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Research article

STAT3 KNOCKDOWN BY siRNA INDUCES APOPTOSIS IN HUMAN CUTANEOUS T-CELL LYMPHOMA LINE Hut78 *VIA* DOWNREGULATION OF *Bcl-xL*

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Abstract: Cutaneous T-cell lymphomas (CTCLs) are non-Hodgkin's lymphomas resulting from clonal expansion and localization of malignant T-lymphocytes to the skin. CTCL cells have defective apoptosis. Signal transducers and activators of transcription (STAT) are a family of transcription factors known to play important roles in the development and progression of several human cancers by promoting cell proliferation and protecting against apoptosis. In this study, we investigated the specific role of STAT3, a major component of the STAT family, in growth and survival of human CTCL cell line Hut78. Western immunoblot analysis showed elevated expression of STAT3 and phospho-STAT3(Y705) in human CTCL cells as compared to freshly isolated peripheral blood lymphocytes (PBLs). Specific knockdown of STAT3 expression in Hut78 cells by RNA interference induced morphological and biochemical changes indicating apoptotic cell death. Moreover, STAT3 inhibition downregulated the expression of Bcl2 family of anti-apoptotic gene *Bcl-xL*. These observations suggest that STAT3 is required for the survival of CTCL cells and strongly indicate that targeting STAT3 using siRNA techniques may serve a novel therapeutic strategy for the treatment of CTCL.

Key words: Apoptosis, Cutaneous T-cell lymphoma (CTCL), siRNA, STAT3

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Abbreviations used: CTCL – cutaneous T-cell lymphoma; MF – mycosis fungoides; PBL – peripheral blood lymphocyte; siRNA – small interfering RNA; SS – Sézary syndrome; STAT – signal transducers and activators of transcription; TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling

INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) is a heterogenous group of lymphoproliferative disorders and represents a spectrum of diseases mainly composed of malignant clonal T-lymphocytes of the CD4 phenotype [1, 2]. The annual incidence of primary cutaneous lymphomas is estimated to be 1:100,000, of which CTCL is the most common type representing approximately 75% of cases [3, 4]. CTCLs are non-Hodgkin's lymphomas characterized by the clonal proliferation of skin-invasive mature T-lymphocytes [3]. The most common and indolent form of CTCL is Mycosis fungoides (MF) and is characterized by patches, plaques, or tumors containing epidermotrophic malignant $CD4^+CD45RO^+$ helper/memory T-cells [5]. In the initial phase, which can endure for several years, MF is found as flat erythromatous skin lesions, resembling non-malignant psoriasis or eczema [3]. In later stages, tumor cells spread to other sites of the body with a fatal outcome. MF may evolve into a leukemic variant Sézary syndrome (SS) with tumour T-cells present both in the skin and in the blood, or transform to large cell lymphoma [3, 6]. As MF/SS advances, the clonal dominance of the malignant cells results in progressive immune dysregulation in patients and further tumor cell growth, ultimately leading to death in 5-10 years [7]. In addition, 50% of patients with advanced disease do not respond to therapy, and >25% of those who respond initially will relapse and progress to fatal disease [8]. Causative roles in the development of CTCL have been suggested for various environmental factors and infectious agents [2], but the etiology of the disease remains unknown. At present, no curative therapy exists. Systemic single-agent or multi-agent chemotherapies are used to treat advanced and aggressive forms of CTCL to palliate patients. Despite moderate response rates, however, no treatment has shown to prolong disease-free or overall survival [3, 5, 9, 10]. Therefore, there is a great need for development of novel emerging therapies.

One of the most important oncogenic properties of cancer cells is their ability to survive and CTCL cells are no exception displaying defective apoptosis [11]. Recent evidences suggest that disturbances in intracellular signalling pathways also play a role in the pathogenesis of CTCL [5, 12-14]. Thus, identification of molecular events that control CTCL cell survival has significant therapeutic implications and reversing resistance to apoptosis may provide a novel therapeutic approach.

Signal transducer and activator of transcription (STAT) proteins are a family of latent cytoplasmic transcription factors activated in response to most cytokines and growth factors and transmit signals from cell surface receptors to the nucleus [15]. Among the seven members of mammalian STAT family identified, STAT3 is the most pleotropic member and most strongly implicated in ongogenesis [16, 17]. It regulates a number of pathways important in tumorogenesis including cell cycle progression, apoptosis, tumor angiogenesis, invasion and metastasis, and evasion of immune response [15-17]. Moreover,

transformation process initiated by diverse oncogeneic protein tyrosine kinase is dependent on STAT3 activation [18]. In healthy individuals STAT3 activation is rapid, transient, and stringently regulated at multiple levels. Aberrant STAT3 signalling was reported in many types of malignancies such as myeloma, head and neck cancer, breast cancer and prostate cancer [18, 19]. Constitutive activation of STAT3 was previously observed *in vivo* and in malignant cell lines derived from the skin and blood from CTCL patients [20-23]. Inhibition of STAT3 signalling in a number of tumor cell types including CTCL cell lines [21] with multiple approaches such as application of synthetic protein tyrosine kinase inhibitor tryphostin AG490 [21, 23] or dominant negative gene constructs [20] has been shown to cause a decrease in cell viability and subsequent apoptosis.

In the present study, we investigated underlying molecular mechanism determining CTCL cell survival. Here we show higher level of STAT3 protein expression and activation in CTCL cells in comparison with peripheral blood lymphocytes (PBLs) isolated from healthy volunteers. Furthermore, we demonstrate that specific knockdown of cellular STAT3 expression by small interfering RNA (siRNA) inhibits CTCL cell growth and induces apoptosis by downregulating the expression of an anti-apoptotic protein Bcl-xL.

MATERIALS AND METHODS

Cell cultures

An established human cutaneous T-cell lymphoma cell line Hut78 [24] developed from peripheral blood of CTCL patients was from American Type Culture Collection (ATCC, Manassas, VA) and cultured as described previously [25]. Briefly, they were cultured in RPMI 1640 medium containing 10% (v/v) heat inactivated foetal bovine serum (Gibco, Grand Island, NY), L-glutamine, and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in 5% CO_2 at 37ºC. PBLs were isolated from healthy volunteers using standard techniques as described [26].

Antibodies

Rabbit monoclonal anti-STAT3, anti-phospho-STAT3(Y705), anti-Bcl-xL, anti-GAPDH, horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies were from Cell Signalling Technology (Danvers, MA). Mouse monoclonal anti-α-tubulin antibody was from Sigma (St Louis, MO, USA).

Small interfering RNA (siRNA) and electroporation

siRNA against STAT3 (SMARTpool® siRNA Reagents) and control siRNAs were from Dharmacon (Lafayette, CO). Hut78 cells in logarithmic growth (5×10^5 cells in 200 μl medium) were mixed with siRNAs and added to 2 mm gap cuvettes. Cells were electroporated at 150 V for 2 ms using BTX ECM830 electroporator (BTX Molecular Delivery Systems, Holliston, MA), transferred to a 12-well tissue culture plate with 2 ml medium and incubated for 24 h.

Transfection efficiency using siGLO labeled siRNA with no significant sequence similarity to human genes (Dharmacon) was estimated to be greater than 80% (data not shown).

Cell viability assay

Cell viability was measured by CellTiter 96® AQ_{ueous} One Solution cell proliferation assay kit according to the manufacturer's instructions (Promega, Madison, WI). Cells (100 µl cultures) were distributed in 96-well microplates and then incubated with 20 µl of MTS tetrazolium solution (provided by the manufacturer) for 4 h. The relative cell viability was calculated by determining the absorbance at 490 nm using a microplate reader (Tecan, Mannedorf, Switzerland).

Cell lysis and Western immunoblotting

The cell lysis was performed as described previously [27]. The protein content of the cell lysates was determined by Bio-Rad protein assay kit according to manufacturer's instructions (Bio-Rad Laboratories GmbH, München, Germany). The cell lysates were boiled with Laemmli sample buffer for 5 min and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferd to polyvinylidene fluoride membrane by semi-dry blotting for 1 h. The membranes were blocked in 5% non-fat dry milk in PBST [0.1% (v/v) Tween20 in phosphate buffered saline] for 1 h at room temperature. After washing, the blots were incubated with the indicated primary antibodies (diluted according to manufacturer's instructions) overnight at 4ºC with gentle rocking. After three washing in PBST, the membranes were incubated with appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and subsequent exposure to Kodak light sensitive film (Cedex, France).

Densitometric analysis

Densitometric analyses of the Western blots were performed by using GeneTools software (Syngene, Cambridge, UK). The relative values of the samples were determined by giving an arbitrary value of 1.0 to the respective control samples of each experiment.

High Content Analysis (HCA) of nuclear morphology

Nuclei were stained with Hoechst. Florescent images were acquired and analyzed using automated microscope (IN Cell Analyzer 1000 HCA platform) equipped with Image Investigator software (GE Healthcare, Buckinghamshire, England).

In situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay was used to monitor DNA fragmentation due to apoptosis. The assay was performed on Hut78 cell suspensions according to manufacturer's instructions (ApoAlert® DNA Fragmentation Assay Kit, Clontech Laboratories, Inc., Mountain View, CA). Fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected by Zeiss confocal workstation attached to Zeiss LSM 510 laser module using 63X oil immersion lens equipped with Zeiss LSM 510 (release 4.0) software (Carl Zeiss, Thornwood, NY).

Annexin-V staining and analysis

Detection of phosphatidlserine on the outer membrane of apoptotic cells was performed by using annexin-V binding and propidium iodide staining according to manufacturer's instructions (Beckman Coulter). Briefly, cells were incubated with FITC-conjugated annexin-V in the presence of 1 mM $CaCl_2$ and 2.5 μg/ml propidium iodide in order to counter-stain nuclei, Ten thousand events per sample were acquired and analyzed using BD FACSCalibur^{TM} system (Franklin Lakes, NJ).

Statistical analysis

The values are expressed as mean ± standard error of the mean. For comparison of two groups, *p*-values were calculated by two-tailed unpaired student's t-test. In all cases $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Expression and activation of STAT3 is elevated in CTCL cells

STAT3 has been shown to be constitutively activated in malignant lymphocytes obtained from skin biopsy from a patient with advanced MF and in tissue sections from patients with skin tumors [20]. To examine the status of STAT3 in CTCL cells, whole cell lysates of Hut78 and PBLs freshly isolated from healthy volunteers were analyzed by Western blotting by use of antibody against STAT3 and tyrosine phosphorylated STAT3 (Fig. 1A). Expression of STAT3 was 2.6 fold higher in Hut78 cells as compared to normal PBLs (Fig. 1B). Moreover, tyrosine phosphorylation of STAT3 was 5.2 fold higher in Hut78 cells in comparison to normal PBLs (Fig. 1C).

siRNA targeting STAT3 specifically inhibits its expression in CTCL cells

The use of RNA interference represents a novel alternative to gene inhibition. Selective oncogene silencing by siRNA shows promise for cancer treatment. siRNA molecules are being used to produce sequence specific gene silencing in mammalian cells for functional analysis of genes [28]. siRNAs have been successfully used for targeting STAT3 in cancer cells [29, 30]. Here, we electroporated Hut78 cells with various concentrations (100 nM, 300 nM or 500 nM) of siRNA against STAT3. STAT3 siRNA resulted in a highly significant and

reproducible reduction of STAT3 expression ranging from 70 to 95% in a concentration dependent manner at 24 h (Fig. 2). The effect of STAT3 siRNA was specific in that it failed to knockdown expression of unrelated proteins α- tubulin or GAPDH.

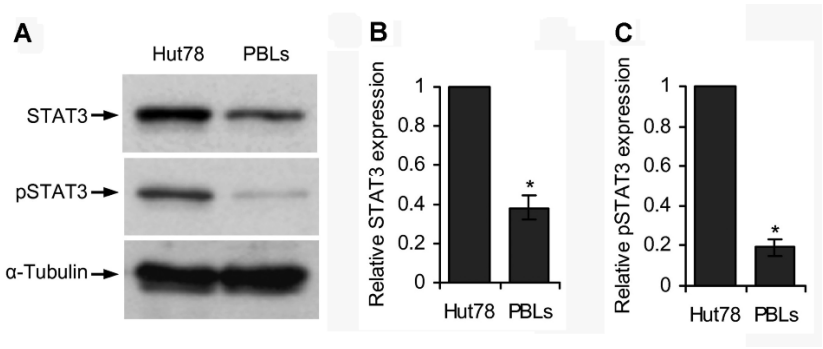

Fig. 1. Expression and activation of STAT3 in CTCL cells. A – Whole cell lysates (20 µg each) of Hut78 CTCL cells and PBLs isolated from healthy volunteers were Western immunoblotted with anti-STAT3 (*STAT3*), anti-phospho-STAT3 (Y705, *pSTAT3*), or anti-α-tubulin (*α-Tubulin*) antibodies. B, C – Relative densitometric analysis of the individual bands of STAT3 and pSTAT3 was performed and presented. Data represent the mean of three independent experiments and bars indicate standard error of the mean. *$P < 0.05$ compared to respective controls.

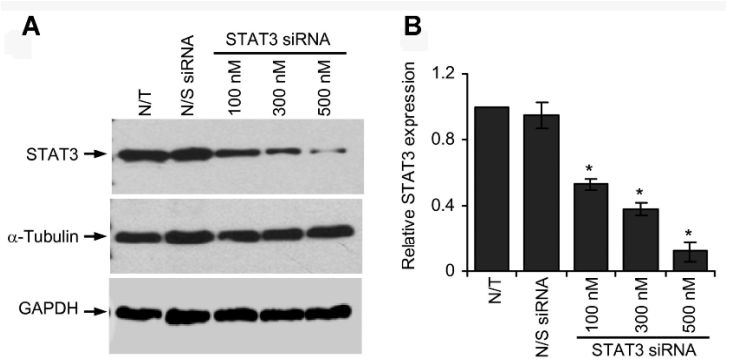

Fig. 2. Specific inhibition of STAT3 expression by siRNA in CTCL cells. A – Hut78 cells (*N/T*) were electroporated with 500 nM non-specific siRNA (*N/S siRNA*), 100 nM, 300 nM or 500 nM siRNA targeted against STAT3 (*STAT3 siRNA*). After 24 h cells were lysed and the whole cell lysates (20µg each) were Western immunoblotted with anti-STAT3 (*STAT3*), anti-α-tubulin (*α-Tubulin*), or anti-GAPDH (*GAPDH*) antibodies. B – Relative densitometric analysis of the individual STAT3 bands was performed and presented. Data represent the mean of three independent experiments and bars indicate standard error of the mean. *$P < 0.05$ compared to control.

STAT3 siRNA inhibits growth of CTCL cells

There are evidences to suggest that malignant cells having constant activation of STAT3 become STAT3 dependent for their survival. Studies have shown that persistently activated STAT3 leads to profound changes in gene expression patterns and promotes uncontrolled tumour cell proliferation [30-32]. Therefore, we sought to determine the effect of inhibiting STAT3 expression on growth of CTCL cells. We noted that siRNA (500 nM) mediated depletion (95%) of STAT3 expression resulted in the inhibition of Hut78 cell growth by 43.8% in 24 h (Fig. 3).

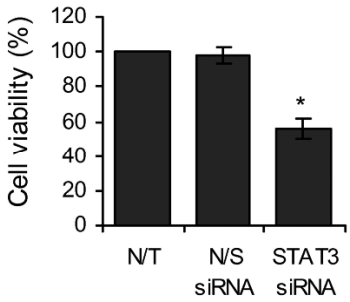

Fig. 3. Inhibition of STAT3 expression inhibits CTCL cell growth. Hut78 cells (*N/T*) were electroporated with 500 nM non-specific siRNA (*N/S siRNA*) or siRNA targeted against STAT3 (*STAT3 siRNA*). After 24 h, percentage cell viability was determined with CellTitre 96^{TM} AQ$_{ueous}$ One Solution Assay and presented. Data represent the mean of three independent experiments in triplicates and bars indicate standard error of the mean. *$P < 0.05$ compared to control.

STAT3 knockdown in CTCL induces apoptosis

To determine whether growth inhibition of CTCL cells by STAT3 siRNA was associated with apoptosis, we examined three indicators of apoptosis. We first analyzed nuclear morphology of Hut78 cells by High Content Analysis. The onset of apoptosis is characterized by shrinkage of nucleus and condensation of nuclear chromatin into sharply delineated masses [33]. Untransfected cells or cells transfected with non-specific siRNA exhibited diffused nuclear staining (Fig. 4A). However, STAT3 depleted cells underwent changes in nuclear morphology such as condensed chromatin and shrunk nucleus (Fig. 4A). High Content Analysis (HCA) for the quantitation of nuclear staining for intensity and shape revealed a significantly higher number of apoptotic nuclei in STAT3 depleted cells as compared to controls (Fig. 4B). Next, TUNEL assay was performed to detect the free ends of DNA after breakage, which is one of the important biochemical hallmarks of apoptosis [33]. STAT3 depleted Hut78 cells showed TdT-labeled nuclei, whereas control cells did not show any TUNEL-positive cells (Fig. 4C). Finally, we investigated the effect of STAT3 siRNA on the externalization of phosphatidylserine in Hut78 cells. Externalization of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to the outer cell membrane is a well-known early hallmark of apoptosis [33]. Annexin-V,

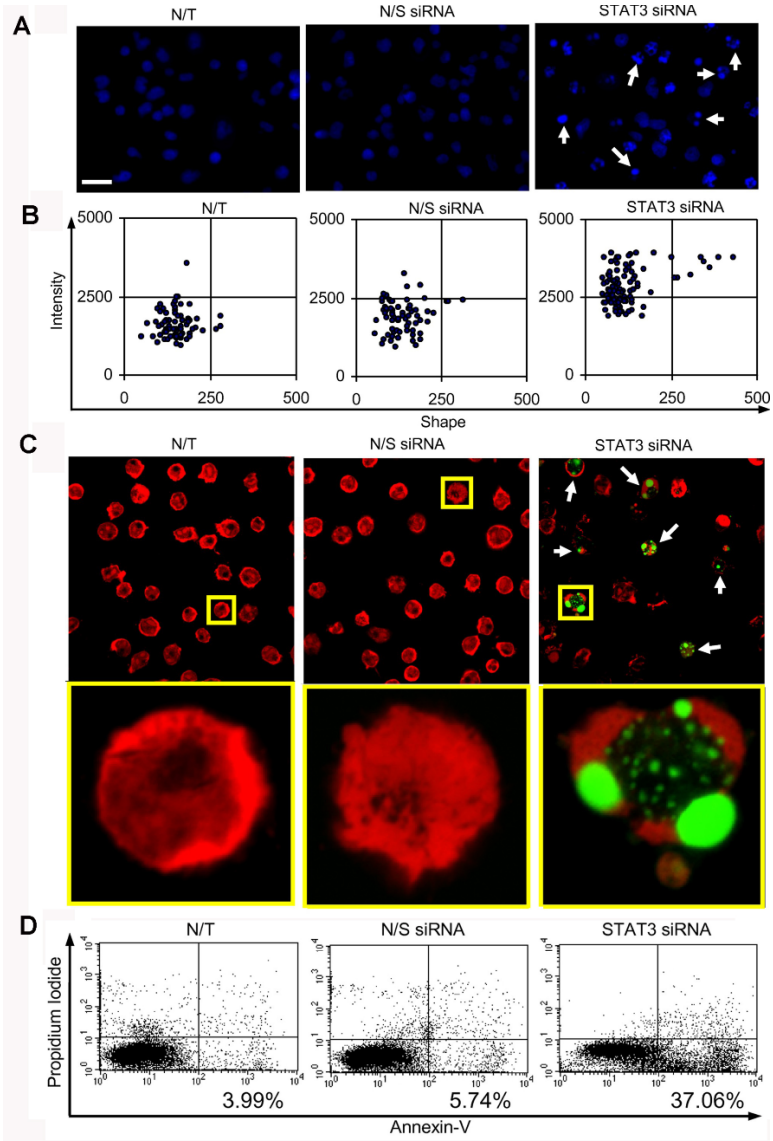

Fig. 4. Inhibition of STAT3 expression induces apoptosis in CTCL cells. Hut78 cells (*N/T*) were electroporated with 500 nM non-specific siRNA (*N/S siRNA*) or siRNA targeted against STAT3 (*STAT3 siRNA*) and incubated for 24 h. A – Cells were stained for nuclei with Hoechst and photographed. Viable cells display diffuse nuclear fluorescence whereas apoptotic cells show the concentrated dense granular fluorescence. Arrow indicates apoptotic nuclei, scale bar 20 μm. B – Cell shape and nuclear intensity was quantified using IN Cell Image Investigator software and presented. Experiments were repeated three times in triplicate and a representative result is shown. C – Cells were stained with ApoAlert® DNA Fragmentation Assay Kit and photographed. Arrow indicates apoptotic cells. D – Apoptotic cells were quantified by flow cytometry following FITC-annexin-V/propidium iodide staining for at least 10,000 events. Lower right corner represents apoptotic cells.

a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues, was used for the detection of apoptosis by flow cytometry analysis. There was no obvious induction of annexin-V positive signal in untransfected cells or cells transfected with non-specific siRNA (Fig. 4D). STAT3 siRNA transfected Hut78 cells showed 37.06% apoptotic cells (Fig. 4D). These observations confirmed that CTCL cell survival is dependent on STAT3 expression and indicate that the cellular demise is triggered through the apoptotic pathway.

STAT3 inhibition downregulates Bcl-xL expression in CTCL cells

Tumor growth requires that pro-oncogenic pathways remain functional [32]. To understand the molecular mechanism by which STAT3 siRNA induces apoptosis in CTCL cells, we examined changes in the expression of apoptosis associated protein. The anti-apoptotic protein Bcl-2 has been demonstrated to play an important role in the regulation of apoptosis and to inhibit the induction of apoptosis in lymphocytes by a variety of signals [34]. Expression of Bcl-2 has been found in CTCL cells and is supposed to increase the survival and the resistance of CTCL cells against various therapeutic options [35]. We addressed whether siRNA mediated STAT3 inhibition was associated with the inhibition of pro-survival member of Bcl-2 family of apoptosis regulator Bcl-xL. Interestingly, we observed that STAT3 depletion (95%) by siRNA drastically reduced the expression level of Bcl-xL by 86% (Fig. 5).

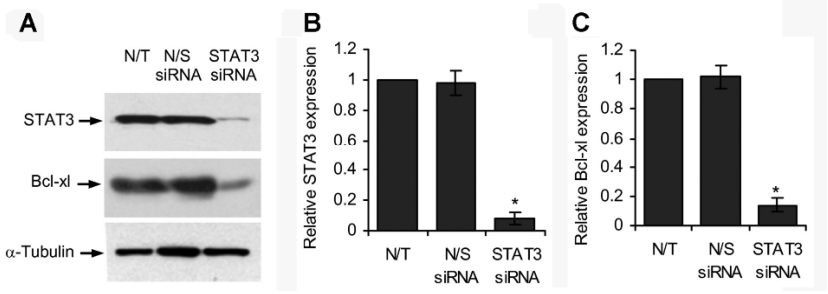

Fig. 5. Inhibition of STAT3 expression inhibits Bcl-xL expression in CTCL cells. Hut78 cells (*N/T*) were electroporated with 500 nM non-specific siRNA (*N/S siRNA*) or siRNA targeted against STAT3 (*STAT3 siRNA*) and lysed after 24 h. (A) Cell lysates (20 μg each) were Western immunoblotted with anti-STAT3 (*STAT3*), anti-Bcl-xL (*Bcl-xL*), or anti-α-tubulin (*α-Tubulin*) antibodies. (B, C) Relative densitometric analysis of the individual bands of STAT3 and Bcl-xL were performed and presented. Data represent the mean of three independent experiments and bars indicate standard error of the mean. **P* < 0.05 compared to respective controls.

Data presented in this paper are consistent with the growing body of evidence suggesting STAT3 may be an important therapeutic target in several human cancers [36] including lymphatic neoplasia. However, no strategy for STAT3 inhibition has reached clinical application [37]. Several modalities have been

employed to achieve effective STAT3 inhibition through either upstream inhibition of cytokine and growth factors, inhibition of STAT3 dimerization, inhibition of STAT3/STAT3 nuclear translocation, or inhibition of DNA binding activity [38]. These approaches utilized small molecule inhibitors, dominant-negative gene constructs, inhibitory peptides, antisense or decoy oligonucleotides. Non-specific effects, poor cellular permeability, insufficient stability and low bioavailability have diminished the enthusiasm and limited their progress from bench to bed side [38]. We note that the above-mentioned approaches to suppress STAT3 activity were by suppression of its nuclear translocation with no inhibition of STAT3 expression levels. In this context, application of siRNA represents a novel and an attractive alternative with a promising therapeutic potential [39]. Theoretically, it is plausible that siRNA can be used to treat any disease linked to an overactive gene or genes with exquisite precision and high efficacy with selective gene silencing [28]. This approach specifically reduces intracellular levels of target protein(s). siRNA based therapy has been used for effective treatment of various diseases, including hepatitis, liver ischemia reperfusion injury, allograft rejection, central nervous system disorders and malignancies. siRNA targeting STAT3 has been applied to induce apoptosis in numerous cancer cell types including human astrocytes, astrocytoma cells [29] and laryngeal cancer cells [30]. However, to our knowledge, no report has been published to date concerning the effect of siRNA against the STAT3 gene in CTCL cells, so our study represents the first report using STAT3 siRNA with CTCL cells.

Several studies established STAT3 involvement in a wide variety of signalling systems mediating a bewildering complexity of responses. STAT3 has recently been shown to control the production of cytokines, which have multiple effects on immune and cancer cells in the tumor microenvironment [40]. Unlike the results with ablation of other STAT family genes, all of which have produced viable mice with relatively limited phenotypes, ablation of STAT3 led to early embryonic lethality [15, 17, 41]. This suggests an essential role of STAT3 in cell survival. A novel link between STAT3 and IL8 has recently been demonstrated, the deregulation of which caused malignant behavior of PTEN-deficient glioblastoma cells *via* NFkB [42]. These findings, along with evidence of STAT3 activation by a wide variety of cytokines, growth factors, and other stimuli [15-17] implied its more generally deployed involvement than other STATs and led to the suggestion that STAT3 might represent a primordial STAT protein. The analysis of conditional loss of STAT3 protein in adult tissues confirmed STAT3 involvement in a wide variety of physiological processes with seemingly contradictory responses [17]. Global chromatin binding surveys revealed that STAT3 binds at least 3,000 different gene promoters [40]. In some cases, STAT3 regulates the induction of a set of important target genes, but in others it may be acting as a repressor (e.g., in thymic epithelium) or as a signalling adaptor without a transcriptional function [16]. Moreover, even in situations where STAT3 appears to function as a transcriptional activator, the

biological effect can involve proliferation, survival, or apoptosis, depending on the target tissue [16, 17, 36]. Thus, although there is a wealth of data on STAT3 functions in various contexts, it is still not possible to describe fully what STAT3 does. Results presented here support the hypothesis that STAT3 exerts its anti-apoptotic action *via* survival genes such as Bcl-xL. Our findings support several other reports that STAT3 may be a key player in the pathogenesis of diverse human cancers and make this molecule a prime target for novel therapies, lending a greater urgency to answering this complex question.

In conclusion, we have demonstrated that STAT3 plays an essential part in the CTCL cell survival. Specific inhibition of STAT3 expression by RNAi approach causes apoptosis of CTCL cell line Hut78 in association with downregulation of pro-survival protein Bcl-xL. These molecular effects have important implications for understanding how STAT controls the cell survival programme in CTCL cells. Since constitutive activation of STAT3 has also been described in other clinical cancers [32, 36], it is possible that dysregulation of STAT3 might have more general role(s) in malignant transformation. Whereas, STAT3 inhibitors have little or no effect on cells lacking STAT3 activation, we demonstrate that it is feasible to inhibit its expression by highly specific siRNA technology. It is tempting to speculate that downregulation of STAT3 could be of therapeutic relevance to CTCL treatment. Current development of tumor-targeted siRNA delivery systems such as assembled nanoparticles [37, 43] may in time become a useful clinical tool for targeting STAT3 in CTCL and other malignant tumors expressing constitutively activated STAT3.

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