Sex differences of NF-κB-targeted therapy for mitigating osteoporosis associated with chronic inflammation of bone

Cite this article:
Bone Joint Res 2024;13(1):
28–39.

DOI: 10.1302/2046-3758.
131.BJR-2023-0040.R3

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Aims

Transcription factor nuclear factor kappa B (NF-κB) plays a major role in the pathogenesis of chronic inflammatory diseases in all organ systems. Despite its importance, NF-κB targeted drug therapy to mitigate chronic inflammation has had limited success in preclinical studies. We hypothesized that sex differences affect the response to NF-κB treatment during chronic inflammation in bone. This study investigated the therapeutic effects of NF-κB decoy oligodeoxynucleotides (ODN) during chronic inflammation in male and female mice.

Methods

We used a murine model of chronic inflammation induced by continuous intramedullary delivery of lipopolysaccharide-contaminated polyethylene particles (cPE) using an osmotic pump. Specimens were evaluated using micro-CT and histomorphometric analyses. Sex-specific osteogenic and osteoclastic differentiation potentials were also investigated in vitro, including alkaline phosphatase, Alizarin Red, tartrate-resistant acid phosphatase staining, and gene expression using reverse transcription polymerase chain reaction (RT-PCR).

Results

Local delivery of NF-κB decoy ODN in vivo increased osteogenesis in males, but not females, in the presence of chronic inflammation induced by cPE. Bone resorption activity was decreased in both sexes. In vitro osteogenic and osteoclastic differentiation assays during inflammatory conditions did not reveal differences among the groups. Receptor activator of nuclear factor kappa B ligand (*Rankl)* gene expression by osteoblasts was significantly decreased only in males when treated with ODN.

Conclusion

We demonstrated that NF-κB decoy ODN increased osteogenesis in male mice and decreased bone resorption activity in both sexes in preclinical models of chronic inflammation. NF-κB signalling could be a therapeutic target for chronic inflammatory diseases involving bone, especially in males.

Article focus

- Sex-dependent osteogenic ability responds to nuclear factor kappa B (NF-κB) modulation during chronic inflammation.

Key messages

- Male mice showed greater osteogenic ability than females during chronic inflammation.
- NF-κB decoy oligodeoxynucleotide (ODN) increased osteogenesis in male

mice and decreased bone resorption activity in both sexes in preclinical models of chronic inflammation.

Strengths and limitations

- This study demonstrated the sex-dependent effects of NF-κB inhibition on osteogenesis during chronic inflammation.
- Further studies are needed using human samples to translate these findings to a clinical setting.

Introduction

Chronic inflammation is associated with numerous orthopaedic conditions including osteoarthritis (OA), rheumatoid arthritis (RA), ankylosing spondylitis, osteonecrosis, osteoporosis, and delayed and nonunion of fractures.[1-8] Transcription factor nuclear factor kappa B (NF-κB) regulates key genes involved in chronic inflammation,[9,10] and the NF-κB signalling pathway has been regarded as a potent therapeutic target for chronic inflammation.[11-13] The NF-κB decoy oligodeoxynucleotides (ODN) are one of the candidates for clinical application that directly prevent the binding of NF-κB to the promoter regions of targeted genes, leading to the attenuation of gene activation.[14] Despite some evidence of preclinical success in in vitro and in vivo studies,[15-17] NF-κB direct-targeted therapy to mitigate chronic inflammation of bone has not been translated yet. Instead, other diagnostic or therapeutic approaches have been pursued.[18] For example, Denosumab inhibits the maturation of osteoclasts by binding to and inhibiting receptor activator of NF-κB ligand (RANKL) on pre-osteoclasts, but does not directly inhibit the inflammatory cascade.[19] To translate NF-κB direct-targeted therapy into clinical success, further preclinical experiments from different points of view should be conducted.

The importance of sex differences in biological processes has recently garnered increasing interest.[20] Females have a different biological hormone cycle compared to males.[21] Sex-based dimorphism is also a consequence of differences in other factors like X-chromosome inactivation, anatomy, sex, or life experiences.[22] In spite of these sex-specific differences, unfortunately, the majority of preclinical research has focused on only male subjects.[23,24] To address this concern, in 2014 the National Institutes of Health in the USA recommended the policy of sex as a key biological variable in preclinical, translational, and clinical studies.[25] There is certain epidemiological evidence of sexual dimorphism in the orthopaedic field.[26-30] In the research field, sex differences in the regulation of osteogenesis in mice have been described.[31,32] Furthermore, sexual dimorphism is also evident in inflammatory conditions.[33,34] Despite these reports, rarely do we see research studies of differentiating responses to immune therapies between sexes, particularly for the purpose of augmenting bone regeneration. To explore this knowledge gap, our study attempted to investigate the sexual dimorphism of immune therapies for bone regeneration.

In this study, we hypothesized that there exist sex-dependent differences in the therapeutic responses to mitigate NF-κB overexpression during chronic inflammation. The inclusion of both sexes in animal research will facilitate mechanistic analyses and drive important discoveries that previous studies which included only each sex could not reach. This study investigated the therapeutic effect of NF-κB decoy ODN in different sexes using in vitro and in vivo murine models of chronic inflammation in bone.

Methods

Animal study

The animal experimental protocol was reviewed and approved by our institution's Administrative Panel on Laboratory Animal Care (APLAC). Institutional Guidelines for the Care and Use of Laboratory Animals were followed in all aspects of this project. All studies were carried out in compliance with the ARRIVE guidelines. All outcome measurements in animal study were evaluated by two blinded independent observers (MT, JK) and averaged (mean) to minimize observer bias.

Mice

Ten- to 12-week-old BALB/c male and female wild-type mice (Jackson Laboratory, USA) were used and randomly assigned in all experiments ($n \geq 5$ in each group). Power analysis was carried out to determine a sufficient number of mice based on detecting a significant difference of 1.5 standard deviations (SDs) (effect size 1.5) with a power of 80% ($\alpha = 0.05$, $\beta = 0.20$). We used all mice assigned, but some were excluded during the sample preparation because of technical difficulties. Mice were housed in a specific pathogen-free facility with a 12-hour light, 12-hour dark cycle and given free access to food and water.

Ultra-high molecular weight polyethylene particles

The polyethylene particles (PE) were obtained as previously described.[35] Briefly, Ceridust 3610 PE (Clariant, USA) were washed with 100% ethanol and filtered using a 20 μm pore membrane. The mean particle size of 4.62 μm (SD 3.76) was confirmed by electron microscopy (Cell Science Image Facility at Stanford University). The particles were vacuum dried for three days and resuspended in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA; Thermo Fisher Scientific, USA). The final concentration of particles was approximately 3.1×10^{10} particles/ml. Lipopolysaccharides (LPS; MilliporeSigma, USA) were used to generate contaminated polyethylene particles (cPE; 1.25% of PE, and 10 ng/ml of LPS) to enhance the inflammatory reaction.[36]

NF-κB decoy oligodeoxynucleotide

The NF-κB decoy ODN was custom synthesized (Integrated DNA Technologies, USA) according to the sequences of 5′-CCTTGAAGGGATTTCCCTCC-3' and 3'-GGAACTTCCCTTCCCTAAAGGGAGG-5'.[37]

Murine continuous femoral particle infusion model

The model was generated as previously described (Figure 1).[38–40] Mice were anaesthetized with inhalation of 1% to 3% isoflurane, and buprenorphine (0.3 to 1 mg/kg) was administered prior to and during the surgery as an analgesic agent. The right distal femur was exposed via a lateral parapatellar approach. Then, a hollow titanium rod (6 mm long, 23 gauge) was press-fit into the distal femoral canal. For the continuous infusion, an osmotic pump containing 10% BSA/PBS with cPE was implanted into the dorsal side of the mouse subcutaneously through a second incision around the right shoulder girdle. This pump was connected to the implanted rod via a subcutaneous vinyl catheter tube. After primary surgery, all mice were infused with cPE for three

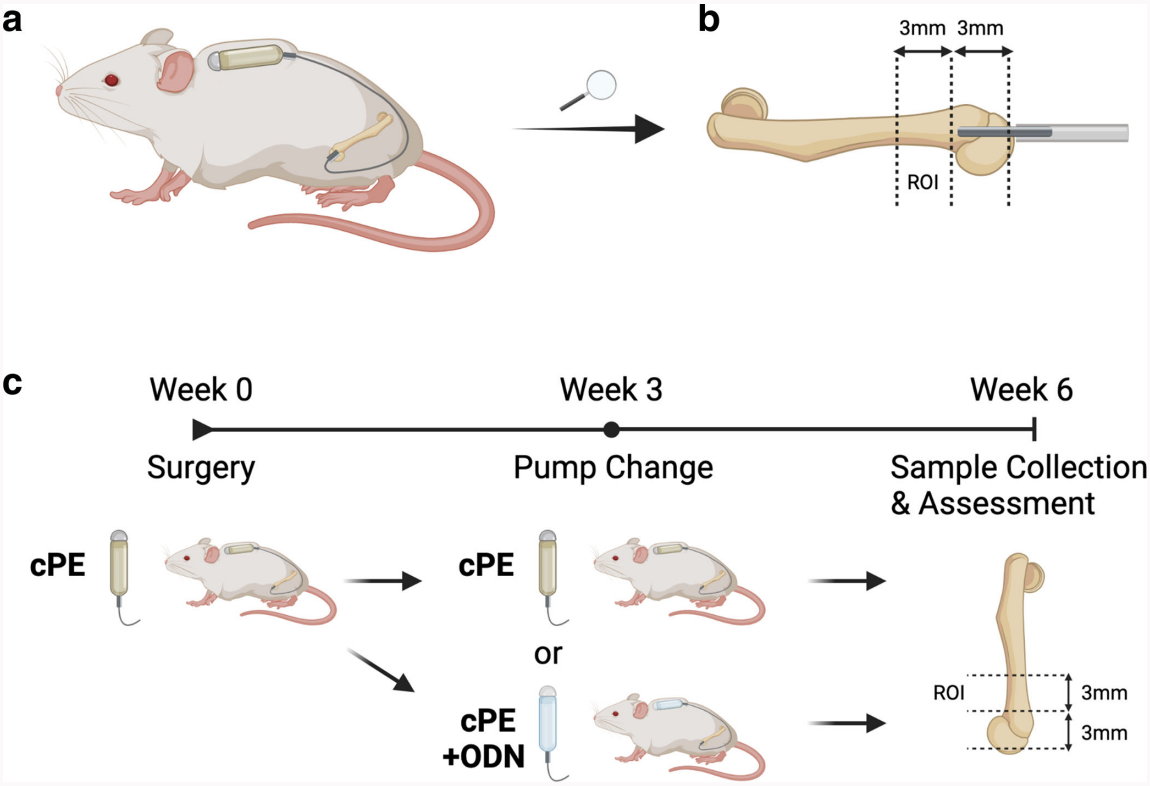

Fig. 1
In vivo experimental design. The images of the murine model of continuous chronic inflammation. The hollow rod was press-fit into the distal femur canal. Timetable of the experimental design is shown. cPE, contaminated polyethylene particles; ODN, oligodeoxynucleotides; ROI, region of interest.

weeks to induce chronic inflammation. The second surgery was performed to replace pumps three weeks after the primary surgery. In the control group, pumps were changed to new ones containing 10% BSA/PBS with cPE. In the ODN group, pumps were replaced with new ones filled with 10% BSA/PBS with cPE and NF-κB decoy ODN (50 μM). All mice were euthanized by CO_2 inhalation three weeks after the second surgery.

Immunofluorescence for macrophage phenotype

Femoral sections were prepared as previously described.[40,41] Briefly, the femora were harvested, fixed overnight in 4% paraformaldehyde, demineralized in 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 7.4), and embedded in optimal cutting temperature compounds. The region of interest (ROI) located 3 mm from the distal end of the femur was cut into 10 μm-thick transverse sections. Immunofluorescent staining for macrophage was performed to confirm the inflammatory conditions. Macrophages were identified by anti-CD11b antibody (1:500; Abcam, USA), M1 proinflammatory macrophages by anti-inducible nitric oxide synthase (iNOS) (1:500; Abcam), and M2 anti-inflammatory macrophages by anti-liver Arginase 1 (Arg1) antibody (1:500; Abcam). The iNOS/CD11b and the Arg1/CD11b double-positive cells were calculated as the M1 and M2 macrophages, respectively, using QuPath (GNU General Public License v3.0).[42]

Histology and immunohistochemistry

Alkaline phosphatase (ALP) staining and tartrate-resistant acid phosphatase (TRAP) staining were performed as previously described.[43-45] Briefly, ALP staining (1-Step NBT/BCIP Substrate Solution; Thermo Fisher Scientific) detected bone formation area, and the percentage of ALP-positive area of the bone was quantified using QuPath. The TRAP staining kit (MilliporeSigma) identified osteoclast-like cells. Multi-nucleated TRAP-positive cells located on the bone perimeter within the resorption lacunae were defined as osteoclast-like cells and counted. Both scores were evaluated by two blinded independent observers (MT, JK) and averaged (median) from three randomly selected areas to minimize observer bias.

Micro-CT

After the mice were euthanized, the titanium rod was removed from the distal femur. Micro-CT scans were performed using TriFoil Imaging CT120 (TriFoil Imaging, USA) with 49 μm resolution. A 4 mm × 4 mm × 3 mm ROI that started 3 mm from the distal end of the femur and proceeded proximally was created.[39,40] The threshold bone mineral density (BMD, mg/mm^3) was quantified using GEMS MicroView software (Parallax Innovations, Canada) (threshold: 700 HU).

In vitro osteogenic differentiation assay

Bone marrow-derived mesenchymal stromal cells (MSCs) were isolated from mice.[14,46,47] Cells were pre-treated with cPE (1.25% of PE, and 10 ng/ml of LPS) for two days to induce the inflammatory condition. Then, cells were subsequently cultured with/without cPE and with/without ODN (0.1 or 1 μM) in α-minimum essential medium (MEM; Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Thermo Fisher

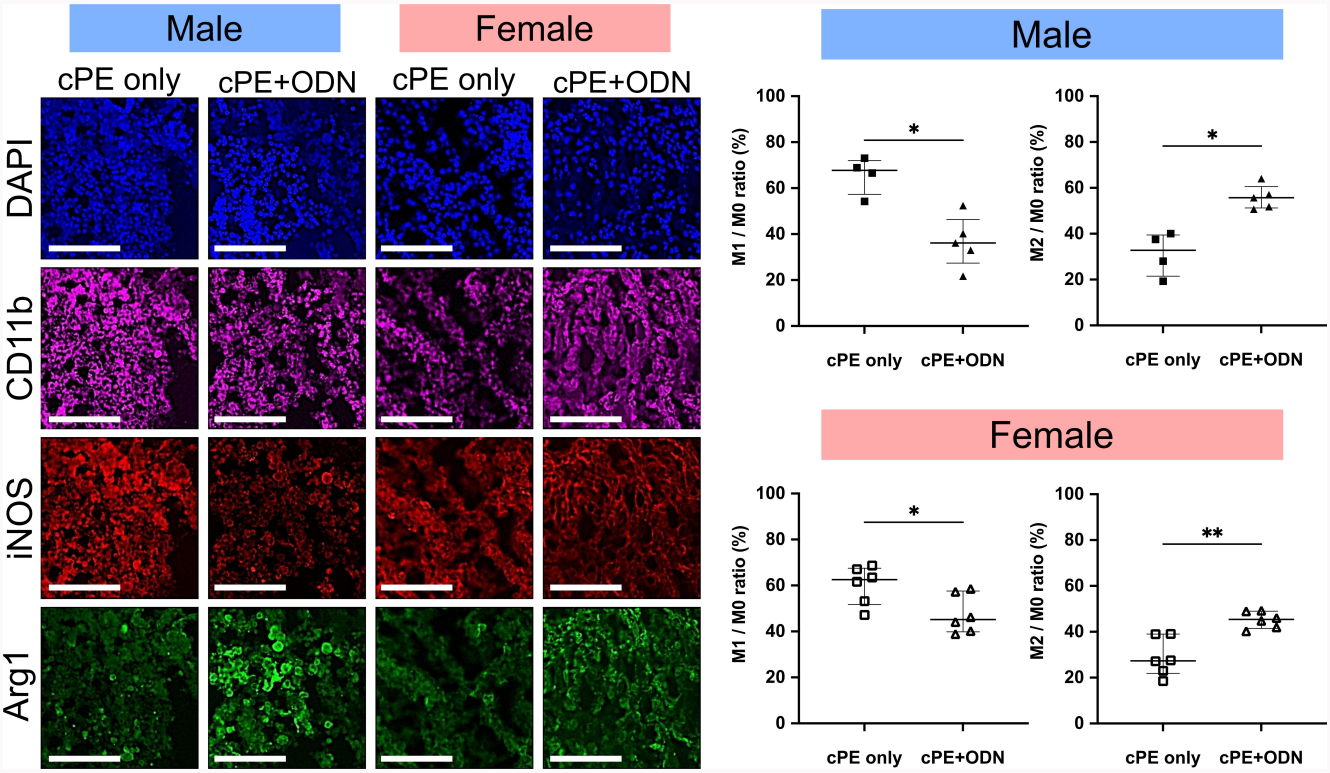

Fig. 2

Immunofluorescence for macrophages in vivo. Representative images of immunofluorescence for macrophages are shown (scale bar = 100 μm). Quantitative assessments of the M1 positive (iNOS positive) cells and the M2 positive (Arg1 positive) cells were performed, on male (control group: n = 4; oligodeoxynucleotide (ODN) group: n = 5) and female mice (control group: n = 6; ODN group: n = 6). *p < 0.05, **p < 0.01, Mann-Whitney U test. Arg1, Arginase 1; cPE, contaminated polyethylene particles; DAPI, 4′ ,6-diamidino-2-phenylindole; iNOS, inducible nitric oxide synthase.

Scientific), 1% antibiotic-antimycotic (A/A; Thermo Fisher Scientific), 10 mM β-glycerol phosphate (MilliporeSigma), 50 μM ascorbic acid (MilliporeSigma), and 100 nM dexamethasone (MilliporeSigma). ALP staining and reverse transcription polymerase chain reaction (RT-PCR) for gene expression were performed on day 7, and Alizarin Red staining for calcified bone matrix on day 21. Whole-well images were captured using BZ-X810 (KEYENCE, Japan), and ALP and Alizarin Red positive areas were measured using QuPath (n = 5 in each group). To compare the osteogenic differentiation ability under different inflammatory conditions, the cells were also pre-treated with ODN. The pre-treatment with cPE simulated the inflammatory exposure to particles similar to our in vivo experiments; pre-treatment with ODN aimed to investigate the preventive ability of ODN prior to exposure to cPE.

In vitro osteoclastic differentiation assay

Primary bone marrow cells were isolated and cultured in α-MEM supplemented with 10% FBS, 1% A/A, and 10 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, USA).[46,47] Following the same protocol described above, cells were pre-treated and cultured in an osteoclastogenic medium containing α-MEM supplemented with 10% FBS, 1% A/A, 10 ng/ml M-CSF, and 50 ng/ml RANKL (R&D Systems). To examine the osteoclastic differentiation ability, TRAP staining was performed on day 7 after the differentiation started. TRAP-positive cells in the three randomly selected areas (1 mm^2) were counted and averaged (median) using BZ-X810 (n = 5 in each group).

Cell proliferation and viability assay

Cell proliferation and viability were evaluated during the osteogenic and osteoclastic differentiation assay using alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Fluorescence was measured using SpectraMax iD3 (Molecular Devices, USA).

Quantitative real-time RT-PCR

Total RNA was extracted on day 7 of the osteogenic differentiation assay using TRIzol reagent (Thermo Fisher Scientific). Total RNA was reverse-transcripted to complementary DNA (cDNA) using iScript Reverse Transcription Supermix (Bio-Rad, USA). Quantitative real-time RT-PCR was performed using QuantStudio 6 Pro (Thermo Fisher Scientific) and PowerTrack SYBR Green Master mix (Thermo Fisher Scientific). Respective data were normalized against the corresponding levels of *Actin* (n = 4 in each group). Primers are summarized in Supplementary Table i.

Statistical analysis

The Mann-Whitney U test was used to compare data between two groups, and the Kruskal-Wallis test with Dunn's multiple comparisons between data in three or more groups. Data were expressed as median with an interquartile range. All analyses were performed using GraphPad Prism 9 (GraphPad Software, USA), and statistical significance was set at $p < 0.05$.

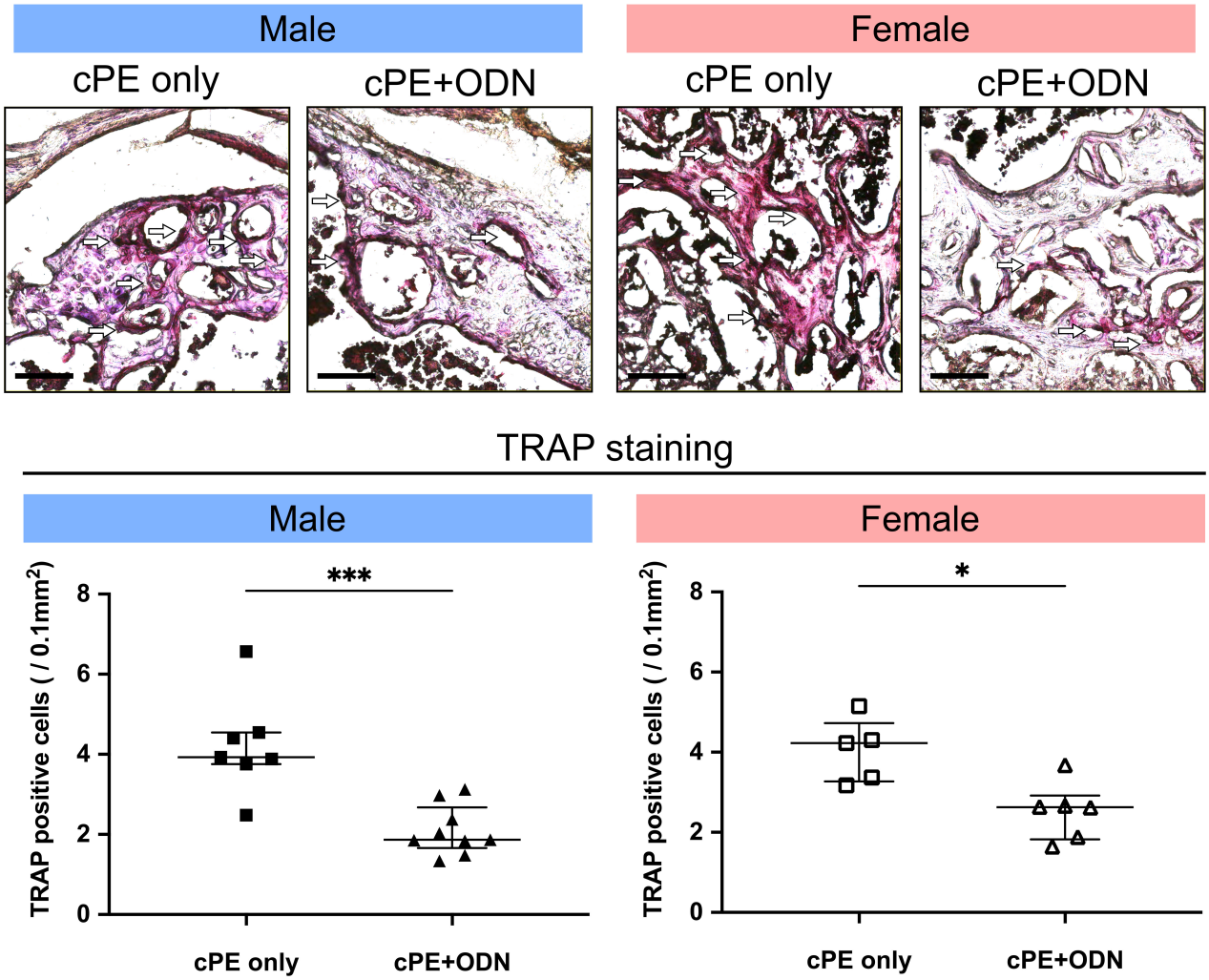

Fig. 3
Tartrate-resistant acid phosphatase (TRAP) staining in vivo. Representative images of TRAP staining and quantitative analysis of TRAP-positive cells (white arrows) are shown (scale bar = 100 μm) for male (control group: n = 7; oligodeoxynucleotide (ODN) group: n = 9) and female mice (control group: n = 5; ODN group: n = 6). *p < 0.05, ***p < 0.001, Mann-Whitney U test. cPE, contaminated polyethylene particles.

Results

Local delivery of NF-κB decoy ODN attenuated chronic inflammation and bone resorption activity induced by continuous cPE infusion in both sexes

It was shown that cPE increased chronic inflammation and bone resorption activity.[43] In both sexes, the M1 cell proportion was decreased and the M2 cell proportion was increased in the ODN group (Figure 2), indicating that NF-κB decoy ODN attenuated cPE-induced chronic inflammation. In the ODN group, there were fewer TRAP-positive cells than in the cPE group in both male ($p < 0.001$) and female ($p < 0.05$, both Mann-Whitney U test) mice (Figure 3).

Local delivery of NF-κB decoy ODN increased osteogenesis in male but not female mice despite chronic inflammation induced by cPE

It was reported that cPE treatment reduced osteogenesis.[43] In male mice, ALP positive area and the BMD in the ODN group were increased compared with the cPE group ($p < 0.05$, Mann-Whitney U test) (Figure 4). However, in female mice, there were no significant differences between the two groups in both ALP positive area and BMD.

Male-derived MSCs exhibited greater osteogenic ability than females but there were no differences among the groups within each sex

To investigate why treatment effects differed between the sexes in vivo, we performed an in vitro osteogenic differentiation assay. Using this assay, cell proliferation was not affected by the different treatments (data not shown). ALP-positive areas were comparable among groups of each sex. However, male mice showed a greater ALP-positive area than female mice ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons). These trends were similar for Alizarin Red staining (Figure 5).

NF-κB decoy ODN attenuated gene expression of *Rankl* in males but not females in the presence of cPE

To explore the mechanisms of inflammation and osteogenesis, we conducted RT-PCR using cells collected during the

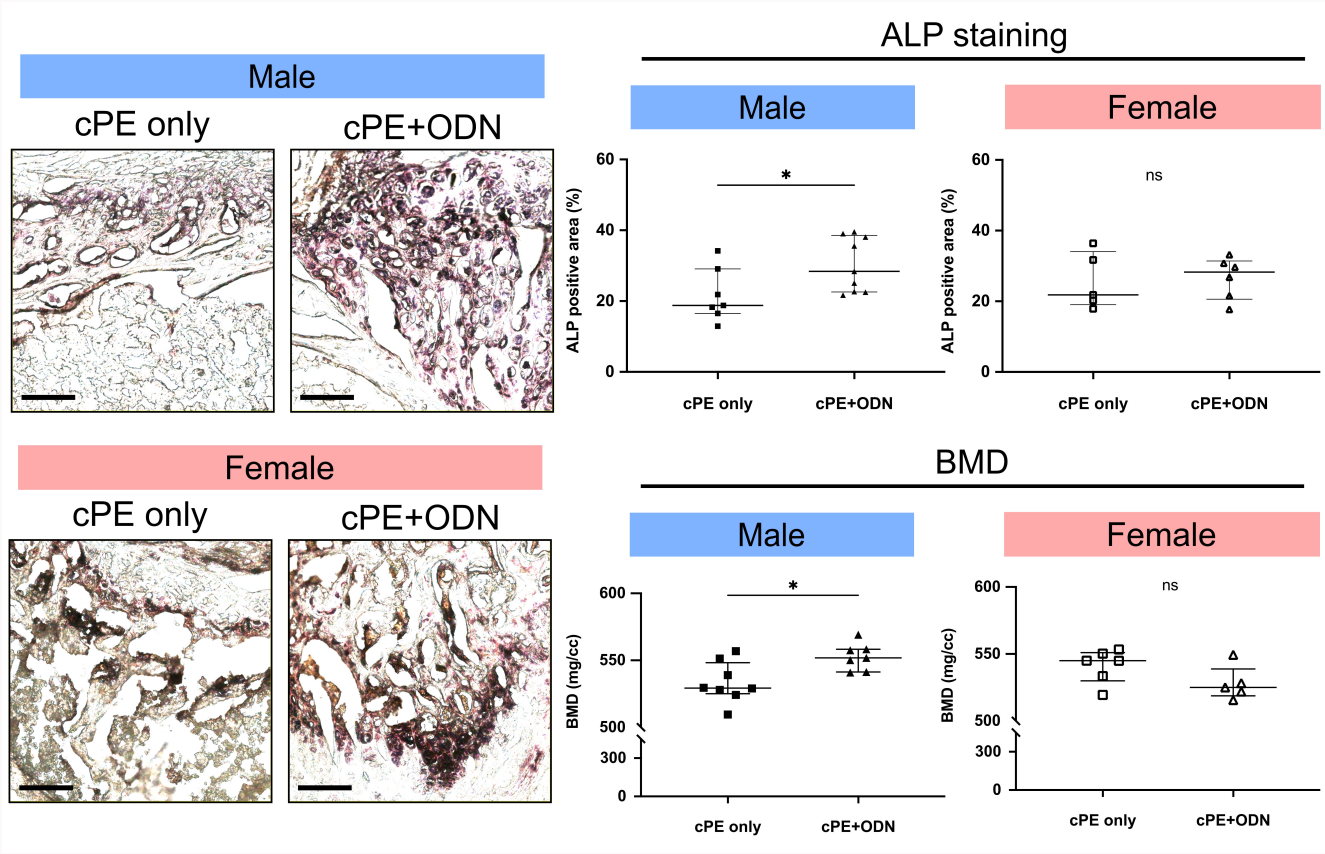

Fig. 4
Alkaline phosphatase (ALP) staining and micro-CT analysis in vivo. Representative images of ALP staining and quantitative analysis of ALP positive area proportion are shown (scale bar = 100 μm) for male (control group: n = 7, oligodeoxynucleotide (ODN) group: n = 9) and female mice (control group: n = 5, ODN group: n = 6). Quantitative assessments of bone mineral density (BMD) are also shown for male (control group: n = 8, ODN group: n = 7) and female mice (control group: n = 6, ODN group: n = 5). *p < 0.05, Mann-Whitney U test. cPE, contaminated polyethylene particles; ns, non-significant.

osteogenic differentiation assay. We checked the osteogenesis-related gene expressions of runt-related transcription factor 2 (*Runx2*), collagen type 1 alpha 1 (*Col1α1*), osteopontin (*Opn*), bone gamma-carboxyglutamate protein 2 (*Bglap2*), and receptor activator of nuclear factor kappa-B ligand (*Rankl*). ODN treatment significantly reduced expression of *Rankl* in males in the presence of cPE ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons) (Figure 6), while no significant difference was noted in females.

ODN treatment decreased the cPE-induced TRAP-positive cells both in male and female mice in vitro

Osteoclastic differentiation assay was used to confirm the ability of ODN to modulate osteoclastic activity in vitro. No significant difference was detected between sexes. Cell viability was not affected by the ODN treatment (data not shown). Co-culture with cPE increased the number of TRAP-positive cells, but ODN attenuated this effect both in males ($p < 0.05$) and females ($p < 0.01$, both Kruskal-Wallis test with Dunn's multiple comparisons) (Figure 7).

ODN pre-treatment increased osteogenesis in males and decreased bone resorption in males and females

We performed another in vitro study using ODN pre-treated cells before inducing inflammation with cPE. We found that only male murine cells pre-treated with ODN showed higher ALP ($p < 0.05$) and Alizarin Red ($p < 0.05$, both Kruskal-Wallis test with Dunn's multiple comparisons) positive areas versus the control group with/without cPE. Following the pre-treatment with ODN, cPE-induced TRAP-positive cells were decreased in both sexes ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons). ODN pre-treatment suppressed subsequent cPE-induced osteoclast differentiation in female cells (Figure 8).

Discussion

Human diseases can affect men and women differently. This sex-based dimorphism is a consequence of differences in X-chromosome inactivation, sex hormones, and other factors such as anatomy, sex, or life experiences.[22] Genetically, X-chromosome inactivation provides compensation for X-linked genes between XX females and XY males. X-inactivation in females results in limited expression of the mutation genes, which gives females protection from immune deficiency. Biologically, sex hormones, such as oestrogen, progesterone, and androgen, mediate sex-based differences in skeletal function[48] and immune responses.[49] While the disease activity of RA is ameliorated during pregnancy, it tends to flare in the postpartum period when oestrogen and progesterone levels fall.[50] Furthermore, there is epidemiological evidence of sexual dimorphism in the orthopaedic field. OA,[27] RA,[28] and

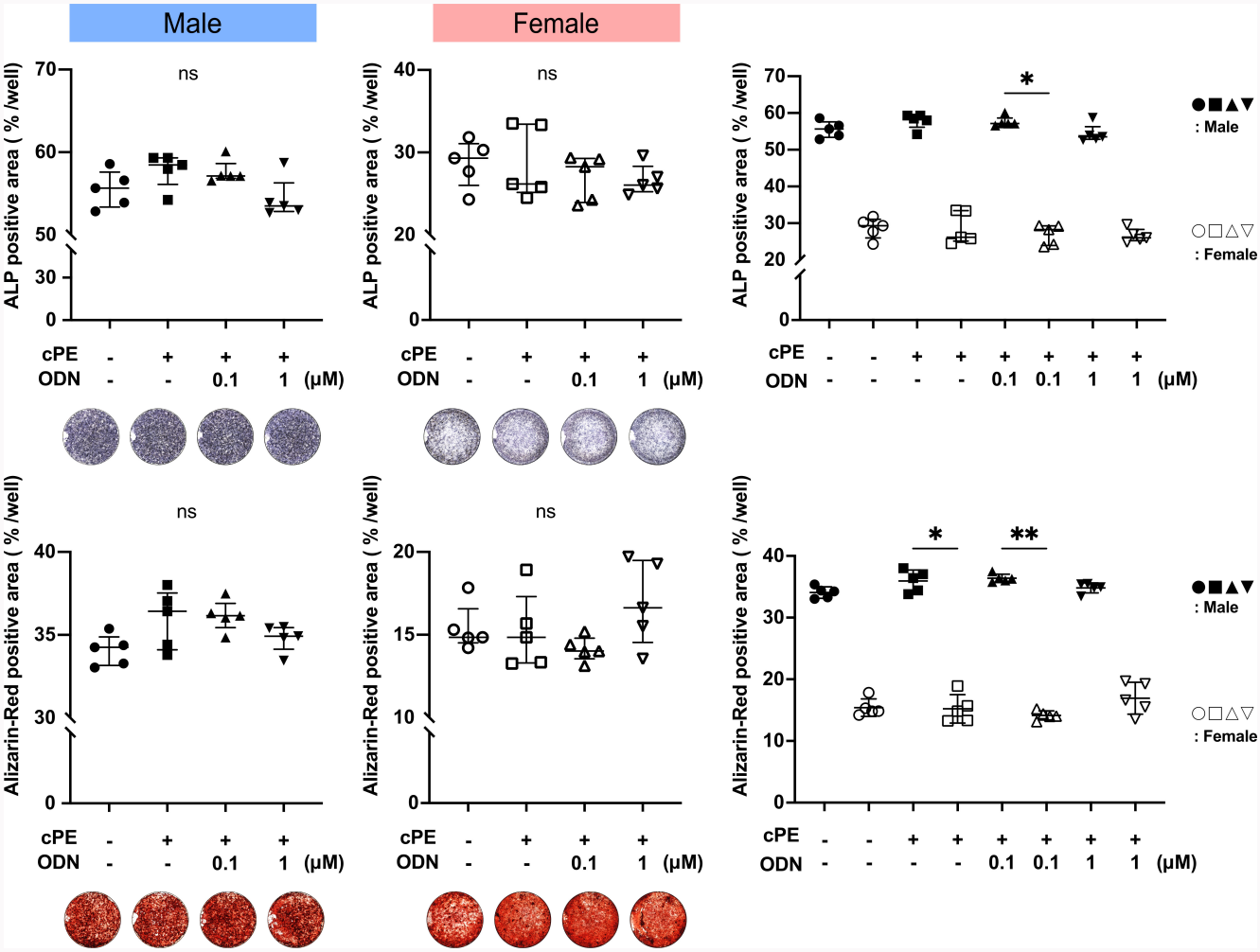

Fig. 5
In vitro osteogenic differentiation assay pre-treated with contaminated polyethylene particles (cPE). Quantitative analysis of alkaline phosphatase (ALP) and Alizarin Red positive area proportion (%/well) and representative whole-well images of each group are shown (n = 5, each group). The comparison of ALP and Alizarin Red positive area proportion (%/well) between male and female cells is also shown (n = 5, each group). *p < 0.05, **p < 0.01, Kruskal-Wallis test with Dunn's multiple comparisons. cPE, contaminated polyethylene particles; ns, non-significant; ODN, oligodeoxynucleotides.

osteoporosis[29] are clinically more common in females, but ankylosing spondylitis[26] and osteonecrosis of the femoral head[30] are predominant in males. In the research field, sexual dimorphism in the regulation of osteogenesis in mice has been described.[31,32] For example, trabecular bone mass of female adult mice was lower compared with male mice.[51] Moreover, the sex-specific difference in osteoclastogenesis has also been reported. Protein kinase C δ (PKC-δ) regulates androgen receptor transcription by binding to its promoter, and PKC-δ conditional knockout in osteoclasts exhibited increased bone mass only in male mice.[52]

Despite reports of sexual dimorphism in bone, the detailed mechanism is poorly understood. Immoderate emphasis has also been placed on females with regard to different ovarian cycle phases. Although oestrogen replacement therapy is effective for postmenopausal osteoporosis, its effectiveness for severe osteoporosis is clinically inferior compared to denosumab.[19] This suggests that factors other than sex hormones might be related to sex differences in osteogenesis. Clinical studies in males often generate different results from females, exemplified by sex differences in response to many drugs.[53] The inclusion of both sexes in animal research will facilitate mechanistic analyses and drive important discoveries in both basic and clinically relevant research.[54] NF-κB direct-targeted therapy to mitigate chronic inflammation of bone has not been translated despite some preclinical success. The aim of our study was to investigate sex-related responses to NF-κB decoy ODN treatment under chronic inflammatory conditions.

In our in vivo experiments, osteogenesis in the NF-κB decoy ODN treatment group was increased compared with the non-treatment group only in male mice. This result highlights different response mechanisms between sexes to the ODN treatment. In our in vitro differentiation assays, male mice exhibited more osteogenic ability than female mice, which is consistent with other previous reports.[31,33] However, in the ALP activity, Alizarin Red, and TRAP assays, there were no differences between groups in both sexes regardless of the ODN treatment. Only *Rankl* expression in osteoblasts was statistically significantly decreased in males under chronic inflammation. The mechanism for differences in in vivo responses to

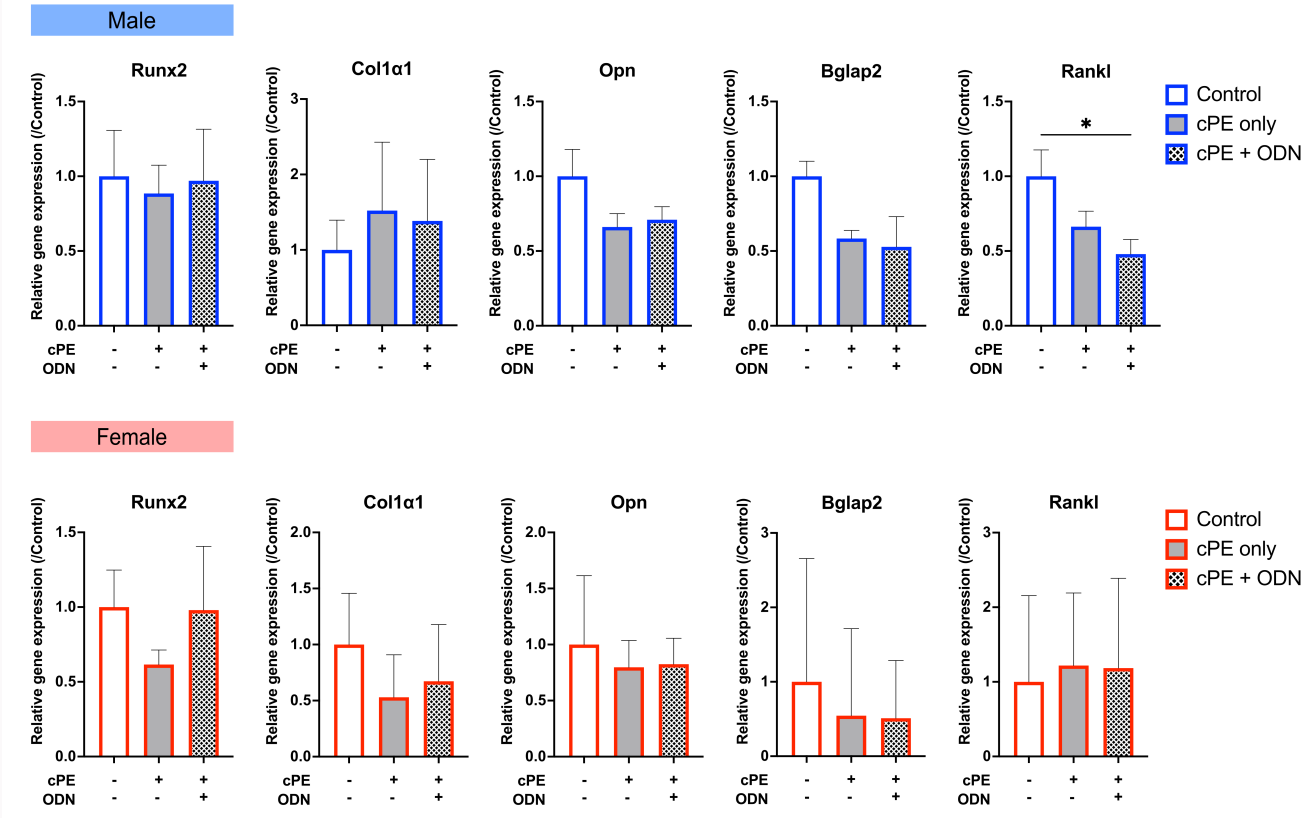

Fig. 6
Gene expressions during the osteogenic differentiation assay pre-treated with contaminated polyethylene particles (cPE). The results of reverse transcription polymerase chain reaction (RT-PCR) on day 7 during the osteogenic differentiation assay are shown. The blue graph shows the results for males and the red for females. Values are shown as relative gene expression levels to the control group. The concentration of nuclear factor kappa B (NF-κB) decoy oligodeoxynucleotide (ODN) was 0.1 μM. n = 4, each group. *p < 0.05, Kruskal-Wallis test with Dunn's multiple comparisons. Bglap2, bone gamma-carboxyglutamate protein 2; Col1α1, collagen type 1 alpha 1; Opn, osteopontin; Rankl, receptor activator of nuclear factor kappa-B ligand; Runx2, Runt-related transcription factor 2.

NF-κB treatment between the sexes is speculative. Osteogenesis was not influenced by sex hormones in vitro, so the differences in sex hormones would be insufficient to explain these findings. Osteocytes are the major sources of RANKL during the bone remodelling process,[55] and the expression of *Rankl* in osteoblasts has been reported to be essential to subsequent osteogenesis. *Rankl* expression by osteoblasts has a function as receptors that recognize RANK released from osteoclasts.[56] This osteoblastic *Rankl* promotes osteoblastic differentiation and osteogenesis. We observed sex-specific responses to ODN treatment in vivo, but not in vitro osteoblast monoculture. One possibility is that the downregulation of *Rankl* genes in males may partially explain the sex differences in our in vivo studies. NF-κB is activated by two separate pathways: the classical pathway and the alternative pathway.[57] In this study, chronic inflammation was induced by PE contaminated with LPS, which is a key activator of the classical NF-κB pathway. Furthermore, several vital regulators in the classical NF-κB pathway such as inhibitor of NF-κB kinase subunit beta (IKKβ) and p65 have been reported to have an association with RANKL, although RANKL activates both classical and alternative pathways of NF-κB in osteoclastogenesis.[58] For example, IKKβ is required for osteoclastogenesis both in vitro and in vivo.[59] Mice lacking p65 in the haematopoietic compartment showed a deficient osteoclastogenic response to RANKL.[60] Based on these facts, the sex-dependent different response to NF-κB decoy ODN in this study is likely to be associated with the classical NF-κB pathway. Another NF-κB inhibitor, parthenolide (PAR), mitigated PE-induced osteolysis in a murine model.[61] Although PAR could not reduce elevated serum levels of type-1 carboterminal collagen crosslinks (CTX-1) and osteoclast-associated receptor (OSCAR), the relatively short-term PAR treatment (intermittent delivery for ten days) was mentioned as one of the possible reasons for this failure. In our in vivo study, we examined the effect of a three-week continuous infusion of NF-κB decoy ODN, which is longer than the previous report.[61] Serum CTX-1 and OSCAR levels were not measured in our study, but the NF-κB decoy ODN could potentially have regulated the above osteoclastogenic factors in our murine model due to the increased duration of drug delivery.

Sexual dimorphism is also evident in inflammatory conditions. Males generally mount a more aggressive inflammatory response to microbial pathogenesis than females, and females have diminished proinflammatory responses compared to males.[34] Furthermore, different strains of mice have different immunological characteristics. BALB/c mice are less resistant to inflammation compared to C57BL/6 mice.[33] Our study was performed in the presence of a chronic

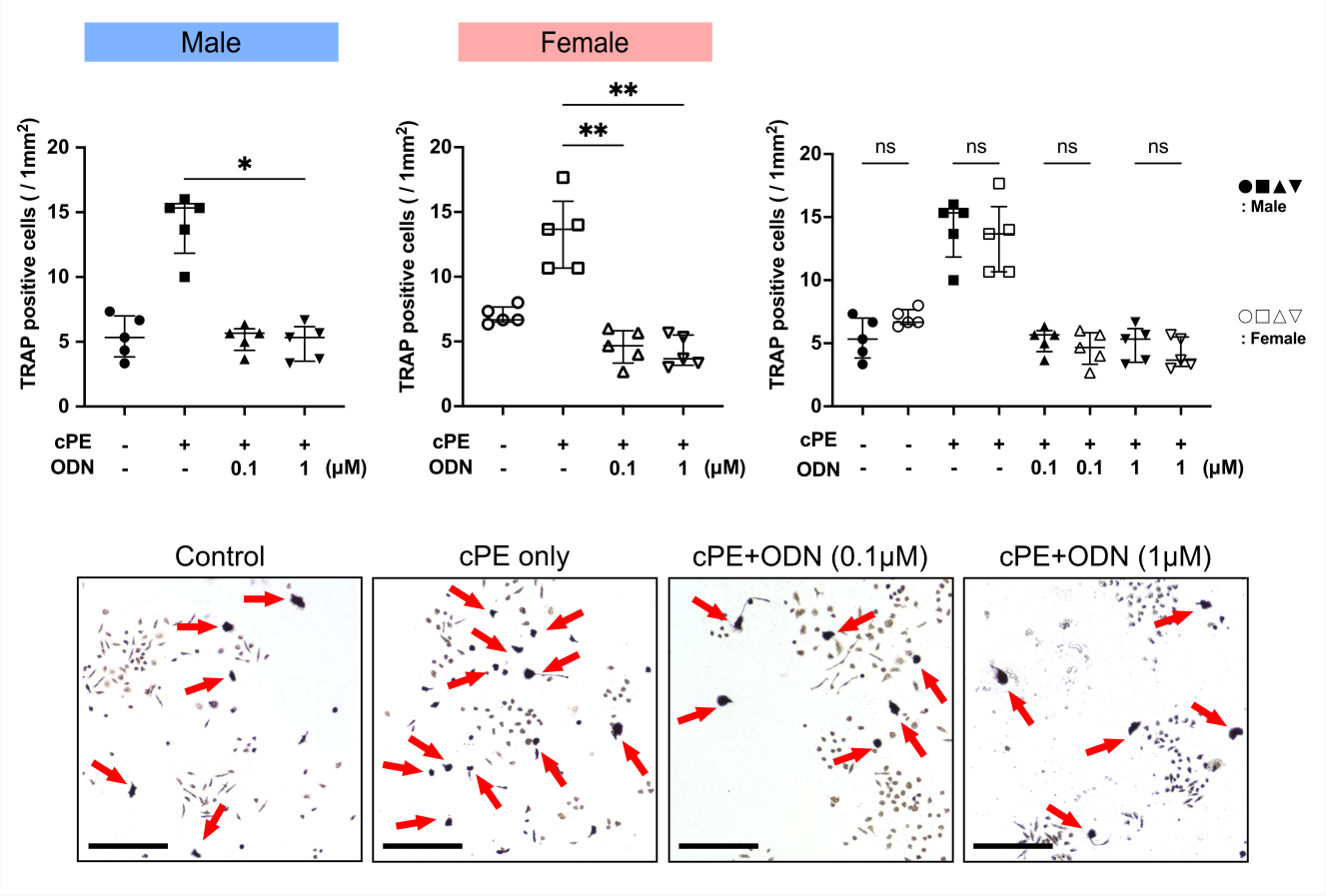

Fig. 7
In vitro osteoclastic differentiation assay pre-treated with contaminated polyethylene particles (cPE). Quantitative analysis of tartrate-resistant acid phosphatase (TRAP) staining in vitro is shown. TRAP-positive cells were evaluated within the range of 1 mm^2 (n = 5, each group). The comparison of TRAP-positive cells between male and female cells is also shown. Representative images of TRAP-positive cells (red arrows) are shown (scale bar = 250 μm). *p < 0.05, **p < 0.01, Kruskal-Wallis test with Dunn's multiple comparisons. ns, non-significant; ODN, oligodeoxynucleotides.

inflammatory stimulus, in which males and BALB/c mice are easily affected.

To investigate the effect of the NF-κB direct-targeted treatment at different stages, we performed another in vitro assay using the NF-κB inhibitor before inflammation was induced. Pre-treatment with the NF-κB decoy ODN upregulated osteogenic differentiation only in male mice, however TRAP-positive cells were decreased in both sexes. Earlier intervention with NF-κB inhibitor decreased the number of cPE-induced osteoclasts, when exposed to a subsequent inflammatory stimulus. These results indicate that NF-κB inhibition would be successful in females to protect them from subsequent bone resorption prior to the onset of diseases, or before the recurrence of inflammation. This concept is consistent with the success of denosumab in postmenopausal osteoporosis. In inflammatory conditions, NF-κB direct-targeted treatment might be more beneficial for males than females in terms of osteogenesis.

The current study has limitations. First, a previous study reported the results of a sham control group in male mice, but not females, therefore we could not anticipate any sex-related differences.[43] Second, the effect of NF-κB decoy ODN in this murine model was evaluated only at six weeks after primary surgery. Additional analyses at different timepoints might provide further information about the functional effects of NF-κB decoy ODN on osteogenesis and bone resorption in chronic inflammation. Third, the sex differences reported in this study were obtained from murine in vitro and in vivo experiments. To translate this finding into the clinical situations, further investigation using human primary samples is required. Future studies using RNA sequencing and other novel methods will also elucidate sex-dependent osteogenic differences during chronic inflammation.

In conclusion, we demonstrated sex-dependent differences in osteogenesis for NF-κB direct-targeted treatment in chronic inflammatory conditions. At different inflammatory stages, NF-κB inhibition could be practical both for males and females. In non-inflammatory conditions, the NF-κB decoy ODN could mitigate subsequent bone destruction in females. NF-κB inhibition was effective in males even in chronic inflammatory conditions, not only by mitigating osteoclastogenesis but also by facilitating osteogenesis. Thus, NF-κB decoy ODN could be a therapeutic target for orthopaedic conditions related to chronic inflammation, especially in males. It is critical to address sex-based differences in the effects of chronic inflammatory diseases on bone to optimize the outcome of drug interventions in both sexes.

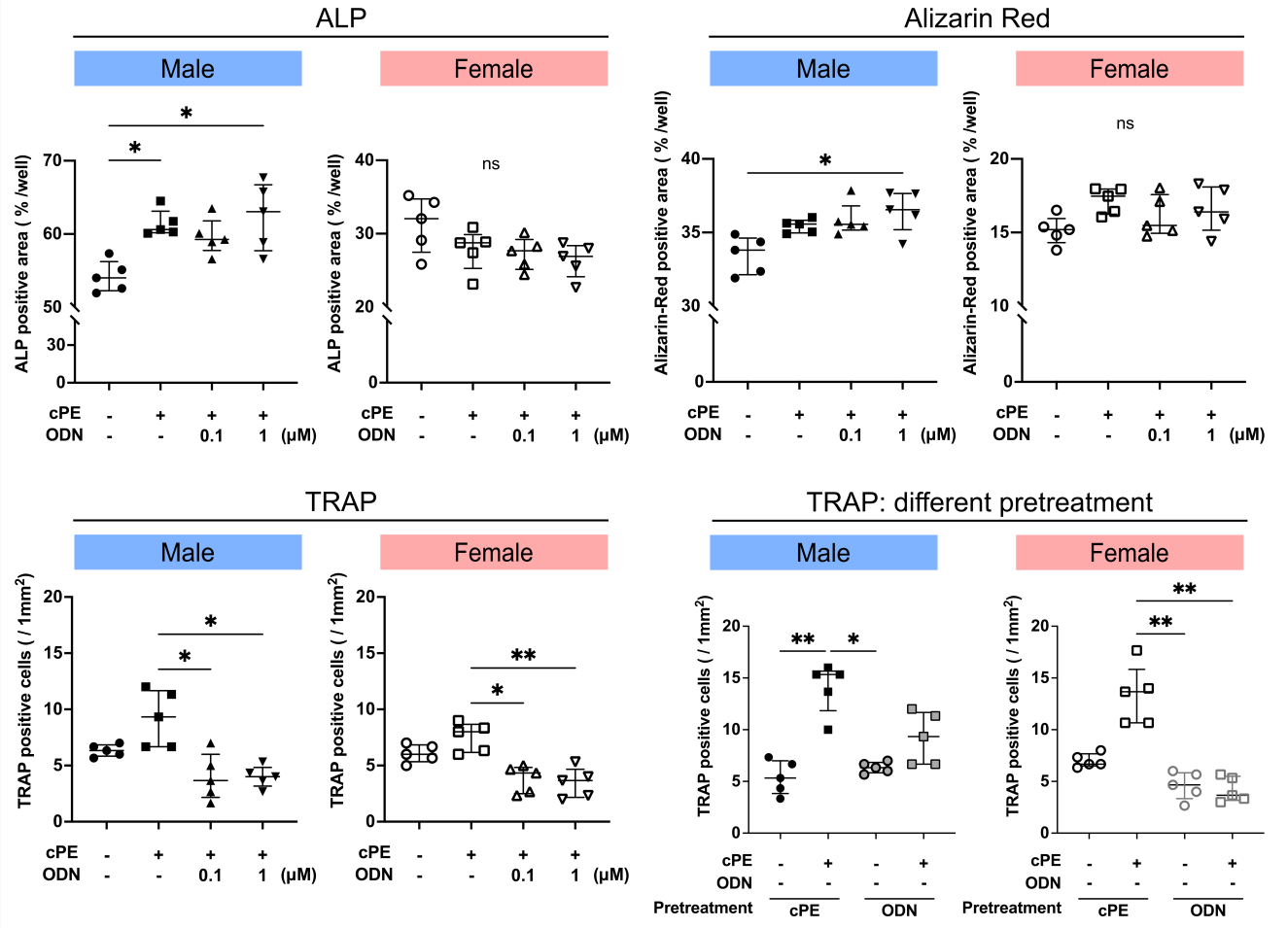

Fig. 8
In vitro osteogenic and osteoclastic differentiation assay pre-treated with nuclear factor kappa B (NF-κB) decoy oligodeoxynucleotide (ODN). Quantitative analysis of alkaline phosphatase (ALP), Alizarin Red, and tartrate-resistant acid phosphatase (TRAP) staining pre-treated with NF-κB decoy ODN are shown (n = 5, each group). Quantitative analysis of TRAP staining between different pre-treatment groups is also shown (n = 5, each group). *p < 0.05; **p < 0.01, Kruskal-Wallis test with Dunn's multiple comparisons. cPE, contaminated polyethylene particles; ns, non-significant.

Supplementary material

Table showing primers for real-time reverse transcription polymerase chain reaction. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

References

1. **Zhou Y**, **Li J**, **Xu F**, **Ji E**, **Wang C**, **Pan Z**. Long noncoding RNA H19 alleviates inflammation in osteoarthritis through interactions between TP53, IL-38, and IL-36 receptor. *Bone Joint Res*. 2022;11(8):594–607.
2. **Liu YD**, **Liu JF**, **Liu B**. N,N-Dimethylformamide inhibits high glucose-induced osteoporosis via attenuating MAPK and NF-κB signalling. *Bone Joint Res*. 2022;11(4):200–209.
3. **Kim MH**, **Choi LY**, **Chung JY**, **Kim E-J**, **Yang WM**. Auraptene ameliorates osteoporosis by inhibiting RANKL/NFATc1 pathway-mediated bone resorption based on network pharmacology and experimental evaluation. *Bone Joint Res*. 2022;11(5):304–316.
4. **Mauro D**, **Thomas R**, **Guggino G**, **Lories R**, **Brown MA**, **Ciccia F**. Ankylosing spondylitis: an autoimmune or autoinflammatory disease? *Nat Rev Rheumatol*. 2021;17(7):387–404.
5. **Ji M**, **Ryu HJ**, **Hong JH**. Signalling and putative therapeutic molecules on the regulation of synoviocyte signalling in rheumatoid arthritis. *Bone Joint Res*. 2021;10(4):285–297.
6. **Goodman SB**, **Maruyama M**. Inflammation, bone healing and osteonecrosis: From bedside to bench. *J Inflamm Res*. 2020;13:913–923.
7. **Robinson WH**, **Lepus CM**, **Wang Q**, **et al**. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2016;12(10):580–592.
8. **Loi F**, **Córdova LA**, **Pajarinen J**, **Lin T**, **Yao Z**, **Goodman SB**. Inflammation, fracture and bone repair. *Bone*. 2016;86:119–130.
9. **Mitchell JP**, **Carmody RJ**. NF-κB and the transcriptional control of inflammation. *Int Rev Cell Mol Biol*. 2018;335:41–84.
10. **Lawrence T**. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*. 2009;1(6):a001651.
11. **Yao Q**, **Wu X**, **Tao C**, **et al**. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. *Signal Transduct Target Ther*. 2023;8(1):56.
12. **Lu R**, **Wang Y-G**, **Qu Y**, **et al**. Dihydrocaffeic acid improves IL-1β-induced inflammation and cartilage degradation via inhibiting NF-κB and MAPK signalling pathways. *Bone Joint Res*. 2023;12(4):259–273.
13. **Lin T-H**, **Pajarinen J**, **Lu L**, **et al**. NF-κB as a therapeutic target in inflammatory-associated bone diseases. *Adv Protein Chem Struct Biol*. 2017;107:117–154.
14. **Lin T-H**, **Sato T**, **Barcay KR**, **et al**. NF-κB decoy oligodeoxynucleotide enhanced osteogenesis in mesenchymal stem cells exposed to polyethylene particle. *Tissue Eng Part A*. 2015;21(5–6):875–883.
15. **Roman-Blas JA**, **Jimenez SA**. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage*. 2006;14(9):839–848.
16. **McIntyre KW**, **Shuster DJ**, **Gillooly KM**, **et al**. A highly selective inhibitor of I kappa B kinase, BMS-345541, blocks both joint

inflammation and destruction in collagen-induced arthritis in mice. *Arthritis Rheum*. 2003;48(9):2652–2659.
17. **Fujihara SM**, **Cleaveland JS**, **Grosmaire LS**, **et al**. A D-amino acid peptide inhibitor of NF-kappa B nuclear localization is efficacious in models of inflammatory disease. *J Immunol*. 2000;165(2):1004–1012.
18. **Grzelecki D**, **Walczak P**, **Szostek M**, **Grajek A**, **Rak S**, **Kowalczewski J**. Blood and synovial fluid calprotectin as biomarkers to diagnose chronic hip and knee periprosthetic joint infections. *Bone Joint J*. 2021;103-B(1):46–55.
19. **Cummings SR**, **San Martin J**, **McClung MR**, **et al**. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. *N Engl J Med*. 2009;361(8):756–765.
20. **Shansky RM**, **Murphy AZ**. Considering sex as a biological variable will require a global shift in science culture. *Nat Neurosci*. 2021;24(4):457–464.
21. **Shansky RM**. Are hormones a "female problem" for animal research? *Science*. 2019;364(6443):825–826.
22. **Fish EN**. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol*. 2008;8(9):737–744.
23. **Yoon DY**, **Mansukhani NA**, **Stubbs VC**, **Helenowski IB**, **Woodruff TK**, **Kibbe MR**. Sex bias exists in basic science and translational surgical research. *Surgery*. 2014;156(3):508–516.
24. **Beery AK**, **Zucker I**. Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2011;35(3):565–572.
25. **Clayton JA**. Studying both sexes: a guiding principle for biomedicine. *FASEB J*. 2016;30(2):519–524.
26. **Ando W**, **Sakai T**, **Fukushima W**, **et al**. Japanese Orthopaedic Association 2019 Guidelines for osteonecrosis of the femoral head. *J Orthop Sci*. 2021;26(1):46–68.
27. **Martel-Pelletier J**, **Barr AJ**, **Cicuttini FM**, **et al**. Osteoarthritis. *Nat Rev Dis Primers*. 2016;2:16072.
28. **Gerosa M**, **De Angelis V**, **Riboldi P**, **Meroni PL**. Rheumatoid arthritis: a female challenge. *Womens Health (Lond)*. 2008;4(2):195–201.
29. **Jordan KM**, **Cooper C**. Epidemiology of osteoporosis. *Best Pract Res Clin Rheumatol*. 2002;16(5):795–806.
30. **Zink A**, **Braun J**, **Listing J**, **Wollenhaupt J**. Disability and handicap in rheumatoid arthritis and ankylosing spondylitis--results from the German rheumatological database. German Collaborative Arthritis Centers. *J Rheumatol*. 2000;27(3):613–622.
31. **Ishida M**, **Kawao N**, **Okada K**, **et al**. Serpina3n, dominantly expressed in female osteoblasts, suppresses the phenotypes of differentiated osteoblasts in mice. *Endocrinology*. 2018;159(11):3775–3790.
32. **Zhong ZA**, **Kot A**, **Lay Y-A**, **et al**. Sex-dependent, osteoblast stage-specific effects of progesterone receptor on bone acquisition. *J Bone Miner Res*. 2017;32(9):1841–1852.
33. **Zanotti S**, **Kalajzic I**, **Aguila HL**, **Canalis E**. Sex and genetic factors determine osteoblastic differentiation potential of murine bone marrow stromal cells. *PLoS One*. 2014;9(1):e86757.
34. **Shiau HJ**, **Reynolds MA**. Sex differences in destructive periodontal disease: a systematic review. *J Periodontol*. 2010;81(10):1379–1389.
35. **Lin T**, **Kohno Y**, **Huang J-F**, **et al**. NFκB sensing IL-4 secreting mesenchymal stem cells mitigate the proinflammatory response of macrophages exposed to polyethylene wear particles. *J Biomed Mater Res A*. 2018;106(10):2744–2752.
36. **Greenfield EM**, **Beidelschies MA**, **Tatro JM**, **Goldberg VM**, **Hise AG**. Bacterial pathogen-associated molecular patterns stimulate biological activity of orthopaedic wear particles by activating cognate Toll-like receptors. *J Biol Chem*. 2010;285(42):32378–32384.
37. **Shimizu H**, **Nakagami H**, **Morita S**, **et al**. New treatment of periodontal diseases by using NF-kappaB decoy oligodeoxynucleotides via prevention of bone resorption and promotion of wound healing. *Antioxid Redox Signal*. 2009;11(9):2065–2075.
38. **Pajarinen J**, **Nabeshima A**, **Lin T-H**, **et al**. * Murine model of progressive orthopedic wear particle-induced chronic inflammation and osteolysis. *Tissue Eng Part C Methods*. 2017;23(12):1003–1011.
39. **Nabeshima A**, **Pajarinen J**, **Lin T-H**, **et al**. Mutant CCL2 protein coating mitigates wear particle-induced bone loss in a murine continuous polyethylene infusion model. *Biomaterials*. 2017;117:1–9.
40. **Lin T-H**, **Pajarinen J**, **Sato T**, **et al**. NF-κB decoy oligodeoxynucleotide mitigates wear particle-associated bone loss in the murine continuous infusion model. *Acta Biomater*. 2016;41:273–281.
41. **Kushioka J**, **Toya M**, **Shen H**, **et al**. Therapeutic effects of MSCs, genetically modified MSCs, and NFκB-inhibitor on chronic inflammatory osteolysis in aged mice. *J Orthop Res*. 2023;41(5):1004–1013.
42. **Bankhead P**, **Loughrey MB**, **Fernández JA**, **et al**. QuPath: Open source software for digital pathology image analysis. *Sci Rep*. 2017;7(1):16878.
43. **Utsunomiya T**, **Zhang N**, **Lin T**, **et al**. Suppression of NF-κB-induced chronic inflammation mitigates inflammatory osteolysis in the murine continuous polyethylene particle infusion model. *J Biomed Mater Res A*. 2021;109(10):1828–1839.
44. **Brooks PJ**, **Glogauer M**, **McCulloch CA**. An overview of the derivation and function of multinucleated giant cells and their role in pathologic processes. *Am J Pathol*. 2019;189(6):1145–1158.
45. **Miron RJ**, **Zohdi H**, **Fujioka-Kobayashi M**, **Bosshardt DD**. Giant cells around bone biomaterials: Osteoclasts or multi-nucleated giant cells? *Acta Biomater*. 2016;46:15–28.
46. **Zhang N**, **Lo C-W**, **Utsunomiya T**, **et al**. PDGF-BB and IL-4 co-overexpression is a potential strategy to enhance mesenchymal stem cell-based bone regeneration. *Stem Cell Res Ther*. 2021;12(1):40.
47. **Nathan K**, **Lu LY**, **Lin T**, **et al**. Precise immunomodulation of the M1 to M2 macrophage transition enhances mesenchymal stem cell osteogenesis and differs by sex. *Bone Joint Res*. 2019;8(10):481–488.
48. **Callewaert F**, **Sinnesael M**, **Gielen E**, **Boonen S**, **Vanderschueren D**. Skeletal sexual dimorphism: relative contribution of sex steroids, GH-IGF1, and mechanical loading. *J Endocrinol*. 2010;207(2):127–134.
49. **Bouman A**, **Heineman MJ**, **Faas MM**. Sex hormones and the immune response in humans. *Hum Reprod Update*. 2005;11(4):411–423.
50. **Olsen NJ**, **Kovacs WJ**. Hormones, pregnancy, and rheumatoid arthritis. *J Gend Specif Med*. 2002;5(4):28–37.
51. **Glatt V**, **Canalis E**, **Stadmeyer L**, **Bouxsein ML**. Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. *J Bone Miner Res*. 2007;22(8):1197–1207.
52. **Li S**, **He T**, **Wu D**, **et al**. Conditional knockout of PKC-δ in osteoclasts favors bone mass accrual in males due to decreased osteoclast function. *Front Cell Dev Biol*. 2020;8:450.
53. **Soldin OP**, **Mattison DR**. Sex differences in pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet*. 2009;48(3):143–157.
54. **Klein SL**, **Schiebinger L**, **Stefanick ML**, **et al**. Opinion: Sex inclusion in basic research drives discovery. *Proc Natl Acad Sci U S A*. 2015;112(17):5257–5258.
55. **Nakashima T**, **Hayashi M**, **Fukunaga T**, **et al**. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med*. 2011;17(10):1231–1234.
56. **Ikebuchi Y**, **Aoki S**, **Honma M**, **et al**. Coupling of bone resorption and formation by RANKL reverse signalling. *Nature*. 2018;561(7722):195–200.
57. **Karin M**, **Greten FR**. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*. 2005;5(10):749–759.
58. **Novack DV**, **Yin L**, **Hagen-Stapleton A**, **et al**. The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis. *J Exp Med*. 2003;198(5):771–781.
59. **Ruocco MG**, **Maeda S**, **Park JM**, **et al**. I{kappa}B kinase (IKK){beta}, but not IKK{alpha}, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. *J Exp Med*. 2005;201(10):1677–1687.
60. **Yip KHM**, **Zheng MH**, **Feng HT**, **Steer JH**, **Joyce DA**, **Xu J**. Sesquiterpene lactone parthenolide blocks lipopolysaccharide-induced osteolysis through the suppression of NF-kappaB activity. *J Bone Miner Res*. 2004;19(11):1905–1916.
61. **Zawawi MSF**, **Marino V**, **Perilli E**, **et al**. Parthenolide reduces empty lacunae and osteoclastic bone surface resorption induced by polyethylene particles in a murine calvarial model of peri-implant osteolysis. *J Biomed Mater Res A*. 2015;103(11):3572–3579.

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Funding statement

The authors disclose receipt of the following financial or material support for the research, authorship, and/or publication of this article: this work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, Grant/Award Numbers: R01AR073145, R01AR063717.

ICMJE COI statement

The authors declare no conflicts of interest.

Data sharing

All data generated or analyzed during this study are included in the published article and/or in the supplementary material.

Acknowledgements

The authors thank Victoria Teissier, Ejun Huang, and Xueping Li for supporting the experiments and manuscript preparation.

Ethical review statement

The animal experimental protocol was reviewed and approved by Stanford's Administrative Panel on Laboratory Animal Care (Protocol number 17566). Institutional Guidelines for the Care and Use of Laboratory Animals were followed in all aspects of this project. All studies were carried out in compliance with the ARRIVE guidelines.

Open access funding

The authors report that they received open access funding for their manuscript via National Institutes of Health (NIH) grants (R01AR073145 and R01AR063717) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS).