New insights into immunomodulation via overexpressing lipoic acid synthase as a therapeutic potential to reduce atherosclerosis

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Abstract

Atherosclerosis is a systemic chronic inflammatory disease. Many antioxidants including alpha-lipoic acid (LA), a product of lipoic acid synthase (Lias), have proven to be effective for treatment of this disease. However, the question remains whether LA regulates the immune response as a

protective mechanism against atherosclerosis. We initially investigated whether enhanced endogenous antioxidant can retard the development of atherosclerosis via immunomodulation.

To explore the impact of enhanced endogenous antioxidant on the retardation of atherosclerosis via immune regulation, our laboratory has recently created a double mutant mouse model, using apolipoprotein E-deficient ($Apoe^{-/-}$) mice crossbred with mice overexpressing lipoic acid synthase gene ($Lias^{H/H}$), designated as $Lias^{H/H}Apoe^{-/-}$ mice. Their littermates, $Lias^{+/+}Apoe^{-/-}$ mice, served as a control. Distinct redox environments between the two strains of mice have been established and they can be used to facilitate identification of antioxidant targets in the immune response.

At 6 months of age, $Lias^{H/H}Apoe^{-/-}$ mice had profoundly decreased atherosclerotic lesion size in the aortic sinus compared to their $Lias^{+/+}Apoe^{-/-}$ littermates, accompanied by significantly enhanced numbers of regulatory T cells (Tregs) and anti-oxidized LDL autoantibody in the vascular system, and reduced T cell infiltrates in aortic walls.

Our results represent a novel exploration into an environment with increased endogenous antioxidant and its ability to alleviate atherosclerosis, likely through regulation of the immune response. These outcomes shed light on a new therapeutic strategy using antioxidants to lessen atherosclerosis.

Keywords

Regulatory T cells; Oxidized LDL autoantibody; Antioxidant mouse models; Atherosclerosis; And immune regulation

1. Introduction

α-Lipoic acid (LA) is a potent antioxidant synthesized by lipoic acid synthase (Lias) in mitochondria [1]. It is a co-factor for several enzymes including pyruvate dehydrogenase complex and alpha-ketoglutarate dehydrogenase complex, both of which participate in ATP production. LA as an antioxidant is well-established [3, 4], but its function in immune regulation remains to be elucidated. In an effort to study the role of LIAS in immune regulation during the development of atherosclerosis, we generated genetic mouse models by modifying the 3′ untranslated regions (3′-UTR) of the *Lias* gene that influence *Lias* mRNA copy number, thus affecting Lias protein expression to obtain $Lias^{H/H}$ mice. $Lias^{H/H}$ mice display ~160% of Lias protein expression confirmed by Western blot, with corresponding low levels of antioxidant and oxidative stress [9]. $Lias^{H/H}$ mice were crossed with an atherosclerosis-prone mouse model, $Apoe^{-/-}$ mice, to test whether enhanced antioxidant capacity can attenuate the onset and development of atherosclerosis in the double mutant $Lias^{H/H}Apoe^{-/-}$ mice. Our novel $Lias^{H/H}Apoe^{-/-}$ mice mimic gain-of-function of antioxidant by creating a strengthened endogenous antioxidant microenvironment. They may amplify subtle changes that may not be apparent under basal conditions and will be helpful to identify LA targeted diagnostic biomarkers in mitochondria, and decipher the mechanisms of immune regulation during development of the disease.

Broad evidence supports that atherosclerosis is a systemic chronic inflammatory disease [10], characterized by the formation of an immune cell infiltration dominated by lipid-laden macrophage foam cells and T cells, and elicits lipid retention and oxidative modification of low-density lipoprotein (LDL) in the arterial wall [11]. These modifications trigger both innate and adaptive immune responses [12]. Adaptive immunity has been shown to participate in all stages of the disease. T lymphocytes are among the earliest cells recruited into the atherosclerotic plaque [13], predominated by a large number of CD4+ T cells in the atherosclerotic plaques in humans and mice [14]. Adoptive transfer of CD4+ T lymphocytes increases the progression of atherosclerosis in immunodeficient mice [15]. In response to the local milieu of cytokines, naïve CD4+ T lymphocytes differentiate into T helper (Th) cell 1 (Th1), Th2, Th17, and Treg lineages. The Th1 response, including the production of interferon-γ (IFN-γ), is prevalent during atherosclerosis [16] and has been shown to promote atherosclerosis [17]. Tregs can suppress the activity of effector T cells and are known to play a critical role in the T-cell homeostasis. Treg cells also mediate suppressor function through the production of IL-10 and TGF-β, respectively [18,19]. As a result, Tregs are considered to be atheroprotective. Evidence shows that numbers of circulating Tregs decline in patients with coronary artery disease or atherosclerosis [20,21]. Since autoimmune antibodies are found in the circulation of patients with atherosclerosis, autoimmune mechanisms may contribute to the pathogenesis of disease [22]. These antibodies react against a wide variety of oxidized lipid-protein adducts, mainly derived from oxidized low-density lipoproteins (oxLDLs). OxLDLs are no longer recognized as self-molecules by the body's immune system after either enzymatic or non-enzymatic modification [23]. 1,4-dihydropyridine-type MDA-acet-aldehyde-Lys adduct (MAA), which is a form of oxLDL, has been proposed to be one of the most potent atherogenic protein adducts caused by lipid peroxidation and appear to play a critical role in atherogenesis [24].

To date, little is known about whether antioxidants can regulate the immune system during the development of atherosclerosis. In particular, how antioxidants regulate T helper cell (Th) lineages during atherosclerosis progression remains to be elucidated. It is well-known that under the influence of their microenvironment, Th cell subsets with their signature cytokine profiles can switch from one lineage to another [25]. In addition, T helper activation is strongly dependent on the redox potential of the microenvironment [26]. We hypothesized that generation of type and amount of T cell subsets, and plasticity of the signature cytokines were influenced and regulated by the antioxidant microenvironment and higher expression of LIAS will promote Treg cell production. In our current study, we found that Tregs and MAA autoantibodies in circulation were significantly increased while lymphocyte infiltration was significantly decreased in aortic walls of $Lias^{H/H}Apoe^{-/-}$ mice compared with $Lias^{+/+}Apoe^{-/-}$ mice. These results raised a possibility that LA alleviates atherosclerosis by manipulating the immune response.

2. Materials and methods

2.1. Animals and experimental design

$Apoe^{-/-}$ mice on C57BL/6 J background were purchased from Jackson Laboratories (B6.129P2-$Apoe^{tm1Unc}$/J, Stock No:002052, Bar Harbor, ME). $Lias^{H/H}$ mice on C57BL/6

background were generated via C57BL/6 embryonic stem cells as we described previously [9]. When the mice were engineered by genetic modification of the 3′-untranslated region (3′-UTR), the endogenous *Lias* gene expression was increased by ~ 160% in *Lias$^{H/H}$* mice [9]. Correspondingly Lias protein expression levels and antioxidant content in *Lias$^{H/H}$* mice increased (9) as confirmed by RT-PCR and Western blot. *Lias$^{H/H}$* mice were then crossed with *Apoe$^{-/-}$* mice on the same genetic background to achieve a double mutant *Lias$^{H/H}$Apoe$^{-/-}$* mouse model. *Lias$^{+/+}$Apoe$^{-/-}$* mice, as controls, were littermates of *Lias$^{H/H}$Apoe$^{-/-}$* mice obtained during the mating process and all mice were sacrificed at 6 months of age. Male animals were used in the study. All animals were bred and housed in a pathogen-free animal facility and fed normal chow in a temperature-controlled environment (22–25 °C) with a 12-h light/dark cycle. The animals received regular chow diet and water ad libitum. Animal care and experimental protocols (19–111; PI: Xianwen Yi) were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill.

2.2. Plasma lipid profile

Blood samples were collected using a submandibular bleeding method and centrifuged ($1500 \times g$, 20 min) to obtain plasma samples. Plasma total cholesterol (Chol) and triglycerides (TG) were quantified using colorimetric kits from Wako (Richmond, VA) based on enzymatic reactions according to the manufacturer's protocols. Plasma high-density lipoprotein (HDL) and low-density lipoprotein (LDL)/Very-LDL (VLDL) cholesterol were separated and measured using HDL and LDL/VLDL cholesterol assay kit (Abeam, Cambridge, MA).

2.3. Oxidative stress status

Plasma lipid peroxide content was determined as 4-hydroxynonenal (4-HNE), a commonly used lipid peroxidation marker, assayed by using an ELISA Kit following the manufacturer's protocol (Cell Biolabs. Inc., San Diego, CA). Blood total antioxidant capacity was measured by Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI). The assay is based on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS® + by metmyoglobin.

2.4. Quantitation of atherosclerotic lesions

Mice were perfused with PBS containing heparin, followed by 10% buffered formalin (pH 7.4) under physiological pressure for histological and immunohistochemical staining. Mouse hearts were collected after perfusion, embedded in optimal cutting temperature (OCT) compound and were snap frozen. Sequential cryosections of OCT-embedded aortic roots were cut such that all three leaflets of the aortic valve could be visualized. The frozen heart blocks were cut at 10 μm thickness and cryosections were first stained with Oil Red O and then counterstained with hematoxylin. Images of aortic root were captured with an Olympus BX61 microscope. The average lesion area from three continuous cross-sections of the aortic root at an anatomical position defined by the presence of all three mitral valves, was measured in each animal. Quantitative morphometric analysis of lesion area using NIH Imaging Software was carried out by a histologist blinded to the sample genotypes.

Atherosclerosis at the remainder of the aortic tree is not sufficiently large in the mice on normal chow and was not used for quantitation.

2.5. Measurement of circulating Tregs using flow cytometry

Mouse peripheral blood was collected by submandibular bleeding into EDTA micro tubes with 500 μl PBS and mixed well. Lymphocytes were obtained by centrifugation over Lympholyte®-Mammal Separation Media (Cedarlane Laboratories Ltd., Burlington, NC) at $800 \times g$ for 20 min at room temperature. The isolated lymphocytes were washed 3 times with FACS wash buffer.

Lymphocytes isolated from blood were stained with CD3-APC-eFluor780, CD4-FITC, CD25-PE-Cyanine 7 for 30 min in the dark. Nuclear staining of forkhead box protein 3 (FoxP3), cells were fixed in Fixation/Permeabilization Buffer (eBioscience) for one hour, followed by staining with FoxP3-PE-eFluor 610 for one hour in the dark. All antibodies were from eBioscience (San Diego, CA). Cells were then examined with Cyan ADP (Dako) and analyzed with Summit software.

2.6. IFN-γ and IL-10 in serum

Serum IL-10 and IFN-γ were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Each sample was assayed in duplicate. Absorbance was measured at 450 nm by means of a spectrophotometer (SpectraMax M5, Molecular Devices).

2.7. Determination of MAA-specific antibody titers

MAA-adducts were synthesized as described previously [27]. Briefly, ɛ-aminocaproic acid (6-ACA), acetaldehyde, and malondialdehyde were incubated at 37 °C for 3 days. MAA-6ACA was purified by HPLC. MAA-6ACA was conjugated with bovine serum albumin (BSA) as reported previously [27]. MAA-6ACA-BSA was seeded in 96-well plates and incubated at 4 °C overnight. After blocking with 3% BSA, serum samples diluted at 1:320 ratio by 1% BSA in PBS were incubated at 4 °C overnight. After washing, peroxidase-labeled secondary antibody (Envision + Single) was used with anti-mouse (IgG)-HRP (Code K4001, Dako North America, Inc., Carpentaria, CA) and incubated for 1 h. After washing the wells, 3,3′,5,5′-tetra-methylbenzidine (TMB)/H_2O_2 substrate was added to the wells and kept at room temperature for 30 min. The plates were then read with a plate reader (Vmax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA) at a wavelength of 650 nm.

2.8. Aortic lymphocyte infiltration

2.8.1. Flow Cytometry analysis of immune cells within mouse aortic walls—
Immune cells within murine aortas were analyzed using flow cytometry following a published protocol [28]. Briefly, entire aortas were perfused and attached adipose tissue was carefully removed under a dissection microscope. After blood was completely flushed out of the aortas, the aortas were dissected and digested with the enzyme cocktail containing 450 U/ml collagenase type I, 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s and

60 U/ml DNase 1 in PBS containing 20 mM Hepes at 37 °C for 1 h. A cell suspension was obtained by mashing the aorta through a 70-μm strainer. The lymphocytes were fractioned by density gradient centrifugation using Lympholyte®-Mammal Separation Media (Cedarlane Laboratories Ltd., Burlington, NC). Aortic cells were stained with CD3-APC and CD4-PE-Cy7 for T cells, and CD45-FITC as a lymphocyte common antigen to identify all lymphocytes in the aortas. Cells were analyzed by flow cytometry. All subsequent analyses were performed on cells gated to CD45.

2.8.2. Immune cell quantification in aortic atherosclerotic lesions—Contiguous 10 μm frozen sections of the ascending aortic root were stained with H&E using standard protocols. Quantification of immune cells was performed on contiguous 10 μm frozen sections of the aortic root by identification of mononuclear cell nuclei apart from surrounding structures using positive pixel intensity defined algorithm. Briefly, H&E stained slides were digitized using a Nikon Eclipse E800 microscope with a Nikon DS-Ri2 camera. The anonymized tiff files were then analyzed by NIS Elements Software BR 4.30.02 (Nikon Instruments Inc.) using the positive pixel intensity algorithm to define positive mononuclear cell nuclei areas in the manually defined region of interest and represented as mean of the percentage of aortic area for positive inflammatory cells by pixel intensity per mouse aortic vessel in a blinded manner. Immune cell quantification in aortic vessels with atherosclerotic lesions was performed in 3 biological replicates per mouse from each group, at the original magnification (4 ×).

2.9. Immunohistochemistry

Immunohistochemical analyses were performed on 10 μm frozen sections of aortic root. Macrophages were detected using rat monoclonal anti-mouse CD68 (Novus Biologicals, Littleton, CO) and rabbit antibody against pMAA-6ACA-BSA antigens. The rabbit anti-MAA polyclonal antibody was kindly provided by Dr. Todd A. Wyatt (University of Nebraska).

All secondary antibodies were conjugated with horseradish peroxidase (HRP). Endogenous peroxidase activity or endogenous biotin sites were quenched with 3% H_2O_2/PBS or with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Images were taken under an Olympus BX61 microscope.

2.10. Statistical analysis

Statistical analysis was performed using Students' *t*-test. All data are represented as the mean ± standard error of mean (SEM). $P < 0.05$ values were considered to be statistically significant.

3. Results

3.1. Reduced atherosclerotic lesion size in Lias$^{H/H}$Apoe$^{-/-}$ mice

Atherosclerotic plaques at the aortic sinus of each mouse fed standard chow were histologically analyzed at 6 months of age. Fig. 1A and 1B are representative photomicrographs of the aortic sinus plaque in *Lias$^{H/H}$Apoe$^{-/-}$* mice (A) and *Lias$^{+/+}$Apoe$^{-/-}$*

mice (B). Lesions are detected in red after **Oil Red O** staining. The mean size of lesions at the aortic sinus was 3.4 ± 0.7 $\mu m^2 \times 10^5$ and 4.9 ± 0.9 $\mu m^2 \times 10^5$ ($P < 0.05$) in 6-month-old $Lias^{H/H}Apoe^{-/-}$ mice and $Lias^{+/+}Apoe^{-/-}$ mice, respectively. $Lias^{H/H}Apoe^{-/-}$ mice had a 31% decrease in atherosclerotic plaque area compared to their $Lias^{+/+}Apoe^{-/-}$ counterparts ($P < 0.05$) (Fig. 1C), suggesting that increased steady-state levels of $Lias$ transcript increased endogenous antioxidant capacity and contributed to a reduction in atherosclerotic lesion size.

3.2. Plasma triglyceride and cholesterol Lias^H/H^Apoe^−/−^ mice

Since lipoic acid has anti-hyperlipidemia function [29] and Treg cells can influence cholesterol metabolism [30], we examined the effect of increased $Lias$ gene expression on plasma cholesterol (Chol) and triglyceride (TG) levels. Plasma TG level was significantly lower (Fig. 1E), but total Chol level was not (Fig. 1D) in $Lias^{H/H}Apoe^{-/-}$ compared to $Lias^{+/+}Apoe^{-/-}$ mice at the end of the observed period. Plasma cholesterol associated with VLDL/LDL was higher in $Lias^{H/H}Apoe^{-/-}$ mice compared with $Lias^{+/+}Apoe^{-/-}$ mice (Fig. 1F) but HDL value was the opposite in the two strains, and both values did not reach significance (Fig. 1G). Since most TG is carried by the VLDL particle, which is the major proatherogenic lipoprotein particle in $Apoe^{-/-}$ mice, higher levels of TG may contribute to the larger atherosclerotic lesions in $Lias^{+/+}Apoe^{-/-}$ mice.

3.3. Decreased oxidative stress in Lias^H/H^Apoe^−/−^ mice

The $Lias^{H/H}Apoe^{-/-}$ mice showed an increased plasma antioxidant capacity (Fig. 1H) and reduced oxidative stress (Fig. 1I) compared with $Lias^{+/+}Apoe^{-/-}$ mice, as measured by Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI) and 4-Hydroxynonenal (4-HNE) Assay Kit (Cell Biolabs, San Diego, CA), respectively.

3.4. Flow cytometry analysis detected T lymphocytes and total mononuclear cell count within aortic walls

Mononuclear cells include macrophages and lymphocytes. Mononuclear cell infiltration is characteristic of inflammatory lesions. We observed more mononuclear cell infiltration in the aortic root of $Lias^{+/+}Apoe^{-/-}$ (Fig. 2A) than in $Lias^{H/H}Apoe^{-/-}$ mice (Fig. 2B) by counting the mononuclear cell nuclei apart from surrounding structures using a positive pixel intensity defined algorithm. The pixel intensity of the (replicate) mean percentage of positive inflammatory cells in the aortic roots in $Lias^{+/+}Apoe^{-/-}$ mouse was significantly higher than in $Lias^{H/H}Apoe^{-/-}$ mice (Fig. 2C), consistent with a reduction in inflammatory cells in atherosclerotic lesions of $Lias^{H/H}Apoe^{-/-}$ mice.

Evidence showed the presence of large numbers of T cells in all stages of lesion development, which may contribute to atherosclerosis [31]. Relative T-lymphocyte density in the aortic wall was analyzed by flow cytometry after enzyme digestion to determine the percentage of CD4$^+$ T lymphocytes of $Lias^{H/H}Apoe^{-/-}$ and $Lias^{+/+}Apoe^{-/-}$ in aortic connective tissues. Staining for the CD45$^+$ common lymphocyte antigen allowed the identification of aortic leukocytes; CD4$^+$ T cells were detected by CD3$^+$ and CD4$^+$ double staining. The percentage of CD3$^+$ / CD4$^+$ T lymphocytes of the total CD45$^+$ cells in the $Lias^{H/H}Apoe^{-/-}$ aortic wall was significantly reduced (Fig. 2G and H) compared with $Lias^{+/+}Apoe^{-/-}$ mice (Fig. 2F and 2H). The data suggest that enhanced endogenous antioxidant

level decreased inflammatory cell infiltration, which may contribute to the suppression of atherogenesis. We also found the positive area stained by antibody against CD68$^+$ monocytes/macrophages are reduced in $Lias^{H/H}Apoe^{-/-}$ mice (Fig. 2I) compared to $Lias^{+/+}Apoe^{-/-}$ mice (Fig. 2J). Quantitative analysis of the area stained with CD68 relative to the total plaque area was significantly less in $Lias^{H/H}Apoe^{-/-}$ mice (8.4 ± 1.2%) compared to $Lias^{+/+}Apoe^{-/-}$ mice (15.4 ± 2.6%, $P < 0.05$) (Fig. 2K). Together these studies demonstrate that increasing $Lias$ expression ($Lias^{H/H}Apoe^{-/-}$) results in decreased CD4$^+$ T cells and CD68$^+$ monocytes/macrophages atherosclerotic plaques compared to $Apoe^{-/-}$ strain-matched controls.

The increased presence of CD3$^+$ CD4$^+$ cells in the aortic walls of $Lias^{H/H}Apoe^{-/-}$ mice. Following enzymatic digestion, cells released from aortic walls were stained with antibodies conjugated to fluorescent dyes for flow cytometry analysis. Cells in the aortic walls were gated by scattering light (D) and CD45 staining (E). Percentage of CD3$^+$ and CD4$^+$ double-positive T cells in lymphocytes of aortic walls in (F) $Lias^{H/H}Apoe^{-/-}$ and (G) $Lias^{+/+}Apoe^{-/-}$ mice. (H) A population of CD3$^+$ and CD4$^+$ double-positive T cells in relation to all T cells (or divided by) all T cells in aortic walls of mice ($n = 6$ for $Lias^{H/H}Apoe^{-/-}$ and $n = 11$ for $Lias^{+/+}Apoe^{-/-}$ mice). The horizontal bars indicate mean ± SE.

Representative immunohistochemical images. CD68 antibody staining in aortic root sections of $Lias^{H/H}Apoe^{-/-}$ (I) and $Lias^{+/+}Apoe^{-/-}$ (J) was detected by staining the aortic root brown with DAPI and counterstaining with hematoxylin. Original magnification 40 ×. Two aortic sections were examined per mouse, five mice per group. (K) Quantification of CD68-positive area relative to plaque area. Results are expressed as mean ± SE.

3.5. Increased Tregs in peripheral blood in Lias$^{H/H}$Apoe$^{-/-}$ mice

It is very demanding to obtain enough aortic lymphocytes to functionally address their characteristics using flow cytometry because there are a very limited number of resident and infiltrating lymphocytes in the aortic walls to acquire reliable analysis of Tregs. Thus, we used peripheral blood lymphocytes to uncover the mechanism behind the observed atherosclerotic phenotypes. The total Treg population was identified as CD3$^+$ CD4$^+$ FOXP3$^+$ cells. The results showed that the proportion of total peripheral blood Tregs was 11.3% in $Lias^{H/H}Apoe^{-/-}$ mice (Fig. 3B) compared to 6.2% in the control $Lias^{+/+}Apoe^{-/-}$ mice (Fig. 3C), reflecting a significant increased Treg population in $Lias^{H/H}Apoe^{-/-}$ mice (Fig. 3D).

3.6. *Circulating IFN-γ and IL-10*

Th1 CD4$^+$ T cell subset secretes pro-inflammatory cytokines such as IFN-γ, whereas Tregs produce the anti-inflammatory cytokine IL-10. Tregs and serum IL-10 are decreased in vulnerable patients that have had recurrent cardiac events in comparison with stable patients [32]. Thus, we measured the level of these two types of cytokines in peripheral blood to determine the influence of two environments of different redox status generated by different levels of $Lias$ gene expression. Furthermore, $Lias^{H/H}Apoe^{-/-}$ mice serum had significantly increased IL-10 and IFN-γ levels compared to $Lias^{+/+}Apoe^{-/-}$ mice (Fig. 3E and 3F).

Therefore, increased antioxidant level in $Lias^{H/H}Apoe^{-/-}$ mice enhances both immunosuppressive Treg and immunosuppressive cytokine IL-10.

3.7. MAA-specific antibody titers in the serum of Lias$^{H/H}$Apoe$^{-/-}$ and Lias$^{+/+}$Apoe$^{-/-}$ mice

To determine the effect of increased *Lias* gene expression on oxLDL autoantibody in peripheral blood, we examined the serum MAA antibody titer using ELISA in $Lias^{H/H}Apoe^{-/-}$ and $Lias^{+/+}Apoe^{-/-}$ mice. The concentration of MAA-specific IgG in the serum of $Lias^{H/H}Apoe^{-/-}$ mice is significantly higher than $Lias^{+/+}Apoe^{-/-}$ mice at 6 months of age (Fig. 4). However, IgM did not show a significant difference between the two groups (data not shown). This result suggests that an enhanced level of antioxidant increased oxLDL autoantibody production, which may contribute to attenuation of atherosclerosis.

4. Discussion

We previously reported that reduced *Lias* gene expression resulted in larger atherosclerotic lesions in $Lias^{-/-}Apoe^{-/-}$ mice (8). To further define the role of endogenous antioxidant, this study was designed to test our hypothesis that strengthened endogenous antioxidant capacity in $Lias^{H/H}Apoe^{-/-}$ mice was able to retard the development of atherosclerosis via immune regulation. Our data showed that $Lias^{H/H}Apoe^{-/-}$ mice with overexpression of the *Lias* gene displayed a correspondingly lower lipid peroxidation level and higher antioxidant capacity in circulation than $Lias^{+/+}Apoe^{-/-}$ mice as a control. Additionally, overexpression of the *Lias* gene significantly alleviated atherosclerosis in the aortic sinus, co-committed with reduced plasma triglycerides, increased number of Tregs, decreased infiltration of CD4$^+$ T cells and elevated MAA antibody in $Lias^{H/H}Apoe^{-/-}$ mice, compared to their counterparts. Thus, our results provide more evidence for potential antioxidant therapy for human atherosclerosis.

Our results indicate that both altered innate and adaptive immune response manipulated by overexpressing *Lias* gene is involved in the attenuation of atherosclerosis. Leukocytes play a pivotal role in modulating atherosclerotic pathogenesis and inflammation. Under healthy conditions, a small number of leukocytes including macrophages, dendritic cells, neutrophils, and lymphocytes reside within the walls of the aorta [33]. Dynamic changes in the leukocyte population occur within the aorta during atherosclerosis [33]. The process of atherosclerotic plaque formation is characterized by the influx of monocytes/macrophages and T cells into lesion sites [34]. To understand the effect of antioxidant on T cell infiltration in arterial walls, critical for the development of atherosclerosis, we quantified the total lymphocyte numbers in atherosclerotic artery walls in the two different endogenous antioxidant environments based on Galkina's method [35]. We found a significant decrease in the percentage of CD4$^+$ T cells within the atherosclerotic wall in $Lias^{H/H}Apoe^{-/-}$ mice, compared with $Lias^{+/+}Apoe^{-/-}$ mice. The result suggests that increased endogenous antioxidant can suppress pro-atherogenesis via decreasing infiltrating CD4$^+$ T cells. This method has its limitation because it releases both resident and infiltrating T cells in the artery walls. Nevertheless, it has been an accepted method [35].

Evidence shows that impaired function or reduced number of Tregs is likely one of the mechanisms for the increased local inflammatory response in atherosclerosis [33]. Our data demonstrate that the frequency of circulating Treg cells in $Lias^{H/H}Apoe^{-/-}$ mice were

significantly increased (vs. $Lias^{+/+}Apoe^{-/-}$ mice) suggesting that the LIAS-altered endogenous antioxidant environment influences Treg differentiation and proliferation. Tregs suppress Th1 that produces IFN-γ and promotes atherosclerosis [17]. Treg cells also mediate suppressor function through the production of IL-10 [18,19]. Thus, increased IL-10 production in $Lias^{H/H}Apoe^{-/-}$ mice provided additional evidence that the activity of Tregs in circulation was elevated. In addition, we previously found that all major organs and cells in $Lias^{H/H}Apoe^{-/-}$ mice including Tregs had upregulated $Lias$ gene expression (160 ± 10% of that in wild type mice). Therefore, it is also possible that because of the upregulation of $Lias$ gene in Tregs, the metabolic changes of Tregs in $Lias^{H/H}Apoe^{-/-}$ mice can better meet the requirement of bioenergetic demand upon activation. Furthermore, our results agree with other studies using a different antioxidant (40). For example, one study revealed that the administration of resveratrol was associated with small but significant increases in the proportion of circulating Treg cells. This result was corroborated by in vitro experiments, which demonstrated the more efficient proliferation of Treg cells when T cell receptor-activated peripheral blood mononuclear cells were cultured in the presence of resveratrol [36]. However, it is still not clear why antioxidants can increase numbers of Tregs. It is reported that T cell commitment to Th1 or Th2 appears to crucially depend on the activation of redox-sensitive signaling cascades, where oxidative stress supports Th1 development while antioxidative milieu leads to a shift to allergic Th2 responses [37,38]. Our animal models provided such an antioxidative milieu for Treg inducement. Moreover, lymphocytes require a reducing milieu for optimal activation. For instance, immunization is associated with a striking increase in free thiols in lymphoid tissue (41; 42). Furthermore, LA is a sulfur-containing compound so it may provide extra protection. Additional studies will be necessary to prove the hypotheses.

Our data demonstrate that MAA autoantibody was significantly increased in the blood of $Lias^{H/H}Apoe^{-/-}$ mice. MAA is a form of oxLDL proposed to be one of the most potent atherogenic protein adducts caused by lipid peroxidation and appears to play a critical role in atherogenesis [24]. Thus, elevated anti-MAA antibody production may represent an attempt by the body to block this antigen and increase clearance of circulating oxidized LDL particles [39].

Our current work disclosed that potential effect of LA attenuated atherosclerosis via immune regulation using our new antioxidant/atherosclerosis mouse model. However, this study is still in its initial stage. We are not certain whether $Lias$ gene expression is also increased in regulatory T cells per se in $Lias^{H/H}$ mice although changes of $Lias$ gene expression occurred at a similar level in all major organs in the genetic mutant $Lias$ mice. In addition, in addition to being an antioxidant, LA is also a cofactor in pyruvate dehydrogenase complex and alpha-ketoglutarate dehydrogenase complex, both of which are mitochondrial enzymes and participate in ATP production. Thus, it is possible that the impact of LA on atherosclerosis is through the regulation of energy homeostasis of Tregs. Furthermore, our results also revealed that plasma TG level was significantly lower but Chol level was not significantly different in the $Lias$ overexpressing mice. Potential mechanisms for LA mediated Chol and TG remains unclear but TG is not thought as a causal risk factor for atherosclerosis [40].

In conclusion, using the novel antioxidant model, we found that overexpression of *Lias* gene and resulting elevation of intrinsic antioxidant levels ameliorates atherogenic properties of *Apoe*$^{-/-}$ mice. One of the plausible mechanisms is the suppression of inflammatory response via potential regulation of the immune system, including the increased population of Treg cells and circulating autoantibody levels and decreased infiltration of T cells. The outcome of this work would provide a potentially novel preventive and therapeutic strategy for clinical applications by lipoic acid treatment.

Funding sources

Abbreviations:

Chol	cholesterol
FoxP3	forkhead box protein 3
4-HNE	4-hydroxynonenal
IFN-γ	interferon-γ
LA	α-lipoic acid
Lias	lipoic acid synthase
MAA	1,4-dihydropyridine-type MDA-acetaldehyde-Lys adduct
oxLDL	oxidized low-density lipoprotein
Tregs	regulatory T cells

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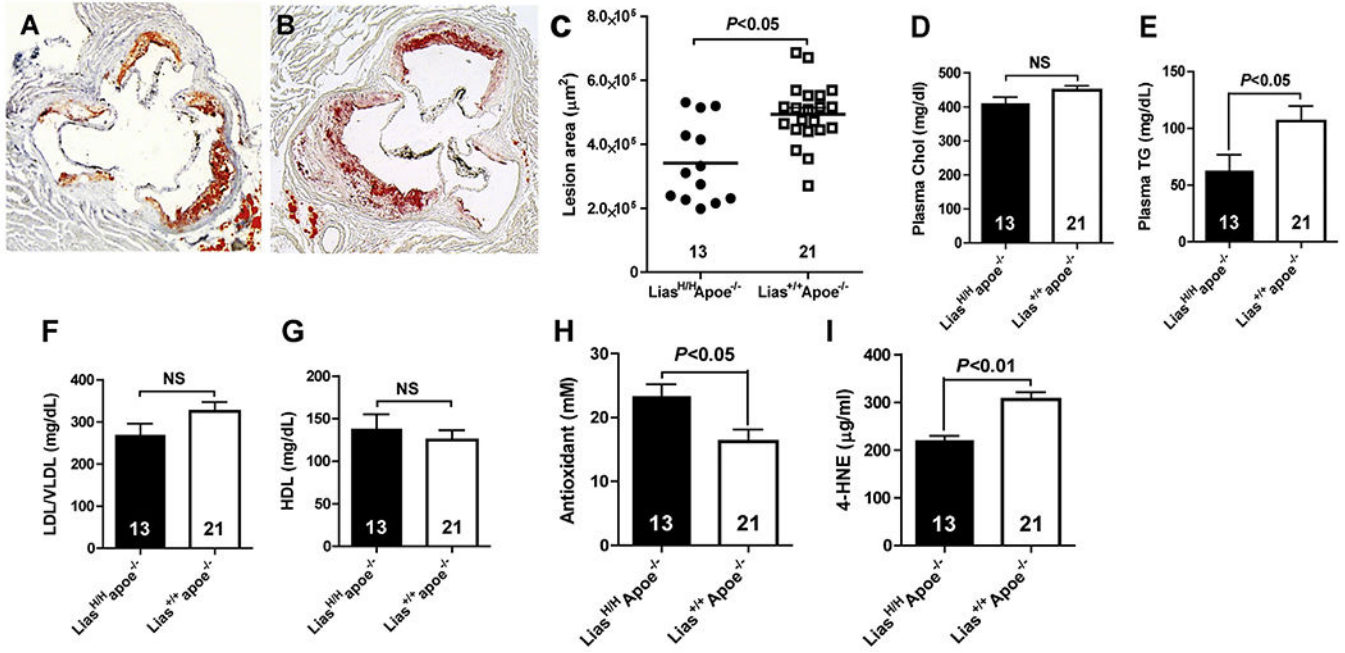
Fig. 1.
Representative light photomicrographs of aortic sinus plaque, plasma lipid profile and redox status. Aortic sinus plaque in $Lias^{H/H}Apoe^{-/-}$ mice (A) $Lias^{+/+}Apoe^{-/-}$ mice (B). Original magnification 40×. Sections are stained with Oil Red O and counterstained with hematoxylin. C) Average atherosclerosis plaques at the aortic roots of 6-month-old male $Lias^{H/H}Apoe^{-/-}$ ($n = 13$) and $Lias^{+/+}Apoe^{-/-}$ ($n = 21$) mice. Each point represents the lesion size of the individual mouse and the horizontal bars stand for mean lesion size. (D) Plasma total cholesterol (Chol). (E) Plasma triglyceride (TG). (F) VLDL/LDL cholesterol. (G) HDL cholesterol. (H) Total antioxidant content and (I) Lipid peroxidation marker, 4-HNE, concentration in 6-month-old male $Lias^{H/H}Apoe^{-/-}$ (closed bars) and $Lias^{+/+}Apoe^{-/-}$ mice (open bars). Numbers inside bars indicate the number of animals. Results are expressed as mean ± SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
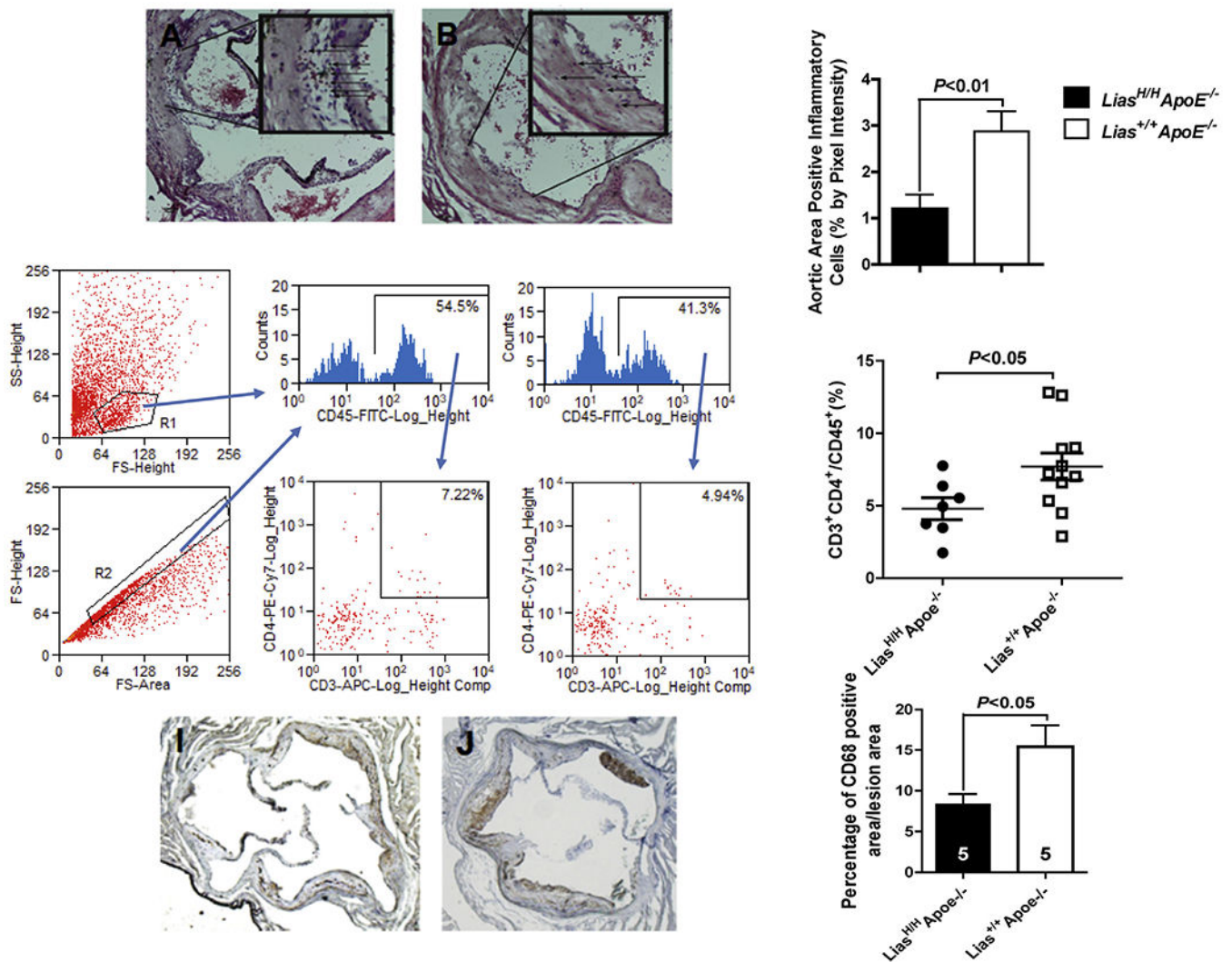
Fig. 2.
Histological analysis of the ascending aortic root. (A). Representative *Lias*$^{+/+}$*Apoe*$^{-/-}$ aortic root (arrows in insert delineate to identified mononuclear cell nuclei). (B). Representative *Lias*$^{H/H}$*Apoe*$^{-/-}$ aortic root (arrows in insert delineate to identified the nuclei). (C). Student's *t*-test was used to determine differences in aortic root cellular nuclei in *Lias*$^{H/H}$*Apoe*$^{-/-}$ compared to controls. Original magnification 40×.
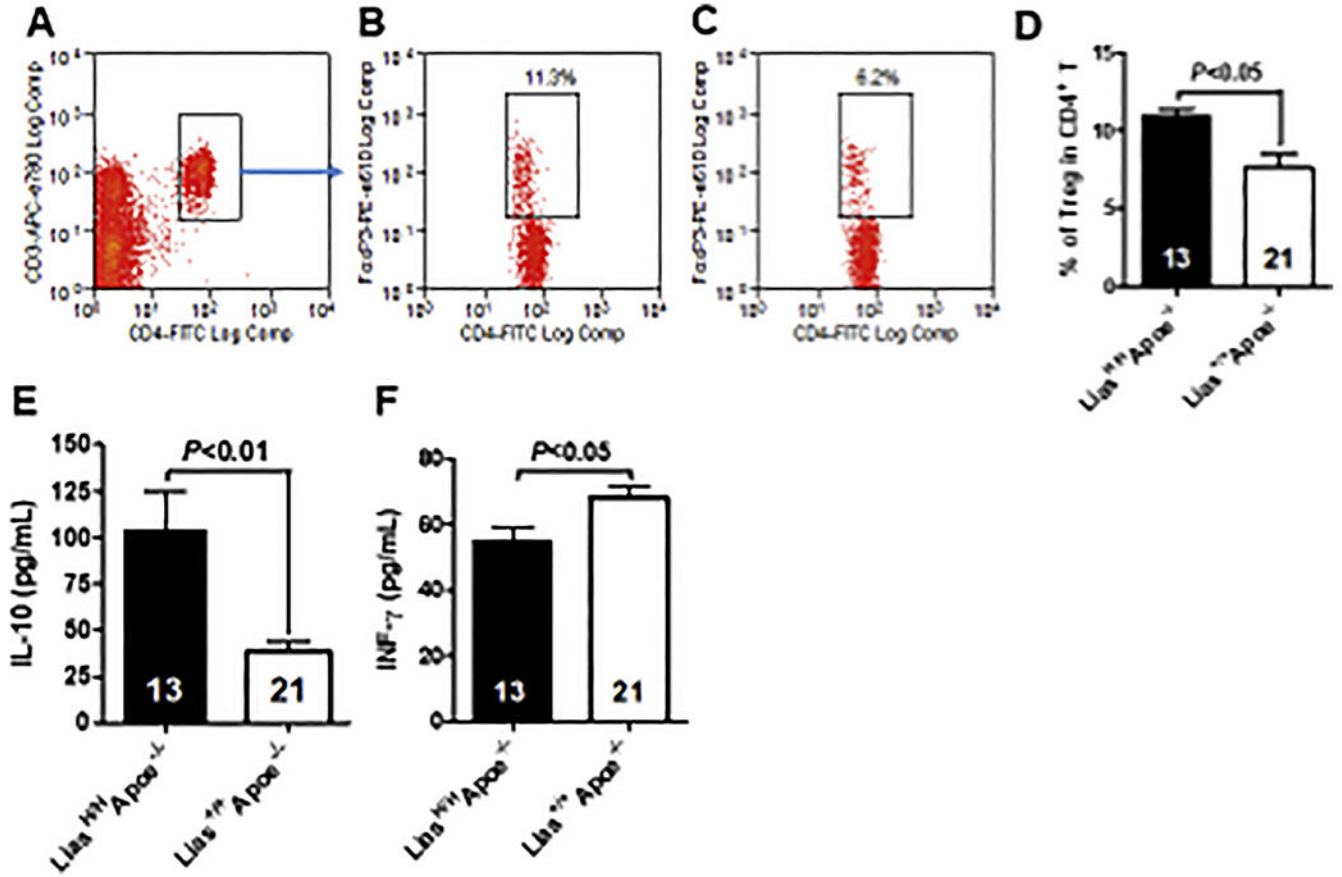
Fig. 3.
Tregs in peripheral blood of *Lias^{H/H}Apoe^{-/-}* and *Lias^{+/+}Apoe^{-/-}* mice. Blood was collected from each experimental group and lymphocytes isolated from blood were stained with CD3-APC-eFluor780, CD4-FITC. Cells were fixed, permeabilized and stained intracellularly with FoxP3-PE-eFluor 610 and flow cytometry analysis. Cells were gated on CD4 and CD3 (A) and FoxP3 with *Lias^{H/H}Apoe^{-/-}* (B) and *Lias^{+/+}Apoe^{-/-}* (C). Quantitative analysis of Tregs in the two groups of mice (D). Results are expressed as mean ± SE. Serum levels of interleukin-10 (E) and interferon γ (F) in 6-month-old male *Lias^{H/H}Apoe^{-/-}* (closed bar) and *Lias^{+/+}Apoe^{-/-}* mice (open bar). Results are expressed as mean ± SE.
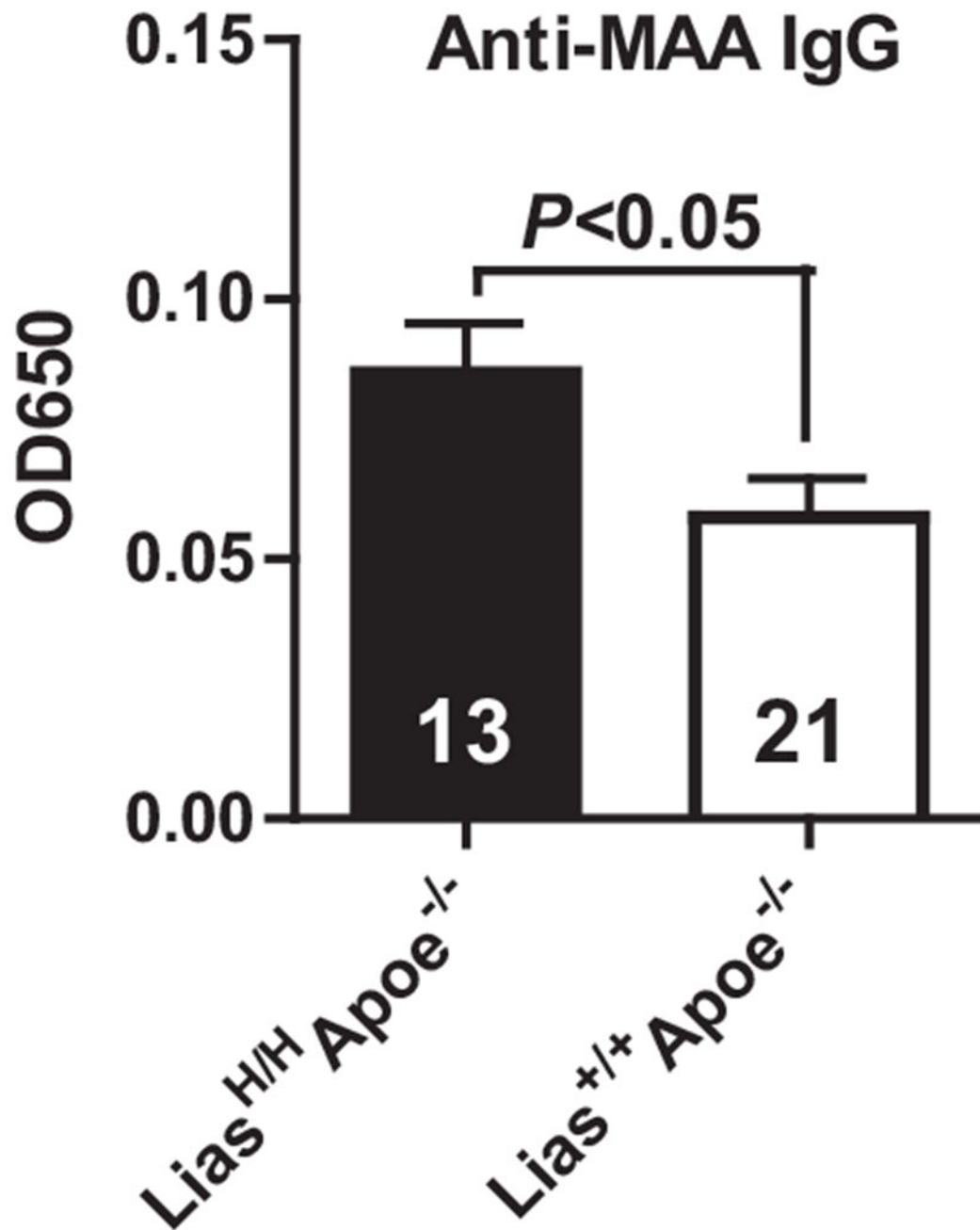
Fig. 4.
An ELISA method for characterizing the concentration of anti-MAA antibodies. Serum IgG antibody levels to MAA were determined by ELISA at 6 months of age in male $Lias^{H/H}Apoe^{-/-}$ (closed bar) and $Lias^{+/+}Apoe^{-/-}$ mice (open bar). Results are expressed as mean ± SE.