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

Tej K. Pareek,¹ Eric Lam,¹ Xiaojing Zheng,² David Askew,¹ Ashok B. Kulkarni,³ Mark R. Chance,² Alex Y. Huang,¹ Kenneth R. Cooke,¹ and John J. Letterio¹

¹Division of Pediatric Hematology/Oncology, Department of Pediatrics, University Hospitals Case Medical Center and The Case Comprehensive Cancer Center, and ²Case Center for Proteomics and Bioinformatics, Case Western Reserve University, Cleveland, OH 44106
³National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892

Cyclin-dependent kinase 5 (Cdk5) is a ubiquitously expressed serine/threonine kinase. However, a requirement for Cdk5 has been demonstrated only in postmitotic 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 nonneuronal Cdk5–p35 activity has not been explored in this context. We describe a previously unknown function of the Cdk5–p35 complex in T cells that is required for induction of experimental autoimmune encephalomyelitis (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 posttranslational modification of coronin 1a and exhibit defective TCR–induced actin polarization and reduced migration toward CCL–19. These data define a distinct role for Cdk5 in lymphocyte biology and suggest that inhibition of this kinase may be beneficial in the treatment of T cell–mediated inflammatory disorders.

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 (CNS; Noseworthy et al., 2000). 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 on 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 posttranslational 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 (Pawson and Scott, 1997). Both tyrosine (Thome and Acuto, 1995) and serine/threonine kinases (Matthews and Cantrell, 2006) 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 (Cohen, 2002).

Abbreviations used: ANOVA, analysis of variance; Cdk5, cyclin-dependent kinase 5; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; IS, immune synapse; MOG, myelin oligodendrocyte glycoprotein; MPM-2, mitotic protein monoclonal 2; MS, multiple sclerosis; NF-H, neurofilament H; PTX, pertussis toxin.

Supplemental material can be found at: http://doi.org/10.1084/jem.20100876

© 2010 Pareek et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License [Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-nd/3.0/].
Cyclin-dependent kinase 5 (Cdk5), a ubiquitously expressed proline-directed serine/threonine kinase, is mainly active in postmitotic neurons as a result of 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 (Dhavan and Tsai, 2001). This perception has been supported by gene KO studies in which germ line deletion of the genes encoding either Cdk5 or p35 lead to inverted cortical neuronal layering during brain development (Ohshima et al., 1996; Chae et al., 1997). Normal activity of Cdk5 is required for proper neuronal migration, synapse formation, and neuronal survival. However, aberrant or hyperactivation of Cdk5 is associated with severe neurodegenerative disorders including Alzheimer’s disease (Shelton and Johnson, 2004).

Recently, Cdk5–p35 has been linked with disease induction in nonneuronal lineages (Rosales and Lee, 2006), with examples which include malignant transformation in cancer (Strock et al., 2004; Lin et al., 2007; Upadhyay et al., 2008), induction of inflammatory pain (Pareek et al., 2006), and other inflammation-mediated disorders (Kitazawa et al., 2005). 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 (Wyss-Coray and Mucke, 2002) and cancer (Coussens and Werb, 2002), 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 (Chen and Studzinski, 2001; Studzinski and Harrison, 2003). However, a role for the Cdk5–p35 complex in the activation and function of normal nontransformed immune cells has not been established. In this paper, 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 WT mice with hematopoietic progenitors from Cdk5-deficient mice embryos (Cdk5–/-/-) after 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 (Ferrari et al., 1999), 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 TCR ligation, and also fail to migrate after 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 MS.

RESULTS

Increased Cdk5–p35 expression in CNS mononuclear infiltrates in EAE

Deregulated Cdk5 activity triggers progressive neurodegeneration and neurofibrillary tangle formation in mice (Cruz and Tsai, 2004). Tau pathology associated with axonal loss during EAE is linked to up-regulation of Cdk5 activity (Schneider et al., 2004). Furthermore, proteomic analysis of chronic active plaques of MS patients has shown significantly increased expression of Cdk5 (Han et al., 2008). In agreement with these results, 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 (Fig. 1 A). 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 (10-fold) and p35 (fivefold) transcripts (Fig. 1 B). The observed increase in expression of Cdk5 and p35 correlated with a more than twofold elevation in Cdk5 activity in total tissue lysates collected from brain stem and lumbar spinal cord (Fig. 1 C). Specificity of Cdk5 kinase activity in this assay was confirmed using Cdk5 WT and KO embryo (embryonic day [E] 16.5) brain lysates (Fig. 1 D). 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 h after CD3/CD28 stimulation and remained elevated until 48 h after stimulation (Fig. 2 A). Although mRNA expression of Cdk5–p35 was consistently high for up to 72 h, the levels of the Cdk5–p35 protein started to decline after 48 h. This late reduction in the levels of Cdk5–p35 protein likely reflects an increase in their degradation, a process which is known to be calcium dependent. We also observed a twoto threefold increase in Cdk5 kinase activity within 24–72 h of CD3/CD28-mediated T cell activation (Fig. 2 B). Similarly, a threefold increase in Cdk5 activity was observed in response to IL-7, a nonredundant cytokine required for T cell survival and development which is also implicated in MS (Lundmark et al., 2007; Fig. 2 C). This effect of IL-7 is enhanced in the presence of CD3/CD28 stimulation. Lastly, protein lysates prepared from total splenocytes (Fig. 2 D) or from CD4+ T cells (Fig. 2 E) collected from mice with EAE showed a three- to fourfold increase in Cdk5 activity. The restimulation of these lymphocytes in vitro with myelin oligodendrocyte glycoprotein (MOG) antigen further increased the activity of Cdk5 when assayed at 72 h. The specificity of Cdk5 kinase activity in these assays was confirmed using Cdk5 WT and KO embryo (E16.5) brain lysates (Fig. 2 F).

To analyze, if the overall increase in Cdk5 activity observed in these experiments is a result of the true increase in specific kinase activity of Cdk5 or merely a result of 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 CD3/CD28, PMA/ionomycin, or allogenic dendritic cells showed a significant decline in their proliferation when treated with 10 µM roscovitine (Fig. S1 A). Results obtained from an MTT assay ruled out the possibility of roscovitine having any adverse effect on lymphocyte viability (Fig. S1 B), and there was no adverse effect of roscovitine on the expression of Cdk5 and p35 protein levels (not depicted). It should be noted that in each kinase assay the activity of Cdk5 is normalized on a per-milligram protein basis. Although 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 (Mapelli et al., 2005), nonselectivity of this compound cannot be ruled out, as this 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 (Ohshima et al., 1996); thus, we generated Cdk5-null immune chimeric mice (Cdk5−/−; Fig. S2). We observed a 60–70% reduction in the proliferative response (as measured
by \(^3\)H-thymidine uptake) of Cdk5\(^{−/−}\)C (Fig. 3 A) or p35\(^{−/−}\)T cells (collected from p35\(^{−/−}\)mice; Fig. 3 B) 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 wk after MOG (35–55)/CFA immunization and subsequently cultured them either in the presence or absence of 33 µg/ml MOG and/or 10 µM roscovitine. 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 (Fig. S1 C). More importantly, encephalitogenic lymphocytes harvested from either the MOG-immunized Cdk5\(^{−/−}\)C mice (Fig. 3 C) or the MOG-immunized p35\(^{−/−}\) mice (Fig. 3 D) did not respond when reexposed to MOG in vitro. Interestingly, we observed a gene dose effect for p35 in these experiments. Proliferation after restimulation 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 4 wk after immunization, the survival of Cdk5\(^{−/−}\)C mice (75%) was significantly greater than that of either the Cdk5\(^{+/+}\)C mice or C57Bl6\(^{+/+}\)C mice (radiation control) in whom survival was <25% as a result of the severity of disease (Fig. 4 A). There was also a significant delay in the development and a reduction in the clinical severity of EAE in Cdk5\(^{−/−}\)C mice (Fig. 4 B). Histological examination of brain and spinal cord of Cdk5\(^{−/−}\)C mice also revealed significantly less inflammation, demyelination, and axonal loss (Fig. 4 C). Immunohistochemical analysis of the immune infiltrates in the CNS demonstrated a significant reduction in the numbers of T cells, macrophages, and neutrophils in Cdk5\(^{−/−}\)C mice (Fig. S3). In 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 (not depicted), despite the objective differences in the proliferative response of lymphocytes reexposed to MOG in vitro (Fig. 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 (Chae et al., 1997). 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\(^{−/−}\) model, as well as the potential influence of the radiation required for establishing the Cdk5 chimeric mice, we next used an adoptive transfer approach, assessing the ability of encephalitogenic lymphocytes from these models to transfer disease to naive animals. Lymphocytes harvested from immunized Cdk5\(^{−/−}\)C mice completely failed to transfer disease (Fig. 4 D). More importantly, disease progression was significantly delayed in recipients of p35\(^{−/−}\) encephalitogenic lymphocytes (Fig. 4 E). This observation is supported by histopathology, which shows reduced inflammatory infiltrates within the spinal cord of recipients of the p35\(^{−/−}\) encephalitogenic lymphocytes (Fig. 4 F).

**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\(^{+/+}\)C and Cdk5\(^{−/−}\)C mice 4 wk after MOG immunization. Deletion of Cdk5 in these cells was confirmed by performing Cdk5-specific RT-PCR (not depicted) and Western blotting (Fig. 5 A). The mitotic protein monoclonal 2 (MPM-2)–specific antibody, which is known to recognize phosphorylated proline-directed serine/threonine residues in proteins (Yaffe et al., 1997), 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\(^{−/−}\)C lymphocytes compared with Cdk5\(^{+/+}\)C lymphocytes (Fig. 5 B). Analysis of these bands with liquid chromatography–tandem mass spectrometry and Mascot database search identified a 51-kD band as coronin 1a (a leukocyte-specific protein) with sequence coverage of 63% (Fig. S4 A) and a Mascot search score of 3557. Our liquid chromatography–tandem mass spectrometry findings clearly confirmed phosphorylation of coronin 1A...
The phosphorylation of threonine 418 was observed in Cdk5−/− lymphocytes and was confirmed by selected ion chromatography (Fig. 5 D). However, Western blot analyses for total coronin 1a protein showed identical expression in Cdk5+/+ and Cdk5−/− lymphocytes (Fig. 5 C), demonstrating that this posttranslational 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+/+ and Cdk5−/− encephalitogenic lymphocytes. A threefold reduction in phosphorylation of threonine 418 was observed in Cdk5−/− lymphocytes and was confirmed by selected ion chromatography (Fig. 5 D). However, Western blot analyses for total coronin 1a protein showed identical expression in Cdk5+/+ and Cdk5−/− lymphocytes (Fig. 5 C), demonstrating that this posttranslational 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+/+ and Cdk5−/− encephalitogenic lymphocytes. Total immunoprecipitates were then assayed by Western blotting with both Cdk5 and coronin 1a antibodies (Fig. 5 E). 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+/+ embryo brain lysates and then subjected to in vitro kinase assays in the
presence of γ-[32P]ATP. Autoradiographs of the kinase assay products clearly show that Cdk5 directly phosphorylates coronin 1α (Fig. 5 F). To confirm the relative importance of threonine residues identified by mass spectrometry analysis, we synthesized 17-mer coronin 1α peptides (416–432) and replaced threonine 418 and/or threonine 424 with alanine (Fig. 5 G). 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 1α peptide compared with WT, with threonine 418 being most important (Fig. 5 G).

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

Genetic deletion of coronin 1α in mice established the absolute requirement of this protein in T cell antigen receptor function (Föger et al., 2006). Coronin 1α has been shown to bind directly to F-actin in vitro and to colocalize with F-actin structures in vivo (de Hostos et al., 1991). To determine the impact of Cdk5-mediated coronin 1α phosphorylation on F-actin and coronin 1α association, we collected lymphocytes from C57BL6+/+C, Cdk5+/+C, and Cdk5−/−C mice after EAE induction. As expected, both normal (Fig. 6 A) and control (Fig. 6 B) lymphocytes showed homogeneous colocalization of coronin 1α and F-actin with slight polarization in control cells but no discernible difference among lymphocytes isolated from the C57BL6+/+C, Cdk5+/+C, and Cdk5−/−C mice. Interestingly, Cdk5+/+C encephalitogenic lymphocytes exhibited a distinct polarization of F-actin and coronin 1α, with most of the coronin 1α clustered on the pole directly opposite of F-actin clustering, and this phenomenon was completely lost in Cdk5−/−C encephalitogenic lymphocytes (Fig. 6, C and D). In contrast, Arp2/3 polarization was not compromised in Cdk5−/−C encephalitogenic lymphocytes (Fig. S5). These results support an essential role for Cdk5-mediated coronin 1α 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 (Proudfoot et al., 2008). Moreover coronin 1α–deficient lymphocytes fail to migrate toward CCL19 (Föger et al., 2006). 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 of Cdk5+ lymphocytes to migrate toward the CNS during EAE (Proudfoot et al., 2008). Moreover coronin 1α–deficient lymphocytes failed to migrate toward CCL19 (Föger et al., 2006). 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 of Cdk5+/+C lymphocytes to migrate toward CCL19 (MIP3−/−) and a 10-fold decrease in migration toward of SDF1α and SDF1β (CXCL12) in the presence of 10 μM roscovitine (Fig. S6).
Figure 6. Disruption of Cdk5 activity diminishes actin polarization and migration toward CCL19. (A–C) Cdk5+/+, Cdk5−/−, and C57BL6+/+ mice were immunized with PBS (normal; A), CFA + pertussis toxin (PTX; control; B), or MOG(35–55) + CFA + PTX (EAE; C) and, 4 wk later, lymphocytes were collected and stained for F-actin 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 six 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+/+ or Cdk5−/− mice. Bars, 100 µm. Data represent three independent experiments of five pairs of mice, mean ± SEM. ***, P < 0.001, one way ANOVA followed by Student’s t test.
Finally, migration of Cdk5−/−C lymphocytes toward CCL19 was significantly reduced when compared with migration of Cdk5+/+C control lymphocytes (Fig. 6 E). Collectively, these results suggest that Cdk5-mediated coronin 1a phosphorylation is required for proper lymphocyte activation and migration.

DISCUSSION

More than a century after the first description of the clinical and pathological characteristics of MS (Pearce, 2005), 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 IS, enabling the cell to migrate toward 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 postmitotic neurons as a result of the predominant expression of its activating partner proteins p35 and p39 in these cells. However, the abundance of Cdk5 expression in nonneuronal 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 nonneuronal 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 (Fu et al., 2004) and suppresses MEF2-mediated transcription (Gong et al., 2003) in neurons, and both of these transcription factors are critical regulators of T cell (Lu et al., 2008) and B cell (Wilker et al., 2008) 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 (Yang and Hinds, 2003), WAVE1 (Kim et al., 2006), WAVE2 (Miyamoto et al., 2008), FAK (Xie et al., 2003), and also Rac and Pak (Nikolic et al., 1998; Rashid et al., 2001). These proteins are similarly known to regulate reorganization of the actin cytoskeleton in immune cells during TCR signaling, lymphocyte differentiation, and migration and also to orchestrate effector function (Burkhardt et al., 2008). Posttranslational modification of these proteins by their phosphorylation is known to regulate cytoskeletal dynamics in immune cells (Kurosaki and Hikida, 2009; Prince et al., 2009; Salmond et al., 2009). 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 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 after 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 3 wk, 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) after 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−/− embryos develop normally, and there are no obvious phenotypic differences in either the number or ratio of immune cell populations in either Cdk5−/− or p35−/− mice compared with their WT littermates (Fig. S2, D and F–H). Similarly, immunophenotyping of the Cdk5−/−C and Cdk5−/−C mice shows no differences in gross anatomical structure, cellularity, or lineage distribution in hematopoietic organs (bone marrow, spleen, thymus, and lymph nodes; Fig. S2 E). However, when these mice are challenged through induction of EAE, the spleen and lymph nodes of Cdk5−/−C mice are significantly smaller with the substantial reduction in tissue weight, cellularity, and disorganized tissue architecture compared with the Cdk5−/−C control mice (unpublished data). Thus, although Cdk5−/−C naive lymphocytes develop normally, their coordinated response to antigenic stimuli is hampered as demonstrated by the delayed development and reduced severity of EAE.

Interestingly, Cdk5−/−C encephalitogenic splenocytes, collected either during the active phase (10 d after EAE induction) or chronic phase (21 d after EAE induction) of EAE, show significant reduction in the production of IL-2, IL-6, IL-17, TNF, and GM-CSF, when compared with Cdk5+/+C cells, after restimulation with MOG antigen in vitro. We did observe a significant drop in IFN-γ cytokine production from these cells during active phase but the difference was
not significant in chronic phase (Fig. S7). The observed difference in cytokine production may reflect a reduction in the number of MOG-specific T cells, impaired TCR stimulation, or a skewing in the differentiation of Cdk5\(^{−/−}\)/C 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 posttranslational 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 (Clemen et al., 2008). Deletion of the coronin 1a gene in mice is associated with alterations in cellular steady-state F-actin formation in lymphocytes (Föger et al., 2006) and reductions in lymphocyte survival, migration, and Ca\(^{2+}\) release from intracellular stores (Mueller et al., 2008). Coronin 1a gene deletions are associated with severe combined immunodeficiency in humans (Shiow et al., 2008, 2009), and nonsense mutation of the coronin 1a gene (Lmb3 locus) protects against the induction of systemic lupus in mice (Haraldsson et al., 2008). The crystal structures of a C-terminal truncated form of coronin 1a (residues 1–402), along with a C-terminal fragment (residues 430–461), have been solved (Appleton et al., 2006); 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 (Gatfield et al., 2005). In this paper, 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 (Huang et al., 1999), has been shown to phosphorylate yeast coronin 1 on multiple sites (Gandhi and Goode, 2008). 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.

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 (Boulanger, 2009). 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 several 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.

**MATERIALS AND METHODS**

**Animals.** 6–8-wk-old C57BL/6 or B6D21 mice were purchased from The Jackson Laboratory 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 Laboratories) and used for generation of chimeric mice. Cdk5\(^{−/−}\) mice were used to generate Cdk5\(^{−/−}\) and Cdk5\(^{+/−}\) embryos, which were further used for collection of brain or hematopoietic cells. 3-mo-old p35\(^{+/−}\), p35\(^{−/−}\), or p35\(^{+/+}\) mice were used for lymphocyte collection and for induction of EAE. All animals were housed in microisolator cages and maintained in climate- and light-controlled rooms (22 ± 0.5°C, 12/12-h 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 EAE kidneys and recognized as C57BL6\(^{+/−}\) and C57BL6\(^{−/−}\) breeder mice. Approximately 10 million cells per mouse were then injected into 12-wk-old lethally irradiated (1,400 rad; Gammacel 137C; J.L. Shepard & Associates) CD45.1 female mice by tail vein injection. 8 wk later, peripheral blood was collected from all recipients and tested for CD45.1 versus CD45.2 expression. All cells were housed in microisolator cages and maintained in climate- and light-controlled rooms (22 ± 0.5°C, 12/12-h 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.

**EAE induction.** EAE was induced in mice by MOG (35–55) immunization and disease was adoptively transferred to unaffected WT animals as previously described (Mi et al., 2007). 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 CFA consisting of 100 µl of incomplete Freund’s adjuvant with 8 mg/ml Mycobacterium tuberculosis and 100 µl PBS (DIFCO Laboratories) or CFA with 200 µg MOG (35–55). At the time of injection and 48 h later, 200 ng PTX (List Biological Laboratories) was injected intraperitoneally in 100 µl PBS and

**Downloaded from jem.rupress.org on December 24, 2017**
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 d after MOG (35–55) immunization and cell suspensions were prepared from spleen and regional lymph nodes (axillary, brachial, and inguinal). About 5 × 10⁶ cells were cultured in the presence of 33 µg/ml MOG (35–55) and 20 ng/ml of mouse IL-12. 3 d later ~2–3 × 10⁷ of the cultured encephalitogenic cells were then injected into 3–mo-old female C57BL6 mice via intraperitoneal injection. 200 ng PTX was injected on the same day and again 48 h 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 paraparenisis; 6, death.

Peptide synthesis. The following peptides were used in this study: MOG (35–55) peptide, MEVGWYRSPSRVHYLRNGK; NF-H peptide, VK-LGTVSVSPK; corinin 1a WT, RATPEPSGTPSSDVTVR; coronin 1a mutant 1, RAAPPSGTPSSDVTVR; coronin 1a mutant 2, RATPEPS-GAPSSDVTVR; and coronin 1a mutant 3, RAAPESCAPSSDVTVR. All peptides were synthesized at 21st Century Biochemical using Fmoc chemistry and were HPLC purified to a minimum >90%, and the mass and sequence were verified by nanospray mass spectrometry and collision-induced dissociation tandem mass spectrometry.

Antibodies. The anti-Cdk5 antibody (C-8), anti-p35 antibody (C-19; Santa Cruz Biotechnology, Inc.), and MMP-2 antibody (Millipore) were each used at 1:200 dilution for Western blots. 5 µg of the anti-Cdk5 antibody (C-8) was used for immunoprecipitation. Hamster monoclonal anti–coronin 1a antibody was provided by A.C. Chen (Genentech, San Francisco, CA) and used at a 1:1,000 dilution for Western blotting and a 1:1,000 dilution for immunohistochemistry at a 1:500 dilution. Alexa Fluor 568 phalloidin (Invitrogen) was used to detect F-actin at a 1:25 dilution. Horseradish peroxidase–conjugated anti–hamster (BD) was used as a secondary antibody at 1:5,000 and horseradish peroxidase–conjugated anti–mouse or anti–rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc.) were 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 (Invitrogen) were used at a 1:200 dilution for immunofluorescence.

Roscovitine. All roscovitine (C19H26N6O; 6-Benzylamino-2-(R)-((1-ethyl)-2-hydroxyethylamino)-9-isopropylpurine) used in this study was purchased from Enzo Life Sciences, Inc. 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 (Thermo Fisher Scientific) containing a protease inhibitor tablet (Roche) and the phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Protein lysates (50 µg of lumbar spinal cord, brain stem, or embryo brain lysates or 1 mg of spleen, lymphocyte, or T cell lysates) were dissolved in lysis buffer to achieve a 1-µg/µl concentration and then precleared with normal rabbit IgG followed by protein A–agarose slurry (Sigma-Aldrich) prepared in lysis buffer. These lysates were then incubated overnight at 4°C with 0.01 µg/µl of anti-Cdk5 IgG. On the next day, the lysates were subjected to a 3-h incubation with 50 µl of a 50% protein A–agarose slurry at 4°C. Immunoprecipitates were washed three times with lysis buffer followed with kinase buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EDTA, 10 µM NaF, and 1 mM Na₃VO₄) and resuspended in 10 µl of 5X kinase assay mixture (100 mM Tris-HCl, pH 7.4, 50 mM MgCl₂, 5 mM EDTA, 50 µM NaF, 5 mM Na₃VO₄, and 5 mM DTT), 30 µl of water, and 20 µl of either of the NF-H peptide or immunoprecipitated coronin 1a or coronin 1a peptides (WT or mutant 1, 2, or 3). Samples were kept at 30°C for 60 min after adding 5 µCi [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 1 mM 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). 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), 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 previously described (Cua et al., 2003). Total lymphocytes were collected from spleen and regional lymph nodes (axillary, brachial, and inguinal) by passing minced tissues through a 40-µm cell strainer (BD) and thereafter incubating with ACK lysing buffer (Lonza) on ice for 5 min to deplete erythrocytes. For further purification, these cells were then subjected to MACS separation columns using a pan T isolation kit (Miltenyi Biotech), as per the manufacturer’s protocol, to isolate total T cells.

Thymidine assay of cell proliferation. Total lymphocytes or T cells were stimulated with CD3/CD28, PMA-sonosycin, allogeneic dendritic cells, or MOG (35–55) for 72 h in a 1% 20% fetal calf serum (FCS) culture media (Invitrogen) containing 50 µM 2-mercaptoethanol and 10% fetal bovine serum. During the last 16–18 h of culture, 1 µCi [³²P]Thymidine was added to each well and cells were harvested on a Unifilter-96 Harvester (Perkin Elmer). Incorporation of [³²P]Thymidine was measured as counts per minute on a 1450 MicroBeta TriLux (Perkin Elmer).

Histology. Histology and immunocytochemical localization were performed as described previously (Pareek et al., 2006). In brief, 4–6 wk after EAE induction, mice were anesthetized and perfused with PBS followed by 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 four parts (cervical, thoracic, lumbar, and cervical).
Flow cytometry analyses. Cells were isolated from bone marrow, spleen, and lymph node by passing tissue through a 40-µm cell strainer. 200,000 cells were first incubated with FACS blocking buffer (DPBS with 0.1% BSA and 10% normal mouse serum) and stained with 1 µg of CD3 (clone 145-2C11), CD4 (clone RM4–5), CD8 (clone 53–67), 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 BD Biosciences (BD) and diluted as recommended by the supplier.

Lymphocyte chemotaxis migration assay. The ability of lymphocytes to move toward a chemotaxant signal was tested in a transmigration assay performed in a 6.5-mm transwell with a 5-µm-pore polycarbonate membrane insert (Costar; Corning). In brief, ∼10 million Jurkat cells, or Cdk5+/− or Cdk5−/− lymphocytes, were serum starved for 4 h and treated with or without 10 µM roscovitine. These cells were cultured in the upper chamber of a transwell plate and the lower chamber was filled with 100 ng MIP-3β/CCL19 (PeproTech). After 15 h 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 Prism (GraphPad Software, Inc.). 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 tests with a Bonferroni α correction for multiple comparisons, where α was set at 0.05.

Online supplemental material. Fig. S1 shows suppression of antigen receptor-mediated lymphocyte activation by roscovitine treatment. Fig. S2 shows the strategy for the generation of immune chimeric mice and presents the immunophenotyping data demonstrating no difference among Cdk5+/− and Cdk5−/−, p35+/− and p35−/−, and Cdk5+/+ and Cdk5−/− at baseline. Fig. S3 shows representative sections of immunostaining performed to characterize immune cell infiltrates in spinal cord after EAE induction. Fig. S4 shows the mass spectrometry spectrum and identification of coronin 1a with peptide mapping. Fig. S5 shows the results of immunostaining for Arp2/3 and F-actin in encephalitogenic lymphocytes revealing normal Arp2/3 polarization in lymphocytes from the Cdk5−/− mice. Fig. S6 provides results of transmigration assays in which migration of Jurkat cells toward CCL19 is suppressed in the presence of roscovitine. Fig. S7 shows the results of assays of in vitro cytokine production by encephalitogenic splenocytes, during both the active and chronic phase of disease, after restimulation with MOG (35–55) ex vivo. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100876/DC1.

We thank Drs. Jean Pieters (University of Basel, Switzerland) and Andrew Chen (Genentech, USA) for providing coronin 1a antibody. Drs. Harish Pant (National Institute of Neurological Disorders and Stroke/National Institutes of Health), Veeranna (New York University) and Sashi Kesavapany (National University of Singapore) for valuable discussions, Michael Sramkoski (Case Comprehensive Cancer Center confocal core facility) for confocal microscopy, and Janet Robinson for experimental help.

We would like to acknowledge the support from the Jane and Lee Seidman Chair in Pediatric Cancer Innovation and The Case Research Institute. The authors have no conflicting financial interests.

Submitted: 3 May 2010
Accepted: 20 September 2010

REFERENCES


Ferrari, G., H. Langen, M. Naito, and J. Pieters. 1999. A coat protein on... 
Kurosaki, T., and M. Hikida. 2009. Tyrosine kinases and their substrates... 
Lin, H., M.C. Chen, Y.M. Song, and S.Y. Lin. 2007. Cdk5... 


Figure S1. Inhibition of Cdk5 activity by roscovitine treatment abrogates antigen receptor-mediated lymphocyte activation. Lymphocyte activation and proliferation was measured by "H-thymidine incorporation. (A) T cells were isolated and activated with CD3/CD28, PMA/ionomycin, or allogeneic dendritic cells in the presence or absence of 10 µM roscovitine. (B) T cell viability was measured with MTT assay, after exposure to roscovitine. (C) Lymphocytes were collected 4 wk after EAE induction and stimulated in vitro with MOG in the presence or absence of 10 µM 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’s t test.
Figure S2. Generation and characterization of immune chimeric mice and their immunophenotyping. (A) Schematic presentation of hematopoietic cell transfer. In brief, 3-mo-old female C57Bl6 (CD45.1) congenic mice were exposed to two doses of 1,000 Gy gamma radiation with a 6-h interval. After 24 h, these mice received hematopoietic cells collected from fetal liver and spleen of Cdk5<sup>+/+</sup>, Cdk5<sup>+/−</sup>, and Cdk5<sup>−/−</sup> littermate embryos via tail vein injection to generate Cdk5<sup>+/−</sup>C, Cdk5<sup>+/−</sup>C, and Cdk5<sup>−/−</sup>C mice. (B) Approximately 4 mo after 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 blotting 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<sup>+/+</sup>, Cdk5<sup>+/−</sup>, and Cdk5<sup>−/−</sup> mouse embryos (E16.5; D) or from spleen and regional lymph nodes of either Cdk5<sup>+/−</sup>C or Cdk5<sup>−/−</sup>C mice (E) and subjected to Immunophenotyping by flow cytometry using specific antibodies against CD3, CD4, CD8, CD11b, CD45R, and CD34. Data represents three independent experiments. (F–H) Total cells were collected from spleen (F), regional lymph nodes (G), and thymus (H) of 8–12-wk-old female p35<sup>+/+</sup> and p35<sup>−/−</sup> mice and subjected to Immunophenotyping by flow cytometry using specific antibodies against CD11b, B220, CD4, and CD8. Data represents four independent experiments. Error bars are mean ± SEM.
Figure S3. Characterization of immune cell infiltrate in spinal cord after EAE induction. Representative sections of lumbar spinal cords from Cdk5+/+ and Cdk5−/− obtained 3 wk 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 min 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; Thermo Fisher Scientific), 2 µg/ml of purified goat polyclonal antiserum to mouse CD4 (lot # EPE02(2); R&D Systems), 10 µg/ml of the rat IgG2b mAb F4/80 for detection of macrophages and microglia, or 2 µg/ml of purified rabbit polyclonal antiserum to myeloperoxidase for detection of granulocytes (lot 373A603A(1); Thermo Fisher Scientific). After TBS washes, slides were incubated for 30 min with 2.5 µg/ml biotinylated rabbit anti–rat IgG secondary antibody (Vector Laboratories), with 2.5 µg/ml biotinylated goat anti–rabbit IgG secondary antibody (Vector Laboratories) to detect CD3 and myeloperoxidase or 2.5 µg/ml biotinylated rabbit anti–goat IgG secondary antibody (Vector Laboratories) to detect CD4, followed by Vectastain ABC Elite (Vector Laboratories) for 30 min. The staining reaction was detected using metal enhanced 3,3′-diaminobenzidine chromogen (Thermo Fisher Scientific) for 4 min. Slides were counterstained with Mayer’s hematoxylin to detect nuclei, dehydrated, mounted, and coverslipped. Bars, 100 µm.
**Figure S4. Coronin 1a identification and peptide mapping.** (A) Primary sequence of coronin 1A. Identified tryptic peptides are indicated by double-headed arrows under the protein sequence. (B) Mass spectrometry spectrum of [band indicated with arrow in Fig. 4C] the triply charged precursor ion at m/z of 660.97. The peptide was identified as coronin 1A phosphopeptide \(^{416}\text{RATPEPSGPSSDTVS}R\). Data represent five pairs of mice of two independent experiments.
Figure S5. Arp2/3 polarization is unaltered in Cdk5<sup>−/−</sup> encephalitogenic lymphocytes. Cdk5<sup>+/−</sup>, Cdk5<sup>−/−</sup>, and C57BL6<sup>+/−</sup> mice were immunized with PBS (normal; A), CFA + PTX (control; B), or MOG(35–55) + CFA + PTX (EAE; C), and lymphocytes were collected from spleen and regional lymph nodes 4 wk after immunization and stained for F-actin using phalloidin (red) and Arp2/3 (green). Bars, 100 µm.
Figure S6. Roscovitine treatment disrupts migration of murine T cells and human Jurkat cells toward CCL19 in a transmigration assay. The histogram shows the percentage of cells present in the lower chamber of a transwell containing 100 ng of either SDF1-α (CXCL12), SDF1-β (CXCL12), or MIP3-β (CCL19) in a standard transmigration assay where the upper chamber contains 10^7 Jurkat cells treated with or without 10 μg roscovitine for 4 h. Data represents three independent experiments, mean ± SEM. **, P < 0.001, one way ANOVA followed by Student’s t test.
Figure S7. In vitro cytokine production by encephalitogenic splenocytes after 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 d after immunization) or the chronic phase (21 d after immunization) of EAE. These cells were cultured for 3 d in vitro in presence or absence of MOG (35–55) and supernatants were analyzed for cytokine production using OptEIA ELISA sets (BD; IL-2, IL-6, IFN-γ, TNF, and GMCSF) and DuoSet ELISA development system (R&D systems; IL-17). The data are representative of three independent experiments of at least four to six mice in each group. All data are mean ± SEM. **, P < 0.01; *, P < 0.05, one-way ANOVA followed by Student’s t test.