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Silencing of either SR-A or CD36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of these receptors

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Aims Macrophage scavenger receptor A (SR-A) and class B scavenger receptor CD36 (CD36) contribute to foam cell formation and atherogenesis via uptake of modified lipoproteins. So far, the role of these scavenger receptors has been studied mainly using knockout models totally lacking these receptors. We studied the role of SR-A and CD36 in foam cell formation and atherogenesis by RNA interference (RNAi)-mediated silencing, which is a clinically feasible method to down-regulate the expression of these receptors.

Methods and results We constructed lentivirus vectors encoding short hairpin RNAs (shRNAs) against mouse SR-A and CD36. Decreased SR-A but not CD36 expression led to reduced foam cell formation caused by acetylated low-density lipoprotein (LDL) in mouse macrophages, whereas the uptake of oxidized LDL was not altered. More importantly, silencing of SR-A upregulates CD36 and vice versa. In LDL receptor-deficient apolipoprotein B100 ($LDLR^{-/-}ApoB^{100/100}$) mice kept on a western diet, silencing of either SR-A or CD36 in bone marrow cells led to a marked decrease (37.4 and 34.2%, respectively) in cross-sectional lesion area, whereas simultaneous silencing of both receptors was not effective.

Conclusion Our results suggest that silencing of either SR-A or CD36 alone reduces atherogenesis in mice. However, due to reciprocal upregulation, silencing of both SR-A and CD36 is not effective.

Keywords Scavenger receptor A • CD36 • Atherosclerosis • RNA interference • Foam cell

1. Introduction

Retention of lipoproteins in the artery wall is one of the primary events in atherosclerosis.[1,2] In the vessel wall, low-density lipoprotein (LDL) is trapped by matrix proteoglycans and becomes susceptible to various modifications that trigger local inflammatory responses leading to recruitment of macrophages in the arterial wall.[3] Uptake of modified LDL by macrophages leads to foam cell formation which is characteristic of early atherosclerotic lesions.[4]

Scavenger receptor A (SR-A) and class B scavenger receptor CD36 (CD36) are expressed in human atherosclerotic lesions,[5,6] and they have been shown to contribute to the uptake of modified LDL.[7,8] Because this uptake of cholesterol through the macrophage scavenger receptors is poorly regulated, excess cholesterol accumulation leads to macrophage-derived foam cell formation. Many studies with SR-A or CD36 knockout mice crossed with atherosclerotic mouse models have proven the pro-atherogenic role of scavenger receptors.[8–10] Additionally, reports using SR-A- or CD36-deficient bone marrow (BM) transplanted to atherosclerotic mice have supported the hypothesis that the lack of these receptors decreases lesion formation.[11,12] However, recently the role of these receptors in atherogenesis has been challenged.[13] Interestingly, Moore *et al.*[13] showed that even though knockout of SR-A or CD36 in $ApoE^{-/-}$ mice led to a reduction in peritoneal macrophage lipid accumulation, there

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was an increase in lesion area in aortic sinus.[13] Consequently, alternative pathways, such as macropinocytosis of LDL, have been proposed to play a role in foam cell formation and atherogenesis.[13,14]

The purpose of our study was to determine the potential of RNA interference (RNAi)-mediated silencing of SR-A and CD36 in reducing atherogenesis in adult mice. In contrast to knockout studies, RNAi offers an opportunity for gene silencing in adult animals and allows studying the role of these receptors when their expression is only partially reduced. Also, we used LDLR$^{-/-}$ApoB$^{100/100}$ mice, where lipoprotein profile mimics human situation with high plasma LDL levels.[15] We constructed lentiviral vectors (LVs) encoding short hairpin RNAs (shRNA), mediators of RNAi, against SR-A and CD36 and show that lesion size decreased in LDLR$^{-/-}$ApoB$^{100/100}$ mice after BM transplantation of SR-A- or CD36-silenced stem cells. However, silencing of one receptor upregulated the other suggesting partly overlapping functions and a delicate balance in the regulation of these receptors.

2. Methods

2.1 Construction of LVs encoding shRNAs

Three individual shRNA sequences against mouse SR-AI/AII (GenBank accession no. NM031195) or CD36 (NM007643) under human U6 promoter were subcloned into LV-1-hPGK-GFP-WPRE plasmid, containing green fluorescent protein (GFP) as a marker. Vectors were constructed as described.[16] The target sequences for SR-A were AAATACCA GTAACGTGGAAAT, AACTCAAAAGCCGACCTTATA, and AAT TGGAGGAACGTGTGTACA for 325, 407, and 660, respectively. For CD36, the sequences were AAATACAGAGTTCGTTATCTA, AAG CTATTGCGACATGATTAA, and AACGACTGCAGGTCAACATAT for 504, 986, and 1420, respectively. The target sequence for shCTRL was AAACCCAAACTCCGAAGACTT.

2.2. Preparation of viral stocks

Third-generation HIV-1-based LVs were prepared by the standard calcium phosphate transfection method in 293T cells as described.[17] Viral titers ranging from 6.0×10^8 to 7.0×10^9 transducing units (TU)/mL were obtained with the average infectivity of 1×10^7 TU/μg p24^{gag}.

2.3 Cell culture and *in vitro* transduction

Mouse macrophage RAW264.7 (ATCC; TIB-71) and mouse monocyte WEHI 265.1 (ATCC; TIB-204) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% penicillin–streptomycin. Viral transductions were made on six-well plates, 24-well plates, or chamber slides with MOI 100 (1×10^7 TU/mL) for 16 h. Virus amounts for each cell line were determined by careful titration to achieve good transduction and silencing efficiencies.

2.4 Real-time RT–PCR

Total RNAs were extracted by Trizol Reagent (Gibco BRL) 1 week after transduction. RNA was reverse transcribed to cDNA by M-MuLV reverse transcriptase (MBI Fermentas). SR-A and CD36 mRNA levels were measured by real-time PCR (ABI PRISM 7700 detection system, Applied Biosystems) using appropriate Taqman® Gene expression assays (Applied Biosystems). Ribosomal 18S RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous amplification control for normalization.

2.5 LDL isolation and modification

LDL was isolated from plasma of healthy normolipidaemic donors by sequential ultracentrifugation.[18] Isolation of LDL conforms the principles of Declaration of Helsinki for the use of human tissue. For foam cell formation studies, LDL was acetylated (AcLDL) or oxidized (OxLDL) as described previously.[18] For degradation studies, LDL was radioiodinated before acetylation or oxidation.[18] Specific activities of labelled LDLs were 65–125 cpm/ng protein.

2.6 Degradation assay

RAW 264.7 cells were plated on 24-well plates, incubated in OptiMem containing 10% lipoprotein deficient serum (LPDS) and transduced with different LVs. After 72 h, cells were incubated for 12 h in media containing 10 μg/mL ^{125}I-AcLDL or ^{125}I-OxLDL. After the incubation, trichloroacetic acid-soluble non-iodine radioactivity in the media was measured.[18]

2.7 Foam cell formation studies

RAW 264.7 cells were plated on chamber slides or six-well plates, incubated in OptiMem containing 10% LPDS and transduced with different LVs. After 72 h, cells were incubated for 24 h in media containing 100 μg/mL modified or native human LDL. Cells were fixed with 4% paraformaldehyde and stained with Oil Red O.[19] For spectrofotometrical assay, Oil Red O was extracted with isopropanol and measured at 492 nm. Results were normalized against total DNA amount.

2.8 Western blot analysis

Proteins were extracted from cell lysates 1 week after transduction, and 30 μg protein was used for electrophoresis. The proteins were transferred to nitrocellulose membrane and incubated with SR-A (Serotec) or CD36 (R&D Systems) or beta-actin antibodies (Cell Signaling). Blots were visualized using HRP-conjugated secondary antibodies and ELC Plus detection system (GE Healthcare) and detected with Typhoon 9400 (GE Healthcare) scanner.

2.9 Experimental animals

LDLR$^{-/-}$ApoB$^{100/100}$ mice[15] were used in the study ($n = 44$). BM cells were extracted from femurs and tibias of the male donor mice. Recipient female mice were 2 months old during transplantation. Mice were anaesthetized by subcutaneous injection of xylazine (10 mg/kg)–ketamine (80 mg/kg) for transplantation. Mice were fed on western-type diet (TD 88173 Harlan Teklad, 42% of calories from fat and 0.15% from cholesterol, no sodium cholate) for 6 weeks. Mice were sacrificed using carbon dioxide anaesthesia and aortas were collected for the histological analysis. All animal experiments were approved by the Experimental Animal Committee of University of Kuopio and conform to the *Guide for the care and use of laboratory animals* published by the US national institute of Health (NIH Publication No. 85-23, revised 1996).

2.10 BM cell handling

Mononuclear cells were extracted by Ficoll Paque PLUS (GE Healthcare, Cat. No. 17-1440-02) gradient purification, followed by removal of dead cells using Dead Cell Removal Kit (Miltenyi Biotec, Cat. No. 130-090-101). Stem cell fraction was enriched using MACS® lineage cell depletion kit (Miltenyi Biotec, Cat. No. 130-090-858) according to manufacturer's instructions. Isolated progenitor/stem cells were transduced overnight with MOI 500 on 24-well plates. Cells were grown in StemSpan-media (Stem Cell technologies, Cat. No. 09650) supplemented with mouse stem cell factor (50 ng/mL, Stem cell technologies, Cat. No. 02731). Thirty thousand cells were transplanted to each recipient irradiated with 9 Gy. For SR-A and CD36 expression studies, cells were stained with antibodies against SR-A (R&D Systems) or CD36 (R&D Systems) and appropriate PE-conjugated secondary antibody. The expression was analysed by fluorescent-activated cell sorting (FACS). For SR-A expression analysis, cells were stimulated with macrophage colony stimulating factor (M-CSF, 50 ng/mL) for overnight.

2.11 Atherosclerotic lesions and immunohistological analysis

For lesion analysis and immunohistochemical stainings, 5 μm thick serial sections of the aortic arch were cut from paraffin-embedded aortas. For histological analysis, modified Movat's pentachrome staining[20] was used or sections were immunostained for macrophages (mMQ AIA31240, 1:6500, Accurate Chemical & Scientific Corp., NY, USA), smooth muscle cells (α-actin, clone 1A4, dilution 1:800, Sigma), CD-31 (Platelet endothelial cell adhesion molecule [PECAM]-1, dilution 1:50, BD Pharmingen), and oxidation-specific epitopes (MAL-2, dilution 1:100) as described.[21] Sections were counterstained with haematoxylin. Lesion volumes were analysed from aortic sinus level and reported as a percentage from total lumen area. Photographs of histological sections were taken using Olympus AX70 microscope (Olympus Optical) and analyses were performed with AnalySIS software (Soft Imaging System GmbH). All analyses were performed three times blindly without the knowledge of the origin of the samples, and average values were reported.

2.12 Statistical analysis

For *in vitro* studies, independent samples Student's *t*-test was used to evaluate the statistical significance. The *in vivo* data were analysed by a linear mixed model's test and *P*-values were Sidak corrected for multiple comparisons. A *P*-value of 0.05 was considered significant. Values are expressed as mean $\pm$ SD.

3. Results

3.1 Lentiviral mediated silencing of SR-A and CD36 *in vitro*

Three *in silico*-selected short-hairpin RNAs against mouse macrophage SR-A (named shSR-A(325), shSR-A(407), and shSR-A(660) according to the target sequence start position) were cloned into LV-1 vector. Two mouse monocyte–macrophage cell lines were transduced with MOI 100 of each vector to determine the silencing efficiency of each construct. shSR-A(660) was the most effective in SR-A silencing, leading to 61 $\pm$ 1% ($P < 0.001$) decrease in SR-A mRNA in WEHI 265.1 cells (*Figure 1A*), whereas the shSR-A(325) and shSR(407) decreased mRNA levels 13 $\pm$ 8 and 12 $\pm$ 5%, respectively. In RAW264.7 cells, all constructs seemed to silence SR-A (*Figure 1B*). shSR-A(660) decreased SR-A mRNA levels 53 $\pm$ 4% ($P < 0.01$), shSR-A(325) 74 $\pm$ 8% ($P < 0.01$), and shSR-A(407) 72 $\pm$ 6% ($P < 0.01$) (*Figure 1B*). Since shSR-A(660) was effective in both cell lines, it was chosen for subsequent studies.

For CD36, three individual target sequences (named shCD36(504), shCD36(986), and shCD36(1420)) were screened. In RAW264.7 cells, shCD36(986) was the best sequence leading to 76 $\pm$ 6% ($P < 0.01$) silencing of CD36 mRNA and it was chosen for subsequent studies (*Figure 1C*).

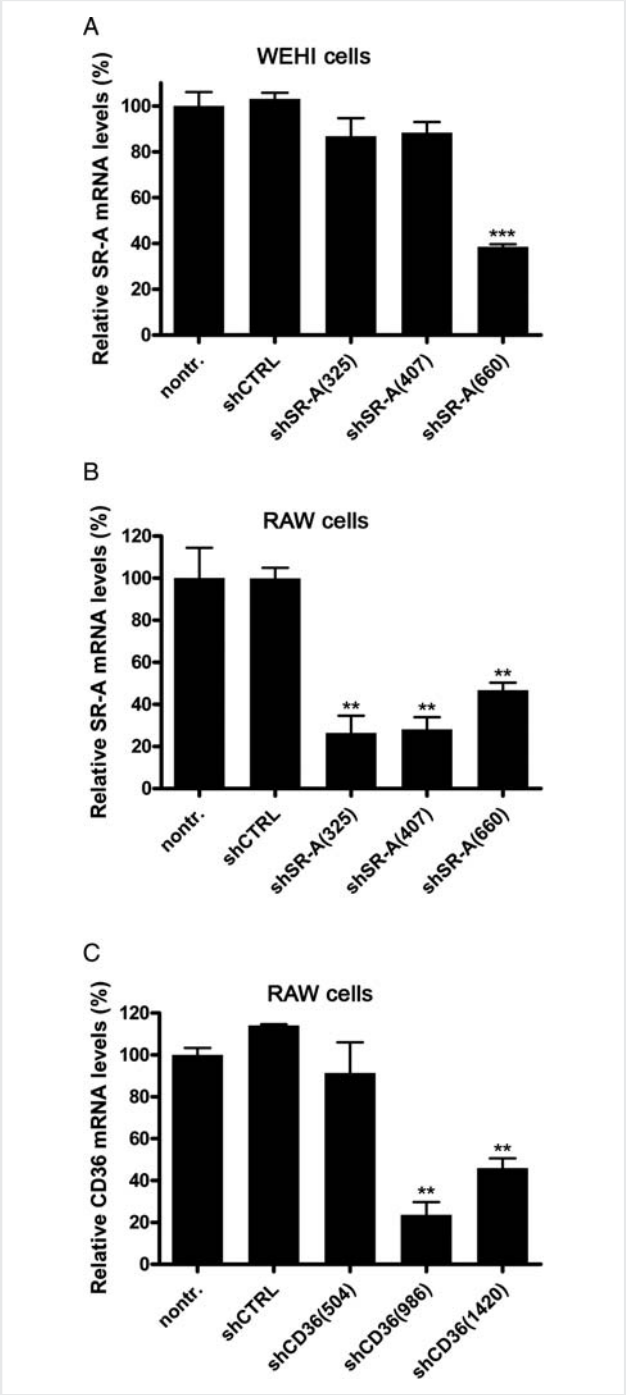

Figure 1 SR-A and CD36 silencing in mouse macrophages. Relative SR-A levels in WEHI cells (*A*) and RAW cells (*B*) after transduction with MOI 100. CD36 levels in RAW cells (*C*) after transduction with MOI 100. All values are mean $\pm$ SD ($n = 4$) (**$P < 0.01$, ***$P < 0.001$ vs. non-treated, Student's *t*-test).

3.2 Effects of SR-A and CD36 silencing on the degradation of modified LDL and foam cell formation

Functional analysis of SR-A and CD36 knockdown was studied using lipoprotein degradation assay. The SR-A knockdown resulted in 75 $\pm$ 1% decrease in the degradation of ^{125}I-AcLDL compared with control cells ($P < 0.001$) (*Figure 2A*), whereas in CD36 silenced cells the degradation of AcLDL was increased 21 $\pm$ 10% ($P < 0.05$) (*Figure 2A*). When both receptors were silenced, the degradation of AcLDL was decreased 64 $\pm$ 5% ($P < 0.001$). On the contrary, the degradation of OxLDL was not changed significantly in CD36 silenced cells; instead, it was increased 34 $\pm$ 8% ($P < 0.05$) in SR-A silenced cells (*Figure 2B*). When both receptors were silenced, the degradation of OxLDL was not changed significantly (*Figure 2B*).

To test whether SR-A and CD36 silencing could alter the uptake of larger quantities of modified lipoproteins, we performed foam cell

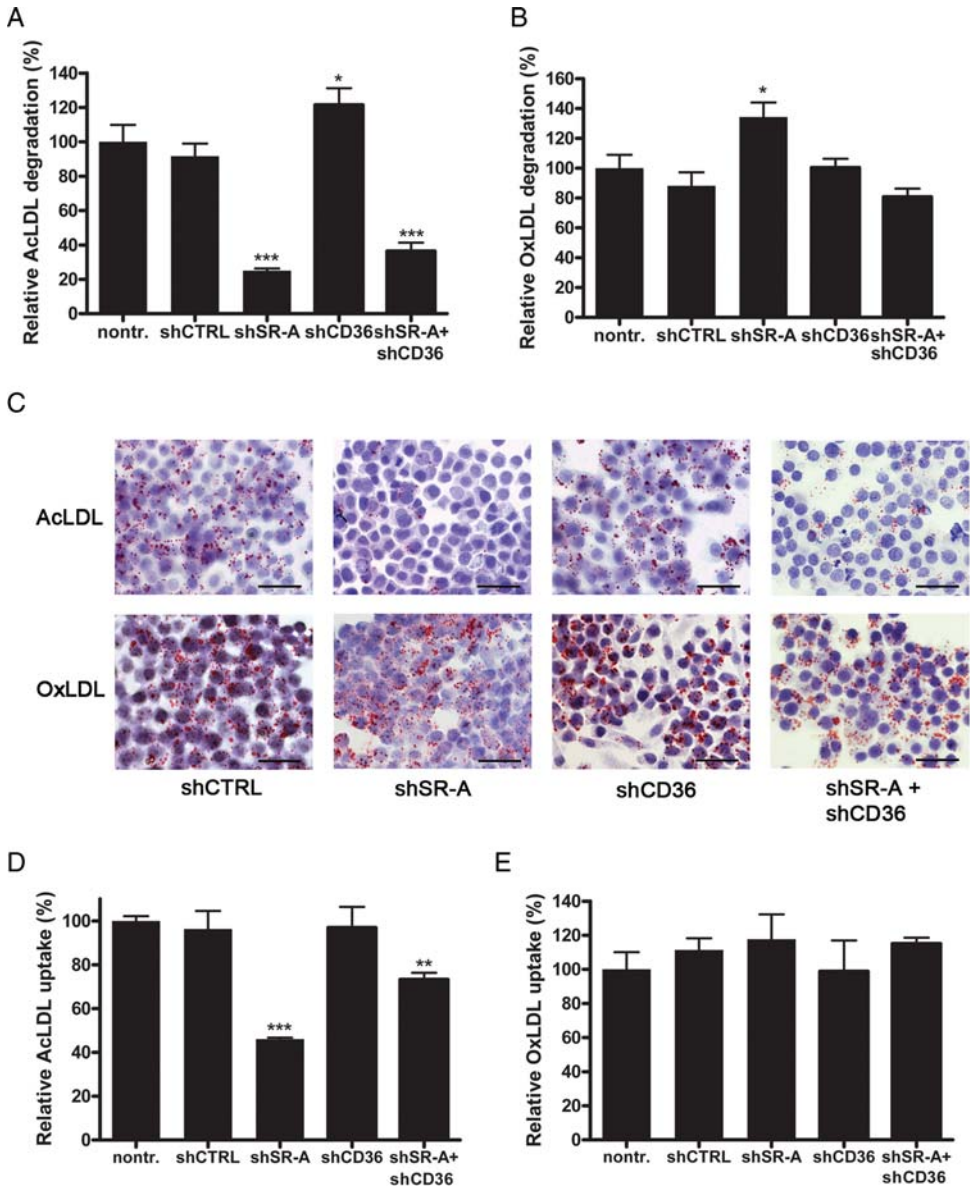

Figure 2 Degradation of modified LDL and foam cell formation studies. For degradation studies, RAW cells were transduced with MOI 100 of different LVs and incubated with 10 μg/mL of ^{125}I-AcLDL (*A*) or ^{125}I-OxLDL (*B*). For foam cell formation studies, cells treated with 100 μg/mL of modified LDL and stained with Oil Red O. Figures of stained cells (original magnification ×400, scale bars 25 μm) (*C*). Quantification of Oil red O uptake after isopropanol extraction of the Oil Red O for AcLDL (*D*) and OxLDL (*E*) treated cells. All values are mean ± SD ($n = 4$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. non-treated, Student's *t*-test).

formation assay in RAW cells using 100 μg/mL of AcLDL or OxLDL. Silencing of SR-A led to a marked decrease in AcLDL-induced foam cell formation, whereas there was no decrease with shCD36 (*Figure 2C*). The uptake of OxLDL was not significantly altered in any group (*Figure 2C*). To quantify these results, we extracted Oil Red O from the cells with isopropanol. SR-A knockdown decreased the AcLDL-induced foam cell formation by 54 ± 1% ($P < 0.001$), when 70% of the cells were transduced, but there was no decrease when CD36 was silenced (*Figure 2D*). In double-silenced cells, AcLDL-induced foam cell formation was decreased by 27 ± 3% ($P < 0.01$). The unaltered uptake of OxLDL was also confirmed by the dye extraction method (*Figure 2E*).

3.3 Silencing of SR-A or CD36 reciprocally upregulates the other receptor

Due to the unexpected results in the degradation and foam cell formation assays, we investigated whether silencing of one receptor and subsequent LDL treatment affects the levels of the other receptor. Interestingly, the silencing of SR-A induced CD36 expression 96 ± 12% ($P < 0.01$) (*Figure 3A*). Conversely, CD36 silencing induced SR-A 62 ± 13% ($P < 0.01$) (*Figure 3B*). Therefore, we also included a group transduced with both viruses for this experiment. However, in this group, SR-A levels were upregulated and reached levels of the control cells (*Figure 3A*). At the same time, CD36

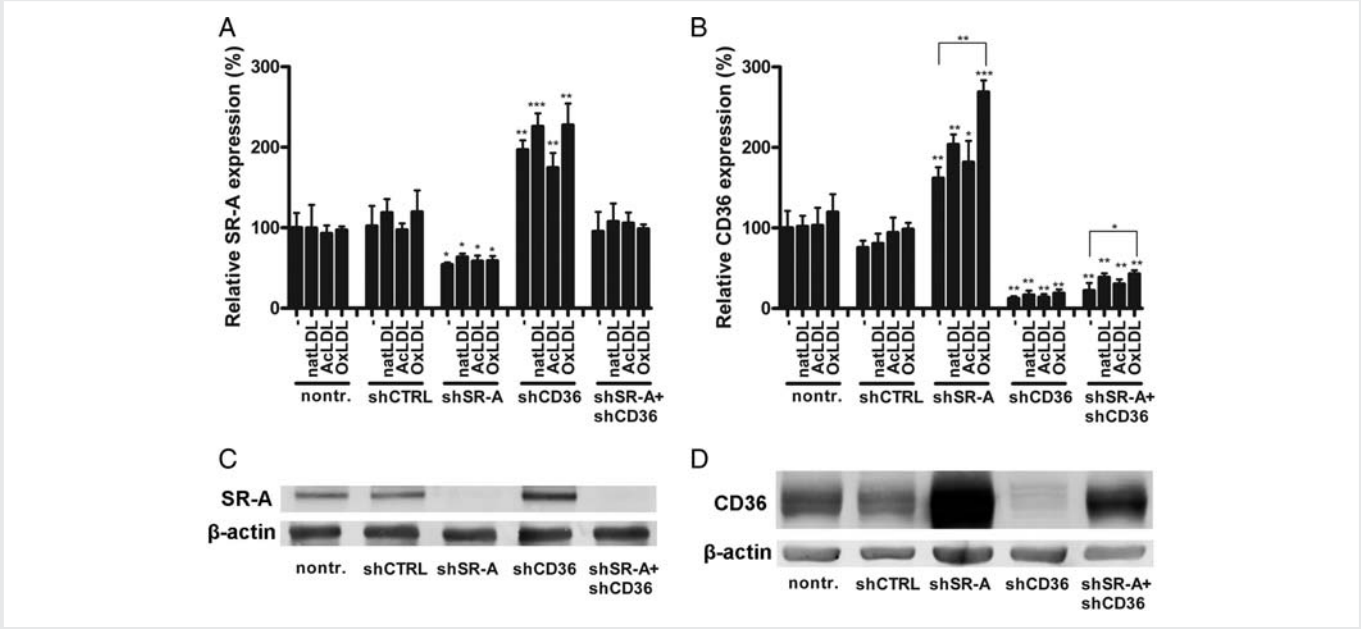

Figure 3 SR-A and CD36 expression after modified LDL treatment. RAW cells were transduced with MOI 100 of different LVs, and incubated with 100 μg/mL of natLDL, AcLDL, or OxLDL. Relative SR-A (*A*) and CD36 (*B*) levels were measured by quantitative real-time PCR. Protein levels for SR-A (*C*) and CD36 (*D*) were determined by western blot. SR-A was blotted in non-reducing conditions and CD36 in reducing conditions. All values are mean ± SD ($n = 3$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. respective non-treated, Student's *t*-test, unless otherwise indicated).

levels remained lower compared with control, but were also upregulated compared with shCD36 group (*Figure 3B*). Furthermore, addition of native LDL (natLDL), AcLDL, or OxLDL did not upregulate SR-A (*Figure 3A*), whereas CD36 was upregulated 66 ± 15% ($P < 0.01$) and 94 ± 20% ($P < 0.05$) after addition of OxLDL in shSR-A and shSR-A + shCD36 groups when compared with respective non-treated control (*Figure 3B*).

The silencing of SR-A and CD36 at protein level was confirmed by western blotting (*Figure 3C* and *D*). Silencing of CD36 upregulated SR-A at protein level even though the upregulation of SR-A in double-silencing group was not evident (*Figure 3C*). Also, CD36 protein was strongly upregulated when only SR-A or both SR-A and CD36 was silenced (*Figure 3D*).

To study genes involved in the regulation of SR-A and CD36, we measured mRNA levels of transcription factors known to be linked to SR-A and CD36 regulation. Transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) levels correlated with CD36 levels, indicating involvement in the regulation of this receptor (*Figure 4A*). The mRNA levels of another transcription factor reported to regulate CD36, nuclear factor erythroid 2-related factor 2 (Nrf2), was upregulated significantly in double-silenced group (*Figure 4B*), although smaller increases were seen in groups where only SR-A or CD36 was silenced. A target gene of Nrf2 and PPARγ, heme oxygenase 1 (HO-1), was induced by OxLDL (*Figure 4C*), but its basal levels were not significantly altered by SR-A or CD36 silencing. However, the induction by OxLDL was higher in shSR-A and shSR-A+shCD36 groups (*Figure 4C*). Finally, the basal mRNA levels of mitogen-activated protein kinase p38 kinase, c-Jun N-terminal kinase 2, or cyclooxygenase 2 were not changed significantly between different virus treatment groups (data not shown).

3.4 Silencing of SR-A or CD36 decreased lesion area *in vivo*

To study the role of SR-A and CD36 silencing in atherogenesis *in vivo*, BM-derived progenitor cells were isolated from LDLR$^{-/-}$ApoB$^{100/100}$ mice and transduced with one or both viruses. The silencing of SR-A and CD36 in BM-derived progenitor cells was analysed by FACS after immunocytochemical staining. SR-A expression was undetectable in BM cells without stimulation (data not shown), but after induction with M-CSF, a reduction in mean fluorescence intensity (MFI) was seen in shSR-A and shSR-A+shCD36 groups (*Figure 5A*). Also, a small increase in SR-A expression was detected in shCD36 group (*Figure 5A*). Further, CD36 was expressed in BM cells without stimulation, being decreased markedly in shCD36 group compared with shCTRL group (*Figure 5A*). While a strong CD36 upregulation was not seen in shSR-A group, in double-silenced group the expression was restored back to the levels of shCTRL (*Figure 5A*).

After transplantation, mice were fed with high-fat western-type diet for 6 weeks. Lesion volumes were analysed from the aortic cross-sections at the aortic sinus level. Silencing of SR-A reduced lesion formation 37.4% ($P < 0.001$) compared with shCTRL (*Figure 5B*). Similarly, decreased lesion size (34.2%, $P < 0.001$) was seen when CD36 was silenced (*Figure 5B*). In the group transduced with both SR-A and CD36 viruses, no change in lesion area was observed (*Figure 5B*).

For histological analysis, Movat pentachrome staining was performed. The smaller lesion areas were clearly visible in shSR-A and shCD36 groups (*Figure 6*). The biggest lesions showed cholesterol crystals in the lesion core. Further, the influence of silencing to the lesion composition was examined by immunological stainings. However, no difference was found in macrophage staining, smooth muscle cell content, or endothelial cell integrity (*Figure 6*). Staining

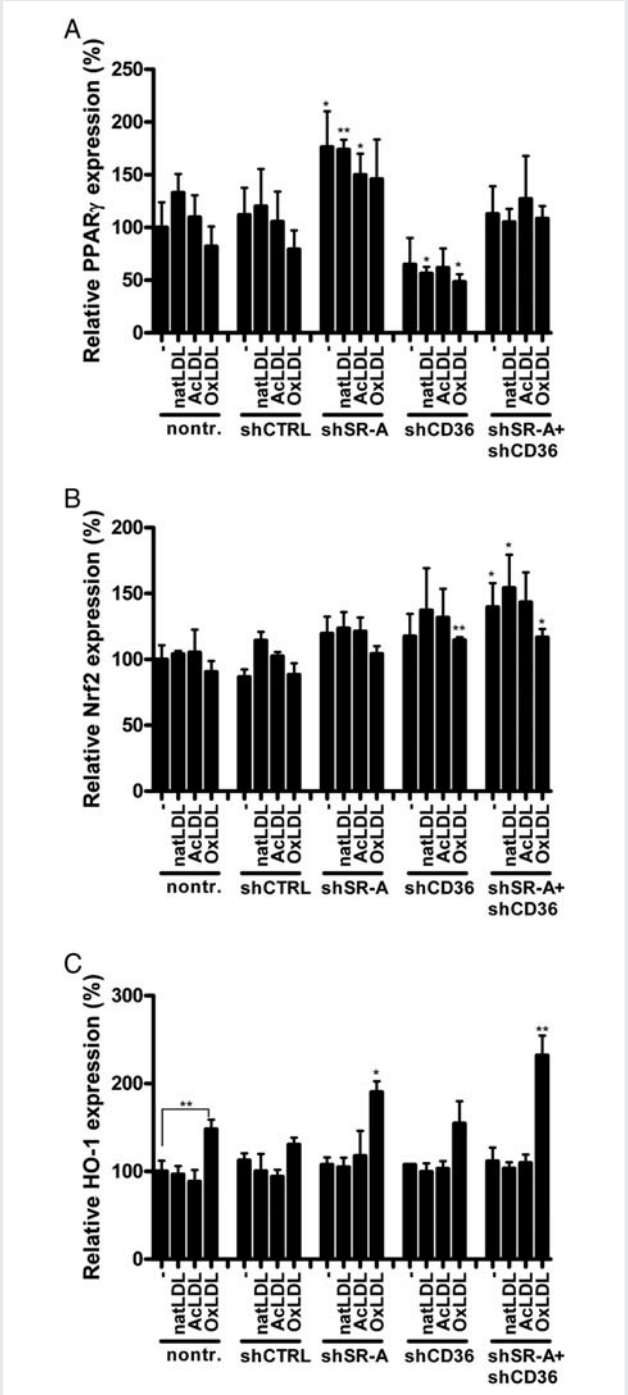

Figure 4 Relative PPARγ (*A*), Nrf2 (*B*), and HO-1 (C) mRNA levels were measured by quantitative real-time PCR. All values are mean ± SD ($n = 3$) (*$P < 0.05$, **$P < 0.01$ vs. respective non-treated, Student's *t*-test, unless otherwise indicated).

for the oxidation-specific epitopes followed the same patterns as macrophage staining and no differences between the groups were detected (data not shown).

4. Discussion

Even though the role of SR-A and CD36 in the uptake of modified LDL and involvement in the pathogenesis of atherosclerosis has been shown in previous studies, recent studies have challenged their role in atherogenesis.[13,22,23] These studies suggest that the role is more complex than previously thought and should be re-evaluated. Therefore, we investigated the contribution of SR-A and CD36 on the uptake of modified LDL *in vitro* and atherogenesis *in vivo* utilizing RNAi-mediated knockdown of SR-A and CD36.

Knockout mice have been invaluable in loss-of-function studies. However, phenotype characteristics may provide conflicting data of the gene function due to the total lack of gene expression. Knockout affects an individual from early embryogenesis and compensatory mechanisms may evolve to compensate for the lack of the gene function. In adults, a given phenotype may represent this accommodation as much as the outcome of the missing gene. In particular, in case of the innate pattern recognition receptors, such as SR-A and CD36, the total deletion of the gene product may have significant consequences.[24] To date, the majority of studies determining the role of SR-A and CD36 have been done with knockout mice or cells totally lacking these receptors. Therefore, RNAi-mediated knockdown using siRNAs/miRNAs provides an alternative way to study the functions of these genes and their role in atherogenesis. Highly efficient third-generation LVs combined with RNAi offer a powerful method to achieve a stable knockdown of a selected gene in adult animals.[16] Genes can also be silenced in BM-derived cells transduced with LVs.[25,26]

The results of this study show that degradation of AcLDL is strongly affected by SR-A levels. Similar results were obtained from foam cell assays, where AcLDL-mediated foam cell formation was almost completely blocked when SR-A was silenced. Earlier studies with soluble SR-A have provided similar results,[27] yet the blockade of foam cell formation through RNAi-mediated silencing seems even more potent. The role of CD36 appears to be minor in AcLDL uptake, since the upregulation of CD36 in SR-A silenced cells does not compensate for AcLDL degradation and foam cell formation. Also, silencing of CD36 did not reduce AcLDL degradation or AcLDL-induced foam cell formation at all, which might also be due to the compensatory upregulation of SR-A. Therefore, these results are in line with earlier observations showing that SR-A is the principal receptor for AcLDL and that CD36 does not play a major role in it.[7]

Many earlier studies have suggested that CD36 is the principal receptor for OxLDL. Interestingly, we did not see any significant changes in the degradation of OxLDL or in the foam cell formation when CD36 was silenced. It cannot be excluded that in contrast to cells totally lacking these receptors, RNAi-mediated silencing which does not completely abolish the receptor activity, might not be sufficient to block the uptake of OxLDL in the foam cell formation assay, where high concentrations of modified LDLs are used. However, since SR-A can also recognize and take up OxLDL, our results suggest that its upregulation in shCD36-treated cells is compensating for the OxLDL uptake. Also, OxLDL-induced CD36 expression significantly only when SR-A was silenced suggesting overlapping functions. Finally, when SR-A was silenced, the degradation of OxLDL was increased, suggesting that upregulated CD36 plays a role there too.

Transcription factor PPARγ has been shown to be required for basal CD36 expression.[28] Our results are in line with this observation since PPARγ levels correlate with detected CD36 levels. Further, our results suggest that this regulation goes also vice versa. In addition, Nrf2 has been shown to be involved in CD36 regulation.[29,30] We saw a small increase in basal Nrf2 levels in the group where both receptors were silenced. HO-1, a target gene of Nrf2 and PPARγ, was upregulated in response to OxLDL when SR-A or both receptors

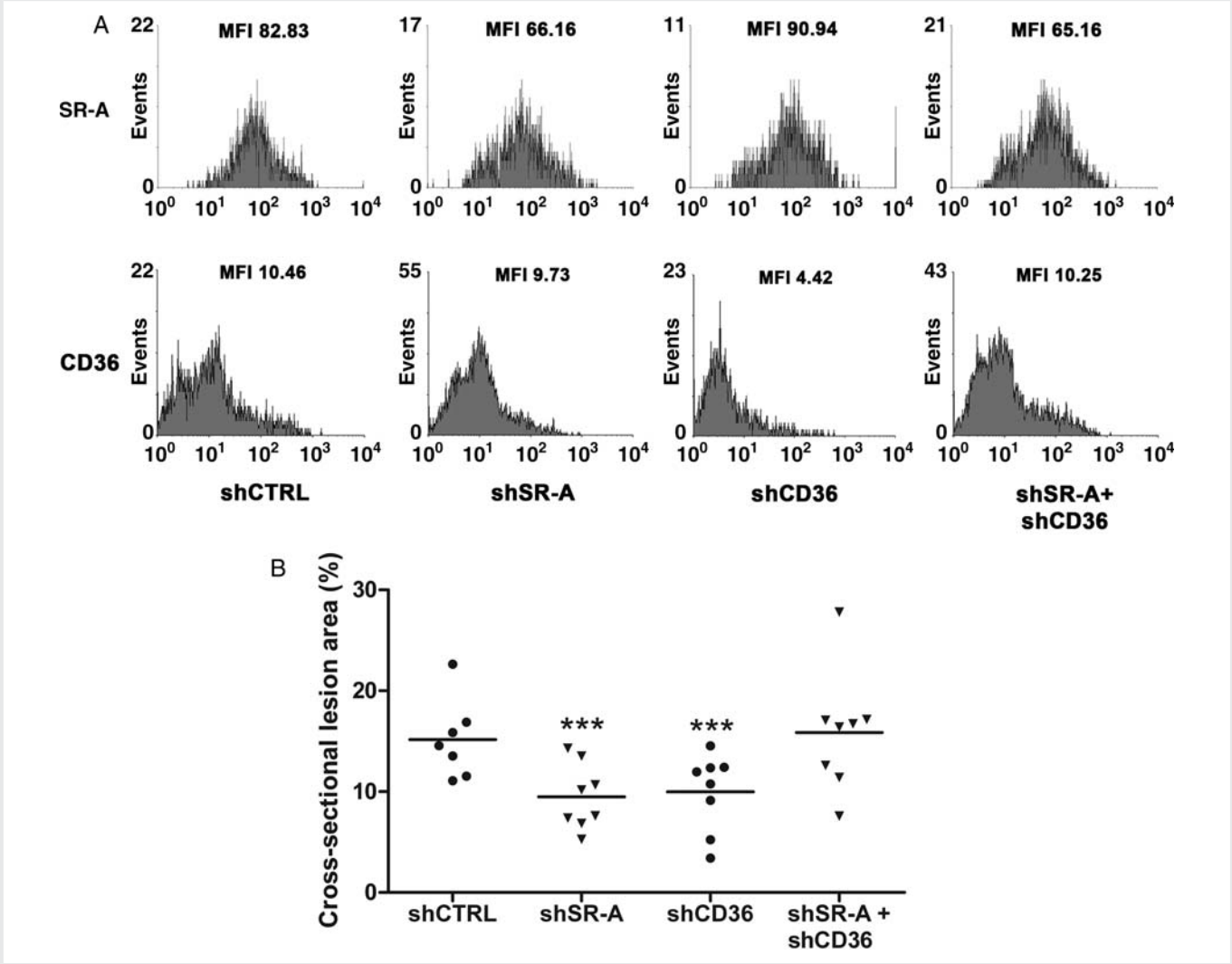

Figure 5 Silencing of SR-A and CD36 in BM-derived stem cells, and lesion areas after transplantation of LV-transduced stem cells. MFIs were analysed by FACS after immunocytochemical staining of SR-A and CD36 (*A*). Cross-sectional lesion areas after 6 weeks on western diet and BM transplantation of LV-transduced stem cells (*B*). Lesion areas were quantified by evaluation of the total lesion areas at the aortic sinus level, which is recognized by three valve cusps. The results for individual mice are presented as scatter plots and the average of each group is indicated as a horizontal bar. ***$P < 0.001$ vs. shCTRL (linear mixed model's test). All groups contained seven to eight mice.

were silenced, which might be due to increased steady-state levels of Nrf2, but it might also reflect increased PPARγ levels especially in shSR-A group. Our results agree with Ishii *et al.*[29] that CD36 is not essential for Nrf2 activation after OxLDL treatment, but proposes that it may be involved in the CD36 regulation.

It has been shown earlier that both SR-A$^{-/-}$ and CD36$^{-/-}$ mice crossbred with ApoE$^{-/-}$ mice develop significantly smaller lesions than ApoE$^{-/-}$ mice.[8,10,31] Also, transplantation of SR-A-deficient BM cells into LDLR$^{-/-}$ mice or CD36-deficient BM cells into ApoE$^{-/-}$ mice results in a profound decrease in atherogenesis.[11,12] In our study, we use LDLR$^{-/-}$ApoB$^{100/100}$ mice where majority of the plasma cholesterol is in the LDL fraction, closely resembling human situation.[15] Since it has been shown that the retention of apoB100 containing lipoproteins contributes to early atherogenesis,[32,33] this model provides novel insights into scavenger receptor-related atherogenesis.

Our results show that the RNAi-mediated partial loss of SR-A or CD36 activity in BM cells reduced the lesion size more moderately when compared with the total loss of SR-A or CD36, supporting a direct expression-related pro-atherogenic role of these receptors. The compensatory mechanism seen *in vitro* does not seem to completely balance and revive the lipoprotein metabolism in the more complex *in vivo* setting. However, in the double knockdown group, the beneficial effect is lost implying that the compensatory upregulation of both receptors is sufficient for the uptake of modified LDLs. Interestingly, two recent studies have provided information about atherosclerosis in ApoE$^{-/-}$ mice lacking both the receptors.[22,34] Firstly, Kuchibhotla *et al.*[34] showed that the absence of SR-A in CD36$^{-/-}$ApoE$^{-/-}$ mice did not provide any additional beneficial effect against atherosclerosis. Secondly, Manning-Tobin *et al.*[22] showed recently that lesion size is not significantly altered when both receptors are knocked out in ApoE$^{-/-}$ mice kept on western

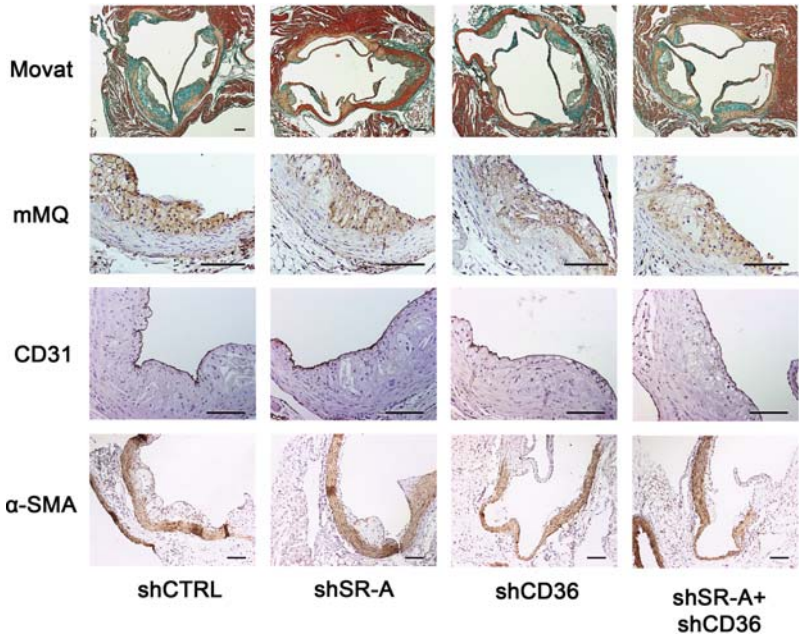

Figure 6 Representative examples of aortic lesion histology in transplanted mice after 6 weeks on western diet and BM transplantation of LV-transduced stem cells. Movat pentachrome staining for aortic histology analysis. Original magnification ×40, scale bars 100 μm. Aortic sections were also stained for macrophages (mMQ), endothelial cells (CD31), and smooth muscle cells (α-Sma). Original magnification ×100, scale bars 100 μm.

diet for 12 weeks. These results are in line with our results in that the silencing of both receptors did not give any additional protection against atherosclerosis. Further, Manning-Tobin *et al.*[22] propose that the principal role of the receptors is in the regulation of inflammatory gene expression, macrophage apoptosis, and plaque necrosis, rather than lipid uptake and foam cell formation. Due to the early time point used in our study, we found only individual lesions with some cholesterol crystals, and thus no necrotic core areas were detected. Also, for the same reason, no changes were found in en face analysis. However, since foam cell formation is considered one of the early events in atherosclerosis, it cannot be ruled out that one mechanism causing these effects is foam cell formation caused by compensatory regulation of SR-A and CD36.

Our group has earlier studied the role of SR-A in atherosclerosis by using a soluble form of SR-A.[27] When overexpressed from adenoviruses or adeno-associated viruses, lesion size was decreased by 19 and 21% in LDLR$^{-/-}$ mice, respectively.[35,36] In the current study, transplantation of SR-A-silenced BM cells led to a comparable effect as achieved with the soluble SR-A injected systemically.[35,36] In agreement with the studies using the soluble SR-A, no clear changes were observed in the histology of the plaques. Similarly, silencing of CD36 did not lead to any alterations in the composition of the lesions. These results imply that the pro-atherogenic mechanisms are probably similar with SR-A and CD36. The unaltered composition of the lesions suggests that the transduced cells are not capable of metabolizing excessive amounts of modified LDLs and therefore not cause any major effects on lesion composition.

To our knowledge, this is the first study exploring the effects of RNAi-mediated silencing of SR-A and CD36 on atherogenesis. Our results support the pro-atherogenic role of these receptors in the early stage of atherogenesis. However, these receptors seem to crosstalk with each other, compensating for the loss of the other receptor. Thus, even though RNAi-mediated treatment strategies of a single scavenger receptor in macrophages might provide new ways to protect arteries against atherogenesis, simultaneous downregulation of both SR-A and CD36 seems not to be beneficial.

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