Knockout of TLR4 promotes fracture healing by activating Wnt/β-catenin signaling pathway

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Research article

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Abstract

Background: The aim of this study was to investigate the effect of Toll like receptor 4 (TLR4) on fracture healing.

Methods: The open tibial fracture models in TLR4 knockout (TLR4 -/-) and wild type (WT) C57BL-6J mice were established. The radiological examination, tartrate-resistant acid phosphatase (TRAP) staining , Micro-CT scan and biological torsion test were performed on 7, 14 and 21 days after operation. Enzyme Linked Immunosorbent Assay (ELISA) kit was used to detect the expression levels of tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β) and interleukin 6 (IL-6). Western blotting was used to detect the expression of β-catenin, Wingless-type MMTV integration site family, member 4 and 5B (Wnt4 and Wnt5B), proliferating cell nuclear antigen (PCNA) and bone morphogenetic protein-2 (BMP-2) of the callus tissue obtained from mice.

Results: TLR4 knockout promoted fracture healing, reduced the number of osteoclasts, increased bone callus volume (BV) and callus mineralized volume fraction (BV/TV%) ($P < 0.05$), increased the maximum torque and torsional stiffness of callus ($P < 0.05$), reduced TNF-α, IL-1β and IL-6 expression ($P < 0.01$), and increased the expression of β-catenin, Wnt4, Wnt5B, PCNA and BMP-2 ($P < 0.01$).

Conclusions: TLR4 knockout reduced inflammatory and promoted fracture healing by activating Wnt/β-catenin signaling pathway.

Background

Fracture is the complete or partial interruption of the bone continuity [1]. Worldwide, the incidence of fractures is increasing in recent years [2]. Fracture healing is a complex physiologic process that involves the coordinated participation of several cell types [3]. It is influenced by many factors (such as inflammation), which leading to delay union or nonunion with a incidence rate of 3–10% [4,5]. Although many efforts have been made on fracture healing mechanism during the process of fracture, the therapeutic effect and recovery rate of fracture is still unsatisfactory.

With the latest advances made in molecular biology and genetics it is now known that fracture healing involves the spatial and temporal coordinated action of hundreds of genes working towards restoring its structural integrity [6,7]. TLRs (Toll like receptors) is an ancient receptor for mediating natural immunity [8]. As a member of the TLRs family, TLR4 has been implicated in inflammation-induced bone destruction in various chronic bone diseases [9]. The overexpression of TLR4 activates the downstream signaling pathways, induces inflammation related genes expression, and further leading to inflammatory reaction [10]. In animal model, bone healing is closed related with higher osteoclastogenesis gene expression in TLR4 knockout mice [11]. In recent years, some researchers suggested that Wnt/β-catenin signaling pathway plays an important role in bone embryonic development [12–14]. Actually, fracture healing is closed associated with the long bone development during embryonic period [15]. A previous study indicates

that activation of Wnt/β-catenin signaling pathway can promote fracture healing [16]. Wnt signaling pathway also plays an important role in inflammatory bone diseases such as rheumatoid arthritis and chronic periodontitis [17]. Interestingly, Vamadevan et al. indicated that TLR4 could inhibit the Wnt/β-catenin signaling in intestinal epithelial cells [18]. The biological function of TLR4 via Wnt/β-catenin pathway has also been revealed in hepatocellular carcinoma [19]. However, whether there is an effect of TLR4 on fracture healing via Wnt/β-catenin signaling pathway is still unclear.

In this study, the open tibial fracture mice model was established based on wild type and TLR4 knockout mice. Radiological examination, tartrate-resistant acid phosphatase (TRAP) staining, micro-CT scan and biomechanical torsion assay were performed on 7, 14 and 21 days after operation. The contents of interleukin–6 (IL–6), tumor necrosis factor- (TNF-a) and IL–1β detected by enzyme-linked immunoassay (ELISA) kit. Furthermore, the expression of β-catenin, Wnt4, Wnt5B, proliferating cell nuclear antigen (PCNA) and bone morphogenetic protein–2 (BMP–2) were detected by Western blot. Our study aimed to reveal the effect of TLR4 on callus remodeling and biomechanical properties in the late stage of fracture healing.

Methods

Establishment of mice open tibial fracture model

A total of 26 SPF C57BL–6J mice (10–12 weeks, 20–30 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Meanwhile, totally 26 TLR4 knockout mice (10–12 weeks, 20–30 g) were purchased from the Jackson Laboratory (US). Briefly, all mice were anesthetized with intramuscular injection of sodium pentobarbital (0.05 mg/g, Chuangdong Co., Chongqing, China), and then laid on an experimental table in the supine position. The right anterior tibial skin of mice was cut along longitudinal axis. The 22G/0.41 mm intramedullary needle was inserted into the tibia bone marrow cavity from the patellar tendon. Then the muscles were separated and the tibia was exposed. After sawing the tibial shaft, 0.9% normal saline was used to rinse the surface of the tibia. Then the intramedullary needle was completely inserted and the fracture was reset. 4 - 0 threads were used to suture the incision by layer-by-layer. After resuscitation, the mice were sent back to the animal room. Tramadol hydrochloride injection (25 mg/l) (Liaoning Tianlong Pharmaceutical Co., Ltd.) paracetamol suspension drops (Tenolin) were added to drinking water 1 day before operation and 3 days after operation for postoperative analgesia (PMID: 23107765). This study was approved by the ethics committee of Qilu Hospitial of ShanDong University (Approval number: ql2019–121D5), and all experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health (Bethesda, MD, USA).*X-ray analysis*

Lateral X-rayfilm was used to evaluate the establishment of fracture model, the shape of intramedullary fixation needle, and the degree of fracture healing. Moreover, X-ray radiographic analysis (with 5.0-kV for

6.0 s, Faxitron X-ray, Wheeling, IL) was performed on days 7, 14 and 21 after operation in mice of each group. All the operations were repeated for 6 times.

After the study, all animals were euthanized. The right hand held the rat tail and pull it back, and the left thumb and forefinger pressed down firmly on the mouse head at the same time. The external force was used to dislocate the cervical spine of the mouse, and the spine and the brain were disconnected. This method can quickly lose consciousness and reduce pain of experimental animals, which is a commonly used method for euthanasia of small experimental animals.

Sample collection

Six mice were randomly selected at 1, 7, 14 and 21 days after operation in each group respectively. The lumen, sculpture and surrounding soft tissues were taken out by cutting in the knee joint space plane and the treading joint space plane. The whole length of tibia and its posterolateral curved fibula could be seen by resecting the muscles around tibia and fibula layer-by-layer from shallow to deep. The tibia and fibula joints are cut gently with sharp blades to observe the fibula. The tibial intramedullary needle was gently removed with a needle holder. Finally, the samples were placed in refrigerator at –80°C.

Micro-CT scan

Micro-CT systems are typically used to examine bones of small animals in vivo. Micro-CT scanning (Scanco Medical co., LTD., Switzerland) and 3D reconstruction of bone callus were performed on fracture specimens. Ethanol was used as the scanning medium. Meanwhile, the potential of X-ray tube was 45 kVp and the voxel size was 10 μm^3. Then, the bone callus volume (BV) was measured, followed by bone callus mineralized volume fraction (BV/TV, %) calculation. The dual threshold method and gold standard was used for measurements of BV/TV [20].

Tissue staining observation

The fracture samples were fixed in the fresh 4% paraformaldehyde for 24 h. Callus tissue was dehydrated in 10% ethylenediamine tetraacetic acid (EDTA) for 4 weeks, dehydrated in ethanol and embedded in paraffin. Then paraffin sections (3–4 μm) were dried at 60°C overnight. Then, Giemsa staining, and tartrate-resistant acid phosphatase (TRAP) staining were used to observe the histological changes under optical microscope (×50). Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to analyze bone tissue area/total callus area, cartilage tissue area/total callus area, and the number of osteoclasts.

Biomechanical torsion assay

Before the test, the intramedullary needle was taken out from the tibial samples. The proximal and distal points of tibia were firmly secured to two aluminous square. Then the tibia was placed on the biological torsion tester at a rate of 1 °/s until callus disruption. The maximum torsion value and stiffness of callus were analyzed by EnduraTec TestBenchTM system (Bose Corp., Minnetonka, MN). After the above operation, the tibial samples were used for Enzyme-linked immunosorbent assay and western blotting

ELISA assay

The callus tissue (100 mg) was ground with a mortar and pestle in phosphate buffer saline (PBS), and the supernatant was taken after centrifugation. The expression levels of TNF-α, IL–1β and IL–6 were determined by the ELISA kit (Diaclone, France). Optical density (OD) value at 450 nm were measured with the microplate reader. The standard curves of OD value and concentration were drawn, and the concentration of the sample was calculated according to the standard curve.

Western blot

The expression of β-catenin, Wnt4, Wnt5B, PCNA and BMP–2 were detected by Western blot. Briefly, the total protein was extracted from callus tissue samples. The callus tissue was ground in liquid nitrogen, and the RIPA buffer was added to the pyrolysis solution. After centrifugation, the total protein was extracted by using kit (Millipore, Billerica, MA, USA). The supernatant was separated and packed in centrifugal tube and stored at - 20 ^{o}C. Then, the protein was added to equal weight and separated by twelve alkyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then the membranes were blocked with 5% defatted milk powder, and incubated with primary antibody including β-catenin antibody (1:300, Abcam, UK), Wnt4 antibody (1:300, Abcam, UK), Wnt5B antibody (1:300, Abcam, UK), PCNA antibody (1:300, Abcam, UK), BMP–2 antibody (1:300, Abcam, UK) and GAPDH (1:2000, Santa Cruz Biotechnology Inc, USA) at 4°C overnight respectively. After that, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:5000, Beijing Zhong Shan Biotechnology Co., Ltd., Beijing, China) at room temperature for 1 h. Proteins were visualized with enhanced chemiluminescence kit and gel imaging system (Invitrogen™ E-Gel™ Imager, ThermoFisher scientific, US). Results were analyzed by Image Tools (Image J, National Institutes of Health, US).

Statistical analysis

Statistical analysis was performed by SPSS version 21.0 (SPSS Inc., Chicago, IL). All data were expressed as mean ± standard deviation (SD). Comparison between two groups was determined by t test (two groups). P-value less than 0.05 was considered to be significantly different. All the experimental data were visualized using Graphpad prism 5.0 software.

Results

Knockout of TLR4 promoted fracture healing

All mice were successfully operated except for 2 WT mice and 2 TLR4$^{-/-}$ mice (Kirschner wire loosening, fracture displacement). X ray results on the remaining 36 mice showed that the fracture lines of the tibia in WT mice and TLR4$^{-/-}$ mice were clear on 7 days after the operation. Fourteen days after the operation, the fracture line of the tibia in WT mice was still clear, but there was a small part of the fracture healing. The bone callus density in TLR4$^{-/-}$ mice was significantly higher than that of WT mice, and the fracture line of the tibia was blurred. The fracture line of the tibia in WT mice was vague, and that in TLR4$^{-/-}$ mice was very blurred and vague on the 21 days after the operation (Figure 1A). Moreover, the bone area/total callus area of TLR4-/- group mice was more than that of WT mice (Figure 1B), and the proportion of cartilage tissue was less than that of WT mice (Figure 1C). Furthermore, TRAP staining was performed on the longitudinal section of callus tissue and found that the number of osteoclasts in TLR4-/- group was less than that in WT mice (Figure 1D and 1E).

Knockout of TLR4 increased BV and BV/TV% in mice with open tibia fracture

Micro-CT reconstructed 3D (Figure 2A) and 2D (Figure 2B) images of fracture callus showed that the callus volume (BV) of TLR4-/- mice was higher than that of WT mice at 14 and 21 days after operation (all $P < 0.05$); there was no significant difference in 7 days after operation ($P > 0.05$) (Figure 2A and 2C). At 7, 14 and 21 days after operation, the volume ratio of callus and total callus (BV/TV%) in TLR4-/- mice was higher than that in WT mice (all $P < 0.05$) (Figure 2B and 2D).

Knockout of TLR4 increased biomechanical properties of tibia

At 14 and 21 days after operation, the maximum torque (Figure 3A) and torsional stiffness (Figure 3B) of the callus in TLR4$^{-/-}$ mice were significantly greater than those in WT mice ($P < 0.05$), but there was no significant difference between the two groups on the 7 days after the operation ($P > 0.05$).

Knockout of TLR4 reduced inflammation of callus tissue

Compared with WT mice, the expression levels of TNF-α (Figure 4A), IL−1β (Figure 4B) and IL−6 (Figure 4C) in TLR4$^{-/-}$ mice was decreased on 7, 14 and 21 days after operation ($P < 0.01$). It indicated that knockout of TLR4 could decrease the expression of inflammatory factors, and promote fracture healing by reducing the inflammatory response and inflammatory injury.

Knockout of TLR4 promoted bone formation by activating the Wnt/β-catenin signaling pathway

Compared with WT mice, the expression levels of β-catenin, Wnt4 and Wnt5B in TLR4$^{-/-}$ mice were increased on 7, 14 and 21 days after operation ($P < 0.01$). It indicated that TLR4 knockout activated the Wnt/β-catenin signaling pathway. In addition, the expression levels of PCNA and BMP−2 in TLR4$^{-/-}$ mice were higher than those in WTmice on 7, 14 and 21 days after operation ($P < 0.01$) (Figure 5).

Discussion

Although TLR4 has been implicated in inflammation-induced bone destruction [11], the detail molecular mechanism of TLR4 on fracture healing is still unclear. In this study,the open fracture model of TLR4$^{-/-}$ and WT mice was successfully established. TLR4 knockout promoted fracture healing, increased BV and BV/TV%, increased the maximum torsion value and stiffness of callus, reduced TNF-α, IL−1β and IL−6 expression, and increased the expression of β-catenin, Wnt4, Wnt5B, PCNA and BMP−2.

Inflammation is an important cause of delayed union or nonunion of fracture [21]. Previous studies have confirmed that TLR4 can mediate inflammatory response in bone [22]. Ye et al. found that down-regulating of TLR4 could attenuated inflammatory pathways by decreasing TNF-α expression in human retinal microvascular endothelial cells [23]. A previous study shows that dioscin can decrease the expression of IL−1β, IL−6 and TNF-α by regulating TLR4 and further inhibit the inflammatory liver injury [24]. Besides, Astragaloside IV prevents inflammatory by inhibiting the TLR4 expression, which further improve renal interstitial fibrosis [25]. In this study, TLR4 knockout reduced inflammatory, which was consistent with the above studies. In addition, we also found that TLR4 knockout promoted fracture healing, suggesting that TLR4 knockout might promote fracture healing by reducing inflammatory. Furthermore, PCNA is a kind of intra nuclear protein, and a cyclical protein related to cell proliferation cycle, which is a good marker for judging cell proliferation [26]. BMP−2 regulates osteoblast differentiation and bone formation, and β-catenin is one of the transcriptional activators of BMP−2 protein [27]. Tsuji et al. showed that BMP2 was an important endogenous mediato for fracture healing [28]. And Bouletreau et al. indicated that up-regulation of BMP−2 could promote fracture healing [29]. Furthermore, upregulation of PCNA can facilitate the cell proliferation and differentiation during fracture healing, which is beneficial for fracture healing [30]. Consequently, TLR4 knockout promotes fracture healing by reducing inflammatory and increasing the expression of PCNA and BMP−2. In addition, Jiao et al. suggested that BMP−2 and Wnt/β-catenin signaling pathway could synergistically facilitate the differentiation of mesenchymal stem cells into osteoblasts [31]. And previous studies have also indicate that upregulation of Wnt/β-catenin signaling pathway can increase the expression of PCNA in rat brains after intracerebral hemorrhage and intestinal stem cells [32,33]. In this study, the expression of PCNA and BMP−2 were increased in TLR4$^{-/-}$ mice. Meanwhile, the Western blot showed that knockout of TLR4 activated Wnt/beta-catenin signaling pathway to promote bone formation. Thus, we speculated that TLR4 knockout might increase the

expression levels of PCNA and BMP–2 by activating Wnt/β-catenin signaling pathway to promote bone formation.

The β-catenin, Wnt4 and Wnt5B were the were all protein associated with Wnt/β-catenin signaling pathway. A previous study shows that Wnt4 can prevent osteoclast formation and bone resorption [34]. Hendrickx et al. found that Wnt4 and Wnt5B participated in bone metabolism [35]. Heras et al. revealed that the expression levels of β-catenin, Wnt4 and Wnt5B were proportional to the osteogenic ability of osteoblasts [36]. Meanwhile, Wnt/β-catenin signaling pathway has been shown to be involved in inflammatory reactions [37]. Silvagarcía O et al. discovered that Wnt/β-catenin signaling pathway could control the inflammatory response in infections caused by pathogenic bacteria [38]. Furthermore, a previous study indicates that Wnt/β-Catenin signaling pathway inhibits inflammatory by decreasing the expression of IL–1β and TNF-α in Parkinson's disease [39]. Actually, Wnt/β-catenin signaling pathway is involved in fracture healing. Huang et al. found that inhibition of β-catenin signaling in chondrocytes can delay fracture healing in mice [40]. Bao et al. showed that a slightly activation of Wnt/β-catenin signaling could ensure better bone fracture repair during the late stage of fracture healing [41]. In this study, the expression of β-catenin, Wnt4 and Wnt5B were increased in TLR4$^{-/-}$ mice, indicating that TLR4 knockout could activate Wnt/β-catenin signaling pathway.

Conclusions

In conclusion, TLR4 knockout reduced inflammatory and promoted fracture healing by activating Wnt/β-catenin signaling pathway. It should be a highlight potential opportunity for bone destruction in inflammatory bone diseases.

Abbreviations

Toll like receptor 4 (TLR4)

TLR4 knockout (TLR4-/-)

wild type (WT)

hematoxylin-eosin (HE)

Enzyme Linked Immunosorbent Assay (ELISA)

tumor necrosis factor-α (TNF-α)

interleukin–1 beta (IL–1β)

interleukin 6 (IL–6)

member 4 and 5B (Wnt4 and Wnt5B)

proliferating cell nuclear antigen (PCNA)

bone morphogenetic protein–2 (BMP–2)

bone callus volume (BV)

Toll like receptors (TLRs)

Optical density (OD)

Declarations

Ethics approval and consent to participate: The ethics committee of Qilu Hospitial of ShanDong University approved the study.

Consent for publication: Not applicable.

Availability of data and material: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: CJZ, TY and QJD designed and analyzed the experiment, and was a major contributor in writing the manuscript. YG, XFY and YZC performed the experiment. All authors read and approved the final manuscript.

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Figures

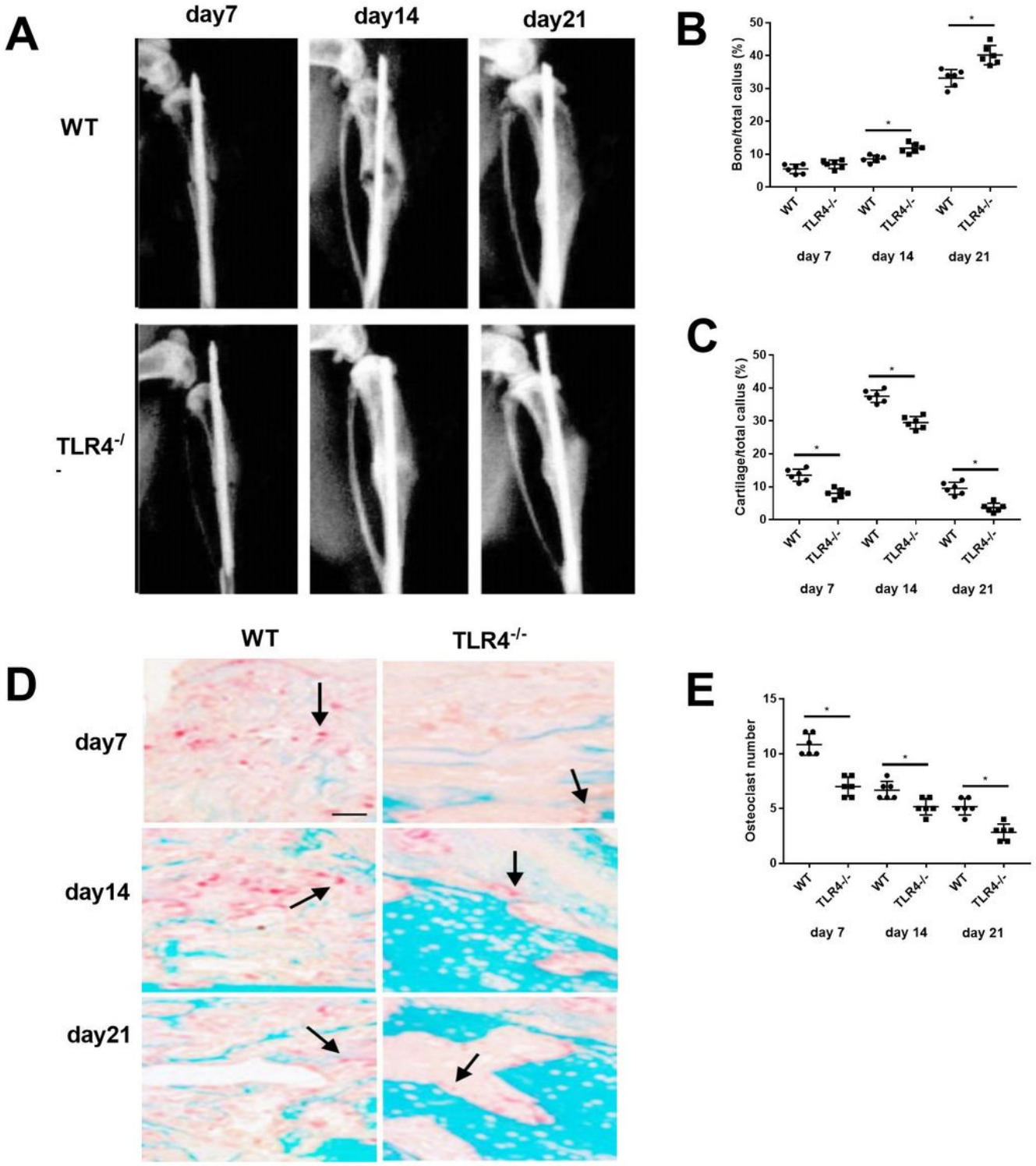

Figure 1

Analysis of X ray and staining. A, X ray results of fracture healing in mice; the arrow showed the fracture of tibia; the experiment was repeated 6 times. B, bone tissue area/total callus area. C, fracture healing process in WT and TLR4-/- mice with cartilage tissue area/total callus area. D, TRAP staining on the longitudinal section of callus tissue; scale bars: 25μm. E, the number of osteoclasts. N=6.

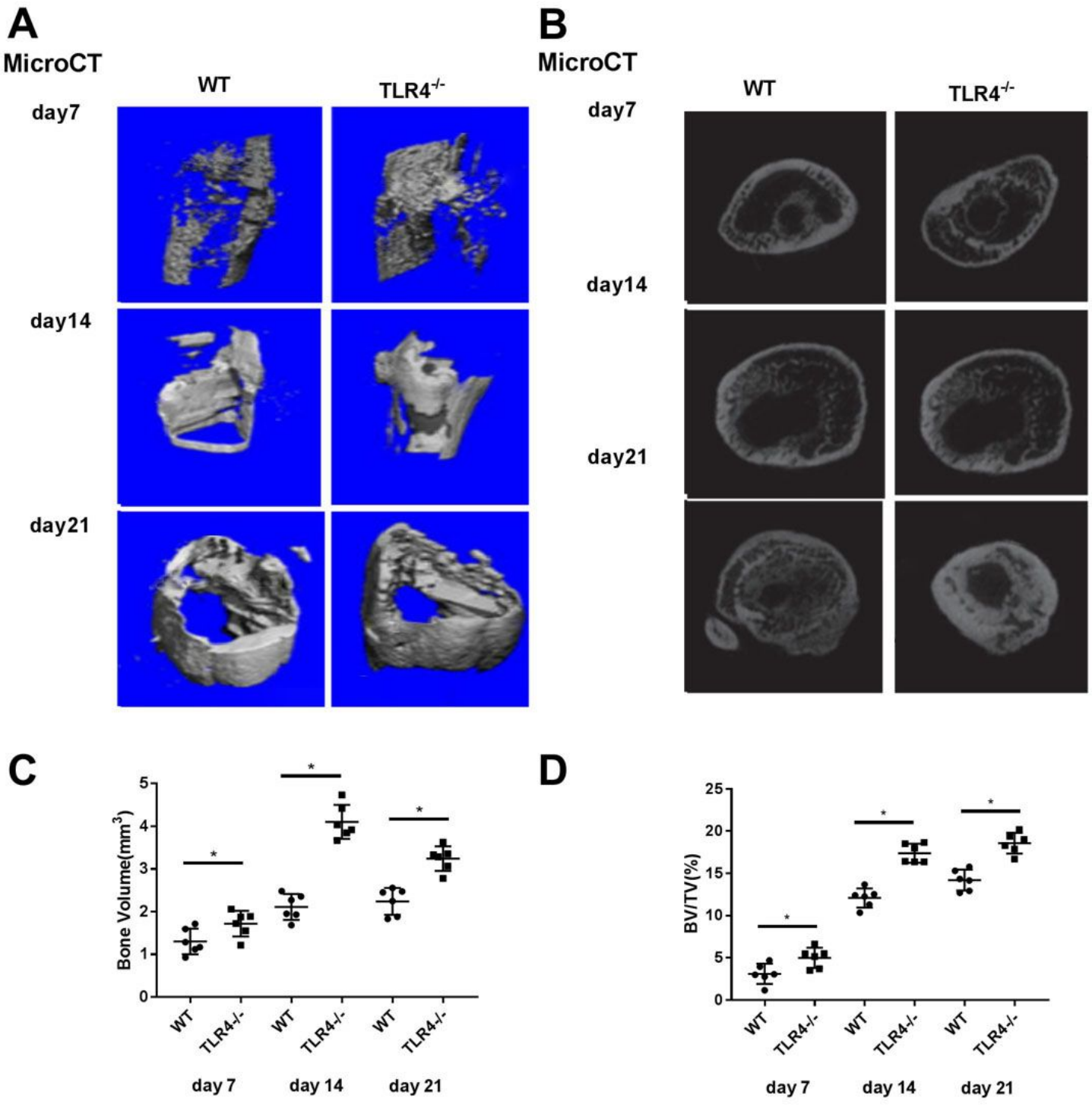

Figure 2

Quantitative analysis of Micro-CT software. A, micro-CT scanning of fracture callus graphics to reconstruct 3D Images. B, micro-CT scanning of fracture callus graphics to reconstruct 2D Images. C, volume analysis of osseous callus. D, results of volume percentage analysis of bone callus. When compared with WT mice, *P < 0.05. All data were expressed as mean ± standard deviation. N=6.

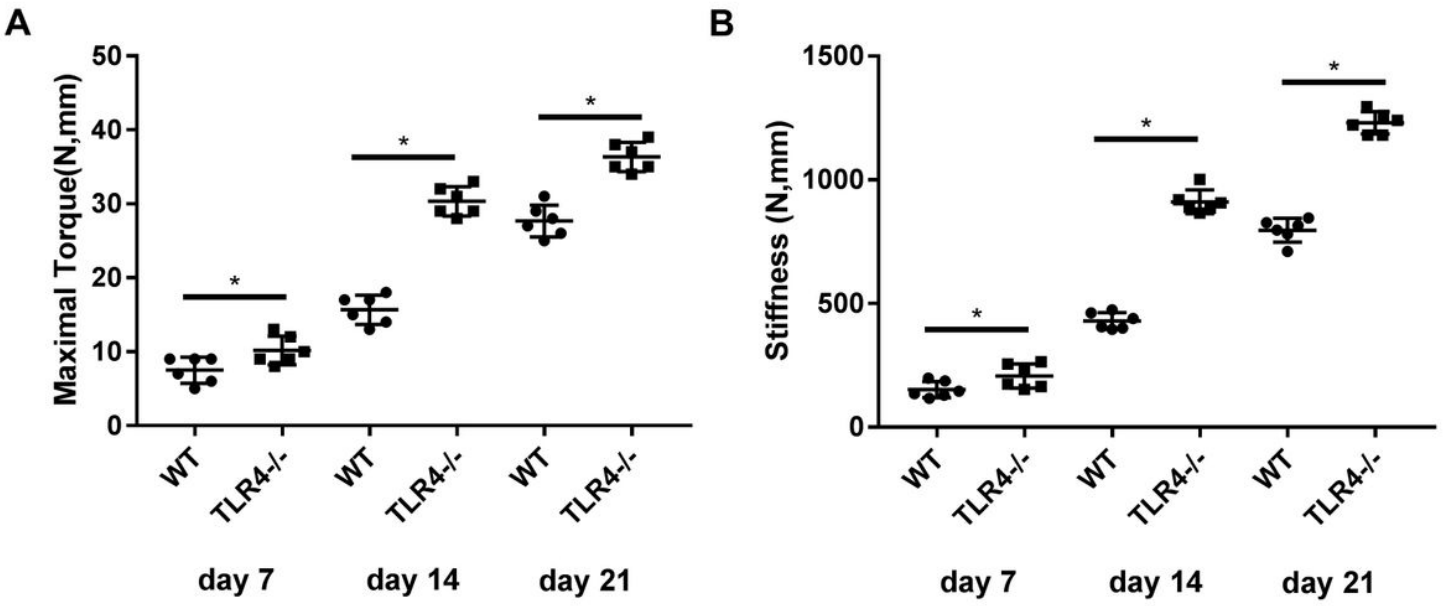

Figure 3

The biomechanical analysis of tibia in mice. A, maximum torque analysis of callus tissue. B, maximum torsion stiffness analysis of callus tissue. All data were expressed as mean ± standard deviation. Compared with WT mice, *P < 0.05. N=6.

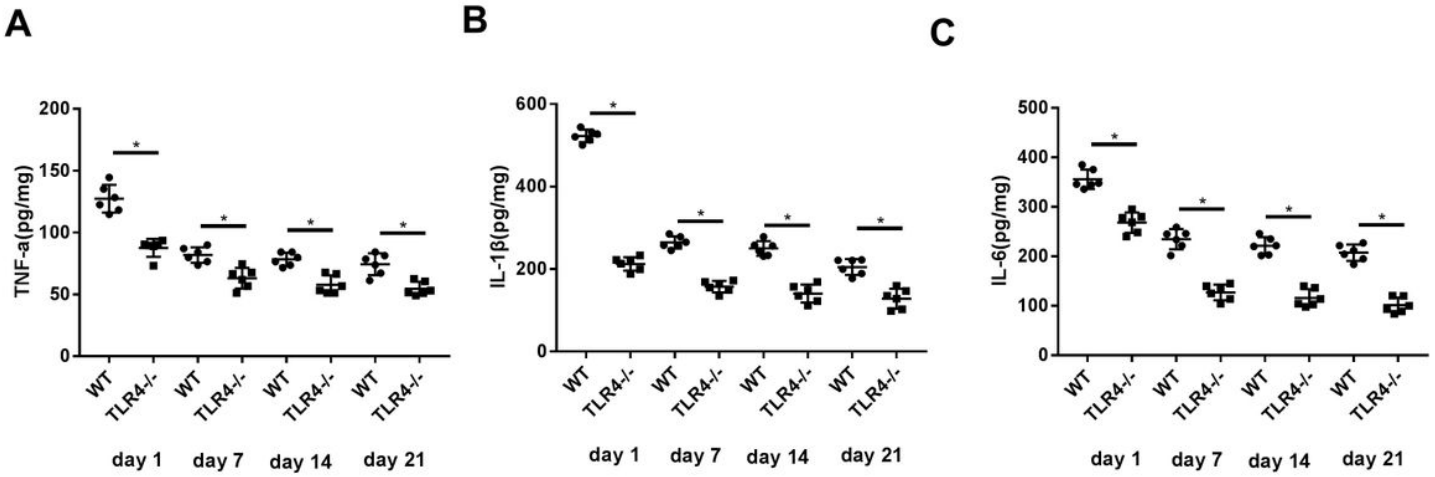

Figure 4

The expression of inflammatory factors in callus of WT and TLR4-/- mice. A, changes of TNF-a level in callus of WT and TLR4-/-mice. B, changes of IL-1β level in callus of WT and TLR4-/-mice. C, changes of IL-6 level in callus of WT and TLR4-/-mice. When compared with WT mice, *P < 0.01. All data were expressed as mean ± standard deviation. N=6.

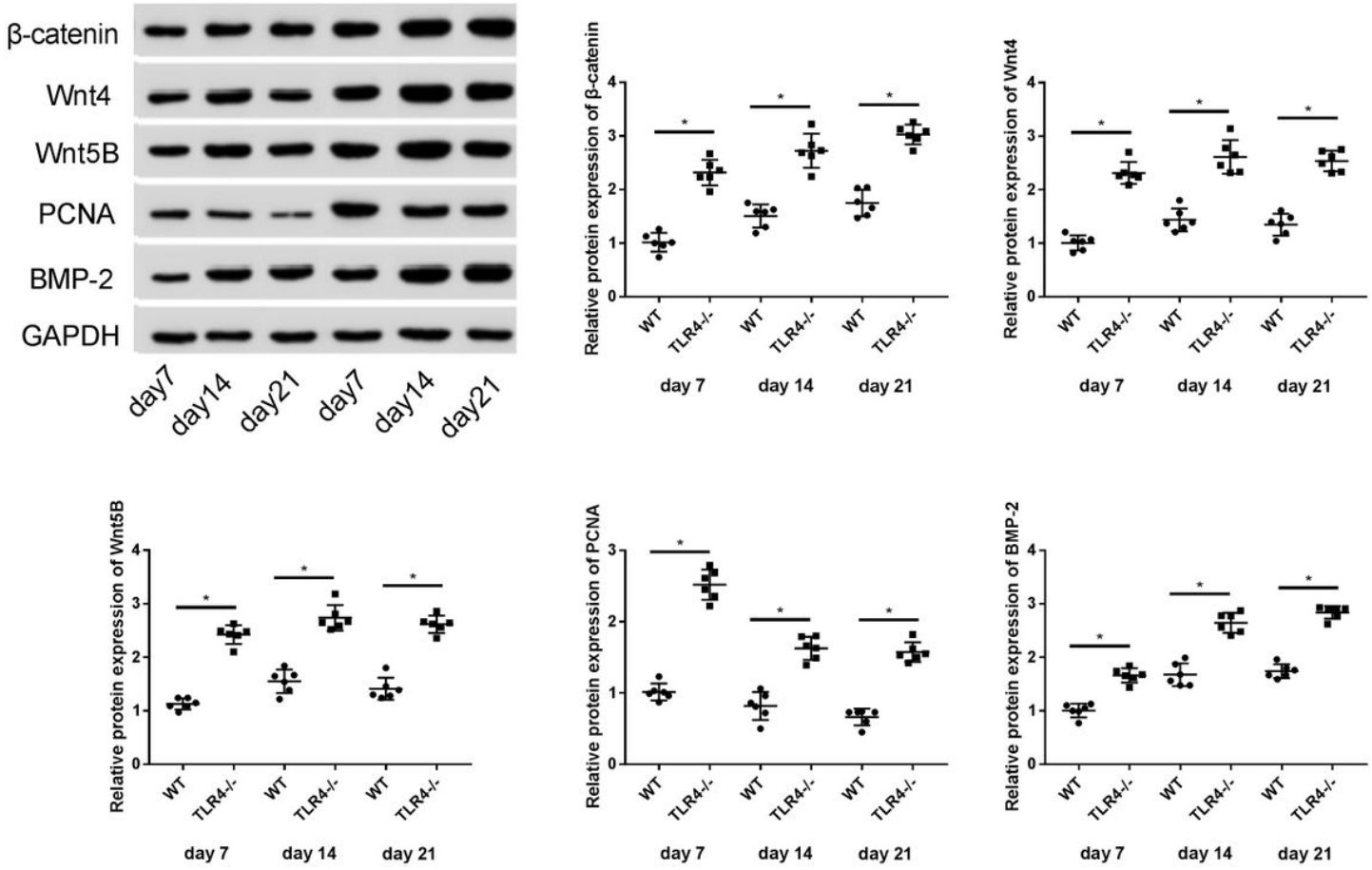

Figure 5

The expression levels of β-catenin, Wnt4, Wnt5B, PCNA and BMP-2. When compared with WT mice, *P < 0.01. All data were expressed as mean ± standard deviation. N=6.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEchecklist.docx