

Letter to the Editor

Functional evaluation of the pathological significance of *MEFV* variants using induced pluripotent stem cell-derived macrophages

To the Editor:

Familial Mediterranean fever (FMF) is the most common hereditary autoinflammatory disorder. It is characterized by recurrent episodes of fever, polyserositis, and abdominal pain. FMF is associated with mutations in the *MEFV* gene, which encodes the inflammasome adaptor pyrin. Pyrin is an inflammasome sensor that detects imbalances in Rho GTPase activity, which can be caused by bacterial effectors or bacterial toxins. Clostridial toxins are glycosylating enzymes that inactivate the RhoA GTPase, which results in activation of the pyrin inflammasome, activation of caspase-1, release of the proinflammatory cytokines IL-1 β and IL-18, and an inflammatory cell death termed pyroptosis.

More than 340 *MEFV* variants have been recorded in *Infevers*, an online database of autoinflammatory mutations. Among the *MEFV* variants, M694V and M694I in exon 10 were demonstrated in a systematic review to be related to a severe disease phenotype.¹ Other *MEFV* variants are associated with variable disease severity. The complexity of the clinical phenotype and its association with *MEFV* variants leads to difficulty in assessing the pathogenicity of variants identified in clinical settings.

Successful use of IL-1 β -blocking therapies in the treatment of FMF indicates that IL-1 β plays a critical role in the pathogenesis of FMF.² Treatment with colchicine, a microtubule polymerization inhibitor, should be started as soon as a clinical diagnosis is made. However, *in vitro* pyrin inflammasome activation and its inhibition by colchicine in patients' hematopoietic cells remain controversial.³⁻⁶ In contrast to a previous report showing hypersecretion of IL-1 β from blood cells derived from patients with FMF,³ Van Gorp et al⁴ found that hypersecretion of IL-1 β did not occur in monocytes from patients with FMF.

To clarify this issue, we evaluated cytokine secretion from monocytes and monocyte-derived macrophages obtained from 5 patients with FMF carrying the M694I mutation (see Table E1 in this article's Online Repository at www.jacionline.org). The monocytes were stimulated with *Clostridium difficile* toxin A (TcdA) without priming with LPS because TcdA is sufficient to activate the pyrin inflammasome in monocytes⁴ and LPS induces IL-1 β secretion through the NLRP3 inflammasome,⁷ complicating the evaluation of pyrin inflammasome activation. In response to TcdA stimulation, monocytes from patients with FMF secreted similar levels of IL-1 β as monocytes from healthy donors (HDs) and asymptomatic carriers (ACs) expressing the E148Q variant (Fig 1, A). Colchicine inhibited IL-1 β secretion from monocytes from HDs and ACs but not those from patients with FMF. We then examined inflammasome activation by evaluating apoptosis-associated speck-like protein containing a CARD (ASC) speck formation. Consistent with the IL-1 β secretion, colchicine abolished TcdA-induced ASC speck assembly in monocytes from HDs and ACs but not those from patients with FMF (Fig 1, B, and see Fig E1 in this article's Online Repository at www.jacionline.org).

Next, the IL-1 β secretion from macrophages derived from monocytes *in vitro* (peripheral blood-derived macrophages [PB-MPs]) was evaluated. Because TcdA stimulation alone did not induce IL-1 β secretion from PB-MPs (data not shown), PB-MPs were primed with LPS before TcdA stimulation. PB-MPs from patients with FMF secreted significantly greater levels of IL-1 β than PB-MPs from HDs or ACs in response to stimulation with LPS and TcdA (Fig 1, C). The IL-1 β secretion from PB-MPs from patients with FMF was inhibited by colchicine. These results suggest that although monocytes from patients with FMF are distinct in their unresponsiveness to colchicine inhibition, PB-MPs from patients with FMF show hyperactivation of the pyrin inflammasome, which is sensitive to colchicine inhibition. Thus, by examining both cell types from the same patients, we revealed the overall picture of the cytokine responses of monocytes and macrophages from patients with FMF.

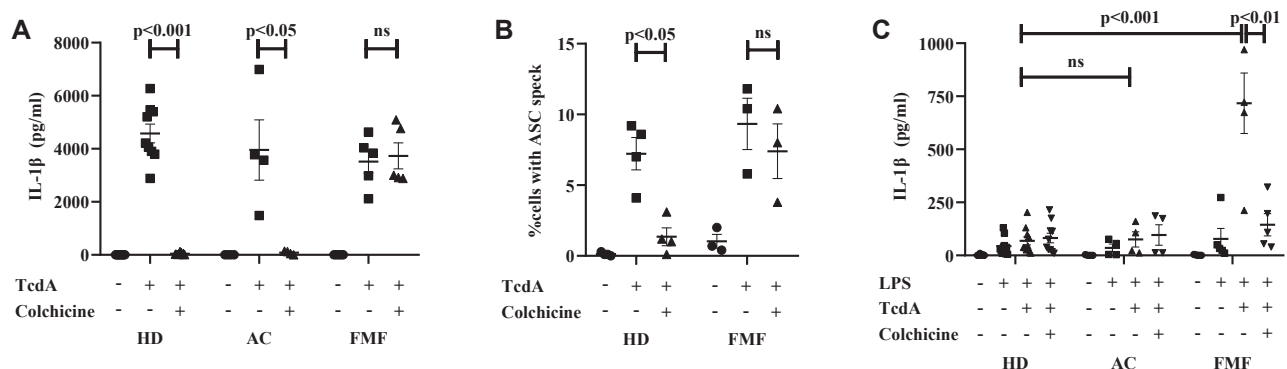


FIG 1. Monocytes and macrophages derived from patients with FMF display distinct pyrin inflammasome activation. **A**, IL-1 β production by primary monocytes from HDs (n = 9), ACs with the E148Q variant (n = 4), or patients with the M694I variant (n = 5) in response to stimulation with TcdA in the absence (squares) or presence (triangles) of colchicine. **B**, ASC speck assembly in primary monocytes. **C**, IL-1 β production levels by monocyte-derived macrophages that were primed with LPS and stimulated with TcdA in the absence (triangles) or presence (inverted triangles) of colchicine. ns, Nonsignificant.

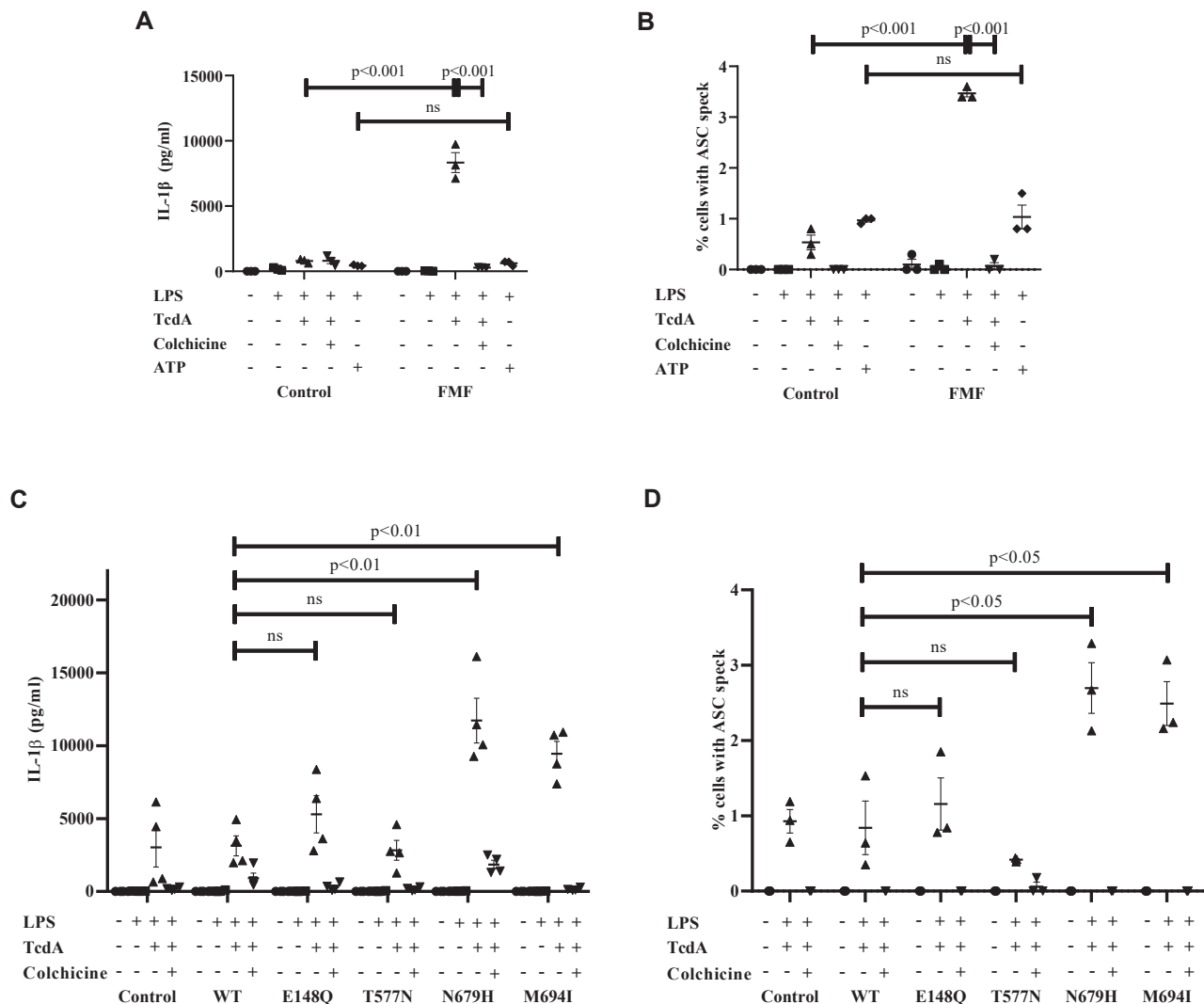


FIG 2. Evaluating the pathogenicity of rare *MEFV* variants using the iPSC model. **A**, IL-1 β production from iPSC-MPs derived from control subjects ($n = 3$) or patients with FMF ($n = 3$) that were primed with LPS and stimulated with TcdA in the absence (*triangles*) or presence (*inverted triangles*) of colchicine or stimulated with ATP (*diamonds*). **B**, ASC speck assembly in iPSC-MPs. iPSC-MPs primed with LPS were stimulated with TcdA in the absence (*triangles*) or presence (*inverted triangles*) of colchicine or stimulated with ATP (*diamonds*). **C**, IL-1 β production from control or transgene-expressing iPSC-MPs that were primed with LPS and stimulated with TcdA in the absence (*triangles*) or presence (*inverted triangles*) of colchicine. Data are from 4 independent experiments. **D**, ASC speck assembly in control or transgene-expressing iPSC-MPs. Data are from 3 independent experiments. *ns*, Nonsignificant.

Induced pluripotent stem cell (iPSC) technology provides the opportunity to analyze the effect of genetic variants free from the influence of medication or differences in genetic background. Therefore we evaluated whether macrophages derived from patients' iPSCs (iPSC-derived macrophages [iPS-MPs]) recapitulated the phenotype of PB-MPs from patients with FMF. iPSC lines from 3 patients with FMF with the M694I and E148Q *MEFV* variants were established (patients 6-8; see Fig E2 and Table E1 in this article's Online Repository at www.jacionline.org) and differentiated into iPS-MPs (see Fig E3 in this article's Online Repository at www.jacionline.org). iPS-MPs with the M694I mutation recapitulated the enhanced pyrin inflammasome activation of PB-MPs, leading to increased IL-1 β secretion (Fig 2, A), ASC speck formation (Fig 2, B), and cell death, which was dependent on *MEFV* expression (see Fig E4 in this article's Online

Repository at www.jacionline.org). By contrast, the *MEFV* mutation did not affect NLRP3 inflammasome activation induced by LPS plus ATP stimulation.

Although epidemiologic studies suggest that patients with FMF carrying *MEFV* exon 10 mutations, such as M694I, have a relatively early-onset severe clinical course,¹ the significance of other *MEFV* variants remains unknown. Therefore we applied our newly established method to 2 additional *MEFV* variants, T577N and N679H, which were identified in 2 families in which autoinflammatory disease with dominant inheritance was suspected. T577N was reported as a disease-causing variant in patients with attacks of fever and chest pain lasting longer than 1 week.⁸ By contrast, the patient carrying the N679H variant experienced typical FMF attacks.⁹ Consistent with the reported phenotype, no patient with the T577N variant in family 1 met

the Tel-Hashomer criteria,^{E1} whereas both patients with the N679H variant in family 2 met the criteria (see Fig E5 and Table E1 in this article's Online Repository at www.jacionline.org). Neither of the variants was registered in the Human Genetic Variation Database or the Exome Aggregation Consortium database, and both of the subsequent amino acid alterations were suggested to be disease causing in at least 1 of the 4 function-prediction programs used (see Table E2 in this article's Online Repository at www.jacionline.org).

iPSC lines stably expressing wild-type (WT) *MEFV* or the E148Q, T577N, N679H, and M694I variants were generated (see Fig E5). iPS-MPs were derived from the genetically engineered iPSCs to evaluate pyrin inflammasome activation. Total and transgenic *MEFV* expression in iPS-MPs were comparable among the clones (see Fig E5). M694I iPS-MPs secreted significantly more IL-1 β than E148Q or WT iPS-MPs (Fig 2, C). Although T577N iPS-MPs secreted comparable amounts of IL-1 β to WT iPS-MPs, N679H iPS-MPs secreted significantly more IL-1 β than WT iPS-MPs (Fig 2, C). ASC speck assembly was also enhanced in N679H and M694I iPS-MPs (Fig 2, D). These data indicate that, like the M694I variant, the N679H variant causes hyperactivation of the pyrin inflammasome.

In summary, we characterized primary monocytes and macrophages from typical patients with FMF. The mechanism underlying the distinctive pyrin inflammasome activation in these cells remains to be elucidated. Gene expression differs considerably between monocytes and macrophages, including expression of the tubulin-related genes.^{E2} The greater expression of tubulin-related genes in macrophages might be related to the observation that pyrin inflammasome activation could be inhibited by the tubulin inhibitor colchicine in these cells. In addition, monocytes and macrophages are somewhat different in terms of inflammasome activation pathways. For example, although both cell types use the canonical NLRP3 inflammasome activation pathway, the alternative⁷ or noncanonical^{E3} inflammasome activation pathway is functional only in monocytes. It is possible that an undiscovered pyrin inflammasome activation pathway is functioning in either monocytes or macrophages but not in both.

After characterizing primary monocytes and macrophages from typical patients with FMF, we introduced a new method to functionally evaluate the pathological significance of *MEFV* variants by using iPS-MPs. Evaluation of diverse *MEFV* variants will be possible through this functional analysis and will facilitate our understanding of FMF pathogenesis.

Macrophage dysfunction is observed in patients with other immune disorders. For example, macrophages are hyperactive in other autoinflammatory diseases, such as NLRC4-associated autoinflammatory diseases, whereas macrophage function is impaired in some immunodeficiencies, such as chronic granulomatous diseases. The current approach of iPSC genetic modification and efficient production of macrophages using iPSC-derived myeloid lineage cells (iPS-MLs) could be used to elucidate the pathogenesis of macrophage-related immune disorders other than FMF.

We thank all participating patients, their families, and the referring physicians for their generous cooperation. Informed consent for genetic and functional analysis of blood samples was obtained from each patient or their guardians in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Kyoto University.

Takeshi Shiba, MD^{a,*}
Takayuki Tanaka, MD, PhD^{a,*}
Hiroaki Ida, MD, PhD^b
Misa Watanabe, MD, PhD^c
Haruna Nakaseko, MD^d
Mitsujiro Osawa, PhD^e
Hirofumi Shibata, MD^a
Kazushi Izawa, MD, PhD^a
Takahiro Yasumi, MD, PhD^a
Yuri Kawasaki, DVM, PhD^e
Megumu K. Saito, MD, PhD^e
Junko Takita, MD, PhD^a
Toshio Heike, MD, PhD^{a,f}
Ryuta Nishikomori, MD, PhD^a

From ^athe Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan; ^bthe Department of Respiratory, Neurology, and Rheumatology, Kurume University School of Medicine, Fukuoka, Japan; ^cthe Department of Pediatrics, Toho University School of Medicine, Tokyo, Japan; ^dthe Department of Infection and Immunology, Aichi Children's Health and Medical Center, Aichi, Japan; ^ethe Department for Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan; and ^fthe Department of Pediatrics, Hyogo Prefectural Amagasaki General Medical Center, Hyogo, Japan. E-mail: tatanaka@kuhp.kyoto-u.ac.jp.

*These authors contributed equally to this work.

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METHODS

Patients and healthy control subjects

Eight patients with FMF with the M694I mutation, 4 ACs with the E148Q variant, 9 HDs with no *MEFV* mutation, and 2 families with rare *MEFV* variants (T577N and N679H) were enrolled in this study (Table E1). All the participants were Japanese. All 8 patients with FMF with the M694I mutation fulfilled the Tel-Hashomer criteria.^{E4} PBMCs obtained from 5 patients with FMF (patients 1-5) were used for *in vitro* cytokine secretion assays, and PBMCs from the 3 other patients (patients 6-8) were used for establishing iPSCs.

Two *MEFV* variants, T577N and N679H, were identified in families in which autoinflammatory disease with dominant inheritance was suspected through analysis of autoinflammatory disease-related genes (Table E3).

The clinical course of family 1 is described elsewhere.^{E1} Briefly, three patients experienced recurrent chest pain that lasted 3 to 6 months and was occasionally accompanied by a low-grade fever. Among the 3 symptomatic patients (I.1, II.1, and III.2), 1 (III.2) received colchicine treatment and had a favorable response. Two patients (II.2 and III.1) in family 2 experienced recurrent fever, unilateral pleuritis, and generalized peritonitis and responded well to colchicine treatment. Although no patient in family 1 met the Tel-Hashomer criteria, both patients in family 2 met the criteria (Table E1).

Cell culture

PBMCs were isolated with Lymphoprep (Alere Technologies, Waltham, Mass). CD14⁺ monocytes were sorted magnetically from PBMCs by using the AutoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Monocytes were cultured in RPMI-1640 (Sigma-Aldrich, St Louis, Mo) supplemented with 10% FBS (Gibco, Carlsbad, Calif) and 50 ng/mL macrophage-colony stimulating factor (R&D Systems, Minneapolis, Minn) for 7 days to obtain monocyte-derived macrophages.

Generation of iPSCs from patients with FMF with the M694I and E148Q variants (patients 6-8) was performed, as described previously.^{E5} Control iPSC lines were provided by the RIKEN BioResearch Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

All iPSCs from patients with FMF carried the M694I and E148Q variants (Fig E2). After confirming the quality of the iPSCs, including marker gene expression, absence of residual vector expression, and pluripotency (Fig E2), iPS-ML lines were established by expressing *MDM2*, *BM11*, and *cMYC* in monocytic lineage cells obtained from iPSCs^{E6} and then differentiated into macrophages (iPS-MPs). The mutant iPS-MLs and iPS-MPs showed comparable morphology and surface marker expression as control counterparts (Fig E3).

piggyBac plasmids encoding WT or missense *MEFV* cDNA together with a blasticidin resistance gene (5 μ g) and a plasmid encoding transposase (5 μ g) were introduced into 1×10^6 control iPSCs with a NEPA21 electroporator (Nepa Gene, Ichikawa, Japan) to generate *MEFV*-reconstituted iPSCs. *PiggyBac* plasmids with missense *MEFV* variants were generated by using PCR-based mutagenesis with the primers listed in Table E4. Three days later, blasticidin (5 μ g/mL; Wako Chemicals, Richmond, Va) was added to the medium for selection of *MEFV*-reconstituted cells.

Quality control of iPSCs

DNA and RNA extraction and cDNA synthesis. Genomic DNA and total RNA were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was diluted to 25 ng/mL in distilled water. cDNA was synthesized with PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) from 500 ng of total RNA and diluted 1:10 in RNase-free water for analysis of *OCT3/4* and *NANOG* mRNA expression.

***OCT3/4* and *NANOG* mRNA expression analysis.** Expression of the pluripotent stem cell markers OCT3/4 and NANOG was confirmed by using TaqMan quantitative RT-PCR with the StepOnePlus Real-Time PCR

System (Applied Biosystems/Thermo Fisher Scientific, Waltham, Mass). Primer and probe sequences are provided in Table E4. Expression of target genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in the same samples and expressed relative to expression in the 201B7 iPS cell line.

Residual plasmid analysis. Residual levels of the plasmids used for iPSC establishment were analyzed by using TaqMan quantitative PCR with the StepOnePlus Real-Time PCR System. Primer and probe sequences for CAG and *EBNA1* were designed on the CAG promoter region and the *EBNA1* coding region of all episomal vectors for iPSC generation, and they are listed in Table E4. Residual plasmid numbers were determined against a standard curve generated with known quantities of pCE-OCT3/4 episomal plasmid by using 50 ng of genomic DNA from iPSCs from patients with FMF at passage 3.

Differentiation of human pluripotent stem cells into the ectoderm, mesoderm, and endoderm. Human pluripotent stem cells were differentiated into ectoderm, mesoderm, and endoderm lineages by using the STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada). Human pluripotent stem cells reaching 70% to 80% confluency were harvested with TrypLE Select Enzyme (Thermo Fisher Scientific) and plated as a single-cell suspension in mTeSR1 medium (STEMCELL Technologies) containing 10 μ Mol/L Y-27632 (Wako Chemicals) on 6-well plates coated with Matrigel (BD Biosciences, San Jose, Calif). The cells were plated at 3.0×10^5 cells/well for differentiation into the ectoderm or mesoderm and 4.0×10^5 cells/well for differentiation into endoderm and harvested at day 7 (ectoderm) and day 5 (mesoderm and endoderm).

Flow cytometric analysis of differentiation. Undifferentiated pluripotent stem cells and human pluripotent stem cell-derived ectoderm, mesoderm, and endoderm (1.0×10^6 cells each) were fixed with 4% paraformaldehyde in PBS (4% paraformaldehyde/PBS) for 20 minutes at 4°C and washed twice with staining medium that contained PBS with 2% FBS. Samples were permeabilized with BD Perm/Wash buffer (BD Biosciences) for 15 minutes at room temperature and stained for 1 hour with the fluorescently conjugated primary antibodies listed in Table E5. Samples were washed with BD Perm/Wash buffer twice and suspended in staining medium. Cells were acquired on an LSR flow cytometer (BD Biosciences). Data were analyzed and graphs were generated by using FlowJo software (FlowJo, Ashland, Ore).

MEFV mRNA silencing

On day 3 of differentiation from iPS-MLs to iPS-MPs, 3.3×10^5 cells were transfected with 60 ng of Silencer Select predesigned small interfering RNA (siRNA; siRNA ID: s502555/s502557; Thermo Fisher Scientific) or Silencer Select Negative Control No. 1 siRNA using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific), according to the manufacturers' instructions.

In vitro activation of monocytes and macrophages

Monocytes were seeded in flat-bottom 96-well plates (Falcon) at 5×10^4 cells/well in RPMI-1640 medium supplemented with 10% FBS. Colchicine (100 ng/mL; Sigma-Aldrich) was added, and cells were incubated for 30 minutes, followed by addition of 1 μ g/mL TcdA (Enzo Life Sciences, Farmingdale, NY). After 4 hours of incubation, supernatants were collected. Macrophages were harvested with Accumax (Innovative Cell Technologies, San Diego, Calif) and seeded in 96-well plates. Colchicine was added, and cells were incubated for 30 minutes. After 2 hours of priming with 1 μ g/mL LPS (InvivoGen, San Diego, Calif), 1 μ g/mL TcdA was added, and supernatants were collected 4 hours later. For NLRP3 inflammasome activation, after priming iPS-MPs with LPS, nigericin (67 μ Mol/L; Sigma-Aldrich) or ATP (1 mmol/L; Sigma-Aldrich) was added, and the supernatants were collected 2 hours later.

Measurement of cytokine secretion

The IL-1 β concentration was measured in duplicates by using the Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories, Hercules, Calif).

Immunofluorescence staining

Monocytes or macrophages were stimulated as in the cytokine secretion assay. After 2 hours of stimulation with TcdA, the cells were attached to slides with a Cytospin 4 Cytoentrifuge (Thermo Fisher Scientific), fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (Nacalai, Kyoto, Japan). Cells were incubated with an anti-ASC antibody (AL177; AdipoGen, San Diego, Calif) and then with an Alexa Fluor 594-labeled antibody to rabbit IgG (A11012; Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (Dojindo, Kumamoto, Japan). Cells were examined by using fluorescence microscopy under a BZ-X710 microscope (Keyence, Osaka, Japan), and BZ-X Analyzer software (Keyence) was used for quantitative analysis.

May–Giemsa staining

Cells were seeded onto glass slides by using Platinum Pro (Matsunami, Bellingham, Wash) and stained with May–Grunwald and Giemsa staining solution (Merck Millipore, Burlington, Mass), according to the manufacturer's instructions. The slides were examined under a BZ-X710 microscope (Keyence), and the BZ-II Viewer software program (Keyence) was used for image acquisition.

Flow cytometry

Expression of surface markers on hematopoietic cells was evaluated on a FACSVerse flow cytometer (BD Biosciences). Primary antibodies for analysis were as follows: CD14–allophycocyanin (Beckman Coulter, Fullerton, Calif) and phycoerythrin–Cy7–CD33 (BD Biosciences). Data analyses were performed with FlowJo software (TreeStar).

Quantitative RT-PCR

Total RNA was extracted from iPS-MPs after stimulation with 1 μ g/mL LPS for 4 hours by using the RNeasy Mini Kit (Qiagen). The RNA was subjected to DNase treatment and reverse transcribed into cDNA by using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR was performed with TB Green Premix Ex TaqII (Tli RNaseH Plus; Takara Bio) and the StepOnePlus Real-Time PCR System, according to the manufacturers' instructions. The copy number of the target

gene was determined against a standard curve of serial dilutions of the *piggyBac* vector. The primers used for detection of total or transgenic *MEFV* are listed in Table E2.

Measurement of cell death

Sytox Green permeability was used to quantify pyroptosis over time. iPS-MPs were seeded in duplicates at 1×10^4 cells per well in flat-bottom 96-well plates in the presence of 1.5 μ M Sytox Green (Invitrogen/Thermo Fisher Scientific). Cells were primed with LPS for 2 hours and then stimulated with 1 μ g/mL TcdA. Data were acquired and analyzed with a fluorescent microscope (BZ-X710; Keyence) over a time span of 2.5 hours.

Statistical analysis

Data are shown as mean $\pm$ SEMs, as indicated in figure legends. All statistical analyses were performed by using the Student *t* test or 1-way ANOVA with GraphPad Prism (version 8.00; GraphPad Software, La Jolla, Calif).

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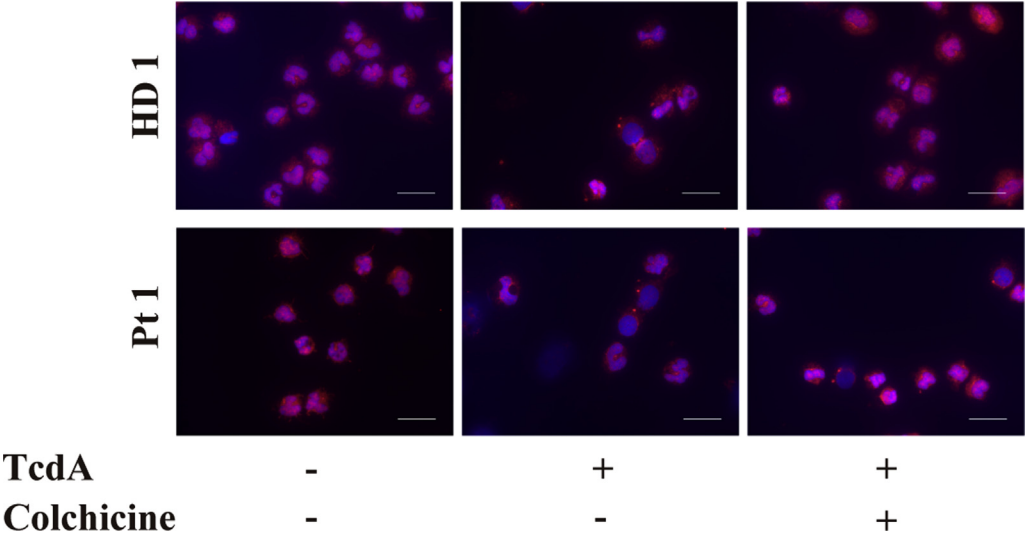


FIG E1. Representative images of monocytes. ASC, Red; nuclei, blue. *Scale bars* = 20 μ m.

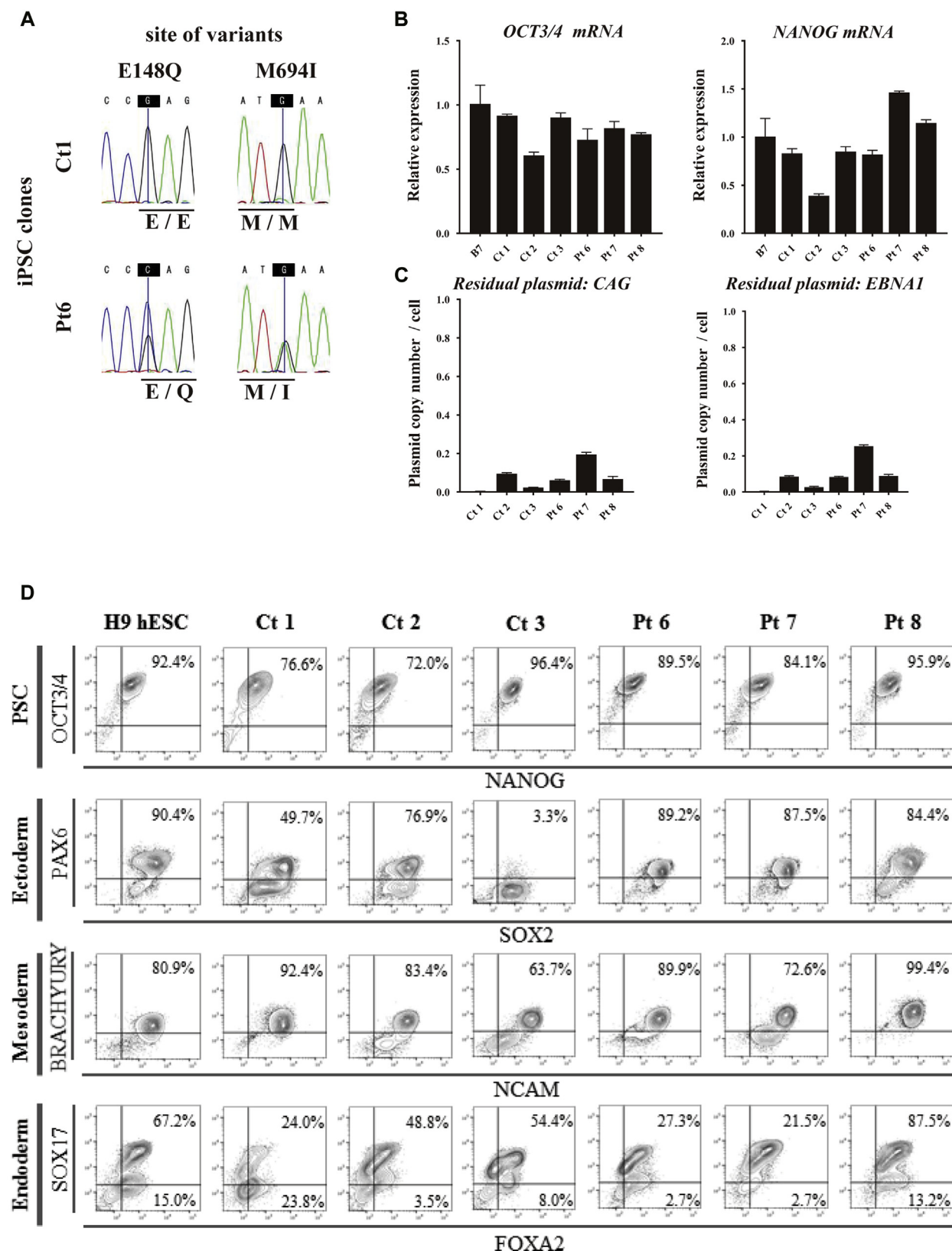


FIG E2. Characterization of iPSCs. **A**, Representative chromatogram of Sanger sequencing of *MEFV*. **B**, Quantitative RT-PCR validation of expression of the pluripotent stem cell-associated genes *OCT3/4* and *NANOG*. Expression is shown relative to that of the 201B7 control clone. **C**, Quantification of the copy number of residual plasmids per cell. Copy numbers of the CAG promoter region and the *EBNA1* sequence were determined. **D**, Flow cytometric evaluation of the *in vitro* differentiation capacity of control embryonic stem cells (H9 hESC), HD-derived iPSCs (Ct 1-3), and patient-derived lines (Pt 6-8).

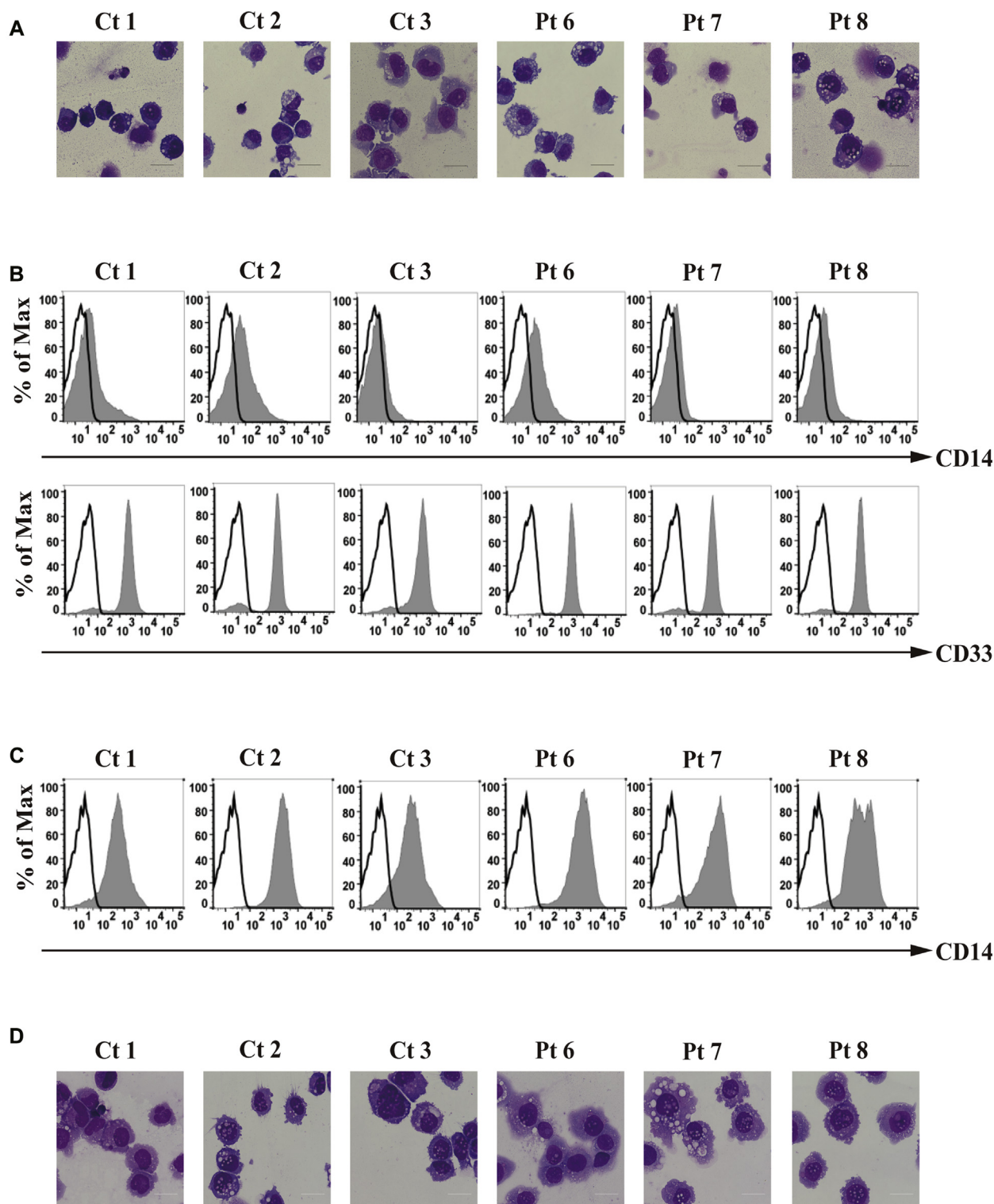


FIG E3. Characterization of iPS-MLs and iPS-MPs. **A**, Representative May-Giemsa staining of iPS-MLs. Scale bars = 20 μ m. **B**, Flow cytometric analysis of iPS-MLs. The white histogram represents the isotype control. **C**, Flow cytometric analysis of iPS-MPs. The white histogram represents the isotype control. **D**, Representative May-Giemsa staining of iPS-MPs. Scale bars = 20 μ m.

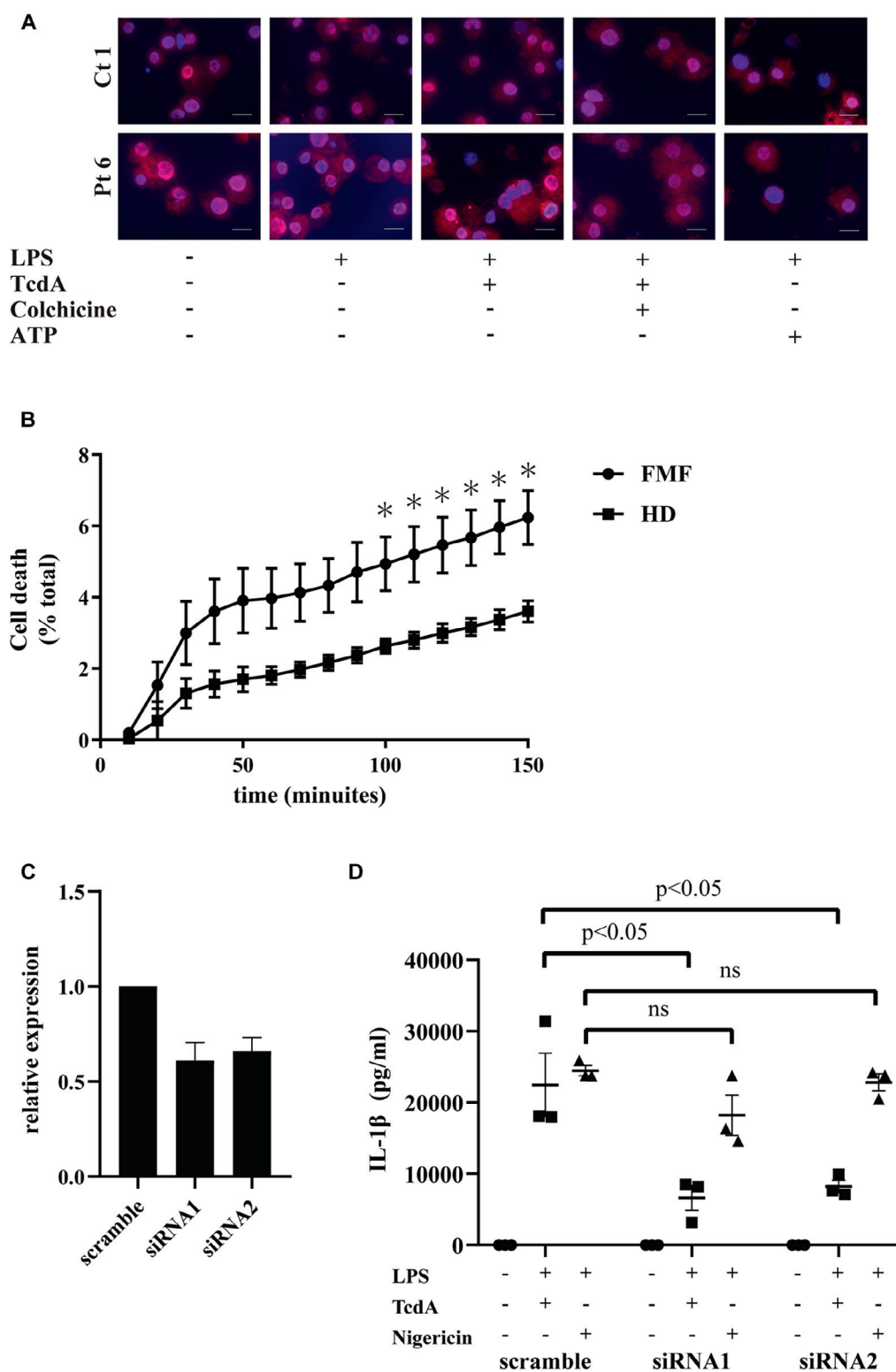


FIG E4. Pyrin inflammasome activation in patient-derived iPS-MPs. **A**, Representative images of iPS-MPs. ASC, Red; nuclei, blue. Scale bars = 20 μ m. **B**, iPS-MPs were stimulated with TcdA, and induction of pyroptosis was quantified over time by monitoring Sytox Green incorporation. $*P < .05$. **C**, Patient-derived iPS-MPs ($n = 3$) were transfected with MEFV siRNA (*siRNA1* and *siRNA2*) or a scrambled control siRNA. Expression of *MEFV* was determined by using real-time PCR. Target gene levels were normalized to levels of *ACTB*, and results are expressed relative to levels in iPS-MPs transfected with scrambled siRNA. **D**, Patient-derived iPS-MPs ($n = 3$) transfected with scrambled control siRNA or *MEFV* siRNA were primed with LPS and then stimulated with TcdA (squares) or nigericin (triangles). IL-1 β levels in the supernatant were assessed. ns, Nonsignificant.

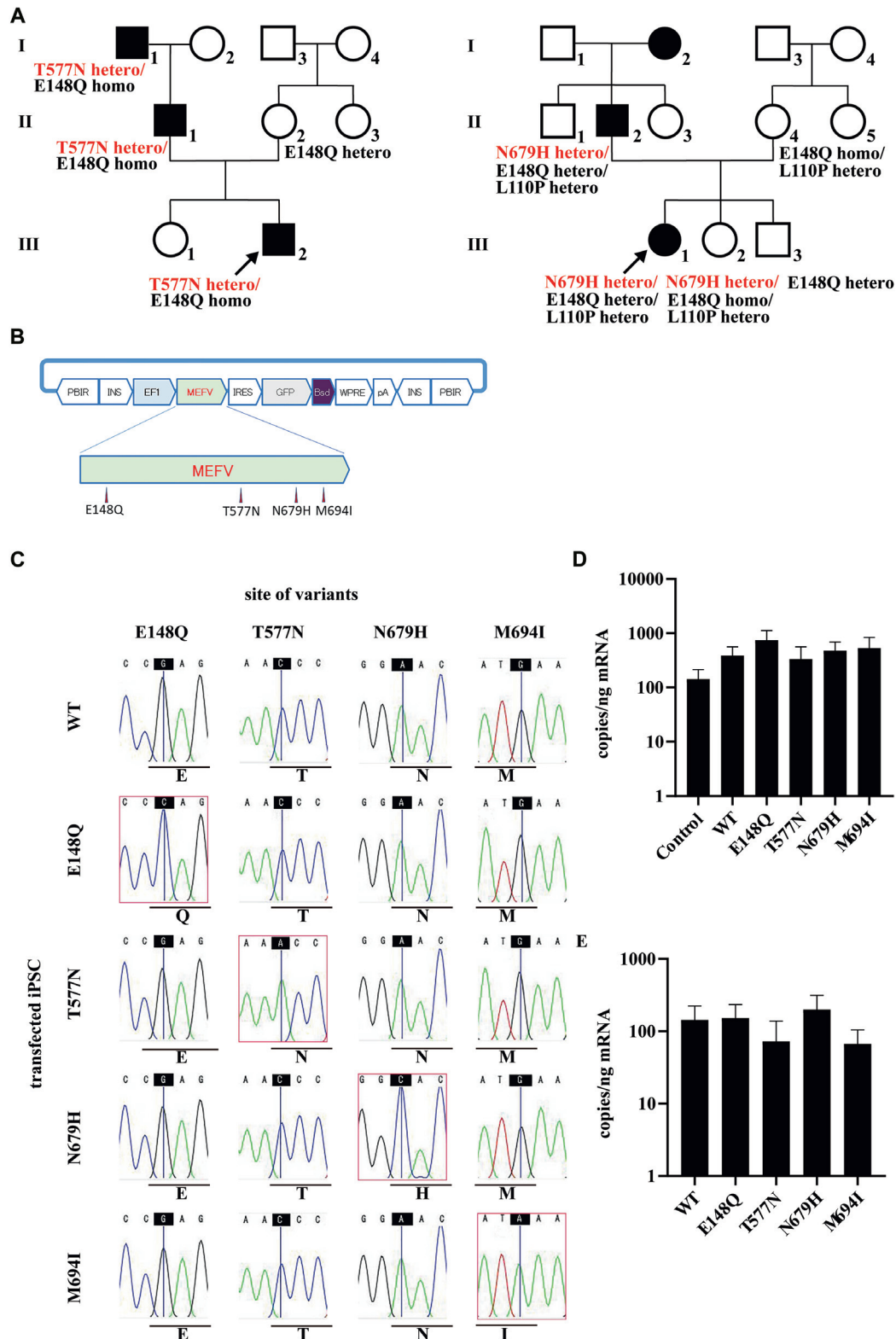


TABLE E1. Genotypes and clinical characterization of patients with FMF and family control subjects

	Patient ID	MEFV genotypes	Inflammatory symptoms	Tel-Hashomer criteria
FMF	Pt 1	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 2	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 3	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 4	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 5	Heterozygosity M694I, L110P, and E148Q	Yes	Yes
	Pt 6	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 7	Heterozygosity M694I and E148Q	Yes	Yes
	Pt 8	Heterozygosity M694I and E148Q	Yes	Yes
Family 1	I.1	Heterozygosity T577N, homozygosity E148Q	Yes	No
	II.1	Heterozygosity T577N, homozygosity E148Q	Yes	No
	II.2	Heterozygosity E148Q	No	No
Family 2	III.2	Heterozygosity T577N, homozygosity E148Q	Yes	No
	II.2	Heterozygosity N679H, L110P, and E148Q	Yes	Yes
	II.4	Heterozygosity L110P, homozygosity E148Q	No	No
	III.1	Heterozygosity N679H, L110P, and E148Q	Yes	Yes
	III.2	Heterozygosity N679H and L110P, homozygosity E148Q	No	No
	III.3	Heterozygosity E148Q	No	No

TABLE E2. Functional predictions of the *MEFV* variants identified in families 1 and 2

Family	Nucleotide change	Amino acid change	SIFT	PolyPhen2	Mutation Taster	PROVEAN
1	c.1730C>A	p.Thr577Asn	Tolerated	Possibly damaging	Polymorphism	Neutral
2	c.2035A>C	p.Asn679His	Tolerated	Probably damaging	Polymorphism	Neutral

TABLE E3. List of the autoinflammatory disease–related genes examined

	Patient ID	Genes
HDs and ACs		<i>MEFV</i> only
Family 1	I.1, II.1, III.2	22 genes*
	II.2	<i>MEFV</i> only
Family 2	II.2, III.1	22 genes*
	II.4, III.2, III.3	<i>MEFV</i> only

*Twenty-two genes: *CECR1*, *COPA*, *FAM105B*, *HMOX1*, *IL1RN*, *MEFV*, *MVK*, *NLR4*, *NLRP12*, *NLRP3*, *NOD2*, *PLCG2*, *POMP*, *PSMA3*, *PSMB4*, *PSMB8*, *PSMB9*, *PSTPIP1*, *RBCK1*, *RNF31*, *TNFAIP3*, and *TNFRSF1A*.

TABLE E4. List of primers

	Forward primer sequence	Reverse primer sequence	Probe sequence	Probe label
Mutagenesis				
E148Q	CCTCCCGGCTGGGGCTGGCTGC	GCAGCCAGCCCCAGGCCGGGAGG		
T577N	TCTGAACGCAGGTTTTCTGAGAAGTACTTTGTGCTC	GAGCACAAAGTACTTCTCAGAAAACCTGCGTTCAGA		
N679H	GGCGACAGAGTCATGTGCCCTTTCCTGCTTATG	CATAAGCAGGAAAGGGCACATGACTCTGTGCGC		
M694I	GGCTACTGGGTGGTGATAATGATAAAGGAAAATGAGTACC	GGTACTCATTTTCCTTTATCATTATCACCACCCAGTAGCC		
Verification of transfected <i>MEFV</i>				
Template amplification	TGACCCTGCTTGCTCAACTC	CTTCTCCCCTGTAGAAATGG		
	TGCACAGGGCTAAGACAGTG	ACCCTGTTATCCCTAGCGGC		
Sequence reaction	ATTCCACACAAGAAAACGGC	CTTCTCCCCTGTAGAAATGG		
	TGCACAGGGCTAAGACAGTG	GAAAGAGCAGCTGGCGAATG		
qPCR				
Total <i>MEFV</i>	TGCACAGGGCTAAGACAGTG	CATTTCTGAACGCAGGGTTT		
Transgene	TATCTGTCCAGTGGGTGGTCA	GCACACCGGCCTTATTCAA		
<i>ACTB</i>	CACCATTTGGCAATGAGCGGTTT	AGGTCTTTGCGGATGTCCACGT		
iPSC characterization				
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	TCTTCTGGGTGGCAGTGATG	ACTCATGACCACAGTCCA	VIC/MGB
<i>CAG</i>	GGCTCTGACTGACCGCGTTA	CAGAAAAGAAACAAGCCGTCATT	TGTAATTAGCGCTTGTT	FAM/MGB
<i>EBNA1</i>	ATCAGGGCCAAGACATAGAGATG	GCCAATGCAACTTGGACGTT	TGTCCGGAGACCCCA	FAM/MGB
<i>hOCT3/4</i>		Hs00999634_gH		FAM/MGB
<i>hNANOG</i>		Hs02387400_g1		FAM/MGB

TABLE E5. List of antibodies for evaluation of iPSC differentiation into 3 germ layers

Antibodies	Company	Catalog no.	Dilution	Application
OCT3/4–Alexa Fluor 647	BD Biosciences, San Jose, Calif	560329	1:25	Primary antibody
NANOG-FITC	BD Biosciences	560791	1:25	Primary antibody
PAX6-FITC	BD Biosciences	561664	1:25	Primary antibody
SOX2-BV421	BioLegend, San Diego, Calif	656114	1:50	Primary antibody
BRACHYURY-PE	R&D Systems, Minneapolis, Minn	IC2085P	1:25	Primary antibody
NCAM-BV421	BioLegend	318328	1:25	Primary antibody
SOX17–Alexa647	BD Biosciences	561589	1:25	Primary antibody
FOXA2-PE	BD Biosciences	562594	1:50	Primary antibody
Mouse IgG1, κ -APC	BioLegend	400120	1:50	Isotype control
Mouse IgG1, κ -Alexa Fluor 488	BioLegend	400129	1:25	Isotype control
Mouse IgG1, κ -PE	BioLegend	400112	1:25	Isotype control
Mouse IgG1, κ -BV421	BioLegend	400158	1:50	Isotype control

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.