Administration of metrnl delays the onset of diabetes in non-obese diabetic mice

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Abstract. Type 1 diabetes is a chronic metabolic disease characterized by hyperglycemia due to progressive destruction of pancreatic beta cells via autoimmune attack. Meteorin-like protein (metrnl) is a secreted protein homologous to the neurotrophin metrn and it is induced after exercise in the skeletal muscle. In our paper published previously, we showed that the serum level of metrnl was significantly correlated with the lipid profile, glucose profile and insulin resistance. In this experiment, we asked whether intravenous administration of metrnl could delay the onset of diabetes in non-obese diabetic (NOD) mice. 4-week-old NOD mice were injected intravenously with metrnl. Blood glucose levels were measured weekly. Insulitis scoring, intraperitoneal glucose tolerance test, adoptive T cell transfer, flow cytometry analysis and real-time PCR were performed to investigate the underlying mechanism. The results showed that intravenous administration of metrnl delayed the onset of diabetes in NOD mice. Histology of pancreas showed a decreased infiltration of leukocytes, which was in association with augmentation of regulatory T cells, suppression of autoreactive T cells and altered cytokine secretion. To sum up, the present study showed that intravenous administration of metrnl ameliorated islet lymphocyte infiltration and modulated immune cell responses, raising the possibility that it might be beneficial in improving islet function clinically.

Key words: Non-obese diabetic mice, Meteorin-like protein, Insulitis, Type 1 diabetes

type 1 diabetes is a chronic metabolic disease characterized by hyperglycemia caused by the progressive destruction of insulin-producing beta cells via autoimmune attack. Nowadays, type 1 diabetes has become a worldwide epidemic, and its long-term complications can be devastating [1]. Clinically, insulin injection therapy is performed as a routine treatment for type 1 diabetes. However, lifelong administration of insulin has failed to prevent the development of severe vascular complications. Immunosuppression strategies that prevent immune-mediated destruction of islets are still the focus of active research [2]. In diabetic animals or human beings, many islet-specific antigens exist. Immune intervention using these antigens is effective in delaying the onset of type 1 diabetes in mice. For example, the incidence of diabetes decreased in NOD mice after intraperitoneal administration of insulin B chain peptide 9–23 emulsified with incomplete Freund’s adjuvant (IFA) [3]. Intraperitoneal injection of glutamic acid decarboxylase (GAD) combined with IFA improved blood glucose and increased the number of interleukin (IL)-4 secreting T helper 2 cells in NOD mice [4]. However, most clinical trials involving the use of these islet specific antigens failed to demonstrate good therapeutic effects [5-7]. Therefore, the discovery of other new methods for treating type 1 diabetes is urgent.

Metrnl is produced by activated macrophages as well as dendritic cells and some granulocytes, but not by any lymphoid populations. On the other side of a coin, metrnl can also regulate the production of several chemokines and cytokines in macrophages. Therefore, metrnl represents an ‘amplification loop’ that promotes the activation of macrophages, possibly leading to anti-inflammatory effects. Scholars showed that metrnl was highly expressed in barrier tissues and found that metrnl could express in several skin or inflammatory diseases [8].
Metrnl is strongly over-expressed in psoriasis, prurigo nodularis, atopic dermatitis and rheumatoid arthritis [9]. Exercise and physical activities can benefit the organs and protect the body against diabetes. Metrnl is a secreted protein that can be induced in skeletal muscle after exercise [10]. Metrnl plays an important role in metabolic homeostasis [11]. Lee reported that serum metrnl in newly diagnosed type 2 diabetic patients was significantly lower in newly diagnosed type 2 diabetic patients than in subjects with normal glucose tolerance or prediabetes [12]. In our previously published paper, we showed that the serum levels of metrnl in newly diagnosed type 2 diabetic patients were significantly correlated with the lipid profile, glucose profile, and insulin resistance [13]. In the present study, we aimed to determine whether intravenous administration of metrnl could ameliorate insulitis in NOD mice, as no previous study has focused on this issue.

The NOD mouse model was established in 1974 [14]. The syndrome observed in NOD mice includes hyperglycemia, hypoinsulinemia, and glycosuria and is similar to that observed in human type 1 diabetic patients. Several checkpoints exist during the progression of diabetes in NOD mice. At approximately 4 weeks of age, female mice begin to demonstrate mononuclear infiltrates surrounding the islet, and this phenomenon is referred to as peri-insulitis. In female mice, overt onset of diabetes typically begins at 12 to 14 weeks of age. The incidence rate of diabetes in female NOD mice reaches 70% to 80% at 6–7 months of age. In this study, NOD mice were injected intraperitoneally with metrnl at 4 weeks of age. Blood glucose levels were measured weekly. Insulitis scoring, intraperitoneal glucose tolerance test, adoptive T cell transfer assay, flow cytometry analysis and real-time PCR were performed to investigate the underlying mechanism.

Materials and Methods

Mice

Female NOD mice, nonobese diabetic (NOD)-severe combined immunodeficient (scid) mice, and NOD.BDC2.5 mice were purchased from Nanjing Institute of model animals (Nanjing, China) and Jackson Laboratory (Bar Harbor, USA). The study protocol was approved by the Ethics Committee of Qilu Hospital, Shandong University (Jinan, China).

Intravenous injection

Four-week-old NOD mice were administered with metrnl intravenously daily through the tail vein at 2 μg/mouse/day for 2 weeks. Mice in the control groups were treated with glutathione s-transferase (GST). Because lipopolysaccharide and other microbial products generated during the production of recombinant proteins might have impact on type 1 diabetes development in NOD mice, we wanted to avoid this confounding factor by injecting the recombinant protein GST into NOD mice in the control groups. Blood glucose levels were monitored weekly. The mice that displayed a blood glucose level of >11.1 mmol/L for 2 days consecutively were considered diabetic.

Insulin immunostaining and insulitis scoring

The pancreas was isolated from non-diabetic NOD mice at 20 weeks of age. Insulin immunostaining and hematoxylin and eosin (HE) staining of paraffin-embedded pancreas were performed. The insulitis score was calculated as follows: 0, normal islet; 1, mononuclear infiltration, less than 25% of the islet; 2, mononuclear infiltration, 25%–50% of the islet; 3, mononuclear infiltration, more than 50% of the islet; and 4, a small, retracted islet containing few mononuclear cells.

Intraperitoneal glucose tolerance test and serum insulin measurement

After fasting overnight, 20-week-old non-diabetic NOD mice were injected intraperitoneally with 2 g/kg glucose. Tail tip was pricked with a needle to obtain several drops of blood. Blood glucose was measured at indicated time points (0, 30, 60, 90, 120, and 180 min). Areas under the curve were subsequently calculated.

In the intraperitoneal glucose tolerance test, serum samples were collected at the time point of 30 min. The levels of insulin were measured using ultra-sensitive mouse ELISA kit according the manufacturer’s instructions.

Adoptive T cell transfer

Five-week-old NOD-scid mice were injected intraperitoneally with splenocytes isolated from non-diabetic NOD mice at 20 weeks of age. Diabetes development was monitored in NOD-scid mice.

Flow cytometry analysis

Splenocytes isolated from non-diabetic NOD mice at 20 weeks of age were used for flow cytometry assay. For the regulatory T cells (Tregs) assay, cells were stained with anti-CD4 and anti-CD25 monoclonal antibodies. Then, the cells were permeabilized and stained using a Forkhead box p3 (Foxp3) staining buffer set (eBiosciences San Diego, CA, USA). Splenocytes were isolated from 20-week-old NOD mice for the cytokine assay. Cells were stimulated with phorbol myristic acid (50 ng/mL) and ionomycin (1 μg/mL) in the presence of GolgiStop reagent for 4 h. The cells were first stained with anti-CD4 (or anti-CD8) and then with anti–interferon (IFN)-γ (or anti–IL-4, anti–
IL-17) (eBioscience, San Diego, CA, USA). The data were analyzed using FlowJo software version 10.0.

In vivo proliferative responses of NOD.BDC2.5 T cells
We performed carboxyfluorescein diacetate succinimidyl ester (CFSE)-splenocyte adoptive transfer experiment using metrnl-treated or GST-treated mice as recipient. NOD.BDC2.5 mice expressed a transgenic T cell receptor (TCR) with specificity for islet antigens. Splenocytes (5 × 10^7/mL) from 8-week-old NOD.BDC2.5 mice expressing a transgenic TCR with specificity for islet antigens were incubated in 5 mmol/L CFSE at 37°C for 30 min, washed in PBS, and suspended in complete medium. A total of 1 × 10^7 CFSE-labeled T cells were intravenously injected into metrnl-treated or GST-treated 20-week-old non-diabetic NOD mice. Five days after this injection, pancreatic lymph node cells of NOD mice were harvested and analyzed by flow cytometry.

Quantitative RT-PCR
Non-diabetic NOD mice at 20 weeks of age were examined. Total RNA was isolated from splenocytes, and cDNA was synthesized. Conditions for real-time PCR were as follows: after initial denaturation at 95°C for 15 min to activate the enzyme, 38 cycles of PCR (denaturation 0.5 min at 94°C, annealing 0.5 min at 56°C, and elongation 0.5 min at 72°C with a final extension 5 min at 72°C) were carried out. We used mouse beta-actin as the control, and relative gene expression was calculated using the 2^-ΔΔCt method. The primers are shown in Table 1 [15].

Statistical analysis
The data are expressed as the mean ± SEM. An unpaired, two-tailed student’s t test and the chi-square test were used to compare the means. The incidence rate of diabetes was analyzed using the log-rank test. Statistical analyses were performed using IBM SPSS Statistics 23 (IBM) software. A p value of less than 0.05 was considered to be statistically significant.

Results

Intravenous administration of metrnl delays the onset of diabetes in NOD mice
Four-week-old NOD mice were injected with metrnl intravenously through the tail vein. As shown in Fig. 1, by 24 weeks of age, 65% of the GST-treated mice became diabetic, whereas only 35% of the metrnl-treated mice became diabetic. By 40 weeks of age, 85% of the GST-treated mice became diabetic, whereas only 60% of the metrnl-treated mice became diabetic. The intravenous administration of metrnl delayed the onset of diabetes in NOD mice.

Intravenous administration of metrnl reduces insulitis severity
As indicated in Fig. 2A, insulin immunostaining of the pancreas revealed that the islets were heavily infiltrated by leukocytes in NOD mice treated with GST. In contrast, lymphocyte infiltration of islets of Langerhans was reduced in metrnl-treated mice. Further analysis of the insulitis spectrum revealed that insulitis severity was markedly reduced by the administration of metrnl (p < 0.05 vs. the GST-treated mice using the Chi-square test) (Fig. 2B).

As indicated in Fig. 2C, glucose tolerance test results revealed that the blood glucose levels remained lower in the metrnl-treated mice than in the GST-treated mice throughout the test period. This result was reflected by the reduction of the area under the curve (Fig. 2D), indicating better preservation of glucose homeostasis. In addition, insulin level was higher in metrnl-treated mice than in GST-treated mice (Fig. 2E). The intravenous administration of metrnl improved glucose homeostasis in NOD mice.

Table 1  Primers

| Genes | Forward primer | Reverse primer | PCR Size (bp) | GenBank accession no. |
|-------|----------------|----------------|---------------|----------------------|
| β-actin | ACC ACA CCT TCT ACA ATG AGC | GGT ACG ACC AGA GGC ATA CA | 184 | NM_007393.2 |
| IL-2   | CCC TTG CTA ATC ACT CCT CA | GAG CTC CTG TAG GTC CAT CA | 217 | NM_008366 |
| IL-4   | CAA GGT GCT TCG CAT ATT TT | ATC CAT TTG CAT GAT GCT CT | 199 | NM_021283 |
| IL-10  | AGT GGA GCA GGT GAA GAG TG | TTC GGA GAG AGG TAC AAA CG | 250 | NM_010548 |
| IFN-γ  | CAA AAG GAT GGT GAC ATG AA | TTG GCA ATA CTC ATG AAT GC | 182 | NM_008337 |
| IL-17  | TGG AAG AGT ATG AGC GGA AC | ATT CAC GCA ACC CAA ACA TA | 209 | NM_019508 |
| FoxP3  | CAG CTG CCT ACA GTG CCC CTA G | CAT TTG CCA GCA GTG GTT AG | 388 | NM_054039 |
Metrnl treatment expands CD4+ CD25+ Foxp3+ Tregs
To assess whether the efficacy of metrnl was associated with the expansion of Tregs, we evaluated the percentage of CD25+ Foxp3+ cells among the CD4+ T cells located in the spleen. Twenty-week-old NOD mice treated with metrnl showed a higher percentage of CD25+ Foxp3+ cells among CD4+ T cells compared with the GST-treated mice (Fig. 3A). The intravenous administration of metrnl modulated CD4+ CD25+ Foxp3+ Treg responses.

Metrnl alters CD4+ IFN-γ+, CD8+ IFN-γ+, CD4+ IL-4+, and CD4+ IL-17+ cells
Total lymphocytes were gated for CD4+ cells. The percentage of IFN-γ+, IL-4+ or IL-17+ cells among the CD4+ T cells was measured using a FACScan flow cytometer. As indicated in Fig. 3B, C, and D, the percentage of IFN-γ+ or IL-17+ cells among the CD4+ T cells was significantly lower in the metrnl-treated NOD mice than in the GST-treated mice. As indicated in Fig. 3E, the percentage of IFN-γ+ cells among the CD8+ T cells was also significantly lower in the metrnl-treated NOD mice than in the GST-treated mice. The percentage of IL-4+ cells among the CD4+ T cells was significantly increased by the administration of metrnl.

Metrnl treatment suppresses autoreactive T cells
As indicated in Fig. 4A, 5-week-old NOD-scid mice were injected with splenocytes from NOD mice treated with metrnl or GST. The NOD-scid mice injected with splenocytes from the metrnl-treated NOD mice exhibited significantly delayed diabetes incidence. Six weeks after the adoptive transfer, the incidence of diabetes was 40% in NOD-scid mice injected with splenocytes from GST-treated NOD donors. In contrast, the diabetic incidence was 20% in NOD-scid mice injected with splenocytes from metrnl-treated NOD donors.

Injection of metrnl suppresses autoreactive NOD.BDC2.5 T cell proliferation
To further determine whether metrnl treatment induced beta cell antigen-specific immunosuppression, CFSE-labeled splenocytes from NOD.BDC2.5 mice expressing TCRs that recognize islet antigenic peptides on CD4+ T cells were intravenously injected into 20-week-old NOD mice. After 5 days, pancreatic lymph node cells were harvested and analyzed by flow cytometry. The percentage of CFSE-labeled NOD.BDC2.5 CD4+ T cells in the pancreatic lymph nodes of metrnl-treated mice was lower than that in the pancreatic lymph nodes of GST-treated mice (Fig. 4B). The injection of metrnl suppressed NOD.BDC2.5 CD4+ T cell proliferation in NOD mice.

Metrnl treatment alters cytokine production
We investigated the expression profile of cytokine genes in the spleen of 20-week-old NOD mice by performing quantitative real-time PCR. As indicated in Fig. 4C, compared with the control groups, the metrnl-treated mice displayed increased the expressions of IL-4, IL-10, and Foxp3. In addition, the down-regulation of IL-2, IL-17, and IFN-γ was also observed. The intravenous administration of metrnl altered the cytokine production of immune cells.
Fig. 2 Metnln treated NOD mice had reduced insulitis and improved glucose homeostasis.

A. Insulin immunofluorescence staining. 20-week-old NOD mice treated with metnln or GST underwent insulin immunofluorescence staining. Representative images are presented. Red arrow shows infiltrating lymphocytes (Original magnification ×200). B. Insulitis scoring spectrum. The percentage of islets within each category (Insulitis scoring) is depicted (20–30 islets per mouse) (p < 0.05 vs. the GST-treated mice using the Chi-square test). C. Intraperitoneal glucose tolerance testing. Blood glucose levels were monitored over a 3-h period. (p < 0.05 vs. the GST-treated mice, at all time points). D. Areas under the curve. Data obtained from intraperitoneal glucose tolerance test were used to calculate the areas under the curve, an index of glucose tolerance. E. Serum insulin. Serum insulin levels in metnln or GST-treated NOD mice were measured by ELISA (N = 5/each group, ** p < 0.01).
Fig. 3  Cell flow assay.
A. Metrnl administration expands CD4⁺ CD25⁺ Foxp3⁺ Tregs in NOD mice. Splenocytes were prepared from 20-week-old NOD mice. CD4⁺ cells were gated. The percentage of CD25⁺ Foxp3⁺ Tregs among the CD4⁺ T cells was measured using a FACScan flow cytometer. B. Metrnl administration alters IL-4 expressing CD4⁺ T cells in the spleen. C. Metrnl administration alters IL-17 expressing CD4⁺ T cells in the spleen. D. Metrnl administration alters IFN-γ expressing CD4⁺ T cells in the spleen. E. CD8⁺ T cells were gated. Metrnl administration alters IFN-γ expressing CD8⁺ T cells in the spleen (Representative flow cytometry plots are shown, N = 5/group, * p < 0.05).
Fig. 4  Metnl suppressed autoreactive T cells and altered cytokine secretion.
A. Splenocytes adoptive transfer assay. Splenocytes from 20-week-old NOD donors treated with metnl or GST were isolated and transferred into 5-week-old NOD-scid mice (N = 10/group). Blood glucose levels were assessed weekly (p < 0.01, using the log-rank test). B. *In vivo* suppression of beta cell antigen–specific CD4⁺ T cell proliferation in metnl–treated NOD mice. Splenocytes were isolated from NOD. BDC2.5 mice. 1 × 10⁷ CFSE-labeled cells were intravenously injected into 20-week-old metnl–treated or GST-treated NOD mice. Five days later, the cells were prepared from the pancreatic lymph nodes of these NOD mice, and the proliferation of CD4⁺ T cells in the CFSE⁺ cell population was analyzed by flow cytometry. The percentages of dividing CD4⁺ T cells and representative flow cytometry pictures are shown (N = 3/group). C. Quantitative RT-PCR analyses. Splenocytes were isolated from 20-week-old NOD mice and real time-PCR assay of cytokines was performed (N = 5/group) (⁎ p < 0.05, ⁎⁎ p < 0.01).
Discussion

Type 1 diabetes is characterized by the lymphocyte infiltration into the pancreatic islets that results in the progressive destruction of insulin-producing beta cells. The genotype associated with the highest risk for type 1 diabetes is the HLA-DR3/4 DQ8, which is closely related to beta cell destruction. Several methods to preserve beta cells have been assessed. However, wide gaps still exist in our ability to delay the onset of diabetes and decrease disease-associated complications. Type 1 diabetes incidence is increasing globally.

Currently, no effective clinical prevention treatment for type 1 diabetes is available [16, 17]. The therapy of type 1 diabetes has been challenging due to the lack of effective methods to prevent the inflammatory injury of the islets of Langerhans. Antigen-specific immune intervention allows the specific inhibition of islet self-reactive immune cells without altering host immunity. A series of autoantigens exists in patients with type 1 diabetes [18-20]. Although advances have been made in the development of antigen specific therapies in animal models, further research is necessary to translate this therapy from bench to bedside. Antigen-specific therapeutic strategies using these antigens have not yielded satisfactory therapeutic effects clinically. For example, studies reported that oral administration of insulin could not delay the progression of diabetes in patients [21-23]. Clinical observation of new-onset type 1 diabetic patients immunized with the insulin B chain in IFA revealed that the treatment had no effect on C-peptide preservation [24]. In addition, a clinical trial showed that injection of GAD-Alum prevented C-peptide loss in newly diagnosed type 1 diabetic patients, but long-term tolerance was not sustained [25]. Moreover, a 1 year clinical trial using a peptide derived from heat shock protein 60 appeared to have no beneficial effect on the preservation of pancreatic beta cell function [26, 27]. Much effort is needed to develop other novel interventions that can improve the treatment effect of type 1 diabetes clinically.

Exercise can effectively relieve chronic diseases, such as diabetes, obesity, and hypertension. Researchers showed that proper exercise leads to a decline in blood glucose in type 1 diabetic patients [28]. Aerobic exercise increases the level of metrnl in muscle. Metrnl is a secreted protein that plays a critical role in both physiological and pathological processes. It is highly expressed in white adipose tissue and is induced after exercise in skeletal muscle. Metrnl could effectively reduce fat accumulation, which could be conducive to the reduction in the risk of obesity [29]. Metrnl also plays important roles in insulin sensitization. Li reported that adipocyte-specific knockout of metrnl exacerbated insulin resistance induced by high-fat diet in mice, demonstrating that metrnl could affect insulin sensitivity through the peroxisome proliferator-activated receptor γ pathway [30]. These evidences suggested that the up-regulation of metrnl might be a novel strategy to improve blood glucose and insulin resistance.

To cure type 1 diabetes, the key elements that need to be addressed include the successful manipulation of immune system and the restoration of beta cell function. Insulitis, which is characterized by an inflammatory lesion consisting of immune cell infiltrates within the islets, is the pathological hallmark of type 1 diabetes. Researchers are focusing on therapies designed to halt progressive destruction of beta cell by inhibiting immune cell infiltration of islets. In this study, histological examination of islets showed that lymphocyte infiltrations were suppressed in metrnl-treated mice compared with the controls. These results suggested that metrnl could ameliorate insulitis through the inhibition of lymphocyte infiltration into the islets, which results in the significantly delayed onset of diabetes.

Type 1 diabetes is perceived as a chronic immune-mediated disease, in which CD4+ T cells play a major role in the process of pathology. Beta cell-specific autoreactive T cell is a critical element of type 1 diabetic pathogenesis and a key mediator of beta cell destruction [31]. In this study, adoptive T cell transfer experiment showed that islet autoreactive T cells were suppressed by the administration of metrnl in NOD mice. Moreover, beta cell-specific CD4+ T cells from NOD.BDC2.5 mice proliferated in recipient NOD mice, and the percentage of CFSE-labeled CD4+ T cells was monitored. The proliferation of injected CD4+ T cells from NOD.BDC2.5 mice was markedly suppressed in the pancreatic lymph nodes of metrnl-treated mice. Autoreactive T cells are usually controlled by a network of Tregs, and a defect in the Treg number can accelerate the development of type 1 diabetes. Foxp3 can regulate the expression of gene correlated with the immune function of CD4+ T cells, and CD4+ CD25+ Foxp3+ Tregs are pivotal for the induction and maintenance of peripheral tolerance. In our study, administration of metrnl resulted in the increase in the percentage of CD25+ Foxp3+ cells in total CD4+ T cells. The intravenous administration of metrnl could ameliorate insulitis through the inhibition of autoreactive T cells at least in part by increasing the proportion of CD4+ CD25+ Foxp3+ Tregs.

Cytokines secreted by immune cells play important roles in the whole process of type 1 diabetes development. Cytokines such as IL-2, IL-4, IL-10, and IFN-γ are generally believed to be important in autoimmune pathogenesis. Studies reported that the administration of recombinant IL-4 protected NOD mice from autoimm-
immune diabetes [32]. IL-10 was described as the cytokine synthesis inhibitory factor, and IL-10 over-expression could inhibit the apoptosis of beta cells [33]. IL-17-expressing cells were involved in many autoimmune diseases, such as rheumatoid arthritis, myocarditis, and type 1 diabetes [34]. The intravenous administration of metrnl altered cytokine secretion of immune cells, resulting in the reduction of pancreatic beta destruction.

The exact underlying mechanism remains unknown and much effort is needed. Several cytokines including TNF-α, IL-17, IL-12 and IL-4 can increase the expression of metrnl. Rao [35] showed that metrnl promoted the activation of macrophages through an eosinophil-dependent increase in IL-4. Metrnl expression in macrophages is also induced by lipopolysaccharide and inhibited by IFN-γ and TGF-β. Furthermore, metrnl may be involved in certain inflammatory responses where macrophages or other metrnl-producing cells are involved. Ushach [36] reported that metrnl played an anti-inflammatory role by modulating cytokine and chemokine production. It raises the possibility that metrnl might be beneficial in ameliorating autoimmune destruction of beta cells clinically. Extensive investigation is necessary to provide more precise information in consideration of the species differences between animals and human beings. Much effort is needed to find novel therapeutic strategies to decrease the lethality of type 1 diabetes.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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