APRIL-dependent lifelong plasmacyte maintenance and immunoglobulin production in humans

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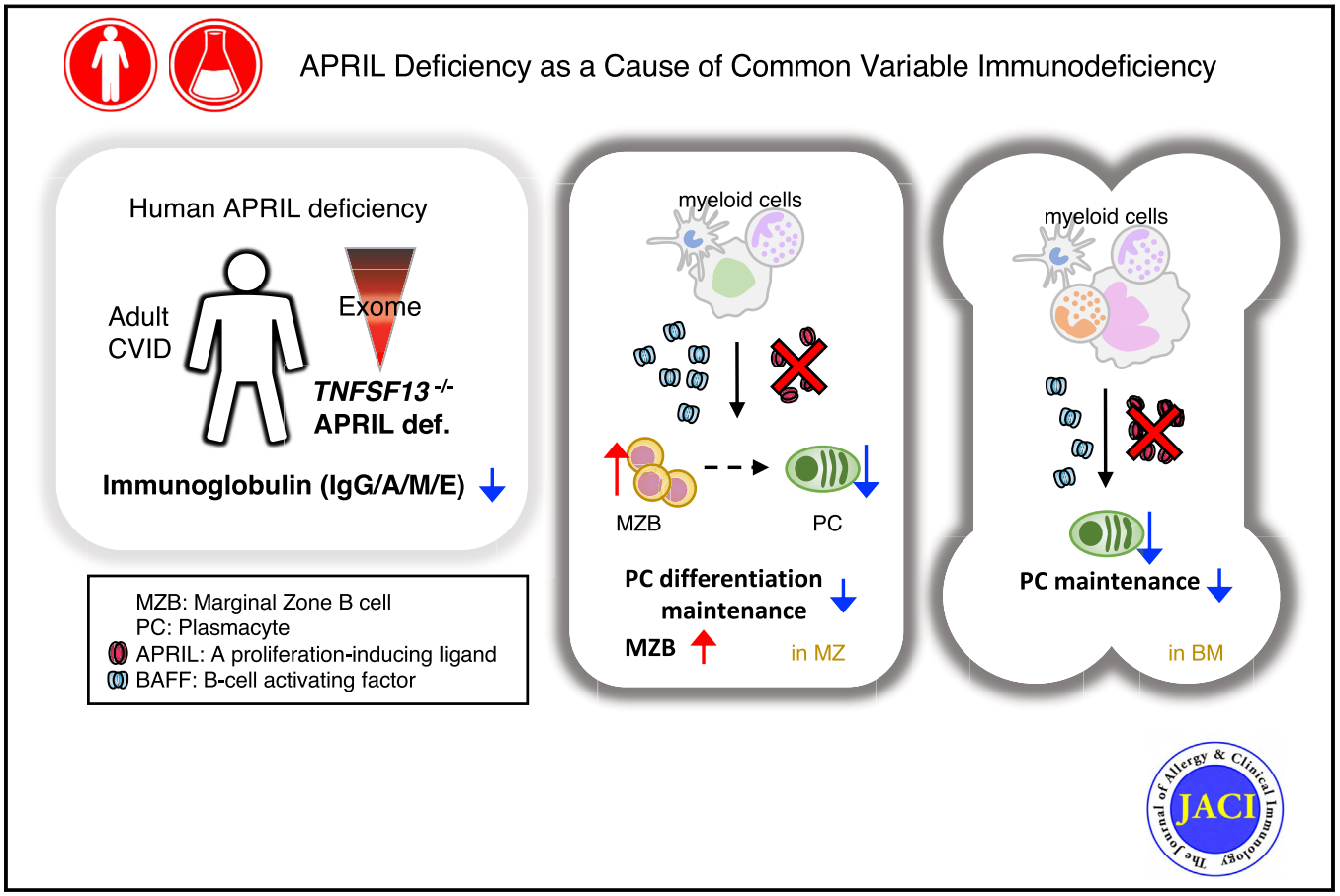
GRAPHICAL ABSTRACT



Background: Interactions between the tumor necrosis factor (TNF) ligand superfamily and TNF receptor superfamily play critical roles in B-cell development and maturation.

A proliferation-inducing ligand (APRIL), a member of the TNF ligand superfamily, is secreted from myeloid cells and known to induce the differentiation of memory B cells to plasmacytes.

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Objective: We sought to elucidate the role of APRIL in B-cell differentiation and immunoglobulin production through the analysis of complete APRIL deficiency in human.
Methods: We performed whole exome sequencing in a patient with adult common variable immunodeficiency. His parents were in a consanguineous marriage. *TNFSF13* mRNA and protein expression were analyzed in the primary cells and plasma from the patient and in cDNA-transfected cells and supernatants of the cultures *in vitro*. Immunologic analysis was performed by using flow cytometry and next-generation sequencing. Monocyte-derived dendritic cells differentiated from induced pluripotent stem cells (iPSC-moDCs) were cocultured with memory B cells from healthy controls to examine *in vitro* plasmacyte differentiation.
Results: We identified a homozygous frameshift mutation in *TNFSF13*, the gene encoding APRIL, in the patient. APRIL mRNA and protein were completely absent in the monocytes and iPSC-moDCs of the patient. In contrast to the results of previous animal model studies, the patient showed hypogammaglobulinemia with a markedly reduced level of plasmacytes in peripheral blood and a clearly increased level of circulating marginal zone B cells. Although iPSC-moDC–induced *in vitro* plasmacyte differentiation was reduced in the patient, recombinant APRIL supplementation corrected this abnormality.
Conclusion: The first APRIL deficiency in an adult patient with common variable immunodeficiency revealed the role of APRIL in lifelong maintenance of plasmacytes and immunoglobulin production in humans. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *APRIL, BAFF, TACI, BCMA, B cell, plasmacyte, somatic hypermutation, class switch recombination, common variable immunodeficiency*

Abbreviations used

APRIL:	A proliferation-inducing ligand
BAFF:	B-cell–activating factor
BCR:	B-cell receptor
CFSE:	Carboxyfluorescein succinimidyl ester
CSR:	Class switch recombination
CVID:	Common variable immunodeficiency
DC:	Dendritic cell
HC:	Healthy control
iPSC:	Induced pluripotent stem cell
iPSC-moDC:	Monocyte-derived dendritic cell differentiated from induced pluripotent stem cells
moDC:	Monocyte-derived dendritic cell
MZ:	Marginal zone
PID:	Primary immunodeficiency
SHM:	Somatic hypermutation
Tfh:	Follicular helper T
TNF:	Tumor necrosis factor
WT:	Wild-type

Primary immunodeficiency (PID) comprises a group of diseases characterized by susceptibility to infections due to congenital abnormalities in genes related to the immune system. Among them, predominantly antibody deficiencies, which include B-cell deficiency, hyper-IgM syndrome due to defects of immunoglobulin CSR, class switch recombination (CSR), and common variable immunodeficiency (CVID), account for nearly half of the patients with PID.[1,2] CVID, the most common form of predominantly antibody deficiencies, is a clinically heterogeneous disorder characterized by hypogammaglobulinemia and increased susceptibility to infections with variable coexisting features, including autoimmunity, autoinflammation, granulomatous diseases, and malignancy.[3] In many cases, affected patients present with normal T- and B-cell counts but show decreased memory B-cell and/or plasmacyte counts.[3-6] Various genes related to B-cell differentiation and activation have been reported as causes of CVID, including costimulatory molecules (eg, *ICOS, CD19,* and *CD20*), PI3K-mTOR pathway (eg, *PIK3CD*–gain of function, *PIK3R1*–loss of function, and *PTEN*–loss of function),[7] NF-kB pathway (eg, *NFKB1, NFKB2, CARD11*), and others (eg, *TRNT1, IKZF1, SEC61A1*). However, the genetic etiology of most patients with CVID is unknown.[8]

A proliferation-inducing ligand (APRIL) and B-cell–activating factor (BAFF) belong to the tumor necrosis factor (TNF) ligand superfamily.[9,10] Interactions between the TNF ligand superfamily (including BAFF and APRIL) secreted from myeloid cells,[11-13] and the B-cell surface TNF receptor superfamily (including BAFFR, TACI, and BCMA) play crucial roles in B-cell development and terminal maturation. Among the superfamily molecule deficiencies, only BAFFR[14] and TACI[15,16] deficiencies have been reported in humans. BAFFR deficiency, reflecting the lack of BAFF stimulation on B cells, impairs B-cell development at the transitional B-cell stage and results in low levels of serum IgG and IgM.[14] Biallelic TACI deficiency abrogates both BAFF and APRIL binding, and B cells from such patients were impaired in CSR and proliferation.[15,16] Furthermore, some heterozygous TACI deficiency is related to the development of CVID.[17] However, defects in APRIL, BAFF, and BCMA, have not previously been identified in humans.

The interactions are ligand specific but mutually compensating; for example, BAFF binds strongly to BAFFR and TACI but weakly to BCMA, whereas APRIL binds strongly to BCMA and weakly to TACI, but not to BAFFR. Moreover, parts of APRIL and BAFF form a BAFF-APRIL heterotrimer.[18,19] BAFF and APRIL are known to be related to the long-term maintenance of B-cell memories in animal models.[20,21] However, because of the functional complexity, it is difficult to discuss the separate, distinct functions of BAFF and APRIL. In particular, their distinct functions in lifelong periods in humans have not been fully elucidated. Therefore, precise description of clinical and immunologic phenotypes in each gene-deficient patient provides significant evidence for human B-cell immunology.

Herein, we report the first APRIL deficiency due to a homozygous single-nucleotide deletion in *TNFSF13* in a patient with CVID. His clinical and immunologic phenotypes and detailed *in vitro* analyses revealed the importance of APRIL in lifelong maintenance of plasmacytes and immunoglobulin production in humans.

METHODS

Genetic analysis

Genomic DNA was extracted from the whole blood of the patient and from the saliva of his parents with the QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands). Exon capture was performed with SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, Calif). Massively

parallel sequencing was performed with an HiSeq1500 system (Illumina, San Diego, Calif), generating 100-bp paired-end reads, followed by analysis using the pipeline for next-generation sequencing data as described previously.[22] Sequence variants were filtered with the following functional prediction tools: Sorting Intolerant from Tolerant (SIFT, Score <0.05 or no value), Polymorphism Phenotyping v2 (Polyphen2, Score >0.85 or no value), and Combined Annotation Dependent Depletion (CADD, Score >20 or no value). The identified nucleotide variants were filtered with our private variant data (MAF <0.01), dbSNP (MAF <0.001), and the Human Genetic Variation Database (HGVD, MAF <0.001); the identified mutation was confirmed by Sanger sequencing.

Written informed consent was obtained from the patient and the parents. We could not obtain the samples from other family members. The study was approved by the Ethics Committee of Tokyo Medical and Dental University.

Quantitative PCR

We isolated the $CD3^+$ T cells and $CD14^+$ monocytes from PBMCs by MACS MicroBead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The total RNA was extracted by using the RNeasy Mini Kit (Qiagen). cDNA was synthesized by using SuperScript VILO Master Mix (Invitrogen, Carlsbad, Calif). The expression of APRIL and BAFF was measured by TaqMan Gene Expression Assays system (Applied Biosystems, Thermo Fisher Scientific, Waltham, Mass). The expression levels of APRIL and BAFF were normalized to *GAPDH* gene expression levels. The following TaqMan probes were used for analysis: Hs00601664_g1 (for APRIL), Hs00198106_m1 (BAFF), and Hs02758991_g1 (GAPDH).

ELISA

Soluble APRIL and BAFF proteins in the plasma were measured by ELISA with use of the LEGEND MAX Human APRIL/TNFSF13 ELISA Kit (Biolegend, San Diego, Calif) and the Human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit (R&D Systems, Inc, Minneapolis, Minn). Patients with X-linked agammaglobulinemia caused by absence of BTK protein showed significantly higher concentrations of APRIL and BAFF, as reported elsewhere.[23]

Plasmid and transfection

We extracted mRNA from PBMCs of a healthy control (HC) and the patient by using the RNeasy Mini Kit and synthesized cDNA with the SuperScript VILO Master Mix. Full-length wild-type (WT) and mutant APRIL cDNAs were amplified by PCR and then subcloned into the pcDNA3 vector containing an N-terminal Flag tag. The primer sequences and PCR conditions are available on request. WT or mutant APRIL was transfected into HEK293T cells and analyzed by immunoblotting.

Immunoprecipitation and Western blot analysis

We used BCMA-Fc (Enzo Life Sciences, Inc, Lausen, Switzerland) for immunoprecipitation. We added BCMA-Fc to the Dynabeads Protein G (Life Technologies, San Diego, Calif) in PBS with 0.02% Tween-20 buffer and incubated them for 10 minutes. The Dynabeads were washed with PBS with 0.02% Tween-20 and were then incubated with plasma or HEK293T cell supernatant for 10 minutes at room temperature. After the beads had been washed 3 times, we added 1× sample buffer and boiled them at 95°C for 5 minutes. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and stained with the following antibodies: anti-APRIL (Enzo Life Sciences, Farmingdale, NY, and Abcam, Cambridge, United Kingdom), anti-FLAG, and β-actin (Sigma-Aldrich, St Louis, Mo).

Flow cytometric analysis

We investigated the immunologic characteristics of the patient by multicolor flow cytometry as previously described.[24] Briefly, we isolated PBMCs from the patient and healthy adult controls. Cells were then stained with various antibodies for 30 minutes on ice and analyzed by using the BD LSRFortessa flow cytometer (Becton Dickinson [BD], Franklin Lakes, NJ). The antibodies used for multicolor flow cytometry are detailed in our previous article.[24] For iIgH isotype and plasmacyte results, PBMCs from the patient and age-matched controls were stained as previously reported.[25]

Repertoire and switch junction analysis

Total DNA and RNA extracted from PBMCs from the patient and HCs were used as a template to amplify the rearrangements at the endogenous IgH loci with use of a Qiagen One Step RT-PCR kit (Qiagen) and Human B-Cell Receptor (BCR) Heavy Chain, V-C Gene primers (iRepertoire Inc, Huntsville, Ala). Purified PCR products were sequenced by using MiSeq (Illumina) next-generation sequencing according to the manufacturer's protocol. Raw data were analyzed by using iRepertoire. Switch junction analysis was performed by using a modified version of the previously described method, referred to as LAM-HTGTS,[26] with a human Sμ biotin primer.

B-cell functional test

Total B cells were isolated from peripheral blood by using a B-cell Enrichment Cocktail (StemCell Technologies, Vancouver, British Columbia, Canada). Cells were then stained with anti-CD19 (BD), anti-CD20 (Beckman Coulter, Calif), and -CD27 (Miltenyi Biotec) antibodies. The $CD19^+CD20^+CD27^-$ naive and $CD19^+CD20^+CD27^+$ memory B cells were further sorted by using the BD FACSAria (BD). Subsets of B cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific). CFSE-labeled cells (4×10^4) were cultured in 96-well *U*-bottom plates with soluble CD40L (sCD40L) and IL-21 (Miltenyi Biotec) for 7 days and stained with CD19 (Miltenyi Biotec). For immunoglobulin production, the supernatant was collected on day 12. Immunoglobulins were measured by using a BD Cytometric Bead Array and Human Immunoglobulin Master Buffer Kit (BD).

iPSC generation from the patients and controls and moDC differentiation

Induced pluripotent cells (iPSCs) in the patient and HCs were established by using PBMCs, as reported elsewhere.[27] Monocyte differentiation from iPSCs was performed by stimulation with GM-CSF and macrophage colony-stimulating factor (PeproTech, NJ) as previously described.[28] MoDCs differentiated from iPSCs (iPSC-moDCs) were obtained by stimulation first with GM-CSF and IL-4 and then with TNF-α and LPS on day 5, as reported elsewhere.[29] Recombinant human APRIL/TNFSF13 (R&D Systems) (1 μg/mL) was added in some experiments involving cocultured iPSC-moDCs and B cells. iPSC-moDCs and CFSE-labeled $CD19^+CD20^+CD27^+$ memory B cells were cocultured for 7 days. B cells were stained with CD24 (BD) and CD38 (Biolegend) and detected by flow cytometry.

Statistical analysis

All the data were analyzed by using GraphPad Prism version 8.0 for Mac (GraphPad Software, San Diego, Calif). The differences between 2 groups were determined by using a Student *t* test or Welch *t* test.

RESULTS

Identification of *TNFSF13* mutation in a patient with CVID

The patient was a 55-year-old Japanese male born to consanguineous parents who were related as second cousins. No obvious family history of PID was documented (Fig 1, *A*).

He was vaccinated without any complications with live smallpox, BCG, and polio vaccines, as well as with inactivated

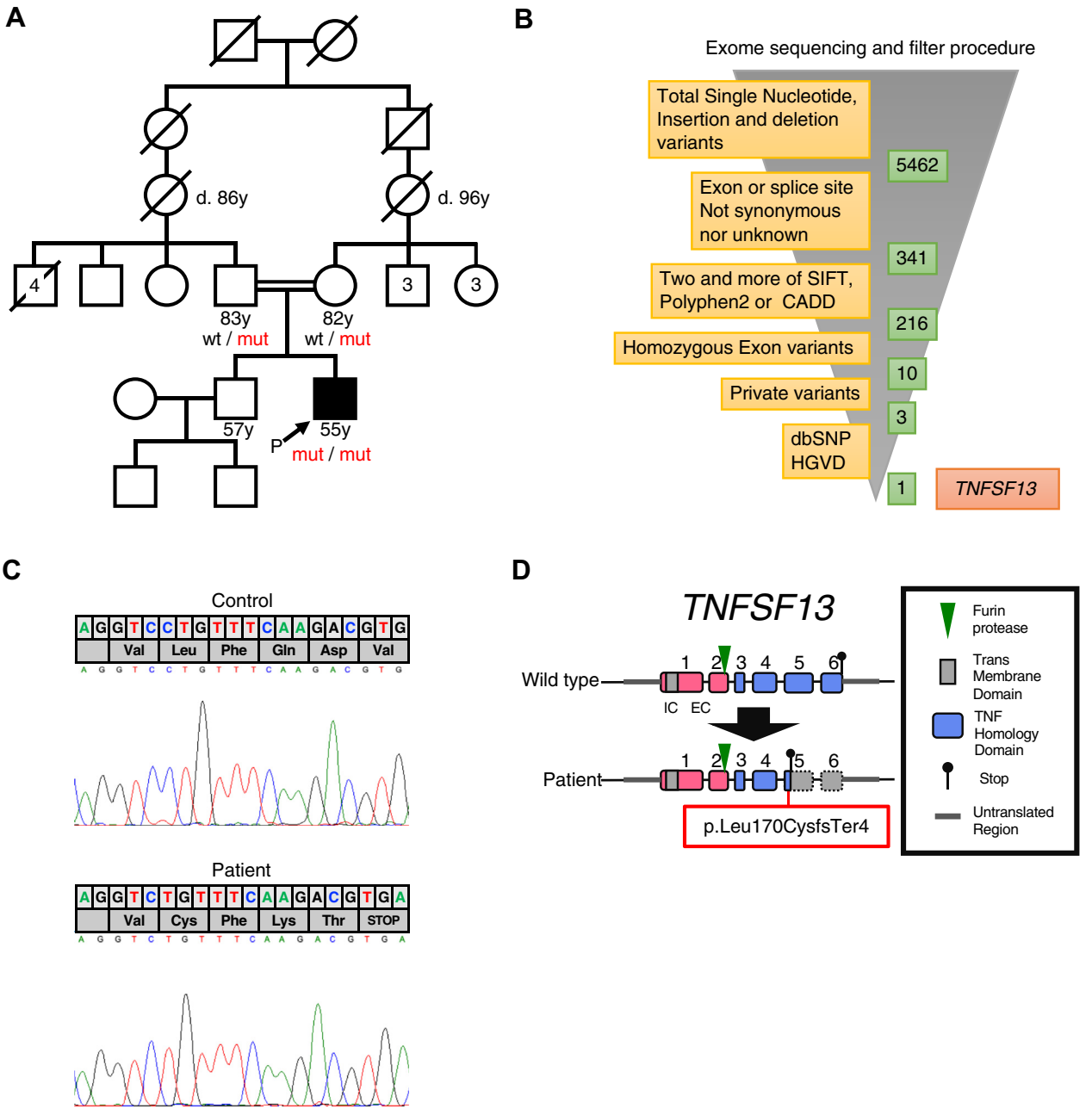

FIG 1. Whole exome sequencing revealed a homozygous *TNFSF13* mutation leading to a defect in APRIL protein. **A,** Family tree of the patient. The patient's parents are in a consanguineous marriage. The patient's father has 5 male and 1 female siblings; 4 of his male siblings died of noninfectious causes. The patient's mother has 3 male and 3 female siblings. **B,** Filtering process of the whole exome sequencing result. **C,** DNA electropherogram shows a homozygous single base deletion mutation, which causes a frameshift and a premature stop codon. **D,** Structure of *TNFSF13* gene and the effect of the mutation on APRIL protein. *CADD,* Combined Annotation-Dependent Depletion; *dbSNP,* Single Nucleotide Polymorphism database; *HGVD,* Human Genetic Variation Database; *mut,* mutant; *Polyphen 2,* Polymorphism Phenotyping, version 2; *SIFT,* Sorting Intolerant from Tolerant.

diphtheria, pertussis, and Japanese encephalitis vaccines. He had experienced varicella, measles, scarlet fever, and rubella in childhood, all of which resolved in normal course. He had experienced otitis media on 1 side at the age of 11 years and then had mild respiratory infections (otitis media, sinusitis, and bronchitis).

At the age of 33 years, he was found to have hypogammaglobulinemia (IgG level, 278 mg/dL) during preoperative screening for bone fracture after an accident. He then underwent immunoglobulin substitution temporarily for the operations; 1 year later, his immunoglobulin levels were as follows: IgG, 333mg/dL; IgA, 3mg/dL; IgM, 10mg/dL; IgD, <0.6 mg/dL; and

TABLE I. Laboratory data of the patient

	Patient's value	Normal range
Differentiation of WBCs (at age 37 y)		
All white blood cells	6,600/μL	3,500-8,700 /μL
Neutrophils	58%	42%-74%
Lymphocytes	36%	25%-45%
Monocytes	3%	4%-7%
Eosinophils	3%	1%-5%
Immunoglobulin level (at age 38 y)		
IgG	310 mg/dL	820-1,740
IgA	4 mg/dL	90-400
IgM	10 mg/dL	31-200
IgE	38 mg/dL	<170
Lymphocyte subpopulation (at age 49 y)		*
T-cell		
$CD3^+$(% of all lymphocytes)	72.2%	68.4% ± 9.3%
$CD4^+$(% of $CD3^+$ lymphocytes)	64.4%	59.7% ± 8.0%
$CD8^+$(% of $CD3^+$ lymphocytes)	28.8%	32.4% ± 7.4%
$CD45RO^+$(% of $CD4^+$ lymphocytes)	43.4%	31.7% ± 6.5%
$CD45RO^+$(% of $CD8^+$ lymphocytes)	53.0%	11.4% ± 5.5%
NK cell		
$CD16^+CD56^+$(% of all lymphocytes)	6.0%	15.4% ± 6.3%
B-cell		
$CD19^+CD20^+$ (% of lymphocytes)	15.4%	6.3% ± 5.6%
$CD24^+CD38^+$ transitional B cells (% of $CD19^+$ lymphocytes)	3.8%	5.2% ± 2.0%
IgM^+CD27^+ memory B cells (of $CD19^+$ lymphocytes)	43.7%	14.2% ± 4.9%
IgD^-IgM^- class switch memory B cells (of $CD19^+$ lymphocytes)	5.6%	15.1% ± 5.0%
IgG^+CD27^+ memory B cells (of $CD19^+$ lymphocytes)	3.3%	2.8% ± 2.6%
IgA^+CD27^+ memory B cells (of $CD19^+$ lymphocytes)	4.9%	4.7% ± 1.0%
$CD24^-CD38^+$ plasmablasts (of $CD19^+$ lymphocytes)	0.1%	1.0% ± 0.27%
$IgM^+IgD^{low}CD27^+$ memory B cells (MZB) (of $CD19^+$ lymphocytes)	33.7%	7.9% ± 5.4%
Monocyte		
$CD16^-CD14^+$ (% of monocytes)	80.8%	77.5% ± 11.3%
$CD16^+$ (% of monocytes)	5.3%	8.1% ± 4.4%
Lymphocyte stimulation tests (at age 39 y)		
PHA	47,472/653 cpm (SI = 72.7)	
ConA	43,540/653 cpm (SI = 66.7)	
T- and B-cell neogenesis marker (at age 49 y)		
TREC	5.3×10^3/μg of DNA	
KREC	7.4×10^4/μg of DNA	

ConA, Concanavalin A; *KREC*, κ-deleting recombination excision circle; *MZB*, marginal zone B cell; *NK*, natural killer; *SI*, stimulation index; *TREC*, T-cell receptor excision circle; *WBC*, white blood cell.
*n = 10.

IgE, 59 IU/mL. After this episode, his hypogammaglobulinemia was observed without severe infection. As he has had no frequent infection, normal T- and B-cell levels, and PHA (stimulation index = 413) and concanavalin A (stimulation index = 190) blastgenesis, he was observed without immunoglobulin substitution. At the age of 43 years, his IgG levels decreased to 241 mg/dL. He had mild infection and was diagnosed with CVID. Intravenous immunoglobulin substitution was initiated, and the infection improved after the treatment. However, he developed multiple alopecia areata at the age of 48 years; it was treated with prednisolone for 3 months and resolved. No relapse was observed until now. He did not show any other autoimmune symptoms.

At the age of 50 years, he was referred to our hospital for further examination and treatment. T-cell receptor recombination excision circles and signal joint immunoglobulin κ-deleting recombination excision circles were within the normal range[30] (Table I). Additional testing of humoral antibody responses was not conducted because the patient was receiving immunoglobulin supplementation. Lung computed tomography revealed mild chronic bronchitis and bilateral small nodules. Lung biopsy was not performed. His trough level of IgG at the first visit was 446 mg/dL (IgA level, <5 mg/dL; IgM level, 3 mg/dL). He continued to receive intravenous immunoglobulin substitution (73-220 mg/kg per month), and his trough level was 550 to 730 mg/dL. We subsequently switched to subcutaneous immunoglobulin (58-87 mg/kg per week), and his IgG level was recently 750 to 850 mg/dL. He had mild chronic sinusitis, which was treated with a low dose of clarithromycin (200 mg per day), and a new quinolone was used when his sinusitis was exacerbated. No bronchitis, pneumonia, or other severe infections have been observed to date.

We performed whole exome sequencing in this patient. We focused on homozygous variants because of his consanguinity and identified a candidate only in *TNFSF13* encoding APRIL after filtration as described in the Methods section (Fig 1, *B* and *C*

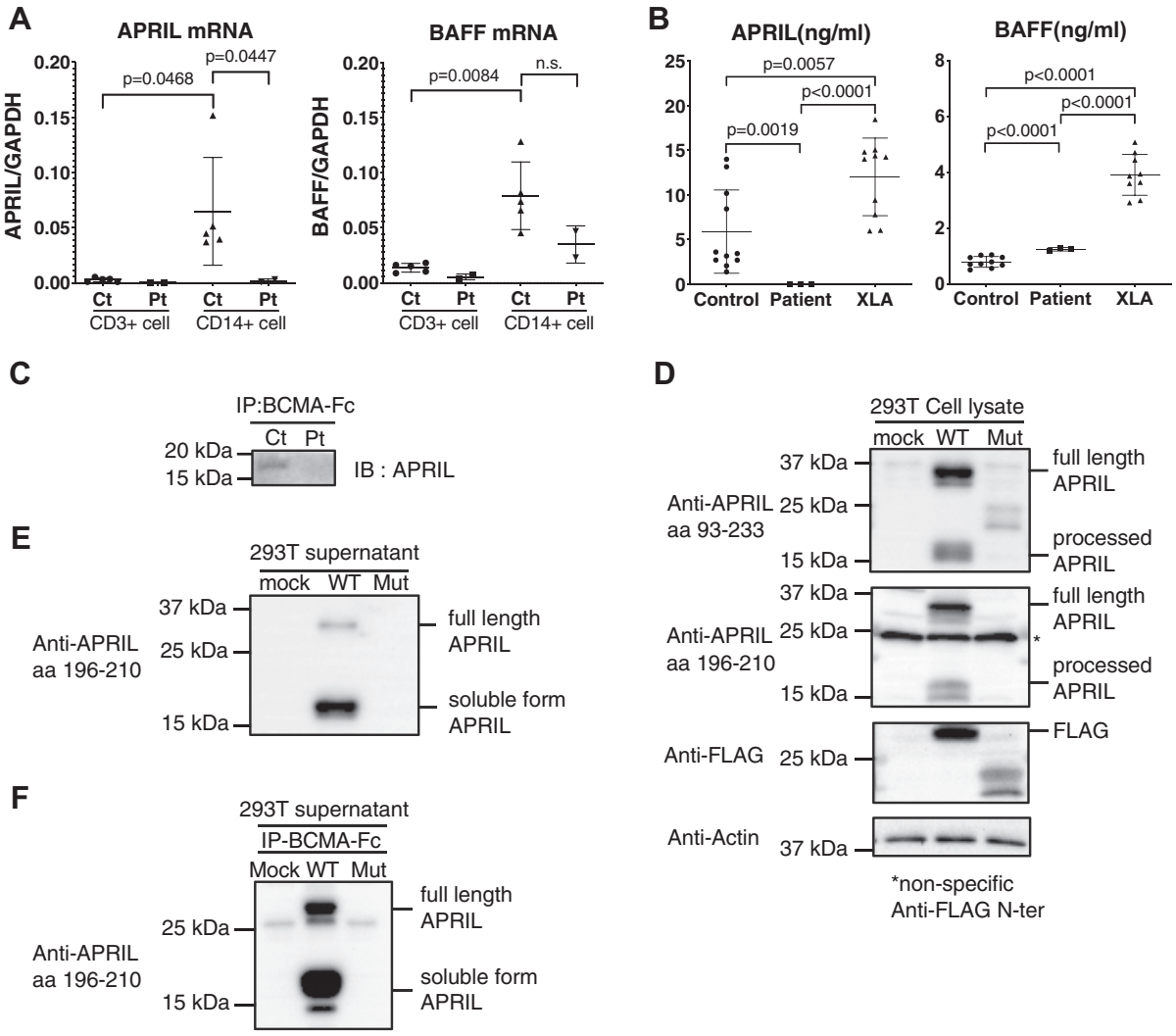

FIG 2. Neither mRNA nor APRIL protein were detected in the patient (Pt). **A,** The transcripts of APRIL (*left panel*) and BAFF (*right panel*) were analyzed by quantitative PCR. Total RNA was purified from MACS-sorted $CD3^+$ T cells and $CD14^+$ monocytes from the healthy controls (Cts) (n = 5) and the patient. **B,** APRIL and BAFF proteins from the plasma of the healthy controls (n = 11), the patient, and the patients with X-linked agammaglobulinemia (XLA) (n = 10) were analyzed by ELISA. **C,** APRIL protein was immunoprecipitated with BCMA-Fc and quantified by Western blotting with APRIL-specific antibody. **D,** APRIL mRNA from the healthy control (WT) and the patient (mutant [Mut]) was transfected into HEK293T cells, and the lysate was immunoblotted with anti-APRIL, anti-FLAG, and anti-actin antibodies. **E,** Immunoblot analysis of the soluble form of APRIL in the supernatants from HEK293T cells transfected with mock vector or WT or Mut APRIL. **F,** A soluble form of APRIL was immunoprecipitated with BCMA-Fc from the supernatants of HEK293T cells transfected with mock vector or WT or Mut APRIL and quantified by immunoblot analysis with anti-APRIL antibody. All statistical analyses depicted in this figure were performed by using the Welch *t* test.

and see Table E1 in this article's Online Repository at www.jacionline.org). APRIL is highly conserved among mammals (see Fig E1 in this article's Online Repository at www.jacionline.org), and this variant in *TNFSF13* (NM_003808.3: c.507_508delC, p.Leu170Cysfs*5) was a frameshift mutation leading to premature termination at Val174 amino acids of the APRIL protein (Fig 1, *D*). The mutation of the patient's parents was also validated by using Sanger sequencing. We found the heterozygous mutation of c.507_508delC in the parents (see Fig E2 in this article's Online Repository at www.jacionline.org). Heterozygous mutation of APRIL in humans may be related to some disease because the probability of loss of function intolerance (pLI) score of APRIL is 0.82 in gnomAD. On the basis of our observation in the patient's family, however, haploinsufficiency does not seem to be related to susceptibility to infections.

mRNA and protein expression of APRIL was absent in the patient's blood cells

We analyzed mRNA expression of *APRIL* and *BAFF* in $CD14^+$ monocytes and $CD3^+$ T cells from the patient and HCs (Fig 2, *A*). Although *APRIL* and *BAFF* were expressed strongly in

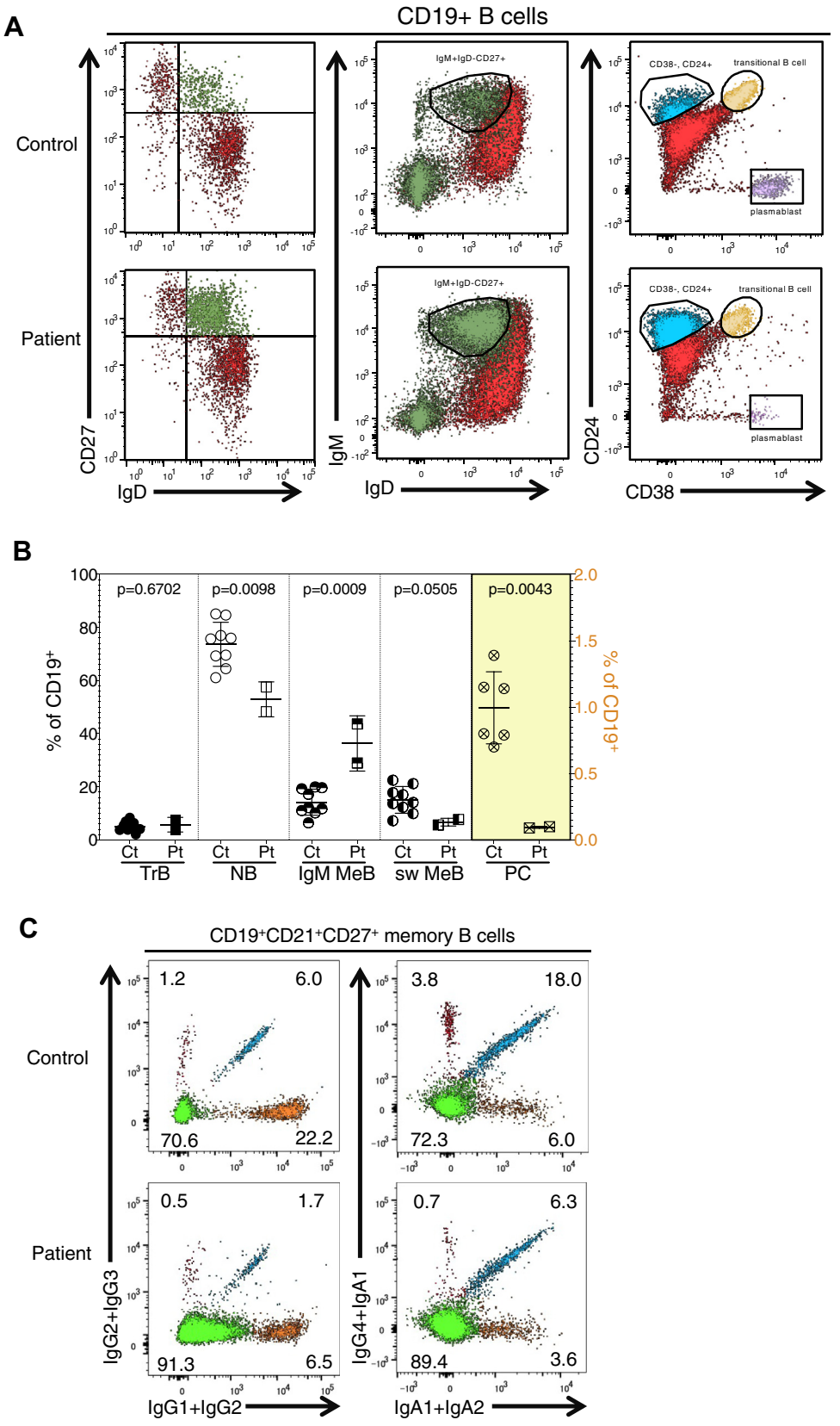

FIG 3. Fluorescence-activated cell sorting analysis of the APRIL-deficient patient. **A,** Representative fluorescence-activated cell sorting analysis of cells from the control (Ct) and the patient (Pt). **B,** Proportion of B-cell subsets from the patient and the control (n = 6 for plasmacytes; n = 9 for the other components of B cells). Yellow background shows data that are plotted on the right *y* axis. **C** and **D,** Comparison of the Ig subset of memory B cells in the controls (n = 4) and the patient. **E,** BAFFR, TACI, and BCMA expression in naive B cells, memory B cells, and plasmacytes in the control and patient. All statistical analyses depicted in this figure were performed by using the Student *t* test.

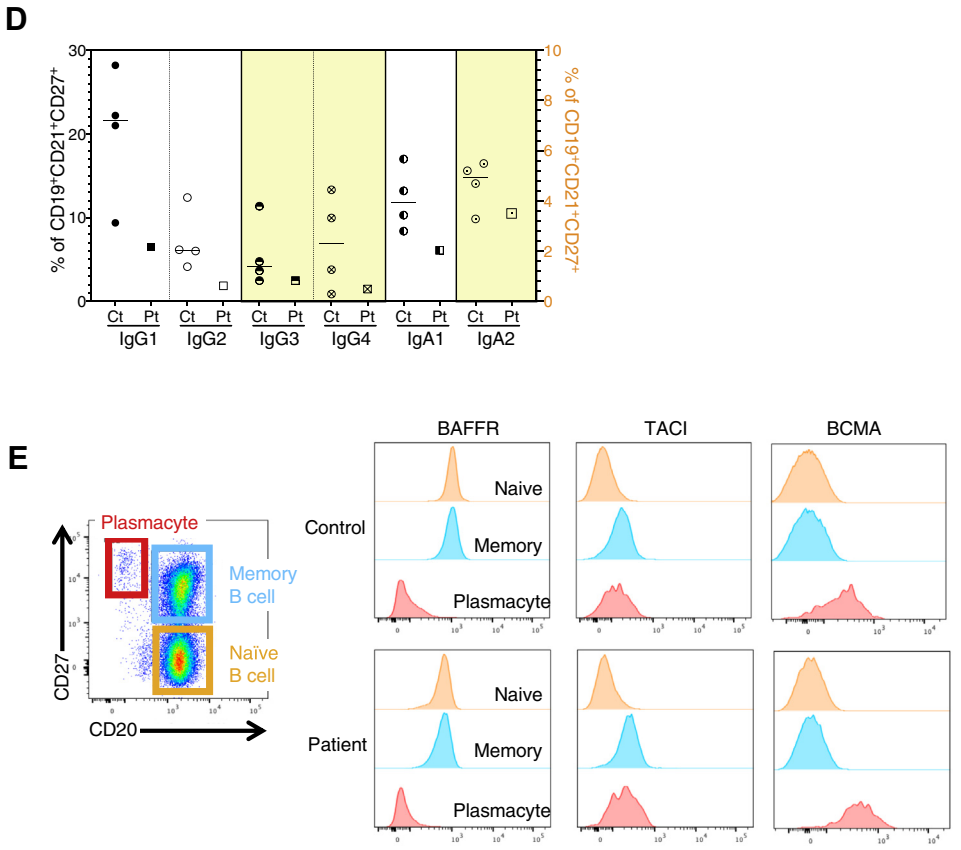

FIG 3. *(Continued).*

monocytes from the HC, the patient monocytes lacked the expression of *APRIL* mRNA. In agreement with this result, the plasma concentration of APRIL protein assessed by ELISA was decreased in the patient compared with in both the HC and the disease control (patients with X-linked agammaglobulinemia), who showed an elevated APRIL concentration as reported previously[23] (Fig 2, *B*). Interestingly, the concentration of BAFF protein was significantly elevated in the patient's plasma. For further verification of the lack of APRIL protein in the patient's plasma, we performed immunoprecipitation by using BCMA-Fc. The APRIL protein was immunoprecipitated from the control plasma, whereas the patient's plasma showed a complete lack of immunoprecipitated APRIL protein (Fig 2, *C*). These findings suggested that *APRIL* mRNA expression was strongly suppressed, likely by nonsense-mediated mRNA decay leading to loss of APRIL protein in patient's blood cells.

To confirm the impact of the mutation on APRIL expression, we further performed transient expression experiments. Both full-length (32-kD) and processed (17-kD) APRIL were detected in WT transfected cells (Fig 2, *D*). In contrast, only a small amount of truncated APRIL protein was detected in mutant transfected cells. We next assessed secreted APRIL and confirmed a complete defect of secreted APRIL protein in the mutant supernatant (Fig 2, *E* and *F*). Taken together, the homozygous frameshift mutation caused severe impairment of APRIL mRNA expression and a complete lack of normal APRIL protein expression.

Lack of plasmacytes and increased marginal zone B cells in the APRIL-deficient patient

The patient showed a normal number of $CD19^+$ B cells (15.4% of his total lymphocytes [425/μL]), whereas his level of IgM^+CD27^+ memory B cells was markedly increased (43.7% compared with the level in the HC; 14.2% of total B cells). In particular, almost all of the IgM^+ memory cells showed diminished expression of IgD, which was a typical finding of circulating marginal zone (MZ) B cells in the blood[31,32] (Fig 3, *A* and *B* and Table I). The frequency of $IgM^-IgD^-CD27^+$ class-switched memory B cells was slightly decreased in the patient (Fig 3, *A* and Table I). The patient also showed an approximately 10-fold decrease in the frequency of $CD24^-CD38^+$ plasmacytes (0.1% compared with 1.0% in the HCs). Immunoglobulin heavy chain isotypes of memory B cells were analyzed by fluorescence-activated cell sorting.[25] The numbers of IgG1, IgG2, IgG4, and IgA1 B cells were lower in the patient than in the HC (Fig 3, *C* and *D*), reflecting an increased percentage of IgM^+ MZ B cells among the total memory B cells. Whereas marked alteration in B-cell subsets was found in the patient, no obvious difference was observed in T cells, natural killer cells, or monocytes between the patient and HC (Table I).

We then analyzed surface expression of TNF receptor family molecules in B subsets (naive, memory, and plasmacytes). The levels of expression of APRIL-binding receptors in the patient were comparable to those in controls (Fig 3, *E*).

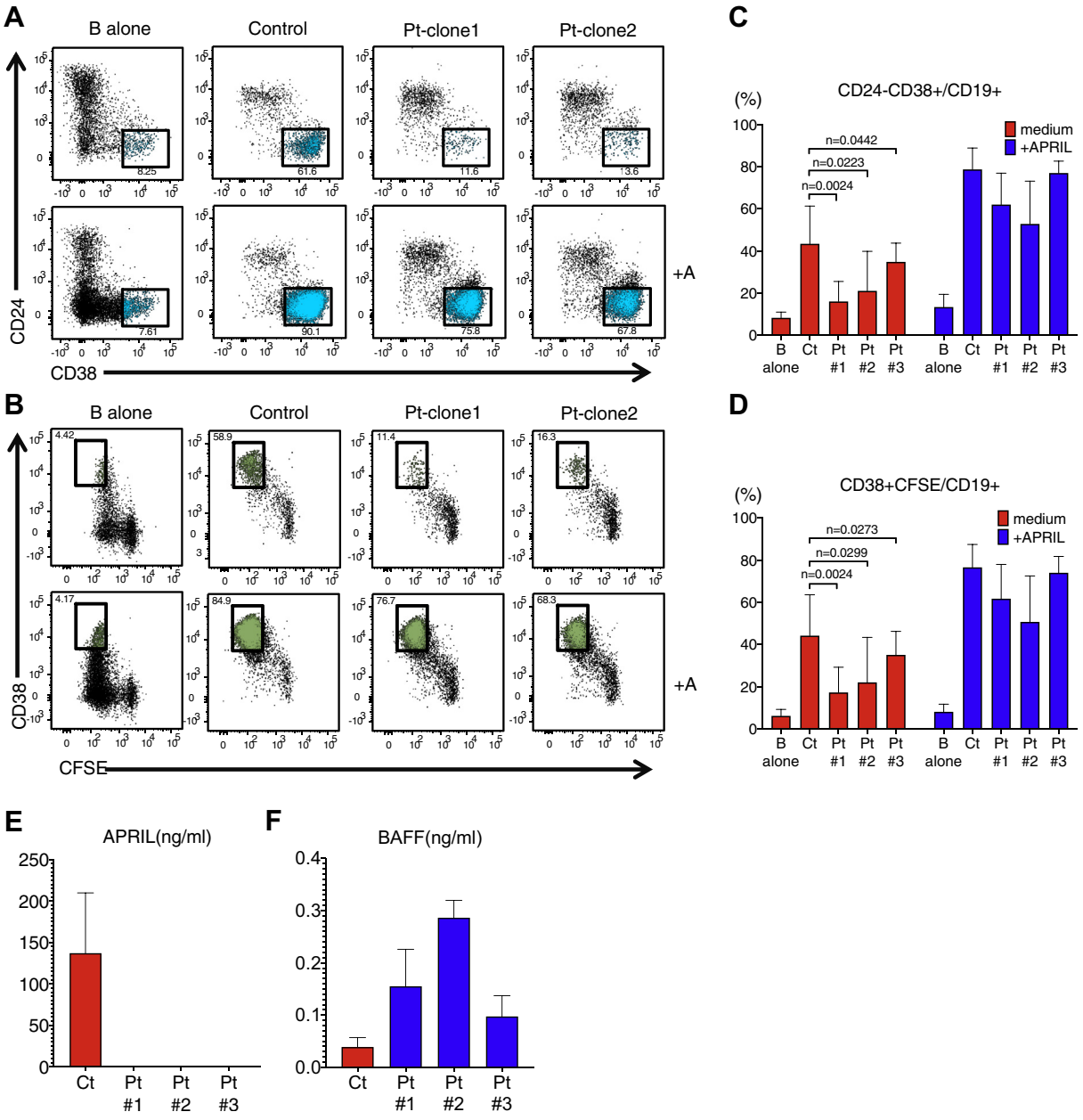

FIG 4. The iPSC-derived moDCs of the patient (Pt) could not induce plasmacyte differentiation from memory B cells. **A,** $CD19^+CD20^+CD27^+$ memory B cells from the control (Ct) were cocultured with iPS-moDCs from a healthy control or the patient (clone 1 and 2) for 7 days. The designator *B alone* indicates the culture without iPS-moDC. The plus sign before the word *APRIL* indicates the addition of recombinant APRIL in the culture. $CD24^-38^+$ cells indicate the plasmacytes. **B,** $CD24^-CD38^+$CFSE-negative cells indicate the proliferating plasmacytes after the culture as described in (**A**). **C** and **D**, Proportions of $CD24^-CD38^+$ cells and $CD38^+$ CFSE-negative cells are indicated (control clone, n = 1; patient clones, n = 3). **E** and **F**, APRIL and BAFF levels in the supernatant of iPS-moDCs from 1 clone of the control and 3 clones of the patient. All statistical analyses depicted in this figure were performed by using the Student *t* test.

Normal B-cell receptor repertoire, somatic hypermutation, and class-switch recombination in APRIL deficiency

To analyze germinal center reaction of B cells from the patient, we performed multiplex PCR–based sequencing of *IgH* genes encoding BCR heavy chain using next-generation sequencing. The results showed that usage of V and J segments in VDJ recombination (BCR repertoire) (see Fig E3, *A* in this article's Online Repository at www.jacionline.org), frequency of somatic hypermutation (SHM) in V segments (see Fig E3, *B*), and CSR profiles (see Fig E3, *C*) were comparable between the patient and control. SHM and CSR occurred in germinal centers of secondary lymphoid organs depending on the antigen-specific T-cell stimulation. According to the results, the APRIL-deficient patient appeared normal with respect to germinal center reaction *in vivo*.

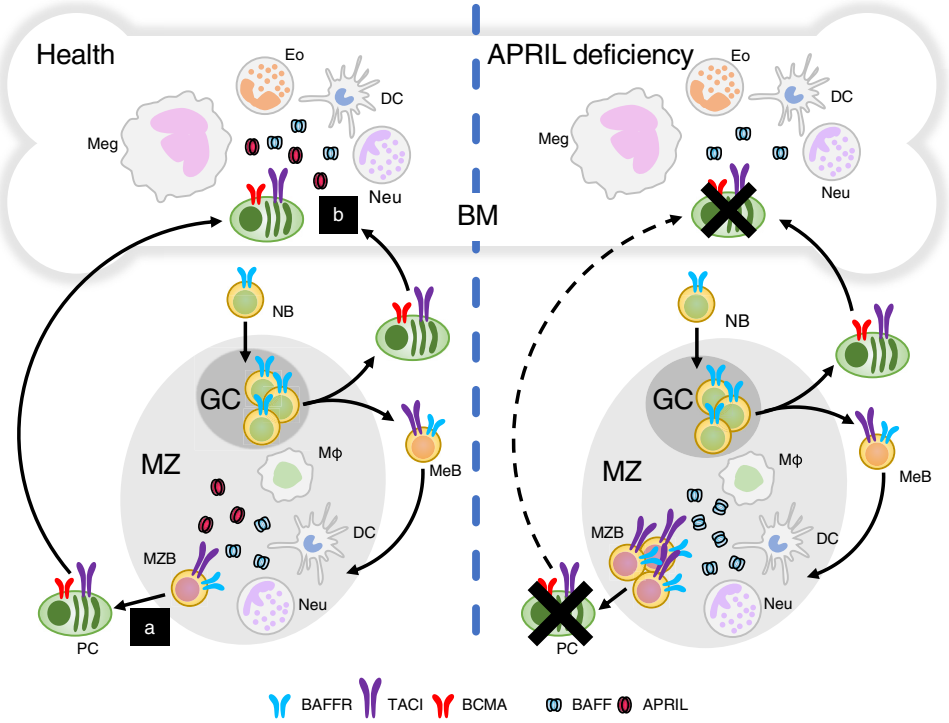

FIG 5. Effect of APRIL deficiency in plasmacyte (PC) differentiation and maintenance. Normal B-cell differentiation from memory B cells or MZ B (MZB) cells to plasmacytes is illustrated on the left side, and defective plasmacyte differentiation and maintenance is depicted on the right side. We assumed that the functional defect in B cells due to APRIL deficiency occurs at plasmacyte differentiation from memory/MZ B cells **(A)** and lifelong maintenance in the bone marrow **(B)**. *Eo*, Eosinophil; *GC*, germinal center; *MeB*, memory B cell; *Mφ*, macrophage; *MZ*, marginal zone; *MZB*, marginal zone B cell; *NB*, naive B cell; *Neu*, neutrophil.

To confirm these findings, we performed *in vitro* CSR assay. We isolated $CD19^+CD20^+CD27^-$ naive and $CD19^+CD20^+CD27^+$ memory B cells from the patient and the HCs and stimulated them with sCD40L and IL-21, mimicking the stimulation from follicular helper T (Tfh) cells at the germinal center. Both naive and memory B cells from the patient showed normal proliferation (see Fig E3, *D*) and immunoglobulin production (see Fig E3, *E*) when compared with proliferation and immunoglobulin production in the controls. Thus, the patient's peripheral blood B cells were able to react to T-cell stimulation normally *in vitro*.

APRIL production from monocyte-linage cells is critical for plasmacyte differentiation from memory B cells

APRIL and BAFF production from MZ dendritic cells (DCs) and macrophages are necessary for maintenance of memory B cells and their differentiation to plasmacytes[33] in a T-cell–independent manner. Thus, we hypothesized that the loss of APRIL stimulation from the myeloid linage cells at MZ would critically affect the B-cell terminal maturation even though BAFF secretion was preserved. To assess this hypothesis, we performed B-cell and monocyte-derived DC (moDC) coculture assay, which was reported to induce plasmacyte differentiation from B cells.[34] First, we differentiated moDCs from control and patient iPSCs. The production of APRIL was confirmed in moDCs from HC iPSCs, whereas moDCs from the patient's iPSCs showed completely abolished APRIL production (Fig 4, *E*). In contrast, BAFF production was preserved in the moDCs from both patient and control iPSCs (Fig 4, *F*). As reported previously,[34] memory B cells proliferated and differentiated to $CD24^-CD38^+$ plasmacytes after coculture with control moDCs. However, this B-cell activation was impaired after coculture with the patient's iPSC-moDCs (Fig 4, *A-D*). To test whether the reduced differentiating efficacy was due to the absence of APRIL from the moDCs, we supplied recombinant APRIL to the coculture system. All patient and control clone experiments were performed at least 5 times. As shown in Fig 4, the supplementation of recombinant APRIL to the patient's iPSC-moDCs rescued the impaired proliferation and plasmacyte differentiation (Fig 4, *A-D*). Few plasmacytes observed after coculture with iPSC-moDCs from the patient were able to be differentiated owing to the presence of BAFF protein.

Collectively, APRIL production from monocyte-lineage cells plays a pivotal role for B-cell differentiation, and this finding partly explains the pathogenesis of APRIL deficiency.

DISCUSSION

APRIL and BAFF are TNF ligand superfamily molecules secreted mainly by monocyte-lineage cells (monocytes, macrophages, and DCs) and they function together to induce B-cell terminal maturation.[21,35,36] Their receptors on the B-cell surface overlap, and their functions are mostly complimentary. Furthermore, these proteins can form an APRIL-BAFF heterotrimer.

Thus, it was difficult to discuss APRIL function separately from BAFF function. However, this study revealed the fundamental roles of APRIL in lifelong immunologic memory and antibody maintenance, which cannot be compensated by BAFF, especially in humans.

According to previous studies performed in mouse models, APRIL deficiency did not result in any gross abnormalities in the immune system *in vivo*. Varfolomeev et al[37] reported normal B-cell immunity, including B-cell differentiation, serum immunoglobulin titer, and antigen-specific antibody secretion. Castigli et al[38] also reported normal or increased antibody reaction after immunization and slightly reduced IgA class switching. Belnoue et al[20] revealed that APRIL from bone marrow cells (mainly from resident macrophages) promoted plasmacyte survival in cell culture assays *in vitro* and artificial cell transfer assays *in vivo*. However, the same study noted that APRIL-KO mice showed a normal number of immunoglobulin-secreting cells (namely, plasmacytes). On the other hand, a defect in the receptors for APRIL and BAFF causes marked B-cell dysfunction in knockout mice. BCMA deficiency leads to defective long-lived plasmacytes in the bone marrow.[39] Another study suggested that antagonizing TACI inhibited plasmacyte differentiation from splenic MZ B cells.[33]

As in the studies in mice, the patient's lymphocyte subset showed a normal number of B cells and lacked plasmacytes. Unlike in the mouse studies, however, the patient developed hypogammaglobulinemia of all isotypes. Interestingly, the patient's circulating $IgM^{+}IgD^{low}CD27^{+}$ MZ B–cell count was significantly increased. MZ B cells are recirculating memory B cells that have passed the germinal center reaction, and they reside in the MZ of the spleen, Peyer patch of the small intestine, and systemic lymph nodes for reacting to secondary stimulations and infections.[40] MZ B cells are continuously activated by APRIL and other stimulation from MZ macrophages, neutrophils, and DCs, depending on continuous pathogen infiltration from a commensal.[41] As a result of lifelong steady-state stimulation, MZ B cells would sustain constant plasmacyte differentiation, maintenance, and baseline immunoglobulin production in an antigen-independent manner (Fig 5, *A*). Although we could not obtain lymphoid tissue from the patient to observe the histologic structure, the increased circulating MZ B cells might indicate a defect in plasmacyte differentiation, resulting in accumulation of this cell fraction owing to lack of APRIL. According to the results, substitution of APRIL protein could be a fundamental therapy for the patient. Moreover, cells from the bone marrow niche, especially eosinophils, were reported as a main source of APRIL, and they were essential for the maintenance of plasmacytes (Fig 5, *B*).[42] As a result, loss of APRIL secretion from bone marrow eosinophils would also contribute to the reduction of plasmacytes in the patient.

When naive B cells encounter specific antigens, they become activated and form germinal centers with cognate Tfh cells. In the germinal center, B cells are stimulated by Tfh cells via the interaction between CD40 and CD40L and cytokines such as IL-21. They then undergo the BCR affinity maturation processes, including SHM and CSR, and become memory B cells. The patient's peripheral blood B cells showed normal BCR repertoire and SHM, and they were able to undergo normal CSR by T-cell stimulation *in vitro*. Taken together, these findings indicate that the APRIL-deficient patient ought to have reacted to the T-cell–dependent antigens in the germinal center and produced antigen-specific antibodies at least for the short term. In line with this speculation, the patient showed milder symptoms without severe infections before immunoglobulin substitution. It is impossible to prove now, but we suspect that the main pathogenicity is not in the germinal center reaction but rather in the differentiation of memory B cells to plasmacytes and their lifelong maintenance. Moreover, the antibody titer in the patient's blood might have been normal had he had a sufficient naive B-cell pool at a young age.

Elevated APRIL concentration has been reported in several autoimmune diseases, such as SLE and rheumatoid arthritis.[43,44] According to this study and others,[34,45] APRIL stimulates memory B cells to differentiate into plasmacytes and secrete antibodies in an antigen-independent manner. This pathophysiology explains how an elevated APRIL level induces the aberrant antibody secretion in such diseases. This study revealed the opposite aspect of APRIL pathology in humans; that is, very low levels of APRIL can also cause disease. Strict regulation of APRIL is thus essential for maintaining lifelong humoral immunity.

In conclusion, APRIL deficiency in a human revealed an essential function of APRIL for lifelong maintenance of plasmacyte differentiation and immunoglobulin production that had not been elucidated in previous animal studies. According to our coculture assays, appropriate substitution of APRIL protein might be the fundamental treatment for this hereditary disease.

We thank to the patient, who provided the samples, as well as Dr Hajime Saito at Hitachinaka General Hospital and the late Professor Emeritus Toshio Miyawaki at University of Toyama, who consulted with us on the patient. We are grateful to Ms Mika Nagase, Ms Naomi Terada-Takahashi, and Ms Yuko Kutami for their technical assistance. We thank the staff of the Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, for supporting this work.

Key messages

- **The first APRIL deficiency was found in an adult patient with CVID.**
- **On the basis of the findings in the APRIL-deficient patient, APRIL is critical for lifelong maintenance of plasmacytes to produce immunoglobulins in humans.**

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Protein Acc.	Organism		Identity (%) Protein (vs. H.sapiens)
NP_003799.1	H.sapiens	1 MPASSPFLLAPKGPPGNMGGPVREPALSVALWLSWGAALGAVACAMALLT 50	-
NP_001192059.1	P.troglodytes	1 MPASSPFLLAPKGPPGNMGGPVREPALSVALWLSWGAALGAVACAMALLT 50	99.6
NP_001192098.1	C.lupus	1 MPASSPSLLAPKGPPGDMGGPVREPALSVALWLSWGAALGAVACAMALLT 50	87.2
NP_001029819.1	B.taurus	1 MPASSPSLLSPKGPQGDMGGPVREPALSVALWLSWGAALGAVACAMVLLT 50	83.6
NP_076006.2	M.musculus	1 MPASSP---------- GHMGGSVREPALSVALWLSWGAVLGAVTCAVALLI 41	81.2
NP_001009623.1	R.norvegicus	1 MPASSP---------- GNMGGSVREPALSVTLWLSWGAVLGAVTCAVALLI 41	79.9
XP_004919464.1	X.tropicalis	1 ---------- MSHMVICGSV ---SLARGLWGGTGAWLGVLICLIALMC 35	38.6
NP_003799.1	H.sapiens	51 QQTELQSLRREVSRLQGTGGPSQNGEGYPWQSLPEQSSDALEAWENGERS 100	
NP_001192059.1	P.troglodytes	51 QQTELQSLRREVSRLQRTGGPSQNGEGYPWQSLPEQSSDALEAWENGERS 100	
NP_001192098.1	C.lupus	51 QQTELQNLRREVARLQRTGGPSEKEEGYPWLSLQEQSPDALEAWENGERS 100	
NP_001029819.1	B.taurus	51 QQTELQTLRREVTRLQRNGGPSEKGEGNPWLNLQEQSPDGTEGQENGERS 100	
NP_076006.2	M.musculus	42 QQTELQSLRREVSRLQRSGGPSQKQGERPWQS LWEQSPDVLEAWKDGAKS 91	
NP_001009623.1	R.norvegicus	42 QQAELQSLRREVSRLQRSGGASQKRGEPPWQSLWEQSPDVLGAWKDGAKS 91	
XP_004919464.1	X.tropicalis	36 QSIHIGNLQVELSLMRKSQEEPRPEVKKHHNTKEEGLAICLRQKRDLTGA 85	
NP_003799.1	H.sapiens	101 RKRRAVLTQKQKKQHSVLHLVPINATSKDDSDVTEVMWQPALRRGRGLQA 150	
NP_001192059.1	P.troglodytes	101 RKRRAVLTQKQKKQHSVLHLVPINATSKDDSDVTEVMWQPALRRGRGLQA 150	
NP_001192098.1	C.lupus	101 RRKRAALIHKQKKKHSVLHLVPINITSKEDSDVTEVMWQPALKRGRGLEA 150	
NP_001029819.1	B.taurus	101 RRRRAVLTRKHKKKRSVLHLVPINITSKEDSDVTEVMWQPALQRGRGLEA 150	
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NP_001192098.1	C.lupus	151 QGYVVRVWDSGIYLLYSQV LFHDVTFTMGQVVSR --EG-QGRQETLFRCI 197	
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NP_076006.2	M.musculus	142 QGDIVRVWDTGIYLLYSQV LFHDVTFTMGQVVSR --EG-QGRRETLFRCI 188	
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XP_004919464.1	X.tropicalis	130 QGVNVTVRNSGIYSVYSQV LYNDSRFTMGHLITRKLEGLPGGEKVLLRCV 179	
NP_003799.1	H.sapiens	198 RSMPSHPDRAYNSCYSAGVFHLHQGDILSVIIPRARAKLNLSPHGTFLGF 247	
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NP_001009623.1	R.norvegicus	188 KSMPSDPDRAYNSCYSAGVFHLHQGDIITVKIPRANAKLSLSPHGTFLGF 237	
XP_004919464.1	X.tropicalis	180 QSMPPNESMAYNTCYSAGVFQLQKGDSISLVIPRSNANLDARGEATFLGL 229	
NP_003799.1	H.sapiens	248 VKL 250	
NP_001192059.1	P.troglodytes	248 VKL 250	
NP_001192098.1	C.lupus	248 VKL 250	
NP_001029819.1	B.taurus	248 VKL 250	
NP_076006.2	M.musculus	239 VKL 241	
NP_001009623.1	R.norvegicus	238 VKL 240	
XP_004919464.1	X.tropicalis	230 VRL 232	

FIG E1. APRIL protein sequence alignment among humans and 6 organisms.

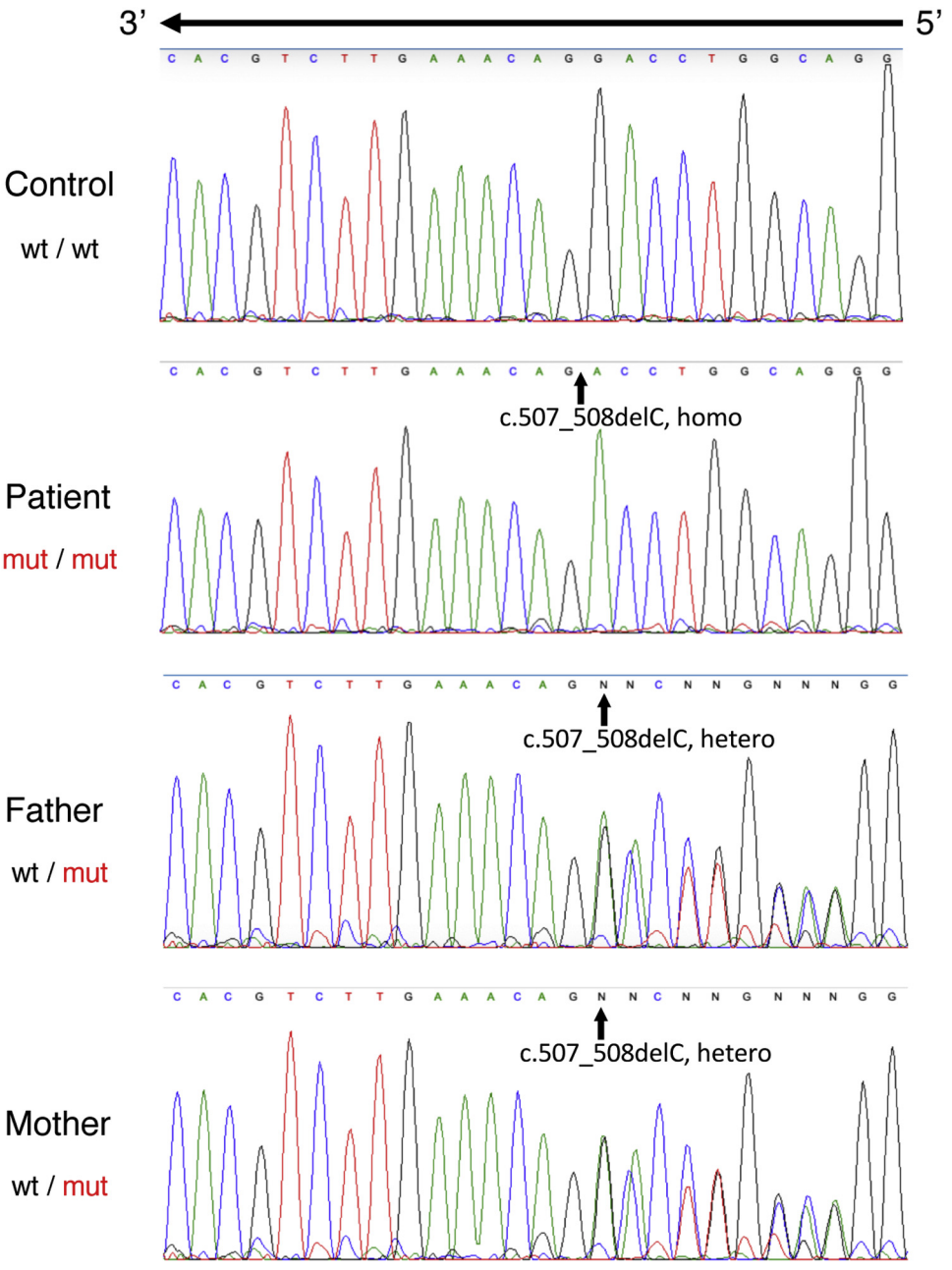

FIG E2. Sanger sequence results of the control, the patient, and his parents. *hetero*, Heterozygous, *homo*, homozygous; *mut*, mutant.

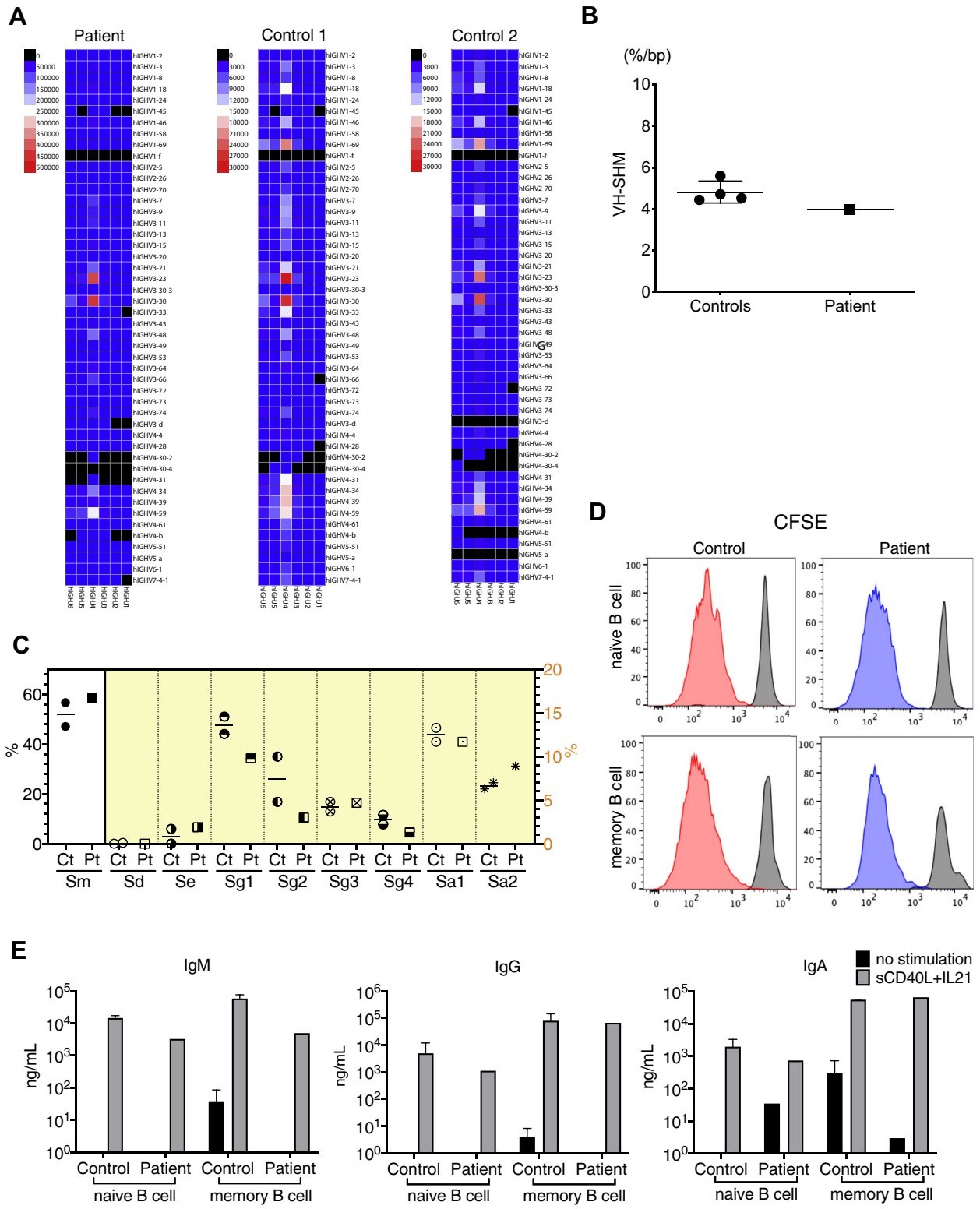

FIG E3. B-cell repertoire, SHM proliferation, and CSR. **A,** The frequency of V and J region combinations is illustrated in the 2-dimensional heatmaps from the patient (Pt) and age-matched controls (Cts) (n = 2). **B,** SHM of V region (VH-SHM) in the top 20 clones in the B cells of the controls (n = 4) and patient. **C,** IgH subclass subpopulations determined by PCR of the switch regions. **D,** Proliferation assay of naive and memory B cells from a healthy control and the patient by using CFSE after stimulation by sCD40L and IL-21 for 7 days (gated on $CD19^+$ cells). **E,** Cytometric Bead Array assay of total IgM, IgG, and IgA production induced by sCD40L and IL-21 in naive and memory B cells from the controls (n = 3) and the patient for 12 days.

TABLE E1. Ten homozygous exon variants of the patient after filtration

Gene	Chr	Start	End	Ref	Alt	Hzyg	AAChange.refGene	HGVD	PV
C6orf223	Chr6	43970503	43970503	—	GCGGCG	Hom	C6orf223: NM_001324369: exon3: c.33_34insGCGGCG: p.R11delinsRAA	—	0.3599
CNOT2	Chr12	70747694	70747694	A	—	Hom	CNOT2: NM_001199303: exon16: c.1622delA: p.X541X	—	0.1648
FADS6	Chr17	72889676	72889676	—	GGCTCCGTAGGTTCCATGGGCTCCGTA GGTTCCATGGGCTCCGTAGGTTCCATC	Hom	FADS6:NM_178128:exon1:c.17_18insGATGGAACCTAC GGAGCCCATGGAACCTACGGAGCCCATGGAACCT ACGGAGCC:p.P6delinsPMEPTEPMEPTEPMEPTEP	—	0.6854
FDFT1	Chr8	11666219	11666224	TCCCAC	—	Hom	FDFT1:NM_001287750:exon1:c.193_198del:p.65_66del	—	0.9863
MYBBP1A	**Chr17**	**4458516**	**4458516**	**A**	**G**	**Hom**	**MYBBP1A:NM_001105538:exon1:c.104T>C:p.F35S**	**0.002737**	**0.0041**
MYH8	**Chr17**	**10310240**	**10310240**	**C**	**—**	**Hom**	**MYH8:NM_002472:exon18:c.2022delG:p.R674fs**	**0.004662**	**0.0041**
RP1L1	Chr8	10467589	10467589	—	CCTCTAACTGCACCCTCTCTTCTTGC AGCCCTTCTATTACTTTAGTCC	Hom	RP1L1:NM_178857:exon4:c.4018_4019insGGACTAA AGTAATAGAAGGGCTGCAAGAAGAGAGGGTGC AGTTAGAGG:p.E1340delinsGTKVIEGLQEERVQLEE	—	0.3805
SLAIN1	Chr13	78272267	78272267	—	GG	Hom	SLAIN1:NM_001242868:exon1:c.219_220insGG:p.A73fs	—	0.9258
TNFSF13	**Chr17**	**7463669**	**7463669**	**C**	**—**	**Hom**	**TNFSF13:NM_003808:exon5:c.507delC:p.V169fs**	**—**	**0.0027**
TRAK1	Chr3	42251577	42251577	—	GGA	Hom	TRAK1:NM_001265609:exon13:c.1841_1842insGGA: p.T614delinsTE	—	0.9794

The 3 variants after exclusion of private variants in Fig 1, *B* are in boldface. For a description of these homozygous exon variants, see the Methods section and Fig 1, *B*.

Alt, Alternative sequence; *Chr*, chromosome; *HGVD*, Human Genetic Variation Database; *Hzyg*, types of zygosity; *Hom*, homozygous; *PV*, private variants; *Ref*, reference sequence.