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Simvastatin Reduces OX40 and OX40 Ligand Expression in Human Peripheral Blood Mononuclear Cells and in Patients with Atherosclerotic Cerebral Infarction

B Liu[1,2,*], G Yu[1,*], Z Yang[2], L Sun[1], R Song[2], F Liu[2], Y Xin[2] and L Zhang[1]

[1]Department of Neurology, The First Affiliated Hospital, and [2]The Fourth Affiliated Hospital, Harbin Medical University, Harbin, China

This study investigated the effect of simvastatin on the expression of OX40 and OX40 ligand (OX40L) *in vitro* and *in vivo*. OX40 and OX40L mRNA and protein levels were measured in human peripheral blood mononuclear cells, using reverse transcription–polymerase chain reaction and Western blot, respectively, in response to simvastatin alone or given in combination with interferon-γ, mevalonate or GW9662, a peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonist. Simvastatin induced down-regulation of OX40 and OX40L mRNA and protein in a concentration-dependent manner, and antagonized the interferon-γ-induced increase in OX40 and OX40L mRNA and protein levels. Mevalonate, but not GW9662, reversed the simvastatin-induced down-regulation of OX40 and OX40L expression, indicating that these effects were mediated through the mevalonate pathway. Serum levels of soluble OX40L and matrix metalloproteinase 9 levels were significantly reduced in patients with atherosclerotic cerebral infarction who were treated for 6 months with routine therapy plus simvastatin (*n* = 46) compared with patients receiving routine therapy alone (*n* = 30). These findings improve our understanding of the anti-inflammatory and immunomodulatory properties of simvastatin treatment for atherosclerotic disorders.

KEY WORDS: Simvastatin; OX40; OX40 ligand; Atherogenesis

Introduction

Simvastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, is used in the clinical treatment of hypercholesterolaemia to decrease plasma low density lipoprotein (LDL)-cholesterol concentration and reduce morbidity and mortality resulting from cardiovascular disease.[1] The beneficial effects of simvastatin are not limited to its ability to lower LDL-cholesterol levels. Recent studies have shown that additional effects of simvastatin include enhanced endothelial differentiation of peripheral blood mononuclear cells (PBMCs)

*These authors contributed equally to this study.

in hypercholesterolaemic patients,[2] reduced serum soluble CD40 ligand (sCD40L) levels in hypercholesterolaemic, hypertensive patients,[3] and reduced vascular endothelial growth factor levels in hypercholesterolaemic patients.[4]

The early stage of atherosclerotic disease is characterized by endothelial dysfunction, but inflammation and immunological processes also play essential roles in the progression of atherosclerosis.[5] Infiltration of blood-derived macrophages and T-cells through the vessel wall seems to be an important feature of the active stage of atherosclerosis,[6,7] and is initiated by signals from antigen-presenting cells (APCs). Several pathways contribute to the initiation and maintenance of inflammation, one of which is signalling by the receptor–ligand pair OX40–OX40 ligand (OX40L), which has recently been shown to be associated with atherosclerosis.[8–10]

OX40, a tumour necrosis factor (TNF) superfamily receptor protein, was first identified as an activator of rat lymphocytes.[11] OX40L is a 34 kDa glycoprotein that is expressed in T-lymphocytes, B-lymphocytes, vascular endothelial cells and dendritic cells.[12,13] The OX40–OX40L-mediated interaction between T-cells and APCs promotes the survival of effector T-cells and is essential for the generation of $CD4^+$ memory T-cells.[14] These interactions have the potential to enhance inflammatory responses in atherosclerotic plaques.

Although simvastatin has been shown to have pleiotropic effects on endothelial cells, vascular smooth muscle cells and macrophages,[15] little is known about its effect on OX40 and OX40L expression. This study examined the effects of simvastatin on the expression of OX40 and OX40L in human PBMCs and the pathways involved, and also investigated the effects of simvastatin treatment on serum levels of soluble OX40L (sOX40L), sCD40L and matrix metalloproteinase 9 (MMP-9) in patients with atherosclerotic cerebral infarction. To the best of our knowledge, there have been no previous reports on the effect of simvastatin on the expression of OX40 and OX40L.

Patients and methods

ISOLATION AND CULTURE OF PMBCs

To study the *in vitro* effects of simvastatin on OX40 and OX40L expression, PBMCs were isolated by Ficoll density-gradient separation (2000 ***g***, 20 min) from anticoagulated blood (40 ml) collected from healthy donors. To separate monocytes from lymphocytes, the PBMCs were allowed to adhere to plastic for 2 h and were subsequently washed. Isolated PBMCs (2×10^6 cells/ml) were resuspended in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and cultured in 75 cm^2 flasks for 24 h. Cell viability was measured by trypan blue exclusion.

Cells were then incubated for 24 h with: (i) 0, 1, 2.5, 5 or 10 μM simvastatin (Sigma-Aldrich, St Louis, MI, USA); (ii) 0 or 10 μM simvastatin and 0 or 1000 U/ml human recombinant interferon-γ (IFN-γ) (Endogen, Rockford, IL, USA); (iii) 0 or 10 μM simvastatin and 0 or 500 μM mevalonate (Sigma-Aldrich), the product of HMG-CoA reductase activity; and (iv) 0 or 10 μM simvastatin and 0 or 10 μM GW9662 (Alexis GmbH, Grünberg, Germany), a peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonist.

EXPRESSION OF OX40 AND OX40L mRNA

Expression of OX40 and OX40L mRNA was determined using reverse transcription–

polymerase chain reaction (RT–PCR). Total RNA was isolated from the cultured cells using TRIzol reagent® (Invitrogen). The RT–PCR for human OX40 and OX40L was performed using a one-step RT–PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Amplification of β-actin cDNA (housekeeping gene) served as an internal standard.

The primers used for amplification are given in Table 1. Gel images were analysed using Quantity One® software, version 1.14 (Bio-Rad, Hercules, CA, USA). The volume rectangle detection method was used to detect bands within the gels.

OX40 AND OX40L PROTEIN EXPRESSION

OX40 and OX40L protein expression was determined in the cultured cells using Western blot analysis. Cells were washed in phosphate-buffered saline (PBS; 10 mM pH 7.2) and harvested by scraping in radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After determining the protein concentration with a Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA), 30 µg of protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline containing 0.5% Tween 20 (TBS-T), 5% dry milk and 1% bovine serum albumin overnight at 4 °C, and then incubated with primary antibody (Santa Cruz Biotechnology), 1:1000 dilution, at room temperature for 2 h. Membranes were washed three times with TBS-T and incubated with secondary antibody (Santa Cruz Biotechnology), 1:5000 dilution, for 1 h at room temperature. The proteins were detected by enhanced chemiluminescence (ECL) using the Pierce ECL™ system (Thermo Scientific Pierce Protein Research Products). The band volumes were analysed using Quantity One® software, version 1.14.

PATIENTS

To study the *in vivo* effects of simvastatin treatment, patients with atherosclerotic cerebral infarction were recruited consecutively from the Department of Neurology, The First Affiliated College of Harbin Medical University between January 2006 and June 2007. The diagnosis of atherosclerotic cerebral infarction was based on the presence of infarct focus, artery stenosis and atherosclerotic plaques on brain magnetic resonance imaging and magnetic resonance angiography. Patients with hepatic or renal dysfunction, diabetes, hypercholesterolaemia, cancer or infection

TABLE 1:
Primers used for the reverse transcription–polymerase chain reaction to determine expression of OX40 and OX40L mRNA

Gene	Primers
OX40	5′-TCAGAAGTGGGAGTGAGCGGAAG-3′ (forward)
	5′-GCAGAGAGCCGGAGGCAGCCATCGGC-3′ (reverse)
OX40 ligand	5′-TGCTTCACCTACATCTGCCTGCA-3′ (forward)
	5′-CTAGTAGGCTCAAGGCAATCTTG-3′ (reverse)
β-Actin	5′-GTATGCCTCTGGTCGTACCACAGGCAT-3′ (forward)
	5′-ACTCATCGTACTCCTGCTTGCTGATCC-3′ (reverse)

were excluded. Patients taking non-steroidal anti-inflammatory drugs, steroids or opiates, or who had an ischaemic cerebral stroke within 3 months of the study were also excluded.

The study protocol was approved by the Research and Ethics Committees of The First Affiliated Hospital of Harbin Medical University, and written informed consent was obtained from all the study participants.

STUDY TREATMENTS AND ASSESSMENTS

The *in vivo* study was of an open-label, randomized, parallel-group design. Patients were separated into two groups matched for age, sex and degree of hypertension and then were randomized to receive simvastatin 40 mg/day plus routine therapy or routine therapy alone without any cholesterol-lowering drugs for the treatment of atherosclerotic cerebral infarction for the 6-month study period. Baseline total cholesterol, triglyceride, high density lipoprotein (HDL)-cholesterol, LDL-cholesterol, blood urea nitrogen, creatinine and alanine aminotransferase levels were recorded in all patients. Blood samples for determination of serum sOX40L, sCD40L and MMP-9 levels were collected at baseline and after 6 months of treatment.

DETERMINATION OF SERUM sOX40L, sCD40L AND MMP-9

Levels of serum sOX40L were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described.[16] The sCD40L concentrations were determined using a commercial kit (Bender Medsystems, Burlingame, CA, USA) according to the manufacturer's instructions. The MMP-9 concentrations were measured using the Human Total MMP-9 Quantikine® ELISA Kit (R&D Systems, Minneapolis, MN, USA).

STATISTICAL ANALYSIS

Statistical evaluation was performed using the Student's *t*-test, with $P < 0.05$ denoting statistical significance. Results are presented as means ± SD.

Results

EXPRESSION OF OX40 AND OX40L mRNA AND PROTEIN IN PBMCs

Simvastatin inhibited expression of both OX40 and OX40L mRNA and protein in PMBCs in a concentration-dependent manner in the range from 1 – 10 µmol/l (Fig. 1).

Levels of OX40 and OX40L mRNA and protein in PMBCs increased after stimulation with 1000 U/ml IFN-γ, however, this increase was antagonized when IFN-γ, was co-administered with 10 µmol/l simvastatin ($P < 0.05$; Fig. 2).

The down-regulation of OX40 and OX40L mRNA transcription (Fig. 3A, 3C) and protein translation (Fig. 3B, 3D) observed with simvastatin was reversed in the presence of mevalonate ($P < 0.05$), whereas treatment with the PPAR-γ inhibitor, GW9662, did not significantly alter the down-regulation of OX40 and OX40L mRNA or protein in the presence of simvastatin compared with that seen for simvastatin alone (Fig. 3).

EFFECTS OF SIMVASTATIN ON SERUM sOX40L, sCD40L AND MMP-9 IN PATIENTS

A total of 76 patients with atherosclerotic cerebral infarction were included in the study; 46 of these received simvastatin plus routine therapy for atherosclerotic cerebral infarction and 30 received routine therapy alone (controls).

The baseline characteristics of the two groups were approximately matched (Table 2). After 6 months' treatment with simvastatin, sOX40L and MMP-9 expression

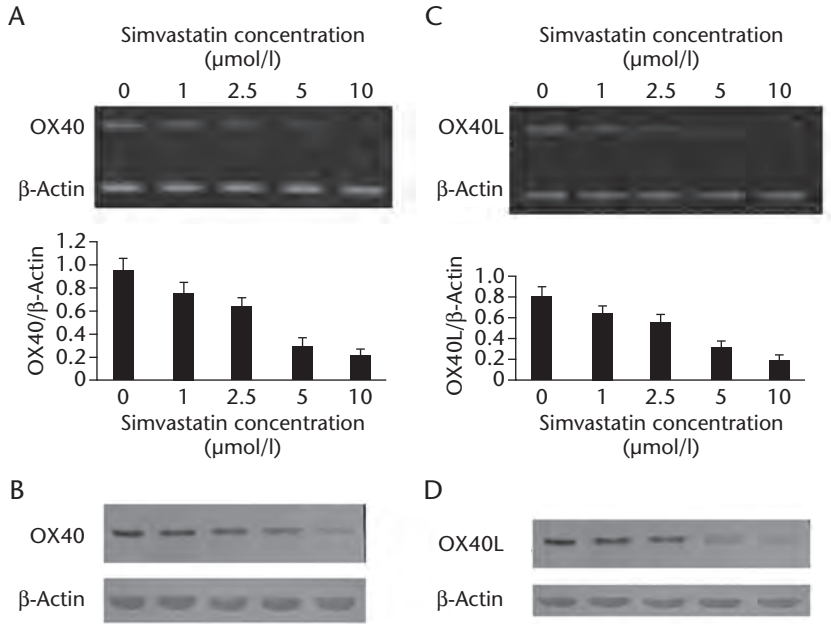

FIGURE 1: Simvastatin 0, 1, 2, 5 and 10 μmol/l decreased OX40 and OX40 ligand (OX40L) mRNA (A and C) and protein (B and D) expression in peripheral blood mononuclear cells in a concentration-dependent manner as measured by reverse transcription–polymerase chain reaction and Western blot analysis, respectively. Data show the mean ± SD from three duplicate experiments, normalized to β-actin

were significantly reduced compared with the control group (3.55 ± 1.01 versus 5.89 ± 2.49 ng/ml for sOX40L; 2.74 ± 0.71 versus 3.52 ± 1.05 ng/ml for MMP-9) ($P < 0.01$; Fig. 4). There was no significant change in sCD40L levels (Fig. 4).

Discussion

Interactions between OX40 and OX40L enhance the generation and survival of memory $CD4^+$ T-cells during inflammation and immune responses. For example, OX40 co-stimulation facilitates the stimulation of T helper 2 (Th2) cells, and OX40L exerts similar effects on T helper 1 (Th1) immunity.[17] IFN-γ is the principal substance produced by Th1 lymphocytes and plays a role in atherosclerotic plaque growth and rupture,[18] which is why the role of IFN-γ was examined in the present study. We found that simvastatin antagonized IFN-γ activity at a concentration of 10 μmol/l. Since simvastatin is an inhibitor of HMG-CoA reductase,[19] we also investigated whether exogenous mevalonate, the product of HMG-CoA reductase activity, could counteract the effects of simvastatin on OX40 and OX40L expression. The results showed that the down-regulation of OX40 and OX40L expression caused by simvastatin was reversed in the presence of mevalonate. In addition, as statins have been reported to activate the PPAR family of transcription factors,[20] the effect of PPAR-γ inhibitor GW9662 activity was investigated. As this inhibitor did not make a significant difference to the effects of simvastatin, it is unlikely PPAR-γ is involved in the down-

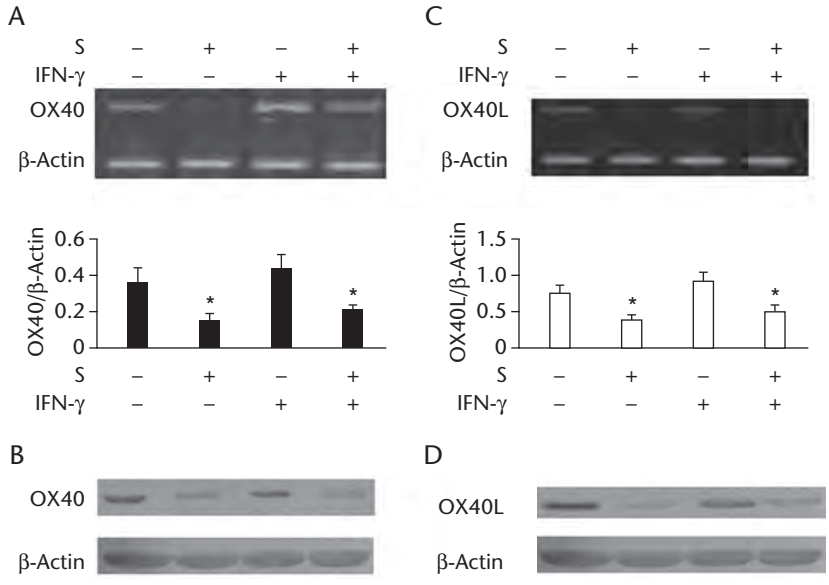

FIGURE 2: OX40 and OX40 ligand (OX40L) mRNA (A and C) and protein expression (B and D) increased in response to treatment with 1000 U/ml interferon-γ (IFN-γ) in peripheral blood mononuclear cells. Addition of 10 μmol/l simvastatin (S) antagonized IFN-γ-induced increases in both OX40 and OX40L mRNA and protein expression (*$P < 0.05$ compared with control). The mRNA and protein levels were measured by reverse transcription–polymerase chain reaction and Western blot analysis, respectively. Data show the mean ± SD of data from three duplicate experiments, normalized to β-actin

regulation of OX40 and OX40L expression caused by simvastatin in human PBMCs.

Inhibition of HMG-CoA reductase by statins for the treatment of hypercholesterolaemia is associated with beneficial effects on the progression and regression of atherosclerosis in animal models as well as in humans.[21] Various clinical data have demonstrated that statins have direct anti-inflammatory effects in addition to their cholesterol-lowering activities. For example, in hypercholesterolaemic patients, simvastatin inhibited expression of pro-inflammatory cytokines in monocytes.[22] Although studies have suggested that simvastatin reduces expression of the pro-inflammatory cytokines interleukin-6, interleukin-8 and monocyte chemo-attractant protein-1 in circulating monocytes from hypercholesterolaemic patients,[23] little is known about the association between simvastatin treatment and soluble OX40L levels. Many mechanisms, other than cholesterol lowering, are proposed to explain these beneficial effects,[24] however to the best of our knowledge, there are no reports on the effect of simvastatin on the expression of OX40 and OX40L. In the present study, simvastatin was shown to reduce the transcription and translation of OX40 and OX40L in PBMCs *in vitro*.

Several studies have examined the effect of statin therapy on expression of the TNF superfamily member CD40 and its ligand, CD40L.[25,26] Long-term simvastatin treatment was also shown to reduce soluble CD40L

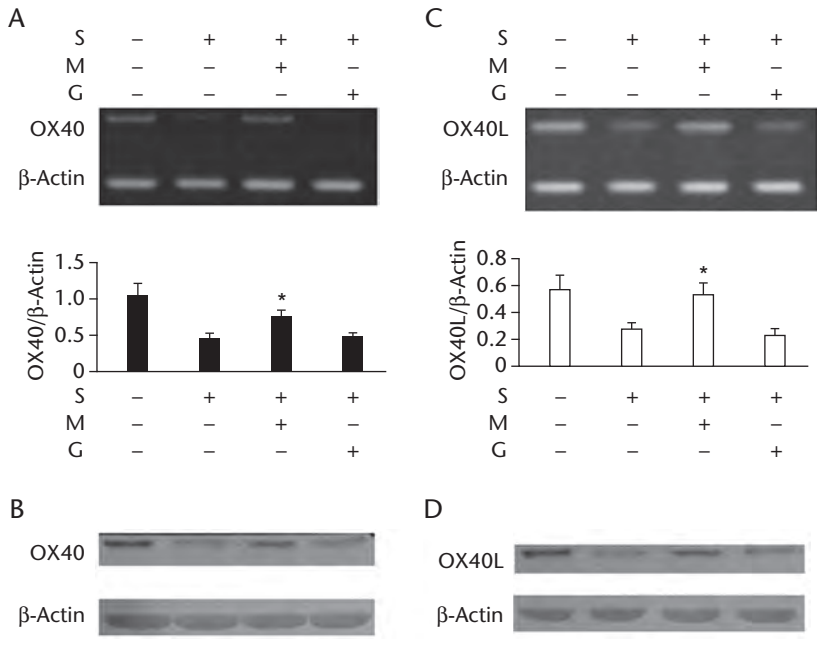

FIGURE 3: Down-regulation of OX40 and OX40 ligand (OX40L) mRNA (A and C) and protein (B and D) expression in peripheral blood mononuclear cells was induced by 10 μmol/l simvastatin (S), and could be reversed by the addition of 500 μM mevalonate (M) (*$P < 0.05$ compared with control) but not by 10 μM GW9662 (G). Levels of mRNA and protein were measured by reverse transcription–polymerase chain reaction and Western blot analysis, respectively. Data show the mean ± SD of data from three duplicate experiments, normalized to β-actin

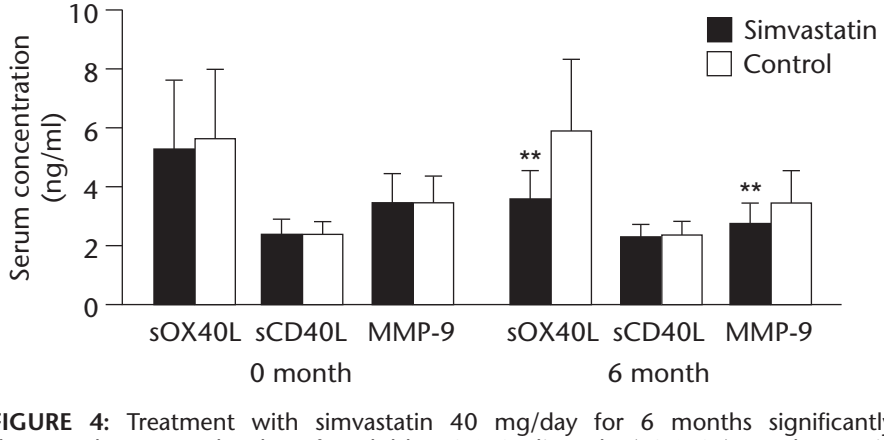

FIGURE 4: Treatment with simvastatin 40 mg/day for 6 months significantly decreased serum levels of soluble OX40 ligand (sOX40L) and matrix metalloproteinase 9 (MMP-9) compared with the control (**$P < 0.01$) in patients with atherosclerotic cerebral infarction, but had no significant effect on soluble CD40 ligand (sCD40L)

TABLE 2:
Baseline characteristics of the two treatment groups of patients with atherosclerotic cerebral infarction

Characteristic	Simvastatin plus routine therapy (*n* = 46)	Routine therapy alone (controls) (*n* = 30)
Age (years)	63.89 ± 5.33	63.53 ± 5.37
Sex		
Male (*n*)	31	19
Female (*n*)	15	11
Body mass index (kg/m^2)	22.42 ± 1.72	23.11 ± 1.47
Systolic blood pressure (mmHg)	136.15 ± 14.73	138.43 ± 13.01
Diastolic blood pressure (mmHg)	80.71 ± 11.15	138.43 ± 13.01
Plasma glucose (mmol/l)	4.90 ± 0.67	4.91 ± 0.70
Total cholesterol (mmol/l)	4.61 ± 0.89	4.75 ± 0.81
Triglyceride (mmol/l)	1.47 ± 0.67	1.39 ± 0.63
HDL-cholesterol (mmol/l)	1.44 ± 0.40	1.46 ± 0.38
LDL-cholesterol (mmol/l)	2.72 ± 0.72	2.76 ± 0.70
Blood urea nitrogen (mmol/l)	4.77 ± 1.09	4.84 ± 1.41
Creatinine (μmol/l)	103.86 ± 15.33	104.01 ± 14.50
Alanine aminotransferase (mmol/l)	23.43 ± 5.66	23.43 ± 4.96

Values are means ± SD unless otherwise stated.
HDL, high density lipoprotein; LDL, low density lipoprotein.

levels in peritoneally dialysed patients.[27] Statins may also play a beneficial role in the treatment of atherosclerosis through the regulation of matrix-degrading enzymes. Atherosclerotic cerebral infarction is precipitated by the rupture of an atherosclerotic plaque. MMPs break down extracellular matrix, are present in atherosclerotic plaques, and appear to be more active in unstable lesions.[28,29] The present report provides evidence for a novel anti-inflammatory pathway by which simvastatin can reduce serum sOX40L and MMP-9 in patients with atherosclerotic cerebral infarction. These results provide an additional model to explain the anti-inflammatory activity of simvastatin in clinical trials.

In conclusion, simvastatin is able to reduce OX40 and OX40L expression at the transcriptional and translational level in human PBMCs. This effect seems to be mediated through the mevalonate pathway rather than via the PPAR-γ pathway. The clinical data from the study suggest that simvastatin reduces expression of the TNF superfamily member, sOX40L, in atherosclerotic cerebral infarction patients. These findings might help explain the anti-inflammatory as well as the immuno-modulatory properties of simvastatin treatment for atherosclerotic disorders.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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Author's address for correspondence
Professor Liming Zhang
Department of Neurology, The First Affiliated Hospital, Harbin Medical University, Harbin 150001, China.
E-mail: zhangliminghy@gmail.com