

# TLR4 antagonist reduces early-stage atherosclerosis in diabetic apolipoprotein E-deficient mice

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## Abstract

Although it has been reported that deficiency of toll-like receptor 4 (TLR4) is associated with reduced atherosclerosis in atherosclerosis-prone mice and attenuated pro-inflammatory state in diabetic mice, it remains undetermined whether treatment with a TLR4 antagonist reduces atherosclerosis in nondiabetic or diabetic mice that have TLR4 expression. In this study, we determined the effect of *Rhodobacter sphaeroides* lipopolysaccharide (Rs-LPS), an established TLR4 antagonist, on early-stage atherosclerosis in nondiabetic and streptozotocin-induced diabetic apolipoprotein E-deficient (*Apoe*<sup>-/-</sup>) mice. Analysis of atherosclerotic lesions of both en face aortas and cross sections of aortic roots showed that administration of Rs-LPS in 14-week-old diabetic *Apoe*<sup>-/-</sup> mice for 10 weeks significantly reduced atherosclerotic lesions. Although atherosclerotic lesions in nondiabetic *Apoe*<sup>-/-</sup> mice appeared to be decreased by Rs-LPS treatment, the difference was not statistically significant. Metabolic study showed that Rs-LPS significantly lowered serum levels of cholesterol and triglycerides in nondiabetic mice but not in diabetic mice. Furthermore, immunohistochemistry studies showed that Rs-LPS inhibited the expression of interleukin 6 and matrix metalloproteinase-9 and reduced the content of monocytes and macrophages in atherosclerotic plaques. Taken together, this study demonstrated for the first time that TLR4 antagonist inhibited vascular inflammation and atherogenesis in diabetic *Apoe*<sup>-/-</sup> mice and lowered serum cholesterol and triglyceride levels in nondiabetic *Apoe*<sup>-/-</sup> mice.

## Key Words

- ▶ toll-like receptor 4
- ▶ atherosclerosis
- ▶ inflammation
- ▶ hyperlipidemia

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## Introduction

Early epidemiological studies showed an association between infection with gram-negative bacteria and atherosclerosis (Mendall *et al.* 1995), suggesting that toll-like receptor 4 (TLR4), a receptor for lipopolysaccharide (LPS) from gram-negative bacteria, is involved in atherosclerosis. It was proposed that bacteria such as *Chlamydia pneumoniae* and *Porphyromonas gingivalis* might promote atherosclerosis by activating TLR4 and TLR4-mediated inflammation via LPS. In recent years, animal

studies with atherosclerosis-prone mice have provided strong evidence that TLR4 plays an important role in atherosclerosis. It has been demonstrated that deficiency of TLR4 or MyD88 in apolipoprotein E-deficient (*Apoe*<sup>-/-</sup>) mice is associated with a significant reduction of atherosclerosis (Bjorkbacka *et al.* 2004, Michelsen *et al.* 2004, Higashimori *et al.* 2011). As no LPS or LPS-enriched gram-negative bacteria was given to the mice, these studies suggested that TLR4 was activated

by endogenous ligands that are likely to be related to hyperlipidemia in *ApoE*<sup>-/-</sup> mice. Indeed, *in vitro* studies have shown that minimally modified LDL (mmLDL) is capable of inducing inflammatory cytokine production in macrophages by engaging TLR4 (Miller *et al.* 2003, 2005). Besides atherosclerosis, studies of diabetes in animal models have shown that deficiency of TLR4 is also associated with attenuation of the pro-inflammatory state of diabetes (Zipris 2010, Devaraj *et al.* 2011, Grieco *et al.* 2011). Thus, TLR4 appears to be a key player in the inflammatory processes contributing to both atherosclerosis and diabetes.

Although the knockout models have shown that the inborn deficiency of TLR4 is related to reduced atherosclerosis, it remains unknown whether administration of TLR4 antagonists in mice with normal TLR4 expression is effective to impede atherosclerosis. Furthermore, as diabetes accelerates atherosclerosis by increasing vascular inflammation (Kanter *et al.* 2012), it is more intriguing to determine whether administration of TLR4 antagonists is also effective to reduce diabetes-accelerated atherosclerosis.

In this study, we treated 14-week nondiabetic and diabetic *ApoE*<sup>-/-</sup> mice with *Rhodobacter sphaeroides* LPS (Rs-LPS), an established TLR4 antagonist (Baker *et al.* 1990, Kirkland *et al.* 1991, Aida *et al.* 1995, Hammad *et al.* 2009, Hutchinson *et al.* 2010), for 10 weeks to determine the effect of TLR4 blockade on the early-stage atherosclerosis. We found that treatment with Rs-LPS reduced atherosclerotic lesions in diabetic *ApoE*<sup>-/-</sup> mice.

## Materials and methods

### Cell culture

Human aortic endothelial cells (HAECs; Invitrogen) were grown in Medium 200 with low serum growth supplement containing 2% fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. HAECs were maintained at 37 °C with 5% CO<sub>2</sub> and 95% humidity and 100% confluent cultures were used for experiments. HAECs were treated with 100 ng/ml LPS (Sigma-Aldrich) in the absence or presence of anti-TLR4 antibody (5 or 10 µg/ml; R&D Systems, Minneapolis, MN, USA) or Rs-LPS (100 or 500 ng/ml; InvivoGen, San Diego, CA, USA) for 24 h. Human U937 mononuclear cells (American Type Culture Collection, Manassas, VA, USA) were cultured in a 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium (Gibco, Invitrogen Corp.) containing 10%

FCS, 1% MEM nonessential amino acid solution, and 0.6 g/100 ml HEPES.

### ELISA

Interleukin 6 (IL6) in medium was quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D System).

### Animals, diet, and treatments

Male *ApoE*<sup>-/-</sup> mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed at the animal facility of VA Medical Center in Charleston, SC, USA. Only male mice were used in this study because female *ApoE*<sup>-/-</sup> mice are resistant to streptozotocin (STZ) treatment (Goldberg *et al.* 2004). The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC). All mice were maintained on a 12 h light:12 h darkness cycle in a pathogen-free environment and had *ad libitum* access to water and regular mouse chow (8604 Teklad rodent diet, Harlan Laboratories, Indianapolis, IN, USA). The study began when mice were 9 weeks old and ended when mice were 24 weeks old. *ApoE*<sup>-/-</sup> mice were randomly divided into four groups: 1) nondiabetic control mice, 2) nondiabetic mice treated with Rs-LPS, 3) diabetic control mice, and 4) diabetic mice treated with Rs-LPS. At the age of 9 weeks, mice in groups 3 and 4 were rendered diabetic by i.p. administration of STZ (50 µg/kg in citrate buffer, 0.05 mol/l, pH 4.5; Sigma-Aldrich) and mice in groups 1 and 2 were given an equivalent amount of citrate buffer for 5 days. Five weeks after STZ treatment, mice in groups 2 and 4 were treated by i.p. administration of Rs-LPS (1 µg/mouse in saline, twice a week for 7 weeks and then once a week for 3 weeks) provided by InvivoGen and mice in groups 1 and 3 were given equivalent amount of saline. This dose of Rs-LPS was reported to be effective in blocking TLR4 by the previous studies (Baker *et al.* 1990, Hammad *et al.* 2009).

### Metabolic measurements

Blood samples were obtained from jugular veins under the fasted condition and glucose level was determined using a Precision QID glucometer (MediSense, Inc., Bedford, MA, USA). Serum cholesterol and triglycerides were measured using Cholestech LDX Lipid monitoring System (Fisher Scientific, Pittsburgh PA, USA). Serum fatty acids were determined using the EnzyChrom free fatty acid kit (BioAssay Systems, Hayward, CA, USA).

### En face analysis

Mice were killed and aortas from heart to the iliac arteries were dissected out, soaked for 24 h in 4% paraformaldehyde for fixation, excised longitudinally and then stained with 0.5% of Sudan IV as described previously (Lloyd *et al.* 2011). After staining, the aortas were laid onto the sponge block surface with the intimal surface up and pinned down using Minutien (Fine Science Tools, Inc., San Francisco, CA, USA). The images of the aortas were taken using an EPSON Perfection 2450 photo scanner and analyzed with Photoshop Software as described previously (Schuyler *et al.* 2011).

### Histological analysis of atherosclerotic lesions

The tissues of aortic root were embedded in Tissue-Tek OCT compound (EMS, Hatfield, PA, USA), immediately frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Starting from the aortic root, cryosections with  $6\ \mu\text{m}$  thickness were cut, and sections with a distance of  $480\ \mu\text{m}$  were collected and mounted on slides. Slides were fixed in 10% formalin for 10 min, stained with Harris modified hematoxylin (Fisher Scientific) for 10 min, and then rinsed in deionized water. Staining of hematoxylin was then developed in tap water for 5 min and differentiated in 1% acid ethanol and blue staining for 50 s. Slides were stained with eosin in Harleco 1% alcohol solution (EMD Chemicals, Gibbstown, NJ, USA) for 2 min. Slides were placed in Coplin jars with 95% ethanol three times for 5 min each and the process was then repeated with 100% ethanol. Slides were further dehydrated in 99.5% xylene (Sigma-Aldrich) and mounted in xylene-based Cytoseal-XYL mounting media (Fisher Scientific). Photomicrographs of tissue sections were taken using an Olympus BX53 digital microscope with Cellsens digital image software (Olympus American, Inc., Center Valley, PA, USA). The area of intima was expressed as the percentage of the total aortic area including intima, media, and lumen.

### Immunohistochemical analysis of protein expression

Aortic roots were fixed in 10% formalin for 10 min and frozen sections were made using a cryostat. Sections were rinsed in 0.01 M PBS, pH 7.4, and then incubated in 0.3%  $\text{H}_2\text{O}_2$ /methanol for 30 min to quench endogenous peroxidase. After being rinsed with 0.01 M PBS, sections were incubated with 3% normal serum in 0.01 M PBS for 1 h to block nonspecific binding. Sections were then blocked with avidin-biotin solution from the ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) for 30 min

and incubated with primary antibodies: rabbit anti-IL6 antibody (1:300; Abcam, Cambridge, MA, USA), goat anti-matrix metalloproteinase-9 (MMP9) antibody (1:100; R&D Systems), or rat anti-CD68 antibody (1:300; AbD Serotec, Raleigh, NC, USA) overnight at  $4^{\circ}\text{C}$ . Sections were incubated with secondary biotinylated antibody (1:250) from the ABC Elite kit (Vector Laboratories) for 1 h and then the ABC reagent (Vector Laboratories) for 30 min. Slides were rinsed in 0.01 M PBS and covered with diaminobenzidine peroxidase substrate solution from the Impack DAB kit (Vector Laboratories) for 2 min and then rinsed in water. Counterstaining was performed with hematoxylin. Slides were then dehydrated using increasing concentrations of ethanol and xylenes and mounted. Staining with normal IgG was used as a negative control. Images were taken and analyzed as described earlier for the histological analysis.

### Image analysis

Images were analyzed with Photoshop Software (version 10; Adobe Systems). The method to use the 'Similar' feature to select a particular color staining on a digitized immunohistochemical image has previously been described in detail (Lloyd *et al.* 2011). Briefly, a standard was created by selecting an area of  $0.5 \times 0.5\ \text{cm}$  from a tissue section that had desired brown color from immunostaining. The cursor of the Magic Wand tool was clicked on the standard to make a selection and the area of the standard was highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection, the Tolerance value in the Magic Wand Options palette was set to 100. Using the 'Similar' command, all the areas with the brown color that is similar to the standard on an image being determined were highlighted. The quantification was done using the Histogram command in the Image menu, which showed the pixels of the highlighted area. The extent of the positive immunostaining was presented as average pixels per cross section of atherosclerotic lesions.

### In situ zymography

The unfixed frozen sections of aortic tissue were incubated for 24 h at  $4^{\circ}\text{C}$  in a developing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM  $\text{CaCl}_2$ , and 0.2 mM sodium azide) containing 250  $\mu\text{g}/\text{ml}$  fluorescein-labeled DQ-Gelatin (Invitrogen) with Tris-borate buffer containing 1% agarose. Sections were rinsed three times with PBS, mounted, and examined with an Olympus BX53 fluorescence microscope.

### Transfection and luciferase activity assay

U937 cells were transfected with 0.5  $\mu$ g NF- $\kappa$ B or AP-1 Cignal reporter firefly luciferase constructs (Qiagen, Inc.) using Fugene HD as the transfection reagent (Roche Diagnostics Corp.) for 20 h. The constitutively expressing renilla luciferase constructs were used as control. The cells were then treated with 100 ng/ml LPS in the absence or presence of 500 ng/ml Rs-LPS for 12 h. After the treatment, the cells were rinsed with cold PBS and lysed with the lysis buffer from the dual-luciferase reporter assay system (Promega). Both firefly and renilla luciferase levels were measured in a luminometer using the dual-luciferase reporter assay reagents (Promega). The firefly luciferase levels were normalized to the renilla luciferase levels.

### Statistical analysis

Data were presented as mean  $\pm$  s.d. Student's *t*-tests were performed to determine the statistical significance of differences of intimal lesion size and protein expression among different experimental groups. A value of  $P < 0.05$  was considered significant.

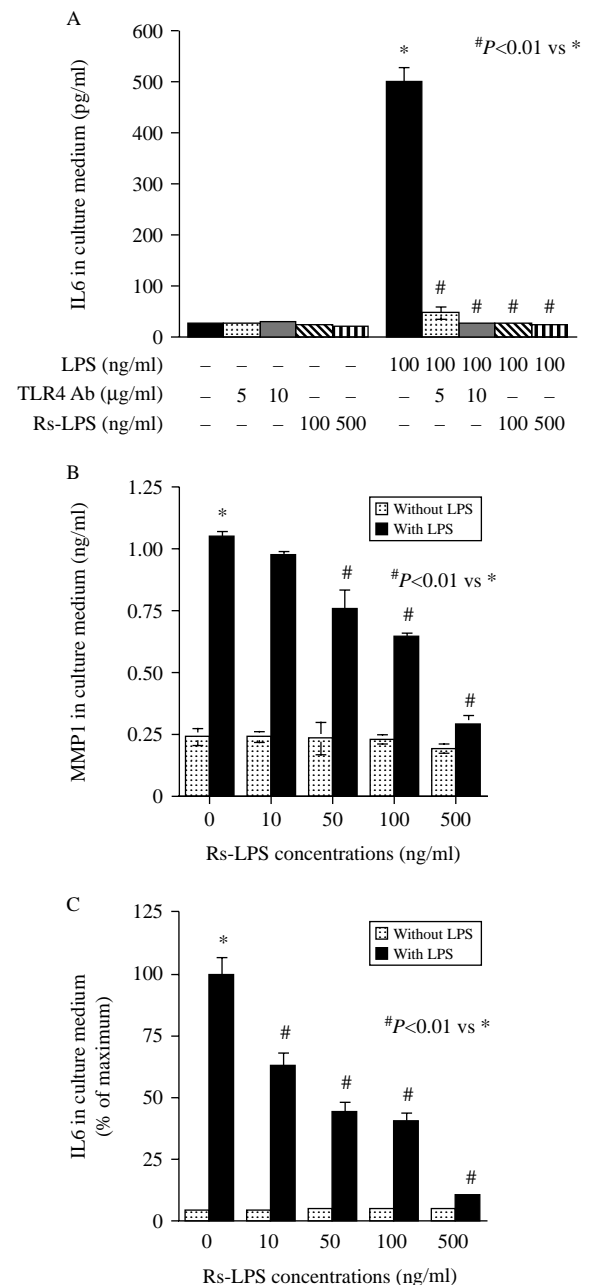
## Results

### Rs-LPS is a potent TLR4 antagonist

To confirm that Rs-LPS is a potent TLR4 antagonist, HAECs were stimulated with 100 ng/ml LPS in the absence or presence of Rs-LPS or TLR4 neutralizing antibody for 24 h, and IL6 in the medium was then quantified. Results showed that while 10  $\mu$ g/ml TLR4 neutralizing antibody blocked LPS-stimulated IL6 secretion as expected, 100 ng/ml Rs-LPS also completely abolished the stimulatory effect of LPS (Fig. 1A). In addition to HAECs, Rs-LPS also effectively antagonized LPS-stimulated MMP1 and IL6 secretion by human U937 mononuclear cells in a concentration-dependent manner (Fig. 1B and C). The  $IC_{50}$  for Rs-LPS to inhibit LPS (100 ng/ml)-stimulated IL6 secretion is between 10 and 50 ng/ml, indicating that Rs-LPS is a potent TLR4 antagonist.

### Rs-LPS reduces cholesterol and triglycerides in nondiabetic mice

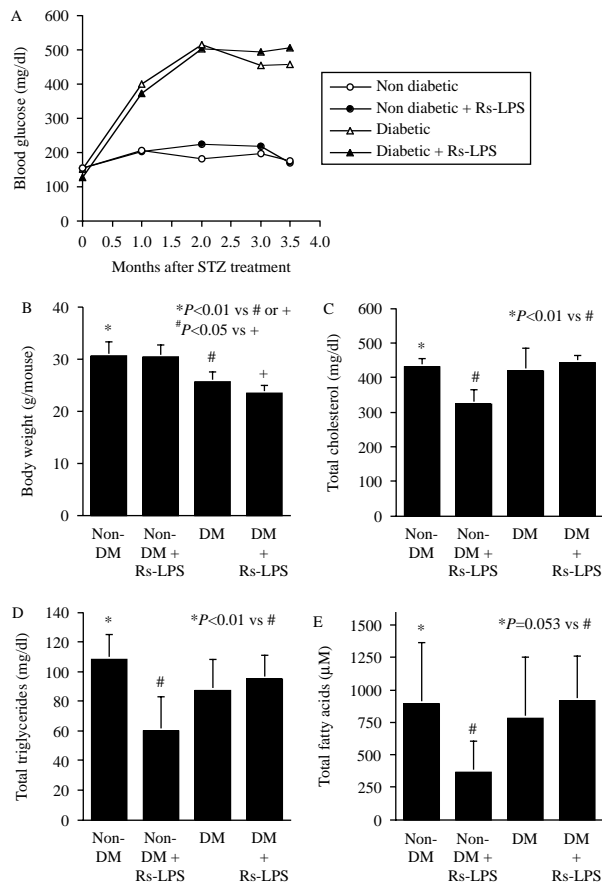
In this study, diabetes was induced by STZ in *Apoe*<sup>-/-</sup> mice and blood glucose was determined 1, 2, 3, and 3.5 months after STZ treatment. Results showed that fasting glucose level was increased significantly at 1 month and stabilized



**Figure 1**

The effect of Rs-LPS on LPS-stimulated IL6 secretion by HAECs (A) and U937 mononuclear cells (B). (A) HAECs were treated with 100 ng/ml LPS in the absence or presence of Rs-LPS (100 or 500 ng/ml) or TLR4 neutralizing antibodies (5 or 10  $\mu$ g/ml) for 24 h. After the treatment, IL6 in culture medium was quantified using ELISA. (B and C) U937 cells were treated with 100 ng/ml LPS in the absence or presence of different concentrations of Rs-LPS (10–500 ng/ml) for 24 h. After the treatment, MMP1 (B) and IL6 (C) in culture medium were quantified using ELISA. In (C), IL6 released by LPS-stimulated U937 cells in the absence of Rs-LPS was designated as 100% (maximum) and IL6 released by cells in other groups was presented as percentage of the maximum. The data (mean  $\pm$  s.d.) were representative of two experiments with similar results.

after 2 months, and Rs-LPS treatment had no significant effect on glucose levels in both nondiabetic and diabetic mice (Fig. 2A). At the end of the study, body weight and serum lipids were determined. Data showed that the average body weight of diabetic mice was about 17–23% lower than that of nondiabetic mice (Fig. 2B), which is consistent with our previous report (Ta *et al.* 2011), and Rs-LPS treatment did not affect body weight significantly (Fig. 2B). Interestingly, Rs-LPS treatment reduced total cholesterol by 25% and triglycerides by 44% in nondiabetic mice (Fig. 2C and D). By contrast, no inhibitory effect of Rs-LPS on cholesterol and triglyceride levels was observed in diabetic *ApoE*<sup>-/-</sup> mice. Similarly, Rs-LPS also marginally reduced serum fatty acid level in nondiabetic mice ( $P=0.053$ ) but not in diabetic mice (Fig. 2E).



**Figure 2** The effects of Rs-LPS on metabolic parameters. (A) Blood glucose was determined 1, 2, 3, and 3.5 months after STZ treatment. (B, C and D) Metabolic parameters including body weight (B), serum total cholesterol (C), triglycerides (D), and free fatty acids (E) were determined at the end of the study. The data presented are mean  $\pm$  s.d. ( $n=5, 7, 9,$  and  $8$  in the groups of non-DM, non-DM + Rs-LPS, DM, and DM + Rs-LPS respectively).

### Rs-LPS inhibits atherosclerosis in diabetic *ApoE*<sup>-/-</sup> mice

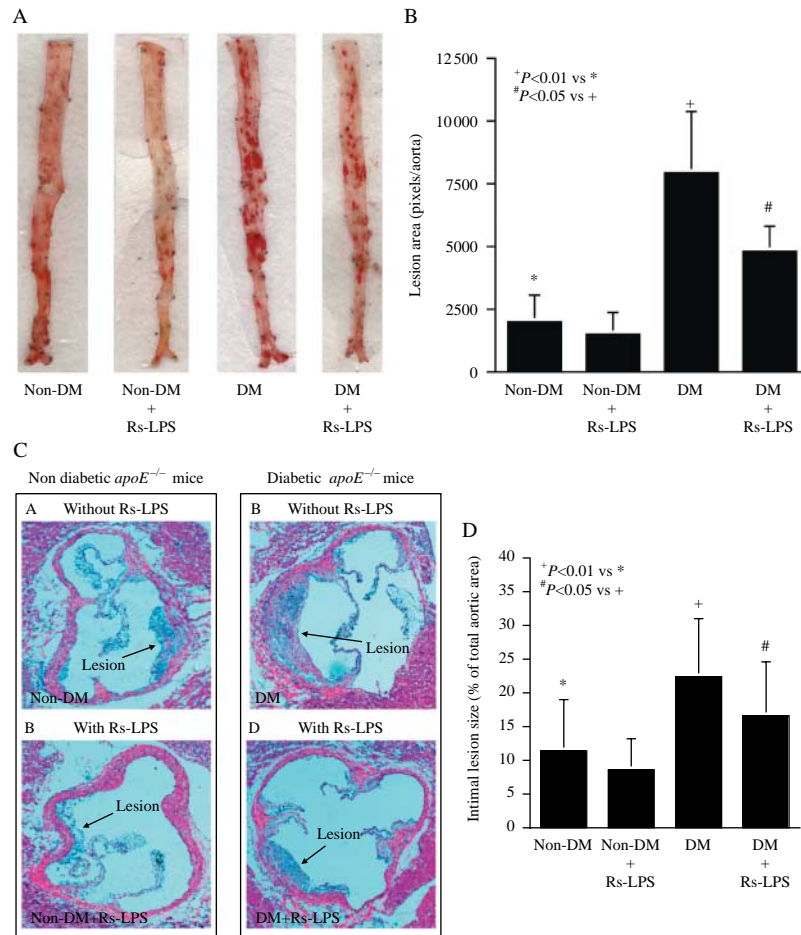
Two approaches were taken to determine the effect of Rs-LPS treatment on atherosclerosis: 1) analysis of en face atherosclerotic lesions in aortas and 2) histological analysis of atherosclerotic lesions in the cross section of aortic roots. As shown in Fig. 3A and B, the en face area of aortas with positive Sudan IV staining was increased significantly in diabetic mice when compared with that in nondiabetic mice. Although the lesion area appeared to be reduced by Rs-LPS treatment in nondiabetic mice, the difference did not reach statistical significance. However, Rs-LPS treatment in diabetic mice significantly decreased atherosclerotic lesions (Fig. 3A and B). Consistent with the observations from the analysis of en face atherosclerotic lesions in aortas, the histological analysis of the intimal lesions on cross sections of the aortic roots also showed that Rs-LPS reduced atherosclerotic lesions significantly in diabetic but not in nondiabetic mice (Fig. 3C and D).

### Rs-LPS inhibits IL6 and MMP9 expression in atherosclerotic plaques in diabetic *ApoE*<sup>-/-</sup> mice

To determine the effect of Rs-LPS on vascular inflammation, we focused on IL6 and MMP9 as both of them have been shown to play an important role in atherosclerosis (Hamirani *et al.* 2008). Results showed that the expression of IL6 (Fig. 4A and B) and MMP9 (Fig. 4C and D) in atherosclerotic plaques was higher in diabetic mice than that in nondiabetic mice, and Rs-LPS treatment led to a significantly decreased IL6 and MMP9 expression in diabetic but not in nondiabetic mice. To determine whether MMP9 detected in atherosclerotic plaques has gelatinase activity, *in situ* zymography using fluorescein-labeled gelatin was performed. Results showed that the core of the atherosclerotic plaques had both high MMP9 expression and gelatinase activity (Fig. 4E). The images also suggest that MMPs other than MMP9 had gelatinase activity, as gelatinase activity was also observed in the shoulder region of atherosclerotic plaque where little MMP9 protein was detected (Fig. 4E).

### Rs-LPS reduces monocyte/macrophage content in atherosclerotic lesions in diabetic *ApoE*<sup>-/-</sup> mice

Monocytes and macrophages in atherosclerotic plaques were detected by immunostaining. Results showed that diabetes increased monocyte/macrophage content significantly and Rs-LPS treatment reduced the content in

**Figure 3**

Reduction of atherosclerotic lesions in diabetic *Apoe*<sup>-/-</sup> mice treated with Rs-LPS. (A) Sudan IV staining of en face aortas from four groups: nondiabetic mice (non-DM), nondiabetic mice treated with Rs-LPS (non-DM + Rs-LPS), diabetic mice (DM), and diabetic mice treated with Rs-LPS (DM + Rs-LPS). (B) Quantification of area with positive staining of the en face aortas ( $n=6, 7, 9,$  and  $8$  for groups of non-DM, non-DM + Rs-LPS, DM, and DM + Rs-LPS respectively). (C) Representative photomicrographs

of atherosclerotic lesions in the aortic roots of non-diabetic or diabetic *Apoe*<sup>-/-</sup> mice treated with or without Rs-LPS. Magnification:  $4\times$ . (D) Quantification of intimal lesion area of atherosclerotic plaques in the aortic roots ( $n=6, 7, 9,$  and  $8$  for groups of non-DM, non-DM + Rs-LPS, DM, and DM + Rs-LPS respectively). The area of the intimal lesions was presented as percentage of the total aortic area.

diabetic mice (Fig. 5A and B). By contrast, Rs-LPS treatment had no significant inhibition on monocyte/macrophage content in nondiabetic mice.

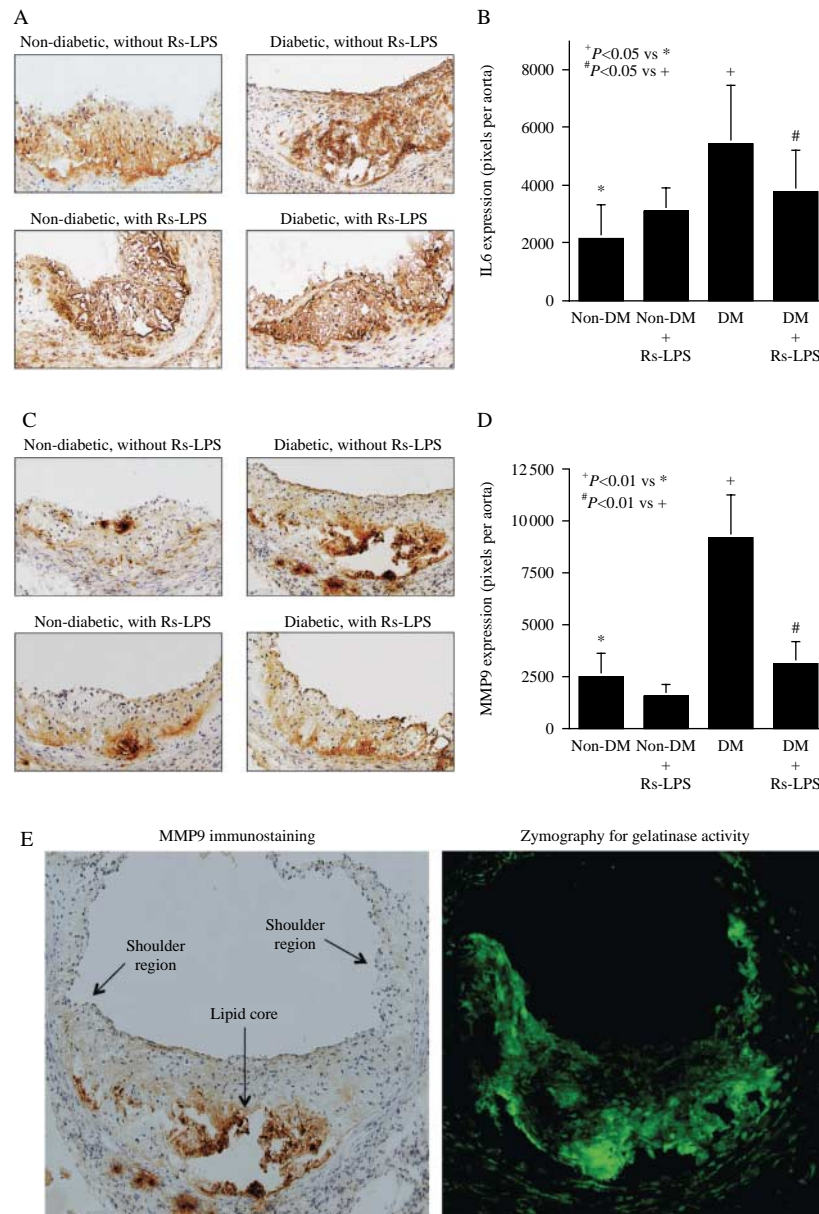
### Inhibition of LPS stimulates NF- $\kappa$ B transcriptional activity by Rs-LPS in mononuclear cells

To better understand the mechanisms by which Rs-LPS inhibited LPS-induced vascular inflammation, we further determined the effect of Rs-LPS on LPS-stimulated NF- $\kappa$ B transcriptional activity in U937 mononuclear cells. Results showed that LPS markedly stimulated NF- $\kappa$ B activity and Rs-LPS inhibited LPS-stimulated NF- $\kappa$ B activity by 90% (Fig. 6A). Furthermore, Bay117085, a specific inhibitor

of NF- $\kappa$ B (Ni *et al.* 2001), inhibited LPS-stimulated IL6 secretion in a concentration-dependent manner (Fig. 6B), indicating an essential role of NF- $\kappa$ B in LPS-induced inflammatory response in mononuclear cells.

### Discussion

The nontoxicity of Rs-LPS in mice and rabbits was first reported by Strittmatter *et al.* (1983). It was shown that over a range of 10–1000 ng/ml, Rs-LPS did not induce inflammatory cytokine TNF $\alpha$  in the RAW264.7 macrophages (Qureshi *et al.* 1991). To understand the mechanisms for Rs-LPS's nontoxicity, analysis of chemical structure showed that Rs-LPS is different from pathological

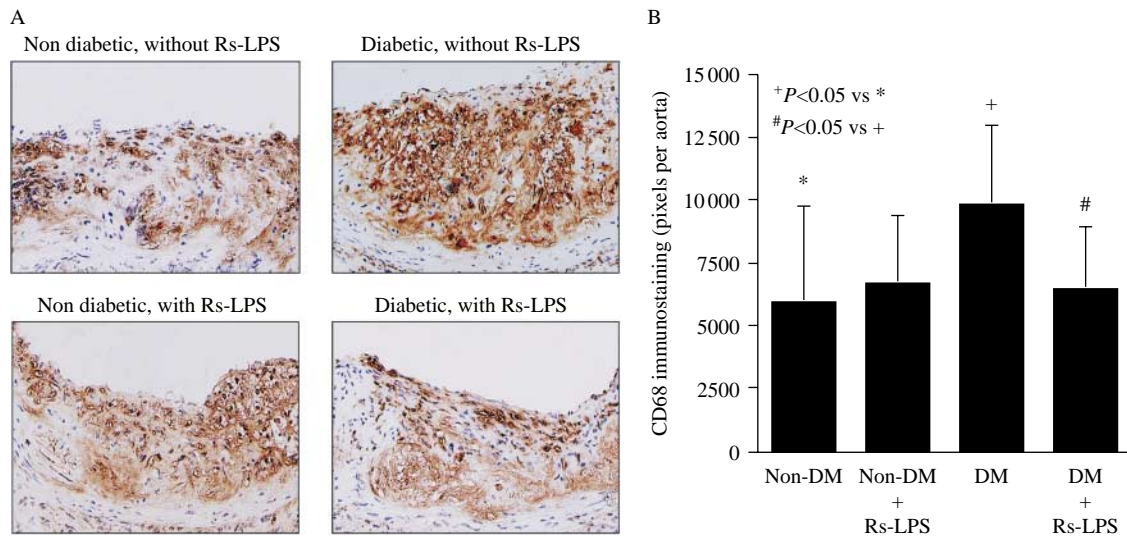
**Figure 4**

Inhibition by Rs-LPS of IL6 and MMP9 expression in atherosclerotic lesions of diabetic *Apoe*<sup>-/-</sup> mice. Mice were killed after the treatment with Rs-LPS and aortic roots were dissected and processed for frozen sections and immunohistochemical analysis of IL6 and MMP9 expression. (A) Immunohistochemistry analysis of IL6 expression. Magnification: 10 $\times$ . (B) Quantification of IL6 protein expression. (C) Immunohistochemistry analysis of MMP9 expression. (D) Quantification of MMP9 protein

expression. IL6 and MMP9 detected by immunostaining were quantified as described in the Materials and Methods section. The animal numbers in the groups are same as those in Fig. 3. (E) Gelatinase activity in atherosclerotic lesions detected by *in situ* zymography. MMP9 immunostaining of aortic cross section from a diabetic mouse. *In situ* zymograph to detect gelatinase activity (green fluorescence) of aortic tissue from the same diabetic mouse.

LPS in number and nature of fatty acyl groups in the lipid A moiety (Kaltashov *et al.* 1997). Further study showed that Rs-LPS is capable of antagonizing TLR4 because it competes with pathological LPS for the same binding site

on MD-2, a TLR4 co-receptor that confers LPS responsiveness (Visintin *et al.* 2005). In recent years, studies have well demonstrated that Rs-LPS effectively and specifically blocks a number of TLR4-mediated pathological effects



**Figure 5**

Inhibition by Rs-LPS on monocyte/macrophage accumulation in atherosclerotic lesions of diabetic *Apoe*<sup>-/-</sup> mice. Mouse monocytes/macrophages were detected using anti-CD68 antibody. (A) Representative photomicrographs of CD68 immunostaining of atherosclerotic plaques.

(B) Quantification of CD68 immunostaining in atherosclerotic plaques. The intensity of the staining was presented as pixels per aorta. The animal numbers in the groups are same as those in Fig. 3.

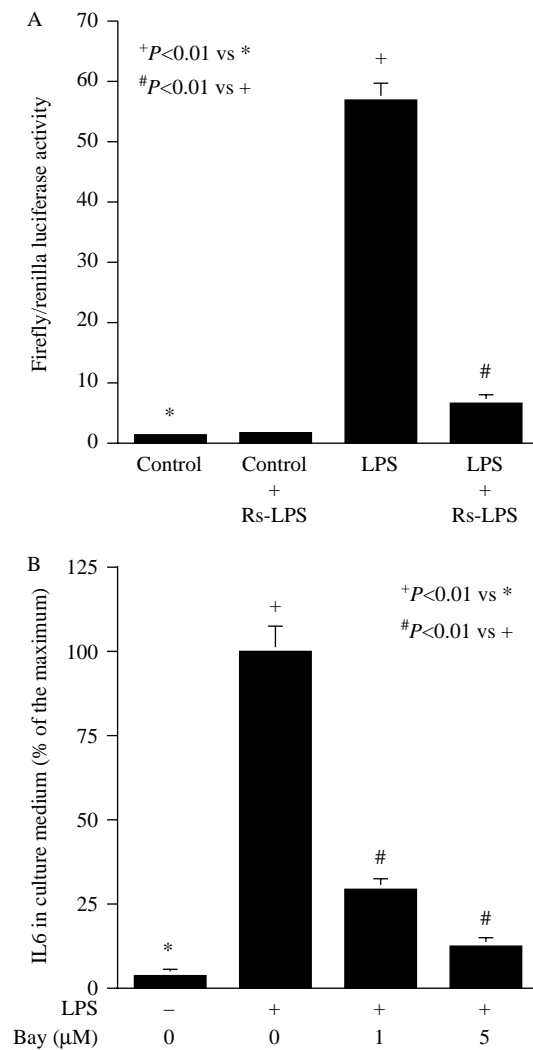
(Baker *et al.* 1990, Kirkland *et al.* 1991, Aida *et al.* 1995, Hammad *et al.* 2009, Hutchinson *et al.* 2010).

In this study, we used Rs-LPS to test our hypothesis that TLR4 blockade attenuated atherogenesis in both nondiabetic and diabetic animal models. We focused on the effect of TLR4 blockade on the early-stage atherosclerosis in *Apoe*<sup>-/-</sup> mice by starting administration of Rs-LPS at age of 14 weeks for 10 weeks of treatment. Inconsistent with our previous reports (Schuyler *et al.* 2011, Ta *et al.* 2011), this study showed that diabetes accelerated the formation of atherosclerotic lesions in *Apoe*<sup>-/-</sup> mice. To understand the mechanisms involved in accelerated atherosclerosis in diabetes, increasing studies have indicated that hyperglycemia promotes a pro-inflammatory state, which plays a central role in diabetes-associated atherosclerosis (Theuma & Fonseca 2003). In agreement with this notion, our present study showed that diabetic *Apoe*<sup>-/-</sup> mice have increased expression of IL6 and MMP9 and content of infiltrated monocytes/macrophages in atherosclerotic lesions when compared with nondiabetic *Apoe*<sup>-/-</sup> mice. As TLR4 plays an important role in inflammation that is responsible for not only atherosclerosis but also diabetes (Bjorkbacka *et al.* 2004, Michelsen *et al.* 2004, Zipris 2010, Devaraj *et al.* 2011, Grieco *et al.* 2011), it is rational to postulate that TLR4 blockade with Rs-LPS in diabetic *Apoe*<sup>-/-</sup> mice would have higher impact than that in nondiabetic *Apoe*<sup>-/-</sup>

mice on inflammation-related atherosclerosis. Indeed, the current study showed that Rs-LPS significantly reduced atherosclerotic lesions in diabetic *Apoe*<sup>-/-</sup> mice.

In addition to the observations that Rs-LPS attenuated early-stage atherosclerosis in diabetic mice, another intriguing finding from the current study is that Rs-LPS reduced serum cholesterol and triglyceride levels in nondiabetic *Apoe*<sup>-/-</sup> mice. This finding suggests the presence of a TLR4-mediated regulatory pathway in cholesterol and triglyceride metabolism in *Apoe*<sup>-/-</sup> mice. In fact, the involvement of a TLR4-mediated pathway in cholesterol and triglyceride biosynthesis in hepatocytes has been reported previously (Victorov *et al.* 1989, Aspichueta *et al.* 2006). Victorov *et al.* (1989) have shown that LPS increased intracellular content of free cholesterol, esterified cholesterol, and triglycerides by 30–40% in the primary culture of rabbit hepatocytes. Aspichueta *et al.* (2006) also reported that rats treated with LPS had increased levels of VLDL-containing apoB, triglyceride, and cholesterol. Our present study demonstrated for the first time that TLR4 blockade is effective in lowering cholesterol and triglyceride levels. Interestingly, Higashimori *et al.* (2011) reported that TLR4 deficiency in *Apoe*<sup>-/-</sup> mice was not associated with reduced serum cholesterol and triglycerides. To explain the controversy between their report and our current study, we believe that as TLR4 deficiency in these mice is inborn,



**Figure 6**

Inhibition by Rs-LPS on LPS-stimulated NF- $\kappa$ B transcriptional activity in U937 mononuclear cells. (A) U937 cells transfected with NF- $\kappa$ B promoter-luciferase reporter constructs were treated with or without 100 ng/ml LPS in the absence or presence of 500 ng/ml Rs-LPS for 12 h. After the treatment, cells were lysed and firefly luciferase activity was determined and normalized to renilla luciferase activity. (B) U937 cells were treated with 100 ng/ml LPS in the absence or presence of 1 or 5  $\mu$ M Bay117085 (Bay) for 24 h and IL6 in culture medium was quantified using ELISA. The amount of IL6 released by cells treated with LPS in the absence of Bay117085 was designated as 100% (maximum). IL6 released by cells in other groups was presented as percentage of the maximum. The data presented are the representative from two experiments with the similar results.

the TLR4-mediated regulation of lipid metabolism in TLR4-knockout mice is compensated by other mechanisms. In our current study, however, TLR4 had been functioning in mice before its blockade with Rs-LPS. Thus, TLR4-mediated regulatory pathways on lipid metabolism are interrupted abruptly and cells are not able to

compensate for this. A recent study showed that TLR4 deficiency in LDL receptor-deficient mice fed a diabetogenic diet was associated with improved plasma cholesterol and triglyceride levels (Ding *et al.* 2012), suggesting that TLR4 deficiency reduces obesity-induced hyperlipidemia in LDL receptor-deficient mice. These findings are consistent with the results from our present study and underscored an essential role of TLR4 signaling in hepatic lipoprotein metabolism.

In our present study, no LPS or gram-negative bacteria were given to mice. Therefore, it is likely that endogenous ligands were responsible for stimulating TLR4-dependent pathways for cholesterol and triglyceride metabolism. Although the particular endogenous TLR4 ligands that are involved in cholesterol and triglyceride metabolism in *ApoE*<sup>-/-</sup> mice remain unknown at this time, endogenous TLR4 ligands such as mmLDL (Miller *et al.* 2003, 2005) have been reported. Obviously, more investigations are warranted to identify endogenous TLR4 ligands involved in alteration of cholesterol and triglyceride metabolism.

Interestingly, our results showed that while Rs-LPS reduced serum cholesterol and triglycerides in non-diabetic *ApoE*<sup>-/-</sup> mice, it failed to do so in diabetic *ApoE*<sup>-/-</sup> mice, suggesting that factor(s) related to hyperglycemia in diabetic mice may abolish the inhibitory effect of Rs-LPS on cholesterol and triglyceride metabolism in liver. We hypothesize that some hyperglycemia-related factors are effective on hepatocytes to abolish the inhibitory effect of Rs-LPS on lipid metabolisms, but ineffective on vascular cells to neutralize the effect of Rs-LPS on vascular inflammation. Therefore, Rs-LPS was capable of inhibiting atherogenesis but failed to reduce serum lipid levels in diabetic mice. Further investigations are necessary to identify the factors that specifically neutralize the effect of Rs-LPS in hepatocytes.

Our results showed that Rs-LPS treatment did not change serum glucose, cholesterol, and triglycerides in diabetic *ApoE*<sup>-/-</sup> mice but inhibited the expression of IL6 and MMP9, two key molecules involved in inflammation, and reduced monocyte/macrophage accumulation in atherosclerotic lesions. Thus, it is strongly suggested that Rs-LPS treatment reduced atherosclerosis by inhibiting vascular inflammation. Given that TLR4 is a receptor for the innate immune response, this study underscored the important role of the innate immune response in diabetes-associated atherosclerosis.

Several TLR4 antagonists have been developed and applied to treatment of sepsis and other diseases in animal models (Fort *et al.* 2005, Sun & Pearlman 2009). For example, it has been shown that treatment with CRS-526,

a lipid A-mimetic, inhibits the development of moderate-to-severe disease in two mouse models of colonic inflammation (Fort *et al.* 2005). Another TLR4 antagonist, Eritoran tetrasodium (E5564), has been effective in the treatment of *Pseudomonas aeruginosa*-induced corneal inflammation (Sun & Pearlman 2009). In recent years, TLR4 antagonists have been successfully applied in the treatment of several infection-unrelated diseases in animal models such as myocardial injury induced by chronic stress (Wang *et al.* 2011). Clearly, TLR4 blockade with TLR4 antagonists was effective in the treatment of various inflammation-associated diseases in animal models.

In conclusion, this study has shown for the first time that the TLR4 antagonist Rs-LPS is effective to reduce vascular inflammation and early-stage atherosclerosis in diabetic mice. The findings from this study suggest that TLR4 is a potential target for anti-inflammatory therapy to reduce cardiovascular complications in diabetic patients. While the findings from this study are interesting, it is noteworthy that there are some limitations in this study. First, the animal model employed in this study is the type 1 diabetic mouse model. Thus, the findings from this study may be different from those from the study using mouse models for type 2 diabetes. Second, our *in vitro* investigations in this study focused on mononuclear cells. Given the crucial role of smooth muscle cell (SMC) proliferation in atherogenesis, it is important to characterize the effect of Rs-LPS on vascular SMC proliferation to better understand the effect of Rs-LPS on atherogenesis.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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