Dendritic cell exosome-shuttled miRNA146a regulates exosome-induced endothelial cell inflammation by inhibiting IRAK-1: A feedback control mechanism

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Abstract. Activation of endothelial cells is the first step of atherosclerosis. The current authors have previously reported that exosomes from mature dendritic cells (mDC-exo) participate in endothelial inflammation and atherosclerosis through membrane tumor necrosis factor-α mediated the nuclear factor (NF)-κB signaling pathway. However, whether mDC-exo shuttled microRNAs (miRNAs/miRs) play a role in endothelial inflammation remains unknown. In this study, mDC-exo were co-cultured with human umbilical vein endothelial cells (HUVECs) and the expression of adhesion molecules, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-Selectin was investigated. Then the expression of miRNAs in DC-exo was explored and the role of miR-146a in endothelial inflammation was investigated. mDC-exos were first demonstrated to increase endothelial expression of adhesion molecules through a quick activation of the NF-κB signaling pathway. Then it was demonstrated that HUVECs resistant to a second stimulation after the first stimulation by mDC-exo. A set of miRNAs were targeted and their expression in HUVECs stimulated with mDC-exo was measured. Finally, it was confirmed that mDC-exo shuttles miR-146a into HUVECs and the shuttled miR-146a contributes to protect HUVECs from a second stimulation through inhibiting interleukin-1 receptor-associated kinase. These data suggest a negative feedback loop of inflammation regulation by DC-exo.

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Introduction

Atherosclerosis is a disease that involves several cell types and a different set of inflammatory mediators (1). The first step is endothelial activation that expresses certain adhesion molecules, including vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-selectin.

A previous study confirmed that dendritic cells (DCs) exist in healthy arteries and participate in diverse pathogenic and protective mechanisms during atherosclerosis (2). It has been demonstrated that DCs participate in atherosclerosis through producing cytokines, including tumor necrosis factor (TNF), interleukin (IL)-6 and IL-12 (3). DCs can be found in the arterial intima of healthy individuals (4) and the number increased in atherosclerotic lesions (5). In mice, DCs are frequently found in the aortic intima which are predisposed to atherosclerosis. Moreover, it has been demonstrated that DCs accumulate in atherosclerotic areas (6-8).

Exosomes are a type of extracellular vesicle which are released by almost all kinds of cells. They have attracted a huge amount of attention because of the ability to transfer information between cells. It has been demonstrated that exosomes contain numerous biological components, including proteins, lipids, RNA and DNA (9). Previous studies showed that DC-derived exosomes (DC-exos) can exert biological effects through membrane proteins or shuttled microRNAs (miRNAs/miRs) (10-12). The authors have reported that mDC-exos increase endothelial inflammation and atherosclerosis through membrane TNF-α mediated nuclear factor (NF)-κB pathway (13). This process was quickly initiated by direct contact between exosomal membrane proteins and human umbilical vein endothelial cell (HUVEC) ligands. However, exosomes also contain a huge number of miRNAs and how these miRNAs influence HUVECs remains unclear. The present study aimed to investigate the role of mDC-exo miR-146a in inflammation of HUVECs. To the best of our knowledge, this study is the first to investigate the successive effect of exosomes on recipient cells in different phases.

The present study first demonstrated that mDC-exos increase endothelial expression of adhesion molecules. Then it was found that HUVECs became resistant to a second

stimulation after the first stimulation by mDC-exo. The change of a set of miRNAs in HUVECs was measured and a significant elevation of miR-146a was observed. Finally, DC-exo was confirmed to shuttle miR-146a into HUVECs and the shuttled miR-146a contributed to protect HUVECs from a second stimulation through inhibiting interleukin-1 receptor-associated kinase (IRAK). The present study demonstrated for the first time that exosomes make up a negative feedback loop to regulate endothelial inflammation.

Materials and methods

Cell culture and cell transfection. Male 6-8-week-old C57BL/6 mice (20-25 g) were purchased from JSJ-lab (http: //www.jsj-lab.com/) and manual cervical dislocation was used to sacrifice the animals. Death was ascertained by observing the cessation of either breathing or the heartbeat. The mice were housed at a constant temperature (22±1˚C) in a 12-h light/dark cycle, with *ad libitum* access to a standard diet and water. A total of four mice were used to obtain DCs for every single experiment and a total of ~40 mice were used in the whole study. As described previously, bone marrow DCs (BMDCs) were from C57BL/6 mice (14). To eliminate the interference of exosomes from fetal bovine serum, X-VIVO 15 was used (Lonza Group, Ltd.) that contains no serum to culture BMDCs (15). Briefly, bone marrow progenitors were washed out and $10x10^6$ cells were placed into every 10 cm dish (generally 4-6 dishes of cells could be obtained in every single experiment). The culture medium was X-VIVO 15 and contains 10 ng/ml granulocyte-macrophage colony-stimulating factor, 1 ng/ml IL-4 (PeproTech, Inc.) and 1% antibiotics (penicillin-streptomycin solution). Non-adherent cells were gently washed out at 48 h since they were not DCs. The remaining clusters were then continually cultured and the medium was changed every other day (half of the original medium was kept to maintain the micro-environment). On day 7, the cultured cells were differentiated into immature DCs and they were ready for subsequent studies. The present study was approved by the Institutional Review Board of the Zhongshan Hospital, Fudan University and Shanghai Institutes for Biological Sciences and were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

For the purpose of exosome isolation, DCs were treated with lipopolysaccharide (LPS; 5 μg/ml) or PBS for 24 h to generate mature or immature DCs. Then they were washed and replaced with new medium. After another 48 h continuous culturing, the culture medium was collected for exosome isolation. For transfection studies, DCs were transfected with miRNA mimic or inhibitor for 24 h and then treated with LPS or PBS for another 12-24 htogenerate mature or immature DCs. Then the cells were washed and replaced with new medium. After another 36-48 h culturing, the culture medium was collected for exosome isolation. For transfection experiments, miR-146 mimics/inhibitors and transfection reagent (riboFECT™ CP) were obtained from RiboBio Co., Ltd. The transfection concentration of miR-146 mimics and inhibitors was 50 and 100 nM, respectively.

HUVECs were obtained from AllCells, LLC and cultured in ECM (Scien Cell Research Laboratories, Inc.). The 8-10th generations of HUVECs were used in this study and they were harvested for mRNA or protein detection after treatment with exosomes for 6 or 24 h, respectively.

Exosomes isolation, analysis, labeling and neutralization. Exosomes were isolated using an exosome precipitation solution (Exo-Quick; System Bioscience) following the manufacturer's protocol with certain modifications (16). Briefly, the medium was harvested by centrifugation at 3,000 x g for 15 min at 4˚C and then 10,000 x g for 30 min (at 4˚C) to eliminate cell debris. Then the supernatant was filtered with a 0.22-μm filter to further eliminate cell debris and large particles. Finally, Exo-Quick was added to the medium at a ratio of 1: 5 and the mixed solution was placed at 4˚C overnight. The solution was then centrifuged at 1,500 x g for 30 min at 4˚C. The exosome pellet was resuspended with PBS and stored at -80˚C for subsequent studies. The ultrastructure was obtained by transmission electron microscopy. For transmission electron microscopy, 10 μl exosome was deposited on TEM grids for 1 min at room temperature and carefully blotted with soft paper. Then 10 μl 2% uranyl acetate was added to the grid for 1 min at room temperature and carefully blotted with soft paper before being left a dry environment for 5-10 min. Finally the grid was observed under transmission electron microscopy at 80 kV. Protein markers, TSG101 (Abcam) and Alix (Santa Cruz Biotechnology, Inc.), were determined by immunoblotting. The miRNA content was measured using quantitative PCR (Qiagen, Inc.).

Purified exosomes were labeled with a PKH67 (green) kit (Sigma-Aldrich; Merck KGaA) according to protocols previously reported (13,17). Briefly, the exosomes were added to 0.5 ml Diluent C. In parallel, 4 μl PKH67 dye was added to another 0.5 ml Diluent C and incubated with the exosome solution for 4 min at room temperature. Then 2 ml 0.5% BSA/PBS (Sangon Biotech Co., Ltd.) was added to bind excess dye. To ensure the exosomes were re-isolated as pure as possible, the labeled exosomes were harvested by centrifugation at 100,000 x g for 70 min (at 4˚C) and then the exosome pellet was suspended with PBS for the next study. Where necessary, labeled exosomes were pre-incubated with 2 μg/ml annexin V (BD Pharmingen; Becton, Dickinson and Company) at 37˚C for 1.5 h (18). After the HUVECs were fixed with 4% paraformaldehyde (Sangon Biotech Co., Ltd.) at room temperature for 15 min, they were visualized with immunofluorescent microscopy (magnification, x400). For immunofluorescence, the HUVEC cytoskeleton was stained with Phalloidin from Sigma-Aldrich (Merck KGaA) according to manufacturer's protocol at room temperature for 30 min. Cell nuclei were then stained with DAPI at room temperature for 10 min.

Reverse transcription-PCR. Total RNA was extracted from cells by TRIzol reagent (Sangon Biotech Co., Ltd.). A ReverTra Ace qPCR RT kit was used (Toyobo Life Science) to generate cDNA from mRNA at 37˚C for 30 min and SYBR Premix Ex Taq (Takara Bio, Inc.) was used for qPCR with the ABI 7500 Real-time PCR system. Primers for human VCAM-1, ICAM-1, E-selectin are as follows: VCAM-1 forward 5'-GCTGCTCAG ATTGGAGACTCA-3', VCAM-1 reverse 5'-CGCTCAGAG GGCTGTCTATC-3', ICAM-1 forward 5'-TCTGTGTCCCCC TCAAAAGTC-3', ICAM-1 reverse 5'-GGGGTCTCTATG

Table I. Forward and reverse primer sequences for the miRs and U6.

miRNAs	Forward (5'-3')	Reverse (5'-3')
miR-146a	GTGAGAACTGAATTCCAT	AACTGGTGTCGTGGAG
miR-222	GCTACATCTGGCTACTGG	TCAACTGGTGTCGTGG
miR-17	AAAGTGCTTACAGTGCAGG	AACTGGTGTCGTGGAG
miR-10a	TACCCTGTAGATCCGAAT	AACTGGTGTCGTGGAG
miR-31	CAAGATGCTGGCATAG	CTCAACTGGTGTCGTG
miR-let-7g	GGGTGAGGTAGTAGTTTGT	CTCAACTGGTGTCGTG
miR-92a	CCTATTGCACTTGTCCC	TCAACTGGTGTCGTGG
miR-221	GCTACATTGTCTGCTGG	CTGGTGTCGTGGAGTC
miR-21a	CCTAGCTTATCAGACTGATG	CTCAACTGGTGTCGTG
miR-217	CTGCATCAGGAACTGATT	TCAACTGGTGTCGTGG
miR-181b	ACGACAACATTCATTGC	AACTCCACACCAGCAC
miR-126	CGTACCGTGAGTAATAATG	CTCAACTGGTGTCGTG
miR-663	GTTTTAGGCGGGGCG	TGGTGTCGTGGAGTCG

miR/miRNA, microRNA.

CCCAACAA-3', E-selectin forward 5'-AATCCAGCCAAT GGGTTCG-3', E-selectin reverse 5'-GCTCCCATTAGTTCA AATCCTTCT-3', GAPDH forward 5'-ATGGGGAAGGTG AAGGTCG-3', and GAPDH reverse 5'-GGGGTCATTGAT GGCAACAATA-3'. To amplify mature miRNA sequences from cells and exosomes, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol and microRNA RT-qPCR syb kit (TaqMan) was used for generating cDNA (at 42˚C for 60 min) from microRNA and qPCR detection. Primers for interested microRNA were purchased from Invitrogen; Thermo Fisher Scientific, Inc., and primer sequences are shown in Table I. The qPCR conditions were as follows: 95˚C for 2 min, 40 cycles of 95˚C for 15 sec and 60˚C for 35 sec. The relative expression levels of the miRNAs were normalized to that of U6 by using the $2^{-\Delta\Delta Cq}$ cycle threshold method (19).

Western blot analysis. The cultured cells were harvested and lysed by RIPA supplemented with complete protease inhibitor cocktail tablets (Roche Diagnostics). Cell debris was removed by centrifugation at 10,000 x g for 20 min at 4˚C. Then the lysates (20 μg) were separated by SDS-PAGE gels (10-15%, 150 V, 70 min) and transferred them to PVDF membranes (Bio-Rad Laboratories, Inc.). After blocking with 5% BSA for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies overnight at 4˚C. Then, the membranes were incubated with HRP-conjugated secondary antibodies (Beyotime Institute of Biotechnology, cat. no. A0208, goat anti-rabbit IgG, 1:1,000) at room temperature for 1 h. After washing, the band intensity was analyzed using DAB Horseradish Peroxidase Color Development kit (Beyotime Institute of Biotechnology) and LAS-3000 Imaging System (Fuji Corporation). Quantity One software (Bio-Rad Laboratories, Inc., version 4.6.2) was used to calculate the expression of protein. An antibody against Alix (1:500; cat. no. sc-53540) was purchased from Santa Cruz Biotechnology, Inc. VCAM-1 (1:1,000; cat. no. ab134047), ICAM-1 (1:1,000; cat. no. ab2213), E-Selectin (1:1,000; cat. no. ab18981), IRAK (1:1,000; cat. no. ab238), TSG101 (1:1,000; cat. no. ab125011) and GAPDH (1:10,000; cat. no. ab8245) were purchased from Abcam. Phosphorylated-NF-κB p105 (1:1,000; cat. no. cst4806) and p105 (1:1,000; cat. no. cst4717) were purchased from Cell Signaling Technology, Inc.

Statistical analysis. All values are presented as the mean ± standard deviation. A Student's t test (2 groups) or one-way analysis of variance (3 or more groups with a least significant difference post hoc test) was used to determine statistical significance between the groups. All statistical analyses were performed by SPSS 17.0 (SSPS Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Successful isolation of exosomes from DC culture medium. The ultrastructure of exosomes was observed using transmission electron microscopy. Exosomes at a typical size of 30-100 nm in diameter were found by electron microscopic analysis (Fig. 1A). Then the expression of exosomes markers, TSG101 and Alix, was confirmed by immunoblotting (Fig. 1B). These results suggest successful isolation of exosomes from the culture medium. To study whether other cells can uptake exosomes, DC-exo was labeled with PKH67 and incubated with HUVECs. The results confirmed that HUVECs could uptake exosomes (Fig. 1C).

mDC-exos increase endothelial expression of adhesion molecules through quick activation of the NF-κB pathway. mDC-exo were co-cultured with HUVECs and it was found that the protein expression of VCAM-1, ICAM-1 and E-Selectin

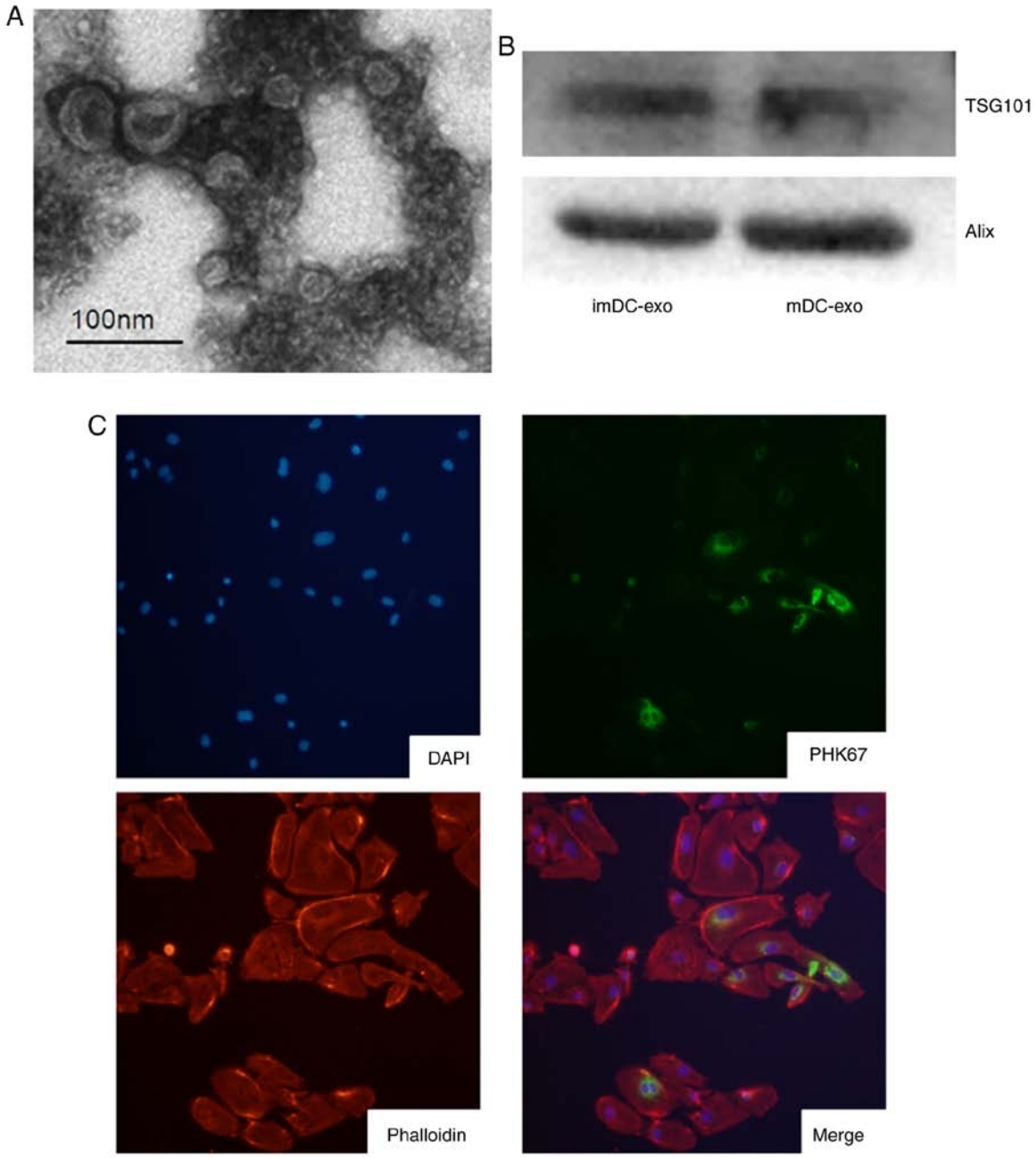

Figure 1. Successful isolation of exosomes from DC culture medium. (A) Ultrastructure of exosome by transmission electron microscopy. (B) Expression of exosomes markers, TSG101 and Alix, confirmed by immunoblotting. (C) HUVECs was incubated with PKH67-labeled exosomes (green) from bone marrow DCs and fixed for confocal imaging. HUVEC was stained with phalloidin (red) and nuclei with DAPI (blue). The incubated time was 24 h. Magnification, x400 HUVECs, human umbilical vein endothelial cell; CD, cluster of differentiation; DC, dendritic cell; exo, exosome; im, immature; m, mature.

significantly increased (P<0.05; Fig. 2A and B). To explore the mechanism of endothelial inflammation, p-p105 of the NF-κB signaling pathway was measured and a quick activation of this pathway (within 0.5 h) was demonstrated (Fig. 2C). The authors' previously published study demonstrated that mDC-exo can quickly activate the NF-κB signaling pathway by direct contact between exosomal membrane proteins and HUVEC ligands (13). The present study aimed to investigate the effect of exosomal miRNAs after the influence of exosomal proteins. The mRNA expression of adhesion molecules at different time (as long as 36 h) was determined and it was confirmed that the first stage effect of mDC-exo on HUVECs gradually vanished 36 h after incubation (Fig. 2D-F).

HUVECs were less vulnerable to a second stimulation and miR-146a might play a role. To investigate the effect of exosomal miRNAs on HUEVCs, the experiments were conducted as follows: HUVECs were pre-treated with mDC-exo for 12 h and then they were allowed a 24-hrecovery period, after which the HUVECs were treated with mDC-exo for another 6 h. According to the findings above, the mRNA expression of adhesion molecules would reduce to a very low level at 36 h. In addition, exosomal miRNAs would have been released into HUVECs and impose an effect on the cells. The second stimulation of mDC-exos could reflect the effect of DC-exosomal miRNAs on HUVECs, whether they are protective or injurious. The results showed that VCAM-1 expression

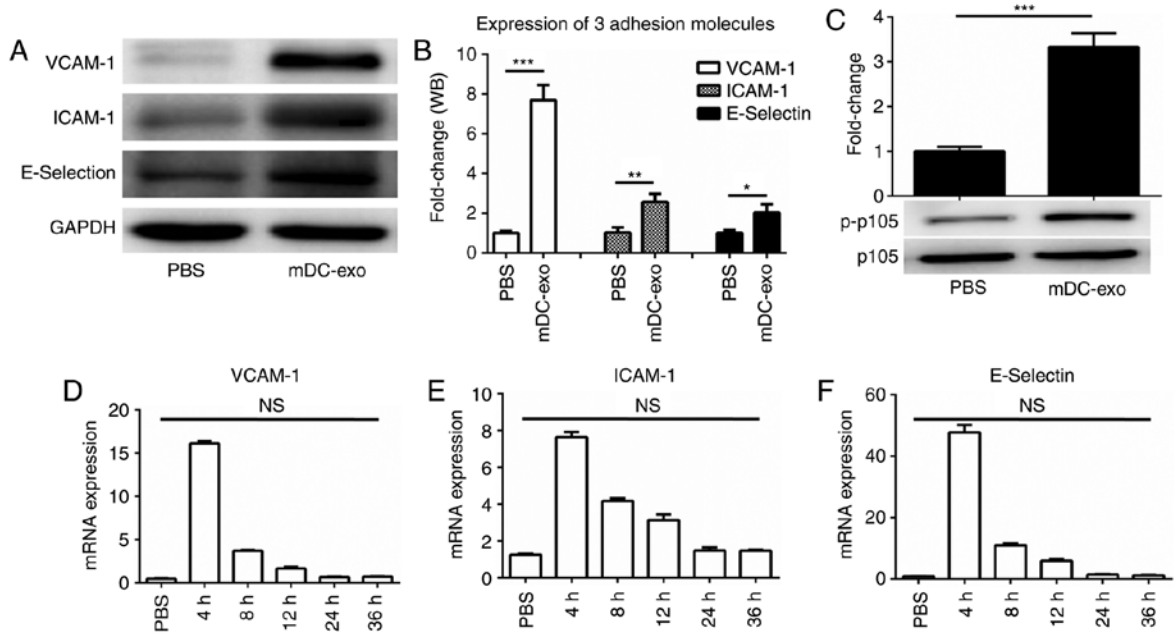

Figure 2. mDC-exo increase endothelial expression of adhesion molecules through quick activation of NF-κB pathway. (A) Western blotting and (B) analysis of exosomes from mDCs were incubated with HUVECs for 24 h and the expression of VCAM-1, ICAM-1 and E-Selectin was detected by immunoblotting. (C) mDC-exo were incubated with HUVECs for 0.5 h and p-p105 of NF-κB pathway was detected by immunoblotting. mDC-exos were incubated with HUVECs for indicated time and the mRNA expression of (D) VCAM-1, (E) ICAM-1 and (F) E-Selectin was detected by quantitative PCR. *P<0.05, **P<0.01 and ***P<0.001. ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; HUVECs, human umbilical vein endothelial cell; DC, dendritic cell; exo, exosome; p, phosphorylated; NF, nuclear factor; NS, not significant; m, mature.

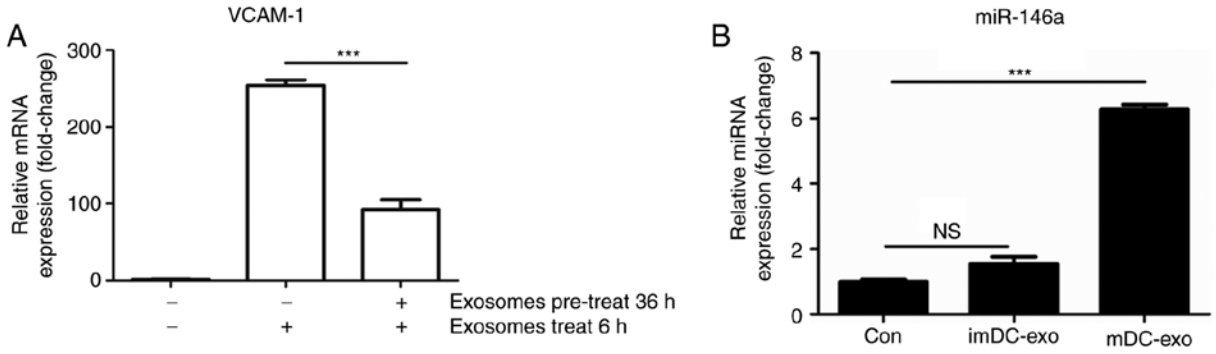

Figure 3. HUVECs less vulnerable to a second stimulation and the possible role of miR-146a. (A) HUEVCs were pre-treated with DC-exo for 12 h and then they were allowed a 24-h recovery period, after which the HUVECs were treated with exosomes for another 6 h. Expression of VCAM-1 was detected by quantitative PCR. (B) Expression of miR-146a in HUVEC treated with imDC-exo or mDC-exo for 24 h. ***P<0.001. HUVECs, human umbilical vein endothelial cell; DC, dendritic cell; exo, exosome; ICAM, intercellular adhesion molecule; VCAM, vascular cellular adhesion molecule; im, immature; m, mature; NS, not significant.

was significantly reduced (P<0.001; Fig. 3A), indicating a protective role of DC-exosomal miRNAs on HUVECs.

Next, which miRNA was involved in the protection of HUVECs was investigated. To this end, a recent review was retrieved, which comprehensively reviewed current evidence of miRNAs in endothelial inflammation and atherosclerosis (20). In that review, the authors concluded that 13 miRNAs are involved in adhesion molecule expression. They are miR-146a/b, miR-181b, miR-126, miR-663, miR-21, miR-217, miR-10a, miR-31, miR-221, miR-222, miR-17, miR-let-7g and miR-92a. their expression in HUVECs were measured after DC-exo stimulation and it was found that miR-146a expression increased 6-fold (Fig. 3B). It was noticed that the expression of miR-222 and miR-17 also slightly increased (Table II). miR-146a, miR-222 and miR-17 were demonstrated to be protective miRNAs (21-24) and miR-146a was chosen as the target of further research since it increased the most significantly.

Elevated miR-146a in HUVECs is partly due to the miRNA cargo in exosomes. Next the present study wanted to investigate whether the miRNAs contained in mDC-exo contribute to the elevation of miR-146a in HUVECs. First, the expression of miR-146a in exosomes was determined and it was found that expression was increased in mature DC-exo compared with in immature DC-exo (Fig. 4A). This result was consistent with a previous study, which performed a microarray to investigate the differential expression of miRNAs

Table II. Expression of 13 miRNAs in immature and mature dendritic cell exosome.

miRNAs	Change	Fold-change
miR-146a	Up	6.28
miR-222	Up	2.15
miR-17	Up	2.06
miR-10a	Up	1.63
miR-31	Up	1.59
miR-let-7g	Up	1.55
miR-92a	Up	1.54
miR-221	Up	1.44
miR-21a	Up	1.27
miR-217	Down	0.99
miR-181b	Down	0.87
miR-126	Down	0.69
miR-663	Down	0.60

miR/miRNA, microRNA.

in immature and mature DC-exos (12). Since miR-146a can be induced in endothelial cells upon exposure to proinflammatory cytokines (21), the direct contact of exosomes and HUVECs induce the NF-κB signaling pathway and it surely will increase miR-146a expression. Therefore, the present study needs to confirm that the elevated miR-146a in HUVECs is partly due to the shuttled cargo in exosomes. Annexin V (AV), a well-known inhibitor of exosome uptake was used to investigate whether the contained miR-146a in mDC-exo would influence its level in HUVECs. The effect of AV was confirmed by immunofluorescence (Fig. 4B). Then the present study found that whether AV was present or not, the HUVECs expression of VCAM-1 induced by mDC-exo remained unchanged (Fig. 4C). This result showed that, although the uptake of exosomes was inhibited, activation of the NF-κB pathway by direct contact of exosomes and HUVECs was not influenced. As a result, the intrinsic expression of miR-146a in HUVECs induced by the NF-κB signaling pathway should be similar. Additionally, it was found that the expression of miR-146a in HUVECs was significantly decreased in mDC-exo-AV group compared with in the mDC-exo group ($P<0.05$), suggesting the inhibition of exosome uptake decreased the shuttling of miR-146a from exosomes to HUVECs (Fig. 4D).

Exosomes can shuttle miR-146a to HUVECs and protect the recipient cells through inhibiting IRAK. To further confirm whether mDC-exo could shuttle miRNAs to recipient cells, imaging experiments were conducted to visualize the transfer of labeled miRNAs between cells. First, DCs were transfected with a Cy3-labeled miRNA control. After 24 h of transfection, DCs were replaced with fresh culture medium. After another 36 h, the culture supernatant was collected for exosome isolation. Then, HUVECs were incubated with the isolated exosomes for 24 h. It was found that DC-exos, which contained the Cy3-labeled miRNA control, were endocytosed by HUVECs, as visualized by fluorescence microscopy (Fig. 5A). To confirm this result, a miR-146a mimic/inhibitor was transfected into BMDCs and co-culture their exosomes with HUVECs. An increase of miR-146a upon mimic transfection and a decrease upon inhibitor transfection was observed (Fig. 5B). To test whether the shuttled miRNA can functionally affect the recipient cells, HUVECs were pre-treated with exosomes derived from DCs transfected with miR-146a mimic or inhibitor for 36 h. Then the HUVECs were once again stimulated with mDC-exo. It was found that exosomes transfected with the miR-146a mimic could attenuate the elevated expression of adhesion molecules and exosomes transfected with miR-146a inhibitor could increase the expression of adhesion molecules (Fig. 5C). A previous study has shown that miR-146a can regulate cell response through targeting IRAK (25). It was confirmed that IRAK-1 expression was significantly inhibited in the miR-146a mimic group and rescued in the miR-146a inhibitor group ($P<0.001$; Fig. 5D). These results indicate that exosomes derived from BMDCs can shuttle miRNA to the recipient cells and the shuttled miR-146a can protect HUVECs from further injury by mDC-exo.

Discussion

In this present study, mDC-exos were shown to increase endothelial expression of adhesion molecules. Then it was found that mDC-exo could shuttle miR-146a into HUVECs and the shuttled miR-146a contributed to protecting HUVECs from a second stimulation. The present study demonstrated for the first time that DCs-derived exosomes make up a negative feedback loop to control inflammation.

It has been demonstrated that DCs play an important role in both pro-atherogenic (3,26,27) and regulatory (7,28) responses within the artery wall. Vascular DCs seem to be involved in endothelial inflammation in three roles as follows: The uptake and storage of lipids; the clearance of lipids, apoptotic cells, and debris from the artery wall, and the maintenance of vascular Treg responses (29). In this study, DCs were shown to regulate endothelial inflammation through released exosomes.

Exosomes can be released by numerous cell types. Classically in cell biology, cells communicate with each other by direct interaction or secreted soluble factors. These factors can act on the cell itself (autocrine signaling) or on both neighboring (paracrine signaling) and distant cells (endocrine signaling). During the past decade, exosomes have been found to be potent vehicles of intercellular communication. In this study, it was shown that DCs can release exosomes and these exosomes can activate endothelial cells.

A major group of exosomal cargos is RNAs (9). Since the beginning of the discovery that exosomes contain RNA, miRNAs shuttled by exosomes have attracted huge attention in all research fields. Previous studies have confirmed that exosomes and exosomal miRNAs play an important role in cardiac repair after myocardial infarction (30-32). However, they are less investigated in inflammation and atherosclerosis. Up to now, ~12 miRNAs have been identified in the process of endothelial injury (20,33). In this study, these miRNAs were detected in HUVECs stimulated by DC-exo and it was found that miR-146a was changed the most. miR-146a has been shown to play a role in numerous diseases, such as

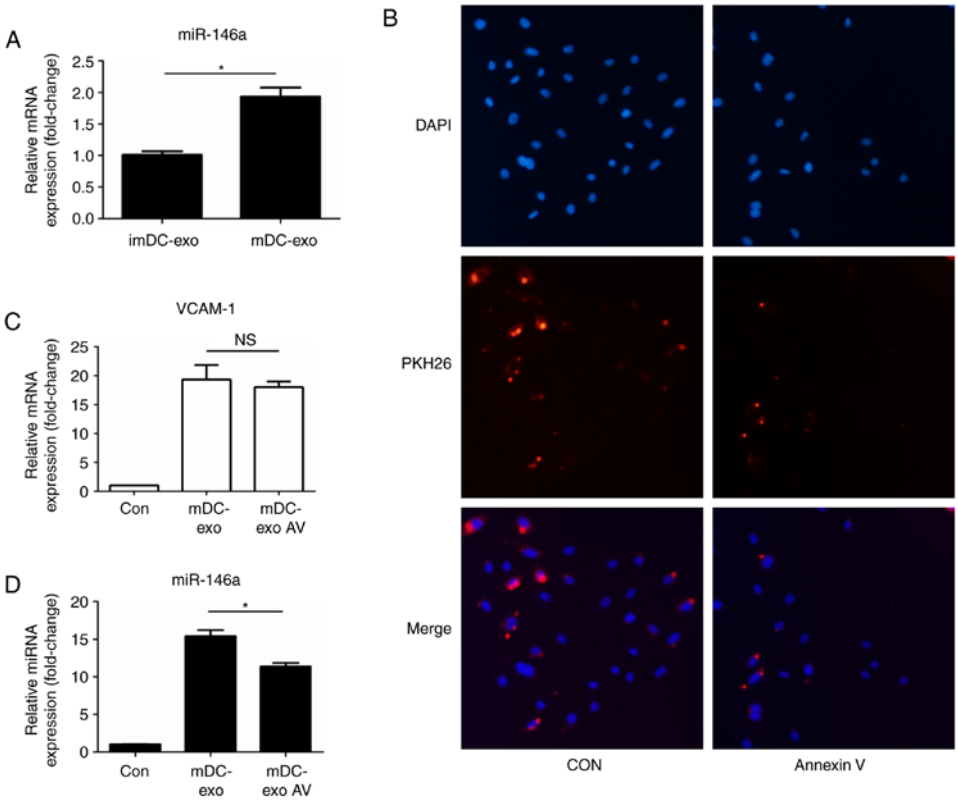

Figure 4. Elevated miR-146a in HUVECs partly due to the miRNAs cargo in exosomes. (A) Expression of miR-146a in imDC-exo and mDC-exo. (B) Fluorescence confocal of HUVECs treated with PKH26-labled exosomes (red) which were preincubated for 1.5 h with annexin V (2 μg/ml). HUVECs were stained with nuclei with DAPI (blue). (C) Expression of VCAM-1 in HUVECs treated with mDC-exo which were pre-incubated with annexin V or not. (D) Expression of miR-146a in HUVECs treated with mDC-exo which were pre-incubated with annexin V. *P<0.05. Magnification, x400. HUVECs, human umbilical vein endothelial cells; DC, dendritic cell; exo, exosome; VCAM, vascular cellular adhesion molecule; microRNA, miR; NS, not significant; im, immature; m, mature; Con, control.

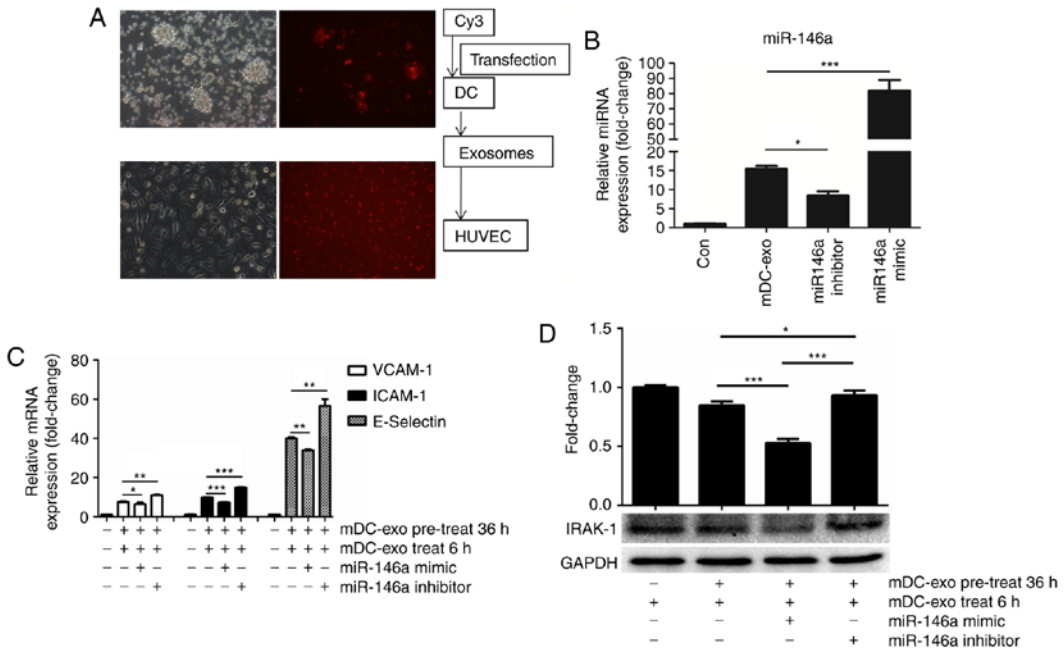

Figure 5. Exosomes can shuttle miR-146a to HUVECs and protect the recipient cells through inhibiting IRAK. (A) Upper two images show that Cy3 was transfected into DCs clusters (the left one was plain image and the right was fluorescence image). In the lower two images, exosomes from Cy3 transfected DCs were co-cultured with HUVECs and uptake by HUVECs are shown. (B) Expression of miR-146a in HUVECs treated with exosomes from DCs transfected with miR-146a mimic or inhibitor. (C) HUVECs were pre-treated with exosomes from DCs transfected with miR-146a mimic or inhibitor for 12 h. After a 24-h recovery period, the HUVECs were once again treated with DCs-exos for another 6 h. Expression of adhesion molecules, VCAM-1, ICAM-1 and E-Selectin, were detected by qPCR. (D) Based on the design of Fig. 5C, expression of IRAK was detected by immunoblotting. *P<0.05, **P<0.01 and ***P<0.001. Magnification, x40. HUVECs, human umbilical vein endothelial cells; DC, dendritic cell; exo, exosome; miR, microRNA; VCAM, vascular adhesion molecule; ICAM, intercellular adhesion molecule; m, mature; IRAK, interleukin-1 receptor-associated kinase.

atherosclerosis, aging and neurodegenerative disease (1,34,35). In the present study, it was then confirmed that DC-exo could shuttle miR-146a into HUVECs and the shuttled miR-146a contribute to protecting HUVECs from a second stimulation.

This is interesting because it is a new kind of negative feedback regulatory loop. A regulatory feedback loop, whether negative or positive, commonly exists in numerous life processes, including the response to injury of endothelial cells. It has been reported that miRNAs provide negative feedback control of inflammation in HUVECs (21,23). With the authors' previous (13) and present findings, the knowledge of this feedback system is extended, indicating that exosomes can exert an influence on recipient cells in a timely manner as follows: Quick and direct effect by direct contact of proteins, slow and indirect effect by shuttled miRNAs.

In conclusion, the present study showed that mDC-exo increased endothelial expression of adhesion molecules. Then after being up-taken by HUVECs, mDC-exo could shuttle miR-146a into recipient cells and the shuttled miR-146a contributes to protecting HUVECs from a second stimulation. These data suggest a negative feedback loop of inflammation regulation by mDC-exos.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and WG designed the study, performed the research and wrote the manuscript. RW performed the molecular biology experiment and immunofluorescence assay. HL helped in the molecular biology experiment and was involved in the immunofluoresence assay. JG designed and supervised the study.

Ethics approval and consent to participate

This research was approved by the Institutional Review Board of The Zhongshan Hospital, Fudan University and Shanghai Institutes for Biological Sciences.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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