

IL-35 improves T_{reg}-mediated immune suppression in atherosclerotic mice

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Abstract. Interleukin (IL)-35 is an anti-inflammatory cytokine that may have a protective role in atherosclerosis (AS). However, the exact role of IL-35 in the disease, and the etiology of AS, remain incompletely understood. The present study aimed to investigate whether exogenous IL-35 was able to attenuate the formation of atherosclerotic lesions in apoE^{-/-} mice, and analyze alterations in the expression levels of forkhead box protein 3 (Foxp3) in peripheral blood and the lesions during the progression of AS. ApoE^{-/-} mice were randomly divided into two groups that received either a basal diet (negative control group) or a high-fat diet (HFD) for 4 weeks. The HFD group was further subdivided into groups that received IL-35, atorvastatin or no treatment for 12 weeks. Diagnostic enzyme assay kits were applied for the detection of plasma lipids, and hematoxylin and eosin staining was used to analyze the severity of atherosclerotic lesions in apoE^{-/-} mice. Immunohistochemistry and flow cytometry were performed to analyze the expression of Foxp3 in the plasma and atherosclerotic plaques. As compared with the negative control group, the plasma lipids were significantly increased, and the lesions were obviously formed, in the HFD groups. Furthermore, the area of the lesion was reduced in IL-35- and atorvastatin-treated groups, as compared with the AS control group. In addition, Foxp3 expression was upregulated in the plasma and lesions of the IL-35- and atorvastatin-treated groups, as compared with the AS control group. The present study demonstrated

that IL-35 improved T_{reg}-mediated immune suppression in atherosclerotic mice, thus suggesting that IL-35 may be a novel therapeutic target for AS.

Introduction

Atherosclerosis (AS) is characterized by the accumulation of lipids in the walls of large and medium-sized arteries, resulting in plaque formation and narrowing of the arterial lumens (1). Although the underlying etiology of AS remains poorly defined, it is generally accepted that AS is not only a disorder of lipids, but also a chronic autoimmune inflammatory disease (2). Evidence from AS-prone models suggested that various immune cells and inflammatory cytokines were present in atherosclerotic lesions, and that a complex imbalance existed between pro-inflammatory and anti-inflammatory factors, indicating that this imbalance may have an important role in AS initiation and progression (3,4). In the immune system, CD4⁺ regulatory T-cells (T_{reg}) are a master subset of regulatory T-cells that have a critical role in limiting the process of AS (5). It has been reported that the transcription factor forkhead box protein 3 (Foxp3) is specifically expressed in CD4⁺ T_{reg} cells, and is a key marker of CD4⁺ T_{reg} cells (6). The functions of T_{reg} cells were deficient in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome and the scruffy (sf) mouse model due to a Foxp3 mutation (7,8), thereby demonstrating the importance of Foxp3 for the functions of T_{reg} cells. Sf is an X-linked recessive mouse mutant resulting in lethality in hemizygous males 16-25 days after birth, and is characterized by overproliferation of CD4⁺CD8⁻ T lymphocytes, extensive multiorgan infiltration and elevation of numerous cytokines. Furthermore, previous studies demonstrated that Foxp3 was indispensable for the development and function of T_{reg} cells (7-9). Therefore, stimulating the expression of Foxp3 and increasing the numbers of T_{reg} cells may be important strategies for the treatment of AS. T_{reg} cells mediate the immunosuppression via cell-to-cell contact and secretion of anti-inflammatory factors, including interleukin (IL)-10, transforming growth factor-β and IL-35 (10).

IL-35, which was identified in 2007 as a member of the IL-12 family, is a heterodimer composed of Epstein-Barr virus-induced protein (EBI)-3 (a subunit of IL-27) and p35

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(a subunit of IL-12) (11,12). Subsequent studies determined that IL-35 is predominantly secreted by CD4⁺ T_{reg} cells (13). Evidence from a mouse model of rheumatoid arthritis demonstrated that IL-35 is an anti-inflammatory cytokine that inhibits the activity of effector T cells (T_{eff}), improves the activity of T_{reg} cells, reduces the secretion of inflammatory factors and suppresses autoimmune diseases (14). In particular, IL-35 was observed to attenuate established rheumatoid arthritis, which indicated that IL-35 has an important role in maintaining the activity of T_{reg} cells (14). In addition, it has been reported that Ebi3 and p35 are strongly co-expressed in the majority of advanced lesions, thus suggesting that IL-35 is associated with AS (15). Previous studies have reported that IL-35 may have a protective effect on the progression of AS (16,17). However, the exact role of IL-35 in AS remains poorly understood. The present study aimed to investigate whether exogenous intervention with IL-35 was able to attenuate the formation of atherosclerotic lesions in advanced AS apoE^{-/-} mice. In addition, alterations in the expression levels of Foxp3 in peripheral blood and atherosclerotic lesions during the progression of AS were analyzed.

Materials and methods

Reagents. Atorvastatin calcium was purchased from AstraZeneca (London, UK). Recombinant human IL-35 was obtained from Sino Biological Inc. (Beijing, China). The peripheral blood mononuclear cell (PBMC) kit was purchased from Tianjin Haoyang Biological Products Technology, Co., Ltd. (Tianjin, China). Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-CD25 antibodies were purchased from eBioscience Inc. (San Diego, CA, USA). Allophycocyanin (APC)-conjugated anti-Foxp3 antibody was obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Anti-Foxp3 antibody was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). SP-9000/9001/9002 SPlink Detection kits were purchased from OriGene Technologies, Inc. (Beijing, China) Diagnostic enzyme assay kits [total cholesterol test kit, cat. no. F002-2; triglyceride test kit, cat. no. F001-2; high density lipoprotein-cholesterol (HDL-C) test kit, cat. no. F003-2; and low density lipoprotein-cholesterol (LDL-C) test kit, cat. no. F004-2] were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and groups. Male apoE^{-/-} mice (age, 8 weeks old; weight, 0.02±0.003 kg) were purchased from Vital River Laboratory in Beijing, China. The mice were maintained at room temperature in a sterilized laboratory with food and sterilized water *ad libitum*. The apoE^{-/-} mice were divided into two groups, as follows: The negative control group (n=8), who received a basal diet, and the high-fat diet (HFD) group (n=24). The normal diet and HFD, constituting 81.85% of the basal diet, 0.15% cholesterol and 18% lard, were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China).

After 4 weeks, the HFD group was further divided into three subgroups (n=8/group), as follows: i) the AS control group, which did not received any treatment; ii) the drug

control group, in which the mice were orally administered with atorvastatin calcium (5 mg/kg); and iii) the exogenous intervention group, in which mice were intraperitoneally injected with IL-35 (1.2 mg/kg) once daily for 12 weeks.

At the end of the experiment and prior to sacrifice of the mice, fresh blood samples were taken intravenously from the epicanthal folds of mice in each group using tubes containing heparin sodium. Subsequently, the tubes were centrifuged at 500 x g for 15 min at 22-25°C to collect PBMCs for flow cytometry. The mice were fasted for 12 h prior to sacrifice. Following anesthetization with 10% chloral hydrate (4.8 ml/kg), blood was collected from the inferior vena cava for biochemical detection. The mice were then sacrificed following chest opening from excessive loss of blood and cardiac arrest. Aortic root sections were generated for hematoxylin and eosin (H&E) staining and immunohistochemical analyses. All procedures complied with and were approved by the Internal Animal Care and Use Committee of Anhui Medical University.

Detection of serum lipids. At the end of the experiment, blood collected from the inferior vena cava of the mice in tubes was incubated for 2 h at room temperature, followed by centrifugation at 1,000 x g for 15 min at 22-25°C to prepare for detection of serum lipids. Total cholesterol (TC), total triglyceride (TG), HDL and LDL levels were detected using diagnostic enzyme assay kits.

H&E staining and immunohistochemistry. Following anesthetization with 10% chloral hydrate, the mice were injected into the apical muscle with normal saline and 4% paraformaldehyde was flushed through the heart vascular system and intercepted thoracic aorta, fixed in 4% paraformaldehyde for 24 h, then dehydrated and embedded in paraffin longitudinally. Aortic root sections (4 µm) were cut from the embedded hearts. To prepare for immunohistochemical analysis, the paraffin-embedded tissue sections were deparaffinized, immersed in phosphate-buffered saline and blocked with 3% H₂O₂ solution for 30 min at room temperature to inhibit endogenous peroxidase activity. Subsequently, the tissue sections were incubated with normal goat serum (included in the SPlink Detection kits) at 37°C for 30 min, followed by incubation with anti-Foxp3 antibody overnight at 4°C. Next, the deparaffinized sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (1:200; cat. no. SP9000-3; OriGene Technologies, Inc.), followed by horseradish-streptavidin complex for 30 min at 37°C. Finally, the sections were incubated with 3,3'-diaminobenzidine and stained with hematoxylin for 2 min. Since Foxp3 is expressed in the nucleus (18), positive staining was indicated by brown coloration of the nucleus. Foxp3 expression was analyzed for the vascular atherosclerotic plaques within every section. Ten visual fields were randomly selected, and the number of positive cells was calculated in each field to obtain the mean. The Image-Pro Plus 5.1 Image Operation system was used to capture images of the sections. The intimal thickness and area of a plaque were measured using the JD-801 Pathological Image Analysis system. The protocols for H&E staining and immunohistochemistry were performed according to previous studies (19,20).

Table I. Lipid levels in the various groups.

Group (n=8)	TC (mmol/l)	TG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
Negative	12.42±1.47	1.68±0.38	2.57±0.36 ^a	2.72±0.27 ^a
AS	17.36±1.25 ^a	2.24±0.18 ^a	3.61±0.15 ^a	3.67±0.18 ^a
Atorvastatin	11.35±1.37 ^b	1.78±0.28 ^c	4.15±0.23 ^c	2.32±0.21 ^c
IL-35	14.13±1.46 ^c	1.96±0.27 ^c	3.96±0.17	3.14±0.24

Data are presented as the mean ± standard deviation. ^aP<0.01, vs. the negative group. ^bP<0.01 and ^cP<0.05, vs. the AS group. TC, total cholesterol; TG, total triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AS, atherosclerosis; IL-35, interleukin-35.

Table II. IMT and plaque areas of apoE^{-/-} mice.

Group (n=8)	IMT (μm)	Plaque area (x10 ⁵ μm ² /cross section)	Plaque/lumen area (%)
Negative	10.63±2.17	0	0
AS	151.54±17.52 ^a	3.01±0.49 ^a	38.13±5.72 ^a
Atorvastatin	36.7±6.37 ^b	0.81±0.05 ^b	10.24±1.14 ^b
IL-35	70.61±9.85 ^b	2.04±0.28 ^b	24.19±4.27 ^b

Data are presented as the mean ± standard deviation. ^aP<0.01, vs. the negative group. ^bP<0.01 and ^cP<0.05, vs. the AS group. IMT, intimal thickness; AS, atherosclerosis; IL-35, interleukin-35.

Flow cytometry. PBMCs were isolated from fresh peripheral blood, and the number of cells was adjusted to a concentration of 1x10⁶. The PBMCs were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 antibodies (1:100) for 30 min at 22–25°C to label cell surface antigens. Subsequently, the cells were fixed and perforated using 1 ml Fixation/Permeabilization solution for 30 min at 4°C in the dark. The cells were repeatedly washed with 2 ml permeabilization buffer, followed by staining with diluted APC-conjugated anti-Foxp3 antibody at 4°C for 30 min in the dark. Finally, the cells were washed repeatedly and resuspended at 1x10⁶ in flow cytometry staining buffer. Flow cytometry was performed using the Beckman Coulter Epics XL™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA), and the image was analyzed using FlowJo 7.6.1 software (<http://www.flowjo.com/download-flowjo/>).

Statistical analysis. Data are presented as the mean ± standard deviation. Using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA), comparison between groups was carried out by one-way analysis of variance. If homogeneity of variance was found, a Student-Newman-Keuls test was performed to analyze differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Exogenous IL-35 downregulates lipid levels in apoE^{-/-} mice. Since lipids are important in the development of AS, the present study analyzed the levels of TC, TG, HDL and LDL in a mouse model of AS. As is shown in Table I and Fig. 1, the levels of TC, TG, HDL and LDL were significantly increased in the AS group (all P<0.01), as compared with the negative group. In addition, a significant reduction in the

levels of TC, TG, HDL and LDL (P<0.05) was observed in the atorvastatin-treated mice, as compared with the AS group. Treatment with IL-35 resulted in a significant decrease in the levels of TC and TG (P<0.05), as compared with the AS group. However, there was no significant difference in the levels of HDL and LDL between the AS and IL-35-treated groups (P>0.05), and between the atorvastatin- and IL-35-treated groups (P>0.05).

Exogenous IL-35 attenuates atherosclerotic lesions. Changes to lesions were analyzed using H&E staining and Image-Pro Plus software. Fig. 2A shows that the vessel wall was smooth, and the elastic plates were clear and neat, in the negative group. In addition, endothelial cells were arranged uniformly and there was minimal evidence of plaque formation. Conversely, in the AS group, eminences were diffused beneath the vascular dissepiments, and hyperplasia of the intima was observed (Fig. 2B). Furthermore, there were a large proportion of foam cells and cholesterol crystals, and a few inflammatory cells; endothelial cells were disordered; the intima appeared discontinuous; rupture and discontinuity of the internal elastic was observed; and the arrangement of smooth muscle cells with spindle-shaped cores was disordered. However, treatment with atorvastatin calcium (Fig. 2C) and IL-35 (Fig. 2D) significantly reduced the proportions of foam cells, cholesterol crystals and inflammatory cells.

In addition, the intima, plaque area and plaque/lumen area were measured. As is shown in Table II and Fig. 3, the HFD diet was associated with thicker intima and larger plaque areas. As compared with the negative group, the mean intima thickness of the AS group was significantly increased (10.63±2.17 vs. 151.54±17.52 μm; P<0.01). Treatment with atorvastatin or IL-35 resulted in a significant reduction in intima

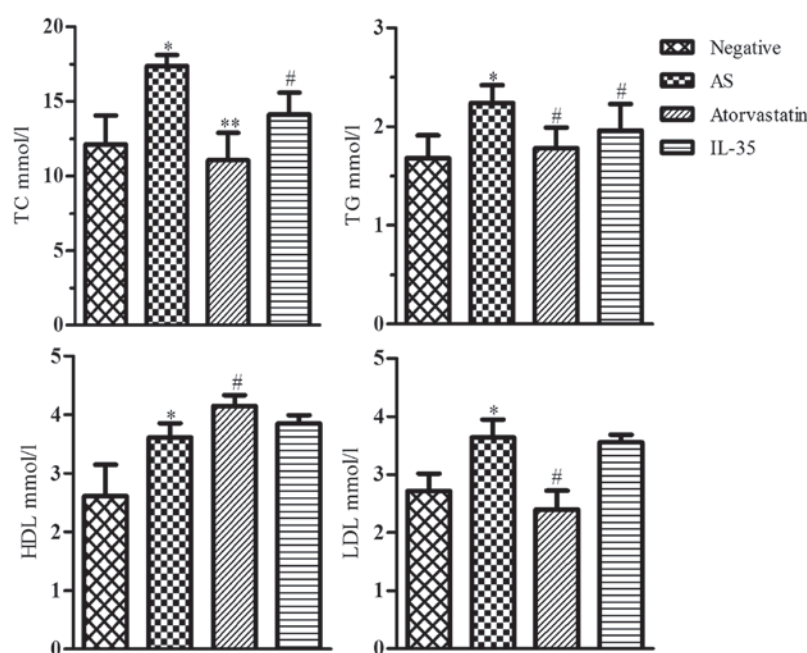


Figure 1. Levels of serum lipids. Blood was collected and centrifuged at 1,000 x g for 10 min to prepare for detection of serum lipids. TC, TG, HDL and LDL contents were analyzed by diagnostic enzyme assay kits. Representative histograms are shown. Data are presented as the mean \pm standard deviation. Comparisons between groups were analyzed by one-way analysis of variance and Student-Newman-Keuls test (n=8). *P<0.01, vs. the negative group. **P<0.01, *P<0.05, vs. the AS group. TC, total cholesterol; TG, total triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AS, atherosclerosis; IL-35, interleukin-35.

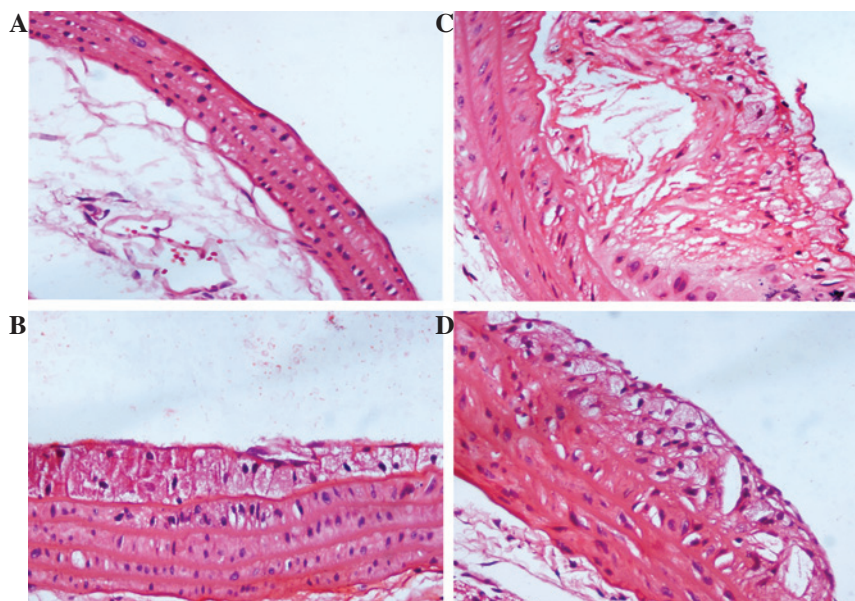


Figure 2. Characteristics of arterial lesions. We analyzed changes in the atherosclerotic lesions by hematoxylin and eosin staining and Image-Pro Plus software (magnification, x400). (A) In the negative group, the vessel walls were smooth, and the elastic plates were clear and neat. Endothelial cells were arranged uniformly, and there was almost no evidence of plaque formation. (B) In the atherosclerosis (AS) group, there were large numbers of foam cells and cholesterol crystals, and a few inflammatory cells. Endothelial cells and the smooth muscle cells with spindle-shaped cores were disordered, and rupture and discontinuity of the internal elastic was observed. In the (C) atorvastatin and (D) interleukin-35 groups, the proportions of foam cells, cholesterol crystals and inflammatory cells were reduced, as compared with the AS group.

thickness, which was reduced to 36.7 ± 6.37 and 70.61 ± 9.85 μ m, respectively (P<0.01). A significant increase in the plaque area and plaque/lumen area were observed in the AS group, as compared with the negative group (P<0.01). Conversely, the plaque/lumen area in the atorvastatin-treated and IL-35-treated mice were reduced from 38.13% in the AS group to 10.24 and

24.19%, respectively. These results suggest that IL-35 attenuates the advancement of atherosclerotic lesions.

IL-35 upregulates the expression of Foxp3 in apoE^{-/-} mice. It has been reported that IL-35 is not only secreted by T_{reg} cells, but is also an inducer of T_{reg} cells and is important

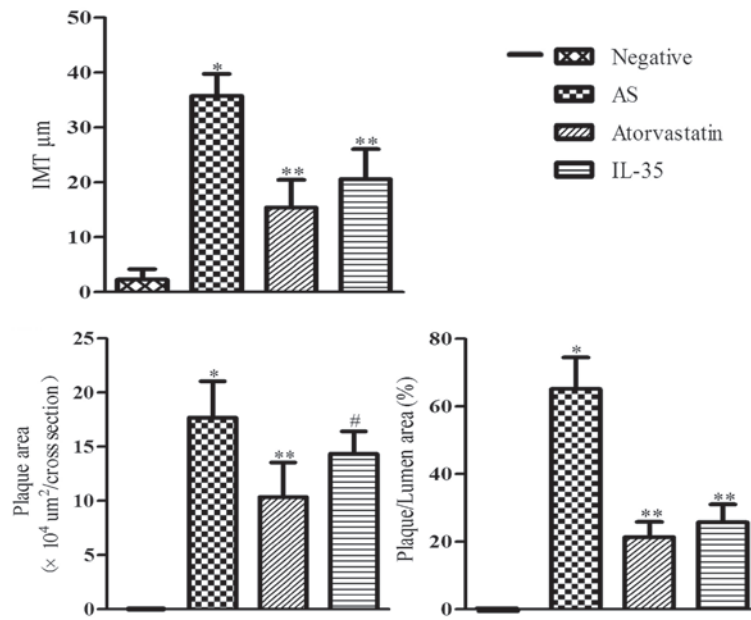


Figure 3. IMT and plaque area of arterial lesions. The IMT and plaque area were measured using the JD 801 Pathological Image Analysis system. Data are presented as the mean ± standard deviation. *P<0.01, vs. the negative group. **P<0.01 and #P<0.05, vs. the AS group. IMT, intimal thickness; AS, atherosclerosis; IL-35, interleukin-35.

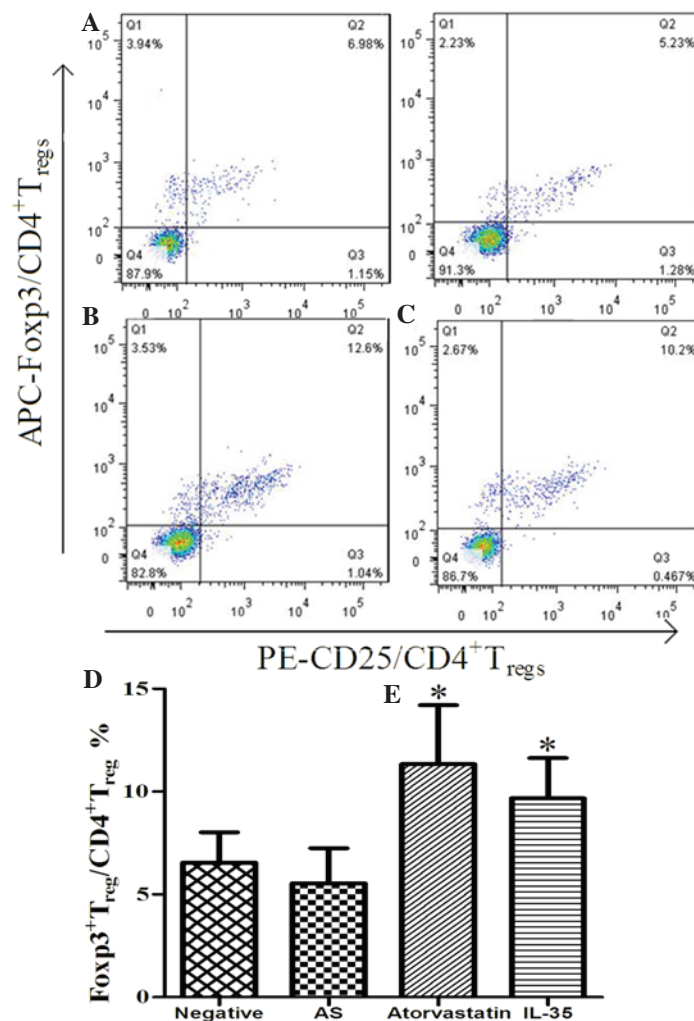


Figure 4. Plasma levels of Foxp3. Peripheral blood mononuclear cells were collected from each group, and the proportions of CD4⁺CD25⁺Foxp3⁺T_{reg}/CD4⁺T_{reg} T-cells were analyzed by flow cytometry, and quantified using FlowJo 7.6.1 software. (A) Histogram. Data are presented as the mean ± standard deviation. *P<0.01, vs. the AS group. (B) Negative group, (C) AS group, (D) atorvastatin group and (E) IL-35 group. Foxp3, forkhead box protein 3; AS, atherosclerosis; IL-35, interleukin-35; APC, allophycocyanin; PE, phycoerythrin.

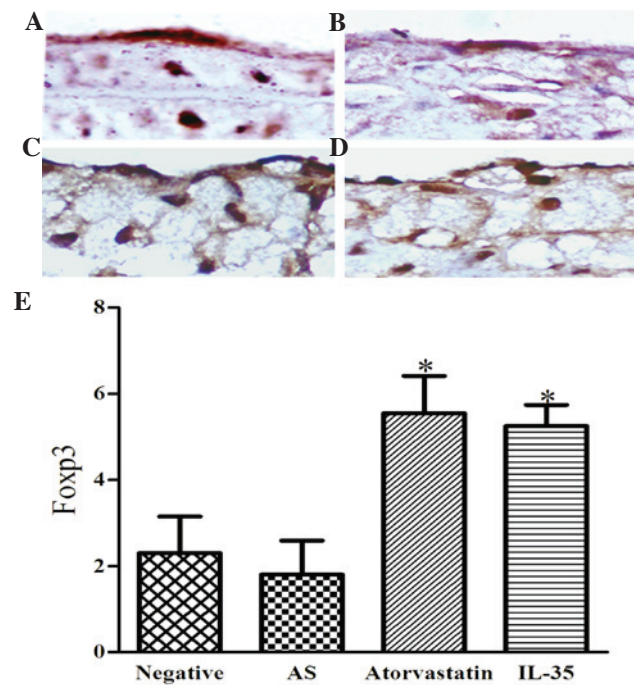


Figure 5. Levels of Foxp3 in atherosclerotic lesions. The deposition of Foxp3 in arteries from various groups was detected by immunohistochemistry (magnification, $\times 400$). Positive Foxp3 was shown as brown nuclei. In the (A) negative and (B) AS groups, the deposition of Foxp3 in the lesions was minimal. Conversely, Foxp3 deposition was increased in the lesions of the (C) atorvastatin and (D) IL-35 groups, as compared with the AS group. (E) This was shown to be significant following quantification. There was no significant difference in the levels of Foxp3 between the atorvastatin and IL-35 groups. * $P < 0.01$, vs. the negative control. Foxp3, forkhead box protein 3; AS, atherosclerosis; IL-35, interleukin-35.

for maintaining the function of these cells (21). Therefore, the present study detected the effect of exogenous IL-35 on the proportions of CD4⁺CD25⁺Foxp3⁺T_{reg}/CD4⁺T_{reg} cells in apoE^{-/-} mice using flow cytometry. As is shown in Fig. 4A-C, there was no significant difference in the proportions of CD4⁺CD25⁺Foxp3⁺T_{reg}/CD4⁺T_{reg} cells between the negative and AS groups, although the ratio of Foxp3⁺T_{reg}/CD4⁺T_{reg} cells appeared reduced in the AS group. However, treatment of the AS mice with atorvastatin or IL-35 resulted in a significant increase in the proportions of CD4⁺CD25⁺Foxp3⁺T_{reg}/CD4⁺T_{reg} cells ($P < 0.01$; Fig. 4A, D and E). There was no significant difference between the mice in the IL-35-treated and atorvastatin-treated groups ($P > 0.05$). These results suggest that IL-35 treatment may upregulate the expression of Foxp3 in the peripheral blood in apoE^{-/-} mice.

To further verify this conclusion, the expression of Foxp3 in atherosclerotic lesions was detected by immunohistochemistry (Fig. 5). The positive expression of Foxp3 in the nucleus was indicated by the formation of brown spheres. Notably, the levels of Foxp3 were markedly reduced in the AS group, as compared with the other groups. Therefore, the levels of Foxp3 were significantly higher in the atorvastatin and IL-35 groups, as compared with the AS group ($P < 0.01$). These results were consistent with the results of the flow cytometry, and suggest that intervention with IL-35 increases the expression of Foxp3 in the peripheral blood and atherosclerotic lesions of apoE^{-/-} mice.

Discussion

At present, the exact mechanism underlying AS is poorly understood. IL-35 is a heterodimer composed of EBI-3 and p35 subunits that is predominantly secreted by CD4⁺ Foxp3⁺

T_{reg} cells (11-13). Previous studies reported that IL-27 α /p28, IL-27 β /Ebi3, IL-12 α /p35 and IL-12 β /p40 were detectable in the majority of established lesions, but only p35 and Ebi3 subunit levels were increased in the lesions following treatment (22,23), thus suggesting that IL-35 was associated with AS. Furthermore, increased expression levels of IL-35 were associated with attenuation of AS in a previous study (17). Therefore, the present study aimed to verify whether exogenous IL-35 was able to attenuate the formation of atherosclerotic lesions in apoE^{-/-} mice. It was demonstrated that advanced lesions were attenuated, and aortic intimal thickness and plaque/lumen area were significantly reduced, following treatment of AS mice with IL-34, thus suggesting that exogenous IL-35 was able to relieve advanced AS.

Immunomodulation is a key factor in the pathogenesis of AS (24). The imbalance between anti-inflammatory and pro-inflammatory factors leads to lipid deposition in the walls of large and medium-sized arteries, causing AS of varying severities (3). The present study used AS mice treated with atorvastatin calcium as the normal drug group, since atorvastatin calcium has been widely used as a traditional lipid-suppressing drug (25). The experimental results demonstrated that atorvastatin calcium and IL-35 treatment were able to significantly attenuate the formation of atherosclerotic lesions. However, atorvastatin calcium and IL-35 were observed to be different in terms of the rate at which they slowed lipid deposition: Although there was a significant difference between the atorvastatin and AS groups, no significant difference was observed between the IL-35 and AS groups. These results suggested that the mechanisms of IL-35 were different from those of atorvastatin calcium. In addition, the expression levels of Foxp3 were significantly increased in apoE^{-/-} mice treated with IL-35, thus

Foxp3 may be a novel target for detecting the benefits of IL-35 and its mode of action.

IL-35 is predominantly secreted by CD4⁺ Foxp3⁺ T_{reg} cells (26). Evidence from rheumatoid arthritis mice suggested that IL-35 was able to inhibit the activity of T_{eff} cells, promote the activity of T_{reg} cells, reduce the expression of inflammatory factors and suppress autoimmune diseases, thereby attenuating the established rheumatoid arthritis (14). These findings indicated that IL-35 has an important role in maintaining the activity of T_{reg} cells (14). The present study demonstrated that, as compared with the AS group, the expression levels of Foxp3 were significantly increased in the plasma of the IL-35 and atorvastatin groups. Furthermore, the expression levels of Foxp3 were significantly increased in the atherosclerotic lesions of the IL-35- and atorvastatin-treated groups, as compared with the AS group. These results suggested that, with the drug alleviating the advanced atherosclerosis plaque, the expression of Foxp3 was improved. Notably, there were no significant differences in the expression levels of Foxp3 in both the plasma and atherosclerotic lesions between the atorvastatin and IL-35 groups. A possible explanation for this is that, since IL-35 is an anti-inflammatory factor, it may not only be secreted by CD4⁺ T_{reg} cells, but also promote the conversion of the conventional T-cells into CD4⁺ T_{reg} cells, which secrete more IL-35 to mediate the immunosuppression (27). Conversely, IL-35 has been demonstrated to promote the conversion of conventional T-cells into a novel Foxp3⁺ T_{reg} cell (iTr35), which is characterized by Foxp3 independence and is dependent on the secretion of IL-35 to exert its function (28). This function for IL-35 was also demonstrated in an experiment involving human rhinoviruses by Seyerl *et al* (29). Further research is required to overcome these challenges. In addition, although both atorvastatin and IL-35 attenuated the atherosclerotic lesions, previous studies have suggested that atorvastatin may cause adverse reactions associated with muscle toxicity (30), and even tumorigenesis (31). Therefore, IL-35 may be a more desirable option for the treatment of AS. In our future studies, we will continue to analyze the association between IL-35 and other inflammatory factors in the process of alleviating advanced AS, so as to further explore its underlying mechanism.

In conclusion, the present study demonstrated that IL-35 may be a novel therapeutic target for preventing and treating AS. Since the specific mechanisms underlying the role of IL-35 in AS are unclear, further studies are required to investigate the mechanism of action of IL-35.

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