T-cell receptor architecture and clonal tiding provide insight into the transformation trajectory of peripheral T-cell lymphomas

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Abstract

While T-cell lymphomas are classified as mature neoplasms, emerging evidence indicates that malignant transformation may occur at an earlier stage of T-cell maturation. In this study, we determined clonal architecture in a broad range of T-cell lymphomas. Our multidimensional profiling indicates that many of these lymphomas do in fact emerge from an immature lymphoid T-cell precursor at a maturation stage prior to V(D)J rearrangement that undergoes branching evolution. Consequently, at single-cell resolution we observed considerable clonal tiding under selective therapeutic pressure. T-cell receptor next-generation sequencing suggested a highly biased usage of TRBV20-1 gene segments as part of multiple antigen receptor rearrangements per patient. The predominance of TRBV20-1 was found across all major T-cell lymphoma subtypes analyzed. This suggested that this particular V gene – independently of complementarity-determining region 3 configuration – may represent a driver of malignant transformation. Together, our data indicate that T-cell lymphomas are derived from immature lymphoid precursors and display considerable intratumoral heterogeneity that may provide the basis for relapse and resistance in these hard-to-treat cancers.

Introduction

T-cell lymphomas are a heterogenous group of malignancies that may reside in lymph nodes, other primary or secondary lymphoid organs or even extralymphatic sites.[1-7] Treatment for this group of diseases is systemic and includes chemotherapy, antibody-drug conjugates as well as stem cell transplantation.[3,8,9] Nevertheless, the prognosis remains poor. Especially after failure of first-line treatment, only up to 25% of patients experience long-term survival.[10-13] There is an unmet clinical need to better understand the biological underpinnings of this group of diseases to establish novel avenues for precision targeting.

T-cell lymphomas are classified as mature neoplasms due to their immunophenotype which closely resembles subsets of normal mature T cells and their rearranged T-cell receptor (TCR) V(D)J genes.[14-17] The TCR rearrangement is an excellent maturation marker since TCRγ, TCRβ, and TCRα loci are sequentially rearranged from a diverse pool of V(D)J genes during various stages of intrathymic development.[18] As a result, each naïve T cell that emigrates from the thymus has a unique TCR gene rearrangement that is retained as the cell divides and differentiates. Prior to the development of advanced technical methods for TCR clonality analysis, the prevailing theory was that T-cell lymphomas are clonal diseases that originate from a single mature T cell that has undergone both positive and negative selection within the thymus.[19] However, recent advancements in sequencing have revealed that several types of T-cell lymphomas are in fact oligoclonal in nature, indicating that they originate from multiple clones with distinct TCR rearrangements, rather than a single clone.[14] This challenges the “maturity” paradigm in lymphoma development and points to a lymphoid precursor as the cell of origin. In angioimmunoblastic T-cell lymphoma (AITL) – one of the major subtypes of T-cell lymphomas and characterized by a T follicular

helper (T_{FH}) phenotype[20,21] – there is additional evidence supporting this view. In this particular subtype, branching evolution appears to start as early as the hematopoietic stem cell.[22] The genetic landscape of AITL suggests that the initial oncogenic event might consist in mutations that are typically found in clonal hematopoiesis such as *TET2* or *DNMT3A*.[20,23] These mutant hematopoietic stem cells may then have the potential to evolve to AITL as well as neoplasms of other hematopoietic lineages.[24,25] This may also explain the frequent coexistence of lymphoid and myeloid cancers in patients with AITL.[26] For other T-cell lymphomas, the transformation trajectory including the acquisition of driver mutations is much less well-defined.[27,28]

Here, we determined clonal architecture in a broad range of T-cell lymphomas and its development with or without selective therapeutic pressure to gain insight into the mechanism of treatment failure for these hard-to-treat cancers.

Methods

Details of the methods are provided in the *Online Supplementary Appendix*.

Patients' samples and tissue microarray

Lymphoma tissue was collected after informed consent as approved by the ethics committee of the University of Halle-Wittenberg (numbers 2021-074 and 2020-033). The distributions of the cases and samples are shown in Table 1 and *Online Supplementary Table S1*.

T-cell receptor immune repertoire sequencing and data analysis

The V(D)J rearranged TRB loci were amplified from genomic DNA isolated from formalin-fixed and paraffin-embedded tissue and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) with a 601-cycle paired-end run and V3-chemistry as described by Schultheiss *et al.*[29] and Simnica *et al.*[30,31] The MiXCR framework[32] v.3.0.12 was used for sequence alignment and assignment of clonotype (considered as one uniqe nucleotide complementarity-determining region 3 [CDR3] sequence). Non-productive reads and clonotypes with fewer than two reads were discarded. TCR repertoires from patients with acute coronavirus disease 2019 (COVID-19) infection were derived from Schultheiss *et al.*[33] For analysis of repertoire metrics, healthy immune repertoires were proportionally normalized to 30,000 productive reads. A more detailed description of immune repertoire data generation, processing and analyses is given in the *Online Supplementary Appendix*.

Tissue microarray staining for TRBV20-1 usage

Available formalin-fixed and paraffin-embedded tissue from our TCR-next-generation sequencing (NGS) cohort as well as additional T-cell lymphoma samples were analyzed using a tissue microarray as reported by Schümann *et al.*[34] The staining was performed on 3 μm tissue sections using TCR Vβ2-PE antibody (diluted 1:100, IM2213, Beckmann Coulter, CA, USA).[35-37] Deparaffinization and peroxidase block was performed as described by Bauer *et al.*[38]

Gene panel profiling

Profiled hotspot mutations associated with T-cell lymphomas are listed in *Online Supplementary Table S2*. Sequencing libraries were constructed using Qiaseq Targeted DNA Custom Panels (Qiagen, Hilden, Germany) and sequenced on a Illumina NextSeq 500 platform with 2 x 150 cycles at an average coverage of 52,700 reads per target region. Variant calling of unique molecular identifiers was performed using CLC Workbench (Quiagen). Mutations were considered as positive if they were found with a variant allele frequency exceeding 10% at a read depth of more than 70 reads. To filter for disease-relevant mutations, common single nucleotide polymorphisms as stated by the dbSNP were discarded, as were synonymous variants.

Cell sorting and single-cell transcriptomic profiling

To enrich lymphoma cells for single-cell analyses using the 10X Genomics platform, we sorted the malignant cells from a patient with T-cell prolymphocytic leukemia based

Table 1. Characteristics of the T-cell lymphoma cohort.

	TCR-NGS cohort	TMA cohort
N of patients (N of samples)	21 (27)	50 (68)
Sex, N		
Female	5	16
Male	16	34
Age in years, median (range)	67 (36-92)	66 (36-92)
Subtype of T-cell non-Hodgkin lymphoma, N		
Angioimmunoblastic T-cell lymphoma	12	15
PTCL, not otherwise specified	6	14
T-cell prolymphocytic leukemia	2	1
T-cell large granular lymphocytic leukemia	1	2
Anaplastic large-cell lymphoma	0	7
PTCL with T follicular helper phenotype	0	3
Sézary syndrome	0	2
Monomorphic epitheliotropic intestinal T-cell lymphoma	0	1
Mycosis fungoides	0	1
Extranodal NK/T cell lymphoma, nasal type	0	1
Post-transplant lymphoproliferative disorder	0	1
Enteropathy-associated T-cell lymphoma	0	1
Subcutaneous panniculitis-like T-cell lymphoma	0	1

TCR-NGS: T-cell receptor next-generation sequencing; TMA: tissue microarray; PTCL: peripheral T-cell lymphoma.

on their aberrant $CD4^+CD8^+$ immunophenotype and cells from an AITL case with aberrant CD3 surface expression from cryopreserved peripheral blood mononuclear cells by using anti-CD3-APC-H7 (clone SK7, BD Biosciences), anti-CD4-PacificBlue (clone RPA-T4, Biolegend) and anti-CD8-FITC (clone SK1, BD Biosciences) antibodies. Sequencing, data processing and analysis were performed as described by Schultheiss *et al.*[39]

Results

T-cell receptor profiling of T-cell lymphomas shows marked oligoclonality and overrepresentation of TRBV20-1 rearrangements

We studied T-cell lymphoma tissue (Table 1, TCR-NGS subcohort; *Online Supplementary Table S1*) by next-generation TCR sequencing to obtain insight into TCR clonality and gene usage of these cases. Most of these patients had AITL or peripheral T-cell lymphomas not otherwise specified (PTCL NOS). As a reference for TCR metrics, we used blood from 121 healthy individuals.

In order to avoid bias, we used only one sample per patient (baseline or earliest available timepoint) to study the broad TCR architecture across this range of lymphomas. In general, T-cell lymphomas showed higher TCR clonality as compared to blood from healthy individuals (Figure 1A). To visualize TCR gene usage in the repertoire, we plotted the frequency of unique V, D and J gene rearrangements on a V(D)J matrix for one healthy sample and oligoclonal and monoclonal cases of T-cell non-Hodgkin lymphoma (Figure 1B). We classified each case as oligoclonal or monoclonal based on the algorithm described in the Methods section taking into account the tumor cell infiltration of each case and the statistical frequency of biallelic rearrangements. According to this definition, 47.4% of cases were oligoclonal, while 52.6% were monoclonal (Figure 1C). Next, we performed principal component analysis to investigate V or VJ gene skewing of the lymphoma TCR repertoire towards specific receptor rearrangements. This analysis revealed a substantial skewing of lymphoma repertoires (Figure 1D). TRBV20-1 was the V gene that contributed most to this skewing (Figure 1D, right side; *Online Supplementary Figure S1*). Nevertheless, as shown in the exemplary case in panel 1B, heterogeneous rearrangements were present without evidence for a specific CDR3 sequence involved. To compare the usage of TRBV20-1, we used blood-derived T cells from healthy donors and patients with acute COVID-19 as controls. The COVID-19 samples were derived from our own previously published dataset[33] and were chosen because of the high systemic inflammatory dysregulation (e.g., excessive IL6, TNF) during acute infection which was linked to TRBV20-1 expansion in some cases.[40] This analysis showed a median TRBV20-1 usage of around 1% in the controls, while the median TRBV20-1 usage was around 40% in T-cell lymphomas (Figure 1E). When examining TCR architecture in samples obtained at initial diagnosis and those collected at disease progression, we did not observe changes in TCR repertoire metrics within our specific cohort (Figure 1F). Furthermore, TCR repertoire metrics were very similar in different lymphoma subtypes, as shown for AITL and PTCL NOS in *Online Supplementary Figure S2*.

Tissue microarray staining confirms preferential usage of TRBV20-1 in the majority of cases

To confirm this striking overrepresentation of TRBV20-1 rearrangements in lymphoma tissue from patients with T-cell lymphoma, we subjected T-cell lymphoma cases, including the majority of the TCR-NGS subcohort (Table 1), to TRBV20-1 staining using a commercial monoclonal antibody (Figure 2A). The samples of this cohort were distributed across three tissue microarrays. Consistently with our NGS results, the majority of samples showed some positivity for TRBV20-1 in the lymphoma tissue (exemplarily shown in Figure 2A). While only very few cases were homogeneously positive for TRBV20-1 throughout the tissue section (exemplarily shown in Figure 2Aa), many samples showed a patchy distribution of TRBV20-1 signals (exemplarily shown in Figure 2Ab) suggesting that TRBV20-1 rearrangements were expressed along with other rearrangements in the lymphoma. About 50% of samples showed no staining for TRBV20-1 at all (exemplarily shown in Figure 2Ac). Matching of NGS and tissue microarray staining data showed concordant results in some cases, but not in all, speaking in favor of spatial intratumoral heterogeneity. Of the four cases with strong and homogeneous positivity for TRBV20-1 on immunohistochemistry, three showed very high TRBV20-1 gene usage, taking up between 87.5% and 100% of the malignant repertoire. Samples with heterogeneous or negative staining results showed variable levels of TRBV20-1 usage (Figure 2B). In the most frequent lymphoma entities of this cohort – AITL, PTCL NOS and anaplastic large cell lymphoma – TRBV20-1-containing rearrangements were about equally prevalent (Figure 2B, right panel).

Genetic heterogeneity extends to alpha chain rearrangements and corresponds with transcriptomic heterogeneity

To better understand genetic and transcriptomic heterogeneity at single-cell resolution, we performed TCR and single-cell RNA sequencing on enriched lymphoma cells from two patients, patient 055 with T-cell prolymphocytic leukemia and patient 056 with AITL.

The integration of these lymphoma cells with T cells from two healthy individuals retrieved from Herrera *et al.*[41] (sample HC1) and resource datasets from 10X Genomics[42] suggest a clear separation between malignant and non-malignant cells (Figure 3A). The cells from the two lymphoma cases clustered together (cluster M1 and M2) although representing different lymphoma subentities (Figure 3A). The

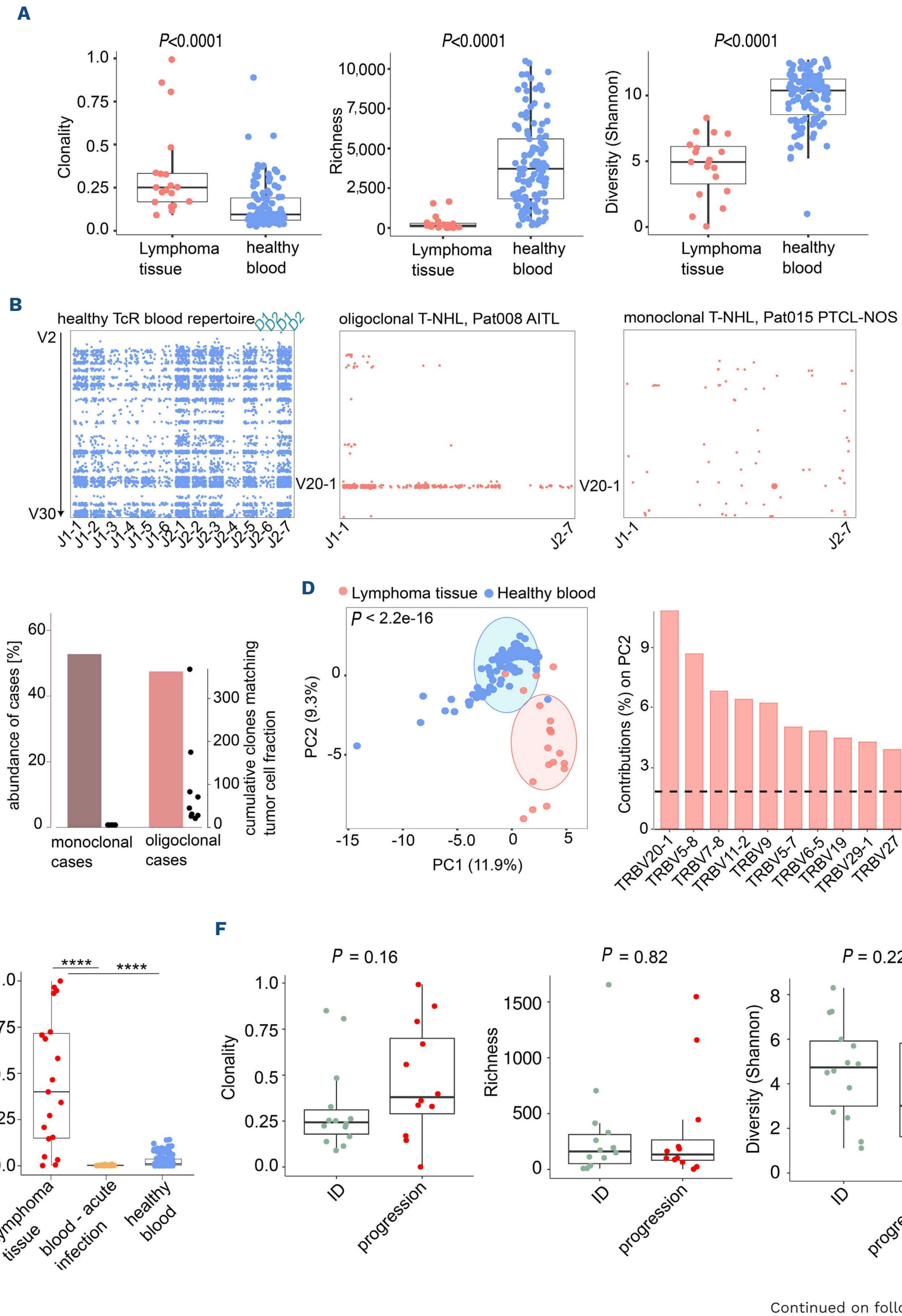

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Figure 1. Immunosequencing of T-cell receptor repertoires of T-cell lymphoma tissue. (A) T-cell receptor (TCR) repertoire metrics of healthy blood (N=121) and lymphoma tissue (N=19). For patients with multiple samples, the earliest timepoint was used. Statistics: analysis of variance (ANOVA). (B) Frequency of unique V(D)J rearrangements in selected individual repertoires. Each dot represents a unique rearranged complementarity-determining region 3 (CDR3) amino acid sequence where the radius reflects clone size in the repertoire. (C) Proportions of mono- and oligoclonal cases within the analyzed T-cell lymphoma cohort (N=19) and number of cumulative clones per repertoire matching tumor cell fraction. (D) Principal component analysis (PCA) of V gene usage in lymphoma tissue samples (red, N=19) and healthy blood samples (blue, N=121). Bars show the top ten contributing V genes responsible for group differences at principal component 2. The dashed line indicates the threshold. Statistics: multivariate ANOVA over PCA with Pillai trace. (E) Frequencies of TRBV20-1 gene usage in the T-cell lymphoma samples (N=19). TRBV20-1 frequencies in the blood of patients with acute COVID-19 infection (N=19) or healthy individuals (N=121) as controls. ****P<0.0001. (F) TCR repertoire metrics for lymphoma samples at initial diagnosis and after progression. Statistics: ANOVA. T-NHL: T-cell non-Hodgkin lymphoma; AITL: angioimmunoblastic T-cell lymphoma; PTCL-NOS: peripheral T-cell lymphoma, not otherwise specified; PC: principal component; ID: initial diagnosis.

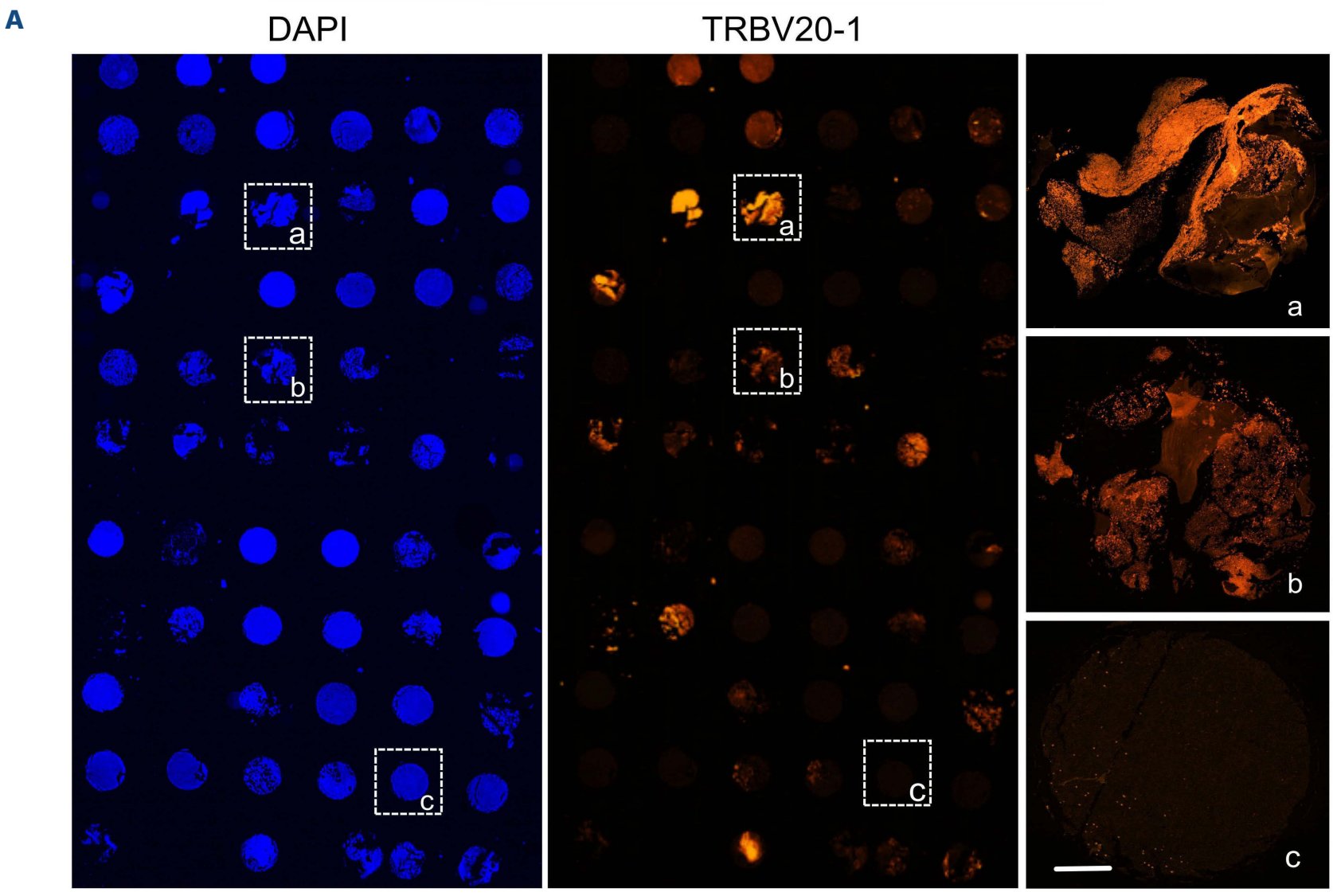

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Figure 2. Tissue microarray staining of T-cell lymphomas for TRBV20-1 expression. (A) DAPI staining was performed to localize section borders. TRBV20-1 gene signals were classified as homogeneously positive (a), distributed patchily (b) or negative (c). Magnification of the indicated examples on the right, 123x. The scale bar represents 200 µm. (B) Left panel: TRBV20-1 frequency (next-generation sequencing) and grouped total cell fluorescence for TRBV20-1. Right panel: summary of detected TRBV20-1 staining pattern per lymphoma entity. DAPI: 4′,6-diamidino-2-phenylindole; AITL: angioimmunoblastic T-cell lymphoma; PTCL, NOS: peripheral T-cell lymphoma, not otherwise specified; ALCL: anaplastic large-cell lymphoma; MEITL: monomorphic epitheliotropic intestinal T-cell lymphoma; T-LGLL: T-cell large granular lymphocytic leukemia; PTCL TFH: peripheral T-cell lymphoma with T follicular helper phenotype; NKTCL: natural killer/T-cell lymphoma; SS: Sézary syndrome; T-PLL: T-cell prolymphocytic leukemia; EATL: enteropathy-associated T-cell lymphoma; SPTCL: subcutaneous panniculitis-like T-cell lymphoma; MF: mycosis fungoides.

integrated lymphoma cells displayed moderate expression of *CCR7* and *LEF1* reminiscent of naïve-like peripheral T cells,[43] but otherwise did not exhibit a clear shared transcriptomic profile relating to a distinct functional canonical T-cell subset (*Online Supplementary Figures S3* and *S4*). Profiling for T_{FH} markers, revealed *CD4* expression in the lymphoma samples (*Online Supplementary Figure S3*) and high levels of *ICOS* and *IL21* in the AITL case (*Online Supplementary Figure S5*). Notably, downregulation of *CCR7* as well as *LEF1* activity are associated with the initiation of T_{FH} differentiation programs.[44,45] In line with the phenotype of circulating T_{FH} cells,[44] we did not detect *BCL6* expression in the majority of cells (*Online Supplementary Figure S3*). Furthermore, lymphoma cells showed expression of T-cell lymphoma-associated genes such as *GATA3* and *TOX* and were characterized by high expression of *EPHB1*, *CYP46A1* and *TLR2* (*Online Supplementary Figures S3* and *S4*). The receptor tyrosine kinase EPHB1 is linked to proliferation and metastasis in solid cancers,[46] CYP46A1 acts in cholesterol biosynthesis linked to T-cell lymphomas[47] and TLR2 is linked with T-cell activation and has been demonstrated to lower the threshold required for TCR stimulation in CD8 T cells.[48,49]

Next, we analyzed the distribution of TCR β and α rearrangements at single-cell resolution in both patients separately (Figure 3B, C). Consistently with the bulk TCR sequencing analysis, the tumor cells of patient 055 showed monoclonality for a TRBV5-1 rearrangement, but the α chain rearrangement was also monoclonal (TRAV13-1 rearrangement). In line with this, the transcriptional profile was rather homogeneous in this case. Module expression of genes regulated by MYC, signal transduction by p53, TNF signaling and NFκB signaling were uniformly high in monoclonal T cells (Figure 3B). In patient 056, single-cell sequencing showed several distinct subclones with different configurations of the β and α chain rearrangements. Two dominant β and α TCR rearrangements were combined either with each other or with other combination partners and some of the cells showed more than one rearrangement per chain in their transcriptome, suggesting expression of different TCR chimera in a single cell. Cells with a combination of the most dominant T-cell receptor β (TRBV3-1) and α (TRAV29/DV5) rearrangements showed transcriptomes that were more driven towards MYC and TGF signaling (Figure 3C) as well as MAP kinase activity, histone modification and cell cycle activity (*data not shown*). In contrast, cells with more aberrant TCR configurations with either more than one expressed rearrangement per locus or different rearrangements at the other locus were more driven towards JAK/STAT signaling and showed more overlap with non-malignant T cells (Figure 3C). This suggests that the transcriptomes expressed in this case were dictated by the respective TCR configuration (Figure 3C).

Together, these data demonstrate the broad spectrum of genetic and transcriptomic complexity in T-cell lymphomas with the TCR configuration dictating downstream cellular programs in these diseases.

Variant allele frequencies of lymphoma driver mutations and T-cell receptor clonality demonstrate the sequence of transforming events

Next, we wanted to assess the relationship between TCR clonality and lymphoma driver mutations. All cases with complete TCR sequencing and TRBV20-1 staining data were subjected to genomic profiling using a gene panel encompassing the most frequent driver alterations for T-cell lymphoma (*Online Supplementary Table S2)*. This analysis revealed a total of 43 genomic alterations in 13 of 17 cases (Figure 4). Consistent with the literature, *TET2* mutations were present in nine of 17 cases and *DNMT3A* in two of 17 cases.[50-53] Although all lymphomas were classified by an experienced reference pathologist, the reported frequent co-occurrence of *IDH2* and *RHOA* mutations in *TET2*-mutated AITL[54,55] was not reflected in our relatively small cohort. A comparison of variant allele frequencies within the same patient revealed distinct patterns: certain genetic alterations were classified as founder lesions, likely present in every lymphoma cell, while others were classified as subclonal, indicating their presence in only a subset of cells. Notably, we observed that the clonal space defined by individual (sub)clonal driver mutations generally surpassed the size of the clonal space defined by specific subclonal TCR rearrangements (Figure 4). This finding suggests that a single driver mutation serves as the foundation for the subsequent development of diverse subclones with heterogeneous TCR rearrangements in the majority of T-cell lymphoma patients.

Clonal tiding

Finally, we explored clonal tiding of T-cell lymphomas over

the course of treatment. The analysis of TCR repertoire overlap between lymphoma tissue from the same patients at different timepoints suggested substantial clonal tiding with only limited clonal overlap at disease progression. This is shown in exemplary patient 010 for whom four samples were available at different timepoints (Figure 5A). While some clones were shared between the timepoints (marked in red), a substantial number of clones did not reappear in consecutive samples. Lymphoma tissue from different patients did not show any clonal overlap, as illustrated in

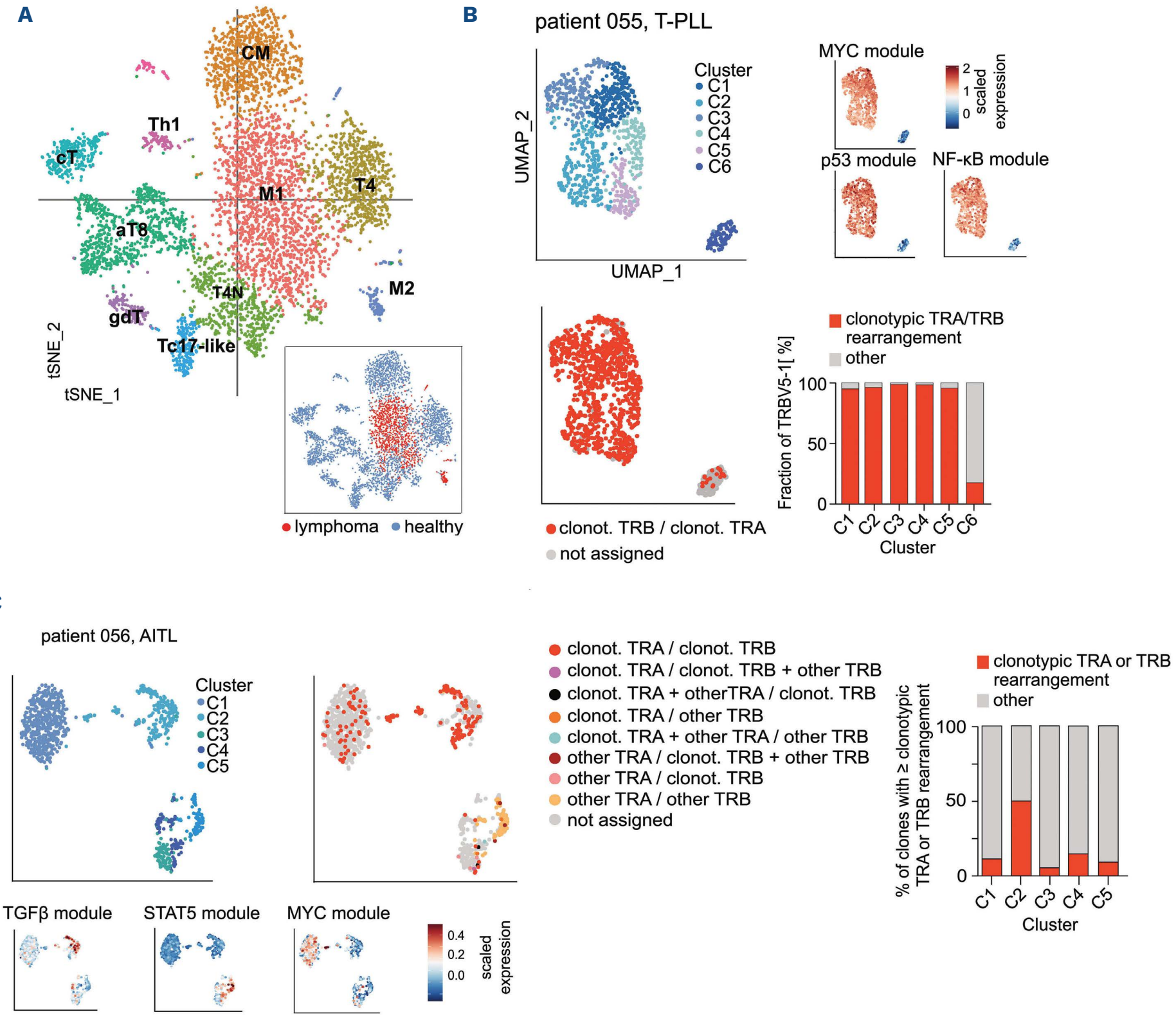


Figure 3. Single-cell analysis of sorted lymphoma cells from two patients. (A) A t-distributed stochastic neighbor embedding (t-SNE) plot of an integrated single-cell RNA dataset encompassing 985 lymphoma T cells from a patient with T prolymphocytic leukemia (patient 055), 479 lymphoma T cells from a patient with angioimmunoblastic T-cell lymphoma (patient 056) and 4,373 T cells from two healthy donors. Cells are colored by cluster or sample origin. (B) Uniform manifold approximation and projection (UMAP) plot of reclustered lymphoma cells from patient 055. Characterizing expression programs of the malignant cells shown as feature plot after scoring (Seurat *addmodulescore* function) of indicated Gene Ontology gene sets. The clonotypic TRA and TRB rearrangements per cluster are shown in red in the lower UMAP. Proportions of these cells within each cluster (C1-6) are depicted in the stacked bar plot. (C) UMAP of lymphoma cells from patient 056 as presented in (B). T cells with at least one clonotypic T-cell receptor rearrangement are highlighted in color within the UMAP. Proportions of cells with at least one clonotypic rearrangement are depicted in the stacked bar plot per cluster (C1-5). tSNE: t-distributed stochastic neighbor embedding; M1: malignant cluster one; M2: malignant cluster two; T4N: naïve CD4⁺ T cells; T4: CD4⁺ T cells; CM: central memory T cells; gdT: γδT cells; aT8: activated CD8⁺ T cells; TC17-like: interleukin 17 producing-like CD8⁺ T cells; cT: cytotoxic T cells; Th1: T helper 1 cells; T-PLL: T-cell prolymphocytic leukemia; AITL: angioimmunoblastic T-cell lymphoma;

the control panel.

To investigate clonal dynamics on selective treatment pressure in more detail, we employed a case of AITL (patient 003) with multiple driver mutations that could be used as clonal barcodes. In this patient, three lymphoma samples were collected at three consecutive timepoints of disease progression. We hypothesized that the cellular co-occurrence of one or more driver lesions with a clonotypic TCR rearrangement would be reflected in synchronous expansion or contraction patterns over the disease course. Of note, while a cell may have multiple distinct driver mutations, it will only have one productive TCR rearrangement. To test for these patterns, we first plotted the dynamics of the 17 driver mutations that were found in this patient. As shown in Figure 5B, these mutations can be grouped into four dynamic patterns that either constantly contracted, expanded

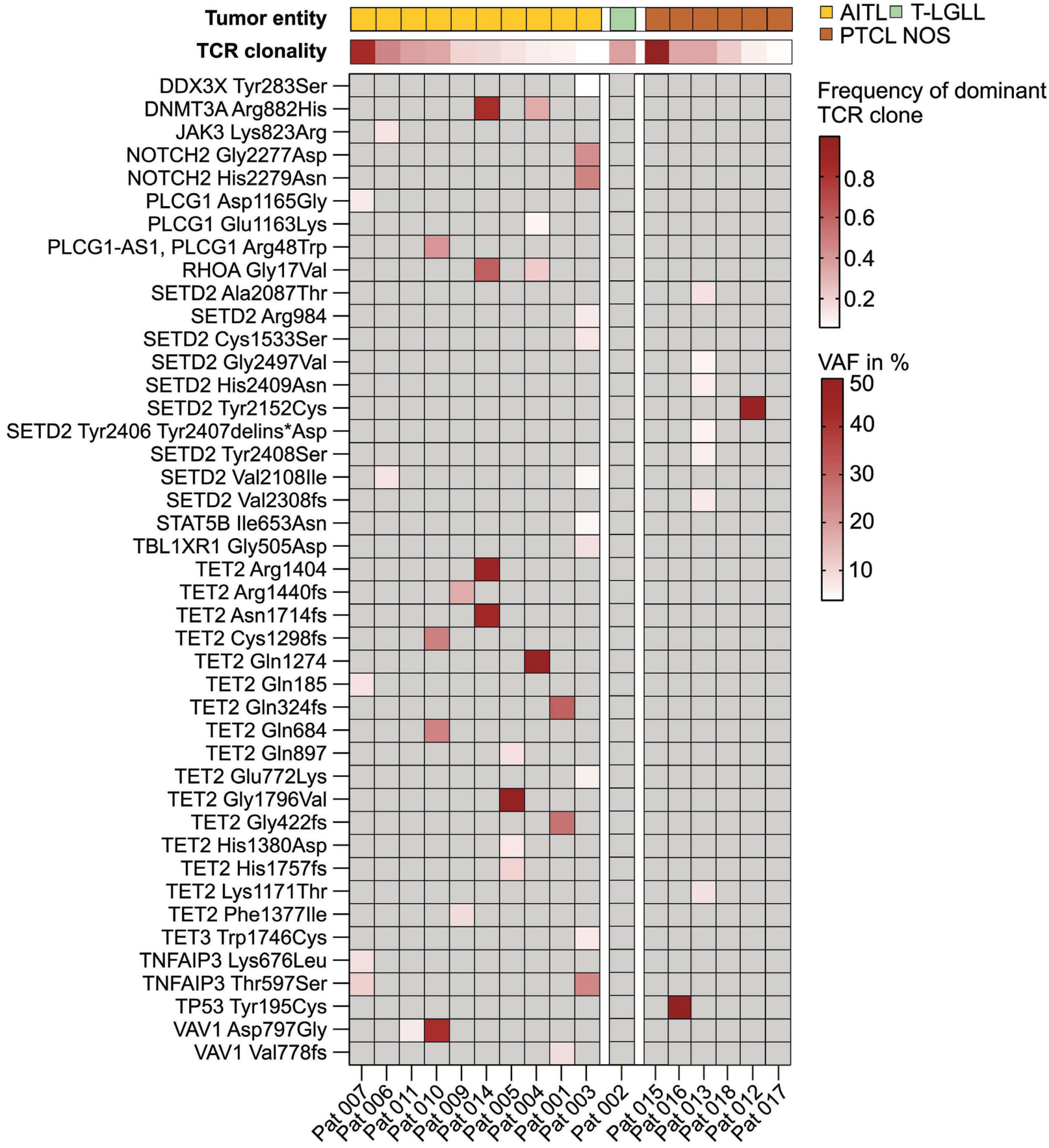
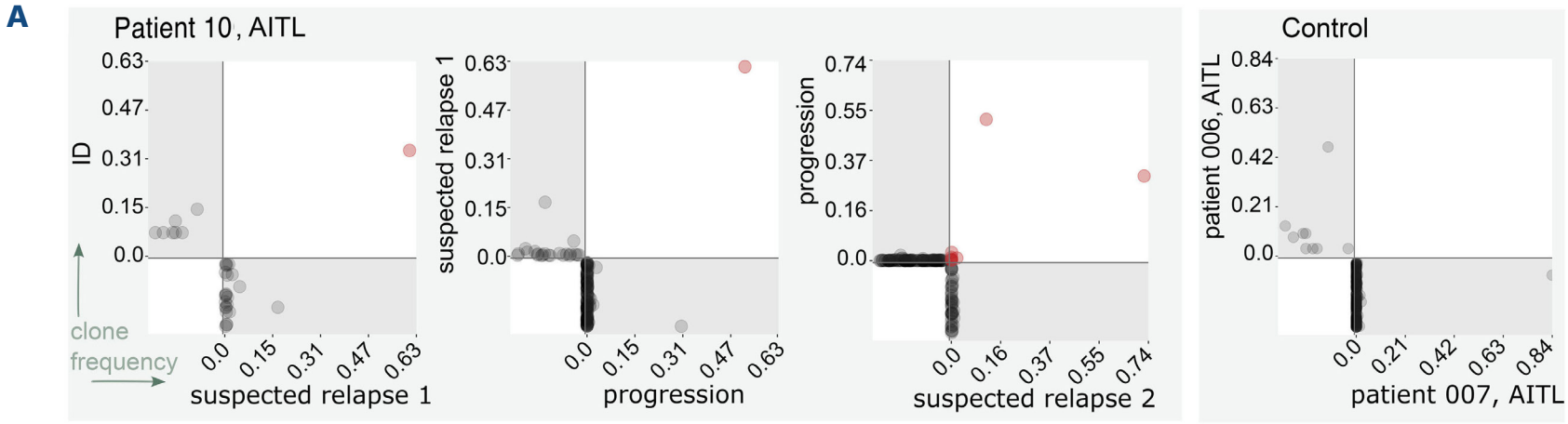
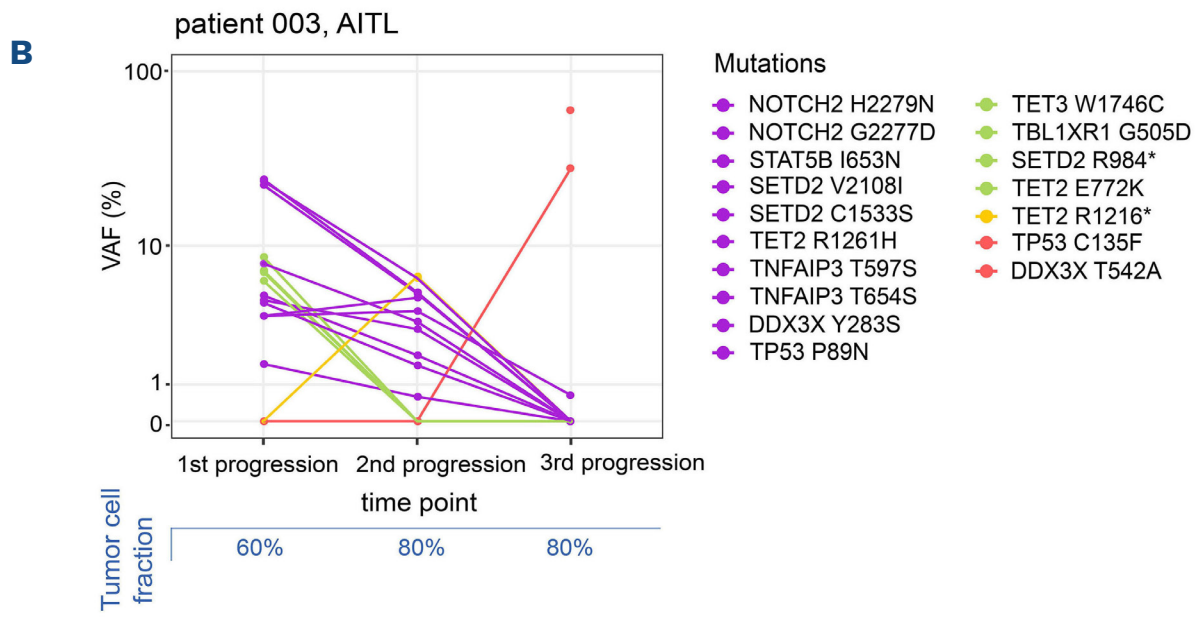
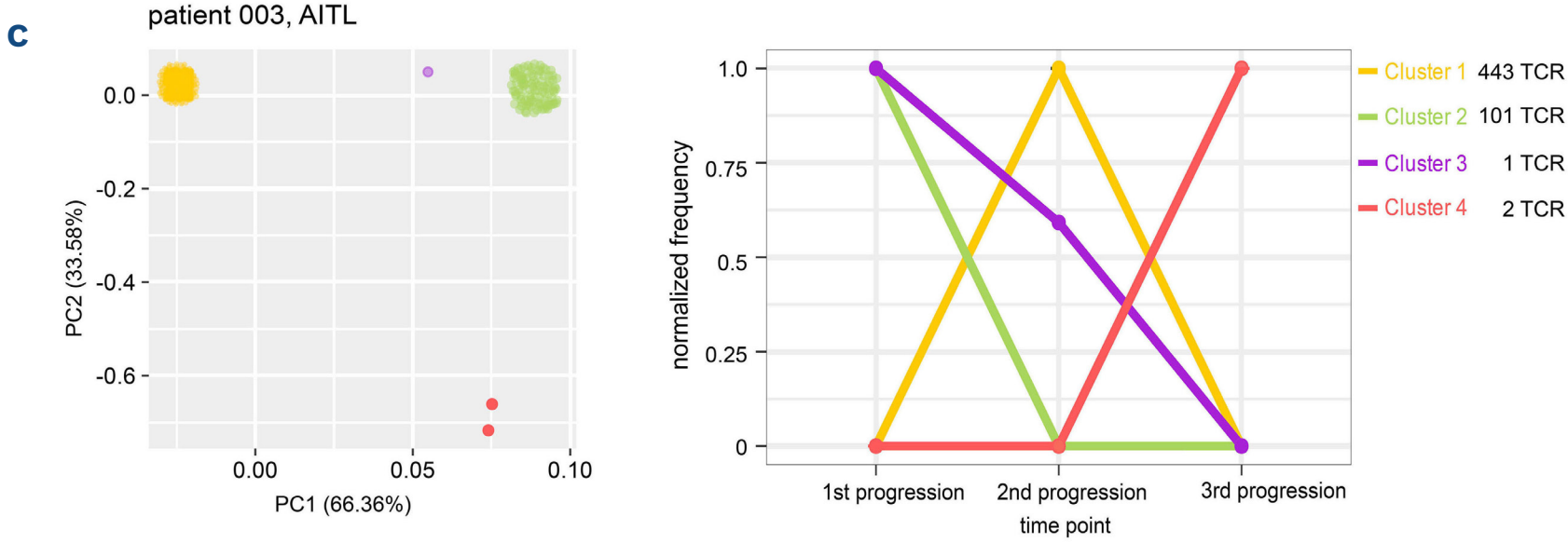


Figure 4. Overview matrix over T-cell receptor clonality of the respective dominant clone and variant allele frequency of each detected single mutation in all evaluable samples. TCR: T-cell receptor; AITL: angioimmunoblastic T-cell lymphoma; T-LGLL: T-cell large granular lymphocytic leukemia; PTCL NOS: peripheral T-cell lymphoma, not otherwise specified; VAF: variant allele frequency.







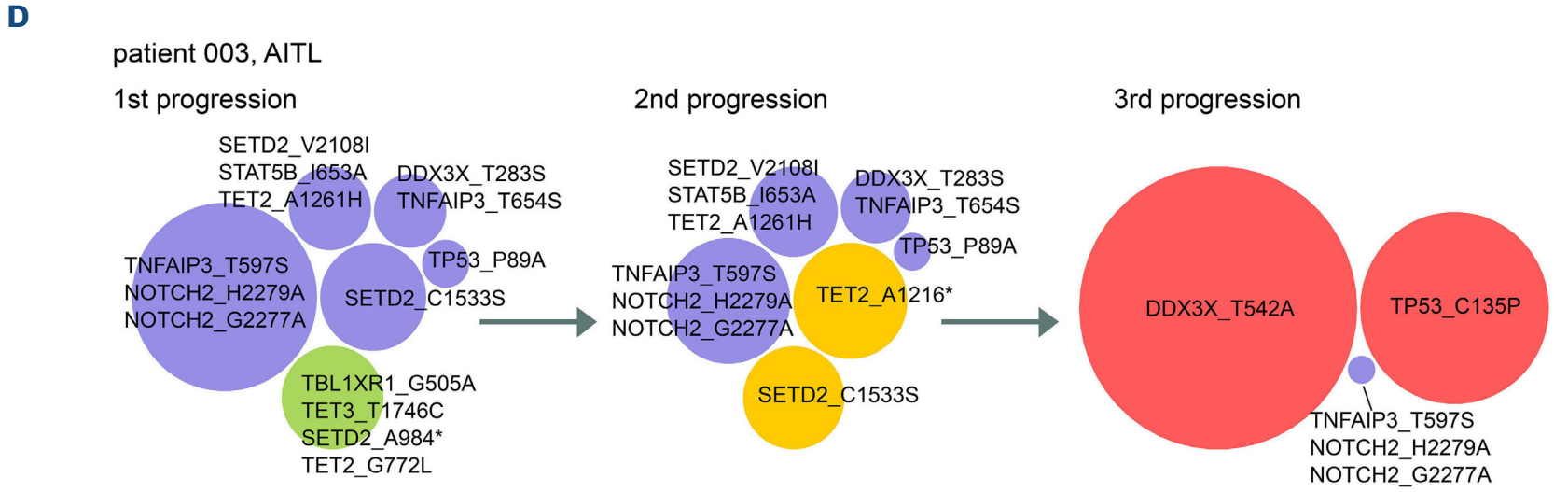


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Figure 5. Clonal tiding of T-cell lymphomas over the course of treatment. (A) Clonal overlap in lymphoma specimens from patient 010 sampled at different timepoints during the course of the disease. Shared identical T-cell receptor (TCR) rearrangements are displayed in red. A control plot shows the lack of TCR overlap between lymphoma tissue from different patients. (B) Four different dynamic patterns (indicated as purple, green, yellow, red) of 17 driver mutations in a case of angioimmunoblastic T-cell lymphoma (AITL) (patient 003) over the course of treatment as determined using gene panel sequencing. (C) Clustering and principal component analysis of the 547 unique TCR clonotypes in the lymphoma tissue of AITL patient 003 present at all sampled timepoints. The dynamic trajectories of the clustered clonotypes are shown on the right. (D) Illustration of the assembled mutational and clonal information over three disease progressions. Circle size represents cumulative variant allele frequency of the mutations. ID: initial diagnosis; VAF: variant allele frequency; PC: principal component.

or showed mixed dynamics during progression. Next, we calculated the dynamics of the 547 unique TCR clonotypes that were found within the patient's lymphoma tissue at one of the sampled timepoints. For this, we used the normalized clonotype frequencies over the time course and generated an Euclidean distance matrix. Using hierarchical clustering, we identified four distinct patterns of clonotype dynamics. We generated a principal component plot based on the normalized clonotype trajectories, color-coded the four different patterns and plotted their mean trajectories (Figure 5C). Surprisingly, the four trajectories matched exactly the four driver mutation trajectories. This implies that driver mutations and TCR clonotypes belonging to the same trajectory were very likely present in the same cell. While cluster 1 was defined by only one driver mutation, but 443 TCR clonotypes, cluster 2 showed four driver mutations along with 101 TCR rearrangements. Cluster 4 showed two driver mutations and two TCR rearrangements. The driver mutations must have been generated at a maturation stage prior to antigen receptor rearrangement in all of these three clusters given the number of TCR rearrangements associated with them. Finally, we assembled the information to illustrate the clonal landscape and its tiding from the first to the third relapse of patient 003 (Figure 5D). Together, this analysis showed that the dominant clone at diagnosis was replaced by alternative clones during the course of treatment.

Discussion

In the past decades, cancer research and especially massive genomic sequencing studies have contradicted the former paradigm of a linear evolution of malignancies. It has become clearer that the clonal evolution of solid tumors is driven by the development of different subclones emerging from immature cells leading to a complex and heterogeneous clonal landscape with its inherent potential for resistance. Hematologic malignancies are traditionally conceived as less complex and an important number of these diseases are believed to derive from a mature cell of origin.

In the work presented, we challenge this view for a broad variety of T-cell lymphoma entities. Almost half of our studied cases showed oligoclonality for the lymphoma TCR rearrangements thereby confirming that the cell of origin of these "mature" neoplasms is in fact an immature precursor lymphocyte. This is in line with recent findings that suggest acquired driver mutations in hematopoietic progenitor cells as the origin of malignant clones in Sézary syndrome.[56]

Moreover, the remarkable overrepresentation of TRBV20-1 rearrangements across all lymphoma subtypes suggests that this receptor configuration acts as a fundamentally relevant tumor driver in the development of these diseases. Furthermore, this work provides insights into the evolution and shaping of the clonal lymphoma architecture under selective pressure of treatment by serial profiling of one lymphoma patient in the course of disease. In this exemplary case, the initially dominant clone was replaced by alternative clones under treatment pressure which could be demonstrated at the timepoint of first disease relapse. In the past years it has been frequently shown that specific treatments do not suppress all existing tumor subclones equally but may select for more aggressive, treatment-resistant clones.[57,58] The pronounced clonal complexity of neoplastic diseases that already exists at the time of diagnosis may provide the cause for this observation. Therefore, in clonally complex diseases, profiling and therapeutic targeting of all coexisting disease subclones may be particularly important in the first-line setting while single-agent therapy may foster resistance. On the other hand, our concept of resistance as a permanent feature ascribed to a specific lymphoma and a specific drug may be revisited in the light of our data. The replacement of the dominant clone by others during the course of treatment may open up the possibility of re-treatment with a regimen on which the patient has previously progressed. The establishment of clonal monitoring techniques that permit real-time clonality snapshots may, however, be a prerequisite for such personalized endeavors of guiding treatment.

Our work on lymphoma clonality may also be of relevance to guide the design of novel treatment approaches such as engineered cell products or bispecific antibodies. The vision of using chimeric antigen receptor T cells for precision targeting of T-cell lymphomas has failed so far because of the lack of targets that are reliably expressed on the malignant T cells, but not on healthy T cells.[59,60] Recent studies demonstrated successful targeting of single TCR variable β chains with either bispecific antibodies[61]

or chimeric antigen receptor T cells[37] while leaving the postulated non-malignant T-cell compartment intact. However, our TCR clonality data call for caution to use a single TCR rearrangement as a reliable target due to the observed widespread oligoclonality and potential for regrowth from a more immature precursor cell with alternative TCR rearrangements.

Beyond its therapeutic implications, our research holds the potential to reshape the interpretation of studies conducted by others. In recent years, numerous single-cell sequencing studies focusing on T-cell lymphomas have been published. However, only a minority of these studies sequenced unequivocal lymphoma cells characterized by an aberrant immunophenotype.[62] In the majority of manuscripts, classification relied predominantly on the identification of the most dominant TCR rearrangement, often leading to the categorization of cells with a divergent TCR rearrangement as non-malignant bystander cells as seen in the research by Zhu *et al.*[63] and Liu *et al.*[64] In the light of the findings presented in our work, this prevailing paradigm must be questioned, urging a reassessment of the results obtained. This includes determination of matched TRA chains as well as TRG/D rearrangements, which were not covered in our analyses. Inclusion of these data may help to pinpoint the differentiation stage of the transformed precursor cell which could help to separate non-malignant bystander clonotypes and may have clinical relevance regarding phenotype plasticity.[14,65]

Taken together, our data confirm the high degree of clonal heterogeneity in T-cell lymphomas with significant clonal tiding under selective therapeutic pressure. These data may foster our understanding of the complex nature of the clonal landscape and resistance to treatment in this group of diseases.

Disclosures

No potential conflicts of interest to disclose.

Contributions

MBi and TW conceived and designed the research project. TW, CW and FLS supplied critical material (e.g., patients' material, cohorts). CS, LP and MBa conducted experimental work. EW, MBi, CS, BT, LP, PS-B and TW analyzed and interpreted data. MBi, EW and BT drafted the manuscript.

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Data-sharing statement

The TCR sequencing data reported in this article have been deposited at the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB72787.

References

1. Alaggio R, Amador C, Anagnostopoulos I, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: lymphoid neoplasms. Leukemia. 2022;36(7):1720-1748.
2. Campo E, Jaffe ES, Cook JR, et al. The International Consensus Classification of mature lymphoid neoplasms: a report from the Clinical Advisory Committee. Blood. 2022;140(11):1229-1253.
3. Fiore D, Cappelli LV, Broccoli A, Zinzani PL, Chan WC, Inghirami G. Peripheral T cell lymphomas: from the bench to the clinic. Nat Rev Cancer. 2020;20(6):323-342.
4. Dummer R, Vermeer MH, Scarisbrick JJ, et al. Cutaneous T cell lymphoma. Nat Rev Dis Primers. 2021;7(1):61.
5. Hague C, Farquharson N, Menasce L, Parry E, Cowan R. Cutaneous T-cell lymphoma: diagnosing subtypes and the challenges. Br J Hosp Med (Lond). 2022;83(4):1-7.
6. Park HS, McIntosh L, Braschi-Amirfarzan M, Shinagare AB, Krajewski KM. T-Cell Non-Hodgkin lymphomas: spectrum of disease and the role of imaging in the management of common subtypes. Korean J Radiol. 2017;18(1):71-83.
7. Broccoli A, Zinzani PL. Peripheral T-cell lymphoma, not otherwise specified. Blood. 2017;129(9):1103-1112.
8. Marks DI, Rowntree C. Management of adults with T-cell lymphoblastic leukemia. Blood. 2017;129(9):1134-1142.
9. Izykowska K, Rassek K, Korsak D, Przybylski GK. Novel targeted therapies of T cell lymphomas. J Hematol Oncol. 2020;13(1):176.
10. Bellei M, Foss FM, Shustov AR, et al. The outcome of peripheral T-cell lymphoma patients failing first-line therapy: a report from the prospective, International T-Cell Project. Haematologica. 2018;103(7):1191-1197.
11. Chihara D, Fanale MA, Miranda RN, et al. The survival outcome of patients with relapsed/refractory peripheral T-cell lymphoma-not otherwise specified and angioimmunoblastic T-cell lymphoma. Br J Haematol. 2017;176(5):750-758.
12. Lansigan F, Horwitz SM, Pinter-Brown LC, et al. Outcomes for relapsed and refractory peripheral T-cell lymphoma patients after front-line therapy from the COMPLETE registry. Acta Haematol. 2020;143(1):40-50.
13. Stuver R, Moskowitz AJ. Therapeutic advances in relapsed and refractory peripheral T-cell lymphoma. Cancers (Basel). 2023;15(3):589.
14. Iyer A, Hennessey D, Gniadecki R. Clonotype pattern in T-cell lymphomas map the cell of origin to immature lymphoid precursors. Blood Adv. 2022;6(7):2334-2345.
15. Marks JA, Switchenko JM, Martini DJ, et al. T-cell receptor gene rearrangement clonality, flow cytometry status, and associated outcomes in early-stage cutaneous T-cell lymphoma. JAMA Dermatol. 2021;157(8):954-962.
16. Vose J, Armitage J, Weisenburger D; International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical

outcomes. J Clin Oncol. 2008;26(25):4124-4130.
17. Zain JM, Hanona P. Aggressive T-cell lymphomas: 2021 updates on diagnosis, risk stratification and management. Am J Hematol. 2021;96(8):1027-1046.
18. Krangel MS. Mechanics of T cell receptor gene rearrangement. Curr Opin Immunol. 2009;21(2):133-139.
19. Attygalle AD, Kyriakou C, Dupuis J, et al. Histologic evolution of angioimmunoblastic T-cell lymphoma in consecutive biopsies: clinical correlation and insights into natural history and disease progression. Am J Surg Pathol. 2007;31(7):1077-1088.
20. Chiba S, Sakata-Yanagimoto M. Advances in understanding of angioimmunoblastic T-cell lymphoma. Leukemia. 2020;34(10):2592-2606.
21. Grogg KL, Attygalle AD, Macon WR, Remstein ED, Kurtin PJ, Dogan A. Angioimmunoblastic T-cell lymphoma: a neoplasm of germinal-center T-helper cells? Blood. 2005;106(4):1501-1502.
22. Yao WQ, Wu F, Zhang W, et al. Angioimmunoblastic T-cell lymphoma contains multiple clonal T-cell populations derived from a common TET2 mutant progenitor cell. J Pathol. 2020;250(3):346-357.
23. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. N Engl J Med. 2012;366(1):95-96.
24. Quivoron C, Couronne L, Della Valle V, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell. 2011;20(1):25-38.
25. Zhang X, Su J, Jeong M, et al. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. Nat Genet. 2016;48(9):1014-1023.
26. Cheng S, Zhang W, Inghirami G, Tam W. Mutation analysis links angioimmunoblastic T-cell lymphoma to clonal hematopoiesis and smoking. Elife. 2021;10:e66395.
27. Sandell RF, Boddicker RL, Feldman AL. Genetic landscape and classification of peripheral T cell lymphomas. Curr Oncol Rep. 2017;19(4):28.
28. Vega F, Amador C, Chadburn A, et al. Genetic profiling and biomarkers in peripheral T-cell lymphomas: current role in the diagnostic work-up. Mod Pathol. 2022;35(3):306-318.
29. Schultheiss C, Simnica D, Willscher E, et al. Next-generation immunosequencing reveals pathological T-cell architecture in autoimmune hepatitis. Hepatology. 2021;73(4):1436-1448.
30. Simnica D, Akyuz N, Schliffke S, et al. T cell receptor next-generation sequencing reveals cancer-associated repertoire metrics and reconstitution after chemotherapy in patients with hematological and solid tumors. Oncoimmunology. 2019;8(11):e1644110.
31. Simnica D, Schliffke S, Schultheiss C, et al. High-throughput immunogenetics reveals a lack of physiological T cell clusters in patients with autoimmune cytopenias. Front Immunol. 2019;10:1897.
32. Bolotin DA, Poslavsky S, Mitrophanov I, et al. MiXCR: software for comprehensive adaptive immunity profiling. Nat Methods. 2015;12(5):380-381.
33. Schultheiss C, Paschold L, Simnica D, et al. Next-generation sequencing of T and B cell receptor repertoires from COVID-19 patients showed signatures associated with severity of disease. Immunity. 2020;53(2):442-455.
34. Schumann FL, Gross E, Bauer M, et al. Divergent effects of EZH1 and EZH2 protein expression on the prognosis of patients with T-cell lymphomas. Biomedicines. 2021;9(12):1842.
35. Meyer-Olson D, Simons BC, Conrad JA, et al. Clonal expansion and TCR-independent differentiation shape the HIV-specific CD8+ effector-memory T-cell repertoire in vivo. Blood. 2010;116(3):396-405.
36. Ciupe SM, Devlin BH, Markert ML, Kepler TB. Quantification of total T-cell receptor diversity by flow cytometry and spectratyping. BMC Immunol. 2013;14:35.
37. Ren J, Liao X, Lewis JM, et al. Generation and optimization of off-the-shelf immunotherapeutics targeting TCR-Vbeta2+ T cell malignancy. Nat Commun. 2024;15(1):519.
38. Bauer M, Vaxevanis C, Bethmann D, et al. Multiplex immunohistochemistry as a novel tool for the topographic assessment of the bone marrow stem cell niche. Methods Enzymol. 2020;635:67-79.
39. Schultheiss C, Paschold L, Willscher E, et al. Maturation trajectories and transcriptional landscape of plasmablasts and autoreactive B cells in COVID-19. iScience. 2021;24(11):103325.
40. Wang P, Jin X, Zhou W, et al. Comprehensive analysis of TCR repertoire in COVID-19 using single cell sequencing. Genomics. 2021;113(2):456-462.
41. Herrera A, Cheng A, Mimitou EP, et al. Multimodal single-cell analysis of cutaneous T-cell lymphoma reveals distinct subclonal tissue-dependent signatures. Blood. 2021;138(16):1456-1464.
42. 10x Genomics. Integrated GEX and VDJ analysis of Connect generated library from human PBMCs by Cell Ranger 6.0.1; 2021. https://www.10xgenomics.com/datasets/integrated-gex-and-vdj-analysis-of-connect-generated-library-from-human-pbm-cs-2-standard-6-0-1. Accessed January 20, 2024.
43. Willinger T, Freeman T, Herbert M, Hasegawa H, McMichael AJ, Callan MF. Human naive CD8 T cells down-regulate expression of the WNT pathway transcription factors lymphoid enhancer binding factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo. J Immunol. 2006;176(3):1439-1446.
44. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. Annu Rev Immunol. 2016;34:335-368.
45. Choi YS, Gullicksrud JA, Xing S, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. Nat Immunol. 2015;16(9):980-990.
46. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer. 2010;10(3):165-180.
47. Mehta A, Ratre YK, Soni VK, et al. Orchestral role of lipid metabolic reprogramming in T-cell malignancy. Front Oncol. 2023;13:1122789.
48. Salerno F, Freen-van Heeren JJ, Guislain A, Nicolet BP, Wolkers MC. Costimulation through TLR2 drives polyfunctional CD8(+) T cell responses. J Immunol. 2019;202(3):714-723.
49. Morrison C, Baer MR, Zandberg DP, Kimball A, Davila E. Effects of Toll-like receptor signals in T-cell neoplasms. Future Oncol. 2011;7(2):309-320.
50. Heavican TB, Bouska A, Yu J, et al. Genetic drivers of oncogenic pathways in molecular subgroups of peripheral T-cell lymphoma. Blood. 2019;133(15):1664-1676.
51. Odejide O, Weigert O, Lane AA, et al. A targeted mutational landscape of angioimmunoblastic T-cell lymphoma. Blood. 2014;123(9):1293-1296.
52. Watatani Y, Sato Y, Miyoshi H, et al. Molecular heterogeneity in peripheral T-cell lymphoma, not otherwise specified revealed by comprehensive genetic profiling. Leukemia. 2019;33(12):2867-2883.
53. Rodriguez M, Alonso-Alonso R, Tomas-Roca L, et al. Peripheral

T-cell lymphoma: molecular profiling recognizes subclasses and identifies prognostic markers. Blood Adv. 2021;5(24):5588-5598.
54. Leca J, Lemonnier F, Meydan C, et al. IDH2 and TET2 mutations synergize to modulate T follicular helper cell functional interaction with the AITL microenvironment. Cancer Cell. 2023;41(2):323-339.
55. Sakata-Yanagimoto M, Enami T, Yoshida K, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat Genet. 2014;46(2):171-175.
56. Harro CM, Sprenger KB, Chaurio RA, et al. Sezary syndrome originates from heavily mutated hematopoietic progenitors. Blood Adv. 2023;7(18):5586-5602.
57. Keats JJ, Chesi M, Egan JB, et al. Clonal competition with alternating dominance in multiple myeloma. Blood. 2012;120(5):1067-1076.
58. 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.
59. To V, Evtimov VJ, Jenkin G, Pupovac A, Trounson AO, Boyd RL. CAR-T cell development for cutaneous T cell lymphoma: current limitations and potential treatment strategies. Front Immunol. 2022;13:968395.
60. Oh BLZ, Vinanica N, Wong DMH, Campana D. Chimeric antigen receptor T-cell therapy for T-cell acute lymphoblastic leukemia. Haematologica. 2024;109(6):1677-1688.
61. Paul S, Pearlman AH, Douglass J, et al. TCR beta chain-directed bispecific antibodies for the treatment of T cell cancers. Sci Transl Med. 2021;13(584):eabd3595.
62. Suma S, Suehara Y, Fujisawa M, et al. Tumor heterogeneity and immune-evasive T follicular cell lymphoma phenotypes at single-cell resolution. Leukemia. 2024;38(2):340-350.
63. Zhu Q, Yang Y, Deng X, et al. High CD8(+) tumor-infiltrating lymphocytes indicate severe exhaustion and poor prognosis in angioimmunoblastic T-cell lymphoma. Front Immunol. 2023;14:1228004.
64. Liu X, Jin S, Hu S, et al. Single-cell transcriptomics links malignant T cells to the tumor immune landscape in cutaneous T cell lymphoma. Nat Commun. 2022;13(1):1158.
65. Iyer A, Hennessey D, O'Keefe S, et al. Clonotypic heterogeneity in cutaneous T-cell lymphoma (mycosis fungoides) revealed by comprehensive whole-exome sequencing. Blood Adv. 2019;3(7):1175-1184.