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Critical Role of Macrophage Migration Inhibitory Factor Activity in Experimental Autoimmune Diabetes

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Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that plays a pivotal role in several immunoinflammatory and autoimmune diseases. In this study we examined the role of MIF in the development of immunoinflammatory diabetes induced in susceptible strains of mice by multiple low doses of streptozotocin. We found that MIF protein was significantly elevated in islet cells during the development of diabetes, and that targeting MIF activity with either neutralizing antibody or the pharmacological inhibitor (*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester, markedly reduced clinical and histopathological features of the disease, such as hyperglycemia and insulitis. Lymphocytes from mice treated with the MIF inhibitors exhibited reduction of both islet antigen-specific proliferative responses and adhesive cell-cell interactions. Neutralization of MIF also down-regulated the *ex vivo* secretion of the proinflammatory mediators, TNF-α, interferon-γ, and nitric oxide, while augmenting that of the antiinflammatory cytokine, IL-10. This study provides the first *in vivo* evidence for a critical role for MIF in the immune-mediated β-cell destruction in an animal model of human type 1 diabetes mellitus and identifies a new therapeutic strategy for the prevention and treatment of this disease in humans that is based on the selective inhibition of MIF activity. (*Endocrinology* 146: 2942–2951, 2005)

TYPE 1 DIABETES MELLITUS (T1D) is a multifactorial disorder caused by the lack of endogenous insulin that is thought to be a consequence of an immune attack mediated by autoreactive T cells and macrophages against pancreatic β-cells. The disease afflicts approximately 4 million people in North America, and epidemiological data concur that the incidence and prevalence of the disease are increasing worldwide (1). The key role played by the immune system in the pathogenesis of the disease has focused much attention on identifying immunotherapeutical approaches that may halt or delay β-cell destruction in prediabetic individuals or those patients with newly diagnosed disease (2). However, although research efforts carried out in both humans and animal models have greatly expanded understanding of the disease pathogenesis, no effective antiinflammatory therapeutics have been approved for the clinical management of T1D (2).

Preclinical models of human T1D, such as the nonobese diabetic (NOD) mouse, the diabetes-prone BB rat, and the mouse made diabetic with multiple low doses of streptozotocin (MLD-STZ), have extensively been used as *in vivo* tools for gaining insights into pathogenic mechanisms and for the screening of immunomodulatory compounds endowed with antidiabetogenic properties worthy of being considered for translation to the clinical setting (3–5).

In particular, repeated injections of susceptible strains of mice with MLD-STZ provokes a condition with clinical, histological, and immunopathogenic characteristics resembling human T1D, including the development of hyperglycemia associated with infiltration of pancreatic islets by T lymphocytes and macrophages (insulitis) (3, 4). As in the NOD mouse, in the diabetes-prone BB rat and probably in humans the immunoinflammatory diabetogenic process triggered by MLD-STZ appears to be related to the preferential production from the islet-infiltrating mononuclear cells of type 1 proinflammatory cytokines IL-1β, interferon-γ (IFN-γ), TNF-α, IL-12, and IL-18 (6–11).

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine produced during immune responses by activated T cells, macrophages, and a variety of nonimmune cells (reviewed in Refs. 12 and 13). Constitutive expression of MIF mRNA and protein is found in various nonimmune cells within normal tissue, such as anterior pituitary cells (14); cardiac myocytes (15); parenchymal cells within liver, brain, or kidneys (16–18); or pancreatic islet β-cells (19). Notably, in many of those tissues the expression and release of MIF are significantly up-regulated under various pathological conditions, such as atherosclerosis, glomerulonephritis, multiple sclerosis, colitis, type 2 diabetes, and pancreatitis

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Abbreviations: Ab, Antibody; Ag, antigen; DM, diabetes mellitus; IFN-γ, interferon-γ; IL-2R, IL-2 receptor; iNOS, inducible nitric oxide synthase; ISO-1, (*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; MIF, macrophage migration inhibitory factor; MLD-STZ, multiple low doses of streptozotocin; NO, nitric oxide; NOD, nonobese diabetic; PC, peritoneal cell; SMNC, spleen mononuclear cell; SN, supernatant; T1D, type 1 diabetes; T/PBS, 0.1% Triton X-100 in PBS.

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(12, 20), thus implicating a role for MIF in the disease process. Indeed, recent studies using neutralizing antibodies (Ab) or MIF-deficient animals demonstrated that MIF is a crucial mediator of several immunoinflammatory disorders in rodents, including Gram-negative and Gram-positive sepsis (reviewed in Ref. 13), delayed-type hypersensitivity (21), leishmaniasis (22), glomerulonephritis (18), arthritis (23), experimental autoimmune encephalomyelitis (24), experimental autoimmune myocarditis (15), and colitis (25).

In contrast to this emerging evidence on the pivotal role of MIF in autoimmune diseases, its role in the pathogenesis of human T1D is still unclear. Although elevated MIF gene expression has been detected in spontaneously diabetic NOD mice during development of the disease, and exogenously administered recombinant MIF exacerbated disease development (26), the circulating levels of MIF were found to be decreased in patients with recent-onset T1D (27). However, despite these conflicting data, MIF possesses biological characteristics that anticipate a role for this cytokine in autoimmune diabetogenesis. These include the capacity of MIF to stimulate delayed-type hypersensitivity responses that mediate β-cell destruction during development of T1D and to up-regulate the production of other proinflammatory cytokines and soluble mediators involved in the pathogenesis of the disease, such as TNF-α, IL-1β, and nitric oxide (NO) (12, 13).

MIF may also influence cell-mediated β-cell destruction through metabolic pathways, because it is constitutively expressed and secreted together with insulin from pancreatic β-cells and acts as an autocrine factor to stimulate insulin release (19). This might contribute to immunoinflammatory diabetogenesis by favoring the expression on β-cells and the presentation to immune cells of antigens (Ag) that are up-regulated when functional activity is augmented (2). Thus, MIF possesses both hormonal and immunological properties that qualify it as a potentially important mediator in the initial events of β-cell dysfunction and destruction.

These observations prompted us to undertake this study evaluating the behavior of endogenous MIF during the development of immunoinflammatory diabetes induced by MLD-STZ as well as the possibility to counteract the diabetogenic process by selective inhibition of MIF activity. The latter aim was achieved by administering either a polyclonal Ab-neutralizing murine MIF or, more importantly for the translation of these findings to the clinical setting, a small molecule that we have developed in our laboratory as a selective pharmacological inhibitor of MIF, (*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) (28).

ISO-1 is the leading compound obtained through a strategy aimed at designing MIF antagonist drugs by targeting the catalytic site of MIF. MIF possesses the unique ability to catalyze the tautomerization of the nonphysiological substrates D,L-dopachrome methyl esters (1) (Fig. 1) into their corresponding indole derivatives (29). The role of this tautomerase-active site in the proinflammatory activity of MIF was investigated by several studies (28, 30–37). We hypothesized that compounds that mimic the indole product (2) (Fig. 1) of MIF's tautomerase catalysis could bind to the active site and be effective inhibitors. To achieve this goal, we synthesized several representative compounds and tested them for dopachrome tautomerase inhibitory activity; we concluded that the isoxazolines represent an attractive scaffold for additional attention, and ISO-1 was found to be the leading compound (28). The crystal structure of MIF complexed to ISO-1 reveals binding in the active site. Additional study of MIF bound with its inhibitor in this manner showed that the active site inhibition is associated with inhibition of MIF proinflammatory properties *in vivo* and *in vitro*, establishing a role for the catalytic active site of MIF in inflammatory activities (28).

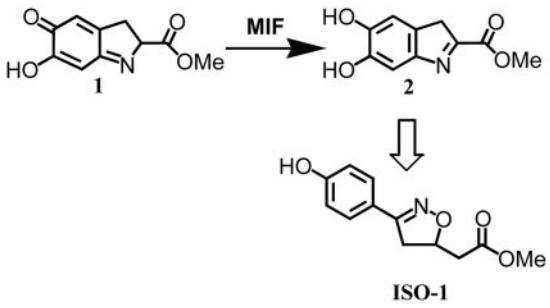

FIG. 1. Structures of dopachrome methyl ester (1), a chromogenic substrate for the measurement of MIF enzymatic activity, its tautomerized, colorless product (2), and ISO-1.

The results of this study show that MIF protein is significantly elevated in islet cells during the development of diabetes and that targeting MIF activity markedly reduced clinical and histopathological features of MLD-STZ-induced diabetes, such as hyperglycemia and insulitis. Protection from diabetes was associated with reduced islet Ag-specific proliferative response of lymphocytes and defective adhesive cell-cell interactions under *ex vivo* conditions. In addition, neutralization of MIF down-regulated the *ex vivo* local and peripheral secretion of the proinflammatory mediators TNF-α, IFN-γ, and NO; simultaneously, the capacity of spleen mononuclear cells (SMNC) to produce the antiinflammatory cytokine IL-10 was significantly increased by *in vivo* abrogation of MIF activity.

Materials and Methods

Mice

Inbred C57BL/6 mice were originally purchased from Charles River Laboratories (Calco, Italy) and then bred by brother/sister mating for up to four generations. Inbred CBA/H mice were obtained from our own breeding colony at the Institute for Biological Research (Belgrade, Serbia and Montenegro). Mice were kept under standard laboratory conditions with free access to food and water. The handling of the mice and the study protocol were approved by the local institutional animal care and use committee.

Reagents

Streptozotocin (STZ; S-0130), sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and irrelevant rabbit IgG were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Antimurine MIF IgG was prepared from rabbit serum raised against murine rMIF (previously shown to react specifically with MIF in tissue sections) and purified by protein A affinity chromatography following the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). ISO-1 was synthesized as previously described (28).

Diabetes mellitus (DM) induction and in vivo treatments

Immunoinflammatory diabetes was induced in adult male mice with MLD-STZ (40 mg/kg body weight·d, ip, for 5 consecutive days) as

previously described (38, 39). In certain experiments to induce nonimmune toxic diabetes, animals were injected ip with a single high STZ dose of 200 mg/kg body weight. The impact of polyclonal Ab against MIF was studied by ip injection of mice (5–10/treatment group) with 5 mg/kg rabbit IgG Ab against mouse MIF on d −3, −1, 2, and 5 in relation to the first STZ dose. ISO-1 was administered for 14 consecutive days by ip injection at a dose of 1 mg/mouse·d under prophylactic and early therapeutic regimes, *e.g.* either 3 d before the first injection with STZ (prophylactic treatment), or 24 h after the last STZ injection (early therapeutic treatment). Control animals received STZ diluent (citrate buffer, pH 4.5), nonimmune IgG, or ISO-1 diluent (DMSO/H_2O). Mice were monitored for diabetes by weekly measurement of blood glucose levels using a glucometer (Sensimac, Imaco, Ludersdorf, Germany). Clinical diabetes was defined by hyperglycemia in nonfasted animals (blood glucose >11.8 mmol/liter). In some experiments a glucose tolerance test was performed by measuring blood glucose levels over a 120-min period after ip injection of 2 g glucose/kg body weight.

Cell incubation and determination of pro- and antiinflammatory mediators

Resident peritoneal cells (PC), SMNC, and pancreatic islets were isolated from individual anti-MIF IgG-treated, ISO-1-treated, or control diabetic mice on d 15 after the first injection of STZ as well as from normal untreated animals. SMNC (5×10^6/well) prepared by Ficoll gradient centrifugation, resident PC (2.5×10^5/well) collected by peritoneal lavage with cold PBS, or pancreatic islets (150–200 islets/well) prepared by collagenase digestion and density gradient purification as previously described (39) were incubated in 24-well Limbro culture plates (Limbro, McLean, VA) in 1 ml RPMI 1640 culture medium containing 5% fetal bovine serum, and cell supernatants were collected after 48 h. The concentration of bioactive TNF-α in culture supernatant was determined as previously described (39) using cytolytic bioassay with the actinomycin D-treated fibrosarcoma cell line L929. The amounts of IFN-γ and IL-10 in cell culture supernatants (SN) were determined by solid phase ELISA using a DuoSet ELISA Development System for mouse IFN-γ and mouse IL-10, respectively (both from R&D Systems, Inc., Oxon, UK), according to the manufacturer's instructions. Nitrite accumulation, an indicator of NO production, was determined in cell culture SN using the Griess reaction (39).

The expression of cytoplasmic MIF or inducible NO synthase (iNOS) was determined by slight modification of a cell-based ELISA protocol (40). Briefly, SMNC (5×10^5/well) or PC (2.5×10^5/well) were allowed to adhere to poly-L-lysine-precoated, 96-well microplates. After fixation with 4% paraformaldehyde and washing with 0.1% Triton X-100 in PBS (T/PBS), endogenous peroxidase was quenched with 1% H_2O_2 in T/PBS, and the reaction was blocked for 1 h at 37 C with 10% fetal calf serum in T/PBS. Subsequently, cells were incubated for 1 h at 37 C with either rabbit antimouse MIF IgG, or rabbit antimouse iNOS (Sigma-Aldrich Corp.) in T/PBS containing 1% BSA. After washing, the cells were incubated for 1 h with the corresponding secondary Ab [goat antirabbit Ig(H+L)-horseradish peroxidase], washed again, and incubated for 15 min at room temperature in the dark with 50 μl of a solution containing 0.4 mg/ml O-phenylendiamine dihydrochloride (Sigma-Aldrich Corp.), 11.8 mg/ml $Na_2HPO_4 \cdot 2H_2O$, 7.3 mg/ml citric acid, 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 in a Titer-Tek microplate reader (Flow Laboratories, McLean, VA).

Ex vivo lymphoproliferative response and adhesion assay

To assess the capacity of lymphocytes for islet Ag proliferation, SMNC (5×10^5/well) from each animal obtained from the same experimental group 15 d after diabetes induction as well as from normal untreated mice were cultured in 96-well microplates in medium alone or restimulated with syngeneic pancreatic islets (1×10^3/well) pulsed for 30 min with 2 mM STZ as previously described (41). Proliferation of SMNC was determined after pulsing the cells with 1 μCi [^{3}H]thymidine (ICN, Costa Mesa, CA) for 24 h (spontaneous proliferation) or after 4 d of restimulation. Incorporated radioactivity in triplicate cultures was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). The analysis of spontaneous adhesion of SMNC (2.5×10^5/well) to a monolayer of L929 fibroblasts, murine MIN6 insulinoma cells (provided by Dr. Jun-ichi Miyazaki, through Dr. Karsten Buschard, Bartholin Instituttet Kommunehospitalet, Copenhagen, Denmark), syngeneic microvascular endothelial cells prepared according to the method described by Issekutz (42), or plastic was performed by using crystal violet assay as previously described (38). The absorbance corresponding to the number of adherent cells was measured at 570 nm.

Abs and flow cytometry

SMNC (1×10^6) were incubated with the rat antimouse monoclonal Ab anti-CD11b (MAC-1)-phycoerythrin or anti-CD25 [IL-2 receptor (IL-2R) α-chain, p55]-biotin (BD Pharmingen, San Diego, CA), followed by streptavidin-phycoerythrin (BD Pharmingen). Each cell suspension of SMNC was a pool from three to five animals obtained from the same experimental group 15 d after diabetes induction as well as from normal untreated mice. Cell surface marker expression was analyzed using a flow cytometer (FACSCalibur, BD Biosciences, Heidelberg, Germany) and CellQuest Pro software (BD Biosciences).

Histological and immunohistochemical analyses

Pancreata were fixed in neutral buffered formalin and then embedded in paraffin. The fixed blocks were sectioned (7 μm thick) and stained with hematoxylin and eosin to assess the incidence and degree of inflammatory changes. Insulitis scoring was performed as previously described (43) by examining at least 15 islets/mouse and was graded in a blinded fashion as follows: 0, intact islet; 1, periislet infiltrate; 2, mild intraislet infiltrate (area of mononuclear cell infiltration within an islet, <25%); 3, heavy intraislet infiltrate (area of mononuclear cell infiltration, >25%), and 4, heavy intraislet infiltrate associated with β-cell destruction or small retracted islet with some residual infiltrate. At least 15 islets were counted for each mouse. A mean score for each pancreas was calculated by dividing the total score by the number of islets examined. Insulitis scores are expressed as the mean ± SD.

Immunohistochemistry was performed on either cryostat sections or paraffin-embedded sections of formalin-fixed tissues (pancreas, kidney, liver, lung, and heart) using a previously described, microwave-based method (18). For MIF immunostaining, a polyclonal rabbit anti-MIF IgG and a control rabbit IgG were used. The examined area of the tissue was outlined, and the percentage of MIF^+ cells was measured using a quantitative Image Analysis System (Optima 6.5, Media Cybernetics, Silver Spring, MD).

Statistical analysis

The blood glucose values are shown as the mean ± SE. Statistical analyses were performed by ANOVA with Bonferroni's adjustment and Fisher's exact test. The other values were expressed as the mean ± SD, and groups of data were compared using Student's paired *t* test. Statistical significance was set at $P < 0.05$.

Results

Anti-MIF prophylaxis suppresses clinical and histological parameters of MLD-STZ-induced DM

To determine whether inhibition of MIF activity modulates disease in MLD-STZ-exposed mice, we first studied the effect of a neutralizing polyclonal Ab against MIF in two DM-susceptible inbred mouse strains. Both C57BL/6 and CBA/H control mice treated with PBS or nonimmune IgG developed sustained hyperglycemia over a 2-wk period after MLD-STZ injections. Although MLD-STZ induced different degrees of hyperglycemia in the two mouse strains, treatment of either C57BL/6 mice (Fig. 2A) or CBA/H mice (Fig. 2B) with anti-MIF Ab from d −3 to d 5 significantly inhibited MLD-STZ-induced hyperglycemia. In addition, histological examination of pancreatic specimens performed on d 15 showed that mice treated with anti-MIF Ab suffered from a

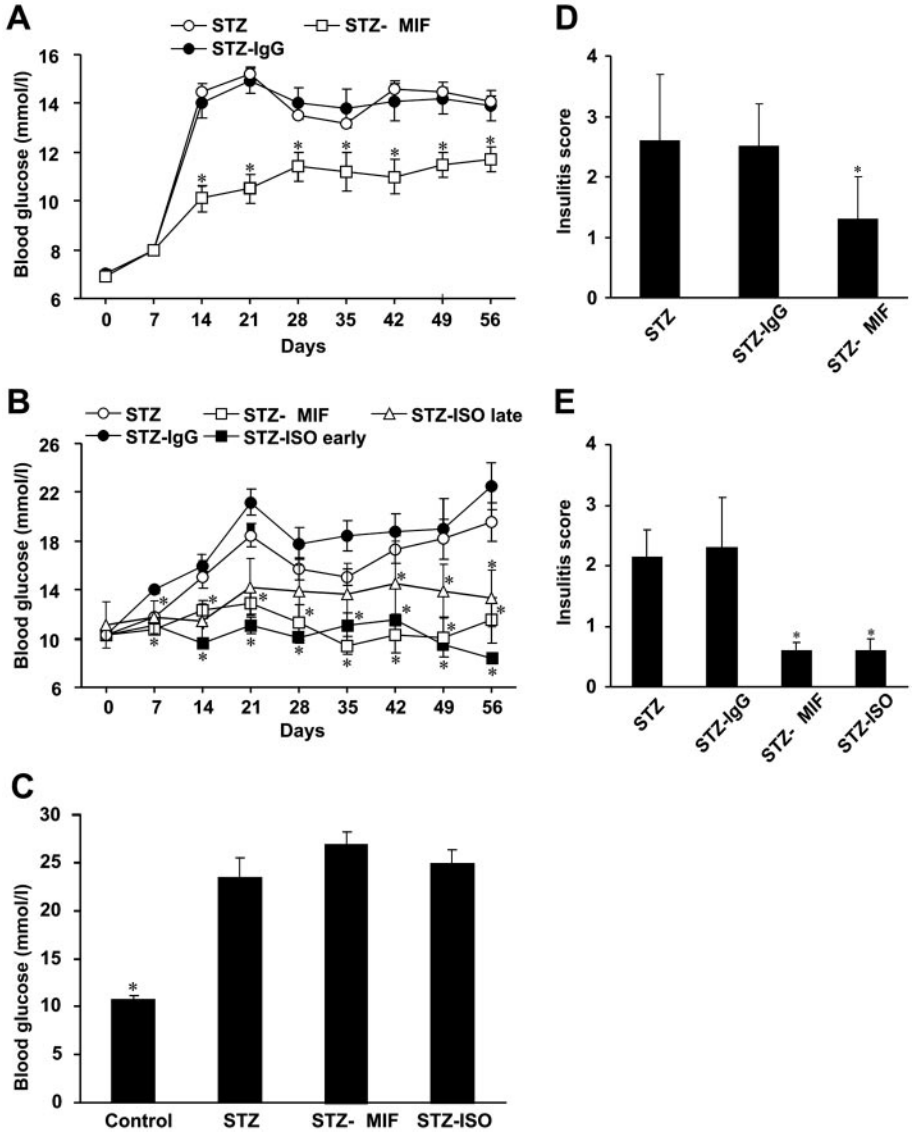

FIG. 2. Effects of MIF targeting on the development of hyperglycemia and insulitis induced by STZ. Blood glucose levels were determined in C57BL/6 mice (A; n = 24/group) or in CBA/H mice (B; n = 5–10/group). Animals received MLD-STZ injections (five injections, 40 mg/kg·d; A) or a single high dose of STZ (200 mg/kg; C) and were treated with vehicle (STZ), nonimmune rabbit IgG (STZ-IgG), anti-MIF IgG (STZ-αMIF), or ISO-1 given as an early (STZ-ISO early) or a late (STZ-ISO late) prophylactic treatment (as described in *Materials and Methods*). Control, Mice without STZ. Blood glucose levels were determined through weekly measurements (A) or 12 d after receiving STZ (C). Histopathological analyses of pancreata from C57BL/6 mice (D) and CBA/H mice (E) are presented as insulitis scores. *, $P < 0.05$ refers to corresponding STZ or STZ-IgG animals.

significantly milder insulitis than control mice treated with irrelevant IgG (Fig. 2, D and E).

The antidiabetogenic effects of the anti-MIF polyclonal Ab were substantiated by the clear-cut protection offered against the development of clinical and histological signs of MLD-STZ-induced diabetes by the pharmacological MIF inhibitor ISO-1, because the mice treated prophylactically with this drug remained euglycemic throughout the 8-wk experimental period (Fig. 2B). The protective effects of anti-MIF Ab and ISO-1 were both long-lasting, with limited variations of blood glucose levels throughout the entire 56-d follow-up period (Fig. 2, A and B). Importantly, neither anti-MIF Ab nor ISO-1 influenced the toxic form of diabetes induced by a single high dose of STZ (Fig. 2C). In addition, when given at a dose of 1 mg/mouse to normal non-MLD-STZ-challenged male CBA/H mice for 14 consecutive days, ISO-1 did not modify either the basal glucose level or glucose tolerance (not shown). Similarly to anti-MIF Ab treatment, MIF blockade by ISO-1 significantly attenuated inflammation of the islets (Fig. 2E).

The capacity of these MIF inhibitors to counteract early diabetogenic pathways of MLD-STZ-induced diabetes prompted us to test the effects of MIF neutralization in a more advanced phase of the disease. Hence, ISO-1 was first administered to mice upon an early therapeutic regimen 1 d after STZ injections were completed. Although all mice were still euglycemic at this time, it is known from literature (4) that β-cells have already suffered from the toxic action of MLD-STZ at this stage, and development of hyperglycemia is usually seen within 7–10 d. Therefore, we considered that starting 1 d after the last of the five injections of STZ was a suitable time point to evaluate the early therapeutic effects of ISO-1 in this experimental model. Figure 2B shows that even when administered under this regimen, ISO-1 significantly reduced blood glucose levels compared with control diabetic mice.

MIF protein expression

To determine whether the MIF protein expression level is altered during immunoinflammatory DM, immunostaining

of MIF was performed in various tissues, including pancreas. Consistently with previous reports (26, 44), significant amounts of preformed MIF were found in both islets of Langerhans (Fig. 3A) and macrophages (not shown) of normal healthy animals. In contrast, markedly increased expression of MIF protein within the islets of MLD-STZ-challenged mice accompanied the progression of the disease (Fig. 3, B and E), whereas exocrine parenchymal cells remained MIF negative. In a similar manner, higher concentrations of MIF were detected in the PC of diabetic mice compared with nondiabetic, control mice (Fig. 3F). The weak to mild constitutive expression of MIF protein found in the kidney, lung, liver, and heart was not influenced by MLD-STZ treatment (not shown).

Upon treatment with either anti-MIF Ab or ISO-1, the expression of MIF in pancreatic islets was down-regulated (Fig. 3, C and D) as it was in PC of anti-MIF Ab-treated animals (Fig. 3F). A similar trend of MIF down-regulation was observed in SMNC of mice treated with anti-MIF Ab or ISO-1 (data not shown). Thus, as a consequence of diabetes induction, increased MIF protein content was observed at the levels of both peripheral and target tissues, and MIF protein level could be attenuated by anti-MIF prophylaxis.

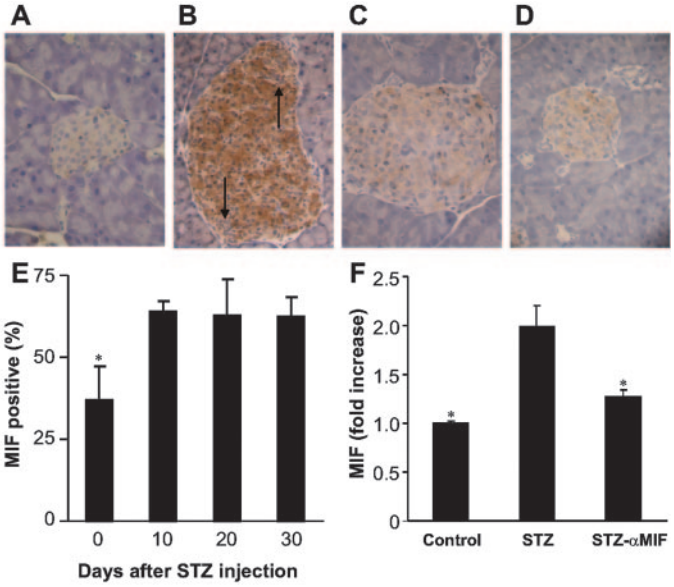

FIG. 3. Immunocytochemical detection of MIF protein expression in the pancreas and PC. A–D, Sections from the pancreas were stained for MIF, detected with a *brown* substrate, and counterstained with hematoxylin. MIF is weakly expressed by islet cells of nondiabetic control mice (A). In pancreatic islets of d 15 MLD-STZ diabetic mice, there is mononuclear cell infiltration (*arrows*), and MIF expression is markedly up-regulated (B). Mild MIF staining by islet cells of d 15 mice treated with MLD-STZ and anti-MIF IgG (C) or with MLD-STZ plus ISO-1 (D) is shown. E, Quantitative image analysis showing different stages of MIF expression by islet cells from diabetic *vs.* nondiabetic control mice. Shown are the mean ± SD percentage of MIF^+ cells per islet (n = 3 mice/group). F, Quantitative analysis of intracellular expression of MIF protein in peritoneal cells of nondiabetic mice (Control), MLD-STZ diabetic mice (STZ), and MLD-STZ diabetic mice treated with anti-MIF Ab (STZ-αMIF), measured by cell-based ELISA performed with MIF-specific Ab. The results are presented as the fold increase in the control absorbance value (OD 492 nm, 0.687 ± 0.013). *, $P < 0.05$ refers to otherwise untreated MLD-STZ diabetic animals.

Anti-MIF treatments reduce adhesive properties, proliferation, and expression of CD11b and CD25 molecules of SMNC

To understand the cellular effects of anti-MIF treatments, *ex vivo* analysis of the functional and phenotype characteristics of SMNC from CBA/H mice was performed during early progression of the disease. Splenocytes from diabetic mice that had been treated with either anti-MIF Ab or ISO-1 were harvested for *ex vivo* analyses on d 15 after MLD-STZ.

Because cell-cell adhesion, mediated by the interaction between CD11b and ICAM-1 (CD54) plays a key role in the immunological processes of T1D (38, 45) by allowing extravasation of leukocytes into the pancreatic tissue, we studied the modifications of adhesiveness of SMNC to various matrices during the development of MLD-STZ and the effects of MIF inhibitors on this assay.

Although the challenge with MLD-STZ greatly enhanced the adhesiveness of SMNC to three types of adherent cells, *i.e.* fibroblasts, microvascular endothelial cells, and insulinoma cells, as well as to plastic surface, the *in vivo* treatment of mice with anti-MIF IgG or ISO-1 strongly inhibited the adhesive properties of SMNC (Fig. 4). Flow cytometric analysis revealed that down-regulation of adhesiveness was associated with changes in CD11b expression. Thus, in comparison with SMNC derived from control diabetic mice, both

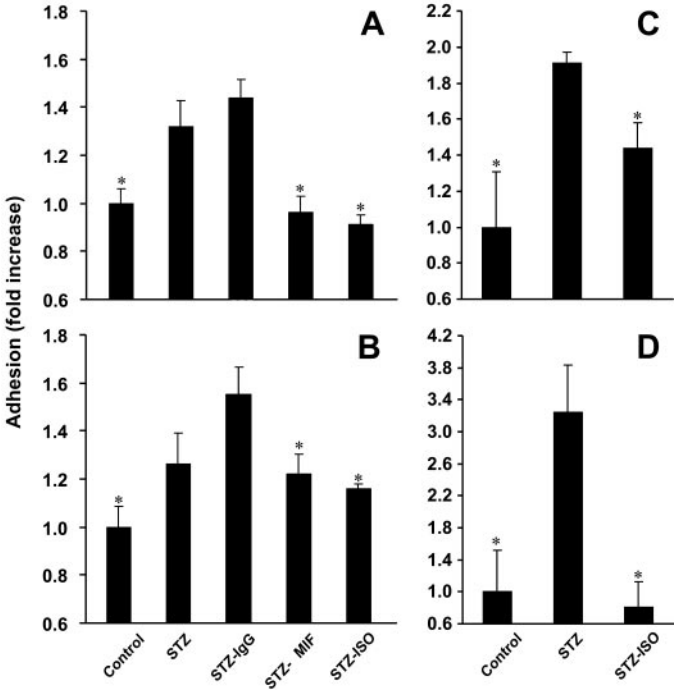

FIG. 4. Neutralization of MIF activity reduces adhesive properties of SMNC. Adhesion to plastic surface (A), L929 fibroblasts (B), microvascular endothelial cells (C), and MIN6 insulinoma cells (D) was determined for SMNC isolated from the mice untreated with STZ (control) or treated with STZ and vehicle (STZ), STZ and nonimmune rabbit IgG (STZ-IgG), STZ and anti-MIF IgG (STZ-αMIF), or STZ and ISO-1 (STZ-ISO). The results are presented as the fold increase in control adhesion to plastic surface (OD 570 nm, 0.316 ± 0.018), L929 fibroblasts (OD 570 nm, 0.905 ± 0.077), microvascular endothelial cells (OD 570 nm, 0.576 ± 0.035), and MIN6 cells (OD 570 nm, 1.120 ± 0.039). * $P < 0.05$ refers to corresponding STZ or STZ-IgG animals.

anti-MIF IgG treatment and ISO-1 treatment (Table 1) reduced the frequency of $CD11b^+$ SMNC and mean density of CD11b molecules (mean fluorescence intensity) to the level in untreated normal mice.

IL-2 is centrally involved in the initiation of a immune response, and negating its action by blocking the interaction with the IL-2R (CD25) system has gained much attention as a possible therapeutic target for immunointervention in both rodent and human disease (46). As shown in Table 1, the increased percentage of $IL\text{-}2R^+$ lymphocytes with higher density of CD25 molecules observed in control mice 15 d after the first of the five injections of STZ was significantly reduced by treatment with either anti-MIF Ab or ISO-1.

Because MIF is a critical component of T cell activation pathways in the Ag-specific immune response (47), we studied whether MIF blockade *in vivo* could affect the priming, clonal expansion, and/or effector function of islet Ag-reactive T cells; to this aim, the proliferative response of SMNC obtained on d 15 after induction with MLD-STZ was measured. As shown in Fig. 5A, proliferation of SMNC from diabetic mice was increased compared with that of SMNC from healthy animals, whereas both anti-MIF Ab and ISO-1 treatments reverted proliferation almost to the control level. Similarly, the proliferative response of SMNC to rechallenge with the STZ-induced islet autoantigen was significantly reduced by ISO-1-treatment (data not shown). This implies that MIF plays a critical role in the T cell proliferative response to islet Ag in the MLD-STZ model of T1D.

Anti-MIF treatments modulate the production of cytokines and NO

Because proinflammatory mediators are believed to play a crucial role in T1D development (6, 8–11, 38, 39), we determined the *ex vivo* effects of MIF inhibition on STZ-associated cytokine release from both local and peripheral immune cells. As shown in Fig. 5B, SMNC obtained from diabetic mice on d 15 during development of MLD-STZ-induced diabetes produced larger amounts of the prototypical type 1 cytokine, IFN-γ, than normal control mice. After *in vivo* administration of ISO-1 or anti-MIF Ab, a significant reduction of IFN-γ was obtained ($P < 0.02$ and $P < 0.004$, respectively, compared with SMNC of diabetic mice). In contrast, SMNC from mice challenged with MLD-STZ secreted less IL-10 than SMNC from normal mice, and this defective production was corrected by both ISO-1 and anti-MIF Ab (Fig. 5C).

Culture SN of pancreatic islets from control diabetic mice or from mice treated with the MIF inhibitors did not contain detectable amounts of either IFN-γ or IL-10 (not shown). In contrast, secretion of the proinflammatory cytokine TNF-α, which was detectable in SN of either pancreatic islets or SMNC from control mice, was reduced to levels close to those seen in healthy non-MLD-STZ-challenged mice by *in vivo* treatment with anti-MIF IgG or ISO-1 (Fig. 6). We also found that intracellular expression of iNOS was significantly reduced in PC isolated from animals treated with either anti-MIF Ab or ISO-1 compared with the relatively high iNOS expression in diabetic animals (Fig. 7A). Similarly, immunostaining of pancreata revealed decreased iNOS protein expression within the islets of ISO-1-treated mice compared with control diabetic mice (data not shown). Thus, MIF blockade abolished subsequent NO production by PC (Fig. 7B) as well as by pancreatic islets (Fig. 7C).

Discussion

We have shown here for the first time that endogenous MIF plays a key role in the development of murine autoimmune diabetes induced by MLD-STZ. Progression of diabetes was accompanied by up-regulated MIF protein expression in both pancreatic islets and peripheral cells. Antagonizing the action of MIF by either anti-MIF IgG or ISO-1 markedly attenuated the clinical and histological manifestations of the disease, and the antidiabetogenic effect of both agents was long-lasting, because the mice remained euglycemic during the 8-wk follow-up period. Anti-MIF therapy profoundly modulated several immune parameters associated with the development of diabetes, including pancreatic and peripheral cytokine production from mononuclear cells, their adhesive properties, and the proliferation of islet Ag-specific T cell. Inhibition of islet Ag-specific T cell expansion, macrophage activation, leukocyte adhesion, and migration into target tissue by these anti-MIF strategies suggests that immunological or pharmacological neutralization of MIF activity may attenuate pathological autoimmune responses *in vivo*.

Autoimmune diabetogenesis is usually accompanied by an increased expression of IL-2R and proliferative response

TABLE 1. MIF antagonists down-regulate the expression of CD11b and CD25 of splenic mononuclear cells

Treatment groups	$CD11b^+$		$CD25^+$	
	%[a]	MFI[b]	%	MFI
A				
Untreated	7.9	44.4	4.4	57.8
STZ + IgG	9.4	72.0	8.5	77.7
STZ + αMIF	6.4	48.6	4.3	70.6
B				
Untreated	10.3 ± 2.7[c]	87.7 ± 20.7[c]	2.9 ± 0.9[c]	81.1 ± 20.5[d]
STZ	18.1 ± 1.8	156.6 ± 16.2	7.9 ± 2.2	121.4 ± 8.8
STZ + ISO-1	11.4 ± 2.8[c]	79.6 ± 15.5[c]	4.5 ± 0.1[d]	84.3 ± 11.8[d]

A, Results from one representative experiment obtained by flow cytometric analysis from the pool of three mouse spleens per group. B, Results from five separate experiments with very similar results, representing a total of three to five mice per group.

[a] Frequency of positive cells.

[b] Mean fluorescence intensity.

[c] $P < 0.01$ *vs.* corresponding STZ animals.

[d] $P < 0.05$ *vs.* corresponding STZ animals.

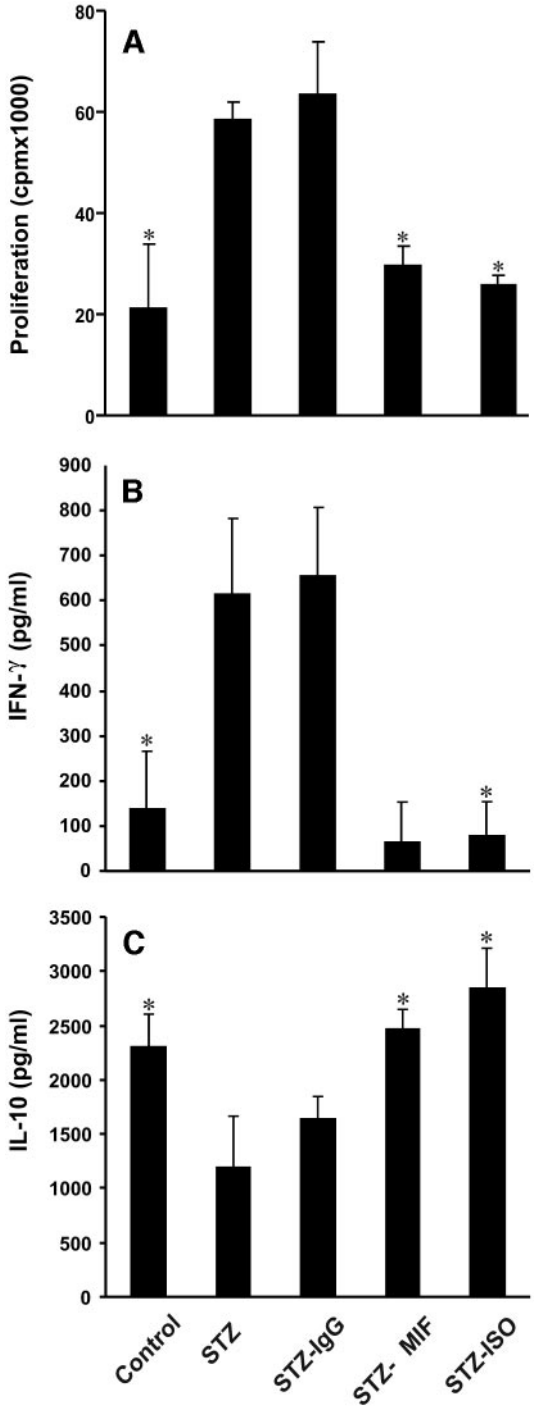

FIG. 5. Neutralization of MIF activity reduces SMNC proliferation and modulates the production of IFN-γ and IL-10. SMNC were isolated from the same groups of mice as described in Fig. 4 on d 15 after DM induction. Incorporation of [^{3}H]thymidine (A) was determined as described in MATERIALS AND METHODS. Production of IFN-γ (B) and IL-10 (C) was measured in 48-h culture supernatants by ELISA. Results are presented as the mean ± SD of three independent experiments with similar results. *, $P < 0.05$ refers to corresponding STZ or STZ-IgG animals.

to pancreatic islet cell Ag (39, 48, 49). In addition, anti-CD25 has been shown to attenuate low dose STZ-induced diabetes in mice (46). Consistent with the role of MIF in T cell activation and mitogenesis (47, 50), we found that MIF blockade

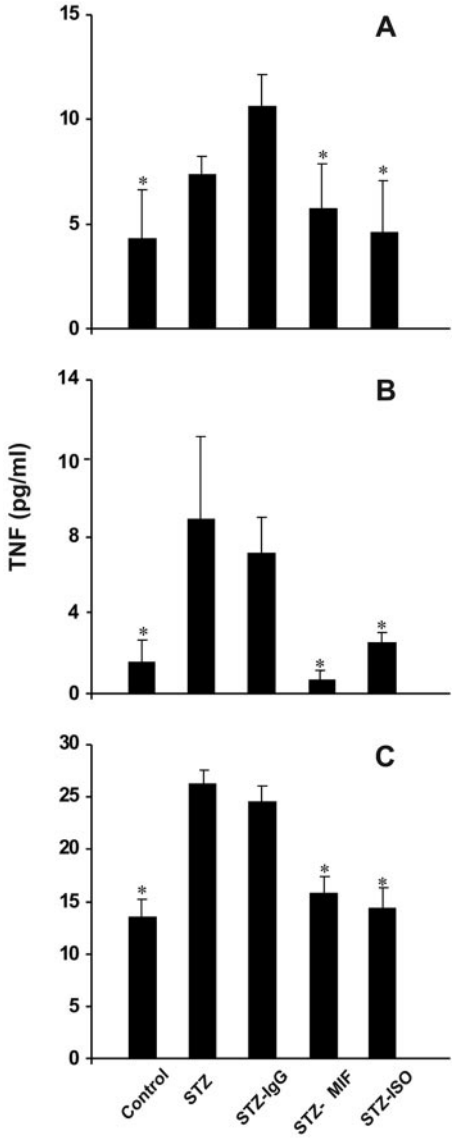

FIG. 6. Neutralization of MIF activity reduces the production of TNF-α. SMNC (A), PC (B), and pancreatic islets (C) were isolated from the same groups of mice as described in Fig. 4 on d 15 after DM induction. TNF production was measured in the 48-h culture supernatants. Results are presented as the mean ± SD of three independent experiments with similar results. *, $P < 0.05$ refers to corresponding STZ-IgG or STZ animals.

considerably decreased IL-2R expression and proliferation of SMNC, which probably reflected the islet Ag-reactive T cell response to blood-borne pancreatic Ag released upon STZ destruction of β-cells (38, 39, 41). The results, therefore, indicate that MIF contributes to the clonal size of T cells reactive to islet Ag, and that blocking the expansion of diabetogenic T clones might be partly responsible for the protective effect of these anti-MIF treatments.

In addition to clonal expansion of islet-Ag reactive cells, the development of T1D is determined by cell-cell interactions mediated by adhesion receptors and ligands in both the induction and efferent phases of the autoimmune response. Sequential adhesion cascade-mediated cell interactions are required for the entry of naive lymphocytes into the lym-

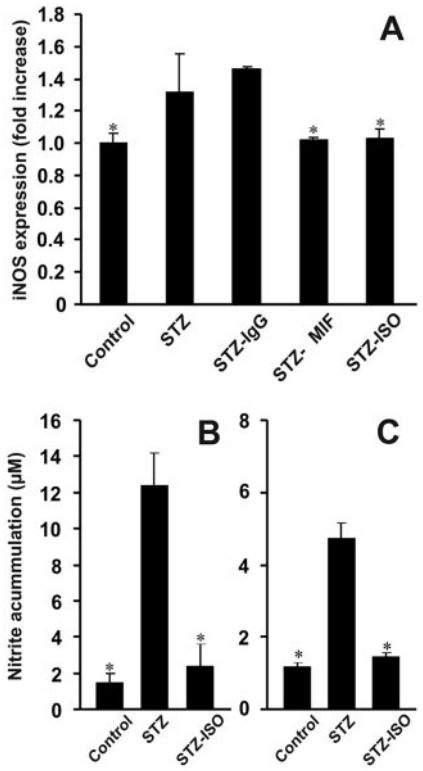

FIG. 7. Neutralization of MIF activity down-regulates the expression of iNOS and NO production. PC (A and B) and pancreatic islets (C) were isolated from mice treated as described in Fig. 4. A, iNOS expression was determined by cell-based ELISA and presented as the fold increase compared with the control value (OD 492 nm, 0.445 ± 0.027). After isolation from mice, PC were cultivated in medium for 48 h (B), and pancreatic islets were cultured in the presence of 250 U/ml IFN-γ plus IL-1β (10 μg/ml) for 72 h (C). Subsequently, nitrite accumulation in cell culture supernatants was determined. Results from three independent experiments with similar results are presented as the mean ± SD for three to five animals per group. *, $P < 0.05$ refers to corresponding STZ or STZ-IgG animals.

phoid tissue in which diabetes-causing lymphocytes are originally primed and for subsequent homing and transendothelial migration of leukocytes into the pancreas (51). Several lines of evidence demonstrate that up-regulation of CD11b, which mediates cellular adhesion to intercellular adhesion molecule-1 (CD54) (52), might have functional consequences on the interactions between cells participating in the immunological processes of T1D, including infiltration of the target tissue (38, 45). Because some adhesion molecules can undergo changes in activity independently of their surface expression, measuring their functional state in addition to their surface expression is a useful approach for elucidating potential effects of test compounds on cell-cell adhesion. The plastic surface is widely used in the assessment of leukocyte function (53). The capacity of our MIF inhibitors to reduce the enhancement of SMNC adhesiveness (38, 45) to plastic as well as to cell populations present within target tissue (*i.e.* vascular endothelial cells, β-cells, or fibroblasts) that occurs during the development of diabetes induced by MLD-STZ and to inhibit the expression of CD11b on SMNC suggests that anti-MIF strategies may impair homing of these cells to the pancreatic islets, thus contributing to the therapeutic benefit of MIF blockade. In favor of this hypothesis, histological analysis showed reduced insulitis in anti-MIF-treated mice. Inhibition of MIF activity also has been shown to down-regulate adhesion molecule-dependent target tissue pathology in glomerulonephritis, experimental autoimmune encephalomyelitis (24), and experimental autoimmune myocarditis (15).

Another important finding of our study in the context of well-known immunopathogenic concepts of T1D (6–8) is the profound inhibitory effect of anti-MIF treatment on the production of the type 1 proinflammatory cytokines IFN-γ and TNF-α, as well as of the cytotoxic mediator NO from both pancreatic islet-infiltrating and peripheral mononuclear cells. The lower local production of TNF-α and NO may result from the reduction of inflammatory cell influx into pancreas. However, based on our *ex vivo* findings, it also seems possible that MIF blockade might directly influence macrophage and T cell effector function, as suggested by the down-regulation of TNF-α, IFN-γ, iNOS, and NO by spleen and/or peritoneal cells. This is consistent with both the *in vitro* ability of anti-MIF Ab to interfere with the production of these mediators (22) and the capacity of MIF to up-regulate the *in vitro* production of TNF-α, reactive oxygen species, and nitrogen metabolites (54). It is therefore conceivable that direct blockade of MIF-mediated iNOS expression and TNF-α synthesis may contribute to down-regulation of tissue-damaging proinflammatory mediators in T1D. Another interesting immunopharmacological property of the antidiabetogenic effect of MIF blockade is the increased production of the antiinflammatory type 2 cytokine, IL-10 (Fig. 5C), which fits with the shift toward a protective type 2 cytokine profile associated with or induced by the treatment. The up-regulation of antiinflammatory cytokines such as IL-10 would also counteract type 1 cytokines and limit ongoing inflammatory events (6, 7). Thus, we hypothesize that the efficacy of MIF blockade in the treatment of MLD-STZ T1D is due to inhibitory effects on the autoimmune/inflammatory response of T cells and macrophages as well as islet cells.

Several populations of $CD4^+$ T cells have been shown to regulate autoimmune diseases. These cells may be identified by their surface phenotype (*e.g.* $CD25^+$) or by unique cytokine profiles characterized by IL-10 (and TGF-β) production (55). Our observation that MIF inhibition reduced CD25-expressing cells and up-regulated the synthesis of IL-10 fits in with the possibility that the $CD25^+$ cells targeted in our model of the disease belong to pathogenic type 1 helper T cells rather than regulatory T cells. These observations also suggest that by enhancing the production of IL-10 while suppressing that of TNF-α, IFN-γ, and NO, the MIF inhibitors may activate regulatory pathways other than Tr activation, which are capable of counteracting type 1 helper T cell-mediated immune responses. For example, keeping in mind the profound ability of MIF to counteract the immunosuppressive effects of glucocorticoids (56), neutralization of MIF activity accompanied with sustained circulating levels of IL-10 could potentiate systemic antiinflammatory effects in treated animals.

Although it has recently been shown that the systemic level of circulating MIF in subjects with recent-onset T1D negatively correlated with autoantibody status (27), the amount of circulating MIF may not necessarily correlate with

local cytokine production. Our observation of both peripheral and local overexpression of MIF during the development of MLD-STZ-induced diabetes concurs with the up-regulation of MIF observed in spontaneously diabetic NOD mice (26). Because the glucose concentration in culture medium potentiates MIF expression in isolated rat islets or the β-cell line INS-1 (19), it is possible that MIF secretion is up-regulated by increased glucose level during disease. However, an interesting issue, observed in the current study and in a model of immunologically induced kidney disease (57), is the effect of anti-MIF treatment on disease-related overexpression of MIF. In both studies the functional blockade of MIF activity resulted in a substantial inhibition of MIF expression by inflammatory macrophages as well as by intrinsic cells of the target tissue. Because the latter model of disease does not include changes in the circulating glucose level (57), it is tempting to speculate that MIF may act *in vivo* in an autocrine manner, amplifying its own production during the inflammatory/autoimmune response. The data thus suggest that MIF is a key player in the pathogenesis of T1D, rather than only a nonspecific marker for illness.

Our study indicates that targeting MIF synthesis/function may represent a novel immunotherapeutical intervention for the prevention and early treatment of human T1D. However, treatment approaches that rely on exogenously administered proteins, including humanized Ab, face several challenges in clinical utility, including potential immunogenicity, the need for iv administration, and the high costs. Moreover, anticytokine Ab can form small inflammatory complexes with cytokines and thereby exacerbate inflammatory responses (58). For these reasons, small, drug-like, inhibitory molecules may be a more suitable approach for the clinical setting.

It is therefore of particular relevance in this context that our small molecule ISO-1, which we have designed as a selective pharmacological inhibitor of MIF, exhibited similar antidiabetogenic properties as anti-MIF Ab in the prophylactic intervention in MLD-STZ-induced T1D and also ameliorated the course of the disease when given under an early therapeutic regimen. ISO-1 may therefore represent a new series of small molecules capable of selectively inhibiting the function of MIF that may be worthy of consideration for their use in the prevention and early treatment of human T1D.

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