blood advances

Subclonal STAT3 mutations solidify clonal dominance

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Key Points

- TCR clones can be followed throughout the disease course with deep NGS sequencing of the TCR Vβ CDR3 and *STAT3*.

T large granular lymphocyte leukemia (T-LGLL) is a clonal lymphoproliferative disorder that can arise in the context of pathologic or physiologic cytotoxic T-cell (CTL) responses. *STAT3* mutations are often absent in typical T-LGLL, suggesting that in a significant fraction of patients, antigen-driven expansion alone can maintain LGL clone persistence. We set out to determine the relationship between activating *STAT3* hits and CTL clonal selection at presentation and in response to therapy. Thus, a group of patients with T-LGLL were serially subjected to deep next-generation sequencing (NGS) of the T-cell receptor (TCR) Vβ complementarity-determining region 3 (CDR3) and *STAT3* to recapitulate clonal hierarchy and dynamics. The results of this complex analysis demonstrate that *STAT3* mutations produce either a sweeping or linear subclone within a monoclonal CTL population either early or during the course of disease. Therapy can extinguish a LGL clone, silence it, or adapt mechanisms to escape elimination. LGL clones can persist on elimination of *STAT3* subclones, and alternate *STAT3*-negative CTL clones can replace therapy-sensitive CTL clones. LGL clones can evolve and are fueled by a nonextinguished antigenic drive. *STAT3* mutations can accelerate this process or render CTL clones semiautonomous and not reliant on physiologic stimulation.

Introduction

Characterized by an increased number of circulating clonal cytotoxic T cells (CTLs), large granular T lymphocyte leukemia (T-LGLL) is frequently accompanied by neutropenia, anemia, or thrombocytopenia, but it often develops silently, without pathologic features or clinical symptoms.[1-5] Many lines of evidence indicate that LGL clonal expansion evolves in the context of physiologic (viruses, tumor surveillance) or pathologic responses (autoimmune conditions) to antigens that drive excessive clonal expansions.[6] This view has been supported by studies of T-cell receptor (TCR) rearrangement and TCR Vβ complementarity-determining region 3 (CDR3) deep sequencing, which has frequently demonstrated extreme expansion of a single immunodominant T-cell clone or multiple codominant clones.[7-10] Discovery of activating *STAT3* mutations in a significant proportion of patients with LGL suggested that such mutations initiated a clonal process yielding a spectrum of hematologic pathologies dependent on the specificity of the transformed/expanded CTL clones. Vβ CDR3 sequences acting as biological bar codes can be used to identify and quantify these expansions and to diagnose LGL.[11-17]

Deep TCR repertoire sequencing can precisely identify and assess CDR3 diversity and quantify immunodominant expansions. Using it in conjunction with next-generation sequencing (NGS) of *STAT3* allows serial monitoring of clonal burden and reconstruction of the process that culminates in LGL.

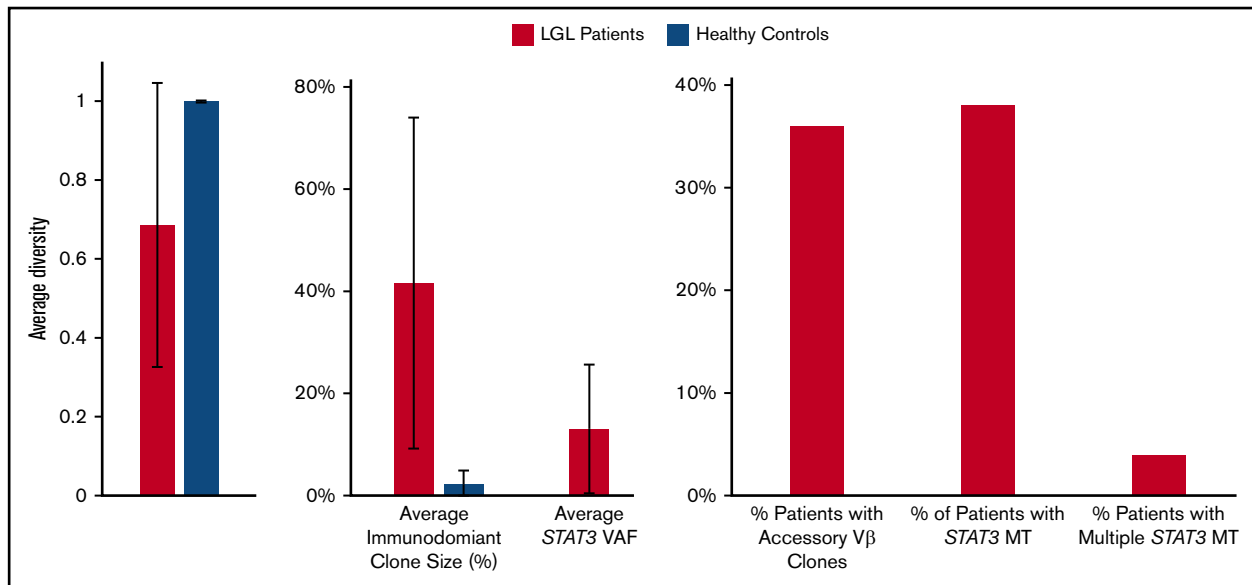The full-text version of this article contains a data supplement.

Figure 1. Characterization of LGL and healthy control patients by deep TCR Vβ sequencing. A diversity value of 1 represents a polyclonal sample.

We used this approach to recapitulate the dynamics of clonal CTL expansions and interactions between individual clones in a large cohort of patients with T-LGLL followed over extended periods of time. Our goal was to determine whether poly- and subsequently oligoclonal immune responses are "anchored" by *STAT3* mutations, most likely occurring in the most proliferative clones, or whether the responses were driven by somatic hits in *STAT3* as the ancestral event. We also set out to characterize the plasticity of the underlying immune response during the course of treatment, including mechanisms of resistance or relapse.

Methods

DNA samples

Blood samples were obtained from patients with LGL seen at the Cleveland Clinic, according to protocols approved by the Cleveland Clinic institutional review board and the Declaration of Helsinki (for diagnostic criteria, see supplemental Table 1).[15,18] DNA was extracted from blood mononuclear cells; the same sample was used for both sequencing methods. Samples were collected from a total of 207 patients with LGL seen at the Cleveland Clinic, 92% T-LGLL, and 8% natural killer LGL. Most patients presented with anemia (46%) and/or neutropenia (46%). Vβ expansion (by Vβ flow cytometry) was present in 94% of cases, with an average LGL count of 2317 k/μL. Clinical features are described in detail in supplemental Table 2.

Deep *STAT3* NGS

All patients with LGL were deep sequenced for the presence of a mutation in exon 21 of *STAT3*, the protein-protein interaction domain.[12] The average coverage was 7500 ± 5700 reads, and hits more than 1% were positive. A *STAT3* mutation was found in 38% of patients in 4 common hotspots: 42% Y640F, 34% D661Y, 11% D661V, and 8% N647I. *STAT3*[MT] VAF was followed over multiple points for 44% of the total cohort. Multiple *STAT3*[MT] were found in 4% of patients. Whole-exome sequencing was performed for some patients, but no recurrent somatic mutations beyond *STAT3* were found.

Deep TCR sequencing

TCR Vβ was sequenced using the Immunoseq assay (Adaptive Biotechnologies), which targets the TCRβ CDR3 region using a multiplex polymerase chain reaction library preparation.[8] Libraries were sequenced on the MiSeq (Illumina), with an average of 40 773 ± 35 667 productive templates. The nucleotide and predicted amino acid sequences, along with the rearranged VDJ regions, were available for every unique CDR3 sequence. TCR deep sequencing was performed on 23% of patients, 10% of which were sequenced at more than 1 point. The diversity of the same represents how monoclonal or polyclonal a sample is as previously defined; values approaching 0 reflect extreme monoclonal samples.[8]

Results

Compared with healthy control patients, the diversity of patients with LGL is much smaller, and they have a higher average max Vβ clone size, suggesting they are more monoclonal (Figure 1). Starting from an initial cohort of 207 patients with LGLL, we selected a representative 18 T-LGLL cases to deep sequence both *STAT3* and TCRB CDR3 longitudinally (Table 1). Patients were sequenced for an average of 4 times (range, 2-8 times). In approximately half the patients, we were able to detect *STAT3*[MT] in previously described canonical positions.[13-15] In all patients, deep TCR NGS identified at least 1 clonotype that was immunodominant over most other clonotypes, with multiple immunodominant subclonotypes identified in about half the patients. For control purposes, we also longitudinally sequenced 2 patients with natural killer LGL; major TCR clonotypic expansions could not be detected in 1 patient with a *STAT3* mutation, but 2 immunodominant T-cell clones were identified in the other patient, consistent with sting antigenic drive also involving T-cell responses (supplemental Figure 1).

In mutant patients, *STAT3* clonal expansions and contractions paralleled those of TCR clones. In some illustrative cases, the TCR clonal

Table 1. Longitudinal T-LGLL characteristics of patients with leukemia

	STAT3^MT	Hematologic presentation	AAD, y	Sex	Therapy	LGL count*	Flow cytometry		TCR deep sequencing			
							VB ID	Clone size*, %	Major clonotype(s)	Clone size*, %	Clonal diversity*†	Clonality*†
CCF1	D661V	Anemia	68	M	CSA, CYC, MTX	2455	NIP	65	CASSLLAGGYNEQFF	42	0.176	0.373
CCF2	D661V	Neutropenia thrombocytopenia	81	M	MTX	1225	TRBV9	72	CASSVGQGSPLHF	75	0.565	0.734
CCF3	Y640F	Neutropenia thrombocytopenia	55	F	CSA	1355	TRBV6-2, TRBV3-1	25, 16	CASSLASISYNEQFF	26	0.068	0.263
CCF4	D661Y, Y640F	Pancytopenia	64	M	CSA, MTX	3672	TRBV10-3	23	CASSAPREGTAYEQYF, CASSPTSGGEQFF	8, 5	0.016	0.222
CCF5	D661Y	Anemia, neutropenia	54	M	CSA, CYC	10 631	TRBV9	99	CASSVGRFQETQYF	44	0.192	0.378
CCF6	Y640F	Neutropenia	47	M	CSA, CYC, MTX	850	TRBV29-1	8, 11	CASSTFAEIRQPQHF, CASSLTENYGYTF	4, 0.01	0.002	0.046
CCF7	D661Y	Neutropenia	40	M	CSA, CYC, MTX	2882	NIP	95	CATSRGTGDSYEQYF	97	0.934	0.938
CCF8	Y640F	Anemia, neutropenia	37	F	CSA	480	TRBV12-3, TRBV12-4	82	CASSSLTGLPPYEQYF	29	0.085	0.247
CCF9	Y640F	Anemia, neutropenia	74	F	CSA, CYC, MTX	496	NIP	96	CASSPEGFQPQHF, CASTSFGTGTDTQYF	91, 0.16	0.819	0.871
CCF10	Negative	Anemia, neutropenia	17	M	CSA, MTX	330	TRBV18	77	CASSPFWTLEKLFF	93	0.871	0.893
CCF11	Negative	Anemia	63	M	CSA, CYC, MTX	980	TRVB28, TRBV19	32, 18	CASSPLGAVGYNEQFF, CAISEFPGGQTSDEGFF	25, 1	0.086	0.471
CCF12	Negative	Neutropenia	47	F	CSA, MTX	188	TRBV12-3, TRBV12-4	45	CASSIAGLGSEQFF, CASSEGQMYNEQFF	12, 3	0.019	0.191
CCF13	Negative	Anemia, neutropenia	52	F	CSA, CYC, MTX	261	TRBV13	97	CASSDLAGMLGRQFF	99	0.983	0.984
CCF14	Negative	Anemia	66	M	CYC	409	NIP, TRBV20-1	84, 24	CASSLEIAGGLIYNEQFF, CASSPGLAGGRETQYF	27, 2	0.093	0.350
CCF15	Negative	Anemia, neutropenia	60	M	CSA, CYC, MDX	4472	TRBV19	97	CASSIGIQPQHF	83	0.686	0.791
CCF16	Negative	Asymptomatic	59	M	SCT, CYC	1515	TRBV27	30	CASSSVGYEQYF	40	0.173	0.478
CCF17	Negative	Anemia	71	F	MTX	2536	TRBV19	20	CASRASGSDEQYF, CARSRDKGTDYGYTF	10, 8	0.024	0.263
CCF18	Negative	Asymptomatic	54	M	None	686	TRBV27, TRVB4-3	12, 8	CASSLGNYEQYF, CASSISQNTEAFF	4, 7	0.026	0.319

AAD, age at diagnosis; CSA, cyclosporine-A; CYC, cyclophosphamide; F, female; FU, follow-up; M, male; MTX, methotrexate; SCT, stem cell transplant.

*At earliest sampling.

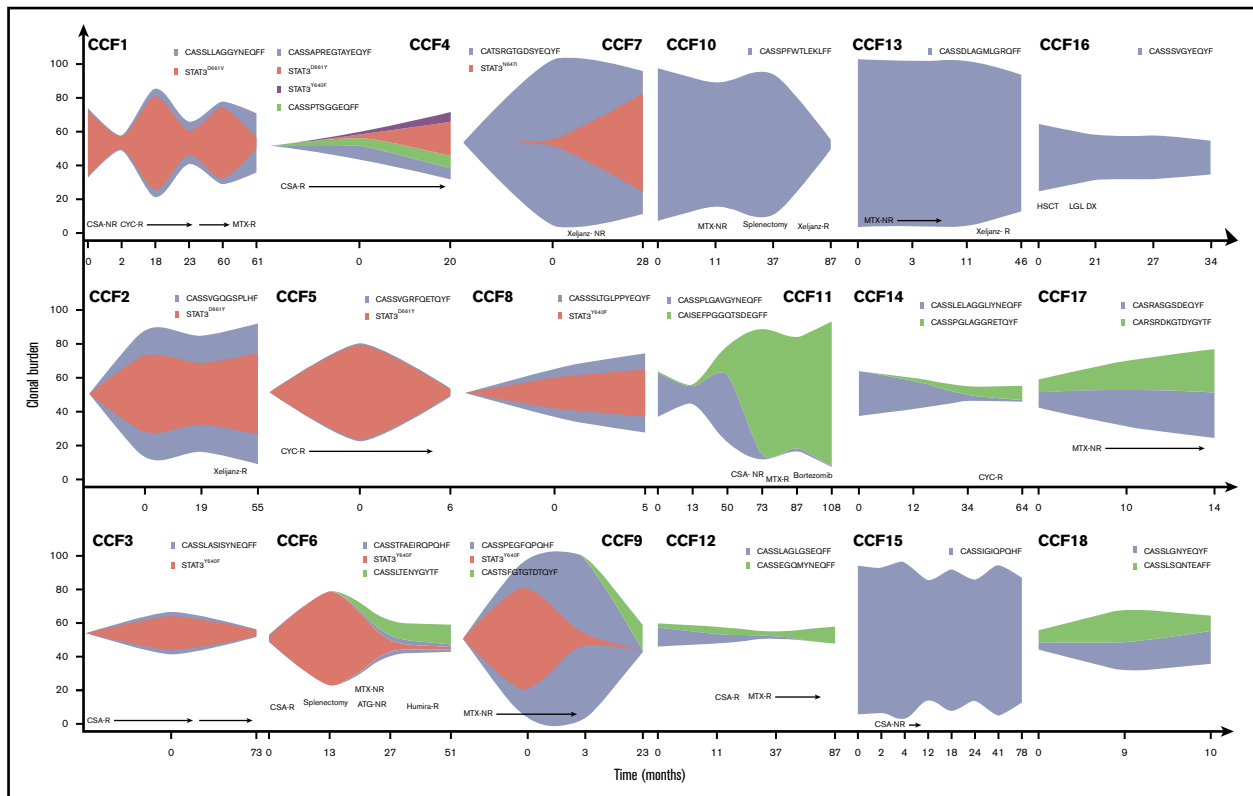†Clonal diversity and Clonality assessed according to Clemente et al.[9]

Figure 2. Clonal dynamics of *STAT3* and TCR clonotypes throughout clinical course of patients with T-LGLL. Fish plots depicting the clonal expansions of 18 patients with T-LGLL. Sampling points are shown along the x-axis, and the clonal burden is shown on the y-axis. NR, no response; R, response.

burden of the dominant clone was higher than that of *STAT3*[MT], suggesting that *STAT3*[MT] is not the ancestral event for clonal expansion (see patients CCF1, CCF2, CCF7, CCF8, and CCF9). Our findings imply that the *STAT3* hit occurs as a secondary event within the preexpanded immunodominant clone. However, in other patients, the clonal burden of both the TCR and *STAT3*[MT] clone were similar (patients CCF3, CCF5, and CCF6), indicating that the *STAT3*[MT] may contribute to autonomous antigen-independent clonal expansion.

Serial samplings illustrated clonal dynamics in response to therapy. More than half of patients with T-LGLL were treated with 1 or more immunosuppressive therapy (IST) regimens, leading to hematologic response in 40% of those treated. Distinct patterns of clonal dynamics were seen after treatment and are illustrated as fish plots (Figure 2). Samples were not available at the first appearance of a clone, but the clonal expansion can be speculated based on samplings at other points. In some patients (4/18), both the *STAT3*[MT] (if present) and major TCR clone decreased in response to treatment (patients CCF1, CCF3, CCF5, and CCF10). In others (3/18), the clones persisted despite a hematologic response (patients CCF2, CCF4, and CCF17), suggesting major clones were functionally silenced in their ability to inhibit/destroy specific hematopoietic progenitors. We also observed a common phenomenon of TCR "clonotype switching" in many patients (6/18), wherein therapy contracts 1 major clonotype while another previously "minor" clonotype expands (patients CCF6, CCF11, CCF12, CCF14, CCF16, and CCF18), likely because of its relative therapy insensitivity. Interestingly, all newly emerging clones were *STAT3*[WT] (wild type), and half the patients with "switching" were resistant to IST therapy. Multiple clonotypes were present at initial

sampling in a few patients without *STAT3*[MT] and persisted at the same rate in subsequent samplings, precluding identification of a truly immunodominant clonotype (4/18). It is thus possible that a small but highly pathogenic clone may have been missed in our initial analysis. Predictably, a stable or increasing clonal burden of both *STAT3*[MT] and VB CDR3 sequence was seen in nonresponders to IST (28%; patients CCF7, CCF9, CCF13, CCF15, and CCF17).

Discussion

Our results demonstrate that *STAT3*[MT] can arise either within an already preexpanded clonotype, or simultaneously with the clonal expansion of the immunodominant TCR Vβ clonotype. This leads us to believe that the *STAT3*[MT] mutation is selected among the originally triggered (responding) T-cell clones, and thus make the response more autonomous by either more persistence or less defendant on accessory signaling while not making the clone totally autonomous. The dynamics of both the *STAT3*[MT] and the TCR Vβ clonotype can be assessed over the course of the disease and in response to treatment regimens, and may demonstrate additional clinical utility when applied to larger prospective clinical trials (supplemental Figure 2). The difficulty in finding a direct correlation between response to a specific IST and a decrease in TCR Vβ clonal burden and *STAT3*[MT] may be a result of variability in time frames between samplings, IST regimens used, and the quality of response. Some large clonotypes may also be asymptomatic/not pathogenic, and thus may be overshadowing signals from smaller truly pathogenic clonotypes. Associations of remission with elimination of immunodominant clonotypes remain unclear. Our results suggest that pathogenic

clones can be contracted to manageable clonal burdens and/or functionally silenced/tolerated. Thus, clonal elimination may not be needed for a complete clinical response.

Acknowledgments

Authorship

Contribution: C.M.K. performed sequencing experiments, collected/analyzed data, and wrote the manuscript; M.J.C collected data, advised on experiments, contributed to study design, and edited the manuscript; P.W.C. assisted with sequencing experiments; B.P., Y.N., V.A., and V.V. contributed to study design; T.R. reviewed the manuscript; A.E.L. and M.A.S. collected patient samples and clinical information; S.M. performed research; and J.P.M. designed and conceptualized the overall research and wrote the manuscript.

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References

1. Loughran TP Jr, Starkebaum G. Clinical features in large granular lymphocytic leukemia. *Blood*. 1987;69(6):1786.

2. Loughran TP Jr, Kadin ME, Starkebaum G, et al. Leukemia of large granular lymphocytes: association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. *Ann Intern Med*. 1985;102(2):169-175.

3. Lamy T, Loughran TP Jr. How I treat LGL leukemia. *Blood*. 2011;117(10):2764-2774.

4. Lamy T, Moignet A, Loughran TP Jr. LGL leukemia: from pathogenesis to treatment. *Blood*. 2017;129(9):1082-1094.

5. Sanikommu SR, Clemente MJ, Chomczynski P, et al. Clinical features and treatment outcomes in large granular lymphocytic leukemia (LGLL). *Leuk Lymphoma*. 2018;59(2):416-422.

6. Sokol L, Loughran TP Jr. Large granular lymphocyte leukemia. *Oncologist*. 2006;11(3):263-273.

7. Qiu ZY, Shen WY, Fan L, et al. Assessment of clonality in T-cell large granular lymphocytic leukemia: flow cytometric T cell receptor Vβ repertoire and T cell receptor gene rearrangement. *Leuk Lymphoma*. 2015;56(2):324-331.

8. Clemente MJ, Przychodzen B, Jerez A, et al. Deep sequencing of the T-cell receptor repertoire in CD8+ T-large granular lymphocyte leukemia identifies signature landscapes. *Blood*. 2013;122(25):4077-4085.

9. Clemente MJ, Wlodarski MW, Makishima H, et al. Clonal drift demonstrates unexpected dynamics of the T-cell repertoire in T-large granular lymphocyte leukemia. *Blood*. 2011;118(16):4384-4393.

10. Wlodarski MW, Schade AE, Maciejewski JP. T-large granular lymphocyte leukemia: current molecular concepts. *Hematology*. 2006;11(4):245-256.

11. Epling-Burnette PK, Liu JH, Catlett-Falcone R, et al. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J Clin Invest*. 2001;107(3):351-362.

12. Andersson E, Kuusanmäki H, Bortoluzzi S, et al. Activating somatic mutations outside the SH2-domain of STAT3 in LGL leukemia. *Leukemia*. 2016; 30(5):1204-1208.

13. Jerez A, Clemente MJ, Makishima H, et al. STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T-cell large granular lymphocyte leukemia. *Blood*. 2012;120(15):3048-3057.

14. Fasan A, Kern W, Grossmann V, Haferlach C, Haferlach T, Schnittger S. STAT3 mutations are highly specific for large granular lymphocytic leukemia. *Leukemia*. 2013;27(7):1598-1600.

15. Koskela HL, Eldfors S, Ellonen P, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med*. 2012;366(20):1905-1913.

16. Rajala HL, Porkka K, Maciejewski JP, Loughran TP Jr, Mustjoki S. Uncovering the pathogenesis of large granular lymphocytic leukemia-novel STAT3 and STAT5b mutations. *Ann Med*. 2014;46(3):114-122.

17. Rajala HL, Olson T, Clemente MJ, et al. The analysis of clonal diversity and therapy responses using STAT3 mutations as a molecular marker in large granular lymphocytic leukemia. *Haematologica*. 2015;100(1):91-99.

18. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20): 2375-2390.