Adenosine receptor activation ameliorates type 1 diabetes

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ABSTRACT **Growing evidence indicates that adenosine receptors could be promising therapeutic targets in autoimmune diseases. Here we studied the role of adenosine receptors in controlling the course of type 1 diabetes. Diabetes in CD-1 mice was induced by multiple-low-dose-streptozotocin (MLDS) treatment and in nonobese diabetic (NOD) mice by cyclophosphamide injection. The nonselective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA) prevented diabetes development in both MLDS-challenged mice and in cyclophosphamide-treated NOD mice. The effect of NECA was reversed by the selective A_{2B} receptor antagonist N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS 1754). The selective A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) and A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA) were less efficacious in ameliorating the course of diabetes. NECA inhibited diabetes in A_{2A} receptor KO mice and the selective A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethyl-amino-5′-N-ethyl-carboxamidoadenosine (CGS21680) had no effect in normal mice, indicating a lack of role of A_{2A} receptors. NECA failed to prevent cytokine-induced β-cell death *in vitro*, but NECA strongly suppressed expression of the proinflammatory cytokines TNF-α, MIP-1α, IL-12, and IFN-γ in pancreata, endotoxin, or anti-CD3-stimulated splenic cells, and T helper 1 lymphocytes, indicating that the beneficial effect of NECA was due to immunomodulation. These results demonstrate that adenosine receptor ligands are potential candidates for the treatment of type 1 diabetes.— Németh, Z. H., Bleich, D., Csóka, B., Pacher, P., Mabley, J. G., Himer, L., Vizi, E. S., Deitch, E. A., Szabó, C., Cronstein, B. N., Haskó, G. Adenosine receptor activation ameliorates type 1 diabetes. *FASEB J.* 21, 2379–2388 (2007)**

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Type 1 diabetes results from an organ-specific immune-mediated attack on pancreatic β-cells. As many as 1.5 million patients have type 1 diabetes in the United States, with some additional 10,000 to 12,000 new cases diagnosed each year (1). At present, no method has been proven to prevent or cure type 1 diabetes, whereas available insulin replacement treatments have only limited success in controlling its devastating consequences. Current therapy is limited to treating the end-stage of the disease with insulin replacement therapy, rather than blocking the immune response to prevent destruction of pancreatic β-cells and preserve insulin secretion. Affected patients, therefore, remain at high risk for the development of long-term complications of the disease. Thus, it has been suggested that extensive research should be carried out in animal models to identify alternative therapeutic approaches to prevent and treat type 1 diabetes (2).

Adenosine is a potent endogenous autocrine antiinflammatory and immunosuppressive molecule that is released from cells into the extracellular space at sites of inflammation and tissue injury. Once released, adenosine binds to specific receptors on the cell surface, which are the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors (3, 4). All four adenosine receptor types have been found on immune cells, and both the innate and adaptive immune systems are subject to regulation by adenosine receptor activation (5). Adenosine receptor activation on monocytes, macrophages, and dendritic cells has been documented to decrease the release of a host of proinflammatory mediators including TNF-α (6–8), MIP-1α (9), IL-12 (10, 11), and nitric oxide (6, 12). These antiinflammatory effects of adenosine can be mediated by all four adenosine receptors, and the

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receptor involved in transducing the antiinflammatory signal depends on the cell type, organism, and model studied (5, 13–15).

Adenosine has been reported to decrease lymphocyte activation in a variety of assay systems including proliferation assays (16) as well as in mixed lymphocyte culture (17). Adenosine receptor stimulation has also been shown to suppress T lymphocyte IL-2 (18) and IFN-γ production (19). A_{2A} and/or A_{2B} receptors may be responsible for the suppressive effect of adenosine on IL-2 and IFN-γ production and lymphocyte proliferation (18–21).

Consistent with its antiinflammatory and immunosuppressive role, protective effects of adenosine receptor stimulation have been observed in various models of autoimmune disease, such as rheumatoid arthritis (9), multiple sclerosis (22), colitis (23), and hepatitis (13). However, the function of adenosine receptors in regulating autoimmune diabetes remains largely unknown. To determine the role of adenosine receptors in regulating type 1 diabetes, we examined the effect of adenosine receptor stimulation in animal models of this disease.

MATERIALS AND METHODS

Pharmacological agents

The nonselective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA), the selective A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA), A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethyl-amino-5′-N-ethyl-carboxamidoadenosine (CGS21680), and A_3 receptor agonist N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA), and the A_{2B} antagonist N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS 1754) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals

Diabetes was induced by multiple low-dose streptozotocin (MLDS) injection in male CD-1 mice that were purchased from Charles River Laboratories (Wilmington, MA, USA). The A_{2A} receptor knockout mice used in the present study (24, 25) were bred on a CD-1 background in a specific pathogen-free facility, using founder heterozygous male and female mice. Female WT and KO littermates of heterozygous parents were used exclusively in all studies. At weaning, a 0.5 cm tail sample was removed for the purpose of DNA collection for genotyping. Genotyping using RT-PCR was performed as described previously (24).

Female NOD mice were purchased at 6 wk of age and allowed to acclimatize to our animal facility for 2 wk prior to cyclophosphamide injection.

All mice were maintained in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" and the experiments were approved by the New Jersey Medical School Animal Care Committee.

Induction of diabetes

Male CD-1 mice were treated with streptozotocin (STZ) (40 mg/kg dissolved in citrate buffer, MLDS) or vehicle for MLDS (citrate buffer) intraperitoneally for 5 consecutive days to induce diabetes, as we have previously described (26). Blood glucose was monitored over the following 21 days using a one-touch blood glucose meter (Accu-Check Active, Roche, Indianapolis, IN, USA). Blood glucose was measured on days 0, 7, 14, and 21 from blood obtained from the tail vein. Hyperglycemia was defined as nonfasting blood glucose level higher than 200 mg/dl. The mice were treated simultaneously with STZ injection and throughout the following 21 days of the experiments, with various adenosine receptor agonists dissolved in 0.1% DMSO. Biopsies of pancreata were removed on day 21 after the last STZ injection for further biochemical analysis.

Diabetes in female NOD mice was accelerated by intraperitoneal injection of a single dose of cyclophosphamide (200 mg/kg). The mice were then treated daily with NECA or its vehicle (0.1% DMSO), both intraperitoneally. Blood glucose was measured on days 0, 7, 14, and 21 after the cyclophosphamide injection from blood obtained from the tail vein. Biopsies of the pancreata were removed on day 21 for histological analysis.

Determination of pancreas insulin content and cytokine levels

The pancreas biopsy was weighed before being placed into 6 ml of acid ethanol and homogenized (26). The pancreas was incubated for 72 h at 4°C before being centrifuged, and the insulin content of the supernatant was determined using a commercially available ELISA kit (ALPCO, Salem, NH, USA). Pancreas insulin content was expressed as nanograms of insulin per milligram of protein. A second sample of pancreas was removed and snap-frozen in liquid nitrogen; the sample was then homogenized in 700 μl of a TRIS-HCl buffer containing protease inhibitors (26). The samples were centrifuged for 30 min, and the supernatant was removed and frozen at 80°C until assay. Cytokine levels were determined using commercially available kits (R&D Systems, Minneapolis, MN, USA).

Histological studies

Pancreata were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm, mounted on slides, and stained with hematoxylin and eosin (26). Coded slides were read by light microscopy. Islet inflammation (insulitis) was graded 0 to 2, according to the extent of islet infiltration by leukocytes: 0 = none, 1 = only peri-islet leukocytes, 2 = intraislet leukocytes. Three sections from each pancreas were examined. A mean insulitis score was calculated by dividing the sum of the insulitis score for individual islets by the number of islets examined. The person scoring the sections was blinded as to the experimental group for each mouse.

Determination of adenosine receptor expression in pancreas

RNA was isolated from the whole pancreata of mice using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA). RNA (5 μg) was transcribed in a 20 μl reaction containing 10.7 μl of RNA (5 μg), 2 μl of 10× PCR buffer, 2 μl of 10 mM dNTP mix, 2 μl of 25 mM $MgCl_2$, 0.5 μl of 50 mM oligo d(T), and 0.3 μl of reverse transcriptase (Roche Molecular Systems). The reaction mix was incubated at 42°C for 15 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated at 99°C for 5 min. cDNA generated by the reverse transcription procedure was used as template in subsequent real-time PCR reactions. The real-

time amplification and analysis were conducted using a LightCycler 2.0 instrument (Roche Applied Science) with software version 4.0. All reactions were performed with the LightCycler FastStart DNA master SYBR green I kit (Roche Applied Science) by using 10 μl of total reaction volume in each capillary. For relative quantification of adenosine receptor or 18S messenger expression, 2 μl of cDNA was added before capillaries were capped, centrifuged, and placed in the LightCycler sample carousel. Amplification conditions consisted of an initial preincubation at 95°C for 10 min (FastStart *Taq*DNA polymerase activation), followed by amplification of the target DNA for 45 cycles (95°C for 8 s, 58°C for 10 s, and extension at 72°C for 20 s) according to the manufacturer's protocol. Melting curve analysis was performed immediately after amplification at a linear temperature transition rate of 0.1°C/s from 65 to 95°C with continuous fluorescence acquisition.

The DNA amplification results were systematically normalized to expression levels of 18S in order to correct variations in nucleic acid quality and quantity. The relative concentration of each sample was calculated by comparing its crossing point (Cp) with the corresponding 18S internal control curve, and relative mRNA expression levels were calculated as a ratio between the expression of adenosine receptor and the reference (18S) gene mRNA. Sequences of all primers used in this study are listed in **Table 1**.

Cell viability studies with β-cell lines

RIN-5F cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in RPMI 1640 medium containing 5.5 mM glucose, penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum. The MIN6 cell line was a kind gift from Dr. Jun-ichi Miyazaki (Kumamoto University, Japan) (27) and cultured in DME medium containing 4.5 g/L glucose, glutamine, and penicillin (50 U/ml), streptomycin (50 μg/ml), as well as 10% fetal bovine serum. RIN-5F and MIN6 cell viability was determined by the reduction of yellow MTT into a purple formazan product by mitochondrial dehydrogenases of metabolically active cells. Confluent cells in 96-well plates were treated with a combination of IL-1β (0.02 μg/ml), TNF-α (0.2 μg/ml), and IFN-γ (0.4 μg/ml) in the presence or absence of adenosine receptor agonists for 48 h. Following this treatment period the media was removed, and 200 μl MTT (1 mg/ml) was added. After 1 h the MTT solution was carefully removed, and the cells were solubilized in 100 μl of DMSO.

TABLE 1. *Primer sequences used to detect adenosine receptors in pancreata of CD-1 mice*

Oligo	Primer sequences
18S sense	5′-GTA ACC CGT TGA ACC CCA TT-3′
18S anti-sense	5′-CCA TCC AAT CGG TAG TAG CG-3′
A_1 receptor sense	5′-GTG ATT TGG GCT GTG AAG GT-3′
A_1 receptor anti-sense	5′-CAA GGG AGA GAA TCC AGC AG-3′
A_{2A} receptor sense	5′-AGC AGT TGA TGA TGT GCA GG-3′
A_{2A} receptor anti-sense	5′-CAC GCA GAG TTC CAT CTT CA-3′
A_{2B} receptor sense	5′-TGG CGC TGG AGC TGG TTA -3′
A_{2B} receptor anti-sense	5′-GCA AAG GGG ATG GCG AAG-3′
A_3 receptor sense	5′-GAC TGG CTT CAG AGA GAC GC-3′
A_3 receptor anti-sense	5′-AGG GTT CAT CAT GGA GTT CG-3′

Solubilized cells were then transferred to an ELISA plate, and absorbance was measured at 550 nm with a 620 nm reference filter. The absorbances obtained from cytokine-treated cells were expressed as a percentage of absorbances obtained from cytokine-untreated cells.

Isolation and treatment of mouse splenocytes

CD-1 mice were killed by cervical dislocation under anesthesia, and the spleens were removed and dissociated in RPMI medium supplemented with 50 mM HEPES, 10% fetal bovine serum, and penicillin/streptomycin. The cell suspension was passed through a 70 μm strainer, and cells were collected by centrifugation at 300 *g* for 10 min. Erythrocytes were lysed by incubating the cells in red blood cell lysis buffer (150 mM ammonium-chloride, 10 mM potassium-bicarbonate, and 1 mM EDTA) at room temperature for 2 min. The splenocytes were washed 3 times and resuspended in complete RPMI medium at a density of 3×10^6 cells per ml. The splenocytes were stimulated with 3 μg/ml plate-bound anti-CD3 and 1 μg/ml plate-bound anti-CD28, or 10 μg/ml LPS in the presence or absence of 100 nM NECA for 24 h. Cytokine levels in the supernatants obtained at the end of the incubation period were determined using commercially available ELISA kits (R&D Systems).

Preparation and treatment of T helper (Th1)1 and Th2 hybridomas

Female BALB/c mice (8–12 wk old; National Institute of Oncology, Budapest, Hungary) were immunized by injecting them with 200 μg of hapten-carrier antigen FITC-KLH (Keyhole limpet hemocyanin from Megathura crenulata conjugated with fluorescein-5-isothyocianate, Sigma) emulsified 1:1 in complete Freund's adjuvant (CFA) containing 1 mg/ml *Mycobacterium tuberculosis* (H37RA, heat-killed and dried, Sigma) into each hind footpad (50–50 μl), tail base (100 μl), and intraperitoneally (800 μl).

At 11 days after immunization, the inguinal and popliteal lymph nodes were harvested and erythrocytes were removed with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA disodium salt, pH 7.2). Lymph node cells (5×10^6/ml) were differentiated into Th1 cells by activation with 25 μg/ml FITC-KLH and 1 μg/ml FITC-LPS in the presence of 0.2 μg/ml IFNγ and 5 μg/ml anti-IL4 or Th2 cells by incubation with FITC-KLH and FITC-LPS in the presence of 0.2 μg/ml IL-4 and 5 μg/ml anti-IFNγ. All monoclonal antibodies and cytokines were purchased from R& D Systems. After 3 days, $CD8^+$ and $Fc\gamma RII/III^+$ cells were removed from the mixture of cells by panning: the cells were incubated with rat anti-CD8 (R&D Systems) and rat anti-Fcγ R (R&D Systems), and then placed onto goat antirat antibody-coated Petri dish. Unbound $CD4^+$ cells were collected by carefully removing them from the dish.

$BW\alpha\beta^-$ thymoma cells (ATCC) were added to $CD4^+$ cells at a ratio of 1:4, and incubated with fusion agent polyethylene-glycol (PEG, Hybri-Max ready-to-use solution, Sigma) for a few minutes. Agglutinated cells were then subjected to limiting dilution by carefully adding GKN buffer (11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na_2HPO4, and 5.5 mM $NaH_2PO_{4*}2H_2O$, pH 7.4). Diluted cells were cultured with feeder thymocytes in 96-well plates in HAT (hypoxanthine-aminopterin-thymidine containing RPMI 1640 medium, Sigma) selection medium. After 10–12 days, when both the nonfused cells and feeder thymocytes died, surviving fused hybridoma cells were placed in normal RPMI 1640 medium, which contained 10% fetal calf serum. Clones that were positive for both CD3 and CD4 as assessed using flow

cytometry were used in later experiments. Hybridomas, which produced primarily IFN-γ were designated as Th1 clones and hybridomas producing high concentrations of IL-4 were designated as Th2 clones.

To determine the effect of NECA on cytokine production by Th1 or Th2 cells, 5×10^4 cells/well were placed in 96-well plates in L-glutamine-containing RPMI 1640 medium (Life Technologies, Inc., San Diego, CA, USA) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.). Cells were then treated with increasing concentrations of NECA (0.001–10 μM) followed by stimulation with 5 μg/ml anti-CD3 and 2.5×10^4 cells/well LK35 B-lymphoma cells (ATCC) 30 min later. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 16–18 h. Thereafter, the cells were centrifuged and supernatants collected for measuring IL-4 or IFN-γ levels using ELISA (DuoSet, R&D Systems).

Statistical analysis

Values in the figures are expressed as mean ± SEM of n observations. Statistical analysis of the data was performed by Student's *t* test or one-way analysis of variance followed by Dunnett's test, as appropriate.

RESULTS

Adenosine receptor stimulation attenuates MLDS-induced hyperglycemia

To investigate the effect of adenosine receptor stimulation on the course of diabetes, we first tested the effect of NECA, a stable adenosine analog and nonselective receptor agonist in the MLDS model of diabetes in male CD-1 mice. We used NECA to stimulate adenosine receptors instead of adenosine itself, because adenosine is subject to rapid metabolism and uptake, making data interpretation with this agent difficult (5). Vehicle-treated MLDS-induced animals developed progressive hyperglycemia (**Fig. 1*A***). MLDS-induced animals treated with NECA at doses as low as 0.01 and 0.03 mg/kg showed significant reductions in the severity of hypergycemia (Fig. 1*A*). By the end of the 3-week observation period, all vehicle-treated mice became diabetic (100%, n=9). NECA treatment reduced the incidence of diabetes to 70% (n=10, P=0.2105, Fisher's Exact Test) and 30% (n=10, P=0.0031, Fisher's Exact Test) at 0.01 mg/kg and 0.03 mg/kg, respectively. As shown in Fig. 1*B*, NECA prevented the MLDS-induced decrease in pancreatic insulin content, indicating a protective effect against the MLDS-induced β-cell loss. Finally, treatment of MLDS-unchallenged mice with NECA for 26 days did not influence blood glucose levels when compared to mice receiving the vehicle for MLDS (data not shown).

We next conducted studies to survey the role of the various adenosine receptors in regulating the development of diabetes. The A_1 receptor agonist CCPA at 0.1 mg/kg decreased MLDS-induced hyperglycemia; however, its suppressive effect was inferior to that one caused by 0.03 mg/kg NECA (**Fig. 2*A***), suggesting that the A_1 receptor is not the primary adenosine receptor in regulating MLD-induced diabetes. Due to the toxicity of CCPA, the experiment was terminated at the end of week 2.

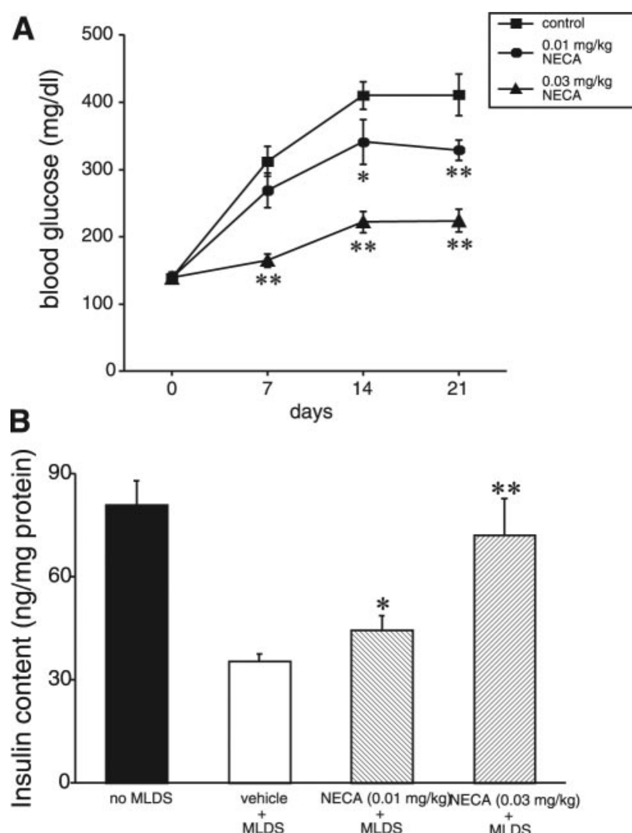


Figure 1. *A)* Daily treatment with NECA (0.01 or 0.03 mg/kg, i.p.) decreases multiple-low-dose-streptozotocin (MLDS)-induced hyperglycemia. Streptozotocin was injected i.p. for 5 consecutive days to induce diabetes. NECA treatment commenced on the first day of streptozotocin injections. Blood glucose was measured on days 0, 7, 14, and 21 after the last streptozotocin injection from blood obtained from the tail vein. *B)* NECA treatment dose-dependently attenuates the inhibitory effect of MLDS on pancreatic insulin content. Pancreas biopsies were taken on day 21, and insulin levels were determined using ELISA. Results are mean ± SEM for n = 9–10 mice/group. Results are representative of two separate experiments. $^*P < 0.05$; $^{**}P < 0.01$ *vs.* vehicle

The A_{2A} receptor agonist CGS21680 (0.1 mg/kg) failed to affect the development of hyperglycemia (Fig. 2*B*). In addition, NECA maintained its suppressive effect on the course of diabetes in A_{2A} receptor knockout mice (Fig. 2*C*). NECA-untreated MLDS-induced A_{2A} receptor knockout mice became hyperglycemic at the same rate as NECA-untreated MLDS-induced A_{2A} receptor wild-type mice. These results indicate that A_{2A} receptors are not involved in the regulation of diabetes.

Since no selective A_{2B} receptor agonist was available, we used the nonselective agonist NECA together with the selective A_{2B} receptor antagonist MRS 1754 to assess the role of the A_{2B} receptor. MRS 1754 (0.5 mg/kg) was injected twice daily with one injection 30 min before NECA treatment and the other 12 h later throughout the experiment. MRS 1754 reversed the suppressive effect of NECA on hyperglycemia, indicating that A_{2B} receptors mediate the effect of NECA (Fig. 2*D*).

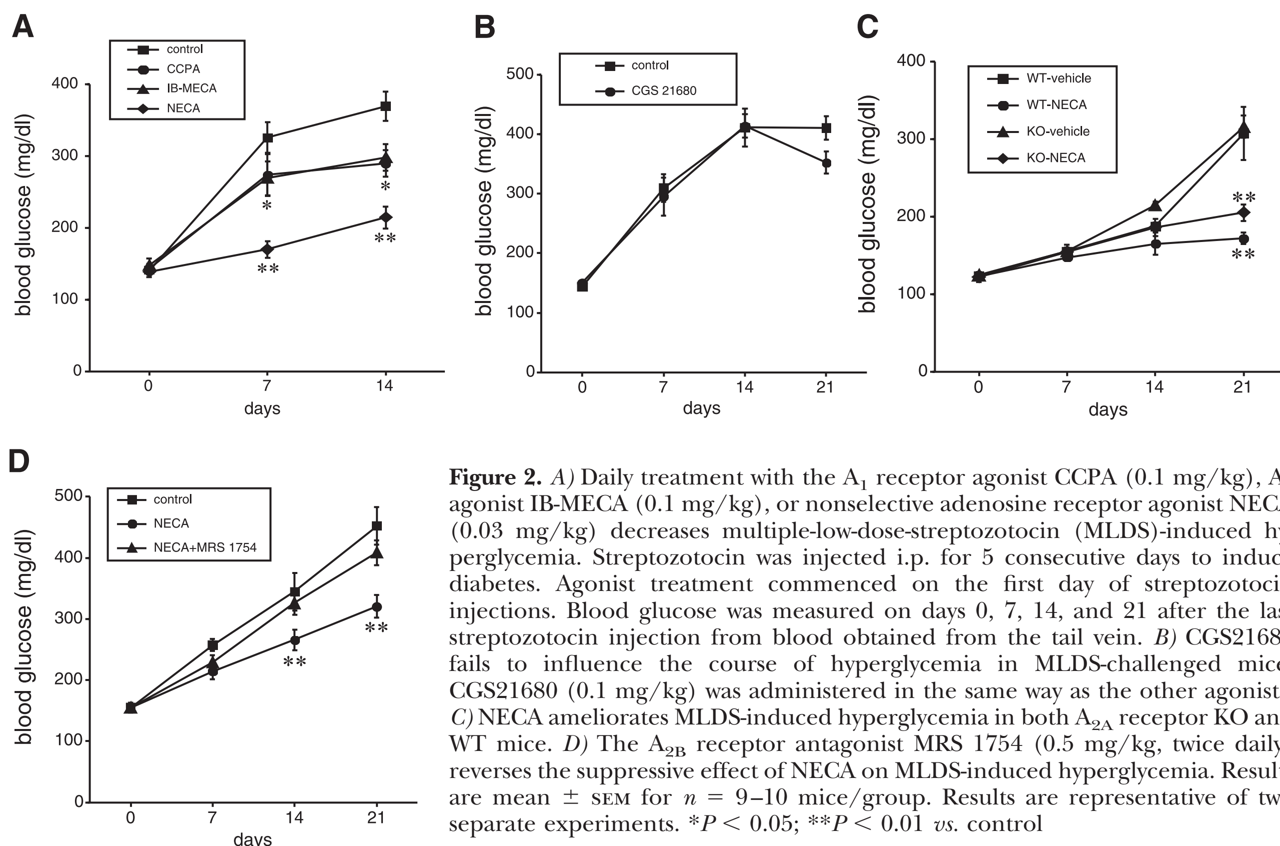


Figure 2. *A)* Daily treatment with the A_1 receptor agonist CCPA (0.1 mg/kg), A_3 agonist IB-MECA (0.1 mg/kg), or nonselective adenosine receptor agonist NECA (0.03 mg/kg) decreases multiple-low-dose-streptozotocin (MLDS)-induced hyperglycemia. Streptozotocin was injected i.p. for 5 consecutive days to induce diabetes. Agonist treatment commenced on the first day of streptozotocin injections. Blood glucose was measured on days 0, 7, 14, and 21 after the last streptozotocin injection from blood obtained from the tail vein. *B)* CGS21680 fails to influence the course of hyperglycemia in MLDS-challenged mice. CGS21680 (0.1 mg/kg) was administered in the same way as the other agonists. *C)* NECA ameliorates MLDS-induced hyperglycemia in both A_{2A} receptor KO and WT mice. *D)* The A_{2B} receptor antagonist MRS 1754 (0.5 mg/kg, twice daily) reverses the suppressive effect of NECA on MLDS-induced hyperglycemia. Results are mean ± SEM for n = 9–10 mice/group. Results are representative of two separate experiments. *$P < 0.05$; **$P < 0.01$ *vs.* control

Similar to CCPA, the A_3 receptor agonist IB-MECA at 0.1 mg/kg limited the extent of hyperglycemia; however, its effect was less pronounced than that of 0.03 mg/kg NECA (Fig. 2*A*). IB-MECA was toxic to the mice and the experiment was stopped at the end of week 2. Collectively, the fact that MRS 1754 prevented the effect of NECA suggests that A_{2B} receptors play a predominant role in regulating MLDS-induced diabetes.

Adenosine receptors are expressed in the pancreas

Figure 3 shows the results of real-time RT-PCR analysis on mRNA isolated from the pancreata of CD-1 mice. mRNA for all 4 adenosine receptors was expressed at comparable levels in the pancreata of mice prior to the initiation of STZ treatment (Fig. 3*A*). In contrast, A_1 receptors were expressed at a higher level than the other receptors at the termination of the experiment 26 days after the first STZ injection (Fig. 3*B*).

NECA decreases the level of proinflammatory mediators in the pancreas of MLDS-treated mice

Inflammatory cytokines play a central role in orchestrating the function of the various immune cell types during development of the diabetic process (28). To study the pancreatic cytokine milieu, we measured pancreatic levels of TNF-α, MIP-1α, IL-12, and IFN-γ. Pancreatic contents of all four proinflammatory cytokines were significantly lower in mice treated with NECA than in vehicle-treated mice (**Table 2**). This observation suggests that NECA alleviates the course of diabetes by reducing the levels of proinflammatory cytokines.

NECA attenuates cyclophosphamide-induced diabetes in NOD mice

We next tested the effect of NECA in the cyclophosphamide-induced diabetes model using female NOD mice after optimizing the dose of cyclophosphamide in this model (data not shown). Here, NOD mice were given a single injection of cyclophosphamide (200 mg/kg), followed by daily injections of NECA (0.01 mg/kg) or vehicle. As seen in **Fig. 4*A***, even this very low dose of NECA almost completely attenuated hyperglycemia in this immune-mediated diabetic model. In addition, insulitis scores of NECA-treated animals were significantly lower than those of vehicle-treated mice (Fig. 4*B*, 0.75±0.13 for NECA-treated mice *vs.* 1.25±0.17 for vehicle-treated mice; $P<0.03$, $n=8$ in both groups). Thus, adenosine receptor occupancy by NECA protects the pancreas from immune-mediated β-cell destruction in the two mouse models tested.

Adenosine receptor stimulation fails to prevent the cytokine-induced death of β-cells

Because our data demonstrated increased insulin content in pancreata of NECA-treated mice, we next tested the hypothesis that adenosine receptor stimulation

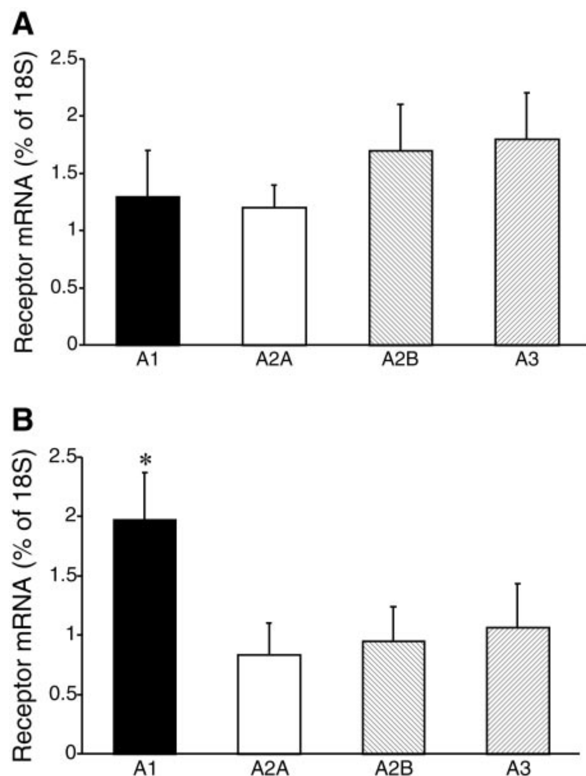


Figure 3. *A)* mRNA expression of adenosine receptors in pancreata of naive CD-1 mice. *B)* mRNA expression of adenosine receptors in pancreata of multiple-low-dose-streptozotocin (MLDS)-treated mice. Streptozotocin was injected intraperitoneally for 5 consecutive days to induce diabetes. Pancreas biopsies were taken on day 21 after the last streptozotocin injection, and mRNA levels of adenosine receptors and 18S were determined using real-time PCR. Data are expressed as percentage of expression of the housekeeping gene 18S. Results are mean ± SEM for $n = 4$ mice/group. $^*P < 0.05$

inhibits diabetes development by preventing β-cell death via a direct effect on β-cells. The effect of adenosine receptor activation on β-cell death *in vitro* was examined by exposing rat RIN-5F or mouse MIN6 β-cells to a combination of TNF-α, IL-1β, and IFN-γ. Although these cytokines induced a significant decrease in cell viability in both cell lines, this cell death was not reversed by 1 μM NECA (**Fig. 5*A, B***) or any of the selective adenosine receptor agonists that were tested previously *in vivo* (data not shown). These results argue against a direct β-cell protective effect of adenosine receptor stimulation.

NECA suppresses the production of proinflammatory cytokines by T cell receptor- or Toll-like receptor 4-stimulated spleen cells

Because we found decreased levels of proinflammatory cytokines in the pancreata of NECA-treated mice, we next evaluated the potential cellular targets of NECA in attenuating cytokine production. NECA (0.1 μM) attenuated the production of TNF-α, MIP-1α, and IFN-γ by cells activated via the T cell receptor (**Fig. 6*A–C***). NECA also inhibited TNF-α, MIP-1α, and IFN-γ production by Toll-like receptor 4-activated spleen cells (Fig. 6*A–C*). We conclude that NECA decreases cytokine production both by T cells and antigen-presenting cells.

NECA decreases IFN-γ and IL-4 production by established Th1 and Th2 cell clones, respectively

It is generally thought that a Th1 response is associated with disease progression in diabetes and that Th2 responses can provide protection against the development of diabetes. We examined the effect of NECA on cytokine production by an *in vitro* established KLH-specific Th1 or Th2 cell clone. Stimulating the Th1 or Th2 clone with KLH (antigen), anti-CD3, and LK35 B-lymphoma cells (antigen-presenting cells) resulted in a predominant IFN-γ or IL-4 response, respectively. Stimulated Th1 cells produced 416.25 ± 38.76 pg/ml IFN-γ and 129.84 ± 14.03 pg/ml IL-4 (n=6), whereas nonstimulated cells produced less than 10 pg/ml of both cytokines. Stimulated Th2 cells produced 193 ± 4.98 pg/ml IL-4 and 18.46 ± 1 pg/ml IFN-γ (n=6). NECA strongly decreased the production of IFN-γ by the Th1 clone with a half-maximal inhibitory concentration of ~10 nM (**Fig. 7*A***). NECA, however, was much less potent in decreasing IL-4 production by the Th2 clone, where the half-maximal inhibitory concentration was in the μM range (Fig. 7*B*). Finally, NECA did not have a toxic effect on either clone as determined using the MTT assay (data not shown).

DISCUSSION

In this study, we found that the general adenosine receptor agonist NECA ameliorated the course of diabetes and protected the pancreas from immune-medi-

TABLE 2. *NECA attenuates the level of proinflammatory mediators in the pancreas of MLDS-treated mice*

Cytokine	Vehicle	NECA (0.01 mg/kg)	NECA (0.03 mg/kg)
TNF-α	1.45 ± 0.33	0.74 ± 0.12**	0.62 ± 0.03**
MIP-1α	2.87 ± 0.91	2.01 ± 0.75	0.47 ± 0.02**
IL-12 (p40)	20.1 ± 6.88	8.81 ± 1.02	5.27 ± 0.05*
IFN-γ	0.37 ± 0.13	0.06 ± 0.009*	0.09 ± 0.02*

Cytokine levels of proinflammatory cytokines in pancreata of MLDS-induced mice treated with either vehicle or NECA. The pancreas was taken 21 days after the last streptozotocin injection. Values are pg/mg protein. Results are means ± SE (n=9–10). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* vehicle-treated mice.

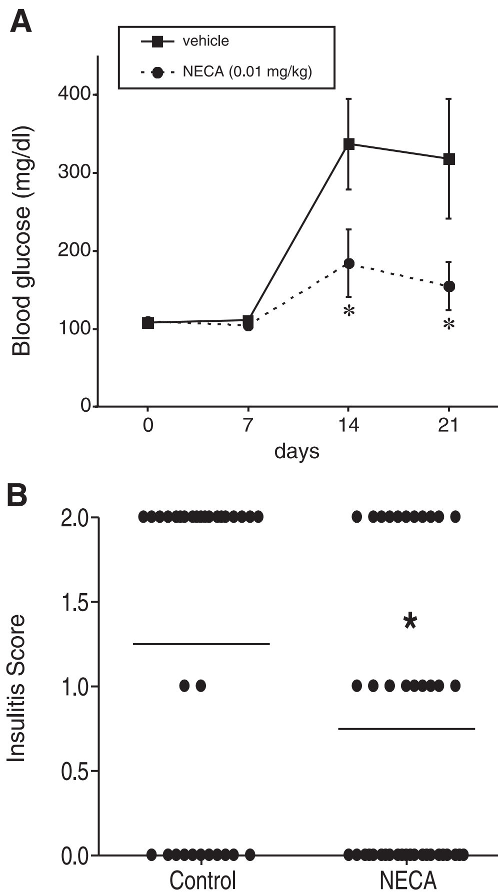


Figure 4. *A)* Daily administration of NECA (0.01 mg/kg, i.p.) ameliorates the severity of disease in female NOD mice in which development of diabetes was accelerated by injection with 200 mg/kg cyclophosphamide at 8 wk of age. NECA injections began on the day of cyclophosphamide treatment. The values on the *x*-axis indicate time elapsed following cyclophosphamide administration. Both the NECA- and vehicle-treated groups contained 8 mice and the experiment shown is representative of two similar experiments. $*P < 0.05$. *B)* Histological analysis of pancreata of NECA- or vehicle-treated cyclophosphamide-accelerated NOD mice (8 in both groups). Pancreas sections taken on day 21 after cyclophosphamide administration were stained with hematoxylin and eosin and slides were read by light microscopy. Islet inflammation (insulitis) was graded 0 to 2, according to the extent of islet infiltration by leukocytes: 0 = none, 1 = only peri-islet leukocytes, 2 = intraislet leukocytes. Three sections from each pancreas were examined. Each closed black circle represents an individual islet. A mean insulitis score was calculated by dividing the sum of the insulitis scores for individual islets by the number of islets examined. The number of islets counted was 32 and 45 for vehicle- and NECA-treated mice, respectively. $*P < 0.05$

ated β-cell destruction in two mouse models of type 1 diabetes.

By conducting detailed pharmacological analysis using the MLDS model, we determined that A_{2B} receptors are likely contributors to the protective effect of NECA against diabetes. This interpretation is suggested by the following findings. Firstly, the protective effect of NECA was reversed by the selective A_{2B} receptor antagonist MRS 1754. Secondly, NECA maintained its protective effect in A_{2A} receptor knockout mice, ruling out a protective role for A_{2A} receptors. Given that NECA is a nonselective adenosine receptor agonist and because the A_1 agonist CCPA or A_3 agonist IB-MECA also alleviated the course of diabetes, a role for additional adenosine receptors cannot be excluded.

The most likely mechanism of action of NECA in limiting β-cell death and the severity of diabetes is immune modulation. NECA attenuated the pancreatic levels of several proinflammatory mediators, which included TNF-α, MIP-1α, and IFN-γ. TNF-α is an important contributor to the immune/inflammatory events resulting in β-cell destruction in the pancreas. Transgenic expression of TNF-α by β-cells results in a progressively severe insulitis in nondiabetes-prone

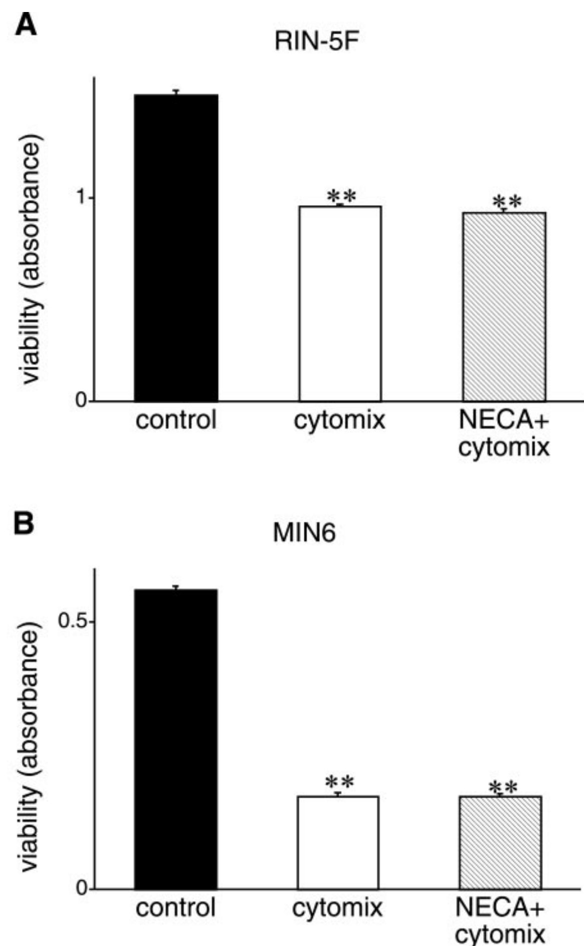


Figure 5. NECA (1 μM) fails to prevent cytokine-induced cell death of β-cells. Cell viability was determined by the MTT assay. RIN-5F (*A*) and MIN6 (*B*) cells in 96-well plates were treated with NECA and then 30 min later they were exposed to a combination of IL-1β (0.02 μg/ml), TNF-α (0.2 μg/ml), and IFN-γ (0.4 μg/ml) for 48 h. Following this treatment period the media was removed and 200 μl MTT (1 mg/ml) added. After 1 h the MTT solution was carefully removed and the cells solubilized in 100 μl of DMSO. Solubilized cells were then transferred to an ELISA plate and absorbance measured at 550 nm with a 620 nm reference filter. Decreased absorbances reflect a decrease in cell viability following cytokine administration. Results are mean ± SEM for $n = 6$ wells/group from three separate experiments. $**P < 0.01$.

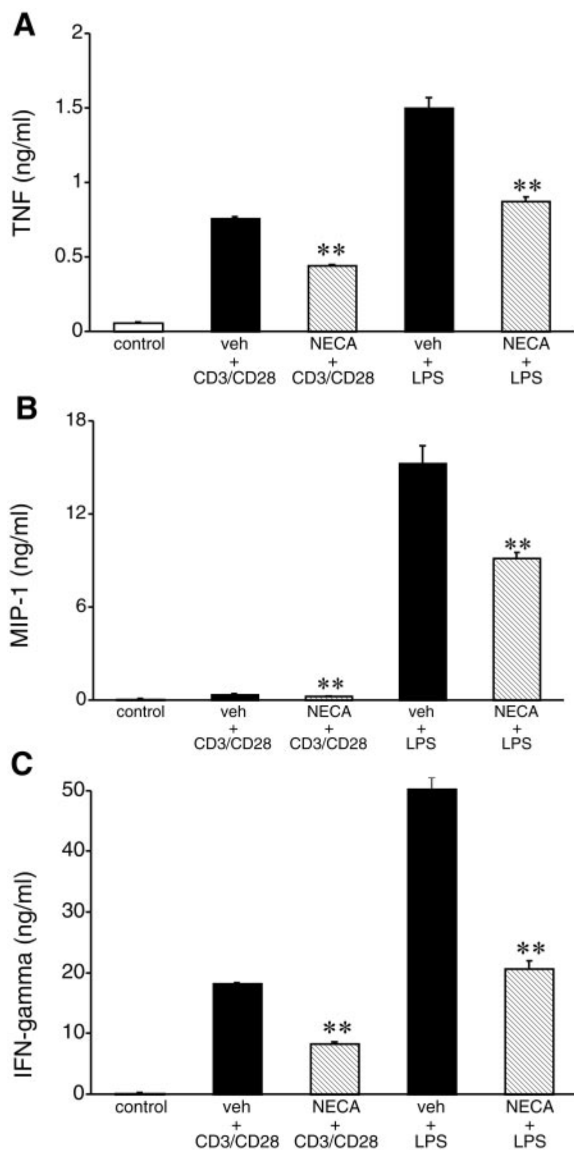


Figure 6. Effect of NECA on TNF-α (*A*), MIP1-α (*B*), and IFN-γ (*C*) production by CD-1 mouse spleen cells. Splenocytes were stimulated with 3 μg/ml plate-bound anti-CD3 and 1 μg/ml plate-bound anti-CD28, or 10 μg/ml LPS (TLR4 agonist) in the presence or absence of 100 nM NECA for 24 h. Cytokine levels in the supernatants obtained at the end of the incubation period were determined using ELISA. Results are mean ± SEM for $n = 6$ wells/group from three separate experiments. $^{**}P < 0.01$ *vs.* vehicle

mice; however, this insulitis does not progress to overt diabetes (30). The fact that deficient expression of MIP-1α in NOD mice protects them from destructive insulitis and diabetes onset suggests that MIP-1α also contributes to the pathogenesis of type 1 diabetes in NOD mice (31). Although the role of IFN-γ is controversial, immunodepletion of IFN-γ prevented the development of diabetes in cyclophosphamide-treated NOD mice (32). These controversial observations reflect the fact that, due to the redundant functions of many of these cytokines, it is unlikely that neutralization of any of these cytokines alone is sufficient to block the autoimmune response (33). Our *in vitro* data confirm that NECA can decrease cytokine production in response to various activating stimuli and by various cellular sources, such as T cell receptor cross-linking on T cells, antigen-specific stimulation of Th1 cells, or Toll-like receptor stimulation on antigen-presenting cells. The facts that NECA decreased the expression of several proinflammatory cytokines *in vivo* and the observation that NECA can inhibit the secretion of proinflammatory cytokines by multiple stimuli and cell types may explain the efficacy of this agent in preventing diabetes. Although A_{2A} receptors are generally viewed as the primary adenosine receptors in inhibiting inflammatory processes, evidence indicates that A_{2B} receptors can also dampen inflammatory cell activation. For example, A_{2B} receptor activation was shown to diminish IFN-γ-induced major histocompatibility (MHC) complex II expression on and nitric oxide production by murine bone marrow macrophages (12). In addition, Kreckler and coworkers recently demonstrated that A_{2B} receptor stimulation suppresses TNF-α production by LPS-stimulated macrophages (7). Recent studies using A_{2B} knockout mice have confirmed the antiinflammatory role of A_{2B} receptors: A_{2B} recep-

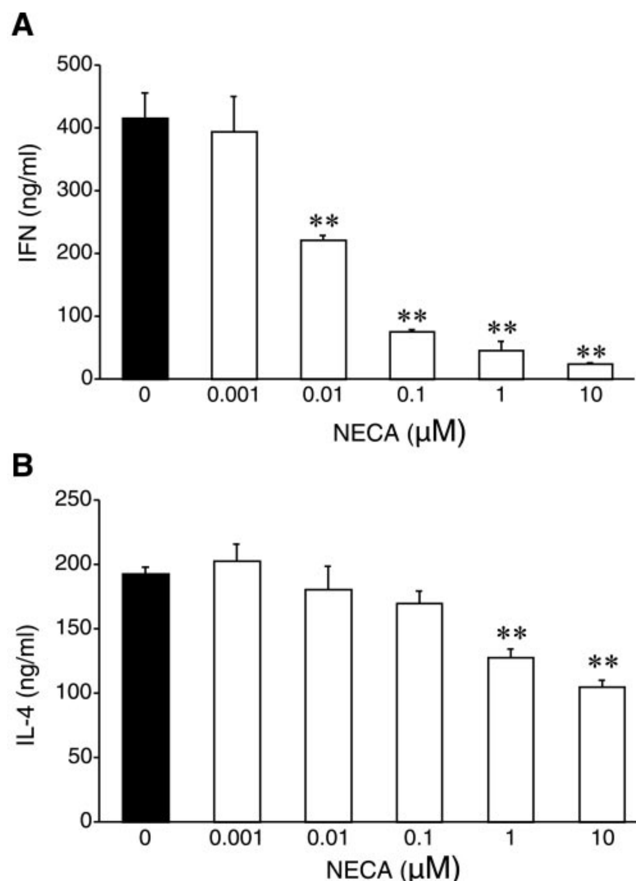


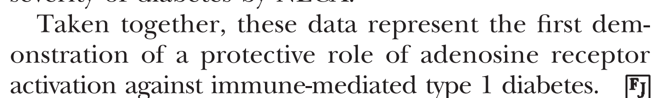
Figure 7. Effect of NECA on IFN-γ production by a stimulated Th1 hybridoma cell line (*A*) and IL-4 production by a stimulated Th2 hybridoma cell line (*B*). Cells were stimulated with KLH (antigen), anti-CD3, and LK35 B-lymphoma cells (antigen-presenting cells) for 16–18 h in the presence or absence of NECA. Cytokine levels were determined from the supernatants using ELISA. Results are mean ± SEM for $n = 6$ wells/group from three separate experiments. $^{**}P < 0.01$.

tor deficiency was linked to increased proinflammatory cytokine production and adhesion molecule expression (15). It is also noteworthy that, although NECA decreased IL-4 production by Th2 cells, a potentially detrimental effect, the potency of NECA in decreasing Th2 activation was markedly lower than in decreasing Th1 activation. This observation indicates that NECA affects Th1 and Th2 cell activation via different receptors, which results in a preferential inhibition of Th1 cell activation. Nevertheless, we should interpret these data with caution, because it is possible that the adenosine receptor profile in these clones may have changed differently during long-term culture.

It is less likely that NECA prevented the development of diabetes by directly protecting β-cells via adenosine receptor stimulation on these cells, because NECA failed to prevent cytokine-induced β-cell death *in vitro*. Adenosine receptors, nevertheless, are expressed on β-cells, because A_1 adenosine receptor activation was shown to inhibit insulin release from the rat pancreatic β-cell line INS-1 and rat islets (34). Although such an effect can potentially complicate the interpretation of our data, our results indicate that NECA did not alter insulin secretion, because NECA had no effect on blood glucose levels in nondiabetic animals.

Adenosine also plays an important role in regulating vascular reactivity in the islet. In one study, adenosine was found to increase vascular conductance and flow in nondiabetic and diabetic rats receiving pancreatic islet grafts (35), and in a recent study adenosine was shown to dilate arterioles supplying single isolated islets (36). Although it is unclear at this point whether the dilatory effect of adenosine was due to an action on endothelial adenosine receptors, this observation raises the possibility that NECA may have affected disease development through endothelial adenosine receptors. In addition, NECA via A_{2B} receptors has been documented to decrease vascular leak and inflammation (37, 38), effects that could have a role in the attenuation of the severity of diabetes by NECA.

Taken together, these data represent the first demonstration of a protective role of adenosine receptor activation against immune-mediated type 1 diabetes. FJ



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REFERENCES

1. Eiselein, L., Schwartz, H. J., and Rutledge, J. C. (2004) The challenge of type 1 diabetes mellitus. *Ilar. J.* **45,** 231–236
2. Kukreja, A., and Maclaren, N. K. (1999) Autoimmunity and diabetes. *J. Clin. Endocrinol. Metab.* **84,** 4371–4378
3. Ralevic, V., and Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50,** 413–492
4. Fredholm, B. B., AP, I. J., Jacobson, K. A., Klotz, K. N., and Linden, J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53,** 527–552
5. Hasko, G., and Cronstein, B. N. (2004) Adenosine: an endogenous regulator of innate immunity. *Trends. Immunol.* **25,** 33–39
6. Hasko, G., Szabo, C., Nemeth, Z. H., Kvetan, V., Pastores, S. M., and Vizi, E. S. (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J. Immunol.* **157,** 4634–4640
7. Kreckler, L. M., Wan, T. C., Ge, Z. D., and Auchampach, J. A. (2006) Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. *J. Pharmacol. Exp. Ther.* **317,** 172–180
8. Nemeth, Z. H., Lutz, C. S., Csoka, B., Deitch, E. A., Leibovich, S. J., Gause, W. C., Tone, M., Pacher, P., Vizi, E. S., and Hasko, G. (2005) Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. *J. Immunol.* **175,** 8260–8270
9. Szabo, C., Scott, G. S., Virag, L., Egnaczyk, G., Salzman, A. L., Shanley, T. P., and Hasko, G. (1998) Suppression of macrophage inflammatory protein (MIP)-1alpha production and collagen-induced arthritis by adenosine receptor agonists. *Br. J. Pharmacol.* **125,** 379–387
10. Hasko, G., Kuhel, D. G., Chen, J. F., Schwarzschild, M. A., Deitch, E. A., Mabley, J. G., Marton, A., and Szabo, C. (2000) Adenosine inhibits IL-12 and TNF-α production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J.* **14,** 2065–2074
11. Khoa, N. D., Montesinos, M. C., Reiss, A. B., Delano, D., Awadallah, N., and Cronstein, B. N. (2001) Inflammatory cytokines regulate function and expression of adenosine A(2A) receptors in human monocytic THP-1 cells. *J. Immunol.* **167,** 4026–4032
12. Xaus, J., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., and Celada, A. (1999) IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J. Immunol.* **162,** 3607–3614
13. Ohta, A., and Sitkovsky, M. (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414,** 916–920
14. Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., Ohta, A., and Thiel, M. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu. Rev. Immunol.* **22,** 657–682
15. Yang, D., Zhang, Y., Nguyen, H. G., Koupenova, M., Chauhan, A. K., Makitalo, M., Jones, M. R., St Hilaire, C., Seldin, D. C., Toselli, P., Lamperti, E., Schreiber, B. M., Gavras, H., Wagner, D. D., and Ravid, K. (2006) The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *J. Clin. Invest* **116,** 1913–1923
16. Sandberg, G. (1983) Regulation of thymocyte proliferation by endogenous adenosine and adenosine deaminase. *Int. J. Immunopharmacol.* **5,** 259–265
17. Nishida, Y., Kamatani, N., Morito, T., and Miyamoto, T. (1984) Differential inhibition of lymphocyte function by 2-chloroadenosine. *Int. J. Immunopharmacol.* **6,** 335–338
18. Koshiba, M., Kojima, H., Huang, S., Apasov, S., and Sitkovsky, M. V. (1997) Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells. *J. Biol. Chem.* **272,** 25881–25889
19. Lappas, C. M., Rieger, J. M., and Linden, J. (2005) A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. *J. Immunol.* **174,** 1073–1080
20. Gessi, S., Varani, K., Merighi, S., Ongini, E., and Borea, P. A. (2000) A(2A) adenosine receptors in human peripheral blood cells. *Br. J. Pharmacol.* **129,** 2–11
21. Mirabet, M., Herrera, C., Cordero, O. J., Mallol, J., Lluis, C., and Franco, R. (1999) Expression of A2B adenosine receptors in human lymphocytes: their role in T cell activation. *J. Cell Sci.* **112,** 491–502
22. Tsutsui, S., Schnermann, J., Noorbakhsh, F., Henry, S., Yong, V. W., Winston, B. W., Warren, K., and Power, C. (2004) A1 adenosine receptor upregulation and activation attenuates neu-

roinflammation and demyelination in a model of multiple sclerosis. *J. Neurosci.* **24,** 1521–1529
23. Odashima, M., Bamias, G., Rivera-Nieves, J., Linden, J., Nast, C. C., Moskaluk, C. A., Marini, M., Sugawara, K., Kozaiwa, K., Otaka, M., Watanabe, S., and Cominelli, F. (2005) Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. *Gastroenterology* **129,** 26–33
24. Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G., and Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388,** 674–678
25. Nemeth, Z. H., Csoka, B., Wilmanski, J., Xu, D., Lu, Q., Ledent, C., Deitch, E. A., Pacher, P., Spolarics, Z., and Hasko, G. (2006) Adenosine A2A receptor inactivation increases survival in polymicrobial sepsis. *J. Immunol.* **176,** 5616–5626
26. Mabley, J. G., Rabinovitch, A., Suarez-Pinzon, W., Hasko, G., Pacher, P., Power, R., Southan, G., Salzman, A., and Szabo, C. (2003) Inosine protects against the development of diabetes in multiple-low-dose streptozotocin and nonobese diabetic mouse models of type 1 diabetes. *Mol. Med.* **9,** 96–104
27. Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990) Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* **127,** 126–132
28. Rabinovitch, A., and Suarez-Pinzon, W. L. (1998) Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem. Pharmacol.* **55,** 1139–1149
29. Feoktistov, I., and Biaggioni, I. (1997) Adenosine A2B receptors. *Pharmacol. Rev.* **49,** 381–402
30. Higuchi, Y., Herrera, P., Muniesa, P., Huarte, J., Belin, D., Ohashi, P., Aichele, P., Orci, L., Vassalli, J. D., and Vassalli, P. (1992) Expression of a tumor necrosis factor alpha transgene in murine pancreatic beta cells results in severe and permanent insulitis without evolution towards diabetes. *J. Exp. Med.* **176,** 1719–1731
31. Cameron, M. J., Arreaza, G. A., Grattan, M., Meagher, C., Sharif, S., Burdick, M. D., Strieter, R. M., Cook, D. N., and Delovitch, T. L. (2000) Differential expression of CC chemokines and the CCR5 receptor in the pancreas is associated with progression to type I diabetes. *J. Immunol.* **165,** 1102–1110
32. Debray-Sachs, M., Carnaud, C., Boitard, C., Cohen, H., Gresser, I., Bedossa, P., and Bach, J. F. (1991) Prevention of diabetes in NOD mice treated with antibody to murine IFN gamma. *J. Autoimmun.* **4,** 237–248
33. Rabinovitch, A. (1998) An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetes. Metab. Rev.* **14,** 129–151
34. Verspohl, E. J., Johannwille, B., Waheed, A., and Neye, H. (2002) Effect of purinergic agonists and antagonists on insulin secretion from INS-1 cells (insulinoma cell line) and rat pancreatic islets. *Can. J. Physiol. Pharmacol.* **80,** 562–568
35. Olsson, R., Jansson, L., Andersson, A., and Carlsson, P. O. (2000) Local blood flow regulation in transplanted rat pancreatic islets: influence of adenosine, angiotensin II, and nitric oxide inhibition. *Transplantation* **70,** 280–287
36. Lai, E. Y., Jansson, L., Patzak, A., and Persson, A. E. (2007) Vascular reactivity in arterioles from normal and alloxan-diabetic mice: studies on single perfused islets. *Diabetes* **56,** 107–112
37. Eltzschig, H. K., Ibla, J. C., Furuta, G. T., Leonard, M. O., Jacobson, K. A., Enjyoji, K., Robson, S. C., and Colgan, S. P. (2003) Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ecto-nucleotidases and adenosine A2B receptors. *J. Exp. Med.* **198,** 783–796
38. Thompson, L. F., Eltzschig, H. K., Ibla, J. C., Van De Wiele, C. J., Resta, R., Morote-Garcia, J. C., and Colgan, S. P. (2004) Crucial role for ecto-5′-nucleotidase (CD73) in vascular leakage during hypoxia. *J. Exp. Med.* **200,** 1395–1405

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