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Apolipoprotein E-knockout mice show increased titers of serum anti-nuclear and anti-dsDNA antibodies

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ABSTRACT

Apolipoprotein E-knockout (ApoE$^{-/-}$) mice, atherosclerosis-prone mice, show an autoimmune response, but the pathogenesis is not fully understood. We investigated the pathogenesis in female and male ApoE$^{-/-}$ mice. The spleens of all ApoE$^{-/-}$ and C57BL/6 (B6) mice were weighed. The serum IgG level and titers of anti-nuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) antibody were assayed by ELISA. Apoptosis of spleen tissue was evaluated by TUNEL. TLR4 level in spleen tissue was tested by immunohistochemistry and Western blot analysis. Levels of MyD88, p38, phosphorylated p38 (pp38), interferon regulatory factor 3 (IRF3) and Bcl-2-associated X protein (Bax) in spleen tissue were detected by Western blot analysis. We also survey the changes of serum autoantibodies, spleen weight, splenocyte apoptosis and the expressions of TLR4, MyD88, pp38, IRF3 and Bax in spleen tissue in male ApoE$^{-/-}$ mice after 4 weeks of lipopolysaccharide (LPS), Toll-like receptor 4 ligand, administration. ApoE$^{-/-}$ mice showed splenomegaly and significantly increased serum level of IgG and titers of ANA and anti-dsDNA antibody as compared with B6 mice. Splenocyte apoptosis and the expression of TLR4, MyD88, pp38, IRF3 and Bax in spleen tissue were significantly lower in ApoE$^{-/-}$ than B6 mice. The expression of TLR4, MyD88, IRF3, pp38, and Bax differed by sex in ApoE$^{-/-}$ spleen tissue. The down-regulation of TLR4 signal molecules induced by LPS led to decreased expression of Bax and increased serum titers of ANA and anti-dsDNA antibody. Therefore, the TLR4 signal pathway may participate in maintaining the balance of splenocyte apoptosis and autoantibody production in ApoE$^{-/-}$ mice.

1. Introduction

Growing evidence indicates that atherosclerosis is associated with autoimmunity, and the related autoantigens include oxidized low-density lipoprotein (LDL), heat-shock protein, β2 glycoprotein and structural components of some microorganisms [1–8]. Apolipoprotein E-knockout (ApoE$^{-/-}$) mice, atherosclerosis-prone mice, show abnormal immune function [9–12]. The levels of serum anti-oxidized LDL and anti-cardiolipin autoantibodies are significantly higher in ApoE$^{-/-}$ than C57BL/6 (B6) mice [13]. Phenotypic analysis of spleen cells showed activation of polyclonal B cells in ApoE$^{-/-}$ mice [13]. However, what induces lymphocytes to play a role in the pathogenesis of autoimmunity in the mice remains to be elucidated.

Toll-like receptors (TLRs) recognize various pathogen-associated molecular patterns and induce an innate immune response. TLRs are also critical for the development of adaptive immunity [14,15]. TLR4 is an important member of the TLR family. Previous studies suggested that TLR4 is associated with atherosclerosis in ApoE$^{-/-}$ mice [16,17]. Lipopolysaccharide (a TLR4 ligand) was found to increase autoantibody levels and the deposition of immune complex in transgenic [18], BALB/C [19] and MRL$^{lpr/lpr}$

Abbreviations: ApoE$^{-/-}$, apolipoprotein E knockout; ANA, anti-nuclear antibody; anti-dsDNA, anti-double-stranded DNA; Bax, Bcl-2-associated X protein; TLR, Toll-like receptor; TUNEL, terminal-deoxynucleoitidyl transferase-mediated nick-end labeling; MyD88, myeloid differentiation factor 88; IRF3, interferon regulatory factor 3; p38, p38 mitogen-activated protein kinase.

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mice [20]. Therefore, TLR4 is associated with both atherosclerosis and autoimmunity and may play an important role in the production of autoantibodies in ApoE$^{-/-}$ mice.

Here, we compared levels of serum IgG, anti-nuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) antibody in ApoE$^{-/-}$ and B6 mice. We also investigated spleen weights, splenocyte apoptosis, and levels of TLR4 and signal molecules in spleen tissue of the mice. Then, we surveyed differences by sex for ApoE$^{-/-}$ mice. Eventually, we investigated the changes of serum autoantibody titers and TLR4 pathway molecule levels in spleen tissue in male ApoE$^{-/-}$ mice after TLR4 ligand administration. This research may provide a new insight into the potential pathogenesis of autoimmunity in atherosclerosis-prone mice.

2. Materials and methods

2.1. Mice

We obtained the first group of mice which included 10 females and males each of B6.129P2-Apoe^{tm1Unc}/J (ApoE$^{-/-}$) and B6 mice, then obtained the second batch of mice which included 12 male ApoE$^{-/-}$ mice from Peking University (Beijing). All mice were 12 weeks old. Use of mice in this study was approved by the Animal Care and Use Committee of Shandong University.

2.2. Lipopolysaccharide (LPS) administration

LPS and physiological saline (NS) were used in the second group of mice. LPS (Sigma Chemical Co.), from *Escherichia coli* 055:B5, was diluted at 0.25 mg/ml with NS. Six male ApoE$^{-/-}$ mice were given a intraperitoneal injection of LPS (10 ml/kg), while other 6 male ApoE$^{-/-}$ mice were given a intraperitoneal injection of NS (10 ml/kg) twice a week for 4 weeks.

2.3. Measurement of serum level of IgG and titers of ANA and anti-dsDNA antibody

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. We diluted 10 μl serum from each mouse at 1:100 to determine the titers of ANA and anti-dsDNA antibody by ELISA kits (Alpha Diagnostic International, San Antonio, TX). Another 10 μl of serum from each mouse was diluted at 1:100,000 and used to determine IgG levels by an ELISA kit (Alpha Diagnostic International).

2.4. Splenomegaly evaluation

Mice were killed, and perfused with NS through the left ventricle to remove blood in organs, and spleens were harvested, weighed and photographed individually.

2.5. TUNEL staining of spleen tissue

Part of the spleen of each mouse in first group of mice was fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, and cut into 4-μm-thick sections for TUNEL staining and TLR4 detection by immunohistochemistry. To investigate splenocyte apoptosis, TUNEL staining involved use of a DNA fragmentation detection kit (Roche, Nutley, NJ) and was analyzed by use of Image Pro Plus (Media Cybemetics). Nine fields were chosen randomly for each specimen. The ratio of positive TUNEL cells to total number of splenocytes was determined by counting the number of TUNEL-positive splenocytes.

2.6. TLR4 detection in spleen tissue by immunohistochemistry

Paraffin sections of spleen tissues were incubated with rabbit polyclonal antibody against mouse TLR4 (1:100, Abcam, Cambridge, MA) for 17 h at 4 °C. After a rinsing in phosphate buffered saline, the sections were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Zhongshan Glodenbridge Biotechnology, Beijing) for 30 min at 37 °C, then ABC reagent and diaminobenzidine chromogen (Zhongshan Glodenbridge Biotechnology). The immunostained areas of TLR4 in spleen tissues were analyzed by use of ImagePro Plus. Nine fields were chosen randomly for each specimen. The percentage of TLR4-positive staining was obtained for each specimen.

2.7. Western blot analysis of levels of TLR4, MyD88, interferon regulatory factor 3 (IRF3), p38, phosphorylated p38 (pp38) and Bcl-2-associated X protein (Bax)

Protein samples were obtained from part of the fresh splenic tissue from each mouse as described [21,22]. In total, 50 μg protein extract was separated by SDS–PAGE, transferred onto polyvinylidene fluoride membranes, and blocked with 5% defatted milk. After a washing, membranes were incubated with the polyclonal rabbit antibodies anti-mouse TLR4 (1:300), MyD88 (1:500), p38 (1:500), IRF3 (1:300), Bax (1:500, all Abcam, Cambridge, UK), pp38 (1:1000), and β-actin (1:1000, both Cell Signaling Technology, Danvers, MA) overnight at 4 °C, washed, then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000–1:10,000) for 2 h at room temperature. After another washing, membranes were incubated with chemiluminescent horseradish-peroxidase conjugated substrate (Pierce, Rockford, IL, USA), and luminescent signals were exposed to films. Signals were quantified by scanning densitometry, and the mean light density was obtained by use of Image Pro Plus. Levels were normalized to that of β-actin.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated by Student's *t*-test. Data analysis involved use of SPSS v16.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Levels of serum IgG, ANA and anti-dsDNA antibody in B6 and ApoE$^{-/-}$ mice

Serum levels of IgG and ANA and anti-dsDNA antibody were higher in ApoE$^{-/-}$ than B6 mice ($P < 0.05$, Fig. 1A, C and E), with a greater increase in female than male ApoE$^{-/-}$ mice ($P < 0.05$, Fig. 1B, D and F).

3.2. Splenomegaly in ApoE$^{-/-}$ mice

ApoE$^{-/-}$ mice showed splenomegaly as compared with B6 mice ($P < 0.05$, Fig. 1G), with greater enlargement and weight increase of spleen for females than male ApoE$^{-/-}$ mice ($P < 0.05$, Fig. 1H and I).

3.3. Splenocyte apoptosis in B6 and ApoE$^{-/-}$ mice

ApoE$^{-/-}$ mice showed less TUNEL staining of splenocytes, for apoptosis, than B6 mice ($P < 0.05$, Fig. 1J). TUNEL staining was lower for female than male ApoE$^{-/-}$ mice ($P < 0.05$, Fig. 1K and L).

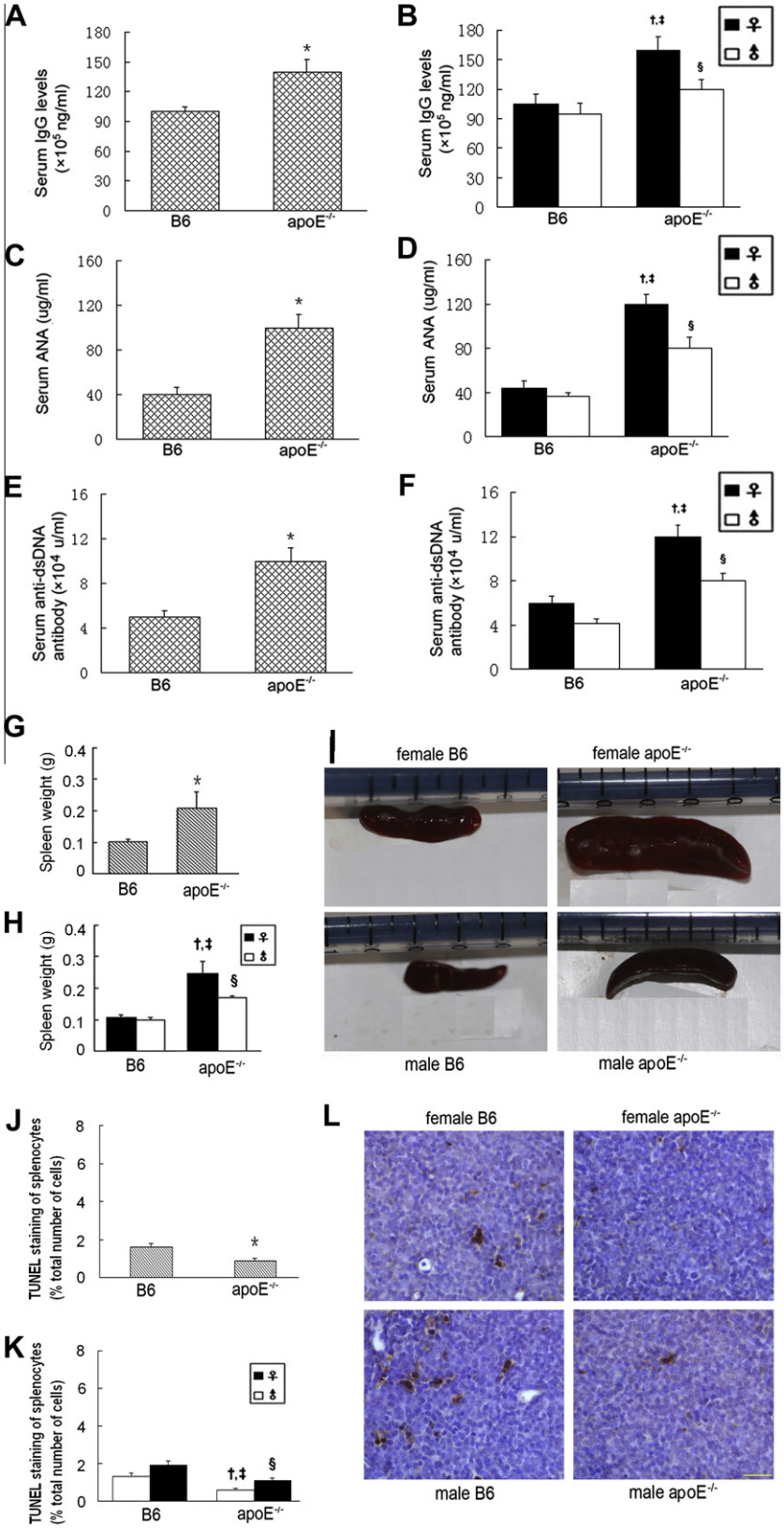

Fig. 1. Serum IgG, anti-nuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) antibody titers higher, spleen greater and splenocyte apoptosis lower in ApoE$^{-/-}$ than C57BL/6 (B6) mice. Quantitative analyses of serum levels of IgG (A and B) and titers of ANA (C and D) and anti-dsDNA antibody (E and F) and sex differences (B, D and F) by ELISA in B6 and ApoE$^{-/-}$ mice. Wet weights of spleens (G and H), sex differences (H), and gross appearance of representative spleen tissues (I) in B6 and ApoE$^{-/-}$ mice. Quantitative analysis of TUNEL staining by immunohistochemistry (J and K) in spleen tissue of B6 and ApoE$^{-/-}$ mice and sex differences (K), and representative photomicrographs (L). Data are mean ± SEM. $^{*}P < 0.05$, compared with B6 mice; $^{\dagger}P < 0.05$, compared with male ApoE$^{-/-}$ mice; $^{\ddagger}P < 0.05$, compared with female B6 mice; $^{\S}P < 0.05$, compared with male B6 mice. Bar = 200 μm.

3.4. TLR4 expression in spleen tissue of B6 and ApoE$^{-/-}$ mice

ApoE$^{-/-}$ mice showed decreased TLR4 expression in spleen tissue than B6 mice by immunohistochemistry and Western blot analysis ($P < 0.05$ and $P < 0.01$, respectively, Fig. 2A and D), with lower levels of TLR4 expression for females than males ($P < 0.05$ and $P < 0.01$, respectively, Fig. 2B, C, E and F).

3.5. TLR4 signal molecules and Bax expression in spleen tissue of B6 and ApoE$^{-/-}$ mice

The relative levels of MyD88, IRF3 and pp38 but not total p38 were lower in ApoE$^{-/-}$ than B6 mice (all $P < 0.01$, Fig. 3A, C, E and G). The relative level of Bax (apoptosis-associated molecule) was also lower in ApoE$^{-/-}$ than B6 mice ($P < 0.01$, Fig. 3J). Furthermore, the relative levels of MyD88, IRF3 and Bax were significantly lower for female than male ApoE$^{-/-}$ mice (all $P < 0.01$, Fig. 3B, D, I, K, and L).

3.6. Serum titers of autoantibodies, spleen weight, and the expressions of TLR4 pathway molecules and Bax in NS- and LPS-challenged male ApoE$^{-/-}$ mice

Serum titers of ANA and anti-dsDNA antibody and spleen weight were significantly increased in LPS-challenged ApoE$^{-/-}$ mice compared with NS-challenged mice ($P < 0.05$, Fig. 4A–D). The relative levels of TLR4, MyD88, IRF3 and pp38 were lower in LPS- than NS-challenged male ApoE$^{-/-}$ mice (all $P < 0.05$, Fig. 4E–H). The relative level of Bax was also lower in LPS- than NS-challenged male ApoE$^{-/-}$ mice ($P < 0.05$, Fig. 4I).

4. Discussion

Significantly increased titers of autoantibody, especially anti-dsDNA antibody, is a characteristic of systemic lupus erythematosis (SLE) [23], a prototypical autoimmune disease. We found ApoE$^{-/-}$ mice with higher serum level of IgG and titers of ANA and anti-dsDNA antibody than B6 mice, which suggested abnormal autoimmunity in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice showed splenomegaly and significantly less TUNEL staining of splenocytes, showing apoptosis, than B6 mice, which suggests decreased splenocyte apoptosis in ApoE$^{-/-}$ mice. In addition, the expression of TLR4 pathway molecules and Bax was lower in spleen tissue of ApoE$^{-/-}$ than B6 mice. The down-regulation of TLR4 pathway molecules and Bax induced by LPS led to elevated titers of ANA and anti-dsDNA antibody. Therefore, TLR4 signal pathway may participate in maintaining the balance of splenocyte apoptosis and antibody production in ApoE$^{-/-}$ mice.

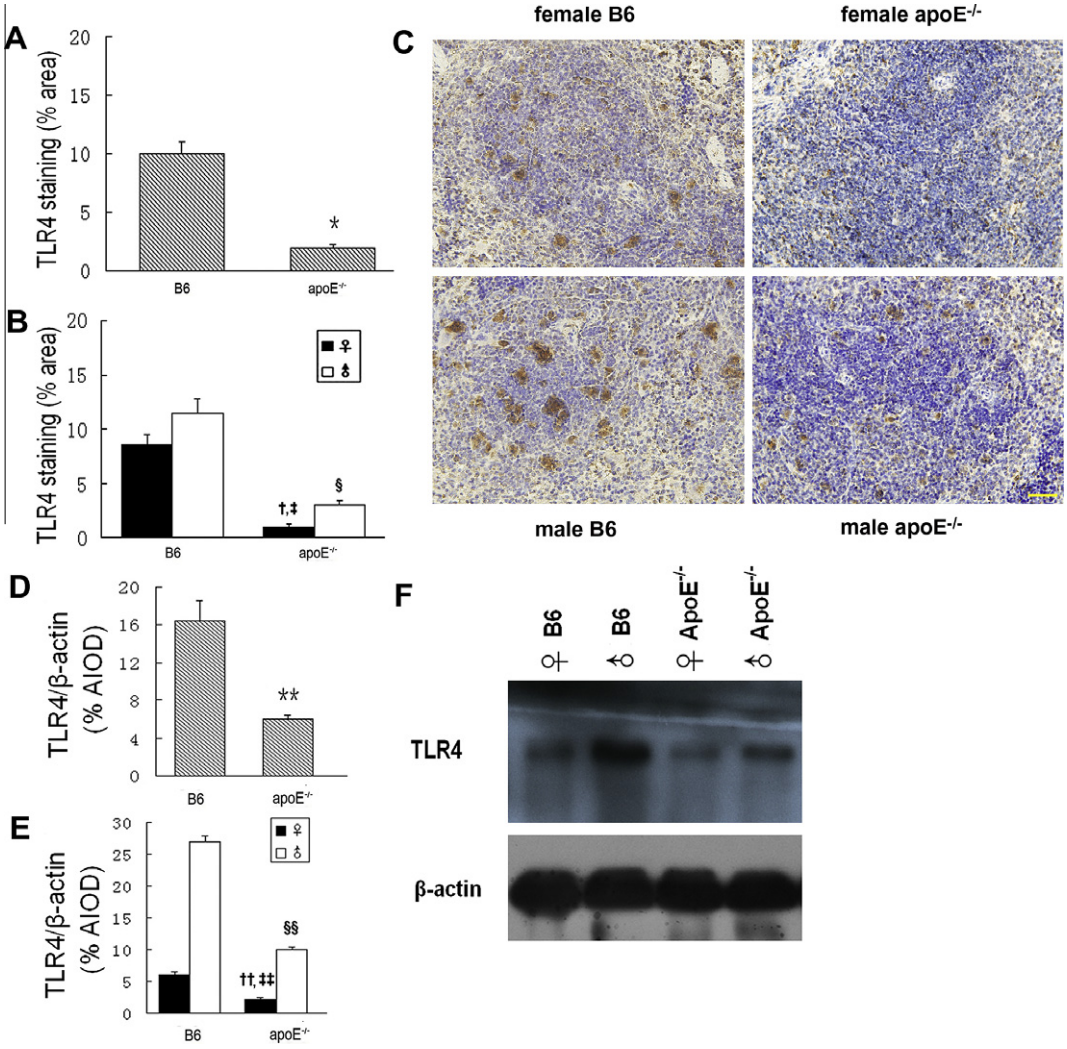

Fig. 2. Toll-like receptor 4 (TLR4) expression in spleen tissue lower in ApoE$^{-/-}$ than B6 mice. Quantitative analysis of TLR4 expression by immunohistochemistry (A and B) and Western blot (D and E) in spleen tissues of B6 and ApoE$^{-/-}$ mice, sex differences (B and E), and representative photographs (C and F). Data are mean ± SEM. $^{*}P < 0.05$, compared with B6 mice; $^{**}P < 0.01$, compared with B6 mice; $^{\dagger}P < 0.05$, compared with male ApoE$^{-/-}$ mice; $^{\dagger\dagger}P < 0.01$, compared with male ApoE$^{-/-}$ mice; $^{\ddagger}P < 0.05$, compared with female B6 mice; $^{\ddagger\ddagger}P < 0.01$, compared with female B6 mice; $^{\S}P < 0.05$, compared with male B6 mice; $^{\S\S}P < 0.01$, compared with male B6 mice. Bar = 200 μm.

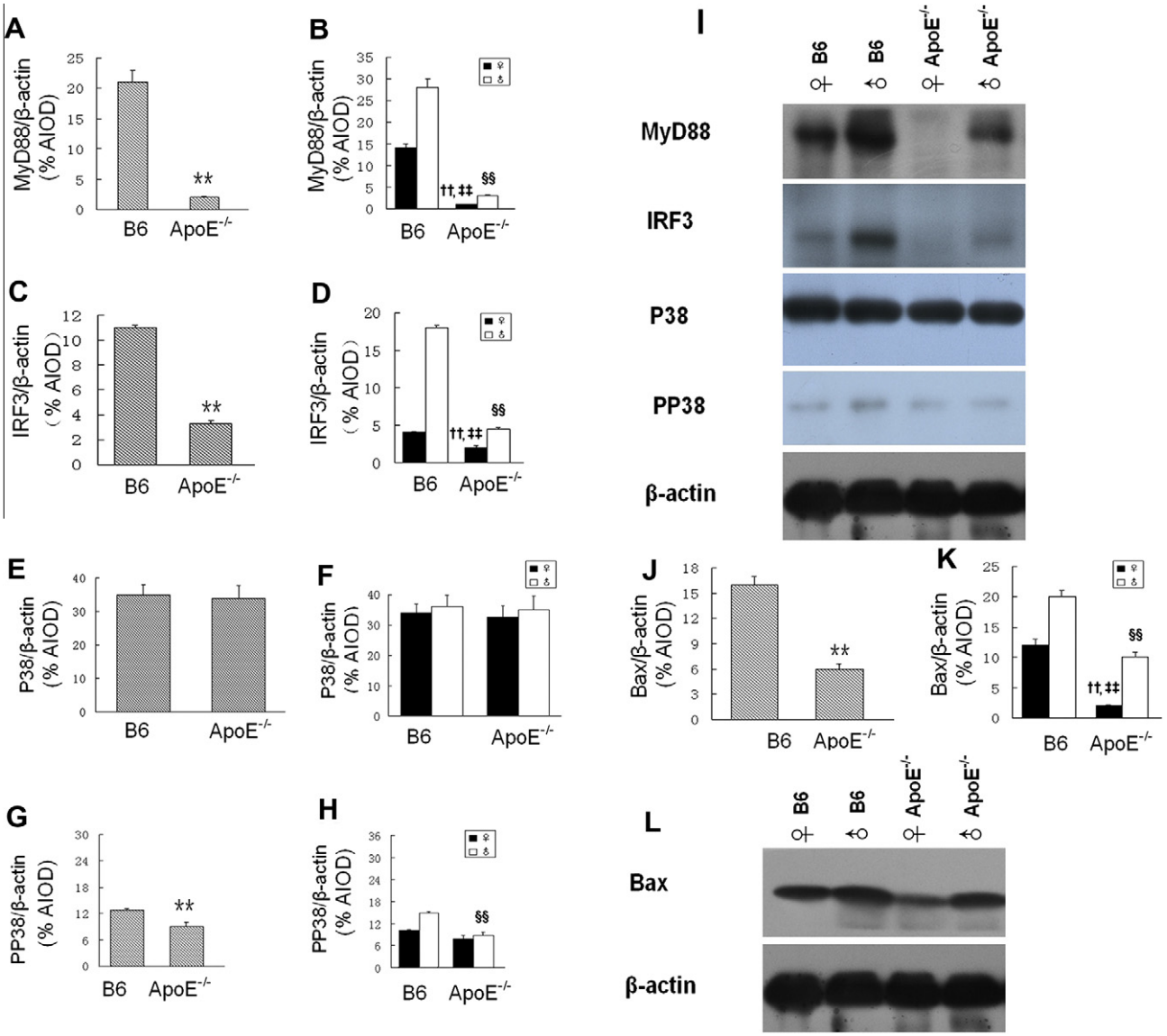

Fig. 3. TLR4 pathway molecules and Bax in spleen tissue lower in ApoE$^{-/-}$ than B6 mice. Quantitative analysis of MyD88 (A and B), IRF3 (C and D), p38 (E and F), phosphorylated p38 (pp38) (G and H) and Bax (J and K) expression in spleen tissue of B6 and ApoE$^{-/-}$ mice, sex differences (B, D, F, H and K), and representative photographs (I and L). Data are mean ± SEM. $^{**}P < 0.01$, compared with B6 mice; $^{\dagger\dagger}P < 0.01$, compared with male ApoE$^{-/-}$ mice; $^{\ddagger\ddagger}P < 0.01$, compared with female B6 mice; $^{\S}P < 0.05$, compared with male B6 mice; $^{\S\S}P < 0.01$, compared with male B6 mice.

Although ApoE$^{-/-}$ mice are known to have an abnormal immune response, significantly increased titers of ANA and anti-dsDNA antibody have never been reported in ApoE$^{-/-}$ mice. Only one study reported that the level of anti-dsDNA antibody did not change significantly with increased levels, although not significant, of total IgG and anti-chromatin antibody titer in ApoE$^{-/-}$ mice [13]. The differences in results may be due to different sample size and different sensitivity of antibodies used.

The spleen, the largest immune organ in the periphery, is the destination for lymphocytes, especially B cells (accounting for 60% of the total number of splenic lymphocytes). It is the main location for the immune response to antigens in blood and the main organ that produces antibodies. Apoptosis of lymphatic cells directly affects the stability of the immune system. Increased apoptosis causes immunodeficiency, and decreased apoptosis causes the proliferation of immune organs and the formation of autoimmunity [24]. Bax, a protein of the Bcl-2 gene family, promotes apoptosis by competing with Bcl-2. Bax is a cytosolic monomer that may insert into the outer mitochondrial membrane and form oligomers [25]. The oligomers lead to the release of cytochrome *c* into cytoplasm and the initiation of apoptosis. We found low expression of Bax and decreased splenocyte apoptosis in spleen tissue of ApoE$^{-/-}$ mice, which suggests that the low expression of Bax probably causes decreased splenocyte apoptosis in ApoE$^{-/-}$ mice.

LPS, the ligand of TLR4, induces apoptosis of endothelial cells [26] and hepatic cells [27] but inhibits that of monocytes [28], neutrophils [29], macrophages [30] and cardiocytes [31]. We found decreased splenocyte apoptosis and TLR4 expression in ApoE$^{-/-}$ mice, which suggests a possible relation of TLR4 regulating splenocyte apoptosis of ApoE$^{-/-}$ mice. IRF3 and p38 are activated via TLR4-TRIF and TLR4-MyD88 pathways, respectively [32–36]. IRF3 and p38 are both associated with apoptosis [37–42], and all may induce apoptosis via Bax [38,42]. MyD88 is an important adaptor molecule in the TLR4 signal pathway. MyD88$^{-/-}$ may inhibit T- and B-lymphocyte apoptosis in mice [43]. We found a significantly lower expression of TLR4, MyD88, IRF3 and pp38 but not p38 in spleen tissue of ApoE$^{-/-}$ than B6 mice, so the TLR4 signal pathway may regulate Bax expression through IRF3 and pp38 and play a role

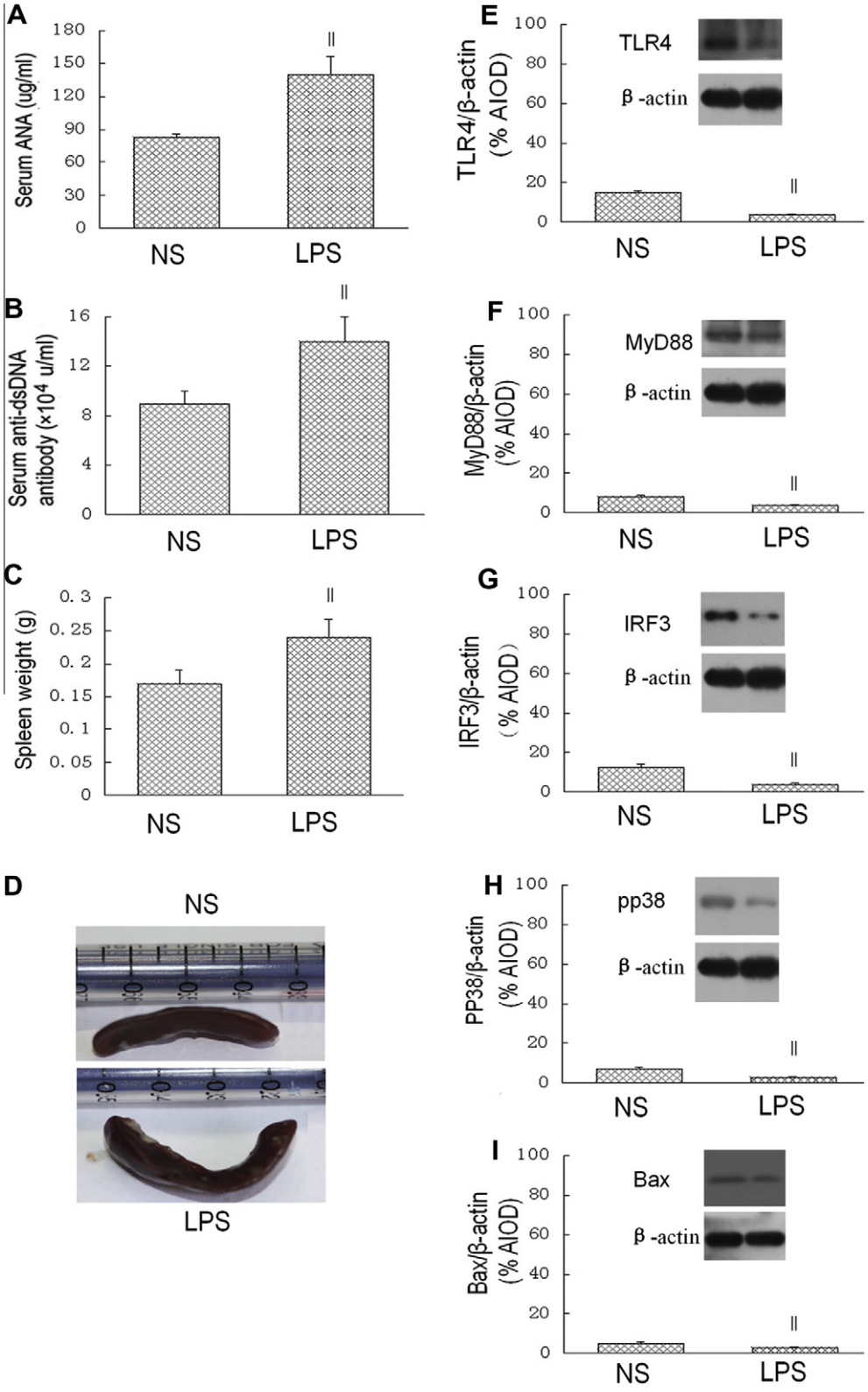

Fig. 4. Serum ANA and anti-dsDNA antibody titers higher, spleen greater, the expressions of TLR4 pathway molecules and Bax lower in lipopolysaccharide (LPS)-than physiological saline (NS)-challenged male ApoE$^{-/-}$ mice after 4 weeks of intraperitoneal injection of LPS or NS. Quantitative analyses of serum titers of ANA (A) and anti-dsDNA antibody (B) by ELISA in NS- and LPS-challenged male ApoE$^{-/-}$ mice. Wet weights of spleens (C) and gross appearance of representative spleen tissues (D) of NS- and LPS-challenged male ApoE$^{-/-}$ mice. Quantitative analysis of TLR4 (E), MyD88 (F), IRF3 (G), pp38 (H) and Bax (I) expression in spleen tissue of NS- and LPS-challenged male ApoE$^{-/-}$ mice. Data are mean ± SEM. $^{\|}P < 0.05$, compared with NS-challenged male ApoE$^{-/-}$ mice.

in splenocyte apoptosis. Furthermore, the down-regulation of TLR4 signal molecules induced by LPS led to decreased expression of Bax and increased serum titers of ANA and anti-dsDNA antibody in male ApoE$^{-/-}$ mice. Therefore, the decreased expression of TLR4

pathway molecules in spleen tissue may have elevated serum titers of autoantibodies in ApoE$^{-/-}$ mice by down-regulating splenocyte apoptosis.

The serum titers of autoantibodies were greater in female than male ApoE$^{-/-}$. SLE is more prevalent among young women, with a female to male ratio of 9:1 [44]. Sex steroid hormones in females were considered to be responsible for this difference in autoimmunity [45]. We found that splenocyte apoptosis and levels of TLR4, IRF3, pp38 and Bax in spleen tissue were lower for female than male ApoE$^{-/-}$ mice. Accordingly, sex steroid hormones in female ApoE$^{-/-}$ mice may boost autoantibody generation by regulating TLR4 signal molecules and the apoptosis of spleen tissue.

We show less splenocyte apoptosis, greater spleen and higher titers of ANA and anti-dsDNA antibody in ApoE$^{-/-}$ than B6 mice. The expression of TLR4 signal molecules in spleen tissue was lower in ApoE$^{-/-}$ than B6 mice and lower for female that male ApoE$^{-/-}$ mice. The down-regulation of TLR4 signal molecules induced by LPS led to decreased expression of Bax and increased serum titers of ANA and anti-dsDNA antibody in male ApoE$^{-/-}$ mice. The TLR4 signal pathway may participate in maintaining the balance of splenocyte apoptosis and antibody production in ApoE$^{-/-}$ mice. Further experiments are needed to explore the connections among the TLR4 signal pathway, splenocyte apoptosis and autoantibodies in ApoE$^{-/-}$ mice.

Conflict of interest

The authors declare that they have no conflicts of interests.

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