Published in final edited form as:

Clin Immunol. 2014 ; 152(0): 48–57. doi:10.1016/j.clim.2014.02.015.

The cyclin dependent kinase inhibitor (*R*)-roscovitine mediates selective suppression of alloreactive human T cells but preserves pathogen-specific and leukemia-specific effectors

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Abstract

Graft versus host disease (GvHD), mediated by donor T cells, remains the primary cause of non-relapse mortality after allogeneic hematopoietic stem cell transplantation and novel therapeutic approaches are required. Cdk2 is a critical node of signal integration and programming of T cell responses towards immunity versus anergy but is dispensable for hematopoiesis and thymocyte development. We examined the effects of pharmacologic Cdk2 inhibition on alloreactive human T cells. Inhibition of Cdk2 blocked expansion of alloreactive T cells upon culture with HLA-mismatched dendritic cells and prevented generation of IFN- γ -producing alloantigen-specific effectors. In contrast, Cdk2 inhibition preserved effectors specific for Wilms' tumor 1 (WT1) leukemia antigen and for CMV as determined by WT1-specific and CMV-specific pentamers. Cdk2 inhibition preserved Treg cells, which have the ability to prevent GvHD while maintaining GvL. Thus, Cdk inhibitors may improve allogeneic HSCT by reducing alloreactivity and GvHD without loss of pathogen-specific and leukemia-specific immunity.

Keywords

T cells; Graft versus host disease; Cdk2; T regulatory cells

1. Introduction

Graft versus host disease (GvHD) is a frequent and severe complication of allogeneic hematopoietic stem cell transplantation (HSCT). GvHD remains the main cause of non-relapse mortality after HSCT and compromises the curative potential of this treatment modality in hematologic malignancies. Recognition of recipient alloantigens by donor T cells forms the basis of GvHD [1]. Numerous interventions have been employed against GvHD, most of which target the number and the function of T cells transferred from the donor to the recipient. Non-specific inhibitors of T cell activation including cyclosporine A

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Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2014.02.015>.

or tacrolimus are administered routinely to all HSCT recipients [2,3]. Experimental strategies target the T cells within the transferred graft, either by negative depletion of CD3⁺ T cells or by transfer of positively selected CD34⁺ stem cells [4–6]. In other cases, polyclonal or monoclonal antibodies have been used to purge the allograft or can be administered to the recipient around the time of graft infusion, including antithymocyte globulin or Campath-1H [7,8]. While these strategies reduce the risk of GvHD, the benefits are often offset by the simultaneous nonspecific reduction of non-alloreactive T cell function, leading to prolonged post-transplantation immunodeficiency and to increased risk of infection and disease relapse [9,10]. Thus, novel practical methods are required to allow specific depletion, suppression or inactivation of alloreactive T cells while sparing other T cell populations, thereby preserving GvL and pathogen-specific immune responses.

Cellular immune responses require expansion of antigen-specific T cell clones from the pool of resting T lymphocytes that perform immune surveillance. Highly controlled regulation of this proliferative potential is critical for defense against pathogens and foreign antigens with simultaneous avoidance of autoimmunity [11,12]. The link between cell cycle progression and T cell effector function has been well documented. T cells that progress through multiple cell divisions during the primary response exhibit strong cytokine production and proliferation upon re-stimulation. In contrast, those cells that do not divide during the primary response fail to produce IL-2 and exhibit growth arrest and unresponsiveness upon rechallenge with antigen. These observations supported the idea that cell cycle progression is necessary to prevent the induction of the anergic state [13]. Consistent with this hypothesis it was determined that the activation of the Cdk2–cyclin E holoenzyme is a critical mediator of signal integration and programming of T cell responses towards immunity versus anergy [14,15].

Although Cdk2 activation is mandatory for the induction of T cell immune responses and prevention of T cell anergy, it is not required for hematopoiesis or thymic development [16]. These properties of Cdk2 make it an attractive therapeutic target for control of GvHD. Previously, we determined that inhibition of Cdk2 suppressed expression and activation of alloreactive T cells in vitro and in vivo and protected from acute lethal GvHD in a mouse model of allogeneic bone marrow transplantation [17]. To evaluate whether pharmacologic inhibition of Cdk2 might control responses of human T cells upon encounter of MHC-mismatched alloreactive stimulators, we employed (*R*)-roscovitine (CYC202), a potent and selective inhibitor of Cdk2–cyclin E with a 50% inhibitory concentration (IC₅₀) of 0.1 μM and a low inhibitory efficiency for complexes of Cdk7–cyclin H, Cdk9–cyclin T1 and Cdk5–p35–p25 [18].

Our studies showed that inhibition of Cdk2 during culture of primary human T cells with allogeneic stimulators resulted in a T cell population that had reduced alloantigen-specific reactivity. Detailed analysis revealed that, by this approach, CMV-specific effectors were retained as determined by their identification with CMV-specific pentamers. Effectors for Wilms' tumor 1 (WT1) leukemia antigen were also retained. In addition, pharmacologic inhibition of Cdk2 preserved and increased Foxp3⁺ Treg cells within the responder T cell populations. These observations suggest that pharmacologic modulation of Cdk2 either alone or in combination with currently established immunosuppressive medications might

reduce the incidence and severity of GvHD without loss of pathogen-specific and leukemia-specific immunity.

2. Materials and methods

2.1. Cell preparations and primary mixed-lymphocyte reaction (MLR)

PBMCs were prepared from leukopacks (platelet apheresis byproduct) obtained at the Dana Farber Cancer Institute and the Children's Hospital of Boston. A protocol for this collection has been approved at the respective Institute's institutional review board. Mononuclear cells were isolated by Ficoll (Amersham-Pharmacia Biotech, Piscataway, NJ) gradient centrifugation. Responder T cells were isolated using negative selection with the Miltenyi Biotec Pan T Cell Isolation Kit (Auburn, CA). Monocytes were isolated by negative selection using the Monocyte Isolation Kit II from Miltenyi Biotec (Auburn, CA). In order to generate dendritic cells these monocytes were cultured in X-VIVO 20 medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) with supplementation on days 0, 2, and 4 with 25 ng/ml of IL-4 (R&D Systems) and 25 ng/ml GM-CSF (R&D Systems). DC maturation was achieved as previously established [19]. Briefly, monocyte cultures were supplemented on days 6 through 8 with IL-4 (25 ng/ml), GM-CSF (25 ng/ml), IL-1 β (10 ng/ml, R&D Systems) and PGE2 (1 μ g/ml; Sigma-Aldrich, St. Louis, MO). Mature DCs were consistently CD11c⁺, CD40⁺, CD83⁺, CD80⁺, CD86⁺, HLA ABC⁺, HLA DR⁺, CD3⁻ and CD14⁻ as determined before using this differentiation/maturation approach [19]. For primary MLR, stimulator DCs were irradiated (5000 Rad) and plated at 1:20 ratio with responder T cells in either roscovitine (10 μ M) or vehicle control in culture media. Roscovitine (CYC202) was kindly provided by Cyclacel (UK).

Stimulation of primary T cells with antibody-coated beads was performed as previously described [20]. Briefly, T cells were resuspended as 10×10^6 cells/ml in pre-warmed RPMI 1640 containing 10 mM HEPES and mixed with equal volume of RPMI/HEPES containing equal numbers of beads (for 1:1 ratio) conjugated with anti-CD3 and anti-CD28 mAbs. T cells were plated at 1.5×10^5 cells/well in 96 well flat-bottom plates with magnetic beads (1.5×10^5 beads/well). Cultures were performed in RPMI 1640 with L-glutamine (Cellgro/Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 10 mM HEPES, 1 mM sodium pyruvate, 50 U/ml Pen/Strep (from Cellgro/Mediatech, Manassas, VA), and 80 μ g/ml gentamycin (from Gibco/Invitrogen, Grand Island, NY). Where indicated, roscovitine (10 μ M) was added at the initiation of the cultures.

2.2. Flow cytometry analysis

Samples were analyzed in a Becton Dickinson FACScalibur (San Jose, CA) with at least 10,000 positive events measured per sample. The following antibodies were used: anti-CD25-APC and anti-CD71-PE (eBioscience) and anti-CD83-FITC, anti-CD11c-PE, anti-CD80-PE, anti-CD14-APC, anti-HLADR-APC, anti-CD4-APC, and anti-CD8-PE (BD/Pharmingen). Intracellular Foxp3 staining was performed using the Human Regulatory T Cell Staining kit (eBioscience).

2.3. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and analysis

CFSE labeling was performed according to standard protocol. Responder T cells were washed with PBS and resuspended in a CFSE solution of 0.5 μ M for 15 min. Subsequently cells were washed two times in MLR culture medium and were subsequently plated in MLR cultures. Assessment of dividing cells was done by flow cytometry and analysis with FlowJo software.

2.4. ELISPOT assay

Interferon gamma and IL-17 ELISPOT (eBioscience) kits and ELISPOT plates from BD Biosciences were used for all ELISPOT assays. ELISPOT plates were prepared according to manufacturer's instructions. Primary MLR was performed and after 48 h responder T cells were purified by negative selection using the Pan T Cell Isolation Kit II (Miltenyi). These cells were rested over 24 h and subsequently, 5×10^4 CD4⁺ cells from each fraction were challenged with the same number of APC from the original stimulators, in 100 μ l of complete medium. Production of IFN- γ and IL-17 was determined by ELISPOT after 48 h of culture. The numbers of spots were counted using ImmunoSpot S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH) and the means of triplicate wells for each culture condition were calculated.

2.5. Analysis of antigen-specific T cells

Responder T cells from HLA-A*0201 expanded in the MLR culture were stained with APC-labeled HLA-A*0201/CMV-pp65 pentamer (NLVPMVATV; ProImmune) or Wilms' tumor 1 (WT1) pentamer (HLA-A*0201/WT1 pentamer RMFPNAPYL; ProImmune) according to manufacturer's instructions. Cells were washed twice in MLR culture medium and were subsequently stained with anti-CD8-FITC and analyzed in a Becton Dickinson FACScalibur flow cytometer. For assessment of EBV-specific responses HLA-A*0201 positive naïve, cord blood T cells were used as responders and were cultured with autologous APC and a pool of EBV peptides corresponding to EBV immunogenic epitope BMLF1, in the presence or in the absence of roscovitine. Detection of EBV-specific T cells was performed using HLA-A*0201/GLCTLVAML-APC conjugated Dextramer (#RX05APC; Immudex, Copenhagen, Denmark), specific for BMLF1.

2.6. Suppression T cell assay

After a 9-day MLR culture in the presence of roscovitine or vehicle control, responder T cells were harvested and were subsequently sorted for CD4⁺CD25^{high} using a FACScalibur cell sorter. Sorted CD4⁺CD25^{high} cells were plated in serial dilutions in a fresh MLR culture generated with a new set of responder stimulator pair. Thymidine uptake was assessed for the last 16 h of a 4-day culture.

2.7. Cell lysate preparation, western blot and in vitro kinase assay

At the indicated time points of MLR culture cells, T cells were isolated and were harvested and protein lysates were prepared by washing cells in PBS and lysing them in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 10% glycerol, and 1% NP-40 supplemented with 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl

fluoride (PMSF), and Protease Inhibitor Cocktail (Thermo Fisher Scientific), according to previously described methods [20]. Samples were analyzed by SDS-PAGE transferred to nitrocellulose membranes, followed by immunoblot with the indicated antibodies. For in vitro kinase reactions, immunoprecipitations were performed with mAb against Cdk2 that was conjugated to agarose (Santa Cruz) followed by in vitro kinase reactions with histone H1 as the exogenous substrate, as previously described [20]. Reactions were analyzed by 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and exposed to film.

3. Results

3.1. Roscovitine inhibits activation of Cdk2 in alloreactive human T cells during allogeneic MLR

The most broadly used ex-vivo approach for generation of alloreactive T cells for experimental and pre-clinical studies has been the co-culture of PBMC used as responders with irradiated unrelated HLA mismatched PBMC used as stimulators in MLR culture. This approach is also established as a conventional way to assess donor/recipient compatibility for clinical purposes [21]. Other groups have chosen to use as allostimulators EBV transformed B cells [22] or activated T lymphocytes [23], because of their ease of collection and expansion to large numbers. However, these approaches may not accurately represent the in vivo events related to the generation of GvHD after transplant. Based on the clinical presentation of GvHD and the preferential organ involvement in this condition, it is most likely that professional antigen presenting cells of the host tissue trigger the responses of donor T cells transferred with the graft, which encounter recipient's alloantigens for the first time [24]. For this reason, we have previously established an in vitro experimental model of human MHC-mediated alloreactivity using as responders primary human T cells and as stimulators in vitro generated dendritic cells (DCs) [19].

First, we performed initial studies to determine the concentration of roscovitine that would not induce non-specific toxicity in human T cells. Purified primary human T cells were cultured with tosylactivated magnetic beads conjugated with anti-CD3 and anti-CD28 antibodies in the presence of titrated doses of roscovitine or vehicle control. Roscovitine used at concentrations as low as 2 μ M resulted in reduction of cell proliferation by 50% (Fig. 1A). Assessment of cell survival analyzed in parallel, indicated that 10 μ M of roscovitine concentration was optimal to avoid non-specific toxicity and to retain cell survival >85% while inhibiting T cell proliferation responses (Fig. 1B). In all subsequent studies we used this concentration of roscovitine.

To determine the effects of roscovitine on Cdk2 activation during stimulation of alloreactive T cells by MHC disparate allo-stimulators we used MLR cultures of responder primary T cells and MHC mismatched stimulator DCs. Assessment of Cdk2 activity by in vitro kinase reaction revealed that roscovitine inhibited the enzymatic activity of Cdk2 in primary human T cells stimulated by alloreactive DC in the MLR experimental system (Fig. 1C).

3.2. Roscovitine inhibits activation, expansion and differentiation of alloreactive human T cells to IFN- γ producing effectors

To determine the functional consequences of Cdk2 inhibition during MLR, we assessed expansion of responder T cells. Addition of roscovitine reduced proliferation of both CD4⁺ and CD8⁺ responder T cells as measured by CFSE dye dilution. This reduced proliferative potential was best observed after six days of MLR culture, at which time alloreactive DCs had induced significant T cell expansion in control cultures that did not contain roscovitine (Fig. 2A). To determine whether this inhibitory effect on cell cycle re-entry mediated by roscovitine also coincided with an altered T cell activation status, we assessed T cell activation by assessment of CD25 and CD71 expression. As shown in Fig. 2B, a significantly smaller proportion of CD4⁺ and CD8⁺ T cells expressed CD25 and CD71 in the presence of roscovitine. Thus, inhibition of cell cycle progression by roscovitine also impacts on the activation status of responder T cells.

To further assess the functional status of alloreactive T cells that were treated with roscovitine during encounter of alloantigens expressed on allostimulator DCs, we analyzed their effector function by evaluating their capacity to produce cytokines in response to rechallenge with specific allostimulators. Alloantigen-primed T cell populations, with or without roscovitine treatment, were incubated with original allostimulator DCs and responses were examined by ELISPOT specific for IFN- γ and IL-17. Strikingly, IFN- γ producing T effector cells were reduced after culture in the presence of roscovitine, whereas IL-17 producing T effector cells were comparable (Fig. 2C). Thus, Cdk2 inhibition prevented the generation of IFN- γ -producing alloreactive effector CD4⁺ T cells while sustaining reprogramming of alloreactive T cells to IL-17 producers.

3.3. Treatment with roscovitine allows preservation of leukemia-specific and pathogen-specific effectors

Graft versus leukemia (GvL) responses are integral to the efficacy of bone marrow transplantation [1]. To determine if roscovitine treatment might compromise leukemia-specific immunity, we assessed responses specific to Wilms' tumor 1 (WT1) protein, a previously established surrogate marker for leukemia-specific responses. Antigens derived from the WT1 protein are overexpressed in acute and chronic myelogenous leukemias. Four HLA-A*0201-restricted WT1-derived epitopes display natural immunogenicity not only in patients with hematologic malignancies but also in healthy donors [25]. To determine whether roscovitine might compromise leukemia-specific immunity we used T cells from HLA-A*0201 homozygous individuals and a previously established approach of in vitro expansion of WT1-specific precursors to levels detectable by flow cytometry [19]. We assayed T cells specific for the WT1 tumor epitope RMFPNAPYL within the entire CD8⁺ T cell population after MLR cultures in the presence of roscovitine or control. Treatment with roscovitine permitted retention of WT1-specific T cells in the CD8⁺ populations (Table 1 and Supplementary Fig. 1).

Morbidity and mortality related to opportunistic infections are serious risks of allogeneic transplantation. Common opportunistic infections related to impaired T cell immunity in a transplantation setting are related to reactivation of latent viruses CMV, VZV and EBV.

Among them, CMV is a major source of post-transplant morbidity due to the high prevalence of infection among healthy populations, leading to frequent reactivation in the post-transplant period due to delayed quantitative reconstitution of T lymphocytes and to the effects of current GvHD treatment drugs, like cyclosporine, which restrain the ability of CD8⁺ T effector cells to function against CMV.

In order to examine the effects of roscovitine treatment on CMV-specific CD8⁺ T cells within the CD8⁺ T cell population, we used fluorescent-labeled pentamer of the HLA-A*0201 allele specific for the immunodominant CMV epitope NLVPMVATV. Using T cells from responders who were homozygous for HLA-A*0201 and positive for CMV, we examined whether addition of roscovitine during MLR culture impacted on the frequency of CMV-specific effectors. Roscovitine did not reduce the frequency of CMV-specific CD8⁺ T cells. Instead, there was rather a small but reproducible increase in the proportion of CMV-specific T cells within the entire CD8⁺ T cell population after MLR culture of alloreactive T cells in the presence of roscovitine as compared to control (Table 1 and Supplementary Fig. 2).

Next, we sought to determine whether during the presence of roscovitine T cells would be able to develop an immune response to a neo-antigen, for which no memory T cells were present in the responder T cell population. As a paradigm, we studied responses against EBV, a virus that has a dual role in the post-transplant patient: one as an infectious agent and second as a cancer-initiating agent because it is responsible for the development of post-transplant lymphoproliferative disorder (PTLD), an aggressive form of lymphoma. For these studies we used naïve T cells from HLA-A*0201 positive umbilical cord blood and we cultured them with autologous APC and a pool of EBV peptides corresponding to immunogenic epitopes of the EBV antigen BLZF1, in the presence or in the absence of roscovitine. Although no EBV-specific T cells were detected prior to culture, after in vitro stimulation with EBV peptides in the absence or in the presence of roscovitine, a fraction of EBV-specific T cells was detected within the CD8⁺ T cell population (Fig. 3). Thus, roscovitine allowed the generation of antigen-specific CD8⁺ T cells upon antigen-specific stimulation of naïve T cells.

3.4. Treatment with roscovitine preserves regulatory T cells

Treg cells are critical for prevention of GvHD while maintaining the GvL effect of allo-HSCT [26]. For this reason, we examined whether treatment with roscovitine might affect the frequency of Treg cells that are present in the responder T cell population upon stimulation with mismatched alloreactive DCs. In human T cells Foxp3 is expressed early after stimulation and under these conditions is not an indicator of Treg development but rather serves as an activation marker [27,28]. In contrast, T cells that remain continuously Foxp3 positive after prolonged stimulation acquire Treg properties and functions [28]. MLR cultures in the presence of roscovitine or control were established for 9 days. At this prolonged time point, a prominent population of CD4⁺CD25^{high}Foxp3⁺ cells emerged among responder T cells treated with roscovitine compared to control responder T cells (Fig. 4A).

To determine whether these CD4⁺CD25^{high}Foxp3⁺ T cells generated upon prolonged culture in the presence of roscovitine were Treg cells, we examined their suppression properties by performing a functional suppression assay. At day 9 of allogeneic MLR culture in the presence of roscovitine, CD4⁺CD25^{high} T cells were isolated by cell sorting and were added in increasing numbers to third party MLR cultures. CD4⁺CD25^{high} T cell populations displayed a dose dependent inhibitory function (Fig. 4B) confirming that CD4⁺CD25^{high} cells generated in the presence of roscovitine have phenotyping and functional properties of Treg cells.

3.5. Roscovitine inhibits induction and phosphorylation of EZH2 in human T cells

The Polycomb group (PcG) protein, enhancer of zeste homologue 2 (EZH2), has an essential role in promoting histone H3 lysine 27 trimethylation (H3K27me3) and epigenetic gene silencing. Cdk1 and Cdk2 phosphorylate EZH2 at Thr 350 in an evolutionarily conserved motif. In cancer cells, EZH2 is constitutively expressed and phosphorylation of Thr 350 during the cell cycle is important for recruitment of EZH2 and maintenance of H3K27me3 levels at EZH2-target loci thereby regulating gene expression by epigenetic mechanisms [29]. To examine whether EZH2 expression and function might be affected by Cdk2 inhibition with roscovitine in primary human T cells, we used an experimental system previously established in our laboratory in which purified primary T cells are cultured with tosylactivated magnetic beads conjugated with monoclonal antibodies against CD3 and CD28 [20]. Strikingly, in contrast to cancer cells, which constitutively express comparable levels of EZH2 during culture [29], unstimulated primary human T cells displayed constitutively low abundance of EZH2 protein, which was upregulated upon stimulation via TCR/CD3 and CD28 receptors. Addition of roscovitine during culture significantly diminished the increase of EZH2 abundance. Phosphorylation of EZH2 on Thr 350 was also diminished (Fig. 5). These results show that, in primary human T lymphocytes, Cdk2 targets a key mechanism governing epigenetic regulation of gene expression via mediating expression of EZH2, which has an essential role in promoting histone H3 lysine 27 trimethylation on EZH2 targeted loci. Thus, inhibition of Cdk2 might alter the functional properties of alloreactive T cells by modulating EZH2-mediated epigenetic gene expression programs.

4. Discussion

In spite of the intense efforts, therapeutic control of GvHD remains incomplete and novel treatment approaches are required. Previously, we determined that inhibition of Cdk2 suppressed expression and activation of alloreactive T cells in vitro and in vivo and protected from acute lethal GvHD in a mouse model of allogeneic bone marrow transplantation [17]. In the present study we examined the effects of the Cdk inhibitor (*R*)-roscovitine (CYC202) on the responses of human alloreactive T cells. Our data showed that roscovitine inhibited expansion and activation of alloreactive T cells in vitro as determined by profound suppression of proliferation responses and by downregulation of activation markers. Furthermore, inhibition of Cdk2 eliminated alloreactive T cell effectors as determined by paucity of responder cells capable of producing IFN- γ upon rechallenge with specific MHC-mismatched allostimulators.

Using T cells from responders who were homozygous for HLA-A*0201 allele we determined that treatment with roscovitine did not induce a concomitant reduction of specific alloreactive T cell effectors. Instead, there was a small but reproducible increase in the proportion of CMV-specific and WT1-specific T cells within the polyclonal responder T cell population that was treated with roscovitine. This is of particular importance because current immunosuppressive medications routinely used for the control of GvHD in clinic also induce non-selective suppression of pathogen-specific and leukemia-specific responses. The increase of CMV-specific and WT1-specific T cells in MLR cultures in the presence of roscovitine might be due to the selective inhibition of activated T cells and the relative expansion of T cell subsets with specificities for other antigens that are not expressed on allogeneic stimulators. Consistent with our observation of a selective inhibitory effect of roscovitine on activated alloreactive T cells but not in quiescent, non-activated WT1-specific and CMV-specific T cells in our system, previous studies showed that roscovitine induces inhibition and apoptosis in proliferating neoplastic cells [30] whereas in non-dividing cells, like neurons and thymocytes, roscovitine has a protective effect [16,31]. These results support the hypothesis that therapeutic inhibition of Cdk2 for prevention of GvHD will spare leukemia-specific T cell responses because inhibition of Cdk2 exerts its effects only on activated T cells. At the time of allogeneic HSCT in humans, there is no residual leukemia left after induction chemotherapy and pre-transplant conditioning. Thus, leukemia-specific donor T cells are not activated and therefore will not be affected by Cdk2 inhibition. Further work will be required to identify the precise mechanism(s) via which inhibition of Cdk2 mediates preservation and increase of pathogen-specific and leukemia-specific effectors while suppressing activation and expansion of alloreactive effectors. Although in the present study we used MHC-disparate responder stimulator pairs, it will also be important to evaluate the applicability of this approach in a matched, haploidentical or single HLA-mismatched setting, which more closely resembles the conditions used in the clinical setting.

An important and unexpected observation of our present studies was the finding that roscovitine preserved and increased the numbers of Treg cells in the MLR cultures. Previously, we examined the molecular regulation of cell cycle progression and the effects of TCR-mediated stimulation on Rb phosphorylation in Treg and non-Treg (Tcon) cells [32]. These studies showed that upon stimulation via TCR/CD3⁺CD28, Tcon cells – which eventually become effectors – displayed robust hyper-phosphorylation of Rb in multiple sites, which is a well-established consequence of the sequential activation of Cdk4/6 in the early G1 phase and the activation of Cdk2 in the late G1 and the S phase of the cell cycle [33]. In contrast, Treg cells had impaired Rb phosphorylation. Specifically, in Treg cells Rb became phosphorylated without displaying a mobility shift [32], a finding consistent with partial phosphorylation of Rb only on sites targeted by Cdk4/6 but not on the additional sites targeted by Cdk2, which leads to Rb hyperphosphorylation and electrophoretic mobility shift. These findings explain why Cdk2 inhibition impacts on Rb phosphorylation and cell cycle progression of T effector cells but has no effect on Treg cells.

In our present studies we employed (*R*)-roscovitine (CYC202), a potent inhibitor of Cdk2–cyclin E with a 50% inhibitory concentration (IC₅₀) of 0.1 μM and a low inhibitory

efficiency for complexes of Cdk7–cyclin H, Cdk9–cyclin T1 and Cdk5–p35–p25 [18]. In the context of primary human T lymphocytes, among these complexes only Cdk2–cyclin E has a functional role because the Cdk7, Cdk9 and Cdk5 are not expressed or activated, in contrast to cancer and normal epithelial cells. Thus our present results predominantly represent consequences of Cdk2 inhibition. Consistent with our findings of the inhibitory effects of Cdk2 inhibition on the expansion, activation and effector function of T cells, previous studies showed that this approach can limit glomerulonephritis in mice with systemic lupus [34] and can prolong survival of kidney allografts in a rat model of fully MHC-mismatched kidney transplantation model [35].

Cyclin-dependent kinases (Cdk), particularly Cdk2, have an essential role in cell cycle re-entry. Studies in our laboratory have indicated that Cdk2 has a central role in the generation of T cell immune responses and inhibition of Cdk2 is mandatory of induction of T cell anergy in vitro and tolerance in vivo [14,15,17]. Subsequent studies indicated that even transient inhibition of Cdk2 activity resulted in a stable state of anergy that eliminated rejection of cardiac allografts in vivo [36]. Cdk2 regulates various pathways and downstream functions: In conjunction with cyclin E, Cdk2 phosphorylates the cell cycle inhibitor p27^{kip1} resulting in ubiquitin-targeted degradation. Cdk2 promotes phosphorylation of Rb on specific sites thereby reversing its ability to sequester E2F [33,37] and allowing for interaction of phosphorylated Rb with histone deacetylases (HDAC) and other chromatin remodeling proteins [38,39]. Cdk2 directly regulates expression of genes including NFκB, Sp1, p300/CBP, and subunits of the RNA polymerase [13]. Cdk2 also phosphorylates Smad3 and antagonizes the antiproliferative function of Smads induced by TGF-β [40]. These pathways have critical roles in regulating the survival, differentiation and inflammatory programs in T lymphocytes [41] and may impact on the fate and function of alloreactive T cells, which induce GvHD. Furthermore, these findings suggest that inhibition of Cdk2 might regulate the fate and function of T cells via additional mechanisms that might not be directly related to its effects on the cell cycle.

Our studies showed that roscovitine abrogated the ability of alloreactive T cells to become IFN-γ-producing effectors upon rechallenge with specific allostimulator DCs. In contrast, upon rechallenge with original allostimulator DCs, roscovitine-treated primed T cells elaborated similar amounts of IL-17 as those produced during rechallenge of control-treated alloreactive T cells. These selective effects on specific cytokine-producing T effector cells might be due to reprogramming of responder T cells by Cdk inhibition. In our studies we identified at least one mechanism via which Cdk2 inhibition might alter the gene expression programs in T cells by regulating abundance and phosphorylation of EZH2, which is important for maintenance of H3K27me3 levels at EZH2-target loci [29]. Notably, modulation of EZH2 by the HDAC inhibitor 3-deazaneplanocin A arrested ongoing GvHD, while preventing GvL in a mouse model of allogeneic bone marrow transplantation [42].

In conclusion, our present data suggest that Cdk2 inhibition may represent a novel mechanism to prevent GvHD without loss of pathogen-specific and leukemia-specific immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants HL107997-01 and T32AI07549, the Leukemia and Lymphoma Society Translational Research Program TRP 6222-11, and the HHV6 Foundation Award 26408.

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Figure 1.

Roscovitine inhibits Cdk2 activation and blocks responses of primary human T cells to stimulation via TCR/CD3 and CD28 or with MHC-mismatched allogeneic dendritic cells. (A) Purified T cells were cultured with tosylactivated magnetic beads conjugated with anti-CD3 and anti-CD28 antibodies in the presence of titrated dose of roscovitine or equal volume of vehicle control (DMSO 0.4% v/v). Proliferative capacity was assessed by incorporation of [3 H] thymidine at day 3 of the culture. Results are expressed as mean $\pm$ standard deviation ($n = 3$) and are representative of four independent experiments. (B) T cells were cultured with anti-CD3 and anti-CD28 antibodies in the presence of roscovitine or vehicle control (DMSO) and viability was determined by PI staining and analysis by flow cytometry at 48 h of culture. (C) Primary human T cells were cultured for 48 h with irradiated allostimulator DC in 20:1 ratio in the presence of roscovitine or vehicle control. Cell lysates were prepared, immunoprecipitation was done with Cdk2-specific antibody conjugated to agarose beads and kinase activity was examined by in vitro kinase reaction using histone H1 as exogenous substrate (top panel). Immunoblot with Cdk2-specific antibody confirmed comparable amounts of Cdk2 protein in the samples (bottom panel).

Unstimulated T cells and irradiated DCs were used as additional negative controls for conditions in which Cdk2 activation should not be induced.

Figure 2.

Inhibition of Cdk2 by roscovitine blocks expansion and activation of alloreactive human T cells and prevents generation of IFN- γ producing effectors while preserving IL-17 producing cells. (A) Responder T cells were plated with MHC-mismatched stimulator DC in a 20:1 ratio. CFSE analysis of CD4⁺ and CD8⁺ T cell subsets was performed at days 2 and 6. (B) Cells were harvested at day 5 of MLR culture and expression of CD25 and CD71 was analyzed on gated T lymphocytes by flow cytometry. T cells cultured in normal media for the same time intervals were used as control. These cells were incubated either with an isotype control antibody (unstained) or with the indicated antibodies (anti-CD71 or anti-CD25). Similar pattern of results was obtained in four separate experiments. (C) Responder T cells were stimulated with MHC mismatched stimulator DC for 48 h and subsequently T cell fractions were isolated, rested in media for 24 h, and subsequently, CD4⁺ cells from each fraction were rechallenged with APC from the original stimulators. Production of IFN- γ and IL-17 was detected by ELISPOT assay after 48 or rechallenge cultures.

Figure 3.

Roscovitine does not affect the ability of UCB T cells to respond to EBV antigens. Naïve T cells isolated from HLA-A*0201 umbilical cord blood were analyzed either fresh or after 5 days of culture with autologous APC and a pool of EBV peptides corresponding to EBV immunogenic epitope BMLF1 in the absence or the presence of roscovitine. EBV-specific T cells were assessed by using HLA-A*0201/GLCTLVAML (BMLF1)-APC conjugated Dextramer. HLA-A*0201/negative control peptide-APC conjugated Dextramer was used as staining control.

Figure 4.

Inhibition of Cdk2 by roscovitine during MLR culture promotes the generation of human T regulatory cells. (A) Responder T cells were plated with stimulator MHC mismatched DC in a 20:1 ratio. On day 9 of culture, expression of Foxp3 on gated CD4⁺CD25^{high} T cells was analyzed by intracellular staining and flow cytometry. Results are representative of four independent experiments. (B) At day 9 of allogeneic MLR culture in the presence of roscovitine, CD4⁺CD25^{high} T cells were isolated by cell sorting and were added in increasing numbers to third party MLR cultures. Proliferation was assessed on day 4 of the culture by ³H-Thymidine incorporation.

Figure 5.

Inhibition of Cdk2 by roscovitine diminishes the abundance and phosphorylation of EZH2 during T cell activation. Purified primary human T cells (1×10^6 cells/ml) were cultured with aCD3/CD28 (1 μ g/ml each) in the presence of vehicle (–) or roscovitine (+) (10 μ M). Cell lysates were prepared and expression of the indicated proteins was assessed by SDS-PAGE and immunoblot.

Table 1

Treatment with roscovitine allows preservation of leukemia-specific and pathogen-specific effectors. Responder T cells from HLA-A*0201 individuals were cocultured with allogeneic HLA mismatched DC for 7 days in the presence of roscovitine or vehicle control. Cells were stained with APC-labeled HLA-A*0201/CMV-pp65 pentamer (NLVPMVATV; ProImmune) or Wilms' tumor 1 (WT1) pentamer (HLA-A*0201/ WT1 pentamer RMFPNAPYL) and with anti-CD8-FITC (ProImmune) and analyzed in a Becton Dickinson FACScalibur flow cytometer.

	Alloactivated control	Alloactivated + roscovitine
<i>A. Expression of WT1-specific CD8+ T cells (% of total CD8+ T cells)</i>		
Donor 1	1.06	0.9
Donor 2	0.8	1.36
<i>B. Expression of CMV-specific CD8+ T cells (% of total CD8+ T cells)</i>		
Donor 1	0.52	0.75
Donor 2	0.4	3.65