The novel involvement of podocyte autophagic activity in the pathogenesis of lupus nephritis

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Summary. Background. Lupus nephritis (LN) is one of the most common and severe complications in Systemic lupus erythematosus patients, and the mechanism underlying the pathogenesis of LN is still unknown. Autophagy plays vital roles in maintaining cell homeostasis and is involved in the pathogenesis of many diseases. In this study, we investigated the role of autophagy in the progression of LN. Methods. Autophagic activities in podocytes of both LN patients (Class IV and V) and mice were evaluated. Podocytes were observed by electron microscopy, and autophagic activity was evaluated by immunofluorescence staining and western blot analysis. Apoptotic activity was evaluated by immunohistochemistry, TUNEL assays and flow cytometric analysis. Results. Significantly greater podocyte injury and discrepant autophagic levels were observed in LN patients. Differentiated mouse podocytes in the LN group showed reduced nephrin expression and increased apoptosis, as well as significantly higher levels of apoptosis-related proteins (cleaved caspase-3 and Bax). In the mice LN group, the increased number of autophagosomes was accompanied by increased LC3-II/LC3-I ratios and decreased p62, suggesting increased autophagic and apoptotic activity in podocytes. Blockade of autophagic activity by 3-MA or siRNA-mediated silencing of Atg5 resulted in decreases in LC3-II/LC3-I ratios, podocyte apoptosis and damage in the mice LN group. Furthermore, Rapamycin treatment increased LC3-II/LC3-I ratios, and enhanced LN-induced apoptosis in podocyte from modal animal. Conclusions. This study demonstrates that autophagic activity of podocytes is a crucial factor in renal injury by directly affecting the function of podocyte; thus, inhibiting this activity during the early stages of LN is implicated as a potential therapeutic strategy for delaying the progression of LN. Also, clinical application in LN needs to consider patients’ pathological type and drugs’ comprehensive effectiveness.

Key words: Lupus nephritis, Autophagy, Podocyte, Apoptosis, Rapamycin

Introduction

Systemic lupus erythematosus (lupus; SLE) is a chronic autoimmune inflammatory disease characterized by loss of tolerance against self-antigens, polyclonal autoantibody production and immune complex formation and deposition in different parts of the body, leading to damaging inflammation and multi-organ dysfunction (Lech and Anders, 2013). Lupus nephritis (LN) develops as a major complication of SLE, occurring in 50-70% of SLE patients during the first 5 years after diagnosis (Maroz and Segal, 2013). The presence of LN significantly reduces overall survival in patients with SLE. Renal damage is involved early in the course of lupus, therefore it is a major predictor of poor prognosis. Although the disease has been known for centuries, the pathogenesis remains unclear. The current
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Clinical treatments for LN rely mainly on immunosuppressants, which have much improved the response rates of acute manifestations. However, at high dose these agents are frequently associated with severe adverse effects, especially with long-term use. Consequently, additional and more effective therapeutic strategies and drugs are expected to modulate the aberrant immunity in LN with fewer side effects.

Podocytes are highly specialized epithelial cells that line the urinary surface of the glomerular capillary tuft in the kidneys, and are involved in ultrafiltration of blood. To maintain this function, podocytes oppose the high intraglomerular hydrostatic pressure forming a molecular sieve, secrete soluble factors to regulate other glomerular cell types, and synthesize the glomerular basement membrane. Impairment of any of these functions following podocyte injury results in proteinuria and possibly renal failure. It is widely accepted that loss of glomerular podocytes is a key feature of the progression of renal diseases, and several lines of evidence have confirmed the role of podocyte injury in the pathogenesis of LN (Kraft et al., 2005; Han et al., 2006). Chronic damaging stimuli, such as infection, metabolic factors and haemodynamic abnormalities, can induce podocyte oxidative stress, leading to podocyte foot-process effacement and loss. Prolonged podocyte injury leads to glomerulosclerosis and the progression of kidney disease (D’Agati, 2008).

Autophagy is a cellular process in which long-lived or aggregated proteins, defective organelles, and various soluble molecules are engulfed, digested and recycled (Mizushima, 2007). This precisely regulated process plays a vital role in maintaining cell homeostasis and is a typical protective, pro-survival response to the initiation of cellular damage. However, if hyperactivated, autophagy causes a type of cell death known as type II programmed cell death. This process has many physiological roles and is involved in the pathogenesis of diverse diseases, including LN (Levine and Kreemer, 2008). There is emerging evidence indicating the relationship between autophagy and lupus nephritis (Wang and Law, 2015). A Genome-wide association study in human revealed that autophagy-relative genes are associated with lupus nephritis (Zhou et al., 2015). Environmental risk factors for lupus nephritis can regulate the autophagy process (Tsokos, 2011). Meanwhile, autophagy can affect functions of diverse immune cells and the immune responses (Wang and Law, 2015).

Mammalian target-of-rapamycin (mTOR) is an evolutionarily conserved protein kinase that plays an important role in signal transduction. This molecule responds to environmental levels of amino acids, ATP, growth factors, and insulin, performing a critical role in the regulation of cell growth (Wu et al., 2013). Under normal conditions, the levels of mTOR in the glomerulus are extremely low, while abnormally high expression of mTOR is found in kidney lesions (Weide and Huber, 2011). Previous studies conducted in animal models of diabetic nephropathy and crescentic nephropathy have shown that mTOR is upregulated in damaged podocytes (Kraft et al., 2005; Huber et al., 2011; Inoki and Huber, 2012). In addition, rapamycin (a drug approved by the United States Food and Drug Administration for clinical use in the prevention of rejection after organ transplantation) binds specifically to mTOR to inhibit mTOR protein kinase activity leading to the activation of autophagy (Colman et al., 2009; Hartford and Ratain, 2007). While the clarification of the precise details of the role of rapamycin in kidney transplants continues to evolve, there is increasing recognition that substantial numbers of patients develop proteinuria when their treatment is changed to rapamycin (Bumbea et al., 2005; Kuypers, 2005).

In this study, we investigated the role of autophagy in the pathogenesis of LN by analyzing autophagic activities in podocytes of LN patients and mice. Also, the potential effects of autophagy on the progression of LN were assessed by either blocking or activating autophagic activity. These results obtained here suggest an important role of autophagy which contributed to the progression of LN.

Materials and methods

Reagents and antibodies

3-Methyladenine (3-MA) and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used: rabbit anti-synaptotodin, anti-Bax, anti-Bcl, and mouse anti-p62, anti-LC3, anti-Pi3K, anti-AKT, anti-mTOR, anti-Atg13, anti-caspase 3, and mouse anti-ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Patient selection and renal biopsies

All LN patients were diagnosed on the basis of renal biopsies carried out at the Department of Nephrology, Zhejiang Provincial People’s Hospital, Zhejiang, China. The patients were selected using the ISN/RPS 2003 classification of LN (Weening et al., 2004). Exclusion criteria were as follows: symptoms of obesity, diabetes mellitus, HBV infection, hepatitis or malignant tumors and patients undergoing continuous renal replacement therapy. A total of 60 LN patients (30 cases of lupus nephritis Class IV, 30 cases of lupus nephritis Class V) and 30 control patients were enrolled in the study. Control patients (with renal carcinoma) had no clinical features of kidney dysfunction, and their glomeruli were pathologically normal. According to the pathological subclassification, of 30 IV LN patients, 3 were IV-S(A), 25 were IV-G(A) and others were IV-G(A/C). Class V lupus nephritis showed advanced sclerosis which did not occur in combination with an active lesion of class III or IV. Control patients (with renal carcinoma) had no clinical features of kidney dysfunction, and their glomeruli were pathologically normal. A total of 60 LN
patients and 30 control patients were enrolled in the study. All protocols concerning the use of patient samples in this study were approved by the Human Subjects Committee of Zhejiang Provincial People’s Hospital. Informed written consent was obtained from all donors.

**Experimental animals**

C57BL/6 (control) and MRL/lpr (LN) mice (aged 8 weeks; body weight 18-20 g) were purchased from the Experimental Animal Center of Zhejiang University. Prior to the study, the animals were maintained under standard conditions with a 12-h/12-h day/night cycle and access to food and water ad libitum. All the mice were sacrificed after 4 weeks. The experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols using animals were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People’s Hospital.

**Podocyte preparation**

Murine glomeruli were resuspended in 2 ml digestion buffer and incubated for 40 min at 37°C on a thermomixer shaking at 1400/min. During this incubation period, the glomeruli were sheared with a 27-G needle. After 40 min, the solution was vortexed and the digestion result controlled by fluorescence microscopy. Samples were put on a magnetic particle concentrator again to eliminate beads and glomerular structures void of podocytes. The supernatant was pooled and the magnetic particles discarded. The cell suspension (2 ml) was sieved through a 40-mm pore size filter on top of a 50-ml Falcon tube, rinsed with 10 ml of HBSS. Cells were collected by centrifugation at 1500 r.p.m. for 5 min at 4°C, resuspended in 0.5 ml of HBSS supplemented with 0.1% bovine serum albumin plus 406-diamidino-2-phenylindole (1 mg/ml). Only viable cells were sorted (laser excitation 380nm, power 80mW). On average, 500,000 podocytes could be obtained per mouse. Efficiency could be easily monitored by evaluating small aliquots under a fluorescence microscope.

**Cell culture, RNA interference and cell transfection**

Mouse podocytes from C57BL/6 and MRL/lpr mouse were differentiated, identified using immunofluorescence methods and cultured as previously described (Wu et al., 2013). Podocytes were maintained in RPMI 1640 medium (Hyclone CA, USA,) supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Podocytes were propagated at 33°C in culture medium supplemented with 10 U/mL recombinant mouse interferon-γ (IFN-γ; PeproTech EC, London, UK) to enhance the expression of a thermosensitive T-antigen (permissive conditions). Subsequently, the cells were incubated for 7 days at 37°C in the absence of IFN-γ (non-permissive conditions) to induce differentiation. In all experiments, podocytes were used after 7-14 days in culture. For autophagy activation or inhibition experiments, cells were cultured in the presence of 3-MA (5 nM) or rapamycin (100 nM) for 48 h. To silence Atg5 expression by RNA interference, podocytes were transfected with siRNA using Lipofectamine 2000 in accordance with the manufacturer’s instructions. A non-specific scrambled siRNA sequence (siRNA-Scr) was used as a negative control.

**Periodic Schiff-methenamine (PASM) Staining**

Kidney samples were fixed with 10% buffered formalin overnight and embedded in paraffin. For histological assessments, sections (5 μm) were deparaffinized, hydrated, and stained with Periodic Schiff-methenamine (PASM).

**Transmission electron microscopy (TEM)**

Morphological analysis utilizing electron microscopy was known to be a reliable approach for monitoring autophagy (Sheng et al., 2014). Kidney samples of patients or MPC5 podocytes were washed twice with PBS and fixed with 2% paraformaldehyde/2% glutaraldehyde/0.5% CaCl₂ (pH 7.4) for more than 6 h; subsequently, they were washed in 0.1 M phosphate buffer, which was followed by 1% OsO₄ (in 0.1M PBS) fixation for 2 h. Then samples were washed and stained with 3% aqueous uranyl acetate for 1 h. After samples were washed again, they were dehydrated with a graded alcohol series, and embedded in Epon-Araldite resin (Canemco, 034). Ultrathin sections were cut by use of an ultramicrotome, stained with 2% (w/v) uranyl acetate and lead citrate, then examined with a Quantum Design LVEM5 transmission electron microscope (Zheng et al., 2008). Autophagosomes in podocytes were identified according to the morphology described previously (Klionsky et al., 2012). The number of autophagosomes was counted in approximately 30 randomly selected podocytes for each group of tissue samples (N=5 patients per group). The average number of autophagosomes per podocyte cell was determined.

**Immunohistochemical staining**

Renal cortex tissues were embedded in Tissue-Tek O.C.T. compound, snap-frozen in liquid nitrogen, and sectioned using a cryostat (Leica CM 3050S, Germany). For immunohistochemical studies, sections were blocked using 5% BSA in PBS and incubated with Beclin 1 antibodies (1:200) for two hours at 37°C followed by incubation with FITC-conjugated secondary antibodies for 30 min. All sections were examined by
immunofluorescence microscopy (Olympus FV1000, Tokyo, Japan).

**Immunocytochemical staining**

Cells were fixed in 4% paraformaldehyde on ice for 30 min, washed, and permeabilized using PBS containing 0.02% Triton X-100, followed by blocking with 5% BSA in PBS. Primary antibodies (1:200 dilution) were added to the cells and incubated for 2 h at room temperature. After extensive washing, cells were incubated with FITC-conjugated secondary antibodies (10 μg/mL) for 1 h at room temperature and then washed. Images were acquired by immunofluorescence microscopy (Olympus FV1000, Tokyo, Japan). The number of LC3-positive punctate signals in the cells was analyzed using the software equipped for immunofluorescence microscopy (FV1000, Olympus, Tokyo, Japan).

**TUNEL assay**

Briefly, after cultivation in growth medium and conditioned media for 24 h, podocytes were washed with PBS and fixed with 4% paraformaldehyde for 30-60 min. After washing once with PBS, the cells were incubated in 0.1% Triton X-100 for 2 min and then in 50 μL TUNEL enzyme and TUNEL label mixture for 1 h at 37°C in a humidified atmosphere in the dark. The cells were washed three times with PBS and then visualized by microscopy (FV1000, Tokyo, Japan).

**Annexin V-FITC/PI apoptosis detection**

Briefly, 1×10⁵ cells were resuspended in 100 μL binding buffer and incubated with Annexin V-FITC and PI for 15 min in the dark. Cellular apoptosis was analyzed with a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA) as previously described (Li et al., 2011) using CellQuestTM Pro software (BD Biosciences).

**Western blotting**

Bax, Bcl, p62, LC3, PI3K, AKT, mTOR, Atg13 and cleaved caspase-3 protein levels were detected by Western blotting analysis with the corresponding antibodies.

The cells were lysed in RIPA buffer (50 Mm Tris-Cl [pH 7.6], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.5% Triton X-100) containing 1 mg/mL protease inhibitors (leupeptin, aprotinin and antipain), 1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. The protein concentration was measured using the Bradford assay. Proteins (total 50 mg) were separated by 6-15% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skimmed milk, the membranes were probed with primary antibodies overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody. Band intensities were quantified using WCIF Image J software, and the results were expressed relative to control (Zhu et al., 2009; Li et al., 2011).

**Cell viability assay**

The viability of cells was measured using CCK-8 assays. Briefly, cells were seeded in 96-well plates (1×10⁵/well) and incubated overnight. The cells were then treated with growth medium and conditioned media for specified time-periods. After treatment, the medium was replaced with 10% CCK-8 reagent dissolved in PBS and cells were incubated for a further 3 h at 37°C in the dark. Absorbance at 450 nm was measured with a Multiskan FC Microplate Reader (Thermo Scientific, USA) and the results were normalized to those of untreated cells.

**Statistical analysis**

All statistical analyses were performed using SPSS 19.0 software (Stanford University, Stanford, CA, USA). Results are expressed as mean ± standard deviation. Group means were compared using Student’s t-test for independent data. All P-values were two-tailed and P<0.05 was considered to indicate statistical significance.

**Results**

**Demographic and clinical characteristics of patients with lupus nephritis**

According to the ISN/RPS 2003 classification of LN, we divided the LN patients into two pathological types, class IV and class V. The mean age of the IV (3 males and 27 females) and V (4 males and 26 females) LN patients was 29.7±11.5 years and 33.3±10.5, respectively. The demographic and clinical data for these patients are listed in Table 1. According to the statistics of the patients manifestations in Table 2, of 30 IV LN patients, 24 had hematuria (80%) and most of the IV and

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>IV (n=30)</th>
<th>V (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>4</td>
<td>1.000</td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td>26</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.7±11.5</td>
<td>33.3±10.5</td>
<td>0.344</td>
</tr>
<tr>
<td>Occult nephritis</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>16</td>
<td>9</td>
<td>0.067</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>11</td>
<td>21</td>
<td>0.010</td>
</tr>
<tr>
<td>Rapidly progressing glomerulonephritis</td>
<td>3</td>
<td>0</td>
<td>0.237</td>
</tr>
<tr>
<td>Podocyte fusion</td>
<td>42.0±12.5</td>
<td>60.7±16.8</td>
<td>0.002</td>
</tr>
</tbody>
</table>
V LN patients median 24-h urine protein exceed standard. The serum autoantibody manifestations of patients are listed in Table 3. A large number of autoantibodies emerged in the progress of LN.

**Podocyte autophagic level differs among the different pathological types**

The kidney biopsy is the gold standard to determine the pathological type of LN. Compared with control biopsies (Fig. 1A), IV LN samples showed significant endothelial cell proliferation and capillary fusion, while V LN samples showed a macroscopic complex precipitation, suggesting much more severe podocyte injury.

To explore the potential role of autophagic level in lupus nephritis, we examined the number of podocyte autophagosomes in controls and LN patients by electron microscopy (EM). EM analysis showed entirely different autophagy manifestation in control and LN samples (Fig. 1B,C). The numbers of autophagosomes were significantly increased in IV LN compared with controls. However, the situation in V LN was inverse.

Further confirmation was obtained by immunohistochemical staining of Beclin 1, a major autophagy regulator (Mizushima et al., 2010), and Immunofluorescence staining of LC3. IV and V LN samples displayed higher and lower levels of Beclin 1 in podocytes than those observed in controls, respectively (Fig 1D). In addition, distended Bowman’s space and widespread vacuolization of podocytes were seen in LN samples. Meanwhile, IF results from double-staining with anti-nephrin and anti-LC3 antibodies were consistent with EM and IHC findings (Fig. 1E)

**Podocyte autophagic activity correlates positively with the podocyte injury and apoptosis in mice with lupus nephritis**

We then investigated the role of autophagic activity in podocyte injury by differentiated mouse podocytes from C57BL/6 (control group) and MRL/lpr (LN group) mice through measuring the expression of podocyte marker proteins, nephrin. Compared with the control group, reduced nephrin levels and increased podocyte apoptosis were observed in the LN group (Fig. 2A,B). This was confirmed by FITC-Annexin V/PI double-staining, which showed significantly higher levels of apoptosis in the LN group (P<0.001 vs. control group: Fig. 2C). Furthermore, Western blot analysis revealed increased apoptosis in podocytes from the LN group with significantly higher levels of cleaved caspase-3, Bax and lower levels of Bcl-2 (P<0.001; Fig. 2D).

We next characterized autophagic activity in podocytes of the control and LN group by analyzing the number of autophagosomes, p62 levels, and LC3-II/LC3-I ratios. LC3 staining (Mizushima et al., 2010) and EM evaluation revealed greatly increased number of autophagosomes in podocytes in the LN group compared with those in the control group (Fig. 3B). Furthermore, Western blot analysis showed significantly increased LC3-II/LC3-I ratios and decreased p62 levels in podocytes from the LN group (P<0.05; Fig. 3C vs. control group). Taken together, these results suggest that LN activates autophagy and increases apoptosis in podocytes.

**Regulation of LN progression by podocytes autophagy**

To determine the effect of podocytes autophagy on LN progression, we first assessed apoptosis of LN-induced podocyte injury by blocking autophagic activity either by siRNA-mediated silencing of Atg5 or treatment with the autophagy inhibitor 3-MA (Chen et al., 2011; Shimizu et al., 2004) (Fig. 4). Western blot revealed decreased LC3-II/LC3-I ratios in podocytes from the LN group, indicating that 3-MA impaired autophagic activity in LN podocytes (Fig. 4A). These results were associated with a decrease of apoptotic marker expression (Fig. 4A). FITC-Annexin V/PI double-staining of apoptotic cells showed that the blockade of podocyte autophagy by 3-MA treatment led to a robust reduction in LN-induced podocyte apoptosis (Fig. 4B). In addition, CCK-8 assays indicated that this autophagy blockade alleviated LN podocyte damage (Fig. 4C).

Table 2. Renal manifestations of patients.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>IV (n=30)</th>
<th>V (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>14 (46.7)</td>
<td>6 (20.0)</td>
<td>0.028</td>
</tr>
<tr>
<td>Hematuria</td>
<td>24 (80.0)</td>
<td>10 (33.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macrohematuria</td>
<td>4 (13.3)</td>
<td>0</td>
<td>0.112</td>
</tr>
<tr>
<td>Urine proteins≥0.5g/d</td>
<td>28 (93.3)</td>
<td>28 (93.3)</td>
<td>1.000</td>
</tr>
<tr>
<td>sCr≥1.3mg/dl</td>
<td>10 (33.3)</td>
<td>2 (6.7)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

For each data in the table, the number outside the parentheses was the number of the patients positive for the specific manifestation; the number inside the parentheses was the percentage of the patients positive for the specific manifestation in each classification group.

Table 3. The serum autoantibody manifestations of patients.

<table>
<thead>
<tr>
<th>Serum data</th>
<th>IV (n=30)</th>
<th>V (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>28 (93.3)</td>
<td>22 (73.3)</td>
<td>0.038</td>
</tr>
<tr>
<td>A-dsDNA</td>
<td>20 (66.7)</td>
<td>8 (26.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>A-Sm</td>
<td>8 (26.7)</td>
<td>6 (20.0)</td>
<td>0.542</td>
</tr>
<tr>
<td>A-RNP</td>
<td>8 (26.7)</td>
<td>6 (20.0)</td>
<td>0.542</td>
</tr>
<tr>
<td>ANCA</td>
<td>2 (6.7)</td>
<td>0</td>
<td>0.492</td>
</tr>
<tr>
<td>SSA</td>
<td>10 (33.3)</td>
<td>12 (40.0)</td>
<td>0.592</td>
</tr>
<tr>
<td>SSB</td>
<td>4 (13.3)</td>
<td>4 (13.3)</td>
<td>1.000</td>
</tr>
<tr>
<td>Low C3</td>
<td>24 (80.0)</td>
<td>16 (53.3)</td>
<td>0.028</td>
</tr>
<tr>
<td>Low C4</td>
<td>14 (46.7)</td>
<td>4 (13.3)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

For each data in the table, the number outside the parentheses was the number of the patients positive for the specific manifestation; the number inside the parentheses was the percentage of the patients positive for the specific manifestation in each classification group.
Podocyte autophagy involved in lupus nephritis

Fig. 1. Podocyte autophagic activity in controls and LN patients. A. Histopathological features of control and LN patient kidney biopsies. B. Autophagosomes (arrows) in the podocytes of controls and LN patients detected by electron microscopy. C. Quantification of podocytes containing autophagosomes in kidney sections from LN patients and controls. D. Representative images of immunohistochemical staining of Beclin 1. E. Immunofluorescence staining of nephrin (red) and LC3 (green). × 400.
FITC-Annexin V/PI double-staining showed that transfection with Atg5-specific siRNA (Fig. 4D) resulted in a similar decrease in LN-mediated cell apoptosis. These results demonstrate that autophagy plays a critical role in LN-induced podocyte injury.

To further characterize the role of autophagic activity in damaging podocytes in LN-induced injury, we treated podocytes with rapamycin, which is an inhibitor of mTOR signaling (Cao et al., 2006; Iwamaru et al., 2007; Lin et al., 2013) used widely to increase autophagic activity. Rapamycin treatment decreased LC3-II/LC3-I ratios in podocytes in the LN group.

Fig. 2. Increased podocyte apoptosis in LN mice. A. Representative image of immunofluorescence staining of nephrin. B. TUNEL staining of apoptosis in podocytes in the control and LN groups. C. FITC-Annexin V/PI double-staining of apoptosis in podocytes in the control and LN groups. D. Western blotting analysis of Bax, Bcl-2, and cleaved caspase-3 protein expression in podocytes in the control and LN groups. ***P<0.001. x 400.
indicating increased autophagic activity (Fig. 4A). FITC-Annexin V/PI double-staining showed that rapamycin-mediated activation of podocyte autophagy led to a robust enhancement of LN-induced podocyte apoptosis (Fig. 4B). A similar effect of rapamycin treatment was observed in Western blot analysis of the expression of apoptosis-related proteins (Fig. 4A).

Western blot analysis of mTOR signaling pathway proteins revealed decreased levels of PI3K, p-Akt, and p-mTOR in the LN group (Fig. 4E), indicating that the damaging effect of rapamycin on podocytes is mediated by inhibition of mTOR signaling.

**Discussion**

It is undisputed that both IV and V LN patients evidence podocytes injury (Fig. 1A). We found that autophagic level in podocytes from IV LN patients’ glomeruli was much higher than controls (Fig. 1), which is consistent with previous reports that injured podocytes display a relatively high level of autophagic activity (Asanuma et al., 2003; Hartleben et al., 2010; Mizushima and Komatsu, 2011). Furthermore, our study discovered that autophagy activity in podocytes was significantly correlated with the pathological types and autophagosome numbers in patients with V LN were significantly lower than controls. On one hand, in order to relieve environmental stress, podocytes from control patients (with renal carcinoma) may experience a relatively higher basal level of autophagic activity than the healthy (Bijian and Cybulsky, 2005). On the other hand, Yang et al. (Stylianou et al., 2011) performed a study showing that autophagic activity experienced a decrease in the progression of heymann nephritis which shows a similar pathological feature with V LN, indicating autophagic level has the ability to adjust itself to the pathological type and progression. The potential for this discrepancy requires further investigation.
Fig. 4. Activation of podocyte autophagy enhances LN-induced podocyte injury and apoptosis. 
A. Activation of LN-induced podocyte injury by treatment with rapamycin (RAP) or 3-methyladenine (3-MA). Podocyte autophagic activity and apoptosis were assessed by Western blot analysis. 
B. Flow cytometric analysis of podocyte apoptosis by FITC-Annexin V/PI double-staining. 
C. Cell viability of podocytes treated with RAP or 3-MA. 
D. Flow cytometric analysis of podocyte apoptosis after transfection with Atg5-siRNA. 
E. Cell viability of podocyte after transfection with Atg5-siRNA. 
F. Relative expression of PI3K, Akt, mTOR, p-mTOR, and Atg13 proteins in podocytes in the control and LN groups (*P<0.05, **P<0.01, ***P<0.001).
Therefore, our results suggest that the level of autophagic activity is related to the progression of podocyte injury in LN patients. Maintaining podocyte health may provide new therapeutic strategies for lupus nephritis.

To clarify the role of autophagy in podocyte injury of LN, we characterized LN-induced podocyte autophagic activity and its damaging effects using differentiated podocytes C57BL/6 (normal) and MRL/lpr (LN) mice. Related data suggest that autophagy was activated together with increased apoptosis in podocytes of LN mice (Figs. 2, 3). These results indicate that autophagy is involved in regulating podocyte injuries. Results from modal animals are similar to IV LN patients samples. It is possibly because MRL/lpr is a common LN modal and pathological feature of V LN is closer to MN. In addition, MRL/lpr mice fail to reflect the degree of LN. Nevertheless, molecular mechanisms of podocyte injuries are not fully clarified, while ER stress, mitochondrial damage, and oxidative stress are implicated in most types of glomerulosclerosis (Bijian and Cybulsky, 2005; Cybulsky, 2013). Further studies focused on ER stress or oxidative stress would provide more mechanistic insight into podocyte injuries and LN progression.

On the other hand, autophagy inhibitors, such as 3-MA and Atg5 siRNA, alleviated podocyte apoptosis, and increased podocyte proliferation, whereas autophagy enhancers rapamycin had the opposite effects in mice (Fig. 4). In addition, PI3K/Akt/mTOR signaling in podocytes of LN mice was abnormally activated, which is one of the most important signalings for the regulation of autophagy and is associated with murine LN progression (Stylianou et al., 2011). Together, results from modal mice suggest that podocyte autophagy is initiated by various factors and is involved in podocyte injury and promoting the progression of LN.

The current treatments for lupus nephritis are mainly dependent on glucocorticoids and immunosuppressants (Chan, 2015). Many drugs for lupus can act as autophagy regulators, however their effects appear paradoxical on the autophagy process. Glucocorticoids can induce autophagy (Harr et al., 2010). Chloroquines (CQ), which are first regarded as antimalarial agents, also present numerous immunomodulatory effects and belong to the first-line therapies for lupus. However, they belong to autophagy inhibitors (Wang et al., 2015).

Activation of the mTOR pathway has been detected in LN and has emerged as a central pathway involved in the pathogenesis (Perl, 2015). Rapamycin is essential not only for the identification of mTOR, but also for elucidating mTOR-dependent signaling events and their role in metabolism and disease (Ballou and Lin, 2008; Rodriguez Perez, 2011). Therefore, the use of this drug for inhibiting the mTOR pathway may serve as a new basis for the treatment of podocyte injury in renal disease. However, our results are in conflict with previous reports showing that treatment with rapamycin and other mTOR inhibitors has displayed protective and beneficial effects in murine LN (Lui et al., 2008; Reddy et al., 2008) and also in patients with LN (Yap et al., 2012). In the present cell experiment, we found that rapamycin treatment promoted podocyte apoptosis and exacerbated podocyte injury (Fig 5). However, the effect on MRL/lpr lupus murine model explained only by autophagy? Some studies discovered the number of podocyte autophagosomes in diabetic mice was decreased. Rapamycin promotes podocyte autophagy and ameliorates renal injury in diabetic mice and other studies found that high glucose concentrations promote autophagy flux in podocyte cultures (Lenoir et al., 2015). In our study, we only treated podocytes with rapamycin in vitro, and there may be some inconsistency between in vitro and in vivo. When it comes to human or mice, the situation is complicated. On one hand, rapamycin generates podocyte injury directly through autophagy. On the other hand, rapamycin acts as an immunosuppressant, which will also benefit patients with lupus nephritis. That is, the clinical application of rapamycin mainly relies on the function of immunosuppression. Our data also well explain why the side effect of rapamycin is proteinuria in clinical practice (Marti and Frey, 2005; Cina et al., 2012).

In conclusion, this study demonstrates that autophagic activity of podocytes is a crucial damaging factor in renal injury; thus, inhibiting this activity during the early stages of LN is implicated as a potential therapeutic strategy for delaying the progression of LN. There is emerging evidence indicating the relationship between autophagy and lupus nephritis. GWAS in human studies reveals autophagy-relative genes associated with lupus nephritis (Graham et al., 2009). Environmental risk factors for lupus nephritis can regulate the autophagy process. Autophagy can affect functions of diverse immune cells and the immune responses. The widely-used first-line drugs and innovative drugs for treatment of lupus nephritis can function as autophagy regulators. Meanwhile, clinical treatment in LN is a complex process that should consider pathological types and overall effect together.

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Authors’ contributions. Jianguang Gong and Qiang He: designed the study. Li Zhao and Yiwen Li: collected samples and clinical information. Wenli Zou and Wei Shen: performed the laboratory assays. Jianguang Gong: performed the statistical analyses, Juan Jin and Meiyu Ye wrote the manuscript. The final version of the manuscript was approved by all
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