

Treatment With Recombinant Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Alleviates the Severity of Streptozotocin-Induced Diabetes

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OBJECTIVE—To evaluate the potential therapeutic effect of recombinant human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment in a model of type 1 diabetes.

RESEARCH DESIGN AND METHODS—Recombinant TRAIL was added in vitro to primary human and mouse peripheral blood mononuclear cells (PBMCs) and isolated human islets to evaluate the expression of the immunoregulatory gene *SOCS1*. Diabetes was induced by five consecutive daily injections of low-concentration (50 mg/kg) streptozotocin (STZ) in C57 black mice ($n = 24$). A group of these mice ($n = 12$) was co-injected with recombinant TRAIL (20 μ g/day) for 5 days, and the diabetic status (glycemia and body weight) was followed over time. After 6 weeks, circulating levels of insulin, TNF- α , and osteoprotegerin (OPG) were measured, and animals were killed to perform the histological analysis of the pancreas.

RESULTS—The in vitro exposure of both PBMCs and human islets to recombinant TRAIL significantly upregulated the expression of *SOCS1*. With respect to STZ-treated animals, mice co-injected with STZ+TRAIL were characterized by 1) lower levels of hyperglycemia, 2) higher levels of body weight and insulinemia, 3) a partial preservation of pancreatic islets with normal morphology, and 4) a lower expression of both systemic (TNF- α and OPG) and pancreatic (vascular cell adhesion molecule [VCAM]-1) inflammatory markers.

CONCLUSIONS—Overall, these data demonstrate that the administration of recombinant TRAIL ameliorates the severity of STZ-induced type 1 diabetes, and this effect was accompanied by the upregulation of *SOCS1* expression. *Diabetes* 59:1261–1265, 2010

While a large amount of data are available about tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) as an anticancer agent (1), some studies have suggested a potential role of endogenous TRAIL in type 1 diabetes (2–4). When TRAIL function was blocked through the systemic administration of a soluble TRAIL

receptor into NOD mice, the onset of diabetes was significantly increased, and the degree of autoimmune inflammation was augmented in pancreatic Langerhans islets as a result of TRAIL blockage (2,3). In addition, normal primary islet cells are resistant to TRAIL-induced cytotoxicity (5,6), and adenovirus-mediated TRAIL gene delivery into pancreatic islets resulted in prolonged normoglycemia in streptozotocin (STZ)-induced diabetic rats compared with animals grafted with mock-infected islets (6).

Our present study aimed to investigate the effects of recombinant TRAIL on the expression of *SOCS1*, a gene known to modulate the sensitivity of both immune cells and pancreatic β -cells to key inflammatory cytokines elevated in diabetes (7–10), in peripheral blood mononuclear cells (PBMCs), and purified human pancreatic islets. Moreover, we have analyzed the in vivo effect of treatment with recombinant TRAIL in the type 1 diabetes mouse model induced by multiple low doses of STZ.

RESEARCH DESIGN AND METHODS

Cell cultures. Human and mouse peripheral blood was obtained from five independent healthy blood donors and four C57 black mice, respectively. Human and mouse mononuclear cells (PBMCs) were isolated by density gradient (Ficoll/Histopaque-1077; Sigma, St. Louis, MO; or Lympholyte-M; Cederlane Laboratories, Hornby, ON, Canada, respectively) and cultured in RPMI-1640 containing 10% FBS (Gibco BRL, Grand Island, NY).

Human islets were obtained from the European Consortium for islet transplantation (ECIT) for the research distribution program (Human Islet Isolation Facility, San Raffaele Institute, Milano, Italy). Islet preparations, with purity <70%, not suitable for transplantation, were obtained and cultured in CMRL medium-1066 (Invitrogen, Carlsbad, CA) with 10% FBS, as previously described (11).

Cell cultures were treated with recombinant TRAIL (1 μ g/ml), prepared as described (12), for 72 h. Cell viability was monitored over time by Trypan blue dye exclusion, and apoptosis was monitored by double staining with Annexin V/propidium iodide, as previously described (13).

RNA and protein analyses. Validation of array results and investigation of specific gene expression were carried out in RNA samples with the real-time thermal analyzer Rotor-Gene 6000 (Corbett, Cambridge U.K.), by using SYBR Green–based technology and the RT-PCR primer set for human *SOCS1* cDNA (SABioscience, Frederick, MD) or mouse *SOCS1* and vascular cell adhesion molecule (VCAM)-1 cDNAs (Qiagen, Hilden, Germany). Gene expression of the target sequences was normalized in relation to the expression of endogenous controls. Each sample was tested in triplicate. (See also the online appendix, available at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-1771/DC1>.)

For Western blot analyses, PBMC samples were lysed as previously described (14). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. The following antibodies were used: monoclonal antibody anti-*SOCS1* and anti-tubulin (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, and Sigma, respectively). After incubation with peroxidase-conjugated anti-mouse IgG, specific reactions were revealed with the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry values were estimated by the ImageQuant TL software (Amersham).

Animal studies. Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international

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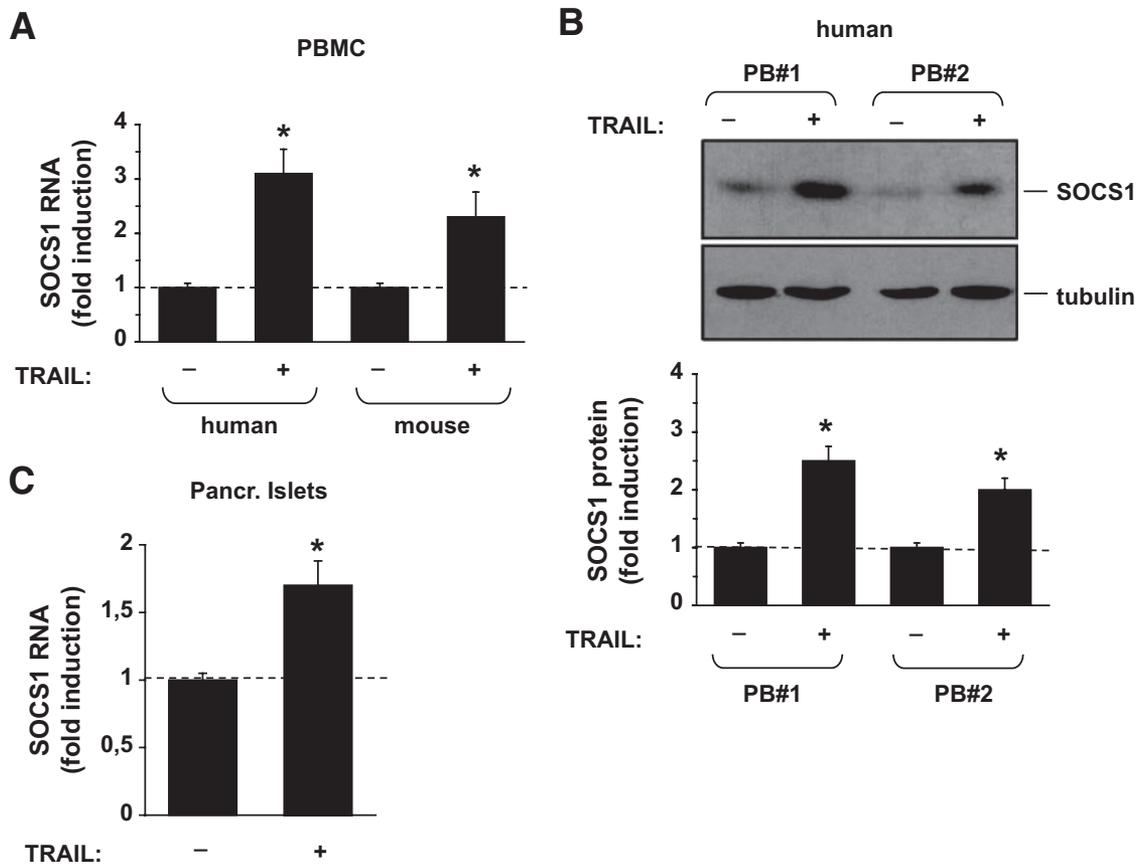


FIG. 1. Effect of TRAIL on SOCS1 expression levels in vitro. PBMCs and pancreatic islet cultures were either left untreated or exposed to recombinant TRAIL. Levels of SOCS1 mRNA and protein were analyzed by quantitative RT-PCR (A and C) and Western blot (B) analyses, respectively. In A and C, after normalization to the level of the housekeeping genes, results were expressed as fold of SOCS1 mRNA induction in TRAIL-treated cultures with respect to the control untreated cultures. Data are reported as means \pm SD of experiments, each performed in triplicate. In B, the levels of SOCS1 protein were assessed by Western blot analysis in cell lysates. Tubulin staining is shown as a loading control. Representative examples of Western blot results obtained from two (PB #1 and PB #2) out of five independent human PBMC cultures are shown. After densitometric analyses, results were expressed as fold of SOCS1 protein induction in TRAIL-treated cultures with respect to the control untreated cultures. * $P < 0.05$ with respect to untreated cells.

laws and policies (EEC Council Directive 86/609, 1987). A total of 24 6-week-old C57 black mice were rendered diabetic by five daily intraperitoneal injections of STZ (Sigma) at a dose of 50 mg/kg. Half of the mice treated with STZ ($n = 12$) were also co-injected daily with recombinant TRAIL (20 μ g/day) (12). Control mice received the vehicle citrate buffer alone (mock treated; $n = 10$). An additional group of nondiabetic mice ($n = 10$) was treated with recombinant TRAIL (20 μ g/day) for 5 consecutive days.

The animals had unrestricted access to water and were maintained on a 12-h light-dark cycle in a non-pathogen-free environment on standard mouse food (Harlan Nossan Corezzana, Milan, Italy). Serum glucose concentrations were determined weekly by an autoanalyzer technique (Hitachi 717; Tokyo, Japan). After 6 weeks, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and killed for blood harvesting and histological examination of pancreas. Blood was immediately centrifuged to remove cells, and serum was collected for enzyme-linked immunosorbent assay (ELISA) assays. Insulin, TNF- α , and osteoprotegerin (OPG) were measured in serum samples using the insulin mouse ultrasensitive ELISA kit (DRG Instruments, Marburg, Germany) and the mouse TNF- α and OPG ELISA kits (both purchased from R&D System, Minneapolis, MN), according to the manufacturer's instructions. Measurements were performed in duplicates.

Pancreas samples were frozen for RNA extraction and were embedded in paraffin for histological examination. The distribution and morphology of pancreatic islets were assessed by analyzing 4- μ m-thick cross-sectional serial sections after staining with hematoxylin and eosin. Pancreatic islets were manually traced on the computer, and area measurements were performed, using a video-based image analysis program (MCID; Imaging Research, St. Catharines, ON, Canada).

Statistical analysis. Data were calculated and shown as mean \pm SD or as median and interquartile range (IQR), according to the distribution. Comparisons between STZ-treated animals and normal controls were performed with Student t test and with χ^2 test. Differences in parameters mean values across

study phases were analyzed using ANOVA for repeated measures. Statistical significance was defined as $P < 0.05$.

RESULTS

In vitro treatment with recombinant TRAIL upregulates the expression of SOCS1 in PBMCs and human islets. Starting from preliminary cDNA microarray analyses of human PBMC cultures exposed to recombinant TRAIL, which identified SOCS1 as one of the most upregulated genes in response to TRAIL, we validated this observation by quantitative RT-PCR for SOCS1 in both human PBMCs and mouse PBMCs (Fig. 1A). The significant upregulation of the steady-state mRNA levels of SOCS1 was confirmed at the protein level by analyzing SOCS1 protein by Western blot (Fig. 1B). Because SOCS1 is known to downregulate the sensitivity of pancreatic β -cells to interferon- γ (IFN- γ) and TNF- α (7–9), the effect of TRAIL was next analyzed in human pancreatic islets. In vitro treatment with recombinant TRAIL upregulated SOCS1 mRNA expression also in human islets (Fig. 1C).

In vivo injection of recombinant TRAIL ameliorates the glycemic levels in diabetic mice. Having demonstrated that recombinant TRAIL promotes the expression of SOCS1 in cultured human and mouse PBMCs, as well as in purified human islets, next experiments were carried out to evaluate the in vivo effect of TRAIL in a relevant

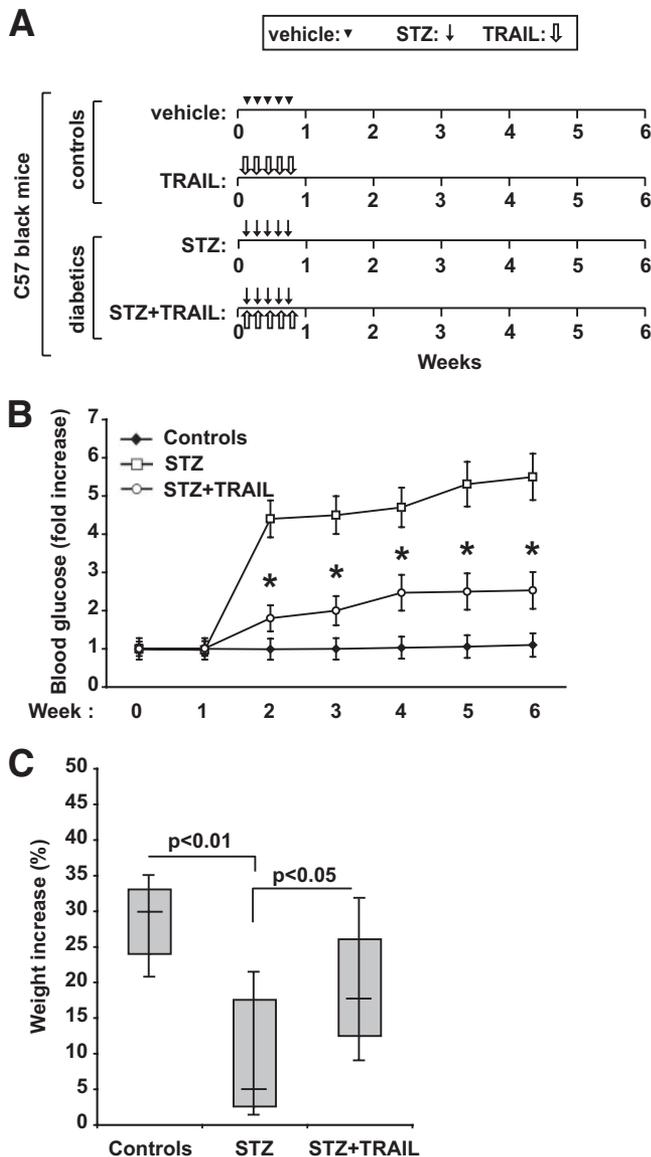


FIG. 2. Effect of in vivo injection of recombinant TRAIL on body weight and glycemia in diabetic mice. **A:** Schematic representation of the treatment protocol for each mice group. **B:** Kinetics of blood glucose levels, reported as fold of increase with respect to time 0, for each group of mice. Results are reported as means. * $P < 0.05$ vs. STZ mice (without TRAIL). **C:** Body weight variation at 6 weeks is reported as percentage of increase with respect with time 0. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, and lines extending from box are 10th and 90th percentiles.

animal model of type 1 diabetes. Five consecutive intraperitoneal daily injections of low-dose (50 mg/kg) STZ were administered to 6-week-old mice ($n = 24$) that were monitored for 6 weeks, in comparison with a group of vehicle-treated control mice ($n = 10$) (Fig. 2A). Half of STZ-injected mice ($n = 12$) also received five intraperitoneal daily injections of recombinant TRAIL (20 μ g/injection) (Fig. 2A). It should be noticed that injection of recombinant TRAIL performed in a group of nondiabetic mice ($n = 10$), following the same schedule of diabetic mice (20 μ g/injection per 5 days, Fig. 2A), did not affect any of the parameters examined in this study (glycemia, body weight, insulinemia). These animals were indistinguishable from vehicle-injected control mice and therefore have been omitted from the figures.

Mice treated with STZ alone displayed a significant increase of blood glucose levels from week 2 onward (Fig. 2B; from 98 + 19 mg/dl at week 0, up to 600 + 15 mg/dl at week 6). Mice co-injected with STZ+TRAIL showed significantly ($P < 0.05$) lower blood glucose levels with respect to the animals injected with STZ alone, although the blood glucose levels of this group remained higher than in controls (Fig. 2B). In addition, mice co-injected with STZ+TRAIL gained significantly ($P < 0.05$) more weight than animals treated with STZ alone, although the body weight of this group of animals remained lower with respect to mock-treated animals (Fig. 2C).

In vivo injection of recombinant TRAIL exhibits anti-inflammatory activity and partially preserves pancreatic islets in diabetic mice. At the end of the experimental period (6 weeks), animals were killed for blood harvesting, to be used for the determination of the circulating levels of insulin, TNF- α , and OPG and for histopathological examination of the pancreas (Fig. 3). While insulinemia was significantly ($P < 0.05$) higher in mice co-injected with STZ+TRAIL with respect to mice injected with STZ alone (Fig. 3A), the levels of the inflammatory markers TNF- α and OPG (8,9,15–17) were lower ($P < 0.05$) in STZ+TRAIL-injected animals (Fig. 3B). Because repeated injections of recombinant TRAIL attenuated hyperglycemia and increased insulinemia in the majority of diabetic mice, we next examined the total area and the morphology of pancreatic islets. While STZ induced a dramatic decrease in the number and size of pancreatic islets (Fig. 3C), the total area of pancreatic islets was significant higher ($P < 0.05$) in STZ+TRAIL-injected animals than in STZ-injected mice (Fig. 3D).

Because the data illustrated above suggested that TRAIL treatment decreases systemic markers of inflammation (TNF- α and OPG), in the last group of experiments, we have analyzed the expression of VCAM-1, a well-defined marker of insulinitis (9), and SOCS1 at the pancreatic level. In vivo treatment with recombinant TRAIL significantly decreased the mRNA steady-state levels of pancreatic VCAM-1 (Fig. 4). On the other hand, similarly to what was observed in vitro (Fig. 1), SOCS1 mRNA levels were higher in the pancreas of animals treated with STZ+TRAIL than in animals treated with STZ alone (Fig. 4).

DISCUSSION

In type 1 diabetes, pancreatic islet inflammation contributes to the progressive loss of insulin-producing β -cells, which eventually renders the patients, many of them children or adolescents, insulin dependent for life (18). In this respect, we have demonstrated for the first time that exogenous recombinant TRAIL, co-injected with STZ, significantly reduced the levels of islet damage with respect to animals injected with STZ alone. Consistently with a protective role of recombinant TRAIL, the levels of insulinemia were significantly ($P < 0.05$) higher in mice co-injected with STZ+TRAIL, and STZ+TRAIL-treated animals showed reduced levels of hyperglycemia throughout the whole period of the study with respect to STZ-injected animals. Moreover, in keeping with previous studies demonstrating that TRAIL displays anti-inflammatory activities in vitro (19,20), the in vivo injection of recombinant TRAIL significantly decreased inflammatory markers, both at the systemic (TNF- α and OPG) and pancreatic (VCAM-1) levels.

The hypothesis that TRAIL might ameliorate STZ-in-

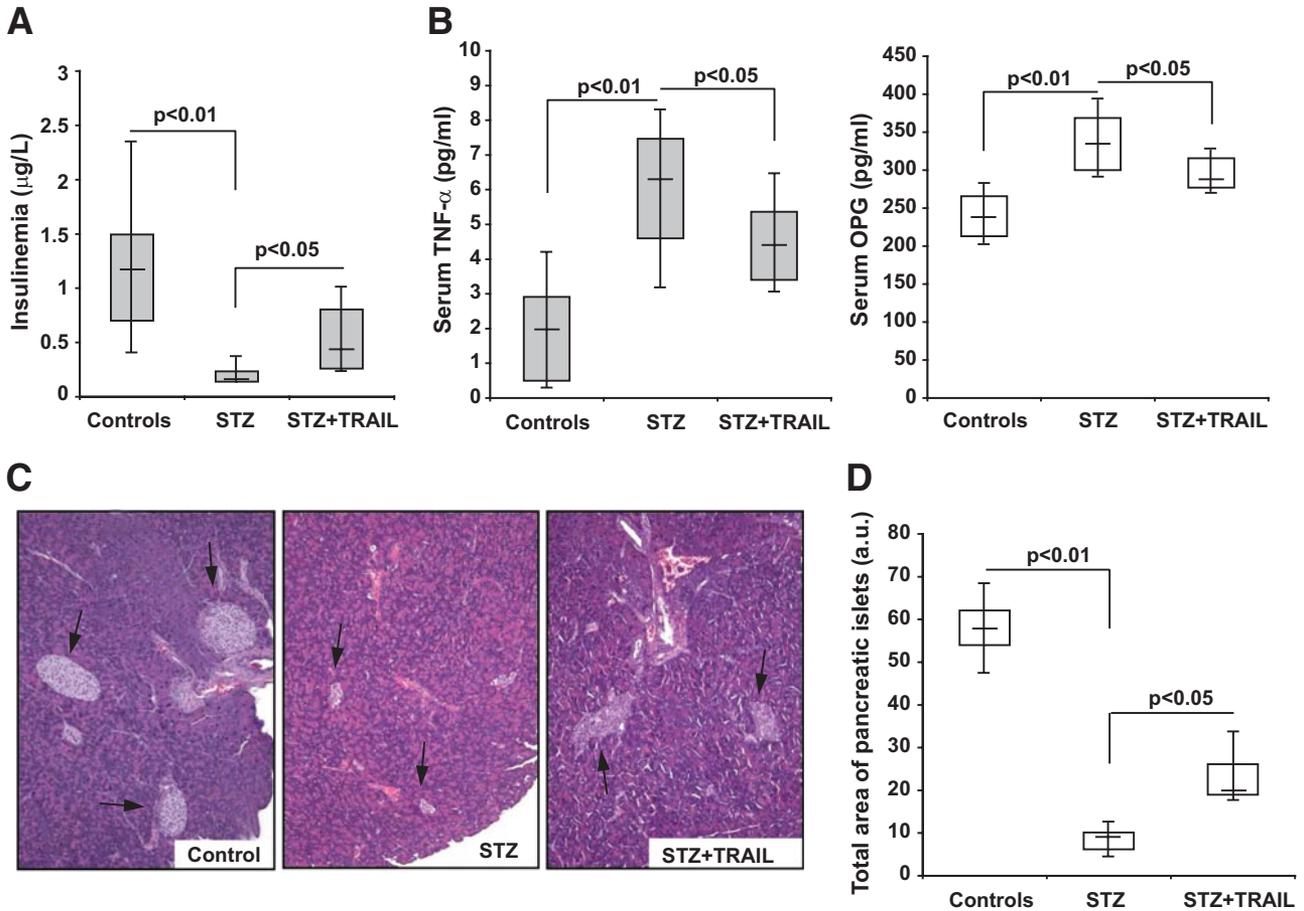


FIG. 3. Effect of in vivo injection of recombinant TRAIL on circulating levels of insulin and inflammatory cytokines in diabetic mice. At the end of the experimental period (6 weeks), blood was harvested for determination of the levels of insulin (A) and TNF- α and OPG (B) by ELISA. In C, representative hematoxylin-eosin-stained histological sections from pancreas of C57 black mice were treated as indicated. Original magnification: 10 \times . Arrows indicate islets. In D, islet area was measured in each mice group, as described in RESEARCH DESIGN AND METHODS, by analyzing at least three independent histological pancreatic sections for each mouse (arbitrary units [a.u.]). In A, B, and D, horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, and lines extending from box are 10th and 90th percentiles. (A high-quality digital representation of this figure is available in the online issue.)

duced insulinitis by modulating the host immune response is consistent with another important finding of our study: the ability of TRAIL to induce SOCS1 expression in both

PBMCs and pancreatic islets. Although we are aware that we have not formally demonstrated the role of SOCS1 in mediating the protective effect of recombinant TRAIL, it is

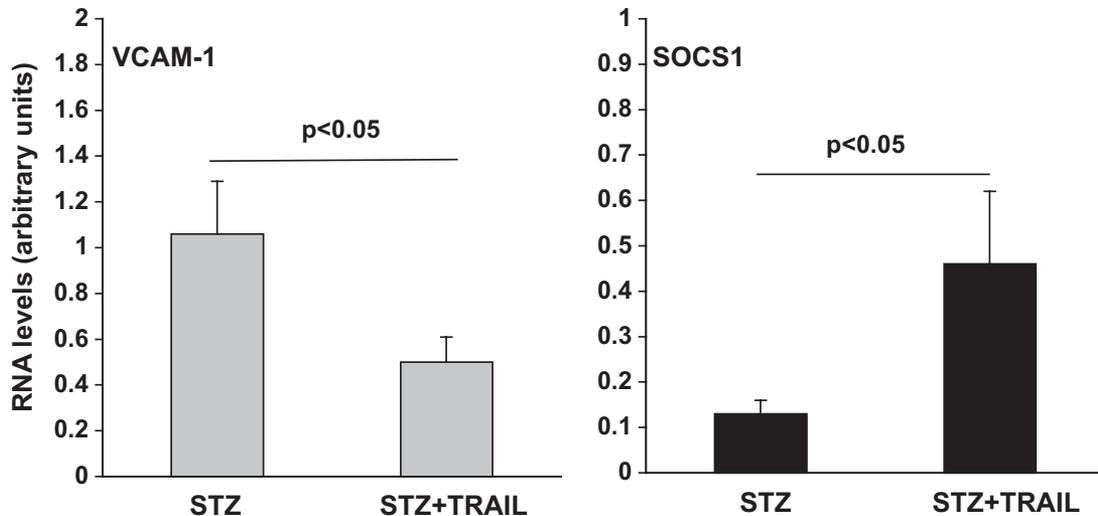


FIG. 4. Effect of in vivo TRAIL treatment on SOCS1 and VCAM-1 RNA levels in the pancreas. Levels of SOCS1 and VCAM-1 were analyzed by quantitative RT-PCR on RNA extracted from pancreas of mice killed at week 6. Data are reported as means \pm SD of quantitative RT-PCR results performed on at least five pancreata for each experimental group.

noteworthy that the SOCS family members play a crucial role in controlling the magnitude and duration of several cytokine signals, which have been involved in the pathogenesis of DMT1, such as in particular TNF- α and IFN- γ (10). Interestingly, we have also shown that recombinant TRAIL might increase SOCS1 expression also at the pancreatic level. This is particularly remarkable, since increasing SOCS1 expression in β -cells has been shown to inhibit TNF-induced Fas expression *in vitro* and prevent the progression to diabetes in NOD mice (21–23), and the overexpression of SOCS1 in islet grafts may inhibit or block the apoptotic pathway and prolong graft survival (22).

Taking into account that 1) proinflammatory cytokines, such as TNF- α and IFN- γ , significantly contribute to initiate β -cell apoptosis in islets; 2) treatment with recombinant TRAIL does not impair the viability of pancreatic islets, even when overexpressed (6); and 3) pancreatic islet transplantation is an important therapeutic option to avoid the need for lifelong hormone injections in type 1 diabetes (24), our current data suggest that systemic treatment with recombinant TRAIL, which is currently under investigation in phase I/II clinical trials for the treatment of several tumors and is usually well tolerated (1), might be considered as a therapeutic option for the treatment of type 1 diabetes. This treatment would be either administered alone in the early onset of type 1 diabetes or in association with islet transplantation in the late phase of type 1 diabetes to attenuate the inflammatory and autoimmune response.

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No potential conflicts of interest relevant to this article were reported.

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