CD27 deficiency is associated with combined immunodeficiency and persistent symptomatic EBV viremia

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Background: CD27 is a lymphocyte costimulatory molecule that regulates T-cell, natural killer (NK) cell, B-cell, and plasma cell function, survival, and differentiation. On the basis of its function and expression pattern, we considered *CD27* a candidate gene in patients with hypogammaglobulinemia.
Objective: We sought to describe the clinical and immunologic phenotypes of patients with genetic CD27 deficiency.
Methods: A molecular and extended immunologic analysis was performed on 2 patients lacking CD27 expression.
Results: We identified 2 brothers with a homozygous mutation in *CD27* leading to absence of CD27 expression. Both patients had persistent symptomatic EBV viremia. The index patient was hypogammaglobulinemic, and immunoglobulin replacement therapy was initiated. His brother had aplastic anemia in the course of his EBV infection and died from fulminant gram-positive bacterial sepsis. Immunologically, lack of CD27 expression was associated with impaired T cell–dependent B-cell responses and T-cell dysfunction.
Conclusion: Our findings identify a role for CD27 in human subjects and suggest that this deficiency can explain particular cases of persistent symptomatic EBV viremia with hypogammaglobulinemia and impaired T cell–dependent antibody generation. (J Allergy Clin Immunol 2012;129:787-93.)

Key words: *EBV, viremia, hypogammaglobulinemia, CD27, immunodeficiency, T cell, B cell, natural killer cell, phenotype*

Abbreviations used
CTL: Cytotoxic T lymphocyte
CVID: Common variable immunodeficiency
FACS: Fluorescence-activated cell sorting
GC: Germinal center
NK: Natural killer
PWM: Pokeweed mitogen
SAC: *Staphylococcus aureus* Cowan I antigen
TT: Tetanus toxoid

CD27, a member of the TNF receptor family, is a transmembrane receptor that is widely used as a leukocyte differentiation marker for subsets of T, natural killer (NK), and B cells.[1-3] Importantly CD27 is recognized as a marker for memory B cells and is held to be of diagnostic/predictive value in patients with common variable immunodeficiency (CVID). In patients with CVID, there can be decreased numbers of switched memory B cells (expressing surface IgA or IgG), which correlates with the presence of splenomegaly and granulomatous disease.[4]

The function of human and murine CD27 has been studied in detail *in vitro* and *in vivo* in murine models.[5,6] Ligation of CD27 by its unique ligand CD70 provides costimulatory signals for T-, B-, and NK cell activation. It furthermore enhances T-cell survival and effector function, NK cell function, B-cell differentiation, and plasma cell function.[7,8] In human subjects an indispensable role of the costimulating signal provided by CD27-CD70 interaction toward immune function and disease susceptibility has not been formally proved. CD27 is a major differentiation/maturation marker for T cells, NK cells[9] and B cells.[10] On the basis of its expression on memory B cells and plasma cells, and the effect of CD27 ligation on *in vitro* B-cell function, *CD27* has been proposed as a candidate gene in patients with CVID, but its expression on B, T, and NK cells suggests that CD27 deficiency might result in a more combined type of immune deficiency.

Primary EBV infection is often asymptomatic in the immunocompetent host. In immunodeficient patients, however, primary EBV infection or secondary reactivation might result in persistent symptomatic EBV viremia, a clinical condition with a prolonged (>6 months) and distinct symptomatic phase with fever, lymphadenopathy, and several other possible features, such as hepatitis and pneumonia. Persistent symptomatic EBV viremia can be associated with lymphoma, lymphoproliferative disease, hemophagocytic lymphohistiocytosis, and aplastic anemia but most typically goes into spontaneous remission.[11]

EBV-specific immunity typically encompasses virus-specific cellular and humoral immune responses, with T cells being most important for long-term control of disease. Several types of cellular immune deficiency can result in an abnormal course of EBV infection, including combined immune deficiencies, X-linked

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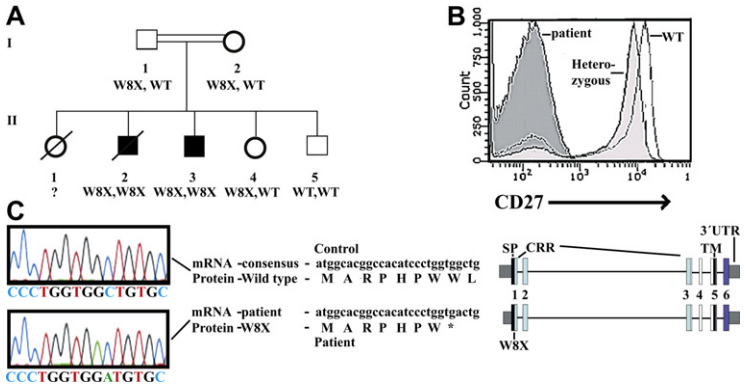

FIG 1. Absence of CD27 expression because of a nonsense mutation in the *CD27* gene. **A,** Family pedigree (/, deceased persons; *double lining*, consanguinity). **B,** FACS analysis of CD27 membrane expression on PBMCs. **C,** Sequence analysis of the *CD27* gene. The mutation *(boldface)* identified in both patients in exon 1 results in a premature stop codon *(X)* in the signal peptide of the protein (NP_001233.1), as depicted in the protein sequence (superimposed on the gene sequence).

lymphoproliferative disease,[12] familial hemophagocytic lymphohistiocytosis,[13] and IL-2–inducible T-cell kinase deficiency.[14] In the majority of persistent symptomatic EBV viremia cases, however, a specific primary immune deficiency has not been identified.

We here describe 2 brothers with CD27 deficiency caused by a homozygous mutation resulting in a premature stop codon in the gene encoding CD27. Clinically, these patients presented as having persistent symptomatic EBV viremia, with lethal aplastic anemia in one and hypogammaglobulinemia with impaired specific antibody function in the other. In the surviving patient the absence of CD27 was associated with an abnormal T cell–dependent B-cell response and disturbed T-cell function.

METHODS

Evaluation of blood, bone marrow biopsy specimens, vaccination responses, and medical records were carried out after written informed consent was obtained in accordance with local medical ethics committee guidelines.

Case report

The index patient, a 21-year-old man of Moroccan descent, was the third child of consanguineous parents (first cousins). At age 2½ years, he experienced fever, severe lymphadenopathy, and hepatosplenomegaly lasting a total of 6 months. EBV seroconversion was noted for early antigen and viral capsid antigen, but during follow-up, no seroconversion for Epstein-Barr nuclear antigen was noted. Immunoglobulin levels were determined longitudinally and were initially increased (IgM, 3.1 g/L; IgG, 15.9 g/L; and IgA, 1.9 g/L; see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). The peripheral blood lymphocyte compartment was phenotyped regularly during the first 1½ years of follow-up, and changes in lymphocyte numbers showed signs compatible with viral infection (see Fig E1, *A*). T-cell proliferation assays showed strongly reduced mitogen- and antigen-specific responses the first 6 months after clinical presentation, and these responses gradually increased to subnormal and normal levels, respectively, during the following year (data not shown). Clinical symptoms disappeared after 6 months, and at the same time, the index patient became hypogammaglobulinemic (IgM, 0.03 g/L; IgG, 4.4 g/L; and IgA, 0.1 g/L). Immunoglobulin replacement therapy was initiated. Since receiving immunoglobulin prophylaxis, he has had an uneventful medical history: no abnormalities were noted in the incidence, type, or course of infections; vaccinations, including live attenuated measles-mumps-rubella, were given without complications; there was normal growth and development; there were no additional hospitalizations; and the index patient did not have autoimmunity or cancer. EBV plasma load was monitored longitudinally in available samples with quantitative PCR and was detectable at low levels but with an increased frequency compared with those seen in healthy control subjects (positive 4/15 times, see Fig E1, *B*); the index patient was asymptomatic at these occasions. CD4 and CD8 T cells, NK cells, and B cells were sorted from a reactive lymph node cell suspension (derived at age 3 years) and from PBMCs (derived at age 21 years). In both cases EBV was detected in the purified B-cell fraction (but compared with other reports in relatively low amounts: viral loads were 3000 and 2050 viral copies/μg of DNA, respectively[15]).

Family history was notable for 2 childhood deaths (Fig 1, *A*). An older sister died during infancy of an unknown illness. She had otherwise had an uneventful medical history, to our knowledge, although details were limited. An older brother was referred at age 3 years with documented EBV-induced lymphadenopathy, fever, hepatosplenomegaly, and EBV-associated uveitis without evidence of malignancy. He had increasing EBV anti–viral capsid antigen and early antigen titers but absence of Epstein-Barr nuclear antigen seroconversion during follow-up. At initial presentation, the serum IgG level was 10.4 g/L, the IgM level was 2.7 g/L, and the IgA level was 0.4 g/L; IgM levels normalized during a period of 8 months. Lymphocyte phenotyping 8 months after initial presentation showed 0.04 × 10E9/L $CD20^+$ B cells, 0.16 × 10^9/L $CD4^+$ T cells, 2.49 × 10^9/L $CD8^+$ T cells (CD4/CD8 ratio, 0.064), and 1.38 × 10^9/L NK cells. Furthermore, mitogenic and antigenic T-cell responses were absent. After a period of 8 months with severe lymphadenopathy and recurrent episodes of fever, he had aplastic anemia, and 1 month thereafter, he died from fulminant gram-positive bacterial sepsis.

Genomic sequencing

Genomic DNA was isolated from PBMCs or snap-frozen lymph node tissue. Exon-specific M13-tagged primers were used to amplify all coding exons, including flanking regions from the genes *CD27* (NM_001242), *SH2D1A* (NM_002351, encoding SH2 domain protein 1A [SH2D1A] or SLAM-associated protein [SAP]), *XIAP* (NM_001167, encoding X-linked inhibitor of apoptosis [XIAP]), and *PRF1* (NM_001083116, encoding Perforin 1); primer sequences are available on request. PCR products were directly sequenced with M13 sequence primers and BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif; www.aplliedbiosystems.com), according to the manufacturer's protocol. Sequencing was performed on a 3130XL genetic analyzer (Applied Biosystems), and sequences were analyzed with SeqScape version 2.5 software (Applied Biosystems).

Flow cytometry

PBMCs were isolated from whole blood by using Ficoll-Hypaque density centrifugation. Antibody staining and fluorescence-activated cell-sorting

(FACS) analysis were performed as described previously (for the antibodies used in the analysis, see Table E1 in this article's Online Repository at www.jacionline.org).[6,16] Custom conjugations of antibodies to Quantum dot nanocrystals (Invitrogen, Carlsbad, Calif) were performed as previously described.[17] For acquisition, PMT voltages were based on isotype-negative controls and compensation on positive controls by using fluorophore-conjugated anti-CD45 or Cytometer Setup and Tracking beads (BD Biosciences PharMingen, San Jose, Calif). Staining with recombinant CD70 was performed with day 3 culture supernatant of soluble Flag-TNC-CD70–transfected HEK cells.[18] Anti-Flag fluorescein isothiocyanate (clone M2; Sigma-Aldrich, St Louis, Mo) was used as a second step to detect binding of the recombinant protein to the cell surface. $CD4^+CD3^+$ and $CD8^+CD3^+$ T lymphocytes were analyzed for their expression of CD45RA in combination with CCR7, CD62L, CD127, CD28, or Fas. The composition of the B-cell compartments was assessed in blood and bone marrow by means of flow cytometry.[19,20]

Immunohistochemistry

Immunohistochemistry was performed on 4-μm-thick paraffin-embedded sections from neutral-buffered formaldehyde–fixed tissue blocks. Immunohistochemical staining for CD3, CD20, CD79a, CD138, Kappa, Lambda, BCL2, and Mib-1 (Dakopatts, Glostrup, Denmark), CD5, CD23, CD27, and BCL6 (Novacastra, Newcastle upon Tyne, United Kingdom) was performed by using a Bond-Max automated staining machine (Vision Biosystems, Norwell, Mass) with the Bond polymer refine detection kit (catalog no. DS9800, Vision BioSystems). Epitope Retrieval Solution 2 (EDTA) was used as antigen retrieval for BCL6 and CD27; for all other reactions, Epitope Retrieval Solution 1 (citrate buffer) was used (Novacastra). Isotype controls were used throughout.

Functional assays

$CD4^+$ cells were isolated with anti-CD4–coated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions. For proliferation analysis, 1.8×10^5/mL PBMCs or isolated $CD4^+$ cells were cultured for 3, 4, or 6 days in RH10 (RPMI 1640 supplemented with L-glutamate and 25 mmol/L HEPES [Invitrogen] containing 10% human AB serum [Sanquin, Amsterdam, The Netherlands], penicillin/streptomycin [100 U/mL, Invitrogen], and 6.0×10^{-5} mol/L β-mercaptoethanol [vol/vol; Calbiochem, Merck, Darmstadt, Germany]) and one of the following stimuli: PHA (5 μg/mL; Wellcome, Beckenham, United Kingdom), concanavalin A (10 μg/mL, Calbiochem; both 3 and 6 days), a cocktail of 3 anti-CD2 mAbs (clone T11.1/1 T11.1/2 T11.1/3, Sanquin; 4 days), pokeweed mitogen (PWM; 1.5 μg/mL, Sigma-Aldrich), tetanus toxoid (TT; 3.5 LF/mL; Netherlands Vaccine Institute, Bilthoven, The Netherlands), purified protein derivative (13 μg/mL; Statens Serum Institute, Copenhagen, Denmark), and *Candida albicans* (7 μg/mL; Hal, Leiden, The Netherlands; 6 days). Tritiated thymidine (1 μCi/96 wells) was added 16 hours before harvesting. Background (medium only) tritiated thymidine incorporation was less than 500 cpm. T-cell costimulation through CD70 and $CD8^+$ T-cell polyfunctionality were tested as previously described.[16,21]

For B-cell differentiation assays, 4×10^5 PBMCs (at 2.5×10^5/mL) were cultured with either *Staphylococcus aureus* Cowan strain I (SAC; 40 U/mL) and IL-2 (50 U/mL) or PWM (1.5 μg/mL, Sigma). After 7 days, cells were harvested, and cytospin preparations (10^5 per sample) were prepared, air-dried, fixed with 95% EtOH/5% acetone, and stained with fluorescein isothiocyanate–conjugated anti-IgM, anti-IgA, and anti-IgG (SBA). Ten fields of 50 cells were analyzed by means of fluorescence microscopy, and the fraction of plasmablasts was calculated.

For NK cell cytotoxicity, NK cells were negatively selected from PBMCs (Miltenyi NK cell isolation kit-II) and added in different ratios to chromium-labeled K562, 721.221, or Raji cells or Raji cells with rituximab, and chromium release was measured after 4 hours, as previously described.[22]

Vaccination studies

The index patient was vaccinated once with 23-valent (polysaccharide) pneumococcal vaccine (Pneumovax; Merck, Sharp & Dohme, Darmstadt, Germany), once with TT-conjugated Men C vaccine (NeissVac; Baxter, Utrecht, The Netherlands), and twice (12 weeks apart) with the human diploid cell rabies vaccine (Institut Pasteur Merieux, Merck Sharp & Dohme), according to standard protocols. During infancy, he was vaccinated 3 times with TT vaccine (Tetanusvaccin; Netherlands Vaccine Institute, Bilthoven, The Netherlands), followed by 2 booster vaccinations in childhood as part of the standard Dutch national vaccination program.

Quantification of serum antibody levels

IgM, IgG, IgA, and IgG subclasses were quantified by means of nephelometry on an Image 800 nephelometer (Beckman Coulter, Fullerton, Calif). Specific antibody levels against vaccination antigens were measured by means of ELISA.[23] Blood samples for antigen-specific antibody determination were drawn at the indicated time points after vaccination. Reference sera for rabies and TT were used as secondary local standards that were calibrated on international standards supplied by the National Institute for Biological Standards and Control (www.nibsc.ac.uk). The response to both antigens was compared with the responses of 20 healthy adults.

Detection of plasma viral EBV load

EBV plasma load was measured as described previously.[24]

Analysis of somatic hypermutation

Hypermutation of immunoglobulin genes was analyzed as previously described.[25] In short, V_H3-Cγ fragments were amplified from randomly primed cDNA and cloned in a pGEM-T Easy Vector (Easy Vector System II; Promega, San Luis, Calif). Fifteen individual clones were sequenced (ABI Prism 3100 Genetic analyzer, Applied Biosystems), analyzed with BioEdit version 5.0.9, and aligned to the germ line with the highest homology within the region from framework regions 1 to 3, as deposited in http://www.ncbi.nlm.nih.gov/igblast. This database contains defined functional V genes. The percentage homology to the germ line V_H gene segment was determined from the beginning of framework region 1 to the end of framework region 3.

RESULTS

Patient identification and genetic diagnosis

Lymphocyte subset analysis was performed in a patient with a confirmed but unexplained diagnosis of CVID and persistent symptomatic EBV viremia. In this patient no $CD27^+$ T or memory B cells were detected, despite the presence of normal percentages of naive ($CD45RA^+$) T cells and switched IgG^+ and IgA^+ B cells (subpopulations normally expressing CD27). Immunohistochemistry (results not shown) and extended flow cytometric analysis with additional anti-CD27 mAbs and a recombinant ligand (CD70) protein confirmed the absence of CD27 expression on all lymphocytes (Fig 1, *B*) in the presence of normal lymphocyte subset distributions, suggesting a genetic defect in CD27. Mutation analysis in the index patient and in the deceased brother revealed a homozygous mutation (c.24G>A) in the gene encoding CD27, resulting in a premature stop codon (W8X; Fig 1, *C*). Both parents and an unaffected brother were asymptomatic heterozygous carriers (Fig 1, *A*). To rule out other known genetic causes of EBV susceptibility, we analyzed both the *SH2D1A* and *XIAP* genes, which can result in X-linked lymphoproliferative disease, as well as the *PRF1* gene, which can cause hemophagocytic lymphohistiocytosis, in the index patient and his mother; no mutations were found.

To better elucidate whether the index patient's CD27 deficiency could explain his clinical course of persistent symptomatic EBV viremia and hypogammaglobulinemia, we evaluated the phenotype and function of the index patient's lymphocytes *in vitro* and *in vivo*.

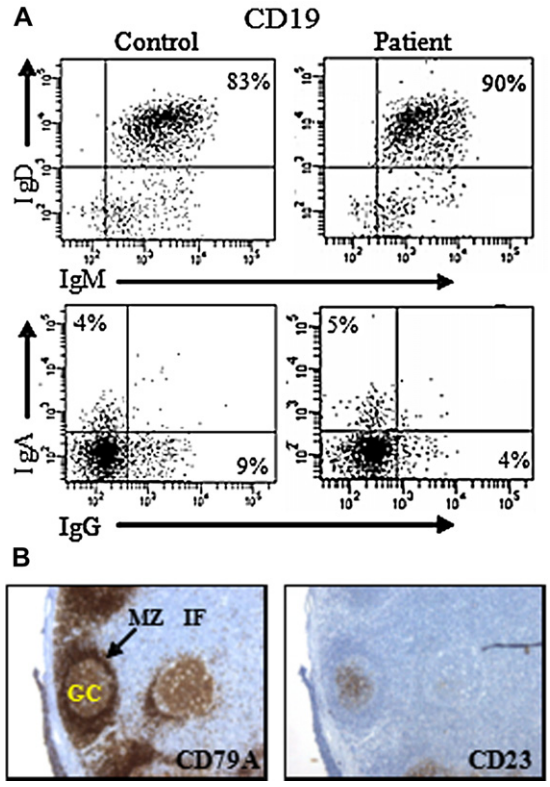

FIG 2. Phenotypic analysis of the index patient's B cells. **A,** The presence of different B-cell subsets was analyzed by using FACS. Within the $CD19^+$ B cells, the fraction of naive (IgM^+ and IgD^+) or memory (IgA^+ or IgG^+) B cells was determined. **B,** The presence of GCs was determined by staining tissue sections with anti-CD79a and anti-CD23 mAb. *IF,* Interfollicular area; *MZ,* mantle zone.

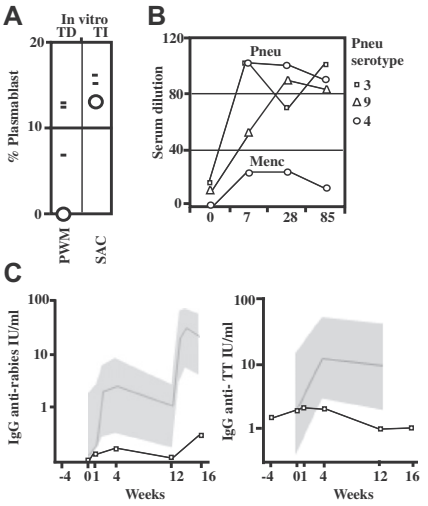

FIG 3. Functional analysis of the index patient's B cells. **A,** PBMCs were tested for their capacity to differentiate into plasmablasts after T cell–independent (*TI*; SAC/IL2) or T cell–dependent (*TD*; PWM) stimulation. The fraction of plasmablasts was determined based on morphology and intracellular immunoglobulin staining on day 7. *O,* Index patient; *-,* control subjects. **B,** Vaccination response of the index patient to polysaccharide vaccine (Pneumovax 23) and conjugated polysaccharide Men C vaccine (NeissVac). **C,** Vaccination response of the index patient to protein antigen (diploid cell rabies vaccine). The mean of healthy control subjects *(black line)* with 95% CIs *(gray region)* is shown.

B-cell compartment

At age 21 years, the index patient had normal B-cell counts, and although he was receiving IgG replacement therapy, decreased serum IgA and IgM values (0.14 g/L [normal 5% to 95% range, 0.70-4.0] and 0.31 g/L [normal 2% to 95% range, 0.40-2.3], respectively) were documented. FACS analysis of early and mature B-cell compartments in the bone marrow (data not shown) and blood (Fig 2, *A*) revealed normal numbers and percentages of all stages of B-cell development, including surface IgA^+ and IgG^+ B cells. The percentage of bone marrow plasma cells, however, was slightly reduced. *In vitro* induction of B cells with the T cell–independent stimuli SAC and IL-2[26] resulted in normal plasmablast formation; however, none were detected by using T-cell dependent stimulation with PWM (Fig 3, *A*). These results reflected the *in vivo* responses because vaccination with pneumococcal polysaccharide and meningococcus C–conjugated vaccines resulted in normal levels of specific antibody production (IgG, Fig 3, *B*; IgM, data not shown). In contrast, repeated protein antigen vaccinations (rabies) and TT booster vaccinations resulted in severely impaired antibody production, suggesting a defect in T cell–dependent B-cell responses (Fig 3, *C*).

Because CD27 and its ligand are expressed in germinal centers (GCs),[27,28] we next tested whether disturbed GC formation could explain the impaired T cell–dependent B-cell responses. Immunohistochemistry on a reactive lymph node, which was obtained for diagnostic purposes from the index patient in infancy during lymphadenopathy, showed a lymph node architecture with a large T-cell zone, primary follicles, and GCs (Fig 2, *B*). Most GCs were small but had a normal constitution. Large numbers of ($CD138^+$) plasma cells were scattered in the lymph node. Analysis of somatic hypermutation, a process occurring in GCs, showed mutation frequencies in the V_H-C_γ transcripts of peripheral blood B cells within the normal range (data not shown), which is comparable with those found in unaffected protein-driven secondary immune responses.[29] Thus although follicles were formed and somatic hypermutation occurred, the relative small size of secondary follicles supported the observation of impaired T cell–dependent responses.

NK cell compartment

Given the critical role of NK cells in defense against herpes viruses[30] and the fact that CD27 is an important marker for NK cell development,[1] NK cells were analyzed phenotypically and functionally. NK cells were present in normal numbers in peripheral blood and appeared to be fully differentiated (see Fig E2 in this article's Online Repository at www.jacionline.org). Importantly, the cytotoxic function of NK cells was also normal because PBMCs or highly enriched peripheral blood NK cells lysed MHC class I–deficient erythroleukemia cells and EBV-transformed B cells and were functional in antibody-dependent cell–mediated cytotoxicity (data not shown and Fig 4). FACS analysis on reactive lymph nodes showed normal (1.5%) and increased (16%) frequencies of NK cell numbers in the index patient and the deceased brother, respectively.

T-cell compartment

CD27 was first recognized as a costimulatory molecule on human T cells, and thus we evaluated the ability of the index patient's T cells to respond to activation. Overall, *in vitro* proliferative responses of the index patient's T cells were reduced in response to mitogens

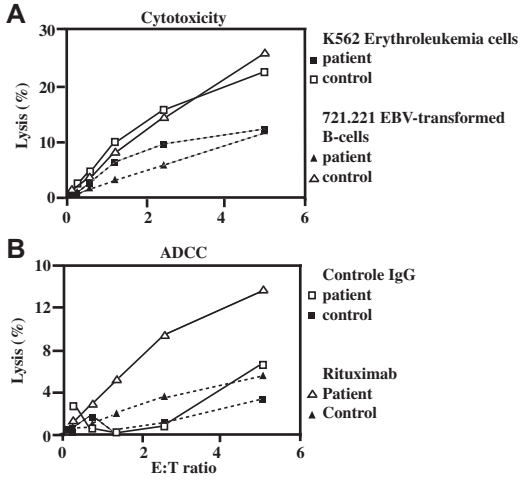

FIG 4. Analysis of the index patient's NK cells. **A,** Cytotoxic activity of highly enriched, negatively selected *ex vivo* PBMC NK cells against the indicated targets. **B,** Antibody-dependent cellular cytotoxicity of Raji cells induced by the addition of rituximab relative to added control IgG.

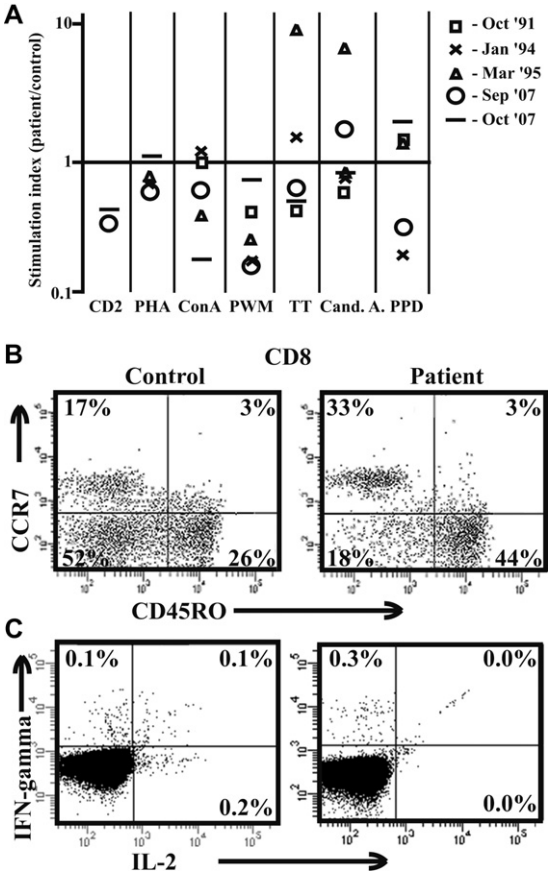

FIG 5. Analysis of the index patient's T cells. **A,** Proliferation assays with PBMCs or $CD4^+$ T cells and the indicated stimuli. Results are presented as the stimulation index (index patient cpm/healthy control subject cpm). **B,** Subset analysis of T cells[32] in PBMCs by means of FACS. Within $CD8^+CD3^+$ T cells, the fractions of naive ($CCR7^+CD45RO^-$), central memory ($CCR7^+CD45RO^+$), effector memory ($CCR7^-CD45RO^+$), and effector ($CCR7^-CD45RO^+$) cells were determined. **C,** Intracellular accumulation of IL-2 and IFN-γ in EBV-specific $CD3^+CD8^+$ T cells after stimulation with HLA-A2–restricted EBV peptide pools.

that strongly depend on CD27 (CD2 and PWM; Fig 4, *A*, and see Fig E3 in this article's Online Repository at www.jacionline.org). Proliferative responses against a variety of recall antigens could be detected *ex vivo*, and after vaccination with TT, the specific proliferative response was increased (data not shown).

Extended phenotypic and functional analyses were performed to determine whether there was an effect of CD27 deficiency on T-cell maturation or specific T-cell subsets. NKT cells and γδ T cells were present in similar numbers and fractions of T cells as in healthy control subjects. γδ T cells produced similar amounts of T-box transcription factor and IFN-γ (after PMA ionomycin stimulation, data not shown). This limited analysis argues against a role for CD27 in γδ T-cell development. For both $CD4^+$ and $CD8^+$ T cells, the phenotypic subset distribution, as defined by CCR7, CD127, and CD45RA (Fig 5, *B*, upper),[31,32] as well as cytokine (IL-2, IFN-γ, and TNF-α), production,[33] after polyclonal stimulation (see Fig E4 in this article's Online Repository at www.jacionline.org) was comparable with that seen in healthy control subjects. *Ex vivo* analysis of T-box transcription factor expression showed comparable percentages and levels in the index patient and control subsets of CD4 and CD8 T cells (data not shown), and HLA-A2–restricted EBV-specific $CD8^+$ T cells were readily detected and amounted to 0.41% of memory $CD8^+$ T cells. These cells degranulated; produced IFN-γ, TNF-α, and macrophage inflammatory protein 1α; and proliferated (see Fig E4) after EBV-specific stimulation *in vitro*. Autologous EBV-transformed B cells were lysed by the index patient's EBV-specific T-cell lines (data not shown). Despite a normal differentiation phenotype, however, the frequency of IL-2–producing EBV-specific CD8 T cells was reduced compared with that seen in healthy control subjects (Fig 4, *B*, lower).[33] This suggests that despite there being normal T-cell differentiation and maturation, the ability to maintain physiologically relevant recall in the $CD8^+$ T-cell population was deficient.

DISCUSSION

We describe 2 cases of CD27 deficiency that presented with persistent symptomatic EBV viremia after primary infection with EBV at a young age. In the one case in which more detailed immunologic evaluation was possible, the immunologic phenotype shared similarities with that of CVID in that there was hypogammaglobulinemia and partially impaired specific antibody function. Overall, our findings demonstrate that CD27 deficiency might be associated with a distinct immunologic phenotype, one of disturbed T cell–dependent B-cell function and subtle but likely clinically relevant abnormalities in T-cell function. CD27 deficiency does not seem to influence NK cell or γδ T-cell development or function, despite being an important lineage-specific marker. The clinical relevance of CD27 deficiency is likely to vary, as reflected by the clinical courses in the 2 patients presented here, one eventually recovering (and currently healthy, although receiving immunoglobulin replacement therapy) and the other succumbing from a presumed complication of persistent symptomatic EBV viremia (ie, aplastic anemia).

Several studies have provided evidence for decreased antibody dependent cellular cytotoxicity, decreased NK cell activity,[34] and lack of NKT cells or EBV-specific cytotoxic T lymphocytes (CTLs)[14,35] during persistent symptomatic EBV viremia.[36] The strongly reduced numbers of NKT cells in SAP-deficient, IL-2–inducible T-cell kinase–deficient, and a large number of

XIAP-deficient patients points toward a role for this cell type in control of EBV infection.[14,35,37,38] The successful treatment of patients with persistent symptomatic EBV viremia using EBV-specific CTL infusions further emphasizes the importance of virus-specific CTLs for EBV clearance.[39] Importantly, our patients had normal numbers of NK cells and NKT cells but demonstrated a subtle CTL abnormality. Investigation of the index patient's immunity at adult age showed polyfunctional EBV-specific $CD8^+$ T cells and killing of autologous EBV-transformed B cells by the index patient's EBV T-cell lines. In line with this EBV-specific immunity, repeated measurements of plasma EBV DNA showed periodic low but detectable viral loads (range, <50-234 copies/mL). When compared with the prevalence of EBV DNA in the plasma of healthy control subjects (no detectable load in 300 healthy control subjects, R. Schuurman, personal communication), the levels detected in the patient are higher than expected. This finding could be a feature of a higher incidence of B-cell activation as a result of (subclinical) infections caused by the index patient's decreased immunoglobulin levels. In keeping with the periodic EBV viremia, sorted B cells from the index patient obtained during viremic periods, as well as during resolution, also demonstrated cell-associated viral loads. This is likely an indication of abnormal EBV-specific immunity.

In murine models the absence of CD27 does not dramatically change *in vivo* immunity[5,40] but does affect distinct aspects of CD8 T-cell responses.[5,41,42] In the index patient naive, memory, memory effector, and terminally differentiated T cells and γδ T and NKT cells were present within the normal range, and memory $CD4^+$ and $CD8^+$ T-cell responses were detected. Thus on the surface, the immune elements required for anti-EBV responses would seem to have been intact. The *ex vivo* fraction of IL-2–producing EBV-specific $CD8^+$ cells, however, was decreased. In line with this observation, the absence of CD27 in mice results in decreased frequencies of IL-2–producing but not IFN-γ–producing CD8 memory cells.[43] In mice NKT cell–dependent induction of the CD70 ligand on dendritic cells[44] enhances CD8 T-cell activation, and lack of CD27 also results in a decreased number of CD8 effector cells at the site of viral infection.[45] Given the importance of CTLs for control of EBV infection, the phenotype of $CD27^{-/-}$ mice is compatible with a subtle yet important role for CD27 in CTL induction and function, which could result in the EBV-related abnormalities observed in our patients. Finally, it is conceivable that the role of CD27 deficiency is more pronounced during the initiation of the cellular immune response, which could preclude identifying additional abnormalities because we were only able to investigate EBV-specific immunity at adult age in the surviving (index) patient.

Phenotypic and functional analyses did not reveal abnormalities of peripheral blood NK cells. Analysis of reactive lymph nodes showed normal (1.5%) to increased (16%) percentages of NK cells in lymph nodes of the index patient and his deceased brother, respectively, which suggests that NK cells were participating in the response against EBV. Recently, CD27 has been identified as a marker that distinguishes both human and murine developmental NK cell subsets[1] and is expressed almost exclusively on immature NK cells. This suggests that CD27 is involved in NK cell maturation into mature cytolytic cells. CD27 also affects NK cell cytolytic capacity because CD27 deficient murine NK cells showed reduced cytotoxicity,[46] although only under activating conditions. However, our analysis showed no abnormalities in the composition of the NK cell compartment, effectively ruling out a requirement for CD27 in the development of human NK cells. Because EBV infection induces interferon production,[47] which can prime NK cells for CD27-dependent and other increases in cytolytic capacity,[46] it is still possible that the lack of a defect in the index patient could represent a cytokine-compensated phenotype. Although this might be possible, the diverse expression of inhibitory and activating receptors on the patient's NK cells, however, strongly suggest that the interaction between CD70 and CD27 is dispensable for human NK cell differentiation, maturation, and cytotoxic function.

In human subjects CD27 is an important costimulator of plasma cell differentiation. The index patient became hypogammaglobulinemic at the time of recovery of lymphadenopathy and, based on his immunoglobulin profile and vaccination responses, could even be classified as having CVID,[48] although the abnormal course of EBV infection suggests suboptimal cellular immunity. In this patient CD27 deficiency did not interfere with B-cell development in the bone marrow, and B-cell numbers and subset composition were normal in peripheral blood. The presence of a normal frequency of IgG^+ B cells carrying hypermutated IgH genes and structurally normal GCs of the index patient implied that CD27 deficiency does not interfere with immunoglobulin isotype switching and somatic hypermutation. Despite the absence of abnormalities in the cellular composition of the B-cell compartment, however, abnormal T-cell dependent immune responses *in vivo* (rabies vaccine) and *in vitro* (PWM) were observed. In contrast, T cell–independent responses against SAC *in vitro* and pneumococcal and meningococcal polysaccharides *in vivo* were effectively induced. This suggests that CD27 deficiency impairs post primary GC terminal B-cell differentiation, possibly by disturbing the balance between induction of memory B cells and plasma cells. The decreased numbers of plasma cells in the bone marrow but increased numbers of plasmablasts in the lymph nodes shows that CD27 is dispensable for plasma cell differentiation but suggest a novel role for CD27 in regulating plasmablast survival, migration, or both. Together, the data of our index patient suggest that CD27 function in human subjects is not essential for any specific stage of the adaptive immune response, as is observed in CD27KO mice. The functions of certain TNF receptor family member functions, such as those of CD27, 4-1bb, and OX40, appear to be largely overlapping. Fine tuning of immune responses through highly restricted expression of their respective ligands appears to be their role. That CD27 deficiency seems to reveal itself during EBV infection might reflect its heightened potential during EBV infection because of the large amounts of EBV-induced CD70 expression.

In conclusion, we describe the clinical and immunologic phenotypes of 2 brothers with CD27 deficiency presenting with persistent symptomatic EBV viremia. Our data suggest that CD27 deficiency is a new molecularly defined primary immunodeficiency disease associated with persistent symptomatic EBV viremia, hypogammaglobulinemia, and impairment in specific antibody function resulting from disturbed $CD8^+$ T-cell and T cell–dependent B-cell responses.

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Clinical implications: In patients with EBV viremia, disturbed T cell–dependent humoral immunity, or both, CD27 expression should be tested in consideration of genetic deficiency.

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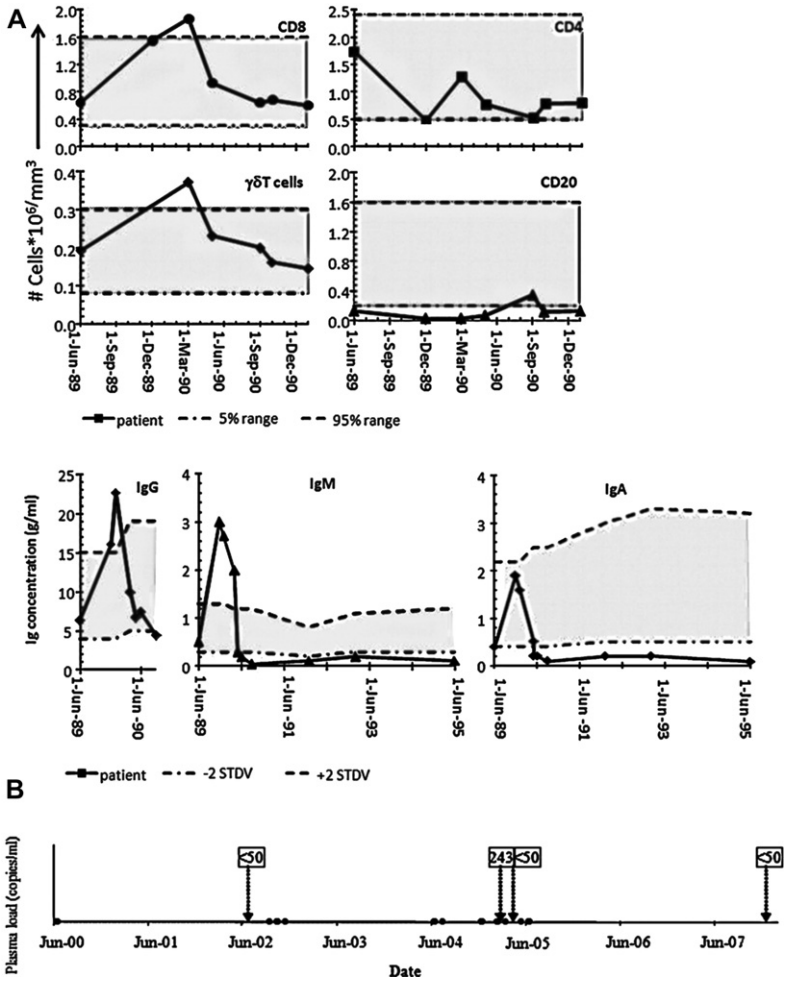

FIG E1. Longitudinal analysis of the index patient's cell numbers, immunoglobulin numbers, and EBV plasma load over time. In the index patient, cell numbers and immunoglobulin levels **(A)** and EBV plasma load (**B**; *solid circles*, no detectable load; *arrows*, indicated load) were determined[E1] at the indicated time points *(symbols)* between 1989 and 1995 (Fig E1, *A*) and 2000 and 2008 (Fig E1, *B*), respectively.

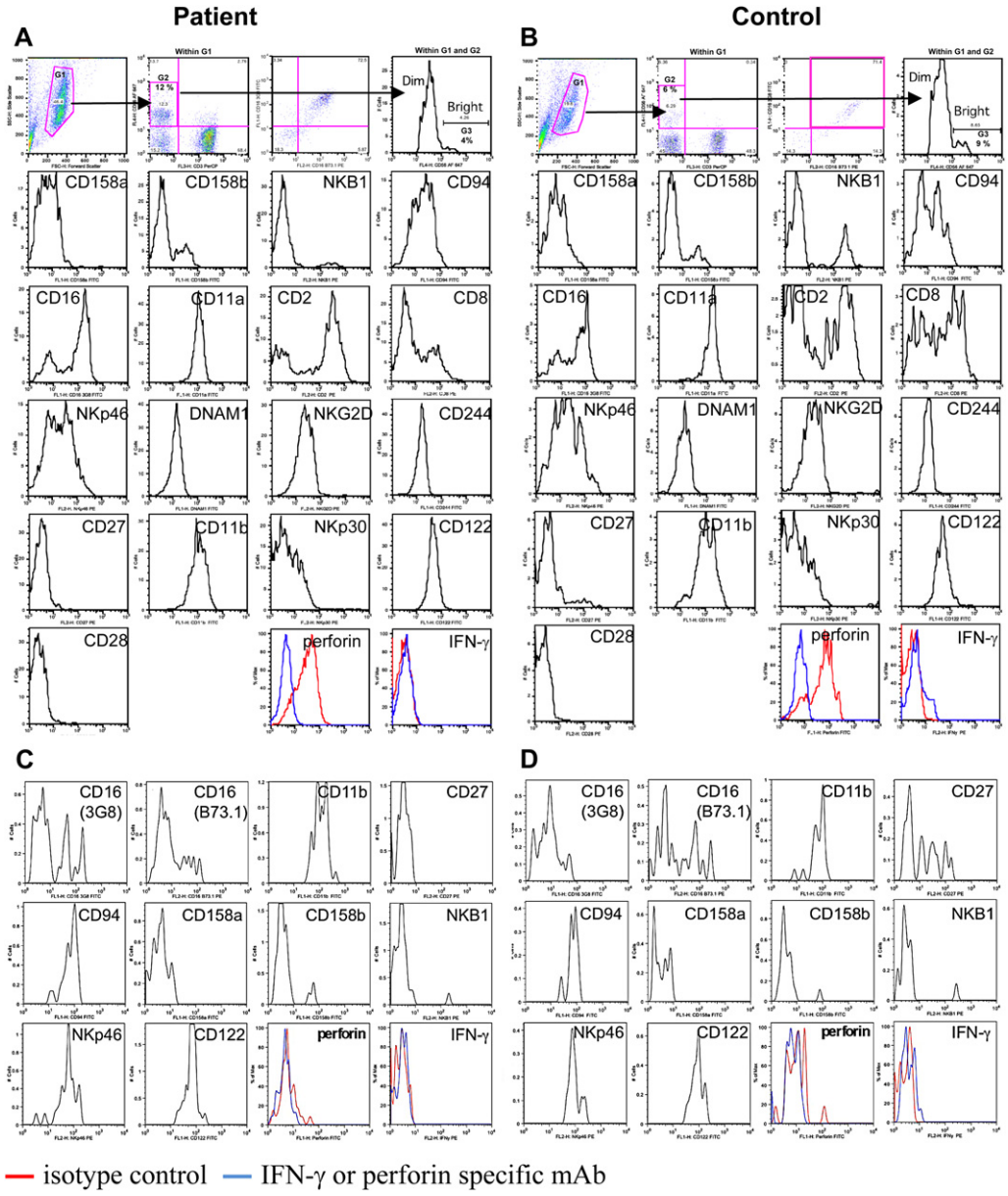

FIG E2. NK cell subset analysis demonstrates appropriate developmental and functional NK cell populations in the index patient's PBMCs. Evaluation of the NK cell subsets in the patient's (**A** and **C**) and control subjects' (**B** and **D**) PBMCs demonstrating populations present among total ($CD56^{+}CD3^{-}$; Fig E2, *A* and *B*) and $CD56^{bright}$ ($CD56^{++}CD3^{-}$; Fig E2, *C* and *D*) NK cells. *Arrows* depict the gating strategy starting at the side scatter/forward scatter plot.

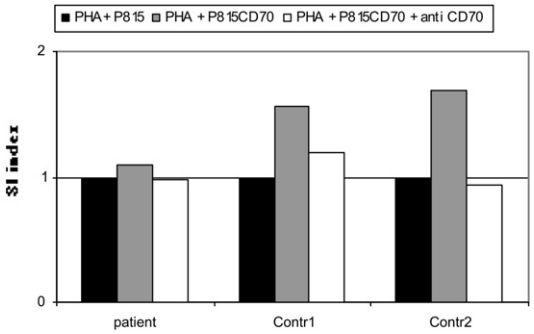

FIG E3. Absence of costimulation through the CD27 ligand CD70 of the index patient's T cells. Purified $CD4^+$ T cells from the index patient and 2 healthy control subjects were stimulated with PHA and mock transfectants *(black bar)*, CD70 transfectants *(gray bar)*, or CD70 transfectants and blocking CD70 mAb. Proliferation was measured based on tritiated thymidine incorporation and is depicted as the stimulation index (ratio [median cpm situation X]/median cpm + P815).

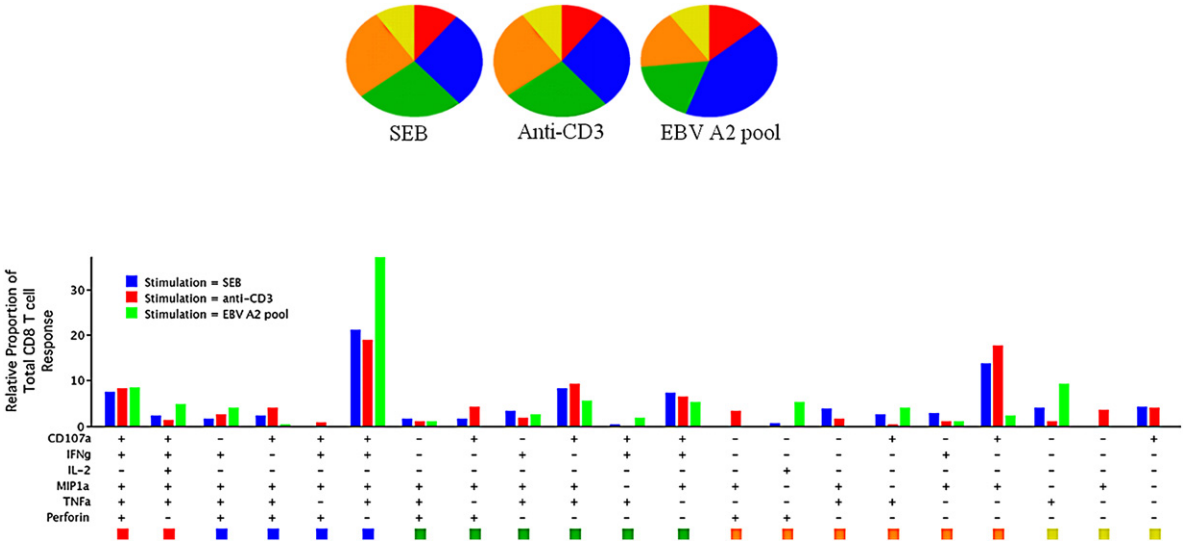

FIG E4. Presence of polyfunctional EBV-specific T cells in the index patient. PBMCs were stimulated, and functional profiles were measured. The *pie plots* depict the fraction of penta *(red)*, tetra *(blue)*, tri *(green)*, bi *(orange)*, and mono *(yellow)* functional cells of the $CD8^+$ T cells. The *bar graphs* represent the relative proportion of the $CD8^+$ T cells having the indicated functions after the different stimulations.[E2] *MIP1a*, Macrophage inflammatory protein 1α; *SEB*, staphylococcal enterotoxin B.

TABLE E1. Antibodies used in analysis of lymphocytes

Antibody specificity	Clone	Conjugation	Manufacturer
BCL2		Unlabeled	Dakopatts, Glostrup, Denmark
BCL6		Unlabeled	Novacastro, Bannockburn, Ill
CCR7	TG8	PerCP/Cy5.5	BioLegend, San Diego, Calif
		Alexa 750	BD Biosciences, San Jose, Calif
CD2	RPA2.10	PE	BD Biosciences
CD3	SK7	PerCP	BD Biosciences
		Qdot 585	Custom
CD4		PE Cy5-5	Invitrogen, Carlsbad, Calif
CD5		Unlabeled	Novacastra
CD8	HIT8a	PE	BD Biosciences
		Qdot605	Invitrogen
CD10	HI10A	PE	BD Biosciences
CD11a	HI111	FITC	BD Biosciences
CD11b	ICRF44	Biotinylated	BD Biosciences
CD14		PB	BioLegend
CD16	B73.1	PE	BD Biosciences
CD16	3G8	FITC	BD Biosciences
CD19	SJ25C1	APC	BD Biosciences
		PB	BioLegend
CD20	L27	FITC	BD Biosciences
		Unlabeled	Dakopatts
CD21	Leu-14	PE	BD Biosciences
CD23		Unlabeled	Novacastra
CD27	M-T271	PE	BD Biosciences
	L128	APC	BD Biosciences
	LG3A10*	FITC	BD Biosciences
		Qdot 665	Invitrogen
	CD27-1	Unlabeled	Sanquin, Amsterdam, The Netherlands
	CD27-2	Unlabeled	Sanquin
		Unlabeled	Novacastra
CD28	CD28.2	PE	BD Biosciences
CD34	8G12	APC	BD Biosciences
CD38	HB7	FITC	BD Biosciences
CD45	H130	FITC	BD Biosciences
	H130	PE	BD Biosciences
	2D1	PerCP	BD Biosciences
	H130	AF647	BioLegend
CD45RA		PE	BD Biosciences
CD45RO		ECD	Beckman Coulter, Fullerton, Calif
CD56	B159	AF647	BD Biosciences
CD57		Qdot 565	Custom
CD62 ligand		FITC	BD Biosciences
CD79a		Unlabeled	Dakopatts
CD94	HP-3D9	FITC	BD Biosciences
CD107-a		PeCy5	BD Biosciences
CD122	Mlk-1	Biotinylated	BD Biosciences
CD127		FITC	BD Biosciences
CD138	MI15	PE	BD Biosciences
		Unlabeled	Dakopatts
CD158a	HP-3E4	FITC	BD Biosciences
CD158b	CH-L	FITC	BD Biosciences
CD226	DX11	FITC	BD Biosciences
CD244	Feb-69	FITC	BD Biosciences
Mib-1		unlabeled	Dakopatts
MIP-1α		FITC	R&D Systems, Minneapolis, Minn
Kappa Lambda		Unlabeled	Dakopatts
IFN-γ	B27	PE	BD Biosciences
		Alexa 700	BD Biosciences
IL-2		APC	BD Biosciences
IgD	Goat polyclonal ($F[ab']_2$)	PE	SBA, Birmingham, Ala
IgG	Goat polyclonal ($F[ab']_2$)	FITC	SBA
IgM	Goat polyclonal ($F[ab']_2$)	FITC	SBA
NKp30	210845	OE	R&D Systems
NKp46	BAB281	PE	Beckman Coulter
NKG2D	149810	PE	R&D Systems
NKB1	DX9	PE	BD Biosciences
Perforin	dG9	FITC	BD Biosciences
		PE	Tepnel, San Diego, Calif
TdT	HT-6	FITC	Dako, Glostrup, Denmark
TNF-α		PECy7	BD Biosciences

APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *MIP-1α*, macrophage inflammatory protein 1α; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex; *TdT*, Terminal deoxynucleotidyltransferase.
*Anti-mouse CD27 mAb that cross reacts with human CD27.