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Original article

Association between Type I interferon and depletion and dysfunction of endothelial progenitor cells in C57BL/6 mice deficient in both apolipoprotein E and Fas ligand

Linyu Geng [a,1], Shiying Wang [a,1], Xia Li [a], Dandan Wang [a], Haifeng Chen [a], Jinyun Chen [a], Yue Sun [a], Weiwei Chen [a], Genhong Yao [a], Xiang Gao [b], Wanjun Chen [c], Songtao Shi [d], Xuebing Feng [a,*], Lingyun Sun [a,*]

[a] *Department of Rheumatology and Immunology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, China*
[b] *Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, 12 Xuefu Road, Nanjing 210000, China*
[c] *Mucosal Immunology Section, OPCB, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892-2190, United States*
[d] *Department of Anatomy and Cell Biology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104-6004, USA*

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ABSTRACT

Patients with systemic lupus erythematosus (SLE) have a tremendously increased risk for cardiovascular disease (CVD), which could not be accounted in entirety by traditional Framingham risk factors. To study whether the accelerated atherosclerosis in SLE patients is mediated by type I interferon (IFN-I) through the regulation of endothelial progenitor cells (EPCs), we created a line of C57BL/6 mice with deficiency in both apolipoprotein E (*ApoE*−/−) and fas ligand (*FasL*−/−, *gld.*). As expected, the resultant *gld. ApoE*−/− mice exhibited both aggravated lupus-like disease and atherosclerosis under normal diet. Increased expression of IFN-I-stimulated genes (ISGs) was closely associated with depletion and dysfunction of EPCs, as well as with accelerated atherosclerotic lesion in *gld. ApoE*−/− mice. While only IFN-α instead of other interventions, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IRS423 and IRS661, impaired EPC function *in vitro*. Mechanistically, activation or inhibition of the TLR7/9 signaling could modulate EPC number and function *in vivo*. Decreased proliferation rate and increased apoptotic rate of EPCs induced by IFN-α might contribute to the results to a certain extent. Thus, our data suggest that excessive expression of IFN-I through the activation of TLR7/9 signaling may induce accelerated atherosclerosis in lupus through the depletion or dysfunction of EPCs, suggesting that targeting IFN-I might have potential therapeutic effects on both lupus disease and premature atherosclerosis in SLE patients.

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Abbreviations: SLE, systemic lupus erythematosus; EPCs, endothelial progenitor cells; IFN-I, type I interferon; FACS, fluorescence-activated cell sorting; FITC-UEA-1, fluorescein isothiocyanate labeled Ulex europaeus agglutinin 1; DII-ac-LDL, 3′-tetramethylindocarbocyanine perchlorate labeled acetylated low-density lipoprotein; CPG-ODN, CPG-oligodeoxynucleiotides; IRS, immunoregulatory sequences; IFN-α, interferon-α; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; TLR, toll like receptor; CVD, cardiovascular disease; C3, complement 3; C4, complement 4; EC, endothelium; *ApoE*−/−, apolipoprotein E deficient; FasL−/−, *Fas* ligand deficient; PBS, phosphate-buffered saline; HDL, high-density lipoprotein; pDCs, plasmacytoid dendritic cells; ISGs, IFN-I-stimulated genes; IRF7, IFN regulatory factor 7; Mx1, myxovirus (influenza virus) resistance 1; Mx2, myxovirus (influenza virus) resistance 2; OAS1, 2′-5′-oligoadenylate synthetase 1; OAS2, 2′-5′-oligoadenylate synthetase 2; SOCS1, suppressor of cytokine signaling 1; IFIT2, interferon-induced protein with tetratricopeptide repeats 2; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN-inducible protein 10; ECM-2, endothelial cell growth medium-2; FBS, fetal bovine serum; RT-PCR, real-time reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor; HGF, hepatic growth factor.

* Corresponding authors.
E-mail addresses: xb.feng@163.com (X. Feng), lingyunsun@nju.edu.cn (L. Sun).
[1] These two authors contributed equally to this work.

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multi-organ involvements with a wide array of clinical manifestations [1]. Patients with SLE show an up to 50-fold increase in developing cardiovascular disease (CVD) compared with age- and sex-matched healthy controls [2]. Traditional Framingham risk factors including hypertension, hypercholesterolemia, diabetes and smoking do not account in entirety for the increased risk, as it remains 8–10-fold higher even after adjustment for these factors [3]. Recently, SLE specific factors, such as disease activity and duration, corticosteroids treatment, presence of anti-phospholipid antibodies, have been suggested to contribute to the development of premature atherosclerosis [4]. However, the etiology of accelerated atherosclerosis in SLE is still largely unknown.

Endothelial progenitor cells (EPCs), released from the bone marrow into the circulation, play a crucial role in the development of atherosclerosis and CVD [5]. In response to signals from vascular injuries, EPCs could migrate to the injury site and differentiate into endothelium (EC) to repair vasculature [5]. During the last decade, depletion of circulating EPCs and abnormal EPCs function have been observed in patients with SLE [6,7]. Specifically, the abnormality of EPCs in lupus could be mediated by various inflammatory factors, especially type I interferon (IFN-I) [8–10]. Mostly, viral infection triggers toll like receptor (TLR) 7/9 on plasmacytoid dendritic cells (pDCs) to secrete IFN-I, also with the secretion of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and so on [11], and the presence of "interferon signature" has been suggested to be integral to the pathogenesis of SLE [12,13]. The depletion of EPCs was more dramatic in patients with elevated levels of IFN-I [9]. When cultured *in vitro*, lupus EPCs have high IFN-inducible gene expression and neutralization of IFN pathways could restore a normal EPCs phenotype [14]. Thus, IFN-I that induced by lupus itself may act through the regulation of EPCs to promote atherosclerosis.

Although a link between IFN-I and EPCs has been established [8,9,14,15], there is no direct evidence showing that excessive endogenous IFN-I in SLE could have direct effects on EPCs and consequently accelerate atherosclerosis *in vivo*. In lupus-prone mice on western diet, loss of IFN-I receptor signaling improves EPCs number and function, yet acute exposure to IFN-α leads to no alteration of EPCs number [8]. To further clarify the role of IFN-I on premature atherosclerosis in SLE with the exclusion of the effect of western diet, we generated *ApoE*−/− and *FasL*−/− (*gld.*) double knockout mice (*gld. ApoE*−/−, C57BL/6 background), which exhibited aggravated lupus and accelerated atherosclerosis under normal diet. Our data showed that increased expression of IFN-I stimulated genes (ISGs) was closely associated with the depletion and dysfunction of EPCs and accelerated atherosclerotic lesion in *gld. ApoE*−/− mice. IFN-I directly inhibited the number and function of EPCs both *in vitro* and *in vivo*, suggesting that overproduced IFN-I through the activation of TLR7/9 signaling had an impact on the development of premature atherosclerosis in SLE.

2. Materials and methods

2.1. Experimental animals

Breeding pairs of *gld.* and *ApoE*−/− mice on C57BL/6 background were purchased from Model Animal Research Center of Nanjing University. The *FasL*+/+ *ApoE*+/+ (wild type), *gld.*, *ApoE*−/−, and *gld. ApoE*−/− mice used in this study were obtained by intercrossing or backcrossing as previously described [16,17]. DNA was extracted from tail tissue using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Genotyping of the wild type versus the *ApoE*−/− allele, the *gld.* allele and the *gld. ApoE*−/− allele was performed following manufacturer's recommendations and previous studies [16,17]. Serum and urine from each genotype female mice were collected at 20-week age (after onset of significant proteinuria or active lupus). Mice at 20-week age were weighed and euthanized by cervical dislocation, with peripheral blood, kidneys, aorta harvested and spleen weighed. Lumbar vertebrae and limbs were excised to get mononuclear cells from bone marrow. All mice were fed with standard chow diet through all age and maintained in a temperature-controlled room with a 12 h light/dark cycle according to the approved protocol by The Affiliated Drum Tower Hospital of Nanjing University Medical School Committee on use and care of experimental animals.

CPG-oligodeoxynucleiotides (CPG-ODN) with immunoregulatory sequences (IRS) that specifically activate/inhibit signaling *via* TLR7/9 were synthesized (Takara, Dalian, China). At 16-week of age, female *gld. ApoE*−/− mice were given phosphate-buffered saline (PBS) (200 μl/20 g), IRS423 (TLR7/9 agonists, 40 μg/20 g) or IRS661 (TLR7/9 antagonists, 40 μg/20 g) twice a week by subcutaneous injection for 4 weeks to detect the role of IFN-I in *gld. ApoE*−/− mice *in vivo* [18,19]. CPG-ODN sequences are as followings: IRS423 5′-TCG ATG ACG TTC CTG ATG CT-3′, IRS661 5′-TGC TTG CAA GCT TGC AAG CA-3′. All were phosphorothioate ODNs and had less than 5 endotoxin U/mg ODN determined by Limulus amebocyte lysate assay (Cambrex) [14].

For *in vitro* experiment, bone marrow EPCs from 20-week old female *gld. ApoE*−/− mice were collected and cultured in the presence of either recombinant human IFN-α (R & D, Minneapolis, MN, 10 KU/mL), recombinant human IL-1β (R & D, Minneapolis, MN, 10 ng/mL), recombinant human TNF-α (R & D, Minneapolis, MN, 10 ng/mL), IRS423 (0.3 μM), IRS661 (0.3 μM) or PBS for 24 h to assess the possible factors influencing EPCs function [14].

2.2. ApoE−/− *and* gld. *strain genotyping*

The genotypes of *ApoE* and *gld.* were determined by polymerase chain reaction (PCR) analysis using DNA extracted from tail samples (Qiagen, Hilden, Germany) [16,17]. The *ApoE*−/− strains were detected using three primers that produced 155-bp or 245-bp products respectively. The primers were as follows: 5′-GCCTAGCC-GAGGGAGAGCCG-3′ and 5′-TGTGACTTGGGAGCTCTGCAGC-3′ for the wild type allele and 5′-GCCGCCCCGACTGCATCT-3′ for the *ApoE*−/− allele. The *gld.* derived progeny was genotyped using 2 primers according to the protocols on the web of Jackson laboratory: 5′-CAAGACAATATTCCTGGTGCC-3′ and 5′-CAATTTTGAGGAATC-TAAGGCC-3′. The mutant = 108 bp, heterozygote = 108 bp, 88 bp and 20 bp, and the wild type = 88 bp and 20 bp.

2.3. Splenic index analysis

Spleens were removed from the 20-week old female mice after the euthanization. With residual blood dried with filter paper, the spleens were weighed. The spleen weight (mg) was divided by the body weight of mice (g), and then multiplied by 10 to get the splenic index.

2.4. Proteinuria measurement

Mice were monitored for proteinuria at 1–2 months and 20 weeks of age. Proteinuria in fresh urine was examined using Albustix (Bayer, Elkhart, IN).

2.5. Serum analysis

Serum samples were diluted 1:50 to measure levels of total IgG, IgG anti-double-stranded DNA (anti-ds-DNA) and IgG anti-nuclear antibody (ANA) using commercial ELISA kit (R&D Systems, Inc, MN) according to the manufacturer's protocol. Serum lipid and blood urine nitrogen (BUN) analysis was performed by biochemistry laboratory of the hospital using a Beckman CX-7 biochemical analyzer.

2.6. Immunohistochemistry

20-week old mice were euthanized, with half of the kidney fixed in 10% formaldehyde and embedded into paraffin. Three micrometer sections of the kidney tissue were cut and observed for various morphologic lesions after periodic acid Schiff staining. The other half of the kidney was embedded in Tissue-Tec OCT medium, frozen in liquid nitrogen, and stored at −70 °C until sectioning. Five micrometer frozen sections were fixed with 4% paraformaldehyde and blocked with 2% BSA. Subsequently, slides were stained with goat anti-mouse IgG (1:150 dilutions; Abcam, Cambridge, UK) or goat anti-mouse complement C3 (1:150 dilutions; Abcam, Cambridge, UK). Stained sections were coded and digitally photographed using a microscope fitted with a digital camera (Cannon Power shot G10, Cannon, Inc). Images were analyzed using Image Pro Plus software (Media Cybernetics) by a blinded observer to measure the size of the glomerular tuft area as described. At least 25 glomeruli were observed for each sample.

2.7. Real-time PCR (RT-PCR) assay for the quantification of ISGs

Total cellular RNA was isolated from bone marrow EPCs using Trizol reagent (Invitrogen, USA) following manufacturer's recommendations, single-stranded

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