

The Modulation of Endoplasmic Reticulum Stress by Chemical Chaperone Upregulates Immune Negative Cytokine IL-35 in Apolipoprotein E-Deficient Mice

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Abstract

Interleukin (IL)-35 is a newly identified immune negative molecule which is secreted by CD4⁺Foxp3⁺ T regulatory cells (Tregs) and contributes to their suppressive capacity. Early data have shown that IL-35 inhibits development of several autoimmune diseases. However, the role of IL-35 in atherosclerosis, a lipid-driven chronic inflammatory disease in arterial wall, remains to be investigated. Here, we found that IL-35 was involved in atherosclerosis in apolipoprotein E-deficient (ApoE^{-/-}) mice. ApoE^{-/-} mice with established atherosclerotic lesion displayed a lower level of IL-35 compared to age-matched wild type C57BL/6 mice without plaque. However, IL-35 expression increased significantly in ApoE^{-/-} mice with attenuated plaque. More importantly, we found that modulation of ER stress treated by chemical chaperone, 4-Phenyl butyric acid (PBA) *in vivo*, mainly upregulated immune negative regulating molecule IL-35, as well as IL-10 and Foxp3, accompanied by increased Tregs. However, no obvious impact on pro-inflammatory molecules such as TNF- α , IFN- γ , IL-17 and IL-23 was observed, which provides new insight into the benefit of ER stress recovery from attenuated plaque. Our results suggest that IL-35 might have a potential value for atherosclerotic therapy.

Citation: Wang B, Dai S, Dong Z, Sun Y, Song X, et al. (2014) The Modulation of Endoplasmic Reticulum Stress by Chemical Chaperone Upregulates Immune Negative Cytokine IL-35 in Apolipoprotein E-Deficient Mice. PLoS ONE 9(1): e87787. doi:10.1371/journal.pone.0087787

Editor: Xi Yang, University of Manitoba, Canada

Received: July 30, 2013; **Accepted:** January 2, 2014; **Published:** January 30, 2014

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Funding: This research was supported by the National "973" program of China (2011CB503906), the National Natural Science Foundation of China (81172863, 81128013, 81202069 and 81072407), Natural Science foundation of Shandong (Z2008C02). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Interleukin (IL)-35 is a newly identified inhibitory cytokine produced by CD4⁺Foxp3⁺ T regulatory cells (Tregs) and is required for suppressive function of Tregs [1,2]. IL-35 belongs to the IL-12 family in which all members including IL-12, IL-23, IL-27 as well as IL-35 exist as heterodimers that are composed of an α chain (p19, p28, p35) and a β chain (p40 and Epstein-Barr virus induced gene 3, EB13). IL-35 (p35 and EB13) shares p35 with IL-12 (p35 and p40) and EB13 with IL-27 (p28 and EB13) [3,4]. On one hand, IL-35 is able to promote the expansion of Tregs and suppress the proliferation of conventional T cells (Tconv cells) and differentiation of Th1 and Th17 *in vitro* and *in vivo* [5,6]. On the other hand, IL-35 can converse naive T cells into strongly suppressive Treg cells called iTreg35 cells, which also function via IL-35 [7]. IL-35 has been shown to inhibit several inflammatory disorders, including inflammatory bowel disorder [8], autoimmune encephalomyelitis [9], autoimmune diabetes [10], collagen II-induced arthritis [11], and airway inflammation [12]. However, the role of IL-35 in atherosclerosis, a lipid-driven chronic inflammation of the walls of large and medium-sized arteries, remains to be investigated. It has been reported that Tregs play an inhibitory effect on development of atherosclerosis [13–16], and the level of plasma IL-35 decreases in patients with acute coronary syndrome (unstable angina pectoris and acute myocardial

infarction, AMI) compared with chest pain syndrome group [17]. Two subunits of IL-35, EB13 and p35, are also strongly expressed in human advanced plaque [18], suggesting that IL-35 may be involved in atherosclerosis.

Endoplasmic reticulum (ER) is an organelle in which newly synthesized proteins are correctly folded and assembled. Once it is perturbed by various pathological conditions, unfolded proteins will accumulate and result in endoplasmic reticulum stress (ER stress), also defined as the unfolded protein response (UPR). Accumulating evidence indicates that the UPR is chronically activated in atherosclerotic lesions by oxidative stress, high levels of intracellular cholesterol and saturated fatty acids [19,20]. The prolonged activation of the UPR further triggers cell apoptosis in atherosclerosis [21,22]. In addition to apoptosis, ER stress also mediates inflammation that contributes to the development of atherosclerosis [23–25]. Furthermore, recovery of ER function is believed to be a critical factor for improvement of atherosclerosis [26]. 4-Phenylbutyric acid (PBA), a chemical chaperone, has been shown to attenuate atherosclerotic lesions through improvement of ER function *in vivo* [27].

The subunits of IL-35, p35 and EB13, are synthesized and assembled in ER [28,29], and it is supposed that modulation of ER stress by PBA can affect production of IL-35. In the present study, we investigated the possible role of IL-35 in atherosclerosis in

Table 1. Summary of the primers for real time-PCR.

Genes	Primers	Sequences
IFN- γ	Forward	CGGCACAGTCATTGAAAGCCTA
	Reverse	GTTGCTGATGGCCTGATTGTC
TNF- α	Forward	CCCTCACACTCAGATCATCTTCT
	Reverse	TGCTACGACGTGGGCTACAG
IL-17A	Forward	CTGATCAGGACGCGCAAAC
	Reverse	TCGCTGCTGCCCTCACTGTA
IL-23	Forward	GGACTTGTGCTGTTCTGTTTTGT
	Reverse	CCTGCTCTGGGGTTTGTTC
TGF- β	Forward	GTGTGGAGCAACATGTGGAACCTCA
	Reverse	CGCTGAATCGAAAGCCCTGTA
IL-10	Forward	GCCAGAGCCACATGCTCCTA
	Reverse	GATAAGGCTTGGCAACCAAGTAA
EBI3	Forward	AGCAGCCTCCTAGCCTTTGTGG
	Reverse	GAGTTCCTGAGGGTGAAAGTCGTG
IL-12 α	Forward	AGCGTTCACACAGCCTCAC
	Reverse	CTCTGGCCGTCTTACCAT
IL-12 β	Forward	TGTCACCAGCAGTTGGTCATCTC
	Reverse	CTCACTGCTCTGGTCCAAGGTC
p28	Forward	GCACAGGCACCTCCGCTTTCA
	Reverse	GCAGCAGCAGGTCCCGAACAG
Foxp3	Forward	CCCAGGAAAGACAGCAACCTT
	Reverse	TTCTACAACAGGCCACTTG

doi:10.1371/journal.pone.0087787.t001

apolipoprotein E-deficient (ApoE^{-/-}) mice fed on a high-fat diet with or without PBA treatment.

Materials and Methods

Induction of Atherosclerosis

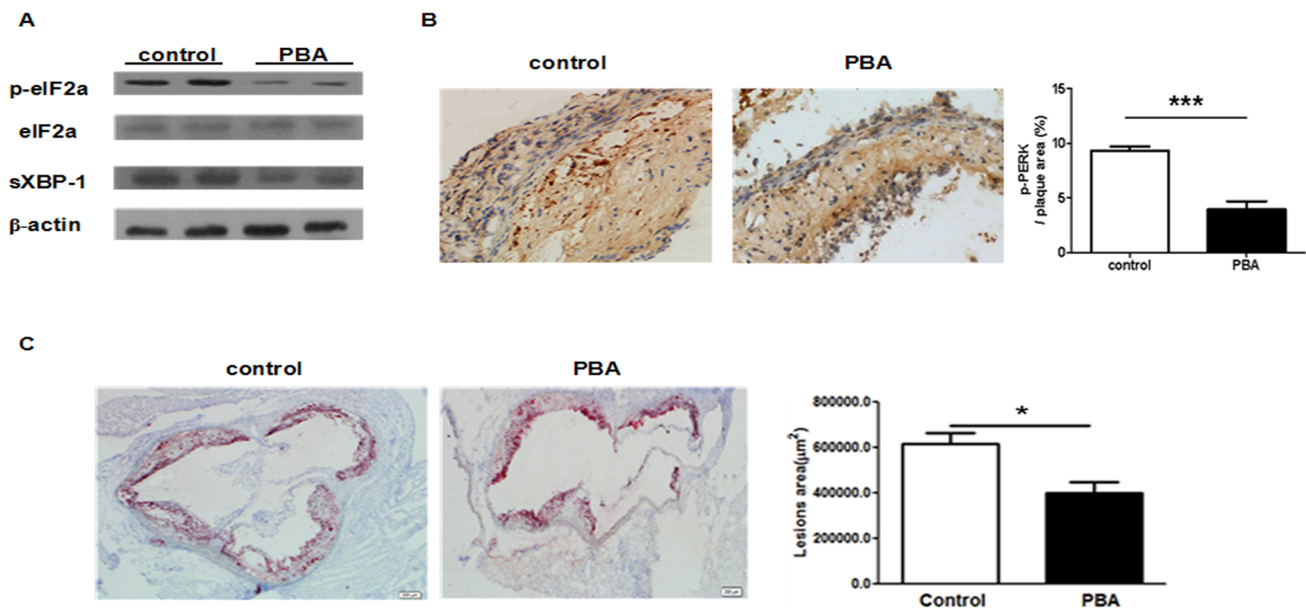
Male C57BL/6 wild type mice and male ApoE^{-/-} mice were purchased from Beijing University and fed on a high-fat diet (0.25% cholesterol and 15% cocoa butter) from 8 weeks of age to 16 weeks to induce atherosclerotic plaques. All animal studies were approved by the Animal Care and Utilization Committee of Shandong University, China.

Application of PBA *in vivo*

ApoE^{-/-} mice (n = 10 for each group) were fed on a high-fat diet (0.25% cholesterol and 15% cocoa butter) from 8 weeks of age. Two weeks after high-fat diet, mice were divided into two groups randomly, one group, named as PBA, was injected intraperitoneally with PBA (P21005, Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS), 100 mg/kg, one time per three days for 5 weeks. The other group was injected intraperitoneally with PBS as control. Mice were sacrificed for analysis one week after the last treatment.

Metabolic Studies

Mice were weighed at the end of experiment. Total plasma cholesterol (TCH), total triglycerides (TGs) and high-density lipoprotein (HDL) levels were determined with automated enzymatic technique (7080, HI TACH, Japan). Low-density lipoprotein (LDL) was detected with an automated chemically modified technique (Roche Modular DPP System, Roche, Switzerland).



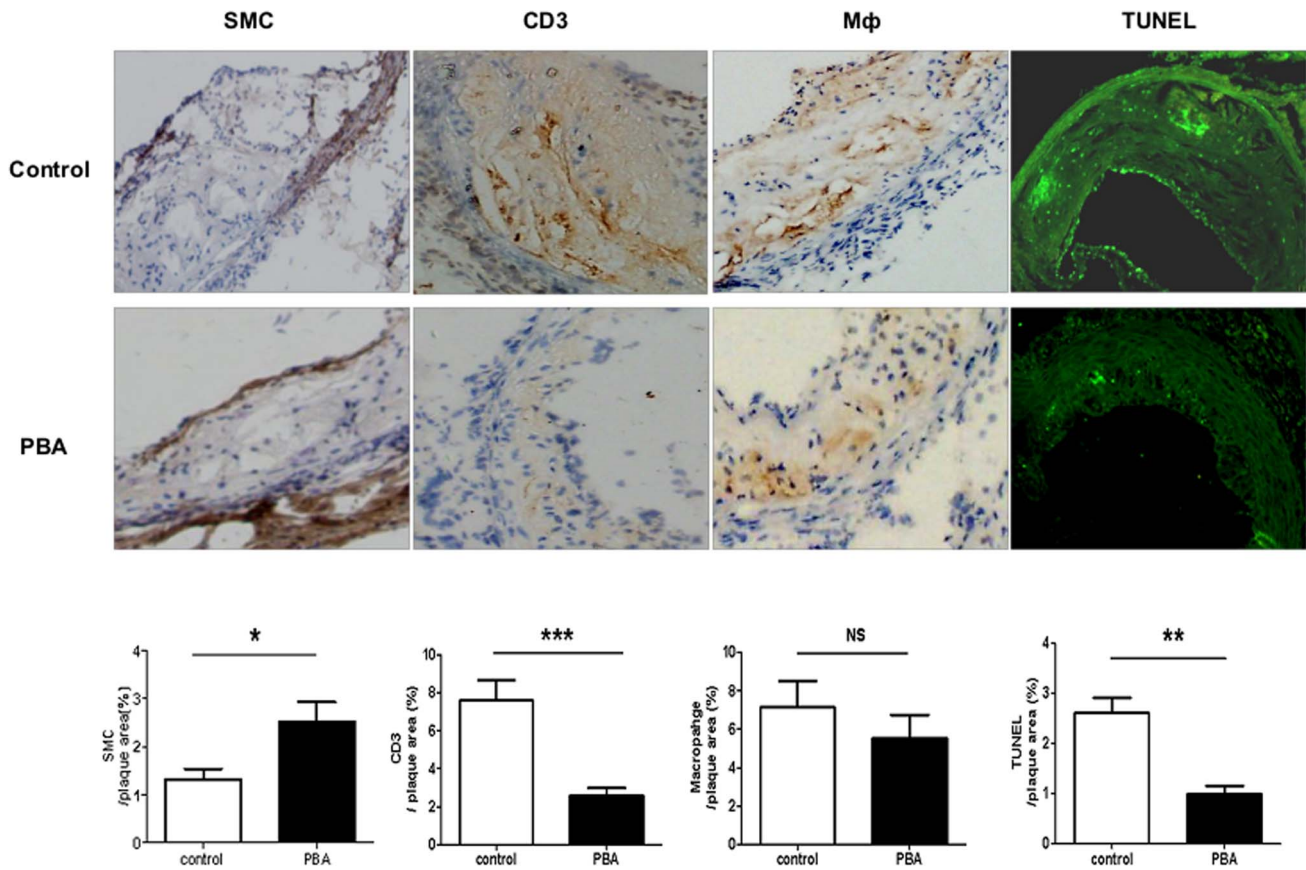


Figure 2. PBA treatment influenced the stability of plaque. Representative photomicrographs and the quantitative analysis of smooth muscle cell (SMC), CD3, macrophage (Mφ), and TUNEL in lesion of aortic root of ApoE^{-/-} mice. The aortic root sections were stained with rabbit polyclonal to α-smooth muscle actin, rat anti-mouse macrophage Moma-2 and rat anti mouse CD3. The content of macrophage, smooth muscle cell, CD3 T cell in lesion was analyzed respectively by immunohistochemistry. For detection of cell apoptosis, sections were incubated with anti-TUNEL antibody and the content of apoptotic cells was analyzed by immunofluorescence. (n = 10 per group), *p<0.05, **p<0.01, ***p<0.001. doi:10.1371/journal.pone.0087787.g002

Western Blots

Proteins obtained from separate thoracic and abdominal aorta, were separated by SDS-PAGE and blotted on PVDF membranes. Membranes were probed with primary antibodies, Phospho-eIF2a (p-eIF2a) (#3597 Cell Signaling Technology, USA), eIF2a (#9722 Cell Signaling Technology, USA), spliced XBP-1 (sXBP-1) (ab37152, Abcam, Hong Kong) and β-actin (Bioworld, Georgia, USA) overnight at 4°C, respectively, followed by secondary antibody conjugated with peroxidase (A0208, Beyotime, China) for one hour at room temperature. After washing, signals were visualized using electrochemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and autoradiography.

Immunohistochemistry and Immunofluorescence

After sacrificing, mice were perfused with PBS through the left ventricle and hearts were fixed in 4% paraformaldehyde solution overnight and embedded in optimal cutting temperature (OCT) (Sakura Finetek, Torrance, CA, USA) medium or paraffin. Aortic root sections were cut from the embedded hearts. Serial cryosection of 6~10 μm or paraffin-section of 4~6 μm were dissected along the longitudinal direction of aortic root vessels using cryotome (HM550, Thermo scientific, USA). Five sections spaced 80 μm apart of each aorta root were stained with oil red “O” staining (O0625, Sigma-Aldrich, USA). Corresponding frozen sections were subjected to immunohistochemical staining according to respective antibody protocols, including rat anti-mouse macrophage Moma-2 (MCA519A647, AbD serotec,

Table 2. PBA treatment did not change body weight and lipid profile.

	Weight (g)	TG (mmol/L)	TCH (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
Control	30.26±0.85	47.74±1.16	4.33±1.24	7.72±0.17	11.25±1.26
PBA	33.10±1.60	46.51±1.39	3.93±0.96	7.65±0.29	10.49±2.44
P value	0.17	0.52	0.82	0.82	0.79

The data are given as the mean±SD. TG:total triglycerides; TCH:total cholesterol; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol. doi:10.1371/journal.pone.0087787.t002

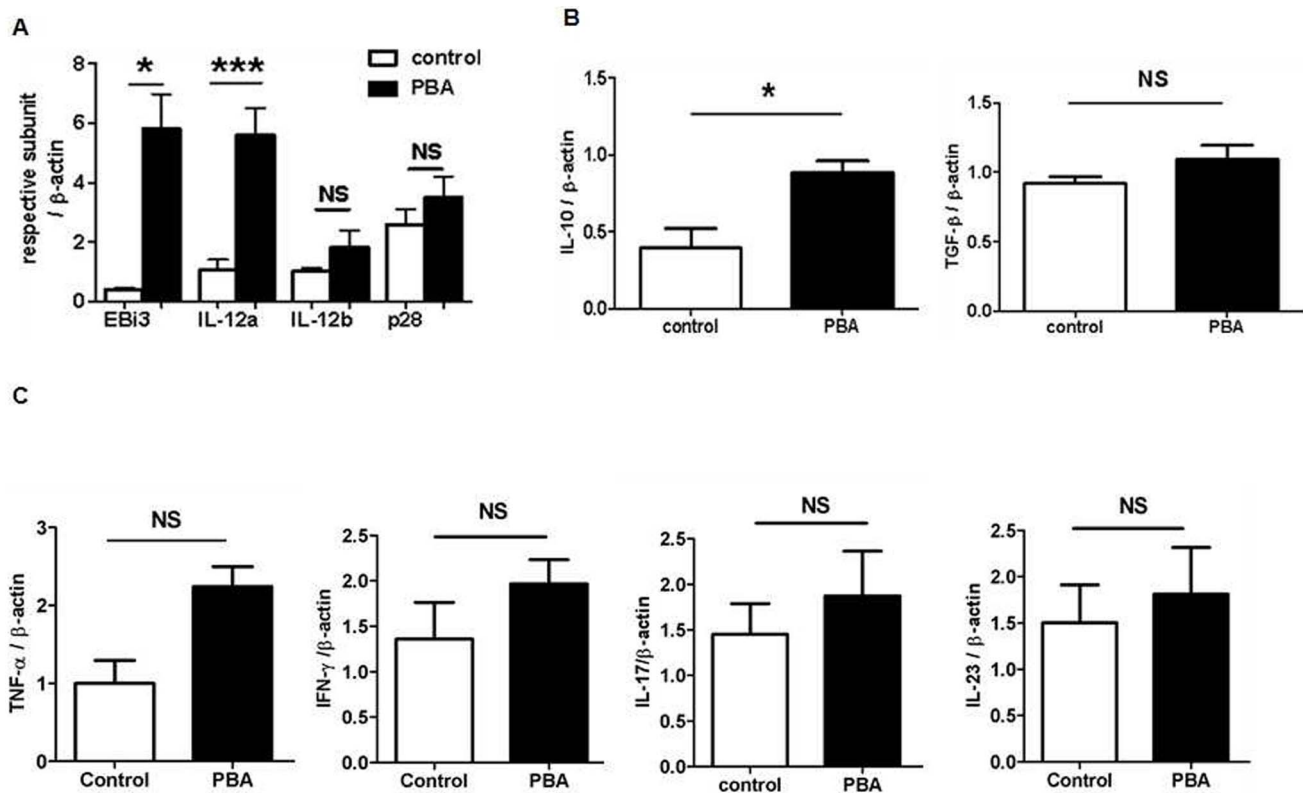


Figure 3. The influence of PBA treatment on cytokines in arterial wall. The expression of cytokine mRNAs in the thoracic and abdominal aorta was quantified by real-time PCR analysis and normalized to β -actin. Fold-changes in expression in PBA treated mice relative to controls are shown. **A.** IL-35-related four subunits, EBI3, IL-12a, IL-12b and p28. **B.** IL-10 and TGF- β . **C.** TNF- α , IFN- γ , IL-17 and IL-23. (n = 6 per group). *p < 0.05, **p < 0.01, ***p < 0.001.

doi:10.1371/journal.pone.0087787.g003

Oxford, U.K.), rabbit polyclonal to α -smooth muscle actin (ab5694, Abcam, Hong Kong), rat anti-mouse CD3 (MCA500G, AbD Serotec, Oxford, U.K.), rabbit anti-mouse Foxp3 (BA2032-1, BOSTER, China) and rabbit anti Phospho-PERK (p-PERK, 3179, Cell Signaling Technology, Danvers, MA, USA) to detect the levels of macrophage, smooth muscle cell, CD3⁺ T cell, Foxp3⁺ cells and p-PERK in lesion respectively. Alexa Fluor 555-labeled goat anti-rabbit IgG (A0462, Beyotime, China) was used to perform indirect immunofluorescence assays of Foxp3. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) was performed on paraffin sections with TUNEL kit (Roche, USA) according to manufacturer's instructions. Images were captured using an Olympus microscope (IX71; Olympus Corporation, Tokyo, Japan). The area of plaque and positive staining were measured using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

RNA Isolation and Real-Time PCR

Total RNA was isolated from thoracic and abdominal aorta using TRIzol reagent (15596-026, Invitrogen, Carlsbad, California, USA). Reverse transcription was performed with RT-PCR quick master mix (PCR-311, TOYOBO, Japan) to get cDNA, and real-time quantitative polymerase chain reactions were performed with Ultra SYBR mixture (CW0956, CW Bio, China) using CFX 96 Real-Time Detection System (Bio-RAD, USA). Sequences of related gene specific primers were included in Table 1. Data of relative molecule expression was presented by real-time quantitative

PCR using the $\Delta\Delta$ Ct model, and our data are reported as the fold change in the experimental group normalized to an endogenous reference gene (ACTB) and relative to the control group.

Flow Cytometry

Splenocytes isolated from PBA-treated and control mice were washed with PBS and stained with PE-Cy5-conjugated anti-CD4 (Anti-Mouse CD4 PE-Cyanine5, eBioscience, San Diego, CA, USA) and then were fixed and perforated by the fixation and permeabilization kit (eBioscience, San Diego, CA, USA) and then stained intracellularly with PE-conjugated anti-Foxp3 monoclonal antibodies (Anti-Foxp3 PE, eBioscience, San Diego, CA, USA). Flow cytometric analysis was performed using a Cytomics FC500 (Beckman Coulter, Brea, CA, USA) and the data were analyzed by CXP2.0 software.

Detection of Cytokines

Serum was collected for detection of TNF- α , IFN- γ , IL-17 and IL-10 using a mouse cytometric bead array (Cytometric Bead Array, BD Biosciences, San Jose, CA, USA) and for assays of IL-23 (EMC114.48, NeoBioscience, China), and TGF- β (BMS608/4, eBioscience, San Diego, CA, USA) using ELISA according to manufacturer's instructions. IL-35 in serum was quantified using IL-35 ELISA kits (RapidBio Lab, USA) according to the manufacturer's instruction and the sensitivity of this assay is ≥ 1.0 pg/ml.

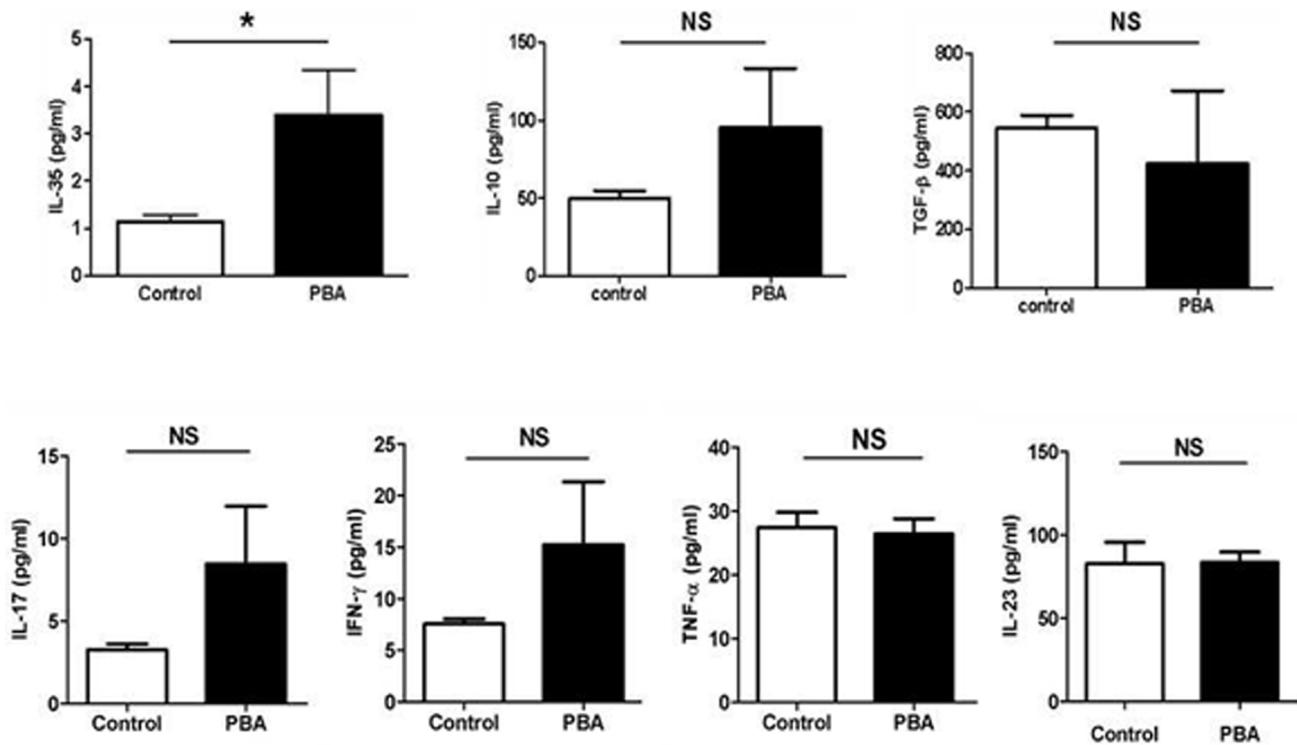


Figure 4. Impact of PBA treatment on circulating cytokines. Serum was obtained from ApoE^{-/-} mice with or without PBA treatment and the concentrations of IL-23, TGF-β and IL-35 in serum were detected by ELISA. The concentrations of TNF-α, IFN-γ, IL-17 and IL-10 were analyzed by cytometric bead array. (n=6 per group), *p<0.05. doi:10.1371/journal.pone.0087787.g004

Statistics

All analyses were done by SPSS 11.0 (SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM. Unpaired *t* tests were used for comparison of different group data. P value <0.05 was considered as significant.

Results

The Modulation of ER Stress and Attenuation of Atherosclerotic Lesion in ApoE^{-/-} Mice Treated by PBA

Previous researches have demonstrated that ER stress is involved in atherosclerosis and recovery of ER function is believed to be a critical factor for improvement of atherosclerosis [30–33]. Here, we firstly observed the impact of ER stress improvement by PBA treatment on atherosclerotic lesion. Consistent with previous research [27], the expression of ER stress signal proteins, p-eIF2α, sXBP-1 detected by western blots (Fig. 1A) and p-PERK by immunohistochemistry (Fig. 1B) were significantly decreased in mice treated with PBA compared to the control. Subsequently, PBA injection led to a decrease in plaque area in aortic root (Fig. 1C). Furthermore, the number of smooth muscle cells (SMC) increased while CD3⁺ T cells and TUNEL positive apoptotic cells (Fig. 2) in lesion decreased in the PBA injection group compared to the control. In addition, as shown in Table 2, there were no significant differences in body weight and plasma lipid profile between the PBA-treated group and the control. This indicates that modulation of ER stress by PBA attenuates the formation of lesion and increases the stability of plaque.

The Increase of IL-35 in ER Stress-attenuated Lesion

We further investigated the change of cytokines in lesion after ER stress modulation by real-time PCR. As shown in Figure 3A, the recovery of ER stress by PBA significantly upregulated the expression of two IL-35-related subunits, IL-12α (p35) and EB13, but had no effect on other subunits, IL-12β and p28, implying an increase in the level of IL-35 after modulation of ER stress. Meanwhile, the modulation of ER stress also increased IL-10 level but had no effect on the level of TGF-β (Fig. 3B) and pro-inflammatory cytokines, TNF-α, IFN-γ, IL-17 and IL-23 (Fig. 3C).

The Increase of Serum IL-35 in ApoE^{-/-} Mice after PBA Treatment

The change in circulating cytokines was detected by cytometric bead array for TNF-α, IFN-γ, IL-17 and IL-10, and by ELISA for IL-23, TGF-β and IL-35. As shown in Figure 4, PBA treatment significantly increased the level of serum IL-35. In addition, the level of IL-10 showed an increasing potential. However, consistent with our findings in lesion, PBA treatment had no effect on other cytokines including TGF-β, TNF-α, IFN-γ, IL-17 and IL-23.

Upregulation of Foxp3⁺ Tregs in ApoE^{-/-} Mice after PBA Treatment

It has been reported that IL-35 is not only an effector molecule of Tregs but also an inducer of Tregs. Therefore, we detected the effect of PBA treatment on Treg cells. The spleen cells were collected from PBA treatment or control group and stained with CD4 and Foxp3 specific antibodies, and then the proportion of Tregs (CD4⁺ Foxp3⁺) in CD4⁺ T cells was analyzed by Flow

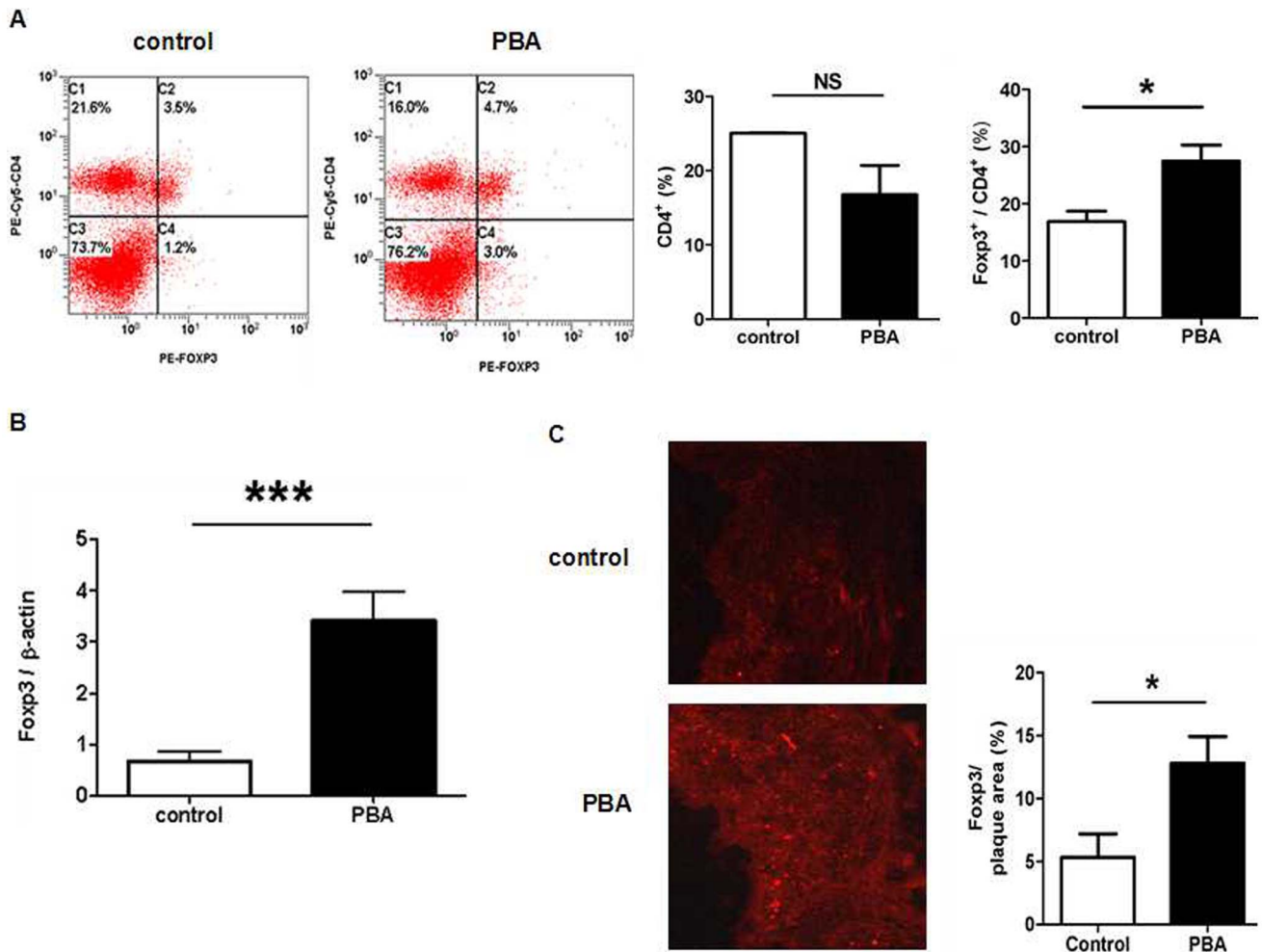


Figure 5. PBA treatment impacted Treg cells. **A.** Spleen cells were obtained from ApoE^{-/-} mice with or without PBA treatment and percentage of CD4⁺ T cells in spleen cells and percentage of CD4⁺Foxp3⁺ Tregs in total CD4⁺ T cells was analyzed by flow cytometry. **B.** The expression of Foxp3 mRNAs in the thoracic and abdominal aorta was quantified by real time-PCR analysis. Normalized to β -actin, fold-changes in expression in PBA treated mice relative to controls are shown. **C.** The expression of Foxp3 in plaque was analyzed by immunohistochemistry and immunofluorescence. The bright red dots in pictures are Foxp3⁺ cells (n = 6 per group). *P < 0.05, ***p < 0.001. doi:10.1371/journal.pone.0087787.g005

Cytometry. As shown in Figure 5A, after PBA treatment, the proportion of total CD4⁺ T cells in spleen showed no significant change but the percentage of Foxp3⁺ Tregs in CD4⁺ T cells markedly increased compared with the control. We further measured the mRNA level of Foxp3, a specific transcriptional factor for Tregs in lesion. As shown in Figure 5B, PBA treatment led to an increase of Foxp3 mRNA in local plaque. Immunohistochemical staining of Foxp3 confirmed the result (Fig. 5C). Collectively, the modulation of ER stress increases the Foxp3⁺ Tregs both in lesion and peripheral immune organ.

The Low Expression of IL-35 in ApoE^{-/-} Mice with Atherosclerotic Plaque

The above results indicate that modulation of ER stress by PBA attenuates atherosclerotic lesion and upregulates level of IL-35 both in arterial wall and in circulation. To further confirm the association of IL-35 with atherosclerosis, we detected the change of serum IL-35 level in development process of plaque in ApoE^{-/-} mice compared with wild type C57BL/6 mice. As shown in Figure 6A, there was a basic level of IL-35 in serum of wild type C57BL/6 as well as ApoE^{-/-} mice without plaque at 8 weeks.

After 8 weeks high-fat diet, while C57BL/6 mice without plaque showed an increased IL-35 level, ApoE^{-/-} mice with established plaque remained low level of IL-35. The mRNA level of IL-35 subunits p35 and EBI3 in artery wall also decreased in ApoE^{-/-} mice with plaque, compared with wild type C57BL/6 (Fig. 6B). This suggests that the low level of IL-35 might be associated with formation of atherosclerosis lesion.

Discussion

Here, we find that IL-35 is involved in atherosclerosis in ApoE^{-/-} mice. ApoE^{-/-} mice with atherosclerotic lesion have lower levels of IL-35 but the level increases in mice with attenuated plaque treated with PBA. More importantly, we find that modulation of ER stress by PBA treatment mainly upregulates immune negative regulating molecules, IL-35 as well as IL-10 and the transcription factor Foxp3, but has no obvious impact on pro-inflammatory molecules, such as TNF- α , IFN- γ , IL-17 and IL-23, which provides a new insight into the benefits of ER stress recovery to attenuated plaque.

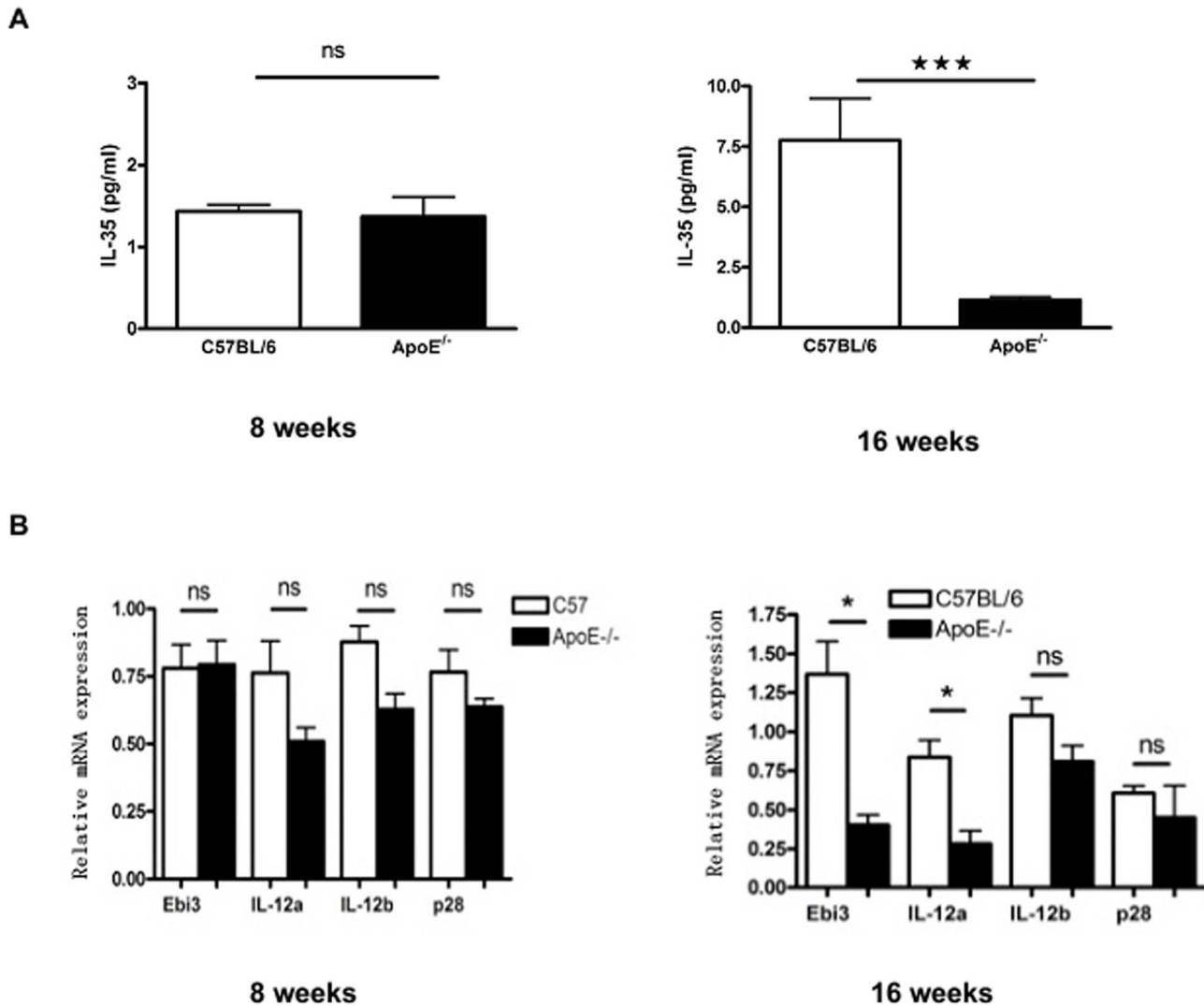


Figure 6. IL-35 expression in ApoE^{-/-} mice with atherosclerotic plaque. A. Serum was obtained from C57BL/6 and ApoE^{-/-} mice at 8 weeks of age fed with normal diet, without plaque (8 weeks) and at 16 weeks of age, after 8 weeks on a high-fat diet, C57BL/6 without plaque and ApoE^{-/-} mice with established plaque (16 weeks). The concentrations of IL-35 (pg/mL) in serum were detected by ELISA. **B.** Total RNA was isolated from thoracic and abdominal aorta of C57BL/6 and ApoE^{-/-} mice at 8 weeks of age fed with normal diet, without plaque (8 weeks) and at 16 weeks of age, for 8 weeks after high-fat diet, C57BL/6 without plaque and ApoE^{-/-} mice with established plaque (16 weeks). The expression of IL-35-related subunits was analyzed by real-time PCR. (n=3 per group), *p<0.05,***p<0.001. doi:10.1371/journal.pone.0087787.g006

To date, little is known about the role of IL-35 in atherosclerosis. Although previous research shows that expression of EBI3 and p35 is detected in human atheroma plaques, but other subunits, IL-27 α /p28 can also be found in plaque [34]. Therefore, it cannot be concluded that IL-35 exists in plaque. Here, we detected four subunits of IL-12 family, p35, p28, p40 and EBI3, and found that only two subunits of IL-35, p35 and EBI3, increased in arterial wall treated with PBA, but other related subunits, p40 (IL12 β) and p28 (IL-27 α), did not change. This suggests an increased level of IL-35 in attenuated plaque treated with PBA.

Atherosclerotic plaques, particularly advanced lesions, contain a large amount of toxic lipids (such as saturated fatty acids or free cholesterol) and pro-inflammatory cytokines (TNF- α , IFN- γ , IL-6, IL-12 etal). The pathophysiological environment can activate UPR which in turn upregulates expression of inflammatory genes (such as IFN- γ and IL-17) accelerating the progress of

atherosclerotic plaque [35,36]. Here, our results showed that modulation of ER stress by PBA application *in vivo* significantly decreased cell apoptosis, inflammation and size of plaque and increased the stability of plaque, which was consistent with previous research. Interestingly, the modulation of ER stress mainly upregulated immune negative regulating molecules such as IL-35, as well as IL-10 and Foxp3 in present research, but had no obvious impact on pro-inflammatory molecules, such as IL-17 and IFN- γ . However, the mechanism of this phenomenon is unknown. Previous research has shown that when overexpressed in cells, EBI3 tends to accumulate in an immature form in the ER associated with the molecular chaperone, calnexin, compatible with the notion that EBI3 associates with another partner that was not sufficiently abundant in these cells to enable its secretion [28,29]. Therefore, one possible explanation is that PBA may help the assemblies of EBI3 with IL-12 α (p35) and then result in an increased IL-35 which in turn amplifies Tregs or induces new Treg

production, as supported by the elevation of IL-10 and Foxp3 expression in plaque and the increase of Foxp3⁺ Treg in spleen.

Although our research did not confirm the causal relationship of IL-35 with atherosclerosis absolutely, it provided a new direction for the research about atherosclerosis. Because IL-35 comprises two subunits, p35 and EB13, which take part in the composition of IL-12 and IL-27 respectively, it is difficult to produce recombinant IL-35 and neutralizing antibody or knockdown/knockout related genes without influencing other cytokines. We believe that further research should overcome those difficulties and the detailed mechanism of IL-35 influencing atherosclerosis would be elucidated in future.

Collectively, our results indicate that IL-35 is involved in the atherosclerosis. The modulation of ER stress by PBA is able to

upregulate cytokine IL-35 which may contribute to attenuation of plaque in ApoE^{-/-} mice.

Acknowledgments

Thanks for the language editing of professor Wanjun Chen of US National Institutes of Health.

Author Contributions

Conceived and designed the experiments: LZ QW FZ. Performed the experiments: BW SD. Analyzed the data: ZD. Contributed reagents/materials/analysis tools: CG YS. Wrote the paper: WB XS LZ.

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