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Restoration of peripheral blood T cell repertoire complexity during remission in advanced cutaneous T cell lymphoma

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Abstract

In advanced stages, cutaneous T cell lymphomas (CTCL) are associated with increased mortality from infections and also increased susceptibility to skin malignancies. In this study, we analyzed the complexity of the peripheral blood T cell repertoire with a sensitive b-variable (BV) complementarity-determining region 3 (CDR3) spectratyping analysis and flow cytometry in three-stage IV CTCL/Sezary syndrome patients who achieved complete clinical remission after therapy. The T cell repertoire of peripheral blood T cells before treatment was profoundly abnormal across multiple BV subfamilies. Following treatment, CDR3 spectratype patterns showed dramatic restoration of normal diversity and complexity. However, absolute CD4 counts across multiple BV families remained low for many months, even after identifiable circulating malignant T cell populations were eliminated. These data suggest that the diversity of the T cell repertoire can be recovered after successful treatment of even advanced CTCL.

Keywords

Cutaneous T cell lymphomas; Complete remission; T cell repertoire; Immune suppression

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Conflicts of interest statement None identified.

Introduction

Cutaneous T cell lymphomas (CTCL) are malignancies of the skin homing memory T cells, and in early stages malignant T cells reside principally in the skin [9]. In some variants of CTCL, such as Sezary syndrome (SS), increasing numbers of the malignant T cells can be observed in peripheral blood [10]. It has recently been appreciated that more advanced CTCL is also associated with significant immunosuppression, and is associated with increased mortality from bacterial and viral infections as well as increased susceptibility to other types of skin malignancies, such as melanoma and squamous cell carcinoma [2,5,8,11,15,16,22]. Studies in patients with CTCL have revealed numerous examples of immune dysregulation related to T cell dysfunction. We have previously reported that the diversity of the T cell repertoire of peripheral blood T cells in patients with advanced CTCL is significantly reduced [29], despite the relatively normal absolute numbers of T cells in peripheral blood [18,21,25]. In addition, we showed that decreased levels of T cell receptor excision circles (TREC), generated by individual T cells during V(D)J gene recombination in the process of T cell maturation, can be observed in non-malignant ostensibly normal T cell populations in CTCL patients [28], suggesting expansion of large numbers of individual T cells. This selective T cell expansion was associated with high plasma interleukin (IL)-7 levels [27].

Some variants of CTCL have also been described as Th2-type malignancies, and clonal CTCL cells have been observed to produce Th2-type cytokines [1,6,24]. Enhanced production of IL-18 in plasma and skin is also seen in patients with CTCL [26] and may be a potent inducer of the Th2 cytokine profile [31]. These features may also contribute to compromised host immune response in CTCL. It has also been proposed that increased numbers of CD4⁺CD25^{high} regulatory T cells (T-reg) may be associated with suppressed functional immune surveillance in patients with CTCL [3,13].

Together, these abnormalities suggest that T cell dysfunction and reduced immunosurveillance contribute to the increase in infection-related mortality and incidence of malignancies observed with disease advancement. Previous reports have shown restoration of some elements of immune function, and a concordant reduction in severe infections in CTCL patients, who achieved complete remission [13,30]. However, studies that address the status of T cell receptor diversity of CTCL patients in remission have not been reported. In this study, we analyzed the complexity of the peripheral blood T cell repertoire with a sensitive b-variable (BV) complementarity-determining region 3 (CDR3) spectratyping analysis and flow cytometry in three successfully treated stage IV CTCL/Sezary syndrome patients.

Materials and methods

Patients

After informed consent, patients with CTCL were recruited for this study from the Cutaneous Lymphoma Program of the Cutaneous Oncology Center at the Dana-Farber Brigham and Women's Cancer Center. All studies using blood samples were approved by the Dana-Farber Harvard Cancer Institute Institutional Review Board, under Protocol 02016 entitled "Collections of tissue and blood specimens and clinical data from patients with cutaneous T cell lymphoma". CTCL was classified and staged according to the WHO TNM classification at the first visit. Three-stage IVA CTCL/Sezary syndrome patients, all of whom had peripheral blood involvement (B2), were included in this study. Diagnoses were based on clinical criteria as well as histologic and immunohistologic assessment of skin specimens.

Blood samples

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation over Ficoll (Histopaque, Sigma, St. Louis, MO). Plasma samples were stored at -80°C prior to use. CD3⁺ T cell populations were separated with immunomagnetic beads following the manufacturer's protocols (Miltenyi Biotec, Auburn, CA). For CD3⁺ T cell selection, after 10 min of incubation with 10 μl of an antibody cocktail mixture, PBMC were incubated for 15 min with 20 μl of magnetic beads per 10^7 cells. CD3⁺ T cells were then isolated from PBMC by negative selection over MiniMACS separation LS columns. Sorted populations were analyzed by flow cytometry, and purity was ranged between 95 and 98%.

Quantification of TRECs

We analyzed the signal-joint delta Rec-J alpha (Rec-J) TRECs. Rec-J rearrangements occur late during T cell differentiation in the thymus after thymocyte expansion; therefore, Rec-J TRECs are considered to be a valid marker for these young T cells. Genomic DNA was extracted from CD3⁺ T cells by means of the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instruction. The construct of primers and probe, and the conditions used in the PCR were mentioned previously [12,17]. The primers and probes for ribosomal RNA (rRNA) were purchased from Clontech (Clontech, Palo Alto, CA), and its copy number was used to standardize for the amount of genomic DNA content. Results were extrapolated to TRECs copy numbers per 1 μg DNA.

CDR3 spectratyping to identify contracted profiles and monoclonal peaks

For spectratyping analysis, total RNA was extracted from 3×10^6 CD3⁺ T cells using the Clontech RNA purification kit (Clontech, Palo Alto, CA). Total RNA was reverse transcribed using oligo-dT primers and PowerscriptTM Reverse Transcriptase (Clontech, Palo Alto, CA). TCR BV segments were amplified with 1 of 38 BV subfamily-specific primers and CB primer recognizing both CB1 and CB2 regions. The sequences of BV primers and fluorescent CB primer were described previously [28]. The size distribution of each fluorescent PCR product was determined by automated 377 DNA sequencer (ABI). Data were analyzed with GeneScan software (ABI) that assigns a size and peak area to the different PCR products. Scoring of CDR3 profiles was performed by determining the number of contracted BV CDR3 size profiles in each subject's T cell CDR3 repertoire. Contracted profiles were defined as follows: normal (>4 peaks), oligoclonal (2–4 peaks), monoclonal (1 peak), or absent (no peaks detectable).

Flow cytometric analyses

The presence or absence of a dominant clone in CTCL patients was determined by flow cytometric analysis. Analysis was performed on PBMC using the monoclonal antibodies to CD3, CD4, and TCR BV chain as described previously [28]. Immunophenotypic analysis of cells was performed with a CellQuest flow cytometer (Becton–Dickinson).

Quantification of cytokines

Plasma cytokine levels were measured by ELISA. ELISA to detect IL-7 and IL-15 was purchased from R&D Systems (Minneapolis, MN). The IL-18 ELISA which detects only 18 kDa mature form was purchased from MBL Co. (Nagoya, Japan).

Results

Patient information

The relevant data on the three patients studied is featured in Table 1. After treatment with extracorporeal photopheresis (ECP) and interferon (IFN) $\alpha 2\text{b}$ ($n = 2$) or denileukin diftitox

(Ontak) ($n = 1$), the skin manifestations improved remarkably and lymph node involvement diminished, and finally all three achieved complete clinical remission.

Blood and plasma data

For all patients, the CD4/CD8 ratio was >10 before treatment, consistent with leukemic CTCL. After therapy, this ratio decreased to a normal range in all three patients. Plasma LDH and IL-18 levels also decreased after treatment (Table 2). Similarly, when the malignant clone could be identified by T cell receptor BV antibodies, this population was decreased to the normal range after treatment (Table 1). However, after remission was achieved, absolute CD4 counts were significantly lower than normal (Fig. 1). As previously described, TREC levels were also lower than normal controls before treatment, as is commonly observed in advanced-CTCL patients. TREC levels did not increase substantially after patients had achieved complete remissions, suggesting that new naive T cells were not being generated, nor were expanded populations of normal cells decreasing in number. Interestingly, plasma IL-7 levels remained elevated after complete remission was achieved (Fig. 1).

Combination of CDR3 spectratyping analysis and quantitative analysis by flow cytometry

Peripheral blood T cells were isolated from these patients, and CDR3 BV spectratyping was performed. Figure 2a shows a representative spectratype from peripheral blood T cells obtained from a normal volunteer. A Gaussian distribution of CDR3 amplicon length is observed in all BV subfamilies indicating a highly diverse T cell repertoire. In clear contrast, an obviously abnormal spectratype was observed in CTCL patient before treatment (Fig. 1b, result from patient A shown in Table 1). Additionally, spectratype patterns suggesting oligoclonality were also noted in certain BV families. Contracted profiles and monoclonal peaks on the spectratyping analysis are increased in CTCL patients particularly those with advanced stages. This was seen in these cases, and had successfully recovered after treatment (Table 2; Fig. 2c).

In order to generate a “global” image that combines both T cell count and reduced T cell repertoire complexity, we have generated topographic TCR “landscapes” that graphically demonstrate the loss of TCR repertoire complexity. The x -axis displays the BV subfamilies, the z -axis shows the CDR3 length distribution (in amino acids), and the y -axis indicates the absolute number of BV+CD4+ T cells/ μ L, as previously described. A diverse T cell repertoire from an individual without CTCL is shown in Fig. 2d. In contrast, a landscape with an identifiable and numerically expanded Vb11 clone was observed in an advanced-CTCL patient (patient A, Fig. 2e). After achieving complete remission in the same patient, the numerically expanded Vb11 clone has disappeared, but there is very little background complexity as compared to the normal T cell repertoire (Fig. 2f vs. Fig. 2d).

Discussion

In this study, we report three patients treated with either ECP and IFN $\alpha 2b$ ($n = 2$) or Ontak ($n = 1$) who achieved complete remissions of their CTCL/Sezary syndrome, as judged by both clinical and laboratory criteria. The CD4/CD8 ratio before treatment was >10 in all three patients, indicating peripheral blood involvement by malignant CD4+CTCL cells; this ratio also returned to the normal range after successful treatment. By flow cytometric analysis with T cell receptor BV antibodies, we could measure the tumor burden in the blood of two of the three patients accurately. After they achieved complete remission, the percentage of the BV family population that contained the malignant clone decreased to normal levels. This dramatic decrease in the actual percentage of malignant T cells was paralleled by a gradual and eventually complete resolution of erythroderma and pruritus. Plasma LDH and IL-18 levels also decreased [14,26].

In parallel, we analyzed TREC levels in peripheral blood CD3+ T cell populations. Because T cell receptor rearrangements occur late during the differentiation in the thymus and after thymocyte expansion, TRECs were originally considered to be a valid marker for individual unexpanded naive T cells. The two other major biological parameters affecting TREC levels are longevity of naive T cells and the dilution of TRECs by cell division. As described previously in CTCL, we have used TREC levels as a proxy marker for proliferation [28]. While TREC levels were increased in patients who achieved remission, they still remained at lower levels compared with age-adjusted normal ranges. This was associated with persistent and relatively high plasma levels of IL-7. This persistent elevation in IL-7 is unlikely to have derived from skin lesions, since these resolved, and may be due to a compensation mechanism controlled by absolute T cell number, as reported in patients with poorly controlled HIV infection [19,20].

As we reported previously [29], contracted BV spectratype profiles and monoclonal peaks on the spectratyping analysis are increased in CTCL patients, particularly those with advanced stage disease. This method provides a good measure of the complexity of the T cell repertoire across multiple BV families. In normal controls, a Gaussian distribution is typically observed in all BV subfamilies, consistent with a highly diverse T cell repertoire. In contrast, the T cell repertoire prior to treatment in our three individuals was profoundly disrupted, with a marked reduction in diversity evidenced by monoclonal and oligoclonal patterns observed in multiple BV subfamilies [28,29]. Following successful treatment of their CTCL, the CDR3 spectratype patterns in each patient recovered dramatically (Fig. 2c). Improvement was noted in both the number of contracted profiles and the number of monoclonal peaks, indicating reconstruction of the T cell receptor repertoire (Table 2). However, equally striking was the very low level of normal T cells (as judged by absolute CD4 counts) in CTCL patients across multiple BV families, even after the identifiable circulating malignant T cell populations were removed (Fig. 2f). The basis of this T cell lymphopenia is unknown. Possible explanations include disruption in lymphoid architecture, as seen in patients with HIV infection, which might disturb normal lymphoid-tissue homeostasis [4,7,23], or impaired production of lymphocyte precursors may prevent the replacement of CD4+ T cells.

For this analysis, we chose patients with stage IV disease involving peripheral blood who achieved complete clinical and laboratory remission, an unfortunately uncommon event. Although the number of patients examined limits interpretation of these results, these observations indicate that CTCL/Sézary syndrome patients that successfully enter remission can experience at least the partial restoration of a more diverse T cell repertoire. Persistent suppression of CD4 counts, however, may indicate that these patients remain at some ongoing risk with regard to immunosuppression, and further investigation is required to understand the basis of this persistent lymphopenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTCL	Cutaneous T cell lymphoma
TREC	T cell receptor excision circle

IL	Interleukin
T reg	Regulatory T cell
BV	b-Variable
CDR3	Complementarity-determining region 3
ECP	Extracorporeal photopheresis
IFN	Interferon

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Fig. 1.

Absolute CD4 count, TREC levels, and plasma IL-7 levels. Absolute CD4 count was lower than normal range after successful treatments. TREC levels were lower than normal controls before treatment, and the levels did not increase enough after patients had achieved complete remissions. Plasma IL-7 levels were still higher than normal after achieving complete remission

Fig. 2.

Restoration of T cell repertoire diversity following induction of remission in CTCL. TCR BV CDR3 spectratyping profiles (a–c) and combination of qualitative (CDR3 spectratyping) and quantitative (flow cytometry) analysis plots (d–f) give a better visual assessment of the global reduction of the TCR diversity in CTCL. The CDR3 spectratype of CD3+ T cells from a representative healthy donor (panel a) shows a highly diverse profile in all BV subfamilies, reflecting a heterogeneous TCR repertoire. The combination plot (panel d) displays BV subfamilies along the x-axis, CDR3 length distribution (in amino acids) along the y-axis, and absolute number of BV+CD3+ T cells/μL along the z-axis as measured by flow cytometry. Analysis of peripheral CD3+ cells from a patient with stage IVA CTCL (Patient A) shows a

markedly reduced spectratype complexity (panel **b**, see also Tables 1 and 2) and a numerically expanded malignant clone (BV 11, panel **e**). Analysis of the same patient after induction of CR shows improved, though still some abnormal, CDR3 spectratype (panel **c**) with the restriction of diverse T cells in the peripheral blood (panel **f**)

Table 1

Patients' information

Patient	A	B	C
Gender	Female	Female	Male
Age	91	53	73
Stage	IVA	IVA	IVA
Skin	Erythroderma	Erythroderma	Erythroderma
Lymph node	Swelling	Histologically confirmed LN involvement	Swelling
Peripheral blood	B2	B2	B2
Dominant clone	BV 11 (93%)	BV 14 (80%)	ND
Treatment to reach CR	ECP	ECP	ECP
	IFN a-2b; 3×10^6 unit 3 times/week	IFN a-2b; $1.5 \sim 3 \times 10^6$ unit 3 times/week	Denileukin diftitox; 6 courses

Three-stage IVA CTCL/Sezary syndrome patients (1 man, 2 women) were recruited for this study. After treatment with extracorporeal photopheresis (ECP) and interferon (IFN) a2b or denileukin diftitox (Ontak), all three achieved complete remission. The percentage of certain BV clonal population was decreased to the normal range

BV b-variable, *ND* not detectable with our panel of antibodies

Table 2

Lab data

Patient	A			B			C		
	Pre-treatment	CR 1 year		Pre-treatment	CR 2 years		Pre-treatment	CR 1 year	CR 2 years
WBC(# × 10 ³ /μL)	16.1	3.9		13.4	4.84		4.95	4.8	5.2
CD4 (#/μL)	9,580	233		4,869	649		649	215	238
CD4:CD8 ratio	48.11	0.82		14.87	1.74		11.01	2.01	1.58
CD7 (#/μL)	31	74		70	61		16	72	72
LDH (U/L)	1,211	481		750	126		1,142	550	479
Plasma IL-18 (pg/mL)	351.4	203.2		357.1	226.2		557.3	385.1	311.1
Contracted profiles (#)	8	3		7	1		17	12	3
Monoclonal peaks (#)	3	2		2	0		5	3	0

In all patients, CD4/CD8 ratio that was over 10 before treatment, decreased to normal range. Plasma LDH and IL-18 levels were also decreased. The increase in the number of contracted profiles and monoclonal peaks on the spectratyping analysis had successfully recovered after treatment CR complete remission