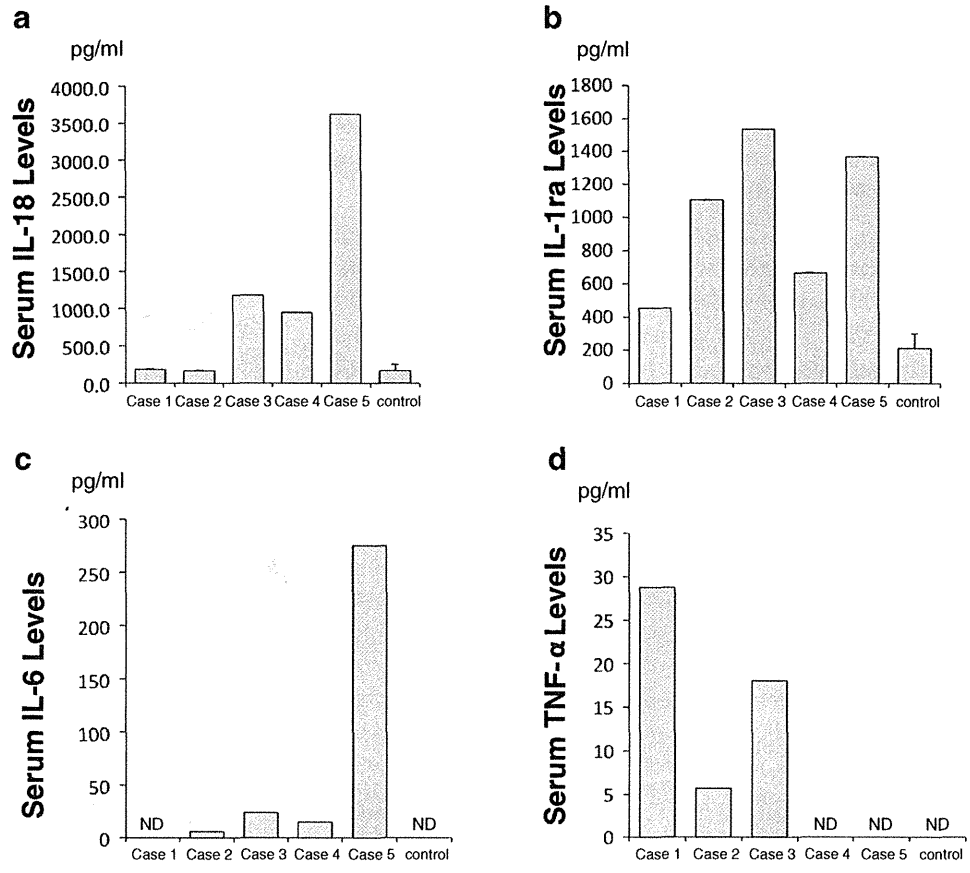

Fig. 3 Serum inflammatory cytokines in the four CAPS cases. IL-1β, IL-6, and TNF-α were not detected in the sera of the control subjects. The means ±D of the serum IL-18 and IL-1ra levels of the healthy control subjects were 169.2±85.7 and 213.4±87.1 pg/ml, respectively (n=10)

(cases 3 and 4) also had no strong deformities of the joints, but had obviously more severe phenotypes than FCAS, such as aseptic meningitis and hearing loss. In the present study, the E688K mutation in the MWS and CINCA/NOMID syndrome patients showed significantly stronger NF-κB activities than the Y563N mutation identified in the FCAS patients. Our findings indicate that the clinical phenotypes and values of the ASC-dependent NF-κB activity assay are well correlated with the genetic mutations, consistent with a previous report [18]. However, the artificial reporter gene assay system used may have little to do with the function of the CAPS pathophysiology, and limited numbers of *NLRP3* variants have been assessed using the assay in the present and previous studies, thereby making it difficult to prove this hypothesis at the present time. Consequently, further experiments including large amounts of pathogenic mutations and accumulation of detailed clinical information about the disease severity of CAPS are necessary to confirm this hypothesis. It should be noted that low-penetrance mutation, G809S, did not show positive activity with this in vitro assay system. But the clinical phenotype of case 3 was obviously more severe than case 4, although the father of case 3, who also was found to have G809S, was as symptomatic. Because of the discrepancy between the patient and the father, it remains unclear whether G809S is a pathogenic mutation or, alternatively, if there is an alternative genetic explanation for disease in the patient not detected by genomic DNA sequencing.

On the other hand, it requires time to build the above-mentioned in vitro experimental system. For the rapid diagnosis and characterization of CAPS, a simple screening system is necessary. In this study, we measured several serum inflammatory cytokine levels in our patients (Fig. 3). The serum IL-6 level is usually used for evaluating the disease severity of rheumatoid arthritis [19]. Moreover, the serum IL-18 level was recently reported to reflect the disease severity of not only JIA but also other diseases such as allergic diseases [20, 21]. In our CAPS patients, the serum levels of IL-18, but not IL-1β, seemed to be correlated with the disease phenotypes. Although the precise reason for this dissociation between the IL-18 and IL-1β levels in the sera is unknown, IL-1β may be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. In fact, serum IL-1ra, which is the counter-regulator of IL-1, was increased in our CAPS patients. Thus, the serum IL-18 levels may be used as an appropriate marker for the evaluation of treatments, although it is unlikely that serum IL-18 can contribute to the differential diagnosis between CAPS and other diseases.

The diagnosis of FCAS seems to be relatively difficult because of its mild phenotypes compared with the other more severe phenotypes of CAPS. The serum inflammatory

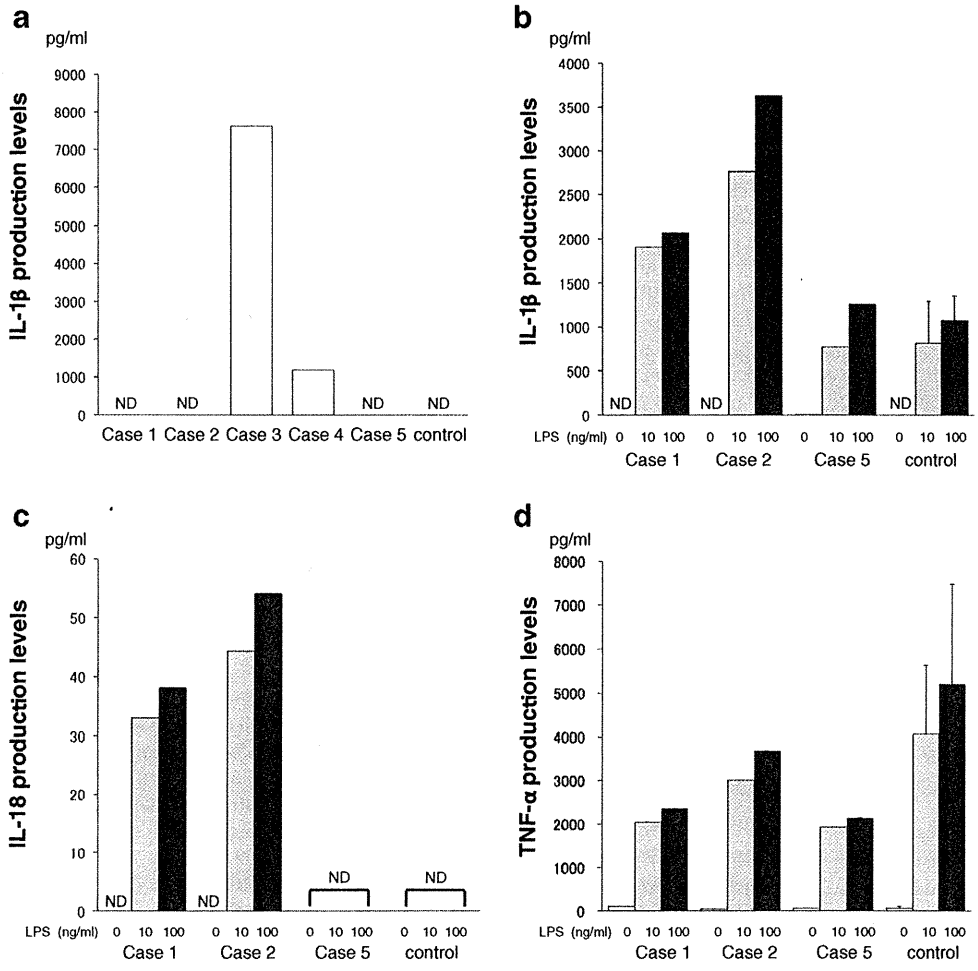

Fig. 4 LPS-induced cytokine production levels in the patients. **a** The *white bars* indicate the spontaneous IL-1β production levels by PBMCs. Increased IL-1β production by PBMCs from case 3 (CINCA/NOMID syndrome) and case 4 (MWS) is detected, whereas no increases are observed for the PBMCs from the control subjects and cases 1, 2 (FCAS), and 5 (JIA). **b**, **c** The LPS-induced IL-1β and IL-18 production levels by PBMCs from the FCAS patients are increased compared with PBMCs from the control subjects. **d** The TNF-α production levels by PBMCs from the FCAS and JIA patients do not show any significant changes. In **b**–**d**, the *white bars* indicate the cytokine production levels without stimulation and the *gray* and *black bars* indicate the cytokine production levels after stimulation by 10 and 100 ng/ml LPS, respectively

cytokine levels in our FCAS patients did not show any typical increases, unlike the case for the CINCA/NOMID patient (Fig. 3), indicating that the establishment of an effective and easy screening method is important for the diagnosis of FCAS. Therefore, we focused on the cytokine production levels in these patients' blood cells. First, IL-1β production by nonstimulated PBMCs was observed in our CINCA/NOMID and MWS patients (cases 3 and 4, respectively), as reported previously [5]. However, no enhancement of spontaneous IL-1β production was observed in our FCAS patients (cases 1 and 2) (Fig. 4a), suggesting that this method may not be suitable for screening of FCAS.

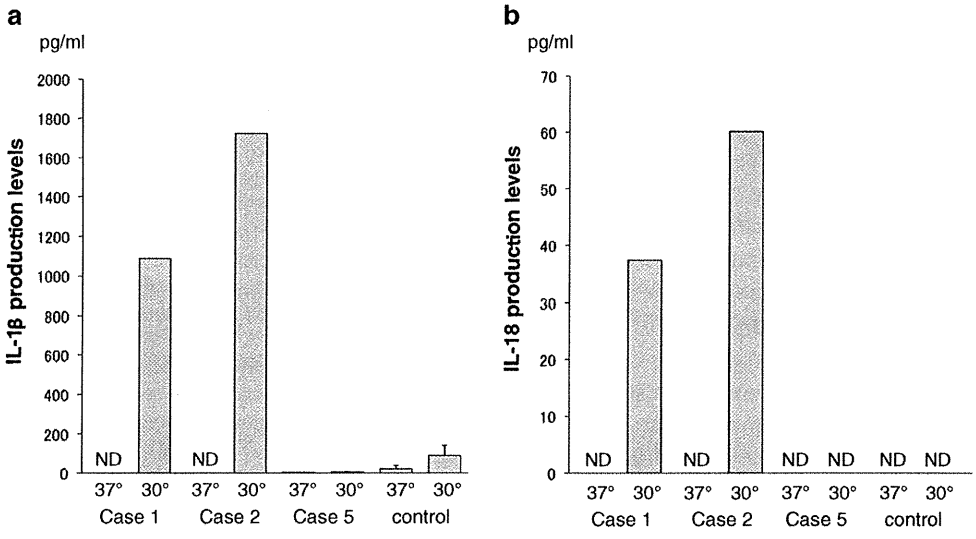

Fig. 5 Hypothermia-induced cytokine production levels by PBMCs from the FCAS and JIA patients. **a**, **b** Comparisons of the cytokine production levels by PBMCs cultured at 30°C and 37°C. The PBMCs from the FCAS patients (cases 1 and 2) show obvious increases in the IL-1β and IL-18 production levels after culture at lower temperature with no stimulation

Furthermore, the LPS- or hypothermia-induced cytokine production levels by the PBMCs showed marked elevation of IL-1β or IL-18 (Figs. 4a–c and 5b), as reported previously [16, 22]. The phenomena for hypothermic culture were similar to the findings in our recent report that NF-κB activity induced by LPS stimulation through TLR4 is enhanced in low-temperature cultures [23], although the precise mechanism of the association between the *NLRP3* variations and the low-temperature stimulation requires further clarification. These findings suggest that the cytokine production assays induced by LPS or hypothermia stimulation should be helpful for the diagnosis of FCAS. It should be noted that the serum IL-18 levels could be detected in all of the non-CAPS subjects, although the production levels of IL-18 from their PBMCs were lower than the detection limit. This might be dependent on the long half-life of IL-18 in human blood compared with the above-mentioned half-life of IL-1β.

The discrimination between CAPS and JIA cases is sometimes difficult because of their similar clinical characteristics. Interestingly, although case 5 had a rare missense variation in *NLRP3* (E378K) and some of her clinical symptoms were similar to those of CAPS (Table I), the E378K variant did not show enhancement of NK-κB activity (Fig. 2). This gene variation was inherited from her mother who did not show any inflammatory symptoms. Case 5 showed strong polyarthritis, continuous fever, and a recurrent generalized urticaria-like erythema as well as symptoms of CAPS. In particular, histopathological examination of a biopsy specimen from her skin rash revealed infiltration of neutrophils and mononuclear cells, representing similar findings to case 1 (Fig. 1). Thus, it was difficult to discriminate CAPS by the clinical symptoms alone in this case.

Therefore, to discriminate between CAPS and JIA in this case, we focused on her cytokine profiles. Her serum IL-6 and IL-18 levels were extremely high compared with not only the healthy controls but also the other CAPS patients (Fig. 3a, c). These observations resembled the serum cytokine pattern of systemic-onset JIA [21, 24]. Furthermore, the LPS-induced and hypothermia-induced IL-1β and IL-18 production levels by PBMCs from case 5 showed no increases compared with the control subjects (Figs. 4b, c and 5a, b). Recently, Saito et al. [5] reported that another screening method, LPS-induced monocyte cell death, was effective for diagnosing CAPS. The monocytes in case 5 did not show LPS-induced cell death. These objective results also supported the diagnosis of case 5 as JIA, rather than CAPS.

In this study, we evaluated several methods for the limited genotypes of patients with *NLRP3* variants. According to comparisons of the clinical phenotypes of previous case reports and our cases, the disease severity seems to be correlated with the serum cytokine levels and the ex vivo and in vitro responses and is almost completely determined by the specific mutations, which appear to suggest that other genetic or epigenetic determinants or environmental factors do not play a significant role.

Conclusions

A precise and easy method for the diagnosis of CAPS has not yet been established. The characteristics of the clinical phenotypes and the identification of proven gene variations of *NLRP3*, as the etiology of CAPS, are very important for diagnosing CAPS. In addition, the serum IL-18 levels and NF-κB activities of patients with the *NLRP3* variants reflect the phenotypes of disease severity. Evaluation of the cytokine profile is also a useful tool for diagnosing and discriminating the severity of CAPS.

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Conflicts of Interest The authors have declared no conflicts of interest.

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ORIGINAL RESEARCH

In Vitro Analysis of the Functional Effects of an *NLRP3* G809S Variant with the co-Existence of *MEFV* Haplotype Variants in Atypical Autoinflammatory Syndrome

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Abstract
Purpose Hereditary periodic fever syndromes have been considered monogenic diseases. However, some recent reports have described patients with co-existence of recurrent fever responsible genes. This study assessed whether a rare variant, found in Japanese children showing atypical autoinflammatory syndrome, located in the leucine-rich repeat domain of Nod-like receptor family, pyrin domain containing 3 (*NLRP3*) with co-existence of Mediterranean fever (*MEFV*) haplotype variants may contribute to a proinflammatory phenotype using a systematic approach.
Methods Cytokine production in serum or from peripheral blood monocytes was measured by ELISA. DNA sequence analysis of genes including *NLRP3*, *MEFV*, mevalonate kinase (*MVK*), and tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) were performed on patient samples. In vitro functional assays determined the effects of the NLRP3 variants and pyrin using NF-κB activation and speck formation assays.
Results A heterozygous genetic variant of *NLRP3*, G809S, was found in samples from both patients. Additionally the previously reported heterozygous *MEFV* variants (P369S-R408Q or E148Q-P369S-R408Q) were also detected in both patients. Serum IL-1ra and sTNFR1 levels increased in the attack phase of the disease in both patients. The production levels of IL-1β from monocytes isolated from both cases were elevated following LPS and IFN-γ stimulation. The *NLRP3* G809S variant demonstrated no increase of NF-κB activity following monosodium urate stimulation, whereas it significantly increased speck formation by interacting with apoptosis-associated speck-like protein with caspase recruitment domain.
Conclusions The phenotype of atypical autoinflammatory disease in patients could be modified by a synergistic effect with two other variants of autoinflammatory-associated genes.

Keywords NLRP3 · leucine-rich repeat domain · autoinflammatory disease · ASC

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a CARD
CAPS	Cryopyrin-associated periodic syndrome
CINCA	Chronic infantile neurologic cutaneous, articular
FCAS	Familial cold-induced autoinflammatory syndrome
FMF	Familial Mediterranean fever
HEK	Human embryonic kidney
IL	Interleukin
MEFV	Mediterranean fever

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MVK	Mevalonate kinase
MWS	Muckle–Wells syndrome
MSU	Monosodium urate
NBS	Nucleotide-binding site
NLRP3	Nod-like receptor family pyrin domain containing 3
NOMID	Neonatal-onset multisystem inflammatory disease
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
TRAPS	Tumor necrosis factor receptor-associated periodic syndrome

Introduction

Autoinflammatory syndromes are characterized by systemic inflammation without the presence of antigen-specific T cells or high-titers of autoantibodies [1]. Many autoinflammatory syndromes are clinically characterized by recurrent or persistent features that include fever, elevation in the levels of acute-phase reactants, and organ-specific complications such as skin rashes and osteoarticular, serosal, neurologic, and ocular manifestations [2]. To date, well-known hereditary periodic fever syndromes are familial Mediterranean fever (FMF), hyperimmunoglobulinemia D with periodic fever syndrome, cryopyrin-associated periodic syndromes (CAPS), and tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS). These syndromes are discriminated by some characteristic phenotypes such as varying age of onset, duration of fever, development of cutaneous manifestations, and several other features.

CAPS include familial cold-induced autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID), also known as chronic infantile neurologic, cutaneous, articular (CINCA) syndrome. FCAS exhibits cold-induced urticaria-like skin rash whereas MWS develops severe phenotypes, such as periodic fever, neural progressive hearing loss and renal amyloidosis. CINCA/NOMID syndrome shows additional more severe phenotypes, such as severe arthritis, patella overgrowth, aseptic meningitis, and mental retardation [3]. CAPS are caused by mutations in the Nod-like receptors (NLRs) family, pyrin domain containing 3 (*NLRP3*) gene, and more than 80 variants are associated with CAPS, in addition to over 50 variants of unclear significance that have been reported in the INFEVERS database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) to date [4].

NLRs recognize microbial molecules such as pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns, which trigger inflammation as well as Toll-like receptor immune responses. NLRP3 protein contains an N-terminal pyrin domain, a central nucleotide-binding site (NBS) domain, and C-terminal leucine-rich repeats (LRR) [5]. Most pathogenic mutations associated with autoinflammatory syndromes are located in exon 3 of *NLRP3*, which encodes the NBS domain. In addition, several mutations outside exon 3 on the LRR domain of *NLRP3*, such as G755R, G755A, and Y859C have been found in patients with CINCA syndrome or atypical autoinflammatory disorders [6–8].

This study reports two cases of Japanese children who presented with atypical periodic fever episodes and who had the variants in the LRR domain of *NLRP3* with co-existence of Mediterranean fever (*MEFV*) haplotype variants. The patients showed periodic prolonged fever and erythema, but lacked symptoms typical of CAPS, FMF, and other common autoinflammatory syndromes. By genetic analysis and functional assays of these variants, the data from this study suggest that the phenotype of atypical autoinflammatory disease in patients could be modified by a synergistic effect with other autoinflammatory-associated genes.

Methods

Subjects

Case 1

The first case was a 9-year-old girl who had experienced recurrent fever episodes approximately three times a year for 6 years from onset at 3 years of age. Although she underwent a tonsillectomy at the age of 5, she still experienced recurrent fever episodes. She presented with mild abdominal pain without signs of peritoneal irritation, peritonitis or pleuritis as typically observed in FMF. High serum C-reactive protein (CRP) levels were observed in the attack phase. She presented with pigmented macules with erythema, which persisted for 6 months, and bilateral petechiae on her legs and dorsa of feet (Fig. 1a). Histological examination of the skin lesion revealed perivascular infiltrate with mononuclear cells in the upper and middle dermis, but vasculitis was not observed (Fig. 1b). Direct immunofluorescence analysis revealed deposits of complement component 3 (C3) at the capillary walls in the upper to middle dermis, but not the presence of immunoglobulin (Ig)A or IgM (Fig. 1c). Rheumatoid factor and autoantibodies were not detected. Colchicine treatment (0.5 mg per day) was effective in treating the erythema and alleviating fever with elevating CRP. Both parents had experienced lasting recurrent fever episodes during their childhood although it was likely that their symptoms were not so severe. The fever episodes of parents resolved spontaneously without specific

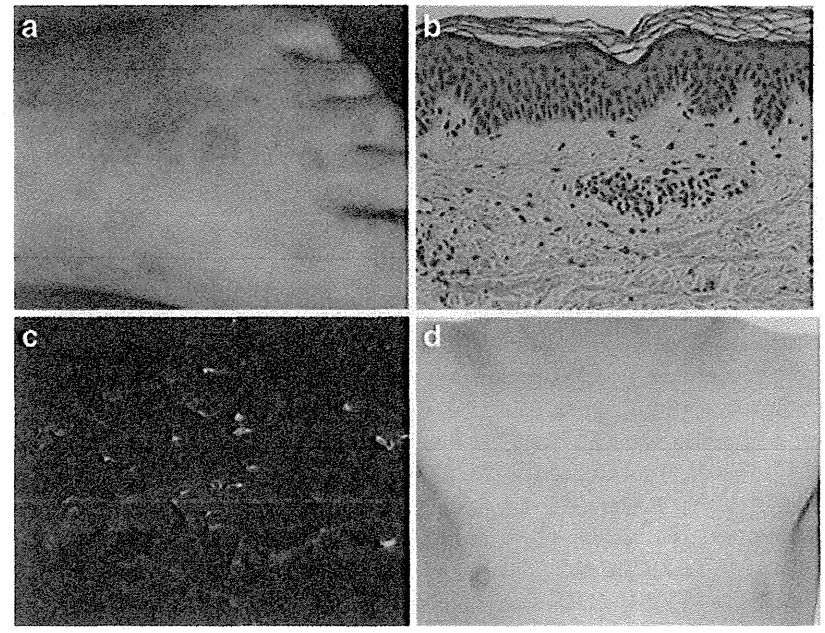

Fig. 1 Presence of skin rash in patients with atypical autoinflammatory syndrome. **a** The clinical appearance of rash on the dorsum of foot in case 1. **b** The histopathological examination of a skin biopsy specimen (hematoxylin and eosin stain, original magnification × 200). Perivascular infiltrate with mononuclear cells was observed in the upper and middle dermis. **c** Direct immunofluorescence demonstrates C3 deposits in the capillary walls (original magnification × 50). **d** The clinical appearance of the skin rash on the breast in case 2

medications such as colchicines and corticosteroids or tonsillectomy when they were about 10 years old. However, they do not remember their childhood in detail as it was over 30 years ago. Their episodes may represent autoinflammatory disease.

Case 2

The second case involved a 4-year-old boy, presenting with recurrent episodes of fever of various duration from a few days to weeks, with or without mild liver dysfunction and multiple erythema without skin itch. The frequency of episodes was at least twice a year. The skin erythema was observed during the fever episodes at 18 months old and at 4 years old (Fig. 1d). The cervical lymphadenopathy and diarrhea were observed in almost all of the fever attack episodes. Although fever duration was 1 week, it resolved immediately following oral administration of 1 mg/kg prednisolone. Rheumatoid factor and autoantibodies were not detected. His parents had no symptoms like periodic fever syndromes or rheumatic diseases. The fever did not recur for a few months after the cessation of oral prednisolone treatment. From 3 years old, colchicine treatment was started because of recurrent fever attacks. However, currently this treatment is not effective.

The genotypes and the clinical profiles of these cases are summarized in Table I. This study was performed according to the Helsinki Declaration. All subjects provided informed consent to participate in the study.

DNA Sequencing

Genomic DNA was extracted from leukocytes using SepaGene (EIDIA, Tokyo, Japan). DNA fragments of the *NLRP3*, *MEFV*, mevalonate kinase (*MVK*), and TNF receptor superfamily, member 1A (*TNFRSF1A*) genes were amplified by polymerase chain reaction (PCR), and analyzed using big dye terminator bidirectional sequencing (Applied Biosystems, Foster City, CA, USA).

Table I Genotype and clinical profiles of cases

	Case 1	Case 2
Initial diagnosis	FMF	TRAPS
Gender	Female	Male
Clinical features		
Age at onset of attacks	3 years	6 months
Duration of episodes	3–5 days	>1 week
Fever	Yes	Yes
Abdominal signs	Yes	Yes
Arthralgia	No	No
Lymphatic signs	No	Yes
Cutaneous manifestations	persistence of rash (pigmented erythema with petechiae)	Two episodes of rash
Hearing loss	No	No
Neurologic signs	No	No
Proteinuria	No	No
Laboratory findings		
WBC (/μl)	11,800	14,620
CRP (mg/dl)	10.1	3.1
ESR (mm/h)	45	32
NLRP3 Genotype	G809S	G809S
MEFV Genotype	P369S, R408Q	E148Q, P369S, R408Q

CRP the serum C-reactive protein level. *WBC* white blood cells. *ESR* erythrocytes sedimentation rate. Laboratory findings were the data in the attack phase

Genotyping

Allelic frequency of NLRP3 G809S (rs141389711) was investigated on a Step One Real-Time PCR System using Custom TaqMan SNP Genotyping assays (Applied Biosystems) in 421 healthy subjects. Further, genotype was confirmed by direct sequence analysis.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of control donors and from patients by gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The CD14-positive cells were cultured in medium consisting of RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 μg/ml). Human embryonic kidney (HEK) 293 T cells and HEK293-ASC cells were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Analysis of Serum Cytokine Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples of patients and controls were stored at −80 °C until assayed. TNF-α concentrations were measured with an Immunoassay Kit (BioSource International, Carlsbad, CA, USA) with a detection limit of 1.7 pg/ml. Similarly, interleukin (IL)-6 and IL-1β concentrations were measured by immunoassay Kit (BioSource) with detection limits of 1.7 pg/ml and 1.0 pg/ml, respectively. IL-1ra and sTNFR1 concentrations were measured by ELISA (R&D Systems) with detection limits of 6.26 pg/ml and 0.77 pg/ml, respectively. IL-18 was assayed by ELISA (MBL, Nagoya, Japan), with a detection limit of 25.6 pg/ml. We defined serum cytokine levels of more than the mean + 2 SD as increasing. Values below the detection limit are shown as not detected.

IL-1β Production from Monocytes

CD14-positive cells were purified from PBMCs using CD14 MACS MicroBeads and MACS magnetic columns according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). The CD14 positive cells were seeded to a density of 3.0×10^5 per ml and cultured with the addition of 1.0 μg/mL LPS O127 (Sigma-Aldrich) and 20 μg/ml IFN-γ (R&D Systems, Minneapolis, MN, USA) for 24 h at 37 °C in a humidified atmosphere at 5 % CO_2 and pulsed with 5 mM ATP (Sigma-Aldrich) for 30 min before harvesting. The cell-culture supernatants were harvested, and stored at −80 °C until assayed. The IL-1β was measured with ELISA. The assay was performed at two different times. The statistical significance between control and each case in the IL-1β production was analyzed using Dunnett's multiple comparison test. *P*-value of <0.05 was considered statistically significant.

Vector Preparations

cDNA encoding *NLRP3* tagged at the C-terminus with a FLAG-epitope (NLRP3-FLAG) was cloned into plasmid pcDNA3.1+ (Invitrogen). NLRP3 mutants (D303N, G755R, G809S and Y859C) were generated using the GeneEditor *In vitro* Site-Directed Mutagenesis System (Promega, Madison, WI, USA). A cDNA encoding pyrin tagged at the C-terminus with an HA-epitope (pyrin-HA) was cloned into plasmid pcDNA3.1+. Pyrin variants (P369S+R408Q) were generated using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). The apoptosis-associated speck-like protein containing a CARD (ASC) variant 1 tagged at the C-terminus with a myc-epitope (ASC1-myc) construct was cloned into pcDNA3.1+. The NF-κB luciferase reporter vector (pGL4.32-luc2P/NF-kappaB-RE/Hygro) and the Renilla luciferase reporter vector (pGL4.74-hRluc/TK) were purchased from Promega.

NF-κB Reporter Gene Activity

HEK293T cells were transfected with 16 ng per well of pcDNA3.1+ control vector or pcDNA3.1+ NLRP3-FLAG (wild type or mutant) or pcDNA3.1+ pyrin-HA (wild type or mutant) in 96-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The pcDNA3.1+ ASC1-myc, NF-κB luciferase reporter, and Renilla luciferase reporter were co-transfected. After transfection, cells were incubated for 24 h. Cells were stimulated with R837 at a concentration of 10 μg/ml (InvivoGen, San Diego, CA, USA) or monosodium urate (MSU) at 250 μg/ml (InvivoGen) for 8 h. Luciferase reporter activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). The statistical significance of differences in luciferase activity between wild-type and mutant gene activity in the NF-κB reporter assays was analyzed using Dunnett's multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

Speck Quantification Assay

HEK293 cells were transfected with ASC-myc and positively selected using 1 mg/ml G418 for 4 weeks. HEK293-ASC cells (1×10^5) were co-transfected with 250 ng of each NLRP3 expression plasmid and pyrin expression plasmid using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. After 24-h incubation, cells were fixed with 3.7 % paraformaldehyde in PBS for 10 min,

and washed with 10 mM glycine in PBS. Fixed cells were permeabilized using PBS containing 0.2 % Triton X-100 for 1 h at room temperature. Cells were then incubated with an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and anti-myc antibody (Invitrogen). Primary antibody binding was detected by incubation with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) secondary antibodies. Fixed cells were incubated with 4'-6-diamidino-2-phenylindole, a nuclear stain, and mounted using Vectashield Mounting Medium (Vector Laboratories Burlingame, CA, USA). The percentage of cells containing ASC specks in the cells expressing *NLRP3* was calculated by randomly selecting at least 10 fields. Differences were analyzed using Dunnett's multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

Results

Detection of NLRP3 and MEFV Mutations in Two Patients with Autoinflammatory Syndrome

In case 1, a heterozygous c.2425G>A (p.Gly809Ser) on LRR in exon5 of *NLRP3* and heterozygous P369S-R408Q in exon3 of *MEFV* were identified (Table I). There are 17 individuals who have the allele of G809S in 421 healthy control subjects. The allele frequency of this variant was 0.02. There were no control subjects carrying P369S-R408Q in *MEFV* in addition to the G809S variant. Interestingly, the same *NLRP3* and *MEFV* haplotype variants were identified in the father of case 1. The heterozygous *MEFV* variant haplotype P369S-R408Q were also observed in the mother of case 1.

Case 2 expressed the same heterozygous *NLRP3* variant found in case 1. In addition, heterozygous E148Q-P369S-R408Q in exon2 and exon3 of *MEFV* were identified (Table I). The G809S variant of *NLRP3* was inherited from his asymptomatic father. His asymptomatic mother was positive for homozygous E148Q and heterozygous P369S-R408Q sequences.

MVK and *TNFRSF1A* mutations were not detected in either case.

The Cytokine Profile of Patients

Serum IL-1β, IL-6 and TNF-α levels were not detected in the sera of healthy control subjects. The mean concentration ± SD of serum IL-18 and IL-1ra in healthy control subjects were 169.2±85.7 pg/ml and 213.4±87.1 pg/ml, respectively [9]. The mean concentration ± SD of serum sTNFR1 in healthy control subjects was 1009±276.4 pg/ml. Figure 2a and b show the serum cytokine profiles from the patients. The serum cytokine concentrations were measured at two different points at least during fever and inter-ictal periods respectively, and average values were calculated. In both cases, serum IL-1β,

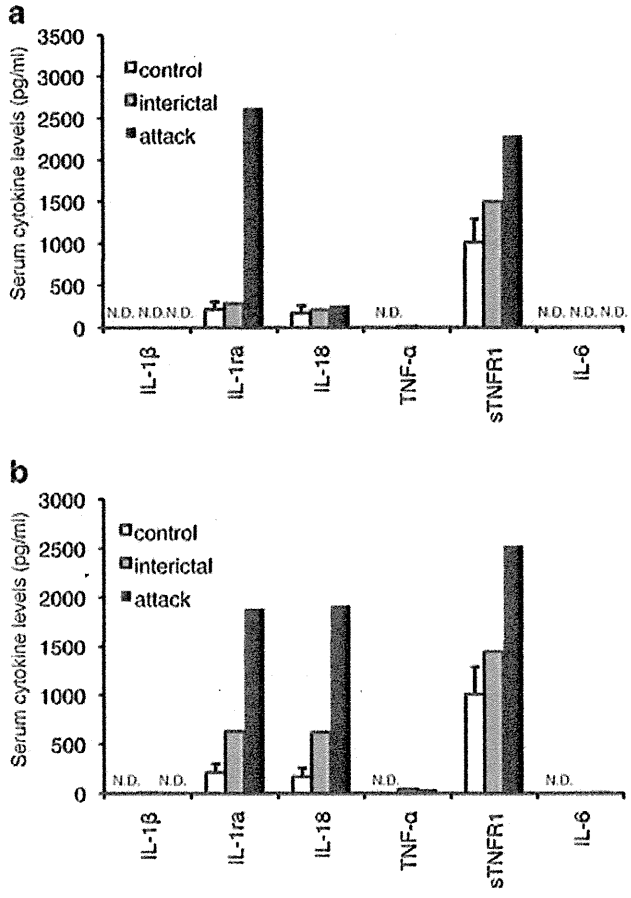

Fig. 2 Inflammatory cytokine levels from two cases during the inter-ictal phase and attack phase. a White bars indicate serum inflammatory cytokine levels of control. Grey bars indicate serum inflammatory cytokine levels of case 1 during the inter-ictal period. Black bars indicate serum inflammatory cytokine levels of case 1 during the attack phase. b White bars indicate serum inflammatory cytokine levels of control. Grey bars indicate serum inflammatory cytokine levels of case 2 during the inter-ictal period. Black bars indicate serum inflammatory cytokine levels of case 2 during the attack phase

TNF-α, and IL-6 did not increase during the fever episodes, whereas serum IL-1ra and sTNFR1 levels were increased. IL-18 levels during the fever episodes were increased in case 2, not in case 1. Interestingly, the serum IL-1ra and IL-18 levels from case 2 were elevated during the inter-ictal period.

Figure 3 shows the production of IL-1β from monocytes with LPS, IFN-γ and/or ATP stimulation. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects ($n=5$) without stimulation were 5.54±4.40 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with 20 ng/ml IFN-γ or 1 μg/ml LPS were 7.74±9.81 pg/ml and 236.0±188.4 pg/ml, respectively. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with 1 μg/ml LPS added 5 mM ATP was 166.0±138.3 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy

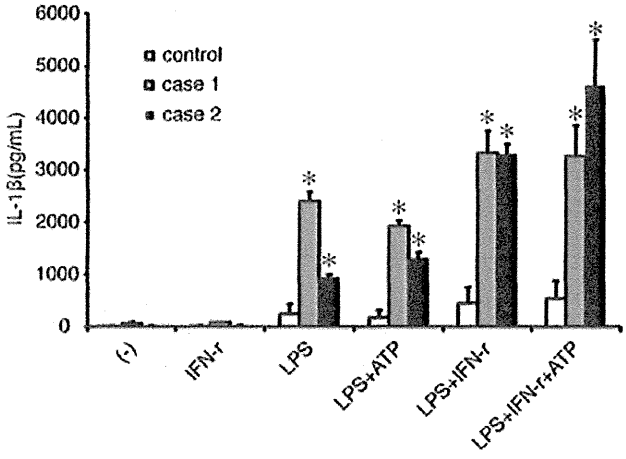

Fig. 3 IL-1β levels from monocytes in case 1 and 2. White bars indicate IL-1β levels in control. Grey bars indicate IL-1β levels in case 1. Black bars indicate IL-1β levels in case 2. IL-1β levels from monocytes from case 1 and 2 were significantly increased compared with controls (n=5). * P<0.05

control subjects stimulated with both 20 ng/ml IFN-γ and 1 μg/ml LPS were 441.3±316.5 pg/ml. The mean concentration ± SD of IL-1β from monocytes of healthy control subjects stimulated with both 20 ng/ml IFN-γ and 1 μg/ml LPS added 5 mM ATP was 549.2±327.3 pg/ml. In both cases, IL-1β secretion was increased compared with the healthy controls when the monocytes were stimulated with LPS and IFN-γ. Additionally, IL-1β from monocytes in case 2 stimulated with LPS and IFN-γ was increased in response to ATP. This was not observed for monocytes from case 1.

NF-κB Reporter Gene Activity of NLRP3 and Pyrin Variants

To assess the function of the *NLRP3* variant G809S on NF-κB signaling, we compared the G809S sequence with those of wild-type and three *NLRP3* mutations (D303N, G755R and Y859C). D303N, G755R, and Y859C were identified in CAPS patients [6, 10, 11] (Fig. 4). When ASC was co-expressed, D303N and G755R mutations increased NF-κB reporter gene activity. However, G809S and Y859C did not lead to significant activation of NF-κB. In the presence of R837, an *NLRP3* inflammasome activator, *NLRP3* D303N and G755R mutations showed enhanced NF-κB activation, whereas G809S and Y859C did not induce any increase in activity. Subsequently, the evaluation of G809S enhanced NF-κB activation in the presence of MSU was measured. MSU induced NF-κB activation of wild-type, D303N and G755R *NLRP3*. However, both G809S and Y859C mutations significantly inhibited NF-κB activation mediated by MSU.

To investigate the role of mutational effect of pyrin in the NF-κB signaling pathway, wild-type or variant pyrin (P369S+R408Q) was expressed in HEK293 cells and co-

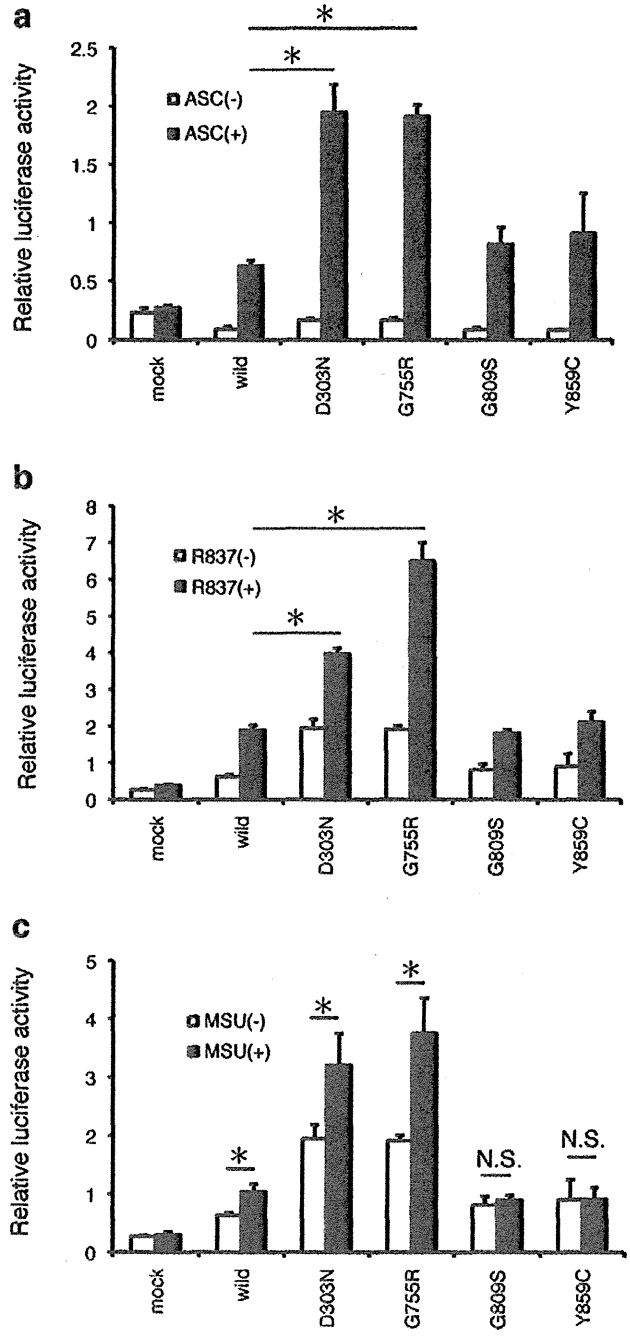

Fig. 4 NF-κB reporter gene activity of *NLRP3* mutants. Bars represent the mean ± SD of triplicate assays. **a** White bars indicate the NF-κB reporter gene activity of the *NLRP3* mutants without co-transfection of ASC. Black bars indicate activity with co-transfection of ASC. ASC-dependent NF-κB reporter gene activity was increased by mutants D303N and G755R. G809S and Y859C did not induce NF-κB reporter gene activity. **b** White bars indicate NF-κB reporter gene activity with co-transfection of ASC. Black bars indicate activity after stimulation with 10 μg/ml R837. **c** White bars indicate NF-κB reporter activity following co-transfection of ASC. Black bars indicate activity after stimulation with 250 μg/ml MSU. * P<0.05

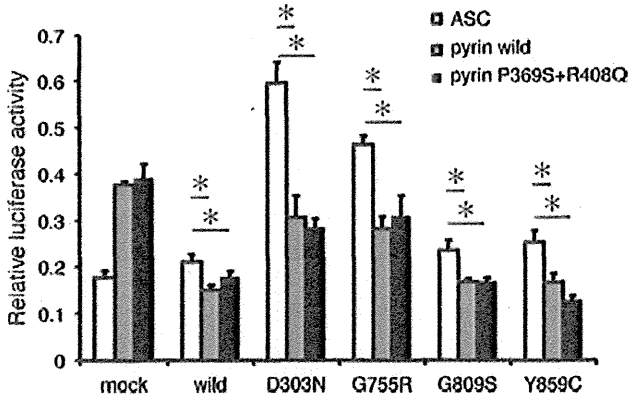

Fig. 5 Pyrin and *NLRP3* mutant-induced NF-κB reporter gene activity. Bars represent the mean ± SD of triplicate assays. White bars indicate NF-κB reporter gene activity with co-transfection of ASC. Grey bars indicate activity with co-transfection of ASC and wild-type pyrin. Black bars indicate activity with co-transfection of ASC and pyrin variant P369S+R408Q. * $P<0.05$

transfected with ASC (Fig. 5). Although both wild-type and variant pyrin inhibited NF-κB activation with co-transfection of wild-type or mutant NLRP3 protein, there was no significant difference in inhibitory capacity between the wild-type and variant pyrin.

Speck Quantification Assay

Previous studies have shown that *NLRP3* LRR variants have an increased ability to induce speck formation in the presence of ASC [6, 12]. To test the effect of G809S on *NLRP3*-ASC interactions and speck formation, wild-type, *NLRP3* variants or empty vectors and pyrin were transiently transfected with cells stably expressing ASC. Cells transfected with *NLRP3* wild-type displayed speck formation (mean ± SD, 36.7± 6.1 %). In comparison, the *NLRP3* D303N, G755R, G809S and Y859C mutants induced significantly higher numbers of speck formation (62.1±8.8 %, 72.6±4.8 %, 53.1±10.1 % and 48.8±13.2 % respectively, Fig. 6).

Discussion

The current study identified a G809S variant within the LRR domain of *NLRP3* with the co-existence of *MEFV* haplotype variants in two unrelated patients with atypical autoinflammatory syndrome. Although we recently reported a CINCA/NOMID patient with the compound heterozygous gene mutations E688K and G809S, it is unclear whether G809S is a pathogenic mutation [9]. To confirm a functional role for the G809S variant, its effect on the NF-κB signaling pathway was investigated in vitro. Although several variants of *NLRP3* show significant increases of ASC dependent NF-κB reporter gene activity in a previous report and as data presented here, no significant increase was observed owing to the *NLRP3* G809S variant in this assay. Kambe et al. demonstrated that the *NLRP3* G755R mutation located within the LRR domain could induce significant NF-κB activation in the presence of an *NLRP3* inflammasome activator, R837 [13]. Therefore,

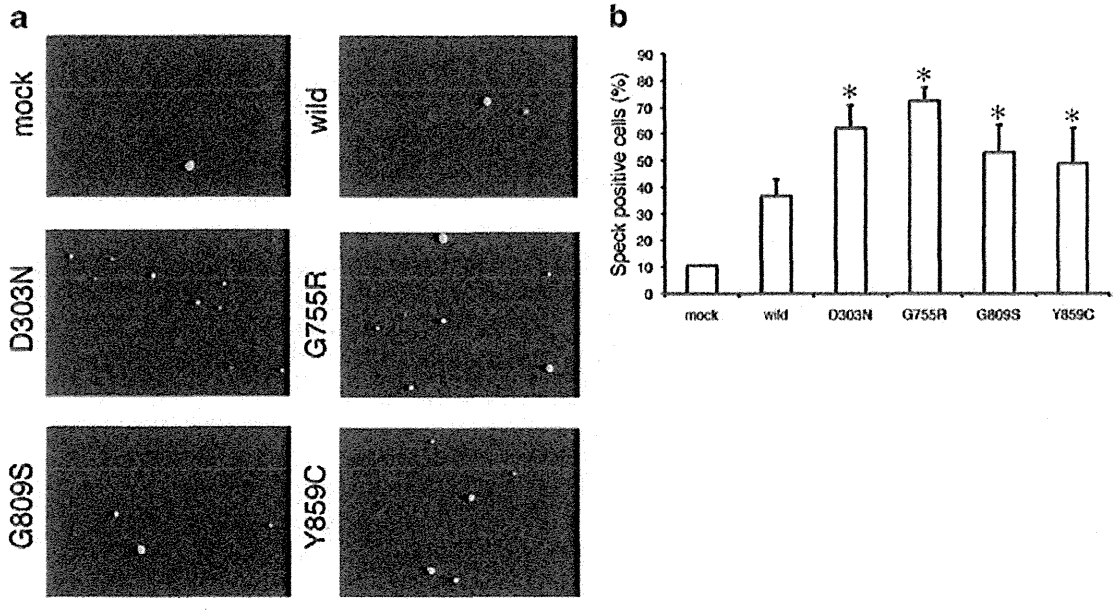

Fig. 6 Effect of the G809S variant on speck formation. Transfection of HEK293-ASC cells with 250 ng each of the *NLRP3* expression plasmids or an empty vector and pyrin expression plasmid was performed. Speck formation was assessed by immunofluorescence microscopy. **a** This panel shows examples of fields obtained by immunofluorescence microscopy. **b** The percentage of cells containing ASC-myc specks was calculated as the mean ± SD percentage of cells. * $P<0.05$

G809S may be expected to enhance NF-κB activation in the presence of R837. However, G809S did not increase NF-κB activity like as Y859C [6] (Fig. 4b). Since the *NLRP3* LRR domain plays a central role in mediating inflammation induced by another inflammasome activator, MSU crystals, we examined whether G809S affected NF-κB activation in the presence of MSU [14]. Interestingly, G809S and Y859C mutations did not show any NF-κB activity responses by MSU stimulation. In contrast, wild-type, D303N and G755R mutations significantly increased NF-κB activity following MSU stimulation. These data suggest the G809S LRR missense variant, which may diminish the responsiveness to PAMPs as NOD2 LRR variant reported in Crohn's disease, has a pathogenic effect on these pathways [15–17].

Jéru et al. recently identified a pathogenic Y859C mutation in the LRR domain of *NLRP3*, which increased speck formation and pro-caspase 1 processing, but which had no direct effect on *NLRP3* mediated NF-κB signaling. The G809S variant also increased speck formation relative to wild-type *NLRP*3. These results suggest that G809S, as well as Y859C in the LRR domain, may be a gain of function variant. It should be noted that although the assays used in this study are sensitive, our findings may provide limited evidence to prove that the G809S variant is pathogenic. However, these results indicate that the variant alters the function of NLRP3.

The two case studies presented here consistently showed elevated IL-1-related serum cytokines, IL-1ra, during the attack phase. In addition, monocytes from case 1 and 2 secreted high levels of IL-1β, which may indicate a gain of function variant in *NLRP3*, associated with inflammasome activation. Additionally, we previously reported a CINCA/NOMID patient positive for the compound heterozygous gene mutations, E688K and G809S [9]. This patient developed severe a phenotype compared with her mother, who carried a single mutation, E688K. This genotype-phenotype correlation suggests that the G809S variant may act as an additional genetic factor associated with the severity of CAPS.

However, in this study IL-1β was not detectable in the serum of patients, as IL-1β might be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. Furthermore, although elevated serum IL-18, which is activated by caspase-1 as well as IL-1β, and IL-6 levels were observed in CINCA/NOMID patient [9], the serum IL-18 levels were increased in case 2 but not case 1, and serum IL-6 levels in both cases did not increase during the fever episodes. Thus, it may be considered that the differences of cytokine profiles and disease phenotypes between case 1 and 2 and typical CINCA/NOMID patients result not only from their genetic background, but also environmental factors.

Additional mutation analysis of our patients also revealed heterozygous variant haplotype of *MEFV*, a gene involved in the pathogenesis of FMF, in addition to G809S in *NLRP3*. Case 1 was heterozygous for P369S and R408Q in *cis* and case 2 was heterozygous for E148Q, P369S, and R408Q in *cis*. Allele frequencies of P369S and R408Q in the Japanese population are 3.6 % and 4.8 %, respectively, according to the International HapMap Project (http://www.hapmap.org/). These frequent variant haplotypes were found to be in strong linkage disequilibrium in the Japanese population. In addition, P369S and R408Q variant haplotype are associated with a variable phenotype and are infrequently associated with typical FMF symptoms [18–21]. Heterozygous P369S and R408Q variant haplotype are also associated with other inflammatory diseases, such as Behçet's disease [18], and systemic lupus erythematosus [21]. Moreover, heterozygous E148Q-P369S-R408Q variant haplotype is more rare, which is associated with chronic recurrent multifocal osteomyelitis [20]. In this report, case 1 and case 2 showed the similar phenotypes as FMF or TRAPS, respectively. Although detailed clinical features and cytokine profiles of the two cases are various, they exhibited a long duration of recurrent fever episodes compared with typical FMF. Thus, these findings suggest that P369S and R408Q variant haplotype may have effects on several inflammatory diseases, but the functional evidence of these variant haplotype remains unclear.

The *MEFV* gene codes for pyrin, that can interact with ASC to induce ASC oligomerization and the activation of procaspase-1, which promotes IL-1β and IL-18 processing [12, 22]. In contrast, some reports have described that pyrin inhibited NLRP3-mediated NF-κB activation by disrupting the NLRP3-ASC interaction [23, 24]. In accordance with the reports, co-expression of NLRP3 and pyrin in HEK293T cells indicated less ASC-dependent NF-κB activation than expression of NLRP3 only, whereas there was no difference in the inhibitory capacity of NF-κB activity between pyrin variants and the wild-type protein. Interestingly, a recent study using pyrin deficient and mutated pyrin knock-in mice demonstrated a gain of function with pyrin variants located in B-Box domains, which caused autoinflammatory phenotypes [22]. Thus, research using knock-in mouse experiments with *MEFV* exon3 variants into pyrin deficient mice would help clarify the pathogenic effects of the *MEFV* variant.

In general, hereditary periodic fever syndromes have been considered monogenic diseases. On the other hand, recent reports have described patients with heterozygous low penetrance variants in two recurrent fever genes [2, 25, 26]. These indicate that oligogenic inheritance has been related to pathogenesis of autoinflammatory diseases. In some cases, patients presented with specific symptoms of both diseases or with a more severe phenotypes. Although the patients in this study were positive for the *NLRP3* variant, they did not present with typical symptoms of CAPS, such as deafness or cold-induced rash. In addition, variants in *MEFV* have been detected in both cases, but they also lacked typical FMF symptoms. However,

both cases had obviously periodic fever episodes. These suggest the presence of oligogenicity and that variants in *NLRP3* and *MEFV* synergistically modify the symptoms of the atypical autoinflammatory diseases.

There are two important limitations in this study when discussing the pathogenicity of low penetrance rare variants. The first limitation is the limited number of patients in the study. Further study using a large number of patients is necessary to confirm our results. Secondly, we only analyzed a limited number of genes. In this study, we concluded that the presence of an *NLRP3* variant with the co-existence of *MEFV* variants contributed to atypical autoinflammatory disease. However, the patients may have had alternative genetic mutations or other rare variants of inflammasome related genes such as *CARD8* [27] elsewhere in the genome, which are truly disease causing, and the two variants described in these patients may be unrelated.

Conclusions

This study describes the molecular analysis of two cases with heterozygous low penetrance variants in exon5 of *NLRP3* and exon3 of *MEFV*. The findings provide in vivo and in vitro evidence for the effect of an *NLRP3* missense variant. Importantly the mutations are within the same signaling pathway and are associated with inflammasome activation. Our observations suggest that oligogenic inheritance may occur in patients with atypical autoinflammatory syndrome. It is therefore important to consider that the phenotypes could be modified by synergistic effects with plural autoinflammatory-associated gene mutations when the patients have atypical autoinflammatory disease.

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Conflict of Interest The authors have declared no conflicts of interest.

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Multiple Reversions of an IL2RG Mutation Restore T cell Function in an X-linked Severe Combined Immunodeficiency Patient

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Abstract Reversion mosaicism is increasingly being reported in primary immunodeficiency diseases, but there have been few cases with clinically improved immune function. Here, a case is reported of X-linked severe combined immunodeficiency (SCID-X1) with multiple somatic reversions in T cells, which restored sufficient cell-mediated immunity to overcome viral infection. Lineage-specific analysis revealed multiple reversions in T cell receptor (TCR) αβ+ and TCRγδ+ T cells. Diversity of the TCRVβ repertoire was comparable to normal and, furthermore, mitogen-induced

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proliferation of the patient's T cells was minimally impaired compared to healthy controls. *In vivo* and *in vitro* varicella antigen-specific T cell responses were comparable to those of healthy controls, although a reduced level of T cell receptor excision circles suggested that recent thymic output was low. During long-term evaluation of the patient's immunologic status, both the number of CD4+ and CD8+ T cells and T cell proliferation responses were stable and the patient remained healthy. This case demonstrates that multiple but restricted somatic reversions in T cell progenitors can improve the clinical phenotype of SCID-X1.

Keywords Severe combined immunodeficiency · reversion · multiple

Introduction

X-linked severe combined immunodeficiency (SCID-X1) is a recessive hereditary disease characterized by a lack of T cells and natural killer (NK) cells. Without stem cell transplantation, persistent infections with opportunistic organisms uniformly lead to death in the first 2 years of life, except in those with atypically attenuated phenotypes [1–3]. Recently, spontaneous genetic reversion has been reported in primary immunodeficiency disorders. Somatic reversion mosaicism is considered to be 'natural gene therapy'; however, few cases are reported with reversions that restore functional immunity [4–9]. Here, an atypical case of SCID-X1 with somatic mosaicism due to multiple reversions in T cells, which restored sufficient T cell immunity, is described.

Materials and Methods

Patient

A male infant was born prematurely at 34 weeks and 4 days of gestation with a birth weight of 1,660 g to healthy parents. There was no family history of consanguinity or immunodeficiency. He was well until 14 months of age, when he started to have recurrent bacterial respiratory tract infections. At the age of 21 months, laboratory tests were performed. Patient results were compared to age-matched normal controls (controls). Examination of serum Ig revealed a decreased level of IgG (IgG, 1.93 g/L [range of controls: 7.15–9.07 g/L]), and normal levels of IgA (IgA, 0.33 g/L [range of controls: 0.22–1.44 g/L]) and IgM (IgM, 0.72 g/L [range of controls: 0.34–1.28 g/L]). His serum IgG was constantly under 2.0 g/L. In addition, he had a reduced number of CD4+ cells (358/μl, [mean of controls: 1,683± 874]) and CD56+ cells (39/μl [mean of controls: 306± 207]), while CD3+ cells (1,803/μl [mean of controls: 2,997±1,751]), CD8+ cells (1,067/μl [mean of controls: 1,683±874]) and CD19+ cells (1,850/μl [mean of controls: 1,114±976]) were within the normal limits. The patient's T cell proliferative response to phytohemagglutinin (PHA) (stimulation index (S.I.) of 172 [range of controls: 105–225]) and to concanavalin-A (Con-A) (S.I. of 140 [range of controls: 68–154]) was within the normal ranges for his age. From these data, he was diagnosed with common variable immunodeficiency (CVID) at that time. Intravenous immunoglobulin therapy was started and he remained in good health thereafter. Without receiving vaccination, varicella infection at 5 years of age did not cause fever, and he was successfully treated with oral acyclovir at an outpatient clinic. At 9 years of age, warts developed and spread over his body, and he was referred to our hospital for assessment of his immunological status. Physical examination revealed neither detectable lymph nodes nor tonsils, and his thymus appeared hypoplastic on CT scan. Before the laboratory studies were performed, informed consent was obtained from the patient and his parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols with a FACSCalibur flow cytometer (Becton Dickinson, USA). The following fluorochrome-conjugated antibodies (Abs) were used for flow cytometric analysis: CD3 (clone SK7), CD4 (clone CK3), CD8 (clone SK1), CD14 (clone M5E2), CD19 (clone SJ25C1), CD56 (clone B159), CD45RA (clone HI100), CD45RO (clone UCHL1) (BD Biosciences Pharmingen, USA), TCRαβ (clone IP26A), TCRγδ (clone IMMU 510) (Beckman Coulter, Inc., USA), CCR7 (clone 150503, R&D Systems Inc., USA), CD27 (clone O323, eBioscience, Inc., USA), CD132 (clone TUGh4, BD Biosciences Pharmingen), and rabbit anti-Human IgD polyclonal Ab (DAKO Japan Co., Japan).

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Sequencing of Genomic DNA and cDNA, and Subcloning Analysis

Peripheral blood mononuclear cells (PBMCs) were obtained from the patient and his parents and various cell lineages were sorted using a FACSVantage (Becton Dickinson). The genomic DNA was isolated from the sorted samples and the cDNA was obtained using reverse transcriptase Super Script II (Invitrogen, USA) with Oligo $(dT)_{20}$ primer. Genomic DNA and cDNA were amplified with the proofreading PCR enzyme, KOD –Plus– (Toyobo, Japan). Direct sequencing analysis of all exons of the *IL2RG* gene, including introns at least 50 bases adjacent, were performed on an ABI 3700 (Applied Biosystems, USA). For analysing revertant subclones in each PBMC lineage, the genomic DNA and the cDNA isolated from sorted cell fractions were amplified by PCR with primer pairs 5′-TCCCAGAGGTT CAGTGTTTTG-3′ and 5′-TTGCAACTGACAGCCA GAAG-3′, and 5′-CGCCATGTTGAAGCCATC-3′ and 5′-TTGCAACTGACAGCCAGAAG-3′, for the region spanning exons 2 and 3 of *IL2RG,* respectively. These PCR products were subcloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced.

T cell Functional Assays

To obtain PHA-induced T cell blasts, PBMCs were stimulated with PHA (Invitrogen) at 1:100 dilution and cultured in RPMI 1640 (RPMI) supplemented with 5 % fetal calf serum (FCS) with recombinant human IL-2 (50 IU/ml, kindly provided by Takeda Pharmaceutical Company, Japan) at 37 °C for 7 days. After being rested in RPMI with 5 % FCS overnight, the T cell blasts were stimulated with various concentrations of IL-2 for 48 h, and [^{3}H]-thymidine uptake assays were performed as previously described [8]. T cell receptor (TCR) Vβ repertoire analysis and CDR3 spectratyping were performed as described [10, 11]. *In vitro* cytokine production against varicella zoster virus (VZV) antigen was performed as previously described [12]. Spots were enumerated automatically using the KS ELISPOT system (Carl Zeiss). The *in vivo* delayed-type hypersensitivity (DTH) reaction to subcutaneous purified VZV antigen (BIKEN, Japan) was performed as previously described [13]. The T cell receptor excision circles (TRECs) from the patient PBMCs were measured as previously described [14].

Tyrosine Phosphorylation of STAT5 by IL-2

PBMCs (1×10^6) were cultured in RPMI with 5 % FCS at 37 °C for 2 h and then treated with or without IL-2 (10,000 U/ml) for 10 min. The cells were fixed and permeabilized with BD Cytofix Buffer and Phosflow Perm Buffer III (BD Biosciences Pharmingen) according to the manufacturer's instructions. After washing with PBS containing 1 % FCS, the cells were stained with mouse anti-pSTAT5 (pY694) (clone 47, BD biosciences), anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry.

Results and Discussion

At the age of 9 years, the patient presented with generalized warts and no detectable lymph nodes and tonsils. This, coupled with his prior hypogammaglobulinemia, prompted a re-evaluation of his immunological status. He showed a decreased level of IgA and a normal level of IgM but no isohemagglutinin. Mitogen-induced proliferation assays showed a slightly reduced response to PHA and Con A (Table I). Surface marker analysis of PBMCs revealed slightly decreased levels of CD3+ and CD4+ T cells, and a normal level of CD8+ T cells (Table II). Naïve CD4+ T cells

Table I Laboratory investigations (patient aged 9 years)

		Patient (IVIG)	Healthy controls
Blood counts			
White blood cells	(count/μl)	7,400	3,600–9,800
Neutrophil	(count/μl)	4,773	3,000–5,000
Lymphocyte	(count/μl)	2,028	2,500–4,500
Monocyte	(count/μl)	340	200–950
Eosinophil	(count/μl)	252	0–700
Basophil	(count/μl)	7	0–150
Red blood cells	($\times10^6$ count/μl)	5.15	4.08–5.07
Hemoglobin	(g/dl)	12.5	11.6–14.1
Platelet	($\times10^3$ count/μl)	275	201–409
Serum Immunoglobulin levels			
IgG	(g/L)	7.69	10.79±2.63
IgA	(g/L)	0.26	2.46±0.91
IgM	(g/L)	1.08	0.83±0.21
IgD	(mg/L)	<6	55±16
IgE	(IU/mL)	<5	<170
isohemagluttinin		Undetectable	
T cell proliferation			
None	(cpm)	163	127–456
Phytohemagglutinin	(cpm)	16,800	20,500–56,800
Concanavalin A	(cpm)	16,600	20,300–65,700
DTH reaction to subcutaneous varicella virus antigen			
Erythematous change (mm in diameter)		18	≧5.0

Control values of blood counts are shown as the range from 95 % of healthy children aged 9 to 12 years. Control values of serum immunoglobulin levels are based on children aged 8 to 10 years and are shown as the mean ± SD. IVIG indicates monthly intravenous infusion of 2.5 g immunoglobulin

Table II Surface marker analysis of peripheral blood mononuclear cells (patient aged 9 years)

	Patient (count/μl)	Healthy controls (count/μl)
CD3+	1,080	2,813±1,197
CD4+	357	1,699±850
CD8+	582	972±457
TCRαβ+	890	2,154±1,004
TCRγδ+	190	324±182
CD4+CD45RA+CCR7+	8	1,290±756
CD8+CD45RA+CCR7+	25	655±503
CD8+CD45RA+CCR7-	114	221±95.3
CD8+CD45RA-CCR7+	33	30.1±27.6
CD8+CD45RA-CCR7-	410	132±87.4
CD19+	894	1,238±605
CD19+CD27+smIgD-	0.4	86.6±61.3
CD19+CD27+smIgD+	14.3	172±123
CD3-CD56+	Undetectable	271±186

Absolute numbers of cells expressing surface markers are shown. Healthy control values are from children aged 2 to 9 years and are shown as mean ± SD

(CD4+/CD45RA+/CCR7+), naïve CD8+ T cells (CD8+/CD45RA+/CCR7+), and both switched memory B cells (CD19+/CD27+/smIgD-) and unswitched memory B cells (CD19+/CD27+/cmIgD-) were scarce. In addition, natural killer (NK) cells (CD3-/CD56+) were absent. This suggested the existence of a genetic defect causing lack of NK cells, such as an *IL2RG* deficiency and *JAK3* deficiency, and therefore the expression of IL2RG (also known as the common gamma chain or CD132) was examined by flow cytometry. Reduced expression was found on B cells and monocytes, although T cells expressed normal levels of CD132 (Fig. 1a). To determine whether CD132-dependent signal transduction was functioning, STAT5 phosphorylation was analyzed on patient CD4+ and CD8+ T cells in response to IL-2. It was found to be comparable with that of normal controls (Fig. 1b). In addition, a proliferation assay of PHA-induced T cell blasts in response to exogenous IL-2 was performed (Fig. 1c). This confirmed that the patient T cells, which were expressing normal levels of CD132, also had intact IL-2 signaling.

To elucidate the genetic cause of the lineage-dependent CD132 expression abnormalities, *IL2RG* genomic sequencing was performed in various cell lineages. Genomic sequencing of *IL2RG* in B cells, monocytes and buccal mucosa revealed a point mutation, c.284-15A>G, in intron 2 of *IL2RG*. This has been reported as a causative mutation of SCID-X1 [15], producing aberrant mRNA with an insertion of 14 bases spanning nucleotide −14 to −1 of exon 3 (Fig. 2a, b). Genomic sequencing of *IL2RG* in T cells showed overlapping bases at and around the mutation sites, while the cDNA of *IL2RG* from

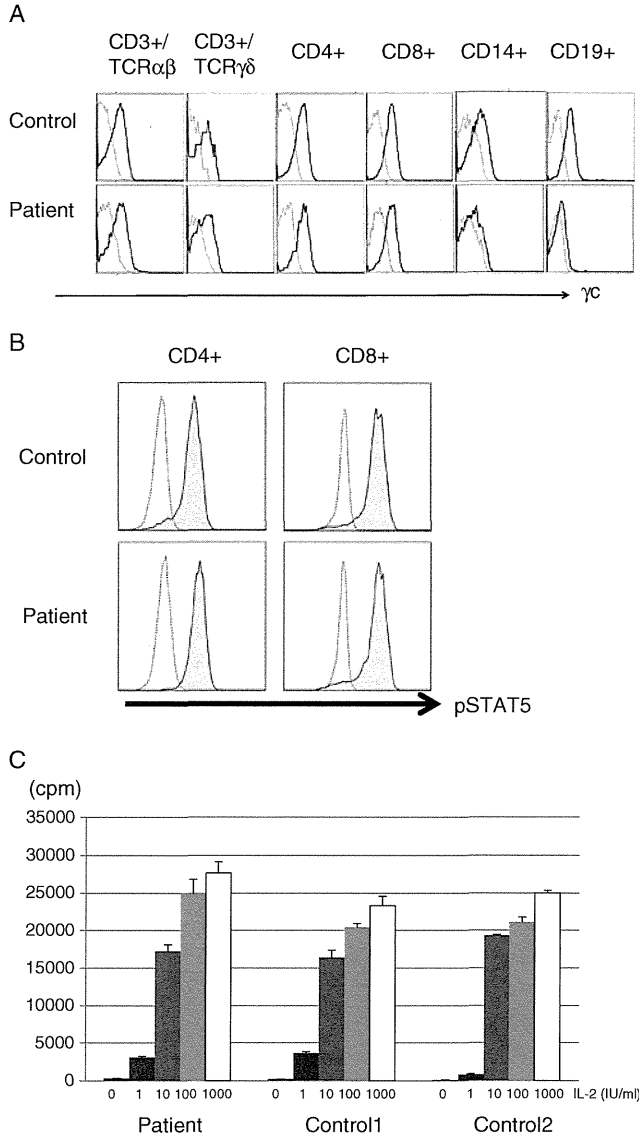

Fig. 1 IL2RG expression and T cell function at 9 years old. **a** Surface expression of IL2RG on PBMCs from the patient and healthy control gated according to the expression of the indicated lineage surface markers. *Black lines* indicate staining for IL2RG (with anti-CD132 Ab) and *gray lines* indicate staining with the isotype control. Data represent one of three independent experiments. **b** STAT5 tyrosine phosphorylation in patient and control CD4+ and CD8+ cells after incubation with (*shaded histograms*) or without IL-2 (*open histograms*). **c** Proliferation of PHA-induced T cell blasts in response to IL-2 stimulation from the patient and two controls. Data are shown as means ± SD

the T cells was normal (Fig. 2a, b). Genomic sequencing of PBMCs from the patient's mother confirmed her as a carrier of the mutation. The possibility of maternal engraftment was excluded by FISH analysis of sex chromosomes (data not shown), and it was concluded that the patient inherited the mutation from his mother and that reversion occurred in the patient's T cells, which led to somatic mosaicism.

To explore the reversions that could have occurred to restore normal *IL2RG* expression in the patient's T cells,

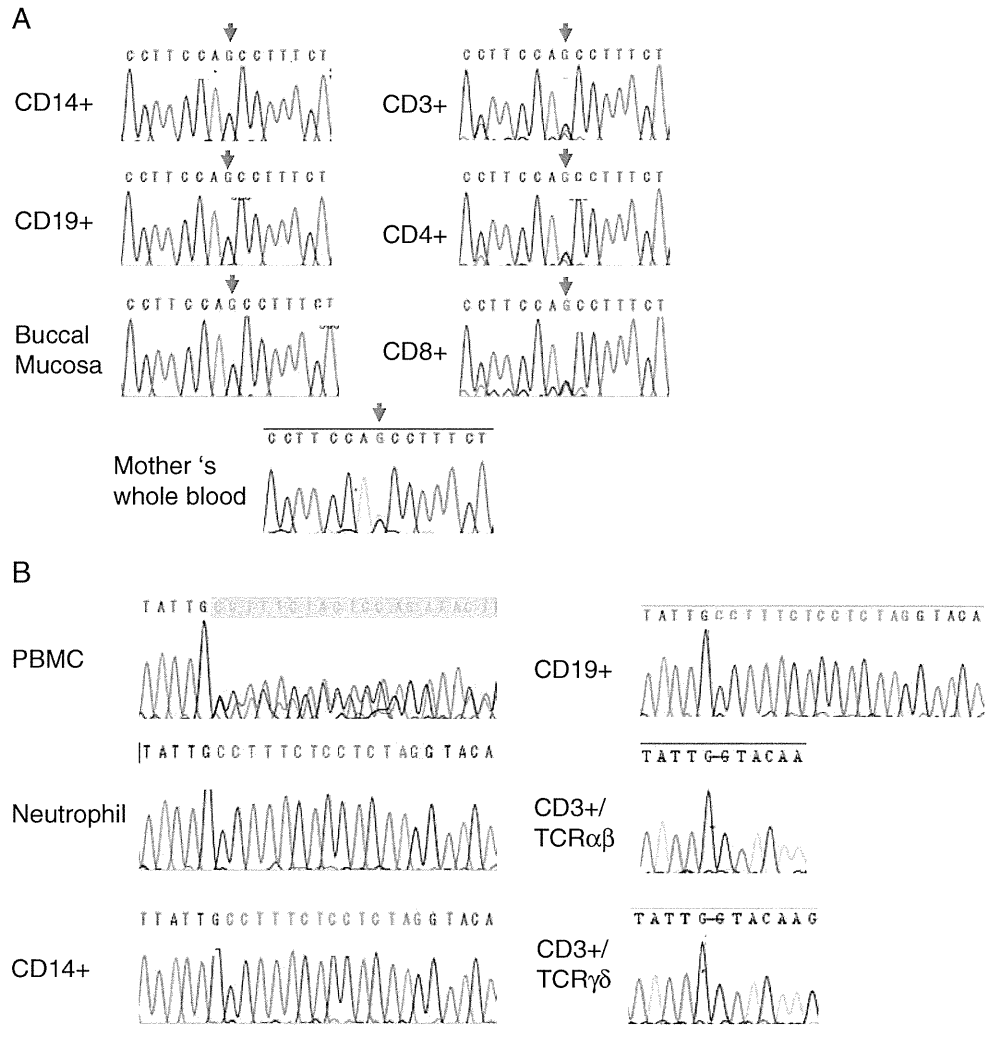

Fig. 2 Genetic analysis of various cell lineages at 9 years old. **a** Sequencing chromatograms of the patient's DNA from various immune cell lineages and buccal mucosa. *Red arrows* indicate the mutated base position c. 284–15. PBMCs from the patient's mother carried the same mutation. The patient's T cells show overlapping base changes at or around the mutated site. Data represent one of three independent experiments. **b** Sequencing chromatograms of the patient's cDNA from various cell types. Re characters indicate the inserted 14 bases spanning nucleotide −14 to nucleotide −1 of exon 3

subcloning and sequencing analysis of genomic DNA and cDNA was performed in various cell lineages. In B cells and monocytes, no reversion was detected and all of the cDNA clones had aberrant splicing (Table III). Analysis of TCRαβ+ cells revealed seven reversions, a true-back reversion, two fully compensating same-site reversions and four second-site reversions, all of which favored a functional reversion according to the splicing analysis software NNSPLICE0.9 [16] (Table IV). None of these base changes were detected in 200 clones from four healthy controls, indicating that the identified intron changes were unlikely to be due to PCR errors. The multiple reversions seen in this case differed from the single reversions seen in other reported cases of reversion mosaicism of SCID-X1 [2, 3]. One possible reason for this is that, compared with the previously reported exonic mutations, an intronic mutation is more likely to acquire additional reversions on top of a true-back mutation. Additionally, the nine-year lifespan of the patient may have provided increased opportunities for extra reversions to occur. TCRVβ V-to-DJ rearrangement is reported to be impaired in some SCID-X1 patients, suggesting that differentiation arrest occurs at the CD4 immature single positive (ISP) stage at which TCRVβ V-to-DJ recombination is completed in normal T cells [17]. Therefore, the

Table III Clonal analysis of *IL2RG* cDNA in various cell lineages

	CD3+	CD4+	CD8+	CD14+	CD19+
Wild-type cDNA	100 % (25/25)	100 % (31/31)	100 % (30/30)	0 % (0/45)	0 % (0/34)
Aberrant cDNA	0 % (0/25)	0 % (0/31)	0 % (0/30)	100 % (45/45)	100 % (34/34)

Data represent the percentages of wild-type or aberrant spliced cDNA subclones in each lineage. The ratio indicates the number of each clone as compared to the total number of clones analyzed, based on subcloning and sequencing analysis

Table IV Multiple additional mutations detected in subclones of the *IL2RG* gene

	Subclones	Mutations
Wild type	TT CCTCT T CCT T CCAACC	Wild type
Inherited mutation	TT CCTCT T CCT T CCAGCC	c.284-15A>G
No.1	TT CCTCT T CCT T CCATCC	c.284-15A>T
No.2	TT CCTCT T CCT T CCACCC	c.284-15A>C
No.3	TT CCTCT T CAT T CCAGCC	c.284-15A>G, c.284-21C>A
No.4	TT **AGAGTGG** CCTCT T CCT T CCAGCC	c.284-15A>G, c.284-29_284-28insAGAGTGG
No.5	TT CCTC **CACCCGCCAAC**	c.284-24_284-14del11ins CACCCGCCAA
No.6	TT CCTCT CAGCC	c.284-23_284-18delTCCTTC

reversions found in the patient's T cells must have occurred before or around the CD4ISP stage. Differences were observed in reversion genotypes between the TCRαβ+ cells and TCRγδ+ cells. TCRγδ+ cells had only one of the second-site reversions found in TCRαβ+ cells in addition to a true-back reversion (No.3 in Table V). The identification of fewer reversions in the patient's TCRγδ+ compared to TCRαβ+ cells may reflect the greater abundance of TCRαβ+ cells, increasing the likelihood of the stochastic occurrence of additional reversions [18]. Although no reversions in the patient's B cells or monocytes were observed, it is possible that the reversions occurred in the progenitor at the stage before commitment to T cells and may reflect the unique nature of T cell proliferation and expansion [19].

Reversion mosaicism has previously been reported in SCID-X1 patients with *IL2RG* mutations, but it was accompanied by reduced T cell number and low proliferative response to mitogens [2, 3]. The paradoxical nature of the patient's cellular immunity, a history of uneventful varicella infection, and the occurrence of widespread warts with very few naïve T cells prompted an evaluation of his T cell function. The TCR Vβ repertoire analysis of CD4+ and CD8+ T cells revealed comparable diversity to the normal controls (data not shown). CDR3 spectratyping analysis revealed the patient CD4+ T cells had as much variety as the normal controls, but his CD8+ T cells displayed restricted diversity (Fig. 3a). To evaluate the antigen-specific response of the patient's T cells, response to VZV was measured. The DTH reaction to subcutaneous VZV antigen and the IFN-γ production from VZV antigen-stimulated PBMCs measured by an ELISPOT assay were comparable to those of normal controls (Table I and Fig. 3b). These data suggested that the patient maintained normal cellular immune responses *in vivo* as well as having normal *in vitro* IFN-γ production ability against VZV antigen. Multiple reversions from intronic mutations could provide a sufficient number of normally functioning T cells and may improve the clinical phenotype compared to previously reported cases with single reversions. However, the number of TRECs in the patient's PBMCs (<10 copies/μg DNA) suggested a low level of recent thymic output, and the restricted diversity of TCRs observed in the patient's CD8+ cells might reflect the exhaustion of the T cell reservoir. To gain further insight, the long-term immunologic status of the patient was evaluated prospectively for 5 years. Absolute counts of CD4+ and CD8+ T cells as well as mitogen-induced T cell proliferation responses were measured every 2–5 months (Fig. 4). Unexpectedly, the number of both CD4+ and CD8+ T cells and mitogen-induced T cell proliferation responses were stable and the patient remained healthy over this period. In recent years, the effector memory subset of CD8+ T cells (CD8+/CD45RA+/CCR7-) has been taken as a marker of cell exhaustion or replicative senescence [20]. However, the majority of CD8+ T cells of the patient were memory CD8+ T cells (CD8+/CD45RA-/CCR7±) and the population of effector memory CD8+ T cells was very small (Table II). These data

Table V Clonal analysis of somatic mosaicism of the *IL2RG* gene in various cell lineages

	Wild type	Inherited mutation	No.1	No.2	No.3	No.4	No.5	No.6
TCRαβ+	21 % (7/33)	12 % (4/33)	9 % (3/33)	12 % (4/33)	21 % (7/33)	9 % (3/33)	6 % (2/33)	9 % (3/33)
TCRγδ+	2 % (1/42)	2 % (1/42)	0 % (0/42)	0 % (0/42)	95 % (40/42)	0 % (0/42)	0 % (0/42)	0 % (0/42)
CD3+	5 % (2/39)	5 % (2/39)	26 % (10/39)	13 % (5/39)	38 % (15/39)	3 % (1/39)	10 % (4/39)	0 % (0/39)
CD4+	32 % (25/79)	3 % (2/79)	13 % (10/79)	19 % (15/79)	16 % (13/79)	3 % (2/79)	3 % (2/79)	13 % (10/79)
CD8+	10 % (7/73)	4 % (3/73)	21 % (15/73)	19 % (14/73)	25 % (18/73)	8 % (6/73)	12 % (9/73)	1 % (1/73)
CD14+	0 % (0/33)	100 % (33/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)
CD19+	0 % (0/30)	100 % (30/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)

Data represent the percentages of each additional mutant subclone in each lineage. The ratio indicates the number of each mutant clone in various cell lineages as compared to the total number of clones analyzed, based on subcloning and sequencing analysis