

Emetine Di-HCl Attenuates Type 1 Diabetes Mellitus in Mice

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Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease characterized by β cell destruction, insulin deficiency and hyperglycemia. Activated macrophages and autoimmune T cells play a crucial role in the pathogenesis of hyperglycemia in NOD murine diabetes models, but the molecular mechanisms of macrophage activation are unknown. We recently identified pigment epithelium-derived factor (PEDF) as an adipocyte-derived factor that activates macrophages and mediates insulin resistance. Reasoning that PEDF might participate as a proinflammatory mediator in murine diabetes, we measured PEDF levels in NOD mice. PEDF levels are significantly elevated in pancreas, in parallel with pancreatic TNF levels in NOD mice. To identify experimental therapeutics, we screened 2,327 compounds in two chemical libraries (the NIH Clinical Collection and Pharmakon-1600) for leads that inhibit PEDF mediated TNF release in macrophage cultures. The lead molecule selected, "emetine" is a widely used emetic. It inhibited PEDF-mediated macrophage activation with an EC₅₀ of 146 nmol/L. Administration of emetine to NOD mice and to C57Bl6 mice subjected to streptozotocin significantly attenuated hyperglycemia, reduced TNF levels in pancreas and attenuated insulinitis. Together, these results suggest that targeting PEDF with emetine may attenuate TNF release and hyperglycemia in murine diabetes models. This suggests that further investigation of PEDF and emetine in the pathogenesis of human diabetes is warranted.

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INTRODUCTION

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease characterized by β cell destruction resulting in insulin deficiency and hyperglycemia. The incidence of T1D is increasing worldwide, and in the United States the prevalence is 1 in 300 by the age of 18 years, with associated annual costs

of \$14.9 billion (1, 2). The chronicity of T1D beginning early in life leads to serious long-term complications affecting the renal, cardiovascular, retinal and neural systems (3). Despite extensive research and significant advances in patient care, the treatment of T1D is mainly limited to multiple daily insulin injections that inadequately prevent

severe hyperglycemia and diabetes related complications.

Experimental models of T1D include the non-obese diabetic (NOD) mice, and streptozotocin (STZ)-induced diabetes in C57Bl6 mice. Studies in NOD mice reveal a role for several immune cell phenotypes in β cell death, including CD4⁺ and CD8⁺ T cells [4–6] and macrophages (7). Macrophages and dendritic cells are the first to infiltrate the pancreas during the development of experimental T1D in NOD mice (8, 9). Non-invasive cytofluorometric analysis shows progressive infiltration of the pancreas by CD11b⁺/CD11c⁻ macrophages in the BDC2.5_NOD T cell receptor transgenic murine model of T1D (10). Inhibition of complement receptor 3 (CR3 or CD11b/CD18) prevents macrophage and T cell infiltration of pancreatic islets and

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ameliorates disease in NOD mice (11). Depletion of macrophages from the pancreas arrests the development of T1D (12, 13). Macrophages mediate the pathogenesis of T1D in this model by secreting tumor necrosis factor (TNF) and other proinflammatory cytokines that modulate β cell cytotoxicity (14, 15). In NOD mice, anti-TNF antibody treatment confers significant protection against the development of diabetes (15). Furthermore, TNF receptor 1 (TNFR1) deficient NOD mice show a reduction in the development of hyperglycemia as well as improved survival (14). Together these data indicate an important role of macrophages and TNF in the pathogenesis of murine diabetes. However, the mechanisms that initiate macrophage activation resulting in increased TNF release are poorly understood.

Pigment epithelium-derived factor (PEDF), also known as serpin F1 (SERPINF1), is a 50 kDa protein member of the serpin family of protease inhibitors that has been studied for its anti-angiogenic, anti-tumorigenic and neurotrophic properties. Recently, we discovered that PEDF as an adipocyte-derived factor that can stimulate macrophage TNF activation and mediate insulin resistance (16). It was initially discovered as a factor secreted by retinal epithelial cells, but we and others have found that PEDF is also released by adipocytes and hepatocytes, and mediates inflammatory phenotype in macrophages (16–18). The cytokine activities of PEDF include activation of macrophages to increase expression of proinflammatory cytokines, differentiation of neurons, inhibition of endothelial cell migration and proliferation, prevention of angiogenesis and suppression of tumor growth (16, 19). PEDF modulates target cell responses by signaling via a family of unrelated high affinity receptors. The anti-angiogenic activity of PEDF is mediated by the laminin receptor (19–21); low-density lipoprotein receptor-related protein 6 (LPR6; Wnt co-receptor) (22); and cell surface F_1F_0 -ATP synthase (23). The macrophage stimulating (16) and neurotrophic activities (24) of PEDF are mediated by adipose

triglyceride lipase (ATGL), also known as patatin-like phospholipase domain containing 2 (PNPLA2; PEDF-RN).

Elevated levels of PEDF have been observed in humans with type 2 diabetes mellitus, obesity, metabolic syndrome, vascular inflammation and cardiovascular diseases (25–27). In type 1 diabetes patients, microvascular complications, vascular stiffness, inflammation and diabetic retinopathy have been significantly correlated with increased serum levels of PEDF (28, 29). Systemic PEDF administration to naïve animals induced activation of proinflammatory cytokine responses, and reduced insulin sensitivity (16, 18). Inhibition or blockade of PEDF in obese, insulin-resistant mice, significantly improves whole-body insulin sensitivity, even when initial dosing of inhibitors was delayed until after the onset of frank obesity (18).

Here we reasoned that the cytokine-like proinflammatory role of PEDF might be exploited as a potential target in T1D. Accordingly, we developed a high throughput screen to identify PEDF inhibitors, and discovered that emetine Di-HCl attenuates PEDF mediated cytokine production and hyperglycemia in NOD and streptozotocin-induced murine diabetes.

MATERIALS AND METHODS

Animals

NOD female mice (15–20 g) and C57Bl/6 male mice (20–25 g) acquired from Taconic were housed at 25°C under light-controlled conditions with a 12-h light/dark cycle. Mice were given free access to water and standard rodent chow and were acclimated to their environment for 1 wk before experimentation. All animal studies were approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, Northwell Health.

Induction of Diabetes

T1D was induced by administration of streptozotocin (Sigma-Aldrich) (50 mg/kg

dissolved in sodium citrate buffer, pH 4.5) to 6 to 8 wk old C57Bl/6 mice intraperitoneally (i.p.) once each day for 5 consecutive days. Mice were given water supplemented with 10% sucrose (Sigma-Aldrich) for six days to prevent sudden hypoglycemia during streptozotocin administration. For time course studies, body weight and blood glucose were measured every other day. Blood glucose was measured with a FreeStyle Lite blood glucose meter (Abbot Diabetes Care, Inc.). For studies with emetine (EMD Millipore), mice received daily administration of emetine dihydrochloride (0.002, 0.02, 0.2 and 2 mg/kg) or saline. Blood glucose with either FreeStyle Lite blood glucose meter (Abbot Diabetes Care, Inc.) or Bayer Contour blood glucose meter (Bayer HealthCare, LLC.) and body weight were measured once weekly. At the end of each study, mice were euthanized by CO₂ asphyxiation. Blood and pancreas were collected for cytokine analysis.

NOD Mice

NOD female mice received i.p. administrations of emetine (0, 0.002, 0.02, 0.2 and 2 mg/kg) daily beginning at 5 wks of age. Blood glucose and body weight were recorded weekly. At the end of the studies, mice were euthanized by CO₂ asphyxiation. Blood and pancreas collected for cytokine analysis.

Histology

Pancreas were fixed in 10% formalin, and processed for paraffin embedding (AML Laboratory and Genecopoeia). 7- μ m-thick sections were stained with hematoxylin/eosin (Genecopoeia) and the degree of insulinitis was evaluated microscopically. Islets were analyzed in multiple sections at 20 \times . Images were captured by Zeiss Axiovert 20-inverted microscope, using the AxioVision V5 software (Zeiss). Insulinitis was scored by infiltration grade 0 to 4 (0 = no visible infiltration; 1 = perivascular/periductular infiltrates with leukocytes touching islet perimeters, but not penetrating; 2 = leukocytic penetration of up to 25% of islet mass; 3 = leukocytic penetration of up to 75% of islet mass; 4 = end-stage insulinitis) (30).

Cytokine Analysis

Pancreas tissues were homogenized with 1:1 zirconium oxide 1.0 mm beads in two volumes of T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc.), supplemented with complete protease inhibitor cocktail (Roche Applied Science). Samples were homogenized using a BBX24 Bullet Blender homogenizer (Next Advance Inc.) at speed 8 for 5 min. Supernatants were collected by centrifugation at 10,000g for 10 min at 4°C. Levels of TNF (R&D systems) and PEDF (BioProducts MD, LLC) were evaluated using commercially available enzyme-linked immunosorbent assay (ELISA) kits as per manufacturer's instructions.

Cell Culture

Murine macrophage-like RAW 264.7 cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-Glutamine (Life Technologies). For high-throughput screening, cells were seeded onto 96-well plates at 5×10^5 cells/mL and incubated overnight in a humidified chamber at 37°C with 5% CO₂. Cells were washed twice with optimum then pre-incubated with compounds from drug libraries (NIH Clinical Collection, Evotec US, Inc. and Pharmakon-1600, MicroSource Discovery Systems, Inc.) for 30 min prior to challenge with his-sumo-rhPEDF (0.05 µg/mL). Supernatant was collected after 2.5 h and TNF levels were analyzed by ELISA. Cell viability was assessed either by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (EMD Millipore) or by quantitating lactate dehydrogenase (LDH) levels (Takara Bio, Inc.) in the supernatants. The IC₅₀ value of emetine was calculated using ED50 Plus v1.0 online software.

Surface Plasmon Resonance

The Biacore T200 instrument (GE Healthcare) was used for real time binding interaction experiments. For binding analysis, recombinant ATGL (Abnova)

was immobilized onto CM5 dextran biosensor chips. The biosensor chip was activated by amine coupling using 0.4 mol/L 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.1 mol/L N-hydroxysuccinimide. Immobilization of a 75 µg/mL concentration of ATGL (Abnova) to the sample flow-cell was accomplished by injection at a flow rate of 30 µL/min until the SPR reached ~ 100 to 300 RU. Binding assays were performed at a flow rate of 30 µL/min for 60 s at 25°C in 1 × PBS buffer, pH 5.5 and 0.1% surfactant p20. For ATGL-emetine binding, increasing concentrations of emetine (0 to 500 µmol/L) were flowed over immobilized ATGL. For ATGL-PEDF binding, increasing concentrations rhPEDF (Abcam) (0 to 1,000 nmol/L) were used as the analyte. A sample of running buffer alone was subtracted from the resulting reaction surface data. Results were analyzed using Biacore T200 Evaluation Software version 2.0 (GE Healthcare). The equilibrium disassociation binding constant (KD) was determined with Biacore T200 Evaluation Software version 2.0 supposing a 1:1 binding ratio.

Statistical Analysis

Data are presented as means ± SEM. A two-way ANOVA (Turkey), one-way ANOVA with Fischer's protected, chi-square test for trend, chi-square test, log rank test or Student *t* test was used where specified; then least significant difference test was conducted.

RESULTS

Pancreatic PEDF and TNF Levels in Murine Streptozotocin (STZ)-Induced Hyperglycemia

To determine the expression kinetics of PEDF in pancreas, we measured PEDF levels in a standard murine model of T1D. In this model, streptozotocin, an alkylating agent, enters the β cell via a glucose transporter (GLUT2) and alkylates DNA, leading to β cell death, resulting in severe insulin deficiency and hyperglycemia (31, 32). Plasma glucose concentrations in streptozotocin-injected

mice were more than >200 mg/dL by d 7, and remained at that level thereafter (Figure 1A). Pancreatic PEDF levels increased in time-dependent manner following streptozotocin administration (Figure 1B). Pancreatic PEDF was >12,000 pg/mg protein by d 12, and remained elevated for the entire observation period. Increasing PEDF levels in pancreatic tissue were significantly correlated with increasing levels of blood glucose (Figure 1C). Moreover, we observed that TNF levels in the pancreas were also significantly increased (Figure 1D), and were strongly correlated with pancreatic PEDF levels (Figure 1E). Serum PEDF and TNF levels did not change significantly in the streptozotocin animals as compared with vehicle control animals (data not shown).

PEDF Induces Inflammatory Cytokine Secretion by Macrophages

Previous studies demonstrated that PEDF induces an inflammatory phenotype and cytokine release in macrophages and other cells (16–18). Here, we analyzed the profile of inflammatory mediators released by macrophages in response to PEDF. Murine macrophage like cells RAW 264.7 were stimulated with increasing concentration of PEDF and an array of cytokines was analyzed using a multiplex assay. PEDF induced a concentration dependent increase in TNF, IL-6, KC/GRO, IL-12p40 and IL-10, but not IL-1β or IFNγ, secretion by macrophages as measured in culture medium at 6 h (Table 1) and 24 h post stimulation (Table 2).

Identification of a Novel PEDF Antagonist

Reasoning that it might be possible to identify PEDF inhibitors, we next established a high-throughput bioassay to screen 2,327 compounds derived from two libraries: the NIH Clinical Collection and Pharmakon-1600. These compound libraries are a collection of small molecules that are either federal drug administration (FDA) approved, or are experimental therapeutics that have been previously

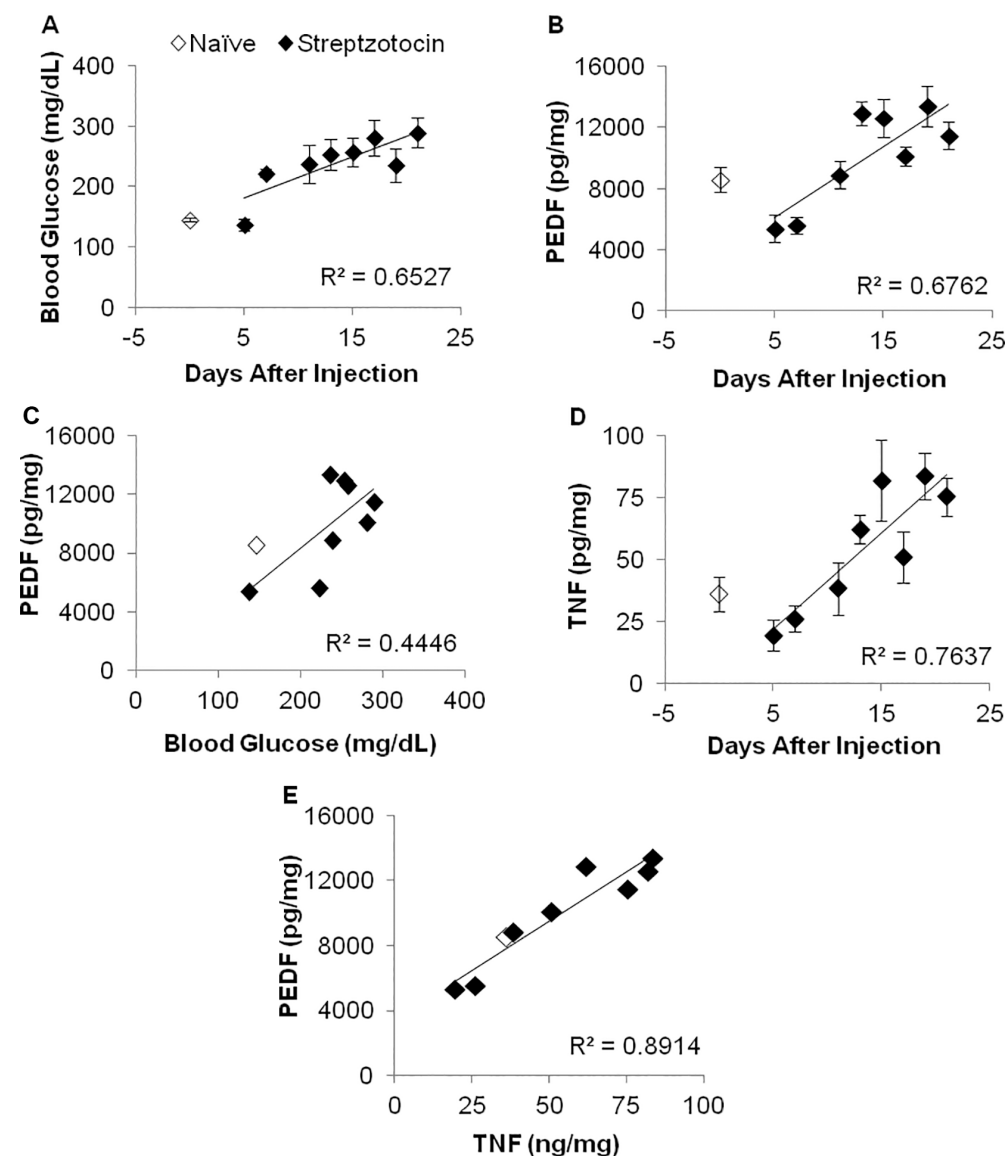


Figure 1. Blood glucose, pancreatic TNF and pancreatic PEDF are elevated in a low-dose streptozotocin model of T1D. (A) Blood glucose, (B) PEDF and (D) TNF levels increase over time in mice streptozotocin-induced type 1 diabetes. Levels of PEDF and TNF and were monitored in blood and pancreas over time. Pancreatic PEDF levels are correlated with (C) blood glucose and (E) pancreatic TNF levels. (A) Days: 0 (n = 84); 7 (n = 62); 5, 11, 13, 15, 17, 19 and 21 (n = 7). (B, D) n = 7. (C, E) Data are shown as mean \pm SEM with a linear regression fit model (n = 7).

used in clinical phase trials. Macrophages were pre-exposed to test compounds for 30 min, and then challenged with PEDF for 2.5 h. TNF levels were subsequently measured in the supernatants.

From the NIH clinical collection we selected 503 compounds that reduced TNF secretion from macrophages by 25% or greater at a concentration of 5 μ mol/L. Next, macrophages were pre-exposed

to increasing concentrations of 503 test compounds, and then stimulated with PEDF. We eliminated compounds that induced cell viability of less than 60% as determined by MTT assay. Next we selected 44 candidates from 503 previously selected compounds that inhibited PEDF induced TNF release (50% reduction) at a concentration of 5 μ mol/L. Finally, we screened increasing concentrations of

these 44 compounds along with an additional 1,600 compounds from the Pharmakon-1600 library to identify candidate drugs that at a ten-fold lower concentration (0.5 μ mol/L) can reduce at least 50% of secreted TNF from PEDF-challenged macrophages (Figure 2A).

Compounds that showed a concentration-dependent reduction in TNF and a viability of greater than 60%

Table 1. PEDF-induced cytokine release at 6 h (pg/mL).

PEDF	0 µg/mL	0.005 µg/mL	0.05 µg/mL	0.5 µg/mL	5 µg/mL
TNF	29	51	1530	12321	17027
KC/GRO	0	0	8	64	202
IL-6	0	11	7	82	1579
IL-1 b	0	0	0	0	13
IL-12	5	0	11	9	390
IL-10	0	0	0	23	368
IFNg	0	0	0	0	2

Table 2. PEDF-induced cytokine release at 24 h (pg/mL).

PEDF	0 µg/mL	0.005 µg/mL	0.05 µg/mL	0.5 µg/mL	5 µg/mL
TNF	38	224	5038	17893	17106
KC/GRO	9	0	27	79	116
IL-6	69	4	66	1979	11533
IL-1 b	8	0	7	22	31
IL-12	167	11	136	401	429
IL-10	18	1	11	276	2591
IFNg	1	0	1	2	3

were selected as lead clinical candidates. Results show that 62% of the identified leading compounds were corticosteroids, while a composite of other known anti-inflammatory compounds represented 11% of these leading candidates (Figure 2B). Among the screened drug candidates, emetine was identified as a lead compound with significant concentration dependent suppression of PEDF-induced TNF secretion and an IC_{50} of 146 nmol/L (Figure 2C). Analysis of supernatants for cell toxicity did not show increased LDH leakage from emetine-treated cells (Figure 2D). Additionally, mitochondrial activity was unaffected by emetine as determined by MTT cytotoxicity assay (Figure 2E) indicating that emetine inhibits PEDF-mediated TNF release without affecting cell viability.

Emetine Binds ATGL with High Affinity

To elucidate the underlying molecular mechanism of emetine-mediated inhibition of PEDF-induced TNF release, we examined whether emetine interferes with the binding of PEDF to its receptor ATGL. Surface plasmon resonance analysis demonstrated that emetine binds to PEDF receptor ATGL with high-binding affinity ($KD = 14.3$ nmol/L) (Figure 3A).

This binding affinity was ten-fold greater than that of PEDF to its receptor ATGL ($KD = 214$ nmol/L) (Figure 3B).

Emetine Administration Attenuates Hyperglycemia and Reduces Pancreatic TNF Levels in T1D

To evaluate the therapeutic potential of emetine, we next studied whether emetine can improve disease severity in T1D mice. C57Bl/6 male mice with streptozotocin-induced T1D received a daily administration of emetine, and changes in body weight and blood glucose were monitored. Emetine did not induce any significant difference in body weight in mice with low-dose streptozotocin model of T1D (Figure 4A). In contrast, administration of emetine not only attenuated blood glucose levels in dose-dependent way but also induced a persistent attenuation of blood glucose levels. Daily administration of emetine dose-dependently attenuated hyperglycemic response by d 21 (Figure 4B). Consistent with this observation, administration of emetine, but not the vehicle control, resulted in a sustained attenuation of blood glucose levels (Figure 4C). Accordingly, mice from the vehicle control group demonstrated significantly increased

insulinitis scores as compared with mice receiving emetine (Figure 4D, E). Levels of TNF in homogenized pancreas were significantly attenuated by emetine administration in doses of 2 mg/kg (Figure 4F).

Next, we assessed the efficacy of emetine in attenuating disease severity in a spontaneous model of T1D. NOD female mice received either saline or emetine once daily for 14 wks, and body weight and blood glucose levels were monitored over time. Body weight was unaffected by emetine administration to animals as compared with control animals exposed to saline (Figure 5A). In the saline group, 80% of animals developed hyperglycemia at 19 wks of age, whereas NOD mice receiving emetine were significantly and dose-dependently protected from hyperglycemia (Figure 5B). Only 40% of the NOD mice receiving emetine developed hyperglycemia (Figure 5B). There was also a time-dependent delay in the onset and progression of hyperglycemia in mice treated with emetine when compared with saline-treated animals (Figure 5C). Although 40% of the animals from the emetine treated group developed hyperglycemia, the levels of blood glucose were significantly lower in emetine receiving animals as compared with the saline group (Figure 5D). We observed a small fraction of non-responders in the emetine treated group (~30%) that maintained high levels of blood glucose even after emetine administration (Figure 5D). Insulinitis scores were significantly reduced in animals exposed to emetine as compared with saline-treated mice (Figure 5E, F, G). In line with our observations in the streptozotocin model, pancreatic TNF levels were also significantly in the emetine treated NOD mice (Figure 5H). Taken together, these results indicate that emetine attenuates hyperglycemia and insulinitis in two independent murine models of diabetes.

DISCUSSION

The principal findings here indicate that PEDF levels are increased in experimental murine T1D, and that administration of a PEDF inhibitor emetine significantly attenuates disease severity. PEDF

is a ubiquitous protein in a wide range of human tissues (33), and PEDF levels are significantly associated with various metabolic diseases (25, 34–36). In the present results from murine streptozotocin-induced diabetes, PEDF and TNF levels are significantly increased in pancreas, and expression significantly correlated with blood glucose levels. Results from high-throughput screenings revealed that emetine is a potent inhibitor of

PEDF-induced macrophage activation. Emetine binds the PEDF receptor ATGL and attenuates TNF release mediated by PEDF. In low-dose streptozotocin and NOD mouse models of T1D, emetine administration attenuates hyperglycemia, reduces insulinitis and decreases levels of pancreatic TNF.

To our knowledge, these are the first results implicating increased pancreatic PEDF in this model of T1D. In agreement

with our prior findings that PEDF stimulates macrophage TNF release, rising PEDF levels in pancreas correlate with increasing levels of pancreatic TNF. On the contrary, circulating levels of PEDF did not change significantly, and did not correlate with levels of blood glucose (data not shown). In an experimental model of ethanol-induced pancreatitis, immunohistochemical analysis showed increased levels of PEDF in the acinus of the inflamed

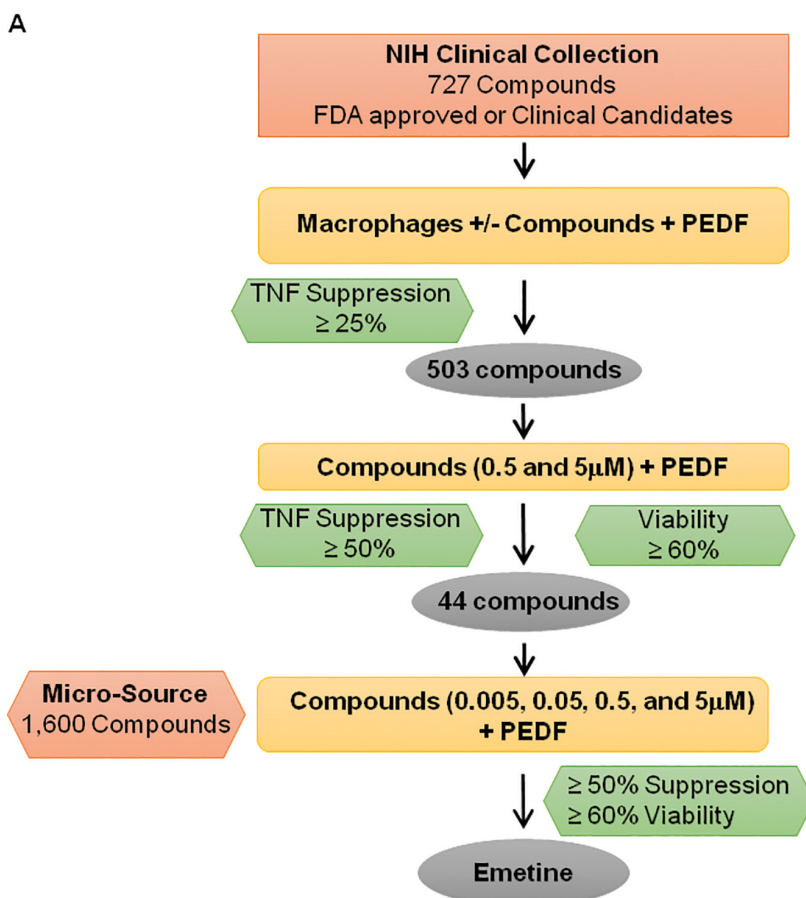


Figure 2. High-throughput screening for antagonists of PEDF-mediated macrophage activation. (A) Raw 264.7 cells were pre-exposed to 5 μM concentrations of compounds from the NIH Clinical Collection library then challenged with his-sumo- rhPEDF (0.05 $\mu\text{g}/\text{mL}$) for 2.5 h. The supernatant was collected for TNF analysis. Five hundred and three compounds that show at least 25% suppression of TNF release were re-tested with 2 concentrations (0.5 $\mu\text{mol}/\text{L}$ and 5 $\mu\text{mol}/\text{L}$), and TNF levels and cell viability was assessed (see Materials and Methods). Forty-four compounds that had at least 50% suppression at 5 μM as well as at least 60% viability by MTT were re-tested at various concentrations along with 1,600 additional compounds from the Pharmakon-1600 library (0.005, 0.05, 0.5 and 5 $\mu\text{mol}/\text{L}$). TNF analysis and viability, by MTT, were analyzed. Three candidate drug targets that yield a concentration-dependent response, at least 50% suppression at 0.5 $\mu\text{mol}/\text{L}$ and at least 60% viability at 0.5 $\mu\text{mol}/\text{L}$ concentration were identified. Emetine is one of the three-candidate drug targets that was studied in detail. (B) Summary of lead compounds. (C) Emetine inhibits PEDF induced TNF release in concentration dependent manner. Horizontal dashed line represents the lower limit of assay detection at 31.5 pg/mL . Emetine does not induce cell toxicity at the concentrations tested. Cell viability was analyzed by (D) LDH Assay and (E) MIT Assay. (D and E) Data are mean \pm SEM. Statistical analysis was measured by one-way ANOVA.

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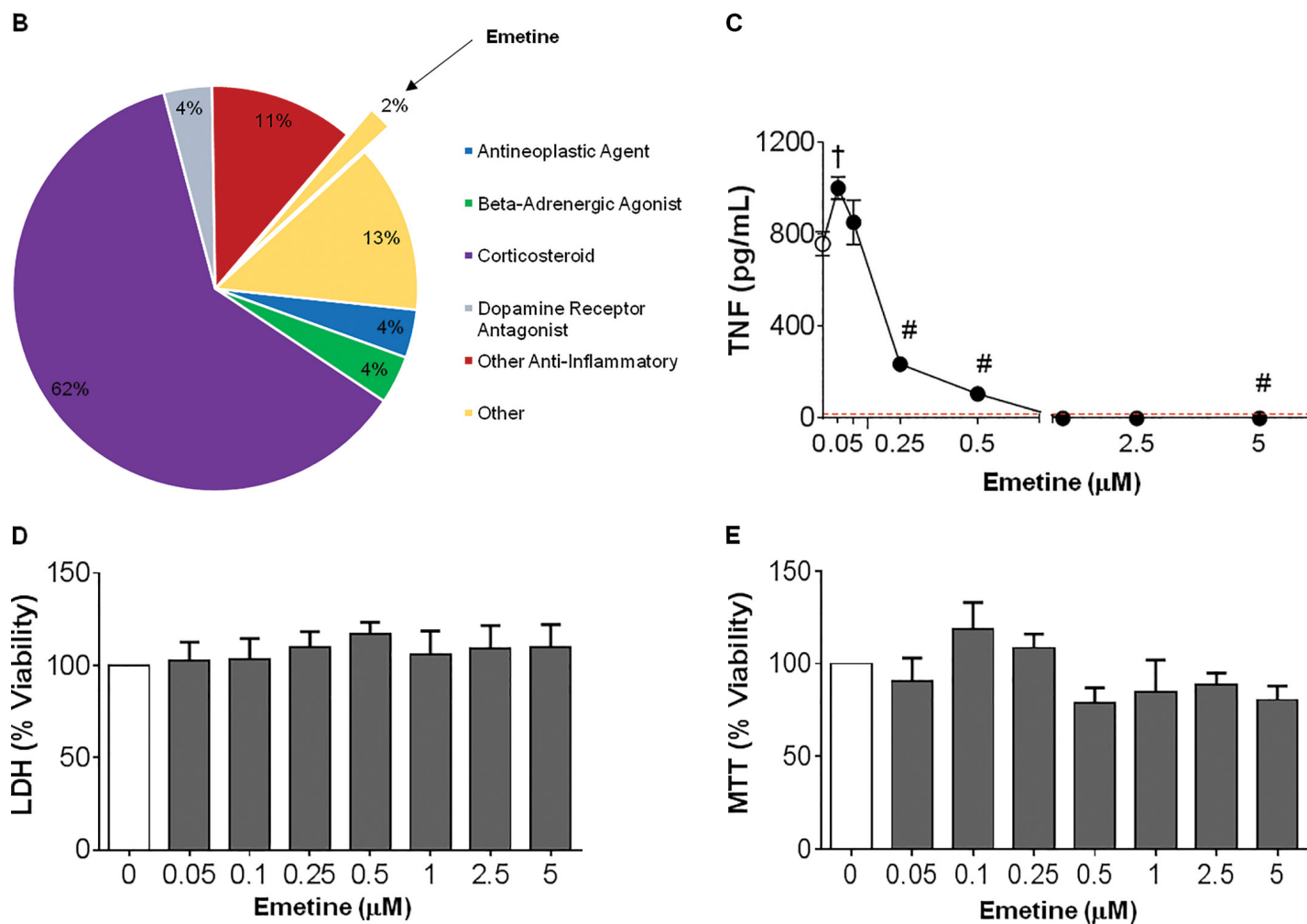


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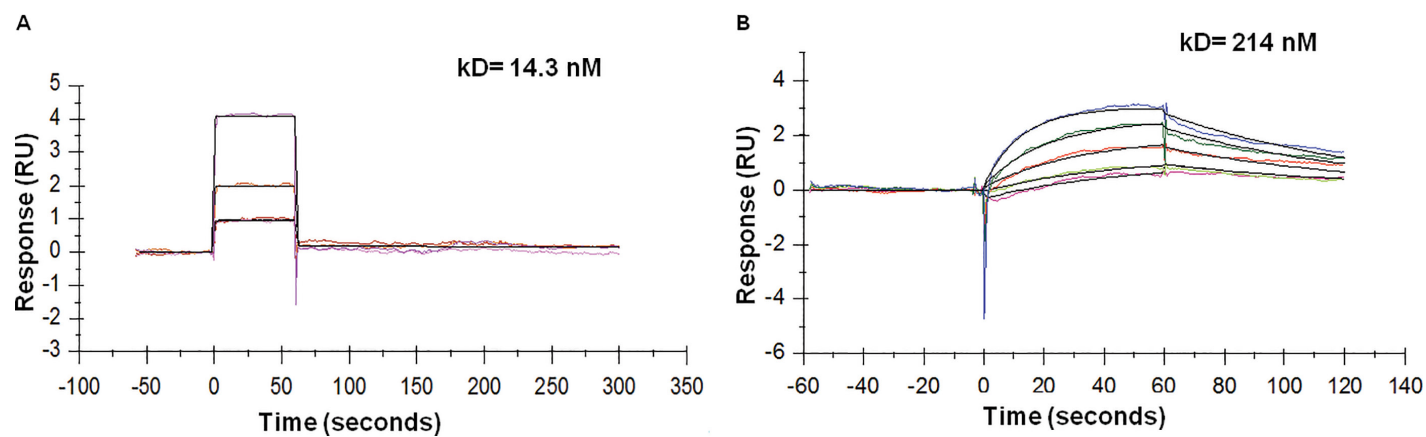


Figure 3. Emetine binds to ATGL with high affinity. Surface plasmon resonance analysis was conducted by immobilizing ATGL on a CM5 dextran biosensor chip surface (see Materials and Methods). (A) ATGL ligand of ~300 RU and emetine analyte (0-500 $\mu\text{mol/L}$). (B) ATGL ligand at ~100 RU, then PEDF (0-1,000 nmol/L) added as an analyte.

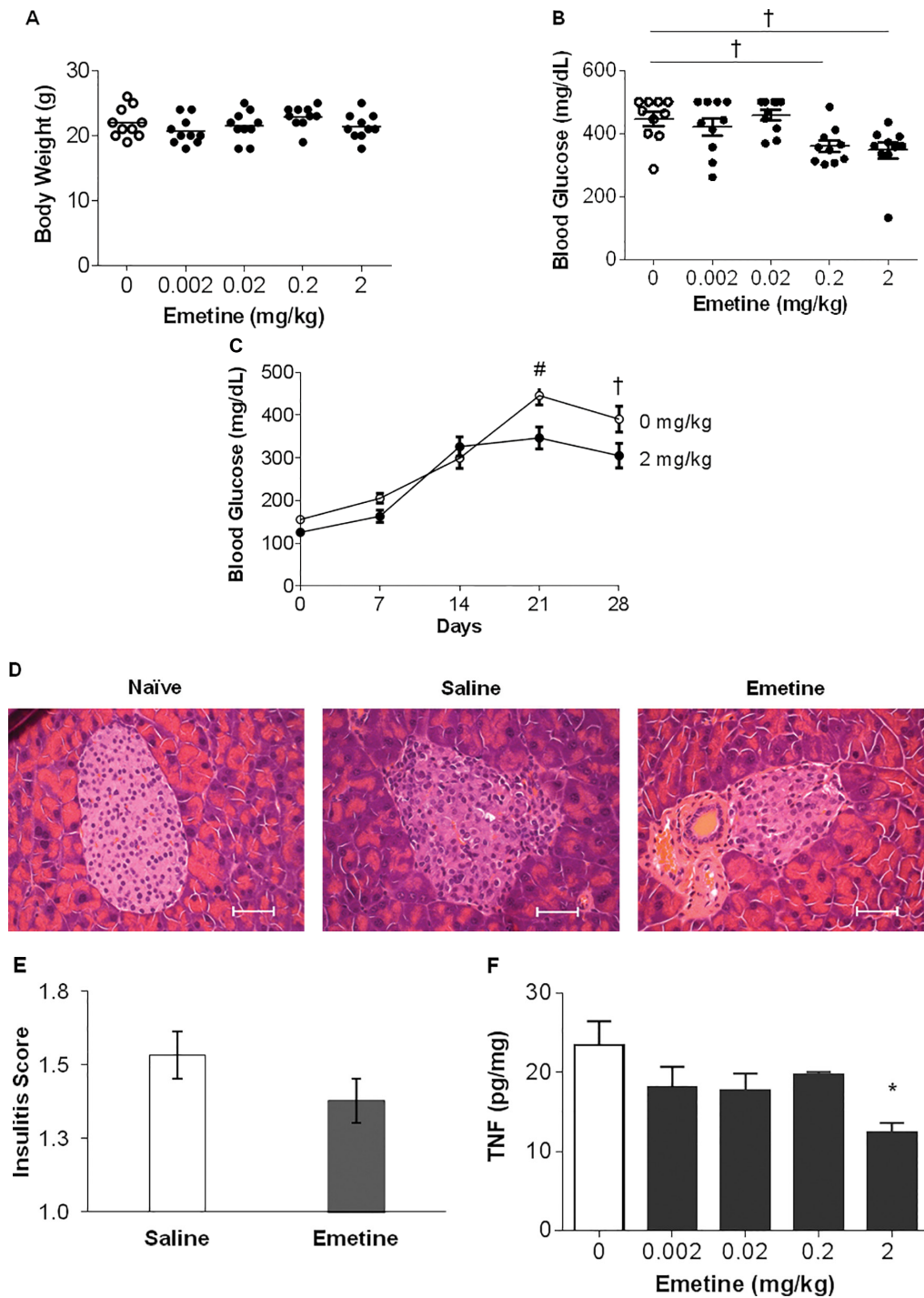


Figure 4. Emetine improves disease severity in a streptozotocin model of T1D. (A) Emetine administration did not induce any significant changes in body weight. Body weights collected at the end of the study are shown here. (B) Emetine administration attenuated blood glucose levels in a dose dependent manner. Blood glucose measured at d 21 in all the dose groups is shown here. (n = 10/group, one way ANOVA: † p < 0.01) (C) Emetine (2 mg/kg) induced a persistent attenuation of blood glucose levels. Data are mean ± SEM. (n = 10/group, two way ANOVA: † p < 0.01, # p < 0.0001). (D) Representative images of hematoxylin and eosin stained pancreatic islets from mice receiving saline or emetine (2 mg/kg). Islet from a naïve animal is shown as a representation of a normal islet. Scale bar-50 µm. (E) Emetine (2 mg/kg) induces decrease in insulinitis severity. Data are mean ± SEM. (n = 5; islets: saline = 81 and emetine = 53, Student t-test, p = 0.72). (F) Emetine induces significant attenuation of pancreatic TNF levels. Data are mean ± SEM. (n = 10/group, one way ANOVA: *p < 0.05).

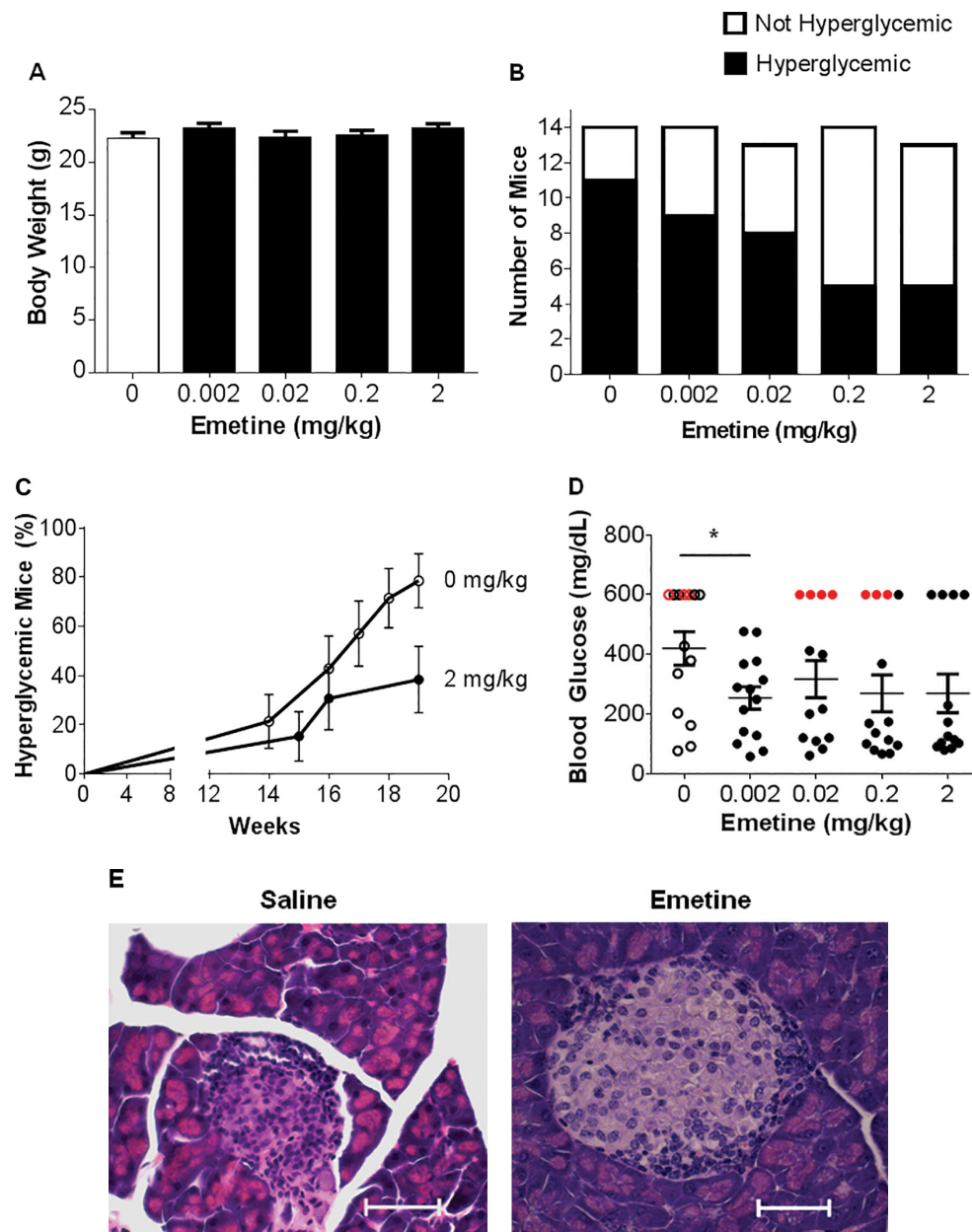


Figure 5. Emetine improves disease severity in a spontaneous model of NOD T1D. (A) Emetine administration did not induce any significant changes in body weight. Body weights collected at the end of the study (19 wks) are shown here. (B) Emetine dose-dependently protects animals from hyperglycemia. Total number of animals, at wk 19, developing hyperglycemia is shown here. Mice with blood glucose levels above 200 mg/dL were considered hyperglycemic, $p = 0.01$. (C) Time course of the percent of mice that are hyperglycemic (200 mg/dL) in saline and emetine (2 mg/kg) treated animals, $p = 0.02$. (D) Individual blood glucose levels measured at 19 wks of age. Red data points signify the last measured blood glucose level of mice that did not survive until the end of the study. (E) Representative images of hematoxylin & eosin (H&E) stained pancreatic islets of Langerhans from either saline or emetine (0.2mg/kg) treated animals. Scale bar, 50 μ m. (F) Insulinitis scores of top slice of H&E stained pancreatic islets from saline and emetine (0.2 mg/kg) treated animals, $p = 0.0037$. (G) Percentage of total islets are sectioned into score categories, $p < 0.0001$. (H) Emetine induces significant attenuation of pancreatic TNF levels. (A–D) 0 mg/kg ($n = 14$), 0.002 mg/kg ($n = 14$), 0.02 mg/kg ($n = 13$), 0.2 mg/kg ($n = 14$), 2 mg/kg ($n = 13$). (H) 0 mg/kg ($n = 12$), 0.002 mg/kg ($n = 14$), 0.02 mg/kg ($n = 11$), 0.2 mg/kg ($n = 12$), 2 mg/kg ($n = 13$). (F, G) $n = 4$ (islets: saline = 29, emetine = 48). Data are mean \pm SEM. Statistical analysis was measured by (A and D, H) one-way ANOVA. (B) chi-square test for trend, (C) Kaplan-Meier curve with a log rank test for trend, (G) chi-square test, and (F) Student t test, $p \leq 0.05$ (*).

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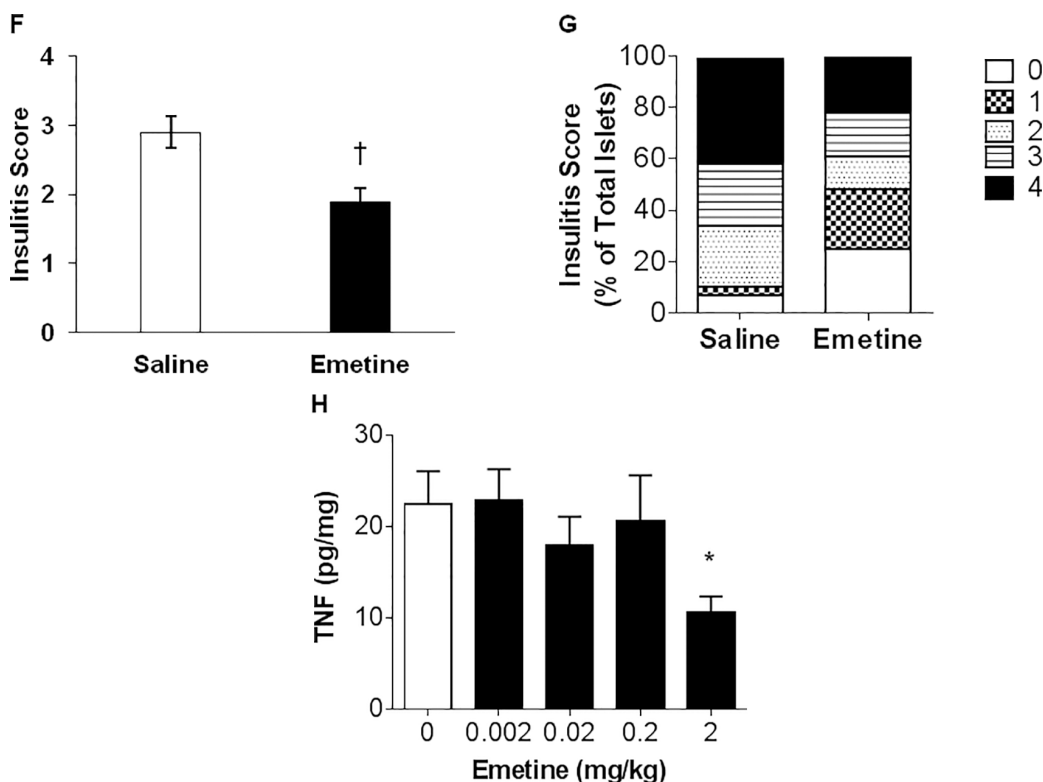


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pancreas (37). It is possible that pancreatic PEDF may be locally derived from acinar cells in experimental T1D, and local accumulation of PEDF may induce inflammatory phenotype. Pancreatic levels of PEDF were positively correlated with both TNF and increased levels of blood glucose, suggesting that PEDF may contribute to the pathogenesis of disease in T1D.

We observed an initial decrease in pancreatic levels of PEDF and TNF within the first week of administration of streptozotocin to animals. This may be a result of an increase in insulin release from cytotoxic β cells (38). Insulin has previously been shown to reduce PEDF expression in adipose tissue of T2D patients (39). It is possible that insulin reduces the levels of PEDF in the pancreas and, as a result, TNF levels are also decreased in the first week of streptozotocin-induced T1D. Together, these findings indicate that PEDF may act as an inflammatory mediator in experimental T1D.

We have previously demonstrated that PEDF activates macrophages and induces the release of TNF (16). At a concentration of 0.05 $\mu\text{g}/\text{mL}$, the concentration of PEDF used for the high-throughput screening of test compounds and *in vitro* studies with emetine, TNF secretion was significantly enhanced from macrophages as compared with other cytokines. At higher concentrations, PEDF also stimulated the release of the inflammatory cytokines IL-6, KC/GRO, IL-12p40 and the antiinflammatory cytokine IL-10. TNF and IL-6 are regulated by both P38MAPK and extracellular signal-regulated kinases (ERK) phosphorylation (16, 40). KC/GRO, IL-12p40, and IL-10 require P38MAPK activation and is necessary for the regulation of macrophage-derived IL-6, KC/GRO and IL-10 (40–42). It is interesting to consider that both proinflammatory cytokines IL-1 β and IFN γ were not increased in this experiment, even at 24 h. IL-1 β and IFN γ production require caspase-1 activation (43, 44). This

suggests that PEDF induces the activation of macrophages and promotes the release of cytokines through P38MAPK and ERK signaling, independent of caspase-1. Collectively, this suggests that PEDF is a specific activator of TNF release from macrophages.

Emetine is an alkaloid that has been tested in Phase I and II clinical trials against solid tumors (45). Emetine has been used as an anti-protozoal drug and a cough suppressant, although its use is limited by cardiotoxicity (46, 47). Exposure of macrophages to emetine reduces PEDF stimulated TNF release with an IC_{50} of 146 nmol/L. At these concentrations, emetine does not induce cytotoxicity as demonstrated by both LDH and MTT assays. Additionally *in vivo*, in the doses used here, emetine did not mediate significant changes in body weight. We have previously shown that macrophages express PEDF receptors, and PEDF mediated its inflammatory phenotype through its receptor ATGL.

Here, we demonstrated that emetine binds to PEDF receptor, ATGL, with almost 15-fold higher affinity than PEDF. It is plausible that emetine mediates its inhibition of TNF release by direct binding to ATGL. The cardiotoxicity of emetine may limit its chronic use in humans, suggesting that a search for other, less toxic PEDF inhibitors is warranted.

The increased levels of PEDF in pancreatic tissue indicate its importance in the pathogenesis of murine type 1 diabetes. To address the role of PEDF, we treated streptozotocin induced T1D mice with a PEDF-antagonist, emetine. As expected, emetine treatment attenuated blood glucose levels and improved insulinitis. Unlike the streptozotocin model of T1D, NOD mice are genetically susceptible to spontaneously develop T1D-like symptoms, including hyperglycemia (31). Similar to both the streptozotocin model and human T1D, NOD mice exhibit autoimmune responses that lead to the development of hyperglycemia (31). Emetine administration not only significantly delayed the onset of hyperglycemia and reduced the total number of hyperglycemic animals, it also attenuated the development of insulinitis. We did notice that some NOD mice failed to respond to emetine, which began at 5 wks of age. The basis of this observation is not clear from our studies. It is plausible that in a subset of NOD mice, the disease onset and progression may have preceded emetine treatment, as macrophage infiltration occurs as early as 3 wks in animals that develop T1D (9). Together, these studies suggests that PEDF as an early mediator in T1D, which may influence immune cell migration into islets. Furthermore, these findings also show that emetine, perhaps through inhibition of TNF from macrophages, can suppress immune cell translocation into the islets of Langerhans.

CONCLUSION

We observed a reduction in pancreas levels of TNF with emetine enterprize. Remarkably, emetine at 2 mg/kg for mice, the highest dose used in these experiments, is equivalent to 0.167 mg/kg

in humans (48). This is 11% of the clinically tested dosage for cancer treatment, which is an intravenous at a dose of 1.5 mg/kg (55.5 mg/m²) (48, 49). Currently clinical trials exist for the prevention of T1D in genetically at-risk patients (50, 51). It is plausible to consider the use of emetine in patients at high risk for T1D. Collectively these data suggest that PEDF is a local inflammatory mediator in T1D and that emetine, or other PEDF inhibitors, may represent a novel therapeutic strategy.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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