

## Original Paper

# Inflammatory Macrophages Promotes Development of Diabetic Encephalopathy

Beiyun Wang Ya Miao Zhe Zhao Yuan Zhong

Department of Gerontology, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China

**Key Words**

Diabetes • Inflammatory macrophages • Diabetic encephalopathy • Streptozotocin (STZ)

**Abstract**

**Background/Aims:** Diabetes and Alzheimer's disease are often associated with each other, whereas the relationship between two diseases is ill-defined. Although hyperglycemia during diabetes is a major cause of encephalopathy, diabetes may also cause chronic inflammatory complications including peripheral neuropathy. Hence the role and the characteristics of inflammatory macrophages in the development of diabetic encephalopathy need to be clarified. **Methods:** Diabetes were induced in mice by i.p. injection of streptozotocin (STZ). Two weeks after STZ injection and confirmation of development of diabetes, inflammatory macrophages were eliminated by i.p. injection of 20µg saporin-conjugated antibody against a macrophage surface marker CD11b (saporin-CD11b) twice per week, while a STZ-treated group received injection of rat IgG of same frequency as a control. The effects of macrophage depletion on brain degradation markers, brain malondialdehyde (MDA), catalase, superoxidase anion-positive cells and nitric oxide (NO) were measured. **Results:** Saporin-CD11b significantly reduced inflammatory macrophages in brain, without affecting mouse blood glucose, serum insulin, glucose responses and beta cell mass. However, reduced brain macrophages significantly inhibited the STZ-induced decreases in brain MDA, catalase and superoxidase anion-positive cells, and the STZ-induced decreases in brain NO. **Conclusion:** Inflammatory macrophages may promote development of diabetic encephalopathy.

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Yuan Zhong

Department of Gerontology, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiaotong University, 600 Yishan Road, Shanghai 200233 (China)  
Tel. +862124056053, E-Mail [zhongyuan1960@163.com](mailto:zhongyuan1960@163.com)

## Introduction

Diabetes and Alzheimer's disease are often associated with each other [1-8], whereas the relationship between two diseases is ill-defined. Many studies indicate that diabetic patients are at higher risk of developing dementia or Alzheimer's disease [1-5]. Indeed, diabetes can cause several complications, including diabetic encephalopathy, which is believed to result from hyperglycemic and inflammatory damages to brain vessels and causes dementia [9-12]. The link between diabetes and Alzheimer's may occur as a result of the complex ways. For example, diabetes may increase the risk of developing mild cognitive impairment, a condition in which people experience more cognitive and memory problems than are usually present in normal aging [9-12]. Mild cognitive impairment may lead to Alzheimer's disease and other types of dementia [9-12].

Although hyperglycemia during diabetes is one major cause of encephalopathy, diabetes may also cause chronic inflammatory complications including peripheral neuropathy [9-12]. Since diabetes is one of the major risk factors for cerebrovascular disease, inflammatory process may take place in central nervous system (CNS). Diabetic encephalopathy is characterized by impaired cognitive functions and neurochemical and structural abnormalities, and may involve direct neuronal damage caused by hyperglycemia [9-12]. However, brain inflammatory events during diabetes may be an important mechanism of brain damage and may be involved in diabetic encephalopathy. In this regard, brain innate immunity mediated primarily by activated and infiltrated macrophages, has been found to contribute to chronic central nervous system neurodegeneration [13]. However, the role and the characteristics of inflammatory macrophages in the development of diabetic encephalopathy remain to be clarified.

Here, we examined the role of inflammatory macrophages in the development of diabetic encephalopathy. Diabetes were induced in mice by i.p. injection of streptozotocin (STZ). Two weeks after STZ injection and confirmation of development of diabetes, inflammatory macrophages were eliminated by i.p. injection of 20 $\mu$ g saporin-conjugated antibody against a macrophage surface marker CD11b (saporin-CD11b) twice per week, while a STZ-treated group received injection of rat IgG of same frequency as a control. We found that saporin-CD11b significantly reduced inflammatory macrophages in the brain, without affecting mouse blood glucose, serum insulin, glucose responses and beta cell mass. However, reduced brain macrophages significantly inhibited the STZ-induced decreases in brain malondialdehyde (MDA), catalase and superoxidase anion-positive cells, and the STZ-induced decreases in brain nitric oxide (NO).

## Materials and Methods

### *Mouse handling*

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the Shanghai Sixth People's Hospital Affiliated to Shanghai Jiaotong University (Animal Welfare Assurance). Mice were housed in Pathogen-free environment. C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME, USA). Sixty male mice were used in the current study. Blood glucose was measured after 3 hours' fasting every morning. Intraperitoneal glucose tolerance test (IPGTT) were performed as described before [14]. The beta-cell toxin streptozotocin (STZ) was i.p. injected at a dose of 150 mg/kg body weight to induce hyperglycemia in 40 mice. The other 20 mice were injected with saline and used as a control (untreated). Two weeks after STZ injection, the 40 mice were randomly divided into two groups (20 each). One group received i.v. injection of saporin-conjugated antibody (20 $\mu$ g; Advanced Targeting Systems, San Diego, CA, USA) against the macrophage surface marker CD11b twice per week to eliminate macrophages (STZ+saporin) [15], while the other group received injection of rat IgG of same frequency as a control (STZ+IgG). Serum insulin levels were determined with an insulin ELISA kit (ALPCO, Salem, NH, USA).

### *RT-qPCR*

RNA samples from mouse brain were prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA), followed with DNase I treatment and the RNeasy column (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. RNA was reverse transcribed by RETROscript™ kit (Ambion, Foster City, CA, USA). Quantitative RT-PCR (RT-qPCR) was performed in the GenAmp 5700 SDS® (Applied Biosystems™ St. Louis, MO, USA), using the default thermocycler program for all genes: 10 minutes of pre-incubation at 95°C followed by 40 cycles for 15 seconds at 95°C and one minute at 60°C. Individual real-time PCR reactions were carried out in 20µl volumes in a 96-well plate (Applied Biosystems™) containing 1× buffer (10×), 3.5 mM MgCl<sub>2</sub>, 200µmol/l dNTPs, different concentrations of sense and antisense primers (Qiagen, Hilden, Germany), 0.025 U/µl enzyme and 1:66000 SYBR GreenI®. All reactions were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). At the end of each reaction, Cycle threshold (Ct) was manually setup at the level that reflected the best kinetic PCR parameters, and melting curves were acquired and analyzed. Data analysis using the 2-ΔΔCt method. F4/80 and GAPDH primers were both purchased from Qiagen. Values of F4/80 were first normalized against GAPDH and then compared to experimental controls.

### *Protein isolation and Western blot*

Protein was extracted from mouse brain by RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) for Western Blot. The supernatants were collected after centrifugation at 12000×g at 4°C for 20min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-F4/80 (1:1000; Invitrogen) and anti-GAPDH (1:2000; Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rat and anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the Figure were representative from 3 repeats. Densitometry of Western blots was quantified with NIH ImageJ software.

### *Immunohistochemistry*

Mouse pancreas and brain were dissected out and fixed with 4% paraformaldehyde for 10 hours, and then cyro-protected in 25% sucrose overnight. Samples were then sectioned in 6µM. Primary antibodies used in immunohistochemistry are guinea pig polyclonal anti-insulin (1:500; DAKO, Carpinteria, CA, USA) and rat polyclonal anti-F4/80 (1:200; Invitrogen). Secondary antibodies were HRP-conjugated antibodies for corresponding species (1:1000; Jackson ImmunoResearch Labs). After immunostaining, hematoxylin counterstaining was performed.

### *Quantification of beta-cell mass*

Insulin staining was used to identify beta cells. The quantification of beta cell mass was done as has been described before [16-18]. Briefly, the pancreas was weighed and then fixed with 4% Paraformaldehyde for 10 hours, and then cyro-protected in 25% sucrose overnight in a way to allow longitudinal sections from tail to head of the pancreas to be obtained. Sections at 120µm intervals from whole pancreas were immunostained for insulin and analysed using NIH Image J software. The relative cross-sectional area of beta cells was determined by quantification of the cross-sectional area occupied by beta cells divided by the cross-sectional area of total tissue. The beta-cell mass per pancreas was estimated as the product of the relative cross-sectional area of beta cells per total tissue and the weight of the pancreas. The beta-cell mass was calculated by examining pancreas from all 20 animals in each group.

### *Measurement of nitrite/nitrate production*

Total nitrite concentration in brain homogenates was used as an indicator of nitric oxide (NO) synthesis. Nitrates in samples were reduced to nitrites by incubating with a nitrate reductase. Nitrite concentration

was measured using a commercial kit following the manufacturer's indications (Cayman Chemical Company, Ann Arbor, MI, USA). Optical density was measured at 570nm and results were expressed as  $\mu\text{mol}/\text{mg}$  of protein.

#### *Measurement of thiobarbituric reactive substances (TBARS)*

Brain malondialdehyde (MDA) content was assessed by the thiobarbituric acid assay. Perfused brain sections were homogenized in a buffer containing 100mmol/l KCl and 3mmol/l EDTA. Afterwards, the homogenates were centrifuged at 600g for 15 min. A volume of supernatant was added to a solution containing 8.1% SDS, 20% acetic acid and 0.8% thiobarbituric acid, at pH=3.5 (Sigma-Aldrich). The complete mixture was heated to 95 °C for 60 min; afterwards, the chromogenic substrate was extracted into the organic phase with butanol/pyridine (15:1). Absorbance of the organic layer was measured at 532nm. As external standard the MDA bisdimethyl acetal (Sigma-Aldrich) was used and results were expressed as nmol per mg of protein.

#### *Measurement of catalase activity*

To determine catalase activity, perfused brain slides from control and diabetic mice were homogenized in 50mmol potassium phosphate buffer at pH=7.2. The homogenates were centrifuged at 600g for 10 min and enzymatic activity was measured in the supernatant. Catalase activity was determined by spectrophotometrical assay by the conversion rate of 7.7mmol/l  $\text{H}_2\text{O}_2$  in 50mmol/l potassium phosphate at pH=7.0 (Sigma-Aldrich). The change in absorbance was read at 250nm and the rate constant of a first order reaction (k) was used. Results are expressed as k per mg of homogenate proteins.

#### *Histological identification of superoxide anion*

Superoxide anion production was determined at cellular levels in the different brain zones by a cytochemical method previously described (Briggs et al., 1986). Briefly, brain frozen sections (10 $\mu\text{m}$ ) from controls and diabetic rats were incubated in a solution containing 0.05mol/l Tris-HCl buffer, 0.05mol/l DAB, 8% NiCl, 10%  $\text{NaN}_3$  and 0.5mol/l  $\text{MnCl}_2$  at pH=7.6 for 60min at 37°C (Sigma-Aldrich). Afterwards, sections were fixed with 10% formalin and counterstained with 1% methyl green. Results were expressed as positive cells per  $\text{mm}^2$ .

#### *Statistics*

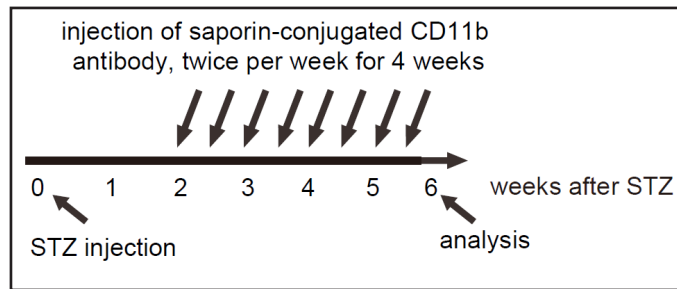
All values are depicted as mean  $\pm$  standard error and are considered significant if  $p < 0.05$ . All data were statistically analysed using one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test, using GraphPad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA, USA).

## Results

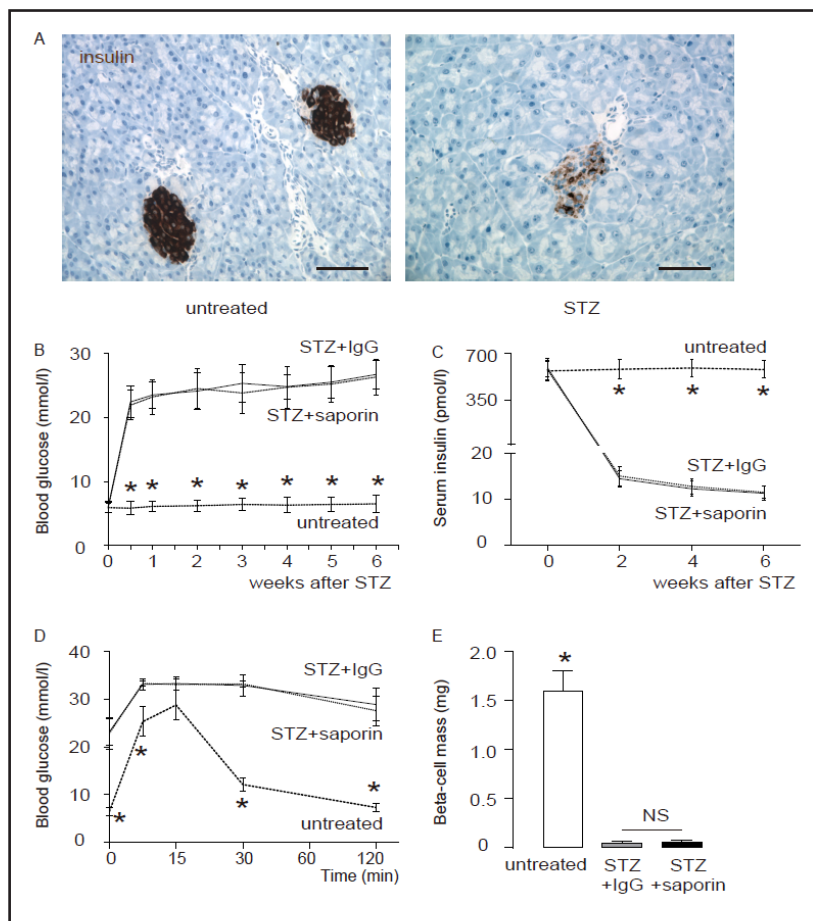
#### *Experimental model*

In order to find out whether inflammatory macrophages may play a critical role in the development of diabetic encephalopathy, we used 60 12 week-old C57BL/6 male mice in this study. The beta-cell toxin streptozotocin (STZ) was i.p. injected at a dose of 150 mg/kg body weight to induce hyperglycemia in 40 mice. The other 20 mice were injected with saline and used as a control (untreated). Two weeks after STZ injection, the 40 STZ-treated mice were randomly divided into two groups (20 each). One group received i.v. injection of saporin-conjugated antibody against the macrophage surface marker CD11b twice per week to eliminate macrophages (STZ+saporin), while the other group received injection of rat IgG of same frequency as a control (STZ+IgG). Another 4 weeks later, the mice were sacrificed for analyses. Blood glucose was measured after 3 hours' fasting every morning every week. Serum insulin levels were determined biweekly. At 6 weeks (the end of experiment), IPGTT were performed. Beta cell mass, macrophages in brain, and the effects of macrophage depletion on brain degradation markers, brain malondialdehyde (MDA), catalase, superoxidase anion-positive cells and nitric oxide (NO) were measured (Fig. 1A).

**Fig. 1.** Schematic of experimental model. Sixty 12 week-old C57BL/6 male mice were used in this study. STZ was i.p. injected to induce hyperglycemia in 40 mice. The other 20 mice were injected with saline and used as a control (untreated). Two weeks after STZ injection, the 40 STZ-treated mice were randomly divided into two groups of 20 each. One group received i.v. injection of saporin-conjugated antibody against the macrophage surface marker CD11b twice per week to eliminate macrophages (STZ+saporin), while the other group received injection of rat IgG of same frequency as a control (STZ+IgG). Another 4 weeks later, the mice were sacrificed for analyses.



**Fig. 2.** Saporin-CD11b does not affect mouse blood glucose, serum insulin, glucose responses and beta cell mass in STZ-treated mice. (A) Representative immunohistochemistry images of the mouse pancreas at 1 week after STZ. (B-C) STZ induced sustained hyperglycemia (B), and significant and constant decreases in serum insulin (C) in mice since 3 days after injection. Saporin-CD11b did not affect mouse blood glucose or serum insulin levels in STZ-treated mice. (D) At 6 weeks, IPGTT showed that STZ-treated mice had significantly impaired glucose response, while saporin-CD11b did not affect the glucose response in STZ-treated mice. (E). At 6 weeks, STZ-treated mice had significantly reduced beta cell mass, which was also not affected by saporin. \*:  $p < 0.05$ . NS: non-significant. Scale bars are 50  $\mu$ m.

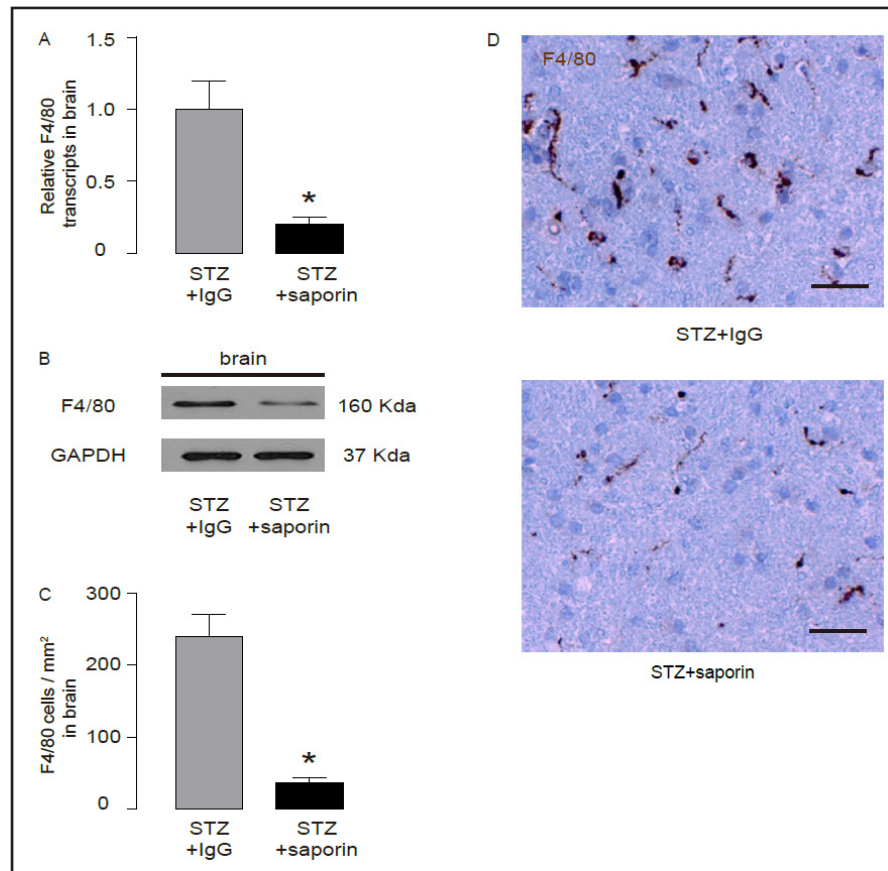


*Saporin-CD11b does not affect mouse blood glucose, serum insulin, glucose responses and beta cell mass in STZ-treated mice*

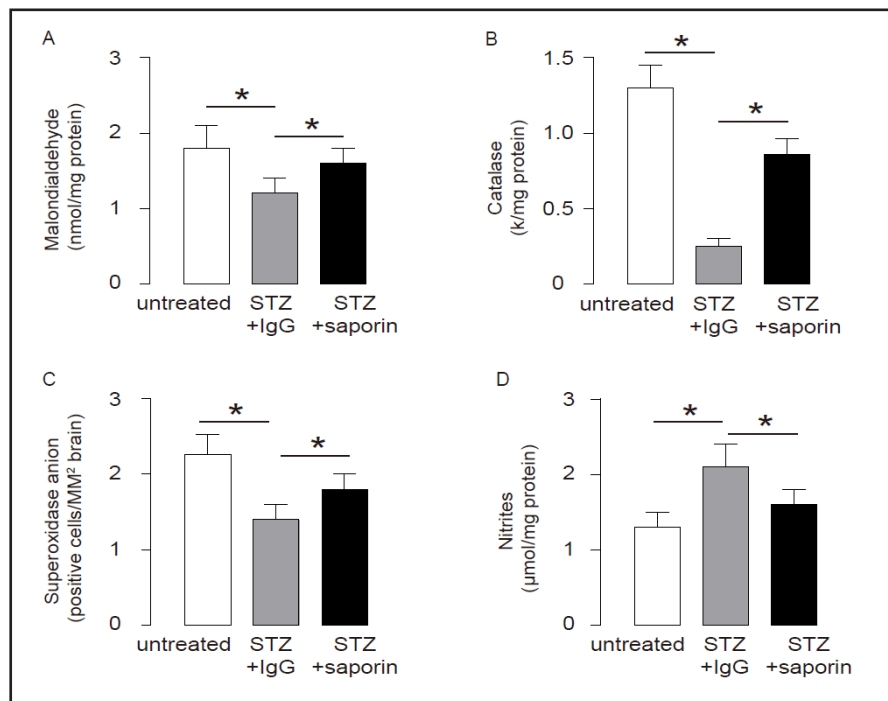
We found that consistent with the immunohistochemistry findings in the mouse pancreas at 1 week after STZ (Fig. 2A), STZ induced sustained hyperglycemia (Fig. 2B), and significant and constant decreases in serum insulin (Fig. 2C) in mice since 3 days after injection. Saporin-CD11b did not affect mouse blood glucose or serum insulin levels in STZ-treated mice (Fig. 2B-C). At 6 weeks, IPGTT was performed. We found that STZ-treated



**Fig. 3.** Saporin-CD11b significantly reduces inflammatory macrophages in the brain. (A-B) Saporin treatments significantly reduced the F4/80 transcripts (A) and protein (B) in the brain from STZ-treated mice. (C-D) Quantification (C) and representative immunohistochemistry images (D) for F4/80 in STZ-treated mice w/o saporin. \*:  $p < 0.05$ . Scale bars are  $40\mu\text{m}$ .



**Fig. 4.** Macrophage depletion rescues brain degradation events in STZ-treated mice. (A-D) Saporin-CD11b significantly inhibited the STZ-induced decreases in brain MDA (A), catalase (B) and superoxide anion-positive cells (C), and the STZ-induced decreases in brain NO (D). \*:  $p < 0.05$ .



mice had significantly impaired glucose response, while saporin-CD11b did not affect the glucose response in STZ-treated mice (Fig. 2D). Moreover, STZ-treated mice had significantly reduced beta cell mass, which was also not affected by saporin (Fig. 2E). Together, these data

suggest that saporin, or elimination of inflammatory macrophages, does not affect mouse glucose metabolism-associated parameters like mouse blood glucose, serum insulin, glucose responses and beta cell mass in STZ-treated mice.

*Saporin-CD11b significantly reduces inflammatory macrophages in the brain*

Then we analyzed whether Saporin-

CD11b altered brain inflammatory macrophages in the STZ-treated mice. We found that saporin treatments significantly reduced the F4/80 (a pan-macrophage marker) transcripts (Fig. 3A) and protein (Fig. 3B) in the brain from STZ-treated mice, which was also supported by immunohistochemistry for F4/80 in these mice, shown by quantification (Fig. 3C) and by representative images (Fig. 3D). Together, these data suggest that Saporin-CD11b significantly reduces inflammatory macrophages in the brain.

*Macrophage depletion rescues brain degradation events in STZ-treated mice*

Then the effects of macrophage depletion on brain degradation markers, brain malondialdehyde (MDA), catalase, superoxidase anion-positive cells and nitric oxide (NO) were measured. We found that saporin-CD11b significantly inhibited the STZ-induced decreases in brain MDA (Fig. 4A), catalase (Fig. 4B) and superoxidase anion-positive cells (Fig. 4C), and the STZ-induced decreases in brain NO (Fig. 4D). These data suggest that macrophage depletion rescues brain degradation events in STZ-treated mice (Fig. 5).

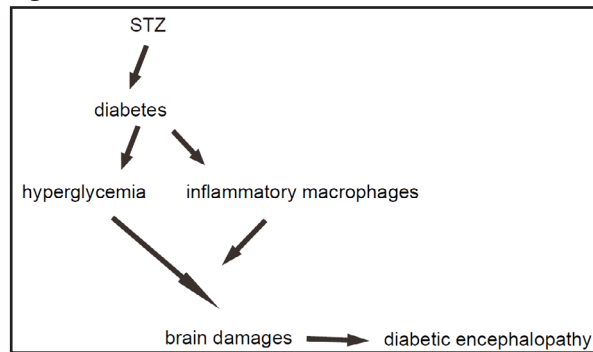
## Discussion

Diabetes is a chronic and prevalent disease that is supposed to predispose development of Alzheimer's disease [1-8]. Indeed, previous studies have demonstrated that diabetic encephalopathy is one of the main complications of severe diabetes, and is believed to result from hyperglycemic and inflammatory damages to brain vessels and causes dementia [9-12]. Moreover, diabetes may increase the risk of developing mild cognitive impairment, a condition in which people experience more cognitive and memory problems than are usually present in normal aging [9-12]. Mild cognitive impairment may lead to Alzheimer's disease and other types of dementia [9-12]. Although great efforts have been made, the previous knowledge on the molecular mechanisms is far from completeness.

Hyperglycemia is the major inducer of encephalopathy, whereas chronic inflammation substantiates the process by impairing normal brain vessel function [9-12]. Thus, brain inflammatory events during diabetes may be an important mechanism of brain damage and may be involved in diabetic encephalopathy. Activated and infiltrated macrophages have been found to contribute to chronic central nervous system neurodegeneration [13]. However, the exact role and the characteristics of these inflammatory macrophages remain unclear.

Here, we used a mouse STZ-diabetes model to examine the role of inflammatory macrophages in the development of diabetic encephalopathy. Diabetes were induced in mice by i.p. injection of STZ. STZ selectively kills pancreatic beta cells in a very short time and beta cells do not regenerate naturally [19], which was confirmed in our study. Two weeks after STZ injection and confirmation of development of diabetes by blood glucose, serum insulin and immunohistochemistry for insulin, which is a beta-cell-specific marker, we eliminated inflammatory macrophages by i.p. injection of 20µg saporin-conjugated antibody against a macrophage surface marker CD11b (saporin-CD11b) twice per week, while a STZ-treated

**Fig. 5.** Schematic of the model.



group received injection of rat IgG of same frequency as a control. CD11b is a specific marker for macrophages. We used a saporin-conjugated antibody against CD11b to eliminate macrophages. Saporin is a ribosome-inactivating protein from seeds of the plant *Saponaria officinalis*. Saporin is safe for laboratory use under normal safety conditions and the LD50 in mice is 4 mg/kg. Moreover, saporin does not have a method of cell entry on its own. Thus, CD11b antibody will direct entrance of Saporin into macrophages to selectively kill them. We found that saporin-CD11b significantly reduced inflammatory macrophages in the brain, without affecting mouse blood glucose, serum insulin, glucose responses and beta cell mass. These data assure that the effects of macrophage depletion on brain are not secondary to changes of systemic glucose metabolism.

However, reduced brain macrophages significantly inhibited the STZ-induced decreases in brain malondialdehyde (MDA), catalase and superoxidase anion-positive cells, and the STZ-induced decreases in brain nitric oxide (NO). All these parameters are related to brain degradation. Thus, macrophage depletion protects the brain from degradation-associated events, or in another word, inflammatory macrophages may play a substantial role in the process of diabetic encephalopathy. Future experiments may be applied to further elucidate the underlying molecular mechanisms.

Together, our study demonstrate that inflammatory macrophages promotes development of diabetic encephalopathy, highlighting inflammatory macrophages may be a promising therapeutic target for treating diabetic encephalopathy to prevent the development of dementia.

## Disclosure Statement

None disclosed

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