Early onset combined immunodeficiency and autoimmunity in patients with loss-of-function mutation in LAT

Baerbel Keller,†* Irina Zaidman,‡ O. Sascha Yousefi,§ Dov Hershkovitz,¶ Jerry Stein,¶ Susanne Unger,† Kristina Schachtrup,† Mikael Sigvardsson,¶ Amir A. Kuperman,∥ Avraham Shaag,∥∥ Wolfgang W. Schamel,†§∥ Polina Stepensky,∥∥ and Klaus Warnatz,∥∥∥ Polina Stepensky

†Center for Chronic Immunodeficiency (CCI), University Medical Center and University of Freiburg, 79106 Freiburg, Germany
‡Department of Pediatric Hematology Oncology, Ruth Rappaport Children’s Hospital, Rambam Health Care Campus, Haifa 3109601, Israel
§Department of Molecular Immunology, Faculty of Biology, BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany
¶Spemann Graduate School of Biology and Medicine (SGBM), Albert Ludwigs University Freiburg, 79104 Freiburg, Germany
∥Department of Pathology, Rambam Health Care Campus, Haifa 3109601, Israel
∥∥Department of Pediatric Hematology Oncology and Bone Marrow Transplantation Unit, Schneider Children’s Medical Center of Israel, Petah-Tikva 49202, Israel
§§Department of Clinical and Experimental Medicine, Experimental Hematopoiesis Unit, Faculty of Health Sciences, Linköping University, 581 85 Linköping, Sweden
¶¶Blood Coagulation Service and Pediatric Hematology Clinic, Galilee Medical Center, Nahariya 22100, Israel
∥∥∥Faculty of Medicine in the Galilee, Bar-Ilan University, Safed 5290002, Israel
**Monique and Jacques Roboh Department of Genetic Research and ** Department of Pediatric Hematology-Oncology and Bone Marrow Transplantation, Hadassah Medical Center, Hebrew University, Jerusalem 91120, Israel

The adapter protein linker for activation of T cells (LAT) is a critical signaling hub connecting T cell antigen receptor triggering to downstream T cell responses. In this study, we describe the first kindred with defective LAT signaling caused by a homozygous mutation in exon 5, leading to a premature stop codon deleting most of the cytoplasmic tail of LAT, including the critical tyrosine residues for signal propagation. The three patients presented from early childhood with combined immunodeficiency and severe autoimmune disease. Unlike in the mouse counterpart, reduced numbers of T cells were present in the patients. Despite the reported nonredundant role of LAT in Ca2+ mobilization, residual T cells were able to induce Ca2+ influx and nuclear factor (NF) κB signaling, whereas extracellular signal–regulated kinase (ERK) signaling was completely abolished. This is the first report of a LAT-related disease in humans, manifesting by a progressive combined immune deficiency with severe autoimmune disease.

T cells are selected during thymic development based on the signal strength elicited through the interaction of the MHC–peptide complex on APCs and the TCR on thymocytes. Although the specificity of the TCR plays a crucial role and allows for positive and negative selection, amplifying or dampening alterations of signaling proteins downstream of the TCR will modify signal strength and, consequently, impact the cellular response and outcome of selection. Multiple examples have illustrated the effect of altered TCR signal strength on the increased survival of autoreactive T cell clones in mice with genetic alterations of signaling molecules like ZAP70 (Sakaguchi et al., 2003; Siggs et al., 2007) or the CD3 signaling unit by deletion of several immunoreceptor tyrosine-based activating motifs (Holt et al., 2008). This signaling machinery downstream of the TCR is composed of a dynamic, fine-tuned network of multiple components that interact in a tightly regulated temporospatial manner. This is achieved by scaffold proteins, which allow for the perassembly of signalosomes to facilitate rapid signal transduction and guarantee signal specificity. Although the lack of certain scaffold proteins like BLNK/SLP65 in B cells (Minegishi et al., 1999) leads to the absence of affected lymphocyte subsets, the lack of others may allow for the development of the respective population but modify their activation or further differentiation. Linker for activation of T cells (LAT) is a transmembrane adapter molecule first discovered in activated T cells. LAT is phosphorylated after TCR triggering at four conserved tyrosine residues that are essential for the recruitment and membrane localization of downstream molecules: human (h)Y132/mouse (m)Y136, hY171/mY175, hY191/mY195, and hY226/mY235 (Balagopalan et al., 2010). LAT knockout mice (Zhang et al., 1999b) and mice with targeted replacement of all four tyrosine residues (Sommers et al., 2001) lack peripheral T cells because of a block at the double-negative 3 stage. These tyrosines serve as docking

*W.W. Schamel, O. Elpeleg, K. Warnatz, and P. Stepensky contributed equally to this paper.
**B. Keller, I. Zaidman, and O.S. Yousefi contributed equally to this paper.
††Correspondence to Klaus Warnatz: klaus.warnatz@uniklinik-freiburg.de; or Polina Stepensky: polina@hadassah.org.il

Abbreviations used: DAG, diacylglycerol; DGK, DAG kinase; ERK, extracellular signal-regulated kinase; ICOS, inducible costimulator; IP3, inositol 1,4,5-trisphosphate; ITK, IL-2–inducible T cell kinase; LAB, linker for activation of B cells; LAT, linker for activation of T cells; NTAL, non–T cell activation linker.

© 2016 Keller et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike License, by http://www.creativecommons.org/licenses/by-nc-sa/3.0/.
sites for PLCγ1, Grb2, Gads, and others, interconnected in positive and negative regulatory plug-ins of (pre)assembled signaling modules (Malissen et al., 2014; Roncagalli et al., 2014) modifying T cell development (Zhang et al., 1999b), specific functions (Ou-Yang et al., 2012), or even terminating T cell activation (Malissen et al., 2014). Mice with a mutation at Y136 of LAT, which is the docking site for PLCγ1, present with hypergammaglobulinemia and severe lupus-like glomerulonephritis and die within 6 wk (Sommers et al., 2002), suggesting an essential role of this docking site for negative regulatory plug-ins. This deletion uncouples the activation of the CD28 pathway from the TCR by allowing for TCR-independent constitutive activation. Because of the distinctive pattern of this dysregulation in affected mice, it was termed LAT signaling pathology (Roncagalli et al., 2010). In contrast to mice, the physiological role of LAT is not known in humans.

Here, we describe for the first time the clinical course and immunological findings in a family with a homozygous loss-of-function mutation in LAT.

RESULTS

Case studies

We evaluated three siblings born to consanguineous parents of Arab origin (Fig. 1). All three patients presented with recurrent infection, lymphoproliferation, and life-threatening autoimmune disease since early infancy. The main clinical and laboratory findings are summarized in Table 1.

Figure 1. Pedigree of the affected family. Circles represent female and squares represent male subjects. Solid symbols show homozygous affected patients, and crossed-out symbols stand for deceased subjects. N, wild type; del, deletion.

Table 1. Summary of major clinical and laboratory findings

<table>
<thead>
<tr>
<th></th>
<th>Patient 1, male</th>
<th>Patient 2, male</th>
<th>Patient 3, female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at presentation</td>
<td>5 mo</td>
<td>6 mo</td>
<td>10 mo</td>
</tr>
<tr>
<td>Infection</td>
<td>Recurrent pneumonia, EBV/CMV viremia, CMV pneumonia</td>
<td>Congenital toxoplamosis, recurrent pneumonia, varicella infection, CMV viremia, Candida pneumonia adenovirus + CMV PCR positive in BAL</td>
<td>Recurrent pneumonia, urinary infections, gastroenteritis, CMV viremia</td>
</tr>
<tr>
<td>Autoimmunity</td>
<td>Coombs+ AIHA, ITP, autoimmune neutropenia</td>
<td>Coombs+ AIHA, ITP</td>
<td>Anti-ADAMTS13+ microangiopathic hemolytic anemia</td>
</tr>
<tr>
<td>Lymphoproliferation</td>
<td>Lymphadenopathy, splenomegaly</td>
<td>Lymphadenopathy, splenomegaly</td>
<td>Lymphadenopathy, splenomegaly</td>
</tr>
<tr>
<td>Lung disease</td>
<td>Chronic lung disease, bronchiectasis</td>
<td>Chronic lung disease, bronchiectasis</td>
<td>Diplegic cerebral palsy due to congenital toxoplamosis, red-brown rash on forearm</td>
</tr>
<tr>
<td>Others</td>
<td>Red-brown rash on face and legs</td>
<td>Steroids, IgG-RT</td>
<td>No</td>
</tr>
<tr>
<td>Treatment</td>
<td>Steroids, IgG-RT, splenectomy</td>
<td>Steroids, IgG-RT</td>
<td>Steroids, plasmapheresis</td>
</tr>
<tr>
<td>Outcome</td>
<td>Died at 9 yr due to disseminated CMV infection</td>
<td>Alive, 8 yr old, 13 mo after HSCT</td>
<td>Died at 2 yr due to TTP</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>Progressive hypergammaglobulinemia</td>
<td>Progressive hypergammaglobulinemia</td>
<td>Hypergammaglobulinemia</td>
</tr>
<tr>
<td></td>
<td>&lt;4 yr: normal</td>
<td>&lt;3.5 yr: normal to rather increased</td>
<td>2 yr:</td>
</tr>
<tr>
<td></td>
<td>IgG 1.6 g/liter (5.4–13.4 g/liter)</td>
<td>IgG 1.3 g/liter (5.4–13.4 g/liter)</td>
<td>lgG 20.45 g/liter (4.7–12.3 g/liter)</td>
</tr>
<tr>
<td></td>
<td>lgA &lt;0.16 g/liter (0.3–1.9 g/liter)</td>
<td>lgA 0.69 g/liter (0.3–1.9 g/liter)</td>
<td>lgA 1.02 g/liter (0.2–1.4 g/liter)</td>
</tr>
<tr>
<td></td>
<td>lgM 0.12 g/liter (0.4–1.7 g/liter)</td>
<td>lgM 0.84 g/liter (0.4–1.7 g/liter)</td>
<td>lgM 0.89 g/liter (0.4–1.5 g/liter)</td>
</tr>
<tr>
<td></td>
<td>lgE 2 IU/ml (&lt;58 IU/ml)</td>
<td>lgE 286 IU/ml (&lt;29 IU/ml)</td>
<td>lgE 85 IU/ml</td>
</tr>
<tr>
<td>Specific antibodies</td>
<td>Hepatitis A+, HSV1+, Hepatitis B+, measles+, mumps+, rubella+</td>
<td>ND</td>
<td>rubella+, measles+, EBV+, CMV+</td>
</tr>
<tr>
<td>Lymphocyte populations</td>
<td>Progressive lymphopenia, high yδ T cells, low CD4 T cells</td>
<td>Progressive lymphopenia, high yδ T cells, low CD4 T cells, low B cells</td>
<td>High yδ T cells</td>
</tr>
</tbody>
</table>

AIHA, autoimmune hemolytic anemia; BAL, bronchoalveolar lavage; HSCT, hematopoietic stem cell transplantation; IgG-RT, Ig replacement therapy; ITP, idiopathic thrombocytopenic purpura; TTP, thrombotic thrombocytopenic purpura. Values in parentheses depict age-matched reference values.
tation, he died because of disseminated CMV infection with pulmonary involvement. He had additionally suffered from chronic lung disease with bronchiectasis and disseminated purple edematous skin lesions on his face and arms.

The index patient (patient 2) presented at the age of 7 mo with diplegic cerebral palsy and megalocystic leukencephalopathy probably caused by perinatal toxoplasmosis infection. From 1 yr of age, he suffered from recurrent respiratory tract infections, which progressed to chronic lung disease with bronchiectasis. Like his brother, he suffered from hepatosplenomegaly, lymphadenopathy, and persistent red edematous nodules on his forearms. From the age of 6 yr, he was treated with steroids for severe Evans syndrome. At age 7 yr, varicella infection was diagnosed, and shortly after, he developed CMV and adenoviral infection with severe progressive deterioration of respiratory function. On antiviral and IgG replacement therapy, he improved gradually, and genetic diagnosis was performed. Currently, at the age of 8 yr, he successfully received an allogeneic stem cell transplantation from his fully matched heterozygous sibling.

The younger sister (patient 3) presented at the age of 2 yr with gastroenteritis, recurrent pneumonia, and urinary tract infection. At the age of 2 yr, she died of anti-ADAMTS13–positive microangiopathic hemolytic anemia and thrombocytopenia.

The histological examination of lymph nodes from patients 1 and 2 showed distorted architecture with poorly formed follicles (not depicted). Aggregates of small, CD20-positive cells were associated with CD21-positive follicular dendritic cells, but Bcl6-positive germinal centers were absent. The spleen of patient 1 resembled the findings in the lymph nodes (not depicted). The skin biopsies showed extensive lymphoid infiltration of the dermis but not the epidermis, mainly by CD8 T cells. In situ hybridization for EBV-encoded RNA in the skin was negative as well as histological examination of lymph nodes from patients 1 and 2 showed distorted architecture with poorly formed follicles (not depicted). The skin biopsies showed extensive lymphoid infiltration of the dermis but not the epidermis, mainly by CD8 T cells. In situ hybridization for EBV-encoded RNA in the skin was negative as well as histological examination of lymph nodes from patients 1 and 2 showed distorted architecture with poorly formed follicles (not depicted).

The immunological evaluation of patient 2 at the age of 2 yr was remarkable for hypergammaglobulinemia and elevated IgE. She had reduced NK cells but normal numbers of B cells and total T cells. The T cell compartment showed reduced relative and absolute CD4 T cells and reduced percentages of CD8 T cells but, like her siblings, strongly increased γδ T cells (Table 2).

In the heterozygous parents, all lymphocyte subset counts and Ig levels were within the normal range, whereas the heterozygous sister had nearly 10% γδ T cells and slightly increased levels of all Ig isotypes (unpublished data).

Molecular characterization of the LAT mutation

The exome analysis of patient 2 yielded 54.1 million confidently mapped reads with a mean coverage of ×62. After alignment to the reference genome (Hg19) and variant calling, we removed variants that were called less than ×8, were off target, synonymous, heterozygous, predicted benign by Mutation Taster software, with minor allele frequencies >0.1% in the dbSNP138 or minor allele frequencies >1% in the Hadassah in-house database. 12 homozygous variants remained, but only chr16:28997725 deletion (del) GG (RefSeq accession no. NM_001014987.1: c.268_269del) segregated with the disease in the family. The mutation was not present in any of the nearly 60,000 exomes deposited by the Exome Aggregation Consortium. This mutation caused a deletion of guanosines 268/269 in exon 5 of LAT, leading to a frame shift and a premature stop codon after 303 bp (Fig. 2 A). The predicted protein of 100 of the 233 aa contains an intact extracellular and transmembrane region but a shortened intracellular region, eliminating the known major phosphorylation sites Y132, Y171, Y191, and Y226 (Fig. 2 B). We did not identify any additional pathogenic mutations by whole exome sequencing.
The relative amount of LAT mRNA in patient’s sorted CD4 CD45R0 T cells was within the range of three different healthy controls (Fig. 2 C), indicating that the mutation does not interfere with transcript stability. The LAT protein, however, could not be detected by flow cytometry using an antibody directed against the intracytoplasmic part of LAT in CD4 T cells (Fig. 2 D) and by Western blotting of patient-derived EBV lines using a polyclonal antibody against LAT (not depicted). Interestingly, LAT staining in the heterozygous sibling showed normal levels of LAT in the majority of cells but a small percentage of cells with low to absent protein expression (Fig. 2 D). To test whether the putative truncated protein can be expressed, LAT-deficient Jurkat-derived J.CaM2.5 cells (Finco et al., 1998) were stably transduced with the mutated form of the protein (J.CaM2.5-LATmut) or with wild-type LAT (J.CaM2.5-LATwt). Both were tagged with a FLAG sequence and cotransfected with ZsGreen1 separated by an internal ribosomal entry site. FLAG expression correlated with ZsGreen1 expression, indicating that translation efficiency of the FLAG-tagged LAT and the ZsGreen1 sequence is identical (Fig. 2 E). Western blotting with anti-FLAG tag but not the polyclonal anti-LAT antibody (not depicted) detected a shortened LATmut protein in the transduced J.CaM2.5-LATmut cells compared with J.CaM2.5-LATwt cells (Fig. 2 F), indicating that the mutated LAT protein can be expressed. The loss of dominant immunogenic epitopes in the truncated LAT might explain the absent recognition by the polyclonal antibody targeting full-length LAT.

**Signaling capacity of mutant LAT in Jurkat cells**

The TCR-induced LAT signaling capacity was analyzed in J.CaM2.5-LATwt or J.CaM2.5-LATmut cells. After CD3

### Table 2. Immune phenotype of patient 1, 2, and 3

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Reference counts</th>
<th>Absolute values</th>
<th>Reference counts</th>
<th>Absolute values</th>
<th>Reference counts</th>
<th>Absolute values</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells²</td>
<td>2.0</td>
<td>85–20.2</td>
<td>37</td>
<td>296–784</td>
<td>3.8</td>
<td>85–20.2</td>
</tr>
<tr>
<td>CD4⁴</td>
<td>4.0</td>
<td>26.5–41.4</td>
<td>74</td>
<td>641–1,453</td>
<td>38.3</td>
<td>13–47</td>
</tr>
<tr>
<td>NK cells²</td>
<td>6.0</td>
<td>2–31</td>
<td>114</td>
<td>70–580</td>
<td>6.0</td>
<td>2–31</td>
</tr>
</tbody>
</table>

**Patient 1 (6 yr)**

B cell subpopulations:

- Transitional: 21.1
- Naive: 68.7
- IgM memory: 3.5
- IgM only: 0.7
- IgA cs memory: 0.0
- IgG cs memory: 0.0
- CD21⁺⁺⁺: 6.3

**Patient 2 (8 yr)**

B cell subpopulations:

- Transitional: 24.4
- Naive: 2.4
- CD45RO: 97.5
- T reg cells: 2.0
- cTFH: 35.0
- Th1⁺⁺Th2⁻⁻-like cTFH: 29.4

**Patient 3 (2 yr)**

B cell subpopulations:

- Transitional: ND
- Naive: 0.3
- CD45RO: 2.4
- T reg cells: 2.4
- cTFH: 1.6
- Th1⁺⁺Th2⁻⁻-like cTFH: 0.3

**CD4 subpopulations**: ND ND ND ND

- RTE: 24.4
- Naive: 2.4
- CD45RO: 97.5
- T reg cells: 2.0
- cTFH: 35.0
- Th1⁺⁺Th2⁻⁻-like cTFH: 29.4

**CD8 subpopulations**: ND ND ND ND

- Naive: 0.3
- Central memory: 2.4
- Effector memory: 65.9
- Terminally differentiated: 1.6

**γδ T cells**: ND ND ND ND

**DN T cells**: ND ND ND ND

**NK T cells**: 0.005 >0.01 ND ND ND ND

**Relative counts are shown as a percentage of parental population. Absolute numbers refer to cells/microliter.**

**CD4 T cell subpopulations**: recent thymic emigrants (RTEs), CD31⁺ of CD45RA⁺ CD4 T cells; central memory, CD45RA⁻ CD27⁺; effector memory, CD45RA⁻ CD27⁻; terminally differentiated, CD45RA⁻ CD27⁻; double-positive (DN) T cells, CD4⁺CD8⁺ of CD3 T cells. NK T cells, Vα24 Vβ11 CD3 T cells, cs, class switched.

³avan Gent et al., 2009.

⁴Shatorjé et al., 2012.

⁵Percentage of naive CD4 T cells.

⁶Internal reference value.

⁷Internal reference value of CD3 T cells.
Figure 2. Molecular characterization of LAT mutation. (A) Genomic DNA sequence around the c.268_269delGG mutation site (arrows) of a patient (top), a parent (middle), and an unrelated healthy control (ctrl; bottom). (B) Schematic view of wild-type LAT showing the extracellular region (ER), the transmembrane domain (TM), and the intracellular region (IR) with the four tyrosine residues phosphorylated downstream of TCR signaling. Below is the mutated form of the protein. The presumptive position of the frame shift starting at 89 aa and the presumed truncation after 100 aa is shown in red. (C) Relative mRNA expression of LAT with hypoxanthine phosphoribosyltransferase as the housekeeping gene is shown in CD4 CD45R0 T cells of patient 2 and three controls normalized to Jurkat cells. (D) FACS plot for LAT expression gated on CD4 CD45R0 T cells and graph of the mean fluorescence intensity (MFI) of LAT in CD4 and CD8 T cells in patient 2, the heterozygous sister, and three healthy controls. (E) Anti-FLAG staining and ZsGreen1 expression are shown in J.CaM2.5 cells expressing the recombinant wild-type (LATwt) or the mutated LAT protein (LATmut). (F) Immunodetection of FLAG-tagged LATwt or LATmut in J.CaM2.5 cells. The immunoblot is representative of three independent experiments.
cross-linking, similar TCR-proximal ZAP70 phosphorylation was observed in LAT-deficient, LAT<sup>wt</sup>-, and LAT<sup>mut</sup>-expressing T cell lines (Fig. 3 A). In line with the literature that PLCγ1 phosphorylation depends on the assembly of the LAT-SLP76 signalosome (Smith-Garvin et al., 2009), PLCγ1 phosphorylation was absent in J.CaM2.5 and J.CaM2.5-LAT<sup>mut</sup> cells (Fig. 3 A). Interestingly, the phosphorylation of IL-2–inducible T cell kinase (ITK), also reported to be dependent on LAT (Shan and Wange, 1999), was not affected by the absence of LAT or the presence of LAT<sup>mut</sup> (Fig. 3 B). TCR-induced Ca<sup>2+</sup> mobilization was restored by LAT<sup>wt</sup> expression but absent in J.CaM2.5 and J.CaM2.5-LAT<sup>mut</sup> cells (Fig. 3 C), indicating that the truncated protein itself is not capable of mediating TCR-induced inositol 1,4,5-trisphosphate (IP₃) production and subsequent Ca<sup>2+</sup> flux. Similarly, extracellular signal-regulated kinase (ERK) phosphorylation and up-regulation of CD69 was strongly reduced in J.CaM2.5-LAT<sup>mut</sup> and J.CaM2.5 cells compared with J.CaM2.5-LAT<sup>wt</sup> cells (Fig. 3 D), showing that the mutation interferes with LAT signaling.

**Signaling in LAT<sup>mut</sup> lymphocytes**

Next, we addressed the effect of the mutated LAT on TCR signaling in primary T cells of patient 2 after stimulation with anti-CD3 or anti-CD3/anti-CD28. As previously reported in LAT-deficient mice (Archambaud et al., 2009), we observed significantly decreased CD3 expression on LAT<sup>mut</sup> CD4<sup>+</sup> T cells (Fig. 4 A), in contrast to mice not on CD8 T cells.
In line with reconstituted J.CaM2.5 cells, phosphorylation of ITK was observed in CD45R0 CD4 T cells of patient 2 after CD3 cross-linking (Fig. 4 B), and the phosphorylation of ERK was absent in LATmut-expressing CD4 T cell subpopulations (Fig. 4 C). Surprisingly, and in contrast to our reconstitution experiments and to mouse LAT-deficient T cells, Ca\(^{2+}\) mobilization was detectable in three independent experiments in CD45R0 CD4 T cells and CD45R0 CD8 T cells of the patient and was predominantly within the range of healthy controls (Fig. 4 D). Ca\(^{2+}\) mobilization in naive CD4 was not evaluable because of extremely low cell counts. In line with the preserved Ca\(^{2+}\) response, I\(\kappa\)B\(\alpha\) degradation, which is also downstream of PLC\(\gamma\)1 activation, occurred normally after CD3/CD28 co-stimulation (Fig. 4 E), indicating that signaling was partially maintained in primary T cells in the absence of LAT. Genomic DNA sequencing of isolated CD45R0 CD4 T cells was performed to exclude reversion of the mutation as a potential explanation for this finding. However, only mutated LAT was detected in this population (not depicted). In addition, the possibility of alternative splicing was addressed by testing the mutant sequence for potential new splice sites. Two possible variants were predicted in silico. However, both variants were not detectable by PCR. Furthermore, RNA sequencing data of sorted CD45R0 CD4, CD8, and y6 T cells showed various fragments covering the genomically encoded 2-bp deletion, but no fragments compatible with the predicted aberrant splicing variants were detected (unpublished data). In T cells of the heterozygous parents and sister, the phosphorylation of ZAP70, ITK, ERK, and Ca\(^{2+}\) mobilization was found to be normal (not depicted), indicating that there is no dominant-negative effect of the mutation in the heterozygous situation.

Expression of alternative adapter molecules in LATmut T cells
Given the preserved Ca\(^{2+}\) response in primary T cells in contrast to J.CaM2.5-LATmut cells, we searched for adapter molecules potentially replacing the function of LAT with regard to Ca\(^{2+}\) mobilization. Expression of the adapter molecules ST, TRIM (TCR-interacting molecule), LAX (linker for activation of X cells), LIME, and PAG did not remarkably differ in patients’ T cells compared with controls in RNA sequencing analysis (Fig. 5 A). Because RNA for non-T cell activation linker (NTAL)/linker for activation of B cells (LAB), the B cell homologue of LAT, was increased, we investigated protein expression in primary T cells but could not detect protein expression (Fig. 5 B).

CD6 was expressed on primary T cells of the patient and controls, whereas it was nearly absent on J.CaM2.5 cells (Fig. 5 C). To investigate the influence of CD6 on Ca\(^{2+}\) induction in the absence of LAT, J.CaM2.5.J.CaM2.5-LATmut and J.CaM2.5-LATmut cells were stably transduced with CD6. Ca\(^{2+}\) mobilization was determined in nonselected CD6-transduced cell lines after gating on CD6-positive or CD6-negative cells. We could not observe an improvement of Ca\(^{2+}\) mobilization in CD6-expressing J.CaM2.5 and J.CaM2.5-LATmut cells, implying that CD6 could not compensate for LAT deficiency in these T cell lines (Fig. 5 D). Thus, CD6 expression in Jurkat-derived cell lines is not sufficient to replace LAT function.

Lymphocyte activation and function
The primary T cells of patient 2 were analyzed for functional impairment caused by the LAT mutation. In ac-
cordance with LAT-deficient mice, a high percentage of LATmut-expressing CD4 T cells constitutively produced IL-4, implying an expansion of the T helper type 2 (Th2) phenotype (Fig. 6 A). In addition, the percentage of IFN-γ–producing CD4 T cells was slightly increased after PMA/ionomycin stimulation. The percentage of IL-17 and IL-2–producing CD45R0 CD4 T cells was similar to the controls after in vitro stimulation with PMA/ionomycin (Fig. 6 A). Anti-CD3 stimulation for 20 h induced only reduced levels of CD69, inducible T cell co-stimulator (ICOS), and CD25 in LATmut T cells, which could not be increased by the addition of anti-CD28 (Fig. 6 B). Similarly, in vitro proliferation of CD4 T cells (Fig. 6 C) and CD8 T cells (not depicted) after stimulation with anti-CD3, anti-CD3/anti-CD28 was abrogated and strongly reduced after PHA compared with the control. Finally, the degranulation of LATmut CTLs after stimulation with anti-CD3/anti-CD28 beads (Fig. 6 D) and of patient-derived NK cells after stimulation with the K562 cell line was reduced (Fig. 6 E), although not absent. Accordingly, NK cells of patient 1 showed diminished cytotoxicity compared with day controls (not depicted). This might reflect previous results from mice that signaling via LAT is not essential but beneficial for the process of degranulation (Ou-Yang et al., 2012; May et al., 2013).

CD4 T cells of the heterozygous parents and one heterozygous sibling displayed normal up-regulation of CD69, ICOS, and CD25 T cells, normal percentage of IFN-γ and IL-17–producing, but an increased percentage of IL-4–positive CD4 T cells in the 9-yr-old sibling (20%). NK cell degranulation in the heterozygous sibling was within the range of healthy controls (not depicted).

γδ T cells in LAT-mutated patient 2
A common finding in all patients was the remarkable increase of γδ T cells. γδ T cells of patient 2 consisted of 24% CD8dim and 76% double-negative (CD4 negative/CD8 negative) γδ T cells, which is comparable to healthy controls. Unlike in healthy individuals, the most abundant circulating Vδ2-positive γδ T cell population was nearly absent in patient 2 (Fig. 7 A), and in line with this finding, Vy9-positive cells were <5% (not depicted). 25% of the patient’s γδ T cells expressed Vδ1, indicating that 74% Vδ1/Vδ2-negative γδ T cells expressed Vδ3, which could not be directly tested because of absent specific antibodies. Consistent with the findings in αβ T cells, Ca2+ mobilization was induced in γδ T cells after CD3 cross-linking and was comparable to healthy controls (Fig. 7 B).

As observed in healthy controls, most CD8–expressing γδ T cells produced IFN-γ upon PMA/ionomycin stimulation (not depicted). Among double-negative (CD4 negative/CD8 negative) γδ T cells, the number of spontaneous IL-4 producers was higher than in the controls, whereas IFN-γ–positive cell counts upon PMA/ionomycin stimulation were similar to healthy controls (Fig. 7 C), again showing the increase in the Th2 phenotype.
Here, we describe the first kindred with a homozygous mutation in LAT presenting with a progressive combined immunodeficiency and profound immune dysregulation. All patients suffered from early onset autoimmune manifestations with normal lymphocyte counts and Ig levels. During the progression of the disease, the immune systems of the two older patients seemed to collapse, lymphocytopenia and hypogammaglobulinemia developed, and opportunistic as well as other infections occurred. The early death of patient 3 at 2 yr of age probably explains why she did not enter the second phase of the disease observed in her siblings. The mutation resulted in the expression of a truncated protein with a preserved extracellular and transmembrane region but lacking a large proportion of the intracellular adapter modules reported to be critically involved in TCR signaling pathways and T cell development in mice (Sommers et al., 2001).

LAT knockout mice (Zhang et al., 1999b) and mice with targeted replacement of all four tyrosine residues (Sommers et al., 2001) lack peripheral T cells because of a block at the double-negative 3 stage, whereas in humans, T cells lacking all four equivalent tyrosine residues were still present, although with a severely disturbed differentiation.

**DISCUSSION**

Here, we describe the first kindred with a homozygous mutation in LAT presenting with a progressive combined immunodeficiency and profound immune dysregulation. All patients suffered from early onset autoimmune manifestations with normal lymphocyte counts and Ig levels. During the progression of the disease, the immune systems of the two older patients seemed to collapse, lymphocytopenia and hypogammaglobulinemia developed, and opportunistic as well as other infections occurred. The early death of patient 3 at 2 yr of age probably explains why she did not enter the second phase of the disease observed in her siblings. The mutation resulted in the expression of a truncated protein with a preserved extracellular and transmembrane region but lacking a large proportion of the intracellular adapter modules reported to be critically involved in TCR signaling pathways and T cell development in mice (Sommers et al., 2001).

LAT knockout mice (Zhang et al., 1999b) and mice with targeted replacement of all four tyrosine residues (Sommers et al., 2001) lack peripheral T cells because of a block at the double-negative 3 stage, whereas in humans, T cells lacking all four equivalent tyrosine residues were still present, although with a severely disturbed differentiation.
Figure 7. γδ T cells in LAT mutation. (A) FACS plots of γδ and αβ T cells in patient 2 and in a representative healthy control (top) and distribution of Vδ1 versus Vδ2 expression gated on γδ T cells (bottom). (B) Ca2+ mobilization in γδ T cells in the patient and a control. The arrow indicates the addition of goat anti-mouse for CD3 cross-linking. The experiment was performed once. (C) Percentage of spontaneous IL-4 producer (IL-4pos) and IFN-γ-positive (IFN-ypos) γδ T cells after PMA/ionomycin stimulation in two independent experiments compared with three healthy controls each. ctrl, control.

In mice, site-directed mutation of the PLCγ1 recruitment site Y136 caused a partial block in TCRαβ T cell differentiation, and over time, these mice developed a lymphoproliferative disorder, systemic autoimmunity, eosinophilia, and elevated IgE and IgG1 levels caused by a prominent Th2 cytokine shift (Aguado et al., 2002; Sommers et al., 2002). This is reminiscent of our patients with regard to lymphoproliferation, autoimmunity, and increased IL-4 production in γδ T cells after PMA/ionomycin stimulation in two independent experiments compared with three healthy controls each. ctrl, control.

Interestingly, the expansion of Vδ3 T cells may contribute to the increased percentage of IL-4–producing γδ T cells (Mangan et al., 2013). A similar expansion of Vδ1 and Vδ3 T cells has been reported in CMV-seropositive patients after kidney and stem cell transplantation (Déchanet et al., 1999; Knight et al., 2010), and a potential involvement of these cells in the anti-CMV immune response has been implied. Of note, patient 2 had CMV viremia, possibly contributing to the skewed Vδ usage seen in this patient.

Thus, LAT-mutated mice and humans resemble each other with regard to severe immune dysregulation and lymphoproliferation associated with an expansion of γδ T cells and a disposition to a Th2 bias. Given the history of our patients, some of the differences compared with the mouse models like the emerging hypogammaglobulinemia and general lymphopenia might be secondary to infection and the increasing immune dysregulation, but the detectable development of mature T cells despite the absence of all four tyrosine phosphorylation docking sites clearly demonstrates a difference between both species.

We therefore analyzed the signaling downstream of the TCR in detail after having excluded a genetic reversion of the LAT mutation and alternative splicing of the mRNA. In mouse T cells and human T cell lines, LAT has been demonstrated to be essential for Ca2+ mobilization and ERK phosphorylation after TCR stimulation (Lin and Weiss, 2001; Balagopalan et al., 2010). Thus, hY132/mY136 phosphorylation is required for the recruitment of PLCγ1, generation of IP3, and subsequent Ca2+ mobilization in mouse T cells (Zhang et al., 2000). Consistent with this finding, the expression of LATwt but not LATmut protein in LAT-deficient J.CaM2.5 cells was able to rescue Ca2+ signaling. However, to our surprise, Ca2+ mobilization after CD3 cross-linking was normal in primary T cells of the index patient, and the sustained T cell differentiation in LAT-deficient patients supports the notion of a preserved residual TCR signal in vivo.

This discrepancy between human T cell lines and primary T cells might be caused by intrinsic differences in the signaling properties of cell lines and primary cells like the
absence of the phosphatases PTEN (phosphatase and tensin homolog) and SHIP-1 in Jurkat cells (Abraham and Weiss, 2004) or the presence of alternative transmembrane adapters replacing LAT function in primary T cells. SIT (Marie-Caridine et al., 1999), TRIM (Bruyns et al., 1998), LAX (Zhu et al., 2002), LIME (Brđičková et al., 2003; Hur et al., 2003), and PAG (Stepean et al., 2014) share structural features with LAT but are expressed in both primary T cells and in Jurkat cells. Moreover, they were not differentially expressed in the primary cells of the index patient compared with controls on the RNA level, rendering the functional substitution for LAT unlikely. NTAL/LAB is not expressed in normal resting T cells (Brđička et al., 2002) but might be up-regulated in activated T cells (Zhu et al., 2006), and LAB expression can partially restore Ca\(^{2+}\) flux in LAT-deficient J.CaM2.5 cells (Janssen et al., 2004). However, we could not detect protein expression in primary T cells of our patient and therefore did not follow up on this protein. Based on the different expression patterns in primary T cells and the J.CaM2.5 cell line, the most potent candidate was CD6, which associates, like LAT, with SLP76 in an activation-dependent manner (Roncagalli et al., 2014). However, in J.CaM2.5 cells and in J.CaM2.5-LATmut cells, the induced expression of CD6 was not sufficient to elicit Ca\(^{2+}\) mobilization upon TCR stimulation. Therefore, it is unlikely that CD6 replaces LAT function in LAT-deficient primary T cells, and the explanation of the preserved Ca\(^{2+}\) flux, NF-κB activation in the patient’s T cells, and the persistent T cell differentiation remains elusive at this time.

In contrast to Ca\(^{2+}\) mobilization, ERK phosphorylation and the up-regulation of CD69 were abolished in primary LATmut-expressing T cells and in LAT-deficient and LATmut-expressing J.CaM2.5 cells. A functional Ca\(^{2+}\) response but defective ERK signaling in the patient’s T cells could be explained by several mechanisms. First, if the LAT-substituting protein allowed TCR-induced activation of PLCγ1, IP\(_3\) and diacylglycerol (DAG) would be produced. IP\(_3\) would cause Ca\(^{2+}\) influx, but DAG would not be sufficient to activate ERK. Indeed, using primary human T cells, it was shown that both Ras-activating proteins RasGRP1 (downstream of DAG) and SOS1 are required to activate Ras and thus the ERK pathway after an activating TCR trigger (Poltorak et al., 2014). In fact, LAT tyrosine residues hY171/mY175, hY191/mY195, and hY226/mY235 are indispensable in recruiting Grb2-SOS1 to the membrane activating the ERK pathway (Finco et al., 1998; Zhang et al., 1999a; Sommers et al., 2001; Balagopalan et al., 2010). In this scenario, the LAT-substituting protein would not recruit Grb2. Hence, our finding again corroborates the nonredundant role for hY171/mY175, hY191/mY195, and hY226/mY235 in the activation of ERK (Balagopalan et al., 2010; Poltorak et al., 2014) in humans and mice. Second, IP\(_3\) and DAG would be produced in the patient’s T cells, but highly active DAG kinases (DGKs), such as DGK\(\alpha\) and DGK\(\zeta\) (Joshi and Koretzky, 2013), would remove DAG so that RasGRP1 could not be activated and, subsequently, Ras would stay inactive. Because we did not find enhanced transcript levels for DGK\(\alpha\) and DGK\(\zeta\) in the patient’s T cells (unpublished data), we find this possibility less likely.

The observed alteration but not absence of TCR-induced signaling illuminates the phenotype, which resembles more the mouse phenotype of LAT signaling pathology in specific LAT mutations than the severe T cell deficiency in knockout mice. Although first proposed to result from defective positive selection and subsequent expansion of autoreactive T cells (Sommers et al., 2005), Roncagalli et al. (2010) subsequently suggested that hyperactivation of normal T cells triggered by peptide–MHC complexes leads to uncontrolled lymphoproliferation and immune dysregulation. Thus, the clinical presentation of human LAT deficiency resembles previously described immunodeficiencies caused by other alterations in TCR signaling like in ZAP70, ITK, or lymphocyte-specific protein tyrosine kinase (LCK) deficiency, all of which are interaction partners of LAT. Despite the immunological resemblance, especially to LCK deficiency, there are clearly distinct features like the prominent reduction of CD8 T cells in ZAP70 deficiency (Roifman et al., 2010), the absent Ca\(^{2+}\) signal in ITK deficiency (Linka et al., 2012), and the preserved ERK signal in LCK deficiency (Hauck et al., 2012) highlighting the complex regulation of the signaling and subsequent effect on T cell differentiation.

Given the severe clinical phenotype and high mortality resulting from the profound combined immunodeficiency and immune dysregulation observed in human LAT deficiency, hematopoietic stem cell transplantation of the index patient was performed. The 1-yr follow-up demonstrated full donor chimerism, resolution of opportunistic infections and autoimmune cytopenias, and disappearance of skin infiltrates without any immunosuppressive treatment. The identification of additional patients with possibly different LAT mutations will shed further light on the full immunological and clinical presentation of human LAT deficiency.

**MATERIALS AND METHODS**

**Patients.** The medical records of three siblings, two males and one female, from one Israeli Arab consanguineous kindred were reviewed for data on clinical presentation, immunological features, genetic findings, treatment, and final outcome. All experiments were performed after obtaining parental written informed consent and approval by the Hadassah and Israeli Ministry of Health ethical review boards.

**Antibodies used in this study.** The following antibodies were used in this study: CD3 AF700, CD4 BV421, CD4 FITC, CD6 APC, CD10 BV605, CD19 APC-Cy7, CD21 PE-Cy7, CD25 PerCP-Cy5.5, CD27 BV421, CD28 PerCP-Cy5.5, CD38 PerCp-Cy5.5, CD45RA APC-Cy7, TCR αβ PerCP-Cy5.5, IL-2 PE, LAT PE, NTAL/LAB APC, and biotin anti–human TCR-Vd2 (BioLegend); TCR-Vd1 APC (Miltenyi Biotec); CD8 APC, CD8 Pacific blue, CD21 PE-Cy7, CD31 PE, CD56 APC, CD69 FITC, CD107a PE, CD127 Alexa Fluor 647, IFN-γ FITC, TCR γδ PE, IgG
Alexa Fluor 700, IkBα PE, ERK1/2 (pT202/pY204) AF647, and ZAP70 (pY319)/SYK (pY352) APC (BD); IgD FITC and IgA PE (SouthernBiotech); CD3 PE-Cy7, CD4 PE-Cy7, CD8 PE, CD16 FITC, CD45RA FITC, CD45 Pacific blue, Vα24 PE, and Vβ11 FITC (Beckman Coulter); CCR7 PE (R&D Systems); Bruton tyrosine kinase/ITK (pY551/pY511) PE, IL-17 PE, IL-4 APC, and ICOS PE (eBioscience); IgM Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Inc.); and PLCγ1 (pY783) and goat anti–rabbit AF647 (Cell Signaling Technology). For immunohistochemistry, CD21 (Dako), CD20 (Invitrogen), CD3 (Cell Marque), CD4 and CD8 (Spring Bioscience), and Bcl-6 (Leica Biosystems) were used. For immunoblotting LAT (sc-7948; Santa Cruz Biotechnology, Inc.), FLAG tag (AHP1074; AbD Serotec), actin (sc-1616; Santa Cruz Biotechnology, Inc.), PLCγ1 (pY783; no. 2821; Cell Signaling Technology), and ZAP70 (pY319; no. 2701; Cell Signaling Technology) were used.

Whole exome sequencing. Exonic sequences were enriched in the DNA sample of our patient 2 using the SureSelect Human All Exon 50 Mb kit (Agilent Technologies). Sequences were determined by HiSeq2000 (Illumina) as 100-bp paired-end runs. Data analysis including read alignment and variant calling was performed by DNAnexus software using the default parameters with the human genome assembly (hg19; GRCh37) as a reference as previously described in the DNA sample of our patient 2 using the SureSelect Human All Exon 50 Mb kit (Agilent Technologies). Sequences were determined by HiSeq2000 (Illumina) as 100-bp paired-end runs. Data analysis including read alignment and variant calling was performed by DNAnexus software using the default parameters with the human genome assembly (hg19; GRCh37) as a reference as previously described (Stepensky et al., 2013). For confirmation of the identified mutation, Sanger sequencing was performed in the index patient, his parents, and two deceased and two living siblings.

Molecular biology. LAT transcript was examined in EBV lines of the index patient and healthy controls. RNA was isolated using the RNeasy Plus Mini kit (QIAGEN), and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific). PCR or quantitative PCR using SYBR green I master mix (Roche) was performed with HiSeq data or quantitative PCR using RNeasy Plus Mini kit (QIAGEN) and cDNA was generated using the RNeasy Plus Mini kit (QIAGEN) and cDNA was generated by using a Superscript II reverse transcription (Thermo Fisher Scientific).
by immunoblotting according to standard protocols via the C-terminal FLAG tag. To measure phosphorylation of PLCγ1 (pY783) and ZAP70 (pY319), 10^6 J.CaM2.5, J.CaM2.5-LAT^ext, and J.CaM2.5-LAT^mut cells were stimulated with 5 µg/ml anti-CD3 (UCHT1) for 3 min at 37°C or left untreated, total cell lysates were separated as before, and protein phosphorylation was detected by immunoblotting using phosphospecific antibodies.

**Cell isolation and cultivation.** PBMCs were isolated from EDTA blood by Ficoll density gradient centrifugation following standard protocols. CD45R0 CD4 T cells were isolated using a memory CD4^+^ T cell isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. LAT-deficient J.CaM2.5 cells and primary PBMCs were cultivated in RPMI 1640 (PAN Biotech) containing 10% FCS, 1% penicillin, and 1% streptomycin.

**Ca^2+ mobilization.** To analyze Ca^2+ mobilization, cells were labeled with 4.5 µM Indo-1 and 0.045% Pluronic F-127 (Thermo Fisher Scientific) for 45 min at room temperature. Cells were washed twice, and for primary cells, cell surface staining for CD4, CD8, and CD45RA or additionally TCR γδ was performed for 15 min at room temperature. Ca^2+ mobilization was determined after incubation with 5 and 0.6 µg/ml anti-CD3 (UCHT1; BD) for 10 min at room temperature. Data acquisition was performed on a flow analyzer (LSR Fortessa; BD). After baseline acquisition for 45 s, cross-linking was performed with 5 µg/ml goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Ionomycin (Sigma–Aldrich) was added as a positive control. J.CaM2.5 cells were stimulated by adding 5 µg/ml anti-CD3 (OKT3) or 5 µg/ml anti-CD3 and preincubated with goat anti-mouse IgG for 30 min at 4°C after a 30-s baseline acquisition. Ca^2+ flux was recorded for 5 min before ionomycin was added as a loading control.

**Signaling assays.** For intracellular flow cytometry, cells were incubated with 5 µg/ml anti-CD3 (OKT3) with or without 5 µg/ml anti-CD28 for 30 min on ice. 5 µg/ml goat anti-mouse IgG was added for an additional 30 min. J.CaM2.5 cells were stimulated with 5 µg/ml anti-CD3 (OKT3). For detection of ZAP70 (pY319), Bruton tyrosine kinase/ITK (pY551/pY511), PLCγ1 (pY783), and ERK1/2 (pT202/pY204) cells were stimulated for 2 min with anti-CD3 and for S6 (pS235/pS236) and IkBa, for 30 min with anti-CD3/anti-CD28. Immediately after stimulation, cells were fixed and subsequently permeabilized using the Phosflow intracellular staining kit (BD) according to the manufacturer’s instructions. Cells were stained with the respective antibodies.

**Cytokine production.** Intracellular cytokines were determined after stimulation of PBMCs with 10 µg/ml anti-CD3 (OKT3) and 2 µg/ml anti-CD28 (Sanquin) or 5 ng/ml PMA and 0.75 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A (all from Sigma–Aldrich). After 4 h, cells were harvested, and fixation and permeabilization was performed using a staining kit for intracellular cytokines (BD) according to the manufacturer’s protocol, and after surface and intracellular staining for 20 min, the cells were analyzed by flow cytometry.

**T cell activation and proliferation.** For T cell activation, 2 × 10^5 cells were stimulated with plate-bound anti-CD3 (OKT3) or anti-CD3/anti-CD28 for 16 h at 37°C. Cell lines were stimulated with 5 µg/ml of soluble anti-CD3 (OKT3) for 6–16 h. Subsequently, cells were harvested and stained with the corresponding antibodies. For T cell proliferation, PBMCs were labeled with 0.5 µM CFSE (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cells were left untreated or stimulated with 1 µg/ml of plate-bound anti-CD3 or 0.3 µg/ml anti-CD3 and 0.5 µg/ml of soluble anti-CD28 or PHA for 5 d at 37°C. Cells were harvested and stained for CD4 and CD8.

**NK cell degranulation.** 2 × 10^5 PBMCs were mixed with 2 × 10^5 target cells of the human erythroleukemia cell line K562 (American Type Culture Collection) in Isocyn’s modified Dulbecco’s medium (Thermo Fisher Scientific) containing 10% FCS. Cells were spun down for 3 min at 30 g and stimulated for 2 h at 37°C. After stimulation, cells were harvested and stained with the corresponding antibodies for flow cytometry to determine CD107 up-regulation on CD56^+ CD3^- NK cells, correlating with target cell lysis.

**ACKNOWLEDGMENTS**

The authors would like to thank Iris Porat, Carmit Lugasy, Ina Stumpf, Liselotte Lenner, the Advanced Diagnostics unit of the Center for Chronic Immunodeficiency, and the BiOSS toolbox for excellent technical assistance. We also thank the team of the Department of Pediatric Hematology Oncology of Ruth Rappaport Children's Hospital for the treatment of the child and the family of the patient for their trust and support. We also thank Burkhart Schraven and Luca Simeoni for discussions.

B. Keller, O.S. Yousef, S. Unger, W.W. Schamel, and K. Warnatz were supported by the German Federal Ministry of Education and Research (BMBF 01EO1303). P. Stepensky and K. Warnatz received funding from the Deutsche Forschungsgemeinschaft [Discovery and Evaluation of New Combined Immunodeficiency Disease Entities; grant DFG WA 1597/4-1]. P. Stepensky was supported by a research grant from the joint fund of the Hebrew University and Hadassah Medical Center. This study was supported in part by the Excellence Initiative of the German Research Foundation (GSC-4, Spremann Graduate School).

The authors declare no competing financial interests.

Submitted: 7 July 2015
Accepted: 4 May 2016

**REFERENCES**


Archambaud, C.A. Sansoni, M. Mingueau, E. Devillard, G. Desol, B. Malissen, and M. Malissen. 2009. STAT6 deletion converts the Th2 inflammatory pathology affecting Lat<sup>Δ148β</sup> mice into a lymphoproliferative disorder involving Th1 and CD8 effector T cells. *J. Immunol.* 182:2680–2689. http://dx.doi.org/10.4049/jimmunol.1803257


