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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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Correspondence: Takahiro Yasumi, Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507 Japan; e-mail: yasumi@kuhp.kyoto-u.ac.jp or Hisanori Horiuchi, Department of Molecular and Cellular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575 Japan; e-mail: horiuchi@idac.tohoku.ac.jp.

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The CD40–CD40L axis and IFN-γ play critical roles in Langhans giant cell formation

Hidemasa Sakai[1], Ikuo Okafuji[1], Ryuta Nishikomori[1], Junya Abe[1], Kazushi Izawa[1], Naotomo Kambe[2], Takahiro Yasumi[1], Tatsutoshi Nakahata[1] and **Toshio Heike[1]**

[1]Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku Kyoto 606-8507, Japan
[2]Department of Dermatology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

Correspondence to: R. Nishikomori; E-mail: rnishiko@kuhp.kyoto-u.ac.jp

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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.

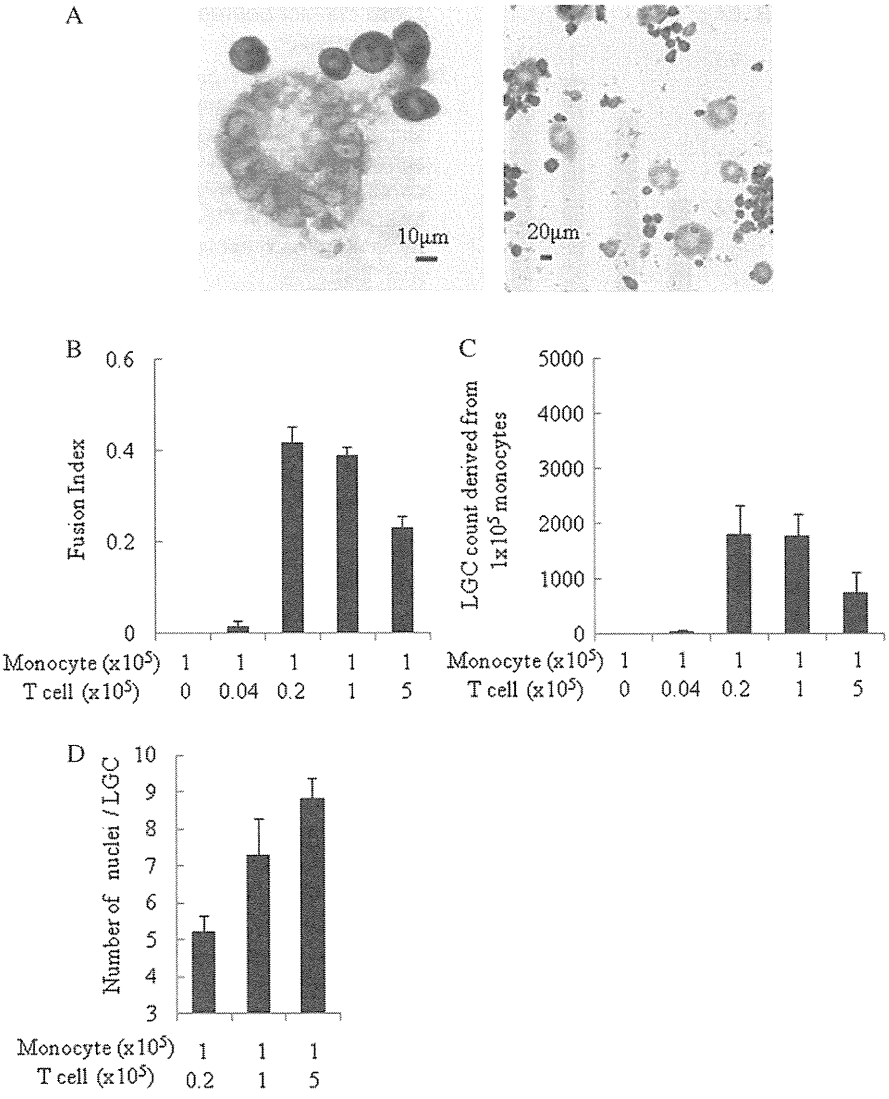

Fig. 1. Co-culture of human monocytes with autologous T cells induces LGC formation. (A) Photomicrographs of Giemsa-stained LGCs induced by co-culture of monocytes and autologous T cells stimulated with ConA. The images show typical LGCs with a circular peripheral arrangement of nuclei. (B–D) T cell-induced LGC formation depends on the ratio of T cells to monocytes. 'Monocyte' refers to the number of monocytes $\times 10^5$ and 'T cell' refers to the number of T cells $\times 10^5$. 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 RNAiMAX (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).

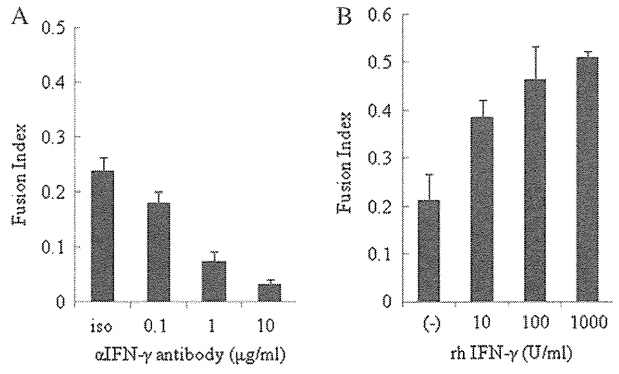

Fig. 2. IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells. (A) An antibody against IFN-γ abrogated LGC formation by the co-culture of monocytes and autologous T cells stimulated with ConA (2.5 μg ml^{-1}). The ratio of monocytes to T cells was 1:1. 'Iso' 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.

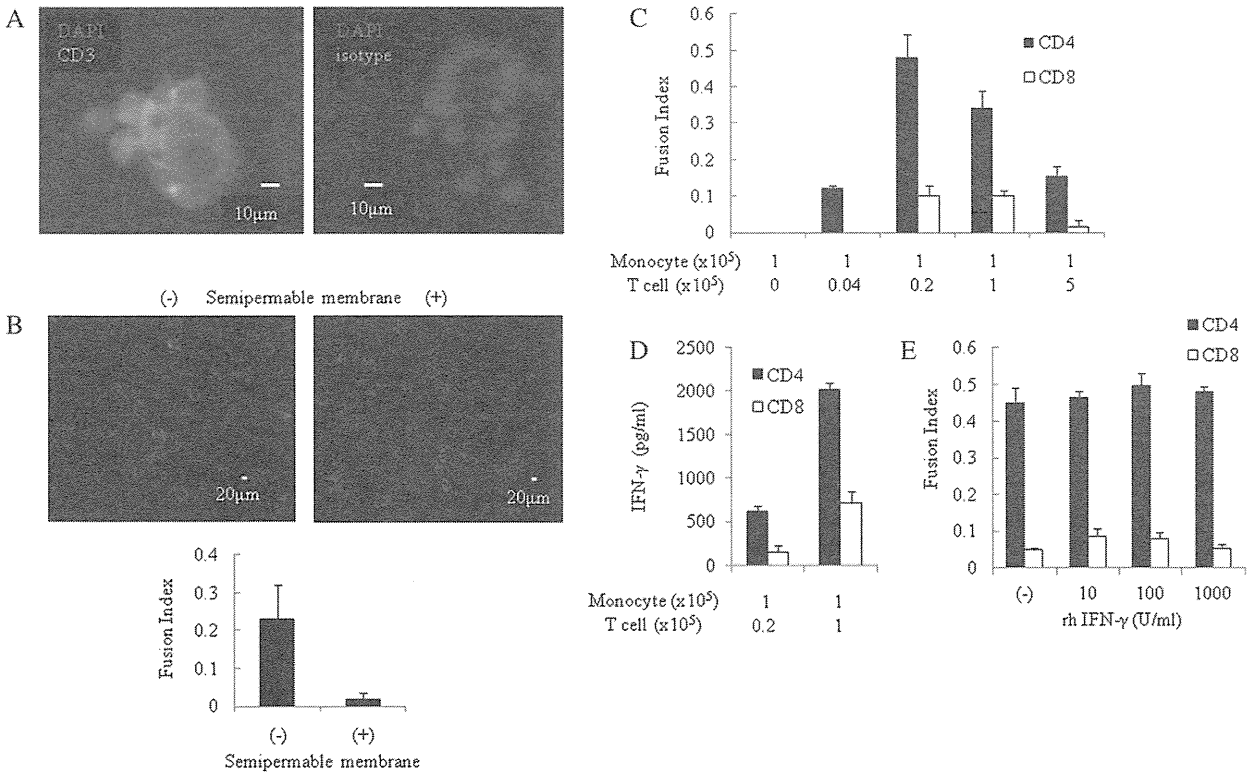

Fig. 3. Direct contact between monocytes and T cells is required for LGC formation. (A) Immunohistochemical staining of LGCs. Co-cultured cells were stained with an antibody against CD3 (green), and nuclei were stained with DAPI (blue). (B) A semipermeable membrane between the monocytes and T cells (1:1 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

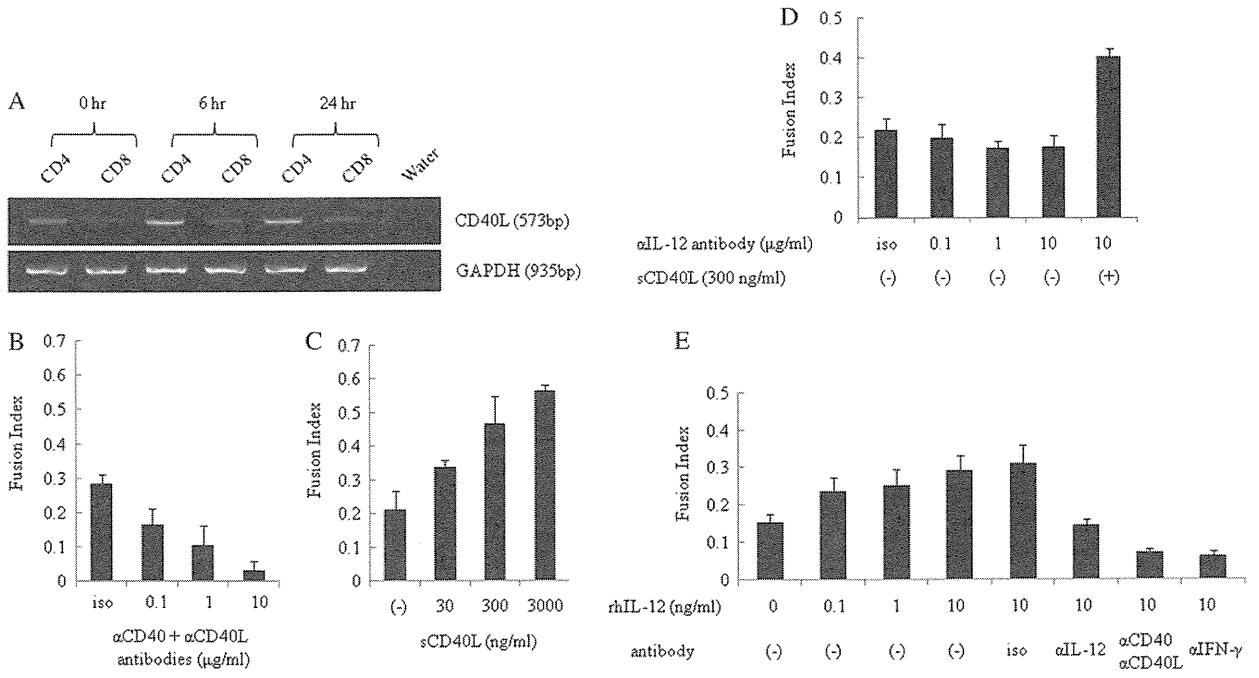

Fig. 4. The CD40-CD40L axis is necessary for LGC formation. (A) CD4+ T cells expressed more CD40L than CD8+ T cells when co-cultured with monocytes stimulated with ConA (5 μg ml^{-1}). RT–PCR analysis for the mRNA expression of CD40L from cells co-cultured for the indicated time periods is shown. (B)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

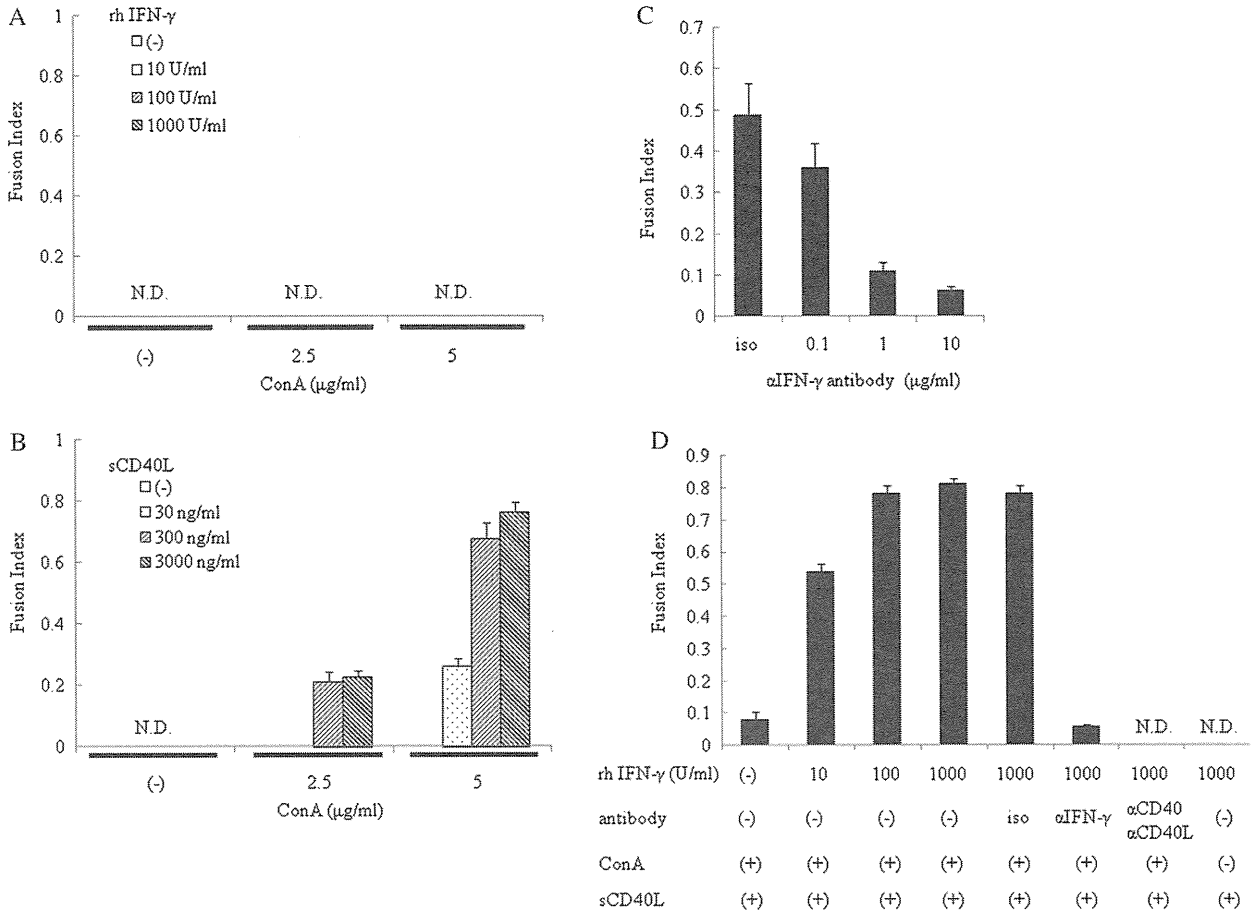

Fig. 5. T cells play a pivotal role in the induction of LGCs by providing IFN-γ and CD40 stimulation. (A) Monocytes stimulated with ConA did not differentiate into LGCs in the presence of exogenous rhIFN-γ. (B) Monocytes stimulated with ConA differentiated into LGCs in the presence of exogenous sCD40L.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

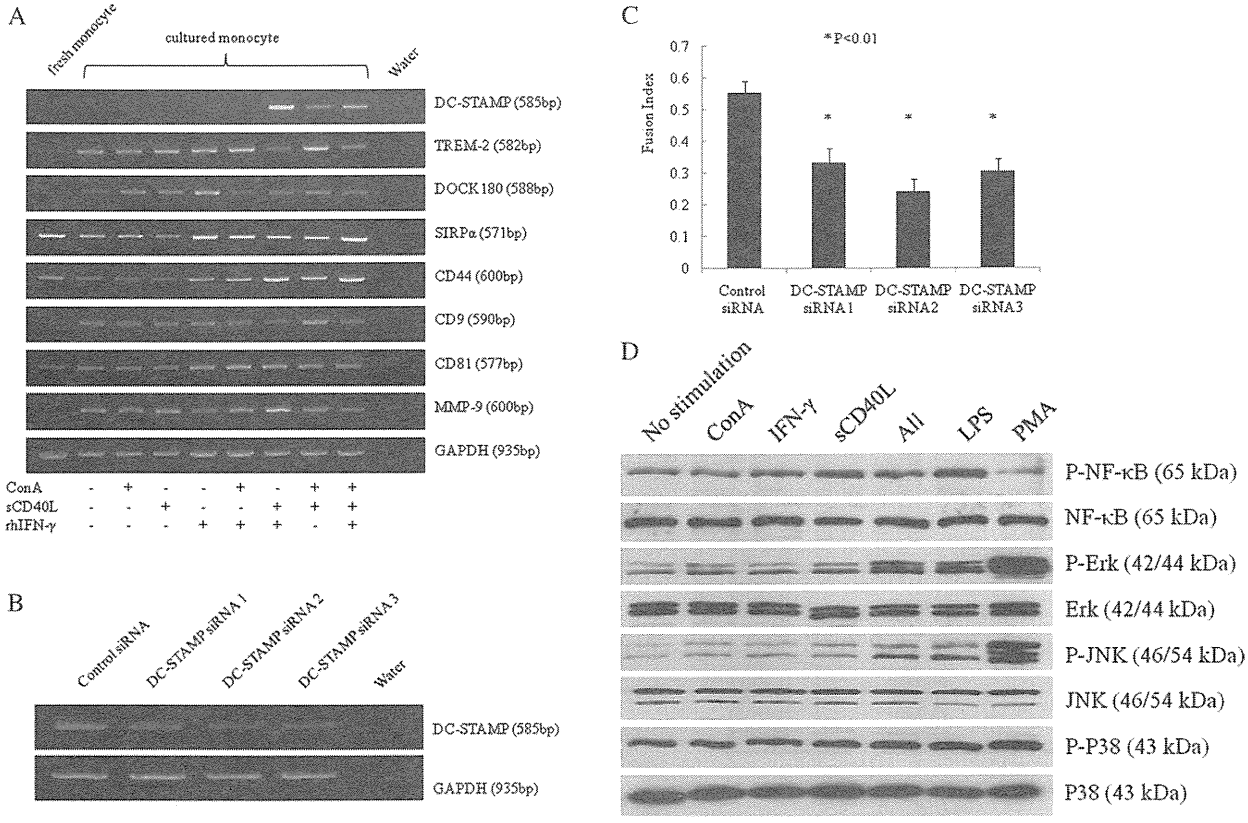

Fig. 6. DC-STAMP is involved in LGC formation. (A) DC-STAMP was up-regulated in LGC formation. RT–PCR analysis of several fusion-related genes in highly purified monocytes stimulated with the indicated reagents (exogenous sCD40L 3 μg ml^{-1}; rhIFN-γ 100 U ml^{-1}; ConA 5 μg ml^{-1}) is shown. (B) and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

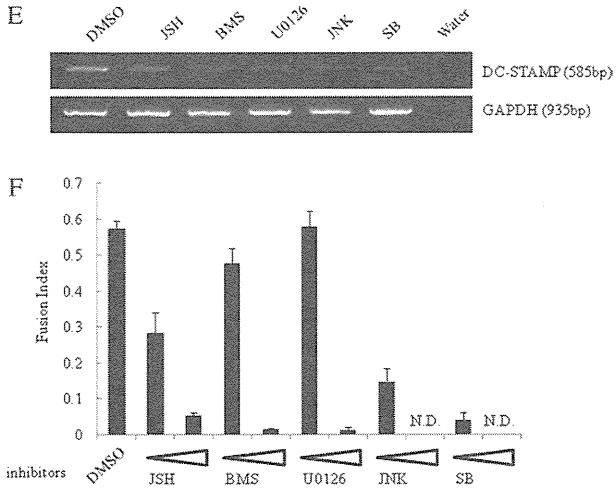

Fig. 6. Continued

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-Dmannopyranoside (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

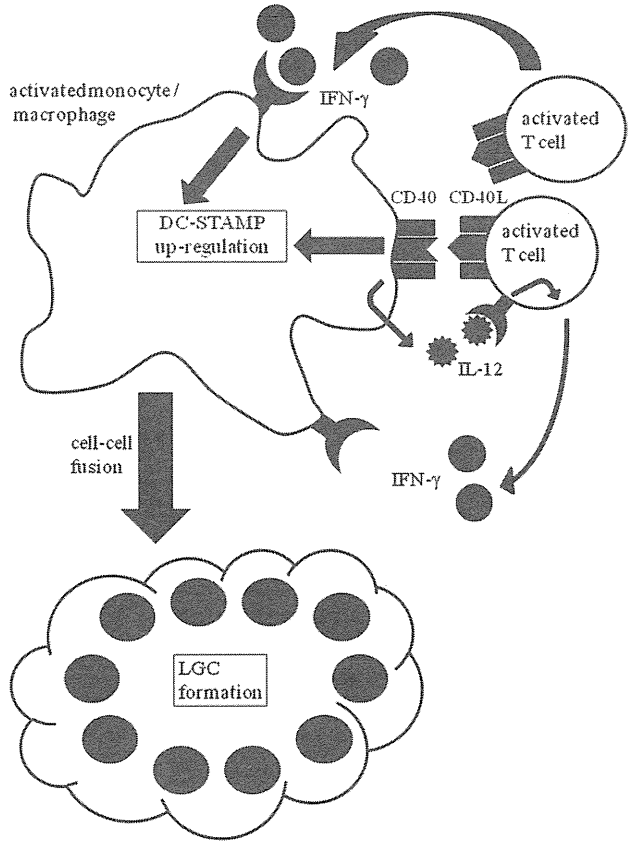

Fig. 7. Schematic presentation of the putative mechanism of human LGC formation. CD40L expressed on activated T cells binds to CD40 on monocytes/macrophages. The activated T cells produce IFN-γ, partially responding to IL-12 production from the activated monocytes/macrophages. Down-stream signaling from 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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Familial Cases of Periodic Fever with Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis Syndrome

Masao Adachi, MD, Aika Watanabe, MD, Atsushi Nishiyama, MD, Yoshinobu Oyazato, MD, Ichiro Kamioka, MD, Masanori Murase, MD, Akihito Ishida, MD, Hidemasa Sakai, MD, Ryuta Nishikomori, MD, and Toshio Heike, MD

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

From the Department of Pediatrics, Kakogawa Municipal Hospital, Kakogawa (M.A., A.W., A.N., Y.O., I.K., M.M., A.I.), and the Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto (H.S., R.N., T.H.), Japan

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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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Reprint requests: Masao Adachi, MD, Department of Pediatrics, Kakogawa Municipal Hospital, 384-1 Hiratsu, Yoneda-cho, Kakogawa, 675-8611, Japan. E-mail: ama-p@rc4.so-net.ne.jp

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ORIGINAL ARTICLE

Novel mutations of MVK gene in Japanese family members affected with hyperimmunoglobulinemia D and periodic fever syndrome

Takahisa Mizuno · Hidemasa Sakai · Ryuta Nishikomori · Koichi Oshima · Osamu Ohara · Ikue Hata · Yosuke Shigematsu · Takashi Ishige · Kazushi Tamura · Hirokazu Arakawa

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Abstract Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS) is a recessively inherited recurrent fever syndrome. We describe a family of eldest son and monozygotic twin younger sisters with characteristic syndrome of HIDS, but normal level of IgD. Mevalonate kinase (MK) activity was deficient in all of them, and analysis of the MVK gene revealed compound heterozygosity for 2 new mutations, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833 T > C, which resulted in exon 4 skipping and p.Val278Ala. This is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. In each case, in which HIDS is clinically suspected, despite normal IgD level, analysis of MK activity and the MVK gene should be performed.

Keywords HIDS · MVK gene · Novel mutation · Compound heterozygous mutation · Splicing mutation · Inherited recurrent fever syndrome

T. Mizuno · T. Ishige · K. Tamura · H. Arakawa (✉)
Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan
e-mail: harakawa@gunma-u.ac.jp

H. Sakai · R. Nishikomori
Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan

K. Oshima · O. Ohara
Laboratory for Immunogenomics, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan

I. Hata
Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

Y. Shigematsu
Department of Health Science, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

Introduction

Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is a rare autosomal recessive auto-inflammatory disorder characterized by recurrent febrile attacks with lymphadenopathy, abdominal distress, skin eruptions, and joint involvement [1–3]. Febrile attacks usually last for 3–7 days and are interrupted by asymptomatic intervals of several weeks' duration [4–6]. Symptoms appear in early infancy and may persist throughout life with gradual increases in serum IgD [7, 8]. The diagnostic hallmark of HIDS is a constitutively elevated level of serum IgD, although parts of the patients have been reported to have normal amount of serum IgD levels.

The HIDS is caused by mutations on mevalonate kinase gene (MVK), which encodes an enzyme involved in cholesterol and non-sterol isoprenoid biosynthesis. We present herein a Japanese family, eldest son and monozygotic twin younger sisters, with HIDS that had compound heterozygous mutations on MVK gene, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. Serum concentrations of IgD were repeatedly within the normal range. These cases demonstrate that detail analysis with more specific diagnostic tests such as urinary excretion of mevalonic acid and MVK genetic analysis should be performed not to miss the correct diagnosis in patients, especially younger children with HIDS.

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Case reports

Patients are the eldest son and monozygotic twin younger sisters of parents of Japanese origin. The eldest son (patient 1) had presented with recurrent fever from 5 months of age. The twin younger sisters (patient 2 and 3) presented with fever from 1 month of age. Vomiting and diarrhea were presented in the younger sister (patient 3). Febrile episodes appeared every 4–8 weeks and lasted for 3–5 days on all the three patients. During febrile episodes, peripheral blood leukocytosis and CRP elevations (more than 10 mg/dl) were observed. In intermittent period between fever episodes, serum CRP levels decreased, but did not always become negative. Their parents had no history of recurrent fever. Sepsis work-up did not show any foci and any pathogens causing the febrile episodes. The repeated bacterial cultures resulted in negative, and administration of the antimicrobial agents did not change the clinical courses of the febrile episodes, indicating that the fever was not induced by pathogen. In addition, immunological analysis such as serum IgA, IgM, IgG, and IgD, lymphocytes counts including T, B, NK cells, and mitogen proliferation assays of peripheral blood mononuclear cells (PBMCs) were normal.

Due to the recurrent high fevers caused most unlikely by pathogen and the heavy family history of the periodic fevers, we suspected hereditary periodic fever syndromes and performed genetic study. After written informed consents approved by institutional review board of the Kyoto University Hospital were obtained, peripheral blood samples were collected from the patients and their parents for isolating genomic DNA and total RNA.

First, we performed genomic DNA sequencing for MEFV gene for familial Mediterranean fever, MVK gene for HIDS, NLRP3 for cryopyrin-associated periodic syndrome, and TNFRSF1A for TNF receptor-associated periodic syndrome. Genomic DNA sequencing analysis of the MVK gene revealed the presence of heterozygous mutations of c.227-1 G > A at the exon/intron border of exon 4 and c.833T > C (p.Val278Ala). Subsequent amplification of the cDNA by RT-PCR showed that the former mutation caused deletion of exon 4 (Fig. 1a). Genomic DNA sequence analysis on their parents revealed that the parents inherited c.227-1 G > A from their father and c.833T > C from their mother, indicating that the three patients were compound heterozygous for MVK gene (Fig. 1b). The patients had markedly elevated excretion of mevalonic acid in urine, especially in febrile periods, and their mevalonate kinase enzyme activities were very low, which confirmed that all the three patients suffered from HIDS (Table 1).

While the patients did not have any mutations on TNFRSF1A and NLRP3, we identified MEFV non-synonymous nucleotide alterations on the elder brother, who was a heterozygote for L110P, E148Q, and R202Q, and the younger twin, who was a heterozygote for R202Q in addition to MVK gene mutations. These MEFV gene nucleotide alterations were regarded as SNPs, and the clinical diagnosis of FMF was not compatible with the patients, although the complex MEFV gene alterations of L110P/E148Q/R202Q have been reported on the clinically-diagnosed FMF patients.

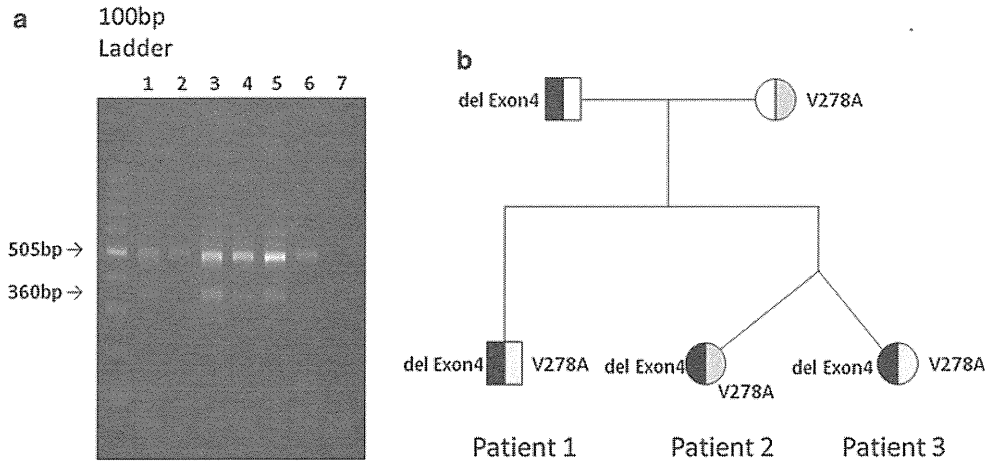

Fig. 1 Molecular genetic findings in the study patients. **a** Agarose gel electrophoresis of RT-PCR products for exon 2 to exon 5 of MVK shows the normal 505-bp alleles in samples from normal healthy control (*lane 6*) and mother (*lane 2*), as well as both the normal allele and the mutant 362-bp allele in the sample from father (*lane 1*), patient 1 (*lane 3*), patient 2 (*lane 4*), and patient 5 (*lane 5*). Subsequent cDNA sequencing confirmed that this 144-bp deletion in cDNA corresponds to codon 303–407 (exon 4). The molecular size marker was a 100-bp ladder. *Lane 7* represents PCR with distilled water added but not with DNA, indicating that there was no background amplification. **b** Pedigree of the affected family. The three patients are heterozygous for del exon 4 and V278A

Table 1 Urinary mevalonic acid and mevalonate kinase levels in the study patients

Patient no.	Mevalonic acid in urine (μg/mgCr)		Mevalonate kinase (pmol/minute/mg)
	Febrile period	Intermittent period	
1	67.9	11.3	3
2	55.6	17.7	2
3	58.8	18.5	2
Control		0.078 ± 0.012[a]	214 ± 62[a]

Control data are given as mean ± SD

[a] Values form healthy subjects were used to obtain a control range for urinary mevalonic acid levels (mean ± SD) and mevalonate kinase levels (mean ± SD)

Discussion

We present herein a sibling of HIDS that demonstrated compound heterozygous for two novel mutations of MVK gene. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833T > C, which resulted in exon 4 skipping and p.Val278Ala. The mutations are novel, especially the splicing mutation of MVK gene was identified at the genomic DNA level.

Cuisset et al. [9] reported that HIDS mutations were evenly distributed along the coding region of the MVK gene, in contrast to mutations causing MA, which clustered between 243 and 334. The sequence variations seen in MA are missense mutations that are in the same region as the variants described in HIDS. Further studies will be needed to clarify the association of phenotypical differences with MVK gene mutations. Over 80% of patients with HIDS were reported to have compound heterozygous mutation in the MVK gene. To our knowledge, both the skipping of exon 4 and V278A mutation have not been reported previously in HIDS. Moreover, this is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. Only few groups reported HIDS patients with the skipping of exon in the cDNA of the MVK gene [10, 11]. They suggested that these exon skipping was probably due to the presence of a potential splice site mutation, but could not identify mutations responsible for these altered splicing through the sequence analysis at the genomic level. Most MVK mutations in patients with HIDS and MA have only been determined at the cDNA level; however, analysis of cDNA sometimes appeared troublesome, probably due to instability of the MVK mRNA. More detailed studies through the sequence analysis at the genomic level lead us to elucidate the role of MVK mutations in HIDS and MA, and expression studies in *E. coli* will be necessary to evaluate the effect of each mutation.

HIDS is classically defined as a high concentration of mevalonic acid in the urine and is characterized by a high serum IgD concentration during each febrile episode, but some reports from the Netherlands stated that high levels of serum IgD levels were not seen and affirmed that other diseases also showed high serum IgD levels [12]. In our cases, the analysis of enzymes and molecular genetics of MVK gene yielded the correct diagnosis, although serum concentrations of IgD were within the normal range. Thus, it should be now common practice to examine the MVK gene in order to diagnose this disease.

In conclusion, we present a Japanese family with HIDS that appeared to have novel mutations of MVK gene. Most of the HIDS cases were reported from European, especially Dutch, whereas only one HIDS case of Japanese patient was reported by Naruto et al. [13], which is only one report of Asian patient. Cases of HIDS may so far have been overlooked or misdiagnosed as infectious diseases or autoimmune disorders in Japan, besides there may be difference in race. It is necessary that accumulation of case in hereditary mutation and in other race leads to solve a detailed cause of HIDS.

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Conflict of interest There is no financial or other potential conflict of interest for each author.

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