ORP4L Facilitates Macrophage Survival via 
G-Protein–Coupled Signaling

ORP4L−/− Mice Display a Reduction of Atherosclerosis

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Rationale: Macrophage survival within the arterial wall is a central factor contributing to atherogenesis. Oxysterols, major components of oxidized low-density lipoprotein, exert cytotoxic effects on macrophages.

Objective: To determine whether oxysterol-binding protein–related protein 4 L (ORP4L), an oxysterol-binding protein, affects macrophage survival and the pathogenesis of atherosclerosis.

Methods and Results: By hiring cell biological approaches and ORP4L−/− mice, we show that ORP4L coexpresses with and forms a complex with Goq/11, and phospholipase C (PLC)–β3 in macrophages. ORP4L facilitates G-protein–coupled ligand-induced PLCβ3 activation, IP3 production, and Ca2+ release from the endoplasmic reticulum. Through this mechanism, ORP4L sustains antiapoptotic Bcl-XL expression through Ca2+-mediated c-AMP responsive element binding protein transcriptional regulation and thus protects macrophages from apoptosis. Excessive stimulation with the oxysterol 25-hydroxycholesterol disassembles the ORP4L/Goq/11/PLCβ3 complexes, resulting in reduced PLCβ3 activity, IP3 production, and Ca2+ release, as well as decreased Bcl-XL expression and increased apoptosis. Overexpression of ORP4L counteracts these oxysterol-induced defects. Mice lacking ORP4L exhibit increased apoptosis of macrophages in atherosclerotic lesions and a reduced lesion size.

Conclusions: ORP4L is crucial for macrophage survival. It counteracts the cytotoxicity of oxysterols/oxidized low-density lipoprotein to protect macrophage from apoptosis, thus playing an important role in the development of atherosclerosis. (Circ Res. 2016;119:1296-1312. DOI: 10.1161/CIRCRESAHA.116.309603.)

Key Words: apoptosis ■ atherosclerosis ■ bcl-XL protein ■ macrophages ■ oxidized low density lipoprotein

Atherosclerosis underlies many chronic disease processes, including cardiovascular diseases, which are the leading cause of death in industrialized societies.1 Macrophage injury and death are crucial for the formation and development of arterial lesions, and a wealth of evidences have demonstrated the importance of macrophage apoptosis in this process.2

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The cells in tissues are usually in contact with a complex network of extracellular matrix such as insoluble carbohydrates, proteoglycans, glycosaminoglycans, and secreted chemokines, which all collectively regulate cell growth, differentiation, and behavior.3,4 The engagement of a chemokine receptor by ligand leads to the activation of phospholipase C (PLC), which triggers Ca2+ signaling.5,6 Although chemokines were originally defined as chemoattractants, a growing body of evidence indicates their additional involvement in control of cell survival.4 Chemokines such as S1P (sphingosine-1-phosphate), SDF-1 (stromal cell-derived factor 1), and MCP-1 (monocyte chemoattractant protein-1) induce upregulation of antiapoptotic protein Bcl-XL in macrophages and thus protect them from apoptosis.5,6,9 Interaction of chemokine CX3CL1 with its receptor CX3CR1 confers an essential survival signal, whose absence leads to increased macrophage death and reduced atherogenesis.4 Thus, targeting chemokines and their receptors or components in their signal transduction pathways is a promising approach to control macrophage survival and atherogenesis.6,12

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The cell injury caused by oxidized lipoproteins was among the first findings that led to the theory that oxidized low-density lipoproteins (ox-LDL) are a key factor in atherogenesis. 15,16 Of note, oxidized lipoproteins and their constituents have numerous harmful effects on cell types found in atherosclerotic lesions, including induction of apoptosis through both the cell death receptor and mitochondrial pathways. 17-21 The toxic lipid constituents of ox-LDL, including a variety of oxysterols, are candidates for the in vivo effectors of this cytotoxicity. 20,21 Liver X receptors, Insigs, and members of the oxysterol-binding protein–related protein (ORP) family have been identified as key cellular oxysterol receptors. 22 However, further study is necessary to identify the critical receptors responsible for oxysterol-induced apoptosis.

The ORP family has been implicated in cellular lipid metabolism, vesicle transport, and cell signaling. 23-26 Certain ORPs bind and transport oxysterols, cholesterol, and phosphoinositides. 27-29 ORP4 (also known as oxysterol-binding protein 2) has been reported to bind oxysterols; it is expressed constitutively in brain, heart, and testis 30,31 and is present as 3 major variants, oxysterol-binding protein–related protein 4 L (ORP4L), ORP4M, and ORP4S. 32,33 Early studies reported that ORP4L was detectable in peripheral blood leukocytes from patients with chronic myeloid leukemia, but not from healthy donors. 33,34 Recent reports consistently indicate that ORP4L is involved in tumor cell proliferation and survival 32 and a target of the natural steroidal saponin with antiproliferative activity, OSW-1. 35 ORP4 knockout mice exhibit sperm apoptosis, indicating that ORP4 is essential for the survival of specific cell types. 31

In this study, we investigate the role of ORP4L in macrophage survival and in the development of atherosclerotic lesions in mice.

Methods

Animal Procedures

All animal experiments were approved by the Ethics Committee for Animal Experiments of Jinan University and performed in compliance with the China government guidelines. Mice were housed in sterilized filter-top cages and given unlimited access to food and water with a 12/12 dark/light cycle. ORP4L−/− mice were crossed with LDL receptor (LDLr)−/− mice on the C57BL/6J background purchased from Jackson Laboratory to generate LDLr−/−ORP4L−/− mice. For analysis of atherogenesis, LDLr−/− and LDLr−/−ORP4L−/− littermates were fed a semisynthetic high-fat diet (HFD), containing 15% (wt/wt) fat and 0.25% (wt/wt) cholesterol (No. 202; Baiyu Animal Diet Factory, Guangzhou, China,) starting at 6 weeks of age for 17 weeks.

Analysis of ORP4L Protein Interactome

Rabbit antibody against human ORP4L was produced by immunizing New Zealand White rabbits with a recombinant protein carrying amino acid residues 382 to 485 of human ORP4L. This antibody was used for proteomics analysis only. RAW264.7 cells were washed twice with ice-cold PBS and incubated for 30 minutes on ice with lysis buffer (50 mmol/L Tris-Cl, 150 mmol/L NaCl, 0.5 mmol/L MgCl2, 10% glyceral, and 0.5% Triton X-100, pH 8.0) supplemented with Protease Inhibitor Cocktail (Roche Group). Cell lysates were centrifuged for 10 minutes at 12,000 g. The supernatants were immunoprecipitated with this anti-ORP4L or control antibody coupled to CNBr (cyanoegen bromide)-activated sepharose (GE Healthcare). The ORP4L and its interacting proteins were eluted and subjected to 10% SDS-PAGE followed by in-gel tryptic digestion and mass spectrometric identification as described previously. 36

Calcium Efflux Assay

Cells were plated on glass-bottomed dishes and incubated with 10 μmol/L Fluo-4 AM (Invitrogen) for 60 minutes at 37°C in extracellular calcium buffer (130 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L CaCl2, 1 mmol/L MgCl2, 25 mmol/L Hepes, pH 7.5, 1 mg/mL BSA, and 5 mmol/L glucose) in dark, after which they were washed by extracellular calcium buffer and additionally incubated for 30 minutes at 25°C to permit dye de-esterification. Cells were excited with a low-intensity 488-nm laser peak. Images were acquired at 2-s intervals under time-lapse mode using confocal microscopy (Zeiss LSM 510 Meta laser system). Fluorescence was imaged for 2 minutes before the C5a (10 nmol/L) was added into the suspension. Image data were subsequently analyzed using ImageJ (National Institutes of Health) and presented as a ratio of F/F0 in final results, where F0 represents baseline fluorescence intensity in each cell.

Computer Docking

The homology model of ORP4L was generated using Molecular Operating Environment (http://www.chemcomp.com) software by using the structure of oxysterol-binding protein homology 3 as a template (PDB entry code: 4IC4). The structure of core sequence of these 2 models is highly conserved (Online Figure VIII). Hydrogen atoms were added to the protein using the MOE modeling suite before carrying out the docking studies. Minimizing contacts for hydrogen, the structures were subjected to an Amber99 energy minimization protocol. The structure of 25-hydroxycholesterol (25-OHC) was minimized, the atomic partial charges were calculated with the MMFF94s force field, and all possible ionization states were generated at pH 7.0 using the MOE suite. 25-OHC was docked into the homology model using MOE software; the binding site was modeled based on previous information on the sterol binding site. 37 The default Triangle Matcher was used as the placement method followed by force field refinement, and London dG scoring was used for the docking. The top scoring conformation of compound was kept for analysis. Ligand interactions were generated by Chimeria. 38

Bone Marrow Transplantation

Recipient LDLr−/− mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using a γ-ray source (Jixing Group) with a 6-mm aluminum filter. Bone marrow cells were isolated from C57BL/6 mice by flushing the femurs and tibias with PBS. Single-cell suspensions were prepared by passing the cell mass through a cell strainer with 27 pond needle. 1×10⁶ cells were infected with either high titer (1×10¹⁰ TU/mL) Lenti-Mock or Lenti-ORP4L lentivirus carrying GFP (green fluorescent protein) tag prepared by Shanghai GenePharma Co. in the presence of 5 μg/mL polybrene (multiplicity of infection=100). At 48 hours post infection, GFP-positive cells were sorted by flow cytometry and injected into the tail vein of the irradiated recipients (1×10⁶ cells per mouse, n=6 mice per group). Starting at 4 weeks post bone marrow transplantation, the mice were fed HFD for 17 weeks.
Figure 1. Oxysterol-binding protein–related protein 4 L (ORP4L) expression profile and effects of ORP4L deficiency on macrophage functions. A, Western blot analysis of ORP4L expression in mice tissues. B, Western blot analysis of ORP4L expression in monocytes and monocyte-derived macrophages. Freshly isolated mice CD115+ monocytes and monocytes stimulated with (Continued)
Atherosclerotic Lesion Analysis

Mice were anesthetized, and blood samples were collected via heart puncture for lipid profile analyses. Then the cardiovascular system was perfused with PBS and fixative (4% paraformaldehyde, 5% sucrose, 0.05% EDTA, pH 7.4). After removal of connective tissue and fat, the aorta was dissected from the aortic root to the iliac artery under a dissection microscope, opened by a longitudinal cut along the ventral surface and immersed in the fixative for 12 hours before being rinsed with PBS and stained with oil red O (Sigma-Aldrich). Stained aortae were washed in 60% isopropanol and imaged with a TOUPCAM industrial digital camera. Quantification of the percentage of aortic surface area occupied by oil-red-O–positive plaques was performed using digital image analysis software (Image-Pro plus 6.0). For analysis of aortic roots, perfused aortas were fixed and embedded in optimal cutting temperature embedding medium. Subsequently, the aortic root area was sliced by a frozen slicer (Leica CM1850). Every 7-μm cross section was collected starting from the end of the aortic sinus. The atherosclerotic lesion areas were stained with oil-red-O–stained and imaged with a TOUPCAM industrial digital camera, and the quantification of atherosclerotic lesion area occupied by oil-red-O–positive plaques was performed using digital image analysis software (Image-Pro plus 6.0). The mean lesion area (in μm²) was quantified by averaging 6 sections per mouse that were spaced 3 sections apart, starting from the base of the aortic root. The aortic sections were also stained with hematoxylin and eosin. Then, the stained aorta were washed in 70% ethanol and imaged with industrial digital camera TOUPCAMTM. The necrosis areas were circumscribed with black lines by using Adobe illustrator CS6.0 (Adobe) based on characteristic morphological features of necrosis (the area of hematoxylin and eosin–negative acellular and anuclear white areas). Plaque necrotic areas were quantified from the average of 6 sections per mouse that were spaced 3 sections apart and normalized to the total lesion area.

Statistics

The data are displayed as means±SD. The n numbers for each group are indicated in the figure legends. All data present in the study fit into a normal distribution and hence a Student 2-tailed t test was used for determining statistical significance between 2 groups, whereas a 1-way ANOVA with Bonferroni correction was applied while evaluating statistical significance between multiple groups. P<0.05 were regarded as statistically significant.

Study Approval

This study was approved by the Institutional Ethics Committee of Jinan University and was performed in accordance with the Declaration of Helsinki. The study was conducted according to an institutional review board–approved protocol, and informed consent was obtained from all subjects. Experiments involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80–23, revised 1996) and according to the institutional ethical guidelines for animal experiments.

Results

ORP4L Is Expressed in Macrophages and Protects Them From Apoptosis

Murine genomic and expressed sequence tag databases indicated that the OSBP2/ORP4 gene encodes ORP4L and 2 potential truncated variants (termed by us ORP4S-1 and ORP4S-2) from alternate transcription start sites in mouse (Online Figure IA). These 2 variants encode 497- and 455-amino acid proteins lacking a PH domain (accession numbers NP_001289560 and NP_001289559), respectively. We first designed PCR primer sets to confirm the presence of ORP4 isoforms in mouse tissues. ORP4L mRNA was present in brain, heart and testis (Online Figure IB, left), whereas the reverse transcription-polymerase chain reaction could hardly detect ORP4S-1 and ORP4S-2 variants in the above 3 tissues (Online Figure IB, right). Therefore, we focused on ORP4L in further functional study of the OSBP2/ORP4 gene.

Similar to previous observations, the ORP4L protein was found constitutively expressed in brain, heart, and testis, but it was virtually absent from other mouse tissues. Surprisingly, we found that the ORP4L protein and mRNA can also be detected in macrophages isolated from the peritoneal cavity of mouse (Figure 1A; Online Figure IB, left). The ORP4L protein was absent in monocytes, but was significantly upregulated in monocyte-derived macrophages differentiated using macrophage colony-stimulating factor (Figure 1B). Heart section staining by immunofluorescence indicated weak expression of ORP4L in cardiomyocytes but strong immunoreactivity in cardiac macrophages (Figure 1C). These results indicated that ORP4L expression is induced during monocyte to macrophage differentiation and may play an important role in macrophage physiology. To study the role of ORP4L in vivo, we generated ORP4L-knockout mice by gene targeting (Online Figure IC and ID) and confirmed the absence of ORP4L protein in macrophages and testes of the knockout animals (Online Figure IE). We examined the cell subsets in peripheral blood and found a similar percentage and
Figure 2. Oxysterol-binding protein–related protein 4 L (ORP4L) reconstitutes a protein complex with Ga_q/11 and phospholipase C (PLC)-β3 and modulates Ca^{2+} signaling in macrophages. 

A, Silver-stained SDS-PAGE gel with anti-ORP4L and control IgG immunoprecipitated proteins from RAW264.7 cells. B, Coimmunoprecipitation analysis of ORP4L binding to Ga_q/11 and PLCβ3 in RAW264.7 cells (top) and peritoneal macrophages (bottom). C, Confocal microscopy analysis of ORP4L (red), Ga_q/11 (blue), and PLCβ3 (green) localization in peritoneal macrophages. Cells were seeded onto coverslips (suspended macrophages) or cultured for 24 h (adherent macrophages). For stimulation, cells were treated with C5a (10 nmol/L) for 3 min before fixation. Scale bars, 10 μm. D, Western blot analysis of ORP4L, Ga_q/11, and PLCβ3 expression in monocytes and during macrophage differentiation. Cells were (Continued)
absolutely numbers of monocytes in wild-type (WT) and knockout animals (Online Figure IF).

Next, we investigated the role of ORP4L in regulation of macrophage responses including migration, phagocytosis, and apoptosis. The effects of ORP4L deficiency on macrophage chemotaxis in response to C5a and SDF-1 were examined: no significant effects were observed on macrophage migration through an endothelial cell monolayer (Figure 1D, left). Likewise, ORP4L deficiency did not affect the ability of macrophages to take up uncoated or ox-LDL–coated FluoSphere beads (Figure 1D, middle) nor did it affect the engulfment of anti–Fas antibody–induced apoptotic Jurkat T cells (Figure 1D, right). As ORP4L deletion has been reported to increase sperm apoptosis,11 we next examined whether ORP4L modulates macrophage apoptosis. When compared with cells from WT mice, a significant increase in macrophage apoptosis was detected in preparations from ORP4L−/− mice (Figure 1E). The combination of tumor necrosis factor-α or lipopolysaccharide with cycloheximide has been shown to induce macrophage apoptosis.9,39 Both inducers induced greater apoptosis of ORP4L-null macrophages than WT macrophages (Figure 1F). Importantly, the increase of apoptosis could be abolished by re-expression ORP4L cDNA in ORP4L-null macrophages (Figure 1F; Online Figure IIA).

The role of ORP4L in regulation of apoptosis was further investigated in peritoneal macrophages overexpressing ORP4L. Oxysterols and ox-LDL are potent inducers of macrophage apoptosis.10,20,40 The number of apoptotic cells was abolished by re-expression ORP4L cDNA in ORP4L-null macrophages isolated from WT or ORP4L−/− mice (n=3 mice per group). Representative changes in [Ca2+] recorded as the F/F0 ratio using Fluo4-AM (left) and average [Ca2+] responses and quantification of [Ca2+] peak amplitudes are shown (right; n=3 mice per group). I, PLC activity (left) and IP3 production (right) in peritoneal macrophages induced by C5a (10 nmol/L, 3 min). Cells were transfected with full-length or truncated ORP4L for 24 h before measurement (n=3 mice per group). J, Calcium efflux induced by 10 nmol/L C5a in peritoneal macrophages on full-length or truncated ORP4L overexpression. Cells were transfected with full-length or truncated ORP4L for 24 h before measurement. The data represent mean±SD from triplicate analyses from each mouse (n=3 mice per group). *P<0.05, **P<0.01, ***P<0.001. NS indicates not significant.
Figure 3. Oxysterol-binding protein–related protein 4 L (ORP4L) maintains ligand-induced Ca²⁺–dependent Bcl-XL expression in macrophages. A, Quantitative reverse transcription-polymerase chain reaction (RT-PCR; left) and Western blot analysis (right) of Bcl-XL expression in peritoneal macrophages isolated from wild-type (WT) or ORP4L−/− mice. B, Quantitative RT-PCR (left) and Western blot analysis (right) of Bcl-XL expression in peritoneal macrophages upon full-length or truncated ORP4L overexpression for 24 h. C, Quantitative RT-PCR (left) and Western blot analysis (right) of Bcl-XL expression in monocytes and during macrophage differentiation. Cells were collected before (D0) and after stimulation with macrophage colony-stimulating factor (50 ng/mL) for 3 d (D3), 5 d (D5), and 7 d (D7). D, Quantitative RT-PCR (left) and Western blot analysis (right) of Bcl-XL expression in peritoneal macrophages isolated from WT or ORP4L−/− mice in the presence or absence of C5a (5 nmol/L) or SDF-1 (stromal cell-derived factor 1; 100 ng/mL) for 24 h. E, Apoptosis analysis of peritoneal macrophages isolated from WT or ORP4L−/− mice (n=3 mice per group) with or without exogenous Bcl-XL expression. Cells were isolated and transfected with Bcl-XL cDNA for 24 h before analysis. F, Confocal microscopy and (Continued)
Figure 3 Continued. cytosolic/nuclear fraction analysis of c-AMP responsive element binding protein (CREB) localization in peritoneal macrophages from WT or ORP4L−/− mice. Line intensity profiles across the cells were obtained in a given image and representative intensity profiles are shown. The relative fluorescence intensity ratio of nuclear area (I_n) vs cytosolic area (I_cyt) is shown (50 cells from three experiments with 10 random fields per experiment) in shown on the right. Scale bars, 10 μm. G, Flow chart showing the inhibitors/ agonists used. H, Western blot analysis of phosphorylated Camk II and CREB levels and Bcl-XL expression in peritoneal macrophages from WT or ORP4L−/− mice. For agonist treatment, cells were incubated with the IP3 receptor agonist adenophostin A (Ada, 1 μmol/L) or the Ca2+ transporter ionomycin (lon, 2 μg/mL) for 16 h. I, Western blot analysis of phosphorylated Camk II and CREB levels and Bcl-XL expression in peritoneal macrophages subjected to ORP4L overexpression and PLC-δ1 expression in peritoneal macrophages subjected to ORP4L overexpression. For inhibitors treatment, cells were transfected with control or ORP4L cDNA for 24 h and then cells were incubated with the phospholipase C (PLC) inhibitor U73122 (1 μmol/L), the IP3 receptor inhibitor xestospongin C (XeC, 1 μmol/L), the Ca2+ chelating agent BAPTA-AM (0.2 mmol/L), the Camk II inhibitor KN-93 (10 μmol/L), or the CREB inhibitor KG-501 (20 μmol/L) for 16 h. J, Western blot analysis of phosphorylated Camk II and CREB levels and Bcl-XL expression in peritoneal macrophages subjected to ORP4L overexpression and PLCδ1 expression. FITC indicates fluorescein isothiocyanate; NS, not significant; PI, propidium iodide; and SSC, side scatter.

(Figure 2H). We then used 2 independent short hairpin RNAs (shORP4L.1 and shORP4L.2) that reduced ORP4L expression with silencing efficiencies of ~80% in RAW264.7 cells (Online Figure IVB). ORP4L knockdown with the short hairpin RNAs reduced PLC activity and IP3 production (Online Figure IVB) in RAW264.7 cells. To demonstrate the specificity and exclude shRNA off-target effects, we performed rescue experiments by re-expression of human ORP4L in these knockdown cells. Full-length ORP4L, but not the truncated protein, rescued the PLC activity, IP3 production, and Ca2+ efflux (Online Figure IVC) dampened on ORP4L knockdown. In contrast, overexpression of full-length ORP4L, but not ORP4LΔ445 to 513 in peritoneal macrophages and RAW264.7 cells increased C5a-induced PLC activity (Figure 2I, left; Online Figure IVD, left), IP3 production (Figure 2I, right; Online Figure IVD, middle), and Ca2+ efflux (Figure 2J; Online Figure IVD, right). In addition, the increased PLC activity and IP3 production on ORP4L overexpression were partly abolished in RAW264.7 cells subjected to Gαq silencing (Online Figure IVE). These results indicated that ORP4L sustains G-protein–coupled ligand-induced Ca2+ signaling in macrophages.

ORP4L Maintains Ca2+-Dependent Bcl-XL Expression

Our recent work evidenced that ORP4L is essential for T-cell acute lymphoblastic leukemia cell survival via sustaining Ca2+-dependent bioenergetics.46 M1 and M2 macrophages use different metabolic programs to fuel their effector function. M1 macrophages mainly use oxidative metabolism for ATP generation.47,48 Different metabolic programs to fuel their effector function. M1 macrophages mainly use oxidative metabolism for ATP generation.47,48

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Figure 4. Oxysterol-binding protein–related protein 4 L (ORP4L) binds to 25-hydroxycholesterol (25-OHC) and counteracts its cytotoxicity. A, Surface representation showing the binding of 25-OHC to ORP4L. B, ORP4L-null peritoneal macrophages were transfected with empty vector, wild-type (WT) or 25-OHC binding site mutant ORP4L (ORP4Lm3) cDNA for 24 h and then (Continued)
sustains Bcl-XL expression through the Ca\(^{2+}\)-dependent CREB pathway.

**ORP4L Binds 25-Hydroxycholesterol and Counteracts Its Cytotoxicity**

Proapoptotic effects of oxysterols have been reported in various cell types, but it has remained unclear whether ORPs are involved. ORP4 has been biochemically demonstrated to bind oxysterols, including 25-OHC.\(^{30,53}\) Ligand interaction studies were performed in silico by using a homology model of the ORP4 ligand-binding domain constructed by using related high-resolution structures as templates.\(^{37}\) As shown in Figure 4A, the interactions between 25-OHC hydroxyl groups and the protein are predicted to be both strong and stable. The 25-OHC hydroxyl groups prefer to establish arene–H interactions with Tyr588 of ORP4L, and the side chains of ORP4L Tyr634 and Lys681 prefer to establish hydrogen bonds with 25-OHC (Figure 4A). In vitro 25-OHC binding assay indicated that ORP4L with the 25-OHC binding site mutated (ORP4L Tyr588Gly, Tyr634Gly, Lys681Gly, designated ORP4Lm3) prevented 25-OHC binding (data not shown). Reoverexpression of the WT ORP4L, but not the ORP4Lm3, dampened apoptosis induced by 25-OHC in ORP4L-null macrophages (Figure 4B). These studies are consistent with the view that 25-OHC binds with high specificity and efficiency within the ORP4 ligand-binding domain of ORP4L.

Excessive treatment with 25-OHC disrupted the complex of ORP4L/Guq/PLC\(\beta3\) as analyzed by coimmunoprecipitation assay in peritoneal macrophages (Figure 4C). Treatment with 25-OHC decreased the Csa-induced PLC activity (Figure 4D, left), IP\(_3\) production (Figure 4D, right), and endoplasmic reticulum Ca\(^{2+}\) efflux (Figure 4E) in peritoneal macrophages, and WT ORP4L overexpression partially rescued these decreases, whereas no rescue was observed in cells overexpressing ORP4Lm3 (Figure 4D and 4E). Treatment with 25-OHC also reduced Camk II and CREB phosphorylation, Bcl-XL expression, and increased peritoneal macrophage apoptosis, which were rescued by WT ORP4L overexpression, but not by the mutant ORP4Lm3 (Figure 4F and 4G). Together, these data suggest that the dysfunction of ORP4L induced by excessive oxysterol leads to the reduction of Bcl-XL expression and subsequent macrophage apoptosis.

**Atherosclerotic Lesions Are Reduced in ORP4L-Deficient Mice**

Knowing that ORP4L-deficient macrophages are hypersensitive to apoptosis, it was reasonable to hypothesize that ORP4L deficiency may affect the formation of atherosclerotic lesions. Therefore, LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice were generated by crossing ORP4L\(^{-/-}\) with LDLr\(^{-/-}\) animals. We visualized ORP4L expression by immunohistochemistry in the aortic lesions of these mice and found significant ORP4L immunoreactivity in LDLr\(^{-/-}\) mice (Figure 5A). Immunofluorescence microscopy showed colocalization of ORP4L with CD68, thus identifying the ORP4L-positive cells in lesions as macrophages (Figure 5A). Importantly, we also found significant ORP4L immunoreactivity in human atherosclerotic vessel wall (clinical information can be found in Online Table II) and colocalization of the ORP4L staining with CD68 (Figure 5B), thus demonstrating the expression of ORP4L in macrophages of human atherosclerotic plaques as well. These observations provide clues for the relevance of our study to human atherosclerosis.

The LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice together with control LDLr\(^{-/-}\) animals were fed a HFD for 17 weeks, and atherosclerotic lesion size in the aortic root and in the aorta en face was analyzed. The LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice displayed a marked reduction in atherosclerotic lesion area when compared with the LDLr\(^{-/-}\) animals: Aortic root lesions in LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice were >50% smaller than in LDLr\(^{-/-}\) mice (Figure 5C). Similarly, knockout of ORP4L led to a significant decrease in plaque burden when compared with the control mice by en face analysis, with an >50% decrease in lesion area (Figure 5D).

To provide additional evidence for a specific role of macrophage ORP4L in atherogenesis, we performed adoptive bone marrow transplantation experiments with hematopoietic cells overexpressing ORP4L. Bone marrow cells from WT mice were transduced with lentivirus-Mock or lentivirus-ORP4L for 48 hours, followed by transplantation into LDLr\(^{-/-}\) mice. The recipient mice were allowed to recover for 4 weeks and then fed a HFD for 17 weeks, followed by analysis of atherosclerotic lesion size in the aortic root. The results demonstrated that ORP4L overexpression in bone marrow cells resulted in a significant increase in aortic root lesion size (Figure 5E, left). ORP4L overexpression in peritoneal macrophages of the animals after 17 weeks of HFD was confirmed by Western blotting (Figure 5E, right). We conclude from the above in vivo observations that ORP4L deficiency suppresses atherogenesis in mice.

**ORP4L Deficiency Is Associated With Decreased Bcl-XL Expression and Increased Macrophage Apoptosis in the Atherosclerotic Lesions**

To examine whether there are differences in Bcl-XL expression in the lesion macrophages of LDLr\(^{-/-}\) and LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice, cells from aortic lesions were dispersed and stained with anti-Bcl-XL, followed by flow cytometry analysis. This revealed a significant decrease of Bcl-XL staining in CD45\(^{+}\)F4/80\(^{+}\)-gated macrophages from aorta of LDLr\(^{-/-}\)ORP4L\(^{-/-}\) mice when compared with the LDLr\(^{-/-}\) animals (Figure 6A). To determine whether ORP4L deficiency increased macrophage apoptosis in atherosclerotic lesions, we first stained serial sections from the proximal aorta with terminal deoxynucleotidyl transferase-mediated dUTP

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**Figure 4 Continued.** incubated without 10 \(\mu\)mol/L 25-OHC for 24 hours and evaluated for cell apoptosis. **C.** Coimmunoprecipitation analysis of ORP4L binding to Goq/PLC\(\beta3\) in peritoneal macrophages treated with or without 10 \(\mu\)mol/L 25-OHC for 24 h. **D-G.** Peritoneal macrophages transected with empty vector, WT, or ORP4Lm3 CDNA for 24 h and then incubated with or without 10 \(\mu\)mol/L 25-OHC for 24 h and evaluated for phospholipase C (PLC) activity (**D**, left), IP\(_3\) production (**D**, right), calcium efflux (**E**) induced by 10 \(\mu\)mol/L Csa, phosphorylated Camk II, and c-AMP responsive element binding protein (CREB) levels and Bcl-XL expression (**F**) and cell apoptosis (**G**). The data represent mean±SD from at least 3 separate experiments each analyzed in triplicate. **P<0.01, ***P<0.001. FITC indicates fluorescein isothiocyanate; NS, not significant; PI, propidium iodide; and SSC, side scatter.
Figure 5. Decreased atherogenesis in mice lacking oxysterol-binding protein–related protein 4 L (ORP4L). A, Sections of lesions from low-density lipoprotein receptor (LDLr)−/− mice were subjected to immunohistochemistry (IHC) using ORP4L antibody. A representative section stained by using antibodies against ORP4L (red) and the macrophage-specific marker CD68 (green) (Continued)
Figure 5 Continued. are shown by immunofluorescence (IF). Scale bars, 10 μm. B, Immunoreactivity is a representative human plaque and a high-magnification view of the same image; immunostaining showed the absence of the ORP4L-positive cells in the aortic ends. C, Representative images of oil-red-O staining of aortic arches from LDLr−/− and LDLr−/−ORP4L−/− mice fed a HFD for 17 wk. Quantification of the lesion area (6 sections per mouse, n=8 mice per group) was performed by using Leica QWin Imaging software. D, Representative images for en face oil-red-O staining of spreads of aortic arches from LDLr−/− (Lenti-Mock) or Lenti-ORP4L→LDLr−/− (Lenti-ORP4L) mice fed a HFD for 17 wk. Quantification of the lesion area (6 sections per mouse, n=6 mice per group) was performed by using Leica QWin Imaging software. Representative Western blot images of ORP4L overexpression in control mice with the use of specific antibodies against ORP4L. E, Representative images of oil-red-O staining of aortic arches from Lenti-Mock→LDLr−/− (Lenti-Mock) or Lenti-ORP4L→LDLr−/− (Lenti-ORP4L) mice after 17 wk HFD are shown. Relative ORP4L overexpression levels in all of the lentivirus-transduced mice were quantified. The data represent mean±SD. *P<0.05, **P<0.01.

nick-end-labeling and the anti–Moma-2 antibody. There were an increased percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling–positive cells in the aortic ends from LDLr−/−ORP4L−/− mice when compared with controls (Figure 6B). As a complementary approach, we quantified the numbers of apoptotic macrophages in the lesions using a fluorescence-activated cell sorter–based assay.54,55 The proportion of CD45/F4/80+ apoptotic macrophages in the LDLr−/−ORP4L−/− mice was significantly higher than that in the controls (Figure 6C). In addition, Moma-2 staining was markedly reduced at the aortic root in LDLr−/−ORP4L−/− mice (Online Figure VI), indicating a reduction in macrophage number. We also quantified the necrosis in the lesions of mice fed a HFD for 17 weeks: significantly less necrosis was found in lesions from LDLr−/−ORP4L−/− mice (Figure 6D). Quantification of the lesion collagen content revealed a slight increase in LDLr−/−ORP4L−/− mice when compared with controls (Online Figure VII). Furthermore, we compared macrophage apoptosis in the mice that received Lenti-Mock or Lenti-ORP4L transduced bone marrow cells. In agreement with the observation of increased lesion size, overexpression of ORP4L in bone marrow cells suppressed macrophage apoptosis in the presence or absence of 25-OHC treatment (Figure 6E). Of note, comparison of serum lipid profiles of the LDLr−/−ORP4L−/− and LDLr−/− mice showed no significant differences in plasma total cholesterol, triglyceride, or choline-containing phospholipid concentrations (Table). Collectively, these results support the notion that ORP4L deficiency is associated with a significant increase in apoptotic macrophages in atherosclerotic lesions, likely because of a reduction of Bcl-XL expression, which in turn contributes to a smaller lesion size and lower numbers of macrophages in the lesions.

Discussion

In this study, we demonstrated that ORP4L is expressed in macrophages and plays an essential role in G–protein–coupled ligand-induced Ca2+ release via forming a complex with Gq/11 and PLCβ3, which contributes to sustained Bcl-XL expression and macrophage survival. Oxysterols execute cytotoxicity via disturbance of a G–protein–coupled ligand-induced Ca2+ signaling pathway at least in part by targeting ORP4L. We further validated the significance of this ORP4L–mediated mechanism in vivo: ORP4L deficiency in macrophages led to a significant reduction of atherogenesis and increased macrophage apoptosis within atherosclerotic lesions.

PLCβ3 has been identified as the predominant enzyme catalyzing IP3 production in macrophages.10 In particular, stimuli from the extracellular microenvironment via the G–protein–coupled receptors that control Ca2+ influx, sustain macrophage survival. In this current study, ORP4L is found expressed in macrophages but not in monocytes. It interacts with Gq/11 and PLCβ3 to regulate both PLC activity and IP3 production. These signaling events maintain Ca2+ release from endoplasmic reticulum and subsequently Bcl-XL expression in response to ligand stimulation, indicating that ORP4L is essential for signaling responses to the microenvironment within the arterial wall.

It is well accepted that chemokines in the microenvironment and their receptors have important roles in atherogenesis;12–14; these roles of chemokines have been largely attributed to their regulation of macrophage recruitment. However, chemokines such as SDF-1 and MCP-1 are able to protect macrophages from apoptosis, suggesting that such chemokines may also contribute to atherogenesis by regulating macrophage survival. Because ORP4L is undetectable in monocytes, we hypothesize that this protein may not contribute to monocyte recruitment to the arterial wall. How ORP4L expression is regulated and whether it plays a role during macrophage differentiation at sites of arterial lesions need further investigation. Although ORP4L deficiency blocks chemokine-induced Ca2+ signaling and increases macrophage apoptosis, it is conceivable to postulate that macrophages develop a protective mechanism supported by ORP4L in a specific physiological environment of the vessel wall. This mechanism may be highly relevant particularly in the atherosclerotic lesions, in which both chemokine ligands and oxysterols/ox-LDL are abundantly present.

Ox-LDL is known to exert cytokotoxic effects and to induce apoptosis in various cell types including macrophages. Recently, studies documented the presence of oxysterols in mouse macrophage foam cells including 7α-hydroxycholesterol, 7-ketocholesterol, 4β-hydroxycholesterol, 22(R)-hydroxycholesterol, 24(S), 25-epoxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (http://lipidmaps.org/#). Consistently, oxysterols are abundant in human atherosclerotic plaques.56–60 Although there is no direct evidence in humans that oxysterols contribute to atherogenesis, they are thought to play an active role in plaque development. To understand the mechanisms underlying oxysterol cytotoxicity, observations by us and others have revealed that oxysterols/ox-LDL regulate cytosolic Ca2+ concentration and oscillations.61–63 In the present study, we built a molecular model to analyze in detail the binding of 25-OHC to ORP4L and to show a specific role of ORP4L in the cellular effects of 25-OHC by using a mutant protein with the binding site inactivated. Excessive stimulation by...
oxysterol interfered with the ORP4L/\(\alpha\)q/11/PLC\(\beta\)3 complex and blocked Ca\(^{2+}\) signaling, which reduced ligand-induced Bcl-XL expression and resulted in macrophage apoptosis. These results are similar to those observed on ORP4L deletion/knockdown. Macrophages from mice lacking ORP4L showed hypersensitivity to apoptosis, whereas overexpression of WT ORP4L, but not the oxysterol-binding–deficient mutant, reduced this hypersensitivity. Importantly, macrophages isolated from atherosclerotic lesions lacking ORP4L also showed hypersensitivity to apoptosis when compared with control cells. These data suggest that selective expression of ORP4L in macrophages supports chemokine ligand-induced

Figure 6. Oxysterol-binding protein–related protein 4 L (ORP4L) deficiency increases macrophage apoptosis in lesions. A, Cells dispersed from mouse aortas were subjected to Bcl-XL intracellular staining; the intensity of the staining was analyzed by flow cytometry. Macrophages were gated by CD45\(^{+}\)F4/80\(^{+}\) staining. Representative flow cytometry images are shown in left. Relative Bcl-XL immunofluorescence intensity (IFI) was determined (n=5 mice per group; right). B, Representative images of the cross sections of aortic roots stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL; green) and Moma-2 (red, left). Scale bars, 20 \(\mu\)m. Arrows indicate typical apoptotic macrophages. Quantification of macrophage apoptosis expressed as the percentage of TUNEL positive cells relative to Moma-2–positive cells are shown (6 sections per mouse, n=8 mice per group). C, Apoptosis percentages of macrophages dispersed from aortas of LDLr\(^{−/−}\), LDLr\(^{−/−}\)ORP4L\(^{−/−}\), LDLr\(^{−/−}\)ORP4L\(^{−/−}\), and LDLr\(^{−/−}\)ORP4L\(^{−/−}\) mice. Representative images of TUNEL positive cells relative to CD45 positive cells. D, Representative histological analysis of cross sections from the aortic sinus stained with hematoxylin and eosin. Macrophages were gated by CD45\(^{+}\)F4/80\(^{+}\) staining. E, Apoptosis percentages of macrophages dispersed from aortas of Lenti-Mock→LDLr\(^{−/−}\) (Lenti-Mock) and Lenti-ORP4L→LDLr\(^{−/−}\) (Lenti-ORP4L) mice (right, n=5 mice per group). Cells were incubated with or without of 10 \(\mu\)mol/L 25-OHC for 24 h before analysis. The data represent mean±SD.*P<0.05, **P<0.01, ***P<0.001. FITC indicates fluorescein isothiocyanate; PI, propidium iodide; and SSC, side scatter.
signaling to maintain Ca\(^{2+}\) homeostasis, but ORP4L function may be disrupted in the lipid-laden macrophages at arterial lesion sites (Figure 7). The above functions of ORP4L were subsequently verified in an in vivo model of atherosclerosis development. Deletion of ORP4L decreased Bcl-XL expression and increased apoptosis of lesion macrophage, associated with a smaller lesion size.

The impact of macrophage apoptosis on the progression of atherosclerosis depends on the stage of lesions and is a remarkably complex issue.\(^{64}\) In the early stages of lesion development, apoptosis of macrophages within the vascular wall seems to be counterbalanced by rapid and efficient engulfment and removal of the apoptotic cells by phagocytic cells, a process termed efferocytosis. Under these conditions apoptosis is suggested to reduce the number of macrophages within the lesion, resulting in the long run in a reduced lesion size.

Examples of such a beneficial effect of macrophage apoptosis on lesion burden are as follows: ABCG1\(^{-/-}\) LDLr\(^{-/-}\) animals,\(^{65}\) ABCA2 deletion in mouse macrophages,\(^{66}\) and ATGL deficiency in mouse macrophages.\(^{67}\) Consistently, deficiency of the proapoptotic key regulator p53 tended to reduce apoptosis, whereas it increased lesion area, lesion macrophage area, and necrotic core area.\(^{68}\)

On the one hand, there is evidence that increased apoptosis of macrophages in advanced lesions, in which the clearance of apoptotic cells is impaired,\(^{69}\) leads to enhanced lesion progression, expansion of the necrotic core, and increased risk of plaque rupture. Under these conditions, macrophage apoptosis can be enhanced by, for example, elevated endoplasmic reticulum stress,\(^{70}\) granulocyte macrophage colony-stimulating factor–induced IL-23 production,\(^{71}\) or excessive accumulation of oxysterols.\(^{72}\) Especially relevant for the present study, PLC\(\beta\)3 deletion results in a reduction in atherosclerotic lesions with increased macrophage apoptosis and reduced Bcl-XL expression.\(^{10}\) On the other hand, Thorp et al\(^{73}\) showed that macrophage deficiency of the antiapoptotic protein Bcl-2 related to Bcl-XL resulted in increased lesional macrophage apoptosis and increased necrotic area, suggesting a critical role of Bcl-2 in macrophage survival in advanced lesions; However, no change of lesion size was observed in that study.

In our study, loss of ORP4L in LDLr\(^{-/-}\) animals resulted in increased macrophage apoptosis and a marked reduction of the size of atherosclerotic lesions. We envision, based on the above literature that the proapoptotic effect of ORP4L deficiency mainly manifests at the early stages of lesion development reducing in the presence of efficient efferocytosis, the number of macrophages within the developing lesions and

### Table. Analysis of Plasma Lipid Levels in LDLr\(^{-/-}\) and LDLr\(^{-/-}\)-ORP4L\(^{-/-}\) Mice

<table>
<thead>
<tr>
<th></th>
<th>LDLr(^{-/-})</th>
<th>LDLr(^{-/-})-ORP4L(^{-/-})</th>
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<tbody>
<tr>
<td>TG mmol/L</td>
<td>Before 0.95±0.29</td>
<td>Before 1.19±0.32</td>
</tr>
<tr>
<td></td>
<td>After 1.97±0.43</td>
<td>After 2.24±0.57</td>
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<tr>
<td>TC mmol/L</td>
<td>Before 14.02±3.37</td>
<td>Before 14.02±4.01</td>
</tr>
<tr>
<td></td>
<td>After 28.23±7.96</td>
<td>After 32.45±5.28</td>
</tr>
<tr>
<td>PL mmol/L</td>
<td>Before 4.25±0.52</td>
<td>Before 4.70±0.49</td>
</tr>
<tr>
<td></td>
<td>After 11.06±1.44</td>
<td>After 12.04±1.55</td>
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Before and after feeding high-fat diet for 17 weeks, plasma of LDLr\(^{-/-}\) and LDLr\(^{-/-}\)-ORP4L\(^{-/-}\) mice were collected for analysis of TG, TC, and choline-containing PL. The data represent mean±SD (n=8 mice/group). PL indicates phospholipids; TC, total cholesterol; and TG, triglycerides.

Figure 7. Model outlining the functions of oxysterol-binding protein–related protein 4 L (ORP4L) in macrophages. ORP4L forms a complex with G\(\alpha\)q11 and phospholipase C (PLC)\(\beta\)3 and facilitates G-protein–coupled ligand–induced PLC\(\beta\)3 activation, IP\(_3\) production, and Ca\(^{2+}\) release, and subsequently Bcl-XL expression for macrophage survival. Excessive stimulation with oxysterols disassembles the ORP4L/G\(\alpha\)q11/PLC\(\beta\)3 complex, disturbs Ca\(^{2+}\) signaling, reduces Bcl-XL expression, and results in macrophage apoptosis via targeting ORP4L. CREB indicates c-AMP responsive element binding protein; and ER, endoplasmic reticulum.
thus slowing down the progression of the plaques. We find it possible that the observed reduction of Bcl-XL in LDLr−/− ORP4L−/− lesions associated with reduced lesion size could reflect a protective function of Bcl-XL at an earlier stage of lesion development than that of Bcl-2.

Albeit the present data suggest that the increased sensitivity ORP4L−/− macrophage may explain the observed lesion phenotype, the present data do not exclusively demonstrate a causal relationship between the 2 phenomena. Thus, further study of the mechanisms underlying the reduced lesion size is warranted. Furthermore, the plaques after 17 weeks’ HFD feeding represent a relatively advanced stage. Changes in their size and composition thus reflect cumulative effects that may have arisen at different stages of the atherogenic process. Therefore, the data do not allow us to draw firm conclusions on the effects of ORP4L deficiency at any specific stage of lesion development.

Protection of macrophages by ORP4L from the proapoptotic effect of 25-OHC may be effective in early lesions in which oxysterol concentrations are moderate, and apoptotic cells can still be efficiently cleared, resulting in reduced macrophage numbers and slowing down of the plaque progression in the absence of ORP4L. In advanced lesions, the accumulation of oxysterols is so excessive and unphysiological that the protective function of proteins such as ORP4L most likely becomes insufficient to prevent further progression of the plaque. Our data suggest that excessive oxysterols/ox-LDL disturb the function of ORP4L, resulting in macrophage apoptosis, which may slow down lesion development at the early stages of atherogenesis but promote the progression of advanced lesions.

In summary, we show that ORP4L is required for G-protein–coupled ligand-induced signaling and Bcl-XL expression in macrophages. ORP4L is thus crucial for macrophage survival. The pathway discovered in this study also offers one mechanistic explanation for the oxidation injury theory of atherogenesis. Our findings point out a key role of ORP4L in the development of atherosclerosis and suggest that this protein represents a potential new target for treatment of this disease.

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Disclosures
None.

References


What Is Known?
• Macrophage survival influences the development of atherosclerotic plaques within the arterial wall.
• Oxysterols/oxidized low-density lipoprotein induce macrophage death.
• Oxysterol-binding protein–related protein 4L (ORP4L) binds oxysterols and is essential for the survival of specific cell types.

What New Information Does This Article Contribute?
• ORP4L is expressed in macrophages and protects them from apoptosis.
• ORP4L forms a complex with Gαq/11 and phospholipase Cβ3 to facilitate Ca2+ signaling and antiapoptotic Bcl-XL expression.
• Excessive stimulation with oxysterol, a major component of oxidized low-density lipoprotein, disassembles the ORP4L/Gαq/11/phospholipase Cβ3 complexes, resulting in macrophage apoptosis.
• ORP4L-knockout mice display increased macrophage apoptosis in atherosclerotic lesions and a reduced lesion size, indicating that this protein represents a potential new target for the prevention/treatment of atherosclerosis.
ORP4L Facilitates Macrophage Survival via G-Protein–Coupled Signaling: ORP4L−/− Mice Display a Reduction of Atherosclerosis

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SUPPLEMENTAL MATERIAL

Detailed Methods

Reagents and antibodies
Fluoro4-AM, TRITol reagent, Alexa Fluor-488 goat anti-mouse IgG, Alexa Fluor-543 goat anti-rabbit IgG and Alexa Fluor-647 donkey anti-goat IgG were purchased from Invitrogen (Carlsbad, CA). 25-OHC, Hoechst 33342, U73122, XeC, C5a, SDF-1, CHX, TNF-α, LPS, M-CSF and Oil red O were from Sigma-Aldrich (St. Louis, MO). Ionomycin and STO-609 were from Merck Millipore (Billerica, MA). KN-93, KG-501 and BAPTA-AM were purchased from Selleckchem. Adenophostin A was from Santa Cruz. Anti-PLCβ3, anti-Gαq/11, anti-IP3R1, anti-IP3R2 and anti-IP3R3 were from Santa Cruz (Santa Cruz, CA). Anti-p-PLCβ3 (ser537), anti-Bcl-XL, Alexa Fluor® 488 anti-Bcl-XL, anti-p-CREB (ser133), anti-CREB, anti-p-CamKII (Thr286), anti-CamK II were from Cell Signaling (Beverly, MA). Anti-CD68 was from Boster (Wuhan, China) and anti-Moma-2 from Abcam. PerCP/Cy5.5 anti-mouse CD45, Alexa Fluor® 647 anti-mouse F4/80 and Anti-H2A was from BioLegend (San Diego, CA). Anti-actin from Proteintech Group (Chicago, IL). Anti-ORP4L from Sigma-Aldrich was used for all of the experiments except for ORP4L-associated proteome analysis.

cDNA constructs
Full length human ORP4L cDNA (NM_030758.3), Bcl-XL cDNA (NM_138578.2), the truncated ORP4L cDNA without aa 445-513 were amplified by PCR and inserted into pcDNA4HisMaxC (Invitrogen) vector. The 25-OHC binding sites mutant ORP4L cDNA (ORP4Lm3) was generated by PCR-based site-directed mutagenesis and inserted into pcDNA4HisMaxC vector. A series of ORP4L cDNA deletion fragments were generated in the bait vector pGBDKT7 (Clontech Laboratories). PLCβ3 cDNA (NM_000932.2) was inserted in the prey vector pGADT7 (Clontech Laboratories). Oligonucleotide primers used can be found in Online Table III.

Cell culture and gene transfer
Peritoneal macrophages were prepared 72 hr after injection of mice with 1 ml 3% thioglycolate broth (Sigma-Aldrich). Peritoneal cavities were lavaged with 10 ml PBS and centrifuged to collect peritoneal macrophages. Peritoneal macrophages were cultured in RPM1640 supplemented with 10% FBS. RAW264.7 cells were cultured in DMEM supplemented with 10% FBS. For gene transfer, peritoneal macrophages were transfected using the Amaxa Mouse Macrophage Nucleofector Kit (Lonza) according to the manufacturer’s protocol. RAW264.7 cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. To knock down endogenous ORP4L, RAW264.7 cells were infected with high titer lentivirus (1 × 10⁹ TU/ml) encoding shORP4L.1 or shORP4L.2 prepared by Shanghai GenePharma Co. Briefly, 1 × 10⁵ RAW264.7 cells were cultured overnight on 6-well culture plates; the following day, 400 μl medium with lentivirus (MOI=50) and 5 μg/ml polybrene were added. Infections were carried out for 6 hr at 37°C, 5% CO₂. At the end of the infection, 1.6 ml medium was added.
Knockdown efficiency was checked by western blotting after 3 days’ culture. The shRNA/siRNA sequences used can be found in Online Table IV.

**Monocyte isolation and macrophage derivation**
CD115⁺ monocytes were isolated from mouse bone marrow by using CD115 MicroBeads Kit (Miltenyi Biotec, Gladbach, Germany). Cells were cultured in RPMI1640 with 10% FBS and 50 ng/ml of M-CSF for 7 days according to the manufacturer’s instructions. Every 2-3 days, half of the medium was removed and replaced with fresh medium plus M-CSF (50 ng/ml). At the day 0, day 3, day 5 and day 7, cells were collected for western blot analysis of ORP4L, Goαq, PLCβ3 and Bcl-XL protein expression. At the day 7, cells were also cultured for further 24 hr with LPS (100 ng/ml) or with IL-4 (20 ng/ml) for M1 and M2 polarization, respectively.

**Generation of ORP4L knockout mice**
Mouse ORP4 genomic DNA sequences were cloned from a C57BL/6-derived genomic DNA and subcloned into the PBR322 vector. The targeting vector contains LoxP-flanked exon 2 along with an Frt-flanked neo selection cassette. The targeting vector was linearized and electroporated into ES cells. Stable clones were selected for G418 resistance. The G418-resistant and correctly targeted clones were confirmed by PCR. Targeted ES cells were microinjected into blastocysts to produce germline chimeric mice. To generate the conditional, loxP-flanked allele (neo-free) for conditional gene targeting, chimeras were crossed to a FLP deleter strain, and the FRT-flanked neo selection marker was excised. Mice harboring the conditional allele were crossed with Cre transgenic mice to generate offspring heterozygous for the conditional allele plus the Cre transgene. To recombine the ORP4L<sup>lox</sup> allele using the Cre-ERT2 transgenic system, Tamoxifen (Sigma-Aldrich) was solubilized at 20 mg/ml in a mixture of 98% corn oil and 2% ethanol and delivered into mice by intraperitoneal injection (0.5 mmol/g body weight, once per day for 5 days). After genotyping by PCR analyses of genomic DNA isolated from tail biopsies, littermate crossing was carried out to obtain offspring with ORP4L stably knocked out.

**Apoptosis assay**
To analyze RAW264.7 cell apoptosis, the cells were incubated with or without 10 μM 25-hydroxycholesterol (Sigma-Aldrich) or 100 μg/ml ox-LDL (Sigma-Aldrich) in DMEM for 24 hr at 37°C and stained with annexin V/propiidium iodide using the AnnexinV-FITC Apoptosis Kit (eBioscience, San Diego, CA). To analyze the peritoneal macrophages, cells were isolated freshly and stained with anti-F4/80 antibody, then stained with annexin V/propiidium iodide and analyzed by flow cytometer (FACSARiaTM, BD). To test sensitivity of macrophages to TNF-α, LPS, 25-OHC or ox-LDL, they were incubated with or without TNF-α (10 ng/ml)/CHX (10 μg/ml), LPS (1 μ g/ml)/CHX (1 μ g/ml), 10 μM 25-OHC or 100 μM/mL ox-LDL for 24 hr before staining. Cells were detected by using flow cytometer (FACSAriaTM, BD Bioscience, San Jose, CA) and the data were analyzed by FlowJo_V10 software.
Co-immunoprecipitation
Aliquots of $10^7$ RAW264.7 cells were washed twice with ice-cold PBS and incubated for 30 min on ice with 1 ml lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 0.5 mM MgCl₂, 10% glycerol, and 0.5% Triton X-100, pH 8.0) supplemented with Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland). Cell lysates were centrifuged for 10 min at 12,000 × g. The supernatant was pre-absorbed for 1 hr at 4°C with 50 µl of Protein G agarose (Invitrogen). The recovered supernatant was incubated with ORP4L antibody (Sigma-Aldrich) or control antibody at 4°C overnight. 50 µl Protein G agarose was added to the lysate-antibody mixture and incubated at 4°C on a roller for 2 hr. Agarose beads were washed four times with lysis buffer and boiled in 30 µl of SDS-PAGE loading buffer. Samples were resolved on 10% SDS-PAGE and subjected to western blot analysis.

Immunofluorescence microscopy
Cells seeded onto coverslips were fixed for 30 min with 4% paraformaldehyde at room temperature, followed by permeabilization with 0.1% Triton X-100 for 5 min, and blocked for 30 min with 10% FBS at room temperature. Cells were then incubated with anti-ORP4L (Sigma-Aldrich), anti-PLCβ3 (Santa Cruz, CA) and anti-Gαq11 (Santa Cruz) in 5% FBS at 4°C overnight. After washing 3 times (10 min each) with PBS, cells were incubated with secondary antibodies conjugated with fluorophore at 37°C for 30 min. Alexa Fluor 543 conjugated goat anti-rabbit IgG (Invitrogen) were used for detection of ORP4L, Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen) for detection of PLCβ3 and Alexa Fluor 647 conjugated donkey anti-goat IgG (Invitrogen) for detection of Gαq11. The specimens were analyzed by confocal microscopy (Zeiss LSM 510 Meta).

Determination of phospholipase C activity
Phospholipase C activity was analyzed by using the EnzChekR Direct Phospholipase C Assay Kit (Molecular Probes) according to the manufacturer’s instructions. Briefly, $1 \times 10^5$ cells in black 96-well plates (Greiner, Germany) were treated with 10 nM C5a (Sigma-Aldrich) for 3 min, then 100 µL PLC substrate was added. The microplates were covered and incubated for 30 min at room temperature, protected from light. Fluorescence was measured in Microplate Reader (Synergy™ 4 Hybrid, BioTek, Winooski, VT). A standard curve was used to determine the sample PLC activities.

Measurement of IP3 production
IP₃ was measured using the HitHunter IP₃ Fluorescence Polarization Assay Kit (Discover Rx Tech, Fremont, CA). Briefly, $2 \times 10^4$ cells in black 384-well plates (Greiner, Germany) were treated with 10 nM C5a (Sigma-Aldrich) for 3 min, and the reaction was terminated by adding 0.2 N perchloric acid. The plate was shaken at 650 rpm for 15 min. The IP₃ tracer was subsequently added to each well, followed by the IP₃ binding protein. The polarized fluorescence from the IP₃ tracer was read on a Microplate Reader (Synergy™ 4 Hybrid, BioTek). The IP₃ value was calculated from an IP₃ standard curve.

Reverse transcription-PCR
Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s
instructions. RNA samples were reverse transcribed using random hexamer primers in the presence of RNase inhibitor (Takara Bio, Mountain View, CA). Genomic and expressed sequence tag databases indicated that the \textit{OSBP2/ORP4} gene encodes ORP4L as well as two truncated variants (termed ORP4S-1 and ORP4S-2) from alternate transcription start sites in mouse. PCR primer sets were designed to amplify specific ORP4 isoforms: ORP4L, ATGATTCTGGGGACGACGAC and AAGATGTAGAGTCTTCCATGGCG with a product of \(534\) bp; ORP4S-1 and ORP4S-2, TCAGCATCAGGGCAGCAGA and AAGATGTAGAGTCTTCCATGGCG with products of \(455\) and \(497\) bp, respectively. PCR conditions were optimized (30 cycles), and the products were separated by \(1.5\%\) (w/v) agarose gel electrophoresis and visualized with ethidium bromide.

**Cell migration assays**

Cell migration assays were performed using 24-well Transwell plates (5-µm pore size; Costar, Washington, DC). Transwell inserts were cultured with a layer of mouse embryonic immortalized endothelial cells and then \(2 \times 10^6\) peritoneal macrophages were loaded into the upper chambers. The lower chambers were filled with DMEM with 1% FBS in the absence or presence of 5 nM C5a or 100 ng/mL SDF-1. The Transwell plates were then incubated at \(37^\circ\text{C}\) for 4 hr. Macrophages attached to the lower surfaces of inserts were trypsinized and collected for counting and FACS analysis after staining with the anti-F4/80 antibody (BioLegend).

**Phagocytosis assay**

Peritoneal macrophages were seeded to culture plates for 3 hr at \(37^\circ\text{C}\). Uncoated FluoSphere beads (Invitrogen) and those coated with ox-LDL were added to the cultures after unattached cells were rinsed away. After 10 minutes of incubation at \(37^\circ\text{C}\), excessive beads were washed away, and the macrophages were detached by trypsinization and analyzed by FACS after being stained with the anti-F4/80 antibody.

For the phagocytosis assay of apoptotic Jurkat T-cells, the apoptosis was induced by incubating Jurkat T-cells with an anti-Fas antibody (Santa Cruz) for 6 hr in \(37^\circ\text{C}\). Apoptosis was verified by annexin V/propidium iodide staining. Apoptotic Jurkat T-cells were then labeled with Vybrant CFDA SE (Invitrogen) and added to peritoneal macrophages. After 3 hr of incubation at \(37^\circ\text{C}\), the unbound Jurkat T-cells were washed away before macrophages were detached and stained with anti-F4/80 antibody. The cells were analyzed by a flow cytometer (FACSAriaTM, BD Bioscience, San Jose, CA). The mean fluorescence intensity of CFDA SE gated for F4/80-positive cells is shown as the number of apoptotic Jurkat T-cells associated with macrophages.

**Yeast two-hybrid assays**

For identification of the interacting region of ORP4L required for PLCβ3 binding, the truncated fragments of ORP4L in bait vector pGBDKT7 and PLCβ3 in prey vector pGADT7 were co-transformed into the yeast strain AH109 before plating on SD/2- (-Leu-Trp) and SD/4- (-Adc-His-Leu-Trp) plates and cultured at \(30^\circ\text{C}\). The colonies that appeared were then transferred onto membrane for X-gal assay.
ATP generation measurement
The ATP levels of macrophages were determined by using ATP Bioluminescence Assay Kit
CLS II (Roche Diagnostics) according to the manufacturer’s instructions.

Isolation of nuclear and cytosolic fractions
Nuclear and cytosolic fractions were isolated by using a Nuclear/Cytosol Fractionation Kit
(Biovision, Milpitas, CA) according to the manufacturer's instructions.

Quantitative real-time PCR
Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s
instructions. RNA samples were reverse transcribed using random hexamer primers in the
presence of RNase inhibitor (Takara Bio). qRT-PCR was performed with SYBR Premix EX
Taq (Takara Bio) using a 7300 Sequence Detection System (Applied Biosystems). A relative
quantification analysis was performed using the ΔΔCt method, with actin as an endogenous
reference. Relative gene expression is presented as the ratio of target gene to reference. The
primer sequences used were: Bcl-XL (sense 5’-CTGTGCGTGAAAGCGTGA-3’,
anti-sense 5’-GTCAGAAACCCGCTTGGA-3’); Actin (sense 5’-
GGCATCCTCACCTGAAGTA-3’, anti-sense 5’-AGGTGTGGTGCCAGATTTC -3’).

Immunohistochemistry and TUNEL staining
For Moma-2 and ORP4L staining, tissue sections were heated in 0.01 mol/L citrate buffer
(pH 6.0) for 10 min in a microwave oven (750 W), and endogenous peroxidase was blocked
with hydrogen peroxide and methanol. Sections were blocked in 10% BSA/PBS for 30 min
and incubated with the macrophage marker Moma-2 (Serotec) or ORP4L (Sigma-Aldrich)
antibodies overnight. After 3 washes (10 min each) with PBS, sections were incubated with
biotinylated secondary antibodies for 30 min, followed by 30 min incubation with
ABCComplex/HRP. Finally, the sections were stained with 3, 30-diaminobenzidine (DAB)
and counterstained with hematoxylin. Then the sections were mounted under glass coverslip
and imaged with a TOUPCAM™ industrial digital camera.

For immunofluorescence double staining, the sections were blocked with 10% BSA/PBS
after microwave antigen retrieval and incubated with the ORP4L and CD68 antibodies
diluted in 5% BSA for 120 min at room temperature. After 3 washes (10 min each) with PBS,
the sections were incubated with fluorescently labelled secondary antibodies for 30 min at
37°C (Alexa Fluor 488 conjugated goat anti-mouse IgG for detection of CD68 and Alexa
Fluor 543 conjugated goat anti-rabbit IgG for detection of ORP4L). The sections were
counterstained with Hoechst 33342 (Sigma-Aldrich), mounted in fluorescence mounting
medium (Invitrogen), and analysed using confocal microscopy (Zeiss LSM 510 Meta).

For TUNEL staining, the In Situ Cell Death Detection Kit (Roche Diagnostics) was used
according to the manufacturer’s protocol: Sections were fixed with 4%
paraformaldehyde/PBS, pH 7.4, for 20 min at RT. After washing 30 min with PBS, slides
were incubated with 3% H2O2 in methanol for 10 min at RT, and then placed in citrate buffer
(0.1M citrate, pH 6.0) at 95°C for 5 min after washing the slides with PBS. The slides were
cooled rapidly by immediately adding 80 ml H2O, and washed with PBS for 3 times,
followed by incubation in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate,
4°C) for 2 min on ice. Finally, the slides were incubated in TUNEL reaction mixture (50 μm label solution and 450 μl enzyme solution) for 1 hr at 37°C and washed with PBS. For Moma-2 staining of the same slide, the slides were incubated in 0.1 M Tris-HCl, pH 7.5, containing 3% BSA and 20% normal bovine serum for 30 min at RT. Then the primary Moma-2 antibody diluted in 5% normal bovine serum was added and incubated for 1 hr at 37°C. After PBS washes, secondary antibody was added and incubated for 30 min at 37°C. Then, nuclei were counterstained with Hoechst 33342 for 10 min. Finally, tri-color immunofluorescence stained (TUNEL, Moma-2 and Hoechst 33342) specimens were photographed by using Olympus VS120 and viewed by Olympus OlyVIA 2.7 software. TUNEL- and Moma-2 positive stained cells were counted. 6 sections from each mouse were stained and quantified. The data are expressed as the number of TUNEL-positive relative to Moma-2-positive cells.

Lesion cell isolation and analysis
Lesions were quickly dissected, minced and incubated with 1 × Aorta Dissociation Enzyme stock Solution (ADES) (125 U/ml collagenase type XI, 60 U/ml Hyaluronidase type 1-s, 60 U/ml DNase I and 450 U/ml Collagenase type I in 2.5 ml of PBS). Cells were separated as described by Butcher et al. To quantify macrophage apoptosis, Cells were treated with or without 10 μM 25-OHC for 24 hr at 37°C before staining with anti-CD45, anti-F4/80 antibody and annexin V/propidium iodide. The apoptotic macrophages were defined by sequential gating beginning with FSC-SSC to select intact cells, subgating on the CD45+/F4/80+ populations, and calculating the numbers of AnnexinV-FITC-positive cells. To quantify Bcl-XL expression of macrophages, cells were stained with anti-CD45, anti-F4/80 antibody, penetrated by using BD cytoFix/Perm kit and stained with anti-Bcl-XL. Cells were detected by using flow cytometer (FACSAriaTM, BD Bioscience, San Jose, CA) and the data were analyzed by FlowJo_V10 software.

Masson’s trichrome staining
For masson trichrome staining, cryosections were fixed with Bouin’s fixative for 8 hours. After washing in water to remove yellow color, the sections were stained in Working Weigert’s Iron Hematoxylin Solution for 5 min. Then the sections were washed in water for 5 min and stained with Ponceau Red Solution for 3 to 5 min. Subsequently, the sections were rinsed in deionized water and placed in Phosphomolybdic Acid Solution for 5 min, followed by staining in Aniline Blue Solution for 5 min. Finally, after washing with 1% acetic acid for 2 min and dehydration in 95% ethanol and xylene, the sections were mounted under glass coverslips and imaged by light microscopy and an industrial digital camera TOUPCAM™. All quantifications were done blind by digital image analysis software (Image-Pro plus 6.0). 4 sections from each mouse were stained and quantified.

Analysis of plasma lipids
Total plasma cholesterol (Cayman Chemical), choline-containing phospholipids (Sigma-Aldrich) and triglycerides (Cayman Chemical) were measured using enzymatic methods according to the manufacturers’ instructions.
Online Figure 1. *OSBP2/ORP4* gene structure, splice variants and the strategy employed to generate ORP4L-knockout mice. (A) The organization of the *OSBP2/ORP4* gene and the predicted splice variants. (B) ORP4 transcripts in mouse tissues analyzed by RT-PCR and variant-specific primers. (C) Schematic representation of the ORP4 gene-targeting strategy. (D) ORP4L-knockout was induced by tamoxifen injection. Heterozygous littermates were then crossed to obtain offspring with ORP4L stably knocked out. (E) Western blot analysis of ORP4L expression in peritoneal macrophages and testes from wild-type (WT) and
ORP4L-knockout (ORP4L<sup>−/−</sup>) mice. (F) Flow cytometric analysis and quantification of monocytes in blood of WT and ORP4L<sup>−/−</sup> mice (n=4). The data represent mean ± S.D. NS, not significant.

**Online Figure II. Decreased sensitivity to induction of apoptosis in peritoneal macrophages with ORP4L overexpression.** (A) Representative images of the FACS analyses for Figure 1F. (B) ORP4L overexpression reduced 25-OHC and ox-LDL induced apoptosis in RAW264.7 cells. After transfection of ORP4L cDNA for 12 hr, cells were incubated with or without 10 μM 25-OHC or 100 μM/mL ox-LDL for 24 hr, followed by determination of apoptosis. The data represent mean ± S.D. from at least three separate experiments each analyzed in triplicate. **p < 0.01.
Online Figure III. Identification of the PLCβ3 binding region in ORP4L. (A) The truncated ORP4L constructs used in the yeast two-hybrid bait. Functional domains of ORP4L, pleckstrin homology (PHD), FFAT motif (FFAT) and OSBP-related domain (ORD) are indicated. (B) The interaction was monitored by growth on SD/4- medium (middle) and the use of X-gal assay (bottom).
Online Figure IV. The role of ORP4L in PLC activity, IP₃ production, Ca²⁺ efflux and Bcl-XL expression in RAW264.7 cells. (A) Western blot analysis of p-PLCβ3 levels induced by C5a (10 nM, 3 min) in RAW264.7 cells upon full-length or truncated ORP4L overexpression. Cells were transfected with empty vector (Mock), full-length or truncated ORP4L (∆445-513) for 24 hr. (B) PLC activity (left panel) and IP₃ production (middle panel) in RAW264.7 cells induced by C5a (10 nM, 3 min) after ORP4L knockdown for 72 hr. Western blot (right panel) indicating ORP4L knockdown efficiency with the shRNAs used. (C) PLC activity (left panel), IP₃ production (middle panel) and Ca²⁺ efflux (right panel) in RAW264.7 cells transfected with shORP4L, shORP4L + ORP4L or shORP4L + truncated ORP4L. (D) PLC activity (left panel), IP₃ production (middle panel) and Ca²⁺ efflux (right panel) in RAW264.7 cells transfected with Bcl-XL, ORP4L, ORP4L (∆445-513) or ORP4L + Bcl-XL.
panel) in RAW264.7 cells induced by C5a. Cells were transfected with full-length or truncated ORP4L for 24 hr before measurement. (E) PLC activity (left panel) and IP₃ production (right panel) in RAW264.7 cells subjected with Gaq/11 knockdown. (F) Upper panel: Western blot analysis of Bcl-XL expression in RAW264.7 cells upon full-length or truncated ORP4L overexpression for 24 hr; Lower panel: Western blot analysis Bcl-XL expression in RAW264.7 cells transfected with shORP4L, shORP4L + ORP4L or shORP4L + truncated ORP4L. Cells were transduced with lent-shNT or shORP4L for 48 hr, then transiently transfected with empty vector (Mock) or full-length or truncated ORP4L for 24 hr. The data represent mean ± S.D. from at least three separate experiments each analyzed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant.

Online Figure V. ORP4L knockout did not affect the ATP levels in macrophages. CD115⁺ monocytes isolated from WT or ORP4L⁻/- bone marrow (n=4 mice/group) were derived with M-CSF (50 ng/ml) for 7 days. The cells were then cultured for 24 hr with or without LPS (M1, 100 ng/ml) or with IL-4 (M2, 20 ng/ml), followed by analysis of ATP contents. The data represent mean ± S.D. NS, not significant.
Online Figure VI. ORP4L deficiency reduced macrophage number in atherosclerotic lesion. Representative images of Moma-2–stained cross-sections of aortic roots from LDLr⁻/⁻ and LDLr⁻/⁻ORP4L⁻/⁻ mice. Quantification of macrophages expressed as the percentage of macrophage number relative to the total cell number in the lesion (5 sections/mouse, n=6 for LDLr⁻/⁻ mice and n=7 for LDLr⁻/⁻ORP4L⁻/⁻ mice). The data represent mean ± S.D. **p < 0.01.

Online Figure VII. ORP4L deficiency increases the collagen content of atherosclerotic lesions. Sections were stained for collagen with Masson’s trichrome, and collagen content was quantified (4 sections/mouse, n=6 mice/group). The data represent mean ± S.D. **p < 0.01.
Online Figure VIII. Similarity of the modeled ORP4L structure with Oxsterol-binding protein homology 3. Overlay of the ORP4L homology model (yellow) with the Oxsterol-binding protein homology 3 structures (green) in ribbon representation.

Supplemental references

## Supplemental Tables

### Online Table I. Putative ORP4L-interacting proteins in Raw264.7 cells

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<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Peptides*</th>
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<tr>
<td>PLCβ3</td>
<td>phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3</td>
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<tr>
<td>Gaq/11</td>
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<td>MRPL39</td>
<td>39S ribosomal protein L39, mitochondrial</td>
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<td>Rho GTPase-activating protein SYDE1</td>
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<td>RPS14</td>
<td>40S ribosomal protein S14</td>
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<tr>
<td>GBF1</td>
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</tr>
<tr>
<td>RAB17</td>
<td>Ras-related protein Rab-17</td>
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<td>LDHA</td>
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<td>ACTB</td>
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<td>TFRC</td>
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*Number of peptides detected by mass spectrometry for each identified protein.
### Online Table II: Clinical information on the human cases used

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<th>NO.</th>
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<th>Age</th>
<th>Diagnosis</th>
<th>Smoking (years)</th>
<th>Alcohol (years)</th>
<th>Hypertension (years)</th>
<th>T2DM (years)</th>
<th>Blood glucose (mM)</th>
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<td>-</td>
<td>5.8</td>
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M, male; F, female; ASO, Arteriosclerosis Obliterans; T2DM, type 2 diabetes; N, blood glucose at normal level
Online Table III. Oligonucleotide primers used for cDNA constructs

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<tr>
<th>Construct</th>
<th>Forward primer 5’- 3’</th>
<th>Reverse primer 5’- 3’</th>
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<tr>
<td>ORP4L-pcDNA4 HisMaxC</td>
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<td>ATTctegaGTGGCGCTCAGAAGATGGTTGGGCGACA TATGCCA</td>
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<td>Bcl-XL-pcDNA4 HisMaxC</td>
<td>ATTgaatTCATGTCTCAGAGCAACCGGGAGGC</td>
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<td><strong>Tyr588Gly-sen:</strong> CCGCCTTCTCTGTGTCCTCCGGCTCAACCA CAGTGACCGCAT</td>
<td><strong>Tyr588Gly-anti:</strong> ATGCAGTGCACTGTGGTGAGCCGGAGGAGC ACAGAAGGCGG</td>
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<td><strong>Tyr634Gly-sen:</strong> CCCCCCCTTCAGCTGCGACCGGTGTTCTCAAGCACAGCT</td>
<td><strong>Tyr634Gly-anti:</strong> AGCCATGCTTTGGAGAACCACCGCTGCGAGCTG AGGGGGG</td>
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<td><strong>Lys681Gly-sen:</strong> AACTCATTCTGTGGAGAGGCACACACCA AAACGTTCACAAC</td>
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<td>ORP4L(A445-513)-pcDNA4 HisMaxC</td>
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<td><strong>Forward514:</strong> TCTTGACTCCCCAAAGGAGAGG ACAGTCCCAACAAAGCCCCAACATACAGCCTT</td>
<td><strong>Reverse444:</strong> AAGGCTGTAGTTGGGCTGTTGGGA CTGTCCTCTCCTTGGGAGTCAAGA</td>
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*Restriction sites are indicated in lower case letters.
Online Table IV. The targeted shRNA/siRNA sequences used

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