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Panax notoginseng saponins reverse steroid resistance in lupus nephritis: Involvement of the suppression of exosomal P-gp levels from lymphocytes to glomerular endothelial cells

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ABSTRACT

Microangiopathy is the most basic pathological manifestation of lupus nephritis (LN), and glomerular endothelial cells (GECs) injury is an important pathological mechanism. LN patients with microangiopathy are prone to steroid resistance (SR). Our previous studies confirmed that Panax notoginseng saponins (PNS) could reverse SR by downregulating the expression of P-gp in SR lymphocytes of LN mice (SLCsL/S). However, the mechanism of how circulating lymphocytes transmit SR information to GECs and thus affect the efficacy of kidney treatment is not clear. Recent studies have found that exosomes (exos) are an important carrier for intercellular bioactive substance communication. But whether exosomes derived from SLCsL/S mediate SR in GECs and PNS interventions. To solve this problem, Exosomes isolated from SLCsL/S were characterized, and in vitro cell coculture was further conducted to investigate the effect of SLCsL/S-derived exosomes in the SR of GECs and PNS intervention. Sequencing was used to define the exosomal miRNA expression profiling of SR GECs. Moreover, the in vivo experiments were performed through the injection of exosomes extracted from SLCsL/S into the tail vein of mice.

Our research results indicate that exosomes derived from SLCsL/S could transmit SR information to GECs and lead to the aggravation of inflammatory injury through conferring P-gp, which were negated by a P-gp inhibitor. Further, we identified higher levels of exosomal miR-125b-5p from SR GECs were associated with SR in LN and could serve as biomarker for the risk of developing SR. PNS could reverse the SR of GECs and alleviate inflammatory injury by suppressing exosomal P-gp levels from lymphocytes to GECs in vitro and in vivo. However, the specific molecular mechanism by which PNS regulates exosomes has not yet been elucidated, and we need to conduct more in-depth research in the future. Overall, Our findings suggest that exosomal transfer of SLCsL/S derived P-gp confer SR to GECs, and PNS can target exosome communication to reverse SR in LN, which provides new ideas and a scientific basis for improving the clinical efficacy of traditional Chinese medicine in the treatment of refractory LN.

1. Background

Lupus nephritis (LN) is the most common and serious complication of systemic lupus erythematosus (SLE) and is associated with considerable morbidity and even mortality [1]. Microangiopathy is the basic pathological manifestation of LN, which often predicts more serious renal function damage and is considered to be an independent risk factor for poor renal prognosis [2]. The key pathogenesis of LN microangiopathy is the activation and injury of glomerular endothelial cells (GECs) [3,4]. LN patients with microangiopathy have a poor response to treatment and need higher doses of steroids, showing insensitivity to steroids or even steroid resistance (SR) [5].

P-glycoprotein (P-gp), a transmembrane protein with a molecular mass of 170 kDa, is produced by a multidrug resistance gene (MDR1) [6]. P-gp is ATP-dependent and can efflux drug substrates to maintain appropriate intracellular levels. The overexpression of P-gp is one key mechanism contributing to SR [7,8]. Our previous unpublished studies confirmed that silent information regulation factor related enzyme 1

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(SIRT1) in SR splenic lymphocytes of LN mice (SLCsL/S) initiates the transcription of MDR1 by upregulating the nuclear transcription factor forkhead box-containing protein of the O subfamily 1 (FoxO1), resulting in high expression of P-gp. However, the mechanism by which circulating lymphocytes transmitting drug resistance information to GECs producing SR is unknown.

Recent studies have shown that cells can communicate with adjacent or distant cells through the secretion of exosomes (exos) [9–11]. Exosomes expelled from drug-resistant cells expressing high levels of P-gp and can deliver the same pattern of P-gp to sensitive cells, which may be a mechanism of exosome-mediated drug resistance [12–14]. miRNAs, a conserved non-coding RNAs, have been found to be closely related to drug resistance. Secreted miRNAs, especially those in exosomes, have natural advantages due to their stable structure and minimal non-specific interference and may serve as better biomarkers to detect SR in LN [15]. Therefore, SLCsL/S may transmit SR signals (such as P-gp) to GECs to produce drug resistance through exosomes. In addition, exosomal miRNAs secreted by SR GECs may be a biomarker for the risk of developing SR in LN.

Panax notoginseng saponins (PNS) is a chemical component extracted from Panax notoginseng herbal medicine that contains a variety of different monomers, such as notoginsenoside R1, ginsenoside Rg1, and ginsenoside Re [16]. It can anti-inflammatory, antioxidative stress and protects vascular endothelial cells [17–19]. Most notably, PNS has an obvious ability to reverse multiple drug resistance including P-gp-mediated multidrug resistance in many studies regarding animals and cells [20–23]. Our previous studies confirmed that PNS could significantly downregulate the levels of P-gp in patients with LN, increase the accumulation of steroids in cells, and synergistically enhance their immunosuppressive effect, which has more advantages than the P-gp inhibitor verapamil [24]. Previous unpublished studies further confirmed that PNS could reverse SR in SLCsL/S through the SIRT1/P-gp pathway. However, it remains unclear whether PNS can reverse the SR of GECs and alleviate microangiopathy by suppressing exosomal P-gp levels from lymphocytes to GECs.

In this study, we confirmed that P-gp-containing exosomes from SLCsL/S could cause GECs to acquire SR and further aggravate inflammatory injury through conferring P-gp. Exosomal miR-125b-5p from SR GECs could be used as predictors for detecting SR in LN. Further investigation demonstrated that PNS could reverse the SR of GECs and alleviate inflammatory injury by suppressing exosomal P-gp levels from lymphocytes to GECs. This study provides a theoretical basis for PNS in the treatment of refractory LN.

2. Methods

2.1. Establishment and grouping of the animal model

Female NZB/WF1 and ICR mice (specific pathogen free, SPF) aged 16 weeks were acquired from Jackson Laboratory by the iBio Logistics Co., Ltd. (Beijing, China) and the Zhejiang Academy of Traditional Chinese Medicine, respectively, and were housed in accordance with relevant guidelines. All procedures involving mice were approved by the Animal Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (approval number: ZATCM-2019045) and were carried out in accordance with the ARRIVE guidelines.

The SR lupus mouse (SRLM) model was established by the intraperitoneal injection of low-dose Meyhylprednisolone (MP, Pfizer, New York, USA) for eight weeks (0.8 mg kg^{-1}·day^{-1}) according to our previous study [25]. Compared with untreated mice, the criterion for the successful construction of the SRLM model was that the expression of P-gp increased and the accumulation of Rh-123 decreased in the splenic lymphocytes of LN mice (SLCsL) (Supplementary Fig. 1). ICR mice were used as a control group (normal saline, 8 weeks). NZB/WF1 mice were randomly divided into the LN group (normal saline, 8 weeks), SR group (0.8 mg kg^{-1}·day^{-1} MP, 8 weeks), SR with PNS (Guangxi Wuzhou Pharmaceutical Co., Ltd, Guangxi, China) (Supplementary Fig. 2) group (0.8 mg kg^{-1}·day^{-1} MP, 100 mg kg^{-1}·day^{-1} PNS, 8 weeks) and SR with P-gp inhibitor Tariquidar (Tariq, Selleckchem, TX, USA) group (0.8 mg kg^{-1}·day^{-1} MP, 8 mg kg^{-1}·day^{-1} Tariq, 8 weeks).

2.2. Preparation of splenic lymphocytes

After the early intervention, five groups of mice were euthanized through CO_2 asphyxiation using slow displacement of chamber air with compressed CO_2 (25 %/min), and their splenic lymphocytes were extracted, namely, the control group (SLCsC), LN group (SLCsL), SR group (SLCsL/S), SR with PNS group (SLCsL/S + PNS), and SR with Tariq group (SLCsL/S + Tariq). Lymphocytes were isolated and cultured as previously described [26].

2.3. Preparation of GECs

The GECs of ICR and NZB/WF1 mice were separated as previously described [27] and cultured in endothelial cell medium supplemented with 10 % FBS at 37 °C with 5 % CO_2.

2.4. Cell co-culture assay

SLCsC and SLCsL, SLCsL/S, SLCsL/S + Tariq (5×10^5/well) were seeded into the upper chamber of a co-culture system and GECs(3×10^5/well) from ICR and NZB/WF1 mice were placed in the lower chamber and incubated for 48 h. Co-cultured cells were separated using Transwell permeable supports (0.4 μm) in poly carbonate membrane (Costar, Cambridge, MA, USA).

2.5. Isolation of exosomes

Exosomes were isolated by ultracentrifugation as described previously [28]. In brief, the culture supernatants of cells were collected and sequentially centrifuged at 300 g for 10 min, 2000 g for 15 min, and 10, 000 g for 30 min to remove cell debris and large vesicles, and then, the cleared sample was further ultracentrifuged for 2 h at 100,000 g to pellet the exosomes. The extracted exosomes were allocated and stored at −80 °C or used for downstream experiments.

2.6. Transmission electron microscopy (TEM)

The morphology of exosomes was observed by TEM [29]. After absorbing onto Formvarcarbon-coated copper mesh grids, exosomes were negatively stained with aqueous phosphotungstic acid for 1–2 min. After air dry, samples were visualized with a Hitachi 7100 transmission electron microscope.

2.7. Nanoparticle-tracking analysis (NTA)

We measured the exosome particle size and concentration using nanoparticle tracking analysis (NTA) at VivaCellBiosceinces with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) [30]. Isolated exosome samples were appropriately diluted using 1× PBS buffer (Biological Industries, Israel) to measure the particle size and concentration. NTA measurement was recorded and analyzed at 11 positions. The ZetaView system was calibrated using 110 nm polystyrene particles. Temperature was maintained around 23 °C and 30 °C.

2.8. Western blot analysis

Exosomes were collected, and total protein was extracted and quantified using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Next, the immunoreactive proteins were detected using the Simon™ machine (ProteinSimple, San Jose, USA). The primary and secondary antibodies used were the following: rabbit anti-

CD63 antibody (Abcam, USA), anti-TSG101 antibody (Abcam), anti-Calnexin antibody (Abcam), anti-P-gp antibody (Abcam) and anti-β-actin antibody (Cell Signaling Technology, USA), anti-rabbit IgG (ProteinSimple). Quantization of detected proteins and image preparation were performed with Compass Software (ProteinSimple).

2.9. Exosome internalization

Exosomes were labeled with the green lipophilic fluorescent dye PKH67 (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions. After coculture with PKH67-stained exosomes for 24 h, GECs were stained with DAPI (Abcam) for the nucleus and phalloidin (Abcam) for the intracellular cytoskeleton F-actin according to the instruction manual and observed under a confocal laser microscope.

2.10. Flow cytometry

GECs were trypsinized and washed twice in 1 × PBS. After resuspension in 100 μl of 1 × PBS, FITC-conjugated antibodies against P-gp (Abcam) and Rh-123 (Sigma) or their respective isotype controls were stained for 30 min at 4 °C. Following washing twice in 1 × PBS, labeled cells were measured by flow cytometry (BD Biosciences) and analyzed with FlowJo software (Tree Star).

2.11. Dexamethasone accumulation

After exosomes intervention, GECs were washed twice in 1 × PBS, and then incubated with 3.0×10^{-8} M ^{3}H-labeled dexamethasone for 20 min at 37 °C. The intracellular dexamethasone concentration was counted with a scintillation counter, according to previously study [31].

2.12. GECs exosomal miRNA sequencing

The total RNA was extracted using the Total RNA Purification Kit (LC Sciences, Houston, USA), according to the manufacturer's protocol. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. Then, the first strand cDNA was synthesized and PCR amplification was performed according to protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA), and Samll RNAs were used for library preparation. Finally, we performed the single-end sequencing (1 × 50 bp) on an Illumina Hiseq2500 at the LC-BIO (Hangzhou, China) following the vendor's recommended protocol [32].

2.13. Animal experiments

To determine the role of SLCsL/S exosomes and PNS in the SR and inflammatory injury of GECs in vivo, 16-weeks-old female ICR and NZB/WF1 mice were injected via the tail vein with exosomes from lymphocytes. ICR mice were used as a control group: SLCsC-exo group (n = 6, SLCsC-derived exosomes treatment). NZB/WF1 mice were randomly divided into four groups: SLCsL-exo group (n = 6, SLCsL-derived exosomes treatment), SLCsL/S-exo group (n = 6, SLCsL/S-derived exosomes treatment), SLCsL/S + PNS-exo group (n = 6, SLCsL/S + PNS-derived exosomes treatment), and SLCsL/S + Tariq-exo group (n = 6, SLCsL/S + Tariq-derived exosomes treatment). All the groups were injected five times every week for three consecutive weeks and then the serum, urine, and renal cortex samples were harvested for further analysis.

2.14. Enzyme-linked immunosorbent assay (ELISA)

GECs supernatant and serum samples were centrifuged at 3500×*g* for 10 min at 4 °C. The concentrations of IL-1β, IL-6, MCP-1, VCAM-1, ANA and anti-dsDNA were detected by using ELISA kits according to the manufacturer's instructions.

2.15. Real-time PCR analysis

The total RNA of exosomes, GECs and tissues was isolated by using E.Z.N.A total RNA kit (Omega Biotech Inc., Norcross, GA, USA) according to the manufacturer's protocol and converted to cDNA with the PrimeScriptTM RT reagent kit (Takara, Dalian, China). Quantitative real-time PCR was performed using SYBR-green mix (TaKaRa) and run on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression levels of mRNA and miRNA were normalized against β-actin and U6, analyzed using the $2-\Delta Ct$ method.

2.16. Hematoxylin and eosin (HE) staining and immunohistochemical analysis (IHC)

To determine renal histopathology, 3-μm-thick paraffin sections of the kidney were stained with hematoxylin and eosin (HE). Renal histology was assessed blindly by an experienced pathologist. Renal histopathological changes were quantitated as described previously [33].

After deparaffinization and rehydration, paraffin sections were blocked with Dual Endogenous Enzyme Block (DAKO, Glostrup, Denmark) for IHC. The primary antibody was rabbit anti-P-gp (or IL-1β, IL-6, MCP-1, VCAM-1) IgG (Abcam). The secondary antibody was polymer-horseradish peroxidase-labeled goat anti-rabbit IgG (Abcam). Finally, sections were incubated with 3, 3′-diaminobenzine-chromogen substrate (DAKO) and counterstained with hematoxylin. The stains were scored by a renal pathologist blinded to the mice grouping.

2.17. Serum and urine analyses

The levels of 24 h urinary protein (24 h Upro), serum creatinine (Scr) and nitrogen concentration (BUN) were assessed using commercially available kits (Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's directions.

2.18. Statistical analysis

The analysis process was performed by SPSS 22.0 software (SPSS Inc., Chicago, IL, USA), and measurement data consistent with a normal distribution are expressed as the mean ± standard deviation. Student's t-test and One-way ANOVA were used to compare differences between two or more groups. A value of $P < 0.05$ indicated statistically significant differences.

3. Results

3.1. SLCsL/S induces the SR of GECs

The levels of P-gp and its transport substrate rhodamine 123 (Rh-123) are the main marker to judge SR [34]. To determine whether SLCsL/S can confer SR to GECs, we cocultured GECs with SLCsL/S in a Transwell insert, which prevents direct cell-to-cell contact. The results showed a significant increase in P-gp expression and a decrease in Rh-123 accumulation in cells cocultured with SLCsL/S compared with those cocultured with SLCsL and SLCsC (Fig. 1). In a word, these data indicated that SLCsL/S prompted the development of SR in GECs.

3.2. Lymphocyte-derived exosomes can be internalized by GECs and PNS downregulates the levels of P-gp in exosomes by SLCsL/S

Many studies have manifested that exosomes could mediate SR by transporting P-gp. To explore the function of SLCsL/S-derived exosomes on SR of GECs, exosomes from lymphocytes were isolated and identified firstly. TEM showed the presence of cup- or sphere- shaped morphology with double-membrane structures (Fig. 2A). The observation of NTA indicated that the diameter of isolated exosomes was 128 nm, and the concentration was 1.3×10^{12} particle/ml (Fig. 2B). WB verified the

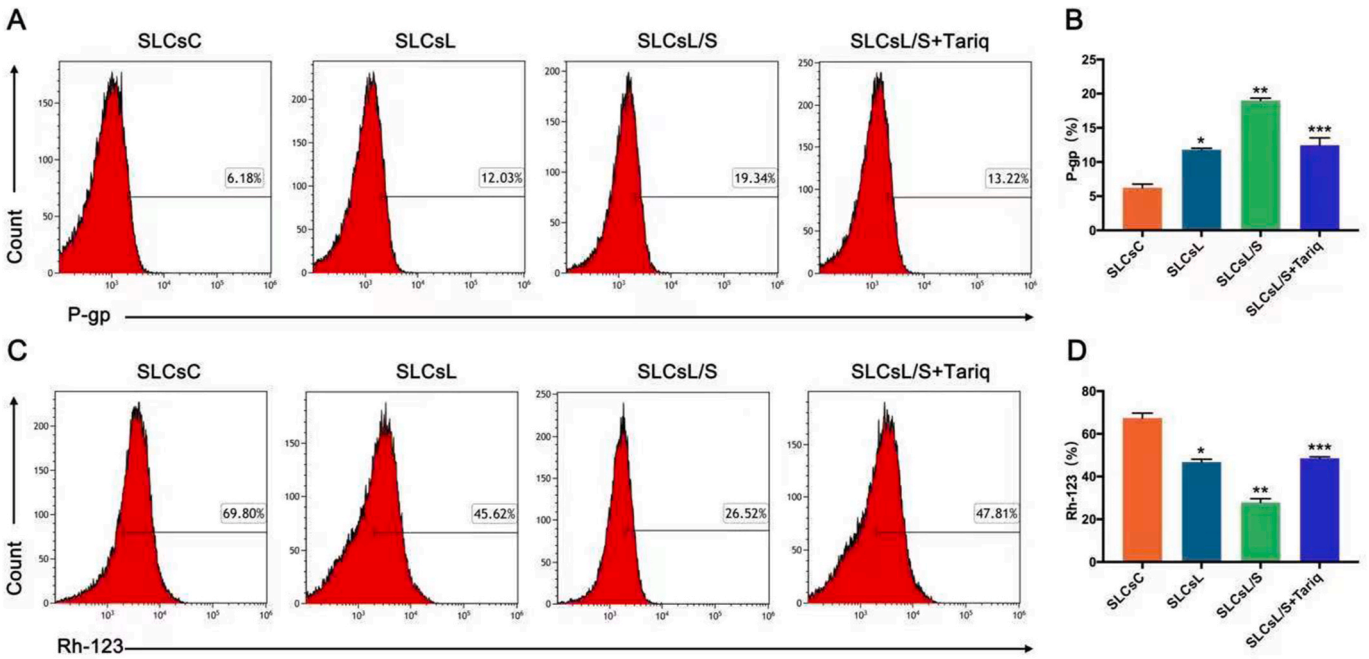

Fig. 1. Effect of SLCsL/S on the SR of GECs. (A) P-gp expression in GECs was measured by flow cytometry. (B) Analysis of P-gp expression. (C) Rh-123 accumulation in GECs was measured by flow cytometry. (D) Analysis of the intracellular accumulation of Rh-123. Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC. **P < 0.05 vs. SLCsL. ***P < 0.05 vs. SLCsL/S.

expression of the marker proteins CD63 and TSG101 in the exosomes (Fig. 2C).

In order to demonstrate lymphocyte derived exosomes could be taken in by GECs, exosomes were firstly labeled with PKH67, a fluorescent cell linker compound, which shows strong green fluorescence under the fluorescence microscope and cocultured with GECs for 24 h. After stained nucleus and cytoskeleton F-actin with DAPI and phalloidin, the uptake of lymphocyte derived exosomes by GECs was visualized with confocal microscopy. The results revealed that PKH67-labeled lymphocyte-exos were mainly localized in the cytoplasm of GECs, indicating that lymphocyte-exos could be taken up by GECs (Fig. 2D).

To determine the impact of PNS on P-gp expression in SLCsL/S-derived exosomes, the exosomal P-gp levels were measured. We found that compared with SLCsL-exos and SLCsC-exos, the expression of P-gp in SLCsL/S-exos increased, while PNS and Tariq significantly downregulated the P-gp levels in SLCsL/S-exos (Fig. 2E and F).

3.3. SLCsL/S-derived exosomes induce the SR and aggravate inflammatory injury in GECs and PNS intervention in vitro

To investigate the functional roles of SLCsL/S-exos in the SR and inflammatory injury of GECs, GECs were incubated with exosomes purified from SLCsC, SLCsL or SLCsL/S for 48 h. First, we tested the expression of P-gp and the accumulation of Rh-123 in GECs by flow cytometry. The results show that, compared with SLCsC-exos and SLCsL-exos, the expression of P-gp was upregulated and the accumulation of Rh-123 was decreased in GECs treated with SLCsL/S-exos (Fig. 3A–D). Furthermore, we demonstrated that intracellular dexamethasone levels in GECs incubated with SLCsL/S-exos were significantly decreased (Fig. 4A). Second, ELISA and RT–PCR were performed to detect the levels of IL-1β, IL-6, MCP-1 and VCAM-1 in GECs. As shown in Fig. 4B and C, incubation with exosomes released by SLCsL/S resulted in increased expression of IL-1β, IL-6, MCP-1 and VCAM-1 in GECs at both the mRNA and protein levels compared to the SLCsC and SLCsL.

To identify differentially expressed miRNAs that change during SR, miRNA profiles assay was performed in exosomes derived from GECs treated with SLCsL-exos and SLCsL/S-exos using Illimina NextSeq 500 technology. After comparing the miRNA profiles of the two groups, there were 3 significantly upregulated miRNAs and 3 significantly downregulated miRNAs(Fig. 4D). We chose two upregulated miRNAs for validation using RT-PCR. The results confirmed significant expression of miR-125b-5p but not miR-128-3p (Fig. 4E and F).

To further determine whether PNS could reverse the SR of GECs induced by SLCsL/S-exos to alleviate the inflammatory injury of GECs, GECs were cocultured with SLCsL/S + PNS-exos for 48 h. The data showed that the expression of P-gp was downregulated and the accumulation of Rh-123 was increased in GECs treated with SLCsL/S + PNS-exos and SLCsL/S + Tariq-exos compared with SLCsL/S-exos, and PNS could increase the levels of intracellular dexamethasone in GECs (Fig. 3A–D). Meanwhile, the protein and mRNA levels of IL-1β, IL-6, MCP-1 and VCAM-1 were decreased in GECs treated with SLCsL/S + PNS-exos and SLCsL/S + Tariq-exos compared with SLCsL/S-exos (Fig. 6A and B).

Taken together, these results demonstrated that exosomes secreted by SLCsL/S induced GECs to acquire SR to aggravate inflammatory injury through conferring P-gp, whereas PNS could reverse the SR of GECs induced by SLCsL/S-exos and alleviate the inflammatory injury by suppressing exosomal P-gp levels from lymphocytes to GECs in vitro.

3.4. SLCsL/S-derived exosomes induce the SR and aggravate inflammatory injury in GECs and PNS intervention in vivo

We next sought to explore the effect of SLCsL/S-derived exosomes on the SR of GECs and inflammatory injury in vivo through the injection of exosomes extracted from SLCsL/S into the tail vein of mice. The results of HE staining showed that GECs proliferated with lymphocyte infiltration in the mice injected with SLCsL/S-exos compared with SLCsC-exos and SLCsL-exos, while SLCsL/S + PNS-exos and SLCsL/S + Tariq-exos could reduce the proliferation of GECs and the infiltration of lymphocytes (Fig. 5A). IHC and RT-PCR results showed that the protein and mRNA levels of P-gp, IL-1β, IL-6, MCP-1 and VCAM-1 were significantly increased in the mice injected with SLCsL/S-exos, but these indicators were decreased when treated with SLCsL/S + PNS-exos and SLCsL/S + Tariq-exos (Fig. 5A and 6A). The levels of 24 h Upro, Scr, BUN, ANA and

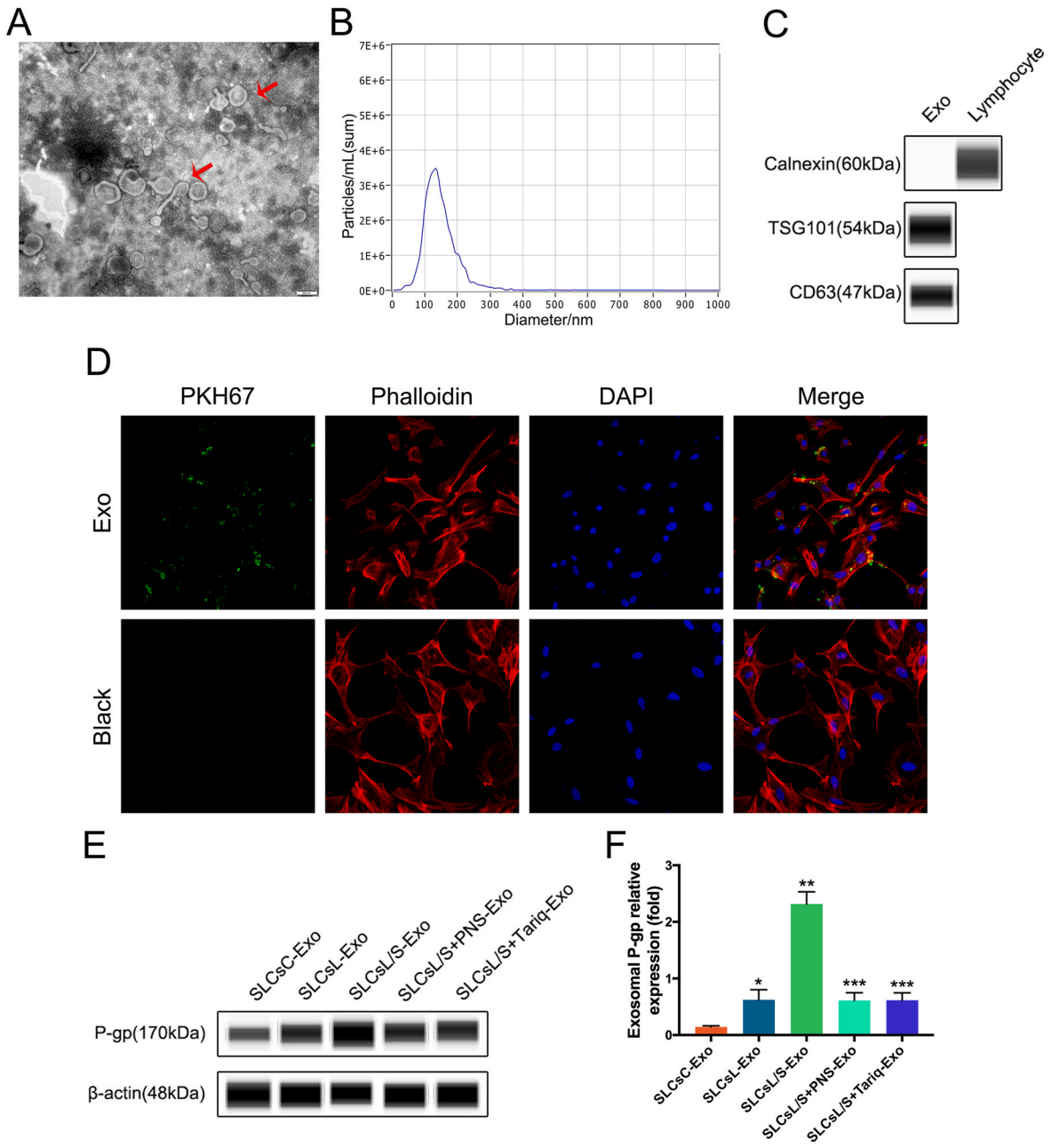

Fig. 2. Internalization of lymphocyte-derived exosomes by GECs and the effect of PNS on exosomal P-gp expression. (A) Lymphocyte-derived exosomes were identified by TEM. Magnification: × 150,000. Scale bar: 200 nm. (B) The particle diameter (nm) of the exosomes. (C) Protein blot of exosomes extracted from lymphocytes using the calnexin antibody, CD63 antibody and TSG101 antibody. (D) The uptake of PKH67-labeled lymphocyte-exos was evident in GECs after 24 h of incubation. No stain was observed in the negative control condition. Scale bars: 20 μm. (E) P-gp expression in exosomes extracted from SLCsC, SLCsL, SLCsL/S, SLCsL/S + PNS and SLCsL/S + Tariq was measured by WB (β-actin was used as an internal reference). (F) Analysis of P-gp expression. Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC-exo. **P < 0.05 vs. SLCsL-exo. ***P < 0.05 vs. SLCsL/S-exo.

dsDNA were increased in the mice injected with SLCsL/S-exos, while treatment with SLCsL/S + PNS-exos and SLCsL/S + Tariq-exos could reduce their levels (Fig. 6B–F).

In summary, our data show that exosomes secreted by SLCsL/S induce kidney-acquired RS to aggravate microangiopathy, whereas PNS could reverse the SR induced by SLCsL/S-exos and alleviate kidney injury through reducing exosomal P-gp levels from lymphocytes to GECs in vivo.

4. Discussion

LN patients with microangiopathy are prone to SR. GECs are the main effector cells of LN microangiopathy. Our previous studies found that there was P-gp-mediated SR in SLCsL, but the mechanism of how lymphocytes transmitted drug resistance information to GECs to produce SR is not clear. Several studies have indicated the marvelous roles of exosomes in drug resistant by conferring functional protein including P-gp [35]. However, whether the exosomes of circulating SR lymphocytes can carry P-gp into GECs and induce SR in GECs has not been explored. In the present study, we first showed that SLCsL/S can transmit SR information to GECs. Then, we further found that exosomes derived from SLCsL/S expressed higher levels of P-gp than those derived from SLCsC and SLCsL, and these exosomes could be taken up into GECs. Exosomes extracted from SLCsL/S, but not from SLCsC and SLCsL, could

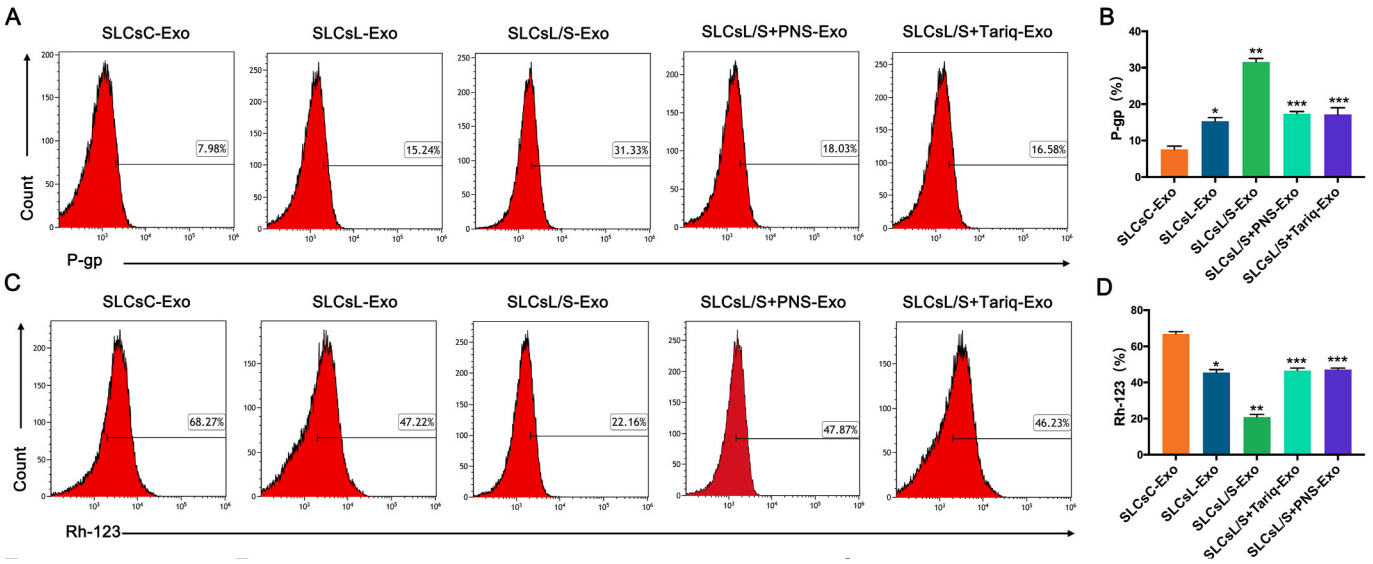

Fig. 3. Effect of SLCsL/S-derived exosomes on SR in GECs and PNS intervention in vitro. (A) P-gp expression in GECs exposed to exosomes was measured by flow cytometry. (B) Analysis of P-gp expression. (C) Rh-123 accumulation in GECs exposed to exosomes was measured by flow cytometry. (D) Analysis of the intracellular accumulation of Rh-123. Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC-exo. **P < 0.05 vs. SLCsL-exo. ***P < 0.05 vs. SLCsL/S-exo.

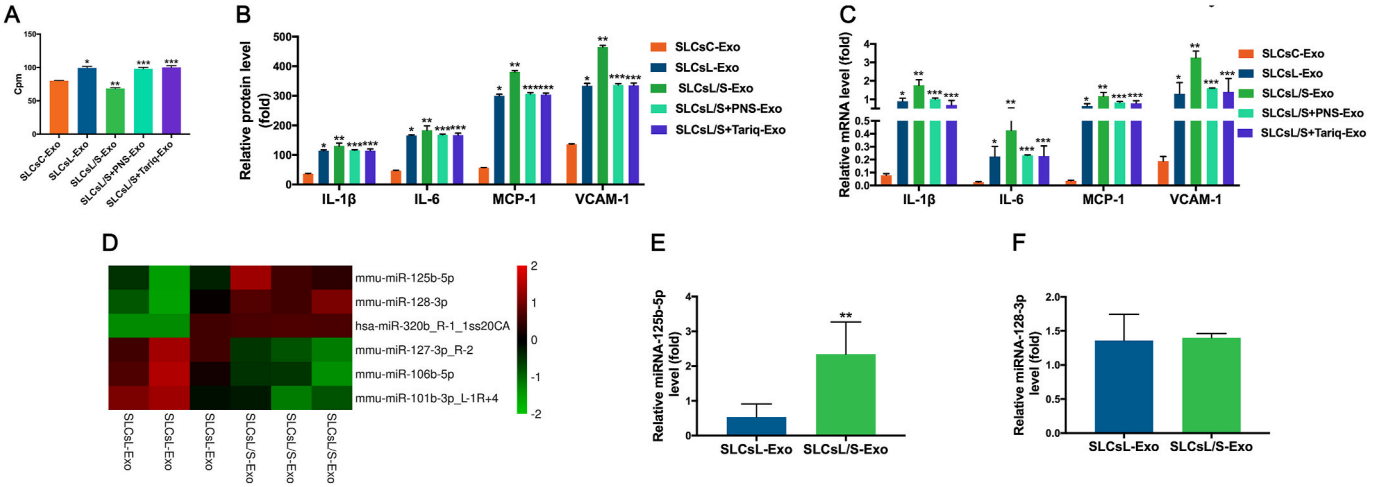

Fig. 4. Effect of SLCsL/S-derived exosomes on inflammatory injury in GECs and PNS intervention in vitro. (A) Intracellular dexamethasone levels in GECs exposed to exosomes were evaluated by scintillation counter. (B,C) Extracellular IL-1β, IL-6, MCP-1 and VCAM-1 were determined after GECs were treated with exosomes for 48 h by ELISA and RT–PCR. (D) Heat map showing the significantly upregulated and downregulated miRNAs in the exosomes derived from GECs after treated with SLCsL-exo and SLCsL/S-exo. (E, F) RT–PCR validation of miRNAs in exosomes from GECs after treated with SLCsL-exo and SLCsL/S-exo. Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC-exo. **P < 0.05 vs. SLCsL-exo. ***P < 0.05 vs. SLCsL/S-exo.

confer SR to GECs by the delivery of P-gp both in vitro and in vivo. Furthermore, intracellular levels of dexamethasone were found to decrease significantly in GECs treated with SLCsL/S-Exos. Moreover, our study found that when GECs acquired SR in vivo and in vitro, their inflammatory injury will be aggravated, and they will secrete more proinflammatory cytokines.

Extensive studies indicated that dysregulation of exosomal miRNA expression has been associated with SR, which show significant potential as first biomarkers for SR and can be highly stable in body fluids [36]. To our knowledge, this is the first study investigating exosomal miRNA profiling of SR GECs in LN mice. The results showed that 6 miRNAs were found to be significantly differentially expressed in the exosomal from SR GECs(3 upregulated and 3 downregulated). After validation by RT-PCR, miR-125b-5p was observed to be significantly upregulated. Therefore, exosomal miR-125b-5p can be used as predictors of steroids treatment efficacy and the patient's risk of developing SR.

To find effective substances to reverse SR, a growing number of researchers have focused their research on the exploration of the active ingredients from natural sources that have the ability to restore relevant indicators to normal levels. Many active components of traditional Chinese medicine have been shown to reverse drug resistance by restraining P-gp expression [37–40]. Our previous studies showed that PNS could inhibit the expression of P-gp without significant cytotoxicity. Taken together, that PNS might have great potential to reverse SR needed to be explored, and thus, it is logical to hypothesize that PNS could reverse the SR of GECs through inhibiting the exosome-mediated intercellular transfer of P-gp in the SLCsL/S-to-GECs direction.

In this study, we first determined the effect of PNS and Tariq on exosomal P-gp levels. The results showed significantly decreased P-gp

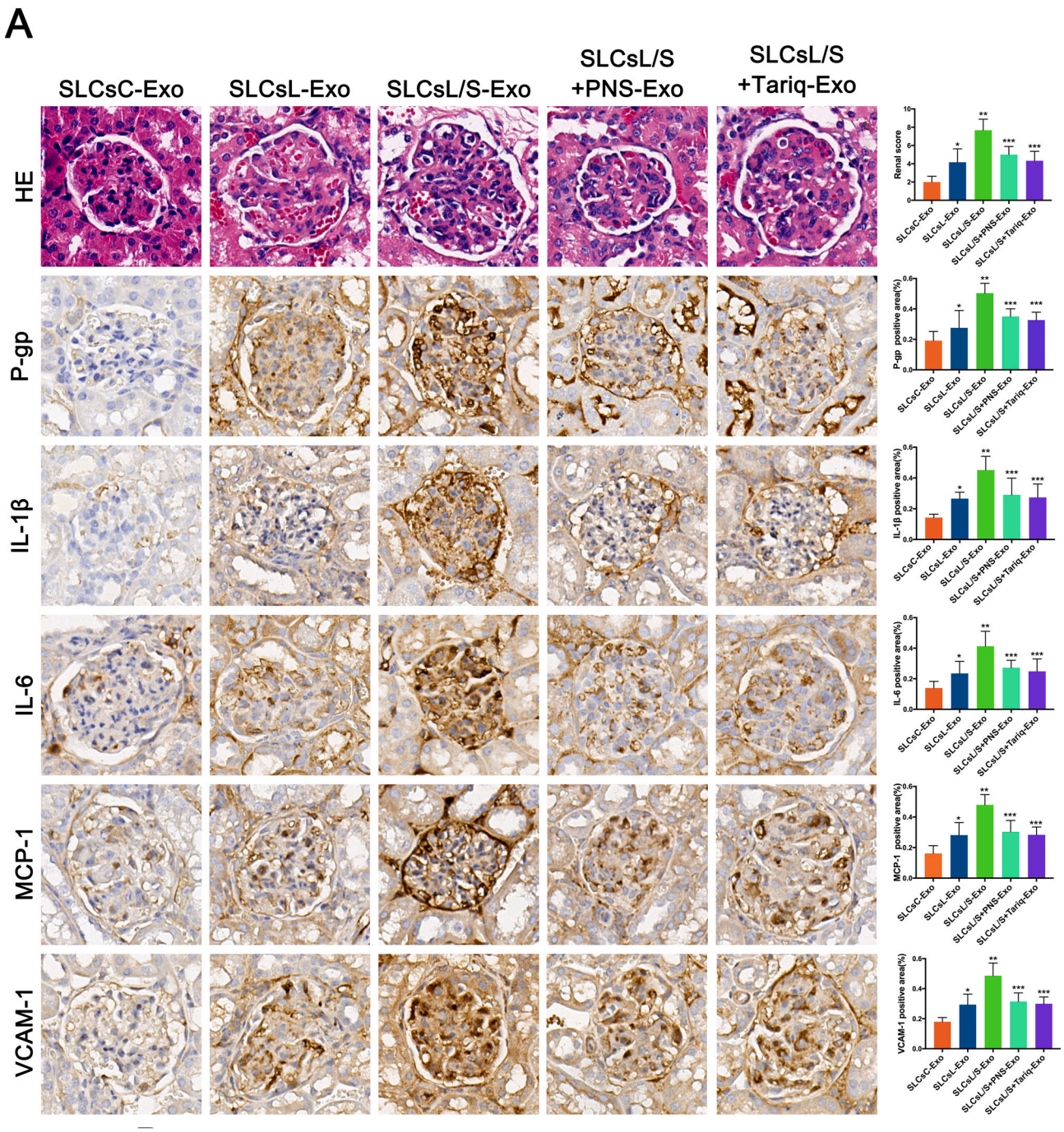

Fig. 5. Effect of SLCsL/S-derived exosomes on SR in GECs and PNS intervention in vivo. (A) Representative photograph for HE and IHC. Scale bars: 50 μm.Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC-exo. **P < 0.05 vs. SLCsL-exo. ***P < 0.05 vs. SLCsL/S-exo.

expression in the exosomes extracted from SLCsL/S treated with PNS and Tariq. The following in vitro experiments further confirmed that compared with SLCsL/S, the expression of P-gp was downregulated and the accumulation of Rh-123 and intracellular dexamethasone were increased in GECs treated with exosomes derived from SLCsL/S + PNS and SLCsL/S + Tariq, and the levels of secreted proinflammatory factors were also reduced accordingly. This shows that PNS could reverse the SR of GECs by inhibiting the expression of P-gp in SLCS/MP-derived exosomes to alleviate inflammatory injury. This conclusion has also been further verified by in vivo experiments. In the present study, we showed that, compared with SLCsL/S-derived exosome intervention, PNS- and Tariq-treated exosomes could reduce the expression of P-gp and proinflammatory-related factors in renal tissue. At the same time, SLCsL/S exosomes treated with PNS and Tariq also alleviated renal pathological injury in mice and reduced 24-h urinary protein, Scr, BUN, ANA and dsDNA.

5. Conclusions

In summary, this is the first report of a P-gp transferred from lymphocytes to GECs by the means of exosomes. Our previous data demonstrate that P-gp-enriched exosomes produced by SLCsL/S may be critical for SR of GECs in LN. Exosomal miR-125b-5p from SR GECs may serve as biomarkers in SR of LN and can be used as predictors of prognosis and steroids treatment efficacy. Meanwhile, we raise the possibility that PNS may be of therapeutic value for treating SR and

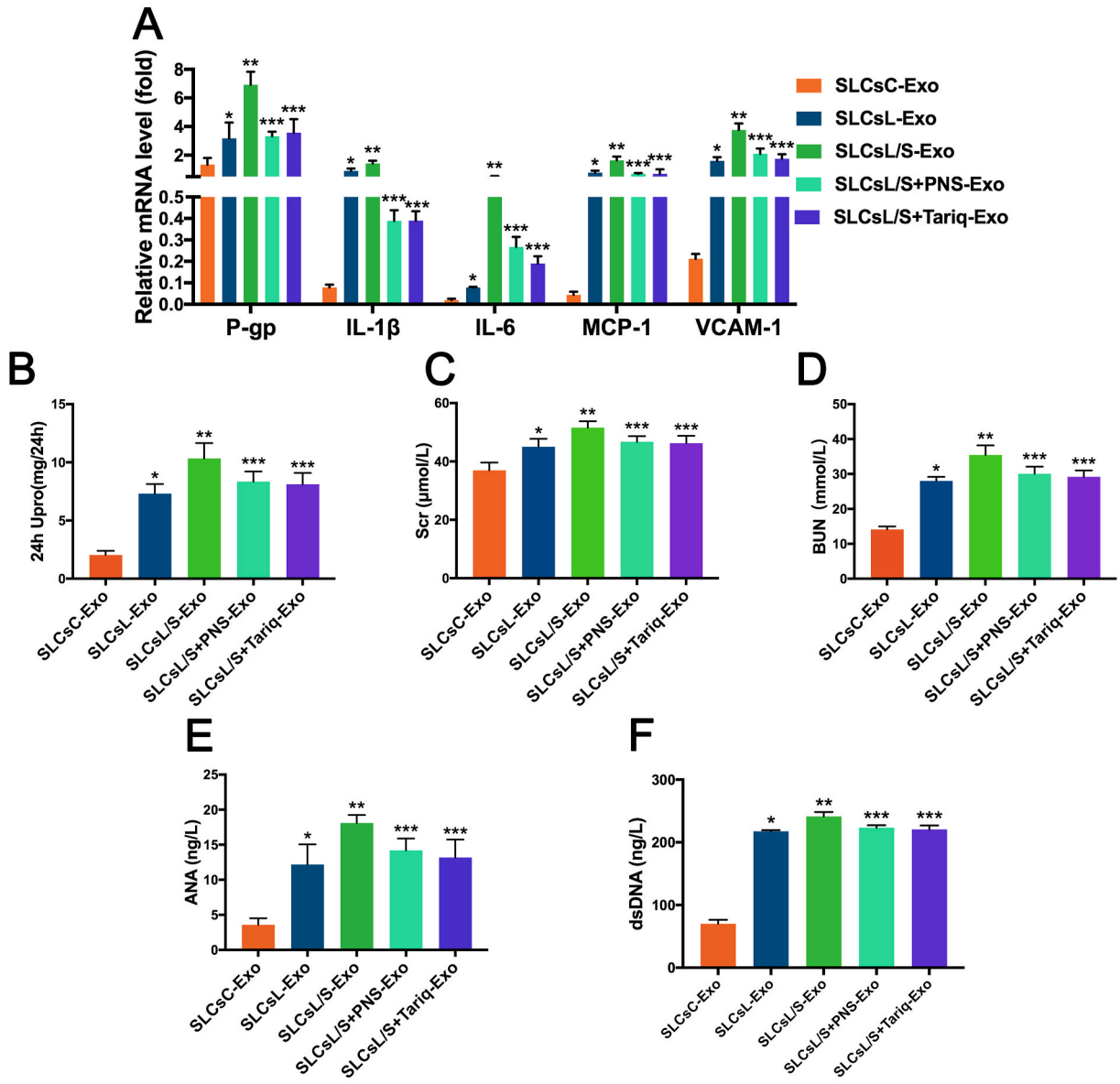

Fig. 6. The effect of SLCsL/S derived exosomes on the inflammatory damage of GECs and the intervention of PNS in vivo. (A) Renal tissue P-gp, IL-1β, IL-6, MCP-1 and VCAM-1 mRNA were determined by RT–PCR. (B,C, D, E, F) The levels of 24 h Upro, SCr, BUN, ANA and dsDNA. Data were repeated three times and expressed as the mean ± SD. *P < 0.05 vs. SLCsC-exo. **P < 0.05 vs. SLCsL-exo. ***P < 0.05 vs. SLCsL/S-exo.

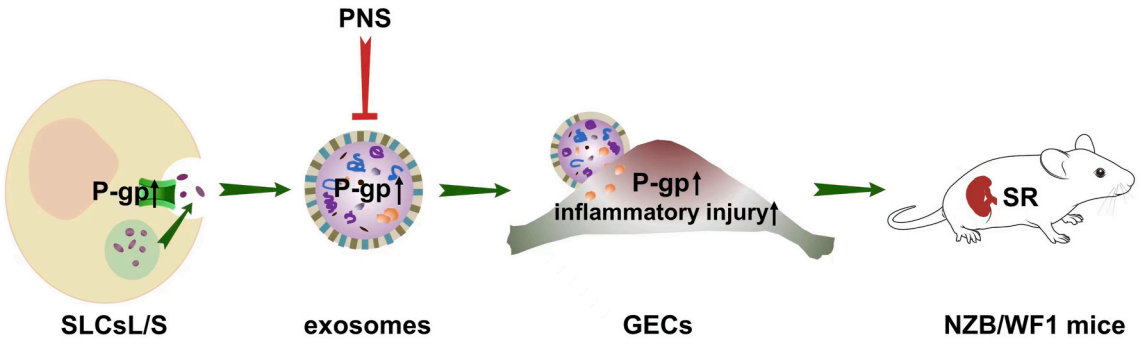

Fig. 7. Schematic illustrations of putative signaling mechanisms of PNS in reversing the steroid resistance through reducing the content of P-gp in the exosomes transferred from SLCsL/S to GECs.

alleviating microangiopathy in LN partly through reducing the content of P-gp in the exosomes transferred from SLCsL/S to GECs(Fig. 7).

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the ARRIVE guidelines and prior approval from the Animal Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (approval no. ZATCM-2019045).

Consent for publication

The authors declare that they agree to submit the article for publication.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author, Ying Lu, on reasonable request.

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Author contributions

FP carried out the experiments and drafted the manuscript. YL designed and coordinated the study, revised the manuscript and submitted it for publication. All authors analyzed and interpreted the data. The author(s) read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Not applicable.

Abbreviations

LN	lupus nephritis
SLE	systemic lupus erythematosus
GECs	glomerular endothelial cells
SR	steroid resistance
P-gp	P-glycoprotein
MDR1	multidrug resistance 1
SIRT1	silent information regulation factor related enzyme 1
FoxO1	forkhead box-containing protein of the O subfamily 1
exos	exosomes
PNS	Panax notoginseng saponins
SLCsL/S	steroid resistance splenic lymphocytes of lupus nephritis mice;
SLCsL	splenic lymphocytes of lupus nephritis mice;
SLCsC	splenic lymphocytes of ICR mice;
Tariq	Tariquidar
Rh-123	rhodamine-123

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101568.

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