A leaky mutation in *CD3D* differentially affects αβ and γδ T cells and leads to a Tαβ⁻Tγδ⁺B⁺NK⁺ human SCID

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T cells recognize antigens via their cell surface TCR and are classified as either αβ or γδ depending on the variable chains in their TCR, α and β or γ and δ, respectively. Both αβ and γδ TCRs also contain several invariant chains, including CD3δ, which support surface TCR expression and transduce the TCR signal. Mutations in variable chains would be expected to affect a single T cell lineage, while mutations in the invariant chains would affect all T cells. Consistent with this, all CD3δ-deficient patients described to date showed a complete block in T cell development. However, CD3δ-KO mice have an αβ T cell–specific defect. Here, we report 2 unrelated cases of SCID with a selective block in αβ but not in γδ T cell development, associated with a new splicing mutation in the *CD3D* gene. The patients' T cells showed reduced *CD3D* transcripts, CD3δ proteins, surface TCR, and early TCR signaling. Their lymph nodes showed severe T cell depletion, recent thymus emigrants in peripheral blood were strongly decreased, and the scant αβ T cells were oligoclonal. T cell–dependent B cell functions were also impaired, despite the presence of normal B cell numbers. Strikingly, despite the specific loss of αβ T cells, surface TCR expression was more reduced in γδ than in αβ T cells. Analysis of individuals with this *CD3D* mutation thus demonstrates the contrasting CD3δ requirements for αβ versus γδ T cell development and TCR expression in humans and highlights the diagnostic and clinical relevance of studying both TCR isotypes when a T cell defect is suspected.

Introduction

T lymphocytes recognize antigens by means of a cell surface complex termed the TCR. The TCR contains 2 variable chains to bind antigens and several invariant chains to support variable chains and to transduce the signals required for T cell differentiation and antigen recognition (1). The invariant chains present in the human TCR are CD3γ, CD3δ, CD3ε, and TCRζ (CD247) (Figure 1A). T lymphocytes belong to either the αβ or the γδ lineage according to the types of variable chains incorporated into their TCR, α and β or γ and δ, respectively.

Mutations in *TCR* or *CD3* genes selectively impair T cell development (2). Unless hematopoietic stem cells are replaced, the mutations frequently cause early-onset SCID and death. When a variable chain such as TCRα is affected, only αβ T cells are impaired, as shown recently (3). When an invariant chain is affected, both αβ and γδ T cells are either absent, as observed in CD3δ or CD3ε deficiency (4, 5), or reduced, as reported for CD3γ or TCRζ deficiency (6, 7). These immunophenotypes are described as $T^-B^+NK^+$ or $T^{\pm}B^+NK^+$, respectively. However, no selective αβ or γδ T cell–deficient patients have been reported for invariant TCR chain deficiencies. We describe 2 SCID cases with severe selective αβ T lymphopenia ($T\alpha\beta^-T\gamma\delta^+B^+NK^+$) caused by a leaky mutation in *CD3D* that titrated the differential CD3δ requirements for human αβ and γδ T lymphocyte development and TCR expression in vivo.

Results and Discussion

Case reports. Two unrelated children from nonconsanguineous Ecuadorian parents showed common clinical and immunophenotypic features. They presented at 13 (AIII.1) and 5 (BII.2) months of age with SCID features, $T^{\pm}B^+NK^+$ phenotype, low CD3 expression, strongly impaired proliferative responses to T cell mitogens (Table 1), severe lymph node T cell depletion, and lack of activated germinal centers. Both received conditioning and haploidentical $CD34^+$ peripheral blood hematopoietic stem cell transplantation at 23 and 8 months, respectively.

Patient AIII.1 was admitted with failure to thrive, bronchopneumonia, severe diarrhea caused by strains of *Salmonella*, *Campylobacter*, and *Cryptosporidium*, oral candidiasis, and atopic dermatitis. CMV and EBV were negative by PCR. Neutrophil, lymphocyte, and platelet numbers and serum and urine biochemistry were normal, including Ig levels except IgE, which was strongly increased (Table 1). NK cell function was normal, whereas T cell–dependent B cell function was impaired. Specific Abs against protein antigens were not induced upon vaccination

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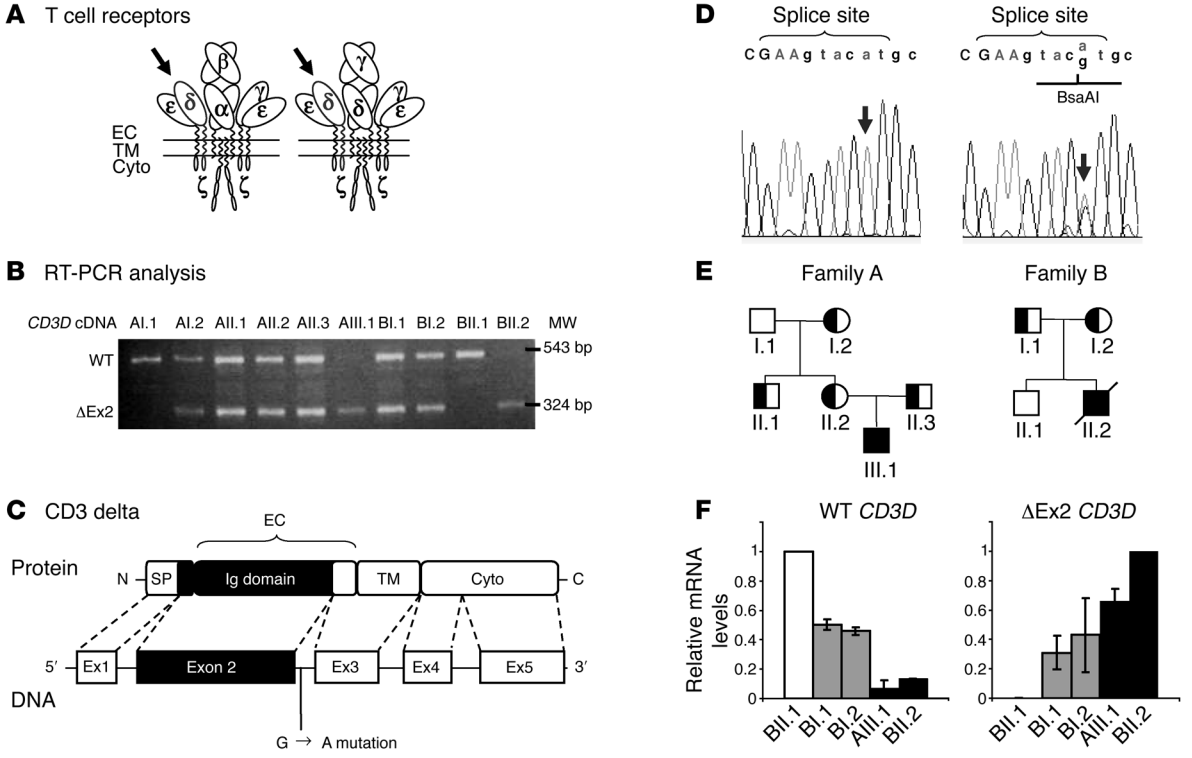

Figure 1

CD3D mutation analysis. (**A**) Structure of the 2 TCR that incorporate the CD3δ chain (arrows). EC, extracellular region; TM, transmembrane region; Cyto, cytoplasmic region. (**B**) *CD3D* RNA RT-PCR amplification products. ΔEx2 bands lack exon 2 (**C**) *CD3δ* protein and gene structure with localization of the G→A mutation. SP, signal peptide. (**D**) *CD3D* electropherograms showing the IVS2+5G→A mutation (arrow) in patient AIII.1 (left) and his father (right), and the BsaAI restriction site. Exon/intron sequences are in upper/lower case, respectively. (**E**) Genetic pedigrees. Circles indicate females; squares indicate males (slashes indicate deceased). Solid symbols denote homozygosity; half-solid symbols denote heterozygosity. (**F**) WT and ΔEx2 *CD3D* transcript levels relative to *CD3E* by quantitative RT-PCR in PBMCs using exon-specific primers and isoform-specific probes. Data represent mean ± SD of at least 2 experiments, relative to the highest value in each data set, which is shown as 1.

and were not detected against common pathogens or autoantigens, whereas natural (IgM) Abs against polysaccharides such as isoagglutinins and heterophile Abs were normal. The patient required total parenteral nutrition, specific antimicrobials, and i.v. Ig therapy. Absence epilepsy developed at 16 months. Sclerosing cholangitis was observed before receiving a maternal transplant. At 4 years of age, he is doing well, with mixed chimerism.

Patient BII.2 was admitted with fever, prostrating diarrhea, and respiratory distress. Urine CMV and nasal adenovirus, discrete lymphopenia, and severe hypogammaglobulinemia were observed. A protein-losing enteropathy was diagnosed, and he started on i.v. Ig and prophylactic Septrin, but developed several lymphadenopathies. After an initial improvement, respiratory function deteriorated and bronchoalveolar CMV was identified, which required antivirals. He underwent paternal transplantation with full chimerism in 3 weeks, but returned to the intensive care unit with respiratory distress, hemodynamic instability, encephalopathy, and liver failure with secondary coagulopathy, and died following multiorganic failure. Necropsy evidenced a rudimentary thymus with conserved reticular structure but complete depletion of lymphocytes and Hassall corpuscles. CMV identified in the liver, brain, heart, and lung was the likely cause of decease.

A novel mutation in CD3D. The decreased CD3 expression observed in both patients suggested a potential TCR defect. We thus analyzed *CD3* and *CD247* RNA by RT-PCR and found short *CD3D* PCR products in several family members (Figure 1B). Sequencing revealed a complete in-frame deletion of exon 2, which encodes the extracellular Ig-like domain of CD3δ (Figure 1C).

Exon skipping suggested a potential splicing defect. Genomic DNA sequencing detected a homozygous G-to-A mutation at position +5 in the 5′ splice donor site of intron 2 (IVS2+5G→A; Figure 1D). The mutation abrogated a restriction site for the enzyme BsaAI, which was used to follow its segregation (Figure 1E). The mutation was causing the immunodeficiency, as it was not found in 140 Spanish or Ecuadorian healthy donors, and its location is strictly conserved in mammals (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI44254DS1).

The patients' parents were carriers of the same *CD3D* mutation and had a similar geographic origin, indicating that they likely shared a founder mutant allele. The analysis of polymorphic microsatellite markers spanning the *CD3* region (which contains *CD3G*, *CD3D*, and *CD3E*) confirmed the presence of a shared core haplotype associated with the mutation (Supplemental Figure 2).

From the data in Figure 1B, it seemed that some normal PCR products might be present in the patients, indicating that the mutation did not abrogate normal splicing at the *CD3D* locus, as shown in similar intronic mutations (8). To analyze *CD3D* splicing, quantitative RT-PCR relative to *CD3E* was performed. The results confirmed the presence of small amounts of WT *CD3D* transcripts in the patients (Figure 1F).

Table 1
Lymphocyte studies in 2 affected children with the SCID disorder

Variables	Patient AIII.1	Patient BII.2	Normal range
Lymphocyte number/μl at	**13 months**	**6 months**	**9–15 months**
T ($CD3^+$)	400	787	1600–6700
B ($CD19^+$)	1987	2183	600–2700
NK ($CD3^-CD16^+/CD56^+$)	636	537	180–1200
T cell proliferation (cpm)[A]			
Medium	201	851	<1000
Phytohemagglutinin	2242	2562	>80,000
Anti-CD3	8020	1327	>50,000
Serum Ig (mg/dl)			
IgG	1170	40[B]	310–1380
IgA	156	38	30–120
IgM	121	44	50–120
IgE (IU/ml)	4019	3	0–120
IgG1	957	ND[B,C]	430–900
IgG2	458	ND[B,C]	30–390
IgG3	<6.4	ND[B,C]	10–80
IgG4	50	ND[B,C]	10–65
Functional Abs		ND[B,C]	
Natural Abs (titer)			
Heterophile Abs	1/256		≥1/64
Isohemagglutinins (anti-B)	1/16		≥1/8
Infectious specificities[D]	Neg		–
Vaccination responses	Before/After		After
Hepatitis B (IU/ml)	NAv[C] / 0		>10
Tetanus toxoid (IU/ml)	0.01 / 0.03		0.04–3.92
Influenza (HI)[E]	8/16		>32
NK cell cytotoxicity (% lysis)[F]		ND[C]	
100:1	34		30–86
50:1	21		20–84
12:1	8		1–57

[A]H^3-thymidine uptake in response to mitogens. [B]Protein-losing enteropathy. [C]Not done or not available (neonatal vaccination). [D]IgG anti-CMV, EBV, HSV, VZV, HIV, HAV, rubella, measles, toxoplasma, and IgE anti-aspergillus. [E]Hemagglutination inhibition. [F]At the indicated effector/target ratios.

From these results, we concluded that a homozygous IVS2+5G→A mutation strongly impaired (around 10-fold), but did not abrogate, normal *CD3D* splicing in both patients.

Reduced CD3δ protein. The small levels of WT *CD3D* transcripts were found to be sufficient to encode for half-normal levels of WT CD3δ proteins in the patients' T cells as shown in family B by Western blotting (Figure 2) or intracellular flow cytometry (Supplemental Figure 3A). In contrast, the dominant ΔEx2 *CD3D* transcripts did not give rise to detectable levels of the predicted headless CD3δ chain, despite being readily detected after transfection in non-T cells (Figure 2B).

We cannot exclude that small amounts of ΔEx2 CD3δ might be expressed below the detection limit of Western blotting. However, when overexpressed in *Drosophila* cells, ΔEx2 did not compete with WT CD3δ in the formation of a TCR complex (Supplemental Methods and Supplemental Figure 4).

From these studies, we concluded that the immunodeficiency was associated with reduced levels of normal CD3δ.

Immunological characteristics. The human CD3δ chain is incorporated into both TCRαβ and TCRγδ (Figure 1A). To establish how the *CD3D* mutation affected lymphocyte differentiation, the numbers of αβ T cells and γδ T cells were determined in both patients (Figure 3A). The results showed a severe selective reduction in peripheral blood αβ T lymphocyte numbers (both $CD4^+$ and $CD8^+$; Supplemental Figure 5), close to 10-fold compared with the median value of healthy age-matched controls. In contrast, γδ T cells as well as B and NK lymphocytes were detected in normal numbers ($T\alpha\beta^-T\gamma\delta^+B^+NK^+$ phenotype; Figure 3A and Table 1). A possible role for CMV in γδ T cell predominance was proposed in some SCID reports (9) but not in others (10). However, CMV-induced γδ T expansion was excluded in patient AIII.1.

The reduction caused by the *CD3D* mutation in αβ, but not γδ, T lymphocyte numbers suggested a differential CD3δ requirement for TCR expression or function in αβ versus γδ T cells. Counterintuitively, TCR expression was around 2-fold lower in γδ than in αβ T cells from the patients using different TCR- or CD3-specific mAbs, both in primary (Figure 3B) and in cultured T cells (Supplemental Figure 3B). TCR downregulation after engagement by anti-CD3, however, was similar in both T cell lineages (Supplemental Figure 6A). In contrast, early activation events such as CD69 or CD25 induction were strongly reduced (Figure 3C and Supplemental Figure 6, B and C). αβ and γδ T cells were nevertheless capable of normal anti-CD3– or phytohemagglutinin-induced short-term proliferation on a per-cell basis (5 days; Supplemental Figure 6D). This was confirmed in culture using allogeneic feeder cells (Figure 3D). However, after day 25 in the same cultures, αβ, but not γδ, T cells showed impaired growth relative to a control. Therefore, the *CD3D* mutation impaired TCR expression and several functions in γδ and αβ T cells, albeit with some contrasting effects: lower TCR expression by γδ T cells but lower in vitro long-term survival of αβ T cells. CD3δ has been reported to bind less strongly to the TCRγδ than to the TCRαβ heterodimer (11), offering a potential mechanism for the observed differential surface levels of TCR in γδ versus αβ T cells when CD3δ is reduced.

The thymus was normal in size at diagnosis (Supplemental Figure 7). To study its function, several studies were performed, including analyses of recent thymic emigrants defined as $CD4^+CD45RA^+CD31^+$ cells, $CD45RA^+$ (naive) and $CD45R0^+$ (memory) T cells, CD25 expression; *TCRB* clonality, and TCRVβ usage (Supplemental Figures 8 and 9). The results indicated that the patients' thymuses produced very few αβ T lymphocytes, and most of these had differentiated into effector memory T cells with an activated phenotype and an oligoclonal TCRVβ repertoire. They may have contributed to the observed in vivo Th2 features in patient AIII.1 (hyper-IgE, eosinophilia, and atopic dermatitis). Similar Th2 immunopathology has been observed in lymphopenic patients with Omenn-like syndrome associated with several primary immunodeficiencies (12) and in mice with partial T cell immunodeficiency (13).

These results indicated that the *CD3D* splicing mutation strongly impaired αβ, but not γδ, T lymphocyte selection in the thymus, resembling the phenotype of CD3δ-KO mice (14). In contrast,

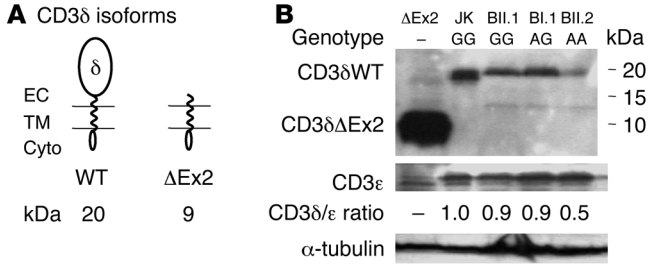

Figure 2
CD3δ protein analysis. (**A**) WT CD3δ and the predicted truncated isoform (ΔEx2). (**B**) Western blotting analysis of CD3δ isoforms in lysates from ΔEx2-transfected 293T cells, Jurkat cells (JK), or T cell lines with the indicated *CD3D* IVS2+5 genotypes using APA1/2 (anti-human CD3δ cytoplasmic tail), anti-CD3ε, or anti-tubulin. The numbers under each lane indicate CD3δWT band intensity relative to CD3ε.

human complete CD3δ immunodeficiencies uniformly lack all T cells (4, 5). Therefore, the leaky mutation, which we believe to be novel, reveals that human αβ and γδ T lymphocytes have differential CD3δ requirements for selection that have not been described for other invariant chains (CD3γ, CD3ε, or TCRζ; Supplemental Table 1). The Tαβ$^-$Tγδ$^+$B$^+$NK$^+$ phenotype has been reported recently in 2 children with TCRα deficiency (3). However, those patients remained relatively healthy for 6–7 years on antibiotic prophylaxis before transplantation; they had activated germinal centers and specific Ab responses against vaccines and autoantigens, likely due to γδ T cell help by homology with the mouse model. The leaky CD3δ SCID patients reported here required very early transplantation (before 1–2 years), lacked germinal centers, and showed very poor T cell–dependent B cell function, perhaps due to the signaling impairment observed also in their γδ T cells (Figure 3C). Thus, CD3δ levels are sufficient for selection, but not for normal function of γδ T cells, as described for CD4$^+$ T cells in Zap70 SCID patients (2).

Two mechanisms may be proposed to explain the selective effect of low CD3δ levels in αβ T lymphocyte development. One is impaired assembly or signaling of the immature precursor of the TCRαβ termed the pre-TCR (15). The second one is impaired signaling through the mature TCRαβ due to reduced interactions of CD3δ with an evolutionarily conserved motif in the TCRα chain membrane-proximal constant region termed the α-chain connecting peptide (16).

Together, the results showed that the leaky *CD3D* mutation reduced CD3δ chains, which in turn blocked αβ rather than γδ T cell selection. In mature T cells, TCRγδ expression was more impaired than TCRαβ expression, but early signaling through both was similarly impaired.

Methods

Further information can be found in Supplemental Methods.

Mutation detection. *CD3* amplimers were generated by RT-PCR of PBMC RNA with specific primers (Supplemental Tables 2 and 3). *CD3D* exons and flanking intronic sequences were amplified from leukocyte DNA by PCR using specific primers and sequenced following standard techniques. Screening for the IVS2+5G→A mutation was performed by RFLP using BsaAI (New England Biolabs). Ecuadorian DNA samples were provided by Antonio Arnaiz-Villena (Complutense University).

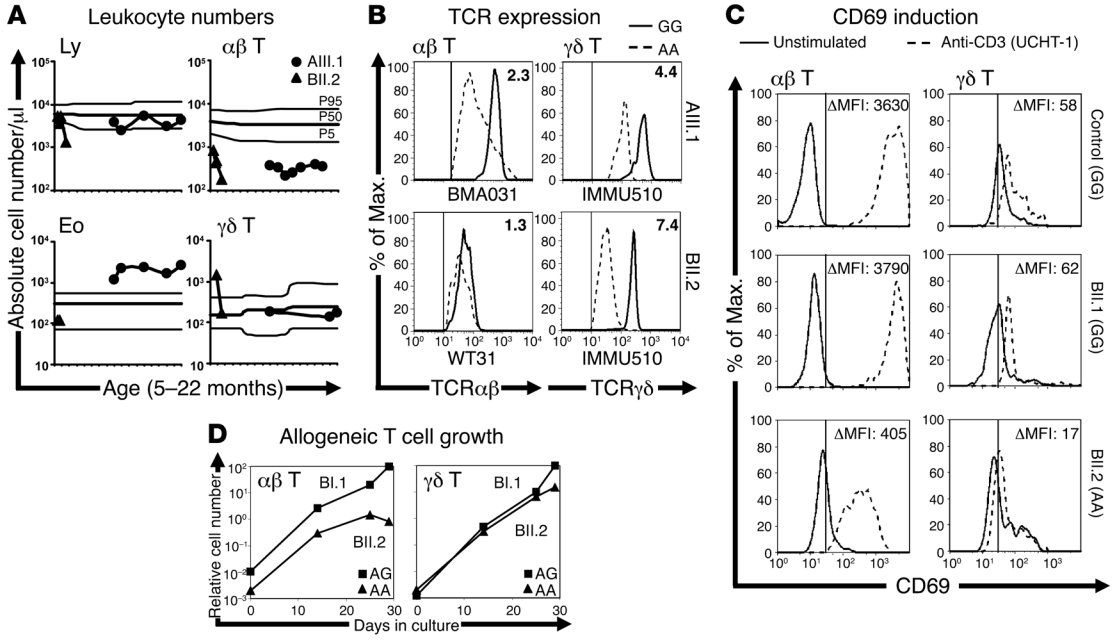

Figure 3
T lymphocyte analysis. (**A**) Absolute cell numbers in patients plotted as a function of age in comparison with the normal age-matched distribution (P5, P50, and P95). The leukocyte subsets are lymphocytes (Ly), eosinophils (Eo), αβ T (CD4$^+$+CD8^{bright}), and γδ T (11F2$^+$) cells. (**B**) TCRαβ and TCRγδ cell surface expression in primary lymphocytes from the patients (dashed lines, AA genotype) in comparison with controls (solid lines, GG genotype), measured using the indicated TCRαβ- and TCRγδ-specific mAb. The numbers in each histogram indicate MFI ratios between control and patient. (**C**) CD69 induction (geometric MFI increments) after 24 hours in anti-CD3–stimulated (dashed lines) versus unstimulated (solid lines) primary αβ T (CD4$^+$) or γδ T (IMMU510$^+$) lymphocytes with the indicated genotypes. (**D**) Patient T cell growth in feeder cell cultures expressed as a percentage of carrier BI.1 T cell numbers.

Quantitative PCR. TaqMan PCR of PBMC cDNA was done using *CD3D* WT– or ΔEx2-specific primers and probes (Supplemental Table 4). Samples were normalized to the Ct of *CD3E*-specific primers and probe 49 from the Universal ProbeLibrary Human (Roche) and to the highest value in each data set, which is shown as 1.

Transfections and Western blotting. 293T cells were transiently transfected using lipofectamine (Invitrogen) with 2 μg of plasmid pIRES-GFP1a (Invitrogen) containing ΔEx2 CD3D cDNA, lysed and analyzed by Western blotting, together with cultured T cells, using APA1/2 (mouse anti-CD3δ cytoplasmic tail IgG mAb; provided by Balbino Alarcón, Centro de Biología Molecular, Madrid, Spain) or M20epsilon (goat anti-CD3ε IgG; Santa Cruz Biotechnology Inc.).

Immunological investigations. Lymphocyte phenotype was determined by flow cytometry using anti-CD3 (Leu4/SK7), anti-CD69 (L78), anti-CD19 (SJ25C1), anti-CD16 (73.1), anti-CD56 (NCAM16.2), and anti-TCRγδ (11F2) from BD Biosciences; and anti-TCRαβ (BMA031 or WT31), anti-TCRγδ (IMMU510), anti-CD4 (13B8.4), and anti-CD8 (B9.11) from Beckman Coulter Immunotech. T cell function was tested by standard overnight H^3-thymidine uptake assays (1 μCi/well) by culturing 10^5 PBMCs with phytohemagglutinin (1 μg/ml; Sigma-Aldrich) or plastic-bound anti-CD3 (10 μg/ml UCHT-1 from BD) for 72 hours. NK cell function was tested by standard ^{51}Cr release assays using PBMCs as effectors and K562 cells as targets. Specific lysis was calculated from cpm as 100 × (sample)/(maximum), after correcting for blank measurements.

T cell lines were generated from PBMCs and expanded weekly with irradiated allogeneic feeder cells (PBMCs and EBV-transformed B cells) at 1:5:5 ratios and final 10^6 cells/ml in IMDM medium (PAA) with 0.1 μg/ml phytohemagglutinin (only at day 0; Sigma-Aldrich), 40 IU/ml rIL-2 (provided by Craig W. Reynolds, Frederick Cancer Research and Development Center, NCI, NIH, Frederick, Maryland, USA), 10% AB^+ human serum, and 1% glutamine (Gibco; Invitrogen).

Study approval. The study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Hospital Clínico Research Ethics Committee, Madrid, Spain. All participants or their guardians provided informed consent for the collection of samples and subsequent analyses.

Statistics. Bar graph data represent mean ± SD. For proliferation and cytotoxicity, median values of triplicates were used.

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