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Original Research

Rapid evaluation of T cell clonality in the diagnostic work-up of mature T cell neoplasms: TRBC1-based flow cytometric assay experience

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ABSTRACT

The identification of mature T cell neoplasms by flow cytometry is often challenging, due to overlapping features with reactive T cells and limitations of currently available T cell clonality assays. The description of an antibody specific for one of two mutually exclusive T cell receptor (TCR) β-chain constant regions (TRBC1) provides an opportunity to facilitate the detection of clonal TCRαβ+ T cells based on TRBC-restriction. Here we prospectively analyzed 14 healthy controls and 63 patients with the flow cytometry protocol currently used for suspected T cell neoplasm implemented with immunostaining targeting TRBC1. Specimens were firstly classified in 3 groups based on clinical records data, laboratory findings and immunophenotypic features. T cell clonality was assessed by TCR Vβ repertoire analysis and the new rapid TRBC1 assay. Results showed that TRBC1 unimodal expression was unequivocally associated with samples presenting with immunophenotypic aberrancies. Moreover, we demonstrated that the use of TRBC1 is useful in solving uncertain cases and confirmed the high sensitivity of the method in identifying small T cell clones of uncertain significance (T-CUS). Finally, we found a high degree of concordance (97%) comparing the currently available clonality assessment methods with the proposed new method. In conclusion, our results provided real-life evidence of the utility of TRBC1 introduction in the flow cytometric clonality evaluation for the routine diagnostic work-up of T cell neoplasms.

Research in context

Flow cytometric diagnostic work-up of T cell malignancies suffers from the absence of both selective markers of phenotypic aberrancies and, differently from B cell neoplasms, an easy-to-use T cell clonality assessment method. Recently, an antibody specific for one of two mutually exclusive T cell receptor β-chain constant region 1, has been described that provides an opportunity to facilitate the detection of clonal TCRαβ+ T-cells. However, to date only few data have been published about the use of this new approach in diagnostic practice. Here we provide additional evidence of the robustness and reproducibility of the method. We also confirmed that TRBC1 assay, due to its high sensitivity, will serve the scientific community on further T cell Clones of Uncertain Significance (T-CUS) knowledge. The very rapid and easy to interpret feature of this new emerging method is very promising to improve and accelerate the diagnostic practice on T cell neoplasms.

Introduction

Mature T cell neoplasms or peripheral T cell lymphomas/leukemia

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(PTCLs) are a group of relatively uncommon disorders arising from clonal proliferation of mature post-thymic lymphocytes with the vast majority being characterized by a TCRαβ+ clonal T cell population. TCRαβ+ PTCLs represent the 80-85% of mature T cell neoplasms and comprise a highly heterogeneous group of entities with variable clinical behavior. Diagnosis of T cell neoplasms relies on clinical manifestation and history in combination with histo-morphological identification of abnormal T cells possibly supplemented by immunohistochemistry, genetic and/or molecular analysis. The contribution of flow cytometry in the diagnostic work-up of mature T cell neoplasms emerged in the last years providing the demonstration of immunophenotypic aberrancies (*i.e.*, differential antigen expression from a normal counterpart population) [1]. The strategy proposed for PTCL immunophenotyping consists of different steps. In a first step, pan-T cell markers that are frequently downregulated are studied, such as CD2, CD5 and CD7. Other markers (or combinations of markers) are selected based on their contribution to a more precise subclassification such as cytotoxicity-associated T cell markers (CD56 and CD57), or activation-related proteins (CD25 and HLA-DR). Maturation-associated T cell markers CD197 (CCR7), CD45RA and CD45RO are also important. In this regard, previous studies have shown that Sézary cells typically display a CD4+ memory T cell phenotype, while T-PLL cells display a phenotype consistent with a naive/central memory T cell and the phenotype of T-LGL leukemia cells overlaps with that of terminally differentiated effector memory T cells [2,3]. Moreover, some T cell neoplasms may lack overt immunophenotypic aberrancies and/or convincing cytological abnormalities, precluding an unequivocal diagnosis. In this complex scenario, the demonstration of clonality will be extremely helpful. Unlike B cell malignancies, where staining of surface immunoglobulin light chains (kappa and lambda) is a rapid method to recognize clonality, in T cells proving clonality have been more challenging in clinical routine settings. Up to now, the demonstration of T cell clonality took advantage of Vβ T cell receptor (TCR) repertoire analysis detected either by Vβ flow cytometry [4–6] or by PCR technique [7,8], these techniques and the required interpretive expertise are not available in all laboratories. In addition, it is important to emphasize that the presence of a clone is expensive, labor intensive, characterized by limited sensitivity and suffers from a difficulty to be interpreted due to the evidence that the presence of a clonal T cell population is not synonymous with the presence of a neoplasm: T cell proliferations can be generated as a part of normal immune response to a specific antigen as well as in autoimmune disorders and with normal aging [9,10] .

The recent finding of a monoclonal antibody (moAb) specific for human TCR β chain constant region 1 (TRBC1) [11,12] opened up the possibility of a low-cost, rapid, and specific T cell clonality test for αβ-positive T-cell malignancies. The rationale of the use of this antibody lay on the evidence that, during αβ TCR rearrangement the choice between the two constant regions C1 and C2 is mutually exclusive: consequently the αβ TCR on any given T cell will have a β chain containing either C1 or C2. Since all daughter cells derived from a T cell undergoing clonal expansion will carry identical TCRs, labeling a clonal population with an antibody specific for TCRβC1 or TCRβC2 will result in either a positive or a negative signal. There is only one commercially available antibody with known specificity for a TCRβ C region 1: the TRBC1 binding monoclonal antibody (clone *JOVI.1*). Appropriate assay design is of utmost importance for the utilization of TRBC1 as a T cell clonality surrogate in clinical flow cytometry practice: it requires the evaluation of several other T cell antigens in the same analysis tube, ideally on an 8 to 10-color flow cytometry set up, allowing for the independent assessment of immunophenotypically distinct T cell subsets and the optimal separation of neoplastic cells from background benign ones. A largely unimodal TRBC1-negative or TRBC1-positive/positive diminished (TRBC1+/TRBC1+dim.) staining pattern is consistent with a restricted (unimodal) TCRβ chain constant region expression, indicative of clonality. Novikov et al. reported for the first time in 2019, mean of TRBC1+ events and 95% confidence intervals for normal total CD4-positive (CD4+), CD8-positive (CD8+) T cells [13]. In 2021, Horna et al. validated and standardized the TRBC1 staining method and published the reference ranges for TRBC1+ percentages as well as the TRBC1+/TRBC1- ratios in healthy subjects' T cell populations [14]. In this last study, the gating strategy employed for the identification of the different TRBC1+ T-cell populations was carried out by selecting the most intense data peak in either a single TRBC1 parameter histogram or a 2D (CD3 or CD4 or CD8 *vs* TRBC1) dot-plot. Similar results were obtained in patients without demonstrable T cell neoplasia, showing a bimodal expression of TRBC1 on CD4+ and CD8+ T-cell subsets gated based on distinct immunophenotypic features, with the exception of occasional small CD8+ T cell subsets with a unimodal TRBC1 expression pattern. Further analysis revealed that these small subsets were truly clonal based on TCR-Vβ-restriction and T cell gene rearrangement molecular studies, consistent with benign immunodominant clonotypes [15]. The unprecedented analytical sensitivity of TRBC1 to confidently detect small clonal T-cell populations allows it to describe T cell clones/clonopathies of unknown significance, termed T-CUS. This approach gives the opportunity to find a possible role of those populations in respect to different stimuli and to study their diagnostic and/or prognostic significance in blood involvement of T lymphopathies or other disorders frequently associated with T cell clonal expansion (autoimmunity, B cell lymphomas) .

Here we described the implementation of TRBC1 antibody in our laboratory routine flow cytometry panel for clonal T cell populations, the potential to improve the diagnostic work-flow of mature T cell neoplasms and verified its correlation with other diagnostic tools.

Materials and methods

Patients and specimen selection

Fresh peripheral blood (PB) and bone marrow aspirates (BM) specimens were obtained from healthy donors (D) enrolled at the transfusion unit or from patients received for routine diagnostic flow cytometric analysis at Careggi University Hospital in Florence and prospectively analyzed between July 2021 and March 2022.

Ethics

Procedures on healthy donors specimens were approved by the Careggi University Hospital Ethical Committee and were in accordance with the declaration of Helsinki. Relevant demographic and diagnostic data were assembled through medical records review, including laboratory test results and clinical notes, according to internal policy of privacy observance.

Flow cytometry immunophenotyping

PB and BM EDTA-anticoagulated samples were analyzed on XN-550 Sysmex Hematological analyzers for WBC count and then immunophenotyped using a direct immunofluorescence stain-and then-lyse technique, within 48h from their withdrawal. An initial assessment on T, B lymphocytes and NK cells subpopulations was performed using a cocktail of moAbs including CD3-FITC, CD56-PE, CD16-PerCP-Cy5.5, CD4-PE-Cy7, CD19-APC, CD8-APC-H7, HLA-DR-V450 and CD45-HV-500. Absolute values of conventional T cells subsets referred as CD4+ for CD3+/CD4+, CD8+ for CD3+/CD8+, as DP for CD3+/CD4+/CD8+ and DN for CD3+/CD4-/CD8- in a 2D CD4 vs CD8 dot plot on CD3-gated events were assessed and CD4/CD8 ratio was also calculated. 50 clustered events were required to consider them as a cell population. T cell subsets were further characterized by using the following two panels:

1) TCRαβ-FITC, TCRγδ-PE, CD3-PerCP-Cy5.5, CD8-PE-Cy7, CD7-APC, CD4- APCH7, CD5 HV-450 and CD45-V500.

2) CD57-FITC, CD7-PE, CD3- PerCP-Cy5.5, CD45RA- PE-Cy7, CD8-APC, CD4-APCH7, CCR7 HV-450 and CD45-V500.

Patients with a history or clinical evidence for cutaneous T cell lymphoma or an increased CD4/CD8 ratio, the second panel was replaced with: CD8-FITC, CD26-PE, CD3-PerCP-Cy5.5, CD45RA-PE-Cy7, CD7-APC, CD4-APCH7, CCR7 HV-450 and CD45-V500.

In addition, for all cases studied, flow cytometric assessment for T cell clonality was performed by using an anti-TRBC1 antibody (clone *JOVI.1*)- including panel as follow: CD5-FITC, CD2-PE, CD3- PerCP-Cy5.5, CD8- PE-Cy7, CD7-APC, CD4-APCH7, TRBC1 (clone *JOVI.1* HV-450) and CD45-V500. Full list of all fluorochrome-conjugated mAbs is in Supplementary Table S1. TRBC1 staining was already standardized and validated by others [16]. Data acquisition was performed using a 3-laser, 8-color flow cytometer (FACS Canto TMII, BD Biosciences, San Jose, CA); data analysis was performed by Infinicyt software (Cytognos SL, Salamanca, Spain).

Flow cytometric evaluation of clonality TCR-Vβ repertoire analysis

All samples were evaluated for T cell clonality by using flow cytometric TCR-Vβ (FC-Vβ) repertoire analysis using a commercial kit, comprising an eight-tube panel containing 24 moAbs known to react with specific TCR-Vβ families, which covers approximately 70% of the TCR-Vβ repertoires of normal T cells (IOTest Beta Mark TCR-Vβ Repertoire Kit; Beckman Coulter, Miami, FL, USA). Each tube contained a mixture of three different antibodies conjugated to PE, FITC, or PE and FITC, thus permitting simultaneous analysis of expression of 3 Vβ families. Monoclonal antibodies anti-CD3, -CD4, -CD8 and -CD45 were used as backbone markers to identify CD4+ and CD8+ cell subsets for analysis as elsewhere described [17]. The FC-Vβ analysis was mainly based on the criteria by Feng et al. [18], defining clonality when (i) a single Vβ was expressed by ≥50% of a gated T cell population, (ii) a Vβ was expressed at a frequency of ≥10 times above its normal value as determined by our specimens from 14 normal controls. In the presence of a percentage ≥70% of gated cells that failed to react to any of the 24 Vβ moAbs we defined the population as "non-reactive" (NR) because presumably characterized by the expression of a TCR-Vβ not recognized by the antibody panel. Oligoclonality was considered when the frequency of at least one of the Vβ families was higher than the mean 3 Standard Deviation (SD) in healthy subjects, as elsewhere described [19]. Data acquisition was performed using a 3-laser, 8-color flow cytometer (FACSCanto TMII, BD Biosciences, San Jose, CA) and then analyzed by FlowJo v10 software (BD Bioscience).

Molecular evaluation of T cell clonality

Gene rearrangement studies were performed using the BIOMED-2 kit (Invivoscribe) as previously described [7,8].

Statistical analysis

The Unpaired, non-parametric Mann–Whitney U test was applied to compare clinical parameters and deviations from mean values of both percentages of TRBC1 expressing T cells or TBRC1+/TRBC1- cells in a specific T cell subset among the three groups of patients. Data wrangling and visualization was done using R (R version 4.1.2 (2021-11-01)) and Rstudio IDE (2021.09.2 Build 382), using the tidyverse package and for tidyHeatmap for heatmap visualization. The non-parametric Fisher exact test (for categorical variables) was used to calculate the difference between frequencies. In all cases, *p values* ≤0.05 were considered significant.

Results

Patients characteristics

In order to define our ability to distinguish aberrant from normal mature αβ+ T cells, a total of 77 individuals were enrolled in this study, including 14 healthy donors (D) used as healthy controls. All samples were processed according to our routinary characterization of specimens with a suspected T cell lymphoproliferative disorder. The immunophenotypic characterization was performed, as described in Materials and Methods section, and the observed features were used to identify T lymphocytes' alterations (*i.e.* CD4/CD8 altered ratio, iper- o ipo-expression of CD4 CD8 or CD3), cross lineage markers expression (*i.e* abnormal expression of CD16 and/or CD56 on CD4+ T cells) and T lymphocytes activation status (*i.e* HLA-DR expression). Of note, only αβ+ T cell subsets were analyzed in all the samples included in the study.

Patients' samples were classified in two main cohorts based on immunophenotype evaluation, patient's history and clinical data (where available), morphological findings and other laboratory records, named Reactive (R) and Pathological (P). A summary of the most relevant findings on all enrolled subjects is shown in Table 1. In detail, reactive patients were defined, in the presence of two compelling evidence of activation among clinical data (*i.e* documented EBV, CMV, HIV and SARS-CoV-2 infections), leuco/lymphocyte counts (abnormal CD4/CD8 ratio, CD4+ or CD8+ T cells lymphocytosis) or immunophenotypic features (*i.e:* CD5 or CD7 diminished expression). The cohort of pathological patients was defined by the concurrent evidence of hematological abnormalities and immunophenotypic aberrancies supported by an overt suspicion of T cell malignancy in the clinical request. As shown in

Table 1
Demographic, clinical and laboratory data of study subjects.

	DONORS	REACTIVE Patients	PATHOLOGICAL Patients
Number of patients	14	26	37
Gender			
Females, *n (%)*	5 (36%)	16 (61%)	21 (57%)
Males, *n (%)*	9 (64%)	10 (39%)	16 (43%)
Age			
Mean Age (±SD), *years*	44 (±13)	49 (±18)	64 (±14)
Complete blood counts (±SD)			
WBC (10^3/μl)	6 (±1)	8.4 (±3.3)	19 (±43)
Neutr. (10^3/μl)	4 (±1)	4.7 (±2)	5(±5)
Hb (g/dL)	15 (±1)	12.6 (±2)	13 (±2)
PLTs (10^3/μl)	230 (±59.6)	270 (±98)	225 (±76)
Lympho. (10^3/μl)	2 (±1)	3 (±2)	14 (±42)
T lymphocytes Flow Cytometry			
CD4/CD8 ratio	2 (±1)	2 (±2)	47 (±207)
Pathological Subtype			
CD4	*na*	*na*	13 (35%)
CD8	*na*	*na*	18 (48%)
Double Positive	*na*	*na*	5 (13%)
Double Negative	*na*	*na*	1 (4%)
TCR-Vβ repertoire pattern evaluation	14	17	37
Policlonal	14 (100%)	7 (41%)	0
Oligoclonal	0	9 (53%)	4 (11%)
Monoclonal	0	1 (6%)	28 (79%)
Non reactive	0	0	5 (13%)
TRBC1 pattern evaluation			
Bimodal	14 (100%)	24 (92%)	1 (3%)
Unimodal	0	2 (8%)	36 (97%)
# Path. Lympho (±SD, 10^3/μl)	*na*	*na*	12 (±42)
% Path. Lympho (±SD, of tot Lymph)	*na*	*na*	53 (±29)

na denotes not applicable.
(WBC= white blood cells; Neutr.= neutrophils; Hb= haemoglobin; PLTs= platelets; Lympho.= lymphocytes)

Supplementary Tables S2 and S3, patients included in the Pathological group were further divided, based on the main T cell subset affected by the immunophenotypic aberrancies. The described criteria allowed us to identify 26 reactive patients and 37 pathological patients: among pathological individuals 13 presented with a CD3+CD4+ aberrant population (PCD4), 18 with a CD3+CD8+ aberrant one (PCD8), 5 showed an expansion of Double Positive (DP) T cells and 1 of Double Negative (DN) T cells.

Gender distribution was quite similar among the three groups with a slight increment of the female component in the reactive one, while the mean age was significantly higher in pathologic patients compared to donors and reactives ($p < 0.01$).

Assessment of TRBC1 expression in healthy, reactive and pathological samples

We first applied the standardized protocol and the analysis strategy indicated by Muñoz-García et al. on healthy donors, in order to define our normal range of polyclonal TRBC1 expression [16].

For this purpose, means ±3 standard deviations (SD), which provide intervals where 99,73% of frequencies of polyclonal cells fall, were used as cut-off values for defining an unimodal *vs* bimodal TRBC1 profile. We also calculated the TRBC1+/TRBC1- ratios for T cell populations on donors (99,73% intervals: in normal CD4+ TRBC1+/TRBC1- ratio= 0.09-2; in normal CD8+ TRBC1+/TRBC1- ratio=0-1.5).

We then moved to the evaluation of TRBC1 percentages distribution on αβ+ T cells in reactive and pathological patients and compared deviations from the mean values of normal ranges between them and donors' ones.

As shown in Fig. 1A we found that deviation from normal was significantly different when comparing donors or reactives with pathological T cell subset from the pathological group on both CD3+CD4+ and CD3+CD8+ T cells. Of note, any difference was found between the healthy counterpart of T cells in pathological subjects compared to healthy and reactive ones. Interestingly, while TRBC1 expression on CD3+CD4+ T cells was not significantly different between donors and reactives, CD3+CD8+ T cells in reactive samples showed a deviation from normal values that was significantly higher compared to donor's ones. As shown in Fig. 1A, two reactive patients presented percentages of TRBC1+ cells out of normal ranges, one on both CD4 and CD8 subsets, the other only on CD4 subset. On the other hand among CD8 pathological cases, several presented with percentages of TRBC1 expression falling into the normal range. As Munoz Garcia et al. reported data on TRBC1 expression, focusing on the aberrant population, we also applied this approach on immunophenotypically abnormal T cells whenever present: this allowed us to get a comparison between the evaluation of TRBC1 expression performed by using the two main analysis approaches previously described. As shown in Fig. 1B, and in the representative plots in Fig. 1C, this way of analyzing samples provided a more robust demonstration of the unimodal pattern of TRBC1 expression of aberrant T cells. Percentages of TRBC1 expressing T cells obtained by applying this strategy, on pathological samples, not only significantly ($p < 0.00001$) deviated from normal ranges but allowed us to exclude from normal ranges almost all the pathological samples with the exception of one. Of note, patients with aberrant DP and DN T cells are not included in the statistics because of their low number (5 DP and 1 DN): however, they showed an evident TRBC1 unimodal pattern of expression on the pathological gated population, as summarized in Table 1 (See also Supplementary Table S3) and represented in one, out of five cases, in Fig. 1D. A summary of patients' classification based on immunophenotype and TRBC1 pattern of expression is reported in supplementary Table S4.

Clonality evaluation with TRBC1 is concordant with TCR-Vβ repertoire and molecular TCR rearrangement

Next, we moved to the evaluation of TCR-Vβ repertoire: testing samples from 14 donors allowed us to establish internal reference ranges based on CD3+CD4+ and CD3+CD8+ T cell subsets. As expected, results showed that our mean values lay within the vendor reference ranges (data not shown).

We referred to our internal ranges to set the criteria, described in Materials and Methods section, in order to define the presence of a clonal T cell expansion, a poly-/oligo-clonal or a "non reactive" profile of TCR-Vβ antigen expression on 54 samples from reactive and pathological classified samples (17 and 36 respectively). As shown in Fig. 2 and Supplementary Tables 2 and 3, reactive patients are characterized by a moderate expansion of some Vβ antigens without reaching none of the criteria to be defined as clonal and providing evidence of their oligoclonal condition. The only exception was represented by one patient (R7) showing a more then ten-fold increase in the higher level of two Vβ family members in the absence of immunophenotypic aberrancies. Most of the pathological patients with suspicious of T cell neoplasm showed a clonal expansion of one Vβ in the relative pathological CD3+CD4+, CD3+CD8+, DP or DN subset, five presented a "non reactive" pattern, with the exception of four patients resulting oligoclonal (see Supplementary Table S3).

We finally evaluated the concordance between TRBC1 assay application and the use of the other currently used techniques. To this end we brought together all the results obtained from TCR-Vβ flow cytometric evaluation and PCR analysis, that was available for 20 samples, and compared them with those obtained by TRBC1 analysis. Of note, in patients presenting with an oligoclonal TCR-Vβ repertoire profile but a clonal TCR molecular analysis, we considered the last one as resolving, based on both specificity and sensitivity properties of the test [8]. As shown in Table 2, concordant results were found in 67 out of 68 cases (97%). The fisher exact test confirmed the statistical weight of this concordance. As a matter of fact, it seems to be an exceeding case among polyclonal specimens due to two discrepant cases within the Reactive group for which we were unable to get PCR analysis of TCR gene rearrangement to confirm the clonality status. Comparing the concordance analysis with Table 1 and Supplementary Table S2, it raises up how R4 presents with a polyclonal TCR profile but a unimodal distribution of TRBC1, on the opposite R7 had a TCR-Vβ expansion in the presence of a bimodal TRBC1 pattern. Concerning clinical data from the two discordant samples, it's noteworthy that they were from patients with chronic CMV and acute phase of EBV infection.

TRBC1 evaluation improves the flow-cytometric work-up in T cell neoplasms

We then searched for a global evaluation of the impact TRBC1 analysis has on our current immunophenotyping ability to classify samples and to distinguish pathological ones. To this end, we derived mean fluorescence intensity (MFI) values of the main T cell associated markers included in our routinary panels from most of the analyzed samples in CD4 and CD8 T cell subsets. MFI values were then normalized and used to build a heatmap.

In Fig. 3, the heatmap highlights how TRBC1 expression (normalized to obtain an unimodal distribution) is the parameter best discriminating between pathological T cell subsets and normal ones. Of note, we confirmed that in the T cell subset free from aberrancies of the same pathological patient TRBC1 expression was normal. Interestingly, this representation of data better pointed out that Pathological CD8+ T cells showed a TRBC1 expression that deviates from normal at a lower extent with respect to CD4+ ones.

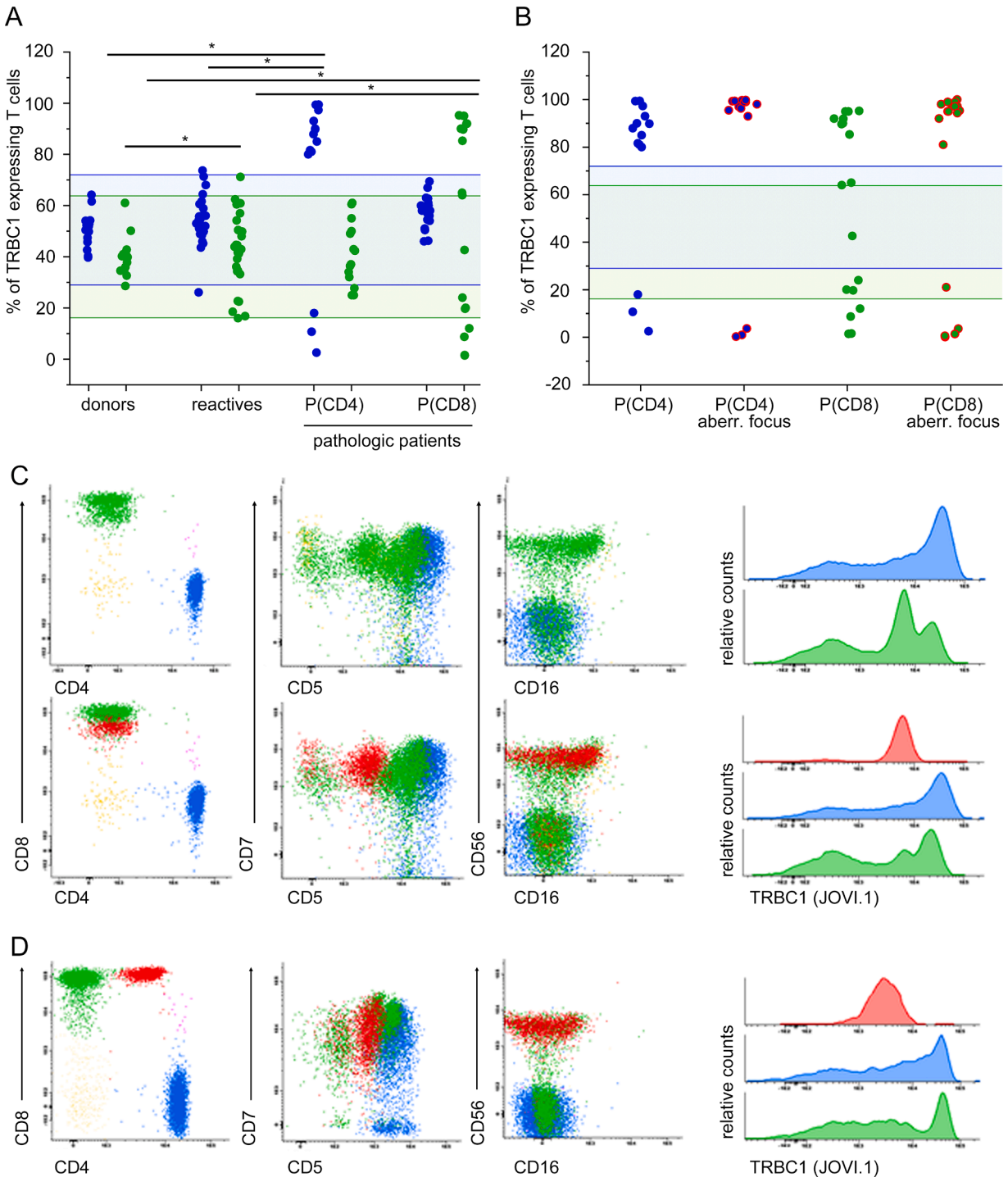

Fig. 1. TRBC1 expression in donors, reactive and pathological patients.
A) Data represent percentages of cells expressing TRBC1 within CD4+ (blu dots) and CD8+ (green dots) subsets in 14 healthy donors, 26 reactive patients and 35 pathological patients further divided in CD4-pathological (=P(CD4)) and CD8-pathological (=P(CD8)) based on the major T cell subset affected by immunophenotypic aberrancies. In the scatter column graphics, dots correspond to individual percentages of TRBC1+ T cells. Light blue and light green areas represent the normal ranges calculated on healthy donors. Mann-Whitney U *t-test* was used to calculate statistics on deviations from mean values between groups. *p* value<0,05 was considered statistically significant. *$p<0{,}001$. B) Representation of percentages of TRBC1 expressing T cells using the two different analytic approaches on pathological patients. Results on CD4+ T cells are in blue and on CD8+ T cells are in green. Red circled dots indicate the results restricted to the aberrant subset. C) Representative flow cytometric dot plots and histograms showing the two different analytic approaches applied to one same specimen; CD4+ T cells are depicted in blue, CD8+ in green and the pathological population is in red. D) One representative analysis of a patient presenting a double positive pathological population. CD4+ T cells are depicted in blue, CD8+ in green and the pathological population is in red.

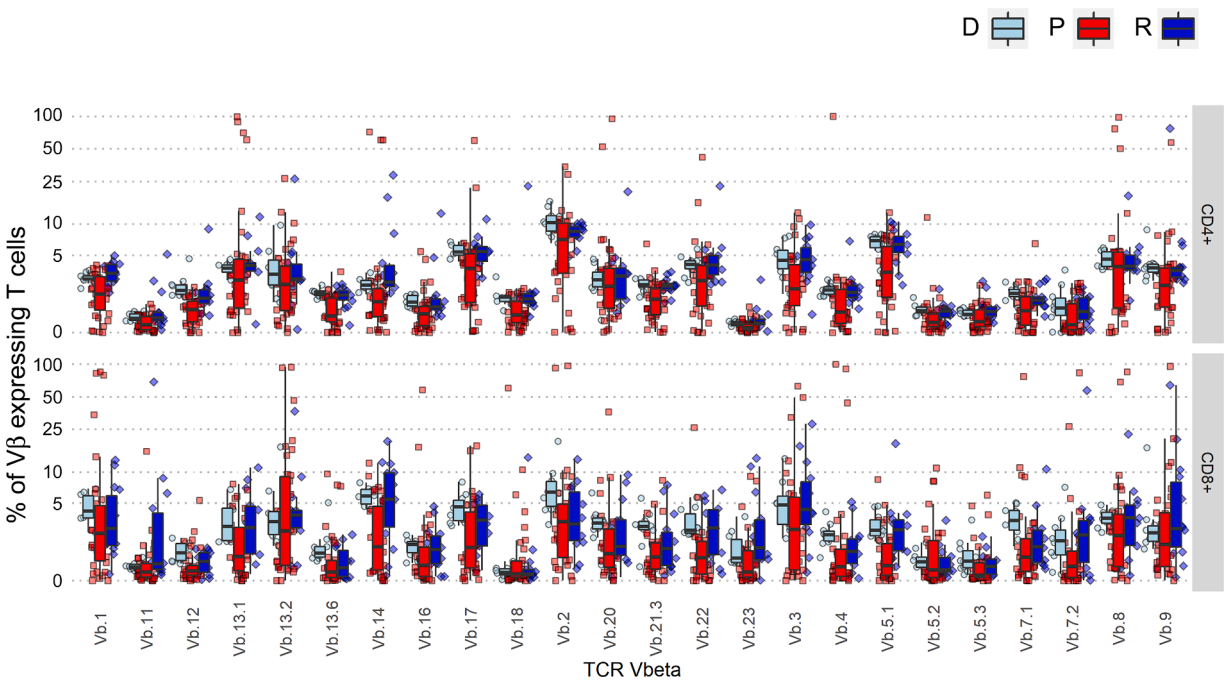

Fig. 2. TCR-Vβ family members expression in donors, reactive and pathological patients.
Percentages of cells expressing a specific Vβ family member within CD4+ and CD8+ subsets in 14 healthy donors (D, light blue dots and boxes), 17 reactive patients (R, dark blue dots and boxes) and 40 pathological patients (P, red dots and boxes). In the box-plot graphics, dots correspond to results from individuals while boxes represent 25th and 75th percentile values, lines inside the box correspond to median values (50th percentile) and whiskers represent 5th and 95th percentile values.

Table 2
Concordance analysis between TRBC1 assay and the reference molecular or Flow Cytometric techniques used to assess Tαβ-cell clonality.

Clonality status by different techniques	TRBC1 expression pattern		(97% concordancy)
	BIMODAL	UNIMODAL	*P Value*
Poly/Oligoclonal (tot.31)	31/31	1/31	**< 0.00001**
Monoclonal (tot.37)	1/37	36/37	

Clonality evaluation with TRBC1 resolve uncertain cases and identify T-CUS in flow cytometric confounding cases

Despite the high level of concordance between the existing methods of clonality assessment and TRBC1 analysis, we observed some discrepancies between TCR-Vβ repertoire and TRBC1 profiling. Among pathological cases, three were found with a discordant result between TCR-Vβ repertoire profile and TRBC1 pattern.

Those specimen's clonality was confirmed by TCR gene rearrangement analysis, thus suggesting that TRBC1 has been resolving the uncertain result of Vβ repertoire. In detail, pathological patients #1, #3 and #14, presenting with a suspicion for a T-LGL and an immunophenotype consistent with a terminally differentiated cytotoxic profile of aberrant T cells (*i.e* CD45RA+CCR7-CD57+), resulted with an oligoclonal TCR-Vβ profile.

In those patients focusing on aberrant T cells highlighted a monotypic TRBC1 pattern (see Fig. 4A and supplementary Fig. S1) that was also confirmed as monoclonal by molecular TCR gene rearrangement evaluation. Finally, in our cohort of patients we had the opportunity to confirm the ability of TRBC1 analysis, associated with our immunophenotyping panels, to identify small subsets of T cells with uncertain significance but certainly clonal. Cut off values for T-cell clones of uncertain significance (T-CUS) identification have been published in two papers by Min S. et al [15,20]. They defined T-CUS a subset of clonal cells, defined by TRBC1 monotypic pattern on immunophenotypically aberrant T cells, representing the 5-20% of lymphocytes and 50-500 cells/μL. Within the pathological group we found five patients (patients #1, 14, 17, 25, 34) that were characterized by the presence of a T-CUS (see Table 2). TCR-Vβ Repertoires of two out of five patients were the above described as oligoclonal (#1 and 14), while one showed a "non reactive" pattern (# 17, see Fig. 4B), with the molecular TCR evaluation confirming clonality and thus supporting the utility and high sensitivity of TRBC1 analysis. Interestingly, as shown in Fig. 4B, the immunophenotype of aberrant T cells in this last patient was characterized by a CD45RA+CCR7- antigen expression profile T-LGL-like.

Discussion

Multiparametric flow cytometry identification of mature T cell neoplasms has been widely demonstrated as a reliable and sensitive method able to detect immunophenotypic aberrancies in the expression of T cell differentiation and functionality markers related to a disease phenotype. The assessment of T cell clonality represents the complementary tool to ascertain the neoplastic nature of a T cell aberrant and abnormally expanded subset and has been traditionally evaluated by multiplexed TCR gene PCR and recently by the flow cytometric evaluation of a certain number of TCR-Vβ families. These techniques have some limitations being relatively labor-intensive, time consuming and requiring interpretative expertise together with expensive reagents. Moreover, a certain risk of false-positive results have been reported for molecular techniques in the presence of small physiologic T cell clones produced by normal immune responses while for TCR-Vβ families the main limit is represented by the lack of a complete coverage of the TCR repertoire. Recently, a monoclonal antibody recognizing one of the two mutually exclusive constant portions of the TCR β receptor chain has been proposed as a surrogate tool for the evaluation of T cell clonality.

In our routinary flow cytometric approach to a suspicion of peripheral T cell proliferative disorder (PTCL), we first apply an immunophenotypic screening panel designed with the main T cell antigens, and, in the presence of aberrancies, we further evaluated the TCR-Vβ repertoire in order to investigate the clonality status. Aimed to introduce the TRBC1 assay as surrogate for clonality, we first assessed our internal cut

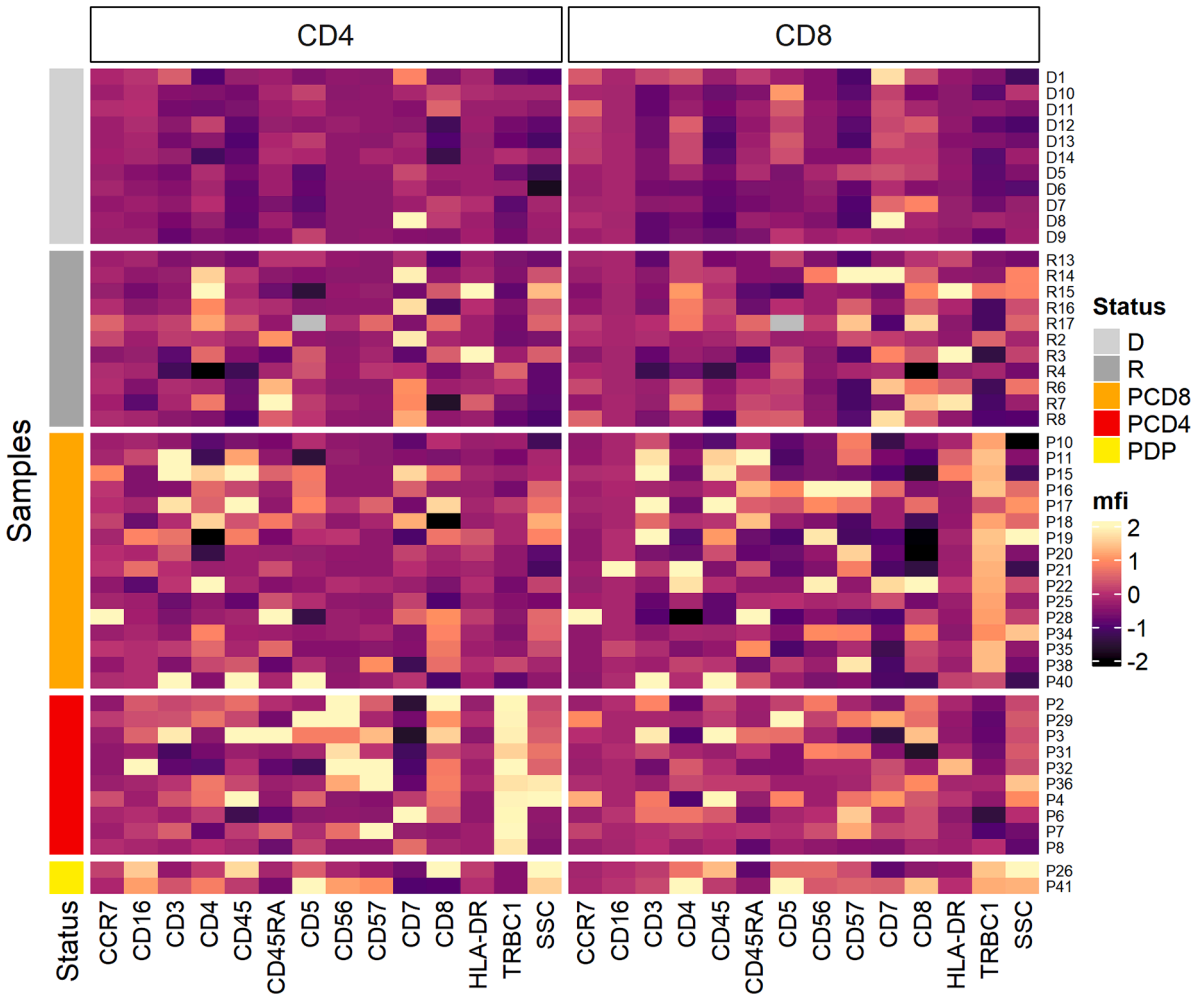

Fig. 3. Impact of TRBC1 analysis into the current immunophenotypic panel.
A) Heat-map representation of mean fluorescence intensities (MFI) of T cell antigens currently evaluated during T cell neoplasm diagnostic work-up. TRBC1 percentages of expression calculated with the two methods were normalized and introduced as unimodal variables in the different identified groups of donors, reactives and pathological (*status*).

off values for both the approaches used for clonality evaluation on 14 healthy donors. Mean frequencies of αβ+ T CD4+ and T CD8+ cells expressing the 24 analyzed Vβ families were comparable to those provided by the vendors. Concerning TRBC1 normal intervals of expression, both in terms of percentages of TRBC1+ cells or TRBC1+/TRBC1- ratios, they were in accordance with those published by Novikov et al. and Munoz-Garcia et al. [13,16], with the CD8+ population presenting a slightly lower rate of expression compared to CD4+ counterpart, thus demonstrating the reliability and reproducibility of this method. Unfortunately, we did not have the chance to reach the number of evaluations necessary to derive the normal ranges on DP and DN subsets of αβ+ T cells because of their rarity: to get statistically reliable data on such a poorly represented cell subset much more samples than 14 would be required. TRBC1 expression on our three cohorts resulted in accordance with data from literature, demonstrating that deviations from mean values were significantly higher in patients of the Pathological group when compared to Donors or Reactives: this further supported the analytical strength of the assay. Applying the normal ranges we derived on CD4/CD8 2D plot gated T cells, enabled us to well distinguish the three groups: however, two reactive cases as well as some patients with an aberrant CD8+ population resulted differently from the expected. This observation raised the already open question on the better approach to be used in analyzing TRBC1 expression. In fact, Munoz Garcia et al. reported data on TRBC1 expression by performing an analysis that focused on the aberrant population; as demonstrated in Fig. 2B, the restriction of the analysis to the aberrant population on our samples, provided a better discrimination of Pathological samples from Reactives. The heathmap in Fig. 3, providing a global representation of our flow cytometric data, further confirmed this last evidence: the association of the TRBC1-including panel to the T cell immunophenotyping improves the discrimination among pathological and healthy or reactive cases. Comparing results from TRBC1 assay and TCR-Vβ repertoire, we elucidated the utility of this new approach in unraveling apparently discordant results. This has been possible thanks to the availability of confirming molecular tests and allowed us to calculate the level of concordance between the already used TCRαβ clonality assessment and the TRBC1 new one: we found a significant high level of concordance between the tests, thus suggesting the improvement deriving from TRBC1 analysis introduction. Interestingly, for one of the two discordant cases showing evidence of monoclonality for TCR-Vβ repertoire in the presence of a bimodal TRBC1 pattern (see Supplementary Table S2), we had the opportunity to evaluate a follow up specimen: the evaluation of TCR-Vβ Repertoire in the same patient, 4 months later, revealed no clonal expansion in any of the 24 Vβ family. This demonstrates the ability of TRBC1 to highlight significant expansion of aberrantly clonal T cell subsets: the expansion of the two families of Vβ in this representative case was probably due to the anti-viral activation status. There is another interesting case among Pathologic

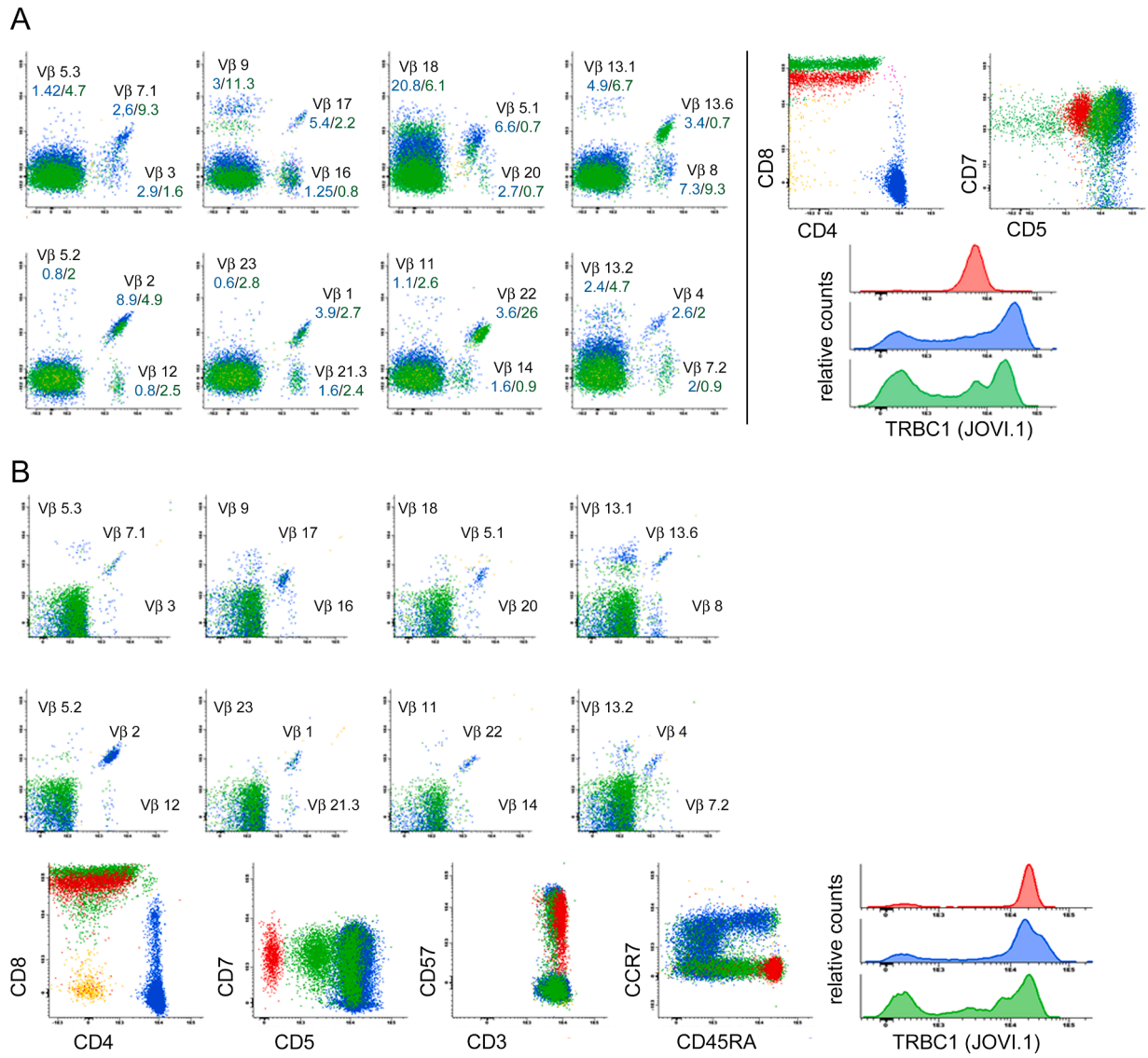

Fig. 4. TRBC1 utility in uncertain cases resolution and T-CUS identification.
(A) Representative flow cytometric plots of an equivocal case showing an oligoclonal TCR-Vβ pattern in the presence of relevant immunophenotypic aberrancies and monotypic TRBC1 profile. (B) Representative flow cytometric plots identifying a T-CUS by an immunophenotypic analysis including TRBC1 in a patient showing a "non reactive" TCR-Vβ pattern on CD8+ T cells. CD4+ T cells are depicted in blue, CD8+ in green and the pathological population is in red. DP and DN T cells are in pink and yellow respectively.

samples we examined that could appear as wrongly classified: P40 patient presented with an aberrant CD8+ T cell phenotype but both ordinary TCR-Vβ repertoire study and TRBC1 assay excluded monoclonality. We deepen the analysis of this particular case by applying an improved panel that associates aberrancy markers to the different TCR-Vβ and TRBC1 monoclonal antibodies. We found, within the CD8+ T cell subset, a subpopulation showing a diminished expression of CD8, CD5 and CD7, 100% positive for the Vβ 21.3 family together with an unimodal TRBC1 pattern (see supplementary Fig. S1). In this case the phenotypic classification seems to be more informative with respect to both the ordinary evaluation of clonality thus suggesting the complexity of T cell proliferative disorder study. This patient was followed in our center from 2014, and showed a CD8+ T cell lymphocytosis that increased in the last years never showing evidence of monoclonality neither by TCR-Vβ repertoire nor with molecular analysis (the last being of 2016) and without evidence of clonality: as shown in Fig. S1 the whole CD8 subset presents an aberrant phenotype and probably includes the expansion of more than one T cell clones that could be elucidated only by a multiparametric enlarged flow cytometric panel.

Results obtained from the comparison between TRBC1 analysis and the currently used ones for clonality evaluation, could pave the way towards a change in the diagnostic algorithm of T cell neoplasms. Moreover, the introduction of TRBC1 could have implications in other laboratories than the flow cytometry laboratory: in fact, we could speculate, for the future, to save for the molecular analysis only the borderline cases as well as apply a TRBC1-based " *watch and wait*" like approach.

TRBC1 assay's application, due to its high sensitivity, led us to identify five cases of T-CUS. Our results are in accordance with and further confirm the ones by Min Shi et al. [15,20], in fact, our T-CUS showed a typical T LGL phenotype too. Interestingly, in three out of five of the T-CUS specimen, the TCR-Vβ repertoire was oligoclonal (patients #1 and 14), as above mentioned, or not conclusive (1 "non reactive", patient #17; Fig. 4B): this was probably due to the low number of aberrant and clonal T cells, thus supporting the higher sensitivity of TRBC1 analysis. Anyway, it must be considered that we conducted a basic TCR-Vβ analysis on the main CD4+ and CD8+ (or DP and DN where expanded) T cell subsets, without focusing on aberrant T cells.

This could explain the different results reported in a recent paper by Munoz Garcia et al. [21]: they found that, in the absence of lymphocytosis, TRBC1 would not be able to identify T-CUS differently from TCR-Vβ repertoire analysis that they performed including T cell aberrancy markers. Of note, we found these rare populations in a cohort of patients that have already been classified, but we could expect, for the future, that the up-grade of our diagnostic panels will highlight the presence of T-CUS more frequently and even in samples without disease evidence. This will offer the opportunity to better investigate the role of this new biological entity. Some authors considered T-CUS as the T counterpart of hematologic populations already demonstrated to have a prognostic relevance in monoclonal gammopathies (M-GUS) or B-cell neoplasms (monoclonal B cell lymphocytosis, MBL) [22]. Data is needed to unravel the role of T-CUS in developing T cell lymphoproliferative disorders and TRBC1 analysis would play a central role in their study. New generations of IVD flow cytometers, by using 12-color antibody panels, will lead us to associate TRBC1 clonality evaluation to a wider immunophenotypic T cell characterization.

In conclusion, our data provide additional evidence that the introduction of TRBC1 expression analysis will improve the diagnostic work-up of mature T cell malignancies and provide a new tool to expand T cell neoplasms flow cytometric diagnostic opportunities and knowledge. We can also state that this high level of multi-parametric flow cytometry analysis, thus producing the most sensitive and specific results, requires a deep expertise and wide knowledge in the field of T cell lymphoproliferative disorders as well as T cells functional properties. Such a level of competence is not available in many flow cytometry laboratories. Anyway, the easy tool represented by TRBC1 staining could provide a chance of improvement even for a less complex diagnostic context.

Data Sharing Statement

Data are available on request due to privacy/ethical restrictions.

CRediT authorship contribution statement

Manuela Capone: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Benedetta Peruzzi:** Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Writing – review & editing. **Boaz Palterer:** Data curation, Formal analysis, Investigation. **Sara Bencini:** Formal analysis, Writing – review & editing. **Alessandro Sanna:** Investigation, Resources. **Benedetta Puccini:** Investigation, Resources. **Luca Nassi:** Investigation, Resources. **Benedetta Salvadori:** Resources. **Marinella Statello:** Methodology. **Alessia Carraresi:** Methodology. **Stefania Stefanelli:** Methodology. **Chiara Orazzini:** Resources, Resources. **Barbara Minuti:** Formal analysis. **Roberto Caporale:** Formal analysis, Supervision. **Francesco Annunziato:** Conceptualization, Supervision.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101552.

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