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Original Article

Genistein alleviates atherosclerosis in apolipoprotein E-deficient mice by interrupting the OX40/OX40L pathway

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Abstract: More and more evidence shows that the OX40/OX40L interaction plays a critical role in the development of atherosclerosis. However, it is not known whether genistein, a natural phytoestrogen with anti-inflammatory effects found in soybean extract, can prevent experimental atherosclerosis by regulating the OX40/OX40L pathway. This study aims to explore the effect and the underlying mechanisms of genistein on the development of atherosclerosis in apolipoprotein E gene knockout ($ApoE^{-/-}$) mice. $ApoE^{-/-}$ mice, fed an atherogenic diet, were treated with genistein (15 and 45 mg kg^{-1} day^{-1}). *In vitro* studies were carried out in oxidized LDL (oxLDL)-stimulated SMCs. Our results show that genistein treatment remarkably reduced atherosclerotic plaque formation and reduced the serum levels of pro-inflammatory cytokines in $ApoE^{-/-}$ mice. Also, genistein promotes plaque stability in $ApoE^{-/-}$ mice, characterized by smaller necrotic core areas of atherosclerotic plaques and reduced MMP-9 protein expression in primary smooth muscle cells (SMCs). Furthermore, when mRNA expression and the protein expression of OX40 were significantly increased, they were inhibited by genistein in response to an atherogenic diet. Notably, $ApoE^{-/-}$ mice with an anti-OX40L antibody presented a significant decrease in atherosclerotic lesion formation, which has no further beneficial effects when combined with genistein. These results suggest that genistein potentially has atheroprotective effects that involve the inhibition of the OX40/OX40L pathway, which could be used to prevent and treat atherosclerosis.

Keywords: Genistein, atherosclerosis, OX40/OX40L, atheroprotection

Introduction

Atherosclerosis is a chronic inflammatory disease in which lipid metabolism disorder and the chronic inflammatory response coexist [1, 2]. Atherosclerosis is also a major cause of death and a critical risk factor for vascular disease, characterized by endothelial cell damage and increased levels of oxidized lipoprotein as white blood cells infiltrate the arteries [3, 4]. These raised, oxidized lipids induce an intense humoral immune response, followed by the production of antibodies specifically against oxidized low-density lipoprotein (oxLDL) and malondialdehyde LDL. However, there are limited agents clinically available for patients with this condition [5, 6].

Genistein is a principal isoflavone extracted from soy which has been reported to produce anti-cancer as well as anti-inflammatory effects [7, 8]. It also possesses cardioprotective effects, such as improving isoprenaline-induced cardiac hypertrophy and pressure overload (PO)-induced cardiac fibrosis [9, 10]. Moreover, a previous study indicated that genistein has the potential of inhibiting aortic atherosclerosis in rabbits [11]. However, the role and underlying molecular basis of genistein in diet-induced atherosclerosis in $ApoE^{-/-}$ mice is poorly understood and is yet to be explored.

Apart from lipids, immune responses also play a key role in the pathogenesis of atherosclerosis. It is generally believed that oxLDL activates the adaptive immune system after being recognized by macrophages or dendritic cells [2]. OX40 and its ligand (OX40L) are costimulatory molecules and belong to the TNF/TNFR family. In mice and humans, tnfrsf4 (OX40, CD134) is a

less well-studied member of the TNF receptor family, which is primarily expressed on activated CD4-positive and CD8-positive T cells [12, 13], but OX40L (Tnfsf4, CD134L) is expressed on many kinds of cells including vascular endothelial cells [14, 15]. OX40/OX40L interaction plays an important role in the proliferation and survival of T cells [16]. Furthermore, the activation of $OX40^+$ T cells promotes a specific isotype switching of B cells by interacting with its ligand OX40L. Current studies suggest that the OX40/OX40L pathway plays a critical role in vasa vasorum formation and in the manipulation of OX40/OX40L, effectively mitigating autoimmune-like diseases, including experimental autoimmune encephalomyelitis (EAE) [17], graft-versus-host disease [18], asthma [16], and arthritis [19]. Moreover, interruption of the OX40/OX40L signaling pathway with anti-OX-40L antibody treatment has been demonstrated to induce the regression of atherosclerosis. Therefore, whether genistein, as an anti-inflammatory agent, could efficiently protect against atherosclerotic lesion development through modulating OX40/OX40L signaling pathway remains unclear.

So, in the present study, we investigated the potential protective effects of early genistein intervention on atherosclerotic lesion formation in $ApoE^{-/-}$ mice kept on a high cholesterol diet (HCD). Additionally, we examined whether the OX40/OX40L pathway was involved in genistein-afforded atheroprotective effects.

Materials and methods

Reagents

Genistein (purity > 95%) was obtained from Xi'an QingYue Biotechnology Co, Ltd (Xi'an, Sha'anxi, China).

Animal experiment

The experimental design was licensed by the Institutional Animal Care and Use Committee and the Ethics Committee of Harbin Medical University and complied with the standards of animal welfare in China, which conform to the NIH guidelines. Male C57BL/6J background $ApoE^{-/-}$ mice and their controls were housed under a 12-h light/dark cycle. Starting with the 6th week, the mice were fed an HCD (containing 10% fat, 1.25% cholesterol and 0% cholic acid) for 9 weeks. 15 or 45 mg kg^{-1} genistein were delivered to the $ApoE^{-/-}$ mice daily via intragastric gavage. All animals received food and water ad libitum. Body weight as well as food intake were monitored during the study. Aortic sinus morphometric and immunohistochemical analyses was performed as described previously [20].

Measurement of serum lipid and inflammatory cytokines

Blood samples were collected using retro-orbital venous plexus bleeding followed by measurement of the lipids and cytokines. Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides in the serum were measured with colorimetric assays as previously reported [20]. Serum pro-inflammatory cytokines were measured using a Bio-Plex Pro Mouse Cytokine Assay Kit (Bio-Rad, USA).

Flow cytometry

Whole blood and spleen white blood cells were separated by density gradient centrifugation according to manufacturer's protocol (Cedarlane Laboratories, Hornby, Ontario, Canada). Cell suspensions from the spleen and blood were taken, incubated with 1% normal mouse serum PBS, and stained with specific surface markers, followed by the flow cytometry analysis as described.

Western blot

Cultured SMAs were lysed in a RIPA buffer. The protein concentrations were examined using the BCA kit. A standard immunoblotting analysis was applied to analyze the lysates. The relative content of the protein was determined by specific antibodies against MMP-9 (1:2000) and anti-GAPDH (1:8000). All specific antibodies were purchased from Cell Signaling Technology or Sigma. An enhanced ECL detection kit was used to visualized the immunoreaction, which was then exposed to film and quantified with a video documentation system (Gel Doc 2000, Bio-Rad, Hercules, CA, USA).

Quantification of mRNA by reverse transcriptase quantitative PCR

SYBR green real-time PCR for the OX40 mRNA level was performed using cDNA generated from total RNA extracted from tissues. The OX40 primers were designed as follows: sense, 5'-TACCTACCCCAGTGGTCACAA-3' and antisense, 5'-ACGGATGACATAGAGTATCCCTG-3'.

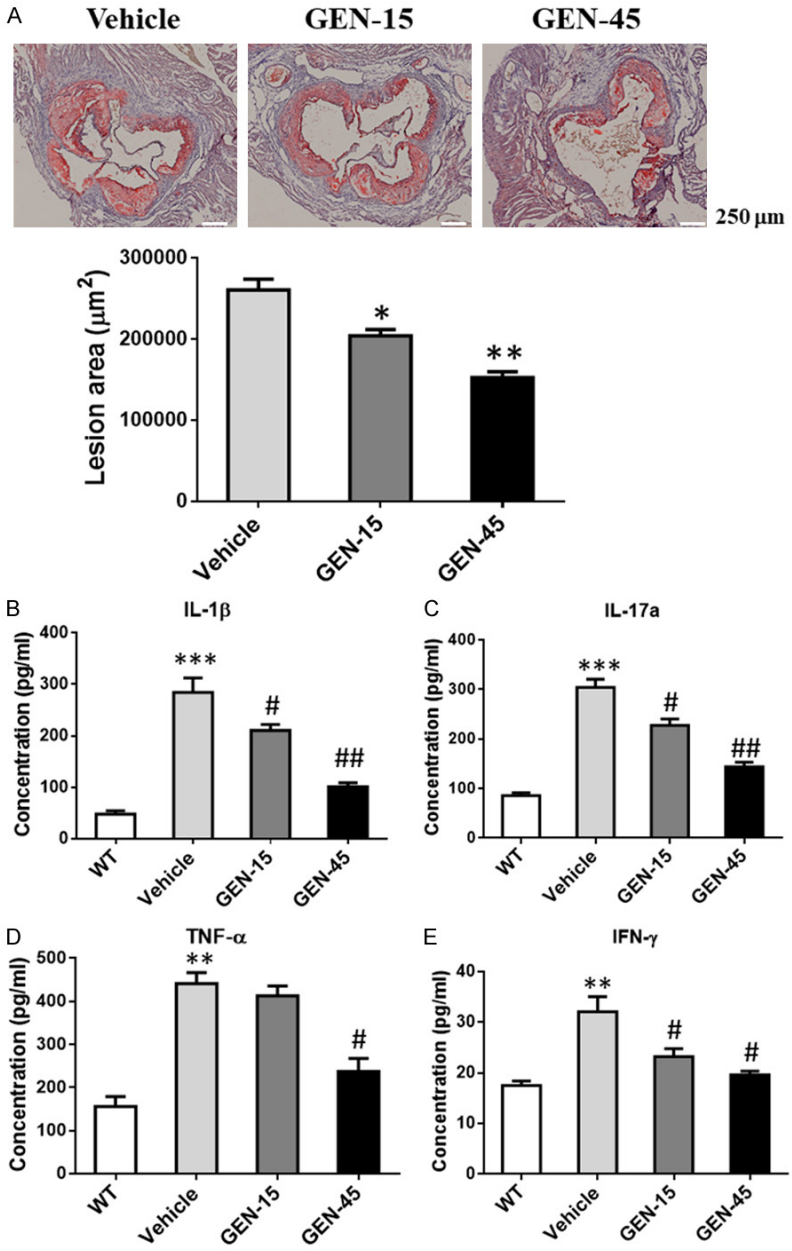

Figure 1. Genistein (GEN) attenuates atherosclerotic lesion size in ApoE$^{-/-}$ mice. A. Representative photomicrographs and morphometric analysis of Oil Red O staining of the atherosclerotic lesions in the aortic sinus. Average sizes of atherosclerotic lesions were calculated from 5 sections in ApoE$^{-/-}$ mice fed an atherogenic diet. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with ApoE$^{-/-}$ group (treated with vehicle control), n = 5-6. B-E. Serum levels of pro-inflammatory mediators in all treatment groups. n = 4 for each group. Data are represented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the WT group. $^{\#}P < 0.05$. $^{\#\#}P < 0.01$ compared with the ApoE$^{-/-}$ group (treated with vehicle control), n = 4.

Statistical analysis

Data were expressed as the means ± SEM. An unpaired, 2-tailed *t* test was used for comparisons between two groups. For multiple comparisons, ANOVA or repeated ANOVA followed by the Bonferroni post hoc test was used with GraphPad Prism® version 6.0 software. A *P* value < 0.05 was considered statistically significant.

Results

Genistein reduces the development of atherosclerotic plaque

ApoE is a class of proteins involved in the metabolism of fats in the body and ApoE knockout mice have been used widely to investigate the development of atherosclerosis. To examine the effects of genistein on atherosclerosis development, 5-week-old ApoE$^{-/-}$ mice were fed an atherogenic diet and were treated daily with or without genistein for 9 weeks. There was no difference observed in body weight (data not shown). But it was clear that genistein treatment attenuated atherosclerotic lesion formation in the aortic sinus compared with vehicle control group of the ApoE$^{-/-}$ mice (**Figure 1A**). Moreover, a biochemical analysis of the serum inflammatory cytokines suggests that genistein remarkably reduced the pro-inflammatory cytokine levels in the serum, including IL-1β, IL-6, IL-17A, IFN-γ, and TNF-α (**Figure 1B**). However, there was no significant change in the serum lipid level (**Table 1**). These results indicate that genistein has potent atheroprotective effects, which are realized through its anti-inflammatory effects rather than by regulating serum lipid levels in the experimental atherosclerosis model.

Table 1. Serum lipid profiles in all the treatment groups

	WT	ApoE$^{-/-}$	ApoE$^{-/-}$ + GEN-15	ApoE$^{-/-}$ + GEN-45
TC (mmol/L)	3.5 ± 0.2	25.3 ± 1.7***	25.1 ± 1.9	23.5 ± 1.4
TG (mmol/L)	1.3 ± 0.1	3.6 ± 0.4**	3.5 ± 0.3	3.1 ± 0.2
HDL (mmol/L)	1.68 ± 0.2	0.54 ± 0.1**	0.57 ± 0.1	0.53 ± 0.2
LDL (mmol/L)	0.9 ± 0.2	11.9 ± 0.8***	12.1 ± 0.9	12.6 ± 1

Data are represented as the mean ± SEM. TC, total cholesterol; TG, triglycerides; LDL, low density lipoprotein-cholesterol; HDL, high density lipoprotein-cholesterol. $^{**}P$ < 0.01, and $^{***}P$ < 0.001 versus WT group (n = 5-6 per group). No statistical significance was observed between the lipid profile of GEN-15, GEN-45 compared to the ApoE$^{-/-}$ group.

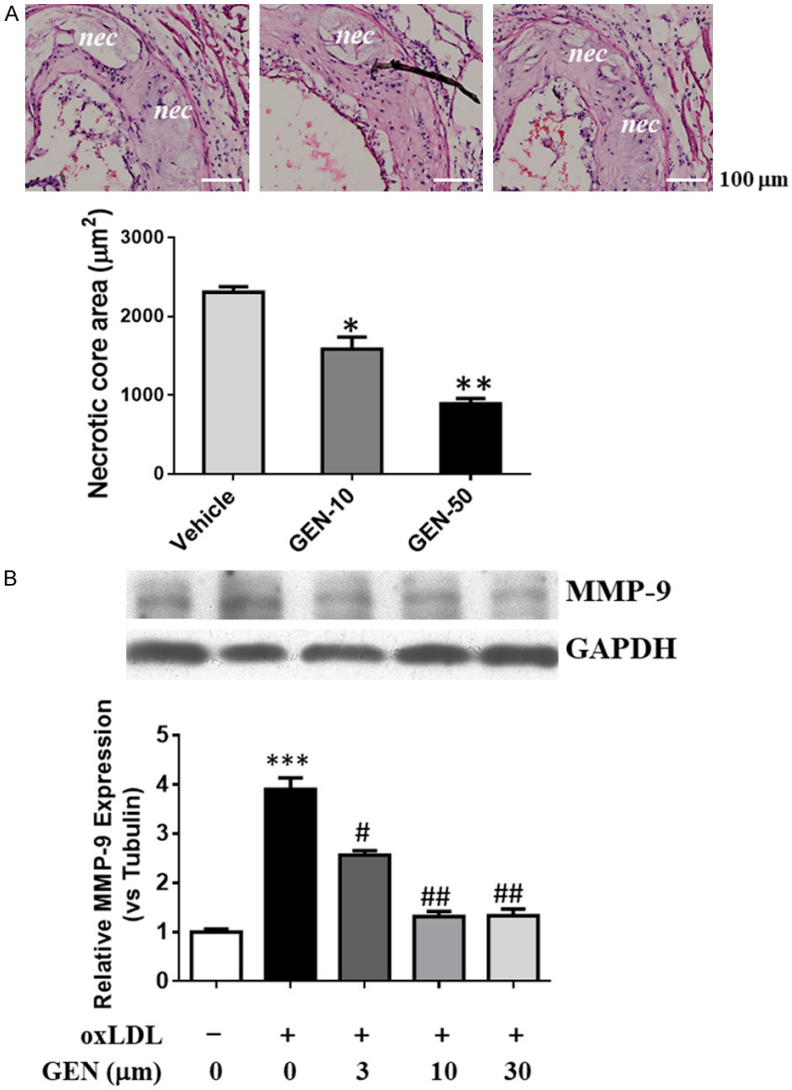

Figure 2. Genistein (EGN) induces features of atherosclerotic plaque stability in ApoE$^{-/-}$ mice. (A) Photomicrographs of sectioned aortic sinuses were stained with H&E to calculate necrotic core area (A, original magnification, ×100). $^{*}P$ < 0.05, $^{**}P$ < 0.01 compared with ApoE$^{-/-}$ group (treated with vehicle control), n = 4. (B) Effect of genistein on oxLDL-stimulated MMP-9 expression and activity in SMCs. Rat aortic SMCs were pretreated with genistein for 2.5 h then stimulated with oxLDL (100 μg mL^{-1}) for 24 h. Whole cell lysates were subjected to MMP-9 protein expression. n = 4. $^{***}P$ < 0.001 compared with the untreated control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 compared with the oxLDL-treated group respectively.

Genistein promotes plaque stability in ApoE$^{-/-}$ mice

We examined plaque composition in ApoE$^{-/-}$ mice to evaluate the effect of genistein on the biology of plaques. Compared with the vehicle group, atherosclerotic plaques in genistein-treated ApoE$^{-/-}$ mice presented smaller necrotic core areas (**Figure 2A**). Next, we determined the effects of genistein on the expression of MMP9, which is a key factor that determines atherosclerotic plaque instability. Notably, there were no significant alterations in the expression of MMP-9 in macrophages with oxLDL treatment (data not shown). While in SMCs, genistein treatment dose-dependently decreased the MMP-9 protein level induced by oxLDL (**Figure 2B**).

Genistein reduces OX40 expression

It was determined that the anti-inflammatory effect is responsible for the genistein-afforded protective effects. Then we investigated the OX40/OX40L pathway, which had been implicated in the atheroprotective effects. Our data indicate that there was a significant increase in the relative mRNA expression of OX40 in the spleens of ApoE$^{-/-}$ mice, which was suppressed by the genistein treatment (**Figure 3A**). In addition, to associate the up-regulated mRNA level of OX40 in the spleen to its protein level, the surface OX40 expression of T cells with the atherogenic diet feeding of ApoE$^{-/-}$ mice was examined.

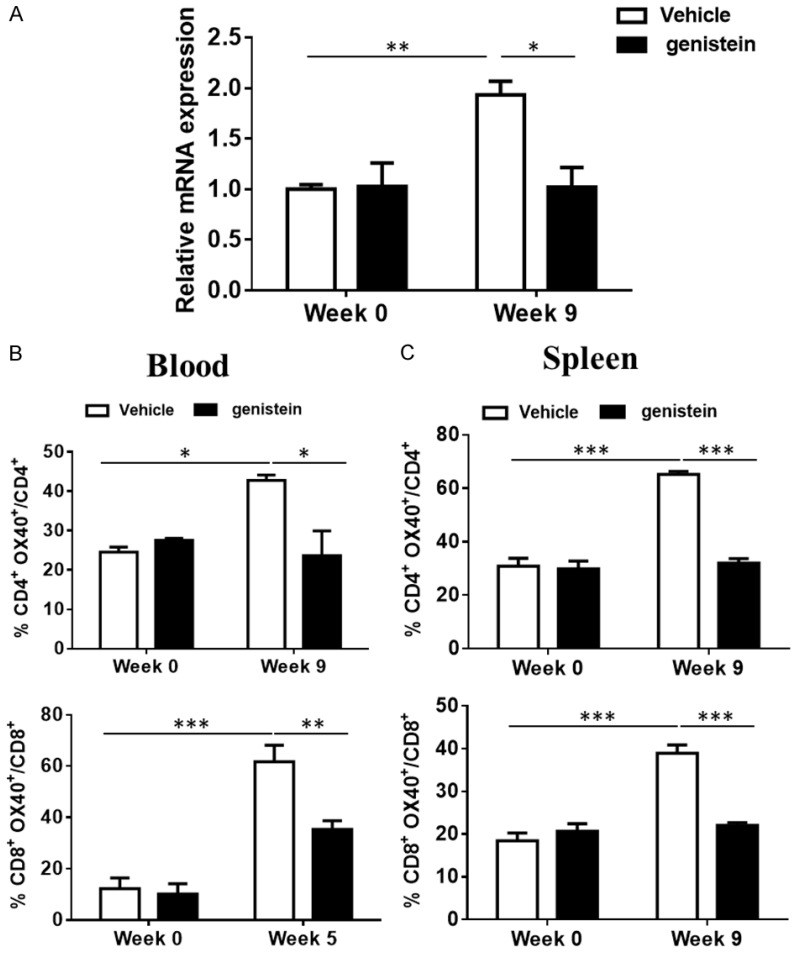

Figure 3. Reduced mRNA expression and protein expression of OX40 in genistein (EGN)-treated mice. A. mRNA expression of OX40 in the spleens of ApoE$^{-/-}$ mice fed a Western type diet for 9 weeks with or without genistein delivery. $^{*}P < 0.05$, $^{**}P < 0.01$ vs indicated group. B, C. Mononuclear cell suspensions from spleen and blood were isolated from the ApoE$^{-/-}$ group treated with vehicle control and genistein. Data are represented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the indicated group.

We performed a FACS analysis to quantify the protein level of OX40 on the spleen T cells as well as the blood. The expressions of CD4, CD8, and OX40 were examined in the ApoE$^{-/-}$ mice at 0 and 5 weeks. The relative percentage of OX40^{+} cells in the CD4^{+} or CD8^{+} positive population was used for a quantitative analysis. There is about a 2-fold increase of CD4/OX40 double-positive and CD8/OX40 double-positive T cells in the spleen after 5 weeks of consuming an atherogenic diet (**Figure 3C**). The induction of OX40 expression in the spleen is associated with increased OX40 expression in the circulation. A remarkable increase in the OX40 positive population cell within the CD4^{+} population was observed in the blood after 5 weeks of atherogenic diet feeding. Also, there is about a 5-fold increase of the percentage of OX40 positive cells in the CD8^{+} population, which shows a 5-fold increase after 5 weeks of being on the atherogenic diet (**Figure 3B**). All these remarkable increases in the expression of OX40 were suppressed in the presence of genistein (**Figure 3B**, **3C**). Taken together, our data convincingly indicate that the OX40/OX40L pathway is involved in the genistein-afforded atheroprotective effects in ApoE$^{-/-}$ mice.

Anti-OX40L antibody administration mimics the beneficial effects of genistein

To determine the effect of a specific blockade of OX40L on the genistein-afforded atheroprotective effects, we used the anti-OX40L antibody in the ApoE$^{-/-}$ mice to block the OX40/OX40L pathway. After 3 weeks of the atherogenic diet, the ApoE$^{-/-}$ mice were furnished with peri-carotid collars followed by treatment twice a week with 300 μg of OX40L antibody for 6 weeks. Our data show that there was a significantly decreased lesion size after the blockade of OX40L and anti-OX40L antibody treatment, which reduced the lesion area by 60% (**Figure 4A**). However, beneficial effects of OX40L on the development of the atherosclerotic plaques are not further improved when combined with genistein.

Next, we determined the mRNA expression of OX40 after the anti-OX40L treatment with and without genistein delivery. There was a significant decrease of OX40 mRNA expression in the spleen after OX40L antibody treatment compared with the control group (**Figure 4B**). Of note, there is no additional beneficial effect

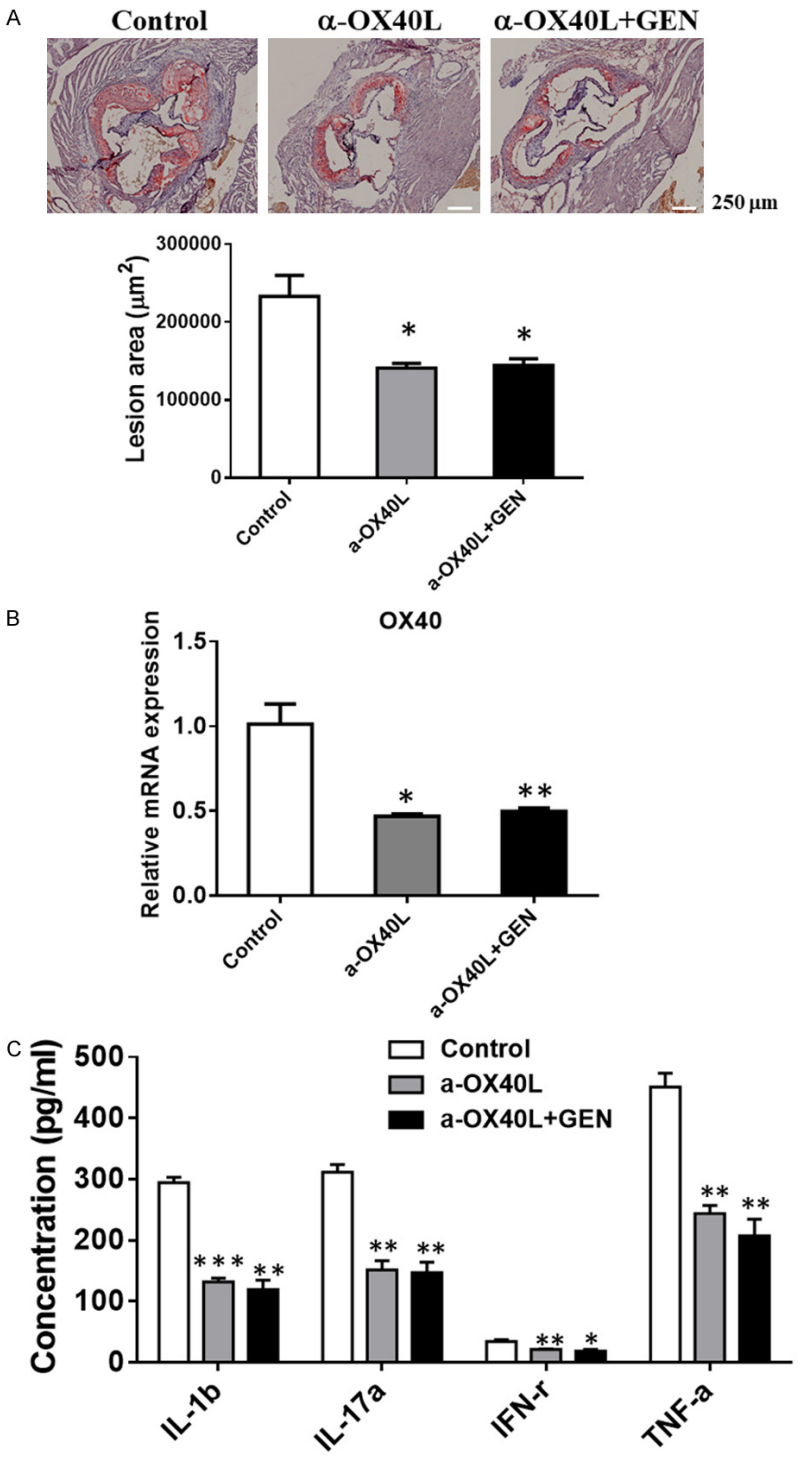

Figure 4. Anti-OX40L synergy with genistein (EGN) induces the regression of atherosclerosis. A. Representative photomicrographs and morphometric analysis of Oil Red O staining of the atherosclerotic lesions in the aortic sinus from ApoE$^{-/-}$ mice fed an atherogenic diet treated with genistein and anti-OX40L antibody. $^{*}P < 0.05$ compared with control group, n = 5. B. mRNA expression of OX40 in spleen. $^{*}P < 0.05$, $^{**}P < 0.01$, vs Control group. n = 3. C. Serum levels of pro-inflammatory mediators in all treatment groups. n = 4 for each group. Data are represented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

with the co-administration of anti-OX40L and genistein (**Figure 4B**). Taken together, these results indicate that the OX40/OX40L plays an important role in mediating the atheroprotective effects of genistein.

Discussion

In the present study, we assessed the beneficial effects of genistein on the development of atherosclerosis in HCD-fed ApoE$^{-/-}$ mice. First, our data showed that genistein treatment results in a significant reduction in the size of the atherosclerotic plaque and promotes plaque stability. Moreover, we showed that genistein effectively interrupts the OX40/OX40L signaling pathway. Finally, we found that the inhibition of the OX40/OX40L signaling pathway is involved in the atheroprotective effects of genistein.

Genistein, a phytoestrogen found in soy, has been demonstrated to afford protective effects against cardiovascular disease partially by regulating blood pressure and improving serum lipid levels [21]. More and more studies suggest that the anti-inflammatory effects of isoflavonoids may be responsible for the beneficial effects on chronic cardiovascular diseases including atherosclerosis [22]. It is widely known that genistein is the major active isoflavonoid in soybean. However, the underlying mechanisms of genistein-afforded atheroprotective effects have not been fully charac-

terized. The present study, for the first time, shows the atheroprotective effect of genistein with HCD-fed ApoE$^{-/-}$ mice, a well-established model for studying atherogenesis. Our study confirmed that treatment with genistein resulted in a significant reduction in the size of the atherosclerotic plaque accompanied by the induction of atherosclerotic plaque stability in ApoE$^{-/-}$ mice. Of note, to assess additional potential effects of genistein which are responsible for the protection against atherosclerosis, the serum inflammatory level was examined. Treatment with genistein significantly reduced the serum levels of pro-inflammatory cytokines, which are tightly related to the development and progression of atherosclerosis in ApoE$^{-/-}$ mice. Those results indicated the potent anti-inflammatory effect of genistein.

The protective effect of genistein on atherosclerosis is ascribed to restraining inflammation, while the underlying mechanism has not been fully clarified. Several pathways are involved in the initiation and maintenance of inflammation, one of which is the receptor-ligand signaling of OX40/OX40L, which has recently been shown to be associated with atherosclerosis. There are several pathways that participate in the initiation and maintenance of inflammation, one of which is signaling triggered by OX40/OX40L, which has recently been shown to be involved in the development of atherosclerosis [23-25]. The OX40/OX40L-mediated interaction between APCs and T-cells improves the survival of effector T-cells, which is critical for the generation of CD4^{+} memory T-cells [26]. Then these interactions may lead to enhanced inflammatory responses in atherosclerotic plaques. Although genistein has been shown to have anti-inflammatory effects, its effects on OX40 and OX40L expression are unknown. Our research examined how genistein regulates the mRNA and protein expression of OX40 on T cells both in the spleens and blood of ApoE$^{-/-}$ mice fed an atherogenic diet. The results show that genistein significantly suppresses the atherosclerosis-simulated up-regulation of the OX40 level. Furthermore, we found that interruption of the OX40/OX40L pathway with the administration of anti-OX40L induces the suppression of atherosclerosis. Moreover, anti-OX40L combined with genistein causes no further regression of atherosclerosis. The present study provides evidence that genistein activates a novel anti-inflammatory pathway which is associated with interrupting the OX40/OX40L pathway.

Conclusion

In conclusion, our results characterize the atheroprotective property of genistein in ApoE$^{-/-}$ mice and indicate a novel mechanism by which genistein prevents atherogenesis. Genistein was demonstrated to suppress the OX40/OX40L signaling pathway, thereby reducing atherosclerotic lesions. These data suggest that genistein has the potential to be an innovative cardiovascular drug to retard the development of atherosclerotic cardiovascular diseases.

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Disclosure of conflict of interest

None.

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