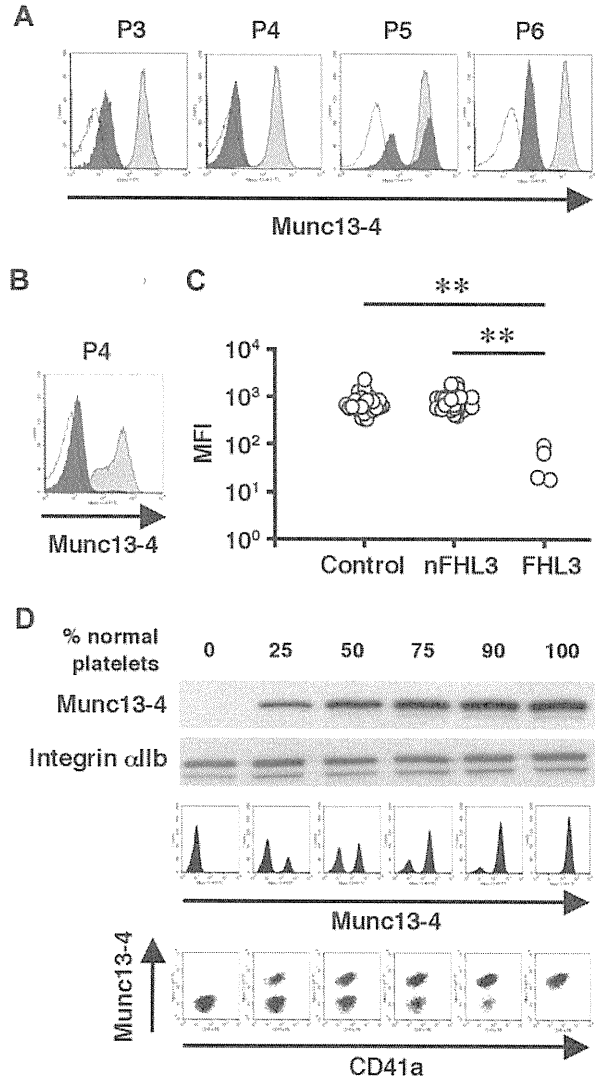

Figure 4. Flow cytometric detection of intraplatelet Munc13-4 protein. Flow cytometric analysis of intraplatelet Munc13-4 expression in 4 FHL3 patients and healthy controls using whole blood samples shipped overnight (A) and in an FHL3 patient (P4) and a healthy control using samples stored at room temperature for a week (B). Dark closed histograms represent platelets from FHL3 patients, whereas light closed histograms represent platelets from healthy controls. Open histograms represent staining with isotype controls. (C) Mean fluorescence intensity (MFI) of intraplatelet Munc13-4 staining for HLH patients and healthy controls. All of the healthy controls (n = 35) were adults. Non-FHL3 (nFHL3) patients (n = 31), as defined in Figure 3, varied in age (2 days-39 years) and included 2 patients with FHL2. Age-related variations in the MFI of Munc13-4 staining were not observed. $^{**}P < .01$ by the Tukey post hoc test. (D) The sensitivities of Western blot and flow cytometric analyses for detecting Munc13-4–deficient platelets were compared.

To determine the sensitivity of the new method, Munc13-4–deficient platelets were mixed with normal platelets at varying ratios. Western blot analysis could not detect Munc13-4–deficient platelets easily, even when the proportion of normal platelets was as low as 25% (Figure 4D). In contrast, flow cytometric analysis easily identified 10% Munc13-4–deficient platelets among 90% normal platelets (Figure 4D), which proved the high sensitivity of the method in diagnosing FHL3.

Discussion

FHL is a rare but life-threatening inherited immune disorder for which mutations in 4 genes have been identified as causative factors. *PRF1* encodes the cytolytic effector protein perforin that forms a pore-like structure in the target cell membrane.[1,12] A mutation in *PRF1* results in FHL2,[7] which accounts for 20%-50% of FHL cases.[4,5] *UNC13D* encodes the protein Munc13-4, which is crucial for the fusion of cytolytic granules to the plasma membrane and the subsequent release of perforin and granzymes.[1,12] Mutations in *UNC13D* result in FHL3,[8] which accounts for 20%-30% of FHL cases.[4,12] FHL4 is caused by mutations in *STX11*, which encodes syntaxin-11.[9] Mutations in *STXBP2*, which encodes Munc18-2, were recently reported to cause FHL5.[10,11] Syntaxin-11 and Munc18-2 also mediate the fusion of cytolytic granules to the plasma membrane.[1,5,12] The ability to screen for FHL2-5 rapidly would facilitate the initiation of life-saving immunosuppressive therapy and the preparation of FHL patients for hematopoietic stem cell transplantation.

In the present study, we found that the Munc13-4 protein is expressed abundantly in platelets (Figure 1A-B). The detection of Munc13-4 protein in platelets by Western blotting (Figure 1C) or flow cytometry (Figure 4A-B) was a reliable screening method to identify FHL3 patients. Munc13-4–deficient platelets were identified easily among normal transfused platelets by flow cytometry, which indicated that this method could be applied to patients who are receiving platelet transfusions during the acute phase of the disease (P5 in Figure 4A). Detection of intraplatelet Munc13-4 was enabled by the use of highly specific antibodies against the full-length human Munc13-4 (supplemental Figure 1).

There is a possibility that FHL3 patients with residual Munc13-4 protein expression could be overlooked by the screening methods described in this study. Most FHL3 patients have mutations that result in the absence or significant reduction of Munc13-4 protein expression,[16,23] as was the case with the patients screened in this study (Figure 1C), which suggests that the mutated Munc13-4 protein is unstable. The NK-cell degranulation assay, which was performed for every referred sample with a sufficient number of NK cells, revealed defective degranulation only in the identified FHL3 patients (date not shown). These results indicate that the majority of mutations in *UNC13D* are likely amenable to rapid detection by the new methods described in this study. Comparative studies on the *UNC13D* genotype, Munc13-4 protein expression, and the lysosomal exocytosis assay must be performed to confirm the reliability of these methods.

It was also investigated whether the analysis of lysosomal release by platelets could be used as an alternative method to screen for FHL3. Profound impairment of lysosomal exocytosis by platelets during the acute phase of the disease and restoration of this impairment after clinical remission was observed in FHL3 and in some secondary HLH patients (Figure 3). It is not clear whether this transient impairment of platelet degranulation is involved in HLH pathogenesis or if it merely reflects in vivo platelet activation by diffuse endothelial damage during the acute phase of the disease that renders them unresponsive to ex vivo stimulation. The release of lysosomal granules by Munc13-4–deficient platelets was impaired only minimally at steady state (Figure 3A and 3C), which is in contrast to a recent study showing the involvement of the Munc13-4 protein in the release of lysosomal granules in mouse platelets.[27] Although the precise reason for this discrepancy is unclear, platelet degranulation is likely to be regulated differentially between species; for example, Munc13-4–deficient mice have bruising and bleeding tendencies[27] that are not commonly associated with human FHL3. Further studies are warranted to elucidate the exocytosis pathways of platelets and their role in the pathophysiology of HLH.

With the development of tools for rapid screening, the diagnostic approach for FHL has changed over the years. Impaired NK cytotoxicity was the first reported signature clinical finding of FHL patients.[13,14] Defective CTL activity was subsequently reported as another hallmark of FHL.[7,8,16,28] However, NK-cell activity is also decreased in some cases of secondary HLH,[15,17-20] and the CTL cytotoxicity assay is not readily accessible to most clinicians. The NK-cell lysosomal exocytosis assay is a comprehensive method to identify patients with a degranulation defect.[10,11,22-24] However, this analysis is not available in some patients with extremely reduced NK-cell numbers, which are often observed during the acute phase of HLH.[19] Although CTLs can be an alternative tool to perform the lysosomal exocytosis assay,[24,28,29] it remains impossible to differentiate FHL3-FHL5.[10,11,23,24] Impairment in these assays warrants the genetic confirmation of FHL, but sequencing all of the candidate genes is not a suitable approach for rapid diagnosis. Flow cytometric detection of perforin expression in NK cells is a reliable and rapid way of identifying patients with FHL2,[21] and the new method described in this study for the detection of Munc13-4 expression in platelets would add to the rapid diagnosis of FHL3.

Platelets could also be used for the screening of FHL4 and FHL5 because they share some granule-transport mechanisms with other types of hematopoietic cells, including CTLs and NK cells.[2,30,31] Indeed, in the present study, both syntaxin-11 and Munc18-2 were expressed abundantly in platelets (data not shown). We are currently using platelet proteins to screen for FHL4-FHL5 by Western blot analysis, although no cases have been found so far because of the extreme rarity of these disorders.

In summary, platelets abundantly express Munc13-4 protein and are a useful tool to screen for FHL3. By detecting intraplatelet Munc13-4 expression by flow cytometry, it is now possible to rapidly screen for FHL3 with a very small sample of whole blood, even in the acute disease phase requiring platelet transfusion. Because platelets share some of their granule transport systems with other types of hematopoietic cells, they could also be used to screen for other types of immune disorders, including FHL4 and FHL5.

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Authorship

Contribution: T.Y., R.N., T.N., H.H., and H.T. designed the research; Y.M., K.I., and M.S. performed the Western blot and flow cytometric analyses; K.O. and O.O. performed the genetic analyses; R.S. and H.H. prepared the anti–Munc13-4 antibodies and started the FHL3 screening; Y.M., T.Y., R.S., K.I., H.S., J.A., N.T., T.K., R.N., E.I., T.N., H.H., and T.H. analyzed and discussed the results; and Y.M., T.Y., and T.H. wrote the manuscript.

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Familial Cases of Periodic Fever with Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis Syndrome

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We report three familial cases of periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis syndrome, including a pair of monozygotic twins and their mother. It suggests that periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis syndrome may have a certain monogenetic background. *(J Pediatr 2011;158:155-9)*

Periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) syndrome was first described in 1987 by Marshall et al.[1] Several subsequent reports have confirmed this syndrome as a distinct clinical entity[2-4] taking the form of periodic fever occurring at intervals and associated with aphthous stomatitis, pharyngitis, and cervical adenitis, beginning usually before the age of 5 years. Diagnostic criteria for PFAPA are shown in a previous report.[3] PFAPA syndrome has been described as a noninfectious, nonautoimmune, and autoinflammatory disease that shows dramatic response to corticosteroid therapy. Most cases of PFAPA syndrome are sporadic, but some previous reports have described cases of nontwin siblings[5] and of siblings and their mother,[6] suggesting that the syndrome may be induced by environmental or genetic factors. We treated three familial cases of PFAPA syndrome, namely, a mother and her monozygotic twins.

Methods

Patients

Case 1. The elder twin was a 2-year-old girl who was referred to our hospital at 1 year and 4 months of age. She was born from healthy and nonconsanguineous parents without any prenatal or postnatal problems. Her neurodevelopment was normal. Her first episode of fever occurred at 11 months of age and was not associated with any other complaints. After this episode, high fevers occurred suddenly and periodically, always lasting for 3 to 5 days. The patient was routinely diagnosed with "pharyngitis or tonsillitis" but always had no upper respiratory tract symptoms and no abdominal complaints (Table I). Each episode of refractory fever continued despite treatment with antibiotics but eventually resolved spontaneously. At first, the intervals between febrile episodes were irregular, but they gradually settled into a regular schedule and then occurred "like clockwork," with about 15 to 20 days between episodes. During the interval periods, the patient exhibited no clinical symptoms.

At 1 year and 4 months of age, the patient visited our hospital during an episode of high fever and tonsillitis. Laboratory examinations (Table I) during febrile episodes revealed elevation of C-reactive protein (CRP) and erythrocyte sedimentation rates (ESR), mild leukocytosis without neutropenia; these results were normal during nonfebrile periods. There were no positive findings in any culture samples or in any virus antigen tests or serum titers. Levels of other inflammatory agents (C3, C4, CH50, antinucleotide and anti-DNA antibody, MMP-3, and PRO-/MPO-ANCA) were all normal. Analysis of immunoglobulin components revealed only immunoglobulin D (IgD) mildly to moderately elevated throughout febrile and symptom-free periods. Only a part of fevers lasted for more than 10 days, but all fevers and some characteristic symptoms (Table I) eventually disappeared spontaneously and completely regardless of treatment with systemic antibiotics.

After about 10 similar clinical episodes of periodic fever attack, we diagnosed the patient with PFAPA syndrome at 2 years of age and introduced oral low-dose prednisolone ([L-PSL] dosage 0.3-1 mg/kg/dose, 1 or 2 doses per day) at the beginning of every fever. The introduction of prednisolone dramatically decreased the duration of each fever to remission in less than 3 hours. After the introduction of L-PSL, the patient's and her family's quality of life improved remarkably, but periodic fever attacks still recur at the same interval of about 15 to 18 days. Cimetidine therapy was refused because of its bad taste, and the parents refused adenotonsillectomy.

CRP	C-reactive protein
ESR	Erythrocyte sedimentation rates
FMF	Familial Mediterranean fever
HIDS	Hyperimmunoglobinemia D syndrome
IgD	Immunoglobulin D
L-PSL	Low-dose prednisolone
MEFV	Familial Mediterranean fever
MVK	Mevalonate kinase
PFAPA	Periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A

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Table I. Clinical features and laboratory findings in familial cases of PFAPA

	Case 1 23 clinical episodes	Case 2 26 clinical episodes	Case 3 10 clinical episodes
Clinical features			
High-grade fever (≥38.5° C)	100%	100%	100%
Pharyngitis	100%	100%	100%
Tonsillitis	100%	100%	100%
Aphthous stomatitis	34.8%	30.8%	60%
Cervical adenitis	56.5%	73.1%	80%
Headache	0%	0%	60%
Abdominal complaints (pain)	0%	0%	0%
Joint pain/muscle pain	0%	0%	0%
Skin rash	0%	0%	0%
Laboratory data			
White blood cell count (/mm^3)*			
No.	12	12	3
Range	8180-23 230	6230-20 000	6570-7500
Mean ± SD	13 475 ± 1170	13 466 ± 1103	
CRP (mg/dL)*			
No.	12	12	3
Range	1.1-18.3	1.4-15.2	0.01-0.5
Mean ± SD	5.0 ± 1.3	6.4 ± 1.1	
ESR at 60 min (mm)*			
No.	12	12	2
Range	15-102	17-102	6-7
Mean ± SD	50.2 ± 6.9	70.5 ± 7.6	
IgM (mg/dL)*			
No.	9	12	2
Range	45-116	51-112	72-102
Mean ± SD	66.4 ± 7.4	68.2 ± 6.1	
IgA (mg/dL)*			
No.	9	12	2
Range	30-79	49-117	98-125
Mean ± SD	47.4 ± 4.9	75.2 ± 6.2	
IgG (mg/dL)*			
No.	9	12	2
Range	633-940	625-970	992-1042
Mean ± SD	775.7 ± 39.1	811.5 ± 28.2	
IgD (mg/dL)*			
No.	2	3	1
Range	35.5-71.6	33.5-41.7	18.2
IgD (mg/dL) (nonfebrile periods)			
No.	3	3	2
Range	46.4-58.2	39.9-66.1	16.5-17.1
Urinary mevalonolactone (during febrile periods)	Normal	Normal	Normal
MK activity (versus normal control)	98%	58%	88%
MVK gene	No mutation detected	No mutation detected	No mutation detected
MEFV gene	Hetero P369S/E148Q#	Hetero P369S/E148Q#	No mutation detected
TNFRSF1A gene	No mutation detected	No mutation detected	No mutation detected

No., Number of times of blood sampling; *#*, not compound single nucleotide polymorphism.
*Measured during febrile periods.

Case 2. The younger patient was the second-born of monozygotic twins from the same parents. Her neurodevelopment also was normal. She had periodic abrupt fevers that occurred only in association with pharyngitis and cervical adenitis beginning at 12 months of age. Antibiotic therapy was not effective; instead, each episode resolved spontaneously about 4 to 5 days after its onset. Aphthous stomatitis appeared late in some episodes, around the time that the fever resolved (Table I).

Clinical examinations (Table I) in our hospital revealed elevated levels of the inflammatory agents including CRP and ESR, and leukocytosis without cyclic neutropenia, but, as in the case of the elder sister (Case 1), no indication of infection or autoimmune disease. This patient exhibited mild to moderate elevation in IgD throughout febrile and nonfebrile periods. After about 12 similar febrile episodes, starting of oral L-PSL resolved each fever dramatically and promptly in 2 to 4 hours after treatment. Although prednisolone has improved the patient's quality of life, she is still experiencing periodic febrile episodes, and the intervals between episodes have gradually shortened to about 14 to 18 days. Cimetidine therapy was discontinued because of its unpleasant taste, and the parents refused adenotonsillectomy.

Case 3 (Cases 1 and 2). The biological mother of twins was a healthy 29-year-old woman. In an interview, she related that she had frequently experienced recurring abrupt high

Table II. Summary of reported familial cases of PFAPA syndrome

	Sampaio et al[5]	Valenzuela et al[6]		Present cases
Patients' background				
Familial history	Two siblings	Two sisters and two brothers in different families		Monozygotic twins and mother
Consanguinity	None	None	None	None
Age/sex	(1) 10 years/boy (2) 4 years/girl	(1) 9 years/girl (2) 7 years/girl	(1) 7 years/boy (2) 3 years/boy	(1) 2 years/girl (2) 3 years/boy (3) 29 years/mother
Race				
Paternal	Unknown	German-Italian-Chilean	Spanish	Japanese
Maternal	Unknown	Jewish Ashkenazi-Spanish-Chilean	Spanish	Japanese
Predisposing factor	Emotional, psychologic factors	None (both)	None (both)	None (all)
Age of onset	(1) 18 months (2) 3 years	(1) 18 months (2) 2 years	(1) 3 years (2) 2 years	(1) 11 months (2) 12 months (3) 2 years
Growth and development	Normal	Normal	Normal	Normal
Clinical symptoms				
Periodicity of fever	Monthly (both)	(1) 5-6 weeks (2) 4 weeks	(1) 3 weeks (2) 16-20 days	(1) 15-18 days (after PSL) (2) 14-18 days (after PSL) (3) 30 days (before PSL)
Aphthous stomatitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Pharyngitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Cervical adenitis	+ (Both)	+ (Both)	+ (Both)	+ (All)
Respiratory complaints	None (both)	None (both)	None (both)	None (all)
Abdominal complaints	None (both)	Vomit (both)	(1) None (2) Vomit, diarrhea	None (all)
Joint pain	None (both)	None (both)	None (both)	None (all)
Laboratory findings				
Leukocytosis	+ (Both)	Mild (both)	Mild (both)	+ (All)
Neutropenia	None (both)	None (both)	None (both)	None (all)
Elevated CRP (or ESR)	+ (Both)	+ (Both)	+ (Both)	+ (All)
Elevated immunoglobulin	None (both)	None (both)	(1) Not described (2) None	Igd (all)
Throat cultures	Negative (both)	Negative (both)	Negative (both)	Negative (all)
Treatment				
Antibiotics	No response (both)	No response (both)	No response (both)	Poor response (all)
Corticosteroids	Dramatic response (all)	Not prescribed (both)	Not prescribed (both)	Dramatic response (all)
Cimetidine	Unknown (both)	Not described (both)	Not described (both)	Discontinued (twins)
Tonsillectomy	Not done (both)	Not done (both)	Not done (both)	Not done (all)
Prognosis				
Age of last attacks	Unknown	(1) 6 years (2) 6 years	(1) 7 years (2) Not described	Continued (all)
Present status	No remission	Cr (both)	Cr (both)	(1) (2) No remission (3) Relapse in adulthood

Cr, Complete remission; *PSL*, prednisolone.

fevers, which were diagnosed as "acute pharyngitis and aphthous stomatitis," between 2 and 10 years of age. Each time, she visited a pediatric clinic and received oral antibiotics, but her fevers failed to respond. After refractory fever had lasted for 3 to 5 days, the patient recovered spontaneously and was symptom-free during nonfebrile periods. After her first pregnancy and delivery of a twins' sister now 5 years of age, abrupt and periodic febrile episodes began again and repeated at about 30-day intervals, always with the same symptoms, including pharyngitis, aphthous stomatitis, and cervical adenitis, and with elevated CRP ranging from 4 to 10 mg/dL. Each episode lasted for 3 to 5 days, did not respond to oral antibiotic therapy, and eventually resolved spontaneously. Serum IgD level during both a febrile and a non-febrile period and found it mildly elevated (Table I). L-PSL (6 mg/doses, only a dose per day) was very effective against her abrupt high fevers, diminishing them dramatically and promptly improved her general condition, although her PFAPA syndrome was an especially unusual type in that her episodes began in childhood, stopped, and then began again in adulthood. In this case, cimetidine therapy has not yet been prescribed.

Genetic Analysis of the Mevalonate Kinase, Familial Mediterranean Fever, and Tumor Necrosis Factor Receptor Superfamily, Member 1A Genes

After written informed consent approved by Institutional Review Board of Kyoto University was obtained, peripheral blood was collected from all the patients and their family members. Genomic DNA was extracted, and all the exons including exon-intron junctions of mevalonate kinase (MVK), familial Mediterranean fever (MEFV), and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A) genes were amplified by polymerase chain reaction and sequenced by ABI3130. No defect was found.

Analysis of Mevalonate Kinase Activity

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of the patients by using Lymphoprep (Axis-Shield PoC, Norton, Massachusetts). Then the PBMCs

were stimulated by PHA to obtain T-lymphocyte, which were harvested to measure mevalonate kinase (MK) activity as previously described by Gibson et al.[7]

Discussion

PFAPA syndrome has recently been identified as a new clinical entity, typically occurring in childhood, characteristically consisting of periodic fever, pharyngitis, aphthous stomatitis, and cervical adenitis, which responds dramatically to corticosteroid treatment, although it is unaffected by antibiotic treatment.[2-4] Among the various autoinflammatory diseases (eg, cyclic neutropenia, hyperimmunoglobinemia D syndrome [HIDS], familial Mediterranean fever [FMF], and tumor necrosis factor–receptor-associated periodic syndrome), only PFAPA syndrome still has an unknown genetic background and pathogenesis. One report has indicated that PFAPA syndrome is associated with abnormal cytokine regulation.[8] The only cytokine measured in these 3 cases was soluble interleukin-2 receptor (s-IL2-R), which was mildly elevated.

The other periodic fever syndrome from which PFAPA syndrome must be differentiated on the basis of these clinical symptoms, and serum IgD levels is HIDS, which consists of nonperiodic or periodic fever, chest complaints, joint pain, abdominal pain, diarrhea, hepatosplenomegaly, and skin rash, with elevation of urinary mevalonolactone levels and serum immunoglobulin A levels during fever periods; none of these conditions are true for cases 1 to 3. We performed immunoassay of MK activity and screening of MVK gene mutation in cases 1 to 3; all results were normal (Table I). Most researchers have reported normal levels of serum IgD in patients with PFAPA, but one report[3] describes mild elevation of IgD levels in 12 of 18 clinical PFAPA cases. Thus it appears that mild elevation of IgD may be characteristic of PFAPA syndrome, but this is not a criterion for diagnosis with PFAPA syndrome.

Among the autoinflammatory diseases listed above, only PFAPA syndrome has been described as a noninherited syndrome; this is because several review articles[2-4] on PFAPA syndrome have included no familial cases. Recently, however, familial cases in which patients are siblings[5] or siblings and their mother[6] have been reported. Thus, this poorly understood syndrome is suspected, but not proven, to be heritable (Table II).

With regard to genetic background of PFAPA syndrome, one article[9] strongly argued against the involvement of MEFV, but another article[10] described that 27% of cases diagnosed as PFAPA syndrome on the basis of clinical criteria[3] exhibited MEFV gene mutations, which are mainly responsible for FMF syndrome. The latter study suggests the involvement of MEFV in PFAPA syndrome and some clinical overlap with FMF syndrome. So we additionally demonstrated screening of MEFV gene in cases 1 to 3, resulting in no significant mutations, except for heterozygous P369S and E148Q (both were variants) only in cases 1 and 2 (Table I). Isolated and typical cases of this syndrome, such as these cases, should be differentiated from those of other monogenic periodic fevers by detecting responsible genes.

The monozygotic twinning of cases 1 and 2 was established through gynecologic findings of their mother at the time of their birth and polymorphisms in the MEFV gene. There are no seasonal or environmental factors triggering the onset of their febrile episodes (data not shown); this observation suggests that the episodes are autoinflammatory responses occurring in the absence of infection. Case 3, their mother, represents an interesting clinical course, because the clinical features of her febrile episodes in childhood were extremely similar to those of her daughters and because she has experienced a recurrence of febrile episodes in adulthood. Recently, one report[11] has demonstrated that adult patients with PFAPA syndrome can be classified into two types: the "early-onset type," which begins in childhood, appears to resolve and then recurs in adulthood (2 of 15 cases), and the "late-onset type," which begins in adulthood (13 of 15 cases). Case 3 in this investigation is believed to belong to the former type.

It is worth noting that another 5-year-old sister born from the same mother as these twins (cases 1 and 2) has frequent episodes of high fever with pharyngitis and highly elevated CRP that resolve spontaneously (no elevation in IgD level). In addition, the elder brother of case 3 and uncle of cases 1 and 2, at 36 years of age also has the same episodes as those of case 3, consisting of periodic fevers and laboratory data (highly elevated inflammatory agents; mildly elevated IgD 9 mg/dL (measured once), and no mutations in MEFV, MVK genes) improving dramatically with L-PSL (6 mg/d) treatment.

Finally, we additionally demonstrated screening of TNFRSF1A gene, recently discussed in HIDS, FMF, and tumor necrosis factor–receptor-associated periodic syndrome, all resulting in the absence of mutations, in cases 1 to 3 and the elder brother of case 3 (Table I).

We report monozygotic twins cases of PFAPA and their family, speculating on the existence of genetic background in PFAPA syndrome. Familial cases of PFAPA syndrome require genetic testing for differential diagnosis and understanding the mechanism of this perplexing syndrome. ■

Immunoassay of MK activity (directed by Dr. Sakai) and genetic analysis of MVK, MEFV, and TNFRSF1A genes (directed by Dr. Nishikomori and Dr. Heike) were performed in the Department of Pediatrics, Kyoto University Graduate School of Medicine (Japan). We thank Dr Yuichi Mushimoto, Department of Pediatrics, Shimane University School of Medicine (Japan), for the analysis of urinary mevalonolactone, and all patients and their families for consenting to be described in this report.

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CASE REPORT

Patient with neonatal-onset chronic hepatitis presenting with mevalonate kinase deficiency with a novel MVK gene mutation

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Abstract A Japanese girl with neonatal-onset chronic hepatitis and systemic inflammation was diagnosed with hyper-immunoglobulinemia D and periodic fever syndrome (HIDS). However, she lacked the typical HIDS features until the age of 32 months. She had compound heterozygous MVK mutations, H380R and A262P, the latter of which was novel. These findings suggest that HIDS patients could lack typical episodes of recurrent fever at the onset and that HIDS should be considered as a possible cause of neonatal-onset chronic hepatitis.

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Keywords Autoimmune hepatitis · Hyper-IgD syndrome · Liver biopsy · MVK gene · Neonatal-onset chronic hepatitis

Abbreviations

HIDS	Hyper-immunoglobulinemia D and periodic fever syndrome
IgD	Immunoglobulin D
MVK	Mevalonate kinase
FMF	Familial Mediterranean fever
MEFV	Familial Mediterranean fever gene
AIH	Autoimmune hepatitis
CRP	C-reactive protein

Introduction

Mevalonate kinase deficiency is an autosomal recessive metabolic disorder caused by mevalonate kinase (MVK) gene mutations. The disorder presents as a phenotypic spectrum in which mevalonic aciduria is the more severe form, with neurological complications, and hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is the milder form. HIDS is characterized by recurrent febrile attacks, with lymphadenopathy, abdominal symptoms, skin eruptions, and joint involvement [1]. In this report, we describe a patient with a severe form of HIDS caused by a novel MVK mutation; the patient had presented with neonatal-onset chronic hepatitis that was temporarily diagnosed as autoimmune hepatitis (AIH). The lack of typical recurrent fever and rashes at the onset of the disease delayed the diagnosis of HIDS, which alerted the clinicians that HIDS could exist in patients with continuous inflammatory episodes even with atypical clinical courses.

Case report

A Japanese girl was born at 36 weeks' gestation with a weight of 2,240 g. Her parents were non-consanguineous and the family history was unremarkable. At birth she had no symptoms, but physical examination revealed hepatomegaly (1.5 cm below the right costal margin) without splenomegaly. No jaundice, ascites, or coagulation abnormalities were present. Laboratory examinations showed increased white blood cell count (45,700/mm^3) and serum C-reactive protein (CRP) (15.8 mg/dl), as well as increased transaminase levels (aspartate aminotransferase [AST] 207 IU/l, alanine aminotransferase [ALT] 96 IU/l), lactate dehydrogenase (LDH) (6,575 IU/l), and biliary enzyme levels (γ-guanosine triphosphate [GTP] 61 IU/l). An increased immunoglobulin M level (53.0 mg/dl) caused us to work on congenital infections, with bacterial cultures of blood, cerebrospinal fluid (CSF), and gastric fluid, and determination of serum β D-glucan, and measurements of serum antibodies against cytomegalovirus, toxoplasmosis, syphilis, rubella, herpes simplex type I and type II, listeriosis, Epstein-Barr virus, adenovirus, hepatitis A and B and C viruses, *Chlamydia trachomatis*, and mycoplasma, all of which were negative. Radiographic work-up with computed tomography (CT), magnetic resonance imaging (MRI), and gallium scintigraphy, as well as bone-marrow aspirate examination, did not reveal any congenital neoplasm. A search for metabolic disorders by measurements of blood amino acids and urinary organic acids was negative.

Without any specific treatments, the hepatomegaly gradually increased, although abdominal MRI revealed diffuse inflammation of the liver. To explore further the cause of the hepatomegaly, a needle liver biopsy was performed at the age of 6 months. The biopsied liver specimen showed the presence of mild lymphocytic infiltration and fibrosing lesions in the portal area, and short septa extending from a slightly enlarged portal tract (Fig. 1a, b), which indicated a diagnosis of chronic hepatitis without specific causes.

At the age of 14 months, splenomegaly appeared, and elevated serum IgG (2,299 mg/dl) as well as anti-smooth muscle antibodies (1:160) were detected, which led us to diagnose the patient as having AIH [2]. The patient received methylprednisolone pulse therapy, followed by prednisolone and azathioprine for the presumed AIH [3]. Serum transaminase levels normalized in response to the treatment, although cervical lymphadenopathy, hepatosplenomegaly, and elevated serum CRP persisted.

The continuous elevation of CRP prompted us to consider autoinflammatory diseases; thus, we performed genetic analysis for familial Mediterranean fever (FMF), tumor necrosis factor (TNF) receptor-associated periodic syndrome, and cryopyrin-associated periodic syndrome, at the age of 26 months. After obtaining written informed consent from the parents and approval from the Institutional Review Board of Kyoto University, peripheral blood samples were collected from the patient and her parents for genetic analysis. The analysis was done by sequencing all the exons, including exon–intron junctions, which showed heterozygous L110P and E148Q missense mutations on the familial Mediterranean fever (MEFV) gene (Fig. 2a) without any mutations of the TNFRSF1A and NLRP3

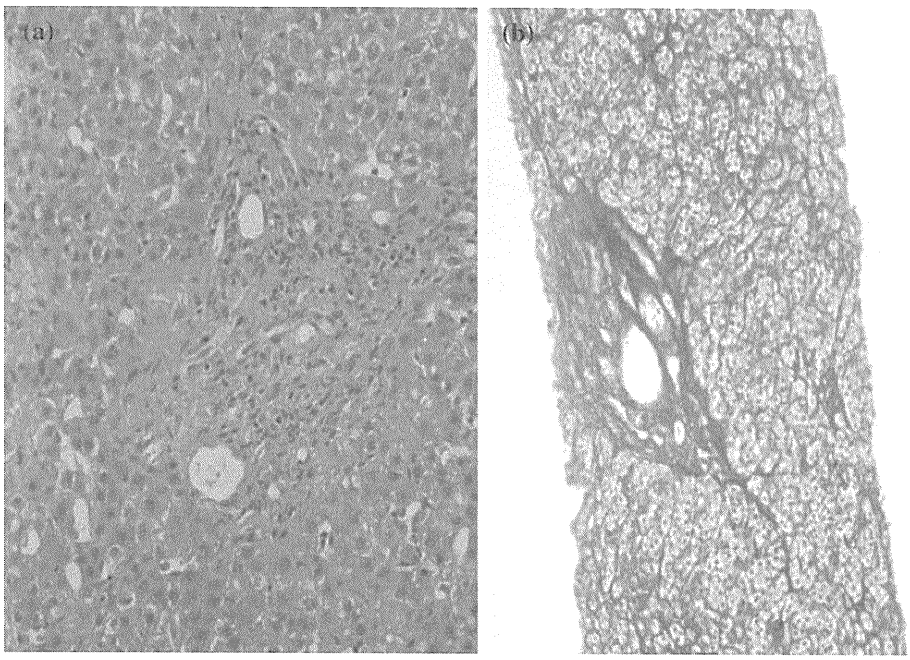

Fig. 1 Liver biopsy specimen showing chronic hepatitis. **a** The portal tract is infiltrated with lymphocytes (H&E, ×200). **b** Short septa extend from the slightly enlarged portal tract (reticulum, ×100)