Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27

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Online Supplementary Appendix

Design and Methods

Immunological functional analyses

Lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMCs; 1^{e5}) from patients and healthy donors were incubated with staphylococcal superantigen A (10 ng/mL; Sigma Chemicals), staphylococcal superantigen B (20 ng/mL; Sigma), phytohemagglutinin (12.5 μg/mL; Sigma Chemicals), soluble CD3 mAb (2 μg/mL; OKT3, Ortho, Raritan, NY, USA), phorbol myristate (10^{-7}μmol/L; Sigma Chemicals) or medium in 96-well round-bottom tissue culture plates for 72 h, after which cells were pulsed with [methyl-^{3}H]thymidine (1 μCi/well)] for another 18 h and processed as described previously.[1]

Cytotoxicity assays

NK-cell cytolytic activity was measured in a standard ^{51}Chromium release [^{51}Cr] assay on K562 targets as described.[2] In brief, ^{51}Cr-labeled K562 target cells were incubated with PBMC effectors directly after density gradient isolation for 4.5h at six different effector-to-target ratios in 96-well U-bottom plates. After determination by FACS analysis, desired NK-to-target ratios were calculated and established. [^{51}Cr]-release into the supernatant was quantified using Packard Cobra II Auto Gamma (Perkin-Elmer).

Quantification of antibody responses to vaccination antigens and of lymphocyte subsets

External routine laboratories using standard techniques such as enzyme-linked immunosorbent assays for the IgG quantification analyzed the concentrations of specific immunglobulins in patient sera. The partly age-specific 'normal' ranges were used as reference as published in Schauer *et al.*,[3] but our data are presented in semi-quantitative terms in Table 1 to simplify their interpretation. For T- and B-cell subpopulations and memory B-cell subsets, age-specific reference ranges were applied.[4-6]

DNA isolation from whole blood

Genomic DNA from EDTA blood of all 5 family members of Family A was isolated using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions

Homozygosity mapping

Homozygosity mapping is an established strategy for mapping single genes, causing autosomal recessive diseases in children from consanguineous marriages. The method involves detection of the disease locus using single nucleotide polymorphisms (SNPs), utilizing the fact that the region surrounding this locus will preferentially be homozygous by descent.[7] To obtain a large number of 'genome wide' SNPs, Affymetrix© 6.0 SNP chip arrays were performed for all family members of Family A, genotyping more than 900,000 SNPs across the genome. Subsequently, genotypes of all family members were analyzed using PLINK (*http://pngu.mgh.harvard.edu/~purcell/plink/*).[8] The analysis was performed to detect intervals showing perfect segregation, meaning that an interval meets the following three criteria: 1) all affected individuals show the same homozygous phenotype; 2) both parents are heterozygous for that specific locus; and in addition, 3) the unaffected individual needs to be heterozygous or homozygous for the other allele at this position.[9]

Considering all individuals bearing the mutation in *CD27*, 6 homozygous regions were detected in Family A, fulfilling the criteria mentioned above (*Online Supplementary Table S1*). For exclusion of a second variant in the most severely affected individual of Family A (Patient 1), a second analysis was performed where only this individual was considered affected. Thus, only regions exclusively homozygous in Patient 1, heterozygous in both parents, and heterozygous or homozygous for the other allele in all other siblings were considered (*Online Supplementary Table S2*).

Primer design and validation using capillary sequencing

Primers for detected variants were designed using PrimerZ (*http://genepipe.ngc.sinica.edu.tw/*) and ordered from Sigma Aldrich, Germany. Primer Sequences for the validation of CD27 position Chromosome 12: 6554611-6554611 (built hg19) Exon 2: Forward: **accttgaagagggcagagaac**; Reverse: **acttgctgtgagccttgaaga**. Primer pair sequences used in Düsseldorf, Germany, were: a) Forward: **gggccaggtgagtggcgaaa**; Reverse: **gcaccccaaacccgtcacc**; b) Forward: **ctgtggggaggcaccaccttga**; Reverse: **acctacacacccttgcccacct.**

Positions of detected variants were PCR amplified using Expand High Fidelity PCR System (Roche, Basel, Switzerland). Samples were sequenced on an Applied Biosystems 3130xL Genetic Analyzer capillary sequencer running 3130xL Genetic Analyzer Data Collection software version 3.0. Bases were called with Sequence Analysis software version 5.2, indicating heterozygous signals with ambiguity code when exceeding a 25% signal intensity threshold. Reads were aligned to reference sequences using DNAStar SeqMan II version 5.08.

Online Supplementary Table 1. Homozygosity mapping of family A displaying shared homozygous intervals between the three affected siblings (Patients 1-3).

Chromosome	Position (Mb)	SNP	SNP
12	6.2-7.9	rs12367162	rs10845821
12	33.8-34.8	rs10844683	rs11053463
12	37.8-39.4	rs3956186	rs11170656
12	110.2-111.2	rs10774911	rs7139221
20	13.2-14.6	rs17263187	rs6034003
20	16.6-17.8	rs6111224	rs11697218

Online Supplementary Table 2. Homozygous regions found exclusively in the most affected patient from family A (Patient 1).

Chromosome	Position (Mb)	SNP	SNP
6	74.8-75.8	rs4132147	rs7758152
9	14.0-15.1	rs7018490	rs7036651
9	27.5-43.3	rs997413	rs7858200
16	28.0-29.2	rs205393	rs7021289
16	29.3-31.8	rs710427	rs11150645
16	46.5-52.2	rs16945380	rs2245948
16	54.0-55.3	rs9935296	rs9938898

Results and Discussion

Patient 1

The first course of anti-CD20 therapy (rituximab) was administered at the age of 34 months (375 mg/m^2/week, four times) because of massive lymphoproliferation, high plasma virus loads of EBV accompanied by systemic inflammatory response syndrome with oliguria, pleural effusions, ascites, and pneumonal infiltrates. Although followed by complete clinical remission of symptoms and normal thriving, EBV-LPD recurred simultaneously with reappearance of peripheral B cells, again responding well to rituximab therapy. At present, 15 months after the second rituximab course, low-level B cells (500/μL) and borderline EBV-positivity (1e^2 copies/mL) were detectable again, so far without symptoms (Table 1).[10] Typical CVID characteristics were met in part with absent CD27$^+$ memory B cells, borderline hypogammaglobulinemia and reduced specific antibody production capacity but present isohemagglutinins (Table 1).[9,10] Genes analyzed to exclude additional causes of primary HLH/EBV-LPD[11] before proceeding to whole exome sequencing included *PRF1, MUNC13-4, MUNC18-2, STX11, ITK*; global and T- and NK-cell specific X-chromosomal inactivation were analyzed to indicate a potential possibility for SAP or XIAP deficiency (all normal; *data not shown*).

Patient 2

An older sister of Patient 1 had only borderline hypogammaglobulinemia with near-normal specific antibody production against vaccines and childhood diseases, including EBV and EBNA antibodies (Table 1). She is 14 years old and except for a reportedly moderately severe course of varicella in her early childhood (varicella zoster virus IgG positive; treated as an outpatient without complications), she has no remarkable history of infections.

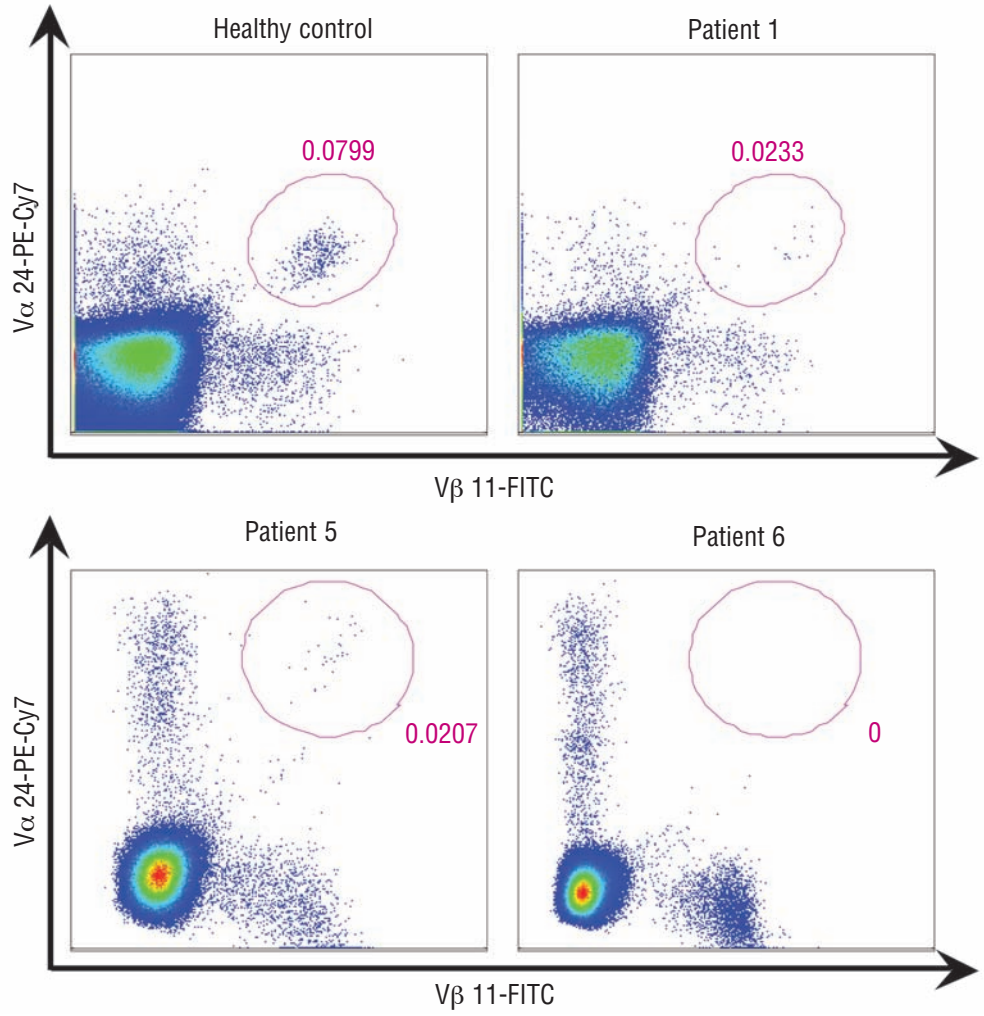
Patient 3

At one year of age, the boy (now 3.5 years old) was severely ill and when hospitalized, tested CD27-negative. Although his immunoglobulin levels were within the low-normal range, we administered prophylactic IgG substitution from the age of 4 to 20 months. During that time, he had contact with EBV and CMV as confirmed by DNA detection in blood and urine, respectively, with intermittent asymptomatic EBV viremia and CMV excretion, but remained EBV-IgG, EBNA and CMV IgG antibody-positive even 18 months after the last IgG substitution, suggesting endogenous specific antibody production ability (Table 1). So far, he has shown no signs of lymphoproliferation or hemophagocytic lymphohistiocytosis. His long-term clinical course needs to be observed carefully, especially given the current EBV reappearance in plasma.

To test specific antibody production under IgG substitution therapy, patients 1 and 3 were immunized against tick-borne encephalitis (TBE), an antigen against which antibodies are not constantly contained within immunoglobulin preparations in sufficiently protective levels.[12] Patient 1 showed borderline positive and Patient 3 completely normal responses to TBE vaccine when antibody levels were compared before and after immunization (Patient 1: 105-155 VIE units; Patient 3: >228-239 VIE units, stable after discontinuation of IgG substitution therapy). Patient 2, who had never needed IgG substitution, had normal TBE IgG concentrations (stable >155 VIE units) after regular basic and booster immunizations against TBE.

Patients 4, 5 and 6

Patients 4, 5 and 6 are from consanguineous Families B and C originating from Lebanon, treated at the Royal Children's Hospital in Melbourne, Australia. Patients 4 and 6 were



Online Supplementary Figure S1. Severe reduction of numbers of iNKT cells in CD27-deficient Patients 1, 5 and 6. The dot plots show iNKT staining of peripheral blood mononuclear cells from a healthy adult control (upper left panel) and Patient 1 during a phase of clinical remission (upper right panel). Similar results were obtained for other patients including Patients 5 and 6 (lower panels; corresponding healthy control not shown). All plots were gated on $CD3^+$ lymphocytes. iNKT cells were defined as $CD3^+$ $TCRv\alpha24^+$ $TCRv\beta11^+$ cells. Note that differences in the appearance of dot plots are due to analysis at two different centers using different flow cytometers and settings (upper row: Vienna, Austria; lower row: Melbourne, Australia).

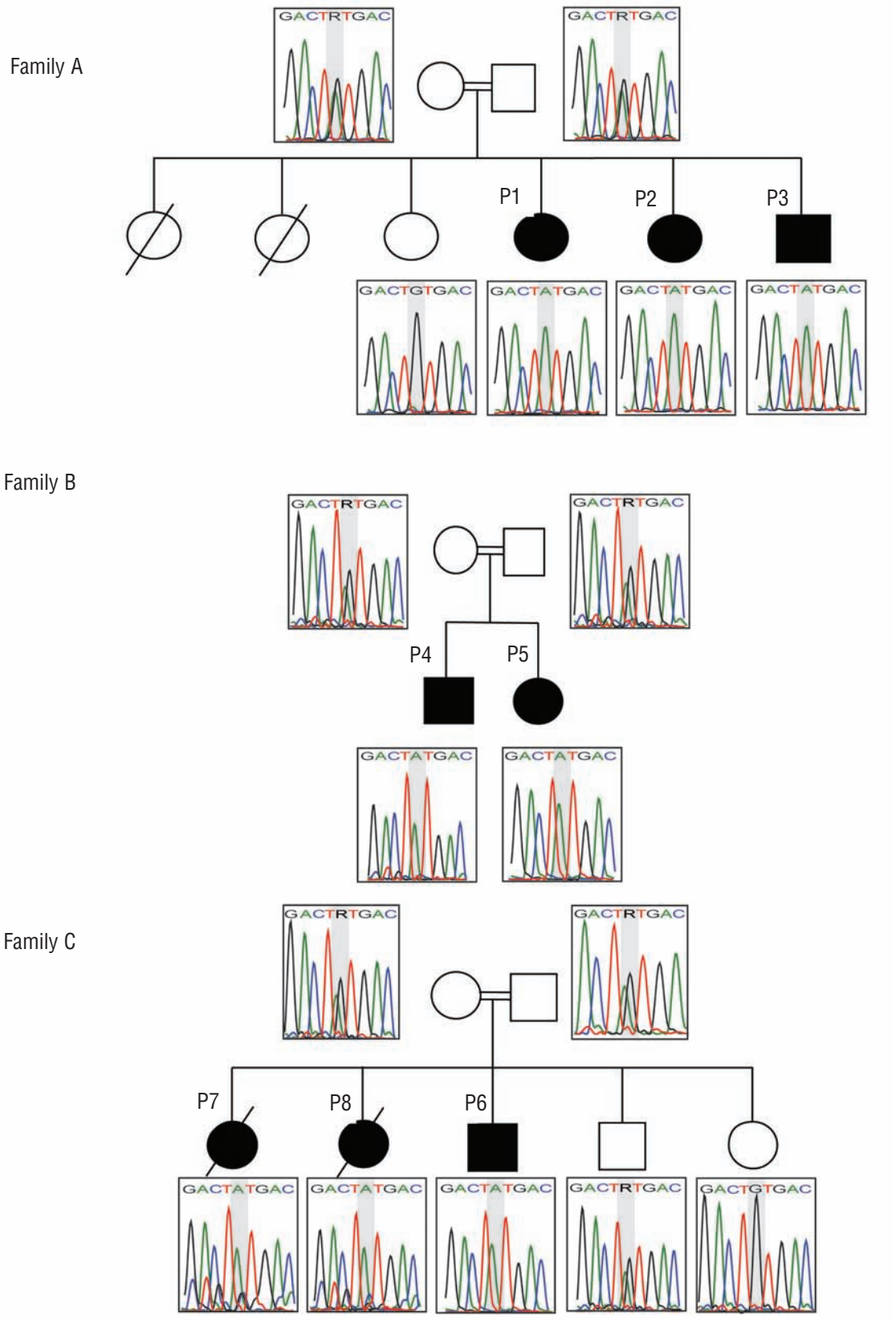
enrolled in the "Registry for EBV-associated lymphoproliferative diseases in childhood" run at the Clinic for Pediatric Hematology, Oncology, and Clinical Immunology at the Heinrich Heine University Medical Center, Düsseldorf, Germany. In Patients 4 and 6, mutations in *SH2D1A, ITK, PRF1* and *MUNC13-4*, and in Patient 4 also mutations in *MUNC18-2* and *STX11*, were excluded before performance of whole exome sequencing.

Patient 5 was recently diagnosed with absent CD27 expression and EBV infection soon after the diagnosis was established in her brother (Patient 4); she currently has normal immunoglobulin levels.

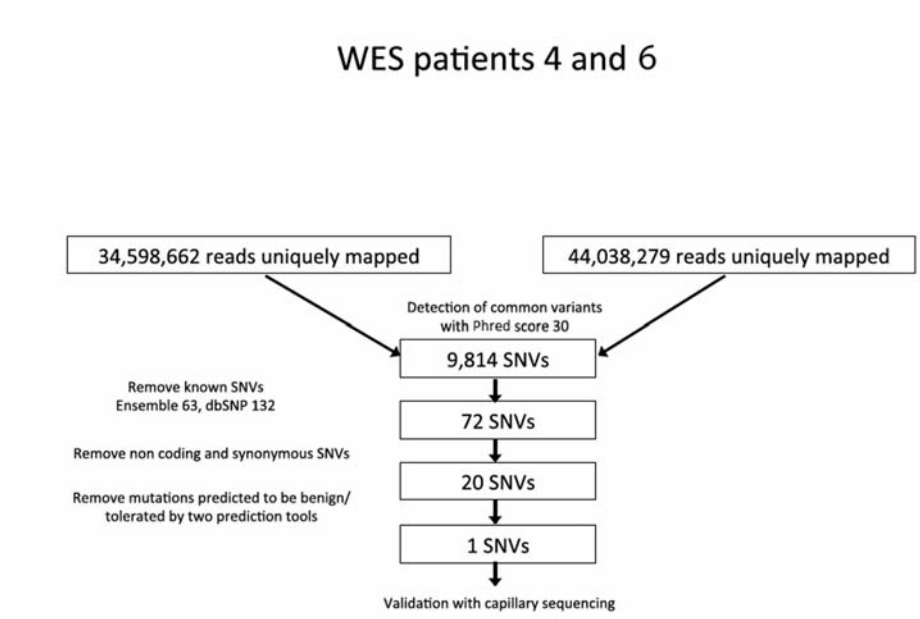
The lymphoma diagnosed in Patient 6 showed the karyotype (45, X,-Y[27]/47,XY,+3[8]/46,XY[1]) in lymph node and T-cell receptor rearrangements in blood and bone marrow and was treated with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP), before proceeding to stem cell transplantation.

Additional immunological aspects

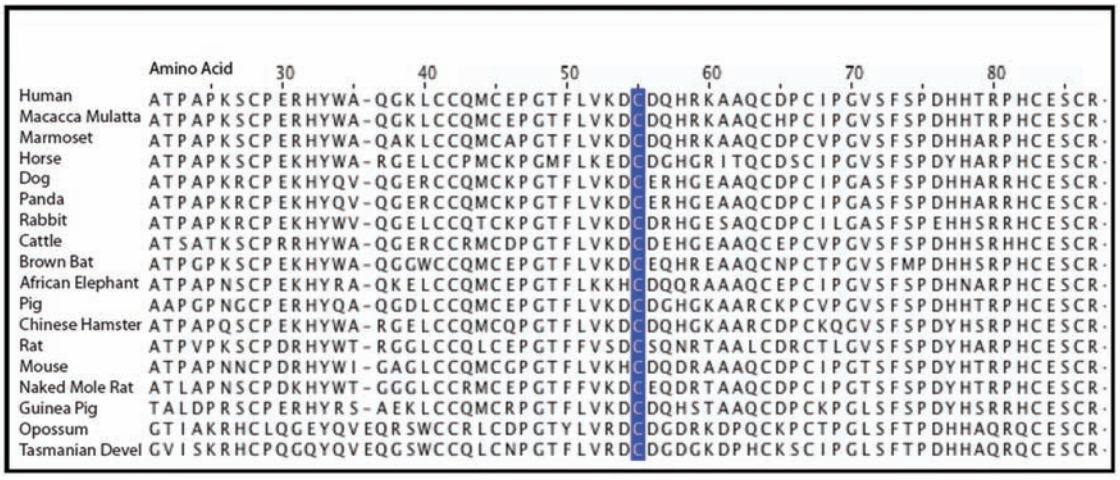
These clinical and laboratory observations indicate that CD27 deficiency due to a complete lack of protein or altered protein conformation and function might cause a new disease entity fulfilling some diagnostic criteria of CVID such as hypogammaglobulinemia, partially diminished specific antibody production / poor response to vaccines,[13,14] and absence of isotype switched and non-class-switched memory B cells (by definition of CD27 positivity).[15,16] Because CD27 is a routine parameter in flow cytometric immunophenotyping and its absence may be easily detected, primary CD27 deficiency most



Online Supplementary Figure S2. Perfect segregation of *CD27* c.G158A mutation in Families A, B and C. The variant allele is marked with a gray bar.



Online Supplementary Figure S3. Identification of CD27 deficiency by whole exome sequencing in Patients 4 and 6. Whole exome sequencing data of Patient 4 (Family B) and Patient 6 (Family C) were assessed for shared homozygous single nucleotide variants (SNV). Removal of known (listed in the Ensemble63/dbSNP132 database), non-coding or synonymous variants from a total of 9814 shared homozygous SNVs yielded a total of 20 hits. Among those, only the mutation in CD27 (c.G158A) was predicted to have a deleterious or damaging impact on protein structure or function using SIFT and PolyPhen prediction tools. The mutation was confirmed by capillary sequencing (Figure 1D).



Online Supplementary Figure S4. Phylogenetic conservation of the mutated amino acid in *CD27* (Cys53Tyr) in different species. Cysteine 53 is marked in blue.

likely will represent a rare condition with a very distinct, variable phenotype and predisposition to persistent EBV infection and EBV-associated complications such as LPD, HLH, and lymphoma. Neither CD27 nor its ligand CD70 were identified in a recent genome-wide association screen for genetic alterations in a large cohort of CVID patients, corroborating the assumption that CD27 deficiency might be recognized as cause of a rare combined immunodeficiency with special phenotypical features rather than represent the cause of a larger proportion of CVID patients.[17]

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