Simivastatin inhibited oxLDL-induced proatherogenic effects through calpain-1/PPARγ/CD36 pathway
Simivastatin inhibited oxLDL-induced proatherogenic effects through calpain-1/PPARγ/CD36 pathway

Xueyan Yang *ab, Meihui Yin *a, Lan Yu ac, Meili Lu a, Hongxin Wang a, Futian Tang a, Yingjie Zhang b

a Key Laboratory of Cardiovascular and Cerebrovascular Drug Research of Liaoning Province, Liaoning Medical University, Jinzhou 121001, China
b Internal Medicine-Cardiovascular Departments, the First Affiliated Hospital of Liaoning Medical University, Jinzhou 121001, China
c Central Hospital of Yingkou Development Areas, Yingkou, 115007, China

Corresponding authors: Futian Tang (email: tangft@163.com) and Yingjie Zhang (email: zhangyingjiejinzhou@126.com)

*These authors contributed equally to this work.
Abstract

We previously reported that simvastatin, an inhibitor of HMG-CoA reductase, inhibits atherosclerosis in rats. The present study was designed to investigate the effect of simvastatin on mouse peritoneal macrophage foam cell formation, the early feature of atherosclerosis and explore its mechanisms. The results showed that simvastatin decreased cholesterol content and Dil-oxLDL uptake, reduced the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the medium, down-regulated the mRNA and protein expression of CD36 and reduced the mRNA expressions of peroxisome proliferator-activated receptor gamma (PPARγ), TNF-α and IL-6 in macrophages treated with oxidized low density lipoprotein (oxLDL). However, PPARγ agonist troglitazone partly abolished the effects of simvastatin on foam cells. In addition, simvastatin reduced the protein expression of calpain-1, Ca²⁺-sensitive cysteine protease, in oxLDL treated macrophages. Furthermore, PD150606, specific calpain inhibitor, reduced mRNA expressions of PPARγ and CD36 in macrophages treated with oxLDL. Combination of simvastatin and PD150606 had no further effects on mRNA expression of PPARγ and CD36 compared with either alone. However, over-expression of calpain-1 in macrophages partly reversed the simvastatin effects including cell cholesterol content, mRNA expressions of PPARγ and CD36. The results suggested that simvastatin inhibits foam cell formation of oxLDL treated macrophages through calpain-1/PPARγ/CD36 pathway.

Key Words Simvastatin; foam cells; CD36; calpain inhibitor; PPARγ
Introduction

Atherosclerotic cardiovascular disease (CVD) is the prime cause of mortality in developed countries, and its incidence in developing countries has been increasing recently (Zelicoff 2015). Oxidative stress has been involved in the development of atherosclerosis (Villa-Belostoa et al. 2013). Macrophage foam cells filled with oxidized lipids mainly derived from oxidized low density lipoprotein (oxLDL) are the characteristics of early atherosclerotic lesion (Barzilay et al. 2015). OxLDL is taken up by macrophages via the scavenger receptors including CD36, SR-A and LOX-1 (Ogura et al. 2016). Mice lacking CD36 do not accumulate esterified cholesterol derived from oxLDL (Kunjathoor et al. 2002) and develop fewer atherosclerotic lesions. Thus, the development of drugs that down-regulate CD36 expression could possibly provide a strong basis for a novel anti-atherosclerotic therapy. We previously reported that Tanshinone IIA extracted from Salvia miltiorrhiza Bunge (Danshen) and vitamin E inhibit the atherosclerosis in apolipoprotein E knockout (ApoE KO) mice through down-regulation of CD36 (Villa-Belostoa et al. 2013).

It has been reported that atherosclerosis is predominantly an inflammatory process (Gu et al. 2011). Various studies have highlighted that pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are the upstream inflammatory cytokines that play important role in propagating the downstream inflammatory response responsible for atherosclerosis (Koga et al. 2004). Considering the importance of TNF-α and IL-6 in the development of coronary artery disease, targeting their actions could prove to be beneficial.

Peroxisome proliferator-activated receptor gamma (PPARγ) is reportedly implicated in the regulation of CD36 by oxLDL (Nicholson 2004; Rutsch et al. 2011). PPARγ is a member of a nuclear hormone superfamily that heterodimerizes with the retinoid X receptor (RXR) (Tontonoz et al. 1995). We previously reported that PPARγ agonists 15-deoxy-Delta12,14-prostaglandin J2 (15d-PGJ2) and troglitazone dose-dependently abolished the down-regulation of CD36 expression by Tanshinone IIA in oxLDL treated macrophages, implying the significance of PPARγ in the
regulation of CD36 expression (Villa-Bellosta et al. 2011).

Calpain, a calcium-dependent cysteine protease, has been shown to be involved in the pathogenesis of atherosclerosis (Baldi et al. 2013; Li et al. 1991). Inhibition of calpain attenuates atherosclerosis in low density lipoprotein (LDL) receptor deficient mice (Devaraj et al. 2007). Calpain-1 and -2, the two major isoforms of the calpain family, are ubiquitously expressed (Shanahan et al. 2011). Calpains are involved in acute inflammatory processes via the activation of nuclear factor kappa B (NF-kB) (O'Neill et al. 2011). Specifically, the protein expression of calpain-1 instead of calpain-2 has been shown to be positively correlated with the contents of TNF-α and IL-6 and the mRNA expression of PPARγ in macrophages (Villa-Bellosta and Sorribas 2011), implying the potential regulatory role of calpain-1 in inflammation and PPARγ mRNA expression.

We and others reported that simvastatin, an inhibitor of HMG-CoA reductase, inhibits atherosclerosis in rabbits, rats and mice (Song et al. 2011; Tang et al. 2006). However, the precise mechanism of the inhibition remains to be investigated. The study demonstrated that simvastatin down-regulates the expression of CD36 at the mRNA and protein levels (Devaraj et al. 2001), suggesting a potential novel target for simvastatin in the prevention of atherosclerosis. The present study was designed to investigate the effects of simvastatin on foam cell formation and to further explore the mechanisms with focus on the calpain-1/PPARγ/CD36 pathway. The results showed that simvastatin inhibits the formation of foam cells by down-regulating mRNA and protein levels of CD36, which might be attributed to the calpain-1 inhibition-mediated antagonism of PPARγ.

**Materials and methods**

**Chemicals and reagents**

Simvastatin was purchased from Merck Co. (Hangzhou, China). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were produced by
Hyclone Company (Beijing, China). Antibodies against CD36 and calpain-1 were obtained from Cell Signaling Company (Danvers, MA, USA), and antibody against GAPDH and PD150606 was from Santa Cruz Biotechnology (Shanghai) Co., Ltd. (Shanghai, China). Other chemicals and reagents were from Sigma Company (Shanghai, China).

Cell culture and adenoviral infection

All authors assured that the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and that the use of animals was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Liaoning Medical University, China (Permit Number: LMU-2014-118). The isolation and culture of macrophages from mouse peritoneal cavities was carried out as reported previously (Tang et al. 2011). Macrophages were infected with adenoviral vectors containing mouse calpain-1 gene (Ad-Cal, provided by Professor Jinshen Huang in Liaoning Medical University) or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection of 10 PFU/cell. Adenovirus mediated gene transfer was implemented as previously described (Chen et al. 2014). Over-expression of calpain-1 in macrophages was confirmed by Western blot. All experiments were performed after 24 hours of adenoviral infection.

Preparation of oxLDL

Human plasma was purchased from the Blood Perfusion Center of the First Affiliated Hospital of Liaoning Medical University after approval of the Human Investigation Committee of the Liaoning Medical University, China (Permit Number: LMU-2013-138). The method for oxidation of LDL was described previously (Tang et al. 2011). Briefly, LDL was separated from human plasma by sequential flotation in NaBr solution containing 1 mg/ml EDTA. Cu$^{2+}$-modified LDL (1.0 mg of protein/ml) was prepared by exposure of LDL to 5 mM CuSO4 for 18 h at 37 °C. The extent of LDL oxidation was determined by thiobarbituric acid reactive substances (TBARS).
and the value of TBARS of oxLDL used in our experiment was 8.8 vs 0.7 nmol/100 mg protein in the native-LDL preparation.

**Preparation of Dil-oxLDL**

The method for preparation of Dil-LDL was described previously (Tang et al. 2011).

**Cholesterol levels in macrophages**

Macrophages were pre-incubated with vehicle or simvastatin (10 µM) in the presence or absence of troglitazone (50µM) for 24 hours, and then incubated with oxLDL (50 µg/ml) for another 24 hours. The cholesterol mass was quantified by enzymatic fluorometric micro-assay as described previously (Tang et al. 2011). Fluorescence was measured with a FLUO star OPTIMA (BMG LABTECH, Germany) (excitation, 320 nm; emission, 410 nm). Cholesterol content in macrophages was expressed as µg/(mg cell protein).

**Dil-oxLDL uptake by macrophages**

Macrophages were pre-incubated with vehicle or simvastatin (10 µM ) in the presence or absence of troglitazone (50µM) for 24 hours and then incubated with Dil-oxLDL (50 µg/mL) for another 24 hours. The cells were washed and then investigated by fluorescence microscopy as described previously (Tang et al. 2011).

**Contents of TNF-α and IL-6 in medium of macrophage**

Medium of macrophages were used for measuring the contents of TNF-α and IL-6 by Enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s protocol.

**mRNA expressions of TNF-α, IL-6, CD36, SR-A, LOX-1 and PPARγ**

The mRNA expression levels were analyzed by quantitative real time RT-PCR using the BioRad iQ5 Real Time PCR system (BioRad Company) as described
previously (Tang et al. 2011). The sequences of the primers (Invitrogen Biotechnology, Shanghai, China) used are: 1) CD36: forward, 5’-GAT GGC CTT ACT TGG GAT TGG A-3’; reverse, 5’-GGC TTT ACC AAA GAT GTA GCC AGT G-3’. 2) PPAR-γ: forward, 5’-GAC CTG AAG CTC CAA GAA TAC CA-3’; reverse, 5’-GAG CTG GGT CTT TTC AGA ATA AGG-3’. 3) TNF-α: forward, 5’-GTG GCC GGC TGC ACT GAA CT-3’; reverse, 5’-ATT GTG AGC GCT GGC CGA CGA GG-3’. 4) IL-6: forward, 5’-GAA CAA CGA TGA TGC TGC ACT TGC-3’; reverse, 5’-CTT CAT GTA CTC CAG GTA GTC ATG GT-3’. 5) GAPDH: forward, 5’-ATG TTT GTG ATG GGT GTG AAC CAI3’; reverse, 5’-TAG CCA TAT TCA TTG TCA TAC CAG G-3’. 6) SR-A: forward, 5’-CCT TGA TTT CGT CAG TCC AGG AAC-3’; reverse, 5’-GTT GCT TTG CTG TAG ATT CAC GG-3’. 7) LOX-1: forward, 5’-TTA CTC TCC ATG GTG GTG CC-3’; reverse, 5’-AGC TTC TTC TGC TTG TG-3’.

**Protein expression of CD36 and calpain-1**

Protein expression of CD36 and calpain-1 in macrophages was determined by Western blot as described previously (Tang et al. 2011).

**Statistical analysis**

Data are presented as the mean ±SEM and analyzed by one-way ANOVA and the Student-Newman-Keuls test using SPSS 16.0 software. A value of $P<0.05$ was considered statistically significant.

**Results**

**Simvastatin reduces cholesterol content and Dil-oxLDL uptake in macrophages**

Macrophages uptaking oxLDL become foam cells. The present study showed that the cholesterol content in oxLDL-treated macrophages increased significantly compared with that in macrophages treated with vehicle. However, the increase in
cholesterol content was significantly inhibited by simvastatin (Fig. 1A). A Dil-oxLDL uptake experiment was conducted to further confirm the inhibition of foam cell formation. As expected, simvastatin decreased the Dil-oxLDL uptake induced by oxLDL (Figs. 2B and 2C). In addition, troglitazone, an agonist of PPARγ partly abolished the effects of simvastatin on cholesterol content and Dil-oxLDL uptake, suggesting the involvement of PPARγ antagonism in the inhibition of foam cell formation by simvastatin.

**Simvastatin down-regulated the expression of CD36 in macrophages**

The uptake of oxLDL by macrophages is mainly mediated through the scavenger receptors including CD36, SR-A and LOX-1. The results showed that mRNA and protein expression of CD36, mRNA expression of SR-A and LOX-1 in oxLDL-treated macrophages is up-regulated significantly compared with that in macrophages treated with LDL (Figs. 2A-2D). However, the up-regulation of CD36 and SR-A expression was significantly inhibited by simvastatin. The inhibition percentage of CD36 mRNA expression (55%) was much higher than that of SR-A (20%). However, simvastatin had no effect on mRNA expression of LOX-1. In addition, troglitazone partly abolished the effects of simvastatin on CD36 expressions, suggesting the involvement of PPARγ antagonism in the down-regulation of CD36 expression by simvastatin.

**Simvastatin decreases the content and mRNA expression of TNF-α and IL-6 in macrophages**

Inflammation is implied in the pathogenesis of atherosclerosis. The present study showed that simvastatin decreased the contents of proinflammatory cytokines TNF-α and IL-6 in the medium of macrophages treated with oxLDL (Figs. 3A and 3B), and down-regulated the mRNA expression of TNF-α and IL-6 in oxLDL treated macrophages (Figs 3C and 3D). The results suggested that anti-inflammation also contributes to the inhibition of formation of foam cells by simvastatin.

**Simvastatin inhibits calpain-1 protein expression and PD150606 down-regulates**
the mRNA expression of PPARγ and CD36 in macrophages

Calpain-1 has been shown to play important role in inflammation and atherosclerosis. We tested whether calpain-1 gets involved in the regulation of PPARγ/CD36 pathway by simvastatin. The present study showed that simvastatin reduced the protein expression of calpain-1 in oxLDL treated macrophages (Figs. 4A and 4B). In addition, PD150606, the specific calpain inhibitor, reduced the mRNA expression of both PPARγ (Fig. 4C) and CD36 (Fig. 4D) in macrophages treated by oxLDL. Combination of simvastatin and PD150616 had no further effects on mRNA expression of PPARγ and CD36 compared with either alone. The results indicated that inhibition of calpain-1 might get involved in the regulation of PPARγ/CD36 pathway by simvastatin.

Over-expression of calpain-1 partly abolishes the effect of simvastatin on the cholesterol content, mRNA expression of PPARγ and CD36 in macrophages

To further clarify the role of calpain-1 inactivation in the inhibition of foam cells formation and the regulation of PPARγ and CD36 in macrophages by simvastatin, the macrophages were infected with adenoviral vectors containing mouse calpain-1 gene. The results demonstrated that over-expression of calpain-1 in macrophages partly reversed the simvastatin effects including cell cholesterol content (Fig. 5A), mRNA expression of PPARγ (Fig. 5B) and CD36 (Fig. 5C) on oxLDL treated macrophages. The results suggested that inhibition of calpain-1 might mediate the down-regulation of PPARγ by simvastatin, subsequently inhibiting the CD36 mRNA expression.

Discussions

The present study demonstrated that simvastatin inhibits the foam cells formation, down-regulates the mRNA and/or protein expressions of CD36, PPARγ, TNF-α, IL-6, calpain-1 in oxLDL treated macrophages. In addition, PPARγ agonist troglitazone partly abolishes the effects of simvastatin. Furthermore, PD150606, the specific calpain inhibitor, reduces the mRNA expression of both PPARγ and CD36. Finally,
over-expression of calpain-1 in macrophages partly reversed the effects of simvastatin including cell cholesterol content, mRNA expression of PPARγ and CD36. These results suggested that simvastatin inhibits foam cell formation through down-regulation of CD36 expression, which might be attributed to the calpain-1 inhibition-mediated antagonism of PPARγ. This in vitro study might add new mechanism by which simvastatin inhibits the atherosclerosis reported previously in our laboratory and others (Liu et al. 2013; Song et al. 2011; Tang et al. 2006).

CD36 is an 88-kD membrane glycoprotein and expressed by monocytes/macrophages, vasculature endothelial cells and smooth muscle cells (Talle et al. 1983). Functional and structural characterization showed that CD36 belongs to the scavenger receptor class B family with a capacity to bind oxLDL (McGregor et al. 1989). The importance of monocytic CD36 in the initiation and perpetuation of atherosclerotic lesions was proved by the reduced size of vascular lesions when CD36 was inactivated in ApoE deficient animals (Kunjathoor et al. 2002; Wallin et al. 2001).

To explore the mechanism by which simvastatin inhibits the formation of foam cells, we investigated the effect of simvastatin on expression of CD36 in oxLDL treated macrophages. The results showed that simvastatin down-regulated the expression of CD36 at the levels of mRNA and protein. The results are in agreement with the following two reports. Han et al found that pitavastatin prevented oxLDL uptake by a murine macrophage cell line J774 cells and murine peritoneal macrophages through inhibition of CD36 expression (Fischer et al. 2003). Luzak et al showed that the incubation of whole blood from healthy volunteers with simvastatin and pravastatin significantly decreased CD36 expression (van Bilsen et al. 2014).

Many reports reveal that the mechanism by which oxLDL induces CD36 is due to its ability to activate the PPARγ (Rutsch et al. 2011; Tontonoz et al. 1995; Tontonoz et al. 1998). PPARγ is a member of a nuclear hormone superfamily that heterodimerizes with RXR. These proteins are transcriptional regulators of genes that encode proteins involved in adipogenesis and lipid metabolism (Tontonoz et al. 1995). To investigate the mechanisms by which simvastatin down-regulates CD36
expression, we observed the effects of simvastatin on PPARγ mRNA expression in oxLDL-treated macrophages. As expected, the results showed that simvastatin down-regulated the mRNA expression of PPARγ. Consistently, we previously reported that PPARγ agonists 15-deoxy-Delta12,14-prostaglandin J2 (15d-PGJ2) and troglitazone dose-dependently abolished the down-regulation of CD36 expression by Tanshinone IIA in oxLDL treated macrophages, implying the significance of PPARγ in the regulation of CD36 expression (Villa-Bellosa et al. 2011). Interestingly, troglitazone, a PPARγ agonist, was reported to inhibit atherosclerosis in ApoE KO mice, although this compound significantly up-regulates CD36 expression in aortas (Chen et al. 2001). The result seemed to be contradictory. The authors explained this phenomenon as following. On one hand, the increased expression of CD36 by troglitazone stimulates the uptake of oxidized LDL, thereby promoting foam cell formation and atherosclerosis. On the other hand, it is possible that the antiatherogenic effects of troglitazone on other factors, such as induction of lipoprotein lipase and cell adhesion, overcome the proatherogenic CD36-inducing effects. This explanation could be understandable because the effects of troglitazone are multiple and comprehensive inside the body. The authors also observed the in vitro effect of troglitazone on CD36 expression in peritoneal macrophages and found that troglitazone increased the protein expression of CD36 in a time-dependent manner. However, the authors did not investigate the effect of troglitazone on foam cell formation. In the present in vitro study, we focused on the role of PPARγ in the down-regulation of CD36 expression by simvastatin and found that troglitazone, the PPARγ agonist, antagonized the effects of simvastatin on CD36 expression and oxLDL uptakes. Different from the multiple factors affecting the atherosclerosis in the in vivo study, PPARγ activation mediated-CD36 up-regulation plays a major role in the uptakes of oxLDL in the in vitro cultured macrophages. Therefore, it is possible that troglitazone partly abolished the inhibitory effect of simvastatin on oxLDL uptakes through PPARγ activation mediated up-regulation of CD36. Taken together, we cautiously concluded that in the in vitro study, antagonism of PPARγ mediated up-regulation of CD36 plays a critical role in the inhibition of oxLDL uptakes in
macrophages by simvastatin. Whether this result can be confirmed in the animal study needs further investigation. Another report showed that pitavastatin, an inhibitor of HMG-CoA reductase, prevents oxLDL uptake by macrophages through inhibition of CD36 expression, which is mediated by decreased expression of PPARγ (Fischer et al. 2003). These counterintuitive effects suggest that activation of PPARγ might have both anti- and pro-atherosclerotic effect.

Inflammation is implied in the pathogenesis of atherosclerosis. Various studies have highlighted that pro-inflammatory cytokines TNF-α and IL-6 are the upstream inflammatory cytokines that play central role in propagating the downstream inflammatory response responsible for atherosclerosis. The present study showed that simvastatin decreased the contents of TNF-α and IL-6 in the medium of macrophages treated with oxLDL, and down-regulated the mRNA expression of TNF-α and IL-6 in oxLDL treated macrophages. The results suggest that anti-inflammation also contributes to the inhibition of atherosclerosis by simvastatin. Consistently, there have been reported that inhibition of TNF-α reduces atherosclerosis in ApoE KO mice (Lomashvili et al. 2008) and that oleoylthanolamide inhibits atherosclerosis in ApoE KO mice by down-regulating the mRNA expression of TNF-α and IL-6 in atherosclerotic tissue (Wallin et al. 2001).

Calpain, a calcium-dependent cysteine protease, has been shown to play important role in inflammation and atherosclerosis (Miyazaki et al. 2013; Miyazaki et al. 2011). Calpains induce proteolytic degradation of ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) in monocytes/macrophages, subsequently causing the impaired cholesterol efflux and subsequent macrophage foam cell formation (Tanaka et al. 2010; Wang et al. 2003). Studies at the animal level showed that inhibition of calpain attenuates atherosclerosis in both ApoE KO and LDL receptor deficient mice (Miyazaki et al. 2011; Subramanian et al. 2012). These studies suggested the potency of calpain as a molecular target in atherosclerosis. Calpains get involved in acute inflammatory processes via the activation of NF-κB (O’Neill et al. 2011; Schibler et al. 1968). Specifically, the protein expression of calpain-1 instead of calpain-2 has been shown to be positively correlated with the contents of TNF-α and IL-6 and the
mRNA expression of PPARγ in macrophages (Villa-Bellosta and Sorribas 2011). However, the causal relationship between calpain-1 and PPARγ has not been reported. To further investigate how simvastatin exerts anti-inflammatory effects and regulates PPARγ mRNA expression, we studied the effects of simvastatin on calpain-1 protein expression. The results showed that simvastatin reduced the protein expression of calpain-1 in oxLDL treated macrophages. In addition, PD150606, the specific calpain inhibitor, reduced the mRNA expression of both PPARγ and CD36 in macrophages treated by oxLDL. Furthermore, combination of simvastatin and PD150616 had no further effects on mRNA expression of PPARγ and CD36 compared with either alone. However, over-expression of calpain-1 in macrophages partly reversed the simvastatin effects including cell cholesterol content, mRNA expression of PPARγ and CD36 on oxLDL treated macrophages. The results suggested that inhibition of calpain-1 might mediate the down-regulation of PPARγ by simvastatin, subsequently inhibiting the CD36 mRNA expression and atherosclerosis.

In summary, the present study demonstrated that simvastatin inhibits the macrophage foam cell formation, which might be largely attributed to calpain-1 inhibition-mediated regulation of PPARγ/CD36 pathway.

**Declarations**

**Conflict of interest**

The authors declare that they have no competing interests.

**Acknowledgment**

This work was supported by National Natural Science Foundation of China (No.81374008), Talent Fund of Liaoning Medical University (No. 2014-18) and Natural Science Foundation of Liaoning Province (No. 2015020325).
References


**Figure legends**

**Fig. 1.** Simvastatin reduces cholesterol content and DiI-oxLDL uptake in macrophages treated with oxLDL. A: cholesterol content; B and C: Representative photographs of DiI-oxLDL uptake and the quantization respectively. Sim: simvastatin; Trog: troglitazone. Data are expressed as the mean ± SEM. *: p<0.05 was considered statistically significant.

**Fig. 2.** Simvastatin down-regulates mRNA and/or protein expression of CD36 and SR-A in macrophages treated with oxLDL. A: mRNA expression of CD36; B: Representative photographs of Western blot of CD36 protein expression (upper panel) and the quantitation (lower panel) respectively. C: mRNA expression of SR-A; D: mRNA expression of LOX-1. Sim: simvastatin; Trog: troglitazone. Data are expressed as the mean ± SEM. *: p<0.05 was considered statistically significant.

**Fig. 3.** Simvastatin decreases the contents of TNF-α and IL-6 in the medium of macrophages treated with oxLDL (A and B), and down-regulates the mRNA expression of TNF-α and IL-6 in oxLDL treated macrophages (C and D). Sim: simvastatin. Data are expressed as the mean ± SEM. *: p<0.05 was considered statistically significant.

**Fig. 4.** Simvastatin reduces protein expression of calpain-1 in oxLDL treated macrophages (A and B), PD150606 down-regulates mRNA expression of PPARγ (C) and CD36 (D) in oxLDL treated macrophages. Sim: simvastatin; PD: PD150606. Data are expressed as the mean ± SEM. *: p<0.05 was considered statistically significant.

**Fig. 5.** Over-expression of calpain-1 partly reversed the simvastatin effects including cell cholesterol content (A), mRNA expression of PPARγ (B) and CD36 (C) on oxLDL treated macrophages. Sim: simvastatin; Cal: calpain-1. Data are expressed as the mean ± SEM. *: p<0.05 was considered statistically significant.
Fig. 1. Simvastatin reduces cholesterol content and DiI-oxLDL uptake in macrophages treated with oxLDL. 
A: cholesterol content; B and C: Representative photographs of DiI-oxLDL uptake and the quantization 
respectively. Sim: simvastatin; Trog: troglitazone. Data are expressed as the mean ± SEM. n=4; *: p<0.05 
was considered statistically significant.

199×139mm (300 x 300 DPI)
Fig. 2. Simvastatin down-regulates mRNA and/or protein expression of CD36 and SR-A in macrophages treated with oXLDL. A: mRNA expression of CD36; B: Representative photographs of Western blot of CD36 protein expression (upper panel) and the quantitation (lower panel) respectively. C: mRNA expression of SR-A; D: mRNA expression of LOX-1. Sim: simvastatin; Trog: troglitazone. Data are expressed as the mean ± SEM. n=4; *: p<0.05 was considered statistically significant.
Fig. 3. Simvastatin decreases the contents of TNF-α and IL-6 in the medium of macrophages treated with oxLDL (A and B), and down-regulates the mRNA expression of TNF-α and IL-6 in oxLDL treated macrophages (C and D). Sim: simvastatin. Data are expressed as the mean ± SEM. n=4. *: p<0.05 was considered statistically significant.

199x166mm (300 x 300 DPI)
Fig. 4. Simvastatin reduces protein expression of calpain-1 in oxLDL treated macrophages (A and B), PD150606 down-regulates mRNA expression of PPARγ (C) and CD36 (D) in oxLDL treated macrophages. Sim: simvastatin; PD: PD150606. Data are expressed as the mean ± SEM. n=4; *: p<0.05 was considered statistically significant.
Fig. 5. Over-expression of calpain-1 partly reversed the simvastatin effects including cell cholesterol content (A), mRNA expression of PPARγ (B) and CD36 (C) on oxLDL treated macrophages. Sim: simvastatin; Cal: calpain-1. Data are expressed as the mean ± SEM. n=4; *: p<0.05 was considered statistically significant.