The genotypes of *NLRP3* and *MEFV* in her asymptomatic mother were the same. It should be noted that E378K and G809S were not present in the *INFEVERS* database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) [14] and were confirmed as rare variants that were not identified in the 100 ethnically matched control subjects.

NF-κB Reporter Gene Activities of the *NLRP3* Variants

Figure 2 shows the ASC-dependent NF-κB activities of the *NLRP3* variants in vitro. The NF-κB reporter gene activities were increased by the Y563N and E688K mutations in *NLRP3*. The activities were higher for D303N (as a positive control *NLRP3* mutation that was previously identified in a CINCA/NOMID patient [5]) and E688K than for the FCAS mutation, Y563N. E378K and G809S did not cause any significant increases in the activities. Initially, we suspected that case 5 had CAPS. However, based on these results, we were able to confirm the diagnosis of case 5 as JIA, rather than CAPS.

Cytokine Profiles of the Patients

The serum IL-1β, IL-6, and TNF-α levels were not detected in the sera of the healthy control subjects. Although we were unable to detect IL-1β in the patients' sera, we clearly detected the serum IL-18 and IL-1ra levels in all cases (Fig. 3a, b). The serum IL-18 levels were extremely high in the CINCA/NOMID (case 3), MWS (case 4), and JIA (case 5) patients compared with the control subjects. The serum IL-1ra and IL-6 levels were increased in cases 2, 3, 4, and 5 (Fig. 3b, c). The serum TNF-α levels were increased in cases 1, 2, and 3 (Fig. 3d).

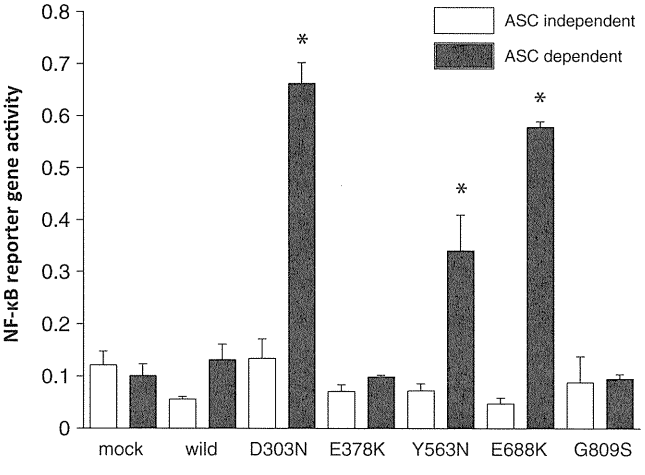

Fig. 2 NF-κB reporter gene activities of the *NLRP3* variants. The *white bars* indicate the NF-κB reporter gene activities of the *NLRP3* variants without cotransfection of ASC, while the *black bars* indicate these activities with cotransfection of ASC. The data shown are the means±SD of triplicate assays. The ASC-dependent NF-κB reporter gene activities are increased for the variants with D303N, Y563N, and E688K. The activities for the CINCA/NOMID mutations, D303N and E688K, are higher than those for the FCAS mutation, Y563N. The variants with E378K and G809S do not show any significant increases in the activities. *$P<0.05$

Interestingly, the serum IL-18 levels in the FCAS patients (cases 1 and 2) did not show any increases compared with the control subjects (Fig. 3a). Furthermore, the levels of spontaneous IL-1β production by PBMCs from the CINCA/NOMID (case 3) and MWS (case 4) patients were increased, whereas those of the control subjects, FCAS patients, and JIA patient (cases 1, 2, and 5) did not show any increases (Fig. 4a).

The lipopolysaccharide (LPS)-induced cytokine production levels by PBMCs from the FCAS and JIA patients are shown in Fig. 4b–d. The IL-1β and IL-18 production levels were increased in the FCAS patients compared with the control subjects. However, TNF-α did not show any significant changes. Comparisons of the cytokine production levels by the PBMCs cultured at 30°C and 37°C are shown in Fig. 5. The PBMCs from the FCAS patients showed obvious increases in the IL-1β and IL-18 production levels after culture at the lower temperature with no stimulation.

Discussion

The diagnosis of CAPS is still based on the clinical symptoms and recognition of a syndrome. Detection of a pathogenic *NLRP3* mutation can confirm the CAPS diagnosis. However, to confirm the diagnosis of CAPS patients with novel identified *NLRP3* variations, some functional experiments regarding the effects of the *NLRP3* mutations, such as the NF-κB luciferase reporter gene assay used in this study, are necessary because of the existence of nonfunctional missense variations of *NLRP3* [7]. Furthermore, although there are many previously reported missense mutations of *NLRP3* associated with CAPS in the *INFEVERS* database [14], the mutations with confirmed functional evidence are limited. In this study, we identified *NLRP3* gene mutations in five patients who were suspected of having autoinflammatory syndromes. Two mutations of *NLRP3*, Y563N and E688K, were previously reported to be disease-causing mutations [15, 16], although in vitro functional assays were not performed. Y563N was first identified in FCAS patients who were diagnosed based on the clinical criteria of FCAS [16, 17]. Our FCAS patients (cases 1 and 2) showed a skin rash, occasional fever, and mild arthritis and did not show any severe symptoms, such as neurological disorders, hearing loss, and renal amyloidosis. On the other hand, E688K was first identified in an Italian male CINCA/NOMID patient [15] who was described as having a skin rash, hearing loss, fever, and transient arthritis without persistent deformities of the involved joints. Our patients with E688K

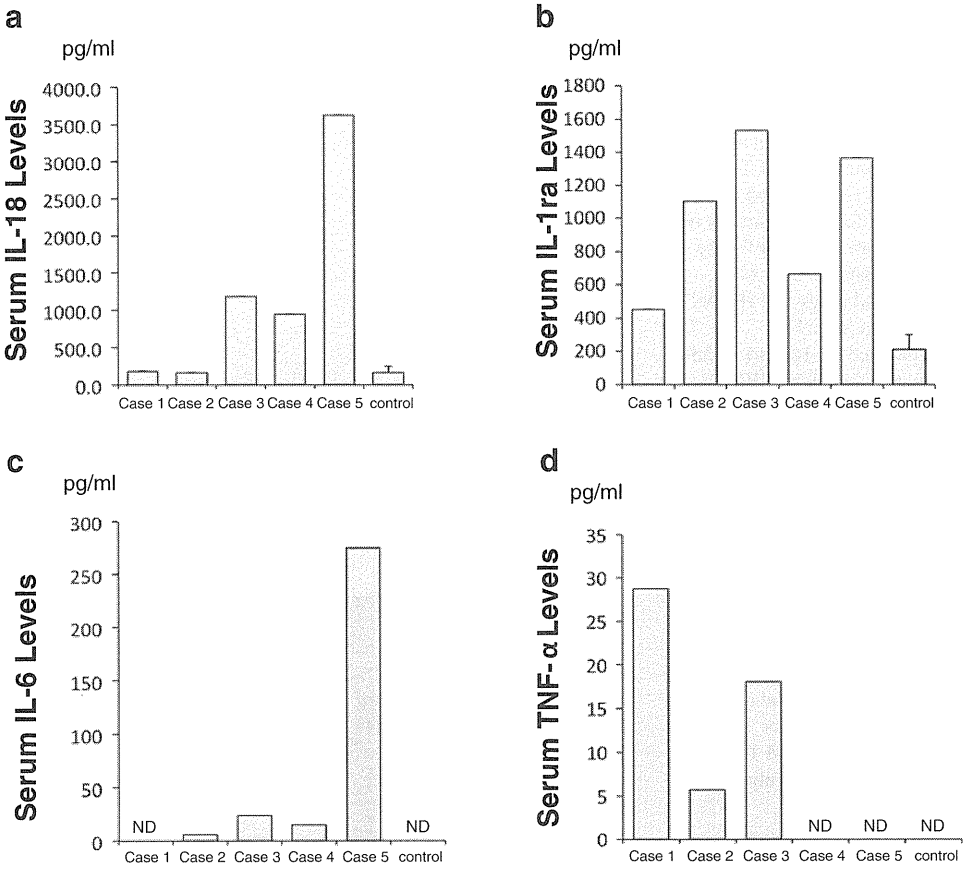

Fig. 3 Serum inflammatory cytokines in the four CAPS cases. IL-1β, IL-6, and TNF-α were not detected in the sera of the control subjects. The means ±D of the serum IL-18 and IL-1ra levels of the healthy control subjects were 169.2±85.7 and 213.4±87.1 pg/ml, respectively (n=10)

(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

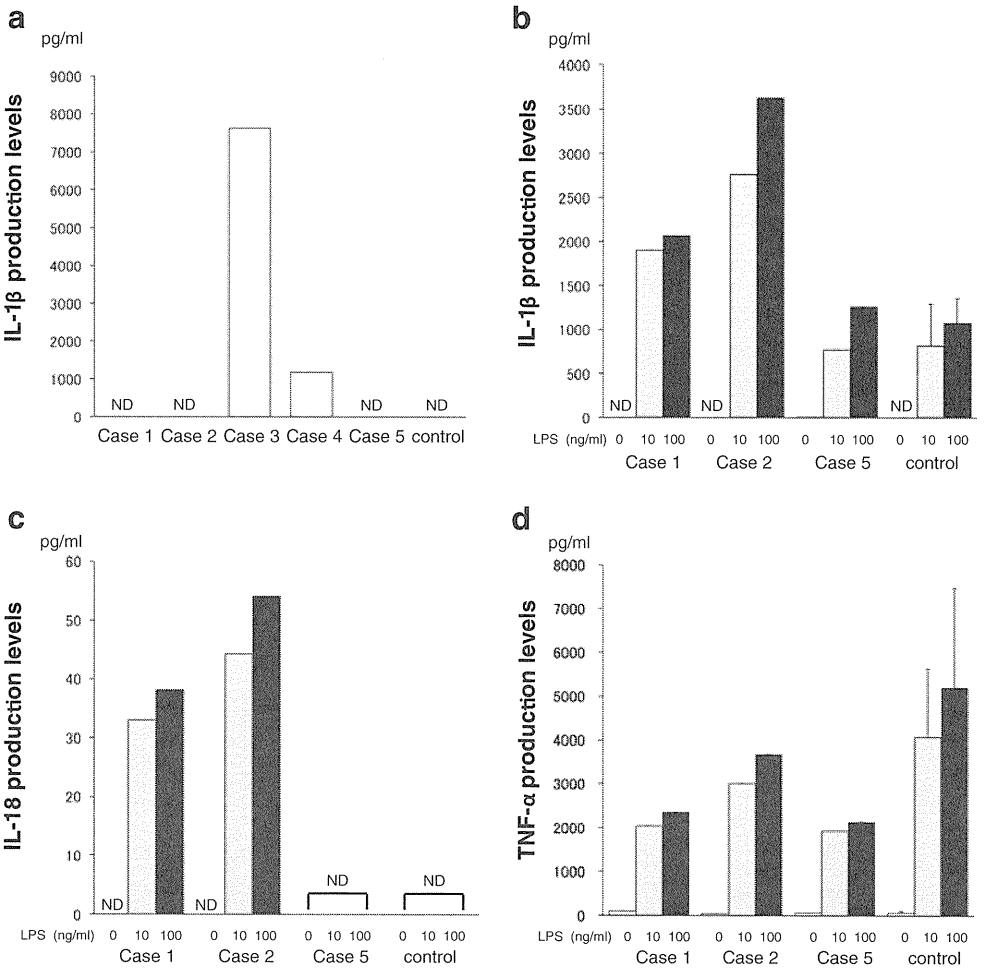

Fig. 4 LPS-induced cytokine production levels in the patients. **a** The *white bars* indicate the spontaneous IL-1β production levels by PBMCs. Increased IL-1β production by PBMCs from case 3 (CINCA/NOMID syndrome) and case 4 (MWS) is detected, whereas no increases are observed for the PBMCs from the 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.

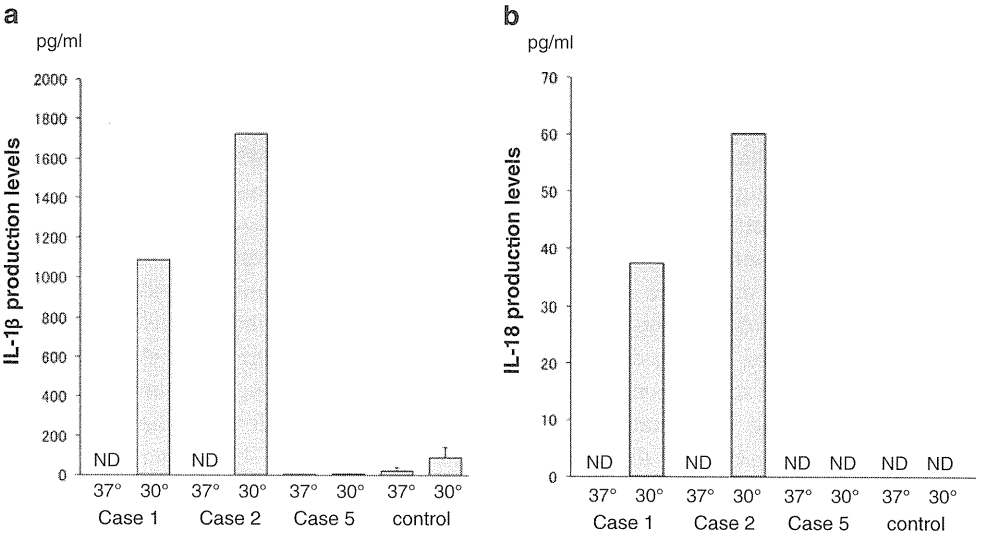

Fig. 5 Hypothermia-induced cytokine production levels by PBMCs from the FCAS and JIA patients. **a**, **b** Comparisons of the cytokine production levels by PBMCs cultured at 30°C and 37°C. The PBMCs from the FCAS patients (cases 1 and 2) show obvious increases in the IL-1β and IL-18 production levels 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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