Utility of TRBC1 Expression in the Diagnosis of Peripheral Blood Involvement by Cutaneous T-Cell Lymphoma

Pedro Horna¹, Min Shi¹, Dragan Jevremovic¹, Fiona E. Craig², Nneka I. Comfere³ and Horatiu Olteanu¹

Peripheral blood involvement by cutaneous T-cell lymphoma is typically assessed by flow cytometry and plays a critical role in diagnosis, classification, and prognosis. Simplified strategies to detect tumor cells (Sezary cells) fail to exclude reactive subsets, whereas tumor-specific abnormalities are subtle and inconsistently present. We implemented a flow cytometric strategy to detect clonal Sezary cells based on the monotypic expression of one of two mutually exclusive TCR constant β chains, TRBC1 and TRBC2. Analysis of CD4⁺ T-cell subsets and TCR variable β classes from healthy donors showed polytypic TRBC1 staining. Clonal Sezary cells were identified by TRBC1 staining in 56 of 111 (50%) samples from patients with cutaneous T-cell lymphoma, accounting for 7–18,155 cells/µl and including 13 cases (23%) lacking tumor-specific immunophenotypic abnormalities. CD4⁺ T-cell subsets from 86 patients without T-cell lymphoma showed polytypic TRBC1 staining, except for five patients (6%) with minute T-cell clones of uncertain significance accounting for 53–136 cells/µl. Assessment of TRBC1 expression within a comprehensive single-tube flow cytometry assay effectively overcomes interpretative uncertainties in the identification of Sezary cells without the need for a separate T-cell clonality assay.

INTRODUCTION

Sezary syndrome (SS) and mycosis fungoides (MF) are two clinically distinct neoplasias of CD4⁺ skin-resident T cells that share remarkable morphologic and immunophenotypic similarities and are commonly referred to as cutaneous T-cell lymphoma (CTCL). MF is characterized by epidermotropic neoplastic T-cell infiltrates, consisting of small- to medium-sized atypical cells with irregular (cerebriform) nuclei (Cerroni et al., 2017). SS is a related condition defined by the triad of erythroderma, generalized lymphadenopathy, and the presence of circulating neoplastic T cells (Sezary cells) (Whitaker et al., 2017).

Detection of neoplastic CD4⁺ T cells in peripheral blood (>1,000 cells/µl) is essential to establish a diagnosis of SS, because the clinical features are often indistinguishable from benign inflammatory dermatoses with erythroderma. In addition, the degree of peripheral blood involvement by MF represents a major prognostic variable and is integrated into the CTCL staging criteria proposed by the International Society for Cutaneous Lymphomas and the cutaneous lymphoma task force of the European Organization for Research and Treatment of Cancer (EORTC) (Olsen et al., 2007, 2011; Olsen, 2015). These blood rating parameters are based on absolute numbers of Sezary cells per µl of blood (B0, <250 cells/µl; B1, ≥250 and <1,000 cells/µl; B2, ≥1,000 cells/µl) and are also utilized in consensus definitions of complete blood response, partial response, and progressive disease for both MF and SS (Scarisbrick et al., 2018).

Flow cytometry is the method of choice for detecting Sezary cells in peripheral blood (Craig, 2020; Morice et al., 2006), largely replacing the less reproducible and subjective morphologic quantitation of abnormal lymphocytes with cerebriform nuclei. The typical immunophenotype of Sezary cells by flow cytometry has been described in numerous studies as CD2⁺, CD3⁺, CD4⁺, and CD5⁺ T cells negative for CD7, CD8, and CD26 (Bernengo et al., 2001; Horna et al., 2014; Kelemen et al., 2008). Based on this profile, a recent iteration of the EORTC blood staging scheme recommended using absolute numbers of CD4⁺ T cells lacking CD7 or CD4⁺ T cells lacking CD26 expression as a direct indicator of peripheral blood tumor burden for staging and monitoring purposes (Scarisbrick et al., 2018). In parallel with these recommendations, a more comprehensive immunophenotypic analysis has been gradually adopted by many flow cytometry laboratories, where Sezary cells are rather identified based on subtle immunophenotypic features strongly associated with neoplasia, such as dim expression of CD3 or CD4 (Craig, 2020; Horna et al., 2020; Jevremovic and Olteanu, 2019). Despite these advances, identification of Sezary cells by flow cytometry remains challenging, given that their most obvious immunophenotypic characteristics (lack of CD7 and CD26) are shared with benign T-cell subsets, whereas more specific immunophenotypic properties are typically subtle, equivocal, or not identified at all.

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Abbreviations: CTCL, cutaneous T-cell lymphoma; EORTC, European Organization for Research and Treatment of Cancer; MF, mycosis fungoides; SS, Sezary syndrome; T-CUS, T-cell clones of uncertain significance; Vβ, variable β

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We recently reported a highly sensitive flow cytometric strategy to detect immunophenotypically distinct clonal T-cell subsets within a comprehensive diagnostic panel (Berg et al., 2020; Shi et al., 2020a). This approach takes advantage of the random utilization of one of two mutually exclusive TCR constant β chain genes during TCR-β T-cell gene rearrangement, and the availability of an antibody (clone JOVI.1) to detect only one of these two gene products (TRBC1) (Maciocia et al., 2017). In this assay, polyclonal CD4⁺ T-cell subsets are characterized by numerically similar TRBC1⁺ and TRBC1-negative (presumptively TRBC2⁺) discrete subpopulations (Figure 1a), whereas clonal CD4⁺ T-cell subsets are readily identifiable by their monophasic TRBC1-positive, dim or negative expression pattern. Each immunophenotypically distinct T-cell subset can be independently and instantly queried for clonality, providing an ideal scenario to evaluate suspected Sezary cell populations identified by various gating strategies. We hereby report our experience implementing this strategy into a diagnostic Sezary cell flow cytometry panel, resulting in the rapid and highly sensitive identification and quantitation of clonal CD4⁺ T-cell subsets compatible with Sezary cells.

RESULTS

CD4⁺ T-cell subsets from healthy donors show a polytypic TRBC1 staining pattern
A total of 24 peripheral blood samples from healthy donors were studied using a previously reported single-tube flow cytometry assay designed to detect Sezary cells based on the identification of TRBC-restricted (clonal) CD4⁺ T cells (Shi et al., 2020a). In all of these samples, each discrete CD4⁺ T-cell subset identified on a CD7 versus CD26 dot plot showed a biphasic TRBC1 expression pattern, with percentages of TRBC1⁺ events well within the previously reported arbitrary thresholds (15% and 85%) for defining polyclonal T cells (Shi et al., 2020a) (Figure 1b and c). In addition, a custom flow cytometry panel to study TRBC1 expression on 21 detectable CD4⁺ variable β (Vβ)-classes from five healthy donors consistently showed a polytypic TRBC1 staining pattern on all Vβ classes (Figure 1d). Thus, TRBC1 expression appeared to be independent of immunophenotype or Vβ class usage, supporting its use as a clonality surrogate in the analysis of CD4⁺ T-cell subsets.

TRBC1 staining facilitates the detection of clonal Sezary cells
We studied 111 peripheral blood samples from 78 patients with previously or subsequently confirmed CTCL, including 72 specimens from 58 patients with MF and 39 specimens from 20 patients with SS. The mean patient age was 65 years old (range = 21–91 years old), and the male to female ratio was 1:1.1. Of these samples, 30 (27%) corresponded to patients with newly diagnosed or untreated CTCL (19 MF and 11 SS), whereas the remainder were from patients with CTCL on therapy. All specimens were studied using a single-tube Sezary cell flow cytometry panel including an anti-TRBC1 antibody, as previously described (Shi et al., 2020a).

Overall, 56 samples (50%) from patients with CTCL showed immunophenotypically distinct CD4⁺ T-cell subsets with a clonal TRBC1 staining pattern consistent with Sezary cells (Figure 2). Most clones were defined by percentages of TRBC1⁺ events outside the previously reported polytypic range (15–85%), whereas six samples from five patients (four with SS and one with MF stage IA) had clones exhibiting a monophasic TRBC1-dim expression pattern that overlapped the fluorescence threshold dividing positive and negative events (Figures 2c and 3a). Calculation of absolute Sezary cell counts based on correlation with the absolute lymphocyte count yielded values ranging from 7 cells/μl to 18,155 cells/μl (mean = 2,087 cells/μl), with a value distribution supporting a lower limit of detection of approximately 10 clonal T cells per μl (Figure 3b). The resulting blood ratings based on these absolute Sezary cell count estimates and overall disease stage of these patients are summarized in Supplementary Table S1.

Of 56 clonal Sezary cell populations identified by TRBC1 staining, 43 (77%) showed one or more immunophenotypic abnormalities strongly associated with neoplasia, namely dim expression of CD2, CD3, CD4, CD5 or CD45, or increased light scatter (Figure 3c). Because these abnormalities were typically subtle (Figure 2), rapid demonstration of clonality by TRBC1 staining often contributed to an unequivocal diagnosis. Importantly, 13 of 56 (23%) Sezary cell populations identified by TRBC1 staining did not exhibit any of the above immunophenotypic abnormalities but were easily identified as clonal by studying TRBC1 staining patterns on discrete CD4⁺ T-cell subsets detected on a CD7 versus CD26 dot plot (Figure 2e and f). Molecular T-cell gene rearrangement studies performed on 22 of 56 samples with Sezary cells detected by TRBC1 were positive (77%) or suspicious (18%) for clonality, except for one sample with only 106 Sezary cells/μl estimated by TRBC1 (Supplementary Table S2).

TRBC1 staining identifies rare small CD4⁺ T-cell clones in patients without T-cell neoplasia
We also studied 88 samples from 86 patients with no prior, current, or subsequently diagnosed T-cell lymphoma based on comprehensive clinical and laboratory evaluation. The mean patient age was 61 years old (range = 6 months–98 years old), and the male to female ratio was 1:2:1. Reported diagnoses included inflammatory dermatoses (59%), B-cell lymphoproliferative disorders (14%), autoimmune disease (10%), reactive leukocytosis (10%), and eosinophilia (5%). This cohort included seven patients with reactive erythroderma (four atopic dermatitis, one allergic contact dermatitis, one pityriasis rubra pilaris, and one erythroderma of uncertain etiology), two of which exhibited a CD4 to CD8 ratio >10. Demonstration of a polytypic TRBC1 staining pattern on CD4⁺ T-cell subpopulations from these patients allowed for a rapid and confident exclusion of peripheral blood involvement by CTCL (Figure 4a and b).

Only five patients (6%) with no evidence of T-cell neoplasia showed small CD4⁺ and CD8-negative T-cell subsets with a TRBC1 expression pattern consistent with clonality (Figure 4c–e). These small clones accounted for 53–136 cells/μl (corresponding to an equivalent of B0 rating), were negative for CD7 and CD26, and lacked tumorspecific immunophenotypic abnormalities (Figure 4e). In addition, one patient harbored a small CD4 and CD8 double-positive clonal T-cell subset (Figure 4d and f). Based on our
recently published experience studying T-cell clonality in patients without demonstrable T-cell neoplasia (Shi et al., 2020b), these small subsets were interpreted as CD4\(^+\)T-cell clones of uncertain significance (T-CUS), likely representative of physiologically expanded clonotypes.

**Comparison of TRBC1 staining with EORTC-recommended Sezary cell estimates**

Absolute Sezary cell counts obtained using the EORTC-recommended gating strategy (maximum value of CD4\(^+\) T cells lacking CD7 and CD4\(^+\) T cells lacking CD26) (Lyapichev et al., 2020; Scarisbrick et al., 2018) correlated linearly and proportionally with those obtained using TRBC1 staining (linear regression slope = 0.99; intercept = 90 Sezary cells/\(\mu\)l; intraclass correlation coefficient = 0.998; \(P < 0.0001\)) (Figure 5a). However, two samples from two patients with a history of MF rated as B1 by TRBC1 staining (617 and 985 Sezary cells/\(\mu\)l; stage IB and IIB, respectively) were assigned a B2 rating by the EORTC gating method (1,103 and 1,010 Sezary cells/\(\mu\)l, respectively; equivalent to stage IVA1). Conversely, a single sample from a patient with SS on therapy was rated as B2 by TRBC1 staining (1,035 Sezary cells/\(\mu\)l) but assigned a B1 rating by EORTC gating (821 Sezary cells/\(\mu\)l). In addition, the EORTC method consistently produced quantifiable Sezary cell counts in the absence of detectable clonal CD4\(^+\) T cells by TRBC1 staining, including three samples from patients with stage IA MF rated as B1 by the EORTC method.

Although there is no accepted laboratory gold standard for the identification of Sezary cells, all 31 samples with more than 250 overtly aberrant T cells per \(\mu\)l (immunophenotypically diagnostic for neoplasia independent of CD7, CD26, and TRBC1 expression) were appropriately rated as B1 or B2 by both the EORTC gating and TRBC1 staining methods, suggestive of a high and comparable diagnostic sensitivity for B1/B2 blood involvement. In 88 samples from patients with no clinical evidence of T-cell malignancy, the EORTC method produced false positive B1 blood ratings in 16
Figure 2. TRBC1 staining confirms clonality of suspected Sezary cells, rapidly resolving diagnostic, and quantitative uncertainties. Peripheral blood flow cytometry plots from patients with (a–c) SS or (d–f) MF. The red events correspond to CD4+ T-cell subsets exhibiting a clonal TRBC1 staining pattern defined as either (a, e, and f) homogenously positive, (b and d) negative, or (c) dim TRBC1 expression (histograms), consistent with Sezary cells. Demonstration of clonality by TRBC1 staining was critical to determine (a, e, and f) which subsets within the CD7 versus CD26 dot plot corresponded to Sezary cells, (d) whether an unusual small subset was actually clonal, and (e and f) to detect Sezary cells with no major diagnostic immunophenotypic abnormalities. Absolute Sezary cell counts, TNMB ratings, and overall staging are shown for each case. Cyan: nonclonal CD4+ T cells; orange: CD8+ T cells. Percentages of TRBC1+ events are displayed within each histogram. Max, maximum; MF, mycosis fungoides; N/A, not applicable; SS, Sezary syndrome; TNMB, tumor-node-metastasis-blood.
specimens (Figure 5b), whereas none of these samples yielded a B1 or B2 blood rating by TRBC1 staining (diagnostic specificity for B1/B2 blood involvement, 82% for the EORTC method vs. 100% for TRBC1 staining). In addition, the 95th percentile of false Sezary cell counts in patients with no T-cell lymphoma (limit of blank) was 445 cells/μl using the EORTC gating strategy (significantly above the B1 rating threshold) but only 46 cells/μl using TRBC1 staining, consistent with a superior capacity of the latter method to discriminate Sezary cells from reactive subsets within the B0–B1 range (Figure 5b).

**DISCUSSION**

We report our experience using a single anti-TRBC1 antibody within a comprehensive Sezary cell flow cytometry panel to identify and quantify TRBC-restricted (clonal) CD4⁺ T-cell subsets compatible with Sezary cells in a large patient cohort. Similar to recent studies (Berg et al., 2020; Novikov et al., 2019; Shi et al., 2020a, 2020b), we show that TRBC1 is able to accurately identify and quantify immunophenotypically distinct clonal T-cell subsets in clinical specimens while ruling out clonality on benign subsets with immunophenotypic features concerning for neoplasia. This approach is particularly useful and well-suited for the detection of Sezary cells, where commonly used strategies based on immunophenotypic analysis are often difficult to interpret, and accurate assessment of peripheral blood tumor burden is of essence for prognosis and monitoring.

Numerous reports have documented the clinical utility of flow cytometry to detect and quantify Sezary cells in peripheral blood, using either simplified gating strategies (Hristov et al., 2011), comprehensive immunophenotypic
Figure 4. TRBC1 staining rapidly confirms the polytypic nature of reactive CD4⁺ T-cell expansions and identifies small CD4⁺ T-CUS. (a) A 70-year-old female with reactive lymphocytosis and increased percentages of CD7-negative and CD26-negative CD4⁺ T-cell subsets, all negative for clonality by TRBC1 staining (histograms). (b) A 59-year-old man with atopic dermatitis, presenting with erythroderma and a markedly increased CD4 to CD8 (CD4:CD8) ratio. All CD4⁺ T-cell subsets were negative for clonality by TRBC1 staining (histograms). (c) Percentages of TRBC1⁺ events for CD4⁺ T-cell subsets defined based on CD7 and CD26 expression for 88 patients without T-cell lymphoma. Dotted lines: Thresholds for clonality. (d) Incidence of CD4⁺ T-CUS in 86 patients without T-cell lymphoma.
analysis (Horna et al., 2014; Novelli et al., 2015; Vaughan et al., 2012), or the detection of selected antigens preferentially expressed in CTCL (Hurabielle et al., 2017; Wysocka et al., 2014). To our knowledge, this is the first study to directly compare the simplified gating strategy recommended by the EORTC staging scheme (Scarisbrick et al., 2018) with a biologically sound assessment of clonal T-cell burden. Of note, the EORTC simplified strategy is unable to exclude common benign CD4+ T-cell subsets lacking CD7 and/or CD26 expression, including subsets readily identifiable in healthy donors and commonly expanded in patients with no history of T-cell neoplasia (Supplemental Figure S1). Consistent with this limitation, we show that the EORTC method for estimating Sezary cells is unable to accurately distinguish neoplastic cells from reactive CD4+ T-cell subsets (Figure 4a).

More recently, an international consensus statement of flow cytometry experts recommended identifying Sezary cells on the basis of a comprehensive single-tube analysis of at least CD3, CD4, CD8 and CD45 expression, in combination with CD7 and CD26 (Horna et al., 2020; Lyapichev et al., 2020). In most cases, comprehensive immunophenotypic analysis using this antigen combination provides accurate and confident estimates of Sezary cell counts based on the identification of immunophenotypic features that are rather specific for neoplasia (namely, diminished CD3, CD4, and CD45 expression and increased light scatter). However, a significant minority of cases lacking tumor-specific immunophenotypic abnormalities (i.e., expanded CD4+ T-cell populations negative for CD7 and/or CD26 in the absence of other distinct immunophenotypic features) remain challenging to interpret. In our cohort, 16 of 56 clonal Sezary cell populations (29%) identified by TRBC1 staining did not show tumor-specific immunophenotypic abnormalities based on the antigens recommended by this international consensus. In our experience, expertise in the analysis of subtle immunophenotypic features, morphologic evaluation of the peripheral blood smear, correlation with separate T-cell clonality assays, and access to detailed clinical information can significantly narrow but not completely eliminate this interpretative gap.

A number of reports have documented the usefulness of TCR-Vβ receptor analysis by flow cytometry in the identification of Sezary cells (Feng et al., 2010; Morice et al., 2006, 2004). This is evidently the approach that most resembles T-cell clonality analysis by TRBC1, because it similarly evaluates for TCR restriction within an immunophenotypically distinct T-cell subset. However, analysis of the TCR-Vβ repertoire has several drawbacks, including its relatively high cost, the complexity of the analysis, and the limited number of antibodies that can be combined for gating and analysis of a particular T-cell subset (Moric et al., 2006, 2004). In contrast, T-cell clonality assessment by TRBC1 can easily be integrated into a routine T-cell flow cytometry panel and performed on all samples evaluated at a much lower cost.

The presence of small CD4+ T-cell clones in 6% of our patients with no evidence of T-cell lymphoma is notable and consistent within our recent definition of T-CUS using a similar flow cytometry assay (Shi et al., 2020b). In that prior study, 26% of patients with no demonstrable T-cell neoplasia had small T-cell clones detected by TRBC1, almost always within the CD8+ T-cell compartment, and showing immunophenotypic features reminiscent of T-cell large granular lymphocytic leukemia. These small clonal T-cell
proliferations are believed to be the product of physiologically normal immune responses to infections, neoplastic processes, or other sources of antigen exposure (Beverley and Maini, 2000; Gil et al., 2015; Maini et al., 1999; Marrero et al., 2016; Rodriguez-Caballero et al., 2008). In this study, inclusion of an anti-CD26 antibody likely facilitated the detection of more CD4\(^+\) and CD8\(^-\) T-cell subsets, because these subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartment. Of note, none of these CD4\(^+\) subsets were characteristically restricted to the CD7\(^-\) and CD26\(^-\) compartments.

In summary, we document the usefulness of TRBC1 assessment in the detection and quantification of Sezary cells in a large cohort of patients with CTCL, including cases without overt diagnostic immunophenotypic aberrancies. A single anti-TRBC1 antibody added to a comprehensive single-tube Sezary cell flow cytometry assay provides a rapid, simple, and low-cost approach to routinely query for clonality within immunophenotypically distinct CD4 T-cell subsets, consistently resulting in unequivocal interpretations in the assessment of peripheral blood involvement by CTCL without the need for a separate T-cell clonality assay.

**MATERIALS AND METHODS**

**Patient and sample selection**

Fresh peripheral blood specimens were evaluated by flow cytometry for diagnostic purposes by the Cell Kinetics Laboratory at Mayo Clinic (Rochester, MN) between December 2018 and December 2019. Electronic medical records, including pathology reports, laboratory test results, clinical notes, and imaging studies, were retrospectively reviewed to identify patients with either (i) no diagnostic clinical or laboratory evidence of a current, prior or subsequent diagnosis of T-cell malignancy, or (ii) a definitive diagnosis of MF or SS. In addition, fresh peripheral blood specimens from 24 healthy adult donors were made available by the Biospecimen Program at Mayo Clinic. Patient consent was not required because the flow cytometric analysis was part of a clinically indicated laboratory workup, and the retrospective review of medical records was deemed appropriate for a waiver of consent. Samples from healthy donors were used with written informed consent. This study was approved by the Mayo Clinic Institutional Review Board.

**Flow cytometry**

A single-tube, 9-color Sezary cell flow cytometry panel was utilized as previously described (Shi et al., 2020a), consisting of a cocktail of fluorescent-labeled antibodies recognizing CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD45, and TRBC1. In short, clonal CD4\(^+\) T-cell populations were identified as immunophenotypically distinct CD4\(^+\) and CD8\(^-\) T-cell subsets exhibiting a monophasic TRBC1 staining pattern defined as either (i) >85% of TRBC1\(^+\) events, (ii) <15% TRBC1\(^-\) events, or (iii) homogenous TRBC1-dim expression. These thresholds were defined arbitrarily to capture all T-cell neoplasms so far evaluated in our practice (over 200 T-cell malignancies) while excluding total benign CD4\(^+\) and CD8\(^+\) T-cell subsets from healthy individuals. Flow cytometric analysis included the independent evaluation of CD4\(^+\) T-cell subsets identified on a CD7 versus CD26 dot plot, in addition to any discrete subset with an antigen expression pattern diverging from that expected for healthy donors (Horna et al., 2020). CD4 and CD8 double-positive events were also studied for clonality based on TRBC1 staining patterns.

A combined TRBC1 and TCR-V\(\beta\) flow cytometry assay was developed based on the previously described iOTeR Beta Mark TCR V\(\beta\) repertoire kit assay (Beckman Coulter, Brea, CA). In short, a cocktail of antibodies recognizing CD2, CD3, CD4, CD5, CD7, CD8, and TRBC1 (conjugated to PerCP-Cy5.5, phycoerythrin-Cy7, APC-R700, APC, Horizon V450, APC-H7, and BV605, respectively) was added to each of the eight tubes of the TCR V\(\beta\) kit assay containing 3 V\(\beta\) class antibodies per tube conjugated to FITC, phycocyanin, and FITC-phycoerythrin. Each TCR-V\(\beta\) specificity detectable on gated CD4\(^+\) and CD8-negative T cells was analyzed separately for clonality based on the pattern of TRBC1 expression, as described previously. Tube 6 of the Beta Mark test (V\(\beta\)23, V\(\beta\)1, and V\(\beta\)21.3) was excluded from the analysis because of suboptimal staining when utilized within this custom panel.

At least 100,000 total events were acquired per analysis tube on a FACScanto II flow cytometer (BD Biosciences, San Jose, CA). All antibodies were obtained from BD Biosciences, except for a FITC-conjugated anti-TRBC1 antibody (clone JOVL1, Ancell Corporation, Bayport, MN) and the anti–TCR-V\(\beta\) antibodies from the Beta Mark kit (Beckman Coulter). All analyses were performed on Kaluza version 2.1 (Beckman Coulter), considering only populations composed of 100 or more events. All cases were analyzed retrospectively by two of the authors (PH and HO) in a blinded fashion to ensure consistency. Absolute Sezary cell counts were calculated by multiplying the percentage of clonal T cells within a CD45 and light scatter lymphocyte gate by the absolute lymphocyte count obtained separately on a Sysmex XN-350 automated analyzer (Kobe, Hyogo, Japan).

**TCR gene rearrangement studies**

Total cellular DNA was extracted and PCR amplification performed in five multiplex PCR tubes with ASR Biomed-2 primers (Invivogen Technologies, San Diego, CA) targeting TCR-V\(\beta\), -D\(\beta\), -J\(\beta\), -V\(\gamma\), and -J\(\gamma\) 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 data plots and summary statistics were performed using GraphPad Prism, version 8.2.1 for Windows (GraphPad Software, San Diego, CA). Passing-Bablok linear regressions and intraclass correlation coefficients were calculated using version 1.4 of the deming package (Therneau, 2018) in R version 3.6.2 (R Core Team, 2019).

**Data availability statement**

There are no relevant datasets related to this article.

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**CONFLICT OF INTEREST**

The authors state no conflicts of interest.
AUTHOR CONTRIBUTIONS
Conceptualization: PH, HO; Data Curation: PH, HO; Formal Analysis: PH, HO; Investigation: PH, HO; Methodology: PH, MS, DJ, HO; Project Administration: PH, MS, DJ, HO; Resources: PH, MS, DJ, HO; Validation: PH, MS, DJ, HO; Visualization: PH; Writing - Original Draft Preparation: PH, HO; Writing - Review and Editing: FEC, NIC

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.09.011.

REFERENCES

www.jidonline.org
Supplementary Figure S1. Simplified flow cytometric analysis of CD4⁺ T-cell subsets from healthy donors and patients with or without CTCL.

Peripheral blood samples corresponding to healthy donors (24), patients with no evidence of T-cell lymphoma (88), and patients with CTCL (111) were studied by flow cytometry. (a, b) Gated CD3⁺ and CD4⁺ T cells were studied for percentage of events negative for CD7 and/or CD26. (c) The CD4 to CD8 (CD4:CD8) ratio was calculated by displaying CD3⁺ T cells on a CD4 versus CD8 dot plot and quantifying the corresponding subsets. Dotted lines depict commonly used thresholds for the detection of Sezary cells: 40% CD7-negative events, 30% CD26-negative events, and CD4-to-CD8 ratio >10, respectively. CTCL, cutaneous T-cell lymphoma.

Supplementary Table S1. ISCL-EORTC Stage and B Ratings on Samples from Patients with CTCL Evaluated by Peripheral Blood Flow Cytometry

<table>
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<th>Overall Disease Stage</th>
<th>Number of Samples (%)</th>
<th>Blood Rating at the Time of Evaluation (Based on TRBC1 Staining)</th>
<th>Number of Patients (%)</th>
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<td>B0 27 B1 0 B2 0</td>
<td>22 (28)</td>
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<td>19 (17)</td>
<td>B0 16 B1 3 B2 0</td>
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<td>6 (8)</td>
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### Supplementary Table S2. Results of T-Cell Gene Rearrangement Studies by PCR Performed on a Subset of Specimens

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<th>Patient Group</th>
<th>CD4⁺ T-Cell Clone by TRBC1</th>
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<th>Total Samples</th>
<th>Samples Studied (%)</th>
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<td>B0</td>
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<td>2 (100)</td>
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</tr>
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<td>CTCL</td>
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<td>B0</td>
<td>55</td>
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</tr>
<tr>
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<td>Yes</td>
<td>B0</td>
<td>17</td>
<td>7 (41)</td>
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<td></td>
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Abbreviation: CTCL, cutaneous T-cell lymphoma.