Development of pristane induced mice model for lupus with atherosclerosis and analysis of TLR expression

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Abstract

Objective

This study was designed to establish a murine model of lupus with atherosclerosis, and to investigate the expression of Toll-like receptors (TLRs) in the aorta and kidney.

Methods

The 9-week-old female ApoE$^{-/-}$ and C57BL/6 mice were randomly divided into a ApoE$^{-/-}$ pristane treated group (group A), ApoE$^{-/-}$ control group (group B), C57BL/6 pristane treated group (group C) and C57BL/6 control group (group D). Each mouse was given either a single intraperitoneal injection of 0.5 ml pristane or saline.

Results

We observed that group A mice specifically had poor spirit, less activity, obvious hair loss, splenomegalia and renomegaly. Levels of ANA, anti-ds-DNA and anti-Sm antibodies were significantly higher than those in other groups. The group A and B mice generally displayed intimal hyperplasia and atherosclerosis mottling in the lumen of the aorta. The kidney tissues from group A, B and C mice showed increased expression levels of TLR2, TLR4, TLR7 and TLR9 proteins in comparison to group D. However, Group A mice did not show any significant difference in TLR2 and TLR4 protein expression levels when compared to group B and C, but displayed higher TLR7 expression than group B and higher TLR9 expression than group B and C mice. In contrast, the group A and B mice apparently expressed TLR2 and TLR4.

Conclusion

We concluded that pristane treated apoE$^{-/-}$ mice exhibited lupus-like phenotype and developed atherosclerosis. The pristane treatment also induced abnormally high expression of TLR2 and TLR4 in the aorta and TLR2, TLR4, TLR7 and TLR9 in the kidney of apoE$^{-/-}$ mice.

Key words

Toll-like receptors, lupus with atherosclerosis, mice model, pristane

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Introduction

The management of SLE had seen major progress in the last decade with significant improvement in patient's prognosis. At present, a 10-year survival rate has increased to 80% in comparison to 20% observed 50 years ago. But, now these long-term survivors face more incidences of premature atherosclerosis (AS) and cardiovascular complications than ever. The imaging studies have established that SLE patients have increased carotid intima-media thickness (IMT) and higher prevalence of carotid plaque and coronary calcification (1, 2). In addition, these patients are at a greater risk for coronary artery disease (CAD), with the incidence rate of at least 2 times higher than the general population (3). The cardiovascular disease morbidity associated with AS in SLE patients has significantly increased, and has thus become the prominent factor affecting the quality of life and long-term mortality. However, the pathogenesis of this accelerated atherosclerosis in lupus remained largely unclear.

It is well known that TLRs signalling pathway plays an important role in the pathogenesis of AS and SLE. Numerous studies have suggested that patients with AS plaques, mainly express TLR2 and TLR4 proteins, more specifically in macrophages and endothelial cells. In addition, animal experiments had also indicated that pharmacologic or genetic blockade of TLR2 or TLR4 activity, significantly reduced the plaque size and vessel stenosis, decreased lipid composition and macrophage infiltration in apolipoprotein E–deficient ($apoE^{-/-}$) mice (4-6). In recent years, the role of TLR7 and TLR9 proteins in the pathogenesis of SLE, has also been highlighted (7). The TLR7 and TLR9 are intracellular receptor proteins, mainly expressed in the cytoplasm of plasmacytoid dendritic cells (pDC). The DNA or RNA containing complexes, respectively, activate pDC, through TLR9 or TLR7 receptors to produce IFN-α. This eventually results in initiation and maintenance of SLE development. Interestingly, stimulation of plaque tissue with CpG ODN, a TLR9 ligand, also increased IFN-α production (8). Indeed, IFN-α functions as an inflammatory amplifier and promote activation of macrophages at atheroma through endogenous TLR2 and TLR4 ligands (8, 9). These observations thus, indicated that TLRs may play a key role in lupus with premature atherosclerosis. Nevertheless, there are very few studies that have addressed the role of TLRs in accelerated atherosclerosis in SLE disease.

In order to explore the pathogenesis of early-onset atherosclerosis in lupus, the presence of an appropriate experimental animal model is important. There have been many efforts to establish a mature model. Recently, a double knockout mice model of SLE with AS was built by breeding and mating the same strains of spontaneous lupus mice ($Fas^{-/-}$ B6, B6/lpr, gld mice) with AS tendency mice ($apoE^{-/-}$ B6 or $LDLr^{-/-}$ B6 mice) (10-13). This model was characterised by high levels of autoantibodies, urine protein, and other lupus-like phenotype. It also displayed significantly increased degree of atherosclerosis. Additionally, cGVH induction in B6.ApoE(-/-) mice or transferring B6.Sle1.2.3-derived bone marrow cells into lethally irradiated $LDLr^{-/-}$ mice, are other adopted/developed murine models (10, 14, 15). However, these models required more complex experimental conditions and long induction times, involved higher cost and also have enhanced mortality rate. Therefore having a more appropriate mice model was critical and absolutely required.

Pristane, one of the hydrocarbons, has been confirmed to be capable of inducing lupus in a variety of strains of non-autoimmune prone mice, even with a single intraperitoneal injection (16). This kind of model exhibited significant SLE phenotype that includes a broad range of autoantibodies production, immune complex deposition in kidneys, nephritis and additional characteristics. Therefore, the primary objective of this study was to establish a murine model of lupus with athersclerosis in apolipoprotein-E deficient mice induced by pristane, and to determine the role of TLRs in the immune response and induction of chronic inflammation in murine lupus with atherosclerosis. So, our

hypothesis was that intraperitoneal injection of pristane to apoE$^{-/-}$ mice, will provide an alternative and appropriate murine research model for SLE accompanied by AS.

Material and methods

Reagents

The different reagents and antibodies used in this study were procured from different sources as follows; Pristane (Tokyo Kasei Co., Tokyo, Japan); dipstick test kit for urine protein measurement (Guangzhou Huadu Gore-Bao Biotechnology Co., Ltd, Guangzhou, China); mouse ANA, anti-ds-DNA antibody, anti-Sm antibody ELISA kit (R&D Systems, Minneapolis, MN, USA); anti-murine TLR2 mAb (Santa Cruz, CA, USA); anti-murine TLR4 mAb (Abcam, Cambridge, MA); rabbit polyclonal Abs for TLR7 and TLR9 (Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China); goat anti-mouse IgG conjugated with DyLight™ 488 (Jackson ImmunoResearch Labs, Pennsylvania, USA); Non-biotin two-step immunohistochemical assay kit, Rabbit hypersensitivity two-step immunohistochemical assay kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing,China).

Mice and their treatment

Female apoE$^{-/-}$ mice and C57BL/6 mice were obtained from Peking University (Beijing). All mice were 9 weeks old and were randomly divided into four groups with seven mice each. Group A, apoE$^{-/-}$ mice, had a single intraperitoneal injection of 0.5 ml pristane. Group B, apoE$^{-/-}$ mice, received a single intraperitoneal injection of 0.5 ml saline. Group C, C57BL/6 mice, were injected with the pristine as in group A, and Group D, C57BL/6 mice were injected with the saline as in group B. The mental state, diet, weight and hair of the mice were regularly observed until before their sacrifice at 8 months. The study was approved by the Animal Care and Use Committee at Fujian Medical University.

Urine protein measurement

The protein in the urine was measured by a semi-quantitative, dipstick test method, once before the injection and thereby after every month. Based on the color level of the reaction zone in the dipstick, the proteinuria was divided into six, (-), (±), (+), (+ +), (+ + +), (+ + + +) levels.

Serum estimation

Mice were first anesthetised with 1% sodium pentobarbital (50μg/g) by intraperitoneal injection. Later, the eyeball blood samples were collected and separated into serum. Circulating ANA, anti-ds-DNA and anti-Sm antibodies were determined (1:1 serum dilution) by ELISA kit (R&D Systems, Minneapolis, MN, USA).

Kidney and aortic histology

After the mice were sacrificed at 8 months, kidneys and aortic root were removed and fixed in paraformaldehyde. This was followed by ethanol dehydration, paraffin embedding, slicing and staining with haematoxylin and eosin stain. Kidney and aortic lesions were observed by optical microscope (Olympus BX51, Japan). The deposition of IgG and C3 (complement component 3 protein) levels in kidneys were examined by direct immunofluorescence staining.

Analysis of TLRs expression

The protein expression levels of TLR2, TLR4, TLR7 and TLR9 in kidney and TLR2 and TLR4 in aorta were detected by immunohistochemical staining. Paraformaldehyde fixed and paraffin-embedded sections from renal or aortic specimens were subjected to specific primary Abs (1:50~200 dilution) incubation, followed by subsequent secondary antibody incubations. Finally the specimens were observed with light microscope and brown signal was regarded as positive staining. The images collected by HPIAS-1000 high-resolution color pathological image analysis system were analysed by Image-Pro plus 6.0 software. Six high-expression fields within the renal cortex were selected randomly from each slice. Each field contained at least one glomerulus and surrounding tubules. The average integrated optical density (IOD) per unit area of positive staining was calculated.

Statistical analysis

The data were presented as mean±SD (standard deviation). Levene's Test, independent samples *t*-test, Welch's *t*-test, Spearman correlation and Pearson Correlation were performed when indicated. The *p*-value of less than 0.05 was considered statistically significant.

Results

Pristane treated apoE$^{-/-}$ mice exhibited lupus-like phenotype

The general observations about the condition of mice from different groups during the experiment revealed some significant differences. The apoE$^{-/-}$ pristane treated mice from group A displayed poor spirit, reduced activity, and one mice even died at about six months. In addition, two weeks after injection of pristane, some of the mice began to lose hair from the head and back as shown in Figure 1A. Subsequently, the rate of hair loss increased, and more number of mice lost their hair. In contrast, none of the mice from group B, C and D showed any hair loss until being sacrificed. The one mouse from the C57BL/6 pristane group (group C) also died at about seven months but other than that all mice from group B, C & D survived and were healthy.

Finally, all mice were sacrificed after 8 months. The volume and weight of their spleens and kidneys were measured. The pristane treated groups (group A and C) displayed splenomegalia (Fig. 1B) and renomegaly. ELISA test was used to detect circulating autoantibodies. The serum levels of ANA, anti-ds-DNA and anti-Sm antibodies in apoE$^{-/-}$ pristane treated mice (group A) were significantly higher than those in other groups (group B, C & D) (Fig. 1 C-D). In addition, the pristane treated mice (group A & C) displayed onset of proteinuria at 3 months of age. Mice underwent a gradual, progressive increase in proteinuria with time. Eventually, at 8 months of age, almost all pristane treated mice developed proteinuria (+~+++), while control groups (group B & D) had a negative to trace amount of protein in their urine (data not shown). Interestingly, the levels of ANA, anti-ds-DNA and anti-Sm antibodies, all correlated positively with the extent of proteinu-

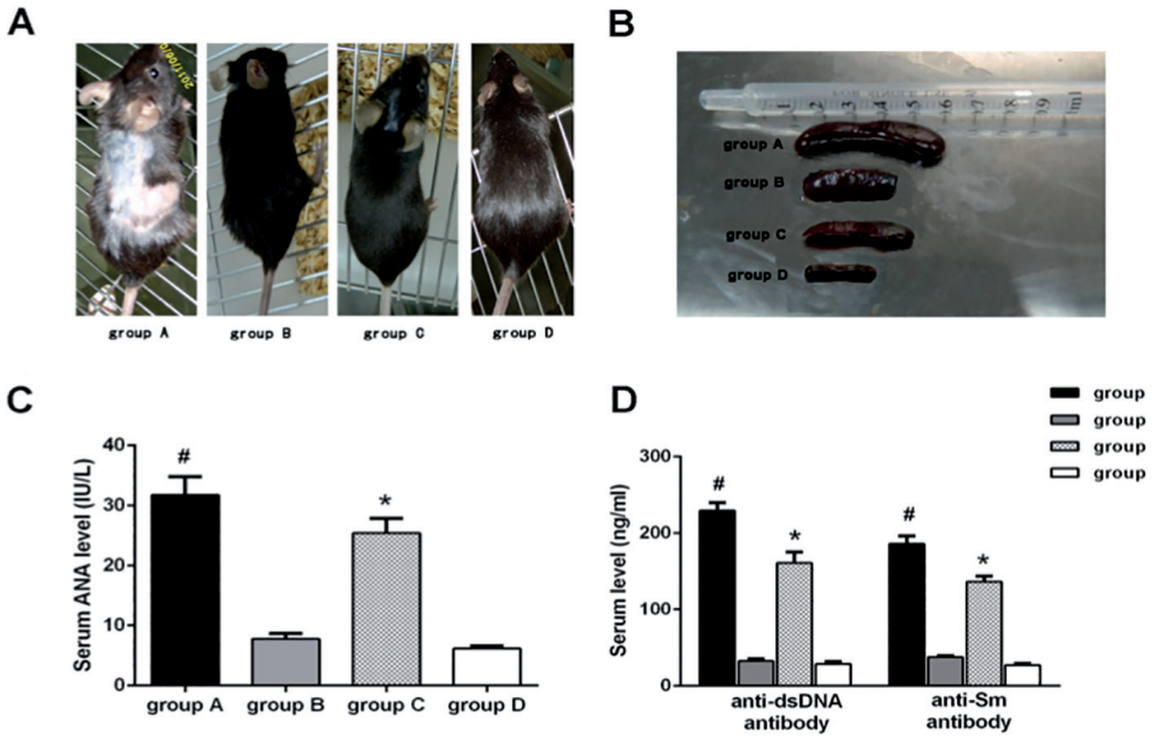

Fig. 1. Pristane treated apoE$^{-/-}$ mice exhibited lupus-like phenotype. Panel, A represents the general condition of mice, especially hair loss in different groups of mice either injected with pristane or saline. Panel, B represents the gross appearance of spleen tissues of mice from different treatment groups. Panel, C depicts the serum titers of ANA from four different groups, whereas Panel, D depicts the serum titers of anti-ds-DNA and anti-Sm antibodies, as analysed by ELISA method. Group A is ApoE$^{-/-}$ mice treated with pristane; Group B is ApoE$^{-/-}$ mice treated with saline; Group C is C57BL/6 mice treated with pristane; and Group D is C57BL/6 mice treated with saline. $^{\#}p<0.05$ *vs*. other groups, $^{*}p<0.05$ *vs*. group D.

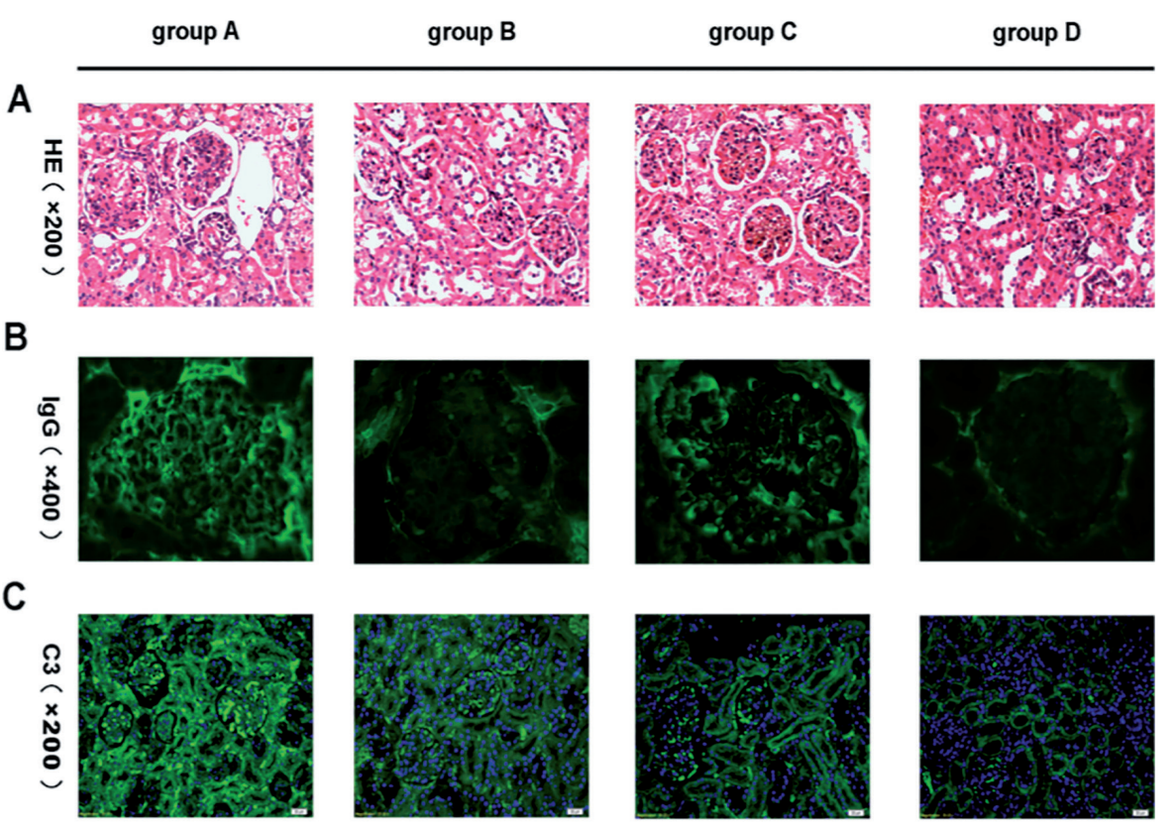

Fig. 2. Pristane-induced glomerulonephritis in apoE$^{-/-}$ mice. Panel, A represents HE staining (200x) of renal tissues from apoE$^{-/-}$ and C57BL/6 mice. Panel, B represents IgG glomerular deposition (400x) in mice from different groups; while Panel C show the C3 (complement 3) glomerular deposition (200x), as analysed by direct immunofluorescence staining.

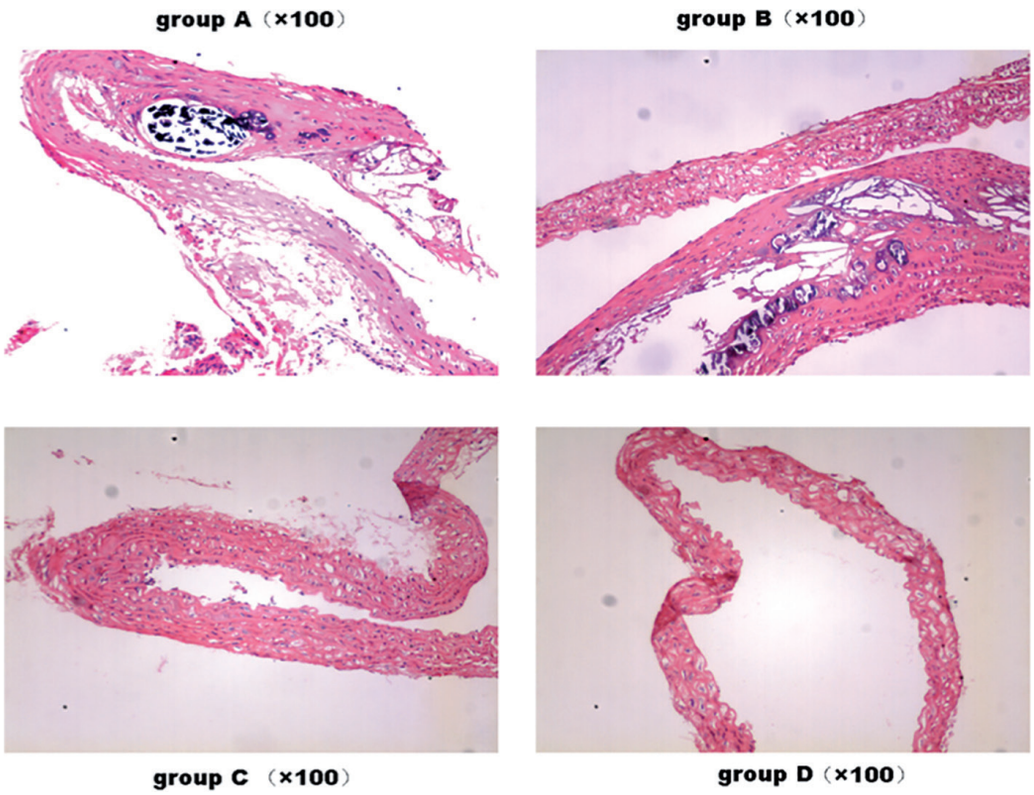

Fig. 3. Pristane treated apoE$^{-/-}$ mice developed atherosclerosis. These representative photomicrographs (100x) represent HE staining of mice aorta from different groups.

ria in group A and C (r=0.575, p=0.002; r=0.767, p=0.000; r=0.663, p=0.000), respectively (data not shown).

Further histological analysis of the group A and C specimens displayed glomerular hypertrophy and hypercellularity, epithelial cell swelling and narrowing of proximal tubule, granular degeneration, edema degeneration of renal tubular epithelial cells, scattered infiltration of lymphocytes into the interstitial tissue and fibrosis, as shown in Figure 2A. Meanwhile, group B and D represented normal nephron histology (Fig. 2A). In addition, pristane treated mice (group A and C) also showed deposition of IgG (Fig. 2B) and C3 (Fig. 2C) in the glomerular capillary loop as analysed by direct immunofluorescence staining. Instead, very weak fluorescence signal was observed in group B and D thus indicating the lack of IgG and C3 deposition.

Pristane treated apoE$^{-/-}$ mice developed atherosclerosis

Next, we analysed the development of atherosclerosis and observed that C57BL/6 mice (group C & D) had smooth and continuous aortic intima. Also, the aorta wall had a uniform thickness, and medial smooth muscle cells lined up in order. In contrast, HE staining of aorta from apoE$^{-/-}$ mice (group A & B) revealed general intimal hyperplasia and atherosclerosis mottling in its lumen. The projection of fibrous cap consisting of cells was thin, and there was a covered amorphous substance and a large spindle shaped cholesterol crystals. In addition, there were fractured muscle fibres, pool of lipids from necrotic cells, and presence of fatty streaks composed of several foam cells. Also, the branches of arteries had plaques (Fig. 3). As well as a great deal of adipose tissue, a few white point-like particles attached to organs roots and mesenteries were also observed in the abdominal cavity of apoE$^{-/-}$ model mice (group A).

Analysis of TLRs expression in kidney tissue

The TLRs expression analysis suggested that renal tissue of C57BL/6 group (group D) mice hardly had any TLR2 or TLR4 protein levels as detected by immunohistochemistry. Instead, group A, B and C all showed significantly increased TLR2 (Fig. 4A) and TLR4 (Fig. 4B) expression in renal tissue as compared to group D. These proteins were mainly located in the glomerular cell membranes (p<0.01). However, among group A, B and C, there were no detectable difference in the TLR2 and TLR4 expression.

Similarly, the mice from group D also displayed almost no or very little TLR7 and TLR9 expression in the glomerulus and renal interstitium but had a weak expression in renal tubular epithelial cells. On the contrary, glomerulus of group A, B and C mice had a significant higher TLR7 and TLR9 (Fig. 4C-D) expression in comparison to group D. Both of these proteins were mainly expressed in glomerular mesangial and capsule cells. Further analysis revealed that the glomerular TLR7 expression was higher in group A than group B (p<0.05), but was not different when compared to group C (p>0.05). Also, the glomerular TLR9 expression was higher in group A than group B and C (p<0.05). In addition, TLR7 and TLR9 expression were also seen in renal tubular epithelial cells, inflammatory cells in perivascular infiltration and some vascular endothelial cells of group A, B and C mice.

Analysis of TLRs expression in aortic tissue

The analysis of aortic tissues for TLR2

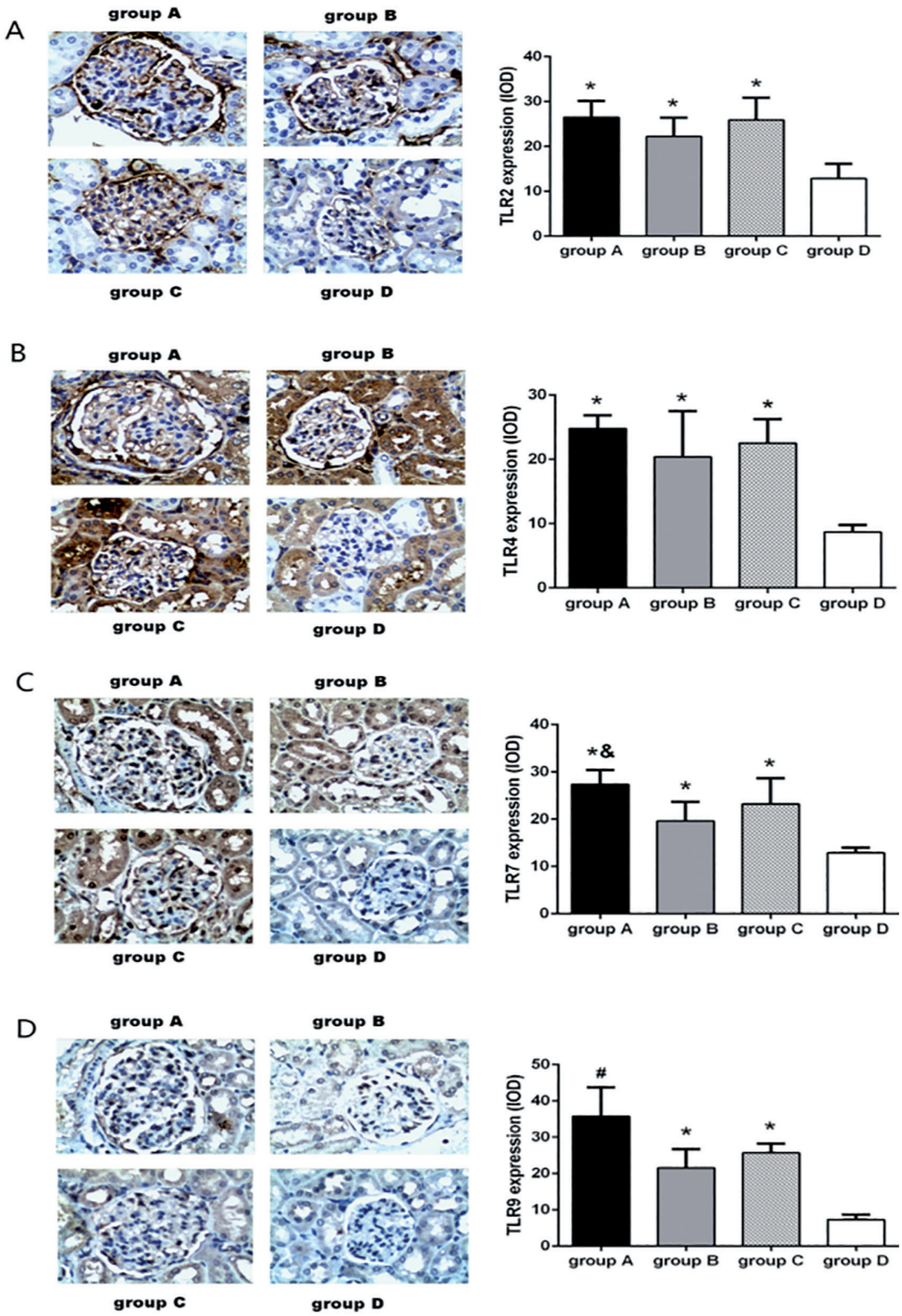

Fig. 4. Analysis of TLRs expression in kidney tissue of apoE$^{-/-}$ and C57BL/6 mice. The panel A, B, C and D represents (400x) photomicrographs (left side) and quantitative analysis (right side) of TLR2, TLR4, TLR7 and TLR9 expression respectively, by immunohistochemistry staining of kidney tissue from four different groups of mice. *p<0.01 *vs*. group D, $^{\&}p$<0.05 *vs*. group B and $^{\#}p$<0.05 *vs*. other groups.

and TLR4 expression suggested that ApoE$^{-/-}$ mice (group A and B) had higher TLR2 and TLR4 (Fig. 5A-B) expression in aortic plaques, especially in the plaque shoulder, lipid-rich plaque and around cholesterol crystals. In addition, the cytoplasm and membrane of macrophages, proliferating smooth muscle cells and endothelial cells in AS plaque, also stained positively (tan or brown) for TLR2 and TLR4 expression by immunohistochemisty. However, the aorta of C57BL/6 mice (group C and D), did not show any TLR2 and TLR4 expression.

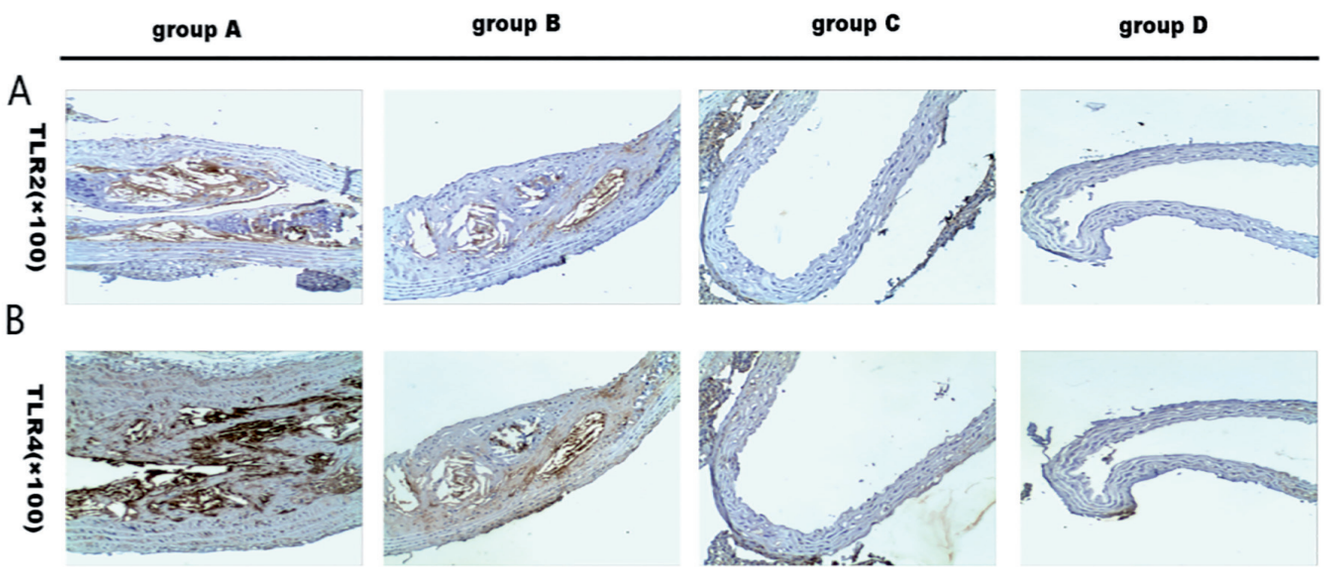

Fig. 5. Analysis of TLRs expression in aortic tissue of apoE$^{-/-}$ and C57BL/6 mice. Panel A and B represents the photomicrographs of TLR2 and TLR4 expression, respectively by immunohistochemistry staining of aorta tissue from four different groups of mice (100x).

Discussion

The availability of very little information about the mechanism of premature atherosclerosis (AS) in lupus (SLE) can be attributed to the lack of good murine model to study its pathogenesis. Therefore, building a simple murine model of SLE with AS will not only facilitate the study of its pathogenesis, but also provide a foundation for the development of new treatment options. In this study, we have developed a mouse model of SLE with AS, for the first-time, by intraperitoneal injection of pristane to female apoE$^{-/-}$ mice. This model exhibited both lupus-like phenotype such as baldness, listlessness, a variety of auto-antibodies production (ANA, anti-ds-DNA and anti-Sm antibodies), proteinuria development, renal immune complex deposition, glomerular sclerosis, and accelerated atherosclerosis. Moreover, this model not only required very simple manipulations and was relatively easy to induce but was also capable of developing typical nephritis and aortic atherosclerosis. Since, it has been suggested that TLRs may participate in the pathogenesis of SLE accompanied by AS, our results have also shown that this mouse model has abundant TLR2 and TLR4 expression in the aorta, and also high TLR2, TLR4, TLR7 and TLR9 expression in the kidney.

In addition, SLE patients have various forms of clinical manifestation. According to the SLICC 2009, modified ACR systemic lupus erythematosus classification standard, patient with biopsy-proven lupus nephritis, accompanied by ANA or anti-ds-DNA antibody can be classified as SLE (17). It has also been reported that in pristane-induced lupus mice model (18, 19), ANA level is gradually increased after pristane injection, and reached a higher peak mainly in the IgM form after one month, followed by its decline until after two months, then again rising back. The levels of anti-ds-DNA and anti-SM antibody also continued to rise two months after pristane administration. Interestingly, our study have also shown that in apoE$^{-/-}$ mice after pristane injection, the serum ANA, anti-dsDNA and anti-Sm antibody levels were significantly higher than controls at 8 months time and developed marked proteinuria. Moreover, they not only displayed renal pathology like, glomerular hypertrophy, glomerular hypercellularity and proliferation of tubular epithelial cell, but also the deposition of immune complex in the glomerular capillary loop. There were also significant atherosclerotic plaques in the aorta. In contrast, the saline treated control apoE$^{-/-}$ mice rarely developed proteinuria, and also had low serum ANA and anti-ds-DNA antibody titer. Their renal glomerular and tubular structure was clear with no abnormal pathology and also no sign of immune complex deposition. Thus, these observations firmly confirmed that the murine model of SLE with AS can be successfully established in apoE$^{-/-}$ mice by pristane injection.

Furthermore, there have been studies linking Toll-like receptor signalling pathway to the SLE pathogenesis. It has been reported that TLR7$^{-/-}$ and TLR9$^{-/-}$ mice, both had significantly fewer anti-snRNP antibodies, less IgG and complement deposition in glomerulus than controls after intraperitoneal injection with pristane. The glomerular inflammation damage was also significantly reduced, but anti-dsDNA antibody level was not affected. The TLR4$^{-/-}$ mice, also displayed significantly decreased levels of anti-RNP and anti-dsDNA antibody and reduced glomerular IgG and complement deposition, after intraperitoneal injection of pristane (20, 21). The study by Urbonaviciute *et al*. showed that the absence of TLR2, in pristane-induced murine lupus, attenuated the production of autoantibodies and the development of renal disease and was reflected by milder proteinuria, reduced glomerular deposition of IgG and complement, and decreased renal infiltration of autoantibody-secreting cells (22). Similarly, our study also hardly detected TLR2, TLR4, TLR7 and TLR9 expression by immunohisto-

chemistry, in kidney of normal control group (C57BL/6 mice). We also did not observe any significant differences in TLR2 and TLR4 expression in kidney from apoE$^{-/-}$ mice groups with or without pristane induction. However, TLR7 and TLR9 expression were largely increased with significant nephritis in apoE$^{-/-}$ mice induced by pristane when compared to apoE$^{-/-}$ mice with normal nephron histology. Thus it can be assumed that TLR7 and TLR9 may only participate in pristane induced lupus nephritis pathogenesis in apoE$^{-/-}$ mice, whereas TLR2 and TLR4 might not be essential. In addition, Lee PY *et al*. demonstrated that pristane (also known as 2,6,10,14-tetramethylpentadecane) could induce IFN-γ production through TLR7/MyD88 signalling (23), while Thibault *et al*. showed that the IFN-γ pathway play an important role in regulating TLR-specific B-cell responses in a murine model of SLE (24). Thus, it would be interesting to analyse the relationship between the IFN-γ pathway and TLRs pathway in this new model and explore if they affect the B cell immune response, production of autoantibodies and the occurrence of atherosclerosis in murine model of SLE with AS. Some studies have demonstrated that in apoE$^{-/-}$Fas$^{-/-}$ C57BL/6 or gld. ApoE$^{-/-}$ mice, levels of ANA and anti ds-DNA antibody were significantly higher than those in apoE$^{+/+}$Fas$^{-/-}$ mice or gld mice. In addition, high-fat diet further increased auto-antibody levels in gld. ApoE$^{-/-}$ mice (25, 26). Our laboratory had previously demonstrated that apoE$^{-/-}$ mice showed increased levels of serum IgG and ANA and anti-dsDNA antibody titers in comparison to C57BL/6 mice and pristane injection, further increased this difference, significantly (27). Thus, it appeared that apoE$^{-/-}$ played a role in promoting prototypical lupus auto-antibodies production in mice, especially stimulated by pristane. In local renal tissue, an increase in TLR2, TLR4, TLR7 and TLR9 expression has been observed as compared to C57BL/6 mice, but no nephritis was observed in apoE$^{-/-}$ mice. Thus, we hypothesised that TLR2, TLR4, TLR7 and TLR9 signalling pathways were partial activated in apoE$^{-/-}$ mice, but the activation was not enough in itself to develop nephiritis without help from other environmental factors. Further, when genetic background of apoE knockout mice encountered the environmental factor, pristane, the TLR2, TLR4, TLR7 and TLR9 signalling pathway became fully activated and resulted in full-blown autoimmunity and kidney injury in experimental lupus. Thus, it was reasonable to suggest that apoE$^{-/-}$ may result in a systemic proinflammatory state in mice.

Some studies have also suggested that there is a close relationship between the expression of TLR2, TLR4 and atherosclerosis. Knocking out of TLR2 and TLR4 genes in apoE$^{-/-}$ mice, resulted in significant reduction in plaque, lipid and macrophage infiltration. The preliminary data in our study was also consistent with this published observation (28, 29). The apoE$^{-/-}$ mice treated with pristane did not show positive expression of TLR2 and TLR4 in aorta plaques, and mainly in macrophages and endothelial cells. But, the small sample size of each group and difficulty in embedding and processing of aortic specimens, posed a limitation to perform a statistical analysis between the above two groups and therefore needs further exploration.

Therefore, humanised murine models will definitely be helpful in better understanding of the mechanisms involved in SLE accompanied by AS. Inflammation and autoimmunity are consequence of gene–environment interactions. In this context, SLE and atherosclerosis both are representative diseases. The current study suggested that pristane induction in apoE$^{-/-}$ mice is a promising method for the establishment of murine model of SLE with AS. A more comprehensive and detailed analysis of atherosclerosis and TLRs in this mouse model will be required, to fully identify how TLR pathway impacted the accelerated atherosclerosis. This in turn will potentially result in a better understanding of the link between lupus and atherosclerosis. In addition, therapies targeting the TLR signalling pathway might also prove to be helpful for SLE with AS.

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