

Exosomal MALAT1 derived from oxidized low-density lipoprotein-treated endothelial cells promotes M2 macrophage polarization

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Abstract. Oxidized low-density lipoprotein (oxLDL)-induced injury and apoptosis of endothelial cells are important initial events in numerous cardiovascular diseases. Following activation by oxLDL, monocytes adhere to endothelial cells, migrate into the subendothelial spaces and then undergo differentiation into macrophages, which subsequently induces the formation of atherosclerotic lesions. However, the mechanisms underlying the activation of macrophage differentiation by oxLDL-treated endothelial cells remain unclear. In the present study, it was demonstrated that exosomal metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was increased in oxLDL-treated human umbilical vein endothelial cells. When co-cultured with monocytes, exosomes extracted from oxLDL-treated HUVECs were endocytosed. Furthermore, exosomes derived from oxLDL-treated endothelial cells were revealed to promote M2 macrophage polarization, as reverse transcription-quantitative polymerase chain reaction, western blotting and ELISA analyses demonstrated increases in the expression of M2 macrophage markers, including macrophage mannose receptor 1 (also termed CD206), arginase-1 and interleukin (IL)-10, and decreases in the expression of the M1 macrophage marker, IL-12. Furthermore, the suppression of MALAT1 expression in monocytes was demonstrated to reverse exosome-mediated M2 macrophage polarization. In conclusion, the results of the present study revealed a novel mechanism underlying the onset of atherogenesis associated

with endothelial cells and macrophages: Exosomal MALAT1 derived from oxLDL-treated endothelial cells promoted M2 macrophage polarization. This result may provide a novel scientific basis for the understanding of atherosclerosis progression.

Introduction

Endothelial dysfunction, which is closely associated with vascular endothelial cell injury, is involved in an array of pathophysiological processes, including coronary artery diseases, diabetes and hypertension (1-4). Oxidized low-density lipoprotein (oxLDL)-induced injury to vascular endothelial cells has been revealed to be an important initial event in atherosclerosis (5-7). It has been reported that the activation of endothelial cells by oxLDL may occur via the induction of the expression of various cell surface adhesion molecules, which mediate the rolling adhesion of leukocytes, including monocytes and T cells (8,9). Following adhesion to the endothelium, leukocytes have been demonstrated to migrate into the intima where monocytes then differentiate into macrophages and induce atherosclerotic lesions (6-11). OxLDL has been reported to induce the onset of atherogenesis involving endothelial cells and macrophages (12). However, the mechanisms underlying the activation of macrophage differentiation by oxLDL-treated endothelial cells remain unclear.

Macrophages are heterogeneous and polarize into specific subsets, such as classically activated proinflammatory macrophage M1 (M1)-like and alternatively activated macrophage M2 (M2)-like macrophages (13). Different markers are employed for the identification of M1 and M2 macrophages. Markers for M1 macrophages include tumor necrosis factor- α , inducible nitric oxide synthase, interleukin (IL)-6 and integrin- α -X (also termed CD11c), while markers for M2 macrophages include macrophage mannose receptor 1 (also termed CD206), arginase-1 and resistin-like- α (14,15). In human atherosclerotic plaques, markers for M1 and M2 macrophages are present in the early and advanced stages of plaque development (16,17). Previous studies in hypercholesterolemic mice have demonstrated that M2 macrophages reduce atherosclerosis, whereas suppression of M2 polarization enhances plaque progression (18-20). Therefore, it may be concluded that macrophage polarization is important for plaque progression in atherosclerosis.

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Exosomes are nano-sized membrane vesicles released by cells, with a diameter of 30–100 nm (21–23). Exosomes are important inter-cellular transporters, carrying bulk biomaterials to regulate the cellular functions of endothelial cells, vascular smooth muscle cells, fibroblasts and leukocytes (24–27). However, the function of exosomes secreted by oxLDL-treated endothelial cells during atherosclerosis remains to be determined.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts that are composed of >200 nucleotides, and studies have demonstrated that certain lncRNAs have important functions in the regulation of numerous biological processes, including cell proliferation, differentiation and death (28–30). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a widely expressed single-exon lncRNA. MALAT1 was initially identified in non-small cell lung cancer and is reported to be involved in the regulation of various biological processes by functioning as a competing endogenous RNA (31–33).

Therefore, the aim of the present study was to investigate the function of exosomal lncRNA-MALAT1 in the process of oxLDL-treated endothelial cell-activated macrophage differentiation.

Materials and methods

Cell culture. THP-1 (American Type Culture Collection, Manassas, VA, USA) human monocyte cells were maintained in RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and human umbilical vein endothelial cells immortalized with telomerase reverse transcriptase (HUVEC/TERT2; American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium/F12 (Thermo Fisher Scientific, Inc.), and each were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C. All cells were confirmed to be free of mycoplasma contamination with the MycoFluor™ Mycoplasma Detection kit (Thermo Fisher Scientific, Inc.).

Exosome isolation and co-culture with THP-1 cells. In order to isolate exosomes, HUVECs (2×10⁷) were treated with 50 µg/ml oxLDL (Shanghai Luwen Biotechnology Co., Ltd., Shanghai, China) for 48 h at 37°C and the supernatant was collected. The supernatant was centrifuged twice (1,000 × g for 10 min and 3,000 × g for 30 min at 4°C) to remove the cells and fragments, added to the Total Exosome Isolation kit (Thermo Fisher Scientific, Inc.) overnight and further centrifuged at 10,000 × g for 1 h at 4°C. Exosomes were resuspended in PBS and stored at -80°C. The concentration of exosomes was determined using a bicinchoninic acid assay. Exosomes (50 ng/ml) were subsequently added to THP-1 cells (1×10⁵) in culture medium for 24 h at 37°C. Exosomes were stained with PKH67 (cat. no. MINI67; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to observe endocytosis, according to the manufacturer's protocol.

Transmission electron microscopic observation of exosomes. The exosome suspension was added to an equal volume of

4% paraformaldehyde at 4°C for 30 min and applied to a Formvar/Carbon film-coated transmission electron microscope grid (Alliance Biosystems, Inc., Osaka, Japan). Subsequently, the sample was fixed by incubation with 1% glutaraldehyde for 5 min at 25°C, washed with PBS and incubated with 1% uranyl acetate for 5 min at 25°C. The sample was subsequently observed under a transmission electron microscope (Hitachi H7650; Hitachi, Ltd., Tokyo, Japan).

Knockdown of MALAT1 in THP-1 cells. The knockdown sequence for MALAT1 was as follows: 5'-ACGGAAGTAATTCAAGATCAA-3'. Lentiviral vectors for human MALAT1 knockdown were constructed by Shanghai Hanyin Industrial Co., Ltd. (Shanghai, China). The recombinant lentivirus and the empty vector negative control (NC) lentivirus (Shanghai Hanyin Industrial Co., Ltd.) were prepared and titrated to 10⁹ transfection U/ml. To isolate stably transfected cell lines, THP-1 cells were seeded in 6-well plates at a density of 2×10⁵ cells/well and infected with the same titer of virus (Shanghai Hanyin Industrial Co., Ltd., Shanghai, China) with 8 µg/ml polybrene (Shanghai Hanyin Industrial Co., Ltd.) on the following day at 37°C. At ~72 h post-viral infection, the RPMI-1640 culture medium was replaced with selection medium containing 4 µg/ml puromycin and the cells were cultured for ≥14 days at 37°C. The puromycin-resistant cells were amplified in medium containing 2 µg/ml puromycin for 7–9 days 37°C and then transferred to medium without puromycin. The knockdown efficiency was confirmed via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) 24 h after co-culture, as described below.

Western blot analysis. Protein concentration was determined using the bicinchoninic acid protein assay method. Membranes were blocked in 5% non-fat milk at 25°C for 30 min. To analyze the expression of the markers CD9, CD63 and CD206 in exosomes (control or oxLDL-treated) or THP-1 cells that were co-cultured with exosomes (control or oxLDL-treated), western blot assays were performed using the following primary antibodies: Mouse anti-human CD63 (cat. no. ab59479; 1:1,000; Abcam, Cambridge, UK), rabbit anti-human CD9 (cat. no. ab92726; 1:1,000; Abcam), rabbit anti-human CD206 (cat. no. 18704-1-AP; 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA) and mouse anti-actin (cat. no. MAB1501; 1:10,000; EMD Millipore, Billerica, MA, USA). Briefly, exosomes or cells were lysed with radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 0.5% Na-deoxycholate] containing protease inhibitors (Complete™, Mini protease inhibitor cocktail; Sigma-Aldrich; Merck KGaA). A total of 20–30 µg lysate were separated on 8–12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were subsequently incubated with the aforementioned primary antibodies overnight at 4°C. The primary antibody incubation was followed by incubation with the following secondary antibodies at room temperature for 1 h: Horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. 7074; 1:10,000; CST Biological Reagents Co., Ltd., Shanghai, China) and HRP-conjugated anti-mouse (cat. no. 7076; 1:10,000; CST Biological Reagents Co., Ltd.). The bound antibodies were detected using an enhanced chemiluminescence kit (cat. no. PI32209; Pierce; Thermo Fisher

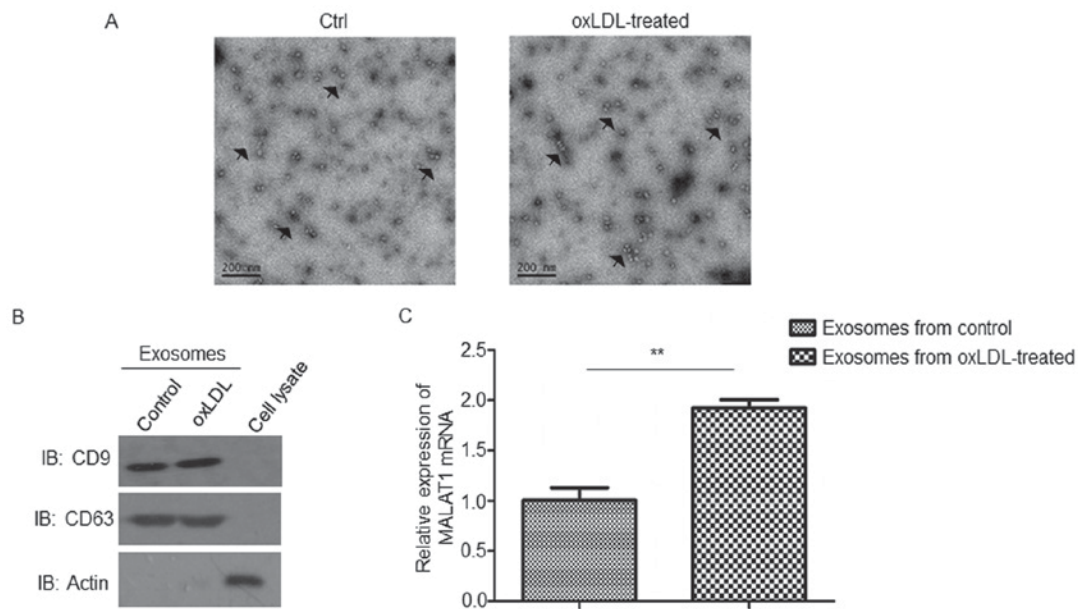


Figure 1. Exosomal MALAT1 is increased in oxLDL-treated HUVEC cells. (A) Representative electron micrographs of exosomes isolated from oxLDL-treated or untreated HUVECs, revealing the typical morphology and size (30-120 nm). Scale bar, 200 nm. Arrows indicate the exosomes. (B) Western blot analysis confirmed the expression of CD9 and CD63, and the absence of actin, in exosomes derived from oxLDL-treated and untreated HUVECs. (C) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated the expression of MALAT1 in exosomes derived from oxLDL-treated or untreated HUVECs. ** $P < 0.01$, as indicated. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; oxLDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; Ctrl, control.

Scientific, Inc.). Quantity One analysis software (version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to quantify the relative band intensities from western blot images.

RT-qPCR. Total RNA was extracted from exosomes (control or oxLDL-treated) or THP-1 cells that were co-cultured with exosomes (control or oxLDL-treated) using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). An equal quantity of total RNA was used for first-strand cDNA synthesis using the reverse transcription system (cat. no. A3500; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The synthesized first-strand cDNA (2 μ l) was used for each qPCR assay. A SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for qPCR experiments. GAPDH was used as an internal reference control. The primers sequences were as follows: MALAT1 forward, 5'-TTGTAGACTGGAGAAGATAGG-3' and reverse, 5'-ACTGAAGAGCATTGGAGAT-3'; CD206 forward, 5'-GGGTTGCTATCACTCTCTATGC-3' and reverse, 5'-TTTCTTGCTGTGTTGCCGTAGTT-3'; Arg-1 forward, 5'-TGGACAGACTAGGAATTGGCA-3' and reverse, 5'-CCAGTCCGTCAACATCAAACT-3'; GAPDH forward, 5'-CATGGCCTTCCGTGTTCTCCTA-3' and reverse 5'-GCGGCACGTCTAGATCCA-3'. qPCR cycling conditions consisted of initial denaturation for 3 min at 95°C, followed by 45 cycles of 95°C for 10 sec and 58°C for 45 sec in a LightCycler® 480 instrument (Roche Diagnostics, Basel, Switzerland). Melt curve analysis was performed at the end of each run between 58-95°C. Data were analyzed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). The $2^{-\Delta\Delta C_q}$ relative quantification method was used to analyze quantitative RT-qPCR data using the housekeeping gene GAPDH for normalization (34).

ELISA. ELISAs were performed on THP-1 cells that had been co-cultured with exosomes from control or oxLDL-treated HUVECs to determine the effect on IL-10 and IL-12 levels. IL-10 and IL-12 ELISA kits were purchased from Abcam (cat. nos. ab185986 and ab46035, respectively), and the analysis was performed according to the manufacturer's protocol.

Statistical analysis. Statistical differences between two groups were determined using the Student's t-test. Experiments were repeated three times. Data are presented as the mean \pm standard error of the mean. SPSS software was used to perform statistical analysis (version 17.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Exosomal MALAT1 is increased in oxLDL-treated HUVECs. To investigate the functions of oxLDL-treated HUVEC-derived exosomes in atherosclerosis, exosomes were isolated from oxLDL-treated or untreated HUVECs. The morphology of isolated exosomes was observed under transmission electron microscopy (Fig. 1A). As revealed in Fig. 1A, the diameters of the exosomes ranged from 30-120 nm. Furthermore, the results of western blot analysis demonstrated that, compared with cell lysates, exosomes were enriched with CD9 and CD63 exosomal markers (Fig. 1B), thus confirming the effective isolation of exosomes.

Following this, levels of MALAT1 mRNA expression were investigated in isolated exosomes via RT-qPCR, and the results revealed that exosomes secreted by oxLDL-treated HUVECs exhibited significantly enhanced levels of MALAT1 mRNA compared with those excreted by untreated controls (Fig. 1C).

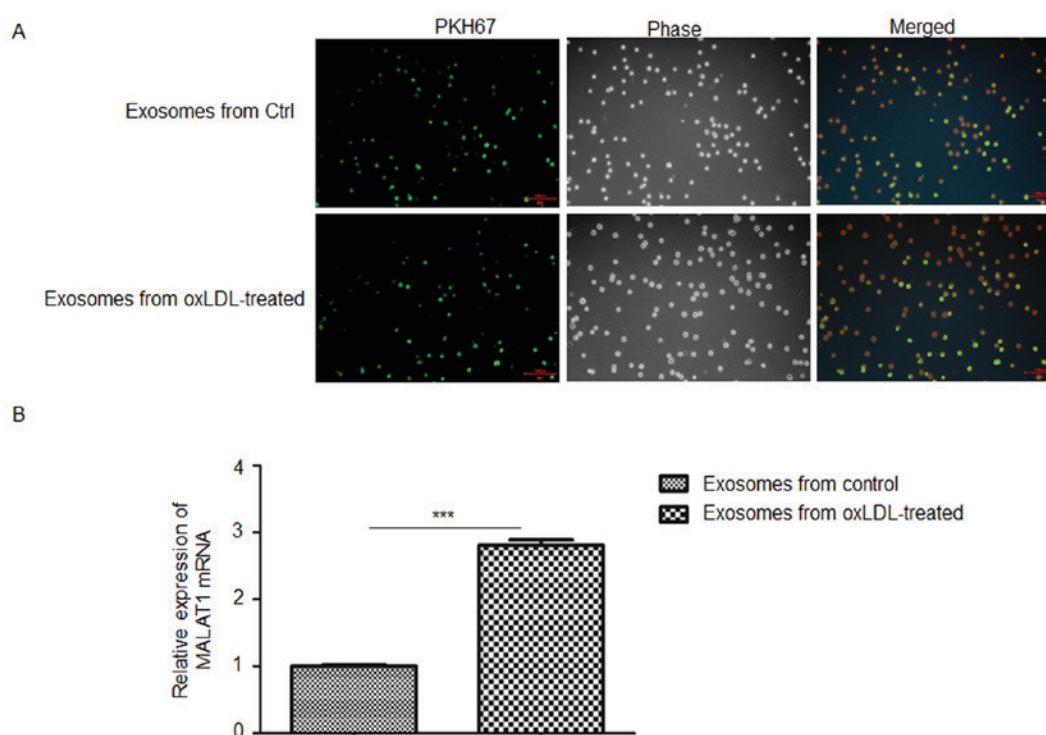


Figure 2. Monocytes endocytose exosomes derived from HUVECs. (A) Fluorescent microscopy observation results of exosomes isolated from oxLDL-treated or untreated HUVECs co-cultured with THP-1 cells. Exosomes were stained with PKH67 (green). Magnification, x200. (B) Reverse transcription-quantitative polymerase chain reaction analysis revealed the expression of MALAT1 in THP-1 cells co-cultured with exosomes derived from oxLDL-treated or untreated HUVECs. *** $P < 0.001$, as indicated. HUVECs, human umbilical vein endothelial cells; oxLDL, oxidized low-density lipoprotein; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Ctrl, control.

Monocytes endocytose exosomes isolated from oxLDL-treated HUVECs. The present study investigated whether exosomes released from oxLDL-treated HUVECs could be taken up by monocytes. Exosomes labeled with PKH67 (green) were co-cultured with THP-1 cells. Following 24 h of co-culture, THP-1 cells were fixed and observed by fluorescence microscopy. As revealed in Fig. 2A, exosomes were observed in co-cultured THP-1 cells, thus indicating that monocytes successfully endocytosed exosomes derived from untreated and oxLDL-treated HUVECs. Furthermore, the levels of MALAT1 mRNA in co-cultured THP-1 cells were investigated via RT-qPCR. The RT-qPCR results demonstrated that THP-1 cells co-cultured with exosomes secreted by oxLDL-treated HUVECs exhibited significantly enhanced levels of MALAT1 mRNA compared with those co-cultured with exosomes excreted by untreated control HUVECs (Fig. 2B).

Exosomes derived from oxLDL-treated HUVECs promote M2 macrophage polarization. In order to investigate the effects of oxLDL-treated HUVEC-derived exosomes on macrophage polarization, THP-1 cells were co-cultured with exosomes isolated from oxLDL-treated or untreated HUVECs and the expression of M1 and M2 macrophage markers were investigated by RT-qPCR, western blotting and ELISA analyses. The results demonstrated that oxLDL-treated HUVEC-derived exosomes enhanced the expression of the M2 macrophage markers (CD206, Arg-1 and IL-10; Fig. 3A-E) compared with oxLDL-untreated HUVEC-derived exosomes, whereas the expression of the M1 macrophage marker (IL-12; Fig. 3F) was

reduced compared with oxLDL-untreated HUVEC-derived exosomes. These results indicate that exosomes derived from oxLDL-treated endothelial cells promoted M2 macrophage polarization.

Suppression of MALAT1 expression reverses exosome-mediated M2 macrophage polarization. To further verify the effect of exosomal MALAT1 on macrophage polarization, MALAT1 expression was knocked down in THP-1 cells and successful knockdown was confirmed by RT-qPCR (Fig. 4A). oxLDL-treated HUVEC-derived exosomes were co-cultured with THP-1 cells with or without MALAT1 knockdown. The results of subsequent RT-qPCR, western blotting and ELISA analyses revealed that knockdown of MALAT1 in THP-1 cells co-cultured with oxLDL-treated HUVEC-derived exosomes led to the reduced expression of M2 macrophage markers (CD206, Arg-1 and IL-10; Fig. 4B-F) and increased levels of IL-12, an M1 macrophage marker (Fig. 4G), compared with those co-cultured with oxLDL-treated HUVEC-derived exosomes without knockdown of MALAT1. These results indicate that the suppression of MALAT1 expression reversed M2 macrophage polarization mediated by oxLDL-treated HUVEC-derived exosomes.

Discussion

Recent studies have demonstrated that exosomes mediate intercellular communication via the transfer of RNAs and proteins, which have important roles in numerous physiological and pathological processes, including cardiovascular

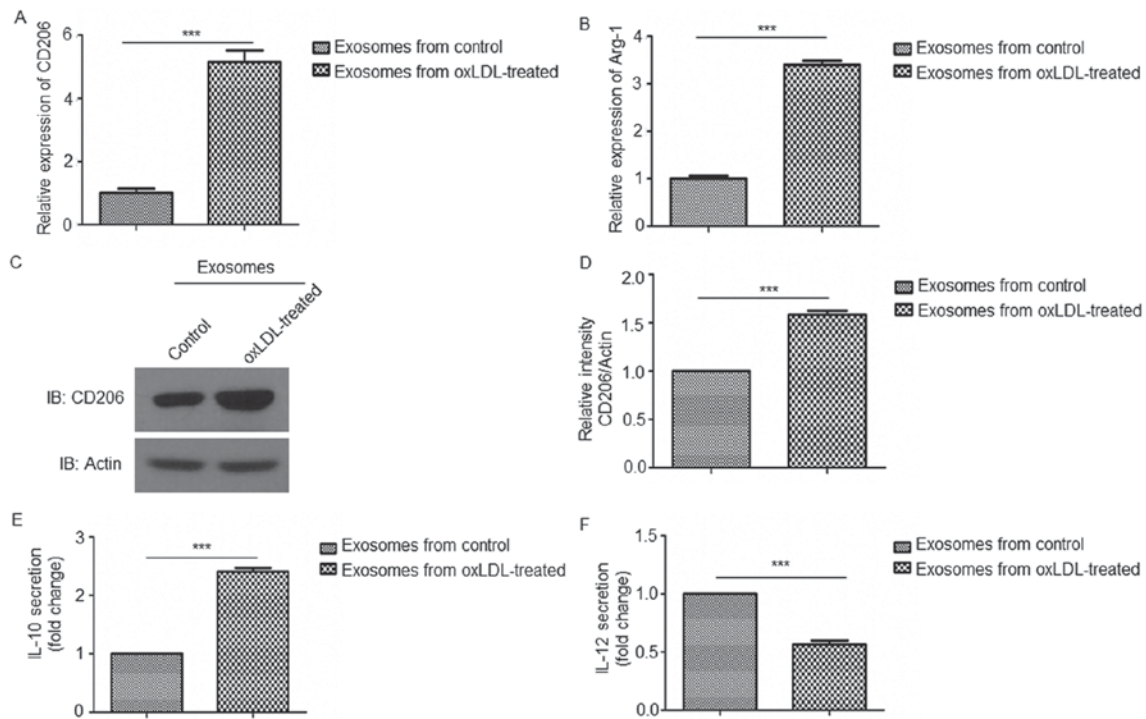


Figure 3. Exosomes derived from oxLDL-treated HUVECs promote M2 macrophage polarization. Reverse transcription-quantitative polymerase chain reaction analyses revealed an increase in the mRNA expression of (A) CD206 and (B) Arg-1 in THP-1 cells co-cultured with exosomes derived from oxLDL-treated HUVECs. (C) Representative western blot bands for CD206 protein expression in THP-1 cells co-cultured with exosomes derived from oxLDL-treated or untreated HUVECs. (D) Western blot analysis revealed an increase in the protein expression of CD206 in THP-1 cells co-cultured with exosomes derived from oxLDL-treated HUVECs. ELISA results demonstrated (E) an increase in IL-10 secretion and (F) a decrease in IL-12 secretion in THP-1 cells co-cultured with exosomes derived from oxLDL-untreated HUVECs. *** $P < 0.001$, as indicated. oxLDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; Arg-1, arginase-1; IL, interleukin.

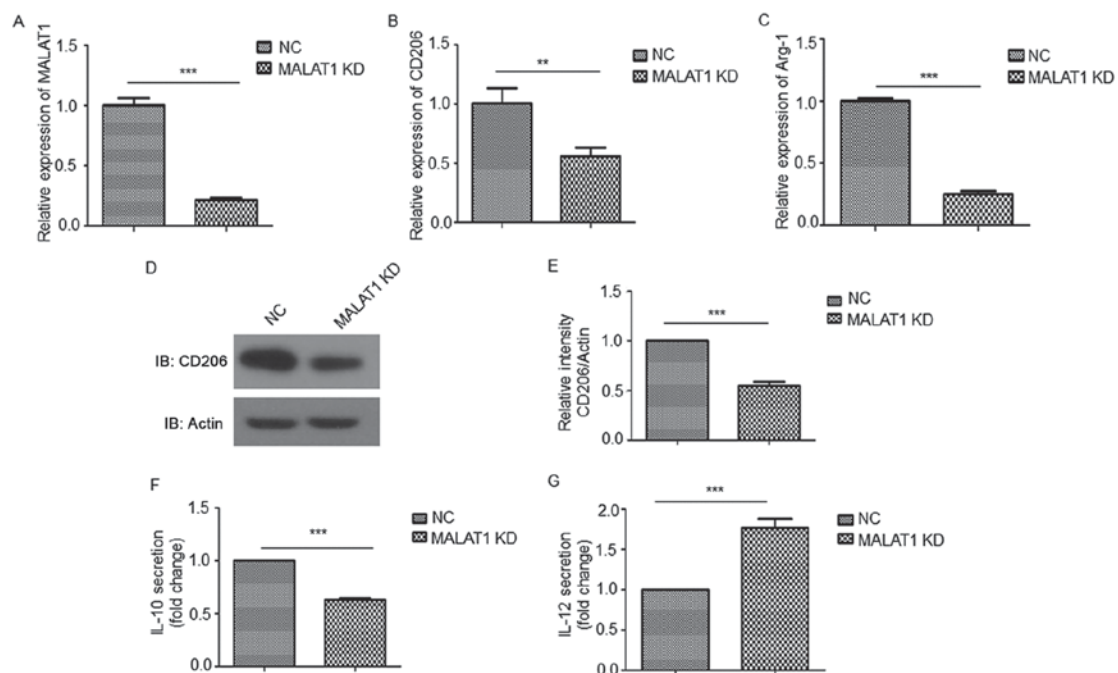


Figure 4. Suppression of MALAT1 reverses M2 macrophage polarization mediated by exosomes derived from oxLDL-treated HUVECs. (A) RT-qPCR analysis was performed to confirm the efficient knockdown of MALAT1 in THP-1 cells. Further RT-qPCR analyses demonstrated a decrease in the mRNA expression of (B) CD206 and (C) Arg-1 in MALAT1 knockdown THP-1 cells co-cultured with exosomes derived from oxLDL-treated HUVECs. (D) Representative western blot bands for CD206 protein expression in THP-1 cells with or without MALAT1 knockdown that were co-cultured with exosomes derived from oxLDL-treated HUVECs. (E) Western blot analysis revealed a decrease in the protein expression of CD206 in MALAT1 knockdown THP-1 cells that were co-cultured with exosomes derived from oxLDL-treated HUVECs. ELISA results demonstrated (F) a decrease in IL-10 secretion and (G) an increase in IL-12 secretion in MALAT1 knockdown THP-1 cells that were co-cultured with exosomes derived from oxLDL-treated HUVECs. ** $P < 0.01$ and *** $P < 0.001$, as indicated. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; oxLDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Arg-1, arginase-1; IL, interleukin; NC, negative control; KD, knockdown.

disease (21-27). Chen *et al* (35) reported that exosomal lncRNA growth arrest-specific 5 regulated the apoptosis of macrophages and vascular endothelial cells in atherosclerosis. In the present study, it was revealed that the expression of exosomal MALAT1 was enhanced in oxLDL-treated HUVECs. Furthermore, the results of the present study revealed that, following co-culture with monocytes, these exosomes were endocytosed and promoted M2 macrophage polarization via enhanced expression of the M2 macrophage markers CD206, Arg-1 and IL-10, and reduced expression of the M1 macrophage marker, IL-12. Additionally, it was demonstrated that the suppression of MALAT1 expression in monocytes reversed M2 macrophage polarization mediated by oxLDL-treated HUVEC-derived exosomes.

OxLDL contributes to atherosclerotic progression via numerous mechanisms, including the induction of endothelial cell dysfunction and macrophage foam cell formation (12). In addition, oxLDL-induced injury in the retinal pigment epithelium was reported to enhance the exosomal and apoptotic bleb release of CD46 and CD59, which are membrane complement regulatory factors, thus indicating that oxLDL stimulation may affect exosomal release (36). Reciprocal interactions between endothelial cells and macrophages have been revealed in angiogenic vascular niches via secreted microvesicles, such as exosomes (37). In the present study, it was demonstrated that oxLDL-stimulated HUVEC-derived exosomes mediated the polarization of macrophages. In addition, in recent studies, exosomes from numerous cell types, including stem cells and cancer cells, have been demonstrated to be involved in macrophage polarization (38-40). However, the underlying molecular mechanisms of this process remain unknown and require further investigation.

It has previously been established that exosomes contain mRNAs, non-coding RNAs and proteins (23-25). It was previously reported that exosomes derived from hypoxic epithelial ovarian cancer deliver microRNA-940 for the induction of M2 macrophage polarization (39). Additionally, exosomal MALAT1 has been reported to have important roles in the regulation of numerous pathological processes, including those involved in cancer and endocrinology (41-43). Serum MALAT-1 exosomal expression was upregulated and demonstrated to promote cell proliferation and migration in non-small cell lung cancer (42). Huangfu *et al* (44) demonstrated that oxLDL induced MALAT1 transcription through the nuclear factor- κ B pathway. Furthermore, Tang *et al* (45) revealed that MALAT1 protects the endothelium from ox-LDL-induced endothelial dysfunction, in part through competition with miR-22-3p for endogenous CXCR2 and AKT mRNA. The results of the present study indicate a novel mechanism for the onset of atherogenesis involving endothelial cells and macrophages. M2 macrophages have atheroprotective function by preventing foam cell formation (17). Thus, the results of the present study demonstrate that exosomal MALAT1 derived from oxLDL-treated endothelial cells may promote M2 macrophage polarization, and this may provide a novel scientific basis for the understanding of atherosclerosis progression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CH and JH designed the study, acquired, analyzed and interpreted the data, and wrote the manuscript. YW, SL, QW and WL collected and analyzed the data. JZ was involved in designing the experiments, interpreting data, and drafting and revising the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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