1-1-2011

Ctla-4 transcriptional activation: regulation of induced expression

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CTLA-4 TRANSCRIPTIONAL ACTIVATION: REGULATION OF INDUCED EXPRESSION

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: IMMUNOLOGY AND MICROBIOLOGY

Approved by:

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Advisor Date

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ACKNOWLEDGEMENTS

I was incredibly fortunate to have a project that was both fun and challenging during my graduate school years. It goes without saying that scientific research, particularly for graduate students, can be both wearisome and frustrating at times, which is why it is critical to have a strong support network and a thoughtful, patient advisor and committee. My experience was not only more bearable but exceedingly rewarding thanks to the wonderful people in my life, and none of the work presented here would have been possible without them.

I want to thank my mentor Dr. Henry Wong for guiding me toward a graduate career. I started my journey in his lab as a technician with a bachelor's degree and a limited understanding of research techniques. He taught me not only how to design and conduct experiments, but more importantly how to think like a scientist. Henry provided an atmosphere where I was able to excel, and for that I am tremendously grateful. His expectations were always challenging but reasonable, and his confidence in me helped develop both my critical thinking as well as presentation skills. It was truly a privilege to be his first graduate student.

Next I thank my committee members: Dr. Wei-Zen Wei, Dr. Yi-chi Kong, Dr. Roy Sundick and Dr. Michael Shaw. I could not have imagined a more balanced team of faculty to provide feedback and questions. It was because of their help and guidance that this project developed into a more thorough analysis. They kept me on my toes during committee meetings which made presentations at conferences all the more painless.

I am also grateful to the department chair Dr. Paul Montgomery and the graduate officers Dr. Harley Tse and Dr. Thomas Holland. Additionally, I would like to thank the office staff Lynette, Nancy and Mary for their hard work and patience. Our lab relocated to Ohio State University, and they did an exceptional job of keeping things running smoothly for me while I was so far away.

I also thank my fellow Wayne State graduate students, in our department as well as others, for providing a fun environment even in stressful situations. My memories of graduate school will be fantastic thanks to these great people. Particularly I want to thank Angela Sosin for engaging in long-winded conversations about our classes and thesis projects. I can only hope to continue these in the future.

Our lab has been fortunate to have a multitude of students and technicians during my years here. Most recently, I thank Timothy Hake and Randi Neubeck for assisting me in experiments and keeping the lab upbeat. I really enjoy training new students and I thank them for giving me an opportunity to teach while being constantly entertained. I wish them the best of luck in their future endeavors. Also I would like to thank Dr. Anjali Mishra, a postdoc at Ohio State, for her invaluable help, guidance, sense of humor and friendship. Without her the transition to OSU would have been far more challenging and far less fun.

Lastly, but most importantly, I would like to thank my family. My brother Brian and sister-in-law Pam were very supportive through the years. My nephew Brody, though only 7 weeks old, was extremely helpful in providing breaks for me while I was writing my thesis. My parents Debbie and Dan always led by example and taught me

the value of hard work, perseverance and integrity. They may not have taught me about DNA or proteins, but it is because of them that I have become the scientist I am today. I could not have asked for better parents. My partner, Katrina has been by my side through the entirety of graduate school. I thank her for putting up with me, even the nights before big deadlines or exams. Without her patience and support I would not have made it this far.

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GENERAL INTRODUCTION

The immune response requires the coordinated function of many cell types to appropriately execute successful defense against extracellular pathogens, infected cells and malignancies. An intricate balance exists between immune activity to clear disease and immune suppression to avoid excessive damage. While rapid and robust induction is necessary to clear the body of destructive pathogens, an over-exuberant response can be equally dangerous. When left unchecked an overactive immune system develops into autoimmunity, leading to the destruction of normal tissue and the onset of diseases such as diabetes mellitus, Hashimoto's thyroiditis and multiple sclerosis among many others. Under these circumstances treatments are directed at suppressing the immune response. On the other hand, treatment of late stage malignancies often requires elimination of immune suppression to boost anti-tumor activity (76). During the onset of cancer the immune system is able to recognize and attack malignant cells; if unsuccessful in clearing the malignancy, the relationship between tumor and immune system shifts in favor of tumor tolerance, a process referred to as immunoediting (35).

Current therapies for a variety of disorders take advantage of the immune response by either targeting activation or suppression pathways. The co-stimulatory molecule cytotoxic lymphocyte associated factor 4, or CTLA-4, acts to control T cell proliferation (116), making it an excellent therapeutic target. CTLA-4 was first discovered in mouse CD8 T cells and its expression was thought to be restricted to activated T and B cells (18). Soon after a human homologue was found and shown to

be structurally similar, indicating these may be the same protein in these two species (33). The CTLA-4 protein is a 223 amino acid T cell surface molecule with an extracellular domain, transmembrane region and short cytoplasmic tail. In humans the CTLA-4 gene is located on chromosome 2, just over 100kb away from the activating receptor CD28, suggesting the CTLA-4 gene may be the result of a duplication event (44, 58). CTLA-4 and CD28 share about 20-30% sequence homology, particularly in exon 2 where the hexapeptide MYPPPY is found on both proteins (23, 58). They each form homodimers and both interact with antigen presenting cell molecules CD80 and CD86, collectively known as B7 (100, 105). However, CTLA-4 binds B7 with at least 10-fold higher avidity (63), which is probably due to its bivalent interaction with B7 versus the monovalent binding of CD28 (100, 105). This phenomenon allows negative T cell regulation to take priority over activation.

The CTLA-4 gene is induced in effector CD4 and CD8 cells upon activation (94), however we detect higher expression levels in memory CD4 populations than CD8 following stimulation (unpublished data). Regulatory T cells (Tregs), which maintain peripheral tolerance and control immune activation, constitutively express CTLA-4 on their cell surface, again demonstrating the role of this gene in immune response control (55, 101, 108). The distinctive methods of expression between regulatory and effector T cells are not well understood; it would be valuable to elucidate what properties direct constitutive versus inducible activation. This work focuses on regulation of the inducible CTLA-4 in human CD4 T cells.

Effects of CTLA-4 dysfunction in mouse models and human disease. The critical role CTLA-4 plays in immune regulation becomes clear when this gene is knocked out in an animal model. Transgenic mice suffer severe lymphoproliferation and infiltration leading to multi-organ failure and death within 3-4 weeks (111, 123). Target organs include liver, heart, lung and pancreas. Knockout T cells proliferate rapidly and robustly, supporting CTLA-4's function as a braking system for T cells which helps maintain homeostasis in the adaptive immunity. The T cell receptor (TCR) associated tyrosine kinases FYN, LCK and ZAP-70 are robustly activated in the transgenic mice (72). Addition of CTLA-4 immunoglobulin (Ig) can block the lymphoproliferative effects in CTLA-4 -/- mice, but this therapy does not have long-term function as T cell activation resumes upon removal of the Ig (112).

In humans, CTLA-4 dysregulation has been linked to autoimmunity. Polymorphisms of CTLA-4 have been discovered, including three within non-coding promoter or regulatory regions at positions -1722, -1661, and -318, one within exon 1 at position +49 and a dinucleotide repeat microsatellite within the 3' UTR. These abnormalities correlate to various immune-mediated diseases including Grave's, systemic lupus erythematosus, Hashimoto's thyroiditis and type 1 diabetes, again implicating its essential role in normal human immune function (9, 56, 61). While the three non-coding polymorphisms likely affect transcription efficiency, the A49G substitution is within the signal sequence and results in atypical processing by the endoplasmic reticulum and thus reduced surface expression (4). A study by Huang, *et al* demonstrated the impact of the AT repeat on T cell activity, showing the length of the repeat was proportional to serum IL-2 levels in their specimens and PBMC isolated from

patients with longer repeats were more proliferative upon stimulation of the TCR and CD28. Those with longer alleles were also more prone to myasthenia gravis development (49). Taken together, these studies have shown the impact dysfunctional CTLA-4 can have on immune modulation.

Splice variants also exist for CTLA-4 and each has an influence on function. Mice may express a variant lacking exon 2, but humans do not generate this protein. Removal of exon 3, where the transmembrane region is located, results in a soluble, secreted form (sCTLA-4). This transcript has been detected in B cells and unstimulated T cells (69, 80). Upon activation, the level of sCTLA-4 decreases in T cells, which then begin to favor the full transcript form. The protein was still capable of binding B7 and inhibiting PBMC proliferation in a mixed leukocyte reaction. Basal levels of sCTLA-4 were measured in a percentage of normal donor sera. Interestingly, in patients with autoimmune thyroid disease, which includes Grave's disease and Hashimoto's thyroiditis, the level of serum sCTLA-4 is significantly higher (79). Similar findings were reported for systemic lupus erythematosus (66). A less characterized splice form containing only exons 1 and 4 is also found in T cells (113). Given that this protein lacks both the transmembrane and B7 interacting regions, it is not clear what the physiological function might be or whether dysregulation of mRNA processing to favor this variant may contribute to disease.

Aside from autoimmunity, where decreases in functional CTLA-4 are observed, CTLA-4 can also play a role in tumor biology. In the case of cutaneous T cell lymphoma (CTCL), a malignancy of skin-homing T cells, CTLA-4 is up-regulated in the tumor population (13, 126). This phenomenon could in part explain the reduced cytokine

production seen in later stages of the disease (29). CTCL may serve as a direct example of a tumor population mediating immune response through CTLA-4. It is unclear if this gene is regulated abnormally in other malignancies.

Mechanisms of T cell suppression by CTLA-4. There are two distinct processes by which CTLA-4 exhibits its suppressive activity. Through its high efficiency interaction with B7 molecules, CTLA-4 sequesters local B7 to prevent stimulation of nearby T cells (82). By use of a CTLA-4 mutant lacking the cytoplasmic tail, Carreno *et al* show B7 binding is sufficient for suppression of IL-2 production and the level of suppression is proportional to the density of CTLA-4 on the cell surface (22). This not only highlights the importance of B7 binding, but shows the level of surface CTLA-4 can be important in immune regulation.

In addition to antagonistic competition for B7, multiple studies have shown that the CTLA-4 cytoplasmic tail contributes to inhibitory signal transduction, but the exact mechanisms remain elusive. A ligand-independent mutant of CTLA-4 was able to reverse the hyperreactivity of CTLA-4 -/- T cells; proliferation, cytokine production and TCR-mediated ERK activation were significantly reduced (26). Current research attributes CTLA-4 inhibitory signaling to a combination of dephosphorylation of TCRζ and TCR-associated kinases, binding and trapping of phosphatidylinositol 3-kinase (PI3K) and inactivation of Ras signaling (27, 93, 109, 110). As CTLA-4 lacks intrinsic enzymatic activity within its cytoplasmic tail, it is likely a series of enzymes interact with CTLA-4 to mediate regulatory functions.

After T cell activation, CTLA-4 binds to the TCRζ chain and recruits tyrosine phosphatase SHP-2 to remove phosphate groups from TCRζ (60). Both CTLA-4 and CD28 cytoplasmic tails contain Src homology 2 (SH2) domains with a similar tyrosine-containing domain. CTLA-4 is also known to cooperate with phosphatidylinositol 3-kinase (PI3K) at the YVKM motif of SH2 on the cytoplasmic tail with similar affinity to the interaction with the CD28 motif YNMN (98). A follow-up study reported phosphorylation of the tyrosine in the CTLA-4 YVKM domain by resting lymphocyte kinase (Rlk) is necessary and sufficient for PI3K interaction (99). It is thought that CTLA-4 binds to PI3K and prevents its contact with CD28. Tyrosine phosphorylation is not required, however, to block IL-2 production, indicating CTLA-4 utilizes its multiple inhibitory mechanisms to regulate T cell response (10).

Beyond TCR kinase activation, Ras is also induced upon T cell stimulation. The serine/threonine phosphatase PP2A, which downregulates the Ras/ERK pathway, associates with CTLA-4 and may affect T cell activation (31). However, the regulatory subunit of PP2A interacts with a 3-lysine repeat on the CTLA-4 cytoplasmic tail and inhibits repression by CTLA-4 as measured by IL-2 production (11). PP2A also interacts at a similar domain on the cytoplasmic tail of CD28 and the exact mechanism of activity remains unclear (31). The Ras pathway is also regulated by CTLA-4 through interaction of tyrosine phosphatase SYP at the YKVM domain of the CTLA-4 cytoplasmic tail (72). The Ras regulator $p52^{SHC}$ is shown by Marengere *et al* to be dephosphorylated upon SYP/CTLA-4 interaction. Simply transducing the cytoplasmic domain has proven effective in the prevention of collagen-induced arthritis,

demonstrating the compelling potential of signaling regulation through the cytoplasmic tail (28).

Control of CTLA-4 activity by intracellular trafficking. Appropriate surface CTLA-4 levels are regulated by trafficking between the cell membrane, endosomes and lysosomes. Though previous studies have implied unstimulated T cells express CTLA-4 in intracellular stores which are quickly transported to the surface upon activation (53), these experiments used cell clones that were cultured with IL-2 and CD3 antibodies, thus activating the cells. A report by Linsley, *et al* developed monoclonal antibodies to CTLA-4 and found surface expression in resting cells was undetectable, as was mRNA transcript (64). In fresh unstimulated primary cells from normal donors we have observed CTLA-4 protein levels are undetectable both at the cell surface and within intracellular stores. We have also found transcriptional regulation mediates protein expression, which does not begin until cells are activated. Regardless, it is clear that transport to and from the cell surface is an extremely important and highly regulated process through which T cells control to what degree CTLA-4 becomes involved in the immune response.

Internalized CTLA-4 is located near the microtubule-organizing center (MTOC), where it can be rapidly polarized to the site of cell-cell interaction upon TCR engagement (62). Recruitment of CTLA-4 is highly dependent on the strength of TCR signaling (37). Low levels of stimulation do not induce CTLA-4 localization to the cell membrane, but strong signaling does. As CTLA-4 functions as an immune suppressor, it is intuitive that it would only be required during potent T cell activation.

The transient surface expression of CTLA-4 is the result of concerted surface budding and internalization into endosomes. CTLA-4 mRNA is targeted to and processed by the rough endoplasmic reticulum and the protein is glycosylated by the Golgi complex to form dimers (18, 33). While in the Golgi, CTLA-4 interacts with clathrin adapter complex AP-1 at the YVKM motif in the SH2 region of the CTLA-4 cytoplasmic tail (97). The AP-1 molecule is involved in transport of excess CTLA-4 to the lysosome for destruction. CTLA-4 interacts at the same motif with the μ2 subunit of adapter AP-2 at the cell surface, but only when the cytoplasmic tail tyrosine residues are not phosphorylated (102). Interestingly, the phosphorylated cytoplasmic tail is capable of interacting with SYP and PI3K, and therefore the phosphorylated species remains on the cell surface (131). Phosphorylation can be mediated by members of the Src family of tyrosine kinases FYN, LYN and LCK, which may ultimately control whether CTLA-4 remains on the cell surface (17, 74).

The medium chain subunit of AP-2, AP50, also interacts with the YVKM domain in an unphosphorylated state and has been shown to direct internalization into endosomes (131). Tyrosine mutation of the CTLA-4 cytoplasmic tail abrogates AP50 interaction and leads to accumulation on the cell surface (30). Upon internalization, CTLA-4-containing endosomes traffic to the lysosome (81), but degradation is not inevitable. Instead, if the cell is appropriately activated the lysosome takes on a secretory function, thus recycling CTLA-4 for reuse to control the immune response (15, 51). The typical half-life of CTLA-4 protein is approximately 2 h (37, 81).

Therapeutic targeting of CTLA-4. Standard autoimmune therapy includes immunosuppressants such as cyclosporin, corticosteroids and methotrexate which dampen the unrestrained immune response. These medications do not exclusively target the immune system; the associated side effects are systemic and can limit the treatment regimen (88, 95, 114). Biologics have more recently come into play, including use of interferons, TNFα inhibitors, costimulatory molecule inhibitors and antibodies to deplete specific populations of immune cells. CTLA-4 has shown promise as a target for autoimmunity, particularly in the case of rheumatoid arthritis (RA). The costimulation modifier abatacept is a fusion protein containing the B7 binding domain of CTLA-4 attached to the constant region fragment (Fcγ) of human IgG1 (91). The Fcγ hinge was modified to eliminate Fc receptor recognition, which would reduce circulating drug levels. Acting as a native CTLA-4 protein, this compound presumably binds to B7 and suppresses immune activation. This therapy has proven effective in RA, even in patients who are refractory to other immune and biologic therapies, and is better tolerated than most alternatives (96). A similar fusion protein, belatacept, substitutes two amino acids to favor a tighter interaction between B7 and CTLA-4, thus acting as a more potent inhibitor. This compound has proven useful in transplant patients, where abatacept was less effective due to insufficient CD86 binding (43).

Tumor vaccination has been utilized in clinical trials for a variety of cancers (39). A potent adaptive T cell immune response subsequent to vaccination is necessary to attack and eradicate malignant cells; however, in many cases late stage patients have developed Treg populations that, in fact, protect the tumor. Efforts to target Tregs using CD25-directed immunotoxins such as RFT5-SMPT-dgA have entered clinical trials, but

reduction is incomplete (90). Instead by targeting CTLA-4, levels of immune response can be increased to help fight the tumor. Fusion proteins with CTLA-4 joined to a tumor antigen of a B cell malignancy were examined in a mouse model in an effort to boost immune activity while simultaneously vaccinating against tumor cells (50). The result of this study was an elevated antibody response, supporting CTLA-4 blockade as a valuable therapy.

Efforts to obstruct CTLA-4 function using antibodies in a mouse model resulted in tumor clearance and immunity to future tumor cell injections (59). Since then, human antibodies to block CTLA-4 activity, particularly the FDA approved drug ipilimumab, have shown considerable success in clinical trials in conjunction with vaccines, chemotherapy and other treatments for metastatic melanoma and prostate cancer to revive the anti-tumor immune response (19, 25, 124). While these efforts have shown promise, many patients experience severe adverse side effects including extensive autoimmunity. Treatments that target CTLA-4 at the transcriptional level may improve efficacy while avoiding overstimulation of the immune response.

Transcriptional regulation of CTLA-4. Analysis of CTLA-4 induction has been limited. An early study found expression was limited to T cells and the first 335bp of the proximal promoter were necessary for maximum activation (87). They also provide evidence that expression is regulated at the level of transcription. A follow-up report found synergistic induction of CTLA-4 occurs with engagement of both the TCR and CD28, which can be blocked with either cyclosporin A or rapamycin (38). In addition, mRNA stability is doubled when CD28 is activated together with the TCR. Work by

Miller *et al* showed a correlation between reduced NFAT1 expression and reduced CTLA-4 (73). Our previous work identified a bona fide NFAT1 binding site in the proximal promoter of CTLA-4 (41). Upon stimulation of cells, NFAT1 binding is detected and histone acetylation occurs. Through discovery of the mechanisms regulating transcription, we feel that distinctive pathways can be targeted to modulate CTLA-4 expression for the novel treatment of autoimmunity and cancer.

MATERIALS AND METHODS

Isolation of primary CD4 T cells and PBMCs: In brief, CD4 T cells were purified from Columbus Red Cross apheresis leukopacks from healthy donors. Cells were collected and incubated with 1.5 mL CD4 T cell Rosette Sep (Stem Cell Technologies) per 50 mL leukopack blood with rocking for 30 min. Afterwards, cells were diluted with PBS, underlayered with Ficoll gradient, and separated by centrifugation at 1600 x *g* for 30 min. The band of CD4s was isolated by aspiration and washed to remove the Ficoll. Residual RBC were lysed by resuspending the CD4 pellet with ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA, pH 7.3) for 5 min. The CD4s were diluted in PBS, centrifuged at 1600 x *g*. Final purities were >90% purity for all donors. PBMCs were isolated using the Ficoll protocol as stated above. Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum.

Cell line culture: The E6-1 clone of Jurkat T cells were maintained at 10^5 to $2x10^6$/mL in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Biochemicals: All stimulations were with 50 ng/mL PMA and 1 μg/mL A23187. Bortezomib (Millennium Pharmaceuticals) was reconstituted in water and maintained at a stock concentration of 2.5 mM. ALLN and ALLM peptides (Sigma) were reconstituted in DMSO at stock concentrations of 10 mM. MAPK inhibitors SB203580 and PD98059 (Sigma) were maintained at stock concentrations of 10 mM in DMSO. TPCA-1 was

reconstituted and stored at a stock of 10 mM in DMSO. Curcumin and garcinol (Sigma) were stored as stock concentrations of 10 mM in DMSO. Cyclosporin A (Sigma) was reconstituted at 10 mg/mL in 100% ethanol. Camptothecin, SN-38 and etoposide (Sigma) were all reconstituted in DMSO at 10 mM stock concentrations. All chemicals were stored at -20^{o}C prior to use.

RNA isolation: Total RNA was isolated from cells using TRIzol (Invitrogen) as recommended by the manufacturer. Briefly, up to 10^7 cells were lysed in 1 mL TRIzol and incubated at room temperature for 5 min. 200 μL chloroform was added followed by vigorous shaking for 15 s. Samples were centrifuged for 15 min at 12 kRPM at 4^{o}C and the aqueous layer was transferred to a new tube. The RNA was precipitated by addition of 600 μL isopropanol, thorough mixing and incubation at room temperature for 10 min. The RNA was pelleted by centrifugation at 12 kRPM at 4^{o}C for 10 min followed by washing with 70% DEPC ethanol. Samples were resuspended in 15 μL DEPC water and heated to 55^{o}C for 5 min prior to quantification by absorption at 260 and 280 nm.

First strand cDNA synthesis: Reverse transcription was performed with up to 4 μg total RNA. Briefly, RNA was diluted to 10 μL with DEPC water and combined with 1 μL oligo dT (Invitrogen) and 1 μL 10 mM dNTP mix (Invitrogen) and incubated at 70^{o}C for 10 min after which the samples were placed on ice. A master mix of 4 μL SuperScript II 5x buffer, 2 μL 0.1 M DTT and 1 μL 50 mM $MgCl_2$ per sample was made and 7 μL was distributed to each sample. Tubes were transferred to 42^{o}C and 1 μL SuperScript II reverse transcriptase was added. After 1 h at 42^{o}C the samples were transferred to

70^{o}C for 15 min to deactivate the enzyme. The volume was then increased to 50 μL per μg RNA initially added and samples were stored at -20^{o}C until use.

Quantitative real-time PCR (qPCR): Total RNA was isolated from cells using TRIzol as recommended by the manufacturer. Reverse transcription was performed with up to 4 μg total RNA to generate cDNA using SuperScript II reverse transcriptase (Invitrogen) and quantitative PCR was performed with the equivalent of 10 ng RNA per sample using an Applied Biosystems 7900HT set for 40 cycles at 95^{o}C for 15 s, 60^{o}C for 1 min/cycle. Primers are listed in Table I and have similar amplification efficiencies. Analysis for relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method (67). The expression of each gene in each sample was performed in duplicates and the level was normalized relative to B2-microglobulin (B2M) for mRNA samples and input DNA for ChIP samples.

Gene	Forward	Reverse
Actin	TGCGTTGTTACAGGAAGTCCC	CTATCACCTCCCCTGTGTGGA
B2M	TCTACTTTGAGTGCTGTCTCCATGT	AAGTTGCCAGCCCTCCTAGAG
CTLA-4	CTACCTGGGCATAGGCAACG	CCCCGAACTAACTGCTGCAA
FoxP3	ATCCGCCACAACCTGAGTCT	GTCCACACAGCCCCCTTCT
GAPDH	CCCACTCCTCCACCTTTGAC	CATACCAGGAAATGAGCTTGACAA
GATA3	TCTGGAGGAGGAATGCCAAT	CCGGGTTAAACGAGCTGTTC
IFN-γ	TCCTGTCACTGTCTCACTTAATCCTT	TTAGGTTGGCTGCCTAGTTGG
IL-2	GCAACCATTGTAGAATTTCTGAACAG	CTGATATGTTTTAAGTGGGAAGCACT
IL-4	CACAGGCACAAGCAGCTGAT	CTCTGGTTGGCTTCCTTCACA
NFAT1	TCCTGGAGATACCCTTGGAGC	AGTCGATGGTTGCCCTCATG
NFκB p50	CAAATAGACGAGCTCCGAGACA	GAGACTCGGTAAAGCTGAGTTTGC
TNF-α	GGAGAAGGGTGACCGACTCA	CTGCCCAGACTCGGCAA
ChIP CTLA-4	GAGGACCCTTGTACTCCAGGAA	CGAAAAGACAACCTCAAGCACTC
ChIP CTLA-4 Cotransfection	GAGGACCCTTGTACTCCAGGAA	AGATCGCAGATCTCGAGGGCT

Table I. PCR Primer Sets

Protein lysates: All lysis buffers were supplemented with protease inhibitor cocktail at 1μL/mL buffer immediately prior to use. Whole cell total protein was prepared from 15 million cells by resuspension of the cell pellet in 100μL RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS). Samples were briefly sonicated to shear genomic DNA and centrifuged to remove cellular debris. Nuclear and cytosolic fractions were isolated by swelling cells in 100μL Buffer A (10mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl) for 15 min on ice followed by centrifugation at 6kRPM for 15 min at 4^{o}C. Supernatant was removed and kept as the cytosolic fraction. The nuclear pellet was then resuspended in 100μL Buffer B (20mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, 25% glycerol) and briefly sonicated to shear genomic DNA. Both the nuclear and cytosolic fractions were then centrifuged to remove cellular debris. All lysates were stored at -80^{o}C prior to use.

Immunoblot analysis: Equal amounts of protein as indicated were denatured in 2x Laemelli buffer and boiled 5 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes in transfer buffer (25 mM Tris base, 192.5 mM glycine, 15% methanol) overnight at 35 mA in 4^{o}C with stirring. The membrane was then blocked in I-Block (ABI) for 1 h at room temperature and incubated overnight at 4^{o}C with one of the following primary antibodies: GATA3 (Abcam), phospho-GATA3 (Abcam), Actin (Santa Cruz), NFAT1 (Santa Cruz), FoxP3 (Abcam), polyubiquitin (Cell Signaling), IκB (Cell Signaling), CTLA-4 (Beckman Coulter), histone 3 (Cell Signaling), phospho-MAPKAPK2 (Cell Signaling) at 1:1000 dilution in I-Block. The membrane was washed five times in

TTBS and the specific protein was detected with an appropriate secondary Ab (Santa Cruz) at 1:2000 dilution in I-Block for 1.5 h. After washing in TTBS five times, protein bands were visualized by chemiluminescence autoradiography with either SuperSignal West Pico or Femto reagents (Pierce).

20S proteasome assay: Cell lysates were prepared from $50x10^6$ cells by resuspension in Proteasome Lysis Buffer (40 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 with addition of ATP to 2 mM immediately before use) and incubation on ice for 30 min, briefly sonicated to shear genomic DNA and centrifuged to remove debris. 10 μg protein was diluted into a total of 40 μL lysis buffer in an opaque 96 well plate. After addition of 10 μL 37.5 mM Suc-LLVY-AMC in DMSO (Enzo Life Sciences), reaction was incubated at room temperature and fluorescence was read every 5 min on a Thermo Fluroskan Ascent FL at an Excitation of 360 nm, Emission of 460 nm and gain of 85. Samples were analyzed in quadruplicate and normalized to substrate in lysis buffer without extract.

Flow cytometric analysis: Cells were stained for extracellular expression of CD4 and CTLA-4 with 1 μL antibody (Beckman Coulter IM2636U and BD 555853, respectively) per 10^6 cells for 20 min prior to washing with PBS and analysis on a FACS Calibur. Intracellular expression was measured by permeabilization and fixation with the FoxP3 staining buffer set (eBioscience). Briefly, 4x permeabilization/fixation concentrate was diluted to 1x with the provided diluent. 10^6 cells were resuspended in 1 mL 1x buffer and were incubated on ice 30 min. Cells were centrifuged and washed 1x with PBS

followed by staining in 100 μL PBS with 1 μL PE-GATA3 (eBioscience 12-9966-42) or 1 μL PE-CTLA-4 (BD 555853) for 3 h. Cells were washed three times with 100 μL PBS, resuspended in 300 μL PBS and analyzed on a FACS Calibur. Data was analyzed with FlowJo software.

Annexin V/PI staining for apoptosis: Staining buffer was diluted to 1x with ddH_2O. 10^5 cells were resuspended in 100 μL 1x staining buffer with 1 μL FITC-annexin V and 1 μL PI, then incubated 15 min at room temperature. Cells were then diluted to 500 μL with 1x staining buffer and transferred to 5 mL tubes followed by analysis by flow cytometry.

Mixed lymphocyte reaction: Primary CD4 T cells were PMA/A23187 stimulated with 0, 0.1 and 10 μM bortezomib for 9 h and allogeneic PBMCs were fixed with 50 μg/mL mytomycin C for 30 min. Cells were then washed with PBS three times for 10 min each with rocking. After counting, cells were plated in a 96-well flat bottom plate at $5x10^5$ PBMCs mixed with $5x10^5$ CD4s per well. Samples were treated with 0.5 μg CTLA-4 blocking antibody or mouse IgG2a control (Beckman Coulter IM2070 and A55763, respectively) and volumes were raised to 100 μL per well with RPMI 1640 medium with 10% fetal bovine serum. After incubating 7 d, proliferation was measured by addition of 20 μL MTS reagent (Promega G5421), 3 h incubation at 37^oC and measurement of absorbance at 570 nm on a Thermo MultiScan plate spectrophotometer.

Plasmids: CTLA-4 promoter constructs were cloned into the pGL3 luciferase vector as previously described (41). The promoterless pGL3 Basic and SV40-driven pGL3 Control were used as controls. The GATA3 expression vector was kindly received from Dr. Gerd Blobel. E1A 12S WT and mutant constructs were described previously (127).

Reporter transcription analysis: Jurkat cells were seeded in 6 well plates at $1.5x10^6$ cells per sample in serum-free RPMI-1640. 1 μg CTLA-4 380bp luciferase construct was transfected into each sample with increasing concentrations (0, 0.5, 1, 2 and 3 μg) GATA3 expression vector or vector control plasmid using Lipofectin (Invitrogen) per manufacturer protocol in triplicate. Cells were lysed in luciferase lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100), with DTT added to 1 mM immediately prior to use, and equal protein concentrations were subjected to luciferase analysis. The luciferase level was determined using a Lumat LB 9501 luminometer as described in *Current Protocols in Molecular Biology* (5).

Chromatin immunoprecipitation (ChIP) assay: Cells were stimulated and treated as indicated, harvested and fixed for 10 min in 1% formaldehyde to crosslink protein and DNA complexes. The reaction was quenched with addition of 1.25 M glycine for 5 min, cells were pelleted and resuspended in sonication RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% SDS). Samples were sonicated to shear DNA fragments to within 200-1000 bp, centrifuged to remove cellular debris and diluted 10-fold with ChIP dilution buffer to reduce SDS concentration. Immunoprecipitations were performed using the ChIP Assay Kit (Millipore). Briefly, after 1 h preclearing with protein A beads,

samples were treated with 10 μg primary antibodies to GATA3 (Santa Cruz), NFAT1 (Santa Cruz) or acetylated histone 3 K9/14 (Millipore) overnight at 4°C with rocking followed by addition of protein A beads for 1.5 h. Samples were washed with Low Salt, High Salt, LiCl and TE buffer per manufacturer protocol. After elution with $NaHCO_3$ and SDS, protein crosslinks were reversed with 5 M NaCl at 65°C overnight. Immunoprecipitated DNA fragments were purified with QiaQuick gDNA columns (Qiagen) and evaluated by PCR or qPCR with primers specific to the CTLA-4 promoter as indicated in Table I.

Agarose gel electrophoresis: PCR samples were stained with 10X xylene cyanol/bromophenol blue dye in 80% glycerol prior to loading onto 1% agarose Tris/Borate/EDTA (TBE) gels stained with 0.5 μg/mL ethidium bromide. Electrophoresis was run at 100V in TBE buffer and DNA fragment sizes were compared to the 1 kb Plus DNA Ladder (Invitrogen). Gels were visualized by UV transillumination.

Nucleic acid electroporation: Primary CD4 T cells were electroporated at 10^7 cells per cuvette using the Amaxa system at a setting of U014. Electroporation solution was 82 μL Nucleofector Solution with 18 μL supplement (Amaxa Human T Cell Kit) for each sample. Expression plasmids for GATA3 or control vector were electroporated at 2 μg/sample. siRNA was electroporated at 20 pmol/sample SmartPool siRNA directed at GATA3 or off-target control (Dharmacon). After electroporation, cells were transferred to RPMI 1640 culture media with 10% fetal bovine serum and allowed to rest 18 h.

Cell sorting: $40x10^6$ bortezomib-treated and/or PMA/A23187 stimulated cells were stained with 20 μL PE-CTLA-4 (BD 555853) in 1 mL PBS for 20 minutes at room temperature. Samples were treated with 100 U/mL DNase (New England Biolabs), diluted to 10 mL PBS and passed through a cell strainer. Cells were then sorted on a FACS Aria for presence or absence of surface CTLA-4 and checked for purity which exceeded 98% for all samples.

Cytometric bead array (CBA): Cell culture supernatant was isolated from cell samples and frozen at -80^{o}C prior to use. The Th1/Th2 CBA kit (BD Biosciences) was used following manufacturer protocol. Briefly, cytokine capture beads were reconstituted in Assay Diluent and combined with 50 μL PE Detection Reagent and either 50 μL culture supernatant or 50 μL serially diluted standards (provided in the kit). After incubation at room temperature for 3 h protected from light, samples were washed with 1 mL Wash Buffer. Samples were resuspended in 300 μL Wash Buffer and analyzed by flow cytometry using the provided template and cytometer setup beads. The resulting data was analyzed with FCAP Array v 1.0.1 software (Soft Flow).

Statistical analysis: Student's t-test (2-tailed, unequal variance) was used to analyze the significance of differences between two experimental groups. Data with a *p* value of 0.05 or less were considered to be significant.

CHAPTER 1

Altered Proteasome Function Leads to a GATA3-Dependent Increase in CTLA-4, a Mechanism That May Provide Insight to CTLA-4 Regulation in CTCL

ABSTRACT

The costimulatory molecule CTLA-4 functions as an immunomodulator generally associated with suppression of T cell proliferation. Though structurally similar to CD28, which is expressed constitutively on T cells, transcription of CTLA-4 is highly regulated. CTLA-4 is elevated in cutaneous T cell lymphoma (CTCL), which may contribute to suppression of anti-tumor response during disease progression. The transcriptional regulator GATA3 is also over-expressed in CTCL, but its significance in CTLA-4 is unclear. We find that both transcript and protein levels of GATA3 are augmented by proteasome inhibition. We show by polyubiquitin immunoblot that the proteasome pathway is dysregulated in CTCL. Here we demonstrate a role for GATA3 in transcriptional regulation of CTLA-4 using the proteasome inhibitor bortezomib, a compound which reversibly binds to and inactivates the catalytic core of the proteasome through its boron atom.

Bortezomib treatment leads to a dose-dependent increase in both GATA3 and CTLA-4 expression in normal CD4 T cells at both the transcript and protein level. We also detect an increase in phospho-GATA3, the activated form, with bortezomib. Flow cytometric analysis confirms the elevated CTLA-4 is properly trafficked to the cell surface. Overexpression of GATA3 into Jurkat T cells by transfection stimulates a

CTLA-4 promoter luciferase construct. The GATA3 expression can enhance CTLA-4 promoter activity in a dose-dependent manner. In primary CD4 cells, we detect specific binding of GATA3 to the CTLA-4 proximal promoter in bortezomib-treated CD4 cells by ChIP assay. Furthermore, depletion of GATA3 with specific siRNA significantly impacts CTLA-4 transcription. These results support a potential mechanism for increased CTLA-4 observed in CTCL T cells where GATA3 is increased. Additionally, this work provides insight into potential effects on T cell function from proteasome inhibition with bortezomib. As GATA3 supports differentiation of Th2 T cells, bortezomib may be useful in immune modulation in diseases associated with Th1 dominance and explain its effectiveness in graft versus host disease.

INTRODUCTION

Cutaneous T cell lymphoma (CTCL) is a malignancy of skin-homing CD4+ CD45RO+ T cells. The leukemic form, Sezary Syndrome, is characterized by an influx of circulating tumor cells in the periphery. An elevated level of CTLA-4 expression is detectable in tumor cells of patients with CTCL, which may contribute to immune suppression and tumor progression (126). The mechanism contributing to the observed augmented CTLA-4 remains unclear. We previously reported a functional NFAT1 binding site in the CTLA-4 proximal promoter (41), but additional factors are likely involved in the complex regulation of this gene. Reports have shown FoxP3 activates expression of CTLA-4 in Tregs (48), but whether FoxP3 plays a role in effector T cell expression is unknown. CTLA-4 is also elevated when Th2-associated transcription factor GATA3 is overexpressed in a mouse model (115), but it is not clear whether the effect is causative. Interestingly, GATA3 levels are also elevated in CTCL (78).

The 26S ubiquitin/proteasome pathway functions as a proficient cellular protein degradation system which enzymatically cleaves a variety of polypeptides (77). Substrates are marked for disposal by the successive addition of ubiquitin groups via a series of ligases. Once a polyubiquitin tail is affixed, the protein is sent through the 19S cap and into the enzymatic 20S core where it is dismantled by a cylinder of proteases. The resulting peptides can either be metabolized, recycled into new proteins or loaded onto MHC I complexes for immune monitoring. Proteasome inhibitors have shown increased cytotoxicity in certain malignancies, including multiple myeloma (117). These compounds block this complex, resulting in stabilization of the substrates. Specifically

in the case of T cell transcription factors, exogenous levels of NFAT1 have been reported to increase in the presence of proteasome inhibitors (130), though an additional study found the inhibitors diminished nuclear NFAT1 (14). NFkB nuclear translocation requires cytosolic ubiquitination and degradation of IkB (86). Blockade of proteasomal activity thus reduces NFkB activity. Additionally, work by Yamashita et al shows proteasome inhibitors cause an increase in GATA3 protein (129).

This chapter investigates whether GATA3 is directly involved in CTLA-4 transcription, particularly in the presence of proteasome inhibitors. We examine whether the proteasome is dysfunctional in CTCL, which may explain why these cells exhibit increased GATA3. Our findings show excessive levels of polyubiquitination in patient samples when compared to normal donors. We then questioned whether proteasome inhibition alone can augment CTLA-4 expression in normal CD4 T cells. Not only do we find elevated CTLA-4 protein and transcript, but we show this increase is functionally able to suppress T cell proliferation. Our findings also demonstrate an increase in activated GATA3, which binds to and induces CTLA-4 transcription. Taken together, this work introduces a novel mechanism for CTLA-4 regulation.

RESULTS

CTLA-4 and GATA3 are abnormally elevated in Sezary T cells. To better understand the role of GATA3 in CTLA-4 regulation, we evaluated the level of CTLA-4 and GATA3 transcript in Sezary Syndrome and normal peripheral CD4 T cells stimulated with PMA/A23187 from normal donors, Sezary patients and psoriasis patients. Psoriasis samples serve as an inflammatory T cell-mediated disease control. The relative expression of CTLA-4 and GATA3 mRNA was analyzed by quantitative RT-PCR (qPCR) as described in Materials and Methods. Though resting T cells express minimal CTLA-4 mRNA, in Sezary T cells, the average level of CTLA-4 transcript is elevated 3.9-fold over normal in unstimulated samples ($p=0.0069$). Upon induction, healthy donor CD4 cells exhibit a peak in CTLA-4 transcription after 2 hours, followed by a gradual decline in expression after longer stimulations. In the Sezary samples analyzed, we detect significant increases in CTLA-4 expression at each of the time points as compared to normal donors (Figure1.1a). Psoriasis samples are similar to normal, indicating its expression is specific to Sezary and not a consequence of the inflammatory response.

Consistent with previously published data, we confirmed that GATA3 transcript is also significantly increased in these same Sezary patients (Figure1.1b). Furthermore, we find GATA3 expression levels remain stable over the stimulation time course ($p< 0.005$). Immunoblot analysis of GATA3 levels in unstimulated whole-cell extract from 5 Sezary patients confirms the elevated mRNA is translated into protein (Figure1.1c). Activation of GATA3 occurs after T cell stimulation and is dependent on phosphorylation

of serine 308 within the protein's nuclear translocation region. Using an antibody specific to phospho-GATA3, we find unstimulated Sezary cells exhibit an elevation in activated GATA3 despite lack of activation (Figure1.1c).

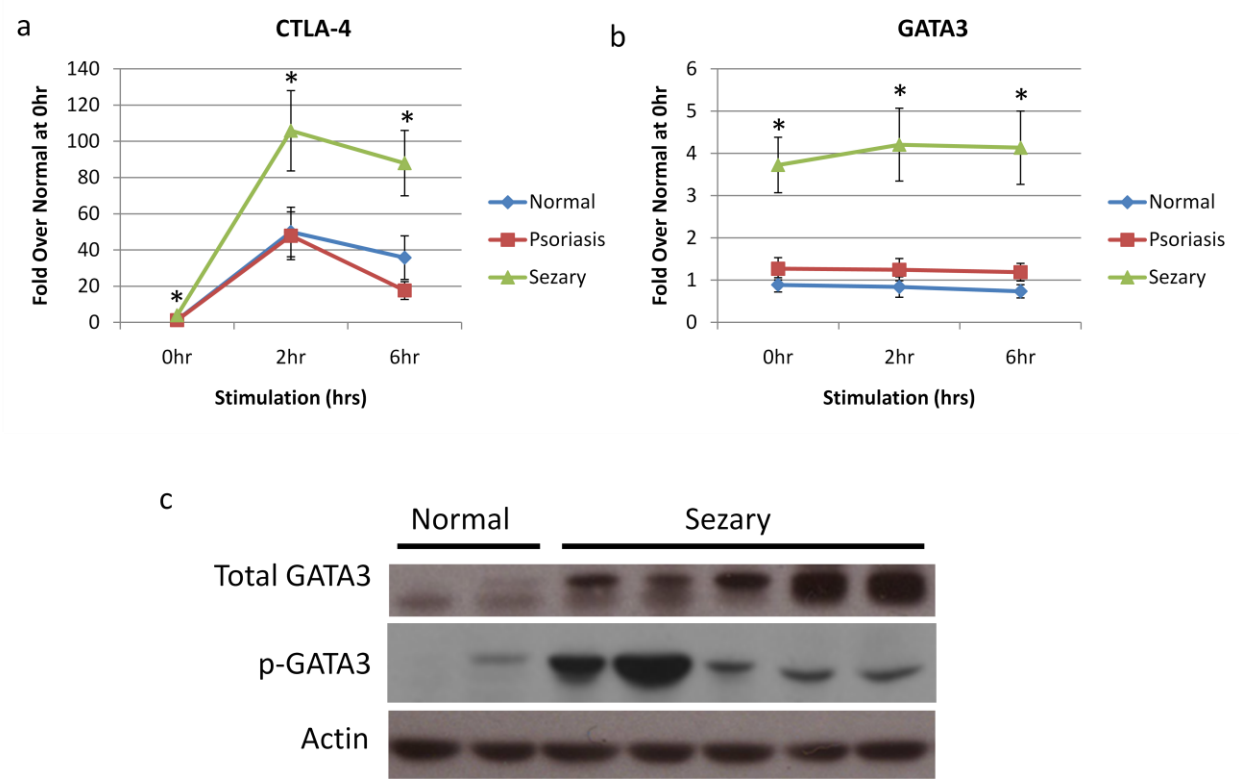

Figure 1.1. CTLA-4 and GATA3 are augmented Sezary and normal T cells. a) PBMCs were isolated from Sezary patients (n = 6), psoriasis (n = 6) and normals (n = 6) and cells were stimulated with PMA/A23187 for the indicated time points. Total RNA was isolated qPCR was performed as described in Materials and Methods with B2M serving as the internal control. Results are shown as the average fold increase over unstimulated normal cells ± SEM (*p<0.05). b) qPCR analysis of GATA3 expression in Sezary, psoriasis and normals using the same samples as in (a). c) Immunoblot analysis of whole cell lysates from unstimulated normal donor (n = 2) CD4 T cells and Sezary (n = 5) patients. PBMCs were purified and protein extracts were processed as described in Materials and Methods. Samples were probed for expression of total GATA3 and phospho-GATA3 (p-GATA3) with specific antibodies as detailed in Materials and Methods. Actin serves as a loading control.

NFAT and FoxP3 are not consistently abnormal in Sezary T cells. We previously identified NFAT1 as an important transcription factor in CTLA-4 expression (41), and FoxP3 has also been identified as a factor involved in CTLA-4 regulation in Tregs (48, 128). Elevated FoxP3 levels were detected in Sezary (119), though another report failed to find an increase (42). To determine whether either of these factors is responsible for the elevated CTLA-4 found in Sezary, we measured levels of mRNA and protein in our Sezary and normal samples. There were no significant differences between normal donors and patients at the mRNA level (Figure1.2a, Fig1.2b), and protein expression was highly variable between patients (Figure1.2c). Given the consistency of increased CTLA-4 in these samples, it is thus unlikely that NFAT1 or FoxP3 contribute significantly to the augmented CTLA-4 found in Sezary.

Total polyubiquitin levels are elevated in Sezary T cells. GATA3 has been shown to be regulated by the ubiquitin-proteasome pathway (129), but proteasome function has not been directly evaluated in Sezary. To assess the proficiency of proteasome function in Sezary, we measured total ubiquitin from whole cell extracts of unstimulated cells by immunoblot and found increased total ubiquitin in Sezary cells compared to normals (Figure 1.3a). The presence of excess ubiquitin in Sezary implies there may be dysregulation of the ubiquitin-26S proteasome pathway. The 20S enzymatic region of the proteasome was assayed by cleavage of a Suc-LLVY-AMC fluorogenic peptide substrate. We did not detect a significant difference between Sezary and normal extracts, though on average Sezary samples were better capable of degrading the peptide (Figure1.3b).

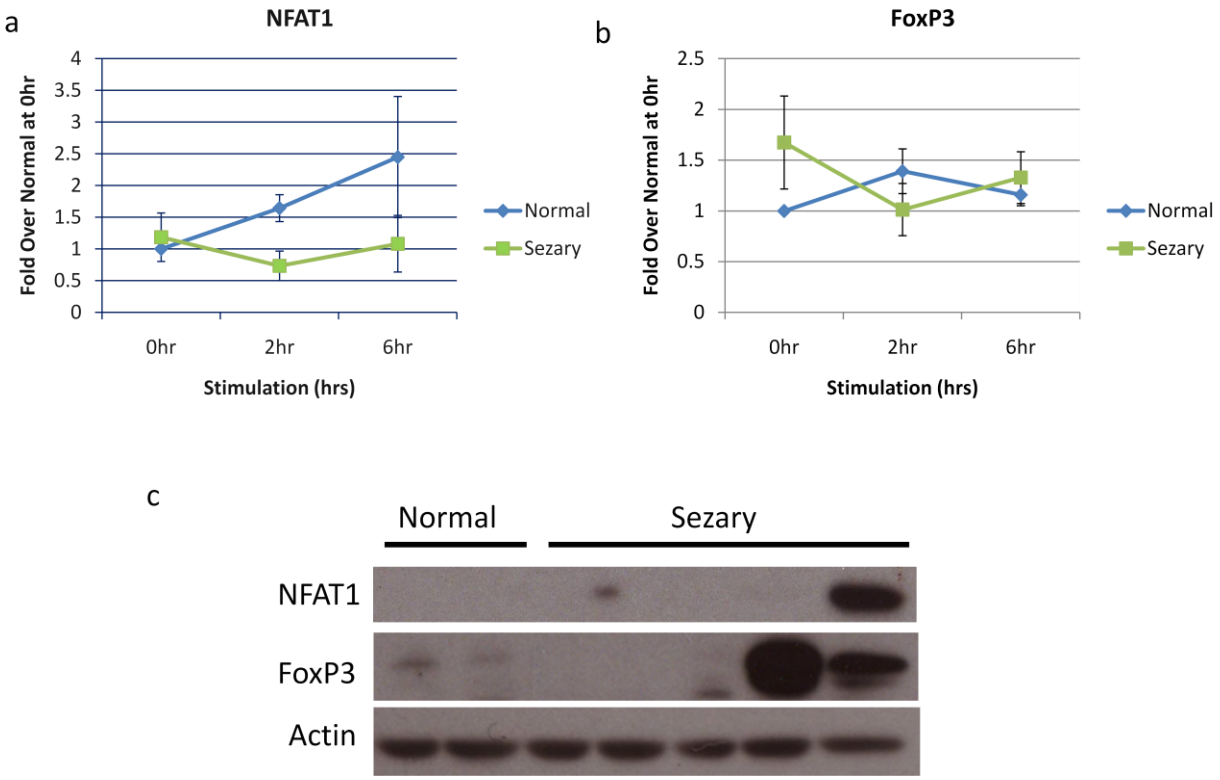

Figure 1.2. NFAT1 and FoxP3 are sporadically dysregulated in Sezary and normal CD4 T cells. CD4 T cells were isolated from Sezary patients (n = 6) and normals (n = 6) and cells were stimulated with PMA/A23187 for the indicated time points. Total RNA was isolated qPCR was performed as described in Materials and Methods with B2M serving as the internal control. Results are shown as average fold increase over unstimulated normal cells ± SEM (* $p<0.05$). qPCR expression analysis of a) NFAT1 and b) FoxP3 in Sezary and normals. c) Immunoblot analysis of whole cell lysates from unstimulated normal donor (n = 2) CD4 T cells and Sezary (n = 5) patients. CD4 T cells were purified and protein extracts were processed as described in Materials and Methods. Samples were probed for expression of NFAT1 and FoxP3 with specific antibodies as detailed in Materials and Methods. Actin serves as a loading control.

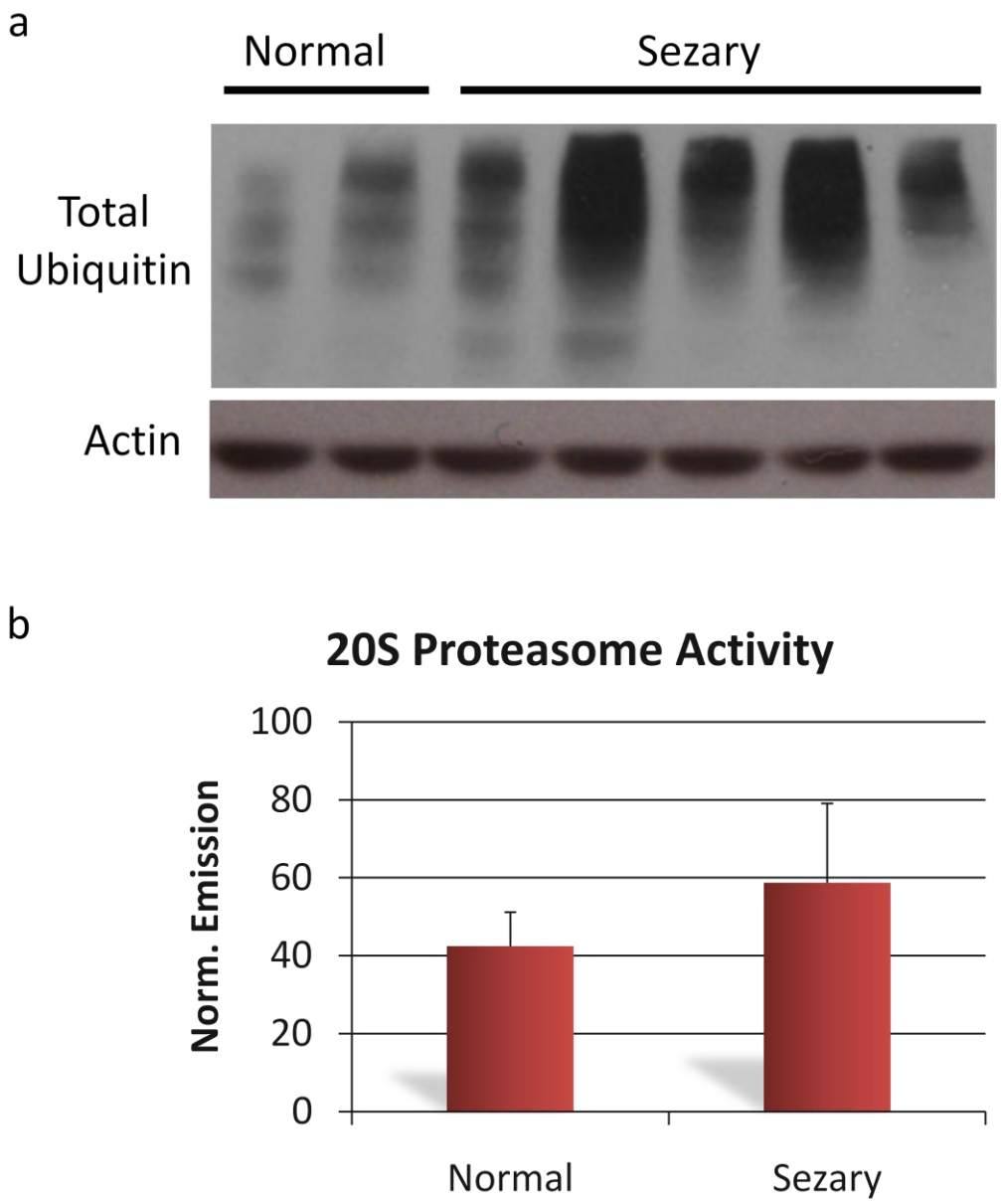

Figure 1.3. Proteasome activity analysis in normal and Sezary cells. a) Immunoblot analysis of whole cell lysates from unstimulated normal donor (n = 2) CD4 T cells and Sezary (n = 5) patients. PBMCs were purified and protein extracts were processed as described in Materials and Methods. Samples were probed for total polyubiquitin with a specific antibody as detailed in Materials and Methods. Actin serves as a loading control. b) 20S proteolytic core activity in Sezary cells and normals. Cell lysates were assayed for 20S proteasome core activity by Suc-LLVY-AMC fluorogenic peptide cleavage. Emission measurements are normalized to background detection levels and are the averages of 5 normal and 5 Sezary CD4 samples ± SEM. The proteasome activities were not significantly different ($p>0.05$).

Surface CTLA-4 expression increases in normal CD4 T cells treated with the proteasome inhibitor bortezomib. Increased ubiquitin levels in Sezary cells suggest altered proteasomal regulation may play a role in enhancing CTLA-4 expression in Sezary. We next study the regulation of CTLA-4 by blocking proteasome function in normal primary CD4 T cells using bortezomib, a dipeptide boronic acid inhibitor of the proteasome (1). Normal CD4 T cells were stimulated with PMA/A23187 and treated with increasing concentrations of the specific proteasome inhibitor bortezomib and surface CTLA-4 was analyzed by flow cytometry. Figure 1.4a shows a representative dot plot of CTLA-4 expression in bortezomib-treated (10 μM) and untreated CD4 cells after 12 hours of stimulation. Cells were stained with PE-conjugated anti-CTLA-4 and PC5-conjugated anti-CD4 prior to analysis. The results demonstrate an overall increase in the quantity of CTLA-4-positive cells after proteasome inhibition, as well as an elevation in the magnitude of surface CTLA-4 expression on each cell.

To understand the kinetics of surface CTLA-4 expression with proteasome inhibition, we conducted a stimulation time-course with treatments of 0, 0.1 and 10 μM bortezomib in CD4 T cells from 4 healthy donors. Untreated CD4 T cells exhibit a peak in percent CTLA-4 expression between stimulation times of 3 and 6 h, after which these levels progressively decline. Treatment with bortezomib leads to sustained CTLA-4 expression which remains stable through 12 h of stimulation (Figure1.4b). Results are also presented graphically as percent CTLA-4 positive cells (Figure1.4c). These data illustrate the potent effect of proteasome inhibition on CTLA-4.

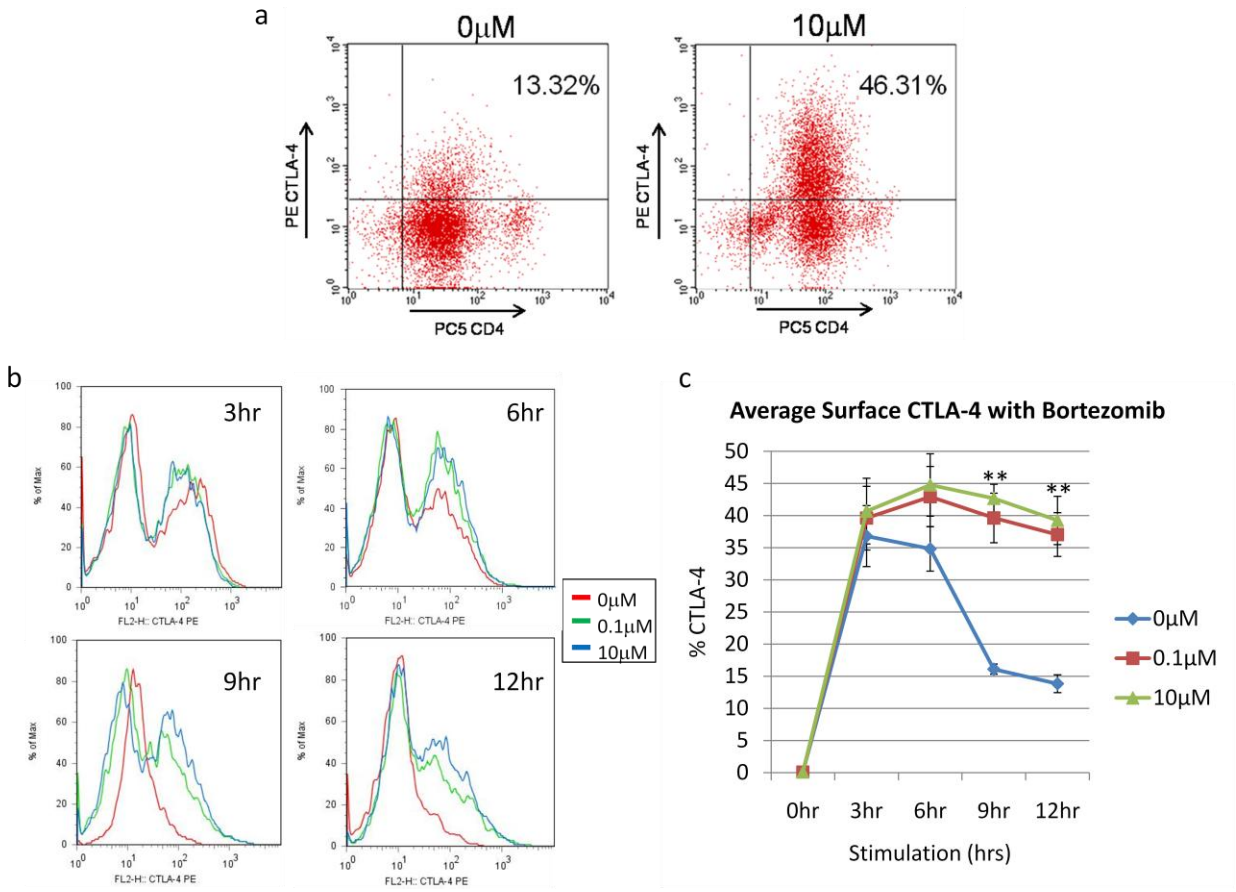

Figure 1.4. Proteasome inhibition augments CTLA-4 surface expression. a) Normal primary CD4 T cells were stimulated 12 h with PMA/A23187 and treated with and without 10 μM bortezomib and analyzed for CTLA-4 expression. Cells were stained with PE α-CTLA-4 and PerCP α-CD4 and analyzed by flow cytometry as described in Materials and Methods. Percentages represent double positive cells. The results are representative of at least 6 independent experiments. b) CTLA-4 expression histogram of one normal donor treated with 0, 0.1 and 10 μM bortezomib over a time course. Cells were stimulated with PMA/A23187 for the indicated time points with and without the inhibitor and % CTLA-4 expression was determined by flow cytometry as described above. These results are representative of 4 independent experiments. c) Graphical representation of average CTLA-4 surface expression ± SEM in (b) (**p<0.005).

Increased CTLA-4 in cells treated with bortezomib is not an artifact of apoptosis. As bortezomib has been used in treatment of multiple myeloma for its cytotoxic effects, we next sought to determine whether apoptosis plays a role in our system. Primary CD4 T cells were treated with bortezomib and stimulated as in Figure 1.4 and analyzed by Annexin V/PI staining (Figure1.5). Our results indicate an increase in apoptosis with stimulation alone. Bortezomib treatment causes a modest increase in apoptosis after 9 h, and only after 12 h at the 10 μM concentration do we detect a significant increase over stimulation alone.

CTLA-4 elevation with bortezomib suppresses T cell proliferation. To determine whether the elevated CTLA-4 in bortezomib-treated cells can suppress T cell proliferation, we conducted a mixed lymphocyte reaction (MLR). Purified primary CD4 T cells were stimulated for 9 h with 0, 0.1 and 10μM bortezomib treatment, which resulted in 8.11%, 14.87% and 23.00% CTLA-4 expression, respectively. Interestingly, CTLA-4 expression diminishes within 12 h after washing in cells with stimulation alone, but at the 10 μM concentration CTLA-4 remains detectable in 9.85% of cells in the bortezomib-treated population after 24 h and 4.49% after 48 h (Figure1.6a). The cells were washed and then used in an MLR as described in Materials and Methods. The bortezomib-treated cells effectively suppressed proliferation, which was reversed with CTLA-4 blocking antibody as compared to IgG control (Figure1.6b). With the CTLA-4 antibody, growth of 0.1 and 10 μM bortezomib-treated cells improved by 51.1% and 42.3%, respectively, while the stimulation alone samples did not significantly change.

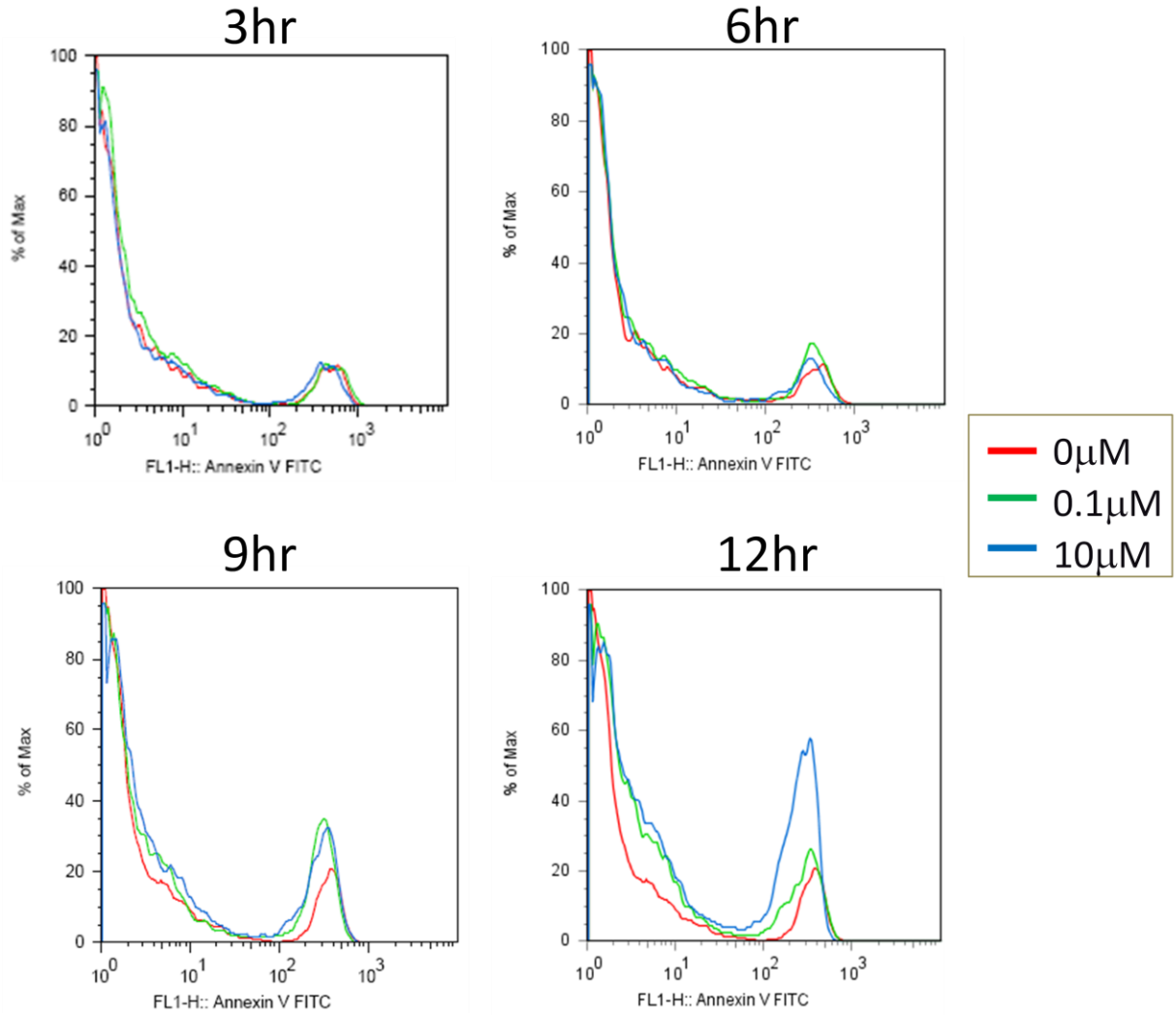

Figure 1.5. Apoptosis levels in normal CD4 T cells treated with bortezomib and stimulated with PMA/A23187. $1x10^6$ fresh CD4 T cells were treated with 0 (red), 0.1 (green) or 10 μM (blue) bortezomib and stimulated for the indicated time periods. Cells were then stained with annexin V and PI as detailed in Materials and Methods prior to analysis by flow cytometry. Results are presented as histograms for intensity of annexin V staining and are representative of three independent experiments.

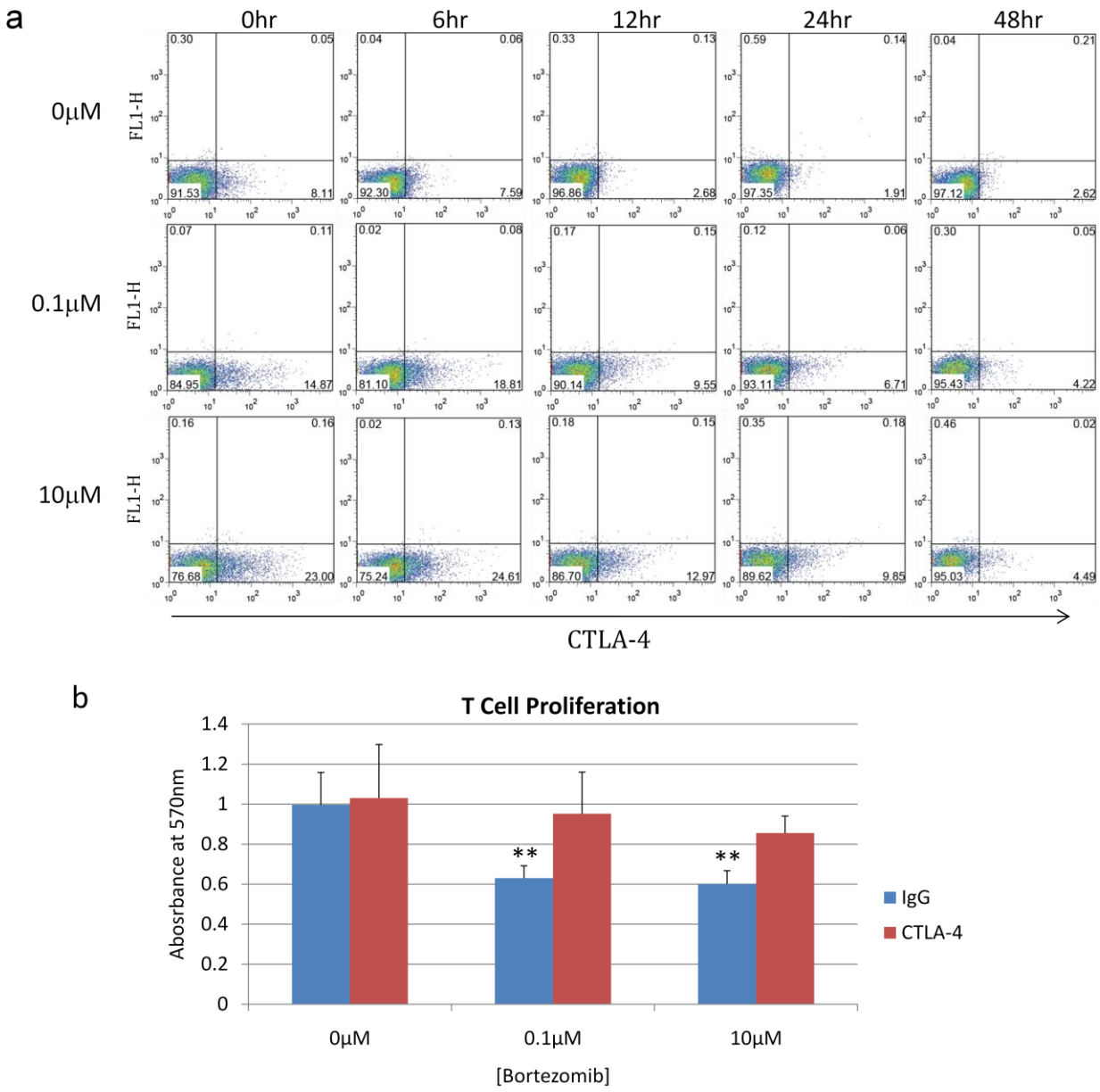

Figure 1.6. Increased CTLA-4 with bortezomib treatment suppresses CD4 T cell proliferation. Primary CD4 T cells were isolated and treated with 0, 0.1 and 10 μM bortezomib during PMA/A23187 stimulation for 9 h. Cells were washed 3x and returned to culture media without bortezomib or PMA/A23187. a) CTLA-4 surface expression by flow cytometry over a time course after washing. CTLA-4 levels were measured at the indicated time points. b) Proliferation was measured by mixed lymphocyte reaction. Washed cells were plated at $5x10^4$/well of a 96-well plate and stimulated with an equal amount of mytomycin C-treated allogenic PBMCs as detailed in Materials and Methods. Samples were supplemented with 0.5 μg CTLA-4 blocking antibody or IgG control. After 7 days, proliferation was measured by MTT assay as described in Materials and Methods. Results are presented as the averages of quintuplicate samples ± SEM and are representative of three independent experiments (**p<0.005).

Bortezomib treatment leads to augmented mRNA expression. We next sought to establish whether the responsible mechanism driving the CTLA-4 increase occurs at the level of transcription, protein regulation or surface trafficking. To determine the effect of proteasome inhibition on mRNA expression of CTLA-4 and selected transcription factors, we treated fresh CD4 T cells from normal, healthy donors with bortezomib at 0, 0.1 and 10 μM. Cells were treated with bortezomib and stimulated with a combination of PMA/A23187 in 3 h increments over 12 h. Expression of mRNA was analyzed by quantitative real-time PCR as detailed in Materials and Methods.

The level of CTLA-4 transcript increases significantly with bortezomib treatment, particularly after extended stimulations (Figure1.7a). Untreated samples follow a pattern where expression peaks at approximately 3 h of stimulation, after which it declines steadily over prolonged activation periods. Cells treated with bortezomib, however, do not exhibit this same decline in CTLA-4 transcription after 3 h. These cells instead maintain and continue to increase expression in a dose-dependent manner through 12 h of stimulation. At the 12 h time point, treatment with 10 μM bortezomib results in more than 7-fold greater CTLA-4 expression over untreated. These results provide evidence that the observed effect of proteasome inhibition on CTLA-4 occurs upstream of CTLA-4 transcription, possibly through specific transcription factors.

We next investigated the relative mRNA transcript levels of NFAT1, FoxP3 and GATA3 in the presence of bortezomib. Neither NFAT1 nor FoxP3 were significantly altered at the level of transcription (Figure1.7b, c). Supporting previously published data, a dose-dependent increase of GATA3 mRNA was detected after proteasome inhibition (Figure1.7d). Given that proteasome inhibition may lead to reduced

degradation of these factors and not necessarily an increase in transcript, mRNA analysis of these genes may not accurately reflect cellular protein levels.

An alternate proteasome inhibitor, ALLN, also enhances CTLA-4 transcription. In order to determine whether our results with bortezomib were due to proteasome inhibition and not an off-target effect of the drug, we utilized an alternative proteasome inhibitor, ALLN. This chemical is a peptide aldehyde that inhibits both the proteasome and the calpain pathways. As a control for calpain inhibition, we included the compound ALLM, which blocks calpain activity without suppressing proteasome function. Cells were treated with 10 μM ALLM, ALLN or bortezomib and stimulated for 9 h. CTLA-4 expression was measured by qPCR (Figure 1.8). Both ALLN and bortezomib increased CTLA-4 transcription over vehicle control, while ALLM did not.

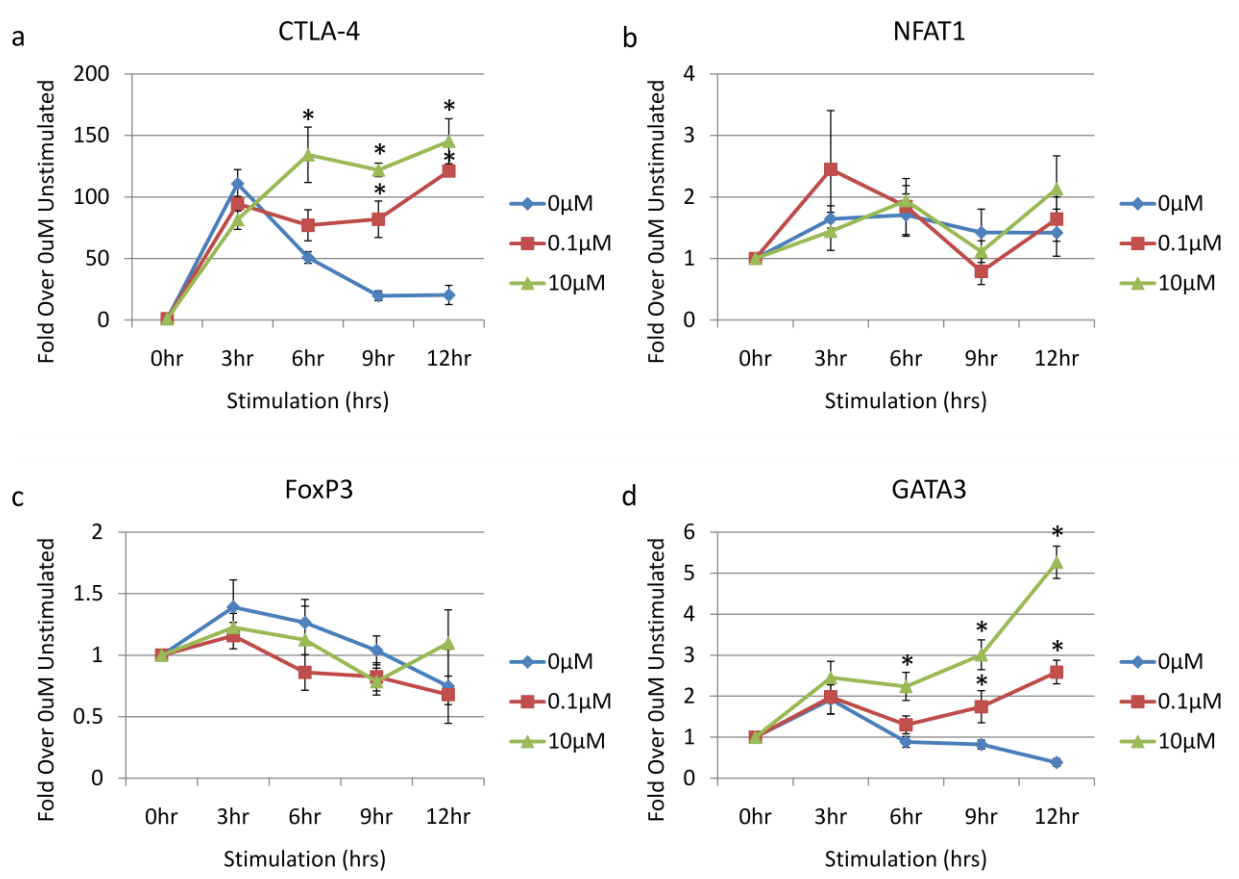

Figure 1.7. Transcript levels of CTLA-4, NFAT1, FoxP3 and GATA3 after bortezomib treatment. Expression of a) CTLA-4, b) NFATc2, c) FoxP3 and d) GATA3 mRNA in normal primary CD4 T cells treated with the indicated concentrations of bortezomib and stimulated with PMA/A23187 over a time course. Normal CD4 T cells were purified using RosetteSep as described in materials methods. Total RNA was isolated for qPCR analysis as previously described. Results are the averages of 4 individual normal donors ± SEM analyzed by qPCR normalized to B2M and presented as the fold increase over unstimulated normal cells (*p<0.05, **p<0.005).

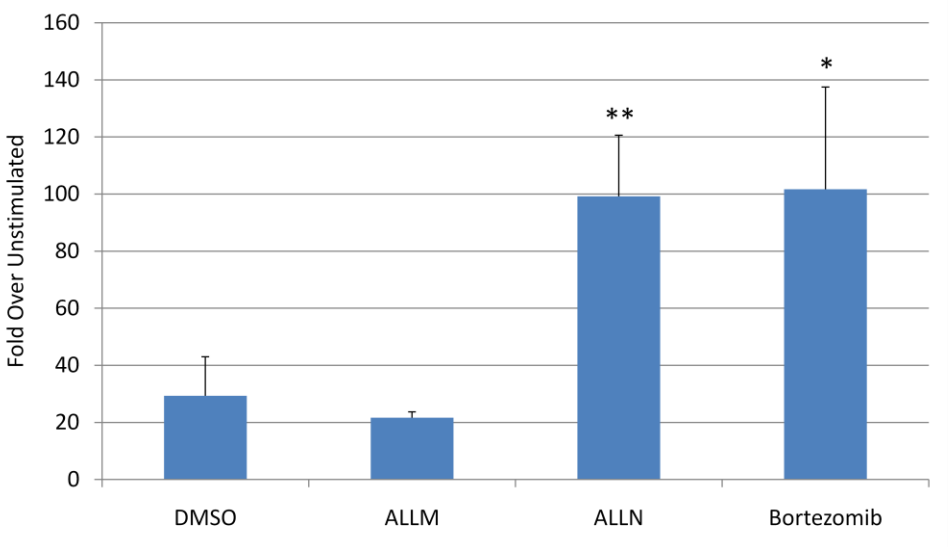

Figure 1.8. CTLA-4 transcript also increases with proteasome inhibitor ALLN. $10x10^6$ primary CD4 T cells were treated with 10 μM ALLM, ALLN, bortezomib or an equivalent volume of DMSO followed by stimulation for 9 h. Total RNA was isolated by Trizol preparation, cDNA was synthesized and qPCR was performed with primers specific to CTLA-4 as described in Materials and Methods. Results were normalized to B2M and are presented as the averages of three independent experiments ± SEM (*$p<0.05$, **$p<0.005$).

Bortezomib-treated cells display transcription factor protein dysregulation. To investigate whether proteasome inhibition leads to increased stability of transcription factors NFAT1, FoxP3 or GATA3, we probed whole-cell lysates from CD4 T cells collected from normal, healthy donors. Cells were treated as they were for transcript studies in Figure 1.7. Levels of IkB serve as a control to demonstrate proficiency of proteasome inhibition. In parallel with flow cytometry data, bortezomib leads to maintained CTLA-4 expression over extended stimulations (Figure1.9). NFAT1 protein was not elevated with bortezomib, indicating this transcription factor is not appreciably regulated by the proteasome pathway. After longer stimulations at higher bortezomib concentrations, we instead detect a modest reduction in the intensity of NFAT1 protein. Untreated samples show enhanced FoxP3 expression over the stimulation time course, but expression is abrogated in cells treated with bortezomib. As early as 6 h after stimulation, FoxP3 levels diminish with proteasome inhibition.

We find that GATA3 protein levels do not increase remarkably, however over longer stimulations with bortezomib we begin to distinguish a second, higher molecular weight band that may represent the phosphorylated, transcriptionally active species. To investigate this further, we isolated nuclear and cytosolic fractions from cells stimulated for 6 h with treatments of 0, 0.1 and 10 μM bortezomib. By immunoblot we probed samples with an antibody specific for phospho-S308 GATA3 (Figure1.10a). In the nuclear fraction, we detect a marked increase in activated GATA3 after bortezomib treatment. Again, total GATA3 levels remain similar, but with proteasome inhibition the higher molecular weight band is more prevalent. Cytosolic levels of total and phospho-GATA3 remain comparatively lower in each sample. As a method of determining the

purity of the fractionation, samples were also probed for total histone 3. The cytosolic fractions do not contain any of this protein. Interestingly, the 10 μM nuclear sample shows elevated histone expression, indicating this protein may be regulated by the proteasome. The included actin loading control verifies equal protein lysates were loaded in the gel.

We next quantified total GATA3 levels by permeabilization and staining with PE-conjugated antibody for intracellular flow analysis (Figure1.10b). Over the time course we find stimulation alone leads to a gradual, stepwise decrease in GATA3 expression from 72.44% in unstimulated cells to 28.73% after 12 h of stimulation. Treatment with bortezomib results in stabilization of GATA3 expression across all time points at an average of 71.31% (+/- 3.55%) for 10 μM samples.

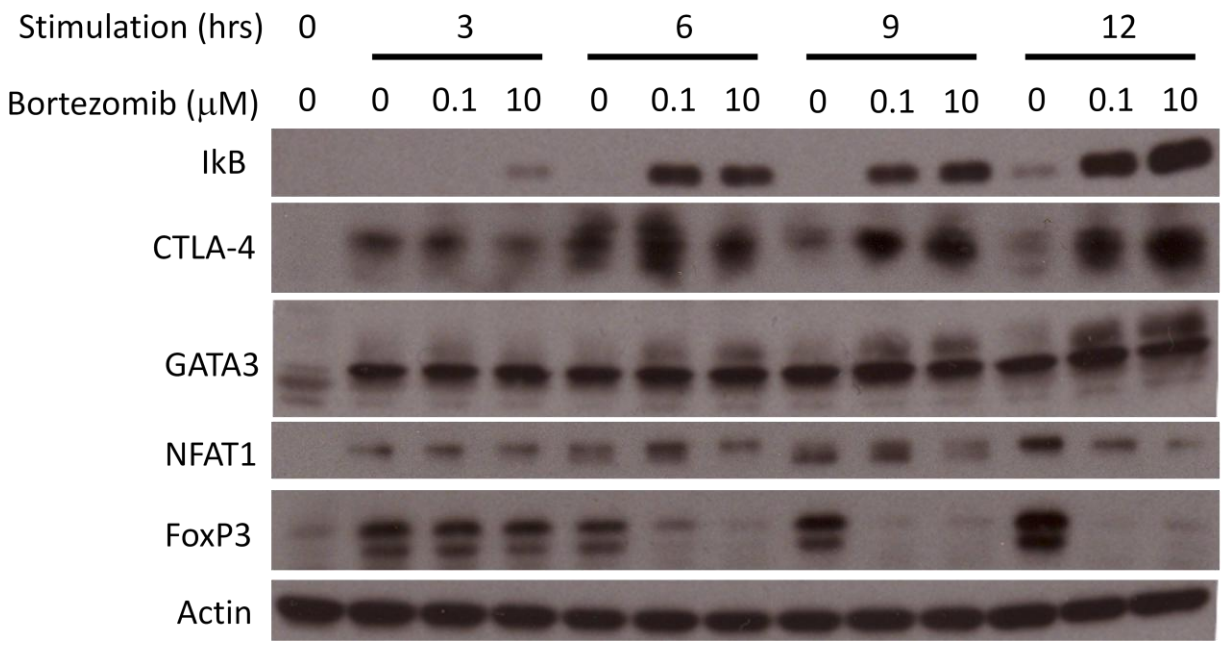

Figure 1.9. Transcription factor protein levels in bortezomib-treated CD4 T cells. Immunoblot analysis of normal CD4 T cells stimulated in a time course with PMA/A23187 and treated with bortezomib for CTLA-4, GATA3, NFAT1 and FoxP3. Whole CD4 T cell extracts were prepared with RIPA buffer and 10 μg total protein was separated on SDS-PAGE gels. Proteins were transferred to PVDF membranes and probed with antibodies specific for IκB (as a proteasome inhibition control), CTLA-4, GATA3, NFAT1 and FoxP3 as described in Materials and Methods. Actin serves as a loading control. Results are representative of 3 independent experiments.

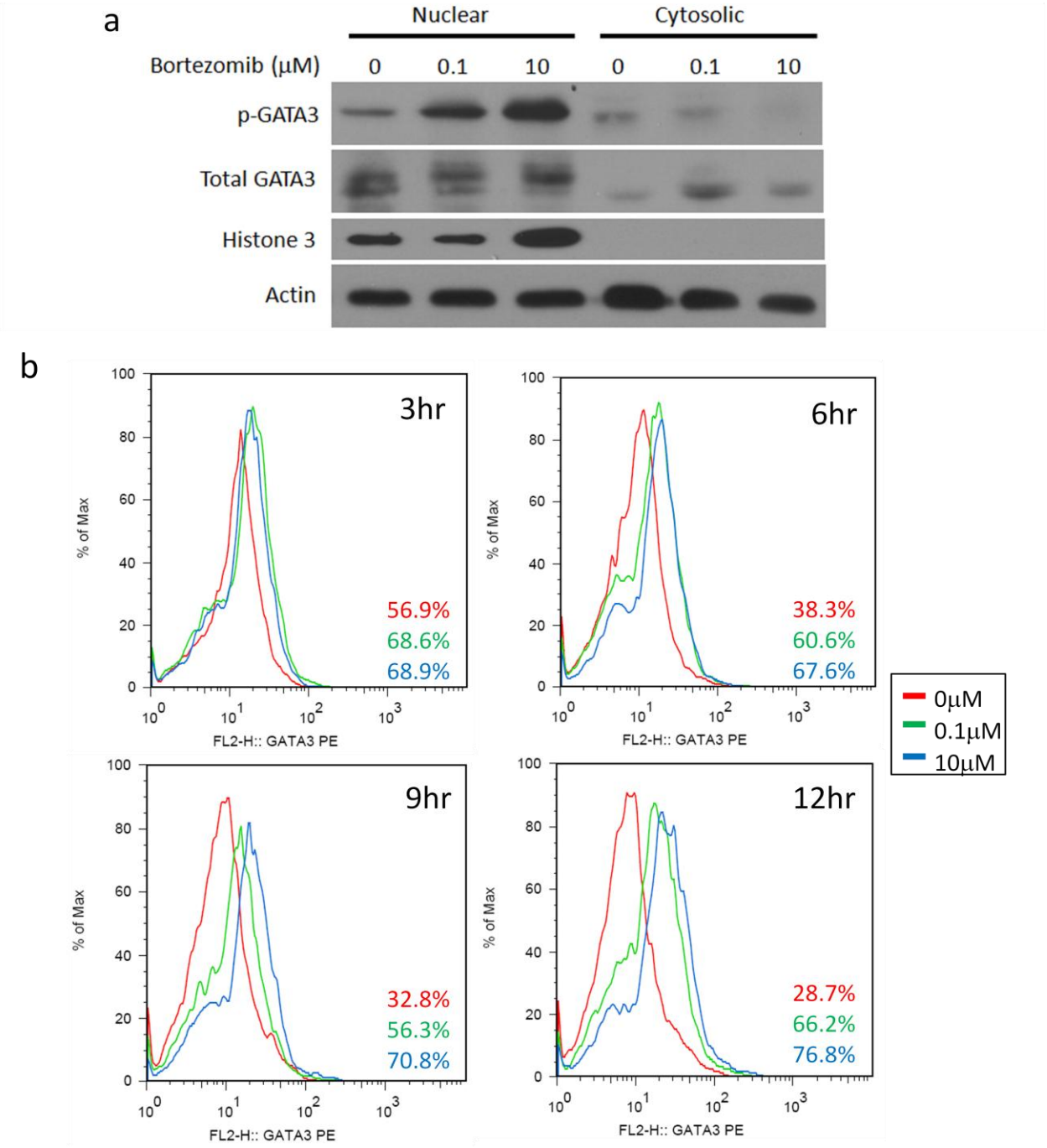

Figure 1.10. GATA3 protein is phosphorylated and stabilized with bortezomib. a) Nuclear and cytosolic fractions were isolated from primary CD4 T cells treated with 0, 0.1 and 10 μM bortezomib and stimulated for 6 h with PMA/A23187 as described in Materials and Methods. Lysates were probed with an antibody specific to phospho-GATA3 (S308) as described above. b) Cells were treated and stimulated as in Figure 1.8, then permeabilized and stained with PE-conjugated antibody to GATA3 as described in Materials and Methods followed by flow cytometric analysis. Percentages of GATA3 positive cells were determined by analysis with FlowJo software and are shown in red (0μM), green (0.1μM) and blue (10μM) text for each time point. Results from (a) and (b) are representative of three independent experiments.

Bortezomib activates the p38 kinase pathway, leading to GATA3 phosphorylation. We have shown that treatment with bortezomib causes a dose-dependent increase in the phosphorylated species of GATA3 protein (Figure 1.10a). Previous work has implicated both the p38 and ERK pathway in GATA3 phosphorylation (70, 129). To establish which mechanism is responsible in our system, we treated fresh primary CD4 T cells with the p38-specific inhibitor SB203580 and the ERK inhibitor PD98059 in addition to bortezomib. After stimulating 9 h, we measured IFN-γ (Figure 1.11a) and IL-4 (Figure 1.11b) transcript levels to determine which kinase was more specific to GATA3. IL-4 is heavily dependent on GATA3 activity whereas IFN-γ is not. We find ERK inhibition suppresses both IFN-γ and IL-4 expression, which indicates this pathway may not be distinctly involved in GATA3 activation. The p38 inhibitor selectively represses IL-4, supporting p38's role in GATA3 regulation.

When we measure CTLA-4 transcript in these samples, we again find ERK inhibition blocks expression (Figure 1.11c). The p38 inhibitor also suppresses CTLA-4 activation, both with and without bortezomib ($p<0.005$). We next examined phospho-GATA3 protein levels with the p38 inhibitor by immunoblot (Figure 1.11d). Bortezomib again increased GATA3 activation, but with the p38 inhibitor the level was reduced. To verify the inhibitor adequately blocked p38 we probed these same samples with an antibody specific to phospho-MAPKAPK2, a direct downstream product of activated p38. The resulting protein bands followed the same trend of phospho-GATA3, further supporting that the p38 pathway phosphorylates GATA3 in cells treated with bortezomib.

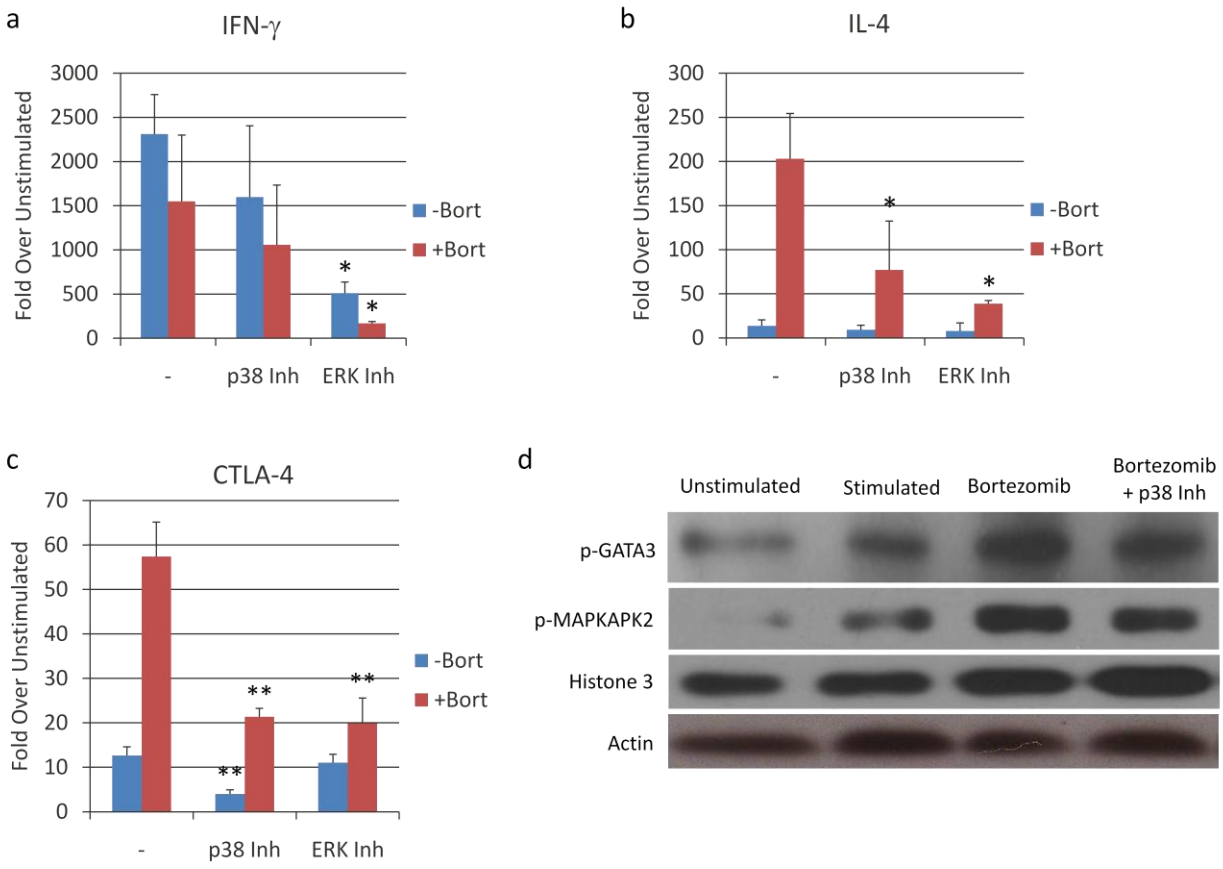

Figure 1.11. The role of the p38 pathway in GATA3 and CTLA-4 expression in CD4 T cells after bortezomib treatment. $5x10^6$ primary CD4 cells were treated with 30 μM p38 inhibitor SB203580, 30 μM ERK inhibitor PD98059 or vehicle control for 1 h prior to stimulation with PMA/A23187 for 9 h. Total mRNA was isolated and analyzed by qPCR as previously described with primers specific for a) IFN-γ, b) IL-4 and c) CTLA-4. Results are presented as the averages of three independent experiments ± SEM (*p<0.05, **p<0.005). d) Immunoblot analysis of stimulated primary CD4 T cells treated with 10 μM bortezomib alone or with 30 μM p38 inhibitor SB203580 as compared to unstimulated and stimulated alone. Cells were pretreated for 1 h followed by stimulation for 9 h with PMA/A23187. Nuclear fractions were isolated as described previously and lysates were probed with antibodies specific to phospho-GATA3 (S308) and phospho-MAPKAPK2 as an indicator of p38 activity. Actin and histone 3 serve as loading controls and results are representative of three independent experiments.

The CTLA-4 promoter is augmented by GATA3. The previous experiments demonstrate a correlation between GATA3 and CTLA-4 expression in normal CD4 T cells treated with bortezomib. To address whether GATA3 can directly affect CTLA-4 transcription, we cotransfected increasing concentrations of a GATA3 expression vector with pGL3 luciferase reporter plasmids containing regions of the CTLA-4 promoter into Jurkat T cells, which we previously demonstrated is dependent on an NFAT1 binding site (41). GATA3 has been shown to directly interact with NFAT1, and addition of exogenous GATA3 leads to a dose-dependent increase in activity of a pGL3 construct with 380 bp of the CTLA-4 promoter by luciferase assay (Figure1.11). The detected RLU values are significantly higher than co-transfections with the same quantity of control vector ($p<0.005$). Similar results were obtained with a 1 kb CTLA-4 pGL3 construct, as well (data not shown). Importantly, co-transfection of GATA3 with the SV40 pGL3 control vector or a promoterless vector did not increase luciferase activity, indicating the effect is specific to the CTLA-4 promoter (data not shown). These results provide evidence that GATA3 is able to augment CTLA-4 transcriptional activity.

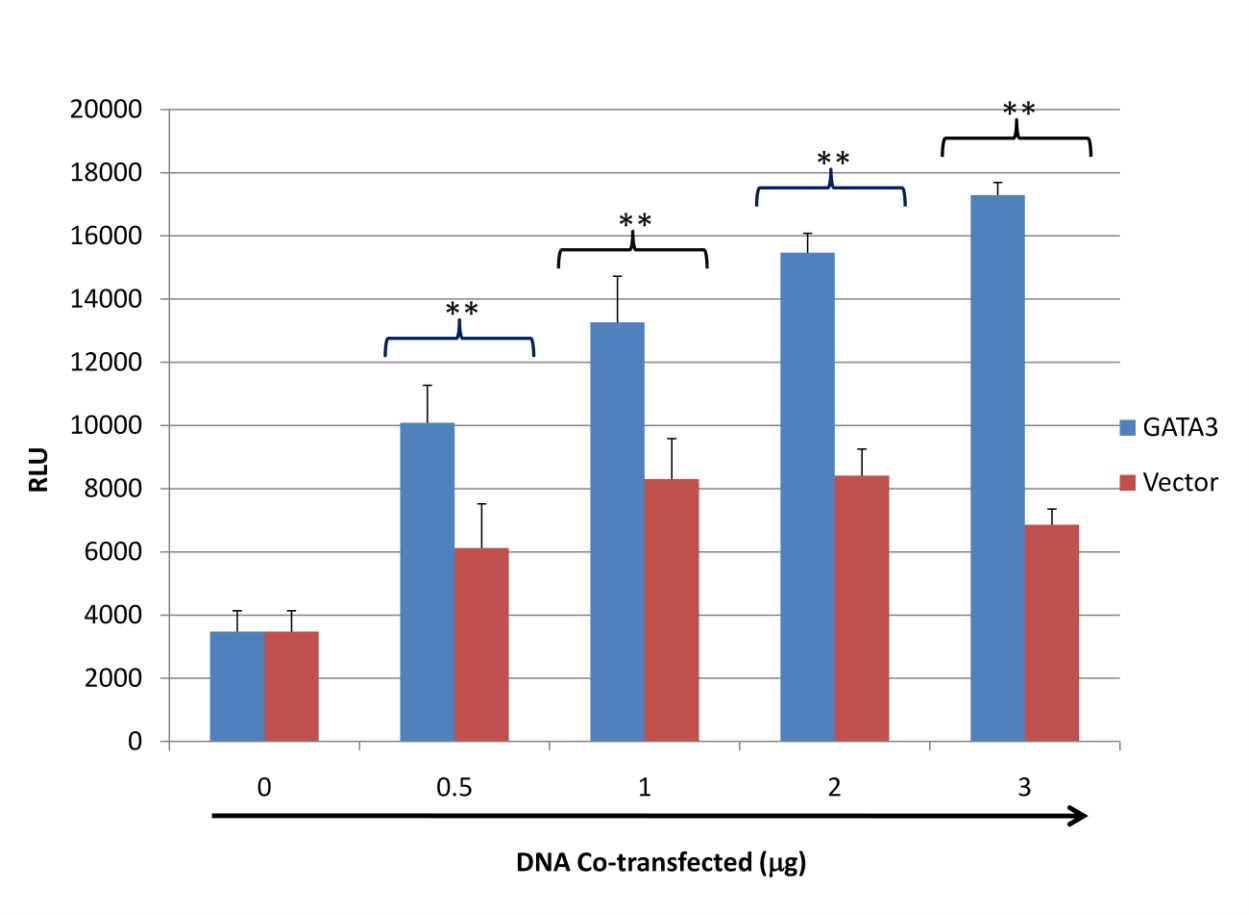

Figure 1.12. Ectopic GATA3 enhances CTLA-4 promoter activity. A 380 bp CTLA-4 promoter luciferase construct (41) was cotransfected with increasing concentrations of GATA3 or vector control into Jurkat cells using Lipofectin as described in Materials and Methods. Luciferase assay was performed and relative light units (RLU) were calculated. Results are averages of three independent experiments ± SEM (**p<0.005).

GATA3 interacts with the endogenous CTLA-4 promoter in bortezomib-treated primary CD4 T cells. To confirm the role of GATA3 at the endogenous CTLA-4 promoter in primary cells, we performed chromatin immunoprecipitation assays (ChIP). Normal CD4 T cells were treated or untreated with 10 μM bortezomib followed by stimulation for 6 h. An unstimulated/untreated sample serves as a negative control. By agarose gel analysis we detect binding of GATA3 to the CTLA-4 promoter in bortezomib-treated samples (Figure1.12a). As a more sensitive method of analysis, we ran quantitative real-time PCR using the same samples and primer set (Figure1.12b). Results are interpreted as fold increase over isotype control, normalized to input samples, and are the averages of three independent experiments. Upon stimulation we detect a 3-fold increase of GATA3 binding ($p=0.028$), however this interaction is markedly enhanced in the presence of bortezomib ($p=0.043$).

Transient overexpression of GATA3 in CD4 T cells does not affect CTLA-4 expression. We next addressed whether addition of ectopic GATA3 alone would be sufficient to induce CTLA-4 to the same extent as proteasome inhibition. Cells were electroporated with a GATA3 expression vector or LacZ control and stimulated for 9 h. We measured intracellular GATA3 (Figure 1.14a) to evaluate the efficacy of our expression vector and found that after 9 h stimulation there was a 17.2% increase from 31.7% in the control LacZ sample to 48.9% with the GATA3 plasmid. Importantly, the level of GATA3 detection in the LacZ population was consistent with what we found in non-electroporated cells in Figure 1.10b. In these same samples, we next measured

extracellular CTLA-4 (Figure 1.14b). We found no change in CTLA-4 expression with the addition of exogenous GATA3.

GATA3 knockdown by siRNA reduces CTLA-4 transcript in bortezomib-treated CD4 T cells. To further assess the role of GATA3 in CTLA-4 expression in bortezomib-treated CD4 T cells, we knocked down GATA3 with siRNA. Primary CD4 T cells were nucleoporated with a set of either GATA3 specific or control siRNA and allowed to rest for 18 h. Samples were then stimulated in the presence or absence of 10 μM bortezomib for 9 h, the point at which CTLA-4 expression becomes distinctly different with bortezomib treatment. GATA3 expression was reduced by approximately 50% with targeted siRNA at both the mRNA and protein level (Figure 1.15 a, b). As a GATA3-dependent positive control, we measured IL-4 mRNA and found a reduction of 40.9% ($p<0.05$) by mRNA qPCR in an average of three independent experiments (Figure 1.15c). Expression of internal control GAPDH was not affected by GATA3-specific siRNA in these same samples (Figure 1.15d), indicating the transcript reduction was not a global effect. Transcript levels of CTLA-4 in bortezomib-treated cells were diminished by 23.6% when GATA3 was depleted ($p<0.05$), but not with stimulation alone (Figure 1.15d).

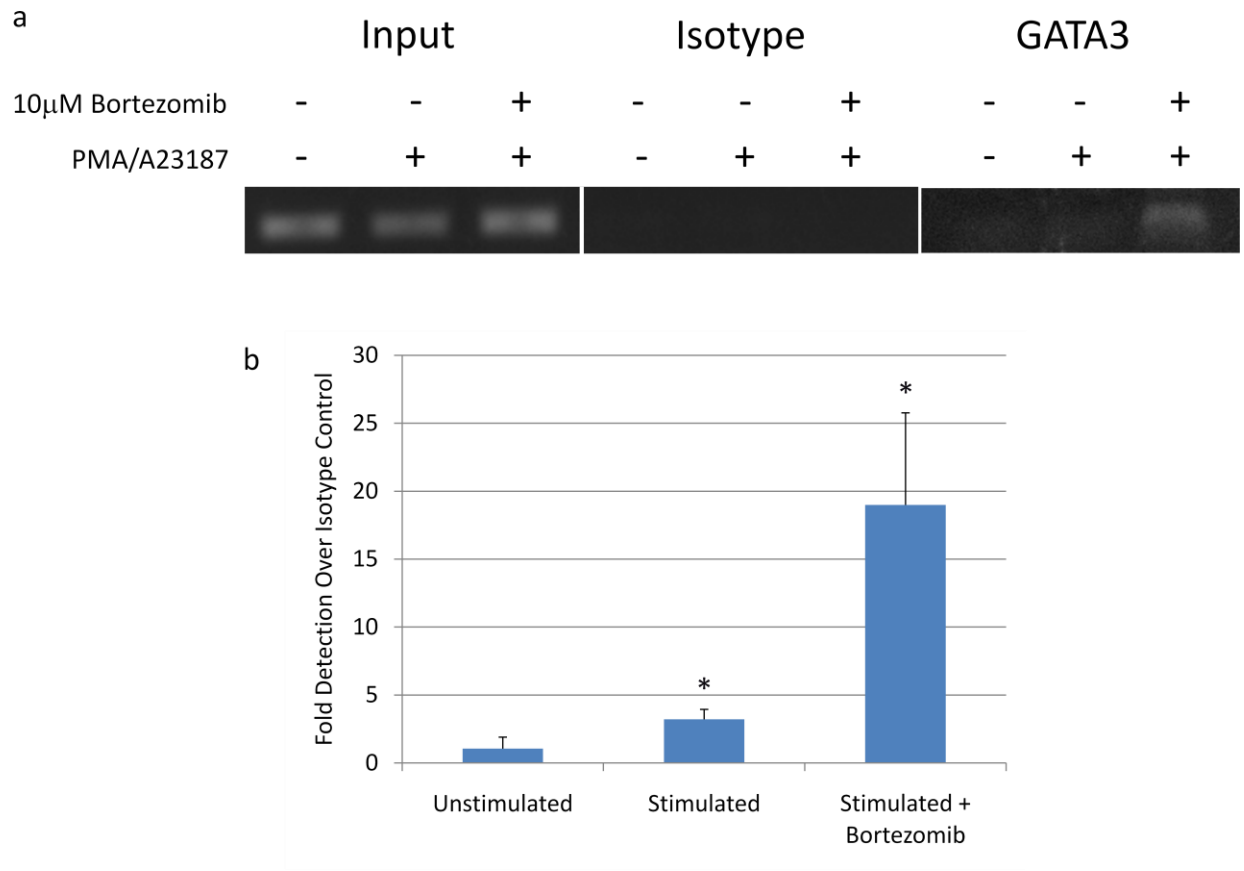

Figure 1.13. GATA3 is detected at the endogenous CTLA-4 promoter with bortezomib. $1X10^6$ fresh CD4 T cells were stimulated and/or treated with 10 μM bortezomib for 6 h as indicated, formaldehyde crosslinked and sheared by sonication. ChIP was performed as described in Materials and Methods with antibodies to GATA3 and an isotype control. Crosslinks were reversed and the DNA was purified for amplification with primers spanning the CTLA-4 promoter. Input samples serve as a loading control. a) Agarose gel of PCR products. b) Quantification by qPCR with primers spanning the proximal promoter where samples are normalized to isotype control and presented as the fold over unstimulated/untreated. Presented results are the average of three experiments ± SEM (*$p<0.05$).

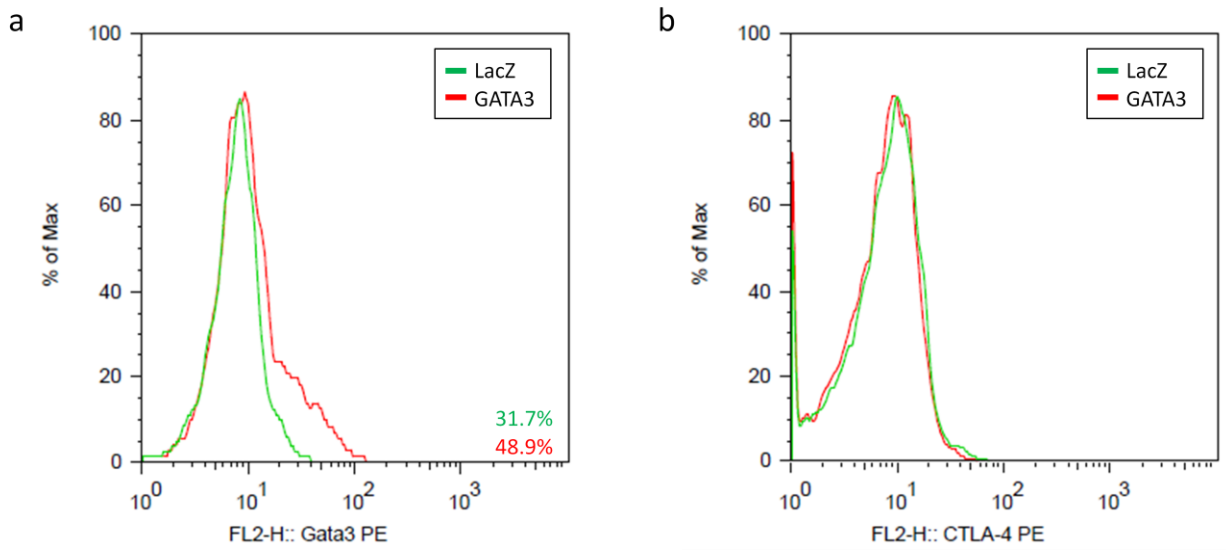

Figure 1.14. Ectopic GATA3 does not transiently affect CTLA-4 expression. $10x10^6$ primary CD4 T cells were electroporated with 2 μg GATA3 or LacZ expression vectors using the Amaxa system as described in Materials and Methods. Cells were cultured 18 h prior to stimulation for 9 h. a) Intracellular GATA3 levels were determined by permeabilization of cells and staining with a PE-conjugated GATA3 antibody as described in Materials and Methods followed by flow cytometric analysis. Histograms were generated with FlowJo software. Percentages of GATA3 expression are shown in green (LacZ) and red (GATA3) text. b) Extracellular CTLA-4 was measured by staining with a PE-conjugated CTLA-4 antibody and analysis by flow cytometry. Results are representative of three independent experiments.

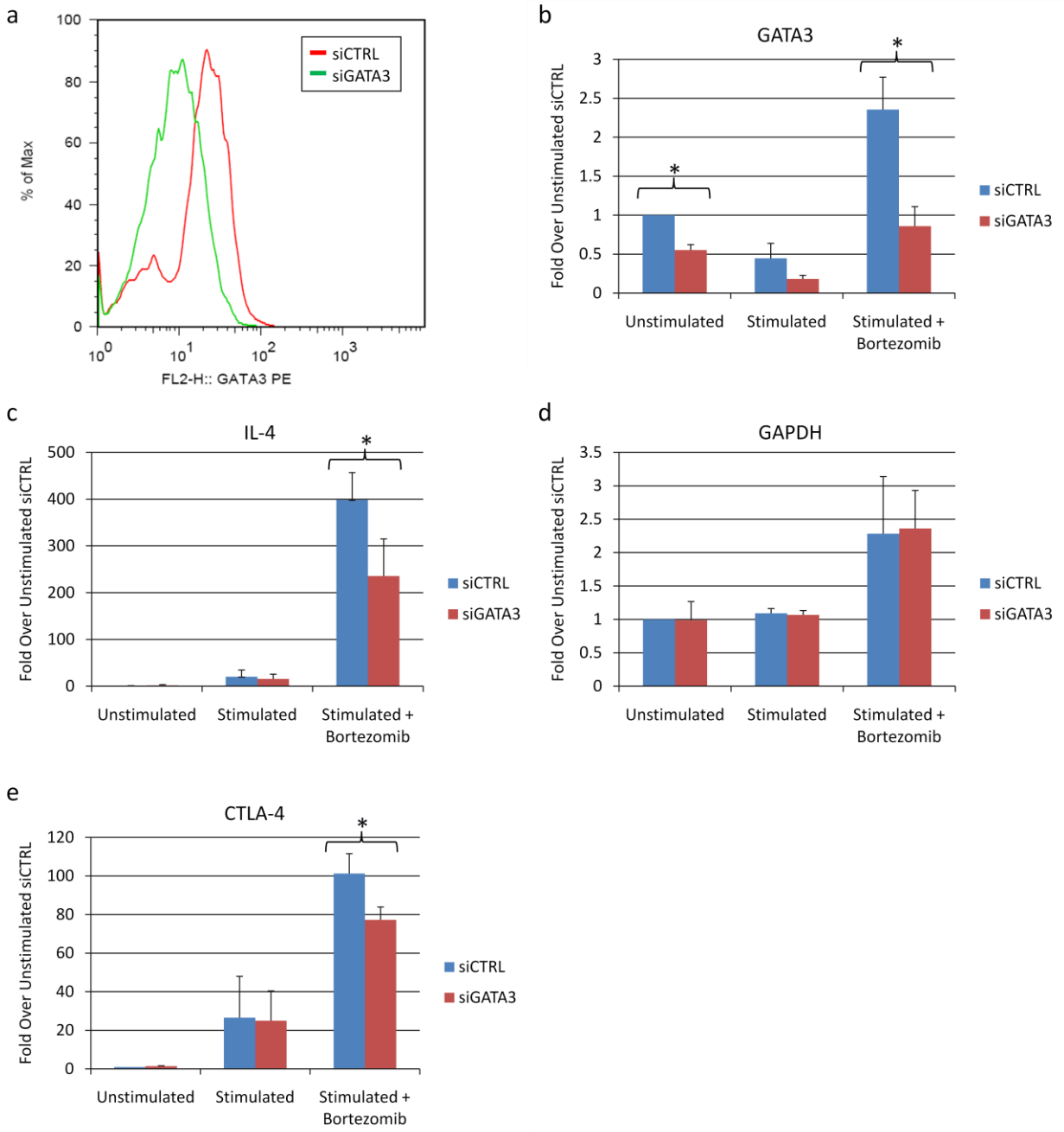

Figure 1.15. GATA3 knockdown by siRNA reduces CTLA-4 mRNA in bortezomib-treated CD4 T cells. Using the Amaxa system, $10X10^6$ fresh CD4 T cells were electroporated with 20 pmol control or GATA3-targeted SMARTpool siRNA (Dharmacon). Cells were rested for 18 h then stimulated 9 h with and without 10 μM bortezomib. Analysis of GATA3 a) protein and b) transcript was conducted by intracellular flow and qPCR, respectively. Levels of c) IL-4, d) GAPDH and e) CTLA-4 mRNA were measured by qPCR. Results are presented as averages of three independent experiments ± SEM (*$p<0.05$).

Elevated GATA3 transcript in bortezomib-treated CD4 T cells does not guarantee surface CTLA-4 expression. To establish whether the cells expressing CTLA-4 also have enhanced GATA3 transcript, we sorted bortezomib-treated and untreated cells for surface CTLA-4 and evaluated their mRNA profile by qPCR (Figure 1.16). Cells were separated into CTLA-4 + and – fractions to a purity greater than 98%. As expected, CTLA-4 mRNA expression is higher in the CTLA-4+ population (Figure 1.16a), and bortezomib treatment nearly doubles what is detected with stimulation alone. Surprisingly, GATA3 expression, though elevated with bortezomib, is not significantly different in the CTLA-4+ fraction (Figure 1.16b). We find NFAT1 levels are increased in the CTLA-4+ population and as we have observed previously bortezomib does not impact NFAT1 expression (Figure 1.16c). FoxP3 is also higher in cells expressing CTLA-4, and there is a modest decrease in the presence of bortezomib (Figure 1.16d).

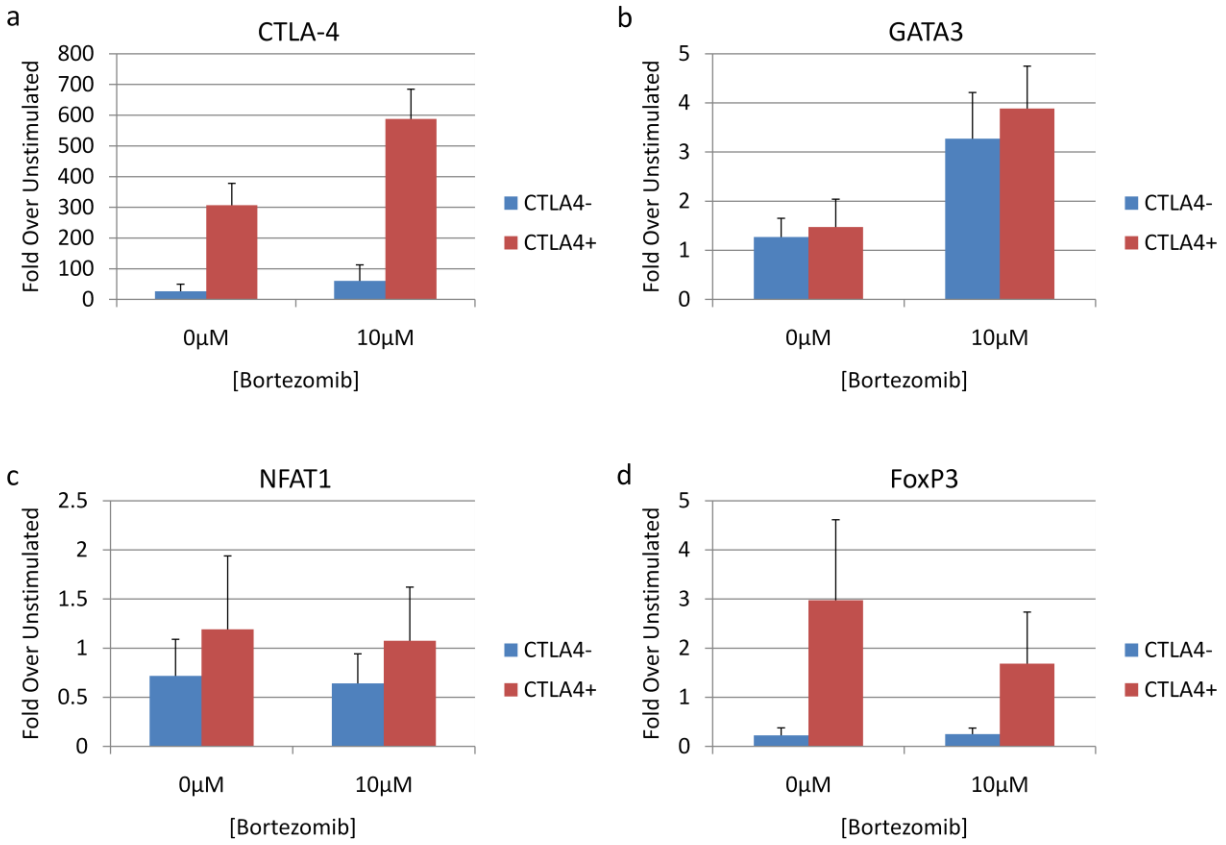

Figure 1.16. Expression profile in cells sorted for surface CTLA-4. $40x10^6$ CD4 T cells were stimulated 9 h with either 10 μM bortezomib or no treatment followed by staining with PE-conjugated α-CTLA-4 and sorting for CTLA-4 + or – fractions. After isolation of mRNA, expression of a) CTLA-4, b) GATA3, c) NFAT1 and d) FoxP3 were measured by qPCR as previously described. Presented results are the averages of three independent experiments ± SEM.

NFκB does not inhibit CTLA-4 transcription. Proteasome inhibition blocks IκB dissociation from NFκB, and thus inhibits NFκB's activity as a transcription factor. As NFκB can function as a transcriptional repressor (16), we next wanted to evaluate whether NFκB can block CTLA-4 activation. This could contribute to the CTLA-4 enhancement we find in cells treated with bortezomib. By inhibiting NFκB directly with the compound TPCA-1 followed by 9 h stimulation, we are able to examine whether NFκB blockade results in increased CTLA-4 transcription (Figure 1.17). To verify NFκB is effectively repressed, we measured transcription of the NFκB-dependent gene TNF-α by qPCR (Figure 1.17a). We find TPCA-1 reduces expression dose-dependently, with significant decreases after 1 and 10 μM treatments. CTLA-4 expression is not affected by TPCA-1 treatment (Figure 1.17b). We include samples treated with bortezomib for comparison, which do exhibit elevated CTLA-4.

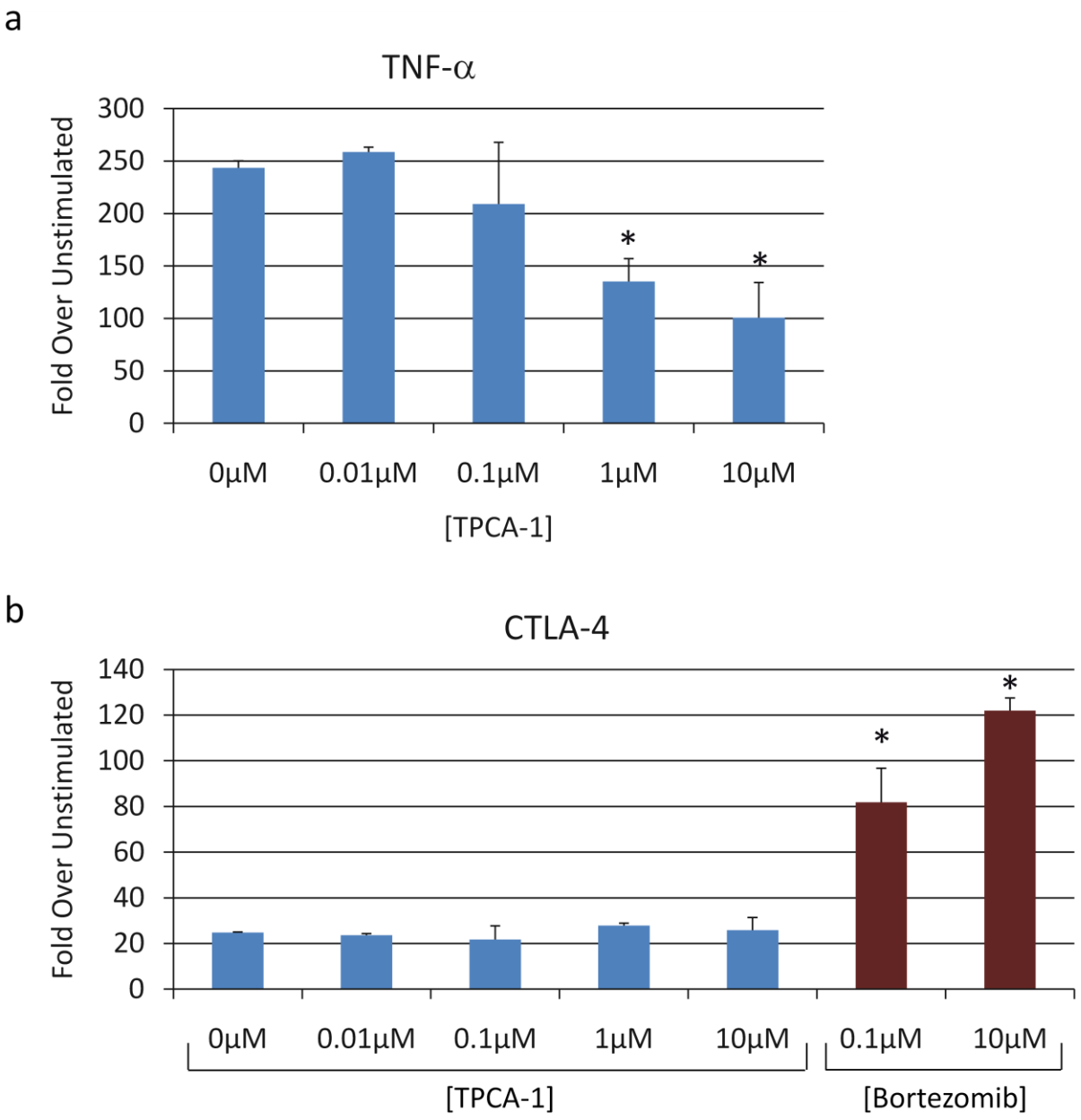

Figure 1.17. NFκB inhibitor TPCA-1 does not affect CTLA-4 expression. 5x10^6 primary CD4 T cells were treated with the indicated concentrations of TPCA-1 and stimulated for 9 h followed by mRNA analysis of a) TNFα (as an NFκB-dependent gene control) and b) CTLA-4 by qPCR as previously described (*p<0.05). Effect of 0.1 and 10 μM bortezomib is shown as red bars in panel (b) for comparison. Results are the averages of three independent experiments ± SEM.

DISCUSSION

Expression of CTLA-4 is elevated in tumor cells of patients with cutaneous T cell lymphoma (126), which may contribute to decreased anti-tumor immunity in later stages of the disease. The mechanism governing the observed transcriptional dysregulation has not been well characterized. In this study we show excessive polyubiquitination of proteins in the malignant cells of CTCL, which implies the ubiquitin-mediated proteasome pathway is defective. These results correlate with elevated CTLA-4 and GATA3 protein and transcript in patient samples, but a link between proteasome activity and expression of these genes had not been previously explored. By treating healthy primary CD4 T cells with bortezomib, we are able to isolate the single element of proteasome deficiency and establish a role for this pathway in CTLA-4 regulation. Our results not only demonstrate a novel T cell response to bortezomib, but also identify a direct role for GATA3 in CTLA-4 transcription. We show GATA3 protein levels are stabilized by proteasome inhibition and we also observe a dose-dependent increase in phosphorylation at S308, the transcriptionally active form. By luciferase and ChIP, we find GATA3 is able to bind to and induce the CTLA-4 promoter, and bortezomib enhances this interaction. After siRNA suppression of GATA3, CTLA-4 induction with bortezomib is significantly reduced. Taken together, these data delineate a mechanism where proteasome inhibition increases CTLA-4 expression through stabilization and activation of GATA3.

This study reveals aberrant proteasome regulation in CTCL, as detected by total polyubiquitin immunoblot. Interestingly, when we assayed the 20S proteolytic core

subunit of Sezary and normal extracts we found it was moderately, though not statistically significantly, more active in patient samples. This is not entirely unexpected given gene array analysis has identified two 26S proteasome regulators, PSMD3 and PSMC5 which are upregulated in Sezary (21). Taken together these data indicate the profuse polyubiquitination detected in Sezary may be due to impaired function of another component within the ubiquitin-proteasome pathway aside from the 20S proteolytic core. Given that a multitude of proteins interact with and direct the function of this pathway (45), further analysis will be required to identify the exact cause.

By inhibiting the proteasome in primary CD4 cells with either bortezomib or ALLN, we show this defect alone can augment CTLA-4 expression. When stimulating with PMA/A23187, we find CTLA-4 transcript levels are rapidly induced but peak at 3 h, after which they steadily decline (Figure 1.7a). Addition of bortezomib prevents this regression, allowing expression levels to remain elevated through 12 h. Interestingly, both GATA3 and CTLA-4 follow a similar kinetic trend when CD4 T cells are stimulated in the presence and absence of the proteasome inhibitor. Our data shows GATA3 is also stabilized in the presence of bortezomib, which substantiates previous studies with alternative proteasome inhibitors (129). Aptly, GATA3 is also overexpressed in CTCL, but further studies will be required to determine whether this is due to proteasome dysregulation. In normal CD4 T cells with stimulation alone, GATA3 protein levels diminish over time (Figure 1.19b). Bortezomib treatment leads to sustained GATA3 expression, which in turn increases transcription of GATA3-dependent genes. CTLA-4 has been implicated as a Th2-associated gene (83), which is further supported here where we show a direct involvement of GATA3 in CTLA-4 transcription.

We were initially concerned that the effects we had observed were a consequence of cytotoxicity from proteasome inhibition. In addition to multiple myeloma, bortezomib has been tested in a variety of other hematological malignancies (46, 47, 85) as well as solid tumors (3, 89, 118, 125) for its ability to induce apoptosis. Only when cells were treated for 12 h at the 10 μM concentration in our system did we detect elevated annexin V staining. Generally stimulation alone caused a slight amount of cell death, likely due to PMA toxicity. The effect of bortezomib on CTLA-4 is evident after 6 to 9 h, time points at which apoptosis levels are minimal. These data indicate the observed increase in CTLA-4 is independent of apoptosis induction.

As CTLA-4 is involved in downregulation of T cell proliferation, we wanted to address whether the increased CTLA-4 in cells treated with bortezomib could functionally suppress growth. Long treatments with bortezomib will induce apoptosis, but since bortezomib is a reversible proteasome inhibitor we are able to vastly reduce the concentration of the compound in the cell culture media by washing. Once the bortezomib and stimulation are removed, CTLA-4 levels begin to decline. However, we found bortezomib-treated cells sustained surface expression over longer intervals. Our mixed lymphocyte reaction showed increased suppression with bortezomib pretreatment. We can determine CTLA-4 is responsible for the observed growth suppression by blocking its activity with a specific antibody. These results identify bortezomib as a T cell growth inhibitor through a mechanism independent of apoptosis, which may in part explain the protective effects of this drug in prevention of GVHD without sacrificing anti-tumor effects (107).

Our results introduce a novel mechanism for CTLA-4 transcriptional regulation where GATA3 interacts with the proximal promoter. GATA3 was loosely connected to CTLA-4 by van Hamburg *et al* (115) with their use of a GATA3 overexpressing mouse model, and our data show a direct role for this transcription factor. In our luciferase study GATA3 augmented promoter activity with as little as 380 bp which indicates GATA3 may be involved with the proximal promoter. Our ChIP assay results support this, as primers were designed for the NFAT1 binding region. Alignment software predicts three potential GATA3 binding sites within the proximal promoter. We were unable to detect GATA3 binding to these sequences by electrophoretic mobility shift assay, however GATA3 may not be directly in contact with the DNA. Indeed, previous studies have shown GATA3 works cooperatively with NFAT1 to activate Th2 cytokines (6), and coprecipitation in cells transfected with expression constructs shows these two proteins can physically interact (57) which may be the case for CTLA-4 regulation. Our efforts to coprecipitate endogenous proteins were unsuccessful, but this may have been due to low protein concentrations.

FoxP3 was also shown to cooperate with NFAT1 to regulate CTLA-4 in the context of regulatory T cells (128), which express CTLA-4 constitutively. This chapter has focused on induced CTLA-4 expression in effector CD4 T cells. Our data does not support a mechanism of FoxP3 involvement in increasing CTLA-4 by proteasome inhibition. Indeed, in the presence of bortezomib FoxP3 levels are suppressed as early as 6 h after treatment and stimulation (Figure 1.8). As FoxP3 is a reliable marker of Treg cells, this would support that proteasome inhibition does not induce a transition to the Treg phenotype. In fact, it has been reported that GATA3 can directly bind to the

FoxP3 promoter and suppress FoxP3 transcription (71). We also assayed our whole cell extracts for FoxP3 expression by immunoblot and found that only 2 of 5 Sezary patients had expression levels higher than normal. This is consistent with previous findings showing erratic expression of FoxP3 in CTCL (42, 119).

Our previous work did not identify a role for NFκB in activation of CTLA-4. If NFκB was required for CTLA-4 transcription, proteasome inhibition would suppress expression. We also wanted to determine if NFκB plays a suppressive role in CTLA-4, and through use of specific inhibitor TPCA-1 we again find NFκB has no effect. Not only does this provide more details to CTLA-4 regulation, but it also suggests therapeutic use of NFκB inhibitors should not directly modulate CTLA-4 expression.

When we sorted cells to isolate those with surface CTLA-4 expression, we found mRNA levels of CTLA-4 were roughly 10-fold higher in cells that were CTLA-4+, regardless whether they were treated with bortezomib. This implies a certain subset of CD4 T cells may be more likely to express CTLA-4. Interestingly, cells treated with bortezomib had about 2-fold higher mRNA and surface protein which suggests trafficking to the cell membrane also increases in this population. Expression of NFAT1 was nearly equivalent among all samples tested, but FoxP3 was specifically expressed in CTLA-4+ cells. A slight but not statistically significant reduction in FoxP3 mRNA was detected in bortezomib-treated cells as compared to stimulation alone, which mimics what we observe at the protein level. We were surprised to find GATA3 levels were not significantly higher in the CTLA-4+ fraction, particularly in the bortezomib-treated samples. This may indicate either another factor is involved or that activation of GATA3 only occurs in a limited collection of cells.

GATA3 activation plays a critical role in bortezomib-induced CTLA-4 expression. We show the p38 kinase pathway mediates GATA3 phosphorylation in the presence of bortezomib. This is not unexpected, given proteasome inhibition triggers a stress response and p38 is activated by cell stress conditions (92). The inhibitor we used, SB203580 did not completely inactivate p38 in our cells, as illustrated by the phospho-MAPKAPK2 blot with our samples. Importantly, the level of phospho-MAPKAPK2 reduction did coincide with that of phospho-GATA3. We did not detect an increase in CTLA-4 upon transient overexpression of GATA3, but this may have been due to a lack of GATA3 phosphorylation under these circumstances. Overexpression in a mouse model did, however, result in CTLA-4 upregulation (115).

Our targeted inhibition of GATA3 by siRNA led to greater than a 50% reduction in GATA3 mRNA and protein. Similar to our other experiments, bortezomib treatment and stimulation for 9 h increased GATA3 roughly 2.5-fold over unstimulated cells when electroporated with control siRNA. GATA3-directed siRNA suppresses mRNA levels in bortezomib-treated samples to just below that of the unstimulated control. This was a functionally adequate reduction, as observed in the measured effect on the GATA3-dependent Th2 cytokine IL-4. The statistically significant reduction in CTLA-4 after GATA3 ablation again validates the important role GATA3 plays in CTLA-4 activation in cells treated with bortezomib. Proteasome inhibition likely triggers the kinase pathway responsible for GATA3 activation as well, as the level of phospho-GATA3 increases dose-dependently with bortezomib. Taken together, these data show that both stabilization of GATA3 protein and increased phosphorylation are essential for the observed augmentation of CTLA-4 transcription.

CHAPTER 2

Identification of the Role of p300 in Activation of T-cell Regulator CTLA-4 by Use of Adenovirus 2 E1A

ABSTRACT

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an important regulator of T cell activation, and thus transcriptional regulation is a tightly controlled process. Expression of the CTLA-4 gene in effector T cells is induced by activation and binding of NFAT1 in the proximal promoter. Upon activation, histones in the CTLA-4 proximal promoter become acetylated at lysines 9 and 14. The presence of these modifications is conventionally recognized as an activated chromatin region. As NFAT1 has no known histone acetyltransferase (HAT) activity, we sought to determine whether a cofactor may be involved by utilizing adenovirus 2 E1A proteins.

Adenovirus 2 E1A 12S contains two distinct conserved regions. The E1A conserved region 1 (CR1) and 2 (CR2) interact with a variety of cellular proteins including histone acetyltransferase p300 and cell cycle regulator pRb, respectively. By co-transfecting luciferase CTLA-4 constructs with wild-type and mutant E1A 12S plasmids in the leukemia cell line Jurkat, we show that the CR1 region, but not CR2, is capable of inhibiting CTLA-4 transcription. During normal activation of this gene, acetylation of histone 3 occurs at lysines 9 and 14. Analysis utilizing chromatin immunoprecipitation (ChIP) assays reveals that this histone acetylation does not occur in the presence of the E1A 12S CR1 region, implicating the potential involvement of

p300. Using the p300 inhibitors curcumin and garcinol, we are able to block transcription of CTLA-4 and demonstrate a role for p300 in CTLA-4 activation. Through use of calcineurin inhibitor cyclosporin A (CyA), we also show NFAT1 binding to the proximal promoter precedes histone acetylation by ChIP assay.

INTRODUCTION

Gene regulation involves the concerted activity of transcription factors and epigenetic modifiers to either initiate or suppress expression. Organization and packaging of genomic DNA is a dynamic process maintained by the wrapping of DNA around nucleosomes made up of core histone proteins H2A, H2B, H3 and H4 (68). A variety of covalent modifications to specific residues of these proteins can further remodel DNA/histone complexes into open, active regions of euchromatin or compressed, transcriptionally inaccessible heterochromatin (54). Specifically, histone 3 acetylation at lysine residues 9 and 14 has been associated with elevated gene expression (2). Addition and removal of acetyl groups at these sites is controlled by the balance between histone acetyltransferases (HATs) or deacetylases (HDACs), respectively (106).

The acetyltransferase p300 was initially identified due to its interaction with the adenovirus early protein E1A (36). Since then, p300's role as a transactivator and HAT has been elucidated. Previous reports have shown p300 interacts with an assortment of transcription factors, including but not limited to AP-1, p53, STAT family members, TFIIB, NFκB, NFAT1 and members of the GATA family, among many others (34). Structurally homologous to p300, CREB-binding protein (CBP) also cooperates with many transcription factors and acetylates many cellular proteins including histones, which leads to the restructuring of chromatin for gene expression. The CBP and p300 proteins are often paired together and can be referred to collectively as p300/CBP. Another related cofactor, p300/CBP-associated factor, or PCAF, associates with p300

and CBP to fulfill its function as a cofactor. Through use of a series of E1A mutants, Liu *et al* established a role for p300 in IL-5 regulation (65) and we employed a similar technique to study CTLA-4 transcription.

More recently, chemical inhibitors to HATs have been discovered and exploited for their impact on gene transcription. The polyphenolic compound curcumin efficiently disrupts p300-dependent histone acetylation without affecting PCAF (8). Less specifically, polyisoprenylated benzophenone, or garcinol, can also block p300 but has a stronger potency toward PCAF (7). The high cellular toxicity of garcinol led to development of its derivative LTK-14, which is specific to p300 but is currently not commercially available. The effect of these compounds on CTLA-4 transcription has not previously been investigated.

Our initial studies identified an NFAT1 binding site in the proximal CTLA-4 promoter in addition to histone acetylation upon T cell induction (41). The main objective of this section was to determine whether p300 is responsible for the detected acetylation at the CTLA-4 promoter through use of a panel of E1A constructs in addition to the above described chemical inhibitors to p300.

RESULTS

The CR1 region of the E1A 12S protein suppresses activity of CTLA-4 reporter constructs. To determine whether E1A proteins can impact CTLA-4 expression, wild-type and mutant 12S E1A vectors were cotransfected with a series of CTLA-4 luciferase promoter constructs in Jurkat T cells as detailed in the schematic in Figure 2.1. The two E1A mutants individually eliminate the two conserved regions (CR), CR1 and CR2 of the wild-type 12S splice variant. CR1 has been shown to sequester cellular proteins including p300, while CR2 is known to bind pRB. For this study, we utilized one 264 bp CTLA-4 promoter construct that omitted the NFAT1 binding site and three constructs with larger promoter regions of 380 bp, 4 kbp and 6 kbp.

After cotransfection, we measured promoter activity by luciferase assay as described in Materials and Methods (Figure 2.2). Our results are presented as relative light units (RLU). As expected due to the lack of NFAT1 binding, the 264 bp construct had undetectable luciferase levels which were unaffected by the presence of E1A vectors. For the remaining constructs we find strong luciferase activity for the promoter alone, but the addition of wild-type 12S E1A significantly suppresses promoter induction. Mutation of the CR1 region, which is known to bind p300, results in the rescue of luciferase activity for each of our promoters. In the 4 kbp and 6 kbp constructs, we actually detect an increase in RLUs with this mutant. However when the CR2 region is mutated, luciferase activity is not recovered.

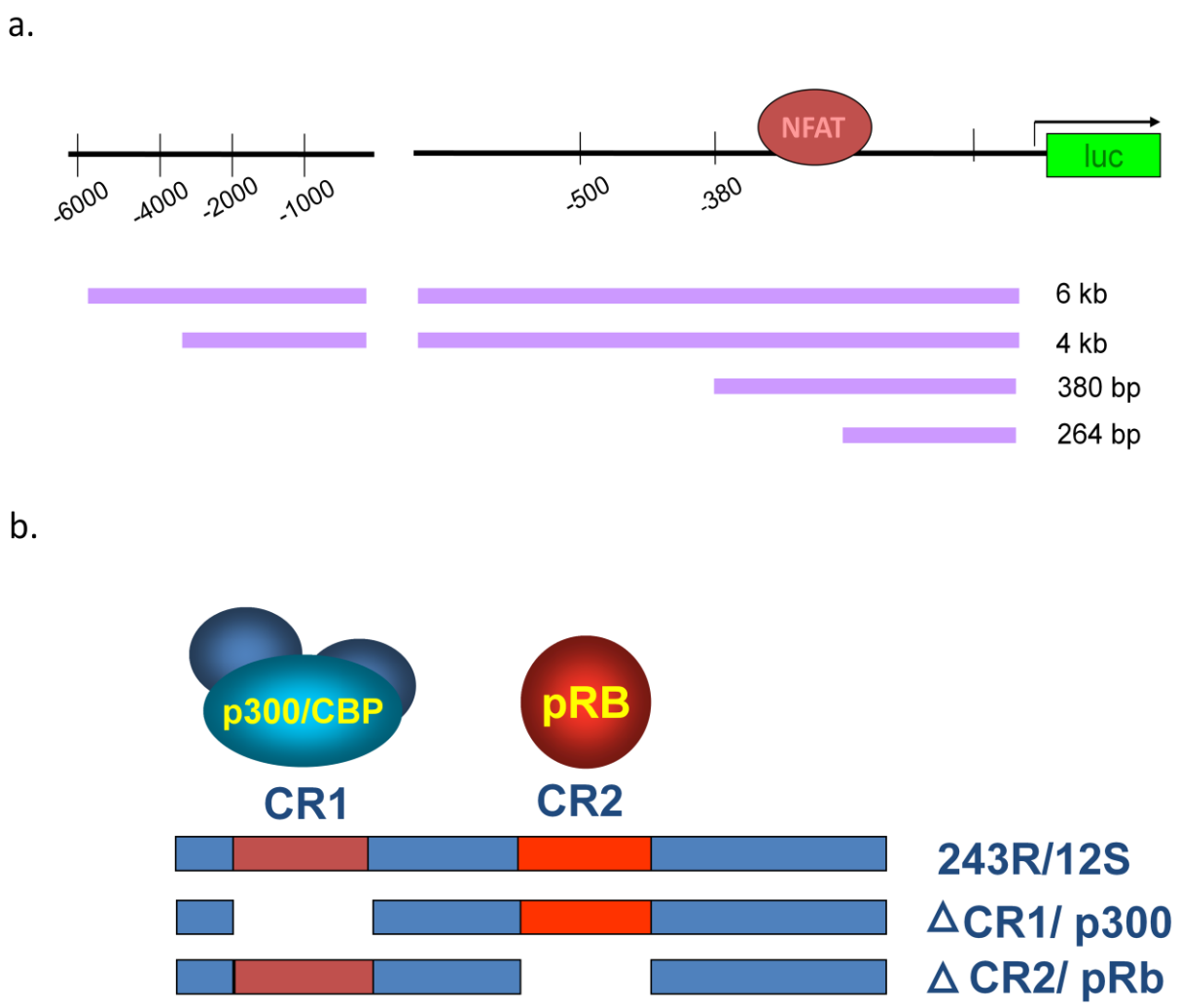

Figure 2.1. CTLA-4 promoter constructs and 12S E1A mutants. a) The CTLA-4 promoter was cloned from genomic DNA and 5' deletion constructs were ligated into luciferase reporter plasmid pGL3. This study used clones of 264 bp, 380 bp, 4 kbp and 6 kbp. As indicated in the top of the schematic, previous work has shown NFAT1 binds to the -280 region of the CTLA-4 promoter. b) Schematic of 12S E1A wild-type and mutants ΔCR1 and ΔCR2. The CR1 region binds p300/CBP while CR2 binds pRB.

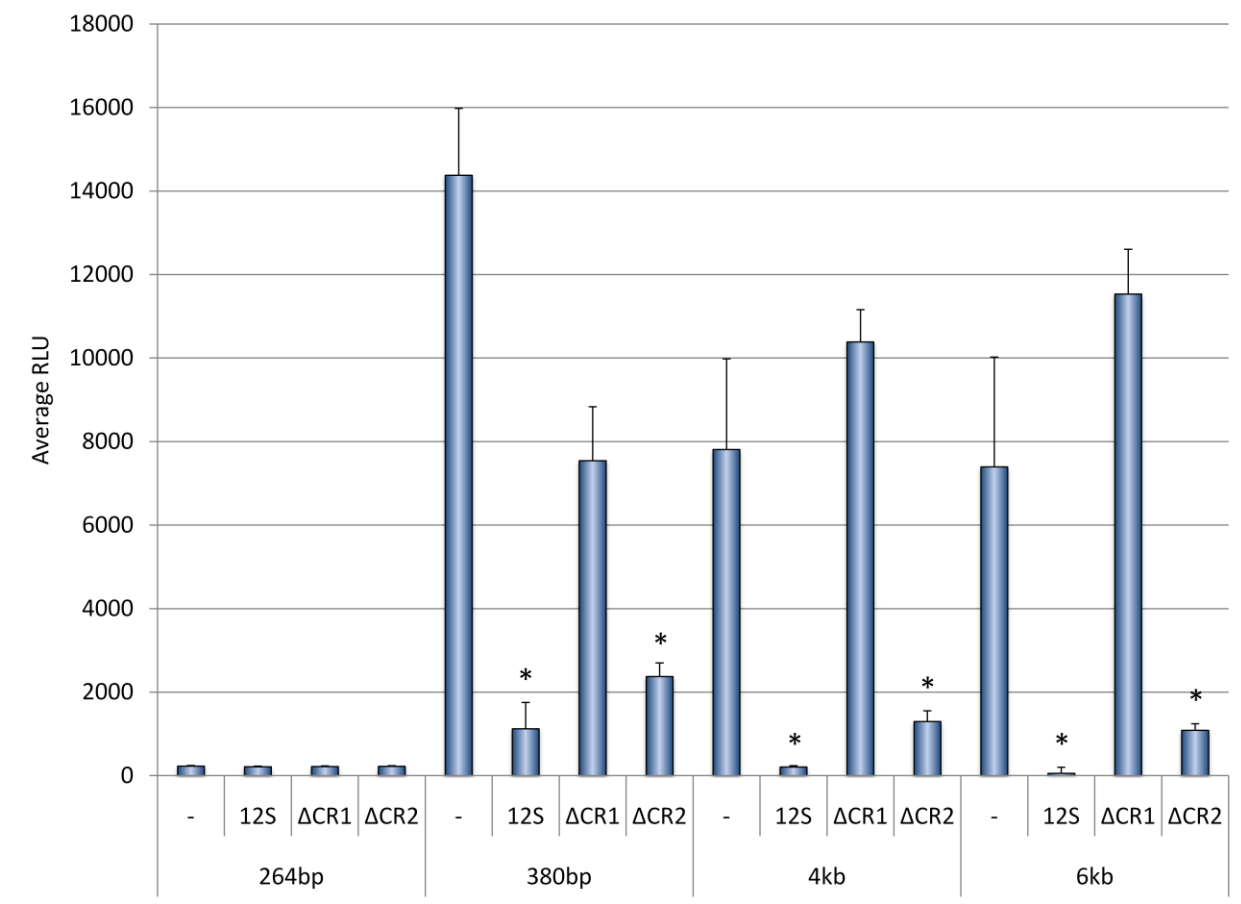

Figure 2.2. CTLA-4 promoter activity with E1A wild-type and mutant plasmids. Jurkat cells were cotransfected with 2 μg each E1A plasmid and in combination with 2 μg CTLA-4 luciferase constructs using Lipofectin as detailed in Materials and Methods. Results are presented as the average RLU of triplicate samples and are representative of three or more independent experiments ± SEM (* $p<0.05$).

Histone acetylation is blocked by wild-type 12S E1A but not ΔCR1. To determine the cause of promoter activity suppression in cotransfected samples, we conducted chromatin immunoprecipitation (ChIP) assays to measure levels of NFAT1 binding and histone 3 acetylation (Figure2.3). After Jurkat cotransfection of E1A plasmids with the 4 kbp CTLA-4 luciferase construct as in Figure 2.2, samples were either stimulated with PMA/A23187 for 4 h or left unstimulated. Chromatin was prepared as described in Materials and Methods and immunoprecipitated with antibodies specific to NFAT1 and acetyl histone 3 at lysines 9 and 14. The resulting DNA was analyzed by real-time PCR with a forward primer specific to the CTLA-4 promoter and a reverse primer within the luciferase gene of pGL3. As Jurkat cells do not express CTLA-4, this primer set allowed us to eliminate background detection of the endogenous promoter.

After stimulation, we detect an increase in NFAT1 binding at the transfected promoter (Figure2.3a). The 2- to 3-fold increase after stimulation is similar to what we previously reported at the CTLA-4 promoter in stimulated PBMCs, indicating the exogenous promoter behaves in a similar manner to what we find in primary cells. Addition of the 12S E1A wild-type and mutant plasmids does not reduce NFAT1 binding at the exogenous promoter. In fact, addition of the wild-type 12S E1A construct results in elevated detection of NFAT1.

Histone acetylation is also enhanced after stimulation, though the degree of increase is much lower than that of NFAT1 (Figure2.3b). Wild-type 12S E1A blocks histone acetylation, reducing detectable levels to 25% of the non-E1A samples. Interestingly, there is an equivalent reduction in the unstimulated cells, which implies acetylated histones accumulate on the plasmid in the absence of activation. When the

E1A CR1 region is eliminated, histone acetylation is no longer inhibited. Similar to the luciferase results in Figure 2.2, our CR2 mutant impedes acetylation to levels comparable to wild-type.

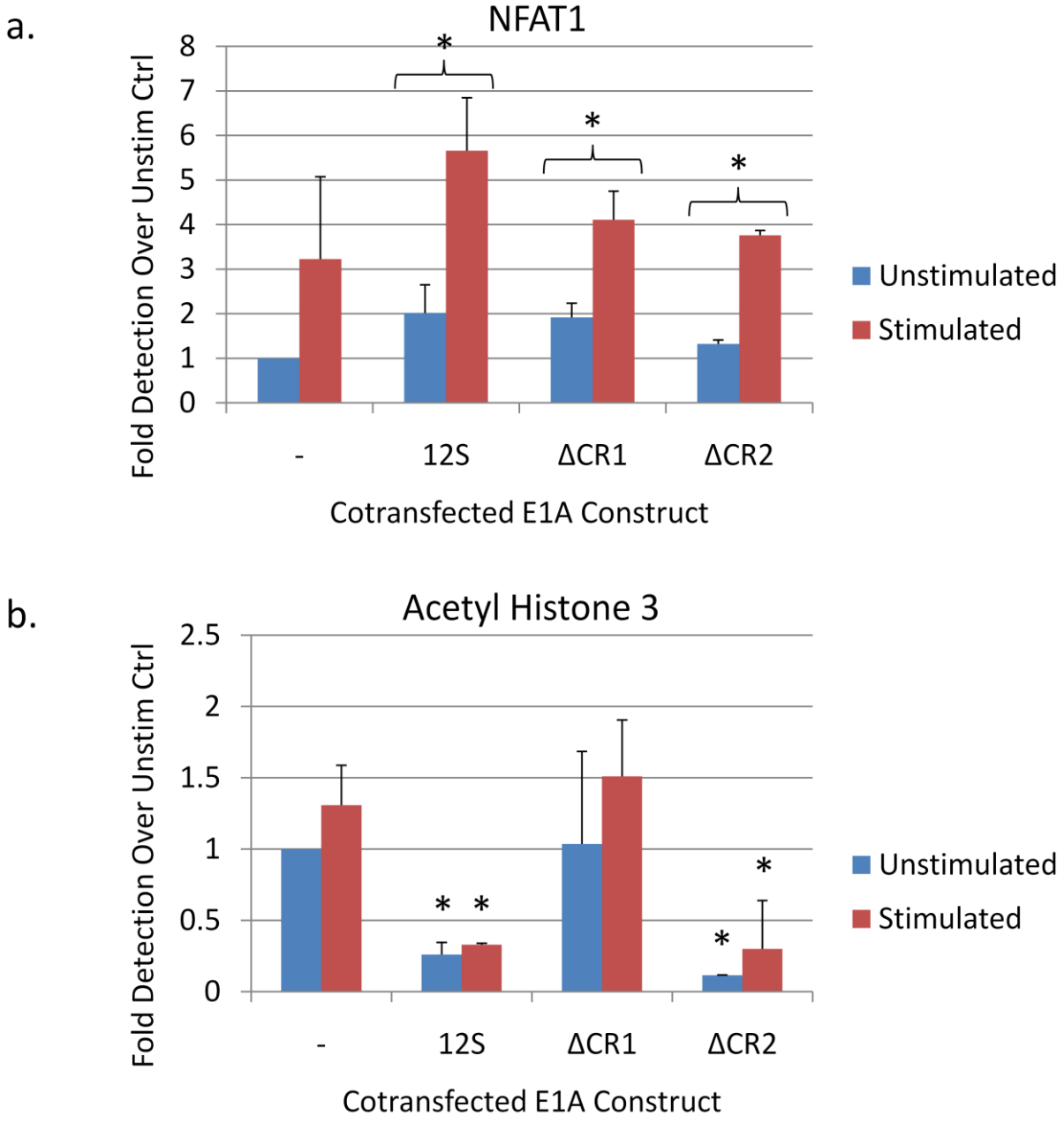

Figure 2.3. ChIP assay analysis of cotransfected Jurkat cells. Jurkat cells were transfected with 2 μg 4 kbp CTLA-4 luciferase plasmid and 2 μg E1A constructs using Lipofectin as described in Materials and Methods. Cells were formaldehyde crosslinked and sheared by sonication. ChIP was performed as described in Materials and Methods with antibodies to a) NFAT1, b) acetylated histone 3 (K9/14) and isotype control. Crosslinks were reversed and the DNA was purified for amplification with primers spanning the NFAT binding region at -280 bp through the 5' region of the luciferase gene. Results are presented as the average fold over unstimulated without E1A normalized to isotype control and are representative of two independent experiments ± SEM (* $p<0.05$).

The E1A 12S construct suppresses CTLA-4 transcription in primary CD4 cells. The previous experiments show our E1A wild-type and mutant constructs can impact an ectopic CTLA-4 promoter in the Jurkat cotransfection system, but our objective is to study the endogenous CTLA-4 promoter. We next transfected E1A into normal primary CD4 T cells to observe the effect on CTLA-4 expression. Using the Amaxa nucleofector system, 2 μg each E1A vector or control plasmid was electroporated into freshly isolated CD4 cells. After resting 36 h, cells were stimulated 4 h, the point at which CTLA-4 expression is at its peak. By real-time PCR we then measured induction of CTLA-4 as compared to unstimulated control plasmid in each of the samples (Figure 2.4a). Similar to our findings with the luciferase assay, we detect a 52.9% reduction of CTLA-4 transcription with wild-type 12S E1A. It is important to note the level of suppression in these experiments is limited to the transfection efficiency, which ranges from 40-60% in these samples by eGFP transfection. When the p300-binding CR1 region is mutated, CTLA-4 expression is recovered. Contrary to the luciferase results, we also find expression is restored with the CR2 mutant.

As the goal of these studies is to find methods to specifically target CTLA-4 expression, we also utilized these samples to measure expression of IL-2 (Figure 2.4b) and GAPDH (Figure 2.4c). We find IL-2 levels are also reduced, by 71.3%, with the 12S construct, indicating the effect is not specific to CTLA-4. The repressive effect of the 12S construct is not global, however. Expression of internal control gene GAPDH is unaffected by this vector, implying the effect may be more specific to induced T cell genes.

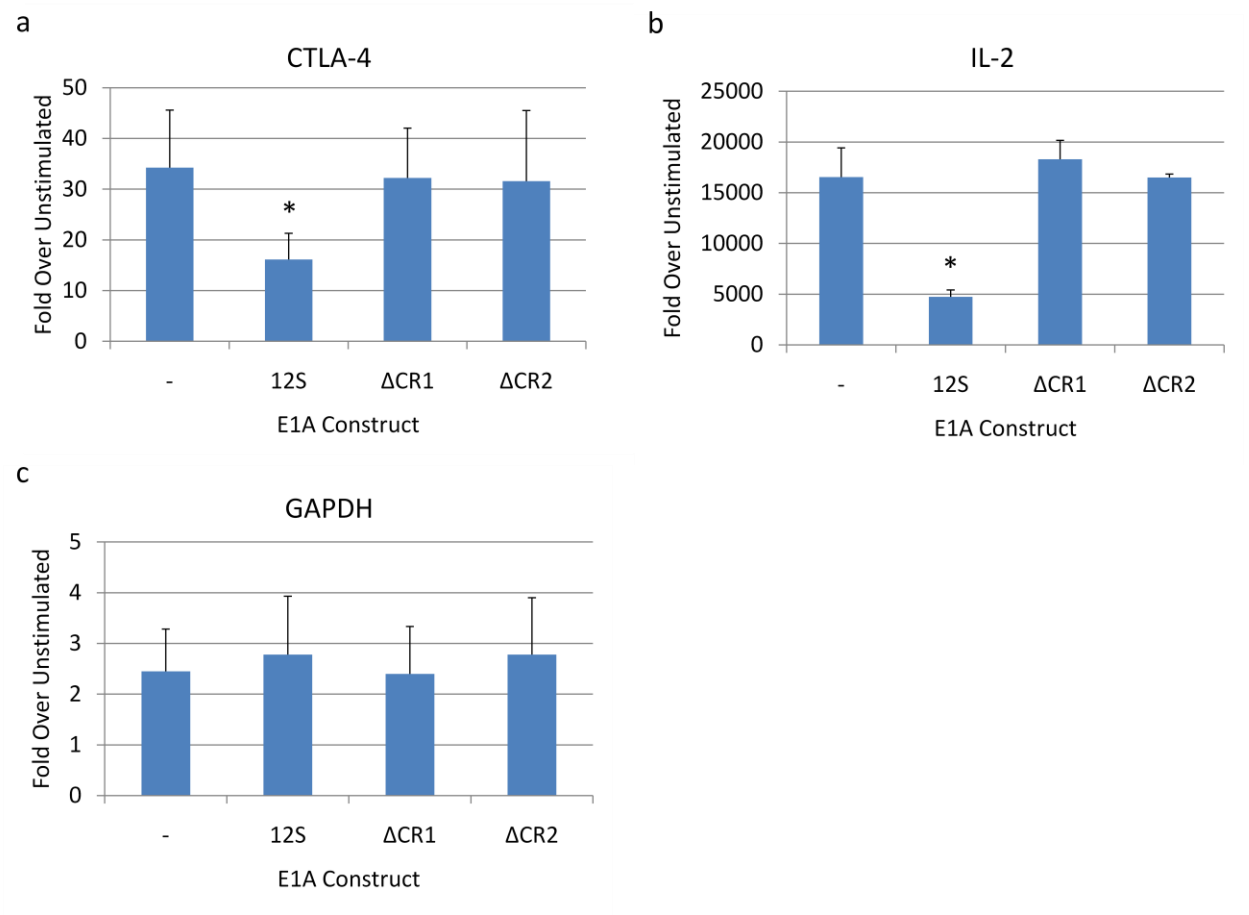

Figure 2.4 Transfection of E1A constructs into primary CD4 T cells. $10x10^6$ primary CD4 T cells were electroporated with 2 μg each of the indicated E1A constructs or a vector control using the Amaxa system as described in Materials and Methods. Cells were then cultured 36 h to allow expression of the constructs, followed by stimulation with PMA/A23187 for 3 h. After RNA was isolated with Trizol, cDNA was synthesized as described in Materials and Methods. qPCR was performed with primers specific to a) CTLA-4, b) IL-2 and c) GAPDH. Results are the averages of 3 independent experiments, presented as the fold over an unstimulated sample ± SEM, normalized to B2M (*$p<0.05$).

NFAT1 binding precedes histone acetylation at the CTLA-4 promoter. NFAT1 nuclear translocation requires activation of the calcium-dependent cytoplasmic phosphatase calcineurin. Cyclosporin A inhibits calcineurin, thus blocking NFAT1 and preventing transcriptional activation of NFAT1-dependent genes (12). To determine the sequential order of NFAT1 binding and histone acetylation at the CTLA-4 promoter in primary cells, we evaluated the effect of NFAT1 blockade on levels of histone 3 acetyl 9/14 by ChIP (Figure 2.5). Freshly isolated PBMC were either untreated or treated with 10 ng/mL cyclosporin. Unstimulated samples were compared to cells stimulated for 2 h with PMA/A23187.

As we have previously observed detection of NFAT1 at the CTLA-4 promoter increased more than 3-fold after stimulation in untreated samples (Figure2.5a). Addition of cyclosporin A inhibited NFAT1 binding; measurable levels were reduced to those of unstimulated cells in these samples. When we assayed for acetylated histone 3, we again saw a 3-fold induction with stimulation alone. Importantly, NFAT1 blockade also negatively impacted the level of histone acetylation, again diminishing detection to that of unstimulated samples. As the cofactor p300 is not directly activated by calcineurin, these data suggest histone acetylation at the CTLA-4 proximal promoter is directly dependent on nuclear translocation of NFAT1.

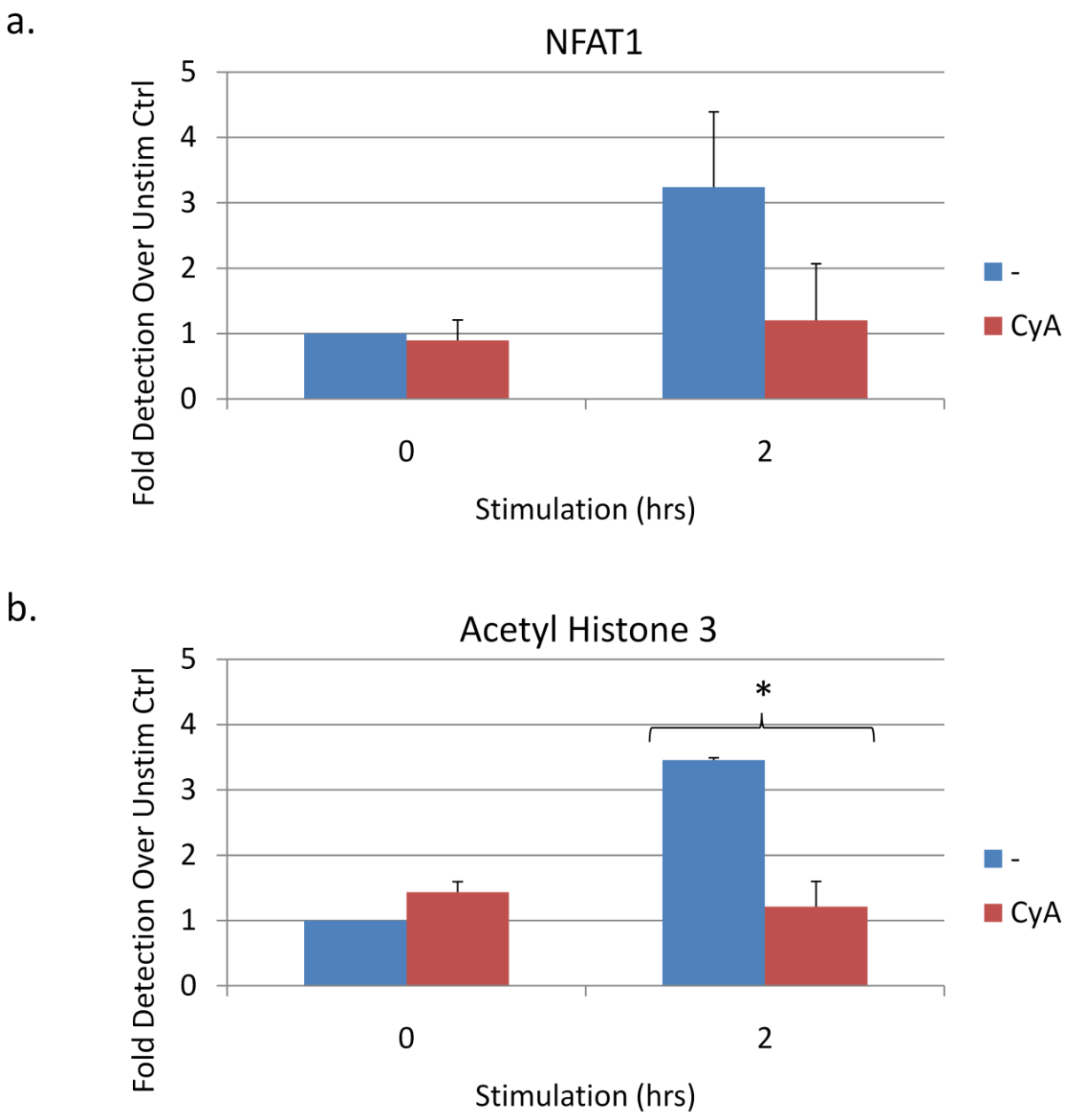

Figure 2.5. ChIP assay analysis of cyclosporin-treated primary PBMC. Primary PBMC were isolated by Ficoll preparation as described in Materials and Methods. $10x10^6$ cells were either untreated or treated with 10 ng/mL CyA for 30 min, after which samples were split in half and either stimulated with PMA/A23187 for an additional 2 h or left unstimulated followed by formaldehyde crosslinking and shearing by sonication. ChIP was performed as described in Materials and Methods with antibodies to a) NFAT1, b) acetylated histone 3 (K9/14) and isotype control. Crosslinks were reversed and the DNA was purified for amplification with primers spanning the CTLA-4 NFAT binding region at -280 bp. Results are presented as the average fold over unstimulated/untreated ± SEM normalized to isotype control and are representative of two independent experiments (*$p<0.05$).

The p300 inhibitor curcumin blocks CTLA-4 transcription in CD4 T cells. Our previous experiments have shown E1A blocks histone acetylation at the CTLA-4 promoter, but it is not clear which HAT is involved. Curcumin has been used to specifically inhibit p300 (8). To determine whether p300 plays a role, we treated primary CD4 T cells with increasing concentrations of curcumin and analyzed the samples for mRNA expression (Figure 2.6). CTLA-4 transcription is repressed dose-dependently, with a significant reduction starting at 20 μM (Figure 2.6a). To establish whether curcumin could serve as a CTLA-4-specific inhibitor, we measured IL-2 transcript levels in the same samples (Figure 2.6b). Unfortunately, IL-2 expression is also abrogated implying curcumin may inhibit multiple aspects of the adaptive immune system. The concentrations needed to suppress expression were similar for both IL-2 and CTLA-4.

To be sure transcription inhibition was not global we also measured mRNA levels of internal control GAPDH (Figure 2.6c). The 20 μM concentration actually caused an increase in GAPDH expression, which was further elevated at 80 μM. The GAPDH gene does not appear to be regulated by p300 and may actually benefit from p300 inhibition, as we observed a dose dependent increase in GAPDH mRNA.

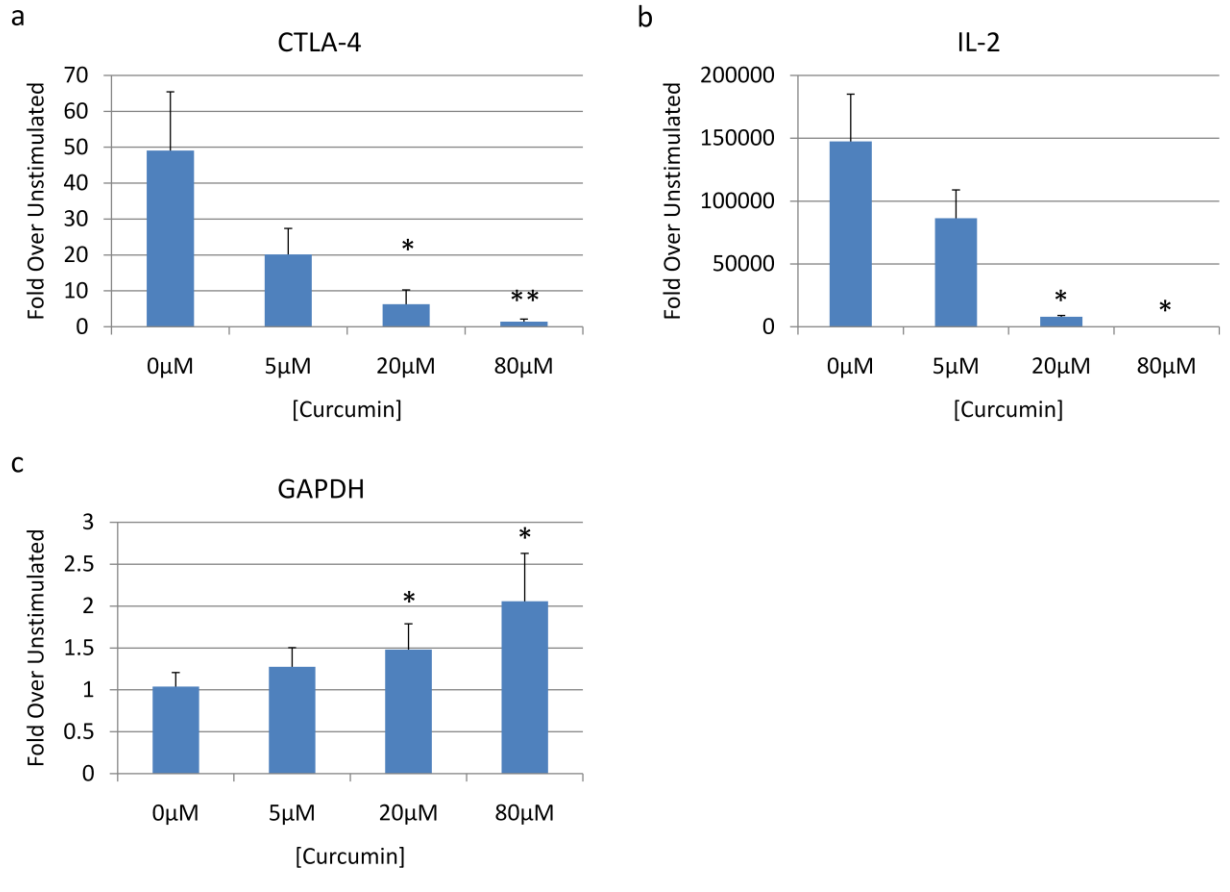

Figure 2.6 Analysis of mRNA after p300 inhibition with curcumin. Primary CD4 T cells were isolated as previously described. $5x10^6$ cells were pre-treated for 1 h with the indicated concentration of curcumin with 0 μM serving as a DMSO vehicle control, then stimulated for 3 h with PMA/A23187. RNA was isolated with Trizol and cDNA was synthesized as described in Materials and Methods. Expression of a) CTLA-4, b) IL-2 and c) GAPDH was measured by qPCR as described in Materials and Methods using specific primers and B2M as the internal control. Results are presented as fold over an unstimulated sample and are the averages of experiments with 3 independent donors ± SEM (*$p<0.05$, **$p<0.005$).

High concentrations of garcinol suppress CTLA-4 expression. The HAT inhibitor garcinol can block both PCAF and p300, with a higher affinity for PCAF (7). We treated primary CD4 T cells with increasing concentrations of garcinol and measured the effect on mRNA expression as we did with curcumin in Figure 2.6. The concentrations necessary to block CTLA-4 were far higher for garcinol than curcumin (Figure 2.7a). We did not detect a significant decrease until 80 μM. IL-2 expression followed the same dose response and was also significantly suppressed with the 80 μM treatment (Figure 2.7b). Again similar to curcumin we found an increase in GAPDH with 80 μM garcinol (Figure 2.7c).

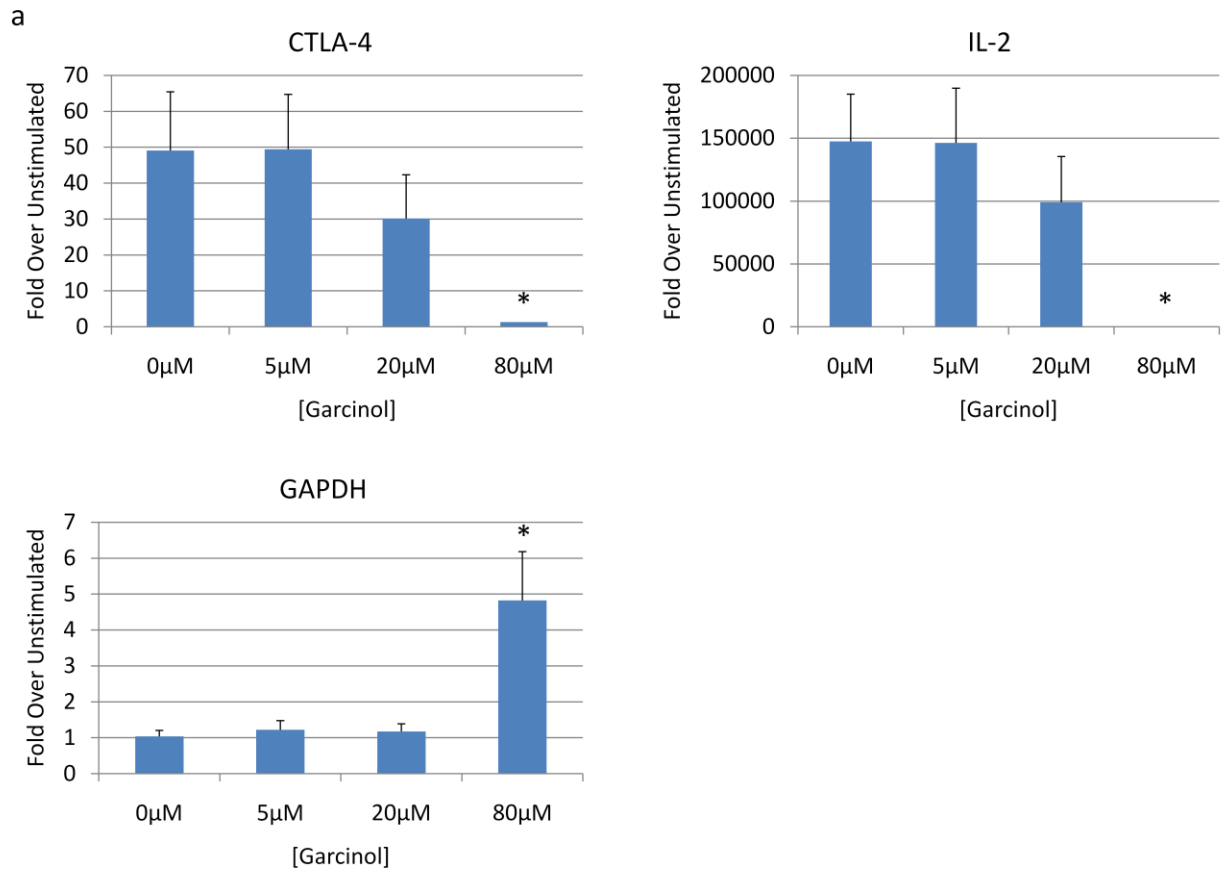

Figure 2.7. Garcinol modulates mRNA expression in primary CD4 T cells. Primary CD4 T cells were isolated as previously described. $5x10^6$ cells were pre-treated for 1 h with the indicated concentration of garcinol with 0 μM serving as a DMSO vehicle control, then stimulated for 3 h with PMA/A23187. RNA was isolated with Trizol and cDNA was synthesized as described in Materials and Methods. Expression of a) CTLA-4, b) IL-2 and c) GAPDH was measured by qPCR as described in Materials and Methods using specific primers and B2M as the internal control. Results are presented as fold over an unstimulated sample and are the averages ± SEM of experiments with 3 independent donors (*$p<0.05$).

DISCUSSION

The work within this chapter has not only identified an NFAT1-dependent cofactor involved in histone acetylation at the CTLA-4 promoter, but through the use of E1A proteins we show this method can be a valuable tool for studying gene regulation. Our initial studies used cotransfection of luciferase CTLA-4 constructs with wild-type and mutant E1A 12S plasmids in the leukemia cell line Jurkat. We show that the CR1 region, but not CR2, is capable of inhibiting CTLA-4 promoter induction. In these experiments, we found the wild-type E1A 12S protein blocked luciferase activity with as little as 380 bp of the promoter. As previously reported, the 264 bp promoter construct was inactive, and addition of E1A proteins had no effect on this region. Use of larger promoter constructs did not improve luciferase production over the 380 bp alone, and again both the wild-type E1A and ΔCR2 constructs abrogate promoter activity in each of these plasmids. These data suggest the involved cofactor binds to CR1 and may be directly interacting with NFAT1.

During normal activation of the CTLA-4 gene, acetylation of histone 3 occurs at lysines 9 and 14. Analysis utilizing chromatin immunoprecipitation (ChIP) assays in our Jurkat cotransfection model reveals that this histone acetylation does not occur in the presence of an intact CR1 region, implicating the potential involvement of HATs p300 or PCAF, which both interact with CR1. Interestingly, we find acetylated histone interacts with the exogenous promoter even in the absence of stimulation in Jurkat cells which may be an artifact of our model. Addition of 12S and ΔCR2 E1A eliminates acetylated histone 3 detection in both unstimulated and stimulated cotransfected Jurkats. When

we immunoprecipitate with antibodies specific to NFAT1, we find stimulation leads to increased NFAT1 binding. Unlike histone acetylation, NFAT1 interaction with the CTLA-4 promoter is not affected by E1A.

Given E1A suppresses histone acetylation but does not block NFAT1 interaction with an ectopic CTLA-4 promoter we wanted to evaluate whether histone acetylation would require NFAT1 at the endogenous promoter in primary cells. Upon induction by PMA/A23187 stimulation, we find both NFAT1 and acetylated histone localize to the proximal CTLA-4 promoter. By pre-treating cells with CyA we block NFAT1 nuclear translocation as evidenced by our ChIP results. Under these conditions we also find histone acetylation is lost. CyA has no direct effect on HATs, and therefore these results provide evidence NFAT1 binding precedes histone acetylation at the CTLA-4 promoter.

Similar to our Jurkat cotransfection experiments, the wild-type 12S E1A construct is also capable of inhibiting CTLA-4 transcription in primary CD4 T cells. The 12S construct reduced expression to approximately 50% of the control sample. Unexpectedly, mutation of either the CR1 or CR2 site revived CTLA-4 induction, which may indicate the involved cofactor is binding to both regions. Another interesting finding is that the 12S E1A protein does not explicitly inhibit CTLA-4 transcription; it also suppresses IL-2. Our objective in these studies is to isolate pathways specific to CTLA-4 in order to modulate the immune response. The suppression of IL-2 in samples electroporated with the 12S E1A plasmid suggests the cofactor may be involved in expression of both activating and regulatory T cell genes. The 12S construct does not

affect global gene expression as evidenced by transcription of GAPDH which was not suppressed with any of the E1A proteins.

E1A is known to bind histone acetyltransferases, but it also interacts with a variety of additional proteins. We next targeted histone acetyltransferases with the specific inhibitors curcumin and garcinol to determine whether the effects of E1A transfection were due to p300/CBP or PCAF activity. We are able to block transcription of CTLA-4 and demonstrate a role for p300 in CTLA-4 activation with curcumin and garcinol. Curcumin was a far more potent CTLA-4 inhibitor in these experiments. Both of these compounds inhibit HAT activity, but their dissimilar chemical structures lead to distinct binding affinities for HAT family members. Curcumin was shown to selectively block p300/CBP without impacting PCAF (8). Conversely, garcinol shows activity against p300 but has a higher potency toward PCAF (7). As curcumin is more effective at suppressing CTLA-4 transcription, our data suggests p300 may be responsible for acetylation at the CTLA-4 promoter.

We also find inhibition of IL-2 transcription with curcumin and garcinol at equivalent concentrations as required for CTLA-4 suppression. This again provides evidence that the same cofactor may be necessary for transcription of both genes. An earlier study using E1A constructs found p300 was important for IL-5 expression (65). Both garcinol and curcumin have been shown to reduce inflammation (52, 84). Taken together with our results, p300 may be a common cofactor for a variety of T cell cytokines. The work in this chapter provides evidence that histone acetylation is necessary for CTLA-4 expression and that targeting the cofactor p300 is an effective method of inhibiting CTLA-4 transcription.

CHAPTER 3

Topoisomerase I Modulates a Subset of Induced T Cell Genes, Including CTLA-4

ABSTRACT

Upon activation, T cells immediately initiate transcription of immune modulating genes to appropriately direct the immune response. The general role of DNA conformation in transcription, including the involvement of topoisomerase enzymes, has been previously explored, but whether this mechanism is specifically involved in induced genes remains unclear. In effector T cells, CTLA-4 transcription is rapidly activated after stimulation. Here we show topoisomerase I but not II is necessary for transcription of CTLA-4 and a subset of induced genes.

By use of the topoisomerase I-specific inhibitors camptothecin and SN-38, we find a dose-dependent reduction in CTLA-4 mRNA when enzymatic activity is blocked. CTLA-4 intracellular and extracellular protein levels are also diminished with topoisomerase I inhibition. We measured expression of a panel of induced genes and found all of them except TNF-α were also inhibited at the transcript and protein levels. The topoisomerase II inhibitor etoposide had no effect on induced gene expression, demonstrating these results are specific to topoisomerase I. When constitutive, internal control genes actin and GAPDH were analyzed, these inhibitors did not modulate expression. Finally, we compared camptothecin to cyclosporin A, an inhibitor of NFAT1 nuclear translocation used clinically for immune suppression. The collection of genes

affected by CyA differs from camptothecin, indicating there may be circumstances where use of topoisomerase I inhibition is a more ideal method of immune suppression.

INTRODUCTION

The previous chapter explored DNA conformation at the level of chromatin organization through histone modifications. We next wanted to investigate whether supercoiling could play a role in CTLA-4 transcriptional regulation. The DNA double helix created by base pairing is further compacted by supercoiling, a process that is regulated by enzymes known as gyrases or topoisomerases. During replication, the two strands of DNA are unzipped by a helicase at the replication fork, resulting in highly coiled DNA upstream of the polymerase machinery. Topoisomerases work to either relax positive supercoils or introduce negative supercoils to relieve the tension caused by helicase activity.

DNA gyrase, a prokaryotic topoisomerase, was first discovered in *E. coli* in 1971 (121). Since then, topoisomerases have been identified in eukaryotes, as well. Aside from replication, these proteins have been implicated in both recombination and transcription (122). The majority of evidence into how topoisomerases regulate transcription has been limited to bacteria and yeast studies, as well as *in vitro* systems with purified proteins and DNA. Whether higher eukaryotes require topoisomerase activity for transcription is still poorly understood. Analysis of eukaryotic cells has demonstrated topo I may be involved in transcriptional elongation or mRNA splicing (20).

There are two main classes of topoisomerases which are differentiated by their mechanisms (24). The ATP-independent type I topoisomerases (topo I) nick one strand of the DNA and relax supercoils before religation. This changes the linking number, or

number of times the two strands wind around each other, by single digits. Type II topoisomerases (topo II) require ATP and function by cutting both DNA strands to change the linking number in increments of two.

Inhibitors to topoisomerases have proven effective in treatment of cancer due to the high replication rates of tumor cells (120). Small molecule inhibitors to topo I and II can either cause excess cleavage of DNA or prevent religation. Topo II can also be inhibited by blocking ATPase activity. In this chapter we will utilize etoposide, a topo II inhibitor, and derivatives of camptothecin, a topo I inhibitor. Camptothecin is a natural compound found in the bark of a Chinese tree known as the “happy tree”. Due to its low solubility, the analog irinotecan was developed. Irinotecan naturally has very little effect on topo I until it is activated by hydrolysis into the metabolite SN-38, a potent topo I inhibitor. Blockade of topo I or topo II with these compounds has been effective in apoptosis induction in proliferating cancer cells. The objective of this chapter is to explore the role of topoisomerase enzymes in the rapid transcriptional induction of CTLA-4 and other T cell genes using the described chemical inhibitors.

RESULTS

Inhibition of topo I, not II represses CTLA-4 mRNA expression. To determine whether topoisomerases are involved in CTLA-4 transcription, we isolated primary CD4 T cells and treated them with increasing concentrations of camptothecin and etoposide to inhibit topo I and II, respectively (Figure 3.1). Cells were pre-treated for 1 h prior to stimulation to ensure the cells had adequately taken up the compounds. We then stimulated the cells for 3 h to induce CTLA-4 to its maximum level, which was 55.5-fold (+/- 9.8) over unstimulated cells as the average of four donors we tested. Compared to DMSO alone, camptothecin significantly reduces CTLA-4 expression dose-dependently. With 100nM expression is suppressed to 40-fold (+/- 10.2), and 10 μM treatment reduces transcript to 10.2-fold (+/- 2.3) on average. With the same concentrations of the topo II inhibitor etoposide, we do not detect a significant effect on CTLA-4 mRNA levels.

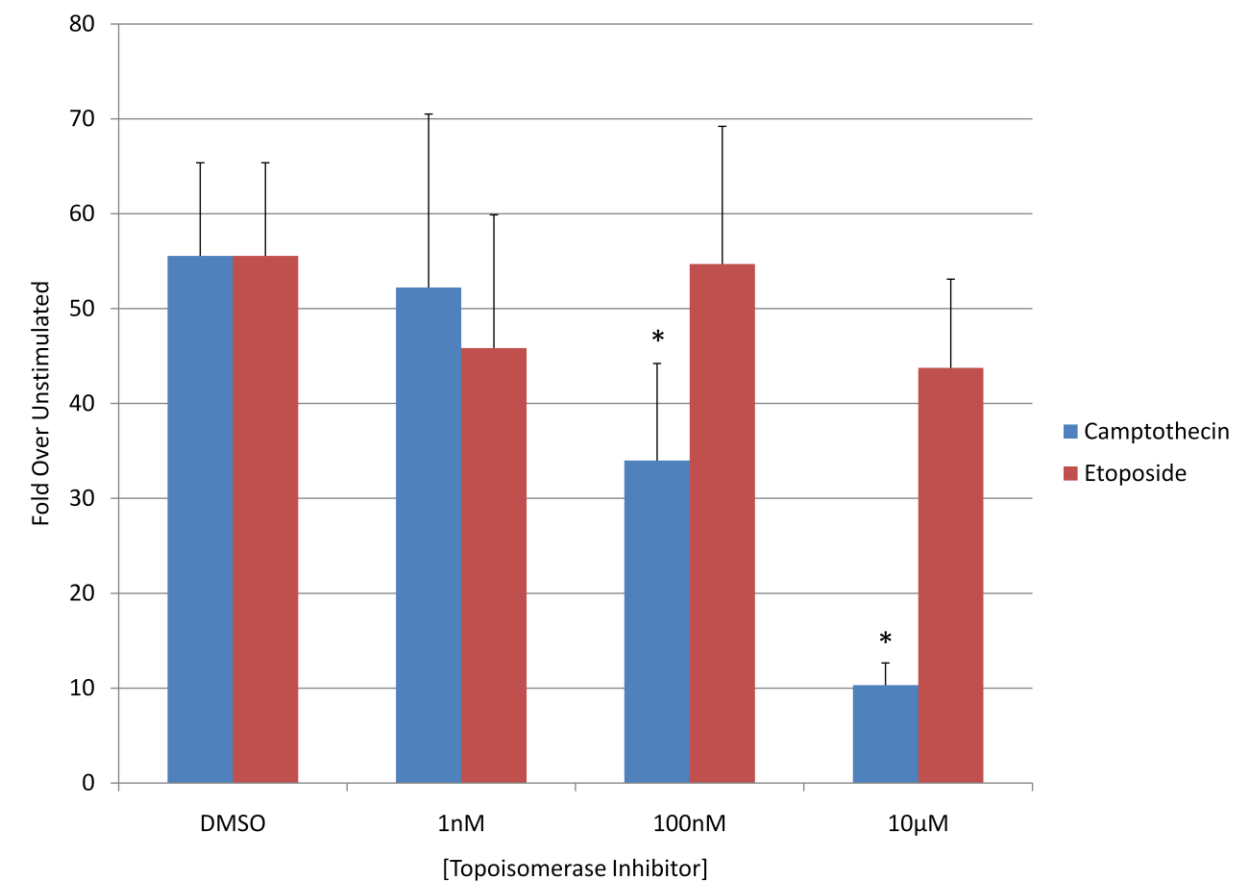

Figure 3.1. Topo I inhibition with camptothecin suppresses CTLA-4 transcript. Primary CD4 T cells were isolated and $5x10^6$ cells were treated with each of the indicated concentrations of camptothecin or etoposide for 1 h prior to stimulation with PMA/A23187 for 3 h. Total RNA was isolated with Trizol, cDNA was synthesized and qPCR was performed as described in Materials and Methods with primers specific to CTLA-4. Results were normalized to B2M and are presented as the average fold over unstimulated cells from four independent experiments ± SEM (*$p<0.05$).

Camptothecin does not induce apoptosis in primary CD4 T cells. Camptothecin and its derivatives have been used to target profusely replicating tumor cells for apoptosis. Based on the raw data of the internal control B2M from our dose curve in Figure 3.1, we were confident the resulting effects were not due to apoptosis in our primary cell system. To verify this experimentally, we treated cells with 0, 0.1, 10 and 100 μM camptothecin as in Figure 3.1, stimulated the cells for 3 and 6 h with PMA/A23187 and measured apoptosis by annexin V/PI staining (Figure 3.2a). We find overall apoptosis levels increase between stimulation for 3 and 6 h, but the addition of camptothecin does not boost cell death over DMSO. Treatment with 100 μM, a 10-fold increase over the highest concentration in our mRNA dose curve, also does not impact programmed cell death in primary CD4 T cells.

To determine the effect of camptothecin on proliferating cells, and as a positive control for our results in Figure 3.2a, we treated Jurkat T cells with 10 μM camptothecin or DMSO for 6 h (Figure 3.2b). With DMSO alone, less than 10% of cells are apoptotic. After 6 h with 10 μM camptothecin, greater than 80% of cells are undergoing programmed death.

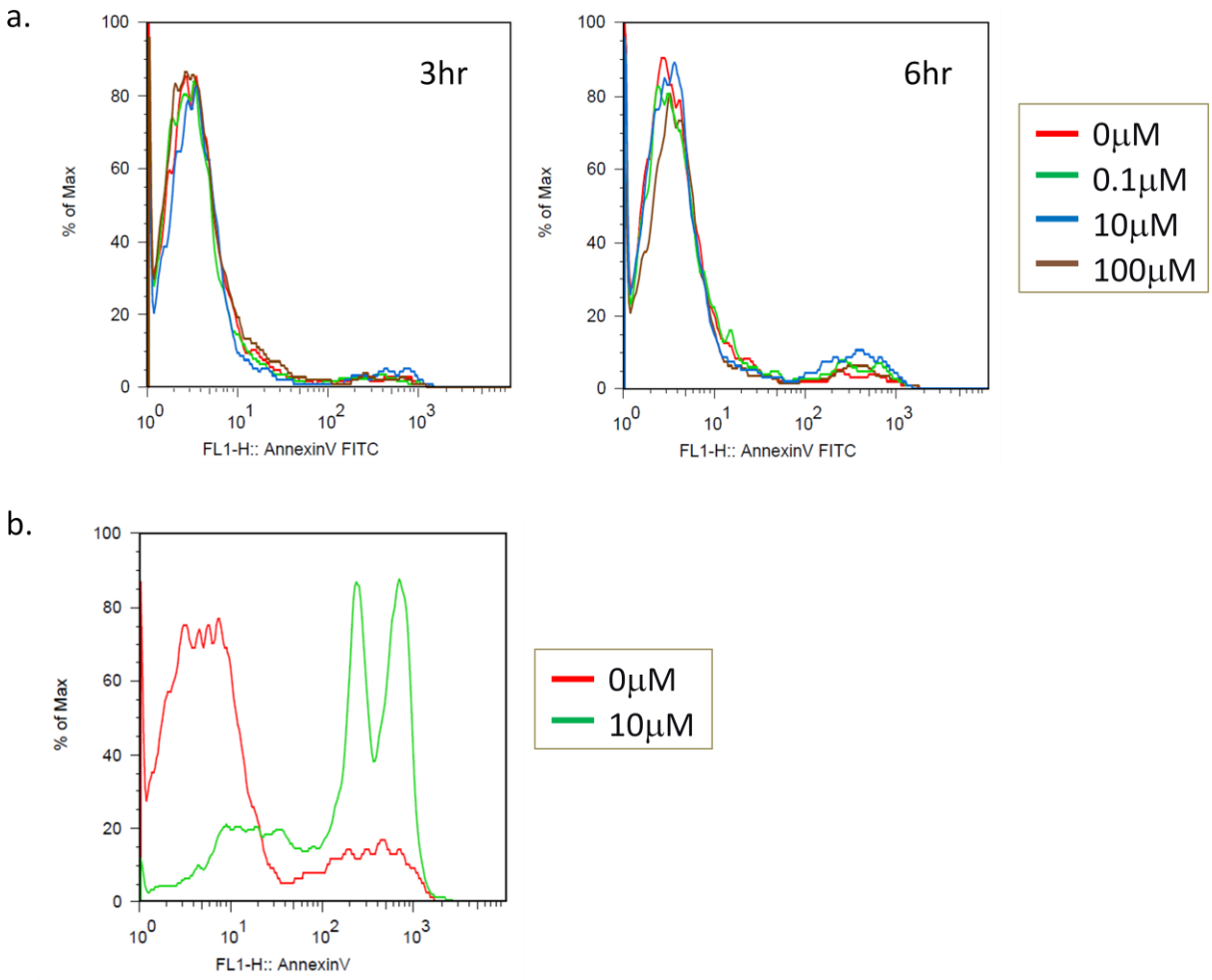

Figure 3.2. Apoptosis does not increase in primary CD4 T cells transiently treated with camptothecin. a) Primary CD4 T cells were isolated and treated with the indicated concentrations of camptothecin for 1 h, followed by stimulation for 3 or 6 h as denoted on the histograms. 10^5 treated cells were stained with 1 μL each of annexin V or PI and positive staining was measured by flow cytometry. Results are presented as histograms generated by FlowJo analysis and are representative of three independent experiments. b) Jurkat T cells were plated in culture at 10^6/mL and treated with 10 μM camptothecin or an equivalent volume DMSO for 6 h, followed by staining and analysis as in (a). Presented results are representative of three independent experiments.

Topo I inhibition suppresses CTLA-4 but not constitutive internal control genes. Irinotecan is a more soluble analog of camptothecin, but its activity as a topo I inhibitor requires its hydrolysis into the metabolite SN-38. In addition to camptothecin, we treated fresh primary CD4 T cells with irinotecan and SN-38 to determine the effect on transcription (Figure 3.3). The calcineurin inhibitor cyclosporin A (CyA) was included as a control for CTLA-4 inhibition and etoposide demonstrates the specificity to topo I. CTLA-4 was suppressed with both camptothecin and SN-38, but not the inactive topo I inhibitor irinotecan (Figure 3.3a). The topo I inhibitors repressed CTLA-4 expression to the same degree as CyA ($p<0.05$). Etoposide again had no impact on CTLA-4 expression.

Previous studies have suggested topoisomerase is necessary for the elongation phase of transcription (20), which would imply all genes would be affected by our inhibitors. We next measured mRNA expression levels of NFAT1 (Figure 3.3b), Actin (Figure 3.3c) and GAPDH (Figure 3.3d). We find the topoisomerase inhibitors have no effect on any of these three non-induced genes. The fact that NFAT1 mRNA is unaffected by topo I inhibition supports that this enzyme directly modulates CTLA-4 activation.

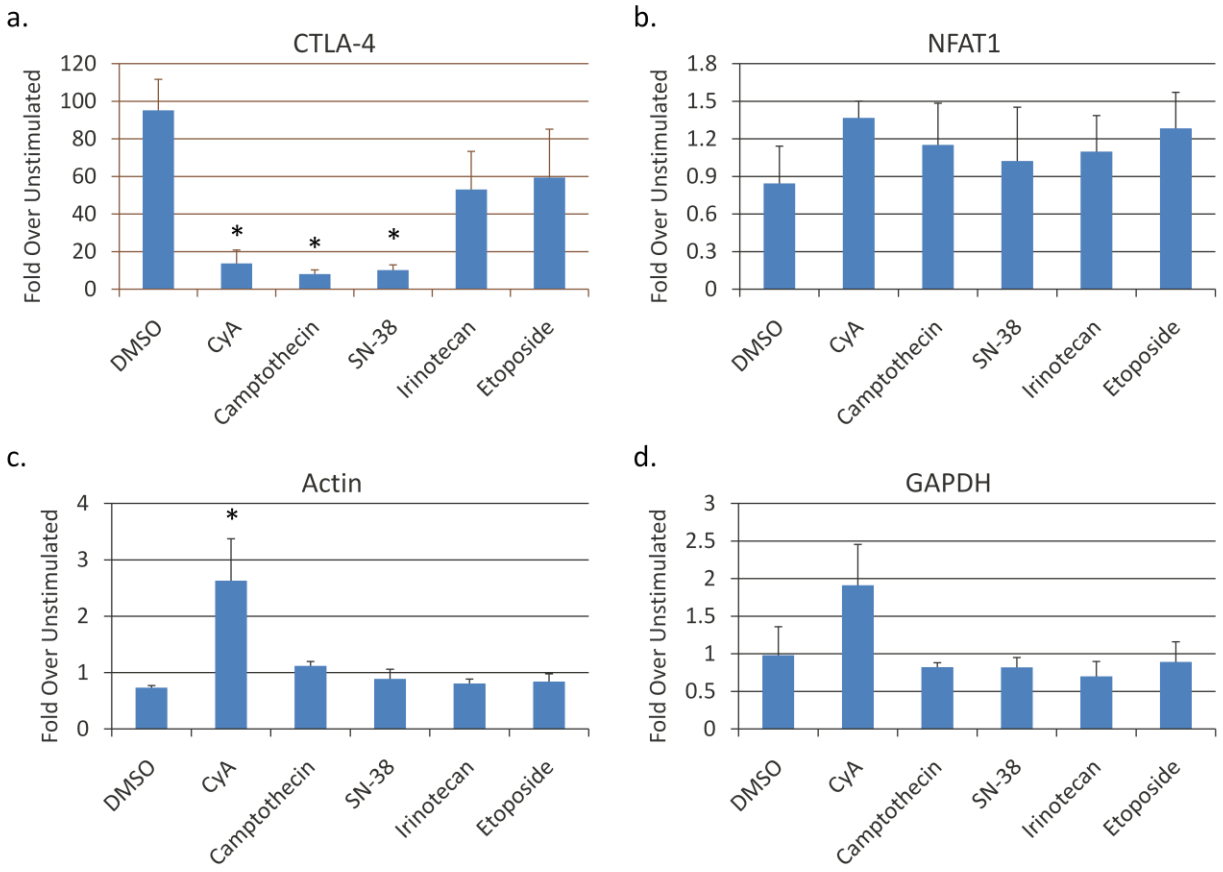

Figure 3.3. SN-38 and camptothecin inhibit CTLA-4 induction but not constitutive genes. Primary CD4 T cells were isolated and 5x106 cells were treated with 10 μg/mL CyA or 10 μM camptothecin, SN-38, irinotecan, etoposide or DMSO as indicated for 1 h prior to stimulation with PMA/A23187 for 3 h. Total RNA was isolated with Trizol and cDNA was synthesized as detailed in Materials and Methods. Samples were analyzed by qPCR using primer sets specific to a) CTLA-4, b) NFAT1, c) Actin or d) GAPDH with normalization to B2M. Results are the averages of three independent experiments ± SEM and represent the fold increase over an unstimulated sample (*$p<0.05$).

CTLA-4 protein expression is reduced with topo I inhibitors. We find that CTLA-4 transcript induction decreases dramatically but incompletely with topo I inhibitors, so we next measured protein expression in primary CD4 T cells to determine the degree of inhibition at this level (Figure 3.4). By immunoblot with whole cell extracts we find strong induction of CTLA-4 with stimulation alone. Addition of CyA blocks expression almost entirely, while camptothecin inhibits CTLA-4 to a slightly lesser degree. Treatment with etoposide has no effect on CTLA-4 and irinotecan also does not impede expression.

As a more quantitative method, we next assayed cells for surface (Figure 3.5a) and intracellular (Figure 3.5b) CTLA-4 expression by flow cytometry. Consistent with the immunoblot results, CTLA-4 protein is minimal in unstimulated cells at both the intracellular and extracellular level. Stimulation induces expression, which can be blocked with CyA or the topo I inhibitors camptothecin and SN-38. The topo I inhibitors were slightly less efficient at suppressing CTLA-4 than CyA. Etoposide and irinotecan do not impede either intracellular or surface CTLA-4.

To establish whether topo I inhibition affects NFAT1 activation or overall protein levels we also probed our immunoblot membrane with an antibody specific to NFAT1 (Figure 3.4). Two species of NFAT1 exist, a higher molecular weight band for the phosphorylated, inactive form and a lower band for the dephosphorylated protein. As expected, the unstimulated sample has a higher band than the stimulated sample. CyA treatment not only blocks dephosphorylation, but also diminishes total NFAT1 protein. The topoisomerase inhibitors do not eliminate NFAT1, nor do they impede dephosphorylation.

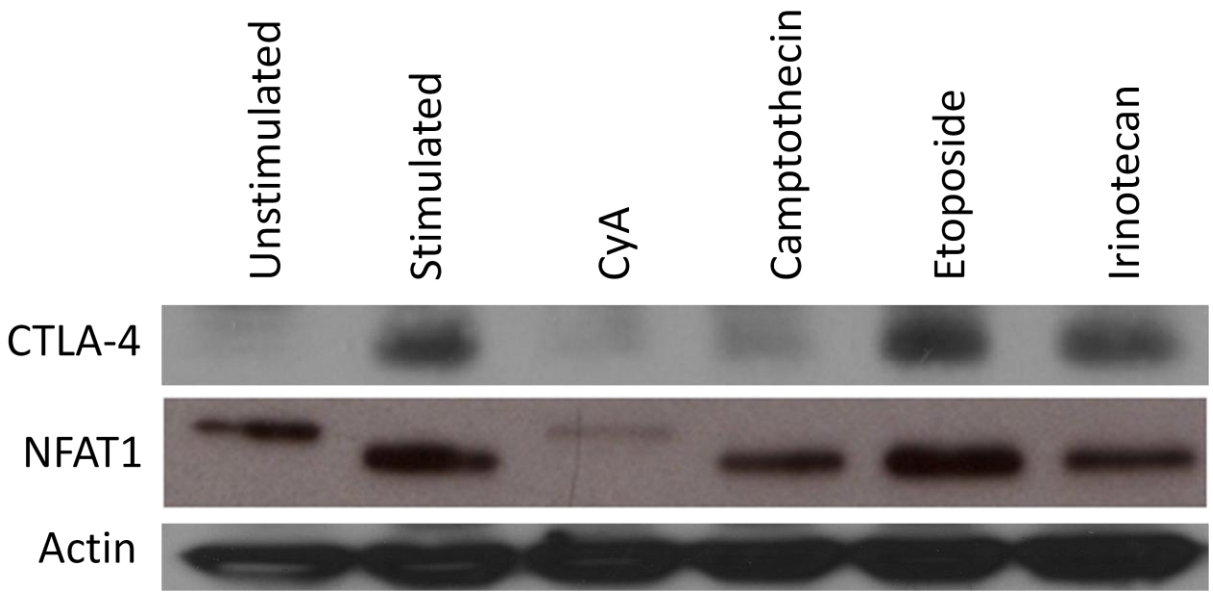

Figure 3.4. Protein analysis of CD4 T cells treated with topoisomerase inhibitors by immunoblot. Primary CD4 T cells were isolated and treated with 10 μg/mL CyA or 10 μM camptothecin, etoposide or irinotecan as indicated for 1 h prior to stimulation for 3 h with PMA/A23187. Total cell lysate was isolated by lysis with RIPA buffer as described in Materials and Methods. Proteins were quantified and 30μg of each sample was separated on a 10% acrylamide gel, transferred to PVDF and probed with antibodies specific to CTLA-4 or NFAT1 as detailed in Materials and Methods. Actin serves as a loading control. These results are representative of three independent experiments.

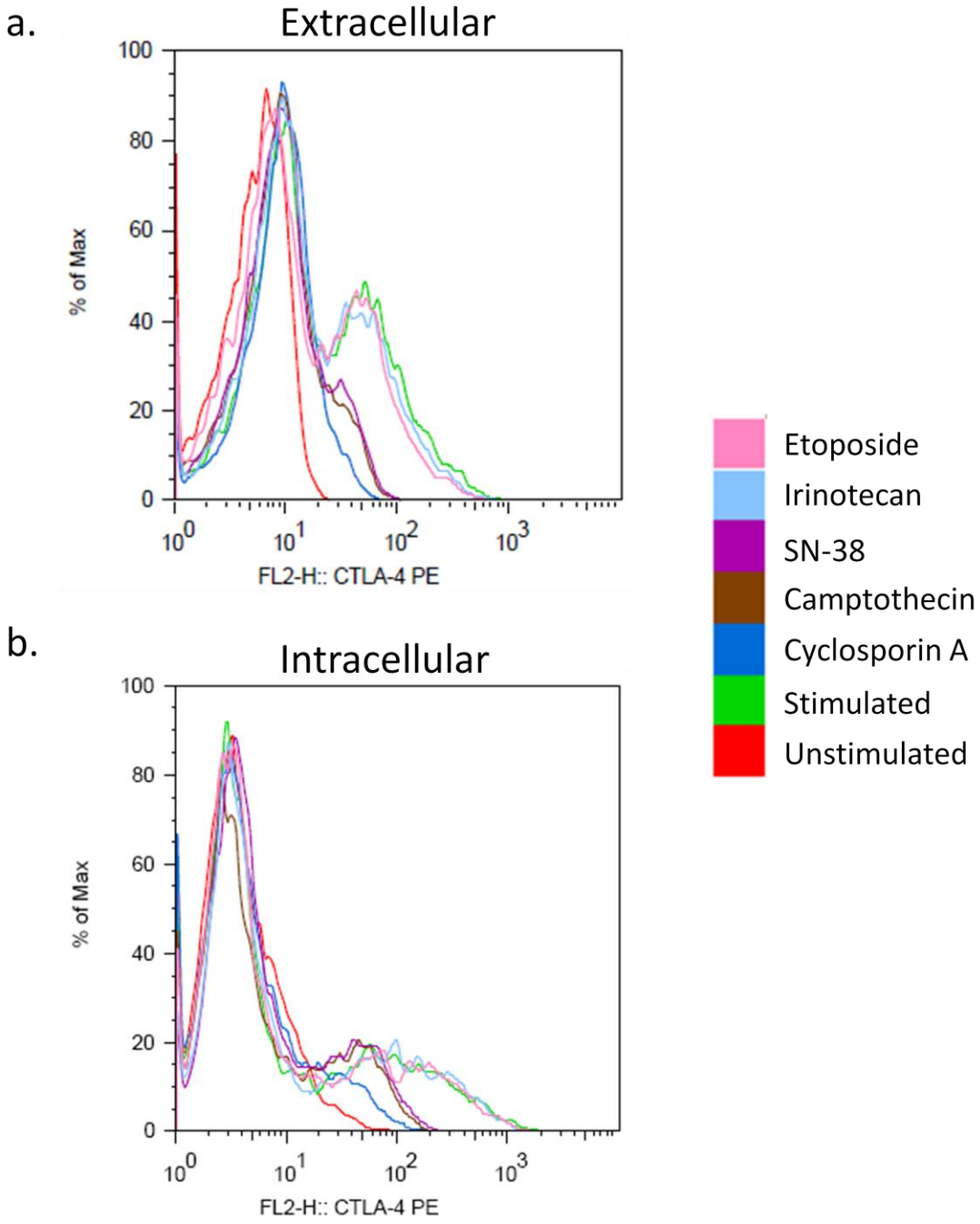

Figure 3.5. Flow cytometric analysis of CTLA-4 expression in CD4 T cells treated with topoisomerase inhibitors. Primary CD4 T cells were isolated and treated with 10 μg/mL CyA or 10 μM camptothecin, etoposide or irinotecan as indicated for 1 h prior to stimulation for 3 h with PMA/A23187. a) 10^6 cells were stained with 1 μL PE-conjugated CTLA-4 antibody and analyzed by flow cytometry as described in Materials and Methods. b) 10^6 cells were fixed/permeablized and stained with 1 μL PE-conjugated CTLA-4 antibody followed by analysis by flow cytometry for intracellular CTLA-4 as described in Materials and Methods. Analysis of the results from (a) and (b) was conducted with FlowJo and the presented histograms are representative of 3 independent experiments.

Topo I inhibitors repress many but not all induced T cell genes. To determine whether topo I inhibition exclusively represses CTLA-4 transcription we measured mRNA expression of additional induced T cell genes using the samples from Figure 3.6. We find IL-2 (Figure 3.6a), IFN-γ (Figure 3.6b) and IL-4 (Figure 3.6c) follow the same trend as CTLA-4, where both camptothecin and SN-38 potently inhibit transcription to nearly the same degree as CyA. Interestingly, TNF-α is unaffected by topo I inhibition, while CyA blocks expression (Figure 3.6d). Conversely, the p50 subunit of NFκB is not affected by CyA but induction is repressed with topo I inhibitors. As with each of the previously measured transcripts, expression levels of all of these genes are unchanged with etoposide or irinotecan.

Inhibition of topo I modulates cytokine secretion in primary CD4 T cells. Given the transcript levels of many of the cytokines were reduced, we wanted to evaluate the effect of topo I inhibition on secreted protein levels. We utilized a flow cytometry based assay to measure cytokine levels in culture media of treated and stimulated cells. Similar to an ELISA, this system uses capture beads to bind and detect each antigen which are then analyzed by flow. Protein concentrations are then calculated based on a standard curve of antigen at known concentrations. Using this assay, we find the secreted proteins follow the expression trend we detected for transcript levels (Figure 3.7). As expected, irinotecan and etoposide did not impact protein secretion.

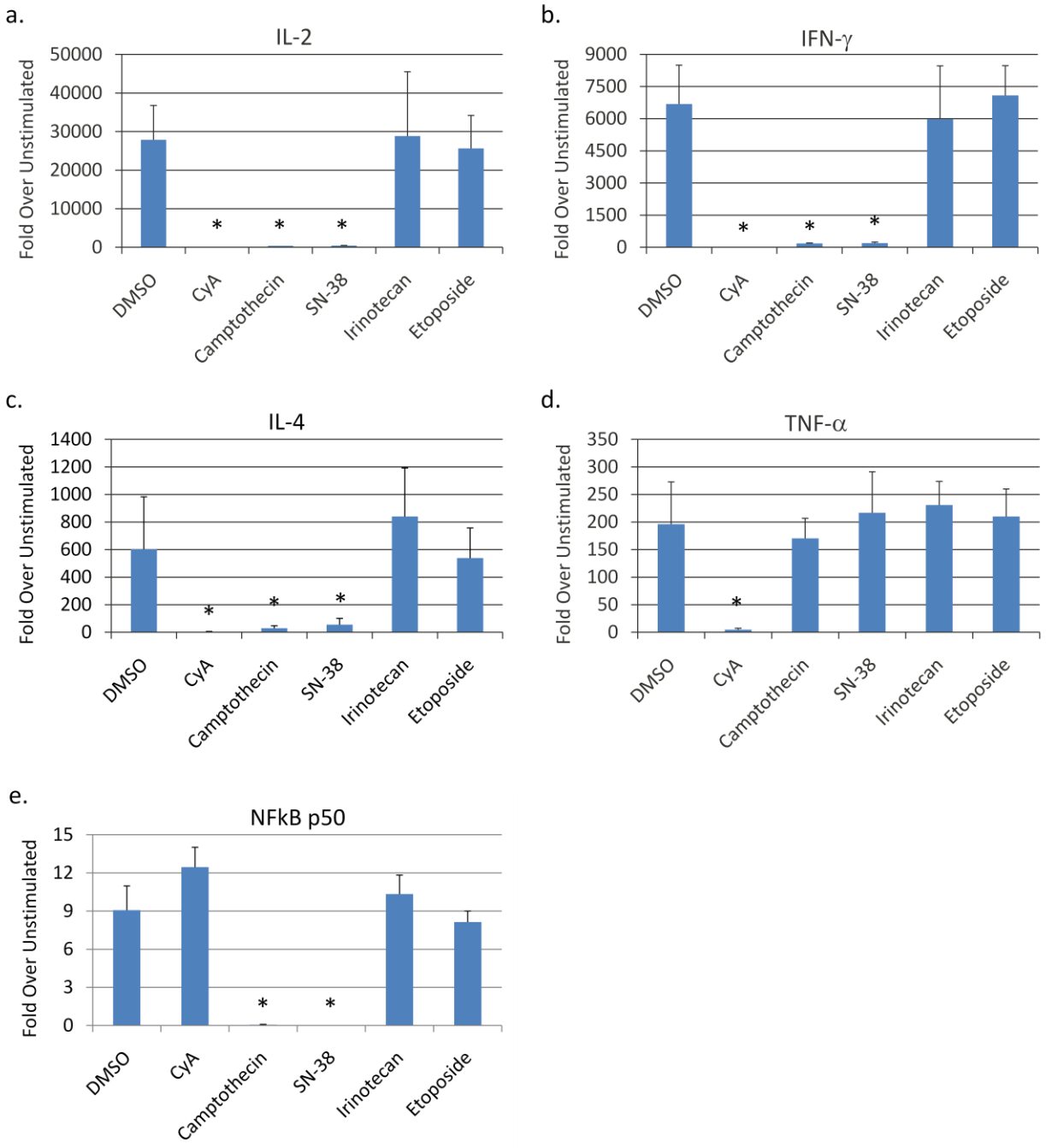

Figure 3.6. SN-38 and camptothecin inhibit many but not all induced genes. Primary CD4 T cells were isolated and $5x10^6$ cells were treated with 10 μg/mL CyA or 10 μM camptothecin, SN-38, irinotecan, etoposide or DMSO as indicated for 1 h prior to stimulation with PMA/A23187 for 3 h. Total RNA was isolated with Trizol and cDNA was synthesized as detailed in Materials and Methods. Samples were analyzed by qPCR using primer sets specific to a) IL-2, b) IFN-γ, c) IL-4, d) TNF-α or e) NFκB p50with normalization to B2M. Results are the averages of three independent experiments ± SEM and represent the fold increase over an unstimulated sample (*p<0.05).

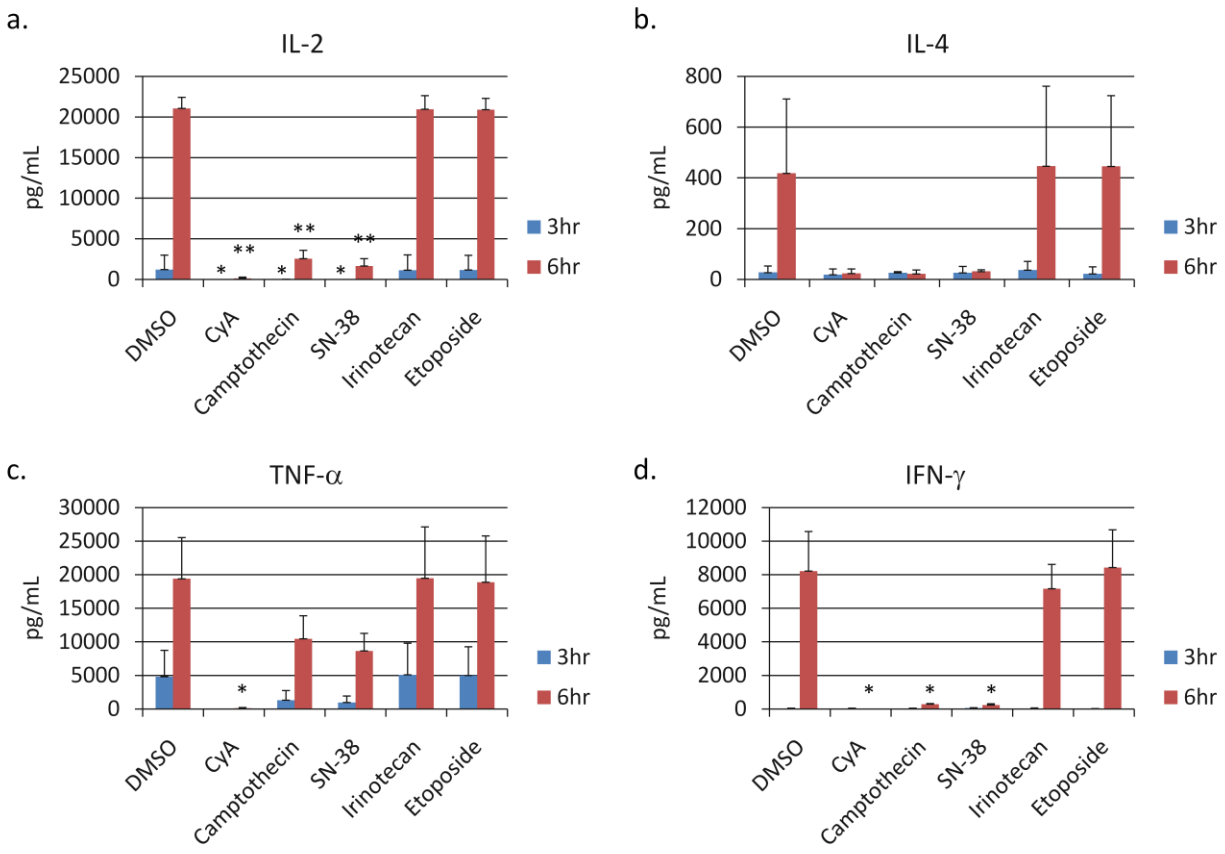

Figure 3.7. Cytokine secretion is suppressed with topo I inhibition. Primary CD4 T cells were isolated and plated at $2x10^6$ cells/mL. Samples were treated with 10 μg/mL CyA or 10 μM camptothecin, SN-38, irinotecan, etoposide or DMSO as indicated for 1 h prior to stimulation with PMA/A23187 for 3 or 6 h. Culture supernatant was collected and analyzed by cytometric bead array as described in Materials and Methods. Samples were quantified based on a standard curve of known antigen concentrations. Presented results are the averages of four independent experiments ± SEM (*p<0.05, **p<0.005).

DISCUSSION

This chapter has identified a novel role for topoisomerases in the rapid induction of certain T cell genes including CTLA-4. It was previously thought that topoisomerase enzymes may take part in transcription, but compelling evidence has remained elusive. We show here that involvement of topoisomerase hinges on the nature of the gene in question. Our results suggest constitutive genes do not require topological modifications to the DNA, while a certain class of induced genes utilizes DNA conformation as a means of regulation. Through use of specific inhibitors, we find topo I activity, but not topo II, is necessary for expression of these genes. Manipulation of this pathway can be a valuable tool for immune regulation, particularly given the group of genes suppressed with topo I inhibitors differs from those blocked by CyA.

Primary T cells present a convenient opportunity in studying topoisomerase function. Cells that are freshly isolated are quiescent, requiring stimulation to promote cell proliferation. Most previous studies tried to demonstrate a role for topoisomerase in transcription using cell lines. This is problematic because cell lines freely replicate, making them targets for apoptosis when topoisomerases are inhibited. High concentrations were required to see any effect on transcription (40), which was not the case in our studies. The alternative was to use an artificial *in vitro* system with purified proteins and DNA, which has shown topo I cooperates at the site of transcription initiation, but it does not represent an accurate depiction of what normally occurs within a cell and *in vivo* studies have had a difficult time reproducing the results (103). Aside from the issue of replication and apoptosis clouding results, part of the obscurity may be

due to the fact that constitutive genes have been the focus of these studies, where we find only induced genes require topo I. Taken together, we have shown primary T cells are an ideal system for studying topoisomerase in transcription due to the lack of replication and the abundance of inducible genes.

Aside from initiation, topoisomerase has also been implicated in both the elongation phase of transcription, particularly for longer transcripts, as well as the processing of mRNA by splicing (20). Our results suggest elongation in general does not involve topo I since the constitutive genes we examined were not affected by inhibitors. We examined NFAT1 expression, which has a long primary transcript of roughly 150 kbp with multiple introns. Not only is the transcript unaffected but protein levels also do not change with camptothecin. From these data we can conclude that elongation, even for longer transcripts, does not always depend on topoisomerase activity. Topoisomerase involvement in splicing is also unlikely given our results with NFAT1, which has multiple splice sites. We do not know the exact mechanism of action for induced genes. It is plausible that the elongation phase of transcription requires topoisomerase for these genes but further experiments will be necessary to establish this conclusively. Another possibility is that the conformation of DNA may be too constrained to allow necessary transcription factors or the RNA polymerase complex to bind for transcription initiation.

In addition to small molecule inhibitors, we sought to deplete topo I with siRNA. As it can be technically challenging to transfect primary T cells with high enough efficiency, our efforts did not result in adequate reduction of topo I protein and we did not detect any change in induced gene expression. Instead we used two related but

chemically distinct topo I inhibitors, camptothecin and SN-38, to verify the specificity of our results.

Mondal *et al* demonstrated topo II is also involved in transcription (75). As both topo I and II have functions in replication in prokaryotes, yeast and higher eukaryotes, it would not be surprising to find overlapping roles in transcription also. We used etoposide, a potent topo II inhibitor that was shown by Mondal *et al* to inhibit chromatin-associated DNA transcription *in vitro* and detected no reduction in transcription for any of the genes we measured.

The topo I inhibitor irinotecan is an improvement over camptothecin due to its higher solubility in aqueous solutions. This compound itself has 100-fold less inhibitory ability than its hydrolyzed metabolite SN-38. Bioactivation of irinotecan into SN-38 requires the activity of carboxylesterases (104). When we treat our primary CD4 T cells with irinotecan we do not observe any effect on transcription, though an equivalent molar concentration of SN-38 inhibits a select group of induced genes. This implies primary T cells do not have the necessary enzymes to convert irinotecan into SN-38, an observation which needs to be recognized for *in vitro* studies.

As expected, when transcript levels of the cytokines IL-2, IFN-γ and IL-4 are blocked by topo I inhibition, we detect a decrease in protein secretion in the culture media. The inhibitor does not have an effect on the secretory pathway, though, based on our results with TNF-α. This gene is neither altered at the transcript level or in its protein secretion by topo I inhibition. This data indicates topoisomerase inhibition only represses a particular subset of induced genes and also does not impact cellular protein processing.

When we compare T cell gene inhibition with CyA to topo I inhibitors we find these compounds suppress different sets of genes. CyA is used for transplant patients to suppress the immune response and prevent graft rejection. It has also shown efficacy in the treatment of autoimmunity. The robust suppression of immune activation with CyA leaves patients susceptible to infection. Though topo I inhibitors have been used in cancer therapy, their capacity in immune regulation has not been investigated. We show many adaptive immunity cytokines are inhibited while the innate immunity cytokine TNF-α remains inducible. More work will be required, but if topo I inhibitors can be used to dampen adaptive immunity without hindering the innate response they could become a valuable tool for autoimmunity and transplant with a reduced risk of infection.

GENERAL CONCLUSIONS

A delicate balance exists in achieving sufficient activation of the immune system without allowing the response to continue unrestrained. Many current cancer therapies strive to enhance immunity through use of vaccines and pro-inflammatory cytokines in an effort to clear malignancy. Recently immune regulatory pathways have become therapeutic targets with the goal of obstructing immune suppression, thus enhancing activation. For example, monoclonal antibodies to NK cell inhibitory KIR molecules as well as T cell regulators PD-1 and CTLA-4 have been investigated experimentally and in clinical trials with considerable promise. In the case of CTLA-4, antibody blockade has been a successful cancer treatment, but rampant autoimmunity is all too commonly a life threatening side effect. CTLA-4 is constitutively expressed on the surface of Tregs and is also induced in effector T cells upon activation. The work of this dissertation has focused on the transcriptional regulation of induced CTLA-4 with the intent to identify pathways to specifically modulate CTLA-4 expression in effector T cells. Ideally down regulation of this gene in these cells would lead to an enhanced immune reaction to tumor with reduced risk of autoimmune side effects.

Conversely, for patients with autoimmunity it would be valuable to augment CTLA-4 expression to quell the uncontrolled immune response. Similar challenges exist for transplant patients where the goal is to reduce the likelihood of graft rejection. Current therapies block inflammatory cytokines with monoclonal antibodies and inhibitors to transcription factors such as CyA, which blocks NFAT nuclear translocation. A CTLA-4 immunoglobulin fusion protein has also shown promise in the treatment of

rheumatoid arthritis, demonstrating the potency of CTLA-4 in an autoimmune setting. By increasing CTLA-4 cell surface expression through modulation of transcription, we may be able to attain more effective suppression of the overactive immune system.

We have shown CTLA-4 is elevated in malignant cells of patients with CTCL both here and previously (126), which presents an interesting opportunity to investigate how these cells differentially regulate expression of this gene. Not only could the pathways used by CTCL cells be targeted to decrease CTLA-4 expression and hopefully revive the anti-tumor immune response, but we can also learn from how these cells augment CTLA-4 transcription and perhaps exploit this mechanism for treatment of both autoimmunity and organ and tissue transplant. Using CTCL as a guide, we have identified a novel method for CTLA-4 regulation where proteasome inhibition leads to elevated expression.

Our initial experiments found an increase in polyubiquitinated proteins in cell lysates from CTCL patients. The proficiency of the 26S proteasome pathway had not previously been investigated in CTCL. Our ubiquitin results suggest either improper function of a component within the proteasome pathway or excessive accumulation of extraneous or misfolded proteins. Due to its involvement in immune monitoring, the proteasome is targeted by viruses including Epstein-Barr to avoid recognition by cytotoxic T cells (32). CTCL may be functioning in a similar manner, but further experiments will be required to determine this. We isolated the single entity of proteasome dysfunction by using specific inhibitors in normal CD4 T cells and found that this caused an increase in CTLA-4 transcription. We identified GATA3 as a necessary factor in augmentation of CTLA-4 in the presence of proteasome inhibitors,

and this transcription factor is also elevated in CTCL. Further experiments will be required to determine whether GATA3 may be responsible for the CTLA-4 increase we observe in CTCL. If this is the case, GATA3 could prove to be a valuable therapeutic target.

Within this work and in combination with our previous study we have established NFAT1 and GATA3 are important transcription factors that promote CTLA-4 induction (41). Our data also suggests NFκB does not contribute to or suppress CTLA-4 expression. GATA3 may be involved only under specific circumstances while NFAT1 is a requirement for CTLA-4 transcription. We were not able to identify a role for GATA3 in CTLA-4 expression in the absence of proteasome inhibitors, though published work by van Hamburg, *et al* shows higher CTLA-4 in a GATA3 over-expressing mouse model (115). Additional expression vector studies with longer term GATA3 overexpression may be a better system for human primary CD4 T cells, but from our transient data it does not appear CTLA-4 is enhanced simply with elevated GATA3 protein. Specific signaling may also be required.

Our kinase inhibitor results suggest the p38 pathway is responsible for GATA3 phosphorylation and this pathway is also activated by proteasome inhibition. This may in part explain why addition of GATA3 alone does not increase CTLA-4. It may also help explain our sorted cell data where cells expressing GATA3 transcript were not destined to express CTLA-4. We did not evaluate kinase activity in these samples, which could be an important future experiment. Altogether, these data provide evidence that the p38 pathway may be a valuable therapeutic target. We show IFN-γ expression is unaffected, while CTLA-4 and Th2 cytokine IL-4 are both suppressed with p38

inhibition. Both IL-4 and CTLA-4 behave similarly in these experiments, again suggesting CTLA-4 is a Th2-associated gene. Treatment with SB203580 or an alternative compound could support a pro-inflammatory immune response, which would be more valuable with cancer therapies.

Aside from the impact on CTLA-4 expression, it is important to note proteasome inhibition, through its increase in the Th2 transcription factor GATA3 and blockade of pro-inflammatory NFκB, can promote a Th2 phenotype. We measure a decrease in transcription of IFN-γ while IL-4 levels rise. Bortezomib has more recently been used for GVHD treatment and prevention (107), but the mechanism behind this effect remains unclear. Our results shed light on the shift from Th1 to Th2 that bortezomib can induce, which in addition to increased CTLA-4 could account for the protective phenotype created by bortezomib to treat GVHD. Other inflammatory diseases could benefit from bortezomib, as well, but further studies will be necessary. Currently clinical trials are focused on using this drug to treat a variety of malignancies, but its influence on the immune response should also be exploited.

As mentioned previously, this work has focused on induced CTLA-4 and not the constitutive expression found in Tregs. FoxP3 is the most consistent marker for Tregs currently known. In the case of proteasome inhibition, we find FoxP3 protein levels are depleted in the CD4 population, indicating the CTLA-4 we measure is strictly expressed on effector T cells. This is important for our mechanistic studies, but it also has implications for understanding regulation of Tregs. It is unclear how proteasome inhibition leads to a reduction in FoxP3 or if this could be an efficient mechanism of Treg

depletion. Further experiments including *in vivo* analysis would be valuable to determine the effects of proteasome inhibition on other immune cell types.

Aside from transcription factors, epigenetic modification in the form of histone acetylation is necessary to induce CTLA-4 expression, as well. This may serve to open the chromatin conformation to allow transcriptional machinery to access the promoter. We find NFAT1 binding precedes and is required for histone acetylation, suggesting the HAT cofactor may interact directly with NFAT1. From our data we can infer p300 is likely the HAT responsible for acetylation at the CTLA-4 promoter. Interestingly, our E1A and inhibitor experiments affected CTLA-4 and IL-2 in a similar manner. A previous report found p300 functions at the IL-5 promoter, as well (65). These and other induced T cell genes, though different in function, may share key steps in their activation. If HAT activity is dependent on transcription factor binding for these genes as it is for CTLA-4, the ultimate regulator may be whether transcription factors are able to access the promoter.

In addition to packaging of genomic DNA into chromatin by its wrapping around nucleosomes, the actual topology of the DNA can also be modified to regulate transcription of certain genes. In humans, topoisomerases have only loosely been associated with transcription, though stronger evidence has been reported for prokaryotes and yeast. We have shown here that transcription of constitutive genes is not impacted by topoisomerase inhibition in primary CD4 T cells, while induced genes require topo I activity. Under normal circumstances T cells are in a quiescent state until they are stimulated. Upon T cell receptor engagement the immune response is quickly activated to clear the responsible pathogen. A cascade of kinase signaling as well as

calcium influx ultimately leads to the activation of transcription factors and production of a repertoire of cytokines and chemokines to direct the immune response appropriately for the type of pathogen involved. Though the activation of transcription factors plays a critical role in the instantaneous induction of many of these genes, we questioned whether the degree of supercoiling in the DNA could also be involved. Resting T cells provide an excellent model for these experiments because in addition to constitutively active genes, they can be stimulated to express many inducible genes. Our results using specific inhibitors *in vivo* define a novel mechanism for topo I, where only a distinct subset of CD4 T cell genes, including CTLA-4, requires topoisomerase activity. As these data support that topo I is not utilized globally for transcription, it is our hypothesis that induction of these genes requires relaxation of the DNA to rapidly initiate expression. Further experiments will be valuable in determining the exact mechanism for topoisomerase at these promoters.

Inhibitors to both topo I and II are used clinically in cancer therapies due to their increased cytotoxicity to highly proliferant tumor cells. Our results provide insight to immune capabilities in the presence of these compounds. Topo II inhibitors may be more favorable therapeutically as they target replicating cells without having an impact on the immune system's cytokine induction. The use of topo I inhibitors may impede the immune response and reduce the rate of tumor clearance by suppressing critical immune signaling. Alternatively, the use of topo I inhibitors to modulate the immune response for autoimmunity and transplant should be investigated. The panel of genes affected by topo I inhibitors varies from those blocked by CyA. As a result it should be

explored whether compounds directed at topo I provide more benefit than current therapies for immune suppression.

This dissertation has focused on mechanisms regulating CTLA-4 transcription. A 2004 study found CTLA-4 was internalized in endosomes in T cell clones and only after stimulation would the protein bud to the cell surface (53). They report that CTLA-4 is pre-formed in the T cell lines they developed, which suggests trafficking is the chief mechanism controlling cell surface expression. As previously mentioned, their model uses T cells that are expanded with IL-2 and CD3 antibody, thus activating the cells. Though intracellular trafficking is an important component of CTLA-4 regulation, our results demonstrate CTLA-4 is not present at considerable levels in freshly isolated unstimulated T cells. This work has utilized primary, resting T cells to study CTLA-4. We find by immunoblot and intracellular flow cytometry that baseline levels of CTLA-4 protein are nearly undetectable. Given we did not deplete Tregs from our CD4 T cell population it is plausible the CTLA-4 we do find in our unstimulated samples is from constitutive expression within the Treg subset but additional experiments would be necessary to support this. It is from our data that we conclude transcription induction is the fundamental switch determining CTLA-4 expression in effector CD4 T cells.

Our goal is to find pathways unique to CTLA-4 transcription that can be exploited to modulate the immune response. Through the use of bortezomib we are able to increase both CTLA-4 mRNA and surface expression to ultimately suppress T cell proliferation in a CTLA-4-dependent manner. These results provide direct evidence that simply altering CTLA-4 transcription is sufficient to modify the immune response. We have defined three distinct levels of CTLA-4 transcriptional regulation which could be

useful targets to adjust T cell activity accordingly for a variety of immune-mediated diseases. Though histone acetylation and topo I involvement did not prove specific to CTLA-4, they can be manipulated to control the immune response. Our proteasome inhibition results suggest GATA3 may be a key component that would be specific to CTLA-4 and the Th2 phenotype without affecting the Th1 response. Through use of proteasome inhibitors or compounds specifically directed at GATA3 or its activation it may be possible to guide T cell activity to be active or suppressive. The discussed transcriptional mechanisms should be investigated further for their use in autoimmune, transplant and cancer therapies.

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ABSTRACT

CTLA-4 TRANSCRPTIONAL ACTIVATION: REGULATION OF INDUCED EXPRESSION

by

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May 2011

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Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a T cell surface protein that is homologous to CD28 and binds to the B7 family of ligands. Unlike CD28, CTLA-4 interaction transmits a negative signal in T cells, leading to suppression of proliferation. CTLA-4 is constitutively expressed on regulatory T cells (Tregs) but is also inducible in effector T cells. The mechanisms driving transcriptional regulation of CTLA-4 are poorly understood. Our previous work identified a *bona fide* NFAT1 binding site in the proximal promoter for effector T cells. In addition, we found histone acetylation occurred after stimulation.

As a result of its role in suppressing T cell proliferation, CTLA-4 is important for regulation of T cell responses. CTLA-4-immunoglobulin fusion proteins have shown efficacy to quell the overactive immune system in various types of autoimmune diseases. Alternatively, blocking antibodies to CTLA-4 have been used in cancer therapies to boost the anti-tumor immune response. Malignant cells of cutaneous T cell lymphoma (CTCL) express elevated levels of CTLA-4, which may contribute to reduced

tumor immunity as the disease progresses. The objective of this work was to identify mechanisms of transcriptional regulation of CTLA-4 to better understand how this gene can be modulated to potentially cater the immune response for a variety of immune-mediated diseases.

The major findings of this work include a mechanism by which proteasome inhibition augments CTLA-4 transcription in normal primary human CD4 T cells. The Th2 associated transcription factor GATA3 is both elevated and activated by phosphorylation after treatment with the proteasome inhibitor bortezomib, which in turn leads to CTLA-4 transcriptional activation in primary CD4 T cells. This finding may in part explain the increase in CTLA-4 found in CTCL, where GATA3 is also more abundant, particularly in its phosphorylated state. The increased CTLA-4 in our primary cell model is capable of suppressing T cell proliferation, demonstrating the potency of transcriptional modulation of this gene. Additionally we explored epigenetic and topological modifications that occur for CTLA-4 activation. We found the histone acetyltransferases p300 is responsible for histone 3 acetylation at the CTLA-4 promoter, and its activity is required for CTLA-4 transcription. We also discovered a previously undiscovered role for topoisomerase I in expression of a variety of induced genes, including CTLA-4. These results define novel mechanisms governing transcriptional activation of CTLA-4 in human effector CD4 T cells.

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2010 Second Place, Poster Competition, TRC3
Ohio State University

PUBLICATIONS:

2010 Tang N, Gibson H, Germeroth T, Porcu P, Lim HW, Wong HK. 2010. T-plastin (PLS3) gene expression differentiates Sézary syndrome from mycosis fungoides and inflammatory skin diseases and can serve as a biomarker to monitor disease progression. Br J Dermatol. 162(2):463-6.

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