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Bortezomib Inhibits Expression of TGF β 1, IL-10, and CXCR4, Resulting in Decreased Survival and Migration of Cutaneous T Cell Lymphoma Cells¹

Tzu-Pei Chang^{*}, Vladimir Poltoratsky[†], and Ivana Vancurova^{2,*}

^{*}Department of Biological Sciences, St. John's University, New York, NY 11439, USA

[†]Department of Pharmaceutical Sciences, St. John's University, New York, NY 11439, USA

Abstract

Increased expression of the immuno-suppressive cytokines, transforming growth factor- β 1 (TGF β 1) and interleukin-10 (IL-10), is a hallmark of the advanced stages of cutaneous T cell lymphoma (CTCL), where it has been associated with suppressed immunity, increased susceptibility to infections, and diminished antitumor responses. Yet, little is known about the transcriptional regulation of TGF β 1 and IL-10 in CTCL, and about their function in regulating the CTCL cell responses. Here, we show that TGF β 1 and IL-10 expression in CTCL cells is regulated by NF κ B, and suppressed by bortezomib (BZ), which has shown promising results in the treatment of CTCL. However, while the TGF β 1 expression is I κ B α -dependent and is regulated by the canonical pathway, the IL-10 expression is I κ B α -independent, and its inhibition by BZ is associated with increased promoter recruitment of p52 that characterizes the non-canonical pathway. TGF β 1 suppression decreases CTCL cell viability and increases apoptosis, and adding exogenous TGF β 1 increases viability of BZ-treated CTCL cells, indicating TGF β 1 pro-survival function in CTCL cells. In addition, TGF β 1 suppression increases expression of the pro-inflammatory cytokines IL-8 and IL-17 in CTCL cells, suggesting that TGF β 1 also regulates the IL-8 and IL-17 expression. Importantly, our results demonstrate that BZ inhibits expression of the chemokine receptor CXCR4 in CTCL cells, resulting in their decreased migration, and that the CTCL cell migration is mediated by TGF β 1. These findings provide the first insights into the BZ-regulated TGF β 1 and IL-10 expression in CTCL cells, and indicate that TGF β 1 has a key role in regulating CTCL survival, inflammatory gene expression, and migration.

Keywords

Bortezomib; cutaneous T-cell lymphoma; CXCL12; CXCR4; IL-10; NF κ B; TGF β 1

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²Address correspondence to: Dr. Ivana Vancurova, Department of Biological Sciences, St. John's University, 8000 Utopia Parkway, Queens, NY 11439, USA. Tel: 718 990-6409; Fax: 718 990-5958; vancuroi@stjohns.edu.

Introduction

Enhanced TGF β 1 and IL-10 expression by T regulatory (Treg) cells is a hallmark of the advanced stages of CTCL, especially mycosis fungoides (MF) and the leukemic variant, Sézary syndrome (SS) (1–5). The increased levels of IL-10 and TGF β 1 have been associated with suppressed immunity, increased susceptibility to infections, and diminished antitumor responses in CTCL patients (6–10). In addition, the advanced stages of CTCL are associated with the increased expression of the chemokine receptor CXCR4 and its ligand CXCL12, also known as stromal cell-derived factor 1 (SDF-1); both CXCR4 and CXCL12 have been linked with the increased CTCL cell proliferation, skin recruitment and accumulation (11–14).

At the transcriptional level, both IL-10 and TGF β 1 expression is regulated by the transcription factor NF κ B (15–21). However, compared to anti-apoptotic and pro-inflammatory genes, the transcriptional regulation of immunosuppressive genes by NF κ B is much less understood. Majority of genes involved in inflammatory response are regulated by NF κ B canonical pathway that is I κ B α -dependent, and involves predominantly p65 and p50 subunits. In contrast, lymphocyte development is regulated by non-canonical pathway, which is I κ B α -independent, and activates complexes containing mostly RelB and p52 (22, 23). Binding of the individual NF κ B dimers, and other transcription factors and co-regulators to different κ B sites provides the transcriptional selectivity of NF κ B responses (24–26). The NF κ B DNA binding activity is constitutively increased in CTCL cells, and has been associated with the high CTCL cell survival and resistance to chemotherapy (27–32). There is no effective strategy to prolong survival of patients with advanced stages of CTCL; thus, novel therapeutic strategies are needed (33–38).

Bortezomib (BZ, Velcade, PS-341) is the first FDA approved proteasome inhibitor that has shown remarkable anti-tumor activity in the treatment of patients with multiple myeloma (39, 40). BZ has shown promising results also in patients with relapsed or refractory CTCL (41–44). However, the precise molecular mechanisms are not fully understood (45, 46). BZ has been originally developed as an inhibitor of the I κ B α -dependent canonical pathway, resulting in the inhibition of NF κ B-dependent anti-apoptotic genes in cancer cells (39, 40). However, we have recently shown that the proteasome inhibition also induces nuclear translocation and accumulation of I κ B α , which has a promoter specific effect on the regulation of NF κ B-dependent genes, depending on the subunit composition of NF κ B proteins recruited to the different κ B binding sites (47–50).

In this study, we report that both TGF β 1 and IL-10 expression in CTCL cells is suppressed by the BZ-mediated proteasome inhibition. However, while the TGF β 1 expression is regulated by the I κ B α -dependent canonical pathway, the IL-10 expression is I κ B α -independent, and its inhibition is associated with the increased promoter recruitment of p52 that characterizes the non-canonical pathway. In addition, TGF β 1 suppression induces apoptosis, and IL-8 and IL-17 expression in CTCL cells, indicating that TGF β 1 regulates survival and pro-inflammatory gene expression in CTCL. Importantly, our results indicate that TGF β 1 mediates the CXCL12/CXCR4-dependent CTCL cell migration, and suggest that BZ inhibits the CTCL migration, at least partly, by inhibiting TGF β 1.

Materials and Methods

Antibodies and reagents

Purified antibodies against human I κ B α (sc-371), NF κ B p65 (sc-372X), p50 (sc-7178X), cRel (sc-71X), RelB (sc-226X), p52 (sc-848X), CXCR4 (sc-9046), T β RI (sc-398), T β RII (sc-17792), and lamin B (sc-6216) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TGF β 1 (240-B-002) and CXCL12 (350-NS-010) proteins, TGF β neutralizing antibody (MAB1835), and the corresponding isotype control antibody (MAB002) were from R&D Systems (Minneapolis, MN, USA). Purified polyclonal antibody against lactate dehydrogenase (LDH; 20-LG22) was from Fitzgerald Industries International (Concord, MA, USA), and actin antibody was from Sigma (St Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Bortezomib was from ChemieTek (Indianapolis, IN, USA). All other reagents were molecular biology grade and were from Sigma (St Louis, MO).

Cell culture

CTCL cell lines, Hut-78 (ATCC $\text{\textcircled{R}}$ TIB-161), H9 (ATCC $\text{\textcircled{R}}$ HTB-176), and HH (ATCC $\text{\textcircled{R}}$ CRL-2105) cells, derived from peripheral blood of patients with SS and non-MF/SS aggressive CTCL were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), and used between the passage numbers 10 and 15. Peripheral blood mononuclear cells (PBMC) from healthy human volunteers were purchased from Zen-Bio (SER-PBMC-200; Research Triangle Park, NC, USA). Cells were maintained at 37 $^{\circ}$ C in RPMI 1640 medium, supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, in a humidified atmosphere with 5% CO $_2$. Prior to cell treatment, cells were seeded (5×10^5 cells/ml) in 6-well plates and grown 24 hours at 37 $^{\circ}$ C with 5% CO $_2$. Bortezomib was dissolved in DMSO and stored at -80° C. An equivalent volume of DMSO was used in all experiments as a solvent control. Cell viability was measured by using Trypan Blue exclusion.

Transfections

Human I κ B α (sc-29360), TGF β 1 (sc-44146), T β RI (sc-40222), T β RII (sc-36657), and non-silencing (sc-37007) small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology. Prior to transfections, cells were seeded (5×10^5 cells/ml) into a 6-well plate and incubated in a humidified 5% CO $_2$ atmosphere at 37 $^{\circ}$ C in antibiotic-free RPMI medium supplemented with 10% FBS for 24 hours. For each transfection, 50 nmol (final concentration) of siRNA was used. Cells were transfected with TransIT-siQUEST transfection reagent (Mirus Bio, Madison, WI, USA) as described (50). After transfection, fresh RPMI medium supplemented with FBS and antibiotics was added and cells were incubated for 48 hours.

Preparation of cell extracts and western blotting

Whole cell extracts (WCE), and cytoplasmic (CE) and nuclear extracts (NE) were prepared as described previously (47–50), and separated on 12% SDS gels. Contamination of nuclear

and cytoplasmic fractions by cytoplasmic and nuclear proteins, respectively, was determined by western analysis using LDH and lamin B as specific markers as described (47–50). To determine equal protein loading, membranes were stripped and re-probed with anti-actin antibody as described (50).

Real time RT-PCR

Total RNA was isolated by using RNeasy mini-kit (Qiagen, Valencia, CA, USA). The iScript one-step RT-PCR kit with SYBR Green (BioRad, Hercules, CA, USA) was used as a supermix and 20 ng of RNA was used as template on a Bio-Rad MyIQ Single Color Real-Time PCR Detection System (BioRad). The primers used for quantification of TGF β 1, IL-10, IL-8, IL-17, CXCR4, and actin mRNA were purchased from SA Biosciences (Frederick, MD, USA).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described (51). Briefly, proteins and DNA were cross-linked by formaldehyde, cells were washed and sonicated. The lysates were centrifuged (15,000 g, 10 min, 4 °C), and the supernatant extracts were diluted with ChIP dilution buffer and pre-cleared with Protein A/G Agarose (Santa Cruz, CA) for 2 hours at 4 °C. Immunoprecipitation was performed overnight at 4 °C, with p65, p50, cRel, RelB or p52 antibodies. Following immunoprecipitation, the samples were incubated with Protein A/G Agarose (1 h, 4 °C), and the immune complexes were collected by centrifugation (150 g, 5 min, 4 °C), washed, and extracted with 1% SDS–0.1 M NaHCO₃. After reversing the cross-linking, proteins were digested with proteinase K, and the samples were extracted with phenol/chloroform, followed by precipitation with ethanol. The pellets were resuspended in nuclease-free water and subjected to real time PCR. Immunoprecipitated DNA was analyzed by real-time PCR (25 μ l reaction mixture) using the iQ SYBR Green Supermix and the Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated by using the human IGX1A negative control primers (SA Biosciences, Frederick, MD, USA), which detect specific genomic ORF-free DNA sequence that does not contain binding site for any known transcription factors. The results were calculated as fold difference in occupancy of the particular protein at the particular locus in comparison with the IGX1A locus. Primers for TGF β 1 and IL-10 were as follows: TGF β 1: Forward, 5'-ATCTCCCTCCACCTCCCT-3' and reverse, 5'-TCCTGCTCGTCTCAGACTCTG-3'; IL-10- κ B1: forward, 5'-CTATGGAATTGAGGCTC-TTGC-3' and reverse, 5'-GCAGAAGCAGTTAGAGGTGC-3'; IL-10- κ B2: forward, 5'-CAGCCAATGTGGAATTCCC-3' and reverse, 5'-GTGGCCAAGGCTCAAACAC-3'; IL-10- κ B3: forward, 5'-TCCCACTCACTTCACGCTG-3' and reverse, 5'-AGATGGAGGGCATTTCAGT-3'.

Apoptosis assay

Apoptosis was evaluated with a cell death detection ELISA kit that quantifies release of nucleosomes into the cytoplasm (Cell Death Detection ELISA^{PLUS}, Roche, Indianapolis,

IN) as described (47). The assay was performed at the indicated time points as per the manufacturer's instructions.

ELISA

TGF β 1, IL-10, and CXCL12 cytokine release was measured in cell culture supernatants by commercially available ELISA kits (R&D, Minneapolis, MN) as previously described (50).

Transwell migration assay

Migration assays were performed using 8- μ m pore polyester Transwell inserts (Corning, MA) equilibrated in PBS buffer. Cells were pre-cultured 12 hours in serum-free RPMI medium, counted, re-suspended (5×10^4) in 100 μ l of serum-free RPMI medium containing 0, 10 or 100 nM BZ, or 300 ng/ml of TGF β neutralizing antibody or IgG1 isotype control, and added to the upper chamber. RPMI medium (650 μ l) containing 10% FBS was added to the lower chamber with or without 50 ng/ml of CXCL12 (12). Migration was allowed to proceed for 6 hours at 37°C in 5% CO₂ incubator. The inserts were then removed, washed with PBS, fixed 10 minutes in 100% methanol, and stained for 30 minutes by using 2% crystal violet. After cells were stained, the inserts were washed with water and allowed to dry before counting the migrated cells. Migrated cells were counted in five random fields. Results are expressed as migration index, which represents the ratio between the numbers of migrated treated and untreated (UT) cells.

Statistical analysis

The results represent at least three independent experiments. Numerical results are presented as means \pm SE. Data were analyzed by using an InStat software package (GraphPAD, San Diego, CA). Statistical significance was evaluated by using Mann-Whitney *U* test with Bonferroni correction for multiple comparisons, and $p < 0.05$ was considered significant.

Results

Proteasome inhibition down-regulates TGF β 1 and IL-10 expression in CTCL cells

Since we have previously shown that proteasome inhibition has a promoter-specific effect on the expression of NF κ B-dependent genes (47, 50), we wanted to determine whether BZ regulates expression of the immunosuppressive cytokines TGF β 1 and IL-10 in CTCL cells. To this end, we first measured TGF β 1 and IL-10 release from CTCL Hut-78 (left panels), H9 (middle panels) and HH cells (right panels) incubated 24 hours with increasing BZ concentrations. All three CTCL cell types release considerable amounts of TGF β 1, and 100 nM BZ, which approximately corresponds to the clinically used BZ concentrations (52), significantly inhibits the TGF β 1 release from all three CTCL cells (Fig. 1A). In contrast, IL-10 is released only by Hut-78 and H9 cells, but not HH cells, and 10 and 100 nM BZ concentrations significantly inhibit the IL-10 release (Fig. 1A).

10 and 100 nM BZ also greatly reduced the mRNA levels of TGF β 1 and IL-10 in all CTCL cells (Fig. 1B). To ensure that the decreased expression of TGF β 1 and IL-10 in BZ-treated cells was not caused by the BZ-induced apoptosis (47), we have analyzed, as a control, expression of the NF κ B-dependent pro-inflammatory genes IL-8 and IL-17. In contrast to

the decreased mRNA levels of TGF β 1 and IL-10, the expression of IL-8 and IL-17 was significantly increased in CTCL cells incubated with 10 and 100 nM BZ (Fig. 1B), demonstrating specificity of BZ effect on the expression of NF κ B-dependent genes. The BZ inhibition of TGF β 1 and IL-10 gene expression in all three CTCL cell types was time dependent (Fig. 1C).

TGF β 1 inhibition is regulated by I κ B α , while IL-10 inhibition is I κ B α -independent

Our previous studies have demonstrated that proteasome inhibition induces nuclear translocation and accumulation of I κ B α , which has a promoter-specific effect on the inhibition of NF κ B-dependent genes (47–50). Thus, we wanted to determine whether the BZ-induced inhibition of TGF β 1 and IL-10 expression is I κ B α -dependent. Hut-78 cells were transfected with control and I κ B α specific siRNA, treated with BZ, and analyzed for I κ B α , TGF β 1 and IL-10 expression. In agreement with our previous observations (47–50), 10 and 100 nM BZ induced the nuclear translocation and accumulation of I κ B α in cells transfected with control siRNA (Fig. 2A, top panel). Transfection with I κ B α siRNA reduced both the cytoplasmic and the nuclear I κ B α levels in BZ-treated cells (Fig. 2A, top panel, bottom gel, exposed together with the control siRNA gel). However, while the TGF β 1 mRNA levels were increased in cells transfected with I κ B α siRNA (Fig. 2B), the IL-10 mRNA levels were not affected by I κ B α suppression (Fig. 2C). These data indicated that the BZ-mediated TGF β 1 inhibition is I κ B α -dependent, while the IL-10 inhibition is not regulated by I κ B α .

TGF β 1 is regulated by NF κ B canonical pathway, while IL-10 is regulated by non-canonical pathway in CTCL cells

The dependence of TGF β 1 expression on I κ B α has suggested that TGF β 1 is regulated by the canonical NF κ B pathway. Using TFSEARCH program, we have identified NF κ B-binding site (GGGGACGCCC) located +94 from the transcription start site in human TGF β 1 promoter (Fig. 3A). Even though we have recently shown that p65, p50 and RelB NF κ B subunits are localized in the nucleus in CTCL cells (50), chromatin immunoprecipitation (ChIP) analysis using p65, p50, RelB, cRel and p52 antibodies has demonstrated that only p65 and p50 are recruited to the TGF β 1 promoter in Hut-78 cells (Fig. 3B). Bortezomib significantly decreased the p65/p50 recruitment (Fig. 3B), correlating with the BZ-reduced TGF β 1 expression (Fig. 1). Together with the data demonstrating that the TGF β 1 expression is I κ B α -dependent (Fig. 2B), these results have indicated that the TGF β 1 expression in CTCL cells is mediated by the classical canonical pathway (Fig. 3C).

The human IL-10 promoter contains three putative NF κ B binding sites: κ B1 (GGGAA-TTTGCCC; –18,337), κ B2 (GGGGAATTCC; –5,734), and κ B3 (GGGGAAACCC; –1,496) (Fig. 4A). p65, p50 and RelB were all heavily recruited to the κ B1 site, and this recruitment was decreased by BZ. Interestingly, BZ concomitantly increased the p52 recruitment (Fig. 4B); this is in an agreement with our recent data demonstrating that proteasome inhibition induces p52 accumulation in the nucleus in CTCL cells (50). The κ B2 site was occupied predominantly by p65 (Fig. 4C), indicating that the κ B2 site is regulated by p65 homodimers (Fig. 4D). Proteasome inhibition by BZ that decreased the IL-10 expression (Fig. 1), significantly reduced p65 recruitment to the κ B2 site, but increased the

recruitment of p52 (Figs. 4C, D). We did not find any significant recruitment of p65, p50, RelB, cRel or p52 to the κ B3 site (data not shown). These results have indicated that the IL-10 expression is regulated by the I κ B α -independent non-canonical pathway, and that the BZ-mediated inhibition of IL-10 expression is associated with increased recruitment of p52 NF κ B that functions as a repressor of IL-10 transcription in CTCL cells (Fig. 4D).

TGF β 1 increases CTCL cell viability, while inhibiting pro-inflammatory gene expression

In most cell types, TGF β 1 has growth inhibitory and pro-apoptotic effects; however, in some tumors, TGF β 1 has tumor-promoting effects that include increased cell proliferation, angiogenesis, and metastasis (53, 54). To investigate the TGF β 1 effect on survival and pro-inflammatory gene expression in CTCL cells, we suppressed TGF β 1 in HH cells that express TGF β 1 in the absence of IL-10 (Fig. 1). HH cells were transfected with control or TGF β 1 specific siRNA, and incubated 24 hours with 0, 10 and 100 nM BZ. As expected, suppression of TGF β 1 with siRNA resulted in significantly decreased TGF β 1 mRNA levels (Fig. 5A) and TGF β 1 release (Fig. 5B). Importantly, TGF β 1 suppression significantly decreased the viability of untreated and BZ-treated HH cells (Fig. 5C), indicating that TGF β 1 induces survival in CTCL cells, and suggesting that the BZ-induced apoptosis is mediated, at least partly, by the BZ-reduced TGF β 1 levels. To test whether the BZ-induced apoptosis is mediated by the reduced TGF β 1 levels, we evaluated apoptosis by the quantitative cytoplasmic nucleosome release assay. As shown in Fig. 5D, suppression of TGF β 1 by siRNA significantly increased the nucleosome release in untreated and BZ-treated HH cells, indicating that TGF β 1 mediates the spontaneous and BZ-induced apoptosis in CTCL cells. To investigate whether TGF β 1 contributes to the BZ increased IL-8 and IL-17 expression in CTCL cells (shown in Fig. 1B), we analyzed IL-8 and IL-17 mRNA levels in HH cells transfected with TGF β 1 and control siRNA. As shown in Figs. 5E and F, cells transfected with TGF β 1 siRNA exhibited significantly higher IL-8 and IL-17 mRNA levels than cells transfected with control siRNA, indicating that TGF β 1 suppresses IL-8 and IL-17 expression in CTCL cells.

The fact that TGF β 1 suppression decreased viability, and increased apoptosis, and IL-8 and IL-17 expression also in BZ-untreated cells, indicated that TGF β 1 increases CTCL cell viability, and inhibits apoptosis and inflammatory gene expression independently of bortezomib. To test this hypothesis, we have analyzed cell viability, apoptosis, and IL-8 expression in HH cells transfected with control siRNA (producing about 350 pg/ml of TGF β 1; Fig. 5B) and TGF β 1 siRNA (producing about 200 pg/ml of TGF β 1; Fig. 5B), and incubated with 0, 200 and 400 pg/ml of exogenously added recombinant TGF β 1 protein. As shown in Fig. 6A, while addition of TGF β 1 did not have any significant effect on the viability of cells transfected with control siRNA, it increased the viability of TGF β 1 siRNA transfected cells; addition of 400 pg/ml of TGF β 1 reversed the decreased viability in TGF β 1 siRNA transfected cells. Similarly, exogenously added TGF β 1 decreased apoptosis (Fig. 6B) and IL-8 mRNA expression (Fig. 6C) in TGF β 1 siRNA transfected cells, while it did not have any significant effect on cells transfected with control siRNA.

To determine whether TGF β 1 increases viability also in other CTCL cells, we tested effect of exogenously added recombinant TGF β 1 protein on the viability of untreated and 100 nM

BZ-treated Hut-78, H9, and HH cells, as well as normal human peripheral blood mononuclear cells (PBMC) used as a control. Addition of recombinant TGF β 1 did not have any significant effect on the viability of untreated cells (data not shown); this is consistent with the results demonstrating that TGF β 1 does not affect viability of HH cells transfected with control siRNA (Fig. 6A). Interestingly, addition of exogenous TGF β 1 significantly increased the viability of all three BZ-treated CTCL cells, but decreased the viability of BZ-treated PBMCs (Fig. 6D), indicating that TGF β 1 has an opposing effect on the viability of BZ-treated CTCL cells and normal PBMCs.

To examine whether TGF β 1 regulates cell viability through its receptors T β RI and T β RII, we have analyzed T β RI and T β RII protein levels in CTCL cells as well as in PBMCs, and tested whether T β RI and T β RII suppression decreases CTCL cell viability, and whether addition of exogenous TGF β 1 reverses this effect. As shown in Fig. 6E, all three CTCL cells as well as PBMCs express both T β RI and T β RII, even though the protein expression of both receptors is lower in Hut-78 and HH cells compared to H9 cells. Importantly, suppression of T β RI and T β RII in HH cells with siRNA (Fig. 6F) significantly decreased cell viability (Fig. 6G) and increased apoptosis (Fig. 6H), and addition of exogenous recombinant TGF β 1 reversed these effects, suggesting that TGF β 1 induces CTCL cell survival through the T β RI/T β RII signaling.

BZ inhibits CXCR4 expression in CTCL cells via TGF β 1-dependent mechanism

Advanced stages of CTCL and skin accumulation of malignant T cells have been associated with the increased expression of the chemokine receptor CXCR4 (11–14). Since previous studies have shown that TGF β 1 can increase the CXCR4 expression on T cells (55–57), we hypothesized that proteasome inhibition, by reducing the TGF β 1 levels, might inhibit the CXCR4 expression in CTCL cells. First, we analyzed the CXCR4 mRNA levels in CTCL Hut-78 and HH cells incubated 24 hours with 0, 10 and 100 nM BZ. As shown in Fig. 7A, BZ significantly decreased the CXCR4 mRNA levels in both CTCL cells; 10 nM BZ decreased the CXCR4 mRNA expression in Hut-78 cells by about 25%, and in HH cells by 90%. 100 nM BZ decreased CXCR4 mRNA levels in both cell types by about 90%. Bortezomib also substantially reduced the CXCR4 protein levels in Hut-78 and HH cells (Fig. 7B). Again, the CXCR4 protein levels were more affected by 10 nM BZ in HH cells than in Hut-78 cells (Fig. 7B), correlating with the mRNA data (Fig. 7A).

To determine whether the CXCR4 expression in CTCL cells is mediated by TGF β 1, we examined the CXCR4 expression in HH cells transfected with control and TGF β 1 siRNA. TGF β 1 suppression significantly reduced both the CXCR4 mRNA (Fig. 7C) and protein levels (Fig. 7D) in HH cells, demonstrating that the CXCR4 expression in CTCL cells is mediated by TGF β 1, and suggesting that TGF β 1 might promote CTCL cell migration.

TGF β 1 and CXCL12 promote CTCL cell migration

Since CXCR4 controls T cell trafficking (58), we hypothesized that suppression of CXCR4 by the BZ-reduced TGF β 1 expression in CTCL cells might decrease their migration potential. First, we tested whether BZ inhibits migration of Hut-78 and HH cells. As shown in Fig. 7E, migration of Hut-78 and HH cells was significantly decreased by 10 and 100 nM

BZ compared to untreated cells. Correlating with the CXCR4 expression (Fig. 7A), 10 nM BZ reduced migration of Hut-78 cells by about 30%, and of HH cells, by approximately 80% (Fig. 7E); 100 nM BZ decreased migration of both cell types by 80–90% (Fig. 7E). To examine whether the decreased migration of BZ-treated cells is mediated by the reduced TGF β 1 levels, we suppressed TGF β 1 expression in HH cells by siRNA, and measured their migration after 6-hour treatment with 0, 10 and 100 nM BZ. Suppression of TGF β 1 significantly reduced migration of untreated HH cells (Fig. 7F), strongly indicating that TGF β 1 promotes CTCL cell migration. TGF β 1 suppression decreased the cell migration also in BZ-treated cells (Fig. 7F), suggesting that the BZ-inhibited CTCL cell migration may be mediated, at least partly, by the reduced TGF β 1 levels.

Since CXCL12 is the major ligand for CXCR4, we wanted to determine whether CXCL12 is released by CTCL cells, whether its expression is regulated by BZ and mediated by TGF β 1, and whether it induces migration of CTCL cells. All three CTCL lines as well as normal PBMCs release considerable amounts of CXCL12 (Fig. 8A). Interestingly however, while 100 nM BZ significantly increases CXCL12 release from all three CTCL cells, it reduces CXCL12 release from PBMCs (Fig. 8A). Suppression of endogenous TGF β 1 by siRNA (Fig. 8B) or addition of exogenous TGF β 1 (Fig. 8C) did not have any significant effect on the CXCL12 release from HH cells, indicating that the CXCL12 expression is not dependent on TGF β 1 in CTCL cells.

To determine whether TGF β 1 and CXCL12 induce CTCL migration, we analyzed migration of serum-starved HH cells towards serum and CXCL12 (50 ng/ml), in the presence of 300 ng/ml of TGF β neutralizing antibody, or isotype control IgG. As shown in Fig. 8D, TGF β neutralizing antibody significantly reduced the HH cell migration both in the absence and presence of CXCL12 added to the lower chamber. Furthermore, CXCL12 increased the HH cell migration more than two-fold (Fig. 8D). Together, these results strongly indicate that TGF β 1 and CXCL12 induce migration of CTCL cells.

Discussion

Progression of CTCL has been associated with the increased expression of TGF β 1 and IL-10 that are believed to be responsible for the suppressed immunity, increased susceptibility to infections, and diminished antitumor responses. Yet, little is known about the transcriptional regulation of TGF β 1 and IL-10 in CTCL, and about their function in regulating the CTCL cell responses. Here, we show that both TGF β 1 and IL-10 expression in CTCL cells is regulated by NF κ B pathway, and suppressed by BZ-mediated proteasome inhibition. However, while the TGF β 1 expression is I κ B α -dependent and is regulated by the canonical pathway, IL-10 expression is I κ B α -independent, and its inhibition is associated with the increased promoter recruitment of p52 that characterizes the non-canonical pathway. Interestingly, our data show that TGF β 1 has opposite effect on the viability of BZ-treated cells; while it enhances the viability of CTCL cells, it decreases the viability of normal PBMCs. TGF β 1 suppression increases IL-8 and IL-17 expression in untreated and BZ-treated CTCL cells, indicating that TGF β 1 inhibits the pro-inflammatory gene expression in CTCL cells. Furthermore, our results show that TGF β 1 mediates CXCR4 expression and

CXCL12-induced CTCL cell migration, and that BZ inhibits the CXCR4 expression and CTCL cell migration (Fig. 9).

Bortezomib is the first clinically approved proteasome inhibitor that has been very effective in the treatment of multiple myeloma (MM), and has shown promising results also in patients with relapsed or refractory CTCL (37–42). However, the mechanisms of BZ function in CTCL remain incompletely understood. Previous studies from our laboratory have shown that BZ induces CTCL cell apoptosis by inhibiting expression of the anti-apoptotic genes cIAP1 and cIAP2 and the proto-oncogene Bcl3, while it concomitantly induces release of the pro-inflammatory cytokines IL-8 and IL-17, and increases their release levels to those seen in healthy adults (47, 50). In this study, we show that BZ inhibits TGF β 1 and IL-10 expression, CXCR4 levels, and CTCL cell migration, indicating that this may represent an additional mechanism of BZ function in CTCL.

Our data indicate that the BZ-induced IL-8 and IL-17 expression is mediated, at least partly, by the BZ-inhibited TGF β 1 release, since TGF β 1 suppression enhances the IL-8 and IL-17 expression in BZ-treated CTCL cells. However, since TGF β 1 suppression increases IL-8 and IL-17 mRNA levels also in BZ-untreated cells (Figs. 5E, F), these results indicate that TGF β 1 inhibits the pro-inflammatory gene expression in CTCL cells independently of BZ. The mechanisms by which TGF β 1 inhibits the pro-inflammatory gene expression in CTCL cells are currently under investigation. The increased IL-8 and IL-17 mRNA levels observed after TGF β 1 suppression are compatible with decreased transcription; however, it is also possible that TGF β 1 regulates IL-8 and IL-17 mRNA stability.

Even though TGF β 1 inhibits survival and proliferation in most cells, some tumor cells become unresponsive to the anti-proliferative effect of TGF β 1, and some actually switch to being stimulated by TGF β 1 (53, 54). Interestingly, our data show that TGF β 1 increases viability of BZ-treated CTCL cells, while it decreases viability of BZ-treated PBMCs (Fig. 6D). Intriguingly, however, this opposing effect on cell viability is observed only in BZ-treated cells (Fig. 6D); TGF β 1 has no effect on CTCL or PBMC cell viability when it is added to untreated cells (data not shown). This is consistent with results shown in Figs. 6A–C, demonstrating that exogenous TGF β 1 increases viability and decreases apoptosis and IL-8 expression in HH cells transfected with TGF β 1 siRNA, but not control siRNA. Since both 100 nM BZ and TGF β 1 siRNA suppression decrease the released TGF β 1 levels in HH cells to about 150–200 pg/ml (Figs. 1A, 5B), these results suggest that cells are responsive to exogenously added TGF β 1 only when the endogenous TGF β 1 levels are reduced. Several signaling mechanisms may be responsible for this “saturation” effect; however, the TGF β 1 receptors, T β RI and T β RII seem to be among the most plausible candidates.

Several studies have shown that CTCL patients have decreased expression of T β RI and/or T β RII, and reduced responsiveness to the growth-inhibitory effects of TGF β 1 (59–64). We have detected T β RI and T β RII in all three CTCL lines, even though Hut-78 and HH cells express considerably lower protein levels of both receptors than H9 cells (Fig. 6E). Since transfection of HH cells with T β RI and T β RII siRNA decreased their viability and increased apoptosis, and addition of exogenous TGF β 1 reversed these effects (Figs. 6F–H), these data suggest that the TGF β 1 receptors in HH cells are, at least partially, functional. However,

since CTCL cells respond to exogenously added TGF β 1 only when the endogenous TGF β 1 levels are suppressed by TGF β 1 siRNA (Figs. 6A–C), or BZ (Fig. 6D), it is possible that the TGF β 1 receptors in CTCL cells are saturated by the high levels of endogenous TGF β 1, and respond to exogenously added TGF β 1 only when the endogenous levels are decreased. In this respect, BZ might have a previously unrecognized effect on the viability of CTCL cells that is mediated by the reduced TGF β 1 levels. However, since the present study used only CTCL established cell lines, and not primary Sezary cells, it will be important in the future to determine whether the same mechanisms are operational in primary Sezary cells as well. Particularly, it would be interesting to investigate whether primary Sezary cells from BZ-treated patients have functional TGF β 1 receptors and signaling.

Importantly, in addition to the inhibition of TGF β 1 and IL-10, BZ inhibits mRNA and protein expression of the chemokine receptor CXCR4 in CTCL Hut-78 and HH cells (Figs. 7A, B). Since TGF β 1 suppression decreases CXCR4 mRNA and protein levels in CTCL cells (Figs. 7C, D), these data indicate that TGF β 1 induces the CXCR4 expression in CTCL cells, and that the BZ-decreased CXCR4 expression is at least partly mediated by TGF β 1. The proposed BZ inhibition of CXCR4 via TGF β 1 signaling is further supported by studies demonstrating that BZ decreases CXCR4 expression in BZ-resistant mouse multiple myeloma cell lines, while it does not inhibit CXCR4 expression in cells that do not express detectable levels of TGF β 1 (65–67). Since the increased CXCR4 expression has been implicated in promoting skin homing of malignant CTCL cells in patients with Sézary syndrome (12), these findings suggest that the BZ-reduced TGF β 1-CXCR4 signaling may represent yet another mechanism responsible for the BZ function in CTCL. Indeed, we have found that BZ significantly reduces migration of CTCL cells (Fig. 7E), and that TGF β 1 suppression decreases HH cell migration (Fig. 7F), indicating that TGF β 1 induces migration of CTCL cells, and suggesting that BZ inhibits the CTCL cell migration by inhibiting TGF β 1 expression. The role of TGF β 1 in inducing migration of CTCL cells is further supported by our data demonstrating that addition of TGF β neutralizing antibody significantly decreases migration of HH cells, both in the absence and presence of CXCL12 (Fig. 8D). Our data show that all three CTCL lines release considerable levels of CXCL12. However, in contrast to CXCR4, which is inhibited by BZ and dependent on TGF β 1, the CXCL12 release is increased by 100 nM BZ in CTCL cells (Fig. 8A), and its expression is not regulated by TGF β 1 (Figs. 8B, C). Since CXCL12 is a potent chemoattractant for lymphocytes, these results suggest that BZ suppresses migration of CTCL cells through the decreased autocrine TGF β 1-CXCR4 signaling, while it may concomitantly increase recruitment of non-malignant lymphocytes by the increased CXCL12 release from CTCL cells. It will be interesting to determine in future if this is indeed the case in BZ-treated CTCL patients.

In cancer cells, the immunosuppressive cytokines TGF β 1 and IL-10 are thought to be responsible for the suppression of anti-tumor immune responses and successful tumor escape (68, 69). Our data indicate that in CTCL, TGF β 1 inhibits apoptosis, blocks IL-8 and IL-17 expression, and promotes CXCR4 expression and CTCL cell migration. Thus, understanding the mechanisms regulating TGF β 1 and IL-10 expression in CTCL cells will likely reveal novel pathways that could be targeted for therapy. Our data demonstrate that the TGF β 1 expression in CTCL cells is I κ B α -dependent (Fig. 2), and correlates with the

p65/p50 occupancy at the TGF β 1 promoter (Fig. 3), indicating that the TGF β 1 expression is regulated by the canonical pathway. In contrast, the IL-10 expression is I κ B α -independent (Fig. 2), correlates with p65, p50 and RelB occupancy at the IL-10 promoter, and inversely correlates with p52 recruitment (Fig. 4). The high p65, p50 and RelB occupancy at the IL-10 promoter, and the increased p52 recruitment in BZ-treated cells, are consistent with our recent study demonstrating high levels of p65, p50 and RelB in the nucleus of CTCL cells, and increased p52 nuclear accumulation in response to proteasome inhibition (50). These results indicate that the IL-10 expression is regulated by non-canonical pathway, and suggest that p52 acts as a transcriptional repressor of the IL-10 expression in CTCL cells. Even though p52 lacks the transactivation domain, it can stimulate transcription by forming complexes with p65, or repress transcription by forming inactive p52/p52 homodimers (70–72), or associating with histone deacetylases (HDAC) (73, 74). Our data suggest that the κ B1 and κ B2 sites of the IL-10 promoter in BZ-treated CTCL cells are occupied by p52 homodimers (Fig. 4); however, it seems plausible that additional recruited transcriptional regulators, such as HDAC (73, 74) or the nuclear onco-protein Bcl3 (50) may contribute to the p52-mediated inhibition of IL-10 expression in CTCL cells.

In conclusion, our study provides a novel insight into the BZ function in CTCL, and demonstrates that in addition to inducing apoptosis, BZ has an important immunomodulatory role by down-regulating expression of the immunosuppressive cytokines TGF β 1 and IL-10, while concomitantly increasing expression of IL-8, IL-17, and CXCL12. Our data show that TGF β 1 regulates the CXCR4 expression and CTCL cell migration, and suggest that it might represent a useful tool to interfere with tumor progression in CTCL and other malignancies characterized by excessive TGF β 1 expression.

Abbreviations used in this paper

BZ	bortezomib
ChIP	chromatin immunoprecipitation
CTCL	cutaneous T cell lymphoma
LDH	lactate dehydrogenase

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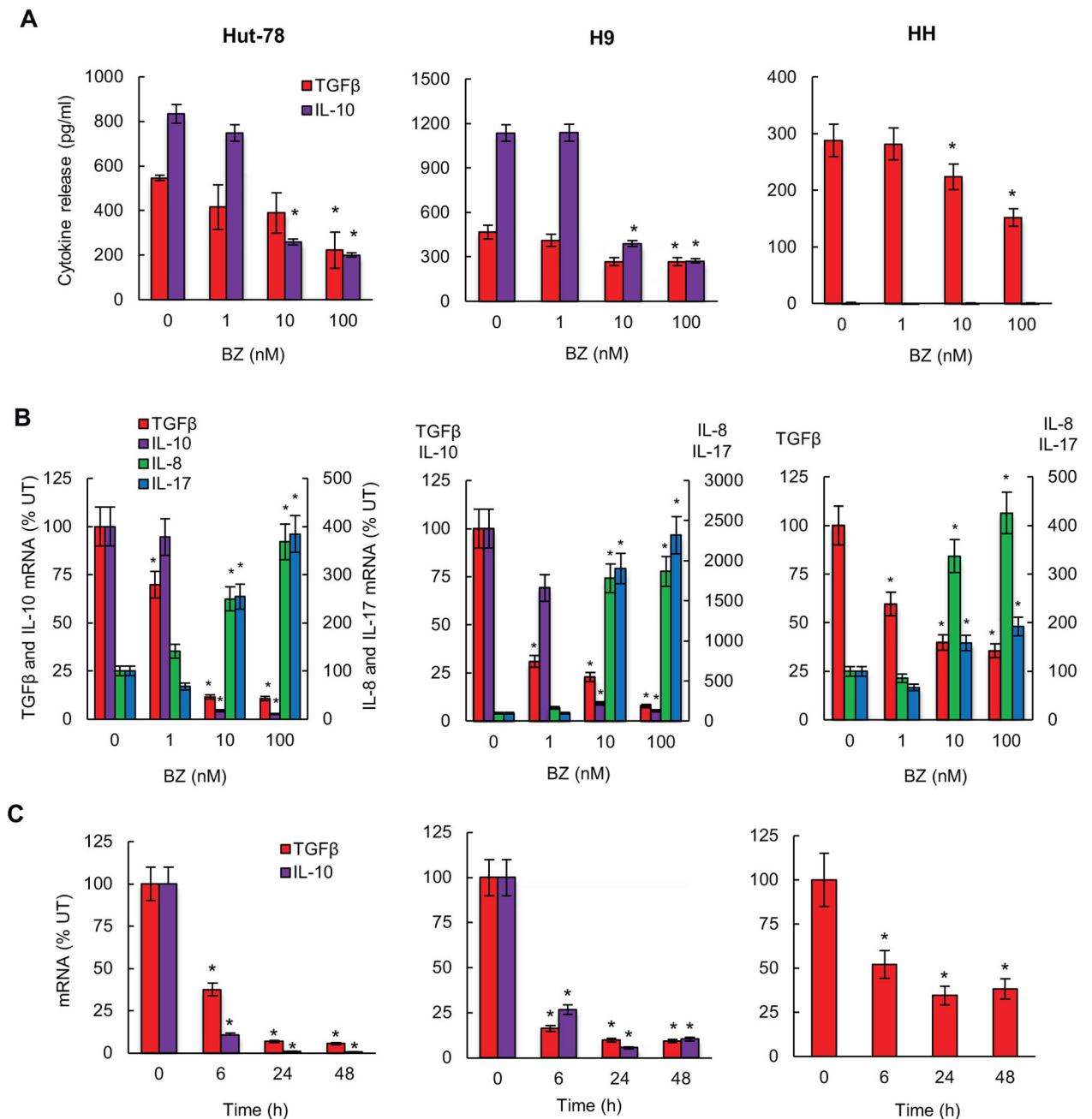


Figure 1. Proteasome inhibition suppresses TGFβ1 and IL-10 expression in CTCL cells
(A) ELISA assay of TGFβ1 and IL-10 release measured in cell culture supernatants of Hut-78 (left panels), H9 (middle panels), and HH (right panels) cells incubated 24 hours with increasing BZ concentrations. **(B)** Real time RT-PCR analysis of TGFβ1, IL-10, IL-8 and IL-17 mRNA levels in Hut-78, H9, and HH cells treated 24 hours with increasing BZ concentrations. **(C)** Real time RT-PCR of TGFβ1 and IL-10 mRNA levels in Hut-78, H9, and HH cells treated 0, 6, 24, and 48 hours with 10 nM BZ. The values in Figs. 1A-C represent the mean \pm SE of four experiments. Asterisks denote a statistically significant ($p < 0.05$) change compared to control untreated (UT) cells.

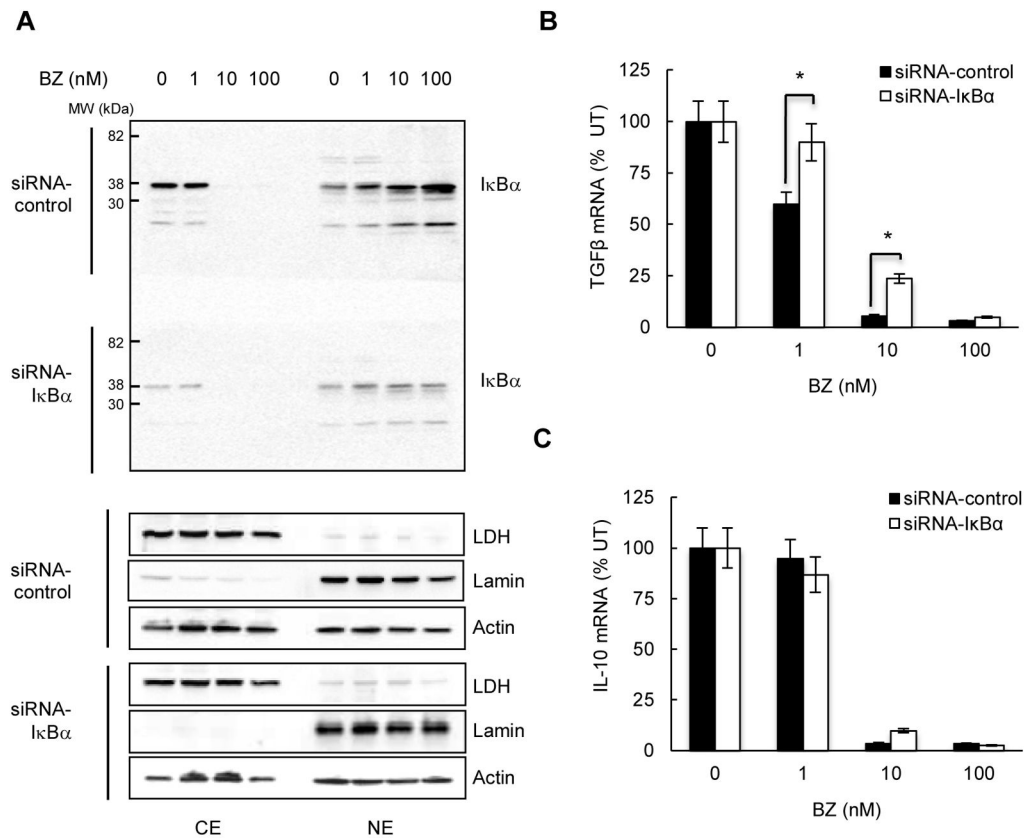


Figure 2. TGFβ1 inhibition is mediated by IκBα, while IL-10 inhibition is IκBα-independent (A) Western blotting of cytoplasmic (CE) and nuclear (NE) extracts prepared from Hut-78 cells transfected with control non-silencing (top gel) and IκBα specific (bottom gel) siRNA, incubated 24 hours with increasing BZ concentrations, and analyzed by using IκBα antibody. Both gels were transferred to a membrane and exposed together at the same time. The purity of cytoplasmic and nuclear fractions was monitored by using lactate dehydrogenase (LDH) and lamin B antibodies. To confirm equal protein loading, the membranes were stripped and re-probed with actin antibody. (B) TGFβ1 and (C) IL-10 mRNA levels in Hut-78 cells transfected with control and IκBα siRNA, and treated 24 hours with increasing BZ concentrations. The values represent the mean \pm SE of four experiments; the asterisks denote a statistically significant ($p < 0.05$) change compared to cells transfected with control siRNA.

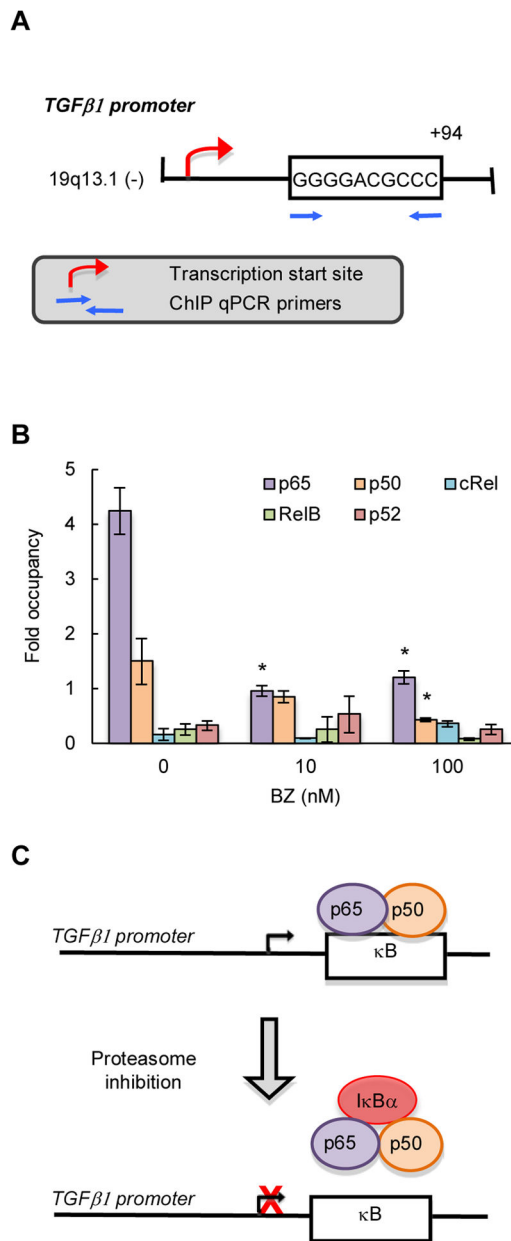


Figure 3. TGFβ1 is regulated by NFκB canonical pathway in CTCL cells

(A) Schematic illustration of NFκB binding site in human TGFβ1 promoter, and the ChIP primers used in the ChIP assay. (B) Recruitment of NFκB p65, p50, cRel, RelB and p52 subunits to TGFβ1 promoter in Hut-78 cells treated 24 hours with 0, 10 and 100 nM BZ was analyzed by ChIP and quantified by real time PCR. The data are presented as the difference in occupancy of each protein between the TGFβ1 promoter and the IGX1A (SA Biosciences) negative control locus, and represent the mean \pm SE of four experiments. Asterisks denote a statistically significant ($p < 0.05$) change compared to control (no BZ) cells. (C) Model of the regulation of TGFβ1 transcription in CTCL cells.

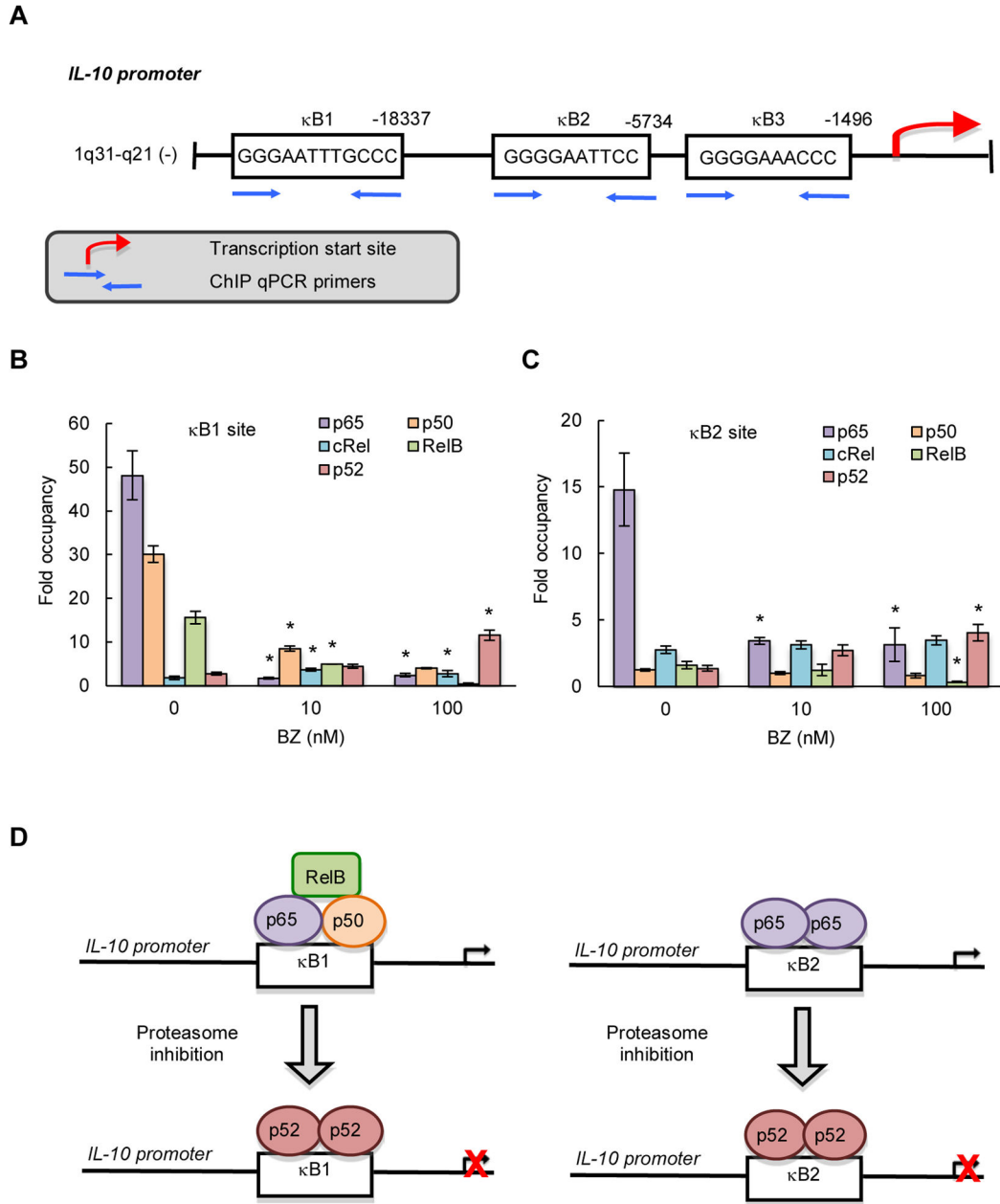


Figure 4. IL-10 is regulated by NFκB non-canonical pathway in CTCL cells

(A) Schematic illustration of NFκB binding sites in human IL-10 promoter, and the ChIP primers used in the ChIP assay. Recruitment of NFκB p65, p50, cRel, RelB and p52 subunits to the IL-10 κB1 (B) and κB2 (C) sites in Hut-78 cells treated 24 hours with 0, 10 and 100 nM BZ was analyzed by ChIP and quantified by real time PCR. The data are presented as the difference in occupancy of each protein between the κB1 and κB2 sites and the IGX1A negative control locus, and represent the mean \pm SE of four experiments. Asterisks denote a statistically significant ($p < 0.05$) change compared to control (no BZ) cells. (D) Model of the regulation of IL-10 transcription by the exchange of NFκB subunits in CTCL cells.

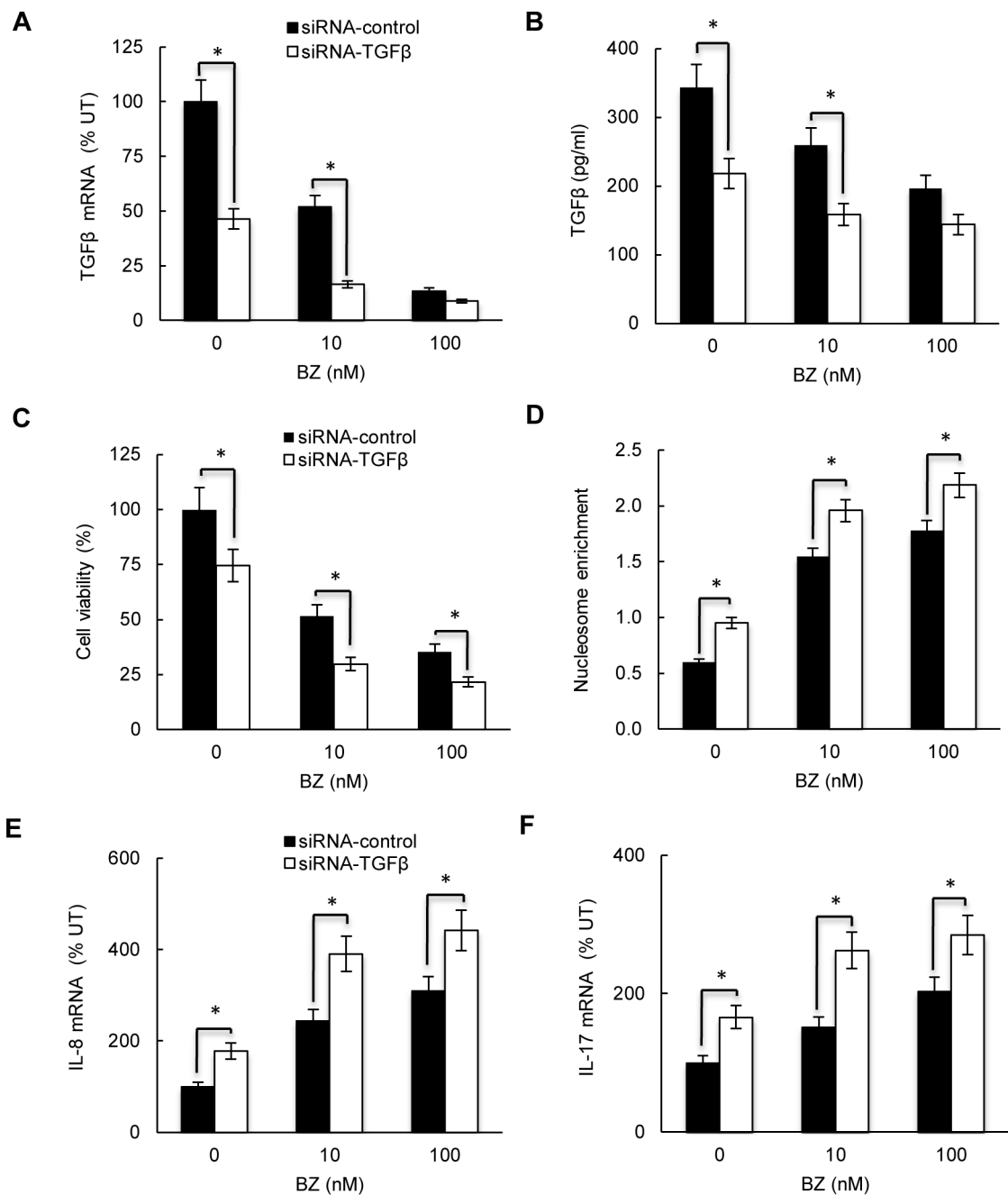


Figure 5. TGFβ1 suppression decreases viability, and increases pro-inflammatory gene expression in CTCL cells

HH cells were transfected with control (full columns) or TGFβ1 specific siRNA (empty columns), treated 24 hours with 0, 10 and 100 nM BZ, and analyzed for TGFβ1 mRNA expression by real time RT-PCR (A), and for TGFβ1 release by ELISA (B). (C) Cell viability measured by Trypan Blue exclusion, and (D) apoptosis analyzed by the cytoplasmic nucleosome enrichment assay in HH cells transfected with control or TGFβ1 specific siRNA. Real time RT-PCR analysis of IL-8 (E) and IL-17 (F) mRNA levels in HH cells transfected with control (full columns) or TGFβ1 specific siRNA (empty columns) and

treated 24 hours with 0, 10 and 100 nM BZ. The values in Figs. 5A-F represent the mean \pm SE of four experiments. Asterisks denote a statistically significant ($p < 0.05$) change compared to cells transfected with the corresponding control siRNA.

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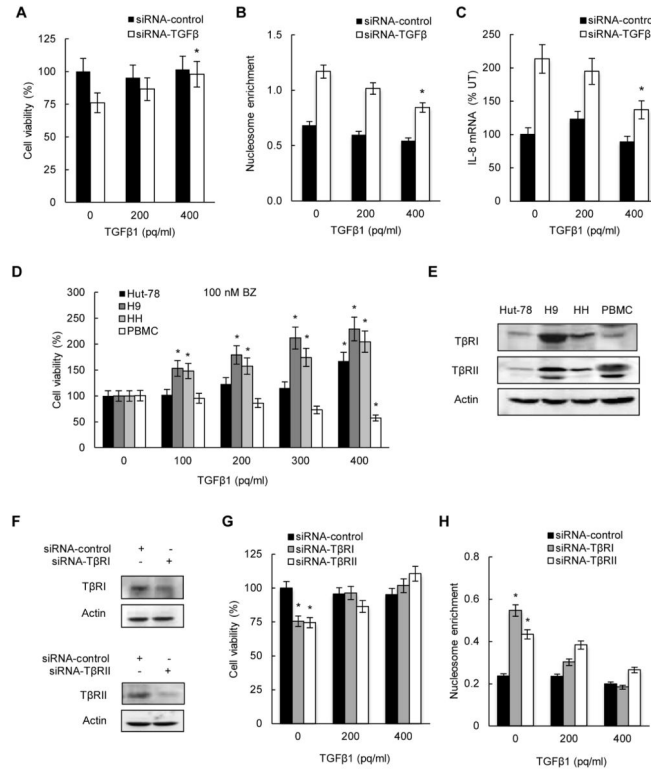


Figure 6. Exogenous TGFβ1 increases viability of BZ-treated CTCL cells

HH cells were transfected with control (full columns) or TGFβ1 specific (empty columns) siRNA, incubated 24 hours with 0, 200 and 400 pg/ml of exogenously added recombinant TGFβ1 protein, and analyzed for cell viability (A), cytoplasmic nucleosome enrichment (B), and IL-8 mRNA levels (C). (D) Viability of Hut-78, H9, HH, and PBMC cells incubated 24 hours with increasing amounts of exogenously added TGFβ1 protein, in the presence of 100 nM BZ. The values in Figs. 6A–D represent the mean \pm SE of four experiments; asterisks denote a statistically significant ($p < 0.05$) change compared to corresponding cells incubated without exogenously added TGFβ1. (E) Western blotting of whole cell extracts (WCE) prepared from untreated Hut-78, H9, HH, and PBMC cells, and analyzed by using TβRI, TβRII, and control actin antibodies. (F) Western analysis of TβRI and TβRII protein levels in WCE of HH cells transfected with TβRI, TβRII, or control siRNA. (G) Cell viability and (H) nucleosome enrichment analyzed in HH cells transfected with TβRI, TβRII, or control siRNA, and incubated 24 hours with 0, 200 and 400 pg/ml of exogenously added TGFβ1. The values in Figs. 6G and H represent the mean \pm SE of four experiments. Asterisks denote a statistically significant change compared to cells transfected with the corresponding control siRNA.

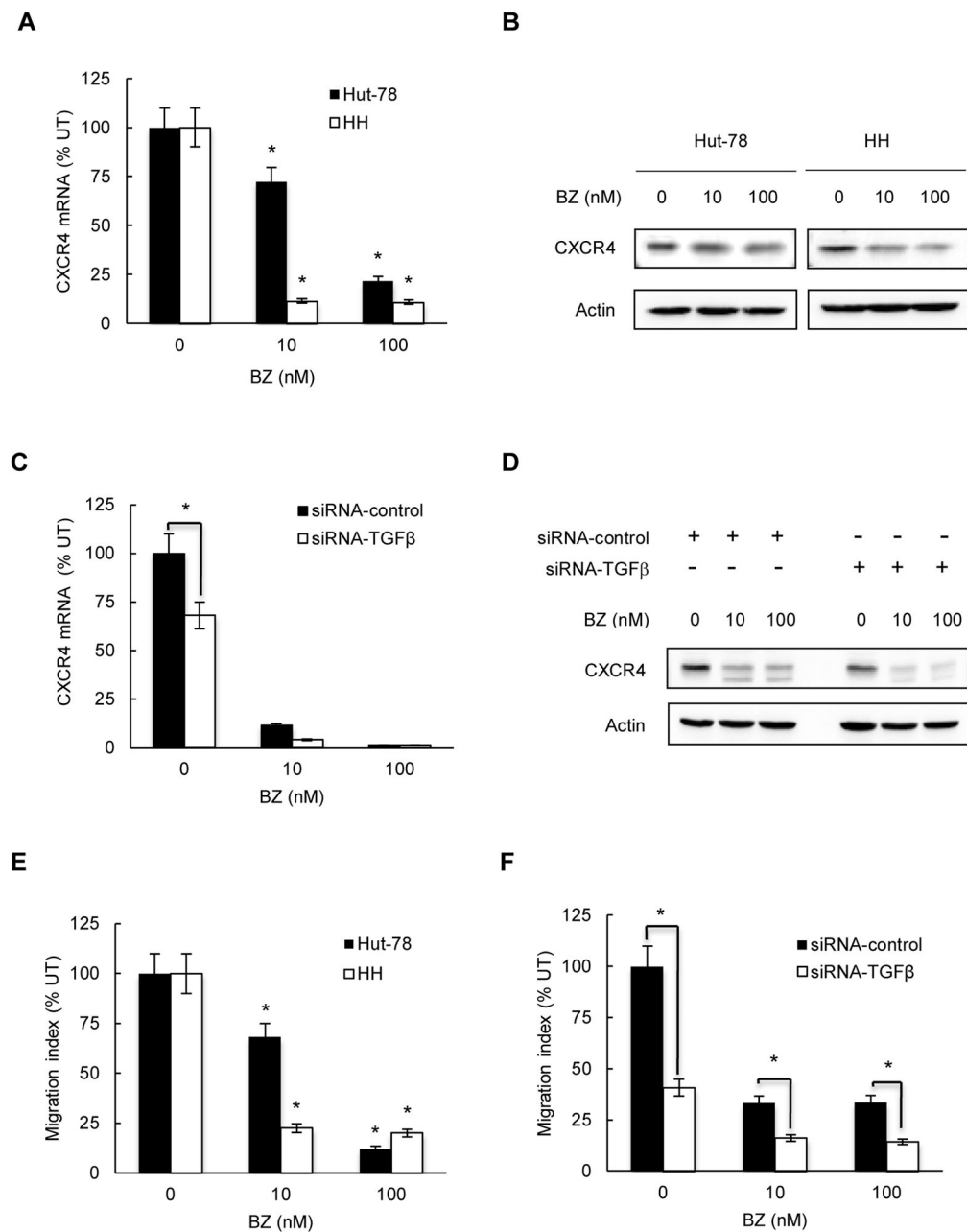


Figure 7. BZ inhibits CXCR4 expression and migration of CTCL cells

(A) Real time RT-PCR analysis of CXCR4 mRNA levels in Hut-78 (full columns) and HH (empty columns) cells treated 24 hours with 0, 10 and 100 nM BZ. The values represent the mean \pm SE of four experiments. Asterisks denote a statistically significant ($p < 0.05$) inhibition compared to untreated cells. (B) Western analysis of CXCR4 protein levels in WCE prepared from Hut-78 and HH cells treated 24 hours with 0, 10 and 100 nM BZ. Equal protein loading was confirmed by re-probing the membranes with anti-actin antibody. (C) Real time RT-PCR of CXCR4 mRNA levels in HH cells transfected with control (full columns) or TGFβ1 specific (empty columns) siRNA and treated 24 hours with 0, 10 and 100 nM BZ. The values represent the mean \pm SE of four experiments. The asterisk denotes

a statistically significant change compared to cells transfected with control siRNA. **(D)** Western analysis of WCE prepared from HH cells transfected with control or TGF β 1 siRNA, treated 24 hours with 0, 10 and 100 nM BZ, and analyzed by using CXCR4 and actin antibodies. **(E)** Migration assay of Hut-78 and HH cells incubated 6 hours with 0, 10 and 100 nM BZ. Results are expressed as migration index that represents the ratio between the number of cells that migrated in the presence of BZ and the number of migrated untreated cells. The values represent the mean \pm SE of four experiments. Asterisks denote a statistically significant inhibition compared to untreated cells. **(F)** Migration assay of HH cells transfected with control (full columns) or TGF β 1 specific (empty columns) siRNA and treated 6 hours with 0, 10 and 100 nM BZ. Results are expressed as migration index representing the ratio between the number of cells migrating in the presence of BZ and the number of untreated migrating cells; the values represent the mean \pm SE of four experiments. Asterisks denote a statistically significant change compared to cells transfected with control siRNA.

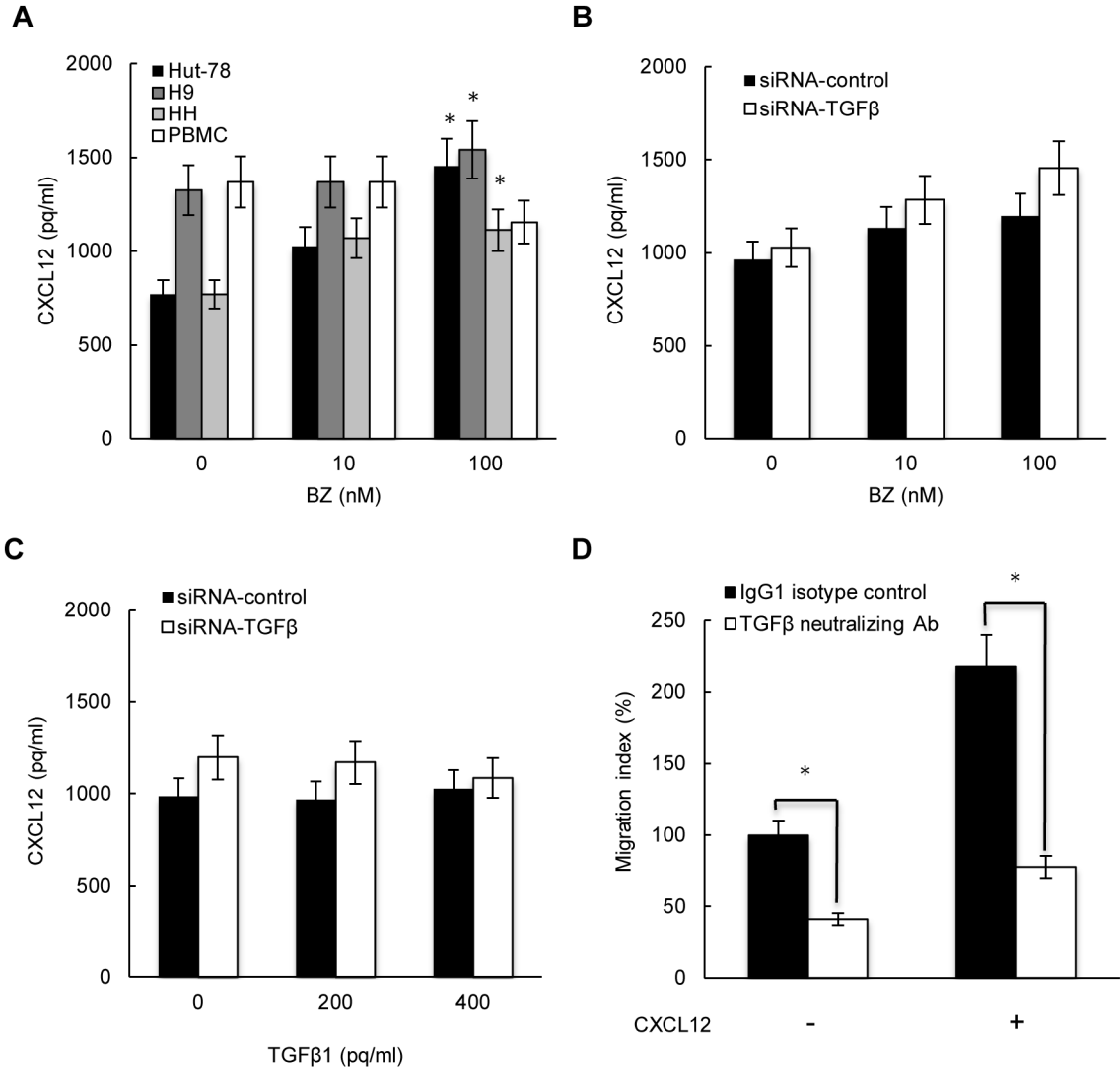


Figure 8. TGFβ1 and CXCL12 promote CTCL cell migration
(A) CXCL12 release measured in Hut-78, H9, HH and PBMC cells incubated 24 hours with 0, 10, and 100 nM BZ. Asterisks denote a statistically significant ($p < 0.05$) change compared to untreated cells. **(B)** CXCL12 release measured in HH cells transfected with control or TGFβ1 specific siRNA, and incubated 24 hours with 0, 10, and 100 nM BZ. **(C)** CXCL12 release measured in control and TGFβ1 siRNA transfected HH cells, and incubated 24 hours with 0, 200 and 400 pg/ml of exogenously added recombinant TGFβ1. **(D)** Migration assay of HH cells incubated 6 hours with 300 ng/ml of TGFβ neutralizing antibody (empty columns) or control IgG (full columns) in the absence and presence of 50 ng/ml of CXCL12 added to the lower chamber. The values represent the mean \pm SE of four experiments; asterisks denote a statistically significant ($p < 0.05$) change compared to cells incubated with control antibody.

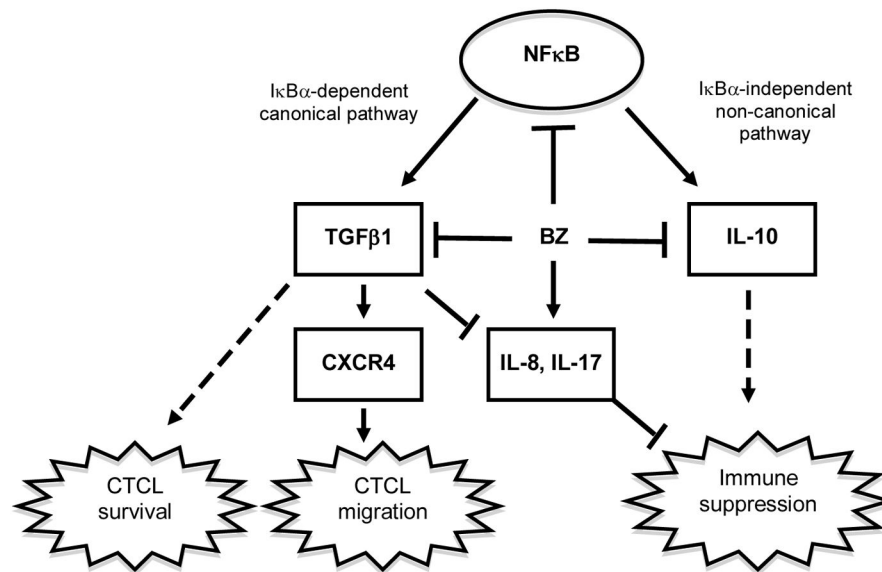


Figure 9. Proposed model of TGFβ1 and IL-10 function and regulation in CTCL cells

In CTCL cells, proteasome inhibition by BZ inhibits expression of the NFκB-dependent immunosuppressive cytokines IL-10 and TGFβ1. However, while the TGFβ1 expression is regulated by the IκBα-dependent canonical pathway, the IL-10 expression is regulated by the IκBα-independent non-canonical pathway. Suppression of TGFβ1 in CTCL cells induces apoptosis and IL-8 and IL-17 expression, while concomitantly inhibiting CXCR4 expression and CTCL migration.