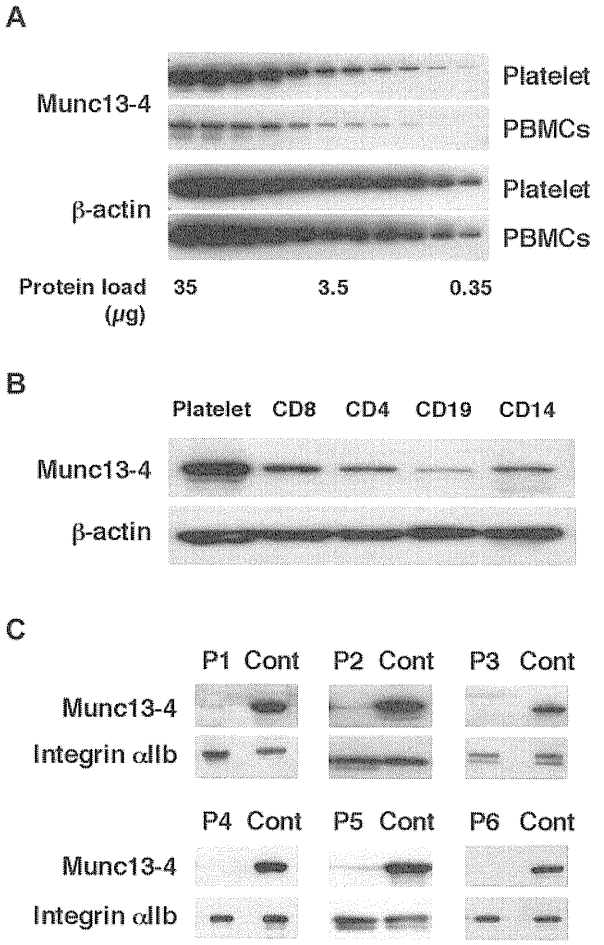

Figure 1. Diagnosing FHL3 by Western blotting using platelet protein. The amount of Munc13-4 protein expression was compared between platelets and PBMCs (A) and among platelets, $CD8^+$, $CD4^+$, $CD19^+$, and $CD14^+$ cells (B) by Western blotting. A representative result of 5 independent experiments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

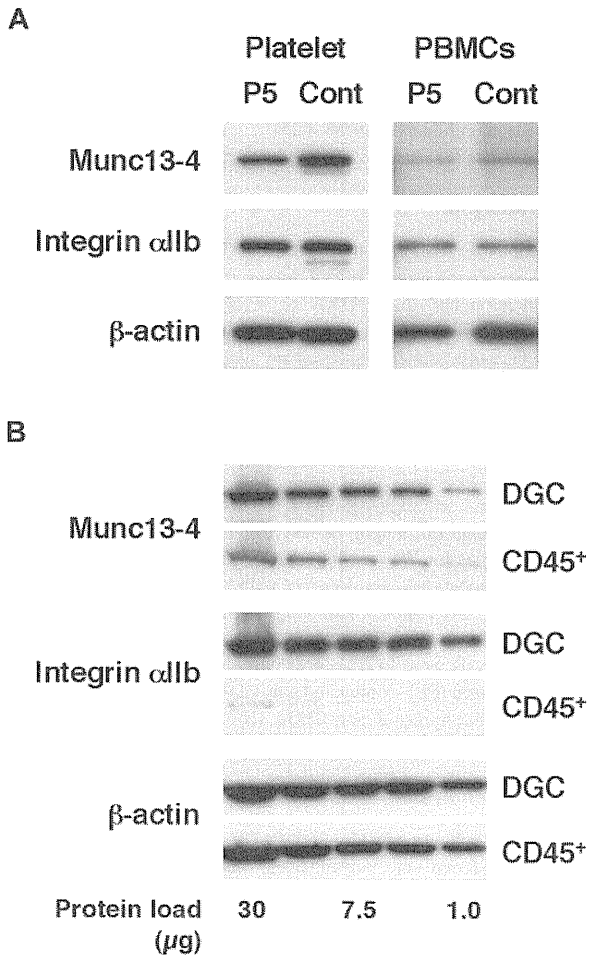

Figure 2. Effect of platelet transfusion on Western blot analysis. (A) Western blotting analysis for Munc13-4 expression using platelets and PBMCs from an FHL3 patient (P5) receiving platelet transfusions during the acute phase of the disease. (B) The expression of Munc13-4 was compared between PBMCs 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.

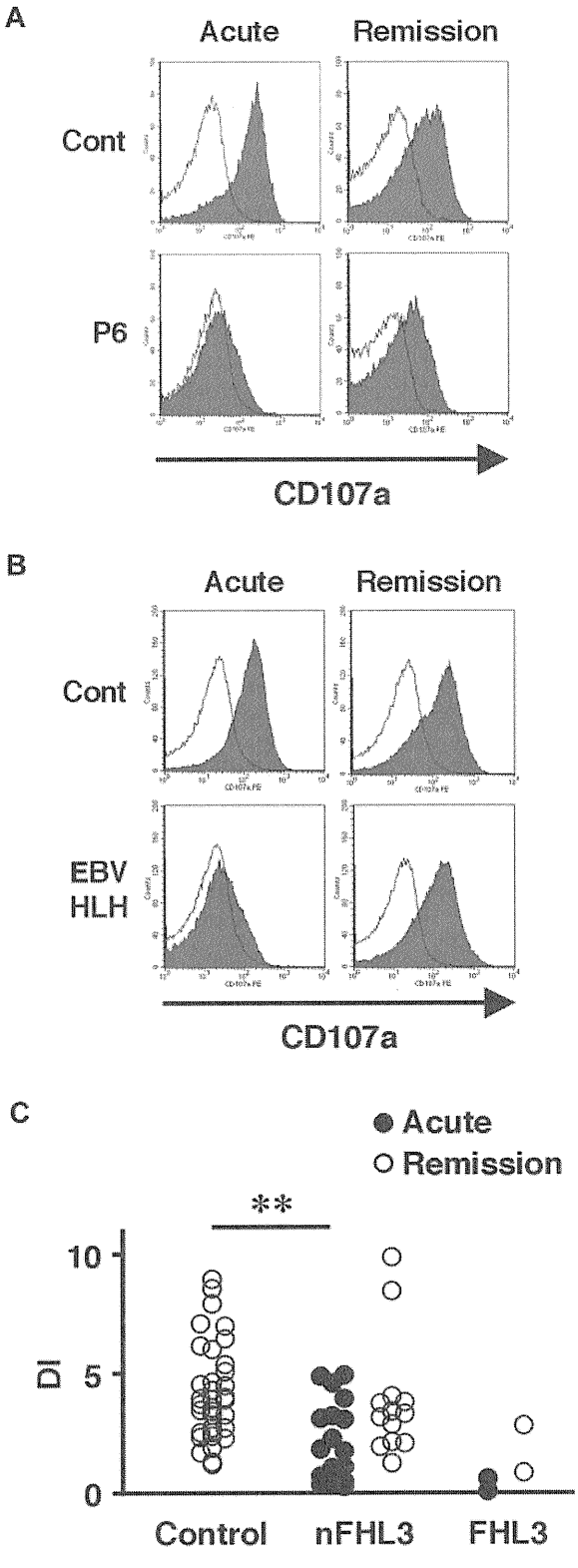

Figure 3. Analysis of lysosomal exocytosis using platelets from HLH patients. Platelets from an FHL3 patient (P6; A) and from a secondary (EBV-associated) HLH patient (B) along with healthy controls were left untreated (open histogram) or were stimulated with thrombin (closed histograms), and the surface 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.

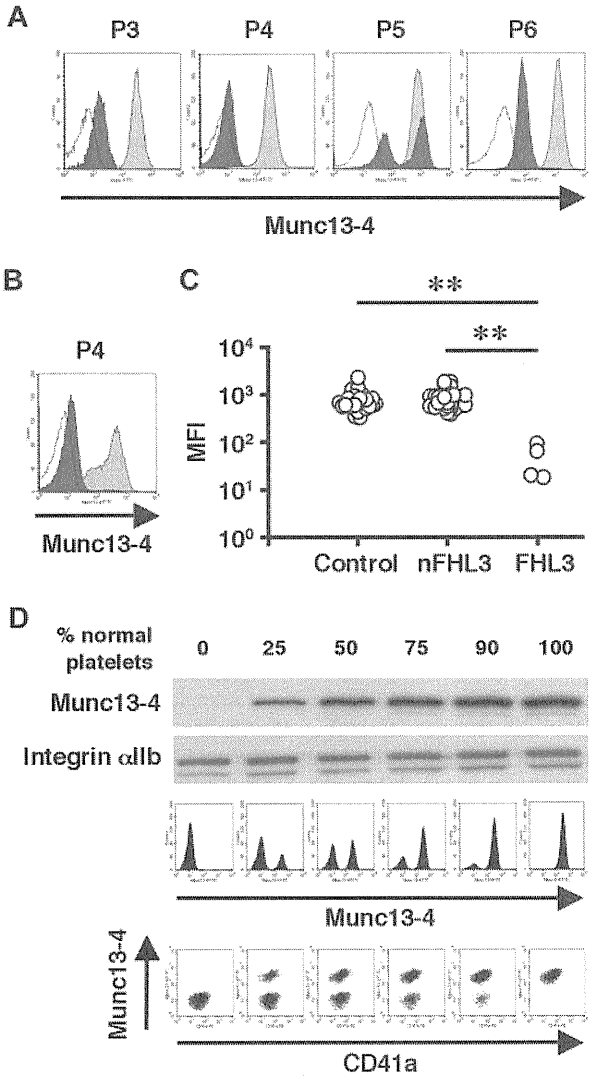

Figure 4. Flow cytometric detection of intraplatelet Munc13-4 protein. Flow cytometric analysis of intraplatelet Munc13-4 expression in 4 FHL3 patients and healthy controls using whole blood samples shipped overnight (A) and in an FHL3 patient (P4) and a healthy control using samples stored at room temperature 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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The CD40–CD40L axis and IFN-γ play critical roles in Langhans giant cell formation

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Abstract

The presence of Langhans giant cells (LGCs) is one of the signatures of systemic granulomatous disorders such as tuberculosis and sarcoidosis. However, the pathophysiological mechanism leading to LGC formation, especially the contribution of the T cells abundantly found in granulomas, has not been fully elucidated. To examine the role of T cells in LGC formation, a new *in vitro* method for the induction of LGCs was developed by co-culturing human monocytes with autologous T cells in the presence of concanavalin A (ConA). This system required close contact between monocytes and T cells, and CD4+ T cells were more potent than CD8+ T cells in inducing LGC formation. Antibody inhibition revealed that a CD40–CD40 ligand (CD40L) interaction and IFN-γ were essential for LGC formation, and the combination of exogenous soluble CD40L (sCD40L) and IFN-γ efficiently replaced the role of T cells. Dendritic cell-specific transmembrane protein (DC-STAMP), a known fusion-related molecule in monocytes, was up-regulated during LGC formation. Moreover, knock-down of DC-STAMP by siRNA inhibited LGC formation, revealing that DC-STAMP was directly involved in LGC formation. Taken together, these results demonstrate that T cells played a pivotal role in a new *in vitro* LGC formation system, in which DC-STAMP was involved, and occurred via a molecular mechanism that involved CD40–CD40L interaction and IFN-γ secretion.

Keywords: concanavalin A, granuloma, multinucleated giant cell

Introduction

Granuloma formation is a specialized inflammatory response observed in infections by certain pathogens such as *Mycobacterium* and *Cryptococcus* (1, 2). Granulomas are also found in immunodeficient conditions such as chronic granulomatous disease (CGD) (3). In addition, this response is a pathological hallmark of non-infectious idiopathic inflammatory disorders including systemic sarcoidosis and Blau syndrome/early-onset sarcoidosis (BS/EOS) (4–7). Granulomas consist of various cell types including macrophages, T cells, plasma cells and epithelioid cells, but the presence of multinucleated giant cells (MGCs) is the cardinal feature of granulomatous inflammation (1, 4). Granuloma formation is generally considered a host defense mechanism against persistent irritants or chronic infection and occurs as a consequence of the failure of the host to eliminate invading pathogens. When the irritants are large and indigestible, MGCs are formed by the fusion of monocyte–macrophage lineage cells (1, 4, 8). However, the pathophysiological mechanism of MGC development and associated granuloma formation is not well understood.

MGCs are morphologically classified into Langhans giant cells (LGCs), which show a circular peripheral arrangement of nuclei, and foreign body giant cells (FGCs), which show irregular scattering of nuclei (9). LGCs are characteristic of systemic granulomatous disorders such as tuberculosis, sarcoidosis and BS/EOS, whereas FGCs are observed as a consequence of a reaction against foreign bodies such as an implant. To explore the mechanisms of MGC formation, particularly LGC formation in systemic granulomatous disease, several *in vitro* systems of human monocyte culture have been developed. These involve the use of stimuli such as phorbol myristate acetate (10–12) and lectins such as concanavalin A (ConA) to induce LGCs (13, 14). However, a considerable degree of concurrent FGC formation is observed in systems that employ ConA.

Cytokines are also used to induce MGCs. For example, IFN-γ (15–19), IL-3 (15, 18, 19) and granulocyte macrophage colony-stimulating factor (15) are frequently used to induce LGCs, while IL-4 is known to induce FGCs (15, 20–23). Notably, IFN-γ has been regarded as a crucial factor

for LGC formation because LGC formation is inhibited by antibodies against IFN-γ (24). However, the roles of other factors and cell types in LGC development have not been elucidated.

T cells are one of the main components of granulomas. Therefore, the present study focused on T cells and investigated their role in the development of LGCs. A novel system was developed in which LGC-dominant MGCs could be generated from freshly isolated monocytes co-cultured with autologous T cells in the presence of ConA, and this system was used to evaluate the role of the CD40–CD40 ligand (CD40L) interaction and IFN-γ in the formation of LGCs. Moreover, the hypothesis that the stimulation of monocytes with exogenous soluble CD40L (sCD40L), recombinant human IFN-γ (rhIFN-γ) and ConA would be sufficient to induce monocyte fusion into LGCs in the absence of T cells was investigated. The findings of this study revealed a pivotal role for T cells in LGC formation and suggest a new pathophysiological mechanism contributing to LGC formation in granulomatous disorders.

Methods

Reagents and antibodies

Blocking antibodies against human CD40 (clone 82102; antagonistic antibody), CD40L, IFN-γ, IL-12 and isotype control IgG and rhIL-12 were purchased from R&D systems (Minneapolis, MN, USA). ConA, LPS and PMA were purchased from Sigma Chemical Co. (St Louis, MO, USA). sCD40L was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was purchased from PeproTech (Rocky Hill, NJ, USA). Inhibitory reagents JSH-23, BMS-345541, U0126, JNK-inhibitor II and SB-203580 were purchased from Calbiochem (San Diego, CA, USA).

Differentiation of MGCs from peripheral CD14+ monocytes

Blood was collected from healthy volunteer donors after informed consent was obtained in accordance with the guidelines of the Institutional Review Board of Kyoto University. PBMCs were isolated from whole blood using the Lymphoprep system (Axis-Shield PoC, Oslo, Norway). PBMCs were incubated with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD14+ cells were isolated using an AutoMACS benchtop magnetic cell sorter according to the manufacturer's protocol (Miltenyi Biotec). Similarly, CD3+, CD4+ and CD8+ cells were collected in a similar fashion, using CD3, CD4 and CD8 beads (Miltenyi Biotec), respectively. The purity of the isolated cells was >95%, as demonstrated by flow cytometry using a FACS Calibur system (BD Biosciences, Franklin Lakes, NJ, USA). In some experiments, CD3+ T cells were first depleted from PBMCs, and CD14+ monocytes were isolated from the negative fraction to obtain highly purified monocytes. (Contaminating T cells comprised <0.5% of the total cell population.)

The isolated CD14+ monocytes were re-suspended at a density of 100 000 cells per well in 48-well plates in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were then cultured for 72 h with various supplemental reagents as described in Results. At the end of the culture period, cells were treated with Giemsa stain to detect nuclei. MGCs were defined as cells with more than three nuclei per cell, according to definitions established by previous studies (9–24).

The stained plates were examined under an Olympus IX70 microscope (Olympus, Tokyo, Japan) using a ×20 or ×10 objective lens with a ×10 eyepiece, and the number of nuclei and MGCs in the representative area was counted. The fusion index was calculated according to the following formula: fusion index = (number of nuclei within MGCs)/(total number of nuclei counted). More than 300 nuclei were counted for each experiment. Images were acquired with an Axio Cam camera (Carl Zeiss, Germany).

Enzyme-linked immunosorbent assay

The concentration of IFN-γ in the culture supernatants was measured by enzyme immunoassay using an OptEIA human IFN-γ ELISA set (BD Biosciences) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical staining was performed using an indirect immunofluorescent technique. Briefly, after fixation with 4% paraformaldehyde, the cells were incubated with a mouse antibody directed against-human CD3 (BD Biosciences), followed by incubation with an Alexa488-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). Cells were then stained with DAPI (Sigma) and visualized with an Olympus laser microscope IX70. Merged reconstruction images were created using Axio Vision SP4 software (Carl Zeiss).

Reverse transciption–PCR

RNA was extracted directly from culture plates using the RNeasy mini kit (Qiagen, Hilden, Germany). After DNase treatment, cDNA was obtained by reverse transcription using the Omniscript RT system (Qiagen). Reverse transcription (RT)–PCR was performed using TaKaRa LATaq (Takara, Shiga, Japan) with the following primer pairs: 5′-GATTGGGTCAGCACTTTTTG-3′ and 5′-CCTCCCAAGTGAATGGATTG-3′ for CD40L; 5′-GGGAGATTTACGTGTCTCCAAG-3′ and 5′-GGGAGGACAACACCTCTGTG-3′ for dendritic cell-specific transmembrane protein (DC-STAMP); 5′-GCCAGCTGGGAGAGAAGG-3′ and 5′-CCTGGTGGGACTTCTCCTG-3′ for triggering receptor expressed on myeloid cells 2 (TREM-2); 5′-TGGAACTCTGCCTCAGGATG-3′ and 5′-ATCACAGCCACTCCAAAAGG-3′ for dedicator of cytokinesis 180 (DOCK180); 5′-GGCAGAGAACCAGGTGAATG-3′ and 5′-GGCATACTCCGTGTGGTTG-3′ for signal regulatory protein (SIRP) α; 5′-CGCAGATCGATTTGAATATAACC-3′ and 5′-GGATTCTGTCTGTGCTGTCG-3′ for CD44; 5′-CAAGAGCATCTTCGAGCAAG-3′ and 5′-TCCTGCTCAGGGATGTAAGC-3′ for CD9; 5′-ATCTGGAGCTGGGAGACAAG-3′ and 5′-CCTCAGTACACGGAGCTGTTC-3′ for CD81; 5′-GAGACCGGTGAGCTGGATAG-3′ and 5′-GCCGTCCTGGGTGTAGAGTC-3′ for matrix metallopeptidase 9 (MMP-9) and 5′-AGGTGAAGGTCGGAGTCAAC-3′ and 5′-ACCTGGTGCTCAGTGTAGCC-3′ for glyceraldehyde 3-phosphate dehydrogenase.

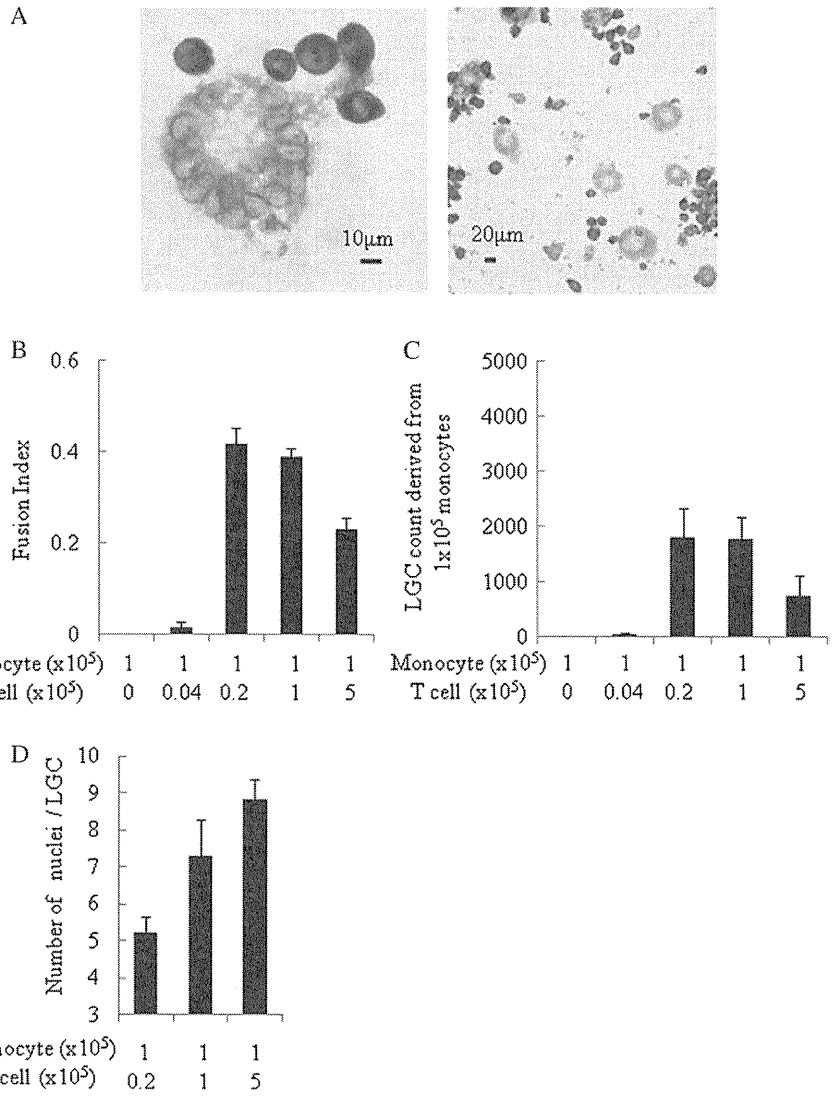

Fig. 1. Co-culture of human monocytes with autologous T cells induces LGC formation. (A) Photomicrographs of Giemsa-stained LGCs induced by co-culture of monocytes and autologous T cells stimulated with ConA. The images show typical LGCs with a circular peripheral arrangement of nuclei. (B–D) T cell-induced LGC formation depends on the ratio of T cells to monocytes. 'Monocyte' refers to the number of monocytes × 10^5 and 'T cell' refers to the number of T cells × 10^5. Values represent (B) the mean fusion index calculated without distinguishing between monocytes and T cells, (C) the mean total number of LGCs derived from 1 × 10^5 monocytes and (D) the mean number of nuclei per LGC. Error bars indicate the standard mean of the error of the indicated ratio of monocytes to T cells stimulated with ConA (5 μg ml^{-1}) (n = 3 independent co-cultures).

siRNA transfection

Pre-designed siRNAs against DC-STAMP or control siRNAs (Applied Biosystems, Carlsbad, CA, USA) were transfected into newly isolated CD14+ cells using Lipofectamine RNAi-MAX (Invitrogen) according to the manufacturers' instructions. Additional transfections were performed after 12 and 24 h of culture.

Western blotting

Antibodies against phospho-(p)-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38, p-p65 and p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Isolated CD14+ cells were incubated without stimulation for 2 h, followed by stimulation with ConA, rhIFN-γ, sCD40L and LPS for 1 h. The harvested cells were lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM orthovanadate) containing a protease inhibitor cocktail (Nacalai, Kyoto, Japan). Equal amounts of total protein were resolved on SDS–polyacrylamide gels and transferred onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA). The membranes were treated with blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.1% tween-20, 0.05% NaN3 and 1% FBS) and then incubated overnight with primary antibody. The membranes were then incubated for 1 h with an HRP-conjugated goat anti-rabbit secondary IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed with ECL Plus Western blotting detection kit (GE Healthcare, Uppsala, Sweden).

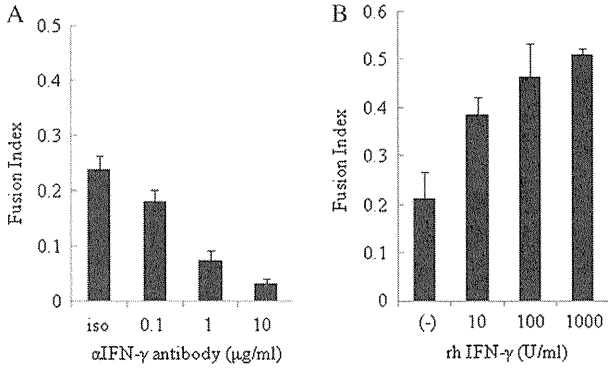

Fig. 2. IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells. (A) An antibody against IFN-γ abrogated LGC formation by the co-culture of monocytes and autologous T cells stimulated with ConA (2.5 μg ml^{-1}). The ratio of monocytes to T cells was 1:1. 'Iso' refers to the isotype control. (B) Addition of rhIFN-γ to the co-culture of monocytes and autologous T cells stimulated with ConA enhanced LGC formation. Values represent the mean fusion index, and error bars indicate the standard mean of the error (n = 3 independent co-cultures).

Statistical analysis

Statistical analysis was performed by applying the Student's t-test, and a P value of <0.05 was considered to be significant.

Results

Co-culturing human monocytes with autologous T cells induces LGC formation

LGCs in granulomas are a specific indication of systemic granulomatous disorders. The observation that T cells are one of the main constituents of granulomas led to the hypothesis that T cells may play a key role in LGC formation. To understand the pathophysiology underlying LGC formation, a new *in vitro* LGC formation system was developed using human monocytes and T cells. When freshly isolated peripheral blood monocytes were co-cultured with autologous T cells under ConA stimulation, LGCs formed within 3 days (Fig. 1A, left), while very few FGCs were detected (Fig. 1A, right). LGC formation, as measured by the fusion index, as well as the number of LGCs per monocyte and the number of nuclei per LGC, increased along with the number of co-cultured T cells, peaking at a monocyte:T cell ratio of 1:1 (Fig. 1B–D).

IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells

The cytokine IFN-γ, which is reported to be critical in LGC formation (15–19, 24), was detected in the supernatants of the ConA-stimulated co-cultures (data not shown). The addition of a neutralizing antibody against IFN-γ to the co-culture system resulted in a dose-dependent inhibition of LGC formation (Fig. 2A). Furthermore, exogenous addition of rhIFN-γ accelerated LGC formation in a dose-dependent manner (Fig. 2B). These results show that IFN-γ is important in LGC formation in this co-culture system of human monocytes and autologous T cells.

Close contact between monocytes and T cells is required for LGC formation

ConA-stimulated T cells secrete several cytokines and up-regulate the expression of surface molecules that can stimulate nearby cells. Notably, differentiated LGCs were surrounded by CD3+ T cells (Fig. 3A), suggesting that direct contact between monocytes and T cells also contributed to LGC formation. The separation of T cells and monocytes by a semipermeable membrane reduced LGC formation (Fig. 3B).

We next co-cultured monocytes with either CD4+ or CD8+ T cells and assessed whether these two T cell subtypes differed in their ability to induce LGC formation. At every T cell-to-monocyte ratio tested, CD4+ T cells induced LGCs more efficiently than an equivalent number of CD8+ T cells (Fig. 3C). Since IFN-γ was required for LGC formation, the IFN-γ concentration was measured in the supernatants of the various culture conditions. The co-cultures with CD4+ T cells contained more IFN-γ than cultures with CD8+ T cells (Fig. 3D). However, the exogenous addition of rhIFN-γ to co-cultures with CD8+ T cells did not enhance LGC formation to the levels observed with CD4+ T cells (Fig. 3E). These results showed that factors other than IFN-γ, potentially surface molecules preferentially expressed on CD4+ T cells, are required for the efficient formation of LGCs in this system.

The CD40–CD40L axis is necessary for LGC formation

A literature search for differentially expressed surface molecules between CD4+ and CD8+ T cells in the expression profiles of activated human T cells (25) identified tumor necrosis factor super family 5 (TNFSF5 or CD40L) as a candidate molecule affecting LGC formation. The preferential expression of CD40L by CD4+ cells was confirmed in our co-culture system (Fig. 4A). To evaluate whether CD40L was indeed important in LGC formation, antibodies that disrupt the CD40–CD40L interaction were added to the co-culture. Blocking antibodies against CD40 and CD40L inhibited LGC formation in a dose-dependent manner (Fig. 4B). Furthermore, exogenous addition of sCD40L to the co-culture system enhanced LGC formation in a dose-dependent manner (Fig. 4C).

The importance of IFN-γ and CD40-CD40L axis in LGC formation suggested that IL-12 might contribute to LGC formation in this co-culture system since IL-12 is produced by monocytes in response to CD40 ligation and causes T cells to produce IFN-γ (26). The blocking antibody against IL-12 failed to inhibit LGC formation (Fig. 4D), although the exogenous addition of rhIL-12 to the co-culture system enhanced LGC formation (Fig. 4E). CD40 ligation also enhanced LGC formation in the presence of anti-IL-12 antibodies (Fig. 4D). Interruption of the CD40–CD40L interaction as well as the neutralization of IFN-γ abrogated LGC formation even in the presence of exogenous IL-12 (Fig. 4E). These data together indicate that the mechanisms through which CD40/CD40L and IFN-γ enhance LGC formation under the co-culture conditions employed in this study are mostly IL-12 independent, although IL-12 may enhance the LGC formation process when it is present in a high concentration.

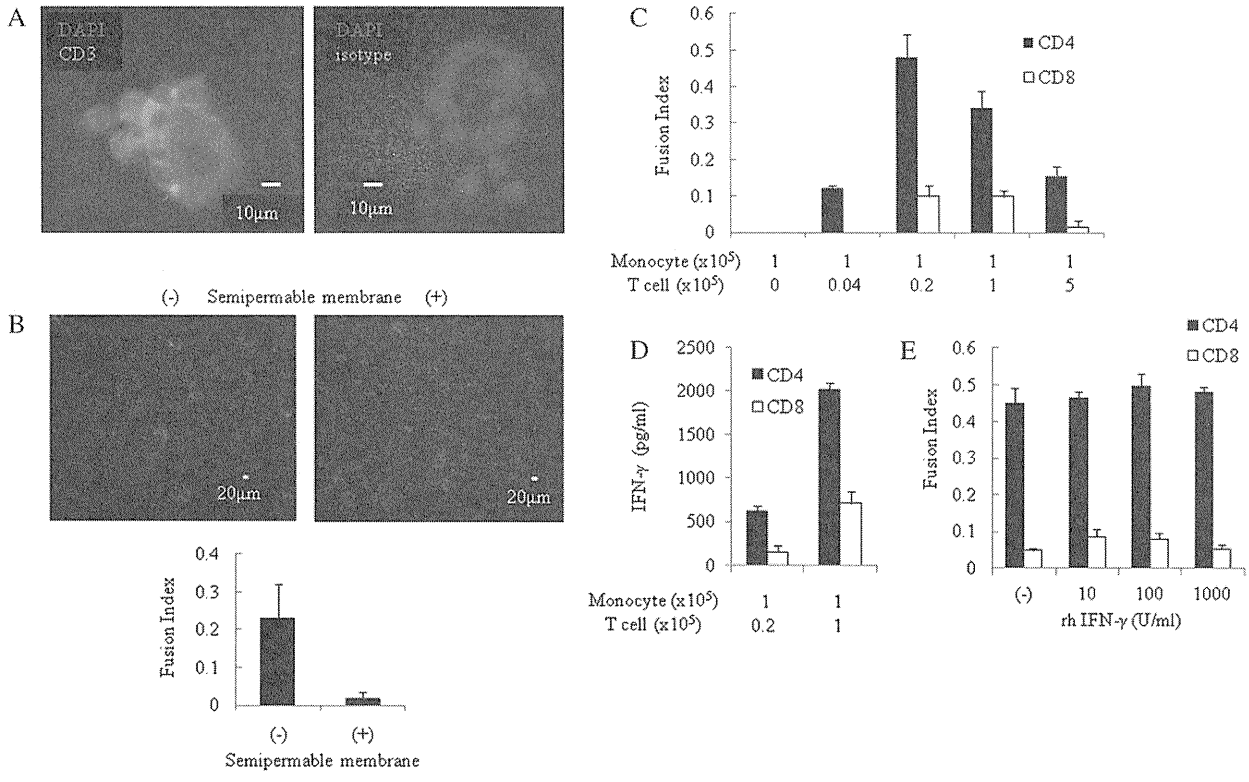

Fig. 3. Direct contact between monocytes and T cells is required for LGC formation. (A) Immunohistochemical staining of LGCs. Co-cultured cells were stained with an antibody against CD3 (green), and nuclei were stained with DAPI (blue). (B) A semipermeable membrane between the monocytes and T cells (1:1 ratio) stimulated with ConA (2.5 μg ml^{-1}) abrogated LGC formation. Nuclei were distributed in a circular pattern in the absence of the semipermeable membrane (upper left), whereas this distribution was disturbed in the presence of the membrane (upper right). The mean fusion index of the cultures are shown, and error bars indicate the standard mean of the error (n = 3 independent co-cultures) (bottom). (C) CD4+ T cells induced LGCs more efficiently than CD8+ T cells. 'Monocyte' refers to the number of monocytes × 10^5, and 'T cell' refers to the number of T cells × 10^5. Values represent the mean fusion index calculated without distinguishing between monocytes and T cells, and error bars indicate the standard mean of the error of the indicated ratio of monocytes to T cells stimulated with ConA (5 μg ml^{-1}) (n = 3 independent co-cultures). (D) CD4+ T cells produced more IFN-γ than CD8+ T cells. The data show the results of triplicate ELISAs for IFN-γ concentration in the supernatants of the experiment shown in Fig. 3(C). Error bars indicate the standard mean of the error of three independent cultures. (E) Addition of rhIFN-γ to the co-culture of monocytes with CD8+ T cells did not enhance LGC formation to the degree observed with CD4+ T cells. The culture condition was the same as Fig. 3(C and D), and the ratio of monocytes to T cells was 1:0.2. Values represent the mean fusion index and error bars indicate the standard mean of the error (n = 3 independent co-cultures).

T cells induce LGC formation by providing IFN-γ and CD40 stimulation

To more specifically evaluate the role of T cells in inducing LGCs, monocytes were cultured with either rhIFN-γ or sCD40L in the presence of ConA without T cells. The addition of rhIFN-γ alone did not induce LGC formation from ConA-stimulated monocytes (Fig. 5A), suggesting that T-cell factors besides or in addition to IFN-γ were required to induce the formation of LGCs. Stimulation with exogenous sCD40L did induce monocytes to differentiate into LGCs (Fig. 5B), indicating the necessity for an interaction between T cell-derived CD40L and monocyte-expressed CD40. Despite the fact that rhIFN-γ alone had no effect on LGC formation from ConA-stimulated monocytes in the absence of T cells, IFN-γ was required for LGC formation when monocytes were co-cultured with autologous T cells under ConA stimulation (Fig. 2A). Furthermore, a blocking antibody against IFN-γ abrogated LGC formation by monocytes cultured with sCD40L and ConA (Fig. 5C), indicating that the LGC formation induced by stimulating cultured monocytes with exogenous sCD40L and ConA required IFN-γ.

Because monocytes do not produce large amount of IFN-γ, it was possible that contaminating T cells were the source of IFN-γ when monocytes were stimulated with exogenous sCD40L and ConA. Indeed, when highly purified CD14+ cells were stimulated with exogenous sCD40L and ConA, significantly less LGC formation was observed than in cultures using crude monocytes (Fig. 5D). In addition, supplementing the culture medium of highly purified monocytes with rhIFN-γ as well as exogenous sCD40L and ConA enhanced LGC formation to the same extent that was observed when crude monocytes were used (Fig. 5D). These results indicate that IFN-γ produced from T cells is required for the efficient induction of LGCs.

The next hypothesis to be evaluated was that the stimulation of highly purified monocytes with rhIFN-γ and sCD40L would be sufficient to induce LGC formation. However, this was not the case; ConA was also required for LGC formation

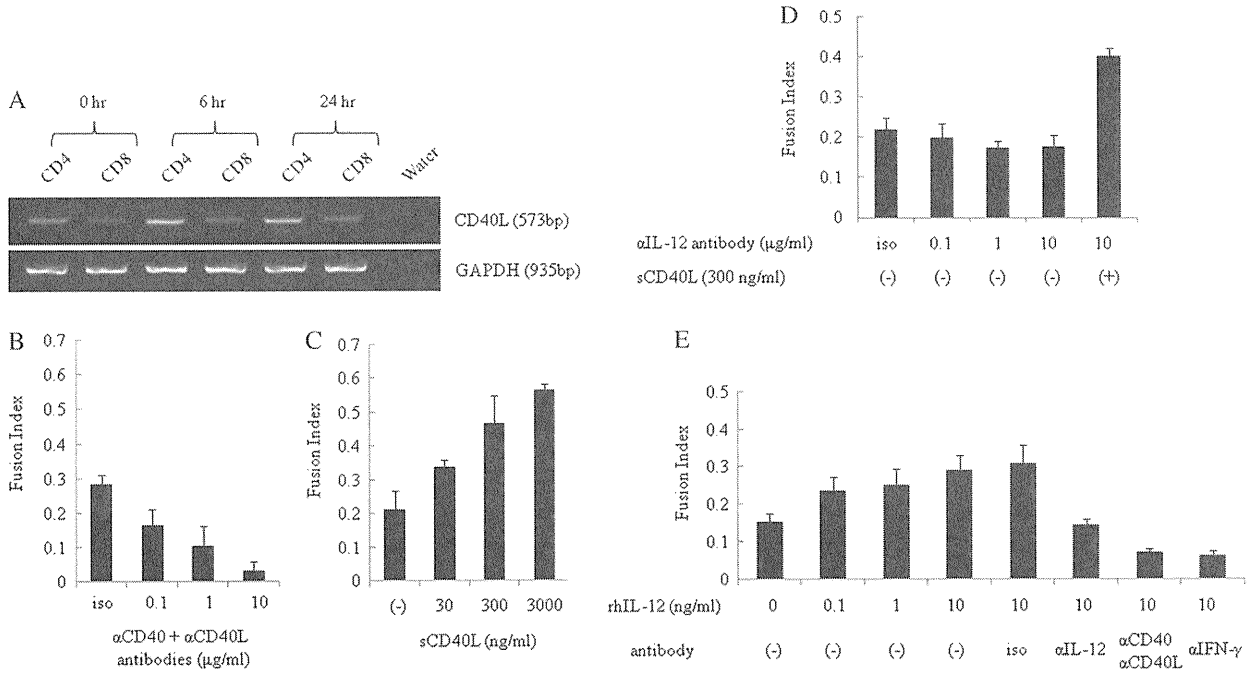

Fig. 4. The CD40-CD40L axis is necessary for LGC formation. (A) CD4+ T cells expressed more CD40L than CD8+ T cells when co-cultured with monocytes stimulated with ConA (5 μg ml^{-1}). RT–PCR analysis for the mRNA expression of CD40L from cells co-cultured for the indicated time periods is shown. (B) 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

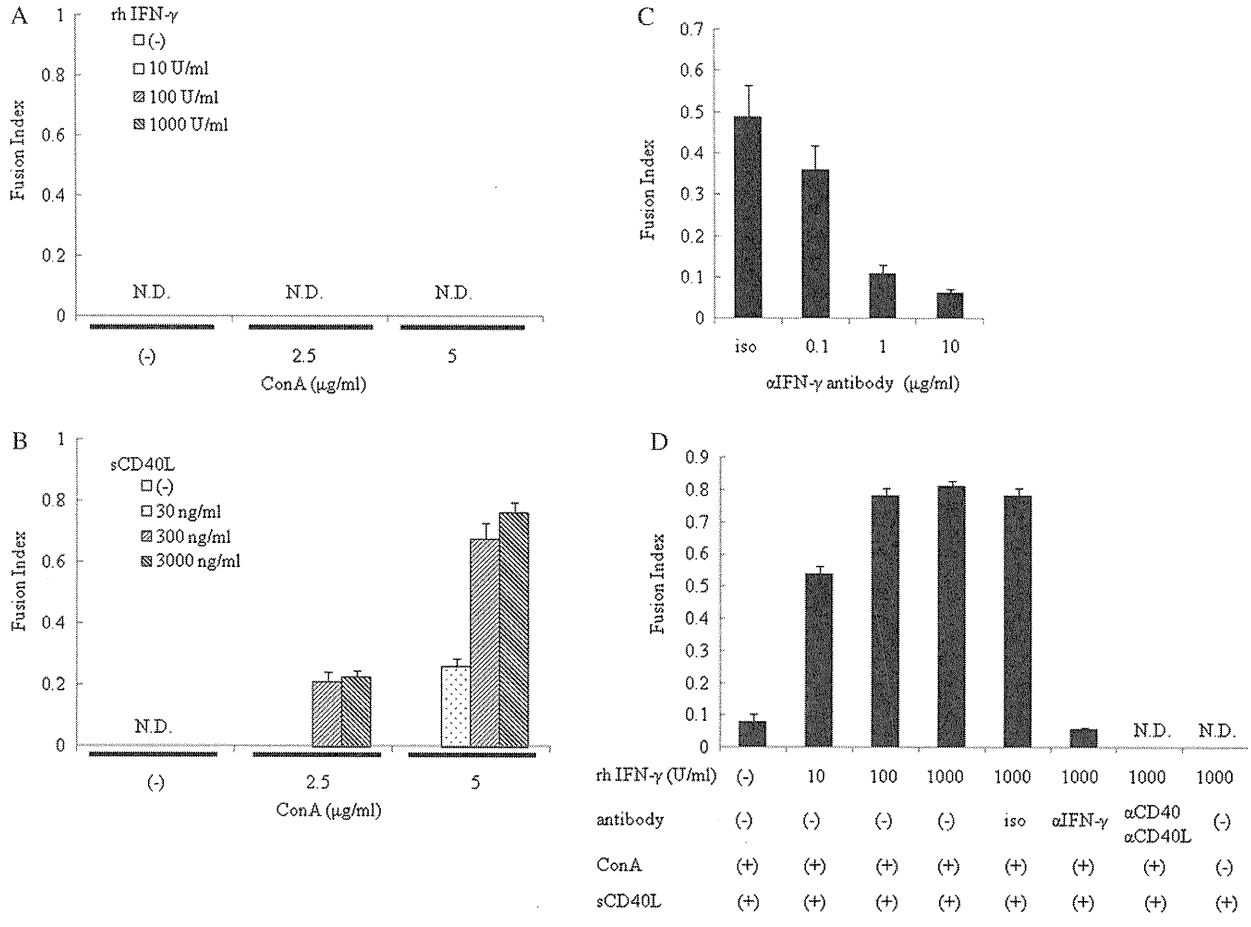

Fig. 5. T cells play a pivotal role in the induction of LGCs by providing IFN-γ and CD40 stimulation. (A) Monocytes stimulated with ConA did not differentiate into LGCs in the presence of exogenous rhIFN-γ. (B) Monocytes stimulated with ConA differentiated into LGCs in the presence of exogenous sCD40L. 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.

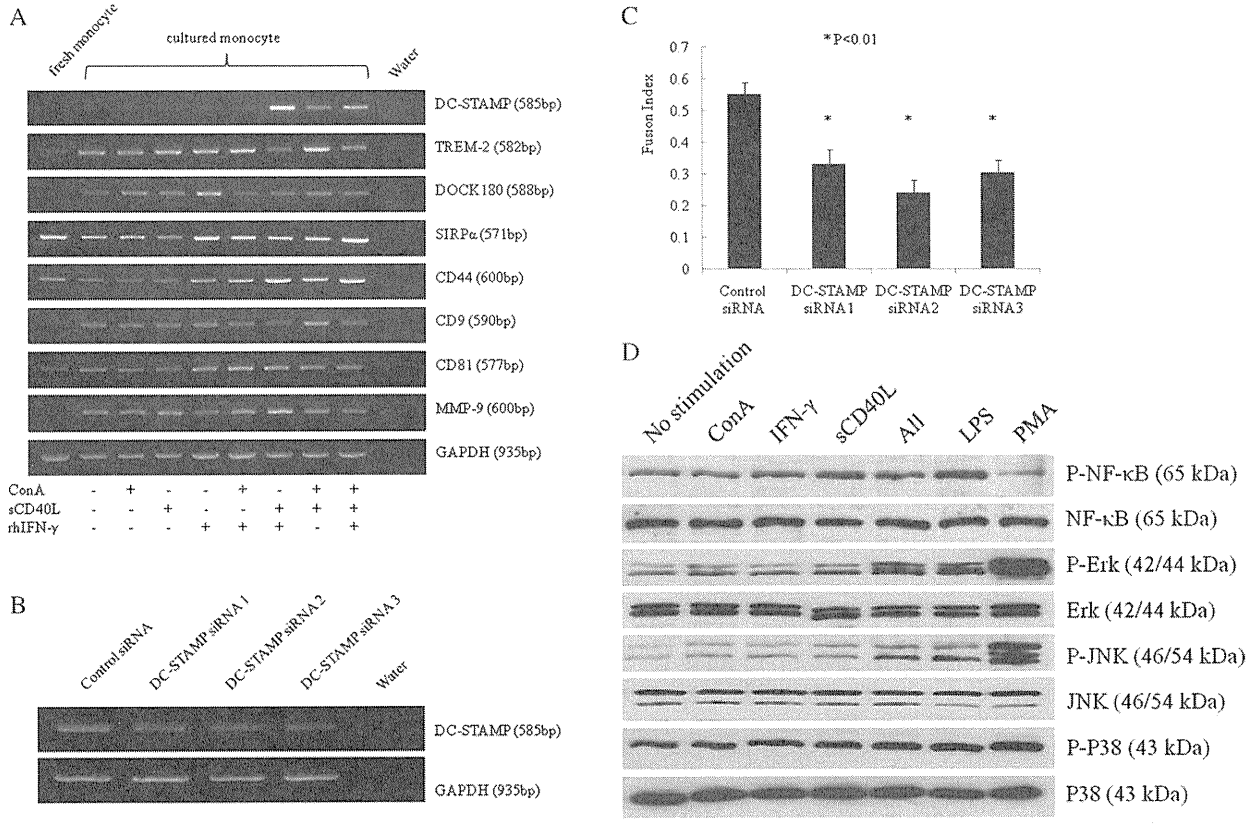

Fig. 6. DC-STAMP is involved in LGC formation. (A) DC-STAMP was up-regulated in LGC formation. RT–PCR analysis of several fusion-related genes in highly purified monocytes stimulated with the indicated reagents (exogenous sCD40L 3 μg ml^{-1}; rhIFN-γ 100 U ml^{-1}; ConA 5 μg ml^{-1}) is shown. (B) and (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,

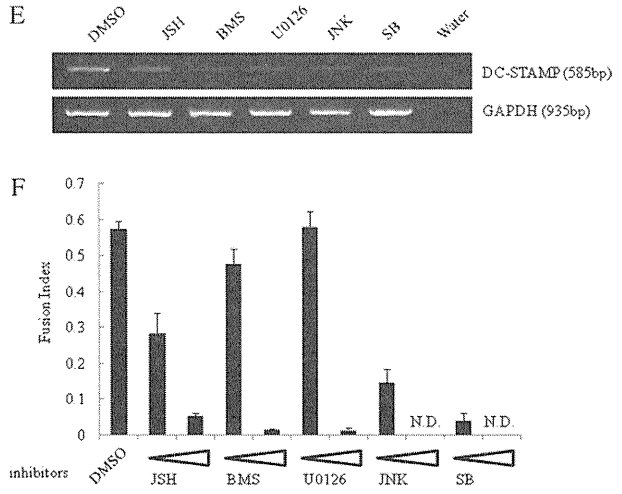

Fig. 6. Continued

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

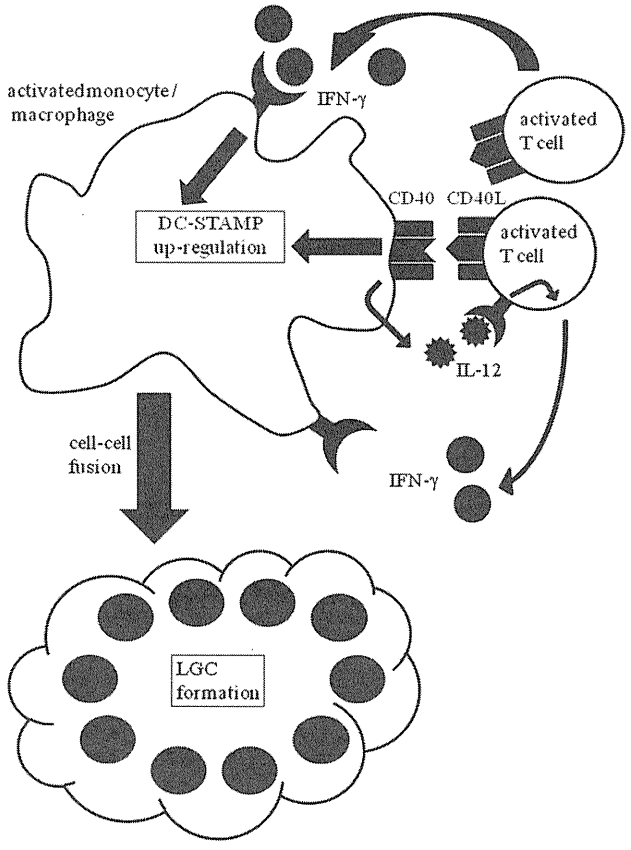

Fig. 7. Schematic presentation of the putative mechanism of human LGC formation. CD40L expressed on activated T cells binds to CD40 on monocytes/macrophages. The activated T cells produce IFN-γ, partially responding to IL-12 production from the activated monocytes/macrophages. Down-stream signaling from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

treated 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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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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