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.7,8 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.9-17

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.
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). 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. 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. STAT3MT VAF was followed over multiple points for 44% of the total cohort. Multiple STAT3MT 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. 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.

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 TCRβ 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 STAT3MT in previously described canonical positions. 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 stig antigenic drive also involving T-cell responses (supplemental Figure 1).
| STAT3<sup>MT</sup> | Hematologic presentation | AAD, y | Sex | Therapy | LGL count* | Flow cytometry | TCR deep sequencing | Clonal diversity† | Clonality‡† |
|------------------|--------------------------|--------|-----|---------|------------|----------------|---------------------|------------------|----------|
| CCF1 D661V       | Anemia                   | 68     | M   | CSA, CYC, MTX | 2455 NIP | TRBV9 65        | CASSLAGGYNEQFF 42, 0.176 0.373 |
| CCF2 D661V       | Neutropenia, thrombocytopenia | 81     | M   | MTX   | 1225 TRBV6-2, TRBV3-1 25, 16 | TRBV10-3 23 | CASSVSSYGSGGSEQF 75, 0.565 0.734 |
| CCF3 Y640F       | Neutropenia, thrombocytopenia | 55     | F   | CSA   | 1355 TRBV6-2, TRBV3-1 25, 16 | NIP 65 | CASSLAGGYNEQFF 26, 0.068 0.263 |
| CCF4 D661Y, Y640F| Pancytopenia             | 64     | M   | CSA, MTX | 3672 TRBV10-3 23 | TRBV9 65 | CASSLAGGYNEQFF 8, 0.016 0.222 |
| CCF5 D661Y       | Anemia, neutropenia      | 54     | M   | CSA, CYC | 10 631 TRBV9 99 | TRBV9 65 | CASSVSSYGSGGSEQF 44, 0.192 0.378 |
| CCF6 Y640F       | Neutropenia              | 47     | M   | CSA, CYC, MTX | 850 TRBV29-1 8, 11 | NIP 65 | CASSLAGGYNEQFF 4, 0.012 0.046 |
| CCF7 D661Y       | Neutropenia              | 40     | M   | CSA, CYC, MTX | 2882 NIP | TRBV18 77 | CASSVSSYGSGGSEQF 93, 0.871 0.893 |
| CCF8 Y640F       | Anemia, neutropenia      | 37     | F   | CSA   | 480 TRBV12-3, TRBV12-4 82 | TRBV18 77 | CASSVSSYGSGGSEQF 93, 0.871 0.893 |
| CCF9 Y640F       | Anemia, neutropenia      | 74     | F   | CSA, CYC, MTX | 496 NIP | TRBV18 77 | CASSVSSYGSGGSEQF 93, 0.871 0.893 |
| CCF10 Negative   | Anemia, neutropenia      | 17     | M   | CSA, MTX | 330 TRBV18 77 | TRBV18 77 | CASSVSSYGSGGSEQF 93, 0.871 0.893 |
| CCF11 Negative   | Anemia                   | 63     | M   | CSA, CYC, MTX | 980 TRBV28, TRBV19 32, 18 | NIP 65 | CASSLAGGYNEQFF 25, 0.886 0.471 |
| CCF12 Negative   | Neutropenia              | 47     | F   | CSA, MTX | 188 TRBV12-3, TRBV12-4 45 | TRBV18 77 | CASSLAGGYNEQFF 12, 3 0.191 |
| CCF13 Negative   | Anemia, neutropenia      | 52     | F   | CSA, CYC, MTX | 261 TRBV13 97 | TRBV13 97 | CASSLAGGYNEQFF 99, 0.983 0.984 |
| CCF14 Negative   | Anemia                   | 66     | M   | CYC   | 409 TRBV20-1 84, 24 | TRBV13 97 | CASSLAGGYNEQFF 27, 0.093 0.350 |
| CCF15 Negative   | Anemia, neutropenia      | 60     | M   | CSA, CYC, MDX | 4472 TRBV19 97 | TRBV19 97 | CASSLAGGYNEQFF 83, 0.686 0.791 |
| CCF16 Negative   | Asymptomatic             | 59     | M   | SCT, CYC | 1515 TRBV27 30 | TRBV19 20 | CASSLAGGYNEQFF 40, 0.173 0.478 |
| CCF17 Negative   | Asymptomatic             | 71     | F   | MTX   | 2536 TRBV19 20 | TRBV19 20 | CASSLAGGYNEQFF 10, 8 0.024 0.263 |
| CCF18 Negative   | Asymptomatic             | 54     | M   | None  | 686 TRBV27, TRBV4-3 12, 8 | TRBV27 12, 8 | CASSLAGGYNEQFF 4, 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
burden of the dominant clone was higher than that of STAT3MT, suggesting that STAT3MT 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 STAT3MT clone were similar (patients CCF3, CCF5, and CCF6), indicating that the STAT3MT 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 STAT3MT (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 STAT3MT (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 STAT3MT 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 STAT3MT and VB CDR3 sequence was seen in nonresponders to IST (28%; patients CCF7, CCF9, CCF13, CCF15, and CCF17).

**Discussion**

Our results demonstrate that STAT3MT can arise either within an already preexpanded clonotype, or simultaneously with the clonal expansion of the immunodominant TCR VB clonotype. This leads us to believe that the STAT3MT mutation is selected among the originally triggered (responding) T-cell clones, and thus make the response more autonomous by either more persistence or less dependent on accessory signaling while not making the clone totally autonomous. The dynamics of both the STAT3MT and the TCR VB 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 VB clonal burden and STAT3MT 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.

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**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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