IDENTIFICATION OF CYCLIN-DEPENDENT KINASE 5 IN T CELLS AND ITS
ROLE IN REGULATING T CELL FUNCTION AND DIFFERENTIATION

By

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Dedication

To my parents, who scarified everything for their children to have a better life, and taught us to work hard, work smart, and to love your family and friends. To my sister who has always inspired me and taken care of me. Thank you.
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Identification of Cyclin-dependent kinase 5 in T cells and its Role in Regulating T Cell Function and Differentiation

Abstract

By

ERIC LAM

Cdk5 is a unique member of a family of serine/threonine cyclin-dependent kinases. Although Cdk5 is ubiquitously expressed, this kinase is predominantly active in post-mitotic neurons where there is abundant expression of its obligate activating partners p35 and/or p39. Consequently, Cdk5 has been historically considered a ‘neuron-specific’ kinase and recognized as an essential regulator of neuronal functions. However, aberrant Cdk5 activity has been associated with a variety of inflammatory neurodegenerative disorders and activity of the Cdk5-p35 complex has been associated with disorders involving non-neuronal tissues, indicating the potential for novel extra-neuronal roles for Cdk5. Our work describes a previously unknown function of the Cdk5-p35 complex in T cells. We demonstrate the activity of Cdk5 is required for the induction of EAE, a T-cell-mediated neuro-inflammatory disease model of multiple sclerosis. Activation of T cells leads to an induction of Cdk5-p35 expression that we show is essential for optimal T cell function. In an effort to define mechanisms mediating this effect of Cdk5, we discovered that Cdk5 controls IL2 gene expression during T-cell activation by modulating the HDAC1/mSin3a repressor complex. We show this effect of Cdk5 is dependent on specific phosphorylation of the mSin3a protein at serine 861. Disruption of Cdk5 activity
in T cells results in enhanced HDAC activity and binding of the HDAC1/mSin3a repressor complex to the IL2 promoter and subsequently leads to the suppression of IL2 expression.

In our pursuit of alternative mechanisms, we focused on Foxp3+ regulatory T cells (Tregs) whose activity is essential for maintaining immune homeostasis and controlling inflammation in the CNS. We show that Cdk5 activity is a determinant of the expression of Foxp3 in CD4+ T cells, effectively regulating the differentiation of Tregs in the presence of pro-inflammatory factors. Our data show the IL-6 induced repression of Foxp3 gene expression requires Cdk5-mediated phosphorylation of Stat3 specifically at residue 727. Taken together, the studies described here define novel and important functions of Cdk5-p35 in T cells, and suggest the potential to exploit this biology through the preclinical/clinical development of Cdk5 as a target for therapeutic intervention against T-cell mediated autoimmune and chronic inflammatory diseases.
Introduction

Protein phosphorylation is an essential process that governs almost all cellular processes including cellular division, differentiation, metabolism, movement, survival and apoptosis. Protein kinases are the enzymes responsible for catalyzing the transfer of a phosphate group to their specific substrate. Although genes that encode for protein kinases constitute only about 2% of the genome in most eukaryotes, they phosphorylate more than 30% of the cellular proteins within an organism\(^1\). As such, phosphorylation of protein substrates is considered one of the most prevalent post-translational modifications that can regulate the structures and functions of cellular proteins. Through the induction of conformational changes or the creation/disruption of protein interaction; phosphorylation events have proven to be indispensable for proper cellular functioning. Therefore, dysregulation of protein kinases and the subsequent phosphorylation of proteins results almost always in a change in cell function and potentially even disease. For example, the aberrant activation of protein kinases is known to result in cancer, immune disorders and neurodegenerative diseases. Therefore, understanding the role and function of specific protein kinases is essential and the effort to do so has the potential to reveal novel therapeutic targets for a wide variety of diseases and disorders.
1.1 Overview of Cyclin-dependent kinase 5 (Cdk5)

1.1.1 History of Cdk5 discovery and introduction to an atypical CDK

Cdk5 was first discovered more than two decades ago in the 1990s, and was initially identified by purification from the bovine brain\(^2\). Since then, a great deal has been uncovered about this unique kinase. Due to its high sequence and structural homology to other CDK members, Cdk5 was named a member of the cyclin-dependent serine/threonine kinase family of proteins. Interestingly, cumulative research throughout the years has proven Cdk5 to be a distinct member of the CDK family of proteins with biochemical and functional characteristics that distinguish it from the other CDKs.

Similar to other CDKs, Cdk5 as a monomeric unit has no enzymatic activity and requires the association of an activation partner for enzymatic activity, however, the main two activators known for Cdk5, p35 and p39, are non-cyclin proteins. Once activated, Cdk5 is a proline-directed serine/threonine kinase (Figure 1.1) that has a strong preference for a basic residue in the +3 position, having the consensus sequence (S/T)PX(K/H/R). Two members of the CDK family, Cdk1 and Cdk2, have an identical specificity as Cdk5 and as expected, share some substrates. However, ectopic expression of active Cdk5 in cells is unable to promote cell cycle progression\(^3\), signifying the unique role of Cdk5 and pointing to the likely importance of substrates distinct from those of the other CDKs.

1.1.2. Regulation of Cdk5 activity
Although Cdk5 is ubiquitously expressed in all tissues, the highest level of Cdk5 activity has been found in the nervous system mirroring the complementary high expression of its activators p35 and p39\(^4\). As such the regulation of Cdk5 activity occurs mainly through association with and expression of its binding partners.

The half-life of the p35 protein is found to be around 20-30 minutes; targeted by the ubiquitin-proteasome pathway\(^5\) for degradation. With its instable nature, the pathways that regulate the expression of p35 are especially important. Initial studies performed on the PC12 neuronal cell line found that stimulation with nerve growth factor (NGF) induces both p35 mRNA and protein expression\(^6\). In these studies, inhibition of the MEK/ERK pathway blocks p35 induction, whereas constitutive activation is sufficient to induce p35 expression. Further, the NGF stimulation induced MEK/ERK activation leading to the binding of early growth response 1 (Egr-1) to the p35 promoter region. Taken together, these data demonstrated that expression of p35 is mediated by the MEK/ERK induction of Egr1 transactivation of the p35 promoter. However, Cdk5 expression has been shown to be regulated through other mechanisms different from pathways responsible for p35 expression control.

Reports indicate that chronic administration of cocaine in rats lead to an increase in the expression of Cdk5 itself. The increase in Cdk5 transcription was found in conjunction with an increase in the transcription factor δFosB. Further studies performed in the striatum and hippocampus of transgenic mice expressing δFosB show elevated
expression of both Cdk5 mRNA and protein\textsuperscript{7,8} and transient overexpression of δFosB in cells lead to increased Cdk5 promoter activity.

Similar to the mitotic CDKs, Cdk5 activity is additionally controlled through phosphorylation of the Cdk5 kinase itself. In mitotic CDKs, phosphorylation of the Thr14 and Tyr15 residues inhibit CDK activity whereas phosphorylation of Thr160 is required for maximal activity\textsuperscript{9}. While these residues are conserved in Cdk5, different kinases are responsible for the phosphorylation. Interestingly, phosphorylation of Tyr15 in Cdk5 is stimulatory and causes significant increase in Cdk5 kinase activity\textsuperscript{10}. Finally, while Thr160 phosphorylation in the T loop of mitotic CDKs is required for maximal kinase activation, the equivalent residue Ser159 in Cdk5 is not required.

Taken together, Cdk5 activity is tightly regulated through different mechanisms including the expression and degradation of its activators and post-translational phosphorylation of Cdk5 itself. Although some similarities exist between the regulation of Cdk5 and mitotic CDKs ultimately, Cdk5 has unique regulatory properties that govern its kinase activity.

1.1.3. Function of Cdk5 in the nervous system

The germ-line deletion of Cdk5 in mice results in death just before or after birth. The initial observations show that mice lacking the Cdk5 gene have severe disruptions in their neuronal development and structure\textsuperscript{11}, accounting for the observed lethality in Cdk5-deficient mice. The layering of the cerebral cortex in these mice is inverted (Figure 1.2),
indicating the necessity for Cdk5 in proper neuronal migration and CNS development. Given this dramatic phenotype of Cdk5-deficient mice and with the pattern of high expression of Cdk5 activators being found in post-mitotic neurons of the nervous system, it is natural that the most well studied and defined roles of Cdk5 has been focused on the nervous system. As previously mentioned, Cdk5 has a crucial role in corticogenesis and therefore neuronal migration. It is hypothesized that Cdk5 influences migration by promoting pro-migratory and subsequently antagonizing anti-migratory signals. In addition to observations of inverted neuronal layering, cultured primary neurons with reduced Cdk5 kinase activity show inhibited neuron outgrowth. Reports indicate that the regulation of actin, microtubule and intermediate-filament components of the cellular cytoskeleton alters the ability for cell adhesion, transportation and intracellular signaling, resulting in the observed altered neural migration.

In addition to Cdk5’s integral role in regulating neuronal migration, there is also evidence of Cdk5’s role in regulating a number of other neuronal processes. Although Cdk5 and its activators are abundantly expressed in the brain, their expression has been found at higher levels at synaptic membranes localizing at both the presynaptic and postsynaptic compartments. Functionally, mice lacking the expression of the Cdk5 activator p35 show increased susceptibility to seizures and numerous synaptic proteins have been shown to be Cdk5 substrates, reinforcing the important role Cdk5 plays in synaptic transmission. In the presynaptic compartment, Cdk5 phosphorylation regulates the substrates synapsin 1, MUNC18 and amphiphysin; these are important proteins that interact with and regulate secretory vesicles. These observations implicate Cdk5 as an important regulator in
neuronal membrane transport and secretion\textsuperscript{16,17}. Post-synaptically, Cdk5 has been found to be critical for the development of the neuromuscular junction; where Cdk5 activity regulates the ERBB neuregulin receptor and where inhibition of Cdk5 blocks expression of the acetylcholine receptor\textsuperscript{18}. Another Cdk5 substrate, DARP32, is an important protein involved in dopamine signaling in neurons, where Cdk5-dependent phosphorylation has been shown to inhibit dopamine downstream signaling. Inhibition of Cdk5 activity augments the dopamine response and in mice this regulation of DARPP32 underlies a role for Cdk5 in drug addiction\textsuperscript{7}. In addition, several reports indicate the importance of Cdk5 activity in regulating axon growth and path finding. A higher accumulation of Cdk5 and p35 expression is found in the shafts and growth cones, which are the migratory tips of growing actions; neurons with inhibited Cdk5 activity show a decreased ability to both grow and maintain their axonal projections.

Finally, Cdk5 has also been implicated in the control of neuronal survival\textsuperscript{19}; for example, the expression and activity of Cdk5 has been shown to increase in PC12 neuronal cells during cell apoptosis\textsuperscript{20}. Furthermore, in several disease models, inhibitors of Cdk5 activity have been shown to be protective against necrotic and apoptotic neuronal cell death\textsuperscript{21}. The exact mechanisms through which Cdk5 regulates cellular death have not been fully elucidated, but the pro-apoptotic functions of Cdk5 have been attributed to its ability to regulate specific transcription factors important for cell death and survival. Recently, Cdk5 has also been implicated in anti-apoptotic functions where Cdk5-dependent phosphorylation of ErbB2 and ErbB3 have been reported leading to enhanced survival signaling in cultured neurons\textsuperscript{22}. Interestingly, data in cultured hippocampal
neurons show that Cdk5 and p35 levels decrease during apoptosis\textsuperscript{23}. Taken together, there is evidence of Cdk5 having both a pro-apoptotic as well as an anti-apoptotic influence, suggesting a multifaceted role for Cdk5 in cell survival that is dependent on cellular context and state.

\textbf{1.1.4. Dysregulation of Cdk5 in disease}

As mentioned above, Cdk5 has crucial roles in a number of neuronal functions including neuronal development, migration, synaptic function and even neuronal survival. However, aberrant Cdk5 activity on the other hand is associated with a number of neurodegenerative disorders such as Alzheimer’s disease and other chronic progressive neurodegenerative and neuroinflammatory conditions. In Alzheimer’s disease, tau is found hyperphosphorylated and polymerizes into fibrillary tangles forming one the distinguishing lesions in the disease. Tau is amongst the first Cdk5 substrates identified and multiple serine and threonine residues on tau with physiological relevance have been identified as Cdk5 phosphorylation sites\textsuperscript{24,25} involved in the formation of the tau pathology in Alzheimer’s. Cdk5 has also been implicated in Aβ processing through phosphorylating and stabilizing presenilin-1, a major component of the γ-secretase complex\textsuperscript{26}. Abnormal Aβ processing and tau phosphorylation is a hallmark of Alzheimer’s disease, and thus firmly implicates the importance of Cdk5 in this disease. Other neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and Parkinson’s disease have also been associated with the aberrant activity of Cdk5; higher
levels of Cdk5 expression and activity are observed in disease models, and a number of functionally relevant Cdk5 substrates have been identified in these model systems\textsuperscript{27}.

1.1.5. The role of Cdk5 in non-neuronal tissues

Upon the initial discovery of Cdk5 more than two decades ago, the predominant expression of Cdk5 and of its activators in neuronal cells led to the assumption that Cdk5 is a neuronal-specific kinase. However, studies reported over the last decade have clearly shown the expression and activity of the Cdk5-p35 complex are not restricted to neuronal cells and are also present in non-neuronal cells and tissues. Cdk5 activity has been found in pancreatic β-cells, monocytes, neutrophils, leukocytes, myocytes, epithelial cells, endothelial cells and adipocytes\textsuperscript{28-30}. Similar to the functions described for Cdk5 in neurons, Cdk5 has been shown to influence phenotypical changes in non-neuronal cells through both direct and indirect effects on cytoskeletal structures\textsuperscript{31}. A number of known Cdk5 substrates (Table 1.1) have now been identified in non-neuronal tissues and in these particular contexts, Cdk5 phosphorylation influences responses that include cell apoptosis, cell migration/adhesion, and gene regulation\textsuperscript{28}.

A role for Cdk5 activity has been implicated for apoptosis in the prostate gland in an inducible cell death system. During androgen withdrawal in male mice, involution and regression of the epithelial compartment of the prostate occurs, accompanied by an increase in apoptosis. Studies show that during the period of increased apoptosis in epithelial cells, an increase of Cdk5 expression and activity is observed, whereas a
subsequent decrease in Cdk5 activity also parallels the decline in the number of apoptosing cells\textsuperscript{32}. In other studies, digoxin, which triggers prostate cancer cell apoptosis, has also been found to increase Cdk5 activity and it is hypothesized that this increased Cdk5 activity is responsible for digoxin-triggered prostate cancer cell death. Inhibitors and siRNA to Cdk5 inhibit digoxin-triggered prostate cancer\textsuperscript{33}. Another example of the regulation of non-neuronal cell apoptosis by Cdk5 is found in promyelocytic immune cells. Treatment of these cells with cAMP selectively upregulates Cdk5 expression as well as enhances cell death. Expression of a dominant negative-Cdk5 has been found to be protective against cell death and this effect of Cdk5 is linked to a role in the cleavage of pro-caspase-3 in cAMP treated cells\textsuperscript{34}.

As mentioned above, Cdk5 plays an essential role in neuronal cell migration. Outside of the nervous system, Cdk5 is also important in regulating lens epithelial cells. Transfection of Cdk5 into lens epithelial cells increase cell spreading and this overexpression of Cdk5 also increases cell-cell interactions within the lens\textsuperscript{35}. In corneal epithelial basal cells, Cdk5 has also been found to be important for cell adhesion and migration. It is believed that Cdk5-dependent phosphorylation of Pak1, a protein kinase linked to cytoskeletal reorganization, causes inactivation of Pak1 and promotes cell adhesion and decreases cell migration by preventing production of contractile forces during migration and cellular movement.

Cdk5 and its activator p35 have been detected in the nucleus of neuronal cells\textsuperscript{36} and a number of nuclear substrates including transcription factors such as p53\textsuperscript{37}, myocyte
enhancer factor 2 (MEF2)\textsuperscript{38}, and Stat3\textsuperscript{39} have been identified. The observed presence of active Cdk5 in the nucleus as well the demonstration of nuclear substrates of Cdk5 further extends into non-neuronal cells, and as such, emerging evidence has implicated Cdk5 as a critical transcriptional regulator of gene expression in both neuronal and non-neuronal cells. For example, the transcription factor Stat3 is a substrate of Cdk5. Cdk5-dependent phosphorylation of Ser727 of Stat3 in both medullary thyroid and colorectal cancer cells regulates their proliferation and survival\textsuperscript{40,41}. Additionally, in pancreatic β-cells, high levels of glucose has been found to increase p35 expression and results in an increase in Cdk5 activity. This increase in Cdk5 activity has also been found to upregulate the transcriptional activity of the insulin gene\textsuperscript{42}, potentially predisposing to the development of type 2 diabetes.

Although Cdk5 activity is most highly present in the post-mitotic neurons, and originally considered a neural specific kinase, the efforts to examine non-neuronal roles for Cdk5 have led to the identification of Cdk5 substrates outside of the nervous system as well as important roles for Cdk5 in non-neuronal cellular contexts. Similar to the role of Cdk5 in disorders of the nervous system, aberrant Cdk5 expression in non-neuronal cells could also lead to the disruption of normal cellular function and underlie disease development. Recent reports have discovered a link between aberrant Cdk5-p35 activity and disorders in a number of non-neuronal cell contexts. For example, Cdk5 has been implicated in the induction of inflammatory pain, where the induction of inflammation results in an increase in Cdk5-p35 activity that is essential in the regulation of pain signaling\textsuperscript{43}, and manifestations of other inflammatory mediated disorders\textsuperscript{44}. In many of the
neurodegenerative disorders associated with aberrant Cdk5 activity, inflammation is a critical component of the pathogenesis of these diseases. However, due to Cdk5’s influence in the neuronal compartment, to this point very little effort has been placed on understanding the role of Cdk5 in inflammation and immunity. It is interesting to note that neurons and immune cells share a number of functional parallels such as the ability to migrate and form a functional synapse with adjacent cells. Furthermore, common molecular mechanisms are often shared between the process of inflammation and the pathophysiology of neurodegenerative diseases\textsuperscript{45}. Thus, I hypothesized that the enhanced Cdk5 expression and activity observed in these disorders, may also include a role for Cdk5 in the immune cells that migrate to the site of the inflammatory lesions in neurodegenerative disease. In this thesis, I focused on understanding the role of Cdk5 in the immune cells, specifically T cells, and the mechanisms through which it exerts its regulation of these lymphocytes.

The presence of Cdk5-p35 activity has previously been reported in the cells of the hematopoietic lineage. For example, in human leukemic cell lines Cdk5 is believed to regulate monocytic differentiation\textsuperscript{46}. However, Cdk5-p35 expression and activity has yet to be described in normal non-transformed immune cells, and no functional significance has been revealed for Cdk5 in this cellular context. This is the focus of Chapter 2 where we provide the first demonstration that Cdk5-p35 activity is present and essential in T cell activation and in the induction of a T-cell mediated disease.

**1.2 The Immune System and Regulatory T cells**
The mammalian body is constantly being exposed to a broad range of foreign pathogens and microorganisms which, if successful in invading the host, can result in serious diseases and disorders. However, the mammalian immune system is an organization of cells and molecules that are specialized in defending against these infections and immunological threats. The mechanisms through which the immune system is able to exert its protection can be separated into two main categories of responses; the innate and the adaptive immune response. The first set of responses is comprised of the innate immune response, which recognizes molecular patterns expressed broadly by large numbers of cells and is initiated early and rapidly. The adaptive immune response on the other hand, is comprised of cells that have evolved with specificity for an individual pathogen or toxin and proliferate after encountering the antigen to mount an effective response. Unique to the adaptive immune response is the ability to create immunological memory after the initial encounter to a specific pathogen, allowing it to contribute prominently to the host response upon re-exposure to the specific antigen.

1.2.1 T cell subsets

Two main types of white blood cells mediate the adaptive immune response. B cells, which develop in the bone marrow, are responsible for the production of antibodies and T cells, so called because of their development in the thymus, are responsible for a number of different immune functions. T cell-mediated immunity is an adaptive process through which the development of antigen-specific T cells contributes to the elimination of viral,
bacterial and parasitic infections, as well immunosurveillance against malignant cells. T cell mediated immunity is an integral component of the adaptive immune response and is largely comprised of a primary response elicited by naïve T cells, effector functions by activated T cells and finally, persistence of antigen specific memory T cells.

The majority of T cells are made up of either CD8+ T cells or CD4+ T cells. CD8+ effector T cells, often referred to as Cytotoxic T Lymphocytes (CTLs) recognize antigen in the context of MHC class I molecules and once activated are extremely effective in the direct lysis of infected or malignant cells. Antigens presented by MHC class II molecules, on the other hand, activate CD4+ helper T cells, and mediate the overall immune response through the secretion of specific cytokines. Upon activation of CD4+ T cells, a network of signaling pathways downstream of the TCR lead to rapid cell proliferation and differentiation into specific effector subtypes including the classical Th1 and Th2 cells as well as newly identified T helper subsets Th17, Treg, Th9, and follicular helper T cells \(^ {48}\). Each specific subset of CD4+ T cells is functionally distinct and the subset-specific differentiation depends on cytokine signaling and on the action of distinct transcription factors working together in a complex network.

1.2.2 The role of IL2 in T cell function and the regulation of IL2 gene transcription.

Interleukin2 (IL2) and its receptor, IL2R, were the first cytokine and receptor combination cloned more than 30 years ago \(^ {49}\). The first functional role attributed to IL2 signaling was the ability to stimulate T cell proliferation and differentiation \textit{in vitro} \(^ {50, 51}\).
and due to this central role in T cell function, IL2 was originally named the ‘T-cell growth factor’. Early research performed in vitro showed that activation of T cells through the TCR and co-stimulatory molecules such as CD28 led to the expression of IL2, and through its engagement with IL2R, with the subsequent downstream signaling driving many immune-enhancing roles. As a result of the recognized importance of IL2 in T cells, a great deal of focus has been placed on understanding the complex function of IL2, specifically its potential to directly boost immunity in cancer or in the setting of immune deficiencies, or to antagonize unwanted immune responses in autoimmune diseases or transplantation rejections\textsuperscript{52}. In vivo examination of the role of IL2 in mice however yielded unexpected lethal autoimmunity in mice lacking IL2, rather than the anticipated lack of immunity\textsuperscript{53}. Subsequent studies revealed the reason for the observed lethal autoimmunity observed in IL2 deficient mice is due in part to the failure of thymic development of Treg cells, that results in an imbalance in immune homeostasis\textsuperscript{54}. Thus it is now clear that IL2 is a cytokine that regulates a vast number of different functions dictated in part by the specific context in which it is expressed, on the subset of cells responding to IL2 and on the other factors in their environment at the time of IL2 exposure. Thus, while it is essential for immune-enhancing effects through the regulation T cell activation, primary T cell responses and differentiation, it also has a key role in down regulating immune responses through facilitating suppressor T cell development.

The transcriptional regulation of the IL2 gene has been extensively studied and a critical proximal promoter of 360-bp located immediately upstream of the transcription start site has been elucidated\textsuperscript{55}. Binding sites for a number of essential transcription factors such
as nuclear factor κB (NFκB), activated protein-1 (AP-1), and nuclear factor of activated T cells (NFAT) are located in this region. Transcriptional expression of the IL2 gene requires the proper combination of these transcriptional factors to form activator complexes on the IL2 promoter\textsuperscript{56}. While the mechanisms promoting the IL2 gene expression has been very clearly defined, the negative regulation of IL2 gene expression has been much less studied. The negative regulation of cytokine expression, especially IL2 expression, is essential for maintaining the gene in an inactive state in naïve T cells as well as for repressing the gene following activation. On the proximal promoter region of the IL2 gene, a negative regulatory element (NRE) has been identified which allows the binding of a repressor complex and subsequent suppression of IL2 gene transcription\textsuperscript{57}. Furthermore, recent studies have highlighted a role for the classical zinc-dependent histone deacetylase, HDAC1, in repressing IL2 gene expression through this specific element\textsuperscript{58}.

Previous reports have implicated Cdk5 as an important regulator of the HDAC1 complex and in neuronal cells, Cdk5 can interact with and inhibit HDAC1 activity\textsuperscript{59}. However, direct phosphorylation of HDAC1 by Cdk5 was not shown and a mechanism to explain how Cdk5 deregulated HDAC1 activity was not demonstrated. Recognizing that HDAC1 is an established negative regulator of IL2 gene expression and that Cdk5 is a regulator of HDAC1 activity, I postulated that a potential regulator role for Cdk5 in the control of IL2 expression. In Chapter 2, our results indicate the presence and importance of Cdk5 activity in activated T cells. Cdk5-deficient T cells exhibited a diminished response to TCR ligation, including a decreased mitogenic response. I hypothesized that Cdk5 may
act through a series of mechanisms to control T cell responses and that this may include the regulation of genes required for T cell activation. Since IL2 is known to be essential for T cell activation, function and differentiation, our aim was to interrogate whether Cdk5 may influence IL2 gene expression, and if true, to then identify the potential mechanisms underlying this regulation. In Chapter 3, I demonstrate that Cdk5 is in fact an important modulator of IL2 gene expression through altering HDAC activity in T cells. These observations implicate Cdk5 as a potential therapeutic target, whose inhibition may serve to impair the progression of T-cell mediated immune disorders.

1.2.3 Regulatory T cells and their role in immune homeostasis and disease

A successful immune response to foreign antigens allows the body to eliminate inciting antigens, thereby allowing for survival in an external environment. Additionally, a productive immune response must eliminate foreign antigens without mounting any detrimental responses to self-antigens and once that response to foreign antigens has subsided, the system must return to a basal level of activity, i.e. normal immune homeostasis. The ability to maintain a state of immune equilibrium between tolerance and response is essential to prevent autoimmune disease and other disorders that may result from the loss of homeostasis.

The majority of either protective or harmful immune responses are mainly mediated by the T and B cells of the immune system. Two broad mechanisms, recessive and dominant, are employed to achieve immune homeostasis. In the recessive mechanism or
cell-intrinsic manner, lymphocytes are programmed to undergo apoptosis upon exposure to self-antigens. Alternatively, they may replace self-reactive receptors with non-reactive ones through receptor editing or they may raise their activation threshold through inhibitory signaling. In the dominant or cell-extrinsic mechanism, a subset of ‘regulatory’ T cells serve to moderate the activation and prevent the expansion of other types of aberrantly active T cells. This T cell-mediated immune suppression has only recently been reported, but it has been found that this mechanism is principally dependent on a novel population of T cells discovered in the late 1990s. Sakaguchi et al., discovered that this specific subset of CD4+ T cells expressing high amounts of the CD25 receptor termed regulatory T cells (Treg) were highly enriched in immune suppressive ability. The significance of this specific subset of Treg cells has been further highlighted by studies which show disruption of Treg development or function being the primary cause of autoimmune and inflammatory diseases both in humans and in other animals. Lower levels of circulating suppressor Tregs were detected in patients with autoimmune diseases such as juvenile idiopathic arthritis, psoriatic arthritis, autoimmune liver disease and systemic lupus erythematosus. Furthermore, the lower level of circulating Tregs was found to correlate with higher disease activity and poorer prognosis.

Although the requirement for Treg in maintaining immune homeostasis is well documented, the specific mechanisms through which Tregs suppresses immune response have yet to be clearly defined. Extensive in vivo and in vitro studies of Treg behavior in both mouse and human have revealed that Tregs may function through multiple mechanisms of suppression and different pathways appear to be dominant under certain
circumstances. These inhibitory mechanisms include suppression through secretion of inhibitory cytokines such as IL-10 and TGF-β, suppression by cytolysis and suppression through targeting dendritic cells. In vivo studies in mice in which IL-10 and/or TGF-β signaling were altered through the use of neutralization antibodies or through receptor deletion in the Treg cells, clearly show a loss in Treg suppressive function. Tregs have also been shown to mediate cytolysis of their target cells through the release of granzymes. In one study, NK cells and cytotoxic T cells were killed by Treg cells in a granzyme-B-dependent and perforin-dependent manner. Tregs also have the ability to down-regulate the capacity of dendritic cells (DC) to mature and may directly inhibit the ability of DCs to activate effector T cells. By expressing lymphocyte activation gene 3 (LAG3), Tregs will engage the MHCII receptor of the dendritic cells and subsequent downstream signaling prevents maturation of the DC. Additionally, through the expression of the CTLA4 molecule by Treg cells, CTLA4+ Tregs engages DC and causes them to express indoleamine 2,3-dioxygenase (IDO). IDO is an enzyme responsible for degrading the essential amino acid tryptophan and can thereby suppresses T cell activation and promote tolerance.

1.2.4 Foxp3+ expression and function in regulatory T cells

Tregs are play a central role in the maintenance of immune homeostasis and the transcription factor Foxp3 is essential for this function and for the development of Tregs. Given the importance of Treg cells in controlling the development of autoimmunity and their therapeutic potential, there remains great interest in understanding the mechanisms
that govern the differentiation and function of Tregs. This effort has led to the discovery of a loss-of-function mutation in the X chromosome that encoded for the forkhead box P3 transcription factor (Foxp3). This mutation causes highly aggressive and fatal systemic immune mediated inflammatory lesions in disorders known as IPEX in humans and scurvy in mice. Further studies revealed the essential role of suppressive T cells in these pathologies as Foxp3-deficient mice failed to develop a suppressive subset of T cells resulting in the systemic inflammatory phenotype. These results raised the question of whether Foxp3 may potentially be a marker for and/or regulate the development of this particular suppressor subset of Treg cells.

Studies performed with a fluorescent GFP-Foxp3 reporter ‘knock-in’ mouse model characterized the expression profile of Foxp3 to be largely restricted to a subset of CD4+ T cells specifically with suppressor function. Fontenot et al. further showed that a conditional deletion of the Foxp3 allele in the T cell lineage resulted in a phenotype of T cell-dependent autoimmune disease with an onset, progression and severity indistinguishable from that observed in mice with a germ line deletion of Foxp3, indicating Foxp3 function to be a Treg lineage-specific factor.

The role of Foxp3 in the development of Tregs was elegantly shown by Sakaguchi’s group with the retroviral induction of Foxp3 into CD4+ naïve T cells. This forced expression of Foxp3 converted the naïve cells towards a Treg cell phenotype as they acquired suppressive function. Separately, Rudensky’s group confirmed the role of Foxp3 in Treg development in experiments in which allelically marked bone marrow
cells from both Foxp3 wild-type and Foxp3 knock-out mice showed that CD4+ Treg cells in the chimeric mice originated only from the Foxp3 expressing hematopoietic precursor cells\textsuperscript{76}. Taken together, this data show Foxp3 to be essential for the development of Treg cells and the use of Foxp3 as a marker for the Treg subset of cells.

**1.2.5 Regulation of Foxp3 expression**

Given the central role of Foxp3 in establishing the function and differentiation of Tregs, the importance of understanding the factors that regulate Foxp3 expression is obvious. However, the physiological factors and pathways responsible for initiating Foxp3 expression remain elusive and poorly understood due to the multitude of pathways and signals that may work in conjunction to regulate Foxp3 expression.

Perhaps one of the most studied aspects of Foxp3 gene regulation is the role of TGF-β signaling in this process. The addition of exogenous TGF-β to CD4+ naïve cells was found to be the major driving force for the induction of high-levels of Foxp3 expression and the gain of suppressive function in T cells\textsuperscript{77}. Although the signaling mechanisms through which TGF-β mediates upregulation of Foxp3 expression remain an intense focus of research, the effort to explore the regulatory elements within the Foxp3 locus has offered insights into the molecular mechanisms controlling Foxp3 expression. Recently, Tone et al., was able to identify a novel enhancer region (enhancer I) where the TGF-β receptor-activated signaling intermediate Smad3 binds, along with the NFAT
transcription factor. Smad3 binding to enhancer 1 and its subsequent activity has been found to be essential for Foxp3 and Treg development.

In addition to factors promoting Foxp3 gene transcription, the negative regulation of its expression is an often-overlooked aspect of Foxp3 gene regulation. In the recent years, a number of cytokines have been found to suppress Foxp3 gene expression in T cells. The pro-inflammatory cytokines IL-6 and IL-27 have been shown to strongly inhibit Foxp3 expression through their shared ability to activate signal transducer and activator of transcription 3 (Stat3) signaling. Although the Foxp3 promoter lacks Stat3 binding sites, Stat3 is now known to bind to a novel enhancer II region downstream of enhancer I. Activation of Stat3 acts as an inhibitor of Foxp3 expression by binding to this silencer element and thereby reduces activated Smad3 binding, the latter being required to activate Foxp3 transcription.

Stat3 is a DNA-binding transcription factor first discovered in IL6 stimulated hepatocytes. Stat3 is associated with a wide variety of physiological processes, has been extensively studied in a number of cell culture systems and is known to be activated by the IL6 family of cytokines which signal through gp130 related receptors. Similar to other members of the Stat family of proteins, Stat3 activity is regulated by phosphorylation of a tyrosine residue proximal to the carboxyl-terminus at residue 705 (Y705). Furthermore, Stat3 is additionally regulated by the phosphorylation of a serine site within the transactivation domain (S727). Although tyrosine phosphorylation of Stat3 mediated by the Janus kinase has been well studied and is required for proper Stat3
dimerization, nuclear translocation and DNA binding\textsuperscript{84}, the functional consequence of serine phosphorylation is still not very clear. Furthermore, a specific kinase responsible for Stat3 serine phosphorylation has never been identified,, mainly due to different activation signals lead to serine phosphorylation by different kinases in different contexts\textsuperscript{85}. Interestingly, specific Stat3 (S727) phosphorylation in T cells have been found to be important in regulating CD4\textsuperscript{+} T cell differentiation into either Th17 or Treg cells\textsuperscript{86}. As mentioned above, Stat3 has been identified as a Cdk5 substrate in a number of different cell types. However, post-translational modification of Stat3 by Cdk5 has not been suggested as a critical event in the regulation of Stat3 function in T cells. Thus, I hypothesized that Cdk5 may regulated the expression of the Foxp3 transcription factor in CD4\textsuperscript{+} T cells and thereby regulate Treg differentiation through the post-translational modification of Stat3. Chapter 4 in this thesis describe a novel role for Cdk5 activity in controlling Foxp3 expression in CD4\textsuperscript{+} T cells, through a mechanism that involves Cdk5 regulation of Stat3 signaling. These observations also provide a rationale for exploiting Cdk5 as a therapeutic target in the therapy of autoimmune and chronic inflammatory diseases. Inhibitors of Cdk5 can potentially serve to regulate immune homeostasis by positively influencing the expression of Foxp3 and Treg differentiation.
**Statement of Purpose**

The studies herein aim to address a novel non-neuronal role for Cdk5 in immune cells and specifically the regulatory role for Cdk5 in T cells. Through this effort I address two major questions: (1) Does enhanced Cdk5 activity observed in many inflammatory neurodegenerative diseases reflect a role for Cdk5 in the immune compartment, particularly T cells and what are the implications of this biology on T cell-mediated disease? (2) What T cell functional phenotype is dependent on Cdk5 activity and through what mechanisms does Cdk5 effect regulation of T cell phenotype?

First I used primary immune cells as well as isolated T cells from the mouse to provide the first demonstration that induction of an active Cdk5-p35 complex is triggered by T cell receptor signaling. We were able to show the relevance of this biology by examining mice with Cdk5-deficient immune cells, in which we found loss of Cdk5 led to amelioration of clinical symptoms associated with experimental autoimmune encephalomyelitis (EAE), suggesting impaired T cell function in these mice. Performing more specific studies with isolated T cells, we found that either genetic disruption or pharmacologic inhibition of Cdk5 activity impaired T cell activation and function phenotype.

We further examined Cdk5-deficient T cells and discovered that disruption of IL2 cytokine expression is a direct consequence of the absence of Cdk5 expression in T cells. Through *in vitro* kinase assays we determined that Cdk5 regulates the mSin3a-HDAC1 repressor complex through direct phosphorylation of the mSin3a protein. I also used
mutant mSin3a protein and determined that Cdk5 specifically phosphorylates mSin3a at serine residue 861. Cdk5-dependent phosphorylation of this residue regulates mSin3a protein abundance and also prevents msin3a-HDAC1 complex formation. Furthermore, I performed ChIP assays and discovered Cdk5-dependent phosphorylation of mSin3a at residue 861 prevents the repressor complex from binding to the IL2 promoter.

Furthermore, the observed decrease of EAE symptoms in mice with Cdk5-deficient immune cells suggested the potential for an imbalance in T cell differentiation, particularly differentiation of Foxp3+ Treg cells. I demonstrated a capacity for Cdk5 to act as an effector pro-inflammatory cytokine mediator of suppression of Foxp3 expression in CD4+ T cells. Mechanistically, I found Cdk5 to phosphorylate the Stat3 protein in T cells and particularly on the serine 727 residue and that Cdk5-dependent Stat3 (S727) phosphorylation is required for Stat3 to properly bind to enhancer II region of the Fop3 gene and subsequently negatively regulate its expression.

In sum, I believe these studies provide a strong groundwork to continue examining the role of Cdk5 in T cell function, activation and differentiation. Most importantly, these studies and the data generated in their pursuit highlight Cdk5 as a potential therapeutic target for the treatment of T cell-mediated inflammation and autoimmune disease.
Figure 1.1  Cdk5 activity and the Cdk5 substrate consensus sequence.  Monomeric Cdk5 kinase is inactive, and requires the association of its activation partners, p35 or p39 to achieve kinase activity.  Upon binding Cdk5 phosphorylates a serine/threonine residue with the particular consensus sequence shown.
Figure 1.1 Cdk5 activity and the Cdk5 substrate consensus sequence.
Figure 1.2. The murine cerebral cortex is inverted in Cdk5-deficient mice. The cortex structure is comprised of layers of post-mitotic neurons produced in successive waves within the ventricular zone during corticogenesis and follows a highly coordinated program of migration. In wild type mice, successive waves of neurons migrate through the cortical plate to take up more superficial positions, such that the cortex is assembled in an “inside-out” gradient. Early-born neurons reside in deeper layers and later born neurons in more superficial layers. In the cerebral cortex of Cdk5-deficient mice (Cdk5-/-), later-born neurons cannot migrate past their predecessors to occupy the more superficial positions and stack up underneath the pre-existing neuronal layers in the intermediate zone. This results in an inversion of the cortex in the Cdk5-deficient mice.
Figure 1.2. The murine cerebral cortex is inverted in Cdk5-deficient mice.
**Figure 1.3 Mechanisms of suppression used by Treg cells.** Various mechanisms of Treg cell suppression of the immune response are depicted here. A) Inhibitory cytokines including TGF-β and IL10 are secreted by Tregs. B) Cytolysis of effector T cells through both granzyme and perforin-dependent mechanisms. C) Targeting DCs to modulate either DC maturation or the induction of IDO, which inhibits effector T cell activation.
Figure 1.3 Mechanisms of suppression used by Treg cells.

A. Release of inhibitory cytokines
B. Cytolysis through granzyme and perforin killing
C. Inhibition of DC maturation and function
Table 1.1 Non-neuronal substrates of Cdk5. A number of extra-neuronal substrates have been recently reported to be phosphorylated by Cdk5. Also listed in the table are the cell/tissue types where Cdk5-dependent phosphorylation occurs and the current known function and related pathology. (Adapted from Contreras-Vallejos et al., *Cellular Signaling*, 2012).
### Table 1. Non-neuronal substrates of Cdk5.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cells/tissues</th>
<th>Function</th>
<th>Possible related pathology</th>
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<tr>
<td>EPRS</td>
<td>Macrophage</td>
<td>Translation</td>
<td>Inflammation</td>
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<td>STAT3</td>
<td>Medullary thyroid carcinoma</td>
<td>Transcription</td>
<td>Cancer</td>
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<td>HEK293 cells, rat renal inner medulas</td>
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<td>Unknown</td>
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<td>mSds3</td>
<td>COS7, NIH3T3, 293 T, and immortalized mouse embryonic fibroblasts</td>
<td>Transcription</td>
<td>Muscular dystrophy</td>
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<tr>
<td>HMGB1</td>
<td>All nucleated eukaryotic cells</td>
<td>Transcription</td>
<td>Inflammation</td>
</tr>
<tr>
<td>cMyc</td>
<td>NCI-H460, NCI-H1299 human non-small cell carcinoma and HCT116 cells</td>
<td>Transcription</td>
<td>Cancer, inflammation</td>
</tr>
<tr>
<td>Titin</td>
<td>Muscle</td>
<td>Assembly of sarcomere</td>
<td>Muscular dystrophy</td>
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<tr>
<td>AATYK1A</td>
<td>Cos7, CHO-K1 cells</td>
<td>Vesicular traffic</td>
<td>Unknown</td>
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<td>Vps34</td>
<td>Hela cells</td>
<td>Vesicular traffic</td>
<td>Cancer</td>
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<td>E-syt1</td>
<td>3 T3-L1 adipocytes</td>
<td>Glucose uptake</td>
<td>Diabetes</td>
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<td>PLD2</td>
<td>Pancreatic β cells</td>
<td>Insulin secretion</td>
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<tr>
<td>β2-syntrophin</td>
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<td>Androgen receptor</td>
<td>Prostate cancer cells</td>
<td>Transcription</td>
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Chapter 2:

Cyclin-dependent kinase 5 activity is required for T cell activation and induction of experimental autoimmune encephalomyelitis

Tej K. Pareek, Eric Lam, Xiaojing Zheng, David Askew, Ashok B. Kulkarni, Mark R. Chance, Alex Y. Huang, Kenneth R. Cooke, John J. Letterio

The work described in this chapter is published in the Journal of Experimental Medicine (2010)

The work described in this chapter was done in collaboration with other authors. Specifically, I contributed to the work in this chapter through designing and performing research which are presented in Figures 2.2A-B, 2.2D-F, 2.3A-D, 2.4A-B, 2.4D-E, 2.6-2.8, 2.10-2.12.

2.1 Abstract

Cyclin-dependent kinase 5 (Cdk5) is a ubiquitously expressed serine/threonine kinase. However, a requirement for Cdk5 has been demonstrated only in post-mitotic neurons where there is abundant expression of its activating partners p35 and/or p39. Although hyperactivation of the Cdk5/p35 complex has been found in a variety of inflammatory neurodegenerative disorders, the potential contribution of non-neuronal Cdk5/p35 activity has not been explored in this context. Here we describe a previously unknown function of the Cdk5/p35 complex in T cells that is required for induction of EAE. T cell receptor (TCR) stimulation leads to a rapid induction of Cdk5/p35 expression that is required for T lymphocyte activation. Chimeric mice lacking Cdk5 gene expression in hematopoietic tissues (Cdk5^{-/-}) are resistant to induction of EAE, and adoptive transfer of either Cdk5^{-/-} or p35^{-/-} encephalitogenic lymphocytes fails to transfer disease.
Moreover, our data reveal a novel mechanism involving Cdk5-mediated phosphorylation of the actin modulator coronin 1a on threonine-418. Cdk5-deficient lymphocytes lack this post-translational modification of coronin 1a, exhibit defective TCR-induced actin polarization and reduced migration towards CCL-19. These data define a distinct role for Cdk5 in lymphocyte biology and suggest inhibition of this kinase may be beneficial in the treatment of T cell-mediated inflammatory disorders.

2.2 Introduction

Multiple sclerosis (MS) is a major cause of neurological disability in young adults and the most common chronic demyelinating disorder of the central nervous system\textsuperscript{87}. Our understanding of the cellular and molecular mechanisms mediating MS has been advanced by studies in the murine MS preclinical model, experimental autoimmune encephalomyelitis (EAE). Development and progression of this and other autoimmune disorders completely depends upon the dynamic nature of immune cells, particularly their ability to migrate and to rapidly form an immune synapse (IS) with antigen presenting cells. Upon stimulation, lymphocytes undergo marked actin-dependent changes in shape that are required for productive cellular interactions and movement during an immune response. The post-translational modification of proteins triggered by TCR signaling is a fundamental requirement for successful IS formation and includes the convergence of several signaling molecules at the plasma membrane. Conformational changes induced by protein phosphorylation affect function by modifying binding motifs essential for recruiting proteins into signaling networks or by placing enzymes within proximity to
substrates. Both tyrosine and serine/threonine kinases have been reported to be key modulators during lymphocyte activation and several novel small molecules designed to inhibit these kinases are currently under investigation in clinical trials involving patients with inflammatory and autoimmune disorders.

Cyclin-dependent kinase 5 (Cdk5), a ubiquitously expressed proline directed serine/threonine kinase, is mainly active in post-mitotic neurons due to abundant expression of its obligate activating partners p35 and/or p39 in these cells. Cdk5 has been considered a neuron-specific kinase and narrowly viewed as an essential regulator of neuronal function. This perception has been supported by gene knockout studies in which germ line deletion of the genes encoding either Cdk5 or p35 lead to inverted cortical neuronal layering during brain development. Normal activity of Cdk5 is required for proper neuronal migration, synapse formation, and neuronal survival. However, aberrant or hyper-activation of Cdk5 is associated with severe neurodegenerative disorders including Alzheimer’s disease.

Recently, Cdk5/p35 has been linked with disease induction in non-neuronal lineages, with examples that include malignant transformation in cancer, induction of inflammatory pain and other inflammation-mediated disorders. Neurons and immune cells share functional similarities, such as the ability to migrate and form a functional synapse with neighboring cells. Understanding that a common molecular mechanism may underlie the link between inflammation and distinct conditions such as neurodegeneration and cancer, we hypothesized that the enhanced Cdk5 activity
observed in these conditions may actually reflect an essential role for Cdk5 in the immune cells that traffic to sites of disease.

Cdk5/p35 activity has been reported in human leukemic cell lines and is thought to play a role in monocytic differentiation\(^98,^99\). However, a role for the Cdk5/p35 complex in the activation and function of normal, non-transformed immune cells has not been established. Here we provide the first demonstration that the Cdk5/p35 complex is essential for T cell activation and for the induction of EAE. We have generated immune chimeric mice (Cdk5\(^{-/-}\)) by reconstituting wild type mice with hematopoietic progenitors from Cdk5-deficient mice embryos (Cdk5\(^{-/-}\)), following lethal irradiation.

Characterization of the Cdk5\(^{-/-}\) and p35\(^{-/-}\) mice shows that Cdk5 activity is dispensable for immune cell development and lineage differentiation. Using phosphoproteomics, we show that Cdk5 phosphorylates coronin 1a, a leukocyte-specific protein and actin modulator\(^100\), at the threonine-418 residue, within a critical actin binding domain. Furthermore, Cdk5-deficient lymphocytes lack this phosphorylation and are hyporesponsive to mitogenic signals, including T cell receptor ligation, and also fail to migrate following CCL19 chemokine stimulation. Most importantly, we show the relevance of these observations by demonstrating the reduced susceptibility of Cdk5\(^{-/-}\) mice to EAE. Encephalitogenic lymphocytes derived from either the Cdk5\(^{-/-}\) or p35\(^{-/-}\) mice fail to transfer disease to normal mice. These results establish Cdk5 as an essential regulator of lymphocyte activation and as a potential therapeutic target for autoimmune disorders, including multiple sclerosis.
2.3 Materials and Method

**Animals.** Six to eight week old C57BL/6 or B6D21 mice were purchased from Jackson Laboratories and used for collection of lymphocytes or allogeneic APC collection and EAE induction. B6-Ly5.2Cr mice (referred to as CD45.1+) were purchased from the National Cancer Institute (Charles River) and used for generation of chimeric mice. Cdk5+/- mice were used to generate Cdk5+/+ and Cdk5-/- embryo, which were further utilized for collection of brain or hematopoietic cells. Three months old p35+/+, p35+/− or p35-/- were used for lymphocyte collection and for induction of EAE. All animals were housed in micro isolator cages and maintained in climate and light-controlled rooms (22 ± 0.5°C, 12/12-hr dark/light cycle) with free access to food and water. Studies were performed in compliance with procedures approved by the Case Western Reserve University School of Medicine’s Institutional Animal Care and Use Committee.

**Generation of Cdk5 null immune chimeric mice.** Hematopoietic cells were collected from liver and spleen of E16.5 Cdk5+/+ and Cdk5-/- littermate embryos, all offspring of male and female Cdk5+/- breeder mice. Approximately ten million cells per mouse were then injected into 12 week old lethally irradiated (1400 rad Gammacel 137Cs source J.L. Shepard and Associates, San Fernando, CA) CD45.1 female mice by tail vein injection. Eight weeks later, peripheral blood was collected from all recipients and tested for CD45.1 vs CD45.2 expression by flow cytometry and PCR analysis for Cdk5 using the following primers 5’ TGA GGG TGT GCC AAG TTC AGC 3’ and 5’GGC ATT GAG TTT GGG CAC GAC 3’. These Cdk5 wild type and Cdk5 null immune chimeric mice were further recognized as Cdk5+/+C and Cdk5-/-C, respectively. C57BL/6 chimeras were generated by injecting lethally irradiated CD45.1 mice with 8 x 10^6 bone marrow (BM)
cells isolated from 10-12 week old C57BL/6 female mice and recognized as C57BL6+/+C.

Upon establishment of chimeric mice, these lines were then maintained by transferring 8 x 10^6 BM cells from each subsequent generation into lethally irradiated CD45.1 female mice. Cdk5 chimeric mice were maintained for a maximum of three generations.

**EAE induction.** EAE was induced in mice by MOG (35-55) immunization and disease was adoptively transferred to unaffected wild type animals as previously described\(^{101}\). In brief, Cdk5\(^{+/+}\), Cdk5\(^{-/-}\) and C57BL6\(^{+/+}\) or p35\(^{+/+}\), p35\(^{-/-}\) and p35\(^{-/-}\) mice were immunized with subcutaneous injections on both flanks with 200 µl of emulsion containing either Complete Freund’s adjuvant (CFA) consisting of 100 µl of incomplete Freund’s adjuvant with 8 mg/ml of *Mycobacterium Tuberculosis* and 100 µl of PBS (DIFCO Laboratories, Detroit, MI) or Complete Freund’s adjuvant (CFA) with 200 µg of MOG (35-55). At the time of injection and 48 hours later 200 ng of pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) were injected intraperitoneally in 100 µl of PBS and mice were monitored daily for clinical signs of EAE. For adoptive transfer of EAE, Cdk5\(^{+/+}\) and Cdk5\(^{-/-}\) or p35\(^{+/+}\), p35\(^{-/-}\) and p35\(^{-/-}\) mice were euthanized 10 days after MOG (35-55) immunization and cell suspensions were prepared from spleen and regional lymph nodes (axillary, branchial and ingunal). About 5 x 10^6 cells were cultured in presence of 33 µg/ml MOG (35-55) and 20 ng/ml of mouse IL12. Three days later about 2-3 x 10^7 of the cultured encephalitogenic cells were then injected into 3 month old female C57Bl6 mice via intraperitoneal injection. PTX (200 ng) was injected on the same day and again 48 hrs later. The following criteria were used to grade clinical scores: 0-no signs of disease; 1-limp tail; 2-moderate hind-limb weakness; 3-severe hind-
limb weakness; 4-complete hind limb paralysis; 5- quadriplegia or premoribund state; 6-death.

**Peptide synthesis.** The following peptides were used in this study: MOG (35-55) peptide: MEVGWYRSPFSRVVHLRYNGK, NF-H peptide: VKSPAKEKAKSPVK, coronin 1a wild type: RATPEPSGTPSSDTVSR, coronin 1a mutant-1: RAAPEPSGTPSSDTVSR, coronin 1a mutant-2: RATPEPSGAPSSDTVSR and coronin 1a mutant-3: RAAPEPSGAPSSDTVSR. All peptides were synthesized at 21st Century Biochemical (Marlboro, MA) using Fmoc chemistry and were HPLC purified minimum >90% and the mass and sequence verified by nanospray MS and CID MS/MS.

**Antibodies.** The anti-Cdk5 antibody (C-8) and anti-p35 antibody (C-19), Santa Cruz Biotechnology, (Santa Cruz, CA) and MPM-2 antibody (Millipore, Billerica, MA) were each used at 1:200 dilution for Western blots; 5 µg of the anti-Cdk5 antibody (C-8) was used for immunoprecipitation. Hamster-monoconal anti-coronin 1a antibody was provided by Dr. Andrew C. Chen (Genentech, San Francisco, CA) and used at a 1:1000 dilution for Western blot and a 1:200 dilution for immunoprecipitation. Rabbit polyclonal anti-coronin 1a serum was provided by Dr. Jean Pieters, University of Basel, Switzerland and used for Western blot at a 1:5000 dilution and for immunohistochemistry at a 1:500 dilution. Alexa Fluor-568 phalloidin (Invitrogen, Eugene, OR) was used to detect F-actin at a 1:25 dilution. Horseradish peroxidase-conjugated anti- hamster (BD Pharmingen, San Jose, CA) was used as a secondary antibody at 1:5000 and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch laboratories Inc, (West Grove, PA) and used at a 1:10,000 dilution for Western blots. The secondary antibodies anti-rabbit Alexa Fluor-488, anti-
mouse Alexa Fluor-546 and anti-mouse Alexa Fluor-633 were purchased from Invitrogen and used at a 1:200 dilution for immunofluorescence.

**Roscovitine.** All roscovitine (C₁₀H₂₆N₆O) [6-Benzylamino-2-(R)-[(1-ethyl)-2-hydroxyethylamino]-9-isopropylpurine; 2(R)-(1-Ethyl-2-hydroxyethylamino) - 6-benzylamino-9-isopropylpurine] used in this study was purchased from Biomol, Enzo life sciences (Plymouth Meeting, PA). A 10 mM stock solution was prepared in DMSO and used at 10 µM for in vitro kinase assays, cell proliferation and cell migration assays, and in the range of 5 to 80 µM for cell viability assays.

**Western blot analyses.** Tissue or cell lysates were prepared in RIPA buffer (Pierce, Rockford, IL) containing a protease inhibitor cocktail tablet (Roche, Mannheim, Germany) as well as phosphatase inhibitor cocktail I and II (Sigma, St. Louis, MO). Proteins were denatured by heating for 10 minutes at 95 °C in sample buffer (2% SDS, 10% glycerol, 80 mM Tris pH 6.8, and 1mM DTT) and 50 to 100 µg of proteins were electrophoresed in 4–20% bis-Tris/polyacrylamide gels, (Invitrogen). Proteins were then transferred to 0.2 µm nitrocellulose membranes (Invitrogen) which were then blocked for 2 hrs in blocking solution (TBS containing 10% nonfat dry milk and 0.05% Tween 20) and incubated overnight at 4°C with primary antibody diluted in blocking solution. On the following day, membranes were probed with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature, and immunoreactivity was detected by using enhanced chemiluminescence (Pierce).

**Immunoprecipitation assays.** Protein lysates (at the concentration of 1µg/µl) were prepared from Cdk5⁺/⁺ and Cdk5⁻/⁻ lymphocytes collected from mice 4 weeks following the induction of EAE. Lysates were pre-cleared with protein A-agarose beads
and then incubated overnight with anti-hamster coronin 1a antibody at 4°C. On the following day, lysates were incubated for an additional 4 hrs at 4°C with 50 µl of a 50% protein A-agarose bead slurry prepared in lysis buffer. After incubation, the protein A-agarose beads were spun down and washed three times with lysis buffer and then subjected to Western blot analysis by using antibody against Cdk5 (C-8) and coronin 1a (anti rabbit coronin 1a).

**Cdk5 kinase activity assay.** Cdk5 kinase activity assays were performed as described earlier\(^\text{102}\). In brief, tissue or cell lysates were prepared in RIPA buffer containing a protease inhibitor tablet (Roche) and the phosphatase inhibitor cocktails I & II (Sigma). Protein lysates (500 µg of either lumbar spinal cord, brain stem or embryo brain lysates or 1 mg of either spleen, lymphocyte or T cell lysates) were dissolved in lysis buffer to achieve a 1 µg/µl concentration and then pre-cleared with normal rabbit IgG followed with 50 µl to 100 µl of 50% Protein A-agarose slurry (Sigma) prepared in lysis buffer. These lysates were then incubated overnight at 4°C with anti-Cdk5 IgG (0.01 µg/µl). On the following day, lysates were subjected to a 3 hrs incubation with 50 µl of a 50% protein A-agarose slurry at 4°C. Immunoprecipitates were washed 3 times with lysis buffer followed with kinase buffer (20 mM Tris-HCl (pH 7.4)/10 mM MgCl\(_2\)/1 mM EDTA/10 µM NaF/1 µM Na\(_2\)VO\(_3\)) and resuspended in 10 µl of 5X kinase assay mixture (100 mM Tris-HCl (pH 7.4)/50 mM MgCl\(_2\)/5 mM EDTA/50 µM NaF/5 µM Na\(_2\)VO\(_3\)/5 mM DTT), 30 µl of water and 20 µM of either the NF-H peptide or immunoprecipitated coronin 1a or coronin 1a peptides (wild type or mutant-1 or 2 or 3). Samples were kept at 30°C for 60 min after adding 5 µCi of (γ-32P) ATP (0.5 mM) and the reaction was stopped by adding 10% trichloroacetic acid in peptide kinase assay buffer and by adding
sample buffer (2% SDS, 10% glycerol, 80 mM Tris pH 6.8 and 1mM DTT) and boiling for the 10 min for the coronin 1a kinase reaction. To detect coronin 1a phosphorylation, 20-µl aliquots were electrophoresed on a 10% polyacrylamide gel, which was then stained with Coomassie blue, destained, dried, and exposed overnight to a phosphoscreen. The phosphoscreen was scanned on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For peptide kinase assay, supernatants were collected and spotted in triplicate onto P81 phosphocellulose squares, air-dried and washed five times with 75 mM phosphoric acid and once in 95% ethanol. Phosphocellulose squares were then soaked in ultima gold liquid scintillation buffer (PerkinElmer, Waltham, MA) and the incorporated γ-32P was counted on a MicroBeta TriLux (PerkinElmer) as a measurement of Cdk5 activity.

**Lymphocyte and T cell isolation.** Mononuclear cells infiltrating mouse CNS tissues were collected by discontinuous percoll gradient as described. Total lymphocytes were collected from spleen and regional lymph nodes (axillary, branchial and ingunal), by passing minced tissues through a 40 µm cell strainer (BD falcon, Franklin Lakes, NJ) and thereafter incubating with ACK lysing buffer (Lonza, Walkersville, MD) on ice for 5 minutes to deplete erythrocytes. For further purification these cells were then subjected to MACS separation columns by using a pan T isolation kit (Miltenyi Biotec Inc, Auburn, CA) as per manufacturer’s protocol, to isolate total T cells.

**Thymidine assay of cell proliferation.** Total lymphocytes or T cells were stimulated with CD3/CD28, PMA-Ionomycin, allogeneic dendritic cells, or MOG (35-55) for 72 hrs in a 96 well plate in RPMI-1640 culture media (Invitrogen) containing 50 µM 2-mercaptoethanol and 10% fetal bovine serum. During the last 16 to 18 hrs
of culture 1μCi $^3$H-thymidine was added to each well and cells were harvested on a Unifilter-96 Harvester (Perkin Elmer). Incorporation of $^3$H-thymidine was measured as counts per minute on a 1450 MicroBeta TriLux (Perkin Elmer).

**Histology.** Histology and immunocytochemical localization were performed as described previously$^{103}$. In brief, four to six weeks after EAE induction, mice were anesthetized and perfused with PBS followed with 4% paraformaldehyde in PBS (PFA-PBS). Spinal cords were collected and fixed in 4% PFA-PBS and embedded in paraffin to obtain 5 µm thick sections. The spinal cord was divided into 4 parts (cervical, thoracic, lumbar, and sacral). At least 5 sections from each part and longitudinal serial sections of all regions were obtained for the entire representation of spinal cord. Hematoxilin/ eosin stain was performed to assess routine histology and inflammation and Luxol Fast Blue, counter stained with Periodic Acid Schiff reagent was used to analyze myelin content. Bielschowsky stain (silver stain) was performed to detect nerve fibers and analyze axonal loss. Images were captured with a D-metrix-40 digital slide microscope (Tucson, AZ) and analyzed with eyepiece software.

**Immunocytochemical localization.** Lymphocytes were collected from spleen and regional lymph nodes and fixed with 4% PFA-PBS for 30 minutes. After 3 washes with PBS, cells were then permeabilized with 1% TritonX100 (MP Biochemicals, Solon, OH) and 10% normal goat serum in PBS for 10 minutes and further washed 3 times with PBS and blocked in blocking solution (10% (vol/vol) normal goat serum/PBS and 0.1% Tween20). After blocking, cells were incubated overnight at 4°C with primary antibodies diluted in blocking solution. The next day, cells were washed 3 times with PBS and incubated for 1 hr at room temperature with secondary conjugated goat anti-
mouse or goat anti-rabbit IgGs. Cells were then mounted on glass slides by using Vectashield mounting medium (Vector laboratories Inc., Burlingame, CA). Immunofluorescence images were acquired by using a confocal microscope with a Zeiss LSM 510 (63µm oil-immersion objective). Images were combined by using Zeiss LSM 510 imaging software and managed in adobe photoshop (Adobe Systems, San Jose, CA).

**Flow cytometry analyses.** Cells were isolated from bone marrow, spleen, and lymph node by passing tissue through a 40 µm cell strainer. Two hundred thousand cells were first incubated with FACS blocking buffer (DPBS with 0.1% BSA and 10% normal mouse serum) and stained with 1µg of CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD11b (clone 37.51), CD45R (clone RA3-6B2), CD45 (clone 30-F11), CD45.1 (clone A20), CD45.2 (clone 104), and CD34 (clone RAM-34) at room temperature and subjected to FACS analysis. All antibodies used for FACS analysis were purchased from either eBioscience (San Diego, CA), or BD Bioscience (San Jose, CA).

**Mass spectrometric analyses.** Following the reduction and S-alkylation, sequencing grade modified trypsin (Promega, San Luis Obispo, CA) was used for the overnight digestion of cut-gel slices at 37 °C. Phosphopeptides were further enriched by MonoTip (GL Sciences Inc., Torrance, CA) according to the manufacturer's protocol. Trypsin-digested samples were next analyzed online using nanoflow high performance liquid chromatography–nanoelectrospray ionization on a LTQ-Orbitrap mass spectrometer (Thermo Finnigan, Waltham, MA) coupled with Ultimate 3000 high performance liquid chromatography (Dionex, Sunnyvale, CA). All data were acquired in positive ion mode. MS/MS spectra were searched against the mouse IPI protein database using the Mascot.
algorithm (Matrix Science Ltd., Version 2.1). Search parameters included a static modification of 57.02146 Da (carboxyamidomethylation) on Cys; dynamic modifications of 79.96633 Da (phosphorylation) on Ser, Thr, and Tyr; and 15.99491 Da (oxidation) on Met. All significant hits were manually inspected.

**Lymphocyte chemotaxis migration assay.** The ability of lymphocytes to move toward a chemo attractant signal was tested in a transmigration assay performed in 6.5 mm transwell with a 5 µm pore polycarbonate membrane insert (Corning Costar, Lowell, MA). In brief, about ten million Jurkat cells, or Cdk5+/−C, or Cdk5−/−C lymphocytes were serum starved for four hours, and treated with or without 10 µM roscovitine. These cells were cultured in the upper chamber of a transwell plate and lower chamber was filled with 100 ng of MIP-3 beta /CCL19 (PeproTech Inc., Rocky Hill, NJ). After 15 hours of culture, cells migrated in the lower chamber were counted by flow cytometry. Data from triplicate samples were plotted as the percent of input cells migrating in response to a chemokine.

**Statistical analyses.** Statistical evaluation was done with Graph Pad Prism (Graph Pad Software, San Diego, CA). Significant differences between experiments were assessed by univariate ANOVA (more than two groups) or unpaired Student's t test (two groups). ANOVA was followed by Student's t tests with a Bonferroni α-correction for multiple comparisons, where α was set at 0.05.

**2.4 Results**

*Increased Cdk5/p35 expression in CNS mononuclear infiltrates in EAE*
Deregulated Cdk5 activity triggers progressive neurodegeneration and neurofibrillary tangle formation in mice\textsuperscript{104}. Tau pathology associated with axonal loss during EAE is linked to up-regulation of Cdk5 activity\textsuperscript{105}. Furthermore, proteomic analysis of chronic active plaques of MS patients has shown significantly increased expression of Cdk5\textsuperscript{106}. In agreement with these reports, we also observed increased expression of p35 protein and of its cleaved product p25 in the lumbar spinal cord and brain stem of mice with EAE (Figure 2.1A). To determine the contribution of inflammatory cells to this process we isolated mononuclear cell infiltrates (including T cells, B cells and macrophages) from these tissues and examined the level of Cdk5 and p35 transcript in these cells. Quantitative RT-PCR demonstrated an increase in both Cdk5 (ten-fold) and p35 (five-fold) transcripts (Figure 2.1B). The observed increase in expression of Cdk5 and p35 correlated with a more than two-fold elevation in Cdk5 activity in total tissue lysates collected from brain stem and lumbar spinal cord (Figure 2.1C). Specificity of Cdk5 kinase activity in this assay was confirmed by using Cdk5 WT and KO embryo (E16.5) brain lysates (Figure 2.1D). These results suggest a potentially important role for Cdk5 in the function of immune cells that may be necessary for induction of EAE.

**Induction of Cdk5 activity is a requirement for lymphocyte activation**

We next determined the presence and importance of this kinase during lymphocyte activation. Although both Cdk5 and p35 were found in very low abundance in normal T cells, their mRNA and protein expression increased within the first 24 hrs following CD3/CD28 stimulation and remained elevated until 48 hrs post stimulation (Figure 2.2A). Although mRNA expression of Cdk5/p35 was consistently high up to 72 hrs, the
levels of the Cdk5/p35 protein started to decline after 48 hrs. This late reduction in the levels of Cdk5/p35 protein likely reflects an increase in their degradation, a process known to be calcium dependent. We also observed a two- to three-fold increase in Cdk5 kinase activity within 24 to 72 hrs of CD3/CD28-mediated T cell activation (Figure 2.2B). Similarly, a three-fold increase in Cdk5 activity was observed in response to interleukin-7 (IL-7), a non-redundant cytokine required for T cell survival and development that is also implicated in MS\(^{107}\) (Figure 2.2C). This effect of IL-7 is enhanced in the presence of CD3/CD28 stimulation. Lastly, protein lysates prepared from total splenocytes (Figure 2.2D) or from CD4\(^+\) T cells (Figure 2.2E) collected from mice with EAE showed a three- to four-fold increase in Cdk5 activity. The re-stimulation of these lymphocytes in vitro with MOG antigen further increased the activity of Cdk5 when assayed at 72 hrs. The specificity of Cdk5 kinase activity in these assays was confirmed by using Cdk5 WT and KO embryo (E16.5) brain lysates (Figure 2.2F).

In order to analyze, if the overall increase in Cdk5 activity observed in these experiments is due to the true increase in specific kinase activity of Cdk5 or merely due to the increased expression of Cdk5 and p35 protein, we repeated these experiments in the presence or absence of a pharmacologic inhibitor of Cdk5 activity (roscovitine), which is known to inhibit Cdk5 activity (by competitive ATP binding) without altering its protein expression. The total splenocytes or purified T cells were stimulated under different conditions in the presence or absence of roscovitine. T-cells stimulated with either CD3/CD28, PMA/ionomycin or allogeneic dendritic cells showed a significant decline in their proliferation when treated with 10 \(\mu\)M of roscovitine (Figure 2.7A). Results obtained from an MTT assay ruled out the possibility of roscovitine having any adverse
effect on lymphocyte viability (Figure 2.7B), and there was no adverse effect of roscovitine on the expression of Cdk5 and p35 protein levels (data not shown). It should be noted that in each kinase assay the activity of Cdk5 is normalized on a per milligram protein basis. While it may also be informative to analyze the specific kinase activity on a per mole basis, we observed a clear increase in Cdk5 kinase activity upon TCR stimulation.

Although roscovitine is viewed as a selective inhibitor of Cdk5 in the dose range used for these experiments, non-selectivity of this compound cannot be ruled out, as is the case for many small molecule inhibitors of kinase activity. Therefore, we next tested the impact of Cdk5 and p35 gene deletion on T cell activation. Germ line deletion of the Cdk5 gene is associated with embryonic lethality in mice, thus we generated Cdk5 null immune chimeric mice (Cdk5−/−) (Figure 2.8). We observed a 60% to 70% reduction in the proliferative response (as measured by 3H-thymidine uptake) of Cdk5−/− (Figure 2.3A) or p35−/− T cells (collected from p35−/− mice; Figure 2.3B) to CD3/CD28 stimulation, suggesting an essential requirement for Cdk5 activity in T cell activation. To determine the potential relevance of this observation in the EAE model, we collected lymphocytes 4 weeks after MOG (35-55)/CFA immunization and subsequently cultured them either in the presence or absence of MOG (33 µg/ml) and/or roscovitine (10 µM). These MOG primed lymphocytes harvested from immunized WT mice exhibited a robust proliferative response when restimulated in vitro with MOG antigen and this effect was completely suppressed by roscovitine treatment (Figure 2.7C). More importantly, encephalitogenic lymphocytes harvested from either the MOG immunized Cdk5−/− mice (Figure 2.3C) or the MOG immunized p35−/− (Figure 2.3D) mice did not respond when re-
exposed to MOG in vitro. Interestingly, we observed a gene dose effect for p35 in these experiments. Proliferation following re-stimulation with MOG in vitro was reduced by 50% in p35+/− lymphocytes and by 70% in p35−/− lymphocytes relative to p35+/+ controls. This represents the first demonstration of true haploinsufficiency for p35.

**Disruption of Cdk5 or p35 gene expression ameliorates clinical signs of EAE**

We next analyzed the susceptibility of Cdk5−/−C and p35−/− mice to EAE. During the first four weeks following immunization, the survival of Cdk5−/−C mice (75%) was significantly greater than that of either the Cdk5+/+C mice or C57Bl6+/+C (radiation control) in whom survival was below 25% due to the severity of disease (Figure 2.4A). There was also a significant delay in the development and a reduction in the clinical severity of EAE in Cdk5−/−C mice (Figure 2.4B). Histologic examination of brain and spinal cord of Cdk5−/−C mice also revealed significantly less inflammation, demyelination and axonal loss (Figure 2.4C). Immunohistochemical analysis of the immune infiltrates in the CNS demonstrated a significant reduction in the numbers of T cells, macrophages, and neutrophil in Cdk5−/−C mice (Figure 2.9). By contrast, there was no significant difference in either disease progression or disease severity among genotypes when EAE was induced in p35+/+, p35+/− and p35−/− mice (data not shown), despite the objective differences in the proliferative response of lymphocytes re-exposed to MOG in vitro (as shown in Figure 2.3). It is important to note that there are significant defects in neuronal development in p35−/− mice, and these mice have a shorter life span. Thus, interpretation of the results of MOG immunization in the p35−/− mice may be complicated.
either by the ability of p39 to compensate for disruption of p35 or by the underlying CNS disease that enhances susceptibility to EAE.

To obviate the impact of these variables that exist in the p35<sup>−/−</sup> model, as well as the potential influence of the radiation required for establishing the Cdk5 chimeric mice, we next utilized an adoptive transfer approach, assessing the ability of encephalitogenic lymphocytes from these models to transfer disease to naïve animals. Lymphocytes harvested from immunized Cdk5<sup>−/−</sup>C mice completely failed to transfer disease (Figure 2.4D). More importantly, disease progression was significantly delayed in recipients of p35<sup>−/−</sup> encephalitogenic lymphocytes (Figure 2.4E); this observation is supported by histopathology which shows reduced inflammatory infiltrates within the spinal cord of recipients of the p35<sup>−/−</sup> encephalitogenic lymphocytes (Figure 2.4F). It is noteworthy that encephalitogenic lymphocytes require restimulation with IL12 prior to adoptive transfer<sup>109</sup>. Therefore it may be interesting to explore whether a Th17 favoring culture condition might restore the encephalitogenic potential to the Cdk5<sup>−/−</sup> T cells.

**Cdk5 directly phosphorylates coronin 1a at threonine-418**

To identify potential biological substrates of the Cdk5 kinase in activated immune cells during EAE, we collected lymphocytes from Cdk5<sup>+/+C</sup> and Cdk5<sup>−/−C</sup> mice 4 weeks after MOG immunization. Deletion of Cdk5 in these cells was confirmed by performing Cdk5 specific RT-PCR (data not shown) and Western blot (Figure 2.5A). The MPM-2 specific antibody, known to recognize phosphorylated proline directed serine/threonine residues in proteins<sup>110</sup> was used to analyze the impact of Cdk5 deletion on proline-directed serine/threonine phosphorylation in lymphocytes. We observed multiple bands with low
abundance on our Western blot in Cdk5<sup>−/−</sup>C lymphocytes compared to Cdk5<sup>+/+</sup>C lymphocytes (Figure 5.5B). Analysis of these bands with LC-MS/MS and Mascot database search identified a 51kD band as coronin 1a (a leukocyte specific protein) with sequence coverage of 63% and a Mascot search score of 3557. Our LC-MS/MS findings clearly confirmed phosphorylation of coronin 1A and pinpointed the phosphorylation site at threonine-418. A 3-fold reduction in phosphorylation of threonine-418 was observed in Cdk5<sup>−/−</sup>C lymphocytes and was confirmed by selected ion chromatography (Figure 2.5D).

However, Western blot analyses for total coronin 1a protein showed identical expression in Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C lymphocytes (Figure 2.5C), demonstrating that this post-translational modification does not affect the abundance of coronin 1a.

To determine whether coronin 1a is a direct substrate for Cdk5, coronin 1a was immunoprecipitated from lysates of Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C encephalitogenic lymphocytes. Total immunoprecipitates were then assayed by Western blot with both Cdk5 and coronin 1a antibodies (Figure 2.5E). The results suggest a direct association of Cdk5 and coronin 1a in lymphocytes. Next, to identify if Cdk5 can directly phosphorylate coronin 1a, immunoprecipitates of coronin 1a obtained from normal lymphocytes were mixed with active Cdk5 complex obtained from Cdk5<sup>+/+</sup> embryo brain lysates and then subjected to in vitro kinase assays in the presence of γp32-ATP. Autoradiographs of the kinase assay products clearly show that Cdk5 directly phosphorylates coronin 1a (Figure 2.5F). To confirm the relative importance of threonine residues identified by mass spectrometry analysis, we synthesized 17mer coronin 1a peptides (416-432) and replaced threonine-418 and/or threonine-424 with alanine (Figure 2.5G). These peptides were then used in an in vitro kinase assay as substrates for Cdk5.
All three mutations were associated with a significant reduction in Cdk5-mediated phosphorylation of the coronin 1a peptide compared to wild type, with threonine-418 being most important (Figure 2.5G).

**Disruption of Cdk5 activity impairs actin polarization and migration of lymphocytes to specific chemokine signals**

Genetic deletion of coronin 1a in mice established the absolute requirement of this protein in T cell antigen receptor function\textsuperscript{111}. Coronin 1a has been shown to bind directly to F-actin *in vitro* and to co-localize with F-actin structures *in vivo*\textsuperscript{112}. To determine the impact of Cdk5-mediated coronin 1a phosphorylation on F-actin and coronin 1a association, we collected lymphocytes from C57BL6\textsuperscript{+/+}C, Cdk5\textsuperscript{+/+}C and Cdk5\textsuperscript{-/-}C mice after EAE induction. As expected, both normal (Figure 2.6A) and control lymphocytes (Figure 2.6B) showed homogeneous co-localization of coronin 1a and F-actin with slight polarization in control cells but no discernable difference among lymphocytes isolated from the C57BL6\textsuperscript{+/+}C, Cdk5\textsuperscript{+/+}C and Cdk5\textsuperscript{-/-}C mice. Interestingly, Cdk5\textsuperscript{+/+}C encephalitogenic lymphocytes exhibited a distinct polarization of F-actin and coronin 1a, with most of the coronin 1a clustered on the pole directly opposite of F-actin clustering and this phenomenon was completely lost in Cdk5\textsuperscript{-/-}C encephalitogenic lymphocytes (Figure 2.6C & D). By contrast, Arp2/3 polarization was not compromised in Cdk5\textsuperscript{-/-}C encephalitogenic lymphocytes (Figure 2.10). These results support an essential role for Cdk5-mediated coronin 1a phosphorylation in actin dynamics, revealing an important mechanism through which Cdk5 may control both T cell activation and migration.
Several chemokines and their receptors have been shown to play a major role in the recruitment of lymphocytes to the CNS during EAE\textsuperscript{113}. Moreover coronin 1a deficient lymphocytes fail to migrate towards CCL19\textsuperscript{111}. Therefore, we next analyzed the impact of either pharmacologic inhibition or genetic deletion of Cdk5 on lymphocyte migration toward the chemokine CCL19. In transmigration assays, there was a 55% reduction in the migration of Jurkat cells toward CCL19 (MIP3-β) and about 10 fold decrease in migration towards of SDF1α and SDF1β (CXCL12) in the presence of 10 µM roscovitine (Figure 2.11). Finally, migration of Cdk5\textsuperscript{+/−} lymphocytes towards CCL19 was significantly reduced when compared to migration of Cdk5\textsuperscript{+/-} control lymphocytes (Figure 2.6E). Collectively these results suggest that Cdk5 mediated coronin 1a phosphorylation is required for proper lymphocyte activation and migration.

2.5 Discussion

More than a century after the first description of the clinical and pathological characteristics of MS\textsuperscript{114}, we have gained immense knowledge regarding the potential molecular and cellular mechanisms mediating disease pathogenesis. Aberrant activation of immune cells is a major hallmark of this autoimmune disease, thus therapeutic efforts in MS patients have been largely directed toward suppression of the activated immune system. The initiation of the immune cell response requires dynamic processing of the actin cytoskeleton and involves the recruitment of different proteins within the cell to form the “immune synapse”, enabling the cell to migrate towards a specific chemokine signal. Several protein kinases have been implicated in this process, but how these
proteins collaborate to enable lymphocyte activation and promote the genesis of an autoimmune response has not been fully elucidated.

In the current study, we explore the role of Cdk5 in immune cells. Our current knowledge suggests that kinase activity of ubiquitously expressed Cdk5 is mainly restricted to post mitotic neurons due to the predominant expression of its activating partner proteins p35 and p39 in these cells. However, the abundance of Cdk5 expression in non-neuronal cells argues against a functional irrelevance of Cdk5 in these lineages and rather points to a potentially important role for this kinase in their normal cell physiology. It is likely that Cdk5 is recruited or activated by cells in response to external stimuli that either influence their state of activation or trigger a differentiated function such as the secretion of cytokines or cell migration. In this context, the absence of Cdk5 kinase activity in non-neuronal cells may not impair normal development or distribution of cells, but rather affect cellular events that are required for host responses to harmful stimuli or to an external challenge.

The relevance of Cdk5 kinase activity in immune cells has not been extensively studied. However, there are several common signaling partners among neurons and immune cells that are participants in the control of gene transcription and cytoskeletal architecture, and the Cdk5-mediated phosphorylation of these proteins is known to modulate their function in neurons. The dependency of these proteins on Cdk5 function in immune cell signaling has not been explored. For example, Cdk5-mediated phosphorylation induces STAT3 transcriptional activity\textsuperscript{115} and suppresses MEF2 mediated transcription\textsuperscript{116} in neurons, and both of these transcription factors are critical regulators of T cell\textsuperscript{117} and B cell\textsuperscript{118} activation. Moreover, Cdk5 is known to modulate actin dynamics in neurons through
phosphorylation of proteins involved in maintaining cytoskeletal architecture and promoting neuronal migration such as the ERM protein ezrin\textsuperscript{119}, WAVE\textsuperscript{120}, WAVE2\textsuperscript{121}, FAK\textsuperscript{122}, and also Rac\textsuperscript{123} and Pak\textsuperscript{124}. These proteins are similarly known to regulate reorganization of the actin cytoskeleton in immune cells during TCR signaling, lymphocyte differentiation and, migration, and to also orchestrate effector function\textsuperscript{125}. Post-translational modification of these proteins by their phosphorylation is known to regulate cytoskeletal dynamics in immune cells\textsuperscript{126,127}. However, the functional relevance of Cdk5 in modulating cytoskeletal proteins during lymphocyte activation has not been explored.

The results presented herein suggest that Cdk5 is a non-redundant kinase in immune cells whose activity is required for lymphocyte activation. When total lymphocytes or purified T cells are activated \textit{in vitro} by antigenic stimulation or by TCR ligation, they demonstrate a significant increase in Cdk5 activity. It is important to note that the up-regulation of Cdk5 activity observed in lymphocytes of MOG/CFA immunized mice is not seen following immunization with CFA alone. The most likely explanation for this difference is that CFA alone induces only a transient activation of lymphocytes that wanes within three weeks, unlike the sustained, adaptive lymphocyte response to MOG. Regardless, it may be informative to see whether there is a short-term induction of Cdk5 activity (either local or systemic) following exposure to CFA alone, and if so, to explore whether this plays a role in the establishment of disease in this model.

Furthermore, pharmacologic suppression of Cdk5 activity or targeted disruption of Cdk5/p35 gene expression impairs the lymphocyte response to TCR ligation or antigenic stimulation. Hematopoietic organs of Cdk5\textsuperscript{-/-} embryos develop normally and there are
no obvious phenotypic differences in either the number or ratio of immune cell populations in either Cdk5<sup>−/−</sup> or p35<sup>−/−</sup> mice compared to their wild type littermates (Figure 2.8D & F-H). Similarly, immunophenotyping of the Cdk5<sup>+/+C</sup> and Cdk5<sup>−/−C</sup> mice shows no differences in gross anatomic structure, cellularity, or lineage distribution in hematopoietic organs (bone marrow, spleen, thymus, lymph nodes) (Figure 2.8E).

However, when these mice are challenged through induction of EAE, the spleen and lymph nodes of Cdk5<sup>−/−C</sup> mice are significantly smaller with the substantial reduction in tissue weight and cellularity, and disorganized tissue architecture compared to the Cdk5<sup>+/+C</sup> control mice (data not shown). Thus, while Cdk5<sup>−/−C</sup> naïve lymphocytes develop normally, their coordinated response to antigenic stimuli is hampered as demonstrated by the delayed development and reduced severity of EAE.

Interestingly, Cdk5<sup>−/−C</sup> encephalitogenic splenocytes, collected either during the active phase (10 days after EAE induction) or chronic phase (21 days after EAE induction) of EAE, show significant reduction in the production of IL2, IL6, IL17, TNF and GM-CSF when compared to Cdk5<sup>+/+C</sup> cells, following restimulation with MOG antigen <em>in vitro</em>. We did observe a significant drop in IFNγ cytokine production from these cells during active phase but the difference was not significant in chronic phase (Figure 2.12). The observed difference in cytokine production may reflect either a reduction in the number of MOG-specific T cells, impaired TCR stimulation, or a skewing in the differentiation of Cdk5<sup>−/−C</sup> lymphocytes. Regardless the observed reduction in Th1 and Th17 cytokines production provides a plausible explanation for the reduced severity of disease in these mice.
The phosphoproteomic studies described herein show that Cdk5-dependent modulation of immune cell function is partially mediated through the post-translational modification of coronin 1a. Coronin is a conserved actin binding protein that promotes different cellular processes that rely on rapid remodeling of the actin cytoskeleton. Deletion of the coronin 1a gene in mice is associated with alterations in cellular steady-state F-actin formation in lymphocytes\textsuperscript{111} and reductions in lymphocyte survival, migration, and Ca\textsuperscript{2+} release from intracellular stores\textsuperscript{128}. Coronin 1a gene deletions are associated with severe combined immunodeficiency in humans\textsuperscript{129, 130} and nonsense mutation of the coronin 1a gene (\textit{Lmb3} locus) protects against the induction of systemic lupus in mice\textsuperscript{131}. The crystal structures of a C-terminal truncated form of conronin 1a (residues 1-402) along with a C-terminal fragment (residues 430-461) have been solved\textsuperscript{132}; however, the structure information with respect to residues 403-429, which contains the Cdk5-targeted threonine-418 phosphorylation site has not been resolved. It is noteworthy that this domain contains a linker region (residues 356-429) of this protein, which is considered essential for interaction of the coronin 1a homotrimer complex with cytoskeleton\textsuperscript{133}. Here we report for the first time that Cdk5 directly phosphorylates coronin 1a on threonine-418. It is quite possible that phosphorylation of threonine-418 is required for the association of a positively charged stretch of linker region with F-actin. Interestingly, Pho85, a functional homologue of Cdk5 in budding yeast\textsuperscript{134}, has been shown to phosphorylate yeast coronin 1 on multiple sites\textsuperscript{135}. In the current study we demonstrate that coronin 1a is directly associated with and is phosphorylated by Cdk5, and that this phosphorylation is required for F-actin nucleation but not for Arp2/3 polarization in activated lymphocytes during EAE.
Finally, specific chemokine receptors have been implicated in MS and their roles have been demonstrated in the EAE model\textsuperscript{136}. In addition to regulating lymphocyte homing to secondary lymphoid tissue, the chemokine CCL19 participates in the pathophysiology of EAE by controlling T cell migration into the central nervous system\textsuperscript{137}. Similar to coronin 1a knockout lymphocytes\textsuperscript{111}, either the pharmacologic suppression or genetic deletion of Cdk5 in lymphocytes impairs migration towards CCL19. Whether these results are a direct and specific consequence of altered Cdk5-mediated phosphorylation of coronin 1a remains to be proven, and thus future studies will explore the functional consequences of Cdk5-mediated phosphorylation of coronin 1a on threonine-418.

In summary, Cdk5 now joins a growing list of proteins that were first discovered in the nervous system, such as Dscam and semaphorins that have since been found to have an important function in immune cells\textsuperscript{138}. This study provides novel insight into the regulation of lymphocyte activation and function through Cdk5-mediated coronin 1a phosphorylation. This discovery of an important “extraneuronal” role for Cdk5 as a biochemical intermediate in T cell signaling expands the repertoire of nonneuronal activities for a molecule that was previously assigned to a predominantly lineage-restricted function in the CNS. Our results have implications for a number of neurodegenerative and mental health disorders wherein immune-mediated activation of Cdk5 may contribute to the predisposition and pathogenesis of diseases including MS. These data establish a new paradigm that links activation of Cdk5 in immune cells to the pathogenesis of disorders associated with inflammation and provide a strong rationale for the development and clinical evaluation of novel inhibitors of Cdk5 in the context of immune-mediated diseases such as MS.
Figure 2.1. Increased Cdk5 activity in the CNS of mice with EAE. Mice were
sacrificed 4 weeks after EAE induction (A) Western blot shows increased p35 and p25 in
lysates of diseased lumbar spinal cord and brain stem. Data represent three independent
experiments. (B) Quantitative PCR for Cdk5 and p35 transcripts shows an increase in
mononuclear cells harvested from affected mice. Data represent 10 pairs of mice
(control) and 6 pairs of mice (EAE) of two independent experiments. (C and D) Cdk5
kinase activity assays were measured as γ-p32 incorporation in Cdk5 specific substrate,
NF-H peptide. (C) Tissue lysates prepared from brain stem and lumbar spinal cord show
a correlation between Cdk5 expression and kinase activity. Data represent three
independent experiments. Specificity of Cdk5 kinase activity was confirmed by using
roscovitine (10 µM) and (D) Cdk5−/− embryo (E16.5) brain lysates as controls. All data
are the mean ± SEM **P< 0.01, student t test.
Figure 2.1. Increased Cdk5 activity in the CNS of mice with EAE.
Figure 2.2. Cdk5 activity is up-regulated in lymphocytes after TCR or antigen specific stimulation. Total T cells were isolated from lymph node and spleen, and then stimulated with CD3/CD28. (A) PCR and Western blot analyses showed increased expression of both Cdk5 and p35 upon stimulation. Data represent three independent experiments of six pairs of mice. (B-F) Cdk5 kinase activity was measured as γ-p32 incorporation in Cdk5 specific substrate, NF-H peptide. (B) Cdk5 kinase activity in T cells was increased following stimulation with or without CD3/CD28. Data represent two independent experiments of six pairs of mice. (C) Addition of IL7 alone or with CD3/CD28 further enhanced Cdk5 activity. Data represent eight pairs of mice. (D) Total splenocytes or (E) CD4+ T cells collected after EAE induction and cultured with or without MOG (35-55) showed increased Cdk5 activity. Data are derived from three independent experiments with six to eight pairs of mice. (F) Specificity of Cdk5 kinase activity was confirmed by using Cdk5−/− embryo (E16.5) brain lysates as a control. All data are the mean ± SEM **P<0.01, one way ANOVA followed by student t test.
Figure 2.2. Cdk5 activity is up-regulated in lymphocytes after TCR or antigen specific stimulation.
Figure 2.3. Disruption of Cdk5 activity abrogates antigen receptor mediated lymphocyte activation. Lymphocyte activation and proliferation were measured by $^{3}$H-thymidine incorporation. (A) Cdk5$^{+/+}$ and Cdk5$^{-/-}$ and (B) p35$^{+/+}$ and p35$^{-/-}$ T cells were isolated and activated with CD3/CD28. Lymphocytes were collected from (C) Cdk5$^{+/+}$, Cdk5$^{-/-}$ and (D) p35$^{+/+}$, p35$^{-/-}$ and p35$^{-/-}$ mice 4 weeks after EAE induction and stimulated in vitro with MOG. Each data set is derived from a minimum of four independent experiments with four to six pairs of mice. All data are mean $\pm$ SEM **P<0.01, ***P<0.001 one way ANOVA followed by student t test.
Figure 2.3. Disruption of Cdk5 activity abrogates antigen receptor mediated lymphocyte activation.
Figure 2.4. Abrogation of Cdk5 activity in lymphocytes ameliorates clinical signs and associates with reduced pathology in EAE. (A) Cdk5\textsuperscript{+/+}C, Cdk5\textsuperscript{−/−}C and C57Bl6\textsuperscript{+/+}C mice were immunized with MOG to induce EAE and routinely observed for survival (Kaplan-Meier survival curve) and (B) for development of clinical signs of EAE. Data represent three independent experiments with a total of 26 pairs of mice. Four weeks post induction of EAE, mice were sacrificed and spinal cord was processed and stained (C) with hematoxilin and eosin to assess inflammation, with Luxol fast blue to analyze demyelination and with Bielschowsky stain to measure axonal loss (each indicated by black arrowheads). Scale bars, 200 µm. Data represent three independent experiments. (D-F) C57BL6 wild type mice received encephalitogenic lymphocytes from either (D) Cdk5\textsuperscript{+/+}C or Cdk5\textsuperscript{−/−}C mice, or from either (E) p35\textsuperscript{+/+}, p35\textsuperscript{+/−} and p35\textsuperscript{−/−} mice and were observed closely for the development of clinical signs of EAE. Data represent two independent experiments with nine pairs of mice (F) Lumbar spinal cord from mice receiving either p35\textsuperscript{+/+} or p35\textsuperscript{−/−} encephalitogenic immune cells was removed and stained with hematoxilin and eosin to evaluate for inflammation. Scale bars, 200 µm. Data represent two independent experiments.
Figure 2.4. Abrogation of Cdk5 activity in lymphocytes ameliorates clinical signs and associates with reduced pathology in EAE.
Figure 2.5. Cdk5 physically interacts with and phosphorylates coronin 1a. Protein lysates were prepared from lymphocytes collected after 10 days post EAE induction in Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C mice and Western blots were probed with anti (A) Cdk5 (B) MPM-2 and (C) coronin 1a antibodies. Data represents three independent experiments with six to eight pairs of mice. (D) Coronin 1a-threonine-418 phosphorylation of Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C encephalitogenic lymphocytes as confirmed by selected ion chromatography. Data represent six pairs of mice of two independent experiments. (E) Coronin 1a protein was immunoprecipitated from Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C encephalitogenic lymphocytes and Western blot was performed using antibodies against Cdk5 (upper panel) and coronin 1a (lower panel). Data represent three independent experiments. (F) Coronin 1a immunoprecipitated from normal lymphocytes was used as a substrate for Cdk5 in a kinase assay. The upper panel depicts the autoradiograph of phosphorylated coronin 1a by Cdk5 and the lower panel is the Coomassie blue stained gel showing total coronin 1a used in the kinase reaction. Data represent three independent experiments. (G) Coronin 1a peptide harboring amino acid sequence of wild type protein from 416-432 (418<sup>T</sup> & 424<sup>T</sup>) or mutant peptide where threonine was replaced with alanine or aspartate. Data represent five independent experiments. (H) Cdk5 mediated coronin 1a wild type or mutant peptide phosphorylation was measured through γ-p32 incorporation. Cdk5 used in this kinase assay was collected from mouse embryo brain and Cdk5 KO brain served as control. Data represent three independent experiments. All data are the mean ± SEM **P<0.01 and *P<0.05, one way ANOVA followed by student t test.
Figure 2.5. Cdk5 physically interacts with and phosphorylates coronin 1a.
Figure 2.6. Disruption of Cdk5 activity diminishes actin polarization and migration towards CCL19. Cdk5+/+C, Cdk5−/−C and C57BL6+/+C mice were immunized with (A) PBS (normal) (B) CFA + PTX (control) or (C) MOG(35-55) + CFA +PTX (EAE) and four weeks later lymphocytes were collected and stained for F-actin by using phalloidin (red) and coronin 1a (green) antibody. The white arrows indicate polarization of F-actin and the yellow arrows indicate polarization of coronin 1a on the opposite end. (D) These polarized cells were counted from 6 different regions by a person blinded to sample identity. The data represent two independent experiments of six pairs of mice (E) The number of cells harvested from the lower chamber containing 100 ng CCL19 in transmigration assays, where the upper chamber contains primary lymphocytes collected from either Cdk5+/+C or Cdk5−/−C mice. Scale bars, 100 µm. Data represent three independent experiments of five pairs of mice, mean ± SEM **P<0.001, one way ANOVA followed by student t test.
Figure 2.6. Disruption of Cdk5 activity diminishes actin polarization and migration towards CCL19.
**Figure 2.7. Inhibition of Cdk5 activity by roscovitine treatment abrogates antigen receptor mediated lymphocyte activation.** Lymphocyte activation and proliferation was measured by $^3$H-thymidine incorporation. (A) T cells were isolated and activated with either CD3/CD28 or PMA/Ionomycin or allogeneic dendritic cells in the presence or absence of 10 µM roscovitine. (B) T cell viability was measured with MTT assay, following exposure to roscovitine. (C) Lymphocytes were collected 4 weeks after EAE induction and stimulated in vitro with MOG in the presence or absence of 10µM roscovitine. Each data represents minimum of five independent experiments. All data are mean ± SEM. **P<0.01, ***P<0.001 one way ANOVA followed by student t test.
Figure 2.7. Inhibition of Cdk5 activity by roscovitine treatment abrogates antigen receptor mediated lymphocyte activation.
Figure 2.8. Generation and characterization of immune chimeric mice and their immunophenotyping. (A) Schematic presentation of hematopoietic cell transfer. In brief, three month old female C57Bl6 (CD45.1) congenic mice were exposed to two doses of 1000Gy gamma radiation with a 6 hr interval. After 24 hrs, these mice received hematopoietic cells collected from fetal liver and spleen of Cdk5\(^{+/+}\), Cdk5\(^{+/−}\) and Cdk5\(^{−/−}\) littermate embryos via tail vein injection to generate Cdk5\(^{+/+}\)C, Cdk5\(^{+/−}\)C and Cdk5\(^{−/−}\)C mice. (B) Approximately 4 months following stem cell rescue, blood was collected from recipient mice and subjected to FACS analysis to test the degree of chimerism by using antibody against CD45.1 and CD45.2 antigen. (C) Lymphocytes collected from these mice were also subjected to RT-PCR and Western blot using primers and antibody against Cdk5, respectively. Equal loading was confirmed by analyzing β-actin levels. Data represents three independent experiments. (D and E) Total hematopoietic cells were collected from Cdk5\(^{+/+}\), Cdk5\(^{+/−}\), and Cdk5\(^{−/−}\) mouse embryos (E16.5) (D) or from spleen and regional lymph nodes of either Cdk5\(^{+/+}\)C or Cdk5\(^{−/−}\)C mice (E) and subjected to Immunophenotyping by flow cytometry using specific antibodies against CD3, CD4, CD8, CD11b, CD45R and CD34. Data represents 3 independent experiments. (F-H) Total cells were collected from spleen (F), regional lymph nodes (G), and thymus (H) of 8-12 weeks old female p35\(^{+/+}\) and p35\(^{−/−}\) mice and subjected to Immunophenotyping by flow cytometry using specific antibodies against CD11b, B220, CD4, and CD8. Data represents 4 independent experiments.
Figure 2.8. Generation and characterization of immune chimeric mice and their immunophenotyping.
Figure 2.9. Characterization of immune cell infiltrate in spinal cord after EAE induction. Representative sections of lumbar spinal cords from Cdk5^{+/+} and Cdk5^{+/+} obtained 3 weeks after EAE induction and immunostained for CD3 and CD4 (T cell), F4/80 (macrophage) and MPO (neutrophil). Arrows indicate positive immunostaining. Data are representative of three independent experiments of similar results. In brief, Slides were incubated for 60 minutes at room temperature with either 7.5 mg/ml of a rabbit anti-human CD3 IgG MAb that cross-reacts with mouse CD3 (clone SP7; Lab Vision; Fremont, CA), 2 µg/ml of purified goat polyclonal antisera to mouse CD4 (lot # EPE02(2); R&D Systems; Minneapolis, MN), 10 µg/ml of the rat IgG2b MAb F4/80 for detection of macrophages and microglia or 2 µg/ml of purified rabbit polyclonal antisera to myeloperoxidase for detection of granulocytes (lot 373A603A(1); Lab Vision; Fremont, CA). After TBS washes, slides were incubated for 30 minutes with 2.5 µg/ml biotinylated rabbit anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA), with 2.5 µg/ml biotinylated goat anti-rabbit IgG secondary antibody (Vector) to detect CD3 and myeloperoxidase, or 2.5 µg/ml biotinylated rabbit anti-goat IgG secondary antibody (Vector) to detect CD4, followed by Vectastain ABC Elite (Vector) for 30 minutes. The staining reaction was detected using metal enhanced 3,3' Diaminobenzidine (DAB) chromogen (Pierce Chemical, Rockford, IL) for four minutes. Slides were counterstained with Mayer’s hematoxylin to detect nuclei, dehydrated, mounted and coverslipped.
Figure 2.9. Characterization of immune cell infiltrate in spinal cord after EAE induction.
Figure 2.10. Arp2/3 failed to polarize in Cdk5<sup>−/−</sup>C encephalitogenic lymphocytes.

Cdk5<sup>+/+</sup>, Cdk5<sup>−/−</sup> and C57BL6<sup>+/+</sup> mice were immunized with (A) PBS (normal) (B) CFA + PTX (control) or (C) MOG(35-55) + CFA +PTX (EAE) and lymphocytes were collected from spleen and regional lymph nodes four weeks post immunization and stained for F-actin by using phalloidin (red) and Arp2/3 (green).
Figure 2.10. Arp2/3 failed to polarize in Cdk5$^{-/-}$ encephalitogenic lymphocytes.
Figure 2.11. Roscovitine treatment disrupts migration of murine T cells and human Jurkat cells towards CCL19 in a transmigration assay. The histogram shows the percentage of cells present in the lower chamber of a transwell containing 100ng of either SDF1α (CXCL12), SDF1β (CXCL12) or MIP-3β (CCL19) in a standard transmigration assay where the upper chamber contains 1 x 10^7 Jurkat cells treated with or without 10 μg roscovitine for 4 hours. Data represents three independent experiments mean ± SEM **P<0.001, one way ANOVA followed by student t test.
Figure 2.11. Roscovitine treatment disrupts migration of murine T cells and human Jurkat cells towards CCL19 in a transmigration assay.
Figure 2.12. *In vitro* cytokine production by encephalitogenic splenocytes following restimulation with MOG (35-55). Immune chimeric mice were immunized with MOG (35-55) and cells were collected from spleen and draining lymph nodes during the active phase (10 days after immunization) or the chronic phase (21 days after immunization) of EAE. These cells were cultured for 3 days *in vitro* in presence or absence of MOG (35-55) and supernatants were analyzed for cytokine production using OptEIA ELISA sets, BD Bioscences (IL2, IL6, IFN-γ, TNF and GMCSF) and Duoset ELISA development system, R&D systems (IL17). The data is representative of 3 independent experiments of at least 4 to 6 mice in each group. All data are mean ± SEM **P<0.01, and *P<0.05 one way ANOVA followed by student t test.*
Figure 2.12. *In vitro* cytokine production by encephalitogenic splenocytes following restimulation with MOG (35-55).
Chapter 3: 

Cdk5 controls IL-2 Gene Expression via Repression of the mSin3a-HDAC Complex

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3.1 Abstract

Cyclin-dependent kinase 5 (Cdk5) is a unique member of a family of serine/threonine cyclin-dependent protein kinases. We previously demonstrated disruption of Cdk5 gene expression in mice impairs T-cell function and ameliorates T-cell-mediated neuroinflammation. Here, we show Cdk5 modulates gene expression during T-cell activation by impairing the repression of gene transcription by histone deacetylase 1 (HDAC1) through specific phosphorylation of the mSin3a protein at serine residue 861. Disruption of Cdk5 activity in T-cells enhances HDAC activity and binding of the HDAC1/mSin3a complex to the IL-2 promoter, leading to suppression of IL-2 gene expression. These data point to essential roles for Cdk5 in regulating gene expression in T-cells and transcriptional regulation by the co-repressor mSin3a.

3.2 Introduction

Cyclin-dependent kinase 5 (Cdk5) is distinguished from other serine/threonine CDKs as it is known to modify a very broad range of protein substrates in a manner that is dependent on the specific co-activator proteins p35 and/or p39 and independent of classical cyclins². These obligate partners of Cdk5 are both constitutively expressed in neuronal stem cells and post-mitotic neurons, suggesting a more lineage-restricted
activity for Cdk5. However, there is increasing evidence suggesting a significant function for Cdk5 exists in other lineages including immune cells and this activity has now been linked to disorders of non-neuronal tissues. Cellular processes known to be regulated by Cdk5 include neuronal cell migration and survival, T cell activation, insulin resistance, and cancer cell invasion and metastasis. Thus, Cdk5 is now recognized as a potential therapeutic target for diseases including neurodegeneration, autoimmunity and cancer. The relevance of Cdk5 activity to these disorders may be attributed to a growing list of Cdk5 substrates that include transcription factors such as Stat3, modulators of cell viability such as Bcl-2, and actin modulators such as coronin-1a and other members of the moeisin family of proteins.

We have recently shown T cells isolated from Cdk5-deficient immune chimeric mice (Cdk5−/−) or p35 knockout mice (p35−/−) exhibit a diminished response to T cell receptor (TCR) ligation, and that induction of Cdk5 activity during T cell activation is necessary for post-translational modification of coronin-1a, an actin co-modulatory protein essential for T cell survival. We hypothesized that Cdk5 must act through a series of coordinated mechanisms to control T cell function and differentiation, and that this may include control of the expression of specific genes required for T cell activation. For example, the suppressed proliferative response of T cells deficient in either the expression or activity of Cdk5 may reflect a defect in the expression of autocrine factors, such as IL-2, that are known to be essential for an optimal mitogenic response following TCR activation. Indeed, the autocrine expression of IL-2 following T cell activation is important for both T-cell differentiation and survival.
Several studies have recently highlighted a role for the classical zinc-dependent histone deacetylases (HDACs) in repressing IL-2 gene expression. Previous reports have implicated Cdk5 as a regulator of the HDAC1 complex although direct phosphorylation of HDAC1 by Cdk5 has never been demonstrated. Here we explore whether the impaired T cell responses observed in Cdk5 deficient T cells reflect a defect in the autocrine expression of IL-2 and whether this may be linked to Cdk5 regulation of the HDAC1 repressor complex. Our data reveal mSin3a, an essential component of the HDAC1-repressor complex, to be a novel substrate of Cdk5. Disruption of either the expression or the activity of Cdk5 enhances HDAC activity and increases occupancy of the IL-2 promoter by the HDAC1/mSin3a complex, ultimately leading to suppression of IL-2 expression. Our data establish an essential role for Cdk5 in regulating gene expression in T cells through post-translational modification of the co-repressor molecule mSin3a. A precise understanding of these mechanisms will provide a rationale for the therapeutic targeting of either Cdk5 or selected Cdk5 substrates in the setting of T cell mediated disease.

3.3. Materials and Methods

Animals. C57BL/6 mice, ages 6-8 weeks, were purchased from The Jackson Laboratory and were used for T cell isolations. Cdk5-null immune chimeric mice used in these studies were generated in our research facility. All animals were housed in micro-isolator cages and maintained in climate/light controlled rooms with free access to food and water. Studies performed were in compliance with the procedures approved by the
Case Western Reserve University School of Medicine’s Institutional Animal Care and Use Committee.

**Antibodies.** The Cdk5 antibody (C8), mSin3a antibody, His-probe antibody and rabbit IgG antibody were purchased from Santa Cruz Biotechnology and used for both immunoprecipitation as well as Western blotting. HDAC1 antibody used for both Western blotting, immunoprecipitation and ChIP assays was purchased from Abcam. The MPM-2 antibody used for both immunoprecipitation and western blot experiments was purchased from Millipore Inc. Monoclonal mouse b-actin antibody was purchased from Sigma.

**Cell Line.** Jurkat T cell lines were cultured in RPMI medium containing 10% (vol/vol) fetal bovine serum and grown according to recommended protocol (ATCC).

**Cdk5 Kinase Assay.** Radioactive Cdk5 kinase activity assays were performed as previously described\(^\text{140}\). In brief, total protein cell lysates were prepared and then incubated overnight at 4ºC with Cdk5 antibody. Afterwards, protein A-Agarose beads were added to samples for 3-5 hours at 4ºC. Following immuno-precipitation, samples were combined with desired substrates and incubated at 30ºC after the addition of ATP. Similarly, non-radioactive Cdk5 kinase assays were performed using ADP-Glo Kinase Assay detection kit purchased from Promega to detect the occurrences of phosphorylation events.

**Chromatin Immunoprecipitation (ChIP) Assay.** Chromatin immunoprecipitation (ChIP) analysis was performed according to the manufacturer’s instructions (Upstate Biotechnology) with T cells under the indicated stimulation and treatment conditions.
Immunoprecipitated samples (DNA) were then subjected to semi-quantitative RT-PCR analysis of the IL-2 proximal promoter region.

**3H-acetyl CoA release assay.** Histone deacetylase activity was analyzed using a histone deacetylase assay kit (Upstate, Millipore) according to procedures described in the manufacturer’s protocol. [3H]-radiolabelled Histone H4 peptides were incubated with cell extracts obtained from T cells under various stimulation conditions. Released [3H]-acetate was assessed on a MicroBeta TriLux Counter to determine the HDAC activity in each sample.

**ELISA.** Cells were first cultured on culture dishes with or without CD3/CD28 stimulation for 48 hours; supernatants of cultured cells were then collected and subjected to Enzyme-Linked Immunosorbent Assays (ELISAs). ELISAs were performed as described in the manufacturer’s protocol for IL-2 ELISA set kits purchased from BD Biosciences.

**Immunoprecipitation assays.** Protein lysates were first diluted to a concentration of 1µg/mL and subsequently pre-cleared with protein A-Agarose beads (Santa Cruz) and then incubated overnight with rotation at 4°C with the immunoprecipitating antibody. Lysates were then incubated with protein A-Agarose beads for 3-5 hours with rotation at 4°C. After incubation, protein A-Agarose beads were collected and washed with lysis buffer. Samples were then resolved by SDS-PAGE and followed by Western blot analysis.

**Semi-quantitative RT-PCR.** Total RNA was extracted using Trizol reagent (Invitrogen) and complementary DNA was then prepared using cDNA first-strand synthesis kit (Invitrogen). PCR was performed with a Px2 Thermal Cycler (Thermo).
**Statistical Analyses.** Statistical evaluations were performed using the Prism computer program (GraphPad Software). Significant differences between experiments were assessed by comparing the means of data sets using the Student $t$ test, with a $p$ value of $<0.05$ considered significant.

**T cell receptor stimulation.** Cell culture plates were coated by incubating with anti-CD3 (3ug/mL) and anti-CD28 (1ug/ML) antibodies (BD Biosciences) diluted in PBS overnight at 4°C. Prior to use, plates were washed with fresh PBS twice.

**Roscovitine.** Roscovitine was purchased from Enzo Life Sciences. Stock solutions of 10mM were first prepared in DMSO and working concentrations of 10µM were used in experiments.

**T lymphocyte isolation.** A mixed population of cells was collected by first isolating cells from spleen and regional lymph nodes of mice and creating single cell suspensions by passing these tissues through a 40µm cell strainer (BD Biosciences). Cells were then incubated with ACK lysing buffer (Lonza) on ice for 5 minutes to deplete the mixed cell population of erythrocytes. T lymphocytes were purified through a process of negative isolation by passing mixed cells through MACS separation columns using a pan T isolation kit (MiltenyiBiotec) in accordance to the manufacturer’s protocol.

**Western blot analysis.** Protein samples were prepared from cellular lysates made in RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail tablet (Roche) in addition to a phosphatase inhibitor phosSTOP (Roche). Proteins were denatured by heating for 10 min at 94°C in sample loading buffer (2% SDS, 10% glycerol 80mM Tris, pH 6.8, and 1mM DTT). 50µg of total protein was separated by electrophoresis in 4-20% Tris-Glycine gels (Invitrogen). Proteins were transferred to a
0.2µm nitrocellulose membrane (Invitrogen) and subsequently blocked for 1 hour in blocking buffer (TBS containing 10% non-fat dry milk and 0.05% Tween 20). Membranes were incubated overnight at 4°C with a primary antibody and probed with horse radish peroxidase-conjugated secondary antibody for 1 hour in room temperature.

3.4. Results

**Cdk5 activity is essential for optimal IL-2 expression during T-cell activation.**

To discern whether induction of Cdk5 activity following T cell receptor (TCR) activation is required for normal T cell production of IL-2, we examined the effects of either Cdk5 gene deletion or pharmacological inhibition of Cdk5 activity on IL-2 production in mouse T-cells (Figure 3.1A). Disruption of Cdk5 activity by the selective Cdk inhibitor Roscovitine (ROS) results in a significant decrease in IL-2 production following T cell receptor (TCR) stimulation. Similarly, T cells deficient in Cdk5 gene expression exhibit a significant reduction in autocrine IL-2 production following TCR stimulation with anti-CD3/CD28 antibodies. This reduction in secreted IL-2 correlates with a decreased abundance of IL-2 mRNA transcripts when naïve wild type T cells undergo TCR stimulation in the presence of Roscovitine (Figure 3.1B). This relationship between Cdk5 activity and IL-2 mRNA expression is also observed when IL-2 expression is quantified following TCR stimulation of either Cdk5 deficient (Cdk5−/−) T cells or wild type (Cdk5+/+) T cells (Figure 3.1C). These results suggest that Cdk5 activity is required for optimal IL-2 expression following TCR stimulation and T cell activation (Figure 3.1D).
Cdk5 interacts with HDAC1 and modulates HDAC activity during T cell activation, but does not alter HDAC1 expression.

Previous reports indicate an essential role for the HDAC1 complex in repression of the IL-2 gene and recent studies implicate Cdk5 in regulation of the HDAC1 complex in neuronal cells. Thus, we decided to examine whether Cdk5 influences HDAC activity in T cells following T cell receptor stimulation. We first examined the physical association between HDAC1 and Cdk5 during T cell activation. A low level of Cdk5 protein is present in HDAC1 immunoprecipitates prepared from protein lysates of primary wild type naïve T cells and this association increases following TCR activation (Figure 3.2A), correlating with the induction of Cdk5 protein expression and activity following TCR stimulation. Next, to determine whether Cdk5 activity modulates the expression of HDAC1 in either resting or activated T-cells, we performed RT-PCR and Western blot analysis for HDAC1 with lysates prepared from naïve cells either before or after TCR-activation of either Cdk5−/− or Cdk5+/− T cells (Figure 3.2B). HDAC1 mRNA and protein expression were similar under all conditions, suggesting HDAC1 gene expression is not under the control or influence of Cdk5 signaling. However, the interaction between Cdk5 and HDAC1 observed in activated T cells suggests the potential for a Cdk5-dependent regulation of HDAC activity.

To address this possibility, we performed an HDAC-specific activity assay to determine whether the reduction in HDAC activity that follows TCR ligation is dependent on the function of Cdk5. A significant decrease in HDAC activity follows TCR ligation in
Cdk5+/+ T cells, however, this suppression not observed in Cdk5−/− T cells (Figure 3.2C). The failure to fully suppress HDAC activity in activated Cdk5−/− T cells supports our hypothesis by directly implicating Cdk5 in the HDAC-dependent regulation of IL-2 gene expression through post-translational modification of the HDAC1 complex (Figure 3.2D).

**Cdk5 phosphorylates the HDAC co-repressor protein mSin3a at serine residue 861.** Although the data above might implicate HDAC1 as a substrate of Cdk5, this possibility has never been directly confirmed. Thus, utilizing an *in vitro* Cdk5 kinase assay, we combined either HDAC1 protein immunoprecipitated from murine T cells or recombinant HDAC1 protein as substrates with Cdk5 isolated from murine T cells (Figure 3.3A). Our data show that HDAC1 is not a direct substrate of Cdk5; neither the recombinant nor the endogenous HDAC1 protein was phosphorylated by the endogenous Cdk5. However, the potential remained that Cdk5 could phosphorylate one of the potent co-repressor proteins that are essential regulators of HDAC activity. Therefore, we examined whether Cdk5 might phosphorylate mSin3a, a crucial component of the HDAC1 repressor complex. Utilizing both recombinant and immunoprecipitated endogenous mSin3a protein as substrates, we found significant phosphorylation of mSin3a by Cdk5 (Figure 3.3B).

Upon further examination of the mSin3a protein sequence, we found one specific site on the mSin3a protein, serin861, which corresponds to the preferred consensus sequence for established substrates of Cdk5 (proline-directed serine residue followed by a basic residue in the +3 position). To further determine the ability of Cdk5 to phosphorylate
mSin3a at this specific site, we created a mutant form of the mSin3a protein (S861A) with its serine861 residue substituted to an alanine (Figure 3.3C). Histidine-tagged wild-type and S861A forms of the mSin3a protein were then expressed following transfection into in HEK293T cells (Figure 3.3D). In Figure 3.3E, wild-type or mutant forms of mSin3a were combined as substrates with Cdk5 in an _in vitro_ kinase assay. Our data show a significant decrease in phosphorylation events when mutant mSin3a was used as a substrate, strongly indicating this specific residue as a site for phosphorylation by Cdk5 (Figure 3.3F).

**Cdk5 activity controls HDAC1/mSin3a complex formation through regulation of mSin3a protein abundance but has no effect on mSin3a mRNA.**

To further understand the effect of mSin3a phosphorylation by Cdk5, we examined both mSin3a mRNA transcript and protein abundance in either wild type or Cdk5\(^{-/-}\) T cells, before and after CD3/CD28 stimulation. As shown in Figure 4.4A, absence of Cdk5 expression does not alter expression of mSin3a RNA transcripts as examined by semi-quantitative RT-PCR. However, examination of mSin3a protein expression following TCR stimulation in wild type T cells reveal a reduction in mSin3a, whereas this reduction in expression is not observed in Cdk5\(^{-/-}\) T cells; implicating an essential role for Cdk5 in mSin3a protein expression. To determine whether phosphorylation of mSin3a by Cdk5 primes mSin3a for proteasomal degradation, cultures of either Cdk5\(^{+/+}\) or Cdk5\(^{-/-}\) T cells were activated in either the presence or absence of the proteasome inhibitor MG132. The presence of MG132 allowed for an accumulation of the mSin3a protein (Figure 4.4B) in both Cdk5\(^{+/+}\) and Cdk5\(^{-/-}\) T cells. To further examine this role of Cdk5 in regulating
mSin3a protein expression, we expressed both the wild-type and S861A forms of mSin3a in Jurkat T cells. In the absence of MG132, the level of expression of the S861A mutant protein observed in lysates of Jurkat cells was significantly higher than that of the wild type mSin3a protein. Addition of MG132 to these cultures of Jurkat T cells increased the abundance of both the endogenous mSin3a protein (Figure 4.4C, lane 4) and the transfected wild type mSin3A (Figure 4.4C, lane 5) to a level comparable to that observed for the S861A mutant in the absence of MG132. This latter observation underlies the augmented expression of mSin3a observed in Jurkat cells transfected with the S861A mutant and exposed to MG132 (Figure 4.4C, lane 6). Taken together, these data suggest that mSin3a phosphorylation at serine861 by Cdk5 regulates mSin3a protein expression through proteasomal degradation (Figure 4.4D).

Phosphorylation of Serine861 in the mSin3a protein disrupts formation of the HDAC1/mSin3a complex and leads to diminished HDAC1 occupancy of the IL-2 promoter.

We hypothesized that phosphorylation of mSin3a by Cdk5 would reduce the capacity to form a complex with HDAC1. This hypothesis is supported by the enhanced abundance of the mSin3a/HDAC1 complex in nuclear extracts of Cdk5−/− T cells following TCR stimulation when compared to extracts of activated Cdk5+/− T cells. (Figure 5.5A). To determine whether mSin3a phosphorylation by Cdk5 influences the capacity of HDAC1 to occupy and thereby repress the IL-2 gene promoter, we performed chromatin immunoprecipitation (ChIP) assays using primary T cells. In unstimulated wild type T cells, HDAC1 clearly occupies the IL-2 gene promoter, however, there is a minimal
detectable binding of HDAC1 to the IL-2 promoter in T cells after TCR stimulation. Consistent with our hypothesis, Cdk5−/− T cells exhibit increased binding of HDAC1 to the IL-2 promoter following TCR stimulation (Figure 5.5B). Finally, we performed the same ChIP assay using Jurkat T cells transfected with either the wild type or S861A mutant form of the mSin3a protein (Figure 5.5C). TCR stimulation in cells expressing wild type mSin3a show no detectable HDAC1 binding to the IL-2 promoter, whereas cells expressing the S861A mutant show significant levels of HDAC1 binding on the IL-2 promoter. In total, the data presented above suggest that Cdk5 phosphorylation of mSin3a negatively influences the occupancy of this region of the IL-2 gene promoter by HDAC1 (Figure 5.5D).

3.5. Discussion

Here we report a novel mechanism through which Cdk5 controls HDAC activity and reveal that T cells require functional Cdk5 in order to attain optimal IL-2 gene expression following TCR stimulation. Autocrine IL-2 expression is essential for optimal T cell activation following TCR stimulation and is required for the differentiation and survival of T cells. The repression of IL-2 gene expression by classical zinc-dependent histone deacetylases (HDACs)149, 151 is relieved during T cell activation. The data shown here demonstrate a requirement for Cdk5-mediated phosphorylation of mSin3a to relieve repression of the IL-2 gene promoter by the HDAC1-mSin3a repressor complex. The relationship between Cdk5 activity in T cells and autocrine IL-2 production is evident as the disruption of either Cdk5 expression or activity results in a significant decrease in IL-
2 production and a commensurate reduction in the abundance of IL-2 mRNA transcripts following TCR stimulation. These data suggest that Cdk5 controls the production of IL-2 through a mechanism that includes regulation of IL-2 gene transcription.

Cdk5 is known to regulate gene expression through several mechanisms and our data support the concept that this includes post-translational modification of the HDAC1 repressor complex\textsuperscript{152,153}. Histone deacetylases regulate gene expression during development and cell differentiation by modulating the accessibility of nucleosomes and by regulating acetylation of proteins within complexes that control gene transcription\textsuperscript{154,155}. For example, HDAC1 is known to associate with mSin3a and other proteins to form functional repressor complexes capable of silencing selected target genes in a context-dependent manner\textsuperscript{156}. Aberrant activity of Cdk5 has been linked to neurodegeneration through mechanisms that include inhibition of HDAC1 activity\textsuperscript{59}, however these studies have not clearly shown HDAC1 to be a direct substrate of Cdk5. Post-translational modification of HDAC proteins can modulate the capacity of HDACs to complex with other co-repressor proteins and potentially influence sub-cellular localization and enzymatic activity\textsuperscript{157,158}. Our data show that HDAC1 is not a direct substrate of Cdk5 as neither recombinant HDAC1 nor endogenous HDAC1 protein precipitated from T cells were directly phosphorylated by endogenous Cdk5 \emph{in vitro}. Thus, while Cdk5 physically associates with HDAC1, we show the modification of HDAC activity by Cdk5 instead is mediated through an effect on the HDAC-associated protein mSin3a.
Members of the mammalian class I HDACs such as HDAC1 repress gene transcription as components of large repressor complexes\textsuperscript{58,159}. HDAC1 associates with mSin3a\textsuperscript{156}, histone binding proteins and mSin3a-associated polypeptides that influence histone deacetylase activity\textsuperscript{160}. \textit{In silico} analysis of the amino acid sequence of the mouse mSin3a revealed an exact Cdk5 target motif at serine 861 and serine 860 in the human mSin3a. The kinase assays described in this report show Cdk5 directly phosphorylates both the recombinant mSin3a protein and endogenous mSin3a immunoprecipitated from T cells when either is used as a substrate in an \textit{in vitro} Cdk5 kinase assay. We further examined direct phosphorylation of mSin3a by Cdk5 by utilizing a S861A mutant form of the mSin3a protein. A significant reduction in phosphorylation of the S861A mutant relative to the wild type mSin3a in this \textit{in vitro} kinase assay with Ckd5 further indicates direct phosphorylation of the mSin3a protein by Cdk5 at the serine 861 residue.

The importance of post-translational modification of mSin3a as a mechanism to regulate mSin3a function has not previously been reported. Our data show that the abundance of mSin3a mRNA in T cells remains constant following TCR stimulation. Specifically, a comparison of mSin3a gene expression in naïve/quiescent T cells versus stimulated T cells shows mSin3a mRNA abundance is not influenced by Cdk5 activity (i.e neither targeted disruption of Cdk5 gene expression in T cells nor pharmacologic inhibition of Cdk5 activity in wild type T cells influences the abundance of mSin3a mRNA). However, the abundance of mSin3a protein decreased during T cell activation, an effect not observed in Cdk5 deficient T cells. These data suggested the potential for Cdk5 activity to regulate the stability of the mSin3a protein, an effect we further confirmed by
the addition of the proteasomal inhibitor, MG132. These data were corroborated in studies in activated Jurkat T cells, where the expression of the wild-type mSin3a protein was reduced relative to that of the S861A mutant. While our data with the proteasome inhibitor confirms that phosphorylation of mSin3a at Serine 861 causes the degradation of mSin3a, there exists the possibility that Cdk5-directed phosphorylation may also cause mSin3a localization to change. Studying the potential for this phenomenon as an additional function of Cdk5-directed phosphorylation would be an important aspect to address in the future.

In addition to an effect on mSin3a abundance, we found formation of the mSin3a/HDAC1 complex in activated T-cells was enhanced in nuclear lysates of T cells lacking Cdk5 when compared to wild type T cells. This effect of Cdk5 on formation of the mSin3a/HDAC1 complex directly impact on the ability for HDAC1 to occupy the IL-2 promoter. A ChIP analysis revealed increased occupancy of HDAC1 on the IL-2 promoter both in Cdk5/− T cells cells in T cells expressing the S861A mutant version of the mSin3a protein. In sum, these observations clearly demonstrate that phosphorylation of mSin3a at Serine861 by Cdk5 leads to degradation of mSin3a, thereby repressing formation of a functional mSin3a/HDAC1 complex during T-cell activation.

Disruption of mSin3a protein expression and function has been shown to impair the normal transcriptional repression of specific target genes by the HDAC1 complex. The significance of this post-translational modification of mSin3a in T cells is supported by previous studies that have shown the repression of IL-2 gene transcription by the lineage
determining factor Ikaros is dependent on the association of Ikaros with mSin3 proteins\textsuperscript{162}, an event which is thought to be important in T cell anergy\textsuperscript{163}. The results presented here reveal a pivotal role of Cdk5 in controlling IL-2 gene expression during T cell activation, through post-translational modification of mSin3a, an event that impairs stability of the mSin3a-HDAC1 complex and thereby relieves repression of the IL-2 gene promoter by HDAC1.

Our data add to accumulating evidence pointing to the importance of the interaction between Cdk5 and the nuclear machinery regulating gene expression. Cdk5 has a prominent nuclear localization\textsuperscript{164} and is now known to modulate the activity of several transcription factors, including p53\textsuperscript{165}, pRB\textsuperscript{2} and Stat3\textsuperscript{40}. A number of reports have also indicated the expression of its activator p35 in the nuclear compartment of cells, albeit at a level lower than in its cytoplasmic fraction\textsuperscript{38,166,167}. For instance, the N-terminal region of p35 has been found to interact and co-localize with the nuclear protein SET in the nucleus of the neurons. Additionally, Cdk5 phosphorylation of its nuclear substrate MEF2 has been shown; where Cdk5 specific phosphorylation of MEF2 inhibits this pro-survival transcription factor. It has been found that p35 nuclear import requires direct interaction with soluble import factors; and Imp-β, Imp-5, and Imp-7 have all been shown to transport p35 into the nucleus through an energy dependent manner. Our data here further corroborates these reports for Cdk5-p35 expression and activity in the nucleus of the cell. A role for Cdk5 in histone modification has been suggested by observations that include the demonstration that the chromatin modulating SET protein affects Cdk5/p35 activity\textsuperscript{166}, the observation that NCoR serves as an adaptor protein that
enhances association of Cdk5 with PPARgamma\textsuperscript{168}, and by the demonstration that the co-activator p35 binds to mouse Sds3, a core component of the HDAC-mSin3a complex\textsuperscript{153}. It is interesting that the latter interaction leads to Cdk5-mediated phosphorylation of Sds3 at serine 228, thereby enhancing transcriptional repression by this complex in neuronal and muscle lineages, suggesting that this control of HDAC function by Cdk5 is very context-dependent and potentially bidirectional.

Previous reports have shown inhibition of HDAC activity in T-cells suppresses IL-2 gene transcription and impairs proliferation and survival of T cells\textsuperscript{149}. These effects of HDAC inhibitors may also be attributed to increased acetylation of non-histone proteins, including transcription factors such as Stat3\textsuperscript{169}, which is a known substrate of Cdk5. Furthermore, the potential clinical utility of HDAC inhibitors in the treatment of autoimmune and inflammatory disorders has also been partially attributed to the ability of these molecules to affect the acetylation status of Foxp3 and the induction of regulatory T-cells\textsuperscript{170}. Acetylation of Foxp3 is also known to enhance the ability of Foxp3 to bind to the IL-2 gene promoter and repress IL-2 expression.

The onset of a number of pathological conditions including chronic inflammation and autoimmunity is often the result of aberrant gene transcription and thus the regulatory role of HDACs in immunity is of significant interest. Recently, roles for HDACs have been studied as important for innate immunity as well as in lymphocyte development and function\textsuperscript{171}. Existing literature has documented a role for class I HDACs in negatively regulating inflammatory cytokine production. By contrast, in the context of immunity, the
multitude of HDAC isoforms are known to exert differential effects and often resulting in both positive and negative regulation of the immune response. Compounds that have been developed to inhibit HDACs act through chelating Zn$^{2+}$ ions at the active site, and they have been examined for therapeutic use in a number of diseases. Three HDAC inhibitors are now in clinical use for T cell lymphoma, epilepsy and bipolar disorders whereas a number of additional inhibitors are in clinical trials. Additionally, ongoing studies of HDAC inhibitor use are being performed on animal models in inflammatory diseases such as graft versus host disease, septic shock and inflammatory bowel diseases. Although these broad-spectrum inhibitors display some degree of selectivity for different HDAC enzymes, ultimately they target multiple isoforms and classes of HDACs. Indeed the pleiotropic and often contrasting effects of the different HDACs on the immune response, it is expected that use of these inhibitors have shows evidence of contraindications. Thus our identification of Cdk5 as a novel target molecule able to modulate the HDAC1-mSin3a repressor complex offers an exciting and novel approach to the treatment of immune mediated disease without the above caveat associated with current HDAC directed therapies. In sum, our data demonstrate the importance of Cdk5 as a modulator of HDAC activity in T-cells and suggest a potential mechanism through which small molecule inhibitors of Cdk5 activity may serve to impair the progression of T-cell mediated autoimmune and inflammatory diseases.
**Figure 3.1. Cdk5 is required for optimal IL-2 expression.** (A) Naïve wild type (Cdk5<sup>+/−</sup>) T cells or Cdk5-deficient (Cdk5<sup>−/−</sup>) T cells were activated with plate bound anti-CD3 and anti-CD28 antibodies for 48hrs in the presence or absence of Roscovitine. Amounts of IL-2 present in T cell supernatants were detected by ELISA. (B) Semi-quantitative RT-PCR analysis was performed to measure IL-2 mRNA expression in T cells activated by CD3/CD28 antibodies for 12hrs in the presence or absence of Roscovitine, and similarly, (C) IL-2 mRNA expression was determined in either Cdk5<sup>+/−</sup> or Cdk5<sup>−/−</sup> T cells following anti-CD3/CD28 activation by semi-quantitative RT-PCR. (D) Summary diagram of IL-2 production after T cell activation with or without the presence of Cdk5 protein or Cdk5 activity.
Figure 3.1. Cdk5 is required for optimal IL-2 expression.
Figure 3.2. Cdk5 interacts with HDAC1 and alters HDAC activity in activated T cells. (A) Immunoprecipitates of HDAC1 protein were prepared from lysates of T cells both before and after CD3/CD28 stimulation for 48hrs. Precipitates were subjected to Western blot for both Cdk5 and HDAC1 protein. (B) The expression of HDAC1 mRNA and protein was examined in both naïve and activated T cells isolated from both wild type (Cdk5+/+) T cells and Cdk5-deficient (Cdk5−/−) T cells both before and after anti-CD3/CD28 stimulation. (C) HDAC activity was measured in both quiescent and anti-CD3/CD28 stimulated Cdk5+/+ and Cdk5−/− T cells. (D) Model depicting how Cdk5/HDAC1 forms a protein complex and inhibits HDAC activity. Cdk5-deficiency does not alter HDAC1 expression but does relieve the suppression of HDAC activity.
Figure 3.2. Cdk5 interacts with HDAC1 and alters HDAC activity in activated T cells.
Figure 3.3. Cdk5 does not phosphorylate HDAC1 but does phosphorylate the mSin3a protein at serine residue 861. (A) Phosphorylation of HDAC1 by the Cdk5 kinase was measured by in vitro kinase assays. Cdk5 protein was immunoprecipitated from murine T cells and combined with either endogenous HDAC1 (End) isolated from murine T cells or with recombinant HDAC1 (Rec). Immunoprecipitates of Cdk5 from Cdk5/− T cells were used as a negative (Neg) control. Neurofilament H (NF-H) is a known Cdk5 substrate and was used as a positive (Pos) control. (B) Phosphorylation of mSin3a by Cdk5 was determined using the previously described kinase assay, in which immunoprecipitates of Cdk5 isolated from murine T cells were incubated with either the endogenously expressed mSin3a (End) protein or recombinant mSin3a (Rec). (C) Schematic of mSin3a protein showing the amino acid sequence in the wild-type and mutant form of the protein used in these studies. In the mutant mSin3a, serine residue 861 was mutated to an alanine residue. (D) Wild-type or mutant forms of mSin3a were transfected into HEK293T cells and examined for expression using Western blot analysis. (E) Phosphorylation of Wild-type and mutant mSin3a protein was measured using a Cdk5 in vitro kinase assay. Immunoprecipitates of Cdk5 isolated from murine T cells were combined with either endogenous (End), Wild-type (WT) or mutant (MUT) mSin3a protein. Positive (Pos) and negative (Neg) controls were the same as described above. (F) Schematic diagram depicting how Cdk5 phosphorylates the mSin3a protein specifically at the Serine861 residue.
Figure 3.3. Cdk5 does not phosphorylate HDAC1 but does phosphorylate the mSin3a protein at serine residue 861.
Figure 3.4. Cdk5 phosphorlyation of mSin3a at Serine861 disrupts the expression of the mSin3a protein. (A) RNA and protein expression for mSin3a were examined with semi-quantitative RT-PCR and Western blot analysis in wild type (Cdk5\(^{+/+}\)) T cells or Cdk5\(^{-/-}\) T cells with or without anti-CD3/CD28 activation. (B) mSin3a protein expression in either Cdk5\(^{+/+}\) or Cdk5\(^{-/-}\) T cells treated with or without the proteasome inhibitor MG132 for 8 hrs. (C) Protein expression for mSin3a were examined in anti-CD3/CD28 activated Jurkat cells transfected with either Wild-type (WT) or mutant (MUT) mSin3a plasmids. Cells were treated in the presence or absence of the proteasome inhibitor MG132. (D) Schematic diagram depicting phosphorylation of the Serine861 residue on mSin3a by Cdk5 and the resultant proteasomal degradation of mSin3a.
Figure 3.4. Cdk5 phosphorlyation of mSin3a at Serine861 disrupts the expression of the mSin3a protein.
Figure 3.5. Phosphorylation of mSin3a by Cdk5 disrupts the formation of the HDAC1/mSin3a complex. (A) HDAC1 immunoprecipitates were isolated from nuclear lysates of primary wild type (Cdk5^{+/+}) T cells or Cdk5^{-/-} T cells either before or after stimulation with anti-CD3/CD28 antibodies. Immunoprecipitates were subsequently probed for mSin3a and HDAC1 expression by Western blot. (B) ChIP analysis was performed to assess the binding of HDAC1 to the IL-2 promoter in either Cdk5^{+/+} or Cdk5^{-/-} T cells, either before or after activation with anti-CD3/CD28 stimulation. (C) Similarly, ChIP analyses were performed on Jurkat cells transfected with either Wild-Type (WT) or mutant (MUT) mSin3a plasmids to determine the binding of HDAC1 to the IL-2 promoter. (D) Diagram depicting the disruption of HDAC1 occupancy of the IL-2 promoter upon TCR stimulation, due to the presence of Cdk5/p35 activity, and the persistence of HDAC1 on the IL-2 promoter when the expression/activity of Cdk5 is disrupted.
Figure 3.5. Phosphorylation of mSin3a by Cdk5 disrupts the formation of the HDAC1/mSin3a complex.
Chapter 4:

Cyclin-dependent kinase 5 represses Foxp3 gene expression and Treg development through specific phosphorylation of Stat3 at Serine 727

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4.1. Abstract

Cyclin-dependent kinase 5 (Cdk5) is a ubiquitous member of the family of cyclin-dependent serine/threonine kinases whose contextual and lineage-restricted activity is regulated by association with highly specific co-activators, including p35 and p39. We previously demonstrated induction of both p35 expression and Cdk5 activity follows T cell receptor stimulation and is linked to post-translational modification of proteins that influence T cell activation and function. Here, we show IL-6-induced repression of Foxp3 gene expression in CD4+ T cells (Treg) requires Cdk5-mediated phosphorylation of the signal transducer and activator of transcription 3 (Stat3) specifically at Serine 727 (p-S727). Lineage-restricted disruption of Cdk5 gene expression in T cells abrogates the IL-6 suppression of TGF-β-induced Foxp3 expression. Phosphorylation at S727 by Cdk5 does not alter either the abundance or nuclear localization of Stat3, nor phosphorylation of Stat3 at tyrosine 705, but it is required for proper Stat3 DNA binding to the Foxp3 gene on the enhancer II region and for the consequent disruption of Smad-dependent TGF-β-induced activation of Foxp3. These data define Cdk5 as molecular mediator of
the repression of Foxp3 gene expression by inflammatory cytokines and suggest the potential to exploit specific inhibitors of Cdk5 to enhance the expansion of endogenous regulatory T cells.

4.2. Introduction

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the cyclin-dependent kinase (CDK) family of proline-directed serine/threonine kinases. In contrast to other CDKs, Cdk5 has no significant role in regulating cell cycle progression and does not require binding to cyclins to achieve kinase activity. Functionally, Cdk5 associates with its binding partners p35 and/or p39, whose constitutive expression is largely restricted to cells of neural crest origin, leading to the presumption that Cdk5 is principally a neuronal-specific regulator of processes including neuronal migration, synapse formation and neuronal survival. For example, the germ-line deletion of Cdk5 in mice results in altered cortical neuronal layering during development, clearly indicating an essential role for Cdk5 in neuronal development and function.

However, aberrant Cdk5 activity is also associated with several neurodegenerative and neuroinflammatory disorders. Furthermore, there are now several reports demonstrating the presence of Cdk5 activity in a number non-neuronal tissues, which suggest the importance of Cdk5 activity in the normal function of pancreatic beta-cells, monocytes, neutrophils, leukocytes, myocytes, epithelial cells, endothelial cells, and adipocytes. Thus, it is not surprising that Cdk5 is now implicated in the
pathogenesis of a number of diseases including cancer, diabetes and inflammation-mediated disorders.\textsuperscript{28}

We recently reported our observations that the expression of both Cdk5 and p35 increases following activation of normal, non-transformed T cells, and that this increase associates with the induction of Cdk5 kinase activity. More importantly, disruption of Cdk5 expression in T cells was protective in the murine model of experimental autoimmune encephalomyelitis (EAE), a T cell-mediated autoimmune pre-clinical model of multiple sclerosis (MS)\textsuperscript{140}. Thus, we hypothesized that Cdk5 may act through a number of different mechanisms to influence both T cell function and differentiation into specific T cell subsets. For example, the reduced susceptibility of Cdk5-deficient mice may result from an imbalance between T effector and T regulatory (Treg) cells. In the EAE model of MS, Foxp3\textsuperscript{+} Treg cells play a central role in controlling inflammation in the central nervous system (CNS) and ultimately their function may be important to improving clinical outcome\textsuperscript{174-176} as even a transient impairment in Tregs can alter the severity of EAE pathology.

A number of factors influence the differentiation of regulatory T cells and the induction of Foxp3, the principal transcriptional regulator of Treg differentiation. For example, the induction of Foxp3 expression that accompanies T cell receptor (TCR) stimulation in the presence of transforming growth factor-beta (TGF-\(\beta\)) is inhibited by a number of pro-inflammatory cytokines including IL-6, IL-27 and IL-21\textsuperscript{80}. These cytokines all share the ability to activate the signal transducer and activator of transcription 3 (Stat3) pathway
and Stat3 is known to inhibit Foxp3 gene expression through binding to a conserved enhancer (enhancer II) region on the Foxp3 gene promoter, effectively preventing proper binding of the activated TGF-β intermediate pSmad3 to the enhancer I region\textsuperscript{81}.

Stat3 is a DNA-binding transcription factor that is associated with a wide range of physiological processes. As with other members of the Stat family of proteins, Stat3 is activated by phosphorylation at a tyrosine residue close to the carboxyl-terminus (Y705). Additionally, Stat3 activity is also regulated by the phosphorylation of serine 727 located in the transactivation domain of the protein\textsuperscript{82}. Interestingly, this specific Stat3 Ser727 phosphorylation in T cell has been highlighted as having a distinct role in dictating CD4\textsuperscript{+} T cell differentiation into Th17 and Treg cells\textsuperscript{86}. Previous reports have shown Stat3 as a Cdk5 substrate in medullary thyroid cancer cells\textsuperscript{40}, prostate cancer cells\textsuperscript{177}, myotubes\textsuperscript{178} and neuronal cells\textsuperscript{179}. However, to our knowledge, there is no evidence suggesting post-translational modification of Stat3 by Cdk5 is a critical step in regulating Stat3 function in T cells. Thus, we hypothesized that Cdk5 may regulate the expression of the Foxp3 transcription factor and impair Treg differentiation through post-translational modification of Stat3.

In this study, we examine how disruption of Cdk5 expression and activity influences the expression of Foxp3 in primary murine CD4\textsuperscript{+} T cells. Our data indicate that the suppression of Cdk5 signaling abrogates IL-6 inhibition of Foxp3 expression. Furthermore, we mechanistically link Cdk5 activity to the regulation of Stat3 signaling and its influence on Foxp3 expression in T cells. Here we show the inhibition of Cdk5
activity (either using a Cdk5-specific inhibitory peptide or through the disruption of Cdk5 gene expression in T cells) leads to decreased Stat3 phosphorylation at Ser727, and this in turn disrupts Stat3 binding to the Foxp3 gene on the enhancer region II. Taken together, our data reveal a novel role for Cdk5 in the differentiation of T cells, as it effectively regulates Foxp3 gene expression and Treg development through specific phosphorylation of Stat3 at Serine 727.

4.3. Material and Methods

Animals. C57BL/6 mice, ages 6-8 weeks, were purchased from The Jackson Laboratory and used for T cell isolations. T cell specific Cdk5-deficient mice used in these studies were generated in our research facility. All animals were housed in micro-isolator cages and maintained in climate/light controlled rooms with free access to food and water. Studies performed were in compliance with the procedures approved by the Case Western Reserve University School of Medicine’s Institutional Animal Care and Use Committee.

Antibodies. The Cdk5 antibody (C8), p-Stat3(Ser727) antibody p-Stat3 (Y705) and rabbit IgG antibody were purchased from Santa Cruz Biotechnology and used for both immunoprecipitation as well as western blotting. The p-Smad2 and p-Smad3 antibodies were purchased from Cell Signaling Technology. The antibody used for total Stat2 and Stat3 was purchased from BD Biosciences. Foxp3 staining kit was purchased from eBioscience and staining of T cells were performed as described by manufacturer.

Chromatin Immunoprecipitation (ChIP) Assay. Chromatin immunoprecipitation (ChIP) analysis was performed according to the manufacturer’s instructions (Upstate
Biotechnology) with T cells stimulated under the indicated treatment conditions.

Immunoprecipitated samples (DNA) were subjected to semi-quantitative RT-PCR analyses of Foxp3 enhancer region II.

**Cytokines and Peptides.** Interleukin 6 cytokine was purchased from PeproTech Inc. Recombinant Interleukin 6 receptor alpha was purchased from Cell Signaling Technology. Cdk5 inhibitory peptide, CIP, was used at 50nM.

**Immunoprecipitation assays.** Protein lysates were first diluted to a concentration of 1µg/µL and subsequently pre-cleared with protein A-Agarose beads (Santa Cruz) and then incubated overnight with rotation at 4ºC with immunoprecipitating antibody. Lysates were then incubated with protein A-Agarose beads for 3-5 hours with rotation at 4ºC. After incubation, protein A-Agarose beads were collected and washed with lysis buffer. Samples were then subjected to western blot analysis.

**Reporter plasmid and luciferase assay.** Luciferase assay were performed in EL4 cells and reporter plasmids were transfected into cells through electroporation. Smad-binding element and Foxp3 enhancer I reporter plasmids were generously provided by Dr. Tone (University of Pennsylvania). Transfected cells were treated under specified conditions for 15 hours and analyzed by Glo Luciferase Reporter Assay System (Promega).

**Semi-quantitative RT-PCR.** Total RNA was extracted using Trizol reagent (Invitrogen) and complementary DNA was then prepared using cDNA first-strand synthesis kit (Invitrogen). PCR was performed with a Px2 Thermal Cycler (Thermo).

**Statistical Analyses.** Statistical evaluations were performed using the Prism computer program (GraphPad Software). Significant differences between experiments were
assessed by comparing the means of data sets using the Student t test, with a p value of
<0.05 considered significant.

**T cell receptor stimulation.** Anti-CD3 (3µg/mL) and anti-CD28 (1µg/ML) antibodies
(BD Biosciences) diluted in PBS were allowed to incubate to become plate bound
overnight in 4°C. Prior to use, plates were washed with fresh PBS twice.

**Transforming Growth Factor Beta (TGF-β1).** TGF-β1 used was purchased from
PeproTech. Working concentrations of 5ng/mL were used to stimulate T cells in all
experiments.

**T lymphocyte isolation.** A mixed population of cells was collected by first isolating cells
from both spleen and regional lymph nodes of mice and creating single cell suspensions
by passing these tissues through a 40µm cell strainer (BD Biosciences). Cells were then
incubated with ACK lysis buffer (Lonza) on ice for 5 minutes to deplete the mixed cell
population of erythrocytes. T lymphocytes were purified through a process of negative
isolation by passing mixed cells through MACS separation columns using a pan T
isolation kit (MiltenyiBiotec) in accordance to the manufacturer’s protocol.

**Western blot analysis.** Protein samples were prepared from cellular lysates made in
RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail tablet
(Roche) in addition to a phosphatase inhibitor phosSTOP (Roche). Proteins were
denatured by heating for 10 min at 94°C in sample loading buffer (2% SDS, 10% glycerol
80mM Tris, pH 6.8, and 1mM DTT). 50µg of total protein was separated by
electrophoresis in 4-20% Tris-Glycine gels (Invitrogen). Proteins were transferred to a
0.2µm nitrocellulose membrane (Invitrogen) and subsequently blocked for 1 hour in
blocking buffer (TBS containing 10% non-fat dry milk and 0.05% Tween 20).
Membranes were incubated overnight at 4ºC with primary antibody and probed with horse radish peroxidase-conjugated secondary antibody for 1 hour in room temperature.

4.4. Results

Disruption of Cdk5 activity prevents suppression of Foxp3 in T cells following stimulation with TGF-β and IL6. To determine whether Cdk5 activity contributes to the suppression of Foxp3 expression in T cells, we utilized primary murine T cells isolated from either wild type mice (Cdk5+/+) or mice with a T cell-restricted Cdk5 gene deletion (Cdk5−/−) and culture conditions specific for Treg induction. Specifically, CD4+ T cells harvested from splenocytes of either Cdk5+/+ mice or Cdk5−/− mice were plated with anti-CD3/CD28 antibody in the presence or absence of TGF-β and IL-6. In cultures of Cdk5+/+ T cells, we also use the CIP peptide inhibitor to achieve specific inhibition of Cdk5 activity and to corroborate data from cultures of T cells from Cdk5−/− mice. As shown in flow cytometry analyses in Figure 4.1A, TGF-β expectedly increased the percentage of CD4+CD25+Foxp3+ cells, whereas the addition of IL-6 effectively suppressed the this induction of Foxp3+ cells by TGF-β. Consistent with our hypothesis, suppression of Cdk5 activity by CIP prevented the suppression in Foxp3 expression observed when CD4+ cells were treated with both TGF-β and IL-6. These data are supported by analyses in Cdk5−/− CD4+ T cells (Figure 4.1B), as IL-6 also failed to suppress the induction of CD4+CD25+Foxp3+ T cells when Cdk5−/− CD4+T cells were activated with anti-CD3/CD28 in the presence or absence of TGF-β and IL-6. The changes in Foxp3 protein expression correlated with changes in Foxp3 mRNA expression.
(Figure 4.1C and 4.1D). When either Cdk5\(^{+/+}\) T cells or Cdk5\(^{-/-}\) T cells were activated in the presence of TGF-\(\beta\), each demonstrated a significant increase in the expression of Foxp3 mRNA as measured by real-time RT-PCR. Correlating with data obtained by flow cytometry, the presence of IL6 in cultures results in a significant suppression of Foxp3 message only in Cdk5\(^{+/+}\) T cells (Figure 4.1C), but not in Cdk5\(^{-/-}\) T cells (Figure 4.1D).

**Cdk5 activity does not inhibit TGF-\(\beta\) dependent activation of Smad proteins in T cells.** The role of Smad-dependent TGF-\(\beta\) signaling in the induction of Foxp3 has been established. Thus we chose to examine whether Cdk5 influenced Smad2/3 signaling through direct phosphorylation Smad proteins. Cdk5\(^{+/+}\) T cells were activated with anti-CD3/CD28 in the presence of TGF-\(\beta\) and IL6, either with or without the CIP peptide inhibitor of Cdk5. We observed a significant increase in phospho-Smad proteins (Figure 4.2A) following TGF-\(\beta\) treatment, whether added alone or in combination with IL6. T cells treated under the same conditions but in the presence of CIP showed similar phosphorylation of the Smad proteins. Similarly, either Cdk5\(^{+/+}\) T cells or Cdk5\(^{-/-}\) T cells (Figure 4.2B) treated with both TGF-\(\beta\) and IL6 also showed no difference in the phosphorylation status of the Smad proteins, again indicating that Cdk5 does not interfere with Smad2/3 phosphorylation in response to TGF-\(\beta\) in T cells.

Although we observed no detectable change in Smad2/3 activation with disruption of Cdk5 activity, this did not rule out the possibility that Cdk5 activity could influence Smad protein binding to DNA through modification of other co-activator or co-repressor proteins. To address this question, we first utilized a luciferase reporter controlled by a
general Smad binding element, which we expressed in the EL4 T cell line (Figure 4.2C). Following TCR stimulation in the presence of TGF-β, either alone or with IL6, we observed a significantly higher level of luciferase activity. Inhibition of Cdk5 activity in these cultures with the CIP inhibitory peptide did not alter luciferase activity. To take this analysis one step further, we analyzed the specific effect Cdk5 activity might have on Smad binding on the Foxp3 gene promoter by utilizing a luciferase reporter driven by the Foxp3 promoter linked in tandem to the enhancer I region (which is known to contain the Smad binding site). As seen in Figure 4.2D, in EL4 cells transfected with this reporter, the basal reporter activity increased significantly in either unstimulated or CD3/CD28 stimulated EL4 cells in the presence of TGF-β. This induction was neither affected by the addition of IL-6, nor by exposure of cells to the CIP inhibitor of Cdk5. Taken together, these data clearly indicate that Cdk5 activity does not directly influence the activation of Smad proteins nor their ability to bind to DNA. Thus, the effect of Cdk5 on Foxp3 expression must be mediated through modulation of a non-Smad pathway that indirectly controls TGF-β induced Foxp3 expression.

**Cdk5 induces Stat3 function in T cells by physical interaction and Stat3 phosphorylation at Serine 727.** Several groups have shown the ability for Cdk5 to interact with Stat3 and furthermore, the ability of Cdk5 to directly phosphorylate Stat3 at the Serine 727 site. However, these previous reports have not assessed the relevance of Stat3 modulation by Cdk5 in the context of T cell activation. Therefore, to determine whether Cdk5 modulates Stat3 function in T cells, we first immunoprecipitated Cdk5 from protein lysates prepared from T cells under various stimulation conditions,
including either with or without anti-CD3/anti-CD28, plus TGF-β and IL6. Probing Stat3 in immunoprecipitates of Cdk5 (Figure 4.3A), we confirmed the presence of a physical interaction between Stat3 and Cdk5. Moreover, the greater abundance of Stat3 observed in immunoprecipitates of TCR stimulated T cells (CD3/CD28) is consistent with the known increase in Cdk5 expression that occurs following TCR stimulation. More importantly, this interaction of Cdk5 with the Stat3 protein leads directly to Stat3 phosphorylation in T cells as shown by an in vitro Cdk5 kinase assay, in which we combined Cdk5 and Stat3 immunoprecipitates obtained from T cells (Figure 4.3B). Since Stat3 phosphorylation mainly occurs on either the tyrosine 705 or serine 727 residues, we examined how Cdk5 activity influences phosphorylation of both residues. As shown in Figure 4.3C, Stat3 Ser727 phosphorylation induced by T cell activation is significantly attenuated by treatment with the CIP peptide inhibitor of Cdk5. However, Cdk5 inhibition had no effect on the phosphorylation of Stat3 on the Tyr705 site and did not alter the total expression of Stat3 (Figure 4.3C). These findings were corroborated in analyses of Cdk5−/− T cells activated under similar conditions (Figure 4.3D). There was a significant decrease in Stat3 serine727 phosphorylation Cdk5−/− T cells, when compared to Cdk5+/+ T cells and once again, Y705 phosphorylation and total Stat3 levels were not affected. In total, these data implicate Stat3 as an important substrate of Cdk5 in T cells, with Cdk5 selectively phosphorylating Stat3 at the Ser727 residue.

**Inhibition of Cdk5 activity in T cells decreases Stat3 binding to the enhancer region of the Foxp3 gene.** Having established the potential for Stat3 to serve as a substrate of Cdk5 in T cells, we next examined whether Cdk5 might affect Stat3 entry into the
Western blot analyses of nuclear and cytoplasmic fractions of T cells for the presence of p-Stat3 (S727) revealed a marked decrease in the abundance of nuclear p-Stat3 when Cdk5 activity was inhibited (Figure 4.3E). However, inhibition of Cdk5 had no discernable effect on total Stat3 expression. Thus, while treatment with CIP suppresses Serine 727 phosphorylation in Stat3, this had no affect on the abundance of Stat3 or on Stat3 entry into the nuclear compartment.

The negative regulation of Foxp3 expression by Stat3 has been attributed to transcriptional repression exerted via binding to the enhancer II region of the Foxp3 gene. Therefore, we next examined whether Cdk5-dependent Stat3 phosphorylation might affect Stat3 DNA binding at the enhancer II region. Lysates prepared from T cells stimulated under various conditions (CD3/CD28, IL6, TGF-β) were subjected to in vitro DNA pull-down assays (Figure 4.4A). Western blot analysis shows Stat3 protein bound to the enhancer II DNA probe in lysates from T cells treated with a combination of CD3/CD28, TGF-β and IL6 together. Consistent with our hypothesis, the binding of Stat3 protein to the enhancer II DNA probe was suppressed in samples from T cells stimulated with CD3/CD28, TGF-β and IL6 together in the presence of the CIP inhibitor of Cdk5 activity. These data suggest Cdk5 activity is necessary for Stat3 to properly bind to the enhancer II region of the Foxp3 gene. Next, we performed a DNA pull-down analysis on samples isolated from the EL4 T cell line (Figure 4.4B). We observed the same DNA-protein interaction as previously mentioned with Stat3 binding to the DNA probe under conditions of CD3/CD28, TGF-β and IL-6 stimulation. Once again, this binding decreased when Cdk5 activity was inhibited by the addition of CIP peptide. To
further confirm whether this effect of Cdk5 on Stat3 is dependent on specific Serine 727 phosphorylation, we transfected the EL4 T cell line with a mutant form of Stat3 in which the serine 727 residue is substituted with an alanine residue. This substitution effectively suppressed Stat3 protein-DNA interaction, even under stimulation conditions including CD3/CD28, TGF-β and IL6. In total, these data clearly implicate a requirement for phosphorylation of Stat3 at the Serine 727 residue by Cdk5 for proper DNA-Stat3 protein interaction.

Finally, to examine the effect of Cdk5 activity on Stat3-DNA binding in the \textit{in-vivo} setting, we performed a ChIP assay designed to measure the ability of Stat3 to occupy the specific enhancer region of the Foxp3 gene under different conditions. As seen in Figure 4.4C, binding of Stat3 to the enhancer II region only occurred in conditions in which CD3/CD28 and TGF-β were combined with IL-6. Most importantly, the addition of the specific Cdk5 inhibitor CIP resulted in a loss of Stat3 binding. Similarly ChIP assays performed with Cdk5-deficient T cells show a lack of Stat3 protein occupying the enhancer II region when compared to ChIP assays with wild type T cells (Figure 4.4D). Together, these results reinforce the concept that Cdk5 plays a central role in regulating the IL6 suppression of Foxp3 expression in CD4$^+$ cells through a direct regulation of Stat3 phosphorylation and consequent DNA binding.

\textbf{4.5. Discussion}
Here we provide the first demonstration that the activity of Cdk5 modulates the expression of the Foxp3 transcription factor in CD4$^+$ T cells, through post-translational modification of Stat3. Either the pharmacologic inhibition of Cdk5 activity or the targeted deletion of the Cdk5 gene in T cells is sufficient to abrogate the suppression of Foxp3 gene expression by IL-6, which requires activation of Stat3 at the Serine 727 site. Our data not only demonstrate that this phosphorylation of Stat3 at Serine 727 requires Cdk5, but also indicate that this phosphorylation of Stat3 at Serine 727 is not necessary for Stat3 entry into the nucleus in T cells. However, nuclear Stat3 lacking this modification at Serine 727 does fail to bind to the enhancer region of the Foxp3 gene, and thus cannot effectively mediate suppression of Foxp3 in response to IL-6 signaling. Our data therefore provide evidence regarding the relevance of Cdk5 as a molecular mediator of regulatory T cell expansion in the context of chronic inflammation.

The Foxp3 transcription factor is essential for control over the differentiation, function and survival of Treg cells. Although a number of factors drive the expression of Foxp3, transforming growth factor-β (TGF-β) is an essential regulator of the inducible Treg population$^{180}$. Induction of Foxp3 by TGF-β requires the action of the Smad3 transcription factor$^{78}$. Activated by the TGF-β receptor, Smad3 binds to a specific enhancer site of the Foxp3 gene and becomes an integral part of the enhanceosome complex required for Treg development$^{181}$. Our data reveal that suppression of Foxp3 by Cdk5 is not mediated through a direct effect on the Smad2/3 pathway. Indeed, Smad2/3 activation by TGF-β is not altered in Cdk5-deficient T cells nor in wild type T cells activated in the presence of a Cdk5 specific inhibitor (CIP peptide). Moreover, utilizing a
luciferase reporter assay linked to a Smad-binding element, we confirmed that treatment of T cells with the Cdk5 inhibitory peptide CIP did not alter the ability for Smad proteins to properly bind to DNA. Finally, our data from assays with a luciferase reporter driven by the Foxp3 enhancer I region show that Cdk5 inhibition does not alter the ability for the Smad proteins to specifically interact with the proper DNA region of the Foxp3 promoter.

In addition to TGF-β, several other factors are known to modulate Foxp3 expression. In particular, IL-6 and IL-27 have been found to be strong inhibitors of Foxp3 expression in T cells activated through the TCR in the presence of TGF-β, and do so by activating Stat3. Xu et al, recently demonstrated a novel enhancer region (enhancer II) of the Foxp3 gene to which activated Stat3 was found to bind\(^81\). These data support a model in which Stat3 acts as an inhibitor of Foxp3 expression by preventing the binding of pSmad3 to enhancer I.

Stat3 has been widely accepted as an essential signaling molecule, selectively interacting with enhancer elements in the promoter region of different genes, thereby affecting a wide variety of physiological processes and responses. The Stat3 molecule is normally a latent cytoplasmic transcription factor that will become activated following cellular stimulation by various extracellular signaling molecules and subsequently translocate into the nucleus ultimately altering transcription of select genes\(^82\). Stat3 is activated by tyrosine phosphorylation (Y705) at a residue close to the carboxy-terminus as well as by a serine phosphorylation (S727) located within the transactivation domain.
Cdk5-mediated Stat3 phosphorylation has been demonstrated in previous reports\textsuperscript{41, 178}. By performing a Cdk5 kinase assay using Cdk5 and Stat3 immunoprecipitated from primary T cell lysates, our results clearly confirm Stat3 as a Cdk5 substrate. Consistent with the demonstrated preference towards phosphorylating proline-directed serine/threonine residues, Cdk5 has been shown to phosphorylate Stat3 at the Serine727 site in specific tissues including myotubes, neurons, colorectal cancer cells and prostate cancer cells. Our data suggest that this modification of Stat3 by Cdk5 plays a very important role in controlling T cell fate by modulating the response to extracellular signaling molecules that control T cell differentiation. Moreover, our data show that either inhibition of Cdk5 activity in wild-type T cells or the disruption of Cdk5 gene expression in T cells lead to a significant decrease in pStat3(S727), but fail to influence either pStat3(Y705) and total Stat3 levels. The data implicate Cdk5 as a required mediator of the Foxp3 repression downstream of IL-6 receptor signaling, and in a manner that cannot be compensated by other signaling intermediates.

Although phosphorylation of Stat3 on Tyrosine Y705 is clearly known to be important for Stat3 dimerization, nuclear translocation and DNA binding, the role of serine phosphorylation of Stat3 is somewhat controversial. There is evidence supporting both a positive and negative role for serine phosphorylation, thus indicating the potential for this modification of Stat3 to define context-dependent function of this transcription factor. In support of this possibility, disruption of Cdk5 activity has no discernible effect on total Stat3 and the decrease in pStat3(S727) caused by a disruption of Cdk5 activity does not alter the ability of Stat3 to translocate into the nucleus.
Prior reports have suggested Cdk5-dependent Stat3 serine phosphorylation alters the ability of this transcription factor to properly bind to DNA. Thus, although Stat3 nuclear translocation was not affected by Cdk5 inhibition in T cells, we explored the possibility that Cdk5 post-translational modification of Stat3 might influence Stat3 DNA binding. In T cells, DNA binding assays revealed phosphorylation of Stat3 at S727 is essential for Stat3 binding to the enhancer region II of the Foxp3 gene, and is thereby critical to the repression of Foxp3 expression in T cells exposed to cytokines like IL-6 and IL-27. Thus, our data not only reinforce the role of Cdk5 as an important post-translational modulator of transcription factor activity, but also demonstrate the importance of this function in controlling T cell fate. In summary, the observations presented here reveal a novel role of Cdk5 in controlling Foxp3 gene expression and suggest that targeting Cdk5 activity may overcome the ability of inflammatory cytokines to repress TGF-β-induced Foxp3 expression, an approach that in the future may be exploited in the therapy of autoimmune and chronic inflammatory diseases.
Figure 4.1. Cdk5 is essential for IL6 suppression of TGF-β induced Foxp3 expression in CD4+ T cells. A) Naïve CD4+ T cells were isolated from wild-type mice and stimulated with anti-CD3 and anti-CD28, with or without TGF-β, IL-6 and the Cdk5 inhibitor CIP as indicated. Foxp3 protein expression and numbers of Foxp3 cells were quantified by flow cytometry. B) Wild-type and Cdk5-deficient T cells were stimulated with anti-CD3 and anti-CD28, with or without TGF-β and IL6 as indicated. Foxp3 protein expression and numbers of Foxp3 cells were quantified by flow cytometry. C) Foxp3 mRNA expression levels in T cells activated in the presence or absence of TGF-β, IL6 and CIP were analyzed with real time RT-PCR. D) Both Cdk5+/+ T cells and Cdk5−/− T cells were treated with or without TGF-β and IL6, and subsequently Foxp3 mRNA expression levels were examined using real time RT-PCR analysis.
Figure 4.1. Cdk5 is essential for IL6 suppression of TGF-β induced Foxp3 expression in CD4+ T cells.
Figure 4.2. Cdk5 activity does not alter Smad activation or binding to the Foxp3 gene. A) Naïve primary T cells were stimulated with anti-CD3/CD28, with or without TGF-β, IL6 and CIP. Whole cell protein lysates were prepared from these samples and western blot analyses were performed to probe for p-Smad2 (Ser465/467), p-Smad3 (Ser423/425) and total Smad2 and total Smad3. B) Similarly, both Cdk5+/+ T cells and Cdk5−/− T cells were stimulated and treated under similar conditions and probed for the presence of p-Smad2/3 and total Smad2/3. C) Luciferase activity was examined in EL4 T cells transfected with the Smad-binding element (SBE) reporter construct and D) the Foxp3 promoter and enhancer I reporter construct. Cells were stimulated with anti-CD3/CD28 with or without TGF-β, IL6 and CIP as indicated.
Figure 4.2. Cdk5 activity does not alter Smad activation or binding to the Foxp3 gene.
Figure 4.3. Cdk5 physically interacts with and phosphorylates Stat3 at Serine 727.

A) Protein lysates were isolated from primary T cells before and after TCR activation in the presence of TGF-β, IL6 and CIP. Samples were then immuno-precipitated with anti-Cdk5 antibody and immunoprecipitates were then probed for the presence of Stat3 and Cdk5. B) A Cdk5-specific in vitro kinase assay was performed to determine the capacity of Cdk5 to phosphorylate Stat3 (the substrate). Neurofilament-H (NFH) as a substrate, was combined with Cdk5 immunoprecipitates isolated from wild type T cells as a positive control (Pos), whereas Cdk5 immuprecipitates isolated from Cdk5−/− T cells served as the negative control (Neg). C) Western blot analysis of protein lysates isolated from T cells with or without anti-CD3/CD28 activation and treatment with or without TGF-β, IL6 and CIP. Expression of total Stat3, p-Stat3 (Y705) and p-Stat3 (S727) were determined by Western blot, and, β-actin expression was probed as a control. D) Lysates from both Cdk5+/+ T cells and Cdk5−/− T cells stimulated under similar treatment conditions were analyzed by Western blot to determine protein expression of total Stat3, p-Stat3 (Y705), p-Stat3 (S727) and β-actin. E) Protein lysates were isolated from the cytoplasmic and nuclear compartments of activated T cells with or without TGF-β and CIP treatment. Western blot analyses were performed probing for p-Stat3 (S727), p-Stat3 (Y705), total Stat3 and Cdk5. Both β-actin and YY-1 were probed as cytoplasmic and nuclear loading controls, respectively.
Figure 4.3. Cdk5 physically interacts with and phosphorylates Stat3 at Serine 727.

A.

B.

***P<0.0001

B.

C.

D.

E.
Figure 4.4. Cdk5 is required to induce Stat3 binding to the Foxp3 gene. A) Nuclear protein lysates were isolated from primary T cells activated by anti-CD3/CD28, with or without TGF-β, IL-6 and CIP treatment. Lysates were incubated with DNA probes that corresponded to the specific Stat3 binding region on the enhancer region II of the Foxp3 gene and allowed to complex in a DNA pull down assay. Western blot analyses were then performed for Stat3 bound to the DNA probes. B) Nuclear protein lysates were prepared from EL4 T cells stimulated under different conditions, and from EL4 T cells expressing the Stat3 (S727A) mutant protein. Lysates were subjected to a DNA pull-down using DNA probes corresponding to the Stat3 binding enhancer II region of the Foxp3 gene. C) ChIP analysis was performed with lysates from primary T cells stimulated with or without anti-CD3/CD28, with or without TGF-β, IL-6 and CIP. PCR was performed to amplify the specific crosslinked DNA corresponding to the Stat3 region on the Foxp3 enhancer II region. D) Similarly, ChIP analyses were performed with lysates from both Cdk5+/+ T cells and Cdk5−/− T cells activated in the presence or absence of either TGF-β and/or IL6.
Figure 4.4. Cdk5 is required to induce Stat3 binding to the Foxp3 gene.

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**A.** WB: Stat3

**B.** (+) DNA Probe  (-) DNA Probe

**C.** ChIP: Stat3  2% Input

**D.**

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<td>IL6:</td>
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ChIP: Stat3  2% Input
Chapter 5: Summary, Conclusions, Discussions and Future Directions

5.1 Summary:
Cyclin-dependent kinase 5 (Cdk5) has historically been most extensively studied in the neuronal lineages due to the predominant expression of its activating partners p35 and p39 in cells of neural crest origin. In addition to the essential roles of Cdk5 in neuronal survival, migration and function, aberrant Cdk5 activity and expression has been associated with a number of inflammatory neurodegenerative diseases. Numerous functional similarities exist between neurons and immune cells, and common signaling partners and substrates among neurons and immune cells exists. Cdk5-mediated phosphorylation of these signaling molecules are known to essentially modulate their functions in neurons, yet the impact of Cdk5-dependent regulation of these proteins in immune cell signaling has not been previously studied. Thus, our data provide the first evidence that Cdk5 activity is inducible and important in immune cells, and particularly in T cells. Mice with Cdk5-deficient immune cells were protected from clinical symptoms associated with experimental autoimmune encephalomyelitis (EAE). Through phosphoproteomic studies, our data indicate that Cdk5-dependent modulation of immune cells is partially mediated through the post-translational modification of coronin 1a and this Cdk5-dependent phosphorylation is required for proper cytoskeletal reorganization of activated T cells. Examining purified primary T cells, we found that activation of these T cells resulted in a significant increase in Cdk5 activity, and that genetic or pharmacological disruption of Cdk5 activity in these T cells impairs their activation and function. We observed significant decrease in the essential IL2 cytokine production by T cells when Cdk5 activity is disrupted, an effect we find to be linked to loss of Cdk5
regulation of the mSin3a-HDAC1 repressor complex. We discovered Cdk5-dependent phosphorylation of mSin3a at serine residue 861 decreases mSin3a protein abundance and also prevents mSin3a-HDAC1 repressor complex formation, thereby decreasing its occupancy on the IL2 promoter. Furthermore, diminished clinical symptoms and decreased susceptibility to EAE induction in mice with Cdk5-deficient immune cells may be a result of an imbalance in T cell differentiation. We found that Cdk5 is an essential regulator of Foxp3+ expression in CD4+ T cells and thus effectively controls differentiation of regulatory T cells (Tregs). We elucidated the mechanism through which Cdk5-dependent phosphorylation of Stat3 at serine residue 727 is required for proper Stat3 DNA-binding on the enhancer II region of the Foxp3 gene. In total, the studies described here provide insights for Cdk5 regulation in the previously unexamined context of T differentiation and function, and highlight Cdk5 as a novel target for therapeutic intervention of inflammatory and autoimmune diseases.

5.2 Conclusions:

5.2.1. Chapter 2: Cdk5 activity is required for T cell activation and induction of experimental autoimmune encephalomyelitis

The data presented in Chapter 2 provide the first demonstration of the presence of an active Cdk5-p35 complex in T cells. Furthermore, these data show the requirement for Cdk5 activity during T cell activation and for the induction of EAE. Cdk5 was originally thought of as a neural specific serine/threonine kinase and aberrant Cdk5 activity has
been linked with a number of neuronal inflammatory disorders. Since neuronal cells and immune cells have a number of functional similarities, we hypothesized that aberrant Cdk5 activity observed in these disorders may potentially reflect an additional role for Cdk5 in immune cells participating in the neuronal inflammation. The results in Chapter 2 suggest that Cdk5 is indeed an essential kinase in immune cells whose activity is required for T cell activation. Higher expression of Cdk5-p35 activity was observed in mononuclear tissues taken from mice with clinically evident EAE when compared to control, non-immunized mice. Additionally, when purified T cells were activated by antigenic stimulation or TCR ligation, a significant increase in Cdk5 expression and activity was observed. Furthermore, pharmacological suppression of Cdk5 activity or genetic disruption of Cdk5-p35 expression impairs T cells response to TCR ligation or antigenic stimulation. To study lymphocyte-specific function of Cdk5 in T cells in an *in vivo* system, we utilized chimeric mice with Cdk5-deficient immune cells challenged with the MOG peptide and show ameliorated clinical signs associated with EAE. Finally, in Chapter 2 coronin 1a is shown to be a direct substrate of Cdk5 on residue threonine-418. Cdk5-dependent phosphorylation of coronin 1a is essential for proper actin polarization and migration of lymphocytes to specific chemokine signals. In summary, the data provided in Chapter 2 are truly significant as they represent the first description of the presence of Cdk5-p35 expression and activity in T cells that is associated with their activation and critical for effector function. This discovery of a novel non-neuronal role for Cdk5 as a mediator for T cell signaling add to a list of functions for a molecule that was previously considered to have a predominantly restricted function in the CNS. These results have implications for several other neurodegenerative and mental health disorders.
where the activation of Cdk5 may contribute to the pathogenesis of the diseases associated with inflammation and thus establish a strong rationale for the development and clinical evaluation of Cdk5 as a therapeutic target against immune-mediated diseases.

5.2.2. Chapter 3: Cdk5 controls IL-2 Gene Expression via Repression of the mSin3a-HDAC Complex

The diminished response to TCR activation in T cells isolated from Cdk5-deficient immune chimeric mice suggested a likely role for Cdk5 in mediating the differentiation and function of T cells, prompting several questions regarding other potential Cdk5 substrates and the contexts in which their phosphorylation by Cdk5 could influence T cell differentiation and function. It was logical to pose that the suppressed proliferative response observed in Cdk5-deficient T cells may have resulted from altered expression of autocrine factors such as IL-2, a cytokine necessary for an optimal mitogenic T cell response. In Chapter 3, our data reveal the requirement for Cdk5 activity in T cells for optimal autocrine IL-2 production, as either the disruption of Cdk5 activity or of Cdk5 gene expression results in significant decrease in both the IL-2 mRNA transcript abundance and IL-2 cytokine expression following TCR stimulation. These findings suggested a role for Cdk5 in controlling IL-2 gene transcription. Cdk5 is known to regulate gene expression through a number of mechanisms and my data support a conclusion that Cdk5 regulate IL-2 expression through post-translational modification of the HDAC1 repressor complex. Either genetic deletion of Cdk5 gene expression in T cells, or pharmacologic inhibition of Cdk5 activity, relieved repression of IL-2 gene expression.
expression by HDAC1 in activated T cells. However, although my results show that Cdk5 associates with HDAC1 in T cells, I found no evidence for direct phosphorylation of the HDAC1 protein by Cdk5. Instead I found that Cdk5 mediates its repression of HDAC activity through direct phosphorylation of the HDAC1-associated protein mSin3a at its serine 861 residue. I further examined the significance of mSin3a phosphorylation by Cdk5 and determined that Cdk5 activity controls HDAC1-mSin3a complex formation through the regulation of mSin3a protein abundance. Finally, through examination of the IL-2 promoter region with ChIP assays, results show that Cdk5 activity disrupts the formation of the HDAC1-mSin3a complex and diminishes HDAC1 occupancy on the IL-2 promoter. In summary, our data reveal a pivotal role for Cdk5 in controlling IL-2 gene expression during T cell activation and add to accumulating evidence that point to the importance of Cdk5 interaction with nuclear machinery responsible for coordinated regulation of gene expression in response to extracellular signals. The regulatory role of HDACs in immunity is of significant interest as the onset of a number of pathological conditions including chronic inflammation and autoimmunity is often the result of aberrant gene transcription. Several reports have documented a role for the class I HDACs in negatively regulating inflammatory cytokine production and a number of HDAC inhibitors are being studied for their use on animal models of inflammatory diseases. Conversely, the multitude of HDAC isoforms has displayed differential effects on the immune system and often resulting in both positive and negative regulation of the immune response. Although the broad-spectrum inhibitors that are currently being studied display some degree of selectivity for different HDAC enzymes, ultimately they target multiple isoforms and classes of HDACs. Due to the pleiotropic and often
contrasting effects of the different HDACs on the immune response, it is expected that use of these inhibitors have shown evidence of contraindications. Our identification of Cdk5 as a novel target molecule able to modulate the HDAC1-mSin3a repressor complex offers a novel approach towards the treatments of immune mediated diseases without the caveats associated with current HDAC-directed therapies.

5.2.3. Chapter 4: Cdk5 regulates Foxp3 expression in CD4+ T cells through modification of Stat3 phosphorylation

In a healthy organism, the immune system is required to maintain a balance between inflammatory responses involving effector cells and stasis which is in part maintained by the immune regulatory cells such as T regulatory cells. An important subset of these regulatory T cells are defined by their expression of the Foxp3 transcription factor, and are found to potently suppress effector T cell function and dampen the inflammatory response. The reduced susceptibility of Cdk5-deficient mice to EAE described in Chapter 2, may reflect either a deficiency in the acquisition of effector function in Cdk5-/- T cells, or a potential enhanced potential for regulatory T cell differentiation in Cdk5-/- T cells. As described in Chapter 4, it was found that dysregulation of Cdk5 signaling prevented the suppression of Foxp3 expression in T cells invariably observed with additional stimulation with IL6. Further examination of the mRNA transcript by real-time RT PCR revealed that the change in Foxp3 expression was concomitantly observed at the mRNA level. Mechanistic studies found no difference in the activation of Smad2/3 nor differences in activated Smad protein binding to the Foxp3 gene in Cdk5-/- T cells or in
wild type T cells exposed to specific inhibitors to Cdk5 activity when treated with conditions to activate Smad signaling. In addition to the known role of TGF-β in promoting Foxp3 expression, the importance of Stats in repression of Foxp3 transcription has also been reported\textsuperscript{185}. As described in Chapter 4, Western blot analysis showed that either the inhibition of Cdk5 activity or targeted deletion of the Cdk5 gene in T cells significantly decreases Stat3 phosphorylation at the Serine727 (S727) residue, but does not affect Tyrosine705 (Y705) phosphorylation. Examining the functional significance of Cdk5-directed Stat3 (S727) phosphorylation in T cells reveal that disruption of Cdk5 activity eliminates the capacity for Stat3 to bind to the enhancer II region of the Foxp3 gene in response to IL-6 stimulation in T cells. In summary, Chapter 4 not only reinforces the importance of Cdk5 as a modulator of transcription factor activity, but also demonstrates a novel role in controlling T cell fate. Furthermore, these results suggest targeting Cdk5 activity may potentially overcome the ability of inflammatory cytokines to repress TGF-β-induced Foxp3 expression. This introduces a novel approach that may be exploited in the therapy of autoimmune and chronic inflammatory diseases.

5.3. Discussion and Future Directions

5.3.1. Chapter 2 – Discussion and Future Directions

Examination of the mechanism responsible for increased Cdk5 activity upon TCR activation in T cells.

Our data clearly and conclusively show that the function of Cdk5 is not just relevant in neuronal tissues, and that it is essential for several steps that regulate the activation and differentiation of T cells. Upon T cell stimulation, we observed a robust increase in
Cdk5 and p35 expression, and an associated increase in Cdk5 activity. Ultimately, the specific mechanism responsible for this increased Cdk5 activity will need to be defined. Multiple reports have indicated that the protein expression level of p35 is one of the limiting factors for Cdk5 activity, thus examining the upstream signaling responsible for the increase in p35 expression is essential and is therefore a focus for my future work.

**Specific Aim 1.1. To elucidate the mechanism responsible for the increased Cdk5 and p35 expression and activity upon activation of T cells.** The expression of p35 requires ERK signaling; the inhibition of this kinase is able to inhibit p35 expression whereas, constitutive ERK signaling is able to induce p35 expression. Studies performed on PC12 neuronal cells have elucidated how the transcription factor Egr1 is induced by the ERK pathway subsequently binds to the promoter of p35, effectively inducing p35 expression\(^6\). Stress, growth factor and cytokines signaling and other factors can activate the MAPK/ERK pathway in a number of different settings and tissues. Similarly, upon TCR-induced T cell activation the MAPK/ERK pathway also becomes activated. Upon TCR activation the tyrosine kinase Zap70 forms a complex and initiates the MAPK/ERK pathway\(^1^8^6\). Thus I hypothesized that upon TCR activation, MAPK/ERK signaling is activated and responsible for upregulating p35 expression and therefore Cdk5 activity.

In order to determine the mechanism behind this increased p35 expression, I propose to compare lysates prepared from naïve and TCR activated T cells (at various time points), and confirm ERK activation and Egr1 induction through western blot analysis. Furthermore, by blocking ERK signaling with the PD98059 inhibitor and examining both
Egr1 and p35 expression I will be able to further determine the role that ERK-Egr1 signaling has in modulating p35 expression in T cells. Finally, by employing a luciferase reporter linked with the p35 promoter, I can assess how TCR activation links to this or other signaling cascades to induce p35 expression. Utilizing the MEK/ERK inhibitor as well as Egr1 directed siRNA I will be able further confirm the direct contribution of this pathway to p35 expression in T cells.

In contrast to ERK1/2 and Egr1 activation, p38 MAPK, JNK and NF-kB signaling pathways have been implicated in exerting a suppressive role for p35 expression. Inhibitors directed to these pathways resulted in increased p35 promoter activity. Although it is unclear how the activation of these three pathways specifically affects the p35 promoter, it is speculated that activation of these pathways may induce an unknown repressor for the p35 promoter that may suppress the expression or activity of Egr1. Thus, in addition to examining the pathways involved with the positive regulation of p35 expression as mentioned above, I propose to also examine the pathways involved with the negative regulation of p35 expression. To examine the involvement of these specific negative regulators I will utilize inhibitors to attenuate each specific signaling cascade and examine the resulting p35 expression through RT-PCR and western blot. The p38 inhibitor SB203580, JNK inhibitor SP600125 and an NF-kB inhibitor will be used to treat TCR-activated T cells. Finally, using a luciferase reporter linked with p35 promoter I will be able to further confirm whether these pathways influence the induction of p35 expression.
Potential of a regulatory role for Cdk5 in acute lymphoblastic leukemia (ALL):

Cdk5 is an important regulator of a number of cellular processes including gene transcription, vesicular and cellular transport, apoptosis, cell adhesion and migration. Through the post-translation modification of downstream proteins Cdk5 signaling is essential for proper cellular function in a number of neuronal and non-neuronal cell types. Furthermore, Cdk5 has been found to be constitutively expressed in several different cancers including pancreatic cancer, medullary thyroid cancer, breast cancer and prostate cancer.

Acute lymphoblastic leukemia (ALL) is the most common form of pediatric cancer and is a highly heterogeneous disease and as such successful treatment of ALL has not been uniform. T-cell ALL accounts for 15-25% of ALL cases\textsuperscript{188}, and this aggressive hematologic tumor results from the malignant transformation of T cell progenitors\textsuperscript{189}. The outcome of T-cell ALL patients with primary resistant or relapsed leukemia remains very poor and current research efforts have placed an emphasis on defining new biological markers for this disease. By discovering new biological markers in T-cell ALL disease activity, it can lead to a better understanding of pathobiology, improved risk stratification and utilization of new therapeutic agents.

The presence and importance of Cdk5 activity in normal T cells has been clearly shown above. I showed that Cdk5 blockade suppresses T cell proliferation in normal primary T cells. However, Cdk5 activity and expression have never been previously examined in
pediatric patients with T-cell ALL. Thus it is important to quantify the expression and activity of Cdk5 in bone marrow and peripheral blood of patients with T-cell ALL and to compare this expression with the bone marrow, peripheral blood and human thymic tissues of healthy subjects. In collaboration with Dr. Irina Pateva, a pediatric oncologist at University Hospitals, we are studying specimens collected from patients at UHCHM and on COG protocols under the IRB approved (CASE1913-05-13-06C) and COG (AALL13B12-Q) protocols to study Cdk5 function in specimens from pediatric, adolescent and young adult patients with ALL. We have screened a number of human leukemic samples for the abundance of Cdk5 and p35 and compared them to normal non-malignant T cells. We observed a significantly higher abundance of Cdk5 and p35 expression in specimens obtained from leukemic patients compared their non-malignant controls (Figure 5.1A,B). Our data therefore point to the potential that Cdk5 may be developed as a marker of disease activity in T-cell leukemias, or exploited for therapeutic intent.

Specific Aim 1.2. To quantify Cdk5/p35 expression and enzyme activity in the bone marrow of patients with T-cell ALL at diagnosis, end of induction/remission and at relapse. Together, the data will be used to correlate with known clinical risk stratification and prognostic markers. In order to determine if there is differential Cdk5 expression and activity in T cell ALL patients compared to healthy controls, we will continue to collect specimens from patients enrolled in the ongoing research study at Rainbow Babies & Children’s Hospital under an IRB approved protocol: UHCMC IRBL CASE1913-05-13-06C. Thymic tissues will be collected from normal volunteers (these
are patients who are undergoing surgery for repair of congenital cardiac abnormalities) to provide a source of normal, developmentally relevant T cell progenitors, as these are not present in bone marrow. Further, blood and bone marrow will be collected from the patients on the day of diagnosis, end of induction/remission as well as at relapse (if available). In addition, we have also obtained bone marrow and peripheral blood specimens that were released from the Children’s Oncology Group biorepository from 10 patients with T-cell ALL. The patient samples will be screen for the expression of Cdk5 and p35 mRNA and protein through RT-PCR and western blot respectively. Analysis of Cdk5 activity will also be analyzed with the Cdk5 specific kinase assay. Through the proposed efforts to characterize the Cdk5 expression and activity in patients with T-cell ALL, this effort will contribute to our understanding of the importance of Cdk5 as both a biological marker of disease activity and potentially as a prognostic indicator.

A specific mechanistic role for Cdk5 in leukemogenesis has not been defined. However, a number of known Cdk5 substrates are essential for this process. The aberrant Cdk5 activity we observed in the leukemic cell lines can potentially lead to dysregulation of different oncogenes or tumor suppressor genes. One potential mechanism I propose involves the activation of Stat3. Amongst the different members of the STAT family, Stat3 is considered highly important in the context of tumorigenesis and considered an oncogene as it mediates the activity of a diverse group of signaling pathways in cancer\textsuperscript{190}. Furthermore, in non-malignant cells, Stat3 activation occurs transiently and activation is strictly controlled to prevent unscheduled gene regulation. In contrast, constitutive activation of Stat3 has been found in a variety of tumor cells including leukemias and
lymphomas where it is essential for the survival and proliferation of the malignant cells. Whereas tyrosine 705 is commonly thought of as the major residue required to be phosphorylated for Stat3 activation, serine residue 727 located on the carboxyl-terminal has also been found to be important. In fact, there are reports that in some leukemias Stat3 is constitutively phosphorylated exclusively on the serine residue and not on the more commonly recognized tyrosine residue. Cdk5 has been reported to phosphorylate Stat3 at the Serine 727 residue in a variety of cells. Our data confirm that Stat3 is a Cdk5 substrate in T cells and that this phosphorylation modulates its transcriptional activity during T cell responses. In my preliminary data, I also examined the expression of total Stat3 and more importantly, the phosphorylation of Stat3 (S727) in the T-cell leukemia cell lines (Figure 5.2). I observed higher abundance of phosphorylated Serine 727 residue on the Stat3 protein in leukemic samples as compared to normal controls. Therefore, I hypothesize that the projected increase of Cdk5 activity in T-cell ALL may result in increased Stat3 (S727) phosphorylation, which may be a mechanism through which Cdk5 promotes leukemogenesis through induction of Stat3 regulated genes.

**Specific Aim 1.3. To interrogate the mechanism through which Cdk5 exerts its mechanistic function in T-cell ALL cell signaling.** Specifically, I will be examining the post-translational modification of Stat3 influenced by Cdk5 in samples of ALL patients and the consequences of this specific phosphorylation. To determine the phosphorylation status of Stat3 in ALL, I will isolate lysates from the bone marrow and peripheral blood from the pediatric patients with T-cell ALL and probe through western blot for p-Stat3 (S727) and total Stat3 expression and compare these samples to normal
control samples. In addition, I propose to examine the mechanistic significance of Stat3 phosphorylation by Cdk5, with a particular focus on the ability of these proteins to translocate into the nucleus in ALL cells compared to nontransformed lymphocytes obtained from normal volunteers. I will separate the total protein lysate into nuclear and cytoplasmic fractions and probe for the presence of Stat3 in these compartments. To confirm the potential role of Cdk5 in ALL, I will examine the effect of inhibition of Cdk5 activity on the nuclear translocation of Stat3 in T-cell leukemia cell lines. Finally, I will also examine the gene expression of select Stat3 regulated genes through RT-PCR and western blot analysis in the presence and absence of Cdk5 inhibitors. By better defining the role of Cdk5 as an activator of Stat3 in T-cell ALL, I will gain a better understanding of the pathobiology of the disease potentially define the utility of Cdk5 inhibitors as a form of targeted therapy in the treatment of T-cell ALL.

5.3.2. Discussion and Future Directions – Chapter 3.

In Chapter 3, my data add to existing evidence that Cdk5 is a regulator of nuclear machinery and gene expression. I show the ability for Cdk5 to regulate IL-2 gene expression in T cells through the disruption of the mSin3a-HDAC1 repressor complex. Furthermore, Cdk5 phosphorylates mSin3a specifically at the serine861 residue and this phosphorylation results in proteasome degradation and a decrease in protein abundance. Finally, this phosphorylation disrupts the formation of the mSin3a-HDAC1 repressor complex and the ability to occupy the IL-2 promoter in activated T cells.
The mSin3a-HDAC1 repressor complex has been reported to serve integral roles in the regulation of gene expression\textsuperscript{192} and has critical roles in the regulation of several prominent transcriptional networks for the proliferation and survival of both normal and neoplastic cells\textsuperscript{193}. Furthermore, the mSin3a corepressor complex is believed to exert its transcriptional regulation through a modular fashion such that various transcription factors and DNA-binding elements may recruit different subsets of mSin3a/HDAC1 components depending on the different cellular contexts and microenvironment conditions. My observation that Cdk5 regulates the mSin3a-HDAC1 repressor complex described in Chapter 3 focused solely on IL-2 gene expression. Thus it would be interesting to also examine whether Cdk5 activity regulates other cytokines important in T cell activation, differentiation and function. I hypothesize that the regulation of the mSin3a-HDAC repressor complex by Cdk5 is not limited to its action on IL-2 expression, but rather that the modulation of this complex by Cdk5 will influence the expression of a number of other important cytokines and other factors that control the development and function of the lymphocyte lineage.

**Specific Aim 2.1. To determine the cytokine expression profile under Cdk5-dependent mSin3a-HDAC1 regulation.** In order to examine whether expression of cytokines other than IL-2 are affected by the regulation of the mSin3a-HDAC1 complex by Cdk5, I propose to utilize a commercially available cytokine assay panel. By culturing and stimulating both wild-type T cells as well as Cdk5-deficient T cells, and subsequently subjecting the supernatants of the cultured T cells, I would be able to determine alterations in cytokine expression. I would then selectively utilize RT-PCR
and ChIP assays to determine whether any observed changes in cytokine expression are due to transcriptional repression and mSin3a-HDAC occupancy. By further examining cytokine expression in T cells, and specifically the influence of Cdk5-dependent mSin3a-HDAC1 phosphorylation on this process, I can then better understand the functional significance of Cdk5 activity in T cells, including whether it is essential for cytokine dependent pro-inflammatory or anti-inflammatory cell differentiation.

**Examination of mSin3a biology after Cdk5-dependent serine861 phosphorylation**

My observations in Chapter 3 convincingly show Cdk5-directed phosphorylation of mSin3a at serine residue 861 leads to decreased mSin3a protein accumulation in both the nuclear lysates and total cellular lysates of activated T cells. However, I hypothesize that mSin3a phosphorylation at Serine 861 may alter the localization of the protein and is a phenomenon that requires further examination.

**Specific Aim 2.2. To determine whether Cdk5-dependent phosphorylation of mSin3a alters the localization of the mSin3a protein.** In order to examine whether the localization of mSin3a changes upon post-translational phosphorylation by Cdk5, I propose to transfect mSin3a into a cell type that does not express the active Cdk5-p35 complex, such as HEK293T cells. Next, using standard transfection strategies that allow temporal regulation, I can constitutively express p35, an approach that allows us to selectively induce Cdk5 activity in any cell. By isolating cytoplasmic and nuclear protein lysates separately from such transfected cells treated with MG132; I will be able to detect
the localization of mSin3a. To further examine whether the localization of mSin3a is affected by Cdk5-dependent phosphorylation, I propose to utilize another mSin3a mutant, specifically, I propose to mutate the serine 861 residue to an aspartic acid residue (S861D) to mimic phosphorylated serine. By expressing either mutant mSin3a (S861A) or mutant mSin3a (S861D) in cells treated with MG132, I can examine the accumulation of the mSin3a protein in either the cytoplasmic or nuclear fraction.

5.3.3. Discussion and Future Directions – Chapter 4.

In our studies, we found mice with Cdk5-deficient T cells were resistant to EAE induction. Since EAE is a T cell mediated autoimmune disease where a breakdown of the balance between regulatory T cells and pathogenic Th17 cells occurs, I wanted to examine how Cdk5 may potentially play a role in influencing this loss of immune homeostasis. I have demonstrated that Cdk5 has an essential role in the control of Foxp3 expression and is required for IL-6 induced repression of Foxp3 gene expression (Chapter 4). When Cdk5-deficient T cells are treated simultaneously with TCR stimulation, TGF-β and IL-6, they retain Foxp3 expression normally present in wild-type T cells without exposure to IL-6. Cdk5 exerts this regulation through post-translational modification of the Stat3 protein, phosphorylating the Serine727 residue thereby disrupting the ability of Stat3 to bind to the Foxp3 enhancer II region. However, the Cdk5 may similarly influence Th17 differentiation, as this T cell subset depends on both TGF-β and IL-6 for development, and thus far this possibility has not been explored.
While Foxp3+ Treg cells play a critical role in suppressing the immune response, the subset of effector CD4+ cells, designated as Th17 cells based on its production of the IL17 cytokine, have proven to also be important in dictating the overall immune response. Naïve CD4+ T cells can be induced to differentiation towards either the Foxp3+Treg phenotype or the Th17 lineage based upon the specific cytokine milieu to which they are exposed. Interestingly, the cytokine combination of TGF-β and IL6 which causes suppression in Foxp3 expression, leads to increased Th17 cell differentiation through upregulation of the nuclear receptor RORγt and expression of IL17. Furthermore, while Stat3 can influence on Foxp3 transcription, it is also a critical transcription factor involved with Th17 differentiation. Activation of Stat3 induces the expression of IL17A as well as the transcription factor RORγt, further driving the differentiation and stable maintenance of Th17 cells. Taken together, I hypothesize that Cdk5 is an important regulator of Th17 differentiation through its ability to influence IL17 and RORγt expression and plays an essential role in maintaining the immune balance between Treg and Th17.

**Specific Aim 3.1. Determine the influence Cdk5 may have on the balance between pathogenic Th17 and regulatory T cells.** In order to determine whether Cdk5 influences the differentiation of Th17 cells, I will first perform *in vitro* studies stimulating both WT and Cdk5-deficient T cells and determine whether the loss of Cdk5 may result in decreased IL-17 and RORγt expression. Although it is known that Cdk5 influences Stat3 signaling through the phosphorylation of the serine 727 residue, it remains unclear how this affects Stat3 in terms of IL-17 and RORγt expression. I will be able to compare the
ability of Stat3 to bind to the promoters of the genes encoding both IL-17 and RORγt by performing both DNA binding assays and ChIP assays using lysates from both wild-type and Cdk5-deficient T cells. Finally, to further confirm the role of Cdk5 in the maintenance of the immune balance in a diseased mice model, I can evaluate the level of IL-17 in the plasma of mice with T cells lacking Cdk5, for example in mice induced to develop EAE by immunization with the MOG peptide. Taken together, examination of the role of Cdk5 in Th17 differentiation and in the regulation of IL-17 gene expression may reveal how Cdk5 might act to modulate the delicate balance between Th-17 and Treg cells and ultimately immune homeostasis. Further, these studies will confirm the potential for Cdk5 as a viable target for future development of therapeutic strategies for the management of chronic inflammatory disease and autoimmune conditions.
Figure 5.1. Increased Cdk5 expression in ALL patient samples. Samples were isolated from healthy controls and from patients with T-cell ALL. (A) RT-PCR was performed to analyze for Cdk5 and p35 mRNA expression. GAPDH was used as a loading control. (B) Western blot analysis was performed on protein lysates isolated from healthy controls and patients with T-cell ALL to determine the protein expression of Cdk5 and p35. β-actin was probed as a loading control.
Figure 5.1. Increased Cdk5 expression in ALL patient samples.

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Figure 5.2. Increased Stat3 phosphorylation detected in ALL patient samples.

Samples were isolated from healthy controls and from patients with T-cell ALL. Western blot analysis was performed on protein lysates isolated from healthy controls and patients with T-cell ALL to determine the protein expression of p-Stat3(S727) and total Stat3. β-actin was probed as a loading control.
Figure 5.2. Increased Stat3 phosphorylation detected in ALL patient samples.
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