TRANSLATIONAL SCIENCE

SGLT2 inhibitors alleviated podocyte damage in lupus nephritis by decreasing inflammation and enhancing autophagy

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ABSTRACT

Objectives The protective role of sodium glucose cotransporter 2 (SGLT2) inhibitors in renal outcomes has been revealed by large cardiovascular outcome trials among patients with type 2 diabetes. However, the effect of SGLT2 inhibitors on lupus nephritis (LN) and its underlying mechanisms remain unknown.
Methods We applied empagliflozin treatment to lupus-prone MRL/*lpr* mice to explore the renal protective potential of SGLT2 inhibitors. An SGLT2 knockout monoclonal podocyte cell line was generated using the CRISPR/Cas9 system to examine the cellular and molecular mechanisms.
Results In MRL/*lpr* mice treated with empagliflozin, the levels of mouse anti-dsDNA IgG-specific antibodies, serum creatinine and proteinuria were markedly decreased. For renal pathology assessment, both the glomerular and tubulointerstitial damages were lessened by administration of empagliflozin. The levels of SGLT2 expression were increased and colocalised with decreased synaptopodin in the renal biopsy samples from patients with LN and MRL/*lpr* mice with nephritis. The SGLT2 inhibitor empagliflozin could alleviated podocyte injury by attenuating inflammation and enhanced autophagy by reducing mTORC1 activity. Nine patients with LN treated with SGLT2 inhibitors with more than 2 months of follow-up showed that the use of SGLT2 inhibitors was associated with a significant decrease in proteinuria from 29.6% to 96.3%. Moreover, the estimated glomerular filtration rate (eGFR) was relatively stable during the treatment with SGLT2 inhibitors.
Conclusion This study confirmed the renoprotective effect of SGLT2 inhibitors in lupus mice, providing more evidence for non-immunosuppressive therapies to improve renal function in classic autoimmune kidney diseases such as LN.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The protective role of sodium glucose cotransporter 2 (SGLT2) inhibitors in renal outcomes has been revealed by large cardiovascular outcome trials among patients with type 2 diabetes.
⇒ A new attempt at additional empagliflozin treatment in five patients with lupus nephritis (LN) also showed inspiring beneficial effects on the kidney manifested by a dramatic reduction in proteinuria.

WHAT THIS STUDY ADDS

⇒ In MRL/*lpr* mice treated with empagliflozin, the levels of mouse anti-dsDNA IgG-specific antibodies, serum creatinine and proteinuria were markedly decreased, and the glomerular and tubulointerstitial damages were lessened in the renal pathology assessment.
⇒ The levels of SGLT2 expression were elevated and colocalised with decreased synaptopodin in the renal biopsy samples from patients with LN and MRL/*lpr* mice with nephritis.
⇒ The SGLT2 inhibitor empagliflozin alleviated podocyte injury by attenuating inflammation and enhanced autophagy by reducing mTORC1 activity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In addition to immunosuppressive therapies to improve immunological disorders, we are optimistic that SGLT2 inhibitors with strong renoprotective and cardioprotective effects will offer significant therapeutic benefit to patients with LN.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterised by the generation of autoantibodies and debilitating inflammation, leading to multiple organ damage. Lupus nephritis (LN) is one of the most common and severe complications of SLE that is characterised by kidney inflammation and eventually leads to end-stage kidney disease (ESKD).[1] Currently, glucocorticoids and other immunosuppressive agents are the frontline therapies. However, non-immunosuppressive drugs with fewer side effects, such as renin–angiotensin–aldosterone system inhibitors, have been verified to have nephroprotective effects and to play a fundamental role in LN treatment. Nevertheless, there are subsets of patients with LN who are at risk of progression to ESKD. Therefore, it is of great value to develop new therapeutic agents focused on renal protective effects by reducing proteinuria and ameliorating progressive renal failure in patients with LN.

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Sodium glucose cotransporter 2 (SGLT2) belongs to the membrane proteins and is located in the proximal tubule, where it accounts for 90% of the reabsorption of glucose that is filtered by the kidney.[2] By blocking glucose reabsorption, SGLT2 inhibitors increase urinary glucose excretion, resulting in improved glycaemic control in patients with diabetes. Inspiringly, the protective role of SGLT2 inhibitors in renal outcomes has been revealed by large cardiovascular outcome trials among patients with type 2 diabetes.[3–6] Moreover, the exact mechanisms underlying the renal protective effect of SGLT2 inhibitors cannot be fully explained by the glucose-lowering effect.[7 8] It is speculated that a reduction in intraglomerular pressure and resident renal cell protection play essential roles.[9] Subsequent trials performed in patients with chronic kidney disease (CKD) demonstrated that SGLT2 inhibitors are highly effective in reducing kidney failure.[10–12] Unfortunately, patients with LN were not included in these trials. Considering the powerful renal protective potential of SGLT2 inhibitors, Morales and Galindo made a remarkable attempt in five patients with LN.[13] Strikingly, the levels of proteinuria were reduced by nearly 50% without a significant influence on reducing the glomerular filtration rates in patients with LN with residual proteinuria despite immunosuppressive therapy.[13] Hence, it is of fundamental interest and of practical importance to explore in depth the mechanisms underlying the renal protective effect of SGLT2 inhibitors in LN.

In the present study, we applied empagliflozin treatment to lupus-prone *MRL/lpr* mice to explore the renal protective potential of SGLT2 inhibitors. In addition to protecting renal tubular cells, a growing number of studies have suggested that SGLT2 inhibitors are capable of ameliorating podocyte injury.[14 15] Given that podocyte injury was common in renal biopsy samples of patients with LN and was associated with a worse prognosis,[16 17] we took a closer look at the effect of SGLT2 inhibitors on podocyte injury in LN. Moreover, we also retrospectively reviewed the renal outcomes in patients with SLE treated with SGLT2 inhibitors in our centre and the results were inspiring.

PATIENTS AND METHODS

Patients and controls

All patients with SLE were diagnosed according to the revised criteria of the American College of Rheumatology[18] and had renal biopsy-proven LN. Twenty-six patients with LN including 13 cases of class III and 13 cases of class IV were used for immunofluorescence staining (online supplemental table 1). Another set of samples with the same amount and pathological types was obtained for immunohistochemistry. Adjacent non-cancerous kidney tissues (n=6) were used as normal control tissues.

Patient and public involvement

Patients and the public were not involved in the design, conduct, reporting or dissemination plans of our research.

Mice

We purchased 26 female MRL/MpJ-Fas<*lpr*>/J (MRL/*lpr*) mice from the Jackson Laboratory (Bar Harbor, Maine, USA) and 3 female C57BL/6 mice from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Six 8-week-old MRL/*lpr* mice were sacrificed by CO_2 inhalation to evaluate kidney impairment pathologically. Standard Hematoxyline & Eosin (H&E), periodic acid–Schiff (PAS) and periodic acid–silver methenamine (PASM) staining were performed and results were assessed by experienced pathologists. MRL/*lpr* mice (10 weeks old, n=10) were orally treated with 10 mg/kg body weight of empagliflozin (S8022 Selleckchem) every day for 10 weeks.[19] The group of vehicle control mice (n=10) was MRL/*lpr* mice that received the same volume of 0.5% carboxymethyl cellulose sodium alone (C104984, Aladdin). After 10 weeks of administration, 24-hour urine was collected using a metabolic cage. Then, 20-week-old female MRL/*lpr* mice were fasted overnight and anaesthetised. Serum was collected from peripheral blood and the kidneys were collected.

ELISA

The levels of total IgG, C3 and anti-dsDNA IgG antibodies in the serum were determined using an IgG ELISA kit (ab151276, Abcam), a C3 ELISA kit (ab157711, Abcam) and an anti-dsDNA IgG ELISA kit (5120, Alpha Diagnostic International) according to the manufacturer's recommendations.

Detection of urine glucose, urine protein-to-creatinine ratio and serum creatinine levels

Urinary glucose, urine protein and urine creatinine concentrations in MRL/*lpr* mice using 24-hour urine samples were measured by a glucose test kit (A154-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), protein quantification test kit (C035-2-1, Nanjing Jiancheng Bioengineering Institute) and creatinine test kit (C011-2-1, Nanjing Jiancheng Bioengineering Institute), respectively. The levels of serum creatinine were also tested using a creatinine test kit (C011-2-1, Nanjing Jiancheng Bioengineering Institute). Assays were performed according to the commercial kits and the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Immunofluorescence

Kidney tissues were fixed with 4% paraformaldehyde (G1101, Servicebio) for 48 hours at room temperature prior to paraffin embedding, sectioning and the preparing of slides. The paraffin slides were dewaxed and repaired with an antigen retrieval solution. Then, endogenous peroxidase was inactivated with 3% H_2O_2 solution for a suitable time, and non-specific binding sites were blocked with 3% bovine serum albumin (BSA) (G5001, Servicebio). The slides were incubated with rabbit antisynaptopodin (ab259976, Abcam) at 4°C overnight and were subsequently incubated with secondary antibody marked with horseradish peroxidase (HRP) (G1213, Servicebio) for 1 hour and tyramide signal amplification (TSA)-fluorescein isothiocyanate (FITC) (G1222, Servicebio) for 10 min at room temperature. After antigen retrieval and blocking, the slides were incubated with rabbit anti-SGLT2 (ab37296, Abcam) at 4°C overnight and were incubated with Cy5-labelled goat anti-rabbit IgG (GB27303, Servicebio) for 1 hour at room temperature.

The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (D8200, Solarbio) and photographed under a laser scanning confocal microscope for observation (NIKON Eclipse Ti; Nikon, Tokyo, Japan).

For C3 evaluation, optimal cutting temperature (OCT)-frozen kidney sections were incubated at 4 °C overnight with the rabbit anti-C3 (ab97462, Abcam) followed by FITC-conjugated secondary antibody (GB22404, Servicebio). The nuclei were stained with DAPI (D8200, Solarbio) and photographed under a fluorescence microscope (Nikon CI-L, Nikon).

Podocyte culture and treatments

The cells were cultured at 33°C in RPMI-1640 (01-101-1A, Biological Industries, USA), containing 10% fetal bovine serum (FBS, 10 100 147 Gibco) and insulin–transferrin–selenium (ITS,

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41400045 Gibco) with 5% CO_2. The podocytes were cultured for 10 days at 37°C without ITS to induce differentiation. After the differentiated of podocytes had matured, the culture was supplemented with purified IgG from serum of patients with LN or commercial IgG (I4506, Sigma). The detailed procedures of the IgG extraction from serum were performed as previously described.[20] Empagliflozin (S8022, Selleckchem) was applied at a concentration of 100 nM.

Construction of SGLT2 and MAP1LC3B knockout (KO) HPC monoclonal cell lines with CRISPR/Cas9

The sgRNA plasmids were designed and constructed by Vigene Biosciences (Shandong, China). The gene sequence for generating sgRNA targeting *SGLT2* is sgRNA5 5′-CACTGTGGGCGGCTACTTCC-3′. The designed sequences were inserted into sgRNA vectors with the spCas9 gene and puromycin resistance gene. The KO effects were tested by the *SGLT2* forward primer (5′-GGATTAGAGCCTGGGTTGCC-3′) and reverse primer (5′-CCCTTTCCTGGAGTTTCCCC-3′). Furthermore, the mixed clones of *SGLT2* KO podocytes were screened by a limited dilution method to obtain a monoclonal cell line. Finally, the *SGLT2* KO podocyte cell line (*SGLT2*-KO-HPC) was obtained. The generation of the *MAP1LC3B* KO podocyte cell line was described previously.[21]

Construction and transfection of overexpression plasmids

The *SGLT2* overexpression plasmid was constructed by ligating the coding sequence of the human *SGLT2* gene into the pDONR223 vector (pDG125154, Unibio). Non-targeting vectors were used as a negative control. Transient transfection of *SGLT2*-KO-HPC cell lines was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. After 48 hours, all transiently transfected cells were collected and the efficiency of transfection was approximately 90%.

Transcriptome analysis

Differentially expressed genes (DEGs) were determined by $|\log2 \text{ fold change (FC)}| \geq 1$ and q value of <0.05. Genes were enriched using Wikipathway (https://www.wikipathways.org/). Gene functional enrichment was performed by Gene Set Enrichment Analysis (GSEA) (http://www.broad.mit.edu/gsea/).[22] The Enrichment Score reflects the extent to which a gene set is overrepresented at the top or bottom of the gene ranking list.

Western blot analysis

Proteins were prepared from human podocytes and renal tissues of MRL/*lpr* mice. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies overnight at 4°C after being blocked with 5% skimmed milk for 2 hours at room temperature. The next day, the membrane was blocked for 2 hours using horseradish enzyme peroxidase-labelled secondary antibodies. Protein expression was detected using ECL chemiluminescent solution (PK10003, Proteintech); images were visualised using a FluorChem R Imaging system (ProteinSimple, USA), and the band intensities were quantified by ImageJ V.1.53.

The antibodies used were listed as follows: rabbit anti-SGLT2 antibody (ab37296, Abcam), rabbit antinephrin (ab58968, Abcam), rabbit anti-NPHS2 (ab50339, Abcam), rabbit anti-NLRP3 (ab263899, Abcam), rabbit anti-caspase1 (PA5-87536, Thermo Fisher), rabbit anti-cleaved caspase1 (PA5-38099, Thermo Fisher), rabbit anti-interleukin (IL)-1 beta (ab283822, Abcam), rabbit anti-LC3B (ab4839, Abcam), rabbit anti-p-RPS6 (Ser240/244) (2215S, Cell Signalling Technology), mouse anti-RPS6 (2317S, Cell Signalling Technology), rabbit anti-p70S6K (2708S, Cell Signalling Technology), rabbit anti-P-p70S6K (Thr389) (9205S, Cell Signalling Technology), rabbit anti-P-eIF4B (Ser422) (3591S, Cell Signalling Technology), rabbit anti-eIF4B (3592S, Cell Signalling Technology) and rabbit anti-α-tubulin (2144s, Cell Signalling Technology).

Immunohistochemistry

For SGLT2, NLRP3, SOD2, anti-NF-κB p65 and LC3B immunohistochemistry analysis, 4% paraformaldehyde-fixed, 4 µm paraffin-embedded kidney sections were used. After antigen repair in citrate buffer, sections were incubated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity, followed by incubation with 5% goat serum (AR1009, BOSTER) for 20 min to reduce non-specific staining. Next, the section was incubated with primary antibody (rabbit anti-SGLT2, ab37296, Abcam; rabbit anti-NLRP3, bs-10021R, Bioss antibodies; rabbit anti-LC3B, ab4839, Abcam; rabbit anti-SOD2, ab137037, Abcam; rabbit anti-NF-κB p65, 8242S, Cell Signalling Technology) overnight at 4°C, washed and incubated with the corresponding secondary antibody for 30 min at room temperature. Stains were developed using a DAB Substrate Kit (G1212, Servicebio). Finally, the slices were sealed with neutral resin and observed under an optical microscope.

TUNEL assay

Samples were fixed with 4% paraformaldehyde (G1101, Servicebio) to prepare paraffin sections. The TUNEL assay (12156792910, Roche) was conducted based on the manufacturer's instructions. Images were acquired using a microscope (Nikon CI-L, Nikon).

Flow cytometry analysis

The level of podocyte apoptosis was determined using the FITC Annexin V apoptosis detection kit (556547, BD Biosciences) according to the manufacturer's recommendations. Then 10 000 cells from each group were collected and analysed by FACScan flow cytometer (Beckman Coulter). The obtained data were analysed by FlowJo V.7.6.

Statistical analysis

Data are expressed as mean±SD. Student's t-test was used to compare data from the two groups. Data analysis was performed and graphs were generated by GraphPad Prism V.8.0 (GraphPad Prism). P values less than 0.05 were considered statistically significant.

RESULTS

SGLT2 inhibitors improved laboratory parameters of MRL/*lpr* mice

In light of the pathological role of SGLT2 and the renal protective effect of SGLT2 inhibitors, we performed in vivo experiments using MRL/*lpr* mice. Administration of vehicles and empagliflozin was started at 10 weeks of age and continued to 20 weeks of age, and the MRL/*lpr* mice were sacrificed at 20 weeks of age (figure 1A). The laboratory parameters and histological alterations of MRL/*lpr* mice are shown in figure 1. By blocking renal glucose reabsorption, the levels of urinary glucose excretion were significantly elevated in the group treated with empagliflozin (figure 1B). Mouse anti-dsDNA IgG-specific

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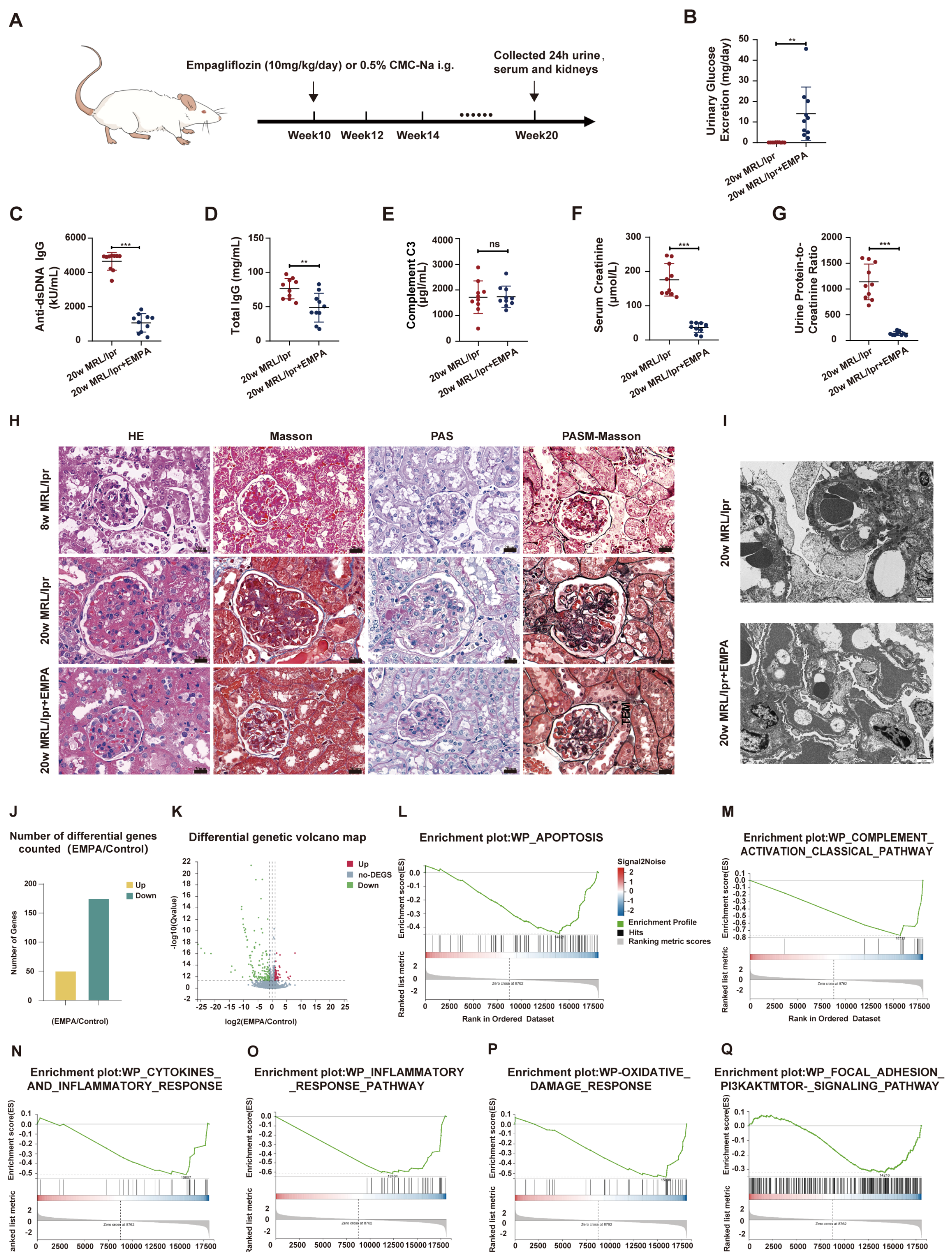

Figure 1 Effects of EMPA on MRL/*lpr* mice. Overall experimental procedure. (A) Levels of urinary glucose excretion, (B) anti-dsDNA IgG, (C) total IgG, (D) complement C3, (E) serum creatine, (F) urine protein-to-creatine ratio, (G) renal pathological alteration by light (H) and electron microscopy, (I) The number of DEGs identified by transcriptome analysis is shown in bar graphs (J) and volcano plots. (K) Gene Set Enrichment Analysis showed the enrichment plot of apoptosis, (L) complement, (M) inflammation, (N,O) oxidative damage (P) and PI3K/Akt/mTOR (Q). **P<0.01, ***P<0.001. DEG, differentially expressed gene; EMPA, empagliflozin; ns, not significant; PAS, periodic acid–Schiff; PASM, periodic acid–silver methenamine.

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antibodies and total IgG were measured using a quantitative ELISA in MRL/*lpr* mice. There was a significant decrease in anti-dsDNA IgG and total IgG titres in the empagliflozin-treated group (figure 1C,D). However, no significant difference was observed in serum complement C3 between the empagliflozin-treated group and the vehicle-treated group (figure 1E).

Renal disease development in MRL/*lpr* mice was characterised by a marked increase in serum creatinine and proteinuria. Treatments with empagliflozin significantly alleviated the levels of serum creatinine in 20-week-old MRL/*lpr* mice (figure 1F). Moreover, the levels of urinary protein excretion were also significantly reduced after treatment with empagliflozin in 20-week-old MRL/*lpr* mice (figure 1G).

SGLT2 inhibitors improved renal histological alterations in MRL/*lpr* mice

To explore whether the decreased level of serum creatinine and proteinuria were associated with renal pathological amelioration, the pathological alterations were assessed by H&E, PAS, Masson and PASM+Masson staining in MRL/*lpr* mice following treatment with vehicle and empagliflozin (figure 1H). Compared with 8-week-old MRL/*lpr* mice, remarkable kidney pathological damage was also observed at 20 weeks (figure 1H). After the treatment with empagliflozin, both the glomerular—hypercellularity and mesangial expansion, and glomerular endothelial cell injury development of crescents—and tubulointerstitial damage were lessened (figure 1H).

Electron microscopy showed prominent granular electron-dense deposits in mesangial, subendothelial and subepithelial in the glomeruli, and partial podocyte foot process effacement in 20-week-old MRL/*lpr* mice treated with vehicles (figure 1). Less electron-dense deposits in the glomeruli were observed and the foot process effacement was alleviated in empagliflozin treated mice (figure 1).

Inflammatory process was downregulated in MRL/*lpr* mice treated with empagliflozin as assessed by transcriptome analysis

To delineate the effective cues of empagliflozin on gene expression, a transcriptome analysis was carried out between MRL/*lpr* mice treated with and without empagliflozin. Genes with $|\log 2\ FC| \geq 1$ and q value of <0.05 were selected as DEGs (figure 1J,K). GSEA revealed that gene sets related to apoptosis (figure 1L and online supplemental figure 1A), complement activation (figure 1M and online supplemental figure 1B), inflammation (figure 1N,O and online supplemental figure 1C) and oxidative damage (figure 1P and online supplemental figure 1D) were significantly downregulated in MRL/*lpr* mice treated with empagliflozin (see detailed pathway enrichment results in online supplemental table 2). More importantly, the PI3K/AKT/mTOR signalling pathway was also significantly downregulated (figure 1Q). These data showed us the way to further explore underlying biological mechanisms.

Expression of SGLT2 was upregulated in the podocyte of MRL/*lpr* mice with renal impairment and patients with LN

Predominantly, SGLT2 is localised to the proximal tubule, but the expression of SGLT2 can also be identified in glomerular in patients with LN.[23] In our present study, SGLT2 colocalised with synaptopodin in podocytes in glomerular. Immunofluorescence staining for SGLT2 was increased and localised in podocytes in 20-week-old MRL/*lpr* mice with nephritis compared with 8-week-old MRL/*lpr* mice without obvious renal impairment (figure 2A). After empagliflozin treatment, SGLT2 expression was reduced in 20-week-old MRL/*lpr* mice, and the expression of synaptopodin was significantly increased compared with vehicle treatment by immunofluorescence staining (figure 2A). As shown in figure 2B, the expression of SGLT2 was significantly upregulated in 20-week-old MRL/*lpr* mice and could be reduced under empagliflozin by immunohistochemical staining.

We also assessed the expression of SGLT2 in the podocytes of 26 patients with LN including 13 patients of class III and 13 patients of class IV. As shown in figure 2C,D, a stronger SGLT2 staining localised in podocytes was observed in patients with LN than in paracancer normal kidney tissues.

These observations strongly indicated that the expression of SGLT2 was upregulated in the podocytes of LN and was associated with podocyte injury.

Empagliflozin reduced podocyte apoptosis

The aforementioned evidence showed that the expression of SGLT2 was upregulated in the podocytes of patients with LN and MRL/*lpr* mice with renal impairment. We further performed in vitro experiments on stimulation with IgG extracted from the serum of patients with LN. Deposition of IgG and immune complexes are believed to be the most common factors in the podocyte injury in LN. On stimulation with IgG extracted from the serum of patients with LN, the levels of nephrin and podocin were reduced, indicating podocyte injury occurred (figure 3A,B). More importantly, the expression level of SGLT2 was significantly upregulated in the podocytes cultured with IgG extracted from the serum of patients with LN in a dose-dependent and time-dependent manner (figure 3A,B).

In accordance with clues from the transcriptome analysis, we identified that the rate of apoptosis was significantly increased after exposure to IgG extracted from the serum of patients with LN and was significantly decreased after empagliflozin treatment (figure 3C).

Inhibition of SGLT2 inhibited NLRP3 inflammasome activation and reduced podocyte injury

To probe the role of SGLT2 in podocyte injury, we used the CRISPR/Cas9 system to generate an SGLT2 KO monoclonal podocyte cell line. The effect of SGLT2 KO is presented in Figure 4A. Figure 4B shows that SGLT2 KO improved the expression levels of podocin and nephrin. Furthermore, using the KO–rescue strategy, the expression levels of podocin and nephrin were decreased transfected with the SGLT2 overexpression plasmid in the SGLT2 KO cell line (figure 4B).

We further explored the underlying inflammatory effect of SGLT2 contributing to LN podocyte injury. After exposure to IgG extracted from the serum of patients with LN, the levels of NLRP3, caspase1, cleaved caspase1, and IL-1β were significantly decreased. On transfection with the SGLT2 overexpression plasmid in SGLT2 KO cell line, the expression of NLRP3, caspase1, cleaved caspase1 and IL-1β was resumed (figure 4C).

In response to empagliflozin, the expression levels of podocin and nephrin were significantly increased compared with those of controls, indicating protection of podocytes from IgG-induced injury (figure 4D). Moreover, the expression of NLRP3, caspase1, cleaved caspase1 and IL-1β was significantly decreased after treatment with empagliflozin, revealing the anti-inflammation effect of SGLT2 inhibitors (figure 4D).

Based on these clues from cell experiments, we further examined the effect of empagliflozin on the NLRP3 inflammasome in animal models. As shown in figure 4E, the expression of NLRP3,

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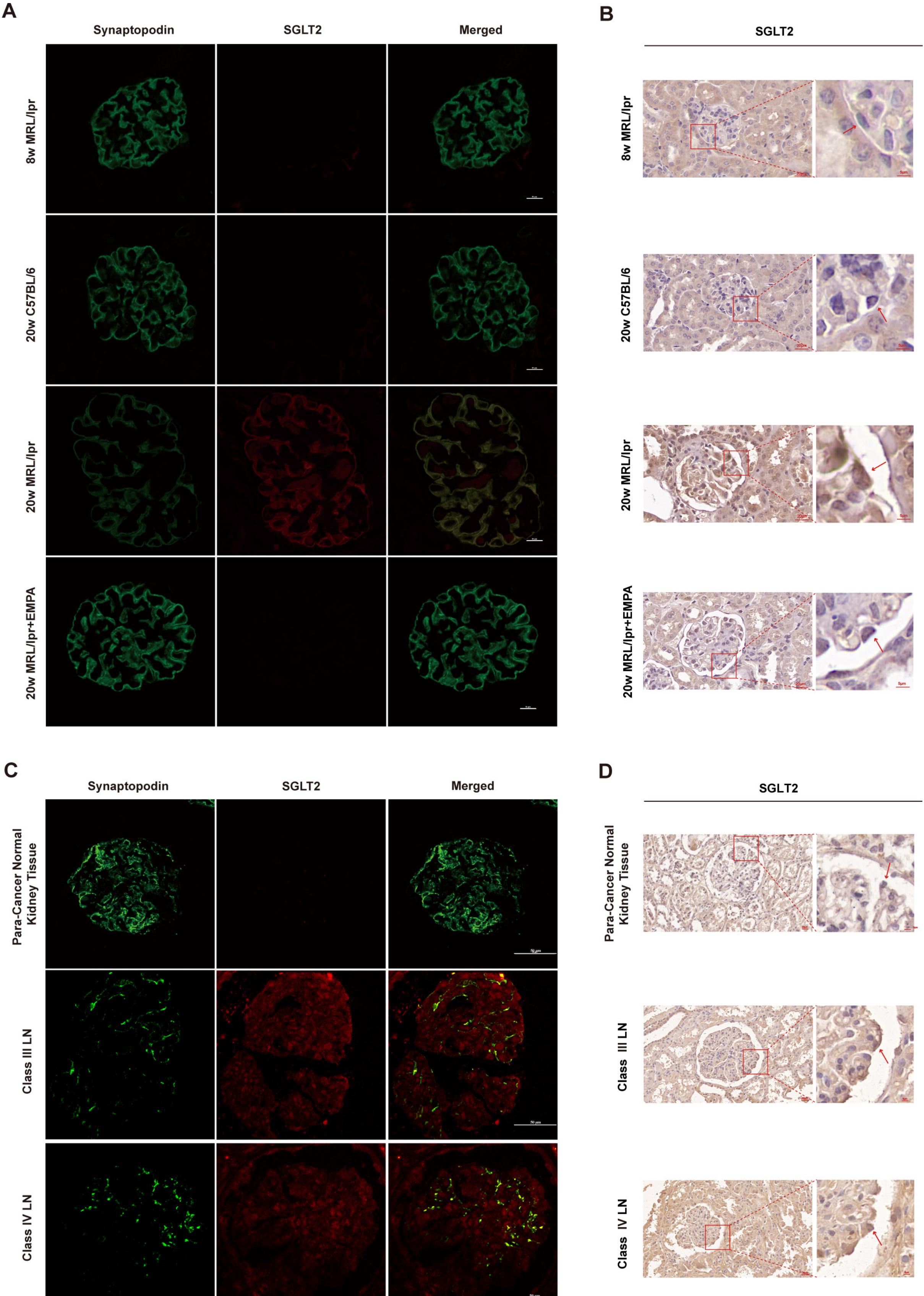

Figure 2 Expression of SGLT2 in podocytes from MRL/*lpr* mice and patients with LN. (A) Confocal immunofluorescence images showing the colocalisation of SGLT2 and synaptopodin in mouse models (scale bar=10 µm); (B) immunohistochemical staining for SGLT2 in kidney tissues in mouse models (scale bar=20 µm, enlarged scale bar=5 µm). (C) Confocal immunofluorescence images for colocalisation of SGLT2 and synaptopodin (scale bar=50 µm) and (D) immunohistochemical staining for SGLT2 (scale bar=20 µm, enlarged scale bar=5 µm) were also performed in renal biopsy samples from patients with LN. EMPA, empagliflozin; LN, lupus nephritis; SGLT2, sodium glucose cotransporter 2.

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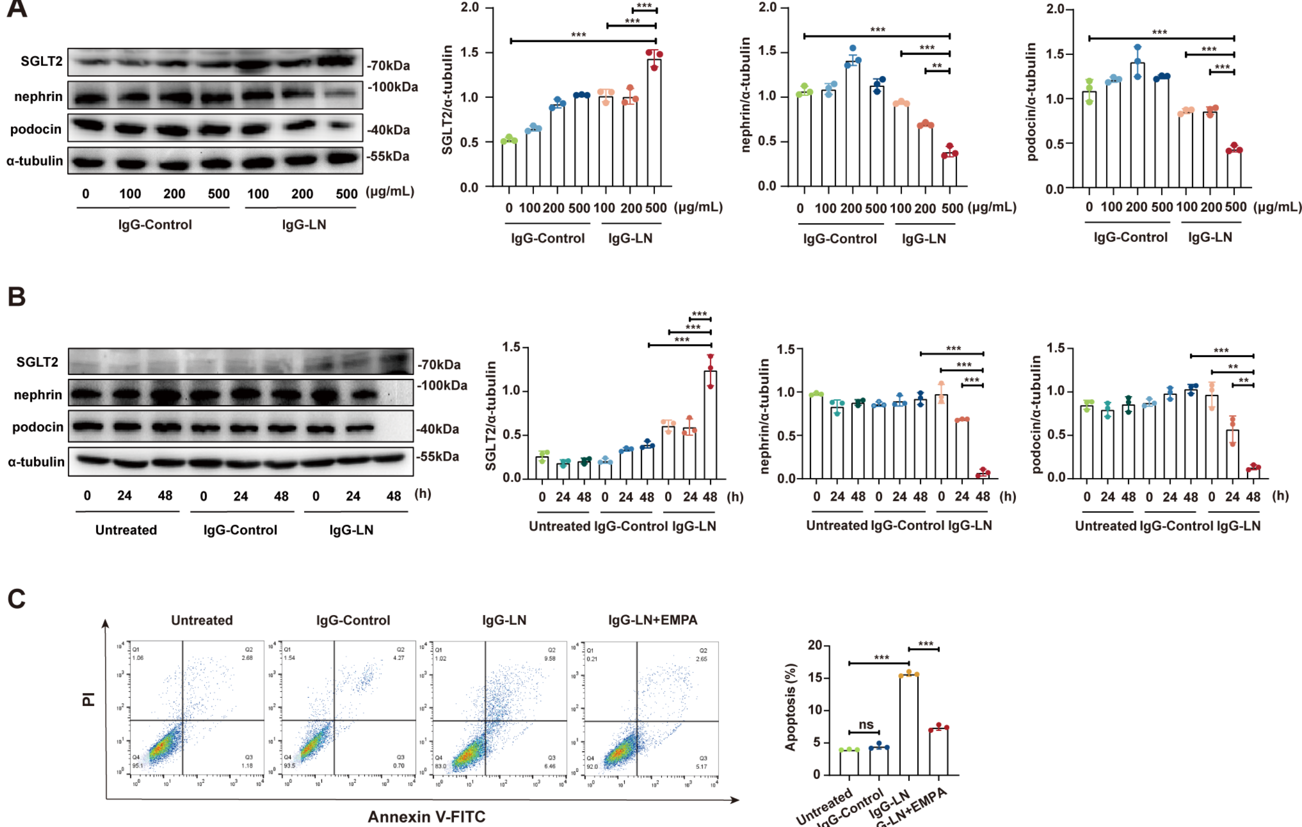

Figure 3 Inhibition of SGLT2 reduced podocyte apoptosis. The dose (A) and time (B) effects of IgG extracted from patients with LN on podocytes. The apoptosis rate of podocytes exposed to IgG extracted from healthy donors or patients with LN treated with or without EMPA (C). *P<0.05, **P<0.01, ***P<0.001. EMPA, empagliflozin; LN, lupus nephritis; ns, not significant; SGLT2, sodium glucose cotransporter 2.

caspase1, cleaved caspase1 and IL-1β from kidney tissues was significantly upregulated in 20-week-old MRL/*lpr* mice with renal impairment compared with 8-week-old MRL/*lpr* mice (which did not present with significant renal impairment pathologically, as shown in figure 1G) and 20-week-old C57BL/6 mice. Empagliflozin treatment significantly reduced the expression of NLRP3, caspase1, cleaved caspase1 and IL-1β in the kidney tissues from 20-week-old MRL/*lpr* mice (figure 4F,G).

Inhibition of SGLT2 reduced podocyte injury by activating autophagy through the reduction of mTORC1

In light of transcriptome analysis, we tested autophagy which would upregulate as the result of PI3K/AKT/mTOR signalling pathway inhibition. Autophagy is known to play an important protective role in LN podocyte injury.[20] Here, we tested the possible relationship between SGLT2 and autophagy. As shown in figure 5A, the expression of LC3B-II was significantly upregulated in the SGLT2 KO cell line (figure 5A). Furthermore, the expression of SGLT2 was not affected by knocking out *MAP1LC3B* using the CRISPR/Cas9 system (figure 5A). Then, we used empagliflozin to inhibit the effect of SGLT2, and we observed that the expression of LC3B-II was significantly increased under empagliflozin treatment (figure 5B). These results indicated that SGLT2 might inhibit autophagy in the podocyte injury in LN.

To elucidate the possible mechanisms by which empagliflozin induces autophagy, we detected the activity of the mTORC1 signalling pathway (figure 5B). The phosphorylation of S6, p70s6k and elF4B was significantly reduced after treatment with empagliflozin, confirming the activation of mTORC1 (figure 5B).

Given the evidence from in vivo experiments, we detected that the level of autophagy was significantly increased in kidney tissues from 20-week-old MRL/*lpr* mice treated with empagliflozin (figure 5C). Moreover, the phosphorylation of S6, p70s6k and elF4B was significantly increased in kidney tissues from 20-week-old MRL/*lpr* mice and could be inhibited by the administration of empagliflozin (figure 5D,E).

Collectively, our data suggested that increased SGLT2 expression contributed to podocyte injury in LN by inhibiting autophagy, and the SGLT2 inhibitors such as empagliflozin enhanced the autophagy process by reducing the expression of the mTORC1 signalling pathway.

Effects of SGLT2 inhibitors on patients with LN

We retrospectively reviewed patients with SLE with renal biopsy-proven LN who were treated with more than 2 months of SGLT2 inhibitors to assess treatment response. The exclusion criteria were as follows: (1) the increased use of steroids or mycophenolate mofetil or tacrolimus; (2) new additional treatment with belimumab or telitacicept within the first 2 months of SGLT2i; and (3) the test results were not collected for analysis if the patients presented with severe infection. A total of nine patients with LN were included in further analysis, and detailed information is presented in table 1. The use of SGLT2 inhibitors was associated with a significant decrease in proteinuria from 29.6% to 96.3%. Furthermore, the eGFR was relatively stable during the use of SGLT2 inhibitors treatment period.

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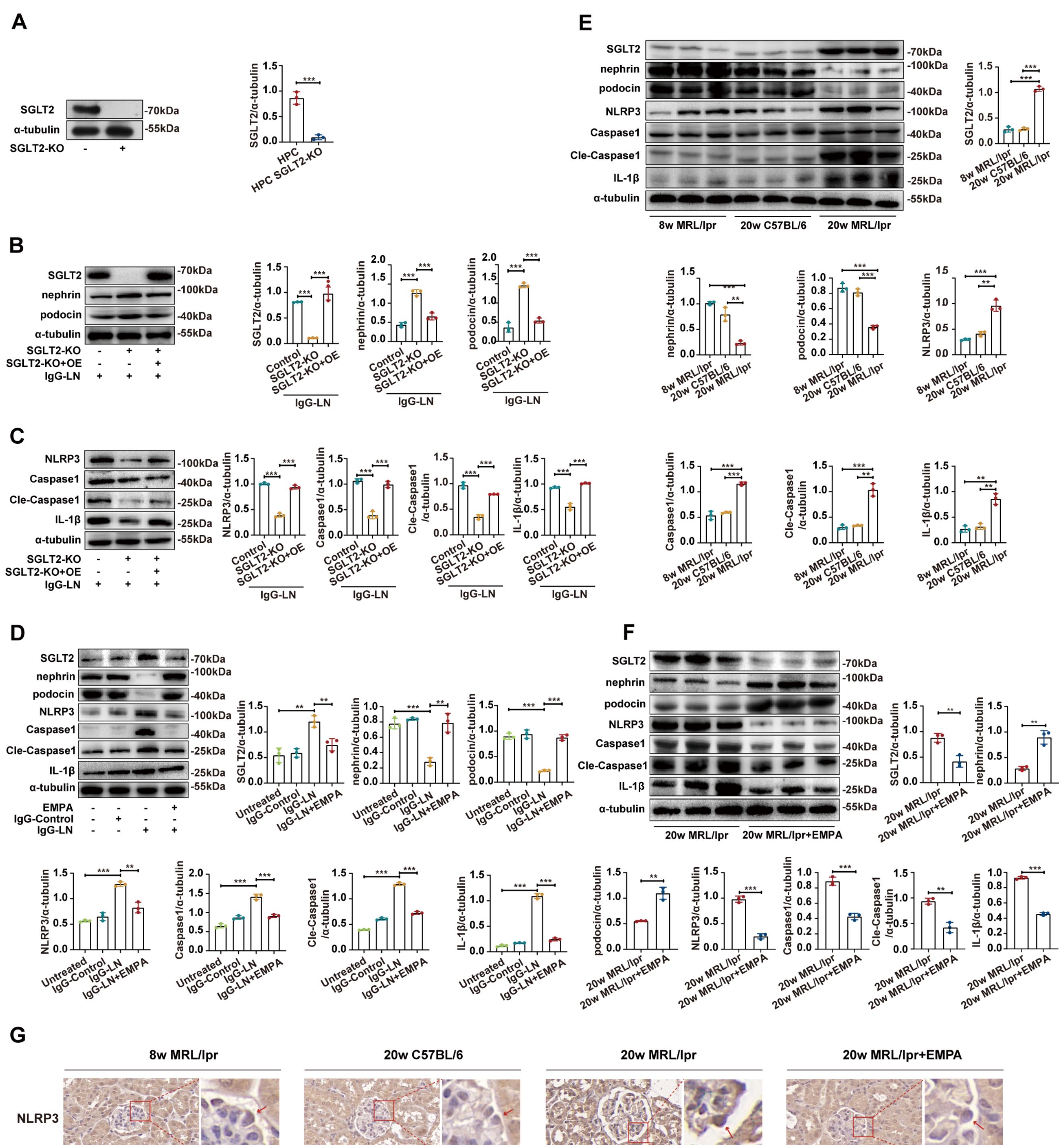

Figure 4 Inhibition of SGLT2 inhibited NLRP3 inflammasome activation and reduced podocyte injury. The efficacy of SGLT2 knockdown was assessed by western blotting. (A) Under administration of IgG extracted from patients with LN, the expression of podocin, nephrin, NLRP3, caspase1, cleaved caspase1 and IL-1β in SGLT2 KO podocytes with and without SGLT2 overexpression (B,C). The expression of SGLT2, NLRP3, caspase1, cleaved caspase1, IL-1β, podocin and nephrin in podocytes exposed to IgG extracted from patients with LN treated with and without EMPA (D). The expression of SGLT2, NLRP3, caspase1, cleaved caspase1, IL-1β, podocin and nephrin in 8-week-old MRL/*lpr* mice, 20-week-old C57 BL/6 mice and 20-week-old MRL/*lpr* mice (E) treated with and without EMPA (F). The immunohistochemical staining (scale bar=20 μm, enlarged scale bar=5 μm) of NLRP3 in the kidney tissues from 8-week-old MRL/*lpr* mice, 20-week-old C57BL/6 mice and 20-week-old MRL/*lpr* mice treated with and without EMPA (G). *P<0.05, **P<0.01, ***P<0.001. EMPA, empagliflozin; IL, interleukin; KO, knockout; LN, lupus nephritis; SGLT2, sodium glucose cotransporter 2.

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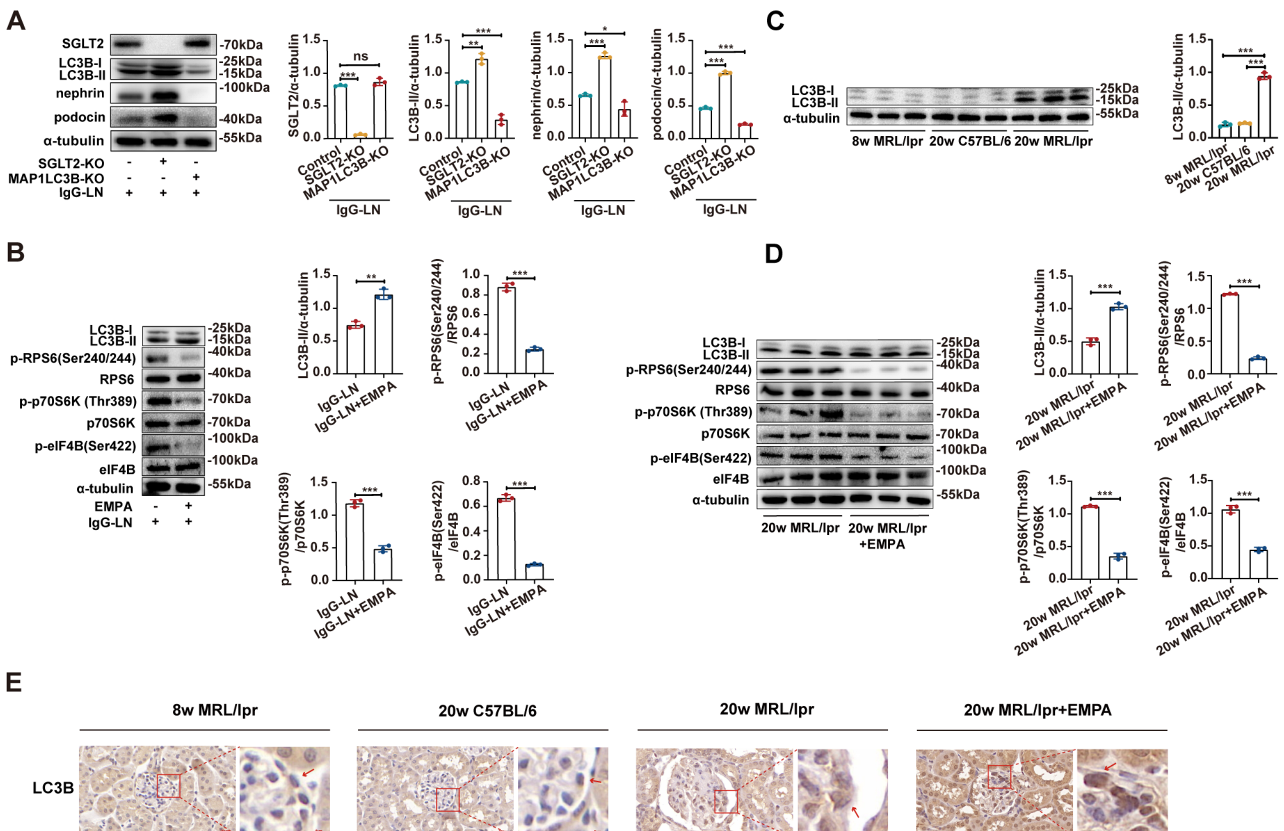

Figure 5 Inhibition of SGLT2 reduced podocyte injury by activating autophagy through the reduction of mTORC1 activity. (A) Expression of SGLT2, LC3B, nephrin and podocin in SGLT2 or MAP1LC3B KO podocyte cell lines. (B) Levels of LC3B and the phosphorylation of S6, p70S6K and eIF4B in podocytes exposed to IgG extracted from patients with LN treated with and without EMPA. (C) Expression of LC3B in 8-week-old MRL/*lpr* mice, 20-week-old C57 BL/6 mice and 20-week-old MRL/*lpr* mice. (D) Levels of LC3B, the phosphorylation of S6, p70S6K and eIF4B in the kidney tissues of 20-week-old MRL/*lpr* mice treated with and without EMPA. (E) The immunohistochemistry staining (scale bar=20 µm, enlarged scale bar=5 µm) of LC3B in the kidney tissues in 8-week-old MRL/*lpr* mice, 20-week-old C57 BL/6 mice and 20-week-old MRL/*lpr* mice treated with and without EMPA. *P<0.05, **P<0.01, ***P<0.001. EMPA, empagliflozin; KO, knockout; LN, lupus nephritis; SGLT2, sodium glucose cotransporter 2.

DISCUSSION

In recent years, there has been a growing appreciation of the renal protective effect of SGLT2 inhibitors among researchers and clinicians. Strong evidence from large clinical trials has demonstrated that SGLT2 inhibitors delayed the progression of renal dysfunction in patients with or without type 2 diabetes.[3–6 10–12] In the prespecified analysis of the DAPA-CKD trial, dapagliflozin therapy reduced the albuminuria by 26% and the risk of CKD progression in IgA nephrology patients.[24] A case series reported that a good response with respect to lowering albuminuria was observed in patients with hereditary focal segmental glomerulosclerosis.[25] A new attempt at additional empagliflozin treatment in five patients with LN also showed an inspiring benefit to the kidney by a dramatic reduction in proteinuria.[13] Consistent with the preliminary clinical study from patients with LN,[13] our data revealed a renoprotective effect of SGLT2 inhibitors by reducing proteinuria and preserving renal function in the murine MRL/*lpr* lupus model.

Podocyte injury is common in LN, ranging from functional impairment (which can be hard to identify pathologically) to extensive effacement of podocyte foot processes (also known as lupus podocytopathy). In patients with LN, SGLT-2 was detectable and predominantly localised to the proximal tubules, and the use of SGLT2 inhibitors would increase the urinary glucose excretion.[23] Additionally, there were indications that SGLT2 was also expressed in glomerular.[23] In the present study, the levels of SGLT2 expression were elevated and colocalised with decreased synaptopodin in the renal biopsy samples from patients with LN and MRL/*lpr* mice with nephritis. After inhibiting SGLT2 with empagliflozin, the expression of synaptopodin was significantly increased in MRL/*lpr* mice. Following in vitro study showed that overexpression of SGLT2 promoted podocyte injury, which could be recovered in the presence of empagliflozin (figure 6).

It has been widely recognised that the activation of the NLRP3 inflammasome induces podocyte injury and promotes proteinuria in patients with LN.[26–29] SGLT2 inhibitors were reported to be capable of modulating NLRP3 inflammasome activity in diabetes,[30] cardiovascular disease[31] and kidney diseases.[15 32] Our data showed that SGLT2 KO and the SGLT2 inhibitor empagliflozin reduced the activity of the NLRP3 inflammasome and protected against podocyte injury.

Autophagy is an important cytoprotective mechanism against diverse stresses in the podocyte injury in LN.[33] A large corpus of consistent data indicates that SGLT2 inhibitors maintain cellular homeostasis by enhancing autophagic flux.[34] The augmentation of autophagy by SGLT2 inhibitors was related to decreased activation of mTOR.[34] However, little is known about the effect of SGLT2 inhibitors on autophagy in podocyte injury in LN. Here, we constructed *SGLT2* and *MAP1LC3B* KO monoclonal podocyte cell lines using the CRISPR/Cas9 system and adopted the MRL/*lpr* mouse model to elucidate the relationship between SGLT2 and autophagy. Our results suggested that SGLT2 could

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Table 1 Clinical features, treatments strategies and effects for patients with LN treated with SGLT2 inhibitors

N	Age (years)*	Gender	Classification of LN	Baseline					SGLT2 (doses/day)	Duration of follow-up (week)	Follow-up				
				IMS (doses/day)	RAASi (doses/day)	eGFR(mL/min/1.73 m^2)	Proteinuria (g/day)	Serum albumin (g/dL)			IMS (doses/day)	RAASi (doses/day)	eGFR(mL/min/1.73 m^2)	Proteinuria (g/day)	Serum albumin (g/dL)
1	33	F	V	Methylprednisolone 24mg+cumulative CYC 0.7 g		98.4	1.35	32.8	Dapagliflozin 10 mg	11	Methylprednisolone 12mg+cumulative CYC 1.1 g		121.0	0.95	39.7
2	25	M	IV	Prednisone 30 mg	Allisartan Isoproxil 240 mg	64.1	3.08	36.0	Dapagliflozin 10 mg	12	Prednisone 30 mg	Allisartan isoproxil 240 mg	99.2	0.60	42.3
3	30	M	IV	Prednisone 37.5 mg+MMF1000 mg	Valsartan 80 mg	92.2	0.78	33.9	Dapagliflozin 10 mg	15	Prednisone 22.5 mg+MMF1000 mg	Valsartan 80 mg	92.7	0.40	43.4
4	45	F	V	Methylprednisolone 14mg+TAC 3 mg	Allisartan isoproxil 240 mg	87.4	0.58	33.9	Canagliflozin 100 mg	17	Methylprednisolone 8mg+TAC 3 mg	Allisartan isoproxil 240 mg	105.5	0.32	37.6
5	31	F	V	Prednisone 45mg+TAC 2 mg		126.8	1.83	37.9	Dapagliflozin 10 mg	12	Prednisone 22.5 mg+TAC 2 mg		131.6	0.73	35.4
6	25	M	IV	Prednisone 22.5 mg+MMF 1000 mg+TAC 2 mg	Valsartan 80 mg	124.7	8.19	37.4	Dapagliflozin 10 mg	16	Prednisone 15mg+MMF 1000 mg	Valsartan 80 mg	121.2	4.50	41.8
7	32	F	IV	Prednisone 60 mg		63.6	4.30	16.5	Canagliflozin 100 mg	21	Prednisone 50 mg		120.3	1.47	26.8
8	55	F	IV	Methylprednisolone 20mg+cumulative CYC 0.6 g	Allisartan isoproxil 240 mg	59.9	6.72	28.2	Canagliflozin 100 mg	11	Methylprednisolone 12mg+cumulative CYC 2.2 g	Allisartan isoproxil 240 mg	74.6	0.25	42.9
9	57	F	V	Prednisone 30mg+cumulative CYC 1.0 g	Sacubitril/valsartan 100 mg	104.2	1.58	39.5	Ertugliflozin 5 mg	18	Prednisone 15mg+cumulative CYC 5.8 g	Sacubitril/valsartan 100 mg	110.0	0.66	41.4

*Age with the first dose of SGLT2 inhibitors.
CYC, cyclophosphamide; F, female; IMS, immunosuppression; LN, lupus nephritis; M, male; MMF, mycophenolate mofetil; RAASi, renin–angiotensin–aldosterone system inhibitor; SGLT2, sodium glucose cotransporter 2; TAC, tacrolimus.

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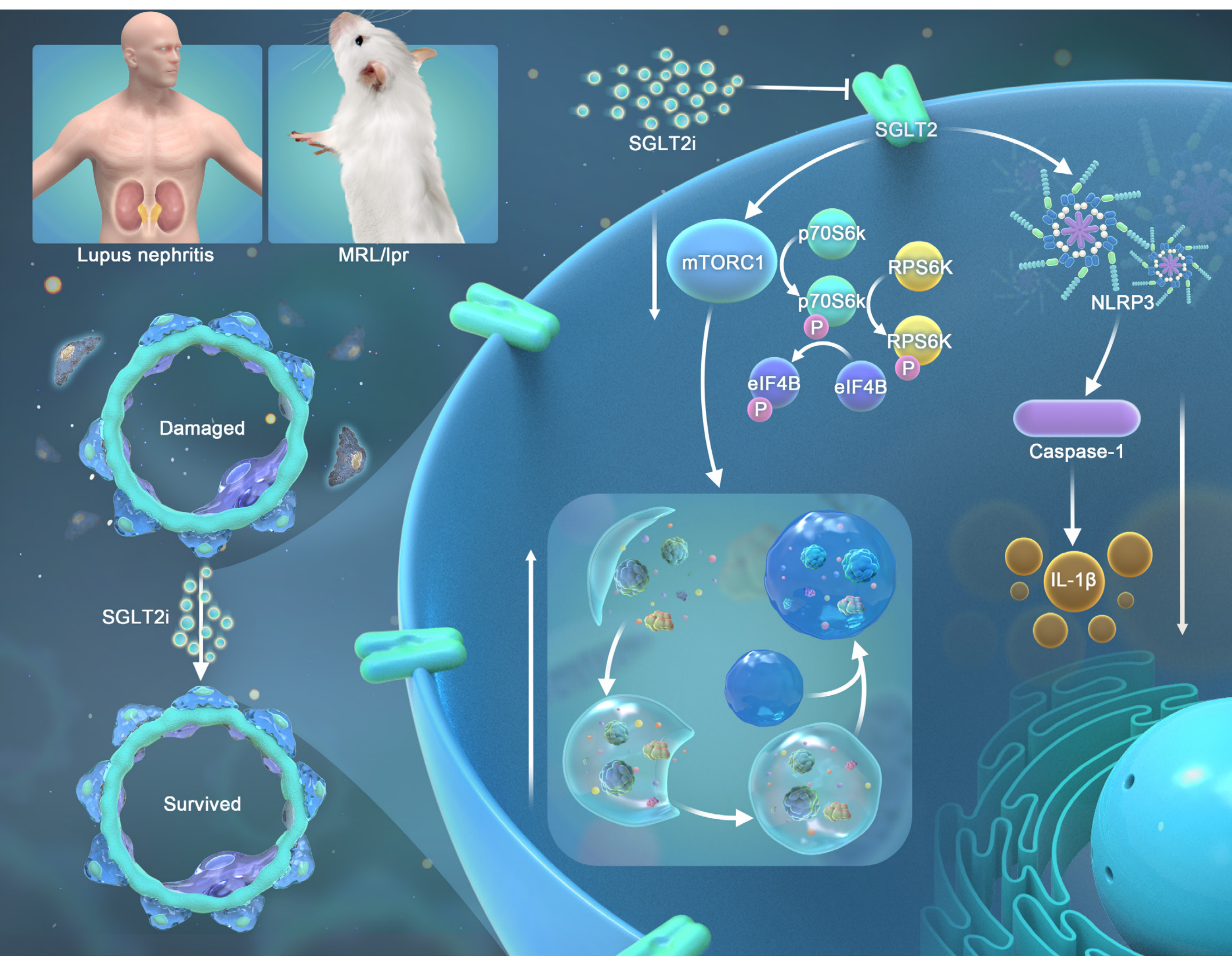

Figure 6 SGLT2 inhibitors alleviated podocyte damage in lupus nephritis by decreasing inflammation and enhancing autophagy.

downregulate autophagy and that using empagliflozin to inhibit the effect of SGLT2 elevated the level of autophagy and reduced podocyte injury in LN. By binding and blocking GLUT1 and GLUT4 within cardiac myocytes, empagliflozin can reduce intracellular glucose, which may inhibit mTORC1 signalling.[35 36] Meanwhile, mTORC1 activity can also be regulated by direct interaction between SGLT2 inhibitors and mTORC1 or sirtuin 1.[37 38] Further in-depth studies will be necessary to determine the mechanisms of mTORC1 activation in LN under the treatment with SGLT2 inhibitors.

Recently, renoprotective effects (with a special focus on podocyte) of SGLT2 inhibitors have been revealed in proteinuric non-diabetic nephropathy, membranous nephropathy and Alport syndrome.[15 39 40] Despite the local renoprotective effects on renal resident cells like podocytes, our data suggested that SGLT2 inhibitors might have systemic immunological regulation effect contributing to renal protection. In the present study, a significant decrease of dsDNA IgG and total IgG titres was observed, and less electron-dense deposits were confirmed by electron microscope in mice treated with empagliflozin. We speculated that SGLT2 inhibitors might have immunomodulatory effect of reducing dsDNA IgG, leading to less immune complex deposition in renal tissues attenuating renal impairment in LN. However, the expression patterns of SGLT2 in immune cells or the effects of SGLT2 inhibitors on systemic immunological regulation remain unknown and require further investigation.

Accumulated evidence also indicated that treatment with SGLT2 inhibitors prevented podocyte injury in diabetic mice, western diet-induced obese mice and proteinuric non-diabetic nephropathy mice.[15 41–44] Clinically, the application of SGLT2 inhibitors among patients with hereditary podocytopathies is also emerging, and satisfactory efficacy and safety performances have been observed.[25] Podocytes are a critical component of the glomerular filtration barrier. Podocyte injury is a major cause of proteinuria, which is associated with an increased risk of ESKD when poorly controlled. Of note, our results indicated that abnormally expressed SGLT2 played a pathogenic role in podocyte injury and that SGLT2 inhibitors mediated podocyte protection in LN. Moreover, the SGLT2 inhibitor empagliflozin could alleviate podocyte injury by attenuating inflammation and enhancing autophagy by reducing mTORC1 activity.

LN is one of the most severely affected organs of SLE, and the progression to ESKD increases disease mortality.[45] ESKD is also a risk factor for cardiovascular disease, which is a major cause of death in SLE.[46–48] Therefore, in addition to immunosuppressive therapies to improve immunological disorders, we are optimistic that SGLT2 inhibitors with strong renoprotective and cardioprotective effects will offer significant therapeutic benefits to

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patients with LN. In light of encouraging indications from case series and basic studies, more evidence from real-world or large clinical trials using SGLT2 inhibitors is expected in patients with LN.

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Contributors Conceived and designed the experiment: Y-yQ; performed the animal experiments: S-sL, L-jY, Y-hG, L-pH, H-dX and Y-cL; performed the cell experiments: X-yZ, Y-xH and FL; collected and analysed the clinical information: X-yZ and Y-yQ; analysed the data: Y-yQ, X-yZ, S-sL, FL and Q-mL; interpretation of the finding: Y-yQ and X-yZ. All the authors contributed to writing the manuscript. Y-yQ is responsible for the overall content as guarantor.

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Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by medical ethics committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-247). The participants gave informed consent to participate in the study before taking part. All experimental procedures were approved by the ethics committee of the Experimental Animal Center of Zhengzhou University (ZZU-LAC20210604).

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