



Vulnerability of atherosclerotic plaques is associated with type I interferon in a murine model of lupus and atherosclerosis

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ABSTRACT. This study aimed to investigate the relationship between type I interferon (IFN-I) and plaque stability in pristane-treated apolipoprotein E-knockout (ApoE^{-/-}) mice. Antinuclear antibody (ANA) and extractable nuclear antigen antibody (ENA) levels were measured by immunofluorescence and enzyme-linked immunospot assay. Atherosclerotic plaques were detected by Sirius red/fast green staining. Cell apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling. Gene expression was determined by real-time PCR analyses. We found that pristane-treated ApoE^{-/-} mice developed a lupus-like syndrome characterized by an increased production of serum ANA and ENA. Pristane treatment decreased the collagen content and increased the number of apoptotic cells in plaques. Moreover, IFN-induced ISG15, IFIT1-1, and IFIT1-2 gene expression was increased in peripheral blood cells and aortic plaques. An IFN- α -stimulated macrophage supernatant inhibited collagen type I, alpha 1 gene expression in vascular smooth muscle cells. We concluded that the vulnerability of plaques was associated

with the activation of IFN-I in pristane-treated ApoE^{-/-} mice. Thus, we speculated that the higher prevalence of cardiovascular events in patients with systemic lupus erythematosus could be due to plaque instability.

Key words: Apolipoprotein E-knockout mice; Plaque vulnerability; Pristane; Systemic lupus erythematosus; Type I interferon

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by elevated levels of autoantibodies and systemic immune disorders leading to multiple-organ damage (Cervera et al., 2003). Recent advances in the diagnosis and treatment of SLE have significantly improved the prognosis, resulting in remarkably prolonged survival. Nevertheless, cardiovascular events as a result of atherosclerosis still importantly affect the long-term survival of SLE patients (Schoenfeld et al., 2013).

Atherosclerotic plaque development is now better understood. Not all cardiovascular events are due to large obstructive plaques. In general, acute coronary syndromes are caused by acute disruption of an unstable atheroma. It is believed that patients with inflammatory diseases, including SLE, are more likely to have vulnerable plaque ruptures (Anania et al., 2010). Type I interferon (IFN-I) has been shown to promote disease in SLE patients, and inhibition strategies are being considered for therapy (Kirou and Gkrouzman, 2013). In addition, previous studies have indicated that IFN-I probably plays a crucial role in endothelial cell damage and cardiovascular disease (CVD) development in SLE patients (Denny et al., 2007; Li et al., 2011; Thacker et al., 2010, 2012). The relationship between plaque vulnerability in lupus and IFN-I has not yet been reported. 2,6,10,14-Tetramethylpentadecane is a hydrocarbon oil also known as pristane. Over the past 15 years, studies have reported that pristane-induced inflammatory responses might provoke a lupus-like disease in mice (Satoh and Reeves, 1994; Satoh et al., 1995). The pathophysiology of the pristane-induced lupus-like disease in mice is believed to be intimately associated with IFN-I as well (Reeves et al., 2009).

In order to better understand the IFN-I pathway in lupus-associated atherosclerosis, the current study aimed to identify the role of IFN-I in the stability of plaques in pristane-treated apolipoprotein E-knockout (ApoE^{-/-}) mice.

MATERIAL AND METHODS

Animals

Female ApoE^{-/-} mice (C57BL/6J background) were obtained from the Peking University Health Science Center. Female C57BL/6 mice were obtained from the Shanghai Laboratory Animal Center. Eight-week-old mice were randomly divided into the following four groups: ApoE^{-/-} pristane group (N = 11), ApoE^{-/-} phosphate-buffered saline (PBS) group (N = 10), C57BL/6 pristane group (N = 10), and C57BL/6 PBS group (N = 5). The mice were injected intraperitoneally with 500 μ L pristane (Sigma) or PBS. Thirty weeks later, the mice were sacrificed and their spleens harvested and weighed. All mice were housed under specific-pathogen-free conditions and were fed a normal chow diet. All experimental procedures were

carried out in accordance with the regulations of the Department of Health, Shanghai, China. The study protocol was approved by the Committee on Use of Human & Animal Subjects in Teaching and Research, Shanghai Jiaotong University School of Medicine. All efforts were made to minimize suffering.

RNA extraction and real-time PCR

Total RNA was extracted by use of TRIzol reagent (Invitrogen) and was reverse transcribed using a PrimeScript RT reagent kit (Takara). The expression of IFN-induced genes (ISG15, IFIT1-1, and IFIT1-2), MMP-9, and tumor necrosis factor alpha (TNF- α) was determined by real-time PCR using SYBR Premix Ex Taq (Takara). The parameters of the amplification cycles included an initial hold at 95°C for 15 s followed by a 2-step reverse transcription-PCR (RT-PCR) program: 95°C for 5 s and 60°C for 30 s for 40 cycles. Postamplification melting curve analysis was performed, and the data were analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR primers were as follows: ISG15, forward (5'-AGAGCAAGCAGCCAGAAG-3') and reverse (5'-CACCGTCATGGAGTTAGTCAC-3'); IFIT1-1, forward (5'-ATGGGAGAGAATGCTGATGG-3') and reverse (5'-AGGAACTGGACCTGCTCTGA-3'); IFIT1-2, forward (5'-GTCAGAAGACAAGGCAATCAC-3') and reverse (5'-GCCCTTTCAGTTTGTAGACTAG-3'); collagen type I, alpha 1 (COL1A1), forward (5'-TTCACCTACAGCACGCTTGTG-3') and reverse (5'-GATGACTGTCTTGCCCCAAGTT-3'); MMP-9, forward (5'-AGACGGCATCCAGTATCTGT-3') and reverse (5'-ACATAGTGGAGGTGCTGTC-3'); TNF- α , forward (5'-AAACACAAGATGCTGGGACA-3') and reverse (5'-TTGATGGTGGTGCATGAGAG-3'); and β -actin, forward (5'-ATGCTCCCCGGCTGTAT-3') and reverse (5'-CATAGGAGTCTTCTGACCCATTC-3').

Autoantibody and urinary protein analyses

Serum samples were collected at week 24 or 30 after pristane or PBS treatment. The serum antinuclear antibody (ANA) level was measured by immunofluorescence using Hep-2-coated slides (Euroimmun). Serum extractable nuclear antigen antibodies (ENA; includes anti-Sm, anti-nRNP, and anti-ribosomal antibodies) and titers of anti-double-stranded DNA (anti-dsDNA) antibodies were measured by an enzyme-linked immunospot assay (Euroassay) and a gamma radioimmunoassay (The Northern Institute of Biotechnology), respectively. The urinary protein level at 24 h was determined by using a BCA protein assay kit (Thermo Scientific, USA).

Collagen staining of atherosclerotic plaques

Sirius red/fast green staining of atherosclerotic plaques was performed per the manufacturer protocol (Chondrex, Inc.). The basal portion of the heart and the proximal aorta were harvested and embedded in Tissue-Tec OCT medium. Frozen sections (7 μ m) of the aorta were obtained and washed with PBS and water. Thereafter, 0.2 to 0.3 mL dye solution was added to completely immerse the tissue section, which was then incubated at room temperature for 30 min. The dye solution was discarded, and the tissue section was rinsed with water until the fluid was colorless. Images were then captured using a digital camera connected to a microscope (Discovery V20; Zeiss) and analyzed by the Image-Pro Plus 6.0 software.

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining

Intimal-layer apoptotic cells of the atherosclerotic lesions were detected by TUNEL, using an *in situ* cell death detection kit (Roche). Tissues were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice. Frozen sections were prepared as previously described. After incubation with a terminal deoxynucleotidyltransferase solution at 37°C for 1 h, sections were treated with DAPI (4',6-diamidino-2-phenylindole) for 30 min. After washing of the sections with PBS, images were captured by confocal microscopy (TCS SP5 microscope; Leica). Apoptotic cells were counted using Image-Pro Plus and are reported as numbers of TUNEL⁺ cells/mm².

Culture and treatment of vascular smooth muscle cells (VSMC) *in vitro*

VSMC were prepared as previously described (Ishida et al., 1999). In brief, the thoracic aorta was obtained from 8-week-old Sprague-Dawley rats and digested with collagenase D for 1 h, and a single-cell suspension was prepared by passage through a 40- μ m cell strainer. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 g/mL streptomycin (Gibco) at 37°C in a 5% CO₂ humidified atmosphere. Medium changes were performed every 2 days until the cells reached 90% confluence. Cells at passages 6 to 10 were used for the experiments.

RAW264.7 cells were maintained in complete Dulbecco's modified Eagle's medium at 37°C in a 5% CO₂ humidified atmosphere until they reached 80 to 90% confluence. Cells were seeded onto a 6-well plate (1 x 10⁶ cells/well) and stimulated with 1 μ g/mL lipopolysaccharide (LPS) (Sigma) or 1000 U/mL IFN- α (PBL Interferon Source). The supernatant was collected after 12 h and added to the VSMC culture medium. After 24 h, the total cellular RNA was extracted, and COL1A1 gene expression was determined using RT-PCR.

Statistical analysis

Data are reported as means \pm standard errors of the means (SE) or medians and interquartile ranges (IQR). Statistical significance was determined by the Student *t*-test for continuous parametric variables and by the Mann-Whitney U-test for nonparametric skewed-distribution variables. P values of <0.05 were considered to be statistically significant.

RESULTS

Development of lupus-like disease in pristane-treated ApoE^{-/-} mice

To examine whether pristane treatment could also cause a lupus-like disease in ApoE^{-/-} mice, we detected the serum antibodies associated with systemic lupus erythematosus (SLE). Pristane-induced ANA production can be detected 4 to 6 months after administration (Reeves et al., 2009); therefore, we examined serum ANA levels at week 24. Both pristane-treated ApoE^{-/-} mice and C57BL/6 mice displayed remarkably varied ANA nuclear patterns. Interestingly, PBS-treated ApoE^{-/-} mice displayed a homogeneous ANA pattern in serum (Figure 1A). Among the four groups, the PBS-treated C57BL/6 mice were the only ones whose serum

ANA level was undetectable.

In order to clarify the antibody specific to SLE, we determined the serum ENA levels at week 24. The positivity rates for serum ENA in the pristane-treated ApoE^{-/-} mice and the C57BL/6 mice were 44.4 and 66.7%, respectively (Table 1). Serum ENA was undetectable in the PBS-treated ApoE^{-/-} mice and C57BL/6 mice.

Mice injected with pristane have a delayed production of anti-dsDNA. Anti-dsDNA titers were measured 30 weeks after pristane injection. The titers of anti-dsDNA in all 4 groups were below 11 IU/mL. ApoE^{-/-} mice have the C57BL/6 background as well, and no statistical significance of the anti-dsDNA titers was observed between the pristane-treated groups and the PBS-treated groups (Figure 1B), consistent with a previous report that C57BL/6 mice failed to develop anti-dsDNA antibodies (Reeves et al., 2009).

The urinary protein level (at 24 h) was quantified at week 28 after pristane injection. Pristane-induced nephritic changes depend on the strain of mice, and nephropathy is much milder in C57BL/6 mice than in other strains (Reeves et al., 2009). In our study, the pristane-treated ApoE^{-/-} mice produced little urinary protein, but the level was not significantly different from that for the ApoE^{-/-} PBS-treated mice (Figure 1C).

At week 30, the pristane-treated C57BL/6 mice had splenomegaly compared to the PBS-treated C57BL/6 mice. Furthermore, the splenic masses of the pristane-treated ApoE^{-/-} mice were substantially larger than those of the PBS-treated ApoE^{-/-} mice and pristane-treated C57BL/6 mice (Figure 1D). These data suggested that the SLE model was successfully set up in the ApoE^{-/-} mice, based on the comparable parameters for the SLE model between the ApoE^{-/-} mice and the C57BL/6 mice.

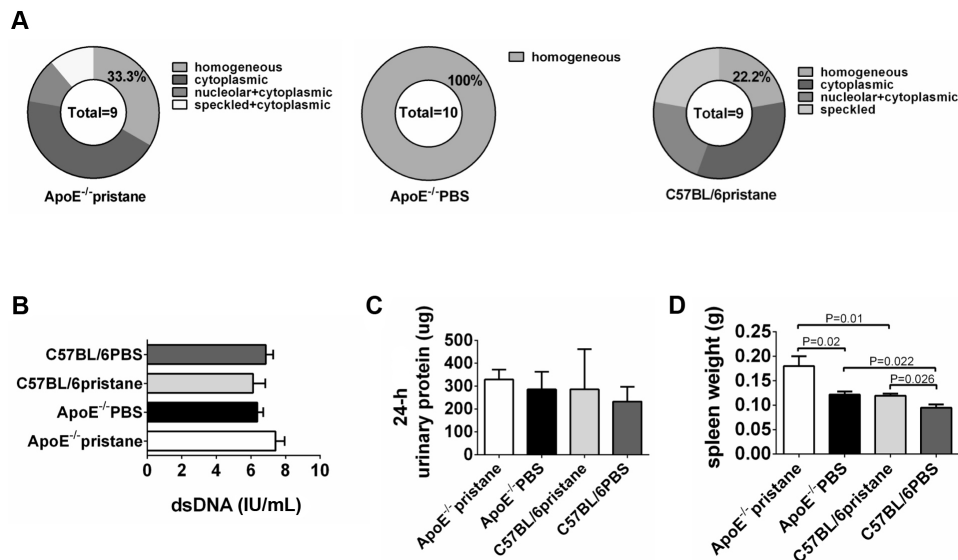


Figure 1. Autoantibody, 24-h urinary protein, and spleen weight changes. **A.** Antinuclear antibody staining patterns classified as homogenous, speckled, nucleolar, or cytoplasmic. The numbers in the circles indicate the total numbers of mice included in the analyses. **B.** Anti-dsDNA titers in the sera of mice 30 weeks after injection. **C.** Urinary protein levels (at 24 h) determined 28 weeks after pristane or PBS treatment. **D.** Spleen weight change in mice receiving PBS or pristane treatment. Data are reported as means \pm SE.

Table 1. Rate of ENA positivity in mice 24 weeks after injection.

Mouse group	No. of ENA(+) mice	No. of ENA(-) mice	Positivity rate (%)
ApoE ^{-/-} -pristane	4	5	44.4 ^{a,b}
ApoE ^{-/-} -PBS	0	10	0
C57BL/6pristane	6	3	66.7 ^c
C57BL/6PBS	0	5	0

P values were calculated by Fisher exact tests. ^aApoE^{-/-} pristane group vs ApoE^{-/-} PBS group; P = 0.033. ^bApoE^{-/-} pristane group vs C57BL/6pristane group; P = 0.637. ^cC57BL/6pristane group vs C57BL/6PBS group; P = 0.031.

Pristane treatment increased the vulnerability of plaques

The concept of a “vulnerable lesion” defines structural and molecular characteristics of an atherosclerotic plaque responsible for its enhanced prothrombotic potential. Stable plaques, even if they are large, may remain silent, while a mildly stenotic but vulnerable lesion may result in a clinically significant event (Aikawa and Libby, 2004). In our study, Sirius red/fast green collagen staining was used to compare collagen contents. The pristane-treated ApoE^{-/-} mice had a significantly decreased collagen content in the plaques of the aortic sinus compared to the PBS-treated ApoE^{-/-} mice (Figure 2A and B). More apoptotic cells were found in the plaque area of the pristane-treated ApoE^{-/-} mice than in that of the PBS-treated ApoE^{-/-} mice by TUNEL staining (Figure 2C and D), indicating that the vulnerability of plaques was increased in the aortic sinuses of ApoE^{-/-} mice after pristane treatment.

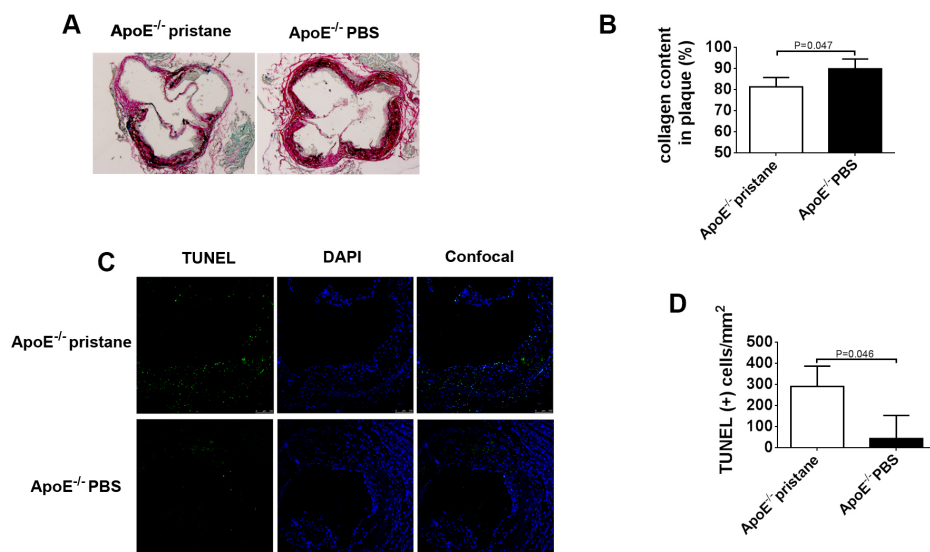


Figure 2. Decreased collagen content and increased numbers of apoptotic cells in plaques of pristane-treated ApoE^{-/-} mice. **A.** Representative collagen staining (magnification, 50X). **B.** Quantification of collagen contents in atherosclerotic lesions of the aortic sinus. **C.** Apoptotic cells in the aortic sinuses of ApoE^{-/-} mice stained by TUNEL (FITC), and nuclei stained by DAPI (blue). **D.** Quantification of TUNEL-positive cells in ApoE^{-/-} mice. Data are reported as medians and IQR.

Vulnerability of plaques associated with activation of IFN-I

Previous studies showed that an enzyme-linked immunosorbent assay was not sensitive for detection of serum IFN- α . A more common method is to measure the IFN-induced gene expression in peripheral blood cells, which mirrors the activation of the IFN-I pathway (Lee et al., 2008; Thacker et al., 2012). Therefore, we assessed the expression of ISG15, IFIT1-1, and IFIT1-2 induced by IFN in peripheral blood cells at week 4 after pristane injection. Compared to the levels in PBS-treated C57BL/6 mice, ISG15, IFIT1-1, and IFIT1-2 gene expression levels were increased in the peripheral blood cells from pristane-treated C57BL/6 mice (Figure 3A). Meanwhile, pristane treatment also led to a significant increase of expression of these three genes in ApoE^{-/-} mice (Figure 3B). These results suggest that pristane treatment evokes an activation of the IFN-I pathway in ApoE^{-/-} mice similar to that in C57BL/6 mice.

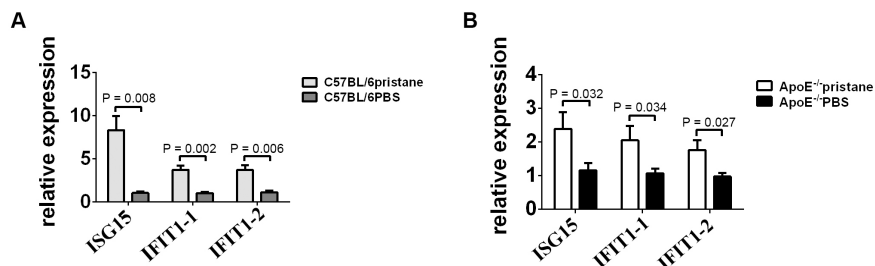


Figure 3. Real-time PCR analyses of IFN-induced genes in peripheral blood cells. The expression levels of ISG15, IFIT1-1, and IFIT1-2 in peripheral blood cells of C57BL/6 mice (A) and ApoE^{-/-} mice (B) are shown. Data are reported as means \pm SE.

To examine whether the IFN-I pathway was activated in the plaque microenvironment, we assessed the gene expression levels of ISG15, IFIT1-1, and IFIT1-2 in the aortic plaques. Compared to the levels in PBS-treated ApoE^{-/-} mice, the gene expression levels of ISG15, IFIT1-1, and IFIT1-2 were increased in the aortic plaques of pristane-treated ApoE^{-/-} mice (Figure 4A). MMP-9 and TNF- α are proinflammatory cytokines associated with plaque instability (Galis and Khatri, 2002; Boyle et al., 2003). It has been reported that IFN- α can intensify the production of TNF- α and MMP-9 (Niessner et al., 2007). After pristane injection, MMP-9 and TNF- α in the aortic plaques of ApoE^{-/-} mice were significantly enhanced at the transcriptional level compared to those in ApoE^{-/-} mice injected with PBS (Figure 4B and C).

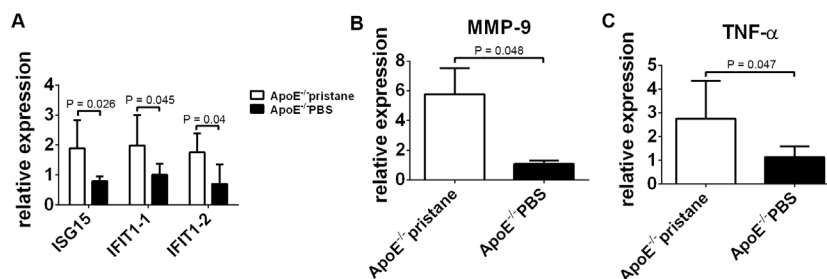


Figure 4. Real-time PCR analyses of IFN-induced genes, MMP-9, and TNF- α in aortic plaques. Expression of ISG15, IFIT1-1, and IFIT1-2 (A), MMP-9 (B), and TNF- α (C) in aortic plaques of ApoE^{-/-} mice. Data are reported as medians and IQR.

IFN- α inhibited COL1A1 gene expression in VSMC

VSMC are responsible for collagen synthesis in atherosclerotic plaques (Newby and Zaltsman, 1999). The extracellular matrix content, particularly the collagen type I content, usually determines the stability and durability of tissues, including arteries (Lee and Libby, 1997). Previous studies have reported that an activated macrophage supernatant can inhibit 1 α (I) procollagen mRNA expression in arterial VSMC (Halloran et al., 1997). To further confirm whether IFN could affect collagen synthesis in VSMC *in vitro*, we incubated VSMC with the supernatant from LPS- or IFN- α -stimulated macrophages. COL1A1 gene expression in VSMC was significantly decreased after incubation with LPS- or IFN- α -stimulated macrophage supernatant for 24 h (Figure 5).

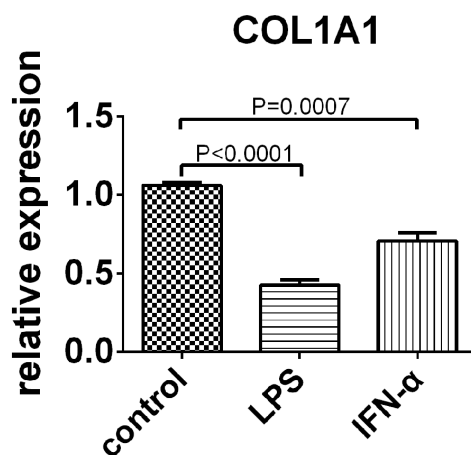


Figure 5. IFN- α inhibited COL1A1 gene expression in VSMC. COL1A1 gene expression levels are shown for VSMC after incubation with LPS- or IFN- α -stimulated RAW264.7 cell supernatant. Data are reported as means \pm SE.

DISCUSSION

Although it is widely accepted that patients with SLE have a marked increase in the prevalence of atherosclerosis, the mechanism of accelerated atherosclerosis in lupus is not well understood.

The pristane-induced lupus model is the first mouse model that expresses the IFN-I signature found in more than half of SLE patients (Reeves et al., 2009). In this study, a mouse model was created in which a single intraperitoneal injection of pristane into ApoE^{-/-} mice, which are susceptible to atherosclerotic lesions, allowed an examination of the impact of IFN-I on atherogenesis. Significantly increased expression of the IFN-I induced ISG15, IFIT1-1, and IFIT1-2 genes in blood cells indicated that the IFN-I pathway was activated in ApoE^{-/-} mice after pristane treatment. Furthermore, pristane-treated ApoE^{-/-} mice also exhibited the traits of SLE, accompanied by increased serum ANA and ENA levels. Although ApoE^{-/-} mice may spontaneously develop ANA, as reported previously (Wang et al., 2012, 2014), pristane treatment resulted in a remarkable change of the ANA nuclear patterns, and the mice developed anti-Sm, anti-nRNP, and anti-ribosomal antibodies. The lupus-specific anti-Sm antibody and

anti-ribosomal antibodies were found only in the ApoE^{-/-} mice treated with pristane, not in those treated with PBS. We also observed more apparent splenomegaly in the pristane-treated ApoE^{-/-} mice than in the PBS-treated ApoE^{-/-} mice. Such observations indicated that the lupus-associated atherosclerosis model was successfully set up in ApoE^{-/-} mice.

It is now well established that the risk of thrombosis depends more on plaque composition than on the degree of luminal obstruction. Vulnerable plaques in coronary and carotid arteries are the main cause of death and disability (Lloyd-Jones et al., 2009). As a prototypic autoimmune disease, SLE can be seen as a model of “vulnerable” patients prone to clinically evident CVD (Von Feldt, 2008). In our study, the lesion characteristics varied, which is potentially suggestive of plaque remodeling. The pristane-treated ApoE^{-/-} mice had less collagen content in the aortic sinus plaques. Collagen is an important component of the extracellular matrix of the arterial wall. There is evidence that the amount and organization of matrix collagen are related to the mechanical stability of the fibrous cap (Halvorsen et al., 2008). We also found that the gene expression level of MMP-9 significantly increased in the aortic plaques of pristane-treated ApoE^{-/-} mice. MMP-9 can cause tissue damage via extracellular matrix degradation, which enables outward remodeling and eventually results in the weakening of the arterial wall (Galis and Khatri, 2002). In a mouse model of plaque rupture, direct induction of VSMC apoptosis in the fibrous cap induces plaque rupture and thrombosis (von der Thusen et al., 2002). Our study did show a significantly larger number of apoptotic cells in the plaques of pristane-treated ApoE^{-/-} mice, suggesting that pristane treatment increased the vulnerability of the plaques by promoting the apoptosis program of cells in aortic plaques. TNF- α , a powerful proinflammatory cytokine, can destabilize plaque tissue integrity by promoting macrophage-induced VSMC apoptosis (Boyle et al., 2003). The expression of TNF- α was upregulated in the aortic plaques of our pristane-treated ApoE^{-/-} mice, indicating the relevance of TNF- α and cell apoptosis in the plaques.

It is known that the intraperitoneal injection of pristane into normal mice leads to a lupus-like autoimmune syndrome and to expression of IFN-I signals (Satoh and Reeves, 1994). The IFN-I pathway in ruptured lesions showed a highly significant upregulation compared to the pathway in stable lesions. At the same time, inhibiting endogenous myeloid IFN-I signals could reduce the formation of necrotic cores, thus directly affecting the stability of plaques (Goossens et al., 2010). In our study, we also found that the expression of IFN-induced genes was upregulated in the aortic plaques of the ApoE^{-/-} mice treated with pristane, as was the gene expression of the proinflammatory cytokines MMP-9 and TNF- α , suggesting that the vulnerability of the plaques had increased.

It has been reported that IFN- α functions as an inflammatory amplifier in atherosclerotic plaques. It sensitizes antigen-presenting cells toward pathogen-derived Toll-like receptor 4 (TLR4) ligands by upregulating TLR4 expression, and it intensifies TNF- α , interleukin-12, and MMP-9 production, all of which are implicated in plaque destabilization (Niessner et al., 2007).

In some clinical applications for hepatitis, it has been found that IFN- α functions not only as an antiviral agent but also as an antifibrotic agent (Okazaki et al., 2002; Papatheodoridis et al., 2005). IFN- α inhibits the activation of type I collagen and thus reduces the synthesis of collagen (Inagaki et al., 2003). We also discovered that the expression of COL1A1 mRNA was remarkably inhibited when VSMC were exposed to IFN- α . We speculate that IFN- α may inhibit the synthesis of collagen, thus reducing the stability of plaques.

In conclusion, the current study suggests that the markedly increased vulnerability of plaques in pristane-treated ApoE^{-/-} mice is due to activation of the IFN-I pathway. The

susceptibility of the plaques to rupture increases cardiovascular risk. We believe that further examination of IFN-I and its downstream molecules will provide clues to the prevention of lupus-associated CVD. More experiments are needed to explore the role of IFN-I in the development of atherosclerosis in the mouse model of lupus.

Conflicts of interest

The authors declare no conflict of interest.

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