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Effect of influenza A virus non-structural protein 1(NS1) on a mouse model of diabetes mellitus induced by Streptozotocin

Decui Pei [a], Jun Dai [a,b], Yu Kuang [a], Huan Wang [a], Laifeng Ren [a], Jingjing Shao [a], Bin Zuo [a], Sha Li [a], Zhonghua Jiang [a], Mingyuan Li [a,c,*]

[a] Department of Microbiology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, China
[b] Department of Pathobiology and Immunology, School of Basic Medicine, Hebei Medical University, Shijiazhuang, Hebei 050091, China
[c] State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, Sichuan 610041, China

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ABSTRACT

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by proinflammatory autoreactive T cells that mediate the selective destruction of insulin-producing β cells via both direct and indirect mechanisms. Many immune cells and proinflammatory cytokines are involved in the pathogenesis of autoimmune diabetes. Immune intervention is effective for the prevention and treatment of T1D by blocking the autoimmune assault to β cells. The non-structural protein 1(NS1) of influenza A viruses is a non-essential virulence factor encoded on segment 8 that has multiple accessory functions, including suppression of innate immunity and adaptive immunity, inhibition of apoptosis and activation of phosphoinositide 3-kinase (PI3K). This research investigated whether the expression of NS1 can prevent and treat diabetes mellitus induced by Streptozotocin (STZ). The NS1 expressing plasmid pEGFP-C2/NS1 was constructed and injected intramuscularly to both thighs of mice. Its effect on mice was observed. Intramuscular delivery of pEGFP-C2/NS1 resulted in reduction in hyperglycemia and diabetes incidence, with an increase in insulin. pEGFP-C2/NS1 could also increase glycogen and regulated serum cytokine levels. In addition, by comparison to the mice treated with empty vector pEGFP-C2, ameliorative insulitis was observed in the mice treated with recombinant plasmid pEGFP-C2/NS1. This result suggests that the expression of NS1 is effective for the prevention and treatment of diabetes mellitus induced by STZ in a mouse model.

1. Introduction

Diabetes, with its attendant complications and related diseases, is one of the most serious, costly and fast-growing health challenges [1]. According to the latest World Health Organization report, 346 million people worldwide suffer from diabetes. In 2004, an estimated 3.4 million people died from consequences of high blood sugar, diabetes deaths will double between 2005 and 2030 [2]. This disease is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces, which results in hyperglycemia with disturbances of carbohydrate, fat and protein metabolism [3,4]. Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, nerves and can lead to related complications [2]. There are two main types of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is a chronic autoimmune disease, in which the pancreatic β-cells (which secrete insulin) are selectively destroyed. Inflammatory cytokines, innate immune cells and adaptive immune cells, have been shown to have a role in β-cell damage [5].

The goal of anti-diabetes therapy is to reduce hyperglycemia to prevent or minimize the complications associated with this disease. Halting the loss of insulin-producing β cells is the most effective way to cure this disease. So far the available therapies for diabetes include insulin and many oral hypoglycemic agents, such as sulfonylureas, biguanides and thiazolidinediones [6]. Insulin is the oldest and most effective medication for controlling blood glucose. However, insulin therapy is resistant, time-consuming, cumbersome, inconvenient and can result in hypoglycemia, which leads to restriction of its use [7–9]. Treatment with sulfonylureas and biguanides is also associated with side effects and fails to significantly alter the course of diabetic complications [10]. There is still a vital need for new therapies in order to prevent or treat diabetes. It has been shown that immune intervention is effective for the prevention and treatment of T1D by blocking or reversing the autoimmune assault [11].

* Corresponding author at: Department of Microbiology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, China.
E-mail address: lmy3985@sina.com (M. Li).

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The NS1 of influenza A viruses is a small (230–237 residue), dimeric, non-essential virulence factor encoded on segment 8 that has multiple accessory functions, including suppression of host immune and apoptotic responses, activation of phosphoinositide 3-kinase (PI3K) [12]. NS1 is involved in regulating the apoptosis of infected cells through IFN, PI3K/Akt, p53 and Scribble [13–17]. NS1 activates PI3K signaling by directly binding to both the iSH2 domain of p85β regulatory subunit and the p110 kinase domain [18,19], which causes the generation of the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3) and the recruitment and phosphorylation of Akt (the key downstream mediator of PI3K). Phosphorylated Akt plays a central role in modulating diverse downstream signaling pathways associated with anti-apoptosis, proliferation, cell cycling, protein synthesis and glucose metabolism including glycogen synthesis, glucose transport and glycolysis [20].

Given that NS1 has been shown to suppress the host innate and adaptive immune response, disrupt inflammatory cytokines, activate PI3K/Akt signaling pathways associated with anti-apoptosis and glucose metabolism, no studies have as yet focused on the role of NS1 in diabetes mellitus. It is therefore interesting to study whether NS1 affects diabetic mice induced by STZ. The aim of the present study was to investigate whether influenza A virus NS1 can prevent and treat diabetes mellitus induced by STZ. In this study, we constructed the NS1 expressing plasmid pEGFP-C2/NS1 and evaluated its effects on STZ-treated mice. Our results indicated that intramuscular delivery with pEGFP-C2/NS1 plasmid could decrease significantly blood glucose levels, increase glycogen content, and regulate cytokines and ameliorate insulitis in the STZ-induced mice.

2. Materials and methods

2.1. Plasmid construction

Total RNA from the influenza A virus (strain A/Puerto Rico/8/34(H1N1)) was extracted with TRIzol (Invitrogen, China) and employed for cDNA synthesis using oligo (dT) primers accorded to the manufacturer's instructions (Fermentas, China). The following primers were used in PCR for influenza A virus NS1 (GenBank ID: NC_002020), forward: 5′-CCGCTCGAGATGGATCCAAACACTGTG-3′ and reverse: 5′-CCGGAATTCTCAAACTTCTGACCTAATTG-3′. The PCR product and vector pEGFP-C2 containing the cytomegalovirus (CMV) promoter were digested by *Xho*I and *Eco*RI, respectively. Then, the two fragments were ligated to construct the expression vector pEGFP-C2/NS1.

2.2. Plasmid transfection and expression analysis

Expression levels of the constructed plasmids in mammalian cells were analyzed after transient transfection of 293T cells. The cells were planted in 6-well plates at a density of 4×10^5 per well in Dulbecco's modified Eagle's medium (DMEM, high glucose, Hyclone) containing 10% fetal bovine serum (Hyclone), 100U/mL of penicillin–streptomycin and 2 mM glutamine under a 5% CO2-humidified air atmosphere at 37 °C. The next day, cells were transfected with pEGFP-C2 or pEGFP-C2/NS1 at the dose of 4.0 μg per well using 6 μl TurboFect™ in vitro Transfection Reagent (Ferments) according to the manufacturer's recommendation. 48 h later, the expression of proteins was assessed by fluorescence microscopy and reverse-transcriptase polymerase chain reaction (RT–PCR).

2.3. Animals and Injection of plasmid DNA

Eight-week-old male BALB/c mice (20–22 g) were obtained from the animal care centre of Sichuan University and were maintained under conventional conditions. All experiments were carried out according to the Regulations for the Administration of Affairs Concerning Experiment Animals of China. All the mice were divided into the following four groups with ten mice in each group: citrate buffer + PBS (normal control), STZ (Sigma) + PBS (diabetic control), STZ + pEGFP-C2, STZ + pEGFP-C2/NS1. All the mice were fasted overnight, but allowed free access to water. STZ was dissolved in sterile 0.1 M citrate buffer (pH4.4), and injected intraperitoneally (i.p.) into mice rapidly at a dose of 150 mg/kg body weight. The equivalent volume of citrate buffer was injected into normal control mice. After 30 min, the plasmids were injected intramuscularly (300 μg per mouse) to the left and right thighs of the mice. Control mice were injected in the same way with a comparable volume of PBS.

2.4. Biochemical analyses

Blood samples were obtained from the tail vein of fasted mice. Serum glucose levels were measured weekly by the glucose oxidase method using commercially available standard assay kits (Maker, Sichuan, China) before and after injection, and diabetes was confirmed when the glucose levels were greater than 11.1 mmol/l in two consecutive determinations. Serum insulin were measured biweekly using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, China) before and after plasmid administration. Muscle glycogen and hepatic glycogen were evaluated at the end of the 8-week study using commercially available standard assay kits (JianCheng, Nanjing, China). All samples were tested in duplicate.

2.5. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed in mice that had fasted overnight at 2 weeks of age. Glucose (1.5 g/kg body weight) was injected intraperitoneally. Tail vein blood was taken at 0, 30, 60, and 120 min time points before and after glucose administration, serum glucose levels were measured as described above and used for establishing glucose tolerance curves.

2.6. Splenic lymphocyte culture and cytokine analysis

Spleen cells were disposed as described previously [21]. Levels of cytokines in the serum and in the supernatants of splenic lymphocytes of the mice were measured using the enzyme-linked immunosorbent assay kits (Boster, Wuhan, China). All samples were analyzed in duplicate on the same plate.

2.7. Histopathologic examinations

The mice were killed by cervical dislocation following anesthetization with methoxyflurane inhalation at the end of the 8-week study and their pancreases were taken for histological analysis. Tissues were taken and fixed in 10% neutral buffered formalin, paraffin embedded, cut, and stained with hematoxylin and eosin for light microscopic examination. All sections were examined histopathologically in a blind manner, and the pancreatic islet histology was ranked according to four classes as previously described [22].

2.8. Statistical analyses

Statistical analysis was performed using SPSS 16.0. Data was analyzed with one-way ANOVA for the comparison between groups, followed by Tukey as a post hoc test. All values were expressed in terms of mean ± SD, and a $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of the recombinant plasmid

To confirm NS1 could be expressed in mammalian cells, 293T cells were transfected with pEGFP-C2/NS1 or pEGFP-C2. 48 h later, the expression of protein was assessed by fluorescence microscopy and RT-PCR. The efficiency of transfection was recorded by counting the number of GFP positive cells under a fluorescent microscope. The transfection efficiency was 80% or higher in 293T cells (Fig. 1A–D). The expression of NS1 was detected by RT-PCR (Fig. 1E). These data showed that pEGFP-C2/NS1 could encode the NS1 in mammalian cells.

3.2. Effect of NS1 on blood glucose and serum insulin

A progressive hyperglycemia with a corresponding increase in diabetes incidence was observed in STZ-treated mice, while intramuscular delivery with pEGFP-C2/NS1 plasmid ameliorated significantly the rise in mean serum glucose levels within 2–8 weeks and reduced the incidence of diabetes within 1–4 weeks (Fig. 2A and B). At week 6, serum glucose started to increase significantly in STZ + pEGFP-C2/NS1 treated mice compared with normal controls, however, serum glucose was significantly lower than untreated diabetic mice and those treated with pEGFP-C2. Diabetes incidence increased significantly in week 5 in STZ + pEGFP-C2/NS1 treated mice and no statistically significant differences were observed compared with STZ + pEGFP-C2 treated mice or STZ + PBS treated mice. The mean serum glucose, however, decreased significantly compared with the latter two groups. NS1 was effective in the delayed onset of hyperglycemia and reduction of diabetes incidence, although blood glucose level and the incidence of diabetes increased in later stages of the study. To determine β-cell function in the pancreas, we measured the serum insulin concentration of four groups by ELISA. The result illustrated that intramuscular delivery with pEGFP-C2/NS1 increased mean insulin contents of STZ-treated mice (Fig. 2C). At week 6, the insulin contents of STZ + pEGFP-C2/NS1-treated mice decreased significantly compared with that of normal controls, which was in accord with the increase of blood glucose levels.

3.3. Effect of NS1 on glycogen content

Muscle glycogen and hepatic glycogen were evaluated at the end of the 8-week study. The glycogen content decreased significantly in liver and muscle in STZ-treated mice. No significant differences in glycogen content were observed between mice treated with STZ + pEGFP-C2 and those treated with STZ + PBS. The mice treated with STZ + pEGFP-C2/NS1 had a significantly greater glycogen content compared with mice treated with STZ + pEGFP-C2, though significantly lower than normal controls (Table 1). Our results suggest that pEGFP-C2/NS1 plasmid increased the glycogen content of liver and muscle in STZ-treated mice.

3.4. Effect of NS1 on intraperitoneal glucose tolerance test

Glucose tolerance was examined using the IPGTT method at 2 weeks of age. The serum glucose levels after glucose loading were significantly lower in STZ + pEGFP-C2/NS1 mice compared to STZ + PBS and STZ + pEGFP-C2 mice (Fig. 3). In the diabetic control group, highly impaired glucose tolerance was evident. However, in the pEGFP-C2/NS1 treated diabetic mice, a better glucose tolerance pattern was observed.

3.5. Effect of NS1 on Cytokines

Cytokine levels of IL-10, TNF-α and INF-γ in the supernatants of splenocytes and serum were measured by ELISA at the end of study. Serum and splenocytes IL-10 level increased significantly in pEGFP-C2/NS1-treated mice compared to the STZ + PBS-treated mice. Intramuscular delivery with pEGFP-C2/NS1 plasmid reduced significantly the levels of TNF-α and IFN-γ in pEGFP-C2/NS1-treated mice compared to the STZ + PBS mice (Table 2). The data described here indicates that intramuscular delivery with pEGFP-C2/NS1 plasmid can up-regulate IL-10 levels and down-regulate TNF-α and IFN-γ levels.

3.6. Histology of the pancreas

Morphological examinations of the pancreas of all study mice revealed obvious insulitis and structural changes to the islets in pEGFP-C2-treated or diabetic control groups compared with the pEGFP-C2/NS1-treated group (Fig. 4). Intramuscular delivery of pEGFP-C2/NS1 plasmid alleviated the degree of insulitis. The islet size and its cell number were reduced in diabetic control mice and pEGFP-C2-treated mice compared to those of normal control mice. But, they were well preserved in mice treated with pEGFP-C2/NS1 plasmid.

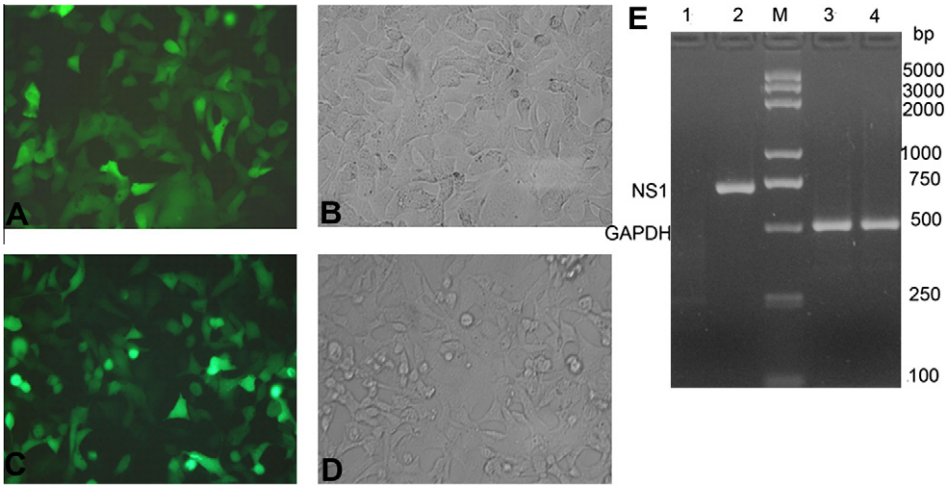

Fig. 1. Expression of the recombinant plasmid in 293T cells. (A and B) Expression of pEGFP-C2 in 293T cells at 48 h after transfection. (C and D) Expression of pEGFP-C2/NS1 in 293T cells at 48 h after transfection. (A and C) Observation under fluorescence microscopy (×400). (B and D) Observation of the same field under light-field microscopy (×400). (E) RT-PCR analysis of NS1 mRNA in 293T cells at 48 h after transfection. Lane 1 and 2, NS1 fragments transfection pEGFP-C2 and pEGFP-C2/NS1; Lane 3 and 4, internal standard GAPDH fragments transfection pEGFP-C2 and pEGFP-C2/NS1; Lane M, molecular weight markers.

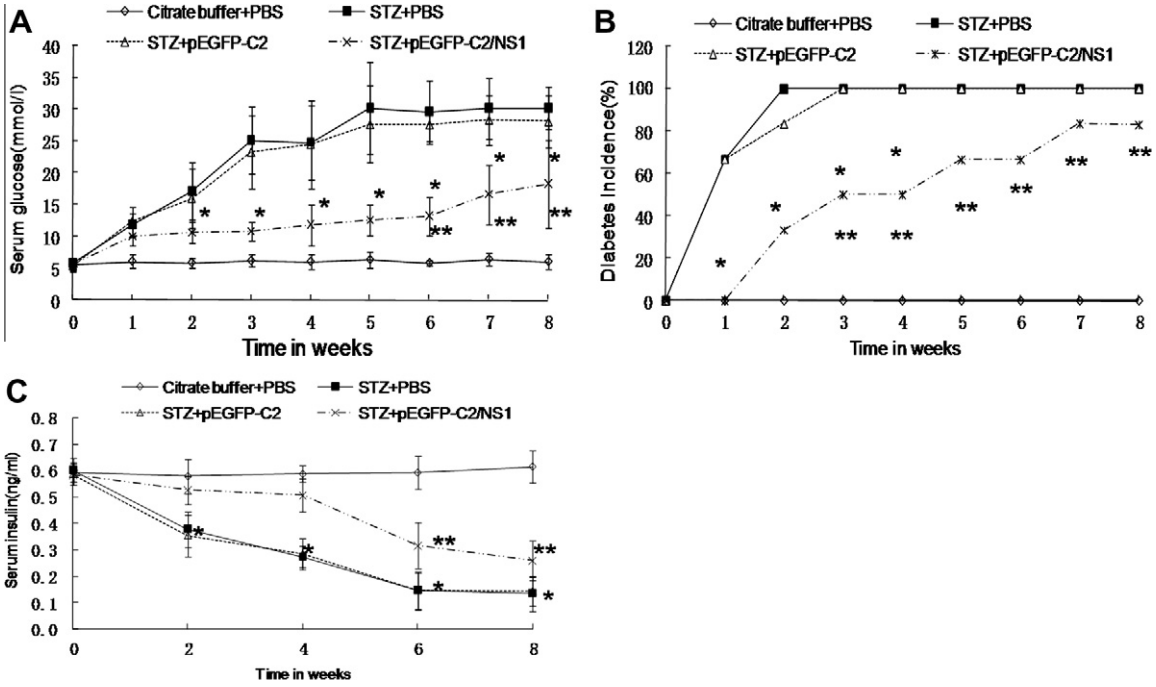

Fig. 2. Effect of pEGFP-C2/NS1 on serum glucose, diabetes incidence and serum insulin. (A) Effect of pEGFP-C2/NS1 on the serum glucose concentration. (B) Effect of pEGFP-C2/NS1 on diabetes incidence. The incidence of diabetes was expressed as a cumulative percentage of mouse serum glucose levels greater than 11.1 mmol/l. (C) Effect of pEGFP-C2/NS1 on serum insulin. Intramuscular delivery with pEGFP-C2/NS1 plasmid ameliorated the rise in mean glucose level, reduced the incidence of diabetes and increased mean insulin contents of STZ-treated mice. Values were presented as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ vs. STZ + PBS, $^{**}P < 0.05$ vs. citrate buffer + PBS.

Table 1
Effects of NS1 on glycogen content.

Parameters	Citrate buffer + PBS	STZ + PBS	STZ + pEGFP-C2	STZ + pEGFP-C2/NS1
Hepatic glycogen content (mg/g tissue)	29.09 ± 2.20	11.81 ± 0.98[a]	11.64 ± 1.16[a]	25.73 ± 1.41[a,b]
Muscle glycogen content (mg/g tissue)	3.87 ± 0.61	1.85 ± 0.49[a]	1.67 ± 0.50[a]	2.95 ± 0.09[a,b]

The dates are analyzed by one-way ANOVA.
Values are expressed as mean ± SD for six mice in each group.
[a] $p < 0.01$ by comparison with citrate buffer + PBS.
[b] $p < 0.01$ by comparison with STZ + PBS.

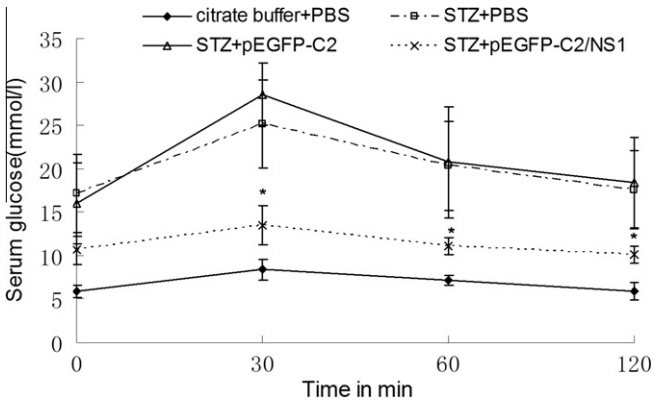

Fig. 3. Effect of pEGFP-C2/NS1 on intraperitoneal glucose tolerance test (IPGTT). The serum glucose levels after glucose loading were significantly lower in STZ + pEGFP-C2/NS1 mice compared to STZ + PBS and STZ + pEGFP-C2 mice. The pEGFP-C2/NS1 plasmid improved glucose tolerance. Values were presented as mean ± SD, $n = 6$ for each group. $*P < 0.01$ vs. STZ + PBS.

4. Discussion

The NS1 of influenza A viruses is a non-essential virulence factor that has multiple accessory functions including inhibition of host innate and adaptive immune responses, and activation of phosphoinositide 3-kinase (PI3K). Limited studies have been tried using the application of NS1, including the development of novel antiviral drugs targeting functions of the NS1, the design and manufacture of influenza vaccines by truncating or mutating NS1, and the manufacture oncolytic influenza A viruses with mutated NS1 gene [12]. In this study, NS1 was used for the first time in diabetic mice induced by STZ. We successfully constructed the NS1 expressing plasmid pEGFP-C2/NS1 and injected it intramuscularly into the left and right thighs of mice after injection of STZ. Our results indicate that the expression of NS1 of influenza A virus has a beneficial effect on hyperglycemia and insulitis of mice induced by STZ.

In our study, NS1 could ameliorate hyperglycemia, reduce diabetes incidence and increase glycogen synthesis, which may be associated with the activation of the PI3K/Akt pathway. NS1 can activate the PI3K/Akt pathway by interacting with the p85 regulatory subunit of PI3K via direct binding to the SH3 and C-terminal SH2 domains of p85 [23,24], and it can also directly interact with Akt and hence enhance Akt kinase activity [15]. Active forms of Akt have been shown to promote the membrane translocation of the glucose transporter GLUT4 and thus increase cellular glucose uptake and glycolysis. In addition, Akt suppresses gluconeogenesis by phosphorylating FoxO1 and promotes glycogen synthesis by phosphorylating and thus inactivating glycogen synthase kinase

Table 2
Effect of NS1 on cytokines.

Parameters	Citrate buffer + PBS	STZ + PBS	STZ + pEGFP-C2	STZ + pEGFP-C2/NS1
Serum				
IL-10(pg/ml)	74.31 ± 8.83	40.66 ± 8.66[a]	40.40 ± 7.76[a]	58.53 ± 7.8[a,b]
TNF-α(pg/ml)	115.57 ± 6.89	216.44 ± 25.31[a]	213.00 ± 34.26[a]	156.11 ± 15.46[a,b]
IFN-γ(pg/ml)	146.47 ± 7.26	272.86 ± 27.37[a]	279.72 ± 27.21[a]	178.78 ± 19.36[b]
Spleen				
IL-10(pg/ml)	147.17 ± 16.68	74.67 ± 8.41[a]	76.17 ± 11.43[a]	99.83 ± 11.41[a,b]
TNF-α(pg/ml)	204.17 ± 24.91	403.67 ± 30.18[a]	400.33 ± 32.65[a]	272.33 ± 43.90[a,b]
IFN-γ(pg/ml)	265.83 ± 35.64	589.83 ± 29.66[a]	585.33 ± 38.20[a]	343.67 ± 49.05[a,b]

The dates are analyzed by one-way ANOVA.
Values are expressed as mean ± SD for six mice in each group.
[a] $p < 0.05$ by comparison with citrate buffer + PBS.
[b] $p < 0.05$ by comparison with STZ + PBS.

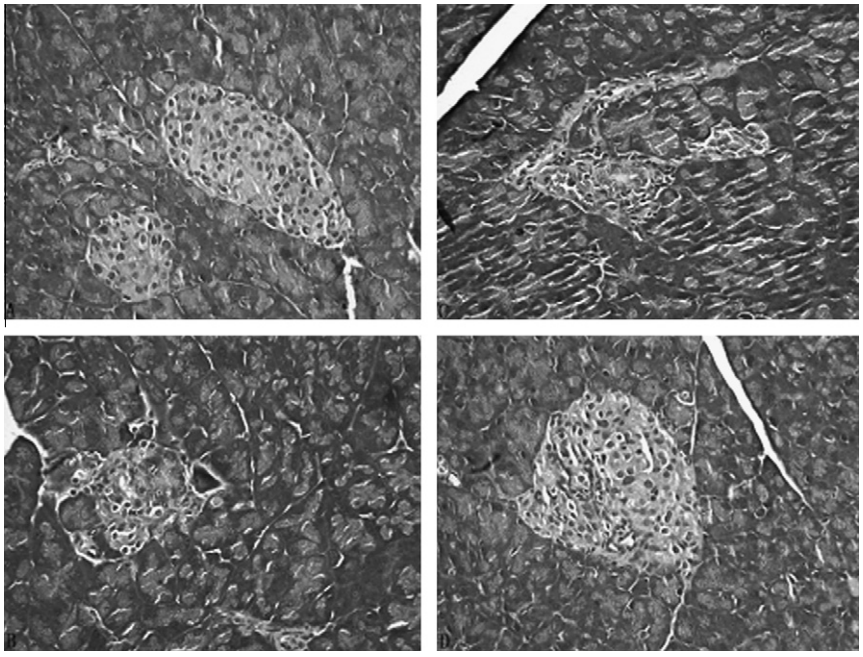

Fig. 4. Morphology of the pancreatic islet with hematoxylin-eosin stain. (A) Citrate buffer + PBS groups: normal islet structure. (B) STZ + PBS group: the pancreatic islet size and its cell number were significantly reduced, necrosis appeared and lymphocytes infiltered. (C) STZ + pEGFP-C2 group: the damage was similar to the damage of STZ + PBS group. (D) STZ + pEGFP-C2/NS1: the pancreatic islet size and its cell number were significantly increased compared to diabetic control and pEGFP-C2-treated mice, minority mononuclear cell invaded into islets. Magnification was ×400.

3 (GSK3), an inhibitory kinase for glycogen synthase [25,26]. Thus, PI3K/Akt pathway activation decreases blood glucose levels and plays a critical role in maintaining glucose homeostasis.

The decrease of blood glucose is also associated with the increase of serum insulin levels. In this study, pEGFP-C2/NS1 injection increased insulin levels in STZ-treated mice, which indicates that NS1 could protect β cells from damage. Morphological examinations of the pancreas also show that the islet size and its cell number were well preserved in mice treated with pEGFP-C2/NS1 plasmid. Progressive insulin-producing β-cell death is the main feature of diabetes and apoptosis is probably the main form of β-cell death [27]. Thus, halting the apoptosis of β cells is a key tactic for the prevention and treatment of both types of the disease. It has been indicated that PKB/Akt activation plays a pivotal role in β cell survival, growth, mass, function and protects pancreatic beta cells from apoptotic damage [28,29]. NS1 can activate the PI3K/Akt pathway, and can also directly interact with Akt and hence enhance Akt kinase activity, which leads to a subsequent inhibition of pro-apoptotic factors such as BAD, caspase 9 and glycogen synthase-kinase 3β(GSK-3β) and limitation of apoptosis [14,15]. The exogenously expressed NS1 was able to associate with the tumor suppressor p53 and inhibit p53-mediated transcriptional activity and apoptosis [16]. In addition, the NS1 has IFN-dependent anti-apoptotic potential, it may elevate the apoptotic threshold and delay the onset of programmed cell death in infected cultures by inhibiting PKR and IRF3 to suppress activation of NF-κB [13]. NS1 may protect β cells from damage by activating the PI3K/Akt pathway to limit apoptosis.

In recent years, the major role ascribed to NS1 has been its inhibition of host immune responses, especially the limitation of interferon (IFN) production by numerous protein:protein and protein:RNA interactions [12]. NS1 can prevent dsRNA- and virus-mediated activation of the IRF-3, NF-κB and c-Jun/ATF-2 transcription factors by forming a complex with RNA helicases retinoic acid inducible gene-I (RIG-I), which limits the induction of proinflammatory cytokines and IFNs by pre-transcriptional processes. In addition, NS1 can also disrupt host cellular mRNA processing and nuclear-cytoplasmic export by interacting with the cleavage polyadenylation specificity factor 30(CPSF30) and poly (A)-binding protein II (PABPII), which limits IFNs production by post-transcriptional (nuclear)

processes. Notably, in addition to inhibition of IFN gene transcription, NS1 promotes the accumulation of IFN pre-mRNA transcripts [30]. In addition to limiting IFN production, influenza A virus NS1 can also control the production of other proinflammatory cytokines, such as IL-6, TNF-α, CCL3 (MIP-1a), IL1β and IL18, through the function of its N- and C-terminal domains [31]. Influenza A virus NS1 is a bifunctional viral immunosuppressor that inhibits innate immunity by preventing the release of IFN and the proinflammatory cytokines TNF-α and IL-6, and inhibits adaptive immunity by attenuating human dendritic cell (DC) maturation and the capacity of DCs to induce T-cell responses [32]. Our studies have also shown that intramuscular injection of pEGFP-C2/NS1 significantly increases the levels of IL-10 and decreases the production of IFN-α and TNF-α compared with the injection of pEGFP-C2. The regulation of cytokines may be beneficial for β cells, as they inhibit the incidence of insulitis and decrease the degree of insulitis. It is possible that NS1 decreases serum glucose levels and ameliorate the symptoms of T1D by regulating the production of inflammatory cytokines and by inhibiting the host immune responses.

This study demonstrated that NS1 is effective in the prevention and treatment of diabetes mellitus induced by STZ in a mouse model, although the precise mechanisms responsible for the response to NS1 requires further examination. The effect of NS1 on STZ-treated mice was diminished 6 weeks after injection, which may be related to the loss of recombinant plasmid or the silencing of episomal transgene expression. Further studies are needed to prolong plasmid DNA expression in mice. In addition, it may be possible to heighten pEGFP-C2/NS1 plasmid's effects on STZ-induced mice by chemically linking the plasmid DNA to special receptors of β cells.

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