Fractalkine Aggravates LPS-induced Macrophage Activation and Acute Kidney Injury via Wnt/ β -catenin Signaling Pathway

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Research

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Abstract

Background: Fractalkine (CX3CL1, FKN) is a CX3C gene sequence inflammatory chemokine, has been found to have pro-inflammatory and pro-adhesion effects. Macrophages are immune cells with a critical role in regulate inflammatory response. The imbalance of M1/M2 macrophage polarization can lead to aggravated inflammation. This study takes an attempt to investigate the mechanisms through which FKN regulates the macrophage activation and the acute kidney injury (AKI) involved in the inflammatory response induced by lipopolysaccharide (LPS) by using FKN-knockout (FKN-KO) mice and cultured macrophages.

Results: In this study, we found that FKN and Wnt/ β -catenin signaling have a positive interaction in macrophages. FKN-overexpression inhibited LPS-induced macrophage apoptosis. However, it enhanced the cell viability and transformed them into M2 type. The effects of FKN-overexpression were accelerated by activation of Wnt/ β -catenin signaling. In the *in vivo* experiments, FKN deficiency suppressed the macrophage activation and reduced AKI induced by LPS. Inhibition of Wnt/ β -catenin signaling and FKN deficiency further mitigated the pathologic process of AKI.

Conclusions: We provide a novel mechanism underlying activation of macrophage in LPS-induced AKI. The positive interaction between FKN and Wnt/ β -catenin signaling pathway may be the therapeutic target in kidney injury.

Introduction

Inflammatory reaction is a clinical pathological process which acts as a self-defense [1]. Immune system regulates the antigens or the immune cells of which when uncontrolled causes serious organ failure and death eventually [2]. Macrophages kill pathogens and promote adaptive immunity through antigen presentation [3]. Macrophages play important roles in the biological progression such as immune homeostats, cytokines signaling transmission and inflammatory response. Identification of key mechanisms involved in the progression of inflammatory macrophage is urgent and of high demand in improving the clinical outcome.

FKN is a CX3C gene sequence chemokine that functions as immune response and adhesion towards its unique receptor CX3CR1-expressing immune cells. FKN divides into two forms that can be converted into each other *in vivo*: membrane-bound and soluble [4, 5]. When inflammation occurs the membrane-bound FKN is broken down into a soluble form. FKN mobilizes immune cells to accumulate to dangerous sites. FKN act as adhesion molecules and chemical primers which regulate the development of inflammatory diseases when tissue injury occurs [6]. Studies suggested that FKN plays critical roles on the pathological process involved in atherosclerosis [7] Osteoarthritis [8] and muscle injury [9]. However, the molecular mechanism of macrophage that interfered with FKN in regulating the development of inflammatory diseases is rarely reported. Moreover, the specific mechanism has been unclear.

Wnt/ β -catenin is a highly conservative signaling pathway that activates the transcriptional activity through β -catenin nuclear translocation [10], regulates synaptic transmission, cells growth, proliferation, differentiation, adhesion and genetic stability [11, 12]. All of these factors play an important role in the progression of inflammatory diseases such as acute lung injury [13], fibrotic disease [14] and systemic lupus erythematosus [15]. However, the potential mechanisms through which Wnt/ β -catenin interacts with FKN in the LPS-induced inflammatory system have not been reported. Study of the molecular mechanism may provide a new direction for clarifying the molecular mechanism of FKN which regulates the development of inflammatory diseases.

The study utilized LPS-induced macrophage and acute kidney injury mice. Results suggested that LPS-induced macrophage activation was accelerated by FKN-overexpression. However, FKN deficiency prevented LPS-induced acute kidney injury through inhibiting macrophage activation in which Wnt/ β -catenin signaling contributed an essential role.

Results

FKN positively interacted with Wnt/ β -catenin signaling pathway in macrophage.

To identify the biological process of FKN in J774A.1 cells, RNA-sequencing was achieved on cells with or without FKN-overexpression (Fig. 1A-B). The biological process showed that synaptic transmission increased with FKN interference (Fig. 1C). GSEA analysis showed that FKN-overexpression was positively correlated with the activation of Wnt/ β -catenin signaling pathway (Fig. 1D). J774A.1 cells lysates were collected for Co-IP analysis confirming the interaction between FKN and Wnt/ β -catenin signaling (Fig. 1E). FKN and β -catenin protein distribution were found throughout the cytoplasm and nuclear after IF staining. FKN-overexpression enhanced the localization of FKN and β -catenin protein. Combination treatment with Wnt3a also enhanced the protein localization in J774A.1 cells (Fig. 1F-G). A positive interaction between FKN and Wnt/ β -catenin signaling pathway in J774A.1 cells was also confirmed.

FKN promoted LPS-induced macrophage vitality via Wnt/ β -catenin signaling pathway.

Cells were treated with different concentrations of Wnt3a (Wnt/ β -catenin signaling pathway activator) and ICG-001 (Wnt/ β -catenin signaling pathway inhibitor) for 48 h. This helped to assess the role of Wnt/ β -catenin in regulating the vitality of J774A.1 cell. Cells viability was detected using CCK-8 assay. Wnt3a increased the viability of macrophages in a concentration-and time-dependent manner while ICG-001 decreased macrophages viability. Wnt3a with 50 ng/ml promoted the proliferation while ICG-001 with 10 μ M/ml inhibited proliferation after 48 h in J774A.1 cells compared to the control group (Fig. 2A-B). The expression of Ki67 protein was upregulated by Wnt3a but downregulated by ICG-001 in J774A.1 cells

after IF staining (Fig. 2C). On this basis, Wnt3a at 50 ng/ml and ICG-001 at 10 μ M/ml were used for 48h in the following studies.

C-myc and Cyclin D1 were critical genes involved in cells proliferation and differentiation as downstream target of classical Wnt/ β -catenin signaling pathway. Results found that LPS increased the expression of FKN, β -catenin, Wnt-4, c-myc and Cyclin D1 protein compared with the controls ($P < 0.05$). Further research found that c-Myc and Cyclin D1 protein abundance were induced by FKN-overexpression. C-Myc and Cyclin D1 combined with Wnt3a significantly enhanced the effects. However, combination with ICG-001 reversed the effects in LPS-induced J774A.1 cells ($P < 0.05$) (Fig. 2D-E). IF staining further identified that LPS increased the content of Cyclin D1 protein in the cytoplasm and nucleus compared with the controls. Wnt3a enhanced the effects of FKN-overexpression that increased the Cyclin D1 protein localization while decreased with ICG-001 (Fig. 2G).

FKN inhibited LPS-induced macrophage apoptosis via Wnt/ β -catenin signaling pathway.

The effects of FKN on the apoptosis of LPS-induced J774A.1 cell were stained with Annexin V-FITC/PI for flow cytometry analysis. As shown in Fig. 3, the apoptosis rate was increased in the LPS group and ICG-001 group compared with the controls. However, Wnt3a group and EX-FKN group inhibited the rate of apoptosis ($P < 0.05$). Furthermore, apoptosis rate increased in the ICG-001 + LPS group and EX-FKN + ICG-001 + LPS group compared with LPS group. However, the apoptosis rate was decreased in the Wnt3a + LPS group, EX-FKN + LPS group and EX-FKN + Wnt3a + LPS group ($P < 0.05$). The data suggested an anti-apoptosis of FKN-overexpression and Wnt3a in LPS-induced J774A.1 cells. ICG-001 played pro-apoptosis in LPS-induced J774A.1 cell.

FKN transformed LPS-induced macrophage into M2 phenotype via Wnt/ β -catenin signaling pathway.

Western blotting, ELISA and IF analysis were used to detected the expression of iNOS, TNF- α , IL-10, ARG-1 in each group. This helped to investigate the driving force of FKN on the polarization process of LPS-induced J774A.1 cells. Figure 4A-D showed the low expression of macrophage polarization cytokines iNOS, TNF α , IL-10, ARG-1 in the control group. However, macrophage polarization was highly expressed after LPS treatment ($P < 0.05$). This confirmed that LPS activates the macrophage polarization process. Furthermore, Wnt3a group and EX-FKN group showed a down-regulation of iNOS and TNF- α protein. IL-10 and ARG-1 protein were upregulated in LPS-induced J774A.1 cells ($P < 0.05$). Combined treatment with Wnt3a (EX-FKN + Wnt3a + LPS group) markedly strengthened the effects of FKN. However, combined treatment with ICG-001 (EX-FKN + ICG-001 + LPS group) reversed the effects of FKN and further increased the expression of iNOS and TNF- α in LPS-induced J774A.1 cells ($P < 0.05$). Through IF staining, iNOS and ARG-1 proteins accumulated in both the nucleus and cytoplasm but were mainly located in the cytoplasm

after LPS stimulation compared with controls. FKN-overexpression and Wnt3a enhanced the localization of ARG-1 while decreased the localization of iNOS in LPS-induced J774A.1 cell. ICG-001 reversed the effects of FKN and Wnt3a (Fig. 4E-F). The preceding data confirmed that LPS-induced macrophage towards the M2 phenotype by FKN-overexpression and activation of Wnt/ β -catenin signaling pathway.

FKN deficiency attenuated LPS-induced acute kidney injury via inhibition of Wnt/ β -catenin signaling pathway.

The above data from J774A.1 cells demonstrated that overexpression of FKN played essential roles in the pathological progression of LPS-induced inflammatory response via activation of Wnt/ β -catenin signaling. FKN deficiency attenuated LPS-induced kidney pathological damage. FKN-KO mice were selected for the study. Serum creatinine (Scr) and blood urine nitrogen (BUN) were analyzed as a marker of renal function. As shown in Fig. 5A-B, the levels of Scr and BUNs in the FKN-KO + LPS mice were markedly reduced compared with the LPS mice ($P < 0.05$). ICG-001-treated cells (FKN-KO + ICG-001 + LPS group) further decreased the Scr and BUN levels. Wnt3a-treated cells (FKN-KO + Wnt3a + LPS group) reversed the action of FKN deficiency ($P < 0.05$). These findings were further supported by the pathological alterations of HE staining and PAS staining in mice kidney tissues. The renal histopathological findings showed that mice stimulated with LPS exhibited glomerular atrophy, glomerular basement membranes proliferation and inflammatory cells infiltration. However, these renal structure changes in LPS group were attenuated through intervention with FKN deficiency or treatment with ICG-001. The combined treatment (FKN-KO + ICG-001 + LPS group) further improved the renal damage (Fig. 5C-D).

FKN deficiency prevented macrophage proliferation and polarization in LPS-induced acute kidney injury via inhibition of Wnt/ β -catenin signaling pathway.

Whether FKN deficiency decreased the progression of macrophage proliferation and polarization in the acute kidney injury after LPS induced were further examined. The protein expression of FKN, β -catenin, Wnt-4, c-myc, Cyclin D1, iNOS, TNF- α , IL-10 and ARG-1 was quantified using Western Blot. After stimulation with LPS, FKN deficiency (FKN-KO + LPS group) showed decreased protein expression of FKN, β -catenin, Wnt-4, c-myc, Cyclin D1, iNOS, TNF- α , IL-10 and ARG-1 compared with the LPS group ($P < 0.05$). The combined treatment with ICG-001 (FKN-KO + ICG-001 + LPS group) further inhibited the expression of these protein. The combined treatment with Wnt3a (FKN-KO + Wnt3a + LPS group) weakly reversed the effects of FKN deficiency ($P < 0.05$) (Fig. 6). Kidney tissues were stained with antibody F4/80, iNOS or ARG-1 to identify macrophage accumulation and polarization progression. LPS augmented the F4/80, iNOS or ARG-1 protein localization in kidney compared with WT mice. Protein localization of F4/80 and ARG-1 was markedly reduced in the FKN-KO + LPS group and FKN-KO + ICG-001 + LPS group mice kidney

tissues compared to LPS group. These results were consistent with Western Blotting results. The experimental data showed that FKN deficiency ameliorates LPS-induced inflammation by suppressing macrophage activation and M2 phenotype differentiation via inhibition of Wnt/ β -catenin signaling (Fig. 7).

Discussion

The mechanism of FKN-regulated macrophage activation during LPS-induced inflammatory response have been reported in this study. FKN promotes LPS-induced macrophage activation. Wnt/ β -catenin signaling pathway plays a major role during macrophage activation process. Furthermore, FKN deficiency suppresses macrophage activation progression by inhibiting Wnt/ β -catenin signaling. This further reduces the pathologic damage of acute kidney injury caused by LPS exposure in mice model.

LPS is effective in triggering robust inflammatory response. LPS is the main microbial mediator leading to septicemia [16] which causes tissue injury by accelerating cells necrosis [17]. LPS is an active component of the cells wall of Gram-negative bacteria. LPS causes host infection and stimulates macrophage polarization [18]. Activated macrophages produce several inflammatory cytokines (iNOS, NO, IL-6, PGE2, COX-2 and TNF- α) which increase the progression of inflammatory response [19]. Moreover, LPS causes metabolic progression and gradually transforms macrophage into M2 phenotype. Expressing profibrogenic cytokines (Arg-1, IL-10 and TGF- β) promote cell differentiation and tissue remodeling [20] thereby accelerating the pathological processes of fibrosis disease [21]. LPS-induced J774A.1 cells and acute kidney injury mice were used as inflammatory model in this study. J774A.1 cells and mice kidney tissue demonstrated a strongly enhanced iNOS, TNF- α , IL-10 and ARG-1 after stimulation with LPS compared with the control group.

Chemokine FKN adheres to immune cells in the process of inflammation and preferentially induce the migration of cytotoxic effector lymphocytes. This migration of cytotoxic effector lymphocytes positively correlates with the activity of inflammatory diseases [22–24]. Recent studies focused on the therapeutic strategy of FKN in inflammatory diseases. Yu et al found that FKN deficiency ameliorates high fructose diet-induced kidney injury [24]. Riopel et al found that FKN expression was elevated *in vivo* and improved glucose tolerance due to enhanced insulin secretion and decreased β -cells apoptosis [25]. In this study, FKN promoted expression of β -catenin and Wnt-4 protein (the signature protein of Wnt/ β -catenin signaling pathway) and inhibited apoptosis in LPS-induced J774A.1 cells. FKN was highly expressed in the cytoplasm and nucleus in LPS-induced J774A.1 cells and mice kidney tissue. Furthermore, FKN promotes the secretion of IL-10 and ARG-1 but inhibits the secretion of iNOS and TNF- α in LPS-induced J774A.1 cells and mice kidney tissue. Mice with FKN deficiency decreased the expression of c-myc, Cyclin D1, iNOS, TNF- α , IL-10 and ARG-1. FKN deficiency ameliorates kidney injury through prevented glomerular atrophy, glomerular basement membranes proliferation and inflammatory cells infiltration.

The molecular mechanism involved in the anti-inflammatory effects of FKN was further explored based on the study results. The molecular mechanism involved in the Wnt/ β -catenin signaling was studied

through RNA-sequencing. This process was adjusted by FKN. Research showed that abnormal transmission of Wnt/ β -catenin signaling plays an important role in the malignant progression of immune inflammatory diseases by regulating the phagocytosis of macrophages [26, 27]. Activation of Wnt/ β -catenin signaling induces macrophage M2 polarized and promotes the progressive of renal fibrosis [28]. Inhibition of Wnt/ β -catenin signaling alleviates organ damage caused by sepsis through reducing macrophage infiltration [29]. However, the study of the relationship between FKN and Wnt/ β -catenin signaling and macrophage activation processes lack detailed research. In this study, we found a positive interaction between FKN and Wnt/ β -catenin signaling in macrophage. LPS-induced J774A.1 cells treatment with Wnt3a showed the same capacity with FKN. FKN-overexpression combined with Wnt3a enhanced LPS-induced J774A.1 cell proliferation, anti-apoptosis and M2 phenotype conversion. For endotoxin-shocked wild type mice, ICG-001 suppressed macrophage proliferation and M2 phenotype conversion which improve the pathological damage of acute kidney injury. The anti-inflammatory role of ICG-001 was clearly consolidated in FKN-KO mice. Study findings suggested that the inhibitory effects of FKN deficiency in inflammation based on the inhibition of Wnt/ β -catenin signaling pathway.

Conclusion

In summary, FKN deficiency exhibits anti-inflammatory activity in LPS-stimulated inflammatory mice model through suppression of macrophage proliferation and polarization (Fig. 8). Wnt/ β -catenin signaling has a potent biological function through which it exerts its anti-inflammatory activity. This finding strongly revealed that FKN could be a potential molecular target for treatment of LPS-induced inflammatory diseases in the future.

Material And Methods

Cell Culture and Treatment.

J774A.1 cells were obtained from Beina Chuang Lian Biology Research Institute (BNCC300973, Beijing, China). Cells were cultured in DMEM medium (Gibco) with 10% fetal bovine serum (Gibco) at 37°C in a 5% CO₂ incubator. J774A.1 cells were infected with lentiviral vector particle-CX3CL1 and Ubi-MCS-3FLAG-SV40-Cherry-IRES-negative control according to manufacturer's protocol (Shanghai Genechem Co., Ltd.) to achieve FKN-overexpression. Stable cell lines were selected by applying puromycin in culture medium. Cells were divided into 12 groups as follows: (1) Control group; (2) LPS group: cells were infected with LPS (L2880, Sigma-Aldrich, St. Louis, MO, USA) 1 μ g/ml for 12 h; (3) Wnt3a (Wnt/ β -catenin signaling pathway activator) group: cells were infected with Wnt3a (ab81484, Abcam, Shanghai, China) 50 ng/ml for 48 h; (4) ICG-001 (Wnt/ β -catenin signaling pathway inhibitor) group: cells were infected with ICG-001 (S2662, Selleckchem, Houston, TX, USA) 10 μ g/ml for 48 h; (5) Wnt3a+LPS group: cells were pretreated with Wnt3a (50 ng/ml, 36 h) and then co-treated with 1 μ g/ml LPS for 12 h; (6) ICG-001+LPS group: cells were pretreated with ICG-001 (10 μ g/ml, 36 h) and then co-treated with 1 μ g/ml LPS for 12 h; (7) EX-FKN group; (8) Ex-FKN+LPS group: EX-FKN cells infected with LPS (1 μ g/ml, 12 h); (9) EX-FKN+Wnt3a group:

EX-FKN cells infected with Wnt3a 50 ng/ml for 48 h; (10) EX-FKN+ICG-001 group: EX-FKN cells were infected with ICG-001 10 µg/ml for 48 h; (11) EX-FKN+Wnt3a +LPS group: EX-FKN cells were pretreated with Wnt3a (50 ng/ml, 36 h) and then co-treated with 1µg/ml LPS for 12 h; (12) EX-FKN+ICG-001+LPS group: EX-FKN cells were pretreated with ICG-001(10 µg/ml, 36 h) and then co-treated with 1µg/ml LPS for 12 h.

Mice.

Two-three months-old (25±3g) specific pathogen-free (SPF) WT C57BL/6 mice (FKN^{+/+}) and FKN-KO C57BL/6 mice (FKN^{-/-}) were purchased from Shanghai Genechem Animal Co. Ltd (NO. SYXK 2015-0008). For FKN-KO mice, CRISPR/Cas9 technology was used to construct sgRNA sequence (CX3CL1 -sgRNA1: CTGGCAGGTTATCACGGGTTGGG; CX3CL1-sgRNA2: TGGCAGTA ACTCATACGTCCTGG) that targets the FKN-gene locus. The surviving embryos after injection with CRISPR/Cas9 mRNA were raised to adulthood and the founders with FKN Knock-out were screened. Mice were housed under 12/12-h light/dark cycle with free access to food and water (*ad libitum*) *for 1 week before the experiments*. All experiments followed the *animal* experimentation ethics at Youjiang Medical University for Nationalities (NO. SYXK 2017-0004) and all procedures were performed according to the National Institute of Health Guidelines.

Six-eight weeks age of mice were randomly grouped (12 groups containing 36 mice) (1) Control group, WT Mice intraperitoneal (IP) injection of 500 µL saline per day; (2) LPS group, WT Mice challenged with LPS (10 mg/kg, 24 h) by IP injection; (3) Wnt3a group, WT Mice Tail vein injection of recombinant Wnt3a protein (2 µg/kg) for 21 consecutive days; (4) ICG-001 group, WT Mice IP injection of ICG-001(5 mg/kg) for 7 consecutive days; (5) Wnt3a+LPS group, WT Mice Tail vein injection of recombinant Wnt3a protein (2 µg/kg) for 21 consecutive days, LPS (10 mg/kg) was IP injected into WT mice on the last day; (6) ICG-001+LPS group, WT Mice IP injection of ICG-001(5 mg/kg) for 7 consecutive day plus LPS (10 mg/kg) was IP injected into WT mice on the last day; (7) FKN-KO group, FKN-KO Mice IP injection of 500 µL saline per day; (8) FKN-KO+LPS group, FKN-KO mice challenged with LPS (10 mg/kg, 24 h) by IP injection; (9) FKN-KO+Wnt3a group, FKN-KO Mice Tail vein injection of recombinant Wnt3a protein (2 µg/kg) for 21 consecutive days; (10) FKN-KO+ICG-001 group, FKN-KO Mice IP injection of ICG-001(5 mg/kg) for 7 consecutive days; (11) FKN-KO+Wnt3a+LPS group, FKN-KO Mice Tail vein injection of recombinant Wnt3a protein (2 µg/kg) for 21 consecutive days plus LPS (10 mg/kg) was IP injected into FKN-KO mice on the last day; (12) FKN-KO+ICG-001+LPS group, FKN-KO Mice IP injection of ICG-001(5 mg/kg) for 7 consecutive days LPS (10 mg/kg) was IP injected into FKN-KO mice on the last day.

Renal Function Measurement

All mice serum was collected for creatinine (Scr) and Blood Urea Nitrogen (BUN) assays used to evaluate the renal function. Scr and BUN levels of mice were examined using the Blood Urea Nitrogen Assay kit

(C013-2-1, Jiancheng Bioengineering *Institute, Nanjing*, China) and Creatinine Assay kit (C011-2-1, Jiancheng Bioengineering *Institute, Nanjing*, China) according to the manufacturer's instructions.

RNA-sequencing Assay

Total RNA was extracted with Trizol Reagent (Invitrogen). Agarose gel electrophoresis was used to assess RNA integrity. Total RNA was purified with Qia Quick PCR kit and PCR amplification was performed. RNA-seq library for sequencing was constructed by Illumina HiSeqTM 2500. ABI Step OnePlus Real-Time PCR System (Life Technologies) was used for quantification and Pooling. The sequence was performed according to the PE150 mode of Hiseq2500.

Co-Immunoprecipitation (Co-IP) Assay

J774A.1 cells were lysed with a pre-cooled IP lysis buffer for 30 min. Cells lysates were preprocessed with magnetic bead and then incubated with FKN antibody or control IgG at 4°C overnight. The antibody was captured on magnetic bead and inspected using Western blot with anti-Wnt-4 and anti-β-catenin.

Cells Viability Assay

Cells viability was analyzed using Cell Counting Kit-8 kit (M4839, AbMole, Beijing, China). J774A.1 cells were seeded into 96-well plates at 5×10^3 cells per well. Cells were treated with Wnt3a (25, 50, 75 ng/ml) and ICG-001 (5, 10, 15 μM/ml) for 24, 48 and 72 h. A total of 10 μl CCK-8 was added to each well and incubated 1h at 37°C in a 5% CO₂ incubator after the treatment with Wnt3a and ICG-001. The TriStar LB 941 multimode microplate reader (Berthold Technologies, Germany) at 450 nm was used in OD assays.

Cell Apoptosis Assay

The fluorescein isothiocyanate-Annexin V/propidium iodide (FITC-Annexin V/PI) apoptosis kit (556547, BD Biosciences, USA) was used to detected cell apoptosis. Cells were seeded into 6-well plates and cultured for 48 h. The cells of each group were collected, rinsed three times with 1×PBS and then centrifuged for 3 min. The cells were then adjusted to 1×10^6 cells/ml and incubated for 15 minutes in a binding buffer containing annexin V-FITC and PI. The apoptosis was detected through flow cytometry using a FACS Canto II (BD Biosciences, USA) within 1 h.

ELISA

Supernatants from all samples were collected. Cyclin D1 (SBJ-M0517, Senbeijia Bioengineering *Institute*, Nanjing, China), TNF-α (E-EL-M0049c, Cusabio Biotech Co., Ltd) and ARG-1 (CSB-EL002005MO, Cusabio

Biotech Co., Ltd) were detected according to manufacturer's instructions using ELISA Kits. The absorbance was measured using the TriStar LB 941 multimode microplate reader at 450 nm.

Western Blotting

All samples which had 40 µg total protein were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the polyvinylidene fluoride (PVDF) membrane. The membrane was incubated overnight at 4°C with rabbit anti-FKN (DF12376, 1:500, Affinity), rabbit anti-iNOS (AF0199, 1:500, Affinity), rabbit anti-Wnt-4 (DF9040, 1:500, Affinity), rabbit anti-β-catenin (AF6266, 1:500, Affinity), rabbit anti-c-Myc (AF0358, 1:500, Affinity), rabbit anti-CyclinD1 (AF0931, 1:500, Affinity), rabbit anti-TNF-α (DF7014, 1:500, Affinity), rabbit anti-IL-10 (DF0175, 1:500, Affinity), anti-ARG-1 (DF6657, 1:500, Affinity), rabbit monoclonal FKN antibody (ab25091, 1:1000, Abcam), rabbit monoclonal anti-Wnt-4(ab262696, 1:1000, Abcam), rabbit monoclonal anti-β-catenin (ab6302, 1:1000, Abcam), mouse anti-β-actin (AF7018, 1:1000, Affinity), mouse anti-β-tubulin (AF7010, 1:10000, Affinity) and mouse anti-GAPDH (AF7021, 1:1000, Affinity). The membrane was then incubated with Goat anti-rabbit IgG (S0001, 1:5000, Affinity), Goat anti-mouse IgG (S0002, 1:5000, Affinity) for 50 min at room temperature. Then the enhanced chemiluminescence substrate (KF003, Affinity) and visualized using Tanon-5200 (Tanon, Shanghai, China).

HE Staining and PAS Staining

Kidney tissue samples were fixed in 10% formalin and then embedded in paraffin for histopathology. A total of 3-4 µm serial sections were stained followed by routine de-waxed and hydrated. These sections were then stained with hematoxylin-imidine Red (HE) or periodic Acid-Schiff (PAS) according to standard procedures. These sections were visualized using a light microscope (H550S, NIKON, Japan).

Immunofluorescence Assay

J774A.1 cells (5×10^3 cells/well) were seeded into glass bottom cell culture dish (IBIDI, Germany) for 72 h. J774A.1 cells were then washed three times with 1×PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. Cells were permeabilized with 1 ml of 0.1% Triton X-100 for 20 min. Cells were incubated with fluorescently labeled primary antibodies (anti-FKN; anti-β-catenin; anti-iNOS; anti-ARG-1; anti-β-tubulin) followed by Goat anti-rabbit IgG (H+L) FITC-conjugated antibody(S0008, 1:200, Affinity) and [Goat Anti-Mouse IgG \(H+L\) Fluor594-conjugated](#) antibody (S0005, 1:200, Affinity).. Finally, cells were incubated with DAPI for 10 min and imaged using an Olympus Fluoview 3000 Confocal Laser Scanning Microscope (FV3000, Olympus and Tokyo, Japan).

Mice kidney sections were prepared using standard procedures. The primary anti-bodies included anti-F4/80, anti-CD11b, anti-iNOS or anti-ARG-1 followed by secondary antibodies staining. All sections were

visualized under Olympus Fluoview 3000 Confocal Laser Scanning Microscope.

Statistical Analysis

All data are expressed in the form of mean±standard deviation. SPSS23.0 and GraphPad Prism 8.0 were used to analyze statistical data. Statistically significance was confirmed as $p<0.05$. Each experiment was repeated three times.

Declarations

Ethics approval and consent to participate

Animal Experiments strictly followed the guidelines of the National Institutes of Health and were approved by the Ethical committee of Youjiang Medical University for Nationalities.

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Competing Interest

The authors declare that they have no competing interests.

Authors' contributions

Qiming Gong, Yan Jiang, and Xiuhong Pan carried out the experimental work and conceived of the study and participated in its design and coordination. Qiming Gong and Yanwu You drafted the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The RNA sequence data reported in this paper have been deposited in the BioProject database, under accession number PRJNA700110 that are publicly accessible at: <http://www.ncbi.nlm.nih.gov/bioproject/700110>. The dataset supporting the conclusions of this article is included within the article.

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Figures

Figure 1

FKN positively interacted with Wnt/ β -catenin signaling pathway in J774A.1 cells. A. Expression profile of FKN-regulated genes using the RNA-seq. B. differentially expressed genes showed in volcano map. C. Biological process were revealed in RNA-sequencing. D. The dataset was analyzed by GESA using the Hallmark collection. The correlation between FKN and Wnt/ β -catenin signaling indicated in GESA. E-G. Co-ip and IF assay detected the interaction between FKN and Wnt/ β -catenin signaling and visualized using confocal microscopy with specific antibodies (green). Nucleus were incubated with DAPI (blue). Cytoskeleton were incubated with β -tubulin (red). Scale bars represent 10 μm .

Figure 2

FKN promoted the viability of J774A.1 cells via Wnt/ β -catenin signaling. A-B. Cells were incubated with Wnt3a (25, 50, 75ng/ml) and ICG-001(5, 10 and 15 μ M/ml) for 24, 48 and 72h. The viability of cells was estimated using CCK-8 assay. C. IF assay for KI67 in J774A.1 cells. D-E. Western Blot assay and quantitative assay showing the protein expression of FKN, β -catenin, Wnt-4, c-myc and CyclinD1 in J774A.1 cell. F. The secretion of CyclinD1 in J774A.1 cell supernatant was detected using ELISA. *P < 0.05 compared with the control group; #P < 0.05 compared with the LPS group. G. The subcellular localization of Cyclin D1 was identified by immunostaining using anti-Cyclin D1 and observed using confocal microscopy. Scale bars represent 10 μ m.

Figure 3

FKN inhibited LPS-induced apoptosis via Wnt/ β -catenin signaling pathway in J774A.1 cells. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the LPS group.

Figure 4

FKN regulated polarization in LPS-induced J774A.1 cells via Wnt/ β -catenin signaling. A-B. Western Blot assay and quantitative assay were performed to detect the expression of iNOS, TNF- α , ARG-1, IL-10 protein in J774A.1 cells. C-D. ELISA was used to detect the content of TNF- α and ARG-1 in the cell supernatant. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the LPS group. E-F.

Immunofluorescence Analysis was used to ascertain the subcellular localization of iNOS and ARG-1. Scale bars represent 10 μ m.

Figure 5

FKN deficiency attenuated LPS-induced acute kidney injury via inhibition of Wnt/ β -catenin signaling. A-B. The BUN and serum creatinine levels. C. H&E stains of kidney tissues. * $P < 0.05$ compared with the WT Mice; # $P < 0.05$ compared with the LPS Mice. D. PAS stains of kidney tissues. a) Control mice; b) LPS mice; c) Wnt3a mice; d) ICG-001 mice; e) Wnt3a+LPS mice; f) ICG-001+LPS mice; g) FKN-KO mice; h) FKN-KO+LPS mice; i) FKN-KO+Wnt3a mice; j) FKN-KO+ICG-001 mice; k) FKN-KO+Wnt3a+LPS mice; l) FKN-KO+ICG-001+LPS mice.

Figure 6

FKN deficiency suppressed macrophages polarization and proliferation in mice kidney tissues via inhibition of Wnt/ β -catenin signaling. A-B. Western Blot and quantitative assay showing the protein expression of FKN, β -catenin, Wnt-4, c-myc, CyclinD1, iNOS, TNF- α , ARG-1 and IL-10 in mice kidney tissues. *P < 0.05 compared with the WT Mice; #P < 0.05 compared with the LPS Mice.

Figure 7

FKN deficiency suppressed F4/80, iNOS or ARG-1 protein localization in mice kidney tissues via inhibition of Wnt/ β -catenin signaling. Scale bars represent 50 μ m.

Figure 8

Molecular mechanism of FKN-Wnt/β-catenin signaling axis in LPS-induced macrophage and mice injury. FKN aggravated LPS-induced macrophage proliferation and polarization in acute kidney injury via Wnt/β-catenin signaling pathway.