The Th9 Axis Reduces the Oxidative Stress and Promotes the Survival of Malignant T Cells in Cutaneous T-Cell Lymphoma Patients

Sushant Kumar, Bhavuk Dhamija, Soumitra Marathe, Sarbari Ghosh, Alka Dwivedi, Atharva Karulkar, Neha Sharma, Manju Sengar, Epari Sridhar, Avinash Bonda, Jayashree Thorat, Prashant Tembhare, Tanuja Shet, Sumeet Gujral, Bhausaheb Bagal, Siddhartha Laskar, Hasmukh Jain, and Rahul Purwar

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 IL9-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/chemotherapy and revealed distinct immune hallmarks of the disease. Importantly, we found a higher frequency of “skin homing” Th9 cells in CTCL patients with early (T1 and T2) and advanced-stage disease (T3 and 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 IL9 receptor (IL9R), and there was negligible IL9R expression on T cells of healthy donors. Mechanistically, IL9/IL9R 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, coexpression of IL9 and IL9R 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). On the basis of Surveillance Epidemiology and End Results registry data, the incidence of CTCL is 6.4 per million individual in the United States 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).
a dose-dependent manner, and the ablation of IL9 by IL9-specific antisense oligomer inhibited the proliferation of Hodgkin lymphoma cells (29). In primary T-cell lymphoma mouse models, IL9/IL9R interaction activated STAT proteins and contributed to in vivo growth of tumor (30–32). However, the roles of IL9 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, 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: (i) T-cell cytokine profile, (ii) T-cell activation, and (iii) 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 coexpression of IL9 and IL9R on malignant T cells of CTCL patients. Functionally, IL9/IL9R 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 in CTCL patients.

**Materials and Methods**

**Study protocol and blood samples**

The study protocol was approved by the Institute Ethics Committee of Tata Memorial Hospital and IIT Bombay and in compliance with the Declaration of Helsinki. The clinical trial (CTRI/2017/04/008356) is registered in the Clinical Trial Registry of India. In this study we recruited primary CTCL patients, mainly mycosis fungoides, 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, 6 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 peripheral blood mononuclear cell (PBMC) from peripheral blood were isolated by ficoll-histopaque (Sigma-Aldrich; 10771) density gradient centrifugation method. Cells (1 × 10⁶ 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, α-selectin, CD45RA, CD45RO, CD25, and CD69) were added in different combinations in tubes and mixed gently by tapping. Similarly, fluorochrome-conjugated IL9R 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 FACSVersa or BD FACS Aria Fusion III (BD Biosciences) 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 with 10% heat-inactivated 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; P8139, 10 ng/mL) and ionomycin (Invitrogen; I24222, 500 ng/mL) in the presence of Brefeldin-A (Golgi Plug- BD Biosciences; 555028, 0.75 µL/mL) for 5 hours 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 1 × Cytofix/Cytoperm Buffer (BD Biosciences; 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 (IL9, 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 resuspended 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, BioLegend, and Thermo Fisher Scientific.

**Cytokine analysis by ELISA**

In T-cell receptor (TCR) stimulation (recall assay), the PBMCs were cultured in T-cell media and were stimulated using anti-CD3/CD28 Dynabeads Human T-cell activator (Read: cell ratio = 1:1, Gibco; 11131D) and incubated for 48 hours. Post 48 hours, IL9 production was quantified in the 100 µL of cell-free supernatant using the human IL9 ELISA Kit (Invitrogen; 88-7958-88) as per the manufacturer’s instructions.

**T-cell sorting**

The PBMCs (1 × 10⁶ 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 resuspended in 250 µL staining buffer for sorting by BD FACS Aria Fusion III (BD Biosciences) 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 (Applied Biological Materials Inc.; G238) as per the 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 patients and healthy donors (0.1 million/mL) were seeded in T-cell media in a 24-well plate. Cells were cultured for 72 hours in the presence and absence of cytokines (IL9: 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**

A total of 0.1 million/mL Jurkat cells were seeded in 12-well plates and cultured in 5% CO2 incubator at 37°C for 72 hours in the presence and absence of cytokines (IL9, 20 ng/mL and IFNγ, 2 ng/mL). At the
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<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>
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<tr>
<td>1</td>
<td>27</td>
<td>M</td>
<td>MF</td>
<td>CTCL early stage</td>
<td>T2A N0 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>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>F</td>
<td>MF</td>
<td>T2 N0 M0, stage I B</td>
<td>T2 N0 M0, stage I B</td>
<td>Pruritus</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>CT scan, multiple subcm retroperitoneal positive; bone marrow, not done; CSF, not done</td>
<td>PUVA</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0, stage I B</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>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0, stage I B</td>
<td>T2 N0 M0, 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>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M</td>
<td>MF</td>
<td>T2 N0 M0, stage I B</td>
<td>T2 N0 M0, stage I B</td>
<td>Itching, macules in bilateral hands, nape of neck, anterior chest wall, and forehead.</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>USG abdomen, no disease; bone marrow, uninvolved; CSF, not done</td>
<td>TSET</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>F</td>
<td>MF</td>
<td>T1 N0 M0, stage IA</td>
<td>T1 N0 M0, stage IA</td>
<td>Hypo pigmented patches on trunk, limb, and abdomen</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>CT scan, normal; bone marrow, not done; CSF, uninvolved</td>
<td>Referred to derma unit</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>M</td>
<td>MF</td>
<td>T1A N0 M0 stage IA</td>
<td>T1A N0 M0 stage IA</td>
<td>Swelling in right forearm</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>CT scan, right paratracheal and subcarinal lymph nodes. BM, likely to be uninvolved. CSF, not done</td>
<td>Observation</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>F</td>
<td>MF</td>
<td>T2A N0 M0 stage I B</td>
<td>T2A N0 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; CSF, not done; USG abdomen, no nodes.</td>
<td>Patient is taking PUVA therapy</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>F</td>
<td>MF</td>
<td>HPR not suggestive of MF</td>
<td>HPR not suggestive of MF</td>
<td>White patch, nonitchy and nonpainful</td>
<td>Extensive macular, scaly lesion all over the body involving chest, back, abdomen, and B/L upper limb and lower limb. No lymphadenopathy. No organomegaly.</td>
<td>PECT/CT, not done; USG abdomen pelvis, no abnormality detected; bone marrow, not done; HPR not suggestive of MF.</td>
<td>Patient is under observation</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>F</td>
<td>MF</td>
<td>T2A N0 M0, stage I B</td>
<td>T2A N0 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>
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<th>Stage</th>
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<th>Clinical examination</th>
<th>Staging</th>
<th>Treatment history</th>
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<tr>
<td>11</td>
<td>68 F MF MF CTCL advanced stage</td>
<td>T3 N0 M0 stage II B</td>
<td>Red patches in B/L upper limb, abdomen, chest, lower limbs, and 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>
<td></td>
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<tr>
<td>12</td>
<td>55 M MF</td>
<td>T3 N0 M0 BO, stage II B</td>
<td>Multiple maculopapular lesions, lesion on left side of the lip with desquamation and serous discharge.</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>PET, not available; marrow, uninvolved; CSF, not done</td>
<td>Gemcitabine pall intent</td>
<td></td>
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<tr>
<td>13</td>
<td>57 F MF</td>
<td>T4 N0 M0 BO, extensive cutaneous disease</td>
<td>Pruritus, fissuring with discharge all over the body, associated with redness, itching, and flaking.</td>
<td>PS-2, axillary lymphadenopathy, diffuse erythroderma, and scaling</td>
<td>PET, not available; bone marrow, not available; CSF, not available</td>
<td>INF plus MTX and RT opinion on TSET</td>
<td></td>
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<tr>
<td>14</td>
<td>31 F 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 15 upper limbs, B/L lower limb, back, and 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, bone marrow, uninvolved; CSF, not done</td>
<td>6 cycles CHOP from Jul 7, 2018</td>
<td></td>
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<tr>
<td>15</td>
<td>63 M CTCL:NOS</td>
<td>T3 N0 M0 BO, stage II B</td>
<td>Ulcerative lesion on right axilla and reddish nodular lesion over left lower limb</td>
<td>PS-1, no lymphadenopathy, no organomegaly</td>
<td>CT scan, right axillary region; distal left thigh, left inferior thyroid, mesenteric nodes</td>
<td>Oral MTX</td>
<td></td>
<td></td>
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<tr>
<td>16</td>
<td>49 F CD30⁺ LPD</td>
<td>T4 N0 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 x 1.4 cm, inguinal node, bone marrow, not done; CSF, not done</td>
<td>Chemotherapy- methotrexate</td>
<td></td>
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<tr>
<td>17</td>
<td>45 M Cutaneous ALC</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; bone marrow, not done; CSF, not done</td>
<td>Observation</td>
<td></td>
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Abbreviations: ALC, anaplastic large cell lymphoma; B/L, bilateral; CSF, cerebrospinal fluid; CD30⁺ LPD, CD30⁺ lymphoproliferative disease; HPR, histopathology report; INF, interferon; MF, mycosis fungoides; MTX, methotrexate; NOS, not otherwise specified; PUVA, psoralen and ultraviolet A; SPTL, subcutaneous panniculitis-like T-cell lymphoma; TSET, total skin electron therapy.
end of the incubation period, cells were harvested and washed with Annexin V binding buffer (1×). The apoptotic cells were stained using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen; 556547) as per the manufacturer’s instructions and acquisition was performed by BD FACSVerse (BD Biosciences). The analyses of FACS data were done using BD FACSuite software. 7ADD was used instead of PE provided in the Apoptosis Detection Kit.

Measurement of oxidative stress

Measurement of intracellular ROS was carried out using 2,7-dichlorodihydrofluorescein diacetate (H$_2$DCF-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-hour treatment of cytokines (IL9, 20 ng/mL and IFNγ, 2 ng/mL), cells were collected, washed with PBS, and stained with 1 μmol/L H$_2$DCFDA for 30 minutes at 37°C and ROS levels were 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 (IL9, 20 ng/mL and IFNγ, 2 ng/mL). Post 24 hours, the supernatant was collected and lactate was measured through the spectrofluorimetric 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 hour 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 Figs. 1–6, comparative data were analyzed by using Student 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, *, P < 0.05; **, P < 0.01; ***, P < 0.001 and ns for nonsignificant.
Results

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

A large proportion of CLA+ skin-resident effector T cells are known to secrete IL9 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+ IL9- IL17+), Th2 cells (CD4+ IL4+ IFNγ- IL9- IL17+), Th9 cells (CD4+ IL9+ IFNγ- IL4- IL17+), and Th17 cells (CD4+ IL17+ IL4- IFNγ- IL-9-) in primary CTCL patients (early and advanced). 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 with healthy donors (Fig. 1D). However, higher numbers of systemic (CLA-) Th9 cells were found in advanced-stage CTCL patients as compared with healthy donors (Fig. 1H).

There was a significantly lower frequency of skin-homing Th1 cells (Fig. 1B) and skin-homing Th17 cells (Fig. 1E) in advanced-stage CTCL patients as compared with healthy donors. There was no difference in the frequency of systemic Th1 cells (Fig. 1F), skin-homing and systemic Th2 cells (Fig. 1C and G), and systemic Th17 cells (Fig. 1I) among healthy donors and CTCL patients. Collectively, this data suggest that advanced-stage 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 IL9 and express high levels of IL9R on their surface

Th9 cells are the major source of IL9, which signals through a γC family receptor (IL9R) on target cells. We examined whether patient-derived T cells secrete increased levels of IL9 upon T-cell activation. The IL9 production was quantified by ELISA in the cell-free supernatant of PBMCs isolated from CTCL patients and healthy subjects upon TCR stimulation using anti-CD3/CD28–coated Dynabeads. Similar to flow cytometry data, IL9 production was higher in CTCL patients as compared with healthy donors (Fig. 2A).

Next, we examined the surface expression of IL9R on malignant T cells. CD3+ T cells were sorted from CTCL patients as well as healthy individuals. We observed a higher expression of IL9R on CTCL patient T cells and negligible expression of IL9R on healthy T cells (Fig. 2B). Similarly, there was increased IL9R expression on Jurkat cells (a T-cell lymphoma cell line). This data collectively suggest the coexpression of IL9 and IL9R expression on malignant T cells.

IL9 promotes the survival of T cells of CTCL patients and Jurkat cells

We further examined the functional relevance of the coexpression of IL9 and IL9R on malignant T cells. IL9 promoted T-cell survival of CTCL patients, Jurkat cells, and had no impact on healthy T cells (Fig. 3A). Because IFNγ is known to be proapoptotic and antiproliferative (33) and has the ability to induce oxidative stress (34) in various cancer cells, we also examined the role of IL9 in IFNγ milieu to further strengthen our observation. IFNγ inhibited the cell survival

| Figure 1. | T cells of patients with CTCL produce increased levels of IL9 and express high levels of IL9R. A, Isolated PBMCs from healthy donors (n = 23) and patients with CTCL (n = 11; 6 early and 5 advanced disease) were stimulated using anti-CD3/CD28-coated Dynabeads (Bead to cell ratio, 1:1). Post 48-hour incubation, IL9 production was quantified by ELISA in the cell-free supernatant as per the manufacturer’s instruction. Bar plots represent means ± SEM. Statistical significance was determined by comparing with healthy donors using Student unpaired t test. B, Sorted CD3+ T cells from CTCL patients (n = 3), healthy donors (n = 3), and Jurkat cells (n = 3) were stained for IL9R and analyzed by flow cytometry. Representative histogram (top) for CTCL, healthy, and Jurkat cells are shown and cumulative data are depicted in the bottom panel. Statistical significance was determined by paired t test (P values are designated as * , <0.05; ns, not significant). |
and IL9 reversed the IFNγ-mediated inhibition of cell survival in CTCL patients and Jurkat cells (Fig. 3A). Next, we examined the mechanism of IL9-mediated increase in tumor cell survival. IL9 reduced the Jurkat cell apoptosis as quantified by Annexin-V/7-AAD staining through flow cytometry (Fig. 3B).

**IL9 reduces oxidative stress and lactic acidosis of T cells of CTCL patients and Jurkat cells**

Because reduced toxic ROS levels are described to promote healthy T-cell survival (35), we examined whether IL9/IL9R 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 IL9 and/or IFNγ and ROS levels were quantified by DCFDA staining using flow cytometry. Interestingly, IL9 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 (Fig. 4A).

Because 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. IL9 decreased the extracellular lactate concentration in T cells of CTCL patients and Jurkat cells, however, had no impact on lactate production in healthy donors (Fig. 4B). Collectively this data suggest that IL9 promotes cell survival by reducing the intracellular toxic ROS levels, lactic acidosis, and apoptosis.

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

An earlier report shows increased migratory memory T cells (TMM) in few CTCL patients and depletion of TMM cells in both the circulation and the skin of CTCL patients treated with alemtuzumab (37). Very recently, TMM cells 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(CM) ref. 38). Here, we quantified the relative percentage of TMM and TCM in CTCL patients as compared with healthy donors. There was an increase in percentage of skin-homing (CLA−) as well as systemic (CLA−) TMM (CCR7+ L-Selectin+) in CTCL patients as compared with healthy donors. However, we observed increased frequency of skin-homing CTCL and healthy donors.
IL9 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 (IL9 and/or IFNγ) or left untreated (control). A, ROS levels were quantified by H2DCFDA staining using flow cytometry. Top panel depicts a representative histogram for each condition. Bottom panel demonstrates 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 ± SEM. Statistical significance was determined by comparing with control using Student paired t test (P values are designated as ‘*’, <.01; ‘+’, <.05; ns, not significant).

To understand the activation state of T cells (CD4+ /CD8+) in CTCL patients as compared with 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 with healthy donors (Fig. 5K) and no difference was observed in the frequency of CD4-CD69+CD25- T cells in CTCL patients as compared with healthy donors (Fig. 5I). Furthermore, CD69- CD25+ T cells (CD4+ and CD8-) frequency was higher in CTCL patients as compared with healthy donors (Fig. 5J and L). 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 reestablished Th1 and Th17 immunity

Finally, we examined the cytokine profile in 6 follow-up CTCL patients [mycosis fungoides 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 (Fig. 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 with 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 maintenance of the disease in CTCL. The disease progression (advanced-stage CTCL: T3 and T4) led to an impaired antitumor immunity, which might have contributed to the infection and persistence of lymphoma in these patients. In addition, there was elevated IL9 production in CTCL patients as compared with healthy donors. Coevolution of Th9 frequency and IL9 production indicates that Th9 cells are the major source of IL9 in CTCL patients. A recent study has reported the presence of CD3+ IL9-producing T cells in skin biopsy of mycosis fungoides lesion, one of the major subtypes of CTCL (19). In this study, we have demonstrated the presence of skin-homing Th9 and elevated IL9 production across different types of CTCL including mycosis fungoides, CD30+ LPD, SPTL, and CTCL-NOS. Importantly, we observed attenuated Th1 immunity in a large cohort of patients with advanced disease. Furthermore, there was an increased 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 RORγt, a transcription factor required for differentiation of IL17, 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 IL9 in tumorigenesis and delineated the underlying mechanisms. Our study revealed the coexpression of IL9 and IL9R on patient-derived T cells, indicating the presence of autocrine role of IL9 on tumor cells. Various studies, using cell lines and murine models, have reported the roles of IL9 in the pathogenesis of different cancers including lung cancer, breast cancer, thyroid cancer, and leukemia (41–44).
We observed that IL9 significantly decreased the rate of apoptosis and ROS levels (oxidative stress) inside the T cells of CTCL patients, thus indicating the role of IL9 in maintaining a steady-state ROS. Concurrently, IL9 increased the malignant T-cell survival, which ultimately resulted in high cell numbers. The state of redox balance in CTCL and the effect of IL9 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 with 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 indicate the role of IL9 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 IL9. We also observed that IL9 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 with 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 IL9 helps in the overall survival and maintenance of malignant T cells in CTCL, generating a protumor response. These findings suggest a possible role of IL9 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 Fig. 6B). In addition to cytokine profile, we examined the skin-homing and systemic immune–phenotyping focusing on T-cell subsets (T\textsubscript{naive}, T\textsubscript{eff}, T\textsubscript{MM}, and T\textsubscript{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\textsubscript{MM} cells in CTCL as compared with healthy donors. In addition, there was decreased frequency of CD4\textsuperscript{+} T\textsubscript{naive} and increased frequency of CD4\textsuperscript{+} T\textsubscript{eff} in...
CTCL patients as compared with healthy donors. Interestingly, the frequency of CD8+ CD69+ CD25+ T cells was higher in CTCL patient and percentage of CD69+ CD25+ T cells (CD4+ and CD8+) was higher in CTCL patient as compared with healthy donors.

In conclusion, this study provides first evidence of the presence of increased skin-homing Th9 cells and elevated IL9 in CTCL. Moreover, it demonstrates the autocrine-positive feedback loop of Th9 axis in CTCL and delineates the role of IL9 in reducing the oxidative stress and rate of apoptosis to promote the survival of malignant T cells (graphical summary: Fig. 6C). Strategies targeting Th9 cells and inhibition of IL9 may harbor significant potential in the development of novel effective therapeutics for these difficult-to-treat malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Jain, R. Purwar

Development of methodology: S. Kumar, B. Dhamija

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kumar, B. Dhamija, S. Ghosh, A. Dwivedi, N. Sharma, M. Sengar, E. Sridhar, A. Bonda, J. Thorat, P. Tembhare, B. Bagal, S. Laskar, H. Jain

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kumar, B. Dhamija, R. Purwar

Writing, review, and/or revision of the manuscript: S. Kumar, B. Dhamija, S. Marathe, H. Jain, R. Purwar

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kumar, N. Sharma, R. Purwar

Study supervision: H. Jain, R. Purwar

Other (helped in performing experiments: procuring blood samples from TML. For assays: T-cell isolation, setting up assays surface and intracellular immunostaining, and setting up assays for ELISA): S. Marathe

Other (helped in performing experiments mainly in flow cytometry): A. Dwivedi

Other (sample collection and performing experiments): A. Karalkar

Acknowledgments

This work was supported by Department of Science and Technology (DST) grant (RD/0119-DST0000-10), Indian Council of Medical Research (ICMR) grant (RD/0119-ICMR000-001), Tata Trust Fund (RD/0117TATAE00-001), Bristol-Myers Squibb (15BMS001), and intramural fund of IIT Bombay to R. Purwar. This work was partially supported by a grant from Department of Biotechnology, Government of India awarded to Wadhwani Research Centre for Bioengineering, and IIT Bombay (BT/INF22/SP32036/2017). We would like to thank the individuals (patients and healthy volunteers) who donated the blood for this study. We also thank family members of the patients, the medical staff of Tata Memorial Hospital, and Central FACS facility of SBSE, IIT Bombay for their support.

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Received September 4, 2019; revised December 9, 2019; accepted January 24, 2020, published first January 29, 2020.

References


Molecular Cancer Research

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


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