5. Saulyte J, Regueira C, Montes-Martinez A, Khudyakov P, Takkouche B. Active or passive exposure to tobacco smoking and allergic rhinitis, allergic dermatitis, and food allergy in adults and children: a systematic review and meta-analysis. PLoS Med 2014;11:e1001611.
6. Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzykowski R, Kim J, et al. Overexpression of IL-10 in atopic dermatitis: contrasting cytokine patterns with delayed-type hypersensitivity reactions. J Immunol 1995;154:1956-63.
7. Ricci G, Patrizi A, Federica B, Calamelli E, Dell'Omo V, Bendani B, et al. Cytokines levels in children affected by atopic and nonatopic eczema. Open Dermatol J 2008;2:18-21.
8. Howell MD, Novak N, Bieber T, Pastore S, Girolomoni G, Boguniewicz M, et al. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. J Invest Dermatol 2005;125:738-45.
9. Ji S, Shin JE, Kim YS, Oh JE, Min BM, Choi Y. Toll-like receptor 2 and NALP2 mediate induction of human beta-defensins by fusobacterium nucleatum in gingival epithelial cells. Infect Immun 2009;77:1044-52.

Available online December 9, 2017.
https://doi.org/10.1016/j.jaci.2017.11.018

Haploinsufficiency of A20 causes autoinflammatory and autoimmune disorders

To the Editor:

A20, which is encoded by the tumor necrosis factor alpha induced protein 3 (*TNFAIP3*) gene, is a negative regulator of the TNF-nuclear factor-κB signaling pathway. It had been reported that several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, psoriasis, Crohn disease, and type 1 diabetes, are associated with *TNFAIP3* gene polymorphisms.[1] Recently, heterozygous germline mutations in the *TNFAIP3* gene have been found to cause the haploinsufficiency of A20 (HA20), which displays an early-onset autoinflammatory disease resembling Behçet disease. Initially, 6 families of patients with HA20 were described by Zhou et al. Subsequently, 3 additional families and sporadic cases were reported.[2] The major phenotype of HA20 is Behçet disease–like symptoms, including a recurrent aphthous stomatitis, genital ulcers, and intestinal symptoms. However, some patients present with not only the symptoms of autoinflammatory disorders but also several autoimmune-like symptoms.[2,3] Therefore, these preceding clinical reports suggested that there might be unexpected phenotypes in HA20. In this study, we performed a multicenter survey investigating the patients with HA20 found in Japan.

A total of 30 patients from 9 unrelated families were enrolled in this study. Twenty-two patients were identified as having mutations in the *TNFAIP3* genes, and the other 8 patients were clinically diagnosed as having HA20 as a result of a Behçet disease–like phenotype with an autosomal-dominant-inheritance trait (Fig 1, *A*). The clinical profiles are described in Table I and in Table E1 in this article's Online Repository at www.jacionline.org. Additional case reports are described in this article's Methods section in the Online Repository at www.jacionline.org. Three mutations in the *TNFAIP3* gene had been previously reported; however, 6 were novel. All these mutations were evaluated to be functionally pathogenic by several *in vitro* assays (see Figs E1-E4 in this article's Online Repository at www.jacionline.org).

HA20 was initially identified as a Behçet disease–like phenotype. The original description showed that all patients with HA20 had recurrent aphthous stomatitis and genital ulcers. The patients included in our study also developed some Behçet disease–like phenotypes; however, 59% of the patients did not fulfill the International Study Group for Behçet's disease 1990 criteria.[4] Intriguingly, 5 patients had only recurrent aphthous stomatitis, and some of them were initially diagnosed as having periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome. Furthermore, patient 4 (P4), showing typical recurrent fever and abdominal pain, was initially diagnosed as having colchicine-resistant familial Mediterranean fever, and she was treated with etanercept. Therefore, in some conditions, HA20 might be difficult to discriminate from other autoinflammatory disorders. Similarly to typical Behçet disease, the symptoms of HA20 tend to increase with aging. In many cases, the initial symptom of HA20 is not a recurrent stomatitis.[5] For example, polyarthritis appeared before the onset of stomatitis, genital ulcers, and intestinal manifestations in P1, P3, and P10. Regarding the relatively low-frequency phenotypes of Behçet disease, uveitis was not found in our series (see Table E2 in this article's Online Repository at www.jacionline.org). Patients with HA20 are juvenile-onset and partially resemble those with Behçet disease, but their phenotypes are distinct from those of classical Behçet disease.

We could not demonstrate genotype-phenotype correlation in our series. In general, autosomal-dominant-inheritance diseases often show variable penetrance in the same family. Some patients are severely affected, whereas other family members are mildly or not affected. This might indicate the possibility of additional effects with other genetic, ethnic, or environmental factors, such as vaccinations or infections. Furthermore, genetic analysis of the asymptomatic individuals, the mother of P2 and the father of P21, revealed low-frequency mosaicism (see Fig E5 in this article's Online Repository at www.jacionline.org). As one of the possible modifier effects, this result suggests that germline mosaicism or somatic reversions might modulate these disease phenotypes as has been observed in other diseases including DOCK8 deficiency.[6]

The patients with HA20 showed an excess production of proinflammatory cytokines. We evaluated the serum cytokine levels between nonflare and flare periods. Proinflammatory cytokines, including TNF-α, sTNFR1, IL-6, IL-18, and IFN-γ–inducible protein-10 (IP-10), were increased during flares, whereas TNF-α, sTNFR1, IL-18, and IP-10 were also increased in nonflare periods (Fig 1, *B*). Furthermore, the production levels of proinflammatory cytokines by PBMCs were increased as previously reported (Fig 1, *C*). These results suggest that the constitutional autoinflammation of patients with HA20 occurs not only in TNF signaling but also in IL-1– or IL-18–related inflammasome activation. In fact, the refractory cases in our cohort were treated with anti–TNF-α agents, and it successfully induced the remission.

A most noteworthy aspect of this study is the association with autoimmune disorders. Zhou et al described the increased differentiation of T_H9 and T_H17 in HA20. However, the excess differentiation of T_H17 cells, but not T_H9 cells, was observed in our study (Fig 1, *C*; see Fig E6 in this article's Online Repository at www.jacionline.org). The differentiation of T_H17 cells was marked with aging. Other genetic factors might therefore affect the differentiation of T_H9 cells. In contrast, T_H17 cells might play a crucial role in the pathogenesis of HA20. T_H17 is well known as playing a role in autoimmunity.[7] Intriguingly, not only autoinflammatory phenotypes but also several autoimmune disorders, including systemic lupus erythematosus, psoriatic arthritis, autoimmune hepatitis, nephritic syndrome, and

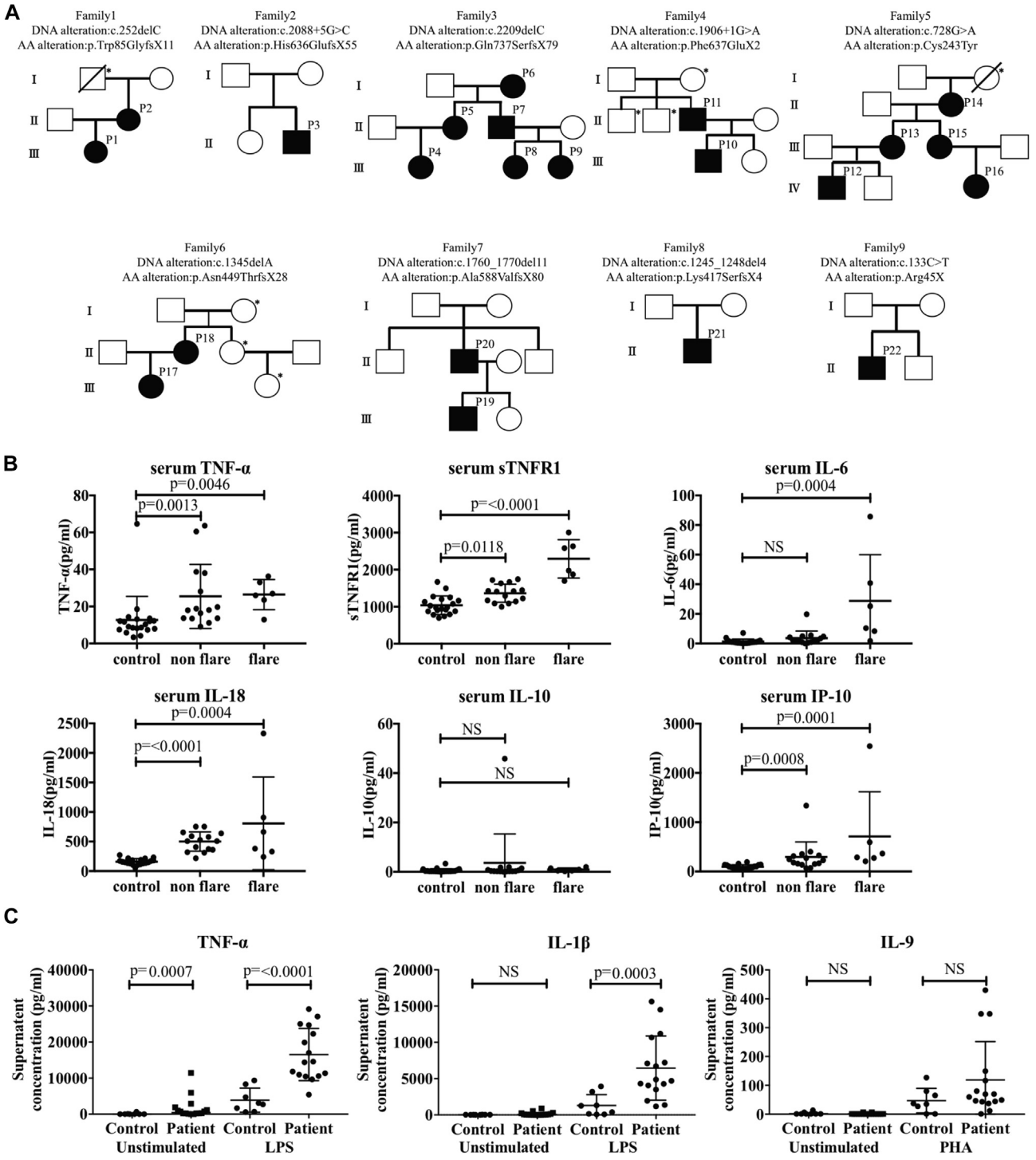

FIG 1. A, Pedigree of the families with heterozygous *TNFAIP3* mutations. An asterisk (*) shows nonanalyzed genetically possible patients. **B,** Cytokine analysis of sera of patients with HA20. The number of controls, patients at the time of nonflare, and patients at the time of flare were 20, 15, and 6, respectively. **C,** Cytokine production levels by PBMCs. The number of controls and patients were 8 and 16, respectively. *NS*, Not significant.

Hashimoto's thyroiditis, were identified in this study (Table I). Severe psoriatic arthritis with the prominent elevation of T_H17 cells was observed in P3. He also had another possible genetic factor, HLA-B27, which is known to cause psoriatic arthritis, ankylosing spondylitis, and reactive arthritis.[8] Symptoms such as psoriasis, spondylitis, and aortic valve insufficiency were specific features of P3. HA20 might drive the other genetic factors associated with autoimmune disorders, such as HLA-B27. These features of human HA20 are compatible with a previous report on aging heterozygous *TNFAIP3* knockout mice.[1,9] Therefore, we consider that autoimmunity is a possible complication of HA20, especially with aging and/or the accumulation of other genetic factors.

TABLE I. Clinical characteristics of patients with HA20

Patient (family) no.	Age of onset	Analysis age	Duration of flare	Frequency of the episode	Criteria of ISGFBD 1990	Initial diagnosis before genetic analysis
P1 (F1)	11 y	17 y	Persistent inflammation with flare attack	Once a month	Fulfilled	RF negative pJIA → intestinal BD
P2 (F1)	9 mo	38 y	3 d	Once a month	Fulfilled	PFAPA-like recurrent fever and stomatitis → intestinal BD
P3 (F2)	1 y	4 y	Persistent inflammation		No	sJIA → PsA
P4 (F3)	6 mo	4 y	1-2 d	Once in 1-3 mo	No	FMF like → TNFRSF1A T61I variant
P5 (F3)	5 y	28 y	1-2 wk	Once in a few years	No	Cervical lymph adenitis and CH → Crohn disease, Hashimoto disease
P6 (F3)	Early childhood	67 y	Unknown	Unknown	No	Recurrent stomatitis, Hashimoto disease
P7 (F3)	Early childhood	34 y	Unknown	Unknown	No	Recurrent stomatitis, Hashimoto disease, HL, Craniopharyngioma
P8 (F3)	1 y	7 y	3-4 d	Unknown	No	Recurrent fever and stomatitis in early childhood
P9 (F3)	2 mo	11 mo	3-10 d	Undetermined	No	Cheilitis, BCG dermatitis, unidentified pneumonia, aseptic meningitis
P10 (F4)	3 y 6 mo	6 y 1 mo	2-5 d	Once in 1-2 mo	No	RF-negative pJIA → PFAPA
P11 (F4)	Early childhood	33 y	Unknown	Once a month	No	IgA vasculitis, recurrent stomatitis
P12 (F5)	17 y	18 y	More than 2 wk	Twice a month	Fulfilled	BD, NS
P13 (F5)	20 y	43 y	More than 2 wk	Once a month	Fulfilled	BD
P14 (F5)	Teens	71 y	Unknown	Once in 2-3 mo	Fulfilled	BD
P15 (F5)	Teens	42 y	1-2 wk	Once in 2-3 mo	Fulfilled	BD
P16 (F5)	12 y	18 y	1-2 wk	Once in 2-3 mo	Fulfilled	BD
P17 (F6)	Infancy	7 y	Unknown	Once a month (stomatitis is sustainable)	No	PFAPA-like recurrent fever and stomatitis, pervasive developmental disorder
P18 (F6)	Early childhood	42 y	Persistent inflammation		Fulfilled	BD
P19 (F7)	Day 5	5 y 2 mo	Persistent inflammation		No	Crohn disease
P20 (F7)	20 y	33 y	1 wk	2-3 times a year	No	PFAPA-like recurrent fever and stomatitis, Graves' disease
P21 (F8)	1 y	4 y	Persistent inflammation with flare attack	3 times a year	No	KD → SLE → AIH → NS → prolonged enteritis → ALPS-U
P22 (F9)	1 y 1 mo	7 y 11 mo	3-5 d	Once a month	Fulfilled	PFAPA → intestinal BD

AIH, Autoimmune hepatitis; *ALPS-U*, autoimmune lymphoproliferative syndrome undefined; *BD*, Behçet disease; *CH*, chronic hepatitis; *FMF*, familial Mediterranean fever; *HL*, Hodgkin lymphoma; *ISGFBD 1990*, International Study Group for Behçet's disease 1990; *sJIA*, systemic juvenile idiopathic arthritis; *KD*, Kawasaki disease; *NS*, nephrotic syndrome; *PFAPA*, periodic fever with aphthous pharyngitis and adenitis; *pJIA*, polyarticular juvenile idiopathic arthritis; *PsA*, psoriatic arthritis; *RF*, rheumatoid factor; *SLE*, systemic lupus erythematosus.

In conclusion, our study demonstrated that HA20 showed unexpected variation in clinical manifestations. Most autosomal-dominant-inheritance disorders typically have a variable clinical phenotype, and HA20 is no exception.

We thank the patients and their families for their participation in this study. Written informed consent was obtained from patients or their parents. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics boards of the Gifu University and Tokyo Medical and Dental University. The clinical pictures of a patient were kindly provided by Dr Goto (Konan Kosei Hospital). The genetic analysis was partially performed by the Initiative on Rare and Undiagnosed Diseases in Pediatrics (IRUD-P) from the Japan Agency for Medical Research and Development.

*Tomonori Kadowaki, MD[a]**
*Hidenori Ohnishi, MD, PhD[a]**
Norio Kawamoto, MD, PhD[a]
Tomohiro Hori, MD, PhD[a]
Kenichi Nishimura, MD[b]
Chie Kobayashi, MD, PhD[c]
Tomonari Shigemura, MD, PhD[d]
Shohei Ogata, MD, PhD[e]
Yuzaburo Inoue, MD, PhD[f]
Tomoki Kawai, MD, PhD[g]
Eitaro Hiejima, MD, PhD[g]
Masatoshi Takagi, MD, PhD[h]
Kohsuke Imai, MD, PhD[h]
Ryuta Nishikomori, MD, PhD[g]
Shuichi Ito, MD, PhD[b]
Toshio Heike, MD, PhD[g]
Osamu Ohara, PhD[i]
Tomohiro Morio, MD, PhD[j]
Toshiyuki Fukao, MD, PhD[a]
Hirokazu Kanegane, MD, PhD[j]

From [a]the Department of Pediatrics, Gifu University Graduate School of Medicine, Gifu, [b]the Department of Pediatrics, Yokohama City University, Yokohama, Kanagawa, [c]the Department of Child Health, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, [d]the Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, [e]the Department of Pediatrics, Kitasato University Hospital, Sagamihara, Kanagawa, [f]the Department of Pediatrics, Eastern Chiba Medical Center, Chiba, [g]the Department of Pediatrics, Kyoto University Hospital, Kyoto, [h]the Department of Community Pediatrics, Perinatal and Maternal Medicine Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, [i]the Department of Technology Development, Kazusa DNA Research Institute, Chiba,

and [i]the Department of Pediatrics and Developmental Biology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan. E-mail:

*These authors contributed equally to this work.

This work was supported by Health and Labour Sciences Research Grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan (grant nos. 17933688 and 17933299).

Disclosure of potential conflict of interest: H. Ohnishi has received Health and Labour Sciences Research Grants. K. Imai has received a grant from the Japanese Ministry of Health, Labour and Welfare and Sony, Inc; has consultant arrangements with CSL Behring KK and Novartis Pharma KK; and has received payment for lectures from CLS Behring and the Japan Blood Products Organization. R. Nishikomori has received payment for lectures from Novartis, Inc. S. Ito has received grants from Astellas Pharma, Inc, Pfizer Japan, Inc, AbbVie GK, Eisai Co Ltd, Sumitomo Dainipponn Pharma Co Ltd, Merck Sharp & Dohme, CSL Behring, and Kyowa Hakko Kirin Co Ltd and has received payment for lectures from Astellas Pharma, Inc, Pfizer Japan, Inc, Sanofi, AbbVie GK, Eisai Co Ltd, Asahi Kasei Parma Corporation, Sumitomo Dainipponn Pharma Co Ltd, Mitsubishi Tanabe Pharma Corporation, and Zenyaku Kogyo Co Ltd. T. Heike has received a grant from the Ministry of Health, Labour, and Welfare. O. Ohara has received a grant from the Jeffrey Modell Foundation. T. Morio has received consultant fees from Novartis, Chugai Pharmaceutical, and Teijin Pharma; has received grants from Asteras, CSL Behring, and Pfizer; and has received payment for lectures from Asteras, AbbVie, CSL Behring, Dainippon Sumitomo Pharmaceutical, JBPO, Tanabe Mitsubishi Pharmaceutical, and Teijin Pharma. T. Fukao has received grants from the Japan Agency for Medical Research and Development, Health and Labour Sciences Research Grants, Sanofi, Pfizer Japan, Inc, Chugai Pharm, Astellas, and Eli Lilly Japan KK. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Ma A, Malynn BA. A20: linking a complex regulator of ubiquitylation to immunity and human disease. Nat Rev Immunol 2012;12:774-85.
2. Aksentijevich I, Zhou Q. NF-kappaB pathway in autoinflammatory diseases: dysregulation of protein modifications by ubiquitin defines a new category of autoinflammatory diseases. Front Immunol 2017;8:399.
3. Duncan CJA, Dinnigan E, Theobald R, Grainger A, Skelton AJ, Hussain R, et al. Early-onset autoimmune disease due to a heterozygous loss-of-function mutation in TNFAIP3 (A20). Ann Rheum Dis June 28, 2017; https://doi.org/10.1136/annrheumdis-2016-210944 [Epub ahead of print].
4. Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. Lancet 1990;335:1078-80.
5. Ideguchi H, Suda A, Takeno M, Ueda A, Ohno S, Ishigatsubo Y. Behcet disease: evolution of clinical manifestations. Medicine (Baltimore) 2011;90:125-32.
6. Jing H, Zhang Q, Zhang Y, Hill BJ, Dove CG, Gelfand EW, et al. Somatic reversion in dedicator of cytokinesis 8 immunodeficiency modulates disease phenotype. J Allergy Clin Immunol 2014;133:1667-75.
7. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol 2014;14:585-600.
8. Ritchlin CT, Colbert RA, Gladman DD. Psoriatic arthritis. N Engl J Med 2017;376:957-70.
9. Tavares RM, Turer EE, Liu CL, Advincula R, Scapini P, Rhee L, et al. The ubiquitin modifying enzyme A20 restricts B cell survival and prevents autoimmunity. Immunity 2010;33:181-91.

Available online December 11, 2017.
https://doi.org/10.1016/j.jaci.2017.10.039

Endolysosomal protease susceptibility of Amb a 1 as a determinant of allergenicity

To the Editor:

Exposure to pollen from short ragweed (*Ambrosia artemisiifolia* [Amb a]) causes severe respiratory allergies worldwide. Amb a 1, a member of the pectate lyase C (pelC) family, is a highly allergenic molecule recognized by more than 90% of ragweed-sensitized individuals and accounts for more than 90% of the allergenic activity in ragweed pollen.[1] However, the factors/features responsible for its high allergenicity (ie, capacity to induce specific IgE antibodies) remain undefined. Efforts to understand allergenicity have lately focused on extrinsic factors normally accompanying the allergen during the initial interaction with the immune system (eg, TLR ligands and lipid mediators).[2] However, data from our previous studies suggested that intrinsic features of Amb a 1 might be linked to its high allergenic potential.[3] Thus, we sought to investigate intrinsic molecular pattern(s) possibly involved in the allergenicity of Amb a 1. As a comparison, we used the nonallergenic pelC homologue from the phytopathogenic *Dickeya chrysanthemi* (previously *Dickeya dadantii* and *Erwinia chrysanthemi*),[4] here termed DC-pelC.

Amb a 1.01, the most allergenic isoform, was purified from ragweed pollen extracts, whereas the bacterial-derived pelC (DC-pelC) was produced as a soluble recombinant protein in *Escherichia coli* (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). After purification, their identity and purity were confirmed by mass spectrometry (Fig E1, *B* and *C*). Despite their relatively low sequence identity (24%), circular dichroism and Fourier-transform infrared experiments indicated the presence of the parallel beta-helix structural element typical for polysaccharide lyases from family 1, with a high beta-sheet content (35.6% to 40.5%) for both molecules (see Fig E2 in this article's Online Repository at www.jacionline.org). These results support the notion that the 2 proteins share some degree of structural similarity (Fig E2, *D*). However, because of low sequence identity, cross-reactivity is not expected.

Because *Dickeya* species are known to cause soft root disease on many crops (eg, bell pepper, tomatoes, potatoes, and onions)[4] and ornamental indoor plants, contact with this phytopathogen has been recently demonstrated by metagenomic sequencing of the microbiota of human buccal mucosa.[5,6] These studies unambiguously identified *Dickeya dadantii* as an oral commensal bacterium. Thus, we performed ELISAs to evaluate immune responses to DC-pelC. None of the 39 ragweed-allergic patients displayed IgE or IgG_4 antibodies recognizing DC-pelC. However, 37.5% of the individuals showed IgG antibodies against DC-pelC, confirming exposure but no sensitization to DC-pelC (see Fig E3 in this article's Online Repository at www.jacionline.org).

To further investigate the sensitization capacity of Amb a 1.01 and DC-pelC, we immunized mice in the presence and absence of aluminum hydroxide (ALUM) as adjuvant (Fig 1, *A*). In both immunization protocols, Amb a 1.01 behaved as a very potent sensitizer, inducing high levels of IgG and IgE antibodies (Fig 1, *B*). These results clearly demonstrate that the intrinsic allergenic properties of Amb a 1.01 were not significantly altered by adsorption to ALUM as adjuvant. In contrast, in the adjuvant-free model, the nonallergenic pectate lyase DC-pelC induced a moderate immune response with low levels of IgG_1 and IgG_{2a} antibodies and failed to initiate B-cell class switch for IgE production. When ALUM was coadministered with DC-pelC, a significant increase in the titers of specific IgG_1 and IgG_{2a} antibodies was observed. In addition, DC-pelC–specific IgE antibody titers increased slightly, showing mediator release capacity in only 2 out of 5 mice (Fig 1, *C* and *E*). The amount of DC-pelC needed to induce half-maximal release in basophils sensitized with sera from ALUM-immunized animals and those without ALUM did not differ significantly (Fig 1, *D*). Our experiments also showed

METHODS

Patients

Family 1. P1 and P2 in Family 1 were genetically confirmed to have HA20.[E1] A deceased maternal grandfather of P1 with Behçet disease might have had HA20. P2 was found to have antithyroid peroxidase antibody at the age of 39 years, even though the thyroid functions were normal.

Family 2. The proband of Family 2 was a 4-year-old boy (P3). He had recurrent febrile episodes following pneumococcal conjugate vaccine (PCV) at the age of 2, 3, and 7 months. He developed swelling of the left middle finger and the left fifth toe from age 6 months. The X-ray disclosed bone thickening at the proximal phalanx of the left middle finger (red arrow) and the left fifth toe (Fig E7, *A*). He was diagnosed as having systemic juvenile idiopathic arthritis and treated with prednisolone (PSL) and methotrexate at the age of 1 year. Enhanced magnetic resonance imaging showed polyarthritis including enthesitis of his left Achilles tendon and thickening of his right knee joint capsule (Fig E7, *B*). In addition, aortic valve insufficiency was found at the age of 1 year and 6 months, and tocilizumab was initiated. Psoriatic skin rash and nail deformation appeared at the age of 2 years and 5 months. A skin biopsy revealed an epidermis with scabbing and neutrophil infiltration, and swelling of dermal papillae with lymphocyte infiltration (Fig E7, *C*). He was diagnosed with psoriatic arthritis, and was treated with adalimumab (ADA) instead of tocilizumab at the age of 2 years and 8 months. ADA was switched to etanercept (ETA) because of the ineffectiveness of ADA caused by anti-ADA antibody. Afterwards, his symptoms were nearly stable. During tapering PSL, he had arthritis of cervical vertebra at the age of 4 years. For that reason, we changed ETA to infliximab. Finally, he was identified as having a heterozygous c.2088+5G>C mutation in *TNFAIP3* (Fig E2). Interestingly, his mother was asymptomatic; however, genetic analysis revealed low-frequency somatic mosaicism of *TNFAIP3* in her peripheral blood (Fig E5, *A*). The frequency of the mutant allele from her peripheral blood was estimated as 10.06% by next-generation sequencing.

Family 3. P4, a 4.5-year-old girl, is the proband of Family 3. P4 and P9 showed relatively severe inflammatory phenotypes, although P5, P6, and P7 had Hashimoto's thyroiditis. P4 had recurrent flare with severe abdominal pain, vomiting, and bloody stool since age 6 months. Abdominal lymphedema and mild intestinal edema were observed on her abdominal contrast-enhanced computed tomography during flare (Fig E7, *D*). First, she was considered as a possible case of familial Mediterranean fever according to the Tel-Hashomer criteria,[E2] but the genetic analysis of the *MEFV* gene was normal. Colchicine was not effective. Then, she was tentatively diagnosed as having TNF receptor–associated periodic syndrome variant due to the heterozygous Thr61Ile variant in the *TNFRSF1A* gene.[E3] At the age of 3 years and 1 month, ETA was started and the flaring events have subsided. Although she was not under suspicion of Behçet disease, the heterozygous c.2209delC mutation in *TNFAIP3* was identified. P9 is an 11-month-old girl, a maternal cousin of P4. She had a febrile episode following PCV vaccinations at the ages of 2 and 4 months. At the age of 9 months, she had a febrile episode with interstitial pneumonia and cheilitis, and CSF analysis showed an increase in mononuclear leukocytes (76/μL), protein (172 mg/dL), and IL-6 (6790 pg/mL). This febrile episode lasted for 10 days. She had a severe crustose lesion on the inoculation site of BCG vaccine after this febrile episode (Fig E7, *E*).

Family 4. P10, a 6-year-and-1-month-old boy, is the proband of Family 4. This family included 2 patients with HA20 (P10 and P11) and 3 possible cases who had Behçet disease–like phenotypes. P10 had arthritis of the right knee joint, both ankle joints, and right toes at the age of 3 years and 6 months. He was initially diagnosed as having polyarticular juvenile idiopathic arthritis, and a combination of PSL and methotrexate therapy was administrated. Although symptoms of arthritis improved, continuous positive serum C-reactive protein levels were noted. After withdrawal of the PSL therapy at the age of 4 years and 2 months, he had periodic fever with lymphadenopathy, exudative tonsillitis, and aphthous stomatitis every 2 to 2 months. At this time, he was diagnosed as having periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome.[E4] Then, the frequency of his febrile attacks was decreased using cimetidine, but his serum C-reactive protein level was continuously positive even in nonflare periods. He had a heterozygous c.1906+1G>A mutation in *TNFAIP3* (Fig E2). P11 is a 33-year-old man, and the father of P10. He had recurrent aphthous stomatitis since his childhood. He was previously diagnosed as having IgA vasculitis at the age of 13 to 14 years. To date, he developed occasional genital and anal ulceration. He also had abdominal pain and diarrhea once a month. The mother, older brother, and younger brother of P11 also showed recurrent aphthous stomatitis, suggesting HA20.

Family 5. P12, P13, P14, P15, and P16 were genetically confirmed to have HA20, and 1 patient in Family 5 with Behçet disease might have had HA20.[E5] Rituximab was used for refractory nephrotic syndrome in P12.

Family 6. Family 6 included 2 patients with HA20 (P17 and P18) and 3 possible cases with Behçet disease–like phenotypes. P17, who was a 7-year-old girl, is the proband of this family. She had a history of RBV-induced infectious mononucleosis with an extreme elevation of peripheral lymphocytes at the age of 1 year and was treated with corticosteroid. She had periodic febrile episodes every month and aphthous stomatitis every day from her infancy. The frequency of her febrile episodes gradually decreased, but stomatitis continued every day afterwards. She felt general fatigue and experienced a fever attack on the day after hard exercise. In addition, she was diagnosed as having pervasive developmental disorder at the age of 7 years. She had a heterozygous c.1345delA mutation in *TNFAIP3*. P18 is a 42-year-old woman, and the mother of P17. She had recurrent aphthous stomatitis since her early childhood and had recurrent fever, abdominal pain, and diarrhea since her teens. She was diagnosed as having Behçet disease at the age of 21 years, because of genital ulcers. Her symptoms were stable for the next 10 years, but relapsed after pregnancy. She developed colon perforation and pneumocystis pneumonia at the age of 39 years. The mother, younger sister, and niece of P18 also showed recurrent aphthous stomatitis since their childhood. Recently, the mother of P18 was diagnosed as having malignant lymphoma in her seventies. The niece of P18 had periodic fever episodes since her early childhood. She was diagnosed as having Crohn disease at the age of 8 years, and treatment with ADA was started. At the age of 14 years, she was diagnosed with Behçet disease due to complications of genital ulcers. Although a genetic test was not performed, she might also have had HA20.

Family 7. Family 7 includes 2 patients with HA20 (P19 and P20). P19, a 5-year-and-2-month-old boy, is the proband of this family. He had a fever and oral ulcer at the age of 5 days. He was initially diagnosed as having sepsis and herpetic stomatitis and treated with an antibiotic and acyclovir. He also had periproctitis during this treatment. He had recurrent aphthous stomatitis once a week since the age of 1 month. He had prolonged fever with periproctitis for 10 days at the age of 5 months. He was frequently hospitalized because of recurrent fever, aphthous stomatitis, and periproctitis thereafter. At the age of 1 year, colonoscopy revealed the ulceration of rectum and colon. Therefore, he was diagnosed as having nonspecific inflammatory bowel disease and treated with mesalazine. He had a heterozygous c.1760_1770del11 mutation in *TNFAIP3*. Colchicine was insufficient. Infliximab treatment was started recently. He had fever episodes after inoculations of PCV at the age of 2, 3, and 5 months. P20 is a 33-year-old man, the father of P19. He had recurrent aphthous stomatitis. He had febrile episodes lasting for 1 week once or twice a year from the age of 20 years. At the age of 20 years, he was also diagnosed as having Graves' disease. Serum autoantibodies associated with the thyroid were detected at the age of 33 years, not only anti–thyroid-stimulating hormone receptor autoantibodies but also anti–thyroid peroxidase antibody and antithyroglobulin antibody.

Family 8. Family 8 included a genetically confirmed patient with HA20 (P21).[E6] At the age of 4 years, because of the prolonged inflammatory bowel disease, ADA treatment was started, and then his symptoms were dramatically improved. Other Behçet disease–like symptoms were not found. It should be noted that his father has not been showing any inflammatory signs, but the genetic analysis showed the low-frequency somatic mosaicism of *TNFAIP3* in his peripheral blood (Fig E5, *B*). The frequency of the mutant allele of his peripheral blood was estimated as 16.7% (5 of 30) on the basis of subcloning and sequencing analysis.

Family 9. The proband (P22) of Family 9 was a 7-year-and-11-month-old boy. He had recurrent flare lasting for 1 week, once for 2 or 3 months, from the

age of 1 year. He was initially diagnosed as having periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome at the age of 2 years. Then, the frequency of flare episodes gradually decreased, but since the age of 5 years the frequency of fever episodes increased. PSL treatment was not effective. He had prolonged fever, aphthous stomatitis, and perianal ulcer at the age of 6 years. Positron emission tomography-computed tomography examination revealed abnormal uptake at the ileocecal region. A colonoscopy showed ileocecal ulceration, and the pathological findings were nonspecific inflammatory lesion (Fig E7, *F*). Colchicine was started, and then the duration of fever was shortened, but the frequency of fever, stomatitis, and perianal ulcer remained once a month. He had a heterozygous c.133G>T mutation in *TNFAIP3*.

Analysis of cytokine levels

Regarding the cytokine profiles, blood samples were obtained from each patient and healthy volunteers. PBMCs were isolated from heparinized blood by gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). PBMCs were seeded at a density of 10^6 cells/mL, and cultured in the presence or absence of 100 ng/mL LPS from *Escherichia coli* 0127:B8 (Sigma-Aldrich, St Louis, Mo) or 10 μg/mL PHA (Gibco, Life Technologies Corp., Grand Island, NY) for 24 hours in 24-well plates at 37°C. Human serum and the culture supernatants, centrifuged in test tubes to remove cells, were stored at −80°C until assayed. The concentrations of TNF-α, sTNFR1, IL-1β, IL-6, IL-9, IL-10, IL-17A, IL-18, and IFN-γ IP-10 were measured using ELISA kits (Invitrogen: TNF-α, IL-1β, IL-6, IL-10; R&D, Minneapolis, Minn: sTNFR1, IP-10; BioLegend, San Diego, Calif: IL-9; MBL, Nagoya, Aichi, Japan: IL-18; and eBioscience, San Diego, Calif: IL-17A). The statistics of serum cytokines and cytokine production levels by PBMCs were analyzed by a Kruskal-Wallis test with Dunn's multiple comparisons test or the Mann-Whitney test using PRISM version 6.0 (GraphPad software, San Diego, Calif), respectively. Values are represented as mean ± S.D. *P* values of less than .05 were considered statistically significant. Nonsignificant *P* values were indicated as NS.

Immunoblot analysis

To detect protein expression, HEK293T cells were transfected with a pcDNA3.1+ control vector or pcDNA3.1+ myc-A20 wild-type (WT) or variants using Lipofectamine 2000 according to the manufacturer's instructions. After a 48-hour incubation, cells were harvested and lysed. 1×10^6 PHA blasts were also prepared. All extracts were adjusted to contain equal amounts of total cellular proteins. The supernatants of cell lysates were analyzed using the western blot. A20 and β-actin proteins were detected with an anti-myc antibody (Invitrogen), anti-A20 antibody (#5630) (Cell Signaling Technology, Danvers, Mass), and anti–β-actin antibody (Sigma-Aldrich, St Louis, Mo) followed by incubation with an antimouse or antirabbit IgG–horseradish peroxidase conjugate (Promega, Fitchburg, Wis). One representative result of 3 independent experiments is shown.

Nuclear factor-κB reporter gene activity

HEK293T cells were transfected with 10 ng per well of pcDNA3.1+ control vector or pcDNA3.1+ myc-A20 WT or variants in 96-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The nuclear factor-κB luciferase reporter and Renilla luciferase reporter vectors were cotransfected. After transfection, cells were incubated for 24 hours, and then stimulated with 20 ng/mL TNF-α (R&D) for 6 hours. Luciferase reporter activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). The activity values of WT and each variant were normalized to that of mock stimulated with 20 ng/mL TNF-α. Values are expressed as mean of 3 independent experiments with technical triplicates ± SD. The statistical significance of differences compared with WT stimulated with TNF-α in luciferase activity was analyzed using 1-way ANOVA with uncorrected Fisher Least Significant Difference multiple comparisons test. *P* values of less than .05 were considered statistically significant. *P* values of less than .05, less than .01, less than .001, and less than .0001 are indicated with *, **, ***, and ****, respectively.

Intracellular cytokine staining

Intracellular cytokine staining was performed to detect T_H1, T_H2, T_H9, and T_H17 cells as previously described.[E7] Flow cytometry plots of IL-17A expression in patients with HA20 (n = 16) were compared with control subjects (n = 40). The cases are grouped into 3 categories: younger than 2 years, 2 years old to 15 years old, and older than 15 years. The statistical significance of differences between the patient and control subjects was analyzed using the Mann-Whitney test. The bars are represented as mean ± SD. *P* values of less than .05 were considered statistically significant.

REFERENCES

E1. Ohnishi H, Kawamoto N, Seishima M, Ohara O, Fukao T. A Japanese family case with juvenile onset Behçet's disease caused by TNFAIP3 mutation. Allergol Int 2017;66:146-8.
E2. Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, Lidar T, et al. Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum 1997;40:1879-85.
E3. Ueda N, Ida H, Washio M, Miyahara H, Tokunaga S, Tanaka F, et al. Clinical and genetic features of patients With TNFRSF1A variants in Japan: findings of a nationwide survey. Arthritis Rheumatol 2016;68:2760-71.
E4. Thomas KT, Feder HM Jr, Lawton AR, Edwards KM. Periodic fever syndrome in children. J Pediatr 1999;135:15-21.
E5. Shigemura T, Kaneko N, Kobayashi N, Kobayashi K, Takeuchi Y, Nakano N, et al. Novel heterozygous C243Y A20/TNFAIP3 gene mutation is responsible for chronic inflammation in autosomal-dominant Behçet's disease. RMD Open 2016;2:e000223.
E6. Takagi M, Ogata S, Ueno H, Yoshida K, Yeh T, Hoshino A, et al. Haploinsufficiency of TNFAIP3 (A20) by germline mutation is involved in autoimmune lymphoproliferative syndrome. J Allergy Clin Immunol 2017;139:1914-22.
E7. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. Nat Genet 2016;48:67-73.

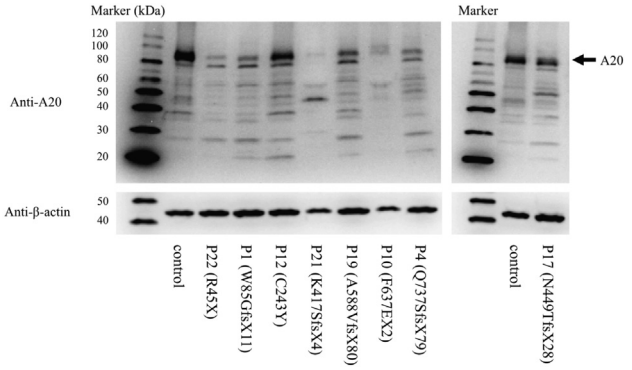

FIG E1. Western blot analysis of the PHA blasts of patients with HA20. The protein expression levels of A20 were reduced except for C243Y.

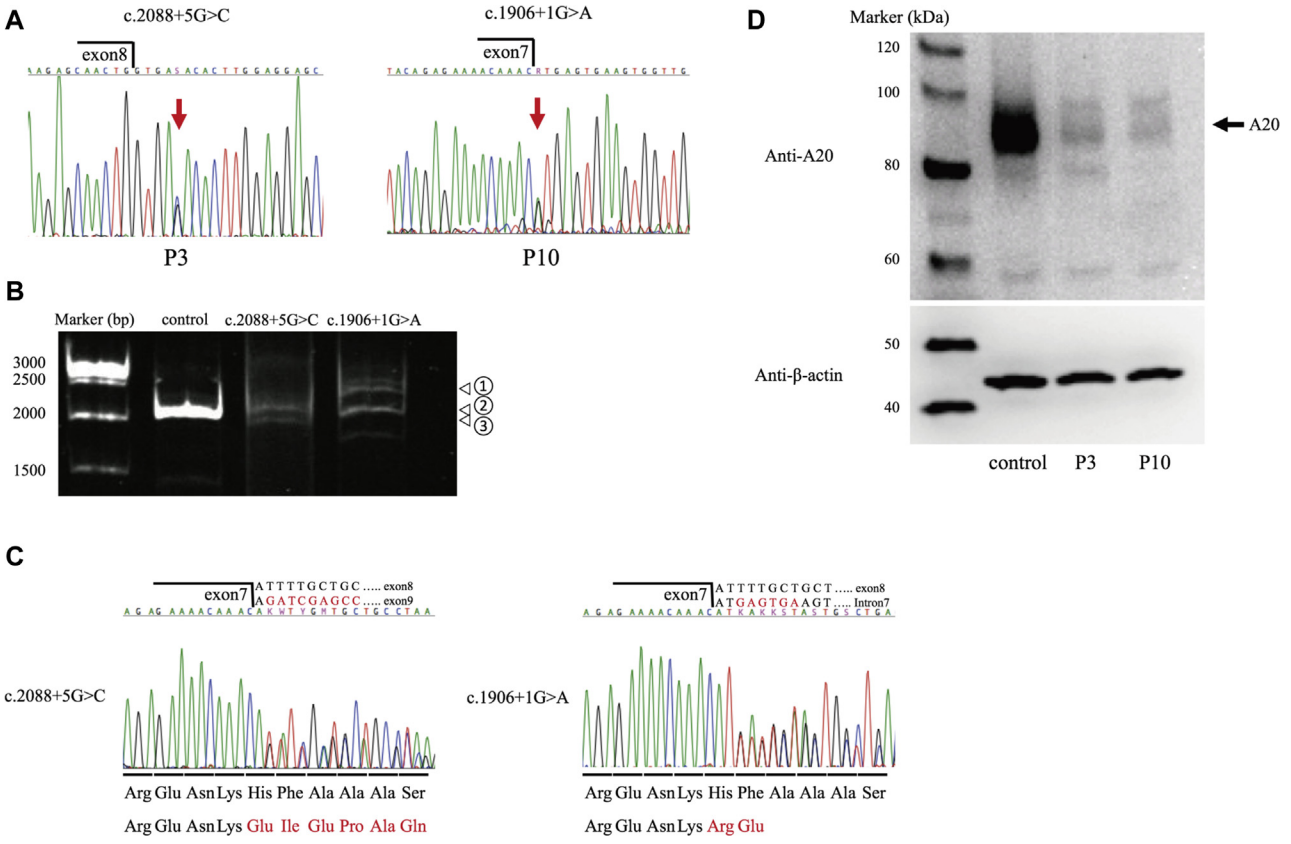

FIG E2. Analyses of the splice site mutations of *TNFAIP3*. **A,** Genomic DNA analysis of P3 and P10. **B** and **C,** Complementary DNA analysis of the splice site mutations. The regions of *TNFAIP3* in which the mutations c.1906+1G>A and c.2088+5G>C are located were amplified by PCR using the forward primer 5′-GAAGTG GACTTCAGTACAAC-3′ and the reverse primer 5′-GGTTACCAAACCTGAGCATC-3′, and then, Sanger DNA sequencing was performed. c.2088+5G>C showed an additional lower band of the predicted size (1998bp), and c.1906+1G>A also showed 1 more upper band. The direct sequencing of these PCR products showed that P3 had a heterozygous exon 8 skipping mutation and P10 had a heterozygous intron 7 insertion mutation. **D,** The western blot analysis of the PHA blasts of P3 and P10 using anti-A20 antibody. The protein expression levels of P3 and P10 were reduced.

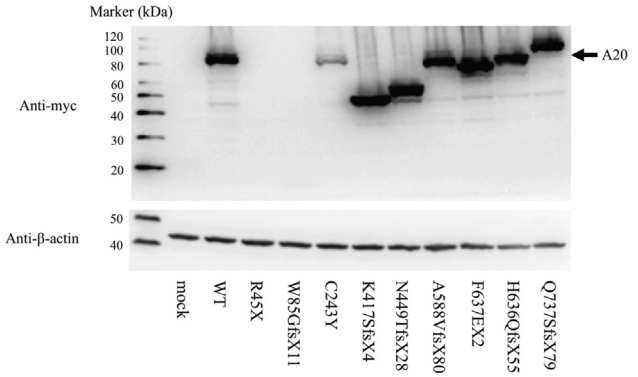

FIG E3. Western blot analysis of the HEK293T cells transfected with pcDNA3.1+ myc-A20 (WT or variants). The control subject showed the protein expression of myc-A20 corresponding to the predicted molecular weight size of 89.5 kDa. The protein expressions of R45X (5.2 kDa) and W85GfsX11 (11.2 kDa) could not be detected because of the detection limit of the gel. The expression level of C243Y was reduced. The other variants were detected as corresponding to the predicted molecular weight.

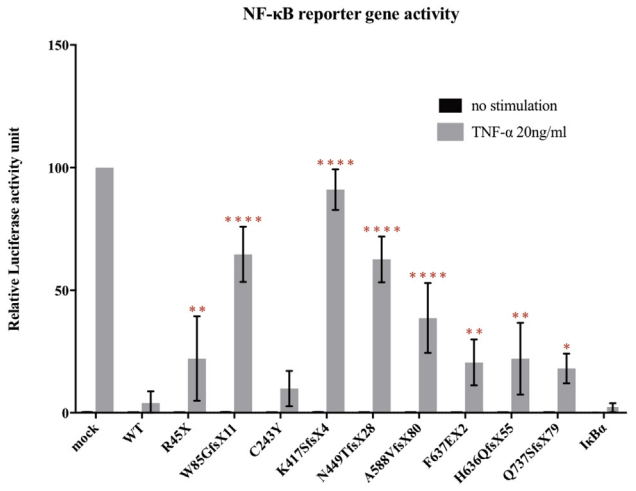

FIG E4. NF-κB reporter gene activity assay. The suppression of TNF-α–induced NF-κB activity by the variants of TNFAIP3 was significantly lower than that of WT, except for C243Y. *NF- κB*, Nuclear factor-κB.

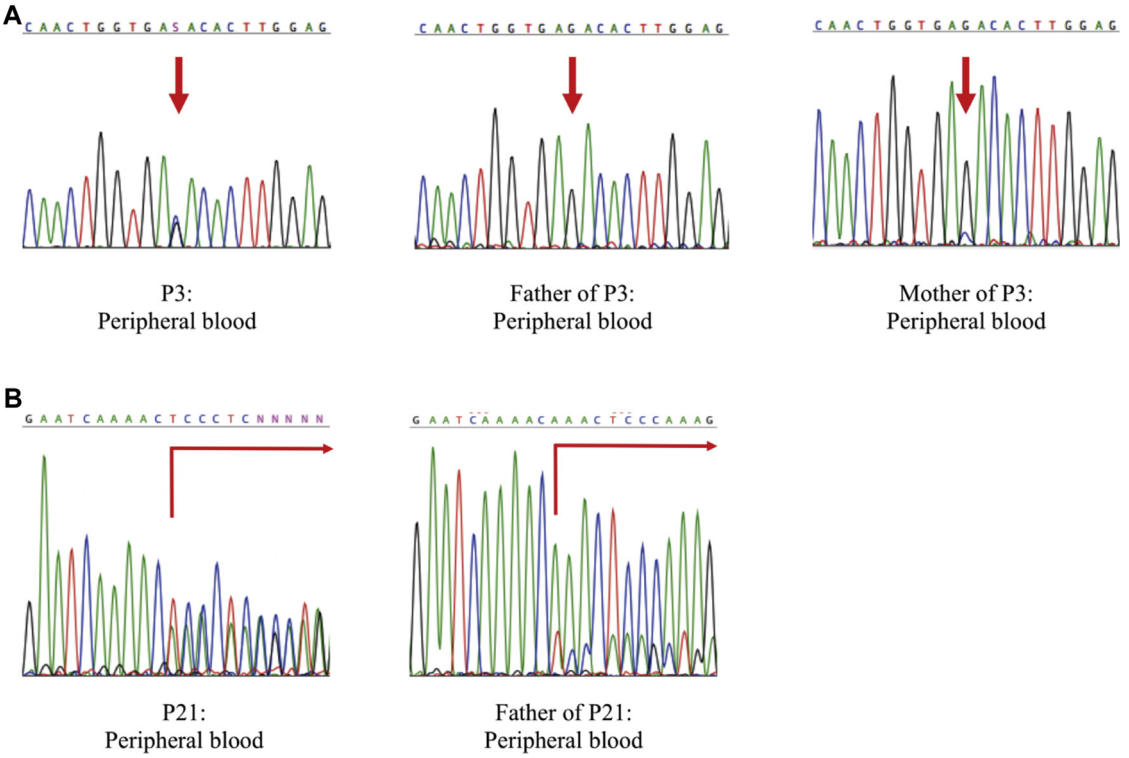

FIG E5. Sanger sequencing of genomic DNA of Families 2 and 9. **A,** P3 had a heterozygous c.2088+5G>C mutation. In addition, the mother of P3 had a low-frequency mutation of *TNFAIP3* in her DNA derived from peripheral blood. **B,** P21 had a heterozygous c.1245_1248del4 mutation. In addition, the father of P21 had a low-frequency mutation of *TNFAIP3* in his DNA derived from peripheral blood.

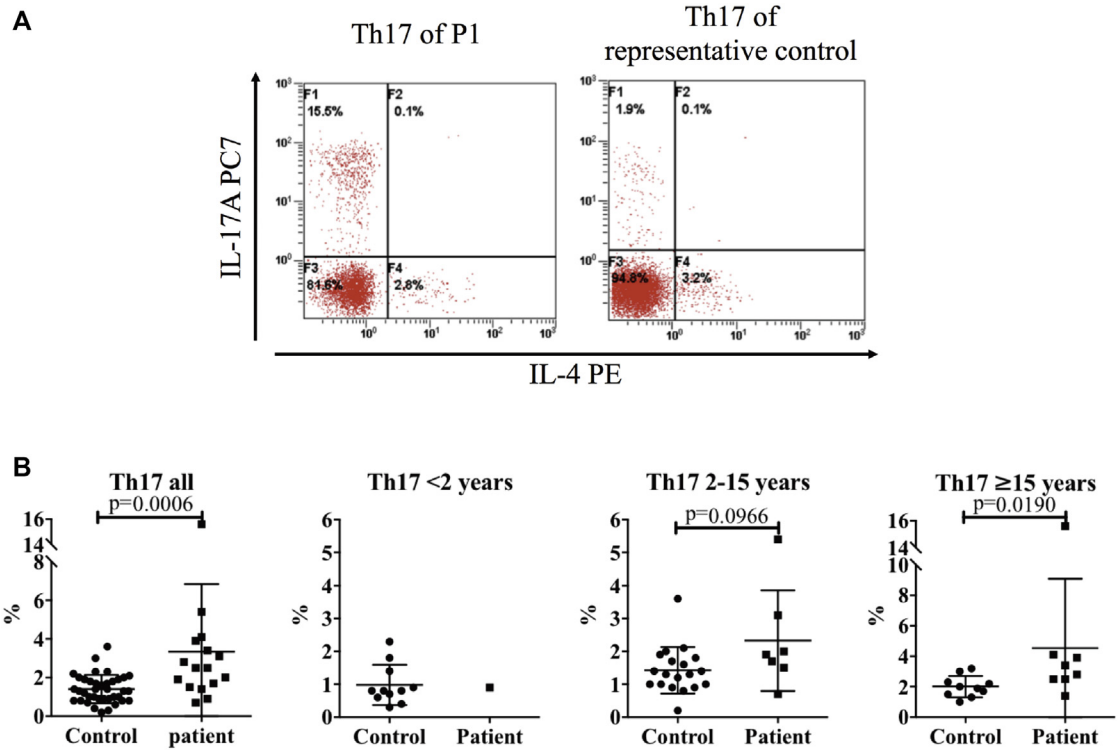

FIG E6. Flow cytometric analysis of T_H17 cells. Upper panels **(A)** are representative flow cytometry plots of IL-4 and IL-17A expression by intracellular cytokine staining. Lower panels **(B)** are flow cytometry plots of IL-17A expression in patients with HA20 (n = 16) as compared with control subjects (n = 40). The cases are grouped into 3 categories: younger than 2 years, 2 years old to 15 years old, and older than 15 years. The proportion of T_H17 cells in patients with HA20 (3.3% ± 3.5%) was significantly higher than that of control subjects (1.4% ± 0.7%; $P = .0006$). T_H9 cells were hardly detected in this study, in any condition.

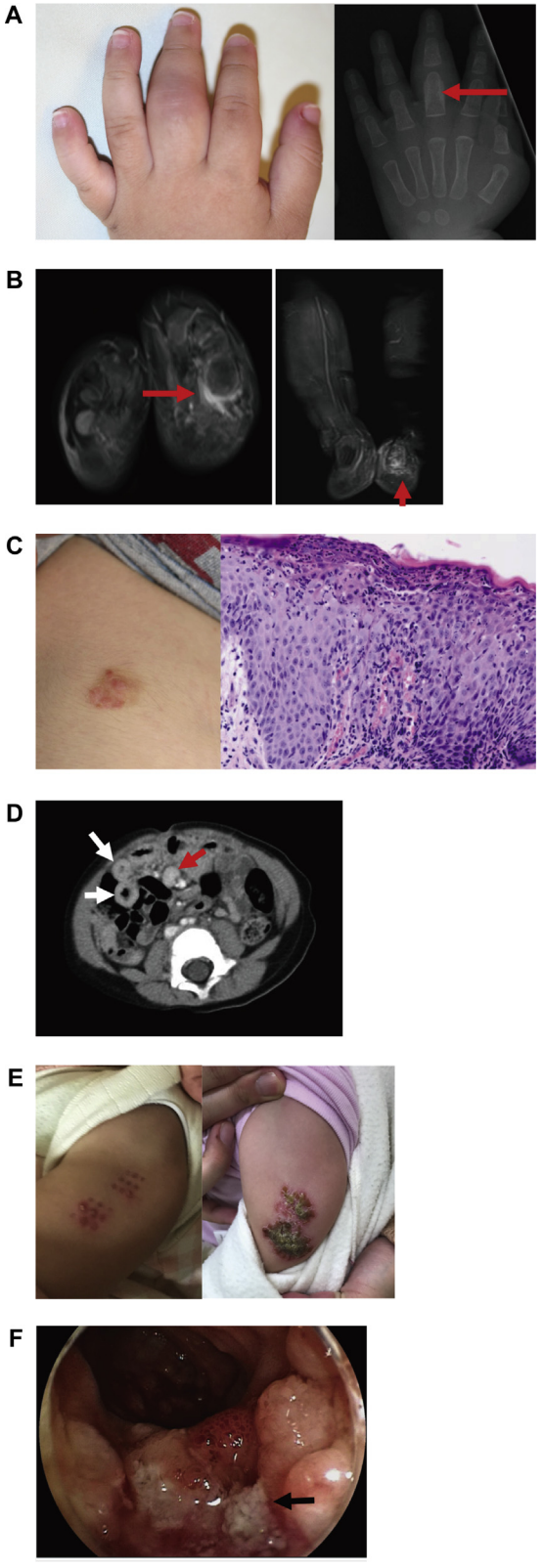

FIG E7. Clinical manifestations of P3, P4, P9, and P22. **A,** P3 had swelling of the left middle finger from age 6 months. The fingers' X-ray findings showed bone thickening at the proximal phalanx of the left middle finger *(red arrow)*. **B,** Gadolinium-enhanced T1 imaging of the lower limbs of P3 showed polyarthritis including the enthesitis of his left Achilles tendon *(red arrows)*. The left and right of the figure show an axial section and a coronal section, respectively. **C,** Psoriatic skin rash of P3 at the age of 2 years and 5 months. The skin biopsy of his psoriatic skin lesion. The epidermis with scabbing and neutrophil infiltration, and swelling of dermal papillae with lymphocyte infiltration were observed. **D,** Abdominal contrast-enhanced computed tomography during flare of P4. Abdominal lymphedema *(red arrow)* and mild intestinal edema *(white arrows)* were observed. **E,** Excess impetigo crustose on the inoculation site of BCG vaccine of P9 after a febrile episode at the age of 9 months. **F,** Colonoscopy findings of P22. Ulceration at the ileum side *(black arrow)* of Bauhin's valve was observed.

TABLE E1. Treatment and other findings in patients with HA20

Patient no.	Colchicine efficacy	Treatment	Vaccination with PCV (PCV7 or PCV13)	HLA	Autoantibody	Upper and colon endoscopy findings
P1	Not effective	Tocilizumab → Adalimumab + PSL + MTX + NSAID + Igratimod	No	B44/B52	No	Small but multiple ulceration of rectal and colon under treatment of tocilizumab
P2	Not effective	PSL 5 mg	No	Not analyzed	Low titer anti-TPO antibody	Duodenal ulcer
P3	Probably effective	Tocilizumab + MTX + PSL + NSAID → Adalimumab + CyA + PSL + NSAID → Etanercept + MTX + PSL + NSAID + Colchicine → Infliximab + MTX + PSL + NSAID + Colchicine	Yes → fever onset with elevated CRP	B27/B51	No	Not analyzed
P4	Not effective	Etanercept (twice a month)	Yes → fever attack occurred after second vaccination	Not analyzed	No	Not analyzed
P5	Unused	Levothyroxine	No	Not analyzed	Anti-TPO antibody	Ulceration of colon and ileocecal region
P6	Unused	Levothyroxine	No	Not analyzed	Anti-Tg antibody	Not analyzed
P7	Unused	Levothyroxine	No	Not analyzed	Anti-Tg antibody, Anti-TPO antibody	Not analyzed
P8	Unused	No treatment	No	Not analyzed	Anti-Tg antibody	Not analyzed
P9	Not determined	LTRA → Colchicine	Yes → fever onset with elevated CRP	Not analyzed	No	Not analyzed
P10	Unused	MTX + PSL → Cimetidine	Yes → No fever	A24/33, B7/44	No	Not analyzed
P11	Unused	No treatment	No	Not analyzed	Not analyzed	Not analyzed
P12	Not effective	Colchicine → MZB + CyA → add PSL → add Rituximab	No	B51 negative	No	Not analyzed
P13	Not effective	Colchicine → PSL	No	Not analyzed	Not analyzed	Findings of Behçet-like disease
P14	Not effective	Colchicine → PSL	No	Not analyzed	Not analyzed	Not analyzed
P15	Not effective	Colchicine → PSL	No	Not analyzed	Not analyzed	Not analyzed
P16	Unused	No treatment	No	Not analyzed	Not analyzed	Not analyzed
P17	Unused	Acetaminophen during fever	No	Not analyzed	No	Mild inflammatory cells infiltration and fibrosis at duodenum
P18	Not effective	Insufficient response for colchicine, adalimumab or infliximab	No	Not analyzed	Not analyzed	Ileocecum perforation
P19	Partial effective	Mesalazine → Colchicine → Infliximab	Yes → fever onset	Not analyzed	Not analyzed	Ulceration of rectal and colon
P20	Unused	No anti-inflammatory treatment	No	Not analyzed	Anti-Tg antibody, Anti-TPO antibody, TSH receptor antibody	Not analyzed
P21	Unused	mPSL pulse → PSL + TAC → PSL + CyA + MMF → PSL + CyA → Adalimumab	Yes → No fever	B51 negative	Anti-DNA antibody, Anti-GBM antibody	Erosion and inflammation, Chronic active colitis with shallow ulcer
P22	Partially effective	Colchicine and PSL (when symptom appeared)	No	B39/44	No	Ulceration of ileocecal region

Anti-GBM antibody, Antiglomerular basement membrane antibody; *anti-Tg antibody*, antithyroglobulin antibody; *anti-TPO antibody*, anti–thyroid peroxidase antibody; *CRP*, C-reactive protein; *CyA*, cyclosporine A; *LTRA*, leukotriene receptor antagonist; *MMF*, mycophenolate mofetil; *MTX*, methotrexate; *MZB*, mizoribine; *NSAID*, nonsteroidal anti-inflammatory drug; *TSH receptor antibody*, thyroid-stimulating hormone receptor antibody.

TABLE E2. Clinical manifestations of Japanese patients with HA20

Symptom	Proportion of symptomatic patients (no. of symptomatic patients)
Recurrent fever	86% (19 of 22)
Recurrent stomatitis	77% (17 of 22)
Genital ulcers	55% (12 of 22)
Abdominal symptoms	55% (12 of 22)
Skin rash	36% (8 of 22)
Arthralgia	14% (3 of 22)
Pathergy	9% (2 of 22)
Central nervous system symptoms	5% (1 of 22)
Ocular symptoms	0% (0 of 22)