The Protective Effects of CD39 Overexpression in Multiple Low-Dose Streptozotocin–Induced Diabetes in Mice

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Islet allograft survival limits the long-term success of islet transplantation as a potential curative therapy for type 1 diabetes. A number of factors compromise islet survival, including recurrent diabetes. We investigated whether CD39, an ectonucleotidase that promotes the generation of extracellular adenosine, would mitigate diabetes in the T cell–mediated multiple low-dose streptozotocin (MLDS) model. Mice null for CD39 (CD39KO), wild-type mice (WT), and mice overexpressing CD39 (CD39TG) were subjected to MLDS. Adoptive transfer experiments were performed to delineate the efficacy of tissue-restricted overexpression of CD39. The role of adenosine signaling was examined using mutant mice and pharmacological inhibition. The susceptibility to MLDS-induced diabetes was influenced by the level of expression of CD39. CD39KO mice developed diabetes more rapidly and with higher frequency than WT mice. In contrast, CD39TG mice were protected. CD39 overexpression conferred protection through the activation of adenosine 2A receptor and adenosine 2B receptor. Adoptive transfer experiments indicated that tissue-restricted overexpression of CD39 conferred robust protection, suggesting that this may be a useful strategy to protect islet grafts from T cell–mediated injury. Diabetes 62:2026–2035, 2013

Pancreas or islet transplantation is a potentially curative therapy for patients with type 1 diabetes (T1D), eliminating the need for exogenous insulin and the inherent risks of life-threatening hyperglycemic and hypoglycemic episodes. Unlike the complex surgery of whole pancreas transplantation, isolated islets are delivered percutaneously into the portal circulation. However such transplanted islets face several challenges. The first is an immediate response, termed the instant blood-mediated inflammatory reaction (IBMIR), involving platelet binding, activation of the complement and coagulation cascades, and infiltration of islets by monocytes and neutrophils. Further loss of islets occurs within a day of transplantation, mediated by activated hepatic natural killer cells and neutrophils (1). Islets surviving these innate immune processes are then subject to the alloimmune response and recurrent autoimmunity.

Recipients of transplanted islets are patients with T1D and as such are already primed for islet autoimmunity. Recurrent autoimmune disease has been demonstrated in animal models and in humans after either islet or pancreatic transplantation (2) and is intertwined with the alloimmune response. There is some evidence that individuals who are particularly prone to autoimmunity are more likely to experience rapid allograft rejection (3). Further, an increase or re-emergence of islet autoantibodies has been observed despite immunosuppression (4,5), irrespective of complete human leukocyte antigen match (6) or mismatch (7).

In animal models, recurrent autoimmune disease potentiates the immune response, resulting in earlier graft rejection after intraportal islet transplantation (8). Indeed, strategies that have been demonstrated to prolong islet allograft survival in chemically induced (high-dose streptozotocin) diabetic recipients have failed to do so in autoimmune NOD mice (9). These data suggest that recurrent autoimmunity can destroy islet allografts even in the absence of an alloimmune response. Although the mechanism of recurrent autoimmunity is not fully defined, T cells are involved primarily through major histocompatibility complex II recognition (10).

We are interested in the role of purinergic catabolism and adenosine signaling in immune and thrombotic responses to islet transplantation. CD39 is a membrane-bound enzyme that hydrolyzes extracellular purinergic nucleotides, including the platelet agonist ADP. We have generated mice overexpressing CD39 (CD39TG) from the H-2Kd promoter, which directs expression to all nucleated cells. Ubiquitous expression has been demonstrated on circulating cells by flow cytometry, and throughout the tissues (including on β-cells) by immunohistochemistry (11,12). We have previously shown that the overexpression of CD39 on murine islets attenuated thrombosis when the islets were mixed with human blood (11), and this approach has been heralded as a potential anti-IBMIR strategy (13). The objective of this study was to examine the impact of CD39 overexpression on diabetes induction using the multiple low-dose streptozotocin (MLDS) model, as a prelude to investigations using CD39TG islets in transplantation where recurrent diabetes may compromise graft survival. Although it shares fewer features with human T1D than the NOD model, we used the MLDS model.

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because it is more convenient to investigate the effect of various genetic modifications and its validity as a model of T cell–mediated diabetes has recently been confirmed (14).

The overexpression of CD39 on the islet surface does not impact glucose homeostasis (11) and thus such islets would be potentially suitable for transplantation. In addition to preventing IBMIR, the overexpression of CD39 on islets may confer additional downstream benefits for islet grafts attenuating T cell–mediated islet graft loss from recurrent autoimmunity and allograft rejection. The end product of CD39’s catalytic activity is AMP, and overexpression of CD39 increases the level of AMP available for conversion to adenosine (12) by the ecto-enzyme CD73 (15). Extracellular adenosine binds to four specific receptors (A1R, A2AR, A2BR, and A3R), promoting a range of effects that are predominantly anti-inflammatory (16). Studies using CD39KO mice have identified a role for ectonucleotidase expression in the regulation of organ-protective adenosine receptor signaling (17). Consistent with this, mice overexpressing CD39 are protected from renal ischemia-reperfusion injury (IRI) by an A2AR-dependent mechanism (18). Kidney isografts from these mice performed significantly better after prolonged cold storage than control isografts, indicating that organ-restricted overexpression of CD39 was sufficient for this protective effect (18). Adenosine receptor signaling influences the development of diabetes in nontransplant settings in the NOD and MLDS models. Activation of adenosine receptors by the non-specific agonist 5'-N-ethylcarboxamidoadenosine (NECA) ameliorated diabetes in both models (19). In the MLDS model, AIR and A3R agonists had a modest protective effect compared with NECA, whereas the A2AR agonist had no effect. The A2BR antagonist reversed the effect of NECA, suggesting that A2BR signaling played the predominant role in regulating MLDS-induced diabetes (19).

In this study, we hypothesized that the level of CD39 expression may affect susceptibility to T cell–dependent diabetes by influencing the rate of proinflammatory extracellular nucleotide catabolism and subsequent generation of anti-inflammatory adenosine. CD39TG and CD39KO were examined for their response to MLDS and compared with wild-type (WT) controls. To examine the utility of tissue-restricted expression of CD39, which is of direct relevance to clinical transplantation, adoptive transfer of bone marrow (BM) between selected lines was performed prior to MLDS treatment. Finally, we examined the importance of adenosine receptor signaling by using mutant mice and pharmacological inhibition of the adenosine receptors.

Adaptive transfer experiments. Male mice (4–5 weeks old) were subjected to 5 Gy of irradiation and received 5 × 10⁶ BM cells intravenously from age-matched mice the next day. Mice received MLDS treatment 5 weeks later.

Immunohistochemistry. Paraffin-embedded pancreas sections (4 μm) were stained with hematoxylin and eosin (H&E). After antigen retrieval, sections were stained with polyclonal guinea pig anti-insulin antibody (Dako, Campbellfield, VIC, Australia) and rabbit anti–guinea pig horseradish peroxidase (Dako) or monoclonal rabbit anti-CD3+ antibody (Abcam, Cambridge, U.K.) and biotinylated donkey anti-rabbit antibody (GE Healthcare, Rydalmere, NSW, Australia). Sections were then developed using 3,3′-diaminobenzidine. TUNEL staining was performed with the Klenow FragEL DNA fragmentation detection kit (Merck Serono, Foresta, New South Wales, Australia).

Flow cytometry. Peripheral blood leukocytes were incubated with fluorescein isothiocyanate–conjugated anti-human CD3 (Ansell, Cambridge, Australia) and analyzed using a FACScan (BD Biosciences, San Jose, CA). The following antibodies were used: CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD44 (clone IM7), CD69 (clone M-A2), and CD103 (clone 8H10A14). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

RESULTS

The level of CD39 expression modulates susceptibility to MLDS-induced diabetes. After MLDS treatment, the mean BGL of WT mice rose steadily after day 6, reaching hyperglycemic levels (15.8 ± 1.2 mmol/L) by day 12 and progressing to diabetes (21.2 ± 2.4 mmol/L) by day 42. CD39KO mice exhibited a heightened susceptibility to MLDS, with a faster and more pronounced rise in BGL. In contrast, CD39TG mice were significantly protected (Fig. 1A). Incidence of diabetes was 71% for WT, 100% for CD39KO, and 14% for CD39TG mice (Fig. 1B).

CD39KO mice have both hepatic insulin resistance, which portends to glucose intolerance (25), and hyperproliferative T cells secondary to dysfunctional regulatory T cells (26). To investigate whether these features contributed to the heightened MLDS response, CD39KO mice were sublethally irradiated and adoptively transferred with WT BM (CD39KOWTBM) prior to treatment with MLDS. Diabetes incidence in CD39KOWTBM was similar to that of WTWTBM mice (Fig. 1C), suggesting that in this model, the dysfunctional T-cell phenotype due to CD39 deficiency rather than hepatic insulin resistance was responsible for the increased susceptibility to diabetes.

To confirm the role of T cells in this model, WT mice were depleted of T cells prior to MLDS. These mice
FIG. 1. Expression levels of CD39 are inversely related to the susceptibility to MLDS-induced diabetes. Nonfasting BGLs (mean ± SEM) (A) and diabetes incidence (B) in CD39KO (▲, n = 6), WT (■, n = 7), and CD39TG (●, n = 6) mice after MLDS. WT mice treated with citrate buffer (X, n = 2). ***P < 0.001 vs. MLDS-treated WT mice. Diabetes incidence (C) of MLDS-treated chimeric CD39KO_WTBM (▲, n = 4) and WT_WTBM (□, n = 6) mice. ns, not significant.

showed a significant reduction in the incidence of diabetes (Supplementary Fig. 1). In a separate experiment, MLDS-treated mice were killed at the onset of diabetes for histological and immunohistochemical analysis of the pancreas. For WT and CD39KO mice, this was performed when BGL first exceeded 20 mmol/L (days 24 and 10, respectively); for CD39TG mice, which never reached this level, the pancreas was harvested on day 30. Although the architecture of islets from MLDS-treated WT mice was largely preserved (Fig. 2A, i), a reduction in the intensity of insulin staining (Fig. 2A, ii) and a CD3+ T-cell infiltrate were observed (Fig. 2A, iii). Consistent with the more severe diabetes in MLDS-treated CD39KO mice, islets from these mice showed disrupted morphology (Fig. 2A, iv), weaker insulin staining (Fig. 2A, v), and a heavier cellular infiltrate (Fig. 2A, vi). In contrast, the architecture of CD39TG islets was preserved (Fig. 2A, vii) and they stained strongly and uniformly for insulin (Fig. 2A, viii), with minimal CD3+ T-cell infiltration (Fig. 2A, ix). The mean insulitis scores were 47, 33, and 20% for the CD39KO, WT, and CD39TG animals, respectively.

CD39 overexpression prevents islet T-cell infiltration and inflammatory gene expression after MLDS. Cytokines released from T cells are critical in the progression of T1D through the inhibition of β-cell function and promotion of apoptosis. Islets were isolated from untreated or MLDS-treated WT and CD39TG mice 10–12 days after MLDS treatment, the time of maximum insulitis in this model (27), and analyzed by qRT-PCR. Expression of the specific T-cell marker CD3γ was detected only in islets from treated WT mice (Fig. 2B), consistent with the T-cell staining pattern (Fig. 2A, iii). Expression of the proinflammatory cytokines IL-1β and IL-6, the adhesion molecule ICAM-1, and the cytokine-inducible enzyme iNOS was significantly upregulated in WT islets after MLDS but was not significantly changed by MLDS in CD39TG islets (Fig. 2C–F).

Overexpression of CD39 on tissues is sufficient to protect against MLDS. CD39TG mice have an enhanced capacity to generate adenosine (12), and isolated islets from these mice have increased nucleoside triphosphate diphosphohydrolase (NTPDase) catalytic activity (11). CD39TG mice also have a selective partial CD4+ T-cell lymphopenia, and the residual T cells are hypoproliferative (28). Adoptive transfer experiments were used to investigate whether the reduced susceptibility of CD39TG mice was due to the CD4+ T-cell lymphopenia or to the effects of CD39 expression on tissues. Transfer of WT BM into sublethally irradiated CD39TG mice produced chimeric mice with CD39 overexpression restricted to the tissues (CD39TG_WTBM). Successful reconstitution was verified by flow cytometric analysis of peripheral blood leukocytes (Supplementary Fig. 2). WT_WTBM chimeric mice were used as controls. The reconstitution of CD39TG BM in irradiated WT (WT_CD39TG) or CD39TG recipient mice (CD39TG_CD39TG) was incomplete (data not shown; 28), and these mice were not further examined.

CD39TG_WTBM chimeric mice were resistant to MLDS (Fig. 3A), and their islets exhibited normal morphology and insulin staining 30 days after MLDS treatment (Fig. 3B). Protection did not appear to be due to an inherent resistance of the islets to proinflammatory cytokines, because islets isolated from CD39TG mice were equally susceptible to proinflammatory cytokines in vitro as islets from WT mice (Fig. 3C). We speculate that in vivo, increased CD39 activity in the tissues enhances adenosine
generation, tempering the host’s immune response and subsequent T-cell infiltration of islets. To investigate this possibility, CD39TG mice were adoptively transferred with BM from CD39KO or A2ARKO mice, both of which have hyperproliferative T cells (26), and treated with MLDS. Chimeric CD39TG\textsubscript{CD39KOBM} and CD39TG\textsubscript{A2ARKOBM} mice remained resistant to MLDS (Fig. 3D and E).

**The protective effect of CD39 overexpression is mediated by A2 receptor signaling.** Adenosine signals via four receptors, the expression of which has been examined in whole pancreas (19). Given that the A2AR is involved in β-cell regeneration (29), we sought to define the expression of these receptors at the transcript level in isolated islets (eliminating acinar and vascular tissue) from untreated and MLDS-treated mice. Basal expression of A1R (Fig. 4A) and A2AR (Fig. 4B) was similar in CD39TG and WT islets, but expression of A2BR was significantly higher in CD39TG (Fig. 4C). MLDS treatment did not significantly affect expression of A1R or A2AR in either mouse line (Fig. 4A and B). The A2BR was upregulated by MLDS in WT but not CD39TG islets (Fig. 4C). No A3R expression was detected on islets from either line, irrespective of treatment.

Antagonism or agonism of the A1R did not alter the susceptibility of the WT mice to MLDS-induced diabetes (Supplementary Fig. 3); however, deletion of A2AR increased susceptibility. Given the hyperproliferative T-cell response of A2ARKO mice (26), A2ARKO mice were adoptively transferred with WT BM, generating chimeric A2ARKO\textsubscript{WTBM} mice that were treated with MLDS. The reverse experiment (A2ARKO BM into WT mice: WTA2ARKOBM) was also performed. Deletion of the A2AR on either the tissues or the circulating cells conferred increased susceptibility to MLDS-induced diabetes (Fig. 5B).

A2ARKO mice were bred with CD39TG mice to generate A2ARKO/CD39TG mice (Supplementary Fig. 4). A2ARKO/CD39TG mice were not protected from MLDS-induced diabetes (Fig. 5A), implicating a role for A2AR signaling in the protection mediated by CD39 overexpression. However, the response to MLDS was less severe than in
A2ARKO mice (Fig. 5A), suggesting that signaling via a second adenosine receptor may also be involved. To address this, WT and CD39TG mice were treated with A2BRi, which led to a greater incidence of hyperglycemia (Fig. 5C) and diabetes (Fig. 5D). CD39TG mice treated with A2BRi rapidly became hyperglycemic with kinetics similar to that of WT mice (Fig. 5C), although they did not progress to diabetes, providing evidence for an independent role for the A2BR in early CD39-mediated protection. Finally, to examine the effect of inhibiting both A2AR and A2BR signaling, A2ARKO/CD39TG mice were treated with A2BRi. Blockade of the A2BR did not further augment the incidence of diabetes in these mice (Fig. 5E).

**DISCUSSION**

The major challenge for islet transplantation as a clinical therapy is to improve the long-term function of the transplanted islets. Currently, the minority of islet recipients...
remain insulin independent, reflecting islet loss over time (30). Significant factors contributing to this progressive loss include IBMIR, recurrent autoimmunity, and the alloimmune response. We have previously reported that overexpression of CD39, a key antithrombotic and anti-inflammatory enzyme, protected mouse islets from IBMIR in vitro (11). In this study, we used the MLDS model to investigate the role of purinergic catabolism and signaling in the regulation of islet inflammation and injury in vivo. Although MLDS is not a perfect model of autoimmunity associated with islet transplantation or T1D, it shares a dependence on the action of T cells in its pathogenesis.

The overexpression of CD39 on β-cells does not perturb islet function (11). Fasting insulin levels in CD39TG mice were comparable to WT mice (data not shown), suggesting normal insulin sensitivity. Reduced insulin sensitivity has been reported acutely after treatment of WT mice with NECA, a nonspecific adenosine agonist (31). We have previously shown that basal levels of adenosine in CD39TG mice are comparable to that of WT but are increased after a proinflammatory insult (12). Although adenosine concentrations were not measured in the current study, the protection of CD39 overexpression was mitigated by A2R inhibition or deletion, implicating a role
CD39 EXPRESSION PREVENTS MLDS-INDUCED DIABETES

for adenosine signaling. It is possible that insulin sensitivity was altered after MLDS; however, persistent normoglycemia in CD39TG mice indicates an adequate β-cell response. The susceptibility of mice to MLDS was strikingly influenced by the level of expression of CD39. Mice lacking CD39 became diabetic faster and with a higher overall incidence than WT mice, whereas mice overexpressing CD39 were significantly protected. The rapid onset of diabetes in MLDS-treated CD39KO mice was likely due to defective regulatory T-cell function (26) rather than impaired glucose tolerance, as reconstitution with WT BM reduced the incidence of diabetes to that of WT mice. In the case of CD39TG mice, chimeric mice reconstituted with a WT immune system retained protection from MLDS, suggesting that tissue-generated adenosine was responsible for reduced susceptibility. In vitro data indicated that CD39TG islets were not inherently resistant to proinflammatory cytokines.

T1D is characterized by the expression of proinflammatory cytokines by islet-invading mononuclear cells (32). Unlike high-dose streptozotocin treatment, which is directly toxic to β-cells, MLDS promotes leukocytic infiltration of islets that peaks at 10–12 days (27), comprised principally of CD3+ T cells. After activation, these cells secrete cytokines such as IL-1β, which contribute to β-cell dysfunction and death (33). Further, cytokine-induced expression of iNOS by the β-cell itself is a key factor in islet cell death. The increase in inflammatory cytokine expression in islets from MLDS-treated WT mice likely reflects T-cell infiltration, which was evident histologically and associated with a reduction of insulin staining (Fig. 2). Islets from MLDS-treated CD39TG mice showed minimal leukocytic infiltration, no upregulation of inflammatory cytokines, and preserved insulin content. Despite a relatively high level of apoptosis in the islets (not shown), CD39TG mice maintained near-normal glycemic control, which may reflect enhanced β-cell regenerative capacity due to increased pancreatic NTPDase activity (29,34). Adenosine signaling has recently been implicated in β-cell–specific regeneration. In a zebrafish model, the nonselective agonist NECA did not protect against β-cell death but promoted β-cell regeneration by increasing proliferation through A2AR-dependent mechanisms (29). Further, NECA treatment did not significantly increase the number of β-cells in normal development but reduced BGL by 30% and increased β-cell mass eightfold in mice treated with 150 mg/kg streptozotocin for 2 days (29).

Ectonucleotidase expression has been defined within the mouse, rat, and human pancreas (35–37). NTPDase1/CD39 is localized to blood vessels and acinar tissue, NTPDase2 to capillaries and connective tissue surrounding islets and acini, and NTPDase3 exclusively to islet cells. NTPDase8 was not detected. CD73 completes the enzymatic cascade for extracellular adenosine generation (38) and has been detected on rat, but not human or mouse, islets (35). CD73 expression is, however, well documented both on leukocytes (39,40) and the vasculature (41). This is similar to rat liver, where CD73 and CD39, and other NTPDases, are expressed by different but adjacent cells in distinct compartments and differentially regulate adenosine generation and signaling (38). Adenosine is a potent systemic anti-inflammatory molecule and inhibits the proliferation of CD4+ T cells in a dose-dependent fashion through the A2AR (26). The A2AR is expressed on regulatory T cells (42) together with CD39 (26) and CD73 (43). Further, A2AR activation on regulatory T cells has been shown to mitigate renal IRI through increased expression of the membrane protein programmed death-1 (42).

The adenosine receptor repertoire has previously been defined at the transcript level within the whole pancreas of CD-1 mice (19). All four adenosine receptors were expressed at comparable levels at baseline, with increased A1R expression after MLDS treatment (19). We analyzed purified islets, thus eliminating the potentially confounding contribution of exocrine tissue in the whole

FIG. 4. Expression of adenosine receptors in isolated islets. RNA from islets isolated at day 10 from untreated (UT) or MLDS-treated (MLDS) mice was analyzed by qRT-PCR for relative expression of A1R (A), A2AR (B), and A2BR (C). Data are expressed as means of four independent experiments ± SEM. *P < 0.05. ns, not significant.
The A3R was not detected and was not investigated further. Although the A1R was expressed on islets, the effect of pharmacological inhibition or activation of A1R on MLDS-induced diabetes was minimal, suggesting a redundant role in this model. This is in contrast to data demonstrating a modest protective effect of A1R activation in the MLDS model (19). However, the effect of the nonspecific agonist NECA in that study was significantly greater than the specific A1R agonist CCPA, suggesting that A1R was not the predominant receptor involved in the protection observed (19).

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A2AR transcripts were detected in islets under basal conditions, with little change after MLDS. The A2AR has well-defined effects on T-cell proliferation (26). In keeping with this, deletion of the A2AR on circulating cells conferred heightened susceptibility to MLDS-induced diabetes (Fig. 5). Additionally, tissue-specific deletion of A2AR increased susceptibility (Fig. 5), implicating a previously unrecognized role for A2AR signaling on the tissues in this model of diabetes. In mice overexpressing CD39, the role of the A2AR on circulating cells was redundant as CD39TG/A2ARKO chimeric mice were fully protected (Fig. 3E). It is possible that the level of adenosine generated is so high that it now signals via different adenosine receptors on T cells or indeed via a different mechanism involving other cell types.

The A2BR is recognized as a low-affinity adenosine receptor with activation occurring only in pathological conditions when the adenosine concentration is substantially increased (44). Expression of A2BR in islets was upregulated after MLDS treatment, and antagonism of A2BR hastened the onset and overall incidence of diabetes (Fig. 5). We speculate that the release of ATP secondary to cellular injury parallels cellular infiltration. CD39 expressed on tissues, together with CD73 expressed on intrasial not capillaries or infiltrating cells, sequentially converts ATP to adenosine, which activates A2BR to limit islet infiltration and destruction. The greater capacity of CD39TG mice to generate adenosine, and higher basal expression of the A2BR in these mice, putatively provides protection against early MLDS-induced injury.

The mechanism underpinning the protective effect of CD39TG expression includes signaling through both the A2AR and A2BR. Blockade or deletion of either receptor mitigated the protective effect of CD39 overexpression. Involvement of more than one adenosine receptor parallels the effects of adenosine in renal IRI, where A2AR signaling predominates on circulating CD4+ T cells (45) and macrophages (46), and A2BR signaling within the renal parenchyma is also important (47). It is intriguing that the treatment of A2ARKO/CD39TG mice with an A2BR antagonist did not further exacerbate the diabetic response. Recent speculation surrounds the requirement for cooperation between the A2AR and the A2BR for each receptor to be fully functional (48), which may account for the lack of exaggerated response in the A2ARKO/CD39TG mice with A2BRi.

Islets that survive the transplant process and recurrent autoimmune response then face destruction by the alloimmune response. A2AR agonists (49) and adenosine (50) prevent the early loss of islets posttransplantation, and adenosine, together with low-dose immunosuppression, prevents islet allograft rejection (50). The overexpression of CD39 is protective in this T cell-mediated model of diabetes by signaling through A2AR and A2BR, which combined with its anti-IBMIR (11,13) and putative immunosuppressive effects, makes CD39 overexpression an attractive strategy for prolonging islet survival after intraportal transplantation.

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J.S.J.C. performed most of the experiments and wrote the manuscript. J.L.M. assisted in islet isolation and provided intellectual input. H.E.T. and A.J.F.d.A. provided intellectual input and reviewed the manuscript. S.F., L.E., and L.M.-S. performed pancreatic perfusion and removal. P.H. scored the insulins in the islets. S.C.R. provided intellectual input and reviewed the manuscript. J.F.C. provided the A2ARKO mice. P.J.C. provided intellectual input, wrote and reviewed the manuscript, and supervised experiments. K.M.D. conceived experimental plans, wrote and reviewed the manuscript, and supervised experiments. K.M.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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