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The IL-2RG R328X nonsense mutation allows partial STAT-5 phosphorylation and defines a critical region involved in the leaky-SCID phenotype

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Summary

In addition to their detection in typical X-linked severe combined immunodeficiency, hypomorphic mutations in the interleukin (IL)-2 receptor common gamma chain gene (*IL2RG*) have been described in patients with atypical clinical and immunological phenotypes. In this leaky clinical phenotype the diagnosis is often delayed, limiting prompt therapy in these patients. Here, we report the biochemical and functional characterization of a nonsense mutation in exon 8 (p.R328X) of *IL2RG* in two siblings: a 4-year-old boy with lethal Epstein–Barr virus-related lymphoma and his asymptomatic 8-month-old brother with a $T^{low}B^{+}$natural killer $(NK)^{+}$ immunophenotype, dysgammaglobulinemia, abnormal lymphocyte proliferation and reduced levels of T cell receptor excision circles. After confirming normal IL-2RG expression (CD132) on T lymphocytes, signal transducer and activator of transcription-1 (STAT-5) phosphorylation was examined to evaluate the functionality of the common gamma chain (γ_c), which showed partially preserved function. Co-immunoprecipitation experiments were performed to assess the interaction capacity of the R328X mutant with Janus kinase (JAK)3, concluding that R328X impairs JAK3 binding to γ_c. Here, we describe how the R328X mutation in IL-2RG may allow partial phosphorylation of STAT-5 through a JAK3-independent pathway. We identified a region of three amino acids in the γ_c intracellular domain that may be critical for receptor stabilization and allow this alternative signaling. Identification of the functional consequences of pathogenic *IL2RG* variants at the cellular level is important to enable clearer understanding of partial defects leading to leaky phenotypes.

Keywords: hypomorphic mutations, IL-2RG, interleukin receptor common gamma subunit, JAK3, JAK1, leaky SCID, STAT-5, whole exome sequencing, X-linked severe combined immunodeficiency

Introduction

X-linked severe combined immunodeficiency (X-SCID) (OMIM 208380) is an inherited disorder of the immune system caused by inborn errors in the interleukin-2 receptor subunit gamma (*IL2RG*) gene, which encodes a common gamma chain, referred to as γ_c or CD132 [1–3]. γ_c is a transmembrane protein shared by various cytokine receptors – interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21 – that transduces signals through a Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathway [4,5]. The IL-2 receptor consists of three subunits: IL-2Rα, IL-2Rβ and IL-2Rγ. When IL-2 binds to IL-2Rα, all the subunits trimerize. IL-2Rβ and IL-2Rγ selectively recruit JAKl and JAK3, respectively, bringing them into close proximity, and they cross-phosphorylate each other, activating STAT-5. Once STAT-5 is phosphorylated, it dimerizes and translocates into the nucleus, transcribing genes involved in proliferation, survival, differentiation and apoptosis of T and natural killer (NK) cells [6].

Because of impaired cytokine signaling, children with complete γ_c deficiency lack T and NK cells. The absence of T cells also leads to non-functional B cells and hypogammaglobulinemia, despite a normal B cell count ($T^-B^+NK^-$ immunophenotype) [7–9]. Patients with X-SCID are prone to severe recurrent and persistent infections caused by certain bacteria, viruses and fungi in their first 6 months of life and may experience chronic diarrhea, skin rashes and failure to thrive [10]. The disease is lethal within the first 2 years of life unless patients undergo prompt hematopoietic stem cell transplantation (HSCT) or gene therapy [7,11,12].

In the 1990s, descriptions emerged of patients with *IL2RG* mutations and a milder phenotype, showing atypical manifestations and prolonged survival. These patients (referred to as having leaky or atypical X-SCID) carry hypomorphic mutations with a less severe pathogenic effect, resulting in lower susceptibility to infections and a moderate reduction in T lymphocytes [13–21]. Because of their poorly defined clinical and immunological phenotype, patients with atypical forms are diagnosed later in childhood or even in adulthood [14]. Most of the hypomorphic mutations found are missense mutations located in the extracellular domain of the γ_c chain, encoded by exons 1–5 of *IL2RG* [12,22–27]. However, the cytoplasmic domain of the protein, encoded by exons 7 and 8, may also be affected. To date, nine cases of atypical X-SCID have been described with mutations in exon 7. Interestingly, the first leaky mutation described in 1990 was within the cytoplasmic tail of γ_c (p.L293Q in exon 7), and it was considered a new X-linked immunodeficiency [22,23]. Until very recently, mutations in exon 8 have been described only in association with the typical X-SCID phenotype [28], thus suggesting a critical role on the C-terminal part of the cytoplasmic domain of γ_c for correct signal transmission. Up to the present, two case reports and a cohort study have reported a hypomorphic mutation located in exon 8 (c.C982T/p.328X) [19,21,29].

Here, we examine the key role of the three amino acid region (YSE) in the γ_c intracellular domain involved in a JAK3-independent signaling pathway that leads to the leaky SCID phenotype. Our study is based on findings in a previously undescribed family with two siblings who are hemizygous carriers of a nonsense mutation in exon 8 (p.R328X) of *IL2RG*. This R328X *IL2RG* hypomorphic mutation was associated with lethal Epstein–Barr virus (EBV)-related lymphoma in the older brother and a $T^{low}B^+NK^+$ phenotype and dysgammaglobulinemia in the younger one. We found that the R328X mutation in *IL2RG* may allow partial phosphorylation of STAT-5 through a JAK3-independent pathway.

Materials and methods

Patient recruitment

Patient II.II (Fig. 1) was recruited through the out-patient clinic of the Pediatric Infectious Diseases and Immunodeficiencies Unit at Hospital Universitari Vall d'Hebron. Information concerning the deceased brother (patient II.I.) was provided by Hospital Clínico Universitario de Valencia. Informed consent for the genetic studies was obtained in accordance with our hospital guidelines. Written informed consent was provided by the patients' parents, according to the procedures of the Institutional Ethical Review Board of Hospital Universitari Vall d'Hebron (PR_AG_134-2011).

Whole exome sequencing analysis and causal variant prioritization

Genomic DNA was extracted from ethylenediamine tetraacetic acid (EDTA)-containing whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA sample quality was assessed using a Colibri spectrophotometer (Titertek-Berthold, Pforzheim, Germany). To increase the quality of the DNA samples, all were precipitated with NaCl.

DNA was sheared to a fragment size of approximately 500 base pairs (bp) using the Covaris sonicator. Whole exome sequencing (WES) libraries were prepared using the Illumina TruSeq and Nimblegen version 3 whole exome enrichment kits. Enriched libraries were pooled to contain six libraries per sequencing run on the Illumina Hiseq2500 (paired-end mode, 125 bp), resulting in an average yield of 300 million paired-end reads per sample. Following the Genome Analysis Toolkit (GATK)

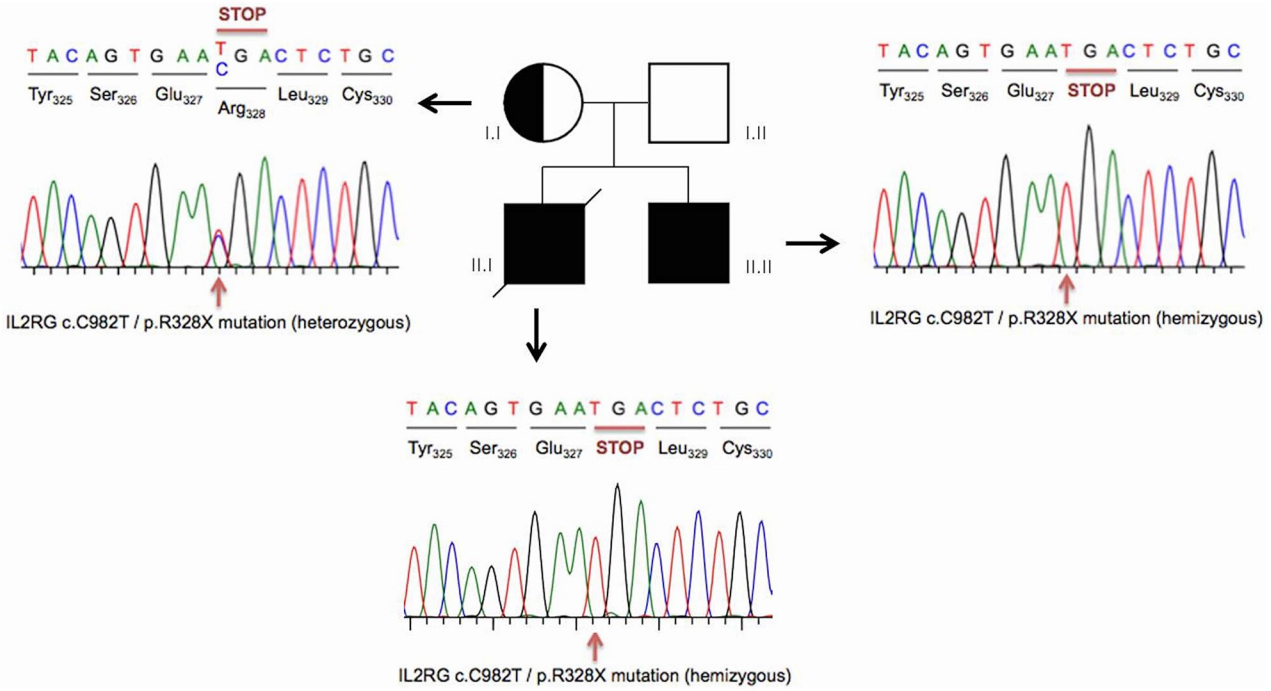

Fig. 1. Sanger sequencing electropherogram of *IL2RG* gene in the family reported. Family pedigree chart with the c.C982T/p.R328X mutation showing an X-linked recessive inheritance pattern. Both patients were hemizygous carriers of the mutation located in exon 8, which was inherited from their asymptomatic mother. Squares = males; circles= females; solid square with line across = deceased patient; bicolor circle = carrier.

best-practice guidelines, next-generation sequencing (NGS) reads were mapped to the human reference genome (hg19) using bwa mem, alignments were post-processed by base quality recalibration, indel realignment and polymerase chain reaction (PCR) duplicate marking and single nucleotide variants (SNVs), and small insertions and deletions (indels) were detected using GATK HaplotypeCaller (3.5.0) in multi-sample calling mode. To identify the disease-causing mutation, all variants were annotated and prioritized using eDiVa [30] (https://ediva.crg.eu/). To pinpoint the causal variants, eDiVA leverages information on minor allele frequencies in the population, various functional impact scores, disease-related knowledge such as previously identified disease genes and phenotypical information. The causal R328X mutation in *IL2RG* was identified by manual inspection of the eDiVA results and verified in the read alignments (BAM files) using the Integrative Genomics Viewer (IGV, https://igv.org/).

L lymphocyte proliferation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin blood by density gradient using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Cells were then stimulated for 72 h at 37°C and 5% CO_2, with several stimuli [2 μg/ml phytohemagglutinin (PHA), 1·25 μg/ml anti-CD3 (muromonab-CD3: OKT3)] and 8 ng/ml IL-2 in 96-well U-bottomed plates. After stimulation, PBMCs were then labeled with radioactive thymidine [methyl-^{3}H] (Perkin Elmer, Waltham, MA, USA), left to stand overnight and counted with a MicroBeta^2 Microplate Counter (Perkin Elmer). Samples from healthy adult donors were always used simultaneously as technical controls. The proliferation ratio was calculated as the median of triplicate counts per minute (cpm) stimulation values/median of triplicate cpm background values.

Flow cytometry

Circulating lymphocyte subpopulations were extensively immunophenotyped by flow cytometry, as previously described [31]. To study expression of CD132 (γ_c), 50 μl of EDTA blood was stained with CD132-phycoerythrin (PE) [Becton Dickinson (BD), Franklin Lakes, NJ, USA], CD127-allophycocyanin (APC) (BioLegend, San Diego, CA, USA) and CD3 Pacific Blue (BD). Briefly, samples were incubated with appropriate concentrations of monoclonal antibodies for 20 min at room temperature in the dark, and then lysed and fixed with fluorescence activated cell sorter (FACS) lysing solution (BD), following the manufacturer's instructions.

The T cell receptor (TCR) repertoire was studied using IOTest Beta Mark (Beckman Coulter, Brea, CA, USA). Acquisition and analyses were performed with FACSDiva software (BD) on a FACS Canto II cytometer (BD).

T cell excision circle quantification

Absolute quantification of TCR excision circles (TRECs) was carried out by quantitative polymerase chain reaction (qPCR) using TaqMan probes. The input sample was 100 ng of genomic DNA from whole peripheral blood. Amplification of a reference gene segment [ribonuclease P (RNase P)] was performed in parallel to ensure an absence of bias in DNA quantification and quality. qPCR was performed using the thermal cycler, TaqMan 7900 fast real-time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were run in triplicate in a 96-well plate. The primer and probe sequences and a complete description of the reaction conditions are available on request.

STAT-5 phosphorylation on flow cytometry

A 100-µl amount of heparinized whole blood from the patient was stained with CD45 Pacific Blue (BioLegend) and CD3 PE (BD) for 20 min at room temperature in the dark. After washing the sample with phosphate-buffered saline (PBS), IL-2 (Novartis, Basel, Switzerland) was added at different concentrations (100, 1000 and 5000 U/ml) and the sample was incubated for 20 min at 37ºC in the dark. Cells were lysed and fixed with lyse/fix buffer 5X (Phosflow; BD) and incubated for 15 min at 37ºC in the dark. Cells were then permeabilized with Perm Buffer III (Phosflow; BD) for 30 min on ice and in the dark. Thereafter, pSTAT-5 (pY694)-Alexa Fluor 488 antibody (BD) was incubated for 30 min at room temperature in the dark. After two washing steps, cells were acquired on a FACS Canto II cytometer (BD). Acquisition and analyses were performed with FACS Diva software (BD).

IL-2RG DNA constructs

The *IL2RG* R328X mutant DNA construct was generated with the GENEART Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, using the pEF6-IL2RGY325X-Myc construct as a template; further verification was performed by DNA sequencing (Macrogen, Seoul, Korea). A pair of primers was used to introduce nine nucleotides encoding the amino acids YSE (primer sequences available upon request). The pEF6-IL2RGWT-Myc, pEF6-IL2RGY325X-Myc and pCDNA3–JAK3 DNA constructs used in this study were kindly provided by Dr Franck Gesbert from the Normal and Pathological Development of Melanocytes group (Institut Curie, Paris, France).

Cell culture and cell transfection

African green monkey kidney (COS-7) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare, Chicago, IL, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate (both from Sigma-Aldrich, St Louis, MO, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Scientific, Waltham, MA, USA). A total of 6×10^5 cells were transiently co-transfected with the previously described DNA constructs using LyoVec Reagent (InVivoGen, San Diego, CA, USA), according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

For the immunoprecipitation studies, transfected COS-7 cells were lysed in ice-cold lysis buffer (1% Triton X-100 in 20 mM HEPES, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail: AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A at 1 µg/ml from Sigma-Aldrich. After 15 min incubation on ice, lysates were centrifuged at 14 000 ***g*** for 15 min at 4ºC and supernatants were further precleared for 30 min at 4ºC using 30 µl of protein G-Sepharose beads (GenScript, Piscataway, NJ, USA) and 1 µg of mouse immunoglobulin (Ig)G (Sigma-Aldrich). An additional preclearing was carried out for 30 min at 4ºC with 30 µl of protein G-Sepharose beads.

To immunoprecipitate γ_c, precleared lysates were incubated with 30 µl of protein G-Sepharose beads and 1 µg of anti-human JAK3 (C-21) rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 3 h at 4ºC. Immunoprecipitated proteins and proteins in the total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels, and transferred onto polyvinylidene fluoride (PVDF) membranes (Thermo Scientific). Membranes were blocked for 1 h with 5% skimmed milk and then immunostained with anti-c-myc-peroxidase (9E10) mouse monoclonal antibody (Roche Applied Science, Penzberg, Germany) and anti-human JAK3 (C-21) rabbit polyclonal antibody, followed by horseradish peroxidase (HRP)-conjugated anti-rabbit goat polyclonal antibody (Santa Cruz Biotechnology). HRP-labeled antibodies on the membranes were visualized using SuperSignal West Pico Chemiluminescent Substrate reagent (Thermo Scientific), and chemiluminescence was detected on X-ray films.

Results

Whole-exome sequencing identification of a nonsense mutation in *IL2RG* (R328X) in a non-consanguineous Caucasian family

Here, we describe a non-consanguineous Caucasian family with two affected brothers (Fig. 1). Patient II.I had a

history of atopic dermatitis, acute otitis media, mononucleosis, persistent cough, recurrent bronchitis and pneumonia, leading to bronchiectasis on chest computerized tomography (CT) scanning. At the age of 4 years, he was hospitalized due to fever and enlarged cervical lymph nodes. During his admission, he developed neutropenia, anemia and increased acute phase reactants, as well as $CD4^+$ lymphopenia (Table 1). Twenty days later he developed fulminant Epstein–Barr virus (EBV)-related B cell lymphoma and sadly died due to multi-organ failure. Lymph node biopsy demonstrated lymphoid proliferation by atypical large cells for line B antigens. The *in-situ* hybridization study evidenced EBV-encoded RNA (EBER). The final diagnosis was diffuse large B cell lymphoma associated with EBV infection with latency III viral gene expression profile (Supporting information Fig. S1). His sibling, patient II.II, an asymptomatic 8-month-old boy, was therefore studied.

Genetic analysis of the family, performed by WES, revealed a nonsense mutation, c.C982T/p.R328X in exon 8 of the *IL2RG* gene (Fig. 1). This mutation generates a stop codon affecting the intracellular domain of the γ_c chain. No additional mutations were found in any other known disease-causing genes. The genetic finding was confirmed by Sanger sequencing of samples from the patient (II.II), the deceased brother (II.I) and their mother (I.I) (Fig. 1). Based on the results, patients II.I and II.II were diagnosed with X-SCID. Anti-microbial prophylaxis and immunoglobulin replacement therapy were started on patient II.II. The patient was in good clinical condition and underwent HSCT, with a protocol consisting of busulfan, fludarabine and anti-thymocyte globulin from an unrelated cord blood donor (5/6) at 4 years of age. Despite the initial occurrence of chronic graft-*versus*-host disease, hematological and immunological recovery was achieved and the patient is alive and well 4 years after transplantation.

IL2RG R328X mutation is associated with T lymphopenia, normal B and NK cell numbers and abnormal lymphocyte proliferation

Functional studies were performed only with samples from patient II.II, as no material was available from the deceased sibling. Initial immunological characterization of patient II.II showed an absence of the thymic shadow on chest radiography (Fig. 2a) and fluctuations in the total lymphocyte count, which eventually indicated severe lymphopenia over time. Flow cytometry analysis of lymphocyte subsets at age 6 months showed a $T^{low}B^+NK^+$ phenotype (Table 1). Percentages of both B and NK cells were increased, although the absolute numbers fluctuated around the lower limit of normality. Extended immunophenotyping showed a normal B cell distribution and increased percentage of $CD56^{bright}$ NK subsets. The extended T phenotype could not be determined because of the low number of T cells (Table 2). The absolute TREC count at the age of 11 months was slightly reduced for this age (1838 TREC/100 ng of gDNA, cut-off > 2800 TREC/100 ng). *In-vitro* proliferation tests for lymphocyte function at 7 months of age showed a proliferation decrease in response to PHA and OKT3 compared to that of a healthy control. However, proliferation was not

Table 1. General immunological characterization of the affected siblings

	Patient II.II					Patient II.II		Patient II.I	
	6 months	7 months	8 months	11 months	Normal range	3 years	4 years	4 years	Normal range
Leukocytes, $\times10^9$/l	↓5·5	↓3	↓3·8	↓2·5	5·8–17·8	↓2·82	↓3·56	4·08	3·9–11·1
Lymphocytes, $\times10^9$/l	1·3	↓0·9	1·3	↓0·7	1·2–3·4	↓0·6	↓0·7	1·36	1·2–3·4
$CD3^+$, %	↓17·6	↓27	↓15	↓16·8	50–77	↓33	↓33·9	n.a.	43–76
$CD3^+$, $\times10^9$/l	↓0·23	↓0·24	↓0·20	↓0·12	2·4–6·9	↓0·20	↓0·22	n.a.	0·9–4·5
$CD4^+$, %	↓16·4	↓25	↓14·4	↓15·1	33–58	27	28	↓8	23–48
$CD4^+$, $\times10^9$/l	↓0·21	↓0·23	↓0·19	↓0·11	1·4–5·1	↓0·17	↓0·18	↓0·11	0·5–2·4
$CD8^+$, %	↓0·9	↓1·1	↓0·7	↓1	13–26	↓5·47	↓4·11	n.a.	14–33
$CD8^+$, $\times10^9$/l	↓0·01	↓0·01	↓0·01	↓0·01	0·6–2·2	↓0·03	↓0·03	n.a.	0·3–1·6
Index	↑18·2	↑22·7	↑20·6	↑15·1	1·6–3·8	↑4·94	↑6·80	n.a.	0·9–2·90
$CD19^+$, %	↑62·2	↑53·4	↑54·1	↑55·4	13–35	↑46	28·94	n.a.	14–44
$CD19^+$, 10^9/l	0·81	↓0·48	0·70	↓0·39	0·7–2·5	0·29	↓0·19	n.a.	0·2–2·1
$CD56^+CD16^+$, %	12	↑18·5	↑26·1	12·1	2–13	20	↑38·2	n.a.	4–23
$CD56^+CD16^+$, $\times10^9$/l	0·16	0·17	0·34	↓0·09	0·1–1·0	0·12	0·25	n.a.	0·1–1
TCR αβ, %	99	n.a.	n.a.	n.a.	83–97	n.a.	89	n.a.	83–97
TCR γδ, %	1	n.a.	n.a.	n.a.	2–15	n.a.	8·4	n.a.	2–15
IgG, mg/dL	308	284	646	874	196–1045	1142·9	n.a.	n.a.	360–1236
IgA, mg/dL	↓< 10	↓< 10	↓< 10	↓< 10	8–90	15·9	n.a.	n.a.	11–153
IgM, mg/dL	↓36	↓35	40	↓32	40–140	↓46·8	n.a.	n.a.	48–245

TCR = T cell receptor; Ig = immunoglobulin; n.a. = not assessed.

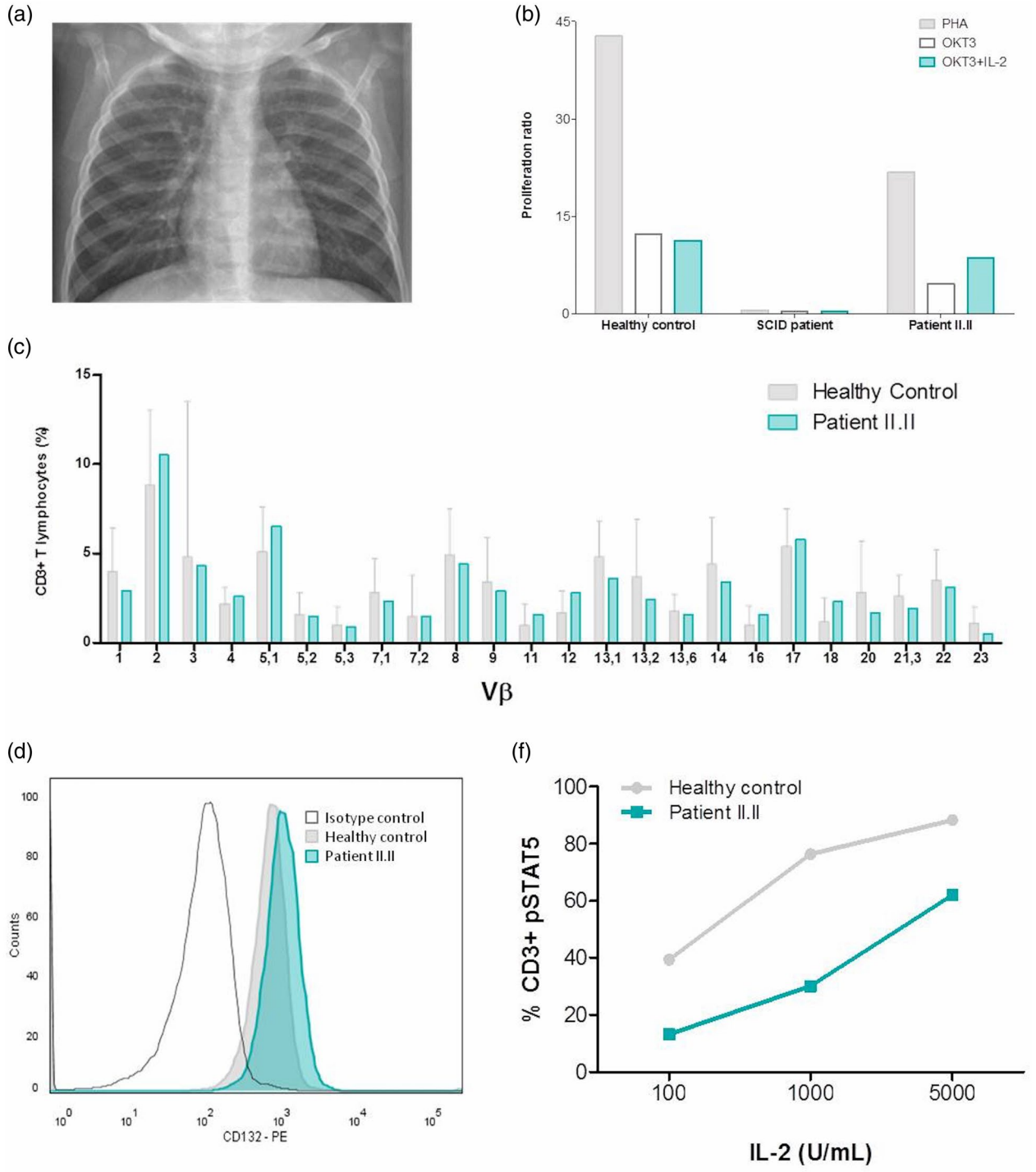

Fig. 2. Immunological studies from patient II.II. (a) Absence of thymic shadow in the chest X-ray. (b) Lymphocyte proliferation assay after phytohemagglutinin (PHA), muromonab-CD3 (OKT3) and OKT3 + interleukin (IL)-2 stimulation compared with a healthy control and a typical severe combined immunodeficiency (SCID patient. (c) Distribution of the T cell receptor-Vb (TCR-Vβ) repertoire in the patient compared with reference values of healthy controls analyzed by flow cytometry in T cells. (d) Expression of CD132 in T lymphocytes in the patient showed mean fluorescence intensity (MFI) and percentage comparable to a healthy control analyzed by flow cytometry. (f) Intracellular staining of signal transducer and activator of transcription-5 (STAT-5) phosphorylation in $CD3^+$ T lymphocytes using increasing concentrations of IL-2 compared with a healthy control analyzed by flow cytometry.

Table 2. Extended immunophenotyping of B lymphocytes, natural killer cells, dendritic cells and monocytes in patient II.II

	%	Normal range
Transitional B cells	↓9·1	10–30
Naive B cells	↑87·2	63–86
Pre-switch memory B cells	4·4	4–13
Switch memory B cells	↓3·5	4–15
Bright NK	↑29·1	2–16
Dim NK	↓66·8	72–91
Classical monocytes	85·8	68–94
Non-classical monocytes	↑11·9	2–9
Myeloid DC	↓34·3	54–83
Plasmacytoid DC	↑56·6	9–40

Extended immunophenotyping of T cells could not be performed because of low cellularity.

DC = dendritic cells; NK = natural killer cells.

absent, as one would expect in patients with typical SCID. Of particular note, proliferation with OKT3 plus recombinant IL-2 was comparable to that of a healthy control (Fig. 2b). Serum immunoglobulin levels showed changes, with reduced IgA and IgM but normal IgG levels (Table 1). The TCR-Vβ repertoire showed a polyclonal pattern on flow cytometry analysis (Fig. 2c) and spectratyping analysis (data not shown).

The R328X mutation in *IL2RG* does not affect the extracellular domain of the protein

As the R328X mutation causes a 42 amino acid truncation of the intracellular domain of γ_c, we investigated whether the mutated γ_c could be properly expressed on the cell membrane. CD132 expression, evaluated by flow cytometry, showed that the patient's lymphocytes expressed the protein with a mean fluorescence intensity (MFI) similar to that of a healthy control. Consequently, the R328X mutation allows surface expression of γ_c and does not affect the extracellular domain of the protein (Fig. 2d).

STAT-5 phosphorylation is partially preserved in *IL2RG* R328X despite impaired IL-2RG–JAK3 binding

Patients with *IL2RG* mutations typically show a decrease or absence of STAT-5 tyrosine phosphorylation in response to IL-2, due to impaired interaction with JAK3 tyrosine kinase. However, we observed partial dose-dependent STAT-5 phosphorylation on $CD3^+$ lymphocytes of patient II.II, at a reduction of approximately 30% compared to a healthy control. The results were reproducible at three different IL-2 concentrations (Fig. 2f).

Signaling through γ_c-containing receptors requires association of JAK3 with the intracellular domain of γ_c. To assess whether the mutant protein R328X interacts correctly with JAK3, we carried out a series of co-immunoprecipitation experiments on three different plasmid constructs (WT, R328X and Y325X). The *IL2RG* WT chain was used as a positive control of the interaction with JAK3, whereas the *IL2RG* Y325X mutant, described by Niemela *et al.* as producing typical X-SCID, was used as a negative control [32]. The results showed that the γ_c chain containing the R328X mutation did not co-precipitate with JAK3, as occurred with the typical X-SCID mutant, Y325X (Fig. 3).

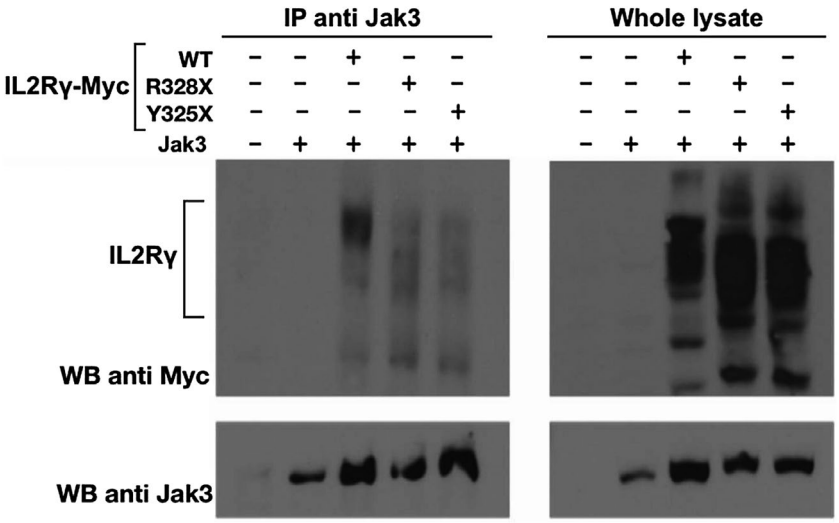

Fig. 3. Co-precipitation of Janus kinase (JAK3) and interleukin (IL)-2RG R328X. African green monkey kidney (COS-7) cells were transiently transfected with Myc-tagged IL-2RG wild-type (WT) or Y325X or R328X mutants in combination with JAK3. Cell lysates were immunoprecipitated with anti-JAK3 in all cases. Western blots were conducted with the indicated antibodies. Whole cell lysates were included as controls.

Discussion

In this study, we report on a family with two brothers carrying a hypomorphic mutation in exon 8 of *IL2RG* (c.C982T/p.R328X), who showed an atypical clinical presentation and a leaky $T^{low}B^{+}NK^{+}$ SCID phenotype. Whereas patient II.II was asymptomatic at the diagnosis, patient II.I showed clinical features suggestive of X-linked lymphoproliferative syndrome and developed lethal fulminant EBV-related B cell lymphoma. We found that this mutant does not interact with JAK3, but is able to partially phosphorylate STAT-5. This result suggests the existence of an independent JAK3 signaling pathway that would explain the leaky SCID phenotype observed in both patient II.II. and the patient reported by Tanita [21], as contrasted with the patients reported by Lim *et al*. [19].

The R328X mutation was first reported in 2017 in a cohort of 147 Asiatic SCID patients [29], but detailed clinical and immunological information has only been described recently in two patients with the same mutation [19,21]. Lim *et al.* reported the R328X mutation in a 16-year-old Kurdish boy with chronic airway hypersensitivity and recurrent sinopulmonary infections [19] and Tanita *et al.* described the same mutation in a 21-year-old Japanese male with recurrent respiratory infections, EBV-associated leiomyoma during childhood and invasive *Haemophilus influenzae* infection [21]. Both the patient reported by Tanita *et al.* and patient II.I from the present study developed respiratory infections and EBV-triggered disease.

A complete loss-of-function mutation in *IL2RG* leads to an arrest of lymphocyte development, resulting in the typical X-SCID $T^{-}B^{+}NK^{-}$ phenotype, whereas patients with hypomorphic mutations often show some T and NK cells, although usually in a reduced number due to partial functioning of γ_c, which allows cell development through IL-7 and IL-15 signaling [31]. The three patients carrying the R328X mutation described to date had low T cell counts and normal or even increased NK cell counts, resulting in an atypical leaky SCID phenotype (Table 3). Differences in these and other immunological parameters contribute to making the diagnosis of leaky SCID difficult. For example, immunoglobulin levels, which are usually absent in X-SCID, varied between the three patients. Patient II.II and the patient reported by Tanita *et al.* showed several degrees of dysgammaglobulinemia, whereas the patient reported by Lim *et al.* had normal immunoglobulin levels. On the contrary, despite the absence of a thymic shadow observed in our patient, the polyclonal T repertoire and slightly reduced TREC levels indicated some residual thymic function. However, the profile of the gamma TCR repertoire in the 21-year-old patient described by Tanita *et al.* showed oligoclonal expansions of ɣ-expressing clonotypes expanded by EBV. These differences and the difficulties in comparing the characteristics of the T repertoire in these cases may have to do with the different ages, infectious profile and type of repertoire studied, as our patient had no infection by EBV, and we studied the TCR beta repertoire. With these data, it is tempting to speculate that mutation carriers may generate a polyclonal repertoire of TCR alpha/beta and it seems plausible that, in the situation of suffering an EBV infection, the virus may have a potential advantage affecting immune function and playing a fundamental role in the evolution of the disease [19,21].

The clinical and laboratory data from patient II.II and the two previous cases suggest a hypomorphic effect of the R238X mutation. Several hypomorphic mutations on *IL2RG* have been reported to date (Fig. 4). However, R328X is the only one located on exon 8 which, together with exon 7, codes for the cytoplasmic domain of γ_c. Various publications have analyzed the role of γ_c in the context of IL-2 receptor signaling. The cytoplasmic domain of γ_c, containing 86 amino acids, is required for JAK1 and JAK3 activation. The R328X mutation leads to a truncated protein lacking 42 amino acids on the cytoplasmic domain containing the JAK3 binding site [33–36]. Lim *et al*. suggested that the R328X mutation affected protein expression, based on the results of Western blot analysis. Nevertheless, we detected normal γ_c levels on the lymphocyte surface by flow cytometry, in accordance with the observations of Tanita *et al*., thus indicating that the mutation does not affect expression of the protein (Fig. 2d).

As γ_c surface expression was normal, STAT-5 phosphorylation was examined to evaluate the functionality of the receptor. STAT-5 phosphorylation was examined in residue Y694 to evaluate the functionality of the receptor. Phosphorylation of residue Y694 is the most widely studied in T cell function experiments in patients with suspected severe combined immunodeficiency and shows a strong correlation with lymphocyte proliferation results, as demonstrated by Bitar *et al* [37]. Our results, using three different concentrations of IL-2, showed an average decrease of 30% in STAT-5 phosphorylation compared to that of a healthy control (Fig. 2f). Similar results were reported by Tanita *et al.* (partial reduction in STAT-5 phosphorylation of 50%), whereas Lim *et al.* reported no STAT-5 phosphorylation (Table 3). This partial response to IL-2 is consistent with the lymphoproliferative response after anti-CD3 stimulation, which recovered after addition of IL-2 (Fig. 2b). It is important to highlight these findings, as they would theoretically exclude defects in the *IL2RG* gene and could lead to an incorrect diagnosis in patients showing these abnormalities.

Several reports have demonstrated the importance of the membrane-proximal region of the cytosolic tail of γ_c

Table 3. Characteristics of reported patients carrying the *IL2RG* R328X nonsense mutation

	Lim *et al.* [19]	Tanita *et al.* [21]	Arcas-García *et al.*
Patient (age, ethnicity)	16-year-old Kurdish male	21-year-old Japanese male	4-year-old and 6-month-old Caucasian males
Consanguinity	Yes	No	No
Medical history	Enteroviral infections with skin rash and diarrhea, chronic cough and fever due to *Moraxella catarrhalis,* otitis, adenopathy and shingles, bronchiectasis and a right middle lobe atelectasis	Recurrent respiratory infections, EBV-associated leiomyoma, complement deficiency, *Yersinia* enteritis, pleurisy, chronic cough, purpura, edema and pain in the lower limbs and leukocytic fragmentative vasculitis	4-year-old atopic dermatitis, acute otitis media, mononucleosis and persistent cough, recurrent bronchitis and pneumonia 6-month-old asymptomatic
Family history	Recurrent infections and several early deaths due to lung failure on the maternal side	No family history suggestive of immunodeficiency	Older brother (4 years old) died of B cell lymphoma associated with EBV infection
Clinical phenotype (at diagnosis)	Chronic airway hypersensitivity and recurrent sinopulmonary infections	Invasive *Haemophilus influenzae* infection and recurrent pneumonia	Asymptomatic*
Immunological findings			
Thymic shadow	n.a.	n.a.	Absence
Blood count	Normal	Normal	Leukopenia with lymphopenia
Immunophenotype	$T^{low}B^{+}NK^{+}$	$T^{low}B^{low}NK^{high}$	$T^{low}B^{+}NK^{+}$
γδT cells	n.a.	Increased	Normal
Extended immunophenotype	n.a.	T cells skewed to the memory phenotype (especially TCM)	Normal distribution of B cell subsets and increased percentage of bright NK cells
Immunoglobulin levels	Normal	Dysgammaglobulinemia	Dysgammaglobulinemia
Lymphocyte proliferation	Variable	Reduced	Variable
TRECs	n.a.	Undetectable	Slightly reduced
TCR-Vβ repertoire	n.a.	Oligoclonal	Polyclonal
Viral examination	High titers of IgG anti-CMV	Positive EBV RT–PCR Positive CMV RT–PCR	Negative EBV RT–PCR Negative CMV RT–PCR Negative adenovirus RT–PCR
Genetic findings	*IL2RG* c.C982T/p.R328X	*IL2RG* c.C982T/p.R328X *C9* c.C346T/p.R116X	*IL2RG* c.C982T/p.R328X
IL-2RG expression	Absence (WB)	Normal (FC)	Normal (FC)
STAT-5 Phosphorylation	Defective (WB)	Partially defective (FC)	Partially defective (FC)
STAT-3 Phosphorylation	n.a.	Partially defective (FC)	n.a.
STAT-6 Phosphorylation	n.a.	Partially defective (FC)	n.a.
JAK3 expression	Absence of the main isoform and expression of the second isoform	n.a.	n.a.
JAK3 phosphorylation	Defective (Western blot)	n.a	n.a.
IL2RG/JAK3 Interaction	n.a.	n.a.	No interaction
Others	n.a.	CD107a expression	
Southern blot analysis of TCR-β chain	n.a.		

CMV = cytomegalovirus; EBV = Epstein–Barr virus; FC = flow cytometry; NK = natural killer; RT–PCR = real-time–polymerase chain reaction; TCM = T cell central memory; TCR = T cell receptor; TRECs = T cell excision circles; WB = Western blot; n.a. = not assessed.

*Data shown in the table correspond only to the 6-month-old patient.

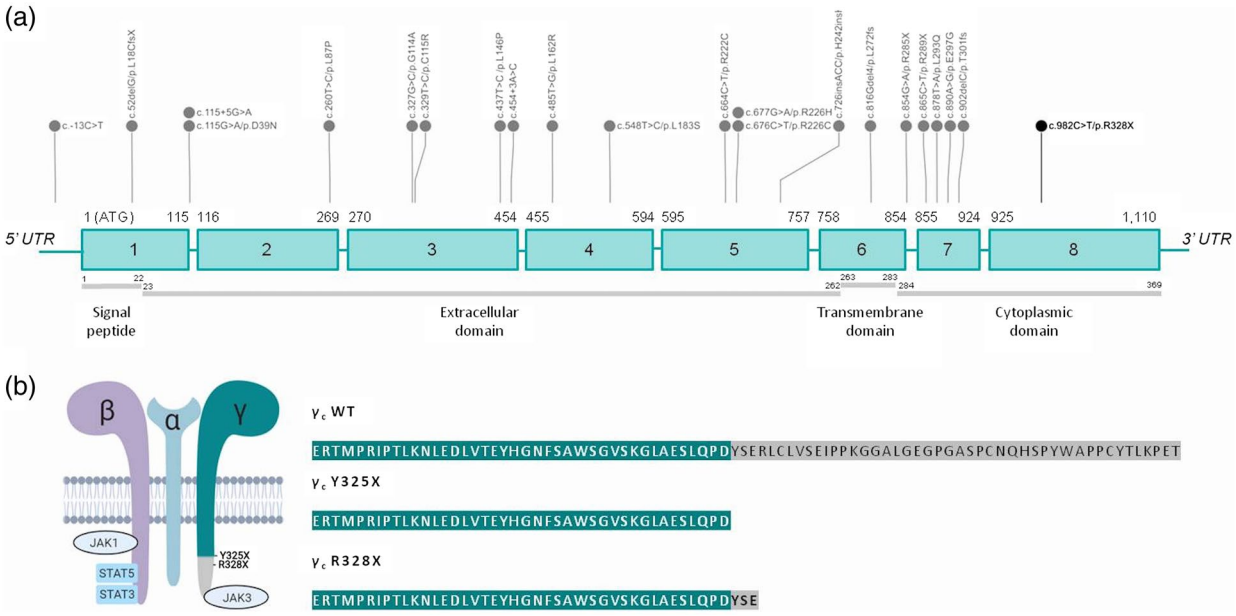

Fig. 4. Gene map of *IL2RG* gene showing leaky mutations and detail of the constructs used in the co-precipitation experiments. (a) Previously reported leaky mutations are indicated with gray dots. R328X mutation is indicated with a black dot. (b) Schematic representation of the interleukin (IL)-2 receptor showing the location and sequence of the mutants used in the co-precipitation experiments (Y325X and R328X). c.-13C>T [40], c.52delG [41], c.260T>C [42], c.890A>G [43]. All other mutations are listed in [19].

for interactions with other proteins involved in signaling [34,35]. As JAK3 binding to the γ_c chain is required for STAT-5 phosphorylation, we wanted to assess whether the R328X mutation would result in partial interaction with JAK3, thus explaining the leaky phenotype and partial STAT-5 function observed. However, the results of co-immunoprecipitation experiments concluded that R328X did not co-precipitate with JAK3, similar to what happens with the typical X-SCID mutant Y325X. Unfortunately, there are no data on the STAT-5 phosphorylation levels associated with Y325X [30], but the severe phenotype of patients with this mutation suggests that phosphorylation is completely lacking.

The mutated protein R328X has three conserved residues (Y325, S326 and E327) compared to Y325X and appears to preserve some of its function, which would be in accordance with the atypical phenotype of SCID (Fig. 4b). Several studies have investigated the impact of mutations on the intracellular tail of γ_c. Nelson *et al.* reported that no JAK3 binding occurs in the absence of the last 46 residues of the intracytoplasmic tail, but when only the last 33 amino acids are absent can the IL-2R complex signal normally [33]. These findings, together with our data, suggest that the 3-amino acid region (YSE) may play a crucial role in allowing STAT-5 phosphorylation by a JAK3-independent pathway. The Y325X mutation, which lacks the YSE region, is associated with a total or near-total lack of STAT-5 phosphorylation, whereas R328X allows some phosphorylation in the absence of an interaction with JAK3. Hence, we hypothesize that the preserved amino acids 326–328 enable stabilization of binding with other kinases to the intracellular area of the receptor, providing partial STAT-5 phosphorylation.

It is well established that both JAK1 and JAK3 are associated with the intracellular domains of cytokine receptors containing γ_c and cooperate in signaling through them [38]. In this case, there was partial phosphorylation of STAT-5 in the absence of JAK3 binding to the γ_c chain intracellular domain, which could indicate that other kinases, such as JAK1, can phosphorylate STAT-5 if a particular region of the proximal γ_c chain is preserved. This would be sustained by studies showing that JAK1 has a dominant role over JAK3 in STAT-5 activation downstream of γ_c-containing cytokine receptors [39]. We then suggest that JAK1 may allow STAT-5 phosphorylation, even though there is no direct binding of JAK3 to R328X. Additional research is needed to further explore these findings.

The diagnosis of X-linked SCID should not be excluded in patients with partially preserved STAT-5 phosphorylation or with a partial proliferative response to stimulation with anti-CD3 and IL-2. Accordingly, the functional outcomes in patients with leaky SCID should be interpreted with caution.

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Disclosures

The authors declare no commercial or financial conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Pathological study of Epstein–Barr virus-encoded small RNAs (EBERs) associated diffuse large B-cell lymphoma in lymph node biopsy. Hematoxylin and eosin (H&E) staining. CD20, CD3, CD10, CD30 and Ki67 staining. EBER positive staining (immune-score 3+) in the nuclei of tumor cells.