Clinical and immunologic consequences of a somatic reversion in a patient with X-linked severe combined immunodeficiency

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X-linked severe combined immunodeficiency is a life-threatening disorder caused by mutations in the gene encoding the interleukin-2 receptor gamma chain (IL2RG). Hypomorphic mutations and reversion of mutations in subpopulations of cells can result in variant clinical phenotypes, making diagnosis and treatment difficult. We describe a 5-year-old boy with mild susceptibility to infection who was investigated for a mutation in IL2RG due to persistent natural killer (NK)– and T-cell lymphopenia. A functionally relevant novel T466C point mutation was found in B, NK, and epithelial cells, whereas α/β and γ/δ T cells showed the normal gene sequence, suggesting reversion of the mutation in a common T-cell precursor. This genetic correction in T cells resulted in a diverse T-cell repertoire and significant immunity despite failure to produce specific antibodies linked to an intrinsic defect of mutant B cells. These observations confirm the potential of revertant T-cell precursors to reconstruct immune function, but questions remain on the longevity of revertant cells implicating the need for careful follow up and early consideration of hematopoietic stem cell transplantation (HSCT). (Blood. 2008;112:4090-4097)

Introduction

Severe combined immunodeficiency (SCID) is a life-threatening disorder due to impairment of T-cell development, function, or both.1,2 The molecular basis of the clinical syndrome is heterogeneous and includes mutations in genes involved in metabolism, signaling, and DNA repair. The most frequent form of SCID is caused by mutations in the gene encoding the common gamma chain of the interleukin-2 (IL-2) receptor (IL2RG).3 In the absence of a functional yc gene, early lymphoid progenitor cells are unable to respond to the cytokine signals of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 that are crucial for the normal development of T cells, natural killer (NK) cells, and late-stage B cells.4 Clinically, this X-linked form of SCID is characterized by severe and persistent infections starting in the first few months of life typically accompanied by diarrhea and failure to thrive.5 Affected infants lack T cells and NK cells and show hypogammaglobulinemia despite normal B-cell counts. Without bone marrow transplantation, the disease is usually fatal within the first year of life.

Not all patients with mutations in the IL2RG gene or other genes associated with SCID, however, show the typical phenotype of severe immunodeficiency, rendering the diagnosis more difficult.6,13 In those patients, variable numbers of residual functional T cells attenuate the phenotype. The most common molecular cause for this phenomenon is hypomorphic mutations allowing sufficient function of the affected protein to generate some T cells. T cells can also be acquired by materno-fetal transfusion.14,15 Usually, however, these maternal T cells are poorly functional and not able to significantly attenuate the clinical phenotype.15 One patient with moderate immunodeficiency has been described who carried a point mutation in the IL2RG gene in all investigated epithelial and hematopoietic cells except for T cells.10 This patient most likely had a genetic reversion in a T-cell precursor. This reversion allowed the generation of a surprisingly large repertoire of T cells, leading to a significant attenuation of the clinical phenotype.16

In this study, we describe the clinical and molecular details of a second patient with a genetic reversion of a point mutation in the IL2RG gene in a common T-cell precursor. In addition, we provide clinical follow-up information on the first patient. The results illustrate that a genetic reversion presumably occurring in a single T-cell precursor is sufficient to provide a T-cell repertoire that can turn an otherwise lethal disease into a mild immunodeficiency. However, close monitoring is required because of uncertainty regarding the stability of the clinical and cellular immunologic phenotype.

Methods

Informed consent for the performed studies was obtained from the patient’s family in accordance with the Declaration of Helsinki and Institutional Review Board approval from the University of Freiburg Ethics Committee.
Immunophenotyping

Lymphocyte phenotyping was performed with 4-color flow cytometry on a FACScan (BD Biosciences, Cowley, United Kingdom) using antibodies from BD PharMingen (San Diego, CA) and Immunotech (Hamburg, Germany) as described.17,18

TCR CDR3 spectratyping

TCR CDR3 spectratyping was performed as described by Pannetier et al for the analysis of the TCR β chain19,20 and for the TCR γ and δ chains as described previously.17 cDNA was prepared from peripheral blood mononuclear cell (PBMC) RNA, amplified using Vβ-, Vγ-, or Vδ-specific primers and the size distribution of polymerase chain reaction (PCR) products was determined by an automated sequencer and GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA). Data on size, height, and area of each peak were extracted and plotted using Plot software version 0.997 (Michael Wesemann, http://plot.micw.eu). Each family of peaks was then graphically amplified until the maximal peak of that family reached a standard height. Therefore, the graphs provide information only about the distribution of CDR3 lengths for a given primer combination, not about the abundance of the PCR product.

Functional T- and B-cell assays

Global T-cell proliferation in response to mitogens and antigens was quantified using a standard [3H]thymidine incorporation assay. Proliferation of single CD4+ and CD8+ T cells was determined in a CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester) proliferation assay.17 For this, “CD3+” T cells from the patient and a healthy control were sorted using a “no touch” magnetic beads procedure according to manufacturer’s instructions (MACS Cell Separation with Pan T Cell Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany). To provide “accessory cells,” which are needed for an appropriate response to phytohemagglutinin (PHA), the T cells were supplemented with autologous CD3-depleted PBMCs at a ratio of 1:1. The cell mixture was labeled with CFSE and plated at 2 × 10^5 cells per well for stimulation with 1.25 μg/mL PHA or with 10 μM anti-CD3/anti-CD28–coated beads (Dynabeads CD3/CD28 T Cell Expander; Dynal Biotech, Hamburg, Germany). After 6 days of culture, the cells were stained with anti-CD4 and anti-CD8 antibodies and the CFSE dilution was determined by flow cytometry. Cytotoxic T lymphocyte (CTL) degranulation and interferon-γ production were studied in day-2 PHA blasts using antibodies against CD107a and CD107b as described previously.17 B-cell function in vitro was tested as described previously.18

STAT5 and STAT6 tyrosine phosphorylation assays

IL-2 (10^4 units; Chiron, Berkeley, United Kingdom) or IL-4 (5 ng/mL; R&D Systems, Abingdon, United Kingdom) was added to 10^6 PBMCs or Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (LCLs), incubated at 37°C for 10 minutes, followed by addition of FACS Lyse/Fix (BD Biosciences) according to the manufacturer’s instructions. After washing the cells with phosphate-buffered saline (PBS)/1% fetal calf serum (FCS), cold Perm Buffer III (BD Biosciences) was added followed by antibodies (STAT5 pTyr Alexa 468, STAT6 pTyr phycocerythrin [PE], and CD4 peridinin chlorophyll protein [PerCP], BD Biosciences) according to the manufacturer’s instructions. The cells were then washed and fixed (FACSfix; BD Biosciences). Ten thousand lymphocyte events were acquired (FACScalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Genetic analysis

Genomic DNA covering the IL2RG gene was amplified with primer pairs for all exons, including intron-exon boundaries yielding overlapping amplimers. These amplimers were sequenced in both directions. Primer sequences for amplification and sequencing are available on request (klaus.schwarz@uni-ulm.de). Lymphocyte subsets were sorted using a MoFlo high-speed cell sorter (Dako, Carpinteria, CA) to more than 90% purity before DNA extraction for genetic analysis. Short tandem repeat (STR) analysis was performed with the AmpF/STR Identifier PCR amplification kit (Applied Biosystems) according to the manufacturer’s description. Three-dimensional modeling of the IL2R common gamma chain was based on the IL2RG structural data deposited in the RCSB-PDB database using the Swiss-PdbViewer, which is available at http://www.expasy.org/spdbv (The Swiss Institute of Bioinformatics).

X-inactivation analysis

To assess the inactivation status of the X chromosome, the methylation status of the fragile X mental retardation gene locus (FMR1)24 was studied, because the androgen receptor locus was not informative in this family. Undigested DNA samples and DNA samples digested with the methylation-sensitive enzyme HpaII were amplified with fluorescence-tagged PCR primers flanking the CGG repeat within the FMR1 gene.24 A PCR fragment derived from a plasmid vector containing 4 HpaII sites as well as DNA from a male donor was included as a control for complete HpaII digestion. PCR products were analyzed on an ABI 3100 capillary genetic analyzer and GeneScan and Genotyper software (Applied Biosystems). The degree of X inactivation was calculated as (p1/p1+p2)/(p1/p1 + pd2/pd2), where pd1 and pd2 represent the peak integrals of the stronger and weaker HpaII-digested allele, respectively, and p1 and p2 are the corresponding peak integrals from the undigested samples. Primer sequences are as follows: FMR1F, 5’-GCTCAAGCTCCGTTCGGTTTCTCACCTCCGGT-3’ and FMR1r, 5’-AGCCCGCACTCCACCACAGCTCTCACA-3’.

Results

Case report

The patient (P2) is the first child of nonconsanguineous healthy German parents. A maternal uncle died at the age of 6 months following recurrent pneumonias. Apart from several episodes of mild obstructive bronchitis, the medical history was unremarkable until the age of 14 months, when he presented with diffuse eruptions of round erythematous papular skin lesions. A biopsy showed granulomas but no evidence for fungal or mycobacterial infection. The lesions spontaneously resolved with some scarification over a period of 8 months. At the age of 21 months he was admitted for septic infection with Salmonella enteritidis that responded well to antibiotic therapy. In the following 4 months, 3 episodes of fever and neutropenia were noted. All of these episodes responded well to antibiotics; microorganisms could not be isolated. Neutropenia was thought to be infection associated and eventually resolved, but lymphopenia of around 10 × 10^9/L (1000 μL) persisted, leading to further immunologic investigations described in “Basic immunologic findings.” The molecular diagnosis was established at 5 years of age. Until then, the boy had been without specific treatment. Measles, mumps, and rubella (MMR) live vaccine was tolerated without complications. He was started on prophylactic cotrimoxazol and on subcutaneous immunoglobulin substitution after the finding of mild bronchiectasis. Under this therapy, the patient is presently 6.5 years old and in excellent health with no more significant episodes of bronchitis.

Basic immunologic findings

Between the age of 5 and 7 years, immunologic investigations of P2 (Table 1) showed a low number of circulating T cells (470-1050/μL) with a CD4/CD8 ratio below 1 and a significantly increased percentage of γδ T cells. The percentage of CD4+ T cells expressing CD45RA was below 10%. The absolute number of circulating B cells was normal, whereas the number of NK cells was severely reduced (12-22/μL). The patient had IgG levels slightly below the normal range, whereas IgM was normal and IgA was elevated. An appropriate vaccination protocol and diagnostic

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booster vaccinations failed to elicit antibody responses to tetanus, diphtheria, pertussis, mumps, measles, and hepatitis B, whereas low antibody titers could be detected after rubella vaccination. T-cell proliferation was reduced to PHA and PWM as well as to tetanus, PPD, and Candida. For comparison, Table 1 also lists the basic immunologic data of a previously published patient with a reversion in the IL2RG gene (P1), who was described in detail by Stephan et al.10 and Bousso et al.16

Genetic analysis

Because of the cellular phenotype (Tlow, NKlow) and the family history, the gene encoding ε was sequenced. A T→C transition in exon 3 at nucleotide 466 was detected. Interestingly, sequence analysis of DNA derived from PBMCs showed a dual signal with about 40% wild-type and 60% mutated sequence. After presence of maternal cells was excluded by STR analysis, we extracted DNA from sorted lymphocyte subpopulations and a buccal swab probe. Wild-type sequence was detected in all αβ and γδ T cells, whereas mutated sequence was detected in DNA isolated from CD19+ B cells, CD3−CD16−CD56+ NK cells, granulocytes, and the buccal swab (Figure 1A). The mutation predicts a Leu151Pro substitution in the extracellular part of the protein (Figure 1B). The same T466C transition was found in a heterozygous state in the mother, whereas the father showed a normal sequence in both alleles. We interpreted these data to reflect a reversion of the ε mutation in a T-cell precursor before versus versus lineage commitment.

Functional relevance of the ε mutation

Since the mutation has not been described previously, we sought to assess its functional relevance. Normal levels of CD132 were

<table>
<thead>
<tr>
<th>Table 1. Basic immunologic data</th>
<th>P1’</th>
<th>P2</th>
<th>Normal range</th>
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<tr>
<td><strong>T cells</strong></td>
<td></td>
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<td></td>
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<tr>
<td>CD3+ /μL</td>
<td>500-2050</td>
<td>475-1059</td>
<td>900-4500</td>
</tr>
<tr>
<td>CD4+ /μL</td>
<td>250-828</td>
<td>105-273</td>
<td>500-2400</td>
</tr>
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<td>CD8+ /μL</td>
<td>360-1860</td>
<td>133-272</td>
<td>300-1600</td>
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<td>% γδ + of CD3+</td>
<td>12</td>
<td>38-51</td>
<td>&lt;10</td>
</tr>
<tr>
<td>% CD45RA+ of CD4+</td>
<td>4</td>
<td>7.0-8.9</td>
<td>50-86</td>
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<tr>
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<td>CD19+/CD20+ /μL</td>
<td>930-2400</td>
<td>257-676</td>
<td>200-2100</td>
</tr>
<tr>
<td><strong>NK cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3−CD16−CD56+/μL</td>
<td>&lt;10</td>
<td>12-22</td>
<td>100-1000</td>
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<tr>
<td><strong>Immunoglobulin</strong></td>
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<td></td>
<td></td>
</tr>
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<td>IgG, g/L</td>
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<td>4.97</td>
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<td>&lt; 100</td>
<td>1950</td>
<td>450-1350</td>
</tr>
<tr>
<td>IgM, mg/L</td>
<td>600</td>
<td>740</td>
<td>460-1900</td>
</tr>
<tr>
<td>Specific IgG</td>
<td>t/d/p–</td>
<td>t/d/p/h/mu/me–; r+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Proliferation, cpm x 10^-3</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>3.3 ± 1.3</td>
<td>24.9</td>
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</tr>
<tr>
<td>PWM</td>
<td>5.0 ± 3.0</td>
<td>6.6</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Tetanus</td>
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</tr>
<tr>
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<td>779</td>
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</tr>
<tr>
<td>Candida</td>
<td>NA</td>
<td>569</td>
<td>&gt;1000</td>
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</table>

PWM indicates pokeweed mitogen; PPD, purified protein derivative of tuberculin; t, tetanus; d, diphtheria; p, pertussis; h, hepatitis b; mu, mumps; me, measles; r, rubella; and NA, not available.

*Patient described by Stephan et al.10

Figure 1. Genetic analysis. (A) DNA sequence analysis in PBMCs from a healthy control (wild type) and various cell populations from the patient obtained either by buccal swab, Ficoll separation (granulocytes), or flow cytometric cell sorting. The wild-type (left) and mutated nucleotide (right) sequences and the resulting amino acid change are shown below. (B) Location of the changed amino acid in a model of the IL-2 receptor with its bound ligand as calculated with the SWISS-MODEL software (The Swiss Institute of Bioinformatics). Brown indicates γ chain; green, β chain; blue, α chain; and purple, IL-2.
detected on the patient’s B cells, indicating that the mutation did not affect protein expression (Figure 2A). CD132-dependent signal transduction was analyzed by determining STAT-5 phosphorylation after IL-2 stimulation and STAT-6 phosphorylation after IL-4 stimulation of EBV-transformed B-cell lines. The use of these cell lines allowed including a control from a patient with a known loss-of-function mutation (c.545G>A [Cys182Tyr]) in the IL2RG gene. We did not detect STAT-5 phosphorylation in response to IL-2 (Figure 2B), but did detect residual STAT-6 phosphorylation in response to IL-4 (Figure 2C). This result was confirmed in fresh B cells (Figure 2D), indicating that the Leu151Pro mutation in CD132 allowed some signaling, at least when paired with the IL-4 receptor alpha chain. The absence of mutated T cells in the patient’s B cells, he failed to produce specific antibodies to most antigens. We therefore determined the differentiation status of his B cells and found a normal fraction of mature IgM+IgD+ B cells. Both stimuli did not induce significant IgM or IgG production, although some IgA production was observed. These data suggest that the impaired responses in the thymidine incorporation assay (Table 1) were due to the low number of T cells and not due to a principal defect in T-cell proliferation. We also analyzed the ability of PHA blasts to produce IFN-γ and to degranulate in response to stimulation with anti-CD3/anti-CD28-coated beads and found no difference compared with control cells (Figure 4C). Overall, the revertant T cells appeared to function normally.

Although residual STAT-6 phosphorylation was detectable in the patient’s B cells, he failed to produce specific antibodies to most antigens. We therefore determined the differentiation status of his B cells and found a normal fraction of mature IgM+IgD+ B cells with few transitional B cells, but few CD27+ non-class-switched and class-switched memory B cells, suggesting impaired terminal B-cell differentiation. (Figure 5A). We then analyzed the ability of the patients PBMCs to respond to stimulation with the mitogens PWM (a T cell–dependent stimulus) and SAC plus IL-2 (T-cell independent for memory B cells). Both stimuli did not induce significant IgM or IgG production, although some IgA production was observed. These data suggest that the lack of specific antibodies was due to an intrinsic defect in the mutated B cells.

**Discussion**

In this study, we describe the clinical and immunologic consequences of a somatic reversion in a common T-cell precursor for a...
child with a novel T466C mutation in the \textit{IL2RG} gene. The mutation impaired tyrosine phosphorylation of STAT-5 in response to IL-2 and led to severely impaired development of T cells as indicated by the absence of mutated T cells in the child and the completely skewed X inactivation in the T cells of the mother.\textsuperscript{26} The mutation also severely impaired NK-cell development.\textsuperscript{27} The few detected NK cells carried the mutation and may reflect either residual NK-cell development in the absence of \(/\beta\textsuperscript{28}\) or some remaining IL-15 signaling.

The \(/\beta\) mutation was found in most hematopoietic cells and cells obtained by a buccal smear, but not in the T cells of our patient. Since maternal engraftment was excluded and the mother carried the same mutation, we interpreted this as somatic reversion of the mutation in a committed T-cell precursor as has been described in P1.\textsuperscript{10} Similar to P1, the genetic correction was due to a C to T reversion in the context of a CG dinucleotide. This dinucleotide pair is the most vulnerable to mutation\textsuperscript{29} and helps explain why a reversion occurred in these 2 patients. Genetic reversions have recently been reported in several different primary immunodeficiencies.\textsuperscript{9,30-36} A common theme is the selective survival advantage of the revertant immune cells over mutant cells, which has been amply discussed in terms of its implications for gene therapy.\textsuperscript{31} Our data confirm the enormous developmental potential of a single T-cell precursor in humans. T-cell repertoire diversity was similar to P1 and included the use of all \(V\beta\) segments analyzed as well as a diverse array of CDR3 lengths as determined by spectratyping. In P2, we also observed a significant expansion of \(\gamma\delta\) T cells with normal \(\gamma\delta\) sequence, indicating that the reversion had occurred in a T-cell precursor before \(\alpha\beta\) versus \(\gamma\delta\) lineage commitment.

The coexistence of revertant T cells and mutant B cells in our patient represents a naturally occurring experiment, allowing for the long-standing question of whether B cells are intrinsically defective in X-SCID to be addressed. It has been shown previously that IL-4–dependent functional responses such as proliferation, IgE secretion, and CD23 expression can occur in mutant B cells.\textsuperscript{37} However, another study showed that the residual IL-4 signaling in the absence of IL2RG is not sufficient for completely normal B-cell function.\textsuperscript{38} In line with these findings, in B cells from P2, STAT-6 phosphorylation in response to IL-4 was detectable, but significantly reduced, and there was no immunoglobulin production in vitro in response to mitogenic stimuli. Previous in vivo observations in X-SCID patients with mixed chimerism who underwent transplantation revealed that in 3 of 13 patients host B cells could cooperate with donor T cells to fully mature into immunoglobulin-producing cells.\textsuperscript{38} This suggested that at least some \(IL2RG\) mutations are compatible with normal B-cell function. In our patient, terminal B-cell differentiation was impaired and apart from antirubella antibodies he did not produce specific antibodies although the autologous revertant T cells appeared fully functional. These data indicate an intrinsic defect of the patient’s B cells in vitro and in vivo.

From a clinical point of view, the most important observation was that the revertant T-cell progenitor provided our patient with significant immunity. He had the phenotype of a very mild immunodeficiency with recurrent bronchitis as its main manifestation. It is well known that patients with point mutations in \(\gamma\delta\) can present...
with a large spectrum of clinical and immunologic findings. This includes normal NK- and T-cell numbers, as documented in patients with R222C, G115A, or L162R mutations,6,8,12,13,39 normal immunoglobulins reported for patients with L271G and G115A mutations,6,39-42 and even normal proliferative responses except to IL-2 in 2 patients with the R222C mutation.12,13 However, all of these patients (apart from 2 patients identified through index patients in a larger pedigree) became manifest with severe infections requiring hospitalization within the first year of life. Our observations therefore underline the need to consider the differential diagnosis of a SCID variant in a very wide spectrum of clinical settings also beyond the neonatal period.

What is the prognosis for our patient and for other patients with primary immunodeficiencies who have a milder clinical course due to genetic reversions? P1, who had a similar immunologic profile (Table 1), initially presented with interstitial pneumonia and a bacillus Calmette-Guérin (BCG) skin abscess at 6 months of age. On intravenous immunoglobulin (IVIG) and antibiotic prophylaxis, he had no severe infections between 2 and 4 years of age. After that, he developed chronic eczema, episodes of prolonged diarrhea, and recurrent respiratory tract infections leading to bronchiectasis and clubbing. At the age of 6 years, the lung disease significantly deteriorated and he received a cord blood transplant after myeloablative conditioning at 6 years, 11 months of age. He is currently 15 years of age and well with moderately reduced lung function. In that patient, T-cell repertoire stability was demonstrated between 3 and 5 years.16 Unfortunately, a close immunologic follow up was not performed thereafter, when the clinical course deteriorated. For P2, a matched sibling donor is not available, but a matched unrelated hematopoietic stem cell transplant donor has been identified. However, under subcutaneous immunoglobulin (SCIG) therapy this patient is presently in perfect health with no more significant episodes of bronchitis. Together with the family we have therefore decided not to perform hematopoietic stem cell transplantation (HSCT) at this time point.

Overall, there is currently insufficient information to answer the important question of whether the T-cell repertoire generated by revertant precursors can be sufficient to provide immunologic protection in the long term. Careful immunologic surveillance will be essential to evaluate the longevity and functionality of the life-saving genetic revertant in our patient.
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Authorship

Contribution: C.S. performed experiments and wrote the paper; U.P. and K.S. performed genetic analysis; E.W. performed CDR3 spectratyping; P.F. performed CDR3 spectratyping analysis; W.F. performed SCT in P1 and provided follow-up information on P1; T.N. provided follow-up information on P1; K.G. performed STAT5/5 phosphorylation assays; K.B. analyzed skewed X inactivation; M.S. performed B-cell phenotyping; H.E. performed structure modeling of IL2R; J.R. performed experiments; A.S.-F. helped in discussion and interpretation of genetic results; U.G.-W. provided clinical care of P2 and provided patient samples; and S.E. designed experiments and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


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