Th2 Cytokine mRNA Expression in Skin in Cutaneous T-Cell Lymphoma

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We have previously demonstrated that peripheral blood mononuclear cells from patients with Sézary syndrome, the leukemic form of cutaneous T-cell lymphoma which is accompanied by erythroderma and lymphadenopathy, have a Th2 cell cytokine [interleukin 4 (IL-4) and interleukin 5] production pattern. In this study, we extend these observations to demonstrate a correlation of the presence of a Th2 cytokine pattern with a malignant T-cell clone in different stages of cutaneous involvement among patients with cutaneous T-cell lymphoma (CTCL). Skin biopsies were obtained from 12 CTCL patients with various disease stages (three patch, three plaque, six tumor), three patients with parapsoriasis, four patients with inflammatory dermatoses, including two psoriasis and two lichen planus, and 12 normal controls. Total RNA was extracted, reverse transcribed, and PCR amplified with IL-2, IL-4, IL-5, interferon gamma (IFN-γ), and β-actin oligonucleotide primers. Although all skin specimens tested had detectable IL-2 and IFN-γ mRNA, only specimens from patients with CTCL or parapsoriasis had demonstrable IL-4 and/or IL-5 mRNA. Specifically, IL-5 mRNA was detected in skin biopsies from five of six tumor-stage CTCL, two of three plaque-stage CTCL, one of three patch-stage CTCL, and 1 of 3 parapsoriasis patients, whereas IL-4 mRNA was demonstrated to be present in five of six tumor-stage, one of three plaque stage, none of three patch-stage CTCL, and none of three parapsoriasis patients. These results indicate that in all stages of cutaneous involvement of CTCL, encompassing patch stage through tumor stage, IL-4 and IL-5 mRNA is variably detectable. In tumor-stage skin lesions, typically characterized by a dense dermal infiltrate of malignant T cells, Th2 cytokine mRNA is virtually always detectable. The ability to detect Th2 cytokine mRNA in the skin of patients with CTCL supports our previous findings that the malignant T cells in CTCL possess a Th2-helper cell phenotype. ***Key words: Sézary syndrome/skin neoplasms/mononuclear leukocytes/interleukin. J Invest Dermatol 103:669–673, 1994***

Distinct subsets of T helper cells, which are delineated on the basis of cytokine-secretion patterns, were initially described in the murine immune system [1–3]. It has become increasingly clear that such subsets of T cells, termed Th1 and Th2, can also be identified in humans [4]. Although the first evidence of Th1 and Th2 cells in humans came from cell lines or T-cell clones [5,6], recent evidence indicates that these subsets are an important component of the specific T-cell response in several different diseases. Infiltrates of interleukin 4 (IL-4)– and IL-5–producing cells (Th2 phenotype) that do not produce IL-2 or interferon-γ (IFN-γ) have been described in atopic individuals in response to dust-mite allergen [7,8]. Bronchial lavage fluids from asthma patients have been shown to contain significantly more Th2-like cells than control subjects [9]. Moreover, Yamamura *et al* have shown using the polymerase chain reaction (PCR) that T cells obtained from lepromatous-type lesions of leprosy patients were positive for IL-4, IL-5, and IL-10 mRNA, the Th2 phenotype, whereas T cells from tuberculoid-like lesions produced cytokine mRNA consistent with the Th1 phenotype (IFN-γ and IL-2) [10]. Parasitic infection, particularly with helminths, has also been shown to evoke a Th2-like cell response [11]. In addition, activation of T cells via the chemical induction of autoimmunity or graft-versus-host disease in rodents appears to most often result in a Th2 cell pattern (reviewed by Goldman *et al* [12]). Although the immunologic factors that lead to the generation of a specific T-helper subset response remain to be defined, it appears that Th1-like cells are primarily responsible for mediating delayed-type–hypersensitivity reactions such as that observed in response to certain infections or contact dermatitis, whereas a Th2-like response is observed in conditions such as parasitic or allergic reactions characterized by eosinophilia and high-antibody titers, particularly IgE.

Patients with Sézary syndrome (SzS), a leukemic form of cutaneous T-cell lymphoma (CTCL) characterized by malignant atypical CD4+/CD45RO+ lymphocytes (Sézary cells) in the skin and peripheral blood, in addition to erythroderma, exhibit an array of immunologic abnormalities that parallel the *in vitro* activities of Th2 cytokines, particularly IL-4 and IL-5 [13]. We have previously shown that peripheral blood mononuclear cells (PBMC) from SzS

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Abbreviations: PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription–polymerase chain reaction; SzS, Sézary syndrome.

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Table I. Comparative Features of Parapsoriasis and Cutaneous T-Cell Lymphoma (CTCL)

	Clinical Presentation	Routine Histology	Cell Marker Analysis	Buffy Coat Analysis
Parapsoriasis	Persistent erythematous scaly patches; trunk and extremities	Individually disposed lymphocytes in a non-spongiotic epidermis; sclerotic papillary dermal collagen splayed by lymphocytes; hyperconvolution of lymphocyte nuclei variable	No loss of pan-T-cell maturation antigens; may have admixture of CD4+ and CD8+ cells in the epidermis and dermis	0–5% Sézary cells in mononuclear component of peripheral blood
Patch-stage CTCL	Erythematous scaly asymmetric patches with irregular borders; trunk and extremities > acral skin	Individually disposed and nested lymphocytes in a non-spongiotic epidermis, many with hyperconvoluted nuclear contours; sparse dermal infiltrate	CD4+:CD8+ cells in the epidermis exceed 6:1; CD4+ cells show partial loss of pan-T cell maturation antigens (CD2,3+/−5); dermal component reactive	From 0 up to 15% Sézary cells in the mononuclear component of peripheral blood
Plaque-stage CTCL	Infiltrated plaques with bizarre arcuate borders; trunk and extremities, also head and neck	Nested lymphocyte with hyperconvoluted nuclear contours in the epidermis and within the superficial dermal stroma	CD4+:CD8+ cells exceed 6:1 in both the epidermis and dermis; there is also loss of pan-T-cell maturation antigen in both sites	Sézary cell count <15% suggests disease localized to skin; if >15%, systemic involvement present
Tumor-stage CTCL	Exophytic nodules ± ulceration anywhere on the body	Epidermotropism of lymphocytes usually lacking; nodules of abnormal lymphocytes (may show blast transformation) in the dermis; may be surrounded by a cuff of reactive lymphocytes	Tumor nodules show preponderance of CD4+ cells that have loss of 1 or more pan-T-cell maturation antigens	Sézary cell count <15% suggests disease localized to skin; if >15%, systemic involvement present
Sézary syndrome (leukemic form of CTCL)	Generalized erythroderma with scaling, often with marked pruritus	Not diagnostic	Not diagnostic	Sézary cell count >15%, usually much higher

patients produce a Th2 cell pattern of cytokine secretion following mitogen-stimulation [14]. Although in that study the source of the Th2 cytokines could not specifically be attributred to the malignant T cell, we observed a direct correlation between the number of Sézary cells and the amount of IL-4 produced, and an inverse correlation between the number of Sézary cells and the production of IL-2 and IFN-γ.

In the present study, we show that these observations can be extended to the detection of Th2 cytokine mRNA in the skin of CTCL patients. Furthermore, we demonstrate that as CTCL progresses from patch to tumor stage, i.e., as the extent of the malignant T-cell infiltrate becomes more marked, the prevalence of Th2 cytokine mRNA in the skin is more frequent.

MATERIALS AND METHODS

Patients and Skin Samples Patients participating in this study were diagnosed with CTCL based on clinical, histopathologic, and immunohistologic criteria **[Table I]** (reviewed in [15]). Skin samples, consisting of 4-mm punch biopsies, were obtained from CTCL patients with different stages of cutaneous involvement (patch, plaque, or tumor), or from volunteers with other skin disorders characterized by lymphoid infiltrates including parapsoriasis, lichen planus, and psoriasis. Skin biopsies were also obtained from normal controls. All CTCL patients were seronegative for human T-cell lymphocytic virus-I antibodies by enzyme-linked immunosorbent assay and had no apparent secondary infections or other dermatologic diseases. All skin samples were procured after informed consent was obtained.

Extraction of RNA and Performance of the Polymerase Chain Reaction (PCR) Total RNA was extracted from skin biopsies, fresh or snap frozen, using RNAzol B (Tel-Test, Inc., Friendswood, TX), a modification of the guanidinium thiocyanate isolation method [16]. Following extraction, the RNA was reverse transcribed (RT) as previously described [17]. Briefly, 3 μg of total RNA, after brief denaturation at 65°C for 5 min, was incubated at 37°C for 30 min in a cocktail containing RT (BRL/Gibco), deoxynucleotide triphosphates (dNTP, Perkin-Elmer Cetus), oligo $(dT)_{16}$ (Sigma), dithiothreitol (BRL), RNase inhibitor (RNAsin, BRL), $MgCl_2$, KCl, and Tris buffer in a total volume of 20 μl. Immediately following RT, 10 μl of the RT cocktail was removed for PCR amplification. PCR amplification of cytokine cDNA was accomplished using sense and antisense primers based on previously published sequence data and by selection using a primer selection program, OSP [18] **(Table II)**. To prevent amplification of any contaminating genomic DNA, all cytokine primers were designed to span introns. To verify the integrity of the RNA and cDNA for each experimental sample, a separate control amplification using β-actin primers was included in the PCR run. The PCR cocktail consisted of Tris buffer, 2.5 mM $MgCl_2$, 1.5 mM dNTP, 500 ng each of the sense and antisense primers, 1.25 U of Taq polymerase (Promega), and deionized water in a total volume of 50 μl. This PCR cocktail was amplified in a forced-hot-air thermocycler (BioOven II, BioTherm Corp., Fairfax, VA) for 30 cycles where a single cycle consisted of 92°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. To avoid carry-over contamination, strict physical and procedural precautions (separate handling areas and dedicated supplies and reagents for pre- and post-amplification) were routinely observed. To monitor for carry-over contamination, a negative control water blank was included in each PCR amplification. Following amplification, a 12-μl sample of the PCR cocktail is sized on a ethidium bromide-containing 3% Nusieve (FMC, Rockland, ME): 1% agarose (Gibco/BRL) gel run at 90 V for 1 h. The gel was visualized under ultraviolet light and the size of any bands present are compared to molecular weight markers run in a parallel lane. The specific cytokine identity of PCR products was verified by size and by autoradiography using a ^{32}P-α-ATP-labeled nested oligonucleotide probe **(Table II)**.

RESULTS

Th1 Cytokine (IL-2 and IFN-γ) mRNA Expression in Normal Skin To evaluate cytokine expression in CTCL, we initially determined the background expression of cytokine mRNA in normal skin. PCR analysis revealed that skin samples from 12 different normal donors all expressed mRNA for IL-2 and IFN-γ; however, IL-4 or IL-5 mRNA was not detected within any of these specimens **(Table III and Fig 1)**. It should be stressed that neither IL-2 nor IFN-γ mRNA were detected in negative control cDNA from HuT 78 cells, a CD4+-transformed T-cell line, or in the negative-control water blanks, indicating that the results obtained in skin samples were not the result of PCR contamination. Thus, normal skin exhibited a Th1 cytokine pattern.

Th2 Cytokine (IL-4 and IL-5) mRNA in Skin Biopsies from CTCL Patients Biopsies of involved skin from CTCL patients

Table II. Oligonucleotides used for PCR and as Probes

Target cDNA	Primer	Basepair Location in cDNA	Oligonucleotide Sequence (5′ → 3′)
IL-2			
	Sense	12–33	TAA TCA CTA CTC ACA TGA ACC T
	Antisense	500–479	TGT TGA GAT GAT GCT TTG ACA
	Nested	384–366	CTA TTA CGT TGA TAT TGC T
IL-4			
	Sense	86–103	TCC CCC CTC TGT TCT TCC
	Antisense	481–462	GCC TTT CCA CGC CGT TTT CC
	Nested	409–385	CGA GAG GAT CCT GTC GAG CCG TTT
IL-5			
	Sense	45–64	ATG AGG ATG CTT CTG CAT TTG
	Antisense	449–430	TCA ACT TTC TAT TAT CCA CTC
	Nested	250–227	TGA AAG ATT TCT TCA GTG CAC AGT
IFN-γ			
	Sense	142–161	AGT TAT ATC TTG GCT TTT CA
	Antisense	497–478	ACC GAA TAA TTA GTC AGC TT
	Nested	419–401	CTT GAT GGT CTC CAC ACT CT
β-actin			
	Sense	285–302	ATG GAG AAA ATC TGG CAC
	Antisense	888–871	TGA TGG AGT TGA AGG TAG
	Nested	703–684	AGG GCG ACG TAG CAC AGC TT

were obtained for evaluation of IL-4 and IL-5 mRNA expression. Specimens were segregated into three separate groups based upon clinicopathologic characterization of lesional skin **(Table I)** and consisted of patch stage (n = 3), plaque stage (n = 3), and tumor stage (n = 6). In addition, biopsies were taken from patients diagnosed with parapsoriasis (n = 3), a frequent precursor of CTCL [19]. Biopsies from patients with patch-stage disease, the earliest stage of CTCL, did not express IL-4 mRNA; however, involved skin from one patient did express IL-5 mRNA **(Table IV)**. In biopsies of plaque-stage disease, one of three specimens expressed IL-4 mRNA, whereas two of three specimens, including the one expressing IL-4 mRNA, had detectable mRNA for IL-5 **(Table IV)**. In the most advanced stage of CTCL, tumor stage, IL-4 mRNA was detected in all six patients evaluated and IL-5 mRNA was present in five of six patients **(Table IV)**. The biopsies from parapsoriasis patients demonstrated a similar pattern to that observed within the samples from the patch-stage CTCL patients, with none of three expressing IL-4 mRNA and one of three having detectable IL-5 mRNA **(Table IV)**.

Non-CTCL Lymphoid Infiltrates, Lichen Planus, and Psoriasis Express a Th1 Cytokine Profile Skin samples were also analyzed from volunteers with benign inflammatory dermatoses unrelated to CTCL characterized by the presence of heavy dermal mononuclear cell infiltrates (psoriasis, n = 2; lichen planus, n = 2). In all four of these specimens, both IFN-γ and IL-2 mRNA were detected. However, in contrast to the findings in CTCL, neither IL-4 nor IL-5 mRNA was detected in any of the psoriasis or lichen planus biopsies **(Table III)**.

DISCUSSION

We have previously shown that PBMC from SzS patients produce increased amounts of IL-4 with a concomitant decrease in IL-2 and IFN-γ production as compared to normal controls [14]. The amount of IL-4 produced correlated with the numbers of circulating Sézary cells. Furthermore, we have demonstrated the ability of exogenous IFN-γ to inhibit the increased IL-4 production by the PBMC from SzS patients [14]. To extend these data and to investigate further the apparent Th2 cytokine pattern in CTCL, we studied the expression of Th1 and Th2 cytokine mRNA in skin lesions from patients with different stages of CTCL. We demonstrate in this study the ability to detect Th2 cytokine mRNA in skin lesions from CTCL patients at all stages of disease encompassing patch stage through tumor stage. However, either IL-4 or IL-5 mRNA, or both, were more frequently detected in densely infiltrated lesions characteristic of a more advanced stage of disease.

Table III. Expression of Cytokine mRNA in Non-CTCL Skin

Patient Dx	n	IL-4	IL-5	IFN-γ	IL-2	β-Actin
Normal Control						
Trunk	5	−	−	+	+	+
Arm, face	7	−	−	+	+	+
Psoriasis	2	−	−	+	+	+
Lichen planus	2	−	−	+	+	+

In addition to finding Th2 cytokine mRNA at various stages of disease, mRNA for both IL-2 and IFN-γ was expressed in both normal and CTCL involved skin. It is unlikely that the amplification of these two cytokines was the result of contamination of reagents because neither water blanks nor cDNA obtained from normal resting PBMCs or HuT78 cells showed amplification of these cytokines. Because there are no known endogenous skin cells that produce IL-2 or IFN-γ, either constitutively or by induction, these data suggest that skin-trafficking T cells, which are observed in perivascular regions of normal skin [20], may be primed to produce low levels of IL-2 and IFN-γ. Furthermore, perivascular T cells in normal skin often express activation markers such as CD25 and class II HLA antigens [20]. Because IL-2 mRNA production precedes the expression of these activation markers in the normal kinetics of T-cell activation and proliferation [21], it is not surprising that mRNA for IL-2 or IFN-γ is detectable by PCR in normal skin biopsies.

Although RT-PCR cannot definitively determine the source of the Th2 cytokines in the involved skin of CTCL, several lines of evidence suggest that IL-4 and IL-5 are being produced by the malignant T-cell clone. First, although the numbers of patients is small, there appears to be a correlation between the number and density of malignant T cells that invade the skin with each successive stage of disease and the likelihood that IL-4 and IL-5 mRNA will be detected. Second, we have previously observed a correlation between the number of circulating malignant T lymphocytes and the amount of IL-4 produced upon activation of PBMCs from SzS patients [14]. Furthermore, PBMC from SzS patients in remission, as well as PBMC from plaque stage patients without circulating malignant T cells, produced IL-4 in amounts comparable to normal controls [14]. Nevertheless, the recent observation by Dummer and colleagues [22] that mitogen-activated PBMCs from plaque-stage patients lacking Sézary cells did produce variably increased amounts

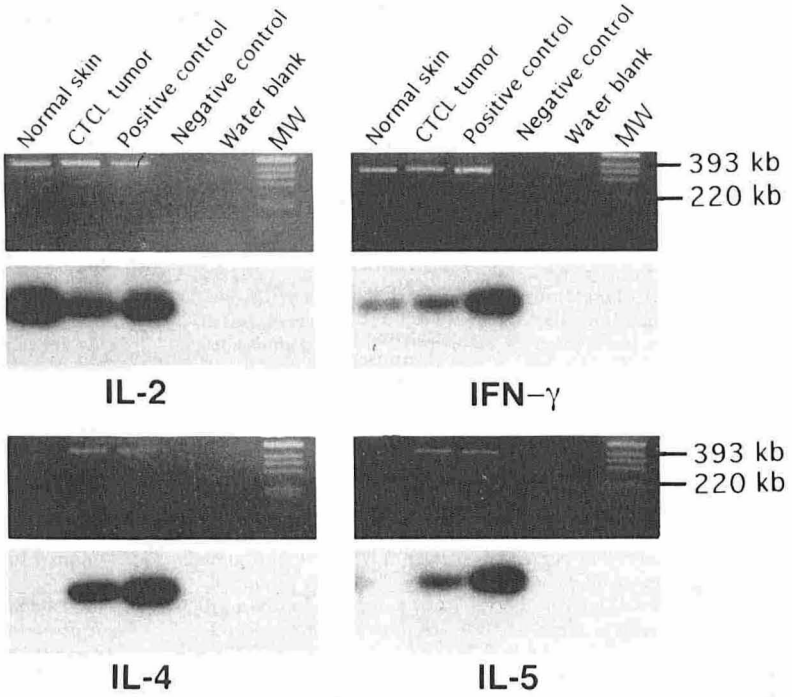

Figure 1. PCR analysis of cytokine mRNAs isolated from skin lesions of CTCL patients and normal volunteers. Samples were analyzed for expression of IL-2, IFN-γ, IL-4, IL-5, and β-actin mRNAs (see *Materials and Methods*). Representative results are shown. Positive and negative controls consisted of Concanavalin A–stimulated normal PBMCs and unstimulated HuT 78 cells, respectively.

of IL-4 suggests that some reactive lymphoid cells in CTCL may be contributing to Th2 cytokine production.

Although our data suggest that the malignant T-cell clone is the source of Th2 cytokine mRNA, other cutaneous cellular sources cannot be ruled out by our studies. Several recent reports have demonstrated that mast cells can synthesize and secrete Th2 cytokines [23]; however, the lack of clinical and histopathologic evidence for mast cell degranulation in these patients suggests that mast cells are not likely to be the source of IL-4 or IL-5. Another potential source of IL-5 in these samples are activated eosinophils, which Broide *et al* have recently shown to be capable of producing IL-5 [24] and are present in increased numbers in Sézary patients. Further investigation into the precise source of the Th2 cytokines is warranted, particularly with the use of more specific techniques, such as *in situ* hybridization, which would permit the precise localization of cytokine mRNA.

Although we are able to detect Th2 cytokine mRNA in clinically involved skin specimens from a large proportion of patients with different histologic stages of CTCL, there are several potential reasons for our failure to detect this pattern in a greater percentage of specimens from early CTCL. One plausible explanation relates to the known interactions between Th1 and Th2 cytokines, which appear to have reciprocal effects on the opposing T-helper subset, with IFN-γ having the capacity to inhibit the expression of Th2 cytokine gene expression [25]. Thus, in early skin lesions of CTCL, a sparse infiltrate of malignant T cells is present upon a background of normal pre-existing, skin-trafficking Th1 cells that may play a role in the host immune response to suppress the activity and proliferation of the malignant cells. In support of this, Wood and colleagues have demonstrated tumor-infiltrating CD8+ cytotoxic T cells in early CTCL [26]. Because cytotoxic T cells are a rich source of IFN-γ production upon activation [27], it is likely that the tumor-infiltrating cells may also play a role in the suppression of the Th2 cytokine transcripts below levels that may be routinely detected by our PCR assay.

Table IV. Expression of Th2 Cytokine mRNA in CTCL Skin

Patient	Stage	IL-4	IL-5	IFN-γ	IL-2	β-Actin
PP1	Parapsoriasis	−	−	+	+	+
PP2	Parapsoriasis	−	+	+	+	+
PP3	Parapsoriasis	−	−	+	+	+
PA1	Patch	−	−	+	+	+
PA2	Patch	−	+	+	+	+
PA3	Patch	−	−	+	+	+
PQ1	Plaque	−	−	+	+	+
PQ2	Plaque	−	+	+	+	+
PQ3	Plaque	+	+	+	+	+
TU1	Tumor	+	+	+	+	+
TU2	Tumor	+	+	+	+	+
TU3	Tumor	+	−	+	+	+
TU4	Tumor	+	+	+	+	+
TU5	Tumor	+	+	+	+	+
TU6	Tumor	+	+	+	+	+

The demonstration of an immunologic milieu characterized by a predominance of Th1 cytokines in early CTCL reflects the clinical and histologic findings at this stage of disease. IFN-γ–mediated suppression of Th2 cell proliferation may be an important factor responsible for the indolent course of patch- and plaque-stage disease. Furthermore, the epidermal expression of intercellular adhesion molecule-1 (ICAM-1) is known to be an IFN-γ–dependent event and is associated with the significant degree of epidermotropism typical of early disease [28]. As lesions evolve and subsequently demonstrate dermal invasion characteristic of tumor-stage disease, both ICAM-1 expression and the degree of epidermotropism become less profound or completely absent. On the other hand, the aggressive course of advanced disease may result from enhanced tumor-cell proliferation mediated by autocrine stimulation of the numerous malignant cells by increased IL-4 production, which additionally suppresses Th1 cell responses. Thus, competing Th1 and Th2 cytokine effects may be important not only in regard to the clinical phenotype and immune defects observed in CTCL [13], but disordered cytokine regulation may also be an essential factor in the events critical to progression of disease.

The presence of a cytokine imbalance that contributes to the progression of CTCL has important therapeutic implications. One obvious strategy might involve replacement of deficient Th1 cytokines via the administration of currently available recombinant proteins. Limited trials of the use of IFN-γ for CTCL have demonstrated some clinical benefit of this cytokine [29]. More importantly, IFN-α, which also mediates Th1 responses by a variety of mechanisms [30], has extremely potent biologic activity in the

treatment of CTCL [31]. We have noted the normalization of the cytokine profile in concert with the normalization of certain critical immune functions while experiencing a complete clinical remission during therapy with IFN-α among patients with Sézary syndrome and elevated IL-4 production [Vowels BR, Lessin SR, Cassin M, Benoit BM, Rook AH: Normalization of cytokine secretion patterns and immune function following disappearance of malignant clone from the peripheral blood of a Sézary syndrome (SzS) patient (abstr). *J Invest Dermatol* 100:556, 1993]. Thus, as additional critical immune factors are identified that favor the evolution of Th1 responses, such as IL-12, which is a potent inducer of Th1 responses ([32], and Rook AH, Kubin M, Vonderheid EC, Cassin M, Vowels BR, Wolfe JT, Wolf S, Trinchieri G, Lessin SR: Interleukin-12 (IL-12) reverses cytokine and immune abnormalities of Sézary syndrome (abstr). *J Invest Dermatol* 102:557, 1994), their therapeutic application in CTCL may prove beneficial.

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