

Mutations in linker for activation of T cells (*LAT*) lead to a novel form of severe combined immunodeficiency

Chiara Bacchelli, PhD,^{a*} Federico A. Moretti, PhD,^{b*} Marlene Carmo, PhD,^b Stuart Adams, PhD,^c Horia C. Stanescu, PhD,^d Kerra Pearce, PhD,^a Manisha Madkaikar, MD, PhD,^{b,e} Kimberly C. Gilmour, PhD,^{b,f} Adeline K. Nicholas, PhD,^g C. Geoffrey Woods, MD, PhD,^g Robert Kleta, MD, PhD,^d Phil L. Beales, MD, PhD,^a Waseem Qasim, MD, PhD,^{b,f} and H. Bobby Gaspar, MD, PhD^{b,f} *London and Cambridge, United Kingdom, and Mumbai, India*

Background: Signaling through the T-cell receptor (TCR) is critical for T-cell development and function. Linker for activation of T cells (*LAT*) is a transmembrane adaptor signaling molecule that is part of the TCR complex and essential for T-cell development, as demonstrated by *LAT*-deficient mice, which show a complete lack of peripheral T cells.

Objective: We describe a pedigree affected by a severe combined immunodeficiency phenotype with absent T cells and normal B-cell and natural killer cell numbers. A novel homozygous frameshift mutation in the gene encoding for *LAT* was identified in this kindred.

Methods: Genetic, molecular, and functional analyses were used to identify and characterize the *LAT* defect. Clinical and immunologic analysis of patients was also performed and reported.

Results: Homozygosity mapping was used to identify potential defective genes. Sanger sequencing of the *LAT* gene showed a mutation that resulted in a premature stop codon and protein truncation leading to complete loss of function and loss of expression of *LAT* in the affected family members. We also demonstrate loss of *LAT* expression and lack of TCR signaling restoration in *LAT*-deficient cell lines reconstituted with a

synthetic *LAT* gene bearing this severe combined immunodeficiency mutation.

Conclusion: For the first time, the results of this study show that inherited *LAT* deficiency should be considered in patients with combined immunodeficiency with T-cell abnormalities. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Severe combined immunodeficiency, linker for activation of T cells, immunodeficiency, T-cell receptor signaling, genetic defect, T lymphopenia

T-cell receptor (TCR) signaling is an essential process, both for the development of T cells and also for their activation and function in the periphery. During thymic development, TCR engagement leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs located in the cytoplasmic tails of TCR-associated subunits through the action of Src family kinases, such as Lck and Fyn. This in turn leads to recruitment and subsequent activation of the ζ chain-associated protein tyrosine kinase of 70 kDa (ZAP-70) kinase, which then phosphorylates specific residues on the TCR adaptor protein linker for activation of T cells (*LAT*).¹ Although it has no intrinsic enzymatic activity, *LAT* performs a critical function in TCR signal transduction by acting as a scaffold protein and coupling the TCR to downstream signaling pathways through enzyme activation and second messengers, including calcium influx, through recruitment of other factors and playing an essential role in intracellular Ca^{2+} mobilization; optimal tyrosine phosphorylation of phospholipase $\text{C}\gamma 1$, Vav, and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76); extracellular signal-regulated kinase activation; CD69 upregulation; activator protein- and nuclear factor of activated T cells-mediated gene transcription^{2,3} and apoptosis.^{4,5}

Severe combined immunodeficiencies (SCIDs) are a group of immunologic disorders that arise from a variety of monogenic defects that lead to an absence of lymphocyte development and function. Overall incidence is estimated to be approximately 1 per 50,000 live births, and patients with SCID do not survive beyond the first year of life unless immunity can be restored.⁶ SCIDs are typically characterized by the abrogation of adaptive immunity and specifically by a low number of autologous T cells (<300 cells/ mm^3). The absence of mature T lymphocytes and B-cell dysfunction commonly leads to lethal complications, such as opportunistic and nonopportunistic infections, chronic diarrhea, and failure to thrive.⁷

A number of genetic defects in the TCR signaling pathway have now been shown to lead to an SCID or a less severe combined immunodeficiency (CID; in which there is greater

From ^aGenetics and Genomic Medicine and ^bInfection, Immunity, Inflammation and Physiological Medicine, UCL Institute of Child Health, London; ^cBone Marrow Transplantation and ^dthe Department of Clinical Immunology, Great Ormond Street Hospital NHS Trust, London; ^ethe Centre for Nephrology, University College London Royal Free Hospital, London; ^fthe Department of Pediatric Immunology and Leukocyte Biology, National Institute of Immunohematology, ICMR, Mumbai; and ^gthe Department of Medical Genetics, University of Cambridge.

*These authors contributed equally to this work.

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Corresponding author: H. Bobby Gaspar, MD, PhD, Infection, Immunity, Inflammation and Physiological Medicine, Molecular and Cellular Immunology Section, UCL Institute of Child Health, 30 Guilford St, London WC1N 1EH, United Kingdom. E-mail: h.gaspar@ucl.ac.uk.

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Abbreviations used

GFP: Green fluorescent protein
 LAT: Linker for activation of T cells
 NK: Natural killer
 OMIM: Online Mendelian Inheritance in Man
 SCID: Severe combined immunodeficiency
 SLP-76: SH2 domain-containing leukocyte phosphoprotein of 76 kDa
 SNP: Single nucleotide polymorphism
 TCR: T-cell receptor
 ZAP-70: ζ Chain-associated protein tyrosine kinase of 70 kDa

T-cell development, although often with abnormal function) phenotype. This includes mutations in the CD3 subunits (CD3 δ , CD3 ϵ , and CD3 ζ), CD45, and ZAP-70; IL-2-inducible T-cell kinase (ITK), ORAI calcium release-activated calcium modulator 1 (ORAI1), stromal interaction molecule 1 (STIM1), magnesium transporter 1 (MAGT1), and LCK.⁸

LAT has not been associated with a human disease, although it has always been considered a strong candidate for SCID, especially because LAT-deficient mice have severe defects in T-cell development but normal B-cell and natural killer (NK) cell populations.⁹ Here, for the first time, we report 5 patients of a multigenerational consanguineous Pakistani family with a well-defined SCID immunophenotype (T⁺B⁺NK⁺ SCID) caused by a frameshift mutation in *LAT*.

METHODS**Study approval**

Genetics studies were approved by the UCL Institute of Child Health Research Ethics Committee. Written informed consent was obtained from the patients' parents.

Genomic studies

Genomic DNA from patients and their relatives was extracted from blood according to standard methods (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany). Genotypes from DNA of 3 affected children (V.1, V.12, and V.13), their parents (IV.4, IV.5, IV.11, and IV.12), and 4 unaffected siblings (V.9, V.10, V.11, and V.14) was generated with single nucleotide polymorphism (SNP) chip arrays (GeneChip Human Mapping 250K Sty; Affymetrix, Santa Clara, Calif), according to the manufacturer's instructions. Haplotype analysis and homozygosity mapping were done with IBDfinder.¹⁰ Oligonucleotide primers flanking the exons of *LAT* (CCDS 10647) and coronin actin binding protein 1A (*CORO1A*) encompassing splice sites were used for the PCR experiments. Primer sequences are listed in Table E1 in this article's Online Repository at www.jacionline.org. PCR products were amplified with the use of Qiagen HotStar Taq DNA polymerase purified by using ethanol/EDTA precipitation and Sanger sequenced with Big Dye v1 (Applied Biosystems, Foster City, Calif) by using forward and reverse primers used for PCR. Sequence alignments were performed and visualized with Sequencher DNA sequencing assembly and analysis software (Gene Codes, Ann Arbor, Mich). SNP searches were performed in the National Council for Biotechnology Information dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>), the 1000 Genomes project (<http://browser.1000genomes.org>), the Exome Sequence Project's Exome Variant Server (<http://evs.gs.washington.edu/EVS>), and the Exome Aggregation Consortium (ExAC; Cambridge, Mass; <http://exac.broadinstitute.org>; December 2015).

Cell culture

The human leukemic Jurkat T-cell line (JE6.1)¹¹ and the derivative LAT-deficient cell lines ANJ3³ and J.CaM2^{12,13} were maintained in RPMI 1640

(Gibco, Carlsbad, Calif) supplemented with 10% FCS (Sigma, St Louis, Mo) and 10 μ g/mL each of penicillin and streptomycin (Gibco) and cultured at 37°C in a 5% CO₂ atmosphere.

Immunoprecipitation, Western blotting, and antibodies

For immunoprecipitation, Jurkat T cells were grown up to a density of 1×10^6 /mL, centrifuged, and serum starved in RPMI 1640 with 0.1% FCS for 15 to 20 hours.¹⁴ Then about 1×10^8 cells were either stimulated with anti-CD3 ϵ antibody (OKT3; BioLegend, San Diego, Calif; 50 μ g/10⁸ cells/mL PBS) for 2 minutes at 37°C or left untreated. Cells were lysed in 2 mL of ice-cold lysis buffer containing 1% Brij-97, 10 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 2 mmol/L EDTA, and a cocktail of protease and phosphatase inhibitors (1 $\times$).³ Proteins of interest were then immunoprecipitated with the Dynabeads Protein G Immunoprecipitation Kit (10007D; Novex by Life Technologies, Carlsbad, Calif), according to the manufacturer's instructions.

For Western blotting, protein samples were resolved on SDS-PAGE according to standard protocols, transferred to polyvinylidene difluoride transfer membrane (Millipore, Temecula, Calif), and immunoblotted with different antibodies. Protein loads were checked by using rabbit anti-actin antibody (A2066, Sigma). Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibodies (GE Healthcare, Fairfield, Conn), followed by ECL (Thermo Scientific, Waltham, Mass).

The antibodies used in the experiments were as follows: mouse monoclonal anti-phosphotyrosine (clone 4G10, Millipore, 05-321), mouse monoclonal anti-LAT (ab57204; Abcam, Cambridge, United Kingdom), rabbit monoclonal anti-Vav proteins (EP482Y; ab40875; Abcam), mouse purified anti-SLP-76 (625002, BioLegend).

Generation of c.44_45insT *LAT* gene-bearing lentiviral vector

The insertion of a T between base 44 and 45 of the *LAT* gene was performed with the GENEART site-directed mutagenesis system (Invitrogen, Carlsbad, Calif), according to the manufacturer's instruction. As a template for mutagenesis, we used a pMA plasmid bearing a synthetic copy of the human *LAT* gene (0957198_LAT_pMA, GENEART; see Fig E1, A, in this article's Online Repository at www.jacionline.org). The mutagenesis reaction was performed by using 2 overlapping primers containing the target mutation (see Fig E1, C), and the correct insertion of a T between base 44 and 45 of *LAT* was verified by means of sequencing, as shown in Fig E1, D and E. The mutant copy of the *LAT* gene was then excised and cloned into a Lent_SF_Ires_GFP_C427 vector (0957199_LAT_Lent_SF_Ires_GFP, GENEART; see Fig E1, B) by using *Bam*HI cloning sites. The Lent_SF_Ires_GFP_C427 vector bearing either the wild-type or mutant copy of the *LAT* gene was then used to generate lentiviruses by transfecting 293T cells.

Lentivirus production

Both the lentiviral vector bearing a wild-type copy of the *LAT* gene (0957199_LAT_Lent_SF_Ires_GFP, GENEART; see Fig E1, B), hereafter called wtLAT-GFP-lent, and the lentiviral vector bearing the mutated copy of *LAT*, hereafter called mutLAT-GFP-lent, were used for producing lentivirus, according to a previously described protocol.¹⁵ Briefly, lentiviral particles were produced by means of transient cotransfection of 293T cells in 16-cm Petri dishes with 10 mL of Opti-MEM medium (Gibco) containing 50 μ g of the corresponding lentiviral construct, 17.5 and 32.5 μ g of the packaging plasmids pMD.G2 (VSVG envelope) and pCMVdr8.74 (gag-pol plasmid; PlasmidFactory, Bielfeld, Germany), respectively, by using 1 μ L of 10 mmol/L polyethylenimine for 4 hours at 37°C in a 5% CO₂ atmosphere. Cells were then washed and incubated with 18 mL of DMEM medium (Gibco) at 37°C in a 5% CO₂ atmosphere. The viral particles were harvested 48 and 72 hours after transfection, cleared of cell debris by means of low-speed centrifugation, and filtered with 0.45-mm Stericup filters (Millipore). The lentivirus supernatant was then ultracentrifuged at 50,000g at 4°C for 2 hours, and the pellet was resuspended in Opti-MEM and stored at -80°C . Viral titers (viral particles per

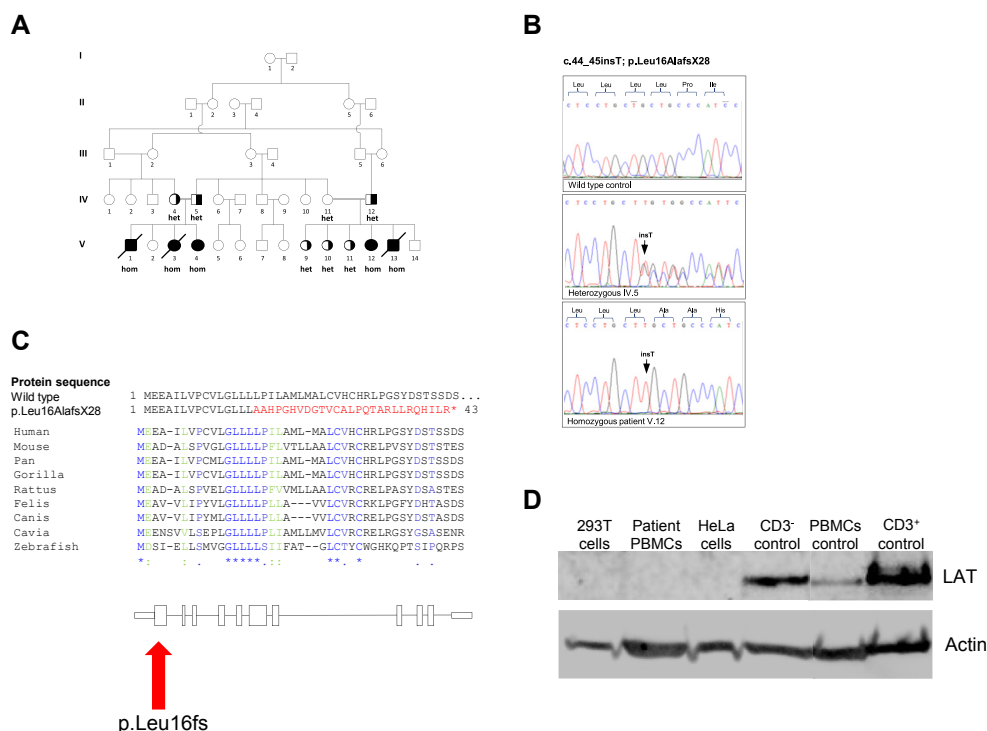


FIG 1. A frameshift mutation in *LAT* leads to loss of LAT expression in patients' T cells. **A**, Extended consanguineous pedigree. **B**, Sequence analysis of genomic *LAT* in a control unaffected heterozygous subject (IV.5) and a homozygous mutation in patient V.12. **C**, *LAT* protein, with the first 43 amino acid showing the frameshift and premature termination codon (*) in red letters in *LAT* exon 1 at position 43. Protein homology of *LAT* in various species. **D**, *LAT* expression in PBMCs from a patient and control subject.

milliliter of concentrated aliquot) were determined in 293T cells transduced with serially diluted lentivirus aliquots by means of flow cytometry to measure the green fluorescent protein (GFP) expression.

Lentiviral transduction of LAT-deficient Jurkat T-cell lines

Both ANJ3 and J.CaM2 cells (2.5×10^5 cells per well) were covered with 0.4 mL of medium (RPMI 1640, 10% FCS, $1 \times$ penicillin/streptomycin) containing either wtLAT-GFP-lent or mutLAT-GFP-lent lentivirus at a multiplicity of infection of 10 (lentiviral particles per cell). After 24 hours of incubation at 37°C and 5% CO₂, the supernatant was exchanged to fresh medium and incubated for an additional 24 hours. Cells were then kept in culture for expansion of cell numbers.

Cytofluorimetric analysis

After lentiviral transduction, cells were harvested at an interval of 6 days, washed, resuspended in PBS, and checked for GFP expression by using a FACSCalibur (BD Biosciences, San Jose, Calif) to quantify the percentage of transduced cells.

Analysis of CD69 expression

Jurkat T cells were either left untreated or stimulated with immobilized anti-CD3ε antibody (OKT3, BioLegend) overnight at 37°C. A 24-well plate was first coated with 2 μg/mL OKT3 for 1 hour at 37°C and washed, and about 1×10^6 cells per well were plated for stimulation. Cells were then stained with allophycocyanin-conjugated anti-CD69 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), washed, and analyzed with a FACSCalibur (BD Biosciences).

Intracellular Ca²⁺ flux measurement

Measurement of intracellular free Ca²⁺ was performed, as described previously.¹⁶ Briefly, Jurkat T cells were harvested from growth medium, and 1×10^6 cells were resuspended in 1 mL of PBS and 0.5% BSA containing 5 μg/mL Indo-1 (Invitrogen) and stained for 30 minutes at 37°C. Cells were then washed and additionally incubated in PBS and 0.5% BSA containing 1 mmol/L Ca²⁺ and 1 mmol/L Mg²⁺. Cells were kept on ice before equilibration at 37°C for 5 minutes directly before measurement. Changes in intracellular calcium were monitored with an LSRII flow cytometer (BD Biosciences). Indo-1 is excited by using a UV laser; its emission depends on whether it is bound to calcium (approximately 420 nm) or free (approximately 510 nm). The ratio of these 2 wavelengths indicates changes in intracellular calcium concentrations. After monitoring baseline activity for 30 seconds, cells were stimulated either with 10 μg/mL anti-CD3ε antibody (OKT3, BioLegend) or 10 μg/mL ionomycin (Calbiochem, San Diego, Calif), and calcium flux was measured for an additional 3.30 minutes. The kinetics of the data acquired were analyzed by using FlowJo 7.2.2 software (TreeStar, Ashland, Ore).

Apoptosis assay

Jurkat T cells were stimulated with immobilized anti-CD3ε antibody (OKT3, BioLegend) overnight at 37°C. A 24-well plate was first coated with 10 μg/mL OKT3 overnight at 4°C and washed, and 0.5 to 1×10^6 cells were plated per well for stimulation. Cells were then stained with anti-human CD95 (APO-1/Fas; eBioscience, San Diego, Calif), anti-human CD178 (Fas ligand; Tonbo Biosciences, San Diego, Calif), and Annexin V Apoptosis Detection Kit APC, according to the manufacturer's instructions (eBioscience), and analyzed with a FACSCalibur (BD Biosciences).

TABLE I. Clinical and immunologic features

Patient	V.1	V.3	V.4	V.12	V.13
Sex	Male	Female	Female	Female	Male
Year of birth	2004	2009	2012	2000	2005
Clinical presentation	Recurrent infections	Diagnosed at birth	Diagnosed at birth	Recurrent infection and failure to thrive	Diagnosed at birth
BMT donor and conditioning	Parental haploidentical First BMT: unconditioned Second BMT: Melphalan only	MUD Busulfan Fludarabine	MUD	MSD	MSD unconditioned
Outcome	Died: multiorgan failure after second BMT	Died: cardiac arrest and multiorgan failure after conditioning	Alive and well	Alive and well	Died: severe acute and chronic GvHD and respiratory compromise
Immunologic features at presentation					
Absolute lymphocyte count (cells/mm ³)	900	960	1190	NA*	2610
CD3 ⁺ T cells (cells/mm ³)	20	10	6	NA*	300
CD19 ⁺ B cells (cells/mm ³)	620	440	428	NA*	1440
CD16 ⁺ /CD56 ⁺ NK cells (cells/mm ³)	140	450	666	NA*	780
CD3 ⁺ CD4 ⁺ cells (cells/mm ³)	10	0	4	NA*	210
CD3 ⁺ CD8 ⁺ cells (cells/mm ³)	0	0	0	NA*	30
PHA response (SI)	1.9	4.8	NA	NA*	3.1
IgG (g/L [normal, 1-3.3])†	NA	8.23	6.09	NA*	6.8
IgA (g/L [normal, 0.07-0.37])	NA	<0.06	<0.07	NA*	<0.07
IgM (g/L [normal, 0.26-1.22])	NA	0.28	0.20	NA*	0.17

BMT, Bone marrow transplantation; GvHD, graft-versus-host disease; MSD, matched sibling donor transplant; MUD, matched unrelated donor transplant; NA, not available; SI, stimulation index.

*Presentation immunology not available because the patient was referred from a remote hospital after transplantation had already been performed.

†Normal ranges were taken from the Mayo Clinic reference range (<http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/8156>).

RESULTS

Clinical outcomes of the study patients

We describe a multigenerational consanguineous Pakistani family with 5 patients affected by an autosomal recessive T⁺B⁺NK⁺ form of SCID (Fig 1, A, and Table I). Both male and female patients were born to 2 sets of first-cousin parents within the same family. Patients V.1 and V.12 presented within the first 3 months of life with severe recurrent infections and failure to thrive, and patients V.3, V.4, and V.13 were given a diagnosis at birth as a result of previous family history. T-cell numbers were low (<300 cells/mm³), with an absent proliferative response to PHA, as might be expected in patients with such low T-cell numbers, but B and NK cell numbers were normal. No NK cell functional analysis was performed. In one patient, V.1, the total number of T, B, and NK cells did not equate to the total lymphocyte count. One possible explanation is that the T-cell analysis performed was specifically for T cells bearing α/β receptors only, and it is possible that there was a significant γ/δ TCR population, which can be seen in cases of dysregulation of T-cell development, although we did not specifically look for this. Immunoglobulin levels were evaluated soon after birth in 3 subjects: V.3, V.4 and V.13. IgG was present most probably as a result of maternal transfer, IgA levels were less than the level of detection, and IgM levels were at the very low end of the normal range. IL-7 receptor α and

CD3 subunit defects were considered unlikely because of haplotype analysis with microsatellite markers (short tandem repeats) and SNP arrays (data not shown). Also, no mutations were found in IL-7 receptor α by means of Sanger sequencing of all exons. Gamma chain expression was normal. All affected subjects underwent bone marrow transplantation, but 3 subjects, V.1, V.3, and V.13, died of transplant-related complications.

Genetic studies

Genomic DNA was available for 3 affected subjects (V.1, V.12, and V.13), their unaffected parents (IV.4, IV.5, IV.11, and IV.12), and 4 unaffected siblings (V.9, V.10, V.11, and V.14) for SNP arrays. Haplotype analysis and homozygosity mapping in the family suggested that the disease gene localizes to a 23.48-Mb region on chromosome 16 flanked by the markers rs4787441 and rs10852513. Two genes within the region were prioritized based on information available in the literature and Online Mendelian Inheritance in Man (OMIM). *CORO1A* (OMIM *605000) was selected as a strong candidate for its causative involvement in immunodeficiency 8 (OMIM #615401) characterized by recurrent infections and decreased number of lymphocytes, but no causative variants were identified. *LAT* (OMIM *602354) also colocalized to this region, and on sequencing of *LAT* coding regions in the

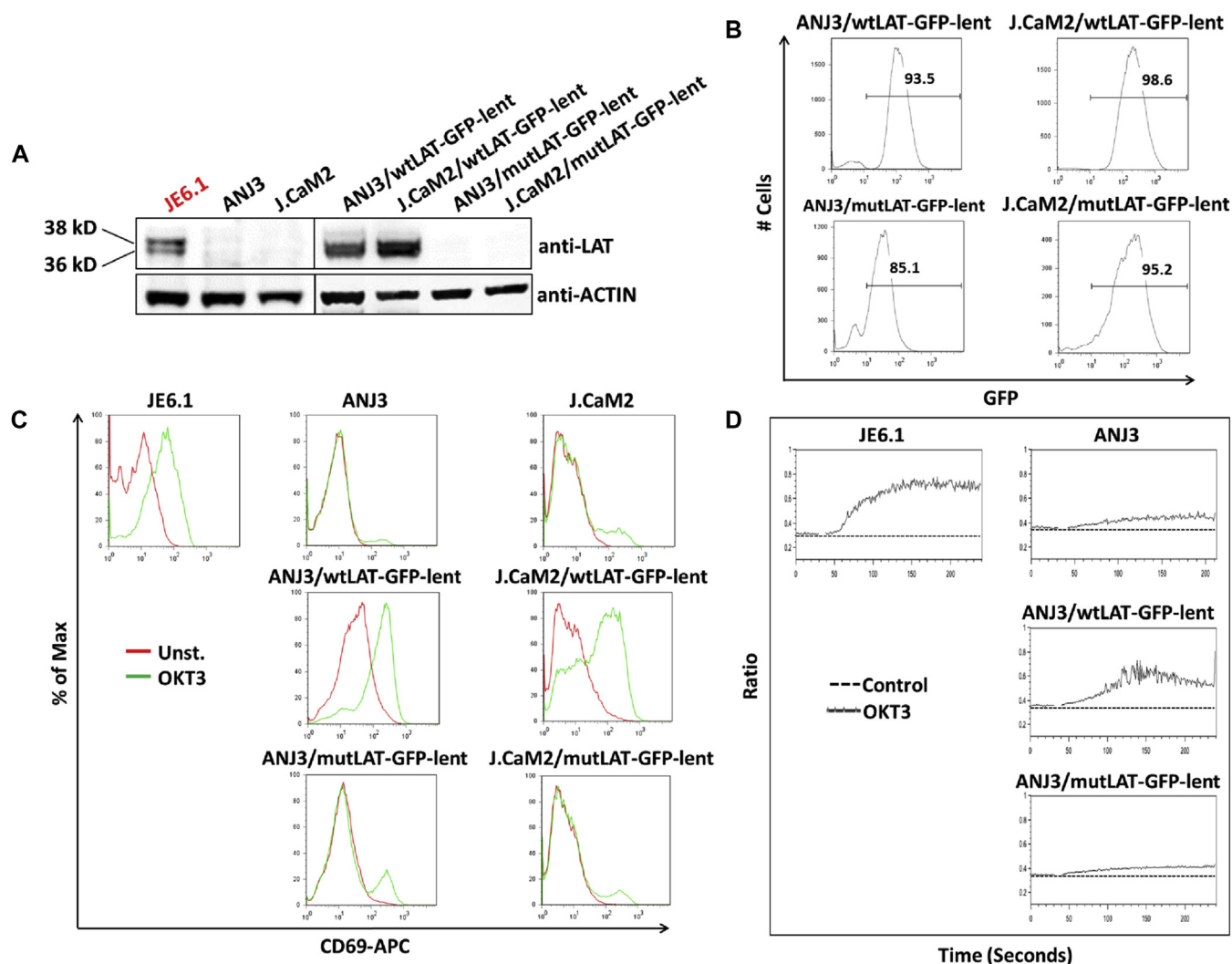


FIG 2. Reconstitution of LAT-deficient Jurkat T-cell lines. **A**, Analysis of LAT expression in Jurkat T-cell lines. **B**, Lentiviral vector gene expression in reconstituted Jurkat T-cell lines by means of fluorescence-activated cell sorting analysis of GFP. **C**, CD69 expression in Jurkat T-cell lines unstimulated (red line) or stimulated (green line) with OKT3. **D**, Ca^{2+} mobilization in JE6.1 and ANJ3 cell lines unstimulated (control) or stimulated with OKT3.

family, we found all patients were homozygous for a recessive frameshift mutation in *LAT* exon 1 and both sets of parents were heterozygous for this variant (Fig 1, A and B). As demonstrated in Fig 1, C, exon 1 is highly conserved across multiple vertebrate species. An insertion of a T between positions 44 and 45 in the coding sequence causes a frameshift and a putative stop codon 28 amino acids downstream (c.44_45insT; p.Leu16A-lafsX28). The nucleotide insertion was not recorded in the publicly available databases National Center for Biotechnology Information dbSNP, 1000 Genomes Project, the Exome Sequence Project's Exome Variant Server, or ExAC. To exclude the possibility of the insertion being a rare polymorphism present in the Pakistani population, genomic DNA from 150 ethnically matched control subjects representative of a total of 300 chromosomes was also sequenced and found to be wild-type for the insertion (data not shown). A further 15 patients with SCID were sequenced but did not reveal any pathogenic variants in *LAT*.

The variant results in a predicted truncation of the LAT protein. LAT is known to be expressed in peripheral blood

lymphocytes, the spleen, and the thymus, as well as in other blood cell types, notably platelets, megakaryocytes, mast cells, and NK cells.¹⁷ Therefore we performed Western blot analysis for the expression of LAT in PBMCs from patient V.4 and from a control blood sample (Fig 1, D). Control PBMCs were further processed for isolation of CD3^{+} and CD3^{-} cells to control for the lack of T cells in the patient sample. For each sample, lysate from 5×10^6 cells was loaded in the gel. 293T and HeLa cells were also used as negative controls. As shown, even in control CD3^{-} cells, LAT can be detected, whereas it is not detectable in patients' PBMCs. It is likely that because the premature termination codon is close to the 5' end of the protein, there is nonsense-mediated decay and complete loss of protein expression.

Reconstitution of LAT-deficient Jurkat T-cell lines

The absence of primary patient material led us to perform *in vitro* studies to demonstrate the functional effect of the

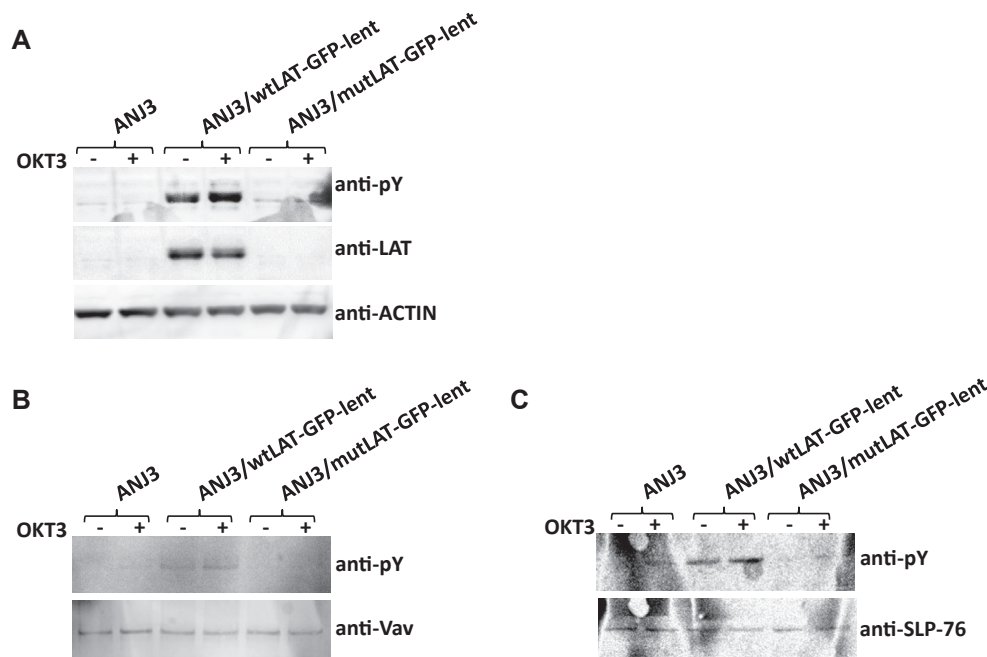


FIG 3. Tyrosine phosphorylation of LAT, Vav, and SLP-76 in ANJ3 cell lines. **A**, Cellular lysates from ANJ3 cell lines, either unstimulated (–) or OKT3 stimulated (+), were analyzed by means of immunoblotting with anti-phosphotyrosine (pY), anti-LAT, and anti-actin. **B** and **C**, Vav (Fig 3, B) and SLP-76 (Fig 3, C) were immunoprecipitated from unstimulated (–) or OKT3-stimulated (+) ANJ3 cell lines with antibodies against each protein. Immunoprecipitates were then analyzed by means of immunoblotting with anti-phosphotyrosine and antibodies against each protein.

observed LAT mutation. We generated a synthetic mutant copy of human *LAT* by using site-directed mutagenesis and generated lentiviruses bearing wild-type (wtLAT-GFP-lent) or mutant (mutLAT-GFP-lent) *LAT*. We then used both *LAT*-encoding lentiviruses to transduce 2 different Jurkat-derived TCR-signaling mutants,² ANJ3³ and J.CaM2,^{12,13,18} both of which lack expression of the *LAT* gene (Fig 2, A). Transduction efficiency was evaluated by means of fluorescence-activated cell sorting analysis of the GFP expressed by lentiviral vectors. One month after transduction, the percentage of GFP-positive cells was more than 85% in all 4 cultured clones, ANJ3 and J.CaM2 transduced by either wtLAT-GFP-lent (hereafter as ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent; Fig 2, B, upper panel) or mutLAT-GFP-lent (hereafter as ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent; Fig 2, B, lower panel) lentivirus. As expected, both ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent clones also efficiently expressed LAT. On the contrary, the 2 clones transduced with the lentiviruses bearing the frame-shift mutation in the *LAT* gene, ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent, were not able to express LAT, as demonstrated by using Western blotting (Fig 2, A). Taken together, these data confirmed the lentiviral vector efficacy in mediating gene transfer and expression in Jurkat T-cell lines and confirmed in an *in vitro* system that the insertion of a T between base 44 and 45 of the *LAT* gene prevents its expression.

CD69 upregulation and intracellular Ca²⁺ mobilization after TCR stimulation

CD69 is a marker for T-cell activation, and its expression is rapidly induced in a Ras-dependent manner after TCR stimulation in Jurkat T cells and easily detectable by means of fluorescence-

activated cell sorting analysis.¹⁹ Stimulation with the anti-CD3ε antibody OKT3 induced CD69 upregulation in JE6.1 control cells but did not induce CD69 expression in ANJ3 and J.CaM2 cells, as shown in Fig 2, C (upper panel), and as previously reported.^{3,18} Both ANJ3 and J.CaM2 cells were transduced with a lentivirus containing a copy of the *LAT* gene (wtLAT-GFP-lent), as described above, to confirm that the failure of CD69 upregulation was due to loss of LAT. Stably transduced cell lines reconstituted with wild-type *LAT*, ANJ3/wtLAT-GFP-lent, and J.CaM2/wtLAT-GFP-lent showed restored expression of CD69 on the cell surface after anti-CD3ε antibody stimulation (Fig 2, C, middle panel). On the contrary, the cell lines ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent reconstituted with 44_45insT *LAT* did not restore full CD69 expression after TCR signaling stimulation with OKT3 (Fig 2, C, lower panel).

Stimulation of TCR signaling with OKT3 also did not induce Ca²⁺ mobilization in the *LAT*-deficient Jurkat T-cell lines ANJ3 (Fig 2, D, upper panel) and J.CaM2 (see Fig E2, A, upper panel, in this article's Online Repository at www.jacionline.org), as previously reported.^{3,18} Similarly, *LAT*-deficient Jurkat T-cell lines reconstituted with the mutant copy of *LAT*, ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent, did not induce calcium flux after OKT3 stimulation (Fig 2, D, and see Fig E2, A, lower panels). On the contrary, control JE6.1 (Fig 2, D, upper panel) and wild-type *LAT*-reconstituted cells, ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent (Fig 2, D, and see Fig E2, A, middle panels), showed a clear Ca²⁺ mobilization response to anti-CD3ε antibody stimulation. As expected, all Jurkat T-cell lines, whether expressing *LAT* or not, were able to increase intracellular calcium levels after stimulation with ionomycin, a pharmacologic agent that mobilizes intracellular calcium in a TCR-independent manner (see Fig E2, B).

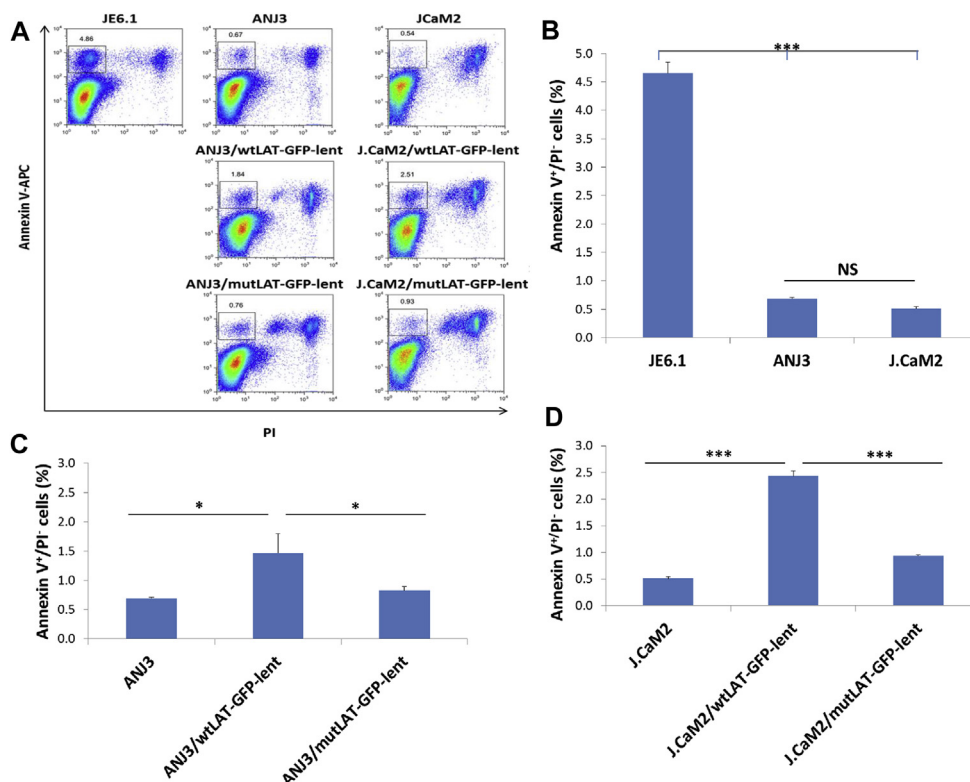


FIG 4. Apoptosis assay on Jurkat T-cell lines. **A**, Jurkat T-cell lines were stimulated with OKT3 and then stained with Annexin V and propidium iodide (PI) and analyzed by using fluorescence-activated cell sorting. **B-D**, Percentages of early-stage apoptotic cells (Annexin V⁺/PI⁺) are plotted. *P* values for statistical significance are indicated as follows: **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001. NS, Not significant.

Altogether, these results show that re-expression of LAT in ANJ3 and J.CaM2 cells after lentivirus transduction restores TCR signaling, confirming the pivotal role of LAT in T-cell activation. The mutant LAT in the described patients with SCID is not able to restore these functions.

Mutant LAT reconstitution does not restore tyrosine phosphorylation on downstream Vav and SLP-76

A clear role for LAT in regulating tyrosine phosphorylation of intracellular proteins during TCR engagement has already been shown.³ Thus cells were stimulated with OKT3 and tyrosine phosphorylation was detected by probing protein samples with anti-phosphotyrosine antibody 4G10 to confirm optimal restoration of TCR signaling in ANJ3 and J.CaM2 lines reconstituted with wild-type LAT (Fig 3 and see Fig E3 in this article's Online Repository at www.jacionline.org). The tyrosine-phosphorylated LAT molecule was not detected in cellular lysates prepared from ANJ3 and ANJ3 cells reconstituted with mutant LAT (ANJ3/mutLAT-GFP-lent), but as expected, this was observed in ANJ3 cells reconstituted with wild-type LAT (ANJ3/wtLAT-GFP-lent; Fig 3, A). Importantly, reconstitution of wild-type LAT in ANJ3 and J.CaM2 cells restored the tyrosine phosphorylation of 2 proteins downstream of LAT, Vav and SLP-76, which are involved in the signal transduction cascade initiated by TCR engagement.² Tyrosine phosphorylation of these proteins was absent in protein samples from stimulated ANJ3 and J.CaM2 cells and cell lines reconstituted with mutant LAT (Fig 3, B and C, and see Fig E3).

Absence of LAT prevents TCR-induced apoptosis and is not restored by mutant LAT

On TCR cross-linking, a number of studies have shown that LAT can be a negative regulator of TCR signaling, including mutant LAT knock-in models, which show a T-lymphoproliferative disease.^{5,20-22} We stimulated JE6.1 and LAT-deficient ANJ3 and J.CaM2 cells with immobilized OKT3 and showed that there was significantly reduced apoptosis in mutant lines than in JE6.1 control cells (Fig 4, A, upper panel, and B). In reconstituted lines introduction of wild-type LAT in ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent led to a significant increase in apoptosis after TCR stimulation, whereas LAT-deficient lines transduced with mutant LAT, ANJ3/mutLAT-GFP-lent, and J.CaM2/mutLAT-GFP-lent did not show any increase in levels of apoptosis (Fig 4, A, middle and lower panels, C and D). Expression levels of death factors, such as Fas and Fas ligand,^{4,23-27} in ANJ3 and J.CaM2 cells were similar to those observed in the wild-type Jurkat line JE6.1 (see Fig E4 in this article's Online Repository at www.jacionline.org). These studies support suggestions that TCR stimulation leads to T-cell apoptosis dependent on functional LAT expression and that expression of the mutant LAT in LAT-deficient lines does not restore apoptosis, whereas wild-type LAT is able to restore apoptosis.

DISCUSSION

Genetic and molecular studies of SCIDs have led to the identification of mutations in many different genes that impair T-lymphocyte differentiation and result in the clinical and

immunologic phenotype of SCID. A number of different mechanisms have been identified, including (1) lymphocyte cell death triggered by purine metabolism abnormalities, (2) defective signaling through the common γ chain-dependent cytokine receptors, (3) defective V(D)J recombination, and (4) defective pre-TCR/TCR signaling.²⁸ Mutations in key proteins involved in pre-TCR/TCR signaling are indeed responsible for rare cases of SCID consisting of pure T-cell deficiencies. These defects account for about 2% of patients with SCID. Patients with defects in the hematopoietic-specific transmembrane protein tyrosine phosphatase CD45, which is required for efficient lymphocyte signaling, are severely immunodeficient and have very few peripheral T lymphocytes unresponsive to mitogen stimulation.^{29,30} Similarly, deficiency of CD3 ϵ ,³¹⁻³⁴ CD3 δ ,^{33,35,36} and CD3 ζ ³⁷⁻⁴⁰ subunits of the pre-TCR/TCR signaling complex cause near-complete arrest of T-cell development and severe immunodeficiency. Further downstream, defects in ZAP-70, IL-2-inducible T-cell kinase (ITK), ORAI calcium release-activated calcium modulator 1 (ORAI1), stromal interaction molecule 1 (STIM1), magnesium transporter 1 (MAGT1), and LCK have been reported, but the immunophenotype in these patients shows evidence of T-cell development, although these T cells are abnormal and in some cases associated with severe immune dysregulatory phenomena.⁸

This report documents the first cases of LAT deficiency in a multigenerational consanguineous Pakistani family caused by an insertion of a T between positions 44 and 45 in the coding sequence (c.44_45insT; p.Leu16AlafsX28), which causes a frameshift and a putative stop codon 28 amino acids downstream. The immunologic phenotype in these subjects is characterized by a significant lack of T-cell development and highlights the critical and nonredundant role of LAT in the regulation of T-cell development.

The human immunophenotype observed here is very similar to that observed in a murine knockout model. In this model there is a complete lack of peripheral T cells, but LAT-deficient mice have normal B-cell populations and no gross abnormality of NK cell numbers or function.⁹ Further analysis of these mice shows that thymocyte development is arrested at the CD4⁺CD8⁺ double-negative and CD4⁺CD8⁺ double-positive stages and that CD4⁺ or CD8⁺ single-positive thymocytes were undetectable. More specifically, the arrest is at the CD25⁺CD44⁺ DN3 stage and indicates the absolute requirement for LAT in the pre-TCR-mediated signal transduction that is required for further thymocyte development. The role of LAT beyond the DN3 stage has also been studied by the generation of LAT knock-in mice in which the LAT gene could be deleted on expression of the Cre recombinase at later stages of thymocyte development.⁴¹ These mice had double-positive thymocytes, but there was a significant block in the single-positive thymocytes and consequently a significant loss of mature peripheral T cells. In addition, there were abnormalities of Treg cell development and function, which is also supported by other studies.⁴² Together, these murine studies highlight the essential role of LAT in early pre-TCR and later thymocyte development.

Mechanistically, it has already been shown that on stimulation of the TCR, LAT becomes phosphorylated at several tyrosine residues on its cytoplasmic tail. This leads to the binding of SH2 domain-containing proteins and their associated molecules and the formation of large multiprotein complexes located at the plasma membrane.^{43,44} These dynamic and highly regulated

signaling complexes facilitate the production of second messengers; activate downstream pathways, such as intracellular Ca²⁺ mobilization; induce actin cytoskeleton polymerization; and stimulate the activity of multiple transcription factors.^{1,2} Thus signaling pathways from the pre-TCR or TCR feed into LAT, which then integrates this information and selectively induces pathways critical for thymocyte development and T-cell activation. In peripheral cells there is evidence to suggest that LAT can also act as a negative regulator of TCR-mediated signaling and that T-cell apoptosis is dependent on a functional LAT protein. In the absence of LAT, there is reduced apoptosis, which leads to lymphoproliferation in murine LAT mutant knock-in models.^{5,20-22}

In the absence of primary material (other than a limited source of PBMCs) from our patients who had either died or undergone hematopoietic stem cell transplantation, we chose to demonstrate the nonfunctional effect of the patient mutation in specific cell lines. We used 2 LAT mutant Jurkat T-cell lines, ANJ3³ and J.CaM2,^{12,13} which are defective in TCR-mediated signal transduction. Although initial TCR-mediated signaling events, such as the inducible tyrosine phosphorylation of the CD3 ζ chain and ZAP-70, are intact, subsequent events, including phosphorylation of LAT downstream proteins, such as SLP-76, Vav, and phospholipase C γ 1; increases in intracellular Ca²⁺, Ras activation, CD69 upregulation, and IL-2 gene expression; and apoptosis, are defective in these mutant cell lines.^{3,18} Through lentiviral gene transfer, we introduced either the wild-type or observed mutant LAT gene into these mutant lines and determined the functional consequences. Both reconstituted cell lines that expressed wild-type LAT had levels comparable with those of Jurkat T cells (JE6.1), and all LAT downstream TCR-inducible signaling events were reconstituted. We also show failure to express LAT protein and loss of TCR-induced signaling and restoration of apoptosis in ANJ3 and J.CaM2 cells transduced by lentiviruses bearing a copy of the LAT gene with the frameshift mutation. Thus we confirmed in an *in vitro* system the loss of LAT expression as a consequence of an insertion of a T between positions 44 and 45 in the coding sequence. The frameshift and a putative stop codon 28 amino acids downstream resulted in a truncation of the LAT protein with complete loss of function. Needless to say, in cell lines in which the patient's mutant LAT was expressed, there was no evidence of recovery of TCR downstream signaling events. Therefore we would infer that there is a similar loss of pre-TCR and TCR signaling in these patients that would lead to and explain the lack of T-cell development.

This is the first report of LAT mutations leading to SCID. The genetic data are highly significant in that they show the presence of a highly deleterious variant that, both in primary patient cells and after *in vitro* expression, leads to the absence of LAT protein expression. The mutation cosegregates with disease and is found in heterozygous form in unaffected carriers and family members. No evidence of this mutation was identified in a number of different human publically available databases. Importantly, when we studied 300 chromosomes from an ethnically matched cohort, the mutation was not identified and demonstrates that this variant is not a polymorphism but is a founder mutation that is carried in this consanguineous pedigree. The expression of this mutant in LAT-deficient cells showed the absence of protein expression and an inability to reconstitute LAT-specific signaling events. Together, these data argue strongly that the LAT mutation identified leads to the SCID immunophenotype in this pedigree.

Although many gene defects have been identified as causing SCID, recent data from prospective SCID screening studies show that there are still more than 10% of patients with SCID who remain genetically undiagnosed.⁶ It is likely that, as in this family, mutations in other genes already known from murine and other studies to be critical for T-cell development will soon be identified.

We thank Professor Weiguo Zhang for providing the ANJ3 cell line and Dr Marco Purbhoo for providing the J.CaM2 cell line.

Clinical implications: Mutations in *LAT* can lead to SCID, and *LAT* should be part of the genetic diagnostic workup in patients with SCID.

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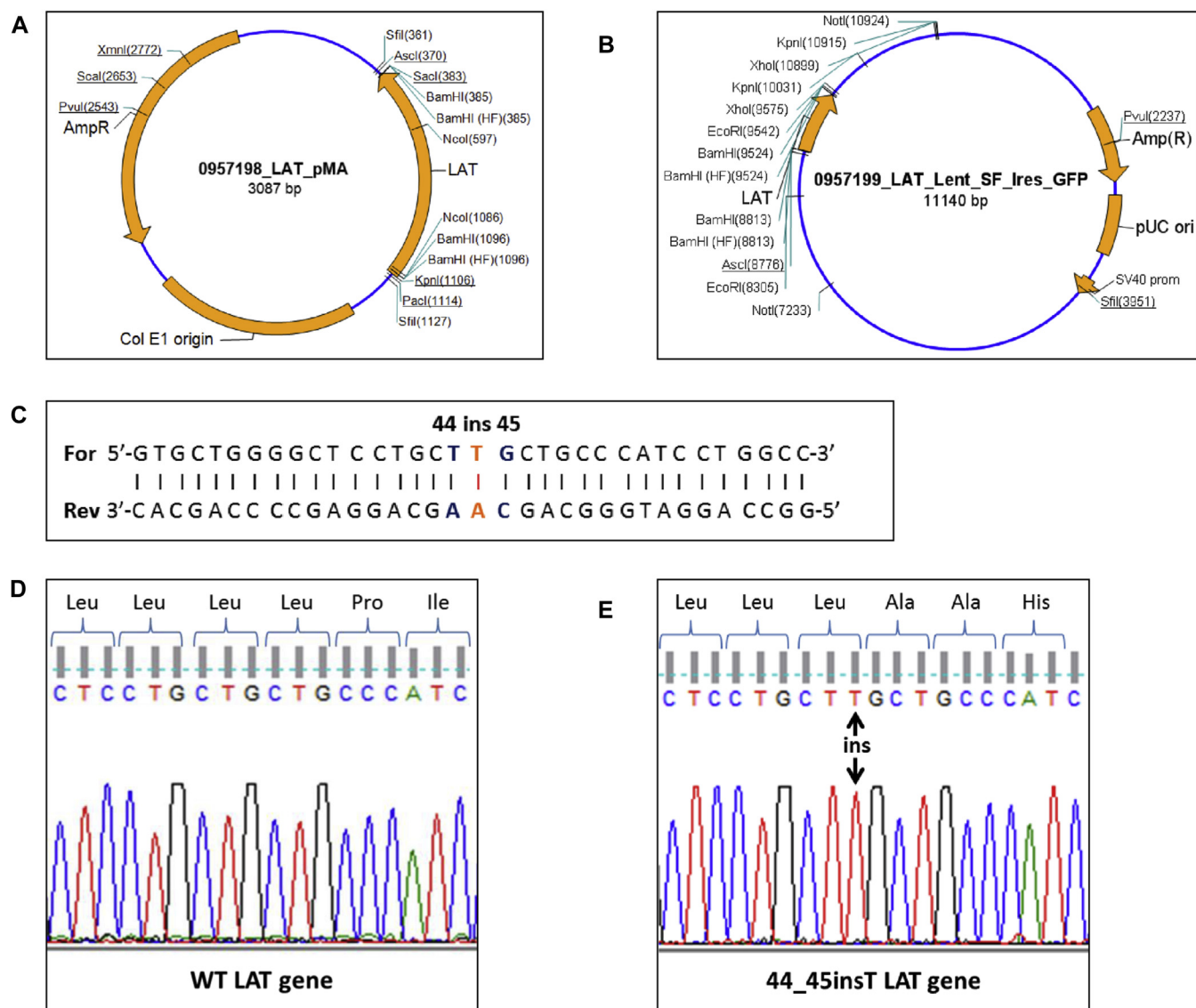


FIG E1. Generation of 44_45insT *LAT* gene-bearing lentiviral vector. **A** and **B**, Map of pMA plasmid bearing a synthetic copy of the human *LAT* gene (Fig E1, A) and lentiviral vector bearing GFP and *LAT* genes (GE-NEART; Fig E1, B). **C**, Overlapping primers designed for side-directed mutagenesis. **D** and **E**, DNA sequences of wild-type (Fig E1, D) and mutant (Fig E1, E) *LAT* gene-bearing lentiviral vectors at the site of insertional mutagenesis.

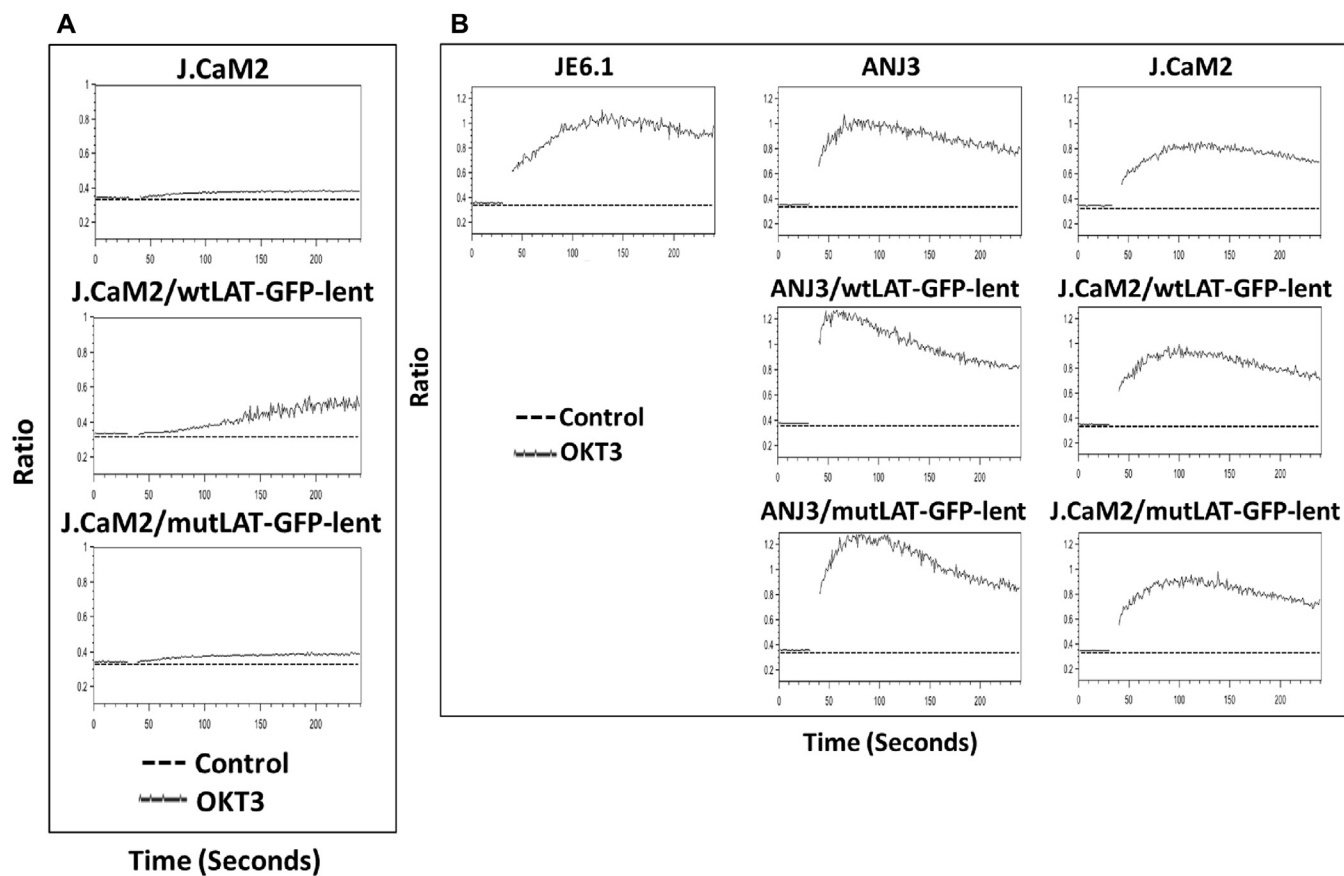


FIG E2. Restoration of calcium flux in J.CaM2 cells and ionomycin-induced mobilization of calcium. Ca^{2+} mobilization in J.CaM2 cell lines loaded with Indo-1 and stimulated with OKT3 or kept unstimulated (control; **A**) and in Jurkat T-cell lines loaded with Indo-1 and stimulated with ionomycin or kept unstimulated (control; **B**). The ratio of the fluorescence emission of Ca^{2+} -bound and Ca^{2+} -free Indo-1 was plotted as a function of time after stimulation.

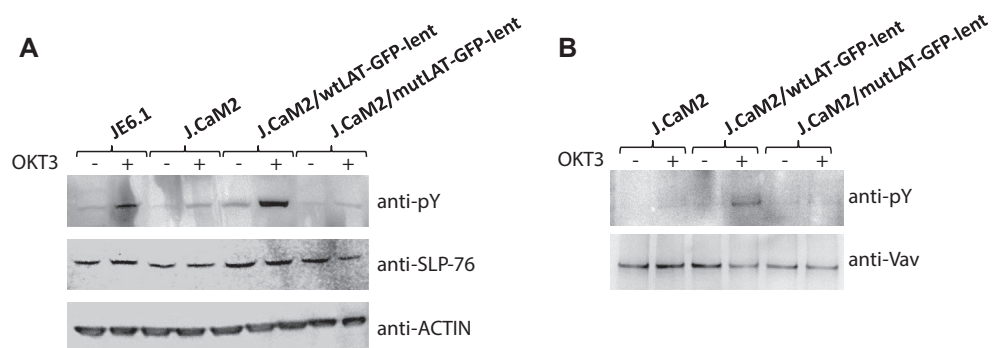


FIG E3. Tyrosine phosphorylation of SLP-76 and Vav in J.CaM2 T-cell lines. **A**, Cellular lysates from JE6.1 and J.CaM2 cells, either unstimulated (–) or OKT3-stimulated (+), were analyzed by means of immunoblotting with anti-phosphotyrosine (pY), anti-SLP-76, and anti-actin. **B**, Vav was immunoprecipitated from unstimulated (–) or OKT3-stimulated (+) J.CaM2 cells with an antibody against Vav protein. Immunoprecipitates were then analyzed by means of immunoblotting with anti-phosphotyrosine and anti-Vav antibodies.

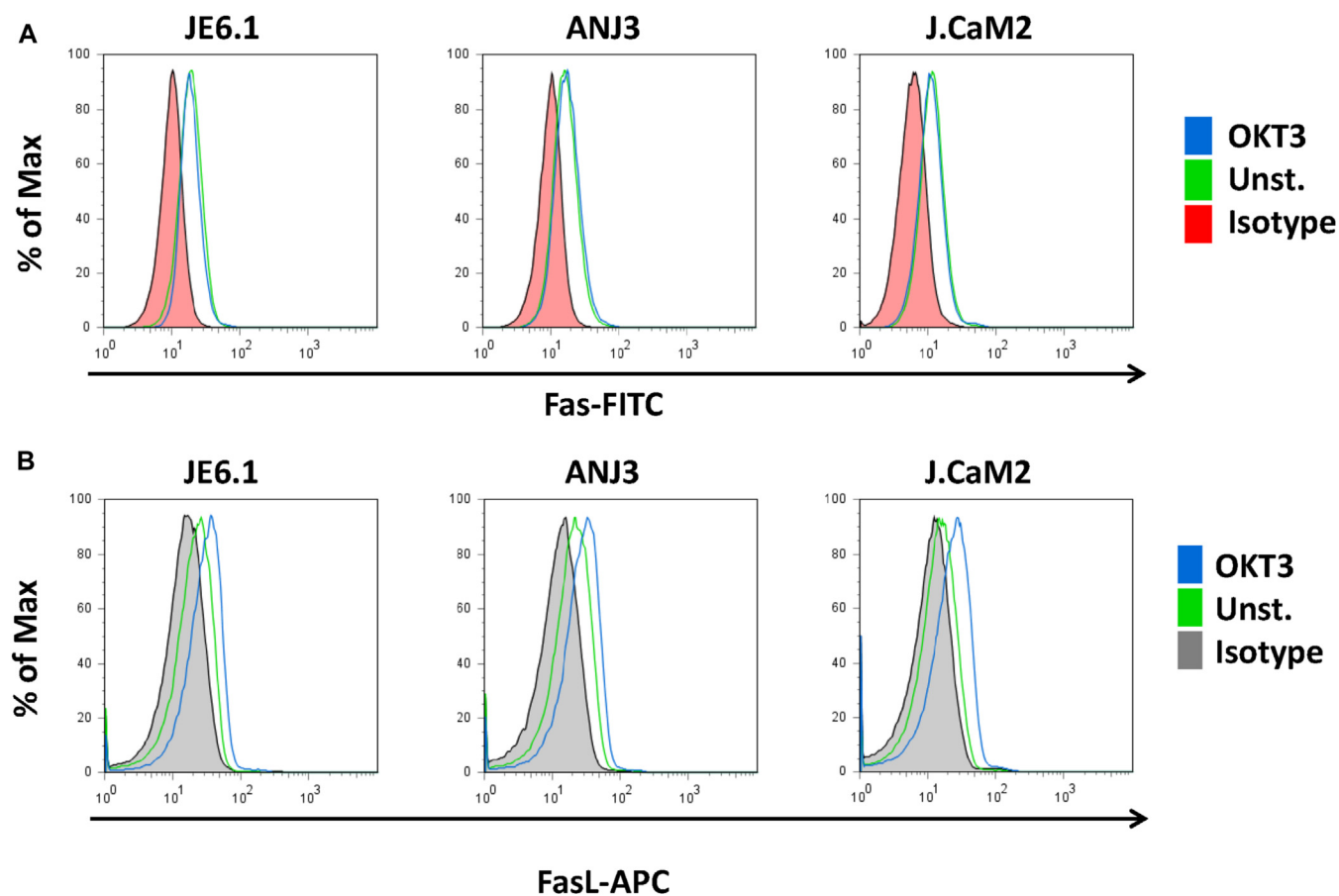


FIG E4. Expression analysis of the death factors Fas and Fas ligand in Jurkat T-cell lines. JE6.1 and LAT-deficient ANJ3 and J.CaM2 cell lines were unstimulated (*green line*) or stimulated with OKT3 (*blue line*) and then stained with antibodies against CD95/Fas (**A**) or CD178/Fas Ligand (**B**) and with isotype controls (*shaded areas*).

TABLE E1. Primers used for PCR amplification and Sanger sequencing of genomic DNA of human *CORO1A* and *LAT* genes

Primer name	Primer sequence	Product size	Tm
<i>CORO1A</i>			
Exon 2F	AGACTGAGGGGTGTCCTGG	362 bp	60
Exon 2R	CACCTAATCAGGACCTGCAC		
Exon 3-4F	GCCTCTCTGAAGGAGGTGTG	448 bp	60
Exon 3-4R	AGTGTGAGAAGCCATGAGCC		
Exon 5-6F	TCAGTGCAGGTGCTGCG	563 bp	60
Exon 5-6R	AGCAGGGACGAAACCTCC		
Exon 7-8F	GTTGTTCCCACTGGTTGGTC	463 bp	60
Exon 7-8R	CTGGTTGGGTGGGCTTG		
Exon 9-10F	ATGCTCCTTGGGCAGTGG	485 bp	62
Exon 9-10R	GATTCCCAATGTTGTGAGTTTG		
Exon 11F	AGGGCTCTAGGGATGGGG	205 bp	62
Exon 11R	CATGTGGCTGGGAATGGG		
<i>LAT</i>			
Exon 1-2-3F	TTCATCTGGCCTTGACTCTG	635 bp	60
Exon 1-2-3R	AAGGTGGCAGGGGAAGTC		
Exon 4-5-6F	GCTTTCAGGGGCTTAGTCTG	596 bp	60
Exon 4-5-6R	GGTAACGACACAGGGGTCAG		
Exon 6-7F	AGAACGAGGGTGCGTCTG	403 bp	59
Exon 6-7R	TACAATGGCACCCTGCAC		
Exon 8-9-10F	CTCTGCATGGCTGAGGTTG	648 bp	60
Exon 8-9-10R	GGTCCTGGAGGGAAGGAG		