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Cyclin-Dependent Kinase 2 Regulates Foxp3 and Regulatory T Cell Function

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Abstract
Foxp3 is a transcription factor required for the development and function of regulatory T cells (Treg). Humans lacking functional Foxp3 are afflicted with uncontrolled systemic autoimmunity. How the Foxp3 protein is regulated post-translationally is unclear. Our previous studies demonstrate cyclin-dependent kinase 2 (CDK2) controls Foxp3+Treg function, but the mechanism by which this occurred was not identified. The CDKs are primarily thought to control cell cycle progression. However, recent studies suggest only CDK1 is required for normal mammalian cell cycle, raising questions about the biological role of the other CDKs. Specifically, mice genetically deficient in CDK2 are viable with no significant defects in cell cycle. We probed the Foxp3 sequence for the presence of CDK motifs, finding four such sites in the amino terminus. We confirmed that Foxp3 is phosphorylated by CDK2 using an in vitro kinase assay and mass spectrometry, as well as a phospho-specific antibody that recognizes one of the phosphorylated Foxp3-CDK motifs. We generated a mutant of Foxp3 lacking all four CDK motifs, which has increased half-life compared to wild-type Foxp3. CD4+ T cells transduced with a Foxp3-CDK motif mutant have increased function compared to cells transduced with wild-type Foxp3 as measured by induction and repression of canonical Foxp3 target genes, as well as the ability to suppress conventional T cell proliferation. These data suggest CDKs negatively regulate Foxp3 protein stability, which has an impact on Foxp3 function. To determine when the CDK cascade was actively regulating Foxp3 in vivo we investigated the role of the CDK2 inhibitor, p27kip1. Recent data shows TGFβ signaling drives expression of p27kip1 in B cells. TGFβ is also required for extrathymic induction of Foxp3 in conventional CD4+ T cells. We show that conventional T cells, which have high CDK2 activity and minimal p27kip1 expression, induce large amounts of p27kip1 along with Foxp3 in the presence of TGFβ. Additionally, T cells lacking p27kip1 have defective TGFβ-dependent Foxp3 induction. We hypothesize that TGFβ signaling is required to activate p27kip1 and stabilize Foxp3 protein levels in developing iTreg by repressing CDK2.

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DEDICATION

This work is lovingly dedicated in memory of Max Boisot, a world-renowned scholar, author, lecturer of economics and strategic management, and my uncle. Max had a tireless pursuit of knowledge and a relentless work ethic. He was a true intellectual, one of my greatest inspirations. Max is sorely missed in this world.

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ABSTRACT

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Andrew D. Wells

Foxp3 is a transcription factor required for the development and function of regulatory T cells (Treg). Humans lacking functional Foxp3 are afflicted with uncontrolled systemic autoimmunity. How the Foxp3 protein is regulated post-translationally is unclear. Our previous studies demonstrate cyclin-dependent kinase 2 (CDK2) controls Foxp3+Treg function, but the mechanism by which this occurred was not identified. The CDKs are primarily thought to control cell cycle progression. However, recent studies suggest only CDK1 is required for normal mammalian cell cycle, raising questions about the biological role of the other CDKs. Specifically, mice genetically deficient in CDK2 are viable with no significant defects in cell cycle. We probed the Foxp3 sequence for the presence of CDK motifs, finding four such sites in the amino terminus. We confirmed that Foxp3 is phosphorylated by CDK2 using an in vitro kinase assay and mass spectrometry, as well as a phospho-specific antibody that recognizes one of the phosphorylated Foxp3-CDK motifs. We generated a mutant of Foxp3 lacking all four CDK motifs, which has increased half-life compared to wild-type Foxp3. CD4+ T cells transduced with a Foxp3-CDK motif mutant have increased function compared to cells transduced with wild-type Foxp3 as measured by induction and repression of canonical Foxp3 target genes, as well as the ability to suppress conventional T cell proliferation. These data suggest CDKs negatively regulate Foxp3 protein stability, which has an impact on Foxp3 function. To determine when the CDK cascade was actively regulating Foxp3 in vivo we investigated the role of the CDK2 inhibitor, $p27^{kip1}$. Recent data shows TGFβ signaling drives expression of $p27^{kip1}$ in B cells. TGFβ is also required for extrathymic induction of Foxp3 in conventional CD4+ T cells. We show that conventional T cells, which have high CDK2 activity and minimal $p27^{kip1}$ expression, induce large amounts of $p27^{kip1}$ along with Foxp3 in the presence of TGFβ. Additionally, T cells lacking $p27^{kip1}$ have defective TGFβ-dependent Foxp3 induction. We hypothesize that TGFβ signaling is

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PREFACE

Forty-five months ago I started this project I called “Foxp3 analysis for CDK2 phosphorylation site”, on the first page of my first Wells lab notebook. It turned out to be a long battle with science, filled with struggles and successes. This paper is the report of those forty-five months. It does not accurately tally the countless hours spent in the lab, nor does it rightly express the emotions felt battling alongside my fellow scientists and friends; the joy of creativity and problem-solving, the hope for good results, the sadness and frustration that came with each failed attempt. Nevertheless, I am proud of those forty-five months and the progress I made, which is laid out on the following pages.

CHAPTER 1 - Cyclin-dependent kinases in T cell biology

Summary

Cyclin-dependent kinases (CDK), their partnering cyclins, and inhibitors have been studied primarily as components of the cell cycle, regulating its initiation, DNA replication, growth, and mitosis. Clonal expansion of an antigen-specific T lymphocyte was thought to depend heavily on the CDK cascade directing cell cycle progression. CDK-driven cell division was also linked to the generation of a productive T cell effector response, while kinase inhibitory proteins were shown to be essential for the onset of anergy and tolerance. In the last decade, genetic studies indicated that only CDK1 is absolutely required for normal cell cycle progression in most tissues. As a result, the focus has shifted to elucidating the cell cycle independent roles for CDKs and their inhibitors in T cell biology. In this chapter, we will discuss the understood and changing roles of the CDK family in T lymphocyte division, expansion, and function. This will establish a groundwork for our hypothesis on the role of CDK2 and its inhibitor, $p27^{kip1}$ in the regulation of T cell tolerance.

The cyclin dependent kinase family & the classical view of cell cycle progression

The cyclin dependent kinases (CDK) are a family of serine/threonine, proline-directed kinases engaged in a large number of different cellular processes. Full-length CDK3 is absent from all inbred mouse strains due to a truncation mutant (1); CDK5 is predominantly known as a neuron-specific kinase (2), but is now appreciated to have a role in T cell function (3); CDK7, -8, and -9, regulate transcriptional complex formation; CDK1, -2, -4, and -6, the interphase and mitotic CDKs, act with their partnering cyclins to engage a complex kinase cascade that drives cell cycle progression (4). Cyclin-dependent kinase inhibitors (CKI) of the ink (i.e. p18ink4c) and cip/kip (i.e. $p27^{kip1}$) families are known to physically interact with and antagonize the activity of these CDKs. This thesis will focus mainly on CDK2 and its inhibitor $p27^{kip1}$ in T lymphocytes.

During the cell cycle, a series of sequential, CDK-directed phosphorylation events allow the cell to pass critical cell cycle checkpoints, resulting in DNA replication, growth and mitosis (4). D-type cyclins are rapidly induced in response to mitogenic stimuli and decline quickly upon withdrawal of these stimuli (5). Mitogenic stimuli also reduce expression of D-type cyclin inhibitors p15ink4b and p18ink4c, freeing CDK4 and CDK6, which are stably expressed, to drive G1 progression (6). CDK4/6-cyclin D complexes reduce the activity of Rb, a negative regulator of S phase entry, by a phosphorylation-dependent mechanism, release E2F to induce cyclin E (7, 8). Next, mitogen induced CDK2 and cyclin E partner to target the degradation of their inhibitor $p27^{kip1}$ which is highly expressed in quiescent cells, while also phosphorylating Rb on sites distinct from CDK4/6 (9). CDK2-cyclin E also phosphorylates histone H1 and activates the SWI/SNF complex, affecting chromatin condensation, then induces a number of factors involved in augmented histone synthesis, centrosome duplication, and protein organization at origins of DNA replication (10, 11). Once these steps are completed a restriction point has been passed. Removal of mitogenic stimuli before this step will reverse cell cycle progression, but once a cell has begun DNA replication it is irreversibly committed to completing division (12, 13). During S phase, CDK2-cycE induce cyclin A, which then takes over as the predominant partner of CDK2 (14). Together, CDK2-cycA increase the stability of complexes involved in mitosis, ensuring only one round of DNA replication occurs during each round of division. Later, during G2/M, CDK1 is

induced and partners with both cyclin A and cyclin B (15-18). Together, these complexes regulate completion of mitosis and the cell cycle.

The complexities of the cell cycle kinase cascade were first studied using fibroblasts and transformed epithelial cells, which continuously cycle, and their division can only be stopped by abnormal methods such as nutrient starvation or harsh drug treatment. Like fibroblasts, lymphocytes provide an easily controllable way to study the cell cycle. They are easily manipulated through the provided mitogenic signals, and enter a natural quiescent state upon the removal of mitogens, allowing cell cycle synchronization (19).

T lymphocyte cell cycle: How essential are the CDKs?

Lymphocyte cell cycle progression is required for clonal expansion, which is a critical aspect of an antigen-specific immune response. An activated lymphocyte expands to create progeny all sharing the same antigen specificity while those lymphocytes bearing receptors for self molecules are being deleted at an early stage (20). Additionally, while a T lymphocyte depends on antigen, costimulatory, and growth factor receptor signaling to begin clonal expansion, these signals are also required to regulate its differentiation and function (21-24). These early studies led to the hypothesis that cyclin-dependent kinases and their partnering cyclins are essential for clonal expansion, differentiation, and effector function of lymphocytes, through the regulation of the cell cycle.

Genetic deletion studies were used to confirm the role of cell cycle proteins. None of the cyclin knockout animals had any serious immune defects except for those lacking cyclin D3, which is required for normal hematopoietic development (25). Mice lacking CDK1 are not able to develop at the embryonic stage due to the loss of DNA rereplication (26). Surprisingly, mice lacking either CDK2 (27), CDK4 (28), or CDK6 (29) are all viable and progress through the cell cycle normally in most tissues and cells including T lymphocytes. CDK2, for example, is not required for normal thymocyte development (30) or mature T lymphocyte clonal expansion (31).

The fact that CDKs are only required for normal proliferation in certain cells was very unexpected. CDK2 is required for meiosis, and mice lacking CDK2 are rendered sterile because of cell cycle defects in spermatocytes (32), although oocyte cycling depends only on CDK1 (33).

CDK4 is essential for pancreatic beta cell proliferation and pituitary lactotrophs (28). CDK6 is needed for normal erythroid cell development (29). Additionally, some tissue development requires multiple CDKs. Cardiomyocyte development is defective in CDK2-CDK4 double knock out mice, resulting in embryonic lethality (34). If, instead, CDK2 and CDK4 are deleted after cardiomyocyte development, the mice are viable with no major cell cycle defects (35). CDK4 and CDK6 double knock mice out have defects in hematopoiesis, but are otherwise normal (29). Mice lacking CDK2, -4, and -6 die at E15.5 and contain decreased liver cellularity as well as cardiomyocyte and hematopoietic cell defects (36). MEFs taken from these mice cycle without major defects and can be immortalized, but cycle slower than wild type controls with an increased requirement for growth factors. These data suggest that only CDK1 is absolutely required for normal cell cycle progression in most tissues and cells, including T lymphocytes.

T cell anergy and tolerance and the cell cycle

In the mid-1990s, immunologists began to employ powerful fluorescent dye approaches to trackd cell division of individual lymphocytes during clonal expansion, both in vitro and in vivo. One of these dyes, carboxyfluorescein succinimidyl ester (CFSE) (37), segregates uniformly between daughter cells upon division and is stably maintained for up to ten days in culture (38). Studies using CFSE revealed that expression of T cell differentiation markers and effector cytokines was cell division dependent (21-23). The number of proliferating cells was shown to depend on TCR signaling, while costimulatory and growth factor receptor signaling accounted for the number of divisions each cell completed, and the quality of their effector response (38). Upregulation of effector differentiation markers CD44, CD45RB, and CD62L, as well as production of key effector cytokines, IFNγ and IL-4, are produced after the third cell division (21, 23). Additionally, the progression of cell cycle, along with the expression of these effector cytokines, contribute to the epigenetic stability of the activated lymphocyte, increasing activating histone acetylation and decreasing inhibitory DNA methylation marks (23).

T lymphocytes that complete more cell divisions become an activated cytokine-producing effector population upon secondary challenge with antigen. Reciprocally, if cell division was limited during initial activation, using CTLA4-Ig costimulatory blockade, the T lymphocytes are

hyporesponsive upon restimulation, and rendered anergic (38, 39). Anergy can be induced during CD4+ T cell activation by pharmacologically blocking G1 progression (40, 41) or IL-2R signaling (42, 43). However, pharmacologic block during S phase fails to induce anergy in T cells. These data suggest the G1 to S phase transition is critical for anergy avoidance. CDK2 and $p27^{kip1}$ were therefore hypothesized to regulate anergy because of their supposed role in regulating G1 to S phase cell cycle restriction point. Surprisingly, although CDK2 is dispensable for G1 to S phase transition because of compensation by CDK1 (44), both CDK2 and $p27^{kip1}$ were found to be involved in T cell anergy and tolerance, suggesting they were involved in cell cycle independent roles.

$p27^{kip1}$ regulates T cell anergy

The CDK inhibitor $p27^{kip1}$ is highly expressed in quiescent naive T lymphocytes, and upon activation forms a scaffold that permits cyclin D-CDK4/6 complex formation, which is required for egress from G0. Afterwards, $p27^{kip1}$ is rapidly degraded in a CDK2-dependent manner in response to CD28 signaling (4, 45). Anergic T cells maintain elevated expression of $p27^{kip1}$ (38, 45-47), while T cells lacking $p27^{kip1}$ are resistant to anergy, and become effectors in response to TCR stimulus in the absence of costimulation (47, 48). This anergy avoidance can occur within 24 hours, showing it is not dependent on cell division. Additionally, the *il2* gene, which is epigenetically silenced in anergic cells, remains epigenetically poised in $p27^{kip1}$-/- T cells stimulated under anergizing conditions. These data suggest $p27^{kip1}$ is a sensor of CD28 signals, and is required to regulate T cell clonal anergy independent of the cell cycle.

CDK inhibitory proteins regulate T cell tolerance

Tolerant T cells induce high levels of the CDK inhibitory protein, $p27^{kip1}$. Mice lacking $p27^{kip1}$ were unable to tolerize to MHC mismatched grafts during systemic costimulatory blockade using antiCD28 and antiCD40 (49). Normally, this regimen of costimulatory blockade is sufficient to induce long-term allograft tolerance in mice (50). However, in the absence of $p27^{kip1}$, rejection occurred rapidly. Cardiac grafts were flooded with IFNγ-producing CD4+ T cells that mediated pathology, accompanied by systemic allospecific lymphocyte expansion. These data are

consistent with in vitro studies of $p27^{kip1}$-deficient T cells showing a role in effector differentiation, and they suggest $p27^{kip1}$ is not only involved in T cell anergy in vitro, but also T cell tolerance in vivo.

Mixed roles in tolerance have been shown for the CDK inhibitory protein, $p21^{cip1}$. Mice lacking p21cip on different genetic backgrounds demonstrate in one case decreased self-reactivity (51), and in another an increased proclivity for autoimmunity (52, 53). These opposing results are not necessarily mutually exclusive. Deletion of $p21^{cip1}$ on the lupus-prone BXSB strain quelled disease progression by inducing a number of pro-apoptotic pathways driving the programmed death of activated autoreactive lymphocytes (51). Contrarily, $p21^{cip1}$ deletion on a C57BL/6 background caused a lupus-like disease in aged female animals highlighted by increased CD4+ T cell activity, anti-nuclear antibody production, and glomerulonephritis (52, 53). It is possible that $p21^{cip1}$ controls apoptotic pathways in autoreactive cells, but also regulates CDK1- or CDK2-mediated substrate phosphorylation that is part of an important, but yet unappreciated tolerance pathway. The studies implicating $p21^{cip1}$ as a tolerance factor did not establish a biochemical component to the developing autoimmunity, including no mention of whether CDK activity or a CDK substrate was dysregulated in the absence of the inhibitor. These remain possibilities that require further study.

CDK2 regulates T cell effector function & tolerance

How does $p27^{kip1}$ induce or maintain anergy? One possibility is through association with JAB1, a c-Jun co-activator. This interaction inhibits AP-1 transactivation and thereby *il2* transcription (45). Another possibility is by CDK2 inhibition. CDK2 interacts with numerous proteins involved in *il2* gene expression. CDK2 is found in complex with NFκB (54), the co-activator and histone acetyltransferase p300/CBP (55), and subunits of the RNA polymerase holoenzyme complex TFIIB and RNApolα (56). CDK2 can phosphorylate Sp1 (57), and Smad3 (58), which all regulate the *il2* gene. Smad3 activity is dyregulated in T cells lacking $p27^{kip1}$, which is an integral reason for their anergy resistance (48). Additionally, CDK2-/- T cells have defects in IL-2 production (31). These data suggest cell cycle independent routes of anergy regulation by the $p27^{kip1}$-CDK2 axis.

T cell anergy and tolerance are regulated in part by the expression and activity of the CDK inhibitor $p27^{kip1}$ as described above. In the absence of $p27^{kip1}$, T cells fail to anergize in vitro, or to form tolerance to heterotopic heart grafts in vivo even in the presence of CD28 and CD40L costimulatory blockade. One way $p27^{kip1}$ could be mediating tolerance is through inhibition of its major target, CDK2. Mice with a germ line deletion of CDK2 are viable with no major cell cycle defects (27). Apart from normal fibroblast cell cycle progression, CDK2-/- mice also have normal lymphocyte development (30) and proliferation (31). In conventional CD4+ T cells, CDK2 is required to sustain normal production of the cytokines IL-2 and IFNγ (31). T cells lacking CDK2 activity either because of genetic deletion, through knockdown by shRNA, or in response to a small molecule inhibitor, produce less IL-2 and IFNγ than their wild type counterparts, a phenotype that is exaggerated in restimulated anergic cells.

In vivo, CDK2-/- mice are able to tolerize to heterotopic heart transplants from fully mismatched donors under conditions that lead to rejection in wild type controls. Immunohistochemical staining of surviving grafts from CDK2-/- recipients revealed a 5-fold decrease in the number of infiltrating IFNγ producing T cells compared to wild type recipients. Wild type recipients also have grafts containing high expression of CDK2 and cyclin A. These data suggest CDK2 is important for the differentiation and effector function of CD4+ T cells, and that the CDK pathway is highly active during allograft rejection.

The long term allograft survival achieved in the absence of CDK2 can be partially explained by the role of this kinase in conventional T cell differentiation and function. However, CDK2 could also affect the balance between immunity and tolerance by acting on regulatory T cells. Immunohistochemical staining of surviving grafts from CDK2-/- recipients revealed a substantial amount of Foxp3, the regulatory T cell lineage specifying transcription factor (31). These data suggest a potential role for CDK2-mediated control of Foxp3 and regulatory T cell driven tolerance. Consistent with this notion, regulatory T cells from mice lacking CDK2 have an increased ability to suppress proliferation of conventional CD4+ T cells in vitro, and are better at ameliorating T cell-mediated colitis than their wild type counterparts (31). These results show that CDK2 regulates the function of regulatory T cells, and that there may also be an effect on Foxp3 expression, but more analysis is necessary to determine the mechanism by which CDK2 is

acting. We hypothesize that Foxp3 is a CDK2 substrate, and that CDK2 kinase activity negatively regulates the expression of Foxp3. This central idea became the basis for my thesis work. How I tested this hypothesis is presented in the following two data Chapters.

CHAPTER 2 - Foxp3 protein stability is regulated by CDK2

SUMMARY

Foxp3 is a transcription factor required for the development of regulatory T cells (Treg). Mice and humans with a loss of Foxp3 function suffer from uncontrolled autoimmunity and inflammatory disease. Expression of Foxp3 is required for Treg development, but whether Foxp3 activity is further subject to regulation by extracellular signals is unclear. Foxp3 contains four cyclin-dependent kinase (CDK) motifs (Ser/Thr-Pro) within the N-terminal repressor domain, and we show that CDK2 can partner with cyclin E to phosphorylate Foxp3 at these sites. Consistent with our previous demonstration that CDK2 negatively regulates Treg function, we find that mutation of the serine or threonine at each CDK motif to alanine (S/T>A) results in enhanced Foxp3 protein stability in CD4+ T cells. T cells expressing the S/T>A mutant of Foxp3 showed enhanced induction (*e.g.*, *CD25*) and repression (*e.g.*, *Il2*) of canonical Foxp3-responsive genes, exhibited an increased capacity to suppress conventional T cell proliferation in vitro, and were highly effective at ameliorating colitis in an in vivo model of inflammatory bowel disease. These results indicate that CDK2 negatively regulates the stability and activity of Foxp3, and implicate CDK-coupled receptor signal transduction in the control of regulatory T cell function and stability.

Introduction

Foxp3, a forkhead box transcription factor, mediates the development of regulatory T cells (Treg), which are involved in immune regulation and tolerance (59-63). Foxp3 translocates to the nucleus to bind DNA and induce (*CD25*, *Gitr*, *Ctla4*) or repress (*Il2*, *Il4*, *Ifn*γ) the expression of various genes. Foxp3 regulates accessibility of these target genes by recruitment of chromatin remodeling factors, which will epigenetically poise or silence certain genes (64, 65). The activity of many transcription factors that contribute to T cell differentiation and function is controlled by antigen, costimulatory, or growth factor receptor signaling (24), however, to what extent extracellular signals regulate Foxp3 function is largely unclear. A number of studies have recently described Foxp3 post-translational modification. Acetylated Foxp3 was first identified in response to TGFβ signaling (66). TIP60, p300, and sirtuin-1 were later also shown to regulate the acetylation status of Foxp3 (65, 67-69). Acetylation of Foxp3 affects its stability (68) and ability to bind to promoters (66). Other stimuli, such as hypoxia, also affect Foxp3 protein expression. The hypoxia induced factor 1α (HIF-1α) can regulate Foxp3 ubiquitination and stability in developing Th17 cells (70). Whether Foxp3 is regulated post-translationally by a specific kinase cascade, however, is not known.

Our analysis shows the primary amino acid sequence of murine Foxp3 contains multiple putative kinase motifs, including four cyclin-dependent kinase substrate motifs concentrated within the N-terminal repressor domain. Our previous studies showed that CDK2-deficient Treg are more suppressive than wild type Treg, as measured by the ability to suppress the proliferation of conventional CD4+ T cells in vitro, and to ameliorate colitis in an in vivo mouse model of inflammatory bowel disease (31). These findings demonstrate an important role for CDK2 in Treg biology, but did not establish the mechanism by which CDK2 functions in these cells. Considering the presence of multiple CDK motifs in the Foxp3 primary sequence, we hypothesized that CDK2 may influence Treg function through phosphorylation-dependent regulation of Foxp3.

In this current study, we find that CDK2 can phosphorylate Foxp3, and that mutation of the N-terminal CDK motifs increases the half-life, steady-state level, and transcriptional activity of *Foxp3*. Furthermore, T cells expressing CDK mutant Foxp3 exhibit increased suppressive

function compared to cells expressing wild type Foxp3. Our results indicate that CDK2 activity controls Treg suppressive function through setting the amount of Foxp3 available in the cell.

Materials and Methods

Mice - Female C57BL/6 (H-2^b) and *Rag1*-deficient mice on a C57BL/6 background were purchased from The Jackson laboratory and maintained in our specific pathogen free facility according to ULAR- and AALAC-approved institutional guidelines on animal care and usage. All mice were used at 6-14 weeks of age.

Full-length Foxp3 purification - Murine Foxp3 cDNA was amplified from wild type C57BL/6 thymus using the following primers: Foxp3 Fwd 5-ccggaattcatgcccaaccctaggccag-3', Foxp3 Rev 5-ccgctcgagtcaagggcagggattggagc-3'. The PCR amplified DNA was then cloned into the protein expression plasmid 6xHis-pET-28a (Novagen). *E.coli* strain BL21 was transformed with 6xHis-Foxp3-pET28a. A single bacterial colony was inoculated in LB containing kanamycin (50µg/mL). The bacterial culture was grown at 37°C and Foxp3 expression induced with IPTG (0.5mM, 12h, 30°C). For the purification of the 6xHis-Foxp3 recombinant protein, cells were harvested from 500mL culture and the recombinant protein was purified using Nickel-nitrilotriacetic acid resin by affinity chromatography under native conditions.

In Vitro Kinase Assay - 0.5µg 6xHis-Foxp3 or 1µg control Histone H1 (Roche) was incubated with CDK2-cycE (Millipore) and 5µCi γ32P-ATP (Perkin-Elmer) in kinase buffer for 30 min at 30°C. The reaction was stopped with 1x SDS-PAGE lysis buffer and analyzed by SDS-PAGE. The gel was dried and exposed to x-ray film. Roscovitine (Cell Signaling) was used to inhibit CDK2 activity.

Mass spectrometry - 6xHis-Foxp3 was incubated with CDK2-cycE and cold ATP and analyzed by SDS-PAGE. The Foxp3 band was then excised, destained, reduced with DTT, alkylated with iodoacetamide (Sigma Aldrich), and digested in-gel with trypsin. The extracted tryptic peptides were analyzed by reverse phase HPLC mass spectrometry using an LTQ-Orbitrap mass spectrometer. The assignment of phosphate to product ions identified was made using a Mascot search of MS2 data against the Oniprot mouse protein sequence database.

Site directed mutagenesis - Ser/Thr>Ala-Foxp3 mutant was generated using the Stratagene QuickChangeII site-directed mutagenesis kit. Mutagenesis primers used: S19A-Fwd 5'-cttggcccttggcccagccccaggag-3', S19A-Rev 5'-ctcctggggctgggccaagggccaag-3', S88A-Fwd 5'-ccgactaggtcccgcaccccacctaca-3', S88A-Rev 5'-tgtaggtggggtgcgggacctagtcgg-3, T114A-Fwd 5'-

gcccatgcccaggcccctgtgctcc-3', T114A-Rev 5'-ggagcacaggggcctgggcatgggc-3', T175A-Fwd 5'-cccacgctcgggtgcacccaggaaaga-3', T175A-Rev 5'-tctttcctgggtgcacccgagcgtggg-3'.

In vivo phosphorylation - HEK293 cells were transfected (Lipofectamine 2000, Invitrogen). 48h post-transfection cells were harvested, lysed, and subjected to FLAG immunoprecipitation according to manufacturer protocol (Sigma Aldrich). Lysis buffer was supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich) and β-glycerophosphate (Sigma Aldrich). Where indicated cells were treated with the proteosome inhibitor MG132 (Sigma Aldrich) and the CDK2 inhibitor Roscovitine (Sigma Aldrich). Affinity purified p+Ser19 Foxp3 rabbit polyclonal antibody (Yenzym) was used to detect phosphorylated species of Foxp3.

Peptide dot blot - Lyophilized Foxp3 peptide (aa12-24) either unmodified or phosphorylated at serine 19, was resuspended at 10mg/mL and serially diluted with PBS. Amounts of peptide from 500µg to 0.05ng were placed on nitrocellulose in log10 dilutions. Western blot was then performed using p+Ser19-Foxp3 (Yenzym).

Retroviral Transduction - Murine Foxp3 cDNA was amplified from wild type C57BL/6 thymus and cloned into the murine stem cell virus (MSCV)-based retroviral vector (13) expressing the reporter gene GFP, MIGR, or human NGFR (CD271), MINR, and containing an in-frame, N-terminal FLAG epitope. For generation of retrovirus, constructs were cotransfected (Lipofectamine 2000, Invitrogen) with the pCLeco (Invitrogen) helper plasmid into the 293T-based Phoenix ecotropic packaging cell line (provided by G. Nolan, Stanford University). Wild type C57BL/6 CD4+CD25- naive T cells were activated with 3ng/mL phorbol 12-myristate 13-acetate (Sigma Aldrich), 1uM ionomycin (Sigma Aldrich), and 10U/mL IL-2 (Roche) for 24h, washed, and transduced by spinfection (71) with supernatants from 48h transfected Phoenix cells. Transduced cells were expanded in IL-2 for 3-4 days. CD4+ T cell transduction efficiencies were ≥90%. Where necessary, transduced T cells were purified to near 100% purity using vector reporter markers.

FACS, ELISA, qPCR, and Western blot analysis of transduced cells - Before analysis, transduced cells were restimulated 4-6 hours with 1µg/mL CD3 (2C11, BioXCell), 1µg/mL CD28 (37.51, BioXCell), and IL-2 (10U/ml, Roche). FACS analysis was performed on a Beckman

Coulter Cyan ADP. Cells were stained for FACS analysis with conjugated mouse anti-Foxp3-APC (FJK-16s, eBiosciences), mouse anti-CD25-APCcy7 (PC61, BioLegend), anti-NGFR-PE (CD271, BD Pharmigen), and anti-CD4-PacificBlue (GK1.5, BioLegend). Supernatants from restimulated cells were harvested and probed for levels of *Il2* protein by ELISA according to the manufacturer protocol (eBiosciences kit). Manufacturer protocols were used to for kits to extract RNA from transduced cells (Qiagen) and convert into cDNA (BioRad). Relative *Foxp3* and *Il2* message levels were determined following quantitative PCR using according to the 2^{ddCt} method after normalization to actin. Whole cell extracts were generated using the radioimmune precipitation buffer lysis kit (Sigma Aldrich) and run on precast 10% SDS-PAGE Criterion gels (BioRad). Western blots were performed using mouse/rat anti-Foxp3 (FJK-16s, eBiosciences) and rabbit anti-p+Ser19-Foxp3 (Yenzym).

Stability assay - Following restimulation of transduced cells with CD3, CD28, and IL-2, cells were harvested, washed, and replated in the presence of 25μg/mL cycloheximide (Sigma Aldrich). Cells were harvested at indicated time points, and whole cell extracts were prepared and analyzed for expression of Foxp3 by western blot as described above.

In Vitro Treg Suppression - CD4+CD25- (Tconv), and CD90-negative APC were isolated from splenocytes of wild type C57BL/6 mice using magnetic bead-conjugated mAbs (Miltenyi). APC (1×10^5) were irradiated (1000 radians, 3 minutes) and plated onto 96-well round-bottomed plates along with Tconv effector cells (5×10^4/well) labeled with CellTrace (Invitrogen Molecular Probes, Violet CFSE equivalent) and 4μg/ml soluble anti-CD3 mAb. T cells transduced with empty vector control, wild type-Foxp3, or Ser/Thr>Ala-Foxp3 (serving as suppressor Treg) cells were added to each well in varying ratios to Tconv cells and cultured for 72h. After 3 days, suppression of responder cell proliferation was measured by flow cytometry to assess the degree of inhibition of CellTrace (Invitrogen, violet CFSE equivalent) dilution as previously published (38).

Adoptive Transfer Colitis - To induce experimental colitis, conventional CD4+25- T cells were purified from naive, wild type C57BL/6 mice and adoptively transferred (1×10^6, i.v.) into *Rag1*-/- B6 recipients (72, 73). Twenty-one days from the initial transfer, groups of three to seven mice then received either PBS, or T cells transduced with empty vector control, wild type-Foxp3, or Ser/Thr>Ala-Foxp3 mutant (2×10^6, i.p.). Recipients were weighed and observed for symptoms

of diarrhea approximately every two days. At the end of the experiment, spleens, mesenteric lymph nodes, and intestines were harvested for examination of gross pathology, and histology.

Gross pathology - Colitis-induced animals were sacrificed and analyzed for signs of gross pathology using a modified version of established methods (74). Scores of 0 (no colitis) to 4 (worst disease) were assigned according to colon rigidity, visible inflammation, and presence of blood in intestines, as well as diarrhea, and presence of fat tissue.

Histopathology - Intestines from colitis-induced animals were fixed in formaldehyde, imbedded in paraffin, sliced, stained with hematoxylin and eosin, and mounted onto glass slides for histological analysis. Blinded evaluation of H&E-stained paraffin sections was performed by a pathologist and scored using established criteria (75) to reach a maximum colitis score of 23. The criteria and scoring used were: Inflammation: 0-3, Mucin depletion: 0-2, Reactive epithelial changes: 0-3, Number of intraepithelial lymphocytes: 0-3, Crypt architectural distortion: 0-3, Number of inflammatory foci per 10 high power field: 1-3, Inflammatory activity: 0-2, transmural inflammation: 0-2, Mucosal ulceration: 0-2.

Statistical analysis - All p values were calculated by Student's paired t test using Prism software (GraphPad).

Results

Foxp3 is phosphorylated by CDK2

We analyzed the Foxp3 amino acid sequence as a primary approach to identifying potential sites of post-translational modification. We found many putative kinase binding elements, including a cluster of four cyclin-dependent kinase (CDK) motifs, Ser/Thr-Pro (Fig. 1). We recently demonstrated that cyclin-dependent kinase 2 is a negative regulator of Foxp3+ Treg function, but a biochemical basis was not established (31). The presence of multiple CDK motifs in Foxp3 suggests CDK2 might control Treg function through a phosphorylation-dependent mechanism. The Foxp3 CDK motifs are concentrated in the N-terminal half of the protein. This segment of Foxp3 (aa1-198) is necessary and sufficient for repression of the *Il2* gene, and is therefore referred to as a 'repressor' domain (76, 77). This domain contains no defined structural motifs, so how it contributes to Foxp3 function is not understood.

To test whether CDK2 can phosphorylate Foxp3, we purified full-length recombinant Foxp3 (Fig. 2a) and performed standard in vitro kinase assays. Recombinant CDK2 together with its G1/S phase binding partner cyclin E was able to phosphorylate Foxp3, and the reaction could be abrogated by the CDK inhibitor roscovitine (Fig. 2b). To map the residues phosphorylated by CDK2-cyclin E, we performed the kinase reaction with cold ATP and subjected the purified Foxp3 substrate to RP-HPLC mass spectrometry (MS). Product ions b14 and y9 from the peptide PAKPMAPSLALGP**SP**GVLPSWK (Fig. 2c) contain an 80 Dalton increase in their expected mass-to-charge (m/z) ratio, indicative of a phosphate group at the serine followed by the proline (aa 19). Product ions containing only the other serine residues in the peptide do not contain an 80 Dalton increase in m/z, and are therefore not phosphorylated (Fig. 2c,e). An 80 Dalton increase is also found in the fragment ion b5 from the peptide SG**TP**RKDSNLLAAPQGSYPLLANGVCK (Fig. 2d,f). The absence of earlier b-series ions or later y-series ions for this peptide precludes specific assignment of the phosphate group to the first serine (aa 173) or threonine (aa 175) residue in the peptide. However, CDK2 is an obligate proline-directed kinase, and therefore must be acting on threonine 175. The phosphorylation of the other two CDK target residues, serine 88 and threonine 114, was not determined as peptides containing these residues could not be isolated during enzymatic digestion preceding RP-HPLC. None of the other serine or threonine residues in

Foxp3 outside these motifs were phosphorylated by CDK2 (data not shown). These data suggest that Foxp3 is phosphorylated by CDK2 on at least two of its CDK motifs, Ser19 and Thr175.

We next generated an antibody that recognizes an N-terminal Foxp3 peptide - aa12-24, PSLALGP**pS**PGVLP - containing phosphorylated serine 19. To confirm that the antibody detects only phosphorylated serine 19, we performed a dot blot using serial dilutions of unphosphorylated and phosphorylated peptide (Fig. 3a). Our results demonstrate that our antibody only recognizes p+Ser19, with the limit of detection in the nanogram range. To determine whether Foxp3 can be phosphorylated on S19 in an in vivo, cellular environment, we transfected HEK293 cells with a control FLAG-tagged vector, wild type Foxp3, or a mutant of Foxp3 in which the serine or threonine of all four CDK motifs was mutated to alanine (Ser/Thr>Ala). After 48h, transfected cells were harvested and lysed in the presence of both phosphatase and proteosome inhibitors. FLAG immunoprecipitation (IP) was performed on all lysates. Whole cell extracts and FLAG-IP eluate were analyzed by western blot for phosphorylated and total Foxp3 (Fig. 3b). Wild type Foxp3 transfected cell lysates and FLAG-IP eluates contain a species of Foxp3 phosphorylated on S19, as detected using the p+Ser19-Foxp3 antibody. No phosphorylated Foxp3 was detected in extracts from Ser/Thr>Ala mutant Foxp3. These data demonstrate Foxp3 is phosphorylated at Ser19 CDK motif by endogenous cellular machinery. However, because the p+Ser-Foxp3 and Foxp3 antibodies recognize different epitopes it is not possible to quantify relative amount of phosphorylated Foxp3 relative to the total pool.

CDK motifs contribute to Foxp3 protein stability

Kinase activity and phosphorylation can be coupled to protein stability (78). To test whether Foxp3 N-terminal CDK motifs contribute to protein stability, conventional CD4+CD25- T lymphocytes were transduced with MSCV-based retroviral vectors encoding wild type Foxp3, or the Ser/Thr>Ala Foxp3 mutant (Fig. 4a). We treated empty vector-, wild type Foxp3-, or Ser/Thr>Ala-Foxp3-transduced CD4+ T cells with cycloheximide (CHX) and measured Foxp3 protein levels over time (Fig. 4b). Wild type Foxp3 was expressed at high levels following transduction, however, inhibition of protein synthesis with CHX revealed rapid turnover of Foxp3 within five hours with a half-life of 2 to 3 hours. Ser/Thr>Ala-Foxp3 exhibited increased protein stability;

following treatment with CHX the S/T>A(ble)-Foxp3 mutant showed a significantly increased half-life of 8 to 9 hours as compared to wild type Foxp3 (Fig. 4c). Importantly, the transcription of S/T>A(ble)-Foxp3 is not elevated over that of wild type-Foxp3 (Fig. 4d). These data indicate that CDK phosphorylation motifs are involved in the regulation of Foxp3 protein stability.

CDK motifs are important for Foxp3-dependent genetic events

Foxp3 drives a specific transcriptional program including the induction of genes like the alpha chain of the *Il2* receptor, *CD25*, and the repression of cytokine genes such as *Il2*. To determine whether these CDK motifs affect the transcriptional activity of Foxp3 we compared CD4+25- naive T cells transduced with either wild type or S/T>A(ble)-Foxp3 mutant. Wild type Foxp3 induced an expected Treg-like signature (59) including high Foxp3 expression, elevated CD25 levels, and decreased *Il2* production as compared to cells transduced with empty vector. Mutation of all four CDK motifs resulted in significant elevation of Foxp3 and CD25 protein levels (Fig. 5a), as well as enhanced repression of *Il2* message (Fig. 5b) and protein (Fig. 5c) as compared to wild type. These data suggest the CDK motifs negatively regulate Foxp3 function.

S/T>A(ble)-Foxp3 expressing cells have increased suppressive function

Mutation of the N-terminal CDK motifs in Foxp3 resulted in elevated protein stability and increased Foxp3-transcriptional activity. Therefore, we next asked whether T cells expressing the S/T>A(ble) mutant of Foxp3 exhibited a gain of suppressive function. To test this, we used a standard, anti-CD3-driven in vitro Treg suppression assay (79). CD4+ T cells expressing wild type Foxp3 were able to suppress the proliferation of conventional T cells compared to empty vector-transduced control cells. However, cells expressing the S/T>A(ble) mutant showed significantly elevated suppressive capacity compared to cells expressing wild type (Fig. 6). From these results, we conclude that the N-terminal CDK motifs restrain Foxp3 activity and suppressive function.

To test whether the N-terminal CDK motifs contribute to the anti-inflammatory capacity of Foxp3-expressing cells in vivo, we used an adoptive transfer model of inflammatory bowel disease (IBD) (72, 73). Immunodeficient *Rag1*-/- mice were injected with congenically marked

(CD45.1+) naive CD4+CD25- T cells and allowed to develop colitis highlighted by diarrhea and weight loss (Fig. 7a). Three weeks after the initial injection, animals were adoptively transferred with congenically marked (CD45.2+) CD4+ T cells transduced with wild type or S/T>A(ble) mutant Foxp3. Control mice receiving either PBS or empty vector-transduced cells developed a severe wasting disease including significant and rapid weight loss (Fig 7a). These animals had Foxp3+ cells in the mesenteric lymph nodes (Fig. 7b), but the expression was restricted to a CD45.1+NGFR- pool (data not shown), representing a population of in vivo induced Foxp3+ cells, which were not sufficient to ameliorate disease. Control mice also developed diarrhea, intestinal wall thickening, and intestinal inflammation and ulceration, including destruction of intestinal architecture as measured by gross and histopathological analysis (Fig. 7c,d). As previously shown (59), mice treated with transduced CD4+ T cells expressing wild type Foxp3 regained weight, no longer exhibited diarrhea, showed decreased intestinal thickness and rigidity, decreased inflammation and restored intestinal architecture. Mice that received S/T>A(ble) mutant-expressing cells experienced a similar degree of weight gain and restored intestinal architecture as compared to wild type treated mice. However, animals that received S/T>A mutant-expressing cells exhibited significantly less colon rigidity and hemorrhaging, and increased mesenteric fat tissue mass as compared to mice receiving wild type Foxp3 transduced cells (Fig. 7d). This decrease in gross pathology was accompanied by an elevated frequency of Foxp3+ donor cells in the mesenteric lymph nodes of S/T>A(ble)-Foxp3 treated animals (Fig. 7b). In addition, mice receiving mutant-expressing cells exhibited fewer outward signs of morbidity; they were more ambulant and had normal grooming behaviors (data not shown), while animals that received wild-type Foxp3-expressing cells were more hunched, less active, and did not exhibit normal grooming behavior. These data demonstrate that Foxp3 CDK motifs are not required for the suppressive activity of Foxp3-expressing cells in vivo, but rather, function to temper Treg anti-inflammatory activity.

Discussion

Foxp3 is a transcription factor necessary for the development of regulatory T cells (59, 61-63). It recruits chromatin remodeling factors to the nucleus to induce or repress the expression of multiple genes (64), but to what extent Foxp3 is subject to regulation by extracellular signals remains unclear. Foxp3 can be acetylated by Tip60 and p300, increasing its stability (65, 67, 68). This effect is countered by the histone deacetylase, sirtuin-1 (67, 69), which negatively affects Foxp3+ Treg function (80). The histone acetyl transferase p300 competes for lysine residues with E3 ubiquitin ligase activity; acetylation, or mutation of target lysine residues to arginine increases Foxp3 stability by inhibiting ubiquitination (67). Foxp3 degradation can also be directed by HIF-1α through a ubiquitin-mediated mechanism. This occurs under hypoxic conditions during Th17 development (70). Phosphorylation of Foxp3 was recently shown to occur at serine 418, affecting Foxp3 transcriptional activity (81). However, the kinase responsible for this modification was not identified. We previously showed cyclin-dependent kinase 2 (CDK2) opposes Treg suppressive function both in vitro and in vivo, but did not determine a mechanism by which CDK2 acts in Tregs (31). We hypothesize that CDK2 modifies Foxp3, and controls Treg function by a phosphorylation-dependent mechanism. Our analysis reveals four putative CDK motifs in the Foxp3 amino acid sequence. We demonstrate CDK2 phosphorylates Foxp3, and that the CDK motifs are required in a process that opposes Foxp3 protein stability and activity. This suggests that Foxp3 is subject to regulation by a kinase cascade, and that the effects of this phosphorylation oppose its function.

Cyclin-dependent kinases are ubiquitously expressed, growth factor receptor-coupled enzymes primarily thought to drive cell cycle progression (82, 83). CDK2 and its binding partner cyclin E were initially described to be indispensable for progression past the G1-S phase checkpoint of cell cycle (84). CDK1, however, is now known to be sufficient for cell cycle progression in most cell types, and can compensate for the absence of other interphase CDK, (44, 85, 86). For example, CDK2-/- mice are viable and present virtually no defect in mouse embryonic fibroblast cell cycle progression (27, 32). T lymphocyte development (30) and proliferation (31) are also normal in the absence of CDK2. These findings suggest that CDK2 could have cell cycle-independent roles in T lymphocytes. We have previously identified such a

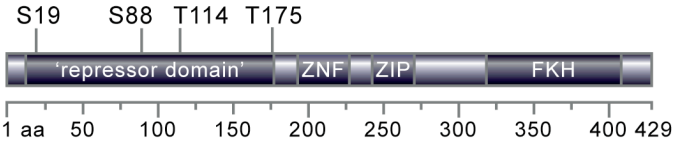
role, showing CDK2 regulates Treg suppressive function (31). Considering our current data we predict CDK2 affects Treg function through phosphorylation and destabilization of Foxp3. However, our studies do not rule out whether other CDKs are acting on Foxp3, as they all recognize the same motif. More work will need to be performed to dissect the effect of each CDK on Foxp3.

Our data suggest Foxp3 is a CDK2 substrate in T lymphocytes, but when is CDK2 active in the cell? Thymic derived regulatory T cells have stable long-term expression of *Foxp3* (87) and may not be subject to regulation by CDKs. Interestingly, a thymic Treg does repress CDK1, CDK2, and CDK6 expression (88). This could be an adapted mechanism to regulate these kinase cascades, and maintain Foxp3 stability. Foxp3 can also be induced peripherally in conventional T cells (89) providing an influx of suppressive cells. CDK2 is active in conventional T cells (31), driving differentiation and cytokine production, and our current data suggest CDK2 negatively affects Foxp3 stability. Therefore, we predict that to generate an extra-thymic Treg, the cell must not only induce *Foxp3*, but must also oppose CDK2 activity. Consistent with this idea, TGFβ signaling not only induces *Foxp3* expression (89), but is known to induce $p27^{kip1}$, an inhibitor of CDK2 (90, 91). In murine B cells, TGFβ induces $p27^{kip1}$, which results in increased association with CDK2 and decreased CDK2 kinase activity (91). We hypothesize that TGFβ-induced $p27^{kip1}$ reinforces the extrathymic Treg cell fate by opposing CDK2-mediated destabilization of Foxp3.

How are CDK motifs involved in regulating Foxp3 stability and function? One possibility is through phosphorylation-dependent ubiquitination. There are numerous examples of this type of crosstalk, in which phosphorylation at one residue can prime ubiquitination at a nearby lysine. Such peptides are referred to as phospho-degrons (78). CDK2 phosphorylation has already been linked to this process, pairing with the E3 ligase SCF/Fwb7 to target cyclin E for degradation (92). We propose the CDK motifs and nearby lysine residues cooperate to form a phospho-degron that regulates phosphorylation-dependent ubiquitination and degradation of Foxp3. This hypothesis is consistent with our data showing S/T>A(ble)-Foxp3 has increased stability as compared to wild type Foxp3. It is also possible that the Foxp3-CDK motifs are involved in subnuclear localization or nuclear export, leading to subsequent cytoplasmic degradation. The CDK2 inhibitor $p27^{kip1}$ is degraded in this manner (93, 94). Either of these possibilities is consistent with our data.

Our data show that CDK2 negatively regulates Treg suppressive function (31), likely through phosphorylation-dependent destabilization of Foxp3. Furthermore, we propose that Foxp3 CDK motifs act to moderate the cell-extrinsic anti-inflammatory activity of Foxp3. Modulating CDK2 activity could therefore be used to affect the balance between immunity and tolerance. Rosocovitine (seliciclib), a pharmacologic inhibitor that targets CDK2, CDK7, and CDK9 (95), is in clinical trials to treat cancer (96), and was shown to ameliorate graft-versus-host disease in mice (97). CDK2 may therefore be a relevant target in the treatment of autoimmunity or during transplantation.

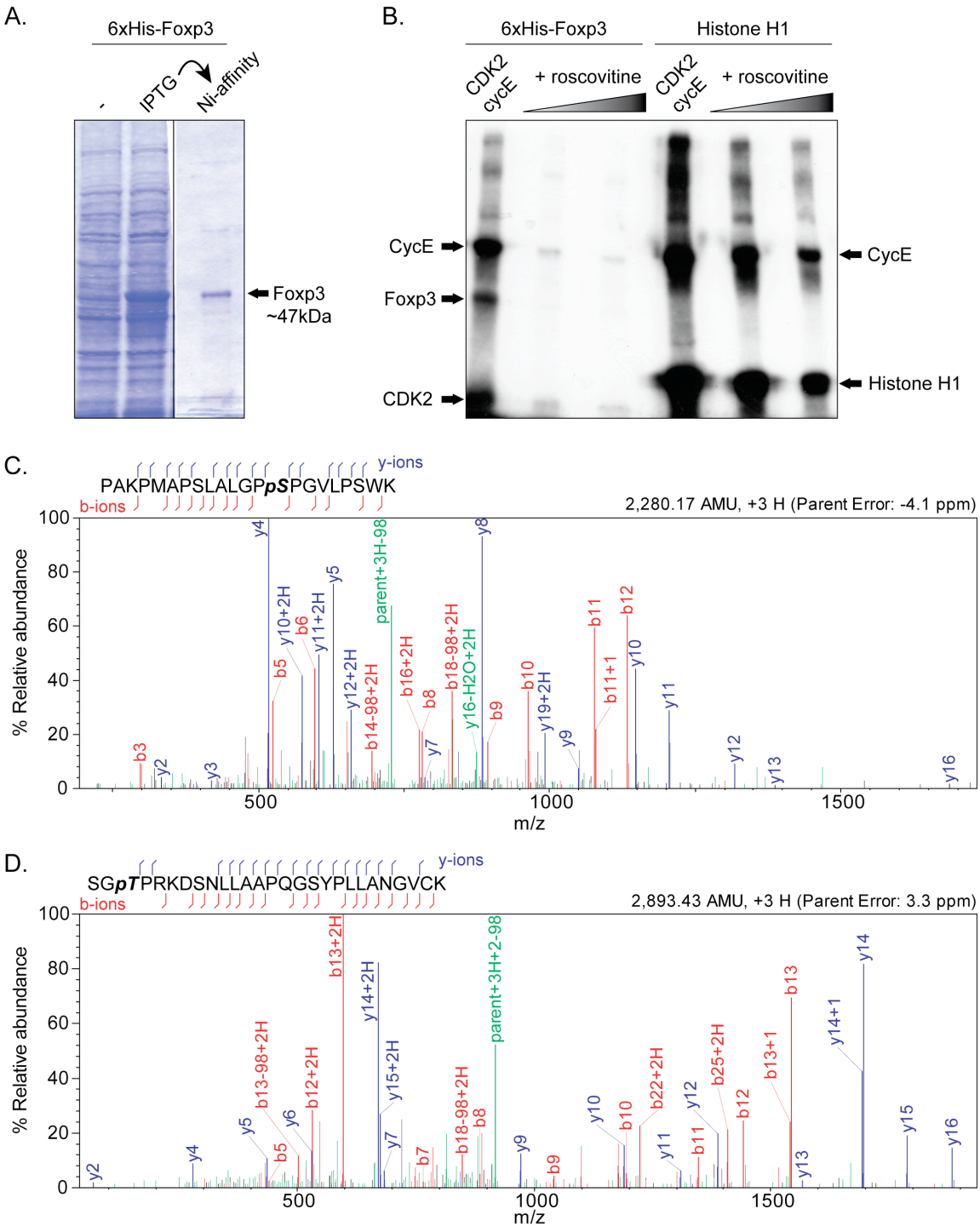
FIGURE 1



Murine Foxp3 CDK motifs (Ser/Thr-Pro)

Murine Foxp3 contains four putative CDK consensus binding sites. Analysis of the murine Foxp3 sequence revealed the presence of four putative cyclin-dependent kinase motifs (Ser/Thr-Pro, S/T-P) in the N-terminal 'repressor' domain. Also shown are the Foxp3 zinc finger (ZNF), leucine zipper (ZIP), and forkhead domains (FKH).

FIGURE 2


A.
6xHis-Foxp3
-
IPTG
Ni-affinity
Foxp3
~47kDa
B.
6xHis-Foxp3
Histone H1
CDK2
cycE
+ roscovitine
CDK2
cycE
+ roscovitine
CycE
Foxp3
CDK2
CycE
Histone H1
C.
y-ions
PAKPMAPSLALGPpSPGVLPSWK
b-ions
2,280.17 AMU, +3 H (Parent Error: -4.1 ppm)
% Relative abundance
m/z
D.
y-ions
SGpTPRKDSNLLAAPQGSYPLLANGVCK
b-ions
2,893.43 AMU, +3 H (Parent Error: 3.3 ppm)
% Relative abundance
m/z

E.

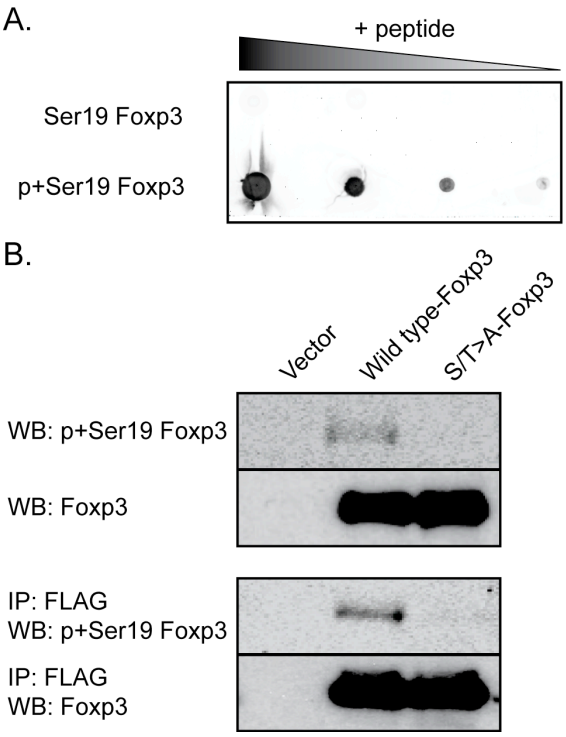
B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	98.1	49.5			P	2,281.2	1,141.1	2,264.2	2,263.2	22
2	169.1	85.1			A	2,184.1	1,092.6	2,167.1	2,166.1	21
3	297.2	149.1	280.2		K	2,113.1	1,057.0	2,096.1	2,095.1	20
4	394.2	197.6	377.2		P	1,985.0	993.0	1,968.0	1,967.0	19
5	525.3	263.1	508.3		M	1,887.9	944.5	1,870.9	1,869.9	18
6	596.3	298.7	579.3		A	1,756.9	879.0	1,739.9	1,738.9	17
7	693.4	347.2	676.3		P	1,685.9	843.4	1,668.8	1,667.9	16
8	780.4	390.7	763.4	762.4	S	1,588.8	794.9	1,571.8	1,570.8	15
9	893.5	447.2	876.5	875.5	L	1,501.8	751.4	1,484.8	1,483.8	14
10	964.5	482.8	947.5	946.5	A	1,388.7	694.9	1,371.7	1,370.7	13
11	1,077.6	539.3	1,060.6	1,059.6	L	1,317.7	659.3	1,300.6	1,299.6	12
12	1,134.6	567.8	1,117.6	1,116.6	G	1,204.6	602.8	1,187.5	1,186.6	11
13	1,231.7	616.3	1,214.7	1,213.7	P	1,147.6	574.3	1,130.5	1,129.5	10
14	1,398.7	699.8	1,381.7	1,380.7	S+80	1,050.5	525.8	1,033.5	1,032.5	9
15	1,495.7	748.4	1,478.7	1,477.7	P	883.5	442.3	866.5	865.5	8
16	1,552.8	776.9	1,535.7	1,534.7	G	786.5	393.7	769.4	768.4	7
17	1,651.8	826.4	1,634.8	1,633.8	V	729.4	365.2	712.4	711.4	6
18	1,764.9	883.0	1,747.9	1,746.9	L	630.4	315.7	613.3	612.4	5
19	1,862.0	931.5	1,844.9	1,844.0	P	517.3	259.1	500.3	499.3	4
20	1,949.0	975.0	1,932.0	1,931.0	S	420.2	210.6	403.2	402.2	3
21	2,135.1	1,068.0	2,118.0	2,117.1	W	333.2	167.1	316.2		2
22	2,281.2	1,141.1	2,264.2	2,263.2	K	147.1	74.1	130.1		1

F.

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	88.0	44.5		70.0	S	2,894.4	1,447.7	2,877.4	2,876.4	27
2	145.1	73.0		127.1	G	2,807.4	1,404.2	2,790.4	2,789.4	26
3	326.1	163.5		308.1	T+80	2,750.4	1,375.7	2,733.3	2,732.4	25
4	423.1	212.1		405.1	P	2,569.4	1,285.2	2,552.3	2,551.3	24
5	579.2	290.1	562.2	561.2	R	2,472.3	1,236.7	2,455.3	2,454.3	23
6	707.3	354.2	690.3	689.3	K	2,316.2	1,158.6	2,299.2	2,298.2	22
7	822.4	411.7	805.3	804.3	D	2,188.1	1,094.6	2,171.1	2,170.1	21
8	909.4	455.2	892.4	891.4	S	2,073.1	1,037.0	2,056.1	2,055.1	20
9	1,023.4	512.2	1,006.4	1,005.4	N	1,986.0	993.5	1,969.0	1,968.0	19
10	1,136.5	568.8	1,119.5	1,118.5	L	1,872.0	936.5	1,855.0	1,854.0	18
11	1,249.6	625.3	1,232.6	1,231.6	L	1,758.9	880.0	1,741.9	1,740.9	17
12	1,320.6	660.8	1,303.6	1,302.6	A	1,645.8	823.4	1,628.8	1,627.8	16
13	1,391.7	696.3	1,374.6	1,373.7	A	1,574.8	787.9	1,557.8	1,556.8	15
14	1,488.7	744.9	1,471.7	1,470.7	P	1,503.8	752.4	1,486.7	1,485.8	14
15	1,616.8	808.9	1,599.8	1,598.8	Q	1,406.7	703.9	1,389.7	1,388.7	13
16	1,673.8	837.4	1,656.8	1,655.8	G	1,278.7	639.8	1,261.6	1,260.6	12
17	1,760.8	880.9	1,743.8	1,742.8	S	1,221.6	611.3	1,204.6	1,203.6	11
18	1,923.9	962.5	1,906.9	1,905.9	Y	1,134.6	567.8	1,117.6		10
19	2,020.9	1,011.0	2,003.9	2,002.9	P	971.5	486.3	954.5		9
20	2,134.0	1,067.5	2,117.0	2,116.0	L	874.5	437.7	857.5		8
21	2,247.1	1,124.1	2,230.1	2,229.1	L	761.4	381.2	744.4		7
22	2,318.2	1,159.6	2,301.1	2,300.1	A	648.3	324.7	631.3		6
23	2,432.2	1,216.6	2,415.2	2,414.2	N	577.3	289.1	560.2		5
24	2,489.2	1,245.1	2,472.2	2,471.2	G	463.2	232.1	446.2		4
25	2,588.3	1,294.6	2,571.3	2,570.3	V	406.2	203.6	389.2		3
26	2,748.3	1,374.7	2,731.3	2,730.3	C	307.1	154.1	290.1		2
27	2,894.4	1,447.7	2,877.4	2,876.4	K	147.1	74.1	130.1		1

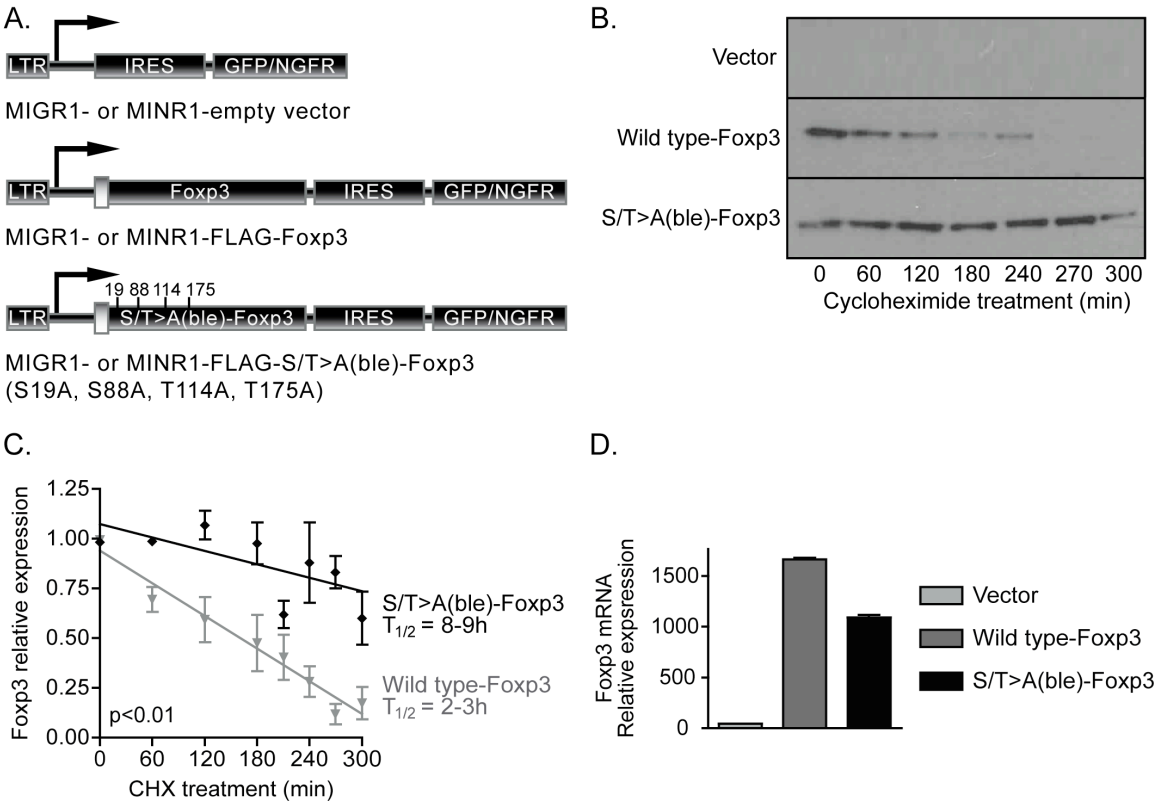
Foxp3 is phosphorylated by CDK2 in vitro. *A*, Recombinant 6xHis-Foxp3 was purified as described under “Materials and Methods”. *B,* A standard in vitro kinase assay was performed using 6xHis-Foxp3 (500ng) or control substrate Histone H1 (1µg), and recombinant CDK2-cycE. Roscovitine was used to inhibit CDK2 activity. The autoradiograms shown are representative of three separate experiments. *C,D*, Purified 6xHis-Foxp3 was digested with trypsin and the resulting peptides were analyzed by RP-HPLC mass spectrometry. Tandem MS spectra for Foxp3 phospho-peptides shown. b- and y-series ions are shown in red and blue, respectively, and ions involving neutral loss of the elements of phosphoric acid, water, or ammonia are shown in green. Phosphorylation site assignments are made for serine 19 (*C*) and threonine 175 (*D*). *E*,*F* Complete ion fragmentation for Foxp3 phospho-peptides shown. Phosphorylation site assignments are made for serine 19 (*E*) and threonine 175 (*F*) The data shown are from two separate mass spectrometry runs with parent error <5ppm.

FIGURE 3



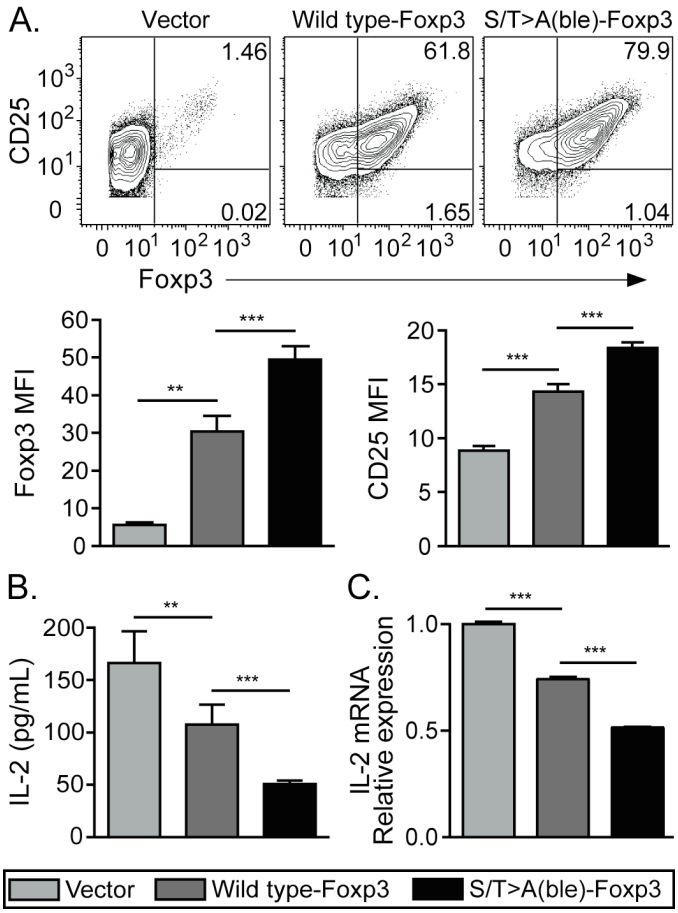
Foxp3 is phosphorylated at CDK motifs in vivo. *A*, Foxp3 peptide (aa12-24) containing either unmodified or phosphorylated serine 19 was serially diluted and used for a dot blot assay. Membranes containing diluted peptides were probed with p+Ser19-Foxp3 antibody. *B*, HEK293 cells were transfected with vector control, wild type Foxp3, or S/T>A-Foxp3 mutant. Whole cell extracts were prepared at 48h post-transfection. FLAG immunoprecipitation and western blotting was performed as described under “Materials and Methods”. Phosphorylated and total Foxp3 is shown. Data are representative of three separate experiments.

FIGURE 4



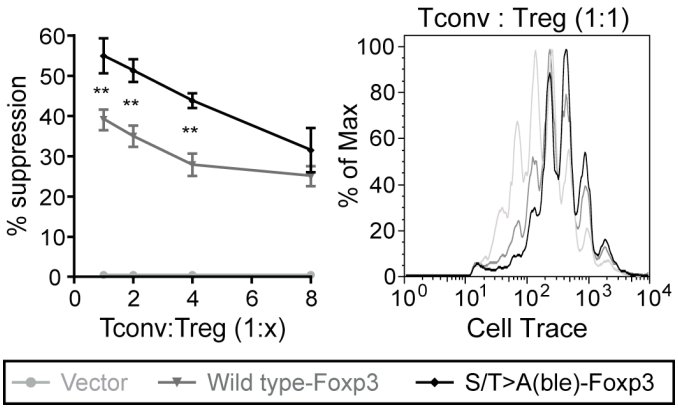
Foxp3 lacking CDK consensus elements has increased protein stability. *A*, CD4+25- T lymphocytes were transduced with vector control, wild type Foxp3, or the S/T>A-Foxp3 mutant. *B*, Transduced cells were rested overnight and restimulated for 4 hours with plate-bound CD3 and CD28 antibodies, incubated with 25μg/mL cycloheximide (CHX), and harvested at indicated times. Whole cell extracts from treated cells were analyzed by SDS-PAGE blotted to membranes, and probed with Foxp3 antibody. One representative image of three experiments is shown. *C*, Average stability of wild type and S/T>A-Foxp3 over three experiments is shown. Half-life was calculated based on these values. *Error bars* are S.E.M. for biological replicate cultures. *D*, *Foxp3* transcription normalized to actin is shown. Message level is represented in arbitrary units, before addition of CHX to transduced cells.

FIGURE 5



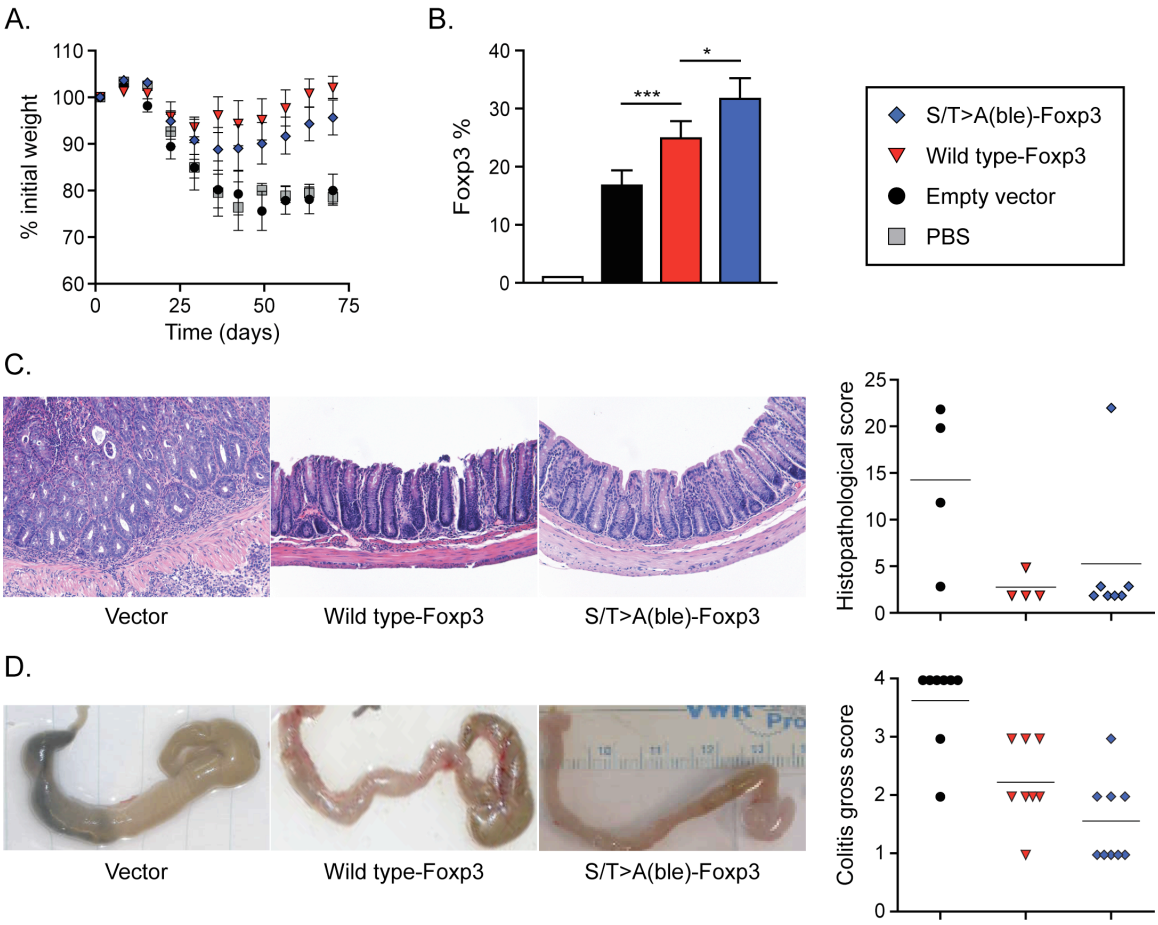
Mutation of the N-terminal CDK motifs results in enhanced Foxp3 expression and transcriptional function. *A*. CD4+25- T lymphocytes were transduced with wild type or S/T>A (ble) Foxp3, restimulated as in Figure 4, and Foxp3 and CD25 expression was assessed by flow cytometry. The mean-fluorescence intensity (MFI) of each parameter was calculated and graphed. *B*, Supernatants from restimulated cells in '*A*' were assessed for *Il2* production by ELISA. *C*, *Il2* transcription normalized to actin is shown. Message level is represented in arbitrary units. The data shown are representative of at least two experiments. *Error bars* are S.E.M. for biological replicate cultures. ***p<0.01, ***p<0.001.*

FIGURE 6



S/T>A(ble)-Foxp3 exhibits enhanced suppressive function in vitro. CD4+25- T lymphocytes transduced with wild type or S/T>A(ble) Foxp3 were used as suppressors in a standard in vitro Treg suppression assay against conventional CD4+25- T cells labeled with CellTrace to track cell division. Suppressors and effectors were cultured with irradiated wild type APCs and soluble CD3 for 72 hours. Proliferation data shown are gated on CD4+ lymphocytes. Percent suppression was calculated as previously described [1]. Data are representative of two separate experiments. *Error bars* are S.E.M. for biological replicate cultures. ***p<0.01, ***p<0.001.*

FIGURE 7



Anti-inflammatory activity of S/T>A(ble) mutant Foxp3 in vivo. *Rag1*-/- mice were injected i.v. with 1 x 10^6 conventional CD4+25- T lymphocytes to induce colitis. After 21 days, mice were administered a therapeutic injection i.p. with 2 x 10^6 transduced cells (vector, wild type-Foxp3, or S/T>A(ble)-Foxp3) or a PBS control. *A*, Mice were weighed every 2-3 days until day 70 post initial injection. *B*, Mice were sacrificed and mesenteric lymph node cells were harvested and stained for CD4 and Foxp3. Shown is percentage Foxp3+ gated on CD4+ lymphocytes. *C*, Large intestines were harvested, prepared for histological analysis, and histopathological scoring was performed blinded. *D*, Gross pathological analysis of sacrificed mice was performed and scored as described under “Materials and Methods”. Each group contained 3-7 mice.

CHAPTER 3 - $p27^{kip1}$ is required for extra thymic Treg cell generation

Summary

Regulatory T cells (Treg) are required to maintain immune homeostasis and tolerance. The development and function of these cells depend on the expression of the forkhead box transcription factor, Foxp3. Thymic derived, 'natural' Treg have highly stable genetic expression of Foxp3. Extra-thymic, 'induced' Treg, however, contain a much less stable Foxp3 gene. While the genetic stability of Foxp3 in each type of Treg has been studied extensively, an understanding of the protein stability of Foxp3 is still lacking. Previously, we showed that Foxp3 is phosphorylated by the cyclin-dependent kinase 2 (CDK2), which leads to a decrease in protein half-life. Our current data show that the CDK2 inhibitor $p27^{kip1}$ is induced in extra-thymic Treg by the same signal that turns on Foxp3 expression. In the absence of $p27^{kip1}$ the conversion of CD4+ T cells into Foxp3-expressing suppressive cells is deficient, and can be partially reversed by the pharmacologic repression of CDK2. Additionally, we find that natural, thymic-derived Treg, downregulate CDK2 expression, and their suppressive function does not depend on the expression of $p27^{kip1}$. These results indicate that induction of $p27^{kip1}$ is required for normal expression of the Foxp3 protein during extra-thymic Treg development, and that thymic-derived Treg may have developed a mechanism to oppose CDK activity.

Introduction

Foxp3 is a forkhead box transcription factor necessary for the development of immunosuppressive regulatory T cells (Treg) (59, 61, 62). Normal Treg development and function depends on Foxp3, while loss or mutation of the gene results in severe autoimmunity in mice and humans (61, 98). Suppressive T cells were first identified as having thymic origin (99) and are referred to as natural Treg (nTreg). Expression of the Foxp3 gene in nTreg is highly stable as a result of demethylation of the intergenic region, CNS2, which occurs during thymic development (100-103). Foxp3 can also be induced peripherally in CD4+25- conventional T cells (Tconv) in response to costimulation with TCR signaling and transforming growth factor β (TGFβ) (89). Peripherally induced Treg (iTreg), have inherently less stable Foxp3, which does not undergo the same degree of demethylation at CNS2 found in nTreg (104). Reports have now emerged citing novel regulatory elements controlling peripheral Treg stability at sites of antigen exposure (105) or in tumors (106). These data show different protein networks can coordinate to affect stability of the peripheral Treg population, but do not specifically address the issue of Foxp3 protein stability within the cell.

We recently demonstrated that cyclin-dependent kinase 2 (CDK2) deficient Treg have a gain in suppressive function, and mice lacking CDK2 are better at accepting MHC-mismatched heart allografts (31). Importantly, CDK2-/- T cells exhibit normal cell cycle progression (31), and CDK1 is now known to be the only CDK both necessary and sufficient for normal cell cycle progression in mammals (36). Additionally, we found that Foxp3 protein stability is negatively regulated by cyclin-dependent kinase 2 (CDK2); Foxp3 lacking CDK putative motifs has an increased half-life and a resulting gain in function (107). These data suggest that CDK2 restricts Foxp3 protein stability and Treg function. What remains unclear is under what circumstance the CDK2 pathway is active in a Treg, and how might it be regulated.

One known mechanism of CDK2 regulation is through inhibition by $p27^{kip1}$ (4). We previously published that $p27^{kip1}$ is required for the acquisition of tolerance to mismatched heart allografts (49), although we did not look extensively at Treg in this model. $p27^{kip1}$ can be induced in B lymphocytes in a TGFβ-dependent manner (91), but it is unclear if this is true in T lymphocytes. Importantly, TGFβ is the same signal that drives Foxp3 expression in this iTreg (89).

Our data indicate that the CDK2 inhibitor $p27^{kip1}$ is induced in developing iTreg in response to TGFβ. Additionally, we find that $p27^{kip1}$-/- T cells have a defect in iTreg conversion as compared to wild type iTreg. However, when analyzing nTreg from these animals in a colitis model, we find no suppressive defect. Together, our data suggest $p27^{kip1}$ plays an important role in iTreg, but not nTreg. These findings represent an important step in appreciating the complex nature of Treg biology.

Materials and Methods

Mice - Female C57BL/6 (H-2^b) and *Rag1*-deficient mice on a C57BL/6 background were purchased from The Jackson laboratory and maintained in our specific pathogen free facility. $p27^{kip1}$-/- (H-2^b) mice were bred in-house by crossing $p27^{kip1}$+/- females with $p27^{kip1}$-/- males (108). Absence of both copies of the $p27^{kip1}$ gene (*cdknlb*) in progeny was confirmed by PCR. Animals were housed according to ULAR- and AALAC-approved institutional guidelines on animal care and usage. All mice were used at 6-14 weeks of age.

Generation of induced regulatory T cells - CD4+25- conventional T cells were isolated from splenocytes of wild type and $p27^{kip1}$-/- animals by magnetic bead-conjugated mAbs (Miltenyi). Purified Tconv cells were incubated for 2-3 days on 5μg/mL plate-bound CD3 (2C11, BioXCell) and CD28 (37.51, BioXCell) along with indicated amounts of TGFβ (Peprotech), 50U/mL IL-2 (Roche), and 5μg/mL blocking Abs against IL-4 and IFNγ (BioXCell). Cells receiving no TGFβ are considered undifferentiated (Th0).

Western blot analysis and FACS analysis of Treg - Before analysis, iTreg cells were restimulated 4 hours with 3ng/mL phorbol 12-myristate 13-acetate (Sigma Aldrich) and 1uM Ionomycin (Sigma Aldrich). FACS analysis was performed on a Beckman Coulter Cyan ADP. Cells were stained for FACS analysis with conjugated mouse anti-Foxp3-PE (FJK-16s, eBiosciences), mouse anti-IL-2-Pacific Blue (JES6-5H4, BioLegend), and anti-CD4-APC (GK1.5, BioLegend). Cell fixation was performed according to manufacturer protocols. Where indicated, 0.2μM roscovitine (Sigma Aldrich) was used to inhibit the activity of CDK2. Whole cell extracts were generated using the radioimmune precipitation buffer lysis kit (Sigma Aldrich) and run on precast 10% SDS-PAGE Criterion gels (BioRad). Western blots were performed using rabbit anti-p+Ser19-Foxp3 (Yenzym), mouse/rat anti-Foxp3 (FJK-16s, eBiosciences), rabbit anti-CDK2 (78B2-Cell Signaling), goat anti-actin (C-11, Santa Cruz), and $p27^{kip1}$ (2552, Cell Signaling).

Detection of phosphorylated Foxp3 - CD4+25- Tconv were converted into Foxp3+ iTreg. After 72h cells were harvested and lysed. Lysis buffer was supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich) and β-glycerophosphate (Sigma Aldrich). Affinity purified p+Ser19 Foxp3 rabbit polyclonal antibody (Yenzym) was used to detect phosphorylated species of Foxp3.

Adoptive Transfer Colitis - To induce experimental colitis, conventional CD4+25- T cells were purified from naive, wild type C57BL/6 mice and adoptively transferred (1 x 10^6, i.v.) into *Rag1*-/- B6 recipients (72, 73), Groups of four or five mice received either simultaneous injection of wild type or $p27^{kip1}$-/- CD4+25+Foxp3+ Treg (2 x 10^6, i.v.), or were left untreated. Recipients were weighed and observed for symptoms of diarrhea approximately every two days. At the end of the experiment, spleens, mesenteric lymph nodes, and intestines were harvested for examination of gross pathology.

Gross pathology - Colitis-induced animals were sacrificed and analyzed for signs of gross pathology using a modified version of established methods (74). Scores of 0 (no colitis) to 4 (worst disease) were assigned according to colon rigidity, visible inflammation, and presence of blood in intestines, as well as diarrhea, and presence of fat tissue.

Statistical analysis - All p values were calculated by Student's paired t test using Prism software (GraphPad).

Results

Induced regulatory T cells contain phosphorylated Foxp3

We previously showed that CDK2 phosphorylates Foxp3 at serine 19 and threonine 175 using an in vitro kinase assay and mass spectrometry (107). In that study, we also generated an antibody reagent against the phosphorylated serine-19 of Foxp3 (p+Ser19-Foxp3), and confirmed that this modification occurs in a cell transfected with wild type Foxp3, but not a mutant Foxp3 lacking the CDK motifs. What remained uncertain was whether this modification occurred in Foxp3+ T cells. To test this we probed whole cell extracts from undifferentiated CD4+25- Th0 cells or extra-thymic induced regulatory T cells (iTreg) for the presence of p+Ser19-Foxp3. We found that iTreg, contain a pool of p+Ser19-Foxp3 (Fig. 8a). Wild type iTreg express CDK2 at levels appreciable to Tconv cells (Fig. 8b). These data suggest Foxp3 is phosphorylated at a CDK motifs and that CDK2 is present in an iTreg.

Induced regulatory T cells upregulate $p27^{kip1}$ to regulate Foxp3 induction

One potential mechanism of suppressing CDK2 activity is through induction of its inhibitor, $p27^{kip1}$. Typically, $p27^{kip1}$ is elevated in quiescent cells, inhibiting cell cycle progression, then rapidly degraded in response to mitogenic stimuli to allow cycling (4). We analyzed whole cell extracts from Tconv, and iTreg cells for the expression of $p27^{kip1}$. Activated Tconv express low amounts of $p27^{kip1}$, while iTreg induce large amounts of the cell cycle inhibitor (Fig. 9). Our iTreg data are consistent with reports showing TGFβ-dependent induction of $p27^{kip1}$ in B cells (91).

TGFβ signaling is sufficient to induce Foxp3 expression in CD4+25- conventional T cells (89). Our data in Figure 9 demonstrate that TGFβ also simultaneously elevates the expression of the CDK2 inhibitor, $p27^{kip1}$ in Tconv. We also know that Foxp3 is phosphorylated at a CDK motif (Fig. 8), and that this modification destabilizes the protein (107). We therefore asked whether expression of $p27^{kip1}$ in developing iTreg is required for normal Foxp3 protein expression. We performed a standard in vitro iTreg conversion assay, incubating both wild type and $p27^{kip1}$-/- CD4+25- Tconv cells with varying amounts of TGFβ. Consistent with the literature, wild type Tconv cells produce high levels of Foxp3 in response to this milieu of signals (Fig. 10a). However, $p27^{kip1}$-deficient Tconv cells upregulated significantly less Foxp3 compared to their wild type

counterparts. This deficiency is evident both in the total number of Foxp3 expressing cells (Fig. 10b) as well as the intensity of Foxp3 protein expression on a per cell basis (Fig. 10c). Additionally, $p27^{kip1}$-/- iTreg exhibit suboptimal repression of IL-2 compared to their wild type counterparts (Fig. 10e,f). These data suggest that normal Foxp3 protein expression and repressive activity in iTreg depends on the induction of $p27^{kip1}$.

$p27^{kip1}$ reinforces Foxp3 protein stability in iTreg

Genetic deletion of $p27^{kip1}$ results in decreased Foxp3 expression in developing iTreg (Fig. 10). Additionally, we know CDK2 phosphorylation decreases Foxp3 protein stability (107). Therefore, we asked whether increased CDK2 activity in $p27^{kip1}$-/- iTreg could account for the changes in Foxp3 protein expression. To do this, we performed the standard in vitro iTreg conversion assay using wild-type and $p27^{kip1}$-/- Tconv cells, in the presence or absence of the CDK2 small molecule inhibitor, roscovitine. At nanomolar doses, roscovitine treatment of wild type iTreg cultures led to a small increase in Foxp3 expression as compared to untreated cells (Fig. 11). These data suggest CDK2 is active in an iTreg. This idea is consistently represented by our data showing CDK2 activity in wild type iTreg as determined by detectable levels of p+Ser19-Foxp3 (Fig. 8). $p27^{kip1}$-/- iTreg cultures treated with roscovitine also showed a small gain in the amount of Foxp3 expression compared to untreated cells, and the gain was larger than that seen in wild type treated cells (Fig. 11). These data suggest that there is more CDK2 activity in $p27^{kip1}$-/- than wild type iTreg, and support the conclusion that $p27^{kip1}$ assists developing iTreg to prevent CDK2 activity and stabilize their Foxp3 protein expression.

Natural regulatory T cells do not require $p27^{kip1}$ for their function

Our data show $p27^{kip1}$ is required to for the expression of Foxp3 during iTreg development. We wanted to know whether nTreg also had any reliance on $p27^{kip1}$. To test this we harvested CD4+25+Foxp3+ nTreg from naive wild type and $p27^{kip1}$-/- mice. We observed no differences in the total number of nTreg between wild type and $p27^{kip1}$-/- animals (data not shown). We then used the nTreg as suppressor cells in an adoptive transfer model of colitis. We found both wild type and $p27^{kip1}$-/- animals are equally capable of suppressing the proliferation of

inflammatory CD4+ conventional T cells in this model. This is clear both from the similarity in their weight curve (Fig. 12a) and gross colitis score (Fig. 12b). We also analyzed nTreg for the expression of CDK2 by Western blot and found that relative to Tconv, wild type nTreg downregulate CDK2 expression (Fig. 13). This finding is consistent with a previous report that nTreg restrict the expression of CDK1, CDK2, and CDK6 (88). Together, these data suggest that nTreg do not require $p27^{kip1}$ for their function, but instead have adapted a mechanism to restrict the activity of the CDK family.

Discussion

Foxp3+ regulatory T cells are required to maintain immune homeostasis and tolerance (60, 98, 109). These suppressive cells can be generated from developing thymoctyes, nTreg (99), or from mature peripheral CD4+25- conventional T cells in response to costimulation with TCR and TGFβ signals, iTreg (89). The expression of Foxp3 in nTreg is inherently stable as a result of demethylation of the Foxp3 intergenic region, CNS2 (100-103). Contrarily, the Foxp3 gene in iTreg does not undergo extensive stabilizing demethylation at CNS2 (104), although treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine can force a more stable phenotype (110). Normally, however, iTreg are considered a particularly 'plastic' T cell lineage, as they can be differentiated into various other T cell subsets, such as Th17 cells, given the right cytokine milieu (111). It has been suggested that depending on whether the iTreg were generated in an inflammatory environment or not can also affect their stability (112). The studies performed have focused on the state of the Foxp3 gene. What remains largely unaddressed is how the stability of the Foxp3 protein, and the networks that regulate it contribute to the function and stability of the Treg lineage.

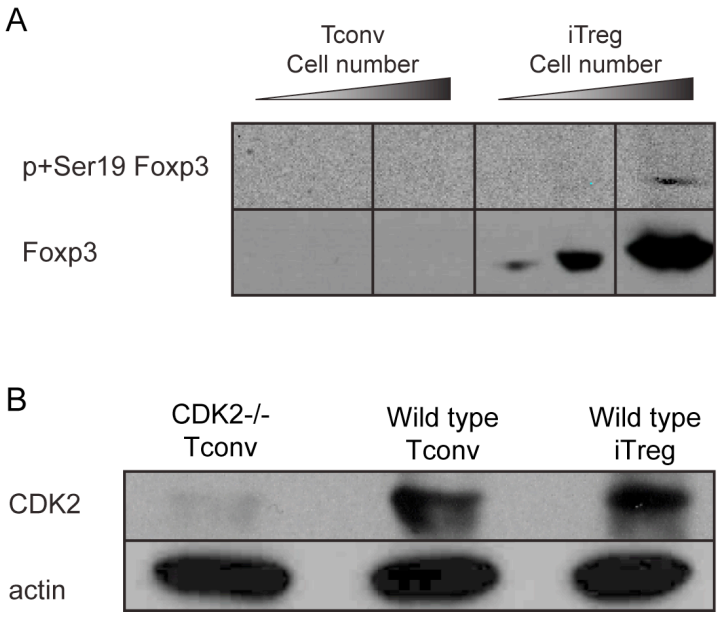
We performed a cursory examination of nTreg from $p27^{kip1}$-/- animals. Our data suggests that nTreg do not depend on $p27^{kip1}$ for their suppressive function. Interestingly, nTreg do downregulate the expression of CDK2 relative to Tconv cells. A recent study also shows thymic-derived nTreg repress the expression of CDK1, CDK2, and CDK6 (88). While CNS2 demethylation of Foxp3 stabilizes the gene in nTreg, tempering the expression of the CDK family represents a possible mechanism by which nTreg can ensure stable Foxp3 protein expression and maintenance of tolerance without reliance on $p27^{kip1}$.

On the other hand, iTreg certainly rely on $p27^{kip1}$ for their development and function. The data we present in this study indicate that induced regulatory T cells express a population of Foxp3 that is phosphorylated by CDK2. We previously showed that CDK2 negatively regulates Foxp3 protein stability in a phosphorylation-dependent manner, resulting in decreased Foxp3 function (107). This suggests that CDK2 activity in an iTreg regulates Foxp3 stability. How does an iTreg modulate this activity of CDK2? One possibility is through utilization of the CDK2 inhibitor $p27^{kip1}$ which actively regulates the activity of CDK2 protein in the cell. Previous studies

show that $p27^{kip1}$ transcription is responsive to TGFβ signaling in B lymphocytes (91). This is intriguing as TGFβ is also the primary signal required for extra-thymic induction of Foxp3 (89). We confirmed that conventional T cells also induce $p27^{kip1}$ in response to TGFβ, and that mice genetically deficient for $p27^{kip1}$ have a significant defect in iTreg conversion. Based on these data we suggest $p27^{kip1}$ is induced in iTreg as a switch to modulate CDK2 activity, contributing to stable Foxp3 expression and suppressive capacity.

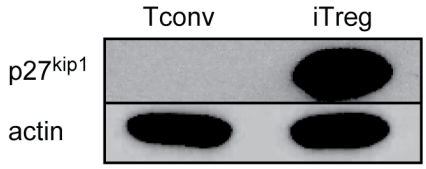
Furthermore, in an attempt to balance immunity and tolerance a cell could modulate the relative expression of $p27^{kip1}$ to allow for either a more stable iTreg, or favor destabilization and conversion into another CD4+ T cell subset. The iTreg-Th17 division is a well documented example of the need for plasticity during an immune response (24), and the pivotal role played by TGFβ in the development of both of these lineages is apparent (113). Interestingly, another cytokine that drives Th17 differentiation, IL-6, is known to induce $p27^{kip1}$ expression in neurons (114). We have preliminary data indicating that $p27^{kip1}$ is required for normal development of the Th17 lineage (Morawski, Wells unpublished observations), but it remains unclear if IL-6 affects $p27^{kip1}$ in T lymphocytes. The CDK2-$p27^{kip1}$ axis may therefore be important for multiple aspects of T cell biology.

Figure 8



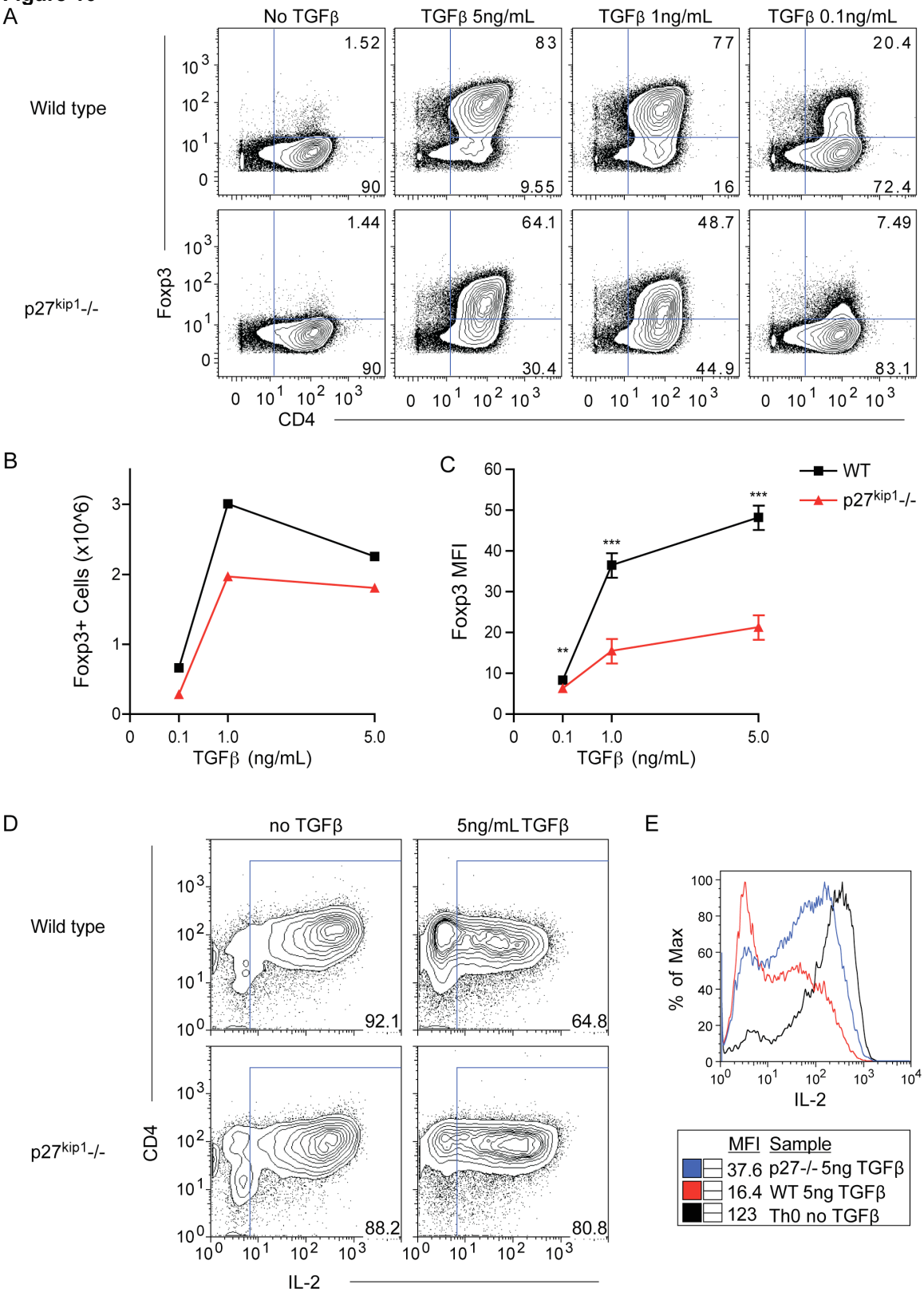
Foxp3 is phosphorylated in vivo. *A*, CD4+ conventional T cells (Tconv) were incubated on plate bound CD3 and CD28 with TGFβ, IL-2, and blocking antibodies against IFNγ, IL-4, and IL-12, to generate induced regulatory T cells (iTreg) as described under "Materials and Methods". After 72h, iTreg were harvested and whole cell extracts prepared. Western blot analysis was performed as described under “Materials and Methods”. Phosphorylated and total Foxp3 is shown. *B*, Tconv and iTreg cells were prepared as in ‘*A*’ and analyzed for CDK2 and β-actin. Tconv cells from CDK2-/- animals are used as a control. Data are representative of two separate experiments.

Figure 9



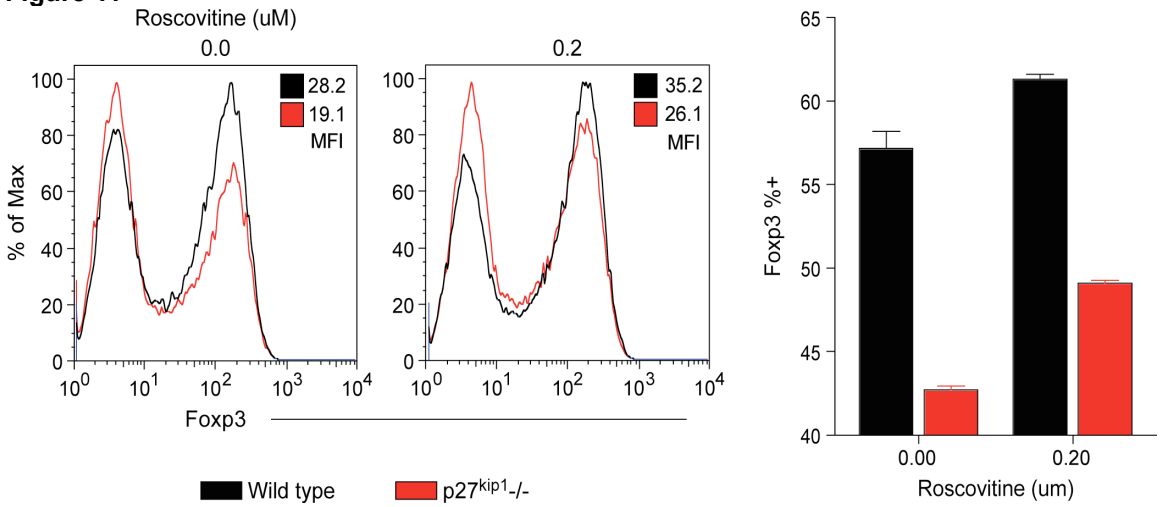
TGFβ signaling induces $p27^{kip1}$ in T cells. Tconv were incubated on plate bound CD3 and CD28 with cytokines and blocking antibodies to generate iTreg or undifferentiated Th0 cells as described under "Materials and Methods". Tconv and iTreg were harvested after 72h and whole cell extracts prepared. Western blot analysis was performed as described under “Materials and Methods”. Membranes were probed for differential expression of the CDK inhibitor, $p27^{kip1}$. Expression of β-actin serves as a loading control. Data are representative of two separate experiments.

Figure 10


A
No TGFβ
TGFβ 5ng/mL
TGFβ 1ng/mL
TGFβ 0.1ng/mL
Wild type
p27kip1-/-
Foxp3
CD4
1.52
90
83
9.55
77
16
20.4
72.4
1.44
90
64.1
30.4
48.7
44.9
7.49
83.1
B
Foxp3+ Cells (x10^6)
TGFβ (ng/mL)
C
Foxp3 MFI
TGFβ (ng/mL)
WT
p27kip1-/-
D
no TGFβ
5ng/mL TGFβ
Wild type
p27kip1-/-
CD4
IL-2
92.1
64.8
88.2
80.8
E
% of Max
IL-2
MFI Sample
37.6 p27-/- 5ng TGFβ
16.4 WT 5ng TGFβ
123 Th0 no TGFβ

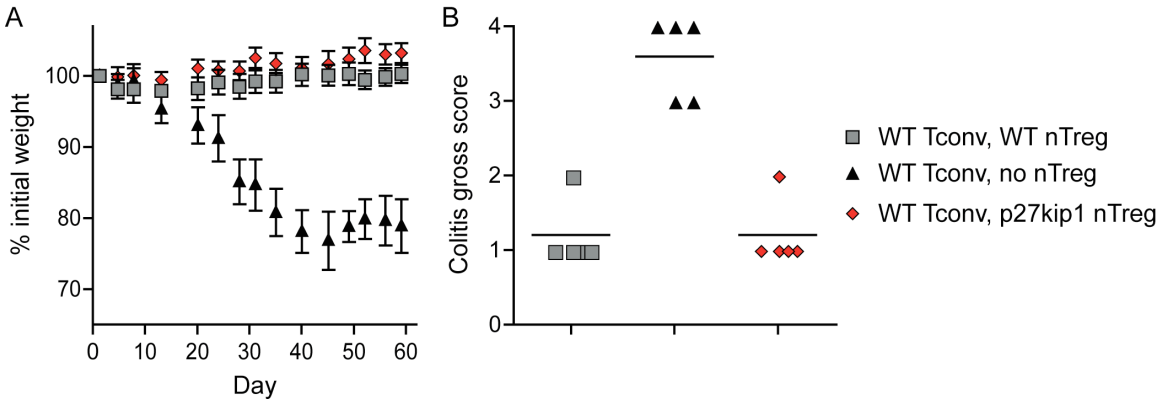
$p27^{kip1}$ is required for normal TGFβ-dependent Foxp3 induction. *A*, Wild type and $p27^{kip1}$-deficient Tconv were incubated on plate bound CD3 and CD28 with cytokines and blocking antibodies to generate iTreg or undifferentiated Th0 as described under "Materials and Methods". Stimulated cells were harvested after 72h and prepared for flow cytometry as described under "Materials and Methods". Expression of Foxp3 and CD4 are shown. *B*, Total number of Foxp3 expressing cells was enumerated and graphed. *C*, Foxp3 mean-fluorescence intensity (MFI) was calculated and graphed. *D*, Expression of IL-2 and CD4 are shown. *E*, IL-2 MFI was calculated and graphed. The data shown are representative of at least two experiments. Error bars are S.E.M. for biological replicate cultures. **p<0.01, ***p<0.001.

Figure 11



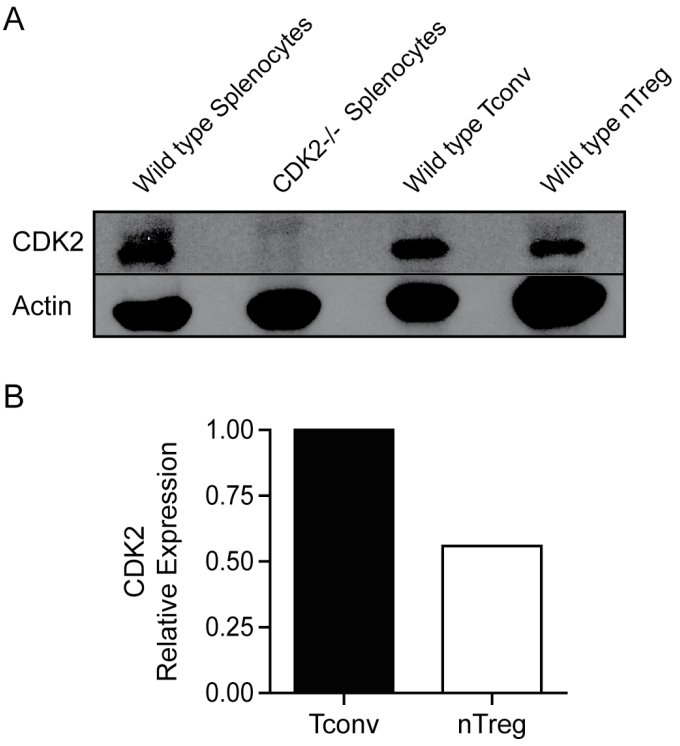
Inhibiting CDK2 increases Foxp3 expression in developing iTreg. Wild type and $p27^{kip1}$-deficient Tconv were incubated on plate bound CD3 and CD28 with cytokines and blocking antibodies to generate iTreg as described under "Materials and Methods". During conversion, cells were incubated in the presence or absence of roscovitine. Stimulated cells were harvested after 48h and prepared for flow cytometry as described under "Materials and Methods". Foxp3 intensity of staining and percent expression is shown. Error bars indicate S.E.M. for three experimental replicates. Data are representative of one experiment.

Figure 12



Suppression by natural regulatory T cells does not require $p27^{kip1}$. Rag1-/- mice were injected i.v. with 1 x 10^6 conventional CD4+25- T lymphocytes to induce colitis. Simultaneously, groups of mice were injected i.v. with either 1 x 10^6 wild type or $p27^{kip1}$-/- CD4+25+ nTreg to prevent disease, or were left untreated. *A*, Mice were weighed every 2-3 days until day 60 post injection. *B*, Gross pathological analysis of sacrificed mice was performed and scored as described under “Materials and Methods”. Each group contained 4-5 mice.

Figure 13



Natural regulatory T cells downregulate CDK2. Wild type and CDK2-/- splenocytes, as well as wild type Tconv and nTreg were purified from mice, stimulated overnight with PMA, Ionomycin, and IL-2, harvested and used to prepare whole cell extracts. *A*, Western blot analysis was performed as described under “Materials and Methods”. Membranes were probed for differential expression of CDK2. Expression of β-actin serves as a loading control. *B*, Relative expression of CDK2 between wild type Tconv and nTreg cells was adjusted for differences in loading and graphed. Data are representative of one experiment.

CHAPTER 4 - Discussion & Future Directions

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The breadth of the CDK2-p27^{kip1} axis in T cells

Cyclin-dependent kinases: A brief review of how we got here

Induction of cyclin-dependent kinase 2 (CDK2) in T cells is dependent on both CD28 and IL-2 signals (4, 115-117), and was originally thought to be required for normal G1 to S phase progression of the cell cycle. Recent genetic studies have demonstrated that only CDK1 is required for normal cell cycle progression in most tissues (35, 36). Specifically, animals lacking CDK2 have a major defect in meioses, causing sterility (32), but have normal cell cycle progression in nearly all other tissues, including T lymphocytes (31), because of the compensatory function of CDK1.

Our initial studies on cell cycle proteins found that competent CD4+ effector T cells could be distinguished from anergic ones based on the number of cell divisions undergone by each following costimulation with TCR engagement (38, 46). Simultaneously, reports emerged showing that cyclin/kinase-inhibitory proteins (cip/kip), such as $p27^{kip1}$ and $p21^{cip1}$, were induced during, and could participate in anergy induction (40, 45, 48). We showed that $p27^{kip1}$ is required for anergy in vitro (47), and for the formation of tolerance to fully MHC mismatched heart allografts (49). These studies also showed that $p27^{kip1}$ has only a mild effect on the clonal expansion of a T cell, but is required to regulate the production of effector cytokines by allospecific effector T cells. We also have new data demonstrating that $p27^{kip1}$-/- Tconv are more difficult to suppress than wild type control Tconv (Fig. 14). We must test whether this is the result of dysregulated clonal expansion or effector cytokine production in the absence of $p27^{kip1}$.

These data suggested that effector differentiation and tolerance induction, but not cell division were the primary processes regulated by $p27^{kip1}$. Based on this idea, we predicted that CDK2 would have the opposite function. Indeed, we found that CDK2 deficient mice are susceptible to transplant tolerance (31). Furthermore, while CDK2-/- T lymphocytes had normal cell cycle progression, their regulatory T cells (Treg) had increased suppressive function in vivo compared to wild type counterparts. These data showed CDK2 negatively regulated transplant tolerance and Treg function, although the mechanism remained unclear. Because Treg development and function are driven primarily by the transcription factor Foxp3, and because CDK2 is by definition a kinase, we hypothesized that CDK2 might phosphorylate Foxp3 as a way to exert influence on the entire Treg lineage.

The data in Chapter 2 demonstrate the validity of our hypothesis. Foxp3 contains putative CDK motifs, and is phosphorylated on at least two of these sites (107). The phosphorylation decreases Foxp3 protein stability and function. In Chapter 3, we show the in vivo relevance of our biochemical finding. The CDK2 inhibitor $p27^{kip1}$ is required for stable expression of Foxp3 in developing extra-thymic induced Treg (iTreg), and the activity of CDK2 may play a role in regulating this process. Simultaneously, we find that CDK2 is downregulated in thymic-derived natural Treg (nTreg), and that $p27^{kip1}$ is not required for normal nTreg suppressive function. Together, the data presented in this thesis support the original hypothesis that CDK2 has a critical cell cycle independent role that controls tolerance and Treg function. However, much work remains unfinished as we attempt to understand more aspects of the biology. Below I will outline several models that encapsulate our data, make predictions based on these models, and attempt to assimilate all these ideas into the larger scope of T cell immunology.

The biochemistry of Foxp3 stability

There is little understood about the post-translational modification of Foxp3. Our data presents the first example of a specific kinase cascade acting on this transcription factor (107). The conclusions from that study stated CDK2 phosphorylation of Foxp3 led to destabilization of the protein (Fig. 15a), and subsequent decreases in induction (e.g. *CD25*) or repression (e.g. *il2*) of canonical Foxp3 genes (Fig. 15b), as well as loss of suppressive function (Fig. 15c). Of these conclusions, the most important one, regarding stability of the Foxp3 protein, is also the one around which the most questions still remain.

Crosstalk between post-translational modifications represents an important step in the regulation of the function and fate of a protein. Acetylation of a substrate competes with ubiquitination at the same lysine residues, while phosphorylation at one site can enhance the likelihood of a nearby ubiquitination (78). We hypothesize that CDK2 phosphorylation of Foxp3 leads to a ubiquitin-mediated destabilization, but we have not yet demonstrated that Foxp3 contains a CDK2-generated phospho-degron. We do know that CDK2 activity is a prerequisite for the SCF/Fwb7-mediated ubiquitination and degradation of Cyclin E (92) and Skp2-mediated ubiquitination and degradation of $p27^{kip1}$ (118), which we believe could be how CDK2 targets

Foxp3 for degradation. Foxp3 is subject to proteasomal degradation following ubiquitin modification (119). The specific E3 ligase has not been identified, but the deubiquitin enzyme (DUB) that reverses this process is USP7. This ubiquitin-mediated degradation is thought to be dependent on p300 and SIRT1 regulated acetylation (68, 69). Moving forward we must ask whether CDK2 phosphorylation of Foxp3 is an added part of this degradation program, and how this additional modification could lead to degradation. Phosphorylation could cause a conformational shift (120) or change in local electrostatic charges (121) that favors binding with an E3 ligase over an acetyl transferase. Alternatively, phosphorylation could affect protein localization. Could p+Foxp3 be subject to nuclear export and directed towards a cytoplasmic proteasome like $p27^{kip1}$ (93, 94)? Perhaps CDK2 drives the sub-nuclear trafficking of Foxp3 towards nuclear degradation machinery (122)? These are all possibilities that should be accounted for, which could suggest additional pharmacologic routes by which Foxp3, and thereby Treg stability, can be modulated in vivo.

When is CDK2 active in a T lymphocyte?

The biggest question emerging from our study on CDK2-dependent regulation of Foxp3 stability is regarding under what circumstances CDK2 engages Foxp3. From our previous work we know CDK2 is highly active in CD4+ T lymphocytes that infiltrate and reject cardiac allografts. CDK2 reinforces the production of IFNγ in infiltrating T cells, and negatively affecting suppressive function (31). In neither of these examples, however, were we looking for the presence of phosphorylated Foxp3. Our recent efforts, laid out in Chapter 3, aim to understand where and when CDK2 acts upon Foxp3. A strong hint came from a published study showing $p27^{kip1}$ could be induced in B lymphocytes by TGFβ signaling (91). We showed that TGFβ, which is the primary signal required for the extra-thymic induction of Foxp3 in conventional T cells (Tconv) (89), simultaneously induces $p27^{kip1}$ in Tconv, and is required for normal development of iTreg. Furthermore, iTreg contain a population of CDK2-phosphorylated Foxp3, and pharmacologic inhibition of CDK2 in iTreg strengthens the expression of Foxp3. These data provide an in vivo link to our earlier biochemical data. Therefore, we propose a model in which simultaneous induction of Foxp3 and $p27^{kip1}$ by TGFβ in a T cell (Fig. 16a) concomitantly serves to initiate the

Treg genetic program (Fig. 16b), and to quell CDK2 activity, thus reducing effector cytokine production and allowing greater Foxp3 protein stability (Fig. 16).

Our data support this adjusted model of TGFβ requirement for iTreg generation, but several key pieces are still missing. First, we must confirm whether the effect we see on Foxp3 expression in $p27^{kip1}$-/- iTreg is exclusively post-translational. We know that the CDK motif mutant of Foxp3 has increased protein stability with no change in transcription of the gene, but we have not yet shown a supporting result in iTreg. We will use both wild type and $p27^{kip1-/-}$ iTreg to measure the comparative message level of Foxp3, and to test the stability of the protein in the presence of cycloheximide with and without continued TGFβ signaling. Our data also suggest that in the absence of $p27^{kip1}$ there is a loss of iTreg function, as the converted cells produce more autocrine IL-2 than wild type controls. However, a complete demonstration of the suppressive capacity of iTreg with or without $p27^{kip1}$ expression is required, and will be performed using our previously described in vitro and in vivo models.

Because of the finding that TGFβ drives $p27^{kip1}$ expression, our studies have focused on iTreg, however, we have begun to account for the roles of CDK2 and $p27^{kip1}$ in nTreg as well. Our data show nTreg suppressive function is normal in the absence of $p27^{kip1}$. One explanation for why nTreg do not require $p27^{kip1}$ to inhibit CDK2 and stabilize their Foxp3 pool is simply that nTreg do not express very much CDK2. Indeed, we show that compared to Tconv, nTreg from wild type animals express about two-fold less CDK2. This is consistent with data from another lab showing nTreg not only down regulate CDK2, but CDK1 and CDK6 as well (88). A possible mechanism by which this occurs is that Foxp3 binds to and represses CDK genes as it does cytokine genes. We believe that repression of CDK family members by nTreg is an adapted mechanism to protect the Foxp3 protein, one that makes much sense. The stability of the Foxp3 gene is specifically enhanced in developing thymic Treg to generate a more lasting suppressive lineage (100-103). If Foxp3 is not present in nTreg then severe autoimmunity will result in an early death in humans (61, 98). We believe that this is a case in which the nTreg simply cannot afford to have high CDK2 activity, which would drive Foxp3 instability and potentially destroy the balance of immunity and tolerance.

How far does the influence of the CDK2-p27^{kip1} axis extend?

Unlike, nTreg, iTreg are appreciated to be naturally unstable. For an iTreg, to be able to modulate Foxp3 expression through regulation of the CDK pathway could be advantageous. It provides a mechanism to assist in lineage plasticity depending on the needs of the organism for a more inflammatory or suppressive environment. A cell could modulate CDK2 activity, and thus Foxp3 expression, allowing either for a more stable iTreg, or for destabilization and subsequent conversion into another CD4+ T cell subset. The Th17-iTreg dichotomy is a well documented example of the need for plasticity during an immune response (24), and the pivotal role played by TGFβ in the development of both of these lineages is apparent (113). Interestingly, we have preliminary data indicating that p27^{kip1} is also required for normal development of the Th17 lineage, although how this is mediated remains unclear (Morawski, Wells unpublished observations).

There are still other signaling events that must be considered that alter the expression of p27^{kip1}. Retinoic acid, which drives Foxp3 expression in the gut (123), and is understood to have a reciprocal role in the iTreg-Th17 axis (124), like TGFβ, has been shown to induce (125, 126) and stabilize (127) the expression of p27^{kip1}. Contrarily, IL-6 signaling is known to oppose expression of Foxp3 in favor of STAT3-driven expression of RORγt, the master transcription factor of the Th17 lineage (110, 128). It is not known whether IL-6 has any affect on the Foxp3 protein, and while IL-6 was shown to drive p27^{kip1} expression in neurons (114), it is unclear if this is the case in T lymphocytes. We hypothesize that inflammatory stimuli inhibit p27^{kip1}, and oppose Foxp3 expression and stability, leading to lineage plasticity (Fig. 16). Many factors are involved in the iTreg-Th17 developmental axis, and we believe that p27^{kip1} can be linked to many of them. More work must be done to dissect the potential effects of TGFβ, retinoic acid, and IL-6 on p27^{kip1}, and how they may contribute collectively to the development and plasticity of iTreg and Th17 cells.

Immunity and peripheral tolerance can be altered in part by the relative balance of iTreg and Th17 cells. A failure to properly stabilize Foxp3+ iTreg could lead to a breakdown of this immune homeostasis and result in autoimmunity. Our data support the important and novel consideration that the CDK2-p27^{kip1} axis is involved in this balance (31, 49, 107). A number of

studies from other groups demonstrate a role for CDK2 and $p27^{kip1}$ in various autoimmune disorders.

Pemphigus vulgaris (PV) is part of a family of blistering autoimmune diseases where pathology is linked to CDK2 activity (129). Inhibition of CDK2 by siRNA or roscovitine treatment could prevent blister formation in epithelial cells and modulate disease in mouse models of PV. The authors conclude that CDK2 is critical for the organization of intracellular signaling during PV progression, but do not propose a mechanism. Other studies demonstrated that $p27^{kip1}$ is downregulated in thyroid epithelial cells during Hashimoto's thyroiditis (HT) (130), and in tissue samples from human systemic lupus erythematosus patients (SLE) (131). The study on HT contains no definitive mechanism to explain the involvement of $p27^{kip1}$. The study on $p27^{kip1}$ on SLE shows hyperactive PI3K/Akt/mTOR signaling in diseased patient samples, as well as increased cell arrest after S phase and high apoptosis. PI3K/Akt/mTOR signaling is known to be detrimental to Treg, as pharmacologic inhibition with rapamycin can stabilize Treg cells (132). Interestingly, many SLE patients also have a deficiency in the generation of extra-thymic iTreg, rendering them less capable of controlling autoreactive lymphocytes (133). According to our model, dysregulated $p27^{kip1}$ expression and subsequent elevation of CDK2 activity can explain the iTreg conversion defect SLE patients. This regulation of iTreg conversion in SLE patients is consistent with our findings from $p27^{kip1}$ deficient animals in Chapter 3. Interestingly, aged $p27^{kip1}$-/- animals develop signs of lupus-like disease including an elevated serum titer of dsDNA antibodies, and glomerulonephritis (134). Our data combined with evidence from several other labs makes a strong case for the importance of the CDK2-$p27^{kip1}$ axis in the prevention and development of autoimmunity. What remains uncertain is why the CDK2-$p27^{kip1}$ axis is dysregulated in patients with an autoimmune disorder like SLE.

In response to costimulation and IL-2 signals, CDK2 will target $p27^{kip1}$ for nuclear export and degradation following a series of phosphorylations (93, 118, 135). The GTPase, Ras-related protein 1 (RAPL), can protect $p27^{kip1}$ from this fate in lymphocytes (136). Interestingly, animals deficient in RAPL have trouble controlling the development of autoimmunity. This network of signals represent one location where the breakdown of $p27^{kip1}$ regulation could occur, and increase susceptibility to autoimmunity. Additionally, it is known that the inflammatory cytokines

that are excessively produced during autoimmunity contribute to worsening of disease by dysregulating the balance of iTreg and Th17 cells. For example, IL-6 produced by autoreactive T and B lymphocytes is known to oppose Foxp3 expression and drive reprogramming of iTreg into Th17 cells (110, 128). SLE patients with defects in iTreg conversion have subsequently high numbers of inflammatory Th17 cells (137), an imbalance thought to contribute to renal pathogenesis. We propose that the inflammatory signals that drive lineage imbalance and disease do so through an effect on CDK2 or $p27^{kip1}$ expression. More experiments must be performed to fully understand the regulation of the CDK2-$p27^{kip1}$ axis in the context of the iTreg-Th17 paradigm.

How is the CDK2-$p27^{kip1}$ axis regulated in vivo during an inflammatory response? A biologically relevant example to answer this question is the CD103+ dendritic cell (DC). CD103 expression on a DC is induced by TGFβ and IL-2 signals (138). It can also be induced and maintained by signaling from CCR9 (139). CCR9 is critical for DC homing in the gut and interaction with the lamina propria (140), which primes DC to be “non inflammatory” (141) through a mechanism that is poorly understood. CD103+ DC induce expression of Foxp3 in conventional T cells through production TGFβ and rentinoic acid (RA) (142) driving tolerance during inflammatory bowel disease (IBD) (143, 144). Contrarily, CD103-negative DC home to the mesenteric lymph nodes and produce large amounts of inflammatory cytokines including IL-6, which destabilizes resident iTreg (142). Thus, the balance of immunity and tolerance in IBD can depend on the presence or absence of CD103+DC. We propose that the cytokine production of each of these two DC subsets not only induces the gene programs of iTreg or Th17, but also regulates the stability of Foxp3, and thereby the balance of immunity and tolerance (Fig. 17).

A broader view of CDK activity and regulation in T cells

Foxp3 is not the only protein regulated by CDKs in T cells. Numerous other proteins critical for T cell function are known CDK substrates, many of them transcription factors. CDK2 and CDK4 regulate Smad3 antiproliferative function (58). The effect of phosphorylation at these motifs is transcriptional, as Smad3-/- cells as well as mutant Smad3 protein lacking the three CDK putative motifs both had an increased ability to induce the cell cycle inhibitor p15 and

consequently to arrest proliferation. CDK8 controls STAT1-dependent cytokine production by phosphorylation S727 of the STAT transcriptional activation domain (145), which was required for DNA binding to the IFNγ promoter and activation of that gene. CDK5 is involved in T cell activation and migration (3). CDK5 phosphorylates coronin1a at threonine 418, driving normal actin polymerization and migration towards CCL-19, allowing efficient T cell activation. Finally, CDK6 is required for Notch-dependent proliferation and differentiation, as well as Akt-driven tumorigenesis (146). These effects are dependent the kinase domain of CDK6 (147), but no substrates have been named to explain the mechanism. However, no CDK6 substrates have yet been named to explain the exact mechanism. These data demonstrate how the CDK family can regulate critical signaling pathways using kinase-dependent mechanisms. However, it remains unclear if CDK modification has any affect on protein stability in these examples, as we show with Foxp3. In addition, many other important T cell transcription factors contain CDK motifs including Tbet, GATA3, RORgt, STAT3, Runx1, Ikaros, DNMT3a, c-Jun, c-Fos, NFAT1, NFAT2. We believe that CDK activity could be part of a critical regulatory network controlling T cell function, lineage specification and stability.

Inhibiting the CDK pathway for clinical benefits

A wide range of important roles for CDKs in T cell biology, including proliferation, effector function, and tolerance have been published including our work presented in the chapters of this Thesis. Modulating CDK activity could be a good way to affect the balance between immunity and tolerance in T cells. A number of drugs now exist that specifically target the CDK family, or certain members of that family. Roscovitine is a pharmacologic inhibitor that targets CDK2, CDK7, and CDK9 (95), and at higher doses can affect the entire CDK family. This drug is in clinical trials to treat cancer (96), and was shown to ameliorate graft-versus-host disease in mice (97). Interestingly, roscovitine can also redirect Th17 cells to the iTreg lineage by reinforcing Foxp3 expression (148).

A potential problem with roscovitine is that it can block global transcription by restricting CDK7 and CDK9 activity (149), which are involved in RNA polymerase II phosphorylation (150), and required for transcriptional complex formation and elongation (151). While roscovitine does

not completely block transcription (152) it does causes complex changes in mRNA levels (153), which could prove problematic during clinical trials.

High sequence homology in the active site of all CDKs explains why roscovitine cannot distinguish between cell cycle and transcriptional CDKs (149). However, there have been successful attempts to generate inhibitors with selectivity for certain CDKs. Purine bioisosteres of roscovitine can enhance the desired biological or physical properties of roscovitine without making significant changes to its chemical structure, and thus its efficacy (154). For example, olomoucine II is a roscovitine analog that contains an additional hydroxyl modification resulting in a tenfold increase in CDK9 inhibition without any differential affects on other CDKs (155). The roscovitine bioisostere pyrazolo has good anti-proliferative properties and bound more specifically to CDK2 (156). Recent research also identified bioisosteres even more potent than roscovitine such as CVT313, H717, and purvalanols (157).

There are many current CDK inhibitors in clinical trials showing varied degrees of success (158). The data we present in this Thesis suggests that these inhibitors may have additional effects on protein stability and Treg function that were not previously appreciated. Additional development of roscovitine analogs and integration into clinical trials may therefore be highly relevant in the treatment of autoimmunity or during transplantation.

Figure 14

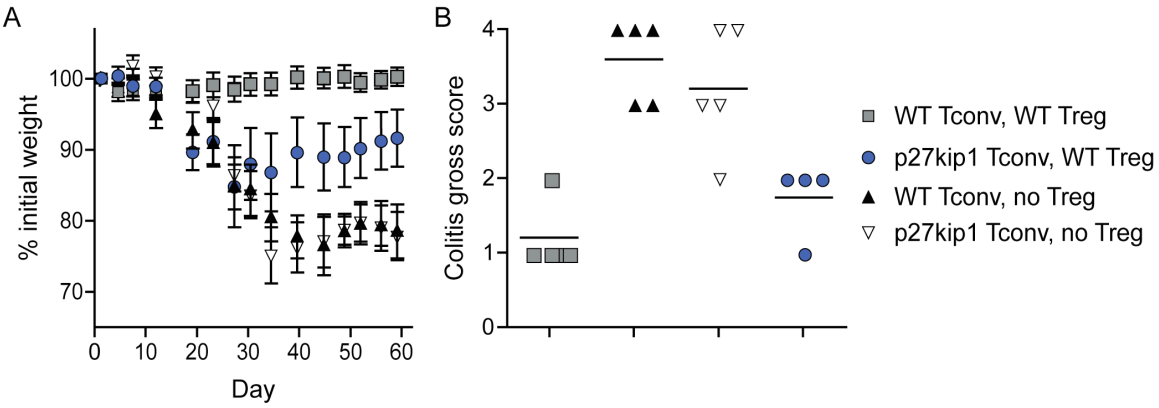


FIGURE 14. The ability of Treg to suppress conventional T cells depends on expression of $p27^{kip1}$. Rag1-/- mice were injected i.v. with 1 x 10^6 conventional CD4+25- T lymphocytes from wild type and $p27^{kip1}$-/- mice to induce colitis. Simultaneously, groups of mice were injected i.v. with 1 x 10^6 wild type CD4+25+ nTreg to prevent disease, or were left untreated. *A*, Mice were weighed every 2-3 days until day 60 post injection. *B*, Gross pathological analysis of sacrificed mice was performed and scored as described previously. Each group contained 4-5 mice.

Figure 15

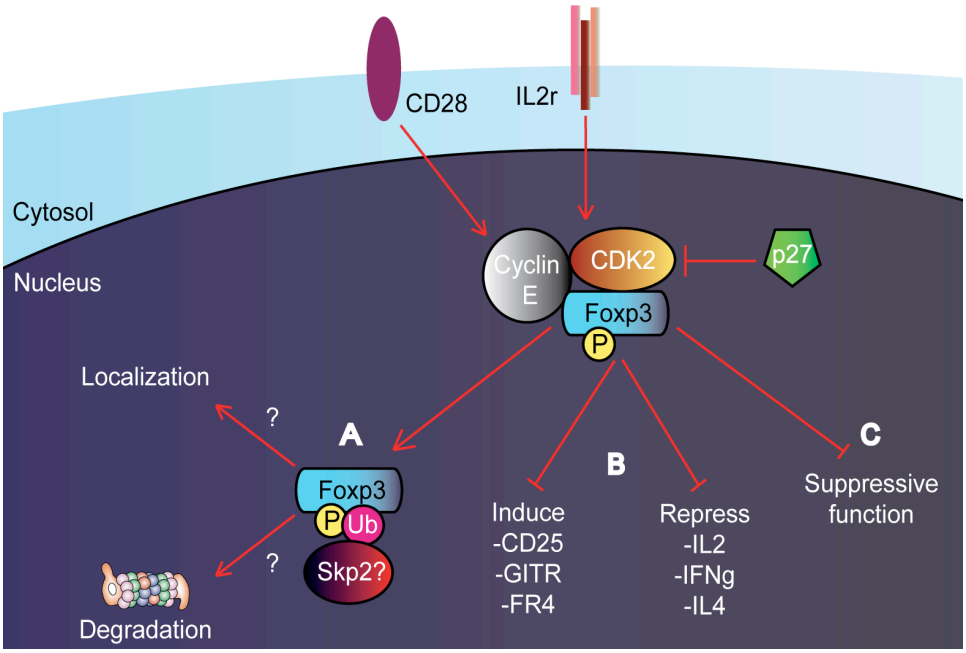


FIGURE 15. CDK2 phosphorylation of Foxp3 affects stability and function. CDK2 is induced in response to IL-2 and costimulatory signals. Together with cyclin E, CDK2 phosphorylates Foxp3, destabilizing the protein, leading to proteasomal degradation or possible changes in localization (*A*). The changes of steady-state Foxp3 levels in response to phosphorylation affect the ability of Foxp3 to induce and repress target genes (*B*), and suppress the proliferation of conventional T cells (*C*).

Figure 16

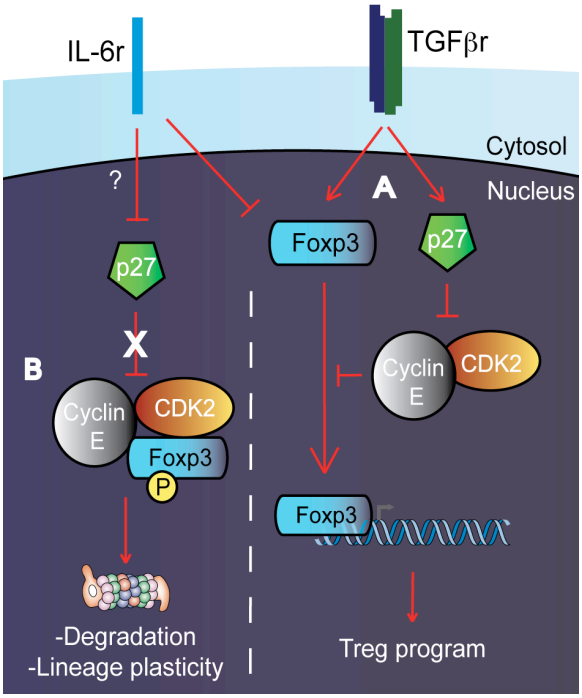
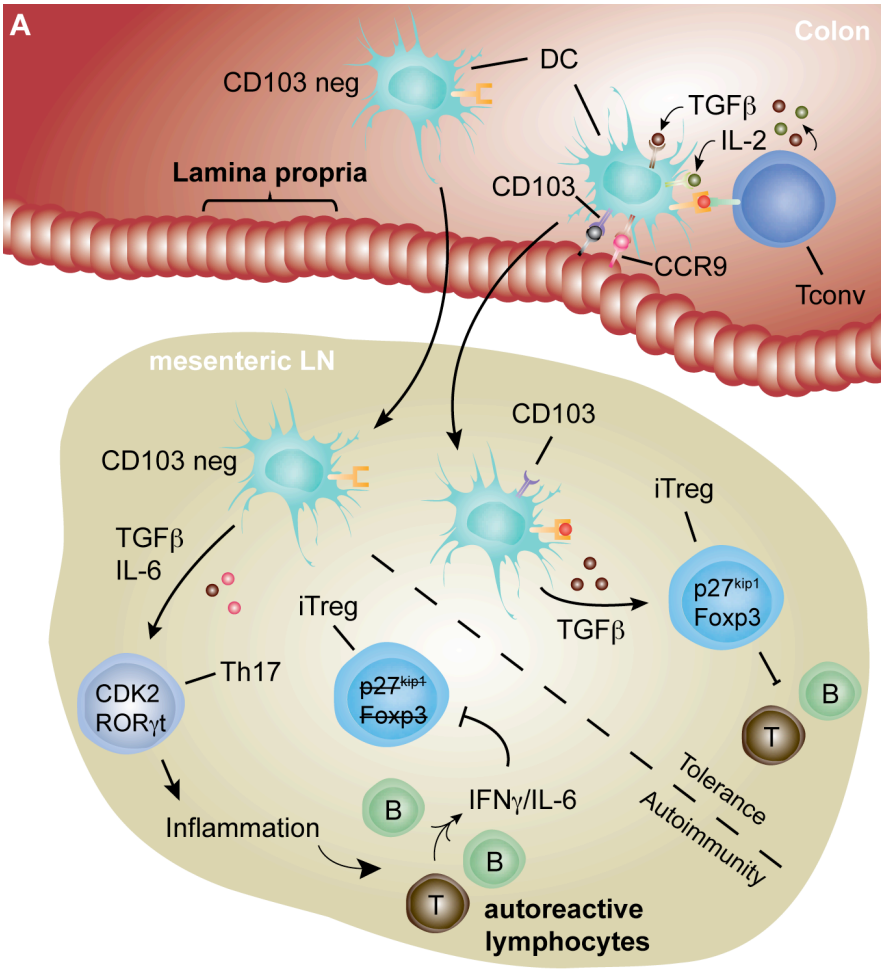


FIGURE 16. Multiple signals target $p27^{kip1}$ in T cells. Foxp3 and the CDK2 inhibitor $p27^{kip1}$ are induced by TGFβ signaling (*A*). Our data suggest $p27^{kip1}$ is required to oppose CDK2 activity and stabilize the Foxp3 protein and ensure a more stable iTreg genetic program. IL-6 is a cytokine signal known to oppose the expression of Foxp3, causing dedifferentiation of iTreg and supporting the development of Th17 cells. It is unclear how IL-6 affects $p27^{kip1}$ in T cells. It is possible that $p27^{kip1}$ is a switch controlling Foxp3 stability and lineage plasticity (*B*).

Figure 17


A
Colon
CD103 neg
DC
TGFβ
IL-2
Lamina propria
CD103
CCR9
Tconv
mesenteric LN
CD103
CD103 neg
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TGFβ
IL-6
p27kip1
Foxp3
TGFβ
iTreg
Th17
CDK2
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Tolerance
Autoimmunity
IFNγ/IL-6
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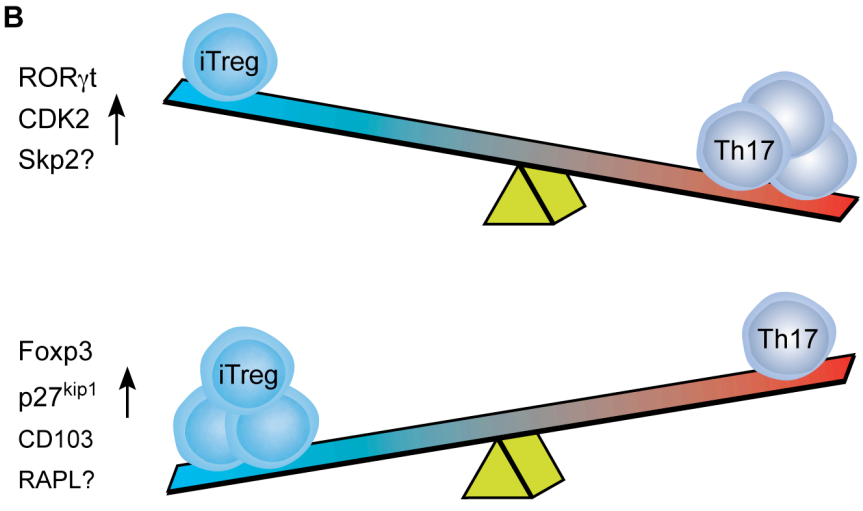
B
RORγt
CDK2
Skp2?
iTreg
Th17
Foxp3
p27kip1
CD103
RAPL?
iTreg
Th17

FIGURE 17. Regulation of the CDK2-p27^{kip1} axis affects the balance of immunity and tolerance. *A*, CD103+ dendritic cells (DC) are primed to be tolerogenic in the gut by epithelial cells of the colonic lamina propria expressing CCR9, or by T cells making IL-2 and TGFβ. The CD103+ DC then home to the mesenteric lymph node (MLN) and drive iTreg conversion. CD103- DC remain inflammatory, travel to the MLN where they support an inflammatory situation driven by Th17 cells. *B*, CD103-dependent DC regulation of Th17-iTreg plasticity is a physiological example of how the CDK2-p27^{kip1} axis could be engaged by cytokine signaling in vivo. Additional regulation of CDK2-p27kip1 expression could be achieved through expression of factors like RAPL and Skp2.

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