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ORIGINAL ARTICLE

FGF-21 Elevated IL-10 Production to Correct LPS-Induced Inflammation

Jun-yan Li,[1] Nan Wang,[1] Mir Hassan Khoso,[1] Cheng-bin Shen,[1] Meng-ze Guo,[1] Xin-xin Pang,[1] De-shan Li,[1,3] and Wen-fei Wang[2,3]

Abstract— Fibroblast growth factor 21 (FGF-21) has been previously judged as a major metabolic regulator. In this paper, we show that FGF-21 has a potential role in anti-inflammation and immunoregulation. *In vivo*, treatment with exogenous FGF-21 can alleviate LPS-induced inflammation. *In vitro*, FGF-21 inhibited LPS-induced IL-1β expression in THP-1 cells. Furthermore, besides the NF-κB pathway, the mechanism of action of FGF-21 was observed to involve the elevation of IL-10 in the ERK1/2 pathway. This study clearly indicates that FGF21 can be used as an attractive target for the management of inflammatory disorders. This piece of research indicates that FGF-21 could have much value in the management of inflammatory disorders.

KEY WORDS: FGF-21; THP-1 cells; IL-10; LPS.

Sepsis, when induced by bacterial infection, has a continuum beginning with a host-pathogen interaction that activates a complex interplay between pro-inflammatory and anti-inflammatory states [1]. At the start of inflammation, the monocytes/macrophages and other inflammatory cells are activated to undergo migration, proliferation, phagocytosis, and secrete endogenous or exogenous substances to defend against infection. However, this beneficial response will become harmful when it is out of control. The overproduction of interleukin-1 IL-1β (IL-1 IL-1β) and other pro-inflammatory cytokines leads to a pro-inflammation/anti-inflammation response imbalance. If this situation is not corrected immediately, the following cascade effect may lead to systemic inflammatory response syndrome (SIRS) or multiple organ dysfunction syndrome (MODS) or even death. In fact, the incidence of cases in which sepsis and severe sepsis lead to as a major common pathway for end-of-life events has increased considerably in the past few decades and is expected to increase further [2, 3].

Fibroblast growth factor-21 (FGF-21), a cytokine which is mainly produced by the liver, has been identified as an important metabolic regulator of glucose and lipids in vertebrates [4–7]. Though some studies indicate that serum C-reactive protein levels, IL-6, TNF-α, and leptin mRNA levels in white adipocytes have been reduced by the administration of FGF-21 [5–8], these beneficial changes are considered to be the result of the improvement of the endocrine system. However, recent studies show that FGF-21 also plays a crucial role in the balance of the pro-inflammation/anti-inflammation system. The possible role of FGF-21 in sepsis has been suggested by the observation of increased serum protein levels of FGF-21 during sepsis in mice and human [1, 9]. The toxicity of lipopolysaccharide (LPS) can be suppressed with the treatment of FGF-

[1] Northeast Agricultural University, School of Life Science, Harbin, Heilongjiang Province, China
[2] Life Science and Biotechnique Research Center, A Division of Northeast Agricultural University, Harbin, Heilongjiang Province 150030, China
[3] To whom correspondence should be addressed to Wen-fei Wang at Life Science and Biotechnique Research Center, A Division of Northeast Agricultural University, Harbin, Heilongjiang Province 150030, China. E-mail: wangwenfei@neau.edu.cn; and De-shan Li at Northeast Agricultural University, School of Life Science, Harbin, Heilongjiang Province, China. E-mail: deshanlee@163.com

21. Moreover, FGF-21 can also reduce the severity of cerulein-induced pancreatitis in mice [10]. These findings most evidently indicate that FGF21 can be implied as a therapeutic tool in the treatment and management of sepsis and inflammatory state. However, the mechanism by which FGF-21 exerts its anti-inflammatory effects is still unclear. Our previous studies proved the production of FGF-21 in monocytes and also showed that FGF-21 can regulate certain bioactivities of monocytes [11, 12]. By evaluating the above results, we hypothesize that FGF-21 may directly suppress the inflammatory response which are elevated by the LPS treatment in leukocytes. The present study was conducted with the aim to evaluate these supposed effects as well as the mechanism of action of FGF-21 in anti-inflammatory responses.

In the present study, FGF-21 effectively alleviated the inflammation induced by LPS *in vivo* and suppressed the IL-1β inflammatory production of substances from monocytes by elevating IL-10 expression. We also provide evidence that FGF-21 induced the expression of IL-10 *via* the ERK1/2 signal pathway. These data suggest that the FGF-21 can be considered as a target for potential treatment of LPS-induced inflammation.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Ethics Committee of Northeast Agricultural University.

Drug and Reagents

Recombinant FGF21 (mFGF21) was expressed from *Escherichia coli* and purified by a Ni Sepharose 6 Fast Flow column in AKTA Purifier (GE Healthcare). Erk1/2 inhibitor FR180204 was purchased from MedChem Express LLC (Princeton, NJ, USA). FBS and RMPI-1640 were purchased from Invitrogen Corporation (Carlsbad, CA, USA). SYBR® Premix Ex Taq™ kit was purchased from TaKaRa Co. (Dalian, China). Penicillin, streptomycin, DMSO, and BSA were purchased from Sigma-Aldrich Shanghai Trading Co. Ltd. (Sigma, Shanghai, China).

Animals and Treatments

Fifty male KM mice were obtained from VITAL RIVER ANIMAL CO. LTD. (Beijing, China). After housing in temperature- (about 22 °C) and humidity- (40–50%) controlled rooms for a week, these mice were then divided into LPS-treated group (5 mg/kg, $n = 12$), LPS and FGF-21 co-treated groups (LPS: 5 mg/kg, FGF-21:0.25 mg/kg, 0.5 mg/kg, 1 mg/kg, $n = 10$), and a vehicle group ($n = 8$). Mice in LPS and FGF-21 co-treated groups were treated withFGF-21 and LPS simultaneously. Mice in the vehicle group were treated with 0.9% saline. All mice were executed after 24 h. Serum and lungs were collected for ELISA assay and histological assessment.

Histological Assessment

The lung tissues were fixed in 4% buffered formaldehyde for 24 h. Each lung was embedded in paraffin and 4-μm sections cut and stained with hematoxylin and eosin (H&E). The 4-μm section was observed with an optical microscope. Pathological changes were assessed by two independent pathologists.

Cell Cultures and Drug Treatments

THP-1 cells were cultured in 1640 medium supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1% antibiotics (Penicillin-Streptomycin). The THP-1 cells were cultured to exponential phase and then cultured in serum-free medium for another 12 h. After that, the cells were treated with different drugs according to the experimental need.

Determination of Cell Viability

The MTT assay was used to determine the cell viability. In brief, THP-1 cells were seeded in 96-well plates and cultured to exponential phase. After that, cells were serum starved 12 h and treated with drug. Then, 100 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h at 37 °C in a CO_2 incubator, incubation supernatant was removed carefully without disturbing colored formazan sediments. To dissolve the formazan sediments, 100 μl of DMSO was added in each well, the plate was kept aside for 1 h to ensure full dissolution, and the absorbance was measured at 570 nm using microplate reader. The survival of THP-1 cells is calculated by (OD treated well [−blank]) / (mean OD control well [−blank]) × 100.

Expression of mRNA by RT-PCR

Total RNA from THP-1 cells were isolated using TRIzol reagent (Invitrogen), and transcribed into cDNA using the reverse-transcription kit (Promega, USA) according to the manufacturer's instructions. This cDNA was used in the RT-PCR as a template. Real-time PCR was

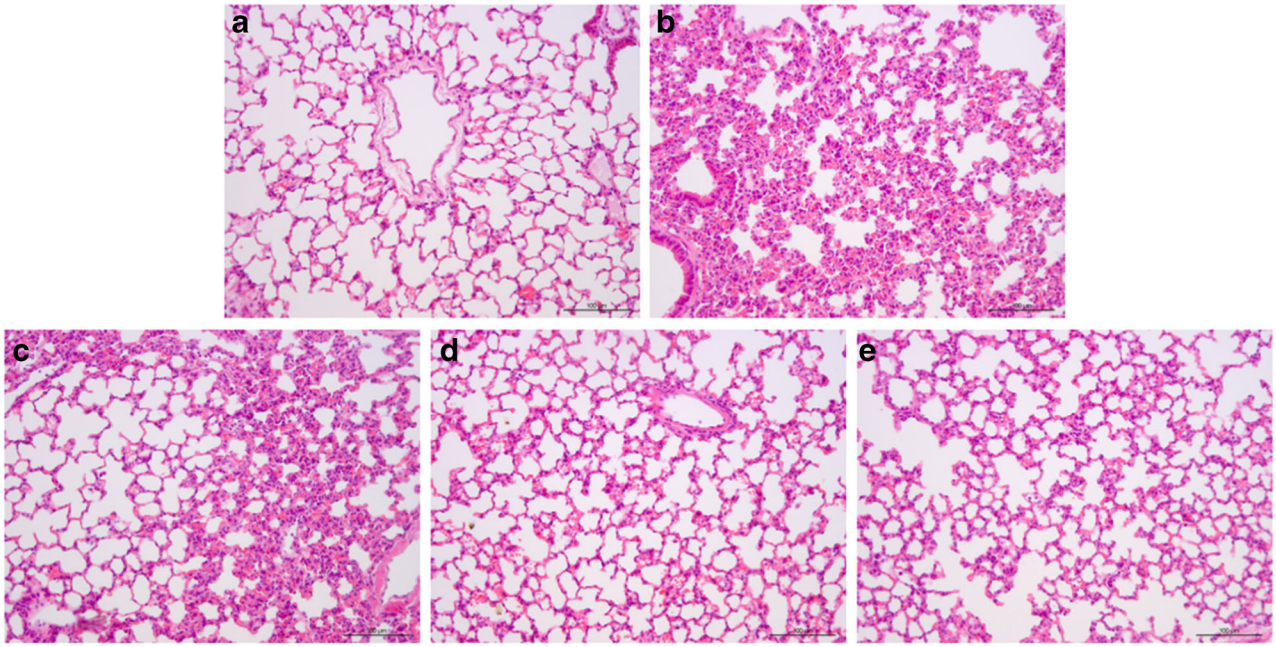

Fig. 1. FGF21 induced alleviation of soakage of inflammatory cells in the lung. HE stained of lung sections showing lungs derived from: PBS (**a**), LPS-treated mice with or without FGF-21 administration: **b** 5 mg/kg LPS, no FGF-21. **c** 5 mg/kg LPS, 0.25 mg/kg FGF-21; **d** 5 mg/kg LPS, 0.5 mg/kg FGF-21; **e** 5 mg/kg LPS, 1 mg/kg FGF-21.

performed with the iTaq SYBR Green Supermix (Bio-Rad, Hercules CA). The following primers pairs were used (sequence 5′-3′):

GAPDH: TTAGCACCCCTGTCCAAGG; CCTACTCCTTGGAGGCCATG

IL-10: GAGAATTCATATGGTCCAGGACAGGG CACCCAG; ATGGATCCTTAGTGCGAATC TTCATTGTCATGTAGCC

IL-1β: TGATGGCTTATTACAGTGGCAATG; GTAGTGGTGGTGGGAGATTCG.

ELISA

Serum IL-1β, TNF-α, and IL-10 levels were tested in supernatants by ELISA Kits (R&D Systems, Oxon, UK) according to the manufacturer's instructions.

Western Blotting Analysis

THP-1 cells were harvested after FGF-21 treatment and washed three times with cold PBS. Total protein was extracted with RIPA lysis buffer with freshly added protease and phosphatase inhibitors PMSF (1 mM). Nuclear extracts were prepared with the Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's instructions. Protein concentration was determined with the BCA protein assay kit. RIPA lysis buffer, PMSF, BCA protein assay kit, and Nuclear and Cytoplasmic Protein Extraction Kit were obtained from Beyotime Biotechnology Corporation (shanghai, China). SDS-PAGE, transmembrane, blocking, and antibody incubation were assessed by the procedure described by Wang WF [12]. Antibodies used for Western blotting was ERK1/2 (CST, Rockford, IL, USA #9101), beta-actin (CST, Rockford, IL, USA #4970), NF-kB (CST, Rockford, IL, USA #8242), and Lamin b (Affinity BF1002).

Statistical Analysis

Values are expressed as mean ± SD of at least three independent experiments and analyzed using one-way ANOVA followed by Duncan's Multiple Range Test for comparisons of group means. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Prism Inc., Chicago, IL, USA). Data were considered as significant at $P < 0.05$.

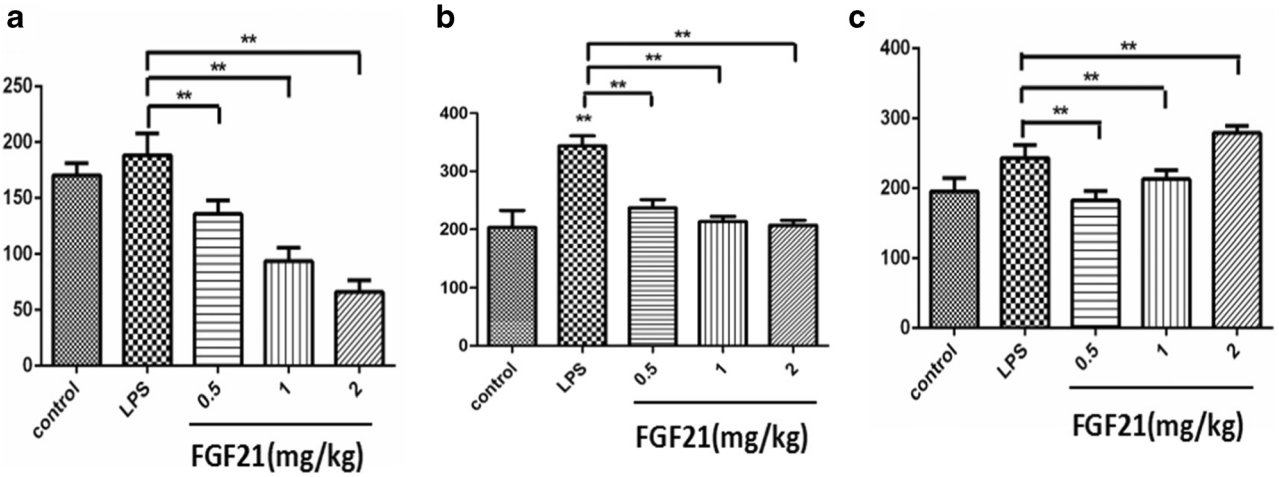

Fig. 2. FGF21 reduced serum levels of inflammatory cytokines in the mice with LPS treatment. FGF-21 reduced IL-1β (**a**) and TNF-α (**b**) protein levels in serum of the LPS-treated mice in a dose-dependent manner. **c** FGF-21 also elevated IL-10 protein levels in the serum of the LPS-treated mice in a dose-dependent manner. All data represent means ± SEM, $n = 10$ per group, $^{**}p < 0.01$ *vs.* normal mice.

RESULTS

Alleviation of Soakage of Inflammatory Lung Cells by FGF21

It was observed that after 24 h of LPS treatment, the capillaries in the lung tissue expanded and became congested due to a significant increase in inflammatory cells. Mice in the 0.25 mg/kg FGF-21 and LPS co-treated group also displayed moderate injury, but the severity was significantly less when compared to mice only given LPS. However, as shown in Fig. 1, the lung injury was significantly alleviated in the 1 mg/kg FGF-21 and LPS co-treated group.

Effect of FGF21 on Serum Inflammatory Cytokine Levels

The influence of FGF-21 on the serum levels of the inflammation in the mice were analyzed by ELISA. The results showed that FGF-21 significantly decreased the serum contents of inflammatory cytokines, such as IL-1β and TNF. IL-10, an anti-inflammatory factor, was significantly elevated by FGF-21 administration (Fig. 2).

Effect of FGF21 on Levels of Inflammatory Factors in LPS-Stimulated THP-1 Cells

THP-1 cells were used as a monocyte model to determine whether FGF-21 has effects on IL-1β expression and production. The THP-1 cells were treated with the indicated concentration of FGF-21, with or without LPS, for 16 h. The amounts of IL-1β mRNA were determined by real-time PCR and Western blotting. FGF-21 inhibited the expression of IL-1β in a dose-dependent manner (Fig. 3b, c). Interestingly, there are two time points in which FGF-21 can significantly reduce IL-1β expression in THP-1 cells (Fig. 3a).

Effect of FGF21 on IL-10 Expression in THP-1 Cells

One concept that is emerging is that as an anti-inflammatory cytokine, IL-10 has an important role in the confinement of the inflammation. Therefore, we detected whether the production of IL-10 is involved in the inhibitory effect of FGF-21 on LPS-stimulated inflammation. As shown in Fig. 4, FGF-21, when administered, induced a significant amount of IL-10 transcription that peaked at the 8-h mark and the effect was that the FGF-21 was dose-dependent.

IL-10 Antibody Inhibited FGF-21-Reduced IL-1β Expression

To further confirm, the IL-10 plays a crucial role in the process of inhibition of IL-1β expression by FGF21. We used IL-10 antibodies as an inhibitor to determine whether IL-10 has any effect on the anti-inflammatory role that FGF-21 may have. The results showed that FGF-21 suppressed the IL-1β expression

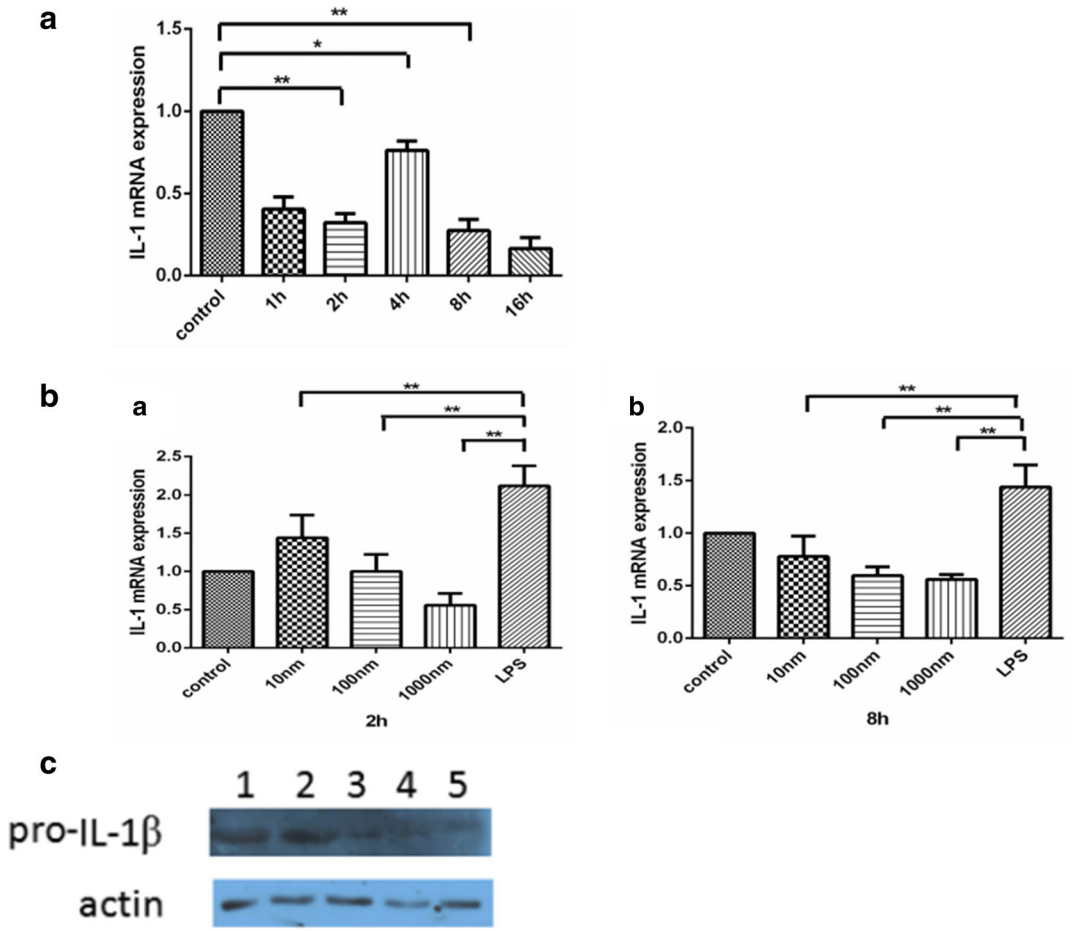

Fig. 3. Reduction of IL-1 mRNA expressions and productions by FGF21 in LPS-stimulated THP-1 cells. For time-courses, THP-1 cells were incubated with 330 nM of LPS and 1000 nM of FGF-21 for different times (0, 1, 2, 4, 8, or 16 h) (B). FGF-21 inhibited LPS-induced IL-1β mRNA expression in THP-1 cells at 2 h (B-a) and 8 h (B-b) *via* a dose-dependent manner. Experiments are duplicated at least six times. **c** THP-1 cells were treated for 8 h with LPS 330 nM and 10, 100, and 1000 nM of FGF-21, followed by Western blotting analysis (1: LPS, 2: LPS, and 10 nM FGF-21, 3: LPS and 100 nM FGF-21, 4: LPS and 1000 nM FGF-21, 5: cells without treating). $*p < 0.05$; $**p < 0.01$.

and was restored by IL-10 antibodies in THP-1 cells at 8 h, but not 2 h (Fig. 5).

ERK1/2- and FGF21-Induced IL-10 Production

Western blot method was used to analyze whether FGF21 induced the expression of IL-10 *via* the ERK1/2 pathway or not. As shown in Fig. 6, FGF21 caused elevation in the expression of ERK1/2 in a dose-dependent manner. Besides, it was also demonstrated that the ERK1/2-specific inhibitor (FR 180204) was also responsible for the inhibition of FGF21-induced IL-10 expression (Fig. 6) without affecting the viability of the THP-1 cells. It was thus concluded that FGF21 stimulates the expression of IL-10 in THP-1 cells *via* the ERK1/2 pathway.

DISCUSSION

Feingold et al. have recently discovered that FGF-21 can lessen inflammation that is induced by LPS. In line with such studies, we tried to define the exact effects of FGF-21 on the anti-inflammatory response and made some new observations, in particular, about the mechanism of FGF-21 modulation of the IL-10 expression in monocytes (Fig. 7). We focused our investigation on monocytes because TLR2 and TLR4, the receptors of LPS, seem to be mainly

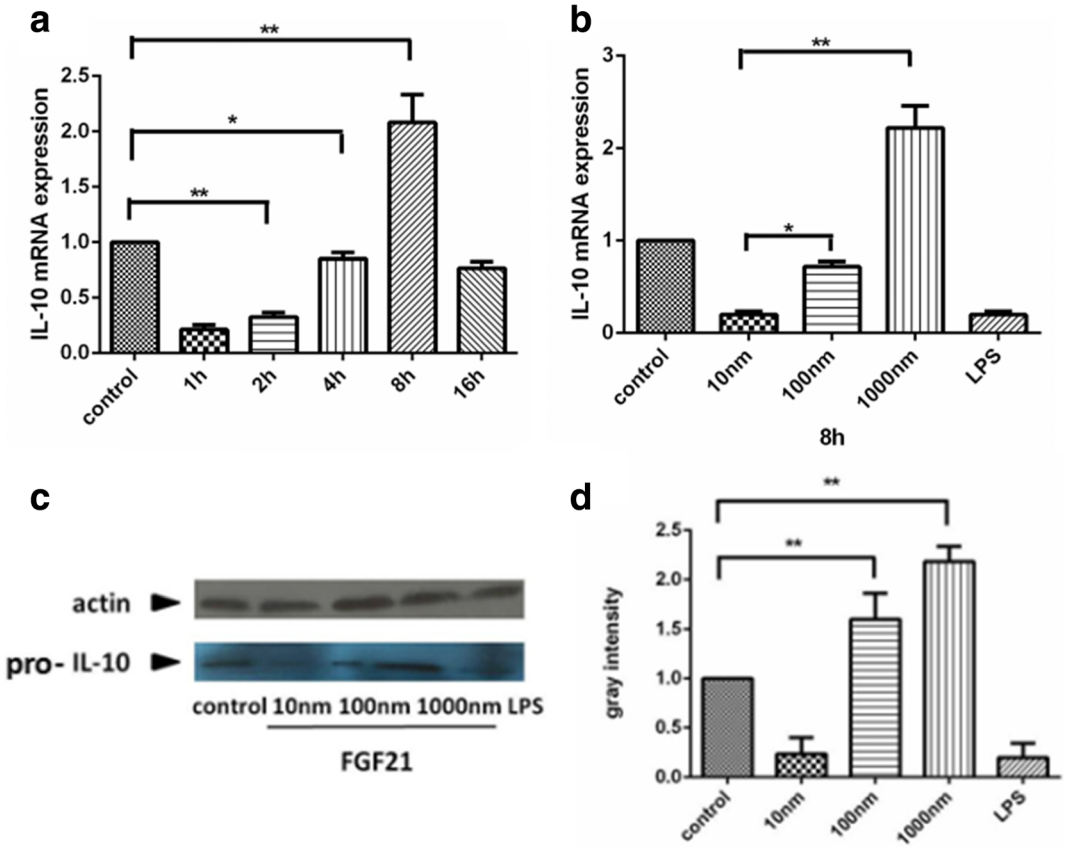

Fig. 4. Elevation of IL-10 expression in THP-1 cells by FGF21. Real-time PCR analysis of IL-10 mRNA expressions in THP-1 cells. **a** THP-1 cells were treated with LPS 330 nM and 1000 nM of FGF-21 for different times (0, 1, 2, 4, 8, or 16 h). **b** THP-1 cells were treated for 8 h with LPS 330 nM and 10, 100, and 1000 nM of FGF-21. Experiments are duplicated at least six times. Experiments are duplicated at least six times. **c** THP-1 cells were treated for 8 h with LPS 330 nM and 10, 100, and 1000 nM of FGF-21, followed by Western blot analysis. **d** Gray intensity analysis of (c). $*p < 0.05$; $**p < 0.01$.

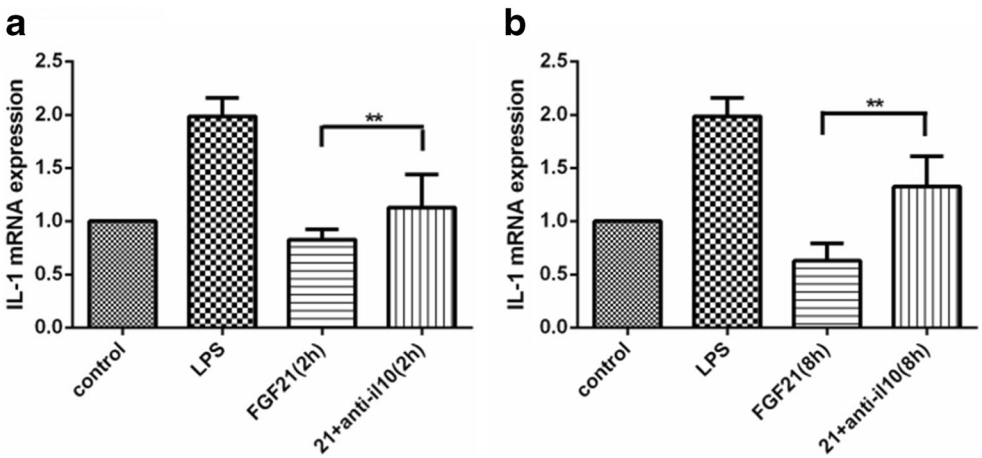

Fig. 5. IL-10 antibody inhibited FGF-21-reduced IL-1 expression. Real-time PCR analysis whether FGF-21 reduced IL-1β mRNA expressions in THP-1 cells that were blocked by IL-10 antibody. **a** IL-10 antibody could not inhibit FGF-21 and reduced IL-1β expression in THP-1 cells at 2 h. **b** IL-10 antibody inhibited FGF-21 and reduced IL-1β expression in THP-1 cells at 8 h. LPS 330 nM, FGF-21 1000 nM, anti-IL-10 antibody: experiments are duplicated at least six times. $**p < 0.01$.

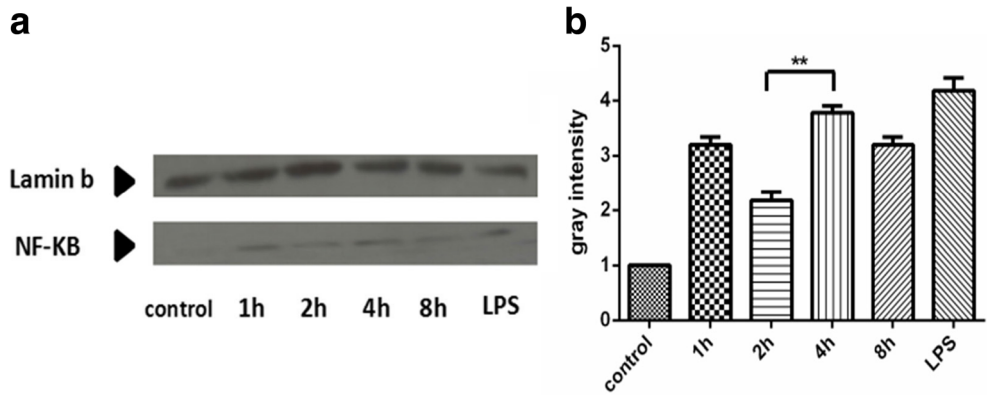

Fig. 6. FGF21 reduced IL-1 production *via* NF-kB pathway at 2 h but not 8 h. **a** Western blots for NF-κB p65 in nuclear extracts of THP-1 cells. **b** Fold change in relative density of NF-κB p65 protein bands. The relative density is expressed as the ratio NF-κB p65/Lamin b1. The bands were analyzed with imageJ. $^{**}p < 0.01$.

expressed on monocytes rather than granulocytes [13], and monocytes are also the main source of inflammatory cytokines. Worth noting, our previous study has identified that FGF-21 has targeted monocytes. In this study, we further investigated the mechanisms underlining the exact immunogenic pathways that are responsible for the inhibition of the inflammatory response in monocytes treated with LPS, especially the ones that are associated with elevated levels of IL-10 expression.

In accordance with Feingold's study, our study has clearly demonstrated that the administration of FGF-21 shows an exert ameliorative effect on LPS-induced inflammation *in vivo* and *in vitro*. *In vivo*, we generated a LPS-induced animal model of acute lung inflammation.

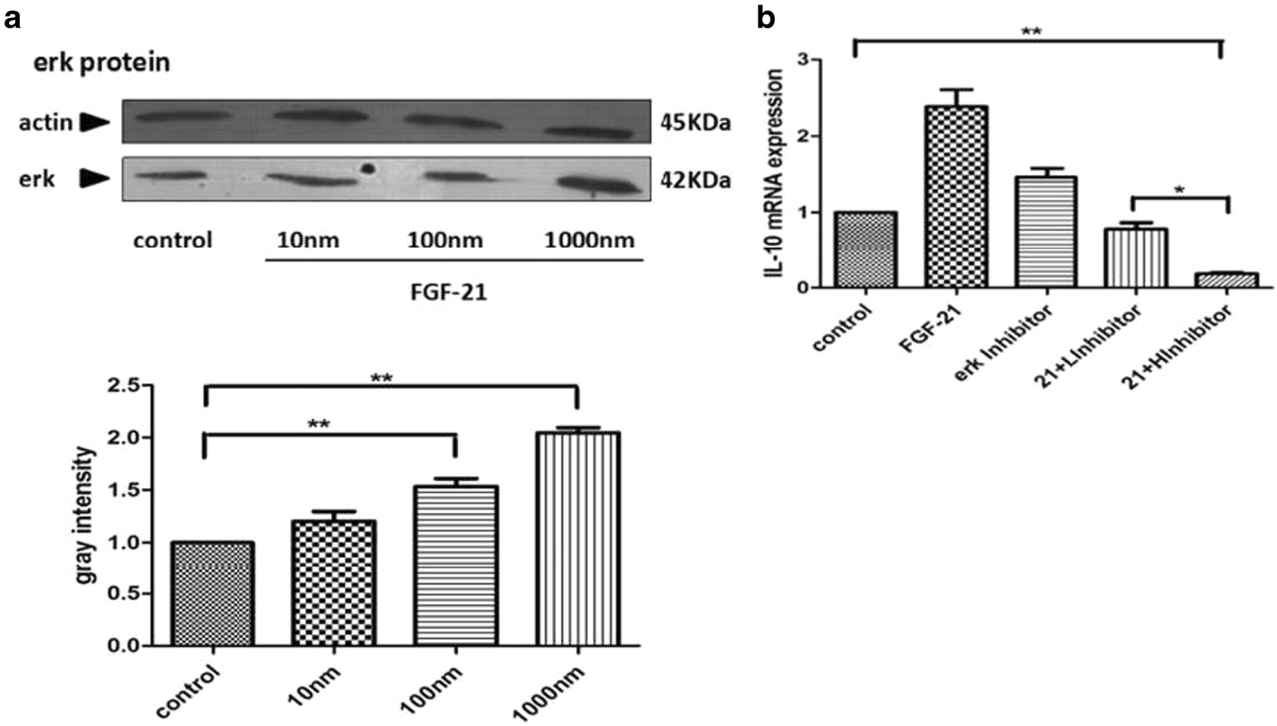

Fig. 7. FGF-21 elevated IL-10 expression *via* ERK pathway. **a** THP-1 cells were treated for 8 h with FGF-21 (control, 10 nM, 100 nM, 1000 nM), followed by Western blot analysis. FGF-21 stimulated the expression of ERK1/2 in THP-1 cells. **b** FGF-21 induced IL-10 expression can be blocked by ERK1/2 inhibitor.

Compared to mice treated with saline, the accumulation of the leukocytes in the lungs was expected in the mice treated with LPS. Notably, in this animal model, the administration of FGF-21 significantly reduced the accumulation of the leukocytes. Therefore, these results further show that FGF-21 has potent anti-inflammatory effects.

IL-1β is a cytokine that plays a crucial role in the progression of inflammation [14]. In mammals, IL-1β activates a protective immune response in moderate concentration [15]. However, the overproduction of IL-1β will lead to dangerous consequences, including hypotension, capillary leak, and acute respiratory distress syndrome [16, 17]. Therefore, regulating the optimal production of functional IL-1 is critical for ensuring proper functioning of the innate immune system.

In the present study, serum concentrations of IL-1β of mice treated with LPS were determined and correlated with the possible effects of FGF21 on inflammation modulation. The results showed that the inflammatory cytokines' mRNA expression and serum protein levels to be markedly reduced by FGF-21 in the LPS stimulated mice. From these results, we conclude that FGF-21 improves the inflammatory reaction through repressing the IL-1β expression in monocytes.

During the *in vitro* experiments, our data showed an intriguing result for FGF-21 downregulated IL-1β expression. For dose–response curves, FGF-21 suppressed IL-1β expression in a dose-dependent manner. However, for the time course, there were two time points when FGF-21 represented an effective inhibitory effect. This suggests to us that FGF-21 may downregulate IL-1β expression *via* two different pathways.

NF-κB is well known as a crucial positive regulator protein for pro-inflammatory factor such as IL-1β [18]. Lee [19] and our previous [20] studies showed that FGF-21 can inhibit the activation of NF-κB. So, in this study, we first studied whether FGF-21 is able to downregulate the IL-1β expression *via* inhibiting the NF-κB signal pathway in THP-1 cells. The Western blotting results showed that FGF-21 can suppress the NF-κB p65 nuclear translocation. However, this effect cannot last more than 6 h. Thus, it was proposed that the long-term anti-inflammation effects of FGF-21 must be another regulatory pathway.

Interleukin-10 is also an anti-inflammatory cytokine that known to be a key substance for preventing inflammatory diseases [21]. Guarda's study has proved that IL-10 inhibits the synthesis of pro-IL-1β as an autocrine agent [22]. In the present study, we observed that elevation of IL-10 expression in FGF21-treated THP-1 cells induced suppression of IL-1β expression. We also showed that treatment with FGF-21 elevated the expression of IL-10 in THP-1 cells at 6 h in a dose-dependent manner. Furthermore, we demonstrated that IL-10 neutralizing antibody can inhibit FGF-21-induced reduction of IL-1β expression, especially for the second time point. These data confirm that IL-10 has an important role in FGF-21 anti-inflammatory effect. The extracellular signal regulated kinase 1 (ERK1) and ERK2 (which are collectively referred to here as ERK) pathway is one of the signaling cascades that is activated in macrophages that results in the expression of IL-10 [23]. In addition, this pathway can be activated by FGF-21 [24]. Thus, we detected whether FGF-21 elevated IL-10 production in THP-1 cells *via* ERK1/2 pathway. The Western blotting results showed that FGF21 can in fact elevate ERK1/2 expression after 8 h of treatment. Furthermore, it was also observed that ERK1/2 inhibitor can block FGF21-induced IL-10 expression.

In conclusion, the major finding of this study is that FGF-21 has a positive effect on LPS-induced toxicity by at least partially elevating IL-10 expression in monocytes. FGF-21 exerts this effect on by at least partially activating ERK1/2 activity. These results may facilitate development of improved therapeutic strategies for the treatment and management of inflammatory diseases by targeting the FGF21.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethics Statement. The study was approved by the Ethics Committee of Northeast Agricultural University.

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