STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T cell large granular lymphocyte leukemia.

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Abstract

Chronic lymphoproliferative disorders of natural killer cells (CLPD-NK) and T-cell large granular lymphocytic leukemias (T-LGL) are clonal lymphoproliferations arising from either natural killer cells or cytotoxic T lymphocytes (CTL) and are associated with autoimmune conditions, including immune cytopenias, rheumatoid arthritis and B cell dyscrasias. We have investigated for distribution and functional significance of mutations associated with in 50 CLPD-NK and 120 T-LGL patients by direct sequencing, allele-specific PCR and microarray analysis. STAT3 gene mutations are present in both T and NK diseases: approximately 1/3 of patients with each type of disorder convey these mutations. Mutations were found in exons 21 and 20, encoding the Src homology 2 domain. Patients with mutations are characterized by symptomatic disease (75%), history of multiple treatments, and a specific pattern of STAT3 activation and gene deregulation, including increased expression of genes activated by STAT3. Many of these features are also found in patients with wild type STAT3, indicating that other mechanisms of STAT3 activation can be operative in these chronic lymphoproliferative disorders. Treatment with STAT3 inhibitors, both in wild type and mutant cases, resulted in accelerated apoptosis. STAT3-mutations are frequent in large granular lymphocytes suggesting a similar molecular dysregulation in malignant chronic expansions of NK and CTL origin. STAT3 mutations may distinguish truly malignant lymphoproliferations involving T and NK cells from reactive expansions.
Introduction

Since its original description, Large Granular Lymphocyte Leukemia (LGL) has been a subject of controversy, as to whether the disorder represents a lymphoid malignancy or an exaggerated reactive T cell process. LGL is a chronic clonal lymphoproliferative disorder that can be phenotypically subdivided into T cell LGL and natural killer LGL.\(^1,2\) Both subtypes, seemingly derived from distinct cell lineages, are morphologically similar, causing an accumulation of large granular lymphocytes that correspond to a mature cytotoxic effector type. The current World Health Organization (WHO) separates chronic proliferations of LGL according to their cell lineage into two entities: chronic lymphoproliferative disorders of NK cells (CLPD-NK) and T cell large granular lymphocytic leukaemia (T-LGL), though no significant differences in clinical features or therapeutic approach are observed between them.\(^3-6\)

Chronic expansions of large granular lymphocytes primarily affects elderly individuals and is likely underdiagnosed. It can be associated with severe cytopenias and other comorbidities, including autoimmune conditions and malignancies, complicating the diagnosis due to the overlap between reactive processes and clear malignant lymphoproliferation.\(^7,8\) While in T-LGL, objective molecular characterization relies on the detection of clonal expansions through the uniquely rearranged TCR, establishing the clonal nature of CLPD-NK is difficult and often disputed.\(^9,10\) For both types, and distinct from typical polyclonal reactions, autoimmune phenomena are mediated by clonal, seemingly malignant immune cells. Recently, the presence of somatic mutations in STAT3 has been described in T-LGL, indicating that a significant proportion of cases represent a true malignant process.\(^11\)

Because of pathogenetic and clinical similarities, we searched for mutations in STAT3 and other related members of the STAT signaling pathway in CLPD-NK, T-LGL and in closely related conditions with possibly reactive expansions.
Methods

Patients

Blood sample collection from patients was performed after informed consent, according to the protocols approved by the Institutional Review Board of Cleveland Clinic, The Pennsylvania State University, and The Helsinki University Central Hospital and in accordance with the Declaration of Helsinki. Thirty-seven T-LGL patients included in a previous work are not incorporated in the present study, so the mutational distribution may not be coincident (for example, 7 D661V mutations were described in the previous report, vs 3 such mutations reported herein). Based on World Health Organization guidelines, the following criteria were used to diagnose T-LGL leukemia: monoclonal TCRγ-chain rearrangement, an LGL count by peripheral blood smear of > 2000 cells/μL (not a critical criteria, patients who met all other criteria but with an LGL count < 2000 cells/μL were included); flow cytometric evidence of an abnormal CTL population characterized by expression of CD2, CD3, TCRαβ (or γδ), CD4 (in 2 cases), CD5dim, CD8, CD16/56 and CD57 with negativity of CD28. Each patient must have met at least 3 of these criteria to be included in the study. In addition, TCR Vβ expansions were detected and quantitated according to criteria described previously. Diagnosis of CLPD-NK was established based on the following parameters: blood LGL count of >700/uL and abnormal immunophenotype pattern including the presence of CD56+CD16+CD2+CD3- and granzyme B expressing cells. Persistence of the condition for more than 6 months was required. Cytopenias were classified as neutropenia (absolute neutrophil count [ANC], < 1.5 × 10⁹/μL), anemia (hemoglobin, < 10 g/dL), and thrombocytopenia (platelet count, < 100 × 10³/μL). Clinical responses were determined following the modified International Working Group criteria for myelodysplastic syndromes, as reported previously. Time to treatment failure was defined as the interval between the start of treatment and the need for initiating a second line of therapy and/or progressive disease (including relapse after remission).

Amplification refractory mutation system (ARMS)-PCR

The presence of D661Y and Y640F mutations non detectable by direct sequencing was determined by a DNA tetra-primer ARMS assay. The primer sequences for the D661Y assay were (5' - 3'): forward inner primer (G allele), AAATCATCATGGGCTATAAGATCACGG; reverse inner
primer (T allele), GGAGACACCAGTATTTGGTAGCGTA; forward outer primer, CCTAGCTGTAGGTTCCATGATCTTTCT; and reverse outer primer, AAAATTAAATGCCAGGAACATGGAAAAT. (Product size two outer primers, 290 bp; G allele, 182 bp; T allele, 161 bp). Primer sequences for the Y640F assay were (5' - 3'): forward inner primer (A allele), ACCCAGATCCAGTCCGTGGAACCTTA; reverse inner primer (T allele), ACATGTTGTTCCAGCTGCTGCTTTGAGA; forward outer primer, AAAAAATGGGCAGTTTTCTCTGAGATGACC; reverse outer primer, CCAGTGAGACACCCAGATATTGGTAGC (Product size two outer primers, 197 bp; A allele, 119 bp; T allele, 131 bp)

Flow cytometry assays

*KIR (killer cell immunoglobulin-like receptor) skewing in CLPD-NK*

In eighteen cases, paired healthy and patient NK cells were analyzed by 2-color flow cytometry using CyChrome-conjugated anti-CD56 (clone B159; BD Biosciences, San Diego, CA) or anti-CD16-fluorescein isothiocyanate (FITC)-conjugated antibodies in combination with the following phycoerythrin (PE)-conjugated antibodies: anti-CD158a (KIR2DL1, KIR2DS1), anti-CD158b (KIR2DL2, KIR2DL3, KIR2DS2), anti-NKB1 (KIR3DL1), anti-KARp50 (KIR2DS4), and anti-NKG2A, which were all obtained from BD Biosciences. A purified antibody was used to stain NKG2D (0.5 μg; R&D Systems, Minneapolis, MN) and KIR2DL4 (0.5 μg; provided by Dr Eric Long, National Institute of Allergy and Infectious Diseases [NIAID], Rockville, MD) followed by rat antimouse immunoglobulin (Ig)-conjugated PE secondary antibody. Six of these patients were included in a previous study.

In seven patients, the NK phenotype was assessed using a 4-color approach with antibodies directed against CD2, CD3, CD57 (Immunotech, Marseille, France) and CD158a (clone EB6; Beckman Coulter, Miami, FL), CD158b/j (clone GL183; Beckman Coulter), CD158e (clone DX9; BD Bioscience, San Jose, CA), and CD158i (clone FES172; Immunotech, Marseille, France), CD159a (cloneZ199, Immunotech, Marseille, France), CD94 (clone Z199, IgG2b; Beckman Coulter) and NKG2D (clone 1D11, Biolegend)
TCR variable β-chain (Vβ) skewing T-LGL

Fresh peripheral blood was stained for Vβ flow cytometry analysis to quantitate the percentage of each Vβ family in the CD4 and CD8 lymphocyte populations. The manufacturer’s instructions (IOTest Beta Mark kit; Beckman Coulter) were modified as follows: 5 μL of phycoerythrin cyanin (PC) 5-conjugated anti-CD4 (Beckman Coulter) and 5 μL of PC7 anti-CD8 (BD Biosciences) monoclonal antibodies were added. Anti-Vβ 6.7 FITC (Pierce Chemical), anti-CD3 FITC, anti-TCRαβ PC5, and anti-TCRγδ PE (Beckman Coulter) also were included in the panel. A 4-color acquisition protocol was used on an FC500 with CXP Version 2.2 software (Beckman Coulter). FCS Express Version 3.0 (De Novo Software) was used for analysis. The lymphocyte gate was set according to forward and side scatter. For Vβ family T-cell repertoire analysis, gates were set on CD4 (two cases) and CD8 bright lymphocyte populations and then analyzed for Vβ distribution. Mean and SD values were provided by the manufacturer of the IOTest Beta Mark kit and are based on a control population of 85 volunteers, as described previously. In addition, a separate Vβ repertoire control group of 69 volunteers was analyzed that did not differ significantly from the Beckman group or previous publications. A significant clonal expansion was defined as an expansion that was greater than the mean ± 3 SD of healthy controls.

Apoptosis assay.

To quantitate apoptosis, we performed three-color flow cytometry with propidium iodide (Sigma), annexin-V APC (Beckman Coulter), and CD3 FITC (Beckman Coulter). Forward and side scatter analyses were used to identify the lymphocyte population in peripheral blood samples from leukemic LGLs and normal PBMCs. This lymphocyte gate then the CD3+ (T-LGL) or CD3- (CLPD-NK) gate was used to examine the population that stained positive for annexin-V APC and propidium iodide in single positive and double positive quadrants, allowing for the calculation of percent early apoptotic, apoptotic, and dead cells. Mononuclear cells were separated from peripheral blood by density gradient centrifugation, then cultured in the presence or absence of 1μM or 10μM STA-21 (Enzo Life Sciences) in RPMI + 10% FCS for 48 hours.

Microarray analysis

Mononuclear cells were separated from peripheral blood by density gradient sedimentation (Mediatech, Herndon, VA, USA). LGL cells were separated by flow cytometric sorting using anti-VB
and CD8 mAb as described previously. Healthy, donor-derived CD8+CD57+ cells were isolated by flow cytometric sorting using CD3, CD8, and CD57 mAb. Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, USA) Phase-Lock gel tubes (Eppendorf, Hamburg, Germany), cleaned RNAeasy columns (Qiagen, Valencia CA, USA), and dissolved diethylpyrocarbonate water. Total cRNA was prepared using the in vitro-transcribed (Affymetrix, Santa Clara, CA, USA) and hybridized to U133 arrays, according to the manufacturer’s instructions (Affymetrix). All microarrays were examined for surface defects, grid placement, background intensity, housekeeping gene expression, and a 3:5 ratio of probe from genes of varying length. Expression analysis was conducted using standard Affymetrix analysis software algorithms (Microarray Suite 5.0). Comparative analysis between expression profiles was carried out on GeneSpring™ software, Version 7.1 (Agilent, Clara, CA, USA). Scanned images of Affymetrix chips were converted spreadsheet numbers using Affymetrix proprietary GeneChip Operating software (GCOS). Gene expression data were normalized in two “per-gene normalization” and “per-sample normalization”. This approach been previously described in detail. Data for this microarray study are uploaded into the Gene Expression Omnibus under accession number: GSE39838.

**Western immunoblotting.**

Peripheral blood mononuclear cells from diagnosis were lysed in a buffer composed of 50 mM Tris-Cl (pH 7.6); 5 mM EDTA; 150 mM NaCl; 0.5% NP-40; 0.5% Triton-X-100 (RIPA) containing 1 µg/ml leupeptin, aprotinin, and antipain; and 1x concentration of phosphatase inhibitor cocktail 2 (Sigma P5726) containing sodium vanadate, sodium molybdate, sodium tartrate (all from Sigma Chemical Co.). Protein concentration was determined in all cell extracts using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Unless otherwise indicated, 25 µg of total protein was loaded per lane in Laemmli SDS-PAGE sample loading buffer and boiled for 5 minutes before separation by 10% SDS-PAGE. Twenty-five micrograms of total protein was found to give ECL signals within the linear range for ß-actin in titration experiments. The proteins were then transferred to a membrane for Western blot analysis. Antibodies were obtained from the following sources and used at the dilutions recommended by the manufacturer: anti-pSTAT3 (Tyr705), 1:1,000 ( #9131 Cell Signaling Technology, Inc., Danvers, MA, USA), and anti–ß-actin 1:25,000 (A3853 Sigma Chemical Co.). Antibody detection was performed by standard ECL.
techniques as recommended by the manufacturer (Amersham Pharmacia Biotech). Protein quantification was performed by densitometry using the ImageJ program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.).

**Polymerase-chain-reaction direct sequencing assays.** PCR primers were designed to amplify and sequence all coding exons in the SH2 domain of the STAT family genes (STAT1, STAT2, STAT4, STAT5a, STAT5b and STAT6) and known mutational hotspot regions in other JAK/STAT pathway genes (gp130 exon 6, JAK2 V617F, JAK3 exons 13-17, RELA exon 5), and are available on request. For each polymerase chain reaction (PCR), 40 ng genomic DNA was used for PCR amplification followed by purification using Montage Cleanup kit (Millipore, Billerica, MA). Sequencing was performed using ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). All STAT3 mutations were detected by bidirectional sequencing and scored as pathogenic mutations on the basis of the observation that they were not detected in nonleukemic cells (CD4+ cells). All mutations were first compared with published SNP data (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP).

**Statistical analysis.** Comparisons of proportions and ranks of variables between groups were performed by chi-squared-test, Fisher’s exact test, Student’s t-test or Mann–Whitney U-test, as appropriate. We used the Kaplan-Meier and the Cox method to analyze overall survival (OS) and progression free survival (PFS), with a two-sided, \( P \) value \( \leq 0.05 \) considered significant. In Cox models, examination of log (-log) survival plots and partial residuals was performed to assess that the underlying assumption of proportional hazards was met.

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Results

Identification of STAT3 mutations.

Using stringent diagnostic criteria we have identified a large cohort of patients with CLPD-NK and T-LGL that have been subjected to a molecular search for mutations associated with STAT3 signaling. We screened 170 cases and identified 49 STAT3 mutations in 48 cases (Fig.1). The cohort included 120 T-LGL and 50 CLPD-NK (Table 1), but no significant differences in distribution of STAT3 mutations were found when comparing cell-lineage subsets. Of note is that no STAT3 mutation was identified in an independent cohort of 31 cases diagnosed with idiopathic neutropenia and identifiable large granular lymphocytes in the blood smear but not fulfilling all diagnostic criteria for T-LGL or CLPD-NK.

All mutations were located in the domain of STAT3 (residues 585–688) that shares homology with Src homology 2 (SH2) domains (Figure 1A). The STAT3 SH2 domain mediates STAT3 dimerization via binding of phosphotyrosine residue Y705. No additional somatic mutations were identified in the SH2 domain of other STAT family members (STAT1, STAT2, STAT4, STAT5a, STAT5b and STAT6) and in other JAK/STAT pathway screened genes’ hotspots (gp130 exon 6, JAK2 V617F, JAK3 exons 13-17, RELA exon 5) in a screening subcohort of 40 STAT3-negative cases (20 CLPD-NKs and 20 T LGLs). All mutations were heterozygous and proved to be somatic, as they were not observed in germline DNA. One patient harbored two STAT3 mutations (D661Y and Y640F). In a case with coexisting NK and CTL components, sorting revealed that the mutation originated in the NK cell subset. Moreover, mutations were not only found in T-LGL with α/β restrictions but also in γ/δ rearranged cases: one out of two γ/δ cases included in this study was mutated (Supplemental Fig.1). Two mutations, Y640F and D661Y, accounted for 80% of the somatic variations found, enabling the design of a more sensitive ARMS-PCR method for each of these alterations, which allowed us to find 7 new mutated cases after analyzing all Sanger negative cases. Sequencing of cloned exon 21 STAT3 DNA products from these cases showed that the mutations were carried by one of the alleles in low proportion (<10% of clones). Titration studies highlighted a higher sensitivity of ARMS-PCR assay compared with Sanger sequencing for detecting mutations, and follow-up analyses confirmed the value of this assay in detecting minimal residual disease throughout the course of the disease in an exemplary CLPD-NK patient.
(Supplemental Fig. 2). In general, ARMS-PCR may increase the diagnostic yield to clones representing less than 10%.

**Proliferation and survival signals in STAT3 mutant LGL cases.**

We then investigated whether mutant and wild type cases show signs of activation along the STAT3 signaling pathway. Western blot analysis in leukemic large granular lymphocytes from mutated or wild type specimens and mononuclear cells in controls showed that phosphorylated-STAT3 (pSTAT3) protein was most abundant in mutated cases, irrespective of the cell lineage, but also expressed at higher levels in WTs when compared with the amount in normal mononuclear cells, suggested constitutive activation in leukemic cells (Figure 2A, top). Aberrant intracellular pSTAT3 signaling also was detected in STAT3 mutant cases of T and NK origin. Counterstaining with anti-cytoplasmic-CD3 identified the lymphoid derivation of this signal (Fig 2A, bottom).

STAT3 signaling has been considered a major intrinsic pathway for cancer inflammation, capable of inducing a large number of downstream genes that are crucial for tumor promotion. Deregulation of the STAT3 pathway has also been specifically implicated in the pathogenesis of T-LGL. Consequently, we examined the gene expression pattern in T-LGL leukemias compared to controls, and the impact of the presence of STAT3 mutations on the transcriptional regulation of a specific set of genes previously described. For this analysis, a global expression microarray analysis was performed on LGL cells from 2 patients with a STAT3 mutation (D661Y and Y640F) and a patient with symptomatic T-LGL, showing a clearly dominant clone as determined by profound TCR Vβ chain usage, but without detectable STAT3 mutations. T-LGL cells were sorted according to the clonal expression of pathognomonic Vβ chain. As expected, sorted clonal cells showed high expression of CD57. Controls derived from flow-sorted CD8+CD57+ CTL effector cells from healthy donors. T-LGL cell populations discordantly expressed 7416 genes: 2966 that were up- and 4450 that were down-regulated (change greater than 2-fold; P=.02 Fig. 2B, bottom). Interestingly, mutated and WT disease samples shared approximately 50% of deregulated genes, implying a role in both of deregulated apoptotic pathways. There was also a striking overexpression of 31 cancer inflammation-related genes described previously to be inducible by STAT3; interestingly, mutated and WT disease samples shared approx. 50% of deregulated genes; showing both mutated and non-mutated patients a preference for deregulation of apoptotic pathways. Focusing on 31 cancer-inflammation related genes described previously to be inducible
by STAT3, there was a striking pattern of overexpression of these genes; interestingly, mutant and non-mutant patients showed significant concordance (Fig. 2B, top), suggesting that, in addition to STAT3 mutations, other mechanisms or lesions can be responsible of deregulation of this pathway.

**Inhibition of STAT3 results in large granular lymphocyte apoptosis.**

We then examined the effects of STAT3 inhibition in CLPD-NK and T-LGL cells using STA-21, a novel synthetic inhibitor of STAT3 dimerization, DNA binding, and STAT3-dependent luciferase reporter activity. STA-21 alone induced an increase in annexin-V-APC binding in leukemic large granular lymphocytes (both NK and T) (Figure 3). Increasing doses of STA-21 induced a dose-dependent increment in the percentage of apoptotic cells in leukemic LGLs but had little effect on normal lymphocytes from the same sample and also from controls. Both leukemic NK and CTL cells showed a much higher sensitivity than their non-malignant counterparts. However, the effect of inhibitor was not specific to STAT3 mutant cells since patients without STAT3 mutations showed similar response to STAT3 inhibition. These results indicate that only the leukemic cells (both mutant and WT), and not non-leukemic cells, are most sensitive to the apoptosis-inducing effects of STA-21.

**Clinical correlates of STAT3 mutations.**

We first studied the correlation between the detection of STAT3 and clonal size as determined by morphologic enumeration of large granular lymphocytes and Vβ TCR repertoire analysis. No correlation was found between the presence of a STAT3 mutation and the absolute large granular lymphocyte count (Table 2). While some patients with otherwise typical cells and extreme clonal expansions did not have STAT3 mutations, overall the STAT3 mutant subset of T-LGLs had more dominant clones, with a remarkable expansion in the panel: all mutated cases the immunodominant clone size exceeded 25% of the whole CD8 compartment, while in the non-mutated cohort 23% of cases did not reach the 25% threshold (Figure 4). In addition, no specific restricted Vβ subset was observed to be characteristic of each group. We performed a KIR expression analysis in half of our CLPD-NK cohort. All STAT3 mutated patients (seven) were included among the 23 out of 25 cases which showed an abnormal pattern of expression (Table 3 and Supplemental Fig.4).
Patients with a somatic mutation in STAT3 were characterized by a higher frequency of symptomatic disease at baseline (75% vs. 40%; P<.001) and the requirement of more lines of therapy through the course of their disease (2.4 vs. 1.2; P=.03; Table 2). Indolent cases (as determined based on "need to treat" bases) were predominantly grouped within the non mutated patients: 59 wild type vs. 6 mutant cases. No significant differences were found when analyzing overall survival between CLPD-NK and T-LGL (Fig. 5A) or between the mutated and non-mutated subsets (Fig. 5B). The STAT3 mutated cohort had a shorter time to treatment failure when compared to those without mutations (Fig. 5C), though no statistical significance was reached (p=0.128). Focusing on comorbidities, both rheumatoid arthritis and autoimmune hemolytic anemia were present in significantly higher frequencies in mutated cases while, surprisingly, pure red cell aplasia cases (PRCA) were invariably related to non-STAT3 SH2 mutant cases (Table 2).

Discussion

Leukemic large granular lymphocytes can arise from either CTLs or NK cells and represents a likely underdiagnosed, often indolent, clonal lymphoproliferation that overlaps with reactive processes via the provisional entity of monoclonal T cell clonopathy of unclear significance. While prognosis is generally good, many patients suffer from chronic morbidities due to cytopenias and the disease is, essentially, not curable. Our discovery of somatic mutations in the SH2 domain of STAT3 in T-LGL, and now also in CLPD-NK, displays its main value both in the diagnostic setting: i) STAT3 mutations may be a useful tool to discriminate malignant NK lymphoproliferations from reactive expansions, in particular to establish clonality using STAT3 mutation as a clonal marker, and ii) the shared altered pathway and clinical behavior, strongly supports the suitability of merging both entities in one disease category; and in pathogenesis: we show that enhanced, and reversible apoptosis, is a hallmark of STAT3 lymphocytes.

Despite a similar cytotoxic effector function, NK cells, unlike T cells, mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity and do not express the surface CD3/TCR complex or go through TCR gene rearrangement. These evident differences led to segregation of chronic large granular lymphocyte leukemias in hematologic tumor classifications according to their cell lineage though no significant disparity in clinical behavior has been established. Here we show that T-LGL and CLPD-NK are associated with somatic STAT3 mutations in a significant proportion of cases, a finding that demonstrates a shared altered signaling pathway mediating
aberrant survival of the clonal cells of T or NK cell origin. A skewed NK receptor expression on NK cells has been described in patients with the natural killer lymphoproliferative disease of granular lymphocytes.\textsuperscript{21} In particular, differences in the repertoire of KIR receptors expressed on NK cells from patients with CLPD-NK in contrast to those of healthy individuals can assist in the difficult process of assessing clonality in this disorder.\textsuperscript{10,22} In our cohort, all of our mutated CLPD-NK patients showed some degree of KIR expression restriction, supporting its value as a marker of malignant clonality.

The presence of the same acquired molecular lesion in T-LGL and CLPD-NK raises the question whether those lesions might distinguish truly malignant, transformed leukemias from reactive processes due to a continuum of cellular immune responses spanning polyclonal, oligoclonal and strict clonal lymphoproliferations. The concerns and difficulties involved in separating, clinically and pathogenetically, those entities have been highlighted by us and others.\textsuperscript{2,7,23} We show herein higher expansions of the Vβ immunodominant clones among mutated patients, corresponding with a higher frequency of symptomatic disease at baseline. However, cases without typical STAT3 mutations are characterized by a great deal of heterogeneity, with indolent and symptomatic patients or cases with either extreme or very modest clonal expansions. One could speculate that some symptomatic patients with clear expansions might harbor other somatic mutations affecting the JAK/STAT3 pathway. That hypothesis led us to screen patients negative for a STAT3 exon 21 mutation by a more sensitive method, to expand the search to the SH2 domain of the rest of the STAT family genes and to genes with reported mutations involved in JAK/STAT3 signaling. Twenty percent of the mutations reported here were found by this search, including an additional 7 cases by allele-specific PCR in exon 21 and 3 cases with a somatic mutation in exon 20 of STAT3. It is therefore likely that increased sensitivity may result in detection of subliminal mutated clones in a higher proportion of cases. Targeted screening of other potentially involved genes did not yield positive results, but it appears that STAT3 mutant negative cases may also display dependence upon STAT3–mediated signals with a stimulatory input, potentially at a more proximal level. Alternatively, this pathway has been demonstrated to be persistently deregulated in cancers, including lymphomas, by mechanisms distinct from acquired alterations in the genetic code. These include mechanisms such as microenvironmental paracrine activation or epigenetic modulation.\textsuperscript{24-26} While acknowledging the cautiousness in the data interpretation due to the relatively scarce number of patients arrayed, our downstream deregulation
and activation studies suggest that STAT3 can be activated independent of key oncogenic driver mutations in its SH2 domain in a subset of patients with large granular lymphocyte disorders.

Reversal of the anti-apoptotic phenotype in response to upstream STAT3 inhibitors occurs in a proportion of patients with T-LGL. 27 We hypothesized that this effect could account for those cases without a STAT3 mutation. A downstream STAT3 selective inhibitor used in our study induced apoptosis of CLPD-NK or T-LGL irrespective of mutational status, and it appears that leukemic cells are more sensitive than their normal counterparts. In this respect, the association of STAT3 mutations with the need for more lines of therapy shown here suggests that targeting the STAT3 mutation may be a viable therapeutic strategy. Of note, dominant negative mutations in the DNA-binding domain of STAT3 can cause Job’s syndrome, a primary immunodeficiency with predisposition to B cell lymphomas; thus, systemic suppression of STAT3 function may have adverse consequences.28-31 Further studies will determine whether there are distinct STAT3 signaling patterns among mutated and non-mutated samples that can measure the response to different Stat3 inhibitors.

We show here that coexistence of RA or AIHA is more frequent in mutant CLPD-NK or T-LGL patients than in wild type cases, and that we could not detect mutations in patients with concomitant PRCA. RA is the most common autoimmune disease associated with these disorders,32 and both entities share essential pathogenic features including expansion of CTLs, constitutive overexpression of cytotoxic molecules and apoptosis resistance. 33 34 35 Our results strengthen the notion of this pathogenic association, which appears closer in mutant cases. For instance, STAT3 promotes survival of RA synovial fibroblasts and, in the therapeutic setting, widely used sulphur-containing gold compounds have been shown recently to depend on the blockade of JAK1/STAT3 signaling. 36 Mutant cases also had a higher prevalence of concurrent AIHA; again, STAT3 constitutive activation might be responsible for apoptotic-resistant autoreactive T cell clones. 37 In contrast, no mutation was found in 7 patients with CLPD-NK or T-LGL and PRCA, where a similar line of argument involving autoreactive apoptotic-resistant immunity could be followed. Further studies are needed to address this apparent controversy; nevertheless STAT3 SH2 domain mutated large granular lymphocytes clones are unlikely to be a major contributor to the development of PRCA. No significant differences between mutant and non-mutant cases were
found when considering other reported associations, such as the presence of B cell processes or concomitant MDS.

In summary, the present study of CLPD-NK or T-LGL shows that STAT3 SH2 somatic mutations can be found in a similar percentage either in NK or T cell entities. Our results strongly suggest a common specific clinical and pathogenic pattern driven by a shared genetic lesion in those cases irrespective of the cell lineage.

Authorship

Contribution: A.J., S.M., T.P.L. and J.P.M. were responsible for overall design, data collection, data analysis, data interpretation, statistical analysis, manuscript preparation, writing and completion and final approval of manuscript; M.C., H.M., H.K., F.L., K.P.N., T.O., B.P., M.A., I.G. K.G., L.D., E.D.H., K.M., D.Z., M.W.W., K.P., M.A.S., and A.L. gathered data, edited and approved the final manuscript and approved the final manuscript. All authors approved the final version of the manuscript and the submission.

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

References


# Tables.

**Table 1. Clinical characteristics at baseline of the cohort and comparison according to the cell lineage.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Whole cohort (n=170)</th>
<th>T-LGL (n=120)</th>
<th>CLPD-NK (n=50)</th>
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<td></td>
<td>Female 45 (86)</td>
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</tr>
<tr>
<td>Anemia, %</td>
<td>30 (52)</td>
<td>31 (37)</td>
<td>30 (15)</td>
<td>0.9</td>
</tr>
<tr>
<td>Thrombocytopenia, %</td>
<td>14 (24)</td>
<td>13 (16)</td>
<td>16 (8)</td>
<td>0.7</td>
</tr>
<tr>
<td>LGL count in PB, x10^9/L</td>
<td>3.4</td>
<td>3.1</td>
<td>3.7</td>
<td>0.5</td>
</tr>
<tr>
<td>LGL count in PB, x10^9/L Range</td>
<td>0.2-22</td>
<td>0.3-22</td>
<td>0.9-13</td>
<td></td>
</tr>
<tr>
<td>Splenomegaly, %</td>
<td>21 (37)</td>
<td>25 (31)</td>
<td>21 (6)</td>
<td>0.4</td>
</tr>
<tr>
<td>Presence of MGUS, %</td>
<td>21 (36)</td>
<td>22 (26)</td>
<td>20 (10)</td>
<td>0.9</td>
</tr>
<tr>
<td>Associated autoimmune disease, %</td>
<td>RA, %</td>
<td>10 (17)</td>
<td>11 (14)</td>
<td>9 (3)</td>
</tr>
<tr>
<td></td>
<td>AIHA, %</td>
<td>7 (12)</td>
<td>5 (6)</td>
<td>12 (6)</td>
</tr>
<tr>
<td>Associated BM disorder</td>
<td>MDS, %</td>
<td>5 (9)</td>
<td>6 (7)</td>
<td>4 (2)</td>
</tr>
<tr>
<td></td>
<td>PRCA, %</td>
<td>5 (8)</td>
<td>6 (7)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Presence of B-Cell malignancy, %</td>
<td>9 (15)</td>
<td>10 (12)</td>
<td>9 (3)</td>
<td></td>
</tr>
<tr>
<td>Treatment lines, Median Range</td>
<td>1.5</td>
<td>1.7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Patients with somatic mutation in STAT3-SH2 Domain, %</td>
<td>28 (48)</td>
<td>27 (33)</td>
<td>30 (15)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Abbreviations: T-LGL, T-cell large granular lymphocyte leukemia; CLPD-NK, chronic lymphoproliferative disorders NK; PB, peripheral blood; MGUS, monoclonal gammopathy of undetermined significance; STAT3, signal transducer and activator of transcription 3; SH2, Src homology 2 domain.

* Absolute neutrophil count ≤1.5 x10^9/L;

† Absolute lymphocyte count ≥ 4 x10 /L

‡ Hemoglobin ≤ 10 gr/dL

§ Platelet count ≤ 100x10^9/L
**Table 2. Clinical differences at baseline between the STAT 3 SH2 domain mutated and non-mutated LGL leukemia patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>STAT 3 SH2 domain mutated patients (48)</th>
<th>STAT 3 SH2 domain Non mutated (122)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Median 63, Range 35-80</td>
<td>Median 64, Range 20-90</td>
<td>0.96</td>
</tr>
<tr>
<td>Sex, %</td>
<td>Male 58 (28), Female 42 (20)</td>
<td>Male 52 (66), Female 48 (54)</td>
<td>0.06</td>
</tr>
<tr>
<td>Symptoms at diagnosis, %</td>
<td>75 (36)</td>
<td>45 (54)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Neutropenia, %</td>
<td>Median 70, Range 34</td>
<td>Median 59, Range 71</td>
<td>0.14</td>
</tr>
<tr>
<td>Lymphocytosis, %</td>
<td>Median 56, Range 27</td>
<td>Median 52, Range 65</td>
<td>0.7</td>
</tr>
<tr>
<td>Anemia, %</td>
<td>Median 50, Range 24</td>
<td>Median 34, Range 28</td>
<td>0.06</td>
</tr>
<tr>
<td>Neutropenia, %</td>
<td>Median 13, Range 6</td>
<td>Median 15, Range 18</td>
<td>0.7</td>
</tr>
<tr>
<td>LGL count in PB, x10^9/L Median</td>
<td>3.9, Range 0.4-20</td>
<td>3.3, Range 0.5-9</td>
<td>0.45</td>
</tr>
<tr>
<td>Splenomegaly, %</td>
<td>Median 33, Range 16</td>
<td>Median 26, Range 32</td>
<td>0.5</td>
</tr>
<tr>
<td>Presence of MGUS, %</td>
<td>Median 14, Range 8</td>
<td>Median 20, Range 28</td>
<td>0.3</td>
</tr>
<tr>
<td>Associated autoimmune disease, %</td>
<td>RA % 22, AIHA % 14</td>
<td>RA % 24, AIHA % 7</td>
<td>0.3</td>
</tr>
<tr>
<td>Associated BM disorder</td>
<td>MDS % 8, PRCA % 0</td>
<td>MDS % 5, PRCA % 6</td>
<td>0.437</td>
</tr>
<tr>
<td>Presence of B-Cell malignancy, %</td>
<td>Median 4, Range 2</td>
<td>Median 10, Range 13</td>
<td>0.162</td>
</tr>
<tr>
<td>Treatment lines, Median Range</td>
<td>Median 2.4, Range 1.2</td>
<td>Median 1.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Lineage, %</td>
<td>T-Cell 28 (33), NK-Cell 30 (15)</td>
<td>T-Cell 72 (89), NK-Cell 70 (35)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Abbreviations: LGL, large granular lymphocyte; PB, peripheral blood; MGUS, monoclonal gammopathy of undetermined significance; STAT3, signal transducer and activator of transcription 3; SH2, Src homology 2 domain.

* Absolute neutrophil count ≤1.5 x10^9/L
† Absolute lymphocyte count ≥ 4 x10^9 /L
‡ Hemoglobin ≤ 10 gr/dL
§ Platelet count ≤ 100 x10^9/L
Table 3. KIR expression and STAT3 mutational status in CLPD-NK patients. Abnormal KIR expression included: homogenous reactivity to a single KIR receptor and/or absence of reactivity to one or more KIR receptor.

<table>
<thead>
<tr>
<th>Patient Code #</th>
<th>Mutation status</th>
<th>Abnormal KIR expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>10</td>
<td>N647I</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>12</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>13</td>
<td>Y640F</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>15</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>17</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>18</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>19</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>23</td>
<td>D661I</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>34</td>
<td>D661Y</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>36</td>
<td>Y640F</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>38</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>63</td>
<td>Y640F</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>83</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>47</td>
<td>D661Y</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>57</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
<tr>
<td>61</td>
<td>No</td>
<td>No</td>
<td>Present Study</td>
</tr>
<tr>
<td>62</td>
<td>No</td>
<td>No</td>
<td>Present Study</td>
</tr>
<tr>
<td>74</td>
<td>No</td>
<td>Yes</td>
<td>Present Study</td>
</tr>
</tbody>
</table>

Note: Shaded cells include those patients analyzed with a 2-colour flow cytometry strategy, not shaded cells correspond to patients tested with the 4-color strategy (please see Methods section).
Figure legends

Figure 1. Distribution of STAT3 mutations throughout gene domains and patient cohort. (a) STAT3 mutations (blue dots, mutations in T-LGL; green dots, in CLPD-NK) found in the Src homology 2 (SH2) domain, necessary for receptor association and tyrosine phosphodimer formation. The major domains of STAT3 are depicted: coiled-coil domain, DNA-binding domain, SH2 domain, and transactivation domain. The lower panel shows a corresponding representative Sanger sequence for each mutation found. B) Percentage of patients with STAT3 mutations. Lesions were observed in 15 of 50 CLPD-NK and 33 of 120 T-LGL patients when using Sanger and AS-PCR (7 cases not detected by Sanger). C) Histograms showing the percentage of cases corresponding to each mutation. D661Y and Y640F accounted for approx. 80% of all mutations found.

Figure 2A. Constitutive STAT3 activation in leukemic cells. (Top) Western blot analysis in leukemic cells from 4 T-LGL patients (2 mutated and 2 wild type), 4 CLPD-NK patients (2 mutated and 2 wild type) and a control. (B) Aberrant intracellular pSTAT3 signal (brown) has been also detected in paraffin sections from bone marrow–biopsy samples STAT3 mutant and non-mutant cases of T and NK origin. Previous immunohistochemical staining with CD8, surface CD3, and CD2 defined the cell lineage of the lymphocyte infiltration. Positive double staining with anticytoplasmic-CD3 (pink) and pSTAT3 (brown) showed aberrant pSTAT3 signal in the infiltrating lymphoid compartment. Finally, a healthy donor tonsil sample shows no brown nuclei in cCD3 positive cells.

Figure 2B. STAT3 pathway related genes deregulated. A) Heat map reflecting color-coded expression levels from a set of genes known to be regulated by STAT3 (columns) in purified T-LGL cells from 3 patients and control samples (rows). B) Pie chart depicting whole genome expression in the 3 T-LGL leukemia patients and overlapping circles showing a high degree of coincidences in deregulated genes in mutated and non-mutated patients. C) Histograms of whole genome expression levels separated by pathways, exposing a predominance of deregulation in apoptosis and cell death, both in mutated and non mutated patients. Upregulated pathway genes are shown in pink (top panel) and downregulated pathway genes in green (bottom panel).

Figure 3. Effect of STA-21 on apoptosis of malignant large granular lymphocytes. Leukemic and control cells were harvested after 48 hours of STA-21 treatment and analyzed with PI and Annexin V staining assays. Top: dose-dependent increase in apoptosis. Bottom: histograms depicting percentage of cells undergoing apoptosis after treatment compared to untreated cells.

Figure 4. Marked immunodominant Vβ expansions can be seen both in STAT3 SH2 domain mutated (orange bars) and non mutated patients (black bars).

Figure 5. Survival outcomes and time to treatment failure in patients with CLPD-NK and T-LGL. P values presented correspond to the Cox regression between the groups indicated. (A) Comparison of survival outcomes according to the leukemic cell lineage. (B) Comparison of
survival outcomes depending on the STAT3 SH2 domain mutational status. C) Differences in the time to treatment failure in patients with or without STAT3 SH2 domain mutation. Time to treatment failure was defined as the interval between the start of treatment and the need for initiating a second line of therapy and/or progressive disease (including relapse after remission).
**STAT3**  Chromosome: 17; (40465343-40540513, complement)

- **Coiled coil domain**
- **DNA binding domain**
- **Src homology 2 domain**
- **Transactivation domain**

Disease: % of patients

- **T-LGL (n=120)**
  - Y640F: 72%
  - S614R: 27%
  - G618R: 30%
  - G618R: 27%
  - D661Y: 72%
  - K658M: 30%
  - N647I: 60%
  - N647I: 90%
  - D661V: 90%
  - K658M: 30%

- **NK-LGL (n=50)**
  - Y640F: 70%
  - S614R: 30%
  - G618R: 60%
  - G618R: 90%
  - D661Y: 70%
  - K658M: 30%
  - N647I: 60%
  - N647I: 90%
  - D661V: 90%

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Y640F MUTANT T-LGL CASE  
D661Y MUTANT NK-LGL CASE  

NO STAT3 SH2-NON MUTATED NK-LGL CASE  
HEALTHY DONOR
| CD8 | LGL mutation | STAT3 mutation | BCLX | MYC | BRCA5 | MMP9 | MMP2 | CND1 | HIF-1α | ICAM1 | TWIST1 | VIM | MCL1 | IL10 | VEGFA | FGFR2 | CXCL12 | CXCL10 | IL11 | IL22A | IL21 | IL17A | IL6 | IL24 | CD80 | CD84 | IFNG | IFNβ | CCL5 | NOS2 | IL8 | IL1B | CCL2 |
|-----|-------------|----------------|------|-----|-------|------|------|------|-------|-------|-------|-----|------|-----|-------|------|-------|-------|------|-------|------|-------|-----|------|-----|------|------|-----|-----|-----|-----|
|     |             |                |      |     |       |      |      |      |       |       |       |     |      |     |       |      |       |       |      |       |     |      |     |      |     |      |     |     |     |     |

### Venn Diagram

There are 712 unique genes in STAT3 mutant samples, 1641 unique genes in STAT3 WT samples, and 577 genes unique to STAT3 mutant samples. The overlap consists of 490 genes.

### Pie Chart

- **Up 8%**
- **Down 12%**
- **No difference 80%**

### Bar Chart

- **Cell death**
- **Apoptosis**
- **Proliferation of lymphocytes**
- **Growth of lymphocytes**
- **Differentiation of lymphocytes**
- **Immune response**

- **STAT3 mutant**
- **STAT3 WT**

### Color Scale

- **0.8**
- **0.5**
- **0.2**
- **0.0**
- **-0.2**
- **-0.4**
- **-0.6**
- **-0.8**
- **-1.0**
Non mutated T-LGLs (n=61)

Mutated T-LGLs (n=24)
Overall survival (months)

Cumulative survival

1.0
0.8
0.6
0.4
0.2
0.0

0 50 100 150 200 250 300

Overall survival (months)

Cumulative survival

1.0
0.8
0.6
0.4
0.2
0.0

0 50 100 150 200 250 300

Time to treatment failure (days)

Cumulative survival

1.0
0.8
0.6
0.4
0.2
0.0

0 1000 2000 3000 4000

STAT3 mutated LGLs (n=39, events 27)
No STAT3 mutated LGLs (n=55, events 36)

p=0.128

T- LGLs (n=120, events 29)
NK LGLs (n=50, events 19)

p=0.6

STAT3 mutated LGLs (n=48, events 12)
No STAT3 mutated LGLs (n=122, events 32)

p=0.8

T- LGLs (n=120, events 29)
NK LGLs (n=50, events 19)
STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T cell large granular lymphocyte leukemia


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