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# Monocytic expression of osteoclast-associated receptor (OSCAR) is induced in atherosclerotic mice and regulated by oxidized low-density lipoprotein in vitro

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ABSTRACT

The osteoclast-associated receptor (OSCAR), primarily described as a co-stimulatory regulator of osteoclast differentiation, represents a potential link between bone metabolism and vascular biology. Previously, we identified OSCAR as an endothelial cell-derived target of the proatherogenic factor oxidized low density lipoprotein (oxLDL). Since monocytes play an important role in the progression of atherosclerosis, we assessed whether atherogenic stimuli also regulate the expression of OSCAR on monocytes. Four-week-old male wild-type (WT), apolipoprotein e knockout (apoe KO), and LDL receptor knockout (ldlr KO) mice were fed a high-fat diet or normal chow for 6 weeks. Peripheral blood mononuclear cells (PBMCs) isolated from the spleen were stained with antibodies against CD14 and OSCAR for subsequent flow cytometric analysis. OSCAR surface expression on CD14-positive monocytes increased 2-fold in PBMCs from apoe KO mice compared to WT mice. Feeding a high-fat diet further increased OSCAR surface expression 1.5-fold in apoe KO mice compared to normal diet. Moreover, OSCAR-positive macrophages were detected in atherosclerotic plaques of apoe KO mice. Interestingly, monocytic OSCAR expression was not altered in ldlr KO mice. In the murine macrophage cell line RAW 264.7, TNFα and oxLDL induced OSCAR mRNA expression by 2-fold and 5-fold ($p < 0.01$), respectively. Blocking the oxLDL receptor LOX-1 and inhibiting the NF-κB pathway prevented OSCAR induction. In conclusion, OSCAR expression in monocytic cells is regulated by proatherogenic stimuli further pointing towards a role in vascular inflammation or plaque vulnerability during atherosclerosis.



## 1. Introduction

Atherosclerosis and vascular calcification are major risk factors for cardiovascular events and are associated with increased morbidity and mortality. Because skeletal and vascular diseases share common signaling pathways, recent studies have focused on bone-specific proteins that not only exert functions in the bone microenvironment but are also localized in the vascular system. For instance, receptor activator of nuclear factor NF-κB ligand (RANKL) and its soluble decoy receptor osteoprotegerin (OPG), two regulators of bone remodeling, have been linked to arterial calcification [1]. RANKL has been shown to respond to inflammatory regulators in microvascular endothelial cells and to regulate bone morphogenetic protein-2 (BMP-2) and matrix Gla protein in human aortic endothelial cells (HAECs) and in smooth muscle cells (HASMCs) [2]. More recently, our group showed that blocking RANKL with a monoclonal antibody diminished aortic calcium load in a mouse model of arterial calcification [3]. By contrast, OPG has been identified as a vasculoprotective protein inhibiting warfarin-induced vascular calcification in rats [4]. OPG deficiency in mice causes osteoporosis and medial calcification of the arteries, further highlighting the link between bone metabolism and vascular homeostasis [5]. More recently, osteoclast-associated receptor (OSCAR) has been identified as a novel osteogenic protein in the vasculature. OSCAR has been described primarily as a co-stimulatory factor in osteoclastogenesis [6], and may serve as an immunological mediator in dendritic cells and monocytes [7,8]. Lately, we and others have identified OSCAR in endothelial cells, responding to inflammatory cytokines and the proatherogenic factor oxidized low-density lipoprotein (oxLDL) [9,10]. Furthermore, OSCAR expression was increased in the aorta of atherogenic apolipoprotein E knockout

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(apoe KO) mice indicating that OSCAR may contribute to the process of atherosclerosis [9,11]. Since OSCAR is expressed by monocytes which are involved in the pathophysiology of atherosclerosis, we assessed whether atherosclerotic stimuli such as oxLDL also regulate OSCAR expression on monocytes.

## 2. Material and methods

### 2.1. Cell culture

All cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany). RAW 264.7 cells (ATCC, TIB-71, Manassas, VA) were cultured in DMEM containing 10% FCS, 1% antibiotic/fungicide at 37 °C and 5% $CO_2$. Similarly, primary murine macrophages generated from bone marrow stromal cells were cultured in DMEM containing 10% FCS, 1% antibiotic/fungicide and 50 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) for 5 days. Stimulation with native LDL (nLDL; 100 μg/ml), oxidized LDL (oxLDL; 10, 50, 75, 100 μg/ml) and TNFα (50, 100 ng/ml; R&D Systems, Minneapolis, MN) was performed in DMEM with 0.1% FCS, 1% antibiotic/fungicide for 6, 24, and 48 h. For signaling experiments cells were preincubated with 1 μM Bay-11-7082 (IκB phosphorylation inhibitor; Calbiochem, EMD Chemicals, Philadelphia, PA, USA), 10 μM Ly-294002 (phosphatitylinositol 3-kinase inhibitor; Cell Signalling Technology, Danvers, MA, USA), 5 μM PD-98059 (MEK1 inhibitor; Calbiochem, EMD, Chemicals), 1 μM 11R-VIVIT (NFATc1 inhibitor; Calbiochem, EMD, Chemicals) or an antibody against lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1; 10 μg/ml; R&D Systems, Minneapolis, MN, USA) for 1 h before n/oxLDL was added. After 24 h of stimulation cells were used for RNA isolation or subsequent flow cytometry.

### 2.2. LDL isolation

Low-density lipoproteins (LDL) were isolated from human plasma by ultracentrifugation as described before [12]. Briefly, the LDL fraction was oxidized for 24 h using 50 μM $CuSO_4$. Oxidation was stopped using EDTA and dialysis against PBS was performed to remove EDTA copper complexes. The oxidation level of LDL was analyzed by gel electrophoresis and spectral photometric measurement of conjugated diene formation at 234 nm.

### 2.3. Animals

Male, 4-week-old wild type mice (C57BL/6J, WT), ldlr-deficient mice (ldlr KO, strain C57BL/6J-Ldlr<Hlb301>/J); both purchased from Charles River Laboratories (Wilmington, MA), and apoe knockout mice (apoe KO, strain B6.129P2-Apoetm1Unc/J; The Jackson Laboratory, Bar Harbor, ME) were fed standard chow (EFR/MCD88137) or high-fat diet (FD EF R/M TD88137; Ssniff GmbH, Soest, Germany) for 6 weeks. For immunofluorescence experiments apoe KO mice were fed standard chow for 26 weeks. The animals were sacrificed under general anesthesia, and all performed experiments were in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The Institutional Review Board of the Technical University Dresden, and the Regierungspräsidium Dresden approved the animal facilities and the experiments according to institutional guidelines and German animal welfare regulations (AZ, 24-9168.24-1 2009-14).

### 2.4. RNA isolation, RT and Real-time PCR

Total RNA from cell culture was isolated using the High Pure RNA Isolation kit (Roche, Mannheim, Germany). The mRNA expression was determined by Applied Biosystems SYBR green-based real-time PCR reactions using a standard protocol (Applied Biosystems, Darmstadt, Germany). The primer pairs used are β-actin (NM_012242; s: ccaaccgcgagaagatga; as: ccagaggcgtacagggatag) and murine OSCAR (NM_175632; s: tggcggtttgcactcttca, as: gatccgttaccagcagttccaga). The results were calculated using the ΔΔCT method, and are presented in x-fold increase relative to β-actin mRNA levels.

### 2.5. Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from mouse spleens by Biocoll density centrifugation. PBMCs and RAW 264.7 cells were stained with antibodies against CD14-APC (1:20; BD Biosciences) and OSCAR (1:50; N16, Santa Cruz Biotechnology, Inc.) with Alexa Fluor 488 (Invitrogen) for subsequent flow cytometric analysis (LSR II, BD GmbH, Heidelberg, Germany). The results were analyzed with the software FlowJo vX 64bit (Tree Star, Inc., Ashland, OR).

### 2.6. Immunofluorescence

Aortic arches of 30-week-old apoe mice were cut into 4 μm cryo-sections and fixed in methanol. After blocking in 2% bovine serum albumin (BSA), sections were incubated with OSCAR antibody (N16, Santa Cruz Biotechnology, Inc.) and the macrophage-specific antibody F4/80 (Santa Cruz Biotechnology, Inc.) for 1 h at RT. After washing three times with PBS, sections were incubated with secondary antibodies Alexa Fluor 488 and Alexa Fluor 594 30 min at RT in the dark. Sections were washed again for three times and after staining with DAPI for 5 min sections were embedded in mounting medium. Using the Zeiss Axio Imager M.1 fluorescence microscope, slides were examined with the AxioVision 3.1 program.

### 2.7. Statistical analysis

Results are presented as means ± SD. Group comparisons were performed using a one-way ANOVA with Bonferroni's post hoc test and single group comparisons using a two-sided Student's *t*-tests (GraphPad Prism 4.02). *P*-values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. OSCAR expression in RAW 264.7 cells is stimulated by oxLDL

We analyzed the expression of OSCAR on RAW 264.7 cells, a murine monocytic cell line. Treatment of RAW 264.7 cells with oxLDL for 6, 24, and 48 h showed increasing mRNA expression of OSCAR over time. OSCAR expression was induced up to 6-fold ($p < 0.001$) after 48 h of stimulation with oxLDL, while nLDL treatment had no effect (Fig. 1A). Additionally, OSCAR expression was increased dose-dependently when RAW 264.7 cells were treated with different concentrations of oxLDL, ranging from 10 to 100 μg/ml (Fig. 1B). Similar to OSCAR mRNA expression, OSCAR was induced by oxLDL at the cell surface. Flow cytometric analysis revealed a significant increase of OSCAR-positive RAW 264.7 cells from 1% to 6% after oxLDL treatment (Fig. 1C and D). Exposure to TNFα (50 ng/ml) increased OSCAR mRNA expression by 2-fold, which was not further increased with a higher TNFα concentration

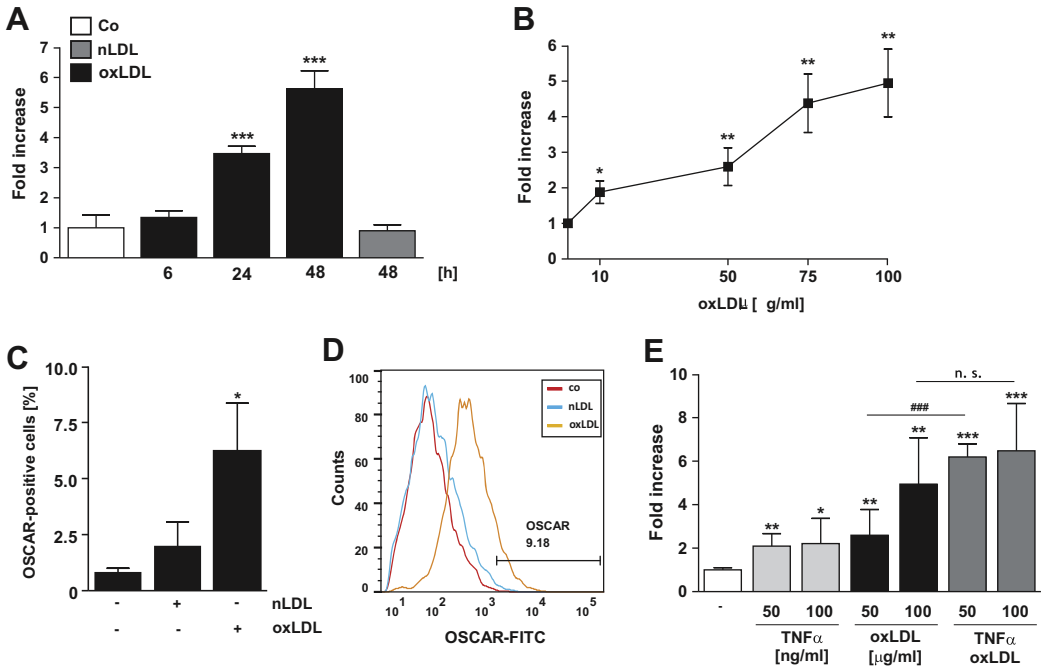


**Fig. 1.** OSCAR expression on RAW 264.7 cells is induced by oxLDL. (A) RAW 264.7 cells were treated with oxLDL for 6, 24, and 48 h. OSCAR expression was induced up to 6-fold after 48 h of stimulation with oxLDL, nLDL treatment had no effect. (B) OSCAR expression was dose-dependently induced by oxLDL after 48 h. (C) OSCAR protein expression was increased up to 6-fold after oxLDL treatment measured by flow cytometry. (D) Representative histogram of OSCAR-positive RAW 264.7 cells. (E) Co-stimulation with TNFα (50 ng/ml) and oxLDL (50 μg/ml) showed an additive effect and increased OSCAR expression 2-fold compared to oxLDL treatment alone. This effect was lost when cells were treated with higher concentrations of TNFα (100 ng/ml) and oxLDL (100 μg/ml). $n = 3$; mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. untreated control; $^{\#\#\#}p < 0.001$ vs. oxLDL (50 μg/ml).

(100 ng/ml). However, when RAW 264.7 cells were treated with TNFα (50 ng/ml) in combination with oxLDL (50 μg/ml) the effects were 2- to 3-fold higher compared to single treatments. High concentrations of both, TNFα (100 ng/ml) and oxLDL (100 μg/ml), did not further increase OSCAR expression (Fig. 1E). Similar results were shown in primary cultured macrophages, generated from bone marrow stromal cells (data not shown).

In order to assess the underlying signaling pathways of oxLDL-dependent OSCAR induction, we exposed RAW 264.7 cells to inhibitors of NF-κB signaling (Bay 11-7082), MAP kinase (PD 98059), phosphatidylinositol 3-kinase (Ly 294002), and NFATc1 signaling (VIVIT). Treatment of RAW 264.7 cells with Bay 11-7082 completely abolished oxLDL-dependent OSCAR induction (Fig. 2A), while MAP kinase, phosphatidylinositol 3-kinase, and NFATc1 had no impact on oxLDL-induced OSCAR expression (data not shown). Pretreatment with a LOX-1 antibody, which blocks binding of oxLDL to the oxLDL-specific receptor LOX-1, further confirmed the specificity of oxLDL to induce OSCAR, as the LOX-1 antibody abolished OSCAR induction (Fig. 2B).

### 3.2. Monocytic OSCAR expression is increased in apoe KO mice

To investigate the functional relevance of OSCAR in vivo, we used two established atherosclerotic mouse models, the apoe KO mouse and the ldlr KO mouse. Four-week-old apoe KO and ldlr KO mice, as well as WT mice were fed a normal chow or a high fat diet for 6 weeks. OSCAR expression on CD14-positive cells was increased 2-fold in PBMCs from apoe KO compared to WT mice (Fig. 3A; $p < 0.05$). Feeding a high fat diet further increased OSCAR surface expression up to 1.5-fold to 9% positive cells in apoe KO mice as demonstrated in the representative dot plot from two apoe mice, either fed normal diet or high-fat diet (Fig. 3B, $p < 0.001$).

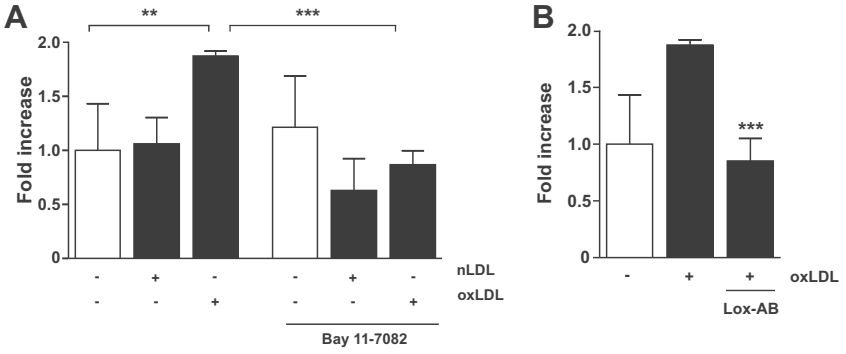


**Fig. 2.** OxLDL-induced OSCAR expression is dependent on LOX-1 binding and NF-κB. (A) Induction of OSCAR was prevented by exposing oxLDL-treated cells to Bay 11-7082 (1 mM) for 24 h. (B) RAW 264.7 cells were treated with oxLDL in combination with a LOX-1 antibody (10 mg/ml), which prevented oxLDL-dependent OSCAR induction. $n = 3$; mean ± SD; $^{**}p < 0.01$; $^{***}p < 0.001$.

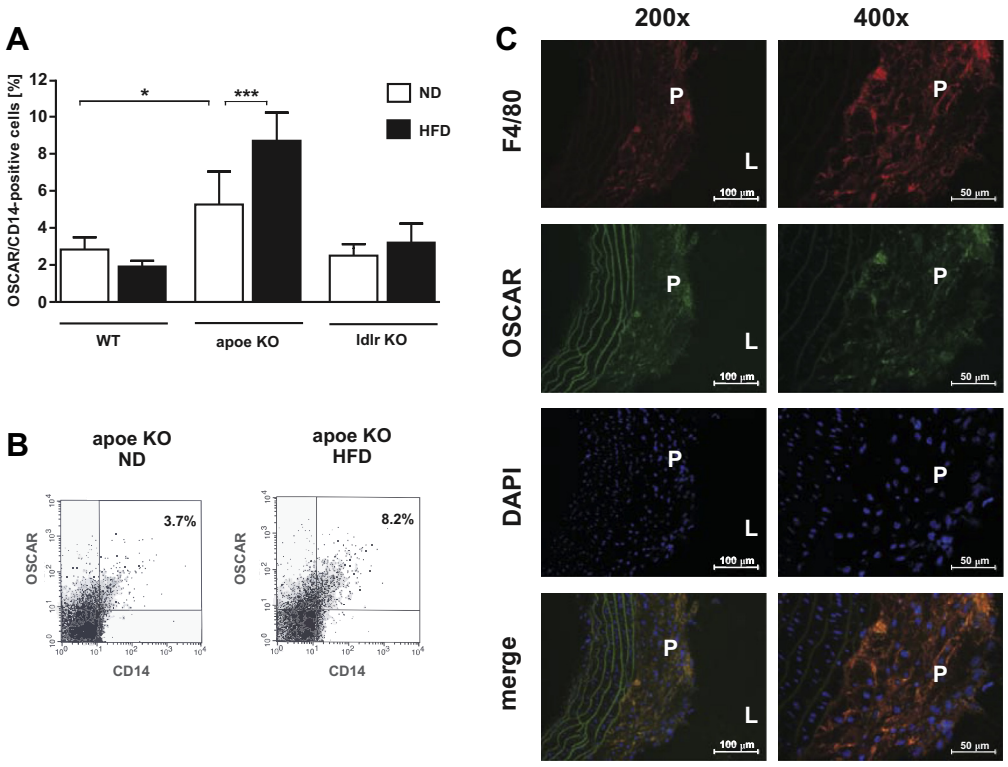


**Fig. 3.** OSCAR surface expression on CD14-positive monocytes from WT, apoe KO, and ldlr KO mice. (A) OSCAR expression levels on CD14-positive cells were increased 2-fold in PBMCs from apoe KO compared to WT mice ($p < 0.01$). Feeding a high fat diet further increased OSCAR surface expression up to 1.5-fold in apoe KO mice ($p < 0.001$), while PBMCs from ldlr KO mice showed no significant differences. $n = 5–8$; mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Representative dot plot from two apoe KO mice, either fed a normal chow or a high fat diet for 6 weeks. (C) Colocalization of OSCAR and F4/80 in aortic arches from 30-week-old apoe KO mice. OSCAR (green), F4/80 (red), DAPI (blue); L, lumen; P, plaque. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OSCAR expression on PBMCs from ldlr KO increased from 2.5% to 3.2% but showed no significant differences (Fig. 3B). In addition, immunofluorescence staining of aortic arches from 30-week-old apoe KO mice revealed that OSCAR and the macrophage-specific marker, F4/80 were co-localized within the atherosclerotic plaque (Fig. 3C).

## 4. Discussion

The receptor OSCAR, initially described as a co-stimulatory factor in osteoclast differentiation and dendritic cell maturation, has recently been described by our group in endothelial cells to respond to atherogenic stimuli like oxLDL [9]. Here, we showed that OSCAR expression induced by oxLDL is affected in monocytic cells in a time- and dose-dependent manner. OxLDL stimulated OSCAR expression via LOX-1, the major scavenger receptor for oxLDL that is present on several cell types, such as monocytes and macrophages [13]. In addition, the inflammatory stimulus TNFα increased OSCAR mRNA expression, which is in line with other studies [10,14]. In fact, in rheumatoid arthritis OSCAR expression is increased in circulating monocytes and correlates with disease activity [14]. Interestingly, co-stimulation with TNFα and oxLDL had an additive effect on OSCAR mRNA expression in RAW 264.7 cells, but only at lower concentrations. High concentrations of TNFα in combination with oxLDL showed no further induction of OSCAR as compared to the single treatment with oxLDL, suggesting that the maximal induction of OSCAR might already have been reached.

Atherosclerosis is characterized by a chronic inflammatory state and an infiltration of monocytes into the vascular wall. This could explain the induction of OSCAR in CD14-positive cells from atherosclerotic mice compared to WT mice especially after feeding a high-fat diet for 6 weeks. Increased OSCAR expression in the whole aorta lysate of apoe KO mice was previously linked to endothelial cells [9]. However our findings show that macrophages also express OSCAR in the plaque of apoe KO mice, suggesting that the monocytic lineage may contribute to enhanced OSCAR expression in atherosclerosis.

Interestingly, OSCAR expression was only increased in apoe KO mice, but not in ldlr KO mice. This might be due to more severe signs of atherosclerosis in apoe KO mice, which develop atherosclerotic lesions already after 10 weeks even on chow diet [15]. Furthermore, apoe exerts anti-inflammatory properties beyond lipid lowering [16,17]. It not only suppresses atherosclerosis by reducing lipid accumulation in circulating monocytes but also the inflammatory state of monocytes/macrophages and the endothelium [18]. Ldlr KO mice have been shown to develop a milder form of atherosclerosis with the first signs of lesions at the age of 5 months and after additional 12 weeks on a high-fat diet [19]. The fact that our mice were only 10 weeks old at the time of analysis might explain why OSCAR expression levels were unchanged in CD14-positive cell. We chose this time point, because previous experiments revealed that OSCAR expression was especially affected during the early inflammatory phase of atherosclerosis [9]. Furthermore, amongst OSCAR/CD14-positive cells, we did not analyze cell type subsets. Future experiments should differentiate between M1- or M2-macrophages, which play opposite roles during inflammation. While M1-macrophages promote inflammation, M2-macrophages take part in the resolution of the inflammatory process, but both types are present in atherosclerotic lesions [20].

To better understand the underlying mechanisms of the regulation of OSCAR by oxLDL, we assessed several possible signaling pathways and identified NF-κB as a major signal transducer in

oxLDL-dependent OSCAR induction. OxLDL has been shown to trigger NFκB activation thereby up-regulating proinflammatory gene expressions and be involved in the initiation and progression of atherosclerosis [21]. In endothelial cells oxLDL induced OSCAR expression by nuclear factor of activated T cells c1 (NFATc1), but was not analyzed referring to NF-κB [9]. Although we do not know if NF-κB is also involved in OSCAR regulation in endothelial cells, both cell types could exert distinct functions in atherosclerosis and thus, may regulate OSCAR via different pathways. For example, it has been discussed that only a long-term inflammatory stimulation of macrophages leads to an activation of the NFAT signaling pathway. Of note, NFAT isoforms are negatively regulated in macrophages to prevent a proinflammatory environment [22,23]. This suggests that in the early phase of oxLDL stimulation OSCAR expression might be only regulated by NF-κB.

In conclusion, OSCAR expression in RAW 264.7 cells and primary murine CD14-positive cells is regulated by the proatherogenic stimuli oxLDL and depends on NF-κB- and LOX-1-signaling pathways. Although the functional relevance of OSCAR in the development of atherosclerosis remains to be elucidated, this pathway may represent an important new player in the triad cross-talk between the vasculature, bone, and the immune system.

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