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Th2 cytokines from malignant cells suppress Th1 responses and enforce a global Th2 bias in leukemic cutaneous T cell lymphoma

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Abstract

Purpose—In leukemic CTCL (L-CTCL) malignant T cells accumulate in the blood and give rise to widespread skin inflammation. Patients have intense pruritus, increased IgE, decreased Th1 responses and most die from infection. Depleting malignant T cells while preserving normal immunity is a clinical challenge. L-CTCL has been variably described as a malignancy of regulatory, Th2 and Th17 cells.

Experimental design—We analyzed phenotype and cytokine production in malignant and benign L-CTCL T cells, characterized the effects of malignant T cells on healthy T cells and studied the immunomodulatory effects of treatment modalities in L-CTCL patients.

Results—12/12 L-CTCL patients overproduced Th2 cytokines. Remaining benign T cells were also strongly Th2 biased, suggesting a global Th2 skewing of the T cell repertoire. Culture of benign T cells away from the malignant clone reduced Th2 and enhanced Th1 responses but separate culture had no effect on malignant T cells. Co-culture of healthy T cells with L-CTCL T cells reduced IFN γ production and neutralizing antibodies to IL-4 and IL-13 restored Th1 responses. In patients, enhanced Th1 responses were observed following a variety of treatment modalities that reduced malignant T cell burden.

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Authorship

E.G. and R.W. carried out experiments, analyzed data, prepared figures and edited the manuscript, J.T., Y.J. M.D., A.G. and C.S. participated in sample preparation, A.H.R. provided funding and advice, K. S. provided reagents and critical advice for dendritic cell isolation, J.A.D., M.T, D.C.F. and T.S.K. supplied patient samples and T.S.K. also provided critical experimental advice. R.A.C. designed the experiments, analyzed data, prepared figures and drafted the manuscript.

Disclosure of Potential Conflicts of Interest

The authors report no potential conflicts of interest.

Conclusions—A global Th2 bias exists in both benign and malignant T cells in L-CTCL and may underlie the infectious susceptibility of patients. Th2 cytokines from malignant cells strongly inhibited Th1 responses. Our results suggest therapies that inhibit Th2 cytokine activity, by virtue of their ability to improve Th1 responses, may have the potential to enhance both anti-cancer and anti-pathogen responses.

Introduction

Cutaneous T cell lymphomas (CTCLs) are a heterogeneous group of non-Hodgkin's lymphomas arising from malignant transformation of T cells that home to and populate the skin (1, 2). In leukemic variants of CTCL (L-CTCL), malignant T cells can accumulate in the blood and lymph nodes and also produce widespread inflammatory skin lesions. L-CTCL is often refractory to multiple therapies and patients often ultimately require hematopoietic stem cell transplantation (3). The median survival for patients with L-CTCL varies with the extent of malignant T cell burden but is generally 2-5 years and patients die most commonly from infection (4-7).

CTCL has been proposed to be a malignancy of three separate T cell populations: FOXP3⁺ Treg, Th2 T cells and Th17 T cells (8-10). Clinically, patients with L-CTCL have abnormalities suggestive of a Th2 driven immunologic process, including decreased antigen specific T cell responses, impaired cell mediated cytotoxicity, peripheral eosinophilia, and elevated levels of serum IgE and IgA (11-13). Prior studies have shown increased levels of Th2 cytokines and Th2 associated genes in T cells from patients with L-CTCL (9, 14-17) but a recent report claims that malignant T cells in the disease are actually Th17 biased (10).

We performed comprehensive analyses of the cytokine production of benign and malignant T cells from L-CTCL with identifiable malignant T cell clones. We report here that both malignant and benign T cells in L-CTCL patients were strongly Th2 biased, that this bias was intrinsic in malignant T cells but extrinsic in benign cells and that inhibition of Th2 cytokines led to recovery of Th1 responses in benign T cells.

Materials and Methods

Blood samples

The protocols of this study were performed in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board of the Partners Human Research Committee (Partners Research Management, Boston, MA, USA). Blood from healthy individuals was obtained as discarded tissue following leukopheresis. 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. L-CTCL patients described in this manuscript met the WHO-EORTC criteria for L-CTCL/SS (18). Patient characteristics are included in Table I; based on the malignant T cell burden in this cohort of patients, expected survival is approximately 5 years (4-7). PBMC were isolated by ficoll centrifugation and clonal and non-clonal CD4⁺ T cells from CTCL patients were isolated using magnetic bead separation (Miltenyi Biotec, Auburn, CA) after staining with TCR V β -specific antibodies (Beckman Coulter Inc., Fullerton, CA).

Flow cytometry

Analysis of T cells was performed using directly conjugated monoclonal antibodies obtained from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), Biolegend (San Diego, CA) or R&D Systems (Minneapolis, MN). V β staining was performed using the IOTest Beta Mark TCR V beta Repertoire kit (Beckman Coulter) as per manufacturer's instructions.

Isotype-matched negative control antibodies were used to set the gates for positive staining. For analysis of cytokine production, T cells were stimulated with either control medium or 50 ng/ml PMA (Sigma Aldrich, Allentown, PA) and 750 ng/ml ionomycin (Life Technologies, Grand Island, NY) plus 10 μ g/ml Brefeldin A (BD) for four hours. Cells were surface stained, fixed, permeabilized, stained with anti-cytokine antibodies, and examined by flow cytometry. Analysis was performed on Becton Dickinson FACSCanto instruments and data were analyzed using FACSDiva software (V5.1).

DC priming of clonal and non-clonal T cells from CTCL patients

The 6-sulfo LacNAc-expressing dendritic cells (slanDC) were isolated as previously described (19). LPS (Sigma Aldrich) was used at 100 ng/ml. For T cell priming, LPS-stimulated dendritic cells were harvested and co-cultured with autologous clonal and non-clonal T cells from CTCL patients in the presence of 1 ng/ml of staphylococcal enterotoxin B (Sigma Aldrich). Following 12 days of culture, T cells were restimulated for intracytoplasmatic FACS analysis with PMA and ionomycin.

Co-cultures of healthy PBMC with L-CTCL cells

2×10^6 /ml PBMC from healthy donors (including lymphocytes, monocytes, basophils and dendritic cells) were co-cultured over 12 days with 1×10^6 /ml fluorescently labelled PBMC from L-CTCL patients in Iscove's Modification of Dulbecco's Media (Mediatech, Manassus, VA) supplemented with 20% heat-inactivated fetal calf serum, antibiotics and L-glutamine. 100 IU/ml IL-2 and 15ng/ml IL-15 (Peprotech, Rocky Hill, NJ) were supplemented every other day. Where indicated, only cell-substrate, but not direct cell-cell, interaction was allowed, and transwell inserts (Corning Inc. Corning, NY) were used to separate L-CTCL from healthy PBMC.

Statistical analysis

For studies described in Fig. 1, the non-parametric Kruskal-Wallis test and Dunn's test were used for comparisons between the three groups. For studies described in Fig. 2-5, a standard two-sample Student's t-test was used. The data were previously tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance. One-way between group analysis of variance (ANOVA) was used for statistical analysis of differences between three or more groups. P values ≤ 0.05 were considered as significant.

Results

Both benign and malignant T cells are Th2-biased in patients with L-CTCL

CTCL has been proposed to be a malignancy of FOXP3⁺ regulatory T cells (Tregs) but more recent studies showed that only a subset of patients had malignant T cells with a Treg phenotype (8, 20, 21). We identified 12 patients in whom the malignant T cell clone could be conclusively identified by staining with commercially available TCR V β antibodies and analyzed benign and malignant T cells by flow cytometry (Fig. 1A). This approach allows direct analysis of the phenotype and functional characteristics of the malignant T cell clone while at the same time providing similar information about remaining benign T cells.

We studied cytokine production and found that both the benign and malignant T cells in L-CTCL patients were strongly Th2-biased. Both benign and malignant T cells from L-CTCL patients produced markedly higher levels of IL-4 than T cells from healthy individuals (Fig. 1 A,B). A mean 42.7% of malignant T cells (SEM 7.8, n=12), and 28.1% of benign T cells (SEM 7.0, n=12), produced IL-4 in patients with L-CTCL, compared with 0.65% of T cells from healthy individuals (SEM 0.18, n=12). IL-13 production was significantly higher in malignant T cells and in the benign T cells of five patients and IL-10 production was also

increased in malignant clones. Th1 responses were globally decreased in both benign and malignant T cells from L-CTCL patients, with significantly reduced production of IFN γ in both populations (Fig. 1). Production of TNF α was also markedly decreased in both the benign and malignant T cells from L-CTCL patients compared to normal controls (Fig. 1). In a subset of patients, malignant T cell clones produced more IL-2 (Fig. 1), consistent with a report that malignant T cells in L-CTCL have a phenotype suggestive of central memory T cells, a cell type known to use autocrine production of IL-2 to maintain survival (22, 23). However, given the heterogeneity among patients, this result was not statistically significant.

Benign T cells show enhanced Th1 and reduced Th2 responses when cultured separately from clonal malignant T cells

Th2 cells can suppress the production of Th1 cytokines by other T cells in vitro (24, 25). To evaluate if clonal malignant T cells suppress benign T cell responses, we separated non-clonal benign from clonal malignant T cells from the blood of L-CTCL patients using magnetic beads and cultured these T cells separately in the presence of autologous LPS-activated DC. IFN γ production increased and IL-4 production decreased in non-clonal T cells cultured away from the malignant clone whereas clonal T cells showed no significant changes in IFN γ or IL-4 production (Fig. 2 A,B). RT-PCR analyses demonstrated that expression of the Th2-associated transcription factor GATA-3 declined and expression of the Th1-associated transcription factor t-BET increased in benign T cells after culture away from the malignant clone (Fig. 2C). In clonal malignant T cells, there was no change in the expression of GATA-3 and t-BET after separate culture. Expression of the Th17-associated transcription factor ROR γ c was low in both malignant and benign T cells before and after culture. T cells isolated from the skin lesions of patients with L-CTCL had similar responses; IFN γ increased in non-clonal T cells after separate co-culture but remained unchanged in malignant T cells (Fig. 2D).

IL-4 and IL-13 produced by L-CTCL T cells suppress Th1 responses of T cells from healthy donors

To evaluate the ability of L-CTCL T cells to suppress non-Th2 cytokine production, we cultured PBMC from healthy individuals with fluorescently labelled PBMC from L-CTCL patients with a high malignant T cell burden. Co-culture was performed in direct contact or in transwell systems which separated healthy from L-CTCL cells. After co-culture with L-CTCL cells, T cells from healthy patients showed reduced production of IFN γ (Fig. 3). Suppression was observed in both direct contact and transwell cultures, suggesting a soluble factor was responsible for Th1 suppression. To determine if Th2 cytokines produced by malignant T cells were responsible for the suppression of IFN γ production in healthy T cells, we included neutralizing antibodies to IL-4 and IL-13 in transwell co-cultures of L-CTCL and healthy PBMC. Results demonstrated that neutralization of IL-4 and IL-13 restored the production of IFN γ to baseline levels (Fig. 4).

A variety of modalities that reduce tumor burden enhance the Th1 responses of benign T cells

It has been previously reported that immunomodulatory therapies for CTCL, including IFN- α 2b and extracorporeal photopheresis, led to enhanced Th1 responses in responding patients (26). IFN- α 2b and extracorporeal photopheresis both have immunomodulatory effects beyond their ability to reduce the number of malignant T cells. If Th2 cytokines produced by malignant T cells are indeed responsible for the decreased Th1 responses, pruritus, susceptibility to infections and benign T cell Th2 skewing observed in these individuals, then a variety of treatment modalities that have in common only the depletion of malignant T cells should all produce enhanced Th1 responses, regardless of the underlying mechanism of action of the therapy used. To study this issue, we evaluated the cytokine production of

benign T cells before and after therapy with a variety of treatment modalities (Fig. 5). We found that successful therapy with a variety modalities, including UVB phototherapy, extracorporeal photopheresis, low dose alemtuzumab and systemic chemotherapy with gemcitabine, all had similar effects on the cytokine production of benign T cells. In all therapies and in all patients examined, when the number of circulating T cells declined, benign T cells subsequently produced less IL-4 and more IL-2 and IFN γ (Fig. 5). In addition to the patients shown, similar findings were observed in six additional patients treated with a combination of therapies that also included topical corticosteroids, electron beam therapy and narrow band UVB (data not shown). In all patients included in this analysis, complete remission of at least 4 months was achieved. With respect to the patients shown in Fig. 5, the first patient (Fig. 5A) remains in remission on UVB and topical steroids but photopheresis was discontinued. Following a complete remission with alemtuzumab therapy, the second patient (Fig. 5B) subsequently relapsed with progressive skin disease not controlled by alemtuzumab and ultimately died from progressive disease. The third patient (Fig. 5C) experienced a complete remission following gemcitabine, and underwent stem cell transplantation.

Discussion

There has been some disagreement regarding the cytokine production and functional polarization of malignant T cells in L-CTCL. L-CTCL has been proposed to be a uniform malignancy of FOXP3⁺ regulatory T cells, Th17 cells and Th2 cells (8-10). All of the patients in our cohort showed a marked Th2 bias in malignant T cells, in agreement with earlier studies that the vast majority of patients with L-CTCL have Th2 biased malignant T cells (9, 14-17, 27). L-CTCL patients have characteristics suggestive of a Th2 driven immunologic process, including elevated levels of IgE and IgA, eosinophilia and reduced Th1 responses (11-13). These patients also suffer from marked pruritus of the skin, high rates of colonization with *Staphylococcus aureus* and a high susceptibility to cutaneous infections, similar to patients with atopic dermatitis, a prototypic Th2 disease (28). We did not observe any patients with malignant T cells of a Th17 phenotype.

Malignant T cells in L-CTCL have a phenotype suggestive of skin tropic central memory T cells (T_{CM}) (22). T_{CM} are highly migratory, recirculate between the blood, skin and lymph nodes, have a high proliferative potential, are markedly resistant to apoptosis and have a variety of cytokine production profiles (23). The T_{CM} phenotype of these cells helps to explain why L-CTCL patients develop erythroderma, peripheral blood disease and lymphadenopathy. However, one might expect that malignant T cells would have a heterogeneous pattern of cytokine production that reflects the diverse population of T_{CM} from which they arise. The fact that the vast majority of patients have strongly Th2 biased malignant T cells is striking. We found that the Th2 bias of the malignant T cells was remarkably stable in vitro, suggesting this bias may result from intrinsic abnormalities of the cell or from autocrine factors, for example galectin-1 produced by malignant T cells which has previously been implicated in the Th2 skewing of the malignant clone (17). It may also be that Th2 malignant T cell clones predominate in L-CTCL because they are remarkably effective in suppressing anticancer Th1 responses and therefore have a survival advantage.

We were surprised to find that the benign T cells remaining in the circulation of patients with L-CTCL were significantly different from those found in normal individuals. There was a striking and consistent Th2 bias even among the benign T cells in these patients, as well as a reduction in the production of Th1 cytokines (Fig. 1). These results demonstrate that a global Th2 bias exists in these patients that leads to skewing of the entire T cell repertoire, both benign and malignant, toward enhanced production of Th2 cytokines. This overproduction of Th2 cytokines by both malignant and benign T cells likely contributes to

the pruritus, reduced Th1 responses and the susceptibility to infections observed in these patients.

The remaining non-clonal benign T cells in patients with L-CTCL would be expected to have a variety of cytokine production capacities. Given the known ability of Th2 cells to suppress Th1 responses and to force a Th2 bias on other T cells (24, 25), we hypothesized that the malignant T cells may suppress the activity of benign T cells, silencing Th1 responses, as has been suggested previously (26). Indeed, when we separated and cultured non-clonal T cells from the blood and skin lesions of patients away from the malignant clone, we observed improved Th1 responses (Fig. 2). When we co-cultured T cells from healthy individuals with L-CTCL T cells, we saw a marked inhibition of Th1 responses (Fig. 3, 4). Incubation of co-cultures with neutralizing antibodies to IL-4 and IL-13 restored Th1 responses, suggesting Th2 cytokines produced by malignant T cells may be directly responsible for the suppression of Th1 responses we observed in our patients and in T cells from healthy donors. If so, successful treatment of patients with CTCL should reduce Th2 and enhance Th1 responses, as a previous study of 3 patients suggested (26).

To study this question, we evaluated L-CTCL patients treated with a variety of modalities that had in common only reduction in the number of circulating malignant T cells. Regardless of the underlying mechanism of action of particular therapies, treatments that reduced malignant T cell burden were invariably associated with enhanced Th1 responses (Figure 5). Although circumstantial, our results suggest that Th2 cytokines produced by malignant T cells play a critical role in suppressing Th1 responses in our patients.

In summary, we report a marked Th2 bias in both the benign and malignant T cells in patients with L-CTCL and we find that Th2 cytokine production by the malignant clone likely suppresses Th1 responses in these patients. Th1 responses improved in vitro when the benign T cells were cultured away from the malignant clone, when IL-4 and IL-13 activities were blocked in vitro with neutralizing antibodies, and in patients themselves after treatment with therapies that reduced the number of circulating malignant T cells. The suppression of Th1 responses in these patients by IL-4 and IL-13 suggests that neutralization of these cytokines may be beneficial in enhancing immune responses. Aeroderm, a recombinant small protein antagonist of the IL-4/IL-13 receptor formulated for skin injection, was previously in clinical trials for the treatment of severe AD (29). By inhibiting Th2 responses and enhancing Th1 responses, IL-4/IL-13 antagonists have the potential to enhance immune responses to both skin pathogens and to the malignant T cells themselves.

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Statement of translational relevance

Patients with leukemic cutaneous T cell lymphoma (L-CTCL) have an average 3-5 year survival, die most commonly from infection and have clinical abnormalities consistent with a Th2-driven immunologic process. We find both malignant and benign T cells in L-CTCL are markedly Th2 biased, demonstrating a global Th2 skewing. Culture of benign T cells away from the malignant clone reduced Th2 and enhanced Th1 responses but separate culture had no effect on malignant T cells. Co-culture of healthy T cells with L-CTCL T cells reduced IFN γ production and neutralizing antibodies to IL-4 and IL-13 restored Th1 responses. In patients, enhanced Th1 responses were observed following a variety of treatment modalities that reduced malignant T cell burden suggesting that Th2 cytokines produced by malignant T cells play a critical role in down-regulating Th1 responses in vivo. Results suggest that neutralization of Th2 cytokines may be beneficial in enhancing immune responses both to pathogens and to the malignancy itself.

Fig. 1. Both benign and malignant T cells are strongly Th2-biased in patients with L-CTCL
 Clonal malignant T cells were definitively identified by staining with TCR V β antibodies specific for each patient's malignant clone. The cytokine production of malignant (clonal) and benign (non-clonal) T cells was evaluated by stimulating cells with PMA and ionomycin, surface staining for CD3, CD4 and the malignant TCR V β clonotype, then performing intracellular cytokine staining followed by flow cytometry analysis. (A) Representative results from patient 330 are shown. Th2 cytokines, including IL-4 and IL-13, were produced at high levels by both benign and malignant T cells. (B) Th2 cytokine production was elevated and Th1 cytokine production was reduced in both the benign (non-clonal) and malignant (clonal) T cells from L-CTCL patients as compared to normal controls. 12 healthy individuals and 12 patients with L-CTCL are shown. Increased IL-4 production and loss of IFN γ and TNF α production were observed in both malignant and benign T cells from patients with L-CTCL. The production of IL-13 and IL-10 was also elevated in the malignant clones. ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. Benign T cells show reduced Th2 and enhanced Th1 responses when cultured separately from clonal malignant T cells

(A, B) Non-clonal benign and clonal malignant T cells were isolated from the blood of L-CTCL patients using magnetic beads. Clonal and non-clonal T cells were cultured separately with LPS-activated autologous DC for 12 days and then assayed for cytokine production by intracellular flow cytometry. IFN γ production increased and IL-4 production decreased in non-clonal T cells cultured away from the malignant clone but clonal T cells showed no significant changes in IFN γ or IL-4 production. Representative dot plots (A) and the mean and SEM of three L-CTCL patients (B) are shown. (C) Changes in the expression of Th1- and Th2-associated transcription factors parallel observed changes in cytokine production in benign and malignant T cells cultured separately. In benign T cells cultured away from the malignant clone, expression of GATA-3 (Th2) decreased and expression of t-BET (Th1) increased whereas expression levels of both transcription factors remained unchanged in malignant T cells. Expression of ROR γ (Th17) was low in both cell populations both before and after culture. (D) Th1 responses are also enhanced in lesional skin T cells cultured away from the malignant clone. T cells were isolated from lesional skin, separated into non-clonal and clonal T cells and then cultured separately with LPS-activated autologous DC. Non-clonal T cells from lesional skin showed a marked enhancement of IFN- γ production after culture away from the malignant clone. * $p < 0.05$.

Fig. 3. Soluble factors from L-CTCL T cells suppress Th1 responses of PBMC from healthy donors

PBMC from healthy donors were co-cultured with PBMC from L-CTCL patients with high malignant T cell burden for 12 days either in direct contact or in a transwell system, then assayed for cytokine production by flow cytometry. Production of IFN- γ was significantly reduced after culture with L-CTCL PBMC in both direct contact and in transwell systems. (A) Representative dot plots and (B) mean and SEM from 3 independent experiments are shown. *** $p < 0.001$.

Fig. 4. Neutralization of IL-4 and IL-13 restores Th1 responses

PBMC from healthy donors were co-cultured with L-CTCL PBMC using a transwell system with or without neutralizing antibodies to IL-4 and IL-13 or appropriate isotype controls. Cytokine production was then determined by flow cytometry. Dot plots are gated to show only healthy CD4⁺ T cells. Representative data from one experiment (A) and cumulative data from 3 independent experiments (B) are shown. **p<0.005, ***p<0.001.

Fig. 5. A variety of treatment modalities that reduce tumor burden enhance Th1 responses of benign T cells in vivo

(A) Clinical photos of patients before and after the indicated therapies are shown. (B) All modalities reduced the number of circulating malignant T cells (red) and (C) all were associated with decreased production of IL-4 and increased production of both IFN γ and IL-2 by benign T cells after therapy (after tx).

Table 1

Clinical patient characteristics.

Patient ID #	Clone	Age	Gender	CD4/CD8 Ratio	Absolute CD4 count	Treatments	Disease duration [years]
395	Vβ17	67	M	31	8,323	I, TS, D, G, ECP	3
326	Vβ7.2	80	M	10.2	2,893	I, ECP, B, A	5
489	Vβ13.2	46	M	17.8	1,991	A, G	1
414	Vβ13.1	72	M	18	2,442	ECP, T, A, V, D, P, R, G	0.5
478	Vβ17	56	F	7.18	2,546	TS, ECP	1
484	Vβ2	56	F	9.43	2,383	TS, ECP, SS	2
279	Vβ13.1	72	M	13.5	2,504	D, B, ECP, PUVA, I, A, V, G,	5
475	Vβ2	70	F	3.1	1,230	UVB, ECP, A	7
382	Vβ1	75	F	2.67	1,029	TS, UVB	9
372	Vβ5.2	64	F	94	11,083	PUVA, ECP, UVB, TS, I	12
365	Vβ17	83	M	23	3,671	B	4
330	Vβ13.1	72	M	18.6	5,028	UVB, TS, B	8
292	Vβ17	67	M	32.3	5,356	PUVA, UVB, TS, I, ECP, B, G	13
119	Vβ13.1	78	F	14.5	2,800	PUVA, D, I, ECP, V, B	6
99	Vβ17	81	F	12.56	3,530	PUVA, UVB, TS	7
44	Vβ21.3	96	F	6.52	1,495	I, SS	16
472	Vβ2	88	M	93	3,798	ECP, B, PUVA, A	3

I – IFN-α-2a, ECP – extracorporeal photochemotherapy, V – vorinostat, B – bexarotene, D – denileukin difitox, TS – topical steroids, SS – systemic steroids, G – gemcitabine, A – alemtuzumab, P – pralatraxate, R – romidepsin, PUVA – psoralen + UVA treatment