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ARTICLE

T-cell clones of uncertain significance are highly prevalent and show close resemblance to T-cell large granular lymphocytic leukemia. Implications for laboratory diagnostics

Min Shi[1] · Horatiu Olteanu[1] · Dragan Jevremovic[1] · Rong He[1] · David Viswanatha[1] · Heidi Corley[1] · Pedro Horna[1]

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Abstract

Benign clonal T-cell expansions in reactive immune responses often complicate the laboratory diagnosis T-cell neoplasia. We recently introduced a novel flow cytometry assay to detect T-cell clones in blood and bone marrow, based on the identification of a monophasic T-cell receptor (TCR) β chain constant region-1 (TRBC1) expression pattern within a phenotypically distinct TCRαβ T-cell subset. In routine laboratory practice, T-cell clones of uncertain significance (T-CUS) were detected in 42 of 159 (26%) patients without T-cell malignancy, and in 3 of 24 (13%) healthy donors. Their phenotype ($CD8^+/CD4^-$: 78%, $CD4^-/CD8^-$: 12%, $CD4^+/CD8^+$: 9%, or $CD4^+/CD8^-$: 2%) closely resembled that of 26 cases of T-cell large granular lymphocytic leukemia (T-LGLL) studied similarly, except for a much smaller clone size ($p < 0.0001$), slightly brighter CD2 and CD7, and slightly dimmer CD3 expression ($p < 0.05$). T-CUS was not associated with age, gender, comorbidities, or peripheral blood counts. TCR-Vβ repertoire analysis confirmed the clonality of T-CUS, and identified additional clonotypic CD8-positive subsets when combined with TRBC1 analysis. We hereby report the phenotypic features and incidence of clonal T-cell subsets in patients with no demonstrable T-cell neoplasia, providing a framework for the differential interpretation of T-cell clones based on their size and phenotypic properties.

Introduction

Clonal T-cell proliferations can be generated as part of a normal immune response, following recognition of a cognate antigen presented within an appropriate major histocompatibility complex, and aided by costimulatory signals [1]. Detection of a T-cell clone in clinical laboratory practice, however, is usually interpreted in the context of the possibility of T-cell malignancy. By far, the most commonly utilized clinical test to detect T-cell clonality is the electrophoretic analysis of polymerase chain reaction (PCR) products using standard BIOMED-2 primers targeting the T-cell receptor (TCR) variable region [2]. While most physiologic T-cell clones produced by normal immune responses are believed to be sufficiently small to remain undetectable by this assay, positive clonality test results in the absence of a T-cell malignancy do occur fairly frequently. Indeed, false-positive results (equivocal or positive clonality testing in the absence of T-cell neoplasia) have been consistently reported in up to 20% of clinical samples of various sources [3–6]. Thus, interpretation of laboratory test results indicative of a T-cell clone remains difficult and occasionally leads to unnecessary laboratory work up or even a misdiagnosis of T-cell neoplasia.

Although the presence of T-cell clones in the absence of T-cell malignancy has been extensively reported [1, 7–10], this knowledge has not yet been consistently translated into a conceptual diagnostic interpretation, mainly due to the limited capacity of most routine T-cell clonality assays to provide immunophenotypic information on small T-cell clones. In contrast, other clonal proliferations of hematopoietic cells not amounting to a malignant process have been clearly defined and extensively studied based on the routine availability of highly sensitive and informative laboratory assays, such as in the case of monoclonal B

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✉ Pedro Horna
Horna.Pedro@mayo.edu

[1] Division of Hematopathology, Mayo Clinic, Rochester, MN, USA

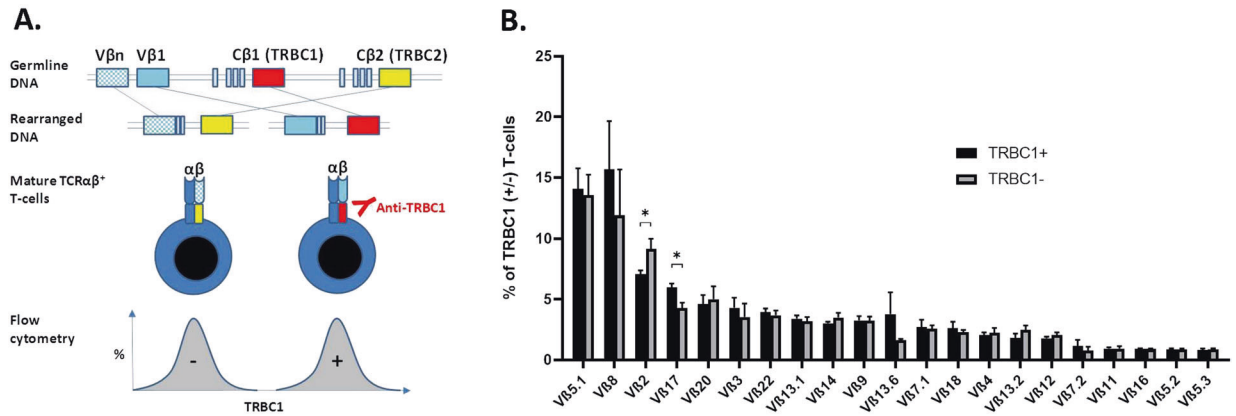

Fig. 1 T-cell receptor β chain constant (TRBC)-1 or TRBC2 gene selection during T-cell receptor (TCR) gene rearrangement is independent of selection of TCR variable β (TCR-Vβ) genes. a Gene rearrangement of the TCR β chain locus involves the selection of one of two mutually exclusive TRBC genes, and one of 52 TCR-Vβ genes. A specific anti-TRBC1 antibody can be used by flow cytometry to distinguish TRBC1 and TRBC2-expressing T-cells. **b** Comparison of the TCR-Vβ repertoire between TRBC1-positive and TRBC1-negative peripheral blood T-cells from five healthy donors, showing remarkably similar distributions. Lines and ranges represent the mean and standard deviation of the mean.

lymphocytosis, monoclonal gammopathy or undetermined significance, and clonal hematopoiesis of indeterminate potential.

We recently reported a novel and highly sensitive flow cytometry assay to routinely detect and immunophenotype clonal T-cell populations [11]. This approach relies on the random utilization of one of two mutually exclusive TCR β chain constant region (TRBC) genes during TCR gene rearrangement, and the capacity of a monoclonal antibody (clone JOVI.1) to detect only one of these constant regions (TRBC1) by flow cytometry [12] (Fig. 1a). A high discriminatory capacity between positive and negative events allows for a clear-cut identification of TRBC1-restricted and TRBC1-negative (thus presumably TRBC2-restricted) T-cell subsets, in a fashion similar to the routine assessment of kappa and lambda immunoglobulin light chain restriction for the detection of B-cell clones. Thus, adding this single antibody to a comprehensive flow cytometry T-cell panel provides a rapid and reliable approach to routinely assess for clonality within gated T-cell subsets, without resourcing to a separate laboratory assay or setting up additional analysis tubes.

While implementing this laboratory test in clinical practice, we noticed a high frequency of small clonal T-cell populations in patients lacking features diagnostic for a T-cell malignancy. We hereby report that these highly prevalent T-cell clones of uncertain significance (T-CUS) exhibit immunophenotypic features closely resembling those of T-cell large granular lymphocytic leukemia (T-LGLL). Moreover, we show that similar minute T-cell clones can be also detected in healthy individuals, expanding the spectrum of clonal T-cell large granular populations to include physiologically normal T-cell subsets.

Materials and methods

Patient and sample selection

Fresh peripheral blood or bone marrow aspirate specimens were received for diagnostic flow cytometric analysis at Mayo Clinic, Rochester, Minnesota, between June 2018 and November 2019. Electronic medical records, including pathology reports, laboratory test results, clinical notes and imaging reports were retrospectively reviewed to identify patients with either (1) no diagnostic clinical or laboratory evidence of a current, prior, or subsequently diagnosed T-cell malignancy, or (2) a definitive diagnosis of T-LGLL, with confirmed disease involvement on the specimen studied. At least three of the four following criteria needed to be met to diagnose T-LGLL: (1) a distinct T-cell population with co-expression of one or more natural killer cell-associated antigens (CD16, CD56, or CD57) and decreased CD2 or CD5 expression; (2) a clonal T-cell population by molecular T-cell gene rearrangement studies or killer cell immunoglobulin-like receptor flow cytometric study; (3) intrasinusoidal cytotoxic T-cell infiltrates in the bone marrow; and (4) persistence of the abnormal T-cell population for more than 6 months associated with unexplained cytopenia. In addition, fresh peripheral blood specimens from 29 adult healthy donors were made available by the Biospecimen Program at Mayo Clinic. This study was approved by the Mayo Clinic Institutional Review Board.

Flow cytometry

A single-tube T-cell panel with fluorescent labeled antibodies recognizing CD2, CD3, CD4, CD5, CD7, CD8, CD45, TCRγδ, and TRBC1, was routinely utilized, as

previously described [11]. In short, CD3-positive/TCRγδ-negative T-cells (TCRαβ T-cells) were displayed on a CD4 versus CD8 dot-plot to identify CD4-positive, CD8-positive, CD4/CD8 double-positive, and CD4/CD8 double-negative subsets. CD4-posiitve and CD8-positive T-cells were separately studied for the presence of immunophenotypically distinct subsets based on CD2, CD5, and CD7 expression using several dot plots and a radar (three-dimensional) plot. Expression of CD3, CD45, CD4, and CD8 was carefully studied on the gating plots to identify additional discrete subsets. The immunophenotype of CD3-negative events (including CD7-positive NK cells) was also evaluated to identify abnormal CD3-negative T-cell subsets. Each identified T-cell subset was evaluated on a histogram of TRBC1 expression. Clonal T-cell populations were identified as immunophenotypically distinct TCRαβ T-cell subsets exhibiting discretely homogenous T-cell antigen expression properties, and a monophasic TRBC1 staining pattern defined as either (1) >85% of TRBC1-positive events, (2) <15% TRBC1-positive events, or (3) homogenous TRBC1-dim expression. These thresholds were arbitrarily defined to capture all T-cell neoplasms so far evaluated in our practice (over 200 T-cell malignancies) using our T-cell panel; while excluding total benign CD4-positive and CD8-positive T-cell subsets from healthy individuals and patients without T-cell neoplasia (with the exception of very rare large CD8-positive T-CUS).

The median fluorescence of CD2, CD3, CD4, CD5, CD7, CD8, CD45, and TRBC1 expression was calculated for each clonal T-cell subset, and expressed as a percentage relative to the median fluorescence of non-clonal background CD4-positive T-cells (for CD2, CD3, CD4, CD5, and CD45), non-clonal background CD8-positive/TCRγδ-negative T-cells (for CD8), NK cells (for CD7), or CD4-positive/TRBC1-positive T-cells (for TRBC1). In all cases of T-LGLL and selected cases of T-CUS, additional tubes were set up to study the expression of the NK-cell-associated antigens CD16, CD56, CD57, CD94, and NKG2A (using antibodies conjugated to PerCP-Cy5.5, Horizon V450, FITC, APC, and PE, respectively), on relevant T-cell subsets gated based CD3 (PE-Cy7) and CD8 (APC-H7) expression.

A combined TRBC1/TCR variable β (TCR-Vβ) flow cytometry assay was developed based on the previously described IOTest Beta Mark TCR-Vβ repertoire kit assay (Beckman Coulter, Brea, CA, USA), combined with antibodies recognizing CD2, CD3, CD4, CD5, CD7, CD8, and TRBC1 (conjugated to PerCP-Cy5.5, PE-Cy7, APC-R700, APC, Horizon V450, APC-H7, and BV605, respectively). In brief, each detectable TCR-Vβ specificity was separately analyzed for clonality based on the pattern of TRBC1 expression within phenotypically distinct subsets, as described above. Tube 6 of the Beta Mark test (Vβ23, Vβ1, and Vβ21.3) was excluded from the analysis due to sub-optimal staining when utilized within this custom panel.

For all flow cytometry assays, at least 100,000 total events were acquired per analysis tube on a FacsCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). All antibodies were obtained from BD Biosciences, except for a FITC-conjugated anti-TRBC1 antibody (clone JOVI.1, Ancell Corporation, Bayport, MN, USA), and the anti-TCR-Vβ antibodies from the Beta Mark kit (Beckman Coulter). All analyses were performed on Kaluza version 2.1 (Beckman Coulter), considering only populations comprised by 100 or more events. All cases were analyzed retrospectively by two of the authors (PH and MS) in a blinded fashion, to ensure consistency.

TCR gene rearrangement studies

Total cellular DNA was extracted and PCR amplification performed in five multiplex PCR tubes with ASR Biomed-2 primers (Invivoscribe Technologies, San Diego, CA, USA) targeting TCR-Vβ, Dβ, Jβ, Vγ, and Jγ regions. The products were separated and detected by capillary gel electrophoresis on the ABI Prism 3130xl genetic analyzer (Applied Biosystems, Warrington, UK).

Statistical analysis

All statistic calculations were performed using GraphPad Prism, version 8.2.1 for Windows (GraphPad Software, San Diego, CA, USA). Comparisons of measurement values between two groups were performed using the Mann–Whitney test (clone size) or an unpaired two-tailed *t* test (age, median fluorescence percentage, TCR-Vβ class percentages, and peripheral blood cell counts). Comparisons of nominal variables were performed using the chi-squared test for expected values equal or greater than 5, and the Fisher's exact test for expected values less than 5. Correlation between two variables was evaluated using the Spearman nonparametric test. A statistical significant *P* value was considered as less than 0.05.

Results

A previously reported single-tube flow cytometry assay for the detection T-cell clones [11] was developed and validated for implementation into laboratory diagnostics. This assay relies on the restricted expression of one of two mutually exclusive TRBCs within immunophenotypically distinct clonal TCRαβ T-cell subsets (Fig. 1a), assuming that selection of TRBC1 or TRBC2 is a random process that occurs independently of TCR-Vβ gene selection. To confirm this hypothesis, we tested five healthy donors with a

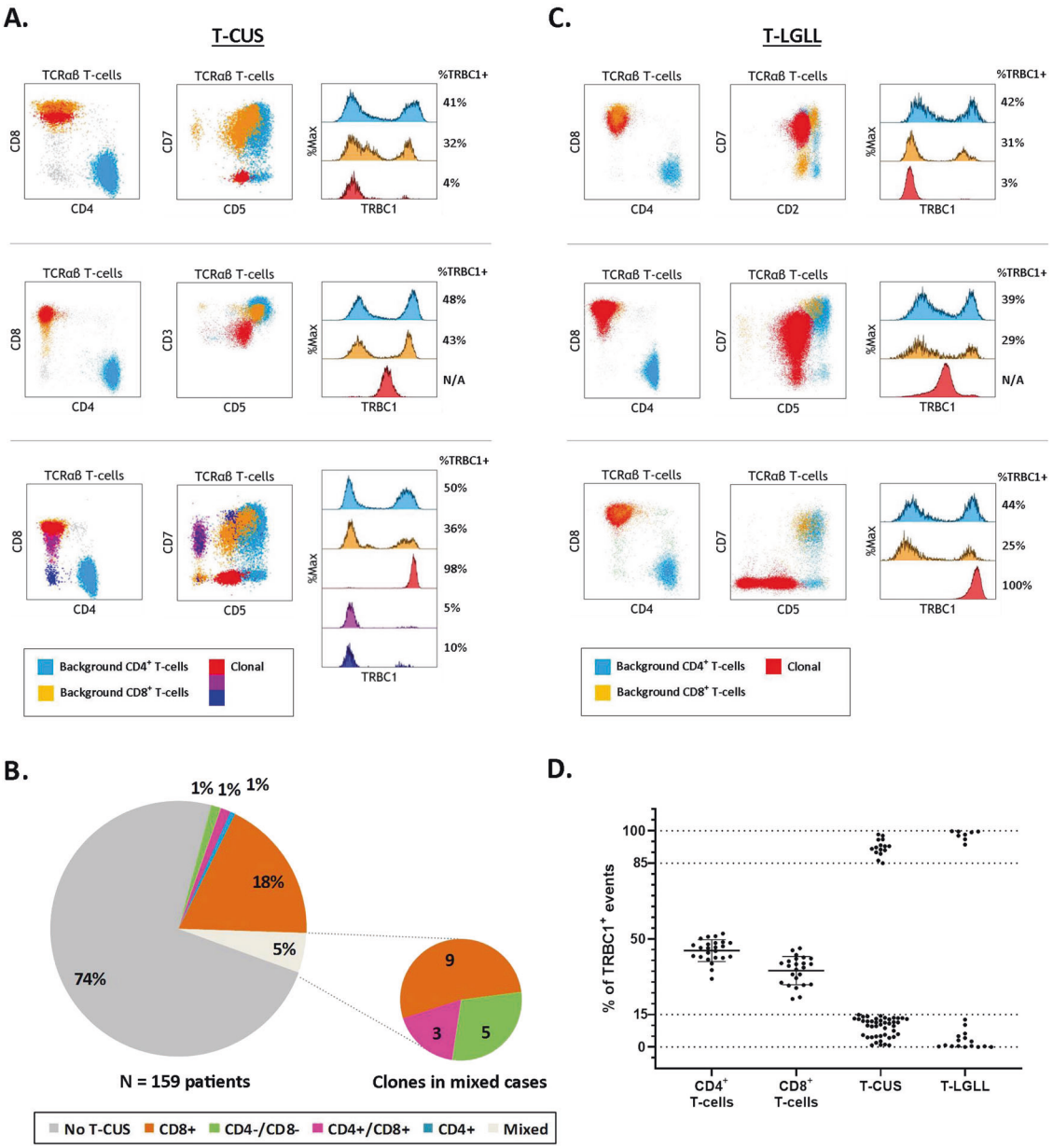

Fig. 2 T-cell clones of uncertain significance (T-CUS) are frequently detected by flow cytometry on patients with no evidence of T-cell neoplasia. a Peripheral blood flow cytometry plots from three patients with T-CUS: A 58-year-old man with a history of hypertrophic cardiomyopathy presenting with diarrhea, dysuria, and pruritus (*top*); a 57-year-old female with acute hypersensitivity reaction to Lamotrigine (*middle*); and a 73-year-old male with newly diagnosed inclusion body myositis and no cytopenias or lymphocytosis (*bottom*). The red events correspond to CD8-positive T-CUS that are homogenously negative (*top*), dim (*middle*), or positive (*bottom*) for TRBC1, accounting for 0.5%, 17%, and 16% of lymphocytes, respectively. The last patient (*bottom*) also features two additional TRBC1-negative T-CUS, which are CD8-positive (*violet*) or CD4/CD8 double-negative (*blue*), and account for 6% and 1.3% of lymphocytes, respectively. Also displayed are background non-clonal CD4-positive (*cyan*) and CD8-positive (*orange*) T-cells, and percentages of TRBC1-positive events for each population adjacent to each histogram. N/A not applicable. **b** Incidence and basic phenotypic features of T-CUS detected in 159 patients with no demonstrable T-cell neoplasm. **c** Three typical cases of T-cell large granular lymphocytic leukemia (T-LGLL) are shown for comparison, featuring similar clonal CD8-positive T-cell populations (red events) on bone marrow (*top* and *bottom*) or peripheral blood (*middle*) specimens, which are homogenously negative (*top*), dim (*middle*), or positive (*bottom*) for TRBC1. **d** Percentages of TRBC1-positive events on CD4-positive and CD8-positive T-cells from 24 healthy donors; and on gated clonal T-cell populations from patients with T-CUS and patients with T-LGLL. Dotted lines depict previously established thresholds for T-cell clonality. TRBC1-dim clonal T-cell populations are not shown.

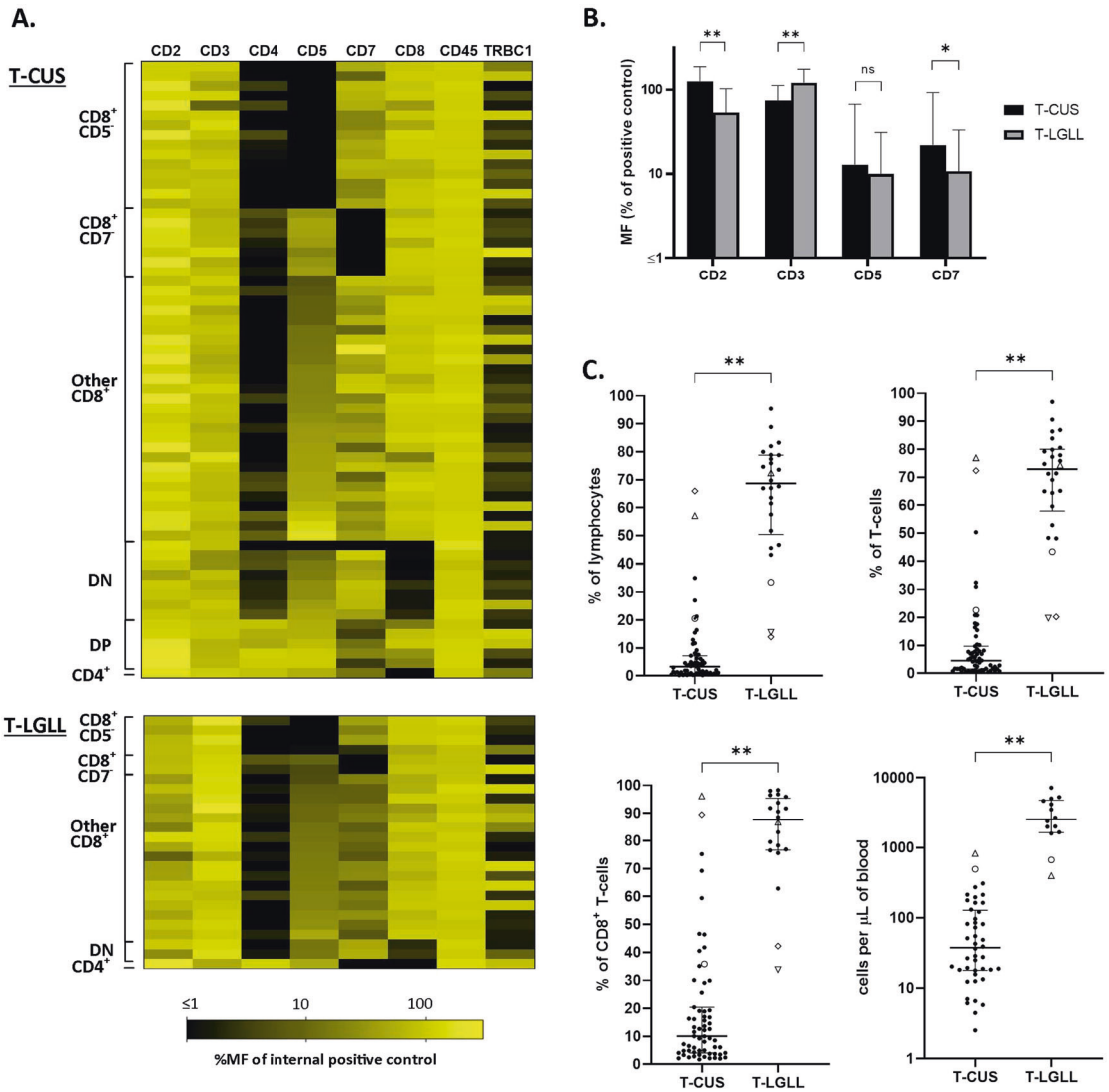

Fig. 3 T-cell clones of uncertain significance (T-CUS) show immunophenotypic features closely resembling T-cell large granular lymphocytic leukemia (T-LGLL) but with a much smaller clone size. a Heat-map of T-cell antigen expression by T-CUS (*top*) and T-LGLL (*bottom*) showing median fluorescence intensities (MF) relative to an internal positive control, and clustered based on their most distinctive immunophenotypic characteristics (*left labels*). **b** Side by side comparison of T-cell antigen expression, showing subtle but statistically significant lower expression of CD2 and CD7, and higher expression of CD3 on T-LGLL cases compared with T-CUS. Bars and ranges depict means and standard deviations, respectively. $*p < 0.05$. $**p < 0.0001$. ns non-significant. MF median fluorescence. **c** Clone size comparison between T-CUS and T-LGLL, based on percentages of total lymphocytes (*upper left*), total T-cells (*upper right*), and CD8-positive T-cells (*lower left*) for peripheral blood and bone marrow specimens; in addition to absolute clonal T-cells per microliter (*lower right*) for peripheral blood specimens only. Clear shapes correspond to outlier values on one or more clone size measurement (individual patients described in "Results" section). Lines and ranges correspond to medians and interquartile ranges, respectively. $**p < 0.0001$.

modified flow cytometry assay where our diagnostic panel was combined with antibodies to detect different Vβ families. Overall, the TCR-Vβ repertoire of TRBC1-positive and TRBC1-negative (TRBC2-positive) T-cells closely mirrored each other, with no detectable selection bias between TCR-Vβ and TRBC expression, except for minor percentage differences for Vβ2 and Vβ17 (Fig. 1b).

A total of 159 patients (104 peripheral blood and 55 bone marrow specimens) with no diagnostic clinical or laboratory evidence of T-cell neoplasia were then studied with our diagnostic T-cell flow cytometry panel including core T-cell antigens and TRBC1. Phenotypically discrete clonal T-cell subsets were detected in 42 patients (26%), with a single clonal T-cell subset observed in most subjects. Eleven patients had two clonal subsets, while three and four clonal subsets were detected in one patient, each (Fig. 2a). Of the 58 total T-cell clones detected in these patients, 45 (78%) were CD8-positive, 7 (12%) were CD4/CD8 double-negative, 5 (9%) were CD4/CD8 double-positive, and only 1 (2%) corresponded to a CD4-positive/CD8-negative T-cell

population (Fig. 2b). Additional phenotyping was performed on T-cell clones from eight patients, showing at least partial expression of CD57 (7 cases), CD56 (5), CD16 (3), CD94 (5), and/or NKG2A (3) in all cases, including one CD4-postive/CD8-negative T-cell clone expressing CD57 (data not shown). The overall immunophenotypic features of these small clonal T-cell subsets were strikingly similar to those encountered in a cohort of 26 cases of confirmed T-LGLL (Figs. 2c and 3a). However, given the absence of other clinical or laboratory features supporting a diagnosis of T-cell malignancy, we favored interpretation of these findings as T-CUS, in the setting of a newly implemented and highly sensitive T-cell clonality assay. A similar immunophenotypic analysis performed on peripheral blood specimens from 24 healthy donors (age 24–76 years old; mean: 46 years old) showed five phenotypically discrete and minute clonal T-cell subsets in 3 (13%) individuals age 39, 42, and 58 years old (four CD8-positive clones, and one CD4/CD8 double-negative clone). This suggests that at least some of these phenotypically distinct subsets might be best interpreted as physiologically expanded clonotypes of a normal immune system repertoire.

We next compared the phenotype and clone size of all 63 identified T-CUS (58 clones from 42 patients, and 5 clones from 3 healthy donors), with that of 26 confirmed cases of T-LGLL (13 peripheral blood samples and 13 bone marrow aspirates). All T-LGLL cases showed a single conspicuously expanded T-cell subset exhibiting a monophasic TRBC1 expression pattern consistent with clonality (Fig. 2c). TRBC1 expression (positive, negative, or dim) was similar between T-CUS (22%, 70%, and 8%) and T-LGLL (31%, 62%, and 8%, respectively; $p = 0.7$), suggestive of a skew towards TRBC2 usage in both settings (Fig. 2d). Arbitrarily assigned phenotypic categories were similarly represented in both T-CUS and T-LGLL, with the exception of CD4/CD8 double-positive clones comprising a small subset of T-CUS (8%) but not represented in our T-LGLL cohort (Fig. 3a). Comparison of fluorescence intensities normalized to internal positive controls revealed subtle but statistically significant phenotypic differences between T-CUS and T-LGLL, including more frequent diminished expression of CD2 and more pronounced loss of CD7 in T-LGLL, in addition to more frequent diminished expression of CD3 in T-CUS (Fig. 3b). As anticipated, the most striking difference between T-CUS and T-LGLL was the much lower clonal burden of T-CUS (Fig. 3c). Indeed, the median clonal burden of T-CUS was only 3.3% of total lymphocytes and 38 cells/μL of blood, compared with 69% of lymphocytes and 2533 cells/μL of blood in cases of T-LGLL ($p < 0.0001$ for both comparisons). Similarly, the five T-CUS detected in three healthy donors accounted for a median of only 1.2% of total lymphocytes (range: 0.2–3.6%).

Despite the overall marked differences in clone size, three patients in which we could not render a definitive diagnosis of T-cell neoplasia (classified as T-CUS in this study) had estimated clones sizes approaching those seen in T-LGLL (Fig. 3c). One patient was an 84-year-old male with a history of mantle cell lymphoma, urinary bladder carcinoma, and lung carcinoma, presenting on cancer remission with persistent eosinophilia of uncertain etiology (2.6×10^3/μL) and otherwise normal blood counts. Peripheral blood flow cytometry showed a CD8-positive/TRBC1-negative T-CUS accounting for 57% of lymphocytes and 829×10^3 cells/μL. The second patient was a 70-year-old male with a history of splenectomy for immune thrombocytopenia and a presumptive diagnosis of eosinophilic bronchitis, recovering from a recent transient episode of acute respiratory distress. Complete blood counts showed mild eosinophilia (0.91×10^3 cells/μL) and otherwise minimal blood count abnormalities, including borderline neutropenia (1.5×10^3 cells/μL) which spontaneously resolved 1 month later. Flow cytometric analysis showed a CD8-positive/TRBC1-negative T-CUS accounting for 21% of lymphocytes and 494 cells/μL; in addition to a second CD4-positive/CD8-dim (CD4/CD8 double-positive) small T-CUS, negative for TRBC1 and accounting for 1.5% of lymphocytes. The third patient was a 66-year-old male with combined variable immunodeficiency and a history of cyclic neutropenia accompanied by high fevers for more than 20 years, directly witnessed during an in-patient evaluation, and without a known etiology after comprehensive genetic evaluation. A complete blood count showed mild anemia and mild thrombocytopenia, but a normal neutrophil count. Flow cytometry on a bone marrow aspirate showed a CD8-positive/TRBC1-negative T-CUS accounting for 66% of lymphocytes, in addition to a second small CD4/CD8 double-negative/TRBC1-positive T-CUS accounting for 6% of lymphocytes. In all patients, T-cell gene rearrangement studies were positive or suspicious for a clonal T-cell population, and bone marrow biopsies showed no atypical lymphoid infiltrates or diagnostic morphologic evidence of malignancy. Despite the presence of a clonal T-cell subset, the lack of unexplained cytopenias and the absence of characteristic bone marrow findings precluded a definitive diagnosis of T-LGLL. Four patients within our T-LGLL cohort showed relatively low clonal T-cell burden: two peripheral blood specimens with T-cell clones accounting for 399×10^6 and 668×10^6 clonal T-cells/μL, and two bone marrow aspirates with T-cell clones accounting for 14% and 16% of lymphocytes (Fig. 3c). In all of these patients, bone marrow biopsies showed obvious intrasinusoidal infiltrates of cytotoxic T-cells which, in the setting of overt unexplained cytopenias and positive T-cell gene rearrangement studies, were morphologically diagnostic for T-LGLL.

Table 1 Clinical and laboratory characteristics of 159 patients with no evidence of T-cell malignancy, with or without T-cell clones of uncertain significance (T-CUS).

	No T-CUS	T-CUS	*P* value
N	117	42	–
Age in years (±SD)	56 (±18)	62 (±18)	0.07
Male:female	0.8:1	1:1	0.7
Diagnoses [*n* (%)]			
Autoimmune	32 (27%)	4 (10%)	**0.01**
Neurologic	4 (3%)	2 (5%)	0.1
Infectious	11 (9%)	1 (2%)	0.1
B-cell/plasma cell neoplasm	23 (20%)	8 (19%)	0.8
Carcinoma/sarcoma	3 (3%)	4 (10%)	0.07
MDS/MPN	7 (6%)	6 (14%)	0.1
Hypereosinophilia	2 (2%)	1 (2%)	0.8
Inflammatory dermatosis	10 (9%)	7 (17%)	0.2
Immunodeficiency	8 (7%)	4 (10%)	0.6
Post allo-HSCT	1 (1%)	0 (0%)	1
Post solid organ transplant	3 (3%)	3 (7%)	0.2
Blood counts [cells × 10^3/μL (±SD)]			
White blood cells	8.4 (±5.9)	7.3 (±4.5)	0.3
Neutrophils	4.7 (±8.2)	3.5 (±2.6)	0.3
Lymphocytes	3.1 (±3.8)	2.4 (±2.0)	0.3
Eosinophils	0.6 (±1.3)	0.4 (±0.8)	0.3
Platelets	228 (±134)	224 (±144)	0.9
Hemoglobin (g/dL)	12.19 (±2.-6)	12.53 (±2.3)	0.5
Peripheral blood subsets analysis			
Blood specimens studied	74	30	–
CD4:CD8	2.7:1 (±3.3:1)	2.0:1 (±1.5:1)	0.3
% NK cells (of lymphocytes)	11.6% (±11.2%)	12.3% (±10.6%)	0.8
% γδ T-cells (of lymphocytes)	4.3% (±8.4%)	3.1% (4.0%)	0.4
T-cell gene rearrangement positive or suspicious	9/43 (21%)	13/15 (87%)	**<0.0001**

SD standard deviation, *MDS/MPN* myelodysplastic and/or myeloproliferative neoplasm, *Allo-HSCT* allogeneic hematopoietic stem cell transplant. Statistically significant differences highlighted in bold.

Given prior reports describing clonal T-cell large granular proliferations in a variety of specific clinical settings, we queried whether an association between T-CUS and certain clinical or laboratory features could be encountered in our cohort. We found no positive association between T-CUS and any particular disease or condition, although an unexplained inverse association between T-CUS and autoimmune disease was noted (Table 1). Also, there was no correlation with age, sex, or peripheral blood counts. As expected, T-cell gene rearrangement studies were positive or suspicious for clonality in most patients with T-CUS on whom this test was performed (13 of 15, 87%), with the exception of two cases where a low clonal burden (<3% of total T-cells) might have precluded the detection of a clonal peak. In our patient population, a positive or suspicious T-cell gene rearrangement study was also fairly common in patients without detectable T-CUS (9/43, 21%), raising the possibility of TCRγδ clones or immunophenotypically indistinct clones that could not be detected by our flow cytometry assay.

To further confirm the validity of TRBC1 staining as an indicator of a clonotypic T-cell population, we studied the TCR-Vβ repertoire in four additional peripheral blood samples from patients with no demonstrable T-cell neoplasia on which a putative clonal T-cell subset was detected by TRBC1 staining. In all samples, these subsets showed either a restricted TCR-Vβ class expression or a lack of expression of all assayed TCR-Vβ classes, confirmatory of T-cell clonality. In addition, a comprehensive study of the TCR-Vβ repertoire in these samples showed one or two additional CD8-positive TCR-Vβ classes exhibiting a TRBC1 staining pattern suggestive of an immunodominant clonotype (Fig. 4a). A similar study on five peripheral blood samples from healthy donors showed two individuals where a minute phenotypically distinct T-cell population suggestive of an immunodominant clonotype could be demonstrated by TRBC1 analysis within a single CD8-positive/TCR-Vβ class-positive subset (Fig. 4b). Remarkably, a similar comprehensive analysis of the CD4-positive T-cell compartment did not yield any clonotypic subsets.

Discussion

T-LGLL is a lymphoproliferative disorder characterized by a persistent clonal expansion of cytotoxic T-cells involving peripheral blood and bone marrow, which frequently results in neutropenia or other cytopenias that may require therapeutic intervention [13]. Chronic antigen stimulation is suspected to be responsible for the initial emergence of a cytotoxic T-cell clone, which is then believed to undergo malignant transformation resulting in clonal overgrowth and suppression of hematopoiesis. The laboratory diagnosis of T-LGLL and its distinction from reactive T-cell large granular lymphocytes can be extremely challenging. Indeed, persistent clonal expansion of large granular lymphocytes have been reported in a variety of clinical settings including myelodysplastic syndromes [14], B-cell lymphoproliferative disorders, plasma cell proliferative disorders, autoimmunity [15], unexplained neutropenia [16], paroxysmal nocturnal hemoglobinuria [17], following allogeneic or autologous hematopoietic stem cell transplantation [18–20], and after solid organ transplantation [21]. Whether these

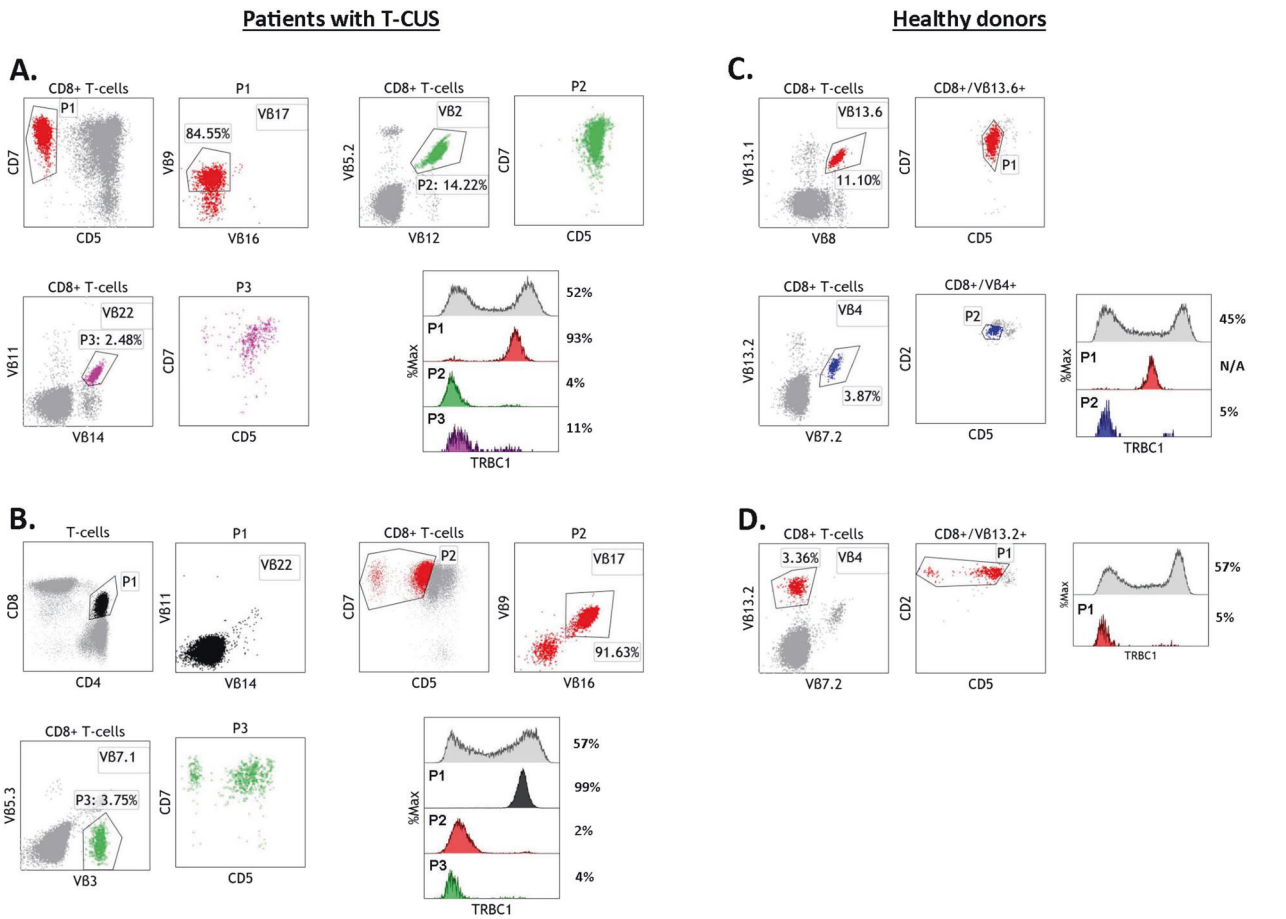

Fig. 4 Combined TRBC1 and TCR-Vβ analysis confirms clonality of T-CUS and reveals additional small immunodominant CD8-positive clonotypes. a Peripheral blood flow cytometry plots from a 67-year-old female with osteoarthritis and mild neutropenia, on hydroxychloroquine for presumptive systemic lupus erythematosus. TRBC1 staining demonstrates a CD8-positive/CD5-negative T-CUS (*P1, red events*) accounting for 4% of lymphocytes ($0.04 \times 10^3/\mu L$), which is shown to be homogenously TRBC1-positive and Vβ9-restricted. In addition, combined TRBC1/TCR-Vβ analysis reveals a TRBC1 staining pattern suggestive of clonality on gated Vβ2-restricted (*P2, green*) and Vβ22-restricted (*P3, violet*) CD8-positive T-cells. **b** Peripheral blood flow cytometry plots from a 77-year-old female with solitary plasmacytoma, stage I breast cancer, mild neutrophilia, mild monocytosis, and no cytopenias or atypical bone marrow lymphoid infiltrates. TRBC1 staining shows a CD4/CD8 double-positive/TRBC1-positive T-CUS (*P1, black events*) lacking staining for any of the assayed TCR-Vβs (only one representative tube shown), and a CD8-positive/CD5-dim/TRBC1-negative T-CUS (*P2, red events*) which is restricted for Vβ17. These subsets account for 36% and 16% of lymphocytes ($1.055 \times 10^3/\mu L$ and $0.469 \times 10^3/\mu L$ of blood), respectively. In addition, combined TRBC1/TCR-Vβ analysis reveals a TRBC1 staining pattern suggestive of clonality on gated Vβ7.1-restricted CD8-positive T-cells. **c**, **d** Peripheral blood flow cytometry plots from two healthy donors showing minute populations of immunophenotypically distinct CD8-positive T-cells with a TRBC1 staining pattern suggestive of physiologic clonotypic expansions, residing within Vβ13.6 (**c** top), Vβ4 (**c** bottom), or Vβ13.2-restricted (**d**) CD8-positive T-cell subsets. TRBC1 staining on background CD4-positive T-cells is shown for comparison (gray histograms), and percentages of TRBC1-positive events for each population are displayed adjacent to each histogram.

clonal large granular proliferations represent a benign T-cell response to chronic antigen stimulation or a very indolent form of T-LGLL has remained a topic of controversy and is best approached on a case by case basis.

Our study expands the understanding of clonal T-cell large granular proliferations by providing a broader picture of the incidence of this phenomenon. For this purpose, we utilized a simple but highly sensitive flow cytometry approach for the detection of T-cell clones, which is already part of our routine clinical practice and likely to be adopted by many other diagnostic laboratories [22]. We found small T-cell clones with phenotypic features corresponding to cytotoxic T-cell subsets in 26% of patients with no diagnostic evidence of T-LGLL or other T-cell neoplasia, and also in 13% of healthy adult donors. While these findings may come as a surprise to those accustomed to interpret positive T-cell clonality test results in support of a diagnosis of T-cell neoplasia, they are actually well in line with a breadth of literature describing the high prevalence of several small CD8-positive clonal T-cell populations in healthy individuals. These clones are already detectable in young individuals, tend to persist on follow-up analyses [23],

become more prominent with aging [24, 25], and are believed to represent physiologic expansions of mostly effector/memory cytotoxic T-cell subsets in response to either highly prevalent chronic infections such as cytomegalovirus and Epstein–Bar virus [8, 26–30], resolved acute infections [31], neoplastic processes, or other sources of antigen exposure. Also in line with our results is the fact that physiologic clonal expansions of CD4-positive/CD8-negative T-cells are much less frequently represented or not detected at all, depending on the sensitivity of the assay utilized, consistent with the concept that clonal expansions of CD4-positive and CD8-positive T-cells are controlled by substantially different mechanisms [1, 32]. Although we did not find a statistically significant correlation between age and the presence (Table 1) or clone size (Supplementary Fig. 1A, B) of T-CUS, the age spectrum of our cohort was too narrow to formally study the dynamics of T-CUS with aging.

We propose utilizing the term T-CUS, modeled after prior reports describing T-cell clones/clonopathies of unknown/uncertain/undetermined significance [33–36], in order to facilitate our understanding of frequently encountered clonal expansions of cytotoxic T-cells that are larger than expected for physiologic T-cell clones but do not meet criteria for T-LGLL at the time of evaluation. Based on our data on healthy individuals, a clonal size of 5% lymphocytes or 50 cells/μL of blood might be an appropriate arbitrary threshold to "screen out" normal clonotypic T-cell subsets which should not be reported in routine laboratory practice as they are detectable in healthy individuals. Higher practical thresholds, such as 20% of lymphocytes or 500 cells/μL of blood, might also be appropriate to prevent unnecessary work up of small T-CUS commonly encountered in patients with unrelated conditions. However, a small but significant overlap in clone size between T-CUS and T-LGLL should be acknowledged, often requiring bone marrow evaluation and clinical follow-up for a final distinction. Although testing for STAT3 mutations was not performed in this study, assessment of these genetic lesions could be contributory to the evaluation of patients where a distinction between T-CUS and T-LGLL cannot be easily determined, as these mutations have been reported in ~40% of patients with T-LGLL [37–39]. However, STAT3 mutations have also been identified in T large granular proliferations from patients with aplastic anemia, myelodysplastic syndrome [40], and Felty syndrome [41], where a diagnosis of T-LGLL could not be unequivocally established.

The definition of T-CUS proposed in this paper encompasses a spectrum of phenotypic variants, mirroring those of T-LGLL. These include previously described indolent CD4-positive largec granular proliferations with variable expression of CD8 [30, 42, 43], classified in our study as CD4/CD8 double-positive T-CUS or a single case of CD4-positive/CD57-positive T-CUS. Recognition of this unique phenotypic variant is important, as it is unlikely to be representative of bona fide T-LGLL. We also describe a significant proportion of CD4/CD8 double-negative T-CUS which has not been as widely recognized but has been encountered in a prior study [35]. While many our patients without T-cell neoplasia had eosinophilia (main indication for flow cytometric analysis in 16% of patients) (Supplementary Table 2), the lack correlation between the degree of eosinophilia and T-CUS, and the rarity of clinical hypereosinophilia in our cohort (Table 1) precludes interpretation of these clones as an abnormality with the spectrum of lymphocytic variant of hypereosinophilic syndrome (LV-HES). Indeed, most (but not all) abnormal T-cells identified in LV-HES correspond to CD4-positive/CD3-negative subsets [44], which markedly differs from the phenotypic spectrum of T-CUS.

Upon comprehensive phenotypic analysis, we found that the degree of CD5 loss by T-CUS is largely indistinguishable from that encountered in T-LGLL, despite the emphasis commonly made on this phenotypic characteristic for the detection of T-LGLL clones [45, 46]. We did however detect some phenotypic differences in the expression of CD2, CD3, and CD7, which were not initially recognized on visual inspection of the flow cytometry plots and currently remain largely unexplained (Fig. 3b). Of interest, a gene expression profiling study also demonstrated a lower level of CD2 transcripts in T-LGLL compared with reactive cytotoxic T-cells [47]. Limited numeric analysis of overall T-cell and NK-cell subsets did not yield significant differences between patients with and without T-CUS (Table 1), but did show decreased numbers of NK cells in patients with T-LGLL (Supplementary Table 1), as previously reported [48].

The high reliance of our assay on immunophenotypic markers and gating strategies to define clonality warrants caution and extensive validation when implementing this analysis in clinical practice. For example, while our initial analysis of a limited number of T-cell malignancies suggested a narrow TRBC1 expression pattern for neoplasia (>97% or <3%) [11], our accumulated experience with many more cases favors using a wider threshold (>85% or <15%) to accommodate for neoplasms on which the phenotypic distinction from background benign T-cells is not as perfect, and the percentage of tumor cells among total T-cells not as high. Given this interdependence between immunophenotypic gating and clonality assessment by TRBC1 staining, selection of accompanying T-cell markers that would best separate neoplastic cells from background benign T-cells is of upmost importance for an optimal test performance.

In addition to estimating percentages of TRBC1-positive events, analysis strategies should also include the identification of monophasic TRBC1-dim expression as a surrogate for clonality, as this often produces falsely normal percentages overlapping the threshold between positive and negative events. We have encountered this pattern in variety of T-cell neoplasms [11] and in T-CUS, always in a minority of cases, and to the best of our knowledge not associated with any particular phenotypic feature, patient demographic, specimen type, or pre-analytical variable. As expected, CD3-negative neoplasms are always TRBC1-negative (based on authors' experience, data not shown) due to lack of expression of the TCR complex. However, we have not found a consistent correlation between dim TRBC1 and dim CD3 expression that would support the hypothesis that this pattern is due to decreased expression of the TCR (Supplementary Fig. 1C). As such, the molecular basis of dim TRBC1 expression remains uncertain.

While most routine T-cell clonality assays rely on the relative abundance of a T-cell clone as detected by a dominant TCR-Vβ class [49, 50] or prominent TCR amplification product [2], our flow cytometry T-cell panel is capable of detecting very small T-cell clones that are not particularly abundant relative to total T-cells, but have distinct immunophenotypic features that allows them to be gated and analyzed separately for clonality. Moreover, we show that a combined TRBC1/TCR-Vβ analysis provides a novel method to detect even smaller clonal T-cell populations that have replaced most of the repertoire of a specific Vβ class, but have not expanded beyond the size of other Vβ classes. Thus, we were able to detect small T-cell clones that would have otherwise been missed by other common laboratory tests, many of which correspond to physiologic clonotypic T-cell expansions of normal immunity. Despite these advantages, our routine flow cytometry panel does not have the capacity to detect TCRγδ T-cell clones or clonal T-cell subsets that lack distinguishable phenotypic features with the antigens studied.

By systematically studying the phenotypic properties of clonal T-cell subsets in patients with no demonstrable T-cell neoplasms and healthy individuals, we provide a framework for the interpretation of laboratory test results where small T-cell clones and/or immunophenotypically distinct T-cell subsets might be detected. The first corollary of our study relates to the rarity of physiologic or reactive CD4-positive/CD8-negative clonotypic T-cell expansions detected by our assay, which should prompt careful evaluation for the possibility of a T-cell lymphoproliferative disorder irrespective of the clone size. Second, a CD4-negative or CD4/CD8 double-positive clonal T-cell subset should be interpreted in the context of clone size, as clones smaller than 20% of total lymphocytes are highly prevalent in patients with no T-cell malignancy (T-CUS) and show no particular disease association in our series, while clones smaller than 5% of lymphocytes are within the expected size for physiologic clonotypic expansions in healthy individuals. Third, it should be noted that the phenotypic spectrum of T-CUS closely resembles that of T-LGLL, which directly relates to the common difficulties in distinguishing a reactive clonal proliferation of T-cell large granular lymphocytes from a bone fide T-cell neoplasm. Finally, the high prevalence of reactive T-cell clones in our series warrants caution in the interpretation of highly sensitive assays of T-cell clonality, as low-positive results might not be contributory to the overall clinical evaluation or may result in misinterpretation of suspicious morphologic, phenotypic, or clinical findings.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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