In conclusion, the *MEFV* variants, viz. *E148Q-L110P*, *P369-R408Q* may be associated with the onset of PFAPA, and some *MEFV* variants may affect the phenotype of PFAPA.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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Safety and efficacy of canakinumab in Japanese patients with phenotypes of cryopyrin-associated periodic syndrome as established in the first open-label, phase-3 pivotal study (24-week results)

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Abstract

Objectives

Cryopyrin-associated periodic syndrome (CAPS), a rare hereditary auto-inflammatory disease, is associated with mutations in the NLRP3 gene resulting in elevated interleukin-1β (IL-1 β) release. CAPS generally occurs in early childhood with most patients presenting with periodic fever, skin rash, osteoarthropathy, aseptic meningitis, sensorineural hearing loss and optic neuritis. Canakinumab, a fully human anti-IL-1β monoclonal antibody which binds selectively to IL-1β, has demonstrated good efficacy with CAPS. This is the first study to evaluate the safety and efficacy of canakinumab in Japanese patients with CAPS.

Methods

In this open-label study, 19 Japanese CAPS patients aged ≥2 years received canakinumab either 150 mg s.c. or 2 mg/kg for patients with a body weight ≤ 40 kg every 8 weeks for 24 weeks. The primary objective was to assess the proportion of patients who were free of relapse at week 24.

Results

A complete response was achieved in 18 (94.7%) patients with some requiring a dose and/or a frequency adjustment to attain full clinical response. The majority of patients (14/18; 77.8%) were in remission, i.e. free of relapse at week 24. Auto-inflammatory disease activity as assessed by physician's global assessment declined from baseline to end of the study (score of absent in 10.5% at baseline versus *31.6% at end of the study). Two patients had serious adverse events (SAEs), which resolved with standard treatment. One patient reported a mild injection-site reaction. No malignancies or deaths were reported during the study.*

Conclusion

Canakinumab 150 mg s.c. every 8 weeks was well-tolerated, highly efficacious and offered a convenient dosing regimen for treating Japanese patients with CAPS.

Key words

canakinumab, cryopyrin-associated periodic syndrome, interleukin-1β, auto inflammatory syndromes

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Competing interests: none declared.

Introduction

Cryopyrin-associated periodic syndrome (CAPS) represents a group of rare inherited auto-inflammatory diseases and encompasses phenotypes of varying severity. An increase in severity is evident between phenotypes: familial cold auto-inflammatory syndrome (FCAS) is the mildest, while Muckle-Wells syndrome (MWS) is predominantly of intermediate severity, and neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurological cutaneous and articular syndrome (CINCA) is the most severe phenotype of CAPS. All phenotypes are characterised by urticaria-like rash, fever, variant degree of central nervous system and tissue inflammation, arthropathy, risk of development of amyloidosis (1) and other constitutional symptoms. CAPS is associated with mutations of the *NLRP3* gene encoding cryopyrin (2-6), an important component of inflammasome. Inflammasone activates caspase-1, leading to enhanced production of the cytokine interleukin-1beta (IL-1β) and subsequent inflammation (7, 8). The pathogenic role of IL-1β in CAPS has been demonstrated by the achievement of complete response after treatment with IL-1β inhibitors (9-13). Positive therapeutic effects of the IL-1 receptor antagonist and anakinra have been hampered by the need for frequent injections (14-17) associated with severe pain, which impairs the quality of life of patients, especially the paediatric population. Canakinumab (ACZ885, Ilaris®, Novartis Pharma), a fully human anti-IL-1β monoclonal antibody (18), has shown prolonged selective IL-1β inhibition (19, 20) and has demonstrated rapid (within hours), complete and sustained response in CAPS patients of mainly Caucasian origin without any consistent pattern of side effects (21). Canakinumab is approved by the US Food and Drug Administration (FDA) for FCAS and MWS (22) only and by EMA for treatment of all three phenotypes of CAPS (23).

At present, there are no approved therapies for CAPS in Japan. The present study was therefore conducted to evaluate safety and efficacy of canakinumab in Japanese paediatric and adult patients with CAPS. Herein we report the study data up to 24 weeks.

Materials and methods

Study design, patients and study definitions

This was an open-label, safety and efficacy study of canakinumab administered for 24 weeks (6 months) in Japanese patients diagnosed with FCAS, MWS or NOMID. Molecular diagnosis showed that 17 (89.5%) patients were positive for *NLRP3* mutations and two (10.5%) patients (one each with MWS and NOMID) were negative for the mutation. The study included an extension phase to provide canakinumab treatment to study patients until canakinumab is marketed in Japan. Two NOMID patients aged 2 and 3 years previously treated with anti-IL-1 agents (anakinra) were enrolled.

Patients received canakinumab 150 mg s.c. or 2 mg/kg for those patients with body weight ≤40 kg for every 8 weeks. In case of residual symptoms, stepwise increase of the dose up to 600 mg s.c. or 8 mg/kg s.c. (≤40 kg) and/or increased dosing frequency were allowed.

After a 6-hour washout period for those patients previously treated with anakinra, 19 patients were included. Ten had received anakinra prior to study initiation, of which five patients had reported a complete response, while the remaining had achieved partial response to anakinra. Patients requiring oral steroids, NSAIDs and/or disease-modifying anti-rheumatic drugs (DMARDs) were enrolled if they were on a stable dose (oral steroids: <20 mg/day or ≤0.4 mg/kg prednisone or prednisone equivalent, whichever applies) for at least 4 weeks prior to the screening visit. Steroid therapy was tapered after the first canakinumab treatment cycle (8 weeks between doses), at the discretion of the investigator. TNF-α inhibitors and IL-6 receptor blockers were not allowed during the study. Women of child bearing potential had to use an accepted form of contraception during the study and for at least 3 months after the last dose. Patients receiving live vaccine within 3 months before recruitment were excluded.

Complete response assessed at day 15 and day 29 was defined by (i) physician's global assessment of no or minimal auto-inflammatory disease (on a 5-point Likert scale ranging from absent, minimal, mild, moderate to severe) and assessment of no or minimal skin disease, and (ii) serological remission defined as serum CRP <1 mg/dL, and/or SAA <10 μg/mL. Patients who did not achieve (or maintain) complete response following canakinumab injection in any treatment period could receive a dose escalation (supporting Fig. 1). The possible step-wise up-titration regimens were: 300 mg s.c. (or 4 mg/kg for patients with a body weight ≤40 kg), 450 mg s.c. (or 6 mg/kg for patients with a body weight ≤40 kg), and 600 mg s.c. (or 8 mg/kg for patients with a body weight ≤40 kg).

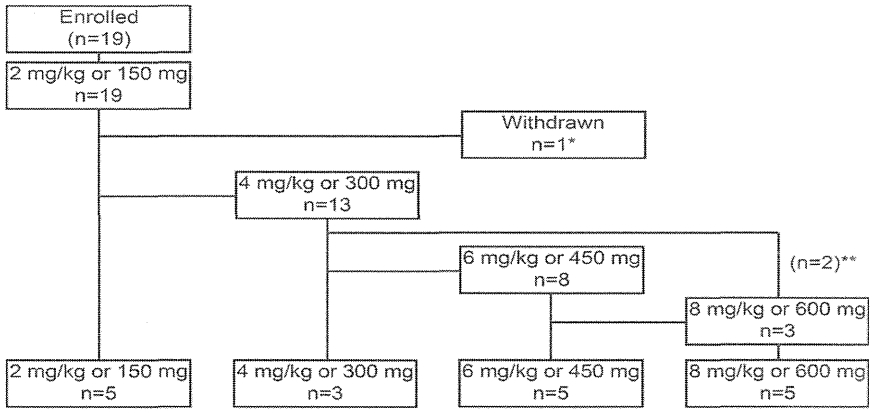
The primary efficacy endpoint was defined as the proportion of patients who did not experience a relapse at week 24. Relapse was defined as clinical relapse (physician's global assessment of both auto-inflammatory disease activity and assessment of skin disease, mild or greater) and serological relapse (serum CRP >3 mg/dL, and/or SAA >30 μg/mL).

Clinical improvement of the central nervous system (CNS) was assessed in NOMID patients only (defined as a mean weekly headache score [from the daily diary] <0.5 and a normal white cell count [≤15 cells/mm^3] in cerebrospinal fluid). Other key secondary endpoints included safety and tolerability of canakinumab, assessed by the occurrence of adverse events (AEs), serious AEs (SAEs) and immunogenicity.

This study was approved by the Independent Ethics Committee for each centre and performed in accordance to the ethical principles of the Declaration of Helsinki. All patients, parents or legal guardians (for patients aged <20 years) provided written informed consent.

Statistical analyses

Safety and full analysis set (efficacy analysis) included all patients who received at least one dose of study treatment. Only 19 patients were enrolled due to the low prevalence of CAPS, hence the estimation of statistical power was not applicable. Descriptive statistics were used to summarise demographics, baseline characteristics, efficacy and safety. Missing values were not imputed.



*One patient withdrew from this study by cancellation of the consent.

**Two patients needed two up titrations till Day 15 due to incomplete response to the first administration of canakinumab. Patients with incomplete response from the standard dosing regimen (2 mg/kg or 150 mg) received step-wise up-titration regimen. Patients who did not achieve complete response or had a relapse before the next planned administration received a dose up-titration.

Fig. 1. Patient disposition and dosing.

Results

Patients, demographic and baseline characteristics

A total of 19 CAPS patients (12 [63.2%] male/7 [36.8%] female) with a diagnosis of MWS (n=7; 36.8%) or NOMID (n=12; 63.2%) were enrolled in this study, of which 18 (94.7%) completed the 24-week study phase. One patient withdrew consent (Fig. 1). At study entry, there were 11 patients (57.9%) aged <16 years and eight patients (42.1%) aged 16 years or older. Median age was 14 years (range 2–48). Of 19 patients, five (26.3%) weighed >40 kg at baseline. Other key demographic and baseline characteristics are summarised in Table I.

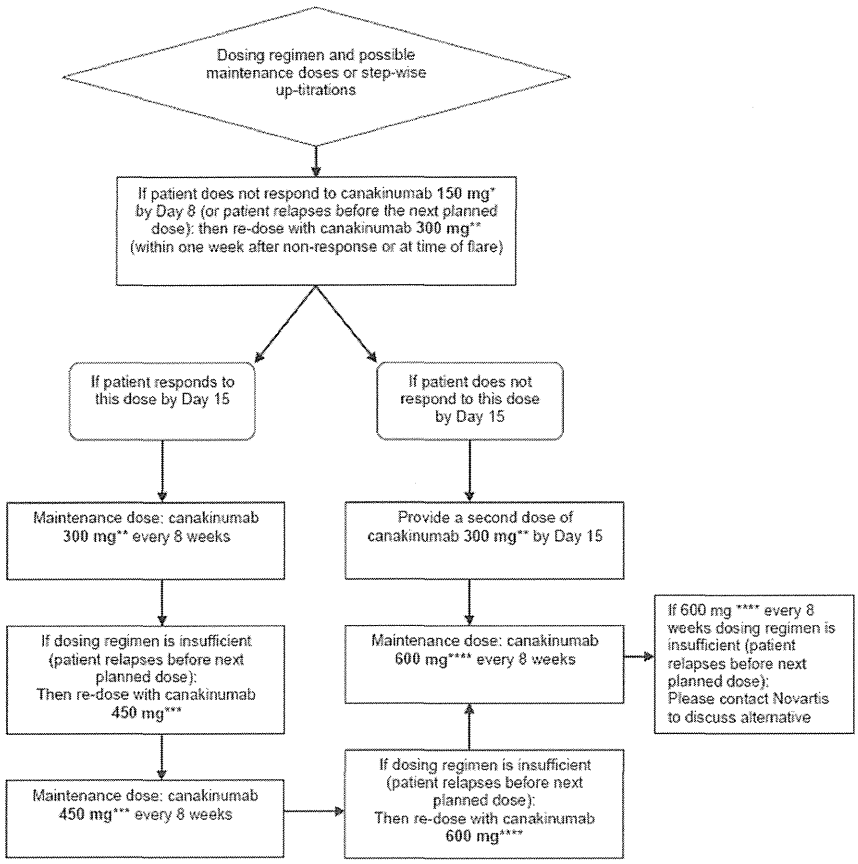
Treatment with canakinumab

At time of the 24-week analysis, the median treatment duration was 168 days (range 59–197 days) and patients received an average of 4.1 injections over 24 weeks of the study; 13 (68%) patients (MWS; n=4 and NOMID, n=9) received an up-titration of their dose, primarily due to absence of a complete response and in one patient the dose frequency was increased to every 6 weeks starting from day 49. In one NOMID patient aged 16 years, the

Table I. Baseline demographics and disease characteristics (safety population).

Characteristics	Canakinumab (n=19)
Sex, n (%)	
Male	12 (63.2)
Female	7 (36.8)
Age (years)	
Mean (SD)	14.8 (11.4)
Median (range)	14.0 (2–48)
≥2– <12 years, n (%)	8.0 (42.1)
≥12– <16 years, n (%)	3 (15.8)
≥16 years, n (%)	8 (42.1)
Weight (kg), n (%)	
≤40	14 (73.7)
>40	5 (26.3)
BMI (kg/m^2)	
Mean (SD)	17.6 (2.2)
Median (range)	17.2 (13.5–21.5)
Diagnosis, n (%)	
FCAS	0
MWS	7 (36.8)
NOMID	12 (63.2)
Molecular diagnosis of	
NLRP3 mutation, n (%)	19 (100.0)
Positive	17 (89.5%)
Negative	2 (10.5%)
Previous use of anakinra, n (%)	10 (52.6)
C-reactive protein (mg/dL) (normal value: <1mg/dL)	
Mean (SD)	4.52 (4.3)
Median (range)	3.3 (0.1–13.2)
Serum Amyloid A (μg/mL) (normal value: <10μg/mL)	
Mean (SD)	324.2 (364)
Median (range)	236 (2.6–1380)

BMI: body mass index; FCAS: familial cold autoinflammatory syndrome; MWS: Muckle-Wells syndrome; NOMID: neonatal-onset multisystem inflammatory disease; NLRP3: NOD-like receptor family, pyrin domain containing3; SD: standard deviation.



* canakinumab 150 mg s.c. for patients whose body weight is > 40 kg (or 2 mg/kg for patients with a body weight ≤ 40 kg)
** canakinumab 300 mg s.c. (or 4 mg/kg for patients with a body weight ≤ 40 kg)
*** canakinumab 450 mg s.c. (or 6 mg/kg for patients with a body weight ≤ 40 kg)
**** canakinumab 600 mg s.c. (or 8 mg/kg for patients with a body weight ≤ 40 kg)
There is currently no long-term safety information for doses greater than 600 mg s.c. available.
The above outlined decision tree may be applied to those patients who either did not achieve a complete response by Day 8 or Day 15 or to those patients who relapse prior to their next scheduled dose.

Supporting Fig. 1. Alternative dosing regimen for CAPS patients who do not experience sufficient symptomatic relief.

canakinumab dose was escalated to the highest dose of 600 mg. Four patients (8–25 years) with baseline body weight ≤40kg received a dose escalation to 8 mg/kg.

Proportionally higher mean last doses of canakinumab were required in patients ≤40 kg (n=12) *versus* >40 kg (n=6) at 6 mg/kg and 250 mg, respectively; in patients weighing >40 kg, the canakinumab dose administered was 350 and 150 mg for NOMID and MWS, respectively.

Efficacy

Relapse assessment. Overall, protocol-defined complete response was achieved in 18 (94.7%) patients. One patient achieved a complete response by day 148. This patient achieved clinical remission by day 29, but the inflammatory markers remained elevated until day 148. One non-responder patient achieved clinical remission, but the patient's CRP and SAA levels remained above normal during the study; however there was a significant decrease by week 24 compared to baseline. Some patients required either a dose escalation and/or a frequency adjustment to attain full clinical response (supporting Fig. 1); 15 (78.9%) patients achieved a complete response within 15 days, 2 patients were up-titrated within 29 days, and 1 patient by day 148. At week 24, the majority of patients (n=14/18 [77.8%]) were in remission, *i.e.* free of relapse (Table II).

Table II. Relapse at week 24 in MWS and NOMID patients (full analysis set).

Characteristics	Canakinumab n=19 n (%)
Number of complete responders by week 24	
Total	18 (94.7)
Day 15*	15 (78.9)
Day 29*	2 (10.5)
Day 148*	1 (5.3)
Relapse at week 24	4 (22.2)
No relapse at week 24	14 (77.8)
MWS patients	6 (85.7)
NOMID patients	8 (72.7)
No clinical/serological relapse at week 24	12 (66.7)
Discontinue prematurely prior to week 24	1 (5.6)

*Patients requiring either a dose and/or a frequency adjustment to attain full clinical response.
MWS: Muckle-Wells syndrome; NOMID: neonatal-onset multisystem inflammatory disease.

Of 12 NOMID patients, 11 achieved complete response by week 24 and nine achieved a complete response by day 15; one achieved complete response with dose adjustment by day 29 and one by day 148. Three (27.3%) out of the 11 complete responders (all NOMID patients) had a relapse at week 24. All patients with MWS (n=7) achieved complete response by week 24, though one patient had a relapse at week 24. All except one patient achieved complete response with canakinumab. All prior responders to anakinra also achieved a complete response with canakinumab.

Auto-inflammatory disease activity

The severity of auto-inflammatory disease activity as assessed by physician's global assessment declined from baseline to the end of the treatment period. This decrease in disease activity was apparent in all the individual symptom components including assessments of skin disease, headache/migraine, conjunctivitis and fatigue/malaise (Fig. 2).

Inflammatory markers

Canakinumab treatment induced a rapid decline in CRP levels within 15 days (Fig. 3a). Overall, mean CRP levels decreased by 2.94±2.99 mg/dL (38% decrease) from baseline to end of the study, day 169 (4.52 mg/dL *vs.* 1.19

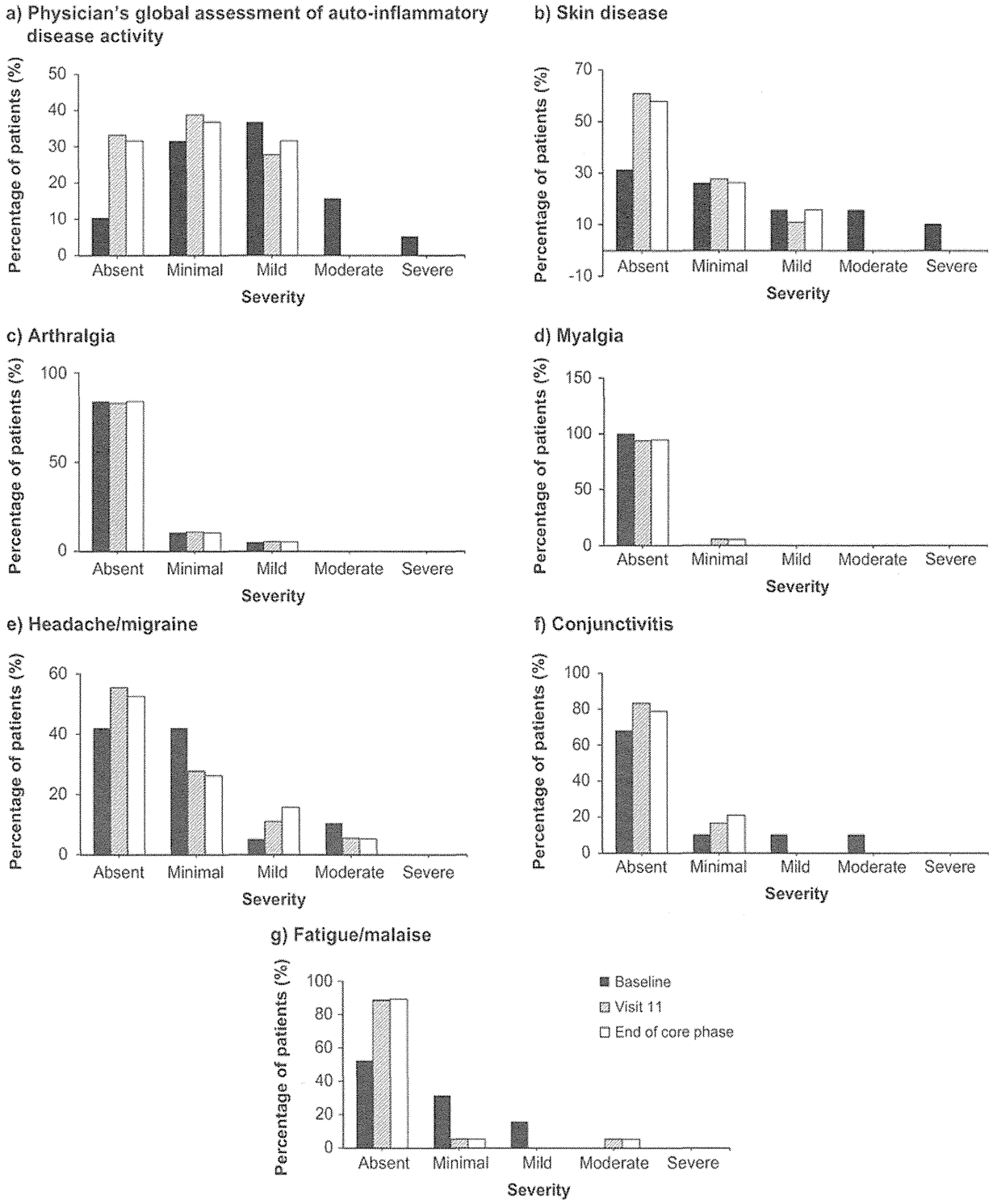


Fig. 2. Summary of assessment of auto-inflammatory disease activity (full analysis set).

mg/dL). A similar trend was observed for mean serum SAA level, which decreased from baseline to end of the study (324.19 μg/mL *vs.* 54.71 μg/mL) (Fig. 3b). On day 57, there was an increase in CRP and SAA levels, however this was driven by measurements from three patients whose mean values were near normal at other time points.

Immunogencity

Of the 19 patients, three were detected with anti-canakinumab binding antibodies during one of the post-dose assessments. However, no anti-canakinumab antibodies were detected afterwards.

Specific assessments in NOMID patients

A protocol-defined CNS remission was achieved in 33.3% (n=4/12) of the NO-

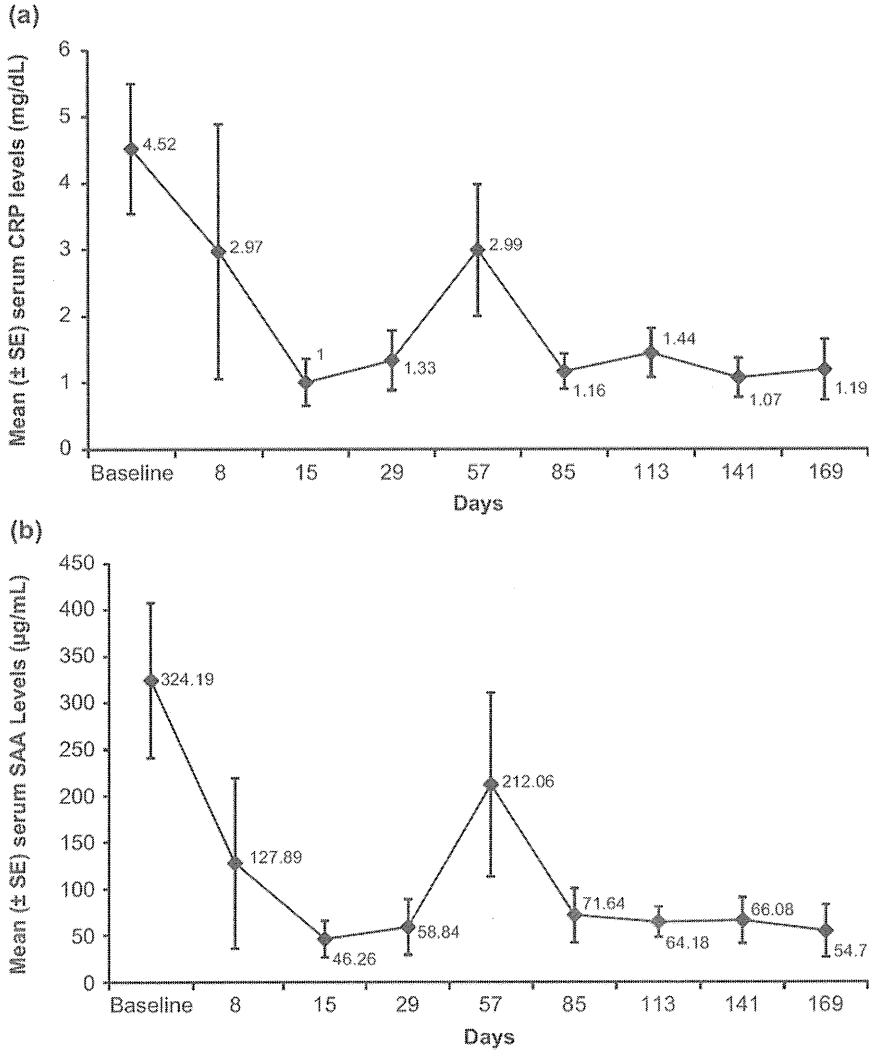


Fig. 3. **(a)** Serum CRP level across time points (full analysis set); **(b)** Serum SAA levels across time points.

MID patients by day 8 and in 41.7% (n=5/12) at the end of study; 9/12 patients had CNS remission at week 24 (with just the headache score). Lumbar puncture was only performed in 7/12 patients, of which five were in CNS remission based on the headache score and normal white cell count. A CNS relapse was reported in two (16.7%) patients on day 57 and in one patient (9.1%) on day 113. Of the three patients with a protocol-defined CNS relapse, one was uptitrated from 4mg/kg to 6mg/kg due to a concomitant clinical and serological relapse. In the other two patients, no uptitration was performed for CNS relapse. In these three patients, there was no association between the CNS relapse and clinical flare. The results of key cerebrospinal fluid assessments in NOMID patients were available in only 6 patients, who had both baseline and week 24 values. In these patients (n=6), mononuclear cells (lymphocytes, macrophages, monocytes) remained unchanged or elevated slightly from baseline to week 24 (normal values: adult ≤5 WBC/mm^3, newborns ≤20 WBC/mm^3). Absolute neutrophils which markedly reduced in two NOMID patients remained largely unchanged in the other three patients, even though it was elevated in one patient at week 24 compared with baseline. None of these patients reported headache, but they were noted to have elevated CRP and/or SAA levels. In addition to elevated SAA levels, one patient had physician's global assessment of autoinflammatory disease activity above minimal and had a relapse at week 24.

Table III. Most frequently occurring (>10%) adverse events regardless of study drug relationship (safety population).

Primary system organ class/ preferred term	Canakinumab n=19 n (%)
Total patients with AEs	18 (94.7)
Gastrointestinal disorders	7 (36.8)
Abdominal pain upper	2 (10.5)
Diarrhoea	2 (10.5)
Stomatitis	2 (10.5)
General disorders and administration site conditions	3 (15.8)
Infections and infestations	16 (84.2)
Nasopharyngitis	7 (36.8)
Gastroenteritis	6 (31.6)
Upper respiratory tract infection	3 (15.8)
Nervous system disorders	2 (10.5)
Respiratory, thoracic and mediastinal disorders	5 (26.3)
Rhinorrhoea	3 (15.8)
Cough	2 (10.5)
Skin and subcutaneous tissue disorders	6 (31.6)
Acne	2 (10.5)
Dry skin	2 (10.5)
Urticaria	2 (10.5)
Vascular disorders	2 (10.5)
Hypertension	2 (10.5)

A patient with multiple occurrences of an AE is counted only once in the AE category. A patient with multiple adverse events within a primary system organ class is counted only once in the total row. AE: adverse event.

Safety

Overall, 18 (94.7%) patients experienced at least one AE. The most commonly reported AEs (≥15% of patients) were nasopharyngitis (n=7, 36.8%), gastroenteritis (n=6, 31.6%), upper respiratory tract infection (n=3, 15.8%), and rhinorrhea (n=3, 15.8%). Twelve (63.2%) patients reported AEs, which were suspected to be study drug-related (Table III). The majority of AEs were mild (n=13, 68.4%) or moderate (n=3, 15.8%) in severity. Severe AEs of diffuse vasculitis and pneumonia were each reported in one (5.3%) patient. All but one MWS patient experienced at least one AE. Nasopharyngitis was reported in a higher proportion of NOMID patients (n=6, 50%) compared to MWS patients (n=1, 14.3%). All other AEs in NOMID and MWS patients occurred at similar frequencies or in less than three patients in each group. Two patients had serious AEs, which were suspected to be treat-

ment-related (Parvovirus infection and Epstein-Barr virus infection [n=1] and pneumonia [n=1]), but resolved with standard treatment. Of the 19 patients, only one reported a mild injection-site reaction. No deaths were reported during the study. Higher canakinumab s.c. doses (>150mg or 2mg/kg q8wks) did not appear to be associated with a differential safety profile.

Discussion

The present study confirms the clinical and serological efficacy of canakinumab in a Japanese population of paediatric and adult CAPS patients presenting with the most severe NOMID and MWS-phenotypes. Eighteen (94.7%) out of 19 patients enrolled in this study have achieved a complete response with some patients requiring either a dose and/or a frequency adjustment to attain full clinical response. For most patients (78.9%), irrespective of CAPS phenotype, a complete response was achieved with the standard subcutaneous canakinumab dose (13), *i.e.* 150 mg (>40 kg body weight) or 2 mg/kg (≤40 kg body weight) every 8 weeks.

All clinical symptoms frequently observed in CAPS patients such as inflammation of skin, eyes, bones, joints and meninges accompanied by recurrent fever, severe fatigue, myalgia and headache, showed an improvement during canakinumab treatment. Improvement in clinical outcomes with canakinumab therapy such as autoinflammatory disease activity, and reduction in the levels of acute phase proteins such as CRP and SAA confirms the pivotal role of IL-1β and its inhibition in CAPS.

The sustained effects of canakinumab on patient's clinical symptoms have been associated with its mean terminal half-life of 26 days and a possibly disease-modifying effect through autocrine down-regulation of IL-1β production (19). The canakinumab administration schedule of one injection every 8 weeks and the low incidence of injection-site reactions, as previously observed in other phase II and III canakinumab CAPS studies (21, 25, 26), may be beneficial, especially to paediatric patients.

In the present study, individualised up-titration in patients with an incomplete response proved to be a safe and an efficacious approach for the majority of patients achieving a complete response within one month. Patients with incomplete response, as shown by changes in clinical symptoms (headache, fever or rash according to CAPS) and raised inflammatory marker levels (elevated CRP >3 mg/dL, and/or SAA >30 μg/mL), had initially received canakunimab titrated up to 8 mg/kg. The dosage interval was shortened by up to four weeks if patients failed to achieve a complete response. There was no clear correlation between the genotype, phenotype, and treatment response. The mean dose requirement for patients ≤40 kg was found to be proportionally higher (6 mg/kg) than for those with a body weight >40 kg (250 mg). In the group of patients with a body weight >40kg, the NOMID patient subgroup required a higher mean dose compared to the MWS patient subgroup, in line with the level of severity of the disease.

At baseline, 12 NOMID patients presented with CNS symptoms that included headache and pleocytosis and 9 showed improvement in these symptoms by week 24. Patients showed no significant changes, either worsening or improvement, based on audiogram and neurological or ophthalmic assessments. Two patients showed normalisation in auditory acuity and one patient showed normalisation in visual acuity. There were no organic changes observed on magnetic resonance imaging (MRI). This may be attributed to the fact that the observation period was relatively short and approximately 53% of patients were pre-treated with anakinra at the time of the study entry.

In the present study, no patients discontinued due to unsatisfactory therapeutic effect, suggesting that an effective individual canakinumab dosing regimen was determined. The safety profile was comparable to that observed in previous canakinumab studies (21, 24), with no new or unexpected safety findings. Consistently with previous studies and other biologics, infections were the most frequent AEs and the patients responded well to standard therapy. There were no deaths, discontinuations nor dose adjustments/or interruptions due to AEs. In 3 out of 19 patients, anti-canakinumab binding antibodies were detected in one of the post-dose visits, however these patients showed no evidence of immunogenicity related AEs or impaired efficacy. The overall safety profile observed in previous canakinumab studies in CAPS was confirmed in this Japanese population including the paediatric and NOMID sub-populations.

The present study has limitations, including the small size of the patient population, the non-controlled design and the relatively short-term observation period, each of which were addressed in previous studies. Additionally, the small sample size and short follow-up period did not allow detailed assessment of side effects related to anti-IL-1 therapy such as malignant disease and autoimmunity. Long-term observation with a large population is needed to address these issues (27).

Conclusion

Canakinumab 150 mg s.c. dosed every 8 weeks proved to be efficacious and provided a convenient dosing regimen for treating Japanese patients with CAPS. Higher canakinumab doses in younger patients and in adult patients with more severe CAPS disease were efficacious in achieving a complete response and were well tolerated without any evidence of increased AEs. While these results for the treatment of CAPS with canakinumab for up to 197 days are encouraging, the long-term safety of canakinumab in CAPS patients will be further evaluated in this ongoing study.

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PLOS ONE

The Contribution of *SAA1* Polymorphisms to Familial Mediterranean Fever Susceptibility in the Japanese Population

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Abstract

Background/Aims: Familial Mediterranean Fever (FMF) has traditionally been considered to be an autosomal-recessive disease, however, it has been observed that substantial numbers of patients with FMF possess only 1 demonstrable *MEFV* mutation. The clinical profile of familial Mediterranean fever (FMF) may be influenced by *MEFV* allelic heterogeneity and other genetic and/or environmental factors.

Methodology/Principal Findings: In view of the inflammatory nature of FMF, we investigated whether serum amyloid A (SAA) and interleukin-1 beta (IL-1β) gene polymorphisms may affect the susceptibility of Japanese patients with FMF. The genotypes of the -13C/T SNP in the 5′-flanking region of the *SAA1* gene and the two SNPs within exon 3 of *SAA1* (2995C/T and 3010C/T polymorphisms) were determined in 83 Japanese patients with FMF and 200 healthy controls. The same samples were genotyped for IL-1β-511 (C/T) and IL-1 receptor antagonist (IL-1Ra) variable number of tandem repeat (VNTR) polymorphisms. There were no significant differences between FMF patients and healthy subjects in the genotypic distribution of *IL-1β -511* (C/T), *IL-1Ra* VNTR and *SAA2* polymorphisms. The frequencies of *SAA1.1* allele were significantly lower (21.7% versus 34.0%), and inversely the frequencies of *SAA1.3* allele were higher (48.8% versus 37.5%) in FMF patients compared with healthy subjects. The frequency of -13T alleles, associated with the *SAA1.3* allele in the Japanese population, was significantly higher (56.0% versus 41.0%, $p = 0.001$) in FMF patients compared with healthy subjects.

Conclusions/Significance: Our data indicate that *SAA1* gene polymorphisms, consisting of -13T/C SNP in the 5′-flanking region and SNPs within exon 3 (2995C/T and 3010C/T polymorphisms) of *SAA1* gene, are associated with susceptibility to FMF in the Japanese population.

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Introduction

FMF is an inherited autoinflammatory disease characterized by recurrent self-limited fever, and serositis [1]. These episodes of inflammation are mainly mediated by a massive influx of neutrophils into serous cavities and are accompanied by an elevation of acute phase reactants [2]. The disease is associated with mutations in the *MEFV* gene that encodes pyrin, and is transmitted in an autosomal-recessive manner [3]. Therefore, heterozygotes are expected to be carriers or lack the clinical phenotype of FMF. However, mutations in the second *MEFV* allele have not been observed in 20–30% of patients with typical FMF [4]. Recent studies suggest that subjects with a single *MEFV* mutation may cross a threshold for the development of an FMF phenotype if they also express a combination of gene polymorphisms that favor increased inflammation [5]. These polymor-

phisms are thought to belong to genes of the interleukin-1β/innate immune system pathways [5]. The IL-1 family of cytokines is critical to the host's response to infection, and induction of innate immunity and acute phase inflammation [6]. The overproduction of IL-1β is responsible for a variety of autoinflammatory syndromes including FMF [7]. IL-1β requires cleavage via caspase-1 for proper secretion, which is facilitated by inflammasome activation [8]. The NOD-like receptor family, pryin domain containing 3 (NLRP3) inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid protein [9].

Recent studies have shown that serum amyloid A (SAA) induced the expression of pro-IL-1β and activated the NLRP3 inflammasome in a cathepsin B and $P2X_7$-dependent manner resulting in secretion of mature IL-1β [10]. SAA is an acute-phase protein, which increases in the serum during inflammation and is susceptible to proteolytic cleavage to amyloid A (AA) protein, the major fibrillar protein in secondary amyloidosis [11]. An allelic variant of *SAA1.3*, was found to be associated with AA amyloidosis in Japanese rheumatoid arthritis (RA) patients [12]. In view of the recent genetic studies in FMF, other modifying genetic factors may contribute to the susceptibility or clinical expression of FMF in addition to *MEFV* mutations. Therefore, we attempted to determine the effect of gene polymorphisms on the susceptibility to FMF in the Japanese population.

Materials and Methods

Patients

In early 2007, a laboratory network collecting the genetic diagnosis of periodic fever was established at the Japan Autoinflammation Association (JAA), and *MEFV* gene analysis was carried out at the Clinical Research Center of National Hospital Organization (NHO) Nagasaki Medical Center. Up to October 2012, 481 consecutive unrelated patients with periodic fever were referred and underwent molecular diagnosis at the NHO Nagasaki Medical Center. All patient, who were originating from Japan, (East Japan n = 36, West Japan n = 47) were asked to complete a questionnaire that included demographics (sex, age of onset), family history (consanguinity of parents, family history of recurrent fever), and the presence of recurrent febrile attacks typical of FMF, including peritonitis, pleuritis, arthritis, and transient inflammatory responses. The genetic analysis of *MEFV* gene was approved by the Ethics Committee of Nagasaki Medical Center, and written informed consent was obtained from each individual. On the basis of Tel-Hashomer criteria [13], we divided the FMF patients in two groups: Group 1, typical FMF exhibiting the presence of 1 or more major criteria independent to the presence of minor criteria; Group 2, incomplete FMF exhibiting the absence of major criteria and 2 or more minor criteria. It is important to stress that response to colchicine was confirmed in almost all patients. As controls, 200 healthy Japanese individuals without pre-existing medical diseases (90 men and 110 women 14 to 64 years, with a mean age of 38.6±13.9 years) from East Japan (n = 86) and West Japan (n = 114) were enrolled in the study after obtaining informed consent.

MEFV gene Mutation analysis

All patients were undergone genetic analysis of *MEFV* gene exons 1, 2, 3 and 10 by direct sequencing. 2 milliliters of blood samples were collected from all subjects. Genomic DNA was extracted from whole blood by means of the Promega Wizard® Genomic DNA Purification Kit (Promega, USA). Mutation analysis was performed by genomic sequencing as described previously [14].

Genotyping

***SAA1* gene.** The genotype of the SAA1 -13C/T in the 5-region of exon 1 (rs11024595) was determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [15]. The primers used for the PCR reaction were 5′-ACATCT TGTTCCCTC AGGTTG-3′ (sense) and 5′-GCTGTAGCTGAGCTGCGG-3′ (antisense).

The 229-bp PCR products were digested with restriction enzyme AciI (BioLabs, Beverly, MA, USA) and electrophoresed on a 12.5% polyacrylamide gel [15].

The *SAA1.1, 1.3*, and *1.5* alleles, corresponding to the T-C, C-T, and C-C haplotypes of the C2995T (rs1136743) and C3010T (rs1136747) polymorphisms were also determined by the PCR-RFLP [15]. The primers used for the PCR reaction were 5′-GCC AATTACATCGGCCTCAG-3′ (sense) and 5′-TGGCCA AA-GAATCTCTGG AT-3′ (antisense).

The 518-bp PCR products were digested with restriction enzyme BclI (Promega, San Luis Obispo, CA, USA) and BanI (Promega) and electrophoresed on a 2.5% agarose gel [15].

The genotype of the *SAA2* (rs2468844) was determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. The primers used for the PCR reaction were 5′-AGAGAATATCCAGAGACTCACAGGC-3′ (sense) and 5′-CAGGCCAGCAGGTCGGAAGT-3′(antisense). The 115 bp PCR products were digested with the restriction enzyme Nco I. The digested products were separated by 3% agarose gels by ethidium bromide staining [15].

IL-1Ra. For the IL-1RA VNTR polymorphism, the region including variable numbers of identical 86-bp tandem repeats was amplified by PCR using the following primers: 5′-CTCAGC-CAACACTCCTAT-3′ (sense) and 5′-TCCTGGTCTGCAGG-TAA-3′ (antisense). PCR products of 240(allele 2, two repeats), 325 (allele 3, three repeats), 410 (allele 4, four repeats), and 500 bp (allele 5, five repeats) were distinguished by agarose gel electrophoresis [16].

IL-1B-511. A fragment containing the *AvaI* polymorphic site at promoter region -511 of the IL-1B gene was amplified by PCR. PCR was carried out with primers, forward primer 5′-GCCTGAACCCTGCATACCGT-3′ (sense). 5′-GCCAA-TAGCCCTTGTCT-3′ (antisense). Fragments were separated by electrophoresis on 3% agarose with ethidium bromide staining using appropriate commercially available size markers for comparison. The C allele was designated if two bands of 92 and 63 bp were obtained, and the T allele was designated if a signal band of the undigested 155 bp was obtained. Genotypes were designated as follows: C/C, two bands of 92 and 63 bp: C/T, three bands of 155, 92, and 63 bp; and T/T, a single band of 155 bp [16].

Statistical Analysis

Results are expressed as mean±SD. Statistical analysis was performed with SPSS18 for windows (SPSS Statistics, Illinois). The statistical significance of differences between groups was calculated by either the chi-square test for categorical data and Mann-Whitney's U-test for quantitative data. Deviation from Hardy-Weinberg equilibrium was assessed using the SNPAlyze software ver. 7.0 (Dynacom, Yokohama, Japan). A p value of <0.05 was considered significant.

Table 1. *MEFV* genotypes, gender, and the presence of amyloidosis in 83 Japanese patients with FMF.

MEFV genotypes	n(%)	Typical	(Male/Female)	Incomplete	(Male/Female)	Amyloidosis	*p* value
M694I/M694I	4(4.8)	4	(1/3)			1	
M694I/normal	4(4.8)	4	(4/0)				
M694I/E148Q	13(15.7)	13	(10/3)				
M694I/P751L	1(1.2)	1	(0/1)				
M694I/E148Q/E148Q	1(1.2)	1	(0/1)				
M694I/E148Q/L110P	5(6.0)	5	(3/2)			2	
P369S/R408Q	4(4.8)			4	(1/3)		
E148Q/P369S/R408Q	3(3.6)			3	(0/3)		
E148Q/E148Q/P369S/R408Q	4(4.8)	2	(2/0)	2	(0/2)		
E148Q/R202Q/P369S/R408Q	1(1.2)			1	(1/0)	1	
E148Q/G304R/P369S/R408Q	1(1.2)			1	(0/1)		
E148Q/E148Q/P369S/P369S/R408Q/R408Q	1(1.2)			1	(0/1)		
E148Q/normal	12(14.5)	6	(3/3)	6	(1/5)		
R202Q/normal	2(2.4)	1	(1/0)	1	(0/1)		
G304R/normal	1(1.2)			1	(1/0)		
E148Q/E148Q	1(1.2)	1	(0/1)				
E148Q/L110P	6(7.2)	1	(0/1)	5	(1/4)		
E148Q/R202Q	1(1.2)	1	(0/1)				
E148Q/E148Q/L110P	3(3.6)	1	(1/0)	2	(2/0)		
E148Q/L110P/R202Q	2(2.4)			2	(0/2)		
E84K/normal	8(9.6)	5	(3/2)	3	(1/2)		
E84K/E148Q	1(1.2)			1	(0/1)		
E84K/G304R	1(1.2)			1	(0/1)		
Normal	3(3.6)			3	(1/2)		
Gender (Male/Fmale)			(28/18)		(9/28)		$P<0.0001$
Age (years)			36.2±18.2		39.9±19.6		$p=0.419$
Total			46		37		

Data are expressed as number (percentage). ± ; standard deviation. *p* values were calculated with chis-square test for qualitative data and Mann-Whitney test for quantitative data.

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Results

Demographic data and MEFV genotypes

We diagnosed 83 subjects, all of Japanese origins, as FMF. Among these patients, 44 were diagnosed as typical FMF and 37 were diagnosed as incomplete FMF. The demographic data of the newly-diagnosed FMF patients are summarized in Table 1. The overall male: female ration in patients with FMF was 0.8 (37:46). In incomplete FMF patients, the more affected sex is female in contrast to typical FMF (Table 1). The mean age ± SD at diagnosis was 37.9±18.8 years. Age at diagnosis of patients with typical FMF was similar to those with incomplete FMF (36.2±18.2 and 39.9±19.6 years, respectively; $p=0.419$; Table 1). By mutation analysis, the *MEFV* gene mutation could not be identified in 3 of 83 patients (3.6%). The distribution of the *MEFV* genotype was heterogenous. The most frequent genotype was M694I/E148Q, followed by E148Q/normal and E84K/normal. AA amyloidosis was histologically confirmed in 4 patients with FMF, whose genotypes were M694I/M694I *SAA1.5/15*, M694I/E148Q/L110P *SAA1.1/1.1*, M694I/E148Q/L110P *SAA1.3/1.5* and E148Q/R202Q/P369S/R408Q *SAA1.3/1.5*.

IL-1β and *IL-1Ra* gene polymorphism

The genotype frequencies of IL-1β-511 (C/T), and IL-1Ra VNTR polymorphisms in FMF patients and healthy subjects are summarized in Table 2. There were no significant difference in the frequencies of these polymorphisms between FMF patients and healthy subjects.

Association between *SAA2* gene polymorphism and FMF

There was no significant difference in the frequencies of the *SAA2* genotype between FMF patients and healthy subjects (Table 2).

Association between *SAA1* gene polymorphisms and FMF

A segment of the genomic *SAA1* gene with polymorphic sites was subjected to PCR/restriction fragment length polymorphism (PCR-RFLP) analysis. Table 3 shows the frequencies of individuals with various genotypes and alleles at the *SAA1* locus in either FMF patients (n = 83) or Japanese healthy subjects (n = 200). The allele frequency of *SAA1.1* was significantly lower in FMF patients compared with healthy subjects (21.7% versus 34.0%). Conversely,

Table 2. Frequencies of the genotypes at the *IL-1β* -511, *IL-1Ra* and *SAA2* loci in patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotype at *IL-1β* -511 locus			
C/C	27(32.5)	59(29.5)	$\chi^2 = 0.934$ $p = 0.627$
C/T	43(51.8)	100(50.0)	
T/T	13(15.7)	41(20.5)	
Genotype at *IL-1Ra* locus			
1/1	73(88.0)	167(83.5)	$\chi^2 = 2.451$ $p = 0.857$
1/2	5(6.0)	20(10.0)	
1/3	0	1(0.5)	
1/4	4(4.8)	7(3.5)	
2/2	0	2(1.0)	
2/4	1(1.2)	3(1.5)	
Genotype at *SAA2* locus			
A/A	62(74.7)	163(81.5)	$\chi^2 = 2.338$ $p = 0.276$
A/G	19(22.9)	35(17.5)	
G/G	2(2.4)	2(1.0)	

IL-1β; Interleukin-*1β* .*IL-1Ra*; Interleukin-1 receptor antagonist .*SAA2*; Serum amyloid A2. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
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the allele frequency of *SAA1.3* was higher in FMF patients compared with healthy subjects (48.8% versus 37.5%).

The -13C/T polymorphism, in the 5′-flanking region of the *SAA1* gene is associated with the *SAA1.3* allele and susceptibility to amyloidosis in Japanese RA patients [17]. We analyzed the frequency of -13C/T polymorphisms in FMF patients and Japanese healthy subjects. Allele frequencies of -13C/T were different among these two groups (Table 4), and -13T allele was significantly increased in FMF patients compared with healthy subjects (56.0% versus 41.0%, $p = 0.001$). These data suggest that the -13T allele is associated with susceptibility to FMF in the Japanese population. Allele frequencies of -13 C/T polymorphisms were also analyzed in typical or incomplete FMF patients. There was no significant difference in the frequencies -13T allele between typical and incomplete FMF patients (Table 5). Among 83 patients with FMF, 30 patients had 0 to 1 *MEFV* mutation (no mutation 3; heterozygous 27) and 53 patients at least 2 mutations (homozygous or compound heterozygous). There was no significant difference in *SAA1* gene polymorphisms between FMF patients with different numbers of *MEFV* mutations (Table 6).

Hardy-Weinberg equilibrium test

Finally, Hardy-Weinberg equilibrium was estimated by chi-square test with Yates' correction. There was no significant difference between observed and experienced frequencies of each genotype (*SAA1* -13C/T, *SAA2*, *IL-1β-511*) in the both FMF patients (Table 7) and healthy subjects (Table 7). These results indicated that these populations had a relatively stable genetic background and were stable for genetic statistical analysis.

Table 3. Frequencies of the genotypes and alleles at the *SAA1* locus of Japanese patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotype at *SAA1* locus			
1.1/1.1	4(4.8)	24(12.0)	$\chi2 = 12.553$ $p = 0.028$
1.1/1.3	22(25.6)	49(24.5)	
1.1/1.5	6(7.2)	39(19.5)	
1.3/1.3	15(18.1)	27(13.5)	
1.3/1.5	29(34.9)	47(23.5)	
1.5/1.5	7(8.4)	14(7.0)	
Allele at *SAA1* locus			
1.1	36(21.7)	136(34.0)	$\chi2 = 9.563$ $p = 0.008$
1.3	81(48.8)	150(37.5)	
1.5	49(29.5)	114(28.5)	

SAA1; Serum amyloid A1. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
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Discussion

FMF is considered to be an autosomal recessive disease [18]. The gene causing FMF is *MEFV*, which encodes pyrin, expressed in the cytoplasm of myeloid cells [2]. Pyrin is postulated to act as a negative regulator of IL-1-mediated inflammation [19]. However, approximately 30% of FMF patients exhibit a single *MEFV* mutation, despite sequencing of the entire *MEFV* genomic region and other autoinflammatory genes [20]. More recently it was demonstrated that pyrin truncation in mice did not show an overt phenotype of FMF, however, pyrin-deficient and FMF-associated B30.2 mutations "knock in" mice showed severe spontaneous inflammatory phenotype, suggesting that FMF may be caused by a gain of function by disease-associated missense changes in pyrin

Table 4. Frequencies of the genotypes and alleles at -13C/T *SAA1* locus of Japanese patients with FMF and healthy subjects.

	FMF patients	Healthy subjects	*p* value
	n = 83(%)	n = 200(%)	
Genotypes at -13C/T *SAA1*			
C/C	13(15.7)	67(33.5)	$\chi^2 = 11.538$ $p = 0.003$
C/T	47(56.6)	102(51.0)	
T/T	23(27.7)	31(15.5)	
Alleles at -13C/T *SAA1*			
T	93(56.0)	164(41.0)	$\chi^2 = 10.682$ $p = 0.001$
C	73(44.0)	236(59.0)	

SAA1; Serum amyloid A1. Chi-square test was used to examine differences of genotype and allele frequencies between FMF patients and healthy subjects.
doi:10.1371/journal.pone.0055227.t004

Table 5. Allele frequencies of *SAA1* gene polymorphisms in typical and incomplete FMF patients.

	FMF criteria		*p* value
	Typical	Incomplete	
	2n = 92(%)	2n = 74(%)	
Allele at *SAA1* locus			
1.1	16(17.4)	20(27.0)	$\chi^2 = 3.733$ $p = 0.155$
1.3	44(47.8)	37(50.0)	
1.5	32(34.8)	17(23.0)	
Alleles at -13C/T *SAA1*			
T	51(55.4)	42(56.8)	$\chi^2 = 0.029$ $p = 0.865$
C	41(44.6)	32(43.2)	

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Table 6. Number of *MEFV* gene mutations and *SAA1* gene polymorphisms in FMF patients.

	Number of mutations		*p* value
	0~1 mutations	≧2 mutations	
	2n = 60(%)	2n = 106(%)	
Allele at *SAA1* locus			
1.1	11(18.3)	25(23.6)	$\chi^2 = 0.955$ $p = 0.620$
1.3	29(48.3)	52(49.1)	
1.5	20(33.3)	29(27.4)	
Alleles at -13C/T *SAA1*			
T	33(55.0)	60(56.6)	$\chi^2 = 0.040$ $p = 0.841$
C	27(45.0)	46(43.4)	

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and that FMF may not be a pure autosomal recessive disease due to the loss of protein function [21]. One explanation is that subjects having a single *MEFV* mutation may develop an FMF phenotype in the presence of other inflammasome-related genes or in the presence of other environmental factors [22]. Therefore, the role of potential modifier genes and polymorphisms within these gene families should be assessed in conjunction with genotype-phenotype association studies. Polymorphisms in genes associated with the inflammasome pathway can affect the development of FMF [5]. For example, TLR2 polymorphisms may be an important factor in the susceptibility of FMF [23,24].

In this study, we investigated the *SAA1* and *IL-1β* gene polymorphisms in Japanese patients with FMF. There was no significant difference in *IL-1β-511* (C/T) or *IL-1Ra* VNTR polymorphisms between FMF patients and healthy subjects in accord to the previous report [25]. However, we demonstrated that *SAA1* gene polymorphisms, which are attributed to AA amyloidosis, might be also responsible for susceptibility to FMF. It is clear that genotypes at the *SAA1* locus are associated with an increased susceptibility to AA amyloidosis [26]. However, the contribution of these genotypes to the occurrence of non-amyloid, inflammatory disease has not been elucidated. In this study, we investigated the allele frequencies of *SAA1.1* and -13 (C/T) polymorphisms of the *SAA1* promoter region in Japanese patients with FMF. Our data demonstrated that the -13T allele polymorphism was a major risk factor and that the *SAA1.1* allele was protective for the occurrence of FMF in Japanese case-control studies.

The presence of 2 single-nucleotide polymorphisms (SNPs) within exon 3 of the *SAA1* gene, 2995 C/T and 3010 C/T, defined 3 haplotypes that corresponded to the *SAA1.1*, *SAA1.3*, and *SAA1.5* isoforms [26]. In Japanese patients with RA, homozygote expression of the *SAA1.3* allele was a proven risk factor, whereas *SAA1.1* appeared to be protective for AA amyloidosis [27]. In contrast, a strong positive association with *SAA1.1* has been established in Caucasian patients with amyloidosis secondary to juvenile idiopathic arthritis and FMF [28–30]. Moriguch *et al.* identified another *SAA1* SNP, the -13T/C SNP in the 5′-flanking region of the *SAA1* gene [17]. They observed the -13T allele was associated with AA amyloidosis, and associated with the *SAA1.3* allele in Japanese RA patients [17]. Interestingly, a polymorphism in the *SAA1* promoter -13T allele was found to be significantly associated with increased AA amyloidosis risk in both populations and to be in linkage disequilibrium with *SAA1.1* and *SAA1.3* in Caucasian and Japanese patients, thus apparently explaining the previous discrepancy [31–35]. Functional studies have demonstrated that the -13T allele is responsible for a higher transcriptional rate [36]. However, this did not result in higher serum levels of SAA, possibly due to increased proteolytic processing rates of *SAA1.1* and *SAA1.3* compared to *SAA1.5* [37]. The mechanisms by which the -13T allele predisposes to FMF remains to be unraveled and many possibilities have been suggested.

The overproduction of IL-1β, induced by NLRP3 inflammasome activation, is responsible for a variety of autoinflammatory syndrome including FMF. The NLRP3 inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid proteins [6]. Recent studies indicated that SAA activates the NLRP3 inflammasome in a cathepsin B and $P2X_7$-dependent manner, resulting in the secretion of mature IL-1β [10]. The accumulation of newly formed AA amyloid fibrils and aberrant processing of SAA is relevant to AA amyloidogenesis [38]. Therefore, in subjects with AA amyloidogenic genetic factors, such as -13T allele, the presence of SAA-derived AA amyloid fibrils may implicate the NLRP3 inflammasome activation pathway, which is thought to be relevant to the pathogenesis of FMF. Jeru *et al.* demonstrated that the *SAA1* genotype influenced the severity of FMF and disease susceptibility through a negative selection process, providing new insights into the role of *SAA1* in the pathophysiology of FMF [39]. Assuming that *SAA1* gene polymorphisms induce the formation of AA amyloid fibrils, this suggests that the polymorphisms may be associated with the NLRP3 inflammasome activation process and susceptibility to FMF. These findings may provide insights into modifier factors, other than *MEFV*, in the development of FMF.

The gender discrepancy (female dominant in incomplete FMF) seen in the present study may result from hormonal or associated environmental factors, which generate a disease of atypical or milder severity in female. For example, the risk for developing amyloidosis had been shown to be higher in male patients with FMF [40,41]. These findings suggest that clinical variability observed in FMF may be party attributed to the influence of environmental factors including gender. The main limitations of the study are its localization to a certain country, and a limited number of patients.

Table 7. Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β* -511 genotypes in Japanese patients with FMF and frequencies of *SAA1* -13C/T, *SAA2*, *IL1β* -511 genotypes in healthy subjects.

Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β*-511 genotypes in Japanese patients with FMF				
Locus	**Genotype**	**Observed number(%)**	**Expected number[a]**	***p* value**
SAA1 -13C/T	C/C	13(15.7)	16.1	$\chi^2=1.292$ $p=0.256$
	C/T	47(56.6)	40.9	
	T/T	23(27.7)	26.1	
SAA2	A/A	62(74.7)	61.6	$\chi^2=0.007$ $p=0.932$
	A/G	19(22.9)	19.8	
	G/G	2(2.4)	1.6	
IL1β -511	C/C	27(32.5)	28.3	$\chi^2=0.144$ $p=0.704$
	C/T	43(51.8)	40.3	
	T/T	13(15.7)	14.3	
Frequencies of *SAA1* -13C/T, *SAA2*, *IL1β*-511 genotypes in healthy subjects				
Locus	**Genotype**	**Observed number(%)**	**Expected number[a]**	***p* value**
SAA1 -13C/T	C/C	67(33.5)	69.6	$\chi^2=0.384$ $p=0.535$
	C/T	102(51.0)	96.8	
	T/T	31(15.5)	33.6	
SAA2	A/A	163(81.5)	162.9	$\chi^2=0.104$ $p=0.747$
	A/G	35(17.5)	35.2	
	G/G	2(1.0)	1.9	
IL1β -511	C/C	59(29.5)	59.4	$\chi^2=0.001$ $p=0.978$
	C/T	100(50.0)	99.2	
	T/T	41(20.5)	41.4	

[a]Expected genotype frequencies based on observed allele frequencies and assuming Hardy-Weinberg equilibrium.

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In occlusion, this study shows a significant prevalence of the -13T allele in Japanese patients with FMF. This comparative case-control study demonstrated that the *SAA1* gene polymorphisms might affect susceptibility to FMF, which is presumed to be a monogenic disease. Further studies are required to determine the impact of *SAA1* gene polymorphisms and the occurrence of FMF in large studies in different geographic areas.

Ethics approval

This study was conducted with the approval of the ethical committees of Nagasaki Medical Center.

Author Contributions

Conceived and designed the experiments: KM KA JM HI AK RU YN . Performed the experiments: YJ YM MY. Analyzed the data: KM M. Nakamura YM. Contributed reagents/materials/analysis tools: SH YI TK M. Nakashima YF FN KE HF TN. Wrote the paper: KM M. Nakamura YM.

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Preferentially Inflamed Tendon Sheaths in the Swollen but Not Tender Joints in a 5-Year-Old Boy with Blau Syndrome

Blau syndrome (MIM #186580), also known as early-onset sarcoidosis (MIM #609464), is an auto-inflammatory condition associated with constitutive active *NOD2* mutation, characterized by a triad of skin rash, uveitis, and symmetrical polyarthritis.[1-3]

A 5-year-old boy underwent ultrasonography for the swollen but not tender fingers, wrists (Figure 1), and ankles. Ultrasound examination revealed markedly thickened tendon sheaths accompanied by increased power Doppler signals and synovial fluid in the fingers, the wrists, and the ankles (Figure 2 and Videos 1-4; Videos 1-4 are available at www.jpeds.com). In contrast, articular-synovitis in the same joint region was absent or minimal.

Although the frequent involvement of tendon sheaths in Blau syndrome has been previously described,[4,5] this is the first report to demonstrate the predominance of teno-synovitis over articular-synovitis using ultrasonography. Because Blau syndrome causes structural damage in painless joints and clinical examination often fails to distinguish between articular- and teno-synovitis, the accurate localization of joint inflammation with ultrasound can be helpful in understanding the pathophysiology and also in establishing the strategy to prevent joint destruction of Blau syndrome. ■

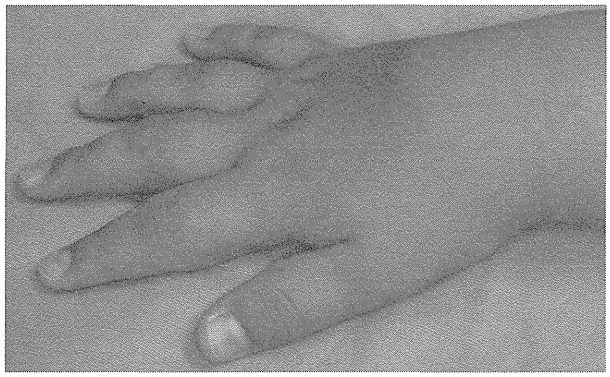

Figure 1. Soft tissue swelling in the right hand.

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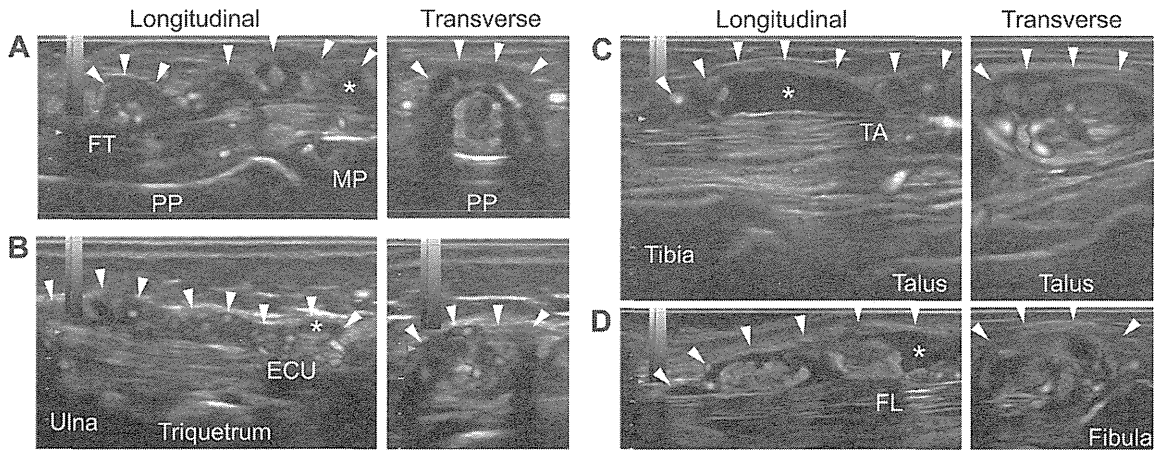

Figure 2. Ultrasound images of the right hand and the right ankle. **A,** The third flexor digitorum in the right hand. **B,** The extensor carpi ulnaris in the right wrist. **C,** The extensor tibialis anterior in the right ankle. **D,** The flexor digitorum in the right ankle. *Arrow heads* demarcate the extended area of synovial sheath. *Asterisks* indicate the synovial fluid. *ECU*, extensor carpi ulnaris; *PP*, proximal phalanx; *MP*, middle phalanx; *TA*, tibialis anterior.

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NLRP3 activation induces ASC-dependent programmed necrotic cell death, which leads to neutrophilic inflammation

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NLR family pyrin domain containing 3 (NLRP3) is a cytoplasmic pattern recognition receptor that regulates innate immune responses by forming a protein complex, the inflammasome. It leads to production of proinflammatory cytokine productions such as interleukin 1β (IL-1β). We and others demonstrated that an induction of activated NLRP3 also induced cell death. However, little is known about the characteristics and mechanisms of the cell death and its involvement in the pathogenesis of inflammatory conditions. In this study, we established cell lines in which NLRP3 was induced by doxycycline using a tetracycline-inducible expression (Tet-on) system. Using this system, the expression of NLRP3 mutants in cryopyrin-associated periodic syndrome (CAPS) patients was sufficient for the induction of necrotic cell death without lipopolysaccharide stimulation or generation of mature IL-1β. We also found that CA074-Me, a cathepsin B inhibitor, blocked cell death before oligomerization of apoptosis-associated speck-like protein containing a CARD (ASC), whereas Z-VAD-fmk, a pan-caspase inhibitor, blocked the cell death after the oligomerization. Silencing of the ASC gene (*Pycard*) by small hairpin RNA treatment inhibited the NLRP3 mutant-induced cell death, but silencing of the caspase-1 gene (*Casp1*) did not. Taken together, these results indicated that ASC was indispensable for NLRP3-mediated programmed necrotic cell death, and that this type of cell death was distinct from 'pyroptosis', which requires caspase-1. Finally, we demonstrated in an *in vivo* model that the programmed necrotic cell death induced by activated NLRP3 could cause neutrophil infiltration, indicating a possible role of cell death in neutrophil infiltration of skin lesions in CAPS patients.
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NLR family pyrin domain containing 3 (NLRP3) is a cytoplasmic protein belonging to the NOD-like receptor (NLR) family.[1] This family is characterized by pattern recognition receptors, and senses microbial molecules and danger signals in the cytoplasm of cells. NLRP3 and apoptosis-associated speck-like protein containing a CARD (ASC) are components of the inflammasome, a multiprotein complex required for caspase-1 activation and interleukin 1β (IL-1β) production. Formation of the NLRP3-inflammasome requires two signals. The first signal (also called the priming step) consists of microbial molecules or endogenous cytokines and is required for the upregulation of NLRP3 itself and the production of targeting cytokines by the inflammasome, including the pro-form of IL-1β (pro-IL-1β). The second signal is provided by extracellular ATP, pore-forming toxins or crystals. These signals activate NLRP3, leading to NLRP3 oligomerization followed by ASC oligomerization, and finally the formation of the NLRP3-inflammasome. Then, the inflammasome induces self-cleavage of pro-caspase-1 into the active form of caspase-1 that cleaves pro-IL-1β into the biologically active p17 form of IL-1β (mature IL-1β).[1]

The mammalian NLR family is structurally related to plant resistance (R) proteins.[2] R proteins detect pathogen-effector or avirulence proteins, leading to production of antimicrobial proteins and programmed cell death that is localized at infection sites.[3] Certain bacterial effectors have evolved the ability to suppress the programmed cell-death responses, allowing pathogen growth and disease in plants, suggesting the importance of programmed cell death in plants' innate immunity.[4] Whereas a large number of recent publications have greatly increased our knowledge of the mechanisms involved in production and processing of IL-1β by the NLRP3-inflammasome, we are only beginning to understand the mechanisms of cell death caused by NLRP3 activation.

NLRP3 (the gene that encodes cryopyrin) was originally identified as the gene responsible for cryopyrin-associated periodic syndrome (CAPS) in which neutrophilic urticarial rash is the most common symptom.[5] CAPS is caused by gain-of-function mutations in *NLRP3* that cause constitutive activation of NLRP3 in the absence of second signals and secretion of IL-1β. Three independent groups, including this laboratory, have reported that monocytes isolated from CAPS

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CAPS, cryopyrin-associated periodic syndrome; HMGB1, high-mobility group box 1; IL, interleukin; LPS, lipopolysaccharide; NLR, NOD-like receptor; NLRP3, NLR family pyrin domain containing 3; shRNA, small hairpin RNA
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patients were selectively induced to undergo cell death upon lipopolysaccharide (LPS) treatment,[6–8] accompanied by mutant NLRP3 expression. Of potential interest are patients with NLRP3 mutation with somatic mosaicism. Monocyte cell death after LPS treatment was only observed in the cells bearing mutant NLRP3 but not in those without mutation.[6–8] Moreover, we previously reported that the transient expressions of CAPS-associated mutant NLRP3 caused necrotic cell death.[9] The observation that the necrosis was inhibited by cathepsin B inhibitor (which also blocks programmed cell death caused by R protein in plants[10]), suggested that it was indeed programmed cell death.

Caution is required in concluding that NLRP3 activation leads to programmed cell death. This is because LPS treatment induces an array of gene products including IL-6, IL-8, and tumor necrosis factor-α in addition to NLRP3 and pro-IL-1β. Thus, it is possible that LPS-induced factors could participate in cell death. In addition, the transfection methodology (i.e., lipid-mediated gene delivery) itself has been shown to activate the inflammasome.[11–14] Moreover, second signals alone are known to induce cell damage or cell death.[15,16] Thus, to conduct studies in an unambiguous manner, we generated cell lines in which NLRP3 was induced upon doxycycline treatment using a tetracycline-inducible expression (Tet-on) system, eliminating the need for LPS treatment or exogenous DNA transfection. In addition, we utilized CAPS-associated mutant NLRP3 to avoid the damage associated with second signals.

Using the Tet-on system, we demonstrated in this study that the expression of CAPS-associated NLRP3 mutants alone was sufficient for necrotic cell death without LPS stimulation. On the basis of the observation that the distribution pattern of ASC in the cytoplasm was quite different after the treatment of two inhibitors, we demonstrated that CA074-Me inhibited the cell death before ASC oligomerization, whereas Z-VAD-fmk inhibited the cell death after ASC oligomerization. We also showed that NLRP3-mediated cell death can recruit neutrophils even in the absence of IL-1β. This characteristic cell death-mediated neutrophil-rich inflammation has wider significance because it is mediated by NLRP3, which responses to not only pathogens but also to danger-associated signals.

Results

Cell death induced by NLRP3 activation was dispensable for IL-1β expression. NLRP3-inflammasome formation and mature IL-1β release requires two distinct signals. The most common examples are LPS as the first signal and ATP as the second signal. The mast cell line MC/9 stably expressed ASC and pro-caspase-1 under unstimulated conditions (Figure 1a, left panels). LPS treatment

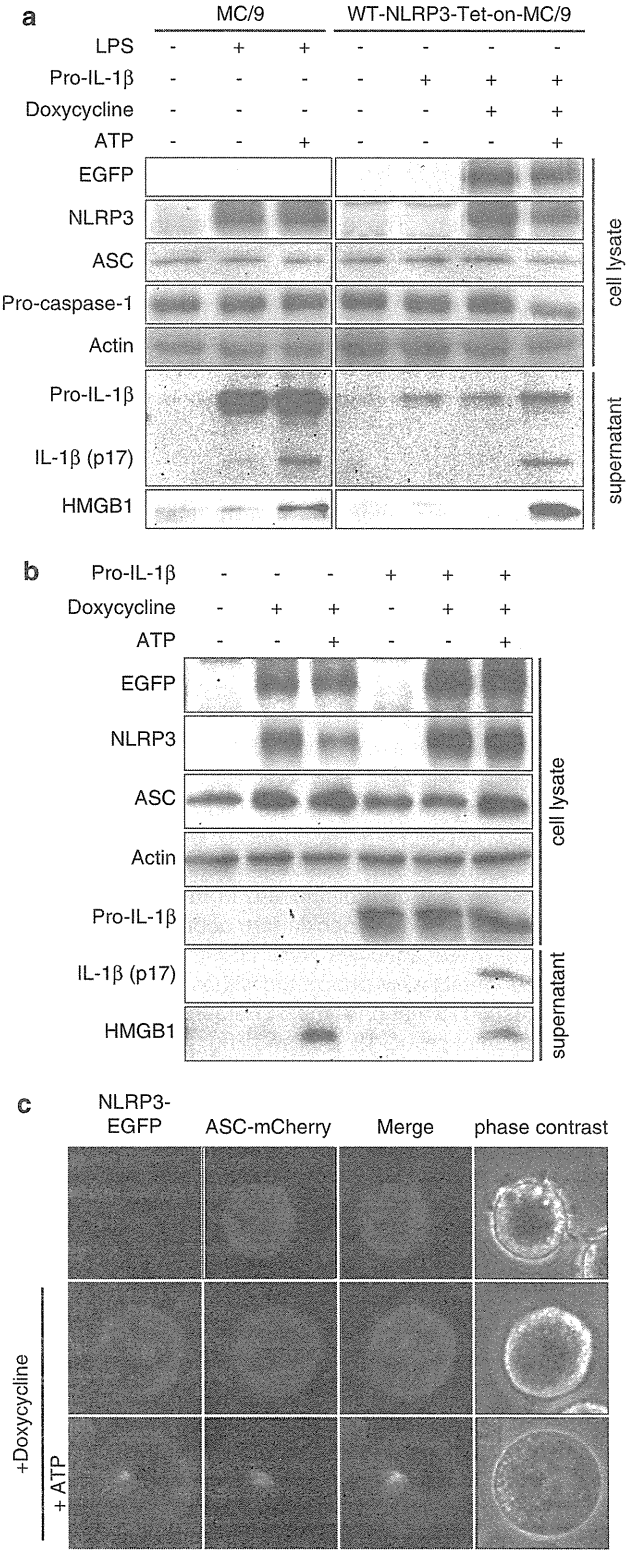

Figure 1 The first signals for the NLRP3-inflammasome can be replaced by expressions of NLRP3 and pro-IL-1β. (**a** and **b**) MC/9 cells (1×10^6) were pretreated with 1 μg/ml LPS for 2 h and stimulated with 5 mM ATP for 45 min. WT-NLRP3-Tet-on-MC/9 cells were transfected with the pro-IL-1β-pMX-IP retroviral vector and incubated for 48 h before ATP stimulation. WT-NLRP3-Tet-on-MC/9 cells (1×10^6) were treated with 1 μg/ml doxycycline for 12 h before ATP stimulation to induce the expression of WT-NLRP3. Cells and supernatants obtained from experiments using MC/9 and WT-NLRP3-Tet-on-MC/9 were harvested 45 min after ATP stimulation, except supernatants for HMHB1 immunoblotting that were harvested 12 h after ATP stimulation. (**c**) WT-NLRP3-Tet-on-MC/9 cells stably expressing ASC-mCherry were pretreated with 1 μg/ml doxycycline for 12 h and stimulated with 5 mM ATP. ATP stimulation induced the co-localization of NLRP3 and ASC speckles, and induced cell swelling, leading to necrotic cell death