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Autoimmunity versus Autoinflammation - Friend or Foe?

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Abstract "Autoimmunity" is a designation dependent on the conventional immunological issue of self/non-self discrimination. Identification of novel target autoantigens is still an important issue ongoing in classical tissue-specific autoimmune bullous diseases and autoimmune connective tissue diseases. In contrast, synchronized with the paradigm shift of the fundamental aspect of immunity to danger sensing/signaling, distinct collagen-like diseases have been defined by the genetic mutations causing dysregulated innate immunity/inflammation and have been designated as "autoinflammatory" diseases. Due to the clinical and etiological similarities, the concept of autoinflammatory diseases has expanded to include non-hereditary collagen-like diseases, tissue-specific chronic idiopathic inflammatory diseases and metabolic diseases. On the other hand, various genetic causes of autoimmune diseases have been identified and the border of these two pathophysiologies is becoming obscure. Instead, a variable mixture of both autoimmunity and autoinflammation can cause each inflammatory phenotype with a variable level of antigen specificity.

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Keywords Autoimmunity · Autoinflammation

Autoimmunität versus Autoinflammation - Freund oder Feind?

Zusammenfassung Unter „Autoimmunität" versteht man konventionell das immunologische Problem der Diskriminierung von Selbst und Nicht-Selbst. Die Identifizierung weiterer Target-Autoantigene bei den klassischen gewebe-spezifischen autoimmunen bullösen Dermatosen und den autoimmunen Bindegewebserkrankungen bleibt auch weiterhin ein wichtiges Anliegen. Dabei hat sich das Verständnis der fundamentalen Aspekte der Immunologie hin entwickelt zum Themenkomplex der Gefahrenerkennung (danger sensing) und Signalübertragung. Bei den Kollagenosen wurden teils genetische Mutationen entdeckt, die verantwortlich zeichnen für Störungen der Immunität und Entzündungskaskade. Die autoinflammatorischen Erkrankungen wurden definiert. Aufgrund klinischer und ätiologischer Ähnlichkeiten wurde das Konzept der autoinflammatorischen Erkrankungen auf nicht hereditäre Bindegewebserkrankungen, gewebe-spezifische chronisch idiopathisch-entzündliche Erkrankungen und metabolische Erkrankung ausgedehnt. Andererseits wurden verschiedene genetische Ursachen der Autoimmunerkrankungen entdeckt, so dass das die Grenzen der klassischen Pathologien verschwimmen. In der Tat kann eine Mischung von Autoimmunität und Autoinflammation nahezu jeden Entzündungs-Phänotyp mit variablem Level der Antigenspezifität auslösen.

Schlüsselwörter Autoimmunität · Autoinflammation

The immune system is working for protection of the living things from harmful things, such as invasive patho-

gen and internal malignancy. At first, discriminating self and nonself had been considered the fundamental aspect of immunity, and "autoimmunity" was designated for immune reaction to the self ("auto") antigens. Identification of major histocompatibility complex and concept of central and peripheral tolerance (clonal deletion and anergy as the mechanism, respectively) explained the basis of self-recognition. However, recent research on regulatory T cells (T_{reg}) and tolerogenic dendritic cells has added further implications in keeping unresponsiveness to the self [1, 2].

Clinically, several kinds of chronic inflammatory disorders have been applied for autoimmune diseases, which target self-antigens. They are divided into two categories: tissue-specific autoimmune diseases caused by type II allergy and immune complex-mediated systemic autoimmune diseases caused by type III allergy. In the skin-specific autoimmune bullous diseases, such as pemphigus and pemphigoid, molecular identification of pathogenic antibodies and the corresponding antigens, desmoglein, and type XVII collagen, respectively, provided insights into the molecular basis of skin structure [3]. Interestingly, another severe bullous disease, staphylococcal scalded skin syndrome is caused by a proteolytic exotoxin, which injures homophilic desmoglein junction [4]. Type VII collagen is a target antigen in autoimmune epidermolysis bullosa aquisita, and is genetically deficient in congenital dystrophic epidermolysis bullosa, suggesting that an autoantibody causes functional defect of the corresponding antigen. By analysis of the cases showing lesional immunoglobulin deposit without detection of known autoantibodies, novel target antigens are still continuously discovered [5]. However, it is mostly unclear how the autoantibodies are generated [6].

Systemic autoimmune diseases include the classical connective tissue diseases (rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, and polymyositis/dermatomyositis) except for rheumatoid fever, in which self-antigen mimicking a part of *Group A Streptococcus* becomes the target after streptococcal infection. Serum antinuclear antibody level is elevated in most of these diseases, and the disease-specific tissue-nonspecific autoantibodies against DNA, DNA-binding proteins, and other nuclear/cytoplasmic proteins, are used as markers for diagnosis and for appreciation of the disease activity. However, their pathogenic role is mostly unclear as compared with the case of skin-specific autoimmune bullous diseases. Nevertheless, recent identification of autoantibodies against novel antigens has defined some specific subtypes of dermatomyositis [7]. Interestingly, antibody against MDA5, which acts as an intracellular receptor for viral RNA and belongs to RIG-I-like receptors, is specific for clinically amyopathic dermatomyositis, which is frequently accompanied with rapidly progressing interstitial pneumonitis [8, 9].

Besides the classical autoimmune connective tissue diseases, some related disorders are still accompanied with specific autoantibodies, such as Sjögren's syndrome, antiphospolipid antibody syndrome, and antineutrophil cytoplasmic antibody-related vasculitis. In contrast, the remaining systemic disorders with chronic inflammation, such as adult Still's disease, Behcet's disease, Sweet syndrome, Weber-Christian disease, and sarcoidosis, are negative for autoantibodies and are driven by activated neutrophils and/or macrophages. Therefore, these diseases are considered to be autoimmune-like, but rather related with nonspecific hyper-reactivity or latent infection. These characteristic features are shared with other tissue-specific chronic idiopathic inflammatory diseases, such as urticaria, psoriasis, and inflammatory bowel diseases including Crohn's disease and ulcerative colitis.

Recently, several distinct diseases, whose phenotypes are similar to these chronic idiopathic inflammatory diseases, have been defined by the causative genetic mutations. As these mutations cause dysregulation of innate immunity/inflammation, the defined diseases have been designated as "autoinflammatory" diseases [10]. These processes have been synchronized with the paradigm shift of the fundamental aspect of immunity, from self/nonself discrimination to danger sensing/signaling [11]. Familial Mediterranean fever (FMF) and related hereditary periodic fever syndromes are the prototypic autoinflammatory disorders and most of them are caused by dysregulated activation of NOD-like receptors (NLR) P3 inflammasome, which senses various dangerous stimuli to induce interleukin (IL)-1β secretion, such as bacterial RNA, imidazoquinolin, and contact allergen [12]. As referred to the membranous toll-like receptors (TLR), NLR have been shown to act as intracellular sensors for various pathogen- or danger-associated molecular patterns [13]. Therefore, it is conceivable that these hereditary diseases resemble infectious or allergic diseases. Although heterozygous *NLRP3* mutations-oriented cryopyrin-induced periodic fever syndromes (CAPS) include formerly called familial cold urticaria, febrile attacks seem to occur periodically or even "automatically", without any apparent triggers. The category of hereditary autoinflammatory diseases has rapidly expanded to include more numbers of diseases, such as pyogenic pustular diseases and systemic granulomatosis [14]. Differentiation of pyogenic arthritis, pyoderma gangrenosum and acne syndrome with *PSTPIP1* mutations among pyoderma gangrenosum and/or cystic acne patients, identification of deficiency for IL-36 receptor antagonist with *IL36RN* mutations among generalized pustular psoriasis patients, and distinction of early onset sarcoidosis with *NOD2* mutations from sarcoidosis have indicated the fact that such monogenic diseases constitute at least a part of sporadic common diseases [15–17].

Then, the previously described autoantibody negative chronic idiopathic inflammatory disorders are considered as acquired autoinflammatory diseases, which share clinical features and the putatively dysregulated inflammatory pathways with hereditary autoinflammatory diseases. Adult Still's disease, Behcet's disease, and Weber-Christian disease show periodic febrile

attacks with skin rash and are similar to CAPS, FMF, and Nakajo-Nishimura syndrome with *PSMB8* mutations, respectively. Sweet syndrome is associated with Majeed syndrome with *LPIN2* mutations [18, 19]. Moreover, Schnitzler syndrome with various clinical similarities to CAPS shows dramatic improvement by anti-IL-1β therapy, suggesting the underlying activation of NLRP3 inflammasome [20]. Thus, autoimmune and autoinflammatory diseases seem apparently distinguished by their clinical and/or genetic features.

Furthermore, some metabolic diseases such as gout, pseudogout, and type 2 diabetes mellitus, which are caused by chronic inflammation due to monosodium urate crystals, calcium pyrophosphate crystals, and hyperglycemia, respectively, are categorized as another class of autoinflammatory diseases [21]. In these diseases, self molecule-induced activation of NLRP3 inflammasome is considered responsible, and anti-IL-1β therapy is effective. Similarly, it has been shown that obesity-induced metabolic syndrome is mediated by TLR4 activation in adipocytes through saturated fatty acid, which can be called as homeostatic inflammation rather than autoinflammation [22].

On the other hand, it is well known that genetic background is important for development of systemic autoimmune diseases. Various mouse strains have been reported to be autoimmune-prone, such as *lpr* (deficient for *fas*), *gld* (deficient for *fas ligand*), *Dnase1-/-*, *Ctla4-/-*, and *Pd1-/-* mice [23, 24]. These mutations are related with dysregulation of apoptosis, clearance of apoptosis, and negative costimulatory (coinhibitory) signaling, which lead to breakage of tolerance. In humans, Aicardi-Goutieres syndrome, which shows the characteristic features of overlapping with cerebellar viral infection and systemic lupus erythematosus, is caused by deficiency for various exonucleases, such as TREX1/DNaseIII [25]. Identification of such various genetic causes for autoimmune diseases is making the border obscure between these diseases and hereditary autoinflammatory diseases.

Interestingly, *Il1rn-/-* mice show rheumatoid arthritis-like autoimmune and psoriasis-like T cell-independent autoinflammatory phenotypes, while *IL1RN* deficiency causes an autoinflammatory syndrome termed deficiency for IL-1 receptor antagonist in humans [26–28]. It may sound strange that both systems can be dysregulated on a monogenic background. However, considering that innate and adaptive immunity not only regulate but also activate each other, it is not surprising that both phenotypes of autoimmunity and autoinflammation are observed in the same body. Actually, elevation of nonspecific autoantibodies can be observed during development of Nakajo-Nishimura syndrome [17]. To develop the autoimmune phenotype (T/B cell-mediated autoantigen-specific hyper-reactivity), autoinflammatory responses (dysregulated signaling from the autoantigen through some pattern recognition receptor such as TLRs) should be involved [29]. Very recently, it has been reported that the repetitive percutaneous stimulation with imidazoquinolin generated a new model of systemic lupus erythematosus with high serum level of anti-double-stranded DNA antibodies [30]. Collectively, a variable mixture of both autoinflammation and autoimmunity can cause the inflammatory phenotype with a variable level of antigen specificity, depending on the lesional tissue, triggering factors, and other genetic backgrounds.

Conflict of interest

None declared.

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Serum Interleukin 18 as a Diagnostic Remission Criterion in Systemic Juvenile Idiopathic Arthritis

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Serum Interleukin 18 as a Diagnostic Remission Criterion in Systemic Juvenile Idiopathic Arthritis

To the Editor:

With the development of new therapeutic agents and combination treatment strategies, more children with systemic juvenile idiopathic arthritis (sJIA) can experience protracted periods of low disease activity levels and, in some cases, complete disease quiescence. These advances create a need for the development of validated criteria that precisely describe the clinical state of disease quiescence.

We previously reported that serum interleukin 18 (IL-18) levels in patients with sJIA were extremely high during the active phase and remained significantly elevated even when other markers of disease activity normalized[1]. We also reported that serum IL-18 levels at birth in a healthy infant born to a woman with active adult-onset Still's disease were markedly increased, and this increase persisted for about 1 month[2]. These findings indicate that it takes several months for extremely elevated serum IL-18 levels to normalize under physiological conditions.

In our study, we serially measured serum IL-18 levels in 11 patients with sJIA (age 10.2 ± 7.6 yrs, male:female = 3:8) until they had relapsed or achieved remission to investigate the kinetics of serum IL-18 levels from the active phase to remission. Further, we investigated the correlation between serum IL-18 and IL-6 levels in these patients. Among 11 patients, 4 patients were enrolled in our previous study[1], while 7 patients were enrolled in our other study[3]. The criteria defining the active phase of sJIA were active arthritis, fever, rash, hepatosplenomegaly, generalized lymphadenopathy, and serositis, as well as increased erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels. The criteria for inactive phase of sJIA were as follows: no active arthritis, no fever; no rash, serositis, splenomegaly, or generalized lymphadenopathy; normal ESR or CRP level, and a physician's global assessment of disease activity indicating clinical disease quiescence[4,5]. The criteria for remission are as follows: remission while receiving medication, a minimum of 6 continuous months of inactive disease while receiving medication, clinical remission while not receiving medication, or 12 months of inactive disease while not receiving any medication[4,5]. Serum IL-18 and IL-6 levels were determined using a commercial ELISA as we previously reported[1]. The limit of detection of IL-18 and IL-6 in our assay were < 12.5 pg/ml and < 3.0 pg/ml. In our assay, serum IL-18 and IL-6 levels of 28 healthy controls (age 8.8 ± 7.3 yrs) were 140.5 (76–255) pg/ml and < 3.0 pg/ml[1], respectively. The protocol of our study was approved by the Institutional Review Board of Kanazawa University, and all the patients provided informed consent. All patients were treated with high-dose steroid, including methylprednisolone pulse therapy (30 mg/kg/day, 3 days). In addition to steroid, 6 patients were treated with cyclosporine and 2 patients were treated with tocilizumab (TCZ; 8 mg/kg, every 2 weeks). Followup periods were at least over 15 months (15 mos–7 yrs). As we previously reported, serum IL-6 levels in patients receiving TCZ therapy are higher compared to those in patients not receiving TCZ therapy because IL-6 receptor-mediated consumption of IL-6 is inhibited by the unavailability of TCZ-free IL-6 receptor[6,7]. Therefore, the correlation between serum IL-18 and IL-6 levels was determined in 9 patients not receiving TCZ therapy.

Of the 11 patients, 5 had no relapse (group A); 3/5 patients achieved remission while not taking medication. Remission while not taking medication was maintained for over 3 years. Two patients achieved remission while taking medication. The other 6 patients experienced relapse during withdrawal of steroid within 12 months after disease onset (group B). Of the 6 patients, 4 experienced relapse during the inactive phase and the other 2 patients experienced relapse during remission while taking medication. As shown in Figure 1A, the longitudinal examination of group A patients showed that serum IL-18 levels decreased to the levels < 1000 pg/ml in inactive phase and normalized in remission phase. In contrast, longitudinal examination of group B patients clearly demonstrated a sustained elevation of serum IL-18 levels (> 1000 pg/ml) during the inactive phase (Figure 1B). The serum IL-18 levels correlated positively with the serum IL-6 levels ($p < 0.0001$; Figure 1C). However, serum IL-18 levels remained elevated even in inactive phase after serum IL-6 levels normalized.

Wallace, *et al* proposed remission criteria for JIA, which acknowledged that patients with JIA may be in 1 of 2 states, either active or inactive disease[4,5]. The following are included in the criteria for inactive disease: no active arthritis, no fever; no rash, serositis, splenomegaly, or generalized lymphadenopathy; normal erythrocyte sedimentation rate or C-reactive protein level, and a physician's global assessment of disease activity indicating clinical disease quiescence. Inactive disease can be further divided into clinical remission while taking medication (a minimum of 6 continuous mos of inactive disease while receiving medication) and clinical remission while not taking medication (12 mos of inactive disease while not receiving any medications).

In our study, serum IL-18 levels in the remission group (group A) were elevated during the inactive phase, but these significantly decreased to normal levels during the remission phase. These findings support the accuracy of the Wallace criteria for clinical remission. Further, serum IL-18 levels may be useful as a diagnostic laboratory criterion for clinical remission in sJIA.

In the clinical setting of sJIA, relapses of acute sJIA flares have occurred during the inactive phase[1]. In our study, 6 patients experienced relapses during the inactive phase or early phase of remission while taking medication. Longitudinal examination in these patients demonstrated sustained elevation of serum IL-18 levels during these phases. These findings indicate that persistent inflammation associated with macrophage activation cannot be controlled. Serum IL-18 levels may reflect the biological activity of the immune system and disease activity of sJIA.

The limitation of our study is the small number of patients with sJIA who were evaluated. It would be necessary to perform larger scale studies to confirm our preliminary data and draw firm conclusions. Despite this limitation, our results indicate that serum IL-18 levels reflect the biological activities of the immune system in sJIA and may predict the prognosis of sJIA. Serum IL-18 levels may be useful as a diagnostic laboratory criterion for clinical remission in sJIA.

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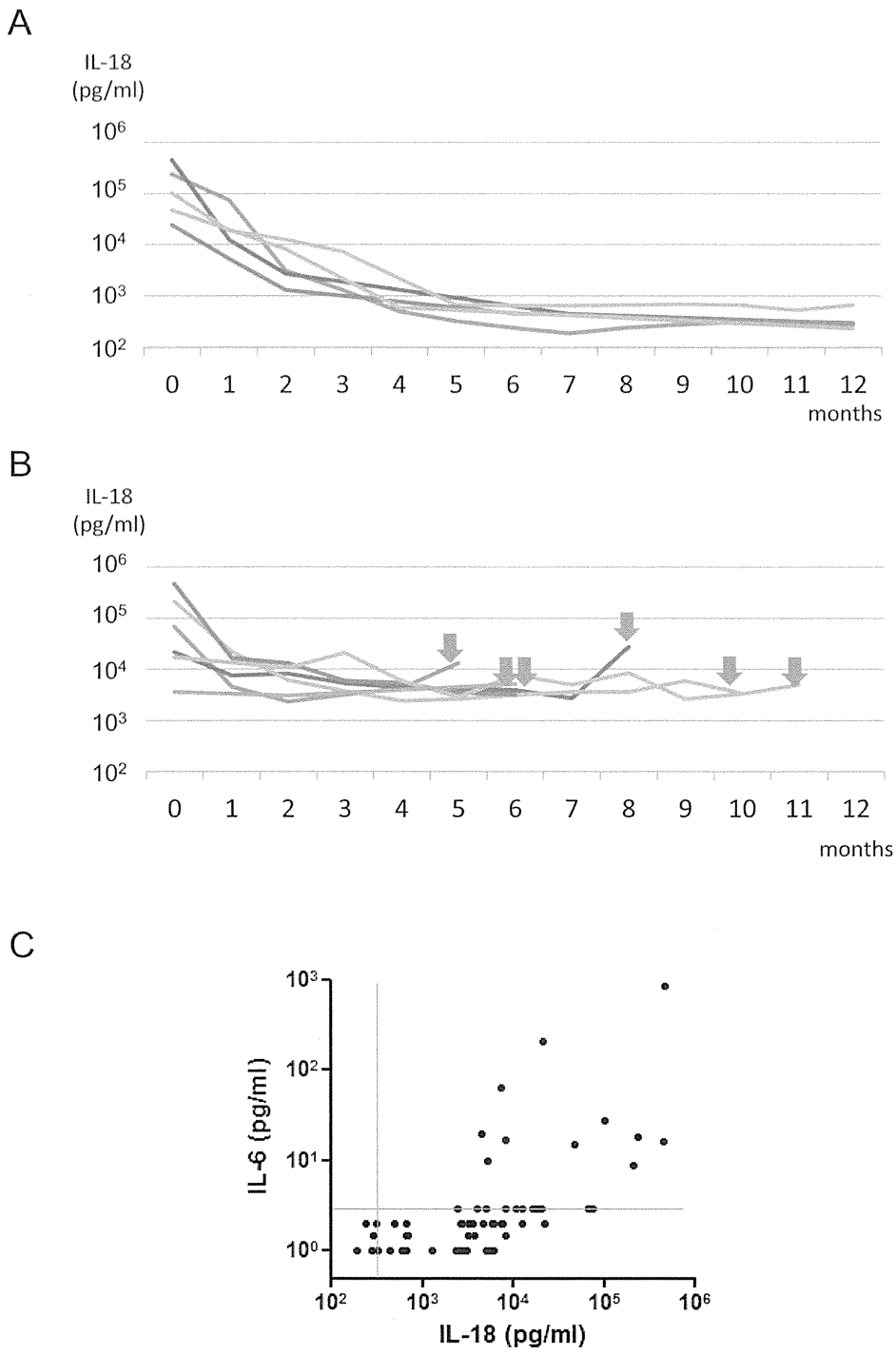

Figure 1. Longitudinal examination of serum interleukin 18 (IL-18) levels in patients with systemic juvenile idiopathic arthritis. A. Group A: patients with remission. B. Group B: patients with relapse. Orange arrows show the timing of the relapses in each patient. C. Correlation between serum IL-18 and IL-6 levels. Serum IL-6 levels are within normal limits whereas IL-18 levels remain elevated.

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Defect of suppression of inflammasome-independent interleukin-8 secretion from SW982 synovial sarcoma cells by familial Mediterranean fever-derived pyrin mutations

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Abstract Familial Mediterranean fever (FMF) is a recessive inherited autoinflammatory syndrome. Patients with FMF have symptoms such as recurrent fever and abdominal pain, sometimes accompanied by arthralgia. Biopsy specimens have revealed substantial neutrophil infiltration into synovia. FMF patients have a mutation in the Mediterranean fever gene, encoding pyrin, which is known to regulate the inflammasome, a platform for processing interleukin (IL)-1β. FMF patients heterozygous for E148Q mutation, heterozygous for M694I mutation, or combined heterozygous for E148Q and M694I mutations, which were found to be major mutations in an FMF study group in Japan, suffer from arthritis, the severity of which is likely to be lower than in FMF patients with M694V mutations. Expression plasmids of wild-type (WT) pyrin and mutated pyrin, such as E148Q, M694I, M694V, and E148Q+M694I, were constructed, and SW982 synovial sarcoma cells were transfected with these expression plasmids. IL-8 and IL-6 were spontaneously secreted from the culture supernatant of SW982 cells without any stimulation, whereas IL-1β and TNF-α could not be detected even when stimulated with lipopolysaccharide. Notably, two inflammasome components, ASC and caspase-1, could not be detected in SW982 cells by Western blotting. IL-8 but not IL-6 secretion from SW982 cells was largely suppressed by WT pyrin, but less suppressed by mutated pyrin, which appeared to become weaker in the order of E148Q, M694I, E148Q+M694I, and M694V mutations. As for IL-8 and IL-6, similar results were obtained using stable THP-1 cells expressing the WT pyrin or mutated pyrins, such as M694V or E148Q, when stimulated by LPS. In addition, IL-8 secretion from mononuclear cells of FMF patients was significantly higher than that of healthy volunteers when incubated on a culture plate. Thus, our results suggest that IL-8 secretion from SW982 synovial sarcoma cells suppressed by pyrin independently of inflammasome is affected by pyrin mutations, which may reflect the activity in FMF arthritis.

Keywords Pyrin · Familial Mediterranean fever · Interleukin-8 · Synovial sarcoma · SW982

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Introduction

Familial Mediterranean fever (FMF) (OMIM#249100) is an autosomal recessive inherited autoinflammatory syndrome [1]. Patients with FMF have symptoms such as recurrent fever and abdominal pain, sometimes accompanied by arthralgia [1]. Patient biopsies have revealed that the cause is arthritis, involving neutrophils infiltrating into synovium [2]. Patients have a mutation in the Mediterranean fever (*MEFV*)

gene, encoding pyrin, which is known to regulate the inflammasome, a platform for processing interleukin (IL)-1β [3–5].

In Middle Eastern countries, FMF patients homozygous for M694V suffer a severe form of the disease, with clinical manifestations including arthritis, while the condition of FMF patients homozygous for M694I, one of the major mutations in Japan, is not so severe [6–8]. In Japan, there were no FMF patients with M694V mutation found by the Japan study group of FMF patients [9]. FMF patients heterozygous for E148Q, heterozygous for M694I, or combined-heterozygous for E148Q and M694I suffer from arthritis, and the severity of E148Q was reported to be lower than in FMF patients with M694V [10]. Interestingly, there was reported to be a corelationship between the concomitant expression of *MEFV* and C5a/IL-8-inhibitor activity in primary cultures of human fibroblasts [11].

Activation of inflammasome is reported to lead to IL-8 production from some cells, as well as that of IL-1β [12]. IL-8 is a chemotactic factor for neutrophils and enhances the trans-endothelial migration of neutrophils by inducing rapid shedding of L-selectin [13, 14]. Focal IL-8 secretion may reflect disease activity [15]. These facts prompted us to evaluate IL-8 secretion from synovial cells.

In this study, we evaluated the secretion of cytokines such as IL-1β, IL-6, IL-8, and TNF-α secretion from synovial sarcoma SW982 cells and monocytic leukemia THP-1 cells transfected with expression plasmids encoding wild-type (WT) pyrin and E148Q, M694V, M694I, and E148Q+M694I mutated pyrin in order to obtain new insight into FMF arthritis. Then, we also confirmed the results were reflected clinically in FMF patients.

Materials and methods

Preparation of expression plasmids

Expression plasmids encoding M694V, M694I, E148Q, E148Q+M694I, or WT pyrin were constructed as follows. The entire open reading frame of pyrin was inserted into the *EcoRI* and *BglII* sites of pFLAG-CMV-4 (Vector) (Sigma-Aldrich, St. Louis, MO, USA) to produce pFLAG-CMV-4-pyrin-WT from pcDNA3-HA-pyrin-WT as a template by polymerase chain reaction (PCR) using primer sets as follows: forward primer Pyrin-EcoRI-F 5-GCGAATTCAGCTAAGACCCCTAGTGACCAT-3 and reverse primer Pyrin-BglII-R 5-GTCAGATCTTCAGTCAGGCCCCTGACCACC-3 [16, 17]. PCR-based site-specific mutagenesis for pFLAG-CMV-4-pyrin-E148Q was generated by two-step PCR using primer sets as follows: forward primer Pyrin-EcoRI-F 5-GCGAATTCAGCTAAGACCCCTAGTGACCAT-3 and reverse primer Pyrin-E148Q-R 5-GGTGCAGCCAGCCCCAGGCCGGGAGGGGGC-3, and forward primer Pyrin-E148Q-F 5-GCCCCCTCCCGGCCTGGGGCTGGCTGCACC-3 and reverse primer Pyrin-BglII-R 5-GTCAGATCTTCAGTCAGGCCCCTGACCACC-3 for the first over-lapping DNA fragment set from pcDNA3-HA-pyrin-WT plasmid as a template [16, 17]. Full-length E148Q mutated-pyrin DNA fragment was amplified by second PCR using a primer set as follows: forward primer Pyrin-EcoRI-F 5-GCGAATTCAGCTAAGACCCCTAGTGACCAT-3 and reverse primer Pyrin-BglII-R 5-GTCAGATCTTCAGTCAGGCCCCTGACCACC-3 from the first over-lapping DNA fragment set as templates, and then inserted into the *EcoRI* and *BglII* sites of pFLAG-CMV-4. pFLAG-CMV-4-pyrin-M694V and pFLAG-CMV-4-pyrin-M694I were generated by the same method. pFLAG-CMV-4-pyrin-E148Q+M694I was also generated by the same method from pFLAG-CMV-4-pyrin-E148Q as a template. Primers and oligonucleotide sequences are listed in Table 1. Mutations were confirmed by sequencing (Fig. 1a).

Transfection of expression plasmids, their expression and generation of THP-1 stable cells

5×10^6 human embryonic kidney (HEK) 293T cells were transfected with 3 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, or pFLAG-CMV-4-pyrin-E148Q+M694I using the calcium phosphate method as described previously [18]. 36 h after transfection, each protein's expression was detected by Western blotting. 5×10^6 synovial sarcoma SW982 cells were transfected with pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, or pFLAG-CMV-4-pyrin-E148Q+M694I using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA) as per the manufacturer's instructions. 1×10^7

Table 1 Primers and oligonucleotide sequences for site-specific mutagenesis of pyrin plasmids

Name	Oligo-nucleotide-sequence
Pyrin-EcoRI-F	5′ GCGAATTCAGCTAAGACCCCTAGTGACCAT 3′
Pyrin-BglII-R	5′ GTCAGATCTTCAGTCAGGCCCCTGACCACC 3′
Pyrin-E148Q-F	5′GGTGCAGCCAGCCCCAGGCCGGGAGGGGGC 3′
Pyrin-E148Q-R	5′GCCCCCTCCCGGCCTGGGGCTGGCTGCACC 3′
Pyrin-M694I-F	5′GGTGGTGATAATGATCAAGGAAAATGAGTA 3′
Pyrin-M694I-R	5′TACTCATTTTCCTTGATCATTATCACCACC 3′
Pyrin-M694V-F	5′GGTGGTGATAATGGTGAAGGAAAATGAGTA 3′
Pyrin-M694V-R	5′TACTCATTTTCCTTCACCATTATCACCACC 3′

Underlines indicate mutation codons for specific amino acids

Double underlines indicate restriction enzyme sites

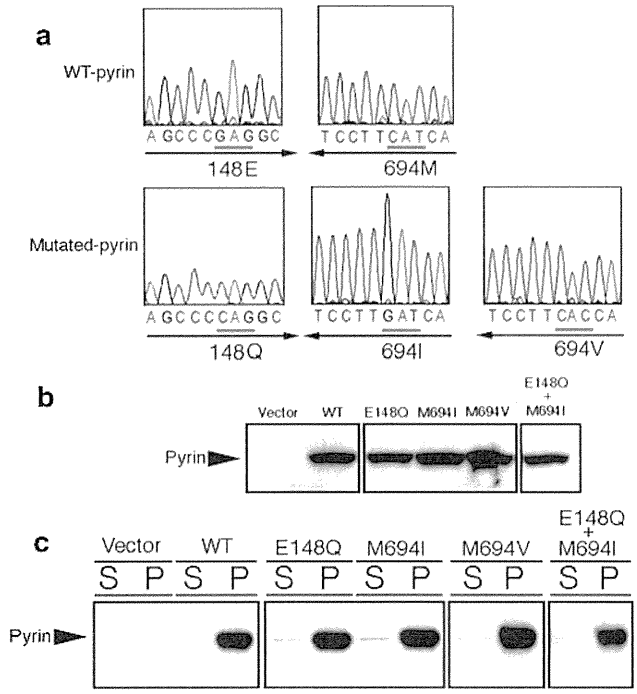

Fig. 1 Chart of sequencing of mutated pyrin plasmids, expression, and fractionation in human embryonic kidney 293T cells. **a** The mutated-pyrin expression plasmids pFLAG-CMV-4-pyrin E148Q, pFLAG-CMV-4-pyrin M694I, and pFLAG-CMV-4-pyrin M694V were sequenced to confirm (from GAG to CAG corresponding to E148Q; from complementary CAT to GAT corresponding to M694I; from complementary CAT to CAC corresponding to M694I) mutations in the appropriate site. **b** 5×10^6 human embryonic kidney 293T cells were transfected with 3 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, and pFLAG-CMV-4-pyrin-E148Q+M694I. 36 h after transfection, 30 μg of each whole cell lysate was subjected to Western blotting. **c** 5×10^6 human embryonic kidney 293T cells were transfected with 3 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, and pFLAG-CMV-4-pyrin-E148Q+M694I. 36 h after transfection, the cells were lysed in 1 % (v/v) NP-40 buffer and fractionated into soluble (*S* supernatant) and insoluble (*P* pellet) fraction. 30 μg of each fractionated protein was subjected to Western blotting

monocytic leukemia THP-1 cells were transfected with 5 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, or pFLAG-CMV-4-pyrin-M694V, using the Amaxa® Nucleofector as per the manufacturer's instructions. After incubation with 500 μg/ml G418 (Sigma) in RPMI 1640 medium including 10 % fetal bovine serum (FBS) (Defined, endotoxin ≦10 EU/ml; Thermo Scientific HyClone, South Logan, UT, USA) for 4 weeks at 37 °C in a humidified atmosphere with 5 % CO_2, THP-1 stable cells expressing WT or mutated pyrin protein were generated.

Fractionation of cell lysates

5×10^6 HEK293T cells were transfected with 3 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, and pFLAG-CMV-4-pyrin-E148Q+M694I. 36 h after transfection, the cells were lysed in 1.0 % (v/v) NP-40 buffer (1 % Nonidet P-40, 142.5 mM KCl, 5 mM MgCl–$6H_2O$, 10 mM HEPES [pH 7.6], 0.2 mM PMSF, 1 mM EDTA) with proteinase inhibitor cocktail Complete™ (Roche Molecular Biochemicals, Mannheim, Germany). The lysate was fully dislodged from the plate surface with a rubber policeman. The lysate from one dish was incubated in a 1.5-ml tube on ice, clarified by centrifugation at 12,000 rpm for 20 min, and separated into soluble (S: supernatant) and insoluble (P: pellet) fractions. Both fractions of the whole cell lysate were subjected to Western blotting using anti-pyrin polyclonal antibody (AL196; ALEXIS Biochemical, Lausen, Switzerland) (Fig. 1c).

Measurement of cytokine secretion from synovial sarcoma SW982 cells and monocytic leukemia THP-1 cells

Human synovial sarcoma SW982 cells and monocytic leukemia THP-1 cells were purchased from American Type Culture Collection, and pre-cultured in 12-well flat-bottomed plates (BD Biosciences, San Jose, CA, USA) to a final cell density of 1×10^6/ml in a volume of 1 ml of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA), including 10 % fetal FBS, for 24 h at 37 °C in a humidified atmosphere with 5 % CO_2. The cells in each well were transfected with 1.67 μg of expression plasmids in the presence of 0.67 μg of pEF1-BOS-β-gal. 8 h after transfection, culture medium was replaced by 1 ml of DMEM alone, or DMEM containing 1.0 ng/ml or 1.0 μg/ml of lipopolysaccharide (LPS) (from *Escherichia coli* O55:B5, cell culture tested, purified by phenol extraction; Sigma-Aldrich, St. Louis, MO, USA). 8 h after medium replacement, the concentrations of IL-1β, IL-6, IL-8, and TNF-α in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) with specific antibodies (BD Biosciences, San Jose, CA, USA). Percentiles of IL-8-related-suppression ratio of mutated pyrin versus WT pyrin were normalized to the transfection efficiency by β-galactosidase activity from triplicate experiments. THP-1-derived stable cells expressing WT or mutated pyrin proteins were pre-cultured in 24-well flat-bottomed plates (BD Biosciences, San Jose, CA, USA) to a final cell density of 2×10^7/ml in a volume

of 300 μl of RPMI1640 Medium (Invitrogen, Grand Island, NY, USA), including 10 % FBS, for 24 h at 37 °C in a humidified atmosphere with 5 % CO_2. Then, culture medium was replaced with 300 μl of RPMI1640 containing 10 ng/ml LPS. 8 h after medium replacement, the concentrations of IL-1β, IL-6, IL-8, and TNF-α in the culture supernatant were measured by ELISA with specific antibodies (BD Biosciences, San Jose, CA, USA).

Western blotting analyses for p38, ERK, and NF-κB pathways

40 μg of SW982 cell lysates were subjected to SDS-PAGE followed by Western blotting analysis for p38, ERK, and NF-κB, pathways. Signals from the same blotting membrane were detected by Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP rabbit monoclonal antibody (Cell Signaling catalog No. #4511) and the p38α MAPK rabbit polyclonal antibody (Cell Signaling catalog No. #9218) for p38 MAPK pathway, or Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit monoclonal antibody (Cell Signaling catalog No. #4370) and p44/42 MAPK (Erk1/2) (137F5) rabbit monoclonal antibody (Cell Signaling catalog No. #4695) for the ERK pathway, or phospho-NF-κB p65 (Ser536) rabbit polyclonal antibody (Cell Signaling catalog No. #3031) and NF-κB p65 (D14E12) XP rabbit monoclonal antibodies (Cell Signaling catalog No. #8242) for the NF-κB pathway.

Cytokine assays for peripheral blood mononuclear cells

Participation of FMF patients and almost age-matched healthy volunteers regarding the analyses of *MEFV* gene and their blood samples with their written informed consents was approved by the institutional review board at the Shinshu University. We obtained peripheral blood mononuclear cells from five FMF patients with definite diagnosis as FMF according to the 'Tel Hashomer' criteria presented a symptom with typical type of FMF, and exhibited a favorable response to colchicine. All of them had *MEFV* mutations; four patients were E148Q/M694I compound heterozygotes (a 30-years-old woman, an 8-years-old boy, a 25-years-old woman and a 22-years-old woman) and one patient was an E148Q/E148Q homozygote (a 7-years-old girl). 5×10^5/ml of peripheral blood mononuclear cells were incubated in 96-well flat plates (Nunc) with RPMI1640 with 10 % heat-inactivated FBS for 6 h. The supernatants were collected and analyzed for cytokine concentration with the Cytometric Bead Array Flex set (BD Biosciences) according to the manufacturer's instructions. For intracellular cytokine staining, 5×10^5/ml of mononuclear cells including BD GolgiPlug protein transport inhibitor (BD Biosciences) were incubated under the same conditions as described above. After 6 h of incubation, adherent cells were collected by pipetting. The cells were fixed using a BD Cytofix/Cytoperm solution for 20 min at 4 °C, then the fixed cells were permeabilized by washing two times in 1 × BD Perm/Wash buffer. Intracellular IL-8 was stained with FITC-conjugated anti-IL-8 monoclona antibody (BioLegend) and APC-conjugated anti-IL-1β monoclona antibody (BioLegend) at 4 °C for 30 min. After washing with 1 × BD Perm/Wash buffer, resuspension in 1 × PBS was carried out, followed by flow cytometric analysis with a FACSCalibur flow cytometer.

Results

Mutated-pyrin expression plasmids were successfully constructed and expressed in HEK293T cells

Site-specific mutagenesis of plasmids, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, pFLAG-CMV-4-pyrin-E148Q+M694I, and pFLAG-CMV-4-pyrin-WT encoding E148Q, M694I, M694V, and E148Q+M694I mutated-pyrin and WT pyrin, generated from pcDNA3-HA-pyrin-WT as a template, was successfully completed and confirmed by sequencing (Fig. 1a). WT pyrin and E148Q, M694I, M694V, and E148Q+M694I mutated pyrin were stably expressed in HEK293T cells transfected with pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, and pFLAG-CMV-4-pyrin-E148Q+M694I, whereas there was an undetectable level of pyrin in HEK293T cells transfected with pFLAG-CMV-4 (Vector) (Fig. 1b).

Wild-type pyrin and E148Q, M694I, M694V, and E148Q+M694I pyrin are detergent-insoluble

HEK293T cells were transfected with the expression plasmids pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, or pFLAG-CMV-4-pyrin-E148Q+M694I, encoding WT, E148Q, M694I, M694V, and E148Q+M694I pyrin, respectively. The cells were suspended in 1.0 % NP-40 buffer and separated into soluble (S: supernatant) and insoluble (P: pellet) fractions by centrifugation at 12,000 rpm for 20 min. Both fractions were subjected to Western blotting. WT pyrin and all mutated pyrins that we tested were fractionated in detergent-insoluble fractions (Fig. 1c; P).

Cytokine secretion from synovial sarcoma SW982 cells

IL-8 and IL-6 were spontaneously secreted from synovial sarcoma SW982 cells (Fig. 2a, b), whereas IL-1β or TNF-α

could not be detected in our ELISA system even when stimulated by LPS (data not shown).

IL-8 secretion from SW982 cells was suppressed by WT pyrin but suppressed much less by mutated pyrins

When SW982 cells were transfected with expression plasmids pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694I, pFLAG-CMV-4-pyrin-M694V, pFLAG-CMV-4-pyrin-E148Q+M694I, and pFLAG-CMV-4-pyrin-WT, IL-8 but not IL-6 secretion from SW982 seemed to be suppressed (Fig. 2a, b). After standardation to the β-galactosidase activity, related % of IL-8 secretion versus WT pyrin suppression, IL-8 secretion was significantly suppressed by WT pyrin but suppressed much less by E148Q, M694I, M694V, and E148Q+M694I pyrin in that order (Fig. 2c). In terms of IL-6 secretion from SW982 cells, there was no significant difference among all the mutations (Fig. 2d).

Neither ASC nor caspase-1 was expressed in 982 synovial sarcoma cells

The expressions of inflammasome components ASC and caspase-1 were analyzed by Western blotting. Although both ASC and caspase-1 were expressed in THP-1 monocytic leukemia cells, they were not expressed in SW982 cells as well as HEK293T cells (Fig. 3).

IL-1β, IL-8, TNF-α but not IL-6 secretion from THP-1 cells was suppressed by stably expressed WT pyrin but suppressed much less by stably expressed mutated pyrin proteins

We generated stable THP-1 cells transfected with expression plasmids pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-E148Q, pFLAG-CMV-4-pyrin-M694 V, and pFLAG-CMV-4-pyrin-WT, which express no pyrin (vector control), or stably express mutant pyrin proteins such as M694V, E148Q, or WT pyrin (Fig. 4a inset). These cells secreted IL-

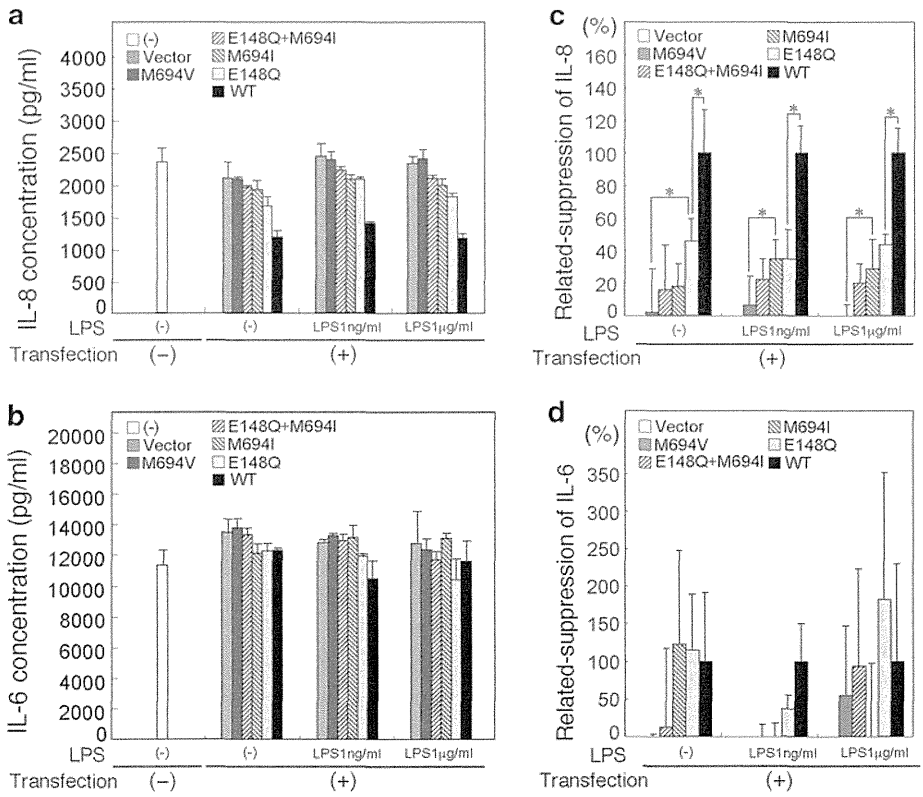

Fig. 2 Interleukin-8 and interleukin-6 secretion from SW982 synovial sarcoma cells transfected with expression plasmids. **a**, **b** 1×10^6 SW982 cells were transfected with 1.67 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-M694V (M694V), pFLAG-CMV-4-pyrin-E148Q+M694I (E148Q+M694I), pFLAG-CMV-4-pyrin-M694I (M694I), pFLAG-CMV-4-pyrin-E148Q (E148Q), pFLAG-CMV-4-pyrin-WT (WT), or left untransfected (−) in the presence of 0.67 μg of pEF1-BOS-β-gal. 8 h after transfection, culture medium was replaced with 1 ml of DMEM alone [LPS(−)], or DMEM containing 1.0 ng/ml or 1.0 μg/ml LPS. 8 h after medium replacement, concentrations of interleukin-8 (IL-8) (**a**) and interleukin-6 (IL-6) (**b**) in the culture supernatant were measured by ELISA. Values are from triplicate cultures. **c**, **d** Percentiles are relative suppression of mutated pyrin versus WT pyrin. Percentiles of relative suppression of IL-8 (**c**) or IL-6 (**d**) secretion from SW982 cells transfected with mutated pyrin versus WT pyrin were normalized to the transfection efficiency by β-galactosidase activity from triplicate cultures. *A p value <0.05 was considered statistically significant

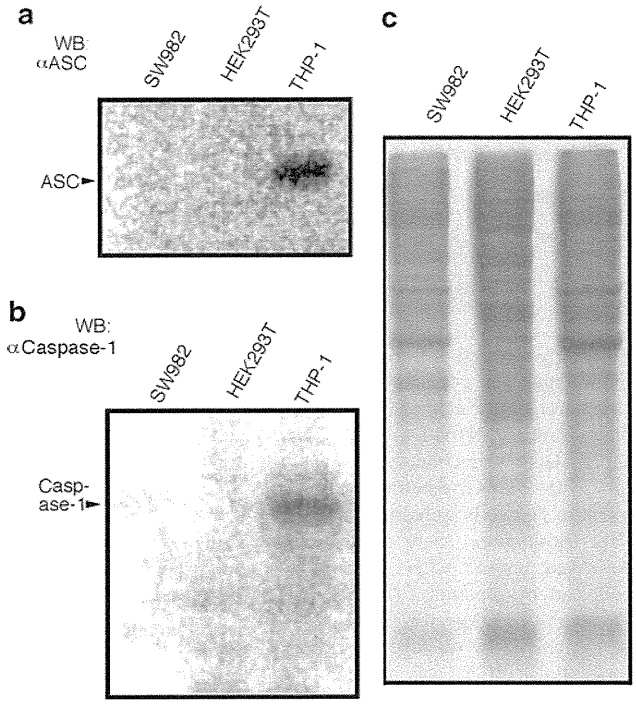

Fig. 3 Expression of ASC and caspase-1 in THP-1 cells, SW982 cells, and HEK293T cells by Western blotting analysis. Thirty μg of whole cell lysates of THP-1 cells, SW982 cells, and HEK293 cells was subjected to Western blotting. **a** Blotting membranes were detected using mouse anti-human ASC monoclonal antibody [26]. **b** Blotting membranes were detected using rabbit anti-human caspase-1 polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA). **c** Gel was stained with Coomassie Brilliant Blue

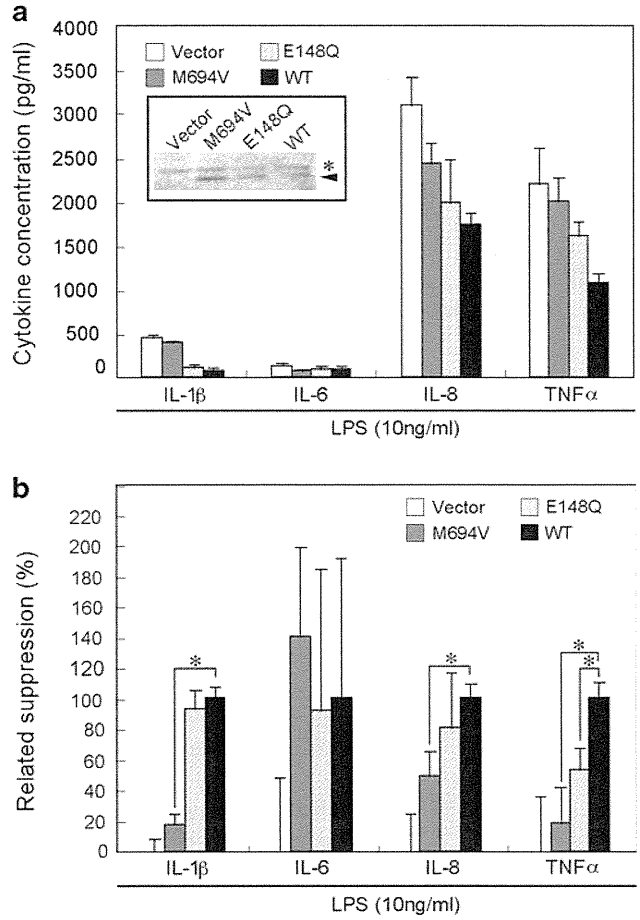

Fig. 4 Interleukin 1β, interleukin-6, interleukin-8, and TNF-α secretion from stable THP-1 cells. **a** 1×10^7 monocytic leukemia THP-1 cells were transfected with 5 μg of pFLAG-CMV-4 (Vector), pFLAG-CMV-4-pyrin-WT, pFLAG-CMV-4-pyrin-E148Q, or pFLAG-CMV-4-pyrin-M694V. After incubation with 500 μg/ml G418 (Sigma) in RPMI 1640 medium including 10 % FBS for 4 weeks to generate stable THP-1 cells. Wild-type (WT) and mutated pyrin expressions were confirmed by Western blotting (*inset*, *arrowhead*; *asterisk* is non-specific band). THP-1-derived stable cells expressing WT and mutated pyrin proteins were pre-cultured in 24-well flat-bottomed plates to a final cell density of 2×10^7/ml in a volume of 300 μl of RPMI1640 Medium including 10 % FBS for 8 h at 37 °C in a humidified atmosphere with 5 % CO_2. Then, the culture medium was supplemented with 300 μl of RPMI1640 containing 20 ng/ml LPS. 8 h after medium replacement, the concentrations of IL-1β, IL-6, IL-8, and TNF-α in the culture supernatant were measured by enzyme-linked immunosorbent assay with specific antibodies (BD Biosciences, San Jose, CA, USA). **b** Percentiles of relative suppression of IL-1β, IL-6, IL-8, or TNF-α secretion from THP-1 cells expressing mutated pyrin versus WT pyrin were calculated from triplicate cultures. *A p value <0.05 was considered statistically significant

1β, IL-6, IL-8, or TNF-α with 10 ng/ml LPS stimulation (Fig. 4a); for IL-1β, IL-8, and TNF-α, each cytokine secretion was significantly suppressed by WT pyrin but suppressed much less by M694V pyrin (Fig. 4a, b). In terms of IL-6 secretion from THP-1 cells, there was no significant difference among all the mutations (Fig. 4a, b).

Pyrin affects ERK1/2 phosphorylation of SW982 cells

We found that p38 and ERK 1/2 were spontaneously phosphorylated even when mutated M694V and E148Q pyrin proteins were ectopically expressed in SW982 cells (Fig. 5a, b). ERK1/2 was found to be less phosphorylated when WT pyrin was ectopically expressed in SW982 cells (Fig. 5b). On the other hand, there was no significant phosphorylation in NF-κB p65 for NF-κB activation (Fig. 5c).

Peripheral blood mononuclear cells from FMF patients secreted IL-8 when incubated on a culture plate

We obtained peripheral blood mononuclear cells from five FMF patients with *MEFV* mutations; four patients were E148Q/M694I compound heterozygotes and one patient was an E148Q/E148Q homozygote. We found a significant difference between five FMF patients and five healthy volunteers in terms of IL-8 secretion from mononuclear cells, even when incubated on a culture plate for 6 h (Fig. 6a, b). Peripheral blood mononuclear cells from FMF

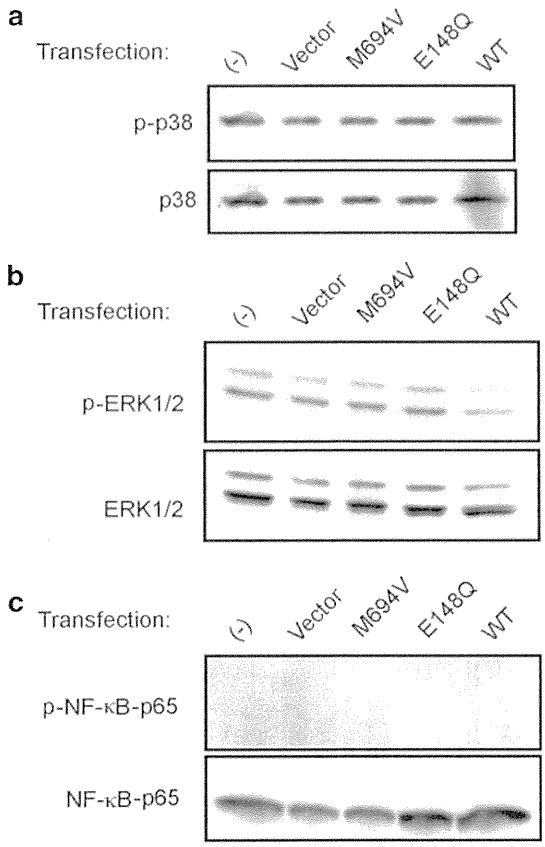

Fig. 5 Western blotting analyses for p38, ERK, and NF-κB pathways. 40 μg of SW982 cell lysates were subjected to SDS-PAGE followed by Western blotting analysis for p38, ERK, and NF-κB pathways. Signals from the same blotting membrane were detected by phospho-p38 MAPK (Thr180/Tyr182) rabbit monoclonal antibody and p38α MAPK rabbit polyclonal antibody for the p38 pathway (**a**), or phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibody and p44/42 MAPK (Erk1/2) rabbit monoclonal antibody for the ERK pathway (**b**), or phospho-NF-κB p65 (Ser536) rabbit polyclonal antibody and NF-κB p65 rabbit monoclonal antibodies for the NF-κB pathway (**c**)

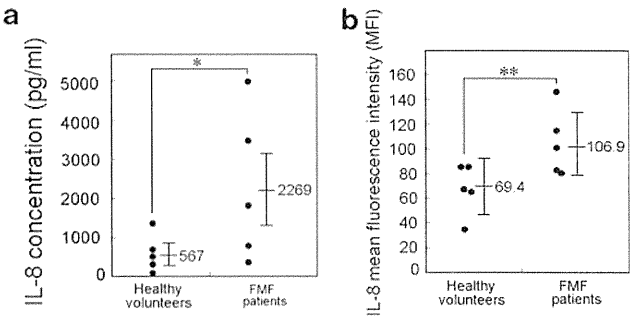

Fig. 6 IL-8 secretion from mononuclear cells from FMF patient with pyrin mutations compared with healthy volunteers. **a** 5×10^5/ml peripheral blood mononuclear cells were incubated in 96-well flat plates with RPMI1640 with 10 % heat-inactivated FBS for 6 h. The supernatants were collected and analyzed for IL-8 concentration (pg/ml) with the Cytometric Bead Array Flex set. **b** 5×10^5/ml mononuclear cells including BD GolgiPlug protein transport inhibitor (BD Biosciences) were incubated under the same conditions as described above. After 6 h of incubation, adherent cells were collected by pipetting. The cells were fixed using a BD Cytofix/Cytoperm solution for 20 min at 4 °C, then fixed cells were permeabilized by washing two times in 1 × BD Perm/Wash buffer. Intracellular IL-8 was stained with FITC-conjugated anti-IL-8 monoclonal antibody and flow cytometric analysis was performed with a FACSCalibur flow cytometer and mean fluorescence intensity (MFI) was calculated. *, **p values <0.05 and <0.01 were considered statistically significant, respectively

patients were found to exhibit higher IL-8 secretion than those from healthy volunteers (Fig. 6a, b). IL-1β concentrations were at an undetectable level under the same conditions (data not shown).

Discussion

We have investigated the relationship between the main pyrin mutations of FMF patients and the suppression of IL-8 secretion from synovial sarcoma SW982 cells. Pyrin was discovered as a causative gene product of FMF, and E148Q, M694I, and E148Q/M694I mutations of pyrin have been found to be the major mutations of Japanese FMF patients [7, 9]. We constructed mutated-pyrin expression plasmids corresponding to the above mutations (Fig. 1a), and found no apparent difference among WT pyrin and mutated pyrin proteins in terms of expression stability and detergent solubility (Fig. 1b, c). We also found that WT pyrin suppressed IL-8 secretion from SW982 cells, but this was less suppressed by E148Q, M694I, M694V, and E148Q+M694I pyrin in that order, and WT pyrin and mutated pyrin proteins did not affect IL-6 secretion from SW982 cells (Fig. 2). Although it is unusual, compared with normal synovia, for SW982 cells spontaneously to secrete IL-8 without any stimulation, it is likely that a similar model is involved in sterile arthritis, which has been found in FMF patients.

Arthritis is one of the major symptoms of FMF patients [8, 19]. The attacks of FMF arthritis are usually acute inflammatory responses, of which the hallmark in the tissue is self-limiting neutrophil infiltration in synovial stroma [20]. Neutrophils are usually recruited by chemotactic factors such as IL-8, which was shown to be induced by epithelial cells or leukocytes in microbial infection or rheumatoid arthritis [21, 22]. However, sterile inflammation in pleura, peritonea, and synovia, which is common in FMF patients, is thought to occur without any microbial infection or rheumatoid factors [23]. FMF-related sterile inflammation is reported to be triggered by dysregulation of inflammasome, an IL-1β processing platform composed of Nod-like receptor (NLR), ASC, and caspase-1 [24]. It was also reported that NLRC4-inflammasome-related NF-κB activation leads to IL-8 secretion from MEIL-8 cells [12]. The NLRC4-inflammasome-related NF-κB activation

is reported to be inhibited by pyrin [17]. Consistent with this, our results indicated that WT pyrin suppresses IL-8 secretion from SW982 cells (Fig. 2). However, unexpectedly, both ASC and caspase-1 were at undetectable levels in SW982 cells (Fig. 3), suggesting that inflammasome may have been dispensable in the mechanism of suppression of IL-8 secretion from SW982 cells in our experiment (Fig. 3).

To investigate whether pyrin can suppress IL-8 secretion from another cell line, THP-1, monocytic leukemia cells, we generated stable THP-1 cells stably expressing WT pyrin or mutant pyrin proteins such as E148Q, M694V, or vector control. We found that pyrin can suppress IL-8 secretion from THP-1 cells as well as IL-1β and TNF-α (Fig. 4). Because pyrin was reported to inhibit ASC-related inflammasome signaling [15, 16], suppression of IL-1β and TNF-α secretion from THP-1 cells may be inflammasome-dependent. Considering the results from SW982 and THP-1, we speculate that pyrin may contribute to the suppression of IL-8 secretion by an inflammasome-independent pathway.

What kind of signaling pathway does pyrin affect? We performed Western blotting analyses for the p38, ERK, and NF-κB pathways of SW982 cells. Interestingly, we found that p38 and ERK were spontaneously phosphorylated (Fig. 5a, b) and just ERK was less phosphorylated when WT pyrin was ectopically expressed in SW982 cells (Fig. 5b). We also found that NF-κB p65 was not phosphorylated (Fig. 5c). Thus, we speculate that pyrin affects at least the ERK pathway independently of inflammasome.

Notably, peripheral blood mononuclear cells from FMF patients exhibit higher IL-8 secretion than those from healthy volunteers, even when plated on a culture dish (Fig. 6a, b), suggesting that only mechanical stress may affect clinical manifestations of FMF patients.

The most frequent mutation of FMF patients in Middle Eastern countries is reported to be M694V, which is associated with arthritis and severe clinical manifestations [25], whereas no M694V mutation was found among Japanese FMF patients [6–9]. Japanese FMF patients exhibit atypical clinical manifestations, and approximately half of FMF patients exhibit E148Q/M694I compound heterozygosity, E148Q heterozygosity, or M694I homozygosity [9]. As for the clinical significance of our results in correlation with the above description, pyrin-M694V hardly suppressed IL-8 secretion from SW982 and THP-1 cells (Figs. 2, 4, respectively), whereas E148Q, M694I, and E148Q+M694I still had the ability to suppress IL-8 secretion from SW982 cells (Fig. 2).

In conclusion, our data demonstrate that FMF-related mutated pyrin proteins have a low ability to suppress IL-8 secretion from SW982 cells independently of inflammasome. Common mutations in Japanese FMF patients of E148Q, M694I, and E148Q/M694I result in retention of the power to suppress IL-8 secretion from SW982 cells, rather than the M694V mutation, which may explain why atypical clinical manifestations are common in Japanese FMF populations.

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Aicardi-Goutières Syndrome Is Caused by *IFIH1* Mutations

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Aicardi-Goutières syndrome (AGS) is a rare, genetically determined early-onset progressive encephalopathy. To date, mutations in six genes have been identified as etiologic for AGS. Our Japanese nationwide AGS survey identified six AGS-affected individuals without a molecular diagnosis; we performed whole-exome sequencing on three of these individuals. After removal of the common polymorphisms found in SNP databases, we were able to identify *IFIH1* heterozygous missense mutations in all three. In vitro functional analysis revealed that *IFIH1* mutations increased type I interferon production, and the transcription of interferon-stimulated genes were elevated. *IFIH1* encodes MDA5, and mutant MDA5 lacked ligand-specific responsiveness, similarly to the dominant *Ifih1* mutation responsible for the SLE mouse model that results in type I interferon overproduction. This study suggests that the *IFIH1* mutations are responsible for the AGS phenotype due to an excessive production of type I interferon.

Aicardi-Goutières syndrome (AGS [MIM 225750]) is a rare, genetically determined early-onset progressive encephalopathy.[1] Individuals affected with AGS typically suffer from progressive microcephaly associated with severe neurological symptoms, such as hypotonia, dystonia, seizures, spastic quadriplegia, and severe developmental delay.[2] On brain imaging, AGS is characterized by basal ganglia calcification, white matter abnormalities, and cerebral atrophy.[3,4] Cerebrospinal fluid (CSF) analyses show chronic lymphocytosis and elevated levels of IFN-α and neopterin.[3–5] AGS-affected individuals are often misdiagnosed as having intrauterine infections, such as TORCH syndrome, because of the similarities of these disorders, particularly the intracranial calcifications.[1] In AGS, etiological mutations have been reported in the following six genes: *TREX1* (MIM 606609), which encodes a DNA exonuclease; *RNASEH2A* (MIM 606034), *RNASEH2B* (MIM 610326), and *RNASEH2C* (MIM 610330), which together comprise the RNase H2 endonuclease complex; *SAMHD1* (MIM 606754), which encodes a deoxynucleotide triphosphohydrolase; and *ADAR1* (MIM 146920), which encodes an adenosine deaminase.[6–9] Although more than 90% of AGS-affected individuals harbor etiological mutations in one of these six genes, some AGS-affected individuals presenting with the clinical characteristics of AGS still lack a genetic diagnosis, suggesting the existence of additional AGS-associated genes.[1]

We recently conducted a nationwide survey of AGS in Japan and reported 14 AGS-affected individuals.[10] We have since recruited three other Japanese AGS-affected individuals, and among these 17 individuals, we have identified 11 individuals with etiologic mutations; namely, *TREX1* mutations in six, *SAMHD1* mutations in three, and *RNASEH2A* and *RNASEH2B* mutations in one each. Of the remaining six individuals without a molecular diagnosis, trio-based whole-exome sequencing was performed in three whose parents also agreed to participate in further genome-wide analyses (Figure 1A). Genomic DNA from each individual and the parents was enriched for protein-coding sequences, followed by massively parallel sequencing. The extracted nonsynonymous or splice-site variants were filtered to remove those with minor allele frequencies (MAF) > 0.01 in dbSNP137. To detect de novo variants, any variants observed in family members, listed in Human Genetic Variation Database (HGVD), or with MAF > 0.02 in our in-house exome database were removed. To detect autosomal-recessive (AR), compound heterozygous (CH), or X-linked (XL) variants, those with MAF > 0.05 in our in-house database were removed (Figure S1 available online). All samples were collected with the written informed consents by parents, and the study protocol was approved by the ethical committee of Kyoto University Hospital in accordance with the Declaration of Helsinki.

After common polymorphisms were removed, we identified a total of 40, 18, 89, and 22 candidate variants under the de novo, AR, CH, and XL inheritance models, respectively, that were present in at least one of the three individuals (Table S1). Among them, missense mutations were identified in *IFIH1* (MIM 606951, RefSeq accession

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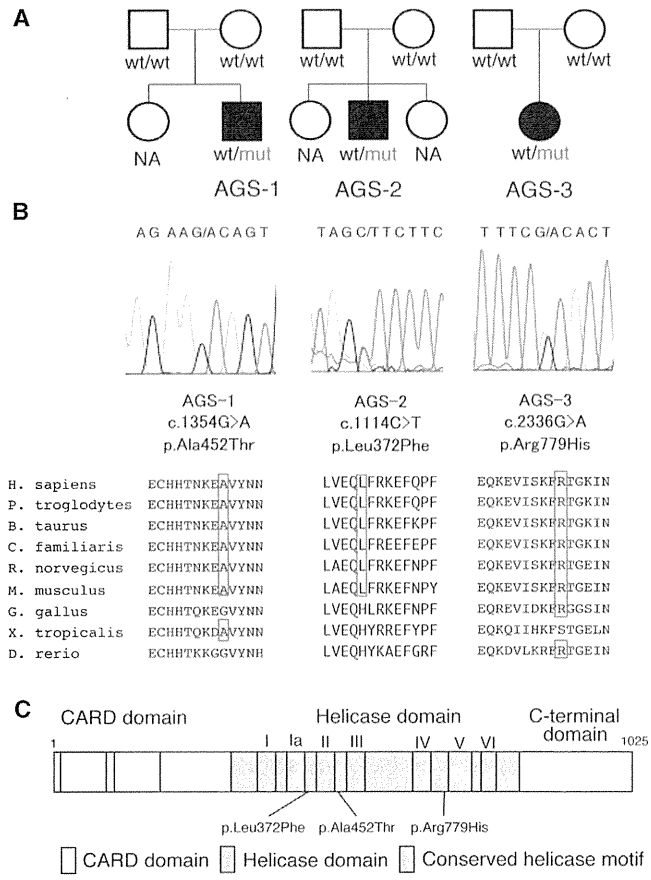

Figure 1. Pedigree Information for the AGS-Affected Individuals and Details of the *IFIH1* Mutations Identified

(A) The pedigrees of the three families indicating the AGS probands.

(B) Sanger sequencing chromatograms of the three *IFIH1* mutations found in the AGS-affected individuals. The locations of these mutations in the amino acid sequence of the MDA5 protein are shown in alignment with the conserved amino acid sequences from several species. This alignment was obtained via ClustalW2. The amino acids that are conserved with human are circled in red.

(C) The MDA5 protein domain structure with the amino acid substitutions observed in these AGS-affected individuals.

number NM_022168.2), which encodes MDA5 (RefSeq NP_071451.2). These missense mutations are c.1354G>A (p.Ala452Thr) in AGS-1; c.1114C>T (p.Leu372Phe) in AGS-2; and c.2336G>A (p.Arg779His) in AGS-3 (Figure 1B). None of the mutations are found in HGVD, including the 1,208 Japanese samples, or our in-house exome database of 312 Japanese individuals. Multiple-sequence alignment by ClustalW2 revealed that each of the amino acids affected by these mutations are conserved among mammals (Figure 1B). The subsequent amino acid alterations were all suggested to be disease causing in at least one of the four function-prediction programs used (Table 1). None of the other genes identified in the de novo inheritance model, or any of the genes identified in the other three inheritance models, were mutated in all three individuals. The *IFIH1* mutations identified were validated by Sanger sequencing. The other coding exons of *IFIH1* were also examined by Sanger sequencing, and no other mutations were found.

MDA5 is one of the cytosolic pattern recognition receptors that recognizes double-stranded RNA (dsRNA).[11] MDA5 consists of N-terminal tandem CARD domains, a central helicase domain, and a C-terminal domain (Figure 1C). When bound to dsRNA, MDA5 forms a closed, C-shaped ring structure around the dsRNA stem and excludes the tandem CARD as well as creates filamentous oligomer on dsRNA.[12] It is hypothesized that the tandem CARD interacts each other and activates MAVS on the mitochondrial outer membrane. Oligomerization of MAVS induces TBK1 activation, IRF3 phosphorylation, and induction of type I interferon transcription, resulting in the activation of a large number of interferon-stimulated genes (ISGs).

The neurological findings of the individuals with these *IFIH1* mutations are typical of AGS (Table S2). They were born with appropriate weights for their gestational ages without any signs of intrauterine infection. However, they all demonstrated severe developmental delay in early infancy associated with progressive microcephaly. No arthropathy, hearing loss, or ophthalmological problems were observed. As for extraneural features, all three individuals had at least one of the following autoimmune features: positivity for autoantibodies, hyperimmunoglobulinemia, hypocomplementemia, and thrombocytopenia. Notably, none of the individuals with *IFIH1* mutations had chilblain lesions, although all the five individuals with *TREX1* mutations and two of the three individuals with *SAMHD1* mutations in the Japanese AGS cohort showed chilblain lesions.[10] Individuals with *SAMHD1* mutations and *IFIH1* mutations both show autoimmune features; however, chilblain lesions have been observed only in individuals with *SAMHD1* mutations.[10]

To predict the effects of the identified amino acid substitutions on MDA5, three-dimensional model structures of MDA5 mutants were generated from the crystal structure of human MDA5-dsRNA complex[12] (Protein Data Bank [PDB] code 4gl2), using PyMOL (Schroedinger) and MOE (Chemical Computing Group) (Figure S2A). The oligomeric model of MDA5 was generated using the electron microscopy imaging data of MDA5 filament lacking CARD domain[13] (Electron Microscopic Data Bank [EMDB] code 5444) (Figure S2B). The three amino acid substitutions in the AGS-affected individuals are all located within the helicase domain (Figures 1C and S2A). Because Ala452 directly contacts the dsRNA ribose O2′ atom, the p.Ala452Thr substitution probably affects the binding affinity to dsRNA due to an atomic repulsion between the side chain of Thr452 and the dsRNA O2′ atom (Figures S2C and S2D). Leu372 is located adjacent to the ATP binding pocket, and the p.Leu372Phe substitution could increase the side chain volume of the binding pocket, affecting its ATP hydrolysis activity (Figures S2E and S2F). In our oligomeric model, Arg779 is located at the interface between the two monomers, which is consistent with the

Table 1. Functional Predictions of the *IFIH1* Variants

Individuals	Nucleotide Change	Amino Acid Change	SIFT	PolyPhen2	Mutation Taster	PROVEAN
AGS-1	c.1354G>A	p.Ala452Thr	tolerated	benign	disease causing	neutral
AGS-2	c.1114C>T	p.Leu372Phe	tolerated	probably damaging	disease causing	neutral
AGS-3	c.2336G>A	p.Arg779His	tolerated	probably damaging	disease causing	deleterious

The potential functional effects of the *IFIH1* variants identified in the AGS-affected individuals were predicted via SIFT, PolyPhen2, Mutation Taster, and PROVEAN.

recent report showing that Lys777, close to Arg779, is in close proximity to the adjacent monomer.[12] Furthermore, in our model, Arg779 is in close to Asp572 on the surface of the adjacent monomer. We speculate that losing the positive charge due to the p.Arg779His substitution would possibly affect the electrostatic interaction between the MDA5 monomers (Figures S2G and S2H).

To connect the identified *IFIH1* mutations with the AGS phenotype, we examined the type I interferon signature in the individuals by performing quantitative RT-PCR (qRT-PCR) of seven ISGs.[14] Peripheral blood mononuclear cells (PBMCs) from the three AGS-affected individuals showed upregulation of ISG transcription (Figure 2), confirming the type I interferon signature in the individuals with *IFIH1* mutations.

To elucidate the disease-causing capability of the identified *IFIH1* mutations, three FLAG-tagged *IFIH1* mutant plasmids containing these mutations were constructed via site-directed mutagenesis. These plasmids were transiently expressed on human hepatoma cell line Huh7 and the *IFNB1* promoter activity as well as endogenous expression of *IFIT1* (MIM 147690) was measured 48 hr after transfection.[15] The three mutant plasmids activated the *IFNB1* promoter in Huh7 cells more strongly than the wild MDA5 and nearby missense variants reported in dbSNP (Figures 3 and S3). The upregulation of endogenous *IFIT1* was also observed in the transfected cells (Figure S4), suggesting that these AGS mutations enhance the intrinsic activation function of MDA5. Recent genome-wide association studies (GWASs) showed association of the *IFIH1* with various autoimmune diseases, such as systemic lupus erythematosus (SLE), type I diabetes, psoriasis, and vitiligo.[16–19] We examined *IFNB1* promoter activity induced by the c.2836G>A (p.Ala946Thr) polymorphism (rs1990760) identified in the GWASs. Although the c.2836G>A polymorphism partially activated the promoter activity, the induced activity was lower than those of the AGS-derived mutants. In addition, the dominantly inherited SLE mouse model in the ENU-treated mouse colony is reported to have the *Ifih1* mutation, c.2461G>A (p.Gly821Ser).[15] These observations suggest that *IFIH1* has strong association with various autoimmune diseases, especially SLE, which also has a type I interferon signature.[20] Because alteration of *TREX1* has been reported to cause AGS as well as SLE,[21] it seems quite plausible for *IFIH1* to also be involved in both AGS and SLE. Interestingly, all the individuals identified with *IFIH1* mutations had autoantibodies, suggesting the contribution of *IFIH1* mutations to autoimmune phenotypes.

To further delineate the functional consequences of the three *IFH1* mutations, we measured the ligand-specific *Ifnb* mRNA induction by stimulating *Ifih1*^{null} mouse embryonic fibroblasts (MEFs) reconstituted with retrovirus expressing the *IFIH1* mutants by an MDA5-specific ligand, encephalomyocarditis virus (EMCV).[22] None of the MEF cells expressing the three mutant *IFIH1* responded to the EMCV, which suggested that the MDA5 variants lacked the ligand-specific responsiveness. The response of the three AGS mutants against the MDA5-specific EMCV was similar to that of the p.Gly821Ser variant reported in the dominantly inherited SLE mouse model with type I interferon overproduction[15] (Figures 4 and S5).

During the revision of this manuscript, Rice et al. identified nine individuals with *IFIH1* mutations, including the c.2336G>A mutation we identified, in a spectrum of neuroimmunological features consistently associated with enhanced type I interferon states including AGS.[23] Although we agree that the *IFIH1* mutations cause constitutive type I interferon activation, Rice et al. show that the mutated MDA5 proteins maintain ligand-induced responsiveness, which was not the case in our study. Because we measured the ligand-specific responsiveness of MDA5 in different experimental conditions, further analysis remains to be performed to reveal the biochemical mechanism of interferon overproduction by the mutated MDA5.

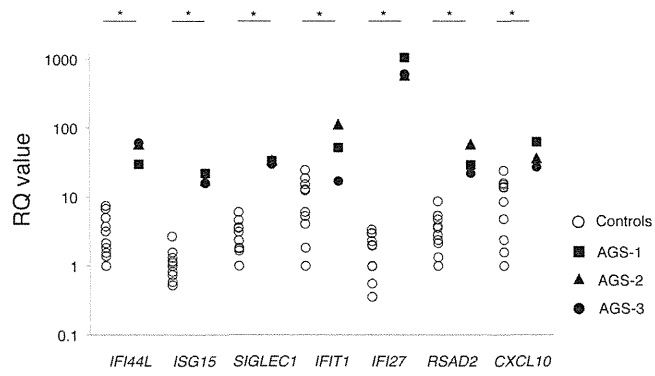

Figure 2. Quantitative RT-PCR of a Panel of Seven ISGs in PBMCs Obtained from the *IFIH1*-Mutated Individuals and Healthy Control Subject

qRT-PCR was performed as previously described.[15] The relative abundance of each transcript was normalized to the expression level of β-actin. Taqman probes used were the same as previous report,[14] except for *ACTB* (MIM 102630). Individual data were shown relative to a single calibrator (control 1). The experiment was performed in triplicate. Statistical significance was determined by Mann-Whitney U test, *$p < 0.05$.