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Regulation of Alloimmune Th1 Responses by the Cyclin-Dependent Kinase Inhibitor p21 Following Transplantation¹

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Abstract

Background—The cyclin-dependent kinase (cdk) inhibitor p21 inhibits cellular proliferation of many cell types, including T cells. However, autoimmune models have yielded conflicting results regarding the role of cdk inhibitors and T cell function. The role of p21 in T cell function following transplantation has not been directly investigated. We hypothesized that p21 plays an important role in alloantigen-driven responses *in vitro* in mixed lymphocyte cultures (MLC) and *in vivo* using the heterotopic murine cardiac allograft model.

Methods—Wild type (WT) and p21-deficient (p21^{−/−}) mice were employed as recipients, and the effects of p21 over expression were assessed by transplanting p21 adenoviral-transfected cardiac allografts. Enzyme linked immunospot (ELISPOT) and 3H-thymidine incorporation were used to evaluate for T cell priming and proliferation *in vitro* while graft histology was evaluated for rejection.

Results—When stimulated with alloantigens *in vitro*, splenocytes from p21^{−/−} mice mounted enhanced proliferative responses and reduced Th2 responses relative to their WT counterparts. No differences in Th1 responses were noted when p21^{−/−} cells were stimulated with alloantigens *in vitro*. However, following cardiac transplantation, Th1 responses were enhanced in p21^{−/−} recipients relative to WT mice. This enhanced *in vivo* Th1 response was associated with exacerbated graft rejection in p21^{−/−} recipients. Interestingly, p21 transfection of WT allografts inhibited graft rejection and Th1 priming.

Conclusion—p21 controls the intensity of the immune response post-transplantation, with over expression inhibiting allograft rejection. Our data demonstrate that p21 controls T cell priming, and also suggests p21 and other cdk inhibitors may serve as potential targets for therapeutic manipulation of alloimmune responses.

Introduction

Cell cycle control has been shown to play a critical role in T cell proliferation, apoptosis, and priming (1–3). Entry into the S phase of the cell cycle is governed by the G₁ cyclins, which assemble with cyclin dependent kinases (cdk) to form functional holoenzymes that phosphorylate key substrates required for the G₁/S phase transition (reviewed in (4–6)). In mammalian cells, cyclin D-cdk4 or -cdk6, cyclin E-cdk2, and cyclin A-cdk2 complexes act sequentially during G₁/S transition and are required for cell cycle progression. p21 and p27

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are considered important regulators of cell proliferation because of their inhibitory interactions with cyclin/cdk complexes (reviewed in (5,6). p21 and p27 belong to the Cip/Kip family of cdk inhibitors, which have the capacity to bind and inactivate many different cyclin/cdk complexes. Hence, the Cip/Kip family of cdk inhibitors is believed to regulate G₁ and S phase cdks *in vivo*. Indeed, p21 and p27 have been shown to be important for cell cycle control of a variety of cell types, including T cells (7–10). Zhang et al. (9) reported that p27 controlled cytokine-induced T cell proliferation, while p21 played a lesser role in this process. One possible explanation for these findings is that p27 may control initial entry of T cells into S phase from G₁, whereas p21 may regulate T cells that are already actively cycling (3). Upregulation of p21 has been observed in memory CD4⁺ T cells and in T cells after several rounds of proliferation and chronic activation (1). Further, p21, along with p27, is upregulated in anergic T cells (10). Thus, cdk inhibitors are considered potent suppressors of immune responses and may therefore be therapeutically important in transplantation and autoimmunity. Indeed, the commonly used immunosuppressant cyclosporine A (CsA) induces expression of p21 in T cells in a TGFβ-dependent manner (11). Hence, p21 may contribute to the anti-proliferative activities of CsA.

Studies in autoimmune models have reported conflicting activities for p21. For example, Balomenos et al. (1) reported that p21^{−/−} mice develop female-prone lupus accompanied by anti-DNA antibodies, lymphadenopathy, and glomerulonephritis while Lawson et al. (12) found no evidence for autoimmunity in an independently derived stock of p21^{−/−} mice. Santiago-Raber et al. (2) reported that p21^{−/−} mice develop mild autoimmunity at a low incidence that is unaffected by gender. Hence, a spectrum of autoimmune disease states has been reported for p21^{−/−} mice. The direct effects of p21 on *in vivo* immune responses have not been completely defined, and the impact of p21 deficiency vs. over-expression in the context of organ transplantation has not been rigorously investigated.

The current study explored the role of p21 in alloimmune responses in an *in vitro* mixed leukocyte culture (MLC) and, most importantly, *in vivo* following cardiac allograft transplantation. As anticipated, p21^{−/−} cells mounted enhanced proliferative responses relative to WT cells in both settings, indicating a role for p21 in regulating clonal expansion of graft-reactive T cells. However, p21 appeared to differentially regulate Th1 and Th2 responses in the *in vitro* versus *in vivo* settings. *In vitro*, p21 deficiency led to reduced Th2 responses but did not alter the magnitude of the Th1 response. Following transplantation, p21 deficiency did not impact Th2 responses, even when Th2 were preferentially induced by *in vivo* depletion of CD8⁺ T cells as previously described (13). In contrast, p21^{−/−} allograft recipients mounted enhanced Th1 responses relative to their WT counterparts, which was associated with exacerbated graft rejection. Over expression of p21 within the graft led to prolonged allograft survival and reduced Th1 responses. These data demonstrate a differential impact of p21 on Th1 versus Th2 responses, with p21 expression level correlating with graft outcome. This study suggests that p21 may provide a target for therapeutic strategies aimed at manipulating Th1 responses following solid organ transplantation.

Materials and Methods

Mice

Female WT and p21^{−/−} C57BL/6/129 (H-2^b) mice and BALB/c (H-2^d) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. Mice were used between 6–12 weeks of age.

Media

The culture medium used in these studies was DMEM supplemented with 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 mM folic acid, 5×10^{-5} M 2-ME (all obtained from Sigma Chemical, St. Louis, MO), 1.6 mM L-glutamine, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% FCS (all obtained from Life Technologies, Grand Island, NY).

Heterotopic cardiac transplantation

WT and p21^{-/-} C57BL/6/129 mice were transplanted with intact BALB/c cardiac allografts, as described (14). In this model, the donor heart is anastomosed to the great vessels of the abdomen, perfused with recipient mouse's blood, and resumes contraction. Transplant function was monitored by daily abdominal palpation, and graft rejection was indicated by cessation of contractions. Histologic evidence of rejection (i.e. leukocytic infiltration, loss of myocyte nuclei and cross-striation) was verified by H & E staining of formalin fixed allograft fragments.

p21 transfection of cardiac allografts

Cardiac allografts were transfected by perfusion with E1/E3 deleted adenoviral vectors encoding human p21 (Adp21) or beta-galactosidase (Ad β -gal) as described (15,16). Adp21 was kindly provided by Dr. Elizabeth Nabel (NIH) and its *in vivo* use has been previously described (8). Stocks of adenoviral vectors were produced for *in vivo* use in the Vector Core at the University of Michigan Medical Center. Prior to *in situ* perfusion of the donor heart, the left superior vena cava and the distal aorta were ligated to retain the perfusate. The aorta was then held in position by suture to facilitate perfusion using a syringe with a 30 gauge needle. The donor heart was flushed with 400 μ l of heparin (250 U/ml), then perfused with 200 μ l of lactated Ringer's solution containing adenoviral vectors (5×10^8 pfu). Following perfusion, donor grafts were harvested and placed in iced Ringer's for approximately 1 hour prior to transplantation.

Verifying p21 transgene expression

Expression of the p21 transgene was verified by RT-PCR as described (8). Adp21 contains an untranslated region of bovine growth hormone proximal to the polyadenylation site. The anti-sense PCR primer is located in this region and therefore is specific for the recombinant p21 and does not amplify endogenous p21 (8). Tissues were homogenized in 1 ml TRIzol® (Invitrogen Life Technologies, Carlsbad, CA) and RNA was isolated as per manufacturer's protocol. Two μ g of total RNA were reverse transcribed using a cDNA Cycle® Kit (Invitrogen Life Technologies) using oligo dT primers and AMV reverse transcriptase to generate cDNA. Primer sequences:

recombinant p21 sense 5' GAG ACA CCA CTG GAG GGT GAC TTC G 3',

anti-sense 5' GGG CAA ACA ACA GAT GGC TGG CAA C 3';

γ actin sense 5' CCA CAC AGA GTA CTT GCG CTC AGG 3',

anti-sense 5' CAC CCT GTG CTG CTC ACC GAG GCC 3'.

Samples were amplified using AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT) in a GeneAmp® PCR System 9700 (Applied Biosystems Inc, Foster City, CA).

In vivo depletion of CD8+ T cells

To deplete allograft recipients of CD8+ cells, mice were injected i.p. with 1 mg of anti-CD8 mAb (hybridoma 2.43, obtained from the American Type Culture Collection Rockville, MD), on days -2, 0, and 5 relative to transplantation. Preparations of anti-CD8 mAb were purified

and re-suspended in PBS by Ligocyte Pharmaceuticals, Inc. (Bozeman, MT). Flow cytometric analysis of splenocytes confirmed CD8⁺ T cell depletion, with <1% of cells staining positive for CD8. Staining with FITC-conjugated rabbit anti-rat IgG (Biosource International, Camarillo, CA) was used to verify that mAb treatment resulted in depletion of CD8⁺ cells, rather than epitope coating with the 2.43 mAb.

MLC

To detect alloantigen-induced proliferation, WT or p21^{-/-} splenocytes (1×10^6 /ml) were stimulated with γ -irradiated (5000R) BALB/c splenocytes (1×10^6 /ml) in microtiter wells for 5 days, including a terminal 8 hour pulse with 0.5 μ Ci ³H-thymidine. Microcultures were harvested by aspiration onto a filter mat (Tomtec Cell Harvester System, Hamden, CT) and ³H-thymidine incorporation determined using a BetaPlate liquid scintillation counter (Wallac, Gaithersburg, MD).

Enzyme-linked immunospot (ELISPOT) assay for cytokine production

The ELISPOT assay used to quantify alloantigen-primed cytokine producing cells has been described previously (17). Capture and detection mAb were obtained from BD-Pharmingen (San Diego, CA). Polyvinylidene fluoride-bottom plates (Cellular Technologies, Cleveland, OH) were coated overnight with rat anti-mouse IFN γ (R4-6A2, 4 μ g/ml) or IL-4 (11B11, 2 μ g/ml) capture mAb, blocked for 90 minutes with 1% BSA in PBS at room temperature, and washed three times with PBS. Irradiated (5000R) BALB/c splenocytes (4×10^5) were added to each well followed by 2×10^5 WT or p21^{-/-} responder splenocytes. Plates were incubated for 24 hours at 37° C, and then washed 3 times with PBS followed by 4 times with PBS-Tween 20 (0.05%). Biotinylated rat anti-mouse IFN γ (XMG1.2, 4 μ g/ml) or IL-4 (BVD6-24G2, 2 μ g/ml) detection mAb were added to each well and incubated overnight at 4° C. Plates were washed 3 times with PBS-Tween 20, then alkaline phosphatase-conjugated anti-biotin antibodies (1/1000 dilution; Vector Laboratories, Burlingame, CA) were added to the IFN γ wells and HRP-conjugated streptavidin (1:2000 dilution; Dako, Carpinteria, CA) was added to the IL-4 wells for 90 minutes at room temperature. Plates were washed 4 times with PBS, developed with NBT/BCIP or 3-amino-9-ethylcarbazole (AEC), washed with H₂O, and air dried. Spots were enumerated using an ImmunoSpot Series 1 ELISPOT Analyzer (Cellular Technologies, Cleveland, OH).

Statistical analyses

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using StatView 5.0.1 software (SAS Institute, Inc., Cary, NC). Analyses of variance or Student's t-tests were performed where appropriate between groups. Fisher's exact tests were performed to determine least significant difference among groups. *p* values of less than 0.05 were considered significant. Significance of allograft survival was assessed by logrank analysis.

Results

Impact of p21 on alloantigen-driven T cell proliferation and Th1/Th2 priming *in vitro*

To begin exploring the role of p21 in alloantigen responses, we first compared the ability of p21^{-/-} and WT splenocytes to proliferate in response to alloantigens *in vitro*. p21^{-/-} cells mounted an approximate 110% increased proliferation response to alloantigens relative to their WT counterparts (p21^{-/-} 210 $\pm$ 31% vs. WT 100 $\pm$ 14%, *n* = 6 experiments, *p* < 0.05). This enhanced proliferative response was not apparent when the polyclonal mitogen Con A was used as a stimulus (data not shown), suggesting that an overwhelming stimulus could over-

ride the impact of p21 inhibition of T cell proliferation and p21 levels primarily affected alloantigen-dependent proliferation.

Since the impact of p21 on T cell priming for cytokine production in response to alloantigens has not been determined, we compared Th1 (IFN γ) and Th2 (IL-4) responses of p21 $^{-/-}$ and WT splenocytes following *in vitro* stimulation. Splenocytes were stimulated in a 3 day MLC and plated in ELISPOT cultures that detect primed, but not quiescent precursor cytokine producing cells (17). Following *in vitro* stimulation with alloantigens, IFN γ responses by p21 $^{-/-}$ and WT cells were similar (p21 $^{-/-}$ 193 $\pm$ 9 IFN γ spots per million cells vs. WT 212 $\pm$ 15 IFN γ spots, one of three representative experiments, p value non-significant), suggesting that p21 plays a minor role in regulating Th1 responsiveness in MLC. In comparison, p21 $^{-/-}$ cells mounted significantly reduced Th2 responses relative to WT cells as measured by IL-4 production (p21 $^{-/-}$ 29 $\pm$ 3 IL-4 spots per million cells vs WT 111 $\pm$ 9 IL-4 spots, one of three representative experiments, p < 0.05). These observations suggest that p21 may play a role in influencing Th1/Th2 balance in this *in vitro* culture system.

Flow cytometry revealed that the CD4:CD8 ratio following *in vitro* stimulation with alloantigen was also impacted by p21. While the percentage of CD8 $^{+}$ cells was comparable following culture of WT and p21 $^{-/-}$ cells, CD4 $^{+}$ cells were preferentially expanded in the p21 $^{-/-}$ MLC with mean a increase over WT of 56% for 4 independent experiments. Percentages of CD4 $^{+}$ and CD8 $^{+}$ T cells in naïve animals prior to stimulation in MLC were similar between WT and p21 $^{-/-}$ mice (data not shown). This is in keeping with the findings of Santiago-Raber et al. (2) who reported that proliferative responses of CD4 $^{+}$ cells are more inhibited by p21 than are those of CD8 $^{+}$ cells in an autoimmune model.

Impact of p21 on cardiac allograft rejection *in vivo*

To assess the influence of p21 on alloimmune responses *in vivo*, WT and p21 $^{-/-}$ mice were transplanted with vascularized cardiac allografts. p21 $^{-/-}$ recipients uniformly rejected BALB/c cardiac allografts by day 7 (Table I), which was slightly accelerated relative to WT recipients (mean survival = 8.5 days, p<0.001). However, differences in the progression of rejection were revealed by histologic evaluation of functioning grafts (Figure 1). On day 5, allografts placed in WT recipients revealed only minor signs of rejection, including a mild mononuclear cell infiltrate with little or no myocyte necrosis (Figure 1A). In contrast, allografts placed in p21 $^{-/-}$ recipients revealed a more intense mononuclear cell infiltrate, early signs of myocyte degeneration, vascular involvement (white arrow) and hemorrhage (black arrows) on day 5 (Figure 1B). As rejection progressed on day 7, histologic differences remained apparent. While grafts in WT recipients revealed a progressive cellular infiltrate and some myocyte necrosis on day 7 (Figure 1C), the signs of rejection were intensified in p21 $^{-/-}$ recipients, with near total myocyte necrosis (as noted by loss of myocyte nuclei) and massive hemorrhage (Figure 1D). These data demonstrate that the rejection process was accelerated in p21 $^{-/-}$ recipients.

Impact of p21 on *in vivo* T cell responses following transplantation

It is generally accepted that immune responses initiated *in vitro* may not accurately reflect the complexity of responses initiated *in vivo* where compartmentalization, microenvironmental parameters, and tissue specific expression of antigens may impact outcome (18–25). Therefore we assessed the ability of WT and p21 $^{-/-}$ mice to mount proliferative and cytokine responses following cardiac transplantation. Donor alloantigen-driven proliferative responses following graft rejection were similar to those initiated *in vitro*, with splenocytes obtained from p21 $^{-/-}$ recipients mounting significantly stronger proliferative responses than that of WT recipients (p21 $^{-/-}$ 182 $\pm$ 25% vs. WT 100 $\pm$ 12%, n = 9–14 mice per group, p < 0.05). However, *in vitro* and *in vivo* cytokine responses in the absence of p21 were disparate. Unlike the *in vitro* initiated response, IFN γ secreting Th1 were significantly increased in p21 $^{-/-}$ allograft

recipients (4830 $\pm$ 777 IFN γ spots per million cells) when compared to WT recipients (1187 $\pm$ 478 IFN γ spots, $p < 0.05$, $n=3$ mice per group for one representative experiment). Further, IL-4 producing Th2 were relatively rare in both groups (p21 $^{-/-}$ 49 $\pm$ 13 IL-4 spots per million cells vs. WT 15 $\pm$ 1 IL-4 spots).

The fact that IL-4 producing Th2 were rare comes as no surprise since we have previously reported that rejection in this model is characterized by a CD8 $^{+}$ Th1 dominated response (13,26–28). Depletion of CD8 $^{+}$ cells reduces IFN γ production and allows Th2 responses to emerge (13,27,28). We next depleted cardiac allograft recipients of CD8 $^{+}$ cells to assess the impact of p21 on Th2 responses to alloantigens *in vivo*. In addition, depleting CD8 $^{+}$ cells would allow us to assess the impact of p21 on CD4-mediated rejection, since CD4 $^{+}$ cells have been reported to be influenced to a greater degree than CD8 $^{+}$ cells by p21 (2). Splenocytes were harvested at the time of rejection from WT and p21 $^{-/-}$ allograft recipients depleted of CD8 $^{+}$ cells to assess the degree of Th1 and Th2 priming by ELISPOT. IL-4 producing Th2 were increased in both groups when compared to Th2 responses observed when CD8 $^{+}$ cells were not depleted. However, there was no difference in the level of Th2 priming in WT and p21 $^{-/-}$ recipients (p21 $^{-/-}$ 122 $\pm$ IL-4 spots per million cells vs. WT 134 $\pm$ 56 IL-4 spots, $n = 5-9$ mice per group, p value non-significant), indicating that p21 played little if any role in regulating Th2 responses *in vivo*. As expected (13), Th1 responses were reduced in recipients that were depleted of CD8 $^{+}$ cells. However, primed Th1 were increased in number in p21 $^{-/-}$ recipients (648 $\pm$ 86 IFN γ spots per million cells) relative to their WT counterparts (321 $\pm$ 46 IFN γ spots per million cells), $n = 5-9$ mice per group, $p < 0.05$, further indicating a role for p21 in regulating Th1 responses *in vivo*.

Over-expression of p21 in cardiac allografts prolongs graft survival and inhibits Th1 priming *in vivo*

To further explore the role of p21 in alloimmune responses and transplant rejection, we transfected WT BALB/c cardiac allografts with adenoviral vectors encoding either p21 (Adp21) or β -galactosidase (Ad β -gal) prior to transplantation into WT C57BL/6 recipients as described (15,16). Adenoviral-mediated transfection with p21 prolonged allograft survival (Figure 2A) with an increase in mean survival from 8 days to 18 days ($p < 0.01$). Indeed, two of the eight p21 transfected allografts continued to function normally until the termination of the experiment (30 days). Histologic evaluation of these two p21 transfected allografts that continued to function at 30 days post transplant revealed only minimal cellular infiltrate and no myocyte necrosis (data not shown).

T cell priming was evaluated at the time of rejection or at the termination of the experiment in recipients of allografts transfected with Adp21 or Ad β -gal (Figure 2B). At the time of rejection, recipients of p21 transfected allografts mounted significantly reduced Th1 responses relative to recipients of β -gal transfected grafts. Further, the two mice bearing functioning p21 transfected allografts on day 30 mounted even smaller Th1 responses (140 ± 14 spots/ 10^6 splenocytes), further indicating that p21 inhibits allograft-reactive Th1 *in vivo*.

To verify that these observations were associated with expression of the p21 transgene, p21 or β -gal transfected allografts were analyzed on day 2 or day 7 post-transplantation. Spleens were also analyzed on day 2, since we have reported that transgene expression may be detected in the spleen at early time points post-transplantation of adenovirally transfected cardiac grafts (15). Figure 2C depicts RT-PCR for p21 transgene expression in Adp21 (Lane 2) or β -gal (Lane 3) transfected 293 cells, illustrating specificity of the PCR primers for recombinant p21 (8).

When allografts were perfused with Adp21 and tissues were harvested on day 2 post-transplant, expression of the p21 transgene was readily detectable in the transplanted heart (Lane 4) and to a lesser degree in the spleen (Lane 5). As expected, recombinant p21 was not expressed in

the heart (Lane 6) or spleen (Lane 7) when allografts were perfused with Ad β -gal. The p21 transgene was expressed within grafts that were perfused with Adp21 (Lane 8) but not Ad β -gal (Lane 9) for at least 7 days post-transplantation. p21 transgene expression was also readily detectable in the two functioning Adp21 transfected grafts that were harvested on day 30 post transplant (data not shown).

Discussion

p21 and p27 are members of the Cip/Kip family of cdk inhibitors and have been shown to inhibit a wide variety of cdk, thus controlling cell cycle progression in a number of cell types (Reviewed in (4–6)). Levels of p27 are high in resting T cells but decline following T cell activation (3,29,30). Costimulation through CD28 as well as responsiveness to IL-2 results in ubiquitination and degradation of p27, thereby allowing the T cell to enter S-phase of the cell cycle (3,31). Wolfram and Letterio (32) have reported that the requirement for CD28 costimulation is markedly reduced in CD8+, but not CD4+ T cells obtained from p27 $^{-/-}$ mice. Further, Rowell et al. (33) have recently reported that p27 $^{-/-}$ mice are less susceptible of co-stimulation blockade-induced allograft acceptance. However, while p27 regulates the expansion and homeostasis of T cells, Shen and Kaplan (34) reported that p27 does not influence Th1/Th2 differentiation. Taken together, these observations indicate that p27 regulates events that occur early in the T cell activation response. The current study reveals a role for p21 in regulating Th1 responses *in vivo*. Unlike p27, p21 levels are low in resting T cells and increase upon activation, where p21 is believed to play a role in controlling actively cycling T cells and IL-2 dependent proliferation (3,31,35). CD4+, rather than CD8+ T cells appear to be more inhibited by p21 (1,2). Finally, high levels of both p27 and p21 are present in anergic T cells (10,36), reflecting induction of p21 as well as a failure to down-regulate p27.

Diverse and controversial activities have been reported for p21 in activated T cells. For example, p21 has been reported to promote T cell apoptosis mediated by Fas/FasL interactions (37) but has also been reported to protect activated/memory T cells from apoptosis (38). In addition to its role as a cdk inhibitor, p21 has been shown to serve as an adaptor protein to promote the association of cdk4 with the D-type cyclins (39). p21 has also been shown to limit T cell responses to chronic stimulation (1). The *in vivo* impact of p21 on immune responses has been studied in models of autoimmunity, where a spectrum of disease states has been reported (1,2,12). Hence, the *in vitro* and *in vivo* effects of p21 on immune responses have not been clearly defined.

Given the diverse activities that have been ascribed to p21 in regulating T cell expansion and function, the current study assessed the impact of p21 on T cell function in response to allogeneic stimulation both *in vitro* and *in vivo*. To this end, we compared proliferative responses and Th1 vs. Th2 cytokine production by splenocytes obtained from p21 $^{-/-}$ and WT mice. We also assessed the impact of over-expression of p21 on allograft survival and Th1/Th2 priming by transfecting grafts with Adp21 prior to transplantation.

When stimulated with alloantigens *in vitro*, p21 $^{-/-}$ cells mounted enhanced proliferative responses when compared to WT cells. While Th1 responses were similar between the 2 groups, Th2 priming was reduced in p21 $^{-/-}$ cells when compared to their WT counterparts. This *in vitro* observation suggested that p21 may positively influence Th2 differentiation, unlike what has been reported for p27 (34). However, an effect on *in vivo* Th2 priming was not observed when p21 $^{-/-}$ mice were transplanted with cardiac allografts. Rather, p21 $^{-/-}$ recipients mounted an exaggerated Th1 response relative to WT mice which was associated with accelerated graft rejection (Table I) and exacerbated graft pathology (Figure 1). These differences in the *in vitro* and *in vivo* scenarios are likely reflected by the fact that there are clear microenvironmental differences present *in vivo* following transplantation with

differential expression of antigens and compartmentalization of responses that may occur (18–25). However, the *in vivo* correlate clearly contains the most relevance as this is most important clinically and reflects the net effect of all of the above variables. It should be noted that unmodified rejection in this transplant model is characterized by a dominant Th1 response and the emergence of Th2 requires manipulations which favor Th2 development (13,40,41). We have previously reported that CD8+ cells represent a major source of IFN γ in this model and depleting CD8+ cells reduces IFN γ production and induces Th2 responses (13,27). Thus, depleting p21 $^{-/-}$ vs. WT allograft recipients of CD8+ cells allowed us to investigate the impact of p21 on *in vivo* Th2 priming and CD4+ T cell mediated rejection (28). While depleting recipients of CD8+ cells did induce measurable Th2 responses in both groups, there was no difference in the level of Th2 priming in p21 $^{-/-}$ vs. WT recipients. However, Th1 priming was significantly increased in p21 $^{-/-}$ recipients even in a setting where Th2 were induced. These data indicate that p21 plays a role in controlling alloantigen-reactive responses following transplantation primarily via a Th1 mechanism. Whether Th1 responses *in vivo* are a result of altered cell cycle progression (39), altered apoptosis (37), or effects on T cell regulation (1) are unclear at the present time. However, many of these studies focused on *in vitro* situations, whereas the current study's focus was to address the net effect of these postulated mechanisms in an *in vivo* transplantation model.

To further explore the role of p21 in modulating alloreactive T cell function *in vivo*, WT cardiac allografts were transfected with Adp21 and transplanted into WT recipients (Figure 2). Over-expression of p21 prolonged graft survival and reduced Th1 priming within the recipients' spleens. This poses the question of how p21 transfection of the vascularized cardiac allograft could influence recipient T cell responses. We have previously reported that transfection of cardiac grafts with adenoviral vectors results in transient transgene expression within the marginal zones of the recipients' spleens (15). Indeed, weak, albeit detectable expression of the p21 transgene was observed in the spleens of recipients of p21 transfected allografts on day 2 post-transplantation (Figure 2C). In this cardiac transplant model, dendritic cells (DC), which are readily transfected by adenoviral vectors (42,43), are known to migrate out of the transplanted heart and localize into the recipient's spleen (44). Therefore transfected DC may serve as a source of transgene product in the spleen. Alternatively, adenoviral vectors may be flushed out of the perfused heart upon revascularization and localize in the recipient's spleen. Since priming of graft-reactive T cells occurs in the secondary lymphoid tissues (18,19), it is possible that p21 expression within the spleen influences Th1 priming. However, T cells are known to be difficult to transfect with adenoviral vectors (45,46). It has also been reported that exogenous p21 protein is capable of entering T cells, localizing in the nucleus, and inhibiting proliferation and proinflammatory cytokine production in response to mitogenic stimulation (47). Thus another possible scenario is that exogenous p21 transgene product secreted by DC or other transfected cells of the spleen could influence T cell behavior. Additionally, other cell types within the allograft that are capable of proliferation and interfacing with the immune system, such as endothelial cells or smooth muscle cells, may also explain some of the observations. Further studies are planned to investigate these potential cellular mechanisms of p21 action.

In summary, we provide evidence that p21 controls the intensity of the immune response using a cardiac transplant *in vivo* model, with over expression of p21 inhibiting allograft rejection. These data have important clinical applications. Immunosuppressive therapies currently used in transplantation are associated with numerous toxicities and side effects (Reviewed in (48, 49)). Hence, new approaches to immunosuppression are being explored. Manipulation of p21 may constitute such an approach. Indeed, the finding that recombinant p21 protein may inhibit T cell responses (47) points to the potential of this approach as a therapeutic modality and certainly warrants further investigation.

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Glossary

Abbreviations used in this paper

cdk	cyclin dependent kinase
CsA	cyclosporine A
DC	dendritic cells
ELISPOT	enzyme-linked immunospot
H&E	hematoxylin and eosin
MLC	mixed leukocyte culture

p21^{-/-}

p21 deficient

WT

wild type

Figure 1. Increased pathology of rejection in p21^{-/-} cardiac allograft recipients

Representative H & E stained sections are depicted of rejecting BALB/c cardiac allografts in WT recipients on days 5 (Panel A) and 7 (Panel C) and in p21^{-/-} recipients on day 5 (Panel B) and 7 (Panel D) post transplantation. Note early vascular involvement (white arrow) and hemorrhage (black arrows) on day 5 in p21^{-/-} recipients (Panel B). Magnification = 200X.

Figure 2. Impact of p21 over-expression on allograft survival and donor-reactive Th1 responses BALB/c cardiac allografts were transfected with 5×10^8 pfu of Ad β -gal or Adp21 or left non-transfected prior to transplantation into WT C57BL/6 mice as described in the Materials and Methods section. **Panel A:** Graft function was monitored by daily palpation and rejection was recorded as the day on which the graft ceased to function ($n = 6-8$ transplants per group). $p < 0.01$ for the mean survival of Adp21 transfected allografts (18.6 days) compared to non-transfected (7.7 days) or Ad β -gal transfected allografts (7.5 days). **Panel B:** Splenocytes were harvested from recipients on the day of rejection and assessed for *in vivo* primed Th1 and Th2 responses by ELISPOT. Data represent the mean number of spots per 1×10^6 cells $\pm$ SEM. $*p < 0.05$ vs. Ad β -gal transfected and non-transfected groups. **Panel C:** RT-PCR was

performed using primers specific for recombinant p21 or γ actin (see Materials and Methods) to verify appropriate expression of p21 following transfection with Ad21, but not Ad β -gal.

Lane 1 – MW ladder. **Lanes 2 and 3** – 293 cells were transfected with Adp21 or Ad β -gal, respectively, at an M.O.I. of 25 and RNA was isolated 24 hours post-transfection. **Lanes 4-7** – Allografts were perfused with Adp21 or Ad β -gal and RNA was isolated from the transplanted hearts (H) and recipient spleens (S) on day 2 post-transplantation. **Lanes 8 and 9** – Allografts were transfected with Adp21 or Ad β -gal, respectively, and RNA was harvested on day 7 post transplant. Results are representative of 3 transplanted mice per group.

Table I

Impact of p21 on allograft rejection

	Allograft Survival (Days)	Mean
WT	8,8,8,9,9,9	8.5
p21 ^{-/-}	7,7,7,7,7,7	7.0*

WT and p21^{-/-} C57BL/6/129 mice were transplanted with vascularized BALB/c cardiac allografts. Graft function was monitored by daily palpation and rejection was recorded as the day on which the graft ceased to function.

* p < 0.001 vs. WT.