Aggravated renal injury through HMGB1 upon TLR2/IL-1 β inflammatory pathway in STZ-induced diabetic mice

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Research Article

Keywords: HMGB, TLR2/IL-1 β signaling pathway, diabetic renal injury, macrophage recruitment, fibroblast proliferation

Posted Date: November 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1086067/v1>

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Abstract

Diabetic kidney disease (DKD) is the major cause of end-stage renal disease and is closely associated with the inflammation. The aim of this study was to investigate the involvement of HMGB upon TLR2/IL-1 β signaling pathway in diabetic renal injury. After intraperitoneal injection of STZ for two consecutive days, mice developed DKD accompanying with remarkable renal injury, manifested with albuminuria and renal histological characteristics. In the kidney, the expressions of HMGB1, TLR2 and p-NF- κ B were obviously increased, and the levels of TNF- α and IL-6 were significantly higher in DKD mice than that in normal mice. The infiltration of macrophages demonstrated by high stain of F4/80 and CD14 in the kidney of DKD mice. Furthermore, fibrosis related marker of Collagen IV, fibronectin and TGF- β 1 showed highly expressions and accumulated in the kidney of DKD mice. *In vitro*, HMGB-mediated inflammatory pathway was activated in HMGB1/IL-1 β -treated HK-2 cell, while C29 (a TLR2 inhibitor) could inhibit the HMGB1 expression. The result indicated that HMGB1-mediated TLR2/IL-1 β signaling pathways facilitated macrophage recruitment and fibroblast proliferation, which resulting in a positive feedback to sustain a self-perpetuating cycle of renal inflammation. Therefore, HMGB1 might be a potential target in DKD therapeutic interventions.

Introduction

Diabetic kidney disease (DKD), one of the serious complications of diabetes, is the major cause of end stage renal diseases^{1–3}. In recent years, the worldwide prevalence of DKD is increasing^{3,4}. However, the underlying pathogenesis of DKD are not fully understood¹, which are required for developing therapeutic strategies in clinical practice⁵. Therefore, the research of identifying and investigating the characters of DKD is urgent¹.

It has been shown that the inflammation involves in the development of DKD^{2,5–7}. Recently, a nuclear DNA-binding protein high-mobility group box (HMGB) -1 has been implicated as a pro-inflammatory cytokine in inflammatory response resulted from tissue injuries. Under status of hyperglycemia and hyperlipidemia, HMGB1 can translocate into the extracellular space⁶. The extracellular HMGB1 acts on its target receptors (e.g. TLR2 and TLR4), and subsequently triggers an inflammatory response through NF- κ B signaling pathway. Thus expressions of pro-inflammatory cytokines (e.g. such as tumor necrosis factor (TNF) - α and interleukin (IL) -6) markedly upregulate⁶. In turn, a variety of pro-inflammatory cytokines, including IL-1 β , TNF- α , Interferon (IFN) - γ , further induce the active secretion of HMGB1 in immune cell including macrophages and dendritic cells, which build a positive feedback loop leading to a self-perpetuating cycle of inflammation in the kidney^{8,9}. Pathogen-associated molecular pattern (PAMPs) or IL-1 β are demonstrated to enhance the effect of HMGB1 on the inflammatory process⁹. As an cytokine, HMGB1 exerts the stimulation of collagen synthesis and accumulation⁶. However, the role of HMGB1 signaling is not clear in the development of DKD.

Streptozotocin (STZ) has been used in different doses to induce diabetic rodent model for research on various complications^{10–13}. Here, we refined STZ-induced diabetic mice model, and aimed to characterize HMGB1 signaling in inflammation and fibrosis contributing to the development of DKD. The research on the mechanism of DKD would be helpful for further exploring nephroprotective agents. Since the principal renal damage show proximal tubular cell (PTC) basement membrane thickening, hyperplasia and hypertrophy in early diabetes^{14,15}, we selected HK-2 cells (human kidney PTC line) to study the effect of HMGB1 via TLR2/IL-1 β signaling on renal injury.

Results

Characteristics of DKD mice induced by STZ. Hyperglycemia was achieved (≥ 16.8 mmol/L) at Day 8 and sustained thereafter in STZ induced groups, compared with the normal group ($P < 0.001$; Fig. 1a). Mice with blood glucose levels higher than 27 mmol/L and weight lower than 16 g were euthanized to minimize the suffering. Survival rates up to Day 64 after STZ treatment were 100% and 50% in the groups of 100 and 150 mg/kg, respectively (Fig. 1b).

At the histological level, the Langerhans' islets of the pancreas exhibited normal circular morphology with healthy cell lining in the normal mice (Fig. 1c). The exocrine glands of the pancreas were well-organized and in normal morphology. In contrast, in the 100 mg/kg STZ group, the islet cell mass appeared atrophied and disorganized. In the 150 mg/kg STZ group, the islets of Langerhans further shrank and were almost indiscernible (Fig. 1c).

Hyperlipidemia is an indicator for the severe diabetes. Compared with the normal group, levels of serum LDL-C in DKD groups (treated with STZ of 100 and 150 mg/kg) were significantly higher ($P < 0.05$, $P < 0.01$, respectively) (Fig. 1d), while the levels of serum HDL-C in DKD group (150 mg/kg STZ) were significantly lower ($P < 0.05$, Fig. 1e).

Renal injuries in DKD mice. Kidney index ($P < 0.001$, Fig. 2a) and serum creatinine level ($P < 0.05$, Fig. 2b) were significantly increased in DKD groups, compared with the normal group. There was no difference in the levels of BUN (Fig. 2c).

The levels of UAER were significantly elevated in the diabetic groups, compared with the normal group, and presented a progressive increase during the experiment ($P < 0.001$, Fig. 2d).

H&E examination indicated the pathological characteristics in the kidney of DKD mice such as expansion of glomerular mesangial region (Fig. 2e). PAS assay showed intensive deposition of saccharides in glomerular mesangial region in the diabetic mice (Fig. 2e).

Renal inflammation in DKD mice. The expressions of HMGB1, TLR2 and TLR4 were upregulated in the kidney of DKD mice, revealed with immunohistochemical assay. Localization of HMGB1 in the cytoplasm was observed in the kidney of DKD mice, while it was mainly in the nucleus in the normal group (Fig. 3a). These proteins were predominantly localized in the renal tubular epithelial cells, and the level of TLR2

was higher than that of TLR4 as demonstrated (Fig. 3a). Quantification of these changes using Western Blot assay showed a marked decrease in HMGB1 expression in nucleus in DKD group (Fig. 3b).

The HMGB1-TLR signaling activation leads to phosphorylation of the NF- κ B (Fig. 3b). As displayed, p-NF- κ B increased in renal tissues of DKD mice based on Western Blot. NF- κ B is an important transcription factor in the TLR signaling to regulate the expression of numerous inflammatory genes¹⁶. The levels of inflammatory cytokines, TNF- α and IL-6 (Fig. 3c), were significantly augmented in the kidney of the DKD groups, compared with the normal group ($P < 0.001$).

Once released to cell outside, HMGB1 potently stimulates macrophage functions¹⁷. The levels of F4/80 and CD14, two biomarkers of macrophages, increased in kidney according to immunohistochemical assay (Fig. 4). As the predominant immune effector cells, macrophages are responsible for both enhancing and suppressing the inflammatory responses¹⁸.

Renal fibrosis in DKD mice. Masson's trichrome assay showed vacuolation of the tubular cells and heavy deposition of extracellular matrix in the tubular interstitial (Fig. 5a). Col IV and FN, indicators of fibrosis, were highly accumulated in the kidney of DKD mice, assessed using immunohistochemistry. Heavy Col IV deposition was seen in the renal tubule interstitial and basement membranes, while FN was highly present in the mesangial of glomerulus and renal tubule interstitial (Fig. 5a). Levels of Col IV and FN elevated significantly in the kidney homogenates of DKD mice, compared with the normal group ($P < 0.05$; Fig. 5b&5c). Increase in the production of TGF- β 1 has been associated with inflammation and fibrosis in DKD. TGF- β 1 increased in the renal tissue of DKD mice (Fig. 5d).

Inflammatory response in HK-2 cells stimulated by HMGB1 or IL-1 β . As shown in Fig. 6, expression of TLR2 and p-NF- κ B were increased in HK-2 cells treated by IL-1 β or HMGB1. Moreover, increment of induced by HMGB1 was dramatic (Fig. 6a). The results were consistent with those in DKD mice. After treatment of C29, the expression of HMGB1 was significantly decreased, indicating that the TLR2 may play an important role in HMGB1 signaling (Fig. 6b).

Discussion

DKD, a common complication of diabetes mellitus, is characterized with renal failure, resulting from tissue injury and functional abnormalities. However, the pathogenesis of DKD is not fully understood. In our study, we optimized the conditions for building a DKD mice model, which presented the characteristics of DKD, including renal tissue injuries, inflammation, and fibrosis. The results showed that STZ of 100 and 150mg/kg both successfully induced DKD mice model, and there was a low mortality in the treatment of 100mg/kg STZ. Therefore, 100mg/kg dose of STZ is recommended to establish the DKD mice model. By means of the model, we demonstrate that HMGB1 upon TLR2/IL-1 β inflammatory pathway involved in aggravating renal injury and contributing to the development of DKD.

In clinical practice, increased excretion of albumin is a most common indicator for diagnosis of renal failure¹⁹. Serum creatinine is also a common marker of renal dysfunction, especially deficient renal

filtration²⁰. Here, the increased levels of UAER and serum creatinine indicated impaired renal function in DKD mice. Elevated BUN is also a marker of renal dysfunction²⁰. In the present study, there was no change of BUN level in diabetic mice. In addition to renal filtration, other factors also contribute to the changes of BUN, such as diet and renal blood flow²¹. Histologically, the characteristic of DKD was further manifested with HE and PAS assays, as mesangial expansion. The increase of kidney index in DKD mice revealed the injury in renal tissues.

The occurrence and development of DKD are a result of multiple factors interaction under hyperglycemic environments²². Accumulating evidence indicates that immune-mediated inflammation plays a significant role in the progression of DKD⁵. As pattern recognition receptors, TLRs exert a fundamental action in the innate immune system. TLRs can be activated by endogenous ligands and trigger inflammatory response to noninfectious stimuli, such as diabetes. Signals from TLR2 and/or TLR4 promote inflammatory response through activating NF- κ B pathways⁶. Subsequently, NF- κ B upregulates the expression of pro-inflammatory cytokines, such as TNF- α and IL-6, resulting in the amplification of inflammation and tissue injuries. Our study shows an increase in HMGB1 expression in the kidney of DKD mice, implying that it may play a role in regulation of TLRs expression. Future work is aimed to investigate the mechanism.

HMGB1 is a nuclear DNA-binding protein that modulates chromatin accessibility²³, and is present variably levels in most cells²⁴. As endogenous ligand, HMGB1 can bind with TLR2 and TLR4 to involve the TLRs/ NF- κ B pathway. HMGB1 is released passively by mechanically ruptured cells or cells that have died necrotically because of energy depletion or pharmacological treatment, and is also secreted by cells of the innate and adaptive immune system that have been activated by a variety of signals, including PAMPs (LPS), IL-1 β , TNF- α , IFN- γ , or phagocytosis of a large number of apoptotic cells⁸. When HMGB1 translocate into the cytoplasm from nucleus and are released into extracellular space, inflammatory reactions is triggered through activation of TLR2 and TLR4 to cause nuclear translocation of NF- κ B⁶. Our results showed that the expression and translocation of HMGB1 were elevated in the kidney of STZ-induced DKD mice, and the increase of TLR2 and TLR4 expression and phosphorylated NF- κ B were also seen. In the kidney of DKD mice, but not the normal mice, HMGB1 was indeed localized in the cytoplasm, suggesting that it was potentially released into the extracellular space of the renal tubular epithelial and glomerular cells, subsequently to activate TLRs/ NF- κ B pathway, leading to an increase of production pro-inflammatory cytokines of TNF α and IL-6. Consistently, our results displayed that the level of F4/80 and CD14 remarkably increased by immunohistochemical staining in the kidney of DKD mice, indicating the appearance of macrophages infiltration. Macrophage is the primary source of inflammatory effectors, which exerts proinflammatory effects through the secretion of cytokines TNF- α , IL-1 β , and IL-6¹⁸. As reported⁸, HMGB1 can be actively secreted by macrophage in response to endogenous inflammatory stimuli, such as IL-1 β . Therefore, macrophages infiltration in the kidney further facilitated a self-perpetuating cycle of renal inflammation.

In terms of immunohistochemical assay, we noticed that the level of TLR2 was higher than that of TLR4. Therefore, we further investigated the expression of TLR2 *in vitro* to discover the interaction between TLR2 and HMGB1. We demonstrated that the treatment of HMGB1 significantly upregulated the expression of TLR2 and phosphorylated NF- κ B in HK-2 cell. The results implied HMGB1 may play a role in regulation of TLR2 expression.

The report indicated that IL-1 β enhanced the expression and release of HMGB, which triggered or sustained inflammation status in the kidney tissue⁹. In agreement with the report, in our study *in vitro*, we found that the stimulus of IL-1 β , significantly promoted the expression of HMGB1, TLR2 and phosphorylated NF- κ B. It is confirmed that HMGB1 causes the inflammatory response via proinflammatory cytokines secretion, and in turn the proinflammatory cytokines promote more expression of HMGB1, suggesting that HMGB1-mediated signaling pathways are amplified by a positive feedback loop involving inflammation²⁵. Furthermore, our data showed that the TLR2 inhibitor reduced the expression of HMGB1, indicating that the TLR2 could play a vital role in the inflammation induced by HMGB1 signaling, which may also be related to the interaction between TLR2 and IL-1 β .

Prolonged inflammation ultimately leads to tubulointerstitial fibrosis and glomerulosclerosis. Renal fibrosis in the mesangial and renal tubule interstitial is thought to be a hallmark of late stages of DKD²⁶. In our study, both Col IV and FN were highly expressed and accumulated in the kidney of diabetic mice, and the level of Col IV and TGF- β 1 increased in kidney homogenate. Such fibrotic condition was correlated with the upregulation of HMGB1 and TLR2/4. It is worth mentioning that FN is also an endogenous ligand of TLR, promoting inflammation and fibrosis²⁷, potentially exacerbating the effects of hyperglycemia-induced TLR2/4 signaling.

Identification of HMGB1 in DKD also suggests a possible mechanism of gene regulation via chromatin modification under hyperglycemia. The participation of other members of the HMGB family in DKD deserves further investigation. Also, the link between HMGB1 and other receptors or proinflammatory cytokines, such as receptor for advanced glycation end product (RAGE), kinases, phosphatases, and transcription factors, need much more attention. Moreover, the interaction between TLR2 and IL-1 β involving in the stimulation of HMGB1 should be further investigated. The understanding of the role of HMGB1-mediated signaling pathways and the inflammatory positive feedback loop of HMGB1 may provide a new insight into anti-inflammatory therapeutic strategies for treating DKD.

Materials And Methods

Establishment of DKD mice model. Male C57BL/6 mice (8-week old) were purchased from Slaccas-Shanghai Lab Animal Ltd. (SPF II Certificate; No. SCXK2012-0005) and kept under specific pathogen-free and normal housing conditions in a 12-h light and dark cycle.

After one week of being adapted to the new environment, mice were intraperitoneally injected with STZ (Sigma-Aldrich) in two doses (100 and 150 mg/kg) for two consecutive days (Days 0 and 1; n = 6 mice

per group). At Day 8, blood glucose levels were monitored using a glucose meter (Abbott). Mice with blood glucose levels higher than 16.8 mmol/L were defined as hyperglycemic and used for experiments²⁸. At Days 29 and 64, blood glucose levels were measured using assay kits, following manufacturers' instructions (Fenghui, Shanghai, China). Survival status of the animals in each group was assessed during the whole experiment. The mice were euthanized and the kidneys were collected for further examination at Day 64.

Biochemical assays. Urine was collected for 5 h via a device in a metabolism cage at Days 32 and 50. The concentration of urinary albumin was tested using an enzyme-linked immunosorbent assay (ELISA) kit, and the urinary albumin excretion rate (UAER) was calculated, indicating the total excretion of urinary albumin in 5 h.

At Day 64, blood was collected via the orbital sinus and centrifuged to obtain serum²⁹. High-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), serum creatinine, and blood urine nitrogen (BUN) were measured using assay kits (Fenghui, Shanghai, China).

The left kidney were weighed to calculate kidney index (kidney weight/body weight, mg/g), and then homogenized in RIPA lysis reagent (Beyotime, Shanghai, China)²⁸. TNF- α , IL-6, COI IV and transforming growth factor (TGF- β 1) in the renal homogenates were measured using ELISA kits (Boatman, Shanghai, China).

Histopathological examination. At Day 64, the pancreas and right kidney were collected, fixed in 10% formaldehyde, and embedded in paraffin wax. The specimens were processed to obtain 4 μ m-thick tissue sections using a microtome. The tissue sections were stained with hematoxylin and eosin (HE)²⁹. Kidney sections were also processed for periodic acid-Schiff's (PAS) and Masson's trichrome (Masson) assays.

Immunohistochemistry examination. The expressions of HMGB1, TLR2, TLR4, F4/80, CD14, collagen IV (Col IV) and fibronectin (FN) in kidney were examined using immunohistochemistry²⁹. The sections were dewaxed, rehydrated, and equilibrated in phosphate-buffered saline (PBS, pH 7.4). After quenching endogenous peroxidase activity with 3% H₂O₂, and blocked with 5% bovine albumin, the sections were incubated with antibodies recognizing HMGB1 (1:400, Proteintech), TLR2 (1:500, Arigo), TLR4 (1:400, Abcam), F4/80 (1:300, Servicebio), CD14 (1:500, Servicebio), Col IV (1:250, Abcam), and FN (1:250, Abcam) overnight. After incubation with a secondary antibody conjugated with horseshoe peroxidase (HMGB1, TLR2, TLR4, Col IV, FN: HRP; 1:1000, Bioworld; F4/80, CD14: HRP; 1:200, Servicebio) for 1 h at 37°C, signals were detected using an HRP substrate diaminobenzidine. The sections were lightly counterstained with hematoxylin and examined under a light microscope (Leica Inc. Switzerland).

HK-2 cells culture. HK-2 cells were obtained from ATCC (SCSP-511, Manassas, VA) and cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere comprised of 95% air and 5% CO₂. The cells were subcultured every 2-3 days. Cell culture medium was changed every 2 days.

Western blotting assay. Kidney specimens were homogenized in RIPA lysis reagent and centrifuged to collect supernatant. HK-2 cells were seeded in 6-well plastic dishes. After cultured for 24 h, Cells were incubated with IL-1 β (20 ng/ml) or HMGB1 (400 ng/ml), or treated with C29 (a TLR2 inhibitor, 100 μ M) for 24 h. The cells were then harvested and incubated in RIPA lysis reagent and centrifuged. The supernatant was collected and mixed with the same volume of 2 $\times$ SDS loading buffer. Total protein was measured by the Bradford assay (Beyotime, Shanghai, China).

Proteins in the samples were resolved in 10% SDS polyacrylamide gel by electrophoresis and blotted onto polyvinylidene difluoride (PVDF) immunobilon membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk (in Tris-buffered saline with Tween-20 (TBST) buffer) for 1 h at room temperature and then probed overnight at 4°C with antibodies against HMGB1, TLR2, p-NF- κ B, FN, PCNA, GAPDH, and β -actin (all 1:1000), followed by incubated with horseradish peroxidase (HRP)-conjugated IgG (1:2000, Beyotime, Shanghai, China) for 2 h at room temperature. Signals were detected by enhanced electrochemiluminescence (ECL) reagent and captured with a camera-based imaging system (BIO-RAD, Santa Clara, CA, USA).

Human and animal rights. No study involving human participants was contained in our study. All mice received humane care in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All operations were performed anesthesia under 10% ethyl carbamate, and all efforts were taken to minimize suffering. When carrying out euthanasia in this experiment, mice were anesthetized with 10% ethyl carbamate followed by cervical dislocation.

Declarations

Ethical approval. All the protocols in this experiment involving animals were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University (approval number 2021-05-YL-WHB-78).

Statistical analysis. The data were expressed as means $\pm$ S.D. Differences between groups were analyzed by one-way analysis of variance (ANOVA) and Fisher's PLSD. *P*-value < 0.05 was considered significant.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request. All data analyzed during this study are included in this published article.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Grant No.82073859, 81673658).

Author contributions

All authors have made a substantial contribution to the study conception and design. Material preparation, data acquisition and analysis were performed by Yan Yuan, Yuchen Feng, Zhenzhen Liu, Lingyu Pan, Yuanxia Liu, Huijing Ye and Hongbo Weng. The original draft of the manuscript was written by Yan Yuan and Yuchen Feng; the manuscript was first revised by Huijing Ye, Zhenzhen Liu, Lingyu Pan, Yuanxia Liu and Hongbo Weng. All authors made a contribution to critical review, commentary and revision. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Characteristics of metabolism and pancreatic histopathology in STZ-induced mice. Mice were treated with STZ at 100 and 150 mg/kg by intraperitoneal injection. The blood glucose levels (a), survival rate (b). Histopathology of the pancreas, photographed at 400 $\times$ magnification (c). The levels of serum LDL-C

(d) and HDL-C (e) in STZ-induced mice, n = 6. Data expressed as means $\pm$ S.D. *P <0.05, **P < 0.01, ***P < 0.001, vs the normal group, tested by one-way ANOVA and the Fisher's PLSD

Figure 2

The renal function parameters in STZ-induced mice. The kidney index (a), serum creatinine (b), blood urea nitrogen level (c), and urinary albumin excretion rate (UAER) (d). Renal histological changes (e). HE, hemotoxylin/eosin; PAS, periodic acid Schiff, photographed at 400 $\times$ magnification, n = 6. Data expressed

as means $\pm$ S.D. *P < 0.05, **P < 0.01, ***P < 0.001 vs the normal group, tested by one-way ANOVA and the Fisher's PLSD

Figure 3

Renal inflammation status in STZ-induced mice. Immunohistochemical staining for of HMGB1, TLR2 and TLR4 in the kidney (a), photographed at 400 $\times$ magnification. Protein expressions of HMGB1 and p-NF- κ B in kidney by Western blot (b), n=3. PCNA and β -actin were used as an internal control. The levels of

inflammatory cytokines TNF- α and IL-6 (c) in renal tissues tested by ELISA method, n = 6; Data expressed as means $\pm$ S.D. *P < 0.05, **P < 0.01, ***P < 0.001, vs the normal group, tested by one-way ANOVA and the Fisher's PLSD

Figure 4

Immunohistochemical staining for CD14 and F4/80 in the kidney of STZ-induced mice, photographed at 400 $\times$ magnification, n = 3.

Figure 5

Renal fibrosis status in STZ-induced mice. Histological assays of the kidney (MASSON, Masson's trichrome), Immunohistochemical staining for Col IV and FN in the kidney (a), photographed at 400 $\times$ magnification. Protein expression of FN in kidney by Western blot (b), $n = 3$. β -actin was used as an internal control. The levels of Col IV (c) and TGF- β 1(d) in renal tissues tested by ELISA method. Data

expressed as means $\pm$ S.D. *P < 0.05, ***P < 0.001 vs the normal group, tested by one-way ANOVA and the Fisher's PLSD

Figure 6

Positive feedback of HMGB1 upon TLR2/IL-1β inflammatory pathway in HK-2 cells. Protein expressions of TLR2 and p-NF-κB in kidney by Western blot (a), n = 3. β-actin was used as an internal control. Cells were stimulated with IL-1β (100 ng/ml) or HMGB1 (400 ng/ml) for 24 h. Protein expression of HMGB1

and p-NF- κ B in kidney by Western blot (b), n = 3. GAPDH was used as an internal control. Cells were stimulated by IL-1 β (20 ng/mL) for 24 h and treated with C29 (a TLR2 inhibitor, 100 μ M) for another 24 h. Data expressed as means $\pm$ S.D. *P < 0.05, **P < 0.01 vs the normal group, ###P < 0.001 vs the IL-1 β group, tested by one-way ANOVA and the Fisher's PLSD