Apolipoprotein M induces inhibition of inflammatory responses via the S1PR1 and DHCR24 pathways

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Abstract. Apolipoprotein M (ApoM) is a type of apolipoprotein. It is well known that high-density lipoprotein (HDL) decreases inflammatory responses via the apoM-sphingosine-1-phosphate (S1P) pathway. The present study further investigated the importance of ApoM in the inhibitory effects of HDL on inflammation. Mice with an apoM gene deficiency (*apoM*$^{-/-}$) were employed to investigate the effects of ApoM on the expression of interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1), S1P receptor-1 (S1PR1) and 3β-hydroxysterol Δ-24-reductase (DHCR24), as compared with in wild-type mice (*apoM*$^{+/+}$). Furthermore, cell culture experiments were performed using a permanent human hybrid endothelial cell line (EA.hy926). Cells were cultured in the presence of recombinant human *apoM* (rec-*apoM*) or were induced to overexpress *apoM* (*apoM*^{Tg}); subsequently, cells were treated with tumor necrosis factor-α (TNF-α), in order to investigate the effects of ApoM on IL-1β and MCP-1. The results demonstrated that the mRNA expression levels of IL-1β and MCP-1 were significantly higher in the liver following administration of lipopolysaccharide in *apoM*$^{-/-}$ mice compared with in *apoM*$^{+/+}$ mice. In cell culture experiments, when cells were pre-cultured with rec-*apoM* or were engineered to overexpress *apoM* (*apoM*^{Tg}), they exhibited decreased expression levels of IL-1β and MCP-1 following TNF-α treatment compared with in normal *apoM*-expressing cells (*apoM*^{TgN}). Furthermore, the mRNA expression levels of IL-1β and MCP-1 were significantly elevated following addition of the S1PR1 inhibitor W146, but not by the scavenger receptor class B type I inhibitor, block lipid transport-1 (BLT-1), in *apoM*^{Tg} cells prior to TNF-α treatment. Conversely, there were no differences in these inflammatory biomarkers under the same conditions in *apoM*^{TgN} cells. The mRNA expression levels of DHCR24 were significantly reduced by the addition of BLT-1 prior to TNF-α treatment in *apoM*^{Tg} cells; however, there was no difference in the expression of this inflammatory biomarker in *apoM*^{TgN} cells. In conclusion, ApoM displayed inhibitory effects against the inflammatory response *in vivo* and *in vitro*; these effects may be induced via the S1PR1 and DHCR24 pathways.

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Introduction

High-density lipoprotein (HDL) is known to exert anti-atherosclerotic effects, which are mediated by the HDL reverse cholesterol transportation system *in vivo* (1). Furthermore, it has been demonstrated that the anti-atherosclerotic effects of HDL may be associated with its anti-inflammatory, antioxidant and endothelial protective properties (2). Apolipoprotein M (ApoM) is a major HDL apolipoprotein (3), which has been suggested to serve an important role in the anti-atherosclerotic effects of HDL (4), possibly via the ApoM/sphingosine-1-phosphate (S1P) axis (5). In addition, it has been reported that the ApoM/S1P axis may have an important role in normal lipoprotein metabolism, lipid disorders and atherosclerosis (5). S1P activates five G-protein-coupled receptors, known as S1P-receptors 1-5 (6), and affects lymphocyte trafficking, angiogenesis, wound repair, virus suppression and possibly cancer progression (7). S1P, combined with S1P receptor-1 (S1PR1), serves an important role in vascular protection (8); impaired pulmonary vascular endothelial cell permeability has been observed in *apoM*-deficient mice in an S1P/S1PR1-dependent manner (9). *In vivo*, ApoM acts as a carrier of S1P (9); therefore, it may be hypothesized that ApoM has a critical role in vascular protection through the S1PR1 pathway.

In mice, 3β-hydroxysterol Δ-24-reductase (DHCR24) is predominantly expressed in the medulla oblongata,

mesencephalon, liver, heart, ovaries and intestinal tissues. DHCR24 is a cholesterol synthetase that is composed of 516 amino acids, which catalyzes the final step in cholesterol biosynthesis, the conversion of desmosterol to cholesterol (10). McGrath *et al* reported that recombinant HDL could inhibit tumor necrosis factor-α (TNF-α)-induced inflammation in human coronary artery endothelial cells (HCAECs) by increasing DHCR24 gene expression (11). A previous study also demonstrated that estrogen can significantly increase *apoM* and DHCR24 expression *in vivo* and *in vitro* (12,13). However, whether ApoM regulates DHCR24 expression remains unclear.

Scavenger receptor class B type I (SR-B1) is a specific receptor for HDL in several cells and tissue types, including liver parenchyma, adrenal cortical cells, platelets, endothelial cells, smooth muscle cells and macrophages (14). It serves crucial roles in cholesterol homeostasis, lipoprotein metabolism, inflammation and atherosclerosis (15-18). SR-B1 also mediates the reverse cholesterol transport of HDL and thus has an anti-atherosclerotic effect (19). Notably, HDL induces cyclooxygenase-2 expression and prostacylin production through the SR-B1-mediated phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt)-endothelial nitric oxide synthase (eNOS) pathway in endothelial cells (20). In addition, SR-B1 signaling helps limit inflammation in atherosclerotic lesions, thereby preventing plaque formation (17). S1PR1 and SR-B1 are co-localized in the caveolae within the plasma membrane (20,21). Lee *et al* (22) demonstrated that HDL-associated S1P can stimulate the molecular interaction of S1PR1 and SR-BI proteins, in order to affect S1P-mediated alterations in cell metabolism. HDL not only exerts anti-inflammatory effects via S1PR1, but also through SR-B1. ApoM is a major HDL apolipoprotein that acts as a carrier of S1P. In addition, SR-BI is expressed in endothelial cells, where it can be combined with ApoM. However, whether it is possible to have two-fold anti-inflammatory action, or if there is an association between the two receptors involved in ApoM-induced inhibition of the inflammatory response requires further exploration Therefore, the present study further investigated the importance of ApoM in the inhibitory effects of HDL on inflammation.

Materials and methods

Animals. A total of 48 male C57BL/6 *apoM* gene-deficient (*apoM*$^{-/-}$) mice (age, 8-10 weeks; weight, 23-25 g) and 48 age- and body weight-matched male wild-type (*apoM*$^{+/+}$) C57BL/6 mice were used in the present study. The *apoM*$^{-/-}$ mice were generated by homologous recombination at the Model Animal Research Center of Nanjing University (Nanjing, China), as previously described (23). All animals were bred under laminar flow at the specific pathogen-free animal laboratory of Soochow University (Changzhou, China), where the conditions were maintained as follows: Interior temperature, 19-24˚C; noise, <60 dB; humidity, 50-60%; 12-h light/dark cycle. Mouse feed (*ad libitum*) and bedding materials underwent high temperature disinfection, and drinking water (*ad libitum*) underwent high temperature sterilization. The present study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and was approved by the Animal Use and Protection Committee of Soochow University. Adequate measures were taken to minimize the number of experimental animals used and to ensure minimal pain or discomfort to the animals throughout the study.

Animal experimental procedure. A total of 48 *apoM*$^{-/-}$ mice were randomly divided into six groups (n=8/group), according to the time of drug administration: 0, 1, 3, 6, 12 and 24 h groups; the 48 *apoM*$^{+/+}$ mice were also randomly divided into the same groups as the *apoM*$^{-/-}$ mice. Mice in each of the six groups received an intraperitoneal (i.p.) injection of 10 mg/kg lipopolysaccharide (LPS; *Escherichia coli* serotype O111:B4 L2630; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 100 μl pyrogen-free 0.9% saline, and the mice were sacrificed at 0, 1, 3, 6, 12 and 24 h, respectively; mice sacrificed at 0 h served as the control. Liver tissues were collected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Cell culture. The permanent human hybrid endothelial cell line, EA.hy926, was purchased from the American Type Culture Collection (Manassas, VA, USA). EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin. Cells were incubated at 37˚C in an atmosphere containing 5% CO_2. Cells were seeded in 6-well plates or 60-mm dishes and were grown to 60-80% confluence prior to use.

Lentiviral infection. EA.hy926 cells were cultured in a 60-mm dish containing DMEM supplemented with 10% FBS under standard culture conditions (5% CO_2, 37˚C). Empty lentivirus (LV) vectors with green fluorescent protein (GFP; *apoM*^{TgN}) and LV-mediated human *apoM* overexpression vectors (*apoM*^{Tg}) with GFP were prepared by Shanghai GeneChem Co., Ltd. (Shanghai, China). Cells grown to 50% and then were infected with LV at a multiplicity of infection of 20 transducing units per cell, in the presence of 10 mg/ml polybrene. After 24 h, cells were washed with fresh complete medium. GFP-positive cells were counted 96 h post-transduction, and underwent RT-qPCR and western blot analysis.

Cell experiments. EA.hy926 cells were incubated under standard conditions, as aforementioned, grown to 60-80% confluence and divided into four groups; the groups were plated in four 6-well plates at $4x10^5$ cells/well. The four groups were as follows: Control group, rec-*apoM* group, TNF-α group and TNF-α + rec-*apoM* group. Cells were incubated in serum-free medium for 12 h prior to treatment. No treatment was administered to the control group cells, whereas the rec-*apoM* group cells were treated with rec-*apoM* [40 μg/ml; Abgent Biotech (SuZhou) Co., Ltd., Suzhou, China] for 1.5 h in 37˚C, and then incubated with PBS for 3 h in 37˚C. The TNF-α group cells were pretreated with PBS for 1.5 h and were then treated with TNF-α (10 ng/ml; Cell Signaling Technology, Inc., Danvers, MA, USA) for 3 h. The TNF-α + rec-*apoM* group cells were treated with rec-*apoM* (40 μg/ml) for 1.5 h prior to treatment with TNF-α (10 ng/ml) for 3 h. Finally, cells were collected for RT-qPCR analysis.

EA.hy926 cells were infected as aforementioned and were divided into two further groups: The *apoM*^{TgN} and *apoM*^{Tg} groups. These were then subdivided into two additional subgroups in two 6-well plates, according to the different treatments administered, resulting in a control subgroup and a TNF-α subgroup for each of the original groups. Cells were incubated in serum-free medium for 12 h prior to drug treatment. The control group cells did not undergo any treatment, whereas the TNF-α subgroup cells were treated with TNF-α (10 ng/ml) for 3 h and were then collected for RT-qPCR analysis.

EA.hy926 cells were infected as aforementioned and divided into two groups: The *apoM*^{TgN} and *apoM*^{Tg} groups, which were further subdivided into two subgroups in two 6-well plates, according to the different drug treatments administered, resulting in a control group and a W146 group. All cells in each of the four groups were treated with TNF-α (10 ng/ml) for 3 h. The control group cells were treated with ethanol, as a vehicle, for 30 min prior to TNF-α treatment. The W146 group cells were treated with W146 (10 μM, Cayman Chemical Company, Ann Arbor, MI, USA) for 30 min prior to TNF-α treatment. After treatment, the cells were collected for RT-qPCR analysis.

In addition, EA.hy926 cells were infected as aforementioned and divided into two groups: The *apoM*^{TgN} and *apoM*^{Tg} groups, which were then subdivided into a control group and a block lipid transport-1 (BLT-1) treatment group. All cells in each of the four groups were treated with TNF-α (10 ng/ml) for 6 h. The control group cells were treated with ethanol, as a vehicle, for 12 h prior to TNF-α treatment. The BLT-1 group cells were treated with BLT-1 (10 μM, Merck KGaA) for 12 h prior to TNF-α treatment. Finally, cells were collected for RT-qPCR analysis.

RNA isolation and RT-qPCR analysis. Total RNA was isolated from mouse liver tissues and cultured cells using the Total RNA Purification kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol; sample quality was determined by measuring absorbance at 260/280 nm. Overall, 2 μg total RNA was reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. The mRNA expression levels of target and reference genes were performed using Immolase™ DNA Polymerase (Bioline USA, Inc., Taunton, MA, USA). GAPDH was used as a reference control. The quantification cycles (24) of the target and reference genes were read through the FAM channel (465-510 nm) and the CY5 channel (618-660 nm), respectively. All primer/probe sets were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) using information derived from GenBank (https://www.ncbi.nlm.nih.gov/genbank/; Table I). Quantification of interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1), S1PR1 and DHCR24 mRNA expression levels was determined relative to GAPDH mRNA expression levels. qPCR was performed using the LightCycler 480® II (Roche Diagnostics) with a final volume of 25 μl. Each well of a 96-well plate contained 2.5 μl PCR buffer (10X), 2.5 μl $MgCl_2$ (25 mM), 0.5 μl dNTPs (10 mM), 0.25 μl Taq DNA polymerase, 0.04 μl 100 μM solutions of each primer and probe, 2 μl cDNA, and 17.01 μl of ddH_2O. Thermal cycling conditions included the following steps: Denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 15 sec.

Western blot analysis. The proteins ApoM, S1PR1 and GAPDH were detected by western blot analysis. Protein samples were extracted from cultured cells using a total protein extraction kit (BestBio, Shanghai, China), and were harvested and washed with ice-cold PBS. Proteins were then quantified using a Bicinchoninic Acid Protein Assay kit (BestBio). Equal amounts of protein (2.4 g/ml) were loaded into all wells, separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked in 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in PBS with 5% Tween-20 at room temperature 1 h. The membranes were then incubated overnight at 4°C with mouse polyclonal anti-ApoM (1:2,000; cat. no. H00055937, Abnova, Taipei, Taiwan), rabbit polyclonal anti-S1PR1 (1:1,000; catalog no. ab11424, Abcam, Cambridge, MA, USA) and mouse polyclonal anti-GAPDH antibodies (1:1,000, cat. no. 10494-1-AP, ProteinTech Group, Inc., Chicago, IL, USA). Subsequently, the membranes were incubated with secondary antibodies [anti-rabbit immunoglobulin G (IgG) (cat. no. SA00001-2, ProteinTech Group, Inc., Chicago, IL, USA) or anti-mouse IgG (cat. no. SA00001-1, ProteinTech Group, Inc.), 1:3,000] for 1 h at room temperature. The proteins were visualized using enhanced chemiluminescence (ECL; ECL Plus Western Blot Detection system; GE Healthcare, Chicago, IL, USA).

H&E staining. Thoracic aorta samples were harvested for histological examination and fixed in 4% paraformaldehyde in room temperature for 12 h then the myocardial tissue was dehydrated with ethanol and embedded in paraffin. Thereafter, the myocardial tissues were cut into serial sections (5 μm) and stained with H&E dye in room temperature for 30 sec. The staining was observed under a light microscope (magnification, x400, Olympus Corporation, Tokyo, Japan).

Evaluation of S1PR1 immunohistochemical staining. Thoracic aorta samples were obtained from 8-10 week old male *apoM*$^{-/-}$ and *apoM*$^{+/+}$ mice, fixed in 3.7% paraformaldehyde 24 h at room temperature and embedded in paraffin 40 min. A series of 2-3 μm sections were collected. Briefly, the sections were pretreated with dimethylbenzene and ethanol, blocked with 5% bovine serum albumin for 20 min at room temperature, and incubated with primary polyclonal S1PR1 antibody (1:50 dilution in PBS; cat. no. ab11424, Abcam) at 4°C overnight. A negative control sample was prepared by omitting the primary antibody. After washing with PBS, the sections were incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (cat. no. 200374. Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). The sections were stained using a Diaminobenzidine Solution kit (OriGene Technologies, Inc., Rockville, MD, USA), counterstained with hematoxylin for 5 min, dehydrated with increasing concentrations of ethanol (30, 50, 70, 80, 90, 95 and 100%) and mounted for 1-3 min at room temperature. The staining was observed under a light microscope (magnification, x400, Olympus Corporation). The sections were then dehydrated, cleared and mounted.

Statistical analysis. All experiment repeated three times, data are expressed as the means ± standard deviation.

Table I. Sequences of primers and probes used for polymerase chain reaction.

Gene	Primer/probe	Sequence (5'-3')
Human		
ApoM	Forward primer	CTGACAACTCTGGGCGTGGAT
	Reverse primer	TGTCCACAGGGTCAAAAGTTGC
	Probe	FAM-AGTTCCCAGAGGTCCACTTGGGCCA-BHQ1
S1PR1[a]	Forward primer 1	GGGCTCTCCGAACGCAAC
	Forward primer 2	GGACCCCGACTCGAGCTG
	Reverse primer	GTTCGATGAGTGATCCAGGCTT
	Probe	FAM-TCCGAGGCCCTCTCCAGCCAA-BHQ1
IL-1β	Forward primer	TCCTGCGTGTTGAAAGATGATAAG
	Reverse primer	ATCGCTTTTCCATCTTCTTCTTTG
	Probe	FAM-CCACTCTACAGCTGGAGAGTGTAGATCCCA-BHQ1
MCP-1	Forward primer	GCTCATAGCAGCCACCTTCAT
	Reverse primer	GCGAGCCTCTGCACTGAGAT
	Probe	FAM-CCAAGGGCTCGCTCAGCCAGAT-BHQ1
GAPDH	Forward primer	GGAAGGTGAAGGTCGGAGTC
	Reverse primer	CGTTCTCAGCCTTGACGGT
	Probe	CY5-TTTGGTCGTATTGGGCGCCTG-BHQ2
Mouse		
S1PR1	Forward primer	CAGCTTCGTCCGGCTTGAG
	Reverse primer	GTTACAGCAAAGCCAGGTCAGC
	Probe	FAM-AGGCTGCTGTTTCTCGGAGGCCTC-BHQ1
IL-1β	Forward primer	GCAGGCAGTATCACTCATTGTGG
	Reverse primer	GAGTCACAGAGGATGGGCTCTTC
	Probe	FAM-TGGAGAAGCTGTGGCAGCTACCTGTGT-BHQ1
MCP-1	Forward primer	GCTGGAGAGCTACAAGAGGATCAC
	Reverse primer	CCTTCTTGGGGTCAGCACAG
	Probe	FAM-CAGCAGGTGTCCCAAAGAAGCTGTAGTT-BHQ1
GAPDH	Forward primer	TCTTGTGCAGTGCCAGCCT
	Reverse primer	TGAGGTCAATGAAGGGGTCG
	Probe	CY5-AGGTCGGTGTGAACGGATTTGGC-BHQ2

[a]S1PR1 forward primer 1 and forward primer 2 are two different transcripts of S1PR1; forward primer 1 has an extra 108 bases compared to forward primer 2. However, the amino acid sequences of proteins translated from these two transcripts are the same. It is unknown whether these two transcripts are regulated differently during transcription and translation; therefore, both forward primers were used, sharing the same reverse primer and probe. ApoM, apolipoprotein M; IL-1B, interleukin-1B; MCP-1, monocyte chemotactic protein-1; S1PR1, sphingosine-1-phosphate receptor-1.

Statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed with one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. Two-way ANOVA was used to analyze the interaction between two factors. Comparisons between two groups were statistically evaluated using a two-tailed Mann-Whitney U test. Two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of apoM on the mRNA expression levels of IL-1β and MCP-1 during inflammation. As shown in Fig. 1A, the mRNA expression levels of IL-1β were similar in *apoM*$^{-/-}$ and *apoM*$^{+/+}$ mice without LPS administration. However, following LPS injection, although the mRNA expression levels of IL-1β were increased at all time intervals in both *apoM*$^{-/-}$ and *apoM*$^{+/+}$ mice; the levels were significantly higher in *apoM*$^{-/-}$ mice compared with in *apoM*$^{+/+}$ mice. The same phenomenon was observed with regards to MCP-1 mRNA expression (Fig. 1B). These data suggested that LPS significantly amplified the inflammatory response in *apoM*$^{-/-}$ mice compared with in *apoM*$^{+/+}$ mice.

Rec-apoM inhibits the expression levels of IL-1β and MCP-1 stimulated by TNF-α in EA.hy926 cells. As shown in Fig. 1C and D, analysis by one-way ANOVA, the mRNA expression levels of IL-1β and MCP-1 were significantly decreased in EA.hy926 cells that had been stimulated by

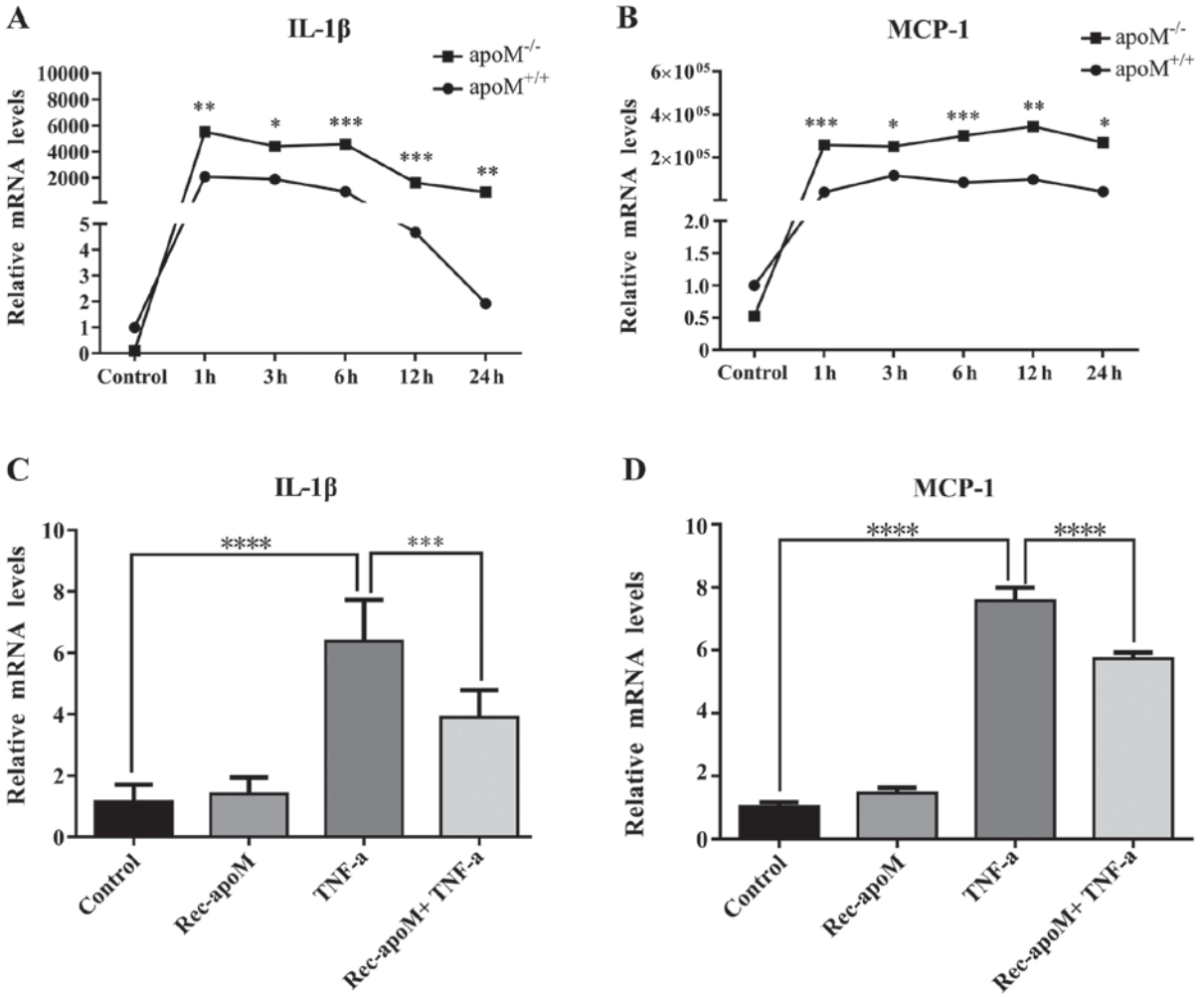

Figure 1. mRNA expression levels of IL-1β and MCP-1 in mice and EA.hy926 cells. The mRNA expression levels of (A) IL-1β and (B) MCP-1 were detected in the livers of *apoM*$^{-/-}$ and *apoM*$^{+/+}$ mice following administration of lipopolysaccharide. The mRNA expression levels of IL-1β and MCP-1 in the control group (*apoM*$^{+/+}$ mice) were set as 1 (n=8, one-way ANOVA followed by Dunnett's test). *P<0.05, **P<0.01, ***P<0.005 vs. control. The mRNA expression levels of (C) IL-1β and (D) MCP-1 were detected in EA.hy926 cells, which were treated with TNF-α (10 ng/ml) and/or rec-*apoM* (40 μg/ml). The mRNA expression levels of IL-1β and MCP-1 in the control group were set as 1 (n=6, one-way ANOVA followed by Dunnett's test). ***P<0.005, ****P<0.001. ANOVA, analysis of variance; *apoM*, apolipoprotein M; IL-1β, interleukin-1β; MCP-1, monocyte chemotactic protein-1; Rec-*apoM*, recombinant human *apoM*; TNF-α, tumor necrosis factor-α.

TNF-α (10 ng/ml) in the presence of rec-*apoM* (40 μg/ml), particularly compared with in cells that were not treated with rec-*apoM* (n=6, P<0.01 and P<0.005). Furthermore, two-way ANOVA demonstrated that *apoM* and TNF-α had additive effects on the mRNA expression levels of IL-1β and MCP-1.

ApoM upregulates the expression of S1PR1 and DHCR24 in EA.hy926 cells and mouse tissue. The effects of ApoM were detected on S1PR1 in cells and mouse tissues (Fig. 2A-E). As shown in Fig. 2B, C and E, the mRNA and protein expression levels of S1PR1 were higher in *apoM*^{Tg} cells compared with in the control group (n=6; P<0.001, respectively). As shown in Fig. 2D, the mRNA expression levels of S1PR1 were significantly decreased in the thoracic aorta of *apoM*$^{-/-}$ mice (n=7-8; P<0.01); immunohistochemical staining further confirmed that the abundance of S1PR1 protein in aortic tissue from *apoM*$^{-/-}$ mice was markedly lower than in *apoM*$^{+/+}$ mice (n=7-8; Fig. 2F and G). These data suggested that ApoM significantly increased the expression of S1PR1 *in vivo* and *in vitro*. In addition, as shown in Fig. 2H and I, compared with in the control group, the mRNA expression levels of DHCR24 were increased in *apoM*^{Tg} cells and *apoM*$^{+/+}$ mouse liver tissues without inflammatory stimulation.

Effects of ApoM on the mRNA expression levels of IL-1β, MCP-1, S1PR1 and DHCR24 in TNF-α-stimulated EA.hy926 cells. As shown in Fig. 3A and B, analysis by one-way ANOVA, the mRNA expression levels of IL-1β and MCP-1 were significantly increased in *apoM*^{TgN} and *apoM*^{Tg} cells following treatment with TNF-α. Compared with in *apoM*^{TgN} cells, the mRNA expression levels of IL-1β and MCP-1 were markedly reduced in *apoM*^{Tg} cells (n=6, P<0.01 and P<0.005). Two-way ANOVA revealed that ApoM and TNF-α respectively affected the mRNA expression levels of IL-1β and MCP-1. These results verified the anti-inflammatory role of ApoM in EA.hy926 cells. As shown in Fig. 3C-E, compared with the increase in IL-1β expression following TNF-α treatment, the mRNA expression levels of S1PR1 and DHCR24 were decreased in *apoM*^{TgN} cells. However, this phenomenon was not observed in apoM^{Tg} cells.

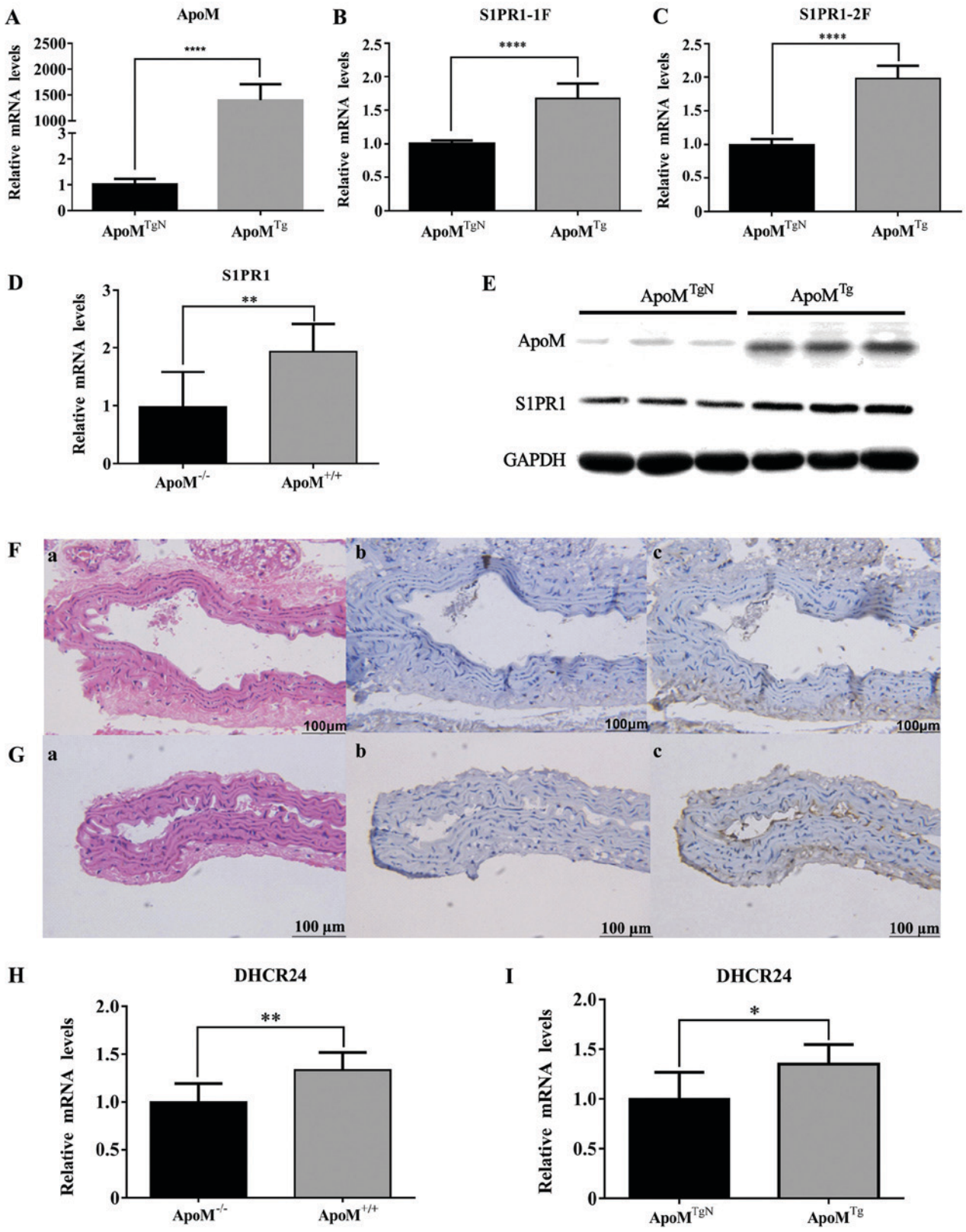

Figure 2. Expression levels of S1PR1 and DHCR24 in mice and EA.hy926 cells. (A) mRNA expression levels of *apoM* were detected in *apoM*^{TgN} and *apoM*^{Tg} transduced cells. (B and C) S1PR1 mRNA levels were significantly increased in EA.hy926 cells when ApoM was overexpressed. S1PR1-1F and S1PR1-2F represent the transcript levels of each fragment of the S1PR1 gene. (D) mRNA levels of S1PR1 in mouse aortas were significantly lower in *apoM*$^{-/-}$ mice than in *apoM*$^{+/+}$ mice. (E) Total protein in *apoM*^{TgN} cells was analyzed by western blotting. Compared with in the control group, S1PR1 protein levels were higher in *apoM*^{Tg} cells. (Fa-c) Expression of S1PR1 in *apoM*$^{-/-}$ mouse aorta. (Ga-c) H&E staining and immunohistochemical staining; original magnification, x400. Immunohistochemical staining of mouse aorta samples indicated that S1PR1 levels were higher in *apoM*$^{+/+}$ mice compared with in *apoM*$^{-/-}$. Expression of S1PR1 in *apoM*$^{+/+}$ mouse aorta. H&E staining and immunohistochemical staining; original magnification, x400. (H) In the absence of inflammatory stimulation, the mRNA expression levels of DHCR24 in the liver of *apoM*$^{-/-}$ mice were lower than in the liver of *apoM*$^{+/+}$ mice. The mRNA expression levels of DHCR24 in the *apoM*$^{-/-}$ mouse group were set as 1. (I) mRNA expression levels of DHCR24 were increased in *apoM*^{Tg} cells. The mRNA expression levels of DHCR24 were set as 1 in the *apoM*^{TgN} group. (n=6-8 mice) *P<0.05, **P<0.01, ****P<0.001. *apoM*, apolipoprotein M; DHCR24, 3β-hydroxysterol Δ-24-reductase; H&E, hematoxylin and eosin; S1PR, sphingosine-1-phosphate receptor-1.

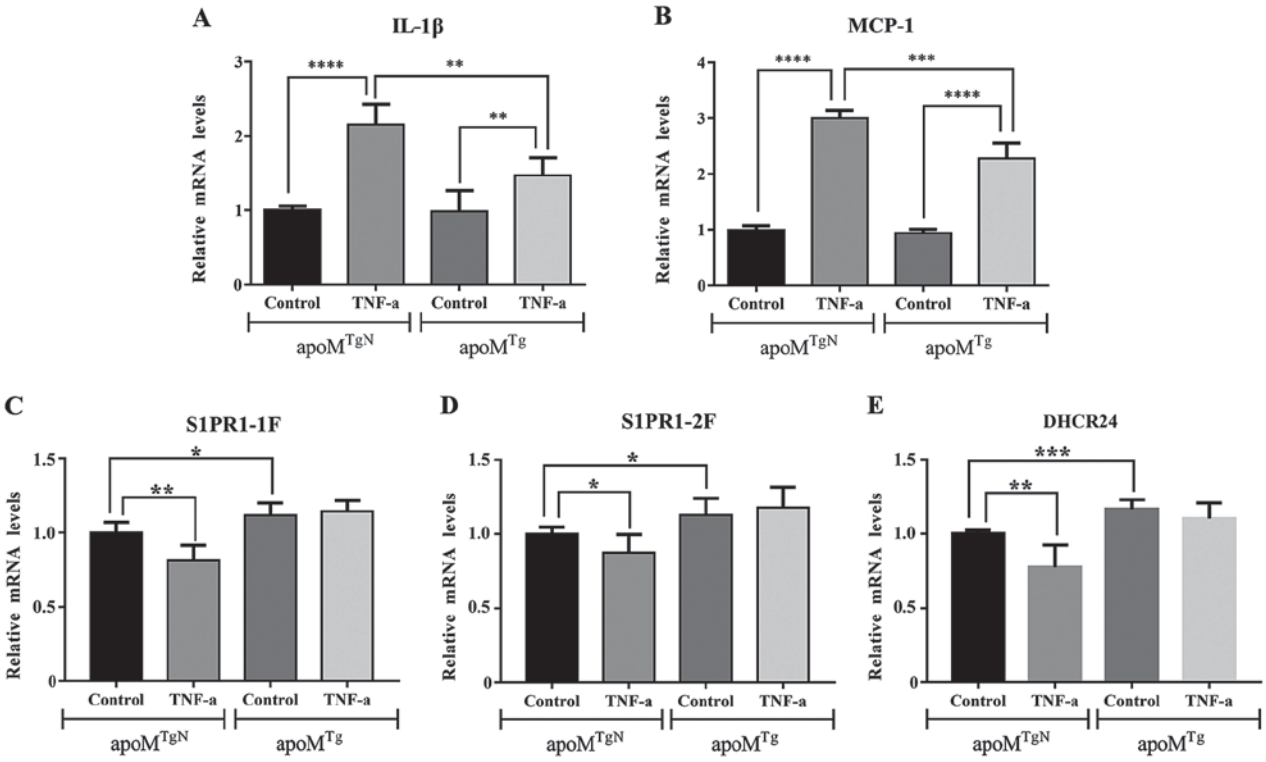

Figure 3. mRNA expression levels of IL-1β, MCP-1, S1PR1 and DHCR24 in *apoM*^{TgN} and *apoM*^{Tg} cells with or without TNF-α treatment. The mRNA expression levels of (A) IL-1β and (B) MCP-1 were increased in *apoM*^{TgN} and *apoM*^{Tg} cells following treatment with TNF-α (10 ng/ml) for 3 h. Compared with in *apoM*^{TgN} cells, the mRNA expression levels were markedly reduced in *apoM*^{Tg} cells treated with TNF-α for 3 h. Two-way ANOVA revealed that ApoM and TNF-α had additive effects on the mRNA expression levels of IL-1β and MCP-1. (C and D) mRNA expression levels of S1PR1 were decreased after treatment with 10 ng/ml TNF-α for 3 h in *apoM*^{TgN} cells, but not in *apoM*^{Tg} cells. (E) mRNA expression levels of DHCR24 were decreased after treatment with 10 ng/ml TNF-α for 3 h in *apoM*^{TgN} cells, but not in *apoM*^{Tg} cells. Two-way ANOVA revealed that *apoM* and TNF-α had no interaction effects on the mRNA expression levels of DHCR24. The mRNA expression levels of IL-1β, MCP-1, S1PR1 and DHCR24 in the control group were set as 1 (n=6, one-way ANOVA followed by Dunnett's test). *P<0.05, **P<0.01, ***P<0.005, ****P<0.001). ANOVA, analysis of variance; *apoM*, apolipoprotein M; DHCR24, 3β-hydroxysterol Δ-24-reductase; IL-1β, interleukin-1β; MCP-1, monocyte chemotactic protein-1; S1PR, sphingosine-1-phosphate receptor-1; TNF-α, tumor necrosis factor-α.

These data suggested that apoM may inhibit the expression of IL-1β and MCP-1 by increasing S1PR1 and DHCR24.

Effects of W146 on the mRNA expression levels of IL-1β, MCP-1 and DHCR24 during inflammation. As shown in Fig. 4A and B, the mRNA expression levels of IL-1β and MCP-1 in the W146 *apoM*^{Tg} group were significantly elevated compared with in the control *apoM*^{Tg} group. Conversely, there were no detectable differences between the W146 *apoM*^{TgN} group and the control *apoM*^{TgN} group. These data suggested that W146 amplified inflammation in EA.hy926 cells. As shown in Fig. 4C, the mRNA expression levels of DHCR24 exhibited no obvious change between the W146 *apoM*^{Tg} group and the control *apoM*^{Tg} group. These data suggested that the S1PR1 inhibitor W146 did not regulate DHCR24 expression during inflammation.

Effects of BLT-1 on the mRNA expression levels of IL-1β, MCP-1 and DHCR24 during inflammation. As shown in Fig. 5A and B, the mRNA expression levels of IL-1β and MCP-1 were no different between the BLT-1 *apoM*^{Tg} group and the control *apoM*^{Tg} group. These data suggested that the SR-B1 receptor inhibitor BLT-1 did not affect the ability of ApoM to reduce IL-1β and MCP-1 during inflammation. As shown in Fig. 5C, the mRNA expression levels of DHCR24 in the BLT-1 *apoM*^{Tg} group were significantly elevated compared with in the control *apoM*^{Tg} group. These data suggested that the SR-B1 receptor inhibitor BLT-1 regulated the mRNA expression of DHCR24 during inflammation.

Discussion

It has been well documented that HDL exerts anti-atherosclerotic effects, which may be partly mediated via its anti-inflammatory properties (2). As an essential component of HDL, ApoM is a novel apolipoprotein, which was initially discovered by Xu and Dahlback in 1999 (3). The anti-atherosclerotic effects of ApoM have been at the forefront of research for many years. Christoffersen *et al* (25) studied ApoM-overexpressing mice and revealed that mice with low-density lipoprotein (LDL) receptor deficiencies exhibit a two-fold increase in *apoM* compared with in control mice, and display decreased occurrence of atherosclerosis. A previous study by our group revealed that deletion of T-855C and C-724del decreases ApoM expression. Furthermore, the occurrence of these two single nucleotide polymorphisms is significantly increased in patients with coronary artery disease (CAD) compared with in non-CAD patients (26). Recent research has revealed that HDL serves a role in minimizing atherosclerosis and may inhibit activation of inflammatory pathways normally activated by cholesterol crystals, through modulating the expression of several key components of the inflammatory process (27).

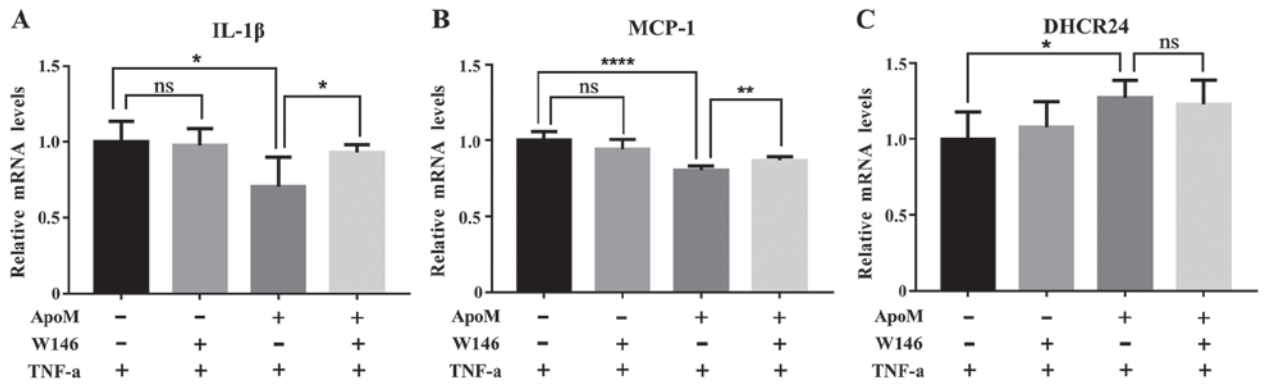

Figure 4. mRNA expression levels of IL-1β, MCP-1 and DHCR24 in *apoM*^{TgN} and *apoM*^{Tg} cells, which were treated with or without W146 prior to TNF-α. The mRNA expression levels of (A) IL-1β and (B) MCP-1 were significantly elevated when W146 was introduced for 30 min prior to TNF-α treatment in *apoM*^{Tg} cells. However, there were no differences in the expression of these inflammatory biomarkers in *apoM*^{TgN} cells. (C) In *apoM*^{Tg} cells, there was no significant difference in the mRNA expression levels of DHCR24 between cells treated with or without W146 before TNF-α. A similar phenomenon was observed in *apoM*^{TgN} cells. The mRNA expression levels of IL-1β, MCP-1 and DHCR24 in the control groups were set as 1 (n=6, one-way analysis of variance followed by Dunnett's test). *P<0.05, **P<0.01, ****P<0.001. *apoM*, apolipoprotein M; DHCR24, 3β-hydroxysterol Δ-24-reductase; IL-1β, interleukin-1β; MCP-1, monocyte chemotactic protein-1; ns, not significant.

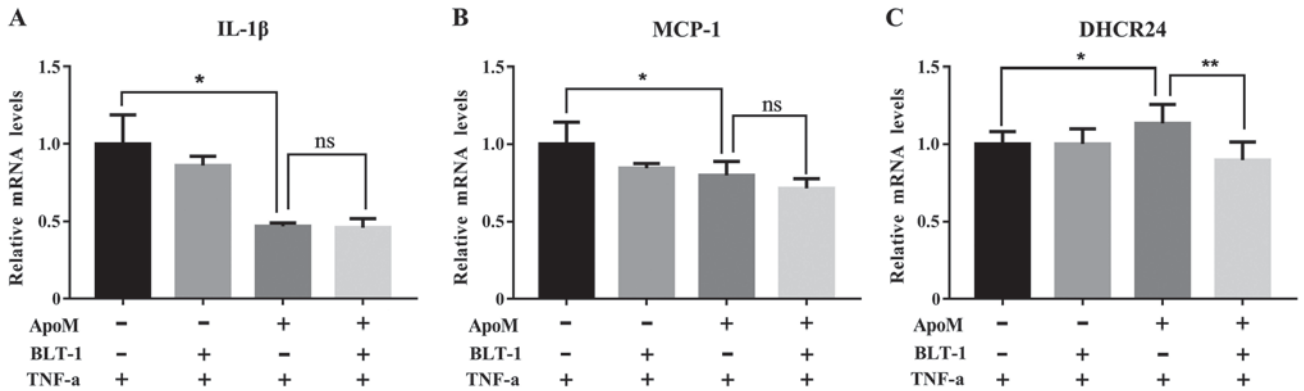

Figure 5. mRNA expression levels of IL-1β, MCP-1 and DHCR24 in *apoM*^{TgN} and *apoM*^{Tg} cells, which were treated with or without BLT-1 prior to TNF-α. Compared with in the control *apoM*^{Tg} cells, the mRNA expression levels of (A) IL-1β and (B) MCP-1 were no different following treatment with BLYT-1 prior to TNF-α exposure. A similar phenomenon was observed in *apoM*^{TgN} cells. (C) mRNA expression levels of DHCR24 were significantly reduced in response to treatment with BLT-1 for 12 h prior to TNF-α in *apoM*^{Tg} cells; however, there were no differences in DHCR24 expression in *apoM*^{TgN} cells. The mRNA expression levels of IL-1β, MCP-1 and DHCR24 in the control groups were set as 1 (n=6, one-way analysis of variance followed by Dunnett's test). *P<0.05, **P<0.01. *apoM*, apolipoprotein M; BLT-1, block lipid transport-1; DHCR24, 3β-hydroxysterol Δ-24-reductase; IL-1β, interleukin-1β; MCP-1, monocyte chemotactic protein-1; ns, not significant.

Previous studies further demonstrated that ApoM is required for pre-β-HDL formation, and lack of *apoM* affects cholesterol reverse transport, leading to atherosclerosis (28,29). ApoM may extend its anti-atherogenic properties via anti-inflammatory effects (4) and the anti-inflammatory effects of HDL may be partially mediated by ApoM. ApoM is mainly expressed in liver cells and renal proximal tubule cells, and its expression is affected by cytokines, leptin, insulin and other hormones (30). In addition, evidence has indicated that serum ApoM concentrations are decreased in patients with infection and inflammation (31-33). Gao *et al* reported that ApoM suppresses TNF-α-induced expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (VCAM-1), via inhibition of the activation pathway for nuclear factor (NF)-κB (34). Our previous study demonstrated that ApoM may regulate cluster of differentiation 4^{+} T lymphocytes and may modify T-lymphocyte subgroups during host immunomodulation in the murine spleen (23). IL-1β expression is secondary to the activation of microglia cells, which is promoted by mediators of inflammation (35) and serves an important role in upregulating the expression of leukocyte adhesion molecules in endothelial cells, vascular smooth muscle cell proliferation, and production of other cytokines that have been implicated in the development of atherosclerosis (36). Compared with normal coronary arteries, arteries with atherosclerotic lesions exhibit higher IL-1β concentrations (37). MCP-1 is a small cytokine that belongs to the CC chemokine family, which can recruit monocytes, memory T cells and dendritic cells to the site of inflammation (23). During early atheroma formation, MCP-1 is considered the link between oxidized LDL (oxLDL) and foam cell recruitment to vessel walls, and oxLDL (but not native LDL) induces MCP-1 expression (38). Other than facilitating migration of macrophages, MCP-1 is also involved in the differentiation of macrophages to foam cells. During this process, the expression of LDL receptors decreases, whereas the expression of scavenger receptors responsible for phagocytosis of oxLDL increases (39). The results of the present study demonstrated that the hepatic mRNA expression levels

of IL-1β and MCP-1 were higher in *apoM*$^{-/-}$ mice compared with in *apoM*$^{+/+}$ mice following LPS injection, whereas two-way ANOVA suggested that there was an interaction effect between LPS and ApoM with regards to the hepatic expression of IL-1β and MCP-1 mRNA. Furthermore, the results of cell culture experiments suggested that ApoM may significantly inhibit the mRNA expression levels of IL-1β and MCP-1 following TNF-α treatment, and a two-way ANOVA deduced the interaction effect between TNF-α and ApoM with regards to the mRNA expression of these factors. *In vivo* and *in vitro*, the mRNA expression levels of IL-1β and MCP-1 were significantly increased following treatment with LPS or TNF-α, whereas ApoM was a negative regulatory factor, thus indicating that ApoM exerts an anti-inflammatory effect; however, the mechanism underlying this phenomenon remains ambiguous.

A recent study (40) have demonstrated that S1P is a bioactive lipid regulator that corresponds with S1PRs, which belong to the G-protein coupled receptor family. There are five subtypes of S1PRs, S1PR1-S1PR5, which are present on the cytoplasmic membrane. A previous study (41) demonstrated that EA.Hy926 cells mainly express S1PR1, S1PR2 and S1PR3. Various S1PRs are usually present in distinct combinations in various cell types to perform specific biological functions. Recent research demonstrated that ApoM expression may be influenced by numerous factors, including TNF-α, epidermal growth factor, vascular endothelial growth factor (VEGF), leptin and insulin levels (30). Christoffersen *et al* reported that in a mouse model with 10-fold and 2-fold increased ApoM concentrations, S1P expression is increased by 267 and 71%, respectively, whereas levels are decreased by 46% in *apoM*$^{-/-}$ mice (9). Sutter *et al* revealed that HDL may facilitate S1P efflux from erythrocytes through ApoM-dependent and ApoM-independent processes in LDL receptor related protein 2$^{-/-}$, chloride voltage-gated channel 5$^{-/-}$, cystinosin, lysosomal cystine transporter$^{-/-}$ and *apoM*$^{-/-}$ mice. Furthermore, ApoM facilitates tubular reabsorption of S1P from urine; however, no effect has been detected on the concentration of plasma S1P in response to ApoM (42). These results suggested that ApoM is not only a physiological carrier of S1P, but may also affect the secretion and clearance of S1P. The present study demonstrated that the mRNA and protein expression levels of S1PR1 in *apoM*^{Tg} cells were increased compared with in the control group. Similarly, aortic S1PR1 mRNA and protein expression in *apoM*$^{-/-}$ mice was reduced compared with in *apoM*$^{+/+}$ mice. As part of the inflammatory response, the mRNA expression levels of S1PR1 were decreased in *apoM*^{TgN} cells, but not in *apoM*^{Tg} cells. These results further confirmed that ApoM may be capable of upregulating S1PR1 and could eliminate resistance to S1PR1. The present findings established that ApoM promoted the expression of S1PR1 in endothelial cells and murine aorta. It was hypothesized that the ApoM-induced increase in S1PR1 expression may lead to enhanced cellular responses to subsequent S1P treatment; therefore, the increase in S1PR1 may be due to the upregulation of S1P by ApoM.

Igarashi *et al* proposed that VEGF may enhance cellular responses to S1P by increasing the expression of S1PR1 and regulating the eNOS signaling pathway, which serves an important role in vascular protection (43). S1P promotes eNOS expression via the S1PR1 and S1PR3 pathways to produce more nitrogen oxide (NO) and decreases the activity of the mononuclear phagocyte system to resist atherosclerosis (8,44). In the present study, ApoM was revealed to upregulate S1PR1 expression; therefore, it was hypothesized that ApoM may have an impact on the eNOS signaling pathway by means of elevation of S1PR1 expression and activation to protect the vasculature. However, the mechanism underlying this phenotype requires further study.

In the present study, mice of the same gender were used to reduce the effects of differences between the genders. Due to reduced hormonal influences, male mice may be considered better models. Notably, our previous studies reported that estrogen upregulates the expression of ApoM *in vivo* and *in vitro*, and DHCR24 may also be regulated by estrogen receptors. In addition, estrogen significantly increases the expression of DHCR24 (12,13). Since estrogen may interfere with the experiments, male mice were used in the present study. This study demonstrated that the mRNA expression levels of DHCR24 were higher in the liver samples of *apoM*$^{+/+}$ mice compared with in *apoM*$^{-/-}$ mice in the absence of inflammatory stimulation. In addition, the mRNA expression levels of DHCR24 were significantly increased in EA.hy926 cells overexpressing *apoM* compared with in control cells. In response to TNF-α treatment in *apoM*^{TgN} and *apoM*^{Tg} cells, the mRNA expression levels of DHCR24 were markedly reduced in *apoM*^{TgN} cells, compared to *apoM*^{Tg}. This phenomenon was very similar to changes observed in S1PR1 expression during inflammation; however, two-way ANOVA revealed that ApoM and TNF-α had no interaction effects on the mRNA expression levels of DHCR24. These data suggested that ApoM may significantly increase DHCR24 gene expression *in vivo* and *in vitro*, although ApoM alone does not induce notable alterations in DHCR24 expression to directly affect the inflammatory response caused by TNF-α. The detailed molecular mechanism underlying the effects of DHCR24 on ApoM-inhibited inflammatory responses require further investigation.

To further elucidate the role of S1PR1 and DHCR24 in the anti-inflammatory response of ApoM, the S1PR1 antagonist W146 was administered prior to TNF-α treatment; the results revealed that IL-1β and MCP-1 mRNA expression was significantly elevated in *apoM*^{Tg} cells that received W146 treatment. However, there were no differences in the expression of these inflammatory biomarkers in *apoM*^{TgN} cells. In addition, the mRNA expression levels of DHCR24 were not affected in W146-treated *apoM*^{Tg} or *apoM*^{TgN} cells. These data suggested that ApoM induced inhibition of the inflammatory response via the S1PR1 pathway, but did not regulate DHCR24 through S1PR1 during inflammation. Furthermore, this result verified the hypothesis that ApoM-mediated anti-inflammation may occur through both S1PR1 and DHCR24, but that they operate in divergent signal pathways. The vascular protective (45), cardioprotective (46) and anti-apoptotic (47,48) properties of HDL are associated with S1PRs. ApoM is a component of HDL and a carrier of S1P; the *apoM*/S1P axis was initially proposed by Arkensteijn *et al* (5). This axis was originally thought to include ApoM, S1P and S1PRs, and it was suggested that an ApoM-S1P complex combined with S1PR1 could regulate the occurrence and development of inflammatory-associated diseases, such as atherosclerosis, diabetes mellitus, venous thromboembolism, hepatic fibrosis

and neuroinflammation (40,49-53). Christensen *et al* suggested that vascular leakage of albumin-sized particles in *apoM*$^{-/-}$ mice is S1PR1-dependent and that this dependency exacerbates the response to inflammatory stimuli (54). Ruiz *et al* demonstrated that ApoM limits endothelial inflammation by delivering S1P to S1PR1 (55). Our previous study revealed that ApoM protects against LPS-induced acute lung injury via S1PR1 signaling (56). The present study further confirmed that ApoM may be an anti-inflammatory molecule that acts on the S1PR1 pathway. Notably, to the best of our knowledge, the present study is the first to reveal ApoM may exert anti-inflammatory properties by regulating the expression of DHCR24, which is independent of S1PR1, and therefore, may be another signaling pathway associated with the anti-inflammatory effects of ApoM. The connections within this pathway require further elucidation.

It has previously been reported that upregulation of DHCR24 gene expression by (A-I) recombinant HDL is independent of cholesterol biosynthesis and the efflux of cholesterol from endothelial cells (11). Furthermore, HCAECs may be activated by TNF-α, and HDL suppresses VCAM-1 promoter activity by inhibiting the NF-κB pathway; suppression of NF-κB and VCAM-1 expression by HDL are dependent on alteration of DHCR24 protein levels. Elevated VCAM-1 and NF-κB expression in small interfering RNA-DHCR24-treated cells is induced by TNF-α, but can no longer be suppressed by HDL (11). Similarly, Patel *et al* reported that ApoA-I inhibits vascular inflammation in New Zealand white rabbits by increasing the expression of DHCR24 (57). HDL inhibits inflammation by increasing DHCR24 expression, which activates PI3K/Akt and induces heme oxygenase-1 (HO-1) expression through the SR-BI pathway (58). The present results demonstrated that ApoM may be involved in the regulation of HDL and expression of DHCR24. Connelly *et al* reported that SR-B1-mediated expression of NO and DHCR24 leads to reduced inflammation, thus reducing monocyte recruitment into the intima (59). SR-B1 interaction with HDL prevents endothelial cell inflammation by controlling eNOS activation and DHCR24 expression (60). S1PR1 and SR-B1 interactions are thought to be involved in the ApoM-mediated anti-inflammatory process. S1PR1 and SR-B1 are co-localized in the caveolar regions of plasma membranes (20,21). Al-Jarallah *et al* demonstrated that inactivation of the expression of SR-B1, PDZ domain containing 1 or Akt1, or antagonism of S1PR1, impairs the ability of macrophages to undergo chemotaxis towards HDL (61). HDL activates eNOS and S1P promotes interaction of SR-B1 with S1PR1 to activate the latter (62). To investigate whether ApoM can regulate DHCR24 through SR-B1 to exert an anti-inflammatory effect, the SR-B1 antagonist BLT-1 was used prior to TNF-α treatment. Under these conditions, DHCR24 mRNA expression was significantly reduced in *apoM*^{Tg} cells, but no differences in DHCR24 mRNA expression were detected in *apoM*^{TgN} cells. These data suggested that ApoM may upregulate DHCR24 via SR-B1 but ApoM may not induce inhibition inflammation via SR-B1. The present study further demonstrated that ApoM not only promoted anti-inflammatory action through S1PR1, but also possibly through DHCR24. ApoM may regulate DHCR24 through SR-B1, but not enough to induce an anti-inflammatory effect through this pathway. It has recently been demonstrated that HDL inhibits inflammation by increasing DHCR24 expression, which activates PI3K/Akt and induces HO-1 through the SR-B1 pathway (55). Whether regulation of DHCR24 through SR-B1 signaling may lead to oxidative stress or other pathways inducing inflammatory changes remains to be determined.

In conclusion, the present study investigated the anti-inflammatory potential of ApoM, and demonstrated that ApoM regulated the expression levels of S1PR1 *in vivo* and *in vitro*, with or without inflammation. Additional studies revealed that ApoM regulated DHCR24 expression *in vivo* and *in vitro*. These results demonstrated that ApoM-induced inhibition of inflammatory responses may occur via the S1PR1 pathway, and suggested that the anti-inflammatory properties of ApoM might be due to its regulation of DHCR24. However, the S1PR1 and DHCR24 pathways of ApoM anti-inflammatory action were not overlapping; therefore, the detailed mechanism of action requires further investigation.

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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

MW and YPZ performed the experiments and wrote the manuscript. XYZ and NX made substantial contributions to conception and experimental design. YY and SY performed the cell experiments, HL, XHZ and BW performed the animal experiment and helped perform the analysis with constructive discussions. GHL and DMD made substantial contributions in acquiring, analyzing and interpreting the data. All authors approved the final version of the manuscript for publication.

Ethics approval and consent to participate

Experimental protocols were approved by the Animal Use and Protection Committee of Soochow University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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