# Detailed analysis of Japanese patients with adenosine deaminase 2 deficiency reveals characteristic elevation of type II interferon signature and STAT1 hyperactivation

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**Background:** Deficiency of adenosine deaminase 2 (DADA2) is an autosomal recessive inflammatory disease caused by loss-of-function mutations in both alleles of the *ADA2* gene. Most patients with DADA2 exhibit systemic vasculopathy consistent with polyarteritis nodosa, but large phenotypic variability has been reported, and the pathogenesis of DADA2 remains unclear.

**Objectives:** This study sought to assess the clinical and genetic characteristics of Japanese patients with DADA2 and to gain insight into the pathogenesis of DADA2 by multi-omics analysis.

**Methods:** Clinical and genetic data were collected from 8 Japanese patients with DADA2 diagnosed between 2016 and 2019. *ADA2* variants in this cohort were functionally analyzed by *in vitro* overexpression analysis. PBMCs from 4 patients with DADA2 were subjected to transcriptome and proteome analyses. Patient samples were collected before and after introduction of anti-TNF-α therapies. Transcriptome data were compared with those of normal controls and patients with other autoinflammatory diseases.

**Results:** Five novel *ADA2* variants were identified in these 8 patients and were confirmed pathogenic by *in vitro* analysis. Anti-TNF-α therapy controlled inflammation in all 8 patients. Transcriptome and proteome analyses showed that upregulation of type II interferon signaling was characteristic of DADA2. Network analysis identified *STAT1* as a key regulator and a hub molecule in DADA2 pathogenesis, a finding supported by the hyperactivation of STAT1 in patients' monocytes and B cells after IFN-γ stimulation.

**Conclusions:** Type II interferon signaling and STAT1 are associated with the pathogenesis of DADA2. (J Allergy Clin Immunol 2021;148:550-62.)

*Key words:* ADA2 deficiency, DADA2, adenosine deaminase 2, cat eye syndrome critical region protein 1, anti-TNF-α, vasculitis, omics, proteome, transcriptome, STAT1, IFN-γ

Deficiency of adenosine deaminase type 2 (DADA2) is an autosomal recessive inflammatory disorder resulting from biallelic loss-of-function mutations in the *ADA2* gene, located at chromosome 22q11.1. The *ADA2* gene encodes ADA2, an enzyme that catalyzes the deamination of adenosine/deoxyadenosine to inosine/deoxyinosine. Low or absent ADA2 activity in plasma is diagnostic for DADA2, as are biallelic pathogenic mutations in *ADA2*.[1,2]

Phenotypically, the characteristics of DADA2 vary widely.[3-5] Most patients with DADA2 develop systemic vasculopathy and

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*Abbreviations used*

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|---|---|
| AGS: | Aicardi-Goutières syndrome |
| CANDLE: | Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature |
| DADA2: | Deficiency of ADA2 |
| DEG: | Differentially expressed gene |
| DEP: | Differentially expressed protein |
| FC: | Fold-change |
| GO: | Gene Ontology |
| IPA: | Ingenuity Pathway Analysis |
| ISG: | Interferon-stimulated gene |
| KO: | Knockout |
| PRCA: | Pure red cell aplasia |
| WT: | Wild type |

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inflammation, characterized by early-onset cerebral infarction, most frequently lacunar infarctions affecting the brain stem and basal ganglia; cutaneous lesions; and chronic/recurrent fever, which are clinically similar to polyarteritis nodosa.[1,2] Other phenotypic characteristics include bone marrow failure, such as pure red cell aplasia (PRCA), common variable immunodeficiency, and autoimmune lymphoproliferative diseases.[4] Although anti-TNF-α therapy has been shown to be effective in treating systemic inflammation,[4] its efficacy in treating other clinical features of DADA2 remains unclear. Hematopoietic stem cell transplantation has been shown to be curative for DADA2,[6] but its potential risks make it suitable only for those not responding to conventional therapies.

The pathophysiology of DADA2 remains unclear. Defect of ADA2 has been reported to be involved in the differentiation of monocytes toward proinflammatory M1 macrophages, which may explain the endothelial cell damage observed in these patients.[1] Recently, Carmona-Rivera et al[7] showed that defect of ADA2 might induce increase of extracellular adenosine levels and result in neutrophil extracellular traps formation. Elevated serum TNF-α concentrations,[8] type I interferon signatures,[9,10] and neutrophil signatures[11] have been observed in some reports about DADA2. However, the molecular mechanisms underlying the effectiveness of anti-TNF-α therapy have not yet been determined.

This study describes the clinical and genetic features of 8 Japanese patients with DADA2. To gain insight into the pathogenesis of DADA2, levels of expression of mRNA and protein were analyzed comprehensively in blood samples obtained from these patients in acute and remission phase. Transcriptome data of these patients were compared with data not only from normal controls but also from patients with other autoinflammatory diseases.

## METHODS
### Patients
Between 2016 and 2019, 38 patients were referred for a possible diagnosis of DADA2 and were screened for plasma ADA2 activity. Patients were diagnosed with DADA2 by lack of ADA2 activity, and their diagnosis was confirmed by genetic analysis. The study protocol was approved by the ethical committee of Kyoto University, and written informed consent was obtained from all participants or their legal guardians according to the Declaration of Helsinki.

### Genetics
DNA was extracted from peripheral blood samples using QIAamp DNA blood kits (Qiagen, Venlo, The Netherlands). Genetic testing was performed using a next-generation sequencing panel.

### mRNA analysis
mRNA was extracted from PHA blasts derived from patient 6 (p.Leu92Val and p.Phe355Leu) and patient 8 (c.753G>A p.Pro251Pro) using RNA easy kits (Qiagen). cDNA was prepared using ReverTra Ace (TOYOBO, Tokyo, Japan) and was PCR amplified using the forward primer 5′-TGCTGCCGGTGTATGAGC-3′ and reverse primer 5′-CAGCACCTGGT-TAGAGATGGG-3′. The PCR products were sequenced on an Applied Biosystems 3730xl DNA analyzer (Foster City, Calif).

### Transfection, Western blotting, and functional assessment of mutations
Full-length constructs of all the novel variants identified, except c.753G>A (p.Pro251Pro), were synthesized. The construct for c.753G>A (p.Pro251Pro) omitted exon 4. HEK293 cells were transfected with each of these constructs, and their supernatants were immunoprecipitated using anti-DDDDK tag mAb Magnetic Agarose (MBL, Nagoya, Japan). Equal amounts of proteins were used for immunoblotting. Briefly, total cell extracts were subjected to SDS-PAGE and transferred onto membranes by electroblotting. After blocking with 5% nonfat dry milk, membranes were incubated with anti-FLAG M2 (Sigma-Aldrich, St Louis, Mo) as primary antibody and with anti-mouse IgG horse radish peroxidase conjugate (Promega, Madison, Wis) as secondary antibody.

To confirm the pathogenicity of c.274C>G(p.Leu92Val) and c.1065C>A(p.Phe355Leu), CRISPR/Cas9 knockout (KO) of ADA2 was performed in U937 cells using guide RNA 5′-GAGCGTCATGAGCCTCTCAT-3′, and the ADA2-KO-U937 cells were transfected with the full-length constructs of wild type (WT), p.Leu92Val, p.Phe355Leu, and p.Leu92Val/p.Phe355Leu using a lentiviral vector. Total cell lysates were subjected to Western blotting, using anti-cat eye syndrome critical region protein 1 (Sigma-Aldrich) as primary antibody and anti-rabbit IgG horse radish peroxidase conjugate (Promega) as secondary antibody. Regarding to the medium of U937 cells, we concentrated equal volume of supernatant using heparin-Sepharose (GE Healthcare, Chicago, Ill) according to the manufacturer's indications, and then used it for Western blotting.

### ADA2 activity analysis
Two isoenzymes, ADA1 and ADA2, are responsible for ADA activity. ADA activity was measured as described[12] in the absence or presence of an ADA1-specific inhibitor, erythro-9-(2-hydroxy-3-nonlyl) adenine.

### Transcriptome and proteome analyses
Transcriptome and proteome analyses were performed using PBMCs collected from patients 2 to 5 before and after starting anti-TNF-α therapy. PBMCs of 4 healthy controls were also analyzed. In addition, transcriptome analysis was performed using PBMCs from 2 patients each with Aicardi-Goutières syndrome (AGS) and cryopyrin-associated periodic syndrome; 3 with TNF receptor–associated periodic syndrome; 1 with pyogenic arthritis, pyoderma gangrenosum, and acne syndrome (PAPA); 1 with chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE); and 1 with pyrin-associated autoinflammation with neutrophilic dermatosis. PBMCs were isolated from peripheral blood samples by Ficoll-Hypaque density-gradient centrifugation. Total mRNAs and proteins were isolated from cell lysates as described.[13] A complete description of these methods is provided in the supplemental Methods in this article's Online Repository at www.jacionline.org.

### Analyses of differentially expressed genes/proteins
The levels of expression of genes identified in the transcriptome were normalized and compared using the Bioconductor R package DESeq2

(www.bioconductor.org). Differentially expressed genes (DEGs) were selected as those with a false discovery rate <0.1 and a minimum 1.5 fold-change (FC) cutoff in comparisons of patients with DADA2 with normal controls, and a false discovery rate <0.5 and FC >1.5 in comparisons of patients with DADA2 and those with AGS. In the proteome, proteins were ranked according to FC. The top 500 most upregulated proteins with a minimum mean FC cutoff of 1.5 were selected as differentially expressed proteins (DEPs) for further analysis. Upstream regulator analysis and network analysis were performed and visualized using Ingenuity Pathway Analysis (IPA; TOMY Digital Biology Co, Ltd, Tokyo, Japan) and Cytoscape (Institute for Systems Biology, Seattle, Wash).

### Cytokine analysis

Multiple proinflammatory cytokines in serum were measured using Multiple Inflammatory Cytokine ELISA kits (Qiagen) and IL-18 ELISA kits (MBL) according to manufacturers' protocols.

### STAT1 phosphorylation

PBMCs from patients 2 to 5 and healthy controls were either left unstimulated or stimulated with IFN-γ (1000 U/mL) or IFN-β (1000 U/mL) for 15 minutes at 37°C, and the expression of surface markers assessed by incubation with CD3-APC (Invitrogen, Waltham, Mass), CD19-PECy7 (BioLegend, San Diego, Calif), and CD14-FITC (Invitrogen). Dead cells were excluded using Zombie Aqua Fixable Viability Kits (BioLegend). The cells were fixed using Lyse/Fix Phosflow buffer (BD Biosciences, Franklin Lakes, NJ) and permeabilized using BD Phosflow TM Perm Buffer III, according to the manufacturer's instructions. The cells were stained with anti-phospho-STAT1-pY701-PE (BD Biosciences) and analyzed by flow cytometry using BD FACSVerse.

### Statistical analysis

Data were presented as means ± SD. Significant differences between 2 groups were determined by one-way ANOVA, with statistical significance defined as $P < .05$.

## RESULTS
### Clinical features of DADA2 in Japanese patients

We identified 8 patients with DADA2 from 6 families in Japan. Their clinical and genetic characteristics are summarized in Table I, and their detailed clinical course and pedigrees are described in Tables E1 and E2 and Fig E1 in this article's Online Repository at www.jacionline. All 8 patients were born to nonconsanguineous Japanese parents; patients 2 and 3 are siblings, as are patients 4 and 5.

Five patients (63%) presented with central nervous system manifestations, including 4 with cerebral infarction and 3 with intracranial hemorrhage. Representative radiographic images are shown in Fig E2, A to C, in this article's Online Repository at www.jacionline. Cutaneous manifestations were present in all 8 patients, including 7 (88%) with livedo racemosa and the 4 with a nonspecific rash. Three (37.5%) also had Raynaud phenomenon. Rashes in patients 4 and 7 were biopsied. The tissue sample from patient 7 showed vasculitis of small-to-medium-size arteries, whereas the sample from patient 4 showed interface dermatitis with predominant infiltration by neutrophils but no evidence of vasculitis. Two patients (25.0%) had acute phase myositis in the right quadriceps muscle. In patient 2, short tau inversion recovery sequence magnetic resonance imaging revealed abnormal signal intensity, and muscle biopsy showed medium-size arteritis (Fig E2, D). Three patients (37.5%) and 5 patients (62.5%) had

low levels of serum IgG and IgM, respectively. None had recurrent or severe infection episodes, and none had granulocytopenia or lymphocytopenia. Two patients (patients 5 and 7) (25.0%) showed PRCA. Bone marrow aspiration on both patients revealed erythroblastopenia (Fig E2, E).

### Treatment

All patients were treated with anti-TNF agents. Of the 8 patients, 7 (88%) were started on anti-TNF therapy after being diagnosed with DADA2. None experienced a central nervous infarction or hemorrhage after initiation of anti-TNF-α therapy. Patients 4 and 6 experienced secondary failure to infliximab. After switching to adalimumab and etanercept, respectively, they were well controlled. Patient 7 experienced PRCA without any inflammatory manifestations, except for livedo racemosa. PRCA in this patient was well controlled by cyclosporine A but was drug-dependent. This patient was recently started on etanercept for vasculitis.[14] Patient 5 also experienced PRCA at age 2 months, but recovered spontaneously without any specific treatment after single transfusion of red blood cells. At age 5 months, however, he started to have systemic inflammation with continuous fever, which were well controlled after starting adalimumab. Patient 1 received regular infusions of fresh-frozen plasma, but these were subsequently discontinued due to lack of efficacy. None of these patients underwent hematopoietic stem cell transplantation.

### *ADA2* mutations

We identified 8 previously described and 3 novel mutations in the *ADA2* gene, with the former including 2 mutations that we reported elsewhere (Fig 1, A).[15] All the *ADA2* variants identified in this cohort are summarized in Table I. Patients 2 and 3 share a pathogenic p.Glu328Lys mutation on 1 allele. Although the expression of the other allele was severely diminished, the causative mutation could not be identified (data not shown). Patient 8 was compound heterozygous for p.Pro251-Leu and c.753G>A (p.Pro251Pro); although the latter was previously reported as pathogenic,[16] its functional consequence was undetermined. Splice-site prediction software (BDGP: Splice Site Prediction by Neural Network; Berkeley Drosophila Genome Project, Berkeley, Calif) indicated that this synonymous mutation abolished the donor splice site. As expected, the c.753G>A mutation resulted in aberrant splicing, with mRNA analysis of PHA blasts derived from patient 8 showing exon 4 skipping (Fig E3, A-B, in this article's Online Repository at www.jacionline.org).

### Effect of *ADA2* mutations on ADA2 activity

The protein expression and enzymatic activity of ADA2 variants were assessed by transfecting cDNA constructs of these ADA2 variants into HEK293 cells and analyzing ADA2 protein expression and enzymatic activity in cell lysates and culture medium. Analysis of cell lysates showed that ADA2 protein expression was reduced following transfection of p.Arg49A-lafs*13 constructs, whereas truncated proteins were expressed in cells transfected with p.Arg248Serfs*18 and c.753G>A p.Pro251Pro (del-Exon 4 in Fig 1, B and C). All variants showed low ADA2 activity except for p.Leu92Val/p.Phe355Leu (Fig 1, B and C). Analysis of ADA2 expression and activity in the culture

**TABLE I.** Clinical characteristics, treatments, and genotypes in the 8 patients with DADA2 in Japan

| Patient no. (family no.) | Sex | Age Onset | Age Diagnosis | Age Current | Mutation | Symptoms | Previous Treatment | Previous Efficacy | Current Treatment | Current Efficacy |
|---|---|---|---|---|---|---|---|---|---|---|
| P1 (F1) | M | 9 y | 17 y | 21 y | p.Tyr227Cys (c.680A>G) p.Gly358Arg (c.1072G>A) | Intracranial hemorrhage Rash (livedo racemosa) Fever Renal infarction | Tocilizumab IVCY Plasma transfusion | PR (recurrent cerebral infarction) | Anti-TNF-α agent (ADA) | CR |
| P2 (F2) | M | 10 y | 22 y | 25 y | p.Glu328Lys (c.982G>A) Unknown | Intracranial hemorrhage Cerebral infarction Rash (livedo racemosa) Frostbite Fever Splenomegaly Renal infarction | High-dose steroids IVIG | PR (recurrent cerebral infarction) | Anti-TNF-α agent (IFX) SCIg | CR |
| P3 (F2) | M | 12 y | 18 y | 22 y | p.Glu328Lys (c.982G>A) Unknown | Diplopia Memory disturbance Rash (livedo racemosa) Frostbite Renal infarction | None | — | Anti-TNF-α agent (IFX) SCIg | CR |
| P4 (F3)* | F | 3 mo | 6 mo | 4 y | p.Glu328Asp (c.984G>C) p.Tyr236del (c.706_708TACdel) | Small lacunar infarction (asymptomatic) Rash (livedo racemosa) Fever Splenomegaly | High-dose steroids Tocilizumab | PR (steroid-dependent) | Anti-TNF-α agent (IFX →ADA) MTX SCIg | CR (switch IFX to ADA due to anti-IFX antibody) |
| P5 (F3) | M | 2 mo | 1 mo | 2 y | p.Glu328Asp (c.984G>C) p.Tyr236del (c.706_708TACdel) | Red cell aplasia Rash | None | — | Anti-TNF-α agent (ADA) MTX SCIg | CR |
| P6 (F4) | F | 5 y | 17 y | 21 y | p.Arg49Alafs*13 (c.144dupG) p.Leu92Val (c.274C>G) p.Phe355Leu (c.1065C>A) | Intracranial hemorrhage Cerebral infarction Rash (livedo racemosa) Renal infarction Splenic infarction | High-dose steroids IVCY Rituximab Colchicine CsA Tac | PR (recurrent cerebral infarction) | Low-dose steroids Anti-TNF-α agent (IFX →ETN) | CR (switch IFX to ETN due to resistance to IFX) |
| P7 (F5)† | F | 13 y | 17 y | 20 y | p.Arg248Serfs* 18 (c.744delG) p.Ile93Thr (c.278T>C) | Red cell aplasia Rash (livedo racemosa) | CsA | PR (relapse when CsA is tapered) | CsA Anti-TNF-α agent (ETN) | CR |
| P8 (F6) | F | 12 y | 12 y | 15 y | p.Pro251Pro (c.753G>A) p.Pro251Leu (c.752C>T) | Rash (livedo racemosa) Fever Myositis Renal hypertention | None | — | Anti-TNF-α agent (ADA) | CR |

*ADA*, Adalimumab; *CR*, complete response; *CsA*, cyclosporine A; *ETN*, etanercept; *F*, female; *IFX*, infliximab; *IVCY*, intravenous cyclophosphamide; *IVIG*, intravenous immunoglobulin; *M*, male; *MTX*, methotrexate; *PR*, partial response; *SCIg*, subcutaneous immunoglobulin; *Tac*, tacrolimus.

The combined annotation-dependent depletion scores of novel variants are as follows: p.Leu92Val c.274C>G (20.9), p.Tyr227Cys c.680A>G (23.3), p.Arg248Serfs*18 c.744delG (22.6). None of them are reported in the gnomAD database.

*Reported in Nihira et al.[15]

†Reported in Keino et al.[14]

medium showed that ADA2 expression and/or activity was lower than WT in all the variants, except for p.Leu92Val/p.Phe355Leu (Fig 1, *B* and *C*). p.Leu92Val, which had not been previously reported in gnomAD (Genome Aggregation Database; https://gnomad.broadinstitute.org), was located on the same *ADA2* allele as p.Phe355Leu in patient 6 (see Fig E4 in this article's Online Repository at www.jacionline.org). Although the level of protein encoding p.Leu92Val was normal when the construct was transiently expressed in HEK293 cells (Fig 1, *D* and *E*), it was reduced after stable expression in U937 cells (Fig 1, *F* and *G*). Taken together, these results indicated that the phenotype in patient 6 was due to p.Leu92Val not p.Phe355Leu.

## DEGs and DEPs in PBMCs

Because the mechanism underlying DADA2 inflammation is not well understood, we performed transcriptome and proteome analyses on PBMCs from 4 patients with DADA2 and 4 healthy controls. Samples were collected from patients with DADA2 before (acute phase) and after (remission phase) the initiation of anti-TNF-α therapy, and DEGs and DEPs were analyzed.

MA plots and heat maps are shown in Fig 2, *A* and *B*. A comparison of samples from patients with DADA2 before treatment with healthy controls showed 261 DEGs, with 220 being upregulated and 41 downregulated in DADA2 samples, whereas a comparison of patients with DADA2 after treatment with healthy
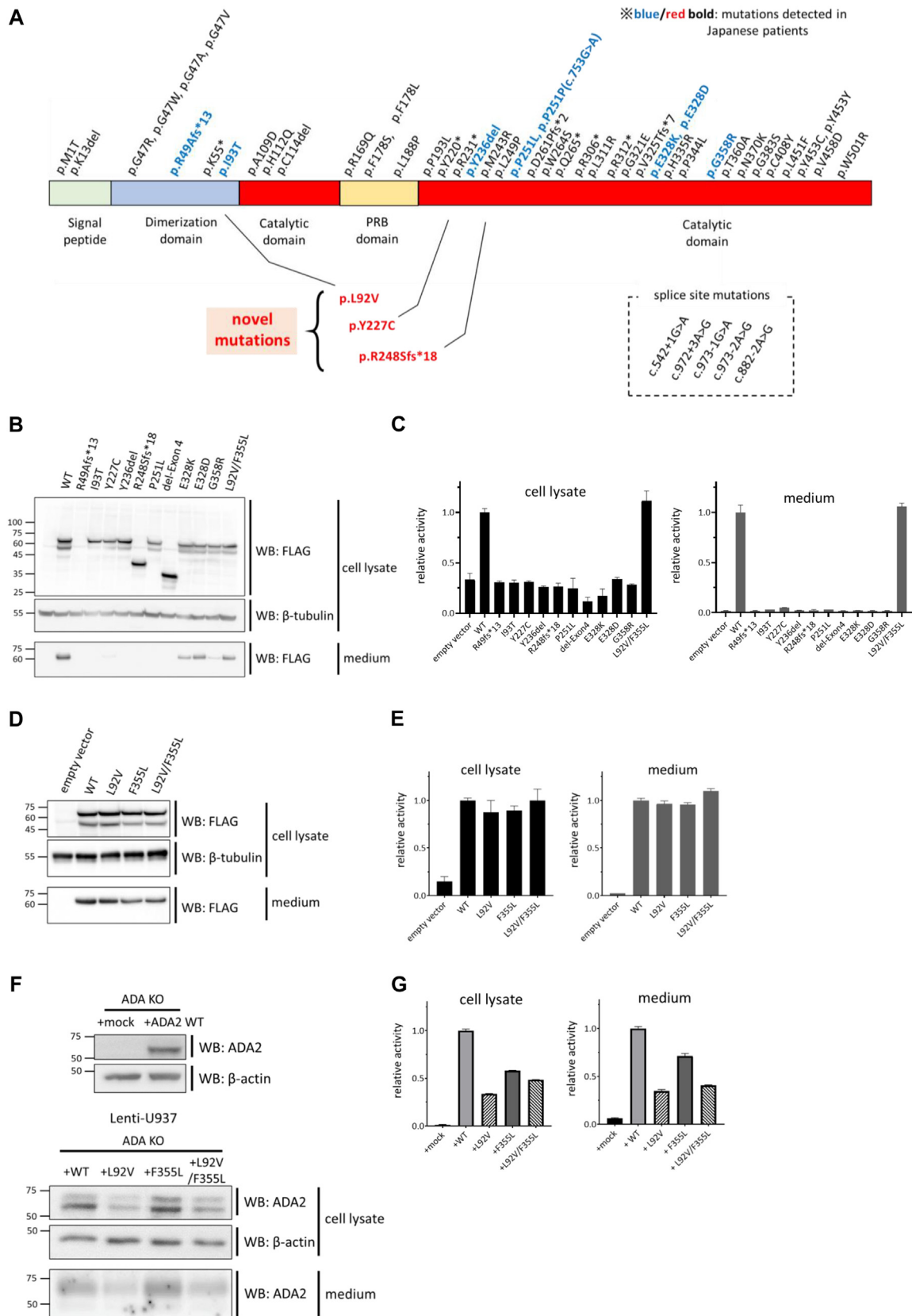
**FIG 1.** *ADA2* mutations and functional analysis *in vitro*. **A,** Schema of disease-associated mutations in *ADA2*. *ADA2* mutations identified in this cohort are shown in bold font (mutations reported before are shown in *blue* bold font and novel mutations in *red* bold font). **B and C,** Western blotting (*WB*) (**B**) and ADA2 activity analysis (**C**) of cell lysates and medium of HEK293 cells transfected with *ADA2* constructs. **D and E,** Western blotting (**D**) and ADA2 activity analysis (**E**) of cell lysates and medium of HEK293 cells transfected with WT, L92V, F355L, and L92V+F355L constructs. **F and G,** ADA2-KO U937 cells were transfected with each *ADA2* construct, including WT, L92V, F355L, and L92V+F355L. Cell lysates and medium of these cells were subjected to Western blotting (**F**) and ADA2 activity analysis (**G**). ADA2 activity was analyzed in triplicate, and the results are expressed as relative activity to WT with mean ± SD. *FLAG*, Flag tag; *PRB*, putative receptor binding.
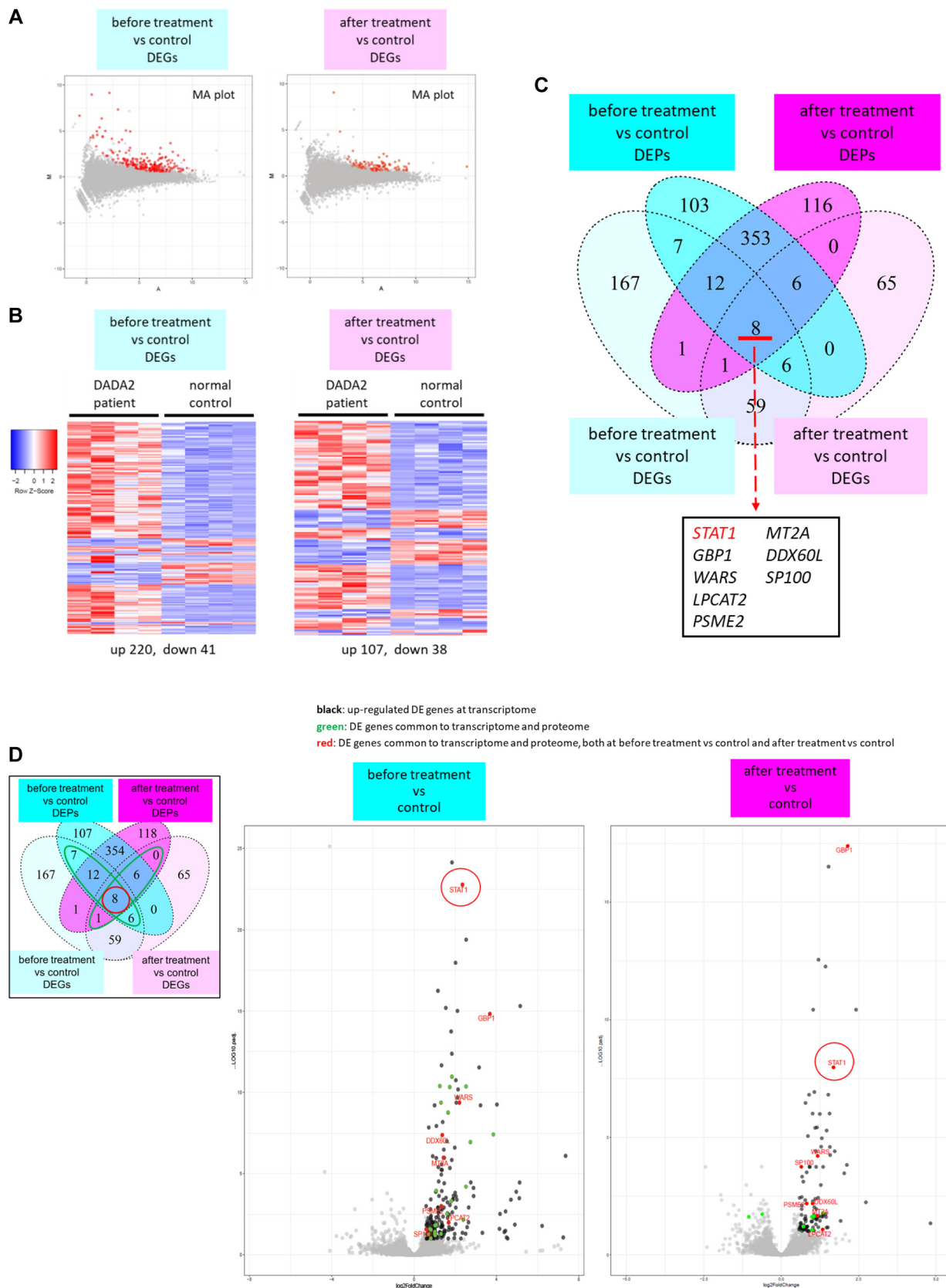
FIG 2. MA plot, heat map, and Venn diagram of DEGs and DEPs. **A** and **B,** MA plot **(A)** and heat map **(B)** of DEGs. **C,** Venn diagrams of DEGs and DEPs in before treatment versus control and after treatment versus control samples. **D,** Volcano plot of transcriptome analysis of comparisons of before and after treatment versus control samples; *black dots* represent upregulated DEGs, *green dots* represent genes common to DEGs and DEPs, and *red dots* represent 8 genes (*STAT1, GBP1, WARS1, LPCAT2, PSME2, MT2A, DDX60L,* and *SP100*) common to integrated datasets.

**FIG 3.** Pathway analysis and upstream analysis of DEGs and DEPs. **A,** Gene enrichment analysis of upregulated genes common to DEGs and DEPs, which utilize GO biological process categories. The top 5 are shown. *Red underlines* indicate GO terms related to the type II interferon; *green underlines* indicate GO terms related to the type I interferon. **B,** Network analysis of common DEGs by Cytoscape using the STRING

controls identified 145 DEGs, with 107 being upregulated and 38 downregulated in DADA2 samples. Seventy-four DEGs were present in both comparisons. In proteome analysis, we ranked all genes by mean FC, and the 500 most upregulated proteins in patients with DADA2 compared with controls were chosen as DEPs for further analysis.

We also compared transcriptome and proteome results of patients with DADA2 samples before and after treatment. Comparison using a P value <.1 and a minimum 1.5 FC cutoff showed 514 DEGs, with 271 being higher and 243 lower in samples obtained before treatment than after treatment. Proteome analysis using a minimum 1.5 FC cutoff showed that the levels of expression of 223 DEPs were higher before treatment than after treatment (Fig E5, A and B, in this article's Online Repository at www.jacionline.org).

### Integrated multi-omics analysis

When integrating the lists of DEGs and DEPs, 33 genes were present in both sets of comparisons of patients with DADA2 before treatment with healthy controls, and 15 genes were present in both sets of comparisons of patients with DADA2 after treatment with healthy controls. Both of these datasets included 8 genes: *STAT1*, *GBP1*, *WARS*, *LPCAT2*, *PSME2*, *MT2A*, *DDX60L*, and *SP100* (Fig 2, C). Fig 2, D shows the statistical significance of DEGs by volcano plot along with FCs. The results of pathway enrichment and network analysis are shown in Fig 3. In Gene Ontology (GO) analysis, type I/II interferon-related pathways were ranked high in comparisons of patients with DADA2 before and after treatment with healthy controls (Fig 3, A). A comparison of samples from patients with DADA2 before and after treatment showed that 17 genes were common to both the DEG and DEP datasets, with GO analysis showing that the levels of expression of those associated with IFN-γ–related pathways were higher before treatment than after treatment (Fig E5, B and C).

### Network analysis and upstream analysis

Network analysis of DEGs using Cytoscape (Fig 3, B) and IPA (Fig 3, C) identified *STAT1* as a hub gene in this dataset. Moreover, upstream analysis by IPA showed that *STAT1* was ranked as the top regulator gene (Fig 3, D). *STAT1* expression levels in the transcriptome and proteome are shown in Fig 3, E and F, respectively. Fig 3, G shows genes and signaling that contribute to the type I/II interferon pathways in IPA.

Fig 3, H shows the heat map of DEGs and DEPs common dataset in before treatment versus control. In the same heat map, we also presented transcriptome data from patients with other autoinflammatory diseases (ie, AGS, cryopyrin-associated periodic syndrome, TNF receptor–associated periodic syndrome, pyogenic arthritis, pyoderma gangrenosum, and acne syndrome, CANDLE, and pyrin-associated

autoinflammation with neutrophilic dermatosis) (Fig 3, H). We found some genes within this dataset were upregulated not only in DADA2 but also in AGS. To reveal the difference in molecular signature, we compared transcriptome data from patients with DADA2 and AGS, a representative type I interferonopathy (Fig 3, I). This analysis identified 30 genes more highly expressed in patients with DADA2 before treatment than in both AGS patients and controls. Though *STAT1* was not included in these 30 DEGs, GO analysis revealed upregulation of the type II interferon signaling pathway (data not shown). Furthermore, even compared with other autoinflammatory diseases, the upregulation of the type II interferon signaling pathway would be characteristic of DADA2.

### Expression of interferon-regulated genes in RNA sequencing analysis

We then assessed the type I interferon signature in patients with DADA2 (Fig 4, A-C). Type I interferon signature and its score are defined as the median FC in levels of expression of 6 type I interferon-stimulated genes (ISGs), *SIGLEC1*, *IFI27*, *RSAD2*, *ISG15*, *IFIT1*, and *IFI44L*, relative to healthy controls. Mean type I interferon scores of patients with DADA2 in RNA sequencing analysis were significantly higher in patients with DADA2 before (mean, 4.27, P = .0064) and after (mean, 3.61, P = .023) treatment compared with healthy controls (mean, 1.11), but the scores before and after treatment of patients with DADA2 did not differ (Fig 4, A and B). In addition, the interferon scores in patients with DADA2 were not as high as those in patients with AGS (mean, 17.3; data not shown). Similarly, although 28 previously described interferon -regulated genes[17] were upregulated in patients with DADA2, their levels of expression were not as high as in patients with AGS (Fig 4, C).

We also assessed the level of expression of *CXCL10*, a representative type II interferon-regulated gene (Fig 4, D). We found that the median FCs of *CXCL10* expression level were higher in patients with DADA2 before (mean, 16.0) and after (mean, 3.75) treatment than in healthy controls (mean, 0.89), and it tended to decrease after treatment; although the differences were not statistically significant (P = .48, .82, .07, respectively).

Several other type I/II interferon signature scoring methods have been used to assess interferon-signaling activity at the mRNA level.[18,19] We therefore assessed IFN-γ–related and IFN-α–related signatures in transcriptome (Fig 4, E and F). In patients with representative interferonopathy, such as AGS and CANDLE, IFN-α scores were equal to or higher than IFN-γ scores, in contrast, IFN-γ scores were higher than IFN-α scores in all before-treatment samples of DADA2, with these IFN-γ scores tending to decrease after treatment with anti-TNF-α agents. Treatment of patients with DADA2, however, did not affect IFN-α scores.

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database (STRING Consortium; http://string-db.org). **C** and **D**, IPA functional networks using the dataset of common DEGs. The highest ranked network **(C)** (the genes in the dataset are shown in *gray*) and list of upstream regulator genes **(D)**. **E** and **F**, Levels of expression of *STAT1* in the transcriptome **(E)** and proteome **(F)**. **G**, Canonical type I/II interferon signaling pathway by IPA. The genes included in the dataset of common DEGs are shown in *gray* and *purple*. **H** and **I**, Comparison of the transcriptome of DADA2 with those of other autoinflammatory diseases. Heat maps of the datasets common to DEGs and DEPs in before treatment versus control samples **(H)** and of the datasets of upregulated genes common to DADA2 before treatment versus AGS and DADA2 before treatment versus control samples **(I)**. *FDR*, False discovery rate.

## Serum cytokine analysis

Serum samples were collected from patients with DADA2 during both acute phase and remission phase, and the levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IL-18, TNF-α, GM-CSF, and IFN-γ were analyzed. IFN-γ and IL-18 levels were increased in patients with DADA2 before treatment and were normalized after treatment (Fig 4, *G*), but no difference was observed in any other of these cytokines.

## STAT1 phosphorylation analysis of PBMCs

To assess whether STAT1 is activated in patients with DADA2, STAT1 phosphorylation was analyzed in PBMCs obtained after the initiation of anti-TNF-α treatment. In the unstimulated state, no significant difference was observed in the levels of phosphorylated STAT1 between patients with DADA2 and normal controls; however, STAT1 phosphorylation in IFN-γ–stimulated monocytes and B cells was markedly higher in samples from patients with DADA2 than in those from healthy controls (Fig 5; and see Fig E6, *A-C, H*, in this article's Online Repository at www.jacionline.org). This tendency was also observed with IFN-β stimulation (Fig E6, *D-G*), but not in T cells (data not shown).

## Transcriptome analysis of U937 cells *in vitro*

We also performed transcriptome analysis of U937-mock and U937-ADA2-KO cell lines, established by CRISPR/Cas9 (Fig E7, *A*), by comparing their mRNA expression of 1152 genes included in the GO term "response to cytokine." DEGs were selected as those with a *P* value <.5 and a minimum 1.5 FC cutoff. Relative to U937-mock cells, 68 genes were found to be upregulated in U937-ADA2-KO cells (Fig E7, *B*). GO analysis of these 68 genes revealed upregulation of the INF-γ and TNF-α pathways (Fig E8, *C*).

## DISCUSSION

To our knowledge, the present study is the first to report that the constitutive upregulation of type II interferon pathway-related genes, including *STAT1*, is a characteristic finding in patients with DADA2. The type II interferon pathway has been associated with other inflammatory diseases and conditions, including psoriasis,[20] systemic lupus erythematosus,[21] and macrophage activating syndrome.[22] Although several studies have reported that the type I interferon pathway is upregulated in patients with DADA2,[9,10] assessment of the type II interferon pathway has yielded conflicting results. One study reported that IFN-γ–induced protein-10 expression was increased significantly in patients with DADA2,[1] whereas another found that *CXCL9* and *CXCL10* were not upregulated, suggesting that the type II interferon pathway was not activated in DADA2.[9]

The present study utilized multi-omics analysis to comprehensively evaluate mRNA/protein expression in patients with DADA2. Even after treatment with anti-TNF-α agents and the induction of remission, the levels of expression of type II interferon-related genes were significantly higher in patients with DADA2 than in normal controls. Furthermore, a comparison of expression in patients with DADA2 before and after treatment revealed that the levels of type II interferon-related genes were lower after treatment. This finding was consistent with the decline

of levels of IFN-γ and *CXCL10* after treatment, likely reflecting the disease activity. The ISG score, which reflects activity of the type I interferon signaling, was higher in patients with DADA2 than in healthy controls, but was not as high as in patients with AGS or CANDLE. ISG scores in patients with typical type I interferonopathies, such as AGS and CANDLE,[23] are at least 10-fold higher than those in normal controls.[9,24,25] The scores in our patients with DADA2 were only several-fold higher than in normal controls, a finding consistent with previous reports.[9,24] Similar trends were observed in both 6- and 28-gene interferon-related genes.[17,26] Moreover, although ISG score has been reported to reflect disease activity in patients with DADA2,[9] the ISG scores in our patients did not change markedly after remission.

In addition, a comparison of patients with DADA2 and those with AGS suggested that upregulation of type II interferon signaling would be a characteristic of DADA2. The use of other type I/II interferon signature scoring in transcriptome also showed that the type II interferon pathway would be more upregulated than the type I interferon pathway in patients with DADA2, compared with patients with AGS/CANDLE. These scoring systems also indicated that type II, but not type I, interferon signatures were reflective of disease activity. RNA sequencing analysis in U937 cells also suggested that the IFN-γ and TNF-α pathways might be upregulated in ADA2-KO cells. Taken together, these findings suggest that type II interferon signaling may play an important role in DADA2 pathogenesis. Regarding the correlation between interferon signature and disease activity, type I interferon signatures in patients with CANDLE have been reported to decrease following the administration of baricitinib.[27] However, this correlation is unclear in patients with SLE.[28,29] Because few studies have assessed the correlation between type I interferon signature with disease activity in patients with DADA2, further studies are needed.

Network analysis of the transcriptome revealed that *STAT1* may be a hub gene and also a top upstream regulator in DADA2. STAT1 is a key molecule in the Janus kinase/signal transducer and activator of transcription signaling pathway. This is a major pathway for a wide range of cytokines, and its activation stimulates a wide variety of cellular functions, such as cell proliferation, differentiation, migration, and apoptosis.[30,31] The type I/II interferon signaling pathway has been associated with STAT1. IFN-γ, a prototypic type II interferon, was originally identified as macrophage activating factor, and macrophages are a major physiological target of IFN-γ.[21] Low-dose IFN-γ priming of monocytes/macrophages, that is subthreshold concentration that does not activate macrophages, increased their level of expression of STAT1 without phosphorylation and their sensitivity to subsequent IFN-γ stimulation.[32] Although the serum concentration of IFN-γ in patients with DADA2 was normalized after anti-TNF-α therapy, constitutive upregulation of type II interferon-related genes, observed even after remission, may result from subthreshold IFN-γ priming. This may also result in increased of STAT1 mRNA and protein expression, as well as hyperphosphorylation and hyperactivation of STAT1 in response to secondary IFN-γ in monocytes. However, IFN-γ priming was also reported to sensitize monocytes to both type I and type II interferons and to induce hyperphosphorylation of STAT1,[32] findings that are consistent with our results.

On the other hand, low-dose IFN-α also has been reported to sensitize primary human monocytes/macrophages to subsequent
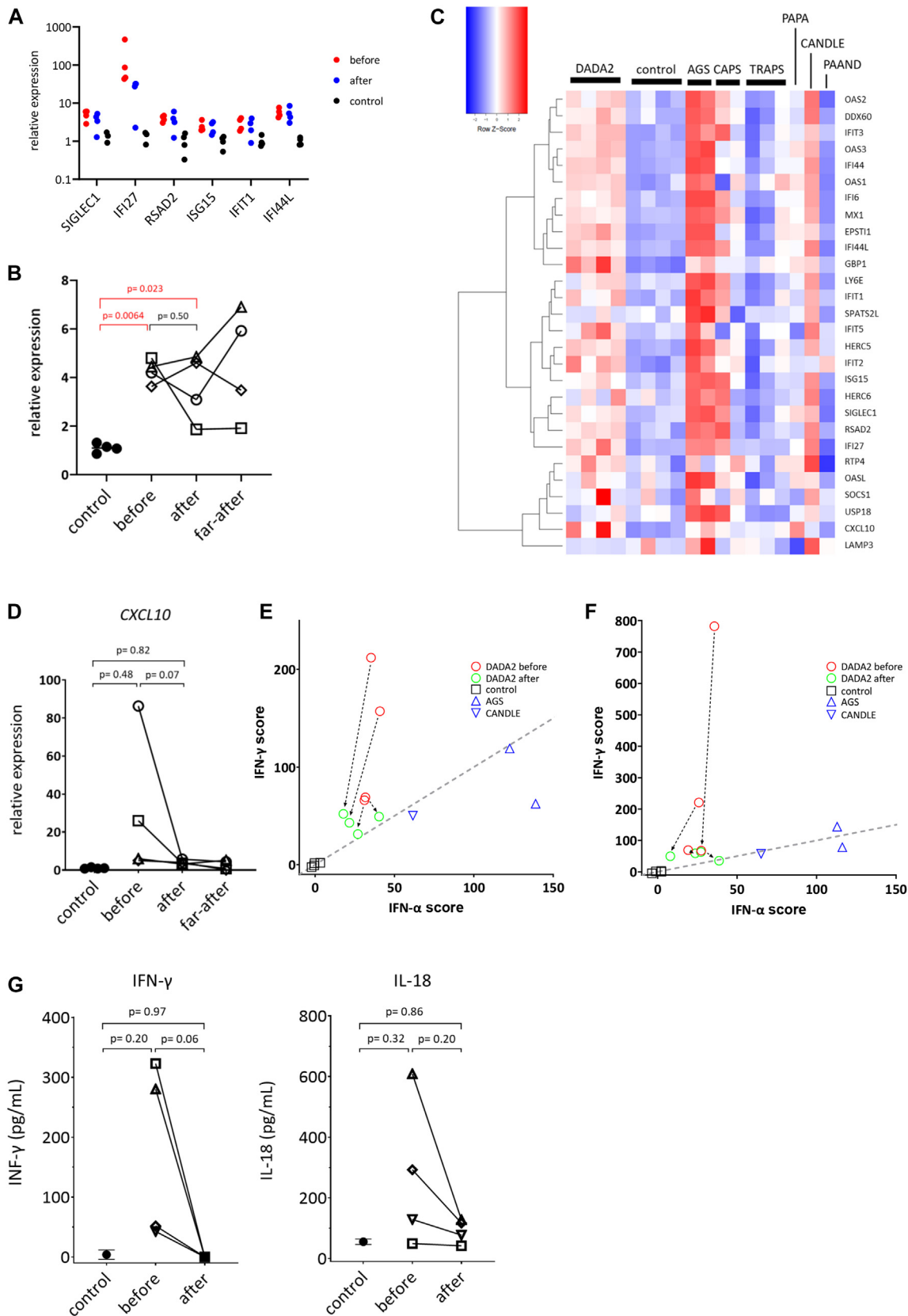
**FIG 4.** Interferon-regulated gene expression analysis. **A** and **B,** Type I interferon-regulated gene (*SIGLEC1,*
*IFI27, RSAD2, ISG15, IFIT1, IFI44L*) expression levels **(A)** and ISG scores **(B)** in the transcriptome. **C,** Heat map
of 28 interferon-regulated genes in comparisons of DADA2 before treatment, normal controls, and patients
with other autoinflammatory diseases. **D,** *CXCL10* expression levels in RNA sequencing. **E** and **F,** Type I/II
interferon signature scoring in transcriptome reported by Kiro et al[18] **(E)** and Liu et al[19] **(F).** In AGS and CAN-
DLE, the IFN-$\alpha$ scores were equal to or higher than IFN-$\gamma$ scores, whereas IFN-$\gamma$ scores were dominant in
patients with DADA2 before treatment, decreasing after treatment with anti-TNF-$\alpha$ agents. **G,** IFN-$\gamma$ and
IL-18 concentrations in plasma. *Before,* Before anti-TNF-$\alpha$ treatment; *after,* after anti-TNF-$\alpha$ treatment; *far-*
*after,* at least 2 years after initiation of anti-TNF-$\alpha$ therapy of patients with DADA2
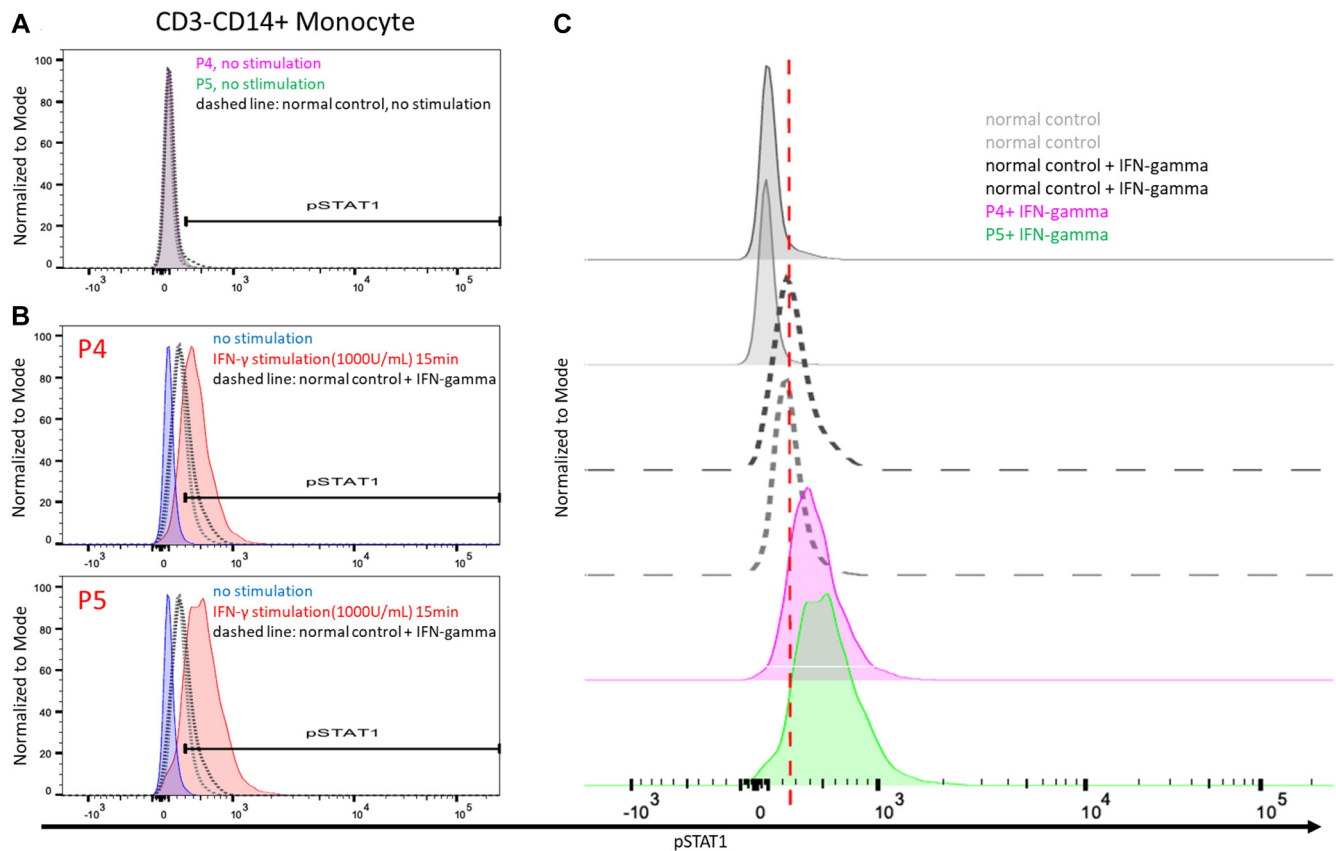
**FIG 5.** Flow cytometry analysis of phosphorylated STAT1 (*pSTAT1*). **A-C,** Levels of expression of pSTAT1 in unstimulated cells **(A)**. STAT1 phosphorylation of unstimulated cells (*blue*) and cells stimulated with IFN-γ 1000 U/mL for 15 minutes (*red*) **(B)**. pSTAT1 in response to IFN-γ is higher in monocytes of patients with DADA2 than in those of healthy controls **(C)**. *P4*, Patient 4.

IFN-γ stimulation.[32] The level of expression of STAT1 was found to be increased in induced pluripotent stem cells of patients with CANDLE, with STAT1 phosphorylation enhanced after IFN-γ stimulation. These findings suggest that CANDLE represents a "primed state" before stimulation.[33] Interestingly, IFN-γ, but not IFN-β, priming of macrophages also increased transcription of inflammatory cytokines, including TNF-α and IL-6, by chromatin remodeling,[34] a finding that may explain the difference between DADA2 and CANDLE. Anti-TNF-α agents have been shown to inhibit both TNF-α and type II interferon pathways. For example, the anti-TNF-α agent infliximab suppressed both TNF-α and IFN-γ expression at both the mRNA and protein levels in PBMCs.[35] In addition, infliximab was shown to give rise to CD4$^+$CD25$^{hi}$FoxP3$^+$ regulatory T cells, to induce CD62L$^-$ regulatory T cell differentiation and to suppress IFN-γ and TNF-α expression in patients with rheumatoid arthritis.[36] This may explain why anti-TNF-α therapy is effective in DADA2 but not in CANDLE, as it controls systemic inflammation by suppressing both the TNF-α and type II interferon pathways.

This report also summarizes the genetic and clinical features of 8 patients with DADA2 from 6 Japanese families. Seven of these patients had polyarteritis nodosa–like vasculopathy, along with recurrent fevers, rashes, and cerebral infarction/hemorrhage, findings that are consistent with a previous case series.[1,2] Similar to other reports, we found that siblings within the same family followed different clinical courses.[3] For example, patient 2 experienced recurrent cerebral infarction/hemorrhage beginning at age 10 years, whereas patient 3 remained asymptomatic until recently, except for elevation of C-reactive protein. Similarly, patient 4 began to experience systemic inflammation in early infancy, whereas patient 5 first presented with PRCA and showed inflammatory symptoms afterward. Interestingly, patient 5 recovered from PRCA after a single transfusion. To our knowledge, this is the first report of spontaneous recovery from PRCA in a patient with DADA2.

All 8 patients were treated with anti-TNF-α therapies, including patient 7 who experienced PRCA without any systemic inflammation. No patient had a cerebral infarction or hemorrhage after starting treatment. Patient 1 received a fresh-frozen plasma transfusion every other week, which had no effect on her inflammation, a lack of efficacy that may have been due to rapid clearance of exogenous ADA2.[37] Patients diagnosed and treated soon after disease onset (patients 4 and 5) have maintained good control without any sequelae for about 3 years. Early diagnosis and treatment are needed because patients with DADA2 may develop cerebral infarction and/or hemorrhage with serious sequelae if not properly managed. Because patients may experience secondary failure of anti-TNF-α agents, it is also necessary to search for alternative therapeutic options that replace or correspond to anti-TNF-α therapy.

Eleven pathogenic *ADA2* variants were found in these 8 patients. In siblings patients 2 and 3, we could detect only 1 mutation (p.Glu328Lys). No other variants were apparent in the exons and adjacent intron regions, but the expression of the other allele was severely diminished (data not shown). Genetic analysis has failed to reveal bi-allelic mutations in some patients with DADA2 with deficient ADA2 activity,[4] emphasizing the importance of evaluating ADA2 activity in diagnosing DADA2. Of the *ADA2* variants identified in these patients, we confirmed pathogenicity of several variants. One is a synonymous mutation, c.753G>A(p.Pro251Pro), which causes aberrant splicing and exon 4 skipping. Although this mutation had been previously reported before,[16] its pathogenicity was predicted only by its base position (the last nucleotide of exon 4), not by a functional assay. It is of great significance that we have confirmed the pathogenicity of this mutation. Another is p.Leu92Val, which showed equivalent protein expression and activity to WT when transiently transfected into HEK293 cells, but had reduced protein expression after being stably transfected into the U937 human monocyte cell line. Regarding patient 6, who carried p.Leu92Val and p.Phe355Leu in tandem, we thought that p.Phe355Leu was pathogenic at first because it had previously been reported.[38] However, the allele frequency of p.Phe355Leu is relatively high in East Asia, constituting 56 of 19,946 alleles (0.28%) in gnomAD and 21 of 9,546 alleles (0.22%) in the Tohoku Medical Bank exome database of healthy Japanese persons, and its expression level and enzymatic activity were normal when transfected into HEK293 cells. These results suggested that p.Phe355Leu may not be pathogenic in patient 6. We therefore focused on the p.Leu92Val variant. When we compared the ADA2 activity of L92V, F355L, and L92V/F355L in the culture supernatants of U937 cells, we found that the activities of both L92V and L92V/F355L were about one-third the activity of those of WT, whereas the activity of F355L was only about 35% lower than WT, indicating that L92V, but not F355L, reduced ADA2 secretion and activity in patient 6. The functions of proteolytic systems, such as proteasome/autophagy, are dependent on tissues and cell types.[39,40] It is very likely that we could confirm pathogenicity of p.Leu92Val for the first time by using human monocytic cell line U937, because monocytes are responsible for production and secretion of ADA2 *in vivo*. On the other hand, it remains uncertain whether F355L also may contribute to pathogenicity in patient 6. The mechanism underlying the regulation of ADA2 production in monocytes remains to be determined.

This study had several limitations. The 4 patients (patients 2-5) included in the omics analyses had mainly inflammatory phenotypes, suggesting that additional studies may be required to assess the pathophysiology of hematological symptoms of DADA2. We also cannot eliminate the possibility that the type I interferon pathway may be involved in the constitutive upregulation of STAT1 in DADA2. Because the sample size in this study was small, it is necessary to increase the number of patients to improve the power of analysis. However, DADA2 is a rare disease of about 300 patients in the world, suggesting the need to create a system that allows samples to be obtained from patients with DADA2 soon after their diagnosis, especially before the start of therapy. In addition, the influence of modification by anti-inflammatory therapy, such as anti-TNF-α agents, cannot be eliminated completely in this study. A system that could verify and analyze these results *in vitro* is required. Moreover, neutrophils were not

analyzed in our study. Although NETosis, a regulated form of neutrophil cell death, is thought to be involved in DADA2, this study focused on the activity and properties of PBMCs and monocytes, the main producers of ADA2.

In summary, this study clarified the genetic and clinical features of 8 Japanese patients with DADA2, finding that both the type I and type II interferon signaling pathways were upregulated in these patients. This study also showed that *STAT1* could be a hub gene in the pathogenesis of DADA2, suggesting that both the type II interferon signaling pathway and *STAT1* may be potential therapy targets in patients with DADA2. Further research is needed to decipher how ADA2 deficiency leads to type I and type II interferon overproduction in DADA2.

**Clinical implications: Type II interferon signaling and *STAT1* may be potential therapeutic targets in DADA2.**

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