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## Intramuscular injection of interleukin-10 plasmid DNA prevented autoimmune diabetes in mice<sup>1</sup>

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**KEY WORDS** interleukin-10; insulin-dependent diabetes mellitus; gene therapy; intramuscular injections; spleen; tumor necrosis factor; interferon type II; mice

### ABSTRACT

**AIM:** To investigate the effect of plasmid coding interleukin-10 (IL-10) DNA on the development of autoimmune diabetes induced by multiple low doses of streptozotocin (STZ) in mice. **METHODS:** Injection of STZ (40 mg/kg, ip) was given daily for five consecutive days. pcDNA3-IL-10 plasmid (IL-10-treated group) or pcDNA3-null plasmid (pcDNA3-null-treated group) (100 µg DNA once a day) were injected into skeletal muscles of mice on d 1 and d 14. Blood glucose concentration was measured. After mice were killed on d 28, serum IFN-γ level was measured by ELISA, and pancreatic IL-1β and TNF-α mRNA expression was detected by semi-quantitative reverse-transcription PCR (RT-PCR). The number of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from spleen was detected using FACS. In addition, pancreatic histology was measured for determination of insulinitis grades. **RESULTS:** Treatment with pcDNA3-IL-10 resulted in the retention and expression of the vector in skeletal muscle, associated with a considerable elevation in the plasma level of IL-10, which was not observed in pcDNA3-null-treated mice. In IL-10-treated diabetic mice induced by STZ, delay-type hypersensitivity responses were suppressed and the glucose level was greatly lower on d 14, 21, and 28 than pcDNA3-null-treated group ( $P < 0.05$  or  $P < 0.01$ ). On d 21 and 28 the incidence of diabetes was 33.3 % and 40.0 %, respectively, which was markedly lower than that of pcDNA3-null-treated group ( $P < 0.05$ ). In IL-10-treated mice pancreatic IL-1β and TNF-α mRNA expression was depressed, and serum IFN-γ concentration and the number of spleen CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes were decreased on d 28. The insulinitis grades of IL-10-treated mice were lower than that of pcDNA3-null-treated group ( $P < 0.01$ ). **CONCLUSION:** Systemic administration of IL-10 plasmid DNA can alleviate insulinitis of experimental autoimmune diabetes in mice and reduce incidence of diabetes.

### INTRODUCTION

Type 1 diabetes is considered to result from an

autoimmune process that selectively destroys β cells within the Langerhans' islets of the pancreas<sup>[1]</sup>. Although the precise mechanisms that lead to this destruction have not yet been elucidated, a cell-mediated immune response has been suggested both in human and one of its animal models, the low-dose streptozotocin (STZ)-induced mouse diabetes<sup>[2-4]</sup>, which is also a classical animal model of autoimmune diabetes. This model

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resembles human type 1 diabetes, and most of its characteristics are infiltration of T-lymphocytes and macrophages to islets.

Interleukin-10 (IL-10) is a pleiotropic cytokine produced mainly by the Th2 subset of helper T-lymphocytes, B-lymphocytes, and macrophages. Many studies suggested that IL-10 played an important role in down-regulating cell-mediated immune responses orchestrated by products of activated Th1 cells<sup>[5]</sup>. Therefore, IL-10 might be a valuable agent for treatment of T cell-mediated autoimmune diseases such as type 1 diabetes. More recently, many studies have shown that recombinant cytokines (IL-4, IL-12) could markedly reduce incidence of autoimmune diabetes<sup>[6]</sup>. However, because the half-lives of these cytokines was very short *in vivo*, these recombinant cytokines need to be administered repeatedly with large amounts<sup>[7]</sup>. Thus, it is an important strategy to administer expression vector coding IL-10 gene to prevent early onset of type 1 diabetes. Recently, one study has provided the first evidence that adeno-associated virus-mediated IL-10 gene transfer can prevent autoimmune diabetes in the nonobese diabetic model<sup>[8]</sup>. However, at relatively low concentrations of plasmid, little or no toxic effect has been reported in mice, rabbits, pigs and nonhuman primates after systemic administration of the naked plasmid DNA coding cytokine genes<sup>[9,10]</sup>, so the purpose of this study was to investigate whether injection of IL-10 plasmid DNA could prevent mice from autoimmune diabetes.

## MATERIALS AND METHODS

**Animals** C57BL/6J male mice aged 6-10 weeks, weighing approximately 30 g (Shanghai Experimental Animal Center, Chinese Academy of Sciences, Grade II, No SYXK-SHANGHAI 2002-0023) were kept under specific pathogen-free conditions in our animal facility.

**Expression vector construction** IL-10 530 bp cDNA fragment was produced by RT-PCR from concanavalin A (Con A)-stimulated human peripheral blood lymphocytes and confirmed by sequencing analysis. The primers (sense primer: 5'-CGGAATTCCACCATGCAC-A-GCTCAGC-3' and antisense: 5'-CGTCTAGAGATG-TCTCAGTTTCGTATC-3') were used by PCR. The amplified IL-10 cDNA 508 bp-fragment containing entire coding sequence was cloned into the pcDNA3 vector at the restriction enzyme sites of *EcoR* I and *Xba* I under control of CMV promoter.

**Plasmid DNA preparation** Large-scale plasmid DNA preparation was produced by alkaline lysis method using a Qiagen kit (Qiagen, CA). All plasmid preparations for im injections were suspended in sterile 0.85 % saline. Spectrophotometric analysis revealed at  $A_{260\text{ nm}}/A_{280\text{ nm}} \geq 1.80$ . Purity of DNA preparations and conformations were confirmed by 1 % agarose gel on electrophoresis.

**RT-PCR** One or two weeks after injection of 100  $\mu\text{g}$  of pcDNA3-IL-10 or pcDNA3-null plasmid DNA into left tibialis anterior (TA) muscle, total RNA was extracted using TRIzol reagent. Total RNA was treated with DNase I, then 0.5  $\mu\text{g}$  RNA was used in a first-strand cDNA synthesis using oligo-dT primer. The PCR reaction generated a 508-bp fragment of human IL-10 by above described primers and a 591-bp fragment of murine- $\beta$ -actin (sense primer 5'-AACGAGCGGTTCCG-ATGCCCTGAG-3' and anti-sense primer: 5'-TGTCGC-CTTCACCGTTCCAGTT-3'). The RT-PCR products were detected by 1.5 % agarose gel on electrophoresis.

**Determination of plasma IL-10 level** To establish the optimal dose of plasmid DNA, different amounts of IL-10 plasmid DNA were injected at different TA muscle sites. Each mouse received a total of 100  $\mu\text{g}$  of IL-10 or pcDNA3-null plasmid DNA injection at left TA muscle. One or two weeks after injection, plasma samples were assayed for IL-10 concentration by ELISA (Shenxiong Biotech Co, Shanghai).

**Induction and measurement of delayed-type hypersensitivity (DTH) response in mice** To examine whether intramuscular injections of plasmid DNA produced sufficient IL-10 to exert biologic effects, DTH model was used. On d 0, C57BL/6J mice were immunized with 200  $\mu\text{g}$  of ovalbumin (OVA) (Sigma, USA) in complete Freund's adjuvant (CFA) (Sigma, USA). On d 5, mice of experimental group were injected with 100  $\mu\text{g}$  of IL-10 plasmid DNA into muscle, and mice in control group received equivalent amounts of pcDNA3-null plasmid DNA. On d 6, mice were injected with OVA in PBS in the right footpad. DTH responses were measured, with calipers, as the increase in footpad thickness 24, 36, and 48 h after OVA Ag recall immunization<sup>[11]</sup>.

**Treatment of experimental groups** C57BL/6J male mice, aged 6-10 weeks, were used. These mice were free access to tap water and pelleted food throughout the experiment. The mice were divided into three groups: STZ (Sigma, USA)+pcDNA3-null plasmid ( $n=14$ ), STZ+IL-10 plasmid DNA ( $n=15$ ), and

saline+pcDNA3-null plasmid ( $n=8$ ). Intramuscular injections of plasmid DNA were done as previously described<sup>[4,11]</sup>. The mice first received either an ip injection of saline (0.2 mL) or STZ (40 mg/kg body weight). After 30 min the mice were given a second intramuscular injection (left tibialis anterior muscle) of either IL-10 plasmid DNA or pcDNA3-null plasmid using a 100  $\mu$ L micro-syringe. The first type (saline or STZ) was given daily for 5 consecutive days, while the second type of injection (IL-10 plasmid DNA or pcDNA3-null plasmid) was given on d 14 after first injection of IL-10 plasmid DNA or pcDNA3-null plasmid. The site of injection was right tibialis anterior muscle of mice. The mice were killed on d 28.

Blood glucose values were detected using One Touch blood glucose metre (Glucocard, USA). The points of measurement were performed on d 0, 7, 14, 21, and 28.

**Flow cytometric analysis** To investigate whether the administration of IL-10 plasmid DNA could affect the number of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from spleen, the number of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes was measured using flow cytometric analysis scan (FACS). Single cell suspensions of spleen cells were prepared from IL-10-treated or pcDNA3-null-treated mice on d 28 after first injection of STZ. Lymphoid cells from spleen by mechanical isolation and selection of hole density-optimized nylon fiber were stained by CD4<sup>+</sup> or CD8<sup>+</sup> molecules. Cells were diluted to a concentration of  $2 \times 10^{10}$  cells/L in FACS buffer (1 % FBS, 0.05 % NaN<sub>3</sub> in PBS pH 7). Antibody [FITC-conjugated anti-mouse CD4<sup>+</sup> or CD8<sup>+</sup> monoclonal antibody (PharMingen, USA)] 1  $\mu$ g was incubated with 50  $\mu$ L of cell suspension at 4 °C for 30 min. Cells were then washed twice in FACS buffer before fixing with 2% paraformaldehyde in PBS at 4 °C for 15 min. Cells were suspended in 300  $\mu$ L FACS buffer and processed with a FACScan (Becton Dickinson, CA).

**Semi-quantitative RT-PCR analysis of IL-1b and TNF- $\alpha$**  Pancreas tissues were removed from IL-10-treated mice and pcDNA3-null-treated mice on d 28 after first injection of STZ. Total RNA was extracted from pancreas tissues using TRIzol reagent. Extracted RNA samples were treated with RNA-free DNase I. PCR were performed to amplify 792-bp fragment of murine IL-1 $\beta$  (sense primer: 5'-AATGCCACCTTTT-GACAG-3' and anti-sense primer: 5'-CCAGCCCATAC-TTTA-GGA-3') and 446-bp fragment of murine TNF- $\alpha$  (sense primer: 5'-AGCCCACGTCGTAGCAA-

CCACCAA-3' and anti-sense primer: 5'-ACACCCATTC-CCTTCACAG-AGCAAT-3'). In addition, 591-bp fragment of murine  $\beta$ -actin (primers shown above) was amplified under the same conditions. PCR amplifications were performed in a total volume of 50  $\mu$ L containing KCl 50 mmol/L, Tris-HCl 10 mmol/L (pH 8.4), 0.01 % gelatin, MgCl<sub>2</sub> 1.5 mmol/L, 200 mmol/L of each dATP, dCTP, dGTP, dTTP, 0.2 mmol/L of each primer and 1 U of *Taq* DNA polymerase. The amplification was performed as follows: incubation for 5 min at 95 °C, 35 cycles of incubation for 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, followed by an extension step of 5 min at 72 °C. When TNF- $\alpha$  gene was amplified, the temperature of annealing was 54 °C. The RT-PCR products were detected by 1.5 % agarose gel on electrophoresis.

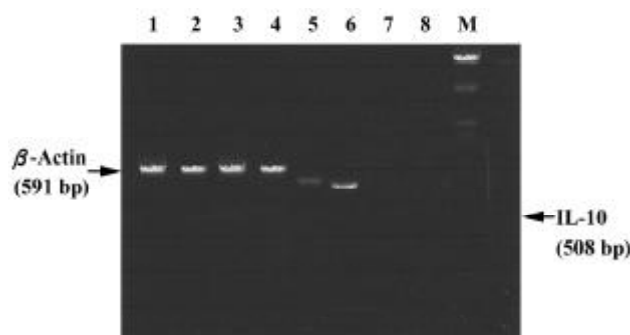
**Measurement of serum IFN- $\gamma$  levels** ConA (20 mg/kg body weight) were intraperitoneally injected to IL-10-treated mice and pcDNA3-null-treated mice on d 28 after first injection of STZ<sup>[12]</sup>. The blood samples were obtained at 8 h after ConA injection. Sera were appropriately diluted and assayed for concentration of IFN- $\gamma$  by ELISA according to the antibody manufacturer recommendation (Shenxiang Biotech Co, Shanghai). Plates were read at  $OD_{490\text{ nm}}$ , and concentration of IFN- $\gamma$  was expressed as ng/L.

**Morphologic examination** Pancreas were removed from mice and fixed in 10 % formalin solution and embedded in paraffin. Sections 5- $\mu$ m-thick were cut and stained with haematoxylin and eosin. The pancreatic islet histology was ranked according to four classes as previously described<sup>[4,13]</sup>: class A: normal islet structure; class B: mononuclear cell infiltration in the periphery of the islets; class C: infiltration of mononuclear cells into a majority of the islets, ie, insulinitis; class D: only a few islets remaining, often with altered histology, for example appearance of pyknotic cell nuclei. Two independent examiners who were unaware of the origin of sections evaluated the morphological change of the sample.

**Statistical analysis** The quantitative data were expressed as mean $\pm$ SD and compared using unpaired *t*-test. The significant differences of incidence of diabetes or grade of insulinitis between IL-10-treated group and pcDNA3-null-treated group were used by chi-square test and two-independent samples test (Mann-Whitney Test), respectively. Statistic treatment was performed by Software SPSS 9.0. Differences were considered to be significant if  $P < 0.05$ .

**RESULTS**

**Detection of expression of IL-10 gene in skeletal muscle** Expression of IL-10 mRNA was detected on d 7 and d 14 at tibialis anterior muscle after administration of expression vector (Fig 1). However, it was not detected on d 28 after gene transfer (data not shown).



**Fig 1.** RT-PCR detection of IL-10 mRNA in muscle injected with IL-10 plasmid DNA. Lane 1 and 2: the amplification of b-actin was served as an internal standard and represented skeletal muscle on d 14 and 7 after injection of IL-10 plasmid DNA; Lane 3 and 4: the fragments of b-actin were detected in skeletal muscle on d 14 and 7 after injection of pcDNA3-null plasmid DNA. Lane 5 and 6: the fragments of IL-10 were obtained in skeletal muscle on d 14 and 7 after injection of IL-10 plasmid DNA; Lane 7 and 8: the fragments of IL-10 were not detected in skeletal muscle on d 14 and 7 after injection of pcDNA3-null plasmid DNA; Lane M: DNA marker.

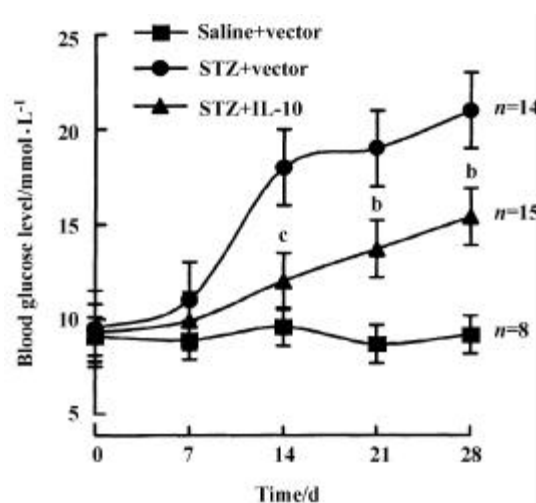
**Increased IL-10 plasma level** Plasma IL-10 concentration was markedly increased on d 7 and d 14 after IL-10 plasmid DNA injection, and the mean plasma concentration of IL-10 on d 7 and 14 was (125±27) ng/L and (69±16) ng/L, respectively. However, plasma IL-10 was not detected in pcDNA-null-treated mice.

**Suppression of DTH responses** DTH responses were suppressed in IL-10-treated mice at 24, 36, and 48 h compared with pcDNA3-null-treated mice (Tab 1).

**Protection of diabetes by IL-10 plasmid DNA administration** The mice treated with multiple low doses of STZ became gradually hyperglycaemic. Treatment with IL-10 plasmid DNA during the observation period reduced the blood glucose level from d 14 ( $P < 0.01$  or  $P < 0.05$ ) (Fig 2). In addition, administration of IL-10 plasmid DNA greatly reduced the incidence of diabetes induced by multiple low doses of STZ (Tab 2). The first case of diabetes occurred 7 d after first injection of STZ in the pcDNA3-null-treated group. On d

**Tab 1.** Suppression of DTH responses by intramuscular injection of IL-10 plasmid DNA.  $n=5$  mice. Mean±SD.  $^cP < 0.01$  vs pcDNA3-null-treated mice.

Group	Increase in footpad thickness/mm		
	24 h	36 h	48 h
pcDNA3-null-treated mice	0.53±0.06	0.65±0.06	0.56±0.07
IL-10-treated mice	0.20±0.02 <sup>c</sup>	0.26±0.03 <sup>c</sup>	0.31±0.02 <sup>c</sup>



**Fig 2.** Effect of muscle administration of IL-10 plasmid DNA on blood glucose level in diabetic mice. Mean±SD.  $^bP < 0.05$ ,  $^cP < 0.01$  vs (STZ+vector)-treated mice.

**Tab 2.** Effect of administration of IL-10 plasmid DNA on the incidence of diabetes. Mouse with blood glucose level  $\geq 16.7$  mmol/L was diagnosed as diabetes.  $^bP < 0.05$  vs pcDNA3-null-treated group. The number in parenthesis represented the percentage of diabetic rats (%).

Group	Number of diabetes			
	7 d	14 d	21 d	28 d
pcDNA3-null-treated mice ( $n=14$ )	1 (7.1)	6 (42.9)	10 (71.4)	12 (85.7)
IL-10-treated mice ( $n=15$ )	0 (0.0)	2 (13.3)	5 (33.3) <sup>b</sup>	6 (40.0) <sup>b</sup>

21 and d 28, the incidence of diabetes were two times higher in pcDNA3-null-treated mice compared with those treated with IL-10 plasmid DNA ( $\chi^2 = 4.209$  and  $6.428$ , respectively.  $P < 0.05$ ).

**Reduced pancreatic IL-1b and TNF-a mRNA expression in IL-10-treated mice** IL-1 $\beta$  and TNF- $\alpha$

mRNA were expressed in the pancreas of pcDNA3-null-treated mice with diabetes. But IL-1 $\beta$  and TNF- $\alpha$  mRNA expression in IL-10-treated mice without diabetes was not detected, respectively (Fig 3).



**Fig 3.** Expression of IL-1 $\beta$  and TNF- $\alpha$  mRNA in pancreas was detected by semi-quantitative RT-PCR analysis. Lane 1, 2, 8, 9: the amplification of  $\beta$ -actin was served as an internal standard. Lane 3, 6 show IL-1 $\beta$  and TNF- $\alpha$  mRNA expression in pancreas from pcDNA3-null-treated mice, respectively. Lane 4, 7 show no IL-1 $\beta$  and TNF- $\alpha$  mRNA expression in pancreas from IL-10-treated mice, respectively. Lane 5: DNA marker.

**Decreased serum IFN- $\gamma$  level** Serum IFN- $\gamma$  level in IL-10-treated mice ( $n=15$ ) was significantly lower than that in pcDNA3-null-treated mice ( $n=14$ ) [(443 $\pm$ 134) vs (782 $\pm$ 172) ng/L,  $P<0.01$ ].

#### Reduced number of CD4 $^+$ or CD8 $^+$ lymphocytes

On d 28 after first injection of STZ, the number of CD4 $^+$  and CD8 $^+$  lymphocytes from spleen of IL-10-treated mice was markedly lower than that of pcDNA3-null-treated mice, respectively [(12.6 $\pm$ 2.1) % vs (17.3 $\pm$ 1.1) % for CD4 $^+$ ; (11.5 $\pm$ 1.8) % vs (20 $\pm$ 3) % for CD8 $^+$ ].

#### Protection from insulinitis in IL-10-treated mice

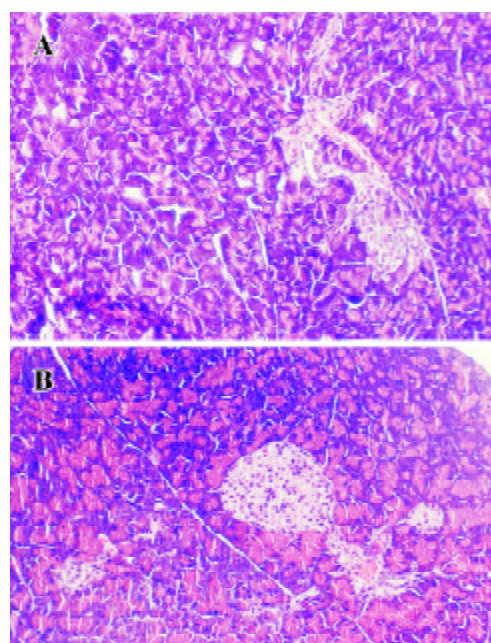
Morphological examinations of the pancreas on d 28 of mice receiving STZ plus pcDNA3-null plasmid DNA revealed an obvious insulinitis and structural changes of the islets (Tab 3). A similar histological pattern around the islets was also found in the (STZ plus IL-10 plasmid DNA)-treated mice, but the number of inflammatory lesions and the degree of insulinitis appeared to be greatly decreased (Fig 4). In addition, normal islets were found in saline plus pcDNA3-null treated mice.

## DISCUSSION

We successfully cloned human IL-10 gene from human peripheral lymphocytes using RT-PCR technique. We found that IL-10 mRNA expression was present on d 7-d 14 at skeletal muscle after injection into mouse.

**Tab 3.** Effects of IL-10 plasmid DNA on pancreatic islet morphology. \* $P<0.01$  vs (STZ+pcDNA3-null) mice. A, B, C, and D was the pancreatic islet history rank, respectively.

Group	Total number of animals	Number of mice in each rank			
		A	B	C	D
Saline+pcDNA3-null	8	8	0	0	0
STZ+pcDNA3-null	14	0	2	11	1
STZ+IL-10 plasmid	15	5 <sup>c</sup>	9 <sup>c</sup>	1 <sup>c</sup>	0



**Fig 4.** Histological observation of pancreas of IL-10-treated and pcDNA3-null-treated diabetic mice. An example of an islet from a pcDNA3-null-treated mouse (A) showed severely destroyed islet. Example of a normal islet from an IL-10-treated mouse (B) displayed no evidence of islet infiltration.

In addition, the serum level of IL-10 was significantly increased on d 7 or d 14 after intramuscular injection of 100  $\mu$ g IL-10 plasmid DNA. Also, we used a DTH model to prove that intramuscular injection of IL-10 plasmid DNA produced sufficient IL-10 to exert biologic effects.

According to expression of IL-10 plasmid DNA *in vivo*, the mononuclear cell infiltration in the pancreatic islets usually reaches its peak on 28 d after first injection of STZ. In the present study d 1 and d 14 were selected to inject IL-10 plasmid DNA after first injection of STZ. Mice were killed on d 28 after first injection of STZ. Our results showed that IL-10 par-

tially protected mice against hyperglycaemia and reduced pancreatic insulinitis. Also, our results demonstrated that injection of IL-10 plasmid DNA markedly reduced pancreatic IL-1 $\beta$  and TNF- $\alpha$  mRNA expression, and decreased release of IFN- $\gamma$ .

Recent studies have demonstrated that the infiltration of autoreactive mononuclear cells into the islet (insulinitis) is due to the imbalance of cytokines secreted by helper type 1 (Th1) and type 2 (Th2) CD4<sup>+</sup> T cells<sup>[14-16]</sup>. Proinflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and IL-1) secreted by Th1 cells increased the inflammatory and cell-mediated immunity, whereas anti-inflammatory cytokines such as IL-4 and IL-10 secreted by Th2 cells stimulated humoral immunity<sup>[4,12,16-18]</sup>. Therefore, IL-10 could reduce insulinitis by suppressing the activation of Th1 cells. Our results demonstrated that the administration of IL-10 gene markedly suppressed Th1 cell-produced cytokines such as IL-1, TNF- $\alpha$ , and IFN- $\gamma$ . Particularly, IFN- $\gamma$  may contribute to islet cell injury, since it has direct toxic effects on islet cells, activates macrophages, and stimulates nitric oxide production<sup>[12,13]</sup>. Many studies found that autoimmune diabetes was delayed in IFN- $\gamma$  gene knockout nonobese diabetic (NOD) mice<sup>[19]</sup>, and prevented by anti-IFN- $\gamma$  mAb treatment<sup>[20]</sup>. In addition, we found that IL-10 plasmid DNA administration decreased the number of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from spleen on d 28 after first injection of STZ. CD4<sup>+</sup> T-cells play an important role in the regulation of immune responses by producing various cytokines. Thus, our result was consistent with down-regulation of the above mentioned cytokines such as IL-1, TNF- $\alpha$ , and IFN- $\gamma$  in this experiment.

In conclusion, systemic administration of IL-10 plasmid DNA could alleviate insulinitis of experimental autoimmune diabetes in mice and reduce incidence of diabetes. It provides evidence to support the possibility of using IL-10 gene therapy to prevent type 1 diabetes in human beings.

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