Therapeutic Effect of Atorvastatin on Interleukin-13-Induced Lung Pathology

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Abstract

PPurpose: Asthma is a common chronic lung disease, in which interleukin (IL)-13 is implicated as a central regulator of IgE synthesis, mucus hypersecretion, airway hyperresponsiveness (AHR), and fibrosis. This study was designed to determine the anti-inflammatory effect of atorvastatin, a widely used lipid-lowering agent, on IL-13-induced lung pathology through the modulation of macrophages. *Methods:* Atorvastatin (40 mg/kg) was given to transgenic mice overexpressing IL-13 (IL-13 TG mice) and their wild-type littermates by oral gavage for 2 weeks. AHR, the number of inflammatory cells in the airway, and cytokine levels in IL-13 TG mice were measured. Using the alveolar macrophage (AM) cell line CRL-2456, the direct effect of atorvastatin on macrophages activated by recombinant IL-13 was assessed. *Results:* Significant reduction in total leukocytes and alleviation of AHR were observed with the administration of atorvastatin in IL-13 TG mice compared to those without atorvastatin treatment ($P < 0.05$). Atorvastatin administration resulted in the upregulation of IL-10 in the lungs of IL-13 TG mice ($P < 0.05$). In addition, mRNA expression of connective tissue growth factor, fibronectin, and type III collagen as well as chord length enhanced by IL-13 overexpression were reduced by atorvastatin administration ($P < 0.05$). M2 macrophage markers were decreased, while the M1 macrophage marker was increased upon atorvastatin treatment ($P < 0.05$). Administration of atorvastatin resulted in improved removal of apoptotic cells ($P < 0.05$). *Conclusion:* The results of this study reveal the potential of atorvastatin as an effective anti-asthmatic agent by reducing IL-13-induced lung inflammation via the modulation of macrophage polarization.

Keywords

Asthma

Interleukin-13

Atorvastatin

Anti-asthmatic agents

Macrophage

1. Introduction

Asthma is a chronic airway inflammatory disease characterized by airway hyperresponsiveness (AHR) and reversible airway obstruction. Pathophysiologically, infiltration of multiple mast cells, lymphocytes, and eosinophils in the airway wall is commonly observed, which is regulated by cytokines secreted by type 2 helper T cells (Th2) [1,2]. Among them, interleukin (IL)-13 is known to play a key role in the pathophysiology of asthma by inducing airway inflammation, fibrosis, AHR, and mucus hypersecretion. A mouse model with lung-specific overexpression of IL-13 confirmed the characteristic inflammatory response of asthma, including AHR, immune cell infiltration in the lungs, and proliferation of mucus-producing cells, and showed chronic airway remodeling, including fibroblast proliferation and increased collagen in the airways [3,4].

Macrophages are the most abundant cells in the lungs and airways, acting as gatekeepers for foreign antigens and regulating the innate immune response in the airways. The phenotype of macrophages is broadly divided into M1 and M2, and their differentiation is dependent on various stimuli, including Th1 and Th2 cytokines, with Th2 cytokines inducing polarization towards M2 macrophages [5,6]. M2 macrophages regulate homeostasis and inflammation and repair damaged tissue, and have recently been reported to contribute to the development of allergic asthma by participating in eosinophil influx and efferocytosis [7,8]. In addition, Th2 cells are recruited by secreting chemokines such as CCL17 and CCL22, and arginase-1 (Arg-1) and Ym-1, known as M2 markers, affect the pathophysiological symptoms of allergic asthma by inducing AHR, airway obstruction, and eosinophil influx, respectively [9]. Therefore, attempts have been made to apply and interpret the association between macrophages and asthma in clinical trials, but more research is still needed on the mechanisms of anti-asthmatic treatment effects using macrophages [10].

Statins are 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors and are used to treat cardiovascular diseases such as hyperlipidemia by inhibiting cholesterol synthesis in the liver [11]. However, in recent years, statins have been shown to be therapeutic in a variety of diseases in addition to inhibiting cholesterol synthesis, which is likely due to their anti-inflammatory and immunomodulatory effects. *In vitro* studies have shown that simvastatin and pitavastatin reduce the antigen-presenting capacity of dendritic cells, suggesting that they may reduce inflammation by interfering with the function and maturation of antigen-presenting cells [12]. However, the exact mechanisms of the various anti-inflammatory effects of statins and their actions in different diseases are still not fully understood.

Reflecting the characteristics of asthma as a chronic airway disease, this study used an IL-13 overexpression model to determine the anti-asthmatic effects of statins in a chronic asthma model rather than short-term ovalbumin (OVA)-induced acute asthma model and confirmed the anti-asthmatic effects induced by oral administration of atorvastatin. In addition, we analyzed the effect of atorvastatin on AMs activation, which has not been well studied before, to confirm the anti-asthmatic effect of atorvastatin through macrophage modulation.

2. Materials and methods

2.1. Mouse model setup and atorvastatin treatment

Lung-specific IL-13 overexpressing C57BL/6 mice and their littermates were maintained under pathogen-free conditions. Both groups used 6-week-old male mice and were performed under the IACUC approval (SNU-140123-5) of the Seoul National University Laboratory Animal Resource Institute.

Atorvastatin was administered by gavage once every other day for 2 weeks at 40 mg/kg for 6 weeks. The effective dose and number of doses were based on the known pharmacokinetics of statins in the literature [13].

2.2. Methacholine airway hyperresponsiveness measurement

AHR was measured the day after the last dose of atorvastatin, using an OCP3000 (Allmedicus, Korea) machine with increasing methacholine concentrations of 6.25, 12.5, 25, and 50 mg/mL. After inhalation for 3 minutes at each step, the enhanced pause (Penh), an index of AHR, was measured at 30-second intervals for 3 minutes at each concentration.

2.3. Asthma inflammatory response and lung tissue analysis

Bronchoalveolar lavage fluid and lung tissue were obtained 24 hours after the AHR assessment and mice were sacrificed. Mice were anesthetized by intraperitoneal administration of ketamine, and after anesthesia, a catheter was inserted into the airway and 2 mL phosphate-buffered saline (PBS) was administered to obtain bronchoalveolar lavage fluid. Cells in bronchoalveolar lavage fluid were attached to slides using a Cytospin machine, and the number of different types of inflammatory cells was determined by Diff-Quik staining (Sysmex Co., Japan) after attachment.

Hematoxylin & eosin (H&E) staining and Masson's trichrome staining were performed to evaluate and compare the pathological changes and characteristics in the lungs. Tissue sections were prepared by expanding the left lungs of mice with PBS, then fixing and embedding them in paraffin. Each staining was performed on the prepared tissue slides and analyzed.

To quantify the extent of lung parenchymal destruction in each group, the stained lung tissue slides were observed and compared between groups by measuring the distance between alveolar walls (chord length) based on the existing literature [14].

2.4. Analysis of macrophage, anti-inflammatory, and lung fibrosis markers

RNA was extracted from lung tissue samples and reverse transcribed into cDNA, and a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with the obtained cDNA. SYBR Green Master Mix (Applied Biosystems, USA) was used to determine the mRNA expression levels of IL-10, macrophage activation markers Ym-1, CD206, inducible nitric oxide synthase (iNOS), lung fibrosis markers connective tissue growth factor (CTGF), fibronectin, and type III collagen. Gene expression in each specimen was normalized to β-actin, and the primer sequences for Ym-1, CD206, iNOS, IL-10, CTGF, fibronectin, and type III collagen used in the experiments were as follows:

(1) IL-10:
forward 5'-CACTGCTATGCTGCCTGCTC-3';
reverse 5'-TGGCCTTGTAGACACCTTGG-3'
(2) Ym-1:
forward 5'-GGGCATACCTTTATCCTGAG-3';
reverse 5'-CCACTGAAGTCATCCCA-3'
(3) CD206:
forward 5'-CAAGGAAGGTTGGCATTT-3';
reverse 5'-CCTTTCAGTCCTTTGCAA-3'
(4) iNOS:
forward 5'-GCTCTACACCTCCAATGTGACC-3';
reverse 5'-CTGCCGAGATTTGAGCCTCATG-3'
(5) CTGF:
forward 5'-ATCCCTGCGACCCACACAAG-3';
reverse 5'-CAACTGCTTTGGAAGGACTCGC-3'
(6) Fibronectin:
forward 5'-CCACCCCCATAAGGCATAGG-3';
reverse 5'-GTAGGGGTCAAAGCACGAGTCATC-3'
(7) Type III collagen:
forward 5'-TGGTCTGCAAGGAATGCCTGGA-3';
reverse 5'-TCTTTCCCTGGGACACCATCAG-3'

2.5. Analysis of the phagocytic capacity of macrophages

Cells extracted from the bronchoalveolar lavage fluid of each mouse were evaluated for apoptosis by Diff-Quik staining, and the phagocytic index, apoptotic

cells, and defective index were derived using the following formula [15]:

(1) Phagocytic index (PI) = number of phagocytosed cells ÷ number of AMs × 100

(2) Apoptotic cells (%) = number of killed cells ÷ (total number of cells – number of AMs) × 100

(3) Defective index (DI) = apoptotic cells (%) ÷ PI

By applying the above formula, DI values were obtained for each group and compared.

2.6. *In vitro* experiments to evaluate the effects of macrophage activation

CRL-2456, an AM cell line, was purchased from the American Type Culture Collection. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotics to form a complete medium, and the medium was changed every 2–3 days. Culture conditions were maintained at 5% CO_2, 37°C in a cell culture chamber, and cultured CRL-2456 were stimulated with mouse recombinant IL-13 protein (20 ng/mL; BioLegend, USA) to differentiate into M2 macrophages. To evaluate the effect of atorvastatin on M2 macrophages, they were treated with 1 μM atorvastatin.

2.7. Statistics

All data were expressed as mean ± standard error of the mean. Statistical significance was determined by Student *t*-test to determine differences between groups, and $P < 0.05$ was presented as statistically significant.

3. Results

3.1. Improvement of airway inflammation and airway hyperresponsiveness by atorvastatin

To determine the extent of lung inflammation in each group, we performed inflammatory cell analysis with bronchoalveolar lavage fluid and found that the total number of leukocytes in bronchoalveolar lavage fluid was significantly reduced ($P < 0.05$) (Figure 1A). Histological analysis of lung tissues by H&E staining confirmed that the inflammatory cell infiltration in the lungs, which was increased when atorvastatin was administered to IL-13 overexpressing mice, was reduced (Figure 1B[d]).

We next measured airway hyperresponsiveness using methacholine and found that Penh values, which were significantly increased in the IL-13 overexpression model, normalized to control levels upon oral administration of atorvastatin six times over two weeks ($P < 0.05$) (Figure 1C), confirming the ameliorative effect of atorvastatin on airway hyperresponsiveness. We then identified cytokines involved in the pulmonary inflammatory response and found that IL-10 expression, which was not increased in the IL-13 overexpression model, was significantly increased by atorvastatin treatment ($P < 0.05$) (Figure 1D).

3.2. Amelioration of lung fibrosis and destruction by atorvastatin

In IL-13 overexpressing mice, pulmonary fibrosis and lung parenchymal destruction were observed as reported in the previous literature [16]. To histologically analyze the amelioration of pulmonary fibrosis by atorvastatin, Masson trichrome staining confirmed the improvement of pulmonary fibrosis seen in the IL-13 overexpression model upon atorvastatin administration (Figure 2A[d]). To confirm the improvement in lung fibrosis at the mRNA level in the lung parenchyma, we determined the mRNA expression of lung fibrosis markers CTGF, fibronectin, and type III collagen. We found that the mRNA levels of each marker, which tended to increase in the IL-13 overexpression model, decreased with atorvastatin treatment, with CTGF showing a statistically significant decrease (Figure 2B).
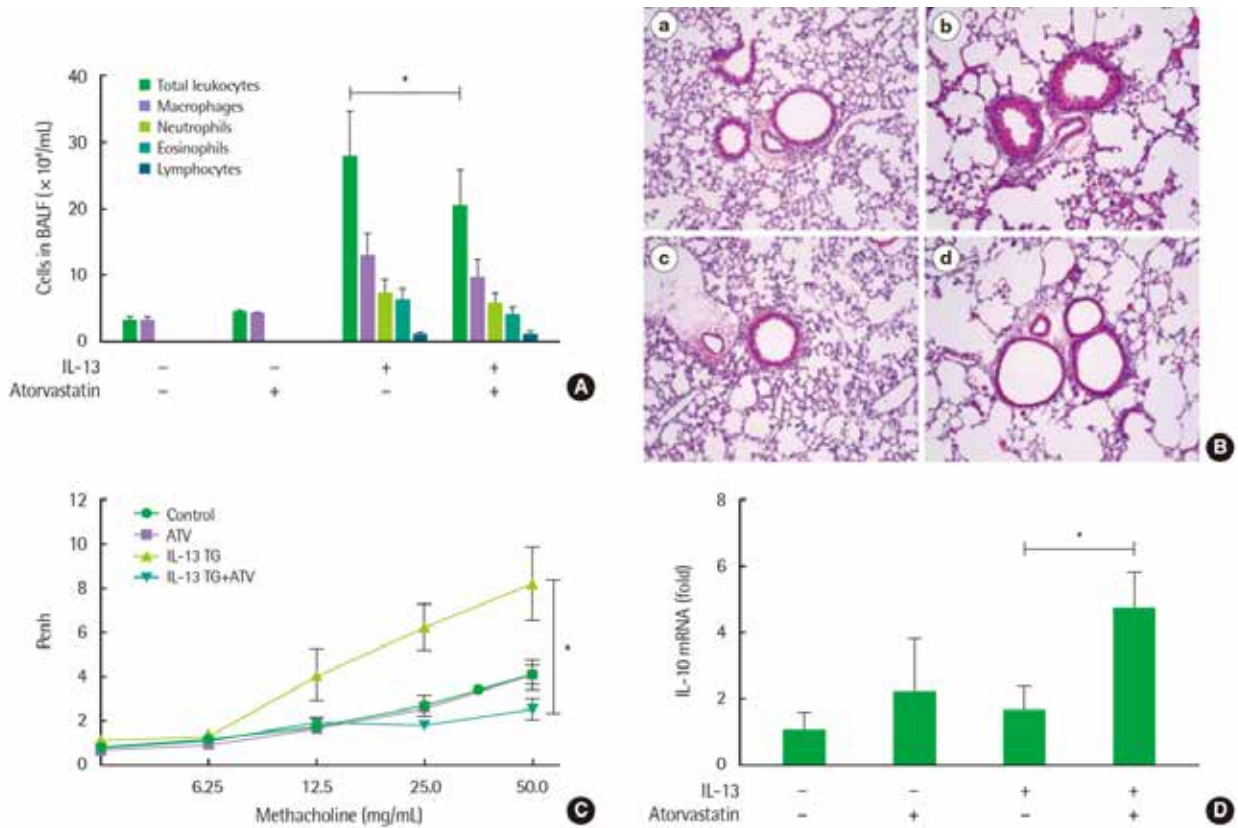
Figure 1. Effect of atorvastatin on interleukin (IL)-13 induced lung inflammation and airway hyperresponsiveness (*$P < 0.05$). (A) The number of inflammatory cells including eosinophils in bronchoalveolar lavage fluid (BALF). (B) Hematoxylin and eosin stain (a-d, ×200) of lung histology in IL-13 transgenic (TG) mouse (a, phosphate-buffered saline [PBS]+control; b, PBS+IL-13 TG mouse; c, atorvastatin [ATV]+control; d, ATV+IL-13 TG mouse). (C) Methacholine hyperresponsiveness was measured 24 hours after the last intranasal challenge. (D) A real-time reverse transcription-polymerase chain reaction was performed to determine the changes in mRNA levels of IL-10 from lung tissues. $n = 6$ for each group, the *$P < 0.05$. All results are representative of at least 3 independent experiments.
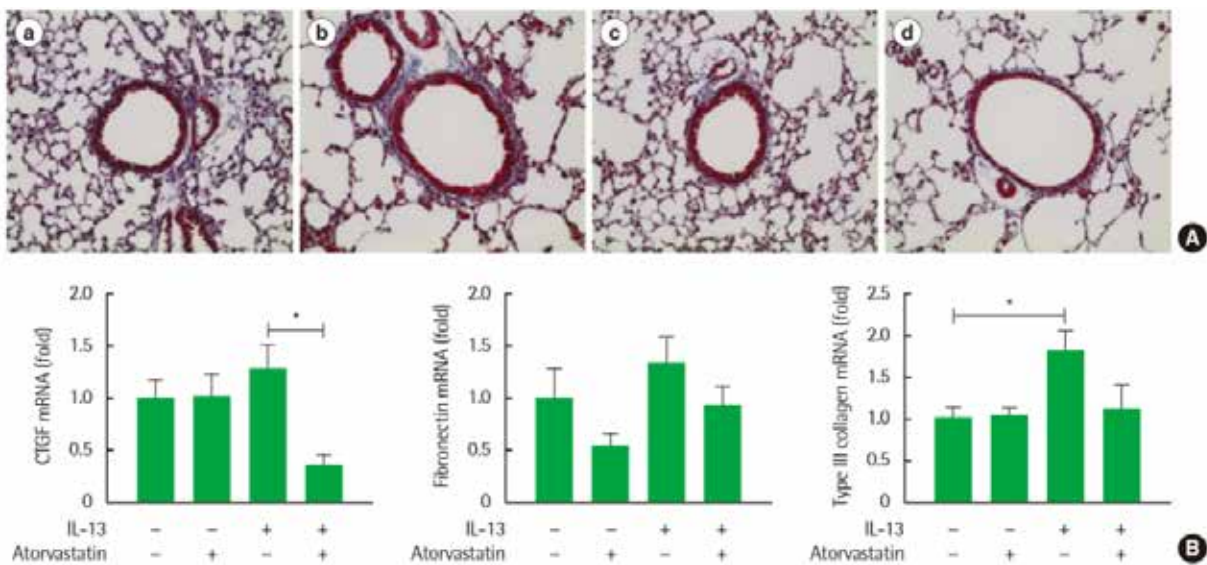

Figure 2. Effect of atorvastatin on interleukin (IL)-13 induced lung fibrosis (*$P < 0.05$). (A) Masson trichrome stain (a-d, ×200) of lung histology in IL-13 transgenic (TG) mouse (a, phosphate-buffered saline [PBS]+control; b, PBS+IL-13 TG mouse; c, atorvastatin+control; d, atorvastatin+IL-13 TG mouse). (B) Real-time RT-PCR to determine changes in mRNA levels of connective tissue growth factor (CTGF), fibronectin, and type III collagen from lung tissue. $n = 6$ for each group, *$P < 0.05$. All results are representative of at least 3 independent experiments.
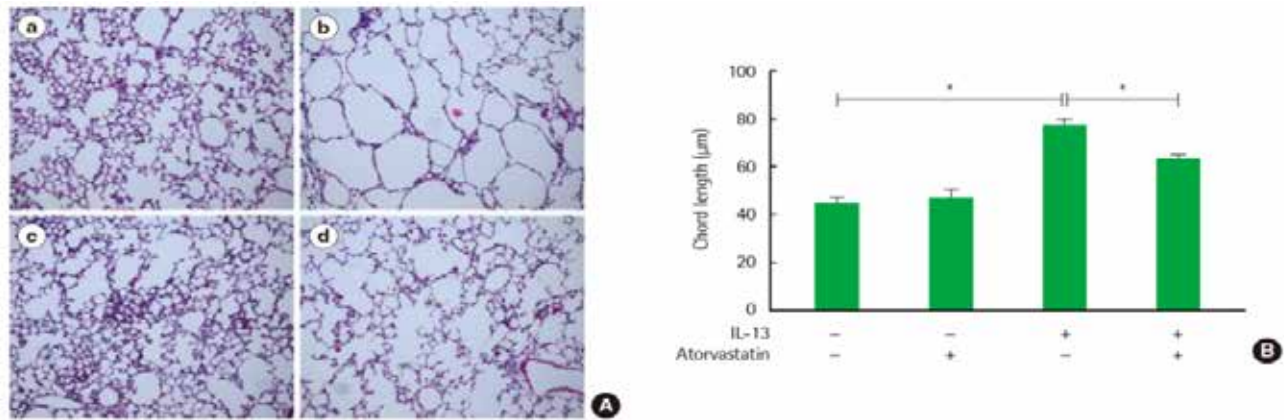
Figure 3. Effect of atorvastatin on interleukin (IL)-13 induced lung destruction (*P < 0.05). (A) Hematoxylin and eosin stain (a-d, ×100) of lung histology in IL-13 transgenic (TG) mouse (a, phosphate-buffered saline [PBS]+control; b, PBS+IL-13 TG mouse; c, atorvastatin+control; d, atorvastatin+IL-13 TG mouse). (B) The levels of Chord length determine the changes in lung destruction from lung tissues. *P < 0.05. Chord length; mean linear intercept length. All results are representative of at least 3 independent experiments.

Next, we determined the effect of atorvastatin on lung parenchymal destruction. For this purpose, we stained lung tissue with H&E and found that the lung tissue destruction identified in the IL-13 overexpression model was improved by atorvastatin treatment (Figure 3A). To quantitatively confirm the improvement in lung destruction, we then determined the changes between groups based on chord length. We found that chord length values, which were significantly increased in the IL-13 overexpression model, were significantly reduced by atorvastatin treatment (Figure 3B). This confirms that atorvastatin treatment has an ameliorative effect on lung fibrosis and destruction.

3.3. Inhibition of M2 macrophages by atorvastatin

To determine the effect of atorvastatin on macrophage activation, we examined the mRNA expression of AM activation markers Ym-1, CD206, and iNOS. We found that the expression of M2 macrophage markers Ym-1 and CD206, which were significantly increased in the IL-13 overexpression model, tended to decrease after atorvastatin treatment, and the expression of M1 marker iNOS, which was also significantly increased in the IL-13 overexpression model, was significantly further increased after atorvastatin treatment (P < 0.05) (Figure 4A).

Next, to determine the direct macrophage-modulating effect of atorvastatin, we evaluated it in vitro using CRL-2456 cells, an AM cell line. First, CRL-2456 cells were Th2-stimulated with recombinant IL-13 to induce M2 macrophage activation and treated with atorvastatin in a concentration-dependent manner. Recombinant IL-13 stimulation increased the expression of Ym-1, and CD206 in AMs, but atorvastatin treatment significantly decreased the expression. iNOS expression was significantly increased by atorvastatin treatment alone, even in the presence of IL-13 stimulation (P<0. 05) (Figure 4A, B). These results confirmed that atorvastatin modulated the activation of macrophages induced by IL-13 stimulation, inhibiting M2 macrophage activation and increasing M1 macrophage activation.

3.4. Improved efferocytosis of macrophages by atorvastatin

To determine the effect of atorvastatin on regulating efferocytosis, cells in bronchoalveolar lavage fluid were first examined for apoptotic cells by Diff-Quik staining and compared between groups. Analysis of bronchoalveolar lavage fluid from the IL-13 overexpression model revealed a large number of apoptotic cells compared with the control group, and the number of apoptotic cells was reduced by
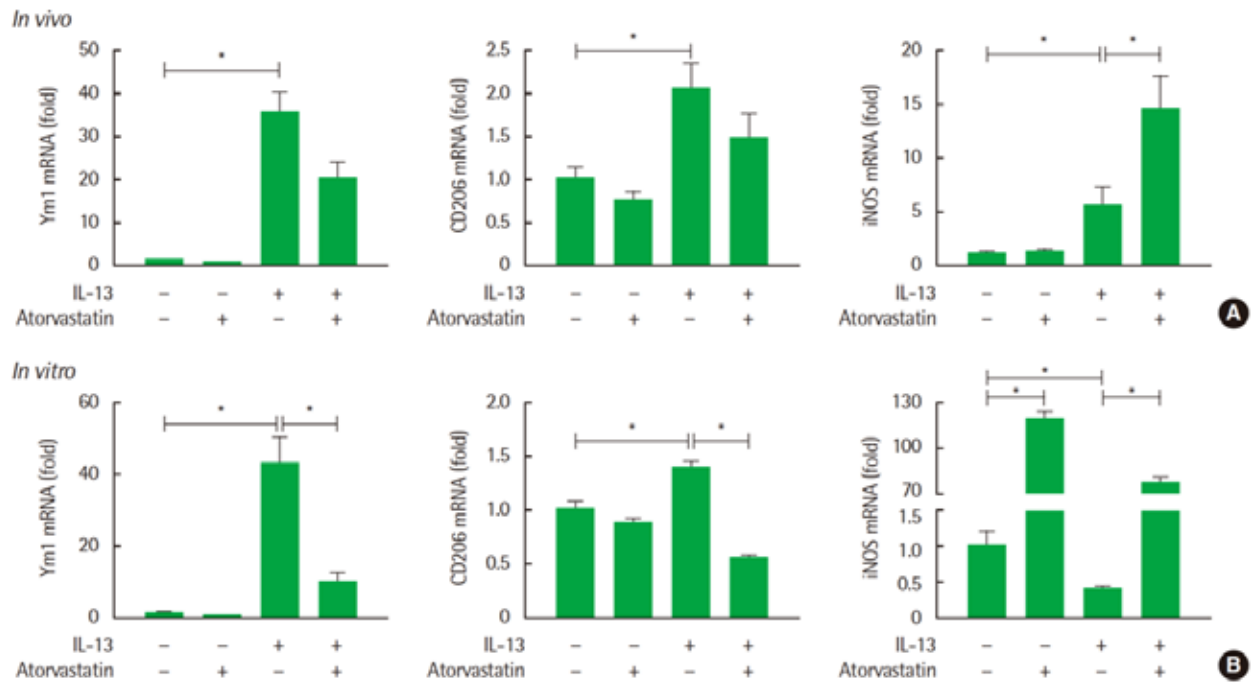
Figure 4. Effect of atorvastatin on macrophage activation markers in vivo and in vitro (*P < 0.05). (A, B) The mRNA levels of CD206, Ym-1, and inducible nitric oxide synthase (iNOS) were detected in lung tissue, and recombinant interleukin (IL)-13 stimulated mouse AM cell line. The levels of mRNA are represented as the ratio to β-actin. *P < 0.05. All results are representative of at least 3 independent experiments.

atorvastatin treatment (Figure 5A). We then evaluated the efferocytosis ability of macrophages in each group using the DI value, which is an indicator to assess the efferocytosis of macrophages and found that the IL-13 overexpression model showed a high DI, but atorvastatin treatment significantly reduced the ability (P < 0.05) (Figure 5B). This confirmed that atorvastatin promoted the efferocytosis ability of macrophages and thus promoted the clearance of apoptotic cells.

4. Discussion

In this study, we demonstrated that atorvastatin ameliorated airway inflammation and AHR in an IL-13 overexpressing mouse model and directly inhibited macrophage activation with histological improvements in the lungs. Furthermore, oral administration of atorvastatin to an IL-13 overexpressing mouse model confirmed the macrophage modulatory ability of atorvastatin by confirming a decrease in mRNA

levels of M2 markers in the lungs and a decrease in M2 phenotype in an *in vitro* assay using AM lines. In addition, atorvastatin enhanced the efferocytosis capacity of macrophages, suggesting that atorvastatin may exert its anti-asthmatic effects by enhancing the apoptotic capacity of macrophages in the context of an excessive inflammatory response.

Macrophages are divided into several classifications based on their resident tissue and origin, among which AMs are responsible for the innate immune system of the airways and constitute the majority of the immune cells present in the airways, so they were expected to play a significant role in the pathogenesis of asthma and have indeed been associated with asthma [17]. Functional and intrinsic classifications of macrophages, including AMs, have been proposed, suggesting that subpopulations of macrophages may be important in analyzing the pathophysiology of asthma [18]. Indeed, we found that CD206+/MHCII+ macrophages, known as markers of M2a macrophages, are increased in
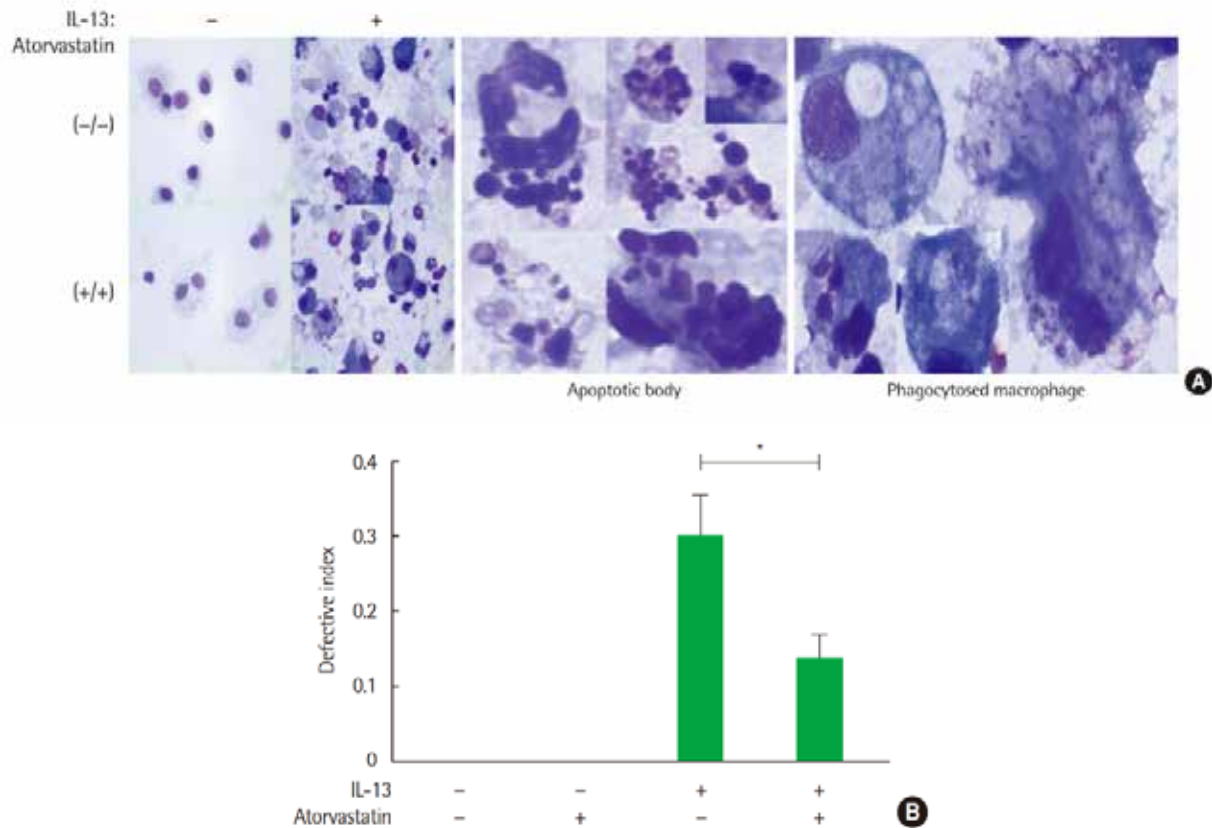
Figure 5. Effect of atorvastatin on IL-13-induced apoptosis and phagocytosed macrophages (*$P < 0.05$). (A) Diff-Quik staining (a-d, ×1,000) of apoptotic cells in bronchoalveolar lavage fluid from interleukin (IL)-13 transgenic mouse. (B) The defective index (DI) was calculated in apoptotic cells and phagocytosed cells. *$P < 0.05$. DI = apoptotic cells (%)/phagocytosis index. All results are representative of at least 3 independent experiments.

asthma patients depending on the severity of the disease, and previous studies from our group reported that these changes in M2 macrophages may apply to the treatment of asthma [19-22]. In the present study, we found that the expression of M2 macrophage markers, which was enhanced in an IL-13 overexpressing mouse model, was reduced by atorvastatin administration and treatment, confirming that the modulation of M2 macrophages by atorvastatin may be a potential anti-asthmatic therapeutic mechanism.

Th2 cytokines are a prominent feature observed in allergic asthma, and their inhibition has been reported to have therapeutic applications in asthma [23]. Indeed, as high expression levels of the IL-13 cytokine are maintained in some patients during inhaled corticosteroid treatment, anti-IL-13 antibodies have been tested in asthma patients and have been shown to

improve lung function and reduce the rate of asthma exacerbations [24]. However, there are still limitations to their application in asthma, including adverse effects on the musculoskeletal and urinary systems and the possibility of co-administration with IL-4 antibodies [25].

Previously reported studies on the anti-asthmatic activity of statins have mainly identified differences in pathophysiological aspects. In a mouse model of OVA-induced asthma, statins decreased eosinophil migration by inhibiting chemokine production and lung tissue intercellular adhesion molecule 1 (ICAM1) expression [26], decreased Th2 cytokines, including IL-13, and increased Th1 cytokines, including interferon-γ [27]. Recently, clinical studies have been reported to confirm the anti-asthmatic effects of statins in asthmatic patients, and in certain patient populations, including smokers with severe asthma, statin administration

improved lung function [28,29]. Furthermore, in asthmatic patients using inhaled steroids, activation of the atypical nuclear factor-kappa B pathway in AMs accompanied by a decrease in Th2 cytokines and consequently increased indoleamine 2,3-dioxygenase transcription and enzyme activity was identified when statins were taken [30]. Thus, a potential therapeutic application of statins in the pathogenesis of IL-13-induced asthma exists [31,32].

Motivated by the fact that statins act on macrophages in atherosclerosis, we sought to determine whether statins affect airway AMs and observed that oral administration of atorvastatin to an IL-13 overexpressing mouse model ameliorated intrapulmonary inflammation, AHR, lung fibrosis, and destruction [33]. Although there was no significant reduction in inflammatory cells such as macrophages and eosinophils in bronchoalveolar lavage fluid, there was a significant difference in the reduction of total inflammatory cell count and activation of AMs, and an increase in IL-10 expression, which has an asthma-modulating effect by inhibiting Th2 cytokines, was identified as the mechanism of action of atorvastatin. In addition, we found significant differences in the differentiation of AMs, with a decrease in the M2 macrophage markers Ym-1 and CD206 and an increase in the M1 macrophage marker iNOS upon atorvastatin treatment, suggesting that atorvastatin inhibits activation into M2 macrophages and promotes activation into M1 macrophages [34].

This study confirmed the anti-asthmatic effect of atorvastatin on the phenotype of asthma induced by overexpression of Th2 cytokines, especially IL-13, and revealed inhibition of Th2 cytokine-stimulated activation of M2 macrophages. However, the inhibition of eosinophilic inflammation was not evident, suggesting that further studies on the anti-asthmatic mechanisms of the statin are warranted, particularly through subtyping of M2 macrophages to investigate the macrophage-modulating effects of atorvastatin and the mechanisms underlying its anti-asthmatic effects. In addition, changes in innate lymphoid cells, which are closely related to macrophages, need to be observed and further investigated.

Taken together, this study used an IL-13 overexpression mouse model to investigate the mechanisms of atorvastatin's anti-asthmatic effects and confirmed the role of atorvastatin in IL-13 cytokine-induced asthmatic inflammation and airway remodeling. In addition, macrophage cell lines were used to determine the effect of atorvastatin on the M2 macrophage activation pathway. These findings suggest that atorvastatin treatment suppresses the IL-13-induced asthma phenotype and inhibits M2 macrophage activation, suggesting the potential of atorvastatin as a treatment for allergic asthma.

Disclosure statement

The authors declared no conflict of interest.

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