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Apolipoprotein E-knockout mice on high-fat diet show autoimmune injury on kidney and aorta

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ABSTRACT

Background: Apolipoprotein E-knockout (ApoE$^{-/-}$) mice is a classic model of atherosclerosis. We have found that ApoE$^{-/-}$ mice showed splenomegaly, higher titers of serum anti-nuclear antibody (ANA) and anti-dsDNA antibody compared with C57B6/L (B6) mice. However, whether ApoE$^{-/-}$ mice show autoimmune injury remains unclear.
Methods and results: Six females and six males in each group, ApoE$^{-/-}$, Fas$^{-/-}$ and B6 mice, were used in this study. The titers of serum ANA, anti-dsDNA antibody and creatinine and urine protein were measured by ELISA after 4 months of high-fat diet. The spleen weight and the glomerular area were determined. The expressions of IgG, C3 and macrophage in kidney and atherosclerotic plaque were detected by immunostaining followed by morphometric analysis. Similar to the characteristics of Fas$^{-/-}$ mice, a model of systemic lupus erythematosus (SLE), ApoE$^{-/-}$ mice, especially female, displayed significant increases of spleen weight and glomerular area when compared to B6 mice. Also, elevated titers of serum ANA, anti-dsDNA antibody and creatinine and urine protein. Moreover, the expressions of IgG, C3 and macrophage in glomeruli and aortic plaques were found in ApoE$^{-/-}$ mice. In addition, the IgG and C3 expressions in glomeruli and plaques significantly increased (or a trend of increase) in female ApoE$^{-/-}$ mice compared with males.
Conclusions: Apolipoprotein E-knockout mice on high-fat diet show autoimmune injury on kidney and aorta.

1. Introduction

Apolipoprotein E-knockout (ApoE$^{-/-}$) mice, a classic model of atherosclerosis (AS), have been widely used in cardiovascular research. In ApoE$^{-/-}$ mice, severe hypercholesterolemia induced by cholesterol feeding contributes to the formation of IgG1 autoantibodies against oxidized LDL [1]. The phenotypic analysis from spleen cells shows that the activation of polyclonal B cell exists in ApoE$^{-/-}$ mice [2]. The titers of serum anti-oxidized LDL and anti-cardiolipin autoantibodies significantly increase in ApoE$^{-/-}$ mice compared with B6 mice [2]. In our previous research [3], we found that ApoE$^{-/-}$ mice showed splenomegaly and higher titers of serum ANA and anti-dsDNA antibody compared with B6 mice. However, whether ApoE$^{-/-}$ mice show autoimmune response and injury remains to be determined.

Systemic lupus erythematosus (SLE) is a classic autoimmune disease, and Fas$^{-/-}$ mouse is a model of SLE-like autoimmune syndromes. In the present study, to investigate the autoimmune response in ApoE$^{-/-}$ mice, we compared serum ANA, anti-dsDNA antibody and creatinine and urine protein levels, IgG and C3 depositions, and macrophage infiltration in ApoE$^{-/-}$ mice with those in B6 and Fas$^{-/-}$ mice.

2. Materials and methods

2.1. Animals

The 3 groups in this study were ApoE$^{-/-}$, B6 and Fas$^{-/-}$ mice. Each group consisted of six females and six males. ApoE$^{-/-}$ and

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B6 mice were 2-month old specific-pathogen-free (SPF) B6.129P2-Apoe^{tm1Unc}/J and SPF C57BL/6J mice respectively, which were obtained from Peking University (Beijing, China). According to the fact that Fas$^{-/-}$ mice on C57BL/6 background exhibit prominent lupus-like autoimmune syndromes in old age, we chose 8-month old SPF B6.MRL-Fas^{lpr}/J mice, which were obtained from Nanking University (Nanjing, China). Above three groups of mice were fed with high-fat diet (0.25% cholesterol and 15% fat) for 4 months. Use of mice in this study was approved by the Animal Care and Use Committee of Fujian Medical University.

2.2. Measurement of serum ANA, anti-dsDNA antibody, creatinine and lipid and urine protein

Mice were anesthetized with 3% pentobarbital and blood was collected from postcava. Serum was separated by centrifugation at room temperature and stored at −40 °C until used. Ten μl of serum from each mouse was diluted at 1:100 and used to determine antibody titers of anti-nuclear antibody (ANA) and anti-dsDNA by ELISA kits (Alpha Diagnostic International, San Antonio, TX). Serum creatinine and urine protein titers were also measured by ELISA method. Blood lipid levels were measured by enzymatic assays.

2.3. Histopathological evaluation

Mice were perfused with physiological saline through the left ventricle to remove the blood in organs, followed by 4% paraformaldehyde. The spleens were harvested, weighed and photographed individually.

Aortas were carefully separated from arteria iliaca and anonyma and extravascular fat tissue was removed, and aortic roots were cut off. The aortic root, remaining part of the aorta and a kidney of each mouse were fixed with 4% paraformaldehyde for 24 h. The kidney was cut into two parts, a part of the kidney tissue of each mouse was prepared for frozen section, the remaining part of the kidney tissue was embedded in paraffin and 4 μm thick sections were cut. The paraffin sections were stained with hematoxylin and eosin (H and E) and used for morphometric analysis. Briefly, glomerulus images were captured with a video camera and analyzed by a computerized image analysis program (Image Pro Plus, Media Cybemetics, Bethesda, MD). The area of the glomerular tuft was measured as previously described [4]. More than 25 glomeruli were examined to calculate the mean area of the glomerular tuft for each mouse. To detect the lesion of AS, paraformaldehyde-fixed aortas (from the aortic origin to the iliac bifurcation) were opened longitudinally, and stained for lipids with oil red O as described previously [5]. The total plaque load in the aorta was determined using Image Pro Plus software as described above.

2.4. Immunofluorescent and histochemical staining

The aortic root and part kidney tissue of each mouse were embedded in OTC, and 6-μm thick frozen sections were cut. For IgG immunofluorescent staining, the sections of the kidney and aortic tissues were incubated with 1: 600 diluted goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) for 17 h at 4 °C, followed by 1:100 diluted fluorescein conjugate anti-goat IgG secondary antibody (SouthernBiotech, Birmingham, AL) for 30 min at 37 °C. For immunohistochemical staining of C3 and macrophages, the frozen sections of the kidney and aortic tissues were incubated with rat monoclonal antibody against mouse monocyte/macrophage (MOMA-2, AbD Serotec, Cambridge, UK) diluted at 1:125, and rat monoclonal antibody against mouse C3 (Abcam, Cambridge, MA) diluted at 1:50 for kidney sections kidney and 1:100 for aorta sections respectively for 17 h at 4 °C. After rinsing in phosphate buffered saline, the sections were incubated with horseradish enzyme-marked anti-rat IgG polymersomes kit (Zhongshan Glodenbridge Biotechnology Co. LTD, Beijing, China) for 30 min at 37 °C, followed by ABC reagent and diaminobenzidine chromogen (Zhongshan Glodenbridge Biotechnology Co. LTD, Beijing, China). The immunostained areas of IgG, C3 and macrophages in kidney and aorta tissues were analyzed using Image Pro Plus software as described above. More than 25 glomeruli were measured for each sample.

2.5. Statistical analyses

Data were analyzed with SPSS software (SPSS Version 16.0, Chicago, IL) and the results were presented as the mean ± standard error of the mean (SEM). The normal distribution test was conducted in the variables. The intergroup differences were analyzed by one-way ANOVA followed by the LSD analysis for multiple comparisons. $P < 0.05$ was considered to be significant.

3. Results

3.1. Body weight detection

ApoE$^{-/-}$ mice body weights were similar to same-sex B6 mice in different phases of the experiment, and simillar to same-sex Fas$^{-/-}$ mice after 4-month fat diet (Supplementary Fig. 1). And the male body weights were bigger than the female in ApoE$^{-/-}$, B6 and Fas$^{-/-}$ mice in diffferent phases of the experiment ($P < 0.05$, Supplementary Fig. 1). After 4-month high-fat diet, all mice weights were significantly increased ($P < 0.05$, Supplementary Fig. 1).

3.2. Levels of blood lipid

Blood cholesterol (CHO), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) titers were higher in ApoE$^{-/-}$ than B6 and Fas$^{-/-}$ mice after 4-month high-fat diet ($P < 0.05$, Supplementary Fig. 2A–C).

3.3. Levels of serum creatinine and urine protein

Serum creatinine and urine protein titers were elevated in both ApoE$^{-/-}$ and Fas$^{-/-}$ mice compared to B6 mice ($P < 0.05$, Supplementary Fig. 3A–D) with a trend of increase in female than in male animals (Supplementary Fig. 3B and D).

3.4. Concentrations of serum autoantibodies

There was no significant difference in serum ANA titers between ApoE$^{-/-}$ and Fas$^{-/-}$ mice. Serum ANA titers were elevated in both ApoE$^{-/-}$ and Fas$^{-/-}$ mice compared to B6 mice ($P < 0.05$, Fig. 1A) with a greater degree of increase in female animals ($P < 0.05$, Fig. 1B).

Similar to ANA antibody, the serum anti-dsDNA antibody titers in ApoE$^{-/-}$ and Fas$^{-/-}$ mice were higher than those in B6 mice ($P < 0.05$, Fig. 1C). No significant difference was observed between ApoE$^{-/-}$ and Fas$^{-/-}$ mice. The serum anti-dsDNA antibody titer increased more significantly in female than that in male ApoE$^{-/-}$ and male Fas$^{-/-}$ mice ($P < 0.05$, Fig. 1D). Neither female nor male mice exhibited significant difference in serum anti-dsDNA antibody titer between ApoE$^{-/-}$ and Fas$^{-/-}$ mice.

3.5. Splenomegaly

The spleen weight in ApoE$^{-/-}$ mouse was similar to that in Fas$^{-/-}$ mouse, but was significantly heavier than that of B6 mouse

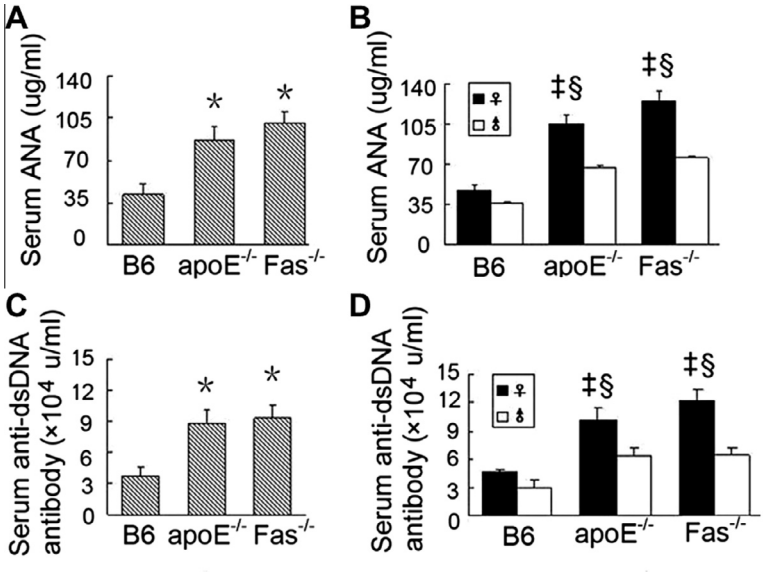

Fig. 1. Serum anti-nuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) antibody titers higher in ApoE$^{-/-}$ and Fas$^{-/-}$ than C57BL/6 (B6) mice, similar in ApoE$^{-/-}$ and Fas$^{-/-}$ mice. Quantitative analyses of serum titers of ANA (A and B), and anti-dsDNA antibody (C and D) and the gender differences (B and D) by ELISA in B6, ApoE$^{-/-}$ and Fas$^{-/-}$ mice. *$P < 0.05$, compared to B6 mice; ‡$P < 0.05$, compared to male ApoE$^{-/-}$ mice; §$P < 0.05$, compared to male Fas$^{-/-}$ mice.

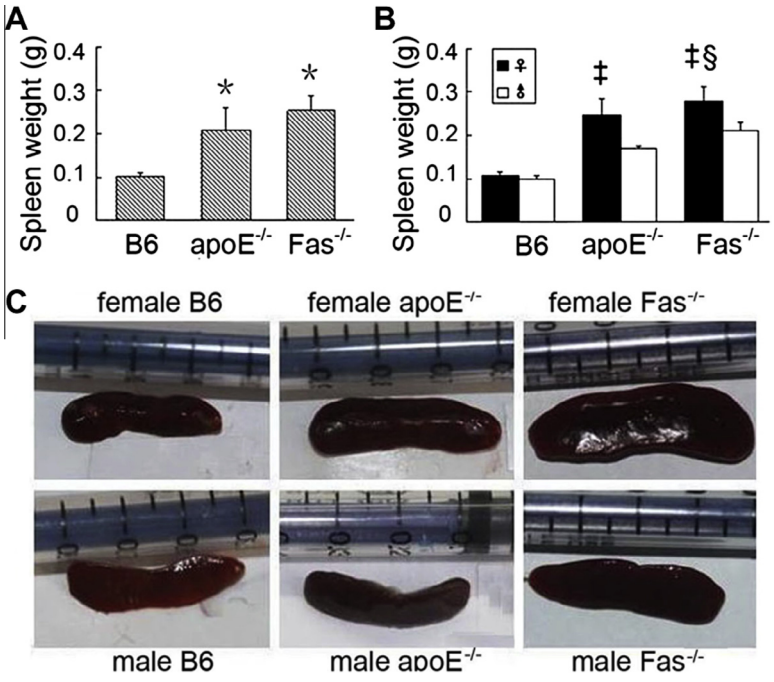

Fig. 2. Spleen greater in ApoE$^{-/-}$ and Fas$^{-/-}$ than C57BL/6 (B6) mice, similar in ApoE$^{-/-}$ and Fas$^{-/-}$ mice. Wet weight of spleens (A and B) and the gender differences (B), and gross appearance of representative spleen tissues (C) in B6, ApoE$^{-/-}$ and Fas$^{-/-}$ mice. *$P < 0.05$, compared to B6 mice; ‡$P < 0.05$, compared to male ApoE$^{-/-}$ mice; §$P < 0.05$, compared to male Fas$^{-/-}$ mice.

($P < 0.01$, Fig. 2A), The enlargement and weight increase of the spleen were more pronounced in female than in male ApoE$^{-/-}$ mouse ($P < 0.05$, Fig. 2B and C).

3.6. Glomerular hyperplasia

To determine whether glomerular hyperplasia exists in ApoE$^{-/-}$ mice, we compared the glomerular area of ApoE$^{-/-}$ mice to that of B6 and Fas$^{-/-}$ mice. The glomerular area of ApoE$^{-/-}$ mice was significantly larger than that of B6 mice ($P < 0.05$, Fig. 3A), but there was no significant difference from Fas$^{-/-}$ mice. Moreover, both ApoE$^{-/-}$ and Fas$^{-/-}$ female mice displayed a significant enlargement of the glomeruli compared to male counterparts (both $P < 0.05$, Fig. 3B and C).

3.7. Immune complex deposition in glomeruli

The area of IgG staining in the glomeruli was larger in ApoE$^{-/-}$ mice than that in B6 mice ($P < 0.01$, Fig. 3D), but there is no significant difference between ApoE$^{-/-}$ and Fas$^{-/-}$ mice. The area of IgG

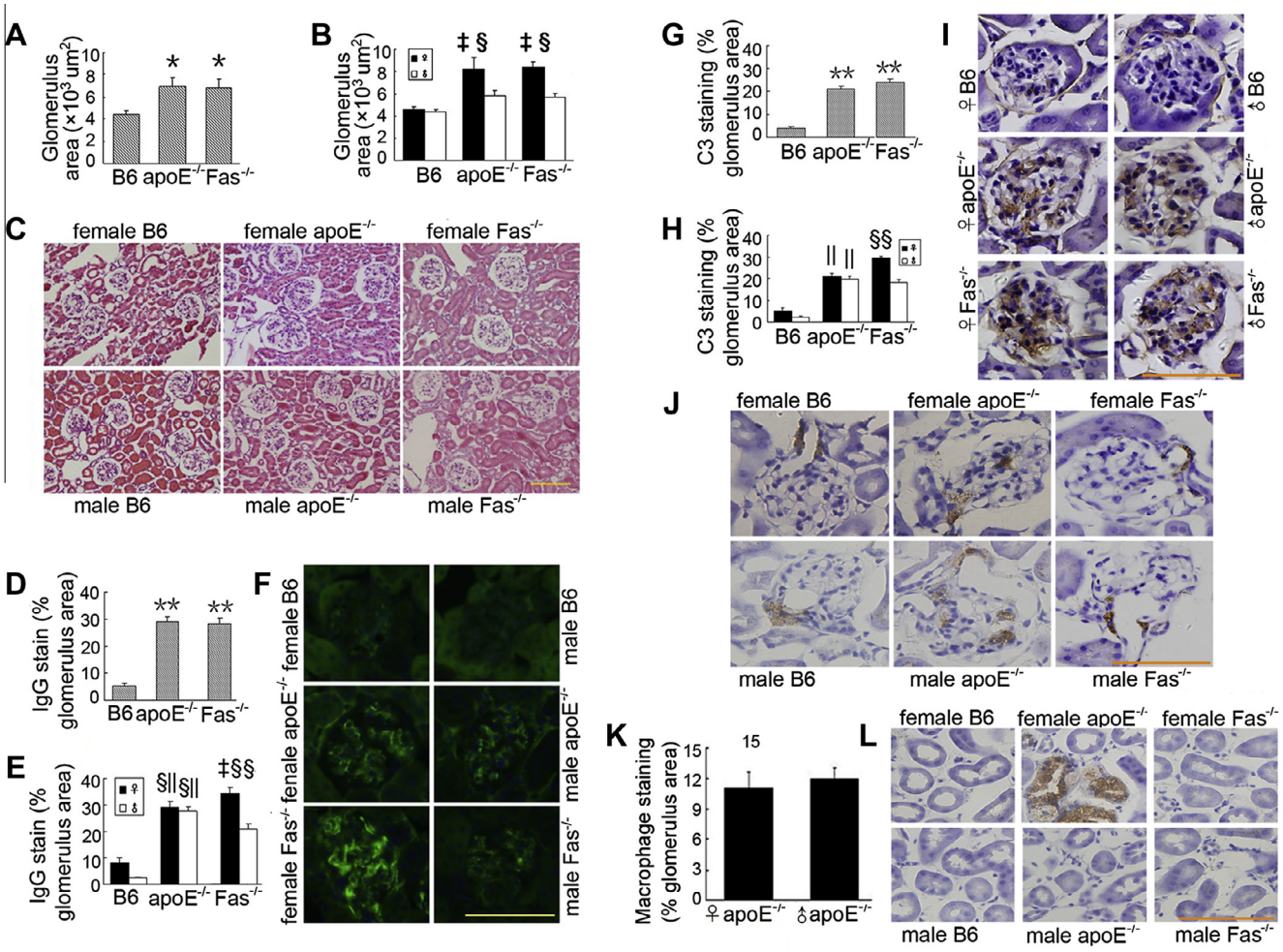

Fig. 3. Glomerular area, IgG and C3 staining areas in glomeruli larger in ApoE$^{-/-}$ and Fas$^{-/-}$ than C57BL/6 (B6) mice, similar in ApoE$^{-/-}$ and Fas$^{-/-}$ mice; macrophage infiltration in glomerulus and renal tubules found only in ApoE$^{-/-}$ mice. Morphometric analysis of glomerular areas (A and B) and the gender differences (B), and H and E stained micrographs of renal cortex (C) in B6, ApoE$^{-/-}$ and Fas$^{-/-}$ mice. Morphometric analyses of glomerulus areas positive for IgG (D and E) and C3 (G and H) immunostaining and the gender differences (E and H). Panels (F and I) are representative micrographs of IgG immunofluorescent (F) and C3 immunohistochemical (I) staining of renal cortex in B6, ApoE$^{-/-}$ and Fas$^{-/-}$ mice. Bar = 100 μm. Immunohistochemical detection of macrophage infiltration in efferent and afferent arterioles (J), glomerulus (J) and renal tubules (L) in B6, ApoE$^{-/-}$ and Fas$^{-/-}$ mice. There is no difference in macrophage infiltration in glomerulus between female and male ApoE$^{-/-}$ mice (K). Bar = 100 μm (C, F and I). Bar = 200 μm (J and L). $^{*,**}P < 0.05$, $P < 0.01$, compared to B6 mice; $^{‡}P < 0.05$, compared to male ApoE$^{-/-}$ mice; $^{§}P < 0.05$, compared to male Fas$^{-/-}$ mice; $^{§§}P < 0.01$, compared to male Fas$^{-/-}$ mice; $^{‖}P < 0.05$, compared to female Fas$^{-/-}$ mice.

staining in the glomeruli was larger in female than that in male Fas$^{-/-}$ mice ($P < 0.01$, Fig. 3E and F). Although the difference between female and male ApoE$^{-/-}$ mice was not statistically significant, there was a trend of increase in IgG deposition in female compared with male ApoE$^{-/-}$ mice ($P = 0.45$, Fig. 3E and F). In addition, we also observed that the IgG deposition in the glomeruli of female and male ApoE$^{-/-}$ mice was higher than that in male Fas$^{-/-}$ mice ($P < 0.05$, Fig. 3E and F).

Similar to IgG expression, the C3 expression in the glomeruli in ApoE$^{-/-}$ mice was higher than that in B6 mice ($P < 0.01$, Fig. 3G), but there was no significant difference between ApoE$^{-/-}$ and Fas$^{-/-}$ mice. There was a trend of increase in C3 expression in the glomeruli of female compared to male ApoE$^{-/-}$ and Fas$^{-/-}$ mice ($P = 0.47$, $P = 0.35$, Fig. 3H and I).

3.8. Macrophage infiltration in glomeruli and renal tubules

Macrophages play important roles in SLE-induced tissues injury, and it is associated with the onset of lupus nephritis [6]. Macrophage infiltration in the efferent and afferent arteriole of glomerulus was detected in every group of mice, and the degree of infiltration was similar among all groups of mice (Fig. 3J). However, the macrophage infiltration in glomerulus was only observed in ApoE$^{-/-}$ mice, and the extend of infiltration was no different between females and males (Fig. 3J and K). The macrophage infiltration in renal tubules was only observed in male ApoE$^{-/-}$ mice, but not in any other groups of mice (Fig. 3L).

3.9. AS lesion, IgG, C3 and macrophage expressions in aortic plaque

The positive staining of Oil red O in aortic intima was only observed in ApoE$^{-/-}$ mice. There was no significant difference in plaque coverage of aorta lining between female and male ApoE$^{-/-}$ mice (Fig. 4A and B). In order to investigate the involvement of immune response in the lesion of AS, we assessed IgG and C3 expression and macrophage infiltration in aortic plaques using immunostaining. The IgG and C3 expressions and macrophage infiltration were all clearly observed in aortic plaques. The immunostaining areas of IgG and C3 were greater in female than in male ApoE$^{-/-}$ mice ($P < 0.01$, Fig. 4C–F), while the degree of macrophage infiltration was lower in female than in male ApoE$^{-/-}$ mice ($P < 0.01$, Fig. 4G and H).

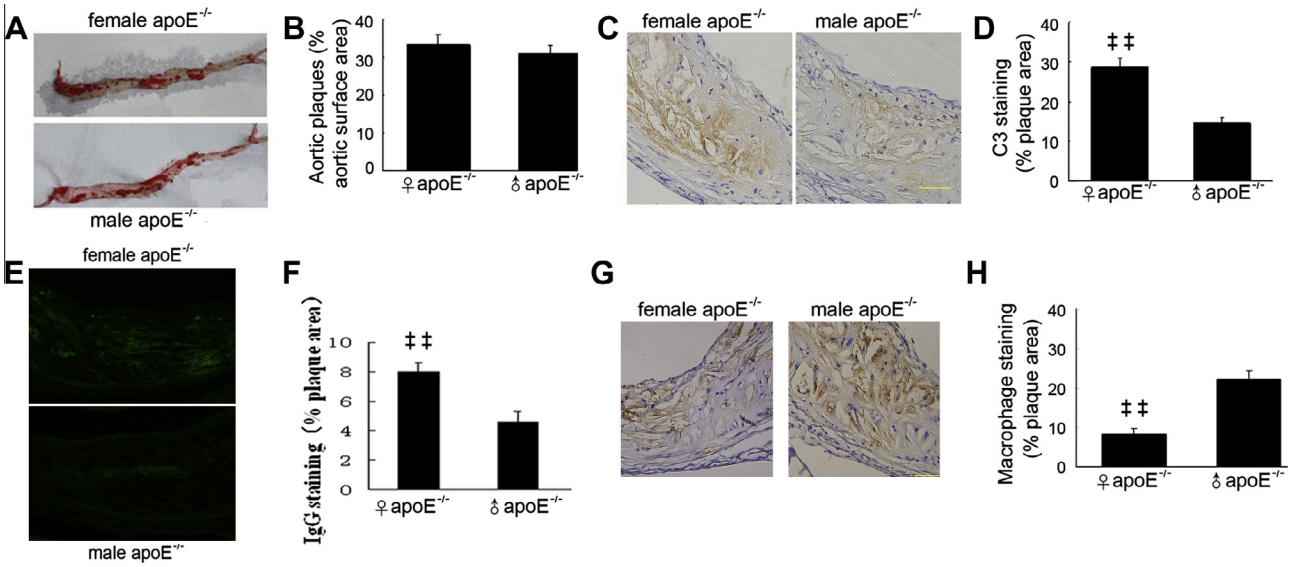

Fig. 4. IgG and C3 staining areas larger, macrophage staining area smaller in aortic plaque in female than male ApoE$^{-/-}$ mice. Representative micrographs of *En-face* aortic lesions stained with oil red O (A), and C3 (C), IgG (E) and macrophage (G) expression in aortic plaques of female and male ApoE$^{-/-}$ mice. Panels (B, D, F and H) are quantitative analyses of plaque areas (B) in aorta lining, C3 (D), IgG (F) and macrophage (H) expression in aortic plaques of female and male ApoE$^{-/-}$ mice. $^{‡‡}P < 0.01$, compared to male ApoE$^{-/-}$ mice. Bar = 200 μm.

3.10. High-fat diet lead to serum autoantibody titers changes

To investigate the effect on ApoE$^{-/-}$ mice serum autoantibody titer from high-fat diet, serum ANA and anti-dsDNA antibody titers were detected for another six females and six males of 6-month old ApoE$^{-/-}$ mice on common diet. We found that ApoE$^{-/-}$ mice on high-fat diet showed higher titers of ANA and anti-dsDNA antibody than those on common diet ($P < 0.05$, Supplementary Fig. 4A and B).

4. Discussion

SLE is a classical autoimmune disease, and characterized by the production of autoantibodies, immune-complex deposition, tissue infiltration of macrophages, and tissue damage [7]. Significant elevation in autoantibodies titers, especially anti-dsDNA antibody may trigger complement cascade, inflammatory cells activation and cause multiple organ damage. In the present study, after 4 month high-fat diet, we observed significant increases in the titers of serum ANA, anti-dsDNA antibody and creatinine and urine protein, spleen weight, glomerulus area, and IgG and C3 expression in glomerulus in ApoE$^{-/-}$ mice compared with B6 mice. These manifestations are resembled to those of the Fas$^{-/-}$ mice, suggesting that autoimmune response and injury exist in ApoE$^{-/-}$ mice.

Long-term existence of high titer of serum autoantibody can cause deposition of immune complex in organs. Immune complex deposition in renal tissue, such as in the glomeruli, renal tubules and matrix, is characteristic of SLE. The activated complements attract inflammatory cells, especially monocyte/macrophage, which can also induce tissue damage. In our experiment, the glomerulus area, IgG and C3 expression in the glomeruli, serum creatinine and urine protein titers were significantly increased in ApoE$^{-/-}$ mice compared with B6 mice, but no significant difference was observed between ApoE$^{-/-}$ and Fas$^{-/-}$ mice. These findings suggest that ApoE$^{-/-}$ mice may suffer similar renal injury as SLE.

We also observed that macrophage infiltration in the glomeruli only existed in ApoE$^{-/-}$ mice and were similar in efferent and afferent arterioles among all the mice. Macrophages are important inflammatory cells and critical players in innate and adaptive immune responses. The infiltrated macrophages in the glomeruli were probably responsible for the damage of kidney tissue. The difference of macrophage infiltration in the glomeruli of ApoE$^{-/-}$ and Fas$^{-/-}$ mice may suggest that there are different injury mechanisms in the glomerulus between ApoE$^{-/-}$ and Fas$^{-/-}$ mice.

It is well known that SLE is more prevalent among young women with a female to male ratio of 9:1 [8], which is thought to be related to sex steroid hormones in females [9]. We found that serum autoantibody titers, spleen weight and kidney injury were more significantly increased in female ApoE$^{-/-}$ mice than those in male ApoE$^{-/-}$ mice. Although the mechanism responsible for this gender difference in immune injury of AoE$^{-/-}$ mice is not clear, we guessed sex steroid hormones have played an important role.

Concurrently, IgG, C3 and macrophage infiltration were positive in aortic plaques of ApoE$^{-/-}$ mice. Growing evidence indicates that AS is associated with autoimmunity [10–17]. The related autoantigens include heat-shock protein, oxidized low-density-lipoproteins, β2 glycoprotein and structural components of some microorganisms [10–17]. At present, whether IgG and C3 in aortic plaque represent the deposition of immune complex in atherosclerosis remains unclear. But the existences of IgG and C3 in aortic plaque of ApoE$^{-/-}$ mice reflected the association between autoimmunity and AS in a certain degree. The association between autoimmune response and AS may be due to those inflammatory cells or other substance attracted by complements and autoantibodies. We also found sex differences in IgG and C3 expression and macrophage infiltration in the plaques. The differences between female and male may due to increased T help (Th) 2-predomination immune response and decreased Th1 response in females, which have been demonstrated in humans [18]. Those differences in AS lesion also mirror the different pathological mechanism of AS between sexes in ApoE$^{-/-}$ mice.

There are different gene knockout backgrounds between Fas$^{-/-}$ and ApoE$^{-/-}$ mice, which lead to different pathogenesises of autoimmunity. Dysregulation of the Fas pathways can alter the function of APCs, thereby leading to SLE pathogenesis in Fas$^{-/-}$ mice [19]. ApoE deficiency leads to impaired clearance of apoptotic cell remnants [20], which results in the increment of autoantigens in the body. In our previous report [3], ApoE$^{-/-}$ mice showed high titer of autoantibody compared with B6 mice, and TLR4 signal pathway participate in maintaining the balance of splenocyte

apoptosis and antibody production. In this experiment, high-fat diet induced more increased titers of serum autoantibodies in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice on high-fat diet showed autoimmune injury on kidney and artery. Accordingly, we infer that autoimmune response exists in ApoE$^{-/-}$ mice and autoimmune injury would be induced when they improperly expose to some inducers such as high fat diet, especially in female ApoE$^{-/-}$ mice. Our findings also strongly suggest that the autoimmune response probably participates the development of AS, which yields the potential clinical significance in prevention and treatment of AS with or without an autoimmune disease.

To the best of our knowledge, this represents the first report that ApoE$^{-/-}$ mice show autoimmune response and injury. Further experiments are needed to explore the autoimmune response and its role in the development of AS in ApoE$^{-/-}$ mice.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.060.

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