Published in final edited form as:

Sci Transl Med. 2015 October 7; 7(308): 308ra158. doi:10.1126/scitranslmed.aaa9122.

TCR sequencing facilitates diagnosis and identifies mature T cells as the cell of origin in CTCL

Ilan R. Kirsch^{#2}, Rei Watanabe^{#3}, John T. O'Malley³, David W. Williamson², Laura-Louise Scott³, Christopher P. Elco⁴, Jessica E. Teague³, Ahmed Gehad³, Elizabeth L. Lowry³, Nicole R. LeBoeuf^{3,6}, James G. Krueger⁵, Harlan S. Robins², Thomas S. Kupper^{3,6,7}, and Rachael A. Clark^{3,6,7}

²Adaptive Biotechnologies, Seattle, WA, USA

³Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

⁴Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

⁵Department of Dermatology, Rockefeller University, New York, NY, USA

⁶Dana-Farber/Brigham and Women's Cancer Center, Boston, MA, USA

[#] These authors contributed equally to this work.

Abstract

Early diagnosis of CTCL is difficult and takes on average six years after presentation, in part because the clinical appearance and histopathology of CTCL can resemble that of benign inflammatory skin diseases. Detection of a malignant T cell clone is critical in making the diagnosis of CTCL but the TCR γ PCR analysis in current clinical use detect clones in only a subset of patients. High-throughput TCR sequencing (HTS) detected T cell clones in 46/46 CTCL patients, was more sensitive and specific than TCR γ PCR, and successfully discriminated CTCL from benign inflammatory diseases. HTS also accurately assessed responses to therapy and facilitated diagnosis of disease recurrence. In patients with new skin lesions and no involvement of blood by flow cytometry, HTS demonstrated hematogenous spread of small numbers of malignant T cells. Analysis of CTCL TCR γ genes demonstrated that CTCL is a malignancy derived from

⁷Correspondence should be addressed to: Rachael A. Clark, Department of Dermatology, Brigham and Women's Hospital, Room 505A, 221 Longwood Avenue, Boston, Massachusetts 02115 or to Thomas S. Kupper, Department of Dermatology, Brigham and Women's Hospital, Room 672, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115. Phone (617) 525-8512; Fax (617) 264-5123; rclark1@partners.org or tkupper@partners.org..

Author contributions: R.A.C. supervised experiments, analyzed data, carried out statistical analyses, drafted figures, wrote and revised the manuscript. T.S.K. helped conceive the study, arranged collaborations, is the PI of the observational clinical trial that provided CTCL patient samples, and edited the manuscript. I.R.K., D.W.W., H.S.R. carried out HTS analyses, analyzed data, carried out statistical analyses, and edited the figures and manuscript. R.W., J.E.T., E.L.L. and A.G. isolated DNA from patient specimens, organized, and helped to analyze patient results, including statistical analyses. J.T.O. and L.L.S. carried out immunofluorescence studies. J.E.T. also provided editorial assistance. C.P.E. and N.R.L. provided skin samples of CTCL, psoriasis and eczematous dermatitis. J.G.K. provided skin samples of psoriasis.

Competing interests: I.R.K., D.W.W., H.S.R., T.S.K. and R.A.C. are named as inventors on two pending patent applications regarding the utility of HTS in cutaneous lymphoma. I.R.K., D.W.W., and H.S.R. hold stock in Adaptive Biotechnologies and I.R.K. and D.W. are employees of Adaptive Biotechnologies. T.S.K. serves on the Scientific Advisory Board of Adaptive Biotechnologies but does not own stock. Other authors have no conflicts.

mature T cells. There was a maximal T cell density in skin in benign inflammatory diseases that was exceeded in CTCL, suggesting a niche of finite size may exist for benign T cells in skin. Lastly, immunostaining demonstrated that the malignant T cell clones in mycosis fungoides and leukemic CTCL localized to different anatomic compartments in the skin. In summary, HTS accurately diagnosed CTCL in all stages, discriminated CTCL from benign inflammatory skin diseases and provided insights into the cell of origin and location of malignant CTCL cells in skin.

Introduction

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous collection of non-Hodgkin's lymphomas derived from skin tropic T cells. CTCL encompasses skin limited variants such as mycosis fungoides (MF) and leukemic forms of the disease, including Sézary syndrome (1). T cells are confined to fixed inflammatory skin lesions in MF. When the disease is limited in extent, MF is often indolent and approximately 80% of patients are expected to have a normal life expectancy (2). A subset of MF patients develop progressive, lethal disease characterized by skin tumors and lymph node involvement. Aggressive MF can involve many sites but peripheral blood involvement is unusual. In contrast, patients with leukemic CTCL (L-CTCL, including Sézary syndrome) present most commonly with diffuse skin erythema, lymphadenopathy and malignant T cells accumulate in the blood, skin and lymph nodes. L-CTCL is typically refractory to therapy and median survival is three years, with death occurring most commonly from infection. Hematopoietic stem cell transplantation is the only potentially definitive cure for both advanced MF and L-CTCL (3).

Early diagnosis of CTCL can be challenging, particularly in MF. The skin lesions of MF can clinically and histologically resemble those of benign inflammatory disorders including psoriasis and atopic dermatitis. The diagnosis of CTCL is based on assessment of a number of factors including the clinical presentation, suggestive histopathology and identification of a clonal T-cell population in blood or skin lesions. However, clonal malignant T cells make up only a small minority of total T cells in MF skin lesions, particularly in early disease (4). The most commonly used clinical test, multiplex/heteroduplex PCR amplification of the TCR V γ chain followed by GeneScan capillary electrophoresis analysis, detects clones in a subset of patients with CTCL but has a significant false negative rate (5, 6). Definitive diagnosis of MF is often delayed and is made on average six years after the first development of skin lesions (7). A more reliable method of discriminating between CTCL and benign inflammatory skin disease would both facilitate timely diagnosis of the disease and help to discriminate CTCL recurrences from unrelated benign inflammatory reactions in the skin.

High throughput sequencing (HTS) of the third complementarity determining regions (CDR3) of T cell receptor β and γ genes provides a comprehensive and quantitative analysis of how many distinct T cell clones are present within a sample, the relative frequency of each clone and the exact unique nucleotide sequences of each clone's CDR3 regions (8). Prior studies have shown this technique can identify malignant T cells in the circulation and may be more sensitive than existing techniques in the detection of skin disease (9, 10).

We present here our findings that HTS of TCR β and γ alleles detected expanded T cell clones in all CTCL patients studied, supporting early definitive diagnosis of CTCL and discrimination of CTCL from benign inflammatory skin diseases. HTS also facilitated the early discrimination of CTCL recurrences from benign inflammation, allowed longitudinal tracking of malignant T cells over time and provided comprehensive information about the nature of T cell infiltrates in CTCL skin that led to additional insights into the immunobiology of CTCL.

Results

High throughput TCR CDR3 region sequencing identifies expanded T cell clones and discriminates CTCL from benign inflammatory skin disorders

We analyzed DNA from punch biopsies of 46 CTCL skin lesions, lesional skin from 23 patients with psoriasis, 11 patients with eczematous dermatitis, 12 patients with contact dermatitis, 12 patients with pityriasis lichenoides et varioliformis acuta (PLEVA) and the skin of 6 healthy donors by HTS for both the TCR V γ and TCR V β genes in order to determine if HTS could provide more accurate diagnosis of CTCL. Clonality values were calculated from entropy of the TCR V β CDR3 frequency distribution and then normalized by log (# unique TCR V β CDR3). Clonality values range from 0 (polyclonal distribution) to 1 (monoclonal distribution). As expected, the aggregate clonality of T cells in CTCL skin lesions increased with advancing stages of disease (Fig. 1A). HTS of the TCR β CDR3 regions identified expanded T cell clones in 46/46 lesional samples of CTCL skin (Fig. 1B-G). The aggregate V gene and J gene usage for a representative patient with stage IB MF is shown (Fig. 1B), as well as the detailed information regarding the specific amino acid sequence of the single expanded T cell clone in the sample (Fig. 1C). However, the top T cell clone expressed as the percentage of total sequenceable T cell genomes in the lesional skin sample was not sufficient to fully discriminate CTCL from other benign inflammatory skin diseases (Fig. 1D, E).

We next calculated what fraction of the total DNA from skin was contributed by the top T cell clone. This calculation reflected what proportion the top T cell clone made up of the total cells in skin, as opposed to evaluating the expanded clone only as the percentage of total T cells present. When the frequency of the top T cell clone was evaluated as the fraction of total nucleated cells (including keratinocytes, fibroblasts and other cell types) HTS readily and effectively distinguished CTCL from benign inflammatory skin disorders and from healthy skin (Fig. 1F, G). PLEVA is an inflammatory skin disorder that is clinically very distinct from CTCL but has been shown to contain clonal T cells in a subset of patients, suggesting it may represent a cutaneous lymphoproliferative disorder (11-13). HTS studies detected a clonal T-cell population in four of 12 patients with PLEVA, in agreement with prior PCR studies demonstrating that T-cell clones exist in a subset of patients (Fig. S1C).

HTS of the TCR V γ CDR3 genes was also carried out in the same skin samples (Fig. 2). Most mature human peripheral blood T cells have two rearranged TCR V γ genes; the average number of rearranged TCR V γ genes for an aggregate population of mature human T cells is 1.8 (14). To account for the two rearranged TCR V γ alleles most often present in a

single T cell, the two most frequent top TCR V γ gene sequences were added in order to calculate the frequency of the top T cell clone in skin samples. Using this approach, there was quite good correlation between TCR β and TCR γ sequencing results in individual samples (Fig. 2A). As expected, some samples showed relatively lower clonal frequency by TCR β analysis (falling to the left of the diagonal line, Fig 2A); these samples represent T cell clones with only one rearranged TCR γ allele, which would be expected to make up 17% of the total T cell population. A notable exception was a single case of $\gamma\delta$ T cell CTCL, which had a clear clonal T-cell population on TCR γ HTS but no detectable clone by TCR β HTS. In agreement with our results, it has recently been reported that human $\gamma\delta$ T cells virtually never have rearranged TCR β alleles (14). We would therefore not expect this variant of CTCL to be detected by TCR β HTS. When expressed as the fraction of total nucleated cells in skin, TCR gamma HTS also readily distinguished CTCL patients from those with benign inflammatory skin disease and from healthy skin (Fig. 2B,C).

HTS diagnoses CTCL in patients with negative clonality assessments by conventional TCR γ PCR

39 patients with clinically confirmed CTCL were evaluated by both HTS and by standard TCR γ PCR, using BIOMED InVivoScribe primers and GeneScan capillary electrophoresis (5, 6). HTS identified T cell clones in 39/39 patients compared to TCR γ PCR which identified clonal populations in 27/39 (70%) of samples. Ten of the twelve patients who had detectable clonality by HTS but not by TCR γ PCR had early stage (IA or IB) disease (Fig. 3A). However, the fraction of nucleated cells of the expanded T cell clone as assayed by HTS was similar in PCR negative and PCR positive patients (Fig. 3B,C), suggesting that TCR γ PCR was negative in these patients for reasons other than a very low predominance of the malignant clone. Similar proportions of samples with DNA extracted from frozen vs. formalin fixed paraffin embedded (FFPE) samples were negative by PCR (Figure S2), suggesting that the source of sample DNA was not a factor. The clinical photographs and HTS results in two representative patients with stage IB CTCL are shown (Fig. 3D,E). HTS demonstrated clear expanded T cell clones but TCR γ PCR was read as negative for clonality in both cases. HTS was particularly helpful in patient 551, a patient with stage IA CTCL in whom three separate biopsies were all read as negative for clonality by TCR γ PCR (Fig. 3F,G, S3). TCR β HTS demonstrated the presence of two high-frequency TCR β sequences (Fig. 3H), suggesting the presence of either a single T cell clone with two rearranged β alleles or two dominant T cell clones. TCR γ HTS demonstrated the presence of four predominant TCR γ sequences in all four biopsies from this patient, demonstrating two expanded T cell clones were present in this patient, each with two rearranged TCR γ alleles (Fig. 3I).

HTS discriminates CTCL recurrences from benign inflammation, provides accurate assessment of responses to therapy and facilitates early diagnosis of disease recurrence in both the skin and blood of patients with CTCL

By virtue of its ability to identify and quantify clonal T cells, HTS can greatly facilitate the care of patients with CTCL. HTS was effective in distinguishing early CTCL recurrences from benign inflammation of the skin, a challenge that occurs when patients develop a cutaneous eruption following what otherwise appears to be successful treatment of their

lymphoma. Patient 247 had stage IB CTCL with peripheral blood involvement that was detectable by both HTS (Fig. 4A) and by clinical flow cytometry analysis. The patient was begun on low dose alemtuzumab along with valacyclovir and sulfamethoxazole/trimethoprim prophylaxis (15). He experienced a rapid improvement in his skin erythema and pruritus but after his ninth cycle of alemtuzumab, he developed an inflammatory cutaneous eruption that was worrisome for recurrence of CTCL (Fig. 4B). Histopathology was suggestive of a drug hypersensitivity response but a T cell dyscrasia could not be ruled out. HTS clearly demonstrated loss of the malignant T cell clone from both blood and skin (Fig. 4C), supporting the diagnosis of a nonmalignant hypersensitivity response. Sulfamethoxazole/trimethoprim was discontinued and the patient recovered completely with topical steroids and narrow band UVB therapy. In agreement with our prior studies, HTS demonstrated the presence of a diverse population of skin resident memory T cells remaining in this patient's skin after alemtuzumab therapy (Fig. 4D).

HTS was also useful for its ability to track individual malignant T cell clones in the blood and skin over months and years in a particular patient. Patient 409 had stage IIA CTCL and was first studied by HTS in 2012, after treatment with electron beam and brachytherapy, and again in 2014, after initiation of gemcitabine. HTS demonstrated an identical clonal T-cell population in the skin at both time points, reduced but still present after gemcitabine therapy (Fig. 4E, F).

In addition, HTS rapidly and efficiently diagnosed early disease recurrences. Pt 425 had a history of recalcitrant stage IIB CTCL with CD30⁺ large cell transformation. HTS performed prior to SCT identified the malignant T cell clone. She underwent a matched donor stem cell transplant and appeared free of disease until 10 months after transplantation, when she developed a new skin lesion on her right abdomen (Fig. 4G). HTS demonstrated recurrence of the same malignant T cell clone demonstrable before SCT (Fig. 4H). Prompt withdrawal of systemic immunosuppression and skin directed narrowband UVB therapy induced a complete and persistent remission.

HTS was also effective in identifying and quantifying expanded T cell clones in blood samples from patients with CTCL. As expected, HTS failed to identify dominant expanded T cell clones in the blood of most patients with MF but routinely detected expanded clonal T cells in patients with clinical evidence of peripheral blood disease (leukemic CTCL, L-CTCL), as evidenced by positive clinical flow cytometry analyses (Fig. 4I). Four patients with benign expanded T cell clones in the peripheral blood were also studied and were clearly discriminated from patients with L-CTCL (Fig. S1).

In patients with new discrete skin lesions and no clinical involvement of peripheral blood, HTS demonstrates hematogenous spread of small numbers of malignant T cells

Patients with skin limited CTCL and no evidence of peripheral blood involvement by clinically available flow cytometry analyses can develop new CTCL skin lesions distant from previously involved areas. Patient 539 had stage IIB CTCL with patchy and nodular skin lesions (Fig. 5A) and no evidence of peripheral blood disease by clinical flow analyses. The skin lesions improved after local radiation therapy but the patient developed a new tumor at a previously uninvolved distant site five months later. HTS studies demonstrated

the same malignant T cell clone in the two lesions biopsied before radiation therapy as well as the new distant tumor (Fig. 5B,C). Evaluation of the peripheral blood at the time of the development of the new skin lesions also demonstrated low but detectable numbers of the same malignant clone within the peripheral circulation. At both time points, clinical flow cytometry studies were negative for peripheral blood involvement. Patient 418 had a long-standing Stage IIB folliculotropic CTCL previously controlled with a number of therapies including narrowband UVB, psoralen and UVA light (PUVA), oral bexarotene, Denileukin diftitox, electron beam radiation, pralatrexate and nitrogen mustard (Fig. 5D). In 2014, he was seen with thickening of existing lesions and development of new skin lesions (Fig. 5E). Several clinical flow analyses were negative for peripheral blood involvement. HTS analysis of blood and skin samples demonstrated the presence of an abundant T cell clone in skin that was demonstrable in low levels in peripheral blood (Fig. 5F). Patient 317 had an over 40 year history of long-standing large plaque parapsoriasis since childhood and was eventually diagnosed with mycosis fungoides in 2007 (Fig. 5G). In 2014, he presented with a 1.5 year history of worsening disease with new areas of involvement. HTS analyses of skin and blood demonstrated the presence of a clonal T-cell population in skin that was also present at a very low level within the peripheral blood (Fig. 5H). In summary, in 3/3 patients with skin limited disease, no evidence of peripheral blood disease by flow cytometry analyses and recent development of new skin lesions, HTS demonstrated the hematogenous spread of low numbers of clonal T cells. HTS also measured the numbers and diversity of malignant and benign T cells in CTCL skin lesions (Fig. 6A,B). The presence of tumor infiltrating lymphocytes has been correlated with better outcomes in a variety of human cancers (16). HTS may therefore be a useful tool in evaluating the health of a patient's T cell repertoire, perhaps also gauging their likelihood to progress.

HTS TCR γ gene studies demonstrate that CTCL is a malignancy derived from mature T cells

CTCL is most commonly a malignancy of CD4⁺ $\alpha\beta$ T cells but the stage of differentiation of the T cell of origin in CTCL has never been definitively established. Prior to rearranging the TCR β genes, $\alpha\beta$ T cells first undergo rearrangement of the TCR γ genes; mature $\alpha\beta$ T cells have on average 1.8 rearranged TCR γ genes (14).

We studied the malignant T cells in CTCL for the prevalence of rearranged TCR γ genes. CTCL patients in whom TCR γ HTS demonstrates the presence of two similarly abundant TCR γ sequences are patients with T-cell clones that have both TCR γ alleles rearranged (bi-allelic, Fig. 6C). Patients with a single most frequent TCR γ sequence by HTS are those who have T cell clones with a single rearrangement of the TCR γ allele (mono-allelic, Fig. 6D). The presence of two independent and distinct TCR γ marker sequences can provide additional verification of the malignant clone. In our specimens, we found 27 CTCL T cell clones that were clearly bi-allelic and six that were clearly mono-allelic (Fig. 6E). On average, each malignant T cell clone had 1.8 rearranged TCR γ alleles, in complete agreement with the values observed in human mature T cells (14). This observation demonstrates that CTCL is a malignancy arising from mature memory T cells that underwent normal thymic maturation and is not a malignancy of immature T cells or lymphoid progenitor cells.

MF and L-CTCL malignant T cells localize to distinct anatomic compartments in the skin

TCR β HTS identifies the TCR V β subunit utilized by the malignant T cell clone. Approximately 60 to 70% of TCR V β subfamilies can be recognized by commercially available monoclonal antibodies, allowing for immunostaining of the malignant T cells in samples of lesional skin or in blood (Fig. 6F-H). We previously reported that malignant T cells in mycosis fungoides have the surface phenotype of skin resident memory T cells (T_{RM}), nonrecirculating T cells with marked effector functions, a subset of which have been reported to localize to the dermoepidermal junction of human skin (17-19). By selectively staining for the TCR V β subunit utilized by the malignant T cell clone, we found that the T cells in a subset of patients with MF selectively localized to the dermoepidermal junction (Figure 7). In contrast, L-CTCL is a malignancy with the surface phenotype of highly migratory skin tropic central memory T cells (T_{CM}) (15, 17). T_{CM} are thought to primarily recirculate between the lymph nodes, blood and skin, and are found in the dermis but not the epidermis of healthy human skin (17, 20). Immunostaining of malignant T cells in patients with L-CTCL demonstrated that malignant T cells localized to the dermis and were excluded from the epidermis and dermoepidermal junction (Fig. 8).

Discussion

Making a timely and accurate diagnosis is one of the greatest challenges in treating CTCL patients

In patients with MF, malignant T cells make up only a small proportion of T cells in skin lesions (4). Spectral karyotyping and CGH studies combined with TCR γ PCR have demonstrated that genetically damaged malignant T cells are present in even the earliest stages of MF (21, 22), confirming that MF is a lymphoma of genetically damaged malignant T cells even in its earliest manifestations. The identification of an expanded T cell clone in skin lesions is a critical piece of information that can, when combined with suggestive histopathology and clinical presentation, establish the diagnosis of CTCL.

In patients in whom the clinically available TCR V γ PCR studies detect a clonal T-cell population, this technique is very valuable in establishing the diagnosis of CTCL (5, 6). However, interpretation of this test is both complex and operator dependant, and each laboratory has its own distinct threshold above which a peak, measured by densitometry, is considered to represent a T cell clone. There are many patients with a suggestive clinical presentation and equivocal histopathology in whom TCR V γ PCR studies are read as polyclonal or otherwise fail to detect a clonal T-cell population. In these patients, definitive diagnosis is often difficult and the institution of definitive and appropriate therapy is delayed, often until disease worsens to the point where the diagnosis is clear but effective treatment is more difficult. On average, it takes six years after the initial onset of skin lesions to make a definitive diagnosis of CTCL (7).

High throughput sequencing of the TCR CDR3 regions, the hyper variable portions of the TCR γ and β genes that make up the antigen recognition domains, has the potential to provide an exact fingerprint for T cell clones in a particular biologic specimen. The exact unique nucleotide sequences that make up each individual T cell clone are identified, the

number of these cells relative to other T cells in the sample is measured and the total number and overall diversity of T cells is provided (8). This single, one step study provides an unprecedented amount of information about the T cell population in a biologic specimen.

Because of its ability to identify and quantify individual T cell clones, HTS is a promising technique to apply to the diagnosis of CTCL. We found that HTS detected an expanded T cell clone in the skin lesions and blood of all CTCL patients studied. The skin lesions of 39 of our patients were studied by both HTS and TCR γ PCR. HTS identified expanded T cell clones in all of these patients whereas TCR γ PCR was positive in only 74%. HTS was particularly effective in detecting expanded T cell clones in patients with earlier stages of disease. Surprisingly, patients who had clones by HTS but not by TCR γ PCR were not necessarily those with lower numbers of clonal T cells in skin lesions, suggesting that factors other than a lower sensitivity of TCR γ PCR may interfere with the detection of clonal T cell populations by this technique. However, taken together, our results suggest that HTS is superior to TCR γ PCR in detecting clonal T cell populations in patients with CTCL.

One of the clinical challenges in diagnosing patients with early-stage disease is discriminating CTCL from benign inflammatory skin disorders such as psoriasis and atopic dermatitis, which these lymphomas can clinically resemble. To be a successful diagnostic test, HTS must therefore not only detect expanded T cell clones in patients with CTCL but must also be able to successfully discriminate CTCL from benign inflammatory skin disorders. Clonal T cell expansion occurs in healthy T cell mediated immune responses and clonal expansion of individual, antigen-specific T cells is the physiologic consequence of antigen recognition. Expanded T cell clones have been detected in benign inflammatory skin disorders and expanded CD8⁺ T cell clones are frequently found in the peripheral circulation of older individuals and are thought to represent expanded populations of anti-CMV specific T cells (23-25). Because many benign inflammatory skin diseases are thought to be antigen driven, for example in response to allergens in atopic dermatitis and contact dermatitis and to autoantigens in psoriasis, it is not surprising that predominant T cell clones have been observed in these disorders.

Indeed, the proportion of the top T cell clone, expressed as a percentage of the total T cell population, did not clearly distinguish between CTCL and benign inflammatory skin disorders (Fig. 1D,E). This analysis evaluates the frequency of the top clone with respect to the remaining T cell population, but it is not a measure of the absolute number of clonal T cells in a particular unit of skin. One way of measuring the absolute number of clonal T cells in a particular skin volume is to determine what fraction of the total DNA derived from all nucleated cells in skin is contributed by the top T cell clone. This “fraction of nucleated cells” is a measure of how many clonal T cells are present in a particular unit of skin. When the data were considered in this way, we found that HTS successfully discriminated CTCL from benign inflammatory skin diseases (Fig. 1F,G, 2B,C). In other words, particular T cell clones may be frequent within the total T cell population in benign inflammatory skin diseases but the absolute number of these cells per unit skin rarely exceeded a certain threshold (~1:1000). In CTCL by contrast, clonal T cells accumulated not only in frequency but also in absolute numbers to levels that exceed those observed in benign inflammatory

skin diseases. It is possible that a homeostatic ceiling may exist for normal T cell expansion in skin, one that is not recognized by the malignant clone in CTCL.

Although it is conjecture, one possible mechanism for this apparent limit to the number of normal T cells in a particular volume of skin could be the dependence of T cells on a growth or survival factor that is present in limited amounts within the skin. For example, the γ chain cytokine IL-15 works together with IL-7 to mediate survival and homeostatic proliferation of memory T cells in blood (26, 27). IL-15 is produced by many cell types in skin, including keratinocytes, fibroblasts, macrophages and dendritic cells (28-30). IL-15 induces the proliferation of both circulating and skin resident T cells and mediates bystander proliferation of CD8⁺ T cells (30-32). Similar to its effects on benign T cells, IL-15 can induce proliferation of malignant CTCL T cells (33-35). However, it is conceivable that malignant T cells may be less dependent than benign T cells on IL-15 or a similar survival factor within the skin. This could lead to malignant T cells outgrowing their cytokine dependent niche whereas benign T cells cannot. However, the signals that maintain T cells long-term in skin remain to be elucidated and differential survival factor dependence of benign vs. malignant T cells is only one possible mechanism for the observed results.

Although CTCL is clearly a malignancy of T cells characterized by their ability to home to the skin, the cell of origin for CTCL has never been conclusively identified. Other hematologic malignancies are more common in patients with CTCL, including Hodgkin's and non-Hodgkin's lymphoma, raising the speculation that a defective common lymphoid progenitor cell could be responsible (36). CTCL patients can have more than one malignant T cell clone, consistent with the possibility that the disease may arise from a pre-T cell prior to rearrangement of the TCR genes (22, 37). By combining findings of TCR β and γ HTS, we calculated that the average malignant T cell clone contained 1.8 rearranged TCR γ alleles (Fig. 6), the exact proportion observed in mature peripheral blood $\alpha\beta$ T cells (14). This observation demonstrates that CTCL is a malignancy of mature T cells and is not a malignancy of immature T cells or lymphoid progenitor cells.

HTS of the TCR β allele allows identification of the V β usage of the malignant clone. This enables selective immunostaining of the malignant T cell clone, permitting evaluation of its phenotype, location within skin lesions and functional characteristics. CTCL is most commonly a malignancy of CD4⁺ cutaneous T cells, although CD8⁺ MF can also occur. We have recently demonstrated that human skin is protected by four distinct populations of T cells, two recirculating and two resident, all of which are presumably susceptible to malignant transformation (20). It is our evolving hypothesis that the distinct clinical presentations of CTCL may represent their derivation from different subsets of skin homing T cells. For example, malignant T cells in stable MF had the surface phenotype of nonrecirculating resident memory T cells (T_{RM}) and classic erythrodermic Sézary syndrome had malignant T cells with a surface phenotype of central memory T cells (T_{CM}), consistent with their tendency to form fixed stable inflammatory skin lesions vs. mobile erythroderma, respectively (15, 17, 38). Our most recent studies identified a novel skin homing subset termed migratory memory T cells (T_{MM}) (20). These cells express CCR7 but lack L-selectin, are present in the blood and skin of healthy individuals and recirculate more slowly out of skin than do T_{CM}. In CTCL patients, malignant T_{MM} gave rise to discrete skin lesions

with ill-defined borders and peripheral blood disease, which in the current classification is referred to as MF with peripheral blood disease. One remaining question is the process by which patients who have long-standing stable MF and years later develop new onset peripheral blood disease. Malignant T cells can accumulate progressive mutations over time and many patients have CTCL for decades. Mutations or expression changes in the molecules that retain T cells in skin could give rise to malignant T cells that can access and accumulate within the circulation. Alternatively, patients with CTCL have been known to develop multiple different malignant T cell clones, arguing that genetic damage and genomic destabilization occur in a group of T cells, some of which give rise to overtly malignant T cell clones (22, 37). Peripheral blood disease in patients with long-standing stable MF could also be caused by two distinct T cell clones. HTS is uniquely well-suited to definitively answer these questions and this is an active topic of investigation in our laboratory.

Distinct skin homing T-cell subsets also localize to different anatomic compartments within the skin. T_{CM} are present in the dermis but excluded from the epidermis in humans whereas T_{RM} can be found both in the dermis and epidermis (20). Moreover, a set of highly protective HSV-specific T_{RM} localize to the dermoepidermal junction in humans (18, 19). We localized the malignant clone by immunostaining biopsy specimens with commercially available TCR V β antibodies. Utilizing this technique, we found that the malignant T cells in a subset of patients with MF and L-CTCL localized to distinct anatomic compartments. In a subset of MF patients, malignant T cells localized to the dermoepidermal junction and malignant T cells in patients with L-CTCL and a T_{CM} phenotype localized to the dermis, the same anatomic compartment of human skin where skin tropic T_{CM} are found (20). These studies raise the intriguing possibility that malignant T cells in some CTCL patients may not only retain the recirculation characteristics of their parent T cell population but also retain their tendency to localize to distinct anatomic positions within the skin. These insights could eventually lead to selective therapies that deplete malignant T cells, spare healthy cells and also give rise to novel insights regarding the behavior of healthy skin homing T cells.

Although HTS is a very useful technique in CTCL, every technology has potential technical problems and limitations. PCR bias can be limited but not entirely removed and it is critical that each source of HTS services used by clinicians, whether academic or commercial, have robust mechanisms for control of PCR bias. Second, measurement of the parameter we find most useful, the fraction total nucleated cells, requires efficient measurement of input DNA and DNA amplifiability and robust measurement of this needs to be a part of the HTS technology. Third, DNA can undergo degradation after formalin fixation and the primer sets and measurements of DNA available for sequencing that function well on FFPE fixed tissues are required if clinicians wish to extract DNA from FFPE samples for these studies. Lastly, it should be remembered that HTS assays need to be evaluated in the context of a patient's history, presentation, histopathology results and other diagnostic tests and we do not propose it as a single diagnostic test for CTCL.

In summary, we found that HTS was a valuable tool in identifying malignant T cell clones and differentiating early stage CTCL from benign inflammatory skin disease. The fraction of malignant T cells per volume of skin was valuable in differentiating CTCL from benign

inflammation but the relative percentage of malignant T cells was not. Our findings suggest that a maximal number of T cells per volume skin exists in benign inflammatory disorders; the signals that maintain retention and survival of T cells in skin are not fully characterized but we hypothesize that malignant CTCL cells are independent of these signals. CTCL T cells contain on average 1.8 rearranged TCR γ genes, suggesting CTCL arises from mature T cells. Lastly, we found that malignant T cells in different subtypes of CTCL localized to distinct anatomic compartments within the skin.

Materials and Methods

Study design / Experimental design

This is an experimental laboratory study performed on human tissue samples. All studies were performed in accordance with the Declaration of Helsinki. Blood from healthy individuals was obtained after leukapheresis, skin was obtained from healthy patients undergoing cosmetic surgery procedures. Blood and lesional skin from patients with CTCL were obtained from patients seen at the Dana-Farber/Brigham and Women's Cancer Center Cutaneous Lymphoma Program. CTCL patients described in this manuscript met the WHO-EORTC criteria for L-CTCL/SS or MF (1). Lesional skin from patients with psoriasis and eczematous dermatitis were obtained from patients seen at the Brigham and Women's Hospital or at Rockefeller University. All tissues were collected with prior approval from the Partners, Dana-Farber, and Rockefeller Institutional Review Boards. Analyses of HTS studies were done in an investigator blinded fashion. Immunostaining studies were performed using in vitro assays without blinding or randomization. Study components were not predefined.

Skin and blood samples

Clinical information for the CTCL patients studied is provided in Table I. Clinical flow analyses assessed for peripheral blood disease by measuring the CD4:CD8 ratio and quantifying putative malignant cells by gating on CD3⁺CD4⁺CD26⁻ cells.

DNA isolation from skin

DNA was isolated from frozen, OCT embedded or formalin fixed, paraffin embedded (FFPE) skin samples as follows. For OCT embedded tissue samples, 30 cryosections of 10 μ m thickness were cut and DNA extraction was carried out using the QIAamp DNA Mini Kit (Qiagen) kit as per manufacturer's instructions with overnight tissue digestion. This method generated 155-3730 ng DNA per sample. For FFPE samples, DNA was extracted from two 40 μ m scrolls using the QIAamp DNA Mini Kit as per manufacturer's instructions with the following modifications. Paraffin was removed from tissue scrolls by two rounds of xylene extraction followed by two ethanol washes prior to overnight tissue digestion. Extra proteinase K was added after overnight digestion if visible tissue still remained. This method generated 272-3656 ng DNA per sample.

HTS analyses

Immunosequencing—For each sample, DNA was extracted from skin biopsies or peripheral blood mononuclear cells, then TCR β CDR3 and TCR γ CDR3 regions were

amplified and sequenced using ImmunoSEQ™ (Adaptive Biotechnologies, Seattle, WA) from 400 ng of DNA template. Bias-controlled V and J gene primers were used to amplify rearranged V(D)J segments for high throughput sequencing at ~20x coverage. After correcting sequencing errors via a clustering algorithm, CDR3 segments were annotated according to the International ImMunoGeneTics collaboration, identifying which V, D, and J genes contributed to each rearrangement (39-41).

Controlling bias in a multiplex PCR—Because accurate quantification of lymphoblast clones for minimal residual disease detection is critical, an approach was developed to ensure minimal bias in multiplex PCR (42). Briefly, each potential VDJ rearrangement of the TCRβ locus contains one of thirteen J segments, one of two D segments and one of 52 V segments, many of which have disparate nucleotide sequences. In order to amplify all possible VDJ combinations, a single tube, multiplex PCR assay with 45 V forward and 13 J reverse primers was used. To remove potential PCR bias, every possible V-J pair was chemically synthesized as a template with specific barcodes (42). These templates were engineered so as to be recognizable as non-biologic and have universal 3' and 5' ends to permit amplification with universal primers and subsequent quantification by HTS. This synthetic immune system was then used to calibrate the multiplex PCR assay. Iteratively, the multiplex pool of templates was amplified and sequenced with TCRβ V/J-specific primers, and the primer concentrations were adjusted to re-balance PCR amplification. Once the multiplex primer mixture amplified each V and J template nearly equivalently, residual bias was removed computationally. The parallel procedure for TCRγ was described previously (42). A schematic of this approach is included (Fig. S4).

PCR template abundance estimation—In order to estimate the average read coverage per input template in the multiplex PCR and sequencing approach, a set of approximately 850 unique types of synthetic TCR analog, comprising each combination of Vβ and Jβ gene segments (42) for TCRβ and approximately 75 for TCRγ was employed. These molecules were included in each PCR reaction at very low concentration so that most unique types of synthetic template were not observed in the sequencing output. Using the known concentration of the synthetic template pool, the relationship between the number of observed unique synthetic molecules and the total number of synthetic molecules added to reaction was simulated (this is very nearly one-to-one at the low concentrations that were employed). These molecules then allowed calculation for each PCR reaction of the mean number of sequencing reads obtained per molecule of PCR template (the amplification factor), and thus estimation of the number of T cells in the input material bearing each unique TCR rearrangement.

Clonal detection—The putative malignant clone was defined by sequence abundance. A clone can have either one or two rearranged alleles. For the majority of clones, both TCRγ alleles are rearranged and for TCRβ, a minority has both alleles rearranged. For consistency, a clone's abundance was defined by summing the abundance of the top two alleles for TCRγ and the top single allele for TCRβ. For TCRγ the method is robust for the cases with only a single rearrangement, as the second largest allele which is present in a non-malignant cell is relatively low.

The percent of T cells consisting of the malignant clone was determined by dividing the abundance of the malignant clone (number of reads) by the total number of T cell reads. For TCR γ , this number was divided by two because most T cells have two TCR γ rearrangements. The fraction of total nucleated cells was determined by multiplying this number by the fraction of T cells in the samples as described above.

Statistical analyses

Primary methods of data analysis included descriptive statistics (means, medians and standard deviations). Differences between two sample groups were detected using the one tailed Wilcoxon–Mann–Whitney test, $\alpha=0.05$. For comparisons of multiple groups, a Kruskal–Wallis one-way analysis of variance with a Bonferroni–Dunn’s post test for multiple means test was used, $\alpha=0.05$.

Cryosection immunostaining

CTCL skin samples were embedded in OCT, frozen, and stored at -80°C until use. 5 μm cryosections were cut, air dried, fixed for 5 min in acetone, rehydrated in PBS, and blocked with 20 $\mu\text{g}/\text{ml}$ of human IgG (Jackson ImmunoResearch Laboratories) for 15 min at room temperature. Sections were incubated with directly conjugated anti-TCR V β antibody (Beckman Coulter) for 30 min, and then rinsed three times in PBS/1% BSA for 5 min. Sections were mounted using Prolong Gold Antifade with DAPI (Life Technologies) and examined immediately by immunofluorescence microscopy. Sections were photographed using a microscope (Eclipse 6600; Nikon) equipped with a 40 $\times$ /0.75 objective lens (Plan Fluor; Nikon). Images were captured with a camera (SPOT RT model 2.3.1; Diagnostic Instruments) and were acquired with SPOT 4.0.9 software (Diagnostic Instruments).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank the patients who made this work possible, both for entrusting us with their clinical care and for donating skin and blood samples. Dr. Thomas Cochran of the Boston Center for Plastic Surgery and Drs. Bohdan Pomahac, Simon Talbot and Elof Eriksson of Brigham and Women’s Hospital generously provided adult human skin samples. The authors thank Dimity Hall for providing DNA from the BWH clinical laboratory.

Funding: This work was supported by a generous charitable contribution from Edward P. Lawrence, Esq., R01 AR063962 NIH/NIAMS (to R.A.C.), R01 AR056720 NIH/NIAMS (to R.A.C.), a Damon Runyon Clinical Investigator Award (to R.A.C.), the SPORE in Skin Cancer P50 CA9368305 NIH/NCI (to T.S.K.), R01 AI097128 NIAID (to T.S.K. and R.A.C.), T32 AR-07098-36 (to T.S.K., supplied salary for J.T.O.). R.W. salary support was provided by a Special Scholar Grant from the Leukemia and Lymphoma Society.

References cited

1. Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, Ralfkiaer E, Chimenti S, Diaz-Perez JL, Duncan LM, Grange F, Harris NL, Kempf W, Kerl H, Kurrer M, Knobler R, Pimpinelli N, Sander C, Santucci M, Sterry W, Vermeer MH, Wechsler J, Whittaker S, Meijer CJLM. WHO-EORTC classification for cutaneous lymphomas 10.1182/blood-2004-09-3502. Blood. 2005; 105:3768–3785. [PubMed: 15692063]

2. Kim YH, Liu HL, Mraz-Gernhard S, Varghese A, Hoppe RT. Long-term outcome of 525 patients with mycosis fungoides and Sezary syndrome: clinical prognostic factors and risk for disease progression. *Archives of dermatology*. 2003; 139:857–866. [PubMed: 12873880]
3. NCCN Guidelines. To view the most recent and complete version of the NCCN Guidelines. 2009 (© 2009 National Comprehensive Cancer Network, Inc. , 2009). go online to NCCN.org.
4. Fivenson DP, Hanson CA, Nickoloff BJ. Localization of clonal T cells to the epidermis in cutaneous T-cell lymphoma. *Journal of the American Academy of Dermatology*. 1994; 31:717–723. [PubMed: 7929915]
5. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuurink E, Garcia-Sanz R, van Krieken JH, Droese J, Gonzalez D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K.* 2003; 17:2257–2317.
6. Ponti R, Fierro MT, Quaglino P, Lisa B, Paola F, Michela O, Paolo F, Comessatti A, Novelli M, Bernengo MG. TCRgamma-chain gene rearrangement by PCR-based GeneScan: diagnostic accuracy improvement and clonal heterogeneity analysis in multiple cutaneous T-cell lymphoma samples. *The Journal of investigative dermatology*. 2008; 128:1030–1038. [PubMed: 17989737]
7. van Doorn R, Van Haselen CW, van Voorst Vader PC, Geerts ML, Heule F, de Rie M, Steijlen PM, Dekker SK, van Vloten WA, Willemze R. Mycosis fungoides: disease evolution and prognosis of 309 Dutch patients. *Archives of dermatology*. 2000; 136:504–510. [PubMed: 10768649]
8. Robins HS, Campregher PV, Srivastava SK, Wachter A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood*. 2009; 114:4099–4107. [PubMed: 19706884]
9. Weng W-K, Armstrong R, Arai S, Desmarais C, Hoppe R, Kim Y. Minimal residual disease monitoring with high-throughput sequencing of T cell receptors in cutaneous T cell lymphoma. *Science Translational Medicine*. 2013; 5
10. Sufficool KE, Lockwood CM, Abel HJ, Hagemann IS, Schumacher JA, Kelley TW, Duncavage EJ. T-cell clonality assessment by next-generation sequencing improves detection sensitivity in mycosis fungoides. *Journal of the American Academy of Dermatology*. 2015
11. Weinberg JM, Kristal L, Chooback L, Honig PJ, Kramer EM, Lessin SR. The clonal nature of pityriasis lichenoides. *Archives of dermatology*. 2002; 138:1063–1067. [PubMed: 12164744]
12. Dereure O, Levi E, Kadin ME. T-Cell clonality in pityriasis lichenoides et varioliformis acuta: a heteroduplex analysis of 20 cases. *Archives of dermatology*. 2000; 136:1483–1486. [PubMed: 11115158]
13. Weiss LM, Wood GS, Ellisen LW, Reynolds TC, Sklar J. Clonal T-cell populations in pityriasis lichenoides et varioliformis acuta (Mucha-Habermann disease). *The American journal of pathology*. 1987; 126:417–421. [PubMed: 3030116]
14. Sherwood AM, Desmarais C, Livingston RJ, Andriesen J, Haussler M, Carlson CS, Robins H. Deep sequencing of the human TCRgamma and TCRbeta repertoires suggests that TCRbeta rearranges after alphabeta and gammadelta T cell commitment. *Sci Transl Med*. 2011; 3:90ra61.
15. Clark RA, Watanabe R, Teague JE, Schlapbach C, Tawa MC, Adams N, Dorosario AA, Chaney KS, Cutler CS, Leboeuf NR, Carter JB, Fisher DC, Kupper TS. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Science Translational Medicine*. 2012; 4:117ra117.
16. Linnemann C, Mezzadra R, Schumacher TN. TCR repertoires of intratumoral T-cell subsets. *Immunol Rev*. 2014; 257:72–82. [PubMed: 24329790]
17. Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. *Blood*. 2010; 116:767–771. [PubMed: 20484084]
18. Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, Wald A, Corey L. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *The Journal of experimental medicine*. 2007; 204:595–603. [PubMed: 17325200]

37. Kaltoft K, Hansen BH, Thestrup-Pedersen K. Cytogenetic findings in cell lines from cutaneous T-cell lymphoma. *Dermatol Clin*. 1994; 12:295–304. [PubMed: 8045039]
38. Watanabe R, Teague JE, Fisher DC, Kupper TS, Clark RA. Alemtuzumab Therapy for Leukemic Cutaneous T-Cell Lymphoma: Diffuse Erythema as a Positive Predictor of Complete Remission. *JAMA dermatology*. 2014
39. Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, Wu Y, Gemrot E, Brochet X, Lane J, Regnier L, Ehrenmann F, Lefranc G, Duroux P. IMGT, the international ImMunoGeneTics information system. *Nucleic acids research*. 2009; 37:D1006–1012. [PubMed: 18978023]
40. Lefranc MP. IMGT, the International ImMunoGeneTics Information System. *Cold Spring Harbor protocols*. 2011; 2011:595–603. [PubMed: 21632786]
41. Lefranc MP. IMGT, The International ImMunoGeneTics Information System, <http://imgt.cines.fr>. *Methods in molecular biology*. 2004; 248:27–49. <http://imgt.cines.fr> [PubMed: 14970490]
42. Carlson CS, Emerson RO, Sherwood AM, Desmarais C, Chung M-W, Parsons JM, Steen MS, LaMadrid-Herrmannsfeldt MA, Williamson DW, Livingston RJ, Wu D, Wood BL, Rieder MJ, Robins H. Using synthetic templates to design an unbiased multiplex PCR assay. *Nat Commun*. 2013; 4

Figure 1. High throughput TCRβ CDR3 region sequencing identifies expanded T cell clones and discriminates CTCL from benign inflammatory skin disorders

DNA was isolated from the lesional skin of patients with CTCL, psoriasis, eczematous dermatitis (ED), allergic contact dermatitis (ACD) and from the skin of healthy individuals (Nml skin) and subjected to high throughput TCRβ sequencing. **(A)** Clonality of lesional skin T cells increased with advanced stage of CTCL. The mean and SEM of clonality scores of 8 stage IA biopsies, 18 stage IB biopsies, 4 stage IIA biopsies, 8 stage IIB biopsies, 2 stage III biopsies, and 5 stage IV biopsies are shown. **(B, C)** TCR sequencing identified

expanded populations of clonal malignant T cells in CTCL skin lesions. The V vs. J gene usages of T cells from a lesional skin sample are shown **(B)**. The green peak includes the clonal malignant T cell population, as well as other benign T cells that share the same V and J gene usage. The individual T cell clone sequence is shown **(C)**, with detailed information on the CDR3 amino acid sequence and V and J gene usage. The nine most frequent benign infiltrating T cell sequences are also shown. In this patient, the malignant T cell clone made up 10.3% of the total T cell population in lesional skin. **(D,E)** The most frequent T cell clone expressed as a percentage of total T cells did not completely discriminate CTCL from patients with benign inflammatory skin disease. The most frequent T cell sequences expressed as a percentage of total T cells is shown for individual samples **(D)** and aggregate data **(E)**. **(F,G)** The most frequent T cell clone expressed as the fraction of total nucleated cells successfully discriminates CTCL from benign inflammatory skin diseases. The most frequent T cell sequence expressed as a fraction of total nucleated cells is shown for individual samples **(F)** and aggregate data **(G)**. This analysis allowed discrimination of CTCL from benign inflammatory skin diseases and healthy skin. 46 CTCL skin lesions, lesional skin from 23 patients with psoriasis, 11 patients with eczematous dermatitis, 12 patients with contact dermatitis, and the skin of 6 healthy donors were evaluated (test: Kruskal–Wallis one-way ANOVA with a Bonferroni-Dunn’s post test).

Figure 2. High throughput sequencing of the TCRγ CDR3 regions also discriminates CTCL from benign inflammatory skin diseases

(A) Skin samples were subjected to deep sequencing of both the TCRγ and TCRβ CDR3 regions. The top TCRβ sequence and the sum of the top two most frequent TCRγ sequences (divided by two because most T cells have two rearranged TCR γ genes) were expressed as the fraction of total nucleated cells and the results from TCRγ and TCRβ sequencing are compared. In general, there was close concordance between TCRγ and TCRβ sequencing results. The exception was one patient with a known γδ T cell malignancy, in whom TCRγ sequencing identified a malignant clone but TCRβ did not, consistent with the known lack of TCR Vβ gene rearrangement in γδ T cells. (B,C) The sum of the top two TCRγ sequences, divided by two, and expressed as a fraction of total nucleated cells discriminates CTCL from benign inflammatory skin diseases. Individual (B) and aggregate (C) data are shown. 46 CTCL skin lesions, lesional skin from 23 patients with psoriasis, 11 patients with eczematous dermatitis and the skin of 6 healthy donors were evaluated (test: Kruskal–Wallis one-way ANOVA with a Bonferroni–Dunn’s post test).

Figure 3. HTS diagnoses CTCL in patients negative for clonality by conventional TCRγ PCR (A) HTS and TCRγ PCR were carried out on skin biopsies from 39 CTCL patients; HTS identified clones in 39/39 CTCL patients compared to TCRγ PCR which identified clonal populations in 29/39 samples. The stages of the ten CTCL patients with clones detected by HTS but negative for clonality by TCRγ PCR are shown. (B,C) Failure of TCRγ PCR to detect clonality was not related to the predominance of the malignant T cell clone within the skin sample. The top TCRβ clone, expressed as the fraction of nucleated cells, is shown. Individual (B) and aggregate (C) data are shown along with psoriasis samples which are

included for comparison. 39 CTCL skin lesions and lesional skin from 23 patients with psoriasis are shown (test: Kruskal–Wallis one-way ANOVA with a Bonferroni–Dunn’s post test). **(D)** Clinical photos and HTS results are shown for Patient 541, who had pathology proven stage IB CTCL but in whom TCR γ PCR did not detect a clonal population. **(E)** In patient 347, TCR γ PCR was negative and pathology was equivocal but HTS demonstrated a clear malignant clone. **(F–I)** In patient 551, four skin biopsies were sent for HTS and three were also studied by TCR γ PCR. All were negative for clonality by TCR γ PCR but 4/4 were positive for clonality by HTS. **(F, G)** TCR γ PCR results are shown for two biopsies; the third is included in Figure S3. Asterisks indicate peaks noted by the pathologist within the expected areas but none were judged significant enough to designate as a clonal population. **(H)** TCRV β demonstrated the presence of two distinct V β clonal sequences, denoting the presence of either a single malignant clone with two rearranged TCR β alleles or two separate malignant T cell clones. **(I)** TCR γ HTS demonstrated the presence of four dominant gamma chain clones in all four biopsies (black circles), confirming that there were two clonal malignant T cell populations in this patient, each with one rearranged TCR β allele and two rearranged TCR γ alleles. The five most frequent benign T cell TCR γ sequences are also shown for comparison (white circles).

Figure 4. HTS discriminates CTCL recurrences from benign inflammation, provides accurate assessment of responses to therapy and facilitates early diagnosis of disease recurrence in both the skin and blood of patients with CTCL

(A-D) HTS distinguishes CTCL recurrences from benign inflammatory disease. Patient 247 had a history of stage IB CTCL, subsequently developed leukemic involvement and was treated with low dose alemtuzumab. Prior to therapy, TCRV β HTS demonstrated a clear malignant clone in blood (A). The patient initially improved on alemtuzumab, then developed the skin eruption shown (B). Histopathology of the lesional skin was suggestive of a drug hypersensitivity reaction but a T cell dyscrasia could not be ruled out. HTS of blood and lesional skin showed clearance of the malignant T cell clone from blood and skin, confirming this was a benign inflammatory dermatitis (C). Bactrim was discontinued and the eruption completely resolved with topical steroids and narrow band UVB therapy. (D) Diverse populations of T cells remain within the skin of alemtuzumab treated patients. TCRV β HTS of the skin of patient 247 while on alemtuzumab is shown. This patient had no circulating T or B cells but a diverse population of T cells remained in skin. (E-F) HTS allows longitudinal observation of disease activity over time and assesses responses to therapy. Patient 409, Stage IIA CTCL, was studied by HTS in 2012, after treatment with electron beam and brachytherapy and again in 2014, after initiation of gemcitabine. HTS demonstrated an identical clonal T-cell population in the skin at both time points, reduced but still frequent after gemcitabine therapy. The malignant clone (black circle) and three most frequent benign T cell clones (white circles) are shown. (G-H) HTS provides early diagnosis of disease recurrence. Patient 425 had recalcitrant stage IIB CTCL with CD30⁺ large cell transformation. She underwent stem cell transplantation (SCT) and appeared well until she developed a new right chest lesion 10 months after SCT (G). HTS prior to SCT demonstrated that presence of a malignant T cell clone (H). Biopsy of the lesion post SCT demonstrated recurrence of the same malignant T cell clone in the skin. The malignant T cell clone (black circles) and the three most frequent benign T cell clones (white circle) are shown. Subsequent withdrawal of systemic immunosuppression and narrowband UVB therapy induced a complete remission. (I) HTS allows accurate assessment of peripheral blood disease. HTS studies of 4 patients without CTCL, 12 patients with MF without evidence of blood disease (MF) and in 7 patients with leukemic disease (L-CTCL) with known blood involvement are shown. (test: Kruskal–Wallis one-way ANOVA with a Bonferroni-Dunn’s post test).

Figure 5. In patients with skin limited disease and no clinical involvement of peripheral blood, HTS demonstrates hematogenous spread of small numbers of malignant T cells
 (A-C) Patient 539, stage IIIB CTCL, had no evidence of peripheral blood disease by clinical flow analysis but was experiencing both patchy (LCT on histopathology) and nodular skin (MF on histopathology) skin lesions (A). The patient improved following local radiation therapy but subsequently developed a new tumor at a previously uninvolved site five months later. HTS studies from all three biopsies are shown (B). Clinical flow was negative for blood involvement on both dates. HTS demonstrated the same clonal T cell population in all three skin biopsies and analysis of the blood demonstrated the presence of small numbers of malignant T cells in the peripheral blood at the time new skin lesions were developing. (D-E) Patient 418 had longstanding stage IIB folliculotropic CTCL (D) with recent thickening of existing skin lesions and new areas of disease (E). Clinical flow analyses were negative for peripheral blood involvement. HTS studies of the skin and blood during the development of the skin lesions demonstrated a clear malignant T cell clone in lesional skin that was also found in low numbers in peripheral blood (F). (G-H) Patient 317 had a longstanding history of large plaque parapsoriasis since childhood for over 40 years. The diagnosis of MF was finally made in 2007 (G). In 2014, he presented with a 1.5 year history of worsening disease with new areas of involvement. Clinical flow analyses showed no evidence of peripheral blood disease. HTS demonstrated a malignant T cell clone in the skin and small numbers were also demonstrated in peripheral blood.

Figure 6. CTCL is a malignancy derived from mature T cells

(A-B) HTS allows comprehensive study of both malignant and benign T cells. (A) The TCR V β HTS profile is shown for patient 541; both the malignant clone and benign infiltrating T cells in the skin lesions are evaluated by this technique. (B) CDR3 length analysis, similar to a spectrotyping graph, provides a rapid assay of T cell diversity via HTS. Shown are the results of TCR γ HTS of lesional skin from a patient with stage III CTCL. The CDR3 lengths of all T cells (left panel, including the malignant clone) and benign infiltrating T cells only (right panel) are shown. The two rearranged TCR γ allele sequences of the malignant clone are indicated by asterisks. A healthy diverse population of benign infiltrating T cells was present. (C,D) HTS demonstrates that CTCL is a malignancy of mature T cells. Prior studies demonstrated that mature $\alpha\beta$ T cells have on average 1.8 rearranged TCR γ alleles (14). We studied 33 CTCL patients with malignant $\alpha\beta$ T cell clones by TCR γ HTS. 27 patients had two rearranged TCR γ alleles, as evidenced by the presence of two similarly frequent TCR γ sequences (C,E) and six patients had a single rearranged TCR γ allele, as evidenced by only a single high-frequency TCR γ sequence (D,E). T cells therefore had on average 1.8 rearranged TCR γ alleles, a proportion characteristic of mature T cells. (F-H) TCR V β HTS provides an unparalleled opportunity to isolate and study malignant clonal T cells. (F) Identification of the TCR V β chain by HTS and subsequent immunostaining using commercially available TCR V β antibodies allows study of clonal malignant T cells. (G,H) Immunostaining of malignant T cells with TCR V β -specific antibodies in two patients with stage IIB MF are shown. The patient shown in panel (H) had a high abundant clone and larger cells as a result of large cell transformation. Scale bars: 100 μ m.

Figure 7. Malignant T cells in MF are localized to the dermoepidermal junction

A clinical image of disease (left panel) and immunostained cryosections for the same patient are shown. Clonal malignant T cells (red) localize to the dermoepidermal junction. The junction is indicated with a broken line in the last panel. Similar results were obtained in three additional MF patients. Scale bars: 100 μ m.

Figure 8. Malignant T cells in L-CTCL are localized to the dermis

A clinical image of disease (left panel) and immunostained cryosections for the same patient are shown. Clonal malignant T cells (red) are localized in the dermis. The basement membrane is indicated with a broken line in the last panel; the dermis is located below this line. Similar results were obtained in three additional L-CTCL patients. Scale bars: 100 μm.

Table 1

Stages of CTCL patient samples.

	Stage IA	Stage IB	Stage IIA	Stage IIB	Stage III	Stage IV	Total
Blood	3	5	1	5	2	8	24
Skin	11	19	5	8	2	5	50