HemaSphere

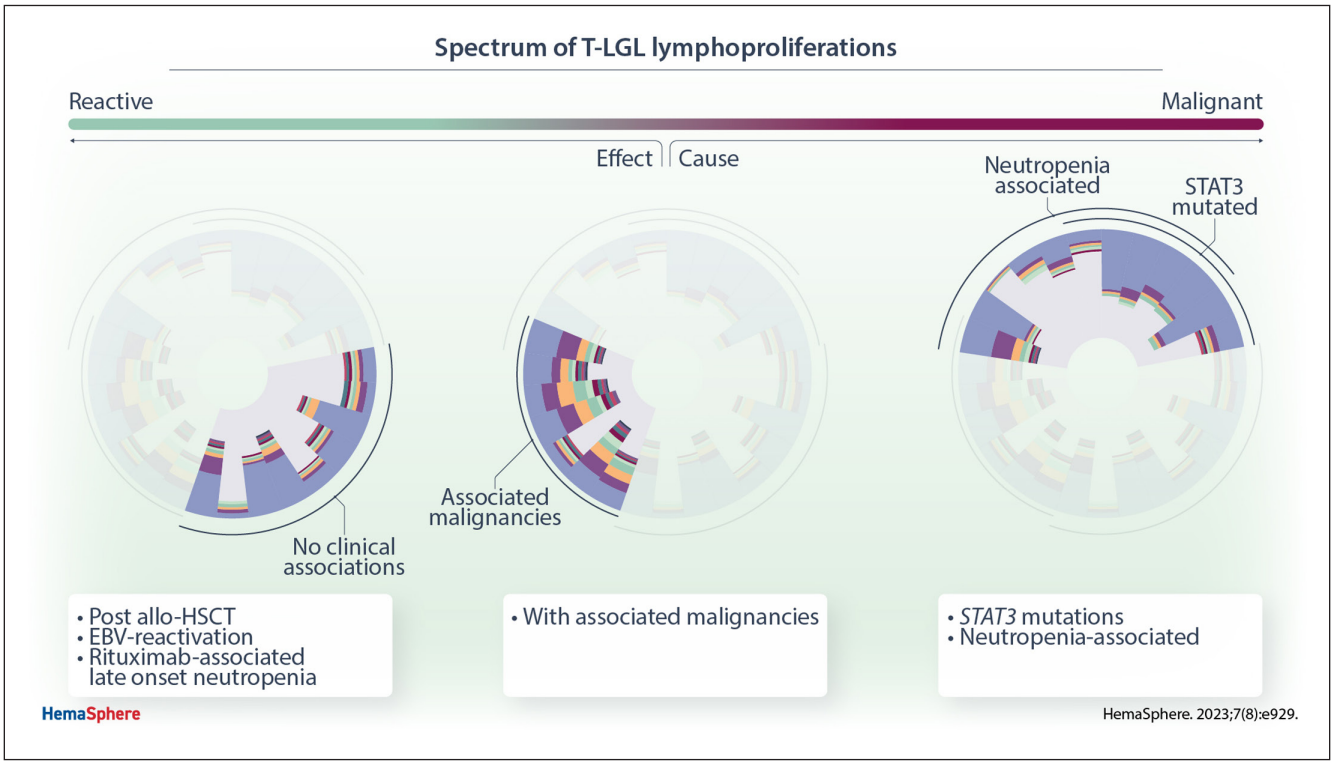
EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
Open Access

Context-dependent T-cell Receptor Gene Repertoire Profiles in Proliferations of T Large Granular Lymphocytes

Jorn L.J.C. Assmann[1], Elisavet Vlachonikola[2], Pieter M. Kolijn[1], Andreas Agathangelidis[2], Nikolaos Pechlivanis[2], Apostolia Papalexandri[3], Kostas Stamatopoulos[2], Anastasia Chatzidimitriou[2], Anton W. Langerak[1]

GRAPHICAL ABSTRACT



HemaSphere

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
Open Access

Context-dependent T-cell Receptor Gene Repertoire Profiles in Proliferations of T Large Granular Lymphocytes

Jorn L.J.C. Assmann[1], Elisavet Vlachonikola[2], Pieter M. Kolijn[1], Andreas Agathangelidis[2], Nikolaos Pechlivanis[2], Apostolia Papalexandri[3], Kostas Stamatopoulos[2], Anastasia Chatzidimitriou[2], Anton W. Langerak[1]

Correspondence: Anton W. Langerak (a.langerak@erasmusmc.nl).

ABSTRACT

T cell large granular lymphocyte (T-LGL) lymphoproliferations constitute a disease spectrum ranging from poly/oligo to monoclonal. Boundaries within this spectrum of proliferations are not well established. T-LGL lymphoproliferations co-occur with a wide variety of other diseases ranging from autoimmune disorders, solid tumors, hematological malignancies, post solid organ, and hematopoietic stem cell transplantation, and can therefore arise as a consequence of a wide variety of antigenic triggers. Persistence of a dominant malignant T-LGL clone is established through continuous STAT3 activation. Using next-generation sequencing, we profiled a cohort of 27 well-established patients with T-LGL lymphoproliferations, aiming to identify the subclonal architecture of the T-cell receptor beta (*TRB*) chain gene repertoire. Moreover, we searched for associations between *TRB* gene repertoire patterns and clinical manifestations, with the ultimate objective of discriminating between T-LGL lymphoproliferations developing in different clinical contexts and/or displaying distinct clinical presentation. Altogether, our data demonstrates that the *TRB* gene repertoire of patients with T-LGL lymphoproliferations is context-dependent, displaying distinct clonal architectures in different settings. Our results also highlight that there are monoclonal T-LGL cells with or without *STAT3* mutations that cause symptoms such as neutropenia on one end of a spectrum and reactive oligoclonal T-LGL lymphoproliferations on the other. Longitudinal analysis revealed temporal clonal dynamics and showed that T-LGL cells might arise as an epiphenomenon when co-occurring with other malignancies, possibly reactive toward tumor antigens.

INTRODUCTION

T cell large granular lymphocyte (T-LGL) leukemia is a rare hematological malignancy characterized by chronic (oligo) clonal proliferation of cytotoxic T cells.[1] Although often asymptomatic, patients may eventually develop symptoms, for example, neutropenia, thrombocytopenia, or anemia.[2] In recent years, T-LGL leukemia has emerged as a paradigmatic example of antigen-driven leukemogenesis, with transitions from poly or oligoclonally reactive responses to monoclonal T-LGL leukemia occurring in a gradual manner.[3] Notably, boundaries between polyclonal, oligoclonal, and monoclonal T-LGL lymphoproliferations are not well-established. In fact, T-LGL clones expand due to persistent antigenic stimulation and characteristic T-LGL cells are seen in various disease contexts, ranging from autoimmune disorders, depletion of B cells through monoclonal anti-CD20 antibodies, solid tumors, and hematologic malignancies to solid organ and allogeneic hematopoietic stem cell transplantation (allo-HSCT).[4–7] Therefore, T-LGL cells may arguably be triggered by various antigens. Eventually, profound immune dysregulation leads to persisting T-LGL clones, with the constitutive activation of the STAT3 transcription activator as a hallmark.[8] Koskala et al first identified activating mutations in exon 21 of the *STAT3* gene, coding for the SH2 domain, whereas other mutations were later identified, all in or around exon 21.[9,10] Although only 30%–40% of all patients with T-LGL leukemia bear mutations in the *STAT3* gene, all patients carry cells that show hyperactive STAT3 signaling.[11]

Based on the concept that antigens drive T-LGL cells to proliferate, much research has been conducted regarding the T-cell receptor beta (*TRB*) gene repertoire looking for evidence of antigen selection.[12,13] Most of this work has been performed with traditional spectratyping methods.[14] Additionally, subcloning of individual *TRBV-TRBD-TRBJ* gene rearrangements followed by Sanger sequencing has also been performed, leading to low-throughput and low-resolution datasets.[12] However, firm conclusions regarding the precise impact of antigenic drive on T-LGL proliferations could not be drawn and, thus, it remains uncertain if T-LGL cells create an environment for

[1]*Laboratory for Medical Immunology, Department of Immunology, Erasmus MC, Rotterdam, Netherlands*
[2]*Institute of Applied Biosciences, Centre for Research and Technology Hellas, Greece*
[3]*Hematology Department and HCT Unit, Papanicolaou Hospital, Thessaloniki, Greece*
JLJCA and EV shared first authorship and have contributed equally to this work. KS, AC, and AWL shared last authorship and have contributed equally to this work.
Supplemental digital content is available for this article.

HemaSphere (2023) 7:8(e929).
http://dx.doi.org/10.1097/HS9.0000000000000929.
Received: January 26, 2023 / Accepted: June 12, 2023

immune-dysregulation disorders to thrive or, instead, arise as a reactive population.[15]

Here, we sought to obtain comprehensive insight into the *TRB* gene repertoire in a series of well-characterized patients with T-LGL lymphoproliferations employing next-generation sequencing (NGS), thus allowing not only to evaluate dominant clonotypes but also to unravel the subclonal architecture. Furthermore, by serial sampling over time and in different disease contexts, we aimed to elucidate if T-LGL lymphoproliferations emerge as reactive populations and how they evolve. We show that the *TRB* gene repertoire of patients with T-LGL lymphoproliferations is profoundly context-dependent, displaying distinct clonality patterns under different circumstances, suggesting that T-LGL proliferations might be triggered by a broad range of antigens. In addition, we argue that large monoclonal populations of T-LGL cells might be the causative factor of neutropenia in patients with T-LGL proliferations (with or without *STAT3* mutations), and that oligoclonal T cell populations might be the consequence of associated malignancies that are often co-occurring in T-LGL patients.

MATERIALS AND METHODS

Study group

Our study group included 27 patients with TRαβ$^+$CD8$^+$ T-LGL proliferations and 22 age-matched healthy controls. The biobanks of the Department of Immunology of Erasmus MC, University Medical Center, Rotterdam, The Netherlands and the Hematology Department and HCT Unit of the G. Papanicolaou Hospital, Thessaloniki, Greece were retrospectively inspected to include persistent TRαβ$^+$CD8$^+$ T-LGL proliferations in peripheral blood (PB). Cases were included based on a combination of clinical, immunophenotypical, and molecular data (Table 1). Five TCRαβ$^+$CD8$^+$ T-LGL leukemia cases were specifically selected for longitudinal analyses. Control samples were obtained from Sanquin Blood Bank (Amsterdam, The Netherlands) and the Institute of Applied Biosciences, CERTH, Thessaloniki, Greece, upon informed consent and anonymized before use. The study was approved by the institutional review boards of the participating institutions (MEC-2015-0617; MEC-2011–0409; CERTH: EHT.COM-45, 21/03/2019). Of note, samples TLGL5 and TLGL15 were removed from all analyses, except the HLA analysis, because of minor laboratory contamination.

PCR amplification of *TRBV-TRBD-TRBJ* gene rearrangements and library preparation

Genomic DNA was isolated from PB mononuclear cells and 500 ng was used for multiplex polymerase chain reaction (PCR) amplification of *TRBV-TRBD-TRBJ* gene rearrangements following the BIOMED-2 protocol (35 cycles).[16] Library preparation was performed as described with an index PCR of 12 cycles.[17]

Bioinformatics

Initial NGS data assessment was performed by the Illumina signal-processing software, including base-calling, quality control, adapter trimming, and demultiplexing, resulting in the exclusion of low-quality and erroneous sequences. A purpose-built, validated bioinformatics algorithm was applied to the raw NGS reads in order to merge and further filter the paired-end reads based on the strict length and quality criteria, as previously described.[18] Merged reads were further annotated using the IMGT/HighV-QUEST tool and the annotated sequences were further characterized by the T-cell Receptor/Immunoglobulin Profiler software. For additional details, see Supplemental Materials and Methods. Data are available under ENA Project ID: PRJEB47814.

Definitions and interpretation of results

In this study, clonotypes were defined as *TRBV-TRBD-TRBJ* gene rearrangements with identical *TRBV* gene usage and TRB CDR3 amino acid (aa) sequence. Clonotypes were characterized as expanded when they comprised ≥2 nucleotide sequences. The most expanded clonotype within a sample is referred to as immunodominant. Clonotypes representing only a single read were characterized as singletons.

For *TRB* gene repertoire analysis, clonotypes rather than single rearrangements were taken into consideration in order to avoid potential biases. Individual *TRBV* gene frequencies within a sample were calculated based on the number of clonotypes using the particular *TRBV* genes over the total number of clonotypes.

For the connectivity analysis as well as the comparisons and clustering of clonotypes based on their sequence identity, only expanded clonotypes were evaluated whereas singletons were excluded. This decision was intentionally taken in order to avoid potentially false estimates of clonotype sharing, as it is very difficult to discriminate whether clonotypes called by a single read represent a true finding or instead arise from sequencing errors or mapping biases (index hopping effect). The latter is an inherent limitation of NGS technology that could erroneously assign sequencing reads to the wrong index during demultiplexing, hence leading to an artificial increase in similarity between samples.

Subclonal connectivity

We interrogated the subclonal architecture of the *TRB* gene repertoire in a given sample by characterizing clonotypes that are clonally related with the immunodominant one that is those with identical CDR3 length as the dominant one yet differing in a single aa position. Pairwise distance calculation was performed and only CDR3s with 1 difference within their aa sequence were connected as clonally related.

Clonotype comparisons and clustering of TRBV-TRBD-TRBJ rearrangements based on TRB CDR3 amino acid sequence restriction

Clonotype comparisons were also performed between the different T-LGL cases and cross-comparisons against a dataset composed of TR clonotype sequences either retrieved from the VDJdb (a curated database of TCR sequences with known antigen specificities[19] or available to our group from previous studies in benign ethnic neutropenia (BEN),[20] chronic idiopathic neutropenia (CIN),[19] chronic lymphocytic leukemia (CLL),[18] monoclonal B lymphocytosis ([MBL], a potentially precursor state to CLL, identified in otherwise healthy individuals),[18] and healthy controls. Additionally, comparisons were undertaken with a published dataset of *TRB* gene rearrangement sequences from patients with T-LGL leukemia reported by Kerr et al.[21]

Furthermore, common immunogenetic signatures between the samples were identified by clustering of *TRBV-TRBD-TRBJ* rearrangements based on the shared TRB CDR3 aa sequence motifs using a modified version of the Teiresias algorithm adapted to the immune repertoire analyses.[22,23] For additional information see Supplemental Materials and Methods.

HLA-A, HLA-B, HLA-C low-resolution typing

Typing of HLA-A, HLA-B, and HLA-C in low resolution was performed with the Olerup SSP HLA typing kits according to the manufacturer's protocol (CareDx, Vienna, Austria).

STAT3 mutation analysis

STAT3 exon 21 was amplified from genomic DNA. Thereafter, PCR products were analyzed using bidirectional Sanger sequencing to assess *STAT3* hotspot mutations. All cases that were found negative by Sanger sequencing were assessed

Table 1

Patient Characteristics of the Present Cohort of Cases With T-LGL Lymphoproliferations

Patient	Age	Gender	% LGL Within CD3	Circulating LGLs (× 10^9/L)	Immunophenotype	Associated Disease	Symptoms	Degree of Neutropenia	Therapy	Sampling Pre/ Post Tx	Impact of Tx on LGL	*STAT3* Mutation
LGL1	70	M	75	3.5	CD8+CD4-CD5-/ CD57+	None	Neutropenia, B-symptoms	Mild	None	Pre	Unknown	No
LGL2	73	M	48	4.4	CD8+CD4-CD57+	Chronic NK-cell leukemia	Neutropenia, recurrent infections	Mild	MTX	Pre	Unknown	D661Y
LGL3	67	M	55	2.0	CD8+CD4-CD2-/ CD57+	RA	Neutropenia, B symptoms, thrombocytopenia	Mild	Plaquenil	Pre	Unknown	Y640F
LGL4	72	M	72	-	CD8+CD4-	None	Thrombocytopenia	No	None	Pre	Unknown	Y640F
LGL5	35	F	52	3.7	CD8+CD4-CD57+	None	Neutropenia	Mild	None	Pre	Unknown	No
LGL6	74	F	41	0.8	CD8+CD4-CD7-/ CD57+	RA, splenomegaly	Neutropenia	Severe	MTX	Pre	Low-level presence	Y640F
LGL7	58	M	6	0.6	CD8+CD4-CD57+	ITP, MGUS	Asymptomatic (with respect to T-cell clone)	No	None (with respect to T-cell clone)	Pre	Unknown	No
LGL8	63	M	50	8.2	CD8+CD4-CD57+	M. Waldenström (2005); T-LGL post allo-Tx	Asymptomatic (with respect to T-cell clone)	No	None (with respect to T-cell clone)	Pre	Unknown	No
LGL9	58	M	29	6.1	CD8+CD4-CD57+	HCL	Asymptomatic (with respect to T-cell clone)	No	None (with respect to T-cell clone)	Pre	Unknown	No
LGL10	64	F	24	0.6	CD8+CD4-CD57+	Unknown	Neutropenia	Mild	Unknown	Pre	Unknown	Y640F
LGL11	38	F	8	0.3	CD8+CD4-CD5-/ CD57+	None	Neutropenia	Severe	MTX	Pre	Unknown	No
LGL12	78	M	20	0.8	CD8+CD4-CD7-/ CD57+	AML	Asymptomatic (with respect to T-cell clone)	No	None (with respect to T-cell clone)	Pre	Low-level presence	No
LGL13	76	F	52	12.0	CD8+CD4-CD57+	None	B symptoms	No	None	Pre	Unknown	Y640F
LGL14	41	F	40	2.5	CD8+CD4-CD7-/ CD57+	PRCA	Anemia, B symptoms	No	CSA	Pre	Unknown	No
LGL15	37	M	40	1.0	CD8+CD4-CD5-CD57+	M. Crohn	Agranulocytosis	Severe	Unknown	Pre	Unknown	Y640F
LGL16	48	F	7	0.3	CD8+CD4-CD57+	DLBCL (2003, CNS loc 2005)	Neutropenia, anemia	Mild	None (with respect to T-cell clone)	Pre	Unknown	No
LGL17	27	M	44	4.2	CD3+/8+/CD57+	None	Pancytopenia	Mild	Yes	Post	Yes	No
LGL18	59	M	24	2.3	CD3+/8+/CD57+	None	Neutropenia, thrombocytopenia	Mild	Yes	Pre	No Tx	No
LGL19	15	M	46	1.1	CD3+/8+/CD57+	Allo-Tx	AIHA	No	Yes	Pre	Yes	No
LGL20	51	F	41	2.2	CD3+/8+/CD57+	None	Neutropenia	Mild	Yes	Pre	Yes	No
LGL21	44	M	69	5.0	CD3+/8+/CD57+	None	None	No	None	Pre	No Tx	D661Y
LGL22			70	6.3	CD3+/8+/CD57+	None	None	No	None	Pre	No Tx	Y640F
LGL23	85	M		0.6	CD3+/8+/CD57+	None	None	No	None	Pre	No Tx	No
LGL24	65	M	17	0.4	CD3+/8+/CD57+	None	Neutropenia	Severe	Yes	Pre	Yes	No
LGL25	35	M	29	0.8	CD3+/8+/CD57+	None	None	No	None	Pre	No Tx	No
LGL26	32	M	23	1.8	CD3+/8+/CD57+	None	None	No	None	Pre	No Tx	No
LGL27	73	M	40	1.6	CD3+/8+/CD57+	Smoldering Myeloma	None	No	None	Pre	No Tx	No

[a]All T-LGL patients were diagnosed based on the cell morphology/immunophenotype and/or molecular genetics.

AIHA = Autoimmune hemolytic anemia; AML = acute myeloid leukemia; CSA = cyclosporine A; DLBCL = diffuse large B-cell lymphoma; HCL = hairy cell leukemia; Het = heterogeneous; ITP = immune thrombocytopenia; MGUS = monoclonal gammopathy of unknown significance; MTX = methotrexate; PRCA = pure red cell aplasia; RA = rheumatoid arthritis; T-LGL = T cell large granular lymphocyte; Tx = transplantation.

for the presence of *STAT3* mutations in exon 21 by NGS with a total coverage >16,000× for each sample.

Statistical analysis

Descriptive statistics for discrete parameters included counts and frequency distributions. For quantitative variables, statistical measures included medians and min-max values. Mann-Whitney test was used to test differences between groups. For all comparisons, *P* values <0.05 were considered statistically significant. Statistical tests and levels of significance are indicated in the figure legends. Shannon diversity index was calculated using the vegan package in R, based on the equation $H = -\Sigma [(p_i) \times \ln(p_i)]$, with p_i being the proportion of each species.

Data visualization tools

Data visualization was performed in the R environment, using the open source data visualization framework RawGraphs. Aligned TRB CDR3 amino acid (aa) sequences were visualized using WebLogo. CDR3 aa positions are shown according to the IMGT numbering for the V domain.[24]

RESULTS

The *TRB* gene repertoire of patients with T-LGL proliferations is largely (oligo)clonally restricted

We sequenced 65 samples from 27 patients with T-LGL proliferations and 22 controls. A general overview on the acquisition of productive *TRBV-TRBD-TRBJ* gene rearrangement sequences and clonotypes is presented in Suppl. Table S1.

Both patients with T-LGL proliferations and healthy controls displayed skewing of the *TRBV* gene repertoire (Suppl. Table S2A). However, cross-comparisons of the respective repertoires revealed 13 differentially used *TRBV* genes (Suppl. Table S2A; $P < 0.05$). Significant differences were also observed between T-LGL patients and healthy controls regarding *TRBJ* genes (Suppl. Table S2B). Regarding *TRBD* genes, the *TRBD1* gene predominated over the *TRBD2* gene in both sample groups (Suppl. Table S2C).

Deep sequencing of the *TRB* gene repertoire allowed us to assess the clonal composition and the clonality of all investigated T-LGL cases. We observed that the immunodominant clonotype had a median frequency of 19.3% (0.4%–52.0%) in T-LGL proliferations versus 6.9% (1.3%–21.5%) in healthy controls (Figure 1A; $P < 0.001$). The median cumulative frequency of the 10 most expanded clonotypes per sample in T-LGL patients accounted for 40.2% (3.5%–64.3%) of the total *TRB* gene repertoire, thus highlighting a monoclonal to oligoclonal repertoire. In contrast, healthy controls were characterized by an oligoclonal to polyclonal profile with a much lower median cumulative frequency of the 10 most expanded clonotypes/sample of 23.7% (4.5%–37.6%) (Figure 1B; $P < 0.01$). Both the immunodominant and the top 10 clonotypes differed greatly in size between T-LGL patients and healthy controls. These repertoire differences were further quantified using the Shannon index, which is used to mathematically calculate the diversity within the *TRB* gene repertoire.[25,26] The median Shannon diversity score of patients with T-LGL proliferations was 6.3, whereas in healthy controls it was 7.8. (Figure 1C; $P < 0.01$).

The *TRB* (sub)clonal architecture of T-LGL proliferations is context-dependent

We then examined whether the *TRB* gene repertoire of patients with T-LGL proliferations might be context-dependent by performing comparative analyses on samples derived from patients with various comorbidities and/or specific clinic-biological features.

Shannon diversity scores of T-LGL patients with neutropenia ($P < 0.05$), *STAT3* mutations ($P < 0.001$), and associated malignancies ($P < 0.001$) were all lower compared with healthy controls, but did not differ from T-LGL counterparts without these characteristics (Figure 2A). Further in-depth analysis of *TRBV* gene usage revealed no preference for certain *TRBV* genes in the groups with neutropenia, *STAT3* mutations, or patients with T-LGL proliferations and associated malignancies either, as compared with the other T-LGL cases.

Next, we assessed the clonality profiles between the different patient groups. Because there was considerable overlap between patients with neutropenia or *STAT3* mutations, these patient categories were grouped in this analysis. Notably, while virtually all *STAT3* mutant/neutropenic T-LGL cases exhibited pronounced monoclonal expansions, the profile of patients with associated malignancies was frequently oligoclonal (Figure 2B). The median frequency of the immunodominant clonotype in neutropenic and/*STAT3* ^{mut} T-LGL cases was 25.6% of the *TRB* gene repertoire (0.6%–52.0%) versus 15.5% (8.2%–21.6%) in T-LGL cases with associated malignancies (Figure 2C; $P = 0.26$). Of note, in this latter group, the top 2–5 clonotypes (ie, top 5 clonotypes minus the immunodominant one) represented a median of 28.6% (6.1%–34.9%) of the total repertoire, whereas in the former group (ie, neutropenic/*STAT3*^{mut}), a median value of only 6.1% (1.6%–26.2%) was observed (Figure 2C; $P < 0.001$), strengthening the argument that the subclonal architecture of T-LGL proliferations is context-dependent.

In the Dutch part of our cohort for which HLA data were available, no clear restrictions were observed toward a certain HLA allele or haplotype in neutropenic patients or patients with coexisting malignancies (Table 2). That said, we did observe that 71% of T-LGL patients bearing *STAT3* mutations (5/7 patients) used the HLA-B15 allele (odds ratio = 21.42; $P = 0.04$); however, the small size of the cohort hinders more definitive conclusions at this point.

Temporal dynamics of the (sub)clonal *TRB* gene repertoire architecture in T-LGL proliferations

Using NGS, we assessed the clonal dynamics of the *TRB* gene repertoire over time (median of 4 time points; range, 2–6) in representative cases of the various groups of T-LGL proliferations in our cohort.

In patients with *STAT3*-mutated T-LGL proliferations, clonal drift of smaller clonotypes was observed (Table 1; Figure 3A). Such clonal drift was also seen in neutropenic T-LGL patients, as exemplified by the case of patient LGL11 (Table 1; Figure 3B).

Regarding T-LGL cases with coexisting clonal conditions, we evaluated multiple time points in patient LGL7 (Table 1) bearing a T-LGL proliferation concomitant with a monoclonal gammopathy of undetermined significance. While the overall shift of the subclonal composition of the *TRB* gene repertoire over the course of 8 years was relatively modest, combined analysis of M-protein levels and *TRB* gene repertoire sequencing provided evidence of correlations between TRB repertoire dynamics and M-protein fluctuations (Figure 3C).

Another intriguing scenario concerns the development of a T-LGL proliferation in the context of allo-HSCT performed in a patient with Ph$^+$ acute lymphoblastic leukemia (LGL19; Table 1), where we observed significant clonal drift temporally connected with various incidents posttransplantation (including Epstein-Barr virus reactivation, neutropenia, and episodes of autoimmune hemolytic anemia), while also documenting expansion of clonotypes present in the repertoire of the donor (Figure 3D).

Finally, we studied a father and a son presenting with cytopenias and splenomegaly in a context of T-LGL proliferation; the clinical features and Sanger-based immunoprofiling of these cases have been reported previously.[27]

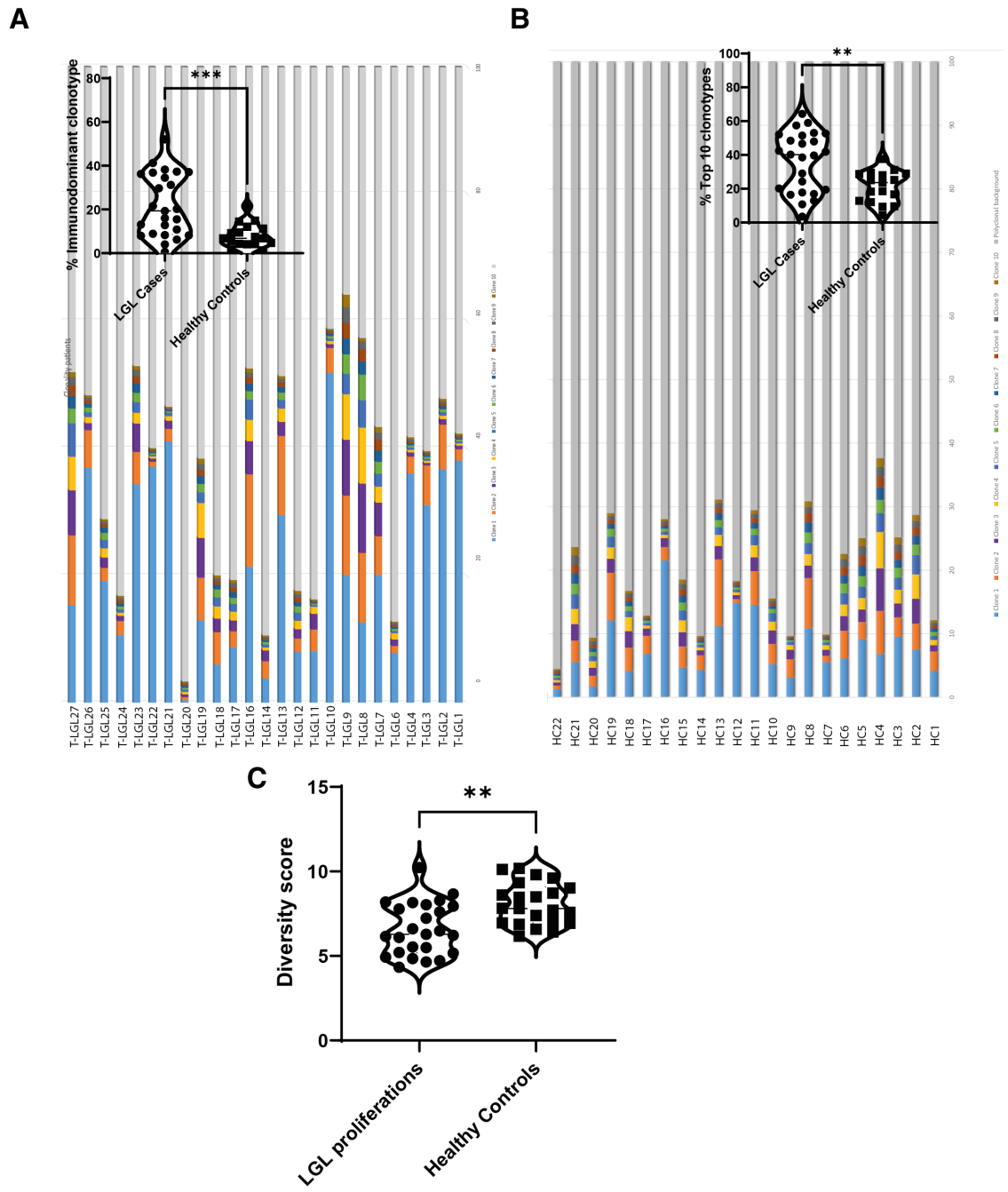


Figure 1. *TRB* gene repertoire diversity in patients with T-LGL lymphoproliferations. (A) Clonotype frequencies of patients with T-LGL lymphoproliferations and % of immunodominant clones. (B) Clonotype frequencies of healthy controls and % of top 10 clonotypes (B). Shannon diversity scores of patients with T-LGL lymphoproliferations and healthy controls. (C) Graphs indicate the mean with SD. Statistical significance was tested using the Mann Whitney *U* test. Level of significance indicated in the plots: ***$P < 0.001$; ****$P < 0.0001$. T-LGL = T cell large granular lymphocyte; TRB = T-cell receptor beta.

The cumulative frequency of the 10 most expanded clonotypes in the diagnostic sample of the father was 20.2% with 2 dominant clonotypes at relative frequencies of 6.2% and 5.1%. In the son, the 10 most expanded clonotypes of his diagnostic samples accounted for almost 19.5% of the total repertoire, rendering it oligoclonal with a dominant clone at a relative frequency of 8.9%. After 5 years, the clonality pattern remained stable; however, expansion of multiple clonotypes was observed. In fact, clonal drift was evident in the son with expansion of certain minor clonotypes over time, as exemplified by the clonotype TRBV29-1 – CSASTGDRSGANVLTF, which started with a relative frequency of 1% at the time of diagnosis and eventually expanded to 6% of the repertoire, representing the second immunodominant clonotype of that particular follow-up sample.

Various degrees of connectivity for the dominant clonotype of patients with T-LGL lymphoproliferations

Seeking to obtain a comprehensive view of the subclonal architecture of the TRB repertoire in the cases under study, next we focused on the related clonotypes to the most immunodominant one. The relation between the immunodominant clonotype and the highly similar clonotypes was visualized as

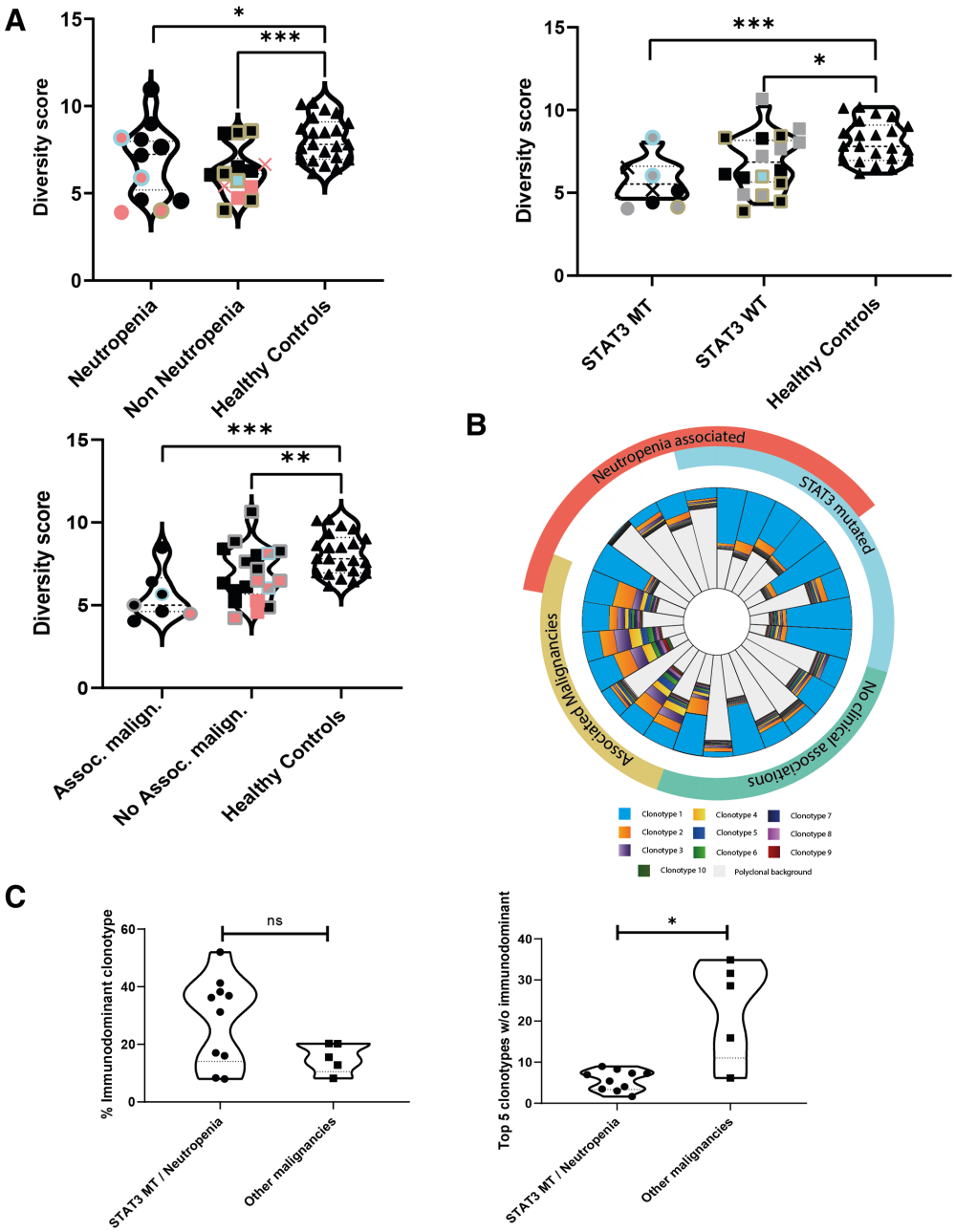


Figure 2. Context-dependent *TRB* gene repertoire of patients with T-LGL lymphoproliferations. (A) Shannon diversity index of subgroups of patients with T-LGL lymphoproliferations (red dots indicate *STAT3*-mutated cases). (B) Clonotype frequencies of neutropenic patients with T-LGL lymphoproliferations, patients with T-LGL proliferations harboring *STAT3* mutations and patients with T-LGL lymphoproliferations with associated malignancies. Comparison of % immunodominant clonotypes and top 2–5 clonotypes of different T-LGL subgroups. (C) Graphs indicate the mean with SD. Statistical significance was tested using the Mann Whitney *U* test. Level of significance indicated in the plots: *$P < 0.05$; **$P < 0.001$. **Patients with *STAT3* mutations are depicted in red (*STAT3* mutated patients without clinical associations are indicated with an X), neutropenic patients are depicted in grey, patients with associated malignancies are depicted in gold, and patients with autoimmune phenomena (especially RA) are depicted in blue. T-LGL = T cell large granular lymphocyte; TRB = T-cell receptor beta.

connectivity graphs where CDR3 aa sequences differing in a particular position were connected with a line. The frequency of the most immunodominant clonotype was re-estimated summing up the frequencies of the highly similar clonotypes. Based on that metric, we observed various degrees of connectivity, ranging from low-level, where the frequency of the dominant clonotype including the highly similar clonotypes was slightly changed, to high-level, where the change observed in the relative frequency of the dominant clonotype was significant (Figure 4A-D). Noteworthy was the pattern in case LGL7 over time, where the connectivity observed for the dominant clonotype was particularly high with a median change in its relative frequency of 10.1% (range, 5.7%–10.9%) (Figure 4E).

Public clonotypes in the repertoire of T-LGL lymphoproliferations

Combinatorial pattern discovery analysis was performed using the TEIRESIAS algorithm and the clonotypes assigned to the clusters formed were compared to the VDJdb,[20] which allows making predictions regarding the possible antigenic specificity of the clustered clonotypes.

Table 2

HLA-A, HLA-B, and HLA-C Profiles in Patients With T-LGL Lymphoproliferations

Patient	HLA A	HLA B	HLA C
LGL1	A01 A26	B38 B44	C05 C12
LGL2	A02	B15 B35	C03 C04
LGL3	A01	B15 B39	C03 C07
LGL4	A03 A32	B41 B55	C03 C17
LGL5	A01 A11	B27 B35	C02 C04
LGL6	A01 A02	B15 B37	C01 C06
LGL7	A01 A02	B08 B44	C07
LGL8	A01	B08	C07
LGL9	A02 A68	B38 B51	C12 C15
LGL10	A02 A24	B15 B27	C02 C03
LGL11	A01 A24	B18 B51	C07 C16
LGL12	A02 A29	B44 B51	C05 C14
LGL13	A02 A03	B07 B15	C03 C07
LGL14	A02	B08	C07
LGL15	A01 A02	B08 B40	C03 C07
LGL16	A30 A68	B39 B44	C07 C12

T-LGL = T cell large granular lymphocyte.

Overall, 3675 of 9073 (40.5%) of all clonotypes with a relative frequency ≥0.1%, were assigned to 1436 distinct clusters. In many cases, the existence of multiple shared patterns within TRB CDR3 sequences led to their concurrent assignment in clusters characterized by more broadly shared sequence patterns, hence, greater individual cluster size. At the end of the process, 1250 clusters were formed including clonotypes that could not be further clustered: the number of cases in each of these final clusters ranged from 2 to 23.

Two distinct types of clusters were identified, characterized by usage of the same or diverse *TRBV* genes, respectively. In both cluster types, the overall TRB CDR3 similarity was very high, that is, most positions in the TRB CDR3 region were extremely, if not entirely, conserved, while only few positions displayed variability (Figure 5).

Finally, the comparison of the clustered clonotypes against the VDJdb database highlighted a potential antigenic specificity for only a minor fraction of clonotypes from the T-LGL dataset (Figure 5).

Cross-entity comparisons highlight the disease-biased nature of the *TRB* gene repertoire in T-LGL lymphoproliferations

We performed cross-entity comparisons between the dataset of expanded clonotypes from the present cohort of patients with T-LGL lymphoproliferations (n = 191,372), a published study of patients with T-LGL leukemia (n = 486,540),[21] and a dataset of 2,079,944 unique TRB clonotypes from various entities. When comparing the 2 T-LGL datasets, 1457 of 677,912 (0.21%) clonotypes were found to be uniquely shared between cases with T-LGL lymphoproliferation from our present series and that of Kerr et al (Suppl. Figure S1A). Within each individual series, the incidence of clonotypes shared by at least 2 cases was overall similar: 7.94% (38,660/486,540) in the Kerr study versus 7.15% (13,697/191,372) in the present study. Furthermore, we used TEIRESIAS algorithm for the combined datasets and we additionally identified 2 high-frequency clusters of shared TRB CDR3 aa sequence motifs: the first connected a case from the Kerr study and case LGL23 from the present cohort, while the second connected 2 cases from the Kerr study. Next, in order to assess the relatedness of these connected clonotypes with other clonotypes comprising the LGL repertoire, we used the CDR3 amino acid sequences of these 2 clusters as baits and searched for CDR3s of the same length within the complete repertoires of all samples of the present cohort that differ in a single amino acid. This led to the identification of a number of additional low-frequent clusters with various degrees of connectivity, further implying shared immunogenetic signatures within different cases with T-LGL lymphoproliferations (Suppl. Figure S2).

Cross-entity comparisons between the 2 datasets of T-LGL lymphoproliferations versus the datasets from other entities revealed 40,741 of 677,912 (6%) shared clonotypes. The largest group included 16,885 of 40,741clonotypes (41.5%) shared between T-LGL lymphoproliferations and healthy controls; closely followed by a group of 11,574 of 40,741 clonotypes (28.6%) shared between T-LGL lymphoproliferations and otherwise healthy individuals with MBL (Suppl. Figure S1B). Relevant to mention, 1587 of 40,741 (3.9%) of these clonotypes have been previously characterized to be specific for viral epitopes (Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus 1, hepatitis C virus, dengue virus, influenza A, Molluscum contagiosum virus, Herpes simplex virus, and human T-lymphotropic virus).

DISCUSSION

We profiled by NGS a cohort of 27 well-established patients with T-LGL proliferations and a publicly available dataset by Kerr et al and aimed at identifying the subclonal architecture of the *TRB* gene repertoire. Moreover, we searched for associations between *TRB* gene repertoire patterns and clinical manifestations, with the ultimate objective of discriminating between T-LGL proliferations developing in different clinical contexts and/or displaying distinct clinical presentation. Clonotype connectivity analysis and combinatorial pattern discovery analysis were used, depicting the disease-biased nature of the *TRB* gene repertoire in T-LGL lymphoproliferations.

Patients with expanded T-LGL cells can remain asymptomatic for prolonged periods of time. Within this time frame, T-LGL cells may eventually shift toward monoclonality and this can be linked to and/or accompanied by the presence of genomic aberrations and/or the emergence of clinical manifestations.[11] This scenario is supported by our present findings, where a clearly monoclonal repertoire was seen exclusively in neutropenic patients and patients carrying *STAT3* mutations. Thus, neutropenic patients typically present at the far end of the spectrum of T-LGL lymphoproliferations; arguably, these larger monoclonal populations secrete higher amounts of FAS-ligand (FASL), resulting in FAS-mediated apoptosis of neutrophils.[28] Nonetheless, patients may develop severe neutropenia without profound (mono)clonal expansions of T-LGL cells due to mechanisms not yet characterized.

Overactivation of STAT3 has been implicated as a central hub in T-LGL lymphoproliferations, driving them toward a more malignant phenotype.[29] Unsurprisingly, therefore, *STAT3*-mutated patients harbor large monoclonal populations, as proliferation and apoptosis resistance is driven by constitutive STAT3 activation.[30] Moreover, T-LGL lymphoproliferations with *STAT3* mutations display more clinical symptoms as well. Neutropenia cooccurs in a large fraction of the *STAT3*-mutated patients, which might be explained by the fact that *STAT3*-mutated T-LGL cells are highly active, thus secreting higher amounts of FASL and proinflammatory cytokines that would eventually lead to symptoms such as neutropenia. Taken together, it seems that the larger, monoclonal T-LGL lymphoproliferations establish an environment in which neutrophils go into apoptosis, thus representing the more malignant T-LGL variant.

Both solid and hematological tumors can be immunogenic, although the adaptive immune system is mostly hindered from mounting an effective response and clearing the tumor due to multiple, frequently coexisting mechanisms of immune escape operating in individual patients.[31] Considering the above, it is not

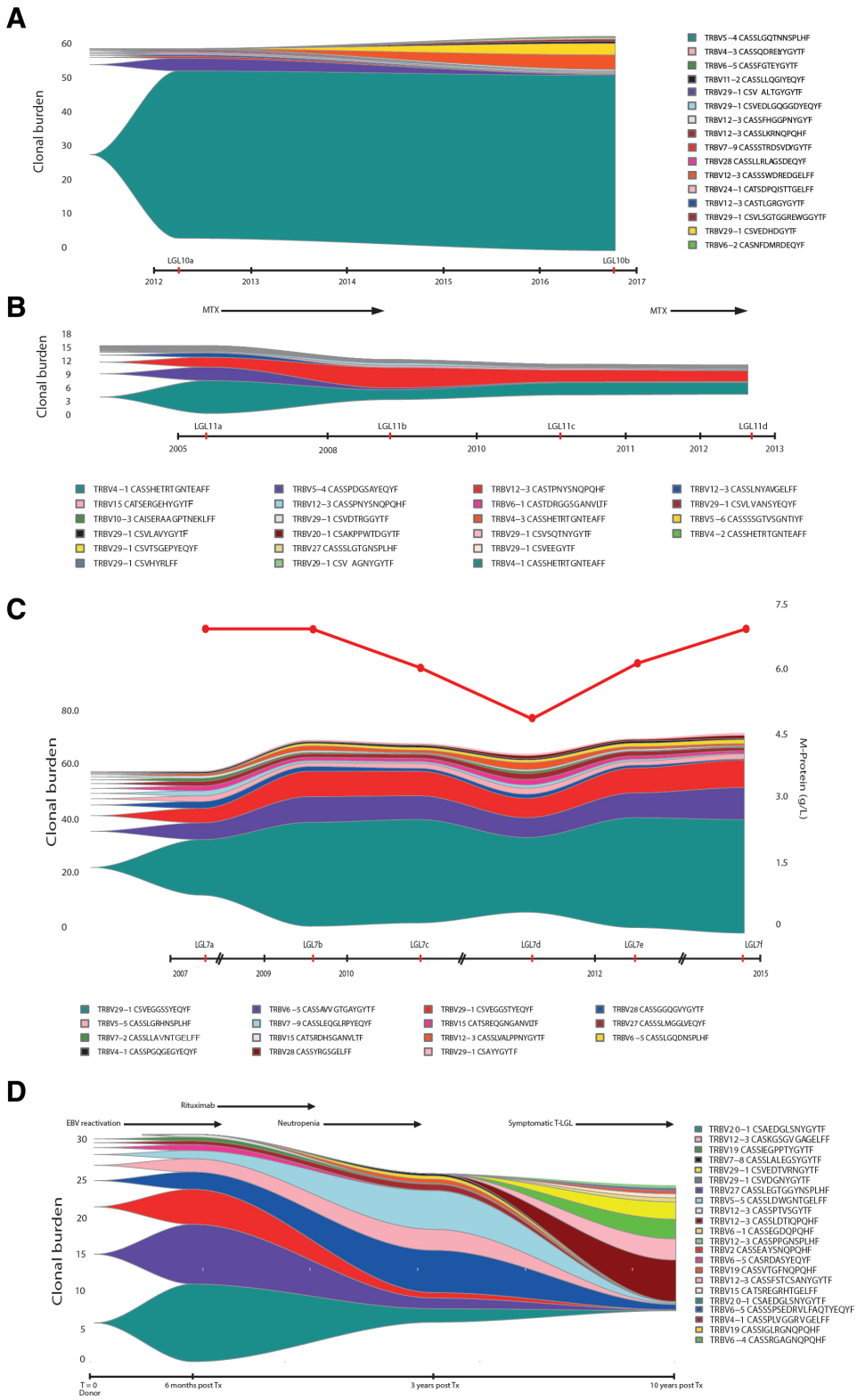
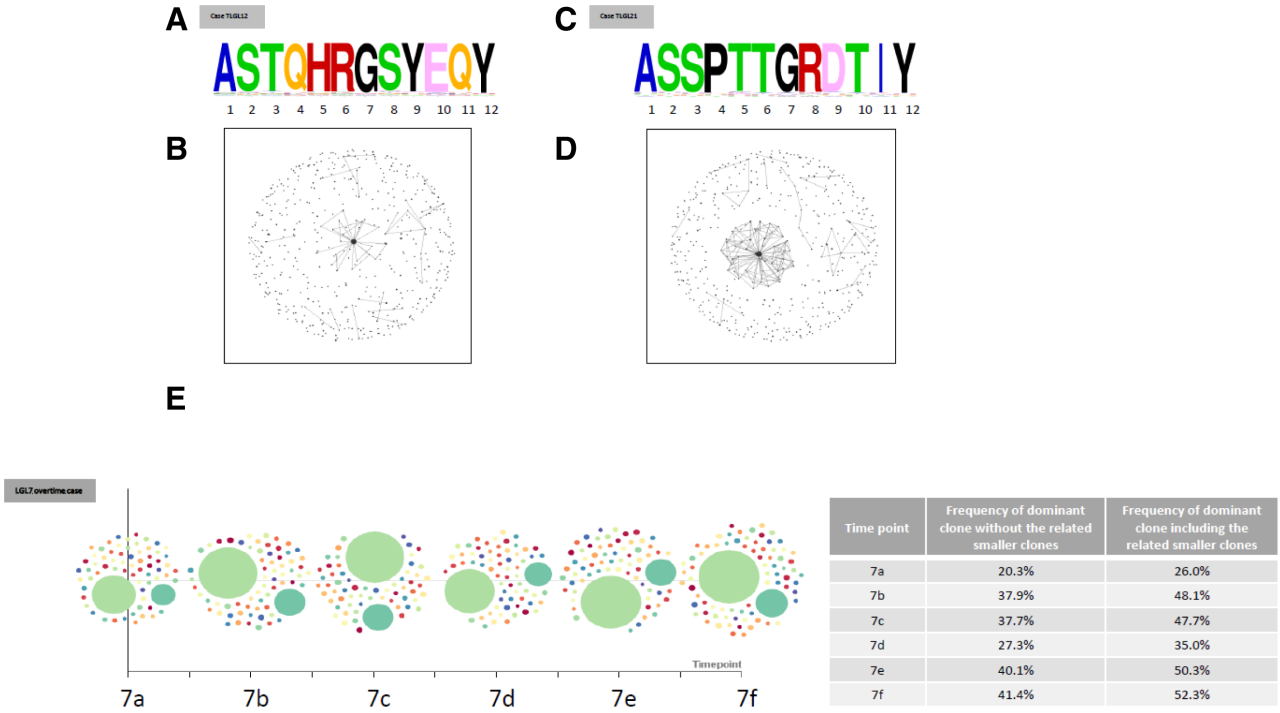


Figure 3. Patients with T-LGL lymphoproliferations display a context-dependent (sub)clonal *TRB* gene repertoire architecture that may shift over time. A. Longitudinal analysis of the subclonal *TRB* gene architecture of a patient showing a *STAT3*-mutated T-LGL leukemia. (B) Clonal dynamics of the *TRB* gene repertoire of a neutropenic patient with T-LGL leukemia under therapy. (C) Complex interplay between the *TRB* gene repertoire and M-protein levels in a case with T-LGL lymphoproliferation and an associated plasma cell malignancy. (D) Dynamics of the *TRB* gene repertoire in a patient developing a T-LGL lymphoproliferation post allo-HSCT. Allo-HSCT = allogeneic hematopoietic stem cell transplantation; T-LGL = T cell large granular lymphocyte; TRB = T-cell receptor beta.

paradoxical that a subgroup of patients with T-LGL lymphoproliferations and associated malignancies in our study displayed clear oligoclonal expansions. Similar clonality patterns have been disclosed with NGS in tumor infiltrating lymphocytes and circulating T cells of patients with both solid tumors[32] and hematological malignancies.[18,33] Hence, the presence of oligoclonality supports



Time point	Frequency of dominant clone without the related smaller clones	Frequency of dominant clone including the related smaller clones
7a	20.3%	26.0%
7b	37.9%	48.1%
7c	37.7%	47.7%
7d	27.3%	35.0%
7e	40.1%	50.3%
7f	41.4%	52.3%

Figure 4. Connectivity networks of the dominant clonotype of patients with T-LGL lymphoproliferations. (A and B) Graphs depicting the connectivity networks formed between the dominant clonotype and other clonotypes of the same length of 2 representative cases with T-LGL lymphoproliferations taking into account the highly similar clonotypes present in each sample. The sequence logo represents the total repertoire of highly similar clonotypes identified. The sequence logo of the CDR3 region of one representative case and the graph depicting low-level connectivity of the dominant clonotype. (C and D) The sequence logo of the CDR3 region of one representative case and the graph depicting high-level connectivity of the dominant clonotype. The large circle depicts the dominant clonotype of the sample and only the CDR3s with one difference are connected. (E) Overtime kinetics and clonal dynamics of highly similar clonotypes in a representative case. T-LGL = T cell large granular lymphocyte.

that T-LGL lymphoproliferations might emerge in response to tumor-associated antigens; it remains to be elucidated whether these T-LGL cells participate in immune surveillance but also how they might be functionally debilitated. Collectively, our data strongly suggest that, in the context of other malignancies, T-LGL lymphoproliferations are more likely a consequence, acting in (ineffective) tumor surveillance. That said, it cannot be a priori excluded that both these B- and T-cell lymphoproliferations are acting toward common viral or autoantigens.[34]

Allo-HCT can create a microenvironment with abundant antigenic triggers for T-LGL clones to expand.[35–37] This is attested by the correlation of clinical events and longitudinal *TRB* gene repertoire analysis in a patient from our study who underwent allo-HSCT and developed clonally expanded T-LGL cells, gradually evolving into overt T-LGL leukemia. This highlights the thin line that segregates reactive T-LGL lymphoproliferations from their leukemic counterparts, while raising the question why the full blown T-LGL leukemia occurred so many years posttransplantation. Arguably, this outcome could reflect a form of profound immune dysregulation or very late onset chronic graft versus host disease, with the T-LGL cells being hyper-reactive to an alloantigen or autoantigen due to failing thymic selection posttransplantation.[38]

Increased sequence identity of different TR CDR3 supports affinity for the same antigens and common evolutionary forces, without, however, excluding the possibility that distinct CDR3 could share common specificity.[39] On the evidence presented herein, the case for (auto)antigenic stimulation is very strong in patients with T-LGL lymphoproliferations, a claim supported by the clonotype connectivity analysis, which revealed clusters of highly related clonotypes coexisting in the same patient and, moreover, displaying over time drift. An observation that was also described by Huuhtanen and colleagues,[40] who demonstrated through single-cell TCR sequencing considerable overlap between the nonleukemic and leukemic part of the repertoire in patients with T-LGL leukemia, with 72% of the leukemic T-LGL clonotypes sharing TCR similarities with their nonleukemic repertoire. This phenomenon of repertoire skewing and TCR similarity within the nonleukemic repertoire was also observed in patients with CD4$^+$ T-LGL leukemia.[41] Along these lines, cross-entity comparisons highlighted the uniqueness of the *TRB* gene repertoire in patients with T-LGL lymphoproliferations, given that only a minor fraction of the total unique clonotypes was found to be shared with other entities, even those presenting with neutropenia (eg, CIN or BEN). This conclusion is also supported by the results from the predicted specificity analysis, where the clonotypes shared between T-LGL lymphoproliferations and other entities were postulated to be specific against viruses. Along these lines, one could speculate that interactions with exogenous antigens and/or auto/neoantigens arising in the context of viral infections (but also cancer or autoimmune disorders) could provide the initial antigenic drive for CD8$^+$ T cells, kicking off the process that eventually leads to the emergence of T-LGL lymphoproliferations. Considering the fact that Huuhtanen and Bhattacharya demonstrated with single-cell sequencing that repertoire skewing is present within the nonleukemic repertoire and, more importantly, that the nonleukemic repertoire also shows TCR similarity with the leukemic repertoire, it seems very plausible that antigenic stimulation provides the initial drive for T-LGL cells to expand.[40,41] In future studies, we will try to solidify this hypothesis by sequencing the repertoires of sorted T-LGL cells and their healthy CD8$^+$ counterparts within the same patient, as it cannot be a priori excluded that some effects are observed by presence of a public repertoire or the non-LGL T-cell population.

In summary, we report that the *TRB* gene repertoire of patients with T-LGL lymphoproliferations is extremely

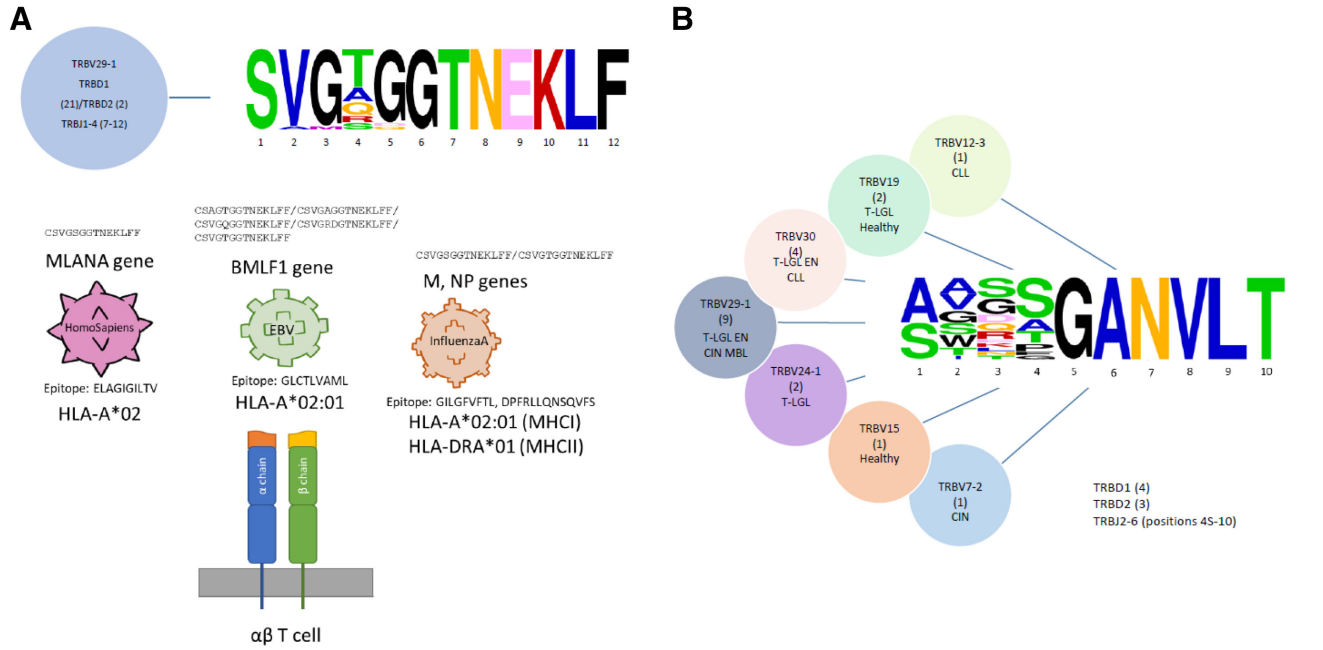


Figure 5. Clusters of cases with restricted TRB CDR3. Two representative clusters are depicted including a sequence logo of the CDR3 regions that clustered together, the *TRB* gene usage and the presumed antigen specificity when a clonotype of those clustered was also found in the VDJdb. One representative cluster including 23 clonotypes with relative frequency ranging between 0.1% and 5.6% characterized by homogeneity regarding the *TRBV* gene usage. The clustered clonotypes derived from 18 different cases (T-LGL, n = 6; EN, n = 1; CIN, n = 3; CLL, n = 2; MBL, n = 4; healthy, n = 2). The clustered clonotypes were identified in the VDJdb and different antigenic specificities can be speculated based on the literature (A). One representative cluster including 20 clonotypes (reactive frequency, 0.1%–3.7%) with heterogeneity regarding the *TRBV* genes used. The clustered clonotypes derived from 17 different cases (T-LGL, n = 7; EN, n = 2; CIN, n = 2; CLL, n = 2; MBL, n = 2; healthy, n = 2). No hits with the VDJdb were found. In brackets the number of clonotypes using the particular gene is indicated (B). CIN = chronic idiopathic neutropenia; CLL = chronic lymphocytic leukemia; EN = benign ethnic neutropenia; MBL = monoclonal B lymphocytosis; T-LGL = T cell large granular lymphocyte; TRB = T-cell receptor beta.

context-dependent, displaying distinct clonality patterns in different disease contexts. We also illustrate that there is a thin line between malignant T-LGL cells causing symptoms such as neutropenia and reactive T-LGL lymphoproliferations and that the diagnosis of T-LGL leukemia has to be established with great caution, as T-LGL lymphoproliferations may or may not give rise to clear symptoms. In addition, our longitudinal analyses revealed profound temporal clonal dynamics, raising the possibility that T-LGL lymphoproliferations might represent an epiphenomenon when co-occurring with other malignancies, being reactive toward, for example, tumor antigens.

AUTHOR CONTRIBUTIONS

JLJCA, AC, KS, and AWL designed the study. JLJCA and EV performed the experiments. EV, KG, and AA performed the bioinformatics analyses. JLJCA, EV, KG, NP, PMK, AC, KS, and AWL analyzed data. JLJCA, EV, KG, AC, KS, and AWL interpreted results and wrote the article. All authors approved the article. AWL and KS were responsible for financial support. AP provided well-defined patient samples.

DISCLOSURES

The authors have no conflicts of interest to disclose.

SOURCES OF FUNDING

The authors declare no sources of funding related to the work described in his manuscript.

REFERENCES

1. Lamy T, Moignet A, Loughran TP Jr. LGL leukemia: from pathogenesis to treatment. . *Blood*. 2017;129:1082–1094.
2. Mohan SR, Maciejewski JP. Diagnosis and therapy of neutropenia in large granular lymphocyte leukemia. *Curr Opin Hematol*. 2009;16:27–34.
3. Langerak AW, Sandberg Y, van Dongen JJM. Spectrum of T-large granular lymphocyte lymphoproliferations: ranging from expanded activated effector T cells to T-cell leukaemia. *Br J Haematol*. 2003;123:561–562.
4. Bockorny B, Dasanu CA. Autoimmune manifestations in large granular lymphocyte leukemia. *Clin Lymphoma Myeloma Leuk*. 2012;12:400–405.
5. Lamy T, Loughran TP. How I treat LGL leukemia. *Blood*. 2011;117:2764–2774.
6. Papadaki T, Stamatopoulos K, Kosmas C, et al. Clonal T-large granular lymphocyte proliferations associated with clonal B cell lymphoproliferative disorders: report of eight cases. *Leukemia*. 2002;16:2167–2169.
7. Papalexandri A, Karypidou M, Stalika E, et al. Skewing of the T-cell receptor repertoire in patients receiving rituximab after allogeneic hematopoietic cell transplantation: what lies beneath? *Leuk Lymphoma*. 2019;60:1685–1692.
8. Zhang D, Loughran TP Jr. Large granular lymphocytic leukemia: molecular pathogenesis, clinical manifestations, and treatment. *Hematology Am Soc Hematol Educ Program*. 2012;2012:652–659.
9. Koskela HLM, Eldfors S, Ellonen P, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med*. 2012;366:1905–1913.
10. Rajala HLM, Porkka K, Maciejewski JP, et al. Uncovering the pathogenesis of large granular lymphocytic leukemia—novel STAT3 and STAT5b mutations. *Ann Med*. 2014;46:114–122.
11. Teramo A, Barila G, Calabretto G, et al. STAT3 mutation impacts biological and clinical features of T-LGL leukemia. *Oncotarget*. 2017;8:61876–61889.
12. Sandberg Y, Kallemeijn MJ, Dik WA, et al. Lack of common TCRA and TCRB clonotypes in CD8+/TCRαβ+ T-cell large granular lymphocyte leukemia: a review on the role of antigenic selection in the immunopathogenesis of CD8+ T-LGL. *Blood Cancer J*. 2014;4:e172–e172.
13. 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;118:4384–4393.

14. O'Keefe CL, Plasilova M, Wlodarski M, et al. Molecular analysis of TCR clonotypes in LGL: a clonal model for polyclonal responses. *J Immunol*. 2004;172:1960–1969.
15. Langerak AW, Assmann JLJC. Large granular lymphocyte cells and immune dysregulation diseases–the chicken or the egg? *Haematologica*. 2018;103:193–194.
16. Van Dongen JJM, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17:2257–2317.
17. Vlachonikola E, Vardi A, Stamatopoulos K, Hadzidimitriou A. High-throughput sequencing of the T-cell receptor beta chain gene repertoire in chronic lymphocytic leukemia. *Methods Mol Biol*. 2019;1881:355–363.
18. Vardi A, Vlachonikola E, Karypidou M, et al. Restrictions in the T-cell repertoire of chronic lymphocytic leukemia: high-throughput immunoprofiling supports selection by shared antigenic elements. *Leukemia*. 2017;31:1555–1561.
19. Bagaev DV, Vroomans RM, Samir J, et al. VDJdb in 2019: database extension, new analysis infrastructure and a T-cell receptor motif compendium. *Nucleic Acids Res*. 2020;48(D1):D1057–D1062.
20. Shugay M, Bagaev DV, Zvyagin IV, et al. VDJdb: a curated database of T-cell receptor sequences with known antigen specificity. *Nucleic Acids Res*. 2018;46(D1):D419–D427.
21. Kerr CM, Clemente MJ, Chomczynski PW, et al. Subclonal STAT3 mutations solidify clonal dominance. *Blood Adv*. 2019;3:917–921.
22. Rigoutsos I, Floratos A. Combinatorial pattern discovery in biological sequences: the TEIRESIAS algorithm. *Bioinformatics (Oxford, England)*. 1998;14:55–67.
23. Darzentas N, Hadzidimitriou A, Murray F, et al. A different ontogenesis for chronic lymphocytic leukemia cases carrying stereotyped antigen receptors: molecular and computational evidence. *Leukemia*. 2010;24:125–132.
24. Lefranc M-P. IMGT, the international ImMunoGeneTics database®. *Nucleic Acids Res*. 2003;31:307–310.
25. Pielou EC. Shannon's formula as a measure of specific diversity: its use and misuse. *Am Naturalist*. 1966;100:463–465.
26. Stewart JJ, Lee CY, Ibrahim S, et al. A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol Immunol*. 1997;34:1067–1082.
27. Stalika E, Papalexandri A, Iskas M, et al. Familial CD3+ T large granular lymphocyte leukemia: evidence that genetic predisposition and antigen selection promote clonal cytotoxic T-cell responses. *Leuk Lymphoma*. 2014;55:1781–1787.
28. Liu JH, Wei S, Lamy T, et al. Chronic neutropenia mediated by fas ligand. *Blood*. 2000;95:3219–3222.
29. Steinway SN, LeBlanc F, Loughran TP Jr. The pathogenesis and treatment of large granular lymphocyte leukemia. *Blood Rev*. 2014;28:87–94.
30. 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;107:351–362.
31. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006;90:1–50.
32. Chen Z, Zhang C, Pan Y, et al. T cell receptor β-chain repertoire analysis reveals intratumour heterogeneity of tumour-infiltrating lymphocytes in oesophageal squamous cell carcinoma. *J Pathol*. 2016;239:450–458.
33. Vlachonikola E, Vardi A, Kastritis E, et al. *Longitudinal t cell immunoprofiling of patients with relapsed and/or refractory myeloma who receive daratumumab monotherapy: A subanalysis of a phase 2 study (the rebuild study)*. Washington, DC: American Society of Hematology; 2019.
34. Wlodarski MW, O'Keefe C, Howe EC, et al. Pathologic clonal cytotoxic T-cell responses: nonrandom nature of the T-cell–receptor restriction in large granular lymphocyte leukemia. *Blood*. 2005;106:2769–2780.
35. Ram R, Ben-Bassat I, Shpilberg O, et al. The late adverse events of rituximab therapy–rare but there!. *Leuk Lymphoma*. 2009;50:1083–1095.
36. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75:555–562.
37. Zambello R, Loughran TP, Trentin L, et al. Serologic and molecular evidence for a possible pathogenetic role of viral infection in CD3-negative natural killer-type lymphoproliferative disease of granular lymphocytes. *Leukemia*. 1995;9:1207–1211.
38. Socié G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood*. 2014;124:374–384.
39. Huisman W, Hageman L, Leboux DA, et al. Public t-cell receptors (TCRs) revisited by analysis of the magnitude of identical and highly-similar TCRs in virus-specific t-cell repertoires of healthy individuals. *Front Immunol*. 2022;13:851868.
40. Huuhtanen J, Bhattacharya D, Lönnberg T, et al. Single-cell characterization of leukemic and non-leukemic immune repertoires in CD8+ T-cell large granular lymphocytic leukemia. *Nat Commun*. 2022;13:1981.
41. Bhattacharya D, Teramo A, Gasparini VR, et al. Identification of novel STAT5B mutations and characterization of TCRβ signatures in CD4+ T-cell large granular lymphocyte leukemia. *Blood Cancer J*. 2022;12:31.