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

Juana Gil,[1] Elena M. Busto,[2] Beatriz Garcillán,[2] Carmen Chean,[1] Maria Cruz García-Rodríguez,[3] Andrea Díaz-Alderete,[1] Joaquín Navarro,[1] Jesús Reiné,[2] Angeles Mencía,[4] Dolores Gurbindo,[1] Cristina Beléndez,[1] Isabel Gordillo,[1] Marlena Duchniewicz,[5] Kerstin Höhne,[5] Félix García-Sánchez,[6] Eduardo Fernández-Cruz,[1] Eduardo López-Granados,[3] Wolfgang W.A. Schamel,[5] Miguel A. Moreno-Pelayo,[4] María J. Recio,[2] and José R. Regueiro[2]

[1]Gregorio Marañón University Hospital, Madrid, Spain. [2]School of Medicine, Complutense University, i+12 and RIER, Madrid, Spain. [3]La Paz University Hospital, Madrid, Spain. [4]Ramón y Cajal University Hospital, IRYCIS and CIBERER, Madrid, Spain. [5]Faculty of Biology, Centre of Chronic Immunodeficiency (CCI), BIOSS Centre for Biological Signaling Studies, University of Freiburg and Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany. [6]Blood Transfusion Centre, Madrid, Spain.

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±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 (Tαβ−Tγδ+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±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
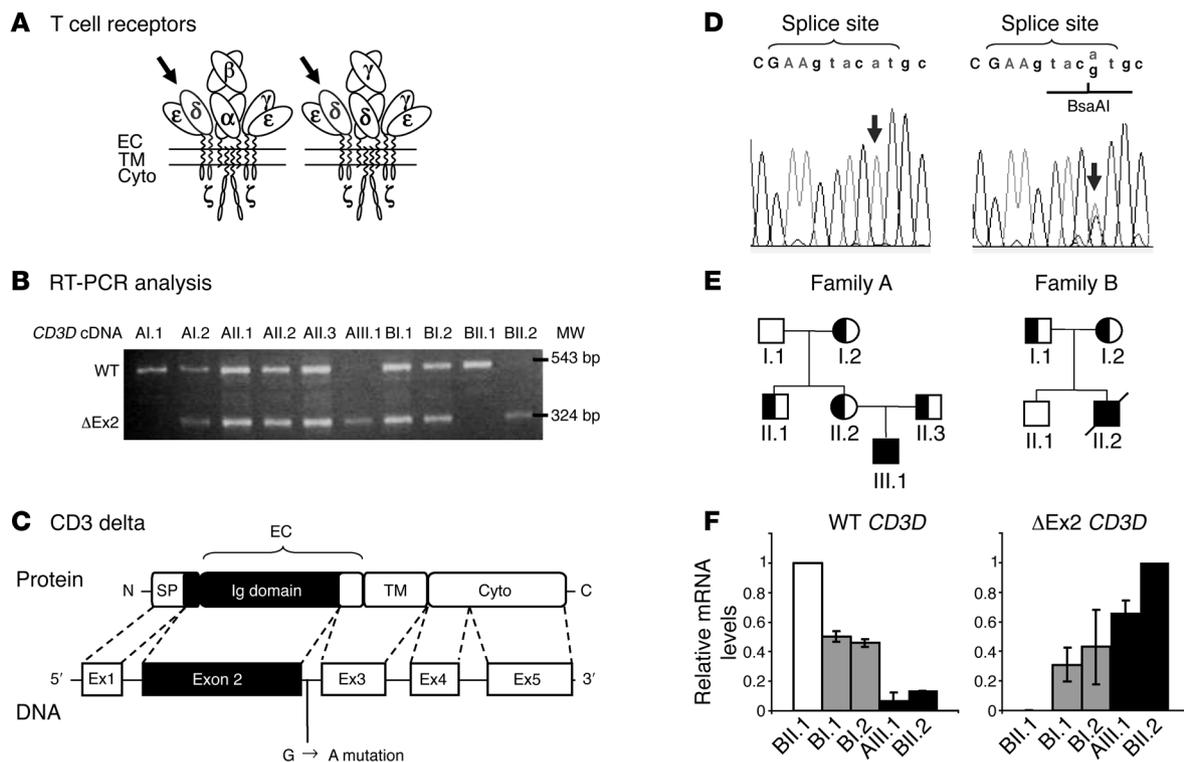
Figure 1

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**) *CD3*δ 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 Lymphocyte number/μl at	Patient AIII.1 13 months	Patient BII.2 6 months	Normal range 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 (Tαβ−Tγδ+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,
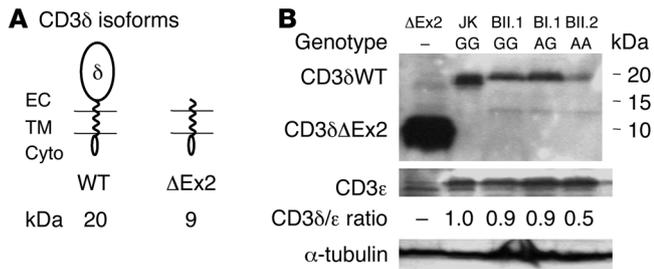
A CD3δ isoforms

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).
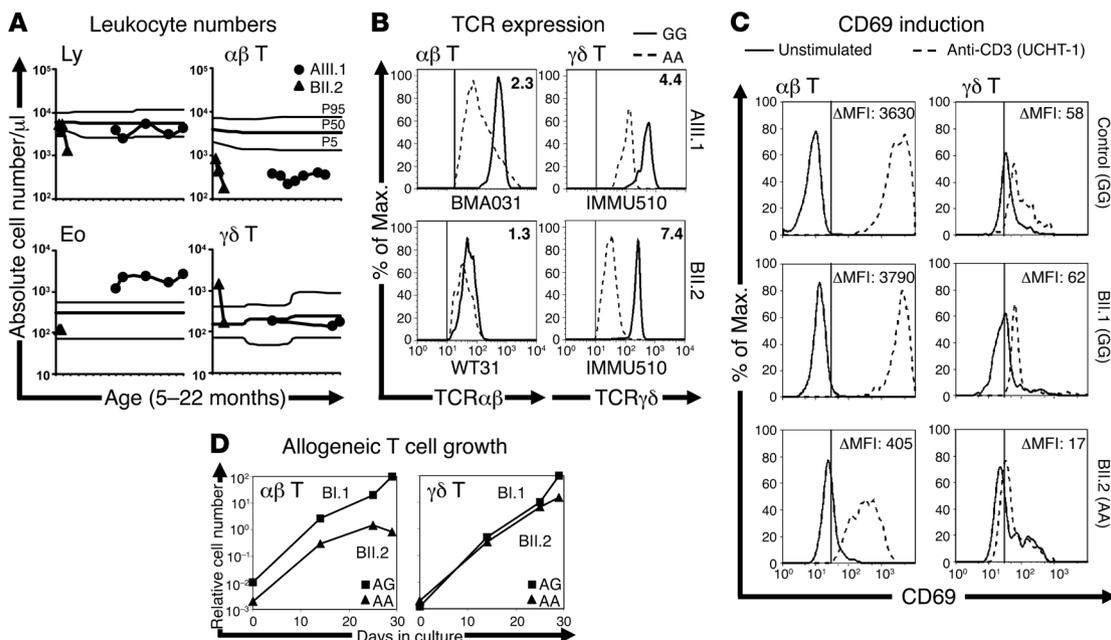

Figure 3

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 H³-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.

Address correspondence to: José R. Regueiro, Inmunología, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain. Phone: 34913941642; Fax: 34913941641; E-mail: regueiro@med.ucm.es.

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SUPPLEMENTAL TABLES

Supplemental Table 1. Human TCR complex deficiencies [A]

Protein	Gene	Chr.	OMIM	References		Number of patients	
				Complete	Leaky	Complete	Leaky
CD3γ	*CD3G*	11	186740	1		5	
CD3δ	*CD3D*	11	186790	2,3,4	[B]	7	2 [B]
CD3ε	*CD3E*	11	186830	3	5	3	1
TCRζ	*CD247*	1	186780	6	7	1	1
TCRα	*TRAC* [C]	14	186880	8		2	
					Total	**18**	**4**

[A] Classified as complete or leaky according to the effect of the mutations. Leaky, but not complete, mutations allow for the synthesis of low amounts of the wild type protein.
[B] Present manuscript. The GenBank accession no. for the cDNA and genomic DNA mutant sequences are JN392069 and JN392070, respectively.
[C] TCRα constant gene segment.

Supplemental Table 2. Primers used for RT-PCR [A]

Gene	Primer	Sequence (5´ to 3´)
CD3G	CD3GF	AAAAAGAATTCTCAATTCCTCCTCAACTC
	CD3GR	AAAAAGGATCCATGGAACAGGGGAAGGG
CD3D	CD3DF	CTGTAGGAATTCACGATGGAACATAGCACGTTTCTC
	CD3DR	CTAGCTCTCGAGTCACTTGTTCCGAGCCCAGTT
CD3E	CD3EF	TTCCTGTGTGGGGTTCAGAAACC
	CD3ER	CCATCAGGCTGAGGAACGATTCT
CD247	CD247F	CTGAGGGAAAGGACAAGATGAAG
	CD247R	AAAGAGTGCAGGGACAACAGTCT

[A] OLIGO Primer Analysis Software version 7 from Molecular Biology Insights was run for the following gene sequences (GenBank accession no.): *CD3G* (NM_000073), *CD3D* (NM_000732), *CD3E* (NM_000733) and *CD247* (NM_0007343.3).

Supplemental Table 3. Primers used for genomic PCR (exons+flanking introns) [A]

Gene	Exon	Sequence (5´ to 3´)	
		Forward	Reverse
CD3D	1	AGCTCTCACCCAGGCTGATAGT	AAGCTCTGGGATTACTGGTGTGA
	2	TGAGCTTCCGCAGAACAAAGG	CACATCCAGAAGCCCTATCCATT
	3	AGGATGGTTCCCTGATCTTAAAGG	CACTCTCATGCTCTGCTCTTCCA
	4-5	GGTGGATCTCACAGTCCCATCT	TATATTTATTGGCTGAGCAAGAAGG

[A] OLIGO Primer Analysis Software version 7 from Molecular Biology Insights was run for *CD3D* (NG_009891.1)

Supplemental Table 4. Primers and probes used for quantitative PCR [A]

Gene	Primer/Probe	Sequence (5´ to 3´)
CD3DWT	Forward	AGGACAAAGAATCTACCGTGCAA
	Reverse	CACGGTGGCTGGATCCA
	Probe	ATTATCGAATGTGCCAGAGC
CD3DΔEx2	Forward	CGTTTCTCTCTGGCCTGGTACT
	Reverse	CACGGTGGCTGGATCCA
	Probe	ACCCTTCTCTCGCAAGTGTGCCAGA
CD3E	Forward	CAAGGCCAAGCCTGTGAC
	Reverse	TCATAGTCTGGGTTGGGAACA
	Probe	49 (Universal ProbeLibrary for Human, Roche)

[A] Primer Express 3.0 from Applied Biosystems was used for *CD3DWT* and *CD3DΔEx2,* and ProbeFinder version 2.40 for Human (Universal ProbeLibrary Assay Design Center) from Roche Applied Science was used for *CD3E*. Sequences as in Supplemental Table 2.

SUPPLEMENTAL METHODS

The S2 *Drosophila* cell reconstitution system

Schneider S2 cells were grown in Schneider's *Drosophila* medium and transfected using CellFectin (Invitrogen Life Technologies) as described (9). Expression vectors for CD3ε, CD3γ and TCRζ were generated as described (10) and for TCRα, TCRβ, CD3δ, ΔEx2 and ΔEx3 is explained below. After 24 h, protein expression was induced by addition of 1mM $CuSO_4$ for another 20 h. Subsequently, cells were either stained for flow cytometry or lysed as described (9, 10). For IP 3 μg of antibody and 5 μl of protein G-coupled Sepharose (GE Healthcare) was incubated with 300 μl of cell lysate overnight at 4°C. Beads were washed 3 times in lysis buffer before standard SDS-PAGE and Western blotting.

Generation of *Drosophila* expression vectors

The cDNAs of the proteins of interest were inserted into the Drosophila expression vector pRmHa-3 containing an inducible metallothionein promotorer (11). pRmHa-3 is abbreviated as pD. The coding sequences of human WT CD3δ, ΔEx2 and ΔEx3 were cloned into the pD vector using EcoRI and XhoI restriction sites from the pIREShrGFPCD3DWT, pIREShrGFPCD3DΔExon2 and pIREShrGFPCD3DΔExon3 plasmids. ΔEx3 CD3δ lacks the transmembrane region and is associated with severe αβ and γδ T lymphopenia and SCID (4). pDECFPhTCRαHA1.7 and pDECFPhTCRβ HA1.7, containing sequences that encode for an N-terminally ECFP-tagged HA-specific human TCRα or TCRβ chain, were derived from the plasmids pJ6omegaTCRαHA1.7 or pJ6omegaTCRβHA1.7, respectively.

Reconstruction of the TCR complex in *Drosophila* S2 cells

To study TCR assembly, we used the S2 *Drosophila* cell reconstitution system that allows co-transfection and expression of more than 5 different vectors (12). We had used this system previously to reconstruct assembly and surface transport of the BCR complex (13). Here, we firstly show that TCRαβ dimers do not come to the S2 cell surface if expressed alone, whereas CD3εδ and CD3εγ do (Supplemental Figure 4A). Co-expression of a CD3 dimer together with TCRαβ leads to surface expression of TCRαβ, showing that some aspects of TCR assembly and transport to the surface can

be studied using this system (Supplemental Figure 4B). Next, S2 cells were co-transfected with plasmids containing cDNAs of WT CD3ε and WT CD3δ or ΔEx2 or ΔEx3. CD3 proteins were IP from the lysates with a mAb specific for folded CD3ε (OKT3) and a CD3δ-specific antiserum (M20δ), separated by non-reducing SDS-PAGE and detected by CD3δ- or CD3ε-specific antisera by Western blotting (Supplemental Figure 4C). ΔEx2, but not ΔEx3, gave rise to a stable protein (bottom left panel) which was able to pair with CD3ε (bottom right panel). The smallest ΔEx2 form might be a disulphide-linked dimer of nearly the same size as a WT CD3δ monomer. CD3ε of the ΔEx2-CD3ε dimers was not folded correctly, since it was not recognized by OKT3 (upper panels), and was aberrantly disulphide bonded to ΔEx2 (lower left panel). Only WT CD3δ produced CD3δε dimers that were recognized by IP using the mAb OKT3 (upper panels) and by flow cytometry using UCHT1 (Supplemental Figure 4D). Furthermore, ΔEx2 could not diminish expression of a TCR complex (Supplemental Figure 4E), although it was expressed at higher levels than WT CD3δ (Supplemental Figure 4C bottom left). Therefore, the head-less CD3δ did not efficiently compete with WT CD3δ in the formation of a TCR complex. From these results we concluded that ΔEx2 was unlikely to impinge on αβTCR assembly or surface expression in the patients' T cells.

TCRB clonality

Clonality at the *TCRB* locus was studied using a commercial kit (Master Diagnostica, Granada, Spain, EC-certified for clinical use), which amplifies genomic TCR VβJβ rearrangements using two primers specific for conserved V and J flanking regions. Polyclonal (normal donor) and monoclonal (Jurkat or MOLT3) control DNAs were included for reference. Amplimers were separated and analyzed in an ABI Prism Genetic Analyzer 3110 using GeneMapper V 4.0 from ABI.
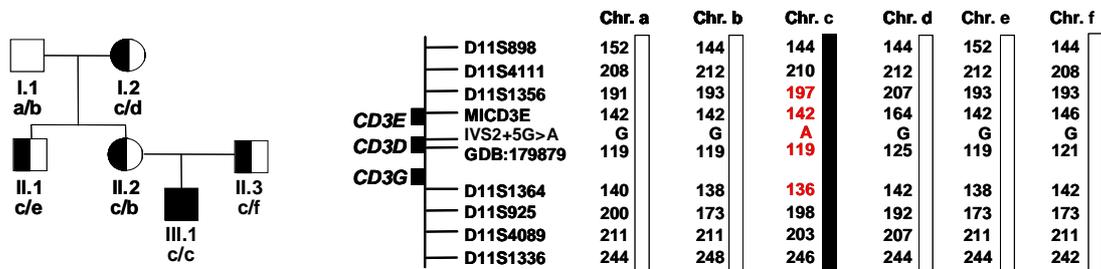
SUPPLEMENTAL FIGURES

Supplemental Figure 1. *CD3D* **intron 2 5′ splice donor site phylogeny.** Multiple alignment of DNA sequences of the gene region surrounding the IVS2+5G>A mutation (arrow) in several mammals (*hominids) reveals that the location of the mutation is conserved. The equivalent location after the exon encoding the extracellular Ig domain is also conserved as a guanine in *CD3G* (not shown).
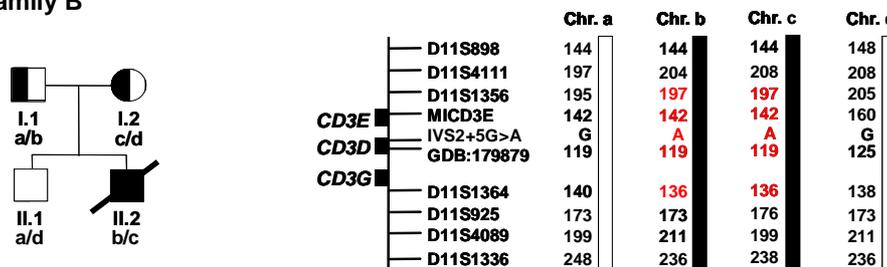
Supplemental Figure 2. Genetic pedigrees and *CD3* haplotype analysis. Genetic pedigrees of the two families with the *CD3D* mutation. Circles indicate females, squares indicate males (slashed when deceased). Solid symbols denote homozygosity for the mutation, half-solid symbols heterozygosity. *CD3* haplotypes under each symbol are based on the indicated polymorphic markers spanning the *CD3GDE* region on chromosome 11q23. The disease-associated chromosomes are depicted in black with the shared core haplotype markers in red. No genotyping inconsistencies were found. The relative order and the physical distances of markers are as previously reported (1). The allele sizes are normalized with respect to individual 134702, available from Center d'Etude du Polymorphisme Humain (14).

Family A

Pedigree:
- I.1 a/b ☐ × I.2 c/d ◐
- II.1 c/e, II.2 c/b, II.3 c/f
- III.1 c/c

	Marker	Chr. a	Chr. b	Chr. c	Chr. d	Chr. e	Chr. f
	D11S898	152	144	144	144	152	144
	D11S4111	208	212	210	212	212	208
	D11S1356	191	193	197	207	193	193
CD3E	MICD3E	142	142	142	164	142	146
CD3D	IVS2+5G>A	G	G	A	G	G	G
	GDB:179879	119	119	119	125	119	121
CD3G	D11S1364	140	138	136	142	138	142
	D11S925	200	173	198	192	173	173
	D11S4089	211	211	203	207	211	211
	D11S1336	244	248	246	244	244	242

Family B

Pedigree:
- I.1 a/b ◑ × I.2 c/d ◐
- II.1 a/d, II.2 b/c (deceased)

|	Marker	Chr. a	Chr. b	Chr. c	Chr. d
	D11S898	144	144	144	148
	D11S4111	197	204	208	208
	D11S1356	195	197	197	205
CD3E	MICD3E	142	142	142	160
CD3D	IVS2+5G>A	G	G	A	G
	GDB:179879	119	119	119	125
CD3G	D11S1364	140	136	136	138
	D11S925	173	173	176	173
	D11S4089	199	211	199	211
	D11S1336	248	236	238	236

Supplemental Figure 3. TCR complex expression in cultured T cells from patients (dashed lines) or controls (solid lines). (A) Lymphocytes were fixed with 2% paraformaldehyde at 3×10^6 cells/ml for 1 hour at 4°C, permeabilized using 0.2% saponin for 15 minutes at room temperature and stained with APA1/2 (anti-human CD3δ cytoplasmic tail mAb, 15) followed by anti-mouse IgG-PE (Beckman Coulter). WT Jurkat cells were compared with CD3δ knock-down (KD) cells using a specific shRNA for CD3δ RNA which showed a 90% reduction in CD3δ by Western blotting (right, shCD3δ 3). The numbers in each histogram indicate the MFI ratios between control and patient or KD. **(B)** Lymphocytes were surface-stained with the indicated TCR- or CD3-specific mAb and analyzed comparatively within the indicated gates. Tαβ cells were defined as BMA031^{+}, CD3^{+}IMMU510^{−} or WT31^{+}, whereas Tγδ cells were defined as IMMU510^{+}. Histogram numbers as in A.

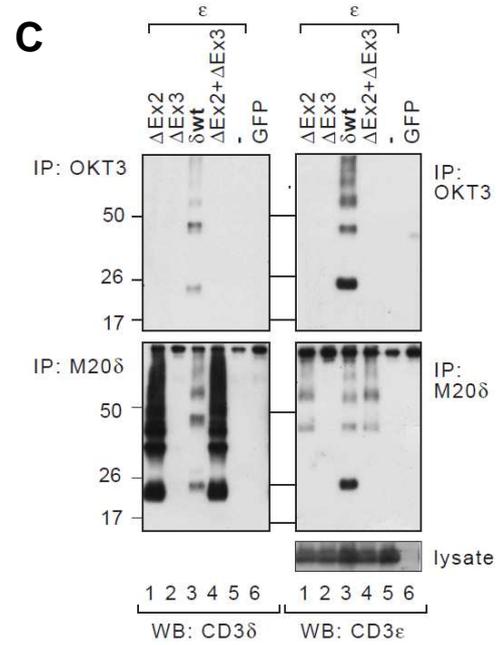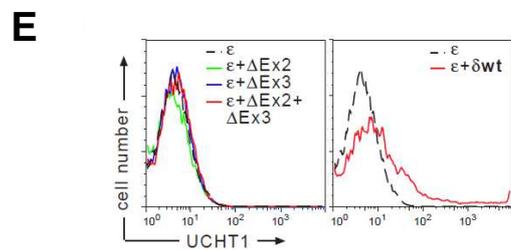## A Intracellular CD3δ expression
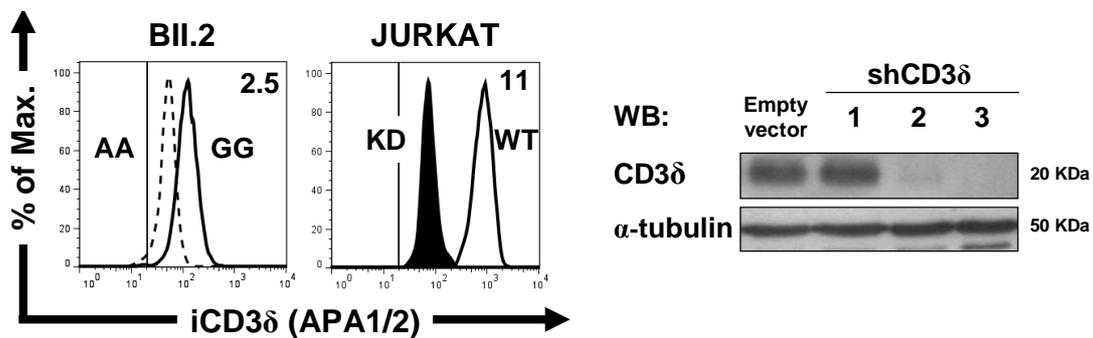
B Surface expression
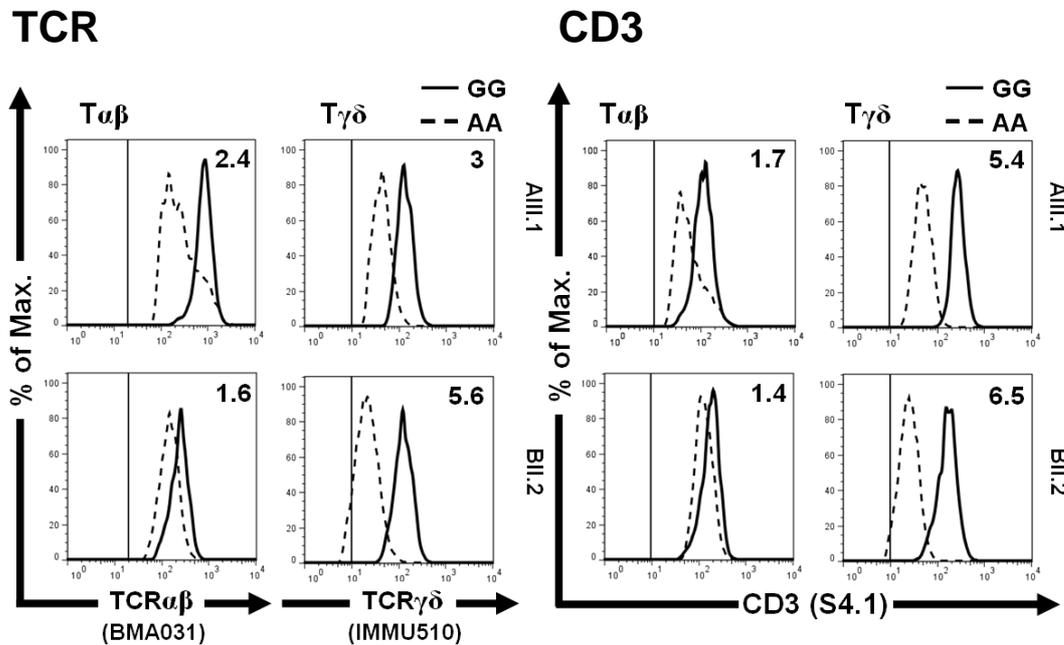
Supplemental Figure 4. Reconstruction of the TCR complex in *Drosophila* S2 cells (A, B, C) to show that ΔEx2 does not compete with WT CD3δ to form a TCR complex (D, E). Refer to Supplemental Methods for details.

(A) *Drosophila* S2 cells were transiently transfected with expression plasmids encoding for the indicated proteins or with the empty plasmid (-). After induction with copper sulfate, cells were stained first extracellularly and then after permeabilisation with saponin intracellularly with anti-TCRβ (Jovi1, left panels) or anti-folded CD3ε (UCHT1, right panel) antibodies and measured by flow cytometry. **(B)** Summary of the expression of TCRαβ (column "αβ") or CD3 (column "ε") on the *Drosophila* S2 cell surface after co-expression of the TCR or CD3 subunits indicated in the left column. Experiments were done as in A) using mouse or human expression plasmids as indicated. - : no expression on the cell surface; + : low and ++ : high expression on the cell surface. As a control, all human TCR and CD3 subunits were expressed as seen by Western blotting (data not shown). Co-expression of a CD3 dimer together with TCRαβ leads to surface expression of TCRαβ, which was not enhanced upon transfection of all six TCR and CD3 subunits. **(C)** S2 *Drosophila* cells were transiently transfected with expression plasmids encoding for the indicated proteins (lanes 1-4 and 6) or with the empty plasmid (lane 5). After induction with copper sulfate, the lysates (lowest panel) or anti-folded CD3ε or anti-CD3δ IP (OKT3, upper panels, or M20δ, lower panels, respectively) were separated by non-reducing SDS-PAGE. Western blotting was performed with anti-CD3δ (M20δ, left panels) or anti-CD3ε (M20ε, right panels) antibodies and the ECL system. **(D)** S2 cells were transiently transfected with expression plasmids encoding for the indicated proteins. After induction with copper sulfate, cells were stained with the anti-TCRβ antibody Jovi3 and measured by flow cytometry. **(E)** S2 cells were transiently transfected with expression plasmids encoding for the indicated proteins (coloured lines) or with the empty plasmid (black lines). After induction with copper sulfate, cells were stained with the anti-folded CD3ε antibody UCHT1 and measured by flow cytometry.

A

stain: anti-β

-     0 / 0

αβ   0 / 100

extracellular ↑ intracellular →

stain: anti-β   stain: anti-ε

-    0 / 0   0 / 0

εδ   0 / 0   79 / 21

αβ εγ εδ ζζ   100 / 0   91 / 9
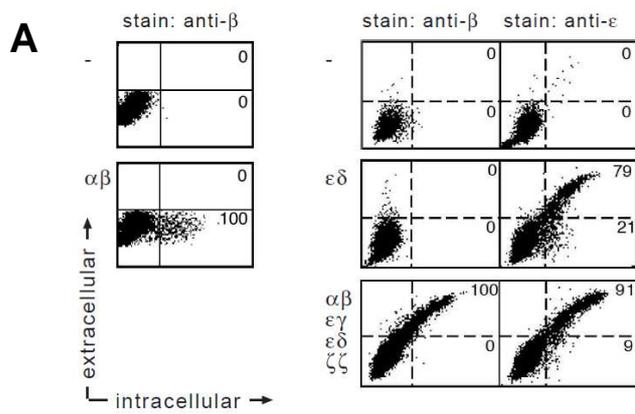
B

co-expression	mouse		human	
	αβ	ε	αβ	ε
αβ	-		-	
εγ		++		++
εδ		++		++
αβ ε	-		-	
αβ γ	-		-	
αβ δ	-		-	
αβ εγ	++	++	+	+
αβ εδ	++	++	++	++
αβ ζζ	-		-	
αβ εγ εδ	++	++	++	++
αβ εγ εδ ζζ	++	++	++	++

C

ε   ε

ΔEx2   ΔEx3   δwt   ΔEx2+ΔEx3   -   GFP

IP: OKT3

50   26   17

IP: M20δ

50   26   17

lysate

1 2 3 4 5 6   1 2 3 4 5 6

WB: CD3δ   WB: CD3ε

IP: OKT3

IP: M20δ

D

αβ+   αβγζ+

-   ε+δ   ε+ΔEx2

αβ+   αβγζ+

εδ   εδ+ΔEx2

cell number

anti-TCRβ →

E

ε   ε+ΔEx2   ε+ΔEx3   ε+ΔEx2+ΔEx3

ε   ε+δwt

cell number

UCHT1 →

Supplemental Figure 5. Tαβ lymphocyte subset numbers. Absolute CD4+ and CD8bright cell numbers in patients (AIII.1 dots, BII.2 triangles) plotted as a function of age in comparison with the normal age-matched distribution (P5, P50 and P95, 16).
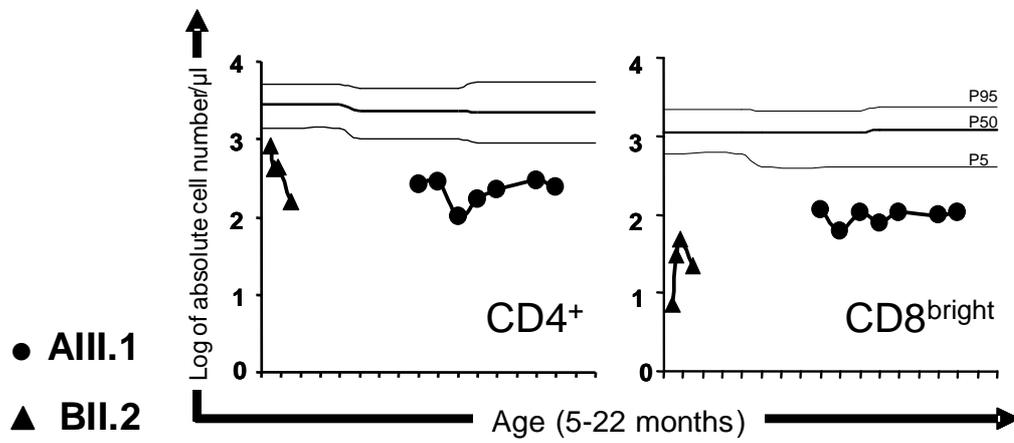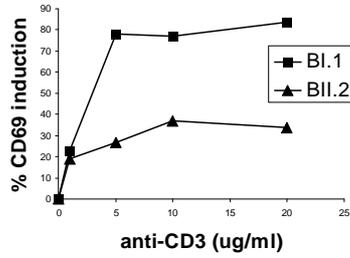
Supplemental Figure 6. TCR complex function. (A) TCR down-regulation after 24 hours in response to anti-CD3 stimulation in primary Tαβ (CD4$^+$) or Tγδ (11F2$^+$) lymphocytes with the indicated *CD3D* IVS2+5 genotypes. The numbers in each histogram indicate CD3 MFI percentages of stimulated (dashed lines) relative to unstimulated cells (solid lines). **(B)** CD69 induction (% expression) in T cell lines from the indicated donors after stimulation with different amounts of UCHT-1 for 24 hours. **(C)** CD25 induction after 36 hours in stimulated (dashed lines) compared to unstimulated (solid lines) primary CD4$^+$ T cells (moslty Tαβ cells). The numbers in each histogram indicate MFI increments normalized to control cell increments. **(D)** Lymphocyte proliferation was evaluated by flow cytometry using CFSE dye dilution (17). CFSE-labeled peripheral blood lymphocytes were cultured for 5 days in the presence (+) or absence (─) of phytohemagglutinin (PHA, left) or the anti-CD3 antibody UCHT-1 (right). Cells were analyzed for CFSE dilution by flow cytometry within the indicated subsets. In this experiment Tαβ and Tγδ lymphocytes were defined as CD4$^+$ (>98% Tαβ cells), and double negative CD3$^+$ (78±6% Tγδ cells) because the TCR was down-regulated after activation.

A TCR down-regulation
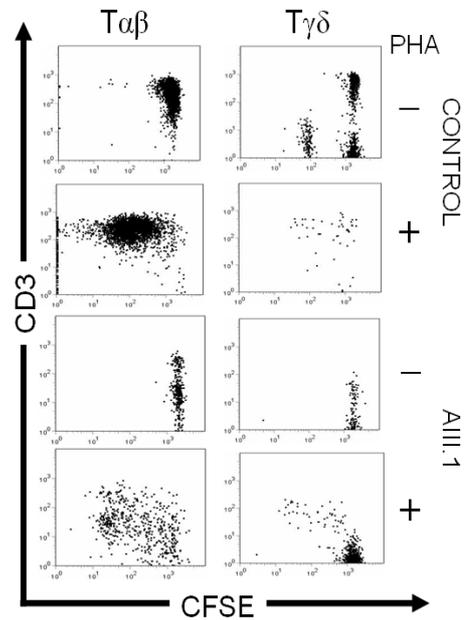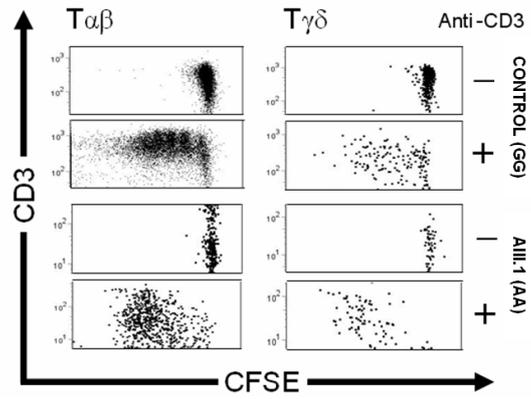
Unstimulated — - - anti-CD3 (UCHT-1)

Tαβ Tγδ

% of Max.

CONTROL (GG) 4% 5%

BII.1 (GG) 3% 6%

BII.2 (AA) 4% 8%

CD3(SK7)

B CD69 induction

% CD69 induction

anti-CD3 (ug/ml)

BI.1
BII.2

C CD25 induction

Unstimulated — - - anti-CD3 (UCHT-1)

% of Max.

CONTROL (GG) 100% BII.1 (GG) 84% BII.2 (AA) 10%

CD25

D Lymphocyte proliferation

Tαβ Tγδ Anti-CD3

CD3

CONTROL (GG) − +

AIII.1 (AA) − +

CFSE

Tαβ Tγδ PHA

CD3

CONTROL − +

AIII.1 − +

CFSE
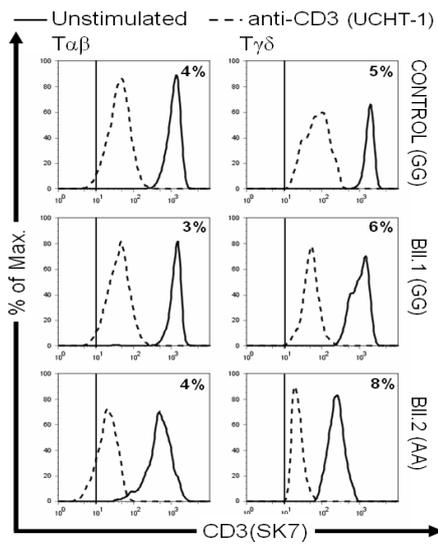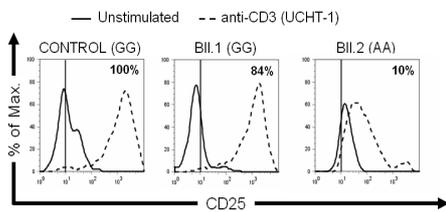
Supplemental Figure 7. Thymus of patient AIII.1 at diagnosis (chest CT scan). Patient AIII.1 showed a thymus of 2.39 x 1.8 cm (1 and 2 in image, respectively), within the normal age-matched dimensions ± SD of 3.13 ± 0.85 x 2.52 ± 0.82 (18). In patient BII.2 the thymus was initially not detected by CT scan, but his necropsy revealed the presence of a thymus remnant of 2 x 1 cm.
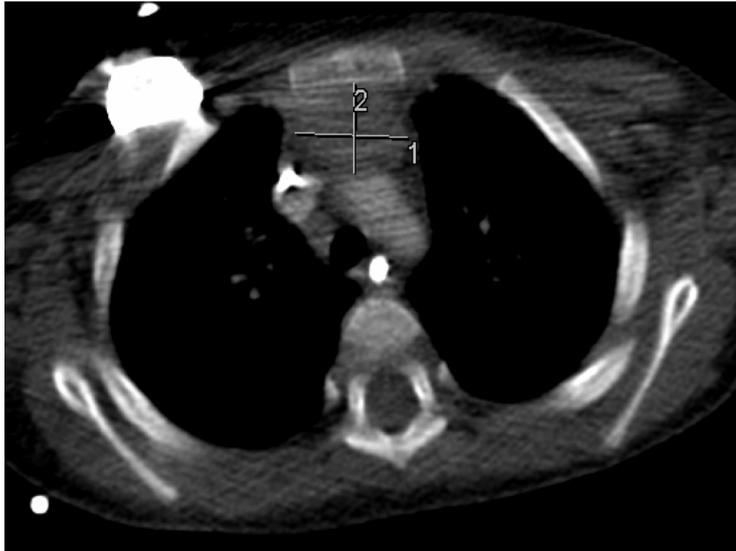
Supplemental Figure 8. T lymphocyte phenotype in the patients to a normal age-matched control. (A) Recent thymic emigrants defined as CD4$^+$CD45RA$^+$CD31$^+$ T cells (19). **(B)** CD45RA$^+$ (naïve) and CD45RO$^+$ (memory) T lymphocytes. **(C)** CD25 expression in patients with *CD3D* IVS2+5 AA genotype (dashed lines) in comparison with controls (GG, solid lines) in Tαβ cells defined as CD4$^+$ and in Tγδ cells defined as 11F2$^+$.
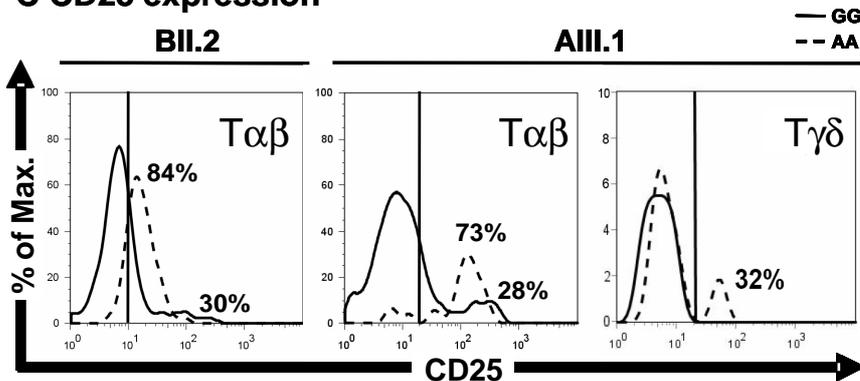
A Recent CD4$^+$ thymic emigrants
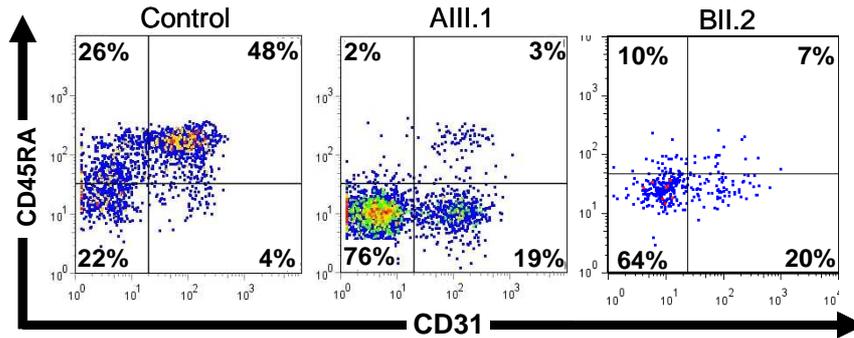
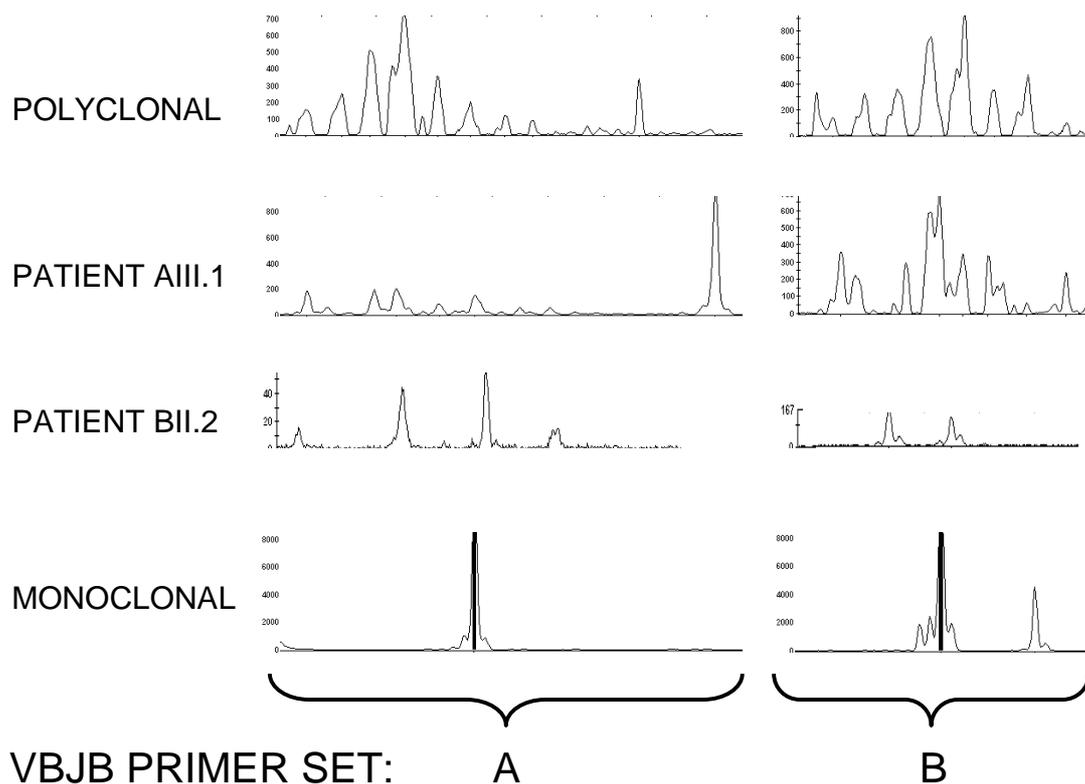## B CD45RA$^+$/CD45RO$^+$ T lymphocytes
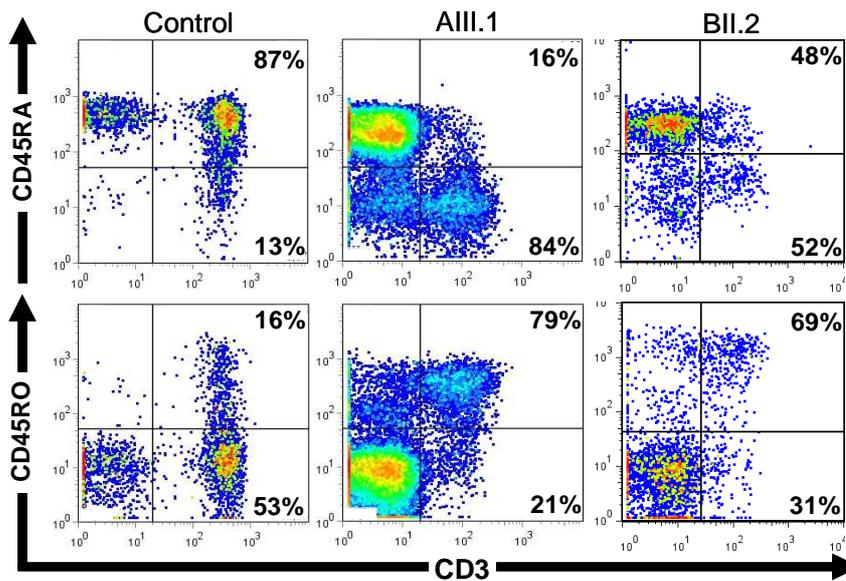
C CD25 expression

Supplemental Figure 9A. *TCRB* **clonality.** Genomic *TCR VβJβ* rearrangements were amplified in the patients using two different primer sets (VBJB-A and –B) and compared with a normal (polyclonal) donor and two tumoral T cell lines (monoclonal) in the 240-280 bp range. The two primer sets are specific for conserved V and J flanking regions, and therefore amplify genomic *TCR VβJβ* rearrangements as fragments of the indicated size range (see Supplemental Methods in page 4). Normal T lymphocytes are polyclonal and thus show a Gaussian fragment distribution (POLYCLONAL in Figure). T lymphoid tumors such as Jurkat or MOLT3 are monoclonal and thus yield a single major peak (MONOCLONAL in Figure). Patients with poor TCRβ diversity show few peaks without Gaussian distribution.

A *TCRB* clonality

POLYCLONAL

PATIENT AIII.1

PATIENT BII.2

MONOCLONAL

VBJB PRIMER SET: A   B

B TCR Vβ repertoire within T lymphocyte subsets
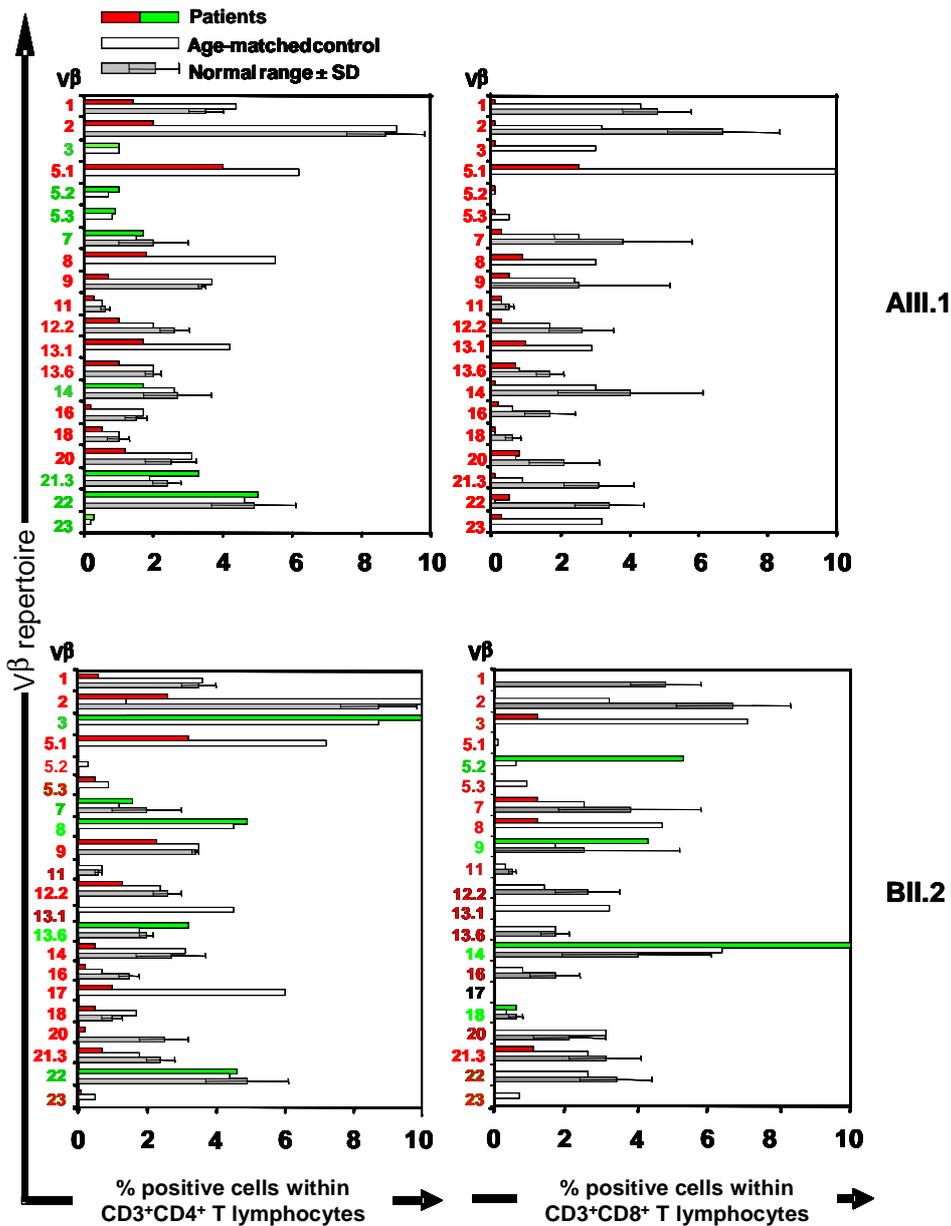
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