

RESEARCH PAPER

Salvianolic acid B suppresses maturation of human monocyte-derived dendritic cells by activating PPAR γ

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BACKGROUND AND PURPOSE

Salvianolic acid B (Sal B), a water-soluble antioxidant derived from a Chinese medicinal herb, is known to be effective in the prevention of atherosclerosis. Here, we tested the hypothesis that the anti-atherosclerotic effect of Sal B might be mediated by suppressing maturation of human monocyte-derived dendritic cells (h-monDC).

EXPERIMENTAL APPROACH

h-monDC were derived by incubating purified human monocytes with GM-CSF and IL-4. h-monDC were pre-incubated with or without Sal B and stimulated by oxidized low-density lipoprotein (ox-LDL) in the presence or absence of PPAR γ siRNA. Expression of h-monDC membrane molecules (CD40, CD86, CD1a, HLA-DR) were analysed by FACS, cytokines were measured by ELISA and the TLR4-associated signalling pathway was determined by Western blotting.

KEY RESULTS

Ox-LDL promoted h-monDC maturation, stimulated CD40, CD86, CD1a, HLA-DR expression and IL-12, IL-10, TNF- α production; and up-regulated TLR4 signalling. These effects were inhibited by Sal B. Sal B also triggered PPAR γ activation and promoted PPAR γ nuclear translocation, attenuated ox-LDL-induced up-regulation of TLR4 and myeloid differentiation primary-response protein 88 and inhibited the downstream p38-MAPK signalling cascade. Knocking down PPAR γ with the corresponding siRNA blocked these effects of Sal B.

CONCLUSIONS AND IMPLICATIONS

Our data suggested that Sal B effectively suppressed maturation of h-monDC induced by ox-LDL through PPAR γ activation.

Abbreviations

ADRP, adipose differentiation-related protein; AP2, activating enhancer binding protein 2; APCs, antigen-presenting cells; GK, glycerol kinase; h-monDC, human monocyte-derived dendritic cells; LXR α , liver X receptor α ; MyD88, myeloid differentiation primary-response protein 88; PAMP, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; PGAR, PPAR γ -angiopoietin related protein; Sal B, salvianolic acid B; TLRs, Toll-like receptors

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall and mechanisms related to immune responses play crucial roles in the initiation and progression of athero-

sclerosis (Ludewig *et al.*, 2002). A growing body of evidence suggests that lipid antigens, such as monophosphoryl lipid A, could activate dendritic cells, one of the major types of antigen-presenting cells (APCs), through selective activation of the MAPK pathway (Ismaili *et al.*, 2002; Cekic *et al.*, 2009)

and enhancement of both Th1- and Th2-specific immune responses (De Becker *et al.*, 2000; Ismaili *et al.*, 2002). Delivery of lipid antigens by human monocyte-derived dendritic cells (h-monDC) and secretion of inflammatory cytokines from these cells are essential for the initiation and progression of atherosclerosis (Lord and Bobryshev, 1999; Nickel *et al.*, 2009). In mice lacking the receptor for low density lipoprotein (LDLR^{-/-} mice), hypercholesterolaemia-induced atherosclerotic lesions could be significantly reduced by depleting intimal CD11c⁺ DCs (Paulson *et al.*, 2010).

Salvianolic acid B (Sal B), a constituent of *Salvia miltiorrhiza*, has been previously reported to exert anti-atherosclerotic activity *in vivo* and *in vitro*, mainly through depressing the inflammatory immune response, including lower expression of inflammatory cytokines, adhesion molecules (Chen *et al.*, 2001; Zhang and Wang, 2006) and attenuating oxidative stress-induced endothelial damage (Shi *et al.*, 2007; Wu *et al.*, 2009). However, the potential mechanisms and related mediators for the vascular protective effects of Sal B are not fully understood. Previous reports from our group suggested that immune maturation of h-monDC might play an important role in the process of atherosclerosis (Luo *et al.*, 2004; Ge *et al.*, 2005), and h-monDC suppression might be one of the anti-atherogenic mechanisms of various vasoprotective agents. Therefore, we tested the hypothesis that the anti-atherosclerotic effect of Sal B might be mediated by suppressing h-monDC maturation. Our results indicated that Sal B could suppress maturation of h-monDC, induced by oxidized LDL (ox-LDL), through activating PPAR γ .

Methods

Preparation of h-monDC

Heparinized venous blood was taken from healthy volunteers from the blood bank of Zhongshan Hospital. Ethical approval was given by the Ethics Committee of Zhongshan hospital. Human peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Histopaque 1077 (Sigma Aldrich) as described previously. (Ge *et al.*, 2005) The monocytes (over 98% pure) were then prepared from PBMCs by positive selection with anti-CD14 magnetic beads (Milteny Biotech Co., Bergisch Gladbach, North Rhine-Westphalia, Germany), seeded into six-well flat-bottom plates with 10⁶ cells per well, cultured in 2 mL RPMI-1640 (Gibco-BRL, Life Technologies, Paisley, UK) containing 100 ng·mL⁻¹ GM-CSF (R&D Systems, Inc., Minneapolis, MN, USA), 40 ng·mL⁻¹ IL-4 (R&D Systems, Inc.) and 10% FBS (Hyclone, Logan, UT). On culture day 6, cells were pretreated with various concentrations of Sal B (0, 10, 50, 100 μ M) and then stimulated by addition of 50 μ g·mL⁻¹ ox-LDL. Cell viability was over 90% as assessed by Trypan blue staining.

H-monDC immunostaining by FACS

H-monDC were washed, resuspended in ice-cold PBS containing 5% FBS (to prevent non-specific binding) then incubated with FITC- or PE-labelled monoclonal anti-CD1a, anti-CD40, anti-CD86, anti-HLA-DR (Invitrogen, Camarillo, CA, USA), biotinylated anti-human TLR4 antibody (R&D Systems) for 30 min at 4°C. Cells were washed twice, and immunofluores-

cence analysis was performed using a FACScan (BD Biosciences, San Jose, CA) and analysed using Cell Quest software.

Measurement of cytokine secretion of h-monDC

TNF- α , IL-10, IL-12 levels in supernatants of cultured h-monDC were measured using ELISA kits (Invitrogen) according to the manufacturer's instructions. The minimum of detectable dose is <18, <15, <18 pg·mL⁻¹ respectively.

RNA Isolation and real-time quantitative PCR

Total RNA was extracted by Trizol (Invitrogen); 5 μ g total RNA sample was reverse-transcribed using oligo-dT and SuperScript III (Invitrogen). PPAR γ was amplified using the up-primer (5'-CTCATAATGCCATCAGGTTTG-3') and down-primer (3'-CCTCGTCTCGTTTCTCCAC-5'), which were designed from the cDNA sequences for PPAR γ (GenBank accession No. NM138712). Quantitative real time PCR using SYBR Green reagent (Applied Biosystems, Foster City, CA, USA) was performed on the Rotor-Gene 3000 Real-time PCR System (Corbett Research, Mortlake, New South Wales, Australia). Gene expression was analysed by corresponding software (version 6.0.19); the copies of each mRNA molecules was determined by the standard curve method.

Western blot analysis

Whole cell extracts (20 μ g) were separated by electrophoresis in 12% PAGE and transferred into PVDF membranes (Millipore Corp., Billerica, MA, USA). Then, the membranes with blotted protein were blocked for 1 h in 0.1% TBS-Tween 20 (TBS-T) containing 5% bovine serum albumin, followed by probing with antibody to the myeloid differentiation primary-response protein 88(MyD88) and antibodies to phospho-p38MAPK, phospho-pJNK1/2, β -actin (Cell Signaling Technologies, Danvers, MA, USA) at 4°C overnight. The membranes were washed three times for 15 min each with TBS-T, incubated at room temperature for 2 h with diluted (1:5000) secondary HRP-marked antibodies. Immunoreactive proteins were identified using Super Signal West Pico Chemiluminescence Substrate (Thermo, Waltham, MA, USA). The ChemiDoc™ XRS gel documentation system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with Quantity One software was used to quantify the immunoreactive proteins. β -Actin was used as the loading control for each lane.

Nuclear PPAR γ detection

Cells (5 $\times$ 10⁶) were obtained for nuclear protein extraction according to the kit protocol supplied by the manufacturer (#K0311, Fermentas Life Sciences, Burlington, ON, Canada). In brief, harvested cells were washed with ice-cold PBS twice and resuspended with 10 volumes of cell lysis buffer. After 10 min incubation on ice, the extracts were centrifuged at 500 $\times$ g for 7 min at 4°C, and the supernatant were removed to separate the cytoplasmic fraction from nuclei. The nuclei pellets were washed with 500 μ L nuclei washing buffer, vortexed briefly and set on ice for 2 min. After adding 50 μ L nuclei lysis reagent, the nuclei pellets were rocked gently for 20 min to allow extraction of nuclear proteins. Finally, the proteins were separated on 12% PAGE and transferred into

PVDF membranes (Millipore Corp.). The blots were detected by probing with anti-PPAR γ (sc-7273, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

RNA interference

Followed by the protocols provided by Santa Cruz Biotechnology, we first seeded cells into six-well flat-bottomed plates (10^7 per well), cultured in 2 mL RPMI-1640 (Gibco-BRL, Life Technologies) containing $100 \text{ ng}\cdot\text{mL}^{-1}$ GM-CSF (R&D Systems, Inc.), $40 \text{ ng}\cdot\text{mL}^{-1}$ IL-4 (R&D Systems, Inc.) and 10% FBS (Hyclone). On culture day 4, the cells were washed once with 2 mL siRNA Transfection Medium (Cat. # sc-29528), then the cells were overlaid with the siRNA duplex solution, which was before mixed gently for 30 min at room temperature, from PPAR γ siRNA (Cat. # sc-29455) or Control siRNA (Cat. # sc-36869) to diluted siRNA transfection reagents (Cat. # sc-29528). For each well, 0.8 mL siRNA transfection mixture was added. The cells were incubated at 37°C for 6 h. siRNA duplex solution was then removed and replaced with RPMI-1640. The cells were incubated for an additional 24 h. Twenty-four hours after transfection, cells were stimulated with ciglitazone ($25 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$). Whole cell lysates were subjected to Western blotting with anti-PPAR γ antibodies (Santa Cruz Biotechnology, Inc.) to confirm the effect of siRNA on alteration of PPAR γ transcriptional expression.

Transfection and luciferase assays

A glycerol kinase (GK) promoter fragment was amplified by PCR from human genomic DNA (GenBank accession no. NW001842360) using the following primers: 5'-CGCTCGAGCTTGTAATCACAGGGCTAT-3'; 5'-CGAAGCTTCACGAGGTCAGGAGTTTG-3'. The resulting fragment was digested with *Xho*I and *Hind*III at 37°C and then was subcloned into *Xho*I and *Hind*III-restricted pGL3-Luc-Enhancer Vector (Promega, Madison, WI, USA). The reconstructed luciferase reporter system was transiently transfected into h-monDC performed by Lipofectamine 2000 reagent (Invitrogen). Briefly, cells were seeded in suspension at a density of 1×10^6 per well. On culture day 4, 1×10^7 cells were harvested and washed twice with PBS, incubated at 37°C for 6 h in 1 mL RPMI 1640 supplemented with $40 \text{ }\mu\text{L}$ of LipofectamineTM 2000, $20 \text{ }\mu\text{g}$ luciferase reporter construct (pGL3-Luc-GK) or pGL3-Luc control vector, along with $5 \text{ }\mu\text{g}$ CMV- β -galactosidase plasmid as internal control. Transfection mixture was removed, washed twice with PBS and re-seeded, and cells were cultured with RPMI-1640 at a density of 1×10^6 per well, and then stimulated with Sal B ($50 \text{ }\mu\text{M}$) and/or ciglitazone ($25 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$). Cell extracts were collected 24 h after transfection, and luciferase activity was analysed using the Dual-Luciferase reporter assay system according to the manufacturer's instruction (Promega). Luciferase activity of each sample was normalized to its β -galactosidase activity.

Statistical analyses

Data are presented as means $\pm$ SD. Group mean values were compared with one-way ANOVA followed by *post hoc* tests using Tukey's procedure for pairwise comparisons. All statistical analyses were performed with SPSS 11.5 statistical software, and a *P*-value < 0.05 was considered statistically significant.

Materials

Sal B was purchased from Shanghai Institute of Materia Medica (IMM), Chinese Academy of Sciences, and was dissolved in PBS (final concentration: $50 \text{ }\mu\text{M}$). Ciglitazone was purchased from Sigma Aldrich (St Louis, MO), SB203580 and SP600125 from Calbiochem (Merck KGaA, Darmstadt, Germany).

Results

Sal B effectively suppressed ox-LDL-induced h-monDC maturation

Cell surface expressions of co-stimulatory molecules CD40, CD86, CD1a and HLA-DR were examined in ox-LDL ($50 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$)-treated h-monDC, exposed to various concentrations of Sal B (0, 10, 50, $100 \text{ }\mu\text{M}$). Pilot experiments showed that Sal B at a concentration of $50 \text{ }\mu\text{M}$ could inhibit, by nearly 70%, the expression of CD40, CD86, CD1a and HLA-DR (Figure S1), and that this effect could last for 24 h and was still visible after 48 h (Figure 1B). Sal B ($50 \text{ }\mu\text{M}$)-induced cell apoptosis was analysed by Annexin V/7AAD double staining after 24 h incubation. Sal B ($50 \text{ }\mu\text{M}$) alone was not toxic to the cells (Figure S2). Sal B at a concentration of $50 \text{ }\mu\text{M}$ was therefore used in the main study. Ox-LDL-induced increased expressions of CD40, CD86, CD1a and HLA-DR were significantly reduced by Sal B (Figure 1A). In addition, ox-LDL-induced IL-12, IL-10 and TNF- α production in culture medium was also significantly reduced by Sal B (Figure 1C–E). These data suggested that Sal B suppressed h-monDC maturation and the inflammatory responses mediated by matured h-monDC.

Effects of Sal B on h-monDC maturation and TLR4/MyD88 signalling

The family of Toll-like receptors (TLRs) is important for pathogen-associated molecular patterns (PAMP) recognition and antigen presenting by functional h-monDC (de Kleijn and Pasterkamp, 2003). The link between TLR4 and h-monDC maturation has been shown in previous reports (Michelsen *et al.*, 2001; Turnbull *et al.*, 2005). In the present study, ox-LDL increased ~ 2.5 -fold the expression of membrane TLR4 in h-monDC and this was attenuated by Sal B (Figure 2A). Up-regulation of MyD88, one of the TIR domain-containing adaptors recruited by TLR4 (Michelsen *et al.*, 2004), induced by ox-LDL, was also significantly down-regulated by Sal B (Figure 2B).

Next, we analysed the effect of Sal B on TLR4/MyD88-mediated MAPK pathway during h-monDC maturation (Ardeshtna *et al.*, 2000; Arrighi *et al.*, 2001). As shown in Figure 2C, Sal B ($50 \text{ }\mu\text{M}$) reduced ox-LDL-induced activation of p38 and JNK1/2, and ox-LDL-induced activation of p38 and JNK1/2 was also inhibited by SB203580 (a specific p38-MAPK blocker) or SP600125 (a specific JNK1/2 blocker). Subsequently, we determined the effects of SB203580 ($20 \text{ }\mu\text{M}$) and SP600125 ($20 \text{ }\mu\text{M}$) on ox-LDL-induced h-monDC phenotype change and IL-12, IL-10 and TNF- α production. Notably, ox-LDL-induced increased expressions of CD40, CD86, CD1a and HLA-DR were attenuated by SB203580

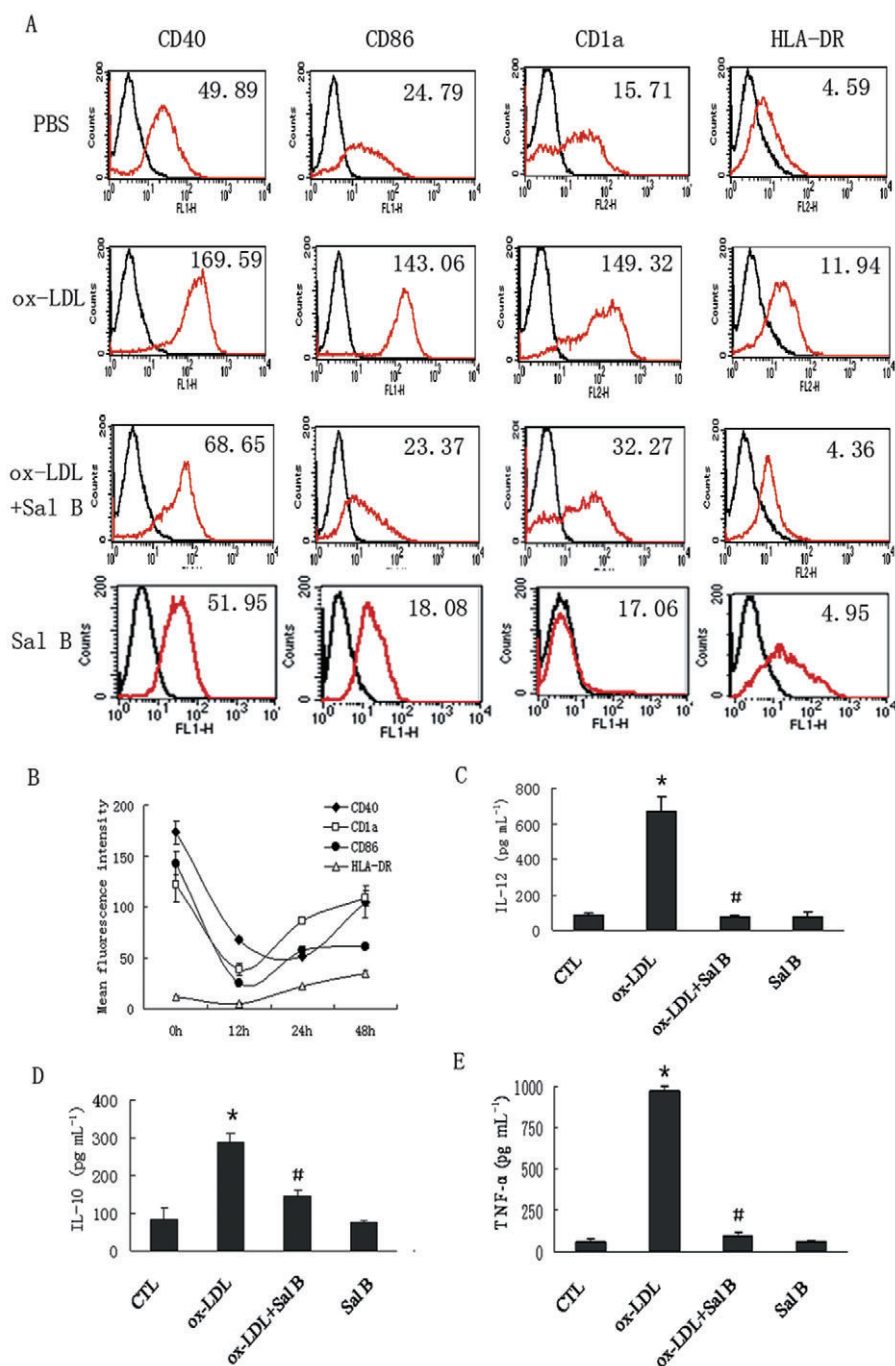


Figure 1

PBMCs were separated and purified with anti-CD14 magnetic beads, then cultured with GM-CSF (100 ng·mL⁻¹) and IL-4 (40 ng·mL⁻¹) for 5 days. On day 6, ox-LDL (50 µg·mL⁻¹) was added to the cells 24 h before analysis in the presence or absence of Sal B (50 µM). The effect of Sal B on h-monDC phenotype changes were analysed by FACS and immune cytokines by ELISA. (A) Ox-LDL-treated h-monDC were analysed for the expression of CD40, CD86, CD1a and HLA-DR in the absence or presence of Sal B by FACS. (B) Time-dependent effect of Sal B on expression of CD40, CD86, CD1a and HLA-DR. (C–E) Sal B reduced levels of IL-12, TNF-α and IL-10 in supernatants of cultured h-monDC 24 h post ox-LDL stimulation by ELISA. Bar graphs show mean concentration results (mean ± SD) of three independent experiments. **P* < 0.05, significantly different from Control (CTL); #*P* < 0.05, significantly different from ox-LDL.

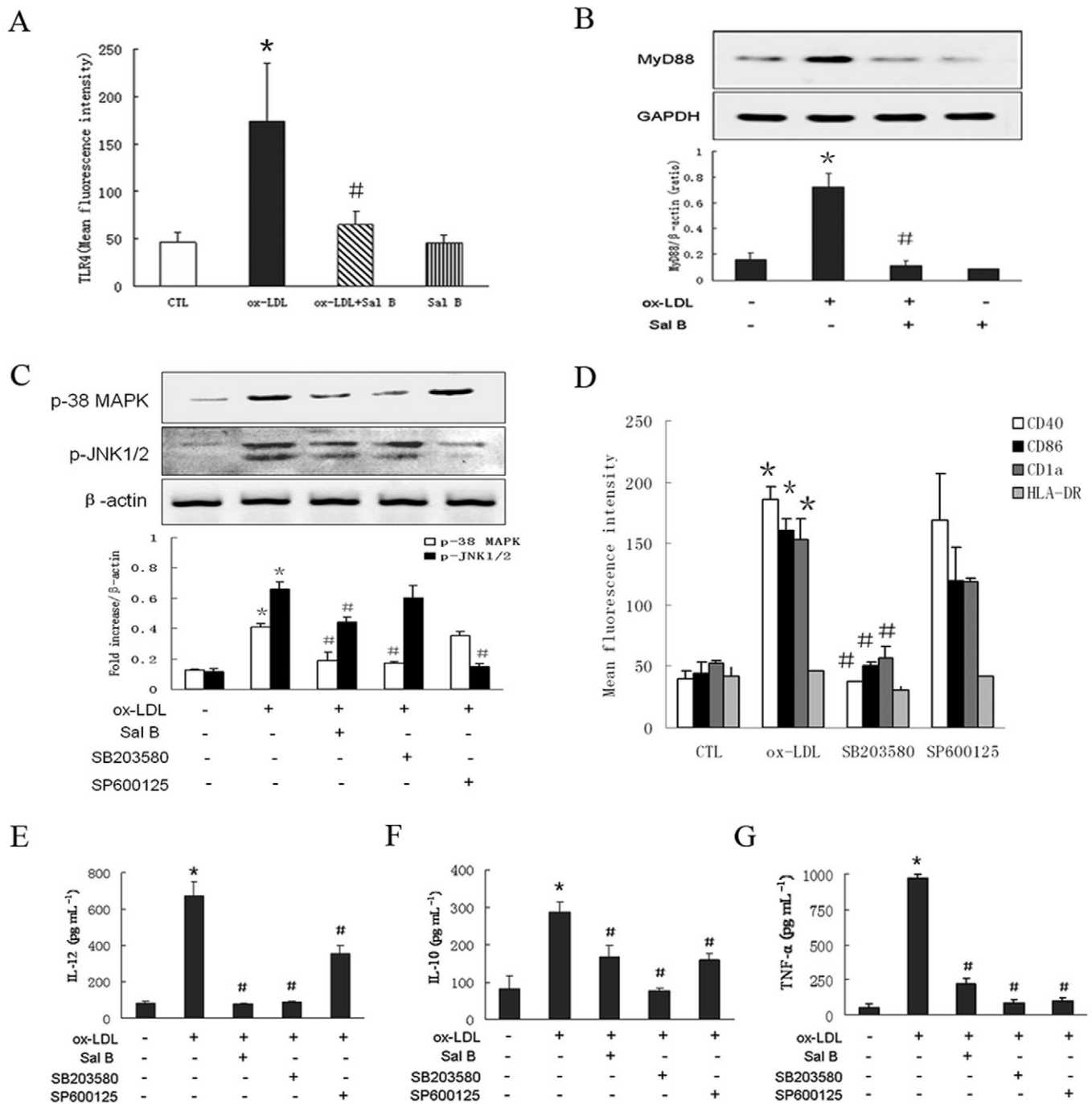


Figure 2

PBMCs derived h-monDC cultured with GM-CSF and IL-4 for 5 days, then stimulated by ox-LDL and/or Sal B for 24 h in the presence or absence of SB203580 (a specific p38-MAPK blocker), SP600125 (a specific JNK1/2 blocker) before analysis. (A) Effect of Sal B on TLR4 expression by FACS. (B) Effect of Sal B on MyD88 protein expression by Western blot. (C) Effect of Sal B on the phosphorylation levels of p38-MAPK and JNK1/2 by Western blot. (D) Effect of SB203580 or SP600125 on ox-LDL-stimulated expression of CD40, CD86, CD1a and HLA-DR by FACS. (E–G) Effect of Sal B on the production of IL-12, IL-10 and TNF- α compared with the application of SB203580 or SP600125 by ELISA. Data were presented as means $\pm$ SD of four independent experiments. * P < 0.05, significantly different from control (CTL); # P < 0.05, significantly different from ox-LDL.

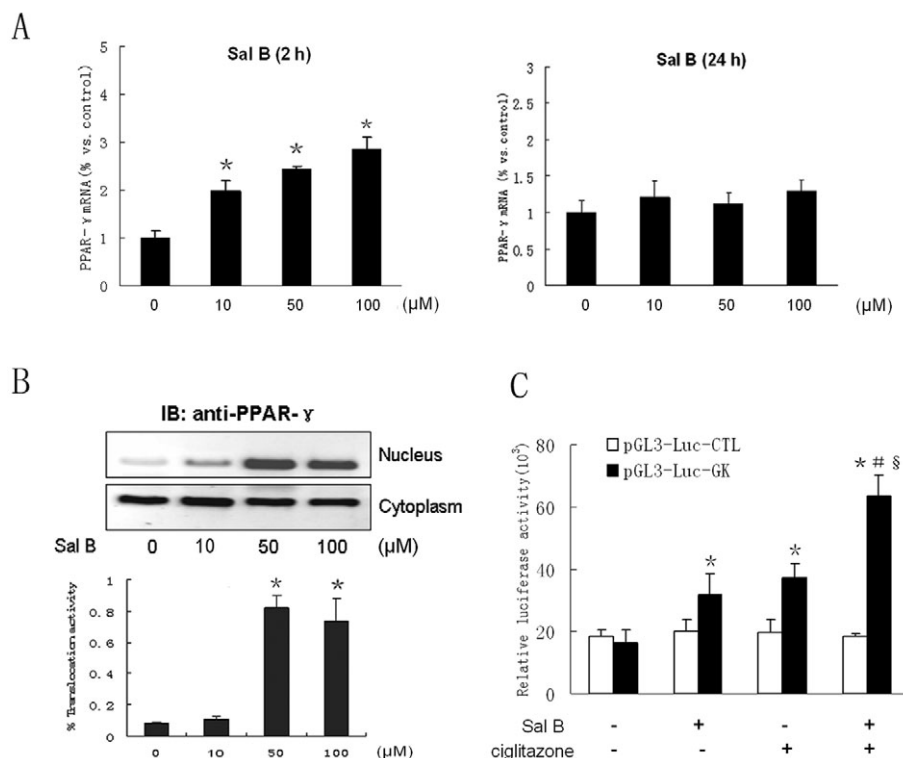


Figure 3

Effects of Sal B on PPAR γ expression and transcriptional activity in h-monDC without ox-LDL stimulation. (A) Cultured h-monDC were treated with various concentration of Sal B for 2 h or 24 h. (B) The mRNA and protein levels of PPAR γ were examined by real-time RT-PCR and Western blot respectively. (C) pGL3-Luc-enhancer report vector with a GK promoter was transfected into h-monDC, and then the cells were treated with vehicle (PBS), Sal B (50 μ M) and/or ciglitazone (25 μ g·mL⁻¹). Cell lysates were harvested, and both luciferase and β -galactosidase activities were analysed using the Dual-Luciferase reporter assay system. Data are shown as related luciferase activity in cells transfected with pGL3-Luc-GK vector or pGL3-Luc control vector after normalized to the β -galactosidase activity. Data were presented as means $\pm$ SD of four independent experiments. * P < 0.05, significantly different from control; # P < 0.05, significantly different from Sal B; § P < 0.05, significantly different from ciglitazone.

but not by SP600125 (Figure 2D), indicating Sal B might inhibit ox-LDL-induced h-monDC maturation through blocking the TLR4-mediated p38–MAPK signalling pathway. Interestingly, SP600125 significantly reduced IL-12, IL-10 and TNF- α production (Figure 2E–G); however, inhibiting JNK1/2 alone did not suppress ox-LDL-mediated h-monDC maturation, suggesting that the JNK1/2 pathway might be only partly involved in the process of h-monDC maturation.

Since other pattern-recognition receptors (PRRs), such as CD36, might also be potential mediators of ox-LDL-mediated signalling transduction in monocyte-derived cells (Rahaman *et al.*, 2006; Stewart *et al.*, 2010), we further examined the possibility that other PRRs could also induce h-monDC maturation after inhibiting TLR4 receptor. First, TLR4 expression was determined by FACS after blocking TLR4 signalling using purified TLR4-neutralizing antibody. As shown in Figure S3, nearly 70% expression of TLR4 was effectively inhibited by TLR4-neutralizing antibody. Then, ox-LDL-induced h-monDC phenotypes were examined after using TLR4-neutralizing antibody. Data showed that ox-LDL-induced up-regulation of CD40, CD86, CD1a and HLA-DR were all attenuated by TLR4-neutralizing antibody (Figure S4), sug-

gesting that TLR4 activation was critically involved in h-monDC maturation induced by ox-LDL.

Sal B enhanced PPAR γ activation and nuclear translocation

Previous experiments from our group showed that the PPAR γ agonist ciglitazone inhibited ox-LDL-induced h-monDC maturation and immune function (Luo *et al.*, 2004). To elucidate if Sal B suppressed ox-LDL-induced h-monDC maturation via PPAR γ signalling, we investigated the expression of PPAR γ gene in the absence or presence of indicated concentrations of Sal B. As shown in Figure 3A, PPAR γ mRNA level was dose-dependently up-regulated by Sal B at 2 h but was reduced to the baseline level at 24 h. However, Sal B (50–100 μ M) effectively up-regulated the PPAR γ protein level at 24 h in the nucleus (Figure 3B), suggesting PPAR γ transcriptional activity was enhanced by Sal B.

We further investigated whether Sal B could modulate DNA binding activity of PPAR γ . Because PPAR γ activation was linked with up-regulation of glycerol kinase expression, we determined the effects of Sal B and ciglitazone on glycerol kinase promoter activity with the luciferase reporter assay. Our result showed that both Sal B and ciglitazone signifi-

cantly enhanced glycerol kinase promoter activity (Figure 3C). Transactivation capacity in pGL3-Luc-Control Vector was also reduced by Sal B, suggesting PPAR γ could be activated by Sal B at the transcriptional level. Furthermore, some of the PPAR γ target genes, including those for activating enhancer binding protein 2 (AP2), adipose differentiation-related protein (ADRP), PPAR γ -angiopoietin related protein (PGAR) and liver X receptor α (LXR α), were identified to confirm that Sal B could actually enhance the PPAR γ -mediated pathway (Figure S5). The results showed that three genes (for ADRP, PGAR and LXR α) were activated by Sal B, suggesting Sal B-induced PPAR γ activation was different from that induced by ox-LDL.

Association between PPAR γ signalling and H-monDC maturation in the presence of Sal B

To determine whether PPAR γ activation was required for the observed effects of Sal B on ox-LDL-induced h-monDC maturation, we tested the effects of Sal B in case of PPAR γ activity inhibition by knocking down PPAR γ with specific siRNA (Figure S6). When PPAR γ was inhibited by specific siRNA, ox-LDL-induced up-regulation of the membrane expression of CD40, CD86, CD1a and HLA-DR (Figure 4D) in h-monDC and increased secretion of IL-12, IL-10 and TNF- α in culture medium (Figure 4A–C) were no longer affected by Sal B, suggesting that PPAR γ activation was essential for the inhibitory effect of Sal B on h-monDC maturation induced by ox-LDL. To exclude the effect of nuclear acids on ox-LDL-induced TLR4 signalling in h-monDC, the effects of PPAR γ siRNA on TLR4 expression in h-monDC were tested. In contrast with the enhanced expression of TLR4 induced by ox-LDL, TLR4 expression was not affected by PPAR γ siRNA alone (Figure 4E). As shown in Figure 4D, TLR4-mediated expression of CD40, CD86, CD1a and HLA-DR was also not affected by PPAR γ siRNA or scrambled siRNA. The association between PPAR γ activation and TLR4-mediated MAPK pathway after knocking down PPAR γ was also investigated. As expected, enhanced expression of phosphorylated p38 in ox-LDL-treated h-monDC was down-regulated by ciglitazone or Sal B (Figure 4F, lanes 3–4) and the effects of both ciglitazone- and Sal B were reversed by PPAR γ siRNA (Figure 4F, lanes 5–6), indicating Sal B-induced PPAR γ activation was likely to be mediated by blocking TLR4-mediated MAPK cascade.

To elucidate whether Sal B could also inhibit h-monDC maturation stimulated by another stimulus, we examined the effect of Sal B on h-monDC maturation induced by LPS. Increased expressions of CD40, CD86, CD1a and HLA-DR in LPS (100 ng·mL⁻¹)-treated h-monDC were also attenuated by pretreatment with Sal B, and these effects could be attenuated by PPAR γ siRNA (Figure 4G). These data suggested that LPS-induced h-monDC maturation could also be attenuated by Sal B via PPAR γ activation.

Discussion and conclusions

Sal B is one of the constituents of extracts of *Salvia miltiorrhiza*, a traditional Chinese medicinal herb. Previous studies suggested its antioxidant activities were associated with the pro-

TECTIVE effects of Sal B on the vasculature (Wu *et al.*, 2009). Besides the oxidative injury, inflammatory and immune injury are the other major mechanisms underlying atherosclerotic vessel disease (Hansson, 1999; Ludewig *et al.*, 2002). In the present study, we demonstrated for the first time that the possible anti-atherosclerotic effects of Sal B might be mediated by suppressing maturation of h-monDC and thus reducing the inflammatory and immune responses mediated by matured h-monDC. Our results showed that Sal B reduce ox-LDL-induced h-monDC maturation through activating PPAR γ and suppressing the TLR4-mediated MAPK signalling pathway.

The TLR4-mediated signalling pathway is important for h-monDC maturation (Turnbull *et al.*, 2005) and in the current study, Sal B attenuated the up-regulation of TLR4 signalling in h-monDC, induced by ox-LDL or LPS. Blockade of TLR4 signalling suppressed transcription of genes responsible for inflammatory chemokine production and h-monDC maturation (Turnbull *et al.*, 2005; Wang *et al.*, 2008), by activating the NF- κ B and MAPK pathways (Appel *et al.*, 2005; Lin *et al.*, 2005). As expected, the enhanced expression of CD40, CD86, CD1a and HLA-DR, which reflects immune maturation of h-monDC (Carucci *et al.*, 2000; Xia and Kao, 2002; Doppeide *et al.*, 2007) and the increased output of pro-atherosclerotic chemokines, such as IL-12, IL-10 and TNF- α (Appel *et al.*, 2005; Hickey *et al.*, 2008), both induced by ox-LDL, were significantly attenuated by Sal B partly through inhibiting TLR4/MyD88 signalling.

Recent studies indicated that CD36, a membrane glycoprotein present on monocytes and macrophages, might work as a co-receptor in facilitating the formation of the TLR complex (Triantafilou *et al.*, 2006; Stewart *et al.*, 2010). The CD36–TLR4–TLR6 signalling complex, induced by ox-LDL, could trigger and activate MyD88-dependent signalling pathways. Both *in vivo* and *in vitro* studies showed that MyD88 deficiency reduced atherosclerosis and attenuated h-monDC maturation by decreasing IL-12 and TNF- α synthesis (Michelsen *et al.*, 2004; Macedo *et al.*, 2008). The role of Sal B in regulating TLR4/MyD88 signalling was explored in our study. Blocking TLR4 receptors with purified TLR4-neutralizing antibody suppressed h-monDC maturation induced by ox-LDL. In fact, blocking the TLR4 signal might also inhibit other PRR-mediated signals, which might be important for synergies between the receptor. For instance, the CD36–TLR4–TLR6 complex is suggested as the ‘first hit’ in the regulation of the innate immune response, which could initiate the progress of differentiation or apoptosis of monocytes and macrophages (Stewart *et al.*, 2010). Recent studies also suggested that CD36 could modulate the functions of h-monDC through triggering TLR4/MyD88-dependent chemokine synthesis and downstream signalling (Taront *et al.*, 2009; Stewart *et al.*, 2010). Further investigations are therefore needed to clarify the role of CD36 in the h-monDC maturation process in responses to atherogenic lipids and the role and interactions of TLR4 receptor and MyD88 protein during these processes.

TLR4-mediated maturation of h-monDC is regulated by different signal transduction pathways, including PI3 kinase, p38MAPK and NF- κ B (Ardeshna *et al.*, 2000). LPS-induced up-regulation of TLR4 could activate all three MAPK cascades. However, the intracellular signals triggered by ox-LDL differ between h-monDC and other monocyte-derived cells because of the different uptake of ox-LDL by

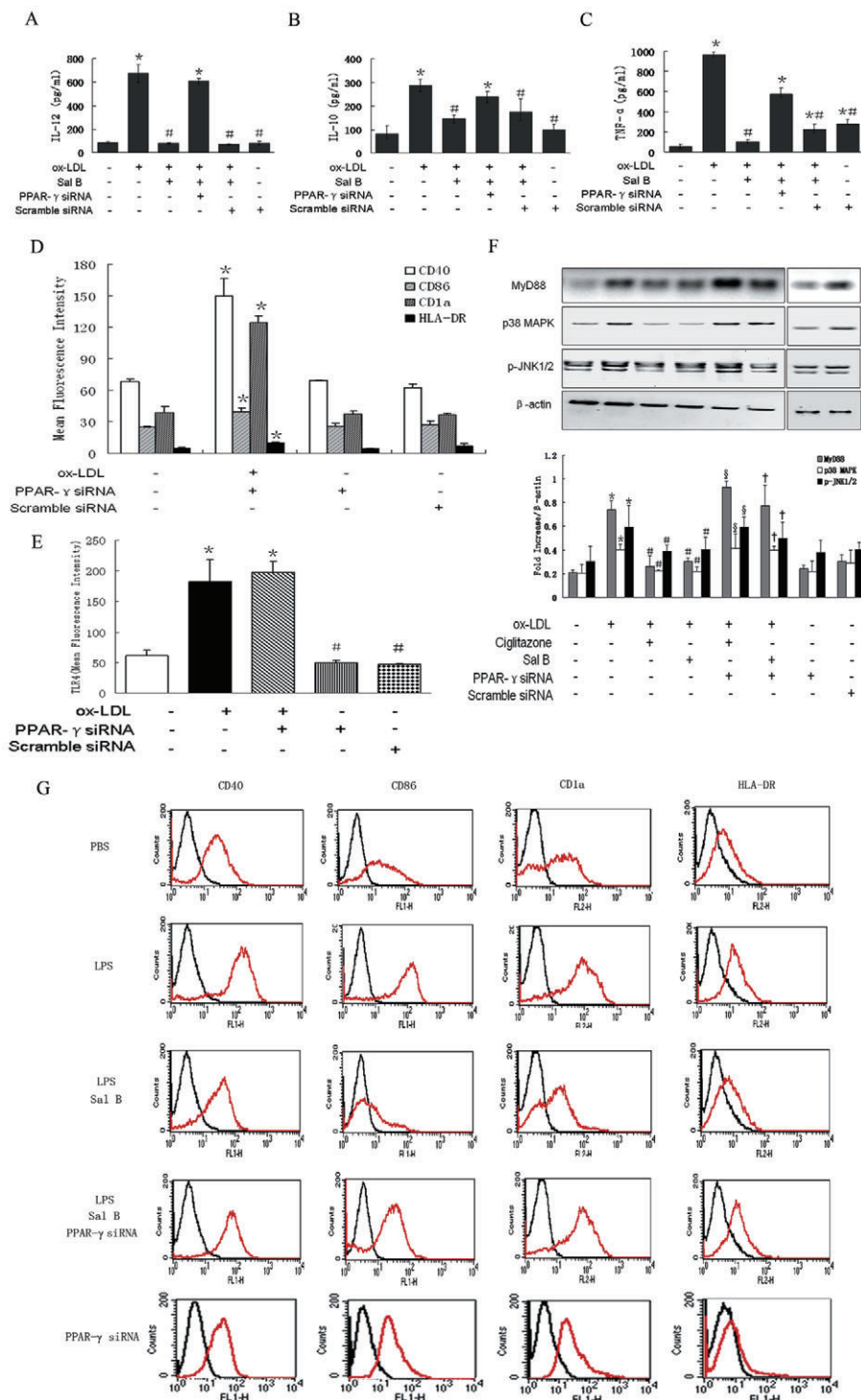


Figure 4

Purified h-monDCs were incubated for 5 days in the presence of GM-CSF and IL-4 with PPAR γ siRNA or scrambled siRNA transfection starting from the fourth day of culture. On day 6, cells were stimulated by ox-LDL for 24 h in the absence or presence of Sal B before harvesting. Effects of PPAR γ siRNA on ox-LDL-induced h-monDC maturation were determined as follows: (A–C) production of IL-12, IL-10 and TNF- α by ELISA. (D) H-monDC phenotype by FACS. (E) Membrane receptor TLR4 expression under ox-LDL stimulated or unstimulated conditions by FACS. (F) Western blot analysis of MyD88, phosphorylated p38-MAPK and JNK1/2 levels pre-incubated with Sal B/ciglitazone. (G) Effect of PPAR γ siRNA on phenotype of LPS (100 ng·mL⁻¹)-stimulated h-monDC by FACS. The results represent the means $\pm$ SD from four independent experiments. * P < 0.05, Sal B.

the different receptors (LOX-1, CD36 and TLR4). JNK1/2 phosphorylation is critically involved in macrophage activation through CD36-dependent formation of signalling complex containing Lyn and MEKK2 (Rahaman *et al.*, 2006; Kennedy *et al.*, 2009). In our study, ox-LDL-induced JNK1/2 phosphorylation was up-regulated in h-monDC, but ox-LDL-induced h-monDC maturation was not suppressed by blockade of JNK1/2, while blocking TLR4 signalling through the p38-MAPK pathway effectively suppressed ox-LDL-induced h-monDC maturation. Taken together, we hold the view that p38-MAPK but not the JNK1/2 cascade was involved in the inhibition by Sal B of h-monDC maturation induced by ox-LDL.

A novel finding of the current study is that PPAR γ activation by Sal B might be the major working mechanism for suppressing ox-LDL-induced h-monDC maturation. There is growing evidence that the anti-atherogenic effects of HMG-CoA reductase inhibitors (statins) are partly mediated by PPAR γ activation (Yano *et al.*, 2007). PPAR γ agonists, such as pioglitazone and rosiglitazone, exert their endothelium-dependent vascular protection through modulating endogenous production of stress-induced receptors such as LOX-1 or of endothelin-1 (Iglarz *et al.*, 2003; Mehta *et al.*, 2003). PPAR activation has been involved in the immune suppression of h-monDC, macrophages, Langerhans cells and other types of APCs (Appel *et al.*, 2005; Dubrac *et al.*, 2007; Yano *et al.*, 2007). Thus, PPAR agonists play critical roles in modulating the process of h-monDC maturation, attenuating release of inflammatory cytokines and cytokine-induced migration of h-monDC (Angeli *et al.*, 2003; Hammad *et al.*, 2004).

The present study shows that Sal B might suppress ox-LDL-induced h-monDC maturation through PPAR γ activation. Earlier studies demonstrated that ox-LDL alone could activate PPAR γ through MAPK-dependent COX-2 expression in macrophages (Taketa *et al.*, 2008; Liu *et al.*, 2010). Notably, ox-LDL-induced COX-2 expression in RAW264.7 macrophages was mediated by activation of ERK1/2, but not p38-MAPK. However, siRNA for COX-2 inhibited ox-LDL-induced PPAR γ activation by only ~50% (Taketa *et al.*, 2008), suggesting ox-LDL-induced COX-2 cascade was only a part of PPAR γ activation in macrophages. In the present study, we showed that PPAR γ target gene ADRP could be activated by both ox-LDL and Sal B, AP2 was activated by ox-LDL, while PGAR and LXR α were only activated by Sal B, suggesting that the mechanism of regulation by ox-LDL was different from that induced by Sal B. In addition, our present data supported the proposal that ox-LDL activated TLR4-dependent h-monDC maturation through p38-MAPK signalling pathway (Xu *et al.*, 2001; Wang *et al.*, 2008), and TLR4-mediated activation of DCs could be attenuated by PPAR γ agonists (Appel *et al.*, 2005). Taken together, we consider that the mode of PPAR γ activation induced by Sal B and ox-LDL was not the same. Moreover, p38-MAPK but not ERK1/2 were involved in h-monDC maturation and could be regulated by Sal B through PPAR γ activation.

Previous studies also demonstrated that Sal B was effective in reducing lipid-related oxidative stress and in the treatment of atherosclerotic vessel disease (Chen *et al.*, 2001; He *et al.*, 2008; Wu *et al.*, 2009), but the potential molecular mechanism for the vasoprotective effect of Sal B is largely unknown. From the present study, we would con-

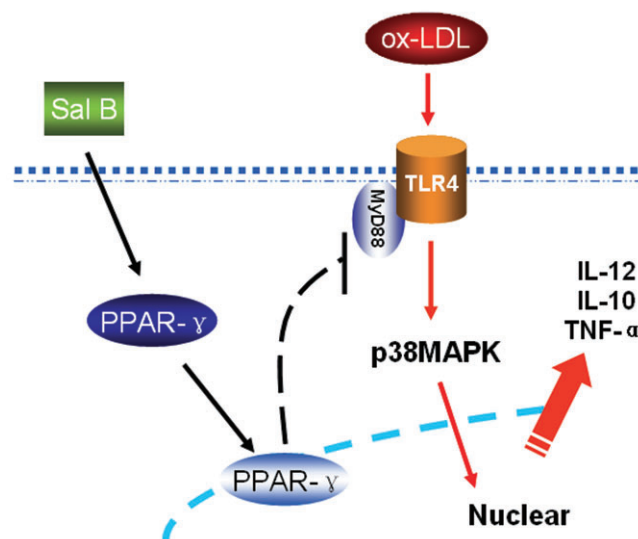


Figure 5

Putative mechanism for Sal B-mediated suppression of h-monDC maturation induced by ox-LDL. h-monDC maturation was stimulated by ox-LDL through TLR4/MyD88-mediated p38-MAPK signalling pathway. This signal cascade could be suppressed by Sal B through up-regulating nuclear translocation and increased transcriptional activity of PPAR γ and subsequently down-regulating of TLR4/MyD88-mediated p38-MAPK pathway.

clude that Sal B promotes PPAR γ activation, and to some extent, Sal B enhances the process of negative regulation induced by ox-LDL. Consistent with the earlier reports of the modulating effects of PPAR γ agonists on inflammatory and immune responses (Klotz *et al.*, 2007), we have shown that the PPAR γ transcriptional activity was enhanced by Sal B. In line with the effects induced by ciglitazone, Sal B also increased PPAR γ nuclear translocation and target gene expression (Leroy *et al.*, 2006). The MAPK signalling pathway is thought to regulate through PPAR γ , which controls the function of h-monDC, or by affecting local cells, such as T cells and Langerhans cells, by paracrine effects (Dubrac *et al.*, 2007; Klotz *et al.*, 2007). In this study, both ox-LDL- and LPS-induced TLR4-dependent MAPK pathway was inhibited by Sal B, suggesting Sal B-mediated PPAR γ activation was important for h-monDC suppression. Also, Sal B-induced PPAR γ activation inhibited the p38-MAPK pathway and reduced expression of CD40, CD86, CD1a, HLA-DR and production of IL-12, IL-10 and TNF- α . These effects were attenuated by PPAR γ siRNA, which implied a causal relationship between the effect of Sal B on h-monDC maturation and PPAR γ activation (Figure 5).

In conclusion, our present study suggests that Sal B suppressed ox-LDL-induced h-monDC maturation through activating PPAR γ and subsequently down-regulating TLR4-mediated p38-MAPK pathways, which might reduce the inflammatory and immune responses of matured h-monDC. Further studies are needed to confirm that Sal B can attenuate atherosclerosis *in vivo* via the mechanisms we have suggested here.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Concentration curves for the effect of Sal B on DCs maturation. Ox-LDL-induced DCs were treated with 0, 10, 50 and 1007 M of Sal B, and the phenotypic changes of CD40, CD86, CD1a and HLA-DR were determined respectively.

Figure S2 PBMCs were separated and purified with anti-CD14 magnetic beads, then cultured with GM-CSF (100 ng·mL⁻¹) and IL-4 (40 ng·mL⁻¹) for 5 days. On day 6, cells were stimulated by Sal B (507 M), and the toxic effects of Sal B were then quantified by FACS analysis after staining with Annexin V and 7-AAD. The results represented the means $\pm$ SD of three independent experiments.

Figure S3 Purified h-monDC was incubated for 5 days in the presence of GM-CSF and IL-4. On day 6, ox-LDL (507 g·mL⁻¹) was added, and the effect of ox-LDL-induced TLR4 expression was determined by FACS after blocking the blocking the membrane TLR4 on matured h-monDC by purified TLR4-neutralizing antibody. Data were presented as means $\pm$ SD of three independent experiments. **P* < 0.05 versus control; #*P* < 0.05 versus ox-LDL.

Figure S4 Purified h-monDC was incubated for 5 days in the presence of GM-CSF and IL-4. On day 6, ox-LDL (507 g·mL⁻¹) was added, and the effect of ox-LDL-induced h-monDC phenotype was determined by FACS after blocking the membrane TLR4 on matured h-monDC by purified TLR4-neutralizing antibody. Data were presented as means $\pm$ SD of three independent experiments. **P* < 0.05 versus PBS; #*P* < 0.05 versus ox-LDL.

Figure S5 PBMCs derived h-monDC cultured with GM-CSF and IL-4 for 5 days, then stimulated by PBS, Sal B, Ciglitazone and ox-LDL, respectively, for 24 h. Then mRNA was extracted and cDNA was reverse-transcribed. The expression of PPAR γ target genes were analysed by RT-PCR using specific primers as follows: LXR- α , 5'-acggtgatgcttctggagac-3' and 5'-tagcaatgagcaaggcaaact-3' (NM_001130101.1); AP-2, 5'-gtctccgccatccctattaac-3' and 5'-ggaatgtgtcgggtgagaaa-3' (NM_001032280.2); ADRP, 5'-agtggaaaaggagcattggata-3' and 5'-ctgtgtgtacaccttgatgttg-3' (NM_001122.3); PGAR, 5'-acaagcacctagaccatgaggt-3' and 5'-ctgaattactgtccagcctccat-3' (NM_001039667.1); GAPDH, 5'-agaaggctggggtcatttg-3' and

5'-aggggccatccacagtcttc-3' (NM_002046.3). (* P < 0.05 vs. control.)

Figure S6 PBMCs derived h-monDC cultured with GM-CSF and IL-4 for 5 days, then cells were transfected with mock conditions (no siRNA), PPAR γ siRNA or scramble siRNA for 24 h respectively.

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