

Original Paper

Triptolide Attenuates Inflammatory Response in Membranous Glomerulonephritis Rat via Downregulation of NF- κ B Signaling Pathway

Ying Zhou Yan Hong Haihua Huang

Wuxi No.2 People's Hospital, Wuxi, China

Key Words

Triptolide • Inflammatory • Glomerulonephritis • Signaling pathway

Abstract

Background/Aims: Triptolide (TPL), a main active ingredient of *Tripterygium wilfordii* has been shown to exert anti-inflammatory effect. The role of TPL on glomerular diseases remains unclear. **Methods:** This study aims to investigate the potential anti-inflammatory effect of TPL in rats with membranous glomerulonephritis (MGN). **Results:** Our data showed that the pathological kidney damage was significantly alleviated by TPL treatment in MGN rats. We also found that MGN rats exhibited significantly higher ($p < 0.01$) level of inflammatory cytokines (TNF- α , IL-1 β and MCP-1) than those in normal group, while these inflammatory cytokines levels were significantly reduced in TPL treatment group compared with model group. Additionally, we found that TPL treatment could significantly decrease the malondialdehyde (MDA) level while enhanced superoxide dismutase (SOD) activity. Meanwhile, we also found that I κ B kinase inhibitor (IMD-0354) could significantly reduce the accumulation of inflammation damage and oxidative lesions. Furthermore, we observed that both TPL and IMD-0354 treatment could block I κ B α degradation and suppress mRNA and protein level of nuclear factor (NF)- κ B p65. **Conclusion:** Together, all above results suggest that inflammatory response could be attenuated by TPL and this is partly due to the inhibition of NF- κ B signaling pathway.

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Introduction

Membranous glomerulonephritis (MGN) is one type of nephrotic syndrome. MGN is commonly characterized histopathologically by thickened glomerular basement membrane (GBM) and subepithelial deposition of immune complexes [1, 2]. Chronic inflammation,

oxidative stress, glomerular and tubulointerstitial lesions are common characteristics of MGN [3, 4]. Generalized edema and asymptomatic proteinuria are clinical presentations of MGN. Approximately 40% of the idiopathic MGN patients exhibit deteriorated renal function and end-stage renal disease [5, 6]. Unfortunately, no specific treatment has been developed to slow the progression of MGN.

Triptolide (TPL) is extracts of *Tripterygium wilfordii* and frequently used to treat inflammatory and/or autoimmune disease, e.g., systemic lupus erythematosus, nephritis, psoriasis, and rheumatoid arthritis [7]. It has been reported that TPL exerts novel chondroprotective and anti-inflammatory effects on rheumatoid arthritis [8]. A clinical study has demonstrated the effect of TPL against rheumatoid arthritis [9]. A larger clinical study echoed the results from the previously mentioned study [10]. Among all the natural anti-inflammatory/immunomodulating product, TPL is one of the most powerful and broadly active ever discovered [11]. It has been reported nanomolar level of TPL could inhibit the various cellular targets such as inflammatory cytokines [12], transcription factors [13] and inducible nitric oxide synthase [14]. Additionally, it has been well known that TPL could inhibit the proliferation of mesangial cell and prolongate renal fibrosis [15]. One recent study showed that TPL displayed a protective effect against renal fibrosis [16]. However, the role of TPL against MGN remains unclear.

In this study, we investigated the effect of TPL on the inflammatory response in MGN rats and further explored its possible anti-glomerulonephritis mechanism.

Materials and Methods

Chemicals and reagents

Aminoguanidine (AG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The superoxide dismutase (SOD) and malondialdehyde (MDA) assay kits were obtained from Nanjing Jiancheng institution of Biotechnology (Nanjing, China). Pierce® BCA Protein Assay Kit was provided by Thermo Scientific. TPL (Zelang, Nanjing, China). IMD-0354 (Tocris Bioscience, Bristol, UK).

Preparation of cationic bovine serum albumin (C-BSA)

C-BSA was prepared as previous report [17]. Ethylenediamine (EDA) solution was prepared by mixing 67 ml of EDA in 500 ml of distilled water, followed by adding 350 ml of 6 N HCl in order to adjust the pH to 4.75, and the solution was cooled to 25 °C in an ice bath. Twenty five milliliters 20% BSA solution was then added to EDA solution, followed by adding 1.8 g of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). With continuous stirring, the reaction was maintained at 25 °C and pH 4.75 for another 120 minutes, then stopped by adding 30 mL of 4 M acetate buffer (pH 4.75). The final solution was dialyzed against distilled water for 48 hours at 4 °C before lyophilized. The final product was stored at -80 °C until further use.

Animal model and treatment

Sixty adult male Sprague-Dawley (SD) rats (weighing 200-250 g) were used in this study. Animals were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and kept in the Animal Resource Facility. All animal-involving procedures were performed in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The animal protocol in this study was approved by the Animal Ethics Committee at Wuxi No.2 People's Hospital, China. Animals were anesthetized using sodium pentobarbital together with all efforts to minimize the suffering in all procedure throughout the study. All animals were housed at 23±2 °C with standard rat chows and ad libitum access to tap water. Firstly, MGN were induced using C-BSA administration for 4 weeks in all rats except 10 rats from the normal group. C-BSA was daily injected intraperitoneally at an increasing dosage from Day 1 to Day 7 (1 mg, 1 mg, 1 mg, 1.5 mg, 1.5 mg, 2 mg and 2 mg.) followed by constant dosage of 2.5 mg for the next 3 weeks. Bradford assay (Bio-Rad, Hercules, CA, USA) was used to confirm the 24 hour proteinuria to ensure that the model was successful. Rats that were successful induced MGN were divided randomly into three groups (10 per

group): MGN model (group A); TPL (0.2 mg/kg/d in 0.9% saline, p.o.) (group B); I κ B kinase inhibitor, IMD-0354 (10 mg/kg/d in 0.9% saline, i.p.) (IMD group C). Rats in normal and model groups were administered saline for 4 weeks at dosage of 10 ml/kg/d. In the meantime, rats of the drug group and model group were daily administered 2.5 mg C-BSA through tail vein injection.

Measurements for oxidative stress

At the end of experiment, animals were euthanized and blood samples and renal tissue samples were collected as previously described [18]. Blood were collected from abdominal aorta, followed by centrifugation at 3,500 rpm at 4°C for 15 minutes. After centrifugation at 3500 rpm at 4°C for 15 minutes, serum was used obtained and used for the following biochemical analysis. The MDA and SOD activity was determined using commercial available assay kits according to manufacturer's instructions. The SOD activity was assessed using a xanthine oxidase method and the value was determined by a microplate reader (Thermo Fisher Scientific, USA). The MDA content was detected by thiobarbituric acid reactive substance (TBARS) test and final reading was taken using a spectrophotometer (PERSEE, T6-1650E). The SOD activity and MDA content were recorded as U/ml and nmol/ml accordingly.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from renal tissue using Trizol (Invitrogen, CA, USA) according to manufacturer's instruction. The gene expression was analyzed using the TaqMan miRNA assays (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Briefly, 10 ng of total RNA was reversed transcribed into cDNA using TaqMan specific primers (Applied Biosystems). Then real-time PCR was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The gene expression levels of all cDNA samples were normalized by using 18S rRNA. The mean levels of the target gene/18S rRNA gene in normal glomeruli were arbitrarily defined as 1.0, and quantitative data were shown as the relative increase/decrease from control glomeruli. The primers used in PCR reaction are below: TNF- α forward 5'-CGA TTT GCC ACT TCA TAC-3' and reverse 5'-GAC TCC GTG ATG TCT AAG-3'; IL-1 β forward 5'-AAT CTC ACA GCA GCA TCT C-3' and reverse 5'-AGC AGG TCG TCA TCA TCC-3'; MCP-1 forward 5'-GCA TCC ACT CTC TTT TCC AC-3' and reverse 5'-AGG CAT CAC ATT CCA AAT CAC-3'; NF- κ B p65 forward 5'-ACG ATC TGT TTC CCC TCA TCT-3' and reverse 5'-TGG GTG CGT CTT AGT GGT ATC-3'; NF- κ B p50 forward 5'-AAC GCA TCC CAA GGT GCT GGAA-3' and reverse 5'-GCA GCT GGA AAA GCT CAA GCCA-3'; I κ B α forward 5'-AAG GAC GAG GAT TAC GAG CAG-3' and reverse 5'-CCC TTC ACC TGA CCA ATC ACT-3'; IL-6 forward 5'-GAC AAC TTT GGC ATT GTGG -3' and reverse 5'-ATG CAG GGA TGA TGT TCTG -3'. The relative expression was analyzed using the comparative Ct method.

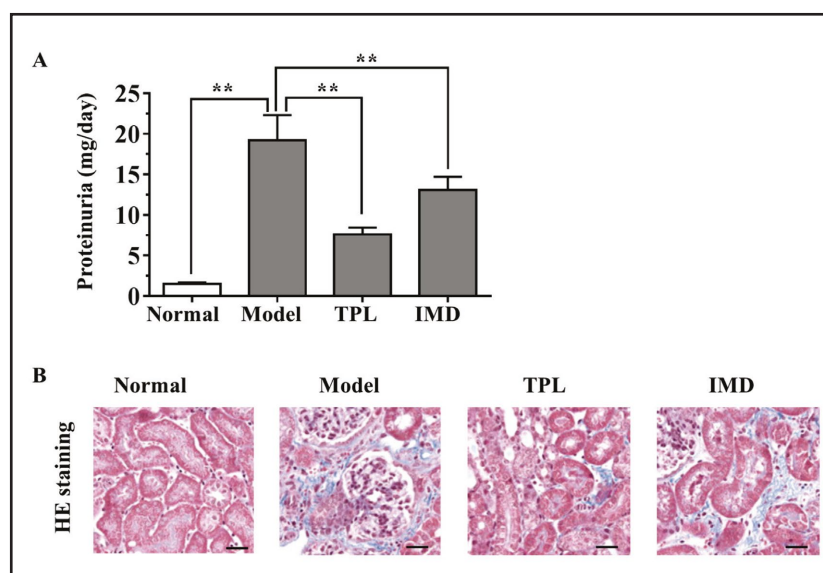
Western blot analysis

The protein levels of phospho-p65 and phospho-I κ B α in renal tissues were determined by Western blotting. β -Actin (Dilution 1:5000) (Santa Cruz, CA) served as an internal control. Briefly, the protein concentration was determined using the BCA method. Equal quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting onto a nitrocellulose membrane. Membranes were incubated with the primary antibodies, including anti-phospho-p65 (Dilution 1:1000), anti-phospho-I κ B α (Dilution 1:2000), anti-p65 (Dilution 1:1000), anti-p50 (Dilution 1:2000) and anti-I κ B α anti-p65 (Dilution 1:2000) (Santa Cruz, CA, USA) at 4°C overnight. After additional TBST washes, membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 hr at room temperature and detected by the enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). Quantitation of signal intensities was performed by densitometry on a Xerox scanner using NIH IMAGEJ software.

Immunohistochemistry

After fixation, paraffin kidney sections (4 μ m thickness) were deparaffinized in 100% xylene and rehydrated in descending ethanol series and water according to standard protocols. Following rehydration, antigen retrieval was carried out by placing the slides in 10 mmol/L sodium citrate buffer (pH 6.0) at 100 °C for 2 min followed by 20 min cooling. Endogenous peroxidase activity and non specific antigens were blocked by peroxidase blocking reagent containing 3% hydrogen peroxide and serum. For HE staining, the sections

Fig. 1. Effect of TPL on proteinuria and glomerular pathomorphology in MGN rats. (A) The 24 h urine collections were taken and proteinuria was detected by the Bradford method. The data are presented as the mean \pm SD from three experiments. $**p < 0.01$ versus model group. (B) The HE staining of the renal tissue from different groups (normal, model, TPL and IMD). Scale bars are 50 μ M.



were stained with hematoxylin for 3 min, washed and then stained with 0.5% hematoxylin-eosin (HE) for another 3 min. The morphological changes in the kidney were blindly evaluated under a light microscope (TE2000, Nikon, Japan) by an experienced pathologist. For immunohistological staining, kidney samples were incubated with antibodies phospho-NF- κ B p65 and phospho-I κ B α (Santa Cruz, CA, USA) overnight at 4 °C. After washing, the sections were incubated with biotin-labeled rabbit anti-goat antibody for 15 min at room temperature, and subsequently were incubated with streptavidin-conjugated horseradish peroxidase (Maixin, Fuzhou, China). The sections were further incubated with 2,4-diaminobenzidine substrate and counterstained with hematoxylin and analyzed using a bright field microscope.

Statistical analysis

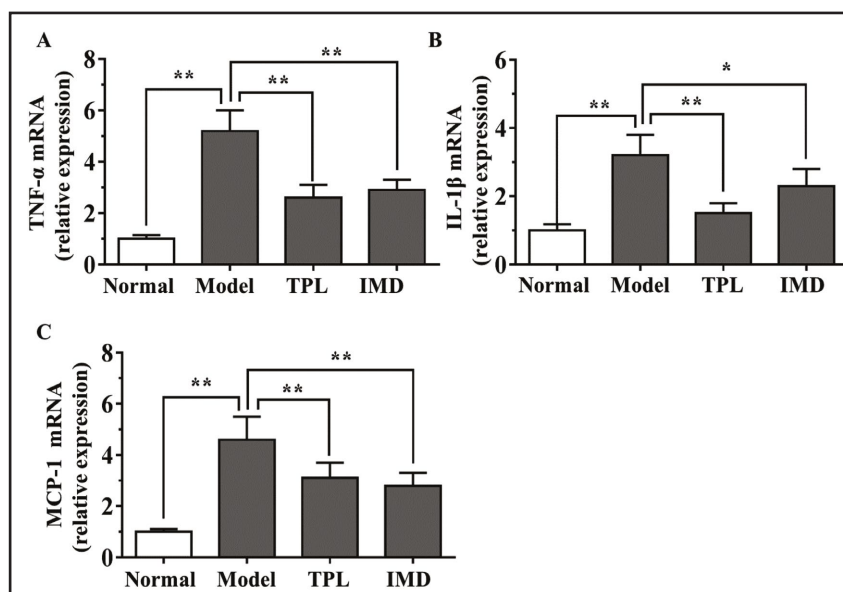
Data were expressed as mean \pm standard deviation unless otherwise indicated. Statistical analysis was performed using SPSS ver. 15.0 (SPSS, Chicago, IL, USA). Differences were determined using one-way analysis of variance (one-way ANOVA). The test was considered significant when $p < 0.05$.

Results

TPL decreased proteinuria excretion and ameliorated glomerular pathomorphology in MGN rats

Twenty four hours proteinuria was found significantly increased in animals with nephritis induction compared with those in normal group; however, TPL treatment significantly reduced the proteinuria excretion (Fig. 1A). It has been well recognized that NF- κ B is actively involved in regulation of expression of many pro-inflammatory genes upon immune response [19]. To see whether the effect of TPL was associated with NF- κ B activity, we designed IMD-0354 (an I κ B kinase inhibitor) treatment group. IMD-0354 has been reported to exert antiproteinuric effect through restoring the downregulated nephrin, synaptopodin, nephrin and synaptopodin proteins [20]. We found that IMD-0354 treatment also significantly reduced the proteinuria excretion (Fig. 1A). Histopathology examination were also done as shown in Fig. 1B. Histological examinations revealed damage in renal tissue of C-BSA-treated rats, while no obvious damage was found in rats from normal group. Interestingly, renal damage was ameliorated in the rats from TPL and IMD groups. Our data suggested that TPL could effectively alleviate the pathological kidney damage in C-BSA-induced MGN rats.

Fig. 2. Effect of TPL on the expression of pro-inflammatory cytokines. Glomerular mRNAs for inflammatory cytokines (TNF- α , IL-1 β , and MCP-1) in the MGN rats were determined by qRT-PCR analysis. The data are presented as the mean \pm SD from three experiments. * p < 0.05; ** p < 0.01 versus model group.



TPL decreased the expression of pro-inflammatory cytokines

The effect of TPL on various pro-inflammatory cytokines in C-BSA-induced MGN rats were assessed using qRT-PCR. Our data showed that glomerular mR-

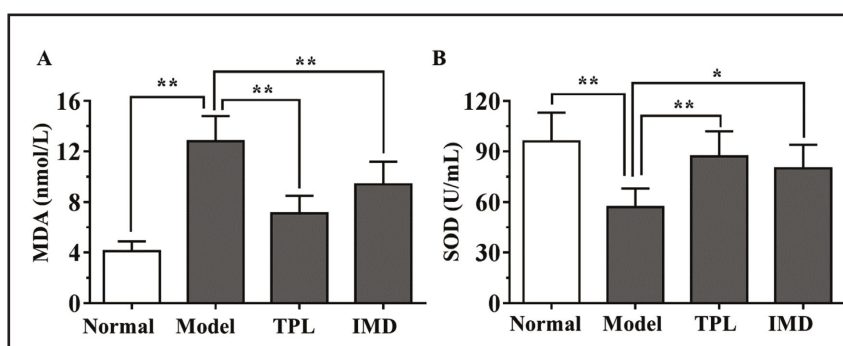


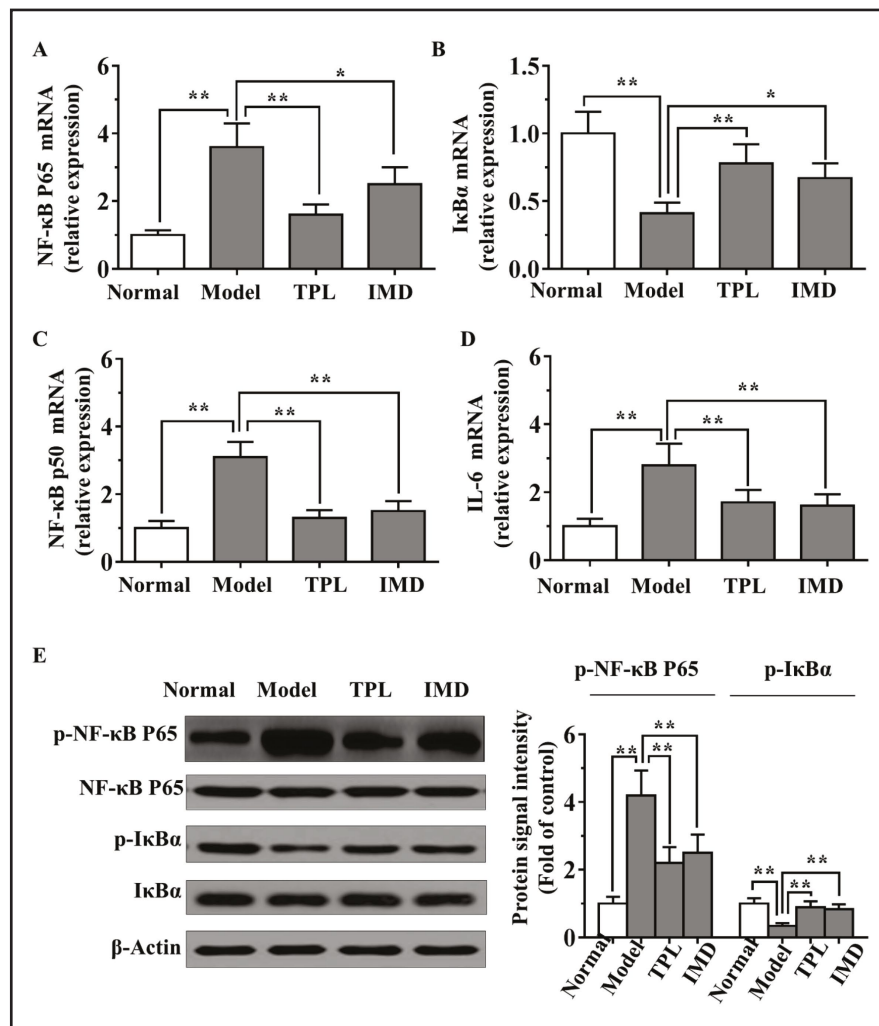
Fig. 3. Effect of TPL on oxidative stress. SOD (A) and MDA (B) levels in the serum from the different groups were detected by commercial kits. The data are presented as the mean \pm SD from three experiments. * p < 0.05; ** p < 0.01 versus model group.

NA of inflammatory cytokines, such as TNF- α , IL-1 β and MCP-1 in the MGN induced rats were significantly higher than those in the normal group (p < 0.01) (Figs. 2A-C). However, MGN rats treated with TPL exhibited significant decrease of TNF- α , IL-1 β and MCP-1 compared with those in model control group. Additionally, significant reduction of inflammatory cytokines was found in IMD group compared with those in model group. These results indicated that inflammatory response could be attenuated by TPL and IMD treatment, and this is probably done through inhibiting the production of TNF- α , IL-1 β , and MCP-1 production.

TPL reduced oxidative stress

To investigate the effect of TPL on oxidative stress, we evaluated the malondialdehyde (MDA) and superoxide dismutase (SOD) levels. C-BSA exposure significantly (p < 0.01) increased the MDA level in induced rats (Fig. 3A). MDA content was significantly reduced in rats of TPL and IMD groups compared to those in model group. On the other hand, SOD level was found significantly reduced in rats of C-BSA-treated group compared with those in normal group (p < 0.01), and this was rescued when rats were treated with TPL and IMD (p < 0.01). The above data indicated that TPL might have an antioxidant effect by down-regulating oxidative stress.

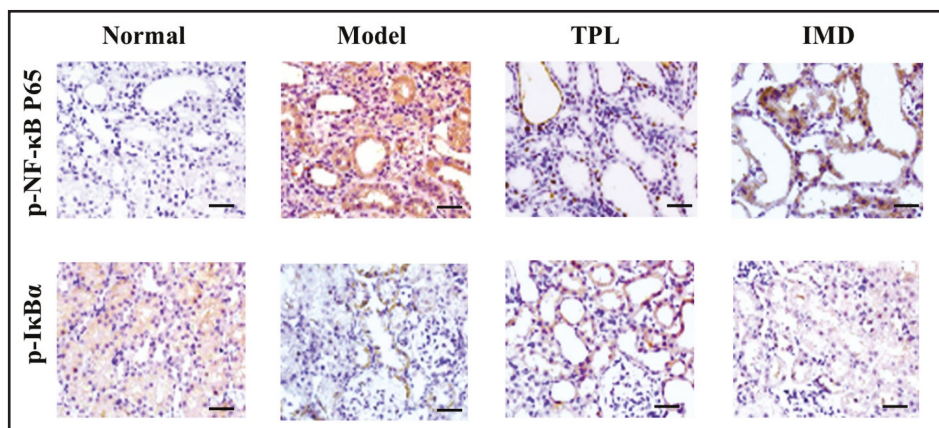
Fig. 4. Effects of TPL on NF- κ B p65 and I κ B α expression. The mRNA levels of the NF- κ B p65 (A), I κ B α (B), NF- κ B p50 (C) and IL-6 (D) in the renal tissue of MGN rats were determined by qRT-PCR analysis. (E) The protein levels of phospho-p65 and phospho-I κ B α were determined by immunoblotting analysis. The quantitation of signal intensities was performed using IMAGEJ software. The data are presented as the mean \pm SD from three experiments. * p < 0.05; ** p < 0.01 versus model group.



TPL suppressed NF- κ B p65 activation and I κ B α degradation

In order to understand the mechanisms of TPL, the expression of a critical gene NF- κ B p65 was assessed. Our data showed significantly higher mRNA level of NF- κ B p65 and lower I κ B α level in C-BSA-treated rats compared with those in normal group (Figs. 4A and B) (p < 0.01). We also found TPL and IMD treatment significantly reduced NF- κ B p65 level and enhanced I κ B α mRNA level compared to the model group (p < 0.01). In addition, the mRNA level of another key component of NF- κ B complex (NF- κ B p50) was also significantly decreased by TPL and IMD treatment (Fig. 4C). Since IL-6 is a well-documented target of NF- κ B [21], in view of the reduced NF- κ B activity by TPL and IMD treatment, we hypothesized that expression of IL-6 would also be reduced. The qRT-PCR result showed that the mRNA level of IL-6 was significantly reduced by TPL and IMD treatment compared with model group (Fig. 4D). This data validated the inhibition effect of TPL on NF- κ B activity. To further explore the mechanisms of TPL, immunoblotting was done to assess the protein expression level of phospho-p65 and phospho-I κ B α in renal tissue. We found that phosphorylated NF- κ B p65 protein was highly up-regulated while I κ B α was down-regulated in the renal tissue from the C-BSA-induced MGN rats compared to those in the normal group (Fig. 4E). Interestingly, phospho-p65 and up-regulate phospho-I κ B α could be down- and up-regulated to different degrees respectively by TPL and IMD treatment. Moreover, the expression of glomerular NF- κ B p65 and I κ B α were determined by immunohistochemical analysis. We consistently

Fig. 5. Immunohistochemical analysis for the effects of TPL on NF- κ B p65 pathway. Renal tissues were processed and performed immunohistochemical analysis for staining phospho-p65 and phospho-I κ B α . Representative staining sections were shown. Scale bars are 50 μ m.



tochemical analysis for staining phospho-p65 and phospho-I κ B α . Representative staining sections were shown. Scale bars are 50 μ m.

found the upregulation of phospho-p65 and downregulation of phospho-I κ B α in renal tissue of MGN rats compared to the normal group. Importantly, the activation of NF- κ B p65 was attenuated and the degradation of I κ B α was blocked by TPL and IMD treatment (Figure 5). Taken together, these results suggest that TPL attenuates inflammatory response, in part, via downregulation the activation of the NF- κ B.

Discussion

MGN is a slow progressive kidney disease and is characterized by immune deposits accumulation [22, 23]. The progression of MGN is contributed by the specific IgGs, e.g., IgG and IgA, and their associated responses, e.g., oxidative stress, inflammation and complement activation [24, 25]. In this study, we found that pathological kidney damage in MGN-induced rats could be alleviated by TPL treatment. Our data also showed that TPL treatment significantly reduced the production of inflammatory cytokines (e.g., TNF- α , IL-1 β and MCP-1) and ameliorated oxidative stress. Meanwhile, we also found that IMD-0354 decrease the accumulation inflammation damage and oxidative lesions. Furthermore, we showed that TPL could suppress the expression of nuclear factor (NF)- κ B p65 and p50, IL-6 and block I κ B α degradation. Together, our results indicated that TPL attenuates inflammatory response, and this is partially done via inhibiting the activation of NF- κ B signaling pathway.

Inflammation is one of the defense mechanism of host against infectious agents and injury which contributes to the pathophysiology of many diseases. Acute inflammation is a short-term process which consists of signs such as pain, swelling, loss of function and heat due to the infiltration of the tissues by leukocytes and plasma [26]. Several pro-inflammatory mediators, e.g., COX-2, IL-6, iNOS and TNF- α were released during the inflammatory response and this is largely depends on the transcriptional activation [27]. TNF- α which promotes inflammatory responses is a member of a cytokine superfamily [28]. Working with IL-1 and IL-6, TNF- α induces autoimmune responses and causes many of the MGN associated problems [29]. In this study, we found serum levels of TNF- α , IL-1 β and MCP-1 were markedly increased in rats in the MGN model group compared to those in normal group, but TPL treatment reduced the TNF- α , IL-1 β and MCP-1 levels. To understand whether the increased cytokine production was associated with NF- κ B levels, we designed an I κ B kinase inhibitor IMD-0354 treatment group in this experiment. It has been shown that IMD-0354, a selective inhibitor of IKK β , inhibited allergic inflammation in an acute mouse model of ovalbumin (OVA)-induced asthma [30]. Ogawa et al. demonstrated that

IMD-0354 inhibited the pathological features of airway remodeling in a mouse model of chronic asthma [31]. One recent study showed that IMD-0354 exerted antiproteinuric effect, and the downregulated nephrin, synaptopodin, nephrin and synaptopodin proteins were restored partially by IMD-0354 [20]. In the present study, we found that both TPL and IMD-0354 could partially regulate TNF- α , IL-1 β and MCP-1. It has been reported that SOD activity and MDA content are two indicators of oxidative stress. It has been found that SODs convert superoxide to hydrogen peroxide, and play a crucial role in reducing oxidative stress and inflammatory responses [32]. MDA is a product of lipid peroxidation and used as a bio-marker of oxidative stress [33]. In this study, both the TPL and IMD-0354 treatments enhanced the SOD activity and decreased the formation of MDA. These results suggest that TPL attenuates inflammatory response associated with inhibition of NF- κ B activity. It is widely accepted that transcription factor NF- κ B is actively involved in regulation of expression of many pro-inflammatory genes upon immune response [19]. Notably, there is close association between NF- κ B activation and inflammatory cytokines and oxidative stress which contribute to the development and progression of MGN. During the progression of glomerulonephritis, complexes of signaling networks are activated in renal cells. As a fundamental intracellular transcription factor system, mammalian nuclear factor (NF)- κ B signaling pathways induced in response to various sources of extracellular stimulation [34]. Accumulating evidences have shown that NF- κ B activation is a critical response to kidney diseases. Upregulation of the canonical (RelA/p50) NF- κ B isoform in tubular epithelial cells, macrophages, podocytes, and mesangial cells has been found mediating acute and chronic inflammatory nephropathy [35]. Protein complex of NF- κ B proteins, p65 and p50 normally binds to inhibitory I κ B α and exists as inactive form in the cytoplasm thereby blocking NF- κ B nuclear translocation. In response to inflammatory stimulation, I κ B α is phosphorylated by I κ B kinase (IKK) and detached from NF- κ B subunits followed by degradation. The free NF- κ B is then translocated into the nucleus and works as transcription factor. In the nucleus, NF- κ B dimers activated the transcription of inflammatory related genes by combining with target DNA elements [36, 37]. During inflammation, TNF- α , IL-1 β , COX-2 and iNOS were regulated by activated NF- κ B [38]. To attenuate inflammation, effort has been made on reducing the production of pro-inflammatory mediators by inhibiting NF- κ B activation. In this study, we showed the involvement of NF- κ B in pathogenesis of MGN and the inflammatory reaction in MGN can be reduced by TPL and IMD-0354 treatment through inhibiting NF- κ B. Previous study have shown that NF- κ B subunit p65 and p50 proteins have critical in vivo functions, and the NF- κ B pathway activation associated with the inflammatory response in renal disease [39, 40]. In agreement with these reports, we also showed that TPL could inactivate NF- κ B (p65 and p50) and blocking I κ B α degradation thus inhibit inflammation, suggesting a potential attenuating effect of TPL on MGN via suppressing NF- κ B signaling pathway.

Conclusion

Our data exhibited the involvement of NF- κ B activation in inflammatory response of C-BSA-induced MGN, suggesting MGN and renal dysfunction that caused by MGN could be attenuated by mediating NF- κ B. Fortunately, MGN induced inflammatory responses could be alleviated by TPL by suppressing the activation of NF- κ B pathway. Future studies might be needed to explore other possible pathways that may contribute to the effect of TPL.

Disclosure Statement

The authors declare that there are no conflict of interests.

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