

# High susceptibility to collagen-induced arthritis in mice with progesterone receptors selectively inhibited in osteoprogenitor cells

**Lixian Liu**

Yunan Vocational and Technical College of Agriculture

**Junjing Jia**

Yunan Agricultural University

**Min Jiang**

Shanghai Ruijin Hospital

**Xueping Liu**

University of California Davis

**Chenlin Dai**

University of California Davis

**Barton Wise**

University of California Davis

**Nancy Lane**

University of California Davis

**Wei Yao** (✉ [yao@ucdavis.edu](mailto:yao@ucdavis.edu))

University of California Davis <https://orcid.org/0000-0002-1198-1161>

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## Research article

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# Abstract

**Background.** Progesterone receptor (PR) affects immunomodulation, and lack of PR in osteoprogenitor cells primarily affects pathways associated with immunomodulation, especially in the males. In this study, we selectively deleted PR from osteoprogenitor cells using Prx1-Cre to evaluate the tissue-specific effects of PR on inflammatory arthritis (IA).

**Methods.** Collagen-induced arthritis (CIA) was used as an IA animal model, which we induced in PR D Prx1 mice and their wild type (WT) littermates were immunized with collagen II (CII) emulsified incomplete Freund's adjuvant (CFA) in both sexes. Joint erosion, inflammation, and cartilage damages were assessed using a semiquantitative histologic scoring system. Bone volume and erosions in knee and ankle joints were quantitated using microCT. We found that knee and paw joint erosions developed in 37.5% and 41.7% of the WT and PR D Prx1 female mice, respectively, and in 45.4 and 100%, respectively, of the male mice. Also, both knee and ankle joint inflammation scores, joint damage scores, and subchondral bone erosions were significantly more severe in male PRcKO mice than in male WT mice. Although of lower severity than in male mice, female PR D Prx1 mice also developed higher inflammation scores and bone erosions than the KO-normal or WT-CIA females. Immunohistochemistry staining of the NLRP3 inflammasome revealed the male PR D Prx1 mice had significantly higher expression of NLRP3 inflammasome in the knee joints.

**Conclusion.** Our observations indicate that the presence of PR in osteoprogenitor cells counteracts the development of collagen-induced arthritis and might help to explain the sex differences observed in human inflammatory arthritis.

## Introduction

Sex dimorphism of Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a systemic autoimmune disease that can affect many organ systems, but notably induces inflammation of synovial tissue, and causes activation of inflammatory cytokines that destroy both cartilage and periarticular bone [1–3]. About 3 million Americans suffer from RA, with nearly three times more women than men affected [4–8]. In women, RA most commonly begins between the ages of 30–60 years, but in men, RA often begins later in life. The mechanism for this sex dimorphism in RA is not clear. Most studies of sex-specific factors affecting RA have focused on the potential effects of sex hormones due to the observation that RA activity is reduced in females during pregnancy and that male RA patients generally have a less severe course of illness and better response to therapy [5, 9, 10]. Estrogen is reported to have both pro-inflammatory and anti-inflammatory effects on the immune system while both progesterone and androgen are anti-inflammatory [11–19]. The effects of hormones are primarily regulated through their hormonal receptors. The presence and proportion of hormone receptors in different tissues and cells, including fibroblasts, chondrocytes, and bone cells, may define their roles in the sexually-dimorphic pathogenesis of RA [20–27].

Potential effects of progesterone on arthritis. Progesterone is a sex-related steroid known mostly for its reproductive system effects. Progesterone's actions are mainly mediated through the progesterone nuclear receptors A and B (PR-A and B), which are ligand-regulated transcription factors [28]. The presence or absence or relative proportion of PR in different tissues may explain the PR's sex dimorphic roles in these tissues [29–31]. In contrast to the estrogen receptor, PR's role may be more important in immunomodulation in female-dominant diseases such as systemic lupus erythematosus, rheumatoid arthritis, and osteoarthritis [32–34]. However, PR as an immunomodulatory regulator in musculoskeletal tissue is not well understood. PR is expressed by cultured osteoblasts, osteoclasts [35–37] and chondrocytes [38] and is present in vivo in mouse bone [37, 39]. Utilizing genetic fate mapping and immunohistochemistry techniques, we observed PR (esp. PR-B) expression in articular cartilage and in the growth plate as well as in bone [39]. We also noted that in PR selective deletion in Prx1 + cells, which give rise to both osteoblasts and chondrocytes, the PR<sup>ΔPrx1</sup> mice also had significantly higher trabecular bone mass as compared to their WT littermates [39]. Additionally, conditional PR deletion in the Prx1 + osteoprogenitor cells significantly suppressed immunomodulatory pathways, especially in the males. The disease pathway analyses from our previous RNA-Seq study suggested that rheumatoid arthritis is a most-likely targeted disease for PR [34]. which prompted further evaluation of the role of PR in the pathogenesis of RA in this current study. Since we observed the lack of PR signaling in OPC significantly regulated pathways involved in immunomodulation pathways previously [34], we set to perform this follow-up study in the PR<sup>ΔPrx1</sup> mice using a CIA model.

## Methods

Mice and collagen-induced arthritis (CIA) model.

PR-flox mice were obtained from Baylor College of Medicine (Houston, TX, USA). A targeting vector designed to replace part of exon 2 of the PR gene with a selectable marker was employed to create a strain of mice carrying a conditional null PR allele [40]. Prx1-Cre mice were purchased from the Jackson Laboratory. Eight-week-old female and male mice were immunized with 100 µg chicken collagen in completed Freund's adjuvant (CFA) (Chondrex Inc. Redmond WA USA). On Day 21, the mice were boosted with 100 µg chicken collagen in in-completed Freund's adjuvant (IFA) subcutaneously. On Day 24, all mice received 50 µg LPS E. coli O111: B4 (Sigma St. Louis, MI USA) via intraperitoneal injection (i.p.) in normal saline. The mice were euthanized on day 48. The onset of the CIA usually occurs on day 26, after initial immunization, and the disease model generally lasts 40 days. [41–45].

PCR-based strategies were used for genotyping mouse genomic DNA. All animal work was done in compliance with the guiding principles of UC Davis's "Care and Use of Animals." Mice were housed in the animal facility under strictly controlled environmental conditions (12-hour light/dark cycle, room temperature 22 °C), and fed ad libidum (food and water). The Institutional Animal Care and Use Committee of the University of California Davis approved the animal protocol.

T-Cell stimulation for FAC.

Total mononuclear cells were collected from peripheral blood using the Ficoll-Paque density gradient method. The cells were then incubated with phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin for three days before running FAC. We used the following key markers for activated T cells CD3/PerCP-Cy5.5 (Total T), CD25/PE-CF594, and CD45RO/PE-Cy7 (R & D Systems, Minneapolis, MN USA).

Measurements of inflammation, bone erosion, and cartilage damage.

Whole knee and ankle joints were fixed, decalcified, embedded in paraffin, and stained with hematoxylin or Safranin-O. Inflammation was scored semi-quantitatively from 0 to 5: 0 = normal; 1 = minimal infiltration of inflammatory cells and/or mild edema; 3 = moderate infiltration; 4 = marked infiltration; and 5 = severe infiltration. For bone erosion, joint sections were stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin (Sigma St Louis, IL USA). A score of 0–5 was assigned for bone erosion: 0 = normal; 1 = minimal (small areas of bone resorption, not readily apparent on low magnification); 2 = mild (more areas of resorption in trabecular and cortical bone); 3 = moderate (obvious bone resorption of trabecular and cortical bone, without defects in cortex or loss of trabeculae); 4 = marked (full-thickness defects in cortical bone and marked trabecular bone loss); 5 = severe (defects in the entire cortex, marked trabecular bone loss) [46–48]. Total TRAP + cells within the subchondral area were counted and presented as TRAP + cell/bone surface. Cartilage damage was calculated by the loss of Safranin-O staining that was scored semi-quantitatively from 0 to 4: 0 = intact; 1 = minor (< 10%); 2 = moderate (10–50%); 3 = high (50–80%); and 4 = severe (80–100%) [49, 50]. Two blinded observers performed all the scorings. Data are presented as the average of the scores of both observers.

Bone mass measurements by microCT

The right knee joints including both the distal femurs (DFM) and the proximal tibiae were scanned and analyzed using VivaCT 40 (Scanco Medical, Bassersdorf Switzerland) with a voxel resolution of 10  $\mu\text{m}$  in all three spatial dimensions and a mono-energetic (70 Kev) X-ray source. We evaluated the entire knee covering a total of 645 mm in length centered around the knee joint to obtain total knee bone volume/tissue volume (BV/TV) ratio [34, 51, 52] using 3D image-registration schemes Gaussian filters of sigma = 0.8, support = 1 and threshold = 180 for total knee and DFM. Gaussian filters of sigma = 1, support = 2, and threshold = 280 were applied to register the paw.

Knee histopathology

The left knee joints were fixed in 10% phosphate-buffered saline formalin for two days, decalcified in 10% EDTA for three weeks, and embedded in paraffin. Sections were stained with Safranin-O – Fast green for measurement of articular cartilage thickness, subchondral bone plate thickness, subchondral trabecular bone number and diameter, and cartilage content using Bioquant Imaging software (Bioquant Imaging System, Nashville, VA USA) [51, 52]. Primary antibodies against NLRP3 (Abcam, Cambridge, MA, USA) were used with Alexa-Fluor 594 - conjugated secondary antibody for immunohistochemistry staining. The sections were counterstained with DAPI. Positively stained cells were counted using the cell count software in Keyence BZ-X9000 all-in-one imaging system (Itasca, IL, USA).

Statistical analysis.

The results are expressed as mean  $\pm$  standard deviation for bone structure measures, bone turnover, and bone strength variables. Two-way ANOVA was used to account for genotype and sex. If significant differences were observed, then a Tukey's multiple comparisons test was used to assess pairwise comparisons. A value of  $p < 0.05$  was considered statistically significant. Data were analyzed using the GraphPad Prism 7 software package (La Jolla, CA, USA).

## Results

Mice with PR conditionally knocked out in osteoprogenitor cells (OPC) had higher systemic activation of T cells and showed higher levels of inflammation.

We found very low levels of circulating activated T-cells marked by CD3e+, CD69+, and CD25 + in the WT - CIA controls, especially in the female WT-CIA mice, at proximately 0.1%. On the other hand, both female and male PR $\Delta$ Prx1 -CIA mice had a significantly higher circulated level of activated T-cells as compared to the WT-CIA mice at day 50 (Fig. 1A). The incidence of arthritis (defined as developing bone erosions viewed by 3D microCT reconstructions of paw images) was 37.5% and 41.7%, respectively, in the female WT and PR $\Delta$ Prx1 mice; and 45.4 and 100%, respectively, in the male WT and PR $\Delta$ Prx1 mice (Fig. 1B). Hence, we observed an increase in inflammation and arthritis prevalence in mice lacking PR in the MSCs, highlighting a possible role of PR in systemic as well as local tissue involvement during RA.

Male PR $\Delta$ Prx1 mice with collagen-induced arthritis had higher levels of bone destruction

MicroCT and histochemical analyses were used to assess the degree of bone erosion in the ankle and the knee joints in WT-CIA and PR $\Delta$ Prx1 -CIA mice and the control mice which did not receive immunizations. In the paws, total bone volume did not differ in WT-CIA mice compared to their WT-non-CIA controls, but was reduced in PR $\Delta$ Prx1 -CIA mice compared to the PR $\Delta$ Prx1 -non-CIA controls (Fig. 2A). Areas of bone erosion were apparent on microCT images of paws, especially in the distal and proximal ends of the metacarpus as well as in the carpus, in the male WT-CIA and PR $\Delta$ Prx1 -CIA mice (Fig. 2B, white arrows). Compared to the non-CIA mice, female PR $\Delta$ Prx1 -CIA, male WT-CIA, and male PR $\Delta$ Prx1 -CIA mice had reduced total bone volume in knee joints (Fig. 3A). The non-CIA mice had smooth and continuous bone surfaces in their knees, while focal peri-articular bone erosions were apparent in both the female and male PR $\Delta$ Prx1 -CIA mice (Fig. 3B, white arrows).

Further histology performed on the same samples using microCT confirmed the absence of trabecular bone loss at the femoral subchondral bone in the female and male WT-CIA mice (Fig. 4A-C). Both female and male PR $\Delta$ Prx1 -CIA mice demonstrated lower trabecular bone volume compared to their sex-matched PR $\Delta$ Prx1 -non-CIA mice, with similar subchondral cortical bone plate thickness across all the groups (Fig. 4C). TRAP histochemistry was used to determine the numbers of osteoclast at the distal femurs, with a focus on the subchondral bone erosions. TRAP + cells were not different in female or male WT-CIA

mice compared to their WT-non-CIA controls. The female PR<sup>ΔPrx1</sup> -CIA mice had a trend of increased TRAP + cells in the subchondral bone area but did not reach statistical significance when compared to PR<sup>ΔPrx1</sup> -normal mice. In contrast, numerous TRAP + cells were present on the femoral subchondral trabecular bone surface in the male PR<sup>ΔPrx1</sup> -CIA mice compared to sex-matched non-CIA mice (Fig. 5, black arrows). Taken together, these results suggest that PR<sup>ΔPrx1</sup> mice, and especially the male PR<sup>ΔPrx1</sup> mice are more susceptible to bone loss in this CIA model of inflammatory arthritis.

Male PR<sup>ΔPrx1</sup> mice with collagen-induced arthritis had worse cartilage damages and inflammation

Cartilage destruction and inflammation were assessed on H&E and Safranin-O stained sections. The overall semi-quantitative scoring on the H&E stained sections revealed higher trends of inflammation and erosion in the male PR<sup>ΔPrx1</sup> -CIA mice versus CIA mice from other groups (Fig. 6). Examination of Safranin-O stained knee samples revealed a loss of articular cartilage, especially in the male CIA mice (Fig. 4A-B). In the male PR<sup>ΔPrx1</sup> -CIA mice, there was an almost complete loss of articular cartilage in areas of subchondral bone erosion where there was scaffolding subchondral bony plate (Figs. 4 & 6). Similar area of subchondral bone erosion and articular cartilage loss was present in the male WT-CIA as well. Cartilage loss in areas of articular cartilage was noted adjacent to inflamed synovium tissues that were present most obviously in the male WT and PR<sup>ΔPrx1</sup> -CIA mice (Fig. 6A-B, \* indicated inflamed synovium tissues). The semiquantitative inflammation, erosion and cartilage damage scores were higher in the male PR<sup>ΔPrx1</sup> -CIA mice than the WT\_CIA mice (Fig. 6C)

To explore the role of the inflammasome NOD-like receptor pyrin domain containing 3 (NLRP3) in inflammation and cartilage degradation following CIA, we performed an immunohistochemistry staining for NLRP3 on knee joint samples. We found NLRP3 was sparsely expressed by subchondral chondrocytes and bone marrow macrophage-like multi-nuclear cells within the bone marrow in the femoral subchondral bone (Supplementary Fig. 1A). CIA increased NLRP3 expressions at the subchondral chondrocytes, bone marrow macrophage-like multi-nucleated cells, and in the synovial tissue, especially in the male PR<sup>ΔPrx1</sup> -CIA mice (Supplementary Fig. 2B). These results indicate that inflammation and cartilage damage that was observed in all the CIA mice was more severe in male PR<sup>ΔPrx1</sup> -CIA mice.

## Discussion

In this study, we report that mice lacking progesterone receptor signaling in the osteoprogenitor cells were more susceptible to collagen-induced arthritis, especially male mice. The male PR<sup>ΔPrx1</sup> -CIA mice had a significantly higher incidence of arthritis, joint inflammation, bone erosion, and cartilage damage compared to the male PR<sup>ΔPrx1</sup> mice or WT-CIA mice. Our findings indicate that under "normal" conditions, the presence of PR in osteoprogenitor cells might be protective for rheumatoid or inflammatory arthritis incidence and might also partially account for the sex differences that are observed in RA patients [53–55].

Some susceptibility genes for RA have been previously identified. The human leukocyte antigen (HLA) is a genetic site controlling immune responses in RA [56, 57]. Several genes outside the HLA region, such as Stat4, the TRAF1-C5 locus, and PTPN22, have been reported to be associated with activation and progression of inflammation in RA [58–62]. There is hardly any information explaining the sex disparities in genetic susceptibility to RA except a polymorphism in the Cyb5a gene, which is related to androgen synthesis, was found to be associated with risk for RA in women but not in men [63]. Recent studies have also suggested a role for epigenetic modifications in the activation and aggressiveness of synovial fibroblasts in RA [64–67] and the X-encoded genes, Timp1 and IL-9R also may play a role [68]. Some of these epigenetic modifications have been linked to X-linked miRNA, and the presence of the second X chromosome in females may affect miRNA expression levels that could account for the development of sex-related autoimmunity [69, 70]. Most of these studies on sex-specific factors affecting RA have focused on the potential effects of sex hormones due to the observation that RA improves during pregnancy and that male RA patients generally have a less severe course of illness and better response to therapy [5, 9, 10]. Estrogen has been reported to have both pro-inflammatory and anti-inflammatory effects on the immune system while both progesterone and androgen are anti-inflammatory [11–19]. The effects of hormones are mainly regulated through their hormonal receptors. The presence and proportion of estrogen and androgen receptors in different tissues and cells, including fibroblasts, chondrocytes, and bone cells, might define their roles in the sexually-dimorphic pathogenesis of RA [20–27]. We and others have found PR expressed in growth plate chondrocytes, osteoclasts, and osteoblasts, and PR plays a critical role in peak bone mass determination [37, 71, 72]. We also have reported that the loss of PR signaling in osteoprogenitor cells regulates key signaling pathways for immune response, especially in males [34]. We identified PR-targeted genes that regulated sex differences, including an "X-inactive specific transcript," Xist, Mtus2, Aldh1a7/1, Tusc5, Cd300c, and Pde3a [34]. The upregulation of Xist is associated with chronic inflammation and pain in females with complex regional pain syndrome [73] and contributes to RA progression [74]. Cd300c and Pde3a were over-presented in RA patients [75, 76] and were associated with inhibition of T cell immunity [77] or response to TNF inhibitors in RA patients [78]. Taken all together, PR appears to regulate susceptibility to RA in mice, targeting several genes that regulate sex differences and which might correlate with clinical observations of sex-differences in RA.

The presence of marginal bone erosions, detected by imaging, predicts a more severe disease course with more disability and increased morbidity. The significance of erosions in RA has been the focus of the development and approval of several agents for modifying the course of RA and has been validated in clinical trials as being able to reduce structural joint damage, including bone erosion and cartilage degradation [79, 80]. The bone erosions in RA show a predilection for specific anatomic sites such as the radial aspects of finger joints, while the ulnar aspects are relatively spared [81]. These focal erosions typically emerge at the site at which the synovium comes into direct contact with the bone which is known as bare areas. Anatomical factors that predispose these skeletal sites for erosion include the presence of mineralized cartilage, the insertion of ligaments at the bone surface, and inflamed tendon sheaths that enable the spread of inflammation from the tendon to the articular synovium. Articular erosion at these "bare areas" represent localized bone loss from osteolysis, which resulted from an

imbalance in which bone resorption by osteoclasts is predominant over bone formation by osteoblasts. Once established, these bone erosions rarely repair despite the use of potent biologic therapeutic strategies including biologics such as TNF, IL-1 or IL-6 receptor blockade [82–85]. Aberrant repair of erosions appeared as sclerosis with new bone apposition at the base of the erosion and might involve the juxta-articular bone marrow. Adipose tissue might populate the erosive area. Bone erosion seemed to correlate with on-going inflammation. Our study provides additional information to better understand the potential PR regulation of the inflammation-bone resorption coupling mechanism in the process of joint and bone damage, and how potentiation of this coupling from lack of PR signaling contributes to bone and joint loss in RA in a sex-dependent manner.

Recent studies have reported that inflammasomes contribute to the pathogenesis of several age-related metabolic diseases, including atherosclerosis [86–89] and Alzheimer’s disease [90–95]. Inflammasome activation resulting in IL-1 $\beta$  production is an essential part of the innate immune response. NLRP3 binds with the adaptor apoptosis-associated speck-like protein (ASC) and triggers the cleavage/activation of pro-caspase-1, which catalyzes the activation of IL-1 $\beta$ , contributing to the progression of inflammation [96]. Genetic variants within the NLRP3-inflammasome are associated with susceptibility to RA [97–101], and enhanced activity of NLRP3 inflammasome has been found in peripheral blood cells of patients with active RA [102]. Inhibition of NLRP3 inflammasome activity alleviated arthritis in CIA mice [103]. Given that NLRP3 was one of the PR-targeted gene [34] and that the lack of PR potentiated NLRP3 inflammasome expression in the synovial tissue of the CIA mice, suggesting that although PR might not alter the underlying cause of disease, it might reduce the production of factors such as inflammasomes which are involved in inflammation, cartilage and bone destructions in RA.

In conclusion, we found that the lack of PR in osteoprogenitors increased susceptibility to IA, especially in male mice. Our findings indicate that the presence of PR in osteoprogenitor cells counteracts the development of collagen-induced arthritis and might also help to explain sex differences observed in rheumatoid arthritis.

## Declarations

**Ethics approval:** the animal studies were approved by UC Davis IACUC under protocol #20064

**Consent for publication:** The data presented in this manuscript have not been submitted or published elsewhere.

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interest:** None

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**Author contributions:** LL, JJJ: study conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. MJ. XPL, CLD: collection and assembly of data, data analysis, interpretation, and final approval of the manuscript. NEL: data interpretation, and final approval of the manuscript. BLW: data analysis, interpretation of the results, editing of drafts, and final approval of the manuscript. WY: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

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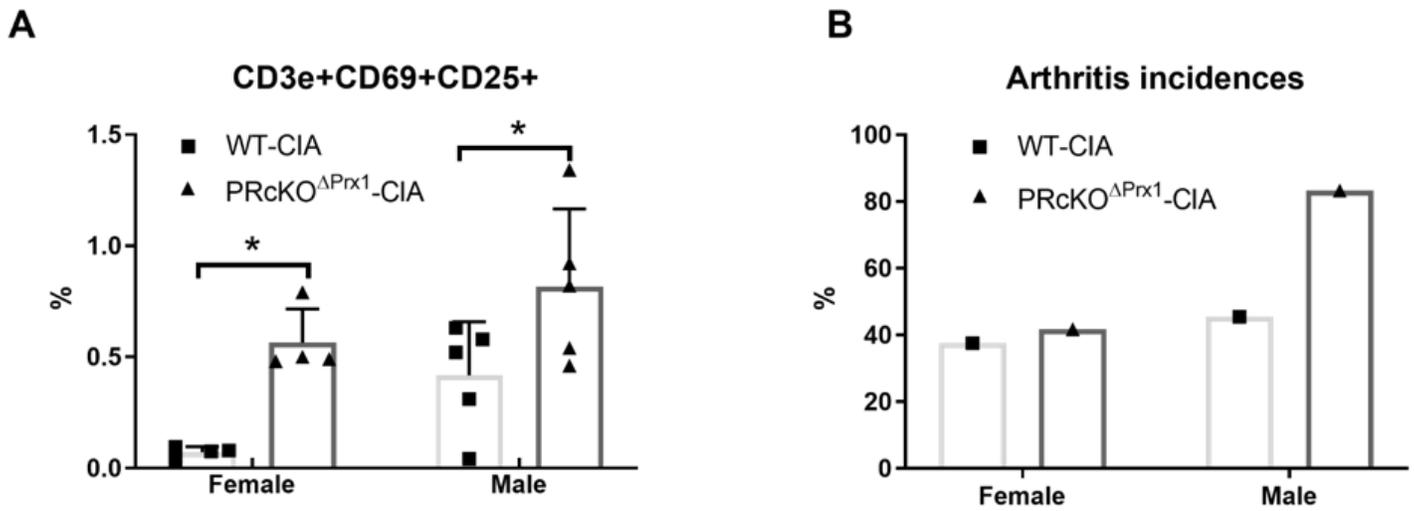
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## Supplementary Figure Caption

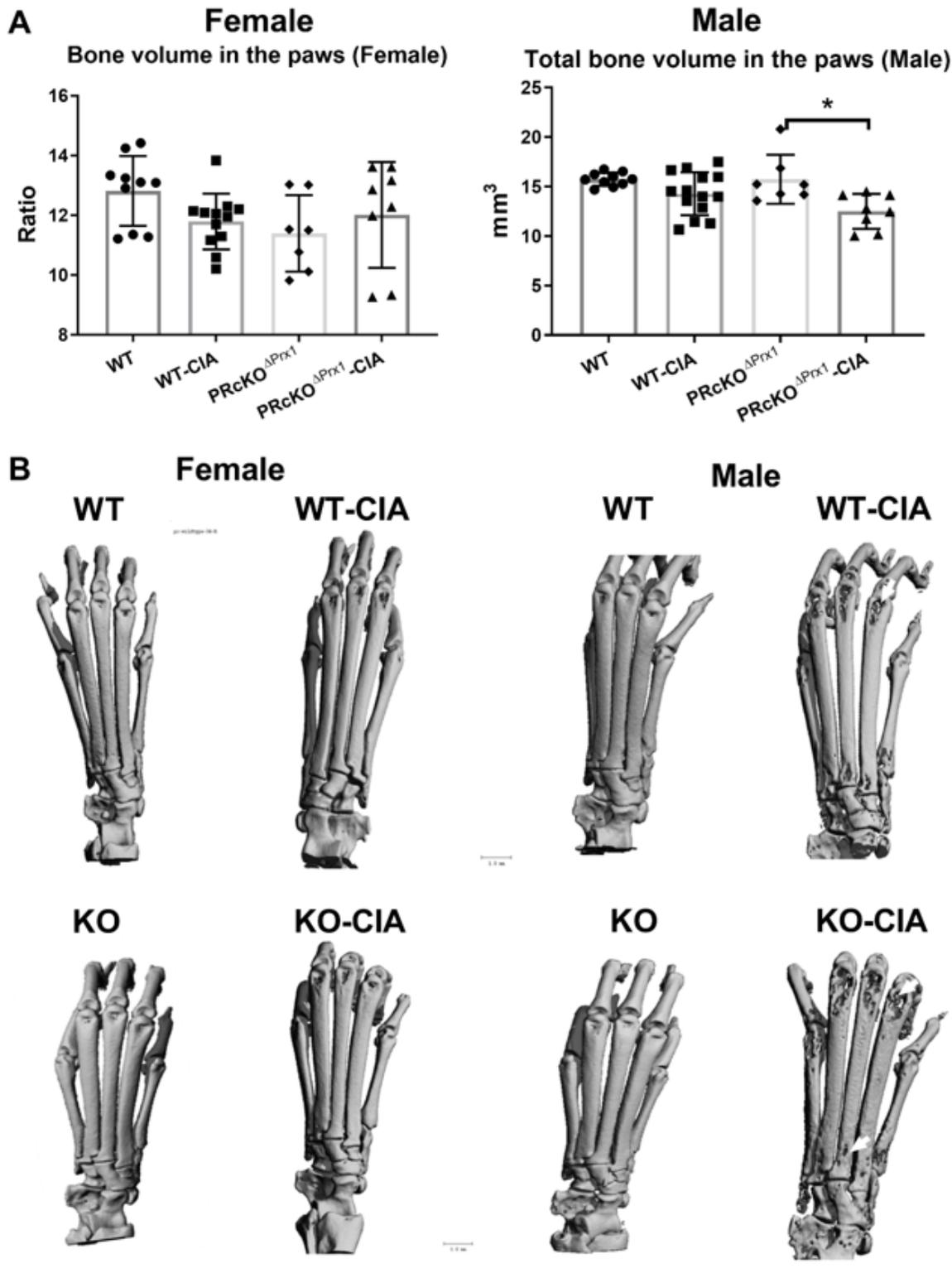
**Supplementary Fig. 1.** Male PR<sup>DPrx1</sup>-CIA mice had higher expression of NLRP3 in the knee joints. (A) Representative anti-NLRP3-Alesa598 stained knee histologic images from female or (B) male in WT or PR<sup>DPrx1</sup> normal or CIA mice. (C) Quantitation of NLRP3 stains normalized by total DAPI + cells at the distal femoral subchondral regions, including the synovial tissue, cartilage, and subchondral bone/bone marrow.

## Figures



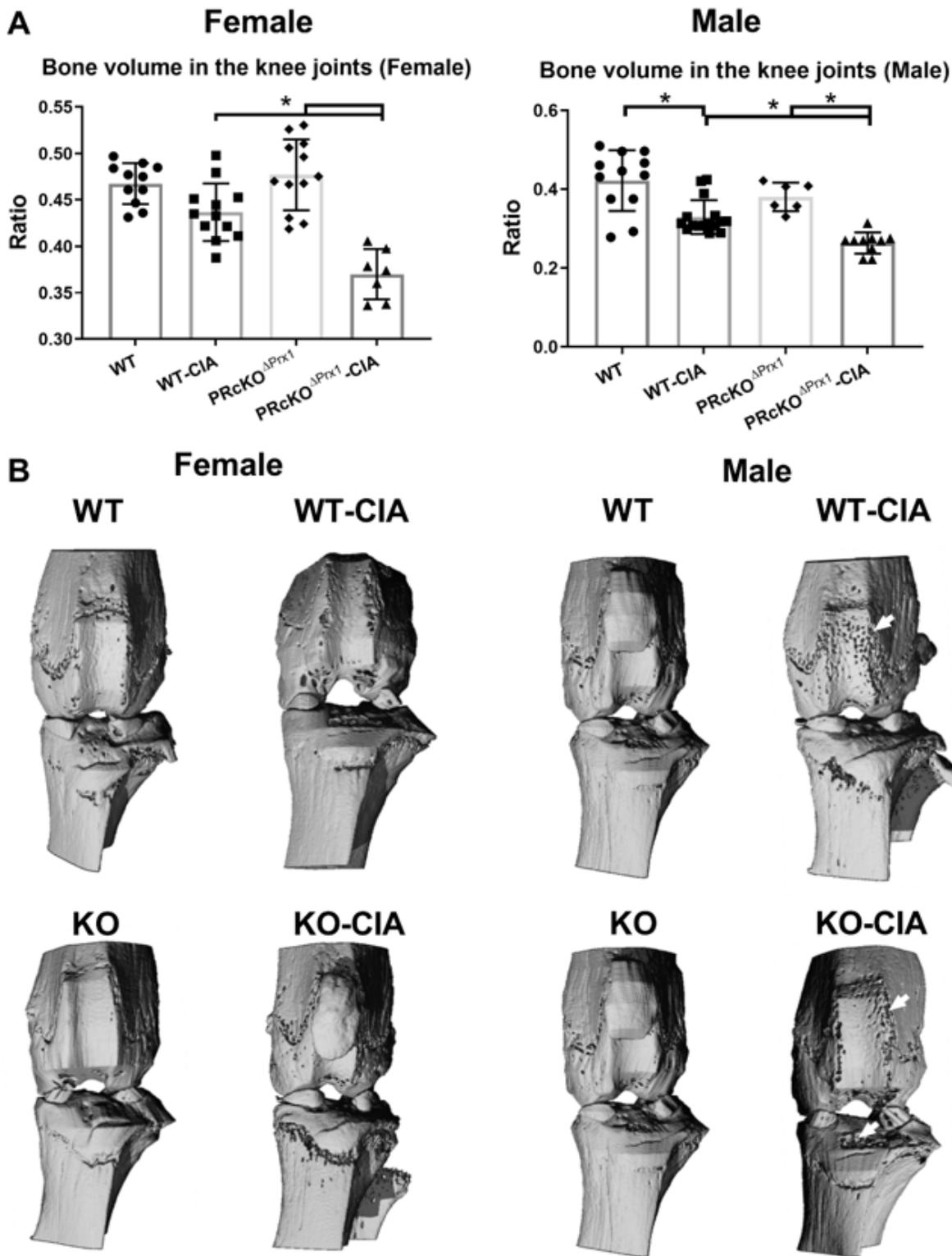
**Figure 1**

Mice with PR conditionally knocked out in osteoprogenitor cells had higher circulating T cells and higher arthritis incidence in male mice. (A) Peripheral blood was obtained in WT-CIA or PR<sup>ΔPrx1</sup>-CIA and subjected to FAC determination of CD3e+, CD69+, and CD25+ T-cells. (B) The incidence of arthritis was scored using 3D images of paws showing bone erosion in at least one of the paws.



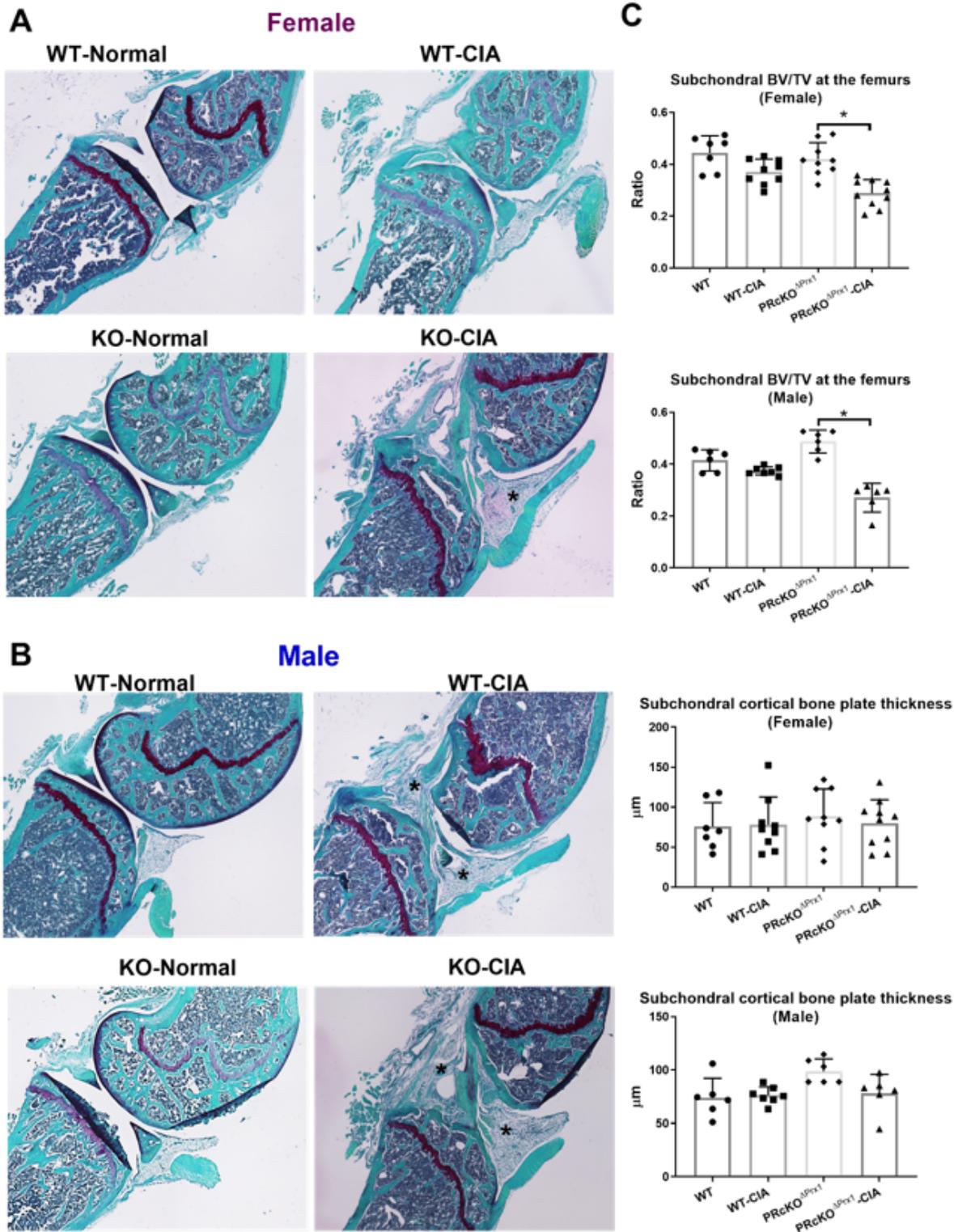
**Figure 2**

Mice with PR conditionally knocked out in osteoprogenitor cells had a lower bone mass in the paws of male PR $\Delta$ Prx1 -CIA mice. (A) The total bone volume of the right paws was measured by microCT in WT and PR $\Delta$ Prx1 normal or CIA mice. (B) Representative microCT paw images from WT or PR $\Delta$ Prx1 normal or CIA mice. White arrows illustrated bone erosion.



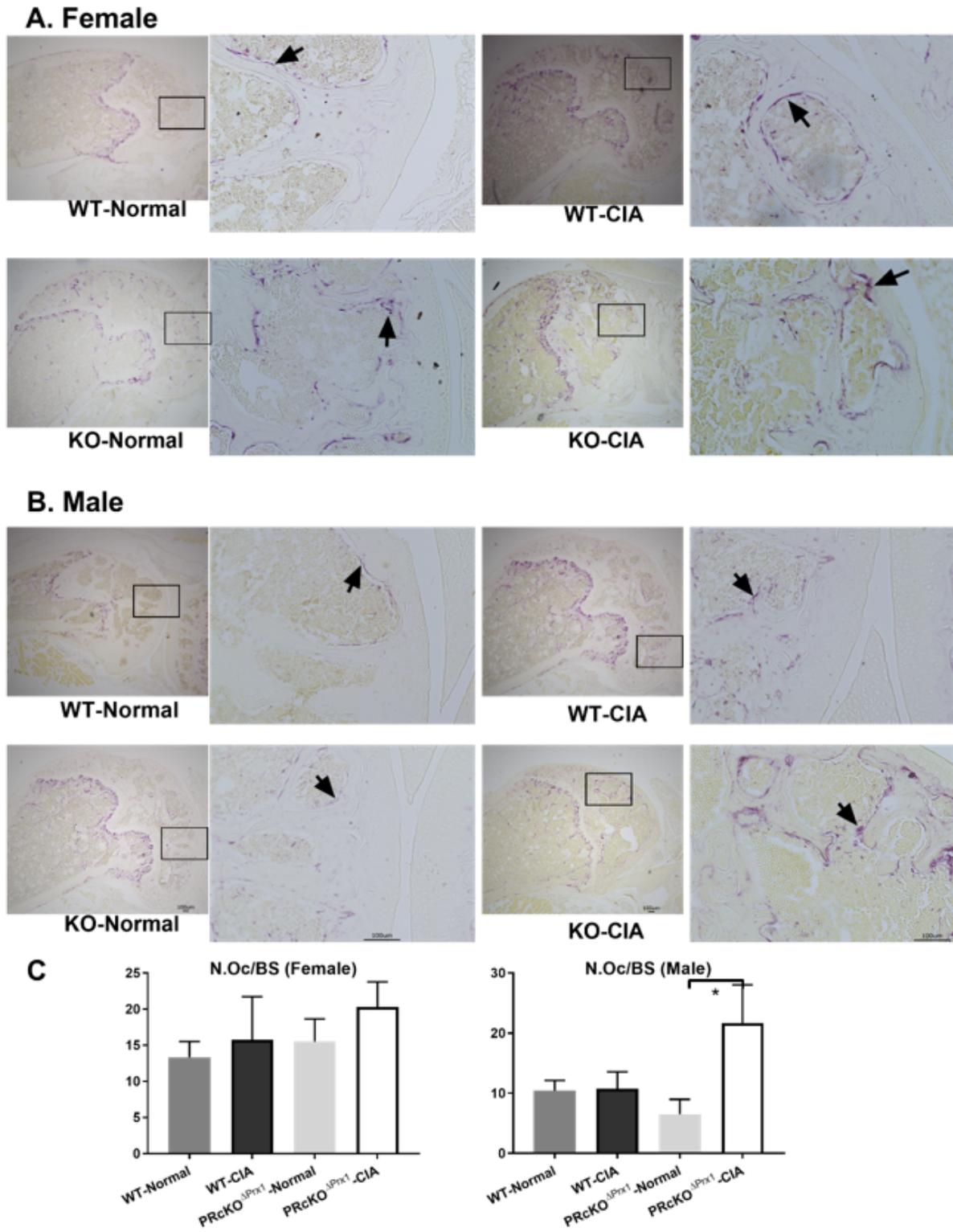
**Figure 3**

Mice with PR conditionally knocked out in osteoprogenitor cells had a lower bone mass in the knee joints of PR $\Delta$ Prx1 -CIA mice. (A) Total bone volume/tissue volume of right knees was measured by microCT in WT and PR $\Delta$ Prx1 normal or CIA mice. (B) Representative microCT knee images from WT or PR $\Delta$ Prx1 normal or CIA mice. White arrows identify bone erosions.



**Figure 4**

Male PR $\Delta$ Prx1 -CIA mice had lower trabecular bone volume. (A) Representative Safranin-O stained knee histologic images from female or (B) male in WT or PR $\Delta$ Prx1 normal or CIA mice. (C) Quantitative measurements of subchondral trabecular bone or cortical plate thickness at the femoral epiphyses. Black stars indicate inflamed synovial tissue.



**Figure 5**

Male PR $\Delta$ Prx1 -CIA mice had higher amounts of surface osteoclasts at the distal femoral subchondral bone. (A) Representative TRAP stained knee histologic images from female or (B) male in WT or PR $\Delta$ Prx1 normal or CIA mice. (C) Quantitative measurements of TRAP+ surface osteoclasts at the distal femoral subchondral bone. Black arrows illustrate TRAP+ cells at bone surfaces

