



Danshensu Promotes Cholesterol Efflux in RAW264.7 Macrophages

Hui Gao^{1,2} · Lingyan Li^{1,2} · Lan Li^{1,2} · Bo Gong^{1,2} · Pengzhi Dong^{1,2} ·
Patrick Asare Fordjour^{1,2} · Yan Zhu^{1,2} · Guanwei Fan^{1,2}

Received: 18 May 2016 / Accepted: 21 July 2016 / Published online: 11 August 2016
© AOCS 2016

Abstract Contemporary research suggests that macrophage foam cell and cholesterol efflux defect play pivotal role in atherogenesis. We reported on the heretofore unknown therapeutic effect of Danshensu (DSS) in reducing intracellular cholesterol level and unraveled the mechanism of DSS promotes cholesterol efflux. Oxidized low-density lipoprotein stimulation of Raw264.7 cells into foam cells, which were treated with DSS and co-treated with Simvastatin and Rosiglitazone. PPAR γ , ABCA1, ABCG1, SR-BI, CD36, and LXR- α mRNA were quantified by Real-Time PCR. Western blotting was used to determine protein expression of PPAR γ , ABCA1 and CD36. Cellular cholesterol handling was studied by measurement of intracellular lipid droplets concentration and cholesterol efflux. DSS significantly reduced scavenger receptor CD36 and its orthologue SR-BI. In addition, DSS stimulated the upregulation of cellular cholesterol exporters ABCA1 and ABCG1 to reduce intracellular lipid accumulation. DSS can reduce lipid deposition in Raw264.7 foam cells by balancing CD36 and ABCA1 protein expression.

Keywords Danshensu (DSS) · Atherosclerosis · Foam cell · Cholesterol efflux

✉ Guanwei Fan
fgw1005@hotmail.com

¹ State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China

² Ministry of Education Key Laboratory of Pharmacology of Traditional Chinese Medical Formulae, Tianjin University of Traditional Chinese Medicine, #312 Anshanxi Road, Nankai District, Tianjin 300193, China

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
CE	Cholesteryl ester
FC	Free cholesterol
HDL	High-density lipoprotein
LXR- α	Liver X receptor alpha
LDL	Low-density lipoprotein
Ox-LDL	Oxidized low-density lipoprotein
PPAR γ	Peroxisome proliferator-activated receptor gamma
RCT	Reverse cholesterol transport
SR-BI	Scavenger receptor class B type I
TC	Total cholesterol

Introduction

Atherosclerosis is a key risk factor for coronary artery disease and is the leading cause of morbidity and mortality in industrialized countries [1, 2]. The atherosclerotic process occurs through infiltration and accumulation of low-density lipoproteins (LDL) in the endothelium. Accumulated endothelial LDL are oxidized and taken up by macrophages through scavenger receptor pathways involving CD36 and SR-BI [3]. This leads to intracellular lipid accumulation and foam cell formation. Thus, foam cell formation is the result of increased macrophage cholesterol levels and impairment of cholesterol efflux. Ox-LDL induced macrophage foam cells are therefore crucial in the development and progression of atherosclerosis.

CD36, a class B scavenger receptor, is a macrophage receptor for Ox-LDL which plays a vital role in the formation of atherosclerotic foam cell. This leads to excess cholesterol buildup and consequently results in vasoconstriction.

The reverse cholesterol transport (RCT) pathway is important for the removal of accumulated lipids from peripheral tissues to the liver for catabolism and excretion. ATP-binding cassette transporter A1 (ABCA1), ABCG1 and scavenger receptor BI (SR-BI) play crucial roles in the reverse cholesterol transport pathway. Studies have shown that ABCA1 promotes cholesterol efflux by interacting with high density lipoprotein (HDL) [4, 5]. The expressions of ABCA1 and ABCG1 are to some extent, regulated by peroxisome proliferator-activated receptor gamma (PPAR γ) activators and liver X receptor alpha (LXR- α). In the present study, we provide evidence to support our contention that DSS can stimulate cholesterol efflux by stimulating the expression of ABCA1 through LXR- α dependent and independent pathways [6]. Hence, DSS amelioration of foam cell formation by attenuating cholesterol influx and improving cholesterol efflux is a worthwhile therapeutic option for the treatment of atherosclerosis.

Salvia miltorrhiza (Danshen), a traditional medicine, is widely used in the management of cerebrovascular and cardiovascular diseases [7]. DSS is the major water-soluble component extracted from *S. miltorrhizae*. Its chemical structure is 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid and could improve microcirculation, suppress the formation of ROS, inhibit platelet adhesion and aggregation, and protect myocardium against ischemia. It has been previously reported that DSS has pharmacological effects such as anti-atherosclerosis, anti-inflammatory, and anti-oxidative damage [8]. However, little is known about the effect of DSS on cholesterol efflux and the potential mechanisms involved in the anti-atherosclerotic effect remain enigmatic.

We report in this study that DSS improvement of cholesterol efflux and attenuation of intracellular lipid accumulation is vital for management of dyslipidemia. Furthermore, we surmise that DSS regulation of intracellular lipid level is due to the reduced uptake of extracellular lipid depots into macrophages by scavenger receptors such as CD36 and SR-BI. In addition, the efflux of intracellular cholesterol from Raw264.7 foam cell by DSS was in part due to the stimulation of ABCA1 and ABCG1. We therefore concluded that DSS prevents Ox-LDL induced Raw264.7 foam cell lipid deposition through PPAR γ /LXR- α /ABCA1 pathway.

Materials and Methods

Reagents

Danshensu was purchased from the Chinese Institute for Drug and Biological Product Control (Beijing, China). Simvastatin, Rosiglitazone and Oil red O were obtained from the Sigma Chemical Co. (St. Louis, MO). Human

Ox-LDL was purchased from Yiyuan biotechnologies (Guangzhou, China). Mycillin and DMEM/high glucose were purchased from Hyclone (USA). DMSO and TriQuick reagent were from Solarbio (Beijing, China). Tissue total cholesterol assay kit, E1015 and tissue free cholesterol assay kit, E1016 were obtained from Applygen Technologies Inc., (Beijing, China). the Foetal bovine serum was purchased from Gibco (USA).

Cell Culture

Raw264.7 cells, a murine macrophage cell line was obtained from the Cell Culture Center of the Chinese Academy of Medical Sciences (Shanghai, China). The cells were cultured in 60-mm petri dishes at a density of 1×10^6 cells/ml in DMEM medium supplemented with 100 IU/ml of penicillin G, 100 μ g/ml streptomycin, 2 mmol/l L-glutamine, and 10 % (vol/vol) FBS, and incubated in a humidified atmosphere of 5 % CO₂ in an incubator at 37 °C. The cells were incubated with 60, 80, 100 μ g/ml Ox-LDL for 48 h. After we successfully build up foam cells, we intervened with 5 μ M Simvastatin and 2 μ M Rosiglitazone for 24 h and intervened with DSS (0.1, 1, 10 μ M) for 24 h.

Oil Red O Staining

Cells were rinsed with PBS and fixed with 4 % formalin for 0.5 h at room temperature. The cells were then rinsed again with 60 % isopropanol for 5–10 min and then incubated with 0.3 % fresh filtered Oil red O solution for 60 min. For analysis, slides were then washed in isopropanol for 10 min, rinsed in tap water, counterstained with hematoxylin and mounted in glycerol/gelatin solution. Images of cells were captured using a light microscope.

Table 1 Primers used in real-time quantitative PCR analysis

Primer	Primer sequence (5'-3')
PPAR γ	Fwd 5'-AGG CCG AGA AGG AGA AGC TGT TG-3' Rev 5'-TGG CCA CCT CTT TGC TCT GCT C-3'
ABCA1	Fwd 5'-AAG CCA AGC ATC TTC AGT TC-3' Rev 5'-CCA TAC AGC AAG AGC AGA AGG-3'
ABCG1	Fwd 5'-ATA CAG GGG AAA GGT CTC CAA T-3' Rev 5'-CCC CCG AGG TCT CTC TTA TAG T-3'
SR-BI	Fwd 5'-GCA AAT TTG GCC TGT TTG TT-3' Rev 5'-GAT CTT GCT GAG TCC GTT CC-3'
LXR α	Fwd 5'-TAG GGA TAG GGT TGG AGT CAG-3' Rev 5'-AGT TTC TTC AAG CGG ATC TGT-3'
CD36	Fwd 5'-TTT CCT CTG ACA TTT GCA GGT CTA-3' Rev 5'-AAA GGC ATT GGC TGG AAG AA-3'
GAPDH	Fwd 5'-TGG TGA AGC AGG CAT CTG AG-3' Rev 5'-TGC TGT TGA AGT CGC AGG AG-3'

Cellular Cholesterol Quantitation Analysis

Cellular cholesterol content was measured using a commercially available quantitation kit (Tissue total cholesterol assay kit, E1015; Tissue free cholesterol assay kit, E1016; Applygen Technologies Inc., Beijing, China) following the manufacturer's instructions.

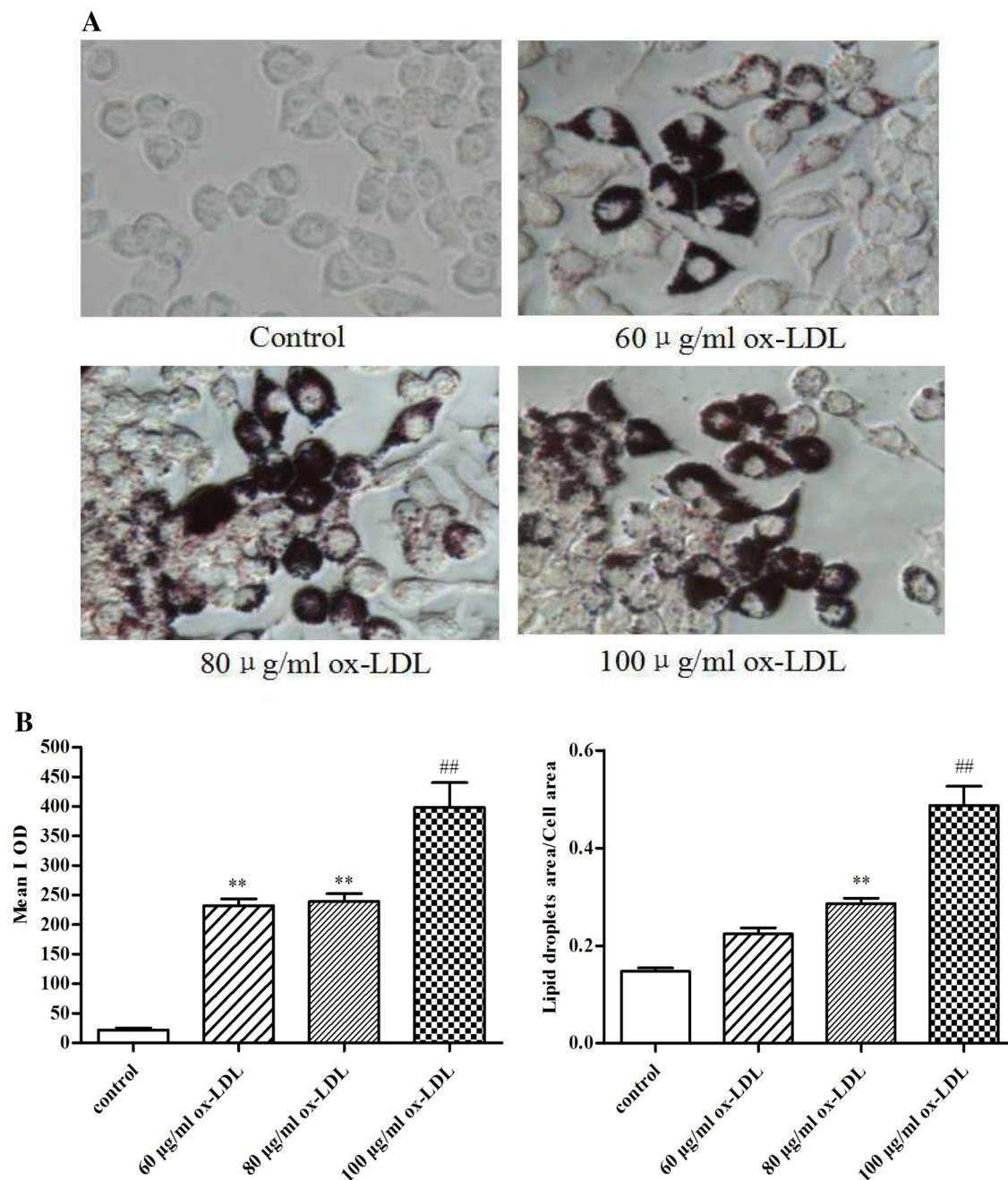


Fig. 1 RAW264.7 cells incubated with different concentrations of Ox-LDL (60, 80, 100 $\mu\text{g/ml}$) transform into foam cells. **a** Oil red O staining of RAW264.7 cells. RAW264.7 cells were incubated with different Ox-LDL concentrations (60, 80, 100 $\mu\text{g/ml}$) for 48 h under staining with ORO ($\times 400$). **b** Different concentrations of Ox-LDL for

Total RNA Extraction, cDNA Synthesis, and RT-PCR Analysis

RAW264.7 cells (1×10^6 cells/well) were incubated with or without the indicated treatment. Total RNA was extracted using trizol, isopropyl alcohol, and chloroform at various treatments. For RT-PCR analyses, the first strand cDNA

the influence of the Mean IOD of intracellular lipid droplets and the area of lipid droplets and cell area ratio. Data represent means \pm SD, ** $P < 0.01$ compared with control, # $P < 0.01$ compared with 60, 80 $\mu\text{g/ml}$ Ox-LDL

synthesis containing 2 mg of total RNA was primed with oligo (dT) and RevertAid first strand cDNA kit under optimized conditions according to the manufacturer's protocol. PCRs were performed using a 2 Taq PCR master mix from Sangon biotech (Shanghai, China) and specific primers for mouse PPAR γ , ABCA1, ABCG1, SR-BI, CD36, LXR- α , and GAPDH, which were designed as Table 1.

PCR was performed on a Real-Time quantitative RT-PCR machine (Real-Time PCR System, Bio-Rad, USA) using the following light cycle conditions: denaturation 95 °C for 3 min, amplification 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, and 72 °C for 7 min for 45 cycles. Melting curve analysis and agarose gel electrophoresis was used to monitor synthesis of the PCR products. The quantitative results for PPAR γ , ABCA1, ABCG1, SR-BI, CD36 and LXR- α , were normalized by the levels of mRNA.

Western Blot Analysis

RAW264.7 cells were plated in 6-well plates at a density of 1×10^6 cells/well in the 6-well plates with 10 % FBS DMEM. The cells were washed twice with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 100 μ l of RIPA lysis buffer with PMSF (1 mM), and subsequently centrifuged at $12,000 \times g$ for 5 min at 4 °C. Supernatants were used as sample proteins. Protein concentrations were determined using the BCA assay. After quantification for the protein concentration of each sample, they were diluted to 2 mg/ml with lysis buffer and 5 \times SDS-PAGE sample loading buffer, boiled at 100 °C for 10 min. Protein samples were applied to 8 % SDS-polyacrylamide gels separately and followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Millipore, MA). Each membrane was incubated for 2 h with 5 % (w/v) skimmed milk in TBST buffer to block nonspecific binding at room temperature, followed by incubation with anti-PPAR γ , anti-ABCA1, anti-CD36, anti-GAPDH primary antibodies overnight at 4 °C. The membranes were then rinsed five times with TBST buffer for 3 min. Washed membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies for 2 h, with continuous shaking. The immuno-reactive proteins

were visualized by chemiluminescence using an ECL plus kit with exposure to X-ray film. Immuno-reactive bands were quantified by using ImageJ analysis software. The data represent the protein variation after treatment of the factors.

Statistical Analysis

Data are expressed as means \pm SD. Significance of differences from control values and comparisons between groups were calculated by one-way ANOVA. Differences were considered to be statistically significant for $P < 0.05$.

Results

Induction of Foam Cell Formation in Cultured RAW264.7 Cells

It is known that macrophages, incubated with Ox-LDL transform into foam cells. RAW264.7 cells were incubated with different concentrations of Ox-LDL (60, 80, 100 μ g/ml) for 48 h under staining with ORO. As shown in Fig. 1a, only 100 μ g/ml Ox-LDL exhibits significant lipid droplets accumulation (indicated by black arrows). We also measured the intracellular lipid droplets of the mean optical density value (Mean IOD) by Image-Pro Plus 6.0 software, to a certain extent, this reflected the degree of cellular foam. The 100 μ g/ml of Ox-LDL not only increased the Mean IOD of intracellular lipid droplets but also increased the ratio of the lipid droplets area to the cell area (Fig. 1b).

The contents of total cholesterol (TC) and free cholesterol (FC) of Raw264.7 in normal cultured cells and foam cells were measured with 60 and 100 μ g/ml after incubation with Ox-LDL for 48 h and calculated ratio of cholesterol ester (CE) to TC. These data show that TC, FC, CE (CE = TC-FC) and CE/TC levels were significantly increased in foam cells incubated with Ox-LDL (100 μ g/ml) for 48 h compared with untreated cells (Table 2). The CE/TC level of untreated cells and 60 μ g/ml Ox-LDL incubated cells less than 50 %, but 100 μ g/ml Ox-LDL incubated cells more than 50 %.

Table 2 Cholesterol content in normal cells and foam cells

Group	FC (μ g/mg protein)	CE (μ g/mg protein)	TC (μ g/mg protein)	CE/TC (%)
Control	36.0 ± 1.1	9.9 ± 0.8	45.9 ± 0.3	21.7
60 μ g/ml ox-LDL	$53.3 \pm 0.8^{**}$	$29.9 \pm 3.0^{**}$	$83.3 \pm 2.4^{**}$	35.9^{**}
100 μ g/ml ox-LDL	$45.0 \pm 0.3^{**}$	$51.5 \pm 2.4^{##}$	$96.5 \pm 2.1^{**}$	$53.3^{##}$

Data are presented as the means \pm SD

** $P < 0.01$ versus control, ## $P < 0.01$ versus 60 μ g/ml Ox-LDL

Different Concentrations of DSS on the Influence of the Content of Lipid Droplets Raw264.7 Cells

Ox-LDL (100 µg/ml) was used to induce Raw264.7 cells for 48 h. Raw264.7 foam cells were then treated with DSS

for 24 h. Oil red O staining results demonstrate that 0.1, 1, 10 µM DSS can significantly reduce lipid droplets (Fig. 2a). DSS (0.1, 1, 10 µM) not only significantly decreased the Mean IOD of intracellular lipid droplets but also decreased the area of lipid droplets and cell area ratio (Fig. 2b). At the

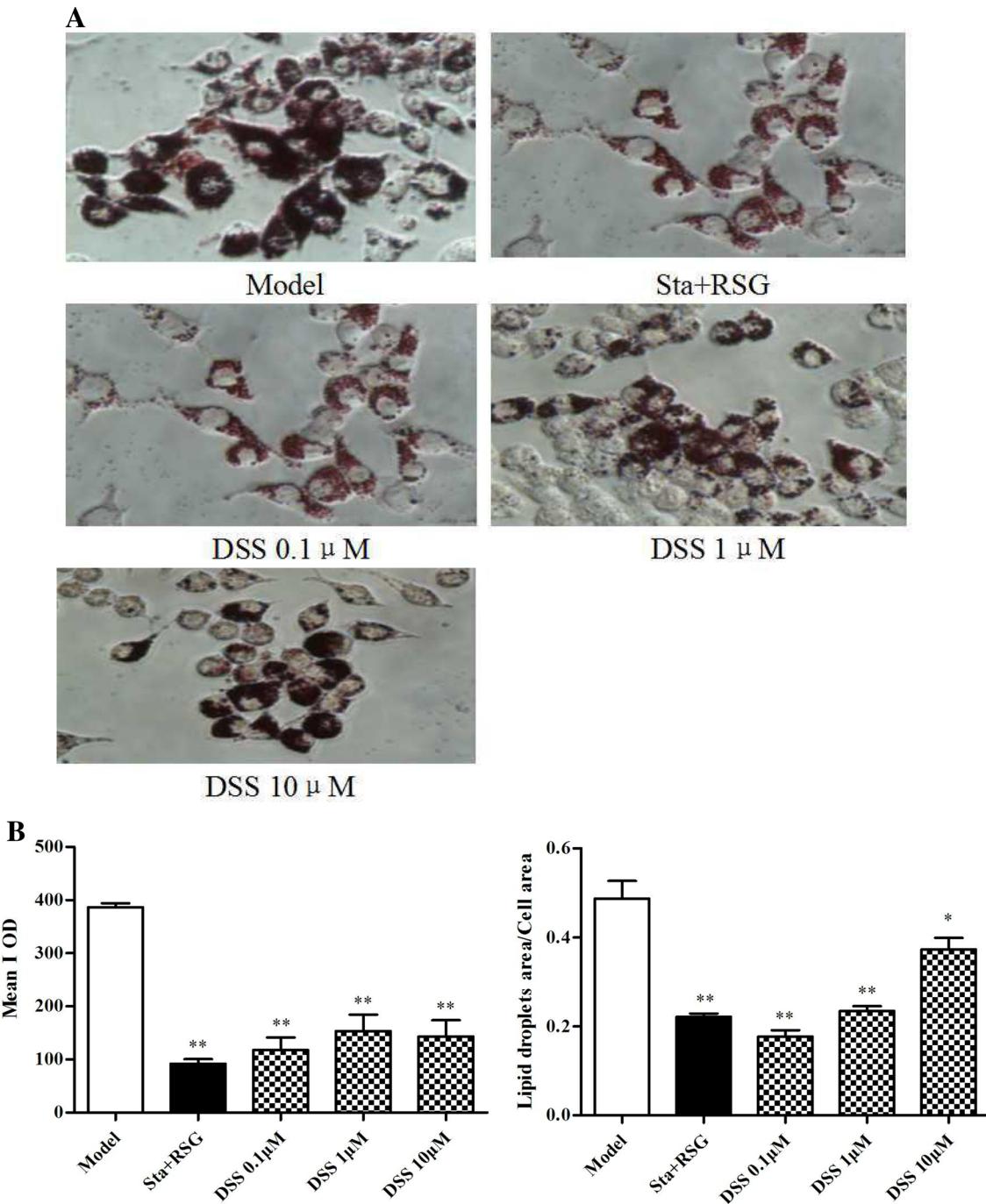


Fig. 2 DSS effect on intracellular lipid droplets. **a** Oil red O staining of RAW264.7 cells incubated with DSS. RAW264.7 cells were incubated with 100 µg/ml Ox-LDL for 48 h under staining with ORO

(×400). **b** DSS effect on the Mean IOD of intracellular lipid droplets and the area of lipid droplets and cell area ratio. Data represent means ± SD, * $P < 0.05$, ** $P < 0.01$ compared with the model

Table 3 Content of cholesterol in foam cells under different concentrations of DSS

Group	FC ($\mu\text{g}/\text{mg}$ protein)	CE ($\mu\text{g}/\text{mg}$ protein)	TC ($\mu\text{g}/\text{mg}$ protein)	CE/TC (%)
Model	45.0 \pm 0.3	51.5 \pm 2.4	96.5 \pm 2.1	53.3
Sta + RSG	48.2 \pm 2.4*	13.8 \pm 3.6**	61.9 \pm 1.3**	22.1**
DSS0.1 μM	50.0 \pm 1.4**	9.3 \pm 1.3**	59.3 \pm 1.0**	15.7**
DSS1 μM	42.0 \pm 0.2	16.0 \pm 0.3**	58.0 \pm 0.4**	27.6**
DSS10 μM	52.7 \pm 0.8**	28.6 \pm 3.8**	81.4 \pm 4.1**	35.1**

Data are presented as means \pm SD

* $P < 0.05$, ** $P < 0.01$ versus model

same time, Treatment with DSS (0.1, 1, 10 μM) and Co-treatment with 5 μM Simvastatin and 2 μM Rosiglitazone significantly reduced the level of CE/TC (Table 3).

Different Concentrations of DSS Alter mRNA Levels of Cholesterol Efflux/Influx-Modulating Related Genes: PPAR γ , ABCA1, ABCG1, SR-BI, LXR- α and CD36 in Raw264.7 Foam Cells

Cholesterol flux in macrophages is tightly regulated by several genes. ABCA1, ABCG1, LXR- α and PPAR γ are key regulators of cholesterol efflux, while CD36, SR-BI are critical scavenger receptors involved in cholesterol uptake. Treatment with DSS (0.1, 1, 10 μM), DSS (10 μM) significantly increased the mRNA level of PPAR γ compared with model (Fig. 3a). DSS (0.1, 1, 10 μM) significantly increased the mRNA level of ABCA1, among DSS (0.1 μM) most significantly increased the mRNA level of ABCA1 (Fig. 3c). DSS (10 μM) increased the mRNA level of ABCG1 and LXR- α (Fig. 3b, d), but did not significantly increase the mRNA level of ABCA1 and SR-BI.

Effect of Different Concentrations of DSS on the Expression of PPAR γ , ABCA1 and CD36 Protein in Raw264.7 Foam Cells

To study the possible mechanisms that are responsible for DSS action, we analyzed the expression of several key molecules involved in cholesterol handling, including CD36, SR-BI, PPAR γ , LXR- α , and ABCA1 in foam cells. 100 $\mu\text{g}/\text{ml}$ Ox-LDL induced Raw264.7 cells for 48 h. Cells were treated with either DSS (0.1, 1, 10 μM) or co-treated with Simvastatin (5 μM) and Rosiglitazone (2 μM) for 24 h. 0.1, 1 μM DSS significantly increased the expression of PPAR γ and ABCA1 compared with the model group (Fig. 4b, c). Conversely, 0.1, 10 μM DSS displayed significantly reduced expression of CD36 (Fig. 4d). These results suggest that DSS may stimulate cholesterol efflux by upregulating the expression of PPAR γ , ABCA1 and reducing the expression of CD36 and SR-BI.

Discussion

Recent reports about atherogenesis suggest that foam cell accumulation and the cholesterol efflux defect lead to progression of atherosclerotic plaque and vasoconstriction. Therefore, the removal of foam cells present in the atherosclerotic lesion through cholesterol efflux has been suggested to play a vital role in atheroprotection. Herein, we provided novel evidence to show that DSS regulates macrophage cholesterol handling and deciphered the potential mechanism through which DSS may improve cholesterol efflux. Notably, excess cholesterol in foam cells can be removed through multiple pathways involving PPAR γ , ABCA1, ABCG1, SR-BI, CD36 and LXR- α .

Our results show that Ox-LDL (100 $\mu\text{g}/\text{ml}$) induced (48 h) [9] the formation of foam cells in RAW264.7 macrophages significantly increased the proportion of cholestryler ester (CE/TC) more than 60, 80 $\mu\text{g}/\text{ml}$ ox-LDL. Duanfang Liao, Chaoke Tang study show that [10] only the proportion of cholestryler ester (CE/TC) over 50 %, we think as they entirely became foam cells. If not, we called it lipid-loaded cells. According to the previous study, the concentration of Ox-LDL was mostly concentrated to 50–100 $\mu\text{g}/\text{ml}$ [11, 12]. In our experiment, we used 60, 80, 100 $\mu\text{g}/\text{ml}$ of Ox-LDL to induce RAW264.7 cells for 48 h, then the cholesterol ester proportion increases with the increase in the concentration of Ox-LDL, but only when the Ox-LDL concentration reached 100 $\mu\text{g}/\text{ml}$, the proportion of cholestryler ester (CE/TC) more than 50 %. On the other hand, our Ox-LDL migrates 2.0-fold further than the native LDL, the low degree of oxidation of Ox-LDL. The degree of oxidation of Ox-LDL was higher, the degree of foam better. Finally, we chose 48 h as the elicited time.

We observed increased foam cell formation and a cholesterol efflux defect caused by Ox-LDL. Furthermore, Ox-LDL inhibited mRNA expression of ABCA1, ABCG1, PPAR γ and LXR- α and increased the expression of both CD36 and its orthologue SR-BI. The relative contributions of these genes to cholesterol efflux have been extensively determined [13]. CD36 and ABCA1 have crucial but

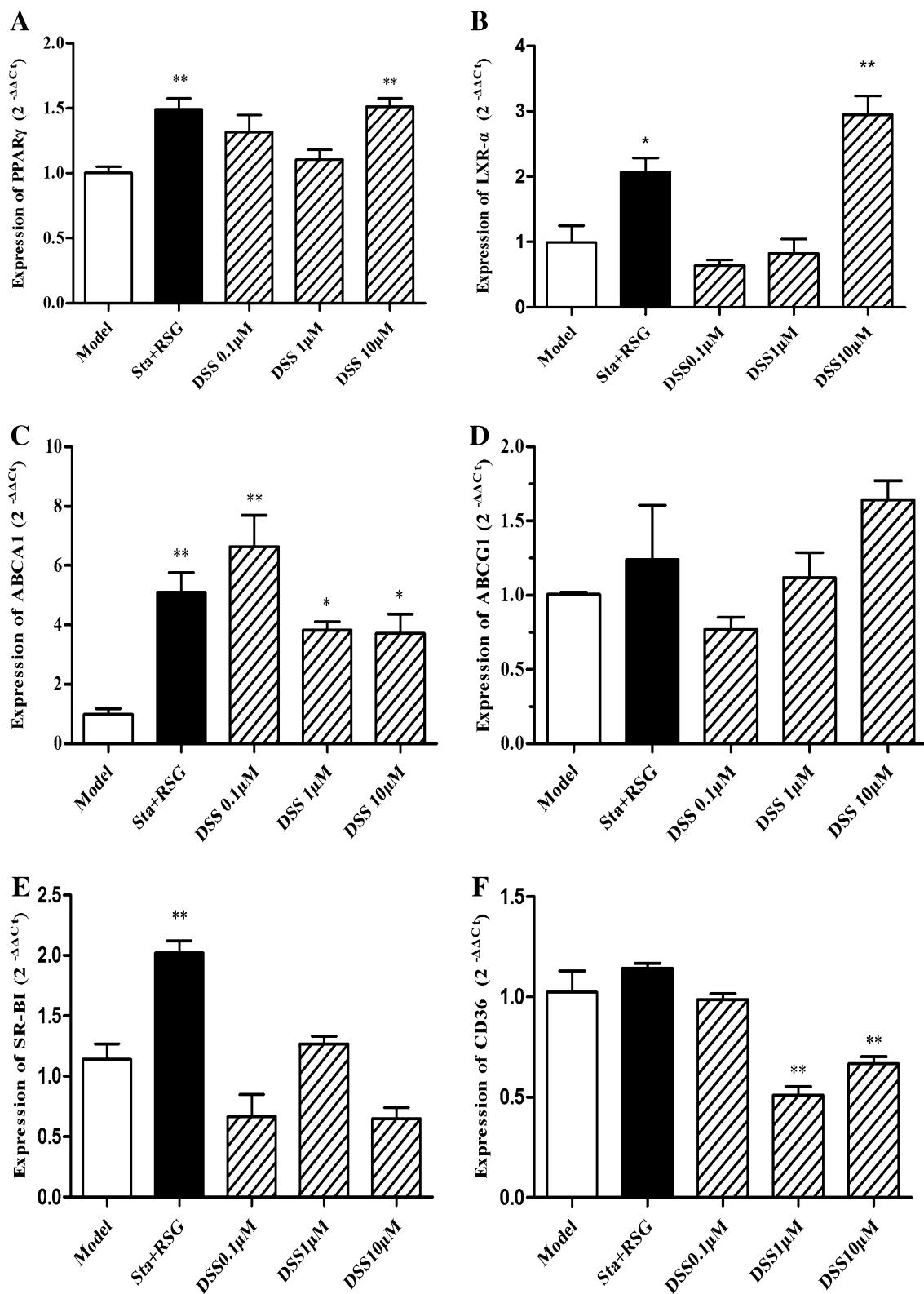


Fig. 3 Effect of different concentrations of DSS on the mRNA expression levels of PPAR γ , ABCA1, ABCG1, SR-BI, LXR- α and CD36 in Raw264.7 foam cells. PPAR γ , ABCA1, ABCG1, SR-BI,

LXR- α and CD36 mRNA levels were detected by RT-PCR. Bars represent means \pm SD; * $P < 0.05$, ** $P < 0.01$ versus the model group

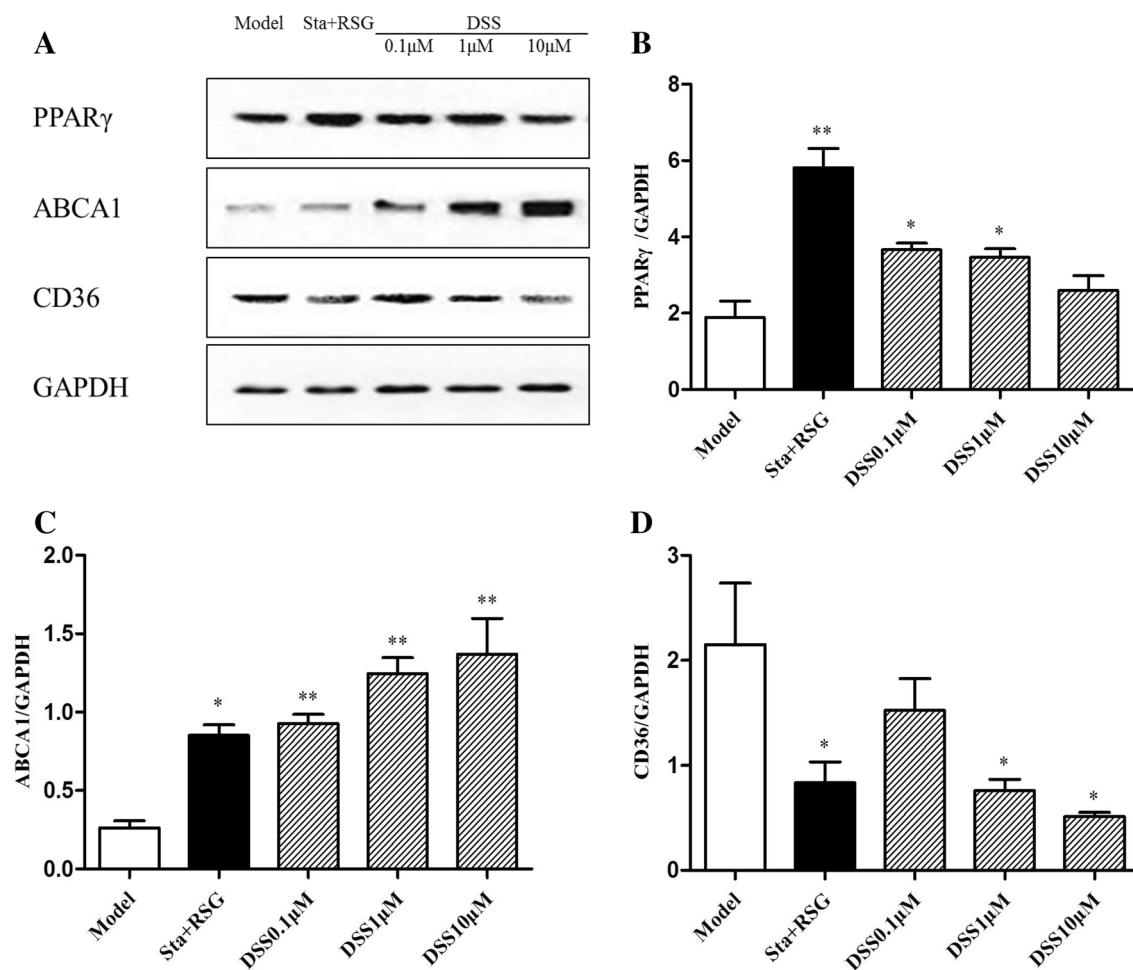


Fig. 4 **a** Effect of different concentrations of DSS on the expression of PPAR γ , ABCA1 and CD36 protein in Raw264.7 foam cells. **b–d** PPAR γ , ABCA1 and CD36 protein levels were assessed by Western

blot analysis. Bars represent mean \pm SD, * P < 0.05, ** P < 0.01 versus the model group

differential effects on macrophage lipid accumulation: an increased expression level of CD36 increases intracellular lipid content while upregulation of ABCA1 decreases cholesterol level [14]. We initially observed that Ox-LDL increases lipid accumulation associated with the elevated levels of CD36 proteins. The increase in CD36 was caused by the Ox-LDL induced impairment of cholesterol efflux and lipid build up in Raw264.7 foam cells. This suggests that Ox-LDL stimulates and binds to CD36 to promote foam cell formation by providing an energy source for lipid storage [15]. Thus, the surrogate inhibition of CD36 mediated uptake of extracellular lipid depots may be considered as a treatment strategy to offset the impaired functioning of lipid-loaded macrophages.

Consistent with our hypothesis, *in vitro* treatment with DSS reduced the lipid level by decreasing macrophage CD36 and SR-BI mRNA expression which is a reflection of improved cholesterol efflux [16, 17]. In addition, DSS

increased ABCA1 proteins which interact with HDL to promote cholesterol efflux from macrophage foam cell. Hence, we infer from previous studies that DSS induction of ABCA1 confers anti-atherogenic action by contributing to the homeostasis of net cholesterol balance which ultimately reduces atherosclerotic plaque and infiltration of inflammatory mediators [18, 19].

In this study, it is evident that DSS confers salutary effects and decreases macrophage foam cell lipid accumulation in a manner similar to Simvastatin (5 μ M) + Rosiglitazone (2 μ M) [20] by activating the PPAR γ -LXR α -ABCA1 pathway. However, Simvastatin + Rosiglitazone paradoxically increased mRNA and protein expression of SR-BI with no significant change in Raw264.7 foam cells expression of CD36. It is noteworthy that SR-BI on one hand promotes free cholesterol efflux to HDL, and on the other hand counteracts this process by increasing the selective HDL cholesteryl

ester uptake into cells to stimulate net cholesterol efflux [21–24]. However, Ox-LDL stimulated SR-BI to promote selective cholesterol ester uptake from mature HDL and subsequently leads to reduced HDL and lipid accumulation [22, 25–28]. Moreover, expression of SR-BI associates with ABCG1 and attenuates the ABCG1-mediated net cholesterol efflux to HDL [29]. Thus, the finding that DSS inhibits SR-BI mRNA expression and stimulates the release of ABCG1 to reduce lipid accumulation in cholesterol-loaded Raw264.7 foam cells is therapeutic.

In summary, DSS increased PPAR γ , LXR- α and ABCA1 mRNA expression to regulate lipid accumulation. Activation of PPAR γ with DSS and Simvastatin + Rosiglitazone enhanced LXR- α , ABCG1 and ABCA1 mediated cholesterol efflux from lipid-loaded cells even in the presence of Ox-LDL. Additionally, DSS inhibition of CD36 and SR-BI expression levels resulted in net reduction of intracellular lipid levels. This reveals the anti-atherogenic mechanism of DSS in regulating lipoprotein and improving cholesterol efflux. These results suggest that DSS could restore the dysregulated lipid level caused by an imbalance between lipoproteins following Ox-LDL treatment. Further studies to elucidate the contribution and therapeutic significance of SR-BI in the pathogenesis of cardiovascular disease is imperative to decipher the intricacies surrounding its regulation.

Acknowledgments This work was support by a grant from the National Key Basic Research Program of China (973 Program) (No. 2014CB542902), the National Natural Science Foundation of China (81273891), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT1276).

References

- Wong ND (2014) Epidemiological studies of CHD and the evolution of preventive cardiology. *Nat Rev Cardiol* 11:276–289
- Fordjour PA, Wang Y, Shi Y, Agyemang K, Akinyi M, Zhang Q, Fan GW (2015) Possible mechanisms of C-reactive protein mediated acute myocardial infarction. *Eur J Pharmacol* 760:72–80
- Padres EA, Febbraio M, Sheibani N, Schmitt D, Silverstein RL, Hajjar DP, Cohen PA, Frazier WA, Hoff HF, Hazen SL (2000) Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest* 105:1095–1108
- Rothblat GH, Phillips MC (2010) High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr Opin Lipidol* 21:229–238
- Sorci-Thomas MG, Thomas MJ (2012) High density lipoprotein biogenesis, cholesterol efflux, and immune cell function. *Arterioscler Thromb Vasc Biol* 32:2561–2565
- Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherosclerosis. *Mol Cell* 7:161–171
- Yu C, Qi D, Lian W, Li QZ, Li HJ, Fan HY (2014) Effects of danshen on platelet aggregation and thrombosis: *in vivo* arteriovenous shunt and venous thrombosis models in rats. *PLoS One* 9:e110124
- Lin TH, Hsieh CL (2010) Pharmacological effects of *Salvia miltiorrhiza* (Danshen) on cerebral infarction. *Chin Med* 5:17–22
- Rubic T, Lorenz RL (2006) Downregulated CD36 and oxLDL uptake and stimulated ABCA1/G1 and cholesterol efflux as anti-atherosclerotic mechanisms of interleukin-10. *Cardiovasc Res* 69:527–535
- Liao DF, Tang CK (2009) Basic and clinical reverse cholesterol transport. *Science Press, Beijing*
- Wu C, Luan H, Zhang X, Wang S, Zhang X, Sun X, Guo P (2014) Chlorogenic acid protects against atherosclerosis in ApoE2/2Mice and promotes cholesterol efflux from RAW264.7 macrophages. *PLoS One* 9:e95452
- Liu QN, Dai ZB, Liu ZQ, Liu XH, Tang CK, Wang Z, Yi GH, Liu LS, Jiang ZS, Yang YZ, Yuan ZH (2010) Oxidized low-density lipoprotein activates adipophilin through ERK1/2 signal pathway in RAW264.7 cells. *Acta Biochim Biophys Sin* 42:635–645
- Rigotti A, Acton SL, Krieger M (1995) The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem* 270:16221–16224
- Isoviita PM, Nuotio K, Sakki J, Turunen R, Ijas P, Pitkaniemi J, Soinne L, Kaste M, Kovanen P, Lindsberg P (2010) An imbalance between CD36 and ABCA1 protein expression favors lipid accumulation in stroke-prone ulcerated carotid plaques. *Stroke J Cereb Circ* 41:389–393
- Park YM (2014) CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med* 46:e99
- Angin Y, Steinbusch LK, Simons PJ, Greulich S, Hoebers NT, Douma K, Zandvoort M, Coumans WA, Wino W, Michaela D, Ouwen M, Glatz J, Luiken J (2012) CD36 inhibition prevents lipid accumulation and contractile dysfunction in rat cardiomyocytes. *Biochem J* 448:43–53
- Lee TS, Lin CY, Tsai JY, Wu YL, Su KH, Lu KY, Hsiao SH, Pan CC, Kou YR, Hsu YP, Ho LT (2009) Resistin increases lipid accumulation by affecting class A scavenger receptor, CD36 and ATP-binding cassette transporter-A1 in macrophages. *Life Sci* 84:97–104
- Yvan-Charvet L, Wang N, Tall AR (2010) Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. *Arterioscler Thromb Vasc Biol* 30:139–143
- Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Alan RT, Danie R (2007) Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *J Clin Invest* 117:2216–2224
- Llaverias G, Lacasa D, Vinals M, Vazque-Carrera M, Sanchez R, Laguna JC, Alegret M (2004) Reduction of intracellular cholesterol accumulation in THP-1 macrophages by a combination of rosiglitazone and atorvastatin. *Biochem Pharmacol* 68:155–163
- Annema W, von Eckardstein A (2013) High-density lipoproteins—Multifunctional but vulnerable protections from atherosclerosis. *Circ J* 77:2432–2448
- Rinniger F, Brundert M, Brosch I, Donarski N, Budzinski RM, Greten H (2001) Lipoprotein lipase mediates an increase in selective uptake of HDL-associated cholesterol esters by cells in culture independent of scavenger receptor BI. *J Lipid Res* 42:1740–1751
- Rader DJ (2006) Molecular regulation of HDL metabolism and function: implications for novel therapies. *J Clin Invest* 116:3090–3100
- Trigatti BL, Krieger M, Rigotti A (2003) Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* 23:1732–1738

25. Barlic J, Zhu W, Murphy PM (2009) Atherogenic Lipids Induce HDL Uptake and Cholesterol Efflux in Human Macrophages by Upregulating Transmembrane Chemokine CXCL16, without Engaging CXCL16-dependent Cell Adhesion. *J immunol* 182:7928–7936
26. Graf GA, Connell PM, van der Westhuyzen DR, Smart EJ (1999) The class B, type I scavenger receptor promotes the selective uptake of high density lipoprotein cholesterol ethers into caveolae. *J Biol Chem* 274:12043–12048
27. Pagler TA, Rhode S, Neuhofer A, Laggner H, Strobl W, Hinterdorfer C, Volf I, Pavelka M, Eckhardt ERM, Westhuyzen DR, Schutz GJ, Stangl H (2006) SR-BI-mediated high density lipoprotein (HDL) endocytosis leads to HDL resecretion facilitating cholesterol efflux. *J Biol Chem* 281:11193–11204
28. Luthi AJ, Lyssenko NN, Quach D, McMahon KM, Millar JS, Vickers KC, Rader DJ, Phillips MC, Mirkin CA, Thaxton CS (2015) Robust passive and active efflux of cellular cholesterol to a designer functional mimic of high density lipoprotein. *J Lipid Res* 56:972–985
29. Song G, Zong C, Liu Q, Si Y, Liu J, Li W, Zhu P, Qin S (2012) SR-BI associates with ABCG1 and inhibits ABCG1-mediated cholesterol efflux from cells to high-density lipoprotein 3. *Lipids Health Dis* 11:111–118