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The analysis of clonal diversity and therapy responses using STAT3 mutations as a molecular marker in large granular lymphocytic leukemia

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Abstract

T cell large granular lymphocytic leukemia and chronic lymphoproliferative disorder of natural killer cells represent an intriguing entity between benign and malignant lymphoproliferation. The molecular pathogenesis has partly been uncovered by the recent discovery of somatic activating STAT3 and STAT5b mutations. Here we show that 43% (75/174) of patients with T cell large granular lymphocytic leukemia and 18% (7/39) with chronic lymphoproliferative disorder of natural killer cells harbor STAT3 mutations when analyzed by quantitative deep amplicon sequencing. Surprisingly, 17% of the STAT3-mutated patients carried multiple STAT3 mutations, which were located in different lymphocyte clones. The size of the mutated clone correlated well with the degree of clonal expansion of T cell repertoire analyzed by T cell receptor beta chain deep sequencing. The analysis of sequential samples suggested that the current immunosuppressive therapy is not able to reduce the level of the mutated clone in most cases warranting the search of novel targeted therapies. These results imply that the clonal landscape of large granular lymphocytic leukemia is more complex than considered before, and a substantial number of patients have multiple lymphocyte subclones harboring different STAT3 mutations, thus mimicking the situation in acute leukemia.
**Introduction**

Large granular lymphocytic (LGL) leukemia is characterized by the increased number of clonal cytotoxic T- or NK cells in blood and bone marrow. Concurrent neutropenia, anemia, and autoimmune disorders such as rheumatoid arthritis further support diagnosis in unclear cases. Leukemic T-LGLs are terminally differentiated effector memory cells (CD3+CD45RA+CD62L\textsuperscript{neg}CD57+) with clonal rearrangement of the T cell receptor (TCR) genes. According to World Health Organization (WHO) 2008 criteria, chronic lymphoproliferative disease of NK cells (CLPD-NK) is a separate entity and differs from aggressive, chemorefractory NK-LGL leukemia.

The phenotype of leukemic cells indicates that the expansion originally developed in response to an antigen. However, unlike their normal counterparts, leukemic LGLs are resistant to subsequent Fas-mediated activation-induced cell death. Current hypothesis postulates that following antigen encounter, the dysregulation of several signaling pathways and cytokines such as IL-15, platelet derived growth factor receptor (PDGFR), and signal transducer and activator of transcription 3 (STAT3) sustains a constitutively active clonal cell population. The importance of these pathways has been emphasized in the light of the recent discovery of somatic mutations in STAT3 and STAT5b genes. The mutations were located in the Src-like homologue 2 (SH2) domain of STAT3 and STAT5b, caused constitutive phosphorylation of the mutated proteins, and increased the transcriptional activity of STAT3 and STATb5 in vitro. Expression of mutated STAT3 in mouse bone marrow led to the development of myeloproliferative neoplasm, and recently similar STAT3 and STAT5b mutations have been discovered also in other hematological malignancies.

In this project we aimed to analyze the frequency of STAT3 mutations and the clonal architecture of expanded lymphocytes using both STAT3 and T cell receptor beta (TCRB) chain deep sequencing methods in a unique cohort of 213 LGL leukemia patients. In addition, the effect of immunosuppressive treatment on the mutated clones was studied with follow-up samples obtained during therapy to assess if quantitative STAT3 mutation analysis could be used to monitor therapy response.
Methods

Additional methods of this study are provided in the Supplementary Appendix.

Study patients

The study was undertaken in compliance with the principles of the Helsinki declaration and was approved by the ethics committees of the Helsinki University Central Hospital (Helsinki, Finland), the Cleveland Clinic (Cleveland, Ohio) and the Penn State Hershey Cancer Institute (Hershey, Pennsylvania). The study population consisted of 213 patients with LGL leukemia as defined by WHO 2008 criteria: 174 patients had CD8+ T-LGL leukemia and 39 patients CLPD-NK. Samples were collected in the Penn State Hershey Cancer Institute (n=93), the Cleveland Clinic (n=89), and Finland (n=31). 112 T-LGL leukemia and 30 CLPD-NK patients included in the current study cohort have also been part of the two previous study cohorts analyzing STAT3 mutation frequency by capillary sequencing. All patients gave written informed consent.

Sample collection and DNA extraction

Mononuclear cells (MNCs) were separated from peripheral blood (PB) using Ficoll gradient separation (GE Healthcare, Pittsburgh, PA, US) and cryopreserved in fetal bovine serum with 10% DMSO. PB MNCs were labeled with antibodies and sorted into CD4+ T cell, CD8+ T cell, and NK cell fractions using antibodies for CD3, CD4, CD8, and CD16/56 (Beckton Dickinson, San Jose, CA, USA). In T-LGL leukemia cases, CD8+ T cell population was further sorted into clonal/non-clonal cells based on the flow cytometry analysis of T cell receptor (TCR) beta variable chain (Vbeta) expression (IOTest® Beta Mark Kit, Beckman Coulter, Brea, CA, US). DNA was extracted using NucleoSpin Tissue or Tissue XS kit (Macherey-Nagel, Dueren, Germany) and the concentration was measured with Qubit (Life Technologies, Carlsbad, CA, US).

Deep targeted sequencing and capillary sequencing of STAT3 exon 21

STAT3 exon 21 was sequenced using deep amplicon sequencing and Illumina Miseq platform as described previously. The data were analyzed with a novel in-house bioinformatics pipeline, which is based on calling of variants with certain count/frequency and filtering out false positives using the estimated error rate and quality data of amplicon reads. STAT3
amplicon sequencing and data analysis are described in detail in the Supplementary appendix. STAT3 exon 21 capillary sequencing was performed as previously described.9

**High-throughput T cell receptor sequencing**
TCRB complementarity determining regions (CDR3β) were amplified and sequenced by Adaptive Biotechnologies Corp (Seattle, WA, US) using the ImmunoSEQ assay Survey level analysis, which is capable of detecting one cell in 40,000 T cells.20 TCRB sequencing method is described in the Supplementary appendix.

**Statistical analysis**
Comparisons of categorical variants between groups were done with chi-square test. Comparisons of parametric variables between groups were performed with 1 way ANOVA and Bonferroni post hoc test, or unpaired t test, as appropriate. A P value <0.05 was considered statistically significant.
Results

**STAT3-amplicon sequencing is a sensitive and reliable method**

Deep, targeted sequencing is a relatively new method, and therefore, we first compared the sensitivity of amplicon sequencing with well-established allele-specific oligonucleotide (ASO) real-time quantitative PCR method. The results of ASO-qPCR for *STAT3* mutations Y640F and D661V and amplicon sequencing of *STAT3* exon 21 were congruent in 7/8 of the cases analyzed (Supplementary Table 1). The only discordant case (patient 14, Figure 1) was faintly positive for Y640F by ASO-qPCR, but even the analysis of sorted lymphocyte fractions did not show Y640F mutation by amplicon sequencing.

As another quality control, we sequenced a dilution series from a CD8+ PB MNC sample (Patient 9, Figure 1) with known *STAT3* D661V (variant allele frequency (VAF) 37%) and Y640F (VAF 1.4%) mutations in the CD8+ fraction: Y640F mutation with low VAF was not seen in the original sequencing of PB MNC sample (Supplementary Table 2, Figure 1). The D661V mutation was detected at the 2% dilution level with variant allele frequency (VAF) of 0.633% (frequency ratio 0.896), whereas with the next dilution of 1% and VAF of 0.433% the frequency ratio dropped under 0.8, being still on the borderline 0.75-0.80. However, the noise exceeded the number of variant alleles and the mutation could not be reliably verified (Supplementary Table 2). The 0.5% cut-off for VAF seemed reasonable and was used in the following analysis.

As a negative control, we sequenced samples from 8 chronic myeloid leukemia (CML) patients with clonal LGL lymphocytosis during dasatinib treatment (a proportion of dasatinib-treated CML patients have drug-induced clonal LGL lymphocytosis). Sorted monoclonal CD8+ expansions from 4 patients (CD8+ Vbeta clone size 10-69%) and total CD8+ fractions from 4 additional cases (CD8+ Vbeta clone size 10-27%) were analyzed by amplicon sequencing. All tested samples were *STAT3* mutation-negative.

The frequency and pattern of *STAT3* mutations in LGL leukemia patients by deep quantitative sequencing

The exon 21 of *STAT3* gene was analyzed both with capillary and amplicon sequencing from a cohort of 213 LGL leukemia patients. DNA samples were extracted either from whole blood (WB), peripheral blood mononuclear cells (PB MNCs), or CD8+ T cells. The frequency of *STAT3* mutations was 23% (50/213) by capillary and 38% (82/213) by amplicon sequencing,
showing the superiority of amplicon sequencing as a screening assay. The mutations were significantly more common in T-LGL leukemia than in CLPD-NK cases (75/174, 43% vs. 7/39, 18%, p=0.0034)\textsuperscript{8,11}. Capillary sequencing did not detect mutations with VAF smaller than 9% (Figure 1, Supplementary Table 3). The patient cohort also included 72 of the 77 T-LGL leukemia patients with large immunodominant clone (mean clone size 78\%, range 32-99\%) described in the original publication of STAT3 mutations in LGL leukemia\textsuperscript{9}, and the frequency of STAT3 mutations was as high as 63\% (45/72) by amplicon sequencing, compared to 43\% by capillary sequencing. The mean clone size in the rest of the patient cohort was 39\% of CD8+ cells (range 6-97\%).

**A substantial proportion of LGL leukemia patients carry multiple STAT3 mutations**

In the amplicon sequencing analysis 22\% (18 of 82) of STAT3-mutated patients harbored multiple mutations in the STAT3 gene (Figure 1). In 17/18 of the cases either Y640 or D661 or both were mutated (Figure 1). As an extreme example, 2 patients harbored 4 different STAT3 mutations (Figure 1). Patients with multiple STAT3 mutations could be divided into two different groups based on the allelic status derived from the amplicon data: A) patients harboring two single nucleotide variants (SNV) in the same STAT3 allele, resulting in one or two amino acid change (n=4, Figure 1, patients 1-4), and B) patients displaying multiple STAT3 mutations in different alleles and lymphocyte clones (n=14, Figure 1, patients 5-18). Thus, 17\% of STAT3-mutated cases (14 of 82) had multiple mutated clones in the original screening.

**Correlation of STAT3 mutations with clinical parameters**

Intrigued by the finding of multiple STAT3 mutations, we studied the correlation between the phenotype of the patients and STAT3 mutation status to assess whether multiple mutations would affect the clinical characteristics of patients (Table 1). Rheumatoid arthritis (RA), which is the most common autoimmune manifestation in LGL leukemia patients\textsuperscript{23}, was significantly more common in T-LGL patients with one or multiple STAT3 mutations: 43\% (6/14) of patients with multiple mutations and 23\% (14/61) with one mutation suffered from RA compared to 6\% (6/99) of unmutated patients (p<0.0001)(Table 1). No statistical difference was seen in the prevalence of RA when patients with one or multiple STAT3 mutations were compared (p=0.18). Patients who had multiple STAT3-mutated clones were almost exclusively males (13/14 (93\%), Table 1). The size of the major expansion in TCR V\beta analysis was the highest among the patients with one STAT3 mutation compared with unmutated cases (p<0.0001) (Figure 2A). Neutropenia is often associated with LGL leukemia\textsuperscript{23}, and T-LGL
leukemia patients with STAT3 mutations had a tendency toward lower neutrophil counts at diagnosis, but the difference was not statistically significant (Table 1). No significant clinical correlations were detected in the CLPD-NK cohort, possibly due to the lower number of patients (Table 1).

The clinical picture was compared in more detail between patients carrying the two most common mutations, Y640F and D661Y (Figure 1). D661Y-mutated patients had lower hemoglobin values at diagnosis (median 104 vs. 122 g/l, p=0.0449), whereas leukocyte and lymphocyte counts were higher (12.4 vs. 5.1 x10⁶/l, p=0.0440 and 7.5 vs. 3.2 x10⁶/l, p=0.0028, respectively) (Figure 2B-D).

**STAT3 sequencing of sorted lymphocyte fractions**

The screening of STAT3 mutations was performed from unsorted samples, and therefore the exact location of the mutations and the clonal hierarchy was further analyzed from sorted lymphocyte fractions.

*Patients with one STAT3 mutation have monoclonal pattern in Vbeta analysis*

First we sorted and analyzed samples from 5 T-LGL leukemia patients with a monoclonal pattern in TCR Vbeta analysis (clone size 27-81%) carrying a single STAT3 mutation in the original screening. In 4/5 cases the mutation was located in the major CD8+ Vbeta expansion, and no other STAT3 mutations were detected in sorted lymphocyte fractions including CD4+ T cells, Vbeta expansion-negative CD8+ cells, and CD3neg NK cells and B cells (Figure 3A, Supplementary Figure 1). In one case (patient 48, figure 1), in addition to the N647I-mutated major Vb21.3+ expansion, three low VAF mutations were detected in the sorted Vb21.3neg CD8+ fraction, which were not discovered in the original screening (Figure 3B).

*Multiple STAT3 mutations in sorted lymphocytes*

Similar analysis was done from 2 T-LGL leukemia patients who harbored a set of multiple STAT3 mutations already in the original screening (Figure 3C, Supplementary Figure 1). The patients carried STAT3 mutations in both detected Vbeta expansions and, in addition, an apparently non-clonal CD8+ population of patient 7 contained two additional mutations, N647Y and D661V (Figure 3C). CD4+ and CD3neg lymphocyte fractions were not harboring STAT3 mutations.
Comparison of clonality analysis by STAT3 and deep T cell receptor beta chain sequencing

Detailed analysis of T cell receptor beta chain (TCRB) CDR3 region rearrangement and clonality was performed from paired samples of 3 T-LGL leukemia patients who had multiple STAT3 mutations. The patients were not HLA-matched. We assumed that in the case of a heterozygous STAT3 mutation, the size of the mutated clone by deep TCRB sequencing would be approximately twice the STAT3 mutation VAF. The number of TCRB CDR3 reads for each sample is shown in Supplementary Table 4.

Deep TCRB sequencing results are in concordance with STAT3 mutation analysis

The results of deep TCRB sequencing were in concordance with STAT3 mutation analysis, and they also explained the observed differences between clone size analysis (flow cytometry) and STAT3 amplicon sequencing. Patient 8, who had Y640F-mutated Vb3+ expansion in the Vbeta flow analysis, had correspondingly one major expansion in the sorted Vb3+ fraction (73% of TCRB sequences)(Figure 4A, Supplementary Table 5). The Vb3neg fraction was also abnormally skewed and consisted of a single clonal TCRB sequence (74% of reads), which explains the high VAF (25-35% in different time-points) of STAT3 I659L in the Vb3neg fraction (Figure 4A). The TCRB rearrangement observed in the Vb3neg fraction appeared also in the sorted Vb3+ sample, due to impurity of the sorting (86% of sorted cells were Vb3+) (Figure 4A).

Patient 48 had large Vb21.3+ clone in Vbeta flow analysis, but the VAF of N647I was only 34% in the sorted Vb21.3+ fraction. The sorted Vb21.3+ population was dominated by a clonal TCRB rearrangement that covered 63% of TCRB sequences, corresponding to the estimated percentage of STAT3-mutated cells from amplicon sequencing (2xVAF of 33%), but in addition, 26% of the sequences came from another rearrangement with TCRBV20-01 gene (Figure 4B). Interestingly, based on the flow cytometry purity analysis the sorted Vb21.3+ fraction seemed monoclonal and contained only Vb21.3+ cells, so both clones with different amino acid sequences were recognized by the same Vbeta antibody (Table 2). The Vb21.3neg population was less skewed: the largest productive TCRB rearrangements were 4%, 2% and 2% of sequences, and probably contained the STAT3 mutations detected in the Vb21.3neg fraction (Figure 4B, Supplementary Table 5).

Patient 14 had two CD8+ expansions (Supplementary Figure 1) and the TCRB analysis of the larger one (Vb17+) showed that 83% of TCRB rearrangement sequences were of a single clone, again corresponding well to the VAF (41%) of D661V mutation (Supplementary Figure
A smaller D661Y-mutated Vb13.6+ clone accounted for 11% of CD8+ cells in Vbeta analysis, but TCRB sequencing could not be done due to the low amount of Vb13.6+ DNA. A corresponding TCRB sequence was seen in the analysis of the CD3+ fraction (Supplementary Figure 1, Supplementary Table 5).

**STAT3-mutated clones did not share homologous TCRB sequences**

In the comparison of the 20 most frequent TCRB CDR3 aminoacid (AA) sequences of each patient, all sequences were unique and not present in either of the two other cases (data not shown). Similar analysis of the clones shown in Supplementary Table 5 was done between patients and 586 healthy individuals (PB MNC sequenced, provided by the Adaptive Biotechnologies), and in each LGL leukemia case at least one of the leukemic clones was seen in healthy control dataset (the prevalence of the amino acid sequence varied between 0-20% in the healthy control dataset, Supplementary Table 5). However, the frequencies of these amino acid sequences in individual healthy control samples were low (0.0001-0.005%), corresponding to a single cell per sample.

In case of multiple STAT3-mutated clones within each patient, the sequences were not apparently homologous, although interestingly the flow cytometry Vbeta antibody recognized two rearrangements in the Vb21.3+ fraction of patient 48, suggesting that although the amino acid sequences in CDR3 region are dissimilar, they may share some homology in the 3D structure of the receptors (Figure 4B, Supplementary Table 5).

**STAT3 mutation analysis can be used in the follow-up of treatment response**

From 6 patients sequential samples were available for STAT3 mutation analysis including patients with one (n=3) or multiple (n=3) STAT3 mutations. Four patients were diagnosed with T-LGL leukemia, one with CLPD-NK, and one patient had both T- and NK cell expansions. Patients were treated either with methotrexate (n=2) or cyclophosphamide (n=3) and one patient was untreated. The median follow-up time was 33 months (range 24-62).

Patient 4 was untreated, and developed anemia and neutropenia during follow-up of 28 months: the CD8 percentage slightly increased concomitant with the development of cytopenias, but the mutated leukemic clone was unchanged and no new STAT3 mutations evolved (Figure 5A). The mutated clone(s) persisted during the course of therapy in two T-LGL leukemia patients treated with methotrexate, although improvement of hemoglobin values was observed in both cases during immunosuppression (Figure 5B, Supplementary Figure 2). However, achieved partial response was lost after the cessation of methotrexate in the case of patient 8 (Figure 5B).
The disappearance of \textit{STAT3}-mutated clones was associated with complete remission in two patients treated with cyclophosphamide (Figure 5C, Supplementary Figure 2). In both cases \textit{STAT3} mutation status was the only reliable marker for clonality, as both patients had an aberrant leukemic CD3\textsuperscript{neg}CD19\textsuperscript{neg}CD16/56\textsuperscript{neg} NK-LGL population carrying a \textit{STAT3} mutation (Figure 5C, Supplementary Figure 2). In the case of patient 44, D661Y-mutated NK-LGL cells were suppressed and normal NK cells were restored at complete remission (Figure 5C). The third patient treated with cyclophosphamide developed marked cytopenias during treatment, and no change was observed in the immunodominant \textit{STAT3}-mutated clone (Supplementary Figure 2).
Discussion

Next generation sequencing (NGS) methods have enabled the discovery of the molecular background and clonal hierarchy in many hematological malignancies. Although LGL leukemia differs from acute leukemia, our results show that clonal diversity plays also a role in this disease. To our surprise, 17% of all LGL leukemia patients with $STAT3$ mutations had multiple mutations in the $STAT3$ gene residing solely in cytotoxic CD8+ or NK cells. The initial mutation-causing event remains undiscovered, but the combined results from TCRB deep sequencing and $STAT3$ mutation analysis suggest that a polyclonal immune response may lay the foundation for mutagenesis.

Ultra-deep amplicon sequencing is a novel method for mutation screening, quantitative analysis of the mutated clones, and for the monitoring of the disease burden. Based on our results, the data obtained from amplicon analysis is in good accordance with ASO-qPCR, and the assay can reliably detect clones down to a VAF threshold of 0.5%, even though higher sensitivities may be reached with ASO-qPCR assays. Compared to ASO-qPCR, the amplicon method can cover a larger part of the gene (one exon, for example, in the case of $STAT3$) and identify previously unknown mutations in a single run, whereas ASO-qPCR is based on mutation-specific primers. However, ASO-qPCR may provide the data of common mutations quickly without the need of NGS facility. With the amplicon method, the $STAT3$ mutation frequency was 38% in our whole LGL leukemia cohort and significantly higher (over 60%) when only patients with large immunodominant clones were analyzed. Based on these results it is likely that LGL leukemia diagnosis done according to WHO criteria may include different disease subtypes and in some cases also reactive LGL proliferations. Similarly, previous publications have shown that $STAT3$ mutation prevalence can be as high as 40-70% in T-LGL leukemia, when patients with monoclonal expansions have been studied.9,11 In other hematological malignancies $STAT3$ mutations are only rarely found.12,15,17,24,25

The actual cause and the reason for the high incidence of $STAT3$ mutations in LGL leukemia is unknown. In the detailed analysis of LGL populations, the occurrence of $STAT3$ mutations was restricted to expanded lymphocyte clones. During CD8 memory cell development, the STAT3 pathway is activated, and it may be exposed to mutagenesis during an initial polyclonal cell proliferation.26,27 As the $STAT3$ mutations in LGL leukemia are gain-of-function mutations and able to cause leukemic transformation in multiple settings,8,9,14,15 they may give survival
advantage to affected cells, leading to the narrowing of TCR repertoire and expansion of certain clones. In most cases, patients with multiple mutations had one immunodominant clone and additional smaller expansions indicating that the clones may have different proliferation capacity or apoptotic resistance, which could be due to the activation potential or temporal variation in the occurrence of the STAT3 mutation in question. The presence of several STAT3 mutations could also be related to the clonal drift phenomenon, where the immunodominant LGL clone changes. In addition, STAT3-mutated clones may carry somatic mutations in other genes, which affect the proliferative capacity of affected lymphocytes. These mutations could also drive the lymphoproliferation in patients without STAT3 mutations. However, it is interesting to speculate that other factors such as dysregulated immune system may be needed to sustain the leukemic LGL expansion. In a proportion of cases, these other abnormalities can even lead to the development of LGL leukemia without a somatic driver mutation. This hypothesis is strengthened by recent publication showing that activated IL6-STAT3 loop had a significant role in LGL leukemia pathogenesis independent of STAT3 mutation status.

The association between RA and multiple STAT3 mutations in our patient cohort points toward the involvement of chronic antigen stimulation in the pathogenesis T-LGL leukemia. Based on our hypothesis polyclonal T cells with autoreactive TCRs may represent the actual starting point for multiple mutated clonal expansions and for the actual RA, but other factors are also likely to contribute. In our patient cohort we were not able to address whether in most cases RA precedes the actual diagnosis of LGL leukemia or vice versa, but in previous studies, symptomatic RA has been demonstrated to occur either concurrently or prior to LGL leukemia. Further studies with paired follow-up samples and genetically modified mouse models are warranted to understand the detailed pathomechanism and the relation of STAT3 mutation formation with RA.

The deep TCRB sequencing data showed that the results of STAT3 mutation analysis were in good accordance with the sizes of the clonal CDR3 rearrangements. STAT3-mutated clones did not share TCRB CDR3 homology even in the case of multiple STAT3 mutations in same patient. However, identical amino acid sequences could be found with the low frequency in healthy controls suggesting them to be common TCR sequences. It has been established earlier that the major clones in CD8+ T-LGL leukemia patients are rarely identical, but the immunodominant clones are sometimes seen with low frequency in other patients when the whole TCRB repertoire is analyzed. It should be though noted that the specific antigen can be recognized by a large number of different TCR types depending on the peptide
presented, and the variation of HLA genes also affects antigen recognition.\textsuperscript{37,38} Therefore, the presence of a shared antigen driving the LGL proliferation is not ruled out, although our current understanding suggests more private antigens.

During the past years LGL leukemia patients have primarily been treated with immunosuppressive regimens with unsatisfactory results, and over half of the patients relapse on therapy.\textsuperscript{3} In clinical studies, the treatment response evaluation has included mainly the follow-up of hematological parameters and TCR PCR testing, both of which are relatively insensitive and unspecific approaches. \textit{STAT3} amplicon sequencing provides a new method to analyze treatment response, and it generates quantitative data of the clone sizes. Although the number of patients we analyzed was relatively small, the sizes of the \textit{STAT3}-mutated clones were in good accordance with clinical results, and the findings may explain why patients relapse. For example, the patients treated with MTX did not show a marked change in the size of the mutated clone during the observation period. The initial partial responses achieved may be due to the immunosuppressive effect of MTX on cytotoxic cell function, but MTX therapy is not able to eradicate the mutated clone. Interestingly though, the mutated clones disappeared in two patients who went into CR during cyclophosphamide treatment. Concordantly, recent results support the use of cyclophosphamide as a first-line therapy: the overall response rate was 71\% (CR 47\%) and relapses were rarely observed.\textsuperscript{39} Low relapse rates could be related to the ability of cyclophosphamide to eradicate LGL clones, but further studies with larger patient cohorts are needed to confirm the preliminary findings.

To conclude, ultra-deep \textit{STAT3} amplicon sequencing is a reliable method, which can be used in the diagnostics and treatment response evaluation in LGL leukemia. A significant proportion of LGL leukemia patients have multiple \textit{STAT3} mutated lymphocyte clones mimicking the clonal diversity observed in patients with acute leukemia. Sequential analysis of samples suggested that the current immunosuppressive therapy is not able to eradicate the \textit{STAT3} mutated clones, and therefore novel targeted therapies are needed to improve treatment results.
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Authorship and disclosures

H.L.M.R designed the study, coordinated the project, performed sequence analysis, did laboratory studies, analyzed the data and wrote the paper. S.M. designed the study, coordinated the project, analyzed the data and wrote the paper. T.O., M.J.C., and A.J. provided patient samples and patient data, participated in the laboratory studies and contributed to write the paper. S.L., P.E., T.L., D.E.H, and E.I.A. performed sequence analysis. S.A.U.Z. and J.M.L.M designed and performed bioinformatics analysis. T.P.L, J.P.M., and K.P. participated in the study design, data analysis and contributed to write the paper. All authors read and approved the final manuscript.

K.P. has received research funding and honoraria from Novartis and Bristol-Myers Squibb. S.M. has received honoraria from Novartis, Bristol-Myers Squibb, and Pfizer. D.E.H is an employee of the company Adaptive Biotechnologies Corp and has equity ownership with the company. T.L. has received honoraria from Bristol-Myers Squibb and Roche.
References


Table 1. Clinical characteristics of the patient cohort.

<table>
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<th>Variable</th>
<th>T-LGL leukemia (n=174)</th>
<th>CLPD-NK1 (n=39)</th>
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<td></td>
<td>≥2 STAT3-mutated clones (n=14)</td>
<td>1 STAT3-mutated clone (n=61)</td>
<td>No STAT3 mutations (n=99)</td>
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<td>65 (31-84)</td>
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<td>Males (%)</td>
<td>13 (93%)</td>
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<td>114 (74-165)</td>
<td>120 (59-172)</td>
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<td>181 (29-596)</td>
<td>215 (9-627)</td>
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<td>Treatment (%)2</td>
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<td>43 (70%)</td>
<td>61 (62%)</td>
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<td>Rheumatoid arthritis (%)3</td>
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<td>14 (23%)</td>
<td>6 (6%)</td>
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</tbody>
</table>

Laboratory values presented in the table are measured at diagnosis. Statistics are compared between three groups of patients (≥2 STAT3-mutated clones vs. 1 STAT3-mutated clone vs. unmutated STAT3) in the case of T-LGL leukemia, and between two groups in CLPD-NK cases.

1Chronic lymphoproliferative disease of NK cells.
2Treatment at any timepoint during follow-up.
3Rheumatoid arthritis diagnosed at any timepoint during follow-up.
**Figure legends**

**Figure 1.** *STAT3* mutations and variant allele frequencies detected by amplicon sequencing. The patients are grouped based on the number and allelic location of *STAT3* mutations, and the LGL leukemia type. *STAT3* mutations detected also by capillary sequencing are marked with “x” in the last column. Abbreviations: NS, nonsense; VAF, variant allele frequency.

**Figure 2.** Comparison of different clinical parameters between LGL leukemia patients divided into groups based on *STAT3* mutation status. Horizontal lines define medians of measured variables in all figures. (A) The sizes of the largest clones in CD8+ lymphocytes detected in T-LGL leukemia patients by flow cytometry (T cell receptor beta chain variable region analysis). T-LGL patients were divided into three groups: those with ≥2 *STAT3* mutation (data available from 13 patients), 1 mutation (n=30) or no mutations (n=62). Hemoglobin (B), absolute leukocyte (C) and lymphocyte (D) counts of T-LGL leukemia patients with either D661Y or Y640F mutation were compared.

**Figure 3.** The flow cytometry Vbeta analysis and *STAT3*-sequencing results of flow cytometry-sorted lymphocyte fractions from 3 representative T-LGL leukemia patients. The monoclonal Vbeta antibodies are conjugated either with FITC (x-axis), PE (y-axis), or both PE and FITC (double positive population), and the Vbeta populations analyzed are marked in each dotplot with the percentage of the clone in CD8+ cells. *STAT3* mutations and their VAFs are indicated with arrows. (A) The results of patient 69 after 1.5 years after cyclosporine treatment. (B) *STAT3* results of patient 48 with 4 different *STAT3* mutations. (C) Patient 7 (Figure 1) had two CD8+ expansions at the baseline, Vb7.1+ 10% and Vb5.1 11% (data not shown), and four years later during methotrexate treatment the expansions were 3% and 13%, respectively, both harboring *STAT3* mutations. This patient had also two minor *STAT3* mutations in Vbeta-negative CD8+ cells. Abbreviations: FITC, fluorescein isothiocyanate; PE, Phycoerythrin; VAF, variant allele frequency.
**Figure 4.** Flow cytometry Vbeta analysis results and TCRB CDR3 repertoire landscape of two T-LGL leukemia patients. STAT3 mutation VAFs are shown in the titles of the analyzed fractions.

(A) Patient 8 had one major Vb3+ expansion (93%) in Vbeta analysis, corresponding to the major TCRB rearrangement in the sorted Vb3+ fraction. Vb3\(^{neg}\) fraction appeared polyclonal in the Vbeta analysis, but TCRB sequencing revealed a single major TCRB clone. The TCRB rearrangement observed in the Vb3\(^{neg}\) fraction was present also in the sorted Vb3+ sample, due to impurity of the sorting (86% of sorted cells were Vb3+).

(B) Patient 48 presented with monoclonal pattern in Vbeta analysis, but TCRB analysis of flow cytometry-sorted Vb21+ cells revealed two TCRB rearrangements with different amino acid sequences, recognized by same monoclonal antibody, whereas Vb21\(^{neg}\) fraction of the same patient showed only minor TCRB rearrangements.

Abbreviations: CDR3, complementarity determining region 3; TCRB, T cell receptor beta chain; VAF, variant allele frequency

**Figure 5.** STAT3 sequencing results and clinical data of three representative LGL leukemia patients. In each case the first figure shows the size of the LGL clone and VAF of STAT3 mutation at different time-points, and the second figure presents the hematological parameters.

(A) The results of untreated patient 4, who developed anemia and neutropenia before the initiation of MTX treatment.

(B) Patient 8 was treated with MTX. Leukemic STAT3-mutated clones persisted during MTX therapy, and the patient developed anemia after the cessation of the therapy.

(C) Patient 44 had aberrant STAT3-mutated CD3\(^{neg}\)CD16/56\(^{neg}\) NK-LGL population, which was suppressed during remission achieved by CTX treatment simultaneously with restoration of normal NK cells. In addition, the patient had two CD8+ clones with unmutated STAT3: Vb20+ clone was suppressed during cyclophosphamide administration, whereas Vb8+ cells were first seen during CR. At the last timepoint the number of NK-LGL cells was too low for DNA extraction and STAT3 mutation analysis.

Abbreviations: CTX, cyclophosphamide; MTX, methotrexate; Tx, therapy; VAF, variant allele frequency.
Figure 1

<table>
<thead>
<tr>
<th>LGL type</th>
<th>Patient no</th>
<th>D661H</th>
<th>D661V</th>
<th>D661I</th>
<th>Y640F</th>
<th>Y657_K658insY</th>
<th>Y657ins NS</th>
<th>N647Y</th>
<th>K658R</th>
<th>P678S</th>
<th>Q643H</th>
<th>D661I</th>
<th>K658H</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLPD-NK</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 SNVs</td>
<td>2 SNVs</td>
</tr>
<tr>
<td>T-LGL</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Multiple SNVs in the same lymphocyte clone

Multiple SNVs in different lymphocyte clones

STAT3 mutation VAF:
- 0.5-5%
- >5%
- >10%
- >20%
- >30%
- >40%
Figure 2

A  Vbeta size

% of CD8+ cells

ns

p<0.0001

ns

2 STAT3  1 STAT3  No STAT3

B  Hemoglobin

g/l

T-LGL D661Y  T-LGL Y640F

p=0.0449

C  Leukocyte count

10^9/l

T-LGL D661Y  T-LGL Y640F

p=0.0040

D  Lymphocyte count

10^9/l

T-LGL D661Y  T-LGL Y640F

p=0.0028
Figure 3

A: Patient 69

B: Patient 48

C: Patient 7

no STAT3 mutations

Vb17 79%

Y640F 44%

Vb16

Y640F 4%

D661V 0.8%

D661Y 0.5%

Vb1

N647I 33%

Vb21.3 64%

Y640F 4%

D661V 0.8%

D661Y 0.5%

Vb3

Vb18

Vb20

D661Y 34%

Y640F 0.7%

N647Y 3%

D661V 4%

Vb23

Vb1

N647I 33%

Y640F 44%

Vb16

Y640F 4%

D661V 0.8%

D661Y 0.5%
**Figure 4**

**A: Patient 8**

CD8+ Vbeta

- **Vb3+:** STAT3 Y640F 45%
  - 13% CASSLAWGINSPLHF
  - 73% CASSSLRSGPMNTEAFF

- **Vb3neg:** STAT3 I659L 30%
  - 74% CASSLAWGINSPLHF

**B: Patient 48**

CD8+ Vbeta

- **Vb21.3+:** STAT3 N647I 34%
  - 63% CASSLRAGGPNEQFF

- **Vb21.3neg:** STAT3 Y640F 4%, D661V 0.8%, D661Y 0.5%
  - 4% CASSRTGILAKNIQYF
Figure 5

A: Patient 4, no therapy

- CD3+CD8+ of lymphocytes
- Vb2+ of CD8+
- Vb2+ Y640F and Q643H VAF

B: Patient 8, MTX

- CD3+CD8+ of lymphocytes
- Vb3+ of CD8+
- Vb3+ Y640F VAF
- Vb3neg I659L VAF

C: Patient 44, CTX

- CD3+CD8+ of lymphocytes
- Vb20+ of CD8+
- Vb8+ of CD8+
- NK-LGL of lymphocytes
- Normal NK cells of lymphocytes
- NK-LGL D661Y VAF
The analysis of clonal diversity and therapy responses using 
*STAT3* mutations as a molecular marker in large granular 
lymphocytic leukemia

Supplementary appendix

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Supplementary methods

Deep targeted sequencing and capillary sequencing of STAT3 exon 21

STAT3 exon 21 was sequenced using Illumina Miseq Platform as described previously 1. Each amplicon was amplified in multiplexed PCR reaction containing locus-specific PCR primers carrying Illumina compatible adapter sequences, Illumina adapter primers carrying Illumina P5/P7 grafting sequences and index sequences, resulting in Illumina -compatible paired-end sequencing templates. The number of PCR cycles was 30, and the input of DNA was 10-20 ng, although it could be less in the case of sorted lymphocyte subpopulations. The genome equivalent, 1ng DNA≈330 genomes, corresponds to 165 cells with dlploid genomes. The DNA input of 10-20ng equals 3300-6600 genomes, meaning that theoretically all of these would be sequenced once when coverage is over 3300-6600. PCR amplicons were sequenced as 151 base pair (bp) or 251 bp paired end reads and two 8 bp index reads using the Illumina MiSeq instrument (Illumina, San Diego, CA, US). Primer sequences are in Supplementary Table 6.

The data was analyzed using an in-house bioinformatics pipeline developed specifically for reporting somatic variants from amplicon sequencing data. Low-quality reads were not filtered out before alignment: after alignment, quality values (i.e. Phred scores) were used to exclude error bases with low quality from further analysis. First, previously reported STAT3 mutations were verified in all STAT3-mapped amplicons: all variants with variant allele count over 5 and variant allele frequency (VAF) over 0.5% were taken into consideration. Second, all variants with variant allele count over 5 and variant allele frequency (VAF) over 0.5% were called from STAT3-mapped amplicons. From these candidates false positives were initially filtered out based on the noise (estimated error rate) level from control sample in every run making use of a binomial distribution to compute p-value for the event that more than the frequency of alternative alleles were observed when the null hypothesis is true. However, variants with VAF over 2% were called independent of the noise. A specific frequency ratio was used to filter out false positive by dividing the ratio of variant calls/number of all the bases (at a position) by the ratio of variant allele quality sum/quality sum of all the bases. All samples with a frequency ratio ≥0.80 were considered to be true mutations, and the variants from both scripts with a borderline frequency ratio between 0.75-0.79 were verified with Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, UK).

High-throughput T cell receptor sequencing

TCRB complementarity determining regions (CDR3β) were amplified and sequenced by Adaptive Biotechnologies Corp (Seattle, WA, US) using the ImmunoSEQ assay. A multiplex PCR system was used to amplify CDR3β sequences from flow cytometry-sorted T-LGL leukemia DNA samples using 52 forward primers for the Vβ gene segment and 13 reverse primers for the Jβ segment. This approach generates a 60 base-pair fragment capable of identifying the VDJ region spanning each unique CDR3β 2. Amplicons were sequenced using the Illumina HiSeq platform (Illumina). Using a baseline developed from a suite of synthetic templates, primer concentrations and computational corrections are used to correct for the primer bias common to multiplex PCR reactions. Raw sequence data was filtered based on the
TCRβ V, D and J gene definitions provided by the IMGT database (www.imgt.org) and binned using a modified nearest-neighbor algorithm to merging closely related sequences and remove both PCR and sequencing errors. Data was analyzed using the ImmunoSEQ analyzer toolset (Adaptive Biotechnologies). The analysis was performed using the Survey level of the ImmunoSEQ assay, which is capable of detecting one cell in 40,000 T cells 3.

Allele-specific oligonucleotide real-time quantitative PCR

In real-time PCR the mutational load was quantified in relation to a known STAT3 Y640F or D661V positive reference sample with high variant allele frequency (VAF) in amplicon sequencing. The reference sample was used for preparing a log-linear dilution series with pooled normal buffy coat DNA and a standard curve was constructed based on the real-time PCR results. The mutational load of samples examined was quantified from the standard curve and data was normalized with an albumin reference gene qPCR assay. ASO-qPCR primer sequences are in Supplementary Table 7.
Supplementary tables

Supplementary table 1

Comparison of ASO-PCR and amplicon sequencing of \textit{STAT3} mutations Y640F and D661V. ASO-PCR results are reported in relation to the positive control sample, and the normalized sensitivity level varied between 0.03%-0.1%. Patient number in brackets refers to Figure 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>STAT3 amplicon result, VAF(^1)</th>
<th>Y640F ASO-PCR</th>
<th>D661V ASO-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control 1 (Patient 57)</td>
<td>Y640F, 27%</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>Positive control 2 (Patient 9)</td>
<td>D661V, 37%, Y640F, 2%</td>
<td>Pos (3% of Control 1)</td>
<td>Pos</td>
</tr>
<tr>
<td>Test 1 (Patient 54)</td>
<td>Y640F, 30%</td>
<td>Pos (75% of Control 1)</td>
<td>Neg (&lt;0.05% of Control 2)</td>
</tr>
<tr>
<td>Test 2 (Patient 7)</td>
<td>Y640F, 2%, D661V, 5%</td>
<td>Pos (1% of Control 1)</td>
<td>Pos (1.5% of Control 2)</td>
</tr>
<tr>
<td>Test 3 (Patient 14)</td>
<td>D661V, 15%</td>
<td>Pos (0.03% of Control 1)</td>
<td>Pos (35% of Control 2)</td>
</tr>
<tr>
<td>Test 4 (Patient 21)</td>
<td>I659L, 5%</td>
<td>Neg (&lt;0.03% of Control 1)</td>
<td>Neg (&lt;0.1% of Control 2)</td>
</tr>
<tr>
<td>Test 5 (Not in Figure 1)</td>
<td>No mutations</td>
<td>Neg (&lt;0.06% of Control 1)</td>
<td>Neg (&lt;0.08% of Control 2)</td>
</tr>
<tr>
<td>Test 6 (Not in Figure 1)</td>
<td>No mutations</td>
<td>Neg (&lt;0.06% of Control 1)</td>
<td>Neg (&lt;0.08% of Control 2)</td>
</tr>
</tbody>
</table>

\(^1\)Abbreviations: VAF, variant allele frequency; pos, positive; neg, negative.
**Supplementary table 2**

Dilution series and *STAT3* amplicon sequencing analysis of a CD8+ T-LGL sample with *STAT3* D661V (chr17:40474419 T>A) and Y640F (chr17:40474482 T>A) mutations. The in-house bioinformatics pipeline does not filter low quality reads prior to alignment, but uses quality values (QV, i.e. Phred scores) to exclude low quality variants (see the methods section of Supplementary appendix). Low average QVs (under 20) are marked in red and those variants are considered as errors.

The D661V mutation is under detection limit with the dilution of 1:100, at which point the number of error calls with low QV probably interferes with the detection of the variant. The Y640F mutation with lower VAF is detected with the dilution of 1:2. Frequency ratio under 0.8 and QVs under 20 are marked in red.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Dilution</th>
<th>Freq. ratio</th>
<th>Total depth</th>
<th>T</th>
<th>T, QV</th>
<th>A</th>
<th>A, QV</th>
<th>G</th>
<th>G, QV</th>
<th>C</th>
<th>C, QV</th>
<th>N</th>
<th>N, QV</th>
</tr>
</thead>
<tbody>
<tr>
<td>D661V</td>
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<td>1.01</td>
<td>11878</td>
<td>56.5%</td>
<td>35</td>
<td>37.3%</td>
<td>33</td>
<td>1.0%</td>
<td>17</td>
<td>0.7%</td>
<td>15</td>
<td>4.5%</td>
<td>2</td>
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<tr>
<td></td>
<td>1:2</td>
<td>1.01</td>
<td>5497</td>
<td>78.0%</td>
<td>35</td>
<td>16.6%</td>
<td>34</td>
<td>0.8%</td>
<td>15</td>
<td>0.3%</td>
<td>14</td>
<td>4.3%</td>
<td>2</td>
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<tr>
<td></td>
<td>1:20</td>
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<td>8132</td>
<td>93.6%</td>
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<td>0.2%</td>
<td>17</td>
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<td></td>
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<td>0.2%</td>
<td>16</td>
<td>4.4%</td>
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<tr>
<td></td>
<td>1:100</td>
<td>0.79</td>
<td>26770</td>
<td>94.5%</td>
<td>36</td>
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<td>27</td>
<td>0.4%</td>
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<td>0.2%</td>
<td>16</td>
<td>4.4%</td>
<td>2</td>
</tr>
<tr>
<td>Y640F</td>
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<td>0.98</td>
<td>15787</td>
<td>97.7%</td>
<td>36</td>
<td>1.4%</td>
<td>35</td>
<td>0.6%</td>
<td>18</td>
<td>0.3%</td>
<td>17</td>
<td>0.1%</td>
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<td>30</td>
<td>0.6%</td>
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<td>0.3%</td>
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<td>25</td>
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<td>46134</td>
<td>98.9%</td>
<td>36</td>
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<td>19</td>
<td>0.5%</td>
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<tr>
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<td>34932</td>
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<td>19</td>
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<td>18</td>
<td>0.3%</td>
<td>17</td>
<td>0.1%</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: Freq, frequency; N, unrecognized base; QV, quality value

1Dilution series made from a sample with both *STAT3* D661V and Y640F mutations.
2The calculation of frequency ratio is explained in the Methods section of this manuscript.
3Number of reads mapped to *STAT3* exon 21.
4The average QV (Phred score) of bases: when FASTQ files are formed, a non-negative quality value (QV) is assigned to each called base using a logged transformation of the error probability. For example, a QV of 30 means that the error probability is 0.1%, whereas a QV of 20 equals 1% error rate. A base with QV under 20 is widely considered as a sequencing error.
5Unrecognized bases with low quality values.
Supplementary table 3

Amplicon sequencing coverage and VAFs of *STAT3* mutations (VAF of the largest variant was used in the analysis in case of multiple mutations).

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>VAF, median (range)</th>
<th>All mapped reads, median (range)</th>
<th>Coverage at the variant allele location</th>
</tr>
</thead>
<tbody>
<tr>
<td>All amplicon sequencing positive samples (n=82)</td>
<td>17% (0.6-51)</td>
<td>14196 (840-565170)</td>
<td>4103 (264-94038)</td>
</tr>
<tr>
<td>Positive by capillary sequencing (n=50)</td>
<td>27% (9-51)</td>
<td>14472 (840-565170)</td>
<td>4060 (264-94038)</td>
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<tr>
<td>Positive only by amplicon sequencing (n=32)</td>
<td>3% (0.6-25)</td>
<td>12616 (1255-309580)</td>
<td>5281 (395-21839)</td>
</tr>
<tr>
<td>All amplicon sequencing-negative samples (n=131)</td>
<td>NA</td>
<td>6931 (1270-804232)</td>
<td>NA</td>
</tr>
</tbody>
</table>

1The total number of amplicon reads aligned to *STAT3* exon 21 (both forward and reverse reads).
2The number of amplicon reads at the location of the variant allele. Most of the *STAT3* mutations detected are in the area covered only by the reverse reads, thus the coverage is approximately half of the total number of *STAT3*-aligned reads.

Abbreviations: NA, not applicable; VAF, variant allele frequency.
**Supplementary table 4**

The number of TCRB CDR3 sequencing reads. Patient numbers are from Figure 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Lymphocyte fraction</th>
<th>Total number of reads</th>
<th>Productive sequences</th>
<th>Unique sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>CD8+Vb3+</td>
<td>2515757</td>
<td>2466380</td>
<td>1134</td>
</tr>
<tr>
<td></td>
<td>CD3+Vb3neg</td>
<td>2493453</td>
<td>2417798</td>
<td>869</td>
</tr>
<tr>
<td>48</td>
<td>CD8+Vb21.3+</td>
<td>2475907</td>
<td>2418665</td>
<td>956</td>
</tr>
<tr>
<td></td>
<td>CD8+Vb21.3+neg</td>
<td>2575304</td>
<td>2226166</td>
<td>14386</td>
</tr>
<tr>
<td>14</td>
<td>CD8+Vb17+</td>
<td>2768966</td>
<td>2687142</td>
<td>598</td>
</tr>
<tr>
<td></td>
<td>CD3+</td>
<td>2908398</td>
<td>2465234</td>
<td>9777</td>
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</table>
## Supplementary table 5

Deep TCRB sequencing results of three T-LGL leukemia patients.

<table>
<thead>
<tr>
<th>Patients(^1)</th>
<th>STAT3 mutations (VAF)</th>
<th>CRD3 nucleotide sequence</th>
<th>CDR3 amino acid sequence</th>
<th>Frequenc y (%)</th>
<th>Count</th>
<th>V-gene name</th>
<th>D-gene name</th>
<th>J-gene name</th>
<th>Healthy(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 8, Vb3+ fraction (TCRBV 28) (Fig. 4A)</td>
<td>Y640F 45%</td>
<td>GCCACGACACACACAGAG</td>
<td>CASSLSRGPMNTEAFF</td>
<td>73</td>
<td>1834805</td>
<td>TCRBV28-01</td>
<td>TCRBD01-01</td>
<td>TCRBJ01-01</td>
<td>0/586 (0%)</td>
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<td></td>
</tr>
<tr>
<td>Patient 8, Vb3neg fraction (Fig. 4A)</td>
<td>N647I 34%</td>
<td>CAGCGCACACAGCAGGAGG</td>
<td>CASSLAWGINSPLHF</td>
<td>73</td>
<td>1844935</td>
<td>TCRBV07-08</td>
<td>TCRBD01-01</td>
<td>TCRBJ01-01</td>
<td>0/586 (0%)</td>
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<tr>
<td>Patient 48, Vb21.3+ fraction (TCRBV 11-02) (Fig. 4B)</td>
<td>Y640F 45%</td>
<td>TGGCGGCCCCTGCCAA</td>
<td>CASSRTGILAKNIQYF</td>
<td>64</td>
<td>1572352</td>
<td>TCRBV11-02</td>
<td>TCRBD02-01</td>
<td>TCRBJ01-01</td>
<td>0/586 (0%)</td>
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<tr>
<td>Patient 48, Vb21.3neg fraction (Fig. 4B)</td>
<td>N647I 34%</td>
<td>GCCCTGCAGCCAGAAGACTCGG</td>
<td>CASSQTSIGGEYNEQFF</td>
<td>2</td>
<td>97873</td>
<td>TCRBV06-02 or TCRBV06-03</td>
<td>TCRBD01-01</td>
<td>TCRBJ01-02</td>
<td>0/586 (0%)</td>
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<tr>
<td>Patient 14, Vb17+ (TCRBV 19) fraction (Supplementary Fig. 1)</td>
<td>D661V 41%</td>
<td>TGGCGGCCCCTGCCAA</td>
<td>CASSPRTGYSNQPQHF</td>
<td>83</td>
<td>2307767</td>
<td>TCRBV19-01</td>
<td>TCRBD01-01</td>
<td>TCRBJ01-01</td>
<td>0/586 (0%)</td>
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<tr>
<td>Patient 14, CD3+ fraction, contains Vb13.6+ cells (TCRBV 06-06) (Supplementary Fig. 1)</td>
<td>D661Y 8%(^2)</td>
<td>TGGCGGCCCCTGCCAA</td>
<td>CASSPRTGYSNQPQHF</td>
<td>10</td>
<td>300013</td>
<td>TCRBV19-01</td>
<td>TCRBD01-01</td>
<td>TCRBJ01-01</td>
<td>0/586 (0%)</td>
</tr>
</tbody>
</table>

\(^1\) Patient numbers are from Figure 1. The TCRBV gene name according to the International ImMunoGeneTics information system (IMGT) nomenclature is written in brackets after each Vbeta clone.

\(^2\) D661Y mutation seen in the smaller Vb13.6+ expansion was seen with manual inspection in the CD3+ fraction with VAF of 0.45%, which is slightly under the detection threshold of the amplicon bioinformatics pipeline.

\(3\) The existence of the amino acid sequence in a cohort of 586 healthy individuals, provided by the Adaptive Biotechnologies company.

Abbreviations: CDR3, complementarity determining region 3; D, diversity; J, joining; V, variable; VAF, variant allele frequency
Supplementary table 6

Locus specific amplicon primer sequences carrying tails corresponding to the
Illumina adapter sequences. Locus specific primer sequences are underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3_exon21-F</td>
<td>5'-ACACTCTTTCCCTACACGACGCTCTTTCCGATCTCCCCAAAAATTTAAATGCAGGA-3'</td>
</tr>
<tr>
<td>STAT3_exon21-R</td>
<td>5'-AGACGTGTGCTTTCCGATCTGGTCCATGATCTTTCTTTCC-3'</td>
</tr>
</tbody>
</table>
Supplementary table 7

STAT3 Y640F- and D661V-specific primer sequences used in ASO-PCR.

<table>
<thead>
<tr>
<th>Primer/Probe name</th>
<th>Primer / Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y640F fwd</td>
<td>5’-ATCCAGTCGGAGACCcTT-3’</td>
</tr>
<tr>
<td>D661V fwd</td>
<td>5’-TCATCATGGCTATAAGATCAaGGT-3’</td>
</tr>
<tr>
<td>STAT3x21-Rev</td>
<td>5’-TCTCTGGCCGACAATACTTTCC-3’</td>
</tr>
<tr>
<td>STAT3x21-Pr</td>
<td>FAM- CCTGACATTCCCAAGGAGGAGGCA –BHQ</td>
</tr>
</tbody>
</table>
Supplementary references


Supplementary figures
Supplementary Figure 1

Supplementary Figure 1. Results of STAT3 amplicon sequencing from sorted lymphocyte populations.
(A-C) STAT3 sequencing results from three monoclonal T-LGL leukemia patients. (D) STAT3 sequencing results of patient 14, who had two Vbeta expansions, Vb17+ (50% of CD8+ cells) and Vb13.6+ (11% of CD8+). Deep TCRB sequencing was done from Vb17+ cells, and the percentage of largest clone (83%) was in good accordance with the VAF of D661V mutation (VAF 41%). Whole CD3+ fraction was sequenced in parallel, and it contained the D661Y-mutated Vb13.6+ clone (4% of reads) in addition to the immunodominant expansion (10% of reads): the percentage of immunodominant Vb17+ cells from total CD3+ fraction was 18% in the Vbeta analysis. If all unproductive sequences are excluded from the TCRB analysis, the proportion of the TCRBV19-01 (Vb17+) clone is 12% and matches with Vbeta results.
Supplementary Figure 2

A: Patient 69, MTX

(B) Patient 1 had aberrant CD3negCD16/56neg NK-LGL expansion, which was D661I-mutated. NK-LGL cells were suppressed and normal NK cells were restored during CTX treatment.

(C) Patient 32 was treated with CTX, and failed to respond to treatment. CTX administration was stopped because of developing cytopenias, and leukemic D661Y-mutated clone (over 90% of CD8+) cells was unchanged. The amount of Vb13.6neg cells was too low for sorting at the last timepoint.

Abbreviations: CTX, cyclophosphamide; CyA, cyclosporine; MTX, methotrexate; Tx, therapy; VAF, variant allele frequency

Supplementary Figure 2. STAT3 mutation analysis and lymphocyte populations at different timepoints during methotrexate (MTX) or cyclophosphamide (CTX) treatment.

(A) Patient 69 was treated with methotrexate, and although the patient achieved partial response (improved Hb values), the size of the STAT3-mutated clone diminished only slightly.

(B) Patient 1 had aberrant CD3negCD16/56neg NK-LGL expansion, which was D661I-mutated. NK-LGL cells were suppressed and normal NK cells were restored during CTX treatment.

(C) Patient 32 was treated with CTX, and failed to respond to treatment. CTX administration was stopped because of developing cytopenias, and leukemic D661Y-mutated clone (over 90% of CD8+) cells was unchanged. The amount of Vb13.6neg cells was too low for sorting at the last timepoint.

Abbreviations: CTX, cyclophosphamide; CyA, cyclosporine; MTX, methotrexate; Tx, therapy; VAF, variant allele frequency