RASGRP1 mutation in autoimmune lymphoproliferative syndrome-like disease

Huawei Mao, MD, PhD,[a,b,c] Wanling Yang, PhD,[b,c] Sylvain Latour, PhD,[d] Jing Yang, PhD,[b,c] Sarah Winter, PhD,[d] Jian Zheng, PhD,[b] Ke Ni, MPhil,[b] Minmin Lv, MPhil,[c] Chenjing Liu, MPhil,[a] Hongmei Huang, MD,[a] Koon-Wing Chan, MPhil,[b] Pamela Pui-Wah Lee, MD,[b,c] Wenwei Tu, PhD,[b,c] Alain Fischer, MD, PhD,[e] and Yu-Lung Lau, MD[a,b,c] *Shenzhen and Hong Kong, China, and Paris, France*

Background: Autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of lymphocyte homeostasis due to impaired apoptosis. It was initially regarded as a very rare disease, but recent studies show that it may be more common than previously thought. Defects in a couple of genes have been identified in a proportion of patients with ALPS, but around one-third of such patients remain undefined genetically.
Objective: We describe 2 siblings presenting with ALPS-like disease. This study aimed to identify the genetic cause responsible for this phenotype.
Methods: Whole-exome sequencing and molecular and functional analyses were used to identify and characterize the genetic defect. Clinical and immunological analysis was also performed and reported.
Results: The 2 patients presented with chronic lymphadenopathy, hepatosplenomegaly, autoimmune hemolytic anemia, immune thrombocytopenia, and the presence of antinuclear autoantibody and other autoantibodies, but normal double-negative T cells. They also suffered from recurrent infections. Novel compound heterozygous mutations of *RASGRP1* encoding Ras guanyl nucleotide releasing protein 1 were identified in the 2 siblings. The mutations impaired T-cell receptor signaling, leading to defective T-cell activation and proliferation, as well as impaired activation-induced cell death of T cells.
Conclusions: This study shows for the first time that *RASGRP1* mutation should be considered in patients with ALPS-like disease. We also propose to investigate the intracellular proteins involved in the T-cell receptor signaling pathway in similar patients but with unknown genetic cause. (J Allergy Clin Immunol 2018;142:595-604.)

Key words: *ALPS-like disease, RasGRP1, immunodeficiency, immune dysregulation, T-cell receptor signaling, genetic defect*

Autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of lymphocyte homeostasis due to impaired apoptosis. It is characterized by nonmalignant lymphoproliferation, autoimmune cytopenia, and increased risk of lymphoma. In addition, the presence of increased $CD3^+TCR\alpha\beta^+CD4^-CD8^-$ ($TCR\alpha\beta^+$) double-negative T (DNT) cells is a hallmark of this disease.[1] Genetic analysis has identified defects in the genes encoding Fas,[2,3] Fas-ligand,[4] and caspase 10[5] in a small number of patients with ALPS. According to the updated diagnostic criteria, patients with autoimmune lymphoproliferation but marginally elevated or normal $TCR\alpha\beta^+$ DNT cells are classified as suffering from ALPS-related disorders. Besides autoimmunity, such patients may present with recurrent infections.[1] Among a proportion of these patients, mutations in caspase 8,[6] NRAS,[7] and KRAS[8] have been identified. ALPS was initially regarded as a very rare disease, but recent studies show that it may be more common than previously thought. It is estimated that around one-third of patients with ALPS(-related) disease remain undefined genetically.

Ras guanyl nucleotide releasing protein 1 (RasGRP1) is a member of Ras guanine nucleotide exchange factors, and specifically activates Ras through the exchange of bound guanosine diphosphate for guanosine triphosphate. It is highly expressed in T cells, and to a lesser extent in B and natural killer cells. Serial studies have demonstrated that RasGRP1 is essential for T-cell development and function through coupling T-cell receptor (TCR) to Ras-mitogen-activated protein kinase signaling pathway. Upon TCR engagement, linker for activation of T cells (LAT) becomes phosphorylated and recruits phospholipase Cγ1 along with other molecules. Activated phospholipase Cγ1 then hydrolyses phosphatidylinositol-(4,5)-bisphosphate into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate. DAG further activates RasGRP1 and then leads to the activation of extracellular signal-regulated kinase (ERK) 1/2, finally the transcription of genes involved in cell proliferation, survival, and other cellular processes.[9-12]

Previous animal studies demonstrated that RasGRP1-deficient mice developed autoimmune lymphoproliferative disease with phenotypes of lymphadenopathy, splenomegaly, and presence of antinuclear autoantibody (ANA).[13-16] The underlying mechanism remains unclear, but the condition was reported to be T-cell–dependent, based on the fact that RasGRP1-deficient nude mice

From [a]the Department of Paediatrics, The University of Hong Kong-Shenzhen Hospital, Shenzhen; [b]the Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Hong Kong; [c]Shenzhen Engineering Laboratory of Primary Immunodeficiency Diagnosis and Therapy, Shenzhen; and [d]the Laboratory of Lymphocyte Activation and Susceptibility to EBV Infection, Inserm UMR 1163 and [e]the Immunology and Pediatric Hematology Department, Necker Children's Hospital, AP-HP, Paris.

This study was supported partly by grants from the National Natural Science Foundation of China (grant no. 81671626 to H.M.), Shenzhen Science, Technology and Innovation Commission (grant no. JCYJ20140411175241066 to H.M.), the Shenzhen Development and Reform Commission (grant no. [2015]164 to Y.-L.L. and H. M.), Hong Kong Health and Medical Research Fund (grant no. 01120846 to W.Y., Y.-L.L., and P.P.-W.L.), and the Society for the Relief of Disabled Children (to Y.-L.L.).

Disclosure of potential conflict of interest: S. Latour is employed by CNRS, France. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 25, 2016; revised September 27, 2017; accepted for publication October 2, 2017.

Available online November 15, 2017.

Corresponding author: Huawei Mao, MD, PhD, Department of Paediatrics, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China. E-mail: maohw@hku-szh.org. Or: Yu-Lung Lau, MD, Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, China. E-mail: lauylung@hku.hk.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749

https://doi.org/10.1016/j.jaci.2017.10.026

Abbreviations used
ALPS: Autoimmune lymphoproliferative syndrome
ANA: Antinuclear autoantibody
DAG: Diacylglycerol
DNT: Double-negative T
ERK: Extracellular signal-regulated kinase
RasGRP1: Ras guanyl nucleotide releasing protein 1
TCR: T-cell receptor
SLE: Systemic lupus erythematous

have significantly reduced serum autoantibodies.[14] Furthermore, patients with systemic lupus erythematous (SLE) were demonstrated to have aberrant *RASGRP1* splice variants, which was correlated with lower levels of RasGRP1 protein in patients' T cells and diminished RasGRP1 activity.[17] These findings strongly suggest that RasGRP1 dysfunction is closely associated with autoimmune disease.

In this study, we report 2 siblings born to nonconsanguineous parents, who presented with ALPS-like disease, including chronic lymphadenopathy, hepatosplenomegaly, autoimmune hemolytic anemia, immune thrombocytopenia, and the presence of ANA and other autoantibodies, but normal TCR$\alpha\beta^+$ DNT cells. They also suffered from recurrent infections. With whole-exome sequencing, compound heterozygous mutations of *RASGRP1* were identified in both patients, resulting in impaired ERK activation, T-cell activation and proliferation, as well as activation-induced cell death of T cells.

METHODS

The family and ethical approval

The 2 patients, their parents, and age-matched controls were included in this study. The study was approved by the Institutional Review Board of the University of Hong Kong-Shenzhen Hospital (IRB reference no. 201603). The informed consent was obtained from the parents for the children and themselves to participate in this study.

Genetic study

Genomic DNA from the patients and parents were extracted from PBMCs. Library preparation, exome capture, and whole-exome sequencing were performed at the Centre for Genomic Sciences, University of Hong Kong. Library preparation and exome capture were done using Illumina TruSeq DNA Library Preparation Kit. Exome sequencing was performed on Illumina HiSeq2000 platform (Illumina, San Diego, Calif). Sanger sequencing was performed to confirm the genetic variations identified by exome sequencing. Additional detailed methods regarding the whole-exome sequencing are provided in this article's Online Repository at www.jacionline.org.

Preparation of PBMCs

The PBMCs were isolated from whole blood obtained from the patients, family members, and healthy normal controls by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The cells were cultured in RPMI 1640 medium plus 10% FBS.

Flow cytometry

The following mAbs were used in this study: anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-TCR$\alpha\beta$, anti-TCR$\gamma\delta$, anti-CD69, anti–active caspase 3, anti-CD21, anti-CD27, anti-Foxp3 (all from BD Biosciences, San Diego, Calif), anti-CD38, anti-IgD, anti-Ki67 (BioLegend), and anti-RasGRP1 (antibodies-online). For surface staining, the cells were stained with the specific antibodies. For intracellular staining, cells were fixed by Lysing Solution, permeabilized by Permeabilizing Solution 2 (both from BD Biosciences), and then labeled with the indicated antibodies. All data were acquired on a BD FACSAria machine with FACS Diva (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, Ore).

Cell proliferation was examined using carboxyfluorescein succinimidyl ester dilution assay as we previously described.[18] Briefly, PBMCs were stained first with 1 mM carboxyfluorescein succinimidyl ester (Invitrogen, Carlsbad, Calif), and then stimulated with LEAF purified anti-CD3 and anti-CD28 antibodies (both from BioLegend, San Diego, Calif). After 4 days, cells were collected and stained with anti-CD4 and anti-CD8 antibodies before the examination by flow cytometry. The number of cell divisions and percentage of cells divided were analyzed using FlowJo software. The intracellular expression of Ki67 was also detected as a surrogate marker for cell proliferation. To examine cell apoptosis, PBMCs were first stimulated with anti-CD3 and anti-CD28 antibodies, or PMA and ionomycin. After 2 days, apoptotic cells were detected with annexin V-fluorescein isothiocyanate and propidium iodide staining by flow cytometry. The percentage of cell death was calculated as [1 − (% of live cells with stimulation/% of live cells without stimulation)] × 100. The intracellular expression of active caspase 3 was also examined for cell apoptosis.

Determination of ERK activation by examining protein phosphorylation

To examine ERK activation, 1 million PBMCs were stimulated with 50 ng/mL PMA for 5 minutes. Cells were then determined for the protein phosphorylation by flow cytometry as described earlier.[19] Briefly, cells were fixed by Fix Buffer I, permeabilized by Perm Buffer III (both from BD Biosciences), and stained with anti-pEKR1/2 (pT202/pY204, BD Biosciences) to detect phosphorylated ERK1/2 expression. Samples were analyzed for the percentage of phospho-protein–positive population by flow cytometry. The increase in the percentage of phospho-protein expression in stimulated cells relative to resting cells was calculated.

Cytokine quantitation

One million PBMCs were stimulated with 1 μg/mL LEAF purified anti-CD3 and anti-CD28 antibodies (both from BioLegend) for 2 days. The supernatants were collected and then determined for the production of IL-10 by using BD Cytometric Bead Array kit (BD Biosciences) according to the manufacturer's instruction. The data were acquired on a BD FACSAria (BD Biosciences) and analyzed using FCAP Array software.

Structural analysis of RasGRP1

The crystal structure of RasGRP1 (4L9M from the protein data bank) was used as the template, which was determined by X-ray diffraction at a resolution of 3.0 Å. The amino acid residue at coordinate 214 was mutated for variant Thr241Ile. The structures were analyzed with Swiss-PdbViewer. The residue 214, together with certain nearby ones within 6 Å, was illustrated. For clear demonstration of interresidue relationship, some residues were highlighted in the indicated colors with the computed hydrogen bonds being labeled.

RESULTS

The patients

The 2 siblings comprising a sister with her younger brother came from a nonconsanguineous kindred with no significant family history (Fig 1, *A*). At age 2 years, the sister had immune thrombocytopenia responsive to intravenous immunoglobulin treatment (Table I). She further experienced 2 episodes of chronic immune thrombocytopenia at the age of 5 and 8 years, respectively, which were refractory to IVIG and steroid therapy.

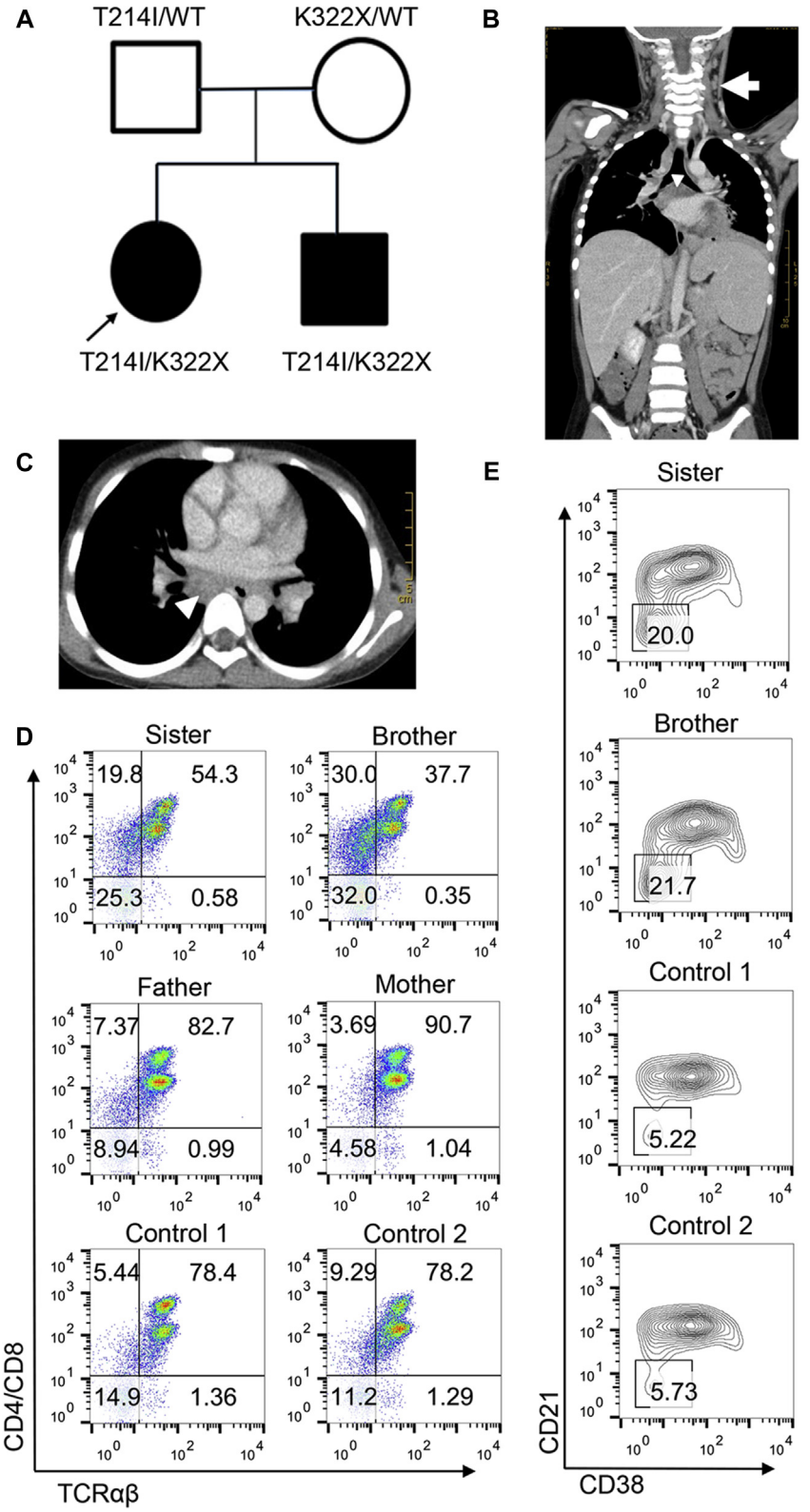


FIG 1. The family pedigree and phenotype of the patients. **A,** The pedigree of the family and the *RASGRP1* genotypes are shown. WT denotes wild type. **B** and **C,** Computed tomography demonstrated hepatosplenomegaly and mediastinal *(triangle)* and superficial cervical *(arrow)* lymphadenopathy in the sister. $TCR\alpha\beta^{+}$ DNT cells **(D)** and $CD21^{low}$ B cells **(E)** in the PBMCs were examined by flow cytometry.

TABLE I. Core clinical features of the 2 patients

Feature	Sister	Brother
Sex	Female	Male
Age of disease onset	2 y	1 mo
Consanguinity	No	No
Autoimmunity		
Immune cytopenia	ITP, AIHA	AIHA
Autoantibodies	ANA (1:10,000 highest), anti-SSA, Coombs, aTPO, TGAb	ANA (1:1,000), anti-C3d, anti-IgG
Gastrointestinal symptom	No	No
Lymphoproliferative disorders		
Lymphadenopathy	Chronic lymphadenopathy	Chronic lymphadenopathy
Splenomegaly	Chronic splenomegaly	Chronic splenomegaly
Hepatomegaly	Chronic hepatomegaly	Chronic hepatomegaly
Infections		
Bacteria	*Moraxella catarrhalis, Hemophilus influenza, Staphylococcus aureus, Mycoplasma pneumonia*	*Staphylococcus aureus, Mycoplasma pneumonia*
Virus	Nil documented	CMV, EBV
Fungus	*Candida albicans*	Pulmonary aspergillosis
Mycobacteria	Nil documented	Left axillary lymph node TB
Other phenotypes	Leiomyoma of adrenal gland and liver, pneumonia	HLH, pneumonia
Immune phenotypes		
Immunoglobulin (reference range)		
IgG (g/L)	29.2 (5.0-10.6)	11.94 (4.53-9.16)
IgA (g/L)	4.96 (0.36-1.72)	1.37 (0.2-1.0)
IgM (g/L)	2.63 (0.44-2.07)	1.58 (0.19-1.46)
IgE (IU/mL)	0.7 (0.0-200.0)	1.5 (0.0-60.0)
Lymphocyte count ($\times 10^9$/L) (reference range)	3.67 (1.15-6.0)	1.98 (1.5-7.0)
Lymphocyte subpopulation % (reference range) Count ($\times 10^9$/L) (reference range)		
$CD3^+$ T	77% (38.6%-70.1%) 2.62 (0.81-4.46)	76% (56%-75%) 1.5 (0.85-4.3)
$CD4^+$ T	21% (14.2%-37.0%) 0.75 (0.35-2.45)	20% (28%-47%) 0.40 (0.5-2.7)
$CD8^+$ T	44% (13.2%-38.5%) 1.60 (0.31-2.08)	32% (16%-30%) 0.63 (0.2-1.8)
$CD19^+$ B	20% (10.9%-28.0%) 0.63 (0.24-1.32)	15% (14%-33%) 0.30 (0.18-1.3)
$CD16^+CD56^+$ NK	2% (7.9%-34.0%) 0.049 (0.21-1.51)	6% (4%-17%) 0.12 (0.061-0.51)
$CD4^+/CD8^+$ ratio	0.47 (0.96-2.05)	0.62 (0.71-2.78)
$CD3^+TCR\alpha\beta^+$ DNT cells	Normal	Normal
RASGRP1 Mutation		
Nucleotide change	c.641C>T, c.964A>T	c.641C>T, c.964A>T
Amino acid change	p.T214I, p.K322X	p.T214I, p.K322X
Domain	Cdc25 domain	Cdc25 domain
Carrier status of parents	Heterozygous carrier	Heterozygous carrier

AIHA, Autoimmune hemolytic anemia; *a-TPO*, anti–thyroid peroxidase antibodies; *CMV*, cytomegalovirus; *HLH*, hemophagocytic lymphohistiocytosis; *ITP*, immune thrombocytopenia; *NK*, natural killer; *SSA*, Sjögren's-syndrome–related antigen A; *TB*, tuberculosis; *TGAb*, antithyroglobulin antibodies.

At age 7 years, she had autoimmune hemolytic anemia. From age 5 years, she had documented chronic lymphadenopathy and hepatosplenomegaly (Fig 1, *B* and *C*). During the first 4 years, the patients had several episodes of pneumonia but without detailed investigation. From age 4 years, she had multiple episodes of pneumonia caused by a spectrum of pathogens including *Mycoplasma pneumonia*, *Moraxella catarrhalis, Hemophilus influenza, Staphylococcus aureus*, and *Candida albicans*. The patient also developed leiomyoma in the right adrenal gland and left lobe of liver at the age of 5 years, which were resected.

At age 1 month, the brother had hemophagocytic lymphohistiocytosis triggered by cytomegalovirus infection. Like his sister, the brother also had documented chronic lymphadenopathy and hepatosplenomegaly since age 1 year (Table I). At age 10 and 17 months, he further developed autoimmune hemolytic anemia, which was treated with steroid and mycophenolate mofetil. He also had several episodes of infections with EBV, *Staphylococcus aureus*, and *Mycoplasma pneumonia*. He further experienced pulmonary aspergillosis and lymph node tuberculosis at age 7 and 17 months, respectively. Investigations showed the presence of hypergammaglobulinemia, ANA and other autoantibodies, and

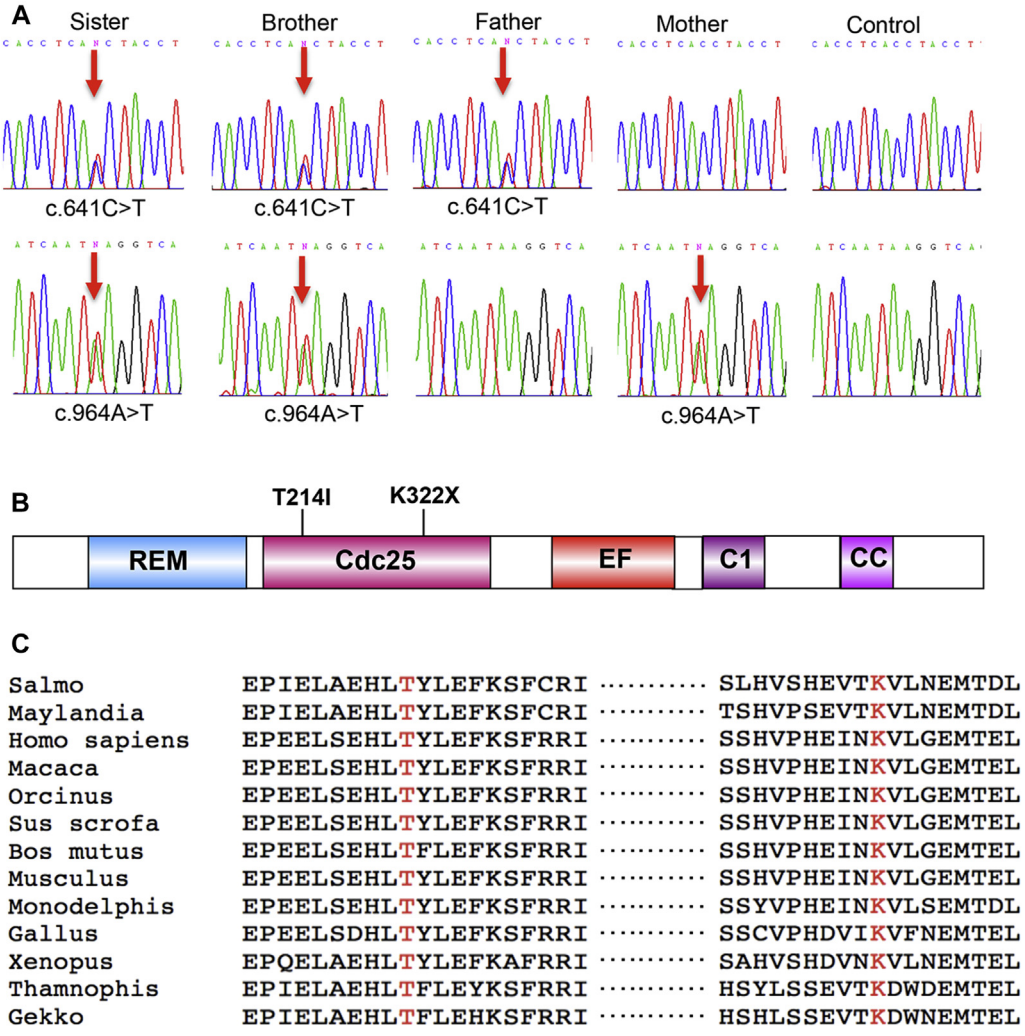


FIG 2. Novel compound heterozygous mutations in *RASGRP1* were identified in the patients. **A,** Compound heterozygous mutations in both patients and heterozygous carrier in the parents were shown in genomic *RASGRP1* DNA. **B,** The schematic structure of RasGRP1. The mutations are located in the Cdc25 domain. **C,** Phylogenetic analysis indicated that the positions of mutations are highly conserved across different species.

normal IgE level in both patients (Table I). They are now stable and followed-up in our clinic.

Immunophenotype

Because both patients presented with chronic lymphoproliferation and autoimmune disease, ALPS was first considered for the working diagnosis. Thus, the presence of TCR$\alpha\beta^+$ DNT cells was examined. As shown in Fig 1, *D*, normal percentage of TCR$\alpha\beta^+$ DNT cells (<1.5%) out of total T cells was observed in the 2 patients. The distribution of TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells was then examined. The patient showed reduced expression of TCR$\alpha\beta$, but largely elevated expression of TCR$\gamma\delta$. Unlike the normal controls in whom most T cells in the peripheral blood were TCR$\alpha\beta^+$ T cells and a few TCR$\gamma\delta^+$ cells, in the patient, TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells each occupied about 50% of the total T cells (see Fig E1 in this article's Online Repository at www.jacionline.org). Regulatory T cells were further examined and found to be not decreased in the patients compared with the normal controls (see Fig E2 in this article's Online Repository at www.jacionline.org). The phenotype of B cells was also analyzed. No obvious difference was detected in the distribution of naive and memory B cells between the patients and normal controls (see Fig E3 in this article's Online Repository at www.jacionline.org). But the percentage of autoreactive CD21^{low} B cells was markedly increased in the patients (Fig 1, *E*).

Whole-exome sequencing identified compound heterozygous *RASGRP1* mutations

To dissect the genetic cause, whole-exome sequencing was performed for the 2 patients and their parents. A total of 10,163 rare variants were detected in the family, of which 645 were missense, nonsense, splice-site, or frameshift variants. Based on a stepwise approaching algorithm routinely practiced in our laboratory, bioinformatics analysis was performed and finally 3 genes that contain either homozygous or compound heterozygous mutations were considered of interest genetically (see Tables E1

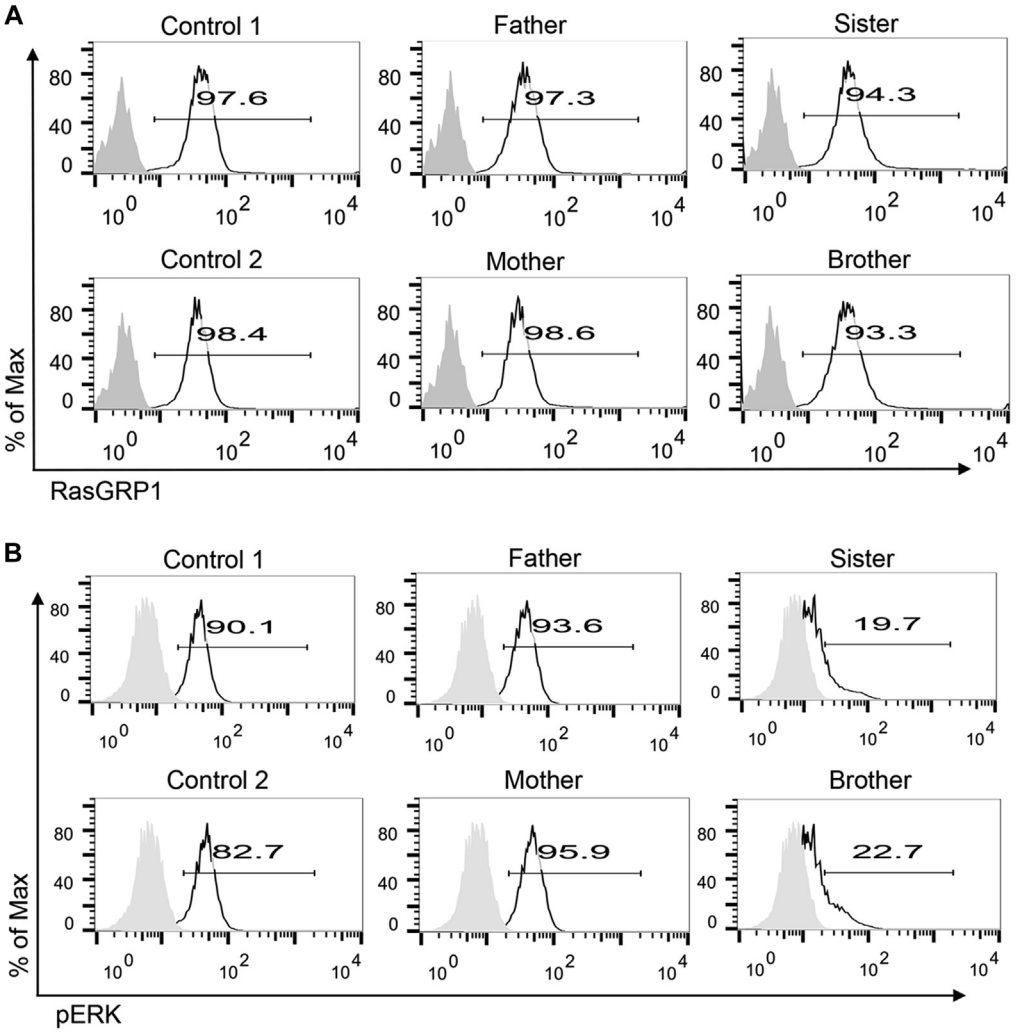


FIG 3. Normal RasGRP1 expression, but impaired ERK activation, in patient cells. **A,** RasGRP1 protein expression in lymphocytes was examined by flow cytometry. Both patients had comparable level of RasGRP1 protein with normal controls. **B,** PMA-induced ERK activation in lymphocytes was examined by flow cytometry. Both patients had markedly lower phosphorylation of ERK compared with normal controls. *pERK*, Phosphorylated ERK

and E2 in this article's Online Repository at www.jacionline.org). The detailed information on exome sequencing and analysis is provided in this article's Online Repository at www.jacionline.org.

The tissue distributions and functions of the 3 genes were further analyzed. Of these, RasGRP1 is a Ras guanine nucleotide exchange factor and mainly expressed in lymphocytes. Through its role in TCR signaling pathway, RasGRP1 is essential for T-cell development and homeostasis. Previous animal studies demonstrated the contribution of RasGR1 in autoimmune disease. Therefore, *RASGRP1* mutations were regarded as the most likely candidate causal mutations for the 2 patients. Sanger sequencing confirmed the heterozygous mutations of *RASGRP1* in the patients' father (c.641C>T, p.T214I) and mother (c.964A>T, p.K322X), and the compound heterozygous nature in the patients (Fig 2, *A*), consistent with the original sequencing finding (see Fig E4 in this article's Online Repository at www.jacionline.org). The 2 mutations are located in the Cdc25 domain (Fig 2, *B*) and not observed in any of the currently available public databases. Phylogenetic analysis indicated that the positions of the 2 mutations are highly conserved across different species (Fig 2, *C*). Both the 2 mutations were predicted to be damaging. Structural analysis showed that a hydrogen bond present between Thr214 and Asn290 in the wild-type RasGRP1 was broken by the Thr214Ile mutation (see Fig E5 in this article's Online Repository at www.jacionline.org), which might affect Ras binding and catalytic activity of RasGRP1 because the residues are located in the catalytic domain. Taken together, considering the similarity between the phenotype of patients described here and that of RasGRP1-deficient mice (see Table E3 in this article's Online Repository at www.jacionline.org), we hypothesized that the compound heterozygous *RASGRP1* mutations are the causal mutations for the 2 patients.

Normal RasGRP1 expression, but impaired ERK activation, in patient cells

We next examined whether the mutations affect the expression of RasGRP1. As shown in Fig 3, *A*, comparable level of RasGRP1 protein was detected between the patients and controls. Because

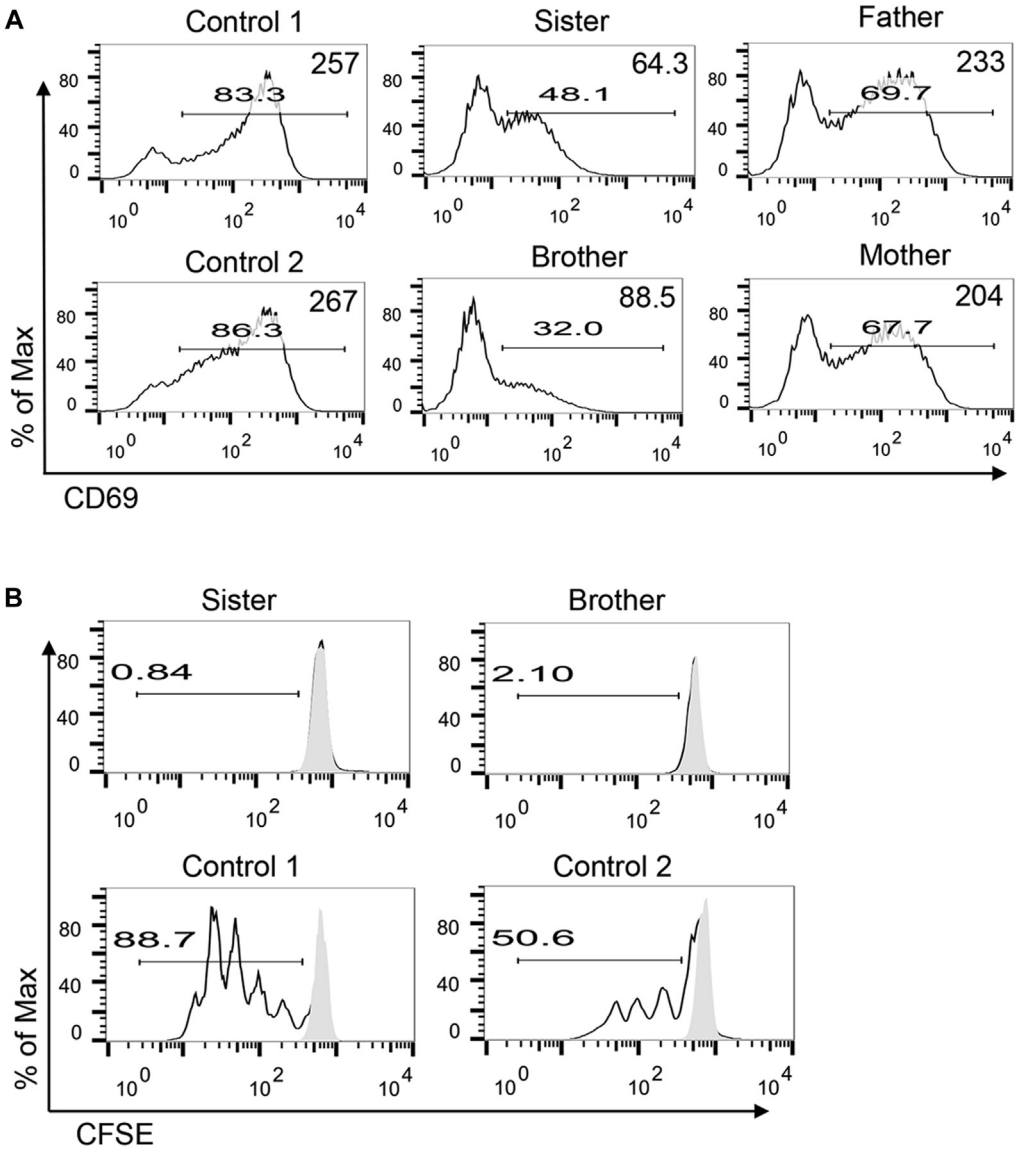


FIG 4. Impaired activation and proliferation in patient T cells. **A,** PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies overnight. The percentage of CD69-positive cells in lymphocytes and intensity of CD69 expression level (right upper corner) were examined by flow cytometry. **B,** PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies, and lymphocyte proliferation was examined by CFSE dilution. *CFSE,* Carboxyfluorescein succinimidyl ester.

RasGRP1 is expressed in both T and B cells, we further examined the protein expression in the lymphocyte subsets. It was shown that the patients had a similar level of RasGRP1 as the controls in either T or B cells (see Fig E6 in this article's Online Repository at www.jacionline.org), which indicates that the compound heterozygous mutations did not alter the protein expression, likely because of 1 mutation being missense.

RasGRP1 acts downstream of antigen receptors of lymphocytes. Upon cognate peptide ligation of lymphoctes, the intracellular messenger DAG is generated and then activates RasGRP1, leading to ERK activation. The effect of *RASGRP1* mutation on ERK activation was further examined. PMA is a synthetic analog of DAG and used in the experiments. Upon PMA engagement, almost all the ERK was phosphorylated in the lymphocytes of normal controls and parents. However, only a negligible level of ERK activation was observed in the patients' cells (Fig 3, *B*). PMA-induced ERK phosphorylation was further examined in T and B cells, respectively. It was found that the impaired ERK activation induced by PMA was observed only in T cells, but not B cells, of the patients. Instead, PMA-induced comparable level of ERK phosphorylation in B cells between the patients and normal controls (see Fig E7 in this article's Online Repository at www.jacionline.org). Taken together, normal RasGRP1 expression, but impaired ERK activation, was revealed in patient cells with compound heterozygous mutations of RasGRP1.

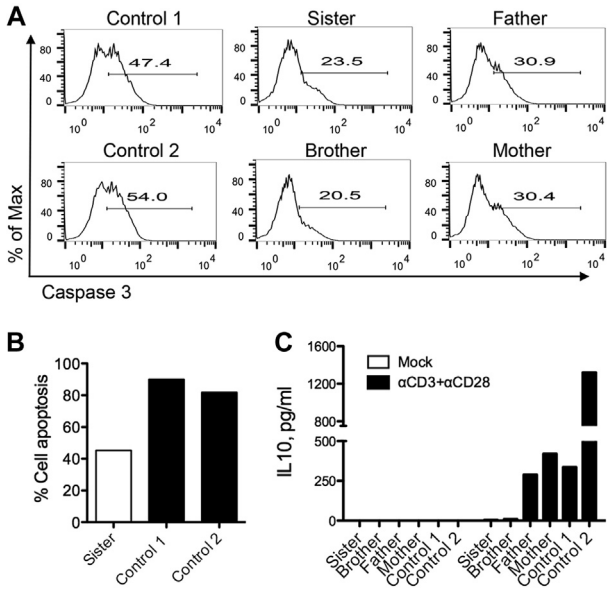


FIG 5. Impaired activation-induced cell death in patient T cells. **A,** After stimulation with anti-CD3 and anti-CD28 antibodies, intracellular expression of active caspase 3 in lymphocytes was examined. **B,** PBMCs were stimulated with PMA and ionomycin. Lymphocyte apoptosis was examined using annexin V and propidium iodide staining. **C,** PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies. IL-10 production in the supernatants was examined.

Impaired activation and proliferation in patient T cells

It is well known that RasGRP1 activates the ERK/mitogen-activated protein kinase cascade through coupling TCR to Ras signaling pathway to regulate T-cell activation and proliferation. After demonstrating that *RASGRP1* mutations blocked ERK phosphorylation, we next investigated T-cell activation induced by TCR ligation. The PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies and then examined for CD69 expression. As shown in Fig 4, *A*, the activation of T cells was significantly depressed in the patients compared with normal controls, as evidenced by the much less percentage of T cells expressing CD69 as well as a significantly lower intensity of CD69 expression level. Of T cells, both CD4 and CD8 T cells showed markedly impaired activation in the patients (see Fig E8 in this article's Online Repository at www.jacionline.org). The cell proliferation was further investigated with carboxyfluorescein succinimidyl ester dilution assay. As indicated in Fig 4, *B*, after stimulation with anti-CD3 and anti-CD28 antibodies, nearly no lymphocytes were demonstrated to undergo proliferation in the patients, in significant contrast to normal controls, where about 3 to 5 cellular divisions were detected in more than half of total lymphocytes. Of T cells, both CD4 and CD8 T did not show proliferation in the patients (see Fig E9 in this article's Online Repository at www.jacionline.org). The cell proliferation was also examined by another method using Ki67 as a surrogate marker.[20] Similarly, significant impairment of T-cell proliferation was detected in the patients compared with normal controls (see Fig E10 in this article's Online Repository at www.jacionline.org). The impaired cell activation and proliferation made the transfection of patient T cells with wild-type RasGRP1-expressing lentivirus quite difficult in our rescue study, whereas the transfection of normal control T cells was successful as an internal control (see Fig E11 in this article's Online Repository at www.jacionline.org). Thus, it made the rescue assay with patient cells unfeasible despite of repeated trials.

Impaired activation-induced cell death in patient T cells

Control of apoptosis in lymphocytes is particularly important, because autoreactive cells must be deleted to avoid autoimmune disorders. RasGRP1 is involved in cell survival through the Ras signaling. The effect of *RASGRP1* mutations on T-cell viability was further examined. The PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies and then examined for the activation-induced cell death of T cells. As shown in Fig 5, *A*, after TCR-mediated activation, the intracellular expression of active caspase 3 was markedly decreased in both patients' cells compared with that in normal controls. Of T cells, both CD4 and CD8 T cells showed significantly impaired activation-induced cell death in the patients (see Fig E12 in this article's Online Repository at www.jacionline.org). The cell apoptosis was further examined by using annexin V and propidium iodide staining. It was also shown that the patient had remarkable decrease in cell apoptosis compared with normal controls (Fig 5, *B*). IL-10 plays an important role in the induction of tolerance and maintenance of immunological homeostasis. The ability of IL-10 production in the patients' cells was next examined. In response to TCR stimulation, the patients' cells produced markedly lower IL-10 as compared with normal controls (Fig 5, *C*).

DISCUSSION

In this study, we identified novel compound heterozygous *RASGRP1* mutations in 2 siblings with ALPS-like disorder with parents being heterozygous carriers. One of the mutations is hypomorphic, resulting in normal RasGRP1 expression but impaired function. The 2 siblings had chronic lymphadenopathy, hepatosplenomegaly, autoantibodies including ANA, and autoimmune cytopenia, but normal TCR$\alpha\beta^+$ DNT cells. They also suffered from recurrent infections.

Series of animal studies have already demonstrated that RasGRP1-deficient mice with either complete deletion[13,14] or truncation[15] of RasGRP1 developed autoimmune lymphoproliferative disorder with the phenotypes of lymphadenopathy, splenomegaly, and the presence of ANA. The similarity between the clinical, immunological, and cellular phenotypes of patients described here and those of RasGRP1-deficient mice (see Table E3) strongly suggests the causative nature of *RASGRP1* mutations in our patients. Indeed, in humans, RasGRP1 has been shown to be associated with some autoimmune disorders. SLE is a complex autoimmune disease characterized by high levels of ANA autoantibodies. A subset of patients with SLE was shown to have aberrant RasGRP1 splice variants, which was correlated with defective RasGRP1 expression in their T cells and diminished RasGRP1 activity.[17] *RASGRP1*-linked single nucleotide variants were also shown to be associated with autoimmune diabetes and thyroid disease.[21,22]

In addition, the phenotype of RasGRP1 deficiency is similar in many aspects to that of *LAT* mutation. LAT is located upstream of RasGRP1 in TCR signal pathway and, together with RasGRP1, contributes to the signaling transmission and final biological

activity. Previous studies showed that LAT deficiency inhibited T-cell development yet induced autoimmune lymphoproliferation. The T cells from *LAT* mutant mice also showed defective TCR signaling and apoptosis.[23,24] Taken together, these findings reveal that the LAT-RasGRP1-Ras signaling axis in T cells plays critical roles in maintaining self-tolerance and homeostasis. Dysfunction of such intracellular signal proteins contributes to the development of autoimmune disorders.

RasGRP1 plays important roles in the host immune defense. Both our data and previous *in vivo* studies have demonstrated that RasGRP1 deficiency impaired T-cell function including activation and proliferation.[10,25,26] In addition, RasGRP1-deficient T cells showed diminished cytokine production upon cognate antigen stimulation.[27] For the innate immunity, RasGRP1 was reported to be required for human natural killer cell function. Knockdown of RasGRP1 blocked cytokine production and cytotoxicity of natural killer cells.[28] RasGRP1-deficient γδT cells were also shown to have diminished IL-17 expression besides defective proliferation.[29] Therefore, the impaired adaptive and innate immunity would render the host susceptible to various infections, which at least partly explains why our patients with RasGRP1 deficiency developed multiple infections. Indeed, a previous study also demonstrated that RasGRP1- deficient mice showed weakened immune system and poor pathogen-specific T-cell response, leading to delayed pathogen clearance when challenged with bacteria and viruses.[30]

Herein, the patients developed ALPS-like disease despite the impaired proliferation of lymphocytes. However, our data and previous report showed that the regulatory T cell is intact, and even the function is enhanced in RasGRP1-deficient mice.[31] To clarify the mechanism underlying the autoimmune lymphoproliferation, we investigated the apoptosis in our patients. A properly functioning immune system is dependent on apoptosis at every stage of lymphocyte development. Apoptosis plays essential roles to maintain tolerance and homeostasis, because self-reactive lymphocytes must be deleted by this means to avoid autoimmune disorders.[32,33] In this study, we showed that patients' cells had markedly impaired activation-induced cell death, which is compatible with previous findings in the mouse study.[10,14] In addition, the patients were shown to have markedly higher $CD21^{low}$ B cells compared with the normal controls. It has been reported that $CD21^{low}$ B cells contain mostly autoreactive clones, and are found enriched in patients with autoimmune disorders. Most $CD21^{low}$ B cells express autoreactive antibodies that recognize nuclear and cytoplasmic structures.[34] We further demonstrated that TCR-stimulated IL-10 production was markedly depressed in the patients. IL-10 plays an important role in the induction of tolerance and maintenance of immunological homeostasis.[35] Collectively, we speculate that the defective apoptosis, IL-10 expression, and increased $CD21^{low}$ B cells may contribute to the development of autoimmune lymphoproliferative phenotype in the patients.

RasGRP1 couples TCR to Ras signaling pathway in T cells, and participates in various cellular processes including cell activation and proliferation.[36,37] In accordance with this, both our data and previous mouse studies revealed that RasGRP1-deficient thymocytes and T cells showed impaired Ras-ERK signaling, leading to defective cell activation and proliferation in response to DAG and TCR stimulation.[10,25,26] RasGRP1 is highly expressed in T cells, though it is also detected in B cells to a lesser extent. In B cells, both RasGRP1 and RasGRP3 act downstream of BCR. RasGRP1-deficient mice do not have a block in B-cell development. Only B cells deficient in both RasGRP1 and RasGRP3 showed defective proliferative response.[10,14,38] Compatible with this, we found that our patients' B cells showed normal activation upon stimulation.

In summary, we identified loss-of-function mutations of *RASGRP1* in 2 siblings with ALPS-like disease. The mutations impair TCR signaling, leading to defective T-cell activation and proliferation, as well as impaired activation-induced cell death of T cells. The similarity between the phenotype of our patients and that of RasGRP1-deficient mice, as well as that of mutation of LAT, a component upstream of RasGRP1 in TCR signaling, clearly indicates the causative nature of *RASGRP1* mutations in our patients. We propose to investigate the intracellular proteins involved in the TCR signaling pathway in similar patients but with unknown genetic cause.

Addendum

During the revision period of our manuscript, an article by Salzer et al[39] was published advance online in Nature Immunology, which reported that 1 patient with homozygous null mutation of RasGRP1 had bacterial and viral infections. It is a different clinical phenotype compared with that in the mouse model with similar RasGRP1 null mutation. In our study, we demonstrated 2 patients with RasGRP1 hypomorphic mutation who developed ALPS-like phenotype in addition to recurrent infections, which is almost the same as what has been reported in RasGRP1-deficient mice with either complete deletion or truncation of RasGRP1[13-15] (Table E3). Our findings are also compatible with the reported association of RasGRP1 with autoimmune disorders in humans, such as SLE, and autoimmune diabetes and thyroid disease.[17,21,22] The reason why the patient described by Salzer et al[39] did not show autoimmune lymphoproliferative features warrants further investigation.

Clinical implications: Mutations in *RASGRP1* are identified in ALPS-like disease. The intracellular proteins involved in the TCR signaling pathway should be part of the genetic diagnostic workup in patients with ALPS-like disorders.

REFERENCES

1. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. Blood 2010;116:e35-40.
2. Drappa J, Vaishnaw AK, Sullivan KE, Chu JL, Elkon KB. Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. N Engl J Med 1996;335:1643-9.
3. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science 1995;268:1347-9.
4. Del-Rey M, Ruiz-Contreras J, Bosque A, Calleja S, Gomez-Rial J, Roldan E, et al. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. Blood 2006;108:1306-12.
5. Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, et al. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell 1999;98:47-58.
6. Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. Nature 2002;419:395-9.
7. Oliveira JB, Bidere N, Niemela JE, Zheng L, Sakai K, Nix CP, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. Proc Natl Acad Sci U S A 2007;104:8953-8.

8. Takagi M, Shinoda K, Piao J, Mitsuiki N, Takagi M, Matsuda K, et al. Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation. Blood 2011;117:2887-90.
9. Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC. RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 1998;280:1082-6.
10. Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, et al. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol 2000;1:317-21.
11. Ksionda O, Limnander A, Roose JP. RasGRP Ras guanine nucleotide exchange factors in cancer. Front Biol (Beijing) 2013;8:508-32.
12. Ebinu JO, Stang SL, Teixeira C, Bottorff DA, Hooton J, Blumberg PM, et al. RasGRP links T-cell receptor signaling to Ras. Blood 2000;95:3199-203.
13. Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. J Immunol 2005;175:7179-84.
14. Coughlin JJ, Stang SL, Dower NA, Stone JC. The role of RasGRPs in regulation of lymphocyte proliferation. Immunol Lett 2006;105:77-82.
15. Fuller DM, Zhu M, Song X, Ou-Yang CW, Sullivan SA, Stone JC, et al. Regulation of RasGRP1 function in T cell development and activation by its unique tail domain. PLoS One 2012;7:e38796.
16. Daley SR, Coakley KM, Hu DY, Randall KL, Jenne CN, Limnander A, et al. Rasgrp1 mutation increases naive T-cell CD44 expression and drives mTOR-dependent accumulation of Helios(+) T cells and autoantibodies. Elife 2013;2:e01020.
17. Yasuda S, Stevens RL, Terada T, Takeda M, Hashimoto T, Fukae J, et al. Defective expression of Ras guanyl nucleotide-releasing protein 1 in a subset of patients with systemic lupus erythematosus. J Immunol 2007;179:4890-900.
18. Tu W, Lau YL, Zheng J, Liu Y, Chan PL, Mao H, et al. Efficient generation of human alloantigen-specific CD4+ regulatory T cells from naive precursors by CD40-activated B cells. Blood 2008;112:2554-62.
19. Lee PP, Mao H, Yang W, Chan KW, Ho MH, Lee TL, et al. Penicillium marneffei infection and impaired IFN-gamma immunity in humans with autosomal-dominant gain-of-phosphorylation STAT1 mutations. J Allergy Clin Immunol 2014;133:894-6.e5.
20. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-22.
21. Qu HQ, Grant SF, Bradfield JP, Kim C, Frackelton E, Hakonarson H, et al. Association of RASGRP1 with type 1 diabetes is revealed by combined follow-up of two genome-wide studies. J Med Genet 2009;46:553-4.
22. Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Wallace C, et al. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. PLoS Genet 2011;7:e1002216.
23. Sommers CL, Park CS, Lee J, Feng C, Fuller CL, Grinberg A, et al. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science 2002;296:2040-3.
24. Aguado E, Richelme S, Nunez-Cruz S, Miazek A, Mura AM, Richelme M, et al. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science 2002;296:2036-40.
25. Kortum RL, Sommers CL, Pinski JM, Alexander CP, Merrill RK, Li W, et al. Deconstructing Ras signaling in the thymus. Mol Cell Biol 2012;32:2748-59.
26. Priatel JJ, Teh SJ, Dower NA, Stone JC, Teh HS. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. Immunity 2002;17:617-27.
27. Priatel JJ, Chen X, Huang YH, Chow MT, Zenewicz LA, Coughlin JJ, et al. RasGRP1 regulates antigen-induced developmental programming by naive CD8 T cells. J Immunol 2010;184:666-76.
28. Lee SH, Yun S, Lee J, Kim MJ, Piao ZH, Jeong M, et al. RasGRP1 is required for human NK cell function. J Immunol 2009;183:7931-8.
29. Chen Y, Ci X, Gorentla B, Sullivan SA, Stone JC, Zhang W, et al. Differential requirement of RasGRP1 for gammadelta T cell development and activation. J Immunol 2012;189:61-71.
30. Priatel JJ, Chen X, Zenewicz LA, Shen H, Harder KW, Horwitz MS, et al. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. J Immunol 2007;179:2143-52.
31. Chen X, Priatel JJ, Chow MT, Teh HS. Preferential development of CD4 and CD8 T regulatory cells in RasGRP1-deficient mice. J Immunol 2008;180:5973-82.
32. Strasser A, Puthalakath H, O'Reilly LA, Bouillet P. What do we know about the mechanisms of elimination of autoreactive T and B cells and what challenges remain. Immunol Cell Biol 2008;86:57-66.
33. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. Immunol Rev 2003;193:70-81.
34. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21− human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010;115:5026-36.
35. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28-50.
36. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37-40.
37. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. Annu Rev Immunol 2003;21:139-76.
38. Stone JC. Regulation and function of the RasGRP family of Ras activators in blood cells. Genes Cancer 2011;2:320-34.
39. Salzer E, Cagdas D, Hons M, Mace EM, Garncarz W, Petronczki OY, et al. RASGRP1 deficiency causes immunodeficiency with impaired cytoskeletal dynamics. Nat Immunol 2016;17:1352-60.

METHODS

Whole-exome sequencing and bioinformatics analysis

The whole-exome sequencing was performed at the Centre for Genomic Sciences, University of Hong Kong. Library preparation and exome capture were done with Illumina TruSeq DNA Library Preparation Kit. Exome sequencing was performed on Illumina HiSeq2000 platform (Illumina). Using software from Illumina (bcl2fastq), sequencing reads were assigned into individual samples, with each sample having an average sequencing volume of 6.8 Gbp. On average, 91% of the bases achieved a quality score of Q30 (accuracy of a base call to be 99.9%). After removal of adapter sequences, low-quality reads, and reads with more than 10% of unknown nucleotides, the clean reads were aligned to the reference human genome build hg19 using Burrows-Wheeler Aligner.[E1] Single nucleotide polymorphisms/Indels were called using GATK.[E2] Annotation of all variants was performed using ANNOVAR.[E3]

Only good-quality variants were included for further analysis. The criteria used included variant mapping quality greater than or equal to 30, coverage depth equal to or greater than 5-fold in both patients, and genotype quality greater than or equal to 30. Taking into consideration the rarity of the phenotype of the patients, we excluded the common variants with population allele frequency higher than 1% in the public databases including the 1000 Genomes Project, dbSNP, NHLBI GO Exome Sequencing Project, and Exome Aggregation Consortium (ExAC: http://exac.broadinstitute.org/).

Whole-exome sequencing detected 10,163 rare variants in this family in total. Of these, 645 were missense, nonsense, splice-site, or frameshift variants by the function filter we adopted on SNV/Indels (Table E1). Based on a stepwise approaching algorithm routinely practiced in our laboratory, bioinformatics analysis was performed and finally 3 genes that contain either homozygous or compound heterozygous mutations were considered of interest genetically. One homozygous missense mutation in *PLIN4* was detected on both patients while parents were heterozygous carrier. Two genes (*RASGRP1* and *CCDC74B*) with compound heterozygous mutations were detected on both patients and parents were also carriers (Table E2). There is no *de novo* mutation detected in either patient.

The tissue distributions and functions of the 3 genes were further analyzed (Table E2). Of these, RasGRP1 is a Ras guanine nucleotide exchange factor and mainly expressed in lymphocytes. Through its role in TCR signaling pathway, RasGRP1 is essential for T-cell development and homeostasis. Previous animal studies have demonstrated the contribution of RasGR1 in autoimmune disease. Therefore, the compound heterozygous mutations in *RASGRP1* were regarded as the most likely candidate causal mutations for the 2 patients. The 2 children inherited a Threonine to Isoleucine missense mutation (T214I) from their father and a nonsense (K322X) mutation from their mother. The 2 mutations are not observed in any of the currently available public databases. The positions of the 2 mutations are highly conserved across different species. Threonine to Isoleucine substitution at position 214 of RasGRP1 was predicted to be damaging or deleterious by all the bioinformatics tools including Polyphen, SIFT, LRT, LR, MutationTaster, Mutation Assessor, and RadialSVM. K322X mutation occurs in the eighth exon of this 17-exon gene and predicted as damaging. This early stop codon may cause nonsense-mediated decay of the mRNA transcript from this allele and lead to abolition of protein expression. Sanger sequencing confirmed the heterozygous mutations of *RASGRP1* in the patients' father (c.641C>T, p.T214I) and mother (c.964A>T, p.K322X), and the compound heterozygous nature in the patients.

REFERENCES

E1. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010;26:589-95.
E2. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297-303.
E3. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.
E4. Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, et al. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol 2000;1:317-21.
E5. Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. J Immunol 2005; 175:7179-84.
E6. Fuller DM, Zhu M, Song X, Ou-Yang CW, Sullivan SA, Stone JC, et al. Regulation of RasGRP1 function in T cell development and activation by its unique tail domain. PLoS One 2012;7:e38796.
E7. Daley SR, Coakley KM, Hu DY, Randall KL, Jenne CN, Limnander A, et al. Rasgrp1 mutation increases naive T-cell CD44 expression and drives mTOR-dependent accumulation of Helios(+) T cells and autoantibodies. Elife 2013;2: e01020.
E8. Coughlin JJ, Stang SL, Dower NA, Stone JC. The role of RasGRPs in regulation of lymphocyte proliferation. Immunol Lett 2006;105:77-82.
E9. Chen Y, Ci X, Gorentla B, Sullivan SA, Stone JC, Zhang W, et al. Differential requirement of RasGRP1 for gammadelta T cell development and activation. J Immunol 2012;189:61-71.
E10. Priatel JJ, Chen X, Zenewicz LA, Shen H, Harder KW, Horwitz MS, et al. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. J Immunol 2007;179:2143-52.

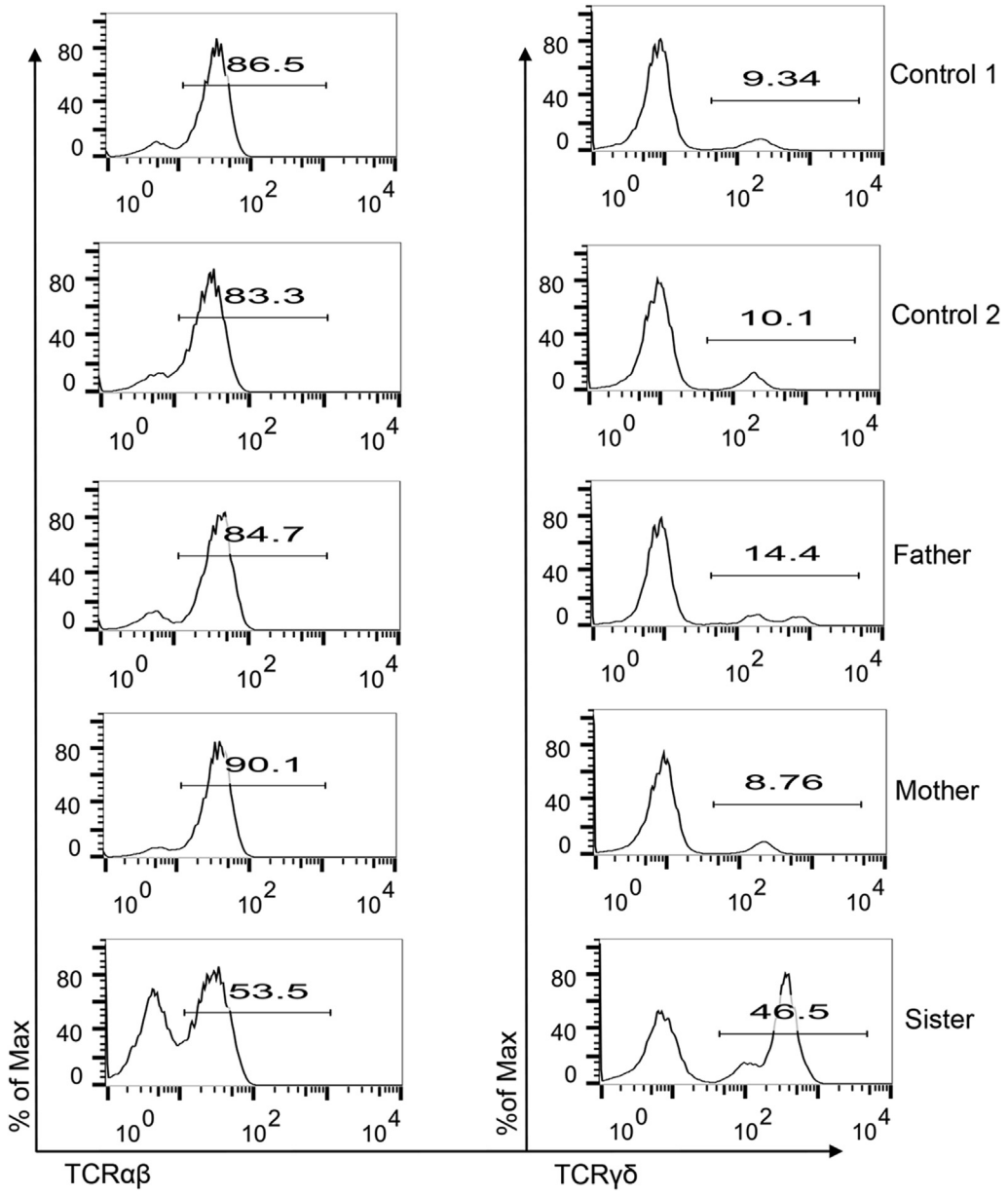


FIG E1. TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells in the patients. The PBMCs were stained for the surface expressions of TCR$\alpha\beta$ and TCR$\gamma\delta$. The percentage of $CD3^+$TCR$\alpha\beta^+$ and $CD3^+$TCR$\gamma\delta^+$ T cells out of total $CD3^+$ T cells was determined by flow cytometry. The patient showed reduced expression of TCR$\alpha\beta$, but largely elevated expression of TCR$\gamma\delta$.

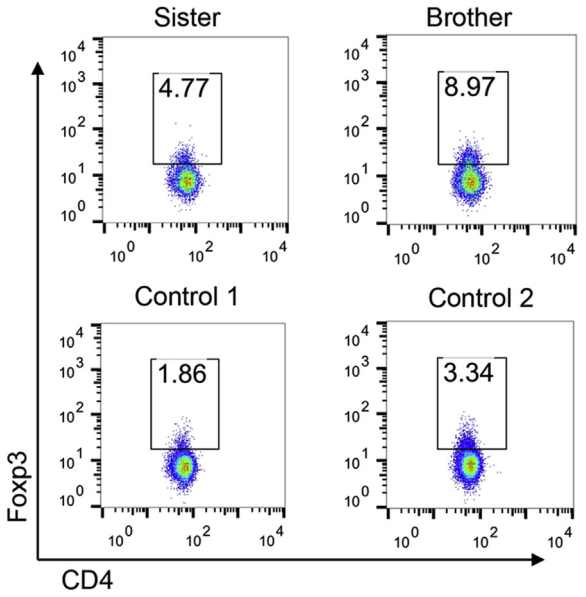


FIG E2. Examination of Treg cells in the patients. The PBMCs were stained for the surface expressions of CD3 and CD4, and intracellular expression of Foxp3. The percentage of $Foxp3^+$ Treg cells out of $CD4^+$ T cells was determined by flow cytometry. *Treg*, Regulatory T.

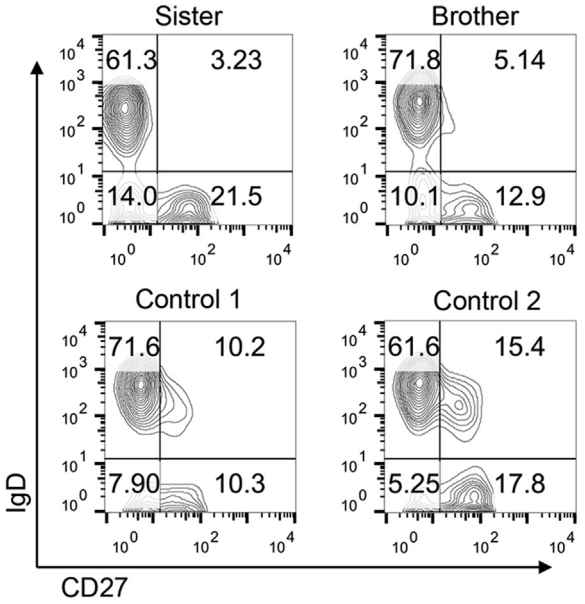


FIG E3. The phenotype of B cells in the patients. The PBMCs were stained for the surface expressions of CD3, CD19, CD27, and IgD. The percentages of naive B cells ($CD3^-CD19^+CD27^-IgD^+$), nonswitched B cells ($CD3^-CD19^+CD27^+IgD^+$), and switched B cells ($CD3^-CD19^+CD27^+IgD^-$) were determined by flow cytometry.

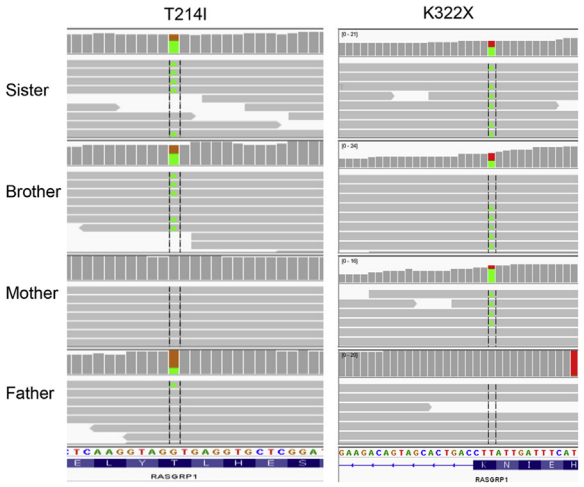


FIG E4. Alignment of sequencing reads around the 2 mutations in *RASGRP1*. This was performed by IGV (Integrative Genomics Viewer) of the raw sequencing reads in the reverse strand. Codon ACC (Thr) was mutated to ATC (Ile) in the father and the 2 affected siblings. Codon AAG (Lys, K) was mutated to TAG (stop codon) in the mother and the 2 siblings. Shown in the IGV are reverse sequences.

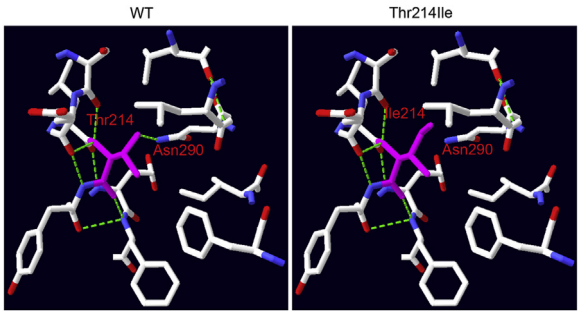


FIG E5. Structure analysis of the mutant RasGRP1. The structural impact of variant Thr214Ile was analyzed on the basis of template of 4L9M from PDB. The residue 214 together with certain nearby residues within 6 Å was illustrated in the wild-type (WT) and variant RasGRP1 by Swiss-PdbViewer. Computed hydrogen bonds are shown as green dashed lines. Residues Thr214/Ile214 are highlighted in pink.

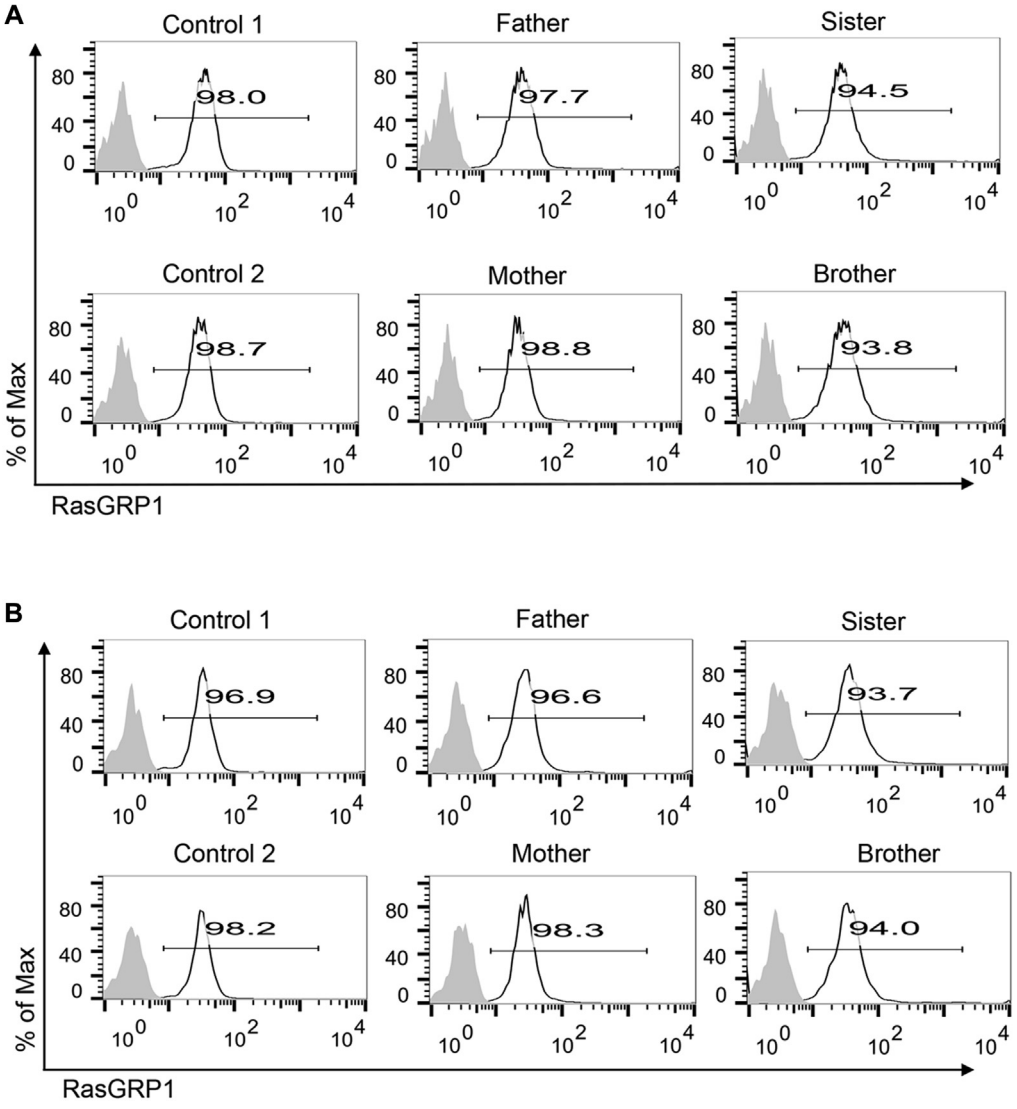


FIG E6. Normal RasGRP1 expression in T and B cells of the patients. The PBMCs were stained first for the surface expressions of CD3 and CD19, and then fixed, permeabilized, and labeled with anti-RasGRP1 antibody. The expression of RasaGRP1 in $CD3^+$ T **(A)** and $CD19^+$ B **(B)** cells was examined by flow cytometry.

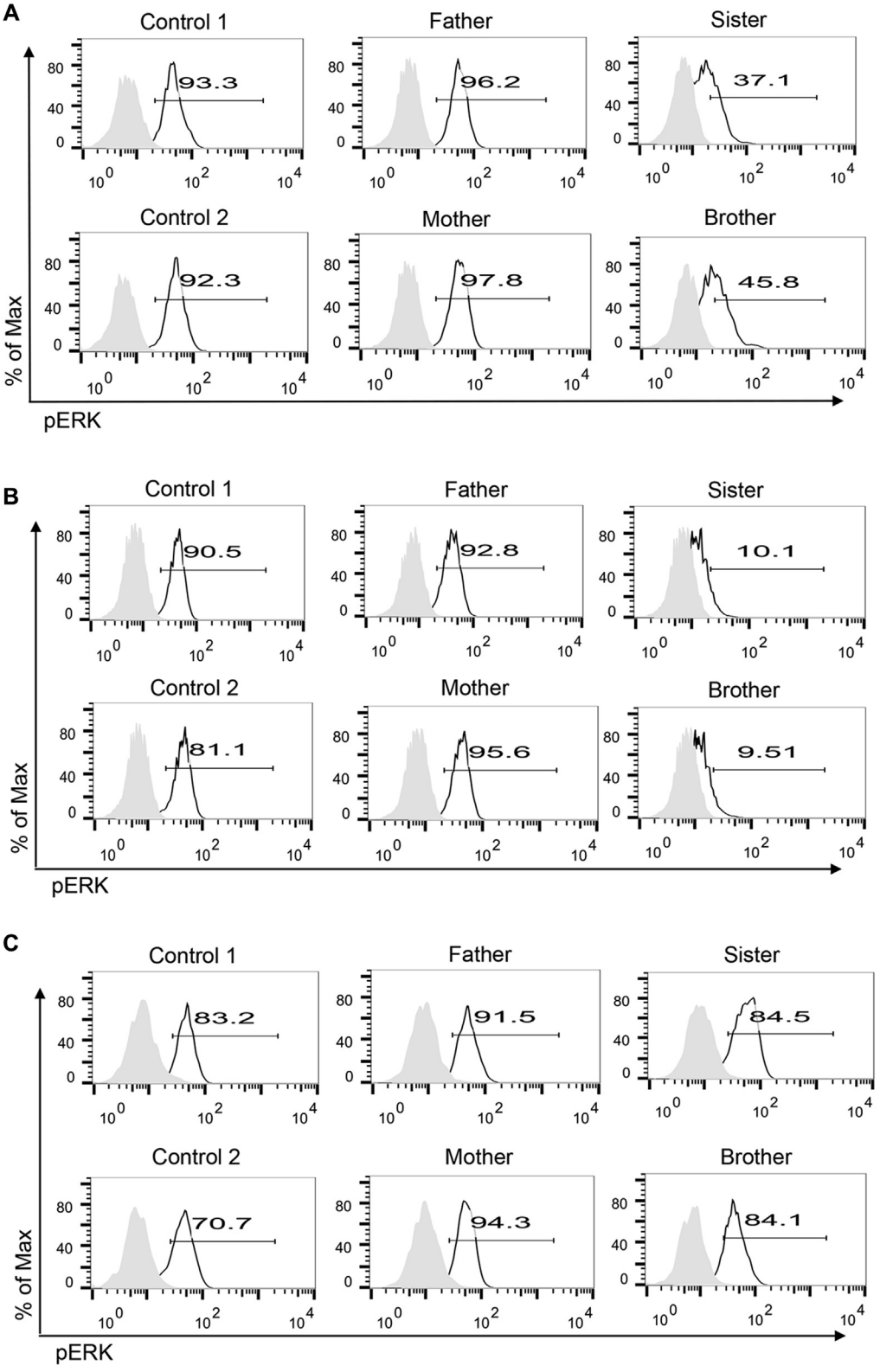


FIG E7. PMA-induced ERK activation was impaired in patient T cells, but not in B cells. PBMCs were stimulated with PMA for 5 minutes. Phosphorylated ERK (pERK) expression was examined by flow cytometry in $CD4^+$ **(A)**, $C8^+$ **(B)** T, and $CD19^+$ B **(C)** cells. The increase in the percentage of pERK expression in stimulated cells relative to resting cells was calculated.

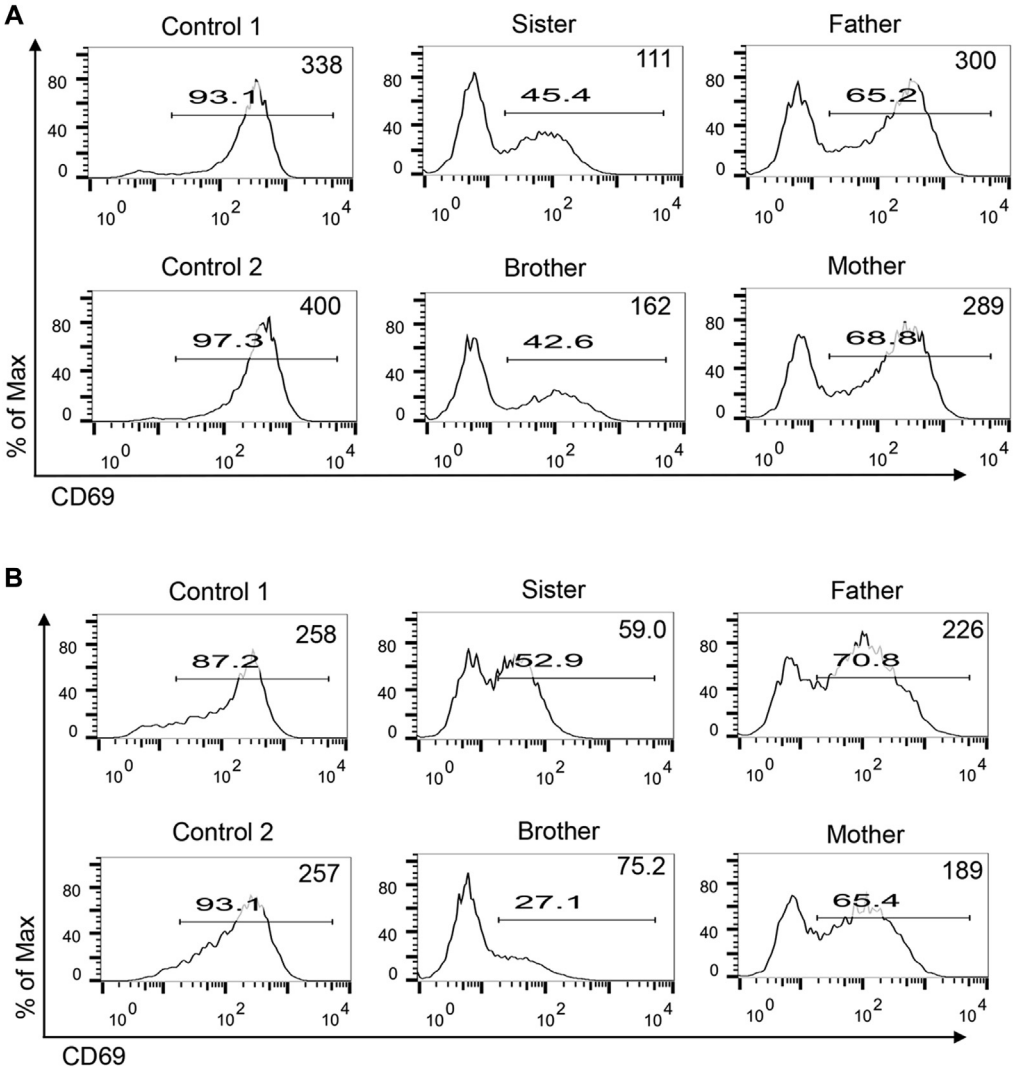
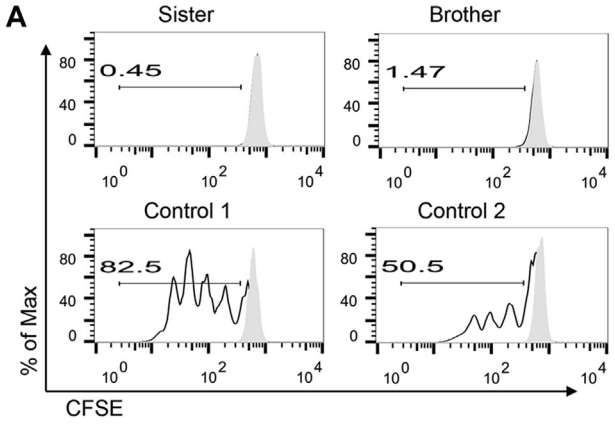


FIG E8. TCR stimulation-induced T-cell activation was impaired in the patients. The PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies overnight. The percentage of CD69-positive cells out of $CD4^+$ **(A)** and $C8^+$ **(B)** T cells was examined by flow cytometry. The mean fluorescence intensity of CD69 expression was indicated at the right upper corner of each histogram.



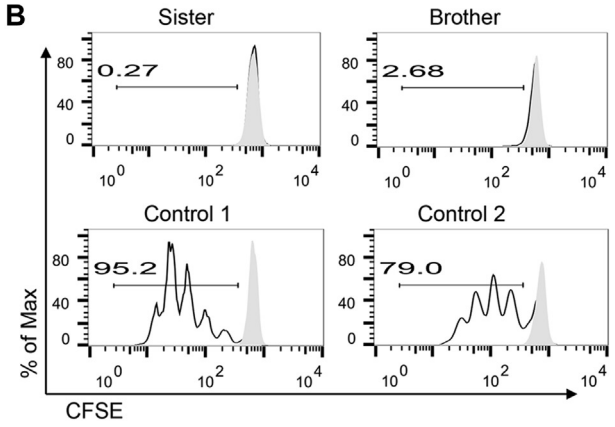


FIG E9. CFSE dilution test showed the impaired TCR-stimulated T-cell proliferation in the patients. The PBMCs were first stained with 1 mM CFSE, and then stimulated with anti-CD3 and anti-CD28 antibodies for 4 days. The proliferation of $CD4^+$ **(A)** and $C8^+$ **(B)** T cells was examined by flow cytometry. *CFSE*, Carboxyfluorescein succinimidyl ester.

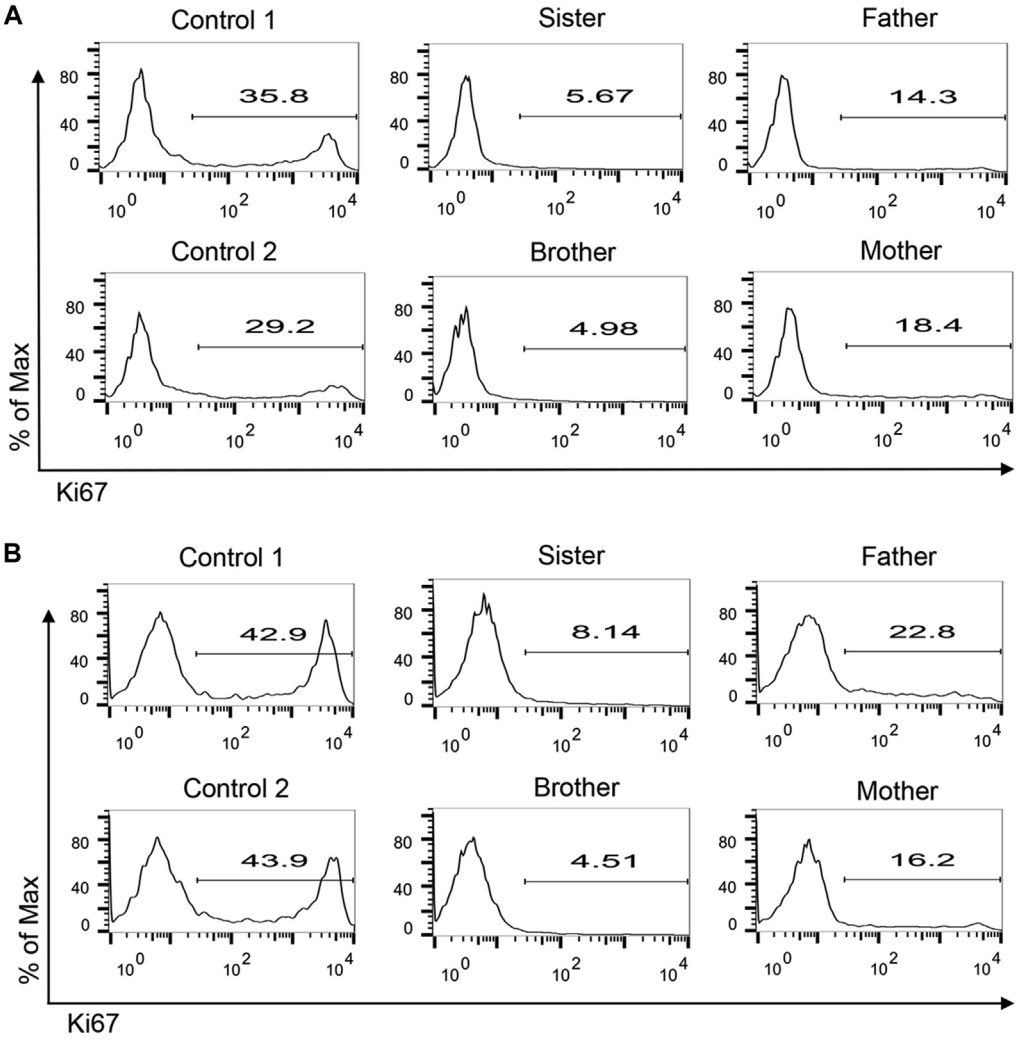


FIG E10. TCR stimulation-induced T-cell proliferation was impaired in the patients. The PBMCs were stimulated with LEAF-purified anti-CD3 and anti-CD28 antibodies for 2 days. The intracellular expression of Ki67 in $CD4^+$ **(A)** and $C8^+$ **(B)** T cells was examined by flow cytometry.

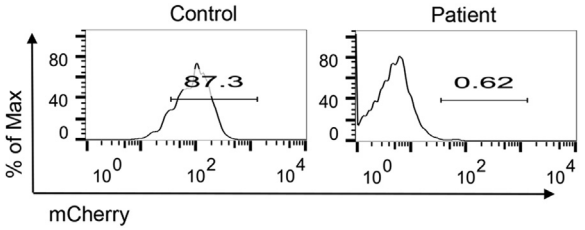


FIG E11. Transfection of T cells by RasGRP1-expressing lentivirus. T cells of normal control and patient were transfected with the lentivirus expressing wild- type RasGRP1 and mCherry. The mCherry expression in T cells was examined by flow cytometry.

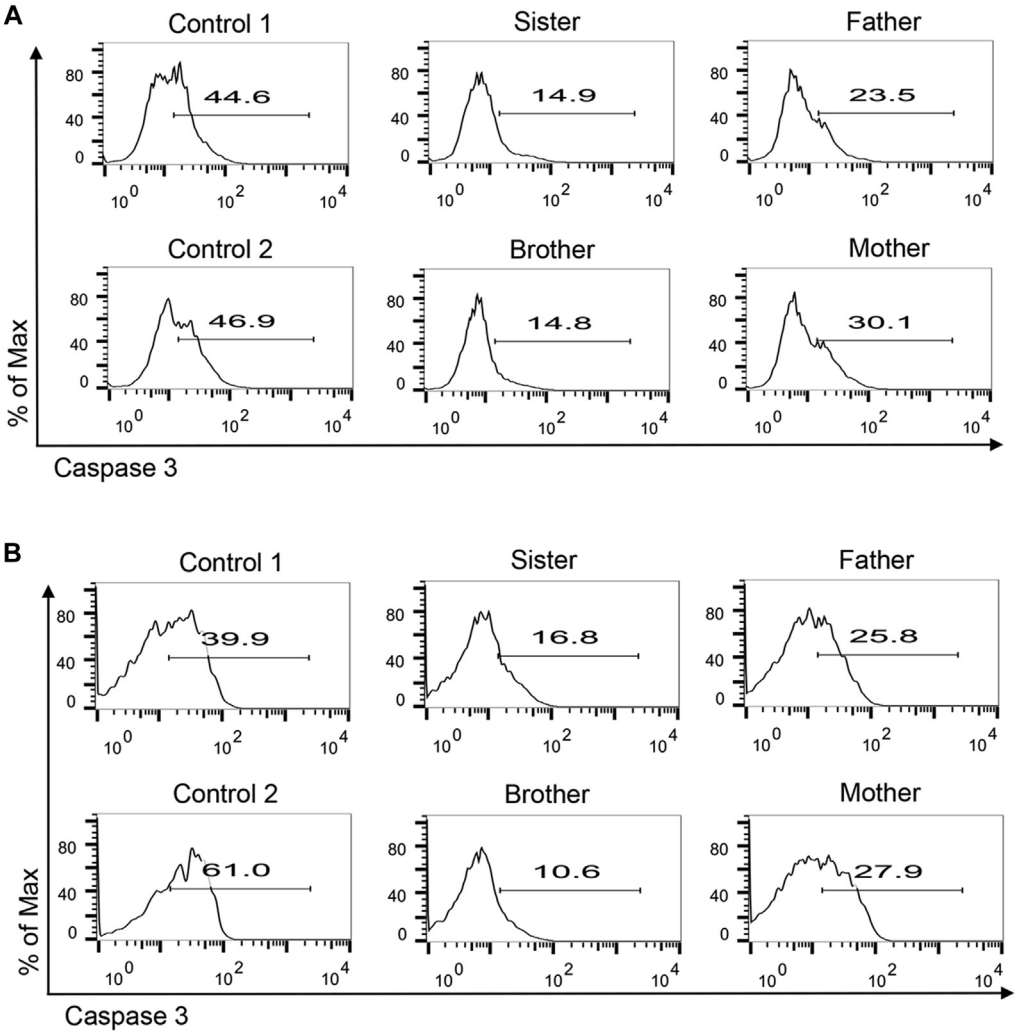


FIG E12. Impaired activation-induced cell death in patient T cells. The PBMCs were stimulated with LEAF-purified anti-CD3 and anti-CD28 antibodies for 2 days. The intracellular expression of active caspase 3 in $CD4^+$ **(A)** and $C8^+$ **(B)** T cells was examined by flow cytometry.

TABLE E1. Quality variants detected by whole-exome sequencing in both patients

Variants detected by whole-exome sequencing	No. of variants
Allele frequency <1% in public databases	10,163
After function filter	645
Missense	591
Frameshift	21
Nonsense	16
Splice-site	17

TABLE E2. The 3 potential candidate genes

Gene symbol	Gene name	Variation	Tissue distribution	Gene function
PLIN4	Perilipin 4	Homozygous	Widely expressed in multiple tissues	Member of the perilipin family, coat intracellular lipid storage droplets
CCDC74B	Coiled-coil domain containing 74B	Compound heterozygous	Not well known	Not well known
RASGRP1	RAS guanyl releasing protein 1	Compound heterozygous	Mainly expressed in lymphocytes and thymus	Activates the ERK/MAP kinase cascade and regulates T- and B-cell development, homeostasis, and differentiation

TABLE E3. Comparison in the phenotype of RASGRP1 deficiency between our patients and mice[E4-E10]

Phenotype	Our patients with RASGPR1 deficiency	Mouse RASGRP1 deficiency
Autoimmunity		
Autoantibody	ANA, other autoantibodies	ANA
Lymphoproliferation		
Lymphadenopathy	Yes	NA
Splenomegaly	Yes	Yes
Hepatomegaly	Yes	NA
Infection	Yes	Yes
T-cell function defect		
ERK signaling	↓	↓
Cell activation	↓	↓
Cell proliferation	↓	↓
Cell apoptosis	↓	↓
Immune phenotype		
T cell	Inverted CD4/CD8 ratio	T lymphopenia
TCRαβ, TCRγδ expression	↓ TCRαβ expression, ↑ TCRγδ expression	Increased γδT in peripheral lymphoid organ
Regulatory T	Normal	↑
Immunoglobulin	↑	↑

NA, Not applicable/available.