Primary T-cell immunodeficiency with functional revertant somatic mosaicism in *CD247*

To the Editor:

T lymphocytes detect antigens with the T-cell receptor (TCR) composed of a variable heterodimer (either αβ or γδ), 2 invariant heterodimers (CD3γε and CD3δε), and an invariant homodimer (CD247 or ζζ).[1] Because of the crucial role of TCR signaling in thymic selection, mutations in *TCR*, *CD3*, or *CD247* selectively impair T-cell development, albeit to different degrees: deficiency of CD3δ or CD3ε, but not of CD3γ or CD247, causes severe T-cell lymphopenia. Their clinical outcome is also disparate, because CD3γ deficiency does not require urgent transplantation. Thus, TCR immunodeficiencies display a range of phenotypes and careful differential diagnosis is essential for appropriate therapy.

We describe an infant born to consanguineous parents with early-onset chronic cytomegalovirus infection, severe immunodeficiency, and extremely low surface TCR levels. Her immunologic characterization at age 11 months is summarized in Table E1 in this article's Online Repository at www.jacionline.org. Briefly, she showed low T- and B-cell counts, selective severe $CD4^+$ T-cell lymphopenia (Fig 1, *A*), low recent thymic emigrants, and naive T cells and poor TCRVβ repertoire, all suggestive of a defect in T-cell development despite a normal-sized thymus (see Fig E1 in this article's Online Repository at www.jacionline.org).

Surface TCR expression was markedly reduced in both αβ and γδ T cells (Fig 1, *B*), suggesting a defect in an invariant chain of the TCR complex. Intracellular (i) flow cytometry showed normal CD3γ, δ, and ε expression but almost absolute absence of CD247 (Fig 1, *C*). cDNA sequencing revealed a homozygous T-to-C mutation at position +2 of exon 1 of *CD247* as reported recently[2] (NCBI/ClinVar: rs672601318), which causes loss of the initiation codon and therefore prevents translation. The patient's parents and 4 additional family members were asymptomatic but heterozygous for the mutation (see Fig E2 in this article's Online Repository at www.jacionline.org). Interestingly, surface TCR expression in mutation carriers was reduced 2-fold (Fig 1, *B*), revealing a clear correlation between surface TCR and CD247 genotype, which was useful for diagnosis and genetic counseling.

Notably, a few T cells in the patient expressed surface TCR levels (Fig 1, *A*) comparable to those of carriers ($CD3\varepsilon^{high}$), which

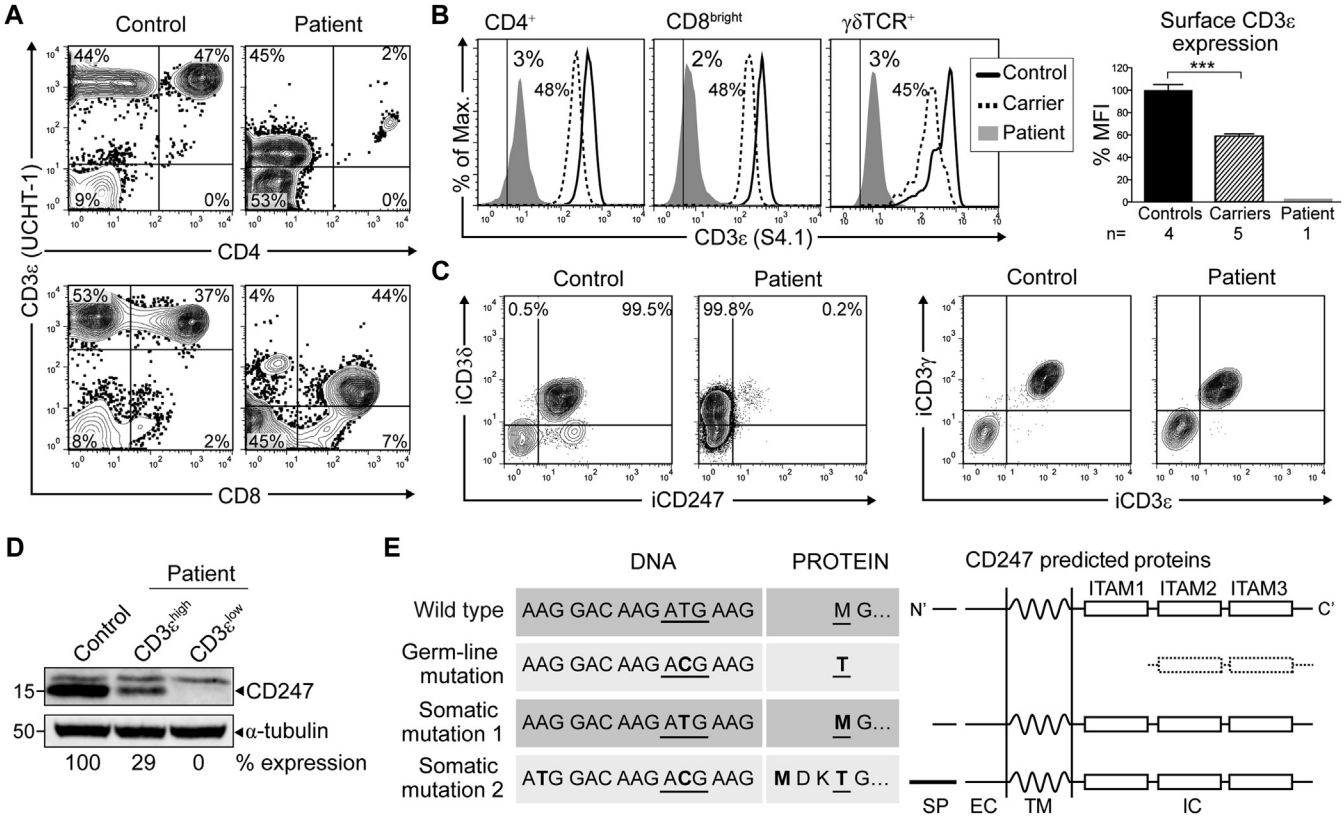

FIG 1. Patient characterization. **A,** Peripheral blood phenotype. **B,** Surface CD3ε expression in the indicated subsets. Numbers indicate % MFI relative to controls. **C,** Intracellular (i) expression of the indicated CD. Numbers indicate % $iCD247^-$ or $iCD247^+$ in $iCD3\delta^+$ (T) cells. **D,** CD247 WB of sorted T cells. Numbers indicate % normalized band intensity relative to control. **E,** Mutations and predicted proteins. *EC*, Extracellular; *IC*, intracellular; *MFI*, mean fluorescence intensity; *SP*, signal peptide; *TM*, transmembrane; *WB*, Western blot. ***$P \leq .001$.

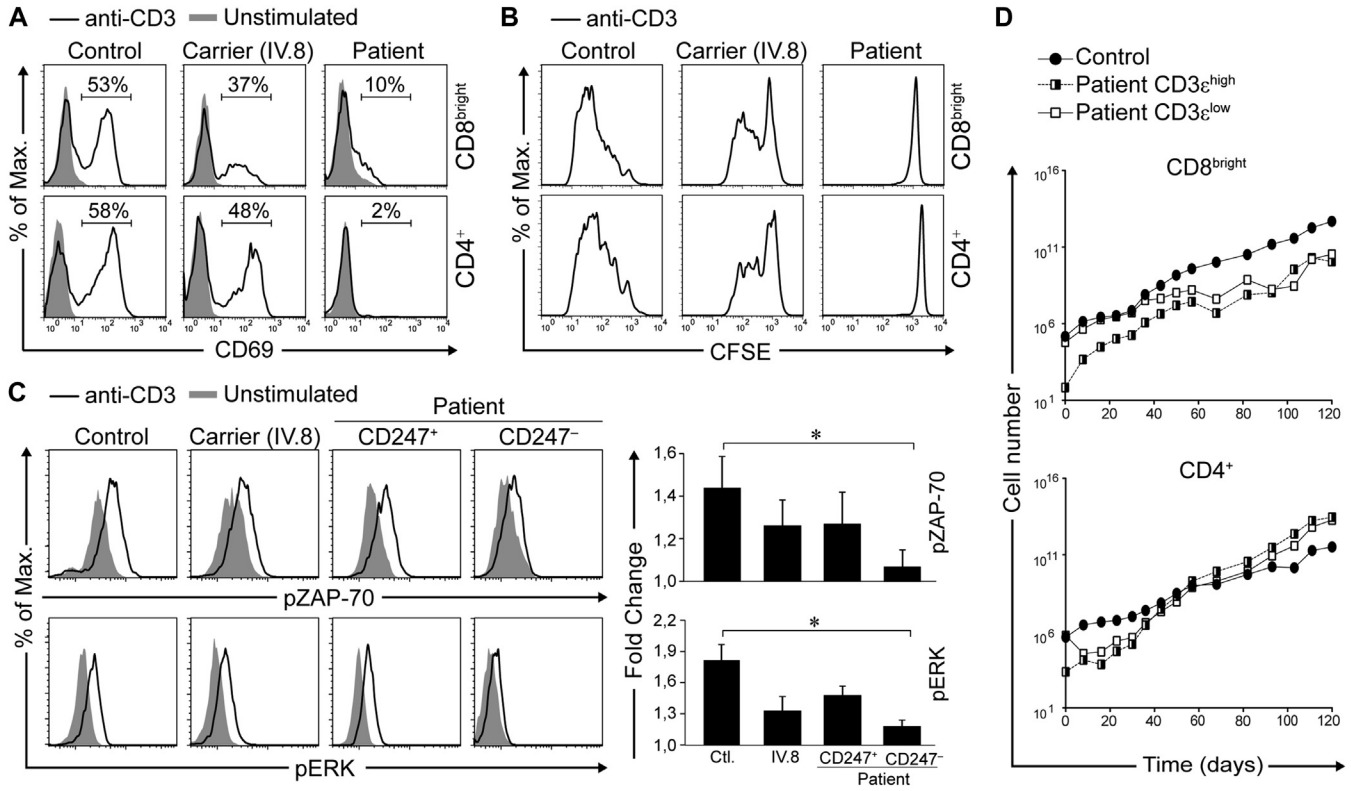

FIG 2. Impaired TCR-induced signaling in primary (**A** and **B**) and cultured T cells (**C** and **D**). % $CD69^+$ cells (Fig 2, *A*) or CFSE dilution (Fig 2, *B*) after stimulation with anti-CD3ε for 24 hours or 5 days, respectively. **C,** Representative pZAP-70 and pERK levels after stimulation with anti-CD3ε *(left)* and MFI + SEM relative to unstimulated cells of 4 independent experiments *(right).* **D,** T-cell growth in allogeneic cultures. *CFSE,* Carboxyfluorescein diacetate succinimidyl ester; *pERK,* phospho-extracellular signal–regulated kinase. $*P \leq .05$.

correlated with the rare $CD247^+$ T-cell subset (0.2%, Fig 1, *C*). To confirm this correlation, T cells from the patient were cultured in allogeneic cultures, where $CD3\varepsilon^{high}$ T cells became prominent (see Fig E3 in this article's Online Repository at www.jacionline.org). Fluorescence-activated cell sorting and CD247 Western blot analysis confirmed that the patient's $CD3\varepsilon^{high}$ T cells had recovered CD247 expression (Fig 1, *D*). RNA sequencing of $CD3\varepsilon^{high}$ T cells revealed 2 independent somatic mutations at or near the germline mutation: a reversion (c.2T>C>T) and a second-site mutation (c.-8A>T) that generates an alternative in-frame initiation codon, 3 codons upstream of the original ATG (Fig 1, *E*). Given the low frequency of revertant T cells *in vivo*, it seems improbable that a single cell would carry both somatic mutations.

Collectively, these results show that a very small percentage of the patient's T cells had undergone somatic mutations able to revert the inherited mutation, allowing CD247 protein synthesis and thus higher surface TCR expression. Revertant T cells showed diversity in TCRCβ1, CD4, and CD8 expression (data not shown), suggesting that the reversion events occurred early in development.

CD69 upregulation and short-term proliferation of primary T cells after anti-CD3 antibody stimulation was impaired in the patient and reduced in carriers (Fig 2, *A* and *B*). ZAP-70 and extracellular signal-regulated kinase (ERK) phosphorylation was also impaired in CD247-deficient T cells, whereas revertant T cells displayed carrier phosphorylation levels (Fig 2, *C*). These results indicate that the reversions could partially rescue TCR signaling *in vitro*. In contrast, the patient's T cells (both $CD3\varepsilon^{low}$ and $CD3\varepsilon^{high}$) readily proliferated when cultured with allogeneic cells and IL-2 (Fig 2, *D*), suggesting that their TCR signaling defect could be overcome if a long-term TCR stimulus together with continuous IL-2 supply were present. This is in line with the *in vivo* expansion of the patient's $CD8^+$ T cells being driven by chronic cytomegalovirus infection, which, in turn, would explain their exhaustion and reduced proliferative response *in vitro* compared with their $CD4^+$ counterparts. Revertant T cells were capable of expansion *in vitro* (Fig 2, *D*) and also, but less efficiently, *in vivo* (data not shown), where they did not suffice to repopulate the T-cell compartment.

The immunologic phenotype of this new patient resembled that of 2 other reported cases of CD247 deficiency (see Table E2 and Fig E4 in this article's Online Repository at www.jacionline.org). In both cases, $CD3\varepsilon^{low}$ and $CD3\varepsilon^{high}$ T cells were also identified. The first study[3] reported 3 second-site somatic mutations in *CD247* that partially rescued TCR expression but not function, as measured solely by anti-CD3–induced ZAP-70 phosphorylation. No molecular analysis for somatic mutations was reported for the second patient.[4] Thus, the presence of revertants along with strongly reduced surface TCR expression is pathognomonic of CD247 deficiency.[5,6]

The new case described here showed complete CD247 protein deficiency due to loss of the initiation codon. The disorder was

associated with strongly reduced surface TCR and multiple developmental and functional T-cell derangements, suggesting that, as observed in mice,[7] CD247 plays a critical role for T-cell selection in the thymus. Yet partial TCR complexes lacking CD247 can signal to some extent for selection. The somatic mutations rescued surface TCR expression as well as proximal and distal TCR-dependent signal transduction, expectedly reaching only mutation carriers' values.

In conclusion, mild lymphopenia and functional revertant somatic mosaicism should not confound the fact that CD247 deficiency is a very severe condition that requires urgent transplantation, but easy to diagnose by intracellular flow cytometry or by the surface TCR phenotype of obligate carriers.

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Early and late B-cell developmental impairment in nuclear factor kappa B, subunit 1–mutated common variable immunodeficiency disease

To the Editor:

The nuclear factor kappa B (NF-kB) signaling pathway plays an important role in immune cell biology.[1] Both the classical (nuclear factor kappa B, subunit 1 [NF-kB1]; p105/p50) and the alternative (nuclear factor kappa B, subunit 2 [NF-kB2]; p100/p52) NF-kB pathways have been largely studied mainly in animal models.[1] Regarding B cells, the role of NF-kB1 was underlined in a murine nf-kb1 knockout model in which peripheral B cells showed defective maturation, defective isotype switching, and impaired humoral immune responses.[1-3] A similar, although more pronounced, immunologic phenotype was observed in the nf-kb2 knockout mice, with defective secondary lymphoid organ development and impaired B-cell development both in early (bone marrow) and in late (periphery) stages with defective humoral responses both to T-dependent and to T-independent antigens.[1] The role of NF-kB2 in human B-cell development was recently defined in patients carrying monoallelic mutations in NF-kB2, leading to common variable immunodeficiency (CVID)-like disease with autoimmunity and defects in late stages of peripheral B-cell maturation.[4,5] Monoallelic mutations in NF-kB1 leading to p50 haploinsufficiency were recently described in a limited number of patients with CVID[6]; however, data regarding the effect of monoallelic NF-kB1 mutations on B-cell development are scarce. We report on 2 patients carrying monoallelic mutations in NF-kB1, one of

METHODS

Case report

The case has been reported in full previously, in connection with her natural killer (NK)-cell dysfunctions in Valés-Gómez et al.[E1] In addition, and because of the association of reduced CD247 expression with several autoimmune disorders, we analyzed serum samples from the patient and several carriers (IV.1, IV.2, IV.5, IV.7, IV.8, IV.10, IV.11, IV.14, IV.15, IV.16) to determine antinuclear antibodies, thyroid autoantibodies (antithyroglobulin and antithyroperoxidase), antibodies against surface antigens, and intracytoplasmic myeloperoxidase and proteinase 3 in neutrophils, which were all found to be negative. Thus, carriers do not seem to be at risk of autoimmunity, despite their slightly impaired TCR functionality. However, the patient showed several positive direct Coombs tests results (November 2013 to June 2014), which became negative after lymphoid engraftment, thus compatible with asymptomatic subtle hemolytic anemia possibly related to the disease itself or to the chronic cytomegalovirus infection. The study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Institutional Research Ethics Committees of the hospitals involved. All participants or their guardians provided informed consent for the collection of samples and subsequent analyses.

PBMC isolation and cell culture

PBMCs from the patient, her family, and healthy controls (age-matched whenever possible) were isolated by centrifugation on a Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, United Kingdom) gradient. Polyclonal T-cell lines were generated by stimulation at day 0 with 1 μg/mL PHA (Sigma-Aldrich, St Louis, Mo), and coculture with irradiated allogeneic feeder cells weekly (PBMC and EBV-transformed B cells, 40 and 65 Gy, respectively) at 1:2 ratio in Iscove's Modified Dulbecco's Medium (IMDM) (GE Healthcare) supplemented with 40 IU/mL recombinant human IL-2 (provided by Craig W. Reynolds, Frederick Cancer Research and Development Center, National Cancer Institute, National Institutes of Health, Frederick, Md), 10% AB^+ human serum, and 1% L-glutamine and Antibiotic-Antimycotic (Life Technologies, Carlsbad, Calif). Cell growth was calculated weekly as the ratio of recovered versus seeded cells, and long-term growth plots were estimated as projections thereof.

Immunophenotype

Multiparametric flow cytometry was performed with mAbs against CD3ε (UCHT-1 and S4.1), CD4 (13B8.2), CD45RA (ALB11), and CD45RO (UCHL-1) from Beckman Coulter (Brea, Calif); CD3δ (EP4426), CD3γ (EPR4517), and CD3ε (EPR5361(2)) from Abcam (Cambridge, United Kingdom); CD247 (6B10.2) from Biolegend (San Diego, Calif); CD247 (H146-968) from Thermo Fisher Scientific (Waltham, Mass); αβTCR (BMA 031) from Miltenyi Biotec (Bergisch Gladbach, Germany); and γδTCR (11F2), CD31 (WM59), CD27 (M-T271), CD56 (B159), and CD8 (RPA-T8) from BD Biosciences (San Jose, Calif). For unlabeled antibodies, an additional step with phycoerythrin-conjugated anti-mouse IgG (H + L) from Beckman Coulter or anti-rabbit IgG (H+L) from Life Technologies was performed. For intracellular staining, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin. Data were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

T-cell function

To analyze CD69 induction after TCR engagement, 0.2×10^6 PBMCs were plated in flat-bottom 96-well plates and stimulated for 24 hours with 10 μg/mL of plastic-coated anti-CD3ε mAb (UCHT-1 from BD Biosciences). CD69 induction was analyzed by flow cytometry with anti-CD69 (L-78 from BD Biosciences). Proliferation was measured by dilution of the cell tracer carboxyfluorescein diacetate succinimidyl ester (Sigma Aldrich). Briefly, cells were stained with 1 μM carboxyfluorescein diacetate succinimidyl ester and stimulated with 1 μg/mL UCHT-1 (eBioscience, San Diego, Calif) for 5 days. Phosphorylation of ZAP-70 and extracellular signal-regulated kinase was determined by intracellular flow cytometry after stimulation of 0.3×10^6 cultured cells with 20 μg/mL anti-CD3ε mAb (OKT3 from eBioscience) at 4°C for 30 minutes cross-linked with 10 μg/mL goat $F(ab')_2$ anti-mouse immunoglobulin (H + L) (Beckman Coulter) at 37 °C for 10 minutes. Phosphorylated (p) proteins were detected by intracellular flow cytometry with rabbit antibodies against pERK (Thr202/Tyr204) and pZAP-70 (Tyr319)/pSyk (Tyr352) from Cell Signaling (Danvers, Mass). A second step with phycoerythrin-labeled anti-rabbit antibody (Life Technologies) was performed.

TCRβ clonality

Clonality at the TCRβ locus was studied using a commercial kit (Master Diagnostica, Granada, Spain, EC-certified for clinical use), which amplifies genomic *TCR VβJβ* rearrangements using 2 specific primers for conserved V- and J-flanking regions. Polyclonal (healthy donor) control DNA was included for reference. Amplimers were separated and analyzed in an ABI Prism Genetic Analyzer 3110 using GeneMapper V 4.0 from Applied Biosystems (Foster City, Calif).

CD3 and *CD247* sequence analysis

Genomic DNA and RNA were obtained from peripheral blood, cultured T cells, or sorted $CD3\varepsilon^{high}$-expressing cells. Primers for *CD3G* and *CD3E* and *CD3D* have previously been described in Recio et al[E2] and Gil et al.[E3] *CD247* cDNA was amplified using specific exon 1–flanking primers (Forward: 5′ ACACCCCAAACCCTCAAACCTC 3′; Reverse: 5′ AGGAGGGCAGGA TTTGAAGGAG 3′) and PCR products were sequenced. For *CD247* cloning, cDNA was amplified by PCR using specific primers (Forward: 5′ GGAGA TCTCCACAGTCCTCCACTTCCTG 3′; Reverse: 5′ GATCCGCGGCCGCA TAGGAAGGCTTTAGCATGCC 3′). DNA fragments were cloned into pJET 1.2 plasmid (CloneJET PCR Cloning Kit, Life Technologies) and transformed into DH5α *Escherichia coli* strain. Colonies containing recombinant plasmids were also sequenced. *CD247* haplotypes were determined by analysis of 4 Sequence Tag Sites in the genetic interval that contains the *CD247* gene on chromosome 1q24.2, essentially as described in Recio et al.[E2]

Western blot

Cells were lysed in buffer containing 0.5% Brij96v; 50 μg of cell lysate was resolved by SDS-PAGE, transferred into polyvinylidene fluoride membranes, and developed with anti–α-tubulin (B5-1-2 clone, Sigma Aldrich) and the rabbit anti-CD247 448 antiserum (specific for the last 34 amino acids of its C-terminal region), kindly provided by Balbino Alarcón, Centro de Biología Molecular Severo Ochoa, UAM-CSIC, Madrid, Spain, and previously described in San Jose et al.[E4] Blots were visualized using an Odyssey infrared imaging system and quantified using Image Studio software (both from LI-COR Biosciences, Lincoln, Neb).

Statistical analysis

To assess statistical significance, the 2-tailed Student *t* test or 1-way ANOVA with Bonferroni multiple comparison test was performed (*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$). Error bars represent SEM.

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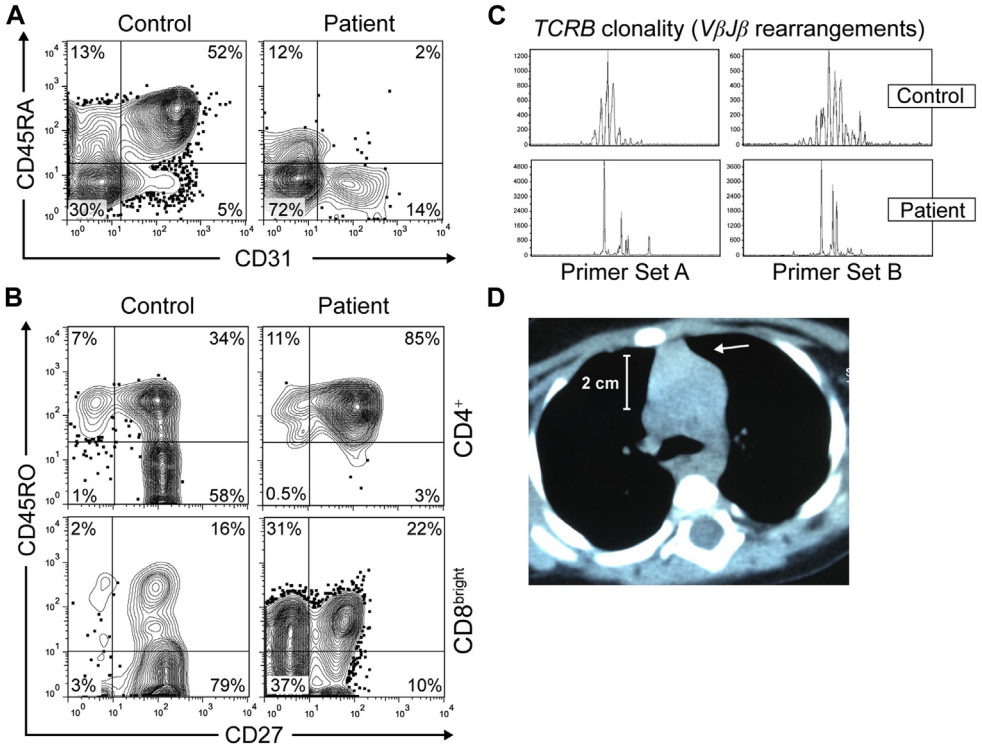

FIG E1. Patient characterization. **A,** Reduced % of recent thymic emigrants defined as $CD4^+$ $CD45RA^+$ $CD31^+$ lymphocytes (age-matched control range,[E5] 77%-96%). **B,** Reduced naive T-cell compartment. T-cell maturation stages in $CD4^+$ and $CD8^{bright}$ cells were defined as naive ($CD45RO^-CD27^+$), central memory ($CD45RO^+CD27^+$), effector memory ($CD45RO^+CD27^-$), or effector ($CD45RO^-CD27^-$). Control in Fig E1, *A* and *B*, was a 20-year-old healthy donor. **C,** Reduced TCRβ clonality in PBMCs. **D,** Chest computed axial tomography showing normal-sized thymus.

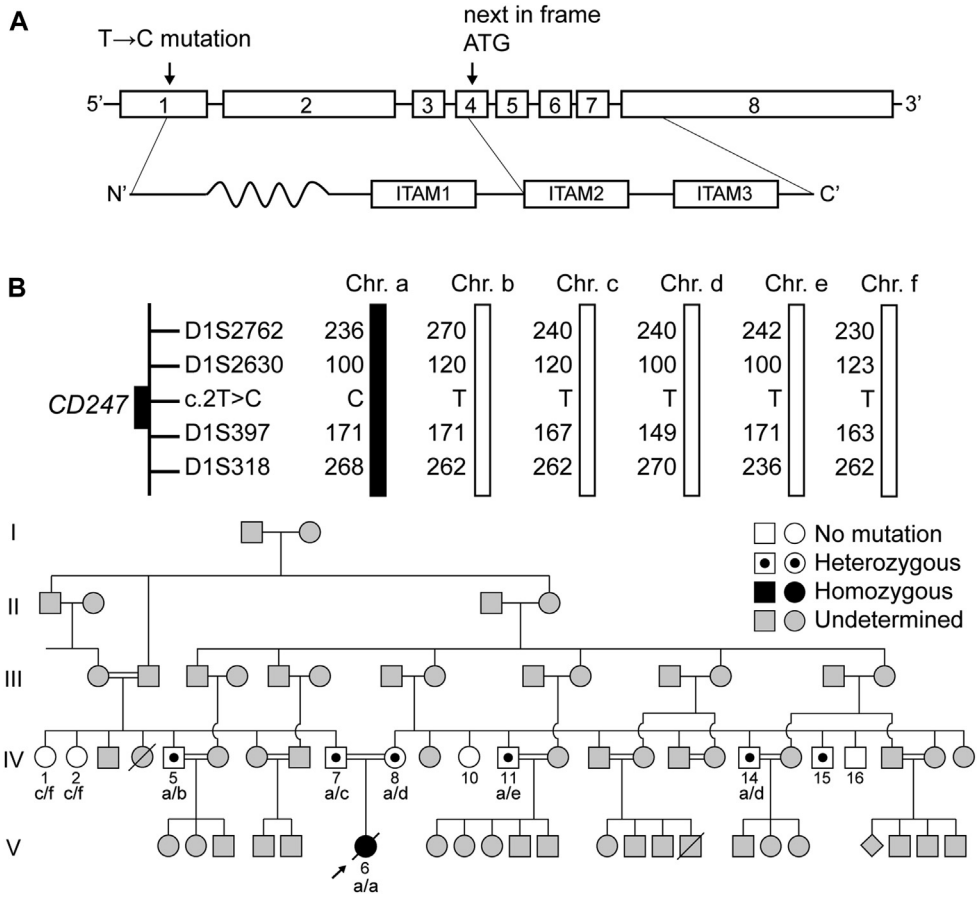

FIG E2. CD247 deficiency due to a homozygous mutation. **A,** CD247 gene and protein, including the germline mutation (NCBI/ClinVar: rs672601318, NM_000734.3:c.2T>C and NG_007384.1:g.5146T>C in exon 1) and the next in-frame ATG. **B,** *Top, CD247* mutation and haplotypes based on the indicated microsatellite markers (*black* = disease-associated chromosome). *Bottom*, pedigree with haplotypes for studied individuals. *Circles, squares*, and *diamonds* indicate females, males, or unknown sex, respectively.

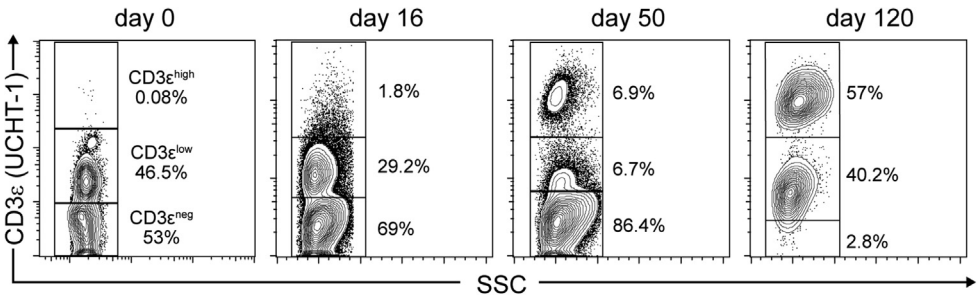

FIG E3. *In vitro* T-cell growth and phenotype. Surface CD3ε expression in cultured T cells with gates and % for $CD3\varepsilon^{neg}$, $CD3\varepsilon^{low}$, and $CD3\varepsilon^{high}$ subpopulations at different time points. *SSC*, Side scatter.

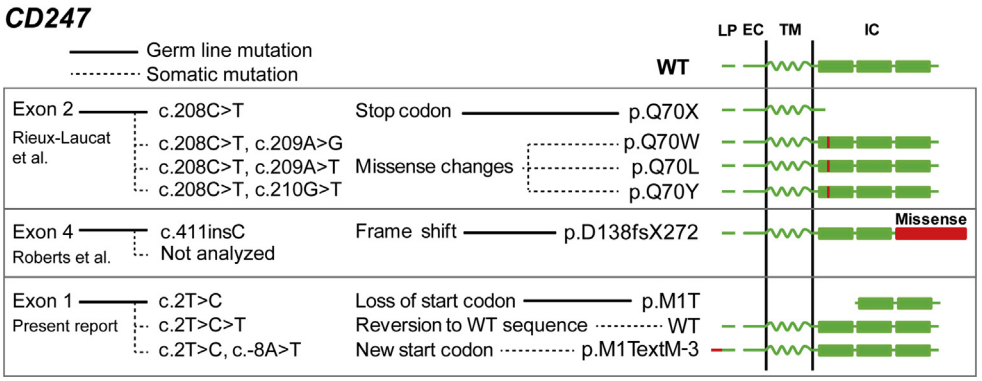

FIG E4. Summary of *CD247* germline and somatic mutations. Molecular basis of the 3 reported CD247-deficient patients, 1 patient/box: Exon 2,[E6] Exon 4,[E7] and Exon 1 (present report). *EC*, Extracellular; *IC*, intracellular; *LP*, leader peptide; *TM*, transmembrane; *WT*, wild-type.

TABLE E1. Immunologic phenotype

Parameter	Patient (11 mo)		Normal range (11-15 mo)
Lymphocytes (cells/mm^3), (%)	3,000		3,200-12,300
	CD3ε^{low}	CD3ε^{high}	
T (CD3$^+$)	**1315** (**44**)	**5** (**0.16**)	2,400-8,300 (56-87)
CD4$^+$	**66** (**5**)	**3** (57)	1,300-7,100 (25-86)
CD8$^+$	1183 (**90**)	**2** (29)	400-4,100 (7-58)
DN (CD4$^-$CD8$^-$)	66 (**5**)	**0.7** (**14**)	12-140 (0.42-2)
TCRγδ$^+$	**7** (**0.5**)	**0** (**0**)	70-630 (1-10)
CD4/CD8 ratio	**0.06**	**1.5**	1.7-3.2
B (CD19$^+$)*	**75** (3)		110-7,700 (3-77)
CD27$^-$ IgD$^+$	**68** (91)		100-7,407 (91-96)
CD27$^+$ IgD$^+$	**0.8** (**1**)		2-316 (1.6-4.1)
CD27$^+$ IgD$^-$	1.5 (**2**)		0-154 (0.1-1.9)
CD27$^-$ IgD$^-$	4.5 (**6**)		1-185 (0.9-2.1)
NK (CD56$^+$ CD16$^+$)	1,020 (34)		71-3,500 (1-64)
Neutrophils (cell/mm^3)	1,500		1,500-8,000
Eosinophils* (cell/mm^3)	200		40-800
Serum Ig (mg/dL)			
IgG	**1,260**		421-1,100
IgA	40		18-154
IgM	138		24-180

Boldface values indicate out of range.
*B cells and eosinophils were determined at 13 and 15 months, respectively. Normal range updated from Ikinciogullari et al,[E8] Schatorje et al,[E5] Morbach et al,[E9] and Aksu et al.[E10]

TABLE E2. Comparative clinical and immunologic features of CD247-deficient patients

Feature	Present report	Rieux-Laucat et al[E6]	Roberts et al[E7]
Sex	Female	Male	Female
Onset	2 mo	4 mo	4 mo
Age at diagnosis (mo)	11	10	11
HSCT (mo)	Haploidentical (19)	Haploidentical (30)	Haploidentical (12, 16)
Present age	33 mo†	13 y*	4 y*†
Cytomegalovirus	+	−	+
Germline mutation			
Type	Truncation	Truncation	Insertion
cDNA	c.2T>C	c.208C>T	c.411insC
Protein	p.M1T	p.Q70X	p.D138fsX272
Serum immunoglobulin levels	High (IgG)	High (IgG, -A, -M, -E)	High (IgG,‡ -A, -M)
T-cell lymphopenia	Mild	Mild	Mild
$CD3\varepsilon^{high}$			
% of T cells	0.36	10	0.6
Lineage	CD4 and CD8	CD4	CD4 and CD8
Somatic mutations	Yes (2)	Yes (3)	NA
Revertant	Yes	No	NA
Second site	Yes	Yes	NA
Functional	Yes (= carriers)	No	NA
$CD3\varepsilon^{low}$			
% of T cells	99.6	90.0	99.4
CD4/CD8 ratio	Inverted (0.06)	Inverted (0.23)	Inverted (0.85)
Functional	No	No	No

IVIG, Intravenous immunoglobulin; *NA*, not analyzed.

*Alain Fischer, Paris Descartes University, and Rebecca Buckley and Joseph Roberts, Duke University (personal communication, 2015).

†Exitus at.

‡Receiving IVIG.