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CONTROL OF THE OF THE FINAL STAGE OF IMMUNE-MEDIATED DIABETES BY ISO-1, AN ANTAGONIST OF MACROPHAGE MIGRATION INHIBITORY FACTOR

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Abstract — We recently showed that attenuation of inflammatory cytokine MIF with pharmacological inhibitor ISO-1 down-regulates the immune-mediated diabetes in mice. Here we explore the effects of MIF neutralization by ISO-1 on the local inflammatory pathway of the disease. In vivo treatment of mice with ISO-1 inhibited the expression of pro-inflammatory cytokines and iNOS in the pancreatic islets. Moreover, ISO-1 affected in vitro cytokine-induced NO production by fibroblasts, endothelial cells, insulinoma cells, and pancreatic islets, and rescued β cells from NO-dependent damage. These results suggest regulatory potential of ISO-1 at the level of the pancreas which can preserve the target tissue from autoimmune attack.

Key words: Macrophage migration inhibitory factor (MIF), autoimmunity, diabetes, (S,R)-3-(4-hydroxyphenil)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), nitric oxide (NO), IL-18, IL-1β

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INTRODUCTION

Insulin dependent type 1 diabetes (T1D) is a chronic inflammatory disorder characterized by the autoimmune destruction of pancreatic β cells. The autoimmune reaction is a multistep process triggered by interactions between immune cells and the islets (reviewed in Kaminitz et al., 2007). Autoimmune insulitis evolves through several discrete stages. Much evidence supports a crucial role for infiltrated immune cells in and around pancreatic islets early in the pathogenesis. Inflammation induces a program of changes within the tissue that promote immune surveillance and recognition. In the process of inflammatory insulitis, there is an accumulation of multiple cytotoxic mediators released by activated macrophages and T cells, other surrounding cells, as well as β cells themselves. Proinflammatory cytokines (such as IL-1β, TNF-α, and IFN-γ) and the free radical nitric oxide (NO) play an important role in the initial destruction of β cells (Cnop et al., 2005). Functional impairment of β cells is induced shortly after exposure to cytokines, and an active process takes place at the β cell level, representing a race between deleterious and protective mechanisms. In this process cytokine production from target cells is specifically required for the transition from insulitis to diabetes (Flodstrom–Tullberg et al., 2003). When the interaction between immune cells and islets crosses a certain point of inflammatory insulitis, it becomes difficult to control the pathogenic pathways and the autoimmune reaction culminates in β cell death and overt diabetes. So far, efforts to identify immunotherapeutic approaches in treatment of T1D have enjoyed limited success. Although results obtained with islet transplantation are promising, interfering with the deleterious autoimmune response by using immunomodulating agents that can target multiple checkpoints or mechanisms within the inflammatory cascade still remains a conceivable strategy for disease treatment.

One of the cytokines involved in the inflamma-

tory cascade during the progression of experimental autoimmune diabetes is macrophage migration inhibitory factor (MIF) (Cvetkovic et al., 2005; Stosic–Grujicic et al., 2008). This is an evolutionarily conserved 12.5-kDa protein mediator with multiple functions in innate and acquired immunity (reviewed in Bernhagen et al., 1998; Cvetkovic and Stosic–Grujicic, 2006). MIF is unique among cytokines because it exhibits certain endocrine properties and has enzymatic activity. MIF has been shown to regulate the expression of numerous genes that play important roles in cellular stress responses, cell growth, and apoptosis. Upon secretion, MIF acts as a typical inflammatory cytokine. As such, MIF is an attractive therapeutic target for the treatment of immunoinflammatory disorders, including diabetes. Thus, blocking MIF or its receptor CD74 may provide new target-specific therapies. In the search for new immunomodulatory drugs, a novel compound, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester or ISO-1, was recently synthesized which was capable of antagonizing MIF activity (Lubetsky et al., 2002). *In vivo* studies, including our own, demonstrated beneficial effects of ISO-1 in prevention and early treatment of a murine model of immune-mediated diabetes (Cvetkovic et al., 2005), experimental autoimmune neuritis (Nicoletti et al., 2005), and sepsis (Al–Abed et al., 2005). As we have shown, ISO-1 dampened immunoinflammatory diabetogenic processes at multiple levels, including autoreactive lymphocyte propagation, adhesive cell-cell interactions, and peripheral secretion of proinflammatory mediators (Cvetkovic et al., 2005). However, realization of MIF's potential as a drug target for T1D prevention also depends on an understanding of the MIF-governed events during the final stages of disease evolution within the target tissue. Our initial observation that MIF negation with ISO-1 protects mice from immunoinflammatory diabetes by impairing peripheral immunological circuits outside the pancreas (Cvetkovic et al., 2005) led to current studies aimed at elucidating MIF-mediated functional interactions between immune cell targets within the local environment. In the present study, we evaluated the direct effects of MIF inhibition by ISO-1 on the local inflammatory pathways in pancreatic islets under conditions that resemble autoimmune T1D. We show that ISO-1 interferes with local cytokine-mediated NO production (which otherwise leads to diabetes progression), thereby suggesting a potentially effective strategy for β cell protection.

MATERIALS AND METHODS

Reagents and drugs

Streptozotocin (STZ, S-0130), collagenase type XI, sulfanilamide, naphthylethylenediamine dihydrochloride, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and fluorescein diacetate (FDA) were purchased from Sigma (St. Louis, MO). Percoll was from Pharmacia (Uppsala, Sweden). RPMI-1640 medium was supplemented with 1 mM Hepes buffer, 5% fetal calf serum (FCS), 1% sodium pyruvate, and 2 mM l-glutamine (all from Flow Laboratories, Irvine, GB); and penicillin/streptomycin and $5x10^{-5}$ M 2-mercaptoethanol (Sigma). (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) was synthesized as described elsewhere (Lybetsky et al., 2002). Recombinant cytokines IFN-γ, IL-1β and TNF-α were from Sigma.

Animals and treatment

Immunoinflammatory (MLD-STZ) diabetes induction and *in vivo* treatment with ISO-1 were performed in CBA/H mice exactly as described elsewhere (Cvetkovic et al., 2005). In brief, STZ was injected i.p. at doses of 40 mg/kg daily for 5 consecutive days. To evaluate the effect of ISO-1, the drug was administered as a continuous 14-day treatment by i.p. injection at a dose of 1 mg/mouse, starting 3 days before the first STZ injection. The handling of animals and the study protocol were in accordance with international guidelines and approved by the local ethical committee.

Cell cultures

Resident peritoneal cells (PC) and pancreases were collected from individual mice given MLD-STZ and treated with either ISO-1 or its vehicle, on day 15 after the first STZ injection, as well as from normal,

untreated mice. The pancreatic islets were isolated by collagenase digestion and Percoll gradient purification as previously described (Stosic–Grujicic et al., 2001). Cell culture supernatants used for *ex vivo* detection of NO production by macrophages were obtained by co-culturing the cells for 5 days in 24-well Limbro culture plates in 1 ml of standard medium ($1x10^5$ macrophages and 100 islets/well). For *ex vivo* detection of cytokine secretion by pancreatic islets, islets were isolated from *in vivo* treated mice and cultured in medium in 24-well flat-bottom plates (100 pancreatic islets/well) for 48 h.

Syngeneic microvascular endothelial cells were prepared as described by Issecutz (1992). Syngeneic pancreatic fibroblasts were obtained according to Pechold et al. (1997). The L929 mouse fibrosarcoma cell line was from the European Collection of Animal Cell Cultures (Salisbury, UK). Rat insulinoma RINm5F (RIN) cells and mouse insulinoma MIN6 (MIN) cells were kindly donated by Dr. Karsten Buschard (Bartholin Instituttet, Copenhagen, Denmark). For the experiments, cells were detached by trypsinization and incubated overnight in 96-well flat-bottom plates ($6x10^4$ L929 cells/well, $4x10^4$ fibroblasts and endothelial cells/well, $8x10^4$ RIN and MIN cells/well) or in 24-well flat-bottom plates (100 pancreatic islets/well), then stimulated with a cytokine combination (IFN-γ + IL-1β, 5 μg/ml of each cytokine) in the presence or absence of various concentrations of ISO-1, as indicated in Results. Cell culture supernatants used for *ex vivo* and *in vitro* detection of cytokines or NO were collected after 48 h, whereas NO production in pancreatic islet cell supernatants was measured after 5 days of cultivation. Subsequently, the viability of cultured β cells was determined in some experiments.

Assessment of cytokine and iNOS expression by intracellular staining

Immunohistochemical examination of pancreases was performed from day 10 to day 52 after MLD-STZ treatment. Pancreases were fixed in 10% buffered formalin and routinely embedded in wax. Deparaffinized sections were boiled in a 750-W microwave in 0.01 M sodium citrate buffer (pH 6.0) to retrieve antigen. After blocking of endogenous peroxidase with 3% H_2O_2 in methanol, the slides were incubated for 1 h with the appropriate dilution of primary antibodies, followed by treatment with the ExtrAvidin rabbit peroxidase staining kit (Sigma) according to the manufacturer's instructions. Primary antibodies were as follows: rabbit anti-TNF-α, anti-IL-1β, and anti-iNOS IgG were from Sigma; and rabbit anti-IL-18 was kindly donated by Dr. C. A. Dinarello, University of Colorado Health Science Center, Denver, CO. Diaminobenzidine was used as a color substrate.

The expression of iNOS in RIN cells was determined by cell-based ELISA, as previously described (Cvetkovic et al., 2005). Briefly, RIN cells ($8x10^4$ cells/well) were allowed to adhere to poly-L-lysine-precoated 96-well microplates. After fixation with 4% paraformaldehide and washing with 0.1% Triton X-100 in PBS, endogenous peroxidase was quenched with 1% H_2O_2, and the reaction was blocked with 10% FCS. The cells were incubated for 1 h with anti-iNOS IgG, and then with goat anti-rabbit Ig(H+L)-horseradish peroxidase. After washing, the cells were incubated for 15 min in the dark with O-phenylenediamine dihydrochloride (0.4 mg/ml), $Na_2HPO_4{\cdot}2H_2O$ (11.8 mg/ml), citric acid (7.3 mg/ml), and 0.015% H_2O_2. The reaction was stopped with 3 N HCl, and the absorbance was measured in a microplate reader at 492 nm.

Assessment of cytokines and NO production in culture supernatants

Cell culture supernatant samples were analyzed in duplicate for IL-12, IL-1β, TNF-α, IFN-γ, and IL-17 by ELISA using anti-mouse paired antibodies (R&D System) according to the manufacturer's instructions.

As an indicator of NO formation, the concentration of the stable NO oxidation product nitrite in the culture supernatant was determined and averaged from triplicate wells using the one-step Griess reagent assay (Stosic–Grujicic et al., 2001) and compared with nitrite standards.

Determination of β cell viability by MTT and FDA assay

Assessment of the viability of MIN and RIN

insulinoma cells was performed exactly as previously described, by FDA assay (Stosic–Grujicic et al., 2007) and MTT assay (Miljkovic et al., 2005), respectively. In the FDA assay, the nonfluorescent molecule FDA is hydrolyzed inside viable cells to fluorescein by the intracellular esterases, whereas hydrolysis is reduced in cells undergoing apoptosis. To discriminate living and dead cells at the end of incubation, MIN cells were pulsed with FDA for an additional 20 min and analyzed on a FACSCalibur flow cytometer using CellQuestPro software (Becton Dickinson, Heidelberg, Germany). FDA-positive cells were considered viable. In the MTT assay, cell respiration, as an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan. Briefly, at the end of incubation, RIN cells were pulsed for an additional hour with 0.5 mg/ml MTT, the culture medium aspirated, and the cells lyzed in DMSO. The conversion of MTT to formazan was monitored on an automated microplate reader at 570 nm.

Statistical analysis

Results are shown as mean values ± S.D. The statistical difference between groups was evaluated using an unpaired Student's *t* test. A level of $p < 0.05$ was considered to be significant.

RESULTS

Our previous study showed that MIF inhibition by *in vivo* administration of ISO-1 produced nearly complete protection against MLD-STZ-induced clinical diabetes in susceptible mouse strains (Cvetkovic et al., 2005). To evaluate the impact of *in vivo* treatment with ISO-1 on immunopathological changes at the level of the target tissue, in the present study we performed immunohistochemical analysis of pancreases in mice treated with MLD-STZ or MLD-STZ + ISO-1 and examined the local expression of proinflammatory mediators. Cytokines predominantly impair β cell function and cause β cell death via up-regulation of the inducible form of NO synthase (iNOS) expression and subsequent NO production (Chambers et al., 2008). We therefore measured the expression of proinflammatory cytokines, as well as iNOS.

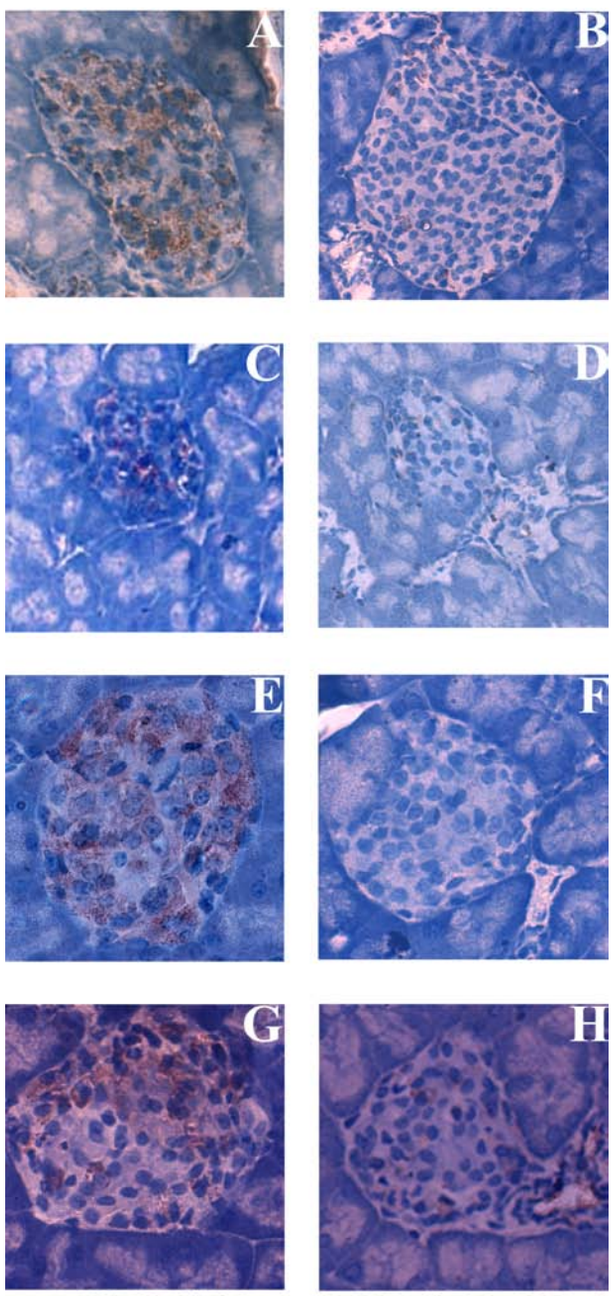

Fig. 1. Immunohistochemical profile of pro-inflammatory molecules of pancreatic islets.
Sections from the pancreas are immunostained for IL-18 (A, B) IL-1β (C, D), TNF-α (E, F), and iNOS (G, H) as indicated in Materials and Methods.
Photos are paired and represent the staining pattern of day-20 control MLD-STZ-treated mice (left panels) vs. MLD-STZ plus ISO-1-treated mice (right panels). Typical sections are shown.

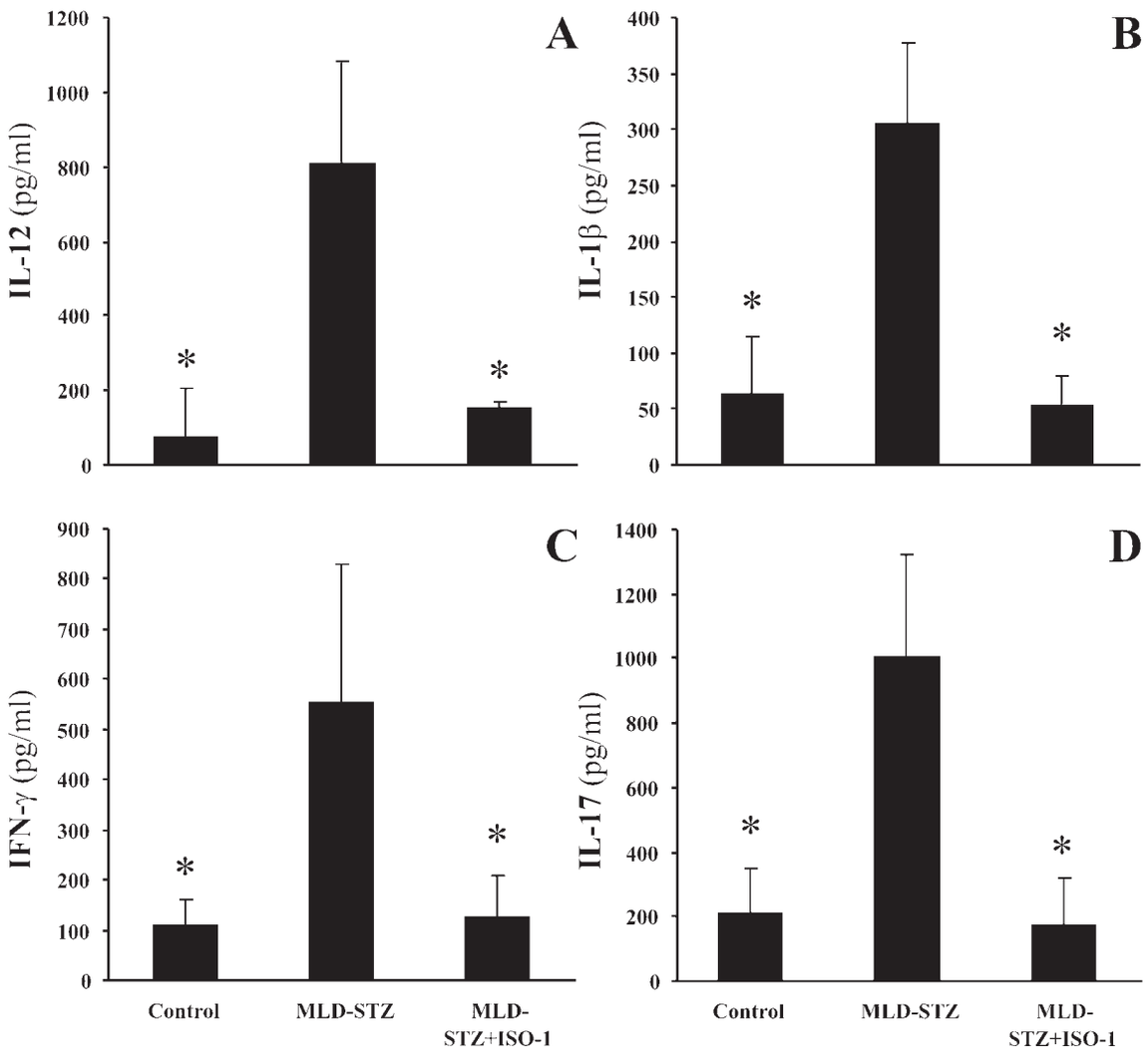

Fig. 2. Pro-inflammatory cytokine protein levels in culture supernatants of isolated pancreatic islets. Pancreatic islets were isolated from day-15 control MLD-STZ-treated mice and from MLD-STZ plus ISO-1-treated mice. IL-12 (A), IL-1β (B), IFN-γ (C), and IL-17 (D) cytokine production was determined by ELISA in 48-h culture supernatants as indicated in Materials and Methods. Each column represents the mean value of five mice per each group; bars indicate SD. * $P < 0.05$ refers to corresponding MLD-STZ-treated control mice.

During MLD-STZ-induced diabetes, in control diabetic mice MNC infiltrates of endocrine pancreas were observed as early as day 10 of the disease, accompanied by scattered IL-18, IL-1β, TNF-α, and iNOS-positive cell staining (not shown). Days 20 and 30 of disease evolution were characterized by more prominent expression of the proinflammatory mediators tested (Fig. 1A-D) and islet necrosis. From this period up to the end of disease monitoring (7 weeks), the destructive process progressed, resulting in islet atrophy. In contrast, administration of ISO-1 concomitantly with MLD-STZ markedly reduced the expression of IL-18, a cytokine responsible for IFN-γ production, as well as that of IL-1β and TNF-α (Fig. 1C-F) in pancreatic islets of these animals. In addition, the expression of iNOS was

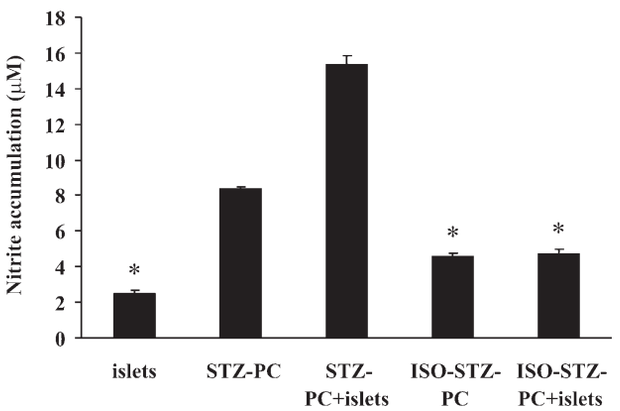

Fig. 3. Effect of in vivo treatment with ISO-1 on macrophage-derived NO production. Peritoneal cells isolated from day-15 control MLD-STZ-treated mice (STZ-PC) or MLD-STZ plus ISO-1-treated mice (ISO-STZ-PC) were cultured alone or in the presence of normal pancreatic islets (islets) for 7 days. Subsequently, nitrite accumulation in cell culture supernatants was determined as indicated in Materials and Methods. Each column represents the mean ± SD for five animals per group. * $P < 0.05$ refers to control PC cultures without ISO-1.

also down-regulated by ISO-1 treatment (Fig. 1H). In conformity with immunohistochemical results, *ex vivo* analysis of proinflammatory cytokine secretion confirmed a marked reduction of IL-12, IL-1β, IFN-γ, and IL-17 in supernatants of pancreatic islets isolated from MLD-STZ+ISO-1-treated mice (Fig. 2). Thus, the drug treatment suppressed immune-mediated destruction and preserved numerous islets from further autoimmune attack.

Total cytokine and NO production of the inflamed islets might originate from both β cells and associated cells of the pancreatic milieu, such as activated macrophages, capillary islet endothelial cells, and/or fibroblasts. To further dissect the effects of ISO-1 on local inflammatory pathways, we assessed the production of inflammatory mediators by both endocrine and nonendocrine cell preparations. Since macrophages are the first cells to accumulate in islets during pathogenesis of T1D, we examined the influence of *in vivo* treatment with ISO-1 on macrophage-derived NO production. In order to test macrophage activity, PC isolated from ISO-1- and/or MLD-STZ-treated mice just before the onset of diabetes (day 15 of diabetes induction) were co-cultured with normal pancreatic islets as a target tissue, and NO release was determined from nitrite accumulation in the culture medium. The results clearly show (Fig. 3) that peritoneal macrophages isolated from MLD-STZ-treated mice produced high levels of NO, while macrophages from ISO-1-treated mice exhibited impaired NO production. After co-culture with

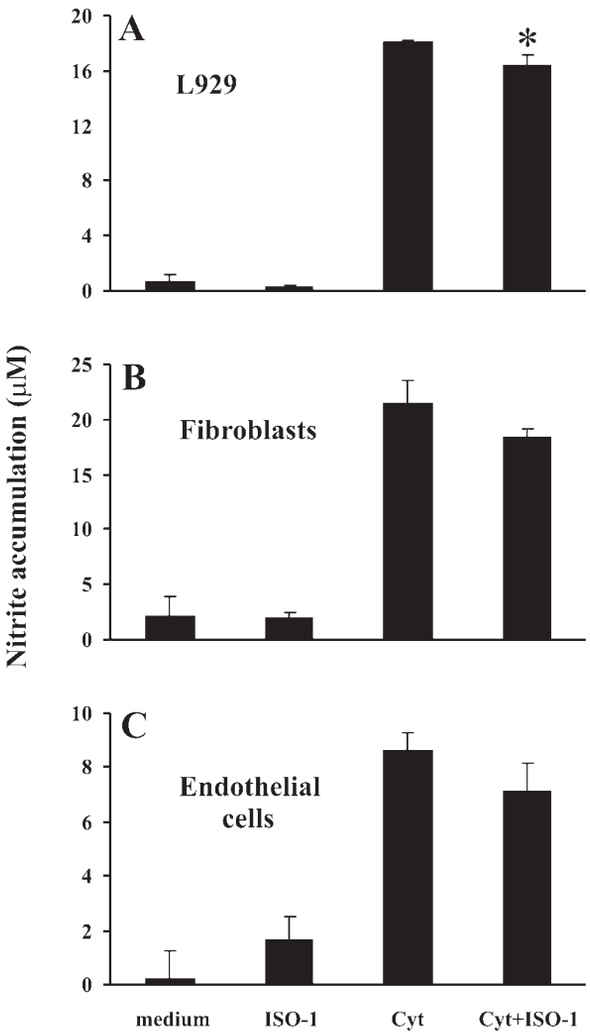

Fig. 4. *In vitro* effects of ISO-1 on NO production of the L929 fibroblast cell line, primary fibroblasts, and primary endothelial cells. (A) L929 fibroblasts (6x104 cells/well), (B) primary fibroblasts (4x104 cells/well), and (C) primary endothelial cells (4x104 cells/well) were cultured overnight, then stimulated with 5 μg/ml of recombinant IL-1β + IFN-γ (Cyt) in the presence or absence of ISO-1 (100 μg/ml) for the next 48 h. Control cells were cultured alone (medium) or in the presence of ISO-1. Nitrite accumulation in cell culture supernatants was subsequently determined as indicated in Materials and Methods. Each column represents the mean ± SD for triplicate cultures. * $P < 0.05$ refers to control cultures without ISO-1.

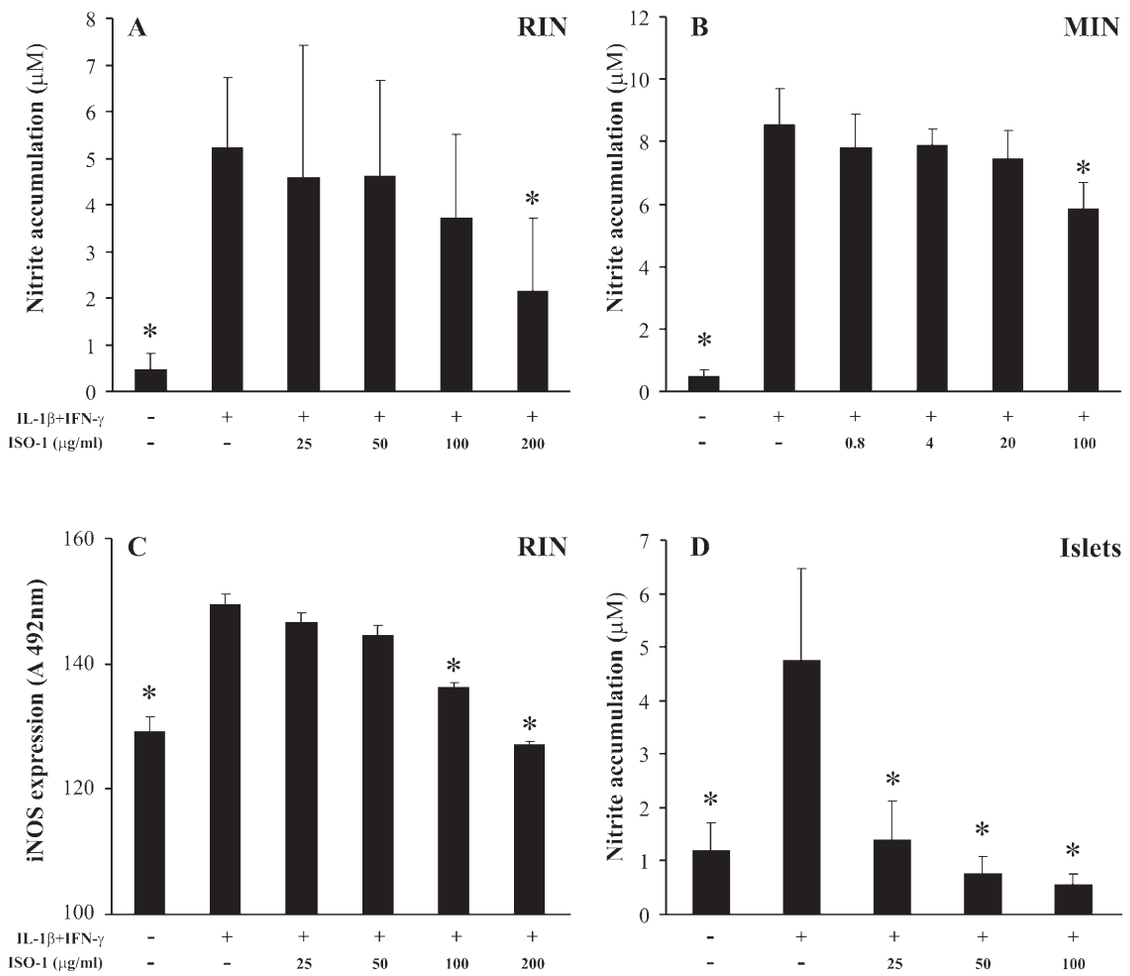

Fig. 5. In vitro effects of ISO-1 on the expression of iNOS and NO production of β cell lines and islet β cells. (A, C) RIN-m5F insulinoma cells (8x104 cells/well), (B) MIN6 insulinoma cells (8x104 cells/well), and (D) pancreatic islets (100 islets/well) were cultured overnight, then stimulated with 5 μg/ml of recombinant IL-1β + IFN-γ (Cyt) in the presence or absence of various concentrations of ISO-1 for the next 48 h (A, B, C) or for 5 days (D). Nitrite accumulation in cell culture supernatants (A, B, D) subsequently was determined as indicated in Materials and Methods. iNOS expression (C) was determined by cell-based ELISA as indicated in Materials and Methods. Each column represents the mean ± SD for triplicate cultures. * $P < 0.05$ refers to control cytokine-stimulated cultures without ISO-1.

pancreatic islets, MLD-STZ-derived macrophages further increased their production of NO, whereas ISO-1-treated MLD-STZ-derived macrophages did not respond to islets and produced the same level of NO as when cultured alone.

In order to see whether ISO-1 directly interferes with MIF-mediated stimulation of NO production in the pancreatic islets, NO production was determined *in vitro* where a combination of inflammatory cytokines was used as a stimulus. We tested the direct *in vitro* effects of ISO-1 on production of NO by endothelial cells, fibroblasts, and β cells. To this end, these different cell preparations were stimulated with a cytokine mixture of IL-1β and IFN-γ to mimic the inflammatory environment within islets attacked by activated mononuclear cells. As expected, exposure to a cytokine mixture of cell monolayers of the L929 fibroblast cell line, primary fibroblasts, and primary endothelial cells significantly increased mean nitrite accumulation above control levels (Fig. 4A-C). The addition of ISO-1 slightly

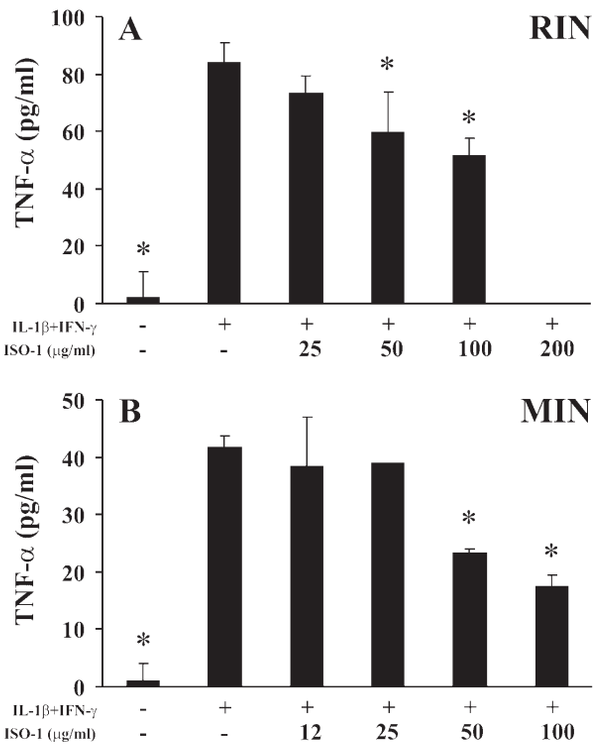

Fig. 6. *In vitro* effects of ISO-1 on the TNF-α production of β cell lines. (A) RIN-m5F and (B) MIN6 insulinoma cells (8x104 cells/well) were cultured overnight, then stimulated with 5 μg/ml of recombinant IL-1β + IFN-γ (Cyt) in the presence or absence of various concentrations of ISO-1 for the next 48 h. Secretion of TNF-α was subsequently determined in cell culture supernatants by ELISA. Each column represents the mean ± SD for triplicate cultures. * $P < 0.05$ refers to control cytokine-stimulated cultures without ISO-1.

inhibited this cytokine-induced NO production, but the differences between ISO-1-treated and nontreated cells reached statistical significance only in L929 cells (Fig. 4A-C). We next examined the efficiency of ISO-1 in modulating NO production in β cells by using rat RIN and mouse MIN insulinoma cell lines as representatives. Incubation of RIN cells (Fig. 5A) and MIN cells (Fig. 5B) with IL-1β + IFN-γ resulted in significant formation of nitrite, while treatment with ISO-1 inhibited cytokine-induced NO release in both cell lines tested. To examine whether ISO-1 inhibits NO release via suppression of iNOS, changes of iNOS protein were investigated in RIN cells by intracellular staining using cell-based ELISA. Cells stimulated with cytokines alone markedly

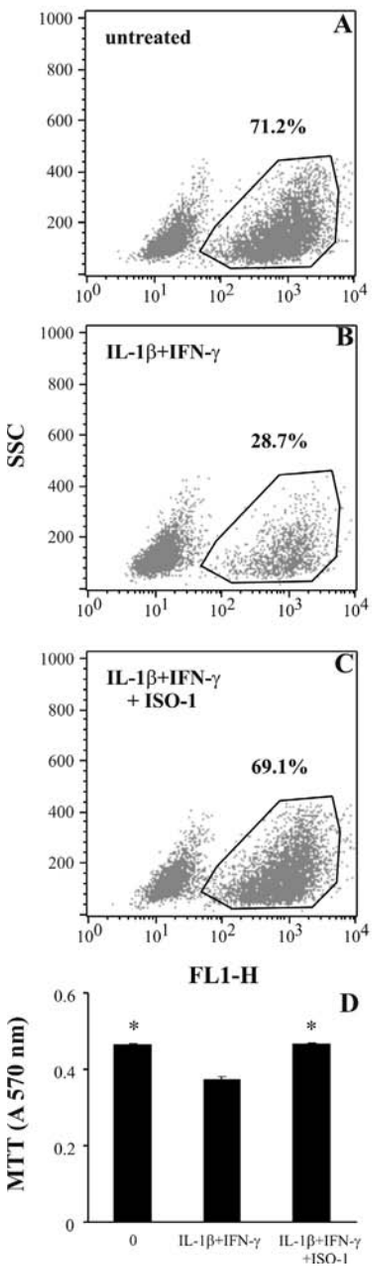

Fig. 7. *In vitro* effects of ISO-1 on survival of β cells. (A-C) MIN6 insulinoma cells (8x104 cells/well) and (D) RIN-m5F insulinoma cells (8x104 cells/well) were cultured overnight, then stimulated with 5 μg/ml of recombinant IL-1β + IFN-γ in the presence or absence of ISO-1 (100 μg/ml). The viability of cells was determined after 48 h by colorimetric FDA assay (A-C) or MTT assay (D), as indicated in Materials and Methods. (A-C) Typical dot plots of flow cytometry analyses are shown. (D) Each column represents the mean ± SD for triplicate cultures. * $P < 0.05$ refers to control cytokine-stimulated cultures without ISO-1.

increased iNOS protein expression in comparison with unstimulated cells, whereas cells treated with cytokines and ISO-1 showed suppressed expression of iNOS (Fig. 5C). Finally, to support the physiological importance of the results obtained in the cell line studies and rule out the possibility that the observed effects were somehow specific for insulinoma cells, we assessed the influence of ISO-1 on NO production using freshly isolated mouse pancreatic islets. Although nitrite accumulation by mouse islets stimulated with IL-1β + IFN-γ was delayed in comparison with insulinoma cells, five-day incubation with a cytokine mixture achieved measurable NO production (Fig. 5D). Similar to results with insulinoma cells, islet treatment with ISO-1 diminished the effects of cytokines and resulted in the basal level of NO production (Fig. 5D).

Besides NO, cytokines produced by β cells could exert autocytotoxic effects. For this reason, we also measured the influence of ISO-1 on TNF-α secretion by insulinoma cells stimulated under the same conditions as in the previous experiments. Compared to the control, the IL-1β + IFN-γ combination induced significant release of TNF-α in both RIN (Fig. 6A) and MIN cells (Fig. 6B). On the other hand, ISO-1 affected in a dose-dependent way the induction of TNF-α in both cell lines (Fig. 6A and B).

Finally, we tested the direct effects of ISO-1 on β cell survival, using both MIN (Fig. 7A-C) and RIN cells (Fig. 7D) for this purpose. In addition to inducing high amounts of NO, treatment with the combination of IL-1β and IFN-γ was apparently toxic for RIN cells, as revealed by MTT assay, whereas ISO-1 significantly reduced cytokine-mediated toxicity (Fig. 7D). Similarly, MIN cells in the presence of ISO-1 were markedly protected from cytokine-induced death, to judge from FDA staining (Fig. 7C).

DISCUSSION

In the current study, we found that treatment of pancreatic islets, insulinoma cells, fibroblasts, and endothelial cells *in vitro* with the pharmacological MIF inhibitor ISO-1 down-regulated cytokine-induced NO production, entailing a direct protective effect on β cells. Furthermore, the ability of ISO-1 to modulate *in vitro* the pattern of cytotoxic mediator generation by potential residents within the pancreas was reflected *in vivo* by down-regulation of macrophage NO-mediated cytotoxic activity and an extinguished inflammatory environment within the pancreas in treated mice. These results suggest that inhibition of the target tissue response substantially contributes to local regulation of the autoimmune process and identify MIF as a stimulator of local inflammatory pathways that acts in a permissive fashion and is able to control the set point of the final stage of disease evolution.

The tissue response to proinflammatory cytokines can promote pathological tissue destruction in autoimmune diabetes (Rabinovitch and Suarez–Pinzon, 1998). These data are complemented by the present results of immunohistochemistry showing *in situ* expression of proinflammatory molecules in the target tissue affected by the disease. Evidence is now emerging which indicates that target cell cytokine responses are required specifically for transition from insulitis to diabetes (for review, see Hill et al., 2007). During evolution of the immune assault, the inflammatory environment acts bidirectionally, further provoking self-perpetuating cycles. In support of complex network interactions, it has been shown that IL-18 is up-regulated in murine β cells *in vitro* upon exposure to IL-1β, TNF-α, and IFN-γ (Frigerio et al., 2002). Furthermore, IL-18 is a potent inducer of IFN-γ, a cytokine which promotes β cell death in combination with TNF-α and IL-1β (Rabinovitch and Suarez–Pinzon, 1998). In this report, we have described how MIF blockade by ISO-1 abrogates target cell cytokine responses responsible for the transition from insulitis to diabetes. Moreover, we demonstrated that both polarizing and downstream cytokines are reduced by ISO-1. This fits well with the already established position of MIF at the pinnacle of the inflammatory cascade, since MIF amplifies the generation of various other proinflammatory mediators involved in functional suppression and destruction of β cells. The local effect of ISO-1 could be attributed to reduced activation of NO-producing inflammatory cells within the islets, as well as to prevention of

the production of iNOS-inducing cytokines such as IL-1β, TNF-α, and IFN-γ. Consequently, decreased production of proinflammatory cytokines and NO generates a less deleterious environment in the islets. Because destructive insulitis is closely associated with Th1-type cytokine and NO production, it is apparent that ISO-1 suppresses T1D by influencing the production of these mediators. Our present observations of reduced pancreatic production of mediators of inflammation after ISO-1 treatment are in line with this. Interestingly, in the present study the resolution of immunoinflammatory diabetes by ISO-1 was associated with the suppression of Th17 products, suggesting that MLD-STZ-induced diabetes is a mixed Th1 and Th17 inflammatory environment. Thus, it seems that downstream effects of MIF include the production not only of IL-1, TNF-α, and IFN-γ, but also of IL-17. This is in accordance with expanding diversity of effector T cell lineages in tissues, which has been linked to autoimmune inflammation (Weaver et al., 2007). Furthermore, we recently showed that IL-17 is an additional factor acting in concert with other inflammatory cytokines to induce nitric oxide synthase-dependent toxicity in mouse beta cells (Miljkovic et al., 2005). In view of the detrimental effects of NO in immune-mediated diabetes, of special interest is the reduced NO generation that we observed after ISO-1 treatment *in vitro*.

It is well recognized that the nitrogen-based free radicals induced by proinflammatory cytokines are involved in the pathological processes leading to β cell dysfunction and death (Chambers et al., 2008). Moreover, in the presence of inflammatory cytokines, NO is a strong predictor of islet cell cytotoxicity (Thomas et al., 2002; Chambers et al., 2008). It is reasonable to expect that the beneficial impact of ISO-1 treatment may result from modulation of the iNOS pathway. Indeed, in this work we have shown that ISO-1 reduces iNOS expression and NO generation in the pancreatic islets, thus entailing a direct protective effect on β cells. In our experimental model, the cellular sources of inflammatory mediators may be both endocrine and nonendocrine cells. *In vivo*, β cells are located in proximity to capillary islet endothelial cells and fibroblasts. Much evidence suggests that the main source of NO after islet stimulation by cytokines are the nonendocrine cells present in and around the islets, such as macrophages, endothelial cells, fibroblasts, and ductal cells (Cantor and Haskins, 2007; Steiner et al., 1997; Pavlovic et al., 1999). In addition to this, it has been shown that β cells themselves contribute some of the cytokines and NO that provoke the immune reaction within the islets (Thomas et al., 2002). In the current study, we found that treatment with ISO-1 influenced inflammatory cytokine and NO production by both nonendocrine cells (macrophages, endothelial cells, and fibroblasts) and pancreatic β cells. This is in line with our previous reports indicating that MIF is necessary for progression of autoimmune diabetes mellitus (Cvetkovic et al., 2005; Stosic–Grujicic et al., 2008) and further suggests that inhibition of MIF in the target organ (the pancreas) may promote the local counter-regulatory pathways that ensure the resolution of inflammation and protect against tissue damage.

Although at physiological concentrations, endogenous free radicals are presumed to play roles in cell signaling (Droge, 2002), they clearly possess the capacity to behave in a destructive fashion. One of the unique features of pancreatic β cells is their relatively low expression and activity of the enzymes involved in antioxidant defense, which renders β cells highly susceptible to NO/ROS-induced damage (Rabinovitch and Suarez–Pinzon, 1998). Although it is well established that high local NO production contributes to the induction of a death cascade in β cells, the role of NO in the mechanism responsible for the loss of β cell viability in response to cytokines is still debatable. Cytokines appear to kill β cells by NO-dependent necrosis that is caspase-independent (Chambers et al., 2008). On the other hand, NO is a mediator that combines with IL-1-β and IFN-γ to cause β cell death by apoptosis that is partly mediated by caspase-3 (Thomas et al., 2002). Consistent with these findings, we recently demonstrated that systemic MIF deletion negatively regulated the NO-induced pathway of apoptosis within the islets (Stosic–Grujicic et al., 2008). In the present study, we provide further evidence indicating that ISO-1 treatment *in vitro*

results in suppression of local cytokine-induced iNOS expression and high output NO production that could otherwise contribute to the impairment of β cell survival. Further studies on molecular interactions may provide an improved understanding of the mechanisms underlying this inhibition.

Current studies suggest that strategies seeking to protect β cells from immune-mediated injury (i.e., attempts to prevent or reverse T1D) might benefit by the administration of ISO-1. Progressive β cell failure and loss is thought to represent a key event in the pathogenesis not only of T1D, but also of T2D. Although the signals and their pathways for β cell death in these two disorders are different (Cnop et al., 2005), NO may play an important role in both diseases. Given that in the present study we demonstrated that treatment with ISO-1 can prevent local NO production, these results may imply a potential of ISO-1 for the treatment of T2D as well.

Collectively, our work points to MIF as an important mediator in the pathogenesis of T1D. MIF's position within the inflammatory cascade may be not only to control the magnitude of the systemic inflammatory response, but also to act in a permissive fashion to control the set point of the final stage of disease evolution leading to destructive insulitis and overt diabetes. It follows that inhibition of MIF's action by ISO-1 may be a powerful pharmacological strategy for the treatment of T1D and similar inflammatory and autoimmune diseases. We believe these studies provide a strong rationale for additional work seeking to identify the potential role of ISO-1 in the pathogenesis of T1D (and possibly T2D) and justify examination of the potential therapeutic benefits of ISO-1 administration for reversing and/or preventing these disorders.

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КОНТРОЛА ФИНАЛНОГ СТАДИЈУМА ИМУНСКИ ПОСРЕДОВАНОГ ДИЈАБЕТЕСА ПОМОЋУ АНТАГОНИСТА ФАКТОРА ИНХИБИЦИЈЕ МИГРАЦИЈЕ МАКРОФАГА (S,R)-3-(4-ХИДРОКСИФЕНИЛ)-4,5-ДИХИДРО-5-ИЗОКСАЗОЛ МЕТИЛ ЕСТРА СИРЋЕТНЕ КИСЕЛИНЕ

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Недавно смо показали да неутрализација инфламаторног цитокина MIF-а фармаколошким инхибитором ISO-1 негативно регулише имунски посредован дијабетес мишева. У овом раду испитивали смо ефекте неутрализације MIF-а помоћу ISO-1 на локалне инфламаторне

путеве болести. In vivo третман мишева помоћу ISO-1 је инхибирао експресију проинфламаторних цитокина и индуцибилне синтазе азот моноксида у острвцима панкреаса. Штавише, ISO-1 је реметио in vitro продукцију азот моноксида индуковану цитокинима у фибробластима, ћелијама ендотела, инсулиномама и панкреасних острваца и тако сачувао бета ћелије од оштећења изазваних азот моноксидом. Ови резултати указују на регулаторни потенцијал ISO-1 на нивоу циљног ткива који штити циљно ткиво од аутоимуног атака.