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Telbivudine antagonizes TLR4 to inhibit the epithelial-to-mesenchymal transition in human proximal tubular epithelial cells in vitro☆

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ABSTRACT

The antiviral drug Telbivudine (LdT) has an extrahepatic pharmaceutical effect that improves renal inflammation and tubulointerstitial fibrosis. However, the exact mechanism of action requires further investigation. Toll-like receptor 4 (TLR4) is involved in several physiological processes, including inflammation, fibrosis, innate immunity, and hepatitis B virus-associated glomerulonephritis. The epithelial-to-mesenchymal transition (EMT) is the characteristic pathological change in tubulointerstitial fibrosis. In this study, we used transforming growth factor-β (TGF-β) to stimulate human proximal tubular epithelial (HK-2) cells to investigate the effects of LdT in EMT. In addition, we treated HK-2 cells with a TLR4 agonist, lipopolysaccharide, to determine the effect of LdT on TLR4. The results indicated that LdT inhibited the expression of TLR4 and its downstream proteins. It also decreased the release of inflammatory factors, downregulated the TGF-β/Smad signaling pathway, and reversed the EMT changes seen in HK-2 cells. In conclusion, LdT antagonized TLR4 to inhibit EMT in proximal tubular epithelial cells.

1. Introduction

Telbivudine (LdT) is a synthetic thymidine nucleoside analog that potently inhibits HBV DNA polymerase. In many clinical studies, LdT has been shown to improve renal function, assessed by estimated glomerular filtration rate (eGFR), with long-term use [1]. However, the exact mechanism that mediates this improvement is still unknown. Some researchers thought that it may be related to an improvement in tubular function or a direct effect on the inflammatory/fibrotic pathways in renal tissue-rather than its inhibition of HBV [2,3]. There are few articles that have studied this effect in renal disease. Kader et al. indicated that LdT could attenuate the levels of cystatin C (a renal function marker) and renal histopathological changes (e.g., glomerular injury, acute tubular necrosis, total injury score) in gentamicin-induced acute kidney injury in rats [4]. Furthermore, our previous study demonstrated that LdT could improve renal fibrosis and inflammation in obstructive nephropathy in rats [5]. Because there are almost no treatments to reverse impaired renal function, more research is needed to determine the exact mechanism of the therapeutic effect of LdT in kidney disease.

Chronic kidney disease (CKD) is defined as decreased renal function shown by a decreased eGFR or expression of kidney damage markers for at least three months [6]. In the clinic, renal function tests are often the first choice for diagnosing CKD. Tubulointerstitial fibrosis and tubular atrophy are the best histological markers to identify declining renal function, as they are directly correlated with the GFR [7,8]. The key feature of tubulointerstitial fibrosis is an excessive accumulation of the extracellular matrix (ECM) produced by fibroblasts. Existing evidence suggests that a large proportion of fibroblasts originate from tubular epithelial cells via the epithelial-to-mesenchymal transition (EMT) [9]. The pathological process of EMT includes a loss of epithelial markers (e.g., E-cadherin) and a transition to a spindle-shaped morphology, concomitant with an acquisition of new mesenchymal markers (e.g., α-smooth muscle actin, α-SMA) [10]. During EMT, cells obtain the ability to release matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1), both of which have important roles in renal disease [11,12]. Tissue injury induces tubular epithelial cells to become mesenchymal cells via EMT, and then production and deposition of ECM in the renal interstitium is increased to promote tubulointerstitial fibrosis and induce renal failure [13]. Therefore, preventing EMT is a key treatment for CKD.

Inflammatory cell infiltration is a feature of tubulointerstitial damage in CKD. Moreover, there is a strong association between inflammation and fibrosis [14,15]. This association has also been

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identified at the molecular level. IκB kinase (IKK) and inhibitor of NF-κB (IκB-α) in the inflammatory NF-κB signaling pathway can act on the TGF-β/Smad signaling pathway to promote renal fibrosis [16,17]. Studies have suggested that inhibiting inflammation, especially the NF-κB pathway, could attenuate tubulointerstitial fibrosis [18,19]. Toll-like receptors (TLRs) are upstream of NF-κB and represent an evolutionarily conserved family of cell membrane receptors (e.g., TLR4), which can be activated by LPS to promote the expression of inflammatory factors. TLR4 is primarily expressed in the proximal and distal tubules [20,21]. It can promote renal fibrosis through inflammasome activation in renal epithelial cells and contributes to a dysregulated immune response related to CKD progression [22]. TLRs sense pathogen-associated molecule patterns and mediate innate immune responses to control hepatitis B virus (HBV) infection [23]. Thus, TLR4 is not only involved in CKD but also plays a role in protecting against HBV infection. Our previous study showed that LdT could downregulate TLR4 expression and attenuate renal inflammation/fibrosis following unilateral ureteral occlusion (UUO) in rats. However, the underlying mechanism for these effects remains unclear.

In this study, we investigated whether LdT could improve EMT by inhibiting inflammation through TLR4. Many signaling pathways can activate the EMT transcriptional program (e.g., TGF-β), which is the most well-characterized canonical signaling pathway in fibrosis [24,25]. We used TGF-β to stimulate human proximal tubular epithelial (HK-2) cells and examined the effect of LdT on EMT. We also used lipopolysaccharide (LPS) to induce inflammation in HK-2 cells and explored the mechanism of renal protection by LdT.

2. Materials and methods

2.1. Chemicals and reagents

Keratinocyte serum-free medium (K-SFM) supplemented with L-glutamine and epithelial growth factor was purchased from Gibco (Carlsbad, CA, USA). LdT was obtained from Sigma (St. Louis, MO, USA), C34 (TLR-4 inhibitor) from Tocris (UK), and TGF-β from Perprotech (USA). LPS and the anti-NF-κB, anti-IKK-α (IκB kinase) and anti-Smad2/3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-TGF-β, anti-α-SMA, and anti-E-cadherin antibodies were from Abcam (Cambridge, MA, USA). The anti-MMP-2, anti-TIMP-1, anti-IL-1, anti-TNF-α, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Proteintech (Wuhan, China). The anti-TLR4 antibody was from Wanlei (Shenyang, China).

2.2. Cell culture and treatment

The HK-2 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated using K-SFM in a humidified atmosphere with 5% carbon dioxide at 37 °C and subcultured at 80% confluence using 0.05% EDTA trypsin. To observe the effect of LdT on EMT, cells from the same passage were divided into five groups: (1) control, HK-2 cells cultured routinely for 48 h; (2) TGF-β, HK-2 cells treated with TGF-β (5 ng/ml) for 24 h and then cultured in K-SFM for 24 h; (3–5) HK-2 cells were treated with TGF-β (5 ng/ml) for 24 h and then treated with LdT dissolved in K-SFM at concentrations of 0.1 μg/mL (LdT-L), 0.5 μg/mL (LdT-M), or 1.0 μg/mL (LdT-H) for 24 h.

To investigate the effect of LdT on inflammation and the fibrosis signaling pathway, cells from the same passage were divided into the following five groups: (1) control, cells had no specific treatment; (2) LPS, cells were treated with 1 μg/mL LPS for 15 min; (3) LdT, cells were pretreated with 1 μg/mL LdT for 24 h and then treated with 1 μg/mL LPS for 15 min; (4) C34, cells were pretreated with 50 μM C34 for 30 min and then treated with 1 μg/mL LPS for 15 min; (5) LdT + C34, cells were pretreated with 1 μg/mL LdT for 24 h, 50 μM C34 for 30 min, and then 1 μg/mL LPS for 15 min.

2.3. Western blot analysis

Proteins were isolated using RIPA lysis buffer (Beyotime, Shanghai, China) containing 10% phenylmethylsulfonylfluoride (PMSF, Beyotime). After a 15-min incubation on ice, the samples were centrifuged at 14,000 rpm for 30 min at 4 °C and the supernatant collected. The protein samples (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (GE Healthcare, USA). The membranes were blocked with 5% BSA for 2 h and then incubated with the following primary antibodies: anti-TGF-β (1:1000), anti-Smad2/3 (1:1000), anti-E-cadherin (1:500), anti-α-SMA (1:250), anti-MMP-2 (1:500), anti-TIMP-1 (1:300), anti-IL-1 (1:500), anti-TNF-α (1:1000), anti-TLR-4 (1:500), anti-IKKa (1:1000), anti-NF-κB (1:1000), or anti-GAPDH (1:3000) overnight at 4 °C. After washing, the blots were incubated with HRP-conjugated secondary antibodies (ZSGB-BIO, Beijing, China; 1:3000) for 1 h at room temperature. Finally, the immune-stained bands were visualized using enhanced chemiluminescence, and the signal intensity was measured using an image analyzer (GE healthcare). Expression levels were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD) and normalized to GAPDH.

2.4. Immunofluorescence staining

Because there was no significant difference between the different LdT treatment groups, only one LdT concentration (1 μg/mL) was used to evaluate the effect of LdT on EMT by immunofluorescence. HK-2 cells were seeded in 6-well plates containing slides and cultured as described above. The slides were washed three times using phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. The slides were blocked with 10% fetal bovine serum (FBS) for 1 h and then co-incubated with E-cadherin (1:100) and α-SMA (1:50) primary antibodies overnight at 4 °C. The slides were washed and incubated in FITC-conjugated goat anti-rabbit IgG (Abbkine, Wuhan, China. 1:100) and Cy3-conjugated goat anti-mouse IgG (Abbkine. 1:100) secondary antibodies for 4.5 h at room temperature. The stained slides were exposed to DAPI for 5 min and then viewed using fluorescence confocal microscopy (400×).

3. Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple groups (GraphPad Prism 5). All data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered significant.

4. Results

4.1. LdT inhibits EMT pathological changes in HK-2 cells

Compared to the control cells, TGF-β induced the downregulation of E-cadherin and the upregulation of α-SMA (Fig. 4.1a). This effect was reduced by treatment with all three concentrations of LdT (i.e., LdT-L, LdT-M, and LdT-H). A similar trend was observed with the immunofluorescence staining (Fig. 4.1b). The control cells almost exclusively expressed E-cadherin and had an oval shape, whereas the TGF-β-treated cells mainly expressed α-SMA and had a long spindle shape. The E-cadherin and α-SMA levels in the LdT-treated cells were intermediate to the levels found in the control and TGF-β cells. The LdT-treated cells were also shorter than the TGF-β-treated cells. These results suggest that LdT inhibited TGF-β-induced EMT in HK-2 cells. However, there were no significant differences between the three LdT groups.

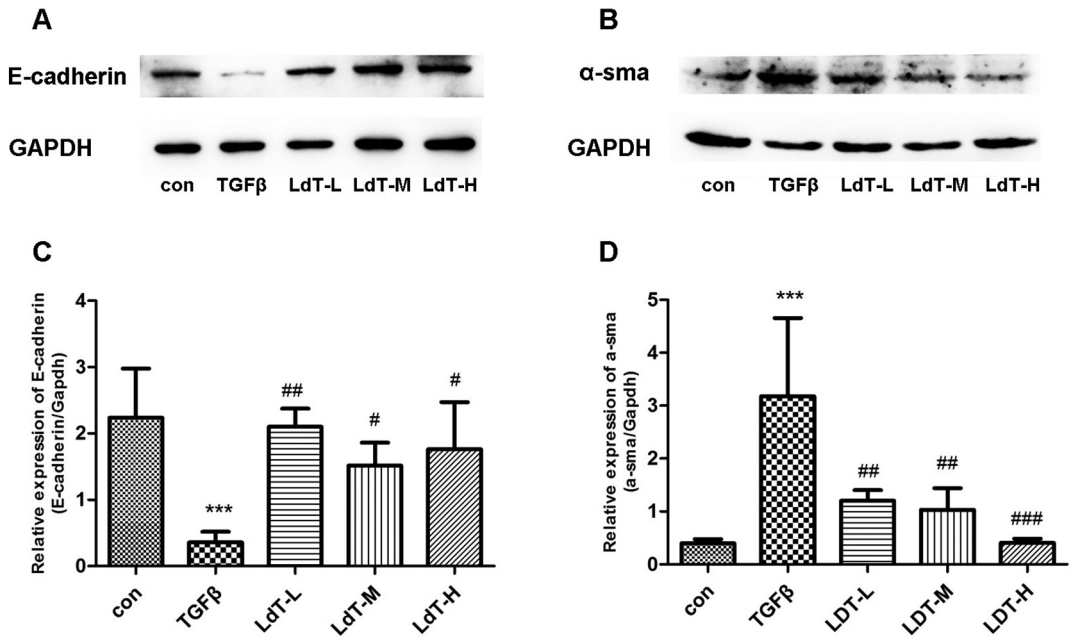

Fig. 4.1a. LdT inhibits EMT in HK-2 cells.
A–B: E-cadherin and α-SMA western blots; C–D: Quantification of E-cadherin and α-SMA levels. Control vs. TGF-β, ***$p < 0.0001$; LdT-L, LdT-M, or LdT-H vs. TGF-β, #$p < 0.05$, ##$p < 0.001$, ###$p < 0.0001$.

4.2. LdT downregulated MMP-2 and TIMP-1 expression induced by TGF-β

We performed western blot experiments to investigate the effects of MMP-2 and TIMP-1 on EMT. As shown in Fig. 4.2, MMP-2 and TIMP-1 expression were both upregulated by TGF-β treatment compared to the control. However, LdT treatment reduced this effect. There were no statistically significant differences between the three LdT treatment groups.

4.3. LdT decreased NF-κB and TNF-α expression during EMT

Western blotting revealed that the control cells expressed little NF-κB or TNF-α, but the levels of these proteins increased following TGF-β treatment (Fig. 4.3). At all three LdT concentrations, TGF-β-induced expression of NF-κB and TNF-α were decreased. The average NF-κB and TNF-α expression levels were not significantly different between the three different LdT dose groups.

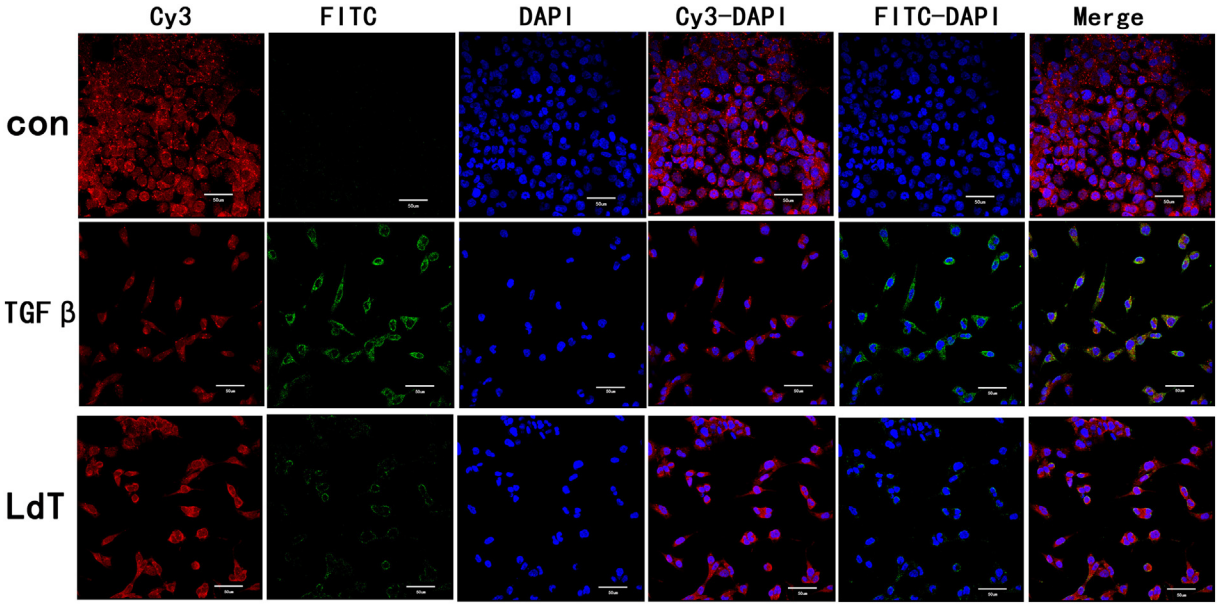

Fig. 4.1b. LdT inhibits EMT in HK-2 cells.
Immunofluorescence staining of HK-2 cells treated with TGF-β and LdT (E-cadherin, red; α-SMA, green) (×400). Scale bar, 50 μm. Control cells mostly expressed E-cadherin. TGF-β-treated cells mainly expressed α-SMA. Expression of E-cadherin was higher and α-SMA was lower in LdT-treated cells compared to the TGF-β-treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

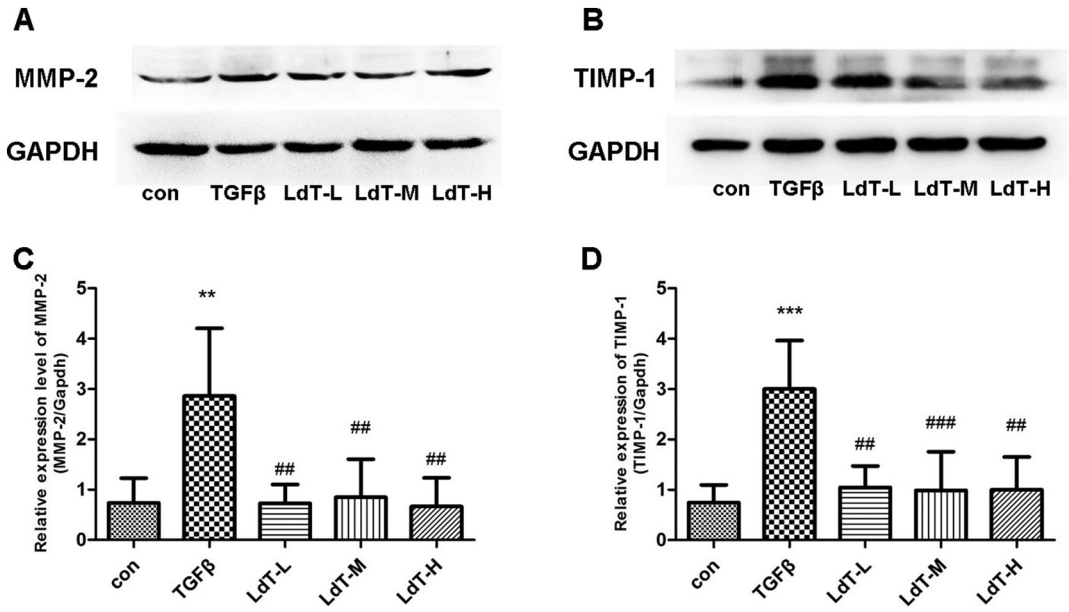

Fig. 4.2. LdT downregulated MMP-2 and TIMP-1 expression induced by TGF-β.
A–B: MMP-2 and TIMP-1 western blots; C–D: Quantification of MMP-2 and TIMP-1 levels. Control vs. TGF-β, $^{**}p < 0.001$, $^{***}p < 0.0001$; LdT-L, LdT-M, or LdT-H vs. TGF-β, ##p < 0.001, $###p < 0.0001$.

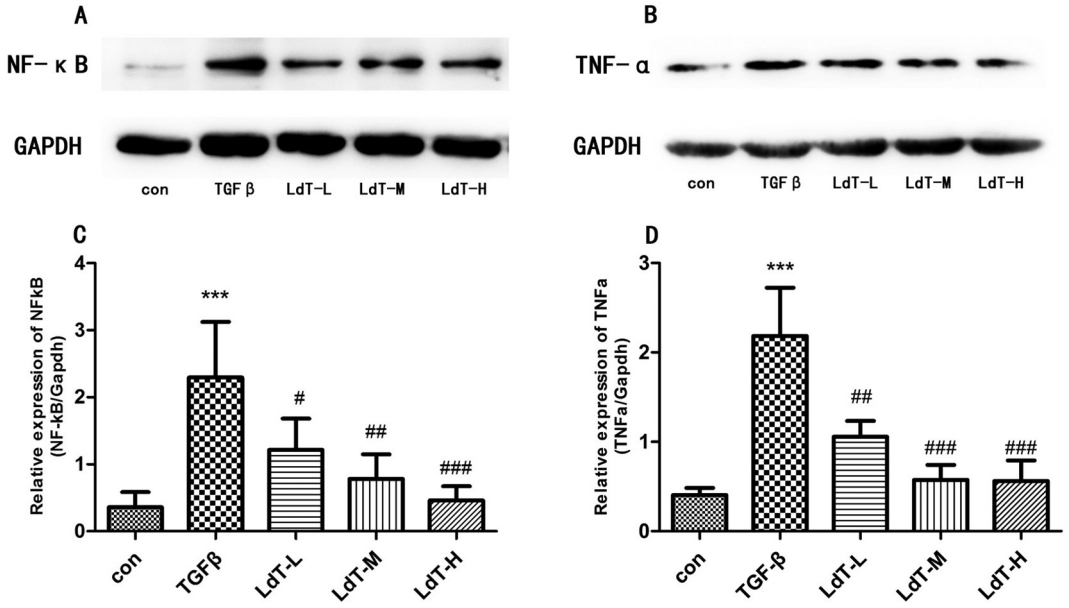

Fig. 4.3. LdT decreased NF-κB and TNF-α expression during EMT.
A–B: NF-κB and TNF-α western blots; C–D: Quantification of NF-κB and TNF-α levels. Control vs. TGF-β, $^{***}p < 0.0001$; LdT-L, LdT-M, or LdT-H vs. TGF-β, $#p < 0.05$, $##p < 0.001$, $###p < 0.0001$.

4.4. LdT suppressed the release of inflammatory factors induced by LPS

The expression of IL-1 and TNF-α could hardly be detected in the control cells but was significantly increased by LPS (Fig. 4.4). However, pretreatment with LdT, the TLR4 inhibitor C34, or a combination of the two (LdT + C34) suppressed the release of these inflammatory factors. The greatest suppression was observed with the LdT + C34 treatment. However, there were no significant differences between the three pretreatment groups.

4.5. LdT inhibited the activation of the TLR4/NF-κB signaling pathway by LPS

TLR4 activates the IKK complex and induces IκBα degradation to release NF-κB for translocation into the nucleus. In this study, TLR4, IKKα, and NF-κB expression were all upregulated by LPS compared to the control cells (Fig. 4.5). In contrast, C34 and LdT inhibited the expression of IKKα and NF-κB by antagonizing TLR4. The expression levels of the three proteins were not significantly different between the LdT, C34, and LdT + C34 treatment groups.

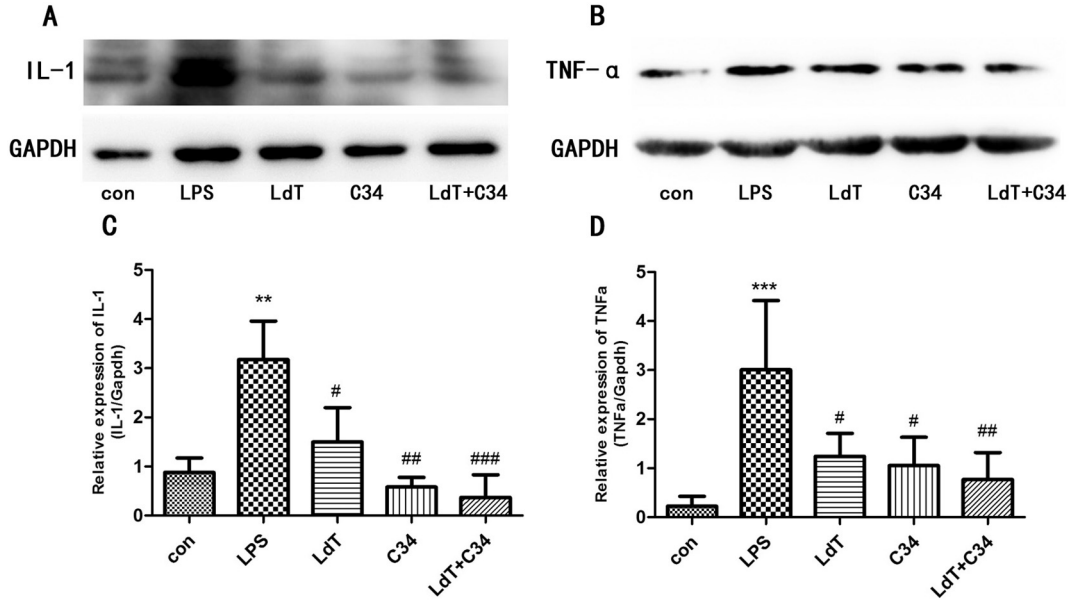

Fig. 4.4. LdT suppressed the release of inflammatory factors induced by LPS.
A–B: IL-1 and TNF-α western blots; C–D: Quantification of IL-1 and TNF-α levels. Control vs. LPS, $^{**}p < 0.001$, $^{***}p < 0.0001$; LdT, C34, or LdT + C34 vs. LPS, $\#p < 0.05$, $\#\#p < 0.001$, $\#\#\#p < 0.0001$.

4.6. LdT inhibited the TGF-β/Smad signaling pathway

TGF-β and its downstream proteins, Smad2 and Smad3, were all significantly activated by LPS compared to the control cells (Fig. 4.6). Pretreatment with LdT, C34, or LdT + C34 inhibited the expression of the TGF-β/Smad signaling pathway proteins, and the greatest effect was observed with the combination of LdT and C34. However, there were no significant differences between the three pretreatment groups.

5. Discussion

Substantial clinical evidence suggests that long-term LdT treatment improves renal function. Our previous study showed that LdT improved UUO-induced renal interstitial fibrosis. Here, we investigated the effect of LdT on EMT in HK-2 cells to understand the mechanism underlying the action of the drug.

Consistent with previous studies, TGF-β induced cellular transition from the normal epithelium into a mesenchymal-like state, and it took on an elongated spindle morphology in this study. However, LdT treatment effectively reversed the EMT changes, resulting in a shorter and oval cellular morphology, as well as higher expression of the epithelial cell marker and reduced expression of the mesenchymal cell marker. The death of renal tubule epithelial cells (TECs) due to kidney injury triggers the proliferation and expansion of surviving cells, which then undergo differentiation to restore kidney function and histological features. However, chronic and repeated TEC injury leads to irreversible loss of function, termed CKD [26]. During this process, a large number of epithelial cells appear dedifferentiated and have mesenchymal-like characteristics, the so-called EMT. As the key mechanism leading to tubulointerstitial fibrosis, EMT is activated to promote the accumulation of ECM [27]. Hence, its inhibition represents a potential anti–fibrotic therapy for chronic renal injury [28,29]. To our best

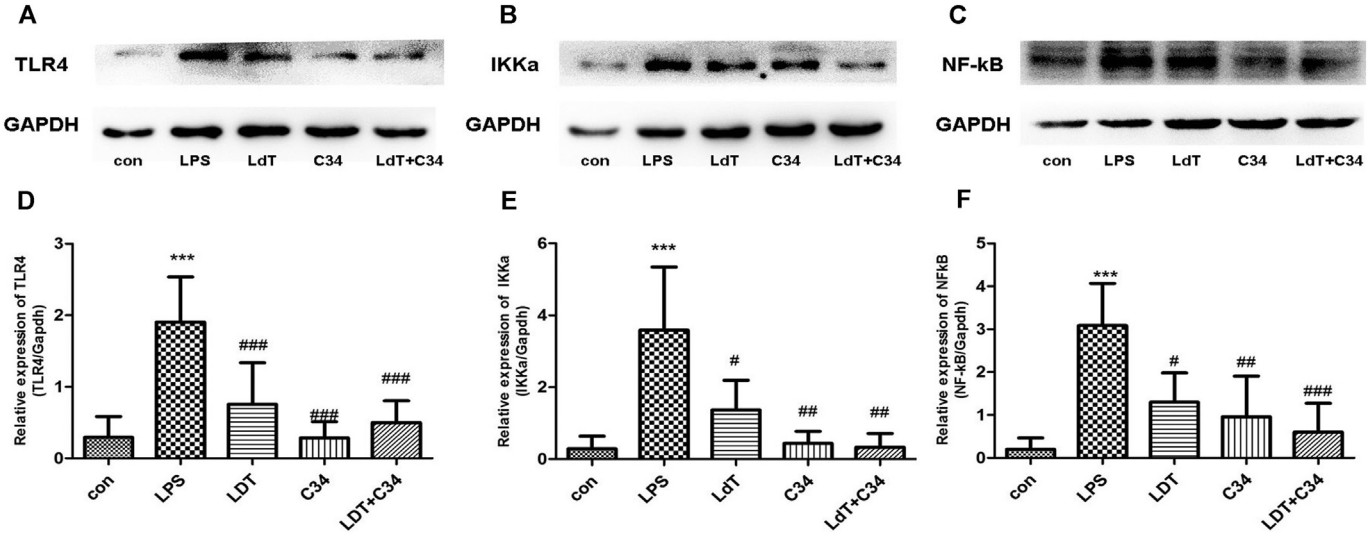

Fig. 4.5. LdT inhibited the activation of the TLR4/NF-κB signaling pathway by LPS.
A–C: TLR4, IKKα and NF-κB western blots; D–F: Quantification of TLR4, IKKα and NF-κB levels. Control vs. LPS, $^{***}p < 0.0001$; LdT, C34, or LdT + C34 vs. LPS, $\#p < 0.05$, $\#\#p < 0.001$, $\#\#\#p < 0.0001$.

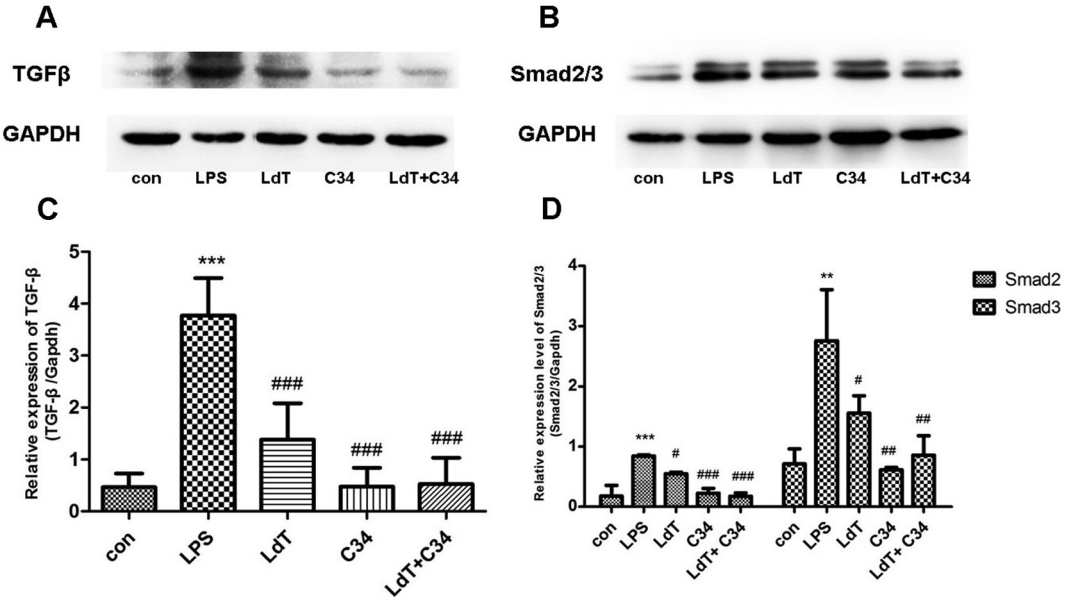

Fig. 4.6. LdT inhibited the TGF-β/Smad signaling pathway.
A–B: TGF-β and Smad2/3 western blots; C–D: Quantification of TGF-β and Smad2/3 levels. Control vs. LPS, $^{**}p < 0.001$, $^{***}p < 0.0001$; LdT, C34, or LdT + C34 vs. LPS, $\#p < 0.05$, ##p < 0.001, $\#\#\#p < 0.0001$.

knowledge, this study is the first to report the effect of LdT on EMT, which may be the key mechanism of drug action to attenuate tubulointerstitial fibrosis.

Previously, we have reported that LdT could suppress the activity of MMP-2 and TIMP-1. Herein, we demonstrated that both MMP-2 and TIMP-1 were upregulated during EMT, which was induced by TGF-β, whereas LdT suppressed this upregulation. MMP-2 is crucial for the development of renal interstitial fibrosis. Its activity promotes the mesenchymal transformation of renal tubular epithelial cells in the early stage of renal interstitial fibrosis [30]. Furthermore, active MMP-2 alone was sufficient to drive the full spectrum of EMT. Zhang et al. found that increased MMP-2 levels were accompanied by a decrease in E-cadherin during EMT [31]. As an inhibitor of MMP-2, TIMP-1 was also confirmed to be increased during EMT, independent of its MMP-inhibitory function [32]. Our results further demonstrated the improvement by LdT of EMT.

It has been widely accepted that inflammation initiates renal damage, and decreased inflammatory responses can attenuate renal tubulointerstitial fibrosis. Therefore, we speculated that the inhibition of EMT by LdT could not be separated from the inflammatory reaction. As our results showed, LdT decreased NF-κB and TNF-α expression. Both of these factors have core regulatory effects on inflammation during EMT. Typical renal injury recovery consists of three phases: inflammation, proliferation, and remodeling. Moreover, chronic inflammation is thought to be one of the most important mechanisms of CKD [33]. Inflammatory responses are characterized by an excessive generation of cytokines, including interstitial proinflammatory cytokines and growth factors, in addition to reactions involving inflammatory cells, renal tubular atrophy, and interstitial fibrosis [34]. Gentle et al. showed that TGF-β induced interstitial inflammation of renal tubular cells [35]. Our results indicated the interaction between inflammation and fibrosis and that the inhibition of EMT by LdT is likely related to its regulation of inflammation.

In this study, the TLR4 agonist LPS induced significantly higher expression of IL-1, TNF-α, and the signaling pathway proteins IKK-α and NF-κB through the activation of TLR4. Both LdT and the TLR4 inhibitor C34 inhibited the inflammatory factors downstream of TLR4. Because chronic inflammation promotes the progression of interstitial fibrosis and plays a vital role in CKD, fibrosis could be reduced by inhibition of inflammation [36]. TLRs play an important role in causing or aggravating renal conditions and combating bacterial infections when activation of TLRs leads to a harmful inflammatory response [37]. The TLR4 pathway has been shown to affect HBV-related nephropathy, renal interstitial lesions, changes in renal function, and inflammatory cell infiltration [38]. Therefore, we hypothesized that the effect of LdT on interstitial fibrosis was related to TLR4, which our study confirmed.

We also observed the expression of the regulatory fibrotic proteins TGF-β and its target protein Smad, which indicates that TGF-β and Smad2/3 could be activated by LPS but inhibited by LdT and C34. It has been reported that TLR4 could activate TGF-β and participate in renal fibrosis [39]. In TLR4-knockout mice, both TGF-β and α-SMA were downregulated, and the tubulointerstitial fibrosis was improved [40]. Chen et al. found that TLR4 accelerated fibrosis and inflammatory factor release by inhibiting NF-κB, whereas TLR4 knockout significantly reduced the risk of renal fibrosis [41]. Kumar et al. reported that the activity of the TGF-β/Smad signaling pathway correlated with LPS-induced inflammation of the microglia [42]. Pulskens et al. found that fibrosis was reduced in TLR4-deficient mice following the stimulation of primary TECs with TGF-β compared to wild-type mice [43]. Our results collectively indicate that LdT antagonizes TLR4 and downregulates fibrosis-related signaling to inhibit EMT.

6. Conclusion

The present study demonstrated that LdT downregulates the expression of TLR4 and reduces inflammation by suppressing the TGF-β/Smad signaling pathway and reversing EMT in HK-2 cells. In summary, LdT attenuates EMT during tubulointerstitial fibrosis by inhibiting the activity of TLR4. Consistent with our previous findings, there were no significant differences among the 3 LdT-treated groups, and we speculate that the drug action may not be related to dose.

Declaration of Competing Interest

None.

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