Supplementary Appendix

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; 1×10⁶) 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 mol/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 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

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 immunoglobulins 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.⁶ 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).
after the second rituximab course, low-level B cells (500/mL) were detected at the same time with reappearance of peripheral B cells, again suggesting endogenous specific antibody production ability (Table 1). So far, he has shown no signs of lymphoproliferation, high plasma virus loads of EBV accompanied by systemic inflammatory syndrome or hemophagocytic lymphohistiocytosis. His long-term clinical course needs to be observed carefully, especially given his 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 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. 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 immunization (Patient 1: 105-155 VIE units; Patient 3: >228-239 VIE units, stable after discontinuation of IgG substitution therapy).
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, and absence of isotype switched and non-class-switched memory B cells (by definition of CD27 positivity). 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.
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
Supplementary References


