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IL6 receptor blockade preserves articular cartilage and increases bone volume following ischemic osteonecrosis in immature mice

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SUMMARY

Objective: Juvenile ischemic osteonecrosis (JIO) of the femoral head is one of the most serious hip disorders causing a permanent deformity of the femoral head in childhood. We recently reported that interleukin 6 (IL6) is predominantly increased in the hip synovial fluid of patients with JIO and that articular chondrocytes are primary source of IL6. This study investigated whether an inhibition of IL6 receptor improves cartilage preservation and bone healing in JIO.
Method: A small animal model (i.e., 6-week-old mouse) of JIO was treated with either saline or tocilizumab, an IL6 receptor blocker, for 6 weeks.
Results: TUNEL-positive chondrocytes in the articular cartilage were reduced by the tocilizumab treatment, concomitant with the increase in cartilage matrix. The levels of a cartilage anabolic marker Sox9 was significantly increased in the articular cartilage of mice treated with tocilizumab. Micro-CT assessment showed tocilizumab treatment significantly increased trabecular epiphyseal bone volume ($P = 0.001$, $n = 10$), thickness ($P = 0.007$) and number ($P = 0.014$) and decreased bone separation ($P = 0.002$) and its deformity ($P = 0.003$). A bone formation marker, BMP2, and an angiogenic marker, vascular endothelial growth factor (VEGF), were both significantly increased by tocilizumab treatment under hypoxia using human chondrocytes while the bone resorption marker, RANKL/OPG ratio, was reduced.
Conclusion: Tocilizumab treatment following ischemic osteonecrosis has cartilage anabolic effect and increases bone volume in JIO mouse model. The findings lead to a possible application of tocilizumab for preclinical study using a large animal model of JIO and a clinical trial to validate this treatment.

Introduction

Ischemic osteonecrosis of the femoral head is one of the most serious conditions affecting pediatric and adult hips. Legg-Calvé-Perthes disease (LCPD), one form of juvenile ischemic osteonecrosis (JIO), is one of the most common pediatric hip disorders causing permanent deformity of the femoral head and leading to debilitating osteoarthritis in 50% of patients[1,2]. Even though the disease was described over 100 years ago, little advancement has been made in terms of developing an effective treatment to prevent the femoral head deformity. In fact, current treatments, including femoral and pelvic osteotomies, do not reliably prevent the femoral head deformity. In order to improve treatment, it is important to understand the pathophysiology and the molecular mechanism of JIO and to develop a mechanism-based therapeutic strategy.

In LCPD, chronic synovitis of the affected hip is common[3]. Our recent study using serial MRI with gadolinium enhancement showed a significant increase of synovitis and synovial effusion in LCPD[4]. Intriguingly, interleukin 6 (IL6) was significantly elevated in the synovial fluids from affected side (500–1,000 pg/ml) compared to the control samples from non-LCPD patients (<30 pg/ml)[4]. In a large animal model of JIO (i.e., piglet), we also found increased IL6 levels in the hip synovial fluid and articular chondrocytes as a major source of IL6 production[5]. Given these observations, we hypothesize that IL6 plays an important role in the pathogenesis of JIO.

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IL6 is known to have multiple pathologic effects on human diseases. In bone metabolism, IL6 has been shown to have a negative role by promoting osteoclast activity while decreasing osteoblast activity under chronic inflammatory conditions, such as rheumatoid arthritis (RA)[6–10]. In the condition of osteonecrosis, we recently reported that IL6 deletion stimulates revascularization and new bone formation following ischemic induction in mice[11]. Therefore, it is interesting to move on the preclinical study investigating the effects of IL6 blockade on bone ischemic conditions including JIO. Tocilizumab, a neutralizing antibody for IL6 receptor, has been used to treat adult patients with RA and to treat patients 2 years of age and older with juvenile idiopathic arthritis (JIA)[12,13].

The purpose of this study was to determine the effects of IL6 receptor blockade using tocilizumab on JIO animal model. Here, we chose to use only available JIO mouse model which we recently established[14] and showed positive effects of tocilizumab on bone volume as well as cartilage anabolism following ischemic osteonecrosis.

Materials and methods

JIO mouse model

The animal protocol for this study was approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. We previously developed a JIO mouse model which is only available in mice with robust incidence of osteonecrosis (ON) at almost 100 %[14]. Using this model, we surgically induced JIO of distal femoral epiphysis on immature 6-week-old C57BL/6 male mice under anesthesia with isoflurane. Briefly, the four blood vessels supplying the right distal femoral epiphysis were identified and cauterized under a surgical dissecting microscope in the ON group. For the sham group, the vessels were identified but not cauterized in the right side. The left side of ON or sham group was designated as the contralateral normal control group, receiving no ON or sham surgery. In this study, additional *in vivo* experiments were performed at post-surgery day one without tocilizumab injection (Supplemental Table).

Administration of tocilizumab into a JIO mouse model

At post-surgery day 3, the first injection of either 100 μl saline or 100 μl tocilizumab solution (i.e., 10 μl tocilizumab plus 90 μl saline) per mouse was performed intraperitoneally into the ON or sham surgery group. The mice received a weekly injection at most six times until endpoint (i.e., at post-surgery day 3, day 10, day 17, day 24, day 31, and day 38). The dose of tocilizumab (i.e., 8 mg/kg) and its frequency were based on clinical protocol, adjusted by mouse growth, with established safety and efficacy[15]. Mice treated with saline or tocilizumab were sacrificed to collect specimens at indicated time point within post-surgery day 42 (i.e., 6 weeks after surgery). The details of the mouse number and endpoint analyzed per each experiment were summarized (Supplemental Table). The number was calculated to obtain approximately 80% power with alpha = 0.05 and effect-size Cohen's $d = 1.2$ based on clinical studies using tocilizumab[16–18]. No overt side effect was observed in the mice following these injections.

Histological analysis

Immediately after sacrifice, mouse legs were gently harvested and fixed with 10% formalin for 5 days followed by micro-CT. After micro-CT scanning, the specimens were decalcified with 10% EDTA for 5 days. Bones around knee joint (i.e., distal femur with proximal tibia) were embedded in paraffin and sectioned at 4 μm thickness. Hematoxylin and eosin (H&E) staining as well as Safranin O staining were performed following a standard protocol at post-surgery day 42 (Supplemental Table).

Terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) staining was performed on paraffin sections from post surgery day 1 using *In Sit0075* Cell Death Detection Kit POD (Roche Applied Science) (Supplemental Figure 1, ON; $n = 6$, sham; $n = 5$) and day 6 (Supplemental Table), as previously reported[14].

The rat polyclonal primary antibody against IL6 (1:500, ab6672, Abcam, Cambridge, MA, USA) was used to detect IL6 protein at post-surgery day 1. The primary antibody for Sox9 (1:1,000, ab71762, Abcam) was used on post-surgery day 6 sections. After applying secondary antibody, the antibody binding was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) before brief counterstaining with hematoxylin (Invitrogen).

Micro-CT analysis of distal femoral epiphysis

Within the ON groups (i.e., saline-treated ON; $n = 11$, tocilizumab-treated ON; $n = 10$), specimens of distal femoral epiphyses were scanned using SKYSCAN 1,172 micro-CT system (Skyscan, Aartselaar, Belgium). Trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) were obtained. All coronal images from each femoral epiphysis were analyzed and the mean ± standard deviation (SD) value for each trabecular parameter was obtained.

The shape of the distal femoral epiphysis was assessed for deformity, and the degree of epiphyseal collapse was evaluated by measuring the height and width of the epiphysis in a medial condyle–coronal plane on micro-CT images. A ratio of the height and width was calculated for each epiphysis in a blinded manner to determine the bone deformity. A lower ratio of height/width would indicate a greater deformity.

Cell culture and tocilizumab addition

Primary chondrocytes were isolated from human articular cartilage of ankle joint obtained from a surgical discard (i.e., 21-month-old) at the time of surgery (IRB protocol Study ID: STU012011-114) as previously reported[19]. In short, the cartilage was digested with collagenase type I (1 mg/ml, Sigma–Aldrich, Saint Louis, MO, USA) and dispase II (2 mg/ml, Roche Applied Science, Indianapolis, IN, USA) in an alpha-minimum essential medium (α-MEM: Invitrogen, Carlsbad, CA, USA) over night. Cells were centrifuged and resuspended with fresh α-MEM containing 20% fetal bovine serum (FBS) at 37°C, 5% CO2. Cells were expanded in α-MEM containing 10% FBS and defined as P1 (passage 1). P2 to P4 cells were used for all experiments.

Cells were pretreated with tocilizumab at either 1 μg/ml or 10 μg/ml concentration for 1 h, followed by interleukin-1β (recombinant human IL1-β; 12.5 ng/ml, R&D systems, Minneapolis, MN, USA) treatment to induce IL6 expression[20] under normoxic (20% oxygen) or hypoxic (1% oxygen) culture condition for 16 h.

RNA isolation and real-time RT-PCR

For in vitro experiments, the RNA was isolated from the cells mentioned above using RNA isolation kit (Qiagen, Germantown, MD, USA). For *in vivo* experiments, the total RNA was isolated from the articular cartilage of distal femur and tibia using a TRIsol Reagent (Invitrogen) at post-surgery day one and day 14 (Supplemental Table). cDNA was synthesized from the RNA using the SuperScript Preamplification System (Invitrogen). PCR reactions, data quantification, and analysis were performed

following TaqMan gene expression assays (ABI PRISM 7,500, Applied Biosystems, Rotkreuz, Switzerland). Predesigned primers for Sox9, bone morphogenetic protein 2 (BMP2), vascular endothelial growth factor (VEGF), receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) were used. The target quantity was normalized to an endogenous HSP90 expression. All measurements were performed in triplicate using the $2^{-\Delta\Delta C(t)}$ method[21].

Statistical analysis

SPSS software (version 25) program was used for the statistical analysis. Continuous variables were first assessed for normality. For data with normal distribution and equal variance assessed by the Shapiro–Wilk test ($P > 0.05$) and Levene homogeneity variance test ($P > 0.05$), Student t test was used for comparison of two groups and the analysis of variance (ANOVA) was used for comparison of more than two groups. When one-way ANOVA showed a significant difference, post-hoc Tukey honestly significant difference (HSD) test was used for further analysis. When a factorial structure was intended, the analysis of two-way ANOVA was performed. The mean differences between two groups were expressed with 95% confidence intervals (CIs). For data with non-normal distribution, nonparametric tests were used. Mann–Whitney U test was used for comparison of two groups. Kruskal–Wallis test, followed by Whitney U test, was used for comparison of more than two groups. Data are expressed as mean ± SD. A P-value of <0.05 was considered statistically significant.

Results

Elevation of IL6 in the epiphyseal articular cartilage in JIO mouse model

We previously showed almost 100% incidence of severe osteonecrosis of the distal femoral epiphysis using TUNEL staining in the JIO mouse model[14]. After 24 h of ischemic induction, TUNEL-positive staining was increased in the epiphyses of the distal femur and the proximal tibia in the ON-right group compared to ON-left groups (Supplemental Figure 1). The staining was not increased in the sham-right group compared to the sham-left group.

Under the condition above, IL6 protein was the most abundantly expressed in the epiphyseal articular chondrocytes of the ON-right group [Fig. 1(A)], consistent with significantly elevated IL6 RNA expression level (ON-right; 93.6 ± 116.4 [95% CI: −91.5–278.8] vs sham-right; 5.5 ± 4.9 [95% CI: −2.3-13.3], $p = 0.021$) [Fig. 1(B)].

Effects of tocilizumab on cartilage anabolism and bone remodeling in JIO mouse model

The early effects of tocilizumab on articular cartilage following ischemic induction were investigated by TUNEL staining at post-surgery day 6. In the ON groups, TUNEL-positive cells were significantly decreased in the tocilizumab-treated ON group (40.2 ± 9.5 [95% CI: 16.6–63.9]) compared to the saline-treated ON group (62.9 ± 5.2 [95% CI: 50.0–75.8]) with a mean

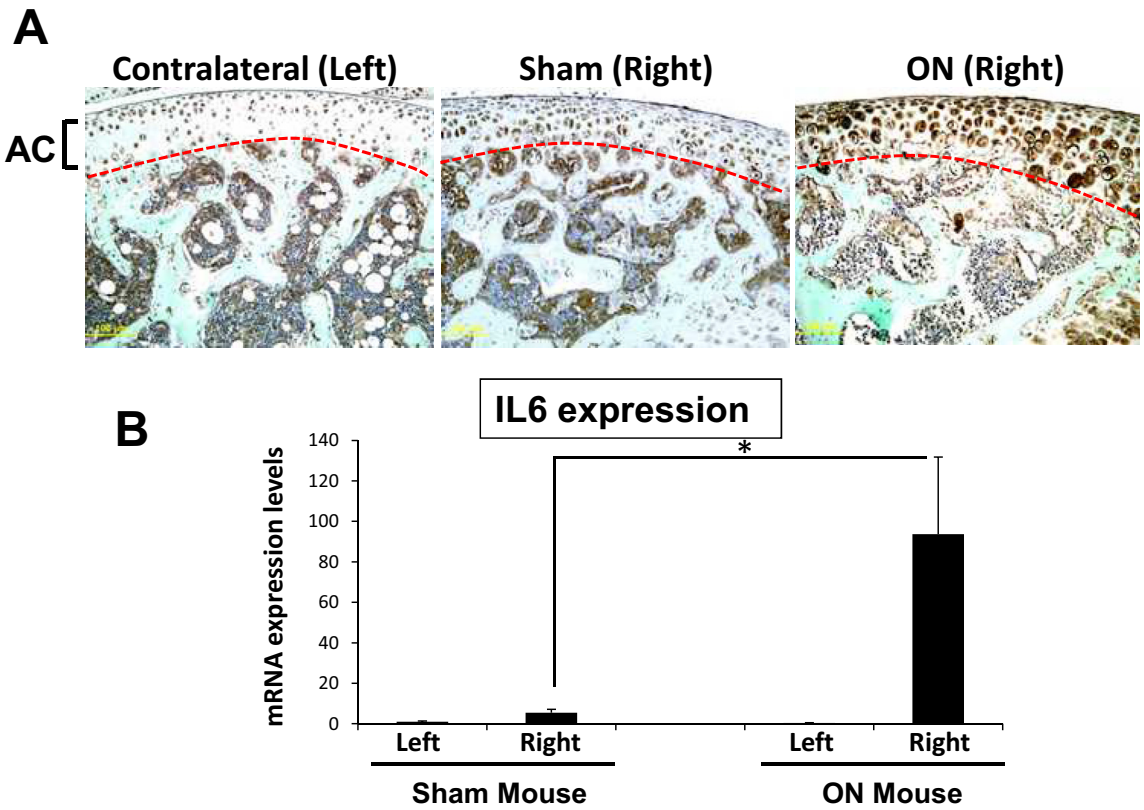

Fig. 1. Increase of IL6 following ischemic induction in JIO mouse model (A) Articular chondrocytes were strongly stained with anti-IL6 antibody after 24 h of osteonecrosis surgery (i.e., ON-right) compared to sham surgery (i.e., sham-right). The data are representative of four mice (ON groups; $n = 4$ mice, sham groups; $n = 4$ mice). Left side is a contralateral control. AC; articular chondrocyte, Bars; 100 μm (B) After 24 h of operation, IL6 expression level obtained from articular chondrocytes in the ON-right group (93.6 ± 116.4 [95% CI: −91.5–278.8%]) was over 15 times higher than that in the sham-right group (5.5 ± 4.9 [95% CI: −2.3–13.3%]). There was a significant difference among four groups as assessed by Kruskal–Wallis test ($n = 4$ mice per each group, $p = 0.008$). The value of contralateral sham-left group was set as 1.0. *$P < 0.05$ by Mann–Whitney U test between the sham-right and ON right groups ($P = 0.021$).

difference of 22.7 (95% CI for mean: 3.2–42.2, $p = 0.024$) [Fig. 2(A) and (B)]. Safranin O staining showed reduced intensity of staining in the articular cartilage of the saline-treated ON group, which was rescued in the tocilizumab-treated ON group [Fig. 2(C)].

To analyze the cartilage changes at a molecular level, we isolated RNA from the articular cartilage and measured the cartilage anabolic marker, Sox9. The Sox9 expression in the tocilizumab-treated ON group (3.6 ± 0.5) was significantly higher compared to the saline-treated ON group (1.0 ± 0.1) with a mean difference of 2.6 (95% CI: 2.0–3.2, $p = 0.0005$) [Fig. 3(A)]. Immunohistochemical analyses showed consistent evidence that Sox9 protein was increased in the nuclei of the articular chondrocytes of the tocilizumab-treated ON group compared to the saline-treated ON group [Fig. 3(B)].

We hypothesized that tocilizumab upregulates Sox9 expression on chondrocytes following IL6 elevation under hypoxic condition. To test this hypothesis, we next cultured human chondrocytes and pretreated them with tocilizumab for 1 h (i.e., 1 μg/ml, 10 μg/ml) followed by normoxic or hypoxic (i.e., 1% oxygen) culture condition for 16 h with IL1-β, as an IL6 stimulator[20]. RT-PCR analyses revealed that Sox9 expression was significantly increased by the addition of tocilizumab under hypoxic condition *in vitro* (IL1; 1.47 ± 0.1 [95% CI: 1.32–1.62] vs IL1+Toci 1 μg/ml; 2.82 ± 0.8 [95% CI: 2.63–3.01], $p < 0.01$) (IL1 vs IL1+Toci 10 μg/ml; 3.81 ± 0.1 [95% CI: 3.66–3.95], $p < 0.01$) [Fig. 3(C)].

Similarly, expression levels of BMP2, VEGF, RANKL, and OPG in the tocilizumab-treated conditions (i.e., IL1+Toci 1 μg/ml, IL1+Toci 10 μg/ml) were significantly increased compared to non-tocilizumab-treated condition (i.e., IL1 alone) under hypoxia [Fig. 4(A)–(D)]. The values from the RANKL to OPG ratio in the tocilizumab-treated conditions were significantly lower compared to non-tocilizumab-treated condition [Fig. 4(E)]. These results suggest that tocilizumab treatment under hypoxic condition can affect expression of molecules related to bone formation and resorption.

IL6 receptor blockade using tocilizumab increases epiphyseal bone volume in JIO mouse model

The micro-CT analysis of distal femoral epiphysis at post-surgery day 42 was performed with reconstructed-images [Fig. 5(A)] and morphometric measurements [Fig. 5(B)–(E)]. For BV/TV, the mean value of the tocilizumab group was significantly higher than that of the saline group (mean difference 5.1 [95% CI for mean: 2.4–7.9], $p = 0.001$, $d = 1.15$, eta squared = 0.26). Within the ON groups, there was a significant increase of BV/TV in the tocilizumab-treated ON group compared to the saline-treated ON group (mean difference 7.3 [95% CI for mean: 3.4–11.2], $p = 0.001$) [Fig. 5(B)]. For trabecular bone thickness (Tb.Th), the mean of tocilizumab group was significantly higher than that of saline group (mean difference 2.7 [95% CI for mean: 1.1–4.3], $p = 0.001$, $d = 1.08$, eta squared = 0.23).

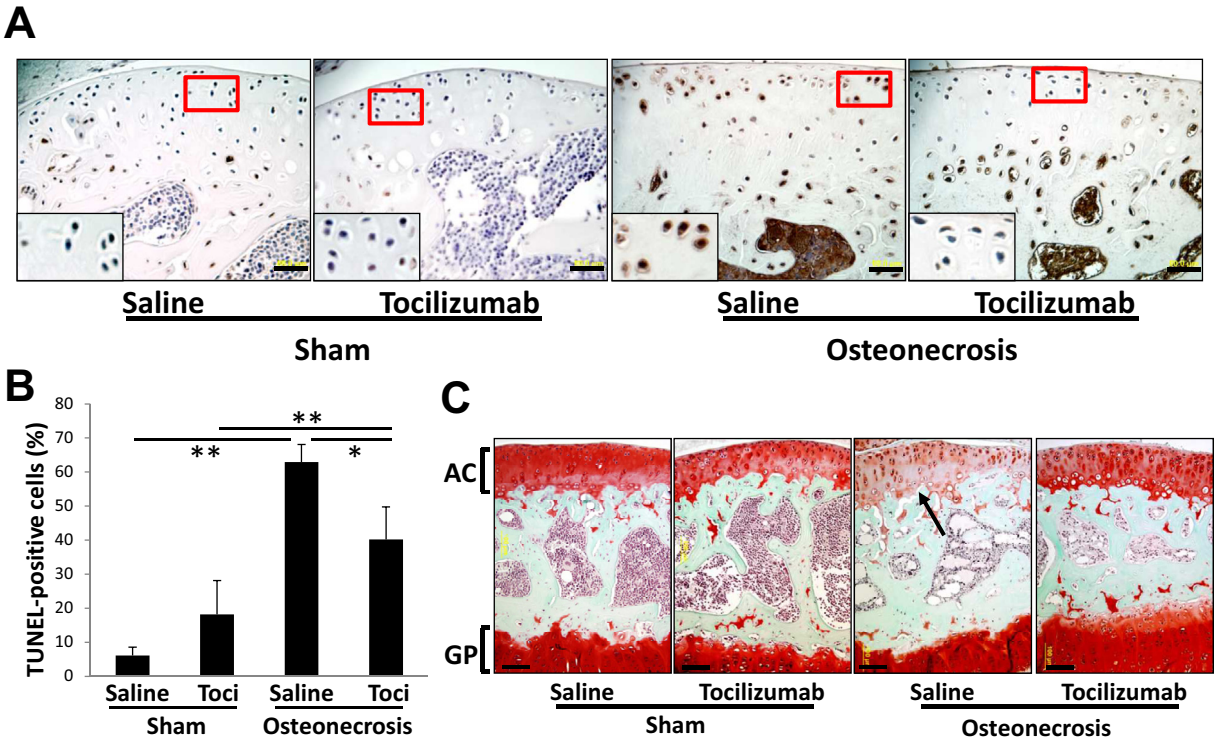

Fig. 2. Effects of tocilizumab on the articular cartilage following ischemic osteonecrosis (A) Three days after initiating tocilizumab or saline treatment (i.e., at post-surgery day 6), TUNEL staining (brown) was performed on the articular cartilage. Red boxes were magnified. The data are representative of three mice ($n = 3$ mice per each group). Bars; 50 μm (B) The number of TUNEL-positive cells was quantified in the articular cartilage. Among four independent groups ($n = 3$ mice per group), there was a significant difference by one-way ANOVA ($P = 0.00007$). Tukey's HSD test revealed a significant reduction in the tocilizumab-treated ON group (40.2 ± 9.5 [95% CI: 16.6–63.9]) compared to the saline-treated ON group (62.9 ± 5.2 [95% CI: 50.0–75.8], mean difference 22.7 [95% CI for mean: 3.2–42.2], $p = 0.024$). The saline-treated ON group was significantly higher than the saline-treated sham group (6.1 ± 2.4 [95% CI: 0.1–12.2], mean difference 56.8 [95% CI for mean: 37.3–76.3], $p = 0.00007$). The tocilizumab-treated ON group was significantly higher than the tocilizumab-treated sham group (18.2 ± 9.9 [95% CI: −6.5-42.9], mean difference 22.0 [95% CI for mean: 2.5–41.5], $p = 0.002$). $^{*}p < 0.05$, $^{**}p < 0.01$ (C) After 6 weeks of sham or ON surgery, Safranin O staining was performed on the articular cartilage. Safranin O staining level, which reflects glycosaminoglycan content of the cartilage matrix, was decreased in the saline-treated ON group (arrow) compared to the tocilizumab-treated ON group while the staining intensity of the growth plate was similar between these groups. The data are representative of three mice ($n = 3$ mice per each group). Bars; 100 μm.

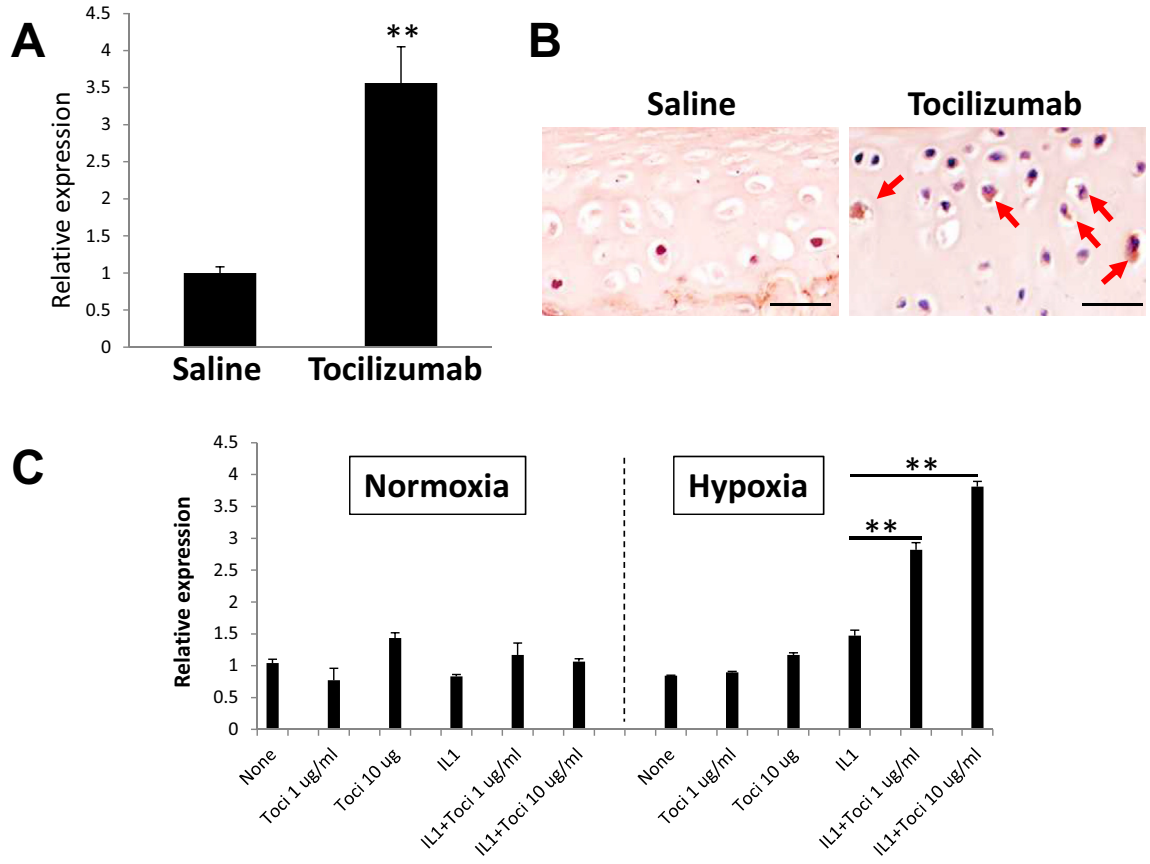

Fig. 3. Increased Sox9 expression in the articular cartilage treated with tocilizumab following ischemic osteonecrosis (A) Articular cartilage was harvested at post-surgery day 14 and RNA was extracted. The expression levels of Sox9 were significantly increased in the tocilizumab-treated ON group (3.6 ± 0.5, $n = 4$ mice) compared to the saline-treated ON group (1.0 ± 0.1, $n = 4$ mice). $^{**}p < 0.01$ by Student t test (mean difference 2.6 [95% CI: 2.0–3.2]) (B) Three days after initiating tocilizumab or saline treatment (i.e., at post-surgery day 6), immunohistochemical staining for Sox9 was performed on articular chondrocytes. The data are representative of three mice ($n = 3$ mice per group). Bars; 50 μm (C) Human chondrocytes were pretreated with tocilizumab (1 μg/ml, 10 μg/ml) for 1 h, followed by IL1-β exposure under normoxia (20% oxygen) or hypoxia (1% oxygen) for 16 h. Expression levels of Sox9 were significantly increased with tocilizumab pretreatment under hypoxic culture condition. The value of the non-treatment group (i.e., no treatment under normoxia) was set as 1.0. $^{**}p < 0.01$ by one-way ANOVA with Tukey's HSD test among IL1-β treated conditions under hypoxia (IL1 vs IL1+Toci 1 μg/ml; mean difference 1.3 [95% CI: 1.2–1.5], IL1 vs IL1+Toci 10 μg/ml; mean difference 2.3 [95% CI: 2.2–2.5], $n = 3$ independent experiments).

Within the ON groups, there was a significant increase in the tocilizumab-treated ON group compared to the saline-treated ON group (mean difference 3.2 [95% CI for mean: 0.9–5.5], $p = 0.007$) [Fig. 5(C)]. For trabecular bone separation (Tb.Sp), the mean of the tocilizumab group was significantly lower than that of the saline group (mean difference 7.4 [95% CI for mean: 2.3–12.4], $p = 0.005$, $d = 0.91$, eta squared = 0.18). Within the ON groups, there was a significant reduction in the tocilizumab-treated ON group compared to the saline-treated ON group (mean difference 11.6 [95% CI for mean: 4.5–18.7], $p = 0.002$) [Fig. 5(D)]. For trabecular bone separation (Tb.Sp), there was a significant increase in the tocilizumab-treated ON group compared to the saline-treated ON group (mean difference 0.0012 [95% CI for mean: 0.000–0.002], $p = 0.014$, $d = 0.60$, eta squared = 0.09) [Fig. 5(E)]. Consistent with micro-CT results, the trabecular bone appeared to be thicker in the tocilizumab-treated ON group compared to the saline-treated ON group on histologic assessment of H&E stained sections [Fig. 6(A)]. In addition, revascularization with new vessel formation was more evident in the tocilizumab-treated ON group compared to the saline-treated ON group (Supplemental Figure 2).

To evaluate the deformity of the epiphysis, we measured the height width ratio (Supplemental Figure 3). The mean ratio of the tocilizumab-treated groups ($n = 16$) was significantly higher than that of the saline-treated groups ($n = 22$) a mean difference of 0.042 (95% CI for mean: 0.013–0.071, $p = 0.005$, $d = 0.99$, eta squared = 0.19). Within the ON groups, the ratio was significantly higher in the tocilizumab-treated ON group ($n = 8$) compared to the saline-treated ON group ($n = 11$) with a mean difference of 0.063 (95% CI for mean: 0.023–0.104, $p = 0.003$) [Fig. 6(B)], indicating less bone deformity following the tocilizumab treatment after ischemic induction.

Discussion

Overall finding

In this study, we investigated a therapeutic strategy for JIO by specifically targeting IL6 receptor using tocilizumab. Here we report three major findings; 1) IL6 was increased in the epiphyseal articular cartilage following the induction of JIO, 2) tocilizumab has an anabolic effect on the cartilage matrix with increased Sox9 expression and reduced chondrocyte apoptosis, 3) tocilizumab has a positive effect on bone remodeling following ischemic osteonecrosis.

IL6 increase in the epiphyseal articular cartilage following the induction of JIO

We observed increased IL6 expression in the articular cartilage following the induction of JIO in mice. In the large animal model (i.e., piglet) of JIO, we demonstrated that articular chondrocytes of

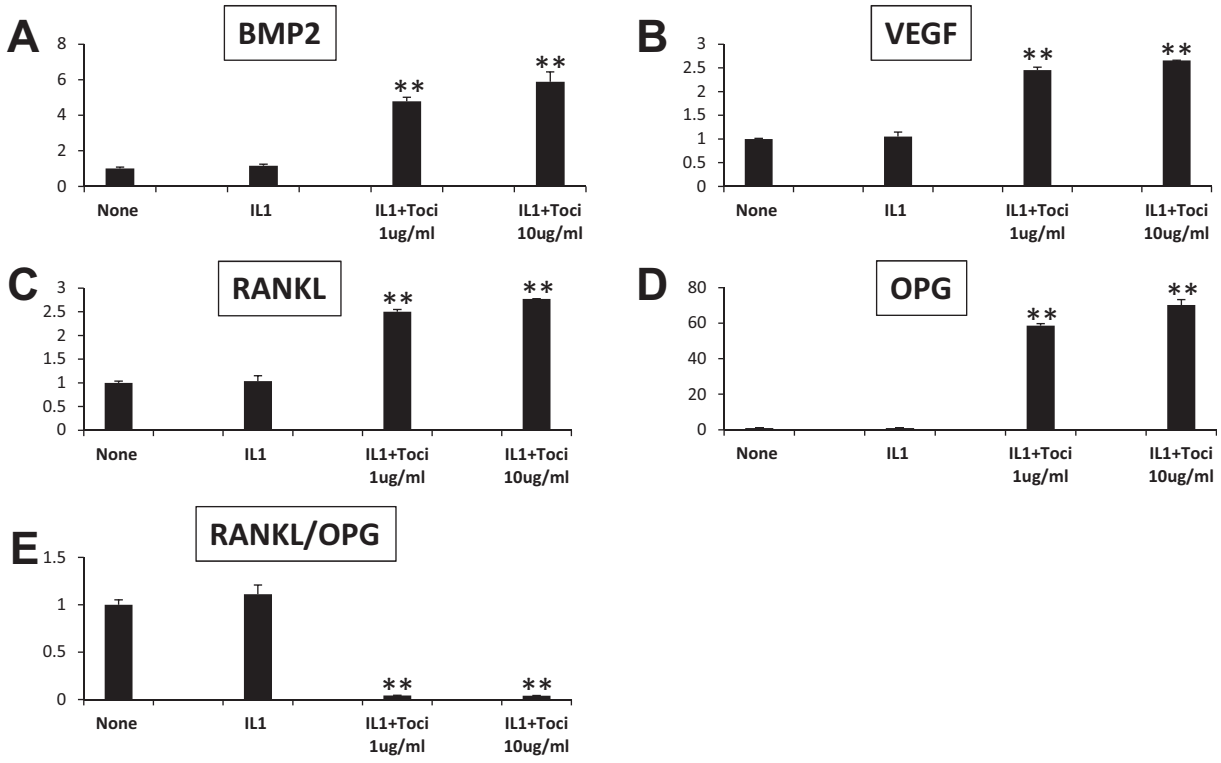

Fig. 4. Molecular changes related to bone metabolism with tocilizumab treatment (A–E) Human chondrocytes were cultured under hypoxia (1% oxygen) for 16 h. Expression levels of BMP2 (A), vascular endothelial growth factor (VEGF) (B), RANKL (C), and OPG (D) were all significantly increased with tocilizumab pretreatment while the ratio of RANKL to OPG (E) was significantly reduced. The value of non-treatment group was set as 1.0. $^{**}p < 0.01$ by one-way ANOVA among IL1-β treated conditions. Tukey's HSD test was further analyzed between IL1 vs IL1+Toci 1 μg/ml and IL1 vs IL1+Toci 10 μg/ml and mean differences with 95% CIs for mean were shown, respectively (A) 3.6 [95% CI: 3.0–4.3], 4.7 [95% CI: 4.1–5.3] (B) 1.5 [95% CI: 1.3–1.6], 1.6 [95% CI: 1.5–1.7] (C) 1.5 [95% CI: 1.3–1.6], 1.7 [95% CI: 1.6–1.9] (D) 57.7 [95% CI: 54.4–61.0], 69.3 [95% CI: 66.0–72.6], and (E) 1.1 [95% CI: 1.0–1.2], 1.1 [95% CI: 1.0–1.2], $n = 3$ independent experiments).

the femoral head were a primary source of IL6 production in a HIF1-dependent manner[5] in the early phase of the model where IL6 was increased in the articular chondrocytes following ischemic induction (data not shown). The chondrocytes in the articular cartilage remain viable in comparison to the cells in the bone marrow of the bony epiphysis which undergo extensive cell death after the induction of osteonecrosis in the epiphysis, as investigated by both mouse and piglet models[14,19,22]. It is postulated that IL6 production from viable chondrocytes in the articular cartilage elevates the IL6 level in the synovial fluid and subsequently increases IL6 secretion from the synovial cells[5].

In human, chronic hip synovitis has been reported in LCPD patients[3], and interestingly, the correlation of synovitis and thickening of the articular cartilage in LCPD patients was documented[23]. The articular cartilage became thicker when ischemic osteonecrosis was surgically introduced in a piglet model as well[24]. In addition, several human studies revealed a genetic association of LCPD in a regional population with *COL2A1*gene mutations[25–27], encoding type II collagen which is abundantly expressed by chondrocytes. MicroRNA may control to promote apoptosis in chondrocytes from LCPD patients[28]. Therefore, several lines of evidence suggest that chondrocytes play an important role in the pathogenesis of LCPD in human.

An anabolic effect of tocilizumab on the cartilage matrix with increased Sox9 expression and reduced chondrocyte apoptosis

After the ischemic induction, TUNEL-positive cells were increased in the articular cartilage where IL6 was abundantly expressed. IL6 enhances acid-induced apoptosis in rat chondrocytes[29], which may be comparable to the condition of ischemic articular cartilage in the present study. We thus postulate that chondrocyte apoptosis is enhanced by ischemia and IL6, and in turn that IL6 inhibition using tocilizumab works to reduce apoptosis. Our study showed that the degree of apoptosis was in fact significantly reduced by tocilizumab treatment. In addition, tocilizumab treatment rescued the loss of Safranin O staining, which reflects glycosaminoglycan content in the articular cartilage matrix[30]. Consistent with these findings, tocilizumab treatment has been shown to reduce neural cell apoptosis in a rat model of cerebral infarction and in oxygen-glucose deprived culture conditions[31]. Similarly, tocilizumab treatment significantly inhibited apoptosis and increased proliferation viability of human cardiac myocytes in culture conditions simulating ischemic reperfusion injury[32].

Sox9 is a transcriptional factor essential for chondrogenesis, which binds to *Col2a1*gene[33]. It is interesting that human chondrocytes respond to tocilizumab by upregulating the cartilage anabolic marker Sox9 *in vivo* and *vitro*. Intriguingly, cell apoptosis is promoted in primary chondrocytes from LCPD patients due to downregulation of Sox9 by microRNA[28]. Furthermore, cell viability and migration is promoted by upregulation of Sox9 in chondrocytes[34]. These observations lead us to postulate a new treatment strategy to improve cartilage viability following the ischemic induction by reducing apoptosis and upregulating Sox9 using tocilizumab treatment.

Positive effect of tocilizumab on bone remodeling following ischemic osteonecrosis

Osteoblasts and osteocytes in the necrotic epiphyseal bone are dead shortly after the induction of ischemia, as indicated by extensive TUNEL-positive cells in the bony epiphysis and empty

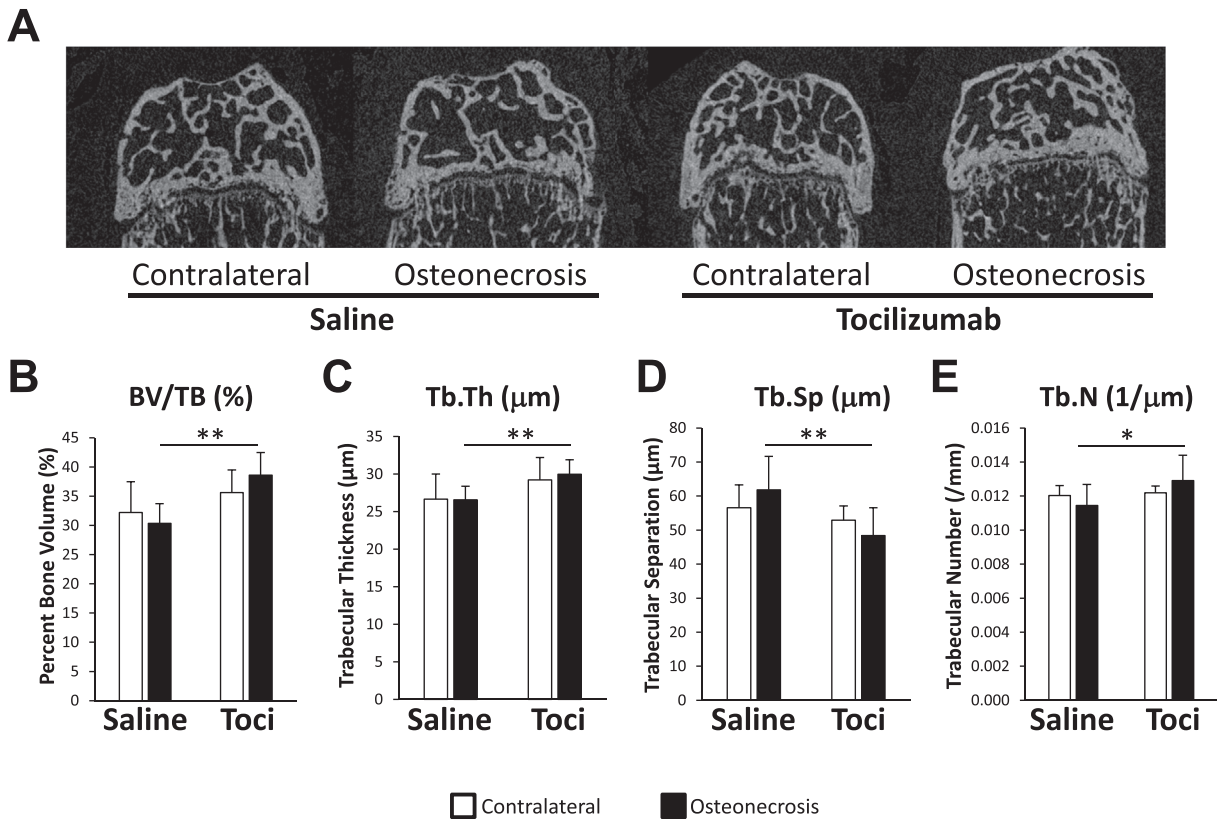

Fig. 5. Increased bone volume by tocilizumab treatment in JIO mouse model (A) Representative micro-CT images of the distal femoral epiphyses after 6 weeks of ischemic induction are shown (saline group; $n = 11$ mice, tocilizumab group; $n = 10$ mice) (B) The mean BV/TV of the tocilizumab group (37.1 ± 4.1 [95% CI: 35.1–39.1], $n = 20$) was significantly higher than that of the saline group (32.0 ± 4.8 [95% CI: 30.1–33.9], $n = 22$) with a mean difference of 5.1 (95% CI for mean: 2.4–7.9, $p = 0.001$). There was no significant difference ($P = 0.56$) between the contralateral ($n = 21$) and ON groups ($n = 21$). Within the ON groups, there was a significant difference between the saline-treated ON ($n = 11$) and tocilizumab-treated ON ($n = 10$) groups (31.3 ± 4.5 vs 38.6 ± 3.9, respectively, mean difference 7.3 [95% CI for mean: 3.4–11.2], $p = 0.001$) while within the contralateral control groups no significant difference was found between the saline-treated contralateral ($n = 11$) and tocilizumab-treated contralateral ($n = 10$) groups (32.7 ± 5.2 vs 35.6 ± 3.9, respectively, $p = 0.14$) as assessed by two-way ANOVA (**$p < 0.01$) (C) The mean trabecular thickness of the tocilizumab group (29.6 ± 2.5 [95% CI: 28.4–30.8], $n = 20$) was significantly higher than that of the saline group (26.8 ± 2.6 [95% CI: 25.7–28.0], $n = 22$) with a mean difference of 2.7 (95% CI for mean: 1.1–4.3, $p = 0.001$). There was no significant difference ($P = 0.71$) between the contralateral ($n = 21$) and the ON ($n = 21$) groups. Within the ON groups, there was a significant difference between the saline-treated ON ($n = 11$) and tocilizumab-treated ON ($n = 10$) groups (26.7 ± 1.8 vs 30.0 ± 1.9), with a mean difference of 3.2 (95% CI for mean: 0.9–5.5, $p = 0.007$) while within the contralateral groups no significant difference was found between the saline-treated contralateral ($n = 11$) and tocilizumab-treated contralateral ($n = 10$) groups (26.9 ± 3.3 vs 29.2 ± 3.0, respectively, mean difference 2.3 [95% CI for mean: −0.0-4.6], $p = 0.053$) as assessed by two-way ANOVA (**$p < 0.01$) (D) The mean trabecular separation of the tocilizumab group (50.6 ± 6.8 [95% CI: 47.0–54.2], $n = 20$) was signficantly lower than that of the saline group (58.0 ± 9.2 [95% CI: 54.5–61.5], $n = 22$) with a mean difference of 7.4 (95% CI for mean: 2.3–12.4, $p = 0.005$). There was no significant difference ($P = 0.91$) between the contralateral ($n = 21$) and the ON ($n = 21$) groups. Within the ON groups, there was a significant difference between the saline-treated ON ($n = 11$) and tocilizumab-treated ON ($n = 10$) groups (60.0 ± 11.1 vs 48.4 ± 8.2) with a mean difference of 11.6 (95% CI for mean: 4.5–18.7, $p = 0.002$) while within the contralateral groups no significant difference was found between the saline-treated contralateral ($n = 11$) and tocilizumab-treated contralateral ($n = 10$) groups (56.0 ± 6.7 vs 52.9 ± 4.2, respectively, $p = 0.39$) as assessed by two-way ANOVA (**$p < 0.01$) (E) For the trabecular number, there was no significant difference between the means of the tocilizumab (0.0126 ± 0.0011 [95% CI: 0.0121–0.0130], $n = 20$) and saline (0.0119 ± 0.0011 [95% CI: 0.0114–0.0124], $n = 22$) groups ($P = 0.055$). There was no significant difference ($P = 0.62$) between the contralateral ($n = 21$) and the ON ($n = 21$) groups. Within the ON groups, there was a significant difference between the saline-treated ON ($n = 11$) and tocilizumab-treated ON ($n = 10$) groups (0.0117 ± 0.0015 vs 0.0129 ± 0.0014) with a mean difference of 0.0012 (95% CI for mean: 0.0003–0.0022, $p = 0.014$) while within the contralateral groups no significant diffference was found between the saline-treated contralateral ($n = 11$) and tocilizumab-treated contralateral ($n = 10$) groups (0.0121 ± 0.006 vs 0.0122 ± 0.0004, respectively, $p = 0.82$) as assessed by two-way ANOVA (*$p < 0.05$).

lacunae in the trabecular bone demonstrated in various animal models of ischemic osteonecrosis[14,35]. As shown in our previous report of the JIO mouse model[14], revascularization and bone healing are initiated and progress over a 6-week time period. In this study, we found increased bone volume in the tocilizumab-treated ON group at 6 weeks post-surgery compared to the saline-treated ON group on micro-CT assessment. Histologic assessment also showed more advanced revascularization and bone marrow restoration with less fatty marrow in the tocilizumab-treated ON group compared to the saline-treated ON group. The fibrovascular tissue invasion of the marrow space was more evident in the tocilizumab-treated ON group. In terms of the epiphyseal bone deformity, the height width ratio was significantly increased in the tocilizumab-treated ON group compared to the saline-treated ON group. These findings lead us to conclude that tocilizumab treatment produces better tissue regeneration with revascularization, which is consistent with the molecular changes discussed below.

We previously reported that chondrocytes in the articular cartilage can express several molecules *in vivo* in addition to IL6[5], including bone anabolic marker BMP2[19] and vascularization marker VEGF[36], by responding to a hypoxic change following the ischemic induction. In the global deletion IL6 knockout mice, VEGF expression and revascularization was enhanced in the epiphyseal bone marrow space following ischemic osteonecrosis compared to the wildtype control[11]. In the current study, both BMP2 and VEGF were upregulated while the bone catabolic indicator, RANKL/OPG ratio was downregulated by tocilizumab treatment. It is possible that tocilizumab treatment induced bone remodeling by increasing BMP2 and VEGF and decreased bone resorption by reducing the

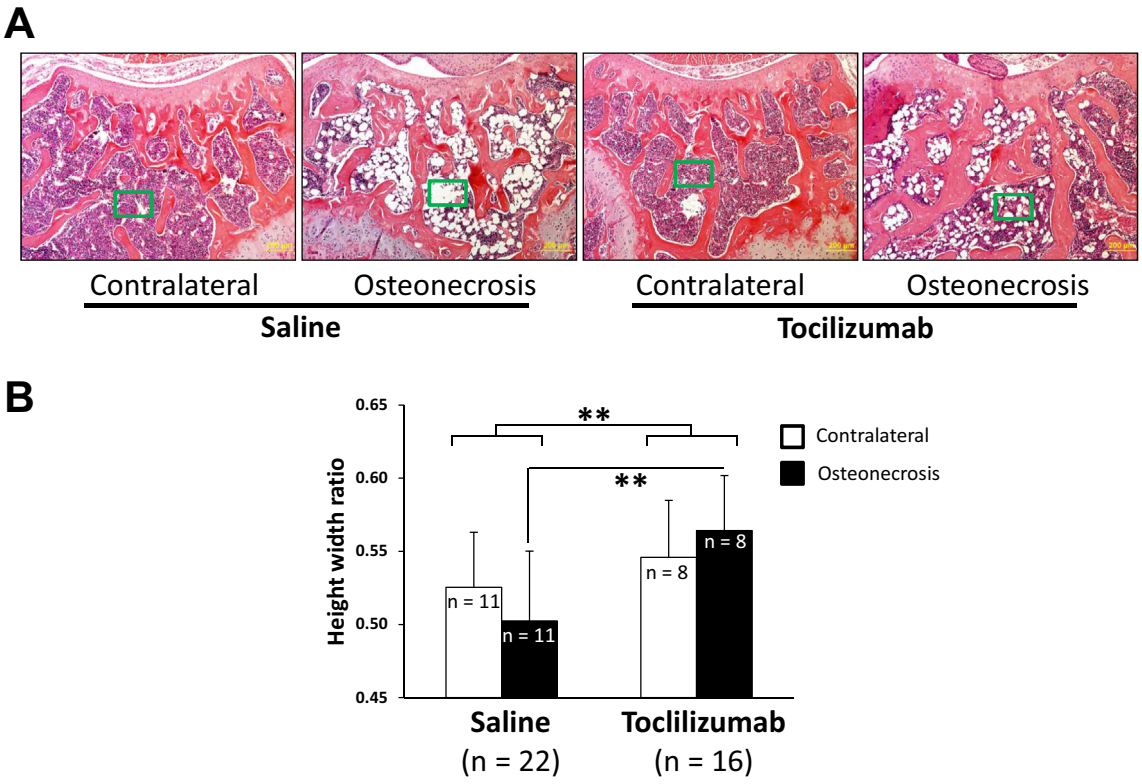

Fig. 6. Effects of tocilizumab on the epiphyseal bone morphology in JIO mouse model (A) After 6 weeks of ischemic induction, histologic assessment was performed on H&E stained sections. The tocilizumab-treated ON group showed increased trabecular bone volume and trabecular thickness compared to the saline-treated ON group. The data are representative of three mice (saline group; $n = 3$ mice, tocilizumab group; $n = 3$ mice). Bars; 200 μm (B) A height width ratio of the medial distal femoral condyle was obtained using the micro-CT images. The mean ratio of the tocilizumab group (0.556 ± 0.038 [95% CI: 0.534–0.577], $n = 16$) was significantly higher than that of the saline group (0.514 ± 0.047 [95% CI: 0.495–0.532], $n = 22$) with a mean difference of 0.042 (95% CI for mean: 0.013–0.071, $p = 0.005$). There was no significant difference ($P = 0.86$) between the contralateral ($n = 19$) and the ON ($n = 19$) groups. Within the ON groups, there was a significant difference between the saline-treated ON ($n = 11$) and tocilizumab-treated ON ($n = 8$) groups (0.502 ± 0.052 vs 0.565 ± 0.037, respectively) with a mean difference of 0.063 (95% CI for mean: 0.023–0.104, $p = 0.003$) while within the contralateral groups no significant difference was found between the saline-treated contralateral ($n = 11$) and tocilizumab-treated contralateral ($n = 8$) groups (0.526 ± 0.039 vs 0.546 ± 0.040, respectively, $p = 0.31$) as assessed by two-way ANOVA (**$p < 0.01$).

RANKL/OPG ratio. In addition, expression levels of MMP13 were increased in the tocilizumab-treated ON group (Supplemental Figure 4). While MMP13 is known to be required for bone formation and vascularization[37], future studies are needed to investigate the mechanisms by which cartilage metabolism following ischemic osteonecrosis affects and alters bone formation and resorption *in vivo*.

The limitation of this study

This study focused on JIO, thus, we specifically chose immature mice to induce ischemic osteonecrosis to mimic JIO in human which occurs in children prior to skeletal maturation. However, the location of osteonecrosis is distal femur, not femoral head. While femoral head is the most common location affected in children with JIO, a reliable mouse model of femoral head osteonecrosis is currently unavailable and no genetic model has been developed. In addition, the distal femoral epiphysis is approximately four times larger than the proximal femoral epiphysis in mice, providing a greater amount of tissue for research. A surgical approach to cauterize blood supply to the distal femoral epiphysis is solely established as a JIO mouse model at this time[14]. Further studies are needed to investigate the efficacy of tocilizumab treatment for ischemic osteonecrosis including a preclinical study using a large animal model of JIO and a clinical trial to validate this treatment.

Another limitation is the imbalance of our study group where the sham group (i.e., saline-treated sham and tocilizumab-treated sham) was not included in the micro-CT experiment in order to reduce the number of animals used in the study.

Lastly, the chondrocytes used in this study were isolated from ankle and not from knee or hip, due to unavailability of these samples from healthy children. The biological difference of chondrocytes by site is unclear, which is also a limitation of this study.

Conclusion

IL6 receptor blockade using tocilizumab produced cartilage anabolic effect and increased epiphyseal bone volume in JIO mouse model.

Contributions

Authors' roles: NK and HK designed experiments, reviewed data. NK wrote the manuscript edited by HK. GK and OA performed the experiments and gathered data.

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Disclosure

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2018.10.010.

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