

Novel mutations in *TNFRSF7/CD27*: Clinical, immunologic, and genetic characterization of human CD27 deficiency

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Background: The clinical and immunologic features of CD27 deficiency remain obscure because only a few patients have been identified to date.

Objective: We sought to identify novel mutations in *TNFRSF7/CD27* and to provide an overview of clinical, immunologic, and laboratory phenotypes in patients with CD27 deficiency.

Methods: Review of the medical records and molecular, genetic, and flow cytometric analyses of the patients and family members were performed. Treatment outcomes of previously described patients were followed up.

Results: In addition to the previously reported homozygous mutations c.G24A/p.W8X (n = 2) and c.G158A/p.C53Y (n = 8), 4 novel mutations were identified: homozygous missense c.G287A/p.C96Y (n = 4), homozygous missense c.C232T/p.R78W (n = 1), heterozygous nonsense c.C30A/p.C10X (n = 1), and compound heterozygous c.C319T/p.R107C-c.G24A/p.W8X (n = 1). EBV-associated lymphoproliferative disease/hemophagocytic lymphohistiocytosis, Hodgkin lymphoma, uveitis, and recurrent infections were the predominant clinical features. Expression of cell-surface and soluble CD27 was significantly reduced in patients and heterozygous family members. Immunoglobulin substitution therapy was administered in 5 of the newly diagnosed cases.

Conclusion: CD27 deficiency is potentially fatal and should be excluded in all cases of severe EBV infections to minimize diagnostic delay. Flow cytometric immunophenotyping offers a reliable initial test for CD27 deficiency. Determining the precise role of CD27 in immunity against EBV might provide a framework for new therapeutic concepts. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: CD27 deficiency, EBV-induced lymphoproliferation, Hodgkin lymphoma, hypogammaglobulinemia, hemophagocytic lymphohistiocytosis

Currently, there are 11 known genetic defects associated with immunodeficiency and the generic category of EBV-associated lymphoproliferative disease (LPD), including *SH2D1A* (SH2 domain protein 1A),¹ *XIAP* (X-linked inhibitor of apoptosis),^{1,2} *ITK* (IL-2 inducible T-cell kinase),³ *WAS* (Wiskott-Aldrich syndrome),⁴ *CORO1A* (coronin, actin binding protein, 1A),^{5,6} *MST1/STK4* (mammalian sterile 20-like kinase-1/serine/threonine protein kinase 4),⁷ *DCLRE1C* (DNA cross-link repair 1C),⁸ *IL10*,⁹ *MAGT1* (magnesium transporter 1),¹⁰ *PIK3CD* (phosphatidylinositol 3-kinase, catalytic, delta)¹¹ and *CD27*,¹²⁻¹⁴ all of which

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Abbreviations used

ADCC:	Antibody-dependent cell-mediated cytotoxicity
CVID:	Common variable immunodeficiency
HLH:	Hemophagocytic lymphohistiocytosis
HLH-2004 protocol:	Dexamethasone, etoposide, and cyclosporine A
HSCT:	Hematopoietic stem cell transplantation
IM:	Infectious mononucleosis
IVIG:	Intravenous immunoglobulin
LPD:	Lymphoproliferative disease
NK:	Natural killer
SNP:	Single nucleotide polymorphism
TBS:	Tris-buffered saline
WB:	Western blotting
WES:	Whole-exome sequencing

function either at the cell-surface level, intracellular level, or downstream to facilitate T-cell receptor activation and natural killer (NK) cell activation.

CD27 deficiency is the most recently reported EBV-associated LPD-associated immunodeficiency.¹²⁻¹⁴ The *CD27* gene (MIM 615122) is located on chromosome 12 in human subjects (12p13.31). CD27, a member of the TNF receptor superfamily, is a type I transmembrane protein with extracellular, intramembranous, and intracellular domains. The extracellular domain of CD27 binds to CD70, regulating the survival, function, and differentiation of T, B, NK, and plasma cells.^{12,15} In T cells, interaction with CD70 provides costimulation, resulting in a signaling cascade important for cell proliferation, long-term maintenance of antigen-specific T cells, antiviral responses, antitumor immunity, and alloreactivity.^{16,17} Disruption of CD27-CD70 interaction in human subjects and mice has been shown to impair immunity and generation of memory responses against pathogens, including EBV, influenza virus, *Listeria monocytogenes*, lymphocytic choriomeningitis virus, and vesicular stomatitis virus.^{12-15,18,19} Siva, a CD27 cytoplasmic tail-binding protein, is thought to play a role in the CD27-transduced apoptotic pathway.²⁰ Ligation of CD27 on B cells by CD70 results in enhanced plasma cell formation and increased IgG production.¹⁷ Expression of CD27 is also used as a marker for memory B cells in the classification of B-cell deficiencies, including common variable immunodeficiency (CVID).^{3,21,22}

The first 10 patients (from 4 kindreds) with CD27 deficiency were identified in consanguineous families and carried homozygous mutations (c.G24A/p.W8X¹² and c.G158A/p.C53Y^{13,14}). This novel genetic defect was termed lymphoproliferative syndrome 2 (OMIM #615122). Here we present 4 novel mutations in 7 patients from 5 kindreds, as well as clinical, genetic, and immunologic data and treatment outcomes of all 17 patients with CD27 deficiency described to date.

METHODS

Patients

Evaluation of medical records was carried out after obtaining written informed consent, which was performed in accordance with research ethics committee guidelines (see the **Methods** section in this article's Online Repository at www.jacionline.org for details).

Genomic DNA extraction

Genomic DNA was extracted by using the salting-out and kit-based methods (see the **Methods** section in this article's Online Repository for details).

Whole-exome sequencing

DNA library preparation, read mapping, variant analysis, and an analysis protocol for whole-exome sequencing (WES) were performed for 2 patients (P15 and P17), as described previously.²³ Read mapping and variant analysis were performed as follows: Sequences were generated as 90-bp pair-end reads. The sequenced reads were aligned to the human genome reference (UCSC hg 19 version, build 37.1) by using SOAP aligner (soap 2.21) software.²⁴ Duplicated reads were filtered out, and only uniquely mapped reads were kept for subsequent analysis. The SOAPSnp (version 1.03) software²⁵ was subsequently used with default parameters to assemble the consensus sequence and call genotypes in target regions. For single nucleotide polymorphism (SNP) quality control, low-quality SNPs that met one of the 4 following criteria were filtered out: (1) a genotype quality of less than 20; (2) a sequencing depth of less than 4; (3) an estimated copy number of more than 2; and (4) a distance from the adjacent SNPs of less than 5 bp. Small insertions/deletions were detected by using the Unified Genotype tool from GATK (version v1.0.4705)²⁵ after alignment of quality reads to the human reference genome using BWA (version 0.5.9-r16).²⁶

Confirmatory sequencing

The methods for DNA library preparation, capillary sequencing, and Sanger sequencing used to validate potential disease-causing variants are provided in the **Methods** section and in **Table E1** in this article's Online Repository at www.jacionline.org.

Flow cytometric immunophenotyping

Intracellular and cell-surface markers were analyzed by using combinations of mAbs (see the **Methods** section in this article's Online Repository for details). For 1 patient (P16) and living family members, flow cytometric immunophenotyping was performed, as previously published.²⁷

Soluble CD27 ELISA

The concentration of soluble CD27 was assessed in serum samples by using the PeliKine Compact ELISA kit (Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions. Twenty-five healthy anonymized control subjects and 10 patients with CVID were included. Statistical analysis was performed with the SPSS software package, version 21 (IBM, Armonk, NY).

RESULTS

Clinical features and mutations

All patients had mutations in the *CD27* gene. Patients P1 and P2 were the index cases, and the other patients were investigated for EBV-related disease or CVID or were related to the probands. Except for patients P11 and P12, all were born to consanguineous parents. An updated summary of the previously published cases (P1-P10) and detailed case reports of new patients (P11-P17) can be found in the **Results** section in this article's Online Repository at www.jacionline.org.

Patient P11 presented at 4 years of age with fever, lymphadenopathy, hepatosplenomegaly, pancytopenia, and EBV encephalitis (**Table I**). Results of both serum and cerebrospinal fluid serology were positive for EBV. Features of secondary hemophagocytic lymphohistiocytosis (HLH) were also present.

The patient was treated with dexamethasone, etoposide, and cyclosporine A (HLH-2004 protocol), with no response.²⁸ Subsequently, rituximab (4 doses, 375 mg/m²/wk) was administered with a favorable response. Five months later, the patient had an EBV-related extranodal lymphoproliferation of the orbit. Histologic examination of this lesion revealed an EBV-associated

inflammatory infiltration with abundant B-cell blasts (B-cell monoclonality), in which EBV latent membrane protein expression was detected and T-cell blasts. This condition also responded well to rituximab treatment. Intravenous immunoglobulin (IVIG) was administered because of secondary hypogammaglobulinemia. Sequencing revealed a novel heterozygous c.C30A/p.C10X nonsense mutation (Fig 1). Despite an extensive genetic analysis, a second disease-causing mutation could not be identified.

Patient P12 presented at 13 years of age. Serology showed a recently acquired EBV infection. She was later repeatedly admitted to the hospital with oral ulcers, posterior uveitis, lymphadenopathy, and recurrent fever. EBV was clinically suspected as the cause of uveitis and oral ulcers because they occurred during active infection, but direct detection was not performed. A persistently positive EBV plasma viral load was consistent with chronic active EBV infection. The patient had mixed cellularity classical Hodgkin lymphoma and was treated according to the EuroNet PHL-C1 protocol (HD 2007/10, Eudra CT no. 2006-000995-33; Table I), which led to complete remission. She subsequently had persistent hypogammaglobulinemia (Table II) and was treated with IVIG. Sequencing revealed a novel compound heterozygous mutation c.C319T/p.R107C and c.G24A/p.W8X (Tables I and II and Fig 1). Her father and brother were heterozygous for c.C319T/p.R107C, and her mother was heterozygous for c.G24A/p.W8X. Currently, she has recurrent bacterial urinary tract infections and a peripheral neuropathy, probably as a result of vincristine treatment.

Patient P13 presented at 7 years of age with recurrent pneumonias, lymphadenopathy, and splenomegaly. EBV viral load was high, and results of EBV serology were positive (Table II). Rituximab therapy (2 courses with a 2-week interval, 500 mg/m²) reduced the viral load. Subsequently, the patient had hypogammaglobulinemia and was treated with IVIG. EBV viremia recurred, and she was treated on 2 more occasions with the same dose of rituximab. Currently, she is clinically well.

Her sister (P14) was hospitalized at 6 years of age because of EBV infection (Table I). She received 1 week of supportive care and recovered spontaneously and is currently asymptomatic. Sequencing of both siblings showed a novel homozygous c.G287A/p.C96Y missense mutation (Fig 1). Their parents and sister were heterozygous for the c.G287A/p.C96Y missense mutation.

Patient P15 (IV.3, see Fig E1, A and B, in this article's Online Repository at www.jacionline.org) presented at 6 years of age (Table I). She was given a diagnosis of CVID at the age of 13 years when immunoglobulin levels were investigated because of recurrent pneumonias (see Table E2 in this article's Online Repository at www.jacionline.org) and was subsequently treated with IVIG. In addition to the lymphadenopathy and hepatosplenomegaly, she later had bronchiectasis, eczema, skin abscesses, and stage 1 nodular sclerosis classical Hodgkin lymphoma (CD15⁺ and CD30⁺, Table I). She died at age 20 years from multiple complications of lymphoma, infections, and hepatic failure. WES revealed a novel homozygous c.G287A/p.C96Y missense mutation (see Fig E1, B).

Her older brother (P16; IV.1; see Fig E1, A and C) had nodular sclerosis classical Hodgkin lymphoma (with the same histologic pattern as P15) at 8 years of age. Early-stage treatment with radiotherapy and chemotherapy led to remission (Table I). Immunoglobulin levels showed a subnormal level of IgA

(Table II). Additional laboratory findings (Table II) suggested a similar pattern of infections as in P15 (see the Results section in this article's Online Repository). Sanger sequencing confirmed that P16 was also homozygous for the mutation, whereas the parents and living siblings were heterozygous carriers (see Fig E1). The heterozygous brother (IV.4; see Fig E2, A, in this article's Online Repository at www.jacionline.org) had recurrent anal ulcers, the father had chronic sinusitis late in life, and the mother has autoimmune thyroiditis.

A younger sister (IV.5; see Fig E1, A) died at age 16 years as a result of EBV infection and nodular sclerosis classical Hodgkin lymphoma (with the same histopathologic pattern as her siblings). Although having Hodgkin lymphoma suggests that she was also homozygous for the mutation, a history of serious infections was not noted.

Patient P17 presented at 8 years of age with upper respiratory tract infection and giardiasis. At the age of 19 years, acute bronchitis led to pneumonia, at which time hypogammaglobulinemia was discovered (Table II) and IVIG treatment was initiated. Later, the patient had infectious mononucleosis (IM) and chronic diarrhea (Table I). She was admitted to the hospital because of severe EBV pneumonia as a consequence of IM but died because of respiratory failure at the age of 25 years. WES revealed a novel homozygous missense mutation, c.C232T/p.R78W (Fig 1).

In addition to recurrent infections, the 7 patients with novel mutations (P11-P17) presented with severe and/or atypical EBV-related features (Table I). The only exception was P14, who, despite carrying the homozygous mutation c.G287A/p.C96Y (as P13, P15, and P16) and being seropositive for EBV, spontaneously recovered and had a negative EBV viral load. Hodgkin lymphoma (3/7), HLH (2/7), and EBV-related extranodal LPD (1/7) were prominent features (4/7) along with recurrent infections (4/7) and hypogammaglobulinemia (5/7).

When comparing all patients with CD27 deficiency (P1-P17), the clinical manifestations of EBV infection were variable, ranging from a benign course (3/17; P4, P5, and P14) to apparent recovery from therapy (6/17) or hematopoietic stem cell transplantation (HSCT; 3/17) to death (5/17). Recurrent infections were a common feature (7/17) and might have also been prominent in patients in whom a detailed history was not obtainable (P9 and P10) or for the deceased sibling of P16 (IV.5; see Fig E1, A). Atypical and/or chronic EBV-related LPD manifestations included uveitis (4/17), which is a rare chronic condition that is usually refractory to conventional treatment with steroids^{29,30}; clinically suspected EBV-induced oral and/or perianal ulcers (4/17), which are rare in immunocompetent hosts³¹; meningitis/encephalitis (2/17); and recurrent pneumonias (2/17). EBV-induced lymphoproliferation was suspected in more than half of the patients (9/17): HLH (4/17), Hodgkin lymphoma (3/17), EBV-induced LPD/lymphoma (3/17), and diffuse large B-cell lymphoma (2/17, Table I). P12 had chronic active EBV, a disorder characterized by chronic/recurrent symptoms resembling IM and abnormal EBV serology and often associated with a poor prognosis.³²⁻³⁴ Interestingly, her symptoms resolved after chemotherapy for Hodgkin lymphoma. The deceased sibling of P16 (IV.5; see Fig E1, A) also had Hodgkin lymphoma.

Ten of the 17 patients received IVIG (Table I). Hypogammaglobulinemia might have been secondary to treatment in some patients (chemotherapy, 1/17; rituximab, 6/17), and primary hypogammaglobulinemia was less common (3/17). However,

TABLE I. Clinical and genetic characteristics of patients with CD27 deficiency*

Kindred (center)	Genetics							
	A (Utrecht)		B (Vienna)			C (Melbourne)		D (Melbourne)
	Moroccan male	Moroccan male	Turkish female	Turkish female	Turkish male	Lebanese Male	Lebanese female	Lebanese male
Patient:	P1	P2	P3	P4	P5	P6	P7	P8
Mutation	HOM, NS c.G24A, p.W8X	HOM, NS c.G24A, p.W8X	HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y
Exon	Exon 1	Exon 1	Exon 2	Exon 2	Exon 2	Exon 2	Exon 2	Exon 2
Clinical characteristics								
Age at onset/ genetic diagnosis (y)	2/21	3/PM	1/1	Not applicable/14	Not applicable/4	1.5/4	1/1	15/15
EBV-related signs and symptoms	Fever, LAD, HSM	Fever, LAD, HSM, uveitis	Systemic inflammatory response syndrome	—	Subclinical EBV	HLH, recurrent infections, oral ulcers	Oral and anal ulcers, uveitis, chronic EBV viremia, EBV meningitis	Oral ulcers, uveitis, chronic EBV viremia
Infections other than EBV	—	Sepsis with gram-positive bacteria (cause of death)	—	Severe varicella (age 4 y)	CMV	—	Recurrent infections	Recurrent infections
Neoplasia	—	—	Recurrent EBV-related LPD	—	—	EBV-related LPD	—	EBV-related LPD, T-cell lymphoma
EBV positivity on histologic sections	Not applicable	Not applicable	Not tested	Not applicable	Not applicable	Not tested	Not applicable	Not tested
Laboratory	Primary HGG	Aplastic anemia	HLH, secondary HGG	Borderline HGG	Borderline HGG	HLH, secondary HGG	Secondary HGG	Secondary HGG
Treatment and outcome								
Treatment	IVIG	Acyclovir	IVIG, rituximab, PDN	None	Prophylactic IVIG	Rituximab HLH-2004, cord HSCT	IVIG, rituximab, cord HSCT	IVIG, rituximab, R-CHOP, cord HSCT
Outcome	Alive, persistent primary HGG	Died aged 4 y	Alive, borderline secondary HGG	Alive, borderline HGG without IVIG	Alive	Alive	Alive	Alive

ABVD, Adriamycin, bleomycin, vinblastine, dacarbazine; CMV, cytomegalovirus; Cord HSCT, Allogeneic cord blood HSCT; DLBCL, diffuse large B-cell lymphoma; EuroNET PHL-C1, vincristine, adriamycin, etoposide, prednisolone; HET, heterozygous; HGG, hypogammaglobulinemia; HLH-2004, dexamethasone, etoposide, and cyclosporine A; HOM, homozygous; HSM, hepatosplenomegaly; LAD, lymphadenopathy; MS, missense; NS, nonsense; PDN, prednisolone; PM, postmortem; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; SM, splenomegaly; URTI, upper respiratory tract infection.

*Boldface type indicates consanguinity for patients and novel status for mutations. Italics indicate nonconsanguineous status.

P3 to P5 had borderline/low-to-normal immunoglobulin levels, which might later develop into hypogammaglobulinemia. Rituximab therapy for EBV-related LPD, HLH, or lymphoma resulted in a favorable (sometimes transient) response (eg, an orbital tumor developed after the first course in P11, and EBV viremia recurred in P13). Response to conventional chemotherapy was variable. Although Hodgkin lymphoma regressed after treatment in P12 and with chemotherapy in P16, the malignant lymphoproliferation/HLH in patients P8 to P11 and P15 responded poorly.

Because CD27 deficiency is a newly identified condition, it was not suspected at presentation (median age, 6 years; range, 1-22 years), leading to a marked delay until a definite diagnosis was

made (median age, 13 years; range, 1-32 years), and only a postmortem diagnosis was available for 5 patients. Causes of death were related to malignancy (P9, P10, and P15), aplastic anemia, gram-positive sepsis (P2), and respiratory failure (P17). Twelve patients are alive, 3 of whom (3/17) have received cord blood HSCT (Table III).

Genetic diagnosis

In addition to the previously reported homozygous mutations c.G24A/p.W8X and c.G158A/p.C53Y, 4 novel mutations were identified. To our knowledge, P12 is the first CD27 deficiency caused by compound heterozygous mutations (c.C319T/p.R107C

TABLE I. (Continued)

Genetics								
D (Melbourne)		E (Krefeld)	F (Krefeld)	G (Rotterdam)		H (Tehran)		I (Tehran)
Lebanese female	Lebanese female	German female	German female	Turkish female	Turkish female	Iranian female	Iranian male	Iranian female
P9	P10	P11	P12	P13	P14	P15	P16	P17
HOM, MS c.G158A, p.C53Y	HOM, MS c.G158A, p.C53Y	HET, NS c.C30A, p.C10X (other not found)	compound HET c.G24A, p.W8X; c.C319T p.R107C	HOM, MS c.G287A, p.C96Y	HOM, MS c.G287A, p.C96Y	HOM, MS c.G287A, p.C96Y	HOM, MS c.G287A, p.C96Y	HOM, MS c.C232T, p.R78W
Exon 2	Exon 2	Exon 1	Exon 1,3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3
Clinical characteristics								
2/PM	22/PM	4/9	14/17	7/7	6/13	6/PM	8/32	8/PM
—	—	LAD, HSM, HLH, EBV encephalitis	Chronic active EBV, uveitis, oral ulcers	LAD, SM, chronic EBV viremia	Primary EBV, fever, prostration, LAD, HSM	Fever, LAD, HSM, chronic hepatitis	EBV infection (positive serology), LAD	Flu-like symptoms, LAD, SM, enlarged tonsils, pharyngitis
—	—	—	—	Recurrent pneumonia and URTI	—	Toxoplasmosis, oral candidiasis, recurrent pneumonia, herpangina	Recurrent influenza	Recurrent sinusitis, otitis media, bronchitis/ pneumonia, giardiasis
DLBCL (suspected EBV-related LPD)	DLBCL (suspected EBV-related LPD)	Extranodal EBV- related LPD	Mixed cellularity classical Hodgkin lymphoma	—	—	Nodular sclerosis classical Hodgkin lymphoma	Nodular sclerosis classical Hodgkin lymphoma	—
Not tested	Not tested	EBV latent membrane protein (+)	Not tested	Not applicable	Not applicable	Not tested	Not tested	Not applicable
—	—	HLH, pancytopenia, secondary HGG	HLH, secondary HGG	Secondary HGG	—	Primary HGG (CVID)	—	Primary HGG (CVID)
Treatment and outcome								
PDN	CHOP	IVIG, HLH-2004, rituximab	IVIG, EuroNET PHL-C1	IVIG, rituximab	None	IVIG	ABVD, radiotherapy	IVIG
Died aged 2 y	Died aged 22 y	Alive, secondary HGG	Alive, secondary HGG, peripheral neuropathy	Alive, secondary HGG on IVIG	Alive and asymptomatic	Died aged 20 y	Alive	Died aged 25 y

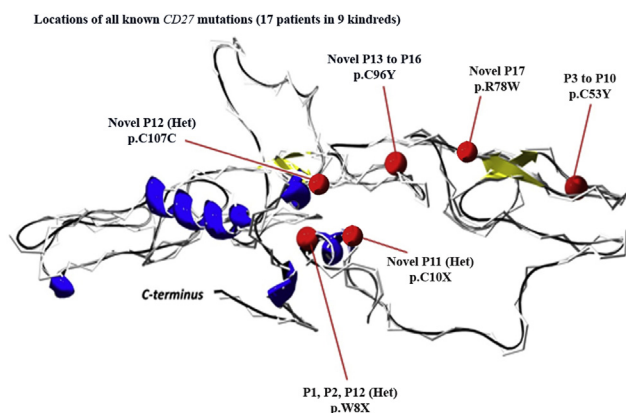


FIG 1. Locations of all known mutations in patients with CD27 deficiency (P1-P17, Table I).

and c.G24A/p.W8X). *In silico* analysis of c.C319T/p.R107C with the Scratch Protein Predictor³⁵ showed that it induces the formation of a disulfide bond between cysteine residues 107 and 112, replacing the bond between cysteine residues 106 and 112 and suggesting production of a misfolded protein. P15 and P16 were homozygous for a novel mutation (c.G287A/p.C96Y) and had severe clinical phenotypes, whereas P13 and P14, carrying the same mutation, had less severe phenotypes. p.C96Y probably abolishes the formation of a conserved disulfide bond involved in protein folding.³⁶ P17 had a novel c.C232T/p.R78W mutation and a severe phenotype. Both SIFT and PolyPhen-2 scores predicted that the p.C96Y (0.04 and 1.000) and p.R78W (0.01 and 1.000) mutations damage protein function. For p.R107C, the SIFT score (0.02) predicted a damaging effect, and the PolyPhen-2 score (0.767) predicted a possibly damaging effect. Other mutations implicated in EBV viremia were excluded by means of WES and conventional sequencing.

TABLE II. Immunologic characteristics of patients with CD27 deficiency

Patient:	P1	P2	P3	P4	P5	P6	P7
CD27 expression and EBV viral load							
CD27 expression	Absent	Not tested	Absent	Absent	Absent	Absent	Absent
Lowest EBV PL (copies/mL)	<50	Not tested	1e2	Not tested	0	0	Not available
Highest EBV PL (copies/mL)	243	Not tested	2e6	Not tested	3.6e2	5e6	8e6
Humoral response							
IgG (g/L) before treatment (RR)	4.4↓ (4.8-15.0)	10.4	4.51 (4.45-15)	8.43 (6.98-11.9)	2.9 (0.55-7.99)	4.67 (2.86-16.8)	11.6 (2.86-16.8)
IgA (g/L) before treatment (RR)	0.1 (0.15-1.2)	0.4 (0.15-1.2)	0.55 (0.21-2.03)	0.67 (0.22-2.74)	0.09 (0-0.64)	1.1 (0.19-1.75)	1.2 (0.19-1.75)
IgM (g/L) before treatment (RR)	0.03↓ (0.2-2.2)	2.7↑ (0.2-2.2)	0.58 (0.36-2.28)	1.34 (0.19-0.99)	0.56 (0.09-0.77)	0.75 (0.43-1.63)	3.05 (0.43-1.63)
Serology/other tests	EA (+) VCA (+) EBNA (–)	EA (+) VCA (+) EBNA (–)	EA (not tested) VCA (+) EBNA (–)	EA (not tested) VCA (not tested) EBNA (+)	After IVIG EA (+) VCA (not tested) EBNA (+)	VCA (+)	VCA (+)
Specific antibodies							
Tetanus toxoid	Low†	Normal	Low	Normal	Low§	Normal	Normal
Diphtheria	Not tested	Not tested	Normal	Normal	Normal§	Not tested	Not tested
Rubella	Not tested	Not tested	Not vaccinated	Normal	Normal§	Not tested	Not tested
Pneumococcus	Positive	Positive after vaccination	Not vaccinated	Positive	Low-normal§	Not vaccinated	Not vaccinated
Other	MCV4 (+) Rabies (+/–)	—	Hib (low) TBE (+)	Hib (+) TBE (+)	Hib (low)§ TBE (+)§	Hib (+)	Hib (+)
White cell count (cells/mL)							
Leukocytes	4,200	16,100	6,680	2,560	15,170	5,700	9,700
Lymphocytes (RR)	2,478 (1,400-4,200)	7,084↑ (1,400-5,500)	4,408 (3,200-12,300)	1,208↓ (1,400-4,200)	9,997↑ (1,400-4,200)	3,990 (1,400-4,200)	6,310 (3,200-12,300)
Lymphocyte subgroups							
CD19 ⁺ B cells/μL (RR)	100-400‡ (300-100)	40↓‡ (300-1,100)	490-1,100 (140-7,700)	520 (120-740)	750-1,040 (180-1,040)	1,120 (180-1,040)	1,700 (140-7,700)
CD4 ⁺ /CD3 ⁺ T cells/μL (RR)	1,700 (1,300-7,100)	160↓ (500-2,700)	1,340 (1,300-7,100)	1,540 (400-2,100)	1,420 (500-2,700)	1,660 (500-2,700)	2,400 (1,300-7,100)
CD8 ⁺ /CD3 ⁺ T cells/μL (RR)	800 (400-4,100)	2,490↑ (200-1,800)	2,150 (400-4,100)	1,210 (300-1,300)	1,060 (200-1,800)	1,250 (200-1,800)	1,580 (400-4,100)
γ/δ T cells/μL	200 (70-630)	Not tested	180 (70-630)	Not tested	160 (27-960)	Not tested	Not tested
NK cells/μL (RR)	Not available	1,380↑ (61-510)	320 (71-3,500)	260 (92-1,200)	310 (61-510)	270 (61-510)	250 (71-3,500)
iNKT cells (% CD3 ⁺)*	0.08	Not tested	0.01-0.02¶	0.08	0.09	Not tested	0.02
Functional assays							
T-cell proliferation	Reduced	Absent	Normal	Not tested	Not tested	Normal	Not tested
NK cell function	Normal	Reduced (improved with IL-2)	Reduced	Not tested	Not tested	Reduced	Normal-reduced††

EA, Anti-EBV early antigen; EBNA, EBV nuclear antigen antibody; EBVC, anti-EBV viral capsid antibody; EBV PL, EBV plasma load copies/mL (minimum-maximum); HBV, hepatitis B virus; Hib, *Haemophilus influenza* type; HSV, herpes simplex virus; iNKT, invariant natural killer T; MCV4, meningococcal polysaccharide vaccine; PVB19, parvovirus B19; RR, reference range; TBE, tick-borne encephalitis; VCA, anti-EBV viral capsid antigen; VZV, varicella zoster virus.

*Invariant natural killer T cells were selected with Vα24 and Vβ11 antibodies in P1, P13, and P14 and with 6B11 mAb in P3 to P5, P7, P8, P11, and 12.

†Poor response to tetanus toxoid booster vaccination.

‡B cells were analyzed based on CD20 positivity.

§Serologic studies were performed 20 months after suspending IVIG.

||Reduced but returned to normal after treatment.

¶Performed first during severe EBV-associated LPD and systemic inflammatory response syndrome (0.01%) and later during clinical remission (0.02%).

*Receiving rituximab.

**Pneumovax after hypogammaglobulinemia.

††Before EBV infection (tested at 6 weeks of age) and then reduced when the patient presented at 1 year.

‡‡Seroconversion was documented after 19 months.

|||See van Montfrans et al¹² and Salzer et al¹³ for details.

¶¶Performed at a later date.

TABLE II. (Continued)

P8	P9	P10	P11	P12	P13	P14	P15	P16	P17
Absent	Not available	Not available	Absent	Reduced	Reduced	Absent	Not tested	Absent	Not tested
0	Not available	0.4	0	22,000	4,560	Negative	Not tested	Not tested	Not tested
5e6	Not available	>2.7e5	1.0e6	3.2e5	2.2e6	Negative	Not tested	Not tested	Not tested
32.4↑ (5.18-17.8)	75 IU/mL (23rd centile)	Not available	24.9 (5.8-17.2)	10.1 (6.4-12.5)	7.6 (5.8-17.2)	7.0 (5.8-17.2)	5.0↓ (6.0-15.0)	7.9 (6.0-15.0)	4.7↓ (6.0-15.0)
1.2 (0.19-1.75)	Trace (5th centile)	Not available	3.6 (0.41-2.5)	2.8 (0.9-2.7)	0.3 (0.8-3.8)	0.7↓ (0.8-3.8)	0.46↓ (0.8-3.8)	0.23↓ (0.8-3.8)	Undetectable
0.8 (0.32-1.35)	51 IU/mL (18th centile)	Not available	2.2 (0.5-2.7)	2.5 (0.4-1.0)	0.42 (0.5-3.7)	1.12 (0.5-3.7)	0.1↓ (0.5-3.7)	0.9 (0.5-3.7)	Undetectable
VCA (+)	Not available	VCA (+)	EA (not tested) VCA (+) EBNA (+)	EA (+) VCA (+) EBNA (–)†‡	EA (+) VCA (+) EBNA (+)	EA (+) VCA (+) EBNA (+)	Not tested	VCA (+) EBVC (+)	Atypical lymphocytosis
Normal	Not available	Not available	Normal (on IVIG)	Normal	Not tested	Low	Not tested	Not tested	Not tested
Not tested	Not available	Not available	Normal (on IVIG)	Not tested	Not tested	Normal	Not tested	Not tested	Not tested
Not tested	Not available	Not available	Normal	Normal	Not tested	Not tested	Not tested	Not tested	Not tested
Negative after vaccination**	Not available	Not available	Positive (on IVIG)	Not tested	Negative	Not vaccinated	Not tested	Not tested	Not tested
Hib (+)	—	—	HBV (+) Hib (+)	HBV (+) Measles (+) PVB19 (+)	Hib (+) VZV (+)	—	—	HSV (+) Toxoplasma (+)	Isohemagglutinin titers: 1/32; blood group: A
17,700	17,000	3,300	3,550	3,600	7,600	5,000	14,000	5,600	9,330
7,430↑ (1,400-4,200)	6,630↑ (1,400-5,500)	1,640 (1,200-4,100)	2,100 (1,200-4,700)	2,052 (1,400-4,200)	2,812 (1,200-4,700)	2,150 (1,200-4,700)	7,700↑ (1,200-4,700)	2,000 (1,200-4,700)	3,732 (1,200-4,700)
84↓# (120-740)	Not available	Not available	441 (100-800)	198 (120-740)	480 (100-800)	500 (100-800)	200 (200-600)	253 (200-1600)	373 (100-500)
796 (400-2,100)	Not available	Not available	914 (400-2,500)	653 (400-2,100)	443 (400-2,500)	Not tested	1,000 (400-2,100)	556 (300-2,000)	757 (300-1,400)
2,724↑ (300-1,300)	Not available	Not available	757 (200-1,700)	911 (300-1,300)	513 (200-1,700)	Not tested	6,100↑ (200-1,200)	636 (300-1,800)	1,824↑ (200-900)
Not tested	Not available	Not available	211 (27-960)	70¶¶ (39-540)	110 (27-960)	Not tested	Not tested	Not tested	Not tested
293 (92-1,200)	Not available	Not available	93 (70-590)	158 (92-1,200)	190 (70-590)	60↓ (70-590)	170 (70-1,200)	364 (90-900)	343 (90-600)
<0.01	Not available	Not available	0.1¶¶	0.01¶¶	0.03	0.3¶¶	Not tested	Not tested	Not tested
Normal	Not available	Not available	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Reduced	Not available	Not available	Not tested	Normal	Not tested	Not tested	Not tested	Not tested	Not tested

TABLE III. Transplantation outcomes for patients P6 to P8

Patient:	P6	P7	P8
Donor	UCD	UCD	UCD
High-resolution tissue typing	7/10 9/10 (by low-resolution typing)	9/10	8/10
ABO grouping	Identical	Minor mismatch	Minor mismatch
Conditioning regimen	RIC (Bu + Cy + ATG)	RIC (Bu + Flu + ATG)	RIC (Cy + Flu + TBI)
GVHD prophylaxis	Cyclosporine, methylprednisolone	Cyclosporine, MMF	Cyclosporine, MMF
Age at transplantation (y)	2.6	2.7	17.8
Days to engraftment*	34	20	5
Posttransplantation infections†	Rotavirus, adenovirus, EBV, HHV6 reactivation	CMV reactivation	HHV6
Acute GVHD	Gut (grade 3-4)	Skin (grade 1-2)	Skin (grade 1-2)
Chronic GVHD	None	Skin (grade 1-2)	None
Donor chimerism‡ (days after transplantation)	100% (day 234)	100% (day 204)	100% (day 84)
Last clinical assessment	1642 d after transplantation	481 d after transplantation	1313 d after transplantation
Current posttransplantation day	1718	577	1553
Current clinical status	Cord colitis syndrome (GIT failure, TPN dependent), infections resolved, recurrent abdominal pain, developmental delay, poor growth, cochlear implant	Well, skin GVHD (resolving) CMV infection and abnormal LFTs (resolved)	Well (no issues) GVHD (resolved)

ATG, Antithymocyte globulin; Bu, busulfan; CMV, Cytomegalovirus; Cy, cyclophosphamide; Flu, fludarabine; GIT, gastrointestinal tract; GVHD, graft-versus-host disease; HHV6, human herpes virus 6; LFT, liver function test; MMF, mycophenolate mofetil; RIC, reduced intensity regimen; TBI, total body irradiation; TPN, total parenteral nutrition; UCD, unrelated cord blood.

*Engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count of $0.5 \times 10^9/L$ or greater.

†There were no active pretransplantation infections.

‡Chimerism was assessed by means of single nucleotide polymorphism polymerase chain reaction.

Protein expression

The living previously published patients described in the article showed absent CD27 expression.

For P11, CD27 lymphocyte surface expression (see Fig E2) was markedly reduced (T and B cells) and absent on Western blotting (WB; data not shown). B cells from both her parents (see Fig E2) were shown to have intermediate expression of CD27, despite the fact that no mutation or any transcriptionally repressive antisense RNA could be identified in her mother (see the Methods section in this article's Online Repository). However, their T cells showed near-normal expression of CD27.

For P12, CD27 expression was markedly reduced on both T and B cells, and expression was more reduced on B cells of the heterozygous parents and brother than on T cells (see Fig E2).

For P13 and P14, lymphocyte surface expression was nearly absent on T cells and reduced on B cells for P14. Expression on B cells could not be determined in P13 because of the rituximab therapy, and all heterozygous family members had reduced T-cell CD27 expression. In P13 absent expression of CD27 was confirmed by means of WB (data not shown).

For P15, CD27 expression studies could not be performed because the diagnosis was made postmortem. B-cell CD27 surface expression was reduced for P16 and all living heterozygous relatives (see Fig E3 in this article's Online Repository at www.jacionline.org). Notably, CD27 expression was markedly reduced on CD19⁺/CD21⁺ memory B cells in P16 ($P < .05$, see Fig E3 and Table E3 in this article's Online Repository at www.jacionline.org). Levels of soluble CD27 were measured in P16 and were markedly reduced (1.82 ± 1.10 U/mL) compared with control values (29.9 ± 9.4 U/mL, $n = 25$, $P < .001$), values in heterozygous family members (8.6 ± 1.4 U/mL, $P = .03$), and values in patients with CVID without known CD27 mutations (44.7 ± 28.8 U/mL, $n = 10$, $P = .02$). The role of soluble CD27 has not been studied

extensively to permit a conclusive functional role, but low levels might correlate with low expression on lymphocytes.

For P17, CD27 expression studies could not be performed because the diagnosis was made postmortem.

Taken together, B-cell surface CD27 expression was absent or near absent in the homozygous (P13-P14 and P16) and heterozygous (P11 and P12) patients and moderately reduced on T cells in heterozygous carriers of p.W8X or p.R107C.

Immunologic studies

Lymphocyte subsets are presented in Tables II and E2 to E4 in this article's Online Repository at www.jacionline.org. Numbers and percentages of CD4 T cells appeared normal, and T-cell proliferation (by mitogen stimulation) was normal in 3 of 5 of the patients. CD8 T cells were expanded in 4 of 7 patients, leading to an inversion of the CD4/CD8 ratio. For P16, T-cell subsets were compared with those of heterozygous family members and healthy control subjects and showed a reduced percentage of CCR7⁺CD45RA⁻CD45RO⁺ (central memory cytotoxic) T cells and T_H17-like CD3⁺ cells ($P < .05$), whereas percentages of regulatory T cells and recent thymic emigrants appeared normal (see Table E3). Invariant NK T-cell percentages varied markedly in the cohort but were mostly found within the normal range in those analyzed (Table II). Of the patients in whom NK cell function was measured, 5 of 7 had reduced function (Table II).^{12,13}

IgG anti-EBV viral capsid antigen was detected in all patients tested (12/17). EBV viral loads were high in all patients tested (11/12), except for P14, who recovered spontaneously from infection.

DISCUSSION

In this article we present all known cases of CD27 deficiency to date presenting with various clinical phenotypes and a high

mortality rate (29%) and describe the clinical and laboratory phenotypes of novel *CD27* mutations.

In healthy subjects EBV infects and replicates in B cells, leading to self-limiting IM that is subsequently controlled by virus-specific cytotoxic T lymphocytes and, supposedly, invariant NK T cells.³⁷ Altered immunity to EBV appears to be the major complication of human *CD27* deficiency.

All mutations led to near-absent expression of T- and B-cell surface *CD27* in the patients or undetectable *CD27* protein by WB. In terms of genotype-phenotype correlation, patients with the same mutation presented with varied phenotypes (Table I). EBV-related LPDs and lymphoma (Hodgkin lymphoma, T-cell lymphoma, or diffuse large B-cell lymphoma) occurred in patients with all mutations (except P17). Although some patients (P1, P5, P7, P13, and P14) have not yet had these conditions, they might be at risk in view of their EBV viremia (Tables I and II). T cells physiologically express *CD27*, and therefore reduced expression in heterozygous relatives would have been expected, but this was not observed. *CD27* partially influences the kinetics of germinal center formation and immunoglobulin production by interaction with *CD70*.^{17,38} Theoretically, a lack of interaction should result in impairment of antibody production. However, most patients with *CD27* deficiency were able to mount specific humoral responses against EBV and other antigens, including live vaccines. However, once the patients had secondary hypogammaglobulinemia, a reduced response to active immunization was observed in some patients (Table II), suggesting a defect in T cell-dependent B-cell responses.^{12,13}

Of the 10 patients who received IVIG, 8 remain alive. IVIG might have provided these patients with additional ability for recovery because IgG from EBV-seropositive donors can protect immunodeficient mice from EBV-induced tumor formation.^{39,40} An *in vitro* study on human lymphocytes has also shown that IVIG alone prevents EBV-induced transformation into lymphoblastoid cell lines.⁴¹ Maximum effectiveness of IVIG in preventing EBV-induced transformation is achieved in the presence of NK cells through antibody-dependent cell-mediated cytotoxicity (ADCC).⁴¹ Whether ADCC is perturbed in *CD27*-deficient patients remains to be investigated, but if ADCC is fully functioning, IVIG might be a valuable therapeutic option.

Cellular immunity to EBV appears to be significantly compromised in patients with *CD27* deficiency. Based on murine studies, *CD27* appears not to be essential for the generation of T and B cells; however, it functions as a costimulatory receptor expressed on memory T and B cells and is required for adequate expansion of antigen-activated immune cells¹⁵ and possibly also NK cell function. We hypothesize that the initial EBV infection in *CD27*-deficient patients is controlled by *CD28*-mediated costimulation, but subsequent generation of memory cytolytic effector cells is markedly impaired.¹⁷ In patients with *CD27* deficiency, we observed an expansion of *CD8* T cells and, in the case of P16, a decreased percentage of central memory cytotoxic T cells (see Table E3). Although the latter cells were not analyzed in other patients, the decreased proportion of *CD8* memory T cells might reflect a reduced ability to generate memory clones reacting against EBV-infected cells.⁴² These clones have been shown to be necessary to suppress the infection in asymptomatic EBV carriers.⁴³ It is unclear whether this decrease is secondary to the EBV infection or a primary manifestation of the *CD27* deficiency. However, decreased

numbers of *CD8* memory T cells are also observed in *CD27*^{-/-} mice after influenza virus challenge.⁴² We observed reduced *CD8* memory T cells in P16 who had recurrent influenza infections. Successful adoptive transfer of EBV-specific T cells in immunocompromised patients is attributed to *CD8* T cells specific for EBV antigens (which are immunodominant targets for the *CD8* memory T cells).⁴⁴ This suggests a vital role of *CD8* memory T cells in controlling the sequelae of a primary EBV infection.⁴⁵

CD27 deficiency might also affect NK cell physiology. The loss of NK cell-mediated control of EBV infection is thought to predispose to EBV-associated lymphoma.⁴⁶ *In vitro* *CD27*-*CD70* interaction enhances human NK cell antiviral activity through a mechanism that involves augmented IFN- γ secretion, which corresponds to findings in murine NK cells.^{47,48} In addition, *CD27*⁺ NK cells (*CD56*^{bright} cells normally located in tonsils and spleen) produce higher levels of IFN- γ compared with peripheral blood *CD27*⁻ NK cells and effectively inhibit EBV-induced B-cell transformation.⁴⁹ A deficiency of *CD27*⁺ NK cells could deprive these patients of cells that would suppress viral replication and subsequently prevent neoplastic complications.^{21,50} In our cohort NK cell function was decreased (5/7), but it is unclear whether this is a primary manifestation of *CD27* deficiency or secondary to EBV infection.^{12,13} Testing for *CD27*⁺ NK cells in tonsillar (and other lymphoid) tissue might provide insights into the pathogenesis of EBV infection in such patients.

In conclusion, the occurrence of EBV infections coupled to other infections with or without hypogammaglobulinemia causes significant morbidity and high mortality. On the basis of our results, we suggest that *CD27* expression on lymphocytes should be included as a diagnostic screening test in all patients with a history of severe EBV infection, lymphomas, or both, as well as in any case of atypical EBV infection. *CD27* deficiency should be considered regardless of consanguinity, and complete serologic, immunologic, and molecular analyses for EBV infection and immunodeficiency (including serial immunoglobulin levels and lymphocyte proliferation analyses) should be conducted in addition to a comprehensive physical examination, with an emphasis on detecting atypical features of EBV infection. These investigations will help elucidate the role of *CD27* in the immunopathogenesis of EBV-related disease and immunodeficiency.

Clinical implications: *CD27* deficiency should be included in the differential diagnosis of any patient with severe EBV infection, Hodgkin lymphoma, or primary antibody deficiency. Lymphocyte flow cytometry is an effective screening tool.

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METHODS

Patients

Participating centers were Wilhelmina Children's Hospital/University Medical Center Utrecht, The Netherlands (P1, P2)^{E1}; St Anna Children's Hospital, Medical University Vienna, Austria (P3-P5); Royal Children's Hospital, Melbourne, Australia (P6-P10); HELIOS Children's Hospital Krefeld, Germany (P11 and P12); Erasmus MC, Rotterdam, The Netherlands (P13 and P14); and the Research Center for Immunodeficiencies, Tehran University of Medical Sciences, Tehran, Iran (P15-P17). Genetic and flow cytometric analysis were performed at Wilhelmina Children's Hospital/University Medical Center Utrecht, The Netherlands (P1 and P2)^{E1}; the Research Center of Molecular Medicine (CeMM), Vienna, Austria, and the University of Graz, Austria (P3-P5)^{E2}; Heinrich Heine University, Düsseldorf, Germany (P6-P10)^{E2}; Erasmus MC, Rotterdam, The Netherlands (P11-P14); the Translational Centre for Regenerative Medicine (TRM), University of Leipzig, Germany (P15 and P16); and Karolinska University Hospital, Stockholm, Sweden (P15-P17).

Evaluation of medical records, physical examination, and paraclinical investigation were performed based on informed patient consent and approval of ethics committees of each institution.

Genomic DNA extraction

For P11 to P14, genomic DNA was isolated from PBMCs with the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, Mo), according to the manufacturer's instructions. Genomic DNA was extracted by using the salting-out method^{E3} for P15 and P17. The Invisorb Spin Blood Mini Kit (Strattec Molecular, Berlin, Germany) was used for P16 and the living relatives.

Genotyping array analysis

Targeted SNP analysis of the *CD27* gene was performed for P11 by using the Human Omni Express 12v1.0 BeadChip (Illumina, San Diego, Calif), which maps approximately 731,442 markers. GenomeStudio software v2010.1/v1.6.3 (Illumina, San Diego, Calif) was used for data analysis. The log R ratio and B allele frequencies were plotted along chromosomal coordinates.

Targeted next-generation sequencing of the *CD27* locus

Targeted next-generation sequencing was performed for P11 to detect a potential second genetic defect. The whole gene locus (7 kb) was amplified through long-range PCR. Long-amplification PCR was carried out with the TaKaRa long-range PCR system (TaKaRa Bio, Otsu, Shiga, Japan), according to the manufacturer's instructions. PCR products were gel purified before sequencing. Targeted sequencing was performed on the Illumina HiSeq2000 platform by using paired-end 100-base reads. Quality control was performed with FastQC software (Babraham Bioinformatics, Babraham Institute, Babraham, United Kingdom). A mean coverage of 80× was achieved. For raw data processing, the raw HiSeq reads were aligned to the reference human genome (hg19) with default parameters. The resulting SAM file was sorted, compressed, and marked for duplicates (PCR artifacts) by using the Picard tools package (Java-based command-line utility). Further quality and metric were assessed with the Picard tools package. Next, the GATK software bundle^{E4} was used for base quality score recalibration.

Confirmatory sequencing

Capillary sequencing was performed for P11 to P14. Exon-specific M13-tagged primers were used to amplify all coding exons, including flanking regions from the *CD27* gene (NM_001242), as previously described.^{E5} PCR products were directly sequenced by using M13 sequence primers with the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif), according to the manufacturer's protocol. Sequencing was performed on a 3130XL genetic analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, Calif), and sequences were analyzed with the DNA Workbench software (CLC Bio, Aarhus, Denmark).

PCR amplification was performed with primers for exon 3 of the *CD27* gene for P15, P16, and their family members (forward TCCCTAGAGG TGGGCTGGGATG and reverse GGGATGGGAAGGCAGAAACAGGC) and for P17 (forward TGAAGAGGGCAGAGAACCAG and reverse ACCA GAGTTACAGTGCCGAC). The PCR reactions were run in a total volume of 15 μ L containing 10 ng of DNA, 2 mmol/L forward and reverse primer, 200 μ mol/L dNTPs, 0.9 mmol/L MgCl₂, and 1 U of GoTaq polymerase (all from Promega, Madison, Wis) in a GeneAmp PCR System 9700 machine (Applied Biosystems, Life Technologies). DNA was denatured at 95°C for 3 minutes, followed by 35 cycles, each of 15 seconds at 94°C, 15 seconds at 64°C for P15 and P16 and their family members, and 59.4°C for both primers for P17, followed by 30 seconds at 72°C and a final extension at 72°C for 7 minutes. Amplified products were resolved on a 1% agarose gel and purified by using the Nucleospin kit (Acheery-Nagel GmbH & Company, Düren, Germany). The resulting PCR products were sequenced by Macrogen (Seoul, South Korea).

Flow cytometric immunophenotyping

In P11 to P14 T and B cells were analyzed at Erasmus MC. Fresh whole blood was stained for 10 minutes at room temperature with the following mAbs: CD27-allophycocyanin (clone L128; BD Biosciences, San Jose, Calif), CD27 (clone O323; BioLegend, San Diego, Calif), CD27–fluorescein isothiocyanate–A (clone L128, BD Biosciences), and CD27–PerCP-Cy5 (clone L128, BD Biosciences). For intracellular staining of CD27, surface markers were stained first, followed by permeabilization with Cytotfix/Cytoperm (BD PharMingen) and staining with the same antibodies. Cells were analyzed with a BD LSR II with BD FACS DIVA Version 6.1.3 (BD Biosciences) and FlowJo software, version 7.6.4 (TreeStar, Ashland, Ore). For P16 and living family relatives, venous blood was collected in Streck Cell Preservative vials (Streck, La Vista, Neb) to preserve cells for transport. The samples were processed within 36 hours. Antibody combinations were used for polychromatic 8-color surface staining panels and optimized, as previously published.^{E6} Analysis of the flow cytometric data was performed with Infinicyte software, version 1.6 (Cytognos, Salamanca, Spain).

RNA isolation, cDNA synthesis, and quantitative RT-PCR expression analysis

Total RNA was isolated from EBV B cells with the GeneElute Mammalian RNA kit, according to the manufacturer's instructions (Sigma-Aldrich). Two micrograms of RNA was used for cDNA synthesis. RNA was dissolved in RNAase-free water and denatured at 70°C for 10 minutes, after which each RNA sample was added to a 23- μ L cDNA synthesis mix containing 10× RT buffer, 25 mmol/L magnesium chloride solution, 100 μ mol/L dithiothreitol, 100 μ mol/L random primers, 40 U/ μ L RNasin, 200 U/ μ L Super Script II reverse transcriptase enzyme, and 20 mmol/L dNTPs. The samples were incubated at 42°C for 45 minutes. The RT enzyme was finally inactivated by means of incubation at 99°C for 3 minutes. Three TaqMan real-time quantitative PCR assays were designed to determine transcript levels of *CD27* and L00678655 in EBV B cells from P11 and a healthy control subject (see Table E1). Two different primer/probe sets were used to estimate the expression level of *CD27*. The first *CD27* primer set was designed to amplify exon 3, with the forward primer located in the end of exon 2 and the reverse primer in the beginning of exon 4. The second *CD27* primer set was designed to amplify exon 4, with the forward primer located in the end of exon 3 and the reverse primer in the beginning of exon 5. All primers and probes were designed with Primer3 software, version 0.4.0.^{E7} The expression levels were normalized with *ABL* as a control gene.^{E8} All reactions were run on the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif), as described previously.^{E9}

CD27-AS1 CD27 antisense RNA 1 is a lincRNA that is transcribed as an antisense to the *CD27* locus. LincRNAs represent a class of long coding RNAs transcribed from intergenic regions. They have been shown to regulate the expression of neighboring genes and distant genomic loci.^{E10,E11} However, we were unable to find any alterations in the expression pattern of lincRNA for P11. This might be due to the multiple splice variants of this RNA, which

complicated the design of optimal primers and probes. It is also likely that different regulatory mechanisms come into play and silence the gene or that a mutation in a distant regulatory element influences its transcription. Full genome/exome sequencing would neither confirm nor reject *CD27* as the only gene involved in the disease phenotype in this patient, and the complete lack of expression of the *CD27* protein product cannot be fully explained with 1 heterozygous mutation.

Generation of *CD27* expression constructs

CD27 cDNA was obtained after enzymatic digestion of the vector pOTB7 (Thermo Scientific, Waltham, Mass) with *EcoRI* and *XhoI*. The digestion products were separated on 1% agarose gel stained with ethidium bromide. The band corresponding to *CD27* cDNA (approximately 1.3 kB) was excised and purified from the gel with the QIAgen Gel Purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The retroviral vector LZRS-IRES-EGFP^{E12} was enzymatically digested with *EcoRI* and *XhoI* and purified from the gel, as described above. The ligation reaction was performed overnight at 16°C. The vector/insert ratio was 3:1. One microliter of T4 DNA ligase (400,000 cohesive end units/mL; New England Biolabs, Ipswich, Mass) was used per 10 μ L of ligation mix.

The ligation product was added to TOP10 *Escherichia coli* (Invitrogen, Carlsbad, Calif) chemically competent bacteria (10 μ L of ligation product to 50 μ L of bacterial suspension). Bacteria were incubated with the ligation product for 20 minutes on ice and then transformed by heat shock at 39°C for 2 minutes. After heat shock, bacteria were kept for 2 minutes on ice, resuspended in 250 μ L of SOC medium (Invitrogen) and incubated at 30°C for 90 minutes in a shaker. Bacteria were plated on ampicillin plates (25 μ g/ μ L) and incubated overnight at 30°C.

Generation of EBV-transformed cell lines and WB

Viral supernatant was collected from the B95-8 cell line after incubation at 37°C for 48 hours and stored at -70°C.^{E13} Post-Ficoll PBMCs were incubated for 1 hour at 37°C with thawed viral supernatant, followed by supplementation with culture medium containing 20% FCS, 2% antibiotics, and 20 pg/mL PHA. After 2 to 4 weeks, clones showing characteristics of EBV-transformed B-cell lines started to grow.

Lysates of 5 million EBV B cells of P11 were heated at 95°C for 5 minutes and separated by means of SDS-PAGE on 10% acrylamide gels. After protein transfer onto polyvinylidene fluoride membranes by means of electroblotting, the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 hour at 4°C. The membranes were then washed once with TBS/Tween and incubated overnight with goat anti-*CD27* (Thermo Fisher Scientific) or rabbit anti- β -actin (Sigma-Aldrich) in 5% nonfat dry milk dissolved in TBS/Tween. The next day, the membranes were washed 6 times in TBS/Tween and incubated with horseradish peroxidase-conjugated secondary antibodies (mouse anti-goat or mouse anti-rabbit) for 2 hours at room temperature. After incubation with the secondary antibody, the membranes were washed 3 times in TBS/Tween and 3 times in TBS. The membranes were then treated with horseradish peroxidase substrate Western Lightning Plus ECL (PerkinElmer, Waltham, Mass).

RESULTS

Clinical features and genetic diagnosis of previously published patients

Case report (P1-P10). Patients P1 and P2 were brothers who presented with fever, lymphadenopathy, hepatosplenomegaly, and high EBV viral loads without evidence of malignancy. Additionally, P2 also presented with uveitis, which was suspected to be EBV induced. P1 later had hypogammaglobulinemia requiring IVIG replacement, whereas P2 had aplastic anemia and died from fulminant bacterial sepsis at age 3 years. Both were homozygous for the c.G24A/p.W8X nonsense mutation.^{E1} An older sister died in infancy from an unknown illness.

Patient P3 presented with severe primary IM and borderline hypogammaglobulinemia and later with recurrent EBV-related LPD. Her siblings, P4 and P5, were found to have immunoglobulins within the low-to-normal range. Clinically, P4 only had a moderately severe course of varicella in early childhood and was otherwise healthy. P5 received prophylactic IVIG from 4 to 20 months of age, despite low-to-normal immunoglobulin levels, because of a severe illness at age 1 year during which EBV viremia and cytomegalovirus excretion in the urine were confirmed. These 3 siblings were found to be homozygous for the c.G158A/p.C53Y missense mutation, whereas their parents were heterozygous.^{E2} An older sister was found to be heterozygous for the mutation. There were 2 older sisters who died from unknown illnesses, but no clinical material was available for testing.

Patients P6 and P7 were siblings who presented with multiple EBV-related features, ultimately requiring cord blood HSCT. P6 presented with EBV-related LPD, HLH, recurrent infections, and oral ulcers. The HLH-2004 protocol and rituximab were used for treatment. Secondary hypogammaglobulinemia developed 4 months later. Relapse of EBV-related LPD necessitated HSCT. His sister, P7, had a more severe phenotype, with recurrent infections, oral/perianal ulcers, uveitis, EBV meningitis, and chronic EBV viremia. EBV was clinically suspected as the cause of uveitis and ulcers, but direct virus detection was not performed. Rituximab treatment was initiated, and secondary hypogammaglobulinemia subsequently necessitated IVIG substitution. Cord blood HSCT was eventually performed. Both siblings were homozygous for the c.G158A/p.C53Y missense mutation.

Patient 8 presented at 15 years of age with oral ulcers, uveitis, chronic EBV viremia, and EBV-related LPD (based on clinical assessment) progressing to T-cell lymphoma (T-cell monoclonality detected based on T-cell receptor rearrangement). EBV was clinically suspected as the cause of uveitis and oral ulcers, but direct virus detection was not performed. Rituximab and chemotherapy were required, but cord blood HSCT was ultimately necessary. Two older sisters, P9 and P10, died of diffuse large B-cell lymphoma, which was suspected to be EBV induced. DNA from these patients was available for a retrospective genetic diagnosis. All siblings were homozygous for the c.G158A/p.C53Y missense mutation.

Case report (P11). Patient P11 is a 9-year-old girl who presented at the age of 4 years with primary EBV infection. Cranial magnetic resonance imaging showed hyperintense corticomedullary lesions, as well as infiltrations of the right maxillary and sphenoidal sinuses. Lactate dehydrogenase, triglyceride, and ferritin levels were increased, and the fibrinogen level was low, suggesting HLH. She received dexamethasone as part of the HLH-2004 protocol, with no response. Shortly thereafter, she had neurologic symptoms (strabismus, nystagmus, intention tremor, ataxia, and meningeal irritation). Treatment with rituximab was started (4 doses of 375 mg/m²/wk), resulting in full recovery. Five months later, she had an orbital tumor. Histologic examination revealed an EBV-associated inflammatory infiltration with abundant B- and T-cell blasts. The tumor responded to rituximab (6 doses at 375 mg/m²/wk). Because of secondary hypogammaglobulinemia, IVIG replacement was begun. Sequencing revealed a novel heterozygous c.C30A/p.C10X nonsense mutation. The absent expression of surface *CD27* on memory B cells and the abnormally low level of *CD27* transcripts in the cells prompted us to look for a mutation

in the second allele. However, sequencing of the exons of *CD27* in the mother and the promoter region of the patient did not reveal the second disease-causing variant. The patient is currently receiving immunoglobulin substitution and remains asymptomatic.

Case report (P12). Patient P12 is a 17-year-old girl who presented at 13 years of age with EBV infection (fever, lymphadenopathy, hepatosplenomegaly, pancytopenia, and increased serum transaminase levels). Results of EBV serology were positive for the early antigens p138 and p54 and negative for Epstein-Barr nuclear antigen IgG and IgM. A persistently positive EBV plasma viral load ($22,000\text{--}3.2 \times 10^5$ copies/mL) was consistent with chronic active EBV infection. At the age of 15 years, she had persistent cervical lymphadenopathy, and a lymph node biopsy revealed mixed cellularity classical Hodgkin lymphoma. A bone marrow aspirate showed signs of hemophagocytosis but no malignant infiltration. Sequencing revealed the novel compound heterozygous mutations c.C319T/p.R107C with c.G24A/p.W8X. She received chemotherapy according to the EuroNet PHL-C1 protocol, leading to complete remission. She subsequently had persistent hypogammaglobulinemia and was started on IVIG.

Case reports (P13). Patient P13 presented at 7 years of age with recurrent pneumonias, lymphadenopathy, and splenomegaly. Chest radiography showed a widened mediastinum and infiltrates in the right middle lobe secondary to compression of the right bronchial tree; computed tomography showed mediastinal lymphadenopathy with right hilar compression and atelectasis of the right middle lobe. Adenoidectomy was performed when she was a toddler because of hypertrophic adenoids. Genetic analysis showed no mutations in *PFR1*, *UNC13D*, *Munc13-4*, *STX11*, and *STXBP2* but revealed a novel homozygous c.G287A/p.C96Y missense mutation in *CD27*. She was treated with 500 mg/m² rituximab (2 courses with a 2-week interval), resulting in a significant reduction of the EBV viral load. Subsequently, she had hypogammaglobulinemia (IgG, 3.1 g/L; IgA, 0.07 g/L; and undetectable IgM), and IVIG was started. After the third course of rituximab, the EBV plasma load has remained stable at around 1×10^3 copies/mL.

Case reports (P15 and P16). Patient P15 was the third child of consanguineous parents of Iranian descent. She presented at 6 years of age with fever, generalized lymphadenopathy, and hepatosplenomegaly. At the age of 13 years, CVID was diagnosed (IgG, 5.0 g/L; IgM, 0.1 g/L; and IgA, 0.5 g/L). IVIG was initiated, almost normalizing IgG trough levels in the subsequent follow-up visits. She later had bronchiectasis, eczema, skin abscesses, chronic hepatitis, oral candidiasis, herpangina, toxoplasmosis, and stage 1 nodular sclerosis classical Hodgkin lymphoma. Serial lymphocyte subset analyses are presented in Table E2. CD8 T-cell percentages and absolute counts were increased.

Her older brother, P16, is a 32-year-old who also had nodular sclerosis classical Hodgkin lymphoma at 8 years of age. Early-stage treatment with radiotherapy and adriamycin, bleomycin, vinblastine, and dacarbazine chemotherapy led to remission. Serum immunoglobulin levels were as follows: IgG, 7.9 g/L; IgA, 0.23 g/L; and IgM, 0.9 g/L. At 32 years of age, his levels were as follows: IgG, 11.6 g/L; IgA, 2.5 g/L; and IgM, 1.2 g/L. This indicated that he did not have CVID. Additional laboratory findings included an anti-EBV viral capsid antigen IgG level of greater than 400 U/mL (normal, <20 U/mL), an anti-HSV I/II IgG level of 187 U/mL (normal, <20 U/mL), and an anti-toxoplasma

IgG level of 277 IU/mL (normal, <1 IU/mL), suggesting a similar pattern of infections as P15.

WES for P15 revealed a novel homozygous c.G287A/p.C96Y missense mutation. Sanger sequencing confirmed the mutation and that P16 was also homozygous for the mutation, whereas the parents and living siblings were heterozygous carriers.

Case report (P17). Patient P17 was a 25-year-old woman, the second child of consanguineous parents of Iranian origin. She had normal development and completed her vaccinations without complications and then presented at the age of 8 years with recurrent sinusitis followed by chronic productive cough, otitis media, bronchitis, and giardiasis. At the age of 19 years, acute bronchitis led to pneumonia requiring hospitalization, at which time hypogammaglobulinemia was discovered and IVIG and prophylactic antibiotics were initiated. Increased absolute counts of CD8 T cells and NK cells were noted. She had several complications, including chronic diarrhea, chronic sinusitis, IM with flu-like symptoms, exudative enlarged tonsils, acute pharyngitis, cervical lymphadenopathy, and splenomegaly. She was admitted to the hospital for severe EBV pneumonia as a consequence of IM but died of respiratory failure at the age of 25 years. WES revealed the novel homozygous missense mutation c.C232T/p.R78W, which was confirmed by means of Sanger sequencing. Screening of family members showed that her sister had selective IgA deficiency.

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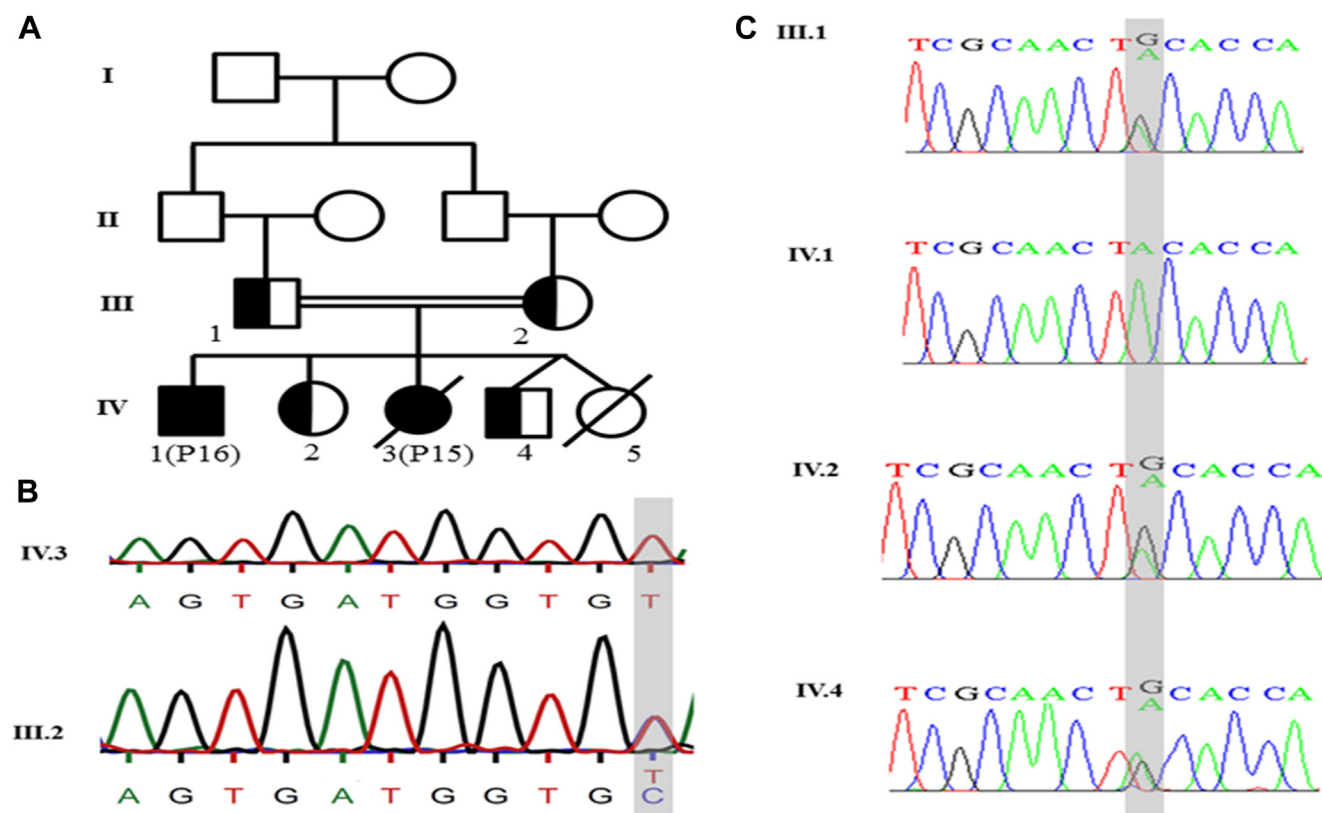
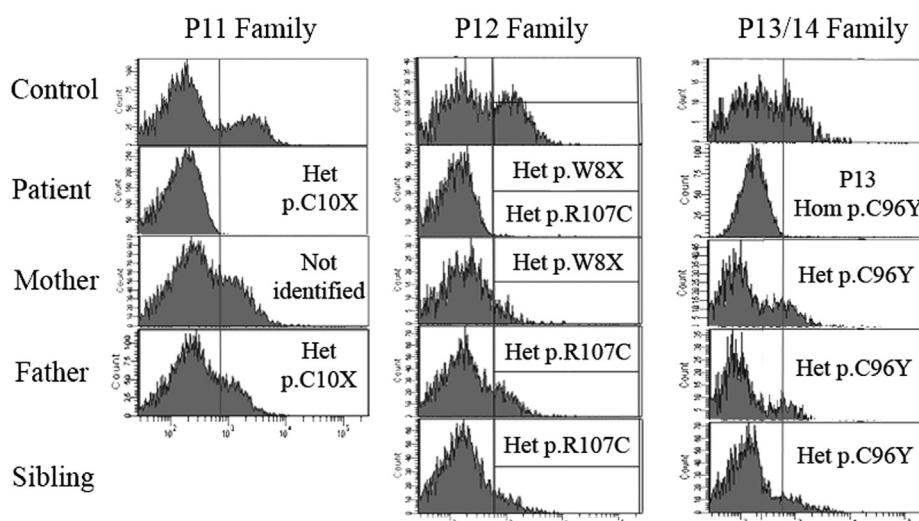


FIG E1. **A**, Family pedigree (P15 and P16). /, Deceased; double line, consanguinity; half-filled symbol, heterozygous; solid black symbols, homozygous; open symbols, unknown. **B**, Sequence analysis of CD27 (reverse primer; c.G287A/p.C96Y mutation in gray): homozygous mutation in P15 and heterozygous mutation in the mother (III.2). **C**, Sequence analysis of CD27 (forward primer; c.G287A/p.C96Y mutation in gray): homozygous mutation in P15 (IV.3) and P16 (IV.1) and a heterozygous mutation in the father (III.1) and 2 siblings (IV.4 and IV.2).

CD27 expression on B-cells



CD27 expression on T-cells

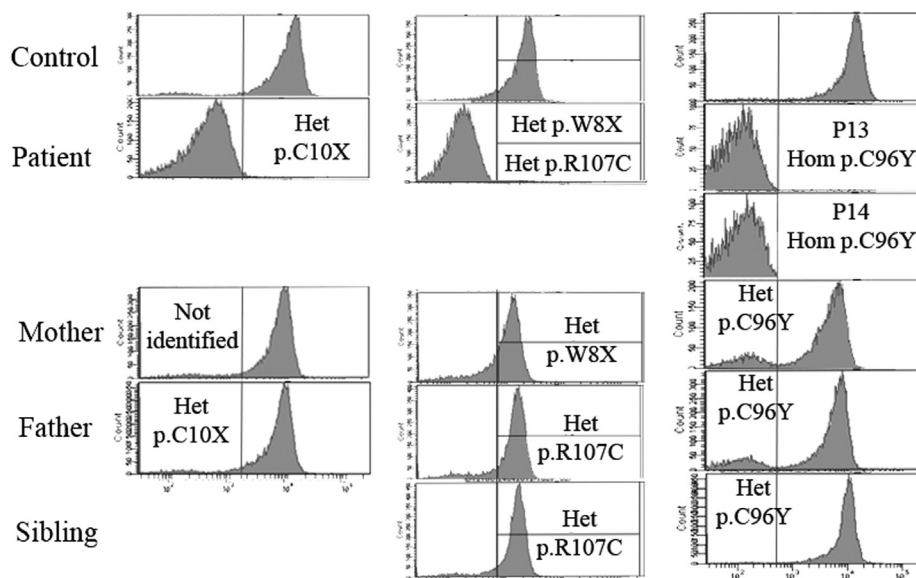


FIG E2. Flow cytometric immunophenotyping in P11 to P14 and relatives with different *CD27* mutations compared with healthy control subjects. *Het*, Heterozygous; *Hom*, homozygous.

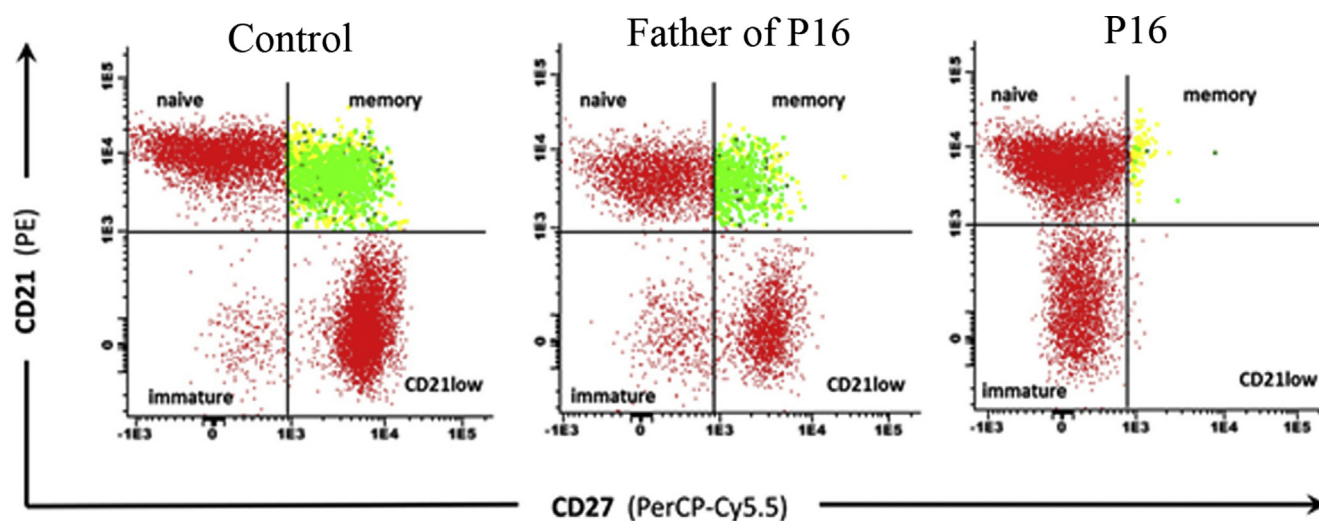


FIG E3. Flow cytometric immunophenotyping showing the fraction of CD21⁺ and CD27⁺ B cells within the CD19⁺ B-cell fraction was determined in a healthy control subject, the father of P16 (heterozygous p.C96Y), and P16 (homozygous p.C96Y). The switched memory B-cell panel shows absent surface CD27 expression for P16. Other heterozygous family members had expression patterns similar to the father. *PE*, Phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

TABLE E1. Sequences of primers and probes used for TaqMan quantitative RT-PCR

Name	Type	Sequence
<i>CD27</i> exon 1/3	Forward primer	CACTACTGGGCTCAGGGAAAGC
<i>CD27</i> exon 1/3	Reverse primer	TGCGACAGGCACACTCAGC
<i>CD27</i> exon 3/5	Forward primer	CCTCAGCCCACCCACTTACC
<i>CD27</i> exon 3/5	Reverse primer	CGGCCAGGGTGAAAACAAGG
lincRNALOC678655 exon 3	Forward primer	AGTGATTCTCCTGCCTCAGC
lincRNALOC678655 exon 3	Reverse primer	ACTCCAGCTTGGGAGACAGA
lincRNALOC678655 exon 3	TaqMan probe	TGGCATCCTTATGGCCCTGTGA
<i>CD27</i> exon 2/3	TaqMan probe	ACACCCGGCCCCACTGTGAG
<i>CD27</i> exon 4/5	TaqMan probe	CCCCTGGCCACCCCAAAGA

TABLE E2. Immunologic evaluation of P15

Parameter	13 y	14 y	17 y	Reference range
IgG (g/L)	5.0 ↓	6.4*	7.2*	6.0-15.0
IgA (g/L)	0.46 ↓	0.40 ↓	0.58 ↓	0.8-3.8
IgM (g/L)	0.1 ↓	0.2 ↓	0.15 ↓	0.5-3.7
WBC ($\times 10^3$ cells/ μ L)	14 ↑	5.8	10.3	4.3-11.4
Lymphocytes (% of total WBC)	55	44	38	28-67
CD3 ⁺ (% of lymphocytes)	90 ↑	97 ↑	63.8	58-82
CD3 ⁺ CD4 ⁺ (% of lymphocytes)	12.9 ↓	12.1 ↓	14.3 ↓	26-48
CD3 ⁺ CD8 ⁺ (% of lymphocytes)	78.6 ↑	73.2 ↑	42.3 ↑	16-32
CD4/CD8 ratio	0.16 ↓	0.16 ↓	0.33 ↓	0.69-2.53
CD19 ⁺ (% of lymphocytes)	2 ↓	2.5 ↓	31.9 ↑	10-30
CD16/CD56 ⁺ (% of lymphocytes)	2.2 ↓	ND	ND	6-27
Absolute no. of lymphocytes	7.7 ↑	2.6	3.9	1.7-5.7
Absolute CD3 ⁺ cells ($\times 10^3$ cells/ μ L)	6.9 ↑	2.5	2.5	1.1-4.1
Absolute CD3 ⁺ CD4 ⁺ cells ($\times 10^3$ cells/ μ L)	1.0	0.3 ↓	0.6	0.6-2.4
Absolute CD3 ⁺ CD8 ⁺ cells ($\times 10^3$ cells/ μ L)	6.1 ↑	1.9 ↑	1.7 ↑	0.4-1.5
Absolute CD19 ⁺ cells ($\times 10^3$ cells/ μ L)	0.2	0.2	1.2	0.2-1.4
Absolute CD16/CD56 ⁺ cells ($\times 10^3$ cells/ μ L)	0.17	ND	ND	0.07-1.20

ND, Not done. Boldface indicates abnormal results.

*Gammaglobulin substituted.

TABLE E3. Distribution of B-cell subsets in P15 and her relatives

B-cell subpopulations (as % of CD19 ⁺ B cells)	Heterozygous father (III.1)	Heterozygous mother (III.2)	Homozygous P16 (IV.1)	Heterozygous sister (IV.2)	Heterozygous brother (IV.4)	Reference range
CD27 ⁻ (naive B cells)	33.1 ↓	54.6	56.9	47.5 ↓	54.8	53.3-86.0
CD27 ⁺ CD21 ⁺ (memory B cells)	4.6 ↓	4.4 ↓	0.7* ↓	2.4 ↓	6.0 ↓	9.1-33.1
CD27 ⁺ IgD ⁻ IgM ⁻ (switched memory B cells)	2.9 ↓	1.3 ↓	0.03* ↓	1.2 ↓	2.4 ↓	4.0-22.1
CD27 ⁺ IgD ⁺ IgM ⁺ (nonswitched memory B cells)	1.3 ↓	2.6 ↓	0.6* ↓	1.0 ↓	2.9 ↓	3.3-12.8
CD38 ⁺ IgM ⁺ (transitional B cells)	1.6	0.8	2.8	2.1	1.0	0.8-6.3
CD38 ⁺ IgM ⁻ (plasmablasts)	2.2	1.3	1.5	1.8	1.8	1.2-4.0
CD21 ^{low} CD38 ^{low} (activated B cells)	58.4 ↑	38.8	38.2	46.3 ↑	36.4	33.2-40.1

Boldface indicates abnormal results.

*Statistically significant ($P < .05$) between homozygous and heterozygous members.

TABLE E4. Percentages of T-cell and NK-cell subsets in living family members of P15

	Heterozygous father (III.1)	Heterozygous mother (III.2)	Homozygous P16 (IV.1)	Heterozygous sister (IV.2)	Heterozygous brother (IV.4)	Reference range
T-cell and NK cell subpopulations						
CD4 ⁺ cell subsets (% of CD4 ⁺ cells)						
CCR7 ⁺ CD45RA ⁺ CD45RO ⁻ (naive helper T cells)	31.0	46.7	55.8 ↑	33.6	37.9	18.2-53.2
CCR7 ⁺ CD45RA ⁻ CD45RO ⁺ (central memory helper T cells)	10.6	9.0	8.0 ↓	8.2 ↓	6.9 ↓	8.3-27.1
CCR7 ⁺ CD45RA ⁺ CD45RO ⁻ (effector helper T cells)	10.0 ↑	8.3	4.0	10.8 ↑	8.7	1.1-9.0
CD38 ⁺ HLA-DR ⁺ CD4 ⁺ (activated helper T cells)	12.7 ↑	10.0 ↑	8.3 ↑	7.3 ↑	9.4 ↑	0.9-4.6
CD8 ⁺ cell subsets (% of CD8 ⁺ cells)						
CCR7 ⁺ CD45RA ⁺ CD45RO ⁻ (naive cytotoxic T cells)	8.5 ↓	11.5 ↓	19.3	19.4	21.6	18.6-70.6
CCR7 ⁺ CD45RA ⁻ CD45RO ⁺ (central memory cytotoxic T cells)	1.6	1.9	0.5 ↓*	1.2	1.0	0.7-5.4
CCR7 ⁺ CD45RA ⁺ CD45RO ⁻ (effector cytotoxic T cells)	52.5 ↑	41.9 ↑	50.3 ↑	33.1 ↑	41.4 ↑	4.0-24.0
CD38 ⁺ HLA-DR ⁺ CD8 ⁺ (activated cytotoxic T cells)	19.1	10.7	35.7 ↑	14.6	15.9	1.4-21.4
NK T-cell subset						
CD3 ⁺ CD16 ⁺ NK-T cells (in % of lymphocytes)	10.0 ↑	6.9	5.6	6.8	4.8	1.7-8.4
Treg cells						
CD25 ⁺ CD127 ^{low} Treg cells (in % of T cells)	5.2	4.4	4.5	5.5	5.1	2.8-6.4
CD25 ⁺ CD127 ^{low} CD45RO ⁺ memory Treg cells (in % of Treg cells)	53.4	40.7	30.9 ↓	40.1	29.6 ↓	36.9-73.6
T _H 17-like CD3 ⁺ T cells (in % of CD45 ⁺ lymphocytes)	17.5	14.7	6.7 ↓*	12.1	11.4	10.8-39.0
Recent thymic emigrants (% of CD4 ⁺ cells)						
CD4 ⁺ CD45RA ⁺ CD62L ⁺ cells	37.0	47.5	55.8	40.3	49.2	27.4-64.7
CD4 ⁺ RTE (CD45RA ⁺ CD62L ⁺ CD31 ⁺) cells	4.9 ↓	9.1 ↓	11.9 ↓	9.8 ↓	11.3 ↓	14.4-38.3
NK cell subpopulations						
CD16 ⁺ CD56 ⁺ NK cells (in % of lymphocytes)	19.6	9.6	18.2	13.9	12.1	4.3-24.6
CD56 ^{bright} CD16 ^{dim} (in % of NK cells)	2.4	1.2	0.7 ↓	0.8	0.4 ↓	0.8-8.3
CD94 ⁺ NKG2D ⁺ CD16 ⁺ CD56 ⁺ (in % of NK cells)	74.3↑	45.9	76.6 ↑	77.4 ↑	56.5	21.8-74.1
CD3 ^{dim} CD56 ⁻ CD16 ⁺ (in % of lymphocytes)	2.1	1.9	2.0	1.9	0.7 ↓	1.6-2.4

RTE, Recent thymic emigrants; Treg, regulatory T. Boldface indicates abnormal results.

*Statistically significant ($P < .05$) between homozygous and heterozygous members of kindred H (Table I).