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- **A concise and informative title**

IRAK4 deficiency presenting with anti-NMDAR encephalitis and HHV6 reactivation

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IRAK4 deficiency presenting with anti-NMDAR encephalitis and HHV6 reactivation

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Abstract

IRAK4 deficiency is an inborn error of immunity predisposing patients to invasive pyogenic infections. Currently, there is no established simple assay that enables precise characterization of *IRAK4* mutant alleles in isolation. Anti-N-methyl-d-aspartate receptor (NMDAR) encephalitis is an autoimmune condition that is characterized by psychiatric symptoms, involuntary movement, seizures, autonomic dysfunction, and central hypoventilation. It typically occurs in adult females associated with tumors. Only a few infantile cases with anti-NMDAR encephalitis have been so far reported. We identified a 10-month-old boy with IRAK4 deficiency presenting with anti-NMDAR encephalitis and human herpes virus 6 (HHV6) reactivation. The diagnosis of IRAK4 deficiency was confirmed by the identification of compound heterozygous mutations c.29_30delAT (p.Y10Cfs*9) and c.35G>C (p.R12P) in the *IRAK4* gene, low levels of IRAK4 protein expression in peripheral blood, and defective fibroblastic cell responses to TLR and IL-1 (TIR) agonist. We established a novel NF-κB reporter assay using IRAK4-null HEK293T, which enabled the precise evaluation of *IRAK4* mutations. Using this system, we confirmed that both novel mutations identified in the patient are deleterious. Our study provides a new simple and reliable method to analyze *IRAK4* mutant alleles. It also suggests the possible link between inborn errors of immunity and early onset anti-NMDAR encephalitis.

Keywords: IRAK4, anti-NMDAR encephalitis, HHV6, autoimmunity

Introduction

Toll-like receptors (TLRs) sense microbial products and play an important role in innate immunity (1). Activation of the TLR response results in increased production of inflammatory cytokines such as IL-6 and type I interferons, a key component of the anti-viral state, and secretion of chemokines to attract innate immune cells (2). Autosomal recessive (AR) interleukin-1 receptor (IL-1R)-associated kinase 4 (IRAK4) deficiency, together with myeloid differentiation primary response gene 88 (MyD88) deficiency, is a primary immune deficiency that impairs IL-1R and TLR family signaling (3). Patients with AR IRAK4 deficiency show a predominant susceptibility to invasive infections with pyogenic bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* in early childhood, with severe and often fatal outcomes (4, 5). However, no deaths have been reported after 8 years of age, which is likely due to the acquisition of humoral immunity and immunologic memory (4). Prophylactic antibiotic treatment, vaccinations against pyogenic bacteria, and intravenous immunoglobulin (IVIG) starting early in life are recommended as prophylactic treatments (1). The diagnosis of IRAK4 deficiency is performed by identification of biallelic mutations in the *IRAK4* gene and cellular assay by testing cytokine production to TLR and IL-1R ligands using a patient's peripheral blood and fibroblasts. However, cells and patients with inherited MyD88 deficiency are indistinguishable from cells and patients with inherited IRAK4 deficiency (4). Furthermore, there is no established simple assay that enables a precise characterization of rare *IRAK4* variants identified in isolation.

Anti-N-methyl-d-aspartate receptor (NMDAR) encephalitis is an autoimmune encephalitis characterized by psychiatric symptoms, involuntary movement, seizures, autonomic dysfunction, and central hypoventilation. Anti-

192 NMDAR encephalitis was originally described in 2007 as a potentially reversible immune-mediated paraneoplastic
193 disorder in a young woman with ovarian teratomas (6). Only a few cases have been reported in the literature in
194 childhood and infants cases although nearly 40% of patients with anti-NMDAR encephalitis are under 18 years of age
195 (7, 8). The precise mechanism of anti-NMDAR antibody production in children still remains unclear. Multiple studies
196 have shown that early treatment of anti-NMDAR encephalitis in children leads to better outcomes (9). In the current
197 study, we report a rare case of IRAK4 deficiency presenting with severe neurological sequelae associated with anti-
198 NMDAR encephalitis and human herpes virus 6 (HHV6) reactivation. We newly established a NF-κB reporter assay
199 system that enabled precise evaluation of *IRAK4* mutations. Using this system, we confirmed that two novel mutations
200 in *IRAK4* identified in the patient are deleterious.
201
202 **Material and Method**s
203 **Case Report**
204 The patient is a 10-month-old Japanese boy and the first child of nonconsanguineous parents. He was born after an
205 uneventful pregnancy and his delivery was normal. There was no family history of neurological or metabolic disorders
206 or immunodeficiency disease. The patient had a history of delayed separation of the umbilical cord. His development
207 was normal and he was fully immunized on schedule until 10 months of age having received three doses each of the
208 diphtheria, tetanus toxoids and acellular pertussis vaccine (DPT), *Haemophilus influenzae* type B (Hib) vaccine, and
209 13-Valent Pneumococcal Conjugate Vaccine (PCV13), and one Bacilli Calmette-Guérin (BCG) vaccine. Although the

patient suffered from otitis media, he had no history of severe invasive bacterial infection. He had a febrile episode that was clinically diagnosed as typical exanthema subitum at the age of 8 months, approximately 2 months before the onset of anti-NMDAR encephalitis. He also had a self-limiting febrile episode 4 weeks before the onset. At the age of 10 months, he was admitted to the local hospital due to a fever of 38.4 degrees and dyspnea. A C-reactive protein (CRP) test was normal. He had a convulsion which was interrupted by midazolam (designated as day 1). However, he required mechanical ventilation for severe respiratory dysfunction after this episode. He also needed continuous intravenous infusion of midazolam not only for sedation, but also to inhibit generalized convulsion. The cerebrospinal fluid (CSF) analysis was clear and negative cultures for any microorganisms without pleocytosis (Table 1). No virus was detected in CSF, throat, or stool. Magnetic resonance imaging (MRI) performed on admission showed no abnormal findings. The absence of anti-NMDAR antibodies in sera obtained at this time was confirmed in a later analysis (the result obtained at day 74). He was weaned off mechanical ventilation on day 9. However, central hypoventilation, which requires respiratory support with a high-flow nasal cannula, became apparent after mechanical ventilation was stopped. He had a fever of 38.2 degrees again on day 19. HHV6 was isolated by virus culture from blood, stool, and throat specimens, which strongly suggested the presence of HHV6 reactivation. After that, his symptoms of central hypoventilation worsened. He was transferred to our hospital on day 31 because of the progression of his neurological symptoms.

At the time of admission to our hospital, his consciousness was impaired. He did not maintain eye contact and his muscular tonus was weak. He needed the support of non-invasive positive pressure ventilation for dyspnea. CSF

examination showed pleocytosis (37 leukocytes/mm^3, reference range (RR) 0-30) with predominant lymphocytosis (lymphocyte; 95%), high protein level (65 mg/dl, RR 15-45), elevated IgG index (1.63, RR 0.33-0.63), and the absence of oligoclonal IgG bands with normal glucose level (64 mg/dl, RR 50-80) (Table 1). The CSF bacterial and virus culture was negative. No viruses, including Herpes simplex virus (HSV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV), were isolated by PCR from CSF. However, we identified anti-NMDA-receptor antibodies (1:20, RR <1:1) in CSF (Table 1) in a later analysis (results obtained 43 days after the examination). Brain MRI revealed hyperintensities in the bilateral thalami in a T2 weighted imaging (T2WI) (Fig 1A, 1B) and fluid attenuation inversion recovery (FLAIR) imaging (Fig 1C, D). In contrast, spinal MRI detected no remarkable findings. EEG revealed diffuse slowing. Nerve conduction studies (NCS), short-latency somatosensory evoked potentials (SSEP), and auditory brainstem responses (ABR) showed no abnormal findings. No solid tumors were detected by whole body CT scanning.

After admission, the deterioration of motor and psychiatric functions was exacerbated. The choreoathetosis of his limbs and trunk, and orolingual-facial dyskinesias had worsened with no social contact, and he needed nasogastric tube feeding. His involuntary movements were treated with haloperidol and trihexyphenidyl hydrochloride. The patient was suspected to have autoimmune encephalitis and started treatment with methylprednisolone pulse therapy (30 mg/kg for 3 days) combined with IVIG (1 g/kg for 2 days) from day 32. Oral prednisolone (0.5 mg/kg/day) was subsequently administered following methylprednisolone therapy. One month later, his involuntary movement was dramatically diminished, and he became able to sit alone and to take milk and food by mouth. At this time, based on the detection of anti-NMDAR antibodies in CSF, the patient was diagnosed with anti-NMDAR encephalitis. Oral

246 administration of prednisolone continued for 6 months (Table 1). Administration of haloperidol and trihexyphenidyl
247 hydrochloride for involuntary movement continued for 5 months. He was discharged from our hospital on day 82.
248 Compound heterozygous mutations c.29_30delAT (p.Y10Cfs*9) and c.35G>C (p.R12P) in the *IRAK4* gene
249 were identified by whole exome sequencing at 12 months of age. The lack of IRAK4-mediated TLR signaling was
250 confirmed by analysis of the patient's peripheral blood and fibroblasts. He was thus given a diagnosis of IRAK4
251 deficiency. Prophylaxis treatment with oral amoxicillin was initiated immediately after the diagnosis of IRAK4
252 deficiency and prophylactic IVIG was started at 27 months of age. A follow-up CSF analysis performed at 15 months
253 of age was negative for anti-NMDAR antibodies (Table 1). Obstructive sleep apnea was identified by
254 polysomnography at 16 months. The B cell immunophenotyping which was performed at 19 months detected no
255 obvious abnormality in the absolute number of B cells and the frequency of naïve, non-switched and switched B cells.
256 The frequency of transitional B cells and plasmablast was also normal. The HLA genotype of the patient was HLA-
257 A*02:01, 33:03, HLA-B*13:01, 44:03, HLA-C*03:04, 14:03, and HLA-DRB1*12:02, 13:02. At 19 months, 9 months
258 after the initial onset of symptoms, he could walk with support. No involuntary movement was noted, however he had
259 not begun talking at this time. At 36 months, he began to take aripiprazole for his irritability, and his psychomotor
260 development was equivalent to an 18 month old. Although he has previously suffered from mild otitis media, he has
261 not had a history of severe invasive bacterial infection with prophylactic treatments.
262
263 **Molecular genetics**

264 Genetic tests were performed after the written informed consent of the participants or their parents was obtained. This
265 study was approved by the Ethics Committees and Internal Review Boards of Hiroshima University. Genomic DNA
266 was extracted from peripheral blood leukocytes and subjected to whole exome sequence (WES) and/or Sanger
267 sequencing. The detailed WES method was described previously (10). We used the pcDNA3+ expression vectors that
268 contain N-terminal FLAG-tagged wild-type (WT) or mutant (p.R12C, p.R20W, or p.Q293*) *IRAK4* genes (11). We
269 generated expression vectors encoding p.R12P and p.Y10Cfs*9 mutant *IRAK4* with PCR-based mutagenesis of the
270 pcDNA3+ WT IRAK4 vector with mismatched PCR primers. The primer sequences and PCR conditions are available
271 upon request.
272 Detailed methods of quantitative real-time-PCR, reverse transcription PCR (RT-PCR), flow cytometry,
273 immunoblot analysis, and TLR testing of patient fibroblasts are shown in previous reports and the Supplemental
274 materials and methods (12, 13).
275
276 **IRAK4-deficient cell preparation**
277 IRAK4-deficient HEK293 cells were created using the CRISPR/Cas9 system. HEK293 cells (purchased from the
278 Japanese Collection of Research Bioresources, Osaka, Japan) were transfected with the IRAK4 CRISPR/Cas9 KO
279 plasmid (h): sc-416405 by Nucleofector II and the Cell Line Nucleofector kit V (Lonza, Basel, Switzerland) using the
280 Q-001 program. Single cell clones adjusted by the limited dilution method were then cultured. Successful *IRAK4*
281 knockout was verified by the detection of a DNA fragment of the target site and the direct sequencing of genomic

DNA from candidate clones along with the detection of endogenous protein expression with an immunoblot.

Luciferase reporter assay

IRAK4-null HEK293 cells were transfected with pcDNA3.1+FLAG-IRAK4 WT or mutant IRAK4 alleles, IL-18RAcPL, Igkcona-Luc (provided by S. Yamaoka), and pRL-TK (Promega, Madison, Wisconsin, USA) using lipofectamine LTX according to the manufacturer's protocol. They were then stimulated with recombinant IL-18 (50 ng/ml) created using a previously described method for 6 h (14). Luciferase reporter gene activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega). The experiments were performed in triplicate and the data are expressed in relative luciferase units (RLU). Three independent experiments were performed to confirm the results.

Protein structure analysis

The ternary structure of the death domain complex of MyD88, IRAK4, and IRAK2 (PDB code: 3MOP) was used as a template (15). The structures of the mutant IRAK4 proteins were built with the MOE software (Molecular Operating Environment 2013.08; Chemical Computing Group Inc., Montreal, Canada, 2013. www.chemcomp. com).

Detection of anti-NMDAR antibody in patients with IRAK4 or MyD88 deficiency

The detection of anti-NMDAR antibodies was performed by live cell-based assay as previously described (16).

300

301 **Results**

302 ***Identification of IRAK4 mutations in the IRAK4 gene***

303 As the development of anti-NMDAR encephalitis in infantile periods is quite rare, we suspected the presence of a
304 genetic background in this patient. We thus performed a comprehensive and unbiased genetic study using WES. After
305 the filtering process, several rare variations were annotated in genes that are reported to be related to inborn error of
306 immunity (Table S1) (17). Among them, rare variants in *EPG5*, *STK4, C5* and *C8A* were unlikely to be disease causing
307 due to their inheritance patterns, clinical phenotypes, and laboratory data that showed normal complement levels. The
308 variations in the *IRAK4* gene, p.Y10Cfs*9 and p.R12P were confirmed by Sanger sequencing and were considered to
309 be the best candidate variation in the list of the annotated variations. No other candidate rare variants that could explain
310 the patient's manifestations were identified by WES. The p.Y10Cfs*9 and p.R12P variations were inherited from his
311 father and mother, respectively (Fig 2A, and 2B). Neither of the variations were found in the Single Nucleotide
312 Polymorphism Database (dbSNP), 1000 Genome Projects, the Exome Aggregation Consortium (ExAc) database, or
313 genome aggregation database (gnomAD). We performed RT-qPCR to analyze *IRAK4* mRNA expression in the
314 patient's peripheral blood mononuclear cells (PBMCs). The expression of *IRAK4* mRNA in the patient was
315 approximately two-thirds compared with a healthy subject (Fig 2C). The expression of *IRAK4* mRNA in the patient
316 was confirmed by RT-PCR (Fig S2A). Sanger sequencing of RT-PCR product revealed that both p.Y10Cfs*9 and
317 p.R12P allele almost equally expressed in mRNA level (Fig S2B).

318

319 ***TNF-α production and IRAK4 protein expression***

320 PBMCs from the patient were stimulated with LPS and intracellular TNF-α production was examined by the

321 intracellular staining of TNF-α according to a previous report (18). As shown in Figure 3A, TNF-α production

322 stimulated by LPS treatment of the patient's $CD14^+$ cells was significantly impaired compared with that of healthy

323 subjects. IRAK4 positive cells were low among in $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD19^+$, and $CD14^+$ cells (Fig 3B).

324

325 ***IL-6 production with the stimulation of fibroblasts with various TLR ligands***

326 IL-6 production was tested by the stimulation with TLR ligands, such as PAM2 (PAM2CSK4, a TLR2/6 agonist),

327 PAM3 (PAM3CSK4, a TLR1/2 agonist), FSL1 (a TLR 1/2 agonist), LTA (a TLR2 agonist), LPS (a TLR4 agonist),

328 MPLA (a TLR4 agonist), poly(I:C), TNF-α, and IL-1β using SV40-immortalized fibroblasts. As shown in Figure 4,

329 defective responses to PAM2, PAM3, FSL1, LTA, LPS, MPLA, and IL-1β were detected in the SV40 fibroblasts from

330 the patient, as well as a disease control with Q293X homozygous *IRAK4* mutation (5). In contrast, normal IL-6

331 production in response to TNF-α was observed in the patient's SV40 fibroblasts. The response of the patient's

332 fibroblasts to poly(I:C) was slightly decreased compared with that of wild-type cells.

333

334 ***NF-κB reporter assay to evaluate function of IRAK4 mutant alleles***

335 The diagnosis of IRAK4 deficiency is usually confirmed by cellular assay using a patient's PBMCs or fibroblasts.

336 However, there is no established simple assay system that enables precise evaluation of *IRAK4* mutations in isolation.
337 We transiently expressed HEK293T cells with an empty vector or with plasmids encoding *IRAK4* p.R12C, a missense
338 mutation (19), p.Q293*, the most common mutation found in Europe (4), or a single nucleotide polymorphism
339 (p.R20W) as well as the *IRAK4* variants p.Y10Cfs* or p.R12P, identified in our patient. We then evaluated the
340 IRAK4 expression by immunoblotting. As shown in Figure 5A, p.Y10Cfs* completely abolished protein expression
341 similarly to the previously reported p.Q293* *IRAK4* mutation (3). The p.R12P variation severely impaired IRAK4
342 protein expression, whereas protein expression was normal for the p.R12C mutation that affects the same amino acid.
343 Further, expression of the IRAK4 p.R20W variant was comparable to that of WT. The immunoblot results were
344 consistent with the flow cytometry results, which showed few IRAK4 positive populations in the patient's PBMCs.
345 Next, we evaluated the functional significance of *IRAK4* variants with a NF-κB reporter assay. Yamamoto et
346 al. previously developed a NF-κB reporter assay system to assess the functional significance of *IRAK4* variants (11).
347 However, in this system, WT IRAK4 decreased IL-18-induced NF-κB activation. This assay successfully segregated
348 WT IRAK4 from four mutants (c.118insA, p.R183*, p.Q293* and p.G298D), but it failed to show molecular defects
349 due to the p.R12C mutant. To resolve these problems, we generated IRAK4-null HEK293T cell lines for use in the
350 NF-κB reporter assay. As shown in Figure 5B, WT IRAK4 increased NF-κB activation in IL-18-treated cells. NF-κB
351 activation was abolished by p.R12P and p.Y10Cfs* variations, displaying lower activity than the p.Q293* and R12C
352 mutations. By contrast, the p.R20W polymorphism showed a comparable level of NF-κB activations to WT. Taken
353 together, we succeeded in establishing a NF-κB reporter assay system that could be used to precisely evaluate the

functional significance of *IRAK4* variations. Both p.Y10Cfs* and p.R12P mutations abolish IRAK4-mediated NF-κB activation by IL-18.

Prediction of the mutational effect of the IRAK4 gene

The R12 residue is located on the surface of the IRAK4 death domain that mediates interaction with MyD88(11). *In silico* analysis was used to study the effect of IRAK4 R12C and R12P substitutions on the interaction with MyD88 (Fig. S2). IRAK4 R12C expression was previously reported to be preserved by the interaction with MyD88 (11). By contrast, *in silico* analysis suggested that unlike R12C, R12P does not create a stabilizing internal hydrogen bond and fails to interact with MyD88 (Fig. S2). The *in silico* study is thus consistent with and may explain the results of our *in vitro* reporter study that showed severely impaired function and expression of the patient's mutated R12P IRAK4 protein (Fig 5A).

Antibody prevalence in other IRAK4-deficient patients

In the current study, the presence of anti-NMDAR antibodies was identified in CSF during the episode of encephalitis. However, a follow-up CSF study performed 5 months later was negative for anti-NMDAR antibodies. We next investigated whether other patients with IRAK4 or MyD88 deficiency have anti-NMDAR antibodies. We measured anti-NMDAR antibodies in sera from patients with IRAK4 (n=5) or MyD88 (n=1) deficiency. However, none of the patients had anti-NMDAR antibodies.

372

373 **Discussion**

374 The case presented herein demonstrated IRAK4 deficiency with anti-NMDAR encephalitis and HHV6 reactivation.
375 IRAK4 deficiency was determined by the identification of compound heterozygous mutations in the *IRAK4* gene, low
376 levels of protein expression for IRAK4 in CD14-positive cells, defective production of TNF-α in CD14-positive cells,
377 and defective NF-κB activation by IL-18 stimulation in *IRAK4* null cells expressing the patient's alleles. To date, 24
378 mutations have been identified in patients with IRAK4 deficiency (Table S2) (3, 4, 11, 18-29). Among them, 20
379 mutations are nonsense, frameshift or splice site mutations that are expected to abrogate their functions. However,
380 four mutations are nonsynonymous which require experimental verification to confirm their pathogenicity. The
381 existing NF-κB reporter assay system can potentially misevaluate the pathogenesis of IRAK4 mutants (11). Moreover,
382 the assay shows WT IRAK4 has a negative impact on IL-18-induced NF-κB activation, although IL-18 upregulates
383 NF-κB via IRAK4 in general. To resolve these problems, we succeeded in establishing a precise assay using CRISPR-
384 generated *IRAK4*-deficient HEK293 cells. To the best of our knowledge it is the first *in vitro* assay system that
385 reproduces IL-18-induced WT IRAK4 mediated NF-κB activation, enabling us to distinguish pathogenic mutations,
386 including p.R12C, not only from WT, but also from the known p.R20W allele. This assay system confirmed that two
387 novel mutations, p.Y10Cfs* and p.R12P, identified in the patient are deleterious. Recent progress in comprehensive
388 genetic studies enabled us to detect pathogenic mutations in previously undiagnosed patients. At the same time, such
389 studies rapidly increased the identification of rare variants that need functional characterization to evaluate their

390 pathogenicity. The evaluation of TNF-α production by flow cytometry is a rapid and reliable functional test to confirm
391 the pathogenesis of rare variants found in IRAK4. However, it requires viable peripheral blood from the patients.
392 Especially under the limited availability of viable patients' samples, the NF-κB reporter assay system that we
393 established in the current study could be a simple and a reasonable tool to evaluate uncharacterized rare variants in
394 the *IRAK4* gene.

395 Picard et al. summarized clinical features and outcomes of 49 patients with IRAK4 deficiency and 22 with
396 MyD88 deficiency (1). The initial infectious phenotypes of the majority of the patients with IRAK4 deficiency were
397 severe bacterial infections, such as *S. pneumoniae*, *S. aureus*, and rarely *P. aeruginosa* and *Shigella sonnei*. However,
398 IRAK4-deficient patients were not particularly susceptible to most microorganisms, including viruses, parasites, and
399 fungi. In contrast, curiously, MyD88- and IRAK4-deficient mice show susceptibility to viruses, including HHV1 and
400 HHV2 (30). It is noteworthy that rare neurological findings associated with anti-NMDAR encephalitis and/or HHV6
401 reactivation in an infant led us to study whole exome sequencing, resulting in the identification of novel compound
402 heterozygous mutations in the *IRAK4* gene. The coexistence of anti-NMDAR encephalitis and HHV6 reactivation in
403 this patient may reveal an unknown manifestation associated with IRAK4 deficiency. It is well known that anti-
404 NMDAR encephalitis is triggered by HSV-1 infection (31-35). The post-infectious autoimmune process that follows
405 the HSV-induced brain damage is thought to be the cause of anti-NMDAR encephalitis (36, 37). HHV6 is a neurotropic
406 DNA virus that establishes chronic latency in brain tissue (38). We suspect that HHV6 reactivation induced some brain
407 damage or dysregulation of host immunity that triggered anti-NMDAR antibody production. The limitation of our

study is a lack of direct evidence that demonstrates the relationship between IRAK4 deficiency and development of anti-NMDAR encephalitis and/or HHV6 reactivation. To date, no cases with anti-NMDAR encephalitis or severe virus infections have been reported in IRAK4-deficient patients. Although we investigated patients with IRAK4 (n=5) or MyD88 (n=1) deficiency, no patients had anti-NMDAR antibodies in sera. Further accumulation of cases are necessary to fully characterize the association of these rare clinical manifestations in patients with IRAK4 deficiency.

TLRs are a key family of pattern recognition receptors (PRRs) involved in driving autoimmune inflammation. The inhibitors of TLR binding or signaling have been applied to potential therapeutic agents for autoimmune and other inflammatory diseases (39, 40). While patients with IRAK4 deficiency accumulate autoreactive B cells in the blood, the inhibition of the TLR signaling pathway is unlikely to develop autoimmune disorders (41). Defective TLR signaling, especially that of TLR7 and TLR9, appears to inhibit activation of these autoreactive B cells, as shown in animal models (42). Collectively, the production of autoantibodies is theoretically suppressed in patients with IRAK4 deficiency irrespective of the presence of large numbers of autoreactive B cells. However, Hugle reported a case of a patient with antinuclear antibody (ANA)-positive juvenile idiopathic arthritis with genetically confirmed IRAK4 deficiency (43). The presence of the previous case, together with the case presented in the current study, suggest that autoimmune diseases can occur in patients with IRAK4 deficiency in conflict with the paradigm of IRAK4 mediated signaling being critically necessary for the development of reactive autoantibodies and autoimmune diseases.

In most patients with IRAK4 deficiency, the first bacterial infection occurs before the age of 2 years. Patients are highly susceptible to life-threatening invasive bacterial diseases caused by *Streptococcus pneumoniae*, and

426 *Staphylococcus aureus*. Delayed diagnosis and inappropriate treatment of patients with IRAK4 deficiency may not
427 only lead to fatal invasive infection, but also to irreversible organ damage later in life (44). Prophylactic treatments
428 such as antibiotic prophylaxis, immunization by vaccines, and IVIG have been significantly effective to avoid invasive
429 bacterial infections in such patients. Thus, early accurate diagnosis of IRAK4 deficiency is important to achieve life-
430 saving treatment. Our patient's atypical clinical manifestation and development of anti-NMDAR encephalitis in
431 infancy led us to sequence his whole exome and identify IRAK4 deficiency. This enabled us to start anti-bacterial
432 prophylaxis before severe bacterial infections could develop. Indeed, the patient has not experienced severe bacterial
433 infections in the first 4 years of his life owing to such prophylactic treatments. The current case also revealed the
434 possibility that genetic studies can contribute to characterizing infantile cases with anti-NMDAR encephalitis. Further
435 accumulation of cases and characterization of the molecular pathogenesis of IRAK4 deficiency are expected to
436 elucidate the risk of viral infections and/or anti-NMDAR encephalitis in patients with IRAK4 deficiency.
437
438 **Appendix**
439
440 **Supplemental materials and methods**
441 **Quantitative real-time-PCR and reverse transcriptional PCR (RT-PCR)**
442 Total RNA was extracted from PBMCs and was subjected to reverse transcription with random primers to generate
443 cDNA. *IRAK4* mRNA levels were determined by quantitative PCR (qPCR) on the cDNA, with the CFX96 Touch

Real-Time PCR Detection System (Bio Rad, USA). We used the following Taqman probes to determine the expression of *IRAK4* (Hs00211610_m1) and *GAPDH* (Hs99999905_m1) (Applied Biosystems, Waltham, Massachusetts, USA). The results were normalized with respect to the values obtained for the endogenous GAPDH cDNA. The RT-PCR was performed using primers spanning the entire coding region of the *IRAK4* gene. The condition of RT-PCR and primers used are available on request.

Flow cytometry

To investigate TNF-α production in response to lipopolysaccharide (LPS) stimulation, PBMCs were stimulated with 100 ng/ml of LPS (Invivogen) for 4 h. The cells were then stained with anti-CD14 antibodies and subjected to intracellular staining of TNF-α using a Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The analysis gate was set for monocytes by forward and side scatter, and CD14 expression. Expression of intracellular TNF-α in monocytes was analyzed using flow cytometry (18). To assess IRAK4 protein expression, PBMCs were suspended at a density of 10^4 cells/μl in RPMI supplemented with 10% FBS. They were then fixed and permeabilized according to the BD Phosflow protocol (Protocol III) and stained with PE-conjugated anti-CD3, FITC-conjugated anti-CD4, CD8, CD19, CD14, Alexa 647-conjugated IRAK4 (BD Becton, Dickinson and Company), and anti-CD19 (BioLegend, San Diego, California, USA) antibodies. The stained cells were subjected to flow-cytometry analysis.

462

463 **Immunoblot analysis**

464 The HEK293T cells were maintained in DMEM supplemented with 10% FBS. The cells were harvested and plated at

465 a density of 2.5×10^5 cells/ml in six-well culture plates. After incubation for a further 24 h, plasmid DNA (5 μg/well)

466 carrying the WT or a mutant *IRAK4* allele was introduced by lipofection using lipofectamine LTX (Thermo Fisher

467 Scientific, Waltham, Massachusetts, USA). The transfected cells were incubated for 24 h, and then subjected to

468 immunoblot analysis. Immunoblot analysis was performed as previously described (12). The following antibodies

469 were used as the primary antibody; anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, Saint Louis, MO, USA) and

470 an anti-β-actin antibody (Sigma-Aldrich).

471

472 **Toll-like receptor (TLR) testing of patient fibroblasts**

473 To assess the impact of the *IRAK4* mutation on TLR signaling, we analyzed SV40 immortalized skin fibroblasts (SV40

474 fibroblast) from the patient as previously described (13). We also used SV40 fibroblasts from a patient with IRAK4

475 deficiency harboring a homozygous Q293* mutation as a disease control. Briefly, SV40 fibroblasts were stimulated

476 with various agonists of TLRs. Twenty-four hours after stimulation, the supernatant was corrected and subjected to

477 ELISA to detect IL-6 production. The experiments were performed in triplicate and two independent experiments

478 were performed to confirm the results.

479

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Author information

Shiho Nishimura and Yoshiyuki Kobayashi contributed equally to this work.

Conflicts of interest:

The authors declare that they have no relevant conflicts of interest.

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622

623

Figure legends

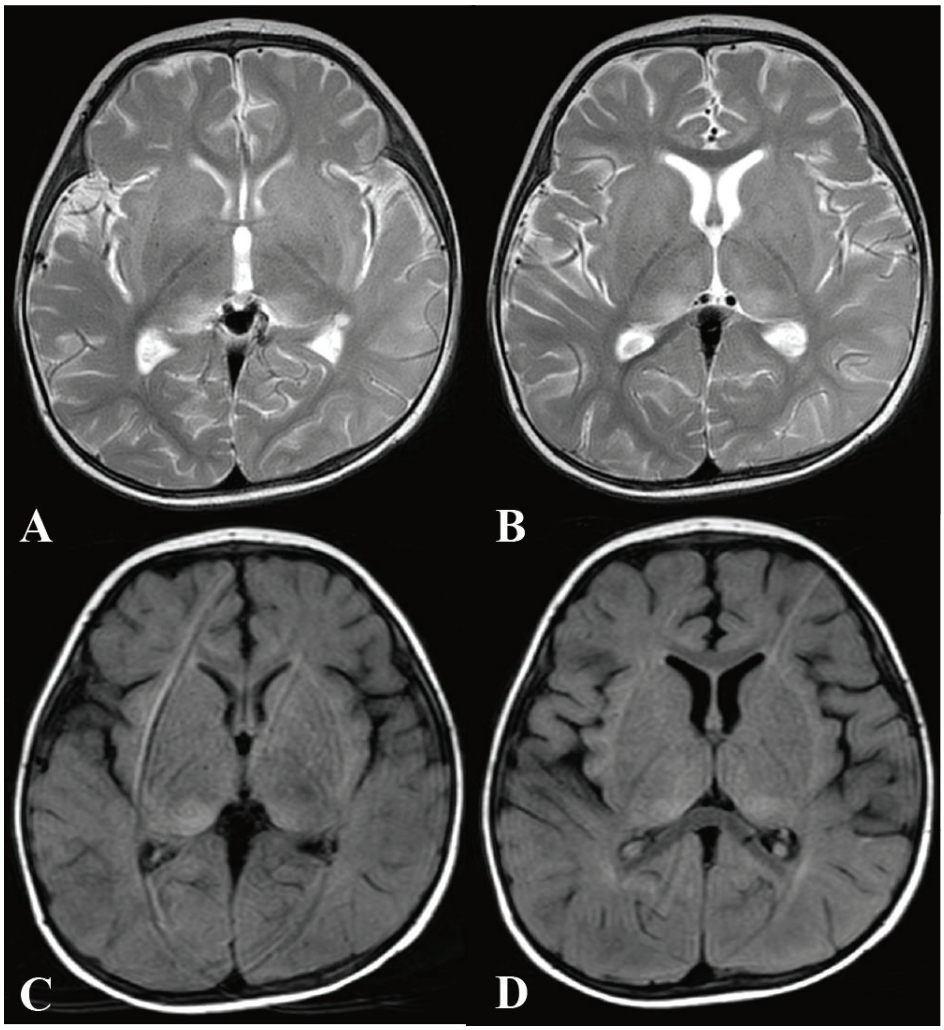


Figure 1

Brain MRI obtained at day 4. (A, B) The axial T2 weighted image (T2WI) showed high intensity regions in the bilateral thalamus. (C, D) The axial fluid attenuation inversion recovery (FLAIR) image showed high intensity regions in the bilateral thalamus.

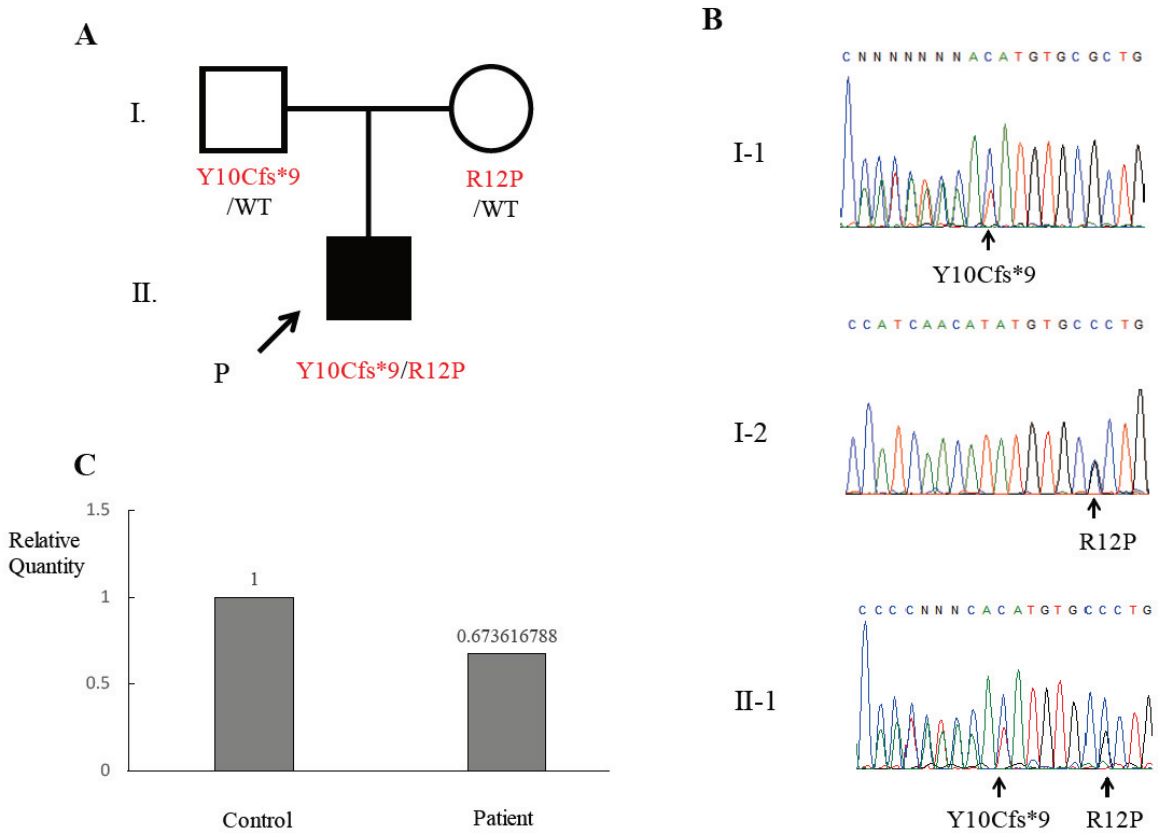


Figure 2

Identification of *IRAK4* mutations and detection of *IRAK4* mRNA expression in PBMCs. (A, B) Familial segregation of *IRAK4* mutations. The novel compound heterozygous mutation in the *IRAK4* gene was detected in the patient (II.1). The Y10Cfs*9 and R12P mutations were inherited from his asymptomatic father and mother, respectively. (C) The expression of the *IRAK4* mRNA was assessed by RT-qPCR from PBMCs of the patient and one healthy control. *IRAK4* mRNA from the patient was about two-thirds lower than that of the healthy control.

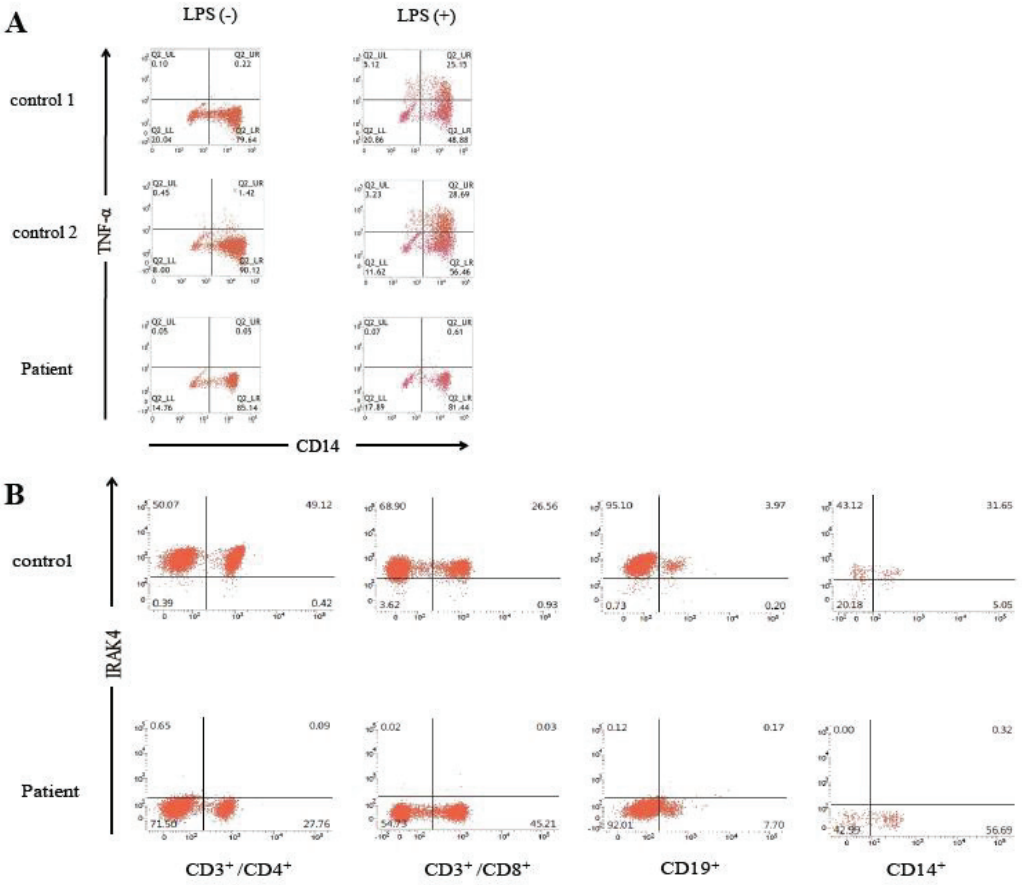
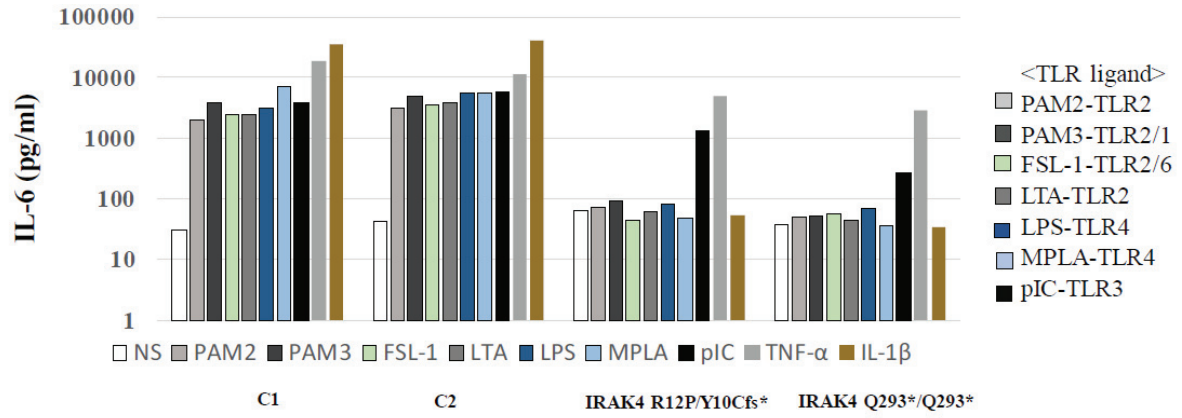


Figure 3

Flow-cytometric analysis of TNF-α production and IRAK4 protein levels of PMBCs. (A) Flow cytometric analysis of intracellular TNF-α production of monocytes in response to LPS. The patient's $CD14^+$ monocytes display impaired TNF-α production in response to LPS stimulation. (B) Flow cytometric analysis of IRAK4 protein expression. IRAK4 expression was abolished in $CD3^+$ /$CD4^+$ T cells, $CD3^+$ /$CD8^+$ T cells, $CD19^+$ B cells and $CD14^+$ monocytes.



651

652 **Figure 4**

653 IL-6 production with the stimulation of various TLR ligands in fibroblasts.

654 IL-6 production by SV40-immortalized fibroblasts from healthy controls and two IRAK4-deficient patients after 24 h

655 of stimulation with various TLR agonists. IL-6 production was defective with the stimulation of TLR1, TLR2, TLR4,

656 and TLR6 but not of TLR3 in cells expressing R12P/Y10Cfs* and Q293*/Q293* alleles.

657

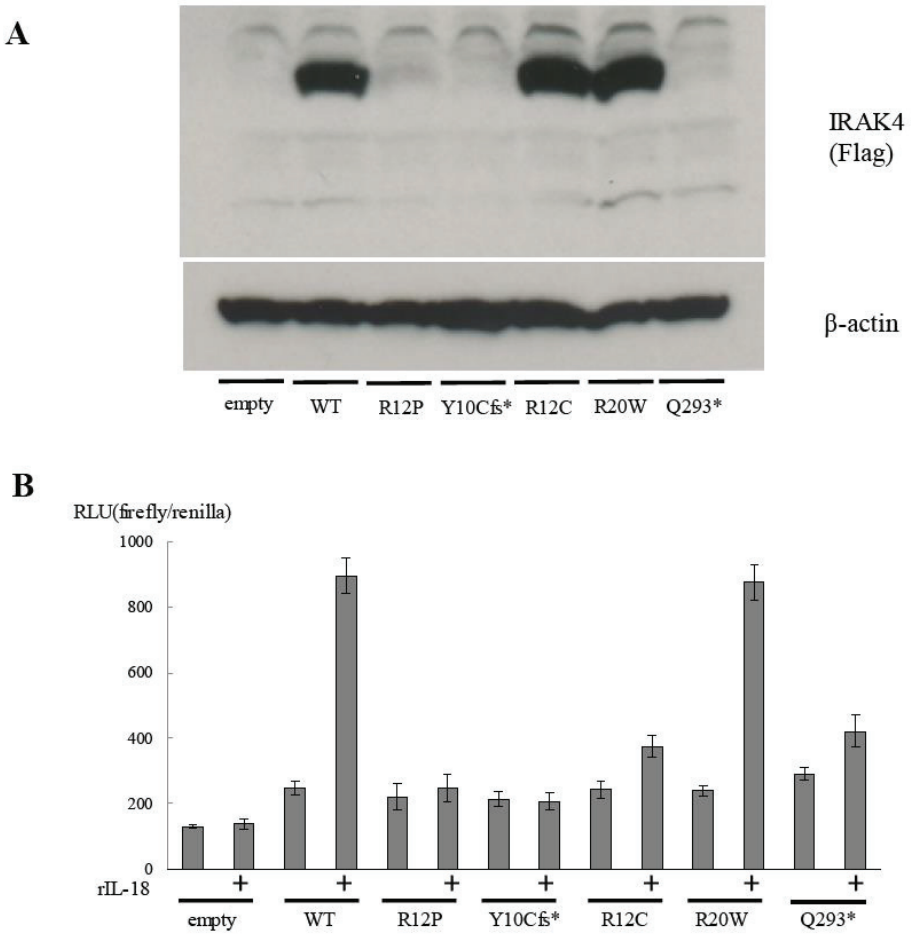


Figure 5

IRAK4 protein expression and IL-18-induced IRAK4-mediated NF-κB activation. (A) IRAK4 and β-actin protein levels in HEK293T transfectants. Both Y10Cfs* and Q293* mutations completely abolished IRAK4 protein expression. The R12P mutation severely impaired IRAK4 protein expression, whereas the protein expression was normally observed in the R12C mutation. The IRAK4 expression of the R20W polymorphism was comparable to that of WT. (B) NF-κB reporter activity in HEK293T transfectants. The R12P, Y10Cfs*, R12C, and Q293* (reported previously) mutant alleles showed severe impairment in IL-18-induced NF-κB activation. The R20W polymorphism showed equivalent levels of IL-18-induced NF-κB activation to WT IRAK4.

669 **Table 1**

670 Cerebrospinal fluid examination results

671

	Day 1	Day 30	Day 32	Day 204	Reference range
Cell count (/ μl)	5	64	37	2	0-20
Differential count (%)					
Neutrophils	20	2	5	0	
Lymphocytes	80	98	95	100	
Monocytes and others	0	0	0	0	
Protein (mg/dl)	21	91	65	24	15-45
IgG index	NA	NA	1.63	0.54	<0.73
Glucose (mg/dl)	76	59	64	58	50-80
Lactic acid (mg/dl)	NA	9.7	12.3	NA	3.7-16.3
pyruvic acid (mg/dl)	NA	0.5	0.87	NA	0.30-0.90
oligoclonal band	NA	NA	positive	negative	
myelin basic protein (pg/ml)	NA	NA	<31.3	<31.3	0-102.0
anti-NMDA-receptor antibodies	NA	NA	positive (1:20)	negative	

672 **Table S1.** Summary of candidate genes by whole exome sequencing

673

Gene		dbSNP	ExAC_ ALL	gnomAD_ ALL	HGVS.c	HGVS.p
IRAK4	Hetero	·	·	·	c. 29_30delAT	p. Tyr10fs
IRAK4	Hetero	·	·	·	c. 35G>C	p. Arg12Pro
EPG5	Hetero	·	·	·	C .6263dupT	p. Leu2088fs
STK4	Hetero	·	·	·	c. 35+8G>A	·
C8A	Hetero	rs56334452	0.0003	0.000256	c. 1654A>G	p. Arg552Gly
C5	Hetero	rs772788429	8.24E-06	0.000012	c. 2737C>T	p. Leu913Phe

674

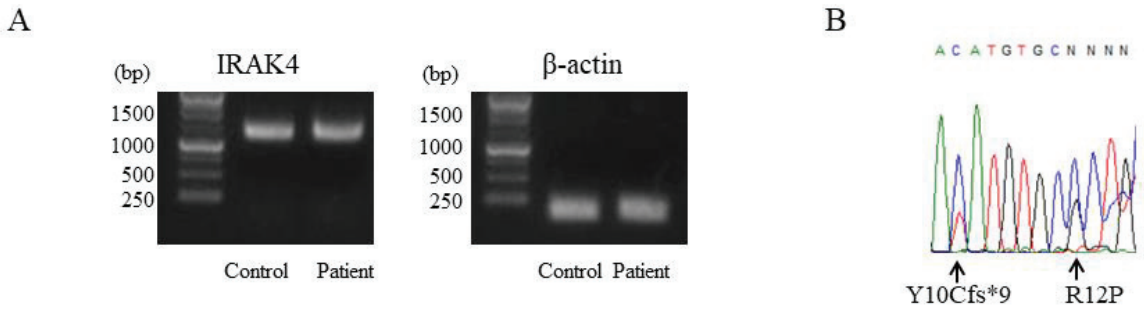
675

676

677 **Table S2**. List of the mutations in patients with IRAK4 deficiency

Nucleotide	Protein	Position	Reference
1-1096_40+23del	unknown	5'UTR	(4, 20)
unknown	M1V	exon2	(4, 23)
34C>T	R12C	exon2	(4, 19)
unknown	Y48*	exon2	(4, 20)
123_124insA	P42Tfs*3	exon2	(24)
547C>T	R183*	exon5	(4, 24)
255_260dup6	D86_87dup	exon3	(22)
573delA	M192Wfs*13	exon5	(25)
593delG	G198Efs*7	exon5	(26)
620_621delAC	T208Nfs*11	exon5	(4, 27)
631delG	A211Qfs*1	exon5	(20)
821delT	L274Pfs*13	exon7	(3, 4)
831+5G>T	unknown	intron7	(4, 19)
877C>T	Q293*	exon8	(3, 4, 27)
893G>A	G298D	exon8	(26)
897_900delCAAT	N300Ffs*43	exon9	(4)
942-1481_1125+547del	unknown	intron9	(20)
1146delT	G383Dfs*14	exon10	(22)
1175G>T	unknown	splicing site of exon9-10	(21)
1188+520A>G	unknown	intron10	(4, 28)
1189-1G>T	unknown	intron10	(4, 28)
1204G>T	E402*	exon11	(4, 29)
1240insA	I414Nfs*1	exon11	(20)
unknown	Y430*	exon11	(21)

678



679

680 **Figure S1**

681 The reverse transcription PCR (RT-PCR)-based detection of *IRAK4* mRNA from PBMCs. (A) The expression of

682 *IRAK4* mRNA in PBMCs from patient and unrelated healthy control. (B) Sanger sequencing of RT-PCR product

683 from A to determine presence of both p.Y10Cfs*9 and p.R12P alleles at mRNA level.

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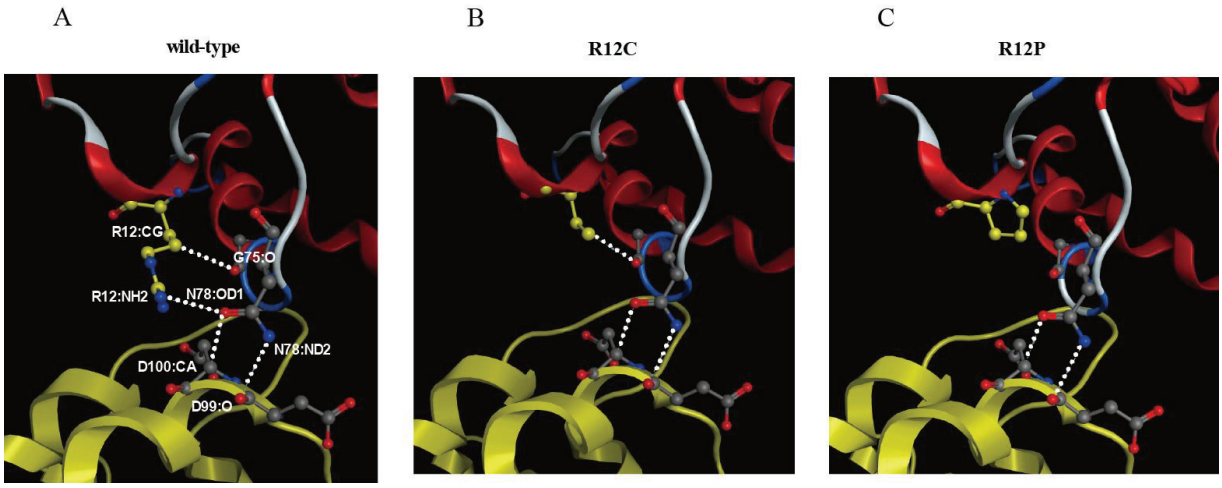

Figure S2

In *silico* analysis of the protein structure of IRAK4. 3D interaction models of IRAK4-death domain (DD) (red) with MyD88-DD (yellow). Residues of R12 appeared to be located on the surface of IRAK4-DD. A protein-protein interaction study was used to assess the mutational effect of these residues. (A) The recombinant proteins of IRAK4-DD+internal domain (ID) WT and MyD88-DD+ID WT formed a higher order oligomeric complex. The surface including R12 directly interacts with MyD88. (B) IRAK4 R12C could keep the molecular structure because of generating new inter molecular interaction with the subdomain of IRAK4, which was possibly formed among the side chains of IRAK4 R12C and G75. (C) IRAK4 R12P failed to interact with MyD88 due to loss of original inter molecular interaction of IRAK4 and lost the interaction to its subdomain.

704

705 **Figure captions list**

706

707 **Figure 1**

708 Brain MRI obtained at day 4.

709 **Figure 2**

710 Identification of *IRAK4* mutations and detection of *IRAK4* mRNA expression in PBMCs.

711 **Figure 3**

712 Flow-cytometric analysis of TNF-α production and IRAK4 protein levels of PMBCs.

713 **Figure 4**

714 IL-6 production with the stimulation of various TLR ligands in fibroblasts.

715 **Figure 5**

716 IRAK4 protein expression and IL-18-induced IRAK4-mediated NF-κB activation.

717 **Table 1**

718 Cerebrospinal fluid examination results

719 **Table S1**

720 Summary of candidate genes by whole exome sequencing

721 **Table S2**

List of the mutations in patients with IRAK4 deficiency

Figure S1

The reverse transcription PCR (RT-PCR)-based detection of *IRAK4 mRNA* from PBMCs.

Figure S2

In *silico* analysis of the protein structure of IRAK4.