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Allergic asthma aggravated atherosclerosis increases cholesterol biosynthesis and foam cell formation in apolipoprotein E-deficient mice

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ABSTRACT

Several studies have demonstrated that allergic asthma can induce atherosclerosis formation in mice. Moreover, allergic asthma and atherosclerosis have been shown to be strongly associated with dyslipidemia. In this study, we investigated the underlying mechanism of allergic asthma-aggravated atherosclerosis-induced cholesterol metabolism disorder in asthmatic apoE^{-/-} mice. We found that allergic asthma increased the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) in the liver and CD36 in the aorta during the acute and advanced stages of atherosclerosis, respectively. These results indicate that cholesterol biosynthesis is increased during acute atherosclerosis and cholesterol uptake and foam cell formation is increased during advanced atherosclerosis. Simvastatin administration significantly ameliorated the aortic root lesion size of asthmatic mice and significantly decreased HMGCR and CD36 expression. However, the expression of the low-density lipoprotein receptor and ATP-binding cassette transporter A1 was markedly increased, indicating that the beneficial effect of statins in allergic asthma and coronary artery disease was mediated, at least in part, by decreasing cholesterol biosynthesis and foam cell formation. In conclusion, allergic asthma aggravates atherosclerosis by regulating cholesterol metabolism in apoE^{-/-} mice. Allergic asthma selectively promotes cholesterol biosynthesis in acute atherosclerosis and increases foam cell formation in advanced atherosclerosis.

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1. Introduction

Allergic asthma is one of the most common chronic inflammatory diseases. Both obesity and high serum cholesterol levels are considered risk factors for allergic asthma [1–3], especially in children. A study conducted on a school student showed a positive correlation between asthma and increased levels of low-density lipoprotein cholesterol (LDL-C), and this correlation increased in overweight and obese subjects [4]. Sun-Hye Ko et al. have shown the positive correlation between hyperlipidemia and asthma prevalence in Korean adolescents [5]. Moreover, obesity can induce low-grade chronic tissue inflammation and reprogram cellular and humoral immunity [6], causing an increased prevalence of asthma and asthma-like symptoms and delayed response to anti-inflammatory therapy [7]. Some experimental studies have also shown increased local inflammation in the lungs of high-fat-fed

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; apo E, apolipoprotein E; TC, total cholesterol; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGCR, HMG-CoA reductase; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; OxLDL, oxidized LDL.

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obese asthmatic mice [8,9]. These studies suggested an association between obesity/dyslipidemia and allergic asthma, or that obesity/dyslipidemia promotes the progression of allergic asthma; however, the causal relationship between them has not been elucidated yet. The ability of allergic asthma to affect serum cholesterol metabolism is unclear.

The relationship between dysfunctional lipoprotein cholesterol metabolism and atherosclerosis development is well known. Several epidemiological studies over the past decades have confirmed that patients with hyperlipidemia exhibit a higher incidence of atherosclerosis; moreover, the independent and opposing roles of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as risk factors for cardiovascular diseases has been reported [10,11]. Elevated serum cholesterol levels, especially LDL-C, can injure vascular endothelial cells to cause infiltration and retention of LDL-C in the arterial intima to initiate an inflammatory response in the arterial wall [12]. Oxidized LDL (OxLDL) plays a vital role in atherogenesis by creating an inflammatory environment and increasing lipid deposition in the arterial wall [13]. Meanwhile, impairment of cholesterol influx and efflux balance in macrophages can cause an excessive intake of OxLDL, resulting in increased foam cell formation [14,15].

Allergic asthma and atherosclerosis have been shown to be positively correlated with dyslipidemia/obesity. It has also been shown that the severity of allergic asthma correlates with the degree of pathological vessel changes in atherosclerosis [16], and that chronic and acute allergic lung inflammation promote atherogenesis and increase the number of aortic lesions [17]. Our previous study has shown that allergic asthma can aggravate atherosclerosis by modulating the balance of effector T cells/Treg cells in apolipoprotein E^{-/-} (apoE^{-/-}) mice [18]. Moreover, clinical studies have demonstrated that the patients with allergic asthma exhibit an increased risk for developing atherosclerosis [19].

In summary, allergic asthma can aggravate atherosclerosis and both are strongly associated with dyslipidemia. This study was aimed at determining whether allergic asthma can aggravate atherosclerosis by affecting cholesterol metabolism.

2. Material and methods

2.1. Allergic asthma mouse model

ApoE^{-/-} mice with a C57BL/6J background were kept in a temperature- and humidity-controlled facility on a 12-h light-dark cycle with free access to food and water. Mice were weaned at 4 weeks of age and then fed normal food. This animal study was approved by the institutional ethics committee for animal experiments of Xi'an Jiaotong University. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Six-to eight-week-old apoE^{-/-} mice were used for this study. Mice were randomly assigned to the control (Con group, n = 6) and asthma (Ast group, n = 12) groups. Sensitization and challenge were performed as previously described [20]. Mice were euthanized 24 h after the last challenge. The asthmatic mouse model was assessed as previously described [18].

2.2. Administration of simvastatin

Simvastatin (Sigma-Aldrich, purity ≥97%) was dissolved in 0.1% sodium carboxymethyl cellulose suspension. Simultaneously with the aerosolised exposure, the asthmatic mice were randomly assigned to two asthmatic subgroups and received either simvastatin treatment (Sim group, 5 mg/kg/d, orally by gastric gavage, n = 6) or an equal volume of vehicle (n = 6) for 2 (acute group) or 8

(advanced group) weeks.

2.3. Detection of lipid in serum

Blood samples were collected before sacrifice and the serum total cholesterol (TC) and LDL-C levels were measured using the respective assay kits according to manufacturers' instructions (LDL-C kit from Rongsheng, TC kit from Dongou, China).

2.4. Quantitative evaluation of atherosclerotic lesions in the aortic root

The aortic root was dissected under a microscope and frozen in optimal cutting temperature embedding medium for serial cryosectioning (7 μm) spanning 500 μm of the root. The first section displayed the three aortic valve leaflets. Every fifth section was harvested on one slide (six sections per slide). Oil Red O staining (Sigma) was used to measure the lesion size and foam cell content. All sections were photographed with identical exposure settings and all images were quantified using Image-Pro plus 6.0 software. The lesion size was calculated as the ratio of the lesion area to the total vessel wall area.

2.5. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the liver or thoracic aorta using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA was reversely transcribed using a ReverAid™ First Strand cDNA Synthesis Kit (TaKaRa, Japan), and analyzed using quantitative real-time PCR with a SYBR® Premix Ex Taq™ II RT-PCR Kit (TaKaRa, Japan). All reactions were performed on the iQ5™ Multicolor Real-Time PCR detection system (Bio-Rad, USA). The housekeeping gene GAPDH was used as an internal control. The primer sequences are listed in Table 1.

2.6. Western blot analysis

Proteins from the liver or thoracic aorta were extracted using RIPA buffer (Cybrdi, Inc, USA) according to the manufacturer's instructions. The total amount of protein was quantified using the BCA protein assay reagent kit (Thermo Fisher, USA). Equal amounts of proteins were separated on SDS-PAGE gels and then transferred onto a PVDF membrane. After blocking with 5% skimmed milk for 1 h, the blots were incubated with primary antibodies against HMGCR (Santa Cruz), LDLR (Abcam), CD36 (Abcam), ABCA1 (Abcam), and ABCG1 (Abcam), and finally with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Abbkine, Inc. USA). All protein signals were detected using an ECL detection system and the band density was determined using Image J. GAPDH were used as a loading control to correct the densitometric values for the target protein.

Table 1
Primer sequences used for real-time PCR.

	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACGGGAAGCTCACTGGCATG	CACCTGTGTGCTAGCCGT
HMGCR	AGCAGATTGCTCGTCTACA	GTCTTCTCGTCTTCGAT
LDLR	GGTCCCACTCGCCAAATT	TTCCCTCTGCCAGCCATGTCAC
CD36	TGGAAAGGATAACATAAGCAAAG	GAACACAGCGTAGATAGACC
ABCA1	GCGGCAACAACGAAAGCTC	GGCACCTGAACCTTCCATTG
ABCG1	CTGAAGTACTGGTACAGCCT	TCCAAGCCGTAGATGGAC

2.7. Statistical analyses

All data were expressed as mean $\pm$ standard deviation. One-way analysis of variance or unpaired Student's *t*-test was used to determine the statistical significance of the differences among the groups if values followed a normal distribution (for other cases, Kruskal-Wallis H statistic or Mann-Whitney *U* test was used). A level of $p < 0.05$ was considered significant.

3. Results

3.1. Atherosclerosis is aggravated and serum lipid levels are increased in apoE^{-/-} mice with allergic asthma

As shown in Fig. 1, the lesion area of the apoE^{-/-} mice with allergic asthma in the acute and advanced atherosclerosis groups was markedly higher than that of the control group mice (Fig. 1A and B, vs. control mice, $p < 0.05$). Meanwhile, serum TC and LDL-C levels were significantly increased in the asthmatic mice of both acute and advanced groups (Fig. 1C and D, vs. control mice, $p < 0.05$).

3.2. Cholesterol biosynthesis is increased in asthmatic apoE^{-/-} mice with acute atherosclerosis

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (HMGCR) is the rate-limiting enzyme in cholesterol biosynthesis.

Therefore, we measured the mRNA and protein expression levels of HMGCR in the liver of asthmatic apoE^{-/-} mice. HMGCR expression was significantly increased in the mice with acute atherosclerosis (Fig. 2A and B, vs. control mice, $p < 0.05$), but was unchanged in those with advanced atherosclerosis. The low-density lipoprotein receptor (LDLR) is expressed in the liver and is responsible for the removal of LDL-C from circulation under physiological conditions. The expression of LDLR was significantly increased in the mice with acute atherosclerosis (Fig. 2A and C, vs. control mice, $p < 0.05$), and markedly decreased in those with advanced atherosclerosis (Fig. 2A and C, vs. control mice, $p < 0.05$). The mRNA and protein expression levels of HMGCR and LDLR were similar (Fig. 2D and E).

3.3. Cholesterol uptake is increased in asthmatic apoE^{-/-} mice with advanced atherosclerosis

We sought to investigate the effects of allergic asthma on foam cell formation and cholesterol uptake and efflux balance. Hence, we measured the expression of related membrane receptors in the aorta of mice. Our results showed that allergic asthma significantly increases CD36 (regulates oxLDL uptake) expression in asthmatic apoE^{-/-} mice with advanced atherosclerosis (Fig. 3A and B, vs. control mice, $p < 0.05$), but exhibits no significant effect on those with acute atherosclerosis. The expression of ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) in the acute and advanced groups of asthmatic mice did not significantly change compared with that in the control mice

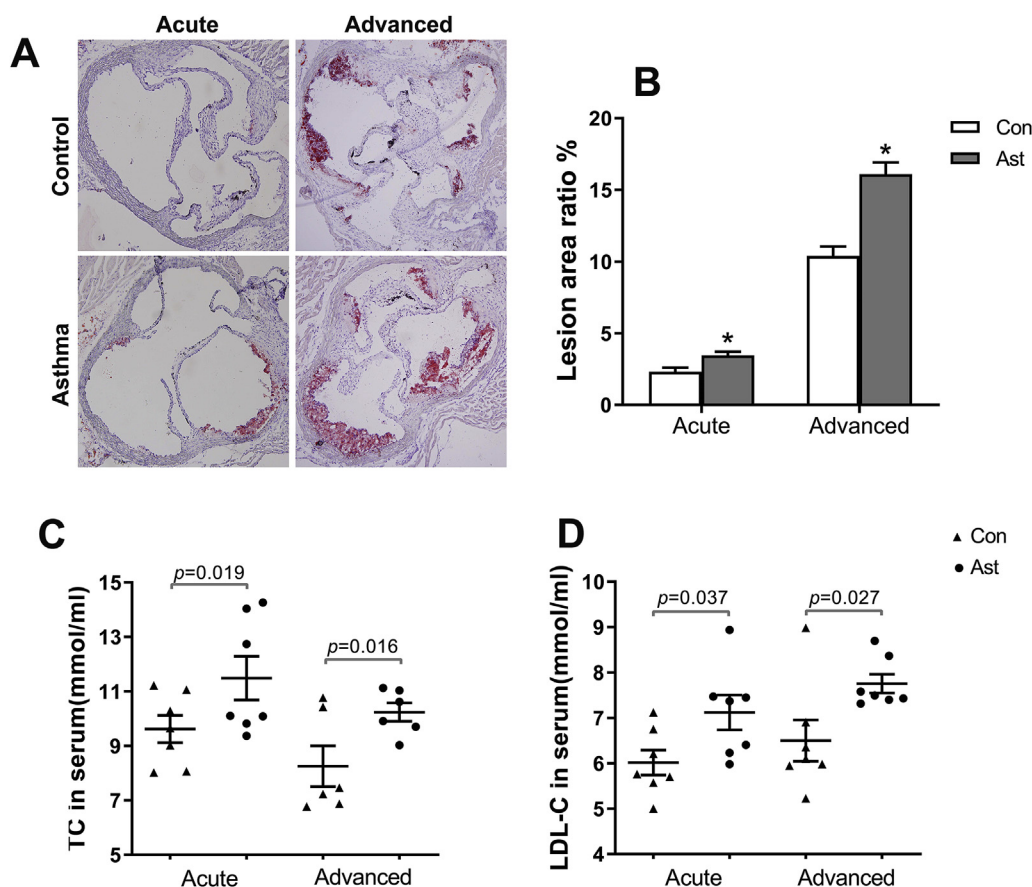


Fig. 1. Effects of allergic asthma on atherosclerosis lesion area and levels of total cholesterol and low-density lipoprotein-cholesterol in apoE^{-/-} mice. ApoE^{-/-} mice were randomly assigned to control and asthma groups. The lesion area was measured by Oil Red O Staining (A and B), magnification $\times 100$. Serum total cholesterol (C) and low-density lipoprotein-cholesterol (D) levels were measured using their respective assay kits. Data are presented as mean $\pm$ SD ($n = 6$). * $p < 0.05$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

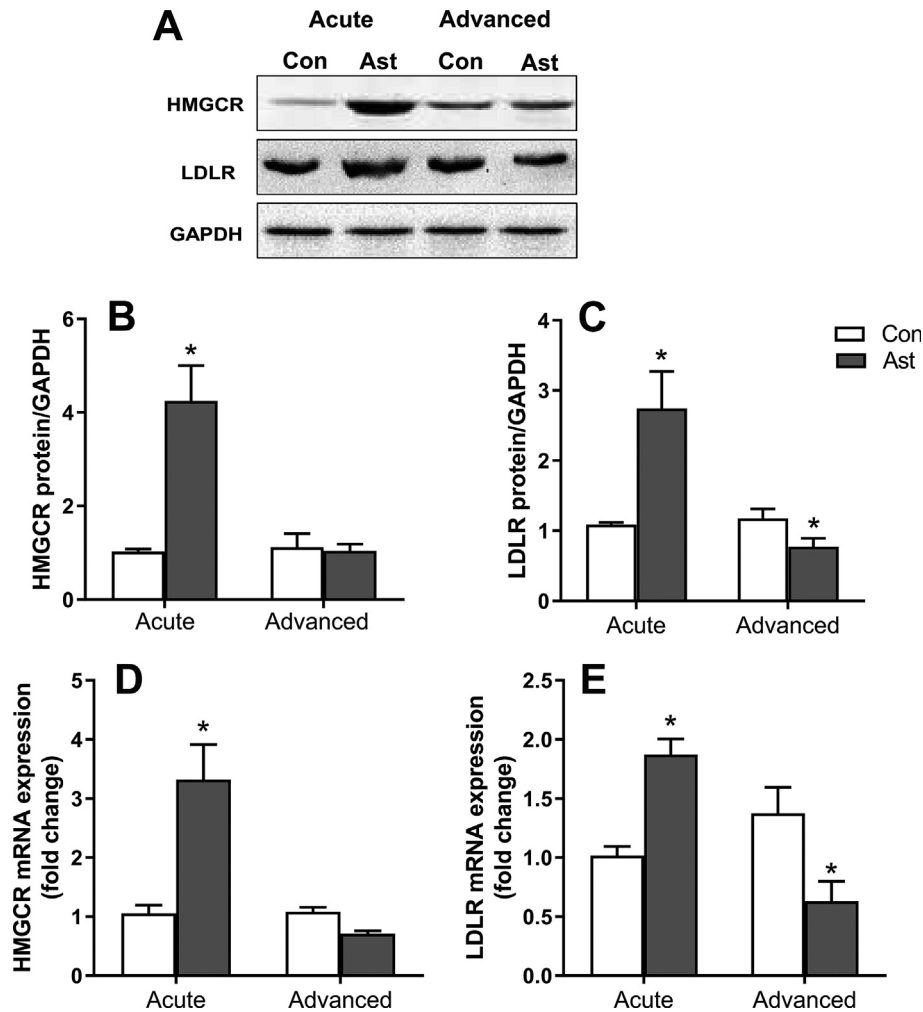


Fig. 2. Effects of allergic asthma on cholesterol biosynthesis and removal in apoE^{-/-} mice. (A) Representative Western blot results of HMGCR and LDLR expression in the liver at different stages. GAPDH was used as a loading control. Average quantification values for HMGCR (B) and LDLR (C), obtained from densitometric analysis. The mRNA expression levels of HMGCR (D) and LDLR (E) at different stages. Data are presented as mean $\pm$ SD (n = 6). * p < 0.05 vs. control group.

(Fig. 3 A, C and D). The mRNA and protein expression levels of CD36, ABCA1, and ABCG1 in the aorta were similar (Fig. 3 E, F and G). These findings suggest that the increased CD36 expression is responsible for increased cholesterol uptake and foam cell formation in the aorta of asthmatic apoE^{-/-} mice with advanced atherosclerosis.

3.4. Administration of statins inhibits LDL-C synthesis and foam cell formation

Statins can reduce cholesterol levels by inhibiting HMGCR expression in the liver. Hence, we administered simvastatin to asthmatic apoE^{-/-} mice 2 (acute group) or 8 (advanced group) weeks. The aortic root lesion area was markedly decreased in both acute and advanced groups of mice (Fig. 4A, vs. asthmatic mice, p < 0.05). Moreover, HMGCR (Fig. 4B and C, vs. asthmatic mice, p < 0.05) and CD36 (Fig. 4E and F, vs. asthmatic mice, p < 0.05) expression were significantly decreased in both acute and advanced groups of asthmatic mice; however, LDLR (Fig. 4B and D, vs. asthmatic mice, p < 0.05) and ABCA1 (Fig. 4E and G, vs. asthmatic mice, p < 0.05) expression were markedly increased both groups. Moreover, the expression of ABCG1 (Fig. 4H) did not change in both groups. The mRNA and protein expression levels were similar (see supplementary material, Suppl. Figure).

4. Discussion

Allergic asthma and atherosclerosis exert an opposite inflammatory effect on CD4⁺ T cells; allergic asthma induces Th2 inflammation [21], whereas atherosclerosis induces Th1 inflammation [22]. Previous studies have shown that both conditions are closely related to cholesterol metabolism disorders, and that allergic asthma can aggravate atherosclerosis [16,17]. However, the mechanism of allergic asthma-aggravated atherosclerosis-induced cholesterol metabolism disorder has not clearly elucidated. Our present study confirmed that allergic asthma can increase the area of atherosclerotic lesions in apoE^{-/-} mice by promoting cholesterol biosynthesis in the acute stage and foam cell formation in the advanced stage. Not only that, several previous studies have demonstrated that high serum cholesterol levels/obesity are risk factors for allergic asthma [1–6]. To our knowledge, ours is the first study to confirm that allergic asthma can promote cholesterol biosynthesis by itself.

HMGCR is a rate-limiting enzyme in cholesterol biosynthesis, which catalyzes the conversion of HMG-CoA to mevalonic acid [23,24]. Increase expression of HMGCR indicates increased cholesterol biosynthesis. Under physiological conditions, a majority of unmodified LDLs are metabolized via the LDLR pathway and a small fraction are metabolized via the mononuclear-phagocytic

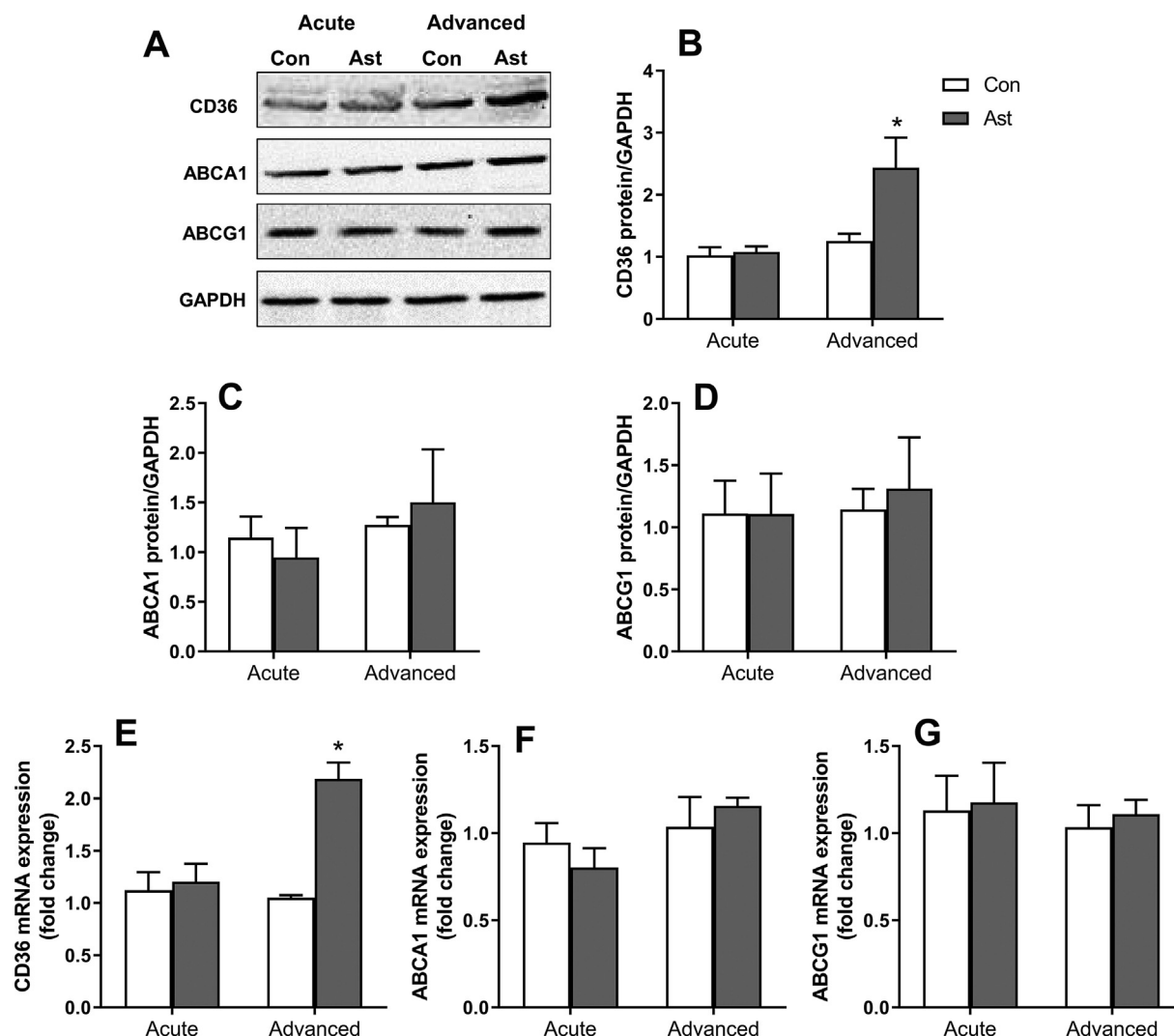


Fig. 3. Effects of allergic asthma on cholesterol uptake and efflux balance in $\text{apoE}^{-/-}$ mice. (A) Representative Western blot results of CD36, ABCA1, and ABCG1 expression in the aorta of mice. GAPDH was used as a loading control. Average quantification values for CD36 (B), ABCA1 (C), and ABCG1 (D), obtained from densitometric analysis. The mRNA expression levels of CD36 (E), ABCA1 (F), and ABCG1 (G) at different stages. Data are presented as mean $\pm$ SD ($n = 6$). * $p < 0.05$ vs. control group.

system [14]. However, modified LDL is primarily cleared upon binding with the scavenger receptors on the surface of macrophages [25]. In the present study, we found that allergic asthma significantly increases HMGR expression in $\text{apoE}^{-/-}$ mice with acute atherosclerosis, but not in those with advanced atherosclerosis. These results suggest that allergic asthma can significantly promote cholesterol biosynthesis in acute atherosclerosis. We also observed that LDLR expression is significantly increased in asthmatic mice with acute atherosclerosis. We believe that this may be caused due to increased cholesterol biosynthesis. Moreover, LDLR expression was markedly decreased in asthmatic mice with advanced atherosclerosis. A possible explanation for this observation is that the modified LDL (mainly oxLDL) is primarily metabolized via the scavenger receptor pathway, but not the LDLR pathway. In addition, elevated LDL can suppress HMGR activity by binding to a receptor and generating secondary messengers [26,27], it is the possible mechanism for HMGR expression decreased to normal level in advanced group.

Foam cell formation is a hallmark of atherosclerosis and plays an important role at all stages of atherosclerotic lesion development [14]. The increased uptake of oxLDL and/or reduced cholesterol

efflux can result in the accumulation of esterified cholesterol in the cytoplasm of macrophages and subsequently trigger foam cell formation [25,28]. Several studies have demonstrated that CD36 is the principal receptor for the binding and uptake of ox-LDL in macrophages, while ABCA1 and ABCG1 mediate cholesterol export from macrophages [25,29,30]. In the present study, we observed that allergic asthma can significantly increase CD36 expression in mice with advanced atherosclerosis; however, there was no significant effect on the expression of ABCA1 and ABCG1. These results suggest that the increased CD36 expression is responsible for foam cell formation in the aorta of asthmatic mice.

To further investigate the underlying mechanisms, we used an HMGR inhibitor simvastatin to treat asthmatic mice. Our results showed that the aortic root lesion area of asthmatic mice is markedly decreased in the acute and advanced stages of atherosclerosis. However, HMGR expression in the acute stage and CD36 expression in the advanced stage were markedly decreased, and LDLR and ABCA1 expression were markedly increased. These results indicate that simvastatin administration can alleviate atherosclerosis in asthmatic mice by decreasing cholesterol biosynthesis and intake as well as increasing cholesterol efflux, which is consistent with the

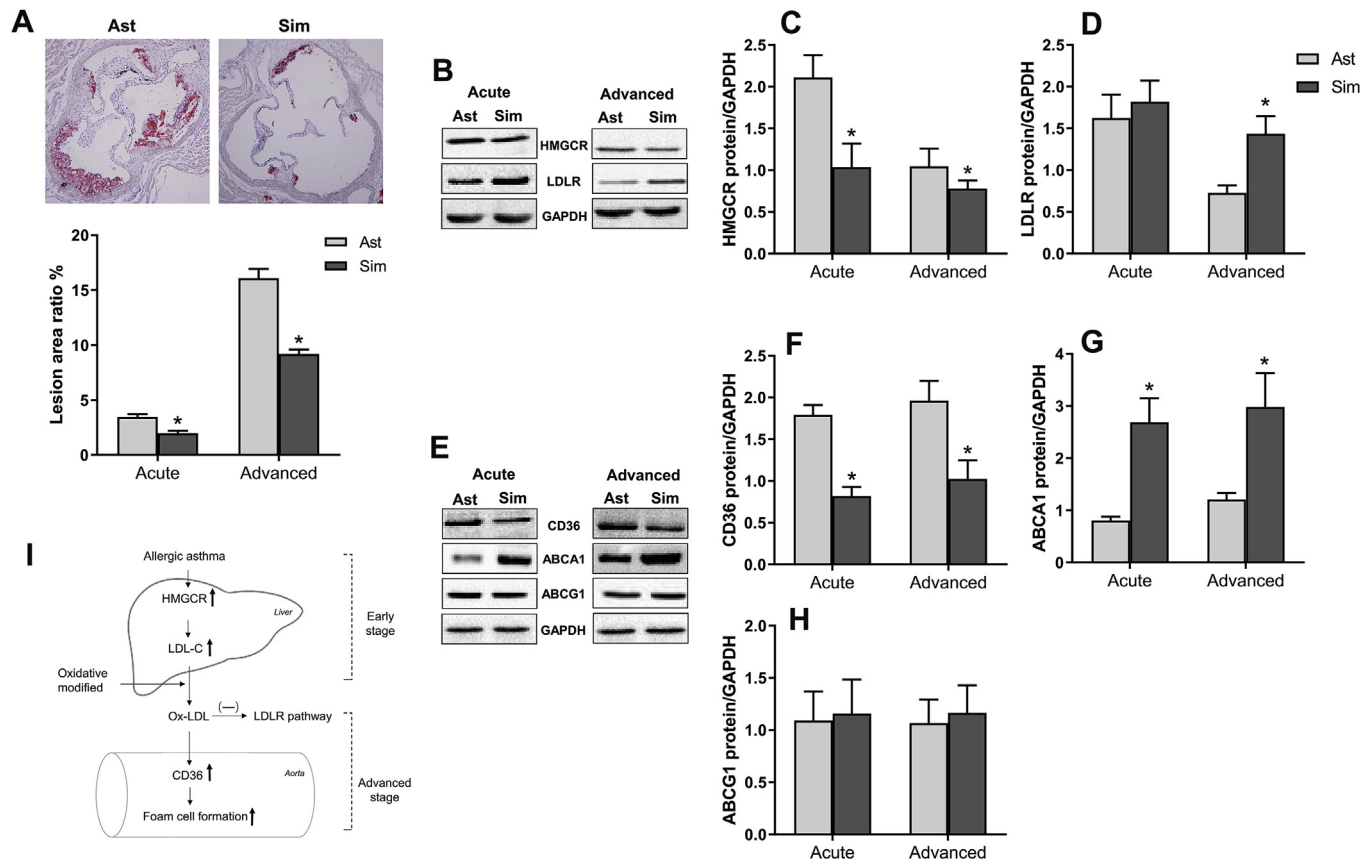


Fig. 4. Administration of statins decreases the lesion area by inhibiting cholesterol synthesis and foam cell formation in asthmatic apoE^{-/-} mice. (A) The aortic root lesion size of asthmatic apoE^{-/-} mice was measured by Oil Red O staining, magnification $\times 100$. (B) Representative Western blot results of HMGCR and LDLR expression in the liver of mice at different stages. GAPDH was used as a loading control. Average quantification values for HMGCR (C) and LDLR (D), obtained from densitometric analysis. (E) Representative Western blot results of CD36, ABCA1, and ABCG1 expression in the aorta of mice. GAPDH was used as a loading control. Average quantification values for CD36 (F), ABCA1 (G), and ABCG1 (H), obtained from densitometric analysis. Data are presented as mean $\pm$ SD ($n = 6$). * $p < 0.05$ vs. asthmatic group. (I) Schematic diagram of allergic asthma-aggravated atherosclerotic lesions characterized by increased cholesterol biosynthesis in the acute stage and increased foam cell formation in the advanced stage. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

results of previous studies [31,32]. Therefore, the protective role of statins in allergic asthma and coronary artery disease [33,34] is mediated, at least in part, by decreasing cholesterol biosynthesis and foam cell formation.

The present study had a few limitations. Firstly, we used simvastatin to reduce cholesterol biosynthesis, but it may be more convincing to use HMGCR knockout animal models because statins can exhibit other effects other than inhibit cholesterol synthesis, such as anti-inflammatory, anti-tumor and improved vascular endothelium. However, HMGCR expression in the liver is essential for the survival of mice and its abrogation causes hepatic steatosis and death [35]. Hence, the choice of the animal model to study the potential mechanism requires further investigation. Secondly, allergic asthma is a heterogeneous disease. Therefore, the model (sensitization with ovalbumin) cannot reflect all forms of asthma or replace clinical studies, but can only study a specific pathway in asthma (the allergen-driven pathway).

In conclusion, we found that allergic asthma aggravates atherosclerosis by modulating cholesterol metabolism in apoE^{-/-} mice. In particular, allergic asthma increases cholesterol biosynthesis in acute atherosclerosis and foam cell formation in advanced atherosclerosis. Additional potential mechanisms warrant further investigation. The present study also partly explains the protective benefits of statins in both allergic asthma and coronary artery disease.

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Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.09.085>

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.09.085>.

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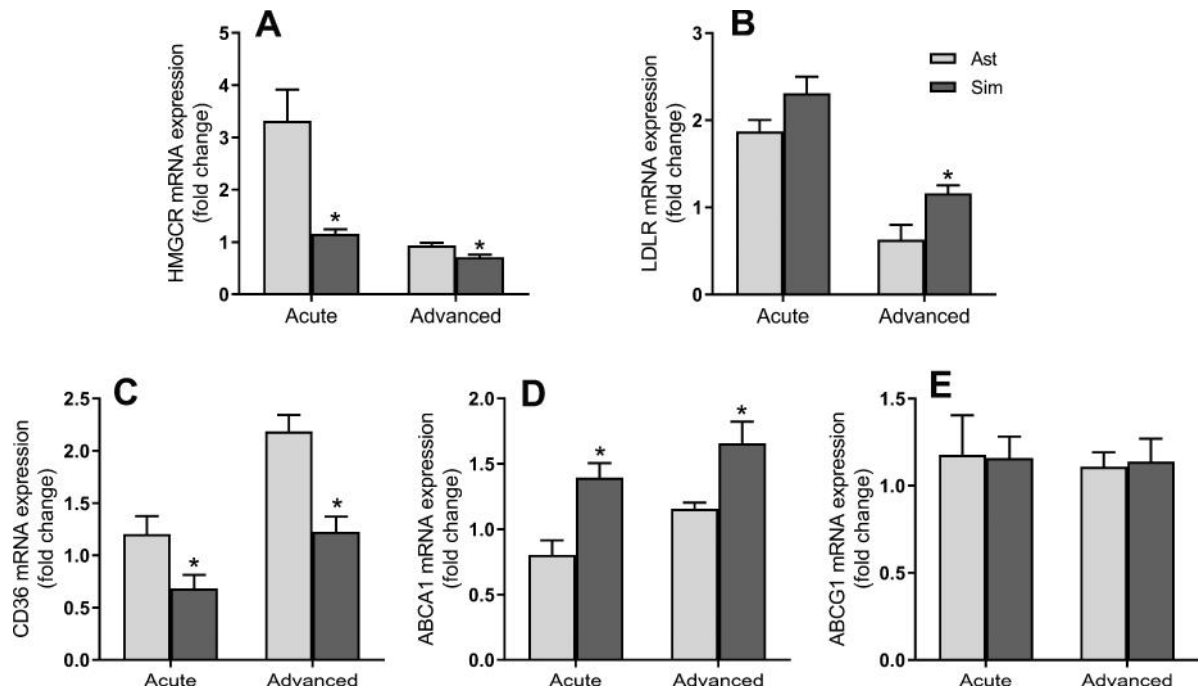


Figure Suppl. The effect of simvastatin on cholesterol synthesis and foam cell formation in asthmatic apoE^{-/-} mice. The mRNA expression levels of HMGCR (A), LDLR (B), CD36 (C), ABCA1 (D), and ABCG1 (E) at different stages were measured by RT-PCR. Data are presented as mean $\pm$ SD (n = 6). * $p < 0.05$ vs. asthmatic group.