

Curcumin ameliorates atherosclerosis in apolipoprotein E deficient asthmatic mice by regulating the balance of Th2/Treg cells

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ABSTRACT

Background: Allergic asthma and atherosclerosis represent different directions of inflammatory responses of CD4⁺ T cells, and allergic asthma accelerates atherosclerosis formation. Curcumin could ameliorate the progression of both atherosclerosis and allergic asthma.

Purpose: We aimed to investigate the roles of curcumin in asthma-accelerated atherosclerosis plaque formation, and the change of CD4⁺ T-cell subsets in this process.

Methods: Six to eight-week-old apolipoprotein E^{-/-} (apoE^{-/-}) mice were sensitized and challenged by ovalbumin (OVA) to establish an allergic asthma model, and then received curcumin or vehicle treatment for 8 weeks.

Results: The accelerated atherosclerosis was induced by allergic asthma accompanied by increased T helper cell (Th)2 and Th17 cells and decreased regulatory T cells (Tregs) in the spleen. After the 8-week treatment with curcumin, the lesion areas in the aortic root in asthmatic mice significantly improved, and the elevated Th2 and Th17 cells significantly decreased, but Tregs markedly increased. Although curcumin treatment markedly reduced the interleukin (IL)-4 and IL-13 in serum and spleen, the elevated IL-17A did not decrease. Moreover, Th1 cells showed no significant change between different groups. The mRNA expression levels of M1 macrophage-related inflammatory factors IL-6, iNOS and IL-1β were markedly elevated in the spleens of asthmatic mice, but significantly decreased after the 8-week treatment with curcumin.

Conclusion: Curcumin ameliorated the aggravation of atherosclerotic lesions and stabilised plaque by modulating the balance of Th2/Tregs in asthmatic apoE^{-/-} mice.

Introduction

The progression of many autoimmune diseases is associated with chronic inflammation, especially the CD4⁺ T cell-related inflammation (Hansson and Libby, 2006). The T helper cell (Th)1/Th2 yin-yang paradigm controls many different inflammatory diseases, including atherosclerosis and asthma (Daugherty and Rateri, 2002). Allergic asthma and atherosclerosis are chronic inflammatory diseases with potential systemic impact and represent different directions of inflammatory responses of CD4⁺ T cells. In atherosclerotic plaques, the Th1 cell immune response prevails, and many pro-inflammatory M1

macrophages are in the lipid-enriched environment. Meanwhile, the decreased anti-inflammatory regulatory T cells (Tregs) and the activated pro-inflammatory Th17 cells also affect the atherosclerosis process, but the importance of Th2 cells in atherosclerosis was not clear (Gisterå and Hansson, 2017; Zhao et al., 2011). Conversely, antigen-specific allergic asthma is classically thought to be a Th2-oriented inflammatory disease (Lloyd and Hessel, 2010). Although the pathogenesis has not been clarified, many inflammatory cells, such as macrophages, mast cells, smooth muscle cells (SMCs), T cells, and various inflammatory mediators that they secrete and synthesise, are present in the bronchoalveolar wall in asthma and in the atherosclerotic aortic

Abbreviations: Th, T helper cell; Tregs, regulatory T cells; Teff, effector T cells; OVA, ovalbumin; IL, interleukin; iNOS, inducible nitric oxide synthase; SMCs, smooth muscle cells; apoE, apolipoprotein E; EAN, experimental autoimmune neuritis; EAM, experimental autoimmune myocarditis; IFNγ, interferon γ; IgE, immunoglobulin E

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wall (Gisterå and Hansson, 2017; Lloyd and Hessel, 2010), suggesting that the two diseases share a similar pathogenesis process, and that there may be an interaction between these two inflammatory diseases. It has been shown that chronic and acute allergic lung inflammations promote atherogenesis and aortic lesion pathology (Liu et al., 2016), and that the severity of asthma correlates with the degree of pathological vessel changes of atherosclerosis (Tuleta et al., 2017). Using apolipoprotein E^{-/-} (apoE^{-/-}) mice, our previous study showed that allergic asthma accelerates atherosclerosis by modulating the balance of effector T cells (Teff)/Treg cells, which are associated with increased Th2 and Th17 cells (Wang et al., 2014). Patients with allergic asthma also show a higher risk of developing atherosclerosis (Guo et al., 2016).

Curcumin, a bioactive ingredient derived from the *Curcuma longa*, could modulate atherogenesis through suppression of aP2 and CD36 expression in macrophages (Hasan et al., 2014), and prevent atherosclerosis by tuning cholesterol homeostasis and modulating inflammatory response in M1 macrophages (Chen et al., 2015). Curcumin also protects against atherosclerosis in apoE^{-/-} mice by inhibiting Toll-like receptor 4 expression (Zhang et al., 2018). However, the roles of curcumin in CD4⁺ T cell-related inflammation appear to be multifaceted and differ according to the disease. Some studies have shown that curcumin converts Tregs to Th1 cells and enhances interferon (IFN)- γ production in cancer patients (Xu et al., 2017; Zou et al., 2018). Others have found that curcumin suppresses the autoimmune response in experimental autoimmune neuritis (EAN) by inhibiting Th1 and Th17 polarisation (Han et al., 2014), and attenuates the scurfy-induced immune disorder (IPEX syndrome) by inhibiting Th1/Th2/Th17 responses (Lee et al., 2017). Moreover, the effect of curcumin on the CD4⁺ T cell balance seems inconsistent in different studies of allergic diseases. Shin et al. reported that curcumin could attenuate food allergy symptoms by reducing Th2-related cytokines and enhancing Th1-related cytokines in a mouse model (Shin et al., 2015), whereas others found that curcumin can markedly attenuate inflammation in an ovalbumin-sensitised asthma model by inhibiting Th17 and increasing Tregs cells (Ma et al., 2013). Furthermore, the effect of curcumin on the balance of CD4⁺ T cell in atherosclerosis progression has not been investigated until now.

In summary, curcumin ameliorates the progression of both atherosclerosis and allergic asthma, and effects the direction of CD4⁺ T cell subset balance in different disease models. Thus, we are interested in the roles that curcumin plays in asthma-accelerated atherosclerosis plaque formation, and the change of CD4⁺ T cell subsets in this process, which is also the purpose of this study.

Material and methods

Allergic asthma mouse model

ApoE^{-/-} mice with a C57BL/6J background were kept in a temperature- and humidity-controlled facility on a 12-hour light-dark cycle with free access to food and water. Mice were weaned at 4-weeks of age and then fed normal food. This animal study was approved by the

institutional ethics committee for animal experiments of Xi'an Jiaotong University. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Six to eight-week-old apoE^{-/-} mice were used throughout the study. Mice were randomly assigned to the control (n = 6) and asthma groups (n = 12). Sensitisation and challenge were performed as previously described (Wang et al., 2014). Mice were euthanised 24 h after the last challenge. The assessment of the asthmatic mouse model was performed as previously described (Wang et al., 2014).

Administration of curcumin

Curcumin (Sigma-Aldrich, purity $\geq$ 99.5%) was dissolved in 0.1% sodium carboxymethyl cellulose suspension. Simultaneously with the aerosolised exposure, the asthmatic mice were randomly assigned to two asthmatic subgroups and received either curcumin treatment (200 mg/kg/d, orally by gastric gavage, n = 6) or an equal volume of vehicle (n = 6) for 8 weeks.

For determining the adequate dosage of curcumin, we performed the initial experiments with various concentration of curcumin; we detected the efficacy of curcumin treatment by measuring lesion area in the aortic root of asthmatic apoE^{-/-} mice, and the 200 mg/kg/d effective dose was selected (see supplementary material, Fig. S).

Cytokine and antibody measurement

Blood samples were taken before euthanasia, and the serum was then collected. Circulating immunoglobulin E (IgE), IFN γ , interleukin (IL)-4, IL-13, and IL-17 were measured using mouse enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions (IgE kit from Cayman Chemical, IL-4 kit from BioLegend, the others from RayBiotech, Inc).

Quantitative evaluation of atherosclerotic lesions in the aortic root

The aortic root was dissected under a microscope and frozen in optimal cutting temperature compound (OCT) embedding medium for serial 7- μ m cryosectioning that covers 500 μ m of the root. The lesion area (lipids) was detected by Oil Red O staining (Sigma). The macrophage content was analysed by immunofluorescence staining with rat anti-mouse CD68 monoclonal antibody (AbDserotec) followed by a FITC-labelled rabbit anti-rat IgG antibody (Abcam). All sections were photographed with identical exposure settings and all images were quantified using Image-Pro plus 6.0 software.

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

Total RNA was isolated from the spleen using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA was reversely transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo), and analysed by quantitative real-time PCR

Table 1.
Primer sequences used for real-time PCR.

	Forward primer 5'–3'	Reverse primer 5'–3'
GAPDH	ACGGGAAGCTCACTGGCATG	CACCCTGTTGCTGTAGCCGT
IFN γ	ACTCAAGTGGCATAGATGTGGAAG	GACGCTTATGTTGTTGCTGATGG
IL-4	ACGGAGATGGATGTGCCAAAC	AGCACCTTGAAGCCCTACAGA
IL-13	CTGACCCCTTAAGGAGCTTATTG	TCGATTTTGGTATCGGGGA
IL-17A	GCTGTTGCTGCTGCTGAG	ACGGTTGAGGTAGTCTGAGG
Foxp3	TGCGACCCCTTTACCTAT	AGGTTGTGCGGATGGCATT
IL-6	TGATGGATGCTACCAACTG	GAGAGCATTGGAAATTGGGG
iNOS	TGTGGCTACCACATTGAAGA	GCCCTCACCATTATCTTTAC
IL-1 β	TCCACCTCAATGGACAGAATA	ATTAGAAACAGTCCAGCCCA

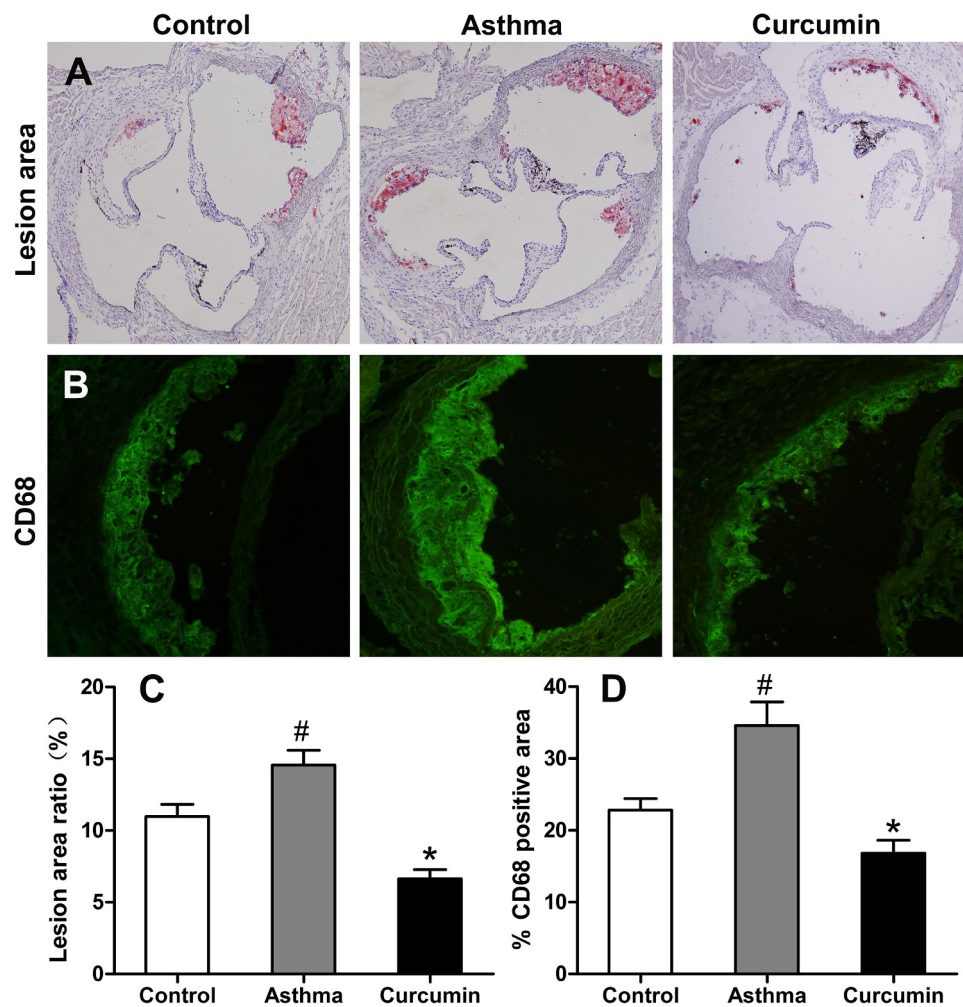


Fig. 1. Effects of curcumin on the lesion area and plaque stability in asthmatic apoE^{-/-} mice. ApoE^{-/-} mice were randomly assigned to control and asthma groups and asthmatic mice were further assigned to the curcumin- and vehicle-treated groups. The fraction area of lesion was measured by Oil Red O Staining (A and C), magnification $\times 100$. The CD68 content representing macrophages were evaluated by immunofluorescence staining (B and D), magnification $\times 200$; Data are presented as mean $\pm$ SD (n = 6). [#] $p < 0.05$ vs. control group, ^{*} $p < 0.05$ vs. vehicle-treated asthma group.

with a SYBR[®] Premix Ex Taq[™]II RT-PCR Kit (TaKaRa, Japan). All real-time reactions were performed on the iQ5[™] Multicolor Real-Time PCR detection system (Bio-Rad, USA). The housekeeping gene GAPDH was used as an internal control. The primer sequences are listed in Table 1.

Flow cytometry

Splenocytes were isolated with a nylon cell strainer, and red blood cells were lysed and subsequently washed. The percentage of CD4⁺CD25⁺Foxp3⁺Tregs in total CD4⁺ T cells was analysed using a Mouse Regulatory T Cell Staining Kit (eBioscience, USA) according to the manufacturer's protocol. To analyse Th1, Th2, and Th17 cells, the activated splenocytes were stained with PE-labelled anti-CD4 and IL4, FITC-labelled anti-CD4, IFN γ and IL-17A, respectively (all Abs were from eBioscience). Analysis was performed on a FACS flow cytometer with the CellQuest software (Becton-Dickinson). Data were analysed as the percentage of cells with positive staining, after adjusting the difference between the cellular fluorescence and non-specific background fluorescence.

Statistical analyses

All data were expressed as means $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the statistical

significance of the differences among the groups if values followed a normal distribution (otherwise a Kruskal–Wallis H test would be used). A level of $p < 0.05$ was considered significant.

Results

Curcumin ameliorates atherosclerosis of asthmatic apoE^{-/-} mice

In the aortic root of asthmatic apoE^{-/-} mice, the fraction area of lesions markedly increased (Fig. 1A and C; vs. control mice, $p < 0.05$), but significantly decreased after 8-weeks of treatment with curcumin (Fig. 1A and C; vs. vehicle-treated asthmatic apoE^{-/-} mice, $p < 0.05$). Similarly, the macrophage content representing the plaque stability significantly increased in asthmatic apoE^{-/-} mice (Fig. 1B and D; vs. control mice, $p < 0.05$), but significantly decreased after 8-weeks of curcumin treatment (Fig. 1B and D; vs. vehicle-treated asthmatic apoE^{-/-} mice, $p < 0.05$). These results indicate that curcumin treatment decreases the atherosclerotic size but increases the plaque stability in asthmatic apoE^{-/-} mice.

Curcumin increases the spleen content of Treg cells

We observed Treg cells and measured the mRNA expression level of their prototypical transcription factor Foxp3 and found that both

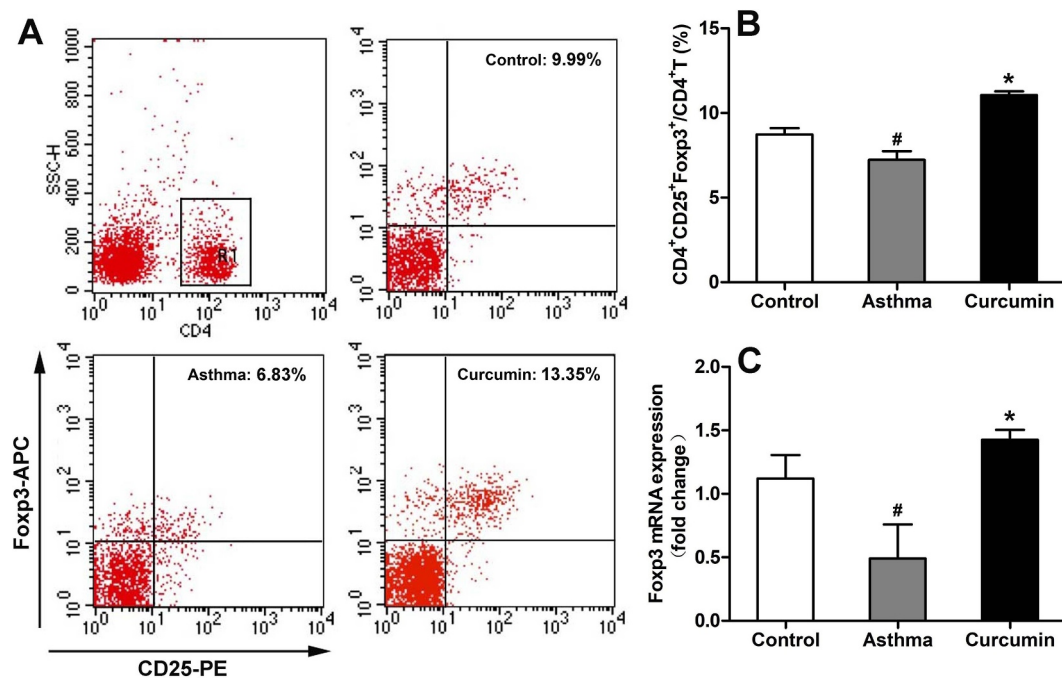


Fig. 2. The effect of curcumin on the spleen contents of Treg cells. (A) Representative FACS results from a single mouse in three groups (gated by CD4⁺ T cells). The percentage of positive cells is shown in each panel. (B) The comparison of the Treg content among the three groups. (C) The mRNA expression level of Fop3 (a prototypical transcription factor of Treg) in the three groups. Data were presented as mean $\pm$ SD (n = 6). [#]*p* < 0.05 vs. control group, ^{*}*p* < 0.05 vs. vehicle-treated asthma group.

significantly decreased in asthmatic mice (Fig. 2B and C vs. control mice, *p* < 0.05); however, 8-weeks of curcumin treatment markedly increased the spleen Treg content and Fop3 expression (Fig. 2B and C; vs. vehicle-treated asthmatic mice, *p* < 0.05).

Curcumin decreases the spleen contents of Teff cells

We found that the contents of Th2 and Th17 cells markedly increased in asthmatic mice (Fig. 3A–D; vs. control mice, *p* < 0.05), and significantly decreased after curcumin treatment (Fig. 3A, B, C, and D; vs. vehicle-treated asthmatic mice, *p* < 0.05). However, Th1 cells were not significantly affected among different groups (Fig. 3E). Consistent with the contents of CD4⁺ T cells in the spleen, the mRNA expression levels of IL-4 and IL-13 (Th2-related cytokine) and IL-17A (Th17-related cytokine) markedly increased in asthmatic mice (Fig. 3F, G and H; vs. control mice, *p* < 0.05). Curcumin treatment significantly decreased IL-4 and IL-13 expression (Fig. 3F and G; vs. vehicle-treated asthmatic mice, *p* < 0.05), but only slightly decreased IL-17A expression (Fig. 3H; no statistical difference), and did not change the expression of IFN γ (Th1-related cytokine) (Fig. 3I).

Curcumin decreases the protein expression levels of IL-4, IL-13, and IgE in serum

The serum concentrations of IL-4, IL-13, IL-17, and IgE in asthmatic mice significantly increased, as measured by ELISA (Fig. 4A–D; vs. control mice, *p* < 0.05). Curcumin treatment significantly decreased the serum level of IL-4, IL-13, and IgE (Fig. 4A and B; vs. vehicle-treated asthmatic mice, *p* < 0.05), but had little effect on IL-17 (Fig. 4C). The level of IFN γ did not show any significant change among different groups (data not shown).

Curcumin inhibits M1 macrophage activation

Macrophage activation is an important indicator of inflammatory disease. To further investigate the effect of curcumin on macrophage

activation, we measured the mRNA levels of M1 macrophage-related inflammatory factors IL-6, inducible nitric oxide synthase (iNOS), and IL-1 β in the spleen of asthmatic apoE^{−/−} mice. These cytokines were significantly elevated in asthmatic apoE^{−/−} mice (Fig. 5; vs. control mice, *p* < 0.05), but significantly decreased after curcumin treatment (Fig. 5; vs. vehicle-treated asthmatic mice, *p* < 0.05).

Discussion

The chronic inflammation of atherosclerosis is dominated by Th1 (Daugherty and Rateri, 2002; Zhao et al., 2011), but allergic asthma is driven by Th2 (Lloyd and Hessel, 2010). Recent studies have shown that allergic asthma accelerates atherosclerosis plaque formation (Liu et al., 2016; Wang et al., 2014), and that curcumin could ameliorate both atherosclerosis and allergic asthma progressions (Chen et al., 2015; Hasan et al., 2014; Ma et al., 2013). The effects of curcumin on asthma-induced atherosclerosis plaque formation and on the change of CD4⁺ T-cell subsets in this process remain unknown. In the present study, we showed that the asthmatic condition accelerates atherosclerosis, but curcumin treatment ameliorates the atherosclerotic plaque in asthmatic apoE^{−/−} mice by decreasing Th2 and increasing Tregs.

It has been shown that curcumin plays cardiovascular protective roles through its anti-inflammatory, anti-coagulant, anti-oxidant, and lipid-lowering activities (Banerjee et al., 2003; Saleh et al., 2016; Wongcharoen and Phrommintikul, 2009). We also found that curcumin ameliorates experimental autoimmune myocarditis (EAM) by reducing the infiltration of inflammatory macrophages and by polarising M0 and M1 macrophages to the M2 phenotype (Gao et al., 2015), and prevents atherosclerosis (Chen et al., 2015). In the present study, we found that the content of plaque macrophages significantly increased in asthmatic apoE^{−/−} mice, but 8-week treatment of curcumin significantly reduced this increase. Consistently, the fraction area of lesion markedly increased in asthmatic apoE^{−/−} mice, but significantly decreased after 8-weeks of curcumin treatment. These results suggested that the curcumin treatment decreases the atherosclerotic size but increases the

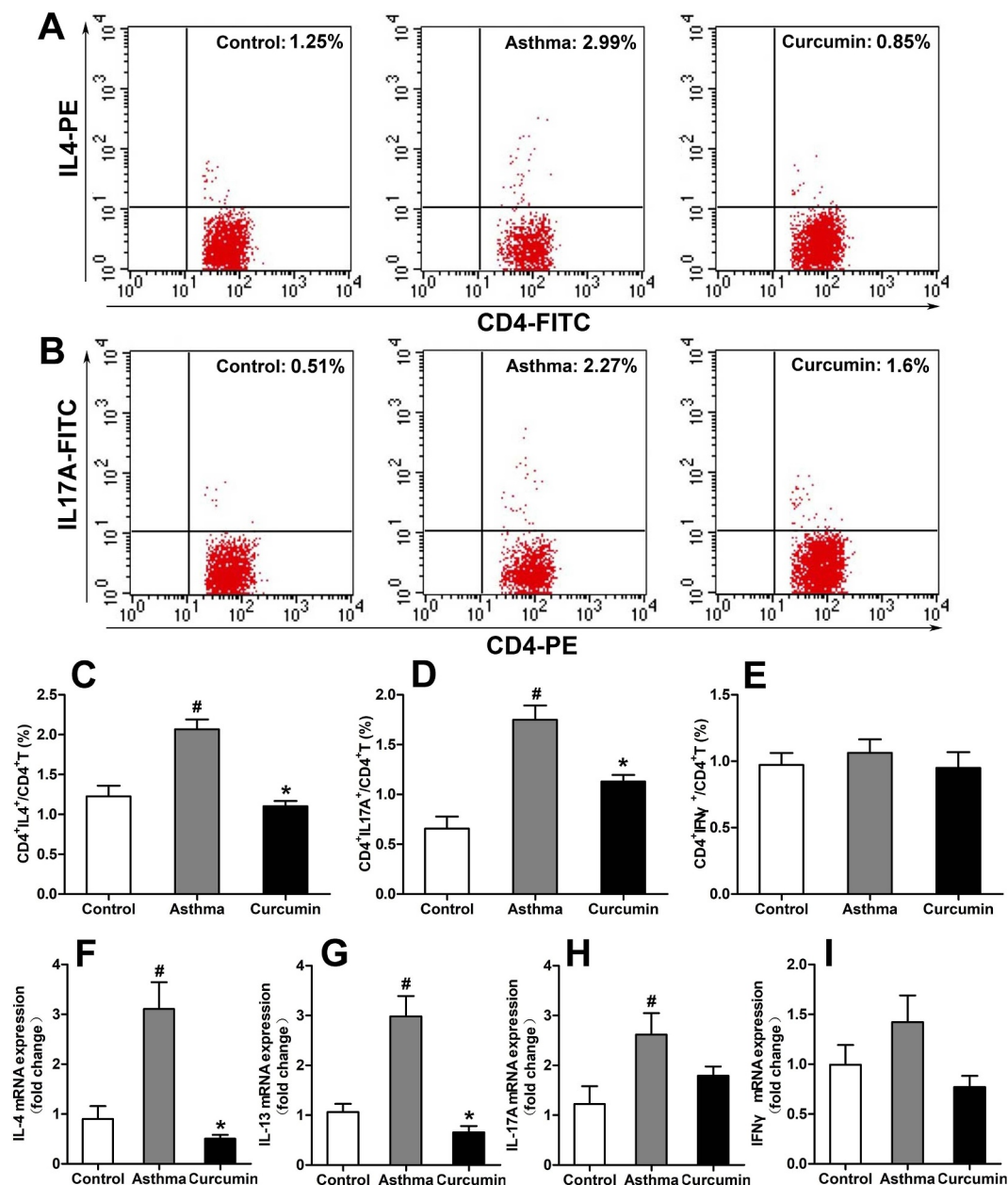


Fig. 3. The effect of curcumin on the spleen contents of Teff cells. (A) Representative FACS results of Th2 from a single mouse in three groups. (B) Representative FACS results of Th17 from a single mouse in three groups. The percentage of positive cells was shown in each panel. Comparisons of Th2 (C), Th17 (D), and Th1 (E) contents among the three groups. Relative mRNA expression levels of Teff markers in the spleen were measured by RT-PCR. IL-4 and IL-13 represent Th2 (F and G), IL-17A represents Th17 (H), and IFN γ represents Th1 (I). Data were presented as mean $\pm$ SD (n = 6). [#]p < 0.05 vs. control group, * p < 0.05 vs. vehicle-treated asthma group.

plaque stability in asthmatic apoE^{-/-} mice.

Curcumin plays multifaceted roles in CD4⁺ T cell-related inflammation in different diseases. Some studies have shown that curcumin could convert Tregs to Th1 cells by repressing Foxp3 expression and enhancing the IFN- γ production in colon cancer and lung cancer patients (Xu et al., 2017; Zou et al., 2018). Others have found that curcumin suppresses Th1 and Th17 cells in autoimmune disease (Han et al., 2014; Lee et al., 2017). Moreover, even in previous studies of allergic diseases, the effect of curcumin on the CD4⁺ T cell balance remains inconsistent (Shin et al., 2015; Ma et al., 2013). In the present study, by aggravating atherogenesis, we showed that the contents of Th17 and Th2 cells and their relative cytokine expression markedly increased but Tregs significantly decreased in the spleen of asthmatic mice, agreeing with previous results (Liu et al., 2016; Wang et al.,

2014). The curcumin treatment clearly decreases the lesion area in the aortic root, and also significantly decreases the elevated Th2 and Th17 cells and markedly increases Tregs. These results suggest that curcumin improves the atherosclerosis in asthmatic apoE^{-/-} mice associated with decreasing Th2 and Th17 cells and increasing Treg cells.

A recent study reported that curcumin attenuates allergic airway inflammation by inhibiting Th17 cells and increasing Tregs in ovalbumin-sensitised mice (Ma et al., 2013). However, in the present study, although the level of IL-4 and IL-13 expression was markedly decreased by the curcumin treatment, the elevated IL-17A was not decreased, indicating that curcumin attenuates atherosclerosis in asthmatic apoE^{-/-} mice, but not by inhibiting Th17. This discrepancy may be due to different inflammatory responses of CD4⁺ T cells in different disease models. In addition, we did not observe an obvious association

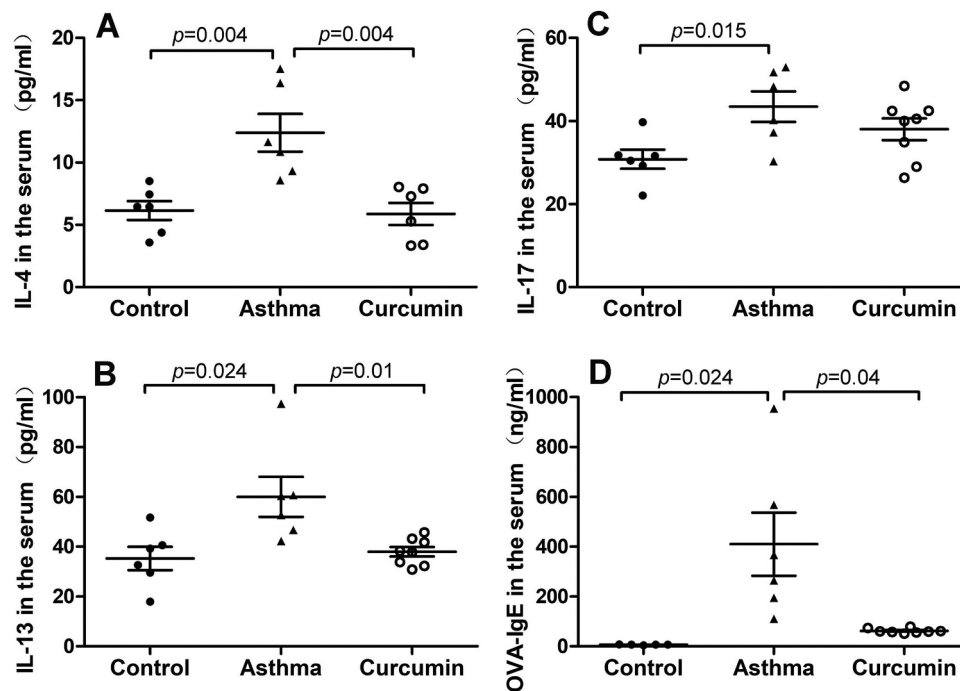


Fig. 4.. The effect of curcumin on the level of cytokine in the serum. The serum concentrations of IL-4 (A), IL-13 (B), IL-17 (C), and IgE (D) were measured by ELISA. Data were presented as mean $\pm$ SD (n = 6).

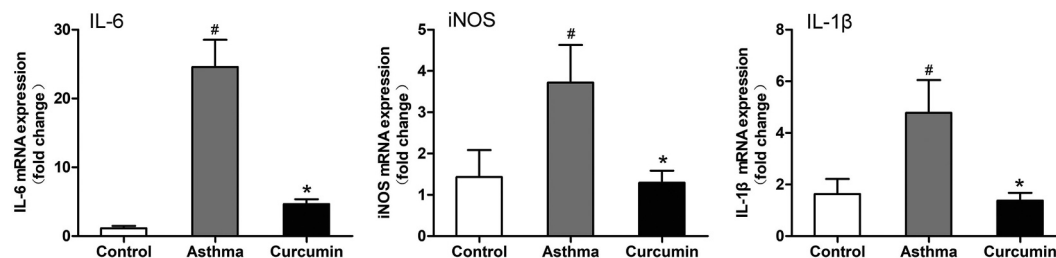


Fig. 5.. The effect of curcumin on the M1 macrophage activation in the spleen. The mRNA expression levels of M1 macrophage-related inflammatory factors IL-6, iNOS and IL-1 β were measured by RT-PCR. Data were presented as mean $\pm$ SD (n = 6). # p < 0.05 vs. control group, * p < 0.05 vs. vehicle-treated asthma group.

between Th1 cells and curcumin-induced anti-atherosclerosis effects, suggesting that Th1 cells may not be involved in the process of curcumin-mediated atherosclerosis plaque amelioration.

Macrophage activation is an important inflammation indicator (Li et al., 2018). Although previous studies have demonstrated that the presence of alternatively activated macrophages (M2) in an allergic airway disease may be merely a consequence of elevated Th2 responses (Nieuwenhuizen et al., 2012), it remains accepted that atherosclerosis is an inflammatory disorder in which macrophages play a central role. Moreover, Sharma et al. suggested that M1-mediated inflammation in the adipose tissue enhances M2-mediated inflammation in the asthmatic lung (Sharma et al., 2018). The elevated Th2 cells and IL-4 in allergic asthma could stimulate the synthesis of IgE (Kim et al., 2010; Wu, 2011), whereas the increased atherosclerotic lesion area and macrophage content in asthmatic apoE^{-/-} mice was partly due to activation of macrophages by IgE-Fc ϵ RI (Wang et al., 2014). In the present study, accompanied by ameliorated atherogenesis, we found that the expression of Th2 and IL-4 was markedly decreased by curcumin treatment in asthmatic apoE^{-/-} mice. Therefore, it would be important to know whether the curcumin treatment also inhibited the activation of M1 macrophages induced by IgE-Fc ϵ RI. To this end, we measured the mRNA expression levels of M1 macrophage-related inflammatory factors IL-6, iNOS, and IL-1 β in asthmatic apoE^{-/-} mice. Compared with the control mice, all of them were significantly elevated in asthmatic apoE^{-/-} mice, but were significantly decreased after 8

weeks of treatment with curcumin, indicating that curcumin-induced anti-atherosclerotic effects are related to the decrease of M1 macrophage activation in asthmatic apoE^{-/-} mice.

In conclusion, we found curcumin ameliorates the aggravation of atherosclerotic lesions and stabilises plaque by modulating the balance of Th2/Tregs in asthmatic apoE^{-/-} mice, which is associated with decreased Th2 cells and increased Treg cells, but not Th1 or Th17. Additional potential mechanisms warrant further investigation. Curcumin might have potential therapeutic implications for coronary artery disease and related manifestations of atherosclerosis induced by allergic asthma.

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Conflict of interest

The authors declare no conflicts of interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2018.09.194](https://doi.org/10.1016/j.phymed.2018.09.194).

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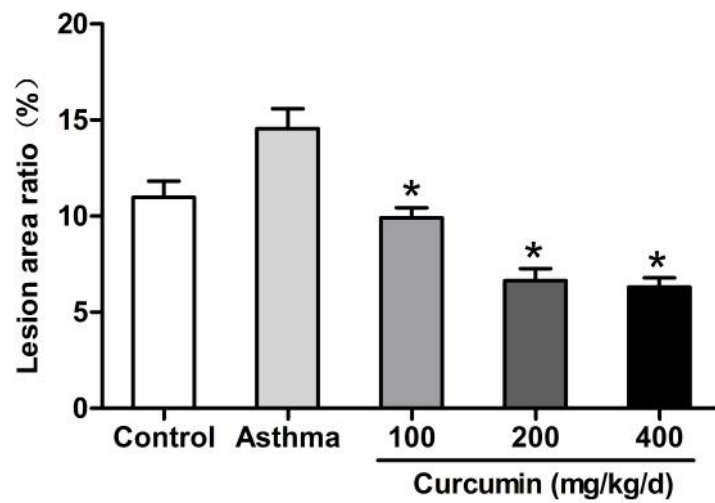


Figure S. Effective dose evaluation of curcumin in apoE^{-/-} mice. ApoE^{-/-} mice were randomly assigned to control and asthma groups, then asthmatic mice were further assigned to various concentration of curcumin treated groups. The lesion area in the aortic root of apoE^{-/-} mice was measured by Oil Red O Staining. Data are presented as mean $\pm$ SD. * $p < 0.05$ vs. asthma group.