Ectoenzymes are membrane-bound proteins capable of regulating the extracellular milieu via enzymatically active sites that protrude from the plasma membrane. CD39 (ecto-nucleoside triphosphate diphosphohydrolase) and CD73 (ecto-5′-nucleotidase) are 2 major ectoenzymes that sequentially phosphohydrolyze adenine nucleotides, leading to adenosine generation.1 CD39 is primarily responsible for the phosphohydrolysis of ATP and ADP to AMP. CD73, which colocalizes with CD39 in membrane caveolae,2 hydrolyzes the phosphate group from AMP to generate adenosine. The adenine nucleotides and adenosine have been shown to play crucial role in many processes, including leukocyte extravasation,3 cardioprotection,4,5 and cellular immunoregulation.6 Signaling via specific adenosine receptor subtypes can mediate a variety of salutary vascular processes, ranging from suppression of vascular leakage and inflammation to promotion of vasodilation.

The experimental observation that CD39 confers a survival advantage to allografts and xenografts through thromboregulatory effects has been confirmed using cardiac transplantation models.7,8 CD73 sits at a more distal checkpoint in the adenosine generation cascade, but very little is known about CD73 as an effector limb of the immune or inflammatory response in the setting of cardiac transplantation. A number of studies have suggested that CD73-generated adenosine plays a beneficial role in modulating processes vital to successful transplantation, including endothelial permeability,9 neutrophil and leukocyte adhesion,10,11 the antithrombotic response,11 immunosuppression,6 and ischemic preconditioning.4 These actions of CD73 might occur either through dissipation of AMP, with attendant reductions in stimulation of putative AMP receptors, or dissipation of the downstream product of CD39 catalytic activity (AMP), which could theoretically increase flux of ADP through the CD39 catalytic pathway; dissipation of ADP would remove a potent stimulus to platelet aggregation and inflammation. As yet another alternative, the phosphohydrolytic actions of CD73 could drive production of adenosine, which could bind to its own...
cognate signaling receptor subtypes and hence affect vascular dilation and inflammation. These adenosine receptors (ARs) include A1AR, A2AAR, A2BAR, and A3AR, with each receptor having a unique tissue distribution, ligand affinity, and signal transduction pathway. The A1AR and A3AR inhibit adenyl cyclase, whereas the A2AAR and A2BAR stimulate this effector system and therefore cAMP production.12 Little is known about the contribution of each subtype receptor to the events surrounding cardiac transplantation.

In the present work, studies examined the role of CD73 on development of cardiac allograft vasculopathy (CAV), the major impediment to the long-term survival of human cardiac allografts. CAV is a rapidly progressive form of atherosclerosis that often leads to reduced blood flow and ischemia of distal tissues. Histologically, CAV is identified by a combination of proliferative myoblasts, macrophages, and T lymphocytes, leading to the formation of a neointima. The mechanism for CAV development is considered to be multifactorial and likely includes both immunologic and nonimmunologic triggers.13 CD73, which sits at an interface position between immune modulator and vascular homeostatic mediator, is an excellent target to consider for involvement in (or protection against) CAV development. Although intimal proliferation mechanisms may differ, a recent study has shown that vascular neointimal formation is increased in CD73-deficient mice after carotid artery injury. In contrast, reconstitution of wild-type mice with CD73-deficient bone marrow did not exacerbate neointimal formation in the artery injury model, indicating that CD73 produced by resident nonhematopoietic cells, rather than by circulating cells, plays an active role in mitigating neointimal hyperplasia.14 However, because CD73 is expressed by leukocytes, as well as by tissue-resident cells including endothelial cells, we hypothesized that CD73 expressed on both local and circulating cells could contribute to preserving vascular homeostasis after cardiac transplantation, at least in part, by modulating the transit of leukocytes across inflamed endothelium.15 The experiments herein examine a role for CD73 and specific adenosine receptor subtypes in modulating leukocyte trafficking and ultimately, rejection and CAV following cardiac allograft transplantation.

**Materials and Methods**

All experiments were performed according to the protocols approved by the University of Michigan Committee on Use and Care of Animals in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Experimental protocols are described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

**Animals**

CD73-deficient mice (CD73−/−) of C57BL/6 (H-2b) background, a generous gift of Dr Linda F. Thompson, have been described previously.12 CD73+/+ littermates were used as the wild-type control. B10.A (H-2b) and CBA/J (H-2b) mice were purchased from The Jackson Laboratories (Bar Harbor, Me).

**Experimental Groups**

Completely allografted murine heterotopic cardiac transplantation was performed for the present study, as we have described previously in detail.12 Two groups were used to study donor sources of CD73 (CD73+/+ or CD73−/− donors into recipients) and another 2 groups were used to study recipient sources (donors into CD73+/+ or CD73−/− recipients).

**In Vitro Experiments**

T lymphocytes were purified from splenocytes of CD73+/+ and CD73−/− mice (H-2b). The BALB/c (H-2k)-derived endothelial cell line bEnd.3 was obtained from American Type Culture Collection (Manassas, Va).

**Statistics**

Database management and statistical analysis were performed with the Statview version 5.0 software (SAS institute Inc, Cary, NC). All values are expressed as means±SEM. Kaplan–Meier analysis was performed to evaluate graft survival, and survival differences were compared by a log-rank test. Comparisons among groups were performed with an unpaired Student t test or 1-way ANOVA where appropriate. Values of P<0.05 were considered statistically significant.

**Results**

**CD73 Deficiency in Either Donor or Recipient Mice Shortens Cardiac Allograft Survival**

To observe the relationship between cardiac allograft survival and CD73 expression in donor and recipient cells, completely allografted heterotopic cardiac transplantation was performed using CD73−/− mice as either donors or recipients. CD73+/+ donor allografts survived between 13 and 20 days (16.3±1.0 days) after transplantation, whereas CD73−/− donor allografts survived for 10.5±0.6 days. CD73+/+ recipient graft survival ranged from 14 to 18 days (16.0±0.6 days), whereas all CD73−/− recipients acutely rejected the donor hearts in less than 14 days (9.0±0.7 days). CD73 deficiency in donors or recipients significantly decreased cardiac allograft survival (P=0.0013, P=0.0005, respectively; Figure 1A).

**CD73 Deficiency Increases Graft Permeability Following Ischemia/Reperfusion Injury**

Based on previous findings indicating that CD73 is critical for control of vascular leakage,9 we evaluated graft permeability in the ischemia/reperfusion (I/R) phase after transplantation. The permeability in cardiac allografts at 4 hours after transplantation was significantly increased in all cases in which CD73 was deficient either in the donor or the recipient (Figure 1B). These data indicate that there is an important role for CD73 in circulating cells, as well as cells resident in or surrounding the cardiac graft. Because the early inflammatory response during reperfusion is initiated by neutrophil infiltration into the allograft,16 we next evaluated the extent of the neutrophil infiltration using immunohistochemical Ly6G staining for direct neutrophil detection and a myeloperoxidase (MPO) activity assay. Compared with experiments in which CD73 was present in either donors or recipients, both the number of graft-infiltrating Ly6G-positive cells and the intragraft MPO activity were significantly increased in grafts involving CD73−/− mice (donors or recipients) (Figure 1C through 1E).

**CD73 Deficiency Accelerates Acute Graft Rejection**

At day 7 posttransplantation, we examined the histology of cardiac allografts to evaluate the acute alloimmune response
Infiltration of mononuclear or polymorphonuclear cells with associated cardiomyocyte damage was greater and more diffuse, and the parenchymal rejection scores were significantly higher, in allografts involving CD73+/H11002/H11002 donors or recipients (Figure 2B). The numbers of infiltrating CD4-, CD8-, and CD11b-positive cells were significantly increased in experiments involving the transplantation of CD73+/H11002/H11002 donors or recipients (Figure 2C through 2E).

CD73 Deficiency Increases Graft Expression of Cytokines, Chemokines, and Adhesion Molecules

At day 7 posttransplantation, we examined whether CD73 expression could modulate the mRNA expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocye chemoattractant protein (MCP)-1, RANTES (regulated on activation normal T cell expressed and secreted), intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 in cardiac allografts. Compared with the CD73+/+ transplantsations, mRNA expression of each of the above genes was significantly increased in the grafts involving CD73−/− donors or recipients (Figure 3).

CD73 Deficiency Aggravates Cardiac Allograft Vasculopathy and Graft Tolerance

To evaluate the severity of CAV development, we examined the histology of cardiac allografts at day 60 posttransplantation using elastin-stained tissue sections (Figure 4A). Compared with CD73+/+ transplantations, the severity of luminal occlusion in the graft coronary arteries involving CD73−/− donors or recipients was significantly increased (75.9±5.4% versus 46.6±2.5% and 70.5±6.5% versus 46.1±2.0%; P<0.0006 and P=0.0051, respectively; Figure 4B). Next, we investigated the impact of CD73 expression on humoral immunity in chronic rejection. CD73 deficiency in donors or recipients resulted in significantly higher levels of donor-reactive alloantibodies in the chronic rejection phase than in transplants between CD73+/+ donors and recipients (Figure

Figure 1. Graft survival and effects of CD73 on cardiac allografts at 4 hours after transplantation. A, Survival of cardiac allograft. *P<0.05. B, Graft permeability. C, Immunohistochemical staining of the neutrophil marker Ly6G in cardiac allograft. Bar=50 μm. D, Quantitative analysis of graft-infiltrating Ly6G-positive cells. E, Graft MPO activity. ΔAbs indicates a change in absorbance. All data are expressed as means±SEM for n=6 mice.

(A Figure 2A). Infiltration of mononuclear or polymorphonuclear cells with associated cardiomyocyte damage was greater and more diffuse, and the parenchymal rejection scores were significantly higher, in allografts involving CD73−/− versus CD73+/+ donors or recipients (Figure 2B). The numbers of infiltrating CD4-, CD8-, and CD11b-positive cells were significantly increased in experiments involving the transplantation of CD73−/− donors or recipients (Figure 2C through 2E).
4C). To further assess the effect of CD73 expression on recipient antidonor cellular immune responsiveness, we evaluated cell proliferation of recipient lymphocytes using an ex vivo 1-way mixed lymphocyte reaction. The cell proliferation was significantly amplified in the transplantation of CD73^{+/−} donors or recipients (Figure 4D).

### Intragraft Expression of CD73 As It Relates to Adenosine Receptor Expression

After cardiac transplantation, recipient circulating cells infiltrate into allografts, thereby promoting the graft injury during the I/R phase and the phases of acute and chronic rejection. To elucidate the impact of CD73 expression in cardiac allografts, we mea-
sured mRNA and protein levels of CD73 in allografts at 4 hours, 7 days and 60 days after transplantation (Figure 5A, 5C, and 5D). In CD73+/H11001/H11001 donors or recipients, CD73 mRNA expression in cardiac allografts was markedly upregulated at 4 hours posttransplantation, upregulated but attenuated at day 7, and finally downregulated at day 60 posttransplantation. CD73+/H11002/H11002 donors or recipients had lower levels of CD73 mRNA in all phases posttransplantation. Because extracellular adenosine produced by CD73 can signal through any of 4 ARs (A1AR, A2AAR, A2BAR, or A3AR), we next measured mRNA and protein levels of each AR in each phase after transplantation (Figure 5B through 5D). Intragraft A2BAR expression was upregulated in all groups at 4 hours and 7 days after transplantation, although CD73+/H11002/H11002 donors or recipient groups had significantly lower levels of upregulation when compared with the CD73+/H11001/H11001 groups. At day 7 posttransplantation, intragraft A3AR

Figure 3. Effects of CD73 on inflammatory molecules in cardiac allografts at day 7 posttransplantation. Intragraft mRNA expression of cytokines (IL-1β, TNF-α, and IFN-γ), chemokines (MCP-1 and RANTES), and adhesion molecules (ICAM-1 and VCAM-1). All data are expressed as means± SEM for n=6 mice.

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expression was significantly upregulated in all groups, though CD73−/− donor or recipient groups showed significantly more upregulation of A3AR, compared with CD73+/+ groups. Although intragraft A2AAR mRNA expression was significantly downregulated throughout posttransplantation, there was no significant difference between the CD73+/+ and CD73−/− groups.

Genetic Deletion or Pharmacological Blockade of CD73 Promotes Activation of Endothelial Cells and T Lymphocytes In Vitro

To further elucidate the effects of CD73 on interactions between endothelial cells and T lymphocytes found in cardiac allografts, we performed an allomismatched coculture of endothelial cells (H-2d) and lymphocytes (H-2b; CD73+/+ or CD73−/−) with or without α,β-methylene ADP (APCP) in vitro. First, we evaluated the contribution of T lymphocytes to endothelial cells on the simple coculture experiments. After a 72-hour coculture, mRNA expressions of endothelial cell TNF-α and VCAM-1 were significantly upregulated in the coculture with CD73−/− T lymphocytes as compared with CD73+/+ T lymphocytes (P=0.0195 and P=0.0270, respectively; Figure 6A). The addition of APCP significantly enhanced these upregulations in the coculture with CD73−/− T lymphocytes (TNF-α and VCAM-1; P=0.0353; Figure 6A). Next, we evaluated the contribution of endothelial cells to T lymphocytes using transmigration coculture experiments. After a 24-hour coculture, the number of T lymphocytes that had transmigrated into endothelial cells significantly increased in CD73−/− T lymphocytes compared with CD73+/+ T lymphocytes (P=0.0004; Figure 6B), and APCP significantly enhanced the transmigration in the coculture of CD73−/− T lymphocytes (P=0.0353; Figure 6B). IFN-γ mRNA expression in the post-transmigrated T lymphocytes was significantly upregulated in all experimental groups when compared with pretransmigrated T lymphocytes, and the IFN-γ mRNA upregulation was significantly higher in the coculture of CD73−/− T lymphocytes compared with the coculture of CD73+/+ T lymphocytes (P=0.0042; Figure 6C). There was no significant enhancement of the IFN-γ mRNA upregulation when APCP was added to the coculture of CD73−/− T lymphocytes.

Influence of Exogenous Adenosine Receptor Modulators on Cardiac Transplantation

To further evaluate the CD73-mediated contribution of specific AR subtypes during allograft rejection or CAV, we performed heterotopic cardiac transplantation using AR modulators given intraperitoneally. First, we examined which AR is acutely responsible for increased vascular leakage in the murine heterotopic cardiac transplantation model. At 4 hours after transplantation, graft permeability tended to increase for each AR antagonist applied (A2B, MRS1754>A2A, MRS1519).
SCH58261 > A_1, DPCPX>A_3, MRS1191), although only the A_2B antagonist MRS1754 caused a statistically significant increase in vascular leakage ($P<0.0001$ versus nontreatment control by ANOVA; Figure 7A). Based on the results of this graft permeability assay, we selected A_2A agonists (A_2A, CGS21680; A_2B, N-ethylcarboxamidoadenosine [NECA]) to establish their potential effects on cardiac allograft rejection or vasculopathy. Both CGS21680 and NECA treatments significantly increased graft survival compared with nontreatment controls, although the survival in NECA-treated recipients was significantly longer than that in CGS21680-treated recipients. When CD73^{+/−} donors or recipients were studied, NECA treatment significantly increased graft survival compared with wild-type nontreatment controls, whereas there was no significant increase in survival between CGS21680 treatment and wild-type nontreatment (Figure 7B). The next set of experiments was designed to measure the role of the A_2B AR in CAV. In both CD73^{+/+} and CD73^{−/−} donors or recipients, the severity of luminal occlusion at day 30 posttransplantation was significantly attenuated by NECA treatment, compared with wild-type nontreatment controls (Figure 7C and 7D). Taken together, these data suggest that

**Figure 5.** Intrgraft expression of CD73 and ARs throughout posttransplantation. A and B, mRNA expression of CD73 (A) and ARs (A_1AR, A_2AAR, A_2BAR, and A_3AR) (B). *$P<0.05$ vs CD73^{+/+} donor, **$P<0.05$ vs CD73^{+/−} recipient, †$P<0.05$ vs native heart. C and D, Protein expression of CD73, A_2BAR, and A_3AR by Western blotting at 4 hours (C) and 7 days (D) after transplantation. The expression of each band was normalized to its corresponding β-actin band. All data are expressed as means±SEM for n=6 mice.
A2AR strongly contributes to CD73-mediated allograft protection in murine heterotopic cardiac transplantation.

**Discussion**

CD73 effects the terminal phosphohydrolysis of AMP, which in turn generates adenosine. In the present experiments, the use of CD73−/− mice as either donors or recipients of heterotopic cardiac allografts allowed us to demonstrate the critical roles that CD73 plays in allograft survival and CAV prevention. Comparisons of CD73−/− mice with CD73+/+ mice showed less rejection and diminished vasculopathy when CD73 was present. These experiments indicate that CD73 promotes graft barrier function, suppresses the inflammatory response, and dampens alloeffect immune responses including the trafficking of leukocytes across allogeneic endothelium. These results could be attributed to the dissipation by CD73 of AMP, resulting in reduced stimulation of putative AMP receptors or by removing the terminal CD39 reaction product (AMP), thereby accelerating ADP catabolism and reducing the procoagulant and proinflammatory effects of ADP. However, it is also quite likely that generation of adenosine as a byproduct of AMP phosphohydrolysis could participate in these salutary vascular effects.

CD73 contributes in a major way to local adenosine concentrations especially at the vascular intimal surface where it is generated. Adenosine in the local vascular microenvironment is known to suppress inflammation, promote vasodilation, and inhibit vascular leakage, each action dependent on the receptor subtype to which it predominantly binds. Use of specific adenosine receptor agonists and antagonists in the present experiments allow us to conclude that the predominant vascular effects of CD73 in murine cardiac allotransplantation are mediated via the A2AR. When an A2AR agonist was given to recipients, graft survival was slightly prolonged (ie, rejection diminished) regardless of CD73 genotype. Interestingly, we have shown that intragraft A2AR mRNA expression was significantly downregulated, possibly suggesting that it might have less involvement in neointimal formation after cardiac allotransplantation. When an A2AR agonist was given to recipients, graft survival was markedly prolonged (ie, rejection diminished) regardless of CD73 genotype. These acute rejection experiments, indicating a dominant immune suppressive role mediated via the A2AR, led us to investigate the effects of chronic A2AR stimulation on development of CAV. Chronic

![Figure 6. Allomismatched coculture of endothelial cells and T lymphocytes in vitro.](http://circres.ahajournals.org/Download)
A2BAR stimulation resulted in a marked suppression of CAV development, and this rescue occurred regardless of whether CD73 was itself absent from the donor or recipient genotype. Taken together, these data clearly demonstrate an antirejection and anti-CAV role for CD73, which is likely to be mediated proximately by the local generation of adenosine and its actions predominantly via the A2BAR.

The microvascular endothelium is a dynamic barrier that regulates the exchange of fluid, solutes, and cells between the vessel lumen and tissues. I/R injury triggers an endothelial barrier dysfunction, characterized by neutrophil infiltration and increased graft permeability, that plays a significant role in the pathophysiology of CAV development in cardiac transplantation. In the present study, a lack of CD73 in donors or recipients attenuated A2BAR expression and promoted an inflammatory cascade involving enhanced graft permeability, neutrophil infiltration, and subsequent MPO release in cardiac allografts during the I/R phase. Previous studies have also shown that CD73-mediated activation of the A2BAR is critical for the maintenance and regulation of endothelial barrier function during hypoxia.4,17 During episodes of inflammation, transendothelial neutrophil infiltration into the tissues has the potential to disturb the barrier function via limitation of the A2BAR activation.17,18 Interestingly, we observed a positive correlation between CD73 and A2BAR mRNA expression in cardiac allografts at 4 hours after transplantation (data not shown).

A2BAR expression in cardiac allografts is still upregulated in the acute rejection phase and, to a lesser degree, in the I/R phase in CD73-deficient transplantsations. It has been reported recently that inflammatory cytokines such as IL-1, TNF-α, and IFN-γ modulate A2BAR expression and function on microvascular endothelial cells19 and that the A2BAR protects against vascular lesion formation via regulation of inflammatory cytokines, chemokines, and adhesion molecules.20,21 We have shown here that CD73 expression in donors or recipients plays an important role in regulating those inflammatory factors in the acute rejection phase of cardiac transplantation. Therefore, our results indicate that activation of A2BAR via CD73-generated adenosine modifies the production of inflammatory molecules. Such interactions could be an important mechanism for dampening endothelial activation and the inflammatory response in the acute allograft rejection.

A2BAR expression in cardiac allografts is also upregulated at 4 hours after transplantation following an administration of AR antagonists (DPCPX, SCH58261, MRS1754, and MRS1191). P<0.05 (by ANOVA) vs control. B. Graft survival following an administration of A2AR agonists (CGS21680 and NECA). P<0.05 (by ANOVA) vs CD73−/− nontreatment, †P<0.05 vs CD73−/− with CGS21680, ††P<0.05 vs CD73−/− with CGS21680. C and D, Effects of A2AR agonist (NECA) on chronic allograft rejection at day 30 posttransplantation. C and D, Elastin staining in cardiac allografts (C) and histomorphometric quantification of luminal occlusion in graft coronary arteries (D). Bar=100 μm. a, Donor CD73+/+ no nontreatment. b, Donor CD73+/+ + NECA. c, Donor CD73+/− + NECA. d, Recipient CD73−/− + no treatment. e, Recipient CD73−/− + NECA. f, Recipient CD73−/− + NECA. Data are expressed as means±SEM for n=4 (A) and n=6 (B, C, and D) mice.
unknown because some studies support a protective role for the receptor, whereas others indicate that it induces myocardial apoptosis.22,23 In the present study, CD73 deficiency in donors or recipients promoted apoptosis in cardiac allografts during the acute rejection phase (data not shown). The effects of A3AR activation appear to depend on the pattern of receptor activation (endogenous or exogenous) and drug administration (dose or duration), and we believe that this relationship between CD73 and A3AR in cardiac transplantation may be explained as a compensatory and protective upregulation of A3AR in response to apoptosis or to a deficiency of CD73. Further studies are needed to further elucidate this complex relationship.

Our present research supports earlier work that showed that adenosine generated by CD73 on T lymphocytes mediates immune suppression in skin allografts and in vitro experiments.6 Using histological studies, we have demonstrated that CD73 deficiency in donors or recipients correlates with intense acute rejection, as evidenced by impressive graft infiltration of both CD4- and CD8-positive T lymphocytes in the acute rejection phase following transplantation. IFN-γ, which enhances antigen presentation and promotes cellular immunity by activated macrophages, natural killer (NK) cells, and Th1 lymphocytes,24 was also significantly upregulated in CD73-deficient cardiac allografts. A critical event during the progression of acute allograft rejection is the recruitment and transmigration of alloantigen-primed CD4- and CD8-positive T lymphocytes into the graft, followed by the release of cytokines by both endothelial cells and T lymphocytes.25 Our in vitro coculture experiments demonstrated that both genetic deletion and pharmacological blockage of CD73 promote the transendothelial migration of T lymphocytes and upregulate expression of TNF-α, VCAM-1, and IFN-γ. In addition, the present studies show that CD73 deficiency in donors or recipients resulted in an increase in the production of donor-reactive alloantibodies and T-lymphocyte proliferation in the chronic rejection phase of cardiac transplantation. Taken together, these results indicate that CD73 regulates allogeneic interactions between endothelial cells and T lymphocytes and thus plays an immunomodulatory role that promotes allograft survival.

T lymphocytes may not be the only effectors relevant to cardiac allograft rejection that are modulated by CD73. NK cells are a type of cytotoxic lymphocytes that are able to kill target cells without prior exposure to antigen. Because their lethal effector functions are triggered without prior antigen priming, they are considered to be an integral constituent cell of the innate immune system and, hence, relevant to cardiac allograft rejection or vasculopathy. CD73 is indeed expressed by NK cells, as well as endothelial cells and other leukocytes.26 Recently, Uehara et al27 demonstrated that NK cells can promote CAV in a murine cardiac transplantation model; however, these cells do so in a milieu that requires T cells and other alloeffectors. Interestingly, the interaction between NK cells and T cells that contributes to CAV likely involves IFNs and other cytokines. In the present study, we focused on the immunologic crosstalk between endothelial cells and T cells in transplant alloresponses. Although we did not specifically evaluate NK cell activity, these cells could indeed be activated in cardiac allografts because intragraft IFN-γ mRNA expression was upregulated at 7 days posttransplantation. IFN-γ mRNA levels were significantly increased in the allografts in which CD73 was absent in either the implanted graft or the recipient compared with wild-type transplants. Therefore, NK cells in CD73-deficient recipients might contribute to CAV development. Further work would need to be performed to understand whether the biological function of CD73+/− NK cells are as same as those of CD73+/- NK cells. Complicating the prediction even further, 1 recent article has shown that NK cells promote transplant tolerance by killing donor antigen-presenting cells.28 Further studies would be needed to understand a role of NK cells in chronic rejection, especially it relates to CD73 on NK cells or their immune targets.

Our study involved both donor (endothelial and parenchymal cells) and recipient (leukocytes) sources of CD73, thus allowing us to explore the contributions that each source makes to the overall transplant milieu. In allotransplant settings, recipient leukocytes attack donor endothelial cells, resulting in acute rejection characterized by endothelial injury and dysfunction, altered endothelial permeability, and neointimal formation (vasculopathy). The alloimmune injury induced by cross−major histocompatibility complex barrier transplantation can be a sustained and severe endothelialitis, which differs from that in mechanical vascular injury (alloeffectors mechanisms do not pertain). In general, the alloimmune vascular injury caused by transplantation is quite brisk and severe. This is an important difference when one considers the work by Zernecke et al,14 in which following carotid wire injury, there was no significant difference in neointimal formation when CD73-null marrow was transplanted into wild-type recipients. Based on knowledge of the effects of CD73 and downstream adenosine and its signaling mechanisms in immune regulation, one could speculate that transplantation of CD73-null marrow might increase allograft vasculopathy. The reasoning behind this hypothesis is that CD73-dependent adenosine generation induces a form of leukocyte−endothelial cell crosstalk that results in reduced leukocyte adhesion to the endothelium and decreased transmigration into tissues in the setting of certain types of inflammatory responses.10 In the present transplant experiments, there was an opportunity to discern whether there was a local vascular effect of CD73 based on its expression on circulating leukocytes or whether the effect was attributable to CD73 present on cells resident in the graft at the time of transplantation. Our data clearly demonstrate that CD73 in either or both locations can play a role in restoring vascular homeostasis to cardiac allografts.

Another recent study has shown that both recipient- and donor-derived cells contribute to the regeneration of damaged cells in cardiac allografts.29 The interaction between endothelial cells and lymphocytes attenuates CD73 activ-
ity,\textsuperscript{30} whereas CD73-dependent adenosine generation induces a novel form of leukocyte–endothelial cell crosstalk that results in reduced leukocyte adherence to the endothelium and decreased transmigration into tissues in the setting of hypoxia-associated inflammatory responses.\textsuperscript{10} Therefore, it is possible that the intragraft level of CD73 expression effects the outcome of cardiac allografts. Because recipient-derived cells infiltrate into allografts over time and injury to the donor-derived cells in allografts is a progressive process, our model results in a total CD73 expression in cardiac allografts that fluctuates with time. It is interesting to note that allotransplants of CD73\textsuperscript{+/−} donors or recipients were found to have lower levels of CD73 expression throughout the posttransplantation period, resulting in increased cardiac graft damage. In our cardiac isograft transplantation experiment, the homologous combination of CD73\textsuperscript{+/−} donors and recipients resulted in an accelerated inflammatory response when compared with a heterologous combination of CD73\textsuperscript{+/−} donors and CD73\textsuperscript{+/+} recipients and vice versa (T. Hasegawa, D.J. Pinsky, unpublished data, 2007). In our in vitro coculture studies involving CD73\textsuperscript{+/−} T lymphocytes and CD73\textsuperscript{+/+} endothelial cells supplemented with APCP (an inhibitor of CD73) significantly enhanced the transendothelial migration of T lymphocytes, as well as TNF-α and VCAM-1 expression. Thus, CD73 expressed on both local and circulating cells could contribute to preserving vascular homeostasis after cardiac transplantation.

In summary, these experiments demonstrate that both local and circulating CD73 contribute to allograft-protection in cardiac transplantation, leading to improved allograft survival and protection against CAV development. Mechanisms underlying this protection likely include (1) the maintenance of graft barrier function resulting from a concurrent upregulation of A2B AR in the I/R phase; (2) suppression of the inflammatory response, possibly attributable to an upregulation of A2B AR; and (3) suppression by CD73 of the transit of effector leukocytes across graft endothelium. These studies point to CD73 as residing at the nexus of inflammatory and vascular reactions that can protect a vulnerable graft and its vasculature from immune attack.

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Disclosures
None.

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Ecto-5′ Nucleotidase (CD73)-Mediated Adenosine Generation and Signaling in Murine Cardiac Allograft Vasculopathy
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MATERIALS AND METHODS

Animals. Inbred male mice between 8 and 12 weeks of age were used for this experiment. CD73-deficient mice (CD73<sup>−/−</sup>) of C57BL/6 (H-2<sup>b</sup>) background, a generous gift of Dr. Linda F. Thompson, have been described previously<sup>1</sup>. CD73<sup>−/−</sup> littermates were used as the wild-type control. B10A (H-2<sup>a</sup>) and CBA/J (H-2<sup>k</sup>) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). The genotypes were confirmed by genomic polymerase chain reaction (PCR). All experiments were performed according to the protocols approved by the University of Michigan Committee on Use and Care of Animals in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Reagents. α, β-methylene ADP (APCP), 8-cyclopentyl-1,3-diprophylxanthine (DPCPX), 5-Amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH58261), 8-[4-[[4-(Cyano)phenylcarbamoylmethyl]oxy]phenyl]-1,3-di-(n-propyl)xanthine (MRS1754), 3-Ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191), 2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS21680), N-Ethylcarboxamidoadenosine (NECA) were purchased from Sigma-Aldrich (St. Louis, MO).

Adenosine receptor modulators. Selective agonists and antagonists of specific adenosine receptor subtypes were used for certain experiments; ANTAGONISTS: A<sub>1</sub>AR antagonist
DPCPX (5 mg/kg)$^2$, A$_{2A}$AR antagonist SCH58261 (3 mg/kg)$^3$, A$_{2B}$AR antagonist MRS1754 (1 mg/kg)$^1$ or A$_3$AR antagonist MRS1191 (1 mg/kg)$^4$ was administered intraperitoneally once just after transplantation as indicated; AGONISTS: A$_{2A}$AR agonist CGS21680 (2 mg/kg/day)$^5$ or A$_{2B}$AR agonist NECA (0.2 mg/kg/day)$^1$ were administered intraperitoneally every 12 hours after transplantation as indicated. Though there is a slight ability of NECA to stimulate the other adenosine receptor subtypes, it is 171 times, 120 times, and 387 times more selective for the A$_{2B}$AR than for A$_1$AR, A$_{2A}$AR, and A$_3$AR, respectively, and is therefore considered a relatively specific agonist for the A$_{2B}$AR$^6$.

**Heterotopic cardiac transplantation.** Completely allomismatched murine heterotopic cardiac transplantation was performed using two rejection models, acute and chronic, as described previously$^7$. The donor heart was transplanted into the recipient mouse’s abdominal cavity. Recipient mice received no immunosuppressive agents in the acute rejection model. Transient pretransplant immunosuppression$^7$ was administered in the chronic rejection model using a brief course of anti-murine CD4 (clone GK1.5) and anti-murine CD8 (clone 2.43) antibodies (Harlan Bioproducts for Science, Indianapolis, IN). No immunosuppressive treatment was given following transplantation.

**Experimental groups.** Cardiac allografts were assessed by using 4 groups (n=6 in each group). Two groups were used to study donor sources of CD73 (CD73$^{+/+}$ or CD73$^{-/-}$ donors into B10A recipients) and two groups were used to study recipient sources (B10A donors into CD73$^{+/+}$ or CD73$^{-/-}$ recipients). In experiments of adenosine receptor
modulators, CBA/J mice were used as donors or recipients instead of B10A mice (n=4 to 6 in each group, as indicated).

**Graft survival and histomorphometric assessment.** Survival of cardiac allografts, histomorphometric quantification of parenchymal rejection (PR) and CAV area were assessed as previously described. Briefly, the graft survival was evaluated by daily palpitation, and absence of pulsation was interpreted as rejection. Graft hearts were fixed in 10% formalin, paraffin-embedded, and sectioned transversely at the maximal circumference of the ventricle. Sections were cut (5 μm) and the PR severity was analyzed by hematoxylin and eosin staining, and graded using a modified form of the Working Formulation of the International Society for Heart and Lung Transplantation (ISHLT) criteria: 0=no rejection, 1=interstitial and/or perivascular infiltration of mononuclear cells without myocyte damage, 2=one focal infiltration of mononuclear cells with associated myocyte damage, 3=multifocal or diffuse infiltration of mononuclear cells with myocyte damage, 4=multifocal or diffuse infiltration of mononuclear and polymorphous cells with extensive myocyte damage. The CAV area was analyzed using elastica van Gieson staining to highlight the internal elastic lamina (IEL). The cross-sectional area of luminal occlusion was calculated using the Image-Pro Plus version 4.5 software (MediaCybernetics, Silver Spring, MA) as follows: Luminal occlusion = [(IEL area – luminal area) / IEL area] ×100 (%).

**Graft permeability assay.** Graft vascular leakage was assessed by intravascular administration of Evan’s blue (Sigma-Aldrich, St. Louis, MO) as described previously.
In brief, Evan’s blue (0.2 ml of 0.5% in PBS) was injected intravenously into recipient mice immediately after transplantation, and then donor graft hearts were harvested at 4 hours post-transplantation. The Evans blue concentrations were quantified after formamide extraction (55°C for 2 hours) by measuring absorbance at 610 nm with subtraction of reference absorbance at 450 nm.

**MPO assay.** Myeloperoxidase (MPO) activity in cardiac allograft was assessed as previously described\(^1\). MPO values were standardized to the protein concentration of each sample as determined by a micro BCA protein assay kit (Pierce, Rockford, IL). Data are expressed as the change in absorbance at 460 nm/min/mg of total protein.

**Immunohistochemistry.** Immunohistochemical staining was performed on frozen sections (5 μm) by using Anti-Ig HRP detection kits (BD Pharmingen, San Diego, CA), with primary antibodies directed against a neutrophil marker (rat anti-mouse Ly6G; SouthernBiotech, Birmingham, AL), T-lymphocyte markers (rat anti-mouse CD4 and CD8; BD Pharmingen) and leukocyte marker (rat anti-mouse CD11b; BD Pharmingen). Diaminobenzidine substrate was used as a chromogen, and cell nuclei were counterstained with hematoxylin. Positive cell numbers were quantified by counting reactive cells in 10 non-overlapping high-power fields using the cell count plug-in of the ImageJ version 1.38 software (National Institute of Health, Bethesda, MD).

**Quantitative real-time PCR analysis.** Total RNA was isolated *in vivo* from graft samples using a RNeasy fibrous tissue mini kit (QIAGEN), and *in vitro* from cells using a RNeasy
Supplement Material  Hasegawa et al., Ecto-5’ nucleotidase (CD73)-mediated adenosine…

mini kit (QIAGEN) according to the manufacturer’s instructions. The RNA was transcribed and amplified to cDNA using a High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis of the mRNA for the genes of interest was performed using ABI Prism 7000 sequence detector system (Applied Biosystems) with TaqMan universal PCR mater mix (Applied Biosystems). TaqMan real-time PCR primers were purchased from Applied Biosystems. The expression levels of each mRNA were divided by levels of mRNA of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin.

**Fluorescent western blot analysis.** Immunoblotting was performed by using iBot Dry Blotting System (Invitrogen, Carlsbad, CA) and Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) per manufacturer’s instructions with primary antibodies; rabbit polyclonal anti-CD73, rabbit polyclonal anti-A2BAR and rabbit polyclonal anti-A3AR (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies used were IRDye 800CW goat anti-rabbit IgG. Images were analyzed by the Odyssey application software, version 2.1 (LI-COR Biosciences). The expression of each band was normalized to its corresponding β-actin band (mouse monoclonal β-actin; Sigma-Aldrich).

**Donor-reactive alloantibodies.** Measurement of donor-reactive alloantibodies was performed as previously described12. A 2-parameter display of fluorescein isothiocyanate (FITC)-conjugated, F(ab’)2, Fc fragment-specific, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and phycoerythrin (PE)-conjugated hamster anti-mouse CD3e monoclonal antibody (BD Pharmingen) was generated using
the BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ), and data were analyzed using CellQuest version 3.3 software (BD Biosciences). Serum from recipient mice sensitized by subcutaneous injection of donor splenocytes was used as a positive control.

**MLR.** Spleens were harvested and minced. The splenocyte suspensions were then passed through a 70-µm cell strainer and treated with a red blood cell lysis buffer (eBioscience, San Diego, CA). A one-way mixed lymphocyte reaction (MLR) was performed using responder splenocytes from recipient mice and mitomycin-C-inactivated stimulator splenocytes from native donors. A total of $5 \times 10^5$ responder cells and an equal number of stimulator cells were cocultured for 3 days. Cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) as previously described\textsuperscript{13}. Control wells contained responder cells in the absence of stimulator cells. Cell proliferation was expressed as the optical density of responder cells.

**Cell isolation and culture.** T lymphocytes were purified from splenocytes of CD73\textsuperscript{+/+} and CD73\textsuperscript{--} mice (H-2\textsuperscript{b}) using a Dynal mouse T cell negative isolation kit (Invitrogen) according to the manufacturer's instructions. The BALB/c (H-2\textsuperscript{d})-derived endothelial cell line bEnd.3 was obtained from American Type Culture Collection (Manassas, VA). Endothelial cells were grown in Dulbecco's minimum essential medium (Invitrogen) containing 100 U/ml penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum at 37°C and 10% CO\textsubscript{2} between passage 4 and 8.
Coculture of endothelial cells and T lymphocytes. Endothelial cells were trypsinized and seeded into 12-well plates (Corning Life Sciences, Lowell, MA). After reaching approximately 90% confluence, T lymphocytes (1×10^6/well) were added to the endothelial cell monolayers with or without a specific CD73 inhibitor, APCP. After a 72-h period of coculture at 37°C in 10% CO₂ / 90% air, endothelial cells were collected and analyzed for mRNA expression as described above.

Transmigration assay. For transmigration studies, cells were placed on 6-well Transwell plates (3 µm pore size, 24 mm diameter, polycarbonate membrane; Corning Life Sciences). Endothelial cells were allowed to grow on the polycarbonate membranes until reaching confluence. T lymphocytes (5×10^6/well) were added to the upper chamber with or without APCP, and were coincubated with the cultured endothelial cells for 24 h at 37°C in 10% CO₂ / 90% air. The number of T lymphocytes that had migrated to the lower chambers was assessed with the Cell Counting Kit-8 (Dojindo Molecular Technologies). Pre-transmigrated T-lymphocytes in the upper chambers and post-transmigrated T-lymphocytes in the lower chambers were collected and analyzed for mRNA expression as described above.

Statistics. Database management and statistical analysis were performed with the Statview version 5.0 software (SAS institute Inc., Cary, NC). All values are expressed as means±SEM. Kaplan-Meier analysis was performed to evaluate graft survival, and survival differences were compared by a log-rank test. Comparisons among groups were
performed with an unpaired Student $t$ test or one-way analysis of variance (ANOVA) where appropriate. Values of $P<0.05$ were considered statistically significant.

Reference List


