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

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ABSTRACT

CD27, a tumor necrosis factor receptor family member, interacts with CD70 and influences T-, B- and NK-cell functions. Disturbance of this axis impairs immunity and memory generation against viruses including Epstein Barr virus (EBV), influenza, and others. CD27 is commonly used as marker of memory B cells for the classification of B-cell deficiencies including common variable immune deficiency. Flow cytometric immunophenotyping including expression analysis of CD27 on lymphoid cells was followed by capillary sequencing of *CD27* in index patients, their parents, and non-affected siblings. More comprehensive genetic analysis employed single nucleotide polymorphism-based homozygosity mapping and whole exome sequencing. Analysis of exome sequencing data was performed at two centers using slightly different data analysis pipelines, each based on the Genome Analysis ToolKit Best Practice version 3 recommendations. A comprehensive clinical characterization was correlated to genotype. We report the simultaneous confirmation of human CD27 deficiency in 3 independent families (8 patients) due to a homozygous mutation (p. Cys53Tyr) revealed by whole exome sequencing, leading to disruption of an evolutionarily conserved cystein knot motif of the transmembrane receptor. Phenotypes varied from asymptomatic memory B-cell deficiency (n=3) to EBV-associated hemophagocytosis and lymphoproliferative disorder (LPD; n=3) and malignant lymphoma (n=2; +1 after LPD). Following EBV infection, hypogammaglobulinemia developed in at least 3 of the affected individuals, while specific anti-viral and anti-polysaccharide antibodies and EBV-specific T-cell responses were detectable. In severely affected patients, numbers of iNKT cells and NK-cell function were reduced. Two of 8 patients died, 2 others underwent allogeneic hematopoietic stem cell transplantation successfully, and one received anti-CD20 (rituximab) therapy repeatedly. Since homozygosity mapping and exome sequencing did not reveal additional modifying factors, our findings suggest that lack of functional CD27 predisposes towards a combined immunodeficiency associated with potentially fatal EBV-driven hemophagocytosis, lymphoproliferation, and lymphoma development.

Introduction

CD27 is part of the tumor necrosis factor receptor family and critical for B-, T- and NK-cell function, survival, and differentiation, respectively.[1-4] After binding to its specific ligand CD70, CD27 plays a co-stimulatory role highly relevant for anti-viral responses, anti-tumor immunity, and alloreactivity.[5] CD27 is routinely used as marker for class-switched and non-class-switched memory B cells ($CD27^+IgD^-$ and $CD27^+IgD^+$) relevant for the classification of B-cell deficiencies including common variable immune deficiencies (CVIDs).[6] Recently, Peperzak *et al*. showed that CD27 signaling is crucial for sustained survival of $CD8^+$ effector T cells in mice,[7] and *Cd27*-/- mice show impaired primary and memory $CD4^+$ and $CD8^+$ T-cell responses.[4] Thus, it may be hypothesized that constitutional lack of CD27 in humans may cause a combined primary immunodeficiency.

In immunocompetent hosts, primary Epstein Barr virus (EBV) infection is often asymptomatic, whereas in primary immunodeficiencies such as IL-2-inducible T-cell kinase (ITK) deficiency, X-linked lymphoproliferative syndromes (XLP1, XLP2), primary hemophagocytic lymphohistiocytosis syndromes (HLH) and others, EBV infection may lead to persistent symptomatic viremia.[8-11] Although EBV-specific immunity involves virus-specific humoral components, $CD8^+$ effector T cells are considered essential for long-term virus control.[12]

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Here, we report 8 pediatric patients from 3 independent pedigrees lacking functional CD27. Clinical manifestations comprised EBV-associated lymphoproliferative disorder (EBV-LPD, n=3) with or without HLH, malignant lymphoma (n=2; +1 after LPD) or absence of overt clinical phenotype (n=3).

Design and Methods

Patients

Material from patients and healthy donors was obtained upon obtaining informed consent in accordance with the Declaration of Helsinki. Family A was analyzed in Vienna, Austria, while Families B and C were assessed in Düsseldorf, Germany. The respective institutional review boards approved the study.

Flow cytometric analysis

Analysis of CD27 surface expression and B-cell class switch was performed as described previously.[13]

Information on DNA isolation, primer design, and functional analyses is available in the *Online Supplementary Appendix*.

Homozygosity mapping

Genome-wide genotyping based on Affymetrix® Genome-Wide Human SNP Array 6.0 was performed for all 5 family members of Family A. For homozygosity mapping, DNA of each core family member was diluted to 50 ng/μL in 12 μL. The protocol was carried out according to the manufacturer's instructions. Raw data were analyzed using genotyping console version 4.0.1.8.6. Loss of heterozygosity (LOH) analysis was performed as well as genotyping, followed by more detailed analysis using PLINK. Homozygous regions in all family members were detected using PLINK (*http://pngu.mgh.harvard.edu/~purcell/plink/*) using a window size of 5000bp with a minimum of 50 SNPs within this region.[14] Adjacent intervals were considered to represent a single interval when the distance between the intervals was less than 1MB in size. Based on these data, homozygous regions present exclusively in the most severely affected patient, as well as homozygous regions present in all 3 affected siblings, were detected (*Online Supplementary Tables S1 and S2*).

Exome sequencing and data analysis

Exome sequencing for Family A was performed in Vienna, while Families B and C were assessed in Düsseldorf, Germany.

For Family A, a 50 base-pair paired read multiplexed whole exome sequencing (WES) run was performed for the most severely affected patient on an Illumina HiSeq2000 Sequencer running on HiSeq Control Software (HCS) 1.4.8, Real Time Analysis Software (RTA) 1.12.4.2. For WES, DNA was diluted to 20 ng/μL in 57 μL and sample preparation was carried out using Illumina TruSeq DNA Sample Preparation Guide and the Illumina TruSeq Exome Enrichment Guide version 2. The multiplexed pool of 6 samples including the patient was run on all lanes of the flowcell. Demultiplexing and raw image data conversion was performed using Consensus Assessment of Sequence and Variation version 1.7 (CASAVA). Reads were aligned with BWA using the algorithm for short reads (up to ~200bp),[15] a gapped global alignment with maximum of a 1bp gap open to a human genome 19 (hg19) reference was performed. Insertion/deletion realignment and GATK base quality score recalibration was performed.[16] Single nucleotide variants (SNVs) and insertions/deletions were called using Unified Genotyper and GATK Variant quality score recalibration (1000Genomes, HapMap, dbSNP131) was performed. All thresholds for GATK tools were based on the GATK Best Practice Variant Detection version 3 recommendations. SNV and insertion/deletion lists were uploaded to SeattleSeq Annotation database. Variants present in 1000 Genomes and dbSNP were excluded and lists were filtered for nonsense, missense and splice-site variants within the homozygous regions detected in the most severely affected patient.

For Families B and C, a similar approach was taken with minor differences referring to the usage of single reads with 100 cycles, and alignment with BWA for long reads and using dbSNP132 dataset. Resulting variation calls were annotated by NGS-SNP17 using a local copy of the ENSEMBL databases, PolyPhen2,[18] SIFT[19] and ConDel[20] before being imported into an SQL database.

Results

Patient reports and immunological findings

Patient 1 (Family A, Figure 1A), a 5-year old girl from a Turkish consanguineous family, presented at the age of 17 months after severe primary infectious mononucleosis with suspected hemophagocytic lymphohistiocytosis (HLH). A brief summary of her clinical phenotype was recently described.[21] She developed fulminant EBV-LPD and systemic inflammatory response syndrome, and was treated repeatedly with high-dose steroids and rituximab over four years. Flow cytometry revealed undetectable $CD27^+$ lymphocytes (Figure 1B), an increase in transitional and $CD21^{low}$ B cells, and near-absent invariant natural killer T (iNKT) cells (Table 1 and *Online Supplementary Figure S1*). EBV-specific T cells were detectable (Table 1), and T-cell receptor Vβ spectratyping showed no major abnormalities in $CD4^+$, but moderate oligoclonality in $CD8^+$ T cells (*data not shown*). Two of her siblings (Patients 2 and 3; Figure 1A), were clinically asymptomatic but showed borderline hypogammaglobulinemia (Table 1 and *Online Supplementary Appendix*). Specific IgG antibody responses against EBV-viral capsid antigen were positive in Patient 1 before initiation of immunosuppressive and immunoglobulin substitution therapy; Patients 2 and 3 showed low-normal antibody responses against viral protein and bacterial polysaccharide antigens including CMV, EBNA1, and vaccine antigens (Table 1).

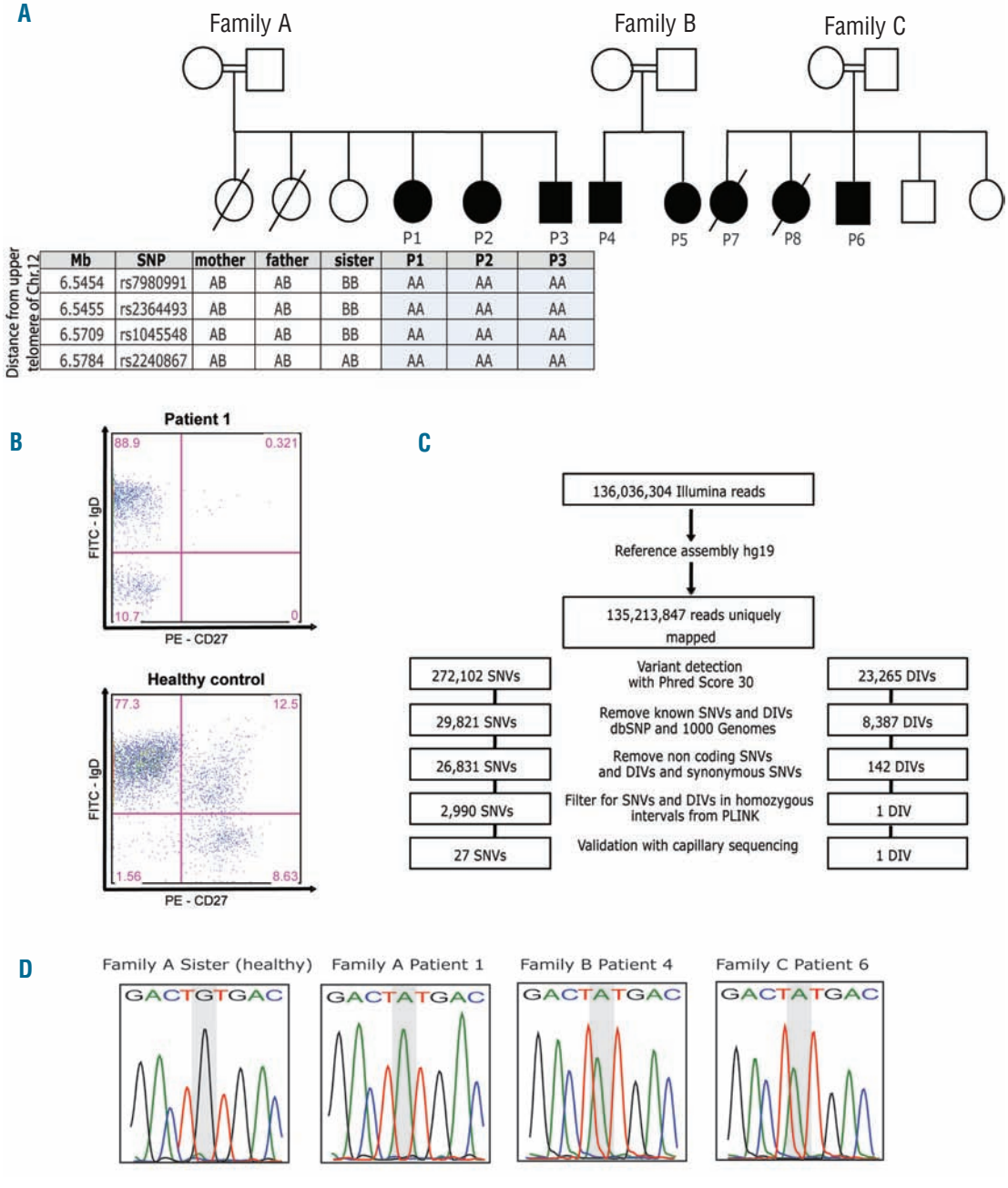
Patient 4 (Family B; Figure 1A) presented at 18 months of age with EBV-LPD and HLH, treated according to the HLH-2004 protocol (including dexamethasone, etoposide, cyclosporine-A) plus an anti-CD20 antibody (rtuximab). Although immunoglobulin levels were normal in Patient 4 at his initial presentation, he became hypogammaglobulinemic four months later. Nine months after initial presentation, EBV-LPD relapsed without signs of hemophagocytosis. He again received HLH treatment and rituximab, followed by matched unrelated cord blood transplantation. His younger sister, Patient 5 (Figure 1A), is 16 months old. She was diagnosed with absent CD27 expression and EBV-infection only after CD27 deficiency had been identified in her brother.

Patient 6 (Family C; Figure 1A) presented at the age of 15 years with EBV-LPD. He responded to rituximab but EBV-viremia recurred three months later. Although he was hypergammaglobulinemic at diagnosis, and his peripheral B cells were again detectable four months after rituximab treatment, immunoglobulin levels slowly decreased. Approximately 20 months after initial presentation, a relapse of EBV-LPD occurred, progressing into T-cell lymphoma within four months, and requiring treatment with

rituximab and chemotherapy (R-CHOP, *Online Supplementary Appendix*) followed by matched unrelated cord blood transplantation. Flow cytometric analysis at relapse revealed absent CD27$^+$ lymphocytes and very low iNKT cells (Table 1 and *Online Supplementary Figure S1*). Only limited clinical history and no immunological data are available for the patient's 2 older sisters (Patients 7 and 8). Both died of suspected EBV-driven lymphoma at two and 22 years of age, respectively (Figure 1A), and the diagnosis of CD27 deficiency was established retrospectively from Guthrie card DNA (*see below*).

Genetics

Because of the variability of phenotypes in Family A, we performed homozygosity mapping and whole-exome sequencing (WES) after earlier detection of the *CD27*



Mb	SNP	mother	father	sister	P1	P2	P3
6.5454	rs7980991	AB	AB	BB	AA	AA	AA
6.5455	rs2364493	AB	AB	BB	AA	AA	AA
6.5709	rs1045548	AB	AB	BB	AA	AA	AA
6.5784	rs2240867	AB	AB	AB	AA	AA	AA

Figure 1. Identification of CD27 deficiency in three kindreds. Three pedigrees with CD27 deficiency were identified. (A) Family A was analyzed by homozygosity mapping, revealing a homozygous interval on Chromosome 12. Two siblings of Patient 1 (Family A) had died at nine months and three days of age, respectively, of unknown causes without molecular diagnosis in Turkey; no material for immunological or genetic analyses was available. (B) Flow cytometry showed absent surface expression for CD27 in lymphoid cells including B-cell subsets, shown here in Patient 1 for IgD *vs.* CD27 staining on CD19-gated B lymphocytes. (C) Exome sequencing was performed in Patient 1 and filtered for homozygous intervals detected exclusively in Patient 1. None of the total of 28 additional single nucleotide or deletions/insertions variants (SNVs or DIVs, respectively) was validated using capillary sequencing, suggesting that the mutation in *CD27* alone is causative of the phenotype in Patient 1. (D) Sanger sequencing confirmed the same mutation in *CD27* (c. G158A, p. Cys53Tyr) in Patients 1-3 from Family A, Patients 4 and 5 from Family B, and Patients 6-8 from Family C, respectively.

mutation by conventional Sanger sequencing (*Wolf et al., manuscript in preparation*), in order to exclude other contributing genetic conditions or, conversely, to define whether the mutation in *CD27* alone was sufficient for the development of a phenotype. Single nucleotide polymorphism (SNP)-array based homozygosity mapping in Family A revealed four intervals which were present only in the affected sibling (Patient 1; *Online Supplementary Table S1*). However, WES did not reveal any additional relevant genetic aberrations (Figure 1C). Analyses of all 3

Table 1. Patients' characteristics and immunophenotypic details of CD27-deficient individuals.

Patient	1	2	3	4	5	6
Center	Vienna	Vienna	Vienna	Melbourne	Melbourne	Melbourne
Gender	Female	Female	Male	Male	Female	Male
Ethnic origin	Turkish	Turkish	Turkish	Lebanese	Lebanese	Lebanese
Current age	5.5 years	14 years	3.5 years	4 years	16 months	19 years
Age at onset	15 months	NA	NA	1 year	1 year	15 years
Symptoms	Recurrent EBV-LPD, SIRS	None, asymptomatic	None, asymptomatic	EBV-LPD, EBV-HLH	Fever, EBV+ ulcers	EBV-LPD, recurrent sinusitis lymphoma
Treatment	IG; Steroids; Rituximab*	None	IG† from age 4-20 months	HLH-2004, cord-HSCT	None	Rituximab, R-CHOP, cord-HSCT
IgG before treatment, g/L (reference range)	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)	32.4 (5.18-17.8)
IgA before treatment, g/L (reference range)	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)	3.93 (0.33-2.67)
IgM before treatment, g/L (reference range)	0.581 (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)	0.85 (0.32-1.35)
EBV plasma load, copies/ mL min-max	1e2-2e6¶	ND	0-3.6e2‡	0-5e6¶	8e6	0-5e6¶
EBNA Ab	NA (IVIG)	Positive	Positive	ND	ND	ND
Response to vaccination antigens§	Tetanus low Pneumococcus not vaccinated HiB low TBE low-normal Rubella not vaccinated	Tetanus normal Pneumococcus normal HiB normal TBE normal Rubella normal	Tetanus low-normal Pneumococcus low-normal HiB low, TBE normal Rubella normal	Tetanus normal Pneumococcus ND HiB normal TBE not vacc. Rubella ND	Tetanus low-normal Pneumococcus ND HiB normal, TBE not vacc. Rubella ND	Tetanus normal Pneumococcus no response** HiB normal TBE not vacc. Rubella ND
$CD19^+$ B cells, number/μL§§	490-1100	520	750-1040	1120	1700	84
IgD^-CD27^+ B cells§§	Absent	Absent	Absent	Absent	Absent	Absent
IgD^+CD27^+ B cells§§	Absent	Absent	Absent	Absent	Absent	Absent
Transitional B cells % $CD19^+$ §§	↑47	ND	↑24	ND	ND	2.6
$CD21^{low}$ B cells, % $CD19^+$§§	↑38	ND	↑18	ND	ND	0.4
$CD4^+$ T cells, number/μL§§	1340	1540	1460	1660	2400	796
$CD8^+$ T cells, number/μL§§	2150	1210	1280	1250	1580	2724
NK cells, number/μL§§	320	260	310	270	250	293
iNKT cells, % of $CD3^+$	0.01%-0.02%##	0.08%	0.09%	ND	0.02	<0.01%
in vitro T-cell proliferation*	Normal	ND	ND	Normal	ND	Normal
NK cell function†	Mildly reduced	ND	ND	Moderately reduced	Mildly reduced	Mildly reduced
EBV-specific T cells‡	present: 0.2% of $CD4^+$ and 9.6% of $CD8^+$ T cells, with highly increased IFNγ secretion of $CD8^+$ T cells	ND	ND	ND	ND	

*NA: not applicable; EBV-LPD: EBV-associated lymphoproliferative disorder; SIRS: systemic inflammatory response syndrome; EBV-HLH: EBV-associated hemophagocytic lymphohistiocytosis; IG: immunoglobulin replacement; CyA: cyclosporin A; cord-HSCT: allogeneic cord blood hematopoietic stem cell transplantation; R-CHOP: rituximab-cyclophosphamide-doxorubicin-vincristine-prednisolone; ND: not determined; reference range: age-specific ranges of immunoglobulin levels in parenthesis; Pneumococcus: pneumococcal polysaccharide vaccine; HiB: Haemophilus influenzae B vaccine; TBE: tick-borne encephalitis vaccine; IFNγ: interferon-γ. §See Online Supplementary Appendix for reference ranges and sources concerning vaccine antibody responses. Briefly, anti-Tetanus IgG was considered 'normal' when >1 IU/mL, 'low-normal' when 0.1-1 IU/mL. Anti-Pneumococcus polysaccharide IgG antibodies were compared before and after vaccination in Patient 6 and measured repeatedly >6-12 months after IgG substitution therapy in Patient 3, being considered normal when >10-30mg/L. Anti-Haemophilus influenzae B IgG were considered normal when >0.16-1mg/L. Anti-TBE IgG were measured as 'Vienna units' and considered normal when >155 VIEunits. Anti-Rubella IgG antibodies were only qualitatively analyzed. §§B and T cell normal ranges and memory B-cell subset reference values were applied according to previous publications (see Online Supplementary Appendix). *In vitro Tcell proliferation was measured as standard lymphocyte function test after 3-day incubation with phytohemagglutinin (in Patients 1, 4 and 6), and concanavalin A, pokeweed mitogen, CD3, Tetanus antigen, and staphylococcal superantigens (Patient 1). †For NK-cell function, cytotoxicity assay with peripheral blood mononuclear cells against NK-sensitive target cells (K562) in six different effector and NK:target-cell ratios (Online Supplementary Design and Methods), and in Patient 1 also CD107 degranulation. ‡For cytokine secretion assay, EBV LMP2A peptivator® (Miltenyi, Bergisch Gladbach, according to the manufacturer's instructions). §Two courses of rituximab in one year. ||Stopped two years ago. ¶Always positive when B cells present. #Variable, asymptomatic. **Performed when patient became hypogammaglobulinemic. ##Performed first during severe EBV-LPD and SIRS (0.01%), and later during clinical remission (0.02%; Online Supplementary Figure S1, right panel). Normal range of iNKT cells was considered >0.02% of $CD3^+$ T cells, and borderline 0.01-0.02%.*

individuals lacking CD27+ cells (Patients 1-3) revealed a total of two overlapping, homozygous candidate intervals (*Online Supplementary Table S2*), including an interval on Chromosome 12 containing the *CD27* gene. The missense mutation in *CD27* (c. G158A, p. Cys53Tyr) was found homozygous in 3 of 4 siblings in this family and heterozygous in both parents (Figure 1D and *Online Supplementary Figure S2*).

The parental consanguinity and shared ethnic background (Lebanese) in Families B and C suggested a common autosomal recessive genetic alteration. Therefore, WES of Patients 4 and 6 was performed which identified the same missense mutation (c. G158A) in *CD27* as the only novel shared homozygous single nucleotide variant predicted to be probably damaging or deleterious by different prediction tools (*Online Supplementary Appendix* and *Online Supplementary Figures S2 and S3*). The mutation was confirmed by Sanger Sequencing in all cases (Figure 1D). It is located within a motif of the ligand-binding domain evolutionarily conserved among different species and various TNFR family members (*Online Supplementary Figure S4*). Retrospective analysis of Patients 7 and 8, who had died years earlier, using DNA obtained from Guthrie newborn screening cards, confirmed the same *CD27* mutation (*Online Supplementary Figure S2*). Unfortunately, no specimens for immunological analyses are available from those patients.

Discussion

In this study, clinical and laboratory observations revealed a novel CD27-linked immunodeficiency predisposing towards an EBV-associated, potentially fatal disease. In parallel, van Montfrans *et al*. recently identified a different homozygous mutation in *CD27* (c.G24A, p.Trp8X) in 2 brothers of a consanguineous Moroccan family, of whom one died from severe infectious mononucleosis at a young age and the other recovered with persistent EBV-viremia and secondary hypogammaglobulinemia.[22] The clinical courses of Patients 1-8 and the patients reported by van Montfrans *et al*. suggest that the immunological/environmental context of the primary EBV infection may play a role in the first occurrence of hypogammaglobulinemia and the severity of the clinical symptoms in CD27-deficient patients,[22] although longitudinal observation of a larger number of patients and, ideally, preemptive monitoring of asymptomatic family members will be needed to confirm this hypothesis. Immunoglobulin levels were normal or borderline-low at onset of symptoms in symptomatic patients and at the time of diagnosis in asymptomatic patients, respectively. To date, hypogammaglobulinemia developed in Patients 1, 4 and 6 who have had relapsing symptomatic EBV infection. In addition, van Montfrans *et al*. observed normal peripheral B-cell differentiation, undisturbed germinal center reactions, and normal T-cell independent B-cell responses, absence of CD27.[22] No EBV-seronegative CD27-deficient individual has been identified until present. It, therefore, remains unclear whether hypogammaglobulinemia is due to the loss of CD27 function alone or, more likely, the result of chronic EBV infection. While 'Timing and Tuning' was described necessary for co-stimulatory signals by Nolte *et al*.,[5] and other TNF- or immunoglobulin receptors (e.g. herpes virus entry mediator [HVEM], CD30, OX40 [CD134], 4-1BB [CD137] or CD28) have partially overlapping functions,[23] the human data presented here suggest that CD27 might not be essential but unique among co-stimulatory molecules in its relevance for the primary immune response against EBV. One could hypothesize that absent function of CD27 affected the normal silencing of *EBV* gene expression in infected cells, thus perturbing the establishment of EBV persistence. Whether there is a cellular reservoir of class-switched/germinal center-derived B cells or potentially other, normally also CD27-expressing, cell types with EBV persistence despite lack of functional CD27 is unclear. Together, the identification of CD27 deficiency in 4 independent families, and the observation that no additional mutations in genes other than CD27 could be identified by WES, suggest that CD27 deficiency alone, either due to a complete lack (p.Trp8X) or perhaps only a deficient surface expression (p.Cys53Tyr), causes disease with a broad clinical variability.

Immunological consequences

The severe reduction in iNKT cells in CD27-deficient patients during massive EBV-LPD (i.e. Patients 1 and 6; Table 1 and *Online Supplementary Figure S1*) may further support a primary role of iNKT cells for EBV-LPD pathogenesis as described previously in SAP, XIAP and ITK deficiency,[9,24,25] implicating that the CD70-CD27 axis acts as a co-stimulatory requirement for development and/or maintenance of iNKT cells, or it may be a secondary phenomenon.

Similar to other subtle T-cell disorders, such as SAP-, XIAP- and ITK deficiency, our data suggest that immunity against other viral infections does not seem as severely compromised in human CD27 deficiency.

Other clinically relevant consequences of CD27 dysfunction might include: i) decreased memory formation to viral (including vaccine protein) antigens;[4,26] and ii) perturbed anti-tumor immunity of T cells,[27] γδT cells,[28] and NK cells,[29,30] potentially leading to an increased risk of other malignomas in addition to EBV-lymphomas. It is likely that more individuals with dysfunctional CD27 will be identified among patient cohorts with hypogammaglobulinemia (with or without EBV-LPD) and absent CD27-expressing memory B cells, potentially leading to the recognition of CD27 deficiency as a novel, albeit probably a rare, combined immunodeficiency.[31-33] Of note, Patients 1 and 3 also showed expansion of transitional and CD21^{low} B cells, which is reportedly associated with increased risks of lymphadenopathy, splenomegaly, and granuloma formation in CVID, similar to XLP patients.[34]

Conclusions

CD27 deficiency should be considered in all patients with hypogammaglobulinemia or unusually severe causes of EBV infection in order to allow for an individualized treatment based upon the experience with this condition so far. Our results illustrate that modern genomic technologies such as WES may identify and confirm disease-causing mutations in monogenetic recessive diseases even with limited numbers of affected individuals. Future studies to clarify the cellular pathomechanistic consequences of CD27 deficiency are warranted.

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