

Letter to the Editor

Autoimmunity due to RAG deficiency and estimated disease incidence in *RAG1/2* mutations

To the Editor:

A growing body of literature demonstrates that recombina-
se activating gene (*RAG1/2*) deficiency is seen in patients with
autoimmune features and variable immunodeficiency, thus
expanding the phenotype beyond T^+/B^- /natural killer $^+$ severe
combined immunodeficiency (SCID) and Omenn syndrome
(OS).^{1,2} Based on data from SCID newborn screening programs
in the United States and Puerto Rico, the estimated incidence of

SCID is approximately 1:50,000.³ Recent data from California's
SCID newborn screening identified *RAG1/2* mutations in
28.6% of SCID/OS cases, for an incidence of about 1:250,000.⁴
However, this may not include individuals with less severe
phenotypes caused by *RAG1/2* defects.

We present a family affected by compound heterozygous
RAG1 mutations that resulted in a combined immunodeficiency
phenotype with autoimmune cytopenias. We performed a
population genetic analysis to estimate the incidence of disease
caused by *RAG1/2* homozygous or compound heterozy-
gous mutations and found that the contribution of immune

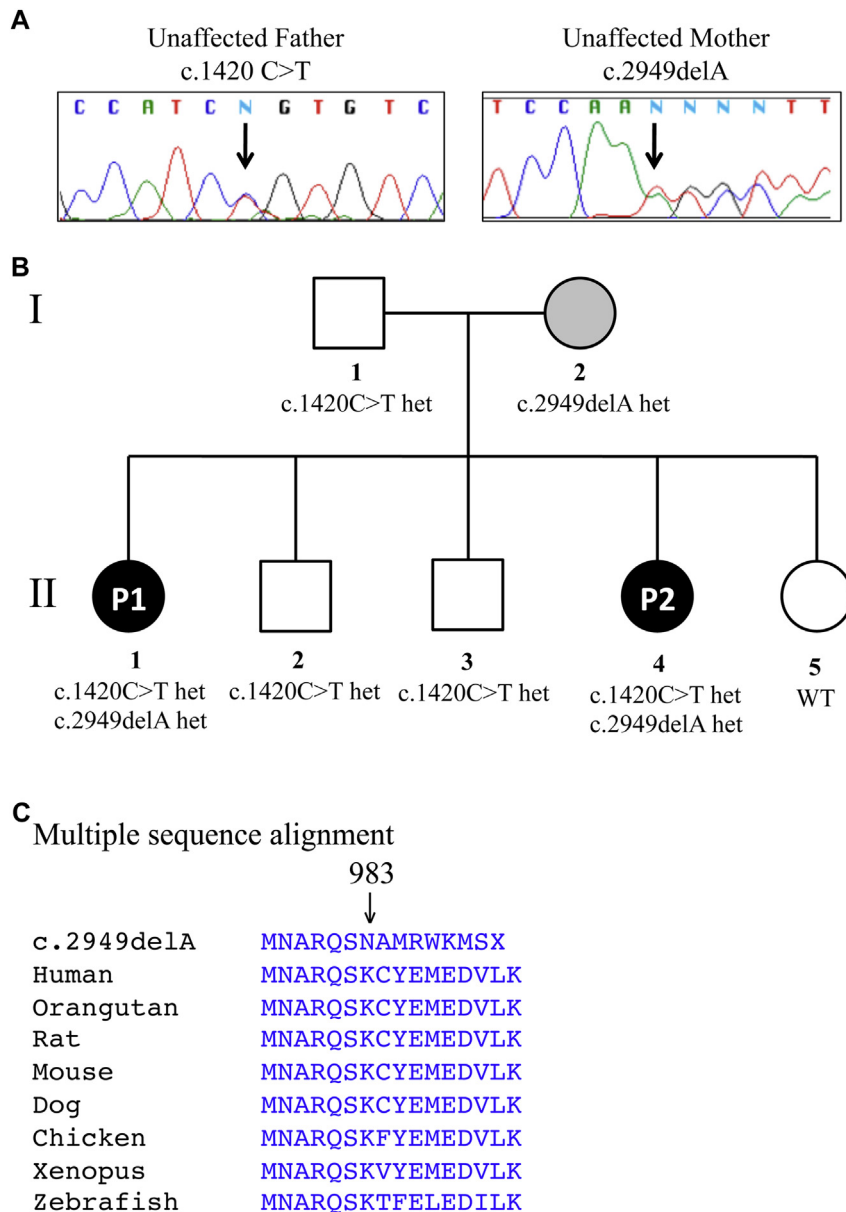


FIG 1. A, Sanger sequencing chromatogram of parents of the affected children confirms *RAG1* mutation carrier status. *Arrow* indicates mutation position. **B,** Pedigree of family with *RAG1* deficiency. Patient 1 (P1, II.1) and patient 2 (P2, II.4) were affected with autoimmune cytopenias and infections. *Het*, Heterozygous; *WT*, wild type. **C,** Multiple sequence alignment of the *RAG1* locus with c.2949delA(p.K983NfsX9) mutation.

TABLE I. Clinical features and key laboratory data

	Patient 1 (II.1)	Patient 2 (II.4)
Infections	Recurrent otitis media Pneumonias (bacterial and viral) Parvovirus aplastic crisis H1N1 Influenza A encephalitis Varicella from vaccinated family contact	Recurrent otitis media RSV bronchiolitis Varicella disease and varicella periorbital cellulitis/keratitis from vaccinated family contact
Clinical autoimmune features	Nephrotic syndrome (18 mo; after childhood immunization; not biopsied but responded to systemic steroids) Autoimmune hemolytic anemia (3 y)	Eczematous rash during infancy Neutropenia (4 mo) Autoimmune hemolytic anemia (15 mo)
Leukocyte counts (cells/ μ L)	Birth: ALC 2700, normal ANC 3.5 y: ALC 1500, normal ANC Eosinophils: normal	4 mo: ALC 6700, ANC 100 26 mo: ALC 600, ANC 100 Eosinophils: 700 (4 mo), then normal
Autoantibodies	Warm IgG agglutinin to RBC Lupus anticoagulant + ANCA; +anti-MPO antibody Thyroglobulin and thyroid peroxidase antibody Anti-IFN- α antibody	Direct Coombs+ (IgG and complement) Antineutrophil antibody Anti-IFN- α antibody
Pathology		Bone marrow biopsy: Expanded $\gamma\delta$ T-cell population (46% of total lymphocytes), granulocyte maturation arrest at promyelocyte stage, myeloid hypoplasia, cellular marrow with trilineage hematopoiesis, and mild increase in CD34 ⁺ myeloblasts. The B-cell light chains were shifted toward lambda predominance, although the kappa:lambda ratio was within normal limits

Time points in parentheses indicate age of onset or age laboratory data were noted.

ALC, Absolute lymphocyte count per microliter; ANC, absolute neutrophil count per microliter; ANCA, antineutrophil cytoplasmic antibody; MPO, myeloperoxidase; RBC, red blood cell; RSV, respiratory syncytial virus.

dyregulatory disease due to *RAG1/2* mutations present in the general population may be much higher than previously estimated.

Patient 1 (P1) is the eldest of 5 siblings born to nonconsanguineous white parents (Fig 1, II.1). Clinical and laboratory data are summarized in Table I and Table E1 (see this article's Online Repository at www.jacionline.org). Briefly, P1 developed early-onset autoimmune hemolytic anemia, recurrent viral and bacterial infections, and nephrotic syndrome. Patient 2 (P2) is the full sibling of P1 (Fig 1, II.4), born full term with an eczematous rash. She developed recurrent bacterial and viral upper respiratory tract infections, early-onset autoimmune hemolytic anemia, and neutropenia with antineutrophil antibodies. The autoimmune cytopenias did not respond to anti-CD20 treatment (rituximab). Both siblings had significantly reduced naive CD4⁺ T-cell counts. Notable family history includes the siblings' healthy mother who had false-positive Rapid Plasma Reagin antibodies and several maternal family members with multiple sclerosis.

P1 underwent a 10/10 HLA-matched sibling bone marrow transplant at 8 years following conditioning with busulfan, fludarabine, and antithymocyte globulin. Graft-versus-host disease prophylaxis included cyclosporine and methotrexate. One year posttransplant, P1 has excellent donor chimerism. Immune globulin replacement was discontinued 16 months posttransplant. P2 received a 10/10 matched unrelated marrow hematopoietic stem cell transplant (HSCT) at 29 months following similar conditioning. She had delayed engraftment of neutrophils with persistence of antineutrophil antibodies. Complications have included grade 3 gastrointestinal graft-versus-host disease 6 months posttransplant, pancytopenia, and concern for graft failure.

To identify an underlying genetic defect, P2 was screened for known mutations associated with SCID. No disease-causing mutations were identified in *JAK3*, *DCLRE1C* (ARTEMIS), *IL7R*, or *RAG2*. She harbored compound heterozygous mutations in exon 2 of *RAG1*, a genotype that was also confirmed for P1 (Fig 1). The c.1420C>T (p.Arg474Cys) mutation has previously been reported in cases of primary immunodeficiency, including SCID, OS, and CD4⁺ T-cell lymphopenia (see Table E2 in this article's Online Repository at www.jacionline.org). The c.2949delA (p.Lys983AsnfsX9) mutation is novel. Sanger sequencing of the remaining family members identified each parent as a *RAG1* mutation carrier (Fig 1). The c.2949delA mutation is located at the carboxy terminus of RAG1, at a RAG2 interaction site within the catalytic core.^{5,6} A homozygous mutation at nearby amino acid position Q981P has been described by de Villartay et al⁷ with clinical features very similar to P1 and P2.

Serum samples from healthy and affected family members were probed against an autoantibody protein microarray. IgG/IgM autoantibodies were increased in P1 and P2, and surprisingly, also in their mother (see Fig E1 and Tables E3-E6 in this article's Online Repository available at www.jacionline.org). Furthermore, serum was tested for anticytokine antibodies that have been reported among patients with thymoma or autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy.^{8,9} Interestingly, our 2 affected patients also produce anti-IFN- α autoantibodies that were able to neutralize IFN- α induction of STAT1 phosphorylation in normal PBMCs, and may comprise a phenotypic signature for certain RAG-deficient patients (see Fig E2 in this article's Online Repository available at www.jacionline.org).

We assessed the European population carrier rate of *RAG1/2* variants that have been identified in primary immunodeficiency diseases (see this article's [Methods](#) section in the Online Repository at www.jacionline.org). Of more than 100 disease-causing *RAG1* variants in the Human Gene Mutation Database, only 2 were present in 379 individuals of European ancestry in the 1000 Genomes database (see [Table E7](#) in this article's Online Repository available at www.jacionline.org). Seven and 3 unrelated individuals were heterozygous for the c.1346G>A and c.3016A>G disease-associated *RAG1* variants, respectively. Therefore, the predicted population frequency based on Hardy-Weinberg equilibrium is 1:5746 individuals of European descent who are homozygous or compound heterozygous, much higher than what is expected for *RAG1/2* SCID or OS cases. The single *RAG2* disease-causing variant present in 1000 Genomes is in linkage disequilibrium with the c.3016A>G variant. Thus, the *RAG1/2* carrier rate does not change. Our population genetic estimate indicates that *RAG1* mutations likely contribute to many more undiagnosed cases of combined immunodeficiency, autoimmune cytopenias, and/or organ-specific autoimmune disease, although it is possible that there is incomplete penetrance of these genotypes. California has a diverse and large Hispanic demographic, and there may be differences when comparing estimates to a predominantly European population. Our estimate does not account for very rare *RAG1* mutations not present in the 1000 Genomes database. Because limited data are available for functional activity in *RAG1* variants identified in patients with primary immunodeficiency diseases, their disease-causing role cannot be easily established.

In this case report, compound heterozygous *RAG1* mutations resulted in a combined immunodeficiency phenotype with autoimmune cytopenias and multiple autoantibodies, including anti-IFN- α antibodies as have been reported in patients with thymoma or autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy. The mechanism for the generation of these autoantibodies is unclear, but it may depend on an underdeveloped thymus with decreased *AIRE* expression. The mother has a history of false-positive Rapid Plasma Reagin and demonstrated presence of elevated levels of autoantibodies that was not seen in the c.1420C>T male carriers. Our findings suggest that the c.2949delA variant, even in heterozygous state, may contribute to the generation of autoantibodies in *RAG1*-related disease.

In patients with autoimmune cytopenias, lymphopenia, and moderate-to-severe infections, screening for *RAG1/2* mutations may well be worthwhile to identify molecular pathogenesis and treatment options. In our cohort, the presence of mild lymphopenia, autoimmune cytopenias, and a skewed naive:memory T-cell ratio led to the diagnosis of *RAG1* deficiency and subsequent HSCT. Earlier identification of a monogenic immune disorder and prompt HSCT during infancy might have spared progressive autoimmune complications. These findings provide additional data that *RAG* has important implications in both immunodeficiency and autoimmune diseases.

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METHODS

Subjects and blood samples

Blood samples were obtained from subjects after written informed consent according to a research protocol approved by the University of Utah Institutional Review Board. A retrospective review of records was completed.

DNA isolation and gene sequencing

DNA was extracted from whole blood by using Gentra Puregene chemistry (Qiagen, Valencia, Calif). Genomic DNA from unaffected family members was PCR amplified at the RAG1 exon 2 locus by using High Fidelity Phusion DNA Polymerase (New England BioLabs, Ipswich, Mass) and the following primers: forward 5'-TGACTTGTTTTCATT GTTCTCAGG-3', reverse 5'-TTGAGTTTCCCTCTGGGTTG-3'. Sanger sequencing was performed by using standard methods (Applied Biosystems Capillary Sequencer; Applied Biosystems, South San Francisco, Calif) with the amplicons and the following primers: 5'-CAACATCTTC TGTCGCTGAC-3' and 5'-GAATCCCTCT GCCAGTACAG-3'.

Autoantibody microarray

Human serum samples obtained from family members with RAG1 deficiency were probed against an autoantibody protein microarray. Age-matched healthy controls and a lupus positive control were also probed. The microarray is a HydroGel slide printed with a selection of 76 glomerular, nuclear, and other autoantigens as well as 6 control proteins in duplicates. The array is probed with serum samples, developed with Cy3 fluorescently labeled anti-IgG and Cy5-labeled anti-IgM, and then scanned at 635 nm and 570 nm fluorescence, respectively. The mean fluorescence intensity was converted into an optimized data set based on the normal control. Data were analyzed for clustering and visualized by using MultiExperiment Viewer, Cluster 3.0 (Open Source software available at <http://mev-tm4.sourceforge.net>), and Mapletree v.0.2.3.2 (Open source software available at <http://mapletree.sourceforge.net>).

Anticytokine autoantibody assays

Patient and control plasmas at 1:100 dilution were screened for autoantibodies by using a bioplex assay against 9 cytokines. Normal PBMCs in the presence of patient or normal plasma (10% concentration) were stimulated for 15 minutes with either IFN- α or IFN- γ . Cells were fixed and permeabilized and evaluated by flow cytometry for the presence of either IFN- α - or IFN- γ -induced phosphoSTAT-1.

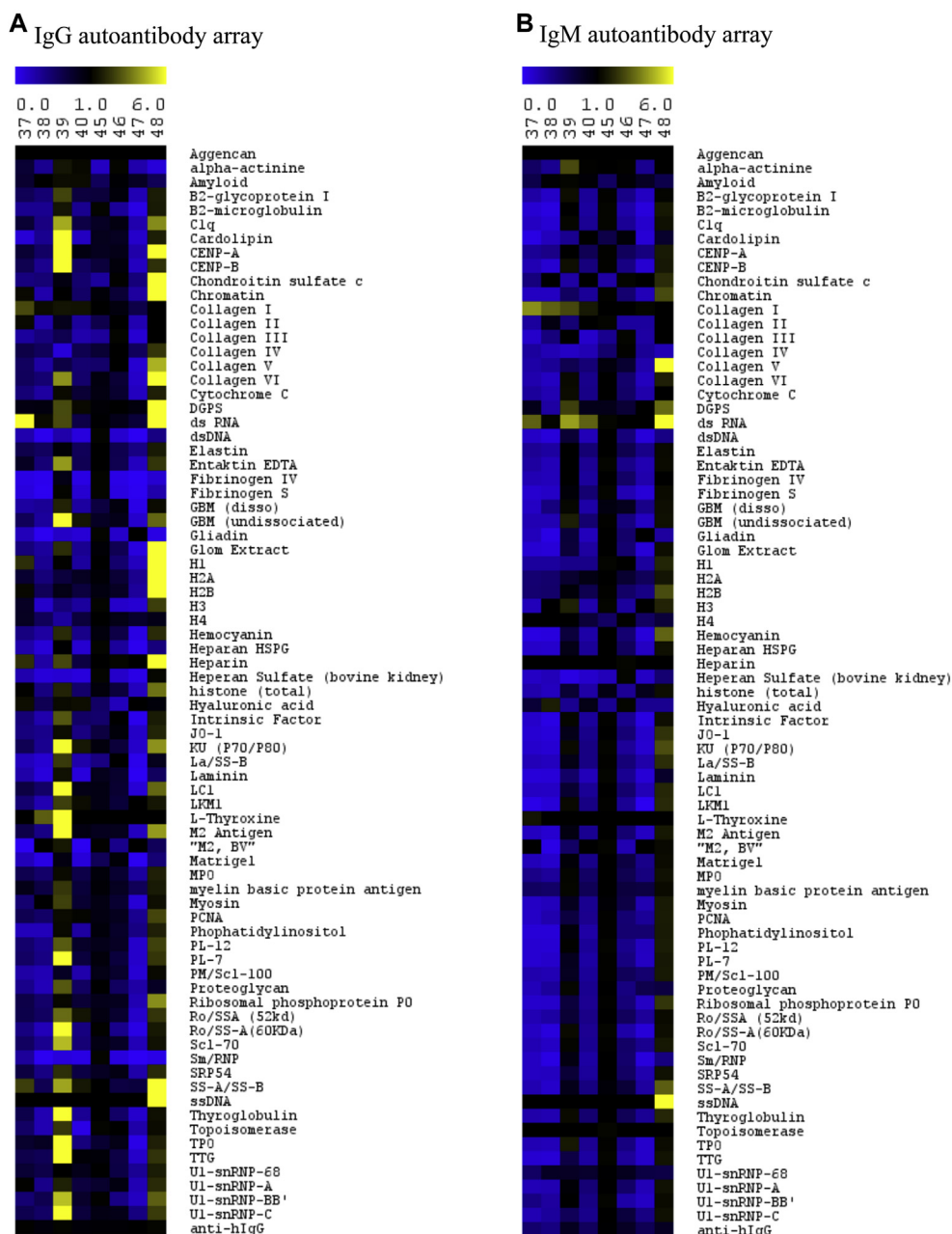
Population genetic disease estimate

RAG1 and RAG2 mutations known to be associated with congenital immune deficiency disorders were procured from the Human Gene Mutation

Database (HGMD) v.2012.3. Variants included missense mutations, nonsense mutations, insertions, and deletions. RAG1 and RAG2 variants in the 1000 Genomes database were identified. The 1000 Genomes database is a comprehensive database of genomes obtained from multiple populations around the world, and it identifies 98% of alleles whose frequency is more than 1% in these populations.^{E10} Lower frequency alleles are also identified in these populations. Variants present in both databases were intersected, resulting in only 2 disease-causing variants present in both the HGMD and the European populations of the 1000 Genomes database. Allele frequencies of the European populations were then estimated from the 1000 Genomes database. Calculations were made focusing on the European 1000 Genomes populations for 2 reasons: (1) The case report involves a family of European descent, and (2) there is a strong bias for European populations in existing mutation data sets such as the HGMD. The references associated with these variants were manually reviewed to ensure that they were correctly associated with a primary immunodeficiency disease.

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Array samples:

- 37. Patient 1 (II.1)
- 38. Patient 2 (II.4)
- 39. Mother (I.2)
- 40. Father (I.1)
- 45. 2-year-old healthy control
- 46. Adult healthy control
- 47. Adult healthy control
- 48. Lupus patient

FIG E1. Heatmaps. Yellow indicates fold increase in signal compared with healthy controls (mean + 1 SD). **A**, IgG autoantibody array using subject samples as indicated. **B**, IgM autoantibody array using subject samples as indicated.

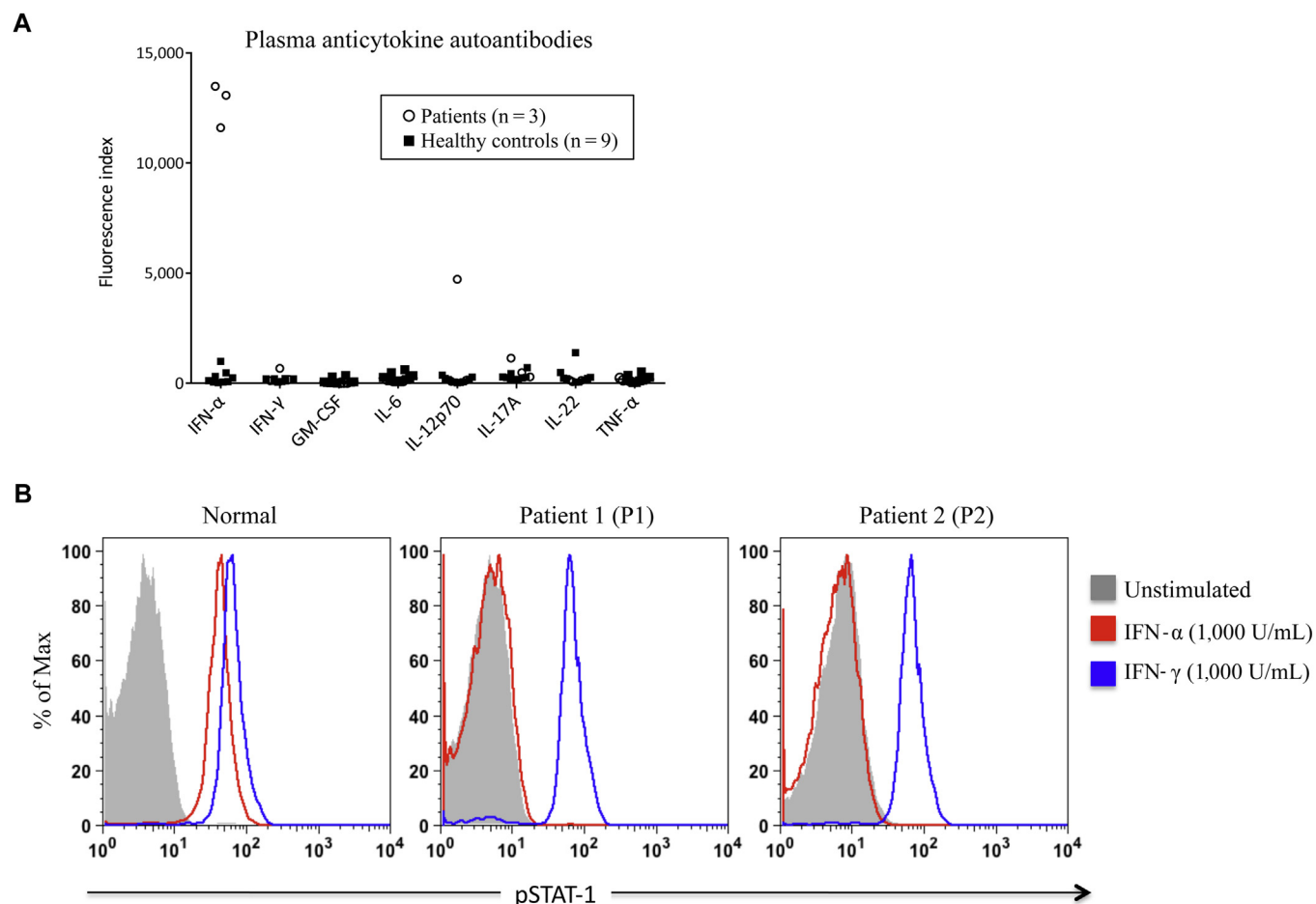


FIG E2. Anticytokine autoantibody evaluation. **A**, Patient and control plasmas were screened for autoantibodies against 9 cytokines. Patient data represent 1 plasma sample from patient P1 and 2 plasma samples from patient P2. **B**, Normal PBMCs in the presence of patient (pretransplant) or normal plasma were stimulated for 15 minutes with either IFN- α or IFN- γ . Cells were fixed and permeabilized and evaluated by flow cytometry for the presence of either IFN- α - or IFN- γ -induced phosphoSTAT-1.

TABLE E1. Clinical laboratory evaluation of patients with RAG1 deficiency

	Patient 1 (II.1) 3.5 y	Reference range (age-adjusted)	Patient 2 (II.4) 2 y	Reference range (age-adjusted)
Lymphocytes cells/ μ L (% of total lymphocytes)	CD3 ⁺ 691 (47%)	684-2170 (58%-87%)	CD3 ⁺ 972 (62%) L	1460-5440 (53%-81%)
	CD4 ⁺ 458 (31%)	381-1469 (32%-62%)	CD4 ⁺ 380 (24%) L	1020-3600 (31%-54%)
	CD4 ⁺ CD45RA ⁺ 46 (3%)	44-869 (3%-28%)	CD4 ⁺ CD45RA ⁺ 47 (3%) L	200-3400 (15%-70%)
	CD4 ⁺ CD45RO ⁺ 427 (30%)	92-1040 (16%-46%)	CD4 ⁺ CD45RO ⁺ 395 (25%)	50-1500 (5%-30%)
	CD8 ⁺ 187 (13%)	196-1060 (4%-27%)	CD8 ⁺ 232 (15%) L	570-2230 (16%-38%)
	NK cells 278 (19%)	76-570 (4%-27%)	NK cells 213 (14%)	80-340 (3%-19%)
	CD19 ⁺ B cells 464 (32% H)	116-613 (5%-23%)	CD19 ⁺ B cells 360 (23%) L	430-3300 (11%-45%)
Lymphocyte mitogen proliferation	Low proliferation to PHA Normal proliferation to Con A and PWM		Normal proliferation to PHA, Con A, and PWM	
Immunoglobulin levels (mg/dL)	IgG 1135		IgG 973	
	IgM 90		IgA 44	
	IgA 98		IgM 70	
	IgE 2		IgE 1 (obtained at 11 mo)	
Additional laboratory data	T-cell spectratyping: Mild skewing for clonal T-cell population			

Abnormal results are given in boldface.

Con A, Concanvalin A; H, high; L, low; NK, natural killer; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

TABLE E2. *RAG1* mutation genotypes in the affected family

<i>RAG1</i> mutation	Exon	Protein	References
c.1420C>T	2	p.Arg474Cys	Villa et al ^{E1} and Sobacchi et al ^{E2} Gruber et al, ^{E3} Kuijpers et al, ^{E4} Asai et al, ^{E5} and Kwan et al ^{E6}
c.2949delA	2	p.Lys983AsnfsX9	Novel mutation

Correlating protein change and references of previously reported cases with the same variant are listed. Full references are available in the Online Repository.

TABLE E3. Patient 2 IgG autoantibody values above 2.0 (fold increase compared with mean of controls + 1 SD)

Antigen	Patient 2	Mother	Patient 1	Control	Lupus
dsRNA	15.18	2.80	1.22	0.86	297.86
Collagen I	2.77	1.52	1.26	0.93	0.00
SS-A/SS-B	2.57	4.48	0.60	0.66	6.37
Heparin	2.23	2.74	0.27	1.10	8.05
H1	2.18	1.05	0.35	0.48	52.57

TABLE E4. Mother IgG autoantibody values above 2.0 (fold increase compared with mean of controls +1 SD)

Antigen	Mother	Patient 1	Patient 2	Control	Lupus
TPO	14.86	0.76	0.82	0.88	1.81
PL-7	13.96	0.52	0.40	0.68	2.38
TTG	9.40	0.62	0.58	0.65	2.31
M2 Antigen	8.92	0.64	0.39	0.85	4.31
Cardiolipin	7.71	0.11	0.35	0.82	1.79
Ro/SS-A (60 KDa)	7.63	0.41	0.21	0.37	1.64
U1-snRNP-C	7.36	0.61	0.52	0.87	2.51
LC1	6.95	0.66	0.51	0.74	3.38
CENP-A	6.69	0.73	0.53	0.87	19.30
GBM	6.26	0.57	0.31	0.86	3.36
L-Thyroxine	6.14	0.00	3.21	0.00	0.00
Thyroglobulin	6.11	0.63	0.23	0.51	1.37
CENP-B	6.01	0.60	0.36	0.95	2.25
Ku (P70/P80)	5.91	0.59	0.36	0.70	4.15
U1-snRNP-BB'	4.94	0.47	0.61	0.40	3.24
Scl-70	4.86	0.53	0.36	0.80	1.42
C1q	4.57	0.73	0.41	0.90	4.05
SS-A/SS-B	4.48	2.57	0.60	0.66	6.37
Entaktin EDTA	4.28	0.81	0.45	0.60	2.33
Collagen VI	4.14	0.56	0.47	0.72	21.46
Ro/SSA (52kd)	3.47	0.62	0.54	0.75	2.33
PL-12	3.15	0.49	0.37	0.80	2.61
Proteoglycan	3.09	0.37	0.21	0.54	0.80
Intrinsic factor	3.05	0.45	0.25	1.01	1.71
DGPS	2.81	0.91	0.93	1.00	11.17
dsRNA	2.80	15.18	1.22	0.86	297.86
Heparin	2.74	2.23	0.27	1.10	8.05
B2-glycoprotein I	2.69	0.58	0.47	0.96	1.73
Myosin	2.61	0.48	0.99	0.74	1.35
LKM1	2.58	0.47	0.23	0.70	1.59
La/SS-B	2.48	0.20	0.11	1.04	0.85
Myelin basic protein	2.40	0.87	0.66	0.82	1.70
Topoisomerase	2.39	0.52	0.23	0.00	1.27
SRP54	2.18	0.66	0.40	0.90	2.07
Glom extract	2.17	0.44	0.31	0.59	10.92
Hemocyanin	2.08	0.41	0.30	0.64	2.11

CENP-A, Centromere protein A; *CENP-B*, centromere protein B; *DGPS*, 1,2-Diacyl-sn-glycero-3-phosphoethanolamine; *GBM*, glomerular basement membrane; *Glom extract*, glomerular extract; *LC1*, liver cytosolic antigen type 1; *LKM1*, liver kidney microsomal antigen 1; *SRP54*, signal recognition particle 54-kDa subunit; *TPO*, thyroid peroxidase; *TTG*, tissue transglutaminase.

TABLE E5. Patient 1 IgM autoantibody values above 2.0 (fold increase compared with mean of controls +1 SD)

Antigen	Patient 1	Mother	Patient 2	Control	Lupus
Collagen I	4.12	2.75	3.29	0.00	0.00
dsRNA	3.29	4.39	1.10	0.00	18.12

TABLE E6. Mother IgM autoantibody values above 2.0 (fold increase compared with mean of controls +1 SD)

Antigen	Mother	Patient 1	Patient 2	Control	Lupus
dsRNA	4.39	3.29	1.10	0.00	18.12
alpha-actinin	2.80	0.52	0.35	1.05	0.00
Collagen I	2.75	4.12	3.29	0.00	0.00
DGPS	2.57	0.86	0.29	0.71	3.43

DGPS, 1,2-Diacyl-*sn*-glycero-3-phosphoethanolamine.

TABLE E7. Carrier rate of *RAG1* disease-associated variants in the European populations of the 1000 Genomes population database

Chromosome	Gene	Base position	Variant	Allele frequency	Carrier frequency	References
11	RAG1	36596200	c.1346G>A (p.Arg449Lys)	0.00923	1:54	Sobacchi et al ^{E2} and Bell et al ^{E7}
11	RAG1	36597870	c.3016A>G (p.Met1006Val)	0.00396	1:126	Sheehan et al ^{E8} and Safaei et al ^{E9}

Variants are known to be associated with immunodeficiency and were identified through the HGMD. Positions are based on human genome reference NCBI build 37.3.