Benign T cells drive clinical skin inflammation in cutaneous T cell lymphoma

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Psoralen plus UVA (PUVA) is an effective therapy for mycosis fungoides (MF), the skin-limited variant of cutaneous T cell lymphoma (CTCL). In low-burden patients, PUVA reduced or eradicated malignant T cells and induced clonal expansion of CD8+ T cells associated with malignant T cell depletion. High-burden patients appeared to clinically improve but large numbers of malignant T cells persisted in skin. Clinical improvement was linked to turnover of benign T cell clones but not to malignant T cell reduction. Benign T cells were associated with the Th2-recruiting chemokine CCL18 before therapy and with the Th1-recruiting chemokines CXCL9, CXCL10, and CXCL11 after therapy, suggesting a switch from Th2 to Th1. Inflammation was correlated with OX40L and CD40L gene expression; immunostaining localized these receptors to CCL18-expressing c-Kit+ dendritic cells that clustered together with CD40+OX40- benign and CD40+CD40L- malignant T cells, creating a proinflammatory synapse in skin. Our data suggest that visible inflammation in CTCL results from the recruitment and activation of benign T cells by c-Kit+OX40L-CD40L+ dendritic cells and that this activation may provide tumorigenic signals. Targeting c-Kit, OX40, and CD40 signaling may be novel therapeutic avenues for the treatment of MF.

Introduction

Cutaneous T cell lymphomas (CTCLs) are a heterogeneous collection of non-Hodgkin’s lymphomas derived from T cells tropic for the skin. Unlike most nodal non-Hodgkin’s lymphomas, approximately 75% of primary cutaneous lymphomas are T cell derived. CTCL patients have inflammatory skin lesions infiltrated by both malignant and benign infiltrating T cells, and variable involvement of the blood, lymph nodes, and visceral organs. Mycosis fungoides (MF), the most common subtype of CTCL, malignant T cells are confined to inflammatory skin lesions (1). Phenotypic studies have suggested that MF is a cancer of nonrecirculating, skin-resident memory T cells (2, 3). Twenty percent of MF patients develop progressive, often lethal skin disease (4). Even in patients with indolent MF, lymphomatous involvement of the skin is lifelong and disease is incurable in the absence of stem cell transplantation. A better understanding of the pathogenesis of MF is needed to develop therapies that can cure or halt progression of the disease.

Psoralen plus ultraviolet A (PUVA) is an immunomodulatory therapy for skin disease in which patients ingest or topically apply 8-methoxypsoralen and are exposed to UVA light. PUVA is an effective treatment for MF and for inflammatory and autoimmune disorders including psoriasis, vitiligo, and graft-versus-host disease (5–7). PUVA induces remission in 72% of MF patients; remission can last up to 10 years with maintenance therapy and PUVA is effective in MF patients who fail to respond to narrowband UVB (1, 8, 9). The mechanisms of action of PUVA are poorly understood and it is not clear why it is effective in treating both a malignant lymphoma and inflammatory and autoimmune disorders such as psoriasis and vitiligo. To address these unanswered questions, we carried...
out translational studies using high-throughput sequencing (HTS) of T cell receptors (TCRs), NanoString-based gene expression profiling, and multiplex immunostaining of biopsies of MF skin lesions before and after PUVA therapy.

**Results**

**PUVA is an effective therapy for MF that can eradicate malignant T cells in patients with low initial tumor burden.** Fifteen patients with stage IA-IIA mycosis fungoides were treated with PUVA twice weekly for 12 to 15 weeks. Clinical severity of disease was assessed in a single index lesion using the Composite Assessment of Index Lesion Severity (CAILS) scoring system and across the entire body surface area using the Modified Severity-Weighted Assessment Tool (mSWAT) scoring system (Figure 1, A and B). Skin biopsies of a single index lesion were obtained before and after therapy and translational studies were carried out using HTS of the TCR CDR3 regions, NanoString-based gene expression profiling, and multiplex immunostaining (Figure 1C). Fourteen out of 15 patients showed reduced skin inflammation in the index lesion and reductions in overall burden of disease after PUVA therapy (Figure 1, D–F). T cell numbers as measured by HTS were significantly reduced after therapy (Figure 1G). However, the changes in the malignant T cell frequency after therapy varied among patients (Figure 1H). There was a wide range of malignant T cell frequencies before therapy but patients clustered into groups that had high or low malignant T cell frequencies after therapy (Figure 1I). We separated patients into different groups based on their initial malignant T cell frequency and found that responses were significantly different in these groups (Figure 1, J–L). Fifty percent of patients with less than 10% malignant T cell clones experienced complete eradication of the malignant T clone after PUVA. Patients with 10%–20% malignant T cells in skin had a mean 97% reduction in the frequency of malignant cells. However, patients with greater than 20% malignant T cells experienced a mean 1.5% increase in the frequency of malignant T cells. To study this in more detail, we measured the absolute number of malignant and benign T cell numbers in skin in low-burden (≤20%) and high-burden (>20%) patients by HTS. The absolute numbers (genomes per 100 ng of DNA) of total T cells, malignant T cells, and benign T cells were all significantly reduced in low-burden patients but varied widely and were not significantly different in high-burden patients (Figure 1, M and N). In summary, PUVA therapy effectively reduced or eliminated malignant T cells in low-burden but not high-burden patients.

Visible inflammation does not reflect malignant T cell burden and reduced inflammation is linked to turnover of benign T cells. We were surprised to find that clinical responses were not significantly different in high-burden and low-burden patients (Figure 2A). High-burden patients had reduced clinical inflammation scores despite the high frequencies of malignant T cells in skin after therapy (Figure 2, B–D). Patient 4 experienced complete clearance of all skin disease clinically but malignant T cells in this patient increased from 51% to 69% after treatment (Figure 2, B and C). Similar responses were seen in 3 other high-burden patients (Figure 2D). Evaluation of the benign T cells by HTS showed that there were marked shifts in the benign T cell populations in high-burden patients after therapy (Figure 2, C and D). Largely nonoverlapping populations of benign T cells, identified by their unique antigen receptors, were present before and after therapy. We correlated improvements in clinical exam scores with various T cell parameters to identify aspects that correlated with reduced visible inflammation. Improvement in inflammation correlated with turnover of the benign T cell populations (Figure 2H [mSWAT] and Supplemental Figure 1D [CAILS]; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.124233DS1) but not with reductions in the number of total T cells, malignant T cells, or total benign T cells (Figure 2, E–G [mSWAT], and Supplemental Figure 1, A–C [CAILS]). These data suggest that visible inflammation was not driven by high numbers of benign or malignant T cells in skin but instead by a specific population of benign T cell clones present before therapy, identifiable by their unique antigen receptors, that was eliminated by PUVA. PUVA also recruited a new population of benign T cells bearing distinct antigen receptors into skin.

**Benign T cells have distinct gene association profiles before and after therapy and PUVA induces a shift from Th2 to Th1 chemokine association.** We studied the expression of a set of inflammation-related genes in skin biopsies before and after PUVA therapy by NanoString. Loss of CD7 on malignant T cells can be a useful marker for distinguishing benign from malignant T cells in MF (10, 11). To identify genes in the codeset associated with benign T cells, we correlated gene expression levels of CD7, which should be expressed on benign CD4+ and CD8+ T cells but lost on malignant T cells, with genes across the data set. CD7 gene expression correlated with the number of benign T cells by HTS, validating this approach (Supplemental Figure 2). To
Figure 1. PUVA is an effective therapy for mycosis fungoides that can eradicate malignant T cells in patients with low initial tumor burden. (A–C) Design of the clinical and translational studies. (A) Fifteen patients were treated with oral 8-methoxypsoralen (8-MOP) and exposed to UVA light. (B) Severity of disease was assessed in a single index lesion using the CAILS scoring system and across the entire body surface area using the mSWAT scoring system. (C) Skin biopsies of a single index lesion were obtained before and after therapy and were studied by HTS, NanoString-based gene expression profiling, and multiplex immunostaining. (D–F) All patients improved clinically with respect to index lesion severity (D) and overall burden of disease (E). Pre-treatment. Individual patients (D and E) and aggregate clinical scores (F) are shown. (G) The number of total T cells in skin as assessed by HTS was significantly reduced after therapy but (H) changes in the malignant T cell frequency varied widely among patients. (I–L) Patients with a low initial malignant T cell burden had the best clearance of malignant T cells. (I) There was a wide range of initial malignant T cell frequencies before therapy but patients tended to cluster into either high or low residual malignant T cell groups after therapy. (J–L) Patients with a low initial malignant T cell frequency (<10% or 10%–20%) had the greatest depletion of malignant T cells. The change in the malignant T cell frequency for each patient (J), the mean reduction in malignant T cell frequency in each group (K), and the percentage of patients in each group with the indicated levels of malignant T cell reductions (L) are shown. (M and N)
PUVA reduced the absolute number of benign and malignant T cells in skin from low-burden but not high-burden patients. The absolute number of total, malignant, and benign T cells (T cell genomes/100 ng of DNA) in skin for low-burden (M) and high-burden (N) patients are shown. The mean and SEM (error bars) are shown. Differences between 2 sample groups were detected using the 1-tailed Wilcoxon-Mann-Whitney test ($P = 0.05$). For comparisons of multiple groups, a Kruskal-Wallis 1-way analysis of variance with a Bonferroni-Dunn post hoc test for multiple means testing was used ($P = 0.05$). All reported $P$ values are adjusted for multiple comparison testing.

identify genes selectively associated with benign CD8+ T cells, we correlated expression of the CD8A gene with other genes in the data set. A list of CD7- and CD8-associated genes and their $r$ and $P$ values is included in Supplemental Tables 1 and 2. Genes associated with total benign T cells had only 1 gene in common before and after PUVA, suggesting a shift in benign T cell gene expression profiles. CD8-associated genes were completely distinct before and after therapy. The number of benign T cells before therapy was tightly correlated with expression of CCL18, a Th2-recruiting cytokine ($r^2 = 0.856$, $P = 0.001$), but this association was lost after therapy (Figure 3B). After therapy, the number of benign T cells instead correlated closely with levels of the Th1-recruiting chemokines CXCL9, CXCL10, and CXCL11 ($r^2 = 0.989$, 0.905, 0.987; $P < 0.0001$, $P = 0.0035$, $P < 0.0001$, respectively; Figure 3B). These findings suggest a shift in benign T cell gene expression before and after therapy. Taken together with HTS results, these findings show that distinct populations of T cells with differing antigen receptors and gene expression profiles are present before and after PUVA therapy and that PUVA may induce a shift in benign T cells from Th2 to Th1.

CCL18 is expressed at high levels in skin (Figure 4). CCL18 was expressed at high levels but did not colocalize with CD163. Instead, CCL18 was strongly associated with the number of malignant T cells after therapy ($r = 0.932$, $P = 0.002$) and its receptor CD40 (OX40L) was strongly associated with malignant T cell number ($r = 0.932$, $P = 0.002$) and its receptor CD40 was associated with CCAI scores of clinical inflammation (Figure 5C). Three of these genes, CD3G, CCR4, and CD5, are widely expressed by both benign and malignant T cells in skin. Two genes, TNFSF4 (OX40L) and CD40 ligand (CD40L), were notable because they correlated with malignant T cell numbers (Figure 5D and Supplemental Table 3) but not with benign T cell subsets (Supplemental Tables 1 and 2). TNFRSF4 (OX40), the receptor for OX40L, was strongly associated with benign T cells before but not after PUVA (Figure 5D and Supplemental Table 1), suggesting a possible interaction between benign and malignant T cells. CD40L was strongly associated with malignant T cell number ($r = 0.932$, $P = 0.002$) and its receptor CD40 was associated with CD4+ T cell and macrophage (CD68 and CD163) but not dendritic cell (CD11c, CCL13, CCL17, CCL20).
CCL22, and HSD11B1) markers (Figure 5D and Supplemental Figure 7). These results suggest that interactions between malignant T cells, benign T cells, and a nonlymphocytic cell type may drive visible inflammation. The antigen-presenting cell–specific (APC-specific) costimulatory gene CD80 was strongly associated with benign T cells, supporting the hypothesis that benign T cells may cluster with a nonlymphoid cell type. OX40 is expressed by benign T cells and OX40L is expressed by c-Kit+ dendritic cells in MF. Stimulation of OX40 on the surface of T cells by OX40L drives chronic T cell activation and cytokine production (16). We immunostained MF skin lesions for OX40 and OX40L to explore the hypothesis that visible inflammation may be driven in part by OX40/OX40L-induced activation of benign T cells. Malignant and benign T cells can be discriminated by costaining for CD3 and the variable (V) region of the TCR β chain (TCR Vβ) expressed by the malignant T cell clone, as identified by HTS (Figure 6A) (17). OX40 was specifically and selectively expressed by benign T cells (Figure 6, B and C), in agreement with the gene association studies. However, OX40L+ cells lacked tryptase and expressed CD11c, confirming their identity as c-Kit+ dendritic cells (Figure 6F and G). OX40L+ cells were specifically and at high levels by c-Kit-expressing dermal cells but not by T cells nor by CD163-expressing macrophages (Figure 6, D and E, and Supplemental Figure 10). OX40L+ cells lacked tryptase and expressed CD11c, confirming their identity as c-Kit+ dendritic cells (Figure 6F and G).

Figure 2. Visible inflammation does not reflect malignant T cell burden and reduced inflammation is linked to turnover of benign T cells. (A) Clinical exam scores in high- and low-burden patients were not significantly different. (B) Two patients are shown in whom visible inflammation (clinical exam scores) improved but the malignant T cell clone remained high after treatment (patient 1, 68%; patient 4, 69%) or even increased (patient 4). (C) Malignant T cell frequency remained high after treatment despite the presence of large numbers of malignant T cells in skin in patient 4, a complete clinical responder. The unique TCR CDR3 sequences of each nonmalignant T cell clone were used to identify which benign T cells persisted after therapy (blue), were eliminated from skin (light green), or were recruited to skin (dark green) after therapy. Persistent benign clones were benign T cell clones that were present in skin both before and after PUVA therapy. (D) Additional patients are shown in whom the malignant T cell burden remained high after therapy despite improvement in clinical inflammation exam scores. (E–H) Improvement in inflammation is correlated with a shift in the benign T cell population but not with depletion of malignant T cells. Improvement in inflammation (mSWAT) did not correlate with reductions in the number of malignant T cells (E), total T cells (F), or benign T cells (G). However, the loss of specific T cell clones from skin and recruitment of a second, distinct T cell population was correlated with reduced inflammation as assessed by mSWAT (H) and CAILS scores (Supplemental Figure 1). Differences between 2 sample groups were detected using the 1-tailed Wilcoxon-Mann-Whitney test (α = 0.05). For correlations, a Pearson’s correlation coefficient with a 2-tailed P value is reported.
Figure 3. Benign T cells have distinct gene association profiles before and after therapy and PUVA induces a shift from Th2 to Th1 chemokine association. (A) Gene expression before and after therapy was studied by NanoString and genes associated with the benign T cell population were assessed by Pearson’s correlations with CD7 and CD8A (CD8⁺ T cells only). CD7 was strongly correlated with the number of benign T cells as measured by HTS, validating the approach (Supplemental Figure 2). Genes associated with total benign cells had only 1 gene in common and CD8-associated genes before and after therapy were completely distinct. Genes in bold font: \( r > 0.9 \), \( P < 0.001 \). (B) PUVA induces a shift from Th2- to Th1-associated chemokines. The number of benign T cells, as assessed by CD7 gene expression levels (top 2 panels), correlated strongly with the expression of the Th2-recruiting chemokine CCL18 before but not after therapy and with the Th1-recruiting chemokines CXCL9, -10, and -11 after, but not before, therapy. Similar results were seen when the number of benign T cells was assessed by HTS (bottom panel). For mRNA expression and association studies, Pearson’s correlations were used to identify genes associated with CD7 or CD8A gene counts (as measured by NanoString). Genes with \( r > 0.8 \) and \( P < 0.01 \) were considered significant. For correlations, a Pearson’s correlation coefficient with a 2-tailed \( P \) value is reported.
Supplemental Figure 4). Benign T cells expressing OX40 and malignant T cells were observed to cluster together with OX40L-expressing c-Kit+ dendritic cells (Figure 6, D–F, and Figure 7D). Clusters of T cells and c-Kit+ cells were frequent in MF but rare in healthy skin (Supplemental Figure 12).

c-Kit+ dendritic cells and malignant T cells express CD40L and CD40 is coexpressed by both malignant and benign T cells in MF. Our association studies also identified CD40L as a driver of skin inflammation. CD40L is a costimulatory molecule expressed by T cells activated through the TCR and by other cell types including mast cells (18). CD40 is expressed on APCs and is also upregulated on the surface of activated T cells, particularly on CD4+ T cells. CD40 and CD40L provide independent and distinct costimulatory signals to T cells (19, 20). We immunostained MF skin lesions for CD40 and CD40L to identify the cell types expressing these receptors. CD40L was expressed by malignant T cells and by c-Kit–expressing CD11c+tryptase+ dendritic cells (Figure 7, A and B, and Supplemental Figure 4), and CD40 was expressed by both benign and malignant T cells (Figure 7C). CD40 was not detectably expressed by macrophages or c-Kit+ cells (Supplemental Figure 11). T cells from healthy skin did not express CD40, CD40L, or OX40L (Supplemental Figure 15).

OX40/OX40L and CD40/CD40L interactions may drive inflammation via an inflammatory synapse created between c-Kit+ dendritic cells, malignant T cells, and benign T cells in MF. Malignant T cells and benign T cells frequently clustered around CCL18-expressing, OX40L+CD40L+c-Kit+ dendritic cells (Figure 7D and Supplemental Figure 12). We postulated that an inflammatory synapse may form between c-Kit+ dendritic cells, malignant cells, and benign T cells and that the resulting activation of benign cells may drive visible inflammation (Figure 7E). In this model, CCL18 secreted by dendritic cells attracts Th2 benign T cells that are then activated by OX40/OX40L dendritic cell interactions and by CD40/CD40L interactions provided by both dendritic and malignant T cells. Malignant T cells also receive a proinflammatory CD40/CD40L signal from dendritic cells and benign T cell activation may provide pro-growth signals to the tumor.

Clearance of the malignant T cell clone after PUVA is associated with recruitment of new CD8+ T cell clones that express markers of antigen-specific activation, are locally expanded, and may be tumor specific. PUVA eradicated malignant T cells from skin in 50% of the patients with less than 10% malignant T cells in skin before treatment, suggesting that the benign T cell shifts that accompany PUVA therapy may enhance tumor clearance in low-burden patients. Tumor antigens have not been identified in MF so we could not test posttreatment skin T cells for their ability to recognize the tumor. As an indirect approach, we identified T cell subsets that were strongly associated with markers of TCR-dependent, antigen-specific activation (CD40L, ITK, LCK, and ZAP-70) in gene expression studies. Before therapy, malignant but not benign T cells were strongly associated with markers of antigen-specific activation (Figure 8A). After therapy, benign T cells and CD8+ T cells in particular were strongly associated with markers of antigen reactivity. These data suggested that PUVA could bring tumor-specific T cells into the skin. If so, recruitment of T cells with new antigen specificities should be associated with tumor clearance. Indeed, the percentage of new T cell receptors recruited into the skin was strongly associated with the percentage reduction in the malignant T cell clone ($r^2 = 0.71$, $P < 0.0001$; Figure 8B). Expanded clonal populations of benign T cells were identified in the skin of MF patients treated with topical resiquimod and were associated with tumor clearance (15). Patients with a greater than 90% reduction in the malignant T cell clone had significantly more expanded benign T cell clones than low-responding patients (19 vs. 1.5, Figure 8C). In summary, markers of antigen-specific T cell activation were strongly associated with T cells recruited into the skin after PUVA, recruitment of T cells with new TCR specificities was strongly associated with clearance of malignant T cells, and expanded clonal populations of benign T cells were present in patients that greatly reduced or eradicated the clone. Our cohort included one exceptional high-burden patient who experienced 95% reduction in the malignant T cell clone after PUVA (Figure 8, D–G). This patient experienced an 85% improvement in both CAILS and mSWAT clinical scores and had a marked reduction in the number of both benign and malignant T cells in skin after PUVA (Figure 8, D and E). Although the number of benign infiltrating T cells was markedly reduced, gene expression studies showed that markers associated with CD8+ T cells (CD8A) and in particular genes associated with the activated, posttreatment CD8+ population (GZMK, KLRK1, and CD6), were increased (Figure 8, F and G). These results illustrate how recruitment of a distinct population of CD8+ T cells can be associated with malignant T cell clearance.
Discussion

PUVA is a therapy for inflammatory skin diseases that works well in both inflammatory disorders and MF, a cancer of skin-resident T cells. Our studies suggest that PUVA targets the benign T cell population in skin. Fourteen out of 15 patients clinically improved after PUVA, in agreement with prior studies. In patients with lower than 10% malignant T cell frequency, PUVA induced benign T cell shifts, malignant T cell reductions in all patients, and malignant T cell eradication in 50% of treated patients. CD8+ T cells bearing new antigen receptors were recruited into skin, clonally expanded in treated lesions, and were strongly associated with markers of antigen-driven activation and malignant T cell clearance. High-burden patients (>20%) appeared to improve clinically but the frequency of malignant T cells increased on average after PUVA. The clinical take-home message is that PUVA is effective and potentially curative in patients with a low malignant T cell burden but suppresses visible inflammation without depleting malignant T cells in high-burden patients.

High-burden patients appeared to improve but silently harbored large numbers of malignant T cells in skin, suggesting that malignant T cells do not directly cause skin inflammation. Improved skin disease was instead linked to benign T cell turnover — i.e., to what percentages of T cell clones present before therapy were eliminated from skin. By contrast, elimination of malignant T cells was tightly linked to recruitment of benign T cells bearing new TCRs into the skin. Thus, reduced inflammation reflected the migration of old benign T cells out of skin and malignant T cell depletion depended on recruitment of new T cells into it.

HTS data showed an almost complete turnover of benign T cells in skin before and after PUVA therapy and expression profiling showed that benign T cells before and after therapy had markedly different gene associations. The number of benign T cells was tightly linked to levels of the Th2-recruiting chemokine CCL18 before therapy and to the Th1-recruiting chemokines CXCL9, CXCL10, and CXCL11 after therapy, suggesting a shift from Th2 to Th1 T cells after PUVA therapy. CCL18 was expressed at levels 115-fold
higher in untreated MF as compared with healthy skin and was highest in low-burden patients in whom benign T cells outnumbered malignant T cells (Supplemental Figure 3). CCL18 is known to be elevated in MF and levels of CCL18 correlate with disease progression (12, 13).

Malignant T cells must ultimately drive skin inflammation; therapies that deplete malignant T cells lead to complete clearance of clinical inflammation and a return to normal-appearing skin (15). We identified a set of genes that were associated with the CAILS score, a measure of visible skin inflammation in the index MF lesion. Of these, OX40L and CD40L were highly correlated with the number of malignant T cells, suggesting they may link malignant T cells to inflammation.

**Figure 5.** Skin inflammation is not linked to malignant T cell number or frequency but is related to the expression of 2 malignant T cell–associated genes. (A and B) Skin inflammation (CAILS) was not correlated with the absolute number (A) or relative percentage (B) of malignant T cells in skin in pretreatment samples from 3 clinical cohorts from 3 different institutions. (C) Genes associated with visible inflammation were identified by correlating pretreatment CAILS with genes across the PUVA treatment data set. Nine genes were identified, 3 of which (CD3G, CCR4, and CD5) are widely expressed by skin T cells and 2 of which (OX40L and CD40LG) were strongly associated with the number of malignant T cells in skin as measured by HTS (D). TNFSF4 (OX40L) was strongly associated with malignant T cell numbers ($r = 0.959, P = 0.0006$) and its receptor TNFRSF4 (OX40) was strongly associated with the number of benign T cells (CD7 gene expression, $r = 0.971, P = 0.0003$). Genes in bold font: $r > 0.9, P < 0.001$. CD40 ligand was strongly associated with malignant T cell number ($r = 0.932, P = 0.0022$) and its ligand CD40 was associated with CD4+ T cell and macrophage (CD68, CD163) but not DC markers (CD11c is shown; CCL13, CCL17, CCL22, and H501181 are included in Supplemental Figure 7). For correlations, a Pearson’s correlation coefficient with a 2-tailed $P$ value is reported. Pearson’s correlations were used to identify genes associated with pretreatment CAILS, CD7, CD40 (as measured by NanoString), or malignant T cell counts (as measured by HTS). Genes with $r > 0.8$ and $P < 0.01$ were considered significant.
Figure 6. OX40 is expressed by benign T cells and OX40L is expressed by c-Kit+ dendritic cells in mycosis fungoides (MF). (A) Malignant and benign T cells can be discriminated by costaining for CD3 and the TCR Vβ expressed by the malignant T cell clone, as identified by HTS. Malignant T cells costain for CD3 and the malignant T cell TCR Vβ (yellow) and benign T cells stain only for CD3 (green). Stained cells from a patient with high-burden stage IIA are shown; a second field from the same donor is shown in the last panel. (B) OX40 is expressed by T cells in MF. A patient with high-burden stage IIA CTCL is shown. A second field from the same donor is shown in the last panel. Similar results were observed in a total of 3 donors. (C) OX40 is expressed by benign but not malignant T cells. OX40 and malignant T cell TCR Vβ antibodies stained distinct populations of T cells. A patient with high-burden stage IIA MF is shown. A second field from the same donor is shown in the last panel. Similar results were observed in a total of 3 donors. (D) OX40L is not expressed by T cells in MF. Antibodies specific for CD3 and OX40L stained distinct cell populations. A patient with low-burden stage IA MF is shown. The last panel is a higher magnification view of the same staining; similar results were observed in a total of 5 donors. (E) OX40L is expressed by c-Kit+ cells in MF. OX40L and c-Kit were colocalized. Patients with high-burden stage IB (first 3 panels) and high-burden stage IA (fourth panel) MF are shown. Similar results were
OX40L is a member of the tumor necrosis factor superfamily that is expressed by activated APCs and lymphocytes. OX40L binds to OX40 on T cells and transmits a costimulatory signal that promotes sustained T cell activation, cytokine production, division, differentiation, and survival (16). OX40 stimulation also suppresses the function and differentiation of regulatory T cells (21). OX40L/OX40 interactions prolong both antigen-specific and nonspecific activation of T cells and its net effect is to sustain chronic inflammation (16). Immunostaining demonstrated that c-Kit+ dendritic cells in MF expressed high levels of OX40L and that T cells formed clusters around OX40L+ cells. T cells and macrophages did not express OX40L. OX40 was specifically expressed by benign but not malignant T cells in MF, suggesting that c-Kit+ dendritic cells provide OX40L stimulation that may drive chronic activation of benign T cells. OX40 could also recruit or retain benign T cells near c-Kit+ dendritic cells, given the finding that OX40 signaling drives the accumulation and retention of activated CD4+ T cells in B cell follicles (22).

T cells upregulate CD40L expression after encounter with a specific antigen and its binding partner CD40 is expressed by APCs, B cells, and activated CD4+ T cells (18). CD40L on T cells can drive APC maturation and B cell class switching, and provides a costimulatory signal to other CD40-expressing T cells that is as potent as CD28 signaling (19). CD40L-expressing T cells also receive a costimulatory signal from CD40L/CD40 ligation that is important for sustaining T cell responses (20). In MF, CD40L was expressed by both c-Kit+ dendritic cells and malignant T cells and CD40 was expressed by both benign and malignant T cells. One prior study reported CD40/CD40L coexpression by malignant T cells in MF (23). However, expression of CD40L by c-Kit+ dendritic cells and CD40 by benign T cells has not been reported in MF.

Our results suggest that c-Kit+ dendritic cells can provide costimulatory signals to both malignant and benign cells via CD40L and that CD40L+ malignant T cells themselves may receive a costimulatory signal while stimulating both benign and malignant CD40-expressing T cells.

Our results show that c-Kit+ dendritic cells produce CCL18 that likely recruits benign Th2 T cells, creating an inflammatory synapse between dendritic cells, benign T cells, and malignant T cells. OX40L/OX40L and CD40/CD40L may drive the antigen-independent activation of benign T cells, creating visible skin inflammation and likely providing growth factors to the tumor. c-Kit+ dendritic cells and benign T cells may also activate malignant T cells via CD40/CD40L interactions. MF skin lesions have been reported to contain increased numbers of predominantly immature dendritic cells (24–26), but to our knowledge, c-Kit+ dendritic cells have not been reported. c-Kit+ dendritic cells are induced by Th2/Th17-polarizing stimuli and act to enhance Th2/Th17 responses and to impair Th1 responses (14). In patients with gastrointestinal stromal tumors lacking c-Kit mutations, Gleevec acted on dendritic cells to block c-Kit signaling, leading to enhanced Th1 cytokine production, activation of NK cells, and enhanced antitumor immunity (27).

Our observation that PUVA targets benign T cells clarifies how it can treat both cutaneous lymphoma and inflammatory disorders such as psoriasis. Other antiinflammatory therapies used in CTCL also inhibit benign T cell activation. Patients with leukemic CTCL are often colonized with Staphylococcus aureus strains producing superantigens that drive the activation and proliferation of benign T cells; treatment of patients with antibiotics reduces cancer progression and improves survival (29, 30). An in vitro study confirmed that bacterial toxins drove the activation and cytokine production of benign T cells that in turn stimulated malignant T cell growth (31, 32). CTCL is the only cancer treated with extracorporeal photopheresis (ECP), an immunosuppressive therapy used to treat inflammatory disorders including allograft rejection and graft-versus-host disease (33). ECP can induce dendritic cell maturation and formation of tolerogenic dendritic cells that enhance regulatory T cell numbers and function (34, 35), presumably suppressing T cell inflammation in skin; it is not clear if malignant or benign T cells are targeted. To our knowledge, CTCL is the only cancer in which the presence of tumor-infiltrating FOXP3+ regulatory T cells is protective and associated with better outcomes (36–40). Increased levels of CCL18, which we believe is responsible for recruiting protumorigenic benign
Figure 7. CD40/CD40L interactions may participate in an inflammatory synapse created between c-Kit+ dendritic cells, malignant T cells, and benign T cells in mycosis fungoides. (A) CD40L is expressed by malignant T cells. A patient with high-burden stage IIA (left 3 panels) and high-burden stage IB (right panel) are shown. (B) CD40L is also expressed by c-Kit+ dendritic cells. A patient with low-burden stage IA (left 3 panels) CTCL is shown; similar results were observed in a total of 3 donors. Costaining of CD40L+ cells with the dendritic cell marker CD11c is shown in the right panel. (C) Both benign and malignant T cells express CD40. The left 2 panels show benign T cells (CD3+Vβ–) expressing CD40. The right 2 panels show malignant T cells (CD3+Vβ+) expressing CD40. Both fields are from the same high-burden stage IIA donor; similar results were observed in a total of 3 donors. (D) An inflammatory synapse is created between dendritic cells, malignant T cells, and benign T cells. Clusters of dendritic cells, benign T cells, and malignant T cells were frequently observed in MF, as illustrated by this costain for OX40L+ dendritic cells, CD3, and the malignant TCR Vβ. A patient with high-burden stage IIB is shown; clusters of these cell types were observed in 5 donors. (E) A proposed model for c-Kit+ dendritic cell, benign T cell, and malignant T cell interactions in MF. Benign T cells are recruited by c-Kit+ dendritic cell-produced CCL18 and activated by OX40/OX40L and CD40/CD40L interactions leading to visible skin inflammation and protumorogenic signals. Dendritic cells may also directly stimulate malignant T cells via CD40/CD40L interactions. All staining was performed on pretreatment skin biopsies. Results from additional patients are shown in Supplemental Figure 13. A color blind–accessible version of this image is provided in Supplemental Figure 14. Scale bars: 50 μm.
Th2 T cells, is associated with tumor progression and worse outcomes in CTCL. Taken together, these results suggest that benign T cells provide critical growth signals to malignant T cells and that depriving malignant T cells of these signals not only makes patients look and feel better, it can decrease disease progression and improve survival.

There are drawbacks to our experimental approach and many questions remain unanswered. Because we had access only to frozen skin specimens, we could not isolate viable immune cells from skin and study them directly. Tumor antigens have not been identified in MF so we were compelled to rely on indirect measures of antigen specificity and were not able to demonstrate conclusively that responding CD8+ T cells in low-burden patients after PUVA were tumor specific. We have not identified how malignant T cells recruit and activate c-Kit+ dendritic cells and little is known about the effect of PUVA on dendritic cells in MF. The protumorigenic signals provided by benign T cell activation have not been identified. Lastly, malignant T cells before treatment are strongly associated with markers of T cell–specific activation. This suggests that malignant T cells are either driven by antigen exposure or the TCR signaling pathway is uncoupled from the TCR, as has been demonstrated in leukemic CTCL (41). These unanswered questions are the subject of ongoing studies in our laboratory.
In summary, our studies demonstrate that visible skin inflammation is driven by benign, not malignant, T cells in MF. PUVA induces shifts in the benign T cell population that both reduce visible inflammation and recruit new benign T cells capable of eradicating malignant T cells in low- but not high-burden patients. Our work provides evidence for an inflammatory synapse between malignant and benign T cells and c-Kit+ dendritic cells. Targeting c-Kit+ dendritic cell and benign T cell recruitment and activation may be novel therapeutic avenues for the treatment of MF.

Methods

**Study design and skin samples.** This is an experimental laboratory study performed on human tissue samples. Patients who were studied met the World Health Organization–European Organization for Research and Treatment of Cancer (WHO-EORTC) criteria for MF (1). Biopsy specimens from 15 stage IA-IIA MF patients before and after 12 to 15 weeks of PUVA therapy were obtained from patients treated at the Medical University of Graz. Patients received 10 mg/20 kg 8-methoxypsoralen 1 hour prior to twice weekly UVA exposure. UVA was administered below 50% of the minimal phototoxic dose at start of treatment. UVA dose was increased weekly by 0%–30%, depending on the absence or presence of erythema after preceding treatments. Evaluation of efficacy was based on improvement from baseline of the CAILS score, which evaluates changes from baseline of the treated target lesions for size, erythema, scaling, and lesion elevation and on change in baseline of the mSWAT score, which evaluates the entire skin surface for percentage involvement with a weighted score based on whether a lesion is a patch, plaque, or tumor. Lesional skin biopsies from 10 patients with stage IA-IIA MF before treatment with topical resiquimod were obtained from the University of Pennsylvania (15). Lesional skin biopsies from 16 patients with stage IA-IIA MF were obtained prior to therapy with low-dose radiation or topical steroids from patients seen at the Dana-Farber/Brigham and Women’s Cancer Center Cutaneous Lymphoma Program. Skin from healthy individuals was obtained from healthy patients undergoing cosmetic surgery procedures. Immunostaining and gene expression studies were performed using in vitro assays without blinding or randomization. Study components were not predefined.

**DNA isolation and HTS.** DNA was isolated from formalin-fixed, paraffin-embedded or frozen, OCT-embedded skin samples and studied by ImmunoSEQ (Adaptive Biotechnologies) using 100–400 ng of DNA template as previously described (17). The percentage 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. The number of malignant T cells per sample was obtained by multiplying the malignant clone frequency and the total number of productive templates. To normalize the samples based on total input DNA, we divided the number of T cells/100 ng input DNA.

**RNA isolation and quantification.** RNA was isolated from healthy skin and CTCL skin samples using the RNeasy FFPE or RNeasy Mini Kit (Qiagen), respectively, as per the manufacturer’s instructions. The expression of 770 inflammation-related genes was measured using the NanoString Human PanCancer Immune Profiling Panel. Normalization and analysis of NanoString data were carried out using nSolver software. Normalization factors were calculated based on 40 reference genes.

**Cryosection immunostaining.** Five-micrometer cryosections were cut from OCT-embedded skin samples. Slides were fixed in acetone, rehydrated in 1× TBS/saponin (Boston BioProducts), blocked with 0.017 μg/μl human IgG, and then stained with relevant antibodies in 1× TBS/saponin for 30 minutes, with 10-minute wash steps between antibodies as necessary. Slides were washed after antibody treatment and mounted in Vectashield Hard Set Mounting Medium with DAPI (Vector Labs). Tissues were imaged immediately after mounting on a Mantra Quantitative Pathology Workstation using Mantra Snap 1.0 imaging software and analyzed with inForm image analysis software (PerkinElmer). Antibodies and the concentrations used are listed in Supplemental Table 4.

**Statistical analyses.** Primary methods of data analysis included descriptive statistics (means, medians, and standard deviations). Differences between 2 sample groups were detected using the 1-tailed Wilcoxon-Mann-Whitney test (α = 0.05). For comparisons of multiple groups, a Kruskal-Wallis 1-way analysis of variance with a Bonferroni-Dunn post hoc test for multiple means testing was used (α = 0.05). All reported P values are adjusted for multiple comparison testing. For mRNA expression and association studies, Pearson’s correlations were used to identify genes associated with malignant T cells (number of cells/100 ng DNA by HTS), or with CD7 or CD8A gene counts (as measured by NanoString). Only genes with r > 0.8 and P < 0.01 were considered significant.
Study approval. All studies were performed in accordance with the Declaration of Helsinki. Written consent was obtained from all patients before study entry and sample collection. All tissues were collected with previous approval from the relevant review boards: Medical University of Graz Ethical Committee, University of Pennsylvania’s IRB, and the Dana-Farber Cancer Institute’s IRB. Translational studies were approved by the IRB of the Partners Human Research Committee. Written informed consent was provided for clinical images appearing in the manuscript.

Author contributions
RAC and PW designed the experiments. RAC supervised experiments, analyzed data, carried out statistical analyses, drafted figures, and wrote and revised the manuscript. PVG, JTO, CHY, JDC, EWS, JET, AMV, AG, ZY, and ELL carried out experiments, analyzed data, and carried out statistical analyses. JET also edited the manuscript. TSW provided expertise on gene expression analyses. JIN assisted with references. PW, JTO, and AHR designed the clinical studies, oversaw the clinical treatment of patients, and provided skin specimens. PW designed and carried out the clinical trial of PUVA in CTCL patients on which these translational studies are based.

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