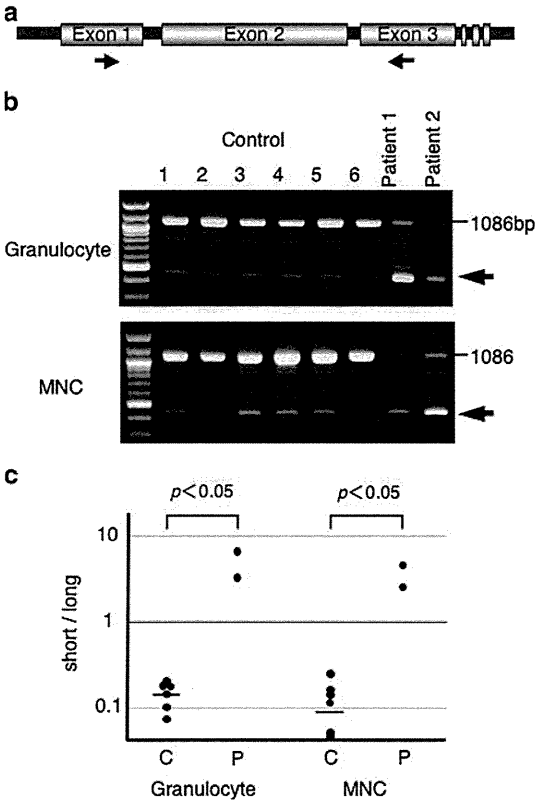

Fig. 2 Alternative splicing of the *MEFV* gene in c.910G>A patients. Both 5′ and 3′ primers were set within exons 1 and 3, respectively (**a**). In both granulocytes and peripheral blood mononuclear cells (PBMCs), full-length (1,086 bp) *MEFV* messenger RNA (mRNA) predominated in controls, and a small number of short product (*arrowheads*) was detectable (**b**). In contrast, in patients, the short product predominated both in granulocytes and PBMCs. Semiquantitative analysis of short to full-length ratio (**c**). Controls (*C*) showed a low short/full-length ratio, whereas patients (*P*) showed a significantly high short/full-length ratio, regardless the cell type

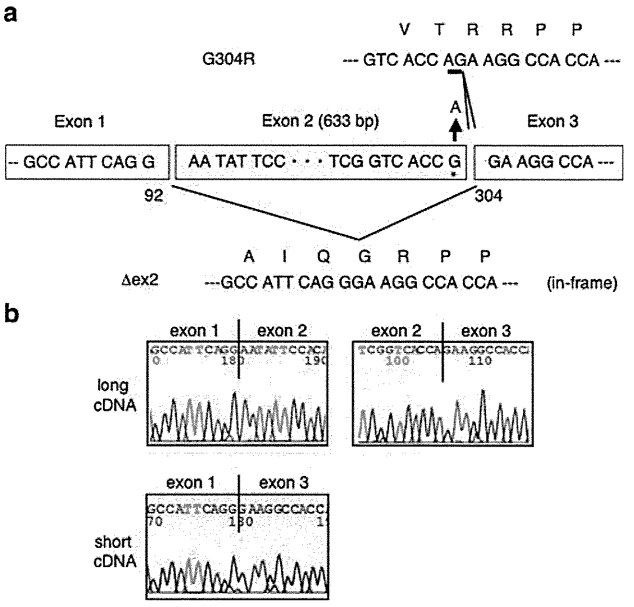

Fig. 3 Nucleotide sequences of alternatively spliced products. Organization of the nucleotide sequences is shown (**a**). Full-length (long) product showed single nucleotide replacement of c.910G>A, leading to the G304R amino acid replacement. Truncated product lacked whole exon 2, leading to an in-frame variant (Δex2). Raw sequencing data from the capillary sequencer (**b**)

sequencing of the short product indicated that the short mRNA transcript reflects the splice variant lacking the 633-bp exon 2 (Fig. 3b), suggesting that the mRNA is transcribed into the pyrin protein with the expected molecular weight of 90 kDa instead of 115 kDa.

MEFV mRNA induction by LPS and IFN-α

LPS or IFN-α stimulation has been reported to result in a significant increase in *MEFV* mRNA in PBMCs. LPS is also known to induce alternative splicing of *MEFV* in in vitro systems. We therefore examined whether enhanced *MEFV* mRNA transcription leads to altered splicing patterns in these patients. LPS and IFN-α treatment enhanced full-length *MEFV* mRNA transcripts in four controls (Fig. 4). Short mRNA transcripts were increased at the same time, but the predominance of full-length *MEFV* mRNA did not change after stimulation. Similar to controls, the short mRNA transcript level was increased after stimulation of patient PBMCs, whereas the full-length mRNA transcript level remained extremely low (patient 1) or paradoxically decreased (patient 2).

In vitro expression of the pyrin protein after *MEFV* gene transfection

To determine whether alternative splicing results in abnormal pyrin protein expression, we transfected normal and variant *MEFV* genes into HEK293T cells. In addition to normal WT cDNA, cDNA with the c.910G>A single nucleotide replacement (G304R), and exon-2-deleted gene (Δex2) were used (Fig. 5a). After transfection, expression profiles of the transfected genes were examined by immunofluorescence (Fig. 5b) and immunohistochemistry (Fig. 5c). GFP fluorescence was diffused within the cytoplasm of both WT and G304R transfectants. In contrast, Δex2 cells showed characteristic uneven clustering of GFP fluorescence. In all transfectants, GFP fluorescence remained within the cytoplasm, with no detection in the nuclei. Immunohistochemical examination of pyrin protein expression showed similar results. Namely, pyrin expression was observed diffusely throughout the cytoplasm of WT, G304R, and Δex2 transfectants but not within the nuclei. Immunoblotting analysis of cell lysates revealed that both WT and G304R transfectants express a pyrin protein of identical size (M_W = 115 kDa) (Fig. 5d), whereas the Δex2 transfectant expresses a truncated pyrin

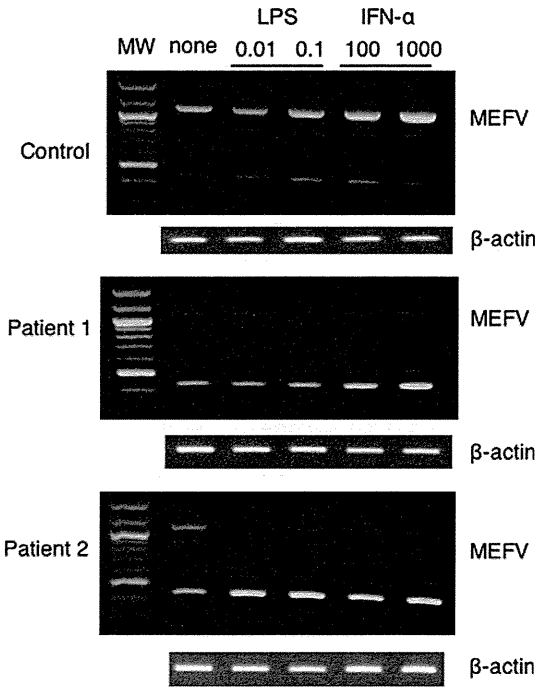

Fig. 4 Lipopolysaccharide (LPS)- and interferon alpha (IFN-α)-mediated regulation of *MEFV* messenger RNA (mRNA) levels. Peripheral blood mononuclear cells (PBMCs) were cultured alone or in the presence of LPS or IFN-α for 72 h. *MEFV* mRNA expression was compared among different samples. Expression of β-actin mRNA was used as the internal control

protein, as expected by the deletion of exon 2 (M_W = 90 kDa) (Fig. 5d). Pyrin was not detectable in nontransfected and mock-transfected cells. The levels of pyrin protein expression were similar among WT, G304R, and Δex2. Ku70 expression was also similar among the different transfectants (Fig. 5d).

Discussion

We report two unrelated cases of the same novel *MEFV* variant. The patients had homozygous c.910G>A variant, which results in excessive alternative splicing of exon 2 of the *MEFV* gene. To date, essentially all *MEFV* mutations of FMF patients are missense mutations [3, 4, 24, 25]. There has been no known report of nonsense mutation or mutation with exon skipping. Although the identical c.910G>A variant has been described as an SNP in the Internet Periodic Fevers (INFEVERS) Web site (rs 75977701), based on a personal communication, the associated phenotype of the reported patient was not described, and the causal role of the variant is unknown [26, 27]. Thus, this is the first report to propose the functional significance of this unique *MEFV* variant as a cause of variant FMF.

It was expected initially that the c.910G>A variant would result in the G304R missense variant with the full-length pyrin protein. To our surprise, most *MEFV* mRNA transcripts were short, and full-length mRNA transcript levels were significantly reduced in both patients. Because the c.910G>A variant is localized at the 3′ end of exon 2, alternative splicing may occur due to splicing errors. Direct sequencing of the short *MEFV* mRNA revealed that exon 2 was indeed deleted. Aberrant splicing of the *MEFV* gene has been reported previously [28, 29]. However, the anomalous splice products remained at low levels in these cases and did not indicate significant functional alterations of total pyrin activity. Similarly to these previous reports, the vast majority of *MEFV* mRNA observed in our control individuals was full length, and short mRNA with exon 2 skipping was seen only occasionally. Therefore, it is unlikely that individuals with heterozygous carrier of c.910G>A mutation show reduced level of full-length *MEFV* mRNA significant enough to have functional impairment of pyrin protein.

Centola et al. [30] previously showed that stimulation of peripheral blood monocytes with IFN-α or LPS resulted in augmented expression of *MEFV* mRNA in vitro, suggesting that an inflammatory environment may alter the expression profile of *MEFV* in vivo. Furthermore, Diaz et al. [29] reported that multiple alternative splicing events, including Δex2, is induced in LPS-stimulated synovial fibroblasts. These findings indicate that *MEFV* mRNA is vulnerable to alternative splicing and the events are induced relatively frequently upon exogenous stimulation, although the frequency of the anomalous splicing remains very low. To rule out the possibility that the enhanced alternative splicing of *MEFV* mRNA seen in our patients is due to sustained exposure to cytokines or endotoxins, we compared the in vitro effect of these agents on the levels of alternative splicing between the two patients and the controls. Stimulation of PBMCs with either IFN-α or LPS did not alter the patterns of *MEFV* mRNA expression in either controls or patients, strongly indicating that excessive levels of short *MEFV* mRNA observed in our patients is the direct result of the c.910G>A variant rather than in vivo augmentation of anomalous splicing, which was induced transiently upon exogenous stimuli. In this respect, the variant that results in significant reduction of full-length *MEFV* mRNA appears to be associated with dysfunction of the pyrin protein. Although it is possible that G304R amino acid substitution itself results in abnormal pyrin activity, its contribution to the total protein function is limited because of the low level of the full-length *MEFV* mRNA. Rather, predominance of the defective mRNA with anomalous splicing might explain the FMF-like clinical profiles seen in our patients.

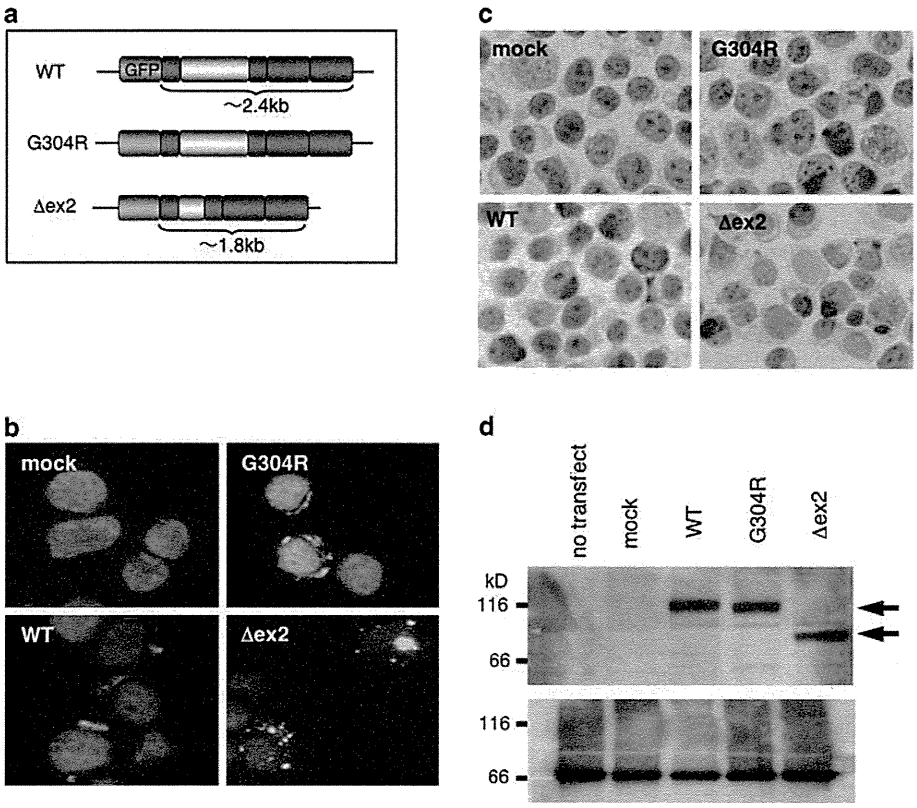

Fig. 5 *MEFV* gene transfection and pyrin expression profiles. HEK293T cells were transfected with plasmid containing the wild type (*WT*), G304R, or Δex2 *MEFV* gene (**a**). Plasmid containing only green fluorescent protein (GFP) was used as a control. Intracellular distribution of GFP was analyzed by immunofluorescence (**b**). Nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI), and GFP fluorescence is localized within the cytoplasm in all transfected cells except mock-transfected cells. Pyrin protein expression was examined by immunohistochemistry. Positive staining is shown in *red* (**c**). The size of the pyrin products within the transfected cells was determined by immunoblotting (**d**). Although WT and G304R transfectants showed full-length protein (*upper gel, higher arrow*), the Δex2 transfectant showed only the truncated protein (*upper gel, lower arrow*). The *lower gel* shows the immunoblotting profiles of Ku70 used as a standard

Papin et al. [28] showed in vitro alternative splicing of exon 2 in the *MEFV* gene and an exon 2 deletion resulting in altered intracellular localization. Chae et al. [12] reported that the 15-aa bZIP basic domain and adjacent sequences, which are encoded by exons 2 and 3, interact with the p65 subunit of NF-κB and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α), respectively, and are important for nuclear translocation of the molecule. These findings indicate that exon 2 of *MEFV* is responsible for nuclear translocation of pyrin and exertion of its function within the nuclei. Notably, native pyrin expression was significantly different among cell types [29]. Namely, the pyrin protein was expressed predominantly within nuclei in dendritic cells, synovial fibroblasts, and neutrophils while being exclusively expressed within the cytoplasm of monocytes in irregularly dispersed patterns. These different patterns of intracellular distribution of native pyrin suggest that only certain types of cells are vulnerable to defective nuclear translocation and that functions of most other cell types remain intact, even when the nuclear translocation machinery is impaired.

Our own experiment using *MEFV* gene transfection failed to show altered intracellular pyrin distribution at least in HEK293T cells. Because HEK293T cells are of embryonic kidney origin, we need to examine the in vitro expression profiles of the transfected *MEFV* genes in other cell types to confirm whether different patterns are observed between Δex2 and control transfectants. Furthermore, native pyrin protein expression profiles should be compared among different cell types in patients when appropriate antipyrine antibody becomes available. The distinct and anomalous patterns of pyrin expression in different cell types in the patients, if present, may explain atypical clinical manifestation of this specific type of *MEFV* variant. Studies are underway to clarify these issues.

Acknowledgments We thank Ms. Harumi Matsukawa and Shizu Kouraba for their excellent technical assistance. This work was

supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.

Conflict of interest None.

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J Infect Chemother
DOI 10.1007/s10156-011-0341-z

CASE REPORT

Recurrent bacterial meningitis by three different pathogens in an isolated asplenic child

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Received: 13 July 2011 / Accepted: 25 October 2011

Abstract Isolated congenital asplenia (ICA) is a rare condition at risk for overwhelming infection. When complicated by invasive infection, the mortality remains high, at greater than 60%. We describe a girl with ICA who developed recurrent meningitis by three different pathogens. The first, meningitis by *Escherichia coli*, occurred 4 days after premature birth. The other two pathogens were serotype 6B *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), at 18 and 25 months of age, respectively. The patient was successfully treated with prompt antimicrobial therapy in all episodes. Serum anti-polyribosylribitol phosphate (PRP) and anti-6B-type pneumococcal antibodies were below the levels for protective activity after natural infections. Although anti-PRP antibody was significantly increased after Hib vaccination, two (6B and 19F) of seven serotype-specific pneumococcal antibodies were not elevated to protective levels after the second 7-valent pneumococcal conjugate vaccine (PCV7). We, therefore, added a third PCV7. To our knowledge, this is the first neonatal ICA patient with invasive infection and the first case of bacterial meningitis occurring three times. Our findings indicate that monitoring of immune responses after natural infections and vaccinations, and reevaluations of vaccine schedule, are important for ICA patients to prevent subsequent invasive infections.

Keywords Isolated congenital asplenia · Bacterial meningitis · Immunological response · Recurrence · Neonate · Vaccine

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Introduction

Congenital asplenia often occurs as part of a recognized malformation syndrome with anomalies of the heart, great vessels, and viscera [1]. The best known among these syndromes is the asplenia/polysplenia syndrome associated with visceroarterial heterotaxy, and its incidence is estimated at approximately 1/10,000 to 1/40,000 live births [2]. In contrast, isolated congenital asplenia (ICA) occurs fairly more infrequently. A recent French nationwide study indicated that the prevalence is 0.51 per million births [2]. Both conditions have an increased susceptibility to overwhelming invasive infections, carrying considerable mortality. However, the diagnosis of ICA is sometimes difficult because of the lack of other anomalies; therefore, such individuals may be unrecognized until postmortem autopsy.

Practice guidelines for the prevention of life-threatening infections in children with hyposplenia and asplenia advocate antibiotic prophylaxis and immunizations against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), the most common causative organisms for

Published online: 17 November 2011

these patients [3]. However, given that several asplenic cases of overwhelming infections that could be considered as vaccine failures have been documented [4, 5], the immunogenicity of vaccination for asplenic patients is still an important concern.

We present here a girl with ICA who developed multiple episodes of meningitis caused by three different pathogens, namely, *Escherichia coli*, *S. pneumoniae* (serotype 6B), and Hib. She was successfully treated with prompt initiation of antibiotics in all episodes. We also present the details of immune responses to natural infections by Hib and serotype 6B *S. pneumoniae* and those to immunizations of Hib conjugate vaccine and 7-valent pneumococcal conjugate vaccine (PCV7).

Case report

A 4-day-old girl, who was born of nonconsanguineous parents as their first child, weighing 1,742 g at the 34th week of gestation, presented with repetitive apnea during admission because of prematurity. Physical examination showed that heart rate was 135/min and body temperature was 37.2°C. Laboratory data showed WBC of 5.8×10^9/l with 28.5% neutrophils, C-reactive protein (CRP) of 4.3 mg/dl, and blood glucose of 95 mg/dl. Cerebrospinal fluid (CSF) examination showed 3,947 cells/μl with 96% polymorphonuclear cells, 197 mg/dl protein, and 44 mg/dl glucose. Two days later, isolates from the CSF and blood were identified as *E. coli* OX:K1:H–, and the same bacterium was also subsequently isolated from the stool of her asymptomatic mother. The patient was diagnosed as having early-onset *E. coli* meningitis that was vertically transmitted. We treated the patient with cefotaxime (CTX) for 21 days. Auditory brainstem response examination at 28 days of age revealed profound hearing impairment at the right ear. The patient was discharged at 38 days of age. Genetic analysis [6, 7] showed that the strain harbored virulence factor genes such as *iroN*, *papG3*, *afa*, and *kps*, but not *cnf1*, *sfa*, or *ibeA*.

At 18 months old, the patient was rehospitalized because of a 6-h history of fever and generalized tonic-clonic convulsion lasting 3 min. On admission, 30 min after the convulsion, heart rate was 170/min and body temperature was 39.4°C. Her consciousness had become clear. Laboratory findings showed WBC of 21.7×10^9/l and CRP of 6.0 mg/dl. CSF examination showed no pleocytosis, with normal concentrations of protein (10 mg/dl) and glucose (85 mg/dl). Treatment with intravenous CTX was empirically initiated under the tentative diagnosis of occult bacteremia. The day after admission, serotype 6B *S. pneumoniae* was isolated from the blood but not from the CSF. Resistance to penicillin was established by microbiological [minimum inhibitory concentration (MIC), 2 μg/ml] and genotypic (mutations in *pbp1a*, *pbp2X*, and *pbp2b* [8]) analyses, and CTX was substituted with panipenem–betamipron. On day 3, prolonged fever and frequent vomiting led us to perform a second CSF examination, showing 14,500 cells/μl, protein of 58 mg/dl, and glucose of 63 mg/dl. The CSF was positive for *S. pneumoniae* antigen test (Binax NOW *S. pneumoniae*; Binax), but yielded no organisms in culture. The blood WBC and CRP were elevated to 21.7×10^9/l and 22.1 mg/dl, respectively. We diagnosed her disease as pneumococcal meningitis following bacteremia and increased the doses of panipenem–betamipron with good clinical response. She received antimicrobial therapy for 14 days and was discharged without any additional sequelae.

At 25 months of ages, the patient was referred to the emergency department in another hospital with a 2-h history of fever, vomiting, and tonic–clonic convulsion of 2-min duration. At arrival, heart rate was 180/min and body temperature was 39.4°C. Her consciousness soon became clear. Laboratory examination showed WBC of 3.5×10^9/l and CRP of 0.6 mg/dl. After blood culture was obtained, the patient received intravenous sulbactam/ABPC. On day 3, the blood culture yielded β-lactamase-non-producing ABPC-resistant (BLNAR) Hib, and the laboratory examinations showed marked deterioration: WBC of 26.6×10^9/l and CRP of 21.5 mg/dl. CSF examination showed 4,992 cells/μl, 164 mg/dl protein, and 34 mg/dl glucose with positive culture for Hib. Thus, the diagnosis of a third bacterial meningitis was made. The patient thereafter received intravenous meropenem for 14 days and was discharged on day 16 after onset without any additional sequelae. Molecular analysis of the strain identified three amino acid substitutions: His-517, Thr-385, and Ile-377, in *ftsI* [9]. This substitution pattern was classified as subgroup III BLNAR by a recent nationwide study of childhood meningitis in Japan [9].

The multiple episodes of meningitis prompted us to evaluate immunological functions. The results after the second episode of meningitis showed that serum levels of IgG (639 mg/dl), IgA (65 mg/dl), IgM (97 mg/dl), IgG_2 (80 mg/dl), C3 (140 mg/dl), C4 (24 mg/dl), and CH50 (36.1 U/ml) were within normal limits. T/B-cell subsets (65/28%), CD3/CD4/CD8 lymphocyte subsets (61%/44%/14%), natural killer cell activity (25%), neutrophil phagocytic activity using fluorescence bead test by flow cytometry (70.0%), and neutrophil bacteriocidal activity (93.4%) were also normal. Computed tomography (CT) of the skull and inner ears did not show any deformity or defects. To screen interleukin-1 receptor-associated kinase 4 deficiency and myeloid differentiation primary response protein 88 deficiency, we performed flow cytometric analysis [10], resulting in normal intracellular tumor necrosis factor-α

production of monocytes after lipopolysaccharide stimulation. After the third meningitis, ultrasonography and CT of the abdomen finally revealed asplenia without visceroarterial anomalies. Howell–Jolly body-containing RBCs were exceedingly rarely found (<0.1% of RBCs) in peripheral blood. Ultrasonographic examinations of her parents detected normal size and normal position of the spleen.

Since the diagnosis of ICA at 26 months of age, chemoprophylaxis with amoxicillin of 20 mg/kg/day was introduced as well as vaccinations of Hib vaccine and PCV7. Subsequent to the introduction of these strategies, the patient has not suffered from any invasive infections for more than 2 years. At 36 months of age, we assessed her neurodevelopmental status using the New Edition of the Kyoto Scale of Psychological Development, indicating a normal developmental quotient of 88 (normal range, >80).

We evaluated immune responses to natural infections with Hib and serotype 6B pneumococcus and those to immunizations of Hib vaccine and PCV7 (Table 1). Despite natural infections, serum anti-polyribosylribitol phosphate (PRP) (0.60 μg/ml) and anti-serotype 6B (0.191 μg/ml) antibodies were below the levels of long-term protective activity (1.0 μg/ml [11] and 0.34 μg/ml [12, 13], respectively) 4 and 6 months after each infection, respectively. At 1 month after administration of the second Hib and PCV7 vaccination, anti-PRP antibody was significantly elevated to 3.15 μg/ml, but two (6B and 19F) of seven serotype-specific pneumococcal antibodies were still below the protection levels. We therefore added a third PCV7. Because antibodies to pneumococcal capsular polysaccharide protect the host by opsonizing pneumococci for phagocytosis, we concomitantly performed the opsonophagocytic killing assay (OPA) [14] after the third PCV7. Table 1 shows significantly high OPA titers against types 6B and 19F were observed, findings inconsistent with the low anti-6B and anti-19F IgG antibody levels. OPA titers against five other types were also elevated to the levels for protection (>8) [12, 13].

Discussion

We report a girl with non-familial ICA with recurrent bacterial meningitis. ICA is a rare anomaly. Mahlaoui et al. [2] recently documented 20 ICA cases in France and reviewed the literature. In addition to the 65 cases in their report and references therein [2], we found reports of 5 other ICA patients [5, 15] in the literature between January 1960 and April 2011 using the Medline database. Thus, we can here review 70 ICA cases in total. Compared with these patients [2, 5, 15], our case is informative and interesting in several respects.

First are the multiple episodes of meningitis caused by three different pathogens. Of the previous 70 cases, 48 (69%) experienced invasive bacterial infection at least once. Of these 48 patients, only 8 had multiple episodes of invasive bacterial infections, two times in 5 cases and three times in 3 cases (Table 2) [2, 16–20]. Our patient is the first described for whom all three episodes were bacterial meningitis. To better understand the underlying pathogenesis, we characterized the causative pathogens by molecular analysis. Penicillin-resistant serotype 6B pneumococcus and BLNAR Hib subgroup III were among the most prevalent strains causing childhood meningitis in Japan [8, 9]. In contrast, *E. coli* is extremely rare among ICA patients, and we are aware of only one such case, which resulted in death at 4 months of age [21]. *E. coli* in our case possessed capsular antigen K1 and the siderophore receptor gene, *iroN*, which contribute to the bacteremic step in *E. coli* neonatal meningitis [7, 22]. Because the same strain was isolated from the stool of her asymptomatic mother, we confirmed the route of contagion. Besides asplenia, prematurity of the host and high pathogenic factors of the *E. coli* strain might have contributed to this infection.

Second is the good prognosis, despite our patient developing meningitis three times, one of which occurred 4 days after premature birth. Our neonatal case is the youngest at the first invasive infection among the previously reported ICA patients. There have been only 3 ICA patients

Table 1 Serum serotype-specific IgG antibody concentrations and opsonophagocytic killing assay titer before and after 7-valent pneumococcal conjugate vaccine

Serotype	4		6B		9V		14		18C		19F		23F	
	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA
Before PCV7 (6 months after natural infection)	0.132	NA	0.191	NA	0.062	NA	0.366	NA	4.229	NA	0.295	NA	0.14	NA
1 month after 2-dose PCV7	2.809	NA	0.263	NA	4.040	NA	6.767	NA	3.949	NA	0.356	NA	0.233	NA
1 month after 3-dose PCV7	1.37	536	0.137	557	1.199	326	5.075	2367	1.89	210	0.295	192	0.471	769

PCV7 7-valent pneumococcal conjugate vaccine, *IgG conc.* anti-serotype-specific IgG antibody concentration (μg/ml), *OPA* opsonophagocytic killing assay (titer), *NA* not assessed (under treatment with antimicrobial agents)

Table 2 Isolated congenital asplenia patients with multiple episodes of invasive bacterial infections

Patient number	Gender	Infectious episodes	Age at onset	Type of infection	Organisms	Outcome	Reference
1	F	1	6 months	Meningitis	*Streptococcus pneumoniae*	Survived	[2]
		2	11 months	Meningitis, purpura fulminans	*S. pneumoniae*	Died	
2	M	1	10 months	Meningitis	*S. pneumoniae*	Survived	[2]
		2	11 months	Purpura fulminans	*S. pneumoniae*	Survived	
		3	1 year 7 months	Purpura fulminans	*S. pneumoniae*	Survived	
3	M	1	1 year 9 months	Meningitis	*S. pneumoniae*	Survived	[16]
		2	2 years 3 months	Meningitis	*S. pneumoniae*	Survived	
4	M	1	1 year 2 months	Meningitis	*S. pneumoniae*	Survived	[17]
		2	15 years	Meningitis	Not available	Died	
5	M	1	1 year	Meningitis	*S. pneumoniae*	Survived	[18]
		2	1 year	Meningitis	*S. pneumoniae*	Survived	
		3	1 year	Osteomyelitis	Culture negative	Survived	
6	F	1	6 months	Meningitis	*S. pneumoniae*	Survived	[19]
		2	2 years 6 months	Sepsis	Not available	Died	
7	F	1	1 year 6 months	Arthritis	*S. pneumoniae*	Survived	[19]
		2	1 year 9 months	Arthritis	*Haemophilus influenzae* type b	Survived	
		3	10 years	Sepsis	*S. pneumoniae*	Died	
8	M	1	5 years	Sepsis	*S. pneumoniae*	Survived	[20]
		2	9 years	Meningitis	*S. pneumoniae*	Died	
9	F	1	0 month (4 days)	Meningitis	*Escherichia coli*	Survived	Present case
		2	1 year 6 months	Meningitis	*S. pneumoniae*	Survived	
		3	2 years 1 month	Meningitis	*H. influenzae* type b	Survived	

who had overt infections under 3 months of age, which include 1 fatal case [21] and 2 with major sequelae (central nervous system deficit [23] or loss of foot and fingers [24]). Of the 45 childhood and adult patients with invasive infections whose outcomes were known, 29 (64%) died and 3 (7%) had serious sequelae [2, 5, 23, 24]. In contrast, our patient showed normal neurological development under non-serious sequelae of unilateral hearing loss. Such favorable outcome may be attributable to the early recognition and hospitalization. Fortunately, the first episode developed during the period of hospitalization under close monitoring because of prematurity. In addition, at both second and third infectious episodes, she could receive immediate antimicrobial treatment.

Finally, we meticulously investigated the immunological responses to natural infections with *S. pneumoniae* and Hib and those to vaccinations. Of the 70 cases we can review [2, 5, 15], there has been no report addressing this issue. The spleen is a pivotal organ for the phagocytosis of encapsulated bacteria and for the production of immunoglobulins against these pathogens [3]. Even after natural invasive infections of Hib and serotype 6B pneumococcus, serum antibody levels were not elevated to the levels of long-term protection against the pathogens, which may reflect the immunocompromised status of asplenia. This concept is supported by findings from Mikoluc et al. [25] that the congenital asplenic patients had significantly lower concentrations of serum anti-pneumococcal antibodies and reduced responses to PCV7, especially to serotypes 6B and 23F. Similar findings were also observed in adult asplenic patients with overwhelming infection caused by *S. pneumoniae*, representing vaccine failures [4, 5]. Serum antibody concentrations against 6B and 19F in our patient were significantly lower than those against five other serotypes. In contrast, when we evaluated OPA titers after the third PCV7 vaccination, they were at sufficient levels for protection against all serotypes including types 6B and 19F. OPA might be a more important indicator for protection against *S. pneumoniae* [13].

In conclusion, we described a girl with a rare case of ICA, who presented with recurrent meningitis caused by three different pathogens, and was successfully treated without severe sequelae. Exact determination of serum antibody concentrations of encapsulated bacteria and reevaluation of vaccine schedules should be important to protect against relevant infections in ICA patients.

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第 42 回日本小児感染症学会シンポジウム 1

IRAK4 欠損症と感染症

高田英俊*

はじめに

自然免疫における分子免疫学的メカニズムが解明されるに伴い，それを構成する分子異常が原因となる原発性免疫不全症も明らかになってきた．2009 年に IUIS（International Union of Immunological Societies）の分類によると，自然免疫不全症として 10 の疾患が分類されている[1]が，これ以外にも自然免疫異常といえる疾患は新たに報告されている．このなかで今回，IRAK4 欠損症およびこれに病態および臨床上類似する疾患である MyD88 欠損症について述べる．

I．Toll-like receptor とそのシグナル伝達

自然免疫は，好中球，マクロファージ，樹状細胞，NK 細胞などによって担われる早期の生体防御機構であり，病原体に特異的な分子パターンを認識するレセプター（pattern recognition receptor：PRR）によってこれらの細胞内にシグナルが伝達される．Toll-like receptor（TLR）は PRR の代表的存在であり，自然免疫のなかで重要な位置を占めている．TLR は細胞外に Leucine-rich repeat を有し，細胞内には IL-1 receptor と TLR とに共通する TIR domain といわれる構造を有する．TLR が種々の病原体の構成成分を認識することによって（**表**）[2]，アダプター分子を介してシグナルが伝達され，NF-κB，MAPK，IRF3，IRF7 の活性化が起こり，炎症，type 1 IFN の産生を引き起こす（**図 1**）[3]．

II．IRAK4 欠損症

Interleukin-1 receptor associated kinase（IRAK）4 は，IRAK1 の homologue として同定され，2002 年にそのノックアウトマウスが発表された[4]．IRAK4 欠損マウスでは，IL-1R，IL-18R 刺激，TLR 刺激（TLR 2，3，4，9）に対する反応が欠損し，ブドウ球菌に対する感染抵抗性が著しく低下していた．この結果を基に，2003 年に，ヒトにおける IRAK4 欠損症が初めて報告され，グラム陽性球菌，特に肺炎球菌に対する易感染性がみられること，肺炎球菌による化膿性髄膜炎で死亡する例が少なくないこと，加齢に伴って易感染性が軽減していくこと，各種の TLR ligand や IL-1 に対する反応性が欠損することなどが示された[5]．Ku らは，日本国内の 1 家系を含めて，28 名 18 家系の IRAK4 欠損症の臨床像を解析している[6]．それによると IRAK4 欠損症患者の 79％が重症肺炎球菌感染症（化膿性髄膜炎や敗血症など）に罹患しており，その 59％に重症肺炎球菌感染症の再発がみられている．患者の 25％が重症ブドウ球菌感染症を起こしている．特に重要な点は，IRAK4 欠損症患者の 43％が感染症で死亡しており，死亡率が高いことである．また興味深いことに，易感染性は乳幼児期に著しく，その後次第に軽減する．実際に 8 歳以上の死亡例はなく，14 歳以上の重症感染例はなかったと報告している（**図 2**）．したがって IRAK4 欠損症患者の管理において，乳幼児期の重症感染を予防することが極めて大切である．その

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表　TLR のリガンドと TLR の細胞内局在

TLR（co-receptors）	細胞内局在	TLR リガンド
TLR1/2	Cell surface	Triacyllipopeptides
TLR2（Dectin-1, C-type lectin）	Cell surface	Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphophatidyl inositol mucin, zymosan
TLR3	Endosome	ssRNA virus, dsRNA virus, respiratory syncytial virus, murine cytomegalovirus
TLR4（MD2, CD14, LBP）	Cell surface	Lipopolysaccharide, mannan, glycoinositolphospholipids, envelope and fusion proteins from mammary tumor virus and respiratory syncytial virus, respectively, endogenous oxidized phospholipids produced after H5N1 avian influenza virus infection, pneumolysin from *Streptococcus pneumonia*, paclitaxel
TLR5	Cell surface	Flagellin from flagellated bacteria
TLR6/2（CD36）	Cell surface	Diacyl lipopeptides from mycoplasma, lipoteichoic acid
TLR7	Endolysosome	ssRNA viruses, purine analog compounds（imidazoquinolines）RNA from bacteria from group B *Streptococcus*
TLR8	Endolysosome	ssRNA from RNA virus, purine analog compounds（imidazoquinolines）
TLR9	Endolysosome	dsDNA viruses, herpes simplex virus and murine cytomegalovirus, CpG motifs from bacteria and viruses, hemozoin malaria parasite

（文献 2）より引用，改変）

ためにも，早期に診断されることが望まれる．IRAK4 欠損症を迅速に診断する方法として，われわれは以下の方法を報告した[7]．すなわち，末梢血全血を Brefeldin A の存在下に LPS で 4 時間刺激し，単球内の TNF-α産生をフローサイトメーターで調べると，健常者では 90%以上の単球が TNF-αを産生するのに対して，IRAK4 欠損症患者では LPS に対する細胞内シグナル伝達が障害されるため，TNF-α産生単球の割合が著しく減少する（**図 3**）．この方法は簡便であり，検査当日に結果が判明する．また同じ TLR のシグナル伝達に重要な分子である MyD88 の欠損症でもこのスクリーニングが有用であると考えられる．この方法で IRAK4 欠損症・MyD88 欠損症をスクリーニングし，遺伝子診断で確定する．この方法で，現在までに国内で 4 家系 7 名の IRAK4 欠損症患者を同定した（**図 4**）のでその臨床像を紹介する．

1．家系 1

発端者（男児）は，出生後の臍帯脱落遅延が認められ，肺炎球菌による化膿性髄膜炎に 1 歳時と 2 歳時の 2 回罹患し，2 回目の髄膜炎が急激な経過をとり死亡している．遺伝子検査で IRAK4 遺伝子の Exon 2 に homozygous な 1 塩基挿入が認められ，その直下にストップコドンが生じていた．その後出生した第 2 子，第 3 子については，出生直後に IRAK4 欠損症と診断され，予防的抗菌剤の投与，肺炎球菌ワクチンの接種，ガンマグロブリンの投与，発熱時の早期抗生剤の静注などで，積極的に感染症を予防し，早期に治療することで，第 2 子（男児），第 3 子（女児）は重症感染症を起こしていない．

2．家系 2

患者（男児）は，生後 5 カ月時と 11 カ月時の 2 回，肺炎球菌による化膿性髄膜炎に罹患したが，抗生剤治療により治癒している．IRAK4 欠損症を疑われフローサイトメーターによるスクリーニング法により単球内 TNF-α産生細胞の割合が低値であったため，遺伝子検査を行った結果，上述の Exon 2 の異常と，Exon 5 と 1 塩基置換の compound heterozygote であり，いずれもストップコドンを生じていた．

3．家系 3

患者（女児）は，生後 6 カ月時にブドウ球菌による皮下膿瘍を繰り返していた．生後 11 カ月時に肺炎球菌による化膿性髄膜炎に罹患し，急速に進行し死亡．遺伝子検査にて，家系 1 と同じ遺伝

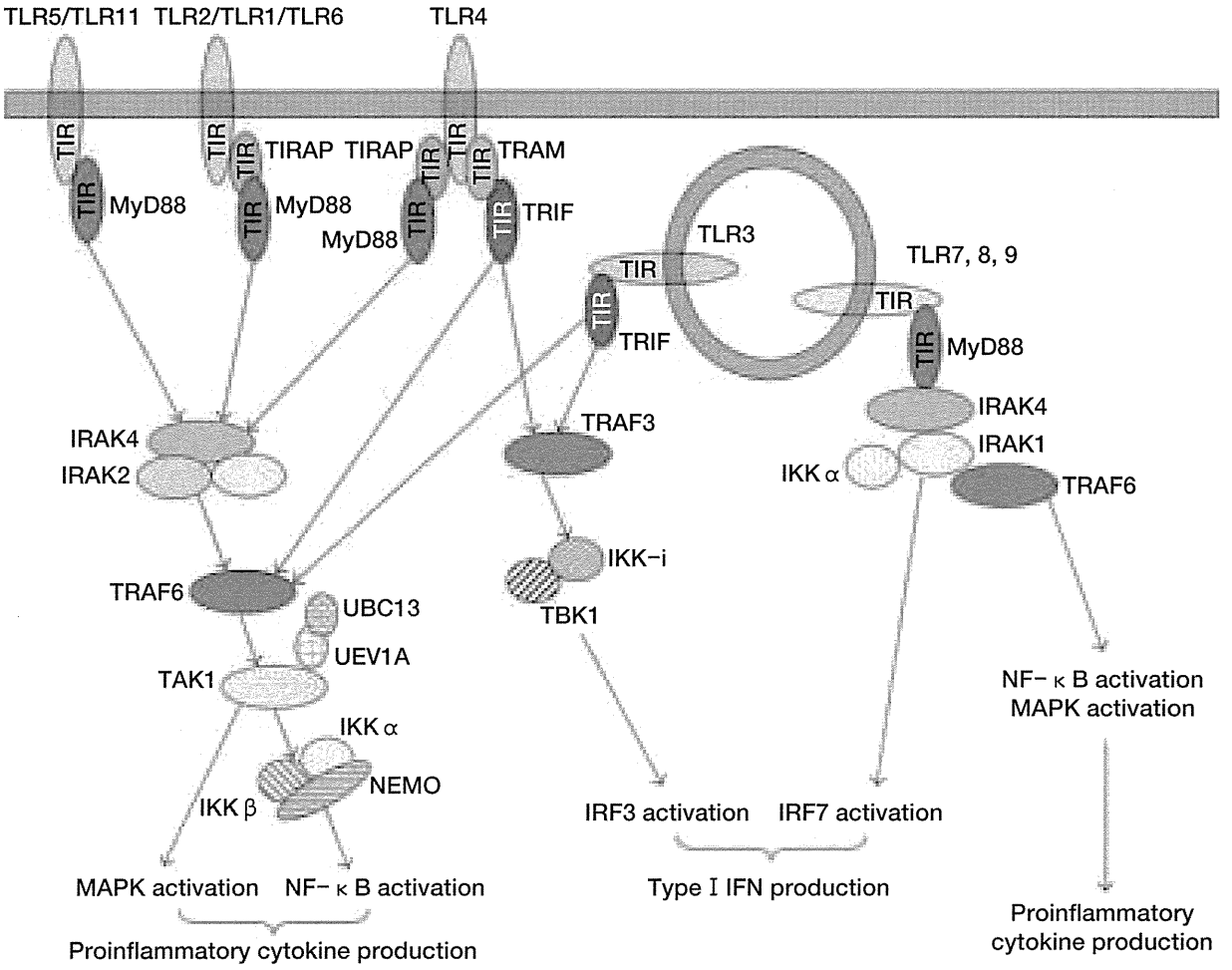

図 1　TLR からのシグナル伝達

TLR は細胞膜あるいは endosome に発現し，そのリガンドを認識することによってシグナルを伝達する．IRAK4，MyD88，NEMO，IκB 分子はこのシグナル伝達において重要な働きを担っており，これらの欠損症および TLR3 欠損症が報告されている．（文献 2）より引用，改変）

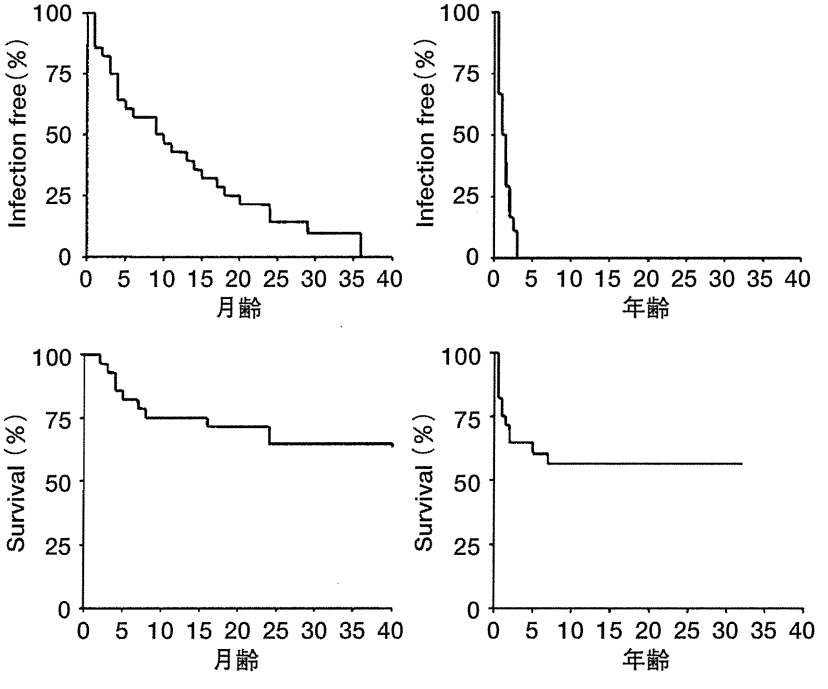

図 2　IRAK4 欠損症の予後

上段：重症感染症の発生．下段：生存率．各々左は月齢，右は年齢で示す．

IRAK4 欠損症患者は乳幼児期に重症感染に罹患することが多く，感染症による死亡もこの時期に多い．

（文献 5）より引用，改変）

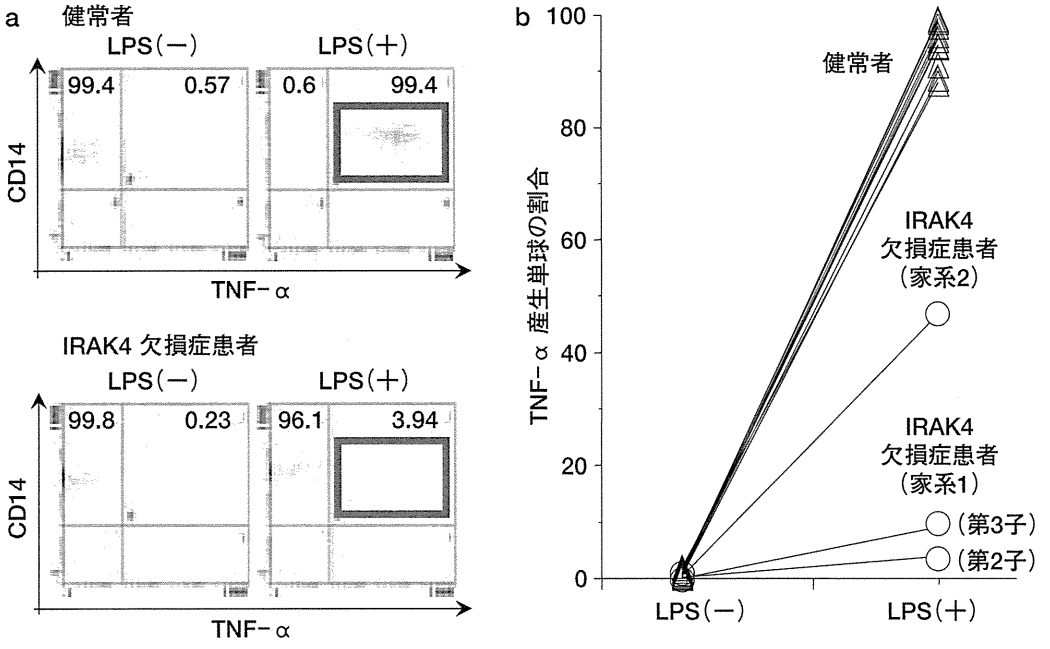

図 3 IRAK4 の迅速診断法

a：末梢血を Brefeldin A の存在下に LPS で刺激すると，健常者では単球のほとんどが細胞内に TNF-αを発現するが（上段），IRAK4 欠損症患者では LPS 刺激に対する反応性が低下するため，単球内の TNF-α産生細胞の割合は著しく低下する（下段）．

b：LPS 刺激後，健常者では 90%以上の単球が TNF-αを産生するが，IRAK4 欠損症では TNF-α産生細胞の割合が低下している．○は IRAK4 欠損症，△は健常者を示す．

（文献 7）より引用，改変）

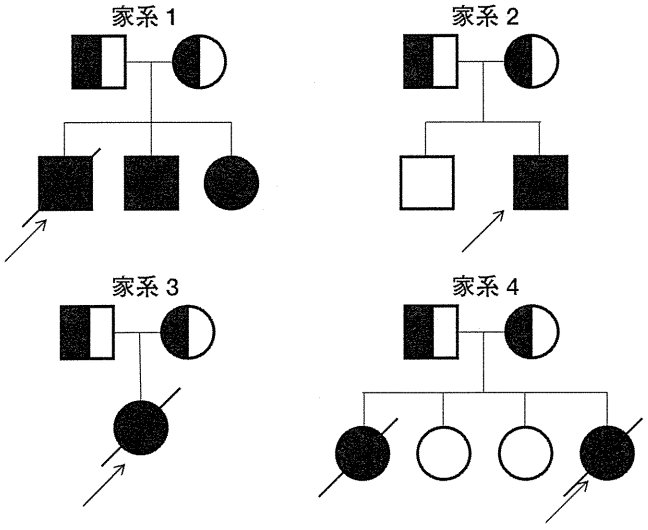

図 4 わが国の IRAK4 欠損症の家系

子変異を認めた．

4．家系 4

発端者は，第 4 子（4 人姉妹）であり，生後の臍帯脱落遅延があった．生後 9 カ月時に緑膿菌による両側下腿の蜂窩織炎，敗血症が急速に進行し死亡．家族歴で，姉（長女）も臍帯脱落遅延があり，生後 7 カ月時に緑膿菌による肝膿瘍・腹腔内膿瘍，3 歳時に肺炎球菌による化膿性髄膜炎に罹患し死亡していたことから，IRAK4 欠損症が疑われた．発端者は遺伝子解析にて家系 1 と同じ IRAK4 遺伝子異常を認め，欠損症と診断された．姉の遺伝子解析はできなかったが，IRAK4 欠損症であると考えられる．

以上のように，国内の IRAK4 欠損症は 4 家系（7 名）あり，肺炎球菌による化膿性髄膜炎を高率に起こしている．また，7 名中 4 名が乳幼児期に死亡している．重症感染症の起こる以前に診断された患者は，抗生剤や肺炎球菌ワクチンの接種などで，重症感染症を免れることができており，早期の診断が重要であることが確認された．

国内の MyD88 欠損症患者はまだ確認されていない．

Picard らは，わが国の患者を含めた 31 家系 48 人の IRAK4 欠損症と MyD88 欠損症の臨床像を

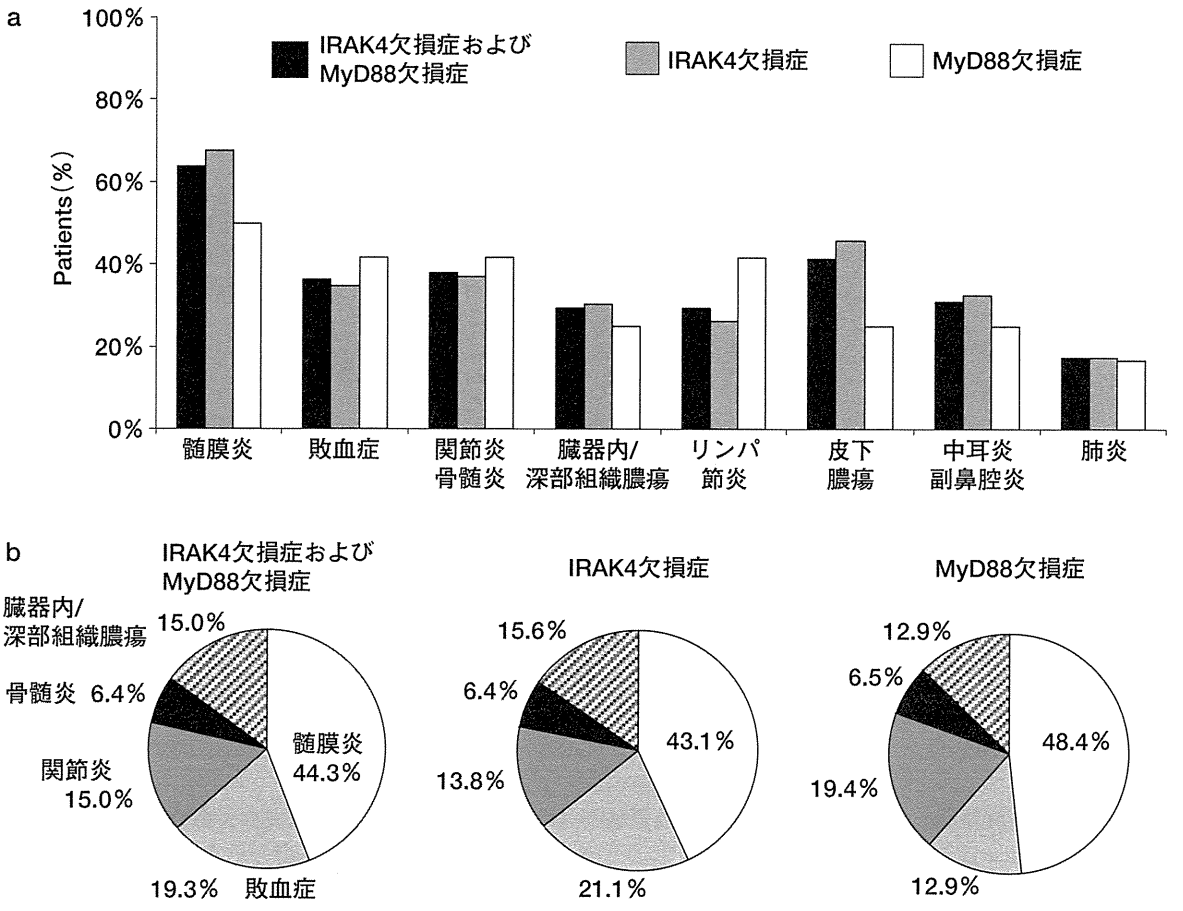

図 5　IRAK4 欠損症および MyD88 欠損症の臨床像（感染症の種類）

a：感染症全体での感染症の種類を示す．b：Invasive bacterial infection での感染症の種類を示す．
（文献 8）より引用，改変）

詳細に検討している[8]．IRAK4 欠損症と MyD88 欠損症は臨床像はほぼ類似している．IRAK4 欠損症ではそのうち 10 名に臍帯脱落遅延があり，臍帯脱落遅延はこの疾患の特徴の一つであることが確認された．

感染症の種類は，髄膜炎，敗血症，関節炎，骨髄炎など，invasive bacterial infection（本来無菌状態である臓器や組織への感染症）が多く（**図 5**），これらの重症感染症が予後を左右する．起炎菌はこれまで報告されていたように肺炎球菌やブドウ球菌が多いが，緑膿菌や連鎖球菌，それ以外のグラム陰性菌も起炎菌になることは注目すべき点である（**図 6**）．

ま と め

IRAK4 欠損症や MyD88 欠損症では，国内外ではほぼ半数が乳幼児期に死亡している．化膿性髄膜炎などの重症感染症が急速に進行して死亡した患者の経過から IRAK4 欠損症が初めて疑われていることが多い．致死的な感染症が起こる以前に診断できた場合，感染予防が可能である．特に乳幼児期の感染予防が重要であり，14 歳以降は感染予防がなくとも重症感染症は起こっていない．加齢によって獲得免疫が得られると易感染性がなくなっていくのであろう．

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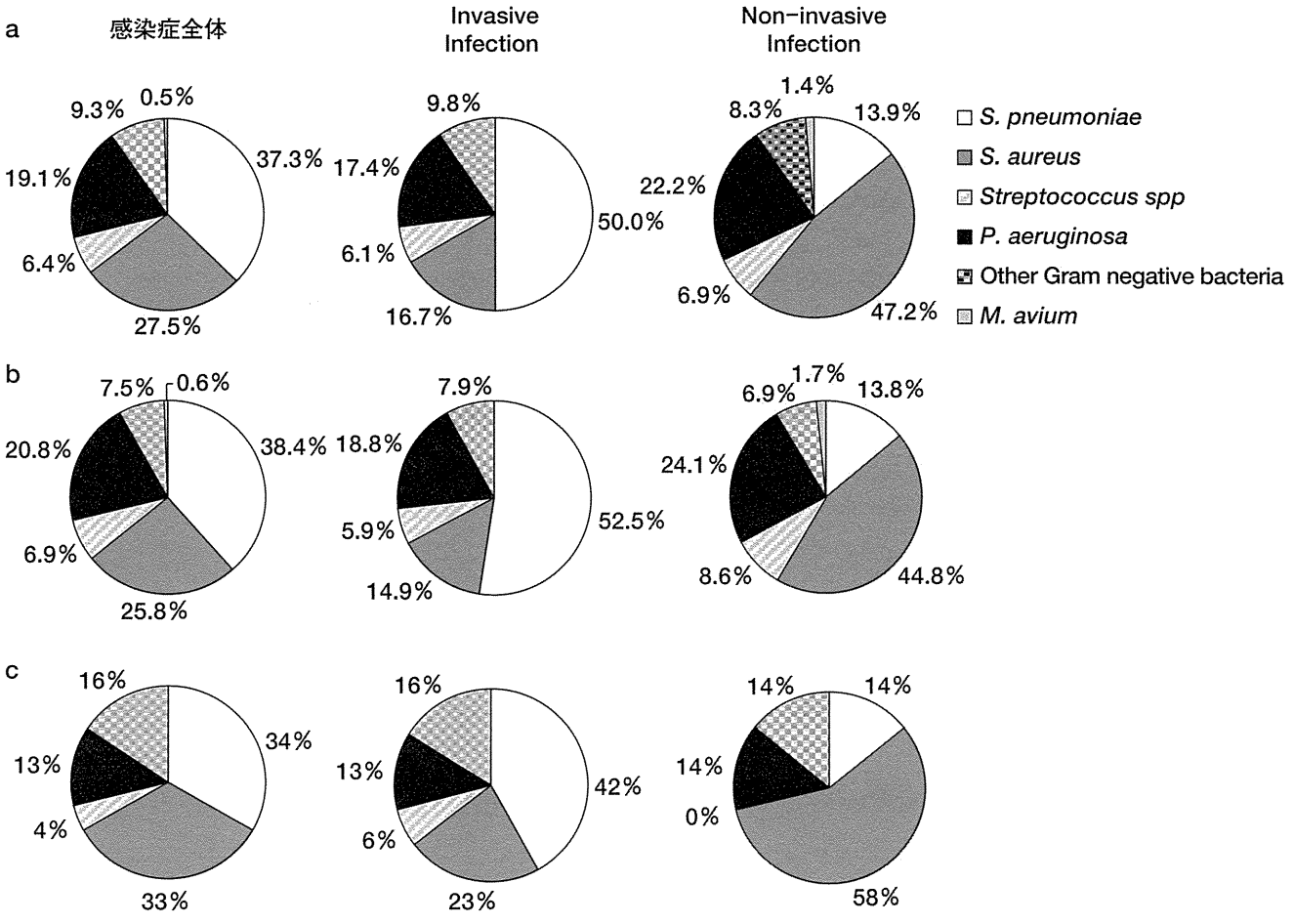

図 6 IRAK4 欠損症および MyD88 欠損症の臨床像（起炎菌の種類）

a：IRAK4 欠損症，MyD88 欠損症における起炎菌の種類． b：IRAK4 欠損症における起炎菌の種類．
c：MyD88 欠損症における起炎菌の種類．（文献 8）より引用，改変）

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IRAK4 deficiency and infection

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According to the recent findings of the molecular mechanism in innate immunity, some new primary immunodeficiency diseases have been clarified. Ten diseases are included in IUIS (International Union of Immunological Societies) classification of primary immunodeficiency. Among them, IRAK4 deficiency and MyD88 deficiency, both of which are closely related in clinical and pathophysiological point of view are described here.

* * *

J Clin Immunol
DOI 10.1007/s10875-011-9600-0

Successful Treatment with Infliximab for Inflammatory Colitis in a Patient with X-linked Anhidrotic Ectodermal Dysplasia with Immunodeficiency

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Received: 23 July 2011 / Accepted: 15 September 2011

Abstract X-linked anhidrotic ectodermal dysplasia with immunodeficiency (X-EDA-ID) is caused by hypomorphic mutations in the gene encoding nuclear factor-κB essential modulator protein (NEMO). Patients are susceptibile to diverse pathogens due to insufficient cytokine and frequently show severe chronic colitis. An 11-year-old boy with X-EDA-ID was hospitalized with autoimmune symptoms and severe chronic colitis which had been refractory to immunosuppressive drugs. Since tumor necrosis factor (TNF) α is responsible for the pathogenesis of NEMO colitis according to intestinal NEMO and additional TNFR1 knockout mice studies, and high levels of TNFα-producing mononuclear cells were detected in the patient due to the unexpected gene reversion mosaicism of NEMO, an anti-TNFα monoclonal antibody was administered to ameliorate his abdominal symptoms. Repeated administrations improved his colonoscopic findings as well as his dry skin along with a reduction of TNFα-expressing T cells. These findings suggest TNF blockade therapy is of value for refractory NEMO colitis with gene reversion.

Keywords NEMO colitis · infliximab · gene reversion

Introduction

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (X-EDA-ID) is a rare inherited disease caused by hypomorphic mutations in the gene encoding nuclear factor-κB

Electronic supplementary material The online version of this article (doi:10.1007/s10875-011-9600-0) contains supplementary material, which is available to authorized users.

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Published online: 13 October 2011

(NF-κB) essential modulator (NEMO), which is the regulatory subunit of IκB kinase [1–3]. Mutations of NEMO can cause an impaired capacity to activate NF-κB, resulting in defects in ectodermal differentiation and innate and adaptive immunity [4, 5]. Affected patients generally show multiple developmental anomalies in ectodermal tissues such as sparse hair, hypodontia with conical teeth, and anhidrosis or hypohidrosis due to lack of sweat glands. These patients also suffer from severe life-threatening infections in various sites caused by Gram-positive or Gram-negative bacteria or mycobacteria. Immunological abnormalities are characterized by defects in the production of proinflammatory cytokines in response to lipopolysaccharide (LPS) stimulation, hypogammaglobulinemia, specific antibody deficiency, and natural killer cell dysfunction. Hematopoietic stem cell transplantation for X-EDA-ID has been employed as a curative treatment [6–10], but has sometimes resulted in engraftment failure.

NEMO colitis, which is inflammatory colitis associated with mutated NEMO protein [11], is found in one fifth of all X-EDA-ID patients [12] and is usually reported as inflammatory bowel disease (IBD), atypical colitis, or Behcet's disease [6, 11, 13]. The onset of inflammatory colitis occurs early in childhood and often causes failure to thrive [2, 5–7, 9, 11–13]. The age of onset of colitis in X-EDA-ID is earlier than that of Crohn's disease, ulcerative colitis, or chronic granulomatous disease [14]. Histological examination reveals active colitis with abundant neutrophilic infiltration, and the colitis usually improves with corticosteroids but not with antimicrobial agents [6, 11]. Susceptibility to colitis remains after hematopoietic stem cell transplantation [6, 9].

Recently, Nenci et al. demonstrated that mice lacking NEMO in intestinal epithelial cells developed spontaneous severe colitis [15]. However, an additional lack of tumor necrosis factor (TNF) receptor-1 in these mice inhibited intestinal inflammation. These interesting findings suggest that TNFα plays a role in the progression of NEMO colitis and that TNF blockade therapy would be a promising treatment.

We describe here an X-EDA-ID boy suffering from severe intractable colitis who improved dramatically following treatment with a chimeric anti-TNFα monoclonal antibody, infliximab. Infliximab administration reduced all symptoms relating to inflammatory colitis, not only frequent diarrhea and severe abdominal pain, but also inflammatory findings by colonoscopy. These effects have lasted for more than 2 years with regular administrations of infliximab.

Methods

Cell Preparation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from our X-EDA-ID patient and his mother using Ficoll-Paque gradient centrifugation. PBMCs were suspended in RPMI 1640 medium (Sigma-Aldrich, USA) and non-adherent cells were used to obtain stimulated T cells. Adherent cells were cultured for 10 days with 500 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, USA) to induce monocyte proliferation. T cells were stimulated for 48 h with 1-μg/mL phytohemaggulutinin (PHA) (Seikagaku Kogyo, Japan) and then for 8 days with 10-U/mL recombinant human interleukin (IL)-2 (Genzyme Techne, USA).

Cytokine Production Assay

PBMCs from our patient and healthy volunteers were incubated with LPS (1 μg/mL) (Sigma-Aldrich) at a concentration of 1×10^6 cells/mL at 37°C for 24 h. The concentration of TNFα in supernatant was measured using human BD OptEIA enzyme-linked immunosorbent assay kits (Becton-Dickinson, USA).

Mutation Analysis and Reversion Analysis

Genomic DNA from our patient and his mother was extracted from PBMCs, stimulated T cells, and stimulated monocytes using Puregene DNA purification kit (Gentra/Qiagen, USA); total RNA was extracted using TRIzol, according to the manufacturer's instructions (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from total RNA with TaKaRa RNA PCR™ Kit (AMV) (Takara, Japan). Polymerase chain reaction (PCR) of genomic DNA and cDNA was performed using TaKaRa LA Taq (TaKaRa) with primers to amplify between exon 2 and exon 4 in the *IKBKG* gene. PCR primers were as follows: c1F, 5′-GCGCTCCTGAGACCCTCCAG-3′; c2R, 5′-GAGGAGAAGGAGTTCCTCAT-3′; G3F, 5′-CCCAGCTCCCCTCCACTGTC-3′; G4R, 5′-AACCCTG GAAGGGGTCTCCGGAG-3′. Genomic DNA was denatured at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and elongation at 68°C for 2 min 30 s, and a final extension for 7 min at 72°C using G3F and G4R primers. cDNA was denatured at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing and elongation at 68°C for 1 min, and a final extension for 5 min at 68°C using c1F and c2R primers. After gel electrophoresis and visualization, targeted bands were extracted and sequenced using ABI Big-Dye Terminator (Applied Biosystems, USA).

To analyze the reversion of mutation, we used our X-EDA-ID patient's PBMCs and stimulated cells. Mononuclear cells sorted with FACSVANTAGE (Becton-Dickinson) were used only at analysis after 12 months of infliximab treatment. PCR products were subcloned using a TOPO

TA cloning kit (Invitrogen) and sequenced as described above.

Reporter Assay for Detecting a Mutant NEMO Function: NEMO-NF-κB Luciferase Reporter Assay

NEMO cDNAs from a healthy volunteer and our patient were subcloned into the p3xFLAG-CMV14 vector (Sigma), respectively. NEMO null rat fibroblast cells (kindly provided by Dr. S. Yamaoka) were plated at a density of 3×10^4 cells/well in a 24-well culture dish and were transfected with 200 ng of plasmid, containing 40 ng of NF-κB reporter plasmid (pNF-κB-Luc; BD Biosciences Clontech, USA), 2 ng of a *NEMO* mutant expression construct, 148 ng internal control for normalization of transfection efficiency (pRL-TK; Toyo Ink, Japan), and the corresponding mock vector, using the FuGENE® HD Transfection Reagent (TOYO-B-Net, Japan) according to the manufacturer's protocol. At 12 h after transfection, the cells were stimulated with 15 ng/mL LPS for 4 h and the NF-κB activity was measured using the PicaGene® Dual SeaPansy assay kit (TOYO-B-NET) according to the manufacturer's protocol. Experiments were performed in triplicate and firefly luciferase activity was normalized to Renilla luciferase activity.

Vβ and Vα Analysis of T Cells

T cell receptor (TCR) β and α chain variable region (Vβ and Vα) repertoires were analyzed by a reverse transcription polymerase chain reaction (RT-PCR) method as described [16]. Briefly, each Vβ fragment (from Vβ1 to Vβ20) or Vα fragment (from Vα1 to Vα18, Vα21, and Vα24) was prepared from a series of HBVT/HBVP or HAVT/HAVP plasmids originating from thymus or peripheral T cells [17] and was dotted on filters. PCR products obtained from the patient by RT-PCR were labeled by α-^{32}P-dCTP and hybridized to the filters. Using densitometry, a semiquantitative assessment of V gene usage was made from the amounts of hybridized products.

Flow Cytometry

Peripheral blood samples were analyzed by three-color flow cytometry. Cells were stained with monoclonal antibodies to the following cell surface markers: CD3, CD4, CD8, CD19 (Becton-Dickinson), and CD14 (eBioscience, USA). Flow cytometry analysis of intracellular NEMO protein was performed as described previously [18]. Flow cytometric data from the stained cells were collected by FACScalibur and analyzed with CellQuest software (Becton-Dickinson).

Intracellular Cytokine Staining

Whole blood samples from our X-EDA-ID patient and healthy donors were stimulated with 1-μg/mL ionomycin (Sigma-Aldrich) and 25-ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) in the presence of 10-μg/mL brefeldin A (Sigma-Aldrich) for 4 h. Cultured cells were stained with monoclonal antibodies against CD4 and CD8 for 30 min at room temperature. Stained cells were fixed and permeabilized with BD Lysing solution (Becton-Dickinson) and incubated with anti-TNFα monoclonal antibody or IgG1 isotypic control (Becton-Dickinson). Cells were analyzed by flow cytometry as described above. Analysis of intracellular TNFα in CD14+ cells was performed after stimulation with LPS (1 μg/mL) at 37°C for 4 h.

Endoscopy and Immunohistochemical Staining

Endoscopy was performed with the consent of legal guardians. Colon biopsies were obtained at regions of visual abnormalities. Formalin-fixed paraffin-embedded tissues blocks were cut into 2-μm sections and stained with hematoxylin and eosin. Subsequently, immunohistochemical analysis using the following primary antibodies with optimized experimental protocols was performed: CD3ε (DAKO, Denmark, rabbit, polyclonal, diluted 1:100, incubated for 24 h at 4°C after microwave heat-induced antigen retrieval for 40 min in pH 6.0 citrate buffer), CD79a (DAKO, mouse, monoclonal, 1:100, microwave for 40 min, pH 6.0), CD68 (DAKO, mouse, monoclonal, 1:50, proteinase K (DAKO) for 10 min at room temperature), CD4 (Novocastra, USA, 1:100, microwave for 40 min, pH 9.0 (NICHIREI BIOSCIENCES, Japan)), CD8 (DAKO, mouse, monoclonal, 1:100, microwave for 40 min, pH 9.0), and TNFα (Santa Cruz Biotechnology, USA, goat, polyclonal, 1:200, microwave for 40 min, pH 6.0). An Envision-HRP Detection kit (DAKO) was used for visualization, except for anti-TNFα, which was visualized using donkey biotin conjugated anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, USA) and LASB2-System/HRP kit (DAKO).

Infliximab Treatment

Infliximab treatment for our X-EDA-ID patient was approved by the medical ethics committee of the University of Miyazaki. We obtained written consent concerning treatment from both the patient and his guardian. Before initiating infliximab, we confirmed that he had no severe infection including tuberculosis according to laboratory data, mycobacterium culture test, skin tuberculin test, and chest computed tomography. Cardiac dysfunction was excluded by echocardiography and electrocardiogram.

Infliximab was given intravenously over 2 h at a dose of 5 mg/kg on 0, 2, and 6 weeks, with follow-up treatments every 7–8 weeks depending on clinical symptoms. The patient was monitored regularly throughout the infliximab treatment.

Results

Case

The patient was born to unrelated Japanese parents after an uncomplicated pregnancy of 41 weeks. There was no history of any first-degree relative diagnosed with incontinentia pigmenti. On the first day after birth, he presented high fever with a markedly increased white blood cell count ($40 \times 10^3/\mu L$) and was treated successfully with antibiotics. He has had a history of recurrent, severe infections including varicella at 3 months of age, penicillin-resistant *Streptococcus pneumoniae* meningitis at 6 months of age, and zoster at 8 months of age. Persistent diarrhea was also observed.

He was introduced to our hospital at 8 months of age for examination of his immunological status. On admission, he showed a marked increase in both white blood cells ($31.9 \times 10^3/\mu L$) and platelets ($872 \times 10^3/\mu L$). Peripheral blood T cell count was decreased (CD3-positive cells, 25.8%), and B cell count was highly increased (CD20-positive cells,: 69.2%). PHA induced a normal proliferation response of T cells, and concentrations of immunoglobulins were within the normal range except IgD (less than 0.2 mg/dL). Natural killer cell activity was markedly impaired. Superoxide-generating ability from neutrophils was intact. LPS-induced TNFα production from patient's PBMC was impaired (Fig. 1a). Interferon (IFN) γ-producing lymphocytes were also reduced apparently at 8 months of age (Table I). All the genes involving in the IL-12 signal pathway, including *IL12RB1*, *IL12RB2*, *JAK2*, and *STAT4* were sequenced, but no mutations were found (data not shown). Surprisingly, both IFNγ-producing T cells and natural killer cells had expanded significantly by 11 months of age (Table I). In addition, we observed that he had ectodermal dysplasia including anhidrosis and conical teeth (Supplementary Fig. 1). A skin biopsy revealed the absence of eccrine sweat glands. When he was 3 years old, a G505C (A169P) missense mutation in his *IKBKG* gene was confirmed and diagnosed as X-EDA-ID. His mother was a carrier. An expression of mutant NEMO protein was not markedly

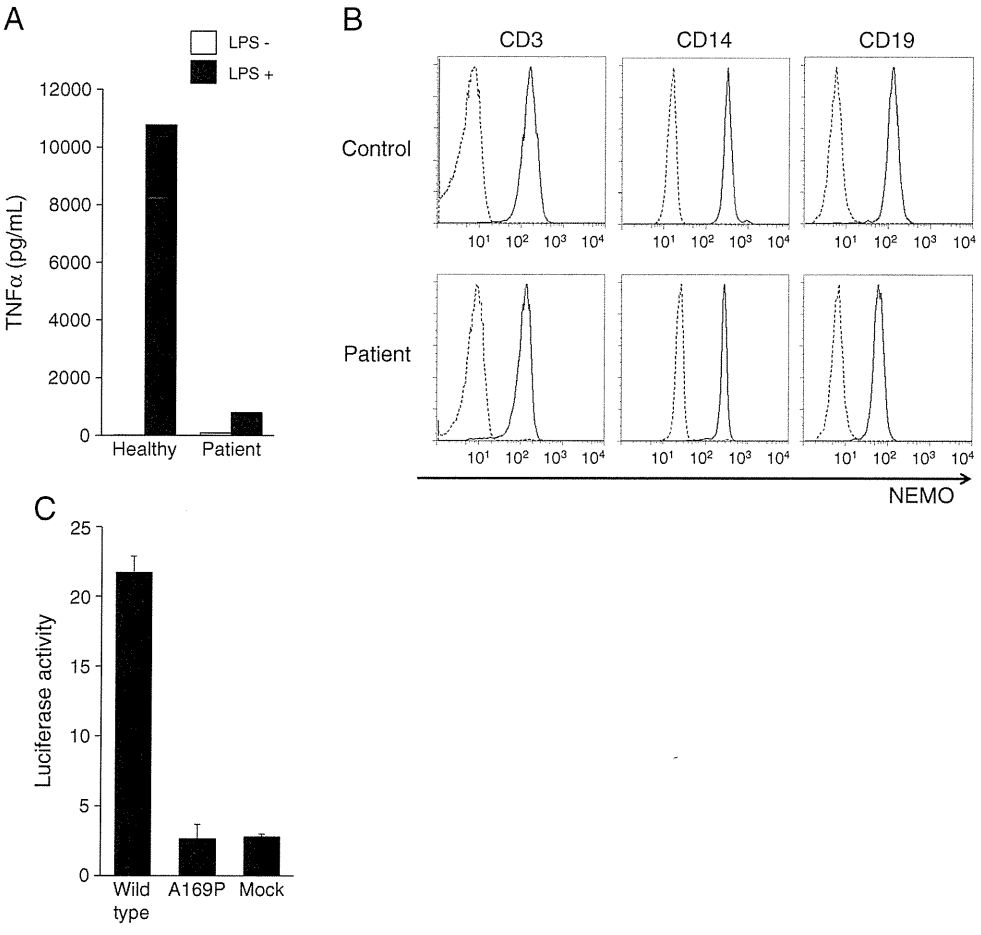

Fig. 1 Analysis of mutant NEMO protein. **a** Reduced production of TNFα from LPS-stimulated PBMCs. PBMCs from our patient and healthy volunteer were stimulated with LPS (1 μg/mL). **b** Analysis of NEMO protein expression using flow cytometry. Intracellular NEMO protein in PBMCs from the patient was not reduced markedly. **c** The result of NEMO-NF-κB luciferase reporter assay. The activity of mutant NEMO in the patient was almost defective. Mock vectors and wild-type NEMO were used as controls. *Error bars* indicate SD