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OmpA-like protein Loa22 from *Leptospira interrogans serovar Lai* is cytotoxic to cultured rat renal cells and promotes inflammatory responses

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Leptospirosis renal disease is one of the common clinical manifestations of leptospirosis, including acute renal failure and tubulointerstitial nephritis. Outer membrane protein A-like protein Loa22 is a lipoprotein from *Leptospira interrogans* and has been suggested to be a corresponding virulence factor. However, the role of Loa22 in leptospiral nephropathy is not yet understood. In the present study, we constructed a vector and artificially expressed Loa22 in *Escherichia coli* BL21(DE)pLysS cells. After extensive purification, along with a GST tag protein control, Loa22 protein was used to test the cytotoxicity in cultured rat proximal tubule cells (NRK52E) and examine its effects on the induction of inflammatory responses. Using morphological examination, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoium hydrixide absorbance, lactate dehydrogenase assays and an analysis of apoptosis via flow cytometry, it was found that Loa22 protein mediates a direct cytotoxic effect on NRK52E cells in a dose-dependent manner. Using real-time PCR, western blotting and immunofluorescence, it was found that Loa22 protein upregulates the expression of toll-like receptor 2 (TLR2), induces nitric oxide synthase and promotes the production of nitric oxide (NO) and monocyte chemoattractant protein-1 (MCP-1) by NRK52E cells. Additionally, using a TLR2 blocking antibody, it was found that enhanced NO and MCP-1 production by NRK52E cells after Loa22 stimulation requires the activation of TLR2. Collectively, our data suggested that Loa22 is a critical virulence factor of *L. interrogans* and is involved in the leptospiral nephropathy through mediating direct cytotoxicity and enhancing inflammatory responses.

Keywords leptospirosis; Loa22; cytotoxicity; tubulointerstitial nephritis; renal proximal tubule cells; toll-like receptor 2

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Introduction

Leptospira, a highly invasive pathogenic spirochete, is the causative agent of leptospirosis, which is a widespread and re-emerging zoonosis that has become a major public health problem in developing countries [1–3]. Rodents, pigs, and dogs may become clinically asymptomatic carriers of leptospire, and *Leptospira* can be shedded into the environment with the urine of the carriers. The chronic infection is restricted to renal tubules. In other instances, such as in human, leptospiral infection results in acute infection. Leptospirosis is transmitted either through direct contact of body fluids from infected animals or by exposure to contaminated water or soil of the urine [4]. More than 500,000 cases of severe leptospirosis occur in the world each year, with a mortality rate of 5–20% [5]. Leptospire is a highly motile bacterium which can penetrate abrasions in skin and intact mucous membranes. Soon after penetrating the contact surface, leptospire rapidly gains access to the bloodstream and spreads to all organs. Following host immune clearance, leptospires remain in immunologically privileged tissues such as renal tubules, brain and the anterior chamber of the eye. In the affected kidney, leptospires further colonize and multiply in proximal tubule cells, resulting in interstitial nephritis, tubular necrosis, and renal failure. Besides the direct invasion into tubule cells of the organism, the effects of leptospiral endotoxins and immunologic responses are considered to contribute to the pathological damage [4,6,7].

Leptospirosis was found more than 100 years ago and its clinical manifestations have been well characterized. However, its pathogenesis is still poorly understood, which in turn hampers the identification of new intervention strategies. To date, only a few proteins have been identified as putative virulence factors of leptospires, such as adhesion molecules [8], hemolysins [9], and lipoproteins [10]. Recently, the genomic sequencing of pathogenic *leptospire* strains [11,12] has provided a foundation for understanding the pathogenesis of leptospirosis by suggesting a variety of

putative virulence factors. It is necessary to explore the function of these putative virulence factors to fully elucidate the pathogenesis of leptospirosis.

Loa22 from *Leptospira interrogans* is a lipoprotein with an Outer membrane protein A (OmpA) domain in the C-terminus. In other bacteria, OmpA acts as a multifunctional protein involved in cell adhesion, tissue invasion [13,14], immune evasion [15], and induction of the immune response [16]. Accordingly, OmpA-like protein, Loa22, may be involved in the interaction of pathogenic leptospire with host cells during the period of infection. Koizumi and Watanabe [17] confirmed that pathogenic leptospire strains could react with anti-Loa22 serum, although Loa22 expression was not detectable in the non-pathogenic strains such as *Leptospira biflexa* and *Leptospira meyeri*, suggesting a correlation between Loa22 and leptospire pathogenicity. Picardeau *et al*. [18] found that Loa22 had an ortholog in *L. biflexa* with a similarity of 73% by genomic sequencing. More recent studies have revealed that Loa22 may play an important role in the infection and immunological responses of leptospirosis [19–21], indicating that Loa22 may be involved in *Leptospira* virulence. However, the role of Loa22 in leptospirosis renal disease has not been elucidated so far.

In the present study, we constructed a vector and artificially expressed Loa22 in *Escherichia coli* BL21(DE)pLysS cells. After extensive purification, along with a GST tag protein control, Loa22 protein was first used to investigate the cytotoxicity to cultured rat proximal tubule cell lines (NRK52E). The data indicated that Loa22 mediates direct cytotoxicity to tubule cells in a dose-dependent manner. Furthermore, since the innate immune response significantly contributes to the pathogenesis of leptospiral tubulointerstitial nephritis, we examined the effects of innate immune response activation by measuring the expression of toll-like receptor 2 (TLR2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), and monocyte chemoattractant protein-1 (MCP-1) by NRK52E cells. Our data indicate that Loa22 triggers proinflammatory responses of proximal tubule cells by upregulating TLR2 and activating the associated signaling cascade, which enhances the production of NO and MCP-1. Our study suggested that Loa22 is a virulence factor of *L. interrogans* and is involved in the pathogenesis of leptospiral nephropathy by mediating direct cytotoxicity and induction of innate immune responses.

Materials and Methods

Expression and purification of recombinant Loa22 protein

Cloning of Loa22 was performed according to a standard recombinant DNA procedure. Briefly, using *Leptospira interrogan serovar Lai* genomic DNA which was extracted by ourselves with a genomic extraction kit of bacterium (Takara Biotechnology, Dalian, China) as a template, we amplified the portion of Loa22 encoding mature protein, in which the signal peptide sequence was excluded through detection with SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). A pair of primers including a *Bam*HI and an *Eco*RI restriction endonuclease site near their 5′ end (underlined) were used for PCR reaction: upstream primer: 5′-TCCGGATCCGAAAAAAAAGAGGAATCC-3′; downstream primer: 5′-CGATGAATTCTTATTGTTGTGGTGCGG-3′. The PCR products were purified with a commercial purification kit (Omega Bio-Tek, Doraville, GA, USA), digested with *Bam*HI and *Eco*RI (Takara Biotechnology, Dalian, China), and ligated into the GST vector pGEX-4T-1 (GE Healthcare, Piscataway, NJ, USA) that has been digested with the same restriction endonucleases. The resulting construct of pGEX-4T-1-Loa22 was transformed into *E. coli* BL21(DE3)pLysS cells (Tiangen Biotechnology, Beijing, China). Isopropyl-β-d- thiogalactopyranoside (IPTG) (0.5 mM, Sigma, St Louis, USA) was added when the OD_{600} reached 0.6 to induce the expression of the GST-Loa22 fusion protein. The GST-Loa22 fusion protein was purified using GST-affinity column (Amersham Biosciences, Uppsala, Sweden), according to the supplier's instructions. Then the purified fusion protein underwent cleavage with thrombin to cut off GST tag and the cleaved protein was repurified with GST-affinity column to remove GST tag. After repurification, the resulting Loa22 protein was subjected to an endotoxin-removing gel to remove contamination of LPS from *E. coli*, according to the protocol provided by the manufacturer (Pierce Detoxi-Gel^{TM}, Rockford, USA). The final purified Loa22 protein was lyophilized and stored at −70°C for experiment. The LPS concentration was determined using a chromogenic substrate Limulus Tachylpleus Amebocyte Lysate (TAL) endpoint assay kit purchased from Xiamen Houshiji, Ltd (Fujian, China), according to the manufacture's instruction. To exclude the possible effects of contamination in Loa22 protein and to verify the specificity of Loa22 protein's biological activity, GST protein from the pGEX-4T-1 vector was also purified as we prepared Loa22 protein and used as a control in all of experiments in this study.

Cell culture

The NRK52E cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hyclone, Logan, USA) in 5% CO_2 humidified atmosphere at 37°C. Experiments were performed on exponentially growing and confluent cells. Cells were shifted to a serum-free medium 24 h before the experiments. Cell morphology was monitored under an inverted microscope

(CKX31, Olympus, Tokyo, Japan) in routine culture or during experiments.

Cytotoxicity and analysis of cell death

NRK52E cells were plated in 96- or 6-well plates (1×10^4 or 1×10^5 cells /well, respectively), incubated for 36 h, then treated with Loa22 protein at a series of concentrations (1, 5, 10, 20, 40 μg/ml) for 48 h. To assess cell growth and viability, cells in 96-well plates were subjected to the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoium hydrixide) assay and cells in 6-well plates for the lactate dehydrogenase (LDH) assay. Propidium iodide (PI) staining and FACS analysis were used to measure the apoptosis. Each assay was repeated four times and purified GST protein from pGEX-4T-1 vector was included as a negative control for Loa22 stimulation in each experiment.

XTT assay

XTT (40 μl; 1 mg/ml) and phenazine methosulfate (PMS, 25 μM, both purchased from Sigma, St. Louis, USA) were added into each well of treated cells in 96-well plates. After incubation for additional 4 h, the absorbance at 450 nm was measured with a Model 680 microplate reader (Bio-Rad Laboratories Inc., Hercules, USA).

Measurement of LDH activity and cell apoptosis

The supernatants from each well of the treated cells in 6-well plates (1×10^5 cells/well) were individually collected and used to measure the LDH activity using an LDH kit (Nanjing Jiancheng Biotechnology Institute, Jiangsu, China), according to the manufacturer's protocol. The LDH activity was calculated using the following formula.

$$\text{LDH activity} = \frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{standard}} - A_{\text{standard blank}}} \times \text{Concentration}_{\text{standard}}$$

At the same time, after supernatant removal, NRK52E cells were washed with PBS, harvested, and fixed in 1 ml 75% ethanol and stained with a PI Kit (Xian Runde Biotecholgy Ltd, Shangxi, China). The resulting cells were analyzed via flow cytometry and the ratio of apoptotic cells was calculated, according to the manufacturer's instructions.

Quantitative real-time PCR

Real-time PCR was used to quantitate the expression of TLR2, iNOS, and MCP-1 mRNA by normalizing their amplification to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). NRK52E cells incubated with Loa22 protein at various concentrations (0.25, 0.5, 1 μg/ml) for 48 h were used to extract total RNA using E.Z.N.A. tissue RNA kit (Omega Bio-Tek, Doraville, USA). Reverse transcription was carried out with a Revert Aid^{TM} first strand cDNA synthesis kit (Fermentas, Shenzhen, China) following the instructions provided by the manufacturer. Using SYBR Green I, the real-time PCR reaction was performed in a Bio-Rad iCycler iQ real-time PCR detection system. The following primer pairs were used to amplify the specific genes. TLR2 primer pair, F: 5′-CTCTTTGACGAGAACAATG-3′ and R: 5′-GTTCATTATCTTGCGCAGTTTG-3′ yields a 102 bp PCR product. iNOS primer pair, F: 5′-CAGGGTCCCCTGCACCA-3′ and R: 5′-GCTGGAAGCCACTGACACT-3′ yields a 100 bp PCR product. MCP-1 primer pair F: 5′-GCAAGATGATCCCAATGAGT-3′ and R: 5′-GTCAGCACAGATCTCTCTCTT-3′ yields a 113 bp PCR product. GAPDH primer pair, F: 5′-TGACATCAAGAAGGTGGTGA-3′and R: 5′-TCATACCAGGAAATGAGCTT-3′ yields a 177 bp PCR product. PCR thermal conditions were as follows: pre-denaturation at 94°C 2 min, followed by 45 cycles of 94°C 20 s, 50°C 20 s and 72°C 30 s for TLR2, or 45 cycles of 94°C 20 s, 52°C 20 s and 72°C 30 s for iNOS, MCP-1 and GAPDH. The relative gene expression level was calculated on the basis of the ΔCt, which is the difference of threshold cycle (Ct) between the gene of interest and GAPDH.

$$\Delta\text{Ct} = \text{Ct}_{\text{GAPDH}} - \text{Ct}_{\text{gene of interest}}$$

Final results were expressed as *N*-fold differences in target gene expression relative to the calibrator, termed 'N_{target}', which were determined as follows.

$$N_{\text{target}} = 2^{\Delta\text{Ct(sample)} - \Delta\text{Ct(calibrator)}}$$

Lysate preparation and western blotting

Using lysis buffer containing 20 mM HEPES, pH 7.2, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethyl sulfonylfluoride and a protease inhibitor cocktail (10 ml/L; Sigma-Aldrich, St. Louis, USA), whole-cell extracts were prepared from NRK52E cells that were treated with Loa22 or GST protein. Protein concentration of the extracts was determined by the bicinchoninic acid method. Fifty micrograms of protein were separated on 10% SDS-PAGE and were electronically transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Richmond, USA). The membrane was detected by a standard western blotting procedure. Briefly, the membrane was blocked with 5% milk in TBST buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20), then probed with a rabbit polyclonal anti-TLR2 antibody, (Santa Cruz Biotechnology, Santa Cruz, USA) (1:200). After washing with TBST buffer, the membrane

was further incubated with HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) (1:20,000). The protein bands were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, USA).

TLR2 immunofluorescence

NRK52E cells were plated onto an uncoated 6-well plate. After treatment with Loa22 protein (0.5 μg/ml) for 48 h, cells were fixed with 4% paraformaldehyde, washed and blocked with 10% normal goat serum, and then incubated with anti-TLR2 or normal rabbit IgG isotype control (Santa Cruz Biotechnology) (1:50) overnight at 4°C. After washing, the cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:400, Zhongshan Biotechnology, Guangzhuo, China), examined and analyzed on an inverted fluorescence microscope (DMI6000B, Leica, Wetzlar, Germany) equipped with a Leica DFX300 FX CCD camera and the Image Pro Plus program.

MCP-1 and NO measurement and TLR2 antibody neutralization

The supernatants were collected from NRK52E cells after being treated with Loa22 or GST protein (0.25, 0.5, 1 μg/ml) for 48 h. MCP-1 was measured with an ELISA kit (BioSource, International, Camarillo, USA) according to the manufacturer's instructions. Nitrite production, measured as an index of NO production in the supernatant, was determined by the Griess reaction using a commercial NO detecting kit (Nanjing Jiancheng Biotechnology Institute, China). Goat anti-mouse TLR2 blocking antibody (Santa Cruz Biotechnology) was added to the cell culture at a concentration of 10 μg/ml for 1 h prior to Loa22 treatment.

Statistical analysis

All measurements were performed at least in triplicate experiments and results were expressed as mean ± SD. Student's *t*-test or one-way ANOVA analysis in SPSS (version 15.0, SPSS Inc., USA) was used for statistical analysis. Differences were considered significant if $P < 0.05$.

Results

Expression and purification of recombinant Loa22

To study the effect of Loa22 on renal tubule cells, it was necessary to prepare a large amount of pure Loa22 protein. Using GST-tag, we successfully expressed and obtained GST-Loa22 fusion protein from *E. coli*. As shown in **Fig. 1**, recombinant Loa22 was expressed in soluble fractions of *E. coli* BL21(DE)pLysS cells. After one-step Gstrap FF purification, the yield of GST-Loa22 fusion protein was approximately 16 mg/L culture. After removal of the GST-Tag with thrombin treatment, the Loa22 protein had a molecular weight of 20 kDa (**Fig. 1**, lane 7). To remove contamination of *E. coli* LPS, Loa22 protein was further purified with an endotoxin-removing gel. The Limulus TAL endpoint assay indicated that LPS levels were less than 0.25 EU/mg of Loa22.

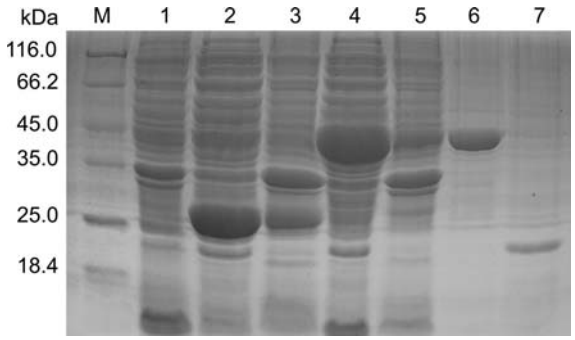

Figure 1 Expression and purification of Recombinant Loa22 in *E. coli* *Escherichia coli* BL21(DE3)pLysS transformed with the pGEX-4T-1 or pGEX-Loa22 plasmid was grown in 2YT-ampicillin medium. IPTG (0.5 mM) was used to induce GST-Loa22 protein expression. Cells were lysed and subjected to electrophoresis. The gel was stained with Coommassie Blue. M: protein marker; lane 1: cellular extracts of cells transformed with pGEX-Loa22 plasmid without IPTG; lanes 2 and 3: the soluble cellular fraction and the inclusion body fraction of cells transformed with pGEX-4T-1 plasmid after induction of IPTG, respectively; lanes 4 and 5: the soluble cellular fraction and the inclusion bodies fraction of cells transformed with pGEX-Loa22 plasmid after induction of IPTG, respectively; lane 6: purified pGEX-Loa22 fusion protein with a GST column; lane 7: purified Loa22 protein after digested with thrombin.

Loa22 mediates cytotoxicity in NRK52E cells

As a putative virulence factor, Loa22 may cause direct cytotoxicity to renal tubule cells. To test this hypothesis, we first examined morphological changes of NRK52E cells after Loa22 treatment. As shown in **Fig. 2**, incubation of Loa22 (20 and 40 μg/ml) with NRK52E cells for 48 h resulted in cell rounding, a decrease in size and some detachment [**Fig. 2(A)**, b,c]. In contrast, the untreated cells and GST protein (40 μg/ml)-treated cells had a normal morphology [**Fig. 2(A)**, a,d]. Then, Loa22-induced cell death was verified with XTT and LDH assays, in which cell death is reflected as a decrease of XTT absorbance and an increase of LDH activity, respectively. As shown in [**Fig. 2(B)**, a,b], significant cell death was observed when NRK52E cells were treated with Loa22 protein at a concentration of 5 μg/ml or higher ($P < 0.05$). Increasing Loa22 protein concentrations resulted in more cell death according to both XTT and LDH assays. In contrast, the untreated cells and GST protein-treated cells did not show any decrease of XTT absorbance or any increase of LDH activity. These data suggested that Loa22 protein mediates

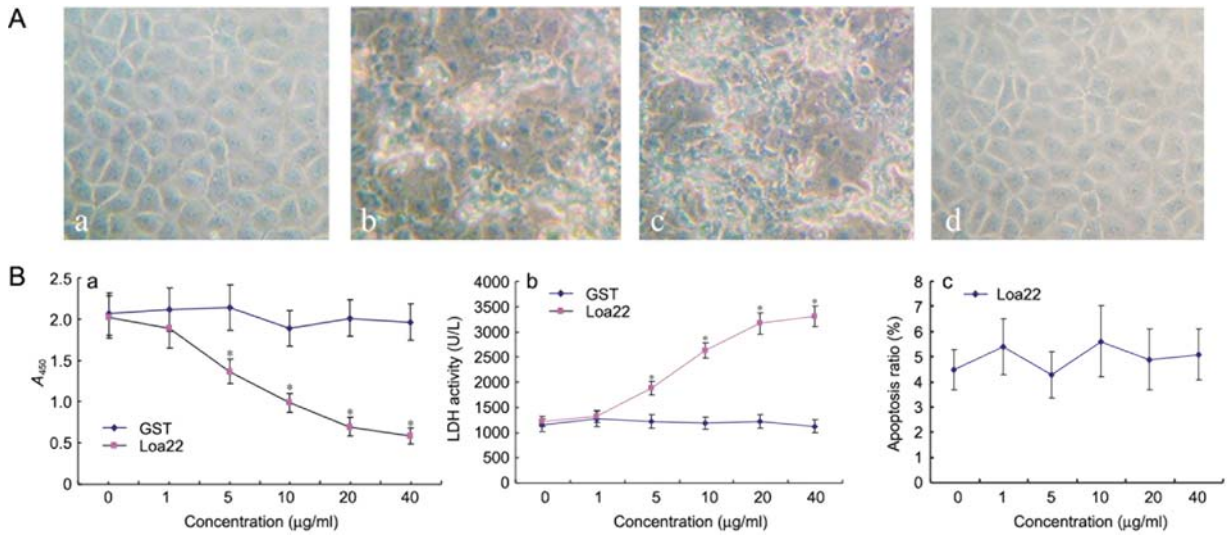

Figure 2 Cytotoxic effects of Loa22 on NRK52E cells (A) Micrographs show morphology of NRK52E cells after treatment with PBS (a) Loa22 at a concentration of 20 μg/ml (b) or 40 μg/ml (c) or GST protein at a concentration of 40 μg/ml (d) (original magnification, ×200). (B) Analysis of cell death with XTT absorbance (A_{450}) (a), LDH activity (b) and apoptosis (c). Data were obtained from five independent experiments and are presented as mean ± SD (*$P < 0.05$ vs untreated cells).

a cytotoxic effect on NRK52E cells in a dose dependent manner. However, in flow cytometry analysis, no difference in apoptosis was detected between untreated cells and cells treated with Loa22 at a variety of concentrations [**Fig. 2(B)**, c]. Collectively, the above data suggested that Loa22 is an important virulent-related factor of pathogenic *leptospire* that causes renal lesions by inducing necrotic cell death.

Loa22 upregulates the expression of TLR2 in NRK52E cells

It has been reported that TLR2 is constitutively expressed in renal tubular epithelial cells [22]. However, it is unknown whether Loa22 influences TLR2 expression in NRK52E cells. To test this hypothesis, we tested the effect of Loa22 stimulation on TLR2 expression at both the mRNA and protein level in NRK52E cells. Using quantitative real-time PCR, it was found that compared with untreated NRK52E cells, TLR2 mRNA expression increased 0.8, 2.6, and 2.3 folds after incubation with Loa22 for 48 h at a concentration of 0.25, 0.5, and 1 μg/ml, respectively ($P < 0.05$) [**Fig. 3(A)**]. TLR2 protein expression was determined by western blotting [**Fig. 3(B,C)**] using a TLR2-specific antibody. In accordance with the increased mRNA expression, TLR2 protein expression was upregulated after incubation with Loa22 for 48 h. In contrast, GST protein did not upregulate TLR2 expression at a similar concentration of Loa22 protein (0–1 μg/ml), suggesting that the above observed upregulation of TLR2 was specifically induced by Loa22 protein and not by contaminated byproducts. In agreement with the results for protein quantification, immunofluorescence for TLR2 expression revealed that TLR2 immunoreactivity on NRK52E cells was markedly upregulated after Loa22 stimulation for 48 h (0.5 μg/ml) [**Fig. 3(D)**]. Taken together, these data suggested that Loa22 stimulation upregulates TLR2 expression at both mRNA and protein level in NRK52E cells.

Loa22 upregulates iNOS and NO expression in NRK52E cells

It was determined that Loa22 protein upregulates TLR2 expression in NRK52E cells, which prompt us to hypothesize that Loa22 protein could induce or upregulate certain pro-inflammatory factors. Since NO has multiple roles during inflammation and maintenance of renal function, the effect of Loa22 on the mRNA expression of iNOS was tested using real-time PCR and release of NO was examined using the Griess reaction. It was found that compared with untreated NRK52E cells, iNOS mRNA expression increased 1.0, 5.4, and 5.1 folds after Loa22 treatment for 48 h at a concentration of 0.25, 0.5, and 1 μg/ml, respectively ($P < 0.05$) [**Fig. 4(A)**]. Consistent with the upregulation of iNOS gene expression, stimulation of NRK52E cell with Loa22 resulted in a dose-dependent increase of NO, and reach maximum at 0.5 μg/ml Loa22 [**Fig. 4(B)**], which was not observed with GST protein alone [**Fig. 4(C)**]. Collectively, these data suggested that Loa22 upregulates iNOS expression, which in turn enhances the release of NO by NRK52E cells.

Loa22 upregulates MCP-1 expression in NRK52E cells

One characteristic feature of tubulointerstitial nephritis caused by leptospire is the infiltration of inflammatory

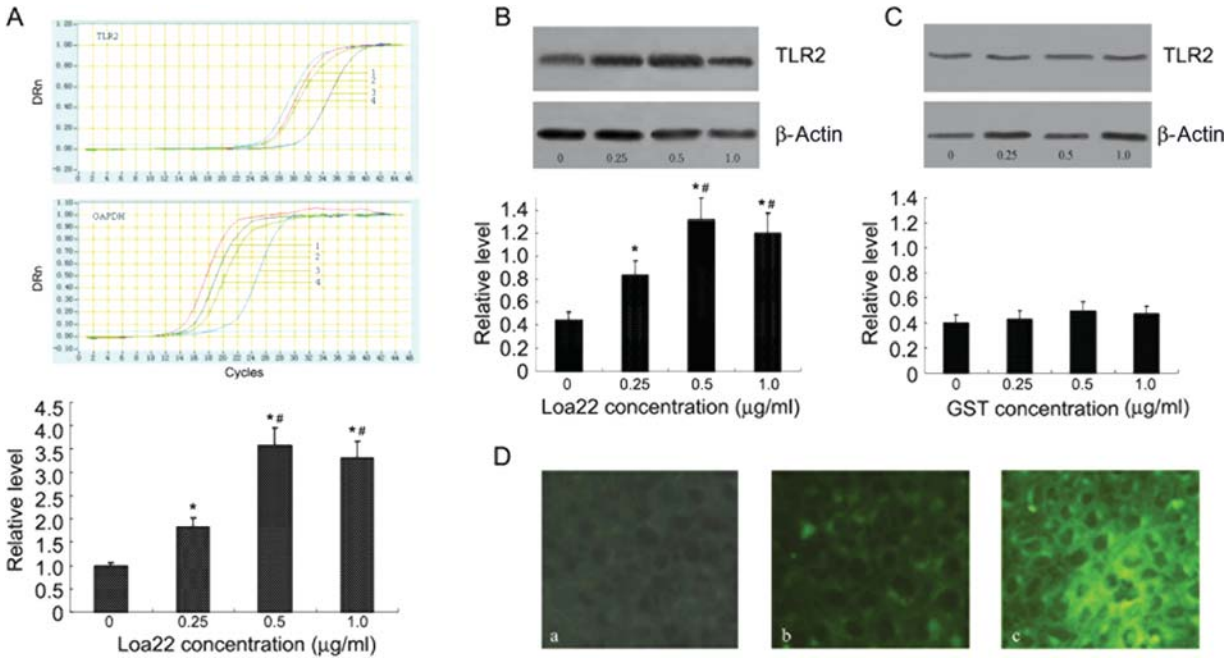

Figure 3 Upregulation of TLR2 expression in NRK52E cells by Loa22 (A) Representative real-time PCR plots show amplification of TLR2 and GAPDH in NRK52E cell cultures after incubation with Loa22 at specified concentrations (1: untreated; 2–4: Loa22 stimulation at 0.25, 0.5, and 1 μg/ml, respectively) (upper panel). Bar graphs represent the *N*-fold differences of TLR2 expression relative to GAPDH (lower panel). (B) Western blotting shows protein bands probed by TLR2 or β-actin antibodies in NRK52E cells treated with Loa22 at specified concentrations (upper panel). Quantitative results of western blotting presented as a TLR2/β-actin densitometry value ratio were shown in lower panel. (C) Western blotting results of TLR2 and β-actin expression in NRK52E cells treated with GST protein at specified concentrations (upper panel). The TLR2/β-actin densitometry value ratio of western blotting (lower panel). The data in (A–C) were obtained from three separate experiments and are expressed as mean ± SD (*$P < 0.05$ vs untreated cells; $^{\#}P < 0.05$ vs 0.25 μg/ml group). (D) Micrographs show immunofluorescence staining of TLR2 in NRK52E cells with or without Loa22 stimulation (0.5 μg/ml for 48 h) (b and c, respectively). Normal rabbit IgG was used as isotype control (a) (original magnification, ×200).

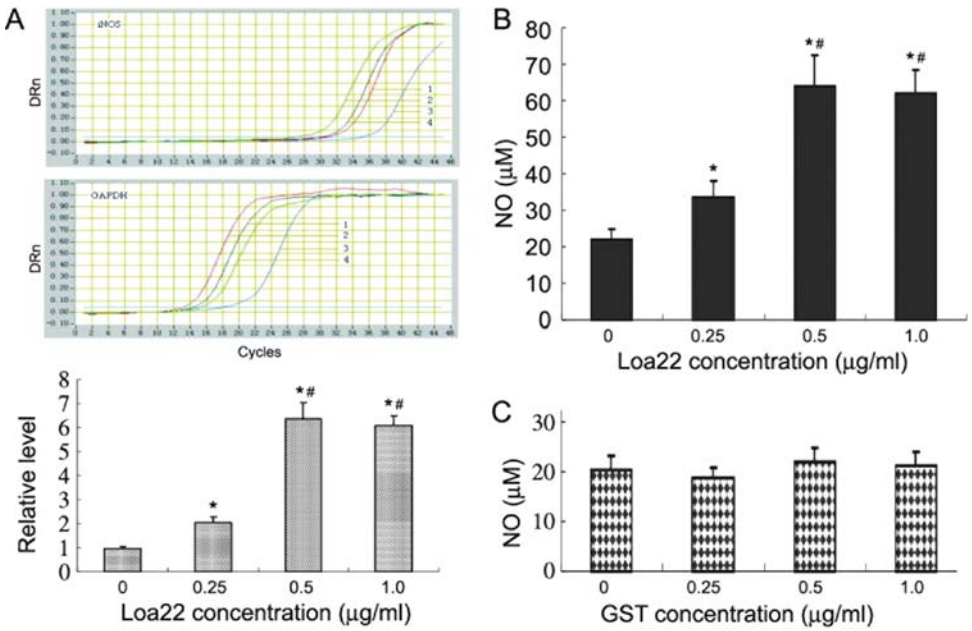

Figure 4 Upregulation of iNOS expression and NO release in NRK52E cells by Loa22 (A) Representative real-time PCR plots show the amplification of iNOS and GAPDH in NRK52E cell cultures after incubation with Loa22 at specified concentrations (1: untreated; 2–4: Loa22 stimulation at 0.25, 0.5, and 1 μg/ml, respectively). Bar graphs represent the *N*-fold differences of iNOS mRNA expression relative to GAPDH. (B) Bar graphs show the release of NO measured by Griess reaction in NRK52E cell cultures after incubation with Loa22 at specified concentrations. (C) Bar graphs show the release of NO in NRK52E cells treated by GST protein at specified concentrations. Data were obtained from three independent experiments and are presented as mean ± SD (*$P < 0.05$ vs untreated cells; $^{\#}P < 0.05$ vs 0.25 μg/ml group).

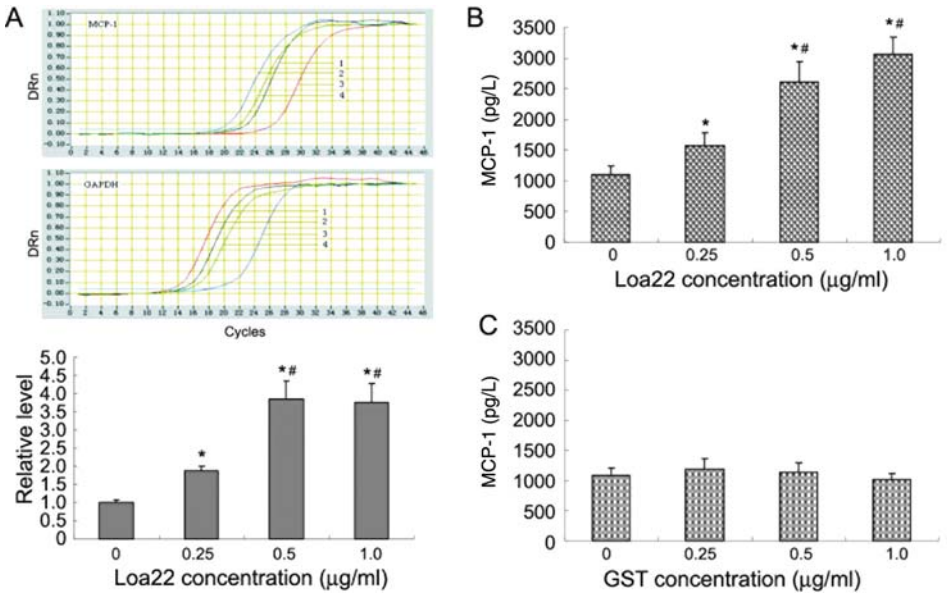

Figure 5 Upregulation of MCP-1 expression by NRK52E cells by Loa22 (A) Representative real-time PCR plots show amplification of MCP-1 and GAPDH in NRK52E cell cultures after incubation with Loa22 at specified concentrations (1: untreated; 2–4: Loa22 stimulation at 0.25, 0.5, and 1 μg/ml, respectively) (upper panel). Bar graphs represent the N-fold differences of MCP-1 mRNA expression relative to GAPDH (lower panel). (B) Bar graphs show the concentration of MCP-1, measured with ELISA in NRK52E cell supernatants after incubation with Loa22 at specified concentrations. (C) Bar graphs show the concentration of MCP-1 in NRK52E cell supernatants after the treatment of GST protein at specified concentrations. Data were obtained from three independent experiments and are presented as mean ± SD (*$P < 0.05$ vs untreated cells; $^{\#}P < 0.05$ vs 0.25 μg/ml group).

cells such as monocytes and macrophages. A potent chemokine associated with monocyte and macrophage recruitment is MCP-1. Thus, whether Loa22 affects MCP-1 expression in NRK52E was tested. Both mRNA [**Fig. 5(A)**] and protein [**Fig. 5(B)**] expression of MCP-1 are upregulated by Loa22 stimulation in a dose-dependent manner similar to the release of NO. The failure of MCP-1 upregulation following GST protein at a similar concentration [**Fig. 5(C)**] confirmed the specificity of Loa22-mediated upregulation of MCP-1 expression.

Loa22 upregulates inos and MCP-1 expression in NRK52E cells via TLR2 signaling

Previous studies have demonstrated that TLR2 is required for OmpA-dependent activation of macrophage and dendritic cells [23]. We hypothesized that TLR2 signaling is also likely to participate in the mechanism of inflammatory mediator production by NRK52E cells following the stimulation of OmpA-domain containing Loa22 protein. To test this hypothesis, we examined whether Loa22-induced upregulation of NO and MCP-1 expression could be abolished by blocking TLR2 activation with an antibody. As shown in [**Fig. 6(A,B)**], Loa22 protein-induced iNOS and MCP-1 mRNA expression were completely inhibited by preincubation of TLR2-blocking antibody, but not by isotype control. Accordingly, the release of NO and MCP-1 expression by NRK52E cells [**Fig. 6(C,D)**] were completely suppressed to basal levels in untreated cells. Collectively, these results suggest that Loa22 induces NO and MCP-1 synthesis through TLR2 activation.

Discussion

In affected kidneys, leptospires may present in renal proximal tubule cells and macrophages, or form large extracellular clumps in the interstitium [24,25]. Loa22 is exposed on the surface of *Leptospira* and the expression of *Loa22* is upregulated during the acute phase of host infection with *Leptospira* [18], suggesting that this protein may be involved in leptospiral nephropathy through interaction with renal cells. Ristow *et al*. [26] confirmed that OmpA-like protein Loa22 is essential for leptospiral virulence. In the present study, we prepared a recombinant Loa22 to further determine its biological activities, focusing on cytotoxicity and the induction of inflammatory molecules. Since the recombinant Loa22 was expressed and prepared from *E. coli*, the *E. coli*-derived contaminants, such as LPS and other lipoproteins that are capable of stimulating inflammation [27], may produce some effects not specific to Loa22. To avoid false positives, we used multiple approaches, such as GST affinity purification and a LPS-removing gel to minimize the contaminants. In addition, to rule out the possible effects of trace contaminants, we used purified GST protein as a control in all experiments. The fact that the purified GST protein consistently showed negative results in all experiments indicated that the trace contaminants in recombinant Loa22 protein

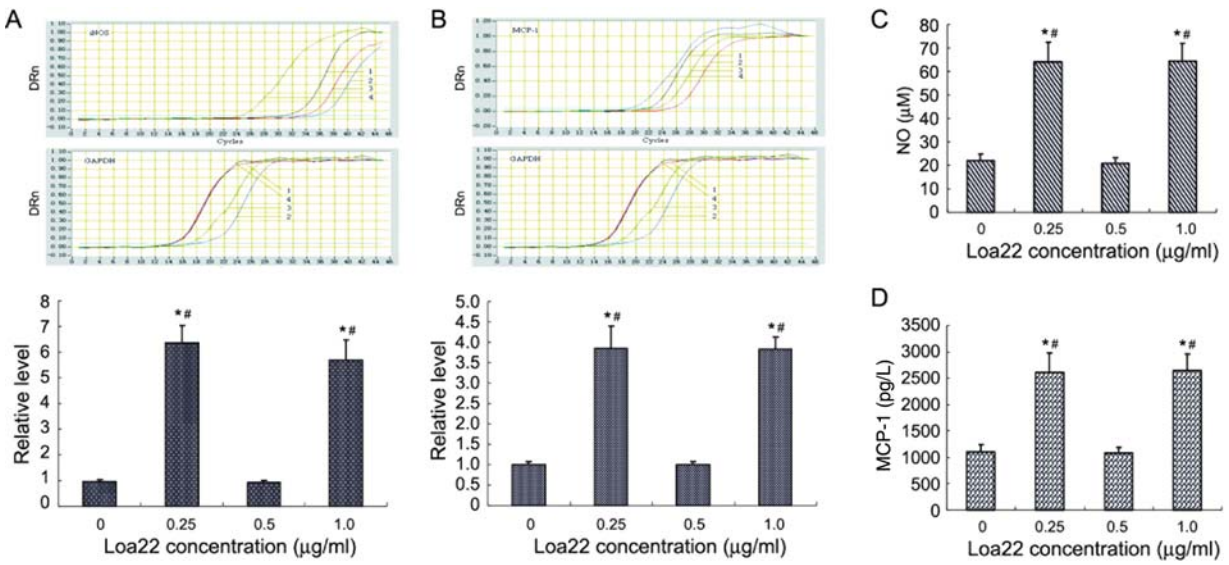

Figure 6 Suppression of Loa22-induced iNOS, NO, and MCP-1 production by a TLR2-blocking antibody (A) Representative real-time PCR plots show amplification of iNOS/GAPDH in NRK52E cells after incubation with Loa22 in the absence or presence of anti-TLR2 antibody (1: untreated; 2: Loa22 at 0.25 μg/ml, 3: Loa22 at 0.25 μg/ml plus anti-TLR2 antibody at 10 μg/ml, 4: Loa22 at 0.25 μg/ml plus goat isotype IgG at 10 μg/ml) (upper panel). Bar graphs represent the *N*-fold differences of iNOS mRNA expression relative to GAPDH (lower panel). (B) Representative real-time PCR plots (upper panel) show the amplification of MCP-1/GAPDH and *N*-fold differences (lower panel) of MCP-1 mRNA expression relative to GAPDH in NRK52E cell after incubation with Loa22 at specified concentrations in the absence or presence of anti-TLR2 antibody (same groups as A). (C,D) Bar graphs show NO release measured by the Griess reaction and MCP-1 protein measured by ELISA from supernatants of NRK52E cells treated with Loa22 in the absence or presence of anti-TLR2 antibody. Data were obtained from three independent experiments and are presented as mean ± SD (*$P < 0.05$ vs untreated cells; #$P < 0.05$ vs the group of 0.25 μg/ml plus anti-TLR2 antibody).

did not induce effects in our experimental system, thus validating the observed effects.

Merien *et al*. [28] reported that virulent *Leptospira* can induce macrophage apoptosis. Recently, Li *et al*. [29] observed that *L. interrogans* induces cell death via necrosis or apoptosis, varying among different host cell types. Furthermore, autopsy data from patients suffering from leptospirosis have revealed significant degeneration and necrosis of renal tubular epithelial cells in the affected kidney [30]. In agreement with previous observations, our study revealed that Loa22 exerts cytotoxicity in cultured NRK52E cells. Morphological characterization, as well as XTT and LDH assays, consistently suggested that Loa22 protein-mediated cytotoxicity at a concentration of 5 μg/ml or higher. However, following Loa22 protein treatment, apoptosis of NRK52E cells did not change, suggesting that the primary NRK52E cell death following Loa22 treatment is not apoptosis but necrosis. Our findings confirm that OmpA-like protein Loa22 is a critical virulence factor of *Leptospira*, which is in agreement with the findings by Ristow *et al*. [26]. With respect to clinical relevance, it has been demonstrated that *Loa22* is expressed on the surface of *Leptospira* [17] and is upregulated during the acute stage of infection [19]. Therefore, Loa22-mediated cytotoxicity on epithelial cells is one potential mechanism underlying the renal damage resulted from leptospiral virulence.

Loa22 contains an OmpA domain, which is a pathogen-associated molecular pattern recognized by pattern recognition receptors, is partly responsible for triggering innate immunity. Toll-like receptors (TLR) are a group of important pattern recognition receptors participating in the initiation of inflammation by sensing a variety of bacteria. Renal tubular epithelial cells are among the non-immune cells that express TLR1, TLR2, TLR3, TLR4, and TLR6 [31,32]. TLR2 and TLR4 are constitutively expressed in both proximal and distal renal tubular epithelial cells *in vivo*. Yang *et al*. [33] found that TLR2 mediated inflammation induced by LipL32, the major leptospiral outer membrane lipoprotein. Jeannin *et al*. [23] confirmed that TLR2 was required for OmpA-dependent activation of macrophages and dendritic cells. In the present study, we found that TLR2 expression on NRK52E cells was significantly upregulated at both mRNA and protein levels following Loa22 stimulation. This supports our idea that Loa22 recognition by TLR2 is involved in the initiation of innate immune responses. Furthermore, using a TLR2 blocking antibody, we found that the activation of TLR2 by Loa22 is necessary for iNOS and MCP-1 gene expression, as well as for NO and MCP-1 synthesis. Collectively, Loa22 plays an important role in inducing inflammatory responses through TLR2, which may underlie the pathogenesis of leptospiral tubulointerstitial nephritis.

It has been established that NO plays important roles in maintaining renal function under normal physiological conditions and promotes inflammation under pathological conditions [34,35]. Furthermore, proximal tubule cells are major sources of NO [35]. In the present study, we found that Loa22 markedly upregulated iNOS mRNA expression and NO production by NRK52E cells. NO may participate in the pathogenesis of tubulointerstitial nephritis via multiple mechanisms. First, although regulating renal oxygenation and preventing hypoxic injury, excessive NO may interact with superoxide and produce reactive nitrogen species such as peroxynitrite. This can promote nitration on important proteins, change signaling pathways and contribute to tissue injury [36]. In addition, NO also exerts potent pro-inflammatory functions by activating multiple signaling cascades and amplifying the scope of inflammatory responses [37]. Furthermore, NO downregulates the Na^+–K^+-ATPase, Na^+/H^+ exchangers and paracellular permeability of proximal tubule cells, which inhibits the transport function of renal proximal tubule and causes interstitial edema [35].

One of the pathological features of leptospirosis renal disease is the infiltration of immune cells including lymphocytes, monocytes, and neutrophils. Chemokines, a group of factors possessing chemotactic activity, are involved in many inflammatory reactions by recruiting inflammatory cells. It has been further demonstrated that chemokines are upregulated during various forms of glomerular and interstitial injuries [38,39]. Furthermore, MCP-1 accounts for 70−80% of monocyte chemotactic activity by cultured human mesangial cells, renal cortical epithelial cells and proximal tubular cells [40–42]. Recently, in a renal fibrosis mouse model, Wada *et al*. [43] found that delivery of the N-terminal deletion mutant of human MCP-1 into skeletal muscle led to a decrease in MCP-1 expression and reduction of macrophage infiltration into kidney. Consequently, we hypothesized that Loa22 may be involved in leptospiral nephritis by promoting MCP-1 expression. Our data indicated that Loa22 stimulation significantly upregulates the expression of MCP-1 by the cultured rat proximal tubule cells at both mRNA and protein levels. We speculated that Loa22 may also induce renal epithelial cells to express MCP-1 *in vivo* in leptospirosis, which may be involved in the immune cell infiltration in leptospiral tubulointerstitial nephritis.

Using the recombinant Loa22 and cultured NRK52E cell model, we demonstrated for the first time that Loa22 mediates a cytotoxic effect on rat renal tubular epithelial cells at high doses and promotes inflammation at low doses. However, it's worth mentioning that infection in rats usually causes a persistent renal carriage of leptospires, therefore these results are of great importance for the maintenance of host infection with leptospires.

In summary, our study revealed that Loa22 is a virulence factor of *leptospire* and may play an important role in the pathogenesis of leptospirosis renal disease by inducing cell death and inducing innate immune responses in tubular epithelial cells.

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