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Dendritic cells aggregate inflammation in experimental osteoarthritis through a toll-like receptor (TLR)-dependent machinery response to challenges

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ABSTRACT

Aims: Dendritic cells (DCs) and Toll-like receptor (TLR) participate in mediating inflammation process. However, the functional role of TLR expressed on DCs in osteoarthritis (OA) development has not been defined yet. The purpose of this study was to investigate the role and mechanism of TLR and DCs in the progression of experimental osteoarthritis (OA).

Materials and methods: Experimental OA model was induced by iodoacetate injection. Expressions of toll-like receptors in DCs of OA mice were detected by qRT-PCR and flow cytometry. TLR agonists lipopolysaccharide (LPS) and R848 or TLR antagonist FP7 were used, and the levels of TLRs and inflammatory cytokines were examined by qRT-PCR and ELISA.

Key findings: The expression levels of TLR family members were increased in DCs derived from synovial fluid of OA mice compared with the sham mice. In vitro, OA mice-derived DCs had increased production of inflammatory cytokine after TLR agonists LPS and R848 challenge, while TLR challenges did not affect DCs maturation. Inhibition of TLR by TLR antagonist FP7 blocked TLR challenges-induced increased inflammation in DCs. In mice, administration of FP7 attenuated LPS-induced inflammatory response and OA condition.

Significance: Increased TLR expression in OA-derived DCs contributes to the inflammation condition and potentially acts as a therapeutic target for osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a common chronic joint disease that characterized by the irreversible destruction of articular cartilage, the activation of synovitis and osteoclasts, and mainly affects middle-aged person with the symptoms of joint pain, joint deformity, and even physical disability [1]. The chronic inflammation and cartilage degeneration are the main characters that promoted OA progression [2]. It has reported that the integrity of resident cells and tissue structure of articular cartilage are associated with inflammatory events [3,4]. A number of studies suggested that the activation of immune inflammatory cells is the cause in pathological progress of OA. The immune cells (T lymphocyte, macrophage, *etc*.) and the secreted inflammatory factors (Interleukin-1 beta (IL-1β), tumor necrosis factor α (TNF-α), IL-6, IL-8, and IL-17) are elevated in the cartilage of patients with OA [4,5]. However, the unknown regulatory mechanism of immune cell activity in infiltrating joints limits the effective control of OA generation and development as well as the development of effective therapeutic drugs.

Dendritic cells (DCs) are a class of bone-marrow-derived cells arising from lymphoid hematopoiesis, and are essential for the innate sensing of pathogens and the activation of adaptive immunity, such as secreting inflammatory mediators, cytokines, and chemokines responding to inflammatory conditions and signal, and displaying antigen

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presentation function [6]. DCs mediate various diseases, such as cancers [7], cardiovascular diseases [8] and neurodegenerative diseases [9]. In addition, immature DCs in the live, and bone marrow can transmit through the blood to vessels to the secondary lymphoid organs [10,11]. In the region of OA, DCs were mostly derived from blood inflammatory monocytes and presented a pro-inflammatory phenotype [12]. The proinflammatoy DCs serve as the essential regulators in immune cells, and mediate inflammatory responses and immune pathology [13]. However, the potential mechanism of DCs on OA pathogenesis is still need further identification.

One or more subtypes of members of the Toll-like receptor (TLR) family probably play a fundamental role in pathogen recognition and activation of innate immunity of DCs [14–17] and are also closely correlated with OA development [18–20]. For example, study showed that genetic polymorphisms of toll-like receptor 10 are associated with susceptibility to joint osteoarthritis in clinical OA patients [21]. Another study showed that TLR4 promotes obesity-induced OA condition [22]. But study with TLR deficient mice demonstrated the dispensable relationship between TLR-1, -2, -4 and -6 and the severity of experimental OA [23]. Taken together, it is necessary to clarify the effect of TLR on pro-inflammatory DCs function and their involvement in OA pathological progress. Our study analyzed the expression of TLR in DCs of experimental mouse OA model and highlighted the effect of TLR challenge induced by TLR agonists on inflammatory cytokine production and OA progression.

2. Materials and methods

2.1. OA mouse model establishment

C57BL/6 mice (aged 8 weeks) were obtained from Shanghai Lab. Animal Research Center (Shanghai, China) and were bred in laboratory animal feeding room. All experimental procedures involving animals were approved by the Animal Care and Use Committee of The Affiliated Lianshui County People's Hospital of Kangda College of Nanjing Medical University. Before OA induction, all 16 mice were anesthetized by 3% isoflurane and then randomly divided into 2 groups. For OA induction procedure, mice were placed to the test bench and subjected to hair remove of hind legs and the injection of 5 μl of 100 mg/mL iodoacetate through the skin incision around joint. The sham mice received the same volume of saline. After three weeks, all animals were sacrificed and mice joints were isolated for RNA extraction.

For evaluating the TLR inhibition on OA development, TLR antagonist FP7 was injected into the joint after OA induction. Mice arthritis score and weight bearing were monitored before animals sacrifice seven weeks later.

2.2. DCs isolation and analysis

DCs were isolated from synovial fluid of adult C57BL/6 mice with OA. Briefly, the DCs were prepared from synovial fluid using Ficoll separation (STEMCELL Technologies, Canada) and sorted by fluorescent activated cell sorting (FACS). Single cell suspensions were stained with biotinylated monoclonal antibodies against CD11c and TLR3 (anti-CD11c, anti-TLR3, eBioscience) for 30 min at 4 °C. Then the cells were evaluated by flow cytometry (FACSCanto BD). Data were analyzed using FlowJo (Tree Star).

2.3. In vitro treatment of DCs with TLR agonists or TLR antagonist

The DCs pools obtained from OA mice and sham-operated mice were challenged by TLR agonists. Briefly, DCs from each pool was seeded in 24-well plates in presence or absence of TLR agonists LPS (100 ng/mL) and R848 (350 nM). For TLR antagonist treatment, DCs from each pool were seeded in 24-well plates supplemented with TLR antagonist FP7 (1 μM) 30 min before the addition of LPS (10 ng/mL) or R848 (350 nM) for indicated times.

2.4. RNA extraction and qRT-PCR

Total RNA was isolated from DCs or joint using Trizol reagent and quantified by using spectrophotometer. The equal quantity of RNA was reversely transcripted into cDNA using the Real MasterMix First Strand cDNA Synthesis Kit (Tiangen) according to the manufacturer's protocols. The cDNA product together with specific primers was processed into real-time PCR with SYBR Premix ExTagTM (Takara, Shanghai, China). Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ calculations, and the expression level of GAPDH served as internal control.

2.5. ELISA assay for inflammatory cytokines detection

DCs were seeded in 24-well plates and were stimulated with TLR agonists, LPS (100 ng/mL) or R848 (350 nM), respectively. The cultural supernatant was harvested for the detection of supernatant levels of IL-6, IL-8, TNF-α and IL-10 by using ELISA kit (Beyotime, Shanghai, China) according to the manufacturer's protocols. Concentration of IL-6, IL-8, TNF-α and IL-10 in synovial fluid of OA mouse joint was detected by using the same ELISA kit.

2.6. Statistical analysis

Student's t-test or one-way analysis of variance was performed to determine significance levels between two groups or among groups using GraphPad Prism. Data are presented as mean ± SD. Differences were considered significant when $p < 0.05$.

3. Results

3.1. TLR expression is increased in dendritic cells of OA mice

To determinate the potential contribution of TLR to OA, the mRNA levels of TLR family members in DCs from synovial fluid of OA mice were analyzed. As is shown in Fig. 1a, TLR1-8 exhibited higher levels in OA group compared with sham group, while there was no significant difference in TLR9 expression between the two groups. And, the expression level of TLR3 showed the most difference between the two groups. Next, flow cytometry was used to further verify whether DCs derived from OA mice had an increased TLR3 expression. The results showed that DCs derived from OA mice had a higher proportion of cells positive for TLR3 compared with that in the sham group (Fig. 1b). The findings indicated that TLR1-8 facilitates the response of OA mice.

3.2. OA-derived DCs had increased inflammatory cytokine production in response to TLR challenges

To further determine the role of DCs in OA development, DCs derived from synovial fluid of different group mice were treated with different TLR agonists LPS, or R848 respectively. The levels of inflammatory cytokines in cell supernatant were analyzed by ELISA. It showed that the levels of IL-6 (Fig. 2a), IL-8 (Fig. 2b) and TNF-α (Fig. 2c) were significantly elevated, and the IL-10 level was significantly reduced in OA mice-derived DCs compared with that in the sham group (Fig. 2d). The data indicated OA-derived DCs exhibited increased inflammation response to TLR challenges.

3.3. TLR challenges did not change DCs maturation

The different functions of DCs depend on its own state, including immature state and mature state. To determine whether the increased inflammation after TLR challenges is contributed by aberrant DCs mature, we determined the mature markers for DCs after TLR agonists

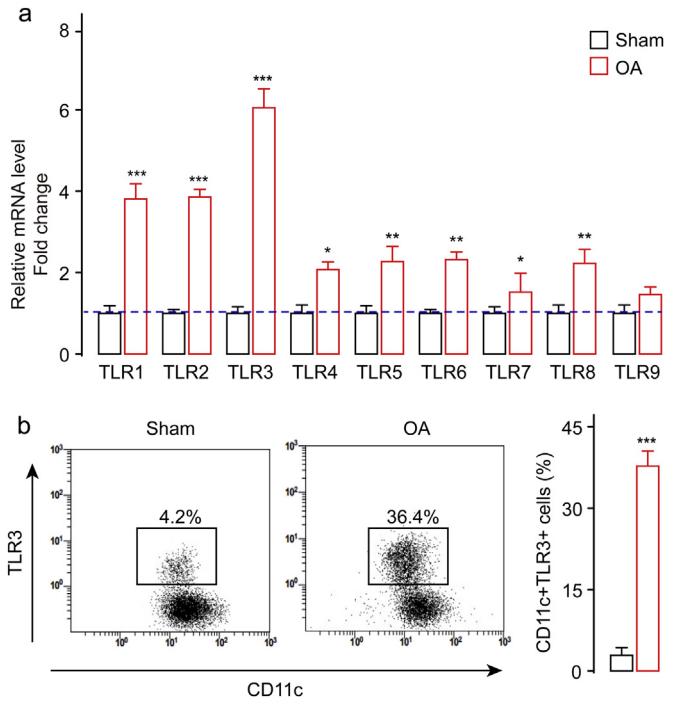

Fig. 1. TLR expression is increased in dendritic cells from OA mice. Expression Profile of TLR in DCs derived from synovial fluid of sham and OA mice. a, the mRNA expression of TLR family members in DCs were examined by qRT-PCR. b, TLR3 expression in DCs were analyzed by flow cytometry. $n = 6$, Results are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with sham group.

challenges. We found that DCs from both sham and OA mice expressed the same levels of CD80 (Fig. 3a), CD86 (Fig. 3b) and CD40 (Fig. 3c), suggesting that TLR activation has no effect on DCs mature and increased inflammation after TLR challenges is not contributed by DCs mature.

3.4. Inhibition of TLR in DCs reverses the inflammatory response to TLR challenges

In order to further demonstrate the role of TLR in the inflammatory activation of DCs, DCs were pretreated with the TLR antagonist FP7 and followed by LPS and R848 challenges. The results showed that the expression levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) were low in the absence of LPS or R848. After TLR challenges, the levels of IL-6, IL-8, TNF-α were significantly higher that was remarkably attenuated by FP7 treatment. (Fig. 4a–c). Inversely, the expression level of the anti-inflammatory cytokine IL-10 was reduced after LPS and R848 challenge that was inhibited by FP7 treatment (Fig. 4d). Taken together, the inflammatory response is attributed to the increased TLRs in DCs.

3.5. Administration of TLR antagonist FP7 reverses LPS-induced inflammatory condition in OA mice

In view of the observation that the expression levels of pro-inflammatory cytokines are inhibited and the expression level of anti-inflammatory cytokines is promoted by TLR inhibition in TLR-challenged DCs, we believed that TLRs of DCs are key molecules in mediating the inflammatory response during the development of OA. Therefore, we further observed the levels of serum inflammatory cytokines in OA mouse treated with FP7 *in vivo* experiment. The results showed that TLR inhibition by FP7 did not change the levels of serum inflammatory cytokines in the sham-operated group, but significantly reduced the elevated levels of serum pro-inflammatory factors IL-6, IL-8 and TNF-α in OA mice, and increased the reduced anti-inflammatory factor IL-10 in the OA group (Fig. 5a–d), that is consistent with the results of the *in vitro* cell experiments.

3.6. Administration of TLR antagonist FP7 attenuated LPS-induced OA development

With the development of OA, the tissue damage of joint site increased gradually and the weight-bearing function of joint was gradually damaged. To determine the functional role of TLR in the progression of OA, we examined the arthritis score and weight-bearing function after treated with TLR antagonist FP7. As shown in Fig. 6a and b, after 7 weeks of administration of FP7, the arthritis score was significantly inhibited and the ability of weight-bearing was significantly increased compared with the OA mice, suggesting that TLR inhibition significantly ameliorated the joint function of OA mice.

4. Discussion

Recently, although much progress has been achieved on OA treatment, there is still no effective method to prevent the occurrence and development of OA due to the largely unknown of underlying multiple pathological mechanisms. Therefore, to explore the potential mechanism underlying OA is essential for its clinical treatment. Our present study highlighted the role and mechanism of DCs and the related inflammatory response on the development of OA through TLR activation in this situation, and the results showed the significant impact of TLR challenges on inflammatory reaction of DCs in osteoarthritis.

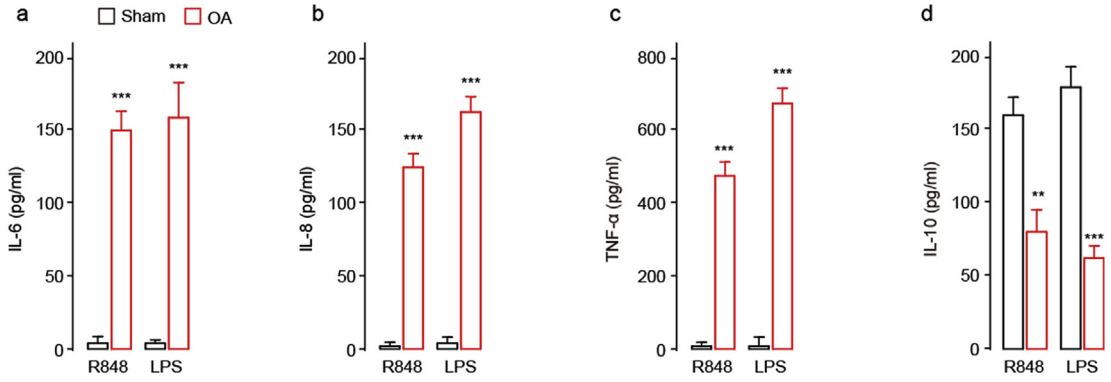

Fig. 2. OA-derived DCs had increased inflammatory cytokine production in response to TLR challenges. DCs were challenged with TLR agonists, LPS (100 ng/mL) or R848 (350 nM) *in vitro*, and cell supernatant levels of IL-6 (a), IL-8 (b), TNF-α(c) and IL-10 (d) were analyzed by ELISA assay. Results are mean ± SD of triplicate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with sham group.

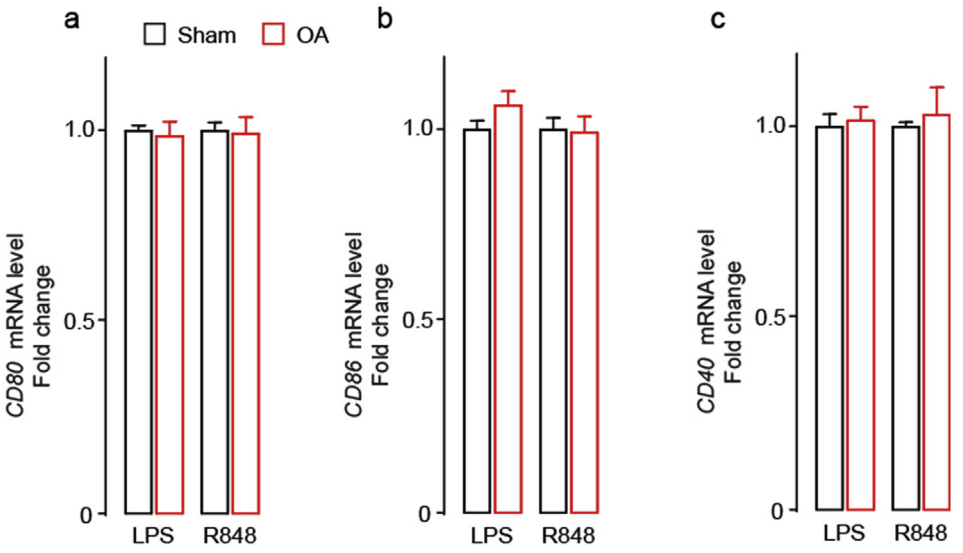

Fig. 3. Determination of the expression of mature DCs biomarkers response to TLR challenges. After incubation with LPS (100 ng/mL) or R848 (350 nM), DCs were harvested for the detection of the mRNA expression of CD80 (a), CD86 (b) and CD40 (c) were analyzed by qRT-PCR. Results are mean ± SD of triplicate experiments.

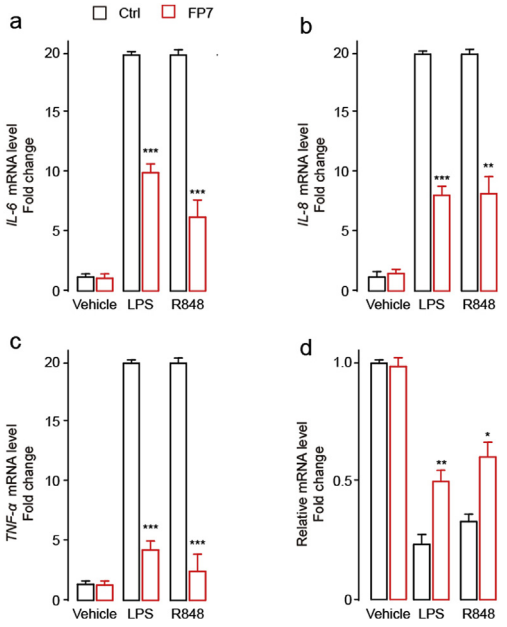

Fig. 4. Inhibition of TLR in DCs reverse inflammatory response to TLR challenges. Sham or OA mice-derived DCs were pretreated with FP7 and followed by subjecting to TLR challenges with LPS and R848. mRNA levels of IL-6 (a), IL-8 (b), TNF-α (c) and IL-10 (d) in DCs were analyzed by qRT-PCR (a, b, c, d). Results are mean ± SD of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group.

Accumulated evidences pointed out the critical role of subtype of TLR in OA pathological progress as well as in inflammatory reaction of DCs [15,16,19,20]. We thus attempted to identify the possible involvement of TLR subtype in joint region of experimental mouse OA. Interestingly, mRNA levels of TLR-1-8 were significantly upregulated to different degrees except TLR9, suggesting TLR-1-8 might be related to OA pathogenesis. Moreover, we further verified the significant higher level of TLR 3 in isolated DCs from experimental OA mouse. These results point to the possible role of TLR 3 in DCs functions and are consistent with previous studies where suggested the important of TLR 3 for OA [24] and for DCs immune activation [25–27].

DCs are the bridge between innate and acquired immunity. The antigen presenting cells of DCs could express recognition receptors, and release inflammatory mediators to regulate immunity responses. The infiltrating DCs are closely associated with the inflammation of the OA pathogenesis [4,28,29], and the members of TLR family are critical for activation of innate immunity of DCs [30,31]. In view of the abnormal high expression of TLR in OA, we challenged OA mice-derived DCs with the TLR agonists to observe the effect on the inflammatory activation by observing the secretion of pro-inflammatory cytokines in DCs. The corresponding experimental results showed that the production of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) in DCs of OA mouse were significantly higher than that in sham mice, while the anti-inflammatory cytokine (IL-10) showed an opposite trend. These data suggested that the activation of membrane TLR is a prerequisite for the inflammatory activity of DCs in OA. Moreover, such activation has no effect on DCs maturity. Commonly, DCs are classified as immature DCs and mature DCs. As immature cells, they express a variety of receptors, including TLR, as well as inflammatory cytokines, while mature DCs act as antigen presenting cells with the help of co-stimulatory molecules (e.g., CD40, CD86) [32]. To further verified the critical role of TLR activation in DCs inflammatory reaction, we next performed experiment of DCs challenged by TLR agonists and treated with TLR antagonist FP7. We found that the inhibition of TLR by FP7 to a great extent abolished TLR agonists-induced pro-inflammatory cytokines production and reduced the levels of anti-inflammatory cytokines, and this phenomenon also occurred in the OA mouse model. Meanwhile, our data showed that the administration of TLR antagonist FP7 attenuated LPS-induced OA development.

In summary, our data defined the role of DCs in mediating OA development, and found that increased TLR expressions in DCs contribute to aggressive inflammatory response in OA. It is proved that TLR hyperactivation in DCs is involved in the pathological development of OA by aggravating inflammatory response that could act as the potential therapeutic target for OA alleviation.

Authors' contribution

CX, QL and FN designed the theme and written the manuscript. FD helped to draft the manuscript and conducted several experiments. FN, BC, and SH performed major experiments. CX and QL analyzed the data and contributed to the quality control. All authors approved this submission.

Declaration of competing interest

There is no conflict of interest to declare.

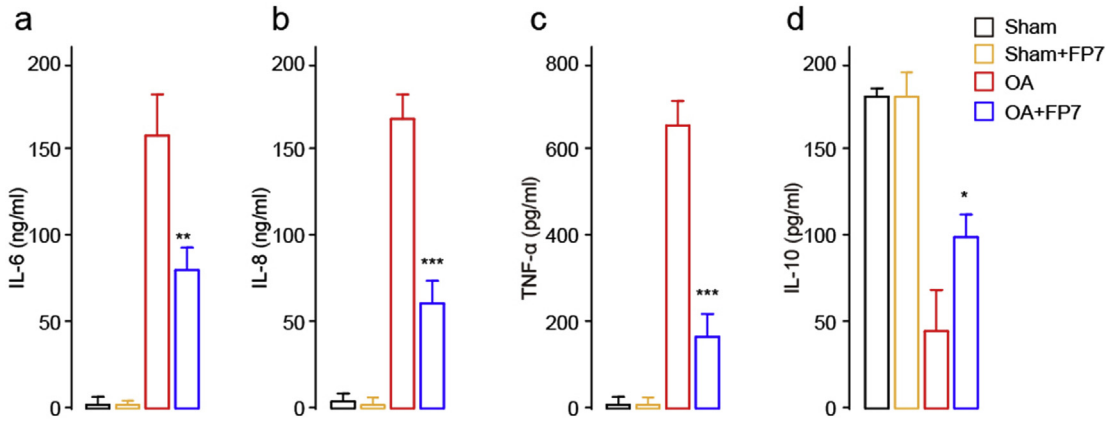

Fig. 5. Effect of TLR antagonist FP7 on LPS-induced inflammatory cytokines production in OA mice. FP7 was administrated to sham and OA mice. Serum levels of inflammatory cytokines IL-6 (a), IL-8 (b), TNF-α (c) and IL-10 (d) were detected by ELISA assay. $n = 6$, Results are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with OA group.

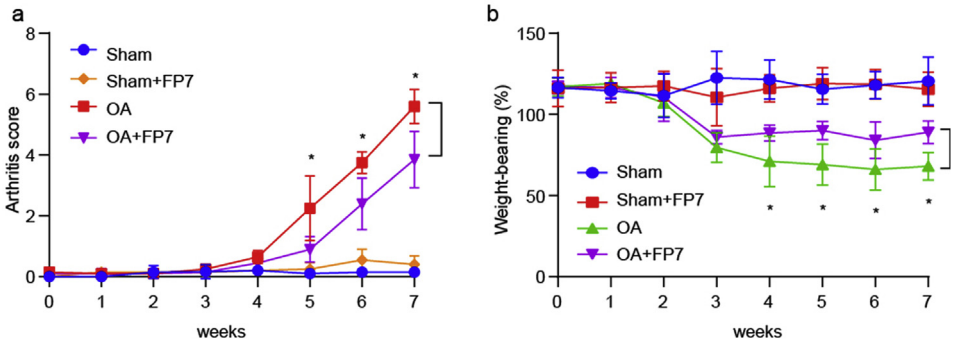

Fig. 6. Effect of TLR antagonist FP7 on the OA development. After administration of FP7, the arthritis score (a) and weight-bearing (b) of each mouse were evaluated at the indicated time. Sham mice and OA mice treated with saline were as control. $n = 6$, Results are mean ± SD. $^{*}p < 0.05$ compared with OA group.

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