CMV-hFasL transgenic mice are sensitive to low doses of streptozotocin-induced type I diabetes mellitus[1]

LIN Bo, ZHANG Zhen-Lin[2], YU Lu-Yang, GUO Li-He[3]

Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031; [2]Center for Preventing and Treating Osteoporosis, Osteoporosis Research Unit, Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai 200233,China

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ABSTRACT

AIM: To investigate the role of Fas-FasL pathway in the pathogenesis of streptozotocin (STZ)-induced type I diabetes mellitus. **METHODS**: Low dose injections of STZ were used to induce type I diabetes mellitus in the CMV-hFasL transgenic mice. Blood glucose concentration was measured with Glucotrand Plus blood glucose test strips. Expression of hFasL was detected by RT-PCR and Western blotting. The severity of insulitis was determined by histological examination. Expressions of IL-1β and TNF-α mRNA in the pancreas were detected by semi-quantitative RT-PCR analysis. Fas expression in apoptotic RIN-5F cells was also confirmed by RT-PCR *in vitro*. **RESULTS**: hFasL was expressed in the islets of CMV-hFasL transgenic mice. The transgenic mice were sensitive to diabetic induction than the control WT mice. IL-1β and TNF-α expressions in the pancreas of CMV-hFasL transgenic mice were far more than that in WT mice. We also found STZ and IL-1β could both induce higher expression of Fas in RIN-5F. The combining of Fas-FasL could lead to the apoptosis of β cells in the CMV-hFasL transgenic mice. **CONCLUSION**: Fas-FasL interaction plays a significant role in the pathogenic mechanism of type I diabetes mellitus.

INTRODUCTION

Type I diabetes mellitus is a T-cell-dependent autoimmune disease resulting in selective destruction of the β-cells in the islets of Langerhans. β-cells apoptosis has been associated with type I diabetes mellitus onset in both animal models and newly diagnosed diabetic patients[1,2].

Various studies have alternatively implicated FasL, perforin, or TNF as effectors of apoptotic islet cell death. However, Fas has been suggested to be the most important effector playing a major role in β-cell apoptosis in type I diabetes mellitus. Much of the evidence for this comes from *in vitro* studies with isolated human or mouse islets of Langerhans[3-6].

Fas (CD95/Apo-1) is a member of the TNFR family that is able to cause apoptosis via a conserved intracellular death domain. Fas is constitutively expressed on a

Abbreviations: FasL: Fas ligand; NOD: nonobese diabetic; *lpr*: lymphoproliferation; *gld*: generalized lymphoproliferation; *scid*: severe combined immunodeficiency; TG: transgenic; WT: wild type; STZ: streptozotocin; TNF-α: tumor necrosis factor α; IL-1β: interleukin-1β; IFN-γ: interferon γ; PBS: phosphate buffered saline.

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[2] Correspondence to Prof GUO Li-He.
Phn 86-21-5492-1392. Fax 86-21-5492-1391.
E-mail lhguo@sunm.shcnc.ac.cn

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number of mouse tissues including thymocytes. FasL is sufficient for induction of apoptosis in Fas-expressing cells. The Fas-FasL interaction leads to activation of an intracellular signaling pathway that results in cell death by caspase-dependent mechanism[7].

Wang *et al* reported the vital role of Fas-FasL pathway in type I diabetes mellitus[3]. Their conclusion came from the following three results. (i) Fas-deficient NOD (NOD-*lpr*/*lpr*) mice failed to develop diabetes. (ii) The incidence of diabetes was markedly reduced following transfer of diabetogenic T cells into irradiated NOD-*lpr/lpr* mice. (iii) The risk of diabetes was increased in RIP-FasL-NOD mice. However, their results had been suggested, by other authors paradoxically, to be due to constitutive expression of FasL by NOD-*lpr/lpr* lymphocytes rather than indicating an essential role[8]. But Wang *et al* provided other evidence that NOD-*lpr/lpr-scid/scid* and NOD-*gld/gld* failed to develop type I diabetes mellitus, which showed a significant role of Fas-FasL pathway in this disease[9].

Fas-FasL mediated programmed cell death in the progression of type I diabetes mellitus was reported with the NOD mouse model. In this report we used hFasL transgenic mice under CMV promoter, which expressed hFasL extensively to determine whether CMV-hFasL transgenic mice were more sensitive to the induction of low dose of STZ.

MATERIALS AND METHODS

Mice C57BL/6J male mice between 6-8 weeks of age were used in all experiments. CMV-hFasL transgenic mice were generated by our group[10]. In brief, hFasL under CMV promoter was microinjected into the pronuclei of fertilized eggs of C57BL/6J F1 hybrid mice (Jackson Laboratory) according to the standard method. Animals were maintained in standard environmental conditions with free access to food and water. They were allowed to adapt to their environment for one week before initiating the experiments. The wild type mice and the transgenic mice were the same genetic background.

Cell culture and induction of apoptosis The rat insulinoma cell line RIN-5F was grown in RPMI-1640 medium containing 15 % heat-inactivated FCS, sodium pyruvate 1.0 mmol/L, glucose 4.5 g/L, and HEPES 10 mmol/L. When it was 70 % confluence, STZ 5 or 10 mmol/L or IL-1β 30U combined with TNF-α 1000 U and IFN-γ 1000 U were added to induce apoptosis and incubated for 24 h.

Animal dosing STZ (Sigma) was dissolved in sodium citrate buffer (pH 4.5) just before use and injected intraperitoneally 45 mg/kg for five daily doses[11].

Diabetes Mice were observed for the onset of diabetes with measurement of the glucose concentration in blood obtained from a tail vain which was measured using Glucotrand Plus blood glucose test strips (Roche). Consecutive readings of blood glucose levels >2500 mg/L (13.9 mmol/L) was considered diagnostic of diabetes onset. It was measured at least twice a week.

Histological examination of pancreata and insulitis scoring Mice were sacrificed when experiment was over at d 29. The pancreata were fixed in 4 % paraformaldehyde in 0.01 mol/L PBS (pH 7.4), processed for paraffin embedding, sectioned (6-μm) and stained with hematoxylin-eosin.

To determine the severity of insulitis, more than 30 pancreatic islets from three or more parallel sections of different cut levels were analyzed per mouse. And the number of islets was counted by one investigator in a blind fashion. The degree of insulitis was classified into four categories: 0, no insulitis; 1, periinsulitis with or without minimal lymphocyte infiltration in islet; 2, invasive insulitis with islet destruction ≤50%; 3, islet destruction >50 %[8].

Immunohistochemistry The pancreata were fixed overnight in 4 % PFA at 4 ºC and embedded in OCT and frozen in liquid nitrogen. Tissues of 10 μm were sectioned. The endogenous peroxides activity was quenched by 3 % hydrogen peroxide methanol for 20 min at room temperature. After a washing with PBS, the slides were incubated with blocking solution (SABC) for 30 min at 37 ºC followed by a washing with PBS. The slides were then incubated with rabbit anti-mouse insulin antibody (Santa Cruz, CA) 1 mg/L overnight at 4 ºC. After washing with PBS, the biotinylated anti-rabbit IgG antiobody (SABC) was added and incubated for 60 min at 37 ºC followed by peroxidase-avidin conjugate (SABC) for another 30 min. Finally the slides were washed with PBS and stained with DAB (SABC) at room temperature for 5-10 min. The color reaction was terminated by repeated washes in distilled water. The slides were counter-stained with hematoxylin for 20 min then dried and mounted with cover slips.

RT-PCR analysis To determine the expression of hFasL, we isolated the islets of 2 month-old wild-type mice and CMV-hFasL transgenic mice using the

standard method. Whole RNA was extracted with Trizol reagent (GIBCO) according to the manufacturer's instructions. Random hexamer primers (Promega) were employed for cDNA preparation using the MMLV reverse transcriptase (Promega). PCR was performed with the following conditions: denaturing at 94 ºC for 40 s, annealing at 58 ºC for 1 min, and synthesizing at 72 ºC for 1 min (Last cycles 10min) for 30 cycles. For detection of FasL, the primers were as follows: forward primer, 5'-AGA TCT ACT GGG TGG ACA GC-3'; reverse primer, 5'-CCA GAG AGA GCT CAG ATA CG -3'. Primers for housekeeping gene β-actin were as follows: forward primer, 5'-AAC GAG CGG TTC CGA TGC CCT GAG-3'; reverse primer, 5'-TGT CGC CTT CAC CGT TCC AGT T-3'.

We also extracted total RNA of diabetic pancreas to determine the expression of inflammatory molecules such as IL-1β and TNFα. Primers for IL-1β were as follows: forward primer, 5'-AAT GCC ACC TTT TGA CAG-3'; reverse primer, 5'-CCA GCC CAT ACT TTA GGA-3'. Primers for TNFα were as followed: forward primer, 5'-AGC CCA CGT CGT AGC AAA CCA CCA A-3'; reverse primer, 5'-ACA CCC ATT CCC TTC ACA GAG CAA T-3'.

Total RNA was extracted from RIN-5F cells to detect the expression of Fas. Primers were as follows: forward primer, 5'-GCA AGG GAC TGA TAG CAT CT-3'; reverse primer, 5'-CTT CCC GTG AGA TTG ATA CCA-3'.

Amplification products (20 %) were separated by electrophoresis on a 1.5 % agrose gel and visualized by ethidium bromide staining.

Western blotting After incubated in liquid nitrogen, the pancrea was smashed then suspended in suspending buffer (NaCl 0.1 mol/L, Tris-HCl 0.01 mol/L, pH 7.6, edetic acid 1 mmol/L, Aprotinin 1 mg/L, PMSF 100 mg/L). Equal volume of 2×SDS loading buffer was immediately added and boiled for 10 min. After centrifugation at 12 000 ×*g* for 10 min, the lysates were sonicated for 10 s and incubated on ice for 10 min. Samples 30 μL were resolved on 10 % SDS-PAGE. Proteins were electro-transferred onto a nitrocellulose membrane. The membranes were blocked with 5 % non-fat milk and probed with rabbit anti human FasL antibody (Santa Cruz, CA). The blots were washed and exposed to HRP-conjugated anti-rabbit IgG secondary antibody (Santa Cruz, CA) and then developed using the ECL reagent. The molecular mass of the protein was estimated relative to pre-stained size marker (Bio-Rad).

Analysis of apoptosis with FACS After RIN-5F cells were induced to undergo apoptosis, cells were fixed in 70 % ice-cold ethanol for 30 min, incubated with propidium iodide (PI) 75 mg/L and RNase 100 mg/L in dark at 4 ºC for 30 min. Then cells were analyzed by FACStar (Becton Dickinson) with excitation at 585 nm. Ten thousand events were acquired in list mode.

Statistical analysis Mean values and difference were analyzed by *t*-test. The *t*-tests assuming two samples were performed with the Microsoft EXCEL 2000 data analysis program. A two-tail *P* value is represented.

RESULTS

Detection of hFasL expression in islets by RT-PCR and Western blotting We detected the expression of hFasL in islets of transgenic mice. A 708-bp DNA fragment was found from the islets of CMV-hFasL transgenic mice with RT-PCR. But no such fragment was detected from the control WT mice (Fig 1A). The hFasL expression in the pancreas of CMV-hFasL

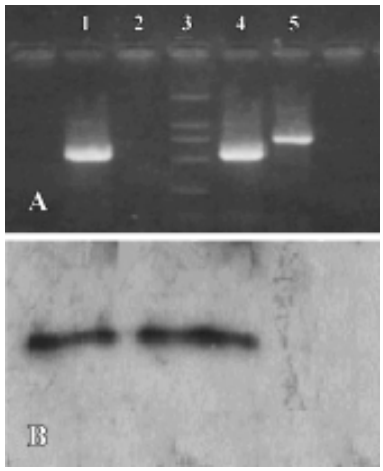

Fig 1. Detection of the expression of hFasL in the islets of CMV-hFasL transgenic mice (TG) and wild type (WT) mice. A: Detection of hFasL with RT-PCR. Lane 1: beta actin in the islets of WT mice; Lane 2: hFasL in the islets of WT mice; Lane 3: DNA marker DL-2000; Lane 4: beta-actin in the islets of CMV-hFasL transgenic mice (591 bp); Lane 5: hFasL in the islets of CMV-hFasL transgenic mice (708 bp). B: Detection of hFasL with Western blotting. Lane 1: positive control of hFasL from HepG2 cells which express hFasL constitutively; Lane 2: hFasL in the islets of CMV-hFasL transgenic mice; Lane 3: hFasL in the islets of WT mice.

transgenic mice was also confirmed by Western blotting (Fig 1B).

Diabetes detection after five daily-injections of STZ Five daily injections of STZ were used to induce type I diabetes mellitus as mentioned in the Materials and Methods.

The blood glucose of CMV-hFasL transgenic mice was much higher than that of WT mice since the d 22 (Fig 2) and showed significant difference. After blind to blind histological examination of pancreatic tissues at d 29, most of CMV-hFasL transgenic mice had more severe insulities than the control WT mice (Fig 3).

We found that the β cells of the diabetic mice were

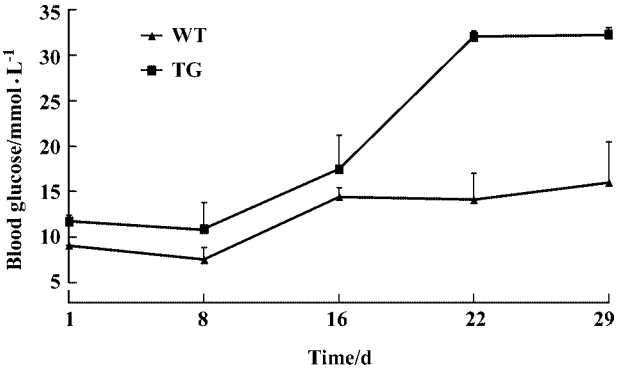

Fig 2. Acceleration of diabetes in CMV-hFasL transgenic mice (TG) induced by STZ five daily injections. The first injection is day 1. Mice were considered diabetes when blood glucose levels were above 13.9 mmol/L. *n*=5. Mean±SD. $^{c}P<0.01$ *vs* WT.

destructed and there were large number of lymphocytes surrounding and intruding into the islets of langerhans. If diabetes were severe, the islets were smaller in size or even distorted in architecture. The histological examination revealed that CMV-hFasL transgenic mice had more severe diabetes than the wild type mice treated with the same condition (Fig 4). This is consistent with the above result in Fig 2 that CMV-hFasL transgenic mice had much higher blood glucose level.

IL-1β, TNF-α expression in pancreas of diabetic mice Expression of IL-1β and TNF-α mRNA in the pancreas were detected using semi-quantitative RT-PCR analysis. IL-1β and TNF-α in the pancreas of CMV-hFasL transgenic mice were apparently higher than that of WT mice. The mRNA level of IL-1β in CMV-hFasL transgenic diabetic mice was 2.9±1.4 (Mean±SD) times of that in WT mice. While the mRNA level of TNF-α in CMV-hFasL transgenic diabetic mice was

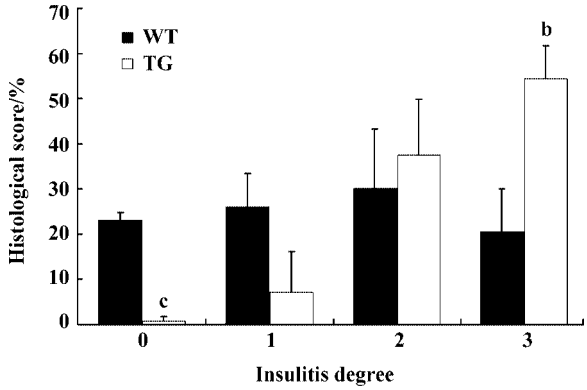

Fig 3. Development of insulitis in CMV-hFasL transgenic mice (TG) and WT mice at d 29 after STZ injection. Total 30 islets from individual mice were counted and scored for the degree of insulitis which was classified into four categories: 0,no insulitis; 1, periinsulitis with or without minimal lymphocyte infiltration in islet; 2, invasive insulitis with islet destruction ≤50 %; 3, islet destruction >50 %. The analysis was performed in a blinded manner. A total of 5 TG mice and 5 WT mice were evaluated. The percentages of 0, 1, 2, and 3 degrees of insulitis were shown. Apparently, the insulitis of TG mice were more severe than that of WT mice. *n*=5. $^{b}P<0.05$, $^{c}P<0.01$ *vs* WT.

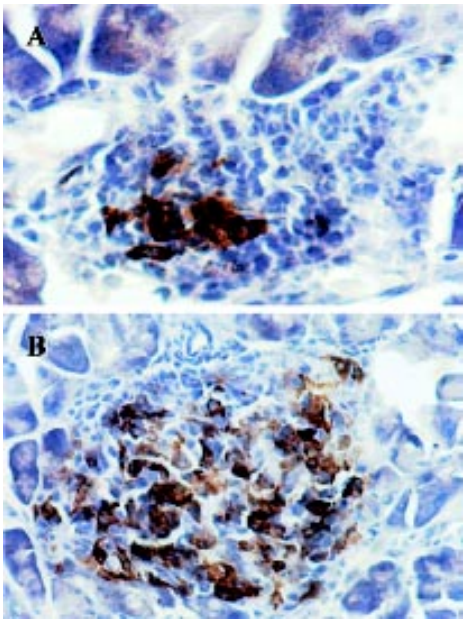

Fig 4. Insulin staining of pancreatic islets from CMV-hFasL transgenic mice and WT mice at the same time after STZ induction (d 29). A: CMV-hFasL transgenic mice pancreatic section; B: WT mice pancreatic section. The yellow staining indicates the insulin. (magnification ×200)

2.3±1.3 (Mean±SD) times of that in WT mice (Fig 5).

STZ and IL-1β plus TNF-α plus IFN-γ could induce apoptosis in RIN-5F cells Both IL-1β plus

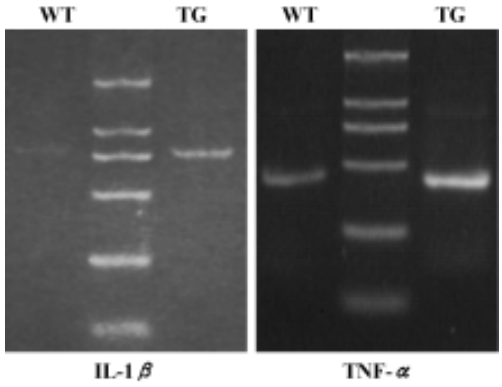

Fig 5. Comparison of TNF-α and IL-1β mRNA levels in pancreas of diabetic CMV-hFasL transgenic mice (TG) and WT mice at d 29 after STZ induction. The mRNA levels were determined with semi-quantitative RT-PCR analysis. After normalization of TNF-α and IL-1β genes to an internal control gene β-actin, mRNA levels of these genes in TG and WT mice were compared.

TNF-α plus IFN-γ and STZ could induce RIN-5F to undergo apoptosis. The percentage of apoptosis has a dose dependent relationship with STZ (Fig 6). The apoptotic rate of normal cell was 12.2 %±1.5 %, while the rate of apoptosis could increase to 57.2 %±2.8 % after STZ 10 mmol/L treatment for 24 h.

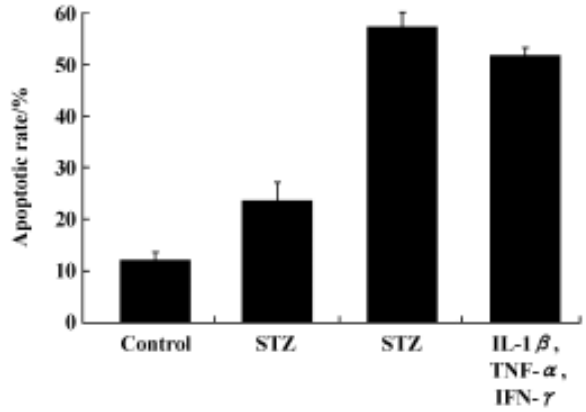

Fig 6. Both IL-1β and STZ could induce apoptosis in RIN-5F cells. Cells were treated with propidium iodide (PI) 75 mg/L including RNase 100 mg/L then analyzed by FACStar with excitation at 585 nm. A: control untreated cells; B: RIN-5F cells treated with STZ 5 mmol/L for 24 h; C: RIN-5F cells treated with STZ 10 mmol/L for 24 h; D: RIN-5F cells treated with IL-1β 30U, TNF-α 1000 U, and IFN-γ 1000 U for 24 h. *n*=3. Mean±SD.

Fas expression in apoptotic RIN-5F cells Fas expression was detected in the apoptotic RIN-5F cells induced by IL-1β or STZ. The induced cells showed a higher level of Fas mRNA compared to the control untreated cells (Fig 7). Background apoptotic cells in the medium may account for the low expression of Fas in the control cells.

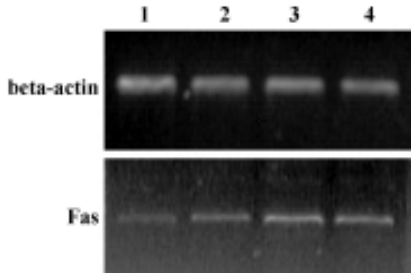

Fig 7. Fas expression was detected in the apoptotic RIN-5F cells induced by IL-1β or STZ. The induced cells showed a higher level of Fas mRNA compared with the control untreated cells. Background apoptotic cells in the medium may account for the low expression of Fas in the control cells. The DNA fragment of Fas was 766 bp. The DNA fragment of beta-actin was 591 bp. Lane 1: normal RIN-5F cells; Lane 2: RIN-5F cells treated with STZ 5 mmol/L for 24 h; Lane 3: RIN-5F cells treated with STZ 10 mmol/L for 24 h; Lane 4: RIN-5F cells treated with IL-1β 30 U, TNF-α 1000 U, IFN-γ 1000 U for 24 h.

DISCUSSION

Multiple injections of low dose of STZ could induce type I diabetes mellitus in mice, which gradually developed diabetes with many of the features of human disease. This model was used to understand the pathogenesis of autoimmune diabetes[11].

In this study, we investigated the role of Fas-FasL pathway in the pathogenesis of STZ-induced diabetes by using CMV-hFasL transgenic mice. Expression of hFasL in islets was confirmed by RT-PCR and Western blotting. The FasL protein function was demonstrated by co-thymocyte killing assay. After overnight coincubation of Jurket thymocytes with fibroblast isolated from the pancreata of CMV-hFasL transgenic mice the apoptosis rate was increased greater than that of non-transgenic mice (23.4 %±3.2 % *vs* 6.6 %±2.1 %). We found CMV-hFasL transgenic mice were more sensitive to develop diabetes with low dose injections of STZ. This was consistent with the report that transgenic expression of FasL on β cells in RIP-FasL-NOD mice resulted in accelerated type I diabetes[3,12].

Fas-FasL was usually mentioned for its disputative role in type I diabetes mellitus[3,8,9,12-15]. Previous reports that diabetogenic lymphocytes did not induce diabetes in NOD-*lpr* mice suggested the critical role of Fas-FasL interaction in pancreatic β cells apoptosis[3].

However, recent works demonstrated that FasL was not an effector molecular in islet β cells apoptosis[12]. Kim *et al* indicated that FasL on abnormal lymphocytes from NOD-*lpr* mice was responsible for the inhibition of diabetes transfer[8]. These results mainly came from the NOD mice. However, our data suggested that Fas-FasL could mediate the destruction of β cells and contribute to the diabetic procession after low dose of STZ treatment.

STZ was a cytotoxic agent that causes DNA and mitochondrial damage and induced the initial destruction of β cells. It could activate the local immune system[11]. Proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ were released by activated mononuclear cells and induced β cells to undergo apoptosis directly[16]. Here it was found that STZ and those proinflammatory cytokines could induce apoptosis in RIN-5F cells with the relatively higher expression of Fas. So if β cells expressed FasL themselves, apoptosis could be accelerated. In our experiment, IL-1β and TNF-α were found expressed with much more amounts in the pancreas of CMV-hFasL transgenic mice than those in WT mice. Recently, FasL was found to induce neutrophil infiltration by inducing the production of IL-1β[17]. These results suggested that FasL in the islets of CMV-hFasL transgenic mice could induce more expression of proinflammatory cytokines when β cells were injured. Then these proinflammatory cytokines could feed back to induce β cells to express more Fas. Thus the conjugation of constitutively expressed FasL with the higher amount of Fas would result in the sensitivity of CMV-hFasL transgenic mice to STZ induction.

In conclusion, Fas/FasL pathway plays a significant role in the STZ-induced type I diabetes mellitus.

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