Mannose-binding lectin reduces oxidized low-density lipoprotein induced vascular endothelial cells injury by modulating autophagy via LOX1

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Abstract

Objective

To investigate the role of mannose-binding lectin (MBL) in modulating autophagy and protecting endothelial cells (ECs) from oxidized low-density lipoprotein (ox-LDL) induced injury.

Materials and Methods

Rapamycin and chloroquine were used to confirm the role of autophagy in ox-LDL induced ECs injury. Dendritic cells (DCs) were co-cultured with ECs, after which inflammatory factors and DCs maturation rate were detected. Autophagy was detected by LC3 and Lamp2a or autophagosomes. Cell viability was analyzed by Cell Counting Kit-8 (CCK-8) assay. Flow cytometry was utilized to analyze cell proliferation and apoptotic rate. ECs transfected with lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)-siRNA or MBL over-expression plasmid were treated with ox-LDL to explore the mechanism of MBL in ECs injury. HE, Oil Red O, TUNEL staining, and immunofluorescence were used to evaluate the atherosclerotic plaque, ECs injury, and autophagy, respectively. Retro-eyeball injections of MBL over-expression adenovirus were conducted every 4 weeks, after which ECs injury, autophagy, the uptake of ox-LDL, and expression of LOX1 were further analyzed.

Results

ECs treated with 100 ug/mL ox-LDL for 24 h significantly increased the expression of LC3, Lamp2a, and ET1. Rapamycin aggravated, chloroquine alleviated ox-LDL induced ECs autophagy and injury. LOX1-siRNA transfected ECs inhibited the uptake of ox-LDL and reduced ECs autophagy and injury compared with siRNA group. MBL over-expression *in vitro* decreased the binding of LOX1 and ox-LDL, ameliorated ECs autophagy and injury compared with the control plasmid group. MBL over-expression *in vivo* alleviated the formation of atherosclerotic plaque in HFD fed ApoE^{-/-} mice, influenced the maturation of DCs, and down-regulated IL-6, IL-12, and TNF- α level compared with the control group. Also, mannan could reverse the protective role of MBL.

Conclusion

MBL exerts a protective role in ox-LDL induced ECs injury and HFD induced atherosclerosis model by regulating DCs maturation and modulating ECs autophagy via blocking the binding of LOX1 and ox-LDL.

Background

Atherosclerosis and subsequent cardiovascular diseases (CVDs) contribute to the leading causes of mortality worldwide [1, 2]. Although they benefit from optimal medical and pharmaceutical care, a

significant portion of CVDs patients still suffer from recurrent cardiovascular events [3]. Increasing evidences suggested that the immune system's activation and the inflammatory response have a crucial role in the initiation and progression of atherosclerosis rather than just lipid deposition [4-6]. Moreover, therapeutic strategies targeted at the immune system, also known as immune-modulatory therapy, have recently attracted extensive attention [7, 8]. Canakinumab, a human monoclonal IL-1b antibody, was confirmed to have a beneficial effect for atherosclerosis in the phase 3 CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) [7]. However, another clinic trial named CIRT (Cardiovascular Inflammation Reduction) revealed that nonspecific anti-inflammation therapy with low-dose methotrexate did not improve CVDs outcomes [8]. Other immune-modulators such as tocilizumab (humanized anti-IL-6R antibody) showed to have a protective effect in non-ST-elevation myocardial infarction patients while still posing the risk of increasing circulating levels of cholesterol and triglycerides [9, 10]. Therefore, selecting an appropriate immunoregulation target along with an optimal treatment window and exploring the underlying mechanisms is urgently needed.

Ox-LDLs were mainly uptaken by ECs through binding with lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), mediating immune reactions and participating in the pathogenesis of atherosclerosis [11, 12]. A previous study revealed that ox-LDL concentration polarization might activate autophagy and apoptosis via the LOX-1 pathway [13], while over-expression of LOX-1 may attenuate ECs protective autophagy and induce apoptotic death of bovine aortic ECs [14]. The contradictory findings might be explained by basal autophagy that has protective effects in the early stage of atherosclerosis, while detrimental effects in advanced atherosclerosis plaques [15]. Therefore, regulating the homeostasis of autophagy might be the treatment target of atherosclerosis.

MBL belongs to the C-type lectins family, which consists of a couple of amino-terminal collagen-like domains, a short neck region, and a carboxy-terminal C-type lectin domain [16]. It can activate the complement system by selectively binding to mannan - rich pathogens and can also trigger opsonophagocytosis through direct binding to cell surface receptors. Several studies have shown that high levels of MBL are harmful to patients with ischemic stroke, diabetic nephropathy, and retinopathy [17-19]. Studies on MBL genotypes revealed that low or deficient MBL was a risk factor for developing CVDs [20, 21]. A recent longitudinally study found that high plasma MBL was associated with lower carotid intima-media thickness (cIMT) [22]. Other studies reported a dual role of MBL in T2DM patients, as both low and high levels of MBL indicate the susceptibility for CVDs [23, 24]. Thus, whether MBL has beneficial or harmful effect in CVDs, as well as relevant underlying mechanisms, remain unclear.

Both MBL and LOX1, which share similar domains, are members of the lectins family. A previous study confirmed that MBL could bind to ox-LDL *in vitro*; however, this recognition step did not activate the lectin complement pathway [25]. In this study, we hypothesized that MBL might competitively inhibit the binding of ox-LDL and LOX1. Our aim was to investigate whether MBL can inhibit ox-LDL induced ECs autophagy and injury.

Methods

Cells culture

Mouse ECs and DCs were purchased from Tongpai (Shanghai, China) and cultured in RPMI-1640 medium (containing 10% fetal bovine serum) at 37 °C and 5% CO₂. Mouse spleen derived dendritic DCs were prepared according to the previously described method [26]. Flow cytometer was used to identify DCs with biomarkers of CD 11c (Bioss, Beijing, China) and CD80 (Biolegend, [San Diego, CA, USA](#)).

To establish ox-LDL induced AS model *in vitro*, mouse ECs (single cultured group) were cultured, DCs and ECs (co-culture group) were co-cultured and then treated with 0, 100, 200 µg/mL ox-LDL (Yiyuanbiotech, Guangzhou, China) for different time durations (0, 24, 48h).

Chloroquine (25uM, TCI, Shanghai, China) and rapamycin (0.02 µg, Gene operation, USA) were pretreated for 1h and then treated with 100 µg/mL ox-LDL for 24h to explore the role of autophagy in ox-LDL induced ECs injury. Mann (6 mg/L, Aladdin, Shanghai, China) pretreated for 48h and then treated with 100 µg/mL ox-LDL for 24h was used to explore the role of MBL in the regulation of autophagy in ox-LDL treated ECs.

Cell transfection

Small interfering RNA (siRNA) against LOX1 (LOX1 siRNA, Forward: 5'-ACTCTGGTCATCCTCTGCCT-3', Reverse: 5'-CCTGCTGAGTAAGGTTTCGCT-3') or negative control siRNA (Forward: 5'-CTCTCTGCTCCTCCCTGTTC-3', Reverse: 5'-AAATCCGTTACACCGACCT -3'), LC3-GFP plasmid, MBL overexpression plasmid (MBL plasmid), and control plasmid, MBL overexpression adenovirus, and empty vector were synthesized by Genepharma (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA) was used for transfection of oligonucleotides or vectors according to the standard protocol.

Cells viability assay

A 300ul ECs suspension (4 x 10⁴ cells/ well) was incubated with different concentrations of ox-LDL for 24 hours and 48 hours. Then a cell Counting Kit-8 (CCK-8) reagents (30 µL/well) (Beyotime, Shanghai, China) were added into each well for 2 hours. The absorbance was measured at 450 nm with a microplate reader.

Cell apoptosis assay

Flow cytometry was used to detect ECs and DCs apoptosis rates. After treated with ox-LDL for 24h, ECs and DCs were resuspended with binding buffer, followed by staining with 5 µL annexin V-FITC (KeyGen Biotech, Nanjing, China) and 10 ul PI (Sigma, St Louis, USA). The apoptotic cells were analyzed by FlowJo software (Tree Star, Ashland, USA).

DCs maturation assay

DCs maturation rates were analyzed by flow cytometry with anti-CD 80 and CD86 antibody (eBioscience, California, USA). 200 μ L DCs (5×10^4 /mL) were incubated at 37°C and 5% CO₂ for 24h, the upper compartment cells were collected and washed twice with PBS (containing 2%FBS); the supernatant was discarded, and 95 μ L PBS and 5 μ L corresponding antibodies were added. Next, they were swirled and mixed, incubated at 4°C for 30 min in darkness, after which 1 mL PBS was added for centrifuge washing for 2 times. The apoptotic cells were analyzed by FlowJo software (Tree Star, Ashland, USA).

Enzyme-linked immunosorbent assay (ELISA)

ET-1, IL-6, IL-12, and TNF- α levels from the supernatants were detected by mouse ET-1 ELISA kit (Cloud-Clone, Wuhan, China), IL-6 ELISA kit (ExCell Bio, Shanghai, China), IL-12 p70 ELISA kit (ExCell Bio, Shanghai, China) and TNF- α ELISA kit (ExCell Bio, Shanghai, China). Operations were performed according to the manufacturer's instructions, and the absorbance was detected at 450 nm wavelength.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cell total RNAs were prepared with Trizol reagent (Invitrogen, Waltham, MA, USA). qPCR was performed using an SYBr green Pcr kit (Thermo, USA). The gene expression levels were quantified with a 7900 Fast Real-Time PCR instrument according to the instruction. Primers used in this experiment were as follows: LOX1 forward: 5'-ACTCTGGTCATCCTCTGCCT-3', reverse: 5'-CCTGCTGAGTAAGGTTTCGCT-3'. MBL forward: 5'-TGCTTACCCAGGCAAGCCTGTG-3', reverse: 5'-TGCTTACCCAGGCAAGCCTGTC-3'.

Western Blot

Proteins were homogenized in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China), then quantified by the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Immunoblot assays were performed using primary antibodies against LC3 (1:1000, Abgent, San Diego, USA), MBL antibody (1:100, Abcam, UK), LOX1 antibody (1:1000, Abcam, UK) and β -actin (Abcam, UK) overnight at 4°C. Following this, the blots were washed in TBS-T and incubated with HRP-conjugated secondary antibody (Jackson111-095-003, USA) for 1 h at room temperature. Image J (National Institutes of Health, Bethesda, Md, USA; version 1.48) was used for semi-quantitative analysis.

Immunofluorescence

LC3-GFP plasmid was transfected into ECs, and 48h later was treated with ox-LDL for 24h, then fixed in 4% formaldehyde for 10-15 minutes and blocked with 5% bovine serum albumin (BSA) within 1% Triton X-100 for 5-10min at room temperature (SenBeiJia Biological Technology, Nanjing, China). Next, it was incubated with Lamp2a primary antibody (1:200, Abcam, UK) at 4°C overnight, after which the mixture of fluorescent secondary antibody and DAPI were diluted at a concentration of 1:500 (Boster Biological Technology, Wuhan, China), and incubated at room temperature in the dark for 30-60 minutes. Finally, a laser confocal microscope was used to take photos (ZEISS, German).

After the aortic vascular endothelial tissue was dehydrated, rendered transparent and paraffin-embedded, serum was added to block antibodies at room temperature for 30min. The blocking fluid was shaken off and incubated with primary antibody CD31 (1:100, Abcam, UK), CD11C (1:100, Abcam, UK), LC3 (1:100, Proteintech Group, Wuhan, China), and LOX1 (1:100, Abcam, UK) at 4°C overnight. Slices were then rinsed with PBS for 3 times, after which diluted fluorescent secondary antibody were added and incubated in a wet box at 20-37 °C for 1 h, and rinsed with PBS for 4 times. DAPI (1:500, Boster Biological Technology, Wuhan, China) were added and incubated at room temperature in the dark for 5 min. The slices were sealed with an anti-fluorescence quenching agent (SouthernBiotech, 0100-01), after which the images were collected under a fluorescence microscope (Olympus,BX53).

Co-Immunoprecipitation (Co-IP) analysis

The treated cells were collected, and proteins were extracted. An ultraviolet spectrophotometer was used to determine the protein concentration. Then, 20 µL of Protein A/G agarose beads, 180 µL of PBS, and MBL antibody at a ratio of 1:100 (Abcam, UK) were mixed and incubated at room temperature for 1.5 h with rotation. Samples were centrifuged at 4 °C for 2 min at 2000 r/min, washed with PBS for 3 times, after which the 400 µL (1mg) protein lysate was added, and the samples were rotated and incubated at 4 °C for 2 h. After centrifugation at 2000 r/min at 4 °C for 2 min, they were washed with PBS for 3 times, the precipitation was collected and added into 1×Loading Buffer and boiled for 5 min. The supernatant was collected after centrifugation at 12000 r/min at 4 °C for 5 min and then prepared for the Western Blot experiment (referred to WB part).

Animals and *ex vivo* experiment

ApoE^{-/-} mice and C57BL/6J mice (6 weeks, male) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). All the animals were housed in an environment with a temperature of 22 ± 1 °C, a relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 hr. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Zhejiang University institutional animal care and conducted according to the AAALAC and the IACUC guidelines.

For atherosclerosis model construction, mice were fed with HFD for 12 weeks. For the MBL intervention group, mice were fed with HFD for 16 weeks; control adenovirus /MBL over-expressed adenovirus was administered with retro-orbital injections every 4 weeks.

Oil Red O and HE staining were performed according to the manufacturer's instructions (Sigma, St Louis, USA). Quantification of Oil Red O staining used NIH ImageJ software (Bethesda, MD, USA). Sections of aortic vascular endothelial tissue were stained with a TUNEL apoptosis assay kit (Roche Applied Science, Switzerland), and the apoptosis of the cells were viewed under a fluorescence microscope (Olympus BX53).

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software 8.0. A multiple t test was used to analyse individual differences. $P < 0.05$ was statistically significant.

Results

Autophagy is involved in ox-LDL induced ECs injury, proliferation, and apoptosis

Vascular ECs were treated with ox-LDL (0, 100, 200 ug/ml) for 24 h and 48 h, after which ECs autophagy, cell injury, and cell proliferation rate were determined. The results showed that ox-LDL induced ECs injury in both time and dose-dependent manner (**Figure 1A, 1B**). Treatment with 100 ug/mL ox-LDL for 24 h significantly increased the LC3 protein expression in ECs compared with 0h (**Figure 1C**). Thus, 100ug/ml ox-LDL treatment for 24h was utilized in the subsequent experiments.

After treated with chloroquine (CQ) and rapamycin (RAPA), the LC3-GFP expression decreased in the EC+CQ+oxLDL group, while it increased in EC+RAPA+oxLDL group compared with the EC+oxLDL (**Figure 1D**). The ET -1 expression (**Figure 1E**) and ECs apoptosis (**Figure 1G**) rate were significantly reduced, while the proliferation rate of ECs (**Figure 1F**) was significantly increased in the chloroquine treated group. In contrast, the ET -1 expression (**Figure 1E**) and ECs apoptosis rate (**Figure 1G**) were significantly increased, while the proliferation rate of ECs (**Figure 1E**) was significantly reduced in the rapamycin treated group compared with the control group. These data suggested that autophagy is involved in ox-LDL induced ECs injury, proliferation, and apoptosis.

DCs aggravate ox-LDL-induced ECs autophagy

DCs were co-cultured with ECs to investigate the role in ox-LDL induced ECs autophagy. The results showed that the co-localization of LC3 and Lamp2a increased both in EC and EC+DC groups after ox-LDL treatment, and compared with the EC group, the co-localization of LC3 and Lamp2a in ECs increased in DCs co-culture group (**Figure 2A**). The electron microscopy also showed that ox-LDL treatment increased the autophagosome formation, and DCs co-culture further promoted autophagosome formation in ECs (**Figure 2B**). When DCs were treated with ox-LDL, we found that the expression of IL-6, IL-12, and TNF- α was up-regulated in a time-dependent manner (Figure 2C). Also, the flow cytometric analysis revealed that the expression of CD86 and CD80 was significantly increased after the treatment of ox-LDL compared with the group without ox-LDL (**Figure 2D**). These data suggested that ox-LDL treatment can induce DCs inflammation and promote DCs maturation, while DCs co-culture can further promote ox-LDL induced ECs autophagy.

Ox-LDL induced ECs autophagy and injury via LOX1 pathway

ECs were treated with ox-LDL, and **Figure 3A** showed that LOX1 was mainly expressed in the cell membrane and cytoplasm. The uptake of ox-LDL into the cytoplasm coincided with the expression of LOX1. Also, ox-LDL uptake was significantly increased at 6 h but did not increase with time, while the expression of LOX1 was strongest at 24h. Consequently, 6 h was selected as detection time. To

investigate the role of LOX1 in ox-LDL induced ECs autophagy, LOX1 siRNA and control siRNA were transfected into ECs; LOX1 mRNA and protein were significantly down-regulated in the LOX1-siRNA group (**Figure 3B** and **3C**).

Figure 3D showed the location of ox-LDL in ECs at 0, 6 h of ox-LDL treatment. The results showed that at 6 h of ox-LDL treatment, red fluorescence appeared, indicating that ox-LDL uptake occurred, while the red fluorescence expression of the LOX1-siRNA group was significantly reduced. These results suggested that down-regulating LOX1 can inhibit ox-LDL uptake. After ox-LDL treatment, the LC3 aggregation increased significantly in the control siRNA and LOX1-siRNA group compared with no ox-LDL treatment groups (**Figure 3E**), thus indicating that ox-LDL can activate the autophagy behavior of ECs. The LC3 aggregation in ECs significantly weakened in the LOX1-siRNA + ox-LDL group compared with the Control siRNA + ox-LDL group (**Figure 3E**), indicating that down-regulating LOX1 in ECs can alleviate ox-LDL induced autophagy.

ECs injury and cell activity results showed that the ET-1 expressions were significantly increased after ox-LDL treatment compared with the control group, while after the interference of LOX1 in ECs, the ET-1 expressions significantly decreased (**Figure 3F**). These results suggested that ox-LDL treatment could promote the secretion of ET-1 in ECs, while LOX1 down-regulation in ECs could inhibit the secretion of ET-1. Similarly, ox-LDL treatment could decrease the activity of ECs, while after LOX1 interference, the activity of ECs increased (**Figure 3G**). These findings suggested that ox-LDL induced ECs autophagy and injury via LOX1 and that downregulating LOX1 can alleviate ox-LDL induced ECs injury.

MBL overexpression *in vitro* may block the binding of LOX1 and ox-LDL, further inhibit ox-LDL induced ECs autophagy and injury

To investigate the role of MBL in protecting ECs from ox-LDL induced injury, we constructed MBL over-expressed plasmid (**Figure 4A**). The results showed that compared with the blank plasmid transfection group, the expression of MBL mRNA and protein in ECs significantly increased after transfection with MBL overexpression plasmid. **Figure 4B** shows the LC3 detection of ECs in each group. After ox-LDL treatment, LC3 aggregation was significantly reduced in the MBL plasmid transfected group compared with the control plasmid transfected group (**Figure 4B**). To further verify the protective effect of MBL on ECs, we used mannan competitive binding to MBL to observe the autophagy and injury of ECs. The results revealed that the aggregation of ECs LC3 significantly increased after treatment with mannan compared with the group not treated with mannan (**Figure 4B**). Moreover, mannan treatment also reversed the protective effect of MBL on ECs activity (**Figure 4C**) and injury (**Figure 4D**). These results confirmed that MBL can protect ECs from ox-LDL induced autophagy and cell damage and that mannan can reverse the protective effect of MBL on ECs.

To further explore the mechanism of MBL in protecting ECs from ox-LDL induced injury, we performed immunofluorescence localization of LOX1 and ox-LDL in ECs. **Figure 4E** shows that there was no red fluorescence without ox-LDL treatment, indicating that ECs did not express ox-LDL and that there was no significant difference in LOX1 expression between the two groups. After ox-LDL treatment, the cells

showed red fluorescence, which indicated that ECs uptake ox-LDL. The LOX1 fluorescence intensity of the ECs increased in the control plasmid group after being treated with ox-LDL, indicating that ox-LDL can promote the expression of LOX1. Compared with the Control plasmid + ox-LDL group, the green fluorescence LOX1 intensity and the red fluorescence ox-LDL intensity of the MBL plasmid + ox-LDL group were reduced, and the overlap between LOX1 and ox-LDL was reduced, thus indicating that MBL over-expression may block the combination of LOX1 and ox-LDL. Further co-immunoprecipitation(co-IP) showed that both ox-LDL and MBL protein expressions were significantly increased in the MBL plasmid + ox-LDL group compared with the Control plasmid + ox-LDL group (**Figure 4F**). These results suggested that ox-LDL and MBL interact with each other. Yet, compared with the MBL Plasmid group, ox-LDL protein expression was decreased after mannan treatment, indicating that mannan could reduce the interaction between ox-LDL and MBL (**Figure 4G**). These data suggested that MBL may block the binding of LOX1 and ox-LDL *in vitro*, thus further inhibiting ox-LDL induced ECs autophagy and injury.

Autophagy was involved in HFD induced atherosclerosis in ApoE^{-/-} mice

ApoE^{-/-} mice fed with HFD were used to establish atherosclerosis models. To investigate the involvement and the mechanism of autophagy in the development of atherosclerosis, intraperitoneal chloroquine injections were performed combined with HFD in ApoE^{-/-} mice every day. After 16 weeks, the thoracic aortas were collected and stained with Oil Red O, HE, TUNEL staining, and immunohistochemical; spleen was collected for DC cells isolation, and serum was collected for inflammatory factors detection. The results showed that the fat mass stained by Oil Red O staining (**Figure 5A**), atherosclerotic plaques stained by HE staining (**Figure 5B**), LOX1 expression (**Figure 5C**), apoptosis rate (**Figure 5D**), uptake of ox-LDL (**Figure 5E**), as well as the expression of LC3 (**Figure 5F**) in ECs of aortas were significantly increased in ApoE^{-/-} mice compared with WT mice. However, the fat mass (**Figure 5A**), the apoptosis rate (**Figure 5D**), and LC3 expression (**Figure 5F**) in ECs were significantly decreased, and the atherosclerotic plaques disappeared (**Figure 5B**) in chloroquine injection group compared with WT mice. These data showed that HFD could increase the expression of LOX1 and uptake of ox-LDL and aggravate the formation of atherosclerotic plaques, while chloroquine can alleviate ECs autophagy and injury and further inhibit atherosclerotic plaques formation, thus indicating that autophagy is involved in the development of atherosclerosis via ox-LDL/LOX1 pathway.

MBL alleviates atherosclerotic plaque formation by competitively inhibiting the uptake of ox-LDL through ECs, influencing the maturation of DCs and down regulating inflammation

To investigate the effect and mechanism of MBL on the development of atherosclerosis, we constructed MBL over-expression adenovirus (**Figure S1**). Different concentrations of MBL over-expression adenovirus were administered with retro-orbital injections into ApoE^{-/-} mice. The results revealed that the expression of MBL in mice liver and serum were significantly increased after 2 weeks of injection and began to decrease after 4 weeks. Also, there was no significant difference between 1×10⁹PFU/mouse and 2×10⁹PFU/mouse of MBL adenovirus injection (**Figure 4G, 4H**). Consequently, 1×10⁹PFU/mouse of MBL/control adenovirus was selected for the following study.

HFD was given to ApoE^{-/-} mice for 16 weeks, and MBL/control adenovirus was administered with retro-orbital injections every 4 weeks. The results revealed that MBL overexpression *in vivo* could significantly reduce the fat mass (**Figure 5A**), the atherosclerotic plaques (**Figure 5B**), the apoptosis rate (**Figure 5D**), and the uptake of ox-LDL and LC3 expression (**Figure 5F**) in mice aorta ECs compared with the control group.

We further investigated the role of DCs in the development of atherosclerosis *in vivo*. DCs were isolated from mice spleen. Flow cytometry analysis showed that the proportion of CD11c+CD80+ and CD11c+CD86+ in spleen-derived DC cells of ApoE^{-/-} mice were significantly higher than that of WT mice (**Figure 6A**). Also, the serum levels of inflammatory factors IL-6, IL-12, and TNF- α were significantly increased (**Figure 6B**). However, after MBL-adenovirus was transfected into ApoE^{-/-} mice, the proportion of CD11c+CD80+ and CD11c+CD86+ in spleen-derived DCs was significantly decreased compared to blank-adenovirus transfected ApoE^{-/-} mice (**Figure 6C**). The serum levels of inflammatory factors IL-6, IL-12, and TNF- α were also significantly decreased (**Figure 6D**). These data indicated that MBL overexpression *in vivo* could influence the maturation of DCs and downregulate inflammation, which may eventually alleviate ECs injury and the progression of atherosclerosis.

Discussion

The current study reported a protective role of MBL in ox-LDL-induced ECs injury *in vivo* and *in vitro*. The main findings of this study are as follows: 1) autophagy is involved in ox-LDL induced atherosclerosis model, and ox-LDL induced ECs autophagy and injury through the LOX1 pathway; 2) MBL can modulate ECs autophagy and DCs maturation, thus further inhibiting ox-LDL induced atherosclerosis. These data further existing knowledge about the role of MBL in CVDs, and provide direction for drug intervention.

MBL ameliorates ECs injury via modulating autophagy by blocking the binding of ox-LDL and LOX1

MBL is widely known as an initiator of the lectin pathway. Previous studies confirmed the role of MBL in complementing activation [27], as well as promoting the complement-independent opsonophagocytosis [28] and clearance of apoptotic cells [29]. Our results showed that over-expression of MBL might alleviate fat mass and atherosclerotic plaque formation in ApoE^{-/-} mice fed with HFD. Moreover, *in vitro* study revealed that MBL could reduce ox-LDL-induced ECs autophagy, injury, and apoptotic rate. Meanwhile, mannan can specifically bind to MBL and finally revise the effects of MBL on ECs autophagy and injury. Previous studies have revealed that proper autophagy is beneficial in atherosclerosis [30, 31], while excessive autophagy may lead to the accumulation of autophagosomes and degradation of vital proteins, which is detrimental to CVDs [15, 32]. Thus, we concluded that MBL protects ECs from ox-LDL induced injury by modulating autophagy.

Ox-LDL was mainly uptaken by ECs via binding with LOX-1. Several studies have shown the vital role of LOX1 in ox-LDL induced ECs autophagy and injury [13, 14]. Coincidentally, both LOX1 and MBL belong to the C-type lectin family. Thus, we hypothesized that MBL modulates ECs autophagy by interfering with the binding of LOX1 and ox-LDL. Our *in vivo* study revealed that ApoE^{-/-} mice fed with HFD had increased

expression of LOX1, while MBL over-expression reduced the expression of LOX1 and ox-LDL uptake. Furthermore, down regulation of LOX1 with siRNA could inhibit the uptake of ox-LDL and alleviate ECs autophagy and injury *in vitro*. These data suggested that MBL ameliorate ECs injury via modulating autophagy by blocking the binding of ox-LDL and LOX1.

MBL alleviates ECs injury by modulating DCs maturation and production of inflammatory factors

MBL also participates in the modulation of inflammation. A previous study reported that MBL could attenuate peptidoglycan induced inflammatory cytokine production and suppress nuclear translocation of NF- κ Bp65, phosphorylation of I κ B α , MAPK p38, and ERK1/2 [33]. MBL may also inhibit autoreactive T cells' activation and proliferation via cell surface calreticulin in patients with silicosis [34]. High concentrations of MBL can inhibit the differentiation of DCs and attenuate LPS-induced maturation of DCs [35, 36]. In this study, we found that ox-LDL or HFD could induce DCs maturation and increase the production of inflammation factors IL-6, IL-12, and TNF- α . DCs co-culturing with ECs can promote ox-LDL induced ECs autophagy and injury. Also, MBL over-expression in HFD fed ApoE $^{-/-}$ mice could reduce the secretion of inflammation factors, as well as affect the maturation of DCs. These data provided further support for the vital role of inflammation in the development of atherosclerosis and provided evidence that MBL may modulate DCs maturation and production of inflammatory factors and further regulate ECs autophagy and injury. Although the protective or detrimental role of MBL in CVDs is still controversial, as both low and high levels of MBL indicate the susceptibility to CVDs [23, 24], our findings provide solid evidence that MBL can be potentially used for immune-modulatory therapy for atherosclerosis.

Study limitations

This study has a few limitations. First, we utilized LOX1-siRNA to investigate the role of LOX1 in ECs injury *in vitro*, while we did not construct LOX1 knockout mice for financial reasons. Second, we did not quantitatively investigate the role of MBL in regulating ECs injury. Thus, more clinical trials and animal studies are needed to investigate the role of MBL in CVDs and determine the optimal level of MBL for CVD patients.

Conclusion

This study showed a protective role of MBL in ECs injury and its potential in inhibiting the development of atherosclerosis. To the best of our knowledge, this is the first study that reported MBL could protect ECs through modulating autophagy by competitive inhibition of the ox-LDL and LOX1 binding. We also found that MBL may modulate DCs maturation and inflammatory factors production, which provide potential of MBL in immune-modulatory therapy for atherosclerosis.

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Figures

Figure 1

ox-LDL induced ECs autophagy and injury. A: ET-1 level in ECs treated with different concentration of ox-LDL with different time durations. B: ECs proliferation rate in ECs treated with different concentration of ox-LDL with different time durations. C: The expression level of LC3 in 100 µg/ mL ox-LDL treatment for different time durations. D: Fluorescence expression of LC3 protein in ECs in each group (400 times). E: ET-1 level in ECs of each group. F: ECs proliferation rate of each group. G: Flow diagram of EC apoptosis. Note: CQ is chloroquine, and RAPA is rapamycin. oxLDL means 100 µg/ mL ox-LDL treatment for 24h; CQ (25 µM) is pretreated for 1 h, and then treated with ox-LDL; RAPA (0.02 µg) is pretreated for 1 h, and then treated with ox-LDL. ** p < 0.01, * p < 0.05.

Figure 2

DC cells promote ox-LDL induced ECs autophagy. A: Co-localization of LC3 and Lamp2a in each group (400 times). B: The electron microscopy of autophagosomes in each group. C: The expression level of inflammatory factors in the supernatant of DCs treated with ox-LDL, D: DCs maturation detection. ** $p < 0.01$ vs. DC group, ## $p < 0.01$ vs. DC+oxLDL group 0 h.

Figure 3

The role of LOX1 in ox-LDL induced ECs autophagy. A: The localization of LOX1 and ox-LDL in ECs treated with ox-LDL at different time points (200 times). B: The localization of ox-LDL in the ECs at 0, 6 h of ox-LDL treatment (200 times). C: Immunofluorescence LC3 detection of ECs in each group (200 times). D: LOX1 mRNA expression levels of ECs in each group. E: LOX1 protein expression level of ECs in each group. F: ET-1 level in the supernatant of ECs in each group. G: Cell viability of ECs in each group. Note: * $p < 0.01$, * $p < 0.05$. Control siRNA means using control siRNA to transfect ECs in the upper chamber and moving to a well plate containing ECs after 48 hours; LOX1 siRNA means using LOX1 siRNA to transfect ECs in the upper chamber and moving to containing ECs after 48 hours in a well plate; oxLDL means 100ug/ml ox-LDL treatment for 24h.

Figure 4

MBL reduce ox-LDL induced ECs injury in vitro. A: The expression of MBL mRNA and protein in transfected ECs. B: Detection of LC3 expression in ECs of each group (200 times). C: ECs viability detection in each group. D: ET-1 level of ECs supernatant detected by Elisa. E: The localization of LOX1 and ox-LDL in ECs treated with ox-LDL for different times (200 times). F: co-immunoprecipitation bands of transfected ECs. G: co-immunoprecipitation bands of transfected ECs treated with mannan. Note: * $p<0.05$, ** $p<0.01$. MBMEC + control/MBL plasmid means ECs were transfected with control/MBL plasmid and incubated for 48h; MBMEC+ control /MBL plasmid + oxLDL indicate transfected ECs incubated for 48h, and then treated with 100 ug/mL oxLDL for 24 h. MBMEC+ control/MBL + mannan means that transfected ECs incubated with mannan for 48h, and then treated with 100 ug/mL oxLDL for 24 h.

Figure 5

Experimental findings in vivo. A: Oil red O staining. B: HE staining; the scale bar indicates 20 μ m. C: LOX1 expression in ECs, the scale bar indicates 20 μ m. D: Apoptosis rate of ECs in each group; the scale bar indicates 20 μ m. E: Uptake of oxLDL in each group; the scale bar indicates 50 μ m. F: LC3 expression in each group, the scale bar indicates 50 μ m. G: MBL level in liver. H: MBL level in serum. **Indicates $p < 0.01$ vs. WT or control, ▨▨Indicates $p < 0.01$ vs. ApoE^{-/-}.

Figure 6

MBL affects inflammatory phenotype and maturation of DCs in vivo. A: DCs maturation rate in ApoE^{-/-} and WT mice. B: serum levels of inflammatory factors in ApoE^{-/-} and WT mice. C: DCs maturation rate in MBL transfected ApoE^{-/-} and ApoE^{-/-} mice. D: serum levels of inflammatory factors in MBL transfected ApoE^{-/-} and ApoE^{-/-} mice. ** p < 0.01.

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