Cyclin-dependent kinase 5 activity is required for allogeneic T cell responses after hematopoietic cell transplantation in mice

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Running Title: Cdk5 activity in GVHD after allogeneic HCT
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• Cdk5 function is required for optimal lymphocyte activation and migration following allogeneic HCT.
• Targeting Cdk5 may be a particularly attractive strategy to reduce GVHD and maintain anti-tumor activity
Abstract (249)

Molecular intermediates in T cell activation pathways are crucial targets for the therapy and prevention of graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation (HCT). We recently identified an essential role for cyclin dependent kinase 5 (Cdk5) in T cell activation and effector function, but the contribution of Cdk5 activity to the development of GVHD has not been explored. Using an established, pre-clinical, murine, GVHD model we reveal that Cdk5 activity is increased in key target organs early after allogeneic HCT. We then generated chimeric mice (Cdk5+/+C or Cdk5−/−C) using hematopoietic progenitors from either E16.5 Cdk5+/+ or Cdk5−/− embryos to enable analyses of the role of Cdk5 in GVHD, as germ line Cdk5 gene deletion is embryonically lethal. The immuno-phenotype of adult Cdk5−/−C mice is identical to control Cdk5+/+C mice. However, transplantation of donor Cdk5−/−C bone marrow and T cells dramatically reduced the severity of systemic and target organ GVHD. This phenotype is attributed to decreased T cell migration to secondary lymphoid organs (SLO), reduced in vivo proliferation within these organs, and fewer cytokine-producing donor T cells during GVHD development. Moreover, these defects in Cdk5−/− T cell function are associated with altered CCR7 signaling following ligation by CCL19, a receptor:ligand interaction critical for T cell migration into SLO. Although Cdk5 activity in donor T cells contributed to GVT effects, pharmacologic inhibition of Cdk5 preserved leukemia free survival. Collectively, our data implicate Cdk5 in allogeneic T cell responses after HCT and as an important new target for therapeutic intervention.
Introduction

Allogeneic hematopoietic cell transplant (allo-HCT) is the only curative therapy for many malignant and non-malignant disorders. In addition to delivering effective anti-cancer treatment, the therapeutic potential of allo-HCT for hematologic malignancies relies on graft-versus-tumor (GVT) effects\(^1\). Successful HCT outcomes are limited by several life-threatening complications; graft-versus-host-disease (GVHD) and malignant relapse are the two primary contributors to mortality. Unfortunately, GVT effects are closely associated with GVHD. Thus, the development of novel strategies that modulate immune dysregulation to reduce GVHD, preserve GVT activity, and facilitate immune reconstitution remain critical to enhancing survival after allo-HCT.

The pathophysiology of acute GVHD is complex\(^2\)-\(^4\). Experimental and clinical data support the hypothesis that immune dysregulation during GVHD occurs in distinct phases\(^3\) involving diffuse damage and inflammation from conditioning regimens, activation of donor T cells by host-derived APCs\(^5\)-\(^7\), and target organ injury by soluble and cellular effectors. Since its inception, components of this paradigm have been challenged and refined\(^4\),\(^8\). Hence, this hypothesis continues to provide a conceptual framework within which novel therapeutic approaches can be explored.

Cyclin dependent kinase 5 (Cdk5) is a unique, ubiquitously expressed, proline directed, serine/threonine kinase whose diverse substrates include transcription factors, cytoskeletal proteins, and signaling molecules\(^9\). Although Cdk5 shares homology with other cyclin dependent kinases, it does not depend on association with cyclins for activity. Rather, Cdk5 interacts with its obligate partner proteins, p35 and p39\(^10\)-\(^13\) whose constitutive expression in post-mitotic neurons is essential for the many known functions of Cdk5 in the regulation of cytoarchitecture, synaptic function and dopamine signaling in the central nervous system. Physiologic Cdk5 activity is essential in neuronal
development memory, and neurogenesis, while aberrant hyperactivity of Cdk5 has been linked with neurodegenerative disorders.

Data from our laboratory and others have implicated Cdk5 in immune dysregulation and inflammatory pain signaling activated by tumor necrosis factor alpha (TNFα), through mechanisms that include transcriptional upregulation of p35. We recently demonstrated a role for Cdk5 in post-translational modification of proteins triggered by TCR and chemokine receptor signaling, and required for optimal immune synapse formation, cellular activation and migratory capacity. Protein phosphorylation by Cdk5 and other kinases can effect conformational changes through modification of binding motifs essential for recruiting proteins into signaling networks or by placing enzymes within proximity to substrates. Both tyrosine and serine/threonine kinases are key modulators during lymphocyte activation and several novel small molecules designed to inhibit these kinases are currently under clinical investigation. Cdk inhibitors have shown activity in experimental models of inflammation and in improving outcomes in pre-clinical models of allo-HCT. However, the specific role of Cdk5 as a mediator of these events remains an important area of investigation.

Here we report a key role for Cdk5 activity in the development of allogeneic T cell responses after HCT. We developed a novel, chimeric, mouse model in which hematopoietic stem cells from Cdk5 deficient (Cdk5−/−) embryos are used to reconstitute lethally irradiated, adult mice (Cdk5−/−C). Transplantation of donor Cdk5−/−C BM and T cells was associated with a significant reduction in the severity of systemic and target organ GVHD. We demonstrate decreased Cdk5−/−C T cell migration to secondary lymphoid organs (SLO), reduced Cdk5−/−C T cell proliferation within these organs, and fewer cytokine producing donor Cdk5−/−C T cells early after HCT. CCR7 signaling following ligation by CCL19 is altered in Cdk5−/−C T cells, which may contribute to defective migration into SLO. Finally,
while Cdk5 activity in donor T cells contributes to GVT effects, pharmacologic inhibition of Cdk5 optimized leukemia-free survival in a manner that includes suppression of Cdk5 activity in leukemia cells in vitro.

Material and Methods

Mice, hematopoietic cell transplantation, lymphocyte assays and statistical analysis

All animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) at Case Western Reserve University (protocol 2010-0076) and Johns Hopkins University (protocol MO13M346). Female C57BL/6J (B6; H-2b) and B6D2F1 (F1; H-2bxd) mice aged 8 to 12 weeks were purchased from Jackson Laboratory (Bar Harbor, ME). Detailed methods for the generation of Cdk5 null hematopoietic chimeras, immunoprecipitation and Cdk5 kinase activity assay, T cell isolation and bone marrow transplant procedures, mixed lymphocyte reactions, T cell proliferation and cytolytic activity, assessment of clinical and target organ GVHD, in vivo GVL models, bio-luminescent imaging techniques, and statistical analysis have been previously described and are outlined in supplemental methods.

In vivo T cell migration, proliferation, and cytokine secretion

Assessment of in vivo T cell migration, proliferation, and cytokine secretion is described in the legend of figure 3 (migration and proliferation) and figure 4 (cytokine secretion) respectively and in supplemental methods. To identify apoptotic cells, single cell suspensions were stained with Annexin-V–APC, 7 amino-actinomycin D (7AAD), and antibodies against CD4 or CD8, prior to analysis by Accuri C6 (BD Bioscience, San Jose, CA).

Western Blot analysis
All tissues and single cell suspensions were treated with Triton-X100 lysis buffer containing complete protease inhibitors (Roche, Indianapolis, IN) to generate protein lysate. Protein levels were measured using the protein BCA assay (Life Technologies, Grand Island, NY) and 2-5 µg of protein from cells were separated by SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes and probed with antibodies against Erk1/2 (Cell Signaling, Technologies, Danvers, MA – rabbit polyclonal), pErk1/2 (Cell Signaling - Thr202/Tyr204 - clone D13.14.4E), pMEK1/2 (Cell Signaling Technologies clone 41G9), Cdk5 (Santa Cruz, Dallas TX clone sc-173), p35 (Cell Signaling Technologies, 2680S), β-actin (Cell Signaling Technologies, 5125S) or GAPDH (Abcam Cambridge, MA clone EPR16891). Antibody detection was performed using West Dura (Life Technologies) and analysis using ChemiDoc and Quantity One software (Bio-Rad, Hercules, CA).

Results

Cdk5−/− T cells develop normally in vivo, exhibit mildly reduced alloreactivity and normal CTL function in vitro.

Germ line deletion of the Cdk5 gene in mice is associated with embryonic lethality. Therefore, we generated Cdk5 null immune chimeric mice (Cdk5−/−C) as described in Supplemental Methods. Three months after embryo transfer, spleen, lymph nodes (LN) and thymus were harvested from fully engrafted, Cdk5+/+C and Cdk5−/−C animals and evaluated anatomically (spleen and LN; Fig. 1A) or by flow cytometry (spleen and thymus; Fig. 1B). No differences between groups were noted in the architecture, cellularity, CD4+ and CD8+ phenotypes, or in the numbers of splenic nTregs (Fig. 1C). Similarly, no differences in Vβ usage were identified between groups (Fig. 1D). In parallel experiments, spleens were collected from Cdk5+/+C and Cdk5−/−C mice and depleted of any remaining CD45.1 cells. T cells were then purified and stimulated in vitro. Functional characterization of Cdk5−/−C T cells in response to allo-antigen stimulation showed only modest reductions in T cell proliferation to
higher concentrations of splenic dendritic cells (DCs) collected from B6D2F1 mice (Fig. 1E) and no differences in cytolytic T lymphocyte (CTL) activity (Fig 1F) or cytokine production (data not shown) were noted between groups.

**Allogeneic HCT using Cdk5<sup>−/−</sup>C donors results in significant reduction of GVHD.**

To define the impact of Cdk5 gene deletion on T cell alloreactivity after HCT we used an established, pre-clinical, model wherein C57BL/6 (B6; H-2<sup>b</sup>) and B6D2F1 (H-2<sup>bxd</sup>) mice serve as HCT donors and recipients respectively. In this model, both CD<sup>4+</sup> and CD<sup>8+</sup> T cells contribute to the development of reproducible systemic and target organ GVHD<sup>33,34,37,38</sup>. We first assessed whether enhanced Cdk5 activity could be detected during early stages of GVHD as described in Supplemental Methods<sup>13</sup>. Cdk5 activity was notably increased by D10 after allogeneic HCT in the spleen and small intestine, established sites of activation and initial recruitment of donor T cells (Fig. 2A).

BM and T cells isolated from Cdk5<sup>+/+C</sup> or Cdk5<sup>−/−C</sup> animals were transplanted into lethally irradiated B6D2F1 mice. HCT recipients of B6 Cdk5<sup>−/−C</sup> donors exhibited a dramatic reduction in mortality and systemic GVHD compared to mice receiving allo-Cdk5<sup>+/+C</sup> HCT (Fig. 2B & C; p < 0.01). These findings were accompanied by a reduction in donor T cell expansion on days 3, 7 and 14 in allo-Cdk5<sup>−/−C</sup> recipients (Fig. 2D) correlating with decreases in day 7 serum IFN<sub>γ</sub> levels (Fig. 2E), and significant reductions in inflammation in key GVHD target organs (Fig. 2F).

Although Cdk5<sup>−/−C</sup> mice show normal hematopoietic cell development<sup>13</sup>, it remained possible that disruption of Cdk5 gene expression in myeloid-derived, BM cells might contribute to the reduction in GVHD severity. We next performed mixing studies wherein T cells from Cdk5<sup>+/+C</sup> or Cdk5<sup>−/−C</sup> donors were added to or “mixed” with either Cdk5<sup>+/+C</sup> or Cdk5<sup>−/−C</sup> T cell depleted BM (in one
of four possible combinations) prior to injection into irradiated F1 recipients. Our results indicate that Cdk5 activity in donor T cells and not BM cells was predominantly responsible for observed reduction in GVHD (Table1).

**Disruption of Cdk5 gene expression impairs T cell migration into SLO and T cell activation in vivo.**

We next assessed the ability of Cdk5⁻⁻ C T cells to migrate to LN and spleen after allo-HCT. Splenic T cells from Cdk5⁺⁺ C (WT) and Cdk5⁻⁻ C (KO) mice were isolated and labeled with either CFSE (WT) or SNARF-1 (KO) and 5 x 10⁶ T cells from each group were co-injected into lethally irradiated F1 animals. A significant reduction in the percentage of Cdk5⁻⁻ C CD4⁺ and CD8⁺ T cells was observed in the spleen and LN at 24 and 48 hrs after injection (Fig. 3A, B). In parallel, T cells from either Cdk5⁺⁺ C or Cdk5⁻⁻ C were labeled only with CFSE and separately injected (5 x 10⁶) into lethally irradiated F1 mice. Spleens and LN from recipients of Cdk5⁻⁻ C T cells analyzed at 72hrs showed significant reductions both in the percent of cells dividing and the number of divisions observed (Fig. 3C). When CFSE⁺ Cdk5⁺⁺ C or Cdk5⁻⁻ C T cells isolated 72hrs after injection were stained with Annexin-V and 7AAD, we found no evidence for increased apoptosis in Cdk5⁻⁻ C T cells in spleen or LN that would account for the marked reduction in proliferation seen in these organs (Suppl Fig 1).

Finally we determined the effect of Cdk5 activity on donor T cell expansion and cytokine production 3, 7, and 14 days after transplant. We found fewer splenic CD4⁺ and CD8⁺ T cells in recipients of allo-Cdk5⁻⁻ C HCT (Fig. 4A), and a reduction in the number of cells producing IFNγ, IL2, and TNFα compared to cells isolated from Cdk5⁺⁺ C HCT recipients (Fig. 4B). Further evaluation of splenic lymphocytes collected from recipients of allo-Cdk5⁺⁺ C HCT on D14 showed a preferential expansion of CD8⁺ cells with an activated phenotype. (Suppl. Fig.2). By D42, the splenic T cell compartment of syngeneic animals was predominately comprised of CD4⁺ cells with a naïve
phenotype (CD44<sup>lo</sup>CD62L<sup>hi</sup>). Splenic T cells in surviving recipients of allo-HCT from Cdk5<sup>+/+</sup>C donors remained predominately CD44<sup>hi</sup>CD62<sup>lo</sup>CD8<sup>+</sup>, whereas recipients of Cdk5<sup>−/−</sup>C T cells showed a phenotype that was similar to syngeneic mice (Suppl. Fig. 2).

**Cdk5 activity regulates CCR7 signaling**

Our data indicate that Cdk5 activity is required for optimal lymphocyte migration and activation *in vivo* (Figs 3 and 4). We have previously shown that Cdk5 gene deletion impairs T cell migration to CCL19 *in vitro*<sup>13</sup>. CCL19 specifically binds to CCR7 and is important in directing the migration of naïve T cells to SLO<sup>39</sup>. Intracellularly, the ERK pathway contributes to CCL19 and CCL21 generated signals governing cell survival and migration<sup>40-42</sup>, and it is known that Cdk5 can suppress ERKs through direct action on a novel site in the MAPK/ERK kinase (MEK)<sup>43</sup>. While ligation of CCR7 by either CCL19 or CCL21 activates the ERK1/2 pathway, CCL19 has been shown to be 4-fold more potent in promoting ERK phosphorylation<sup>44</sup>. We found no difference in the surface expression of CCR7 on peripheral blood T cells from Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C mice when examined by flow cytometry (Fig 5A). To determine how Cdk5 contributes to CCL19 induced changes in ERK 1/2 phosphorylation, purified T cells from Cdk5<sup>+/+</sup>C (WT) or Cdk5<sup>−/−</sup>C (KO) mice were incubated with CCL19 and cell lysates were examined by western blot to measure expression of pERK1/2 to and total ERK1/2 (Fig 5B1). The relative increase in the expression of pERK/ERK in both Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C T cells (Fig 5B2) and the percent reduction of pERK expression in Cdk5<sup>−/−</sup>C T cells were determined at each time point (Fig 5B3). Both Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C T cells expressed similar levels of total ERK-1/2 protein. However, analysis of the kinetics of ERK1/2 phosphorylation shows both delayed and reduced total phosphorylation of ERK1/2 in Cdk5<sup>−/−</sup>C T cells when compared with Cdk5<sup>+/+</sup>C T cells. ERK activation is regulated by MEK, and the MEK-ERK axis is also known to be modulated by Cdk5<sup>45,46</sup>. We observed a similar delay in the kinetics of MEK phosphorylation in Cdk5<sup>−/−</sup>C T cells following exposure to 100 ng/ml CCL19 (Fig 5C).
The contribution of Cdk5 to graft-versus-tumor activity

Given the observed impact disruption of Cdk5 activity had on donor T cell responses after allo-HCT, it was critical to define how loss or inhibition of Cdk5 activity would affect GVT effects. Irradiated B6D2F1 mice received HCT from either allogeneic B6 (Cdk5\(^{-/-}\) or Cdk5\(^{+/+}\)) or syngeneic (F1) donors, and a reduction in systemic GVHD was again noted (Fig 6A). Next, an initial dose of 250 P815 tumor cells (H-2\(^{d}\)) was co-infused with the BM inoculum on day 0. Survival was monitored daily and the cause of each death was determined to be either GVHD or tumor. All syn-HCT recipients uniformly died of widely disseminated, P815 tumor cell infiltration by day 25 whereas recipients of Cdk5\(^{+/+}\) allo-HCT effectively rejected their tumor but died of GVHD. By contrast, allo-HCT recipients of Cdk5\(^{-/-}\) donors exhibited a consistent reduction in GVHD severity, preservation of GVT activity, and improved survival (Fig 6B). To better ascertain the potency of these GVT effects, we doubled the tumor cell inoculum. Allo-HCT from Cdk5\(^{-/-}\) donors still resulted in significant anti-tumor activity, but the survival advantage was reduced (Fig 6C).

Recent studies have uncovered a specific role for Cdk5 in cancer biology and tumor invasiveness\(^{47-49}\) and Cdk5 is highly expressed in a variety of tumors\(^{47,50-52}\). These observations suggest that pharmacologic inhibition of Cdk5 activity might optimize outcomes after HCT not only through suppression of T cell alloreactivity and GVHD, but also through direct anti-tumor effects. The latter may in fact serve to compensate for any reduction in GVT activity as a consequence of Cdk5 gene deletion. Western blot analysis demonstrated the expression of Cdk5 and p35 on P815 and EL4 tumor cell lysates (Fig 6D). To assess the impact of Cdk5 inhibition on tumor cell replication and viability \textit{in vitro}, tumor cells (\(10^{4}\) cells/well) were incubated with \(^{3}\)H-Thy for 24 hrs in the presence or absence of a non-selective Cdk inhibitor (roscovitine) or the Cdk5 Inhibitory Peptide (CIP) which is specific for Cdk5\(^{53}\). As shown in figure 6, a dose dependent reduction in proliferation of both cell lines was
observed in each scenario. In parallel experiments, we found that exposure to these Cdk5 inhibitors also increases the percentage of early apoptotic cells as measured by Annexin-V and 7AAD (Fig 6G).

In a final set of studies, irradiated F1 mice received HCT from either syngeneic (F1) or allogeneic, (naïve B6) donors. The P815 cells used in these experiments expressed a luciferase reporter\textsuperscript{36} and were added to the donor cells on day 0. Subsets of HCT mice received roscovitine 10 mg i.p. daily x 21 days and all animals were followed for survival, clinical score and tumor burden by bioluminescence imaging as described in \textit{Supplemental Methods}. As anticipated, susceptible, syngeneic animals rapidly succumbed to tumor whereas allo-HCT recipients benefited from significant GVT activity (Fig 7A). Consistent with previous reports\textsuperscript{32}, allogeneic mice treated with roscovitine maintained anti-tumor effects comparable to allogeneic controls and had a significant reduction in GVHD clinical score (Fig 7B). Importantly, the dose and schedule of roscovitine administered non-selectively inhibited Cdk5 activity in spleens of allogeneic-HCT recipients 7 days after HCT (Fig 7c) and had no significant effect on survival of syngeneic mice receiving P815; all animals died from tumor dissemination before D20 (median survival time: 16D for Syn + P815 + Rosco and Syn + P815).

\textbf{Discussion}

GVHD and malignant disease relapse remain the primary causes of death following allo-HCT, and novel strategies that modulate alloreactivity without compromising GVT activity are being pursued. We examined whether targeted disruption of Cdk5, a serine-threonine kinase known to be involved in neurodegenerative and inflammatory disorders, might mitigate the toxicity of GVHD while preserving anti-tumor effects. We first generated chimeric mice that lack Cdk5 expression in all immune cells since germ-line deletion of Cdk5 is embryonically lethal\textsuperscript{13}. Immuno-phenotyping of these hematopoietic chimeras (Cdk5\textsuperscript{-/-C}) showed no difference in structure, cellularity, or lineage distribution in hematopoietic organs (bone marrow, thymus, spleen, or lymph node) compared to
Cdk5<sup>+/−</sup>C mice. While immunologically intact, our prior studies demonstrated that Cdk5<sup>−/−</sup>C mice are less susceptible to experimental autoimmune encephalomyelitis (EAE) <sup>13</sup>. Proteomic analysis of activated T cells revealed that a major target of Cdk5 phosphorylation is coronin-1a, an adapter protein that links cytoskeleton dynamics to the TCR and TCR signaling. Specifically, phosphorylation of a critical threonine-418 was absent in T cells of Cdk5<sup>−/−</sup>C mice and associated with a reduction in F-actin-mediated polarization of the T cell membrane following TCR ligation. As with lymphocytes deficient in coronin1a<sup>54</sup>, pharmacological suppression and genetic deletion of Cdk5 resulted in reduced migration in response to CCL19, a major ligand for CCR7.

Data presented herein demonstrate that while lymphocytes from Cdk5<sup>−/−</sup>C mice exhibit slight reductions in proliferation to allo-antigen and no decrements in cytolytic function <em>in vitro</em>, they are significantly impaired in their capacity to initiate and sustain the clinical hallmarks of GVHD <em>in vivo</em>. These findings were associated with a reduction in T cell migration into SLO, diminished proliferation, and lower numbers of cytokine producing T cells in these tissues. The difference in the number of Cdk5<sup>−/−</sup>C T cells present in SLO did not reflect changes in T cell survival during the early phase of activation; both Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C T cells show similar percentages of cells that are annexin-V<sup>+</sup>. Analysis of splenic T cells from mice surviving 6 to 7 weeks after HCT suggests a failure of Cdk5<sup>−/−</sup>C T cells to maintain an effector phenotype, and donor chimerism is robust in both Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C BMT recipients (97 ± 3% vs. 99 ± 0.7% respectively) at this time. While Cdk5<sup>+/+</sup>C and Cdk5<sup>−/−</sup>C T cells were predominately CD8<sup>+</sup> and CD44<sup>hi</sup> 2 weeks after HCT, there was a shift towards CD44<sup>lo</sup> cells by 6-weeks post-transplant with Cdk5<sup>−/−</sup>C T cells. The reduction in the expression of CD44 suggests that these cells are naïve, as a transition from effector to memory cells will maintain CD44 expression<sup>55</sup>. In aggregate, our results demonstrate that Cdk5 activity contributes to optimal lymphocyte activation and migration and suggest a potential role for Cdk5 in the generation and maintenance of memory T cells. Studies are planned to more definitely test this hypothesis.
The SLO are a major site of T cell activation during GVHD. Factors that can influence interaction between host APCs and donor T cells in SLO reduce GVHD severity. Several chemokines contribute to the recruitment of lymphocytes to SLO in HCT and non-HCT settings (reviewed in39). CCR7 is a G protein–coupled receptor expressed on naive T cells (both conventional and regulatory), B cells, and dendritic cells (DCs) that binds to two structurally unique ligands, CCL19 and CCL2156-58. CCR7 is the central regulator of homing and trafficking of lymphocytes into SLO including LNs and the splenic white pulp. Ligation of CCR7 on T cells by CCL21 allows for lymphocyte firm arrest on LN high endothelial venules (HEV)56. After egress from the circulation, CCR7 also direct T cells to appropriate sub-regions within LN and spleen in response to CCL1939. Our observations are very similar to those reported using mice deficient in CCR7 as allo-HCT donors59. CCR7−/− T cells also exhibit an impaired ability to traffic to recipient lymph nodes and a reduced ability to undergo in vivo expansion in the spleen59. Moreover, we found that phosphorylation patterns of key down-stream intermediates of CCR7 signaling (following CCL19 binding) are significantly altered (ERK1/ERK2) or delayed (MEK) in T cells isolated from Cdk5−/−C mice (Fig 7). We did not complete similar studies in peripheral tissues, semi-quantitative. However, subset analyses show that mononuclear cell infiltration into the gut and liver was reduced in mice receiving BMT from Cdk5−/−C donors compared to littermate controls (intestine: 5.0 ± 0.4 vs. 8.6 ± 0.5; liver: 2.3 ± 0.3 vs. 4.4 ± 0.2. p < 0.05). It remains unresolved whether a reduction of lymphocytes into GVHD target organs is secondary to diminished donor T cell expansion in SLO or a direct effect of Cdk5 on effector cell migration to inflamed tissue.

An emerging body of data implicate Cdk5 activity in processes and conditions as diverse as neurodegenerative disease11,17, inflammation13,22,23,31, cancer biology47,50-52, and tumor invasiveness48,49. Cdk5 was the top hit of 37 genes that when silenced synergistically potentiate effects of proteosome inhibition in myeloma cells47, and a cell-based high-content screening assay determined
that Cdk5 was the biologic target of several cyclin-depending kinase inhibitors that could modulate
cancer cell invasion capacity. Cdk5 is highly expressed in a variety of tumors and several
clinical trials studying the use of roscovitine and other non-selective Cdk5 inhibitors in patients with
cancer have either been completed or are ongoing. Our studies demonstrated that the Cdk5 specific
inhibitor CIP resulted in a dose dependent reduction in tumor cell proliferation and an increase in the
percentage of early apoptotic cells in culture. The optimal formulation of CIP for administration has yet to be determined. A limitation to our in vivo GVL studies is that roscovitine is a broadly acting Cdk inhibitor potently inhibiting Cdk1, 2, 5 and 7. Via its effects on Cdk2, roscovitine has been shown to reduce human alloreactive responses without impairing key responses to tumor (WT-1) or vial (CMV, EBV) antigens. Furthermore, defective activation of Cdk2 (along with Cdk4 and 6) has been associated with tolerance induced by IL-10 and TGFβ and with a reduction in experimental GVHD. These data notwithstanding, the administration of roscovitine at the dose and schedule delivered did effectivity abrogate Cdk5 activity and consistent with a previous report was associated with a reduction in clinical GVHD and preservation of GVL effects. Finally, any strategy to modulate GVHD severity that focuses on T cell activation, or migration (to SLO or peripheral organs) may influence (negatively) responses to opportunistic infection. While Nellore and colleagues assessed the effects of roscovitine on human T cell responses to CMV and EBV in vitro, future lines of investigation to study the impact Cdk5 gene deletion has on infectious immunity would have significant merit.

The potential utility of these agents in the setting of inflammatory and immune disorders is high given the increasing appreciation for the roles that cyclin dependent kinases play in T cell biology. For example, Foxp3 stability is regulated by Cdk2 through phosphorylation of four cyclin-dependent kinase motifs (Ser/Thr-Pro) within the N-terminal repressor domain, and we have shown
the capacity of interleukin-6 to suppress activation of Foxp3 expression by TGF-β requires Cdk5-dependent phosphorylation of STAT3 on Serine 727. Moreover, we have also shown that Cdk5 controls IL-2 gene expression via repression of the mSin3a-HDAC complex during T cell activation. Finally Cdk5 activity may also significantly contribute to inflammation engendered by the innate immune response, which is also operative during GVHD. Cdk5 activity was notably increased in macrophages stimulated by LPS through the synthesis of its binding partner p35. Specifically, Cdk5 activity enhances the inflammatory function of macrophage by regulating MAPK-dependent production of suppressor of cytokine signaling (SOCS)-3 and IL-10. While experiments mixing BM from Cdk5-/-C donors with Cdk5+/+C T cells resulted in a modest improvement in survival after allo-HCT (33% vs. 13%, table 1), systemic GVHD as measured by clinical score was not different between groups underscoring the predominant role of Cdk5 activity in the T cell compartment as a determinant of GVHD severity.

In summary, the accumulating evidence regarding Cdk5-dependent T cell function has important implications for a broad-spectrum of immune and inflammatory disorders. The data presented here suggest that targeting Ckd5 may be a particularly attractive strategy to reduce GVHD and maintain (or enhance) anti-tumor activity. Hence our results not only further illuminate the role and mechanism of Cdk5 in modulating GVHD but also provide a rationale for developing innovative strategies using (selective or non-selective) small molecule inhibitors of Cdk5 to prevent or treat this lethal complication in the context of carefully controlled clinical trials. The relevance of the latter is quite high with an expanding industry effort to develop specific Cdk inhibitors, including small molecules already in early phase cancer trials.
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<td>2.4 ± 0.3 *</td>
<td>2.5 ± 0.4 *</td>
<td>3.0 ± 0.5 *</td>
<td>73*</td>
</tr>
<tr>
<td>Allo KO T + Allo WT BM → F1</td>
<td>3.2 ± 0.8 *</td>
<td>2.6 ± 0.5 *</td>
<td>2.7 ± 0.4 *</td>
<td>60*</td>
</tr>
</tbody>
</table>

**Table 1:** Lethally irradiated B6D2F1 received HCT from either syngeneic B6D2F1 or allogeneic Cdk5+/+C (Allo WT) or Cdk5−/−C (Allo KO) donors as described in Figure 2. Two additional allogeneic groups were evaluated in this “mixing” experiment as described in Methods: allogeneic KO BM cells mixed with allogeneic WT T cells and allogeneic WT BM cells mixed with allogeneic KO T cells. Survival was monitored daily and GVHD clinical scores were assessed weekly as described; n = 8 to 16 per group. *p < 0.01 compared to recipients of Cdk5+/+C HCT.

**Figure Legends**

**Figure 1:** *Immune reconstitution is intact in Cdk5−/−C hematopoietic chimeras.* The spleen, lymph nodes (LN) and thymus were harvested from naïve, fully engrafted, Cdk5+/+C (WT) and Cdk5−/−C (KO) mice and sectioned and either stained for anatomical evaluation – spleen and LN (A) or enzymatically digested, dispersed into single cell suspensions, stained for CD4 and CD8, and examined by flow
cytometry – spleen and thymus (B). No differences between groups were noted in architecture, cellularity or CD4+ and CD8+ phenotypes. The numbers of nTregs in spleen were determined by staining single cell suspension with CD4 and CD25 on surface followed by intracellular staining of FoxP3 (C). Vβ usage in T cell populations was determined by staining single cell suspension of splenocytes with Vβ antibodies in combination with ant-CD4 and anti-CD8 (D). No differences in splenic Treg numbers or Vβ usage were noted. N = 3 to 6 mice per group. CD4+ and CD8+ T cells from or Cdk5−/− mice were cultured with B6D2F1 splenic DCs for 96 h and examined for proliferative capacity (E) and cytotoxicity (F) when added 3H-thymidine labeled, allogeneic (P815) or syngeneic (EL4) tumor cells. Data are representative of 1 of at least 3 replicate experiments. *p < 0.05

**Figure 2:** *Cd5k activity is critical for GVHD induction*. Lethally irradiated B6D2F1 mice received HCT from either syngeneic B6D2F1 or allogeneic (B6) mice as described in *Supplemental Methods*. Cdk5 kinase activity was significantly increased in the spleen and small intestine by D10 after HSCT (A). Allo-HCT using Cdk5−/− (Allo Cdk5 ko) donors results in significant reduction in GVHD severity as measured by survival (B), clinical score (C), splenic T cell expansion (D), serum IFNg levels (E) and target organ histopathology (F) compared to recipients of Cdk5+/+ (Allo wt) donors. Data are from 2 to 3 comparable experiments, n = 3 to 4 mice per group (A), n = 12 to 24 mice per group (B and C) and 4 to 8 mice per group (D, E, F). *p < 0.01 for all comparisons.

**Figure 3:** *Cdk5 regulates migrational and proliferative capacity of donor T cells after allogeneic HCT*. Isolated Cdk5+/+ (WT) T cells were stained with 2.5 μl CFSE and Cdk5−/− (KO) T cells were stained with 5 μl SNARF-1. Equal numbers of WT and KO T cells (3-5 x 10^6) were co-injected into lethally irradiated B6D2F1 mice. Spleens and lymph nodes were isolated from recipient mice at 24 and 48h after injection and cells were stained with CD4 or CD8 fluorescent antibodies and examined by
flow cytometry. The percentage of CD4+ and CD8+ from WT (CFSE+) and KO (SNARF-1+) and percentage reduction of cells from KO mice were determined (A, B). In vivo T cell proliferation was assessed in parallel experiments. T cells from Cdk5+/+C or Cdk5-/-C were labeled with CFSE and 5x10^6 T cells were separately injected i.v. into lethally irradiated B6D2F1 mice. 72h later mice were sacrificed and the spleens were removed. Proliferating cells from WT or KO donors were identified based upon decreased staining for CFSE (C, D). Percent reduction at 72hr likely reflects effects of Cdk5 on both migration to secondary lymphoid organs (SLO) and subsequent proliferation once present. N = 4 mice per group and represent one of at least three replicate experiments.

**Figure 4:** Loss of Cdk5 expression results in fewer donor-derived, cytokine–producing T cells early after HCT. Lethally irradiated B6D2F1 received HCT from either syngeneic (B6D2F1) or allogeneic Cdk5 (Cdk5 wt) or Cdk5-/-C (Cdk5 ko) donors as described in Figure 2. HCT recipients were sacrificed and single cell suspensions were prepared from spleen of mice 3, 7, 14 days post-transplant. Total numbers of donor-derived, CD4+ and CD8+ T lymphocytes were determined in Kd- or Kd+ cell populations in spleens of mice receiving allo- or syngeneic HCT respectively (A). Cells were also incubated on anti-CD3 coated plates for 6 h in RPMI-10% FBS containing 1 μg/ml brefeldin A, subsequently stained with anti-CD4 and anti-CD8, and incubated with fluorescent anti-cytokine antibodies. Total numbers of donor-derived, CD4+ and CD8+ T cells were counted and examined for the production of IFNγ, IL-2 and TNFα (B). Data are representative of one of 3 replicate experiments, N = 3 to 4 mice per group. *p < 0.01, #p < 0.05 or as otherwise noted.

**Figure 5:** Patterns of phosphorylation of known intermediates of CCR7 intra-cellular signaling are altered in CDk5 deficient T cells. Cell surface expression of CCR7 on peripheral blood CD4+ and CD8+ T cells was examined by flow cytometry after labeling cells with CCL19-Fc followed by anti-
human IgG-PE and either CD4-APC or CD8-APC (A). Next, purified T cells from Cdk5^{+/+} (Cdk5 WT) or Cdk5^{-/-} (Cdk5 KO) mice were incubated with 100 ng/ml CCL19 for 0 to 4 min and then lysed with triton-X100 buffer containing phosphatase/protease inhibitors. Total cell lysate (4μg) was separated on a 4-12% Bis-Tris gel and transferred to nitrocellulose. Cell lysates were examined by Western blot using antibodies against pErk1/2 to measure levels of phosphorylated protein and Erk1/2 to measure levels of pErk1/2 to and total Erk1/2 loaded (B1). The relative increase in expression of pERK/ERK in lysates from either WT or KO T cells was determined at each time point using time 0 for respective samples (e.g. 100%) as baseline (B2). The percent reduction of pERK expression in KO T cells was also determined at each time point (B3). In separate experiments, purified T cells from WT or KO mice were again incubated with 100 ng/ml CCL19 and cell lysates were examined for expression of pMEK1/2 and GAPDH (C). Data shown are from one of 3 replicate experiments.

**Figure 6:** *Effects of Cdk5 gene deletion on GVL activity:* Lethally irradiated B6D2F1 mice received HCT from either syngeneic (B6D2F1) or allogeneic Cdk5^{+/+} (Allo Cdk5 WT) or Cdk5^{-/-} (Allo Cdk5 KO) donors as described in figure 4. Consistent with previous experiments, animals receiving HCT from Cdk5^{-/-} donors have significantly reduced mortality from GVHD (A). In subsequent experiments, groups of HCT recipients received 250 or 500 P815 tumor cells at time of transplant. Recipients of BM and T cells from Cdk5 KO donors effectively eliminate low dose tumor challenge and show improved leukemia free survival (B). At higher tumor dose, significant GVL activity remains, but some Cdk5 KO HCT recipients succumb to tumor (C). The expression of Cdk5 and p35 was determined on P815 and EL4 tumor cell lysate using Western Blot analysis (D). Tumor cells (10^4 cells/well) were incubated with ^3H-Thy for 24 h in the presence or absence of Roscovitine or CIP and cell proliferation was determined (E, F). In parallel experiments, tumor cells incubated with Roscovitine or CIP for 24 h were stained with annexin-V and 7amminoactinomycin D (7AAD) and the
percentage of early apoptotic cells was determined (G). Data are from at least 2 combined experiments, n = 8 to 12 per group (A-C), or one of at least two comparable experiments (E-F). *p < 0.01.

**Figure 7:** Pharmacologic Inhibition of Cdk5 after HCT reduces clinical GVHD severity and while maintaining potent GVL effects. Lethally irradiated B6D2F1 mice received HCT from either syngeneic B6D2F1 or allogeneic (B6) mice as described in Figure 2. Groups of mice received 500 P815 cells previously transduced using a lentiviral vector carrying a luciferase reporter that allows visualization of proliferating cells using bioluminescence imaging (BLI). Syngeneic and allo-HCT recipients were monitored for tumor progress (A) and clinical GVHD severity (B). Roscovitin, at the dose and schedule administered after HCT effectively inhibited Cdk5 activity measured in spleens of mice collected on D7 post-transplant (C). Data shown are representative of two replicate experiments, n = 5 to 12 mice per group; P < 0.01. Note: a single mouse in the allo-HCT group receiving roscovitine unexpectedly died following anesthesia for BLI and was censored “C”. Radiance is expressed as p/sec/cm2/sr. Color scale: Min = 2.00e4, Max = 2.00e5.
Figure 1. Askew et al.
A. CDk5 activity

B. Survival

C. Clinical GVHD

D. T cell expansion

E. Serum IFNγ

F. Target organ GVHD

Figure 2. Askew et. al
Figure 4. Askew et al.
Figure 5. Askew et al.
Figure 6. Askew et. al
A. Tumor burden by BLI

B. Systemic GVHD

C. Splenic Cdk5 activity

Figure 7. Askew et. al
Cyclin-dependent kinase 5 activity is required for allogeneic T cell responses after hematopoietic cell transplantation in mice

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