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Title: Evaluation of T-cell clonality by anti-TRBC1 antibody-based flow cytometry and correlation with T-cell receptor sequencing

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PCN analysed data and wrote the paper. PCN, TN, IST, KB, VN, NC, PB, and DAW acquired and analysed data. PCN and DAW conceived and designed the study. All authors approved the final manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval statement

This study was undertaken with approval by the Human Research Ethics Committee (HREC) of the Peter MacCallum Cancer Centre (Project no: 03/90, Title: Ethical oversight of pathology activity) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Key words

T-cell clonality, TRBC1, flow cytometry

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Abstract

Flow cytometry (FC) incorporating the T-cell receptor β constant chain-1 (TRBC1) has been recently proposed as a new standard in T-cell clonality assessment. While early studies demonstrated high sensitivity in samples with conspicuous tumour burden, performance in real-world samples, including those with low tumour burden, and correlation with molecular methods has been limited. We evaluated TRBC1-FC performance and correlated the results with high-throughput *TRB* sequencing and a targeted next generation sequencing gene panel. Our cohort consisted of 90 evaluable samples from 57 patients. TRBC1-FC confirmed T-cell clonality in 37 out of 38 samples (97%) that were involved by a mature T-cell neoplasm (MTCN). T-cell clonality was also identified in nine samples from patients lacking a current or prior diagnosis of MTCN, consistent with the emerging entity T-cell clonality of uncertain significance (T-CUS). TRBC-FC was polyclonal in all samples negative for disease involvement by standard pathology assessment. However, correlation with *TRB* sequencing in 17 of these samples identified two cases that harboured the known clonal sequence from index testing, indicating the presence of measurable residual disease not otherwise detected. Our study provides real-world correlative validation of TRBC1-FC, highlighting the strengths and limitations pertinent to its increasing implementation by general diagnostic laboratories.

Introduction

T-cell clonality is a fundamental property of mature T-cell neoplasms (MTCNs) that can be evaluated to provide adjunctive evidence of disease involvement.[1] The laboratory assessment of an MTCN requires an integrated approach that combines morphology, flow cytometry (FC), immunohistochemistry, and molecular results;[2] however, these investigations are inconclusive in 10% of cases.[3,4] For example, reactive T cells with aberrant antigen expression can be difficult to distinguish from malignant cells.[5] Additionally, MTCNs with an immunophenotype resembling that of normal T cells can be mistaken as benign.[6] T-cell clonality can provide ancillary evidence of neoplasia that may be necessary in equivocal cases.[4]

The different methods used to detect T-cell clonality are based on the genetic rearrangement or protein structure of the T-cell receptor (TCR).[7-10] PCR-based assays are the most commonly utilised method of T-cell clonality assessment.[11] This is often performed using consensus BIOMED-2 primer sets that target conserved regions adjacent to the VDJ segments of *TRB* and *TRG* genes.[12,13] Limitations include incomplete coverage of VDJ rearrangements, difficulty in distinguishing clonal peaks, and benign conditions associated with clonal patterns.[14-18] T-cell clonality can also be assessed using high-throughput sequencing, providing nucleotide-level resolution of the TCR rearrangement.[19,20] This is more sensitive but less widely available than the BIOMED-2 method.[21] Finally, FC can be used to evaluate the Vβ repertoire, employing commercially available antibodies targeting ~70% of Vβ proteins.[22,23] Limitations include being expensive, labour-intensive, and restricted to αβ T cells.[24]

Recently, a new FC method to assess T-cell clonality has been proposed, utilising an antibody targeting the T-cell receptor β constant chain-1 (TRBC1).[25-28] TRBC1-FC is based on the β-chain constant region and allows for a more simplified determination of T-cell clonality, analogous to light chain restriction in B lymphoproliferative disorders.[29] This method is based on random and mutually exclusive selection of either TRBC1 or TRBC2 during VDJ recombination.

Early studies have demonstrated high sensitivity in the detection of T-cell clonality using TRBC1-FC.[25,27,28] In the initial study, TRBC1-FC detected clonality in 100% of patients with conspicuous MTCN involvement (32-97% of total lymphocytes).[27] The analytical limit of detection has been assessed in a small number of studies using serial dilutions.[28,30] However,

more studies are required to determine the analytical sensitivity at low tumour burden, particularly in real world samples with measurable residual disease (MRD). Additionally, several studies have correlated TRBC1-FC with other methods of T-cell clonality assessment, typically TCR gene rearrangement studies using the BIOMED-2 PCR based method.[28,30-33] However, this assay has limited sensitivity in samples with low tumour burden and does not provide a clonal marker for sequential analyses.

We evaluated the performance of TRBC1-FC in diagnostic samples from our laboratory, reporting on a wide range of tumour burdens, including MRD and negative cases. Molecular correlation was performed using a targeted next-generation-sequencing (NGS) gene panel and high-throughput sequencing of the *TRB* gene, leveraging a clonal marker or nucleotide-level sequence from sequential samples to determine the true clonal status in discordant cases. To the best of our knowledge, this correlative analysis has not been previously reported and provides a framework for TRBC1-FC implementation as it becomes increasingly adopted in the evaluation of MTCNs.

Methods

Patient samples and diagnostic criteria

TRBC1-FC was implemented in the diagnostic haematology laboratory of the Peter MacCallum Centre in September 2020. Over the subsequent 12 months, a total of 110 consecutive TRBC1-FC samples from peripheral blood (PB), bone marrow (BM), and lymph node compartments were evaluated for known or suspected MTCN. Samples were excluded if the T-cell clone was γδ in subtype (n=7) or lacked CD3 expression (n=3), since negative TRBC1 expression does not indicate TRBC2 selection in these settings. Other samples were excluded due to an insufficient lymphocyte count (n=4) and missing clinical information (n=6).

At the patient level, clinical and longitudinal pathology records were retrospectively reviewed to identify patients with a confirmed diagnosis of MTCN. At the sample level, disease involvement was based on an integrated pathology assessment that included standard-of-care morphology, flow cytometry, and immunohistochemistry. T-cell clonality using TRBC1-FC and high-throughput *TRB* sequencing was evaluated against this integrated assessment.

TRBC1 flow cytometry

T-cell clonality was evaluated using a custom single-tube TRBC1 panel consisting of 10 monoclonal antibody-fluorophore conjugates: CD2-APC, CD3-PE, CD4-PE-Cy7, CD5-ECD, CD7-V450, CD8-APC-H7, CD16-APC-AF700, CD26-PerCP-Cy5.5, CD45-V500, and TRBC1-FITC. An additional 8-colour T-cell panel was performed in selected cases, consisting of CD3-V450, CD4-Pe-Cy7, CD26-PerCP-Cy5.5, CD27-APC-AF750, CD45-V500, CD94-APC, TCR αβ-FITC, and TCR γδ-PE conjugates. The anti-TRBC1 antibody was obtained from the Ancell corporation (Bayport, MN), while all others were obtained from BD Biosciences (San Jose, CA). Samples undergoing TRBC1 analysis were acquired on a Navios flow cytometer (Beckman Coulter) while the 8-colour panel was performed on a BD FACSCanto II instrument.

Post-acquisition FC data was analysed using Kaluza software (Beckman Coulter). The proportion of TRBC1 positivity was assessed in multiple T cell compartments that included the aberrant population (if present) as well as normal CD4+ and CD8+ T cells (Figure 1). Additionally, T cells subsets characterised by CD4+CD26+, CD4+CD7-, CD4+CD26-CD7+, and CD8+CD5- expression were routinely interrogated for evidence of clonality. Thresholds for T-cell clonality were defined as TRBC1 positive events >85% (positive restriction) or <15% (negative restriction), consistent with the reported literature.[26] An aberrant T cell population was defined as a distinct cluster (>20 events) exhibiting at least one marker with different antigen expression compared to normal T cells.

TRB sequencing

High-throughput sequencing of the *TRB* locus was performed using the LymphoTrack *TRB* assay (Invivoscribe, San Diego, CA). DNA libraries were prepared, pooled, and sequenced on an Illumina MiSEQ instrument according to the manufacturer's instructions. Each sample had a minimum input of 50 ng of DNA. Sequencing data was analysed using the provided LymphoTrack Software package. T-cell clonality was defined according to the manufacturer's interpretation criteria (Supplemental Methods).

Targeted NGS gene panel

Targeted NGS gene panel sequencing was performed using a unique molecular index (UMI) incorporated single-primer extension panel as previously described.[34] Libraries were prepared

using the standard protocol for QIAseq targeted DNA panel (QIAGEN) as per manufacturer's specifications. Pooled libraries were sequenced on an Illumina NextSeq 500 on a NextSeq 500/550 Mid-Output v2.5 kit (300 cycles; Illumina) using a 151-cycles paired-end protocol. Alignment and variant calling against the hg19 reference genome was performed using the QIAGEN CLC Genomic Workbench (v12.0.2) using the QIAseq DNA Somatic Variant workflow. All variant calls were manually inspected in integrated genome viewer (IGV). In the absence of paired germline samples, variants were assumed somatic in origin based on population frequency (gnomAD database), variant allele frequency (VAF), presence or absence in cancer databases (COSMIC), and variant characteristics (eg. predicted consequence on protein function, nature of amino acid change). Somatic variants were interpreted according to our standard diagnostic reporting pipeline which included evaluation of the VAF, disease burden, and published literature.

Results

Patients and samples

The primary cohort (n=90) consisted of all evaluable TRBC1-FC samples after exclusion (Table 1). Samples were categorised based on disease involvement (integrated assessment, Supplemental Table 1) and MTCN diagnosis (clinical history and integrated assessments from all longitudinal samples). This resulted in four groups: (1) disease-positive samples diagnostic of an MTCN (disease+/MTCN+, n=38); (2) samples with an aberrant T-cell population detected by flow cytometry, but with insufficient evidence to diagnose an MTCN (flow+/MTCN-, n=9); (3) disease-negative samples in patients previously diagnosed with an MTCN, reflecting either disease remission or involvement at another site (disease-/MTCN+, n=21); and (4) disease-negative samples in patients without a history of MTCN (disease-/MTCN-, n=22).

Across all samples, the median tumour burden was 12% of total white blood cells (WBCs, range: 0.2-94%). The median sensitivity was 10^{-4} based on a median of >20,000 WBCs analysed. The most frequent indications for testing samples from patients without an MTCN diagnosis (flow+/MTCN- and disease-/MTCN- groups) were lymphadenopathy (38%) and rash (13%, Supplemental Table 2).

The correlation cohort (n=38) was a subset of samples from the primary cohort in which DNA was available to perform *TRB* sequencing and a targeted NGS panel.

TRBC1 flow cytometry

Using clonal thresholds of <15% and >85% TRBC1 positive events, TRBC1-FC demonstrated T-cell clonality in 97% of disease+/MTCN+ samples (Table 2). The one exception demonstrated a CD4+CD26- T-cell population (with no other antigenic aberrancy) accounting for 32% of lymphocytes with 78% TRBC1 positive events. This lymph node sample was diagnosed with disease involvement based on the previous detection of the same immunophenotype and concurrent histology showing known nodal peripheral T-cell lymphoma with T follicular helper phenotype (PTCL-TFH). All samples in the flow+/MTCN- group were clonal, while all samples in the disease-/MTCN+ and disease-/MTCN- groups were polyclonal. In a parallel analysis of samples with disease burden <5% of total WBCs, TRBC1-FC demonstrated clonality in all samples of the disease+/MTCN+ and flow+/MTCN- groups (Supplemental Table 3).

TRBC1 restriction was often far more skewed than the clonal thresholds (Figure 2). In the disease+/MTCN+ group, positive and negative clones exhibited a median TRBC1 expression level of 97% (range: 78-100%) and 0.6% (range: 0.1-4%), respectively. Similarly, positive and negative clones in the flow+/MTCN- group demonstrated a median TRBC1 expression level of 90% (range: 87-92%) and 1% (range: 0.3-2%), respectively. TRBC1 expression was also evaluated in normal CD4+ and CD8+ T cells. Across all four groups, normal T cells displayed a polytypic pattern of expression that did not overlap with the clonal population. Median TRBC1 expression was 43% (range: 26-76%) in CD4+ T cells and 38% (range: 16-61%) in CD8+ T cells (Figure 2).

In addition to the clonal thresholds of <15% and >85% TRBC1 positive events in our diagnostic implementation, we also analysed TRBC1-FC using the 99.7% confidence interval derived from normal CD4+ (13-73%) and CD8+ (9-61%) across all samples of our primary cohort. Using these different thresholds, TRBC1-FC was clonal in all samples (100%) of the disease+/MTCN+ group; however, three samples were also classified as clonal in the disease-/MTCN+ group (data not shown).

T-cell clonality of uncertain significance (T-CUS)

The flow+/MTCN- group consisted of nine (10%) samples collected from six patients without a diagnosis of MTCN (Table 3). These were all clonal by TRBC1-FC, indicative of the diagnostic label T-cell clonality of uncertain significance (T-CUS). Median tumour burden was lower compared to disease+/MTCN+ samples (7% vs 12%) and ranged from 0.14 to 19% of total WBCs. The median number of circulating TRBC1-FC clonal T cells was 0.3 $x10^9$ cells/L (range: 0.0003-0.6). These samples all underwent additional immunophenotyping (Table 3). In all samples, at least one aberrancy was noted, with the most common being dim or bright expression of CD3 and/or CD2.

The median patient age was 63 years (range: 38-84). Four patients (67%) had a history of autoimmune disease that included primary biliary cirrhosis, immune thrombocytopenic purpura, autoimmune haemolytic anaemia, and IgA vasculitis. Longitudinal TRBC1 testing was available for analysis in three patients. Follow-up testing in patient one (129 days) and four (529 days) demonstrated resolution or near resolution of the T-cell clone, respectively (Supplemental Figure 1). In contrast, the T-cell clone in patient three persisted at a stable clone size with no progression to an MTCN (328 days).

TRB sequencing

Thirty-eight samples (42%) in the primary cohort had stored DNA available for *TRB* sequencing and targeted NGS gene panel testing (Table 2). This correlation cohort was used to evaluate T-cell clonality with reference to the four groups. In the 17 correlative samples from the disease+/MTCN+ group, merged reads corresponding to monoclonal, oligoclonal, and polyclonal T-cell populations were observed in 13, 1, and 2 samples, respectively (Table 2). In the flow+/MTCN- group, *TRB* sequencing was performed in four samples in which three were monoclonal and one was polyclonal by the interpretation criteria. In these two groups (disease+/MTCN+ and flow+/MTCN-), a total of three patients with T-cell clonality by TRBC1-FC exhibited an incongruent polyclonal pattern by *TRB* sequencing. These samples displayed a small T-cell clone that accounted for 0.2%, 0.5%, and 2.7% of total WBCs. The spectratype from these three cases were visually inspected; while no evidence of clonality was seen in two cases, expanded clonotypes were identified in the sample with 2.7% disease burden (correlation sample 19, Table 4, Supplemental Figure 2).

In the disease-/MTCN+ and disease-/MTCN- groups, where TRBC1-FC was polyclonal in all samples, *TRB* sequencing demonstrated incongruent monoclonality in six samples from six different patients (Table 2 and 4). In two of these discordant cases, the clonal sequence was known from a different correlation sample in which disease involvement was apparent. Interestingly, the known clonal sequence was detected in both of these discordant samples (correlation sample 24 and 28, Table 4), indicating the presence of MRD that was not detected by TRBC1-FC. Here, the T-cell clones exhibited an immunophenotype overlapping with reactive physiological subsets (CD4+ CD26-) with no antigenic aberrancy. While previous TRBC1-FC demonstrated abnormal expansion and TRBC1 restriction, small disease burden and/or expansion of reactive T cells may dilute the degree of TRBC1 restriction and account for the polyclonal result in these two cases. Indeed, the TRBC1 positive percent was 75% in correlation sample 24 (Table 4) and would have been classified as TRBC1-FC clonal using the 99.7% confidence interval. The remaining four discordant cases did not have previous *TRB* sequencing to correlate with, and their true clonal status remains unknown. However, it is worth noting that in two of these, one had active disease detected at another site, while the other had a longstanding rash with indeterminate biopsy results (Supplemental Table 4).

Targeted NGS gene panel

Targeted NGS gene panel testing was also performed in the correlation cohort. Assumed somatic variants were detected in nine samples from the disease+/MTCN+ group (Table 4). The majority of these variants were attributed to the MTCN with the exception of a *SETBP1* variant ascribed to clonal haematopoiesis of indeterminate potential (CHIP, correlation sample 11, Table 4)[35] and a pre-lymphomatous *TET2* lesion in the context of PTCL-TFH (correlation sample 4, Table 4).[36] In the flow+/MTCN- (T-CUS) group, variants in *TET2, DNMT3A,* and *SF3B1* were detected in 3 (75%) patients, aged between 69 and 84 years. These variants were present at a low VAF and most consistent with CHIP. Variants were also detected in samples from disease-/MTCN+ and disease-/MTCN- groups. Here, *TET2* variants with a high VAF (44% and 49%) were attributed to pre-lymphomatous lesions in the context of PTCL-TFH.[36] Noting the VAF, a germline TET2 variant is also possible; however, this could not be confirmed in the absence of a germline sample. Interestingly, sample 24 demonstrated the same low-level *TET2* variant (4%) detected in at the time of disease involvement (sample 4, Table 4), consistent with MRD demonstrated by *TRB* sequencing.

Discussion

We report on the performance of TRBC1-FC in our diagnostic laboratory. TRBC1-FC detected T-cell clonality in 97% of samples involved by an MTCN (disease+/MTCN+ group). The median tumour burden was lower compared to previous studies in which samples with conspicuous disease involvement were selected.[27] *TRB* sequencing demonstrated high concordance with T-cell clonality identified by TRBC1-FC in the disease+/MTCN+ and flow+/MTCN- negative groups. The three exceptions demonstrated low tumour burden, which were likely below the limit of detection for *TRB* sequencing. In the disease-/MTCN+ and disease-/MTCN- groups, where TRBC1-FC was polyclonal in all samples, *TRB* sequencing ascribed clonality in six cases. Notably, two of these cases were confirmed to be clonal, containing the known nucleotide-level clonal sequence detected in a different sample with significant disease involvement. Correlative analysis involving *TRB* sequencing has not been previously reported, highlighting both the strengths and limitations of TRBC1-FC applied to a diagnostic setting.

The thresholds used to define TRBC1 clonality has varied in the reported studies to date. Shi et al. established arbitrary thresholds of <15% and >85% TRBC1 positive events to define clonality, finding 100% sensitivity in all MTCNs.[26,27] Other studies have defined T-cell clonality based on the normal reference ranges of TRBC1 positive events in normal CD4+ and CD8+ T cells, with populations outside the 99.7% confidence interval (three standard deviations) considered clonal.[28,30,31] We used a diagnostic threshold of <15% and >85% TRBC1 positive events and found that this detected T-cell clonality in 97% of samples involved by an MTCN (disease+/MTCN+ group). This improved to 100% when using the 99.7% confidence interval, which also correctly reclassified TRBC1-FC as monoclonal in one disease-/MTCN+ sample in which the known clonal TRB sequence was detected. More studies are required to evaluate these different thresholds.

The routine implementation of TRBC1-FC in the assessment of T-cell clonality has identified a high frequency of T-CUS among healthy donors.[26,37] Shi et al. recently reported T-CUS in 26% of patients without an MTCN. The immunophenotype was predominantly CD8+ and closely resembled T-cell large granular lymphocytic (T-LGL) leukaemia.[26,37] Similarly, Blomme et al reported an almost exclusive T-LGL immunophenotype in samples with no

clinical or laboratory evidence suspicious of T-LGL leukaemia.[33] It is unclear whether T-CUS represents a more indolent form of T-LGL leukaemia, a broader phenomenon in which different T-cell subsets proliferate in response to antigen exposure, or a combination of both.

In our study, T-CUS was identified in six patients (11%). Noting small numbers, this group demonstrated an equal number of CD4+, CD8+, and CD4/CD8 double positivity immunophenotypes. Two patients exhibited a T-LGL immunophenotype (Table 3). Two patients, one with IgA vasculitis and with no significant medical history, exhibited a CD4+ immunophenotype, which has rarely been reported in T-CUS and may arise from different antigenic stimuli compared to infection or malignancy where CD8+ responses are dominant.[38,39] Notably, the clone size in T-CUS samples overlapped with those involved by an MTCN, ranging from 0.01 to 20% of total WBCs. Therefore, one of the challenges facing TRBC1-FC is the high prevalence of T-CUS and its pathologic overlap with disease,[37] and selective testing in cases with a sufficient pre-test probability may avoid unnecessary follow-up, particularly while the natural history of T-CUS remains to be clarified.

The correlation between TRBC1-FC and orthogonal molecular techniques has not been widely reported.[28,30,31,33,40] Novikov et al. performed TRBC1-FC and the BIOMED-2 *TRG* assay in samples with equivocal routine FC.[28] Discordant cases, in which the *TRG* assay indicated oligoclonality while TRBC-FC indicated polyclonality, were resolved in favour of TRBC-FC and considered polyclonal. However, it is possible that TRBC1-FC was falsely polyclonal, particularly in samples with low tumour burden and in cases with minimal immunophenotypic aberrancies. In other studies, TRBC1-FC was evaluated against a combined BIOMED-2 PCR and Vβ repertoire approach, showing high rates of concordance.[30,31] The final diagnosis in discordant cases was based on the integrated clinical context.

In our study, T-cell clonality by TRBC1-FC was correlated with *TRB* sequencing and a targeted NGS gene panel. TRBC1-FC was highly concordant with *TRB* sequencing and more sensitive in detecting T-cell clonality in samples with low tumour burden. While the *TRB* LymphoTrack assay can achieve a sensitivity of 10^{-6}, this level of detection requires significantly more input DNA than was available in our samples.[41] Notwithstanding, *TRB* sequencing was able to detect disease in two cases in which TRBC1-FC was polyclonal, leveraging the known clonal sequence identified on a different sample. Notably, the neoplastic population in these two cases exhibited an immunophenotype (CD4+ CD26-) that could not be clearly distinguished from

normal/reactive CD4+ T cell subsets. In these cases, TRBC1 restriction can be diluted by normal T cells and appear polyclonal, particularly with low tumour burdens encountered in MRD assessment. This highlights both a limitation of TRBC1-FC, where the sensitivity depends on the detection of an abnormal immunophenotype, and the value of molecular correlation in such cases. It is also worth noting that the detection of an abnormal immunophenotype is contingent on the TRBC1 panel and the inclusion of relevant markers. In our study, the TRBC1 panel did not include a γδ antibody and small clonal CD4-CD8- αβ T cells may be missed.

In summary, the incorporation of anti-TRBC1 antibody into routine clinical flow cytometric testing provides a simple and accurate assessment of T-cell clonality. While advantageous in samples with low tumour burden, the high degree of sensitivity will also lead to the frequent identification of T-CUS, the natural history of which remains to be clarified, particularly in the context of autoimmune disorders, pathogenic subsets, and relationship with CHIP. Additionally, *TRB* sequencing may be valuable in cases with minimal immunophenotypic abnormalities; however, further validation is required to establish its role in routine diagnostics.

TABLES AND FIGURES

Table 1. Clinical and laboratory features of the primary cohort. Samples are classified into four groups based on disease involvement and history of current or prior mature T-cell neoplasm. Abbreviations: MTCN = mature T-cell neoplasm, AITL = angioimmunoblastic T-cell lymphoma, ALCL = anaplastic large cell lymphoma, PTCL = peripheral T-cell lymphoma, TFH = T follicular helper, T-LGL = T large granular lymphocytes, PLL = prolymphocytic leukaemia.

	Total	Disease+ MTCN+	Flow+ MTCN-	Disease- MTCN+	Disease- MTCN-
Patients	57	17	6	14	20
Samples	90	38	9	21	22
Age, median	58	50	69	53	65
Female sex, patients	24	7	2	5	10
MTCN, patients/samples					
Sézary syndrome	11/28	7/20	-	4/8	-
AITL	1/2	0/0	-	1/2	-
ALK-negative ALCL	1/4	1/4	-	0/0	-
Mycosis fungoides	3/3	2/2	-	1/1	-
Nodal PTCL with TFH phenotype	3/8	1/4	-	2/4	-
PTCL NOS	6/6	3/3	-	3/3	-
Subcutaneous panniculitis like TCL	2/2	0/0	-	2/2	-
T-LGL leukaemia	1/3	1/3	-	0/0	-
T-PLL	2/2	2/2	-	0/0	-
Not classifiable	1/1	0/0	-	1/1	-
Specimen type, samples					
Peripheral blood	48	23	5	8	12
Bone marrow	37	13	4	13	7
Lymph node	5	2	0	0	3
Peripheral blood counts, median					
WBC count ($x10^9$/L)	7.7	8.4	7.8	5.4	8.1
Lymphocyte count ($x10^9$/L)	1.5	1.9	1.8	0.8	1.6

Table 2. T-cell clonality evaluated in samples of the primary and correlation cohort. The correlation cohort consisted of samples from the primary cohort in which both TRBC1 flow cytometry and *TRB* sequencing were performed on the same sample. T-cell clonality was defined as the percentage of TRBC1 positive events being >85% or <15% for TRBC1 flow cytometry. *TRB* sequencing was defined using a modified interpretation criteria based on the manufacturer's recommendation (Supplemental methods). Percentages refer to the number of samples as a proportion of total samples from that particular cohort (primary or correlation) and group (disease/MTCN status).

	Disease+ MTCN+	Flow+ MTCN-	Disease- MTCN+	Disease- MTCN-
Primary cohort, n	38	9	21	22
TRBC1-FC, n (% total)				
Monoclonal	37 (97)	9 (100)	0 (0)	0 (0)
Polyclonal	1 (3)	0 (0)	21 (100)	21 (100)
Correlation cohort, n	17	4	11	6
TRBC1-FC, n (% total)				
Monoclonal	17 (100)	4 (100)	0 (0)	0 (0)
Polyclonal	0 (0)	0 (0)	11 (100)	6 (100)
TRB sequencing, n (% total)				
Monoclonal	13 (76)	3 (75)	3 (27)	3 (50)
Oligoclonal	1 (6)	0 (0)	0 (0)	0 (0)
Polyclonal	2 (12)	1 (25)	8 (73)	3 (50)
Failed	1 (6)	0 (0)	0 (0)	0 (0)

Table 3. Characteristics of the flow+/MTCN- group. Abbreviations: AIHA = autoimmune haemolytic anaemia, ITP = immune thrombocytopenic purpura, PBC = primary biliary, cirrhosis, MCD = multicentric Castleman disease, NA = not applicable, NT = not tested. *TRB sequencing in this sample was further reviewed by evaluating the spectratype, showing expanding clonotypes suggestive of clonal disease (Supplemental Figure 2).

Patient	Age	Gender	Comorbidities	Clone size, %WBC	TRBC1 positive (%)	Targeted NGS	TRB sequencing
1	38	M	Renal transplant, IgA vasculitis	0.1	92.0	NA	NA
2	51	M	Renal transplant, ITP	0.6	90.4	No mutations	Monoclonal
3	78	F	PBC, DLBCL	2.7 3.8	2.0 1.0	*TET2* NT	Polyclonal* NT
4	84	F	Warm AIHA, MCD	17.2 17.1	1.0 0.3	*DNMT3A* NT	Monoclonal NT
5	57	M	Nil significant	12.6	87.2	NA	NA
6	69	M	Nil significant	7.1 19.6	1.0 1.0	*SF3B1*, *DNMT3A* NT	Monoclonal NT

Table 3 continued.

Patient	Immunophenotype
1	CD3+dim, CD4+, CD8-, CD2+, CD5+br, CD7+, CD16-, CD26-, CD27+, CD94-, TCRαβ+
2	CD3+dim, CD4-, CD8+, CD2+br, CD5+, CD7+, CD16-, CD26-, CD27-, CD94-, TCRαβ+
3	CD3+br, CD4-, CD8+, CD2+dim, CD5+, CD7+, CD16-, CD26-, CD27-, CD94-, TCRαβ+
4	CD3+, CD4+, CD8+dim, CD2+, CD5+, CD7+dim, CD16-, CD26-, CD27-, CD94-, TCRαβ+
5	CD3+, CD4+, CD8+dim, CD2+, CD5+, CD7-, CD16-, CD26-, CD27-, CD94-, TCRαβ+
6	CD3+dim, CD4+, CD8-, CD2+dim, CD5+, CD7+, CD16-, CD26-, CD27+, CD94-, TCRαβ+

Table 4. T-cell clonality in the correlative cohort in which both TRBC1-FC and molecular evaluation were performed. Assumed somatic variants unrelated to MTCN reflect either clonal haematopoiesis of indeterminate potential or pre-lymphomatous lesions occurring outside the T cell compartment (seen in PTCL-TFH). Abbreviations: MTCN = T-cell neoplasm, AITL = angioimmunoblastic T-cell lymphoma, ALCL = anaplastic large cell lymphoma, PTCL = peripheral T-cell lymphoma, TFH = T follicular helper, LGL = large granular lymphocytic, PLL = prolymphocytic leukaemia, SPCL = subcutaneous panniculitis like T-cell lymphoma. **TRB* sequencing was unsuccessful with an insufficient total number of reads for analysis. **The *TET2* variant with high VAF likely represents a pre-lymphomatous lesion occurring in the common myeloid/lymphoid progenitors noting the PTCL-TFH context and low disease burden demonstrated by TRBC1-FC. ***The same low-level *TET2* variant was observed in sample 4 (4%) and 24 (4%) and attributed to MTCN involvement, noting minimal disease involvement in sample 4 and the same clonal sequence detected by *TRB* sequencing in both samples.

Sample	Disease	TRBC1-FC	TRB sequencing	Assumed Somatic Variants	
				Related to MTCN (%VAF)	Uncertain or unrelated to MTCN (%VAF)
Disease+/MTCN+					
1	ALK- ALCL	Monoclonal	Monoclonal	No mutations	
2	Mycosis fungoides	Monoclonal	Polyclonal	No mutations	
3	Mycosis fungoides	Monoclonal	Monoclonal	*TP53* (10)	
4	PTCL-TFH	Monoclonal	Monoclonal	*TET2* (4)***	*TET2* (48)**
5	PTCL-NOS	Monoclonal	Monoclonal	*CBL* (6), *DNMT3A* (13)	
6	PTCL-NOS	Monoclonal	Monoclonal	*STAT3* (24)	
7	PTCL-NOS	Monoclonal	Polyclonal	No mutations	
8	Sézary syndrome	Monoclonal	Monoclonal	No mutations	
9	Sézary syndrome	Monoclonal	Monoclonal	No mutations	
10	Sézary syndrome	Monoclonal	Monoclonal	*TP53* (37)	
11	Sézary syndrome	Monoclonal	Monoclonal	*JAK3* (3, 7, 10)	*SETBP1* (9)
12	Sézary syndrome	Monoclonal	Oligoclonal	No mutations	
13	Sézary syndrome	Monoclonal	Monoclonal	*CARD11* (3)	

14	Sézary syndrome	Monoclonal	NA*	No mutations	
15	T-LGL	Monoclonal	Monoclonal	*STAT3* (6), *DNMT3A* (3)	
16	T-PLL	Monoclonal	Monoclonal	*PIGA* (2)	
17	T-PLL	Monoclonal	Monoclonal	No mutations	
			Flow+/MTCN-		
18	NA	Monoclonal	Monoclonal	No mutations	
19	NA	Monoclonal	Polyclonal		*TET2* (4)
20	NA	Monoclonal	Monoclonal		*DNMT3A* (9)
21	NA	Monoclonal	Monoclonal		*SF3B1* (8), *DNMT3A* (3)
			Disease-/MTCN+		
22	AITL	Polyclonal	Polyclonal	No mutations	
23	PTCL-TFH	Polyclonal	Polyclonal		*TET2* (11, 44)
24	PTCL-TFH	Polyclonal	Monoclonal	*TET2* (4)***	*TET2* (49)
25	PTCL-NOS	Polyclonal	Polyclonal	No mutations	
26	PTCL-NOS	Polyclonal	Monoclonal	No mutations	
27	PTCL-NOS	Polyclonal	Polyclonal		*TP53* (29)
28	Sézary syndrome	Polyclonal	Monoclonal	No mutations	
29	Sézary syndrome	Polyclonal	Polyclonal	No mutations	
30	SPTCL	Polyclonal	Polyclonal	No mutations	
31	SPTCL	Polyclonal	Polyclonal	No mutations	
32	Mycosis fungoides	Polyclonal	Polyclonal	No mutations	
			Disease-/MTCN-		
33	NA	Polyclonal	Monoclonal	No mutations	
34	NA	Polyclonal	Monoclonal		*TET2* (6)
35	NA	Polyclonal	Polyclonal	No mutations	
36	NA	Polyclonal	Monoclonal	No mutations	
37	NA	Polyclonal	Polyclonal	No mutations	
38	NA	Polyclonal	Polyclonal	No mutations	

Figure 1. Illustrative plots representing the gating strategy for evaluating T-cell malignancy in a normal sample (A) and a sample with known involvement by Sézary syndrome (B). T-cell subsets are analysed for T-cell clonality by TRBC1 expression. Polytypic expression is observed for small reactive subsets indicating polyclonal origin (A, panel 3). In contrast, a restricted pattern of TRBC1 dim expression (in red) is observed for immunophenotypically aberrant populations indicating disease involvement (B). The intermediate TRBC1 expression in this case is likely related to decreased surface CD3+ expression (not shown).

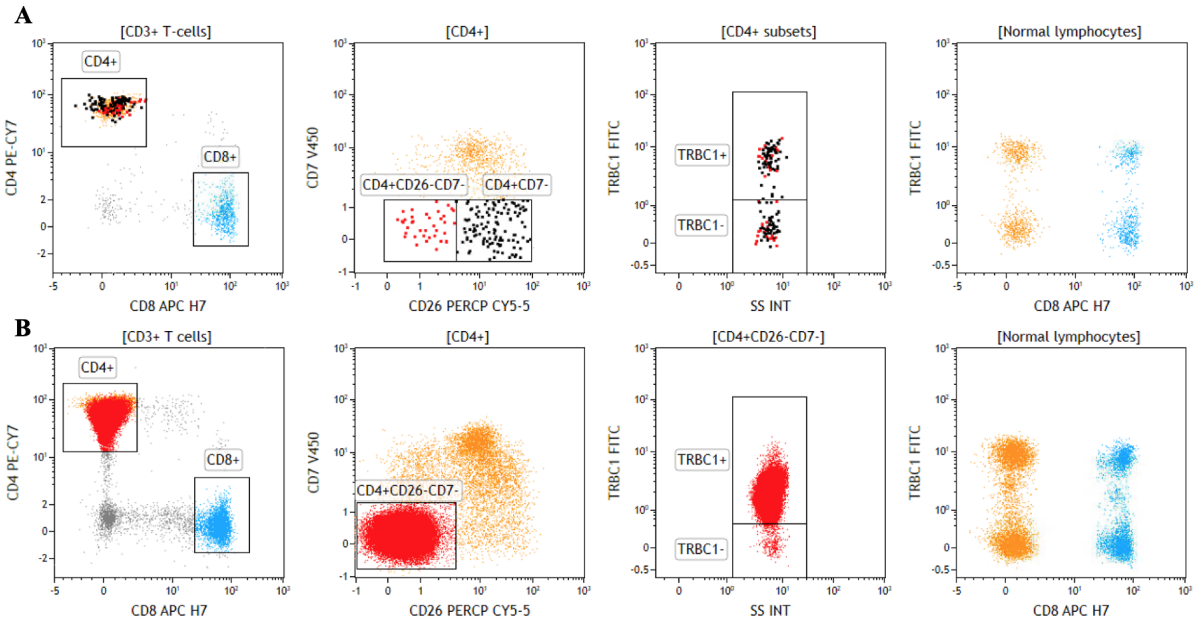
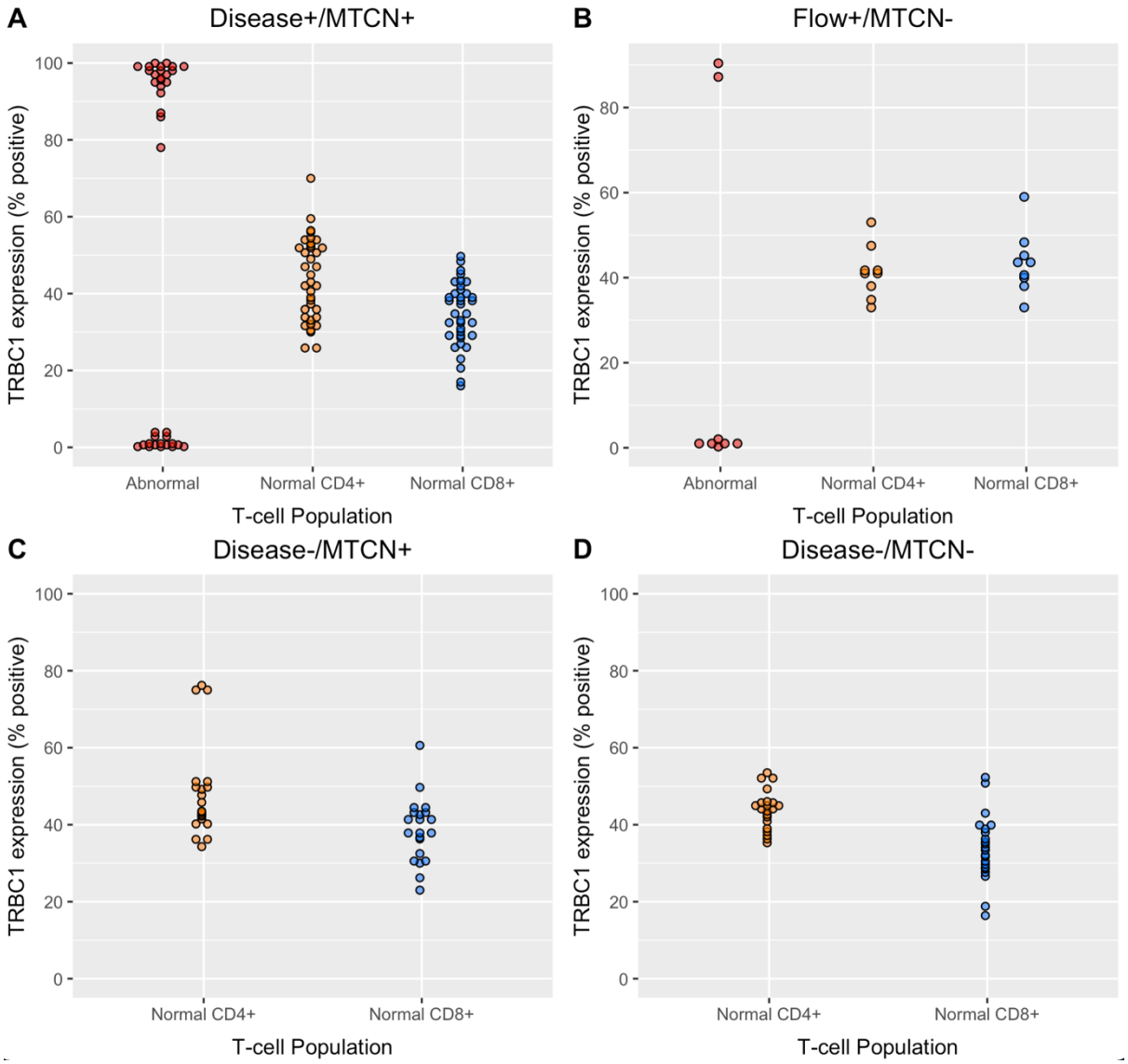


Figure 2. TRBC1 expression of abnormal (A, B) and normal background T cells (A-D) across the primary cohort. Abnormal populations demonstrated a highly restricted pattern of TRBC1 expression. In contrast, normal CD4+ and CD8+ T cell populations demonstrated a polytypic pattern of TRBC1 expression that did not overlap with the clonal populations.



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Data availability statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethics approval statement

This study was undertaken with approval by the Human Research Ethics Committee (HREC) of the Peter MacCallum Cancer Centre (Project no: 03/90, Title: Ethical oversight of pathology activity) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Patient consent statement

Not applicable

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Not applicable

Clinical trial registration (including trial number)

Not applicable

Authorship contribution

PCN analysed data and wrote the paper. PCN, TN, IST, KB, VN, NC, PB, and DAW acquired and analysed data. PCN and DAW conceived and designed the study. All authors approved the final manuscript.