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The Cyclin-Dependent Kinase Inhibitor p27kip1 Is Required for Transplantation Tolerance Induced by Costimulatory Blockade

Emily A. Rowell,* Liqing Wang,† Wayne W. Hancock,*† and Andrew D. Wells2*†

The cyclin-dependent kinase (CDK) inhibitor p27kip1 is an important negative regulator of the cell cycle that sets a threshold for mitogenic signals in T lymphocytes, and is required for T cell anergy in vitro. To determine whether p27kip1 is required for tolerance in vivo, we performed cardiac allograft transplantation under conditions of combined CD28/CD40L costimulatory blockade. Although this treatment induced long-term allograft survival in wild-type recipients, costimulatory blockade was no longer sufficient to induce tolerance in mice lacking p27kip1. Rejected allografts from p27kip1−/− mice contained more CD4+ T lymphocytes and exhibited more tissue damage than allografts from tolerant, wild-type mice. Infiltrating p27kip1-deficient T cells, but not wild-type T cells, exhibited nuclear expression of cyclins E and A, indicating uncontrolled T cell cycle progression in the graft. The failure of tolerance in p27kip1−/− mice was also accompanied by markedly increased numbers of allospecific, IFN-γ-producing cells in the periphery, and occurred despite apparently normal regulatory T cell activity. These data demonstrate that the CDK inhibitor p27kip1 enforces the costimulatory requirement for the expansion and differentiation of alloimmune effector T lymphocytes in vivo, and point to CDKs as novel targets for immunosuppressive or tolerance-inducing therapies. The Journal of Immunology, 2006, 177: 5169–5176.

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3 Abbreviations used in this paper: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein.

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donors (H-2b) were transplanted heterotopically into wild-type or p27kip1-/- mice on the C57BL/6 background (H-2b). The donor aorta was anastomosed to the recipient abdominal aorta, and the donor pulmonary artery was sutured to the recipient inferior vena cava, as described (17). Graft function was monitored by abdominal palpation, and grafts were harvested at the time of rejection or as indicated. Hearts were deemed rejected when cardiac contractility was no longer detected, and rejection was confirmed visually at the time of graft harvest. At time of harvest, portions of graft were fixed in formalin for paraffin sectioning or snap frozen for subsequent immunohistologic and gene expression studies. H&E-stained paraffin sections were evaluated using the recently revised International Society of Heart and Lung Transplantation standardized criteria for grading of cardiac allograft biopsies (18). Immunohistologic labeling of cryostat sections was performed using mAbs, polyclonal Abs, and respective Envision kits (Dako/Cytomation), as described (19). Quantitation of Foxp3 staining within six consecutive fields/allograft section and three samples/group was performed using Photoshop software (Adobe Systems) (20).

Tolerating protocols for transplant recipients
Transplant recipients received combined costimulatory blockade, as indicated, consisting of anti-CD154 mAb (MR1; 200 μg/mouse; BioXpress) administered i.v. on the day of transplant, and CTLA-4Ig (BioExpress; 200 μg i.p.) administered i.p. on days 0, 2, and 4 following transplant.

ELISPOT assay
ELISPOT plates (Millipore) were prewet with 70% EtOH, washed three times in sterile PBS, then coated with IFN-γ capture Abs in sterile PBS at 4°C overnight, according to the manufacturer's instructions. The next day, plates were blocked at room temperature for 2 h with 2% nonfat milk in PBS and washed three times with sterile PBS. Splenocytes in from transplant recipients (1 × 10^5, 2 × 10^5, or 4 × 10^5 cells/well) were placed in each well with or without T-depleted splenocytes as stimulator cells. The optimal stimulator/responder ratios were determined for combined costimulatory blockade to be 1:1, and 1:5 for untreated transplant recipients. The cells were cultured at 37°C in a 7% CO_2 environment overnight (16–24 h) to allow adequate time for cytokine production, but not to allow for significant amounts of proliferation. Following incubation, supernatant was removed from the plate, and 100 μl of PBS-0.1% Tween 20 was added to the plate for 10 min at 4°C; then the plate was washed three times with 100 μl each PBS-0.1% Tween 20. Detection Ab was then added in PBS containing 1% BSA at 4°C. The plate was then washed three times with PBS-0.1% Tween 20. Streptavidin-Alkaline phosphatase was added to the wells for 1 h at 37°C. Following three more washes, spots were visualized by addition of 5-bromo-4-chloro-3-indolyl phosphate/NBT reagent. Spot formation was monitored, and the reaction was stopped by rinsing the plate well with distilled water. After drying, spots were read on an ImmunoSpot reader (Cellular Technology).

Parent into F1 in vivo one-way MLR
As described previously (21), CFSE-labeled donor splenocytes from p27kip1-/- (H-2b) mice or p27kip1-/- (H-2b) mice were retrovirally injected into B6 × DBA F1 (H-2ab) recipient mice (20 × 10^6 per recipient). Recipients were either administered i.v. anti-CD154 mAb (200 μg/mouse) on day 0 in combination with CTLA-4Ig (i.p. 200 μg/mouse) on days 0 and 2, or were treated with equivalent doses of control IgG. Recipients were sacrificed on day 3, and spleens were harvested for subsequent flow cytometric analysis. Donor cells were differentiated from recipient cells by staining for differences in H-2 expression, and the frequencies of CD4+ alloreactive donor cells were determined by gating on CD4+ T cells that had diluted their CFSE.

Suppression assays
MACS-purified CD4+CD25+ T cells were CFSE labeled and seeded at 5 × 10^5 per well in 96-well round-bottomed dishes. Irradiated syngeneic T-depleted splenocytes were added to the wells at 1 × 10^6 per well along with 0.5 μg/ml soluble anti-CD3ε mAb. Cells were cultured for 3 days at 37°C in 7% CO_2 in the presence or absence of MACS-purified CD4+CD25+ regulatory T cells (rT cells) to responder ratios of 2:1, 1:1, 1:2, or 1:3 and 1:10. After 3 days, suppression of responder cell proliferation was determined by flow cytometrically assessing the degree of inhibition of CFSE dilution.

Statistics
For graft survival, Kaplan-Meier survival graphs were constructed and log-rank comparison of the groups was used to calculate p values. For ELISPOT assays, p values were calculated with Student’s t test. Significance in the parent into F1 studies was determined with a paired one-tailed t test. Statistical analyses were performed with Prism software (GraphPad). Differences were considered significant at p < 0.05.

Results
p27kip1 is required for in vivo tolerance induced by costimulatory blockade
We have demonstrated previously that T cells from p27kip1-/- deficient mice are resistant to in vitro anergy induction (15); however, the role of p27kip1 in tolerance induced in vivo has not yet been investigated. To explore this question, we studied the alloresponses of T lymphocytes in a murine cardiac allograft tolerance model using costimulatory blockade (22, 23). C57BL/6-p27kip1-/- and C57BL/6-p27kip1-/- mice (H-2b) were transplanted with fully MHC-mismatched cardiac allografts from BALB/c-p27kip1-/- mice (H-2b). Transplant recipients were either untreated, or were administered CTLA-4Ig and anti-CD154 mAb to block the CD28 and CD40 costimulatory pathways. In the absence of treatment, the kinetics of allograft survival in the wild-type and p27kip1-/- mice were equivalent, with both sets rejecting their grafts within 8 days of transplantation (n = 3) (Fig. 1A). Treatment with combined costimulatory blockade resulted in long-term allograft acceptance in wild-type recipients, with all recipients retaining their allografts for >100 days (n = 5). In contrast, combined costimulatory blockade failed to induce allograft tolerance in p27kip1-deficient recipients, with transplants in these mice rejecting with a mean of 50 days (n = 5, p < 0.0001) (Fig. 1A). Histological examination of allografts collected from untreated recipients at day 6 posttransplantation revealed more severe damage to the heart tissue in the p27kip1-/- recipients than in wild-type mice (Fig. 1B, left panels). Allografts from wild-type mice showed mild rejection with multifocal mononuclear cell infiltrates without myocyte damage (grade 1R), whereas sections from p27kip1-/- recipients showed severe acute rejection (grade 3R, AMR1) with diffuse mixed leukocytic infiltrates, extensive myocyte necrosis plus focal interstitial hemorrhages, and vasculitis (Fig. 1B, left panels). This was associated with more IgM and IgG alloantibodies in the sera of p27kip1-deficient recipients than in wild-type recipients (data not shown). Conversely, allografts from wild-type mice treated with CTLA-4Ig/anti-CD154 mAb exhibited only minor mononuclear cell infiltration at day 21 posttransplant (grade 1R), and myocardial architecture was well preserved (Fig. 1B, right top panel). In contrast, cardiac allografts from p27kip1-/- recipients showed extensive mononuclear cell infiltration and multifocal myocyte necrosis characteristic of acute cellular allograft rejection (grade 3R) (Fig. 1B, right bottom panel). Neither wild-type nor p27kip1-/- recipients exhibited significant serum alloantibody under conditions of combined costimulatory blockade (data not shown). These data suggest that p27kip1 functions to dampen allograft rejection and promotes in vivo tolerance induced by costimulatory blockade.

p27kip1 regulates alloimmune effector cell generation in vivo
p27kip1 is important for the normal development of most mammalian tissues (16), and regulates the capacity of naive T lymphocytes to proliferate to mitogenic stimulation in vitro, particularly under conditions in which costimulation or growth factors are limiting (15, 24, 25). Therefore, we examined whether costimulation-independent graft rejection by p27kip1-/- mice was characterized by
an augmented alloimmune response in the periphery. Although the naive peripheral lymphoid compartment is increased in p27kip1-deficient mice (16), we find no differences in the frequencies of naive or memory CD4+ and CD8+ T cells as compared with wild-type animals, and naive p27kip1−/− mice have a normal frequency of alloreactive T cell precursors (Fig. 2A). Following cardiac allograft transplantation, wild-type mice exhibited a ~20% increase in splenocyte number (Fig. 2B, □), while p27kip1−/− allograft recipients exhibited a ~50% increase and accumulated double the number of splenocytes as wild-type recipients (Fig. 2B, ▫). Co-stimulatory blockade during organ transplantation induces apoptosis and deletion of alloreactive T cells (26), which is required for the induction of long-term donor-specific tolerance (27). This was reflected in these studies by a nearly 20% reduction in splenocyte number (Fig. 2B, ▪), and p27kip1−/− recipients exhibited a 50% increase in cellularity, accumulating roughly the same number of spleen cells as untreated recipients and 3-fold more than wild-type mice (Fig. 2B, ▩). These results are consistent with in vitro studies showing that p27kip1 sets a costimulatory threshold for T cell proliferation (15, 24), and suggest that alloreactive lymphocytes that lack p27kip1 can expand in vivo independently of costimulation.

T cell differentiation and proinflammatory cytokine gene expression are linked to the cell cycle (28–30); therefore, we investigated whether the CDK inhibitor p27kip1 regulates the generation of effector cells during alloimmune vs tolerant responses in vivo. The frequency of allospecific IFN-γ producers in the spleens of wild-type (□) and p27kip1−/− (▫) transplant recipients was determined by ELISPOT for untreated (day 6, left panel) or CTLA4-Ig/anti-CD154-treated mice (harvested at time of rejection by p27kip1−/− recipient; right panel). Separate ELISPOT assays were performed on sets of transplant recipients as depicted in each plot. Shown is mean ± SEM for quadruplicate wells. The fractions in each panel are the mean alloresponder frequencies of the groups. D, The absolute numbers of IFN-γ producers per spleen were determined from the frequencies in C and the cell numbers in B. Numbers in each panel represent the fold increase of p27kip1−/− over wild type.

**FIGURE 1.** p27kip1 regulates cellular mechanisms of allograft rejection. A, Kaplan-Meier survival curves for untreated wild-type (n = 4) (●) and p27kip1−/− (n = 3) (■) transplant recipients, and recipients treated with CTLA-4Ig (200 μg/mouse i.p.) on days 0, 2, and 4, and a single i.v. dose of anti-CD154 mAb (200 μg/mouse) on day 0 wild type (n = 8) (▲), and p27kip1−/− (n = 8) (◇). Results were analyzed by nonparametric log-rank test (p < 0.0001). B, H&E staining of grafts (magnification, ×350). Three mice per group were sacrificed for histology at day 6 posttransplant for untreated recipients, or at day 21 for recipients receiving combined costimulatory blockade. Representative fields from three mice are shown.

**FIGURE 2.** p27kip1 regulates alloreactive effector cell generation in vivo. A, CD62L and CD44 expression by T cells from naive wild-type (left plot) and p27kip1−/− (right plot) mice. The alloreactive T cell precursor frequency in wild-type and p27kip1−/− mice was determined by adoptive transfer of CFSE-labeled donor T cells into B6 × DBA2 F1 recipients, as described previously (21) (right graph). B, Splenic cellularity was assessed in wild-type (□) and p27kip1−/− (▫) mice that did not receive transplant (naive), transplant recipients that received no treatment (day 6), and transplant recipients receiving CTLA-4Ig/anti-CD154 mAb. C, The frequency of IFN-γ producers in the spleens of wild-type (□) and p27kip1−/− (▫) transplant recipients was determined by ELISPOT for untreated (day 6, left panel) or CTLA4-Ig/anti-CD154-treated mice (harvested at time of rejection by p27kip1−/− recipient; right panel). Separate ELISPOT assays were performed on sets of transplant recipients as depicted in each plot. Shown is mean ± SEM for quadruplicate wells. The fractions in each panel are the mean alloresponder frequencies of the groups. D, The absolute numbers of IFN-γ producers per spleen were determined from the frequencies in C and the cell numbers in B. Numbers in each panel represent the fold increase of p27kip1−/− over wild type.
in 60 lymphocytes in the spleens of untreated wild-type allograft recipients at the time of rejection (Fig. 2C, left panel, filled symbols), while the frequency of IFN-γ producers in untreated p27kip1−/− allograft recipients was 2-fold higher (1 in 30 splenocytes; Fig. 2C, left panel, open symbols). These data suggest that p27kip1 normally acts to oppose alloreactive effector cell differentiation on a per-cell basis during an allograft response. Moreover, the enhanced cellularity and IFN-γ-producer frequency evident in untreated p27kip1−/− allograft recipients translated to a 4- to 5-fold increase in the absolute number of allospecific IFN-γ-producing cells present in the spleen at the time of rejection as compared with wild-type recipients (Fig. 2D, left panel). Combined costimulatory blockade reduced the frequency of allospecific IFN-γ-producing cells in wild-type allograft recipients by at least 30-fold, to an average of 1 in 2000 spleen cells (Fig. 2C, right panel, filled symbols). In p27kip1−/−-deficient allograft recipients, however, inhibition of CD28 and CD40 costimulation decreased the frequency of IFN-γ producers to 1 in 500, a frequency 4-fold higher than in wild-type treated recipients (Fig. 2C, right panel, open symbols). These differences in both frequency and cellularity lead to a 5- to 7-fold increase in the absolute number of allospecific IFN-γ-producing cells in p27kip1−/− recipients as compared with wild-type recipients treated with costimulatory blockade (Fig. 2D, right panel).

*p27kip1* regulates T cell and leukocyte proliferation within allograft tissue

To determine whether the enhanced alloimmune responses observed in the periphery of p27kip1−/−-deficient mice were likewise reflected in the graft, we conducted further analyses of the mononuclear cells infiltrating the cardiac allografts in wild-type and p27kip1−/− recipients treated with CTLA-4Ig and anti-CD154. Rejecting allografts from p27kip1−/− recipients exhibited increased infiltration by CD8+ and particularly CD4+ T cells as compared with tolerant allografts from wild-type animals (Fig. 3, A and B). On a molecular level, mononuclear cell infiltrates in the CTLA-4Ig/anti-CD154-treated wild-type recipients exhibited intense p27kip1 staining (Fig. 3C, left panel and inset), demonstrating that this CDK inhibitor is normally expressed by tolerant cells in the allografts in this model. Although the rejected BALB/c-p27kip1+/+ cardiac allografts in C57BL/6-p27kip1−/− recipients showed diffuse parenchymal expression of p27kip1, the infiltrating mononuclear cells from these recipients did not exhibit p27kip1 staining (Fig. 3C, right panel and inset). To determine whether the lack of p27kip1 expression by infiltrating mononuclear leukocytes leads to enhanced proliferation of these cells in the allografts, we measured in situ expression of the G1 and S phase cyclins E and A. Allografts from p27kip1−/− recipients contained more CD3+ cells than allografts from wild-type mice, but for this analysis we imaged regions of each allograft that contained comparable concentrations of focal T cell infiltrates (see Fig. 3F). Very few of the T cells and mononuclear cells infiltrating tolerant allografts from CTLA-4Ig/anti-CD154-treated wild-type mice were positive for cyclin E or A (Fig. 3, D and E, left panels), confirming that these cells were not actively cycling at 3 wk posttransplantation. Conversely, rejecting allografts from p27kip1−/− recipients contained large numbers of proliferating mononuclear cells in the G1 and S phases of the cell cycle, as indicated by strong expression of both cyclin E and cyclin A (Fig. 3, D and E, right panels). Many of these proliferating cells were T cells, as determined by CD3e staining of serial sections (Fig. 3F). CDK/Cyclin complexes must localize to the nucleus to promote cell cycle progression, while p27kip1 can inhibit these complexes by associating with them in the nucleus (31). An analysis of the subcellular localization of these proteins in mononuclear allograft infiltrates from wild-type tolerant recipients showed that p27kip1 was present in both the nucleus and the cytoplasm, while the majority of the cyclin E and A protein in the few positive mononuclear cells in the grafts of tolerant, wild-type recipients was localized to the cytoplasm (Fig. 3, D and E, left insets). Conversely, the infiltrating T lymphocytes and mononuclear cells that did not express p27kip1 exhibited strong nuclear localization of both cyclin E and cyclin A (Fig. 3, D and E, right insets). Together, these results show that the

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**FIGURE 3.** Immunohistochemical analysis of mononuclear cell infiltrates from allografts harvested on day 21 posttransplantation from CTLA4-Ig/anti-CD154 mAb-treated wild-type (left panels) and p27kip1−/− (right panels) recipients (magnification, ×350). Expression of CD4 (A), CD8 (B), p27kip1 (C), cyclin E (D), cyclin A (E), and CD3e (F). Insets in C–E represent ×500 images of the regions denoted by red arrows. D–F, Analogous regions from serial sections, selected by similar staining for CD3e. Representative fields from three mice are shown.

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CDK inhibitory protein p27kip1 is an important negative regulator of alloreactive effector cell proliferation and differentiation, and operates as a crucial sensor of costimulatory signals during the induction of transplantation tolerance.

The findings of the allograft studies above suggest that T lymphocytes from p27kip1−/− mice are capable of undergoing greater expansion in response alloantigen. Because all cells in the p27kip1−/− mouse lack p27kip1, it is unclear from these studies whether our observations are due to a T cell-intrinsic phenomenon. To address this issue, we performed in vivo one-way MLR. In these experiments, equal numbers of CFSE-labeled donor CD4+ T lymphocytes from p27kip1−/− or wild-type mice were transferred to allogeneic B6 × DBA2 F1 hosts (21). The hosts were then treated with control Ig or CTLA-4Ig/anti-CD154 mAb, and the dividing alloreactive cells were enumerated by flow cytometry. From these experiments, we were able to determine whether differences in p27kip1 expression in CD4+ T cells alter their expansion in a T cell autonomous manner. We found little difference between the expansion of wild-type and p27kip1−/− deficient T cells with no costimulatory blockade (Fig. 4A), most likely because wild-type T cells efficiently down-regulate p27kip1 when strongly stimulated (15). Under conditions of combined costimulatory blockade, there was ~1-log decrease in the recovery of alloreactive cells as compared with the control Ig-treated hosts. However, under these conditions, the number of alloreactive p27kip1−/− CD4+ T cells recovered was nearly 2-fold greater than that of wild-type donor cells (Fig. 4B). These data indicate that p27kip1 influences T cell proliferation in a cell-intrinsic manner, and that, similar to T cell responses in vitro (15), p27kip1 is a critical factor in determining the requirement for costimulation.

Failure of tolerance in the absence of p27kip1 is not associated with a defect in regulatory T cell function

Long-term donor-specific tolerance induced by costimulatory blockade is known to require regulatory T cells (32); therefore, it is possible that the failure of tolerance in the p27kip1−/− mice is due to a lack of regulatory T cell-mediated suppression. To address this possibility, we tested whether CD4+CD25+ regulatory T cells isolated from p27kip1−/− mice were able to suppress CD4+CD25+ responder T cells in vitro, and likewise, whether naive CD4+CD25+ responder T cells isolated from p27kip1−/− mice were susceptible to in vitro suppression by CD4+CD25+ regulatory T cells. There were no marked differences in the frequency of CD25+CD4+ T cells in wild-type vs p27kip1−/− mice (Fig. 5A), or in the ability of p27kip1−/− regulatory T cells to suppress the proliferation of p27kip1−/− vs wild-type responder CD4+CD25+ T cells (Fig. 5B). This was seen across various regulatory T cell-to-responder ratios, and demonstrates that elimination of p27kip1 in CD4+CD25+ T cells has no effect on their ability to be suppressed by regulatory T cells. Similarly, p27kip1−/− deficient regulatory T cells exhibited no defect in suppression of CD4+CD25+ T cells (Fig. 5C). Together, these results indicate that neither an overt defect in regulatory T cell function nor a defect in the ability of naive T cells to be suppressed is likely to explain the failure of tolerance in p27kip1−/− mice. p27kip1 may differentially influence alloreactive regulatory cells induced during CTLA-4Ig/anti-CD154 mAb therapy; therefore, we also measured the number of Foxp3+ regulatory T cells within the allografts of wild-type and p27kip1−/− deficient recipients at day 21 posttransplantation. Wild-type allografts that go on to long-term acceptance show significant numbers of infiltrating Foxp3+ cells (Fig. 5D, left panel). However, we observed just as many Foxp3+ cells infiltrating the allografts of p27kip1−/− recipients around the initiation of graft rejection in these animals (Fig. 5D, right panel). These data suggest that immunoregulatory mechanisms are operative in p27kip1−/− allograft recipients treated with CTLA-4Ig/anti-CD154 mAb, but that these mice are able to overcome both costimulatory blockade and regulatory T cell-mediated suppression to mediate graft rejection.

Discussion

We have demonstrated an obligatory role for the CDK inhibitor p27kip1 in the establishment of in vivo tolerance induced by costimulatory blockade. Unlike in wild-type mice, inhibition of the CD28 and CD40 costimulatory pathways failed to induce tolerance to cardiac allografts in p27kip1−/− recipients. Rejection was associated with increased graft infiltration by actively cycling, p27kip1−/− deficient T lymphocytes, and with more severe myocyte damage as compared with tolerant, wild-type mice. Augmented allograft infiltration and inflammation were also accompanied by increased expansion of p27kip1−/− alloreactive T cells within the allografts of wild-type and p27kip1−/− recipient mice. The failure of tolerance in p27kip1−/− alloreactive T cells does not appear to result from a defect in regulatory T cell function, but is instead associated with a T cell-intrinsic reduction in the requirement for costimulation. These studies do not preclude a role for p27kip1 in regulating the functions of other leukocytes during an alloimmune response. In fact, acute allograft rejection in untreated p27kip1−/− mice was accompanied by increased serum alloantibody compared with rejecting wild-type animals, and alloantibody in these recipients showed pathological evidence of Ab-mediated vascular damage that was not observed in allograft tissue from wild-type recipients. However, graft rejection in p27kip1−/− deficient recipients treated with combined costimulatory blockade occurred without significant alloantibody, and instead was associated with enhanced T cell responses in both the graft and the periphery. These studies suggest that p27kip1 promotes allograft tolerance by limiting the number of effector cells able to infiltrate the graft and cause damage. A similar role for p27kip1 in promoting immune cell infiltration, inflammation, and vascular disease was found recently in a syngeneic model of mechanical arterial injury (33). Our previous studies have shown that an apoptotic program

![FIGURE 4](http://www.jimmunol.org/)

Absence of p27kip1 in T cells leads to a cell-autonomous increase in costimulation-independent, alloreactive CD4+ T cell expansion. A total of 20 × 10^6 B6 CFSE-labeled wild-type (○) or p27kip1−/− (□) donor cells was transferred into B6 × DBA2 F1 recipients. Recipients were administered control Ig (n = 2) (A) or CTLA-4Ig (200 μg/mouse) (B) on days 0 and 2 and a single i.v. dose of anti-CD154 mAb (200 μg) on day 0 (n = 3). Mice were sacrificed on day 3, and the absolute number of divided donor cells was determined by flow cytometry. Significance was determined by a paired one-tailed t test (p < 0.02) (n = 3 for CTLA-4Ig/anti-CD154 mAb-treated recipients). Results are representative of two separate experiments.
A CELL CYCLE REGULATOR IS REQUIRED FOR TOLERANCE

Why is p27kip1 crucial for tolerance? An important aspect of p27kip1 function in T cells is its role in determining the costimulatory threshold for activation. T cell function is critically dependent on costimulatory interactions between APCs and T cells (1). p27kip1 is expressed at high levels in resting T cells, and is degraded upon activation by signals from CD28 and IL-2R (4, 15, 35, 36). Down-modulation of p27kip1 is required for efficient T cell cycle entry (5, 6, 12, 15, 37), and in the absence of this CDK inhibitor, T cells exhibit increased CDK2-mediated phosphorylation of Rb (16) and undergo a significant degree of costimulation-independent cell division (15, 24). The development and differentiation of many tissues have been linked to proliferation, suggesting the existence of a mitotic clock that specifies developmental cell fates through a cell division counting mechanism. A similar mechanism is operative during T cell differentiation, in which the frequency of cells able to produce effector cytokines such as IFN-γ increases with successive cell divisions, while changes in the expression of homing, adhesion, and cytokine receptors important for effector/memory differentiation are also tied to the cell cycle (11, 28–30). This link between cell division and T cell differentiation is currently thought to represent a requirement for epigenetic changes in DNA methylation and chromatin structure for the expression of genes important for T cell effector function (38–40). p27kip1 may therefore inhibit T cell differentiation by limiting cell division and clonal expansion. Inhibition of costimulation or cell cycle progression during T cell activation results in elevation of p27kip1 levels and the induction of anergy (8, 10–13, 15, 41–43), suggesting a link among p27kip1 expression, anergy induction, and cell cycle progression. Indeed, forced expression of p27kip1 in vitro promotes anergy in the presence of costimulation (12), while genetic deletion of p27kip1 renders T cells resistant to anergy induced by costimulatory blockade (15). p27kip1 is therefore an important intracellular sensor of costimulatory signals that may promote tolerance by restricting T cell division.

p27kip1 possesses multiple functions in the cell, but its primary role is as a negative regulator of CDK. The fact that p27kip1 is required for T cell anergy and tolerance implies that CDK activity normally opposes tolerance induction. How might CDK activity influence tolerance? As outlined above, CDK may indirectly promote T cell differentiation by driving cell division. CDK4, CDK6, and CDK2, the principal CDK regulated by p27kip1, promote cell cycle progression by phosphorylating and inactivating the tumor suppressor pRb. This derepresses the E2F transcription factors, which are required for the induction of the E- and A-type cyclins and CDK1/Cdc2 during the S phase transition (44). In addition to regulating pRb phosphorylation, CDK2 also has multiple functions related to the replication of DNA during S phase. It regulates the stability of Cdc6 and promotes loading of MCM and Cdc45 proteins onto origins of DNA replication (45, 46). CDK2 also phosphorylates histone H1 and activates NPAT (nuclear protein mapped to the AT locus), a regulator of histone synthesis, events important for chromatin condensation and remodeling during mitosis (47–49). However, CDK can also influence the function of proteins that are not direct components of the cell cycle machinery.
For instance, CDK2 phosphorylates the tumor suppressor p53 in early S phase, stimulating its DNA-binding and transcriptional activity (50). It also inactivates Smad3, a transcription factor that mediates the antiproliferative effects of the TGF-β receptor, via phosphorylation (51). CDK2 has been shown to directly regulate the transcriptional activity of p300/CREB binding protein (52), as well as the RelA and c-Rel components of NF-κB (52, 53). Less is known about similar roles for CDK4 and CDK6, but there is evidence for cross-talk between NF-κB and CDK4/6 pathways (54, 55). RelA, c-Rel, and p300/CREB binding protein are each known to regulate the expression of proinflammatory cytokine genes in T cells (56–58), and could therefore potentially be involved in the capacity of CDK activity to oppose tolerance and of p27kip1 to enforce tolerance.

Our results demonstrate that p27kip1 is a key molecule that sets the costimulatory requirement for T cell responses in vivo, and is required for the induction of transplantation tolerance by treatments that block T cell costimulatory pathways. These studies imply that CDK and their inhibitory proteins may represent important new therapeutic targets for promoting tolerance in autoimmune and organ transplantation.

Disclosures
The authors have no financial conflict of interest.

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