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CD200R1 agonist attenuates LPS-induced inflammatory response in human renal proximal tubular epithelial cells by regulating TLR4-MyD88-TAK1-mediated NF-κB and MAPK pathway

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ABSTRACT

Previous studies have revealed the anti-inflammatory effect of CD200Fc, an agonist of CD200R1 in autoimmune disease. However, little is known about its anti-inflammatory effects in kidney diseases. The aim of this study is to assess the function of CD200Fc in regulating lipopolysaccharide (LPS)-induced inflammatory response in human renal proximal tubular epithelial cells (hRPTECs) and the possible mechanisms. LPS reduced the CD200R1 expression in hRPTECs, and this effect was attenuated by CD200Fc in a dose-dependent manner. In addition, CD200Fc inhibited LPS-induced expressions of TLR4 and its adapter molecule (MyD88 and phosphorylation of TAK1), and abolished its interactions with MyD88 or TAK1 in hRPTECs cells. CD200Fc also attenuated LPS-induced phosphorylation of IκB, NF-κB-P65 translocation to nucleus, and increased phosphorylation of ERK1/2, p38 and JNK in hRPTECs. Moreover, CD200Fc suppressed the LPS-induced release of pro-inflammatory mediators in hRPTECs, including IL-1β, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, TNF-α, INF-α and INF-γ. Our results suggested that CD200Fc could inhibit the TLR4-mediated inflammatory response in LPS-induced hRPTECs, thus might be beneficial for the treatment of renal disease, such as lupus nephritis.

1. Introduction

Lupus nephritis (LN) affects up to 70% of patients with SLE and is a major cause of mortality [1,2]. Despite there were many advances in the diagnosis and management of LN, the incidence of end-stage renal disease secondary to SLE was not decreased in the last 20 years [3]. LN is associated with inflammation caused by renal deposition of immune complexes comprise of auto-antibodies. If not resolved, renal inflammation can lead to renal injury, dysfunction and failure [4].

CD200 is a member of the immunoglobulin super-family of glycoproteins, and is expressed in a wide variety of cells [5,6]. CD200 exerts its effect by binding to a structurally similar CD200 receptor family (CD200R1-R4), triggering intracellular signaling cascades, such as Ras/MAPK pathways [7]. CD200-deficient mice express an inflammatory phenotype exhibiting increased macrophage or microglial activation in models of arthritis [8,9], encephalitis [10,11], and uveoretinitis [12]. CD200R1 agonists (CD200Fc) down-regulate pro-inflammatory cytokine production by activated macrophages and microglia in a model of multiple sclerosis [13]. These studies suggest that CD200 and its receptor play a key role in the modulation of inflammatory responses.

Recent studies also showed that systemic lipopolysaccharide (LPS)-induced microglial activation resulted in different temporal reduction of CD200 and CD200R gene expression in the brain [6]. In addition, LPS exerted more profound effects on release of the pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and TNF-α in glia isolated from CD200-deficient mice compared with wild type mice [5]. Moreover, expressions of TLR4 and TLR2 were increased in glia prepared from CD200-deficient Mice [5]. Based on this information, we postulated that CD200R1 agonists (CD200Fc) play a key role in the attenuation of inflammatory responses in LPS-induced human

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renal proximal tubular epithelial cells (hRPTECs), which may be associated with TLR4-dependent pathways. The purpose of this research is to clarify the anti-inflammatory effects and further mechanisms of CD200Fc on LPS-induced hRPTECs.

2. Materials and methods

2.1. Cell culture

Primary hRPTECs were purchased from FMG-Bio (Sciencell, Shanghai) and cultured in suggested medium: REBM/REGM bullet kit (Biowhittaker Inc, Walkersville, MD) or RECGM2 (Promocell, Heidelberg, Germany), as described [14]. Confluent cells between passages 6 and 9, derived from three different single donor preparations were used in experiments. We purchased LPS from Sigma–Aldrich Co (St. Louis, MO), and CD200Fc (a CD200 fusion protein consisting of the extracellular domain of murine CD200 fused to the Fc domain of murine IgG2a) was provided by Genentech Inc. (San Francisco, USA).

2.2. Cell viability assay

hRPTECs were plated into 96-well clusters at a density of 5×10^4 cells/well. After incubation under group-specified experimental conditions, the clustered hRPTECs were processed for detection of cell viability by MTT assays. Briefly, 100 μl of MTT stock solution was added to each well and incubated at 37 °C for 4 h, after which 100 μl of dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 570 nm with a microplate reader. Percent inhibition of cytotoxicity was calculated as a fraction of control (with DMSO) and expressed as percentage of cell viability.

2.3. Immunoprecipitation (IP)

To examine protein–protein interactions, hRPTECs were lysed in 1 mL buffer consisting of 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM NaF, 1 mM Na_3VO_4, 10 g/mL leupeptin, 10 g/mL aprotinin and 20 mM PMSF after harvesting. Aliquots of the cellular lysates (containing 500 μg proteins) were incubated with proper primary anti-TLR4 antibodies with rocking overnight at 4 °C. The immune complexes were allowed to bind to 40 μl of Recombinant Protein G Agarose beads (Invitrogen, USA) at 4 °C for 2 h, and the beads were washed three times with lysis buffer. The washed beads were re-suspended in electrophoresis sample buffer and boiled for 10 min. After centrifugation, the supernatants were obtained as immunoprecipitates for western blotting analysis.

2.4. Preparation of cytosolic and nuclear extracts

hRPTECs were collected by centrifugation at 800 rpm for 5 min at 48 °C and washed with ice-cold PBS. The Cell nuclear and cytoplasmic fractions were prepared using a nuclear/cytosol fractionation kit of Biovision Inc. (Mountain View, CA) according to the manufacture's direction. After centrifugation, the supernatants were obtained for Western blotting analysis.

2.5. Western blotting analysis

Protein samples from hRPTECs extracts were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary antibodies, which were purchased from these companies: Santa Cruz (TLR4, Lamin A and β-actin), R&D Systems (CD200R1, CD200R2, CD200R3 and CD200R4), and Cell Signaling (p38, ERK, JNK, p-p38, p-ERK, p-JNK, IκB, p-IκB, NFκB-P65, p-NFκB-P65, MyD88, TAK1 and p-TAK1). After washing with TBST, HRP-conjugated secondary antibodies (goat anti-rabbit IgG, Amersham Pharmacia Biotech; donkey anti-goat IgG, Santa Cruz Biotechnology) were applied. The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Densitometry analysis of bands was performed with the Image Master™ 2D Elite software, version 3.1 (Amersham Pharmacia Biotech).

2.6. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

hRPTECs was isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, each reaction contained 2 μl of cDNA (0.1 μl of RNA equivalent), 0.8 μl primer, 2.2 μl of H_2O, and 5 μl of Sofast EvaGreen supermix. qRT-PCR was performed (BioRad) in a three-step program (95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s for 50 cycles). qRT-PCR data were analyzed by CFX manager software (BioRad, Hercules, CA, USA). Gene expression fold change was obtained by dividing treated group signal by that of base expression level signal of corresponding genes in untreated cells. Results were normalized using qRT-PCR signal from β-actin of respective samples. The primers of target genes were showed in supplementary material (Table 1).

2.7. Statistical analysis

Statistical calculations of the data were performed using an unpaired Student's t-test and ANOVA analysis. Statistical significance was at $P < 0.05$.

3. Results

3.1. CD200Fc attenuates the decreased expression of CD200R1 in LPS-induced hRPTECs

As shown in Fig. 1A–C, incubation of hRPTECs with LPS (0, 0.1, 1, or 10 μg/ml) for 90 min induced dose-dependent reduction of CD200R1 both in mRNA and protein levels. However, incubation of hRPTECs with LPS (0, 0.1, 1, or 10 μg/ml) for 90 min had no effect on the mRNA and protein expression of CD200R2-4. As illustrated in Fig. 1D–F, the mRNA and protein expression of CD200R1 reduced by 10 μg/ml LPS was up-regulated gradually when pre-treated with 1 and 10 μg/ml CD200Fc and maintained at highest levels when pre-treated with 100 μg/ml CD200Fc. However, CD200Fc had no effect on the mRNA and protein expression of CD200R2-4 in LPS-induced hRPTECs. In addition, 100 μg/ml CD200Fc pre-treatment only increased the mRNA and protein expression of CD200R1, but had no effect on the mRNA and protein expression of CD200R2-4 in hRPTECs under normal conditions (Fig. 1G–I). Moreover, we detected the decrease of cell viability after incubation of hRPTECs with 10 μg/ml LPS for 24 h CD200Fc pre-treatment induced strong up-regulation of cell viability reduced by 10 μg/ml LPS in a dose-dependent manner, with maximum activation of cell viability detected after 100 μg/ml CD200Fc pre-treatment (Fig. 1J).

3.2. CD200Fc reduces LPS-induced TLR4 expression and its interactions with MyD88 or TAK1 in hRPTECs

hRPTECs cultured were pre-treated with 100 μg/ml CD200Fc for 24 h and then exposed to LPS for 90 min. Western blotting analysis showed that 10 μg/ml LPS induced strong activation of TLR4, and pre-treated with CD200Fc (0, 1, 10, or 100 μg/ml) induced dose-dependent reduced of TLR4 expression in LPS-induced hRPTECs

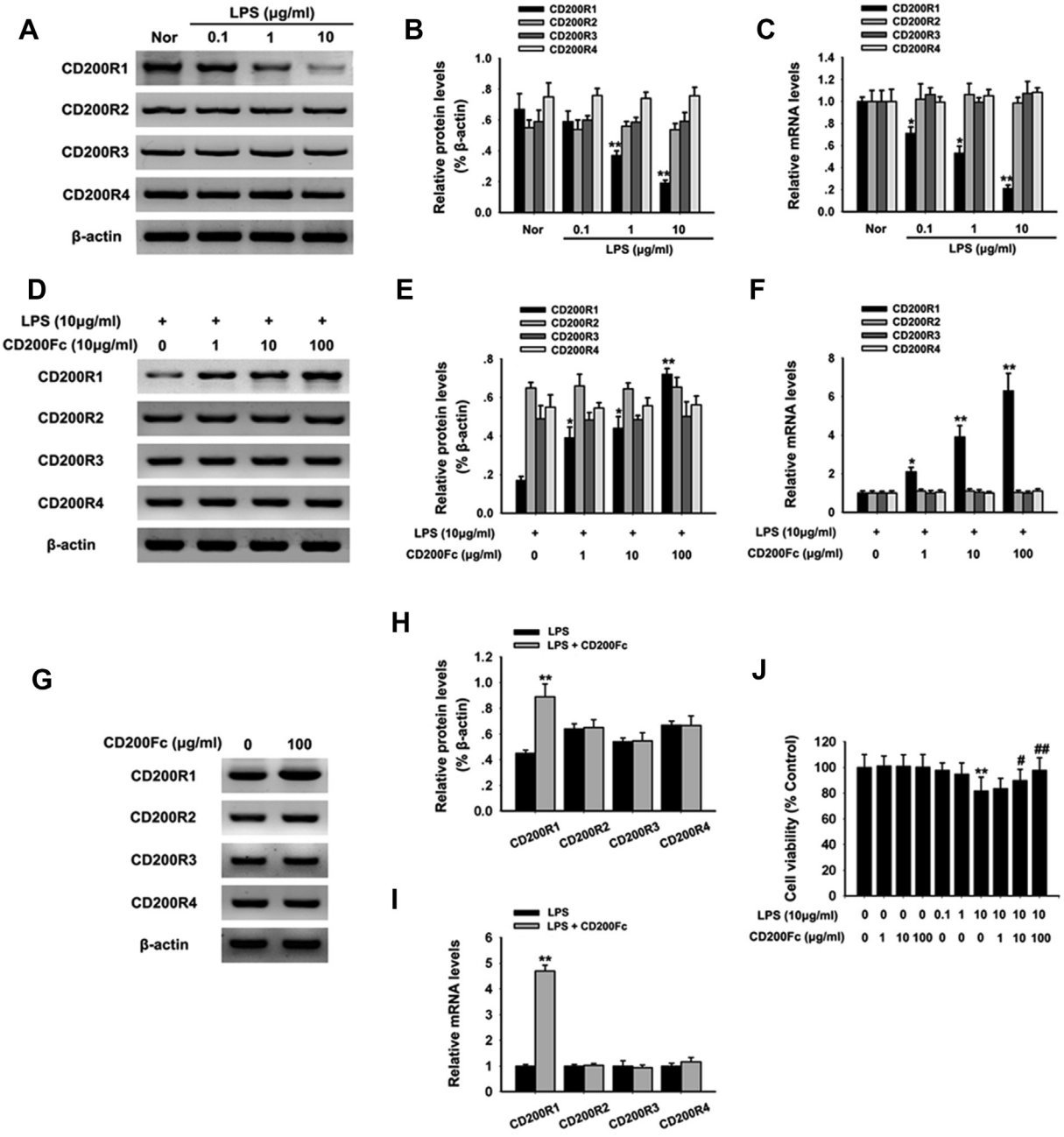

Fig. 1. Effect of CD200Fc on CD200R1-4 expression in LPS-induced hRPTECs. A–C. hRPTECs were cultured 90 min after treatment with LPS (0, 0.1, 1, or 10 μg/ml) A. CD200R1-4 protein expression was examined by Western blotting analysis. B. Densitometric analysis showed the effects of LPS on expression of CD200R1-4 protein. C. CD200R1-4 mRNA expression was examined by qRT-PCR. Data were shown as mean ± SEM (n = 3, *P < 0.05; **P < 0.01 compared to control group). D–F. hRPTECs were cultured 24 h after pre-treated with CD200Fc (0, 1, 10, or 100 μg/ml) and then induced by 10 ng/ml LPS for 90 min. D. CD200R1-4 protein expression was examined by Western blotting analysis. E. Densitometric analysis showed the effects of CD200Fc on LPS-induced expression of CD200R1-4 protein. F. CD200R1-4 mRNA expression was examined by qRT-PCR. Data were shown as mean ± SEM (n = 3, *P < 0.05; **P < 0.01 compared to LPS-induced group). G–I. hRPTECs were cultured 24 h after pre-treated with 100 μg/ml CD200Fc and then induced by 10 ng/ml LPS for 90 min. G. CD200R1-4 protein expression was examined by western blotting analysis. H. Densitometric analysis showed the effects of 100 μg/ml CD200Fc on expression of CD200R1-4 protein. I. CD200R1-4 mRNA expression was examined by qRT-PCR. Data were shown as mean ± SEM (n = 3, **P < 0.01 compared to control group). J. hRPTECs were cultured 24 h after pre-treated with CD200Fc treatment (0, 1, 10, or 100 μg/ml) and then induced by LPS (0, 0.1, 1, or 10 μg/ml) for 24 h. Cell viability were determined by MTT assays. Data were shown as mean ± SEM (n = 3, **P < 0.01 compared to control group; #P < 0.05, ##P < 0.01 compared to LPS-induced group).

(Fig. 2A and B). In addition, 100 μg/ml CD200Fc pre-treatment had no effect on the protein expression of TLR4 in hRPTECs under normal conditions (Fig. 2A and C).

Moreover, we investigated the effect of 100 μg/ml CD200Fc on the interaction of TLR4 with adapter molecule MyD88 and TAK1 in LPS-induced hRPTECs. As shown in Fig. 2D–E, LPS stimulation of hRPTECs for 90 min caused an increase in MyD88 and p-TAK1 expression, which was significantly inhibited after pre-treated with 100 μg/ml CD200Fc for 24 h. However, there were no apparent differences in MyD88 and p-TAK1 expressions among control and 100 μg/ml CD200Fc pre-treatment in normal conditions. Moreover, as shown in Fig. 2F–G, the formation of TLR4/MyD88 and TLR4/TAK1 complex significantly increased after LPS stimulation. Pre-treated with 100 μg/ml CD200Fc showed a reduction in the

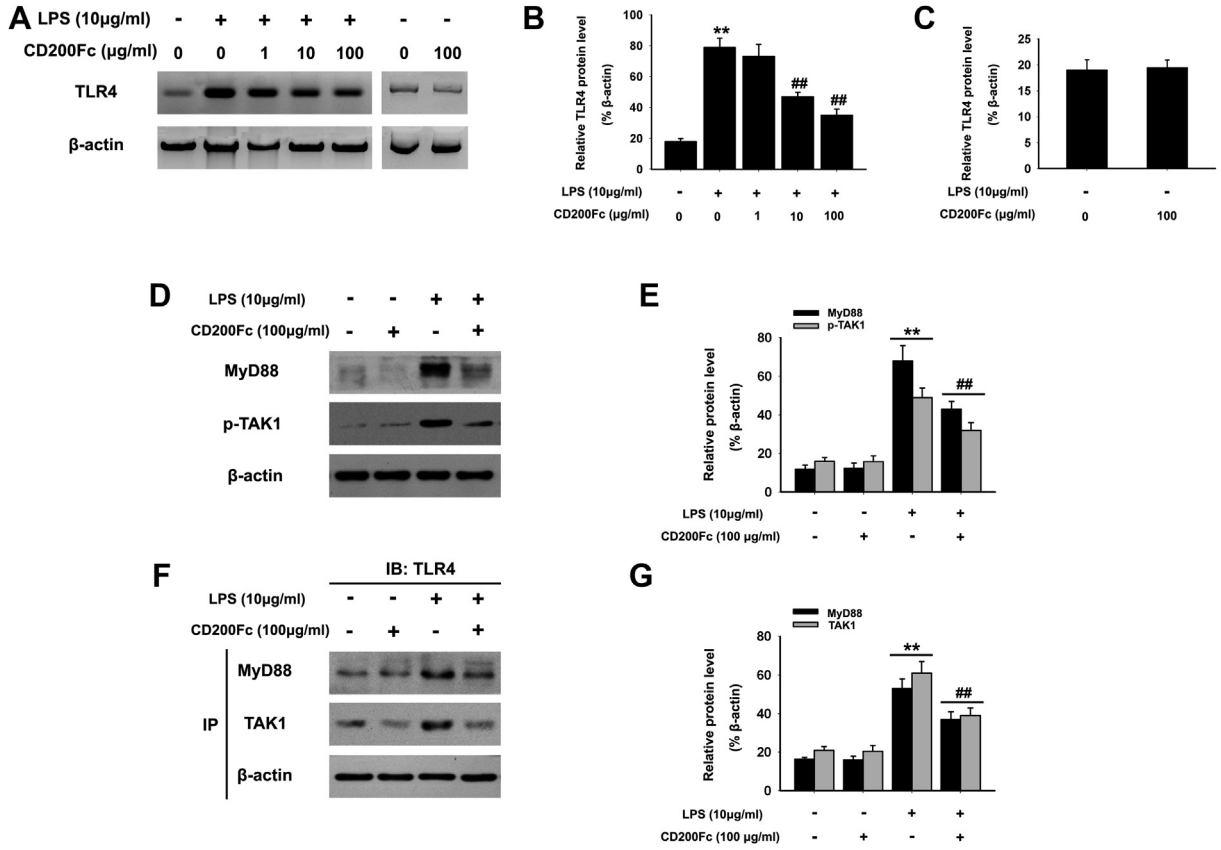

Fig. 2. Effect of CD200Fc on TLR4 expression and its interactions with MyD88 or TAK1 in LPS-induced hRPTECs. A–C. hRPTECs were cultured 24 h after pre-treated with CD200Fc (0, 1, 10, or 100 μg/ml) and then induced by 10 ng/ml LPS for 90 min. A. TLR4 protein expression was examined by Western blotting analysis. B. Densitometric analysis showed the effects of CD200Fc on LPS-induced expression of TLR4 protein. C. Densitometric analysis showed the effects of 100 μg/ml CD200Fc on expression of TLR4 protein under normal conditions. Data were shown as mean ± SEM (n = 3, $^{**}P < 0.01$ compared to control group; $^{\#\#}P < 0.01$ compared to LPS-induced group). D–G. hRPTECs were cultured 24 h after pre-treated with 100 μg/ml CD200Fc and then induced by 10 ng/ml LPS for 90 min. D. MyD88 and p-TAK1 protein expression was examined by western blotting analysis. E. Densitometric analysis of effects of CD200Fc on expression of MyD88 and p-TAK1. F. The complexes of TLR4/MyD88 and TLR4/TAK1 were precipitated by antibody against TLR4 first and then analyzed by western blotting analysis. The protein levels of MyD88, TAK1 and β-actin in whole cell lysate were analyzed by western blotting analysis. G. Densitometric analysis of effects of CD200Fc on interactions of TLR4 with MyD88 and TAK1. Data were shown as mean ± SEM (n = 3, $^{**}P < 0.01$ compared to control group; $^{\#\#}P < 0.01$ compared to LPS-induced group).

intensity of the MyD88 and TAK1 band co-immunoprecipitated using anti-TLR4 antibody. However, there were no apparent differences in the formation of TLR4/MyD88 and TLR4/TAK1 complex among control and 100 μg/ml CD200Fc pre-treatment in normal conditions.

3.3. CD200Fc inhibits the activation of NF-κB and MAPK pathway in LPS-induced hRPTECs

Because NF-κB is an important downstream modulator of TLR4, the effects of CD200Fc on NF-κB activity were investigated using Western blotting analysis. As shown in Fig. 3A–B, IκB was phosphorylated and degraded 90 min after LPS treatment. Pre-treated with 100 μg/ml CD200Fc decreased to phosphorylation and degradation of IκB in LPS-induced hRPTECs.

To further evaluate the nuclear translocation of NF-κB-P65, nuclear and cytoplasmic protein fractions were extracted from hRPTECs. As shown in Fig. 3C–D, total NF-κB-P65 in the nucleus was significantly high in LPS-induced hRPTECs. The increase in NF-κB-P65 was decreased significantly by pre-treating hRPTECs with 100 μg/ml CD200Fc. However, there were no apparent differences in NFκB-P65 in the nucleus among control and 100 μg/ml CD200Fc treatment in normal conditions. On the other hand, we failed to detect any change of total NF-κB-P65 in the cytoplasm among different groups. These result suggested that CD200Fc could block LPS-triggered NF-κB signaling pathway.

In order to further demonstrate the inhibitory effect of CD200Fc on MAPK signaling pathway, Western Blotting analysis was utilized to examine the expression of p-ERK1/2, p-p38 and p-JNK in LPS-induced hRPTECs. As shown in Fig. 3E–J, 100 μg/ml CD200Fc apparently inhibited the up-regulated expression of p-ERK1/2 (Fig. 3E–F), p-p38 (Fig. 3G–H) and p-JNK (Fig. 3I–J) induced by LPS in hRPTECs, which was consistent with the effect of CD200Fc in LPS-triggered NF-κB signaling pathway.

3.4. CD200Fc suppresses the LPS-induced release of pro-inflammatory mediators in hRPTECs

In order to confirm that LPS-induced inflammatory mediators release in hRPTECs could be inhibited by CD200Fc, we tested the mRNA expression levels of pro-inflammatory mediators, including IL-1β, IL-4, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, TNF-α, INF-α, INF-γ, TGF-β1 and TGF-β1, which we employed as biological markers for inflammation. hRPTECs cultured were pre-treated with 100 μg/ml CD200Fc for 24 h and then exposed to LPS for 24 h. The qRT-PCR results showed that the mRNA expression levels of IL-1β, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, TNF-α, INF-α and INF-γ were increased in LPS-induced hRPTECs. Conversely, these markers were decreased

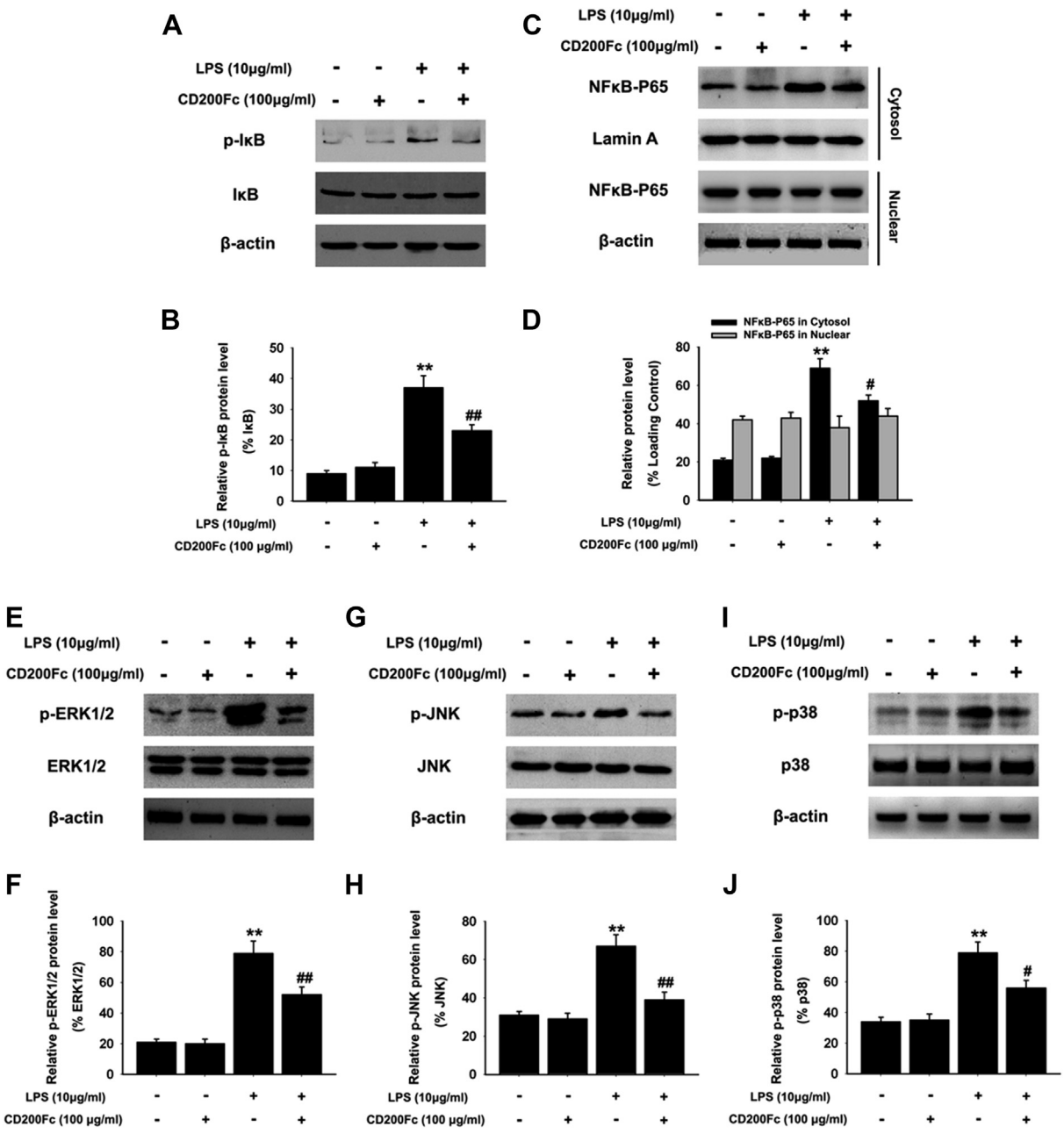

Fig. 3. Effect of CD200Fc on the activation of NF-κB and MAPK pathway in LPS-induced hRPTECs. hRPTECs were cultured 24 h after pre-treated with 100 μg/ml CD200Fc and then induced by 10 ng/ml LPS for 90 min A. p-IκB and IκB protein expression was examined by western blotting analysis. B. Densitometric analysis of effects of CD200Fc on p-IκB expression. C. The nuclear and cytoplasm extracts were separated. Effect of CD200Fc on the NF-κB-P65 nuclear translocation induced by LPS was examined by Western blotting analysis. D. Densitometric analysis of effects of CD200Fc on the NF-κB-P65 nuclear translocation. E–J. Effects of CD200Fc on the protein expression of p-JNK (E), p-ERK (G) and p-p38 (I) were examined by western blotting analysis. Densitometric analysis of effects of CD200Fc on expressions of p-JNK (F), p-ERK (H) and p-p38 (J). Data were shown as mean ± SEM (n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to LPS-induced group).

after 100 μg/ml CD200Fc pre-treatment (Fig. 4A, C–J). There were no apparent differences in the mRNA expression levels of IL-4, TGF-β1 and TGF-β1, among control and CD200Fc pre-treatment in normal or LPS-induced hRPTECs (Fig. 1D). These findings suggested that CD200Fc could inhibit the activation of the inflammatory cascade in LPS-induced hRPTECs.

4. Discussion

Recent studies suggest that inflammation is a major aggravating factor in the decline of renal function in patients with SLE [4,15]. Specifically, It is well addressed that RPTECs are the cells most susceptible to inflammation, which results in tubular necrosis [16]. Accordingly, the control of the inflammatory response has been found to be beneficial in attenuating kidney injury in several animal models of SLE [4,17]. Our present studies showed that CD200Fc shut down the up-regulation of pro-inflammatory molecules such as IL-1β, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, TNF-α, INF-α and INF-γ in LPS-induced RPTEC. Moreover, CD200Fc exerted potent NF-κB and MAPK inhibiting effects via TLR4-MyD88-TAK1 complex mediated signaling pathway. Collectively, these results indicated that CD200Fc could be optimal and safe anti-inflammatory therapies to prevent or treat kidney injury and forestall progression of LN.

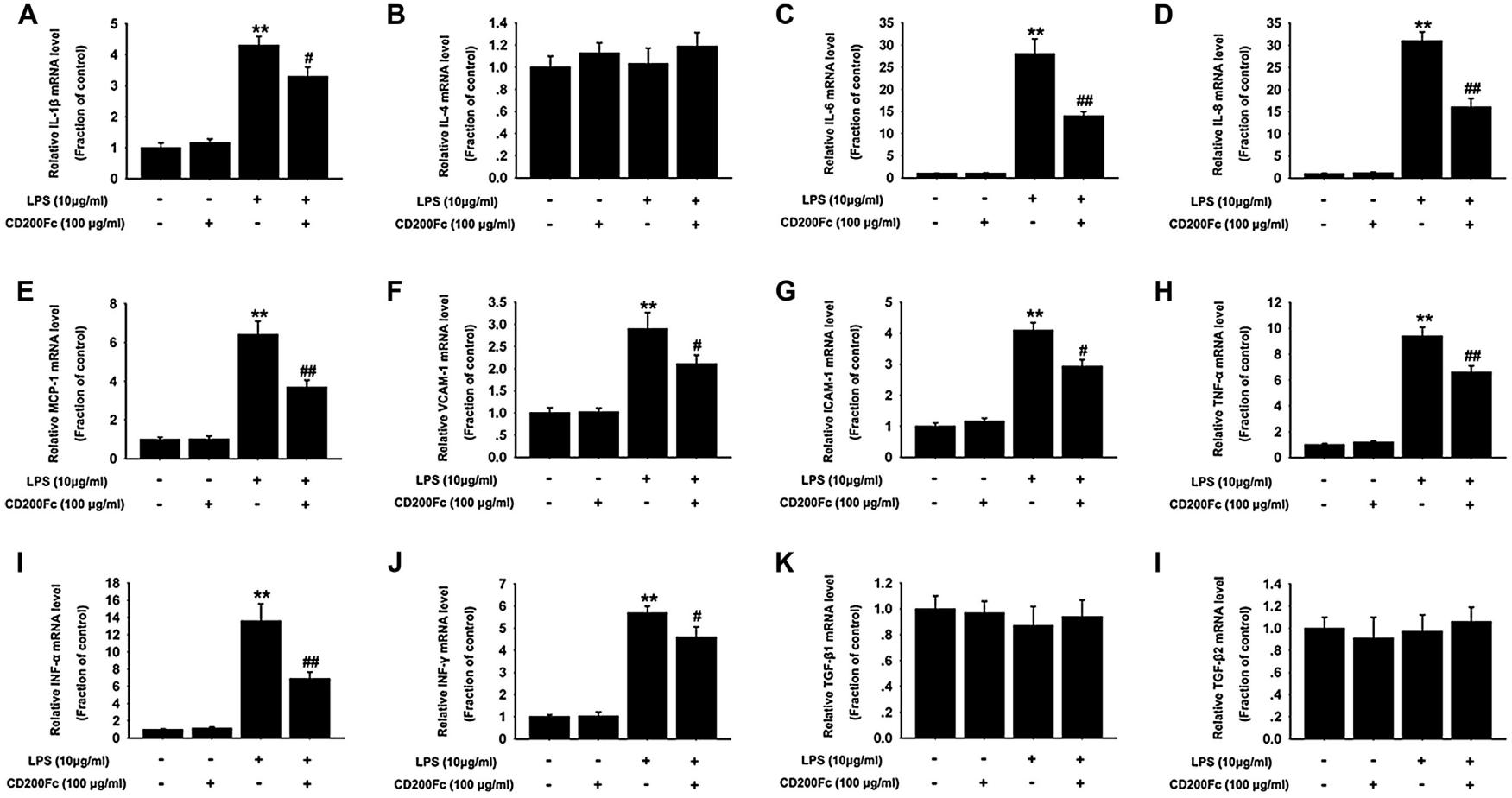

Fig. 4. Effect of CD200Fc on the expression of inflammatory mediators in LPS-induced hRPTECs. hRPTECs were cultured 24 h after pre-treated with 100 μg/ml CD200Fc and then induced by 10 ng/ml LPS for 24 h. The mRNA expression levels of pro-inflammatory mediators, including IL-1β (A), IL-4 (B), IL-6 (C), IL-8 (D), MCP-1 (E), VCAM-1 (F), ICAM-1 (G), TNF-α (H), INF-α (I), INF-γ (J), TGF-β1 (K) and TGF-β2 (L) were examined by qRT-PCR. Data were shown as mean ± SEM (n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to LPS-induced group).

Toll like receptors (TLRs) have been identified as therapeutic targets in autoimmune diseases, including LN. LPS can exacerbate the severity of LN [18], suggesting TLR4 is involved in renal inflammation and injury. Recent studies showed that TLR4-deficient lupus prone mice demonstrated a more global decrease in immune responses, with less cytokine production, less autoantibody production and attenuation in renal injury [19,20]. Together these data supported the idea that TLR4 could be a potential therapeutic target of LN. Recent studies have indicated that RPTECs are central players in tubulointerstitial inflammation, being both producers of inflammatory cytokines and chemokines and early casualties of inflammation and apoptosis [21,22]. In our study, we demonstrated the potent pro-inflammatory effects of LPS on hRPTECs, thus confirming the relevance of previous findings as LPS induced the inflammation of RPTECs, which could be a well-established model to research renal inflammation and injury of LN in vitro.

The cellular and tissue distribution of CD200-CD200R suggests that this pathway plays a largely inhibitory role in the modulation of inflammation. Previous studies showed that CD200R agonists down-regulate pro-inflammatory cytokine production by activated macrophages [23,24] and microglia [6]. Moreover, compared with wild type mice, LPS induced the expressions of TLR4 and TLR2, and promoted the release of the pro-inflammatory cytokines in glia from CD200-deficient mice [5]. Collectively, all recent studies have demonstrated a positive correlation between CD200-CD200R and TLR4-mediated inflammatory response. However, the anti-inflammatory effects and further mechanisms of CD200Fc on LPS-induced hRPTECs are not clear. Our present study showed that the protein and mRNA levels of CD200R1, not CD200R2-4 were up-regulated after LPS induction, the pre-treatment with CD200Fc reduced expression of CD200R1 in LPS-induced hRPTECs. Moreover, pre-treatment with CD200Fc induced dose-dependent reduced of TLR4 expression, which might be a reason for anti-inflammatory effects in LPS-induced hRPTECs.

The cellular receptor that transduces LPS signaling has been identified as TLR4. When LPS binds to TLR4, it triggers intracellular signaling, most notably via NF-κB pathway. NF-κB is an important nuclear transcription factor, which initiates transcription of genes associated with the fibrotic and inflammatory process in hRPTECs, that is a hallmark for progression of chronic kidney disease [25,26]. Inhibition of NF-κB activity has been shown to have a therapeutic effect on chronic kidney disease [25,26]. Our data indicated that CD200Fc inhibited LPS-activated NF-κB pathway through down-regulating IκB degradation and NF-κB-P65 nuclear translocation.

The activation of MAPK signaling pathway, such as ERK1/2, p38, and JNK, is triggered by TLR4 when binds to LPS, and plays a critical role in the fibrotic and inflammatory process in hRPTECs [25,26]. TLR4 stimulates ERK1/2, p38, and JNK, which in turn activate transcription regulators of inflammation in the nucleus. Our present studies showed that CD200Fc elicited an obvious inhibitory effect of p-ERK1/2, p-p38, and p-JNK in LPS-induced hRPTECs.

Stimulation of the TLR4 extracellular domain by LPS sequentially triggers the intracellular association of TLR4-MyD88-TAK1 complex with their cytosolic domains. MyD88 and TAK1 were recruited to TLR4 after being stimulated by LPS, after that it dissociated from the receptor presumably to bifurcate the signal into two important branches, the MAPK and NF-κB-dependent cascade. The present results showed that CD200Fc significantly suppressed the LPS-induced MyD88 expression and phosphorylation of TAK1 in hRPTECs. Moreover, CD200Fc also decreased the formation of the complexes of TLR4 with MyD88 or TAK1, which indicated that CD200Fc could disturb the linkage of TLR4 with its adapters (MyD88 and TAK1), leading to inactivation of TLR4 in LPS-induced hRPTECs.

In conclusion, exposure of hRPTECs to LPS leads to inflammation through activation of TLR4-dependent signaling pathways. We also demonstrated dose-dependent inhibitory effects of CD200Fc on inflammation in LPS-induced hRPTECs. The inhibitory effects of CD200Fc against inflammation are considered important, and it appears to be a potent regulatory element in TLR4-MyD88-TAK1 complex mediated NF-κB and MAPK signaling pathways.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.026.

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