

Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis syndrome is linked to dysregulated monocyte IL-1 β production

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Background: The exact pathogenesis of the pediatric disorder periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) syndrome is unknown.

Objectives: We hypothesized that PFAPA might be due to dysregulated monocyte IL-1 β production linked to genetic variants in proinflammatory genes.

Methods: Fifteen patients with PFAPA syndrome were studied during and outside a febrile episode. Hematologic profile, inflammatory markers, and cytokine levels were measured in the blood. The capacity of LPS-stimulated PBMCs and monocytes to secrete IL-1 β was assessed by using ELISA, and active IL-1 β secretion was visualized by means of Western blotting. Real-time quantitative PCR was performed to assess cytokine gene expression. DNA was screened for variants of the *MEFV*, *TNFRSF1A*, *MVK*, and *NLRP3* genes in a total of 57 patients with PFAPA syndrome.

Results: During a febrile attack, patients with PFAPA syndrome revealed significantly increased neutrophil counts, erythrocyte sedimentation rates, and C-reactive protein, serum amyloid A, myeloid-related protein 8/14, and S100A12 levels compared with those seen outside attacks. Stimulated PBMCs secreted

significantly more IL-1 β during an attack (during a febrile episode, 575 ± 88 pg/mL; outside a febrile episode, 235 ± 56 pg/mL; $P < .001$), and this was in the mature active p17 form. IL-1 β secretion was inhibited by ZYVAD, a caspase inhibitor. Similar results were found for stimulated monocytes (during a febrile episode, 743 ± 183 pg/mL; outside a febrile episode, 227 ± 92 pg/mL; $P < .05$). Genotyping identified variants in 15 of 57 patients, with 12 *NLRP3* variants, 1 *TNFRSF1A* variant, 4 *MEFV* variants, and 1 *MVK* variant.

Conclusion: Our data strongly suggest that IL-1 β monocyte production is dysregulated in patients with PFAPA syndrome. Approximately 20% of them were found to have *NLRP3* variants, suggesting that inflammasome-related genes might be involved in this autoinflammatory syndrome. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis syndrome, recurrent fever, autoinflammatory disease, interleukine-1 β , inflammasome

Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) syndrome is characterized by fever flares accompanied by pharyngitis, adenitis, and/or aphthous stomatitis without evidence of infection, asymptomatic intervals between the flares, and onset before the age of 5 years.¹⁻⁴ The precise incidence of the syndrome is not known, but it is probably the most common cause of recurrent fever in children in Western European populations, as shown by the recent report on the Eurofever registry.¹⁻⁵ The diagnosis is based on clinical criteria and the exclusion of other causes of recurrent fever because there are no specific laboratory tests for PFAPA syndrome. Generally, these febrile episodes resolve spontaneously before adulthood. The rapid response of the fever attacks to a single dose of corticosteroid points to an inflammatory origin, and the absence of an infectious or autoimmune cause supports the hypothesis that PFAPA syndrome is an autoinflammatory disease (AID).

AIDs are characterized by recurrent or chronic febrile episodes caused by a defect in the regulation of the inflammatory response by innate immune pathways. Monogenic origins have been established for some AIDs, including familial Mediterranean fever, TNF receptor–associated periodic syndrome, hyper-IgD syndrome, and cryopyrin-associated periodic syndrome (CAPS).⁶ In most of these diseases, the genetic defect is or might be related to a molecular complex called the inflammasome, which is involved in the regulation of IL-1 β activation, a cytokine with a central role in innate immunity. The inflammasome activates caspase-1 to cleave pro-IL-1 β to IL-1 β .⁷⁻⁹ Thus far, 4 different inflammasomes have been described, namely the *NLRP1*, *NLRP3*, *IPAF*, and *AIM2* inflammasomes.⁷

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Abbreviations used

AID:	Autoinflammatory disease
CAPS:	Cryopyrin-associated periodic syndrome
IL-1Ra:	IL-1 receptor antagonist
IP-10:	Interferon-inducible protein 10
MCP:	Monocyte chemoattractant protein
MRP:	Myeloid-related protein
PFAPA:	Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis
PFAPA-IN:	Patient with PFAPA syndrome during a febrile episode
PFAPA-OUT:	Patient with PFAPA syndrome outside of a febrile episode
SoJIA:	Systemic-onset juvenile idiopathic arthritis

The inflammatory mechanisms underlying PFAPA syndrome are currently unknown. A first study on cytokine profiles in the sera of patients with PFAPA syndrome showed increased serum IL-1 β and IL-6 levels compared with those seen in control subjects and a reduction in anti-inflammatory cytokine levels, specifically those of IL-4 and IL-10.¹⁰ Another group found that during PFAPA-related fever flares, serum levels of IL-6, but not of IL-1 β and TNF- α , were higher compared with those seen in healthy children.¹¹ Unlike the monogenic recurrent fever syndromes, PFAPA was considered a sporadic disease. Recently, we showed that a familial aggregation was observed in almost 50% of patients with PFAPA syndrome, suggesting a potential genetic origin.¹² This observation raised the question of what genetic variants predisposing to PFAPA because identifying the underlying genes might indicate the pathogenesis of PFAPA. We hypothesized that, as in the other AIDs, IL-1 β might play a central role in the pathogenesis of PFAPA secondary to a genetic defect, leading to a dysregulation of IL-1 β secretion.

Patients with PFAPA present with the interesting feature of alternating highly inflammatory states with totally asymptomatic periods. We aimed to further investigate the role of IL-1 β activation by monocytes in the pathogenesis of the disease in relation to genetic variants. Thus we compared the laboratory parameters of inflammation, cytokine levels, and LPS-induced IL-1 β production by mononuclear cells during the flares versus asymptomatic periods, and we characterized the genetic variants for the loci involved in 4 monogenic periodic fever syndromes.

METHODS**Subjects**

Children with PFAPA syndrome attending the pediatric rheumatology clinic in Lausanne were asked to participate in this study. Inclusion criteria were as follows: PFAPA diagnosis according to the previously published criteria,⁵ with other monogenic recurrent fever syndromes excluded clinically. The patients had no prior genetic analysis for the monogenic periodic fevers and no regular treatment with corticosteroids, immune suppressors, or biological agents. After a parent's written informed consent was obtained, patients were asked to come to the clinic for a blood draw during the first 24 hours of the fever flare and at least 14 days after the disappearance of the last flares' symptoms. Patients were instructed to take the prednisone dose only after the blood draw. All patients with a diagnosis of PFAPA syndrome and attending our clinic since 2006 were asked to participate in the genetic part of the study. Four healthy control children were selected from the allergy clinic (median age, 72 months; 3 boys and 1 girl); they were undergoing investigations for allergies (food allergy, rhinoconjunctivitis, perennial rhinitis, and drug allergy). All subjects received code numbers to guarantee anonymity with regard to the

laboratory results. Approval for the study was obtained from the Cantons' Ethical Committee in Lausanne.

Sample preparation

Blood from the subjects was collected in S-Monovette tubes containing EDTA or, for the isolation of sera, clot activators (Sarstedt, Nümbrecht, Germany). For cytokine determinations (see below), sera were stored in sterile tubes at -80°C before analysis. Anticoagulated blood was collected for DNA extraction and genetic analysis.

Complete blood cell count and acute-phase reactant levels

Complete blood cell count and white blood cell differential were determined by using a Sysmex XE 2100 cell counter (Laboratory of Hematology, Lausanne University Hospital, Lausanne, Switzerland). ELISA was used to measure C-reactive protein (Laboratory of Chemistry, Lausanne University Hospital, Lausanne, Switzerland) and serum amyloid A (Institut central des Hôpitaux Valaisans, Sion, Switzerland) levels.

PBMC and monocyte isolation and stimulation

PBMCs were isolated by using Ficoll-Paque Plus (BD Healthcare, Franklin Lakes, NJ), according to the manufacturer's instructions. Human monocytes were further purified from PBMCs with the MACS Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were primed overnight with ultrapure LPS (200 ng/mL; InvivoGen, San Diego, Calif) to induce pro-IL-1 β production. The following day, cells were stimulated for 6 hours with 1 $\mu\text{g/mL}$ crude LPS (Sigma-Aldrich, Buchs, Switzerland), which is an LPS preparation that also contains muramyl dipeptide, a potent inflammasome activator that induces caspase-1 activation, one enzyme responsible for the processing of pro-IL-1 β into its active form. This double stimulation allowed for maximal proinflammatory mediation production from the PBMCs. Because monocytes have constitutively active caspase-1, the experiments with purified monocytes were stimulated only with LPS Ultrapure at 200 ng/mL overnight. Cells were also treated with the pan-caspase inhibitor z-YVAD-fmk (50 $\mu\text{mol/L}$, ZYVAD; Sigma-Aldrich) in addition of LPS stimulation. Supernatants were collected from all of the stimulation experiments.

Western blotting

Mature IL-1 β p17 was detected with an antibody recognizing the precursor and mature forms (no. 2022s; Cell Signaling, Danvers, Mass). As a positive control, we took supernatants from THP1 cells (a human acute monocytic leukemia cell line) stimulated with LPS.

Cytokine measurements

Human IL-1 β ELISA was purchased from eBioscience (CBI, Baar, Switzerland). Other cytokines and 2 chemokines (monocyte chemoattractant protein 1 [MCP-1] and interferon-inducible protein 10 [IP-10]) were measured with the Luminex assay (Luminex, Austin, Tex).

Caspase-1, myeloid-related protein 8/14 (S100A8/S100A9), and S100A12 ELISAs

Human caspase-1 ELISA was obtained from BenderMedSystems (Vienna, Austria). The concentrations of myeloid-related protein (MRP) 8 (S100A8) and 14 (S100A9) complexes and S100A12 were determined by using ELISA, as described previously.¹³ Internal control sera were used as a reference in all ELISA studies for comparison with earlier studies. The readers of the laboratory assay were blinded for the diagnosis and the inflammatory activity of the subjects. The normal upper limit of MRP8/14 is 450 ng/mL and that of S100A12 is 150 ng/mL in serum.

TABLE I. Clinical characteristics of the 15 patients with PFAPA syndrome with laboratory investigations (12 PBMC stimulations and 3 monocyte stimulations)

Patient no.	Age at onset (mo)	Male/female	Major symptoms	Other symptoms	Positive family history	NLRP3 variants	Other variants	Response to steroids
1	11	Male	P, AD, APH		Father: recurrent fever, tonsillectomy Mother: recurrent tonsillitis	Q703K	No	NA
2	18	Male	P, AD, APH		Father: tonsillectomy	R488K	No	NA
3	72	Male	P, AD	AR	None	No	No	NA
4	18	Male	P, AD, APH	D	Grandmother: tonsillectomy	Q703K	No	2 doses
5	6	Female	P, AD	AbdP, M, AR	None	No	No	12 h
6	66	Male	P, AD, APH	AbdP, AR, R	Brother: suspicion of PFAPA Uncle: recurrent fever, tonsillectomy	No	No	12 h
7	9	Male	P, APH	AbdP, M, AR, H	None	No	No	12 h
8	4	Male	P, AD	M	None	No	No	12 h
9	21	Male	P, AD, APH		Father: recurrent fever	No	No	12 h
10	11	Female	P, AD, APH	R	Mother and father: tonsillectomy	No	No	12 h
11	6	Female	P, AD, APH	AbdP, AR	None	Q703K	No	12 h
12	12	Male	P, AD, APH	AbdP, AR	Mother and father: tonsillectomy Uncle: recurrent fever	No	No	12 h
13	33	Male	P		None	No	No	24 h
14	18	Male	P, AD, APH	AbdP	Father: pharyngitis, recurrent fever, tonsillectomy	No	No	12 h
15	27	Female	P	AbdP	None	No	No	12 h

Patients 1 to 12 = experiments on PBMCs; patients 13 to 15 = experiments on monocytes.

AbdP, Abdominal pain; AD, adenitis; APH, oral aphthosis; AR, arthralgia; D, diarrhea; H, headache; M, myalgia; P, pharyngitis; R, rash; NA, not available, patients never treated with steroids; 2 doses, a second dose was necessary to interrupt the flare; 12 h, response within 12 hours; 24 h, response within 24 hours.

Isolation of RNA and cDNA synthesis from patient's monocytes for real-time PCR

RNA was extracted and reverse transcribed into cDNA by using TRIzol (Invitrogen, Carlsbad, Calif) and RNA Clean & Concentrator5-Zymoresearch and Superscript II (Invitrogen). Relative gene expression of *IL1B* (5'-CTGGACCTCTGCCCTCTGG-3' and 5'-TCCATGGCCACAACAACCTGA-3') was quantified with the LightCycler480 system (Roche Applied Science, Mannheim, Germany) by using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5'-CTCATGACCACAGTCCATGC-3' and 5'-CACATGGGGG TAGGAACAC-3') and TATA box binding protein (*TBP*; 5'-ACAGTGAATCTTGGTTGTAACTTGACCTA-3' and 5'-TGGTTCGTGGCTCTC TTATCCT-3') as reference genes.^{14,15} Relative gene expression, determined by using the $2^{-\Delta\Delta Ct}$ method, was normalized against unprimed and unstimulated monocytes from the same patient.

Genetic analysis

Genomic DNA was extracted from EDTA blood (Autopure; Qiagen, Hombrechtikon, Switzerland). Mutation hotspots in the 4 major genes associated with monogenic periodic fevers¹⁶ were analyzed by using PCR and DNA sequencing, according to our routine diagnostic procedures (BigDye Terminator 3.1 Cycle Sequencing and 3500 Genetic Analyzer, Applied Biosystems, Rotkreuz, Switzerland; Agencourt CleanSeq purification; sequences were analyzed with GenSearch v3.63b, Phenosystems, Belgium). The regions analyzed (coding sequences ± 10 intronic bases) were as follows: *MEFV* exons 2 and 10 (detects >95% of known pathogenic mutations); *TNFRSF1A* exons 2, 3, 4, and 6 (detects close to 100% of pathogenic mutations); *MVK* exons 9 and 11 (detects approximately 70% of pathogenic mutations); and *NLRP3* exon 3 (detects close to 100% of pathogenic mutations).

Statistical analysis

Data are reported as means $\pm$ SEMs. Differences between groups were analyzed by using 1-way ANOVA or the unpaired *t* test. Observed and

expected frequencies of genetic variants were compared by using a χ^2 test with 2 criteria (normal or variant) and 1 *df*. Statistically significant results are annotated as follows: **P* < .05, ***P* < .01, and ****P* < .001.

RESULTS

Fever episodes are characterized by increased neutrophil and monocyte counts and increased markers of inflammation

Fifteen patients (median age, 59 months; range, 17-133 months; male/female ratio, 2.8/1) agreed to participate in the full *ex vivo* study; their clinical characteristics are shown in Table I. The length of the fever attacks ranged from 2 to 5 days, and the asymptomatic intervals ranged from 2 to 6 weeks. The median age of the control subjects was 72 months (range, 27-165 months). Only 7 patients had a negative family history: 8 children had at least 1 close relative with recurrent fever or tonsillectomy. In 12 patients the fever responded to prednisone; in 3 patients the parents chose not to treat their child with steroids. Patients' blood was taken during a fever flare (PFAPA-IN) and during the asymptomatic interval (PFAPA-OUT). Healthy control children were also included in the study.

PFAPA-IN patients displayed significant changes in their PBMC profiles compared with those seen in PFAPA-OUT patients and healthy control children. Specifically, we observed a significant increase in neutrophil counts and decreased lymphocyte and platelet counts (Fig 1 and data not shown). There were no differences in hemoglobin content between PFAPA-IN and PFAPA-OUT patients.

During fever flares (PFAPA-IN patients), the serum C-reactive protein concentrations, erythrocyte sedimentation rates, and

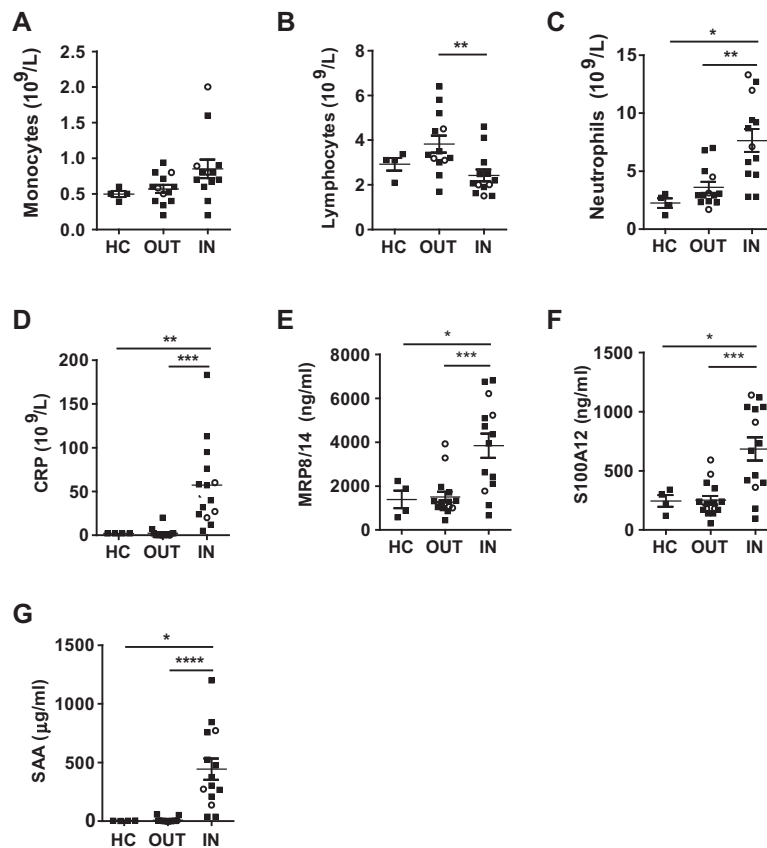


FIG 1. Differential blood cell counts and increased inflammation markers during PFAPA syndrome-related febrile episodes. Levels of monocytes (**A**), lymphocytes (**B**), and neutrophils (**C**) were determined in blood from healthy control children (HC; $n = 4$) and patients with PFAPA syndrome ($n = 13$) during (IN) and out (OUT) of a febrile episode. Serum levels of C-reactive protein (CRP; **D**), MRP8/14 (**E**), S100A12 (**F**), and serum amyloid A (SAA; **G**) are shown. Significance was determined by using the Student *t* test as follows: * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$. Open circles denote patients with an identified *NLRP3* polymorphism.

serum amyloid A levels were significantly higher than outside of flares (PFAPA-OUT patients) and those seen in the healthy control children (Fig 1). Serum MRP8/14 and S100A12 levels were also higher during than outside the fever flares. Interestingly, MRP8/14 and S100A12 levels were greater than the normal upper limit in the PFAPA-OUT patients.

FEBRILE EPISODES ARE CHARACTERIZED BY INCREASED SERUM PROINFLAMMATORY CYTOKINE LEVELS

Serum caspase-1 (Fig 2, A), IL-6 (Fig 2, C), and IP-10 (Fig 2, D) levels were significantly increased in PFAPA-IN patients, whereas no significant differences in TNF- α or MCP-1 levels were observed (Fig 2, B and E). IL-1 α could be detected in only 2 PFAPA-IN patients of all the subjects included in the study (data not shown). IL-1 receptor antagonist (IL-1Ra) could be detected in only 1 patient during the symptom-free period, whereas 5 patients had positive results during a febrile episode (data not shown). Serum IL-1 β could not be detected in any of the tested subjects (data not shown). In healthy control children mean caspase-1, TNF- α , IL-6, IP-10, and MCP-1 levels were lower compared with those seen in PFAPA-OUT and PFAPA-IN patients, although none of these differences were significant.

Increased secretion of active IL-1 β and IL-1Ra by LPS-stimulated PBMCs during a febrile episode

LPS-stimulated PBMCs from PFAPA-IN patients secreted higher amounts of IL-1 β (575 ± 88 pg/mL; Fig 3, A) compared with those seen in both PFAPA-OUT patients (235 ± 56 pg/mL) and healthy control children (63 ± 12 pg/mL). Both IL-1 α and IL-1Ra levels were increased after LPS stimulation of PBMCs, but significance was only attained for IL-1Ra (Fig 3, C and D). In contrast, nonstimulated PBMCs from patients with PFAPA syndrome, either during asymptomatic periods or during flares, had very low expression of IL-1 α , IL-1 β , and IL-1Ra and were indistinguishable from levels seen in healthy control children (Fig 3, A, C, and D). LPS-induced IL-1 β secretion was almost completely abolished by using ZYVAD, a pan-caspase inhibitor (Fig 3, A), indicating that pro-IL-1 β was cleaved by caspase-1 and secreted as active p17 IL-1 β . LPS-stimulated PBMCs from patients with PFAPA syndrome secreted active IL-1 β (p17), whereas no active IL-1 β was detected in supernatants of nonstimulated PBMCs (Fig 3, B). The secretion of the active form of IL-1 β was similar, regardless of the presence or absence of *NLRP3* variants (results not shown). LPS-stimulated PBMCs from PFAPA-IN patients secreted higher levels of the proinflammatory mediators TNF- α , IL-6, IP-10, and MCP-1 (Fig 4, F and G, and data not shown) compared with those seen

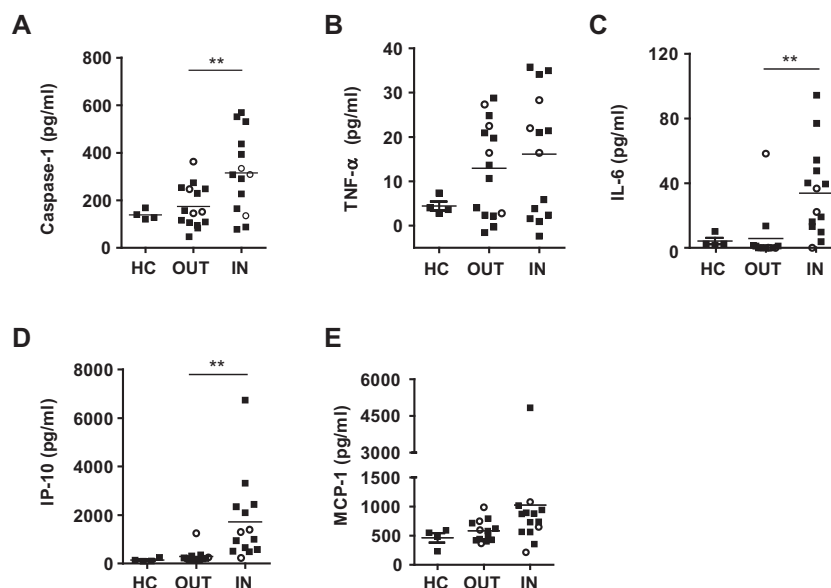


FIG 2. Increased circulating cytokine and chemokine levels during PFAPA syndrome–related febrile episodes. Levels of caspase-1 (**A**), TNF- α (**B**), IL-6 (**C**), IP-10 (**D**), and MCP-1 (**E**) were determined in sera from healthy control children (HC; $n = 4$) and from patients with PFAPA syndrome in (IN) and out (OUT) of a febrile episode ($n = 13$). Significance was determined by using the Student t test as follows: ** $P \leq .01$. Open circles denote patients with an identified *NLRP3* polymorphism.

in PFAPA-OUT patients and healthy control children, but only TNF- α was significant ($P < .05$).

To rule out that differences in secreted IL-1 β levels between PBMCs from PFAPA-IN and PFAPA-OUT patients were due to differences in the proportion of monocytes, we purified monocytes. In agreement with our PBMC results, LPS-stimulated monocytes from PFAPA-IN patients secreted significantly more IL-1 β than those from PFAPA-OUT patients (PFAPA-OUT, 227 ± 92 pg/mL; PFAPA-IN, 743 ± 183 pg/mL; Fig 4, A). In monocytes caspase-1 is constitutively activated, and mature IL-1 β is released after a single stimulation with Toll-like receptor ligands.¹⁷ After a single stimulation with LPS, monocytes isolated during a febrile episode (PFAPA-IN patients) secreted significantly more IL-1 β (Fig 4, C) and TNF- α (Fig 4, D) than monocytes isolated out of a febrile episode (PFAPA-OUT patients). Interestingly, when monocytes were stimulated in the presence of ZYVAD, TNF- α levels were diminished (data not shown). In contrast, IL-6 (Fig 4, E) and caspase-1 (Fig 4, F) levels were the same during and out of a febrile episode. To assess whether the differences in secreted IL-1 β levels could be accounted for by differences in mRNA expression, we performed quantitative PCR on RNA extracted from LPS-stimulated monocytes. No differences in RNA levels of IL-1 β (Fig 4, B) were found between monocytes from PFAPA-IN and PFAPA-OUT patients.

Identification of genetic variants in patients with PFAPA syndrome

DNA of 57 patients with PFAPA syndrome (including 1 pair of affected siblings and all 15 patients from the PBMC and monocyte stimulation studies) were analyzed for the 4 genes most commonly associated with monogenic periodic fevers. Fifteen (25%) patients carried a total of 18 variants, all in heterozygosity. Specifically, 12 patients carried variants in

NLRP3, 4 in *MEFV* (3 carried both *NLRP3* Q703K and *MEFV* E148Q), and 1 each in *TNFRSF1A* and *MVK* (Tables I and II). Eight (53%) of the 15 patients with a variant presented with all 3 major PFAPA symptoms compared with 12 (29%) of 42 for the other patients. Family history was more often positive in patients presenting a variant (12/15 [80%] vs 23/42 [55%]). Forty-one patients responded to steroids, which were not given to 16 patients.

All the genetic variants identified are of uncertain clinical significance, being variously considered as mild, of reduced penetrance, or both or without functional effect. We compared the frequency of *NLRP3* variants in our cohort with published frequencies in the healthy population.¹⁸ All 3 variants were more frequent than expected in patients, although considered singly, only R488K reached statistical significance (V198M: expected frequency = 0.0148, observed frequency = 2/57, $P = .205$; Q703K: expected frequency = 0.10, observed frequency = 9/57, $P = .145$; R488K: expected frequency = 0.0028, observed frequency = 1/57, $P = .035$). When all 3 are considered, there is a significantly higher than expected occurrence of *NLRP3* variants in patients with PFAPA syndrome (expected frequency = 0.118, observed frequency = 12/57, $P = .029$).

Both familial cases of PFAPA were concordant for the variants identified in the probands: siblings 20 and 21 (Table II) both carried *NLRP3* Q703K plus *MEFV* E148Q, and 2 cousins (patient 24 and cousin, not included) were heterozygous for *TNFRSF1A* R92Q.

DISCUSSION

On the basis of its typical clinical features and recurrent fever flares, PFAPA syndrome can be considered an AID, and recent data lead us to suggest a genetic predisposition for this syndrome.¹² On this basis, we hypothesize that a minor trigger,

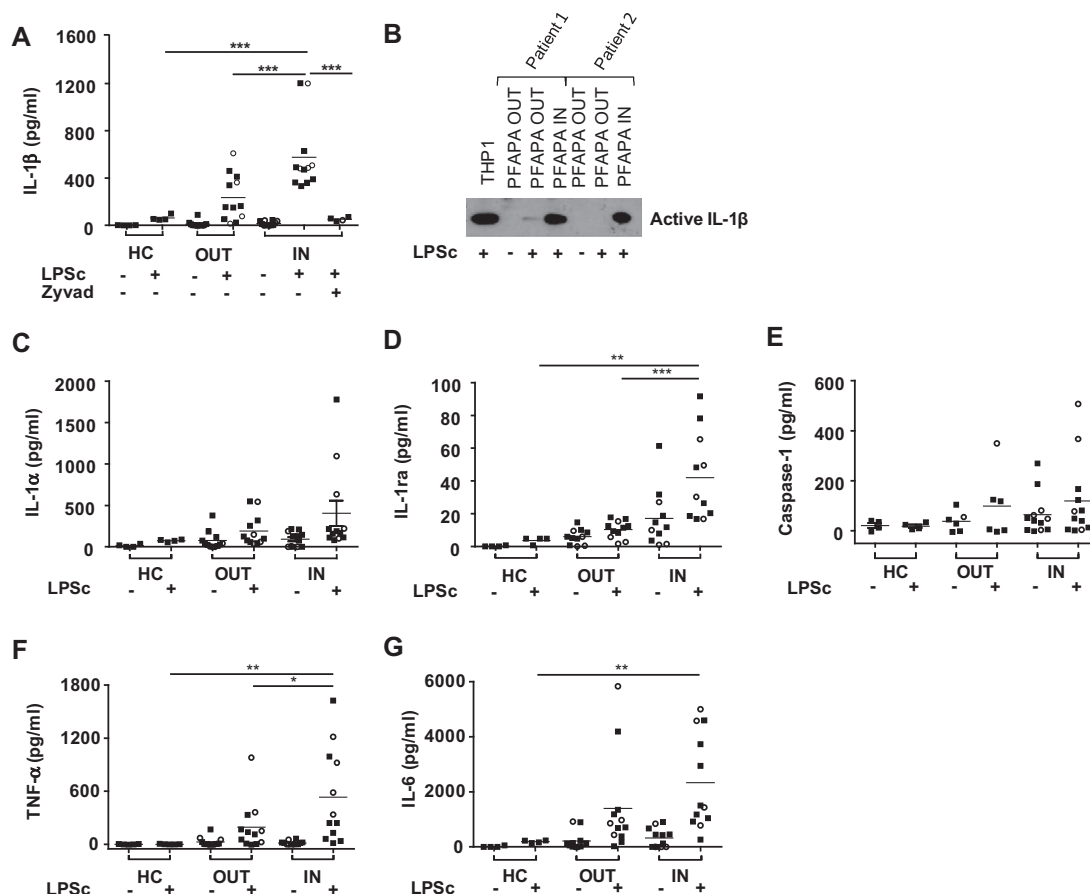


FIG 3. Increased cytokine secretion by PBMCs derived from patients with PFAPA syndrome during a febrile episode. PBMCs isolated from patients with PFAPA syndrome ($n = 12$) in (*IN*) or out (*OUT*) of febrile episodes and from healthy control children (*HC*; $n = 4$) were primed and stimulated with LPS crude (*LPSc*) for 6 hours with or without z-YVAD-fmk and compared with unstimulated control cells. IL-1 β (**A**), IL-1 α (**C**), IL-1Ra (**D**), caspase-1 (**E**), TNF- α (**F**), and IL-6 (**G**) levels were quantified by using ELISA. By using immunoblotting (**B**) of collected supernatants from 2 patients, THP1 was included as a positive control, with an anti-IL-1 β (p17 kD) to detect the active form. Significance was determined as follows: * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$. Open circles denote patients with an identified *NLRP3* polymorphism.

normally insufficient to induce systemic inflammation, could lead to activation of innate immune pathways to induce a fever flare in a patient with a susceptible genetic background.

During a flare, our patients showed an increase in the absolute number of monocytes and neutrophils and a marked increase in inflammatory marker levels, suggesting an activation of the innate immune system, which is similar to results described by Brown et al.¹¹ Increased absolute neutrophil counts have also been shown in patients with systemic-onset juvenile idiopathic arthritis (SoJIA), an AID without clear genetic cause, and higher neutrophil numbers have been correlated with a good response to IL-1 blockade by anakinra.¹⁹ The decreased lymphocyte numbers during the flare might be due to lymphocyte migration into the affected tissues. During a flare, we also found very high levels of MRP8/14 and S100A12, 2 proteins expressed in early-stage differentiated neutrophils, monocytes, and macrophages. High serum levels of MRP8/14 have also been documented during acute flares in patients with SoJIA and in patients with CAPS, with levels most increased in patients with SoJIA.^{20,21} As expected, while out of fever flares, patients with PFAPA syndrome have similar MRP8/14 and S100A12 levels compared with those seen in healthy control children. These observations emphasize

the similarities between PFAPA and the recognized autoinflammatory syndromes and implicate activation of innate immune cells, such as monocytes, in promoting systemic inflammation during the fever flares.

To understand the mechanisms underlying this activation state, we measured the changes in proinflammatory cytokine levels in the serum in and out of a flare. We did not detect measurable serum levels of IL-1 β during PFAPA-related fever flares, which is similar to previous findings in patients with PFAPA syndrome and other autoinflammatory disorders.²¹ However, we found that flares increased serum levels of caspase-1, suggesting activation of the inflammasome to produce IL-1 β and increased levels of IP-10. Taken together, our results suggest that activation of the T_H1 axis is characteristic of a PFAPA flare, as recently proposed.²²

We went on to characterize the regulation of IL-1 β production in more detail in patients with PFAPA syndrome. Primed PBMCs and monocytes from patients during a febrile episode showed an exacerbated secretion of IL-1 β when stimulated by LPS compared with that seen in cells from patients out of a febrile episode. In contrast, LPS-stimulated PBMCs from healthy children had a lower IL-1 β secretion compared with that seen in patients with

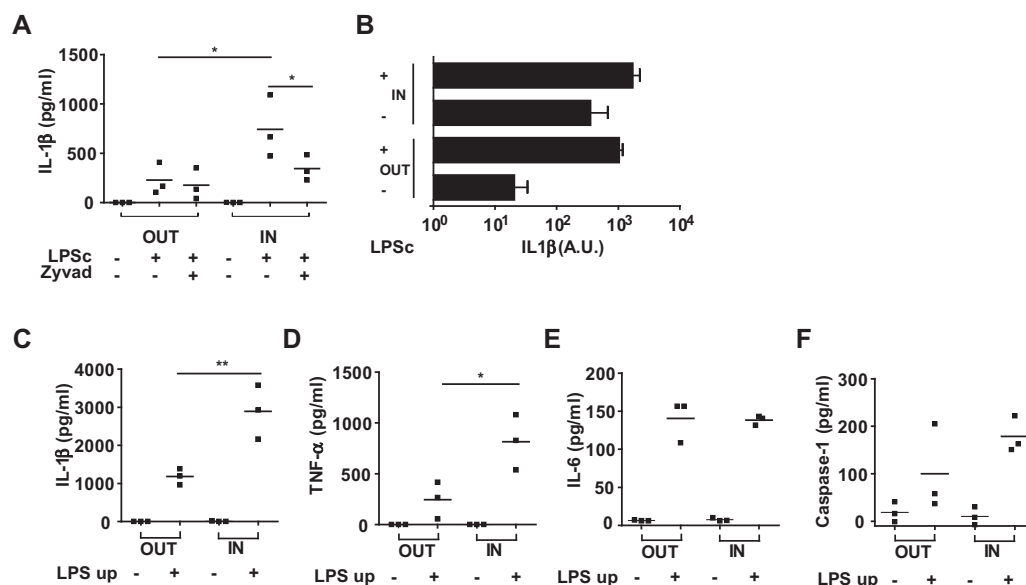


FIG 4. Increased cytokine secretion by purified monocytes from patients with PFAPA syndrome during febrile episode. Monocytes isolated from patients with PFAPA syndrome ($n = 3$) in (IN) or out (OUT) of febrile episodes were primed and stimulated with LPS crude (LPS_c) for 6 hours, the levels of IL-1 β were determined by means of ELISA (A), and the relative expression levels (AU) of *IL1B* mRNA were determined by using quantitative RT-PCR (B). Results were calculated by using the $2^{-\Delta\Delta CT}$ method with the reference genes *GAPDH* and *TBP*, and the data were normalized against unprimed and unstimulated monocytes. Isolated monocytes were stimulated with LPS Ultrapure (LPS_{up}) overnight, and the levels of IL-1 β (C), TNF- α (D), IL-6 (E), and caspase-1 (F) were quantified by using ELISA.

PFAPA syndrome during or outside of the flare. In unstimulated cells IL-1 β production was at basal levels and did not vary in or out of a flare. That initiation of IL-1 β processing in mononuclear cells from patients with PFAPA syndrome is dependent on inflammasome and caspase-1 activation was shown by the almost totally abrogated secretion of IL-1 β by ZYVAD, a caspase-1 inhibitor. Interestingly, in these cells ZYVAD also partially inhibited TNF- α secretion, suggesting that IL-1 β secretion can trigger TNF- α release, possibly through autocrine binding to the IL-1 receptor, a mechanism already reported in THP1 cells.²³ Altogether, our results strongly suggest that dysregulation of IL-1 β secretion is an intrinsic phenotype of monocytes from patients with PFAPA syndrome and that alterations in inflammasome activation underlie this phenomenon.

We found that 15 of 57 patients with PFAPA syndrome had heterozygous variants in 1 or more of the genes *NLRP3* (12 variants), *MEFV* (4 variants), *TNFRSF1A* (1 variant), or *MVK* (1 variant), which are known to be involved in monogenic periodic fever syndromes. *NLRP3* variants are responsible for approximately 60% of cases of CAPS, a disease linked to dysregulated IL-1 β production that responds well to IL-1 inhibitors.²⁴ All 3 *NLRP3* variants that we found are recurrent missense variants, the potential roles of which in CAPS are unclear, being variously considered as reduced-penetrance pathogenic variants or benign. R488K is a rare variant (allelic frequency, 0.0014¹⁸), as described in both patients with familial cold autoinflammatory syndrome and those with Muckle-Wells syndrome, as well as in occasional asymptomatic family members¹⁸; V198M has similarly been found in patients with familial cold autoinflammatory syndrome and those with Muckle-Wells syndrome and asymptomatic subjects and has also been implicated in excessive IL-1 β secretion in a patient with Schnitzler syndrome.²⁵ Q703K (also described in

publications as Q705K) is a polymorphism with an allele frequency of approximately 3% to 5% in white subjects²⁶; however, this does not preclude the variant from having an effect on the regulation of inflammasome activation, as suggested by its reported involvement in patients with celiac disease²⁷ and those with Crohn disease²⁸ and in 3 cases of atypical CAPS.⁶

Three patients with PFAPA syndrome (including 2 siblings) were transheterozygous for the variants *NLRP3* Q703K and *MEFV* E148Q. As with the other variants identified, the role of E148Q is uncertain: E148Q was formerly considered a common but mildly pathogenic variant, but others suggest it might be a benign variant.²⁹ The expected frequency of double heterozygotes can vary from approximately 1 in 40 to less than 1 in 100, principally because of the varying frequency of E148Q. Patient 17 is of Indian origin, and in such patients E148Q is a common polymorphism that appears to be associated with AA amyloidosis and other inflammatory disorders.³⁰ Patients 20 and 21 are Swiss, with 1 Northern Italian ancestor; E148Q is rare in Swiss but common in Italian subjects.

The unexpectedly high frequency of *NLRP3* and other variants in patients with PFAPA syndrome is intriguing and further strengthens our suspicion that dysregulated inflammasome activation is the pathologic mechanism of PFAPA. The concordant genotypes in the recurrent familial cases of PFAPA for Q703K+E148Q and for *TNFRSF1A* R92Q, a mild low-penetrance mutation,³¹ further support the concept that periodic fever gene variants can contribute to PFAPA. The association between heterozygous *NLRP3* variants with PFAPA suggests that they might have a gain-of-function effect, resulting in increased maturation of pro-IL-1 β , as has been described for CAPS.³²⁻³⁵ In support of this, the Q705K polymorphism in *NLRP3*, the most abundant polymorphism found in patients with PFAPA

TABLE II. Clinical characteristics in the 15 patients with PFAPA syndrome (of 57 tested) with variants in the 4 major genes associated with monogenic periodic fevers

Patient no.	Age at onset (mo)	Male/female	Major symptoms	Other symptoms	Positive family history	<i>NLRP3</i> variants	Other variants
1	11	Male	P, AD, APH		Father: recurrent fever, tonsillectomy Mother: recurrent tonsillitis	Q703K	=
2	18	M	P, AD, APH		Father: tonsillectomy	R488K	=
4	18	M	P, AD, APH	D	Grandmother: tonsillectomy	Q703K	=
11	6	F	P, AD, APH	AbdP, AR	None	Q703K	=
16	15	M	P		Mother and father: tonsillectomy	=	<i>MVK</i> c.769-7T>G
17	10	M	P, AD	AbdP, M	None	Q703K	<i>MEFV</i> E148Q
18	96	F	P, AD, APH	AbdP	None	Q703K	=
19	12	M	P, AD, APH	AbdP	Father: recurrent fever, tonsillectomy Mother: recurrent fever	V198M	=
20	60	M	P	AbdP, D	Sister (patient 21): PFAPA	Q703K	<i>MEFV</i> E148Q
21	54	F	P		Brother (patient 20): PFAPA	Q703K	<i>MEFV</i> E148Q
22	72	F	AD, APH		Brother: recurrent pharyngitis	Q703K	=
23	2	F	AD, APH		Brother: recurrent fever Uncle and great-uncle: rheumatism in childhood	V198M	=
24	24	F	P		Cousin: PFAPA	=	<i>TNFRSF1A</i> R92Q
25	10	M	P, AD, APH		Uncle: pharyngitis, recurrent fever, tonsillectomy	=	<i>MEFV</i> c.*9C>T
26	12	M	P, AD, APH	AR	Mother and uncle: recurrent pharyngitis	Q703K	=

Patients 1, 2, 4, and 11 are also part of Table I. Patients 20 and 21 are siblings. All variants were in the heterozygous state. Numbering according to National Center for Biotechnology Information reference sequences is as follows: NM_000243.2 (*MEFV*), NM_001065.3 (*TNFRSF1A*), NM_000431.2 (*MVK*), and NM_001243133.1 (*NLRP3*). Variants coded according to Human Genome Variation Society nomenclature are as follows: *NLRP3* V198M, c.592G>A; R488K, c.1463G>A; Q703K, c.2107C>A; *MEFV* E148Q, c.442G>C; *TNFRSF1A* R92Q, c.362G>A.

=, No genetic variant identified; *AbdP*, abdominal pain; *AD*, adenitis; *APH*, oral aphthosis; *AR*, arthralgia; *D*, diarrhea; *H*, headache; *M*, myalgia; *P*, pharyngitis; *R*, rash.

syndrome, has very recently been identified as a gain-of-function alteration leading to an overactive *NLRP3* inflammasome.²³

Finally, the clinical and genetic overlap of PFAPA with some CAPS raises the question of whether PFAPA is a distinct syndrome or whether it represents a mild presentation of the spectrum of CAPS and *NLRP3*-associated pathologies.

Interestingly, increased expression of *AIM2* and caspase-1 transcripts during flares has recently been reported,²² suggesting increased expression and activation of the *AIM2* inflammasome.³⁶ We can speculate that the *AIM2* inflammasome, other caspase-1-dependent inflammasomes, or both might be involved in PFAPA syndrome. Recent publications have described somatic *NLRP3* mutations in 70% of patients with mutation-negative chronic infantile neurologic, cutaneous, articular syndrome, with estimates of the level of mosaicism ranging from 4.2% to 35.8% of hematopoietic and nonhematopoietic cells.^{37,38} Our study demonstrated that 12 of 57 of our patients with PFAPA syndrome had positive results for one of the 3 different *NLRP3* variants (V198M, R488K, and Q703K). Expansion of our genetic studies is required to identify whether the remaining 45 of 57 patients could display other, as yet unidentified inflammasome-associated variants. It might also be possible that mosaicism could occur in patients with PFAPA syndrome who did not show any heterozygosity for an *NLRP3* mutant. Finally, we cannot rule out the possibility that in patients without inflammasome involvement, other mutations in currently unrecognized genes will be found. To solve this important question, whole-genome sequencing is warranted.

An important part of the evidence for the role of IL-1 β in autoinflammatory disorders is the efficacy of IL-1 β blockade. Three anti-IL-1 β agents have been extensively studied in the treatment of AIDs: anakinra, rilonacept, and canakinumab.²⁴ For patients with PFAPA syndrome, clinical responsiveness to IL-1 β blockade will be crucial in identifying the involvement of IL-1 β in the pathogenesis of this syndrome. Recent data suggest that treatment with anakinra reduces the duration of an acute flare in a small uncontrolled study.²² Our observation that IL-1 β dysregulation might play a key role in the pathogenesis of PFAPA syndrome will open new therapeutic perspectives for these patients experiencing years of recurring fever attacks.

We would like to express our sincerest thanks to Dr Laeticia Kolly, who devoted a great amount of energy to this project despite a long-standing disease that she supported courageously. She was still engaged in the research until a few days before her death.

Key messages

- Monocytes of patients with PFAPA syndrome produce more LPS-stimulated IL-1 β during fever flares.
- A significant number of patients with PFAPA syndrome present inflammasome-related gene variants.
- The observation of IL-1 β dysregulation opens new therapeutic perspectives in patients with PFAPA syndrome.

REFERENCES

1. Tasher D, Somekh E, Dalal I. PFAPA syndrome: new clinical aspects disclosed. *Arch Dis Child* 2006;91:981-4.
2. Long SS. Syndrome of periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA)—what it isn't. What is it? *J Pediatr* 1999;135:1-5.
3. Hofer M. A child with a systemic febrile illness—differential diagnosis and management. *Best Practice Res Clin Rheum* 2006;4:627-40.
4. Thomas KT, Feder HM Jr, Lawton AR, Edwards KM. Periodic fever syndrome in children. *J Pediatr* 1999;135:15-21.
5. Toplak N, Frenkel J, Ozen S, Lachmann HJ, Woo P, Kone-Paut I, et al. An International registry on Autoinflammatory diseases: the Eurofever experience. *Ann Rheum Dis* 2012;71:1177-82.
6. Kastner DL, Aksentijevich I, Goldbach-Mansky R. Autoinflammatory disease reloaded: a clinical perspective. *Cell* 2010;140:784-90.
7. Schroder K, Tschopp J. The inflammasomes. *Cell* 2010;140:821-32.
8. Hoffman HM, Wanderer AA. Inflammasome and IL-1 β -mediated disorders. *Curr Allergy Asthma Rep* 2010;10:229-35.
9. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* 2010;10:210-5.
10. Stojanov S, Hoffmann F, Kery A, Renner ED, Hartl D, Lohse P, et al. Cytokine profile in PFAPA syndrome suggests continuous inflammation and reduced anti-inflammatory response. *Eur Cytokine Netw* 2006;17:90-7.
11. Brown KL, Wekell P, Osla V, Sundqvist M, Savman K, Fasth A, et al. Profile of blood cells and inflammatory mediators in periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome. *BMC Pediatr* 2010;10:65.
12. Cochard M, Clet J, Le L, Pillet P, Onrubia X, Gueron T, et al. PFAPA syndrome is not a sporadic disease. *Rheumatology* 2010;49:1984-7.
13. Foell D, Roth J. S100 proteins in monitoring inflammation: the importance of a gold standard and a validated methodology. *J Immunol* 2005;175:3459, author reply 3460.
14. Delaloye J, Roger T, Steiner-Tardivel QG, Le Roy D, Knaup Reymond M, Akira S, et al. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. *PLoS Pathog* 2009;5:e1000480.
15. Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, Schutz F, et al. *Leishmania* RNA virus controls the severity of mucocutaneous leishmaniasis. *Science* 2011;331:775-8.
16. Shinar Y, Obici L, Aksentijevich I, Bennetts B, Austrup F, Ceccherini I, et al. Guidelines for the genetic diagnosis of hereditary recurrent fevers. *Ann Rheum Dis* 2012 [Epub ahead of print].
17. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1 β in monocytes and macrophages. *Blood* 2009;113:2324-35.
18. Aksentijevich I, Putnam C, Remmers EF, Mueller JL, Le J, Kolodner RD, et al. The clinical continuum of cryopyrinopathies: novel CIAS1 mutations in North American patients and a new cryopyrin model. *Arthritis Rheum* 2007;56:1273-85.
19. Gattorno M, Piccini A, Lasigle D, Tassi S, Brisca G, Carta S, et al. The pattern of response to anti-interleukin-1 treatment distinguishes two subsets of patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2008;58:1505-15.
20. Frosch M, Ahlmann M, Vogl T, Wittkowski H, Wulffraat N, Foell D, et al. The myeloid-related proteins 8 and 14 complex, a novel ligand of toll-like receptor 4, and interleukin-1 β form a positive feedback mechanism in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2009;60:883-91.
21. Lachmann HJ, Lowe P, Felix SD, Rordorf C, Leslie K, Madhoo S, et al. In vivo regulation of interleukin 1 β in patients with cryopyrin-associated periodic syndromes. *J Exp Med* 2009;206:1029-36.
22. Stojanov S, Lapidus S, Chitkara P, Feder H, Salazar JC, Fleisher TA, et al. Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) is a disorder of innate immunity and Th1 activation responsive to IL-1 blockade. *Proc Natl Acad Sci U S A* 2011;108:7148-53.
23. Verma D, Sarndahl E, Andersson H, Eriksson P, Fredrikson M, Jonsson JI, et al. The Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to excessive interleukin-1 β and IL-18 production. *PLoS One* 2012;7:e34977.
24. Lachmann HJ, Quartier P, So A, Hawkins PN. The emerging role of interleukin-1 β in autoinflammatory diseases. *Arthritis Rheum* 2011;63:314-24.
25. Loock J, Lamprecht P, Timmann C, Mrowietz U, Csernok E, Gross WL. Genetic predisposition (NLRP3 V198M mutation) for IL-1-mediated inflammation in a patient with Schnitzler syndrome. *J Allergy Clin Immunol* 2010;125:500-2.
26. Ting TV, Scalzi LV, Hashkes PJ. Nonclassic neurologic features in cryopyrin-associated periodic syndromes. *Pediatr Neurol* 2007;36:338-41.
27. Pontillo A, Vendramin A, Catamo E, Fabris A, Crovella S. The missense variation Q705K in CIAS1/NALP3/NLRP3 gene and an NLRP1 haplotype are associated with celiac disease. *Am J Gastroenterol* 2011;106:539-44.
28. Schoultz I, Verma D, Halfvarsson J, Torkvist L, Fredrikson M, Sjoqvist U, et al. Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *Am J Gastroenterol* 2009;104:1180-8.
29. Marek-Yagel D, Bar-Joseph I, Pras E, Berkun Y. Is E148Q a benign polymorphism or a disease-causing mutation? *J Rheumatol* 2009;36:2372.
30. Booth DR, Lachmann HJ, Gillmore JD, Booth SE, Hawkins PN. Prevalence and significance of the familial Mediterranean fever gene mutation encoding pyrin Q148. *QJM* 2001;94:527-31.
31. Pelagatti MA, Meini A, Caorsi R, Cattalini M, Federici S, Zulian F, et al. Long-term clinical profile of children with the low-penetrance R92Q mutation of the TNFRSF1A gene. *Arthritis Rheum* 2011;63:1141-50.
32. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1 β -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 2004;20:319-25.
33. Gattorno M, Tassi S, Carta S, Delfino L, Ferlito F, Pelagatti MA, et al. Pattern of interleukin-1 β secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum* 2007;56:3138-48.
34. Aksentijevich I, Nowak M, Mallah M, Chae JJ, Watford WT, Hofmann SR, et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheum* 2002;46:3340-8.
35. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, Rubin BI, et al. Neonatal-onset multisystem inflammatory disease responsive to interleukin-1 β inhibition. *N Engl J Med* 2006;355:581-92.
36. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 2009;458:514-8.
37. Arostegui JJ, Lopez Saldana MD, Pascal M, Clemente D, Aymerich M, Balaguer F, et al. A somatic NLRP3 mutation as a cause of a sporadic case of chronic infantile neurologic, cutaneous, articular syndrome/neonatal-onset multisystem inflammatory disease: novel evidence of the role of low-level mosaicism as the pathophysiologic mechanism underlying Mendelian inherited diseases. *Arthritis Rheum* 2010;62:1158-66.
38. Tanaka N, Izawa K, Saito MK, Sakuma M, Oshima K, Ohara O, et al. High incidence of NLRP3 somatic mosaicism in patients with chronic infantile neurologic, cutaneous, articular syndrome: results of an International Multicenter Collaborative Study. *Arthritis Rheum* 2011;63:3625-32.