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Received: 25 May 2023 | Revised: 23 August 2023 | Accepted: 12 October 2023
DOI: 10.1002/cyto.b.22147

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Integration of T-cell clonality screening using TRBC-1 in lymphoma suspect samples by flow cytometry

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Abstract

Background: The diagnosis of T-cell non-Hodgkin lymphomas (NHL) is challenging. The development of a monoclonal antibody specific for T-cell receptor β constant region 1 (TRBC1) provides an alternative to discriminate clonal T cells. The aim of this study was to evaluate the diagnostic potential of an anti-TRBC1 mAb for the identification of T-NHL.

Methods: We performed a cross-sectional diagnostic analytic study of samples tested for lymphoma. All samples sent for lymphoma screening were first evaluated using the standard Euroflow LST, to which a second additional custom-designed T-cell clonality assessment tube was added CD45/TRBC1/CD2/CD7/CD4/TCRγδ/CD3. Flow cytometry reports were compared with morphological and molecular tests.

Results: Fifty-nine patient samples were evaluated. Within the T-cell population, cut-off percentages in the CD4+ cells were from 29.4 to 54.6% and from 23.9 to 52.1% in CD8+ cells. Cut-off ratios in CD4+ T cells were from 0.33 to 1.1, and in CD8+ cells between 0.22 and 1.0. Using predefined normal cut-off values, 18 of 59 (30.5%) samples showed a restricted expression of TRBC1. A final diagnosis of a T-NHL was confirmed clinically and/or by histopathological studies in 15 of the 18 cases (83.3%). There were no cases of T-NHL by morphology/IHC with normal TRBC1 expression. Non-neoplastic patient samples behaved between predefined TRBC1 cut-off values.

Conclusions: Expression of TRBC1 provides a robust method for T-cell clonality assessment, with very high sensitivity and good correlation with complementary methods. TRBC1 can be integrated into routine lymphoma screening strategies via flow cytometry.

KEYWORDS
flow cytometry, T-cell lymphoma, TRBC1

1 | INTRODUCTION

The diagnosis of T-cell non-Hodgkin lymphomas (NHL) is a clinical and pathological challenge. Approximately 10%–15% of all non-Hodgkin lymphomas (NHLs) are of T-cell or Natural Killer (NK)-cell origin (Jiang et al., 2017). According to the WHO classification, these neoplasms include a broad group of entities under the name of “T-cell and NK-cell lymphoid proliferations and lymphomas” (Alaggio et al., 2022; Swerdlow et al., 2016). The diagnosis relies on a combination of morpho-phenotypic alterations that identify abnormal T cells in a clinical context. However, the identification of pathological T cells is often difficult due to overlapping features with reactive T cells and limitations of currently available diagnostic assays (Herrera et al., 2014).

Abnormal T cells in NHL can be recognized by several parameters. Size and complexity of the cells are frequently augmented, and a disruption of the tissue structure is followed (Laurent et al., 2017).

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The immunophenotype is variable, but lineage markers are sometimes lost, including CD3, CD4, CD7 or CD8. Specific T-NHL variants may express markers such as CD10, CD25, CD30 or PD-1, among others, which help identify entities like T-follicular helper NHL, HTLV-associated T-NHL or large-cell anaplastic T-NHL (Alaggio et al., 2022; Hsi et al., 2014). Even with the help of large immunophenotypic panels the histopathology is not convincing in 15%–20% of the cases, leading to potential misdiagnosis or misclassification (Hsi et al., 2014; Laurent et al., 2017). In cases where the morphology or the immunophenotype are unclear a clonality assessment of the T cell population is recommended (Fanny et al., 2020). T-cell clonality is mostly evaluated via molecular biology testing for Vβ-gamma rearrangement (Mahe et al., 2018). However, T-cell clonality determination has also limitations. It requires interpretive expertise, it has limited sensitivity, and its costs make it unavailable in many laboratories.

An alternative to Vβ-gamma rearrangement determination is the assessment of the T-cell receptor (TCR) β constant region (TRBC). Because mature T-cells express either of two constant regions of the TCR (TRBC1 or TRBC2), an immunophenotypic approach opened the possibility of a low-cost, rapid, and specific T-cell clonality test for mature (TCR-positive) T-cell malignancies using flow cytometry (Horna, Shi, et al., 2021; Maciocia et al., 2017; Novikov et al., 2019). Similar to kappa or lambda light chain restriction among mature B-cell malignancies, T-cell NHL also exhibits a restriction of TRBC1 or TRBC2. This finding shows promising results in the evaluation of T cell clonality. Flow cytometric detection of TRBC1 shows sensitivity of 96% and a correlation coefficient of 0.999 with molecular Vβ-gamma assessment (Muñoz-García et al., 2021). How this can be integrated in a diagnostic screening of T-NHL is still unclear.

We designed a protocol to determine if TRBC1 expression can be incorporated into routine lymphoma screening using flow cytometry. We prospectively analyzed blood, bone marrow, pleural fluid and tissue samples using an anti-TRBC1 monoclonal antibody in a complementary characterization tube to the standard EuroFlow Lymphoma Screening Tube (LST).

2 | METHODS

2.1 | Patients and sample selection

Fresh peripheral blood, bone marrow aspirate, and tissue samples were received for routine flow cytometric analysis at Clínica Alemana de Santiago. Specimens were collected between November 2021 and July 2022. As controls, we selected 10 PB samples from adult healthy donors (HD) with confirmed normal range blood counts. Clinical and laboratory information was collected from electronic medical record. This study was approved by our local IRB and Clinical Ethics Committee (ID1025).

2.2 | Flow cytometry

Peripheral blood and bone marrow samples were received in EDTA or sodium heparin anticoagulant and kept at room temperature for up to 24 h before analysis. Lymph node and tissue samples were received in ice-cold RPMI media, grinded and filtered through a tube with a 35 μm nylon mesh, to produce a cell suspension in wash buffer (0.2% bovine serum albumin) and kept at 4°C for up to 24 h after collection. Body fluid samples were received and kept at 4°C for up 24 h after collection. All samples were washed three times with wash buffer before staining. Aliquots of 100 μL were incubated in the dark for 15 min at room temperature with a cocktail of monoclonal antibodies including CD45 V500c, CD4 V450, CD8/λ FITC, CD56/κ PE, CD5 PerCP-Cy5.5, CD19/TCRγδ PE-Cy7, CD3 APC and CD38 APC-H7 for the lymphoma screening tube (Flores-Montero et al., 2019; Table 1). The T-cell NHL tube was custom designed, using CD45 V500c, CD4 V450, TRBC1 FITC, CD2 PE, CD5 PerCP-Cy5.5, TCRγδ PE-Cy7, CD7 APC and CD3 APC-H7 (Table 2). Except for the anti-TRCB1 antibody (clone JOVI-1, Ancell Corporation, Bayport, Minnesota), all other fluorochrome-conjugated antibodies were BD Biosciences, San Jose, California. Stained cells were lysed with BD FACS lysing solution (BD Biosciences) and washed with buffer. Finally, cells were resuspended in 300 μL of buffer, and at least 100.000 events were acquired on a FACSCanto II flow cytometer (BD Biosciences) equipped with FACSDiva software for data acquisition. Calibration and daily quality control, as well as monitoring, were performed according to the established EuroFlow protocols (Kalina et al., 2012).

2.3 | Data analysis

List mode files were analyzed on Infinicyt™ 2.0 (Cytognos, Salamanca, Spain). In brief, sequential gating and real-time color coding was used to identify each major T and NK cell population, based on the most informative and biologically relevant parameters. Neoplastic cells were identified as immunophenotypically abnormal T-cell subsets with discretely homogeneous fluorescence properties. TRBC1 expression was studied on a log scale histogram, and a fluorescence channel intermediate between discrete TRBC1-positive and TRBC1-negative normal T cells was visually selected as a threshold to estimate the percentage of TRBC1-positive events. TRBC1 expression was evaluated on CD4-positive and CD8-positive. All cases were analyzed by two independent reviewers.

2.4 | Statistical analysis

Statistical analyses were carried out in GraphPad Prism, v.9 (GraphPad Software, San Diego, CA). Mean percentages of TRBC1-positive events and ratio TRBC1+/− were compared using a two-tailed test, and by one-way ANOVA for comparisons between more than two groups. A statistically significant *p*-value was considered of less than 0.05.

3 | RESULTS

3.1 | Patient characteristics

A total of 59 patient samples from 57 patients were evaluated, in addition to 10 peripheral blood samples from healthy controls.

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TABLE 1 Monoclonal antibodies, showing clone, isotype, and conjugated fluorochrome used for flow cytometry in samples with suspect lymphomas.

Antibody	Clone	Isotype	Fluorescence labeling
Anti-Lambda	F(ab')2	Goat IgG1, κ	FITC
CD8	SK1	Mouse IgG1, κ	FITC
CD56	MY31	Mouse IgG1, κ	PE
Anti-Kappa	TB28-2	Mouse IgG1, κ	PE
CD5	L17F12	Mouse IgG2a, κ	PerCP-Cy5.5
CD19	SJ25C1	Mouse IgG1, κ	PE-Cy7
Anti-TCRg/d-1	11F2	Mouse IgG1, κ	PE-Cy7
CD3	SK7	Mouse IgG1, κ	APC
CD38	HB7	Mouse IgG1, κ	APC-H7
CD4	RPA-T4	Mouse IgG1, κ	V450
CD20	L27	Mouse IgG1, κ	V450
CD45	2D1	Mouse IgG1, κ	V500-C

TABLE 2 Monoclonal antibodies, showing clone, isotype, and conjugated fluorochrome used for flow cytometry on samples to assess T clonality.

Antibody	Clone	Isotype	Fluorescence labeling
Anti-TCR Cb1	Jovi-1	Murine IgG2a, κ	FITC
CD2	S5.2	Mouse IgG2a, κ	PE
CD5	L17F12	Mouse IgG2a, κ	PerCP-Cy5.5
Anti-TCRg/d-1	11F2	Mouse IgG1, κ	PE-Cy7
CD7	M-T701	Mouse IgG1, κ	APC
CD3	SK7	Mouse IgG1, κ	APC-H7
CD4	RPA-T4	Mouse IgG1, κ	V450
CD45	2D1	Mouse IgG1, κ	V500-C

Overall, individuals had a median of 52 years of age (range, 11–90; interquartile range [IRQ], 35–70 years) and 53.6% were female. A summary of the demographic data are shown in Table 3.

Most patient samples were tissue samples (Onuoha et al., 2018), which were comprised by fine needle aspiration (FNA; 5), incisional biopsies (Fanny et al., 2020), and excisional biopsies (Muñoz-García et al., 2021). The rest of the samples are peripheral blood (Muñoz-García et al., 2021), bone marrow (Novikov et al., 2019), breast implant-associated seromas (Hsi et al., 2014), and pleural fluids (Alaggio et al., 2022). Two patients had concurrent samples analyzed. Cases 46 and 47 correspond to a single patient with a bone marrow sample and an excisional tissue biopsy. Cases 58 and 59 correspond to a single patient with an incisional lung biopsy and a pleural fluid sample.

3.2 | Normal range of TRBC1 expression in peripheral blood

To define the normal biological range of TRBC1 expression, we first performed flow cytometric immunophenotypic analysis of 10 peripheral blood samples from healthy donors. Samples were selected based on WBC differential counts within normal limits (5000–10,000 WBC/μL). TRBC1 expression on CD4+ and CD8+ T cells was assessed. All 10 HD PB samples showed a polytypic pattern of TRBC1 expression. Within the T-cell population, a mean (±SD) of 42% (4.2%) of TαβCD4+ cells and 38% (4.7%) of TαβCD8+ cells expressed TRBC1. This resulted in a TRBC1+/TRBC1-ratio of 0.72 ± 0.129 for CD4+ and 0.61 ± 0.130 for CD8+ T cells. These percentages and ratios were used as our normal reference values for polyclonal T cells and were used as a reference for testing all patient samples.

Cut-off values were defined as a mean TCRBC1 expression ±3 standard deviations (SD). The intervals in which 99.7% of the expected normal percentages for TRCB1 expression in the CD4+ T-cells was from 29.4 to 54.6%, and for the CD8+ T-cells from 23.9% to 52.1% (Figure 1a,b). The normal range considered for the TRBC1+/TRCB1- ratio for CD4+ T cells was between 0.33 and 1.1, and for CD8+ T cells between 0.22 and 1.0 (Figure 1c,d). These confidence intervals were used to assess the expression of TRBC1 in all subsequent experiments.

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TABLE 3 Baseline patient characteristics.

Characteristic	Donors	Non-neoplastic	T-cell NHL
n	10	41	18
Age, median (years; IQR)	42 (35–45)	53 (32–71)	56 (44–66)
Sex (*n*; %)			
Female	7 (70%)	22 (54%)	8 (44%)
Male	3 (30%)	19 (46%)	10 (56%)
Sample (*n*)			
PB	10	3	9
BM	0	7	3
Tissues	0	24	5
Seroma	0	6	0
Pleural fluid	0	1	1
CD4/CD8 ratio			
Abnormal	0	23 (56%)	12 (67%)
TRBC1			
Polytypic	10 (100%)	41 (100%)	0
Monotypic	0	0	18 (100%)

Abbreviations: BM, bone marrow; IQR, interquartile range; NHL, non-Hodgkin's lymphoma; PB, peripheral blood.

3.3 | Monotypic TRBC1 expression in patient samples

18 of 59 (30.5%) samples showed a restricted (monotypic) expression of TRBC1. 6/18 were monotypic positive (33.3%) and 12/18 were monotypic negative (66.6%). Monotypic expression of TRBC1 was bright and homogeneous in most cases with positive expression, although in some cases TRBC1 expression was low (Figure 2). Negative monotypic expression of TRBC1 contrasted the polytypic expression of TRBC1 in normal residual T cells, and in most cases, the abnormal TRBC1-negative population displayed T-cell lineage markers (Figure 3). In addition, most cases with a monotypic TRBC1 expression also exhibited aberrant expression of one or more T-cell markers (CD2, CD3, CD5, CD7) and/or an abnormal CD4/CD8 ratio (67%) (Table 4).

Because the calculated normal ranges of TRBC1 expression and the TRBC1+/− ratio were defined in peripheral blood, we evaluated our cut-off values in the patient samples. All monotypic TRCB1 expression and ratio fell outside the pre-defined normal ranges (Figure 4).

A final diagnosis of a T-cell neoplasm was confirmed clinically and/or by histopathological studies in 15 of the 18 cases (83.3%), and 3 of the 18 monotypic TRBC1 expression cases were discordant results (16.7%). In six cases the flow cytometry report was confirmed positive by a concomitant biopsy, including three cases of an anaplastic large-cell lymphoma (ALCL), two cases of a peripheral T-cell lymphoma non-otherwise specified (PTCL-NOS), and one case of a T-cell large granular leukemia-lymphoma (T-LGLL) in the BM. In nine cases, the diagnosis was made clinically. There were five cases of Sezary syndrome, in which all patients had a previous cutaneous biopsy positive for Mycosis fungoides; three cases of T-LGLL without confirmation in the BM, and one case of ALCL in leukemic phase in a patient with a previous diagnosis of the ALCL. Of all studies, a molecular TCR gene rearrangement assay was available only in 3 of 15 cases, all of which confirmed clonality.

Of the three discordant cases, the first was a tissue sample from a core biopsy of a mediastinal mass where flow cytometry showed a dominant CD8-positive clone, with monotypic positive expression of TRBC1. The definitive biopsy showed a lung adenocarcinoma. The second and third cases were two complementary samples from the same patient. One of pleural tissue and the other of pleural fluid. By flow cytometry, both samples were monoclonal negative for TRBC1, with expression of cytoplasmic CD3, partial expression of CD7, and expression of CD30 and CD38. The definitive biopsy of pleural tissue confirmed a primary effusion lymphoma (PEL).

3.4 | Polytypic TRBC1 expression in patient samples

Using our predefined normal expression ranges, polytypic expression of TRBC1 was identified in 41 of the 59 samples tested. All non-neoplastic T cells also displayed normal T-cell antigen expression.

Non-neoplastic samples included PB and BM ($n = 10$), tissues ($n = 24$), seromas/pleural fluid ($n = 7$). CD4+ cells were positive for TRBC1 expression in 44% (±3.8%), 42% (±4.6%) and 40% (±7.4%) of PB/BM, tissue, and seroma/pleural fluid, respectively. CD8+ cells were positive for TRBC1 expression in 35% (±8.2%), 35% (±5.9%) and 34% (±7.9%) of PB/BM, tissue and seroma/pleural fluid, respectively (Figure 5a). Differences in the TRBC1 expression between sample types were not significant.

Mean TRBC1+/TRBC1- ratio among CD4+ cells were 0.80 (±0.12), 0.73 (±0.13) and 0.70 (±0.20) for PB/BM, tissue and seroma/pleural fluid, respectively. For CD8+ cells the ratios were 0.56 (±0.2), 0.55 (±0.16) and 0.53 (±0.18) for PB/BM, tissue, and seromas/pleural fluid, respectively (Figure 5b).

None of the non-neoplastic samples had evidence of a T cell neoplasm. The bone marrow samples had adequate cellularity and one had an angioleiomyoma. Among the tissues, diagnosis by IHC included Hodgkin lymphoma (Hsi et al., 2014), granulomatous lymphadenitis or sarcoidosis (Herrera et al., 2014), diffuse large B-cell lymphoma (Herrera et al., 2014), dermatopathic lymphadenopathy (Jiang et al., 2017), reactive lymphoid hyperplasia (Jiang et al., 2017), melanocytic nevus (Jiang et al., 2017), mesenteric panniculitis (Jiang et al., 2017), large cell neuroendocrine carcinoma (Jiang et al., 2017), Kaposi Sarcoma (Jiang et al., 2017), basaloid carcinoma (Jiang et al., 2017) and negative for neoplastic cells (Swerdlow et al., 2016). The remaining 10 samples which included 3 PB, and 7 seromas were clinically confirmed as non-clonal.

4 | DISCUSSION

The diagnosis of a T lymphoproliferative neoplasm demands to prove one or multiple aberrations in the immunophenotype, in the tissue

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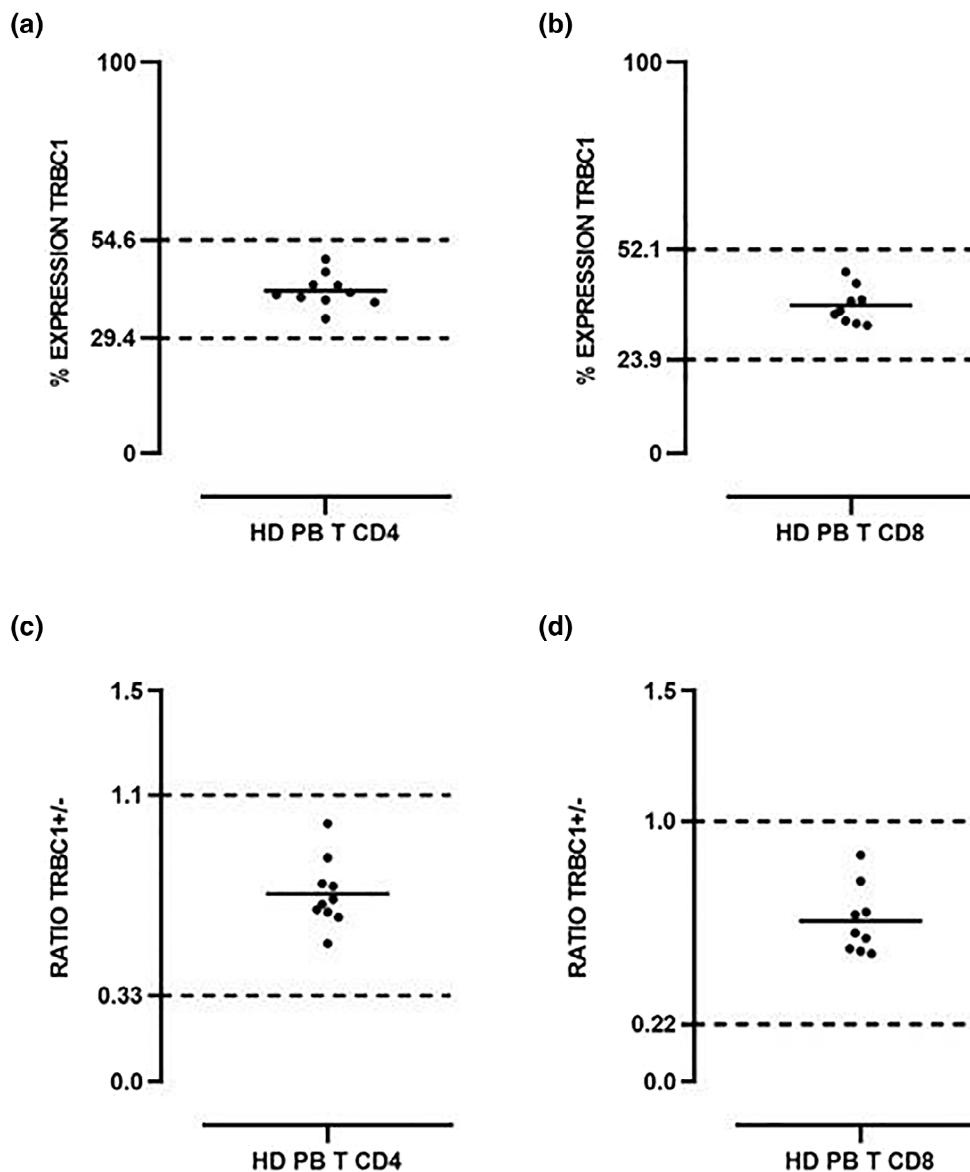

FIGURE 1 TRBC1 expression patterns on benign CD4-positive and CD8-positive T-cell subsets from peripheral blood from healthy donors. Percentages range of TRBC1-positive events on benign CD4-positive (a) and CD8-positive (b) subsets identified. Ratio TRBC1+/− on benign CD4-positive and CD8-positive T-cell subsets from peripheral blood from healthy donors. Ratio range on benign CD4-positive (c) and CD8-positive (d) subsets identified. Graph shows data from 10 HD PB (p = ns).

structure or composition, and ideally to demonstrate clonality. Extensive immunotypic characterization is expensive, time-consuming and, as such, unfit for a screening method (Capone et al., 2022; Waldron et al., 2022). Molecular clonality assays are also of high cost, laborious, and there is significant time-to-response involved (Jevremovic & Olteanu, 2019). In our study, we have validated TRCB1 in the routine characterization of T cells. We also show that TRBC1 can serve as a clonality assessment in any sample, from standard peripheral blood or bone marrow samples to solid fresh tissues. Moreover, TRBC1 expression can easily be integrated into EuroFlow LST, with the potential to improve time to final diagnosis and to decrease T-NHL misdiagnosis.

Our results validate what previous studies have reported regarding normal and pathological values of TRCB1 by flow cytometry (Berg et al., 2023; Horna, Shi, et al., 2021; Muñoz-García et al., 2021; Novikov et al., 2019). Normal results of TRBC1 expression are consistent among different groups, with normal positivity values of 44% (±4.3%) and 39% (±11%) for CD4+ and CD8+ T cells, respectively. The distribution of TRBC1 expression in CD4+ and CD8+ T cells, considering a 99.7% confidence interval, is 32%–57% and 7%–71%, respectively (Novikov et al., 2019). As a conservative estimate, a range of positive events for TRBC1 between 15% and 85% can be used to define polyclonal cells (Shi et al., 2020). The results are in line with these recommendations and with previous studies, with all our polyclonal samples falling within these ranges, none of which ended as a false-negative result. However, we did have three discordant results suggesting a potential for false-positive diagnosis by flow cytometry. In the first case, the sample was an incisional biopsy of a mediastinal tumor with a dominant clone of large CD8+ T-cells with monoclonal-positive TRBC1 expression. Here, the pathological cells were large and had high complexity, which may have determined significant nonspecific binding of some of the markers. The second and third cases, both from the same patient, showed the same pattern of double-negative monotypic-negative TRBC1 T cells suggesting a PTCL-NOS. The pleural tissue biopsy concluded a PEL due to Epstein–Barr Virus

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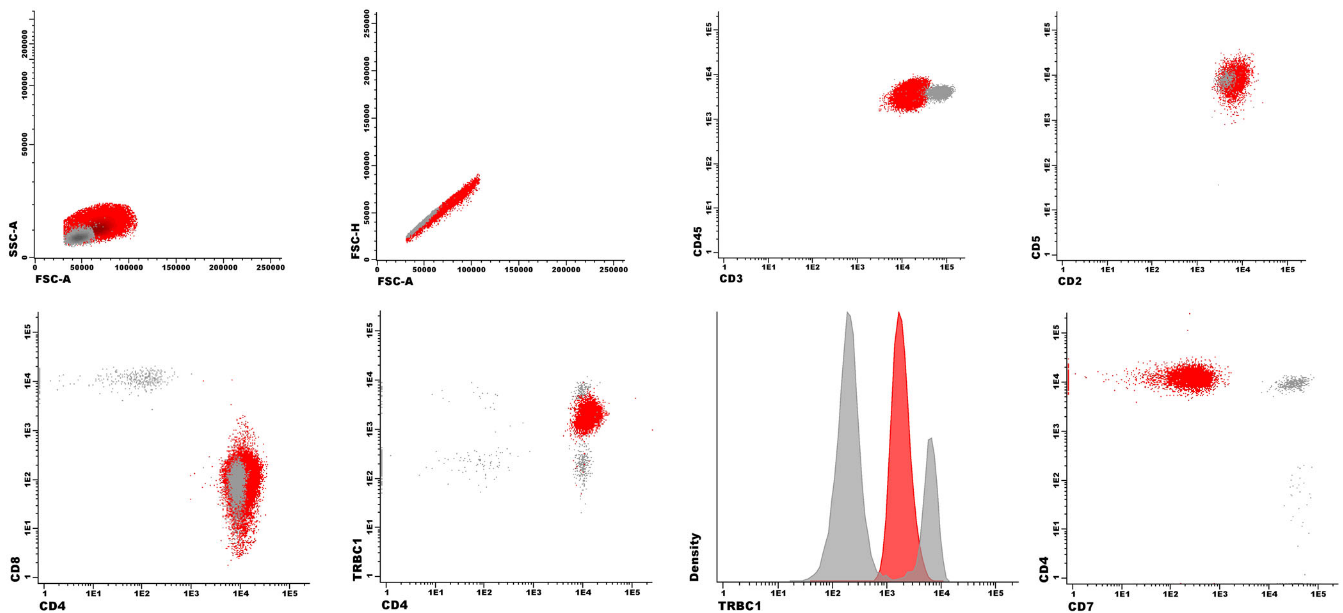

FIGURE 2 TRBC1 expression in a PB sample involved by SS (case #42). The neoplastic T cells (red) are surface CD3 (weak), CD4 (+), CD45 (+) and surface TRBC1 (dim). Also shown are subsets of normal T cells (gray), with polytypic expression TRBC1 in the surface membrane. [Color figure can be viewed at wileyonlinelibrary.com]

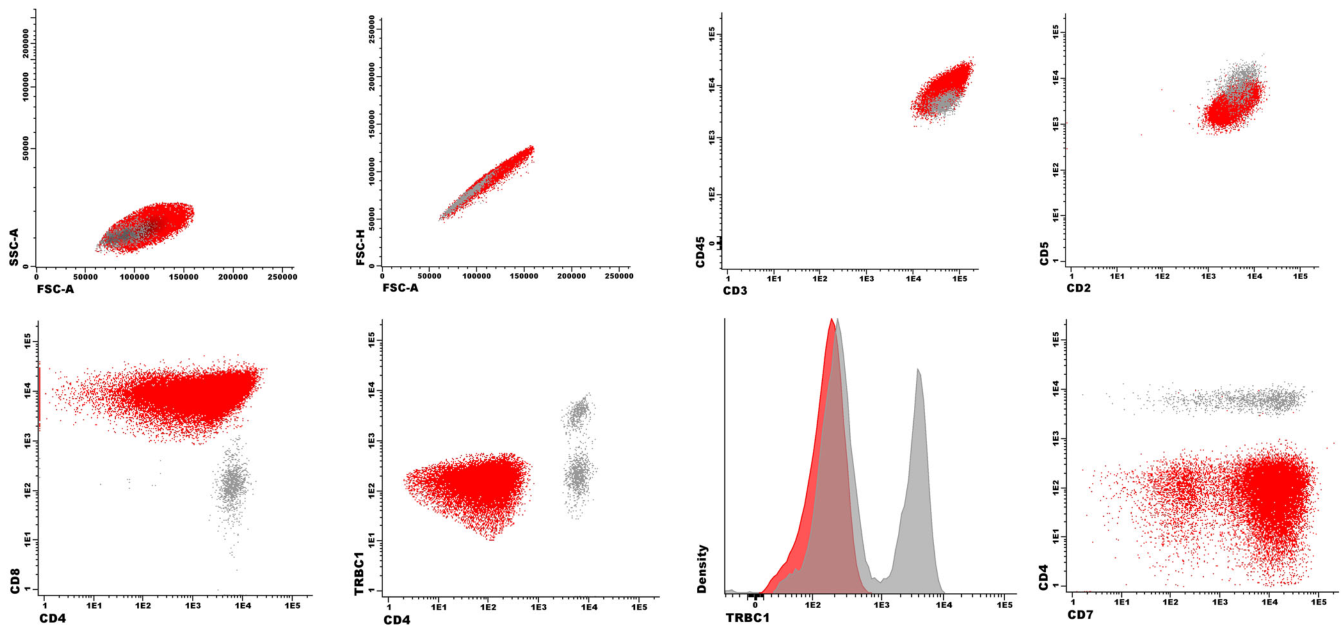

FIGURE 3 TRBC1 expression in a BM sample involved by T-cell large granular lymphocytic leukemia (case #35). The neoplastic T cells (red) are surface CD3 (+), CD8 (+), CD45 (+) and surface membrane TRBC1 (−). Also shown are subset of normal CD4+ T cells (gray), with polytypic expression TRBC1 in surface membrane. [Color figure can be viewed at wileyonlinelibrary.com]

(EBV) positive expression by IHC, without the presence of B-cell markers (CD20 and PAX5), as commonly reported (Liu et al., 2022). Because the presence of Human Herpes Virus (HHV)-8 was not performed to confirm PEL, and two different samples showed a similar immunophenotypic pattern, we believe that this case may have been a true PTCL (Hu et al., 2021; Alkhasawneh et al., 2022). In these cases, a molecular T-cell clonality study should have been performed to clarify these discrepancies.

The extent of the utility of TRBC1 in the diagnosis of T-NHL can still increase and provide novel patterns in the diagnosis of specific entities. In the diagnosis of T-LGLL the determination of TRBC1 expression by flow cytometry has a sensitivity of 100%, which could

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TABLE 4 Clinical and laboratory characteristics in cases with T-cell NHL by flow cytometry.

Case (#)	Age (years)	Sex	Sample	Clonal T-cells (%)	Positive marker expression	smTRBC1	MFC report diagnosis	Clinical/IHC diagnosis
2	56	F	PB	23.27	smCD3 CD2 CD5 CD4	Positive	CD4+ T-cell lymphoma	Cutaneous T-cell lymphoma
20	82	M	FNA	63.54	smCD3w CD2 CD7 CD4 CD30	Negative	Anaplastic large cell lymphoma	Anaplastic large cell lymphoma
24	64	M	PB	40.47	smCD3 CD2 CD5p CD7p CD8	Negative	CD8+ T-cell lymphoma	T-cell large granular lymphocytic leukemia
27	53	M	Tissue	64.85	smCD3 CD2 CD5w CD7 CD8	Positive	CD8+ T-cell lymphoma	Lung adenocarcinoma[a]
29	74	F	PB	37.00	smCD3 CD2w CD5 CD4	Negative	CD4+ T-cell lymphoma	Sézary syndrome
31	71	F	Tissue	4.25	smCD3 CD2 CD7	Positive	DN T-cell lymphoma	Anaplastic large cell lymphoma
35	58	M	BM	68.27	smCD3 CD2 CD5 CD7p CD8	Negative	CD8+ T-cell lymphoma	T-cell large granular lymphocytic leukemia
36	48	M	PB	74.75	smCD3 cyCD3 CD2 CD5p CD4	Positive	CD4+ large T-cell lymphoma	Anaplastic large cell lymphoma
40	51	F	PB	5.20	smCD3 CD2 CD5 CD7p CD8	Negative	CD8+ T-cell lymphoma	T-cell large granular lymphocytic leukemia
42	34	F	PB	61.40	smCD3w cyCD3 CD2 CD5 CD4	Positive	CD4+ T-cell lymphoma	Sézary syndrome
46	44	M	BM	15.20	cyCD3 CD2 CD5 CD7	Negative	Low-grade T-cell lymphoma	Peripheral T-cell lymphoma[b]
47	44	M	Tissue	57.05	smCD3w CD2 CD5 CD7	Negative	DN T-cell lymphoma	Aggressive Peripheral T-Cell Lymphoma[b]
50	61	F	PB	2.70	smCD3 CD2 CD5w CD8	Positive	CD8+ T-cell lymphoma	Peripheral T-Cell Lymphoma[b]
51	74	F	PB	40.42	smCD3 CD2 CD5 CD4	Negative	CD4+ T-cell lymphoma	Sézary syndrome
53	46	M	BM	19.69	smCD3 CD2 CD5 CD4	Negative	CD4+ T-cell lymphoma	T-cell prolymphocytic leukemia
57	58	F	PB	5.19	smCD3 CD2 CD5 CD7 CD4	Negative	CD4+ T-cell lymphoma	Sézary syndrome
58	42	M	Tissue	26.44	cyCD3 CD7p CD30 CD38	Negative	DN T-cell lymphoma	Primary effusion lymphoma EBV +[a]
59	42	M	PF	81.36	cyCD3 CD7p CD30 CD38	Negative	DN T-cell lymphoma	Primary effusion lymphoma EBV +[a]

Abbreviations: BM, bone marrow; cy, cytoplasm; DN, double negative; NHL, non-Hodgkin's lymphoma; p, partial; PB, peripheral blood; PF, pleural fluid; sm, surface membrane; w, weak.
[a]Discordant case.
[b]Molecular TCR gene rearrangement assay.

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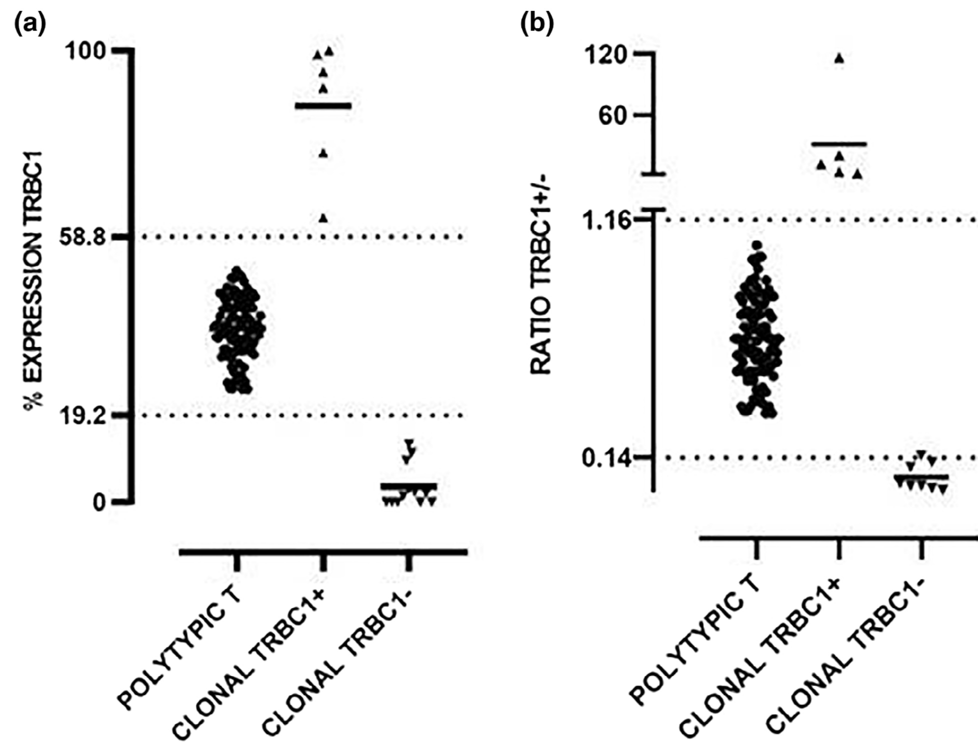

FIGURE 4 TRBC1 expression patterns in patients with malignant mature T-cell tumors. Range percentages of TRBC1-positive events on benign CD4-positive and CD8-positive subsets identified in polyclonal specimens (a). Ratio TRBC1+/− in patients with malignant mature T-cell tumors. Ratio range on benign CD4-positive and CD8-positive subsets identified in polyclonal specimens (b). The graph shows data from 53 polyclonal samples and 18 samples with evidence of T-cell malignancy.

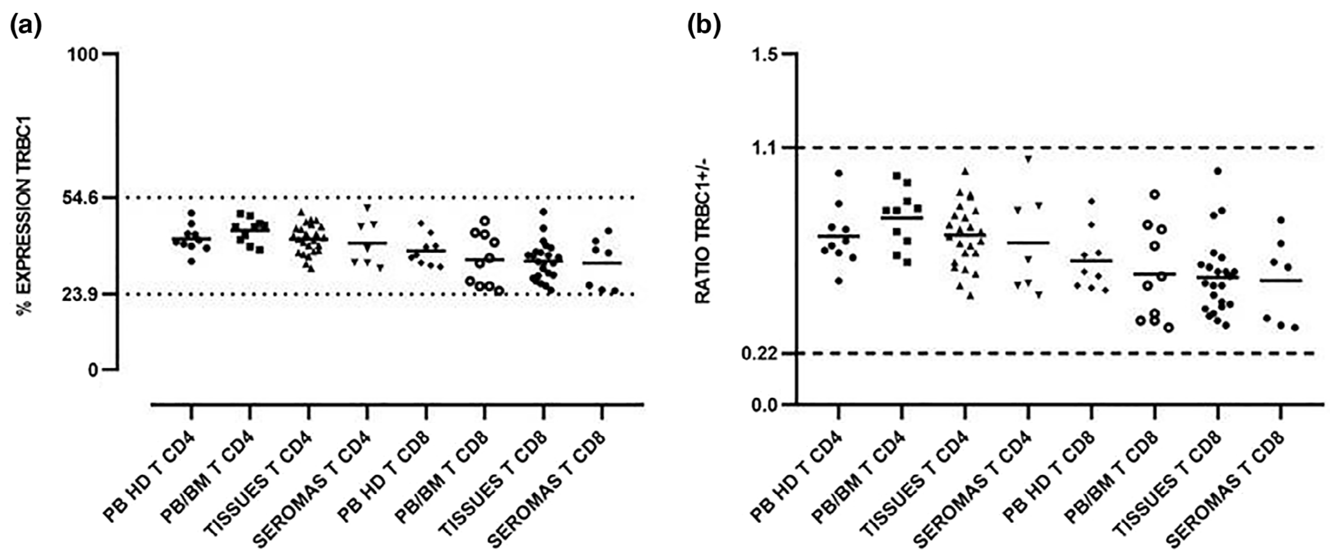

FIGURE 5 TRBC1 expression patterns on benign CD4-positive and CD8-positive T-cell subsets from patients with no evidence of a T-cell malignancy. Range percentages of TRBC1-positive events on benign CD4-positive and CD8-positive subsets identified (a). Ratio TRBC1+/− on benign CD4-positive and CD8-positive T-cell subsets from patients with no evidence of a T-cell malignancy. Ratio range on benign CD4-positive and CD8-positive subsets identified (b). The graph shows data from 10 HD PB, 10 PB/BM, 24 tissues and 7 seromas with no evidence of T-cell malignancy (p = ns).

replace the need of complementary molecular or invasive studies (Horna, Olteanu, et al., 2021; Muñoz-García et al., 2022). In our study, we were able to detect and confirm two cases of T-LGLL, until then with a suspected diagnosis. Other relevant diagnostic additions could include the detection of small clones of chronic or smoldering HTLV1 adult T-cell lymphoma-leukemia (ATLL) or intestinal T-cell neoplasms, often hard to diagnose. Similarly, the high sensitivity of TRBC1 expression can provide a significant tool to immunologists seeking to rule out the diagnosis of a T-NHL in patients with immune dysregulations or T-cell proliferation.

The limitations of TRBC1 expression include first the need to replace a marker in the panel. Current EuroFlow panels include a maximum of 8–10 colors, which allows a limited number of markers to be included. Although now there are several fluorochromes are available

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for TRBC1, we used a FITC because it was reported to have better staining stability after sample collection of up to 72 h (Muñoz-García et al., 2021). We created our own secondary tube so as not to modify the EuroFlow recommended tube, which does not limit other laboratories from standardizing their own tube. We replaced CD8 with TRBC1 in FITC in the second tube. CD8+ T cells were analyzed in the first LST tube and in the second tube they were analyzed as the CD3+/CD4-/TCRγδ-cells. A second clear limitation is the lack of an anti-TRBC2 mAb. Monoclonal negative or intermediate cases open the question of false negative samples, which forces a repetition of the analysis, ideally with cytoplasmic expression of TRBC1.

Intracellular evaluation of TRCB1 (cyTRBC1) can provide a highly sensitive and specific evaluation of clonality among T-cell precursor neoplasms. Aberrant T-cell acute lymphoblastic leukemia (T-ALL) clones display cyTRBC1 restriction, which is not seen in normal thymocytes (Horna et al., 2022; Wheeler et al., 2022). In our study, we did not include patients with suspected T-ALL, but plan to incorporate it in future analysis of mediastinal masses and in the evaluation of acute leukemias in bone marrow samples. However, we present two cases of double-negative T-NHL with null surface TRBC1 expression, clonally confirmed by molecular biology. These cases demonstrate how critical it is to permeabilize the sample and to demonstrate whether there is cytoplasmic restriction of TRBC1. It has also been reported that in those cases that lose CD3 on the surface it is necessary to evaluate clonality in differentiation trajectories using CD45RA and CD45RO (Pu et al., 2022).

Future directions include the necessary evaluation of TRBC2. Several groups are working on the development of an anti-TRCB2 mAb (Maciocia et al., 2018; Onuoha et al., 2018). This antibody would likely improve the sensitivity and specificity of flow-guided T-cell clonality, such as kappa and lambda immunoglobulin light chains in the current routine identification of clonal B cells. Although not commercially available yet, one group has reported the modification of the JOVI.1 clone to change its specificity from TRBC1 to TRBC2 (Ferrari et al., 2020).

5 | CONCLUSIONS

In conclusion, our results show that the flow cytometry-based expression of TRBC1 is a robust and quick method for T-cell clonality assessment. TRBC1 expression can easily be integrated into routine lymphoma screening strategies using flow cytometry, and across several sample types.

ACKNOWLEDGMENTS

We thank Mirentxu Iruretagoyena for her outstanding work in our laboratory and for having always provided temperance and harmony to our group.

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How to cite this article: Castillo, F., Morales, C., Spralja, B., Díaz-Schmidt, J., Iruretagoyena, M., & Ernst, D. (2024). Integration of T-cell clonality screening using TRBC-1 in lymphoma suspect samples by flow cytometry. *Cytometry Part B: Clinical Cytometry*, *106*(1), 64–73. https://doi.org/10.1002/cyto.b.22147

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