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ORIGINAL ARTICLE

Endophilin A2, a Potential Therapeutic Target for Lupus, Promotes Lupus Progression

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ABSTRACT

Objective: Endophilin A2 (EPA2) is a member of the Endophilin family. The relationship between EPA2 and SLE pathogenesis is unclear.

Methods: Plasma levels of EPA2 in SLE patients and healthy controls were detected by ELISA, and EPA2 mRNA levels of SLE patients were explored by qRT-PCR. EPA2 siRNA adenovirus was further injected into pristane-induced lupus mice, and the histological and serological changes were observed. In vitro, EPA2 siRNA adenovirus was transfected to human umbilical vein endothelial cells (HUVECs) in the presence of growth differentiation factor 15 (GDF15), and the proliferation, migration, and tube-forming ability of HUVECs were discussed.

Results: Plasma EPA2 levels were significantly higher in SLE patients than in healthy controls ($p < 0.001$), and EPA2 mRNA levels were significantly higher in SLE patients than in healthy controls as well ($p = 0.030$). Lupus mice exhibited splenomegaly, severe histologic damage, and high levels of autoantibodies (antinuclear antibody (ANA), anti-double-stranded DNA antibody (anti-dsDNA), and immunoglobulin G (IgG)) (vs. the control group, all $p < 0.05$). After injection of EPA2 siRNA adenoviruses, the lupus mice showed a lower proportion of $CD11b^+LY\text{-}6C^+$, $F4/80^+$, $CD11c^+$, $CD19^+$, $CD8^+$, $Th1^+$, $Th2^+$, $Th17^+$ cells and reduced expression of pro-inflammatory cytokines, and autoantibodies (vs. the adenoviral empty vector group, all $p < 0.05$). Addition of EPA2 siRNA adenovirus to HUVECs resulted in decreased GDF15 mRNA levels and reduced cell proliferation, migration and tube formation. However, in the presence of GDF15, EPA2-mediated effects were reversed, and the proliferation, migration, and tube formation ability of HUVECs were enhanced.

Conclusion: EPA2 may regulate angiogenesis through GDF15, and then involve in SLE pathogenesis.

1 | Introduction

Systemic lupus erythematosus (SLE) is a common rheumatic disease associated with a variety of autoantibodies as well as immune complex deposition, which ultimately leads to multi-organ damage and a lot of clinical manifestations [1, 2]. Many patients with SLE have cardiovascular comorbidities, and part of the mortality in SLE patients is induced by cardiovascular-related death [3, 4]. To date, the clear etiology of SLE has not been elucidated, and available studies suggest that genetic and environmental factors may correlate with SLE pathogenesis [5]. It is known that the risk of SLE may be attributed to genetics, and there is a higher prevalence of SLE in women than in men [6]. Homozygous twins with SLE have a higher concordance

rate than heterozygous twins [7]. This suggests that the risk of developing SLE may be highly heritable.

Endophilin A2 (EPA2) is a membrane-bound protein encoded by the SH3GL1 gene. EPA2 is closely related to cellular endocytosis [8] and is involved in the regulation of a variety of biological functions, such as autophagy [9–11]. Current studies have found that EPA2 is involved in tumor metastasis, neuromodulation, viral body production, and vascular function. EPA2 is highly expressed in neoplastic diseases such as colorectal cancer, breast cancer, and osteosarcoma, as well as rheumatoid arthritis (RA) [12–15]. EPA2 was found to be a disease-associated protein in adolescent idiopathic scoliosis [16, 17]. Therefore, EPA2 may be a biomarker for some diseases. The study by Norin et al. revealed that EPA2 may regulate T cells in RA patients, and there were higher serum EPA2 levels in RA patients than in healthy controls.

Angiogenesis, or the development of new capillaries from existing vessels, is associated with inflammation. Effector cells in the inflammatory phase release pro-angiogenic molecules that promote the formation and invasion of new blood vessels, allowing inflammatory cells to invade. Studies have shown that EPA2 is a regulator of endothelial cell migration during angiogenesis. EPA2 is expressed in endothelial cells, which are required for developmental and pathological angiogenesis. EPA2 promotes endothelial cell migration by regulating VEGFR2 internalization and downstream signaling [18]. Thus, EPA2 may be involved in regulating angiogenesis, and excessive angiogenesis may promote the development of SLE. A previous study by our group showed that SLE patients had elevated levels of growth differentiation factor 15 (GDF15) [19]. GDF15 was accepted to be involved in angiogenesis, and GDF15 stimulates the proliferation of human umbilical vein endothelial cells (HUVECs) and promotes vascular development. Since the proliferation of endothelial cells plays an important role in angiogenesis, GDF15 may be a potential angiogenic factor for tissue regeneration [20–23]. Indeed, SLE is a disease significantly associated with impairment of vascular function. Based on this, we speculate that EPA2 may affect endothelial cells migration and angiogenesis by regulating GDF15 signaling, which in turn promotes the development of SLE.

To date, there are no studies of EPA2 associated with SLE. Therefore, the aim of this study was to investigate the association of EPA2 with the pathogenesis of SLE. In this study, we first discussed plasma EPA2 levels in SLE patients and EPA2 mRNA levels in SLE patients. Second, we conducted a lupus mice model to confirm the role of EPA2 in lupus development. Finally, in vitro experiments, we discussed the role of EPA2 on HUVECs proliferation, migration, and tube formation through regulating GDF15. This study revealed the potential of EPA2 as a biomarker for SLE and demonstrated the role of EPA2 in promoting lupus development by regulating GDF15.

2 | Materials and Methods

2.1 | Study Population

For population study, we included 3 independent case–control studies. The first was to analyze plasma levels of EPA2 in SLE patients, and this part included 81 SLE patients and 81 healthy controls. The second part was to discuss EPA2 mRNA levels in 34 SLE patients and 34 healthy controls, which was used to demonstrate expression profile of EPA2 in SLE plasma. Moreover, we validated EPA2 plasma levels from an independent SLE cohort (81 SLE patients and 81 healthy controls). Diagnosis of SLE was made according to the classification criteria of the American College of Rheumatology (ACR) in 1997. Data about clinical and laboratory characteristics of SLE patients were collected. Systemic lupus erythematosus disease activity index (SLEDAI) was calculated according to the SLE Disease Activity Index 2000 version (SLEDAI-2K). All SLE patients were from the Department of Rheumatology and Immunology of the Affiliated Hospital of Southwest Medical University, and healthy controls were from the Center for Disease Control and Prevention of Jiangyang District and the Health Examining Center of Affiliated Traditional Chinese Medicine Hospital of Southwest Medical University.

2.2 | Animal Models

Thirty-two female C57BL/6J mice (6–8 weeks) were purchased from GemPharmatech (Chengdu, China). After 1 week of acclimatization feeding, the mice were randomly divided into 4 groups of 8 mice each using completely randomized grouping. Three groups of mice were injected intraperitoneally with 0.5 mL pristane (Sigma Aldrich, St Louis, USA) to induce lupus. The other group was injected with phosphate-buffered saline solution (PBS). After 3 months' observation, EPA2 siRNA adenovirus and empty vector adenovirus were diluted in sterilized saline and injected into part of the pristane-induced lupus mice. Different kinds of adenoviruses were injected into the tail vein for 5 and 7 days, and then EPA2 mRNA levels in the spleen were evaluated. Five days' injection of EPA2 siRNA adenovirus showed the best knockdown efficiency (Figure S1). In the following study, all mice were euthanized after injection of EPA2 siRNA adenovirus for 5 days. Cardiac blood was collected and centrifuged to obtain serum. The liver, spleen, and kidneys were weighed and photographed.

2.3 | Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma from each patient and control subject was separated, and then the plasma was stored in a −80°C freezer until use. Plasma EPA2 was detected using an ELISA kit (Abebio, Wuhan, China). In addition, mouse serum was collected after centrifugation for detection of antinuclear antibody (ANA), anti-double-stranded DNA antibody (anti-dsDNA), and immunoglobulin G (IgG) (CUSABIO, Wuhan, China). The data were measured at 450 nm using an enzyme marker.

2.4 | Quantitative Real-Time PCR Analysis (qRT-PCR)

Total RNA from the whole blood of the patient and control subjects was extracted using the Whole Blood Total RNA Kit (Sangon Biotech, Shanghai, China), and total RNA from cells was extracted using the Ultra Pure Total RNA Extraction Kit (Sangon

Biotech, Shanghai, China). cDNA was synthesized using reverse transcriptase reagent (Vazyme, Nanjing, China). cDNA was amplified using SYBR dye (Vazyme, Nanjing, China). All qRT-PCR analysis was performed in triplicate and normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Relative mRNA levels of EPA2 and GDF15 were calculated for each sample using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were shown in Table S1.

2.5 | Flow Cytometry

Mouse spleens were ground using a 70 μm cell filter to prepare single-cell suspensions. Leukocytes were obtained by removing erythrocytes on ice using erythrocyte lysis buffer (Beyotime, Shanghai, China). Monocytes, dendritic cells, B cells, macrophages, and T cells were detected using CD11b-FITC, LY-6C-PE-CY7, CD11c-APC, CD19-PE, F4/80-BV421, CD3-BV510, and CD8-percp-cy5.5. For Th1, Th2, Th17, and Treg cells, the proportion of the cells were detected by markers of CD4-BV421, interferon-γ (IFN-γ)-FITC, interleukin-4 (IL-4)-PE-CY7, IL-17A-PE, and Foxp3-AF647. All antibodies were purchased from BD Biosciences (California, USA). All data were analyzed by FlowJo software (v10.8.0, Treestar, USA).

2.6 | Cytometric Bead Array (CBA)

The BD Cytometric Bead Array Mouse Cytokine Kit was used to detect expression of inflammatory cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α in mouse serum. Experiments were performed according to the CBA kit instructions. Briefly, 50 μL of serum samples, 20 μL of mixed microspheres, and 20 μL of PE detection antibody were gently mixed and incubated for 2 h at room temperature, protected from light. Data were collected using a BD FACSCantoII flow cytometer. A standard curve was obtained, and the sample content was calculated using FCAP Array software.

2.7 | Histopathology and Immunofluorescence Analysis

Mice kidneys were prepared by dehydration, trimming, embedding, sectioning, staining, sealing, and microscopic examination; hematoxylin–eosin (HE) staining and Masson staining were performed to observe the renal histopathology and renal fibrosis. HE and Masson scores were calculated according to the degree of renal tissue damage and the percentage of area of renal fibrous tissue expression, respectively [24, 25]. The site and intensity of immune complex expression in the mice's kidneys were observed by immunofluorescence staining under a laser confocal microscope.

2.8 | Cell Culture and Processing

Twenty-five milliliters of fetal bovine serum, 5 mL of endothelial cell growth supplement, and 5 mL of penicillin–streptomycin were added to 500 mL of ECM (Sciencell, USA) basal medium, which was configured as fresh medium. HUVECs were cultured in a 37°C incubator at 5% CO_2. HUVECs were transfected with the EPA2 siRNA adenoviral vector. For discussing the role of EPA2 in HUVECs proliferation, migration, and tube formation by regulating GDF15, we first transfected HUVECs with EPA2 siRNA adenoviruses, and then we treated the cells with recombinant human GDF15. The concentration is 100 ng/mL with a 24 h intervention.

2.9 | Proliferation

Cell proliferation capacity was evaluated using a cell counting kit (CCK-8; Sigma). HUVECs were spread in 96-well plates at a density of 2×10^3 cells for 24 h. Ten microliters of CCK-8 solution was added to each well, and the absorbance at 450 nm was measured by an enzyme marker.

2.10 | Migration

Migration was determined using transwell cell culture inserts (8um, Corning). HUVECs (3×10^4 cells) were placed in the upper layer of the transwell, and serum-containing ECM was placed below the cell-permeable membrane. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde for 15 m, washed, and then stained with 0.1% crystal violet for 20 m. Three fields of view were photographed in each well, counted using ImageJ, and the average value was taken for statistical analysis.

2.11 | Tube Formation

Each well in a 24-well plate was coated with 80 μL matrigel (Lablead, Beijing, China), and then HUVECs were added to the matrigel-coated wells at a density of 5×10^4 cells per well. After 8 h, photographs were taken with a light microscope, and the number of nodes and total tube length were calculated using the Angiogenesis Analyzer plug-in for ImageJ.

2.12 | Determination of Sample Size

The sample size was determined based on the research experience of the subject group and the ability of the group to actually collect samples. After completing the inclusion of samples, power was applied to conduct post hoc analysis as a means of calculating the actual test efficacy of the included sample size. The test efficacy for EPA2 plasma levels in the two case–control studies was both 1.000, and the test efficacy for EPA2 mRNA levels for qRT-PCR analysis was 0.970.

2.13 | Statistical Analysis

All data were analyzed using SPSS 26.0 statistical software. Quantitative data were expressed as mean ± standard deviation (SD) according to the Shapiro–Wilk test if the data were normally distributed. Median (interquartile spacing) was used for non-normal distribution data. Qualitative data were expressed as frequencies and (or) percentages. The rank correlation coefficient was used to evaluate the relationship between two variables. The specificity and sensitivity of plasma EPA2 as a diagnostic marker for SLE were measured by receiver operating characteristics (ROC) curves. $p < 0.05$ was statistically significant.

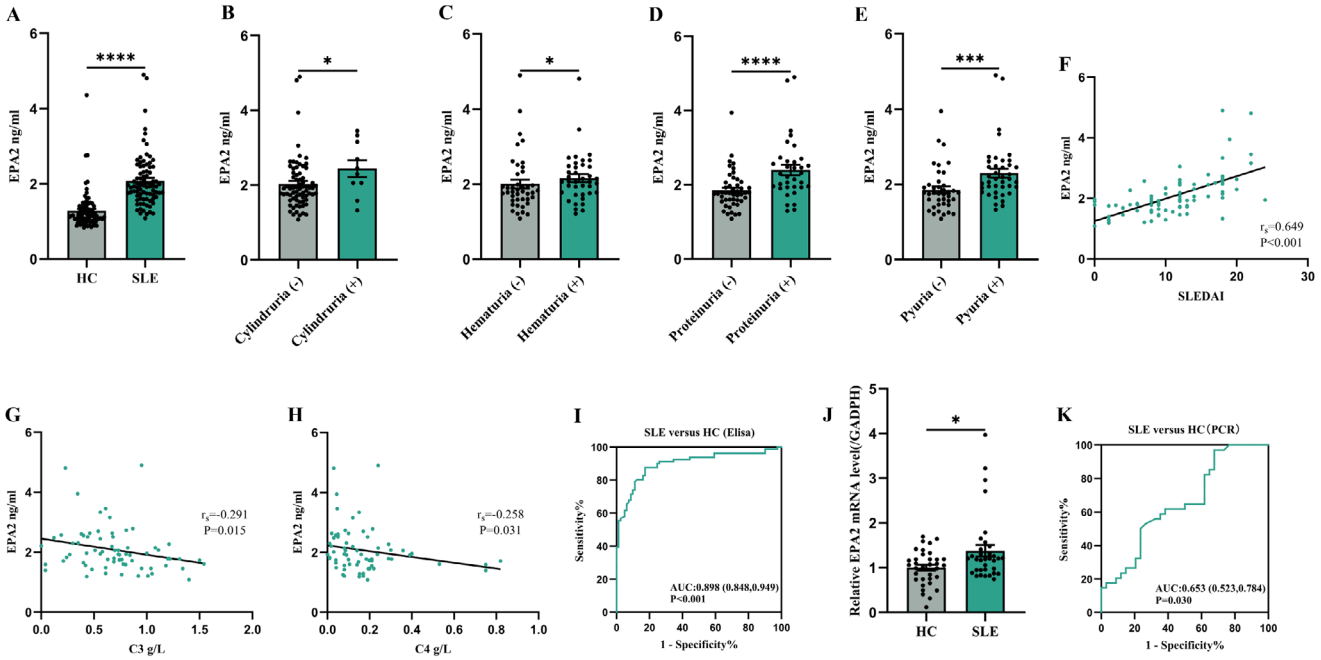

FIGURE 1 | Plasma and mRNA levels of EPA2 in SLE patients and healthy controls. (A) Plasma EPA2 levels in 81 SLE patients and 81 healthy controls were detected by ELISA. Comparison of EPA2 plasma levels between the two groups. (B–E) EPA2 plasma levels are associated with some clinical characteristics of SLE patients. (F–H) Correlation of plasma EPA2 levels with SLEDAI, C3, and C4. (I) Exploration of the potential of EPA2 plasma as a biomarker for SLE by receiver operating characteristics (ROC) curve analysis. (J) The mRNA levels of 34 SLE patients and 34 healthy controls were detected by qRT-PCR. (K) Exploration of the potential of EPA2 mRNA as a biomarker for SLE by receiver operating characteristics (ROC) curve analysis. *$p<0.05$, ***$p<0.005$, ****$p<0.001$.

3 | Results

3.1 | Correlation Between Plasma Levels of EPA2 and SLE Patients

Clinical and laboratory characteristics of SLE patients and healthy controls for ELISA analysis are shown in Table S2. Plasma EPA2 levels were higher in SLE patients compared with healthy controls ($p<0.001$, Figure 1A). Plasma EPA2 levels were significantly higher in SLE patients complicated with some clinical and laboratory features (including cylindruria, hematuria, proteinuria, and pyuria) compared to patients without these features (All $p<0.05$, Figure 1B–E). Correlation analysis showed that plasma EPA2 levels were associated with a number of clinical and laboratory features, including SLEDAI ($r_s=0.649$, $p<0.001$, Figure 1F), C3 ($r_s=-0.291$, $p=0.015$, Figure 1G), and C4 ($r_s=-0.258$, $p=0.031$, Figure 1H). ROC curve analysis showed that plasma EPA2 distinguished SLE patients from healthy controls with an area under the curve of 0.898 (95% CI: 0.848–0.949, $p<0.001$, Figure 1I). In addition, other clinical and laboratory characteristics were not significantly associated with plasma EPA2 levels ($p>0.05$, Tables S3 and S4). We assessed the ability of plasma EPA2 to distinguish SLE from healthy controls. The diagnostic efficiency of plasma EPA2 was evaluated (Table S5). At the cut-off value of 1.533 ng/mL, the sensitivity, specificity, +LR, −LR, Youden's index, accuracy, PPV, and NPV were 0.827, 0.877, 6.724, 0.197, 0.704, 0.852, 0.870, and 0.835, respectively. To validate plasma levels of EPA2 in SLE patients, we used another independent SLE cohort, which included 81 SLE patients and 81 healthy controls (Table S6). We found that SLE patients also had significantly higher plasma EPA2 levels than healthy controls ($p<0.001$, Figure S2A). Plasma EPA2 levels were correlated with some clinical and laboratory characteristics of SLE patients (Figure S2B–I, Tables S7 and S8). ROC curve also found that plasma EPA2 may differentiate SLE from controls (Figure S2J).

3.2 | Peripheral Blood EPA2 mRNA Levels Were Higher in SLE Patients

Another cohort of SLE patients and healthy controls was conducted to discuss the expression profile of EPA2 mRNA. The characteristics of the patients and controls were shown in Table S9. There was no statistically significant difference in the age and gender of the study subjects in both groups. By exploring the differences between peripheral blood EPA2 mRNA levels in SLE patients and healthy populations, it was found that mRNA levels of EPA2 were higher in SLE patients than in healthy controls ($p=0.030$, Figure 1J). ROC curve analysis showed that EPA2 mRNA levels distinguished SLE patients from healthy controls with an area under the curve of 0.653 (95% CI: 0.523–0.784, $p=0.030$, Figure 1K). Correlation between EPA2 mRNA levels and SLE patients' clinical and laboratory features was listed in Tables S10 and S11.

3.3 | EPA2 siRNA Adenovirus Inhibited Kidneys Swelling in Lupus Mice

The weights of the liver, spleen, and kidneys were significantly increased in pristane-induced lupus mice compared to controls. Treatment with EPA2 siRNA adenovirus decreased the weights

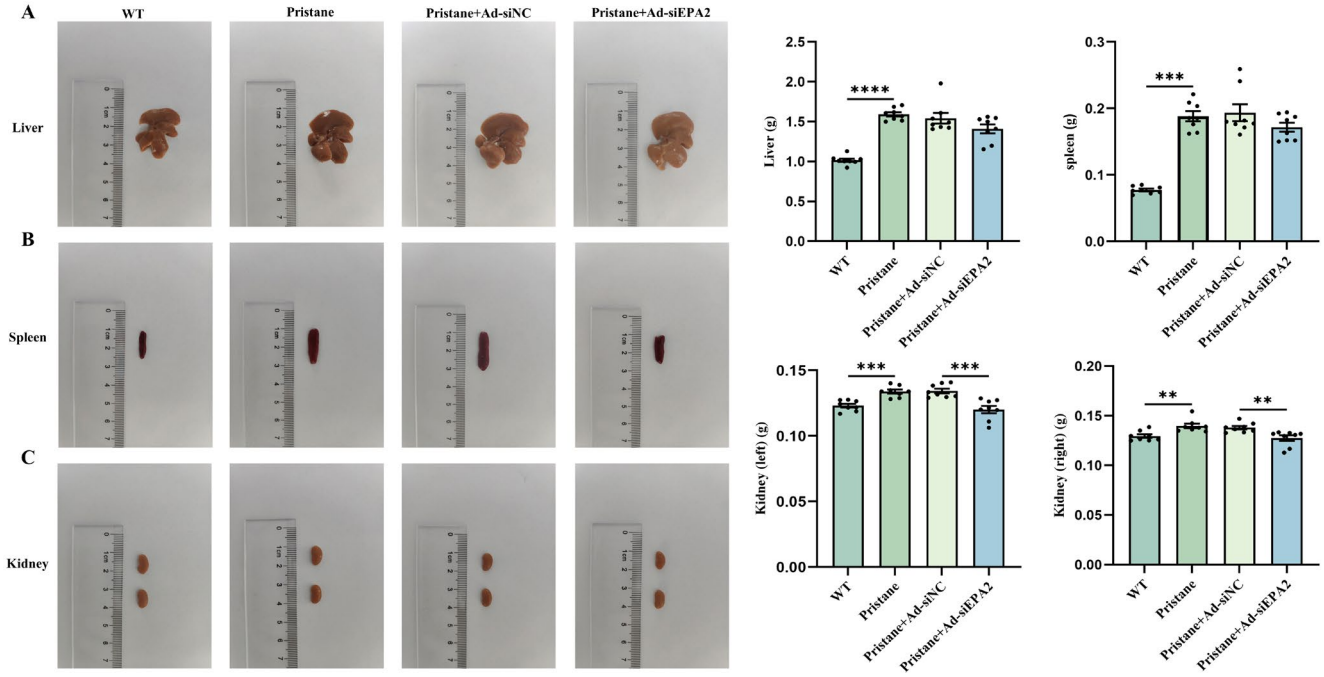

FIGURE 2 | Reduction of liver, spleen, and kidney enlargement in lupus mice after injection of EPA2 siRNA adenovirus. (A–C) Lupus mice (model group) were constructed by intraperitoneal injection of pristane into wild-type (WT) C57BL/6J mice, and control mice were constructed by injection of PBS into WT mice. The lupus mice were injected with an adenoviral empty vector or EPA2 siRNA adenovirus. Representative pictures of liver, spleen, and kidney of four groups of mice (left) and statistical analysis of organ weights (right). Error bars are shown as mean ± standard deviation. 8 mice/group. $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

of kidneys but did not significantly decrease the weights of liver and spleen in lupus mice (Figure 2A–C).

3.4 | EPA2 siRNA Adenovirus Attenuates Renal Injury in Lupus Mice

Compared with controls, pristane-induced lupus mice exhibited glomerulosclerosis and necrosis, thickening of the basement membrane of the renal capsule, narrowing of the capillary lumen, localized crescent formation, nuclear deletion of renal tubular epithelial cells, dilatation of part of the renal tubule, thinning of the tubular wall, lymphocyte infiltration, and fibrous tissue hyperplasia. In addition, glomerular IgG deposition was significantly increased in lupus mice compared with control mice. However, HE, Masson, and immunofluorescence intensity scores indicated that the EPA2 siRNA adenovirus had a significant therapeutic effect in lupus mice (all $p < 0.05$, Figure 3A–C).

3.5 | Inhibition of EPA2 Reduces Inflammatory Cytokines and Autoantibodies Production

Pristane-induced lupus mice had significantly higher levels of ANA, anti-dsDNA, and IgG compared to control mice. Silencing of EPA2 in lupus mice significantly reduced ANA, anti-dsDNA, and IgG levels compared to adenoviral empty vector-treated lupus mice (Figure 4A–C). In pristane-induced lupus mice, serum IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α levels were higher than those in the control group (Figure 4D–I). The expression of all inflammatory cytokines was down-regulated after silencing EPA2 in the lupus mice.

3.6 | Silencing EPA2 Reversed Dysregulation of Immune Cells in Lupus Mice

The percentages of $CD11b^+LY\text{-}6C^+$, $F4/80^+$, $CD11c^+$, $CD19^+$, $CD8^+$, $Th1^+$, $Th2^+$, and $Th17^+$ cells in the spleen of lupus mice were significantly higher than in control mice, and the proportion of Treg cells was lower in the lupus mice. After inhibition of EPA2, the lupus mice showed lower percentages of $CD11b^+LY\text{-}6C^+$, $F4/80^+$, $CD11c^+$, $CD19^+$, $CD8^+$, $Th1^+$, $Th2^+$, $Th17^+$ cells, and a higher percentage of Treg cells (All $p < 0.05$, Figure 5A–I).

3.7 | EPA2 siRNA Adenovirus Down-Regulates the mRNA Levels of GDF15

It is known that EPA2 may regulate angiogenesis. Therefore, we conducted in vitro experiments to discuss whether silencing EPA2 may regulate the function of HUVECs. Moreover, in our previous study, we found that GDF15 may be involved in SLE pathogenesis by regulating angiogenesis. In this study, we also discuss the effects of EPA2 on HUVECs by regulating GDF15.

First, we discussed which titer (MOI = 50, MOI = 100, MOI = 200, MOI = 500) of adenoviral transfection of HUVECs may have the best effect, and immunofluorescence observation of the cells showed MOI = 100 to be the best (Figure 6A). Then, EPA2 siRNA adenoviral transfection of HUVECs was further conducted to discuss which time silencing EPA2 may inhibit EPA2 expression the best. Results showed that expression of EPA2 was mostly inhibited after 48 h (Figure 6B). In addition, qRT-PCR analysis showed that the GDF15 mRNA expression was significantly reduced in

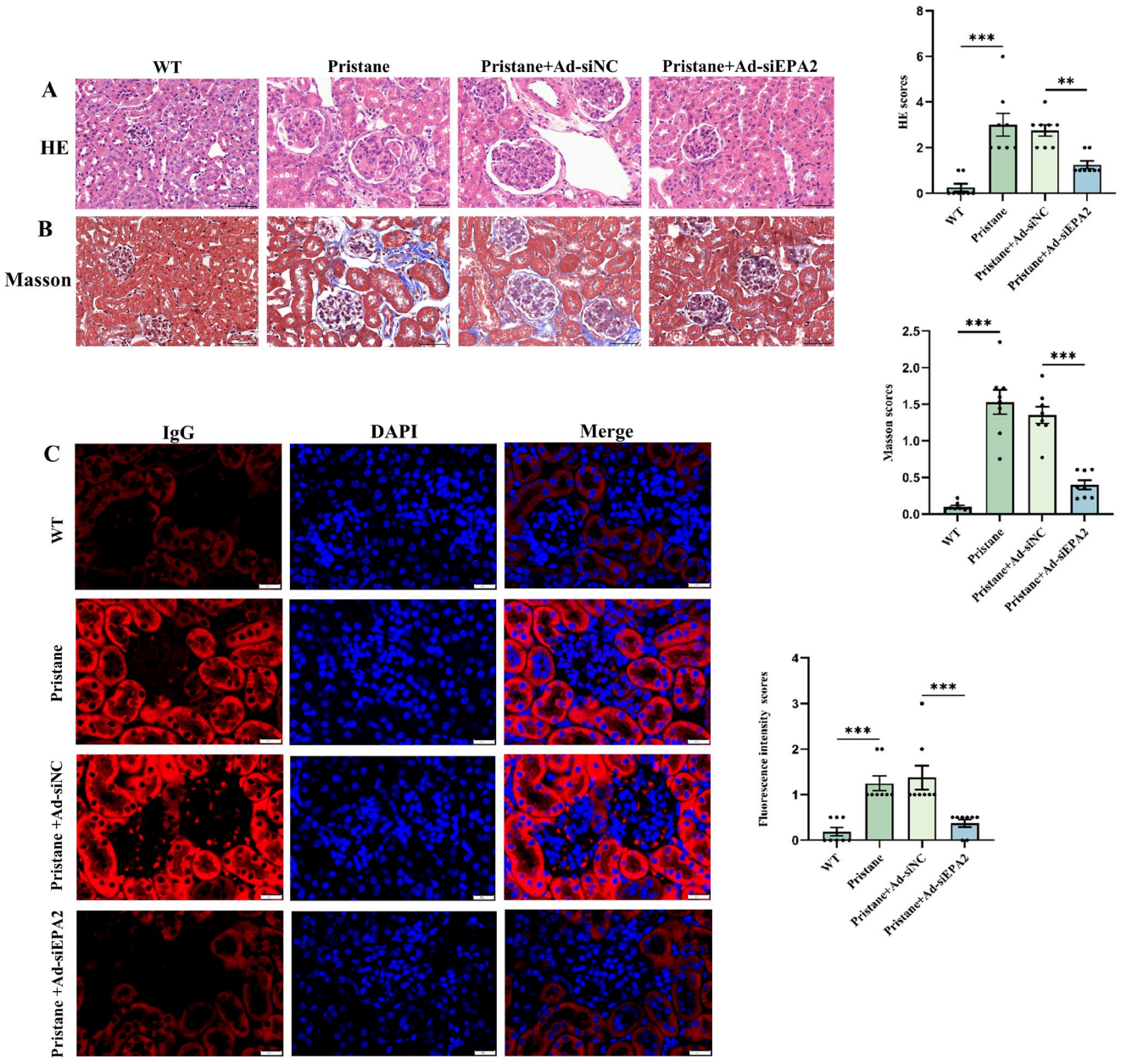

FIGURE 3 | EPA2 silencing attenuates kidney injury in lupus mice. (A–C) HE staining, Masson staining and immunofluorescence staining of the kidneys of mice in the control group, pristane-induced lupus group, adenovirus empty vector, and EPA2 siRNA adenovirus-injected groups. Acquisition of ×400 micrographs. Representative graphs of HE staining, Masson staining, and immunofluorescence of each group are shown on the left, and statistical scores of HE score, Masson score, and immunofluorescence score are shown on the right. Error bars are shown as mean ± standard deviation. 8 mice/group. $^{**}p < 0.01$, $^{***}p < 0.005$.

the EPA2 siRNA adenovirus group compared with the control and empty vector adenovirus groups ($p < 0.05$, Figure 6C).

3.8 | Inhibition of HUVECs Proliferation by EPA2 Inhibition

HUVECs were transfected with empty vector adenovirus (MOI = 100) and EPA2 siRNA adenovirus (MOI = 100), and the proliferation of HUVECs was detected by CCK8. The results showed that the proliferation ability of HUVECs was significantly reduced in the EPA2 silencing group compared with the control and empty vector groups ($p < 0.05$, Figure 6D). Interestingly, addition of GDF15 to the EPA2-silenced group significantly enhanced the proliferation ability of HUVECs ($p < 0.05$).

3.9 | Inhibition of HUVECs Migration by Suppression of EPA2

We discussed the effects of EPA2 on HUVECs migration. Transwell assay of HUVECs migration showed that the number of crystal violet staining was significantly reduced in the EPA2-silenced group compared with the control group and the empty vector group ($p < 0.05$, Figure 6E), suggesting that EPA2 silencing significantly reduced the migratory ability of HUVECs. After

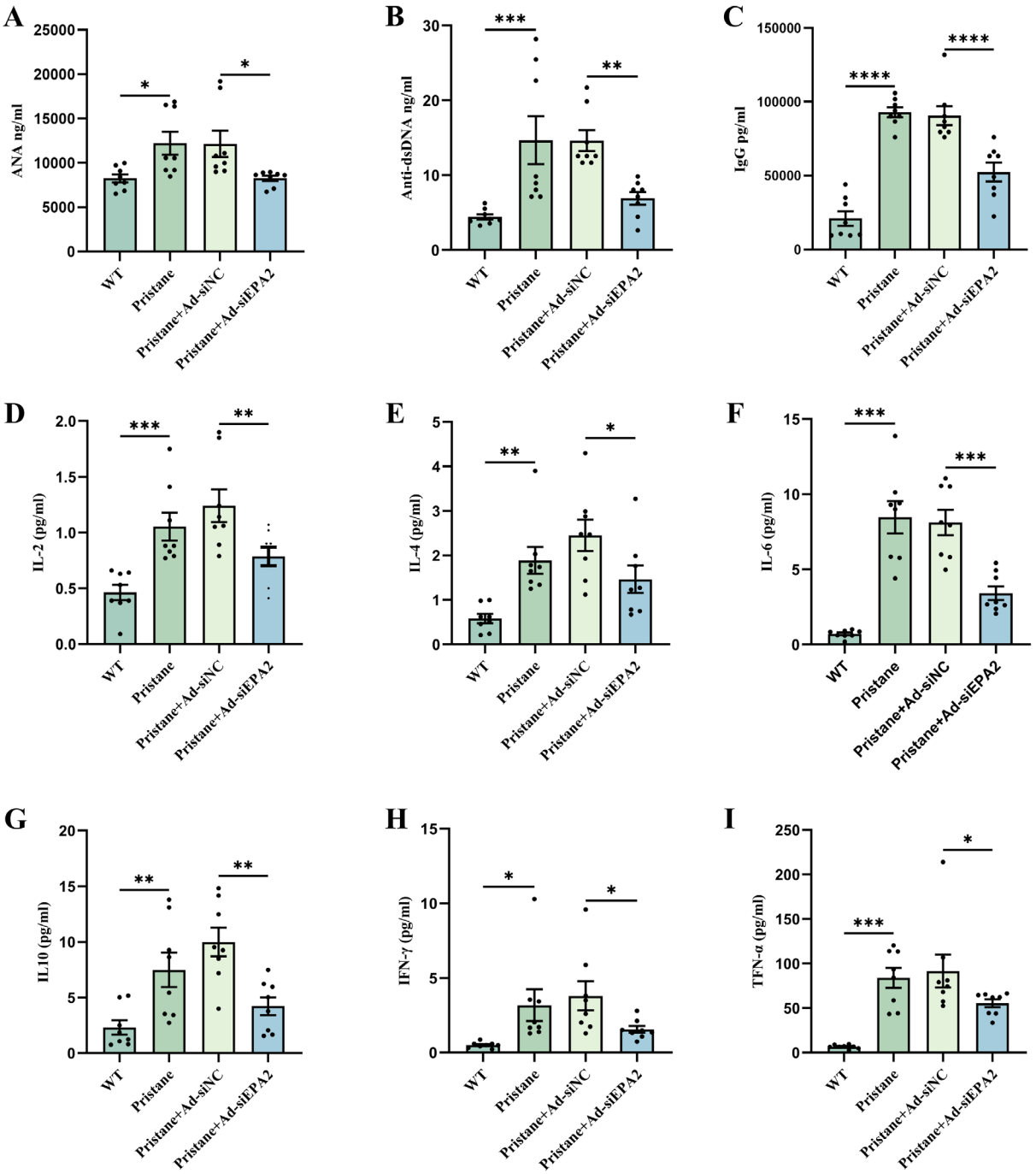

FIGURE 4 | Inhibition of EPA2 reduces autoantibodies and inflammatory cytokines production in lupus mice. (A–C) Concentrations of ANA, anti-dsDNA, and IgG in four groups of mice were measured by ELISA analysis. (D–I) Concentrations of inflammatory cytokines, including IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, in four groups of mice were measured by CBA. Error bars are shown as mean ± standard deviation. 8 mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

the addition of GDF15 to the EPA2-silenced group, the migratory ability of HUVECs was significantly enhanced ($p < 0.05$).

3.10 | Inhibiting EPA2 in HUVECs Reduces the Generation of Lumen-Like Structures

Based on the above-mentioned regulatory effects of EPA2 silencing on HUVECs' proliferation and migration, we discussed the effects of EPA2 on the angiogenesis of HUVECs. The results showed that compared with the empty vector adenovirus group, the number of nodes in the EPA2-silenced group was significantly reduced, and the tube length was significantly shortened ($p < 0.05$, Figure 6F). On the contrary, the addition of GDF15 reversed EPA2 silencing-mediated effects. It is suggested that EPA2 silencing significantly inhibited the generation of lumen-like structures in HUVECs.

4 | Discussion

In the present study, 3 population-based case–control studies were conducted to discuss not only the correlation between

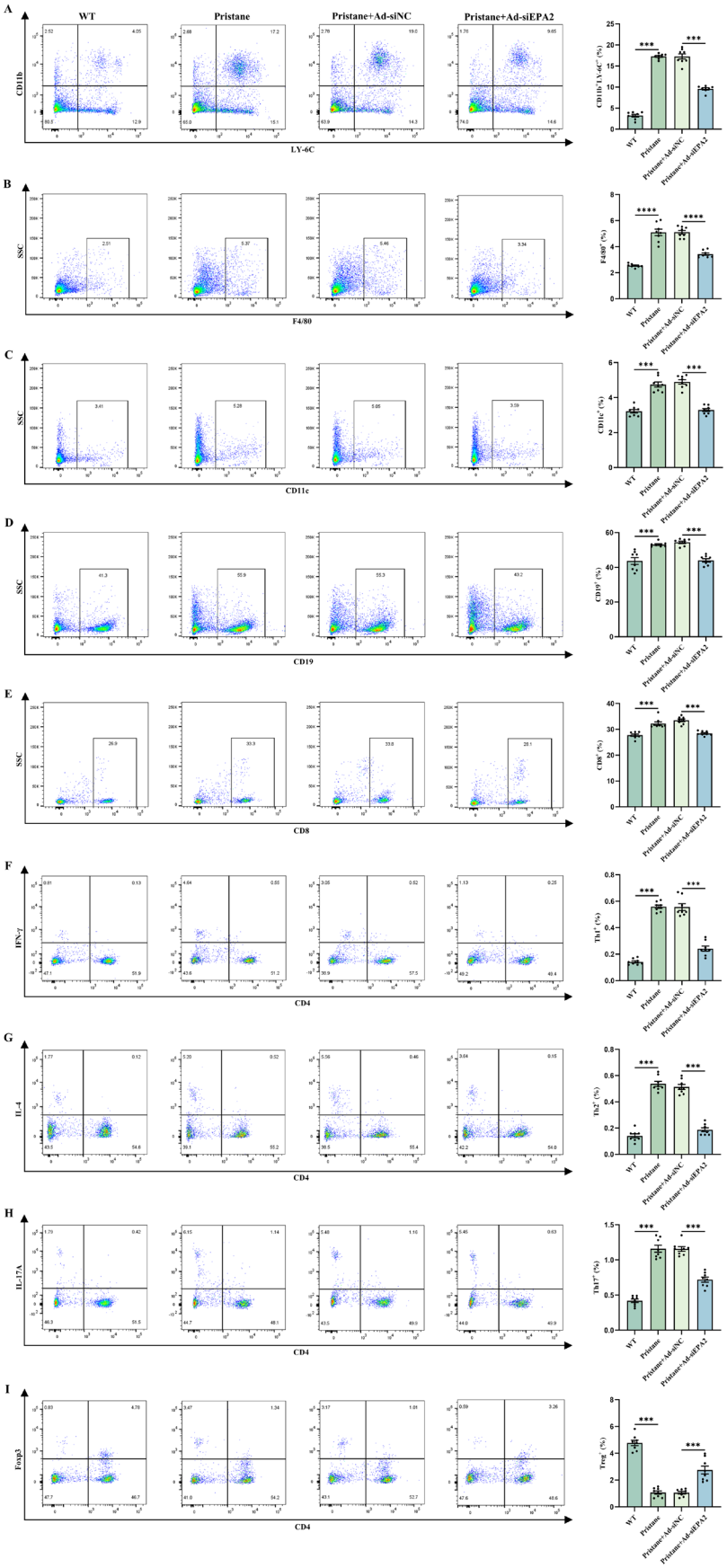

FIGURE 5 | Legend on next page.

FIGURE 5 | Distribution of immune cells in lupus mice after inhibiting EPA2 expression. Expression of different immune cells was analyzed using flow cytometry in four groups of mice, including wild-type mice injected with PBS, pristane-induced lupus mice, and pristane-induced lupus mice injected with adenoviral empty vectors or EPA2 siRNA adenovirus. (A) CD11b-FITC, LY-6C-PE-CY7; (B) F4/80-BV421; (C) CD11c-APC; (D) CD19-PE; (E–I) T cell subsets, and all cell proportions were first labeled with $CD3^+$. Representative flow plots are shown on the left, and statistical analysis of the corresponding immune cells is shown on the right. Error bars show mean ± standard deviation. 8 mice/group. ***$p < 0.005$, ****$p < 0.001$.

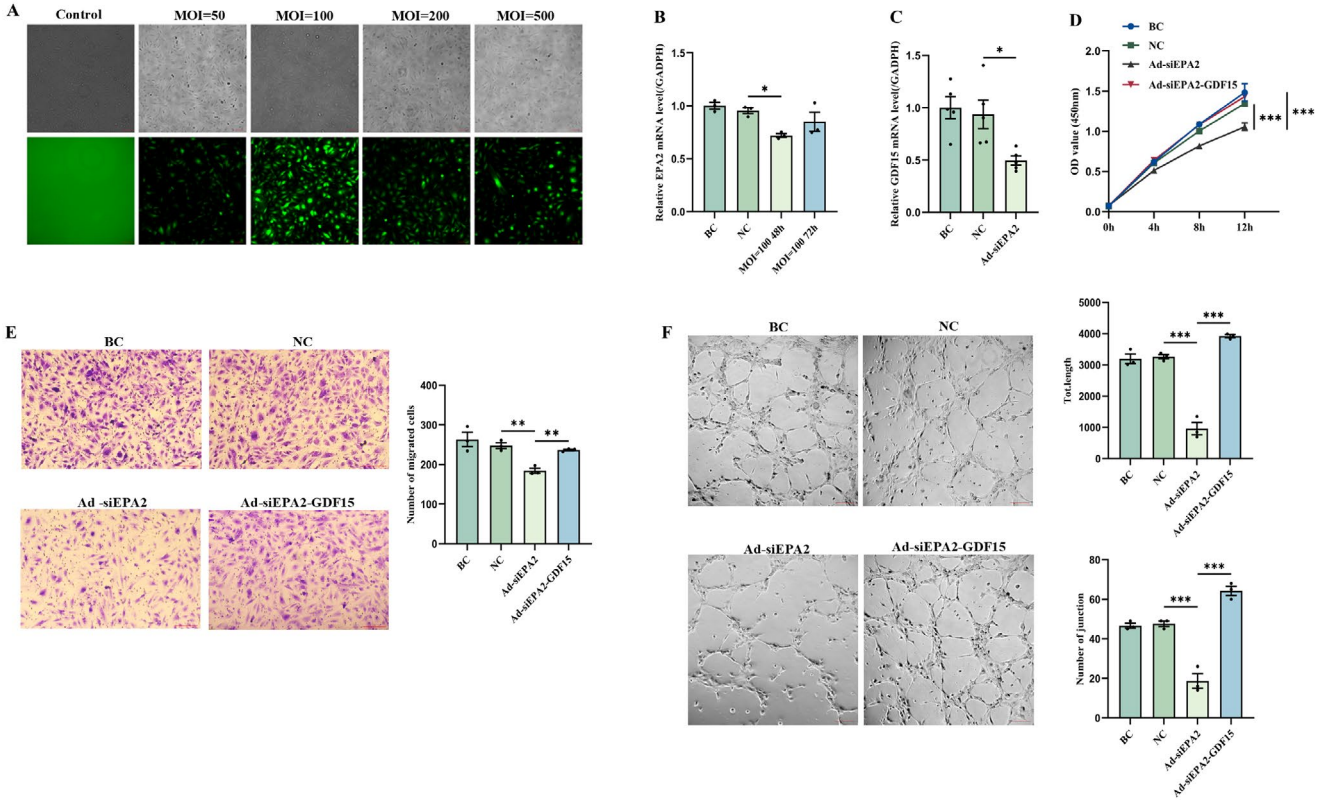

FIGURE 6 | Suppressing EPA2 inhibits proliferation, tube formation, and migration of HUVEC cells. (A) HUVECs were transfected with EPA2 siRNA adenovirus at different titers (MOI = 50, MOI = 100, MOI = 200, MOI = 500), and the transfection efficiency was observed under a fluorescence microscope at 48 h of transfection. (B) The optimal transfection time was detected by the qRT-PCR method ($N = 3$/group). (C) The relative mRNA levels of GDF15 in the control, adenovirus empty vector group, and EPA2 siRNA adenovirus group were detected by the qRT-PCR method ($N = 5$/group). (D) EPA2 siRNA transfection of HUVECs was carried out for 48 h for the proliferation assay. Cells were seeded in 96-well plates, and GDF15 recombinant protein was added; CCK-8 reagent was added after 24 h of incubation, and the absorbance at 450 nm was measured by an enzyme marker ($N = 5$/group). (E) HUVECs were transfected with EPA2 siRNA adenovirus, and a transwell assay was carried out and photographed after 48 h. Scale bar: 200 μm. For each experiment, 3 fields of view were randomly selected for cell counting, and the mean value was taken for statistical analysis. Each experiment was repeated 3 times. (F) HUVECs were transfected with EPA2 siRNA adenovirus to knock down EPA2 expression, and tube-forming experiments were performed at 48 h of transfection and photographed after 8 h. Scale bar: 200 μm. The total length of tube formation and the number of nodes were statistically analyzed. Each experiment was repeated 3 times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

plasma EPA2 levels and SLE pathogenesis but also the association between peripheral blood EPA2 mRNA levels and SLE risk. The role of EPA2 in the development of lupus was further demonstrated by the establishment of a lupus mouse model, in which we explored the role of EPA2 in the development of lupus by treating lupus mice with EPA2 siRNA adenovirus. The results showed that both plasma and mRNA levels of EPA2 were higher in SLE patients, and silencing EPA2 significantly reduced kidney weight, slowed down kidney injury, and restored immune cell abnormalities. In in vitro experiments we demonstrated that EPA2 is associated with angiogenesis and may be involved in angiogenesis through regulating GDF15.

Dysregulation of T and B cell activation is a major feature of SLE. EPA2 is the only Endophilin family member expressed in hematopoietic cells, which is important for B cell-mediated humoral immunity and pathology [26, 27]. EPA2 can abnormally activate self-reactive T cells by regulating T cell receptor internalization, triggering, and driving RA. EPA2 deficiency was protective against autoimmune encephalomyelitis [15, 28]. Since SLE is also an autoimmune disease, we speculated that EPA2 might be involved in the pathogenesis of SLE. Our study confirmed that plasma EPA2 levels were much higher in SLE patients, and clinical subgroup analysis revealed that EPA2 expression was associated with a number

of indicators associated with acute kidney injury, such as tubular urine, hematuria, proteinuria, and pyuria. Our study also found a negative correlation between C3, C4, and EPA2 expression in SLE patients. Interestingly, EPA2 expression was significantly correlated with SLEDAI score. Plasma EPA2 levels could differentiate between SLE patients and healthy controls. In addition, we found that peripheral blood EPA2 mRNA levels were significantly higher in SLE patients than in healthy controls, and peripheral blood EPA2 mRNA showed the ability to differentiate SLE from healthy controls. All these suggest that EPA2 may be associated with SLE pathogenesis and may be a biomarker to distinguish SLE patients from healthy individuals. The diagnosis and treatment of SLE are highly dependent on biomarkers, but existing markers have significant shortcomings in terms of specificity and sensitivity. For example, the specificity of ANA is not high, the sensitivity and specificity of anti-dsDNA are not enough, and the levels of complement C3 and C4 are correlated with disease activity and may be affected by other factors such as infection. Therefore, there is a need to find new biomarkers to improve the efficiency and accuracy of early diagnosis of SLE. EPA2 has a good diagnostic ability with sensitivity and specificity of 0.827 and 0.877, respectively.

To further demonstrate the role of EPA2 in SLE pathogenesis, lupus mice were injected with EPA2 siRNA adenovirus. The results showed that EPA2 silencing could attenuate kidney injury and reverse the abnormality of immune cells, autoantibodies, and inflammatory cytokines in lupus mice. Interestingly, pristane-induced lupus mice exhibited enlarged livers, spleens, and kidneys, as well as renal injuries. The hepatomegaly, splenomegaly, and nephrometastasis were reduced in the lupus mice after silencing EPA2, and glomerulonephric necrosis was reduced. Renal lymphocyte infiltration was inhibited, and IgG deposition was reduced in lupus mice injected with EPA2 siRNA adenovirus. EPA2 silencing also significantly reduced ANA, IgG, and anti-dsDNA autoantibodies production in lupus mice. This confirms the role of EPA2 in promoting renal immune complex deposition. Some studies showed that EPA2 was positively correlated with the expression of IL-6 and TNF-α [29, 30]. It is well recognized that an imbalance of these pro-inflammatory factors is associated with SLE disease activity. In this study, lupus mice exhibited high levels of inflammatory cytokines, including IL-6 and TNF-α. After injecting with EPA2 siRNA adenovirus, the lupus mice showed down-regulated levels of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFNγ. The findings suggest that silencing EPA2 inhibits the production of autoantibodies and inflammatory cytokines. In pristane-induced lupus mice, the frequencies of $CD11b^+LY\text{-}6C^+$, $F4/80^+$, $CD11c^+$, $CD19^+$, $CD8^+$, $Th1^+$, $Th2^+$, and $Th17^+$ cells were higher, and the frequency of Treg cells was lower. It is known that the imbalance of these cells promotes the pathogenesis of SLE. When injecting with EPA2 siRNA adenovirus in lupus mice, the imbalance of the immune cells was reversed. Interestingly, there are some potential off-target effects of EPA2 inhibition. First, EPA2 has homology with EPA1 and EPA3, and its function may be interfered with if the sequence is silenced. Second, EPA2 is involved in synaptic vesicle recycling and membrane remodeling, and its silencing may affect neuronal synaptic transmission, leading to behavioral abnormalities or cognitive impairment. Third, EPA2 is associated with apoptosis regulation, and silencing EPA2 may lead to unintended cell death or abnormal proliferation by affecting mitochondrial membrane stability or caspase activation pathways. Fourth, EPA2 may indirectly regulate other pathways, leading to aberrant activation of signaling such as MAPKs and PI3K/AKT after EPA2 silencing.

To date, some studies have shown that the pathogenesis of cardiovascular diseases such as atherosclerosis, myocardial infarction, and heart failure may relate to insufficient angiogenesis. EPA2 may block subcutaneous deposition of oxLDL proteins when treating mice with atherosclerosis. EPA2 has a protective effect on cardiac function in ischemia-induced injury by attenuating cardiomyocyte apoptosis and decreasing the endoplasmic reticulum stress in response to myocardial infarction injury [31]. EPA2 may promote angiogenesis and may be protective against cardiovascular disease, suggesting a potential role in vascular function [32]. However, excessive angiogenesis may promote the development of autoimmune diseases and tumors. In glioblastoma, EPA2 may lead to activation of the MAPK cascade response and result in altered expression of genes associated with cell proliferation [33]. Reduced EPA2 expression was effective in inhibiting the phosphorylation activation of the AKT/GSK-3b/FAK signaling pathway and regulating cell proliferation [14]. A study found that silencing EPA2 reduced cancer cell migration [13]. In addition, a study showed that EPA2 promotes endothelial cell migration and angiogenesis. SLE pathogenesis was related to angiogenesis. Thus, we discussed whether EPA2 may regulate HUVECs function. Moreover, our previous study found that GDF15 correlated with SLE pathogenesis, and GDF15 may induce angiogenesis [29]. Therefore, in this study, we also explored whether EPA2 plays a role in angiogenesis through regulating GDF15. The results showed that HUVECs transfected with EPA2 siRNA had reduced proliferation, migration, and tube-forming ability, which was enhanced by the addition of GDF15 recombinant protein. It suggests that EPA2 may be involved in angiogenesis through regulating GDF15.

There are some innovations in the study. First, this study explored the plasma levels of EPA2 in SLE patients for the first time, revealing the ability of EPA2 as a diagnostic biomarker for SLE. Moreover, the potential of EPA2 as a biomarker for SLE was further verified by analyzing the expression of EPA2 in PBMCs of SLE patients and another independent cohort discussing EPA2 plasma levels, which will help the early detection and diagnosis of SLE in the future. Second, the functional study of the association between EPA2 and SLE pathogenesis was explored by carrying out animal experiments, which will provide a basis for targeting EPA2 for the treatment of SLE in the future. Third, this study revealed the role of EPA2 in angiogenesis by regulating GDF15, which provides a preliminary basis for future elucidation of whether EPA2 is involved in lupus progression by affecting GDF15.

There are several limitations in this study. First, plasma EPA2 levels correlated with some clinical and laboratory characteristics of SLE patients, and functional studies are needed to investigate how EPA2 modulates these characteristics. Second, in vitro studies have shown that EPA2 may be involved in angiogenesis through regulating GDF15, but whether EPA2 is involved in the

development of lupus through regulating GDF15 in vivo needs to be confirmed. Third, multicenter studies are needed to confirm the potential of EPA2 as a biomarker for SLE.

In summary, our study suggests that EPA2 was associated with the pathogenesis of SLE, EPA2 may be a biomarker for SLE, and EPA2 may be involved in SLE development by regulating GDF15.

Author Contributions

Study conception and design: L.-Q.Y., Y.-Y.L., Y.-Q.W., S.-Y.F., W.-D.X., A.-F.H. Acquisition of data, analysis, and interpretation of data: L.-Q.Y., Y.-Y.L., Y.-Q.W., S.-Y.F., W.-D.X., A.-F.H. Drafting the article: L.-Q.Y., W.-D.X., A.-F.H. Final approval of the version of the article to be published: all authors, and that all authors agree to be accountable for all aspects of the work.

Acknowledgments

The authors have nothing to report.

Ethics Statement

Animal studies were approved by the Animal Ethics Committee of Southwest Medical University (swmu20240050). All animal housing and experiments were conducted in strict accordance with laboratory animal care and use regulations. Population studies were approved by the Ethics Committee of Affiliated Hospital of Southwest Medical University (KY2020180). We certify that the study was performed in accordance with the World Medical Association's Declaration of Helsinki.

Consent

This study was conducted by obtaining written informed consent from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

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