Statin or PCSK9 inhibitor alleviates airway hyperresponsiveness and lung fibrosis in high-fat diet-induced obesity mouse model

LIANG LIN

Department of Medicine

Graduate School

Yonsei University

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Advisor Park Jung-Won

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LIANG LIN

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This Certifies that the Dissertation of LIANG LIN is Approved

Committee Chair	**Lee, Jae-Hyun**
Committee Member	**Park, Jung-Won**
Committee Member	**Lee, Sook Young**
Committee Member	**Kang, Hye-Ryun**
Committee Member	**Park, Chang Ook**

Department of Medicine
Graduate School
Yonsei University
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"Knowing what things to keep, and what things to release. You get what you get. "

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ABSTRACT

Statin or PCSK9 inhibitor alleviates airway hyperresponsiveness and lung fibrosis in high-fat diet-induced obesity mouse model

Background: Obesity induces airway hyperresponsiveness (AHR) and lung fibrosis, which may cause patients with asthma and obesity to respond poorly to typical asthma medications. Statins and blocking proprotein convertase subtilisin/kexin-9 (PCSK9) decrease serum cholesterol, renin-angiotensin system (RAS) activity, free fatty acids (FFAs), and anti-inflammatory effects, which may contribute to lung pathologies associated with obesity.

Objective: This study examined whether statin or anti-PCSK9 monoclonal antibody (mAb) administration alleviated obesity-induced lung pathologies using high-fat diet (HFD)-induced obese mice.

Methods: HFD-induced obesity was produced in male C57BL/6 mice, which were fed an HFD for 16 weeks. Transforming growth factor (TGF)-β1 transgenic mice were fed a normal diet. Mice were administered a statin (atorvastatin) or alirocumab and analyzed for AHR, lung histology, pro-inflammatory mediators, and lipid metabolites in the lungs.

Results: HFD mice had increased cholesterol, RAS activity, and FFAs, which were suppressed by lipid-lowering agents. The HFD obesity model also had enhanced AHR, macrophages in bronchoalveolar lavage fluid, lung fibrosis, epithelial-to-mesenchymal transition (EMT) markers, NLRP3 inflammasomes, cholecystokinin, pro-inflammatory interleukin (IL)-1β, IL-6, IL-17a, and TGF-β1 in the lungs. Atorvastatin and alirocumab attenuated the obesity-associated lung pathologies, pro-inflammatory mediators, EMT markers, RAS activity, NLRP3 inflammasomes, cholecystokinin, and TGF-β1 expression. Lipid-lowering agents also suppressed pro-inflammatory immune responses and lung fibrosis in TGF-β1 overexpressing transgenic mice fed a normal diet.

Conclusions: Serum lipid-lowering treatment may alleviate obesity-induced AHR and lung fibrosis through inhibition of the NLRP3 inflammasome, RAS, and cholecystokinin activity. Lipid-

lowering strategies may prove beneficial in treating asthma patients with obesity who exhibit a poor response to typical asthma medications.

Key words : Obesity, statin, PCSK9, asthma, alirocumab

I. INTRODUCTION

Obesity is closely linked to metabolic impairments and to both the increased incidence and exacerbation of asthma symptoms [1]. According to the 2022 Global Initiative for Asthma report, obesity-associated asthma is now considered a distinct clinical phenotype. As a result, individuals with coexisting obesity and asthma often exhibit more severe symptoms, show reduced responsiveness to corticosteroids, and present with non-eosinophilic airway inflammation [2].

Animal studies have shown that a high-fat diet (HFD) can induce obesity in mice, subsequently leading to airway hyperresponsiveness (AHR) and pulmonary fibrosis [3]. Moreover, obesity is associated with higher systemic levels of pro-inflammatory markers, including C-reactive protein, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, leptin, and interleukin (IL)-6 [4].

In addition, multiple studies have identified the NLRP3 inflammasome as a central mediator of obesity-induced inflammatory diseases. Under these conditions, damage-associated molecular patterns (DAMPs)—including elevated glucose concentrations, reactive oxygen species (ROS), oxidized low-density lipoprotein (LDL), and cholesterol crystals—can activate NLRP3, eliciting pro-inflammatory responses via IL-1β. This cascade eventually culminates in insulin resistance and metabolic syndrome [5]. Increased oxidative stress in the bloodstream, together with NLRP3 inflammasome activation in the respiratory tract, is also thought to contribute to asthma symptoms in obesity-associated asthma[6,7]. Furthermore, research on HFD-induced obese mouse models has demonstrated increased levels of renin and angiotensin II [8]. The renin–angiotensin system (RAS) is recognized to be activated, potentially contributing to fibrosis in multiple organs, including the lungs [9,10]. Evidence also supports an autocrine loop between RAS activity and TGF-β1 signaling, indicating potential crosstalk between these pathways. Additionally, NLRP3 is central to reactive oxygen species (ROS) production triggered by angiotensin II and TGF-β in mouse fibroblasts, thereby driving fibrotic changes [11]. Consequently, simultaneous targeting of RAS activation and inhibition of NLRP3 and TGF-β1 expression may provide novel therapeutic approaches for patients with coexisting asthma and obesity.

Statins and the anti-proprotein convertase subtilisin/kexin-9 (PCSK9) monoclonal antibody are well-known LDL-cholesterol lowering agents. LDL-cholesterol induces inflammation by activating the NLRP3 inflammasome and Toll-like receptors (TLRs) [13]. Consequently, these lipid-lowering

agents can have several anti-inflammatory effects. A recent study showed increased free fatty acid (FFA) levels in HFD-induced obese mice, which led to elevated secretion of cholecystokinin (CCK), a well-known gall bladder smooth muscle contractor, and enhanced CCK receptor expression was found in smooth muscle in the lungs. The study also showed that a CCK receptor antagonist blocked AHR, which suggested that AHR development may be due to the CCK system in the HFD-induced obesity mouse model [12].

PCSK9 accelerates the degradation of the LDL receptor, thereby diminishing LDL clearance [14, 15]. Previous studies have also linked obesity to elevated PCSK9 levels [16]. Moreover, multiple investigations show that the inflammatory cytokine TGF-β1 can upregulate PCSK9 secretion across various human organs [17]. In addition, deleting PCSK9 in cardiomyocytes reduces NLRP3-driven inflammatory signaling [18]. Furthermore, statins inhibit both the NLRP3 inflammasome and TLR signaling, suggesting potential therapeutic benefits for airway inflammatory disorders [19-22].

This study aims to assess the impact of statins or PCSK9 inhibitors on AHR and lung fibrosis in an HFD-induced obesity mouse model. In addition, we seek to clarify how these lipid-lowering agents modulate RAS activation, NLRP3 inflammasome function, pro-inflammatory cytokine release, and CCK expression.

II. MATERIALS AND METHODS

1. Study scheme and animals

Male C57BL/6 mice were randomly assigned to one of two dietary regimens for 16 weeks: a normal diet (ND) providing 10% of calories from fat (Diet D12450, Research Diets Inc.) or a high-fat diet (HFD) delivering 60% of calories from fat (Diet D12492, Research Diets Inc.). Body weight was measured weekly throughout the study.

Administration of the anti-PCSK9 monoclonal antibody alirocumab or atorvastatin commenced at week 5. Alirocumab was injected weekly at doses of 3 or 10 mg/kg for the remaining 16 weeks, whereas atorvastatin was administered orally at 10 mg/kg five times per week over the same duration.

Triple-transgenic TGF-β1 mice, male and female transgene+ mice, and transgene− littermates aged 6–8 weeks were fed water containing 0.5 mg/mL doxycycline ad libitum for 4 weeks. During the same period, these mice received intraperitoneal injections of alirocumab (10 mg/kg) once weekly and oral atorvastatin (10 mg/kg) five times weekly. All animal procedures were conducted in strict compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Yonsei University College of Medicine.

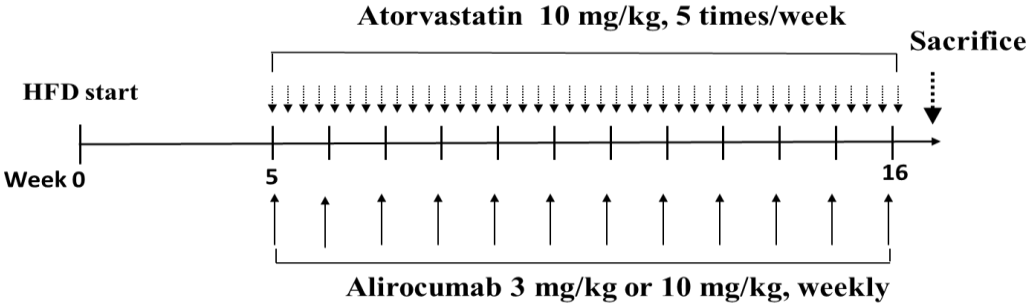

Figure 1. This figure shows the scheme of PCSK9 inhibition or statin treatment in HFD or ND mice. Male C57BL/6 mice were fed a ND or HFD for 16 weeks. The mice were weighed every week. At week 5, the administration of PCSK9 blocking alirocumab or atorvastatin began. Alirocumab was injected 3 mg/kg or 10 mg/kg weekly for 16 weeks. Atorvstatin was orally administered five times per week for the same 16-week period at a dosage of 10 mg/kg.

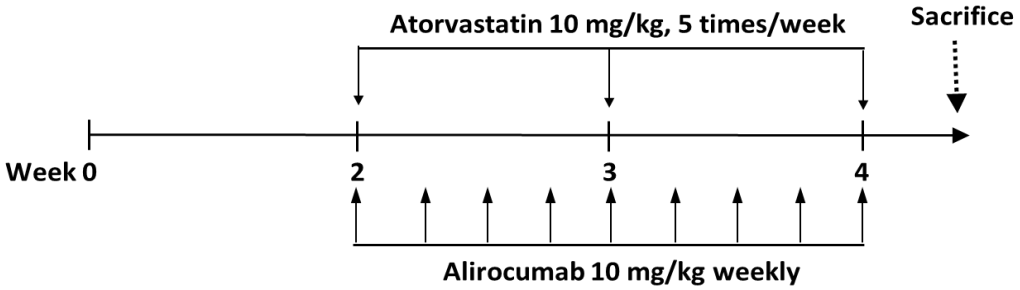

Figure 2. Experimental scheme of the TGF-β1 transgenic mouse study. Male and female transgene+ mice and transgene− littermates aged 6–8 weeks were fed 0.5 mg/ml doxycycline in water ad libitum for 4 weeks, with intraperitoneal injection of alirocumab (10 mg/kg) weekly and oral administration of atorvastatin (10 mg/kg) five times per week.

2. *In vitro* Bronchial Epithelial Cell Stimulation with TGF-β1

Human bronchial epithelial BEAS-2B cells were grown and maintained in 6-well plates using bronchoepithelial basal medium (Lonza). After 24 hours of serum starvation, cells were treated with alirocumab (10 μg/mL) or atorvastatin (20 μM) for 24 hours, followed by a 10-minute stimulation with TGF-β1 (10 ng/mL). Real-time PCR analysis was conducted.

3. Measurement of AHR

AHR was assessed using the FlexiVent system (SCIREQ, Montreal, QC, Canada). To determine baseline airway resistance (Rrs), mice first received a 3-minute inhalation of nebulized phosphate-buffered saline. They then underwent sequential nebulized methacholine (MCh; Sigma-Aldrich, St. Louis, MO, USA) challenges at 6.25, 12.5, 25, 50, and 100 mg/mL, delivered by an ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA). Each test lasted for 3 min, and average Rrs values were calculated for each MCh concentration.

4. Bronchoalveolar lavage

Before tracheostomy, mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharma Co., Seoul, Korea). A silicone tube was then inserted into the trachea using a 23-gauge needle and connected to an 800 μL tuberculin syringe, delivering 1 mL of Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA) into the lungs. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 10,000 rpm for 3 minutes at 4 °C, and the supernatant was stored at −70 °C. The cell pellet was resuspended in HBSS, and cell smears were made via cytocentrifugation (Thermo Scientific Cytospin 3, Marshall Scientific, Hampton, NH, USA) followed by Diff-Quick staining (Sysmax, Kobe, Japan). Finally, the percentages of macrophages, eosinophils, lymphocytes, and neutrophils were determined by counting 500 leukocytes in randomly selected microscopic fields.

5. Serum biochemical assays

After clot formation and centrifugation, serum was collected and analyzed for cholesterol, triglycerides, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) using an automated clinical chemistry analyzer (Dri-Chem 4000i, Fujifilm, Japan).

6. Enzyme-linked immunosorbent assays (ELISAs)

For cytokine quantification, 100 mg of the right lung tissue was homogenized in RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA) using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA). After a 30-minute incubation on ice, the homogenate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered through a 0.45-μm membrane (Gelman Science, Ann Arbor, MI, USA) and stored at −80 °C. Cytokine, angiotensin II, and angiotensin II receptor type 1 levels were measured and normalized to tissue weight. Commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-17a, TGF-β1, TNF-α, and leptin in lung homogenate and serum.

Oxidative stress was assessed by measuring malondialdehyde (MDA) in lung homogenates and serum using an ELISA (DoGenBio, Seoul, Korea). In addition, cholecystokinin (CCK) was detected with a CCK Enzyme Immunoassay Kit (Biorbyt, Durham, NC, USA), and free fatty acids (FFAs) were quantified with an FFA Assay Kit (DoGenBio). All procedures were performed in accordance with the manufacturers' protocols.

7. RNA purification, reverse transcription, and real-time PCR amplification

Total RNA was isolated from lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was then synthesized according to the manufacturer's instructions using an RNA-to-cDNA EcoDry premix kit (Takara Bio, Kusatsu, Japan). Quantitative RT-PCR was performed with a Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on a StepOnePlus™ PCR System (Applied Biosystems). Target gene expression was normalized to β-actin.

Table 1. Sequences of quantitative RT-PCR primers

Genes	Forward primers sequence (5'→3')	Reverse primers sequence (5'→3')
Collagen 1_Ms	TGGGATTCCCTGGACCTAA	GCTCCAGCTTCTCCATCTTT
Collagen 3_Ms	ATCTGAGGGCTCGCCCGGT	CAATGGCAGCACCGCCACCA
Fibronectin_Ms	TCAGAAGAGTGAGCCCCTGA	GGAAGGGTAACCAGTTGGGG
NLRP3_Ms	GCTGCTGAAGATGACGAGTG	TTTCTCGGGCGGGTAATCTT
Caspase-1_Ms	TCATTTCCGCGGTTGAATCC	CCAACAGGGCGTGAATACAG
ASC_Ms	ATGCCAACCAAAGCCAGAAG	CCTTGGGGTTGGAGAGATGA
IL-1β_Ms	GGCTCATCTGGGATCCTCTC	TCATCTTTTGGGGTCCGTCA
β-actin_Ms	CGCCACCAGTTCGCCATGGA	TACAGCCCGGGGAGCATCGT
GAPDH_Hm	CACATCGCTCAGACACCATG	TGACGGTGCCATGGAATTTG
NLRP3_Hm	TCTCATGCTGCCTGTTCTCA	CAAGGAGATGTCGAAGCAGC
Caspase-1_Hm	CCGAGCTTTGATTGACTCCG	TTCTGAGCCTGAGGATGTGG
IL-1β_Hm	GGAGAATGACCTGAGCACCT	GGAGGTGGAGAGCTTTCAGT

(Ms: mouse, Hm: human).

8. Histopathology and immunohistochemistry

Lung tissues from the left lobe were fixed via perfusion with 4% paraformaldehyde and then processed for paraffin embedding. To evaluate inflammatory changes, sections were stained with hematoxylin and eosin (H&E), whereas periodic acid–Schiff (PAS) staining was performed to detect goblet cell hyperplasia and submucosal gland enlargement. Fibrosis was assessed by Masson's trichrome (MT) staining. All slides were examined with an Olympus BX40 microscope (BX53F, Center Valley, PA, USA) outfitted with an Olympus U-TV0.63XC digital camera, and images were acquired using the cellSens Standard 1.6 software. The fibrotic area was quantified by measuring the color-pixel density above a predefined threshold in regions containing multiple bronchial tubes at 200× magnification, using ImageJ.

For immunohistochemical detection of NLRP3 and caspase-1paraffin-embedded lung sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed by autoclaving in citrate buffer (pH 6.0) at 120 °C for 15 minutes. Sections were then treated with 3% hydrogen peroxide for 15 minutes to block endogenous catalase activity. Next, they were incubated overnight at 4 °C with antibodies against NLRP3 (1:100, SAB, USA), caspase-1 (1:200, Abcam, USA), and α-smooth muscle actin (α-SMA, 1:100, Abcam). Visualization was achieved via streptavidin-horseradish peroxidase, and the percentage of positive staining was measured using ImageJ software.

9. Statistical analysis

Data analysis was conducted using Prism (GraphPad Inc., San Diego, CA, USA). Differences in airway hyperresponsiveness (AHR) and body weight were evaluated by repeated-measures ANOVA with a Bonferroni post hoc test. Other parameters were compared by one-way ANOVA with Bonferroni correction. A P-value < 0.05 was considered statistically significant.

III. RESULTS

1. PCSK9 inhibition or statin administration induced weight loss and altered serum biochemical marker levels in HFD-induced obese mice

Compared with a standard chow diet, HFD led to significant weight gain (Fig. 3A). In HFD-fed mice, treatment with 10 mg/kg alirocumab ($P = 0.005$) or statins ($P = 0.024$) reduced body weight, whereas a lower 3 mg/kg dose of alirocumab produced no change.

The HFD significantly increased serum cholesterol ($P < 0.001$), triglycerides ($P = 0.001$), and glucose ($P < 0.001$). Administration of 10 mg/kg alirocumab lowered these parameters to levels comparable with the ND group, while the 3 mg/kg dose had no effect (Fig. 3B–3D). Although statin therapy modestly lowered overall lipid concentrations while markedly reducing serum glucose ($P < 0.001$), alirocumab produced a significantly greater reduction in triglycerides than statins ($P < 0.001$).

Moreover, the HFD markedly elevated ALT levels ($P < 0.001$); 10 mg/kg alirocumab reduced ALT to ND-group values, whereas 3 mg/kg alirocumab had no effect (Fig. 4A). By contrast, AST and ALP levels were unchanged in all HFD groups, irrespective of alirocumab or statin treatment (Fig. 4B– 4C).

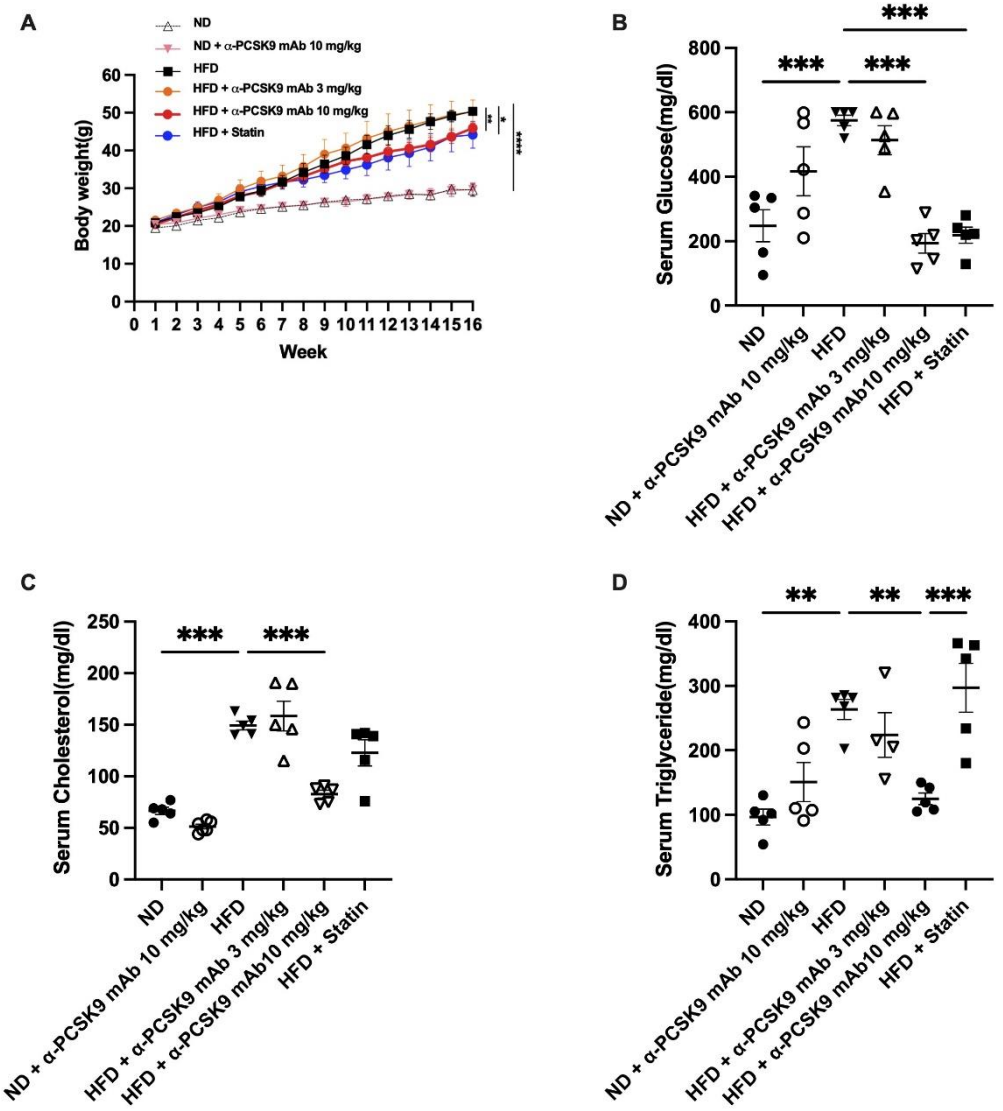

Figure 3. Effects of PCSK9 inhibition or statin administration on body weight and serum biochemical marker levels in HFD-induced obese mice. (A) Body weight in mice on the ND or HFD. (B) Glucose, (C) Cholesterol, (D) Triglyceride levels were measured in serum. The results are expressed as the mean±SEM (n=5 per group). Statistical analysis of body weight changes was performed using repeated-measures ANOVA, and other analyses were performed with one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$. ND: normal diet; HFD: high-fat diet.

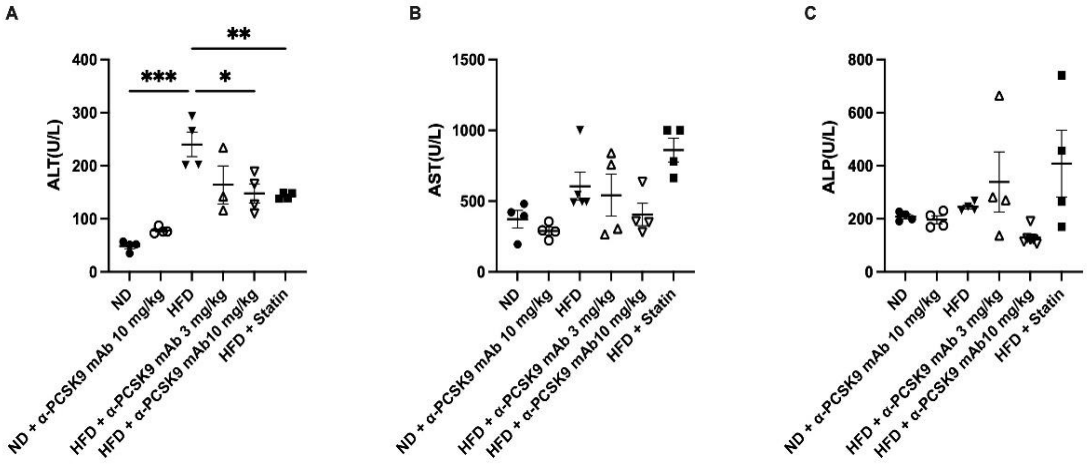

Figure 4. Effects of PCSK9 inhibition or statin administration on liver function status in HFD-induced obese mice. (A) Alanine aminotransferase (ALT), (B) Aspartate aminotransferase (AST), and (C) Alkaline phosphatase (ALP) levels were measured in serum. The results are expressed as the mean±SEM (n=5 per group). Statistical analysis of body weight changes was performed using repeated-measures ANOVA, and other analyses were performed with one-way ANOVA with Bonferroni correction. *: P<0.05, **: P<0.01, and ***: P<0.001. ND: normal diet; HFD: high-fat diet.

2. PCSK9 inhibition or statin administration suppressed AHR and monocytosis in BALF on the HFD mice

AHR was significantly higher in HFD-fed mice than in those on a ND ($P = 0.005$). In HFD-fed mice, AHR was significantly reduced by treatment with alirocumab at 3 mg/kg ($P = 0.018$) or 10 mg/kg ($P = 0.003$), as well as by statins ($P = 0.022$) (Fig. 5A). Moreover, total cell counts and macrophage numbers in bronchoalveolar lavage fluid (BALF) were significantly elevated in HFD-fed mice ($P < 0.001$ for both) compared with ND-fed controls, whereas eosinophil, neutrophil, and lymphocyte levels were unchanged. In addition, administering PCSK9 inhibitor or statin markedly decreased total cellularity and macrophage counts in the BALF of HFD-fed mice (Fig. 5B).

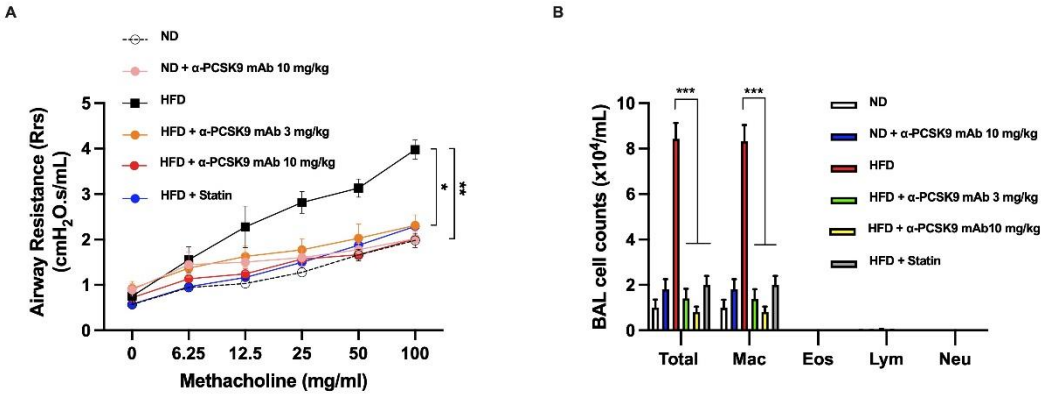

Figure 5. Effects of PCSK9 inhibition or statin administration on AHR and airway inflammation in HFD-induced obese mice. (A) AHR was measured as the Rrs at 24 h after the final treatment. (B) Effect of the HFD on cell counts in BALF. Mice were sacrificed 24 h after the final treatment, and BALF cells were isolated. The results are expressed as the mean±SEM (n=5 per group). Statistical analysis of AHR was performed using repeated-measures ANOVA, and other analyses were performed using one-way ANOVA with Bonferroni correction. *: P<0.05, **: P<0.01, and ***: P<0.001

3. PCSK9 inhibition or statin administration suppressed systemic pro-inflammatory mediators in the lungs of HFD mice

Compared with mice on a ND, those fed HFD showed markedly elevated levels of IL-1β, IL-6, IL-17a, IL-18, TGF-β, and TNF-α in the lungs, as well as higher serum leptin. Treatment with 10 mg/kg alirocumab significantly reduced these inflammatory markers (Fig. 6A–6G). Similarly, 3 mg/kg alirocumab lowered most cytokines except TNF-α ($p=0.100$) and serum leptin ($p=0.050$). Statin therapy also attenuated these parameters, although IL-17a ($p=0.070$) and TNF-α ($p=0.460$) were not different significantly.

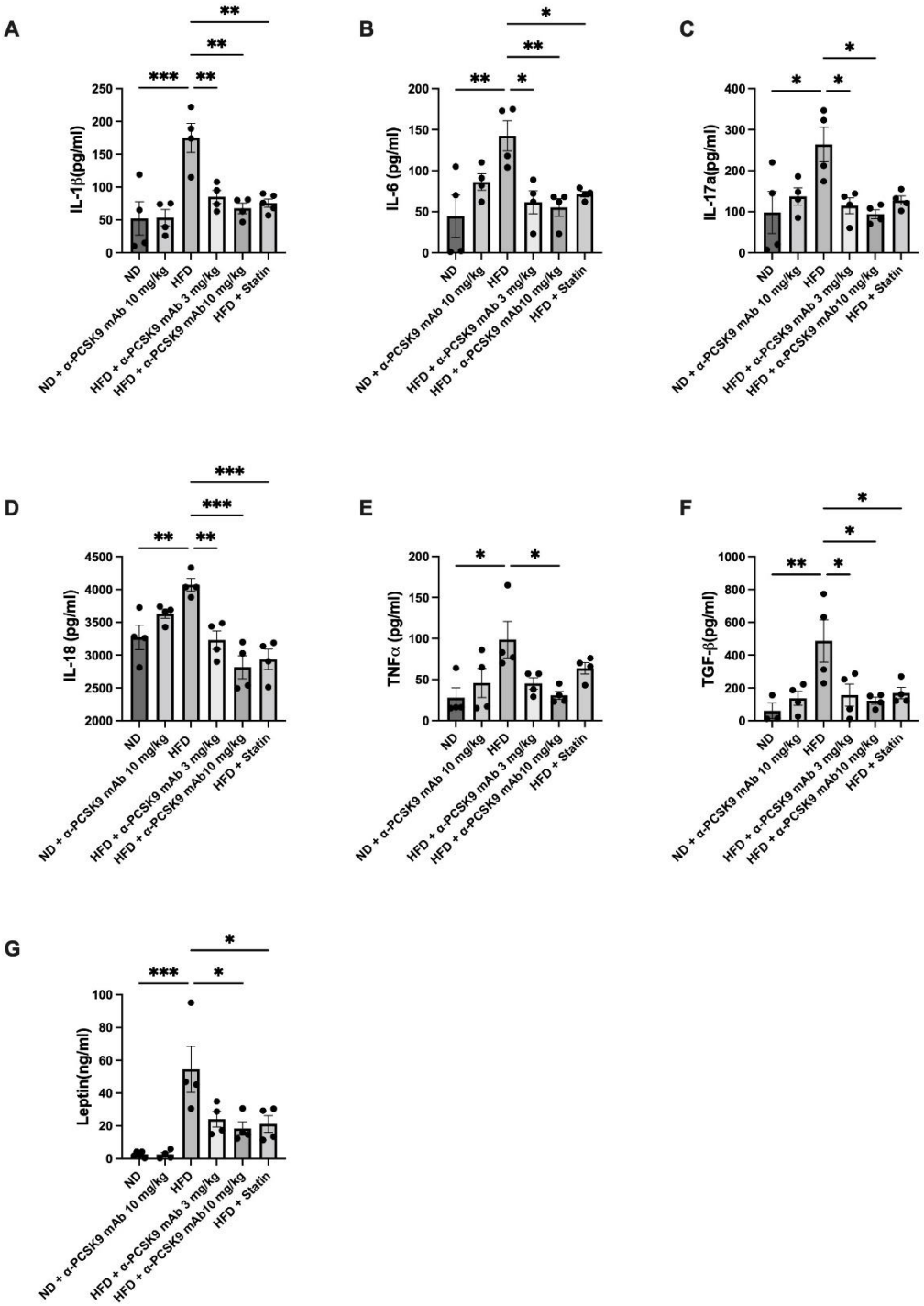

Figure 6. Effects of PCSK9 inhibition or statin administration on systemic pro-inflammatory mediators in HFD-induced obese mice. (A–G) IL-1β (A), IL-6 (B), IL-17a (C), IL-18 (D), TNF-α (E), and TGF-β (F) levels in lung homogenates and leptin (G) levels in serum were evaluated by ELISAs. The results are expressed as the mean±SEM (n=4 per group). Statistical analysis of AHR was performed using repeated-measures ANOVA, and other analyses were performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$.

4. PCSK9 inhibition or statin administration attenuated fibrosis in HFD mouse lungs

Histopathological assessment revealed no eosinophilic inflammation or goblet cell hyperplasia in the HFD group compared with ND controls (Fig. 7A), suggesting that neither alirocumab nor statin influenced these features. However, Masson's trichrome staining showed increased peribronchial and perivascular fibrosis in HFD-fed mice, which was substantially reduced by either alirocumab or statin administration (Fig. 7A–7B). Similarly, α-SMA immunostaining indicated heightened peribronchial and perivascular expression in HFD mice, effectively attenuated by both 3 and 10 mg/kg alirocumab, as well as by statin treatment (Fig. 7A,7C).

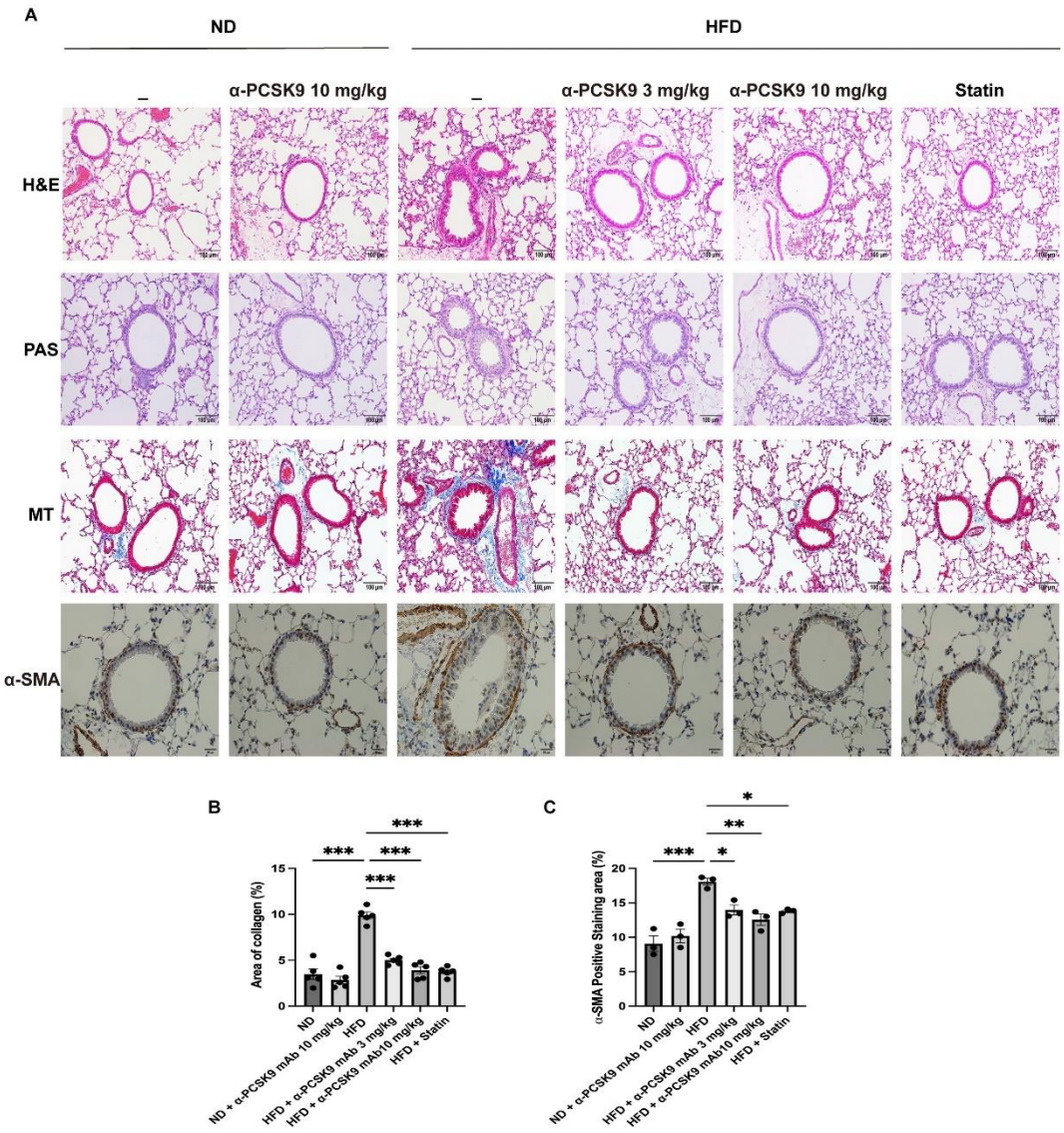

Figure 7. Effects of PCSK9 inhibition or statin administration on fibrosis in HFD mouse lungs. (A) Paraffin-embedded lung tissue sections underwent H&E, PAS, MT (original magnification: 100×), and α-SMA IHS (original magnification: 50×) staining. (B-C) Quantitative analyses of the fibrosis and peribronchial α-SMA-positive staining areas were performed using an image analysis system. The results are expressed as the mean±SEM (n=5 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: P<0.05, **: P<0.01, and ***: P<0.001. EMT: epithelial–mesenchymal transition, H&E: hematoxylin and eosin; IHS: immunohistochemical staining; MT: Masson-trichrome; PAS: periodic acid–Schiff; α-SMA: alpha-smooth muscle actin.

5. PCSK9 inhibition or statin administration attenuated epithelial-mesenchymal transition (EMT) markers in HFD mouse lungs

To support the histological observations, we measured mRNA expression of collagen I, collagen III, and fibronectin in lung homogenates (Fig. 8A–8C). The HFD significantly upregulated collagen I ($P = 0.002$), collagen III ($P = 0.030$), and fibronectin ($P < 0.001$). Administration of alirocumab at both 3 and 10 mg/kg or statin consistently lowered these EMT markers.

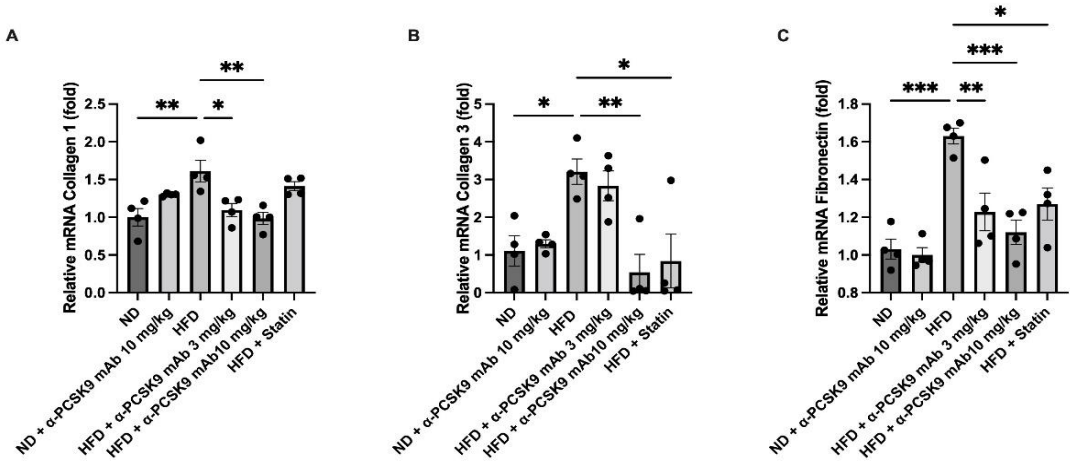

Figure 8. Effects of PCSK9 inhibition or statin administration on EMT markers in HFD mouse lungs. Quantification of EMT markers in the lungs was performed using mRNA expression of collagen 1 (A), collagen 3 (B), and fibronectin (C). The results are expressed as the mean±SEM (n=4 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: P<0.05, **: P<0.01, and ***: P<0.001. EMT: epithelial–mesenchymal transition.

6. PCSK9 inhibition or statin administration suppressed RAS activation in HFD mouse lungs

RAS fulfills multiple functions in adipose tissue [23]. Its key effector, angiotensin II, is associated with adipose expansion and insulin resistance, which can lead to activation of NADPH oxidase and production of ROS.[8-10] Compared with ND controls, HFD-fed mice showed significantly higher levels of angiotensin II ($P = 0.003$) and angiotensin II receptor type 1 ($P = 0.001$). Notably, alirocumab at 3 or 10 mg/kg effectively reduced these elevations, whereas statin therapy had a milder effect on RAS activity in HFD mice (Fig. 9A–9B).

In addition, the oxidative stress marker malondialdehyde (MDA) was markedly increased in both serum ($P = 0.008$) and lung homogenates ($P = 0.003$) of HFD-fed mice compared with ND controls. Administration of either alirocumab (3 or 10 mg/kg) or statins significantly lowered MDA levels both locally and systemically (Fig. 9C–9D).

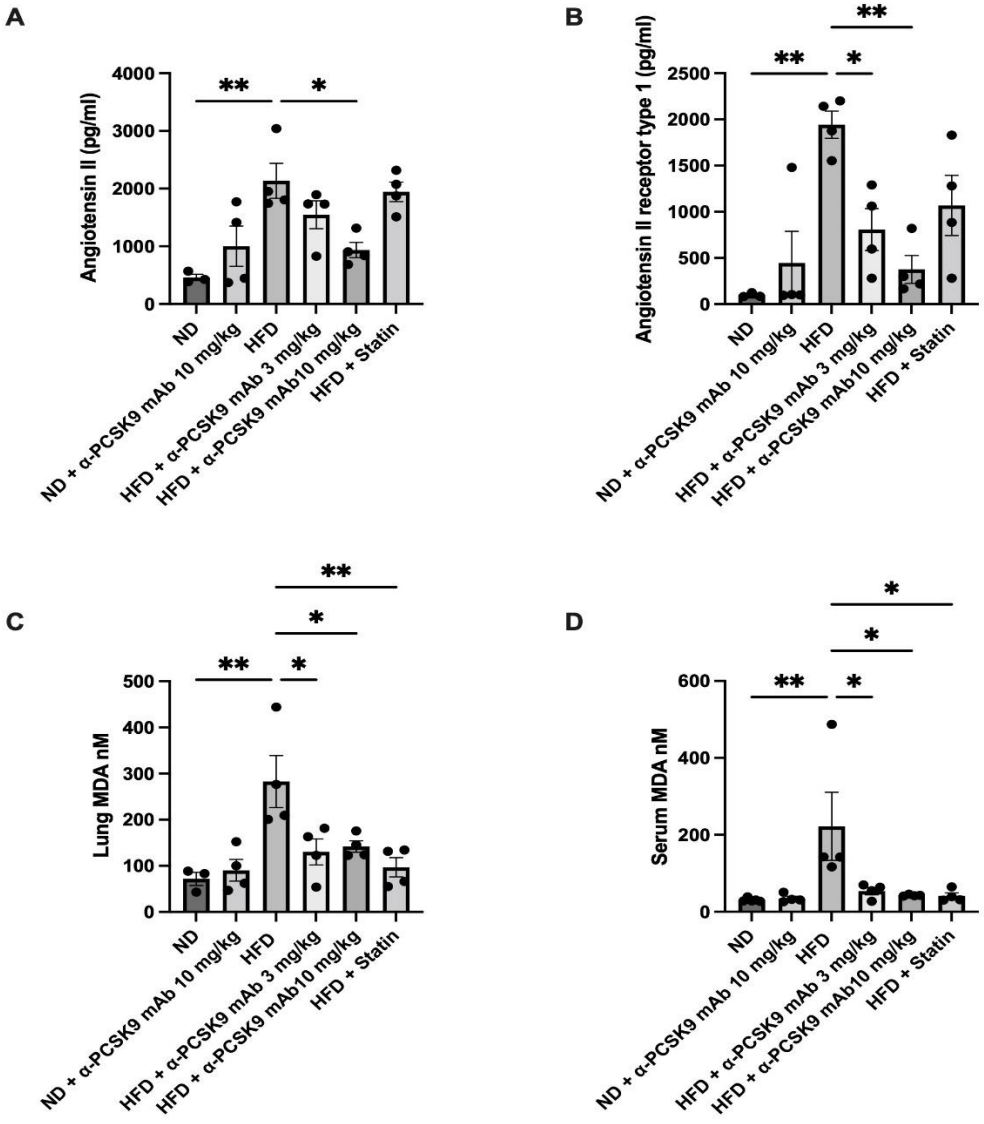

Figure 9. Effects of PCSK9 inhibition or statin administration on RAS activation in HFD mouse lungs. Protein expression of angiotensin II (A), angiotensin II receptor type 1 (B), and MDA levels (C) in lung homogenates and MDA (D) in serum were measured by ELISAs. The results are expressed as the mean±SEM (n=4 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$. MDA: malondialdehyde.

7. PCSK9 inhibition or statin administration decreased CCK expression and FFA levels in HFD mouse lungs

Obesity is often accompanied by elevated free fatty acids (FFAs), which can trigger airway hyperresponsiveness [24, 25]. Earlier studies have also shown that fatty acids can upregulate cholecystokinin (CCK) expression in airway smooth muscle cells. In our study, both serum and lung tissues from HFD-fed mice exhibited higher FFA and CCK levels than those from ND controls. Notably, administering alirocumab (3 or 10 mg/kg) or statins significantly lowered FFA and CCK levels in the lungs (Fig. 10A–10D).

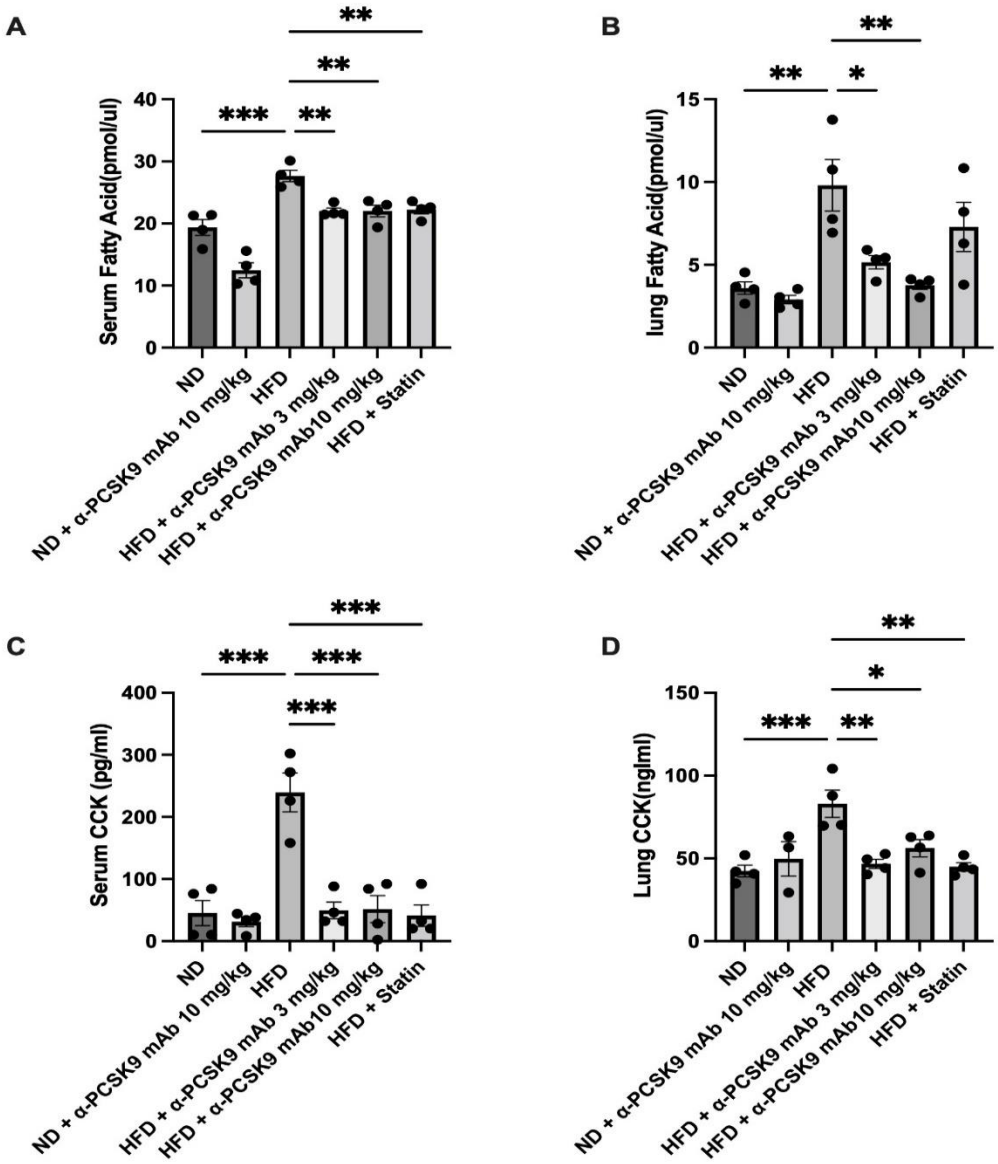

Figure 10. Effects of PCSK9 inhibition or statin administration on CCK expression and FFA levels in HFD mouse lungs. FFA (A–B) and CCK (C–D) protein expression in both lungs and serum were measured by ELISAs. The results are expressed as the mean±SEM (n=4 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$. FFA: free fatty acid, CCK: cholecystokinin.

8. PCSK9 inhibition or statin administration decreased NLRP3 inflammasome activity in HFD mouse lungs

Previous evidence indicates that NLRP3 inflammasome activation depends on cholesterol levels [13]. Moreover, NLRP3 is an important contributor of ROS production induced by angiotensin II and TGF-β in mouse fibroblasts, thereby contributing to fibrosis[11]. Assuming that HFD upregulates NLRP3 inflammasome activity in the lungs, we tested whether PCSK9 inhibition or statin therapy could suppress this inflammatory cascade. Immunohistochemical staining showed that NLRP3 and caspase-1 were both elevated in the respiratory epithelium of HFD-fed mice; treatment with alirocumab (3 or 10 mg/kg) or statins significantly reduced their expression (Fig. 11A–11C).

In line with these findings, quantitative real-time PCR revealed significantly higher lung mRNA levels of NLRP3 ($P < 0.001$), caspase-1 ($P = 0.004$), ASC ($P < 0.001$), and IL-1β ($P < 0.001$) in HFD-fed mice. Both statin and alirocumab therapies markedly attenuated these increases (Fig. 11D–11G).

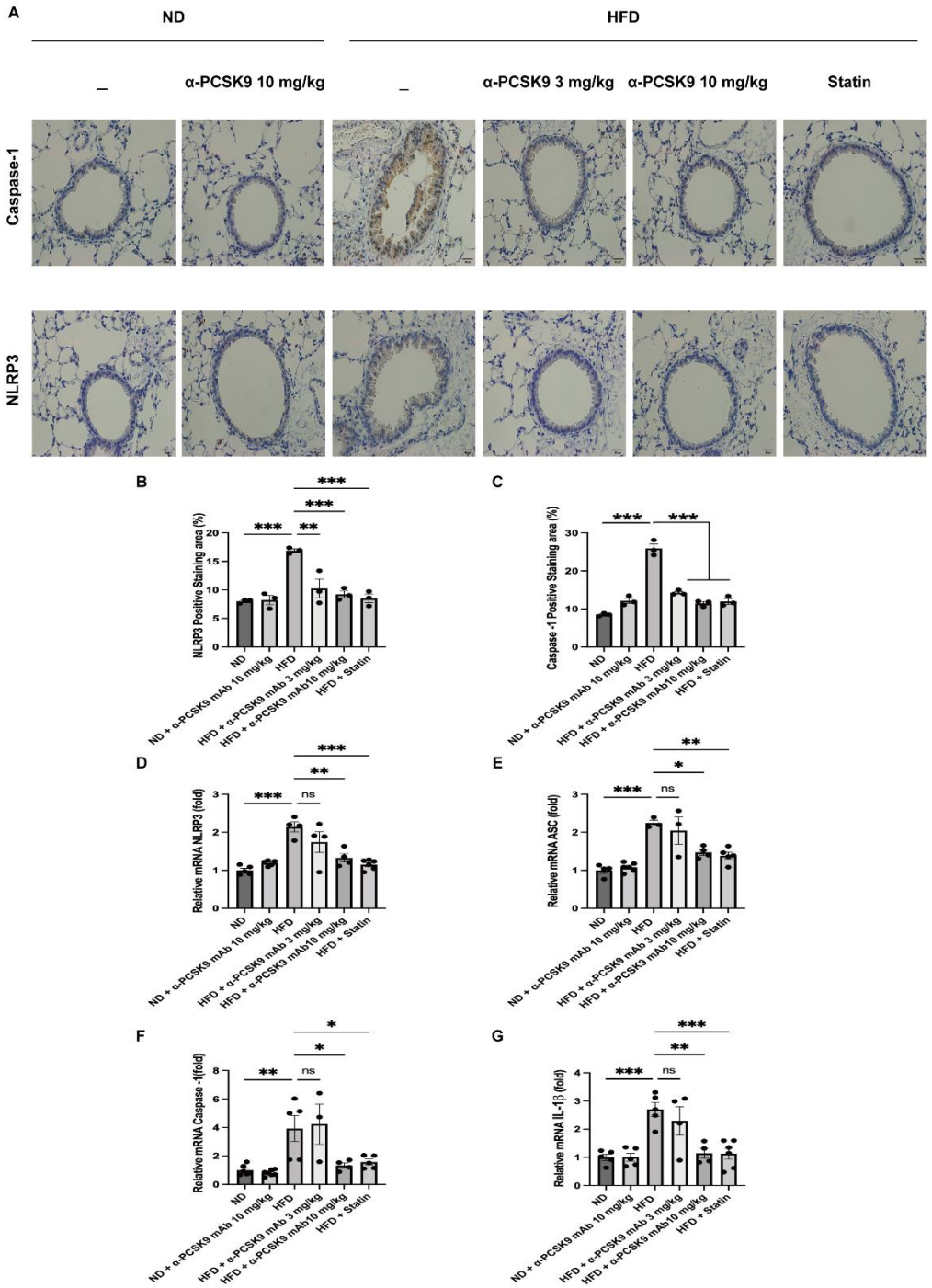

Figure 11. Effects of PCSK9 inhibition or statin administration on NLRP3 activity in HFD mouse lungs. Representative photomicrographs of caspase-1- and NLRP3- positive areas (A) in lung sections from mice of the different treatment groups are shown (50×). Quantification of NLRP3- (B) and caspase-1-positive (C) areas was performed using an image analysis system. Quantitative RT-PCR measurement of NLRP3 (D), ASC (E), caspase-1 (F), and IL-1β (G) mRNA expression in the lungs is shown. The results are expressed as the mean±SEM (n=4-6 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$.

9. PCSK9 inhibition or statin administration decreased NLRP3 production of respiratory epithelium in the BEAS-2B cells

Multiple studies show that TGF-β1 upregulates NLRP3 expression, ultimately promoting fibrosis in major organs [26, 27]. In our experiment, BEAS-2B cells were stimulated with TGF-β1, and mRNA levels of NLRP3 and related mediators were quantified. TGF-β1 significantly elevated NLRP3 ($P < 0.01$), caspase-1 ($P < 0.001$), and IL-1β ($P < 0.001$). Alirocumab treatment markedly decreased NLRP3 ($P = 0.02$), caspase-1 ($P < 0.001$), and IL-1β ($P = 0.001$). Although statin therapy reduced both NLRP3 and caspase-1, it unexpectedly led to higher IL-1β levels (Fig. 12A–12C).

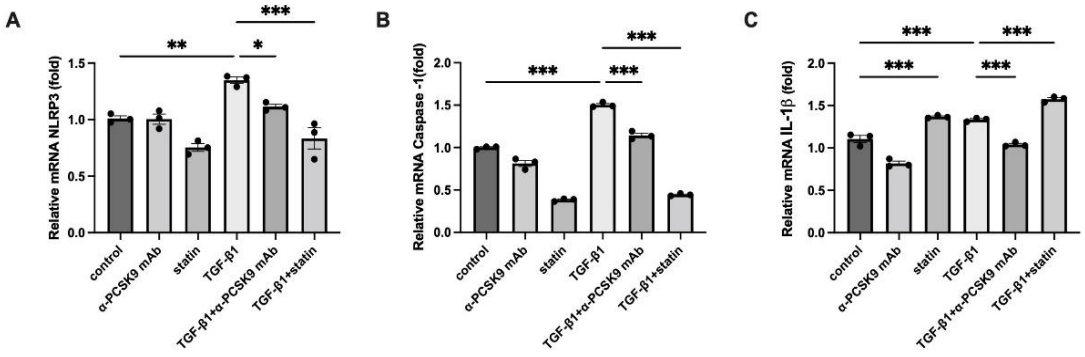

Figure 12. Effects of PCSK9 inhibition or statin administration on the NLRP3 inflammasome activity in respiratory epithelial cells stimulated by TGF-β1. The mRNA expression of NLRP3 (A), caspase-1 (B) and IL-1β (C) are shown. The respiratory epithelium was stimulated with 10 ng/ml of TGF-β1. Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$.

10. PCSK9 inhibition or statin administration decreased NLRP3 production in TGF-β1 overexpressing transgenic mice

TGF-β/Smad signaling stimulates NLRP3 production and phosphorylates Smad2/3 [28]. Moreover, our previous work showed that HFD–induced obesity greatly activates TGF-β1 signaling [3]. Furthermore, TGF-β1 enhanced NLRP3 expression in BEAS-2B cells. Based on these findings, we examined whether alirocumab or statin treatment could reduce TGF-β1 expression through NLRP3 attenuation, and mitigate lung fibrosis in TGF-β1 transgenic mice. In these mice, TGF-β1 overexpression coincided with elevated mRNA levels of NLRP3 ($P < 0.001$), caspase-1 ($P < 0.001$), ASC ($P < 0.01$), and IL-1β ($P < 0.01$). Treatment with alirocumab or statins significantly lowered these inflammatory markers (Fig. 13A–13D).

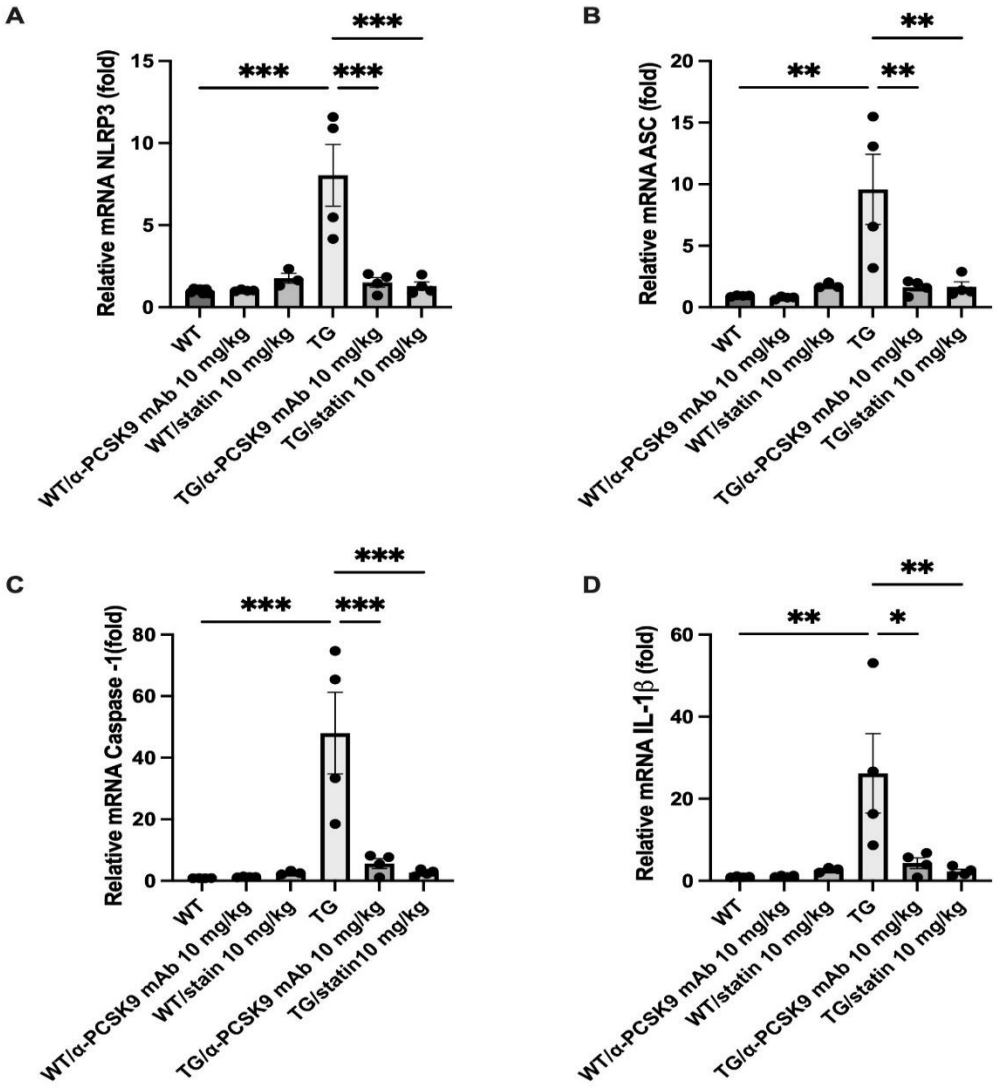

Figure 13. Effects of PCSK9 inhibition or statin administration on NLRP3 activity in TGF-β1 overexpressing transgenic mice. Quantitative RT-PCR measurement of NLRP3 (A), ASC (B), caspase-1 (C), and IL-1β (D) mRNA expression in the lungs is shown. The results are expressed as the mean±SEM (n=4 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: P<0.05, **: P<0.01, and ***: P<0.001. DC: doxycycline; WT: wild-type.

11. PCSK9 inhibition or statin administration improved lung fibrosis and epithelial-mesenchymal transition (EMT) markers in TGF-β1 overexpressing transgenic mice

Masson's trichrome staining showed that TGF-β1–overexpressing mice developed marked peribronchial and perivascular fibrosis. However, treatment with alirocumab or statins significantly alleviated this fibrotic change (Fig. 14A–14B). Furthermore, mRNA levels of EMT markers—collagen I, collagen III, and fibronectin—were substantially reduced following alirocumab or statin therapy (Fig. 14C–14E).

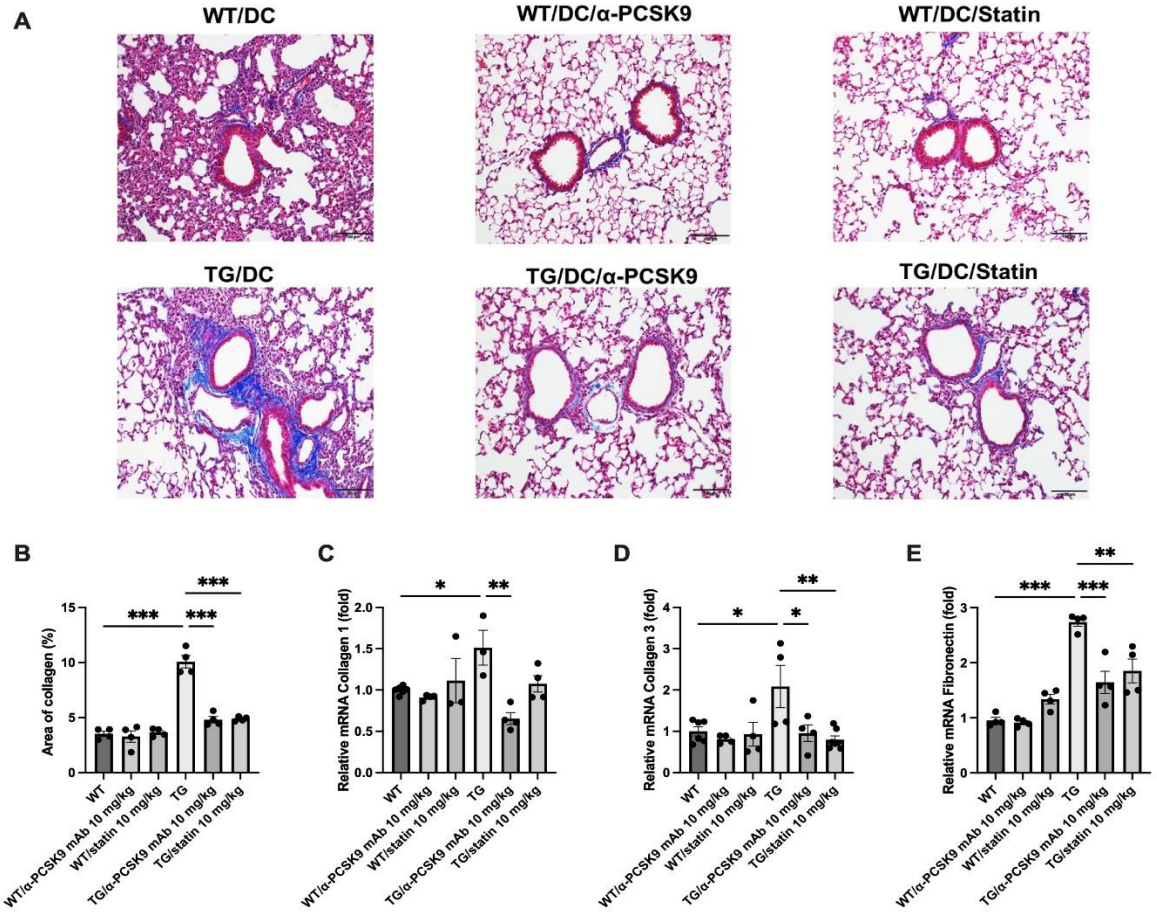

Figure 14. Effects of PCSK9 inhibition or statin administration on fibrosis and EMT markers in TGF-β1 overexpressing transgenic mice. (A) MT staining also showed inhibition of peribronchial and perivascular fibrosis by PCSK9 inhibition or statin administration in transgenic mice (original magnification: 100×). (B) Quantitative analyses of the fibrosis area were performed using an image analysis system. Quantification of EMT markers in the lungs was performed using mRNA expression of collagen 1 (C), collagen 3 (D), and fibronectin (E). The results are expressed as the mean±SEM (n=4-6 per group). Statistical analysis was performed using one-way ANOVA with Bonferroni correction. *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$. DC: doxycycline; WT: wild-type

IV. DISCUSSION

Obesity is a common feature in patients with severe asthma and is associated with decreased lung function, poor responsiveness to β-adrenergic agonists and corticosteroids, and an increasing rate of lung function decline over time [2]. Moreover, obesity in the absence of allergic sensitization can cause severe asthma with Th2 low, non-eosinophilic characteristics [29, 30]. These effects have been attributed to a variety of pathways, including mechanistic impairment of lung elastance, metabolic dysfunctions, and activation of innate immunity, especially pro-inflammatory cytokines [30, 31]. Therefore, a pressing need exists for the development of effective therapeutic strategies for patients with asthma and obesity.

Our study highlights the potential usefulness of the cholesterol-lowering drug alirocumab and statins in treating patients with asthma and obesity. Alirocumab is a new type of lipid-lowering drug that inhibits PCSK9, a liver enzyme that degrades LDL receptors on the surface of liver cells [32]. This is the first study to demonstrate that alirocumab can improve AHR and fibrosis in an HFD-induced obesity model. Statins have been reported to be effective for asthma treatment using an in vivo model and in a retrospective clinical study [19, 20]. However, the impact of these drugs on therapy for patients with asthma with obesity remains unknown [21]. Obesity is commonly linked to elevated levels of systemic pro-inflammatory mediators such as C-reactive protein, TNF-α, TGF-β1, leptin, and IL-6 [29, 33], and we have shown that suppression of TNF-α and TGF-β1 improved AHR and fibrosis using the same HFD-induced obesity model [3, 33]. Factors such as lipopolysaccharide, oxidized LDL, TNF-α, and IL-1β have been reported to induce PCSK9 secretion in various organs, which in turn induces hyperlipidemia, atherosclerosis, diabetes, and hypertension [17]. Several investigators showed that intracellular accumulation of cholesterol or intracellular or extracellular cholesterol crystal deposition activates the NLRP3 inflammasome in myeloid cells and macrophages and then plays a key role in producing inflammatory lesions in atherosclerosis plaques [13, 34]. In our HFD-induced model, only macrophages were increased in BALF and were reduced by both lipid-lowering agents. These findings indicate that the anti-inflammatory properties of these agents may involve the suppression of NLRP3 activity in macrophages. Previously, we showed that depletion of lung macrophages attenuated AHR in an HFD-induced obesity model [33], supporting the crucial role of lung macrophages in AHR development. However, obesity is a systemic disease, and pro-inflammatory mediators were also increased in the serum. The precise contribution of

inflammation, including systemic inflammation, local lung inflammation, or a combination of both, to the development of lung fibrosis and AHR remains uncertain.

Several clinical studies have indicated a significant correlation between serum levels of PCSK9 and pro-inflammatory cytokines including IL-6, IL-1β, TNF-α, and hsCRP [35-37]. TLRs and the NLRP3 inflammasome are activated in the intermediary steps, leading to PCSK9 production and release [38]. We also revealed a decrease in systemic pro-inflammatory mediators including TNF-α, TGF-β, leptin, IL-6, and IL-17a associated with obesity upon treatment with lipid-lowering agents. Nonetheless, given obesity's broad systemic impact and elevated serum pro-inflammatory markers, it remains uncertain whether lung fibrosis and AHR primarily arise from systemic influences, local pulmonary processes, or both.

Moreover, administering 10 mg/kg of alirocumab or a statin produced a modest but statistically significant reduction in body weight relative to HFD-fed controls. It should be noted that the observed improvement in AHR could partly stem from mechanical alterations induced by weight loss. Indeed, previous research has linked obesity-related biomechanical changes, reduced lung function, and heightened AHR—implying that this relationship extends beyond airway inflammation [35]. Nonetheless, we propose that the minor weight decrease seen in HFD mice treated with alirocumab or statins was insufficient to cause substantial mechanical shifts in this study.

Two interactive pathways, the NLRP3 inflammasome and the RAS, synergistically initiate a cascade that fosters chronic inflammation, tissue damage, and ultimately fibrosis [39]. Angiotensin II, a principal effector of RAS, induces ROS, thereby activating the NLRP3 inflammasome. Once the NLRP3 inflammasome is activated by ROS, a downstream cascade triggers TGF-β secretion from mouse cardiac fibroblasts [40]. Our previous findings indicate that obesity induced by HFD substantially upregulates RAS activity and insulin resistance, thereby intensifying TGF-β1 signaling and promoting lung fibrosis in mice [3]. Building on these observations, our current study suggests that pharmacologically reducing specific biochemical mediators—through statins or anti-PCSK9 agents—can attenuate inflammasome activation and potentially improve pulmonary function. Our findings are consistent with a recent report demonstrating that direct inhibition of NLRP3 by MCC950, a selective NATCH domain inhibitor, significantly attenuates AHR and inflammatory cell infiltration in obesity-induced models [41]. Targeting these interrelated pathways therefore presents promising opportunities to develop therapeutic strategies that could decelerate or even halt the progression of lung fibrosis. Further

evidence from an animal study indicates that selective PCSK9 deletion in cardiomyocytes suppresses NLRP3 inflammasome activity [18]. In our study, lipid-lowering therapy inhibited collagen I, collagen III, fibronectin mRNA, and smooth muscle actin (SMA) protein expression. Accordingly, we propose that alirocumab or statin treatment can prevent lung fibrosis driven by excessive TGF-β/Smad signaling.

Remarkably, our *in vitro* experiments using respiratory epithelial cells demonstrated that TGF-β1 significantly enhances NLRP3 expression downstream, suggesting autocrine activation of TGF-β1 within the NLRP3 signaling pathway in the obesity model; importantly, this amplification was effectively attenuated by PCSK9 inhibition or statin treatment. These observations were further corroborated by studies employing conditional transgenic mice overexpressing TGF-β1, which exhibited pronounced pulmonary fibrosis along with elevated mRNA levels of NLRP3, caspase-1, ASC, and IL-1β, despite no significant change in body weight. However, these elevations declined upon the administration of alirocumab or statins. Consequently, we suggest that alirocumab or statin therapy could protect against lung fibrosis by suppressing pro-inflammatory cytokines, inhibiting RAS and the NLRP3 inflammasome, and downregulating TGF-β1 signaling—even in the absence of obesity.

Nevertheless, this study had certain limitations, particularly its focus on the NLRP3 inflammasome and associated downstream pathways in TGF-β1–overexpressing transgenic mice. Future studies using this model should investigate the precise mechanisms by which TGF-β influences both the initial priming phase and the secondary activation of the NLRP3 inflammasome.

Elevation of plasma FFAs is well known in patients with obesity and is due to the increased release of FFAs from adipose tissue or impaired clearance mechanisms [42]. FFAs can induce mitochondrial ROS through NLRP3 inflammasome activation, which may play a crucial role in inflammation and endothelial dysfunction [43]. Interestingly, one study showed an autocrine stimulatory loop of CCK-activated, CCKA receptor -mediated airway smooth muscle contraction, which could be enhanced by elevated FFAs in an HFD-induced obesity model [12]. We observed increased FFA and CCK levels in HFD mice, both in the serum and lungs. Furthermore, studies have shown that angiotensin II can increase CCK expression at both the mRNA and protein levels in cardiomyocytes [44]. Therefore, increased lung CCK levels are more likely to result in altered lung function and heightened AHR in the context of obesity. Moreover, our findings demonstrated that both alirocumab and statin administration significantly reduced FFAs and subsequently CCK levels in the lungs and

serum of HFD mice. This suggests that inhibiting the FFAs-CCK pathway via lipid-lowering agents could represent a promising strategy for treating individuals with asthma and obesity.

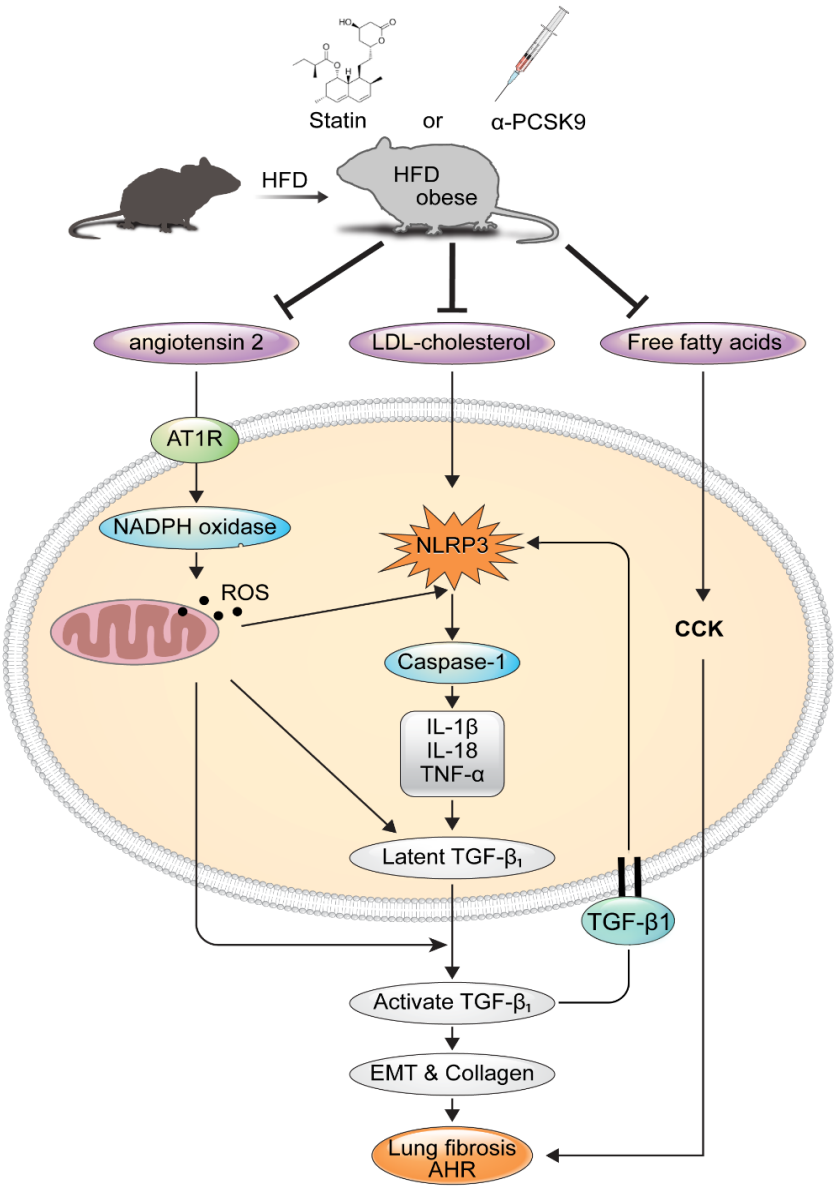

Figure 15. Proposed mechanisms of action for statins and PCSK9 inhibitors in treating obesity-induced lung pathologies.

In summary, our findings demonstrate that obesity induced by HFD is associated with enhanced activity of pro-inflammatory cytokines, the RAS, the NLRP3 inflammasome, and CCK, potentially mediated by elevated levels of cytoplasmic LDL-cholesterol and FFAs. Collectively, these molecular alterations ultimately result in increased TGF-β1 expression, subsequently driving pulmonary fibrosis and AHR. Alirocumab and statins likely exert pleiotropic effects that interfere with these pathological cascades, thereby preventing the progression of pulmonary fibrosis and AHR in the HFD-induced obesity model. Further investigations employing specific inhibitors targeting these molecular pathways are necessary to elucidate the precise underlying mechanisms. Finally, we suggest that lipid-lowering agents may represent a promising therapeutic strategy for patients with obesity-associated asthma who exhibit poor responsiveness to conventional asthma therapies. Nevertheless, validation through large-scale real-world epidemiological studies and long-term randomized clinical trials is required to confirm the clinical efficacy and applicability of this approach.

V. CONCLUSION

Our findings indicate that serum lipid-lowering treatments can attenuate obesity-induced AHR and pulmonary fibrosis via anti-inflammatory mechanisms involving suppression of the RAS, NLRP3 inflammasome activation, and CCK activity. Lipid-lowering strategies may therefore represent a beneficial therapeutic approach for managing asthma in patients with obesity, particularly those exhibiting poor responsiveness to conventional asthma treatments.

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Abstract in Korean

지방식으로 유도된 비만 쥐 모델에서 기도과민반응과 폐섬유화에 대한 statin 또는 PCSK9 저해제의 효과

배경: 비만은 기도과민반응과 폐섬유증을 유발하여, 이는 천식과 비만이 동반된 환자에서 일반적인 천식 치료제에 잘 반응하지 못하게 할 수 있다. 스타틴과 전단백질 전환효소 서브틸리신/케신-9(PCSK9)은 혈청 콜레스테롤, 레닌-안지오텐신 시스템(RAS) 활성, 유리지방산(FFAs)을 감소시키고 항염 효과를 높여, 비만과 관련된 폐 병리학적 변화에 기여할 수 있다.

목적: 본 연구는 고지방 식이(HFD)로 비만을 유도한 생쥐 모델을 이용하여, 스타틴 또는 항-PCSK9 단클론항체가 비만에 의해 유발된 폐 병리 현상을 완화할 수 있는지 평가하고자 하였다.

방법: 수컷 C57BL/6 생쥐에 16주간 HFD를 먹여 비만 모델을 확립하였으며, 트랜스포밍 성장 인자(TGF)-β1 트랜스제닉 생쥐는 일반 식이를 먹였다. 이후, 생쥐에게 스타틴(아토르바스타틴) 또는 PCSK9 억제제(알리로쿠맙)를 투여한 뒤, 기도과민반응과 폐섬유화에 미치는 영향을 분석하였다.

결과: HFD 생쥐는 콜레스테롤, RAS 활성, FFAs 수치가 증가하였으며, 이는 두가지

지질 강하제 모두를 통해 억제되었다. 또한 HFD 비만 모델에서는 기도과민반응, 기관지폐포세척액내 대식세포 증가, 폐섬유증, 상피 전환 마커, NLRP3 인플라마좀, 콜레시스토키닌, IL-1β, IL-6, IL-17a 및 TGF-β1이 모두 상승하였습니다. 아토르바스타틴과 항-PCSK9 단클론항체는 이러한 비만 관련 폐병리 현상과 전염증성 매개체, 콜레시스토키닌, TGF-β1 발현을 완화시켰다. 또한 지질 강하제는 정상 식이를 급여받는 TGF-β1 형질전환 생쥐에서도 염증성 면역 반응과 폐 섬유증을 억제하였다.

결론: 혈청 지질 강하 치료는 NLRP3 인플라마좀, RAS, 콜레시스토키닌 활성을 억제함으로써 비만에 의해 유발된 기도과민반응과 폐섬유증을 완화할 수 있다. 따라서, 일반적인 천식 약물에 대한 반응이 저조한 비만 동반 천식 환자들에게 지질 강하 전략이 도움이 될 것으로 기대된다.

핵심되는 말: 비만, 스타틴, PCSK9, 천식, 알리로쿠맙