Nuevas estrategias de diagnóstico de clonalidad T por citometría de flujo

A novel flow-cytometry-based assay to assess T-cell clonality

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Background

Diagnosis of T-cell chronic lymphoproliferative disorders (T-CLPD) in cases with lymphocytosis or suspected T-cell populations is often challenging, due to the lack of fast and reproducible routine diagnostic assays for T-cell clonality, together with the fact that malignant/clonal T-cells and normal (reactive) polyclonal T-cells may show morphologic and immunophenotypic similarities[1]. Accordingly, despite flow cytometry (FCM)-based T-cell receptor Vβ (TCRVβ) repertoire and/or polymerase chain reaction (PCR)-based TRB and/or TRG gene rearrangement analysis assays are used to assess T-cell clonality in the diagnostic work-up of T-CLPD[2,3], both approaches show limitations for routine implementation and/or they are not available in many diagnostic laboratories. Therefore, the availability of a simple, fast, and reliable approach for screening/assessment of T-cell clonality is strongly welcomed to be used in clinical settings.

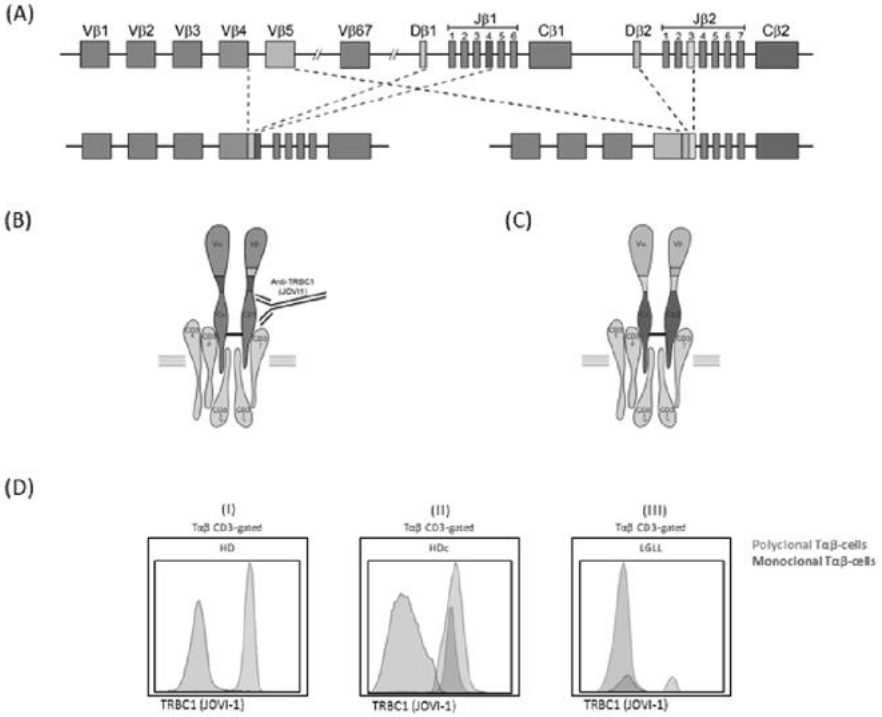
Recently, a single antibody (anti-TRBC1; JOVI-1 antibody clone) against one of the two mutually exclusive T-cell receptor β-chain constant domains has been identified as a FCM marker potentially useful to assess Tαβ-cell clonality[4], based on the monotypic vs. polytypic expression observed in monoclonal vs polyclonal cells, respectively (Figura 1). Despite recent reports had preliminarily shown the potential utility of this antibody for assessment of Tαβ-cell clonality in T-CLPD vs. normal/reactive conditions[4-6], optimization of the staining protocol for routine use in diagnostic laboratories, as well as normal reference values for both normal and reactive Tαβ-cells (and their different subsets) had not been provided. Similarly, the demonstration of both the specificity and (analytical) sensitivity of FCM assessment of the TRBC1-expression profile of Tαβ-cells for detecting clonal Tαβ-cells, including the validation of the assay against the gold standard

(i.e., PCR), were still missing at the beginning of our study. Therefore, we aimed to optimize the flow cytometric method for routine use of anti-TRBC1 to assess T-cell clonality in a fast and robust manner, and to validate it in a large series of normal and pathological samples, including T-large granular lymphocyte leukemia (T-LGLL) cases.

Optimization and validation of the TRBC1-FCM protocol approach for detecting clonal Tab cells

The potential steric hinderance between surface membrane CD3 and TRBC1 was tested in paired aliquots of 11 blood samples from healthy donors (HD) under four different staining conditions [1]: (a) staining with TRBC1 only; and with both CD3 and anti-TRBC1 reagents, where CD3 was added; (b) 10 min after the anti-TRBC1 reagent; (c) simultaneously or (d) 10 min before TRBC1. We showed that TRBC1 labeling significantly improved in the presence of CD3, and that the best resolution to accurately identify TRBC1+ cells was achieved by adding the CD3 antibody either simultaneously or after TRBC1, but not before [1]. Further confirmation of mutually exclusive TRBC1 vs. TRBC2 gene usage in different

Figura 1. Schematic representation of TRB gene rearrangement and interpretation of the TRBC1 antibody (JOVI-1 clone)-based flow cytometry approach: **(A)** Mutually exclusive TRBC selection during TRB gene rearrangement in the thymus; **(B,C)** Representation of the two resulting TRB complex structures, composed of either the TRBC1 **(B)** or the TRBC2 **(C)** proteins, and specific binding of the anti-TRBC1 antibody to TRBC1 but not to TRBC2; **(D)** Illustrative histograms of TRBC1 staining of blood Tαβ-cells from: (I) one representative adult HD, showing the bimodal TRBC1 expression pattern, typical of polyclonal Tαβ-cells (TRBC1^{+} in light blue and TRBC1^{-} in green); (II) one HDc showing a minor TRBC1^{+} (clonal) Tαβ-cell population (in red), among a majority of polyclonal Tαβ-cells; and (III) one LGLL case with a major population of TRBC1^{-} monoclonal Tαβ-cells (in red) with a minor background of polyclonal Tαβ-cells. Monoclonal T-cells were selected by the presence of a phenotypic aberrancy and/or expression of a single TCRVβ family, both in HDc (e.g., CD8^{+}TCRVβ16^{+}) and in LGLL (e.g., CD8^{+}CD279^{++}) cases. *Abbreviations (alphabetical order):* HD, healthy donor; HDc, healthy donor with a small Tαβ-cell clone in blood; LGLL, large granular lymphocytic leukemia.



From: Fig 1 Muñoz-García N, et al. Cancers (Basel). 2021 Aug 30;13(17):4379 [1]

populations of Tαβ-cells with a TRBC1-positive vs. TRBC1-negative phenotype was assessed in genomic DNA extracted from 95 FACS-sorted Tαβ+ cell populations -collected from HD and patients with confirmed T-CLPD-; as expected, purified TRBC1+ and TRBC1– Tαβ-cells rearranged TRBJ1 in 44/47 (94%) and TRBJ1+TRBJ2 in 48 of 48 (100%) populations, respectively, confirming the high specificity of the TRBC1-FCM assay.

Analysis of the TRBC1+/TRBC1– ratio in distinct subsets of polyclonal (normal and reactive) Tαβ-cells according to the Tab-cell lineage (TCD4+, TCD8+, Tαβ double positive and double negative cells), the TCR-Vβ family repertoire and the maturational stage (naïve, central memory, effector memory and effector cells)

Here we provide TRBC1+/TRBC1– ratios within the different Tαβ-cell subsets (TCD4+, TCD8+, Tab double positive and double negative cells) as reference for polyclonal cells. The percentage of TRBC1+ cells and the TRBC1+/TRBC1- ratio of 65 HD plus 18 reactive lymphocytosis was calculated and used to derive normal range values for polyclonal (normal and reactive) cells, which are specified in Table 1. Importantly, we also calculated ranges defined by the mean ± 3 standard deviations, which define intervals where 99.7% of TRBC1+/TRBC1- ratios from polyclonal cells fall, to provide reference cut-off values for defining monoclonal vs. polyclonal Tαβ-cell profiles (Tabla 1).

We also analyzed the $TRBC1^{+}/TRBC1^{-}$ ratios within the different TCR-Vβ families (stained with the IOTest® Beta Mark TCRVβ Repertoire Kit) and for all the 24 TCR-Vβ families identified with the kit, a bimodal pattern of TRBC1 expression profile was found. Accordingly, despite different median $TRBC1^{+}/TRBC1^{-}$ ratios were found among cells expressing each of the TCR-Vβ families (especially in reactive cases), TRBC1 expression appeared to be independent of the specific TCRVβ family expressed among HD, as well as reactive lymphocytosis patients, since a polytypic (bimodal) pattern was found for all families.

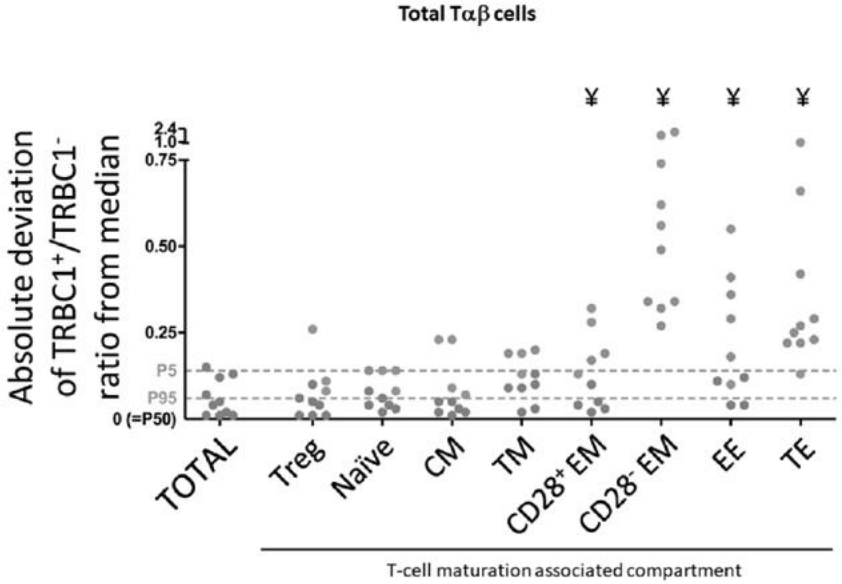
Further, we investigated the $TRBC1^{+}/TRBC1^{-}$ ratio distribution within different maturation-associated compartments of Tαβ-cells from a subgroup of 10 adult HD (Figure 2). Our results showed that at earlier maturation stages (i.e., naïve, central memory and transitional memory Tαβ-cells) most samples were within the 5th and 95th percentiles observed for the total population of Tαβ-cells in normal/reactive blood. In contrast, at the more mature stages of effector memory, early effector and terminal effector Tαβ-cells, a statistically significant number of cases were outside the normal (5th and 95th percentile) range observed for total Tαβ-cells (i.e., more dispersed TRBC1+/TRBC1- ratios) (Figure 2)[1].

TABLE 1. Ranges for polyclonal (normal and reactive) total Tαβ-cells and their major Tαβ-cell populations in blood (n=83) as defined by the mean percentage of TRBC1+ cells and the mean TRBC1+/TRBC1- ratio ± 3 standard deviations (3 SD).

Tαβ-cell subset	% TRBC1+ cells*		TRBC1+/TRBC1- ratio		Probability (%) of finding a clonal Tαβ expansion when TRBC1+/TRBC1– ratio is outside the range mean ± 3 SD (P-value)
	Mean ± 1 SD	Range (Mean ± 3 SD)	Mean ± 1 SD	Range (Mean ± 3 SD)	
Tαβ cells	40 ± 6.7	20–60	0.66 ± 0.071	0.25–1.4	99.73% (<0.001)
Tαβ CD4+	43 ± 6.3	24–62	0.75 ± 0.067	0.31–1.6	
Tαβ CD8+	35 ± 8.8	8.3–61	0.53 ± 0.096	0.091–1.6	
Tαβ DP	36 ± 12	1.6–71	0.57 ± 0.13	0.016–2.5	
Tαβ DN	29 ± 10	0-61	0.41 ± 0.12	0–1.5	

Abbreviations (alphabetical order): DN, doble negative; DP, double positive; PB, peripheral blood; SD, standard deviation; TR, T-cell receptor [1 Muñoz-García N, et al. Cancers (Basel). 2021 Aug 30;13(17):4379].

FIGURE 2. TRBC1+/TRBC1− ratio of normal total Tαβ-cells according to their maturation stage. TRBC1+/TRBC1− ratio observed among normal Tαβ-cells from 10 adult healthy donors, distributed into different maturation-associated compartments, represented both in individual ratio values per maturation stage. Dots correspond to results from individual experiments, while the notched box represent 25th and 75th percentile values, lines inside the box correspond to median values (50th percentile) and whiskers represent minimum and maximum values. The continuous horizontal and dotted lines that cover the entire graph correspond to median values (percentile 50) and both the 5th and 95th percentiles (P5 and P95), respectively. Cases below P5 are depicted in blue, while cases above P95 are colored as orange dots. ¥P-value ≤0.05 vs. total Tαβ-cells.



Abbreviations (alphabetical order): CM, central memory; EE, early effector; EM, effector memory; TE, terminal effector; TM, transitional memory; Treg, regulatory T-cells.
From: Fig 3B Muñoz-García N, et al. Cancers (Basel). 2021 Aug 30;13(17):4379 [1]

Validation of the TRBC1-FCM approach for detecting clonal Tab cells through comparison with the reference TCR-Vβ-FCM and/or molecular techniques for assessment of Tαβ-cell clonality

Upon comparing the TRBC1-FCM assay with the reference TCRVβ-FCM and/or molecular techniques for assessment of Tαβ-cell clonality, concordant results were found in 112 of 117 cases (96%). Concordant cases corresponded to 21 of 24 poly/oligoclonal cases (87%) that showed a polytypic TRBC1 profile by FCM and 91 of 93 monoclonal samples (98%) that displayed a monotypic TRBC1-FCM pattern (p<0.0001). There were only five of 117 discrepant cases (4.3%), either because cases that were classified as poly/oligoclonal by PCR showed a monotypic pattern of TRBC1 (n=3) or because monoclonal cases by PCR showed a polytypic TRBC1 pattern (n=2).

Evaluation of the analytical sensitivity of the TRBC1-FCM approach for detection of progressively low numbers of clonal Tαβ cells

Serial dilution experiments (n=8 blood samples) of clonal Tαβ-cells in normal leukocytes, performed both directly and *in silico* showed a high degree of correlation between the percentage of clonal Tαβ-cells identified among cells that displayed an aberrant/suspicious phenotype by monotypic expression of TRBC1 vs. expression of a specific TCRVβ region (R^2 = 0.966; p < 0.001) with a sensitivity of at least 10^{-4} in seven of eight (88%) dilutional experiments. Further identification of clonal Tαβ-cells based on both the pattern of expression of TRBC1 and a specific TCRVβ family (vs. TRBC1 alone) showed a slightly improved correlation (R^2 = 0.999; p < 0.0001), with a sensitivity of at least 10−4 in eight of eight experiments.

Validation of the TRBC1-FCM approach to assess Tαβ-cell clonality in Tαβ-large granular lymphocytic leukemia (T-LGLL)

Since we had observed for the first time that the more mature polyclonal Tαβ large granular lymphocytes (Tαβ-LGL) show broader TRBC1$^+$/TRBC1$^-$ ratios (vs. total Tαβ cells) [1], we further aimed to validate the utility of this assay specifically for the diagnosis of T-cell clonality of T-LGLL, taken into consideration that most T-LGLL derive from effector memory and particularly terminal effector cells (i.e., large granular lymphocytes, LGL). For this purpose, we compared the distribution and absolute counts of TRBC1$^+$ and TRBC1$^-$ Tαβ LGL in blood containing polyclonal (n=25) vs. clonal (n=29) LGL. Our results showed that TRBC1-FCM assay is also a fast and easy method for detecting T-cell clonality in T-LGLL based on altered (increased or decreased) percentages of TRBC1$^+$ Tαβ cells of LGL suspected of T-LGLL presenting with lymphocytosis, whereas in the absence of lymphocytosis (or in TαβCD4-LGLL), the detection of increased absolute cell-counts of more precisely defined subpopulations of T-LGL expressing individual TCRVβ families would be required to detect clonal Tab cells with the TRCB1-FCM assay [7].

CONCLUSIONS

Our results support implementation of the optimized TRBC1-FCM approach as a fast, simple, cost-effective and accurate method for assessing T-cell clonality, and at the same time it shows a high specificity (96% of concordance with the gold standard for assessing T-cell clonality) and sensitivity (of at least 10-4, once used in combination with tumor-associated aberrant immunophenotypes) for detection of monoclonal Tαβ-cells in patients suspicious of T-CLPD, including those with T-LGLL. Therefore, appropriate integration of the TRBC1/CD3 reagents into comprehensive lymphocyte screening panels for the diagnostic work-up of patients presenting with lymphocytosis is strongly recommended, as well as into the current T-CLPD classification and measurable/minimal residual disease (MRD) monitoring panels.

Conflictos de interés: La autora declara no poseer conflictos de interés.

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