Title: The Th9 axis reduces the oxidative stress and promotes the survival of malignant T-cells in cutaneous T-cell lymphoma patients

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Running Title: Th9 axis promotes T-cell survival in CTCL

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Abstract

Immune dysfunction is critical in pathogenesis of cutaneous T-cell lymphoma (CTCL). Few studies have reported abnormal cytokine profile and dysregulated T-cell functions during the onset and progression of certain types of lymphoma. However, the presence of IL-9 producing Th9 cells and their role in tumor cell metabolism and survival remain unexplored. With this clinical study, we performed multidimensional blood endotyping of CTCL patients before and after standard photo/chemo-therapy and revealed distinct immune hallmarks of the disease. Importantly, there was a higher frequency of “skin homing” Th9 cells in CTCL patients with early (T1, T2) and advanced-stage disease (T3, T4). However, advanced-stage CTCL patients had severely impaired frequency of skin-homing Th1 and Th17 cells, indicating attenuated immunity. Treatment of CTCL patients with standard photo/chemotherapy decreased the skin homing Th9 cells and increased the Th1 and Th17 cells. Interestingly, T-cells of CTCL patients express IL-9 receptor (IL-9R), and there was negligible IL-9R expression on T-cells of healthy donors. Mechanistically, IL-9/IL-9R interaction on CD3+ T-cells of CTCL patients and Jurkat cells reduced oxidative stress, lactic acidosis, and apoptosis and ultimately increased their survival. In conclusion, co-expression of IL-9 and IL-9R on T-cells in CTCL patients indicates the autocrine positive feedback loop of Th9 axis in promoting the survival of malignant T-cells by reducing the oxidative stress.

Implications: The critical role of Th9 axis in CTCL pathogenesis indicates that strategies targeting Th9 cells might harbor significant potential in developing robust CTCL therapy.
Introduction

Cutaneous T-cell lymphoma (CTCL) represents a diverse group of non-Hodgkin lymphomas derived from mature malignant T-cells that traffic to the human skin (1,2). Based on Surveillance, Epidemiology, and End Results (SEER) registry data, the incidence of CTCL is 6.4 per million individual in USA and the highest incidence has been reported among African-Americans (3). According to the World Health Organization/European Organization for Research and Treatment of Cancer (WHO-EORTC) classification, primary CTCL include multiple variants with distinct clinical manifestations (4). The pathogenesis of CTCL is not clear, however, the immune dysregulation is believed to have a vital role in the pathogenesis of lymphoma (5-7). Few studies reported the abnormal production of various cytokines during the onset of certain lymphomas (8).

Interleukin 9 (IL-9) is a pleiotropic cytokine and IL-9/IL-9 receptor (IL-9R) interaction promotes T-cell growth (9) and is responsible for diverse functions in inflammatory and immune responses (10,11). Multiple cell types including Th2, Th17, Treg, mast cells, dendritic cells and natural killer (NK)/T-cell have been reported to secrete IL-9 (12-16). Later, IL-9 producing Th9 cells, a distinct T helper (Th) subset was described. The Th9 cells are reported to be “skin tropic” as a majority of Th9 cells express cutaneous lymphocyte antigen positive (CLA+) (17) and their presence was demonstrated in the various skin diseases including psoriasis and atopic dermatitis patients (17,18). Increased levels of IL-9 were demonstrated in biopsies or sera in multiple types of peripheral T-cell lymphomas (PTCL) including adult T-cell leukemia (ATLL), anaplastic large cell lymphoma (ALCL), Hodgkin lymphoma (HL) and nasal NK/T-cell lymphoma patients (19-24). A recent study reported the presence of CD3+ IL-9 producing T-cells in the dermis and dermo-epidermal junction in mycosis fungoides (MF) skin lesions (19). However, the presence of Th9 cells as well as IL-9 production by malignant T-cells in an early and advanced-stage of CTCL patients have not been reported so far.
The role of IL-9 in tumor development depends on multiple factors including the nature of tumor and tumor microenvironment. We and others have reported the anti-tumor role of Th9 cells in a murine model of melanoma, which were mediated by promoting CD8\(^+\) cytotoxic T-cells and mast cell functions (25,26). Unlike melanoma, the pro-tumor activity of IL-9 has been reported in hematological cancers using murine models or cell lines. For example, transgenic mice over-expressing IL-9 have been shown to develop thymic lymphoma at the age of 3-9 months (27). Moreover, IL-9 stimulation of mouse thymic lymphoma cells induced by N-methyl-N-nitrosourea or by X-ray irradiation showed increased proliferative responses (28). IL-9 promoted the proliferation of human HL cell lines in dose-dependent manner, and ablation of IL-9 by IL-9 specific antisense oligomer inhibited the proliferation of HL cells (29). In primary T-cell lymphoma mouse models, IL-9/IL-9R interaction activated STAT proteins and contributed to in vivo growth of tumor (30-32). However, the roles of IL-9 in malignant T-cells survival in CTCL patients have not been probed.

We performed blood endotyping of early and advanced-stage CTCL patients (Mycosis Fungoides (MF), CD30\(^+\) lymphoproliferative disease (CD30\(^+\) LPD), subcutaneous panniculitis-like T-cell lymphoma (SPTL) and CTCL, not otherwise specified (NOS)) focusing on three major components of “skin-homing” and “systemic” T-cell mediated immune responses: 1) T-cell cytokine profile, 2) T-cell activation and 3) T-cell subsets. We demonstrated the increased frequency of “skin-homing” Th9 cells in early as well as advanced-stage CTCL. Interestingly, we observed the co-expression of IL-9 and IL-9R on malignant T-cells of CTCL patients. Functionally, IL-9/IL-9R interaction reduced the toxic reactive oxygen species (ROS) production, lactic acidosis and apoptosis, and ultimately increased the survival of malignant T-cells. In conclusion, Th9 axis promotes malignant T-cell survival by reducing the oxidative stress of T-cells.
Materials and Methods

Study protocol and blood samples

The protocol of this study was approved by the Institute Ethics Committee (IEC) of Tata Memorial Hospital and IIT Bombay and is in compliance with the Declaration of Helsinki. The clinical trial (CTRI/2017/04/008356) is registered in the Clinical Trial Registry of India (CTRI). In this study we recruited primary CTCL patients, mainly MF, CD30+ LPD, SPTL and CTCL-NOS. Table 1 describes the patient’s details, risk status, symptoms, clinical examination, staging and treatment history of CTCL patients at the baseline. In brief, de novo and previously treated adult patients (≥ 18 years) suffering from CTCL (n=17) were recruited, provided they were not on any active immunosuppression at least 4 weeks prior to the sampling. Among these, six patients of CTCL were enrolled as a follow-up study after standard photo/chemotherapy. The treatment details and patient information of follow-up patients are described in Supplementary Table S1. CTCL diagnoses were established in accordance with the WHO-EORTC classification. Good clinical practice guidelines were followed and written consent was obtained from all patients for blood sample collection. Whole blood was collected from 17 CTCL patients (10 early stage and 7 advanced-stage patients) and 19 healthy donors in BD Vacutainer® EDTA tubes.

Surface marker and intracellular cytokine analysis by flow cytometry

The PBMCs from peripheral blood were isolated by ficoll-histopaque (Sigma-Aldrich, Missouri, USA; 10771) density gradient centrifugation method. Cells (1x10^6 cells/tube) were washed in FACS staining buffer (2% FBS in PBS) and resuspended in 50 µl staining buffer. The fluorochrome-conjugated surface antibodies (CD3, CD4, CD8, CLA, CCR7, L-selectin, CD45RA, CD45RO, CD25, and CD69) were added in different combinations in tubes and mixed gently by tapping. Similarly, fluorochrome-conjugated IL-9R antibody was added to Jurkat cells (Source:...
National centre for cell science, Pune, India) and sorted T-cells. All tubes were incubated for 40 minutes in dark on ice and then washed twice with staining buffer and then resuspended in 250 µl staining buffer for acquisition by BD FACSVerse or BD FACS Aria Fusion III (BD Biosciences; CA, USA) and analyses were done using BD FACSuite software.

For intracellular staining, the PBMCs were cultured in T-cell media (Iscove's Modified Dulbecco's Medium (IMDM) with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine). PBMCs were stimulated in 1 ml of T-cell media in 24-well plate with PMA (Merck, Darmstadt, Germany; P8139, 10 ng/ml) and Ionomycin (Invitrogen, CA, USA; I24222, 500 ng/ml) in the presence of Brefeldin-A (Golgi Plug- BD Biosciences, CA, USA; 555028, 0.75 µl/ml) for 5 h at 37 °C. The PBMCs were first stained with fluorochrome-conjugated surface antibodies (CD4 and CLA) and then surface stained cells were washed twice with staining buffer and cells were resuspended in 250 µl 1X Cytofix/Cytoperm buffer (BD Biosciences, CA, USA; 555028), mixed gently and incubated at room temperature for 20 minutes. After incubation, cells were washed twice with 500 µl perm/wash buffer. Fluorochrome-conjugated antibodies for intracellular marker (IL-9, IL4, IL17, and IFNγ) were added in different combinations in the respective tubes and mixed gently by tapping in perm/wash buffer (total volume not exceeding 100 µl). All tubes were incubated for 30 minutes in dark at room temperature and then washed twice with perm/wash buffer and re-suspended in 250 µl staining buffer for acquisition by BD FACSVerse or BD FACS Aria Fusion III (BD Biosciences) and the analyses were done using BD FACSuite software.

All antibodies were procured from BD Biosciences (San Jose, CA, USA), BioLegend (San Diego, CA, USA) and Thermo Fisher Scientific (Waltham, MA, USA).

**Cytokine analysis by ELISA**
In TCR stimulation (recall assay), the PBMCs were cultured in T-cell media and were stimulated using anti-CD3/CD28 Dynabeads Human T-cell activator (Bead: cell ratio= 1:1, Gibco, MA, USA; 11131D) and incubated for 48 h. Post 48 h, IL-9 production was quantified in the 100 µl of cell free supernatant using the human IL-9 ELISA kit (Invitrogen, CA, USA; 88-7958-88) as per manufacturer’s instructions.

**T-cell sorting**

The PBMCs (1x10^6 cells/tube) were washed using FACS staining buffer (2% FBS in PBS) and resuspended in 50 µl staining buffer. The Fluorochrome-conjugated CD3 antibodies were added in tubes and mixed gently by tapping. The tubes were incubated for 40 minutes in dark on ice and then washed twice with staining buffer and then re-suspended in 250 µl staining buffer for sorting by BD FACS Aria Fusion III (BD Biosciences; San Jose, CA, USA) and purity analyses were done using BD FACSDiva software.

**Cell survival analysis**

Jurkat cells were procured from National centre for cell science, Pune, India and mycoplasma testing was performed using PCR mycoplasma detection kit (abm Inc., Canada; G238) as per manufacturer’s instruction. No additional cell line authentication was performed. Jurkat cells (0.1 million/ml) were suspended in RPMI media with 10% FBS and 1% penicillin/streptomycin and seeded in a 24 well plate. Similarly, sorted T-cells of CTCL patient and healthy donors (0.1 million/ml) were seeded in T-cell media in a 24 well plate. Cells were cultured for 72 h in the presence and absence of cytokines (IL-9: 20 ng/ml and IFNγ: 2 ng/ml). Post incubation, cells were harvested and stained for trypan blue and live cells were counted.

**Flow cytometric apoptosis assay**

0.1 million/ml Jurkat cells were seeded in 12 well plates and cultured in 5% CO₂ incubator at
37 °C for 72 h in the presence and absence of cytokines (IL-9: 20 ng/ml and IFNγ: 2 ng/ml) At the end of the incubation period, cells were harvested and washed with Annexin V binding buffer (1X). The apoptotic cells were stained using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA; 556547) as per manufacturer’s instructions and acquisition was performed by BD FACSVerse (BD Biosciences). The analyses of FACS data were done using BD FACSuite software.

**Measurement of oxidative stress**

Measurement of intracellular ROS was carried out using 2, 7-dichlorodihydrofluorescein diacetate (H₂DCF-DA), a dye that after hydrolysis by intracellular esterases reacts with superoxide, hydroxyl or oxygen radicals and forms fluorescent green product dichlorofluorescein (DCF). After 24 h treatment of cytokines (IL-9, 20 ng/ml and IFNγ, 2 ng/ml), cells were collected, washed with PBS and stained with 1µM H₂DCFDA for 30 minutes at 37 °C and ROS levels are measured by BD FACSVerse or BD FACS Aria Fusion III (BD Biosciences).

**Lactate measurement in supernatant**

Jurkat cells and sorted T-cells from patients and healthy individuals were cultured in the presence and absence of cytokines (IL-9: 20 ng/ml and IFNγ: 2 ng/ml). Post 24 h, the supernatant was collected and lactate was measured through the spectro-fluorimetric method. Lactate dehydrogenase (1 U/ml) was used to convert lactate to pyruvate, further generating NADH which was measured at 340 nm using ELISA plate reader. Incubation was carried out in 96-well ELISA plates for 1 h at 37°C. The pyruvate generated was trapped using hydrazine present in glycine-hydrazine buffer (pH -9.0), to prevent reverse production of lactate.

**Statistical analysis of individual immune features**

For studies described in Figures 1-6, comparative data were analyzed by using Student’s t-test.
The specific statistical test chosen for each experiment is mentioned in the figure legend. Prism 7.02 software was used to perform the statistical analysis. In the figures, * stands for a p-value <0.05, ** for p<0.01, *** for p<0.001 and ns for non-significant.
Results

Increased skin-homing Th9 cells in blood of CTCL patients as compared to healthy controls.

A large proportion of CLA⁺ skin resident effector T-cells are known to secrete IL-9 with distinct Th9 phenotype in healthy and inflamed skin (17). In this study, we quantified skin-homing (CLA⁺) and systemic (CLA⁻) Th1 cells (CD4⁺ IFNγ⁺ IL-9⁻ IL4⁻ IL17⁻), Th2 cells (CD4⁺ IL4⁺ IFNγ⁻ IL-9⁻ IL17⁻), Th9 cells (CD4⁺ IL-9⁺ IFNγ⁻ IL4⁻ IL17⁻) and Th17 cells (CD4⁺ IL17⁺ IL4⁻ IFNγ⁻ IL-9⁻) in primary CTCL (early and advanced) patients. Figure 1A represents the gating strategy to quantify the skin-homing (CLA⁺) and systemic (CLA⁻) Th9 cells and Th1 cells. Similar strategies were used for gating Th2 and Th17 cells. In early as well as advanced-stage CTCL patients, there was an increased frequency of skin-homing (CLA⁺) Th9 cells as compared to healthy donors (Figure 1D). However, higher numbers of systemic (CLA⁻) Th9 cells were found in advanced-stage CTCL patients as compared to healthy donors (Figure 1H).

There was a significantly lower frequency of skin-homing Th1 cells (Figure 1B) and skin-homing Th17 cells (Figure 1E) in advanced-stage CTCL patients as compared to healthy donors. There was no difference in the frequency of systemic Th1 cells (Figure 1F), skin-homing and systemic Th2 cells (Figure 1C and 1G) and systemic Th17 cells (Figure 1I) among healthy donors and CTCL patients. Collectively, this data suggest that advanced CTCL patients have attenuated skin immunity (Th1 and Th17 responses), and Th9 cells are increased in early and advanced-stage CTCL patients.

CTCL patient derived T-cells secrete increased levels of IL-9 and express high levels of IL-9R on their surface

Th9 cells are the major source of IL-9, which signals through a γC family receptor (IL-9R) on target cells. We examined if patient derived T-cells secrete increased levels of IL-9 upon T-cell
activation. The IL-9 production was quantified by ELISA in the cell-free culture supernatant of PBMCs isolated from CTCL patients and healthy subjects upon TCR stimulation using anti-CD3/CD28 coated dynabeads. Similar to flow cytometry data, IL-9 production was higher in CTCL patients as compared to healthy donors (Figure 2A).

Next, we examined the surface expression of IL-9R on malignant T-cells. CD3⁺T-cells were sorted from CTCL patients as well as healthy individuals. We observed a higher expression of IL-9R on CTCL patient T-cells and negligible expression of IL-9R on healthy T-cells (Figure 2B). Similarly, there was increased IL-9R expression on Jurkat cells (a T-cell lymphoma cell line). This data collectively suggest the co-expression of IL-9 and IL-9R expression on malignant T-cells.

**IL-9 promotes the survival of T-cells of CTCL patients and Jurkat cells**

We further examined the functional relevance of the co-expression of IL-9 and IL-9R on malignant T-cells. IL-9 promoted T-cell survival of CTCL patients, Jurkat cells and had no impact on healthy T-cells (Figure 3A). Since IFNγ is known to be pro-apoptotic and anti-proliferative (34) and has the ability to induce oxidative stress (35) in various cancer cells, we also examined the role of IL-9 in IFNγ milieu to further strengthen our observation. IFNγ inhibited the cell survival and IL-9 reversed the IFNγ mediated inhibition of cell survival in CTCL patients and Jurkat cells (Figure 3A). Next, we examined the mechanism of IL-9 mediated increase in tumor cell survival. IL-9 reduced the Jurkat cell apoptosis as quantified by Annexin-V/7-AAD staining through flow cytometry (Figure 3B).

**IL-9 reduces oxidative stress and lactic acidosis of T-cells of CTCL patients and Jurkat cells**

Since reduced toxic ROS levels are described to promote healthy T-cell survival (33), we examined if IL-9/IL-9R interaction impacts metabolic alterations, which would be beneficial for the survival of malignant T-cells. Sorted CD3⁺ T-cells from CTCL patients and healthy donors
were cultured in the presence and absence of IL-9 and/or IFNγ and ROS levels were quantified by DCFDA staining using flow cytometry. Interestingly, IL-9 alone and/or in presence of IFNγ significantly reduced the ROS levels inside the T-cells of CTCL patients and Jurkat cells, however there was no significant reduction in ROS levels in healthy T-cells (Figure 4A).

Since lactic acidosis promotes ROS release in cancer cells and increases oxidative stress inside the cells (36), we quantified extracellular lactate in cell-free supernatant of CTCL patients, Jurkat cells and healthy donors. IL-9 decreased the extracellular lactate concentration in T-cells of CTCL patients and Jurkat cells, however, had no impact on lactate production in healthy donors (Figure 4B). Collectively this data suggest that IL-9 promotes cell survival by reducing the intracellular toxic ROS levels, lactic acidosis and apoptosis.

**T-cell subsets and immune activation profile of CTCL patients and healthy donors**

An earlier report shows increased T<sub>MM</sub> in few CTCL patients and depletion of T<sub>MM</sub> cells in both the circulation and the skin of CTCL patients treated with alemtuzumab (37). Very recently, migratory memory T-cells (T<sub>MM</sub>) were demonstrated to be the connecting link between skin and lymph nodes and critical to the pathogenesis of L-CTCL, a malignancy of central memory T-cells (T<sub>CM</sub>) (38). Here, we quantified the relative percentage of T<sub>MM</sub> and T<sub>CM</sub> in CTCL patients as compared to healthy donors. There was an increase in percentage of skin-homing (CLA<sup>+</sup>) as well as systemic (CLA<sup>-</sup>) T<sub>MM</sub> (CCR7<sup>+</sup> L-Selectin<sup>-</sup>) in CTCL patients as compared to healthy donors. However, we observed increased frequency of skin-homing T<sub>CM</sub> (CCR7<sup>-</sup>L-Selectin<sup>+</sup>) in advanced-stage CTCL patients as compared to healthy donors (Figure 5A-D). In addition, decreased numbers of CD4<sup>+</sup> naïve T-cells (CD4<sup>+</sup> T<sub> naïve</sub>) and increased CD4<sup>+</sup> effector T-cells (CD4<sup>+</sup> T<sub> eff</sub>) were found in CTCL patients as compared to the healthy donors (Figure 5E and 5F). However, there was comparable frequency of CD8<sup>+</sup> naïve T-cells (CD8<sup>+</sup> T<sub> naïve</sub>) and CD8<sup>+</sup> effector
T-cells (CD4+ Teff) in CTCL patients and healthy donors (Figure 5G and 5H).

To understand the activation state of T-cells (CD4+/CD8+) in CTCL patients as compared to healthy donors, the expression and frequency of CD69+ and CD25+ T-cells were quantified. CD69 is an activation marker which appears very early while CD25 appears a bit late on the surface of lymphocyte’s plasma membrane. Interestingly, the frequency of CD8+CD69+CD25− T-cells was higher in CTCL patients as compared to healthy donors (Figure 5K) and no difference was observed in the frequency of CD4+CD69+CD25− T-cells CTCL patients as compared to healthy donors (Figure 5I). Further, CD69− CD25+ T-cells (CD4+ & CD8+) frequency was higher in CTCL patients as compared to healthy donors (Figure 5J and 5L). This data indicate that CTCL showed increased early activation in CD8+T-cells.

Patients under standard photo/chemotherapy exhibit attenuated skin-homing Th9 cells and re-established Th1 and Th17 immunity

Finally, we examined the cytokine profile in six follow-up CTCL patients (MF early-stage (n=2), CD30+ LPD advanced-stage (n=1) and SPTL advanced-stage (n=3)) who were under standard photo/chemotherapy. The treatment and patients’ clinical details are provided in Supplementary Table S1. Interestingly, in these patients there was a significant reduction in the frequency of skin-homing Th9 cells. However, the frequency of Th1 and Th17 cells significantly increased upon treatment in follow-up patients (Figure 6A).

Discussion

With this study, we have observed a distinct cytokine profile in early and advanced-stage CTCL
patients. Early-stage CTCL patients had an increased frequency of skin-homing Th9 cells and comparable numbers of other effector Th cells as compared to the healthy individuals. In contrast, patients with advanced disease had increased frequency of Th9 cells (skin-homing and systemic) and impaired frequency of skin-homing Th1 and Th17 cells. This indicates that skin-homing Th9 cells may play a role in initiation as well as in maintenance of the disease in CTCL. The disease progression (advanced-stage CTCL: T3, T4) led to an impaired anti-tumor immunity, which might have contributed to the infection and persistence of lymphoma in these patients. In addition, there was elevated IL-9 production in CTCL patients as compared to healthy donors. Co-elevation of Th9 frequency and IL-9 production indicate that Th9 cells are the major source of IL-9 in CTCL patients. A recent study has reported the presence of CD3+ IL-9 producing T-cells in skin biopsy of MF lesion, one of the major subtypes of CTCL (19). In this study, we have demonstrated the presence of skin-homing Th9 and elevated IL-9 production across different types of CTCL including MF, CD30+ LPD, SPTL and CTCL-NOS. Importantly, we observed attenuated Th1 immunity in a large cohort of patients with advanced disease. Further, there was an increase in Th1 cell frequency upon photo/chemotherapy treatment. A recent study has reported that increase in Th1 response reduces the malignant T-cell burden (39). Our observation of impaired Th17 in advanced CTCL directly correlates with a previous study where deficiency of RORγ, a transcription factor required for differentiation of IL-17, was shown to promote rapid development of T-cell lymphoma (40). Finally, we observed the reversal of cytokine profile, increase in frequency of skin-homing Th1 and Th17 cells as well as a significant decrease in Th9 cells upon standard photo/chemotherapy in a subset of follow-up CTCL patients. Collectively, this reveals the interplay of cytokines in pathogenesis of CTCL and posits that strategies promoting the Th1 and Th17 responses and inhibiting Th9 cells might be beneficial and effective in treating CTCL.

Next, we demonstrated the role of IL-9 in tumorigenesis and delineated the underlying
mechanisms. Our study revealed the co-expression of IL-9 and IL-9R on patient derived T-cells, indicating the presence of autocrine role of IL-9 on tumor cells. Various studies, using cell lines and murine models, have reported the roles of IL-9 in the pathogenesis of different cancers including lung cancer, breast cancer, thyroid cancer and leukemia (41-44).

We observed that IL-9 significantly decreased the rate of apoptosis and ROS levels (oxidative stress) inside the T-cells of CTCL patients, thus indicating the role of IL-9 in maintaining a steady-state ROS. Concurrently, IL-9 increased the malignant T-cell survival, which ultimately resulted in high cell numbers. The state of redox balance in CTCL and the effect of IL-9 in regulating ROS were not known previously. The ROS are chemically reactive oxygen-containing species that are generated directly or indirectly from free oxygen. In cancer cells, ROS is relatively higher as compared to the normal cells, which help them in inducing tumorigenesis (45). However, ROS generation higher than the steady-state level can be toxic even for the cancer cells (46). Once the balance is disturbed, it can lead to oxidative stress inside the cells. Our data indicates the role of IL-9 in maintaining ROS levels in CTCL to promote the malignant T-cell survival. Rapid increases in intracellular ROS may lead to cellular transformation, DNA damage and activation of p53, which can ultimately lead to apoptosis (46). ROS levels have been shown to be elevated in various cell types when the extracellular environment is rich in lactate (36). The decrease in ROS thus could be due to the less acidic environment (reduced lactate levels) generated by IL-9. We also observed that IL-9 stimulation reduced the extracellular lactate levels in the T-cell culture supernatant of CTCL patients, indicating less acidosis. This could be due to the fact that Warburg effect is energetically inefficient as compared to oxidative phosphorylation, and could limit the tumor growth in glucose depleted environment, where the cells would then start utilizing lactate as an alternative nutrient source (47,48). Thus, the alteration in redox balance caused by elevated IL-9 helps in the overall survival and maintenance of
malignant T-cells in CTCL, generating a protumor response. These findings suggest a possible role of IL-9 in reducing the oxidative stress, which otherwise could be detrimental for the cancer cells and can lead to apoptosis instead of helping in tumorigenesis. Another interesting observation was identifying the unique immune hallmarks of CTCL (summarized in Figure 6B). In addition to cytokine profile, we examined the skin-homing and systemic immune-phenotyping focusing on T-cell subsets (T_{naive}, T_{eff}, T_{MM} and T_{CM}) and T-cell (CD4/CD8) activation profile (CD69/CD25).

Surprisingly, in our study, we have found an increase in skin-homing as well as systemic T_{MM} cells in CTCL as compared to healthy donors. In addition, there was decreased frequency of CD4^{+} T_{naive} and increased frequency of CD4^{+}T_{eff} in CTCL patients as compared to healthy donors. Interestingly, the frequency of CD8^{+}CD69^{+}CD25^{+} T-cells was higher in CTCL patients and percentage of CD69^{+} CD25^{+} T-cells (CD4^{+} & CD8^{+}) was higher in CTCL patients as compared to healthy donors.

In conclusion, this study provides first evidence of the presence of increased skin-homing Th9 cells and elevated IL-9 in CTCL. Moreover, it demonstrates the autocrine positive feedback loop of Th9 axis in CTCL and delineates the role of IL-9 in reducing the oxidative stress and rate of apoptosis to promote the survival of malignant T-cells (graphical abstract: Figure 6C). Strategies targeting Th9 cells and inhibition of IL-9 may harbor significant potential in the development of novel effective therapeutics for these difficult-to-treat malignancies.

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References


Authors' Contributions

R.P. and H.J. conceptualized and supervised the project. R.P. and S.K. designed the protocol and experiments. H.J., N.S., M.S., E.S., A.B., J.T., P.T., T.S., S.G., B.B. and S.L. recruited patients and provided the blood samples. S.K., S.M., and A.K. collected the sample from TMH. S.K. performed PBMCs isolation, Surface and intracellular staining, Sample acquisition, T-cell sorting, Gating and ELISA. S.K. and B.D. performed lactate assays, measurement of ROS and cell viability analysis. B.D. performed flow cytometric apoptosis assay. S.M., S.B., A.K. and A.D. helped in performing experiments. S.K. performed data analysis. S.K. prepared the figures. S.K., B.D. and R.P. interpreted the results and wrote the manuscript.
Figure 1: Increased skin homing Th9 cells in CTCL patients with early and advanced stage disease: Human peripheral blood mononuclear cells (PBMCs) of CTCL patients (early (T1, T2): n=10, advanced disease (T3, T4): n=7) and healthy individuals (n=19) were stimulated with PMA/ionomycin in presence of brefeldin-A and stained as described in materials and methods. (A) Representative dot plots and gating strategy for identification of Th9 and Th1 cells are depicted. (B-I) Cumulative data of skin homing (CLA+: B-E) and systemic (CLA−: F-I) Th9 cells, Th1 cells, Th2 cells and Th17 cells are shown. Bar plots represent means ± standard error of mean. Statistical significance was determined as compared to healthy donors by Student unpaired t test; P-values are designated as ***<0.001, **<0.01, *<0.05.

Figure 2: T-cells of CTCL patients produce increased levels of IL-9 and express high levels of IL-9R: (A) Isolated PBMCs from healthy donors (n=23) and CTCL patients (n=11; 6 early and 5 advanced disease) were stimulated using anti-CD3/CD28 coated Dynabeads (Bead to cell ratio; 1:1). Post 48 h incubation, IL-9 production was quantified by ELISA in the cell free supernatant as per manufacturer instruction. Bar plots represent means ± standard error of mean. Statistical significance was determined by comparing with healthy using Student unpaired t test. (B) Sorted CD3+ T-cells from CTCL patients (n=3) and healthy donors (n=3) and Jurkat cells (n=3) were stained for IL-9R and analyzed by flow cytometry. Representative histogram (upper panel) for CTCL, healthy and Jurkat cells are shown and cumulative data is depicted in the lower panel. Statistical significance was determined by paired t test. P-values are designated as ***<0.001, **<0.01, *<0.05.

Figure 3: IL-9 promotes survival of T-cells of CTCL patient and Jurkat cells and reduces the apoptosis in Jurkat cells: CD3+ sorted T-cells from CTCL patients and healthy donors and Jurkat cells were cultured in presence of cytokines (IL-9 and/or IFNγ) or left untreated (control). (A)
Numbers of live cells were quantified to assess the cell survival (n=3). (B) Representative images of Jurkat cells apoptosis after IFNγ and IL-9 treatment for 72h. Apoptotic cells were stained for Annexin and 7-AAD and analyzed by flow cytometry. Live cells are shown in the lower left quadrant (7-AAD−/Annexin−); early apoptotic cells are shown in the lower right quadrant (7-AAD−/Annexin+); apoptotic cells are shown in the upper right quadrant (7-AAD+/Annexin+) and upper left quadrant (7-AAD+/Annexin−). Percentage of dead cells (7-AAD+/Annexin+ and 7-AAD+/Annexin−) were plotted and cumulative data of four individual experiments are depicted. Statistical significance was determined by comparing with control using Student paired t test; P-values are designated as ***<0.001, **<0.01, *<0.05.

Figure 4: IL-9 reduces toxic ROS levels and lactic acidosis of T-cells of CTCL patient and Jurkat cells: CD3+ sorted T-cells from CTCL patients and healthy donors and Jurkat cells were cultured in presence of cytokines (IL-9 and/or IFNγ) or left untreated (control). (A) ROS levels were quantified by H2DCFDA staining using flow cytometry. Upper panel depicts a representative histogram for each condition. Lower panel demonstrate the cumulative data of CTCL patients (n=4), healthy donors (n=3) and Jurkat cells (n=8). (B) Lactate concentration was measured in cell-free supernatant upon appropriate cytokine treatment as shown in figure in CTCL (n=4), healthy donors (n=4) and Jurkat cells (n=5). Bar plots represent means ± standard error of mean. Statistical significance was determined by comparing with control using Student paired t test; P-values are designated as ***<0.001, **<0.01, *<0.05.

Figure 5: T-cell subsets and immune activation profile of CTCL patients and healthy donors: Human PBMCs isolated from CTCL patients (early (T1, T2): n=9, advanced disease (T3, T4): n=6) and healthy donors (n=19) were stained for surface markers (CD3, CD4, CD8, CD45RA,
CD45RO, CLA, CCR7, L-Selectin, CD69 and CD25) as described in materials and methods. (A-L) Cumulative data of skin homing T\textsubscript{MM} (CD4\textsuperscript{+} CLA\textsuperscript{+} CCR7\textsuperscript{+} L-Selectin\textsuperscript{−}) (A), systemic T\textsubscript{MM} (CD4\textsuperscript{+} CLA\textsuperscript{−} CCR7\textsuperscript{+} L-Selectin\textsuperscript{−}) (B), skin homing T\textsubscript{CM} (CD4\textsuperscript{+} CLA\textsuperscript{+} CCR7\textsuperscript{+} L-Selectin\textsuperscript{+}) (C), systemic T\textsubscript{CM} (CD4\textsuperscript{+} CLA\textsuperscript{−} CCR7\textsuperscript{+} L-Selectin\textsuperscript{+}) (D), CD4\textsuperscript{+} T\textsubscript{naive} cells (CD4\textsuperscript{+} CD45RA\textsuperscript{+} CD45RO\textsuperscript{−}) (E), CD4\textsuperscript{+} T\textsubscript{eff} cells (CD4\textsuperscript{+} CD45RO\textsuperscript{+} CD45RA\textsuperscript{−}) (F), CD8\textsuperscript{+} T\textsubscript{naive} cells (CD8\textsuperscript{+} CD45RA\textsuperscript{+} CD45RO\textsuperscript{−}) (G), CD8\textsuperscript{+} T\textsubscript{eff} cells (CD8\textsuperscript{+} CD45RO\textsuperscript{+} CD45RA\textsuperscript{−}) (H), CD4\textsuperscript{+} CD69\textsuperscript{+} CD25\textsuperscript{−} T-cells (I), CD4\textsuperscript{+} CD69\textsuperscript{−} CD25\textsuperscript{+} T-cells (J), CD8\textsuperscript{+} CD69\textsuperscript{+} CD25\textsuperscript{−} T-cells (K) and CD4\textsuperscript{+} CD69\textsuperscript{−} CD25\textsuperscript{+} T-cells (L) are shown for healthy donors and CTCL patients. Bar plots represent means ± standard error of mean. Statistical significance was determined by Student unpaired \(t\) test; \(P\)-values are designated as \(***<0.001, **<0.01, *<0.05.\)

**Figure 6: Cytokine profile after standard photo/chemotherapy, immune hallmarks of disease and graphical abstract of the study.** CTCL Patients were treated with standard photo/chemotherapy as described in Supplementary table S1. (A) Skin homing (CLA\textsuperscript{+}) and systemic (CLA\textsuperscript{−}) Th1, Th2, Th9 and Th17 cells were quantified. Cumulative data of 6 follow-up patients are depicted. Statistical significance was determined by Student paired \(t\) test; \(P\) values are shown. (B) Summary of blood endotyping represents hallmarks of immune features in CTCL. (C) Graphical summary depict the role of Th9 cells in CTCL patient and healthy individual.
**Table 1: Patients information**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Risk Status</th>
<th>Symptoms</th>
<th>Clinical Examination</th>
<th>Staging</th>
<th>Treatment history</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>M</td>
<td>MF</td>
<td>T2 A NO M0, Stage I B</td>
<td>Lymph Node swelling and Rash</td>
<td>PS-1, No Lymphadenopathy, No organomegaly</td>
<td>PET CT scan - No nodal disease, Bone Marrow - Not Done, CSF - Not Done</td>
<td>PUVA</td>
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<tr>
<td>2</td>
<td>23</td>
<td>F</td>
<td>MF</td>
<td>T2 N0 M0, Stage I B</td>
<td>Pruritus</td>
<td>PS-1, No Lymphadenopathy, No organomegaly, Skin; Patches over trunk, thighs, Upper Limbs</td>
<td>CT Scan- Multiple subcm Retroperitoneal positive, Bone Marrow - Not Done, CSF - Not Done</td>
<td>PUVA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0, Stage I B</td>
<td>Excessive Dryness, Occasional itching</td>
<td>PS-1, No Lymphadenopathy, No organomegaly,</td>
<td>No staging done. Biopsy showed no evidence of Lymphoma</td>
<td>Off protocol. Referred to dermatologist and cardiologist opinion.</td>
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<tr>
<td>4</td>
<td>53</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0 B0, Stage I B</td>
<td>Erythematous Plaques on chest abdomen and thighs present.</td>
<td>PS-1, No Lymphadenopathy, No organomegaly.</td>
<td>PET/CT - Not Done, Bone Marrow- Uninvolved, CSF- Not Done</td>
<td>On observation</td>
<td></td>
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<tr>
<td>5</td>
<td>61</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0 B0, Stage I B</td>
<td>Itching, Macules in bilateral hands, nape of neck, anterior chest wall, forehead.</td>
<td>PS-1, No Lymphadenopathy, No organomegaly.</td>
<td>USG abdomen-No disease, Bone Marrow- Uninvolved, CSF-Not Done</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>31</td>
<td>F</td>
<td>MF</td>
<td>T1 N0 M0, Stage I A</td>
<td>Hypo pigmented Patches on trunk, limb and abdomen</td>
<td>PS-1, No Lymphadenopathy, No organomegaly.</td>
<td>CT-Norma, BM- Not done, CSF- Uninvolved</td>
<td>Referred to derma unit</td>
<td></td>
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<tr>
<td>7</td>
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<td>M</td>
<td>MF</td>
<td>T1 A NO M0 Stage I A</td>
<td>Swelling in right forearm</td>
<td>PS-1, No Lymphadenopathy, No organomegaly.</td>
<td>CT- Right paratracheal and subcarinal lymph nodes. BM- Likely to be uninvolved. CSF- Not done</td>
<td>Observation</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>F</td>
<td>MF</td>
<td>T2 A NO M0, Stage I B</td>
<td>Hyper pigmented patch seen in the left breast, erythematous patch seen on the left chest wall, depigmented patch seen on the lower abdominal wall, legs (popliteal fossa)</td>
<td>PS1, No peripheral lymphadenopathy, No hepatosplenomegaly chest clear. Macular lesions, &gt;10% BSA</td>
<td>PET/CT - Not Done, Bone Marrow- Not Done, USG Abdomen- Not nodes.</td>
<td>Patient is taking PUVA therapy</td>
<td></td>
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<tr>
<td>9</td>
<td>32</td>
<td>F</td>
<td>MF</td>
<td>HPR not suggestive of Mycosis Fungoides</td>
<td>White patch, non-itchy and non-painful</td>
<td>Extensive macular, scaly lesion all over the body involving chest, back, abdomen, B/L upper limb and lower limb, No lymphadenopathy. No organomegaly.</td>
<td>PET/CT/CT- Not Done. USG Abdomen Pelvis: No abnormality detected. Bone marrow - Not done. HPR not suggestive of Mycosis Fungoides.</td>
<td>Patient is under observation</td>
<td></td>
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<td>10</td>
<td>55</td>
<td>F</td>
<td>MF</td>
<td>T2 A NO M0, Stage I B</td>
<td>--</td>
<td>PS-1, No Lymphadenopathy, No organomegaly</td>
<td>PET Scan- B/L Cervical Nodes, B/L Axillary nodes, external iliac nodes, B/L inguinal nodes</td>
<td>Chemotherapy- Gemcitabine. Changed to CEOP after cycle of Gemcitabine.</td>
<td></td>
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<tr>
<td>11</td>
<td>68</td>
<td>F</td>
<td>MF</td>
<td>T3 N0 M0 Stage II B</td>
<td>Red patches in B/L upper limb, abdomen , chest, lower limbs, nodular swelling in lower abdomen and right inguinal region</td>
<td>PS-3, No Lymphadenopathy, No organomegaly</td>
<td>Not done</td>
<td>TSET</td>
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<tr>
<td>12</td>
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<td>M</td>
<td>MF</td>
<td>T3 N0 M0</td>
<td>Multiple maculopapular</td>
<td>PS-1, No Lymphadenopathy, No organomegaly</td>
<td>PET-Not available, Marrow-</td>
<td>Gemcitabine Pall intent</td>
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<tr>
<td>ID</td>
<td>Age</td>
<td>Gender</td>
<td>Diagnosis</td>
<td>Stage</td>
<td>Lesions</td>
<td>PS</td>
<td>Involvement</td>
<td>Treatment</td>
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<tr>
<td>13</td>
<td>57</td>
<td>F</td>
<td>MF</td>
<td>B0, Stage II B</td>
<td>Lesion on left side of the lip with desquamation and serous discharge.</td>
<td>PS-2, Axillary Lymphadenopathy, Diffuse erythroderma and scaling</td>
<td>PET- NA, BM-NA, CSF-NA</td>
<td>INF plus MTX and RT opinion on TSET</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>31</td>
<td>F</td>
<td>SPTL</td>
<td>T3B N0 M0</td>
<td>Fever, subcutaneous edema over B/L upper and lower limbs, multiple nodules palpable over left cervical, B/L 15upper limbs, B/L lower limb, back, anorexia</td>
<td>PS-2, No Lymphadenopathy, Splenomegaly</td>
<td>PET- B/L axillary nodes, B/L external iliac nodes, B/L inguinal nodes, retroperitoneal nodes, BM- Uninvolved, CSF- Not Done</td>
<td>6 cycles CHOP from 7/07/2018</td>
<td></td>
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<tr>
<td>15</td>
<td>63</td>
<td>M</td>
<td>CTCL :NOS</td>
<td>T3 N0 M0 B0, Stage II B</td>
<td>Ulcerative lesion on right axilla, reddish nodular lesion over left lower limb</td>
<td>PS- 1, No Lymphadenopathy, No organomegaly</td>
<td>CT- Right Axillary region, distal left thigh, left inferior thyroid, mesenteric nodes</td>
<td>Oral MTX</td>
<td></td>
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<tr>
<td>16</td>
<td>49</td>
<td>F</td>
<td>CD30+ LPD</td>
<td>T4 N1 M0, Stage III A</td>
<td>Fever, weight Loss, Pruritus, Lymph Node swelling</td>
<td>PS- 1, Lymphadenopathy- B/L axillary nodes, No organomegaly, Skin; Diffuse erythroderma, extensive scaling and plaques</td>
<td>PET CT Scan- B/L axillary nodes : 2.4*1.4 cm, Inguinal node, Bone Marrow - Not Done, BM- Not Done</td>
<td>Chemotherapy-Methotrexate</td>
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<tr>
<td>17</td>
<td>45</td>
<td>M</td>
<td>Cutaneous ALCL</td>
<td>T3 N0 M0, Stage II B</td>
<td>Skin Lesions on chest wall</td>
<td>PS-0, No Lymphadenopathy, No organomegaly.</td>
<td>CT/PET- Not Done, BM- Not Done, CSF- Not Done</td>
<td>Observation</td>
<td></td>
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</tbody>
</table>
A. Gating Strategy

B. CLA− Th1 cells
C. CLA− Th2 cells
D. CLA+ Th9 cells
E. CLA+ Th17 cells

F. CLA− Th1 cells
G. CLA− Th2 cells
H. CLA− Th9 cells
I. CLA− Th17 cells

Figure 1
Figure 2

A.

IL-9 (pg/ml)/10^6 cells

Healthy | Total | Early | Advanced

CTCL

B.

CTCL patients

Healthy donors

Jurkat cells

IL-9R

Count

Fluorescence Intensity

Isotype | IL-9R

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. CLA+ (Skin homing) and CLA- (Systemic) T cell frequencies in Th1, Th2, Th9, and Th17 cells.

B. Table showing the cytokine profile and skin homeing of CTCL and hallmarks of immune features in CTCL.

C. Diagram showing the differences between healthy and CTCL states.
The Th9 axis reduces oxidative stress and promotes the survival of malignant T-cells in cutaneous T-cell lymphoma patients

Sushant Kumar, Bhavuk Dhamija, Soumitra Marathe, et al.

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