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Full Length Article

Interleukin 4 promotes anti-inflammatory macrophages that clear cartilage debris and inhibits osteoclast development to protect against osteoarthritis

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ABSTRACT

Objective: Osteoarthritis (OA), the leading cause of joint failure, is characterized by breakdown of articular cartilage and remodeling of subchondral bone in synovial joints. Despite the high prevalence and debilitating effects of OA, no disease-modifying drugs exist. Increasing evidence, including genetic variants of the interleukin 4 (IL-4) and IL-4 receptor genes, implicates a role for IL-4 in OA, however, the mechanism underlying IL-4 function in OA remains unknown. Here, we investigated the role of IL-4 in OA pathogenesis.

Methods: Il4-, myeloid-specific-*Il4ra-*, and *Stat6*-deficient and control mice were subjected to destabilization of the medial meniscus to induce OA. Macrophages, osteoclasts, and synovial explants were stimulated with IL-4 *in vitro*, and their function and expression profiles characterized.

Results: Mice lacking IL-4, IL-4Ra in myeloid cells, or STAT6 developed exacerbated cartilage damage and osteophyte formation relative to WT controls. *In vitro* analyses revealed that IL-4 downregulates osteoarthritis-associated genes, enhances macrophage phagocytosis of cartilage debris, and inhibits osteoclast differentiation and activation *via* the type I receptor.

Conclusion: Our findings demonstrate that IL-4 protects against osteoarthritis in a myeloid and STAT6-dependent manner. Further, IL-4 can promote an immunomodulatory microenvironment in which joint-resident macrophages polarize towards an M2 phenotype and efficiently clear pro-inflammatory debris, and osteoclasts maintain a homeostatic level of activity in subchondral bone. These findings support a role for IL-4 modulation of myeloid cell types in maintenance of joint health and identify a pathway that could provide therapeutic benefit for osteoarthritis.

1. Introduction

Osteoarthritis (OA), the most common type of arthritis, is a debilitating joint disease for which no disease-modifying therapy is available [1]. The rapidly increasing incidence of osteoarthritis due in part to the aging population and obesity epidemic [2] highlights the need to further understand its underlying pathobiology in order to develop disease-modifying therapeutics.

Osteoarthritis was previously thought to be a mechanical 'wear-and-tear' disease. However, accumulating evidence implicates chronic, low-grade inflammation as a key driver of osteoarthritis development, including identification of inflammatory mediators in a number of osteoarthritic tissues [3]. Further, the presence of synovitis, characterized by immune cell infiltration into the synovium, is considered a hallmark of osteoarthritis and correlates with clinical symptoms, disease severity [3] and progression of structural disease [4].

During the past decade, interleukin 4 (IL-4), a type 2 inflammatory cytokine best known for its roles in promoting atopic disease and tissue healing, has been linked to osteoarthritis. Genomic analyses identified associations between single nucleotide polymorphisms (SNPs) in the IL-4 receptor (*IL4R*) gene and hand osteoarthritis [5], knee osteoarthritis [6], and hip osteoarthritis [7]. SNPs in the IL-4 (*IL4*) gene, have also been associated with knee osteoarthritis [8]. Further, levels of soluble IL-4 receptor, an IL-4 modulator, are elevated in serum from patients with osteoarthritis [9].

Despite these genetic associations, the underlying mechanism for IL-

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4 in osteoarthritis pathogenesis has yet to be explained. Multiple IL-4-responsive cell types, including macrophages and osteoclasts, are present in the joint and are known to be dysregulated in osteoarthritis. Further, macrophages and macrophage-related cytokines and chemokines have been implicated in osteoarthritis pathogenesis [10–12], and activated macrophages are present in multiple tissues of human osteoarthritic joints [13]. Osteoclasts, another joint-resident myeloid cell type, have also been implicated in osteoarthritis both in animal models [14–19] and in humans [20–23]. A key role for osteoclast biology in osteoarthritis development is further supported by the emergence of osteoclast-targeting therapies [24,25].

Most cell types are capable of responding to IL-4, and it is well-established that macrophages polarize to an "anti-inflammatory" M2 phenotype in response to IL-4 [26]. These immune-modulatory M2 macrophages are associated with wound healing, debris clearance, and other anti-inflammatory responses [27]. In addition to its role in innate immunity, IL-4 also potently inhibits osteoclast differentiation and activation [28–30].

Osteoarthritis pathogenesis lays at the intersection of immune system and musculoskeletal biology, particularly in cases of osteoarthritis arising following a history traumatic joint injury which involve cycles of chronic tissue injury and repair. Thus, IL-4-dependent macrophage polarization and osteoclast inhibition could be critical determinants in osteoarthritis development. In this study, we investigate the role of IL-4 in osteoarthritis pathogenesis, and the effects of IL-4 signaling on osteoclasts and macrophages. Our findings suggest that IL-4 modulation of myeloid cell types promotes the maintenance of joint health and reveals a pathway that could provide therapeutic benefit for osteoarthritis.

2. Materials and methods

2.1. Animals

C57BL/6 J, BALB/cJ, IL-4$^{-/-}$ (BALB/c-*Il4*^{tm2Nnt}/J), STAT6−/− (C·129S2-*Stat6*^{tm1Gru}/J), and IL-4Rα−/− (BALB/c-*Il4ra*^{tm1Sz}/J) mice were purchased from The Jackson Laboratory. IL-13Rα1−/− (C57BL/6-*Il13ra1*$^{tm1.2mrl}$) mice were cryorecovered from Taconic. Myeloid-specific IL-4Rα−/− (LysM$^{WT/Cre}$IL-4Rα$^{fl/fl}$) mice were provided by Aida Habtezion (Stanford University) [31].

2.2. Mouse model of osteoarthritis

All experimental and control animals were age and sex matched using 20-week-old male mice for all experiments. Destabilization of the medial meniscus (DMM) was performed as previously described [32–34]. All animal studies were performed under protocols approved by the Stanford Committee of Animal Research and in accordance with National Institutes of Health guidelines.

2.3. Study approval

Human samples studied were under protocols approved by the Stanford Institutional Review Board (IRB) and with the subjects' written informed consent.

2.4. Human synovial explant culture

Human synovial tissue explants were obtained from the knees of patients undergoing total knee arthroplasty (TKA) for end-stage osteoarthritis, stimulated with recombinant human IL-4 (Peprotech), and subsequently harvested for gene expression analysis.

2.5. Human macrophage culture

Human macrophages were cultured from peripheral blood monocytes as previously described [35] and stimulated with recombinant human IL-4 (R&D). Supernatants were collected for cytokine quantification, and cells were harvested for gene expression analysis. Macrophages were also cultured with human osteoarthritic cartilage debris that was harvested from patients undergoing TKA and that was labeled with amine-reactive, pH-sensitive pHrodo Red dye (ThermoFisher). Macrophages were then stained for CD11b and analyzed by flow cytometry for CD11b and pHrodo Red positivity to assess phagocytic capacity. All experiments utilized three independent biologic samples, each with two technical replicates per sample, and results were repeated in three independent experiments. Data shown are means and standard deviations of biologic replicates from a representative independent experiment. The sample size used was modeled after recent publications that employ similar methodology for *in vitro* macrophage functional assays [35].

2.6. Mouse macrophage culture

Mouse bone marrow-derived macrophages were isolated and cultured from long bones of 8-week-old male mice according to standard protocols. Macrophages were stimulated with IL-4 and cells collected for gene expression analysis.

All experimental conditions were performed in triplicate and were repeated in at least three independent experiments each using cells harvested from new mice. Data shown are means and standard deviations for the three technical replicates from a representative experiment. All experiments were performed using age-, sex-, and background-matched controls.

2.7. Mouse osteoclast culture

Mouse bone marrow-derived osteoclasts were isolated and cultured from long bones of 8-week-old mice as previously described [36]. Osteoclasts were stimulated with IL-4, resulting cells were stained for tartrate-resistant acid phosphatase (TRAP), and supernatants were assayed for TRAP activity as previously described [37] (Sigma). Cells were collected for gene expression analysis.

All experimental conditions were performed in triplicate and were repeated in at least three independent experiments each using cells harvested from new mice. Data shown are means and standard deviations for the three technical replicates from a representative experiment. All experiments were performed using age-, sex-, and background-matched controls.

2.8. Gene expression analysis

RNA was extracted from cell and tissue lysates using RNeasy system (Qiagen) and cDNA generated using High-Capacity cDNA Reverse Transcription Kit (AppliedBiosystems). Gene expression analysis was performed by quantitative polymerase chain reaction (qPCR) using TaqMan Gene Expression Assays (AppliedBiosystems) and TaqMan probes (Supplementary Table 1). Expression levels were quantified as fold change relative to unstimulated controls, using the $2^{-\Delta\Delta Ct}$ method [38].

2.9. Statistics

For two group comparisons, data were analyzed using two-tailed, unpaired Student's *t*-test in Prism (Graphpad, version 8.1). For multiple group comparisons, data were analyzed using one-way ANOVA and *post hoc* comparison with Tukey correction in Prism. $p < 0.05$ was considered statistically significant.

Detailed methods are described in online supplementary materials.

3. Results

3.1. IL-4, acting on myeloid-derived cell types and through its STAT6 signaling intermediate, protects against the development of osteoarthritis in mice

To investigate the role of IL-4 in osteoarthritis pathogenesis we utilized the destabilization of medial meniscus (DMM) model, a widely-accepted murine model of osteoarthritis development induced by surgical transection of the medial meniscus [33]. We performed DMM in mice globally deficient for the *Il4* gene ($Il4^{-/-}$) and *Il4*-sufficient ($Il4^{+/+}$) wild-type control mice. Twenty weeks after surgery, $Il4^{-/-}$ mice showed significantly exacerbated cartilage damage and osteophyte formation compared to $Il4^{+/+}$ mice (Fig. 1 A and B). We also analyzed osteoarthritis development in mice deficient for the IL-4Ra subunit of the IL-4 receptor complex in myeloid cell types ($LysM^{Cre}Il4ra^{fl/fl}$) and in IL-4Ra-sufficient ($LysM^{Cre}Il4ra^{+/+}$) wild-type control mice following DMM. Twenty weeks after surgery, $LysM^{Cre}Il4ra^{fl/fl}$ mice showed significantly increased cartilage damage and osteophyte formation compared to $LysM^{Cre}Il4ra^{+/+}$ mice (Fig. 1 C and D). Finally, we analyzed osteoarthritis development in mice deficient for *Stat6* ($Stat6^{-/-}$) and Stat6-sufficient mice ($Stat6^{+/+}$) wild-type control mice following DMM. Twenty weeks after surgery, $Stat6^{-/-}$ mice showed significantly increased cartilage damage and osteophyte formation compared to

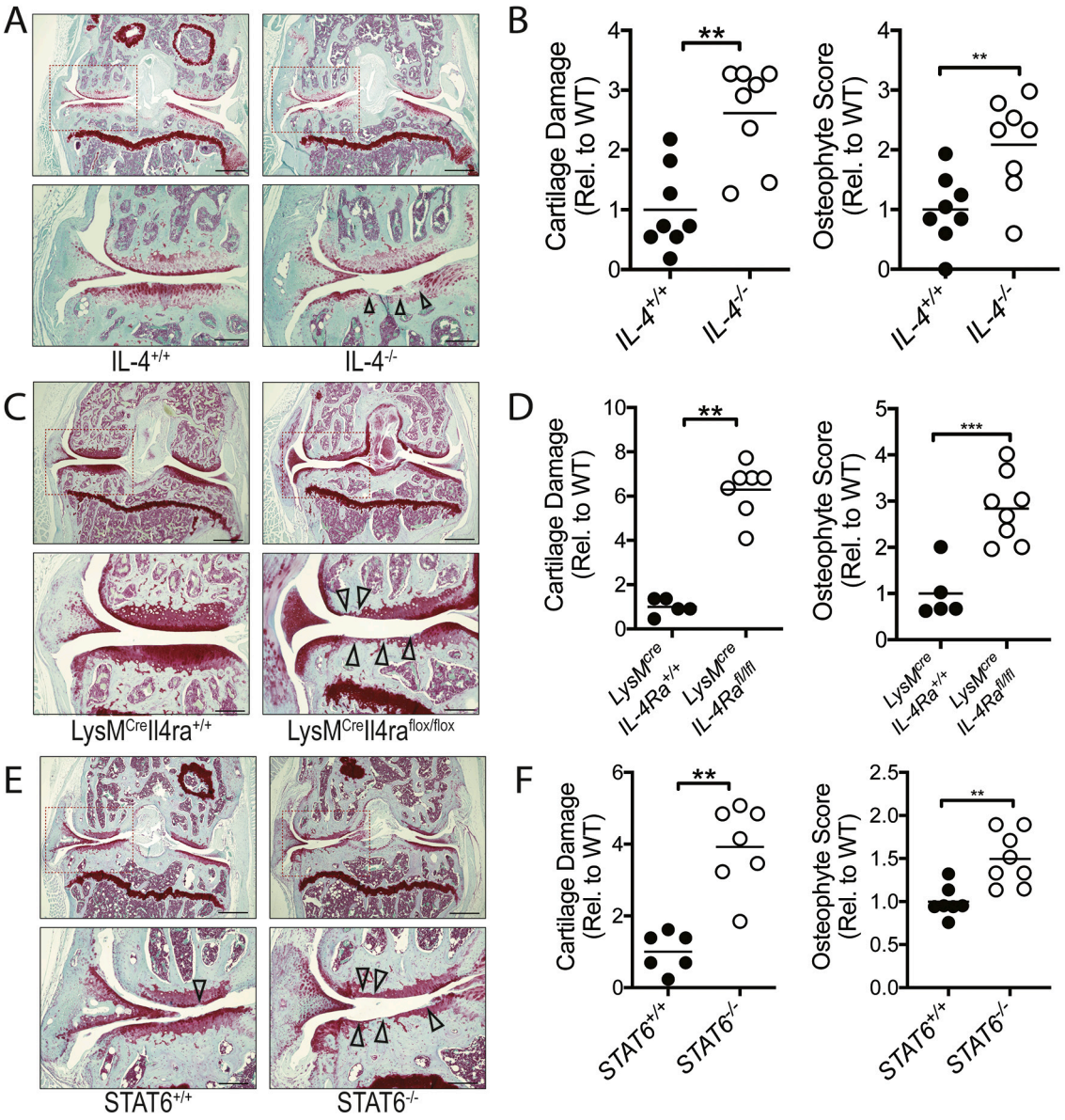

Fig. 1. IL-4, acting on myeloid-derived cell types and through the STAT6 signaling intermediate, protects against the development of osteoarthritis in mice.
(A) Representative low-magnification (upper) and high-magnification (lower) histologic images of cartilage degeneration in Safranin O-stained sections of the medial region of the femoro-tibial joint from wild type ($IL\text{-}4^{+/+}$) and IL-4 deficient ($IL\text{-}4^{-/-}$) mice subjected to DMM. (B) Quantification of the cartilage damage and osteophyte formation from (A) ($n = 8$ mice per group). (C) Representative histologic images of cartilage degeneration in Safranin O-stained sections of the medial region of the femoro-tibial joint from $LysM^{Cre}Il4ra^{+/+}$ and $LysM^{Cre}Il4ra^{fl/fl}$ mice subjected to DMM. (D) Quantification of the cartilage damage and osteophyte formation from panel C ($n = 5$ mice $LysM^{Cre}Il4ra^{+/+}$; $n = 8$ $LysM^{Cre}Il4ra^{fl/fl}$). (E) Representative histologic images of cartilage degeneration in Safranin O-stained sections of the medial region of the femoro-tibial joint from $STAT6^{+/+}$ and $STAT6^{-/-}$ mice subjected to DMM. (F) Quantification of the cartilage damage and osteophyte formation from (E) ($n = 6$ mice $STAT6^{+/+}$; $n = 7$ $STAT6^{-/-}$). Scale bars in the low-magnification (upper) images, 500 μm; scale bars in the high-magnification (lower) images, 200 μm. *P* values were calculated using two-tailed, unpaired Student's *t*-test; $*p < 0.05$, $**p < 0.01$.

Stat6$^{+/+}$ mice (Fig. 1 E and F). Together these data demonstrate a critical role of IL-4 in protection against post-traumatic OA, potentially through signaling in myeloid cell types.

3.2. IL-4 stimulation of human macrophages promotes M2 polarization and enhanced phagocytosis of cartilage debris

IL-4 is known to polarize macrophages towards the M2, anti-inflammatory, phenotype. We confirmed this effect, by stimulating peripheral blood monocyte cell (PBMC)-derived macrophages from healthy human donors ($n = 3$) with recombinant IL-4 protein. Analysis of expression of genes and secretion of cytokines known to be associated with the M1 and M2 phenotypes revealed that 24 h after stimulation of PBMC-derived macrophages with recombinant IL-4 protein, expression of the M2-associated gene *CD206* was increased over unstimulated control cells (Fig. S1A). Similarly, production of the M2-associated cytokines CCL24 and CCL18 [39] was increased in IL-4-stimulated macrophages as compared to unstimulated control cells (Fig. S1B). Together, these results recapitulate previous findings [40] that indicate that stimulation of unpolarized macrophages with IL-4 polarizes them towards the M2 phenotype.

Cartilage debris is present in osteoarthritic synovial fluids and joint tissues, as a result of cartilage breakdown [41]. Evidence from prior studies suggest that phagocytosis of cartilage debris by macrophages, or

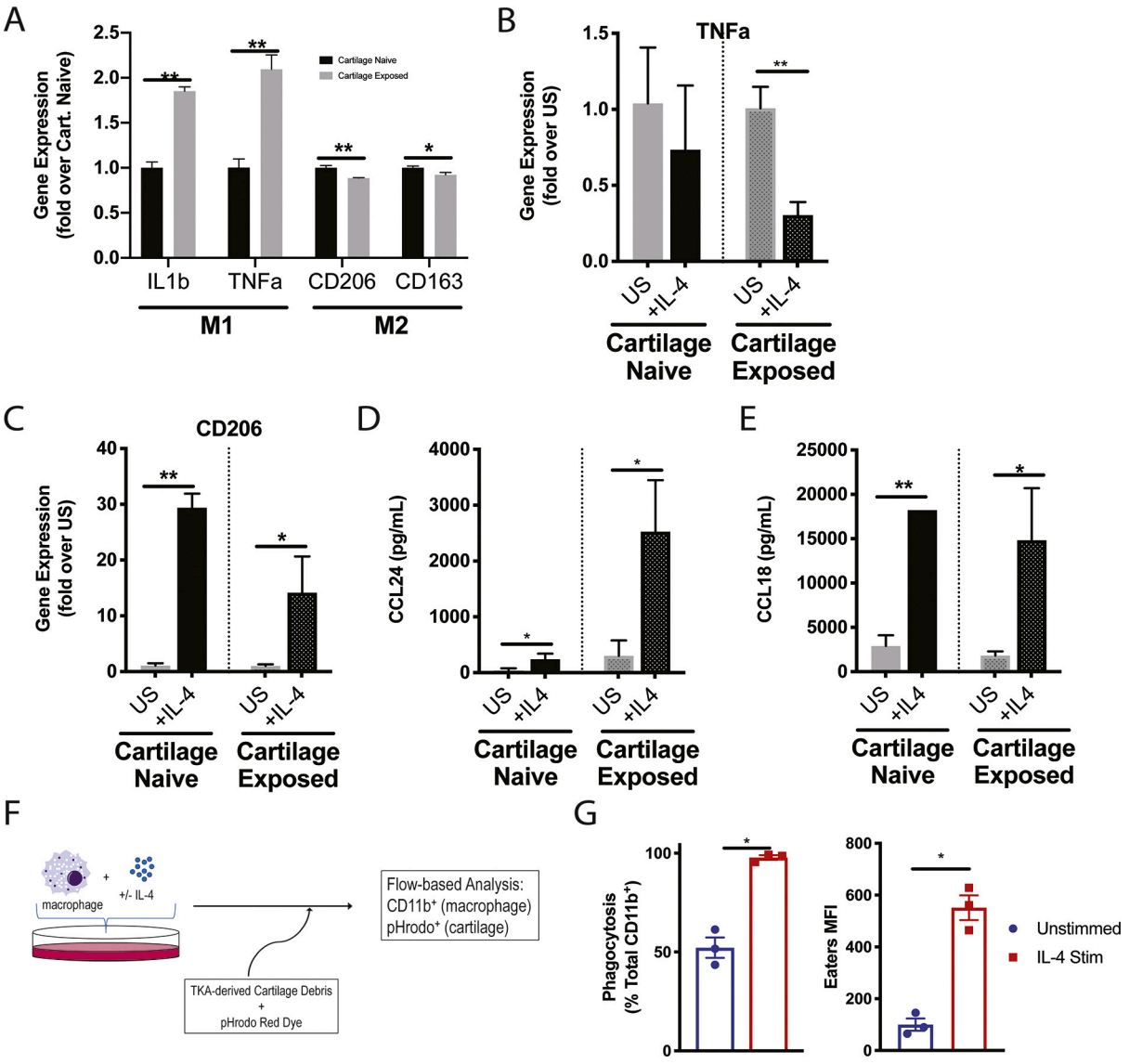

Fig. 2. IL-4 stimulation of human macrophages promotes M2 polarization and enhanced phagocytosis of cartilage debris. (A) Gene expression of M1 and M2-associated genes, as measured by quantitative PCR analysis of relative mRNA expression, in macrophages exposed to cartilage debris (Cartilage Exposed) or not (Cartilage Naïve) for 24 h. Expression is reported as fold change over the cartilage naïve condition. (B) Gene expression of M1-associated gene, TNFa, and (C) M2-associated gene, CD206, as measured by quantitative PCR analysis of relative mRNA expression, in US or + IL-4 macrophages exposed to cartilage debris (Cartilage Exposed) or not (Cartilage Naïve). (D,E) Supernatant cytokine concentration of M2-associated cytokines, CCL24 and CCL18, as measured by Luminex magnetic bead assay, in US or + IL-4 macrophages exposed to cartilage debris (Cartilage Exposed) or not (Cartilage Naïve). (F) Schematic of the macrophage phagocytosis assay in which IL-4 treated or untreated macrophages are exposed to pHrodo Red-labeled cartilage debris derived from patients undergoing total joint arthroplasty (TKA) and subsequently analyzed by flow cytometry. (G) Flow cytometry-based assessment of phagocytosis of labeled cartilage debris by IL-4 stimulated and unstimulated macrophages; results are shown as percentage of CD11b$^+$ macrophages that are CD11b$^+$pHrodo$^+$(left) and mean fluorescence intensity (MFI) of CD11b$^+$pHrodo$^+$ (termed: 'Eaters', right). *P* values were calculated using two-tailed, unpaired Student's *t*-test; $*p < 0.05$, $**p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other cell types including phagocytic chondrocytes, may be protective against osteoarthritis development [42,43]. We tested whether cartilage debris can promote polarization and phagocytic function of macrophages, by exposing PBMC-derived macrophages from healthy human donors to cartilage debris derived from end-stage osteoarthritis joints. Exposure of unpolarized macrophages to osteoarthritis joint cartilage debris resulted in elevated expression of the M1-associated genes *IL1b* and *TNFa*, and decreased expression of the M2-associated genes *CD206* and *CD163* (Fig. 2A). These findings suggest that cartilage debris is a proinflammatory stimulus that promotes the M1 macrophage phenotype associated with production of pro-inflammatory cytokines, typically for host defense against intracellular pathogens [40].

Upon finding that cartilage debris induces the M1 phenotype in macrophages, we next assessed whether polarization to the M2 phenotype, prior to exposure to cartilage debris, was sufficient to maintain the M2 phenotype in response to a pro-inflammatory stimulus. We stimulated PMBC-derived human macrophages from healthy donors ($n = 3$) with recombinant IL-4 protein followed by exposure to cartilage debris derived from end-stage osteoarthritic joints. Following exposure to osteoarthritis cartilage debris, IL-4-stimulated macrophages exhibited decreased expression levels of the M1-associated gene TNFa as compared to macrophages that were not stimulated by IL-4 (unstimulated; US) prior to exposure to cartilage debris (Fig. 2B). Further, IL-4-stimulated macrophages had increased expression levels of the M2-associated gene *CD206*, following exposure to cartilage debris, as compared to macrophages that were unstimulated prior to exposure to cartilage debris (Fig. 2C). Similarly, macrophages that were pre-stimulated with IL-4 had increased levels of production of the M2-associated cytokines, CCL24 and CCL18, as compared to macrophages that were unstimulated prior to exposure to cartilage debris (Fig. 2D,E). These findings suggest that the M2 phenotype can be maintained in the presence of a pro-inflammatory stimulus, if exposed to IL-4 prior to exposure to the stimulus.

We further analyzed the potential function of M2 macrophages in osteoarthritis by assessing whether IL-4 stimulation of macrophages could enhance phagocytosis of cartilage debris. We treated PMBC-derived human macrophages from healthy donors ($n = 3$) either with or without recombinant IL-4 protein, followed by exposure to osteoarthritis cartilage debris that was covalently labeled with a pH-sensitive fluorescent dye (pHrodo Red). Flow cytometry was used to quantify all macrophages ($CD11b^+$) as well as macrophages that took up cartilage debris through phagocytosis ($CD11b^+$, pHrodo Red^+) (Fig. 2F). Nearly all IL-4-stimulated macrophages stained positive for pHrodo Red, a significant increase as compared to the approximately half of unstimulated macrophages that stained positive for pHrodo Red following exposure to the labeled cartilage debris (Fig. 2G). Additionally, among $CD11b^+$pHrodo$^+$ cells (termed 'eaters'), IL-4 stimulated macrophages phagocytosed significantly more cartilage debris than unstimulated macrophages, as measured by mean fluorescence intensity (MFI) of pHrodo Red staining (Fig. 2G). This result suggests that phagocytosis of cartilage debris is stimulated to a greater degree in macrophages that have been exposed to IL-4.

3.3. IL-4 stimulation of murine macrophages downregulates osteoarthritis-associated genes and polarizes macrophages to the M2 state via the type 1 receptor

IL-4 can signal through two heterodimeric receptors, the type I and type II receptor. The type I receptor is composed of the IL-4Rα and the common gamma chain (γC) subunits, while the type II receptor is composed of the IL-4Rα and IL-13Rα1 subunits. IL-4Rα is widely expressed on most cell types, but availability of γC and IL-13Rα1 on the responding cell's surface determines which receptor type will be formed [26]. To gain further insights into which receptor IL-4 uses to promote clearance of cartilage debris in osteoarthritic joints, we cultured bone marrow (BM)-derived macrophages from mice deficient for genes encoding the IL-4Ra receptor subunit (*Il4ra*$^{-/-}$), the IL-13Ra1 receptor subunit (*Il13ra*$^{-/-}$), and mice sufficient for the IL-4 receptor (WT). The WT and receptor subunit-deficient macrophages were stimulated with recombinant murine IL-4 protein. Expression analysis revealed that IL-4 stimulation resulted in decreased expression of the osteoarthritis-related genes *Tnfa*, *Cxcl1* and *Cox2* in WT macrophages (Fig. 3A) and in *Il13ra*$^{-/-}$ macrophages (Fig. 3B), but not in *Il4ra*$^{-/-}$ macrophages (Fig. 3C). Expression analysis also revealed that IL-4 stimulation resulted in increased expression of the M2-associated genes, *Mrc1* and *Arg1,* and decreased expression of the M1-associated gene, *Tnfa* in WT macrophages (Fig. 3D) and *Il13ra*$^{-/-}$ macrophages (Fig. 3E), but not in *Il4ra*$^{-/-}$ macrophages (Fig. 3F). Of note, increased *Tnfa* expression is associated with both proinflammatory M1 macrophage phenotype [40,44] and OA [45], underscoring the understanding of the OA joint as a proinflammatory environment. These findings suggest that IL-4 can modulate the proinflammatory genes associated with both macrophage polarization and OA *via* the Type 1 receptor.

3.4. IL-4 potently inhibits murine osteoclast formation in a dose-dependent manner

IL-4 is known to potently inhibit osteoclast formation [28]. To validate this effect of IL-4 on osteoclasts, we stimulated mouse bone marrow (BM)-derived osteoclasts with recombinant IL-4 over six days of differentiation (Fig. 4A). Tartrate-resistant acid phosphatase (TRAP) staining revealed robust formation of multinucleated, TRAP-positive ($TRAP^+$) osteoclasts in the unstimulated condition, while very few multinucleated, $TRAP^+$ osteoclasts were generated in the IL-4 stimulated condition (Fig. 4B). Quantification of TRAP in cell culture supernatants revealed that IL-4 stimulation significantly inhibited TRAP secretion from osteoclasts(Fig. 4C). qPCR analysis revealed that IL-4 stimulation resulted in decreased expression of the *Il4ra*, Il2rg, and *Il13ra1* genes, which comprise subunits of the IL-4 receptor complexes (Fig. 4D). IL-4 stimulation also resulted in decreased expression of the osteoclast differentiation-associated genes *Nfatc1*, *Tnfrsf11a* and *Acp5* (Fig. 4E) and osteoclast activation genes *Ctsk*, *Mmp9*, *Calcr*, and *Car2* (Fig. 4F).

We also investigated the potency of IL-4 in inhibition of osteoclastogenesis by testing multiple doses of recombinant IL-4 stimulation. Stimulation with either 1 ng/mL or 10 ng/mL recombinant IL-4 resulted in lower measurable supernatant TRAP as compared to 0.1 ng/mL IL-4. No significant differences in supernatant TRAP levels were observed between stimulation with either 1 ng/mL or 10 ng/mL (Fig. 4G). Similarly, stimulation with either 1 ng/mL or 10 ng/mL recombinant IL-4 resulted in dramatic reductions in the expression of the osteoclast differentiation gene, *Acp5,* and the osteoclast activation gene, *Ctsk,* as compared to unstimulated osteoclasts, while stimulation with 0.1 ng/mL IL-4 resulted in relatively less inhibition of these genes (Figs. 4 H and I). These findings suggest that IL-4 can inhibit osteoclast differentiation and activation in a dose-dependent manner.

We further investigated the role of these effects of IL-4 on osteoclasts observed *in vitro* in our *in vivo* DMM model of post-traumatic OA through immunohistochemical staining of the monocyte marker CD68 [46] and osteoclast activity marker cathepsin K [47]. *Il4*$^{-/-}$ mice showed increased $CD68^+$ osteoclasts (Fig. 4J) and cathepsin K (Fig. 4K) staining compared to wild-type control mice following DMM. These findings demonstrate that osteoclast number and activity are increased in osteoarthritic joints of IL-4 deficient mice, reinforcing the relevance of our *in vitro* findings.

3.5. IL-4 inhibits osteoclast formation via the type 1 receptor

We further parsed the role of IL-4 in inhibition of osteoclastogenesis by determining whether IL-4 signals through the Type 1 or Type 2 receptor. We stimulated BM-derived osteoclast precursors (Fig. 4A) cultured from WT, *Il4ra*$^{-/-}$, and *Il13ra*$^{-/-}$ mice with recombinant IL-4 for six days during differentiation. IL-4 inhibited the formation of

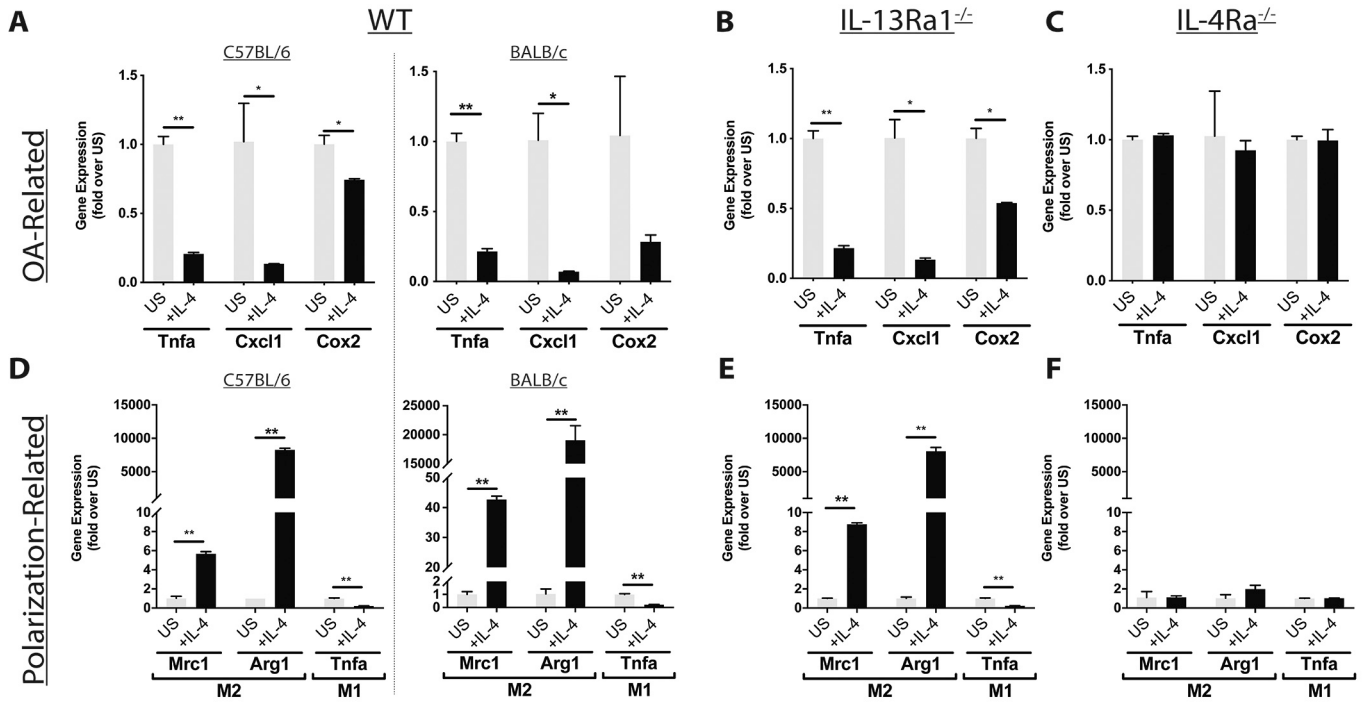

Fig. 3. IL-4 stimulation of murine macrophages downregulates osteoarthritis-associated genes and polarizes macrophages to the M2 state *via* the Type 1 receptor. (A-C) Expression of osteoarthritis-related genes (TNFa, Cxcl1, and Cox 2), as measured by quantitative PCR analysis of relative mRNA expression, in both C57BL/6 and BALB/c WT macrophages (A), IL-13Ra1-deficient (IL-13Ra1$^{-/-}$) macrophages (B), and IL-4Ra-deficient (IL-4Ra$^{-/-}$) macrophages (C) stimulated with (+IL-4) or without (US) recombinant IL-4 for 24 h. (D—F) Expression of macrophage polarization-related genes (M2: Mrc1, Arg1; M1: Tnfa), as measured by quantitative PCR analysis of relative mRNA expression, in both C57BL/6 and BALB/c WT (D), IL-13Ra1$^{-/-}$ (E), and IL-4Ra$^{-/-}$ (F) US or + IL-4 macrophages. Gene expression is reported as fold change over the unstimulated condition. *P* values were calculated using two-tailed, unpaired Student's *t*-test; $*p < 0.05$, $**p < 0.01$.

multinucleated, TRAP^{+} osteoclasts in cells derived from WT and *Il13ra*$^{-/-}$ mice (Fig. 5 A and B), but not in cells derived from *Il4ra*$^{-/-}$ mice (Fig. 5C). Quantification of cell culture supernatants revealed significantly decreased levels of TRAP in IL-4-stimulated cells derived from *WT* and *Il13ra*$^{-/-}$ mice (Fig. 5D and E) as compared to unstimulated cells. Levels of supernatant TRAP were also decreased in cells derived from *Il4ra*$^{-/-}$ mice, but to a lesser extent than observed in *WT* and *Il13ra*$^{-/-}$ cells (Fig. 5F).

We further tested the role of IL-4 in osteoclastogenesis by looking at its effect on expression of genes involved in the regulation of osteoclast differentiation and activation. IL-4 stimulation led to decreased levels of the osteoclast differentiation genes *Nfatc1*, *Tnfrsf11a* and *Acp5* in cells derived from *WT* and *Il13ra*$^{-/-}$ mice (Fig. 5 G and H) but not in cells derived from *Il4ra*$^{-/-}$ mice (Fig. 5I). Similarly, genes associated with osteoclast activation, *Ctsk, Mmp9, Calcr, and Car2* exhibited significantly decreased expression in cells derived from *WT* and *Il13ra*$^{-/-}$ mice following stimulation with IL-4 stimulation (Fig. 5 J and K), while we did not observe significant changes in the expression of these genes following IL-4 stimulation of cells derived from *Il4ra*$^{-/-}$ mice (Fig. 5L). Together these findings indicate that IL-4 likely signals through the Type 1 receptor complex in osteoclasts and may mediate its effects in osteoarthritis.

3.6. IL-4 pathway components are dysregulated in human osteoarthritis synovium

We next sought to determine whether IL-4 signaling was dysregulated in human osteoarthritis joints. We analyzed IL-4 protein levels in synovial fluids derived from human subjects with osteoarthritis ($n = 12$) and non-osteoarthritis arthritides (rheumatoid arthritis $n = 4$, psoriatic arthritis $n = 2$, reactive arthritis n = 1, and crystal-induced arthritis n = 2) by ELISA. The concentration of IL-4 was significantly higher in synovial fluids derived from individuals with osteoarthritis as compared to synovial fluids from individuals with non-osteoarthritis arthritides ($p < 0.01$; Fig. 6A).

We also investigated the effect of IL-4 on expression of inflammatory and degradative mediators in human synovial tissues, as macrophages reside in this tissue. We stimulated human osteoarthritis synovial tissue explants with recombinant IL-4 protein for 24 h. qPCR analysis revealed that IL-4 stimulation resulted in decreased expression of a panel of cytokine, chemokine, and enzyme genes known to be associated with osteoarthritis (Fig. 6B). These findings suggest that IL-4 expression in synovial tissues may downregulate expression of mediators that contribute to joint dysfunction in osteoarthritis.

4. Discussion

The objective of this study was to investigate the role of IL-4 in the development of osteoarthritis. The association of SNPs in the *IL4* and *IL4Ra* genes [5–8], along with the dysregulation of IL-4 in osteoarthritis synovial fluid suggest that this pathway, typically associated with Type 2 immunity and atopic diseases, plays a role in the development of osteoarthritis. Here we show that, in a mouse model of post-traumatic osteoarthritis, IL-4 deficiency exacerbates disease with worse cartilage damage and osteophyte formation. Further, we show that STAT6 deficiency and IL-4Rα deficiency on myeloid cells also exacerbates osteoarthritis in mice. Taken together, these data suggest that IL-4 protects against osteoarthritis *via* signaling through its IL-4Rα subunit and its STAT6 signaling intermediate in myeloid cell types.

In vitro analyses of IL-4 effects on human synovium and joint-resident myeloid cell types revealed that IL-4 can inhibit osteoarthritis-related phenotypes. In macrophages, IL-4 stimulates improved phagocytosis of cartilage debris, a potential mechanism by which macrophages can modulate the DAMP-driven inflammatory microenvironment of a

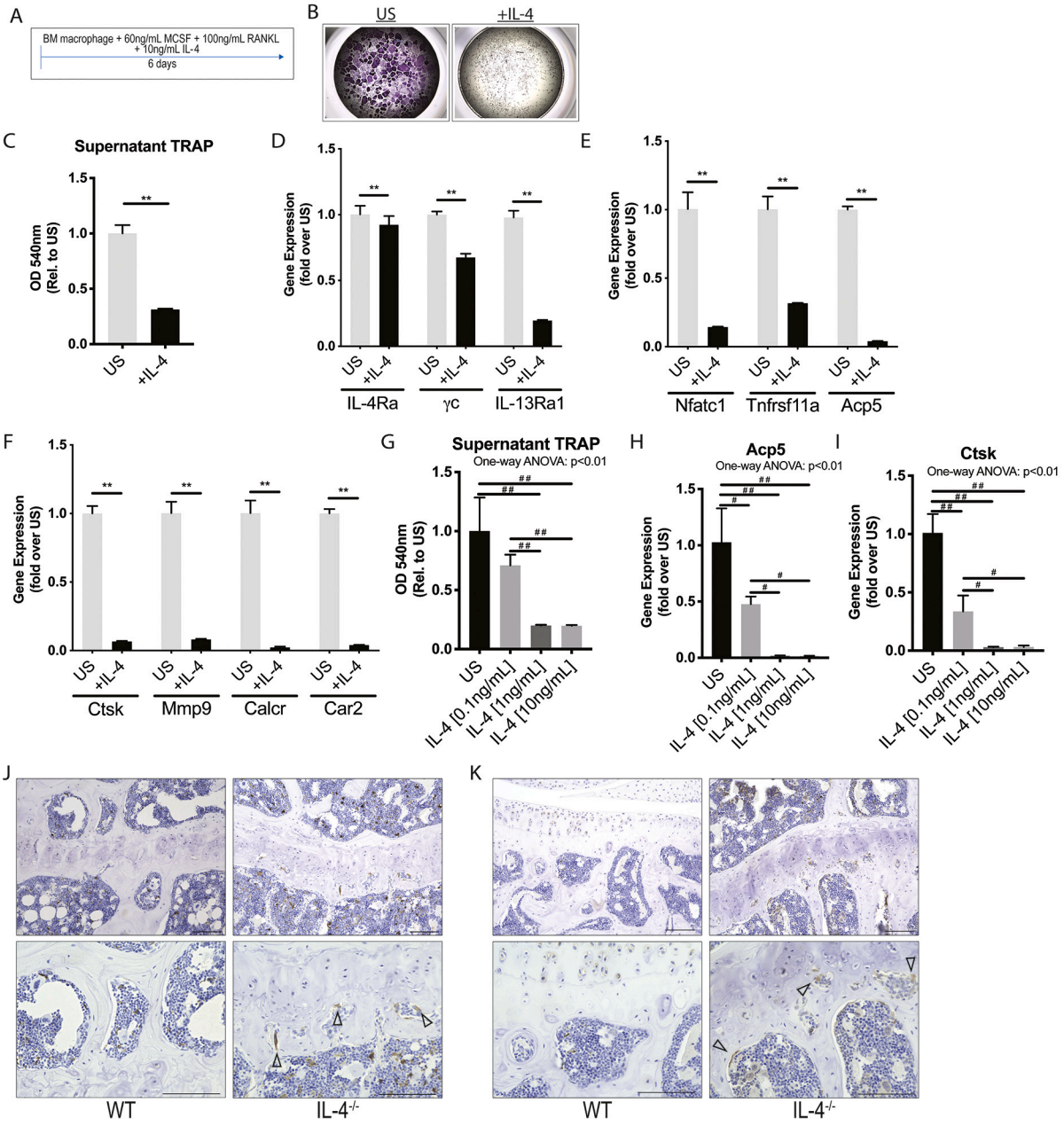

Fig. 4. IL-4 potently and dose-dependently inhibits murine osteoclast formation.
(A) Experimental design of osteoclast stimulation with recombinant IL-4 throughout six days of differentiation. (B) TRAP staining of unstimulated (US) and IL-4 stimulated (+IL-4) osteoclasts. (C) Quantification of supernatant TRAP activity from US and + IL-4 osteoclasts. TRAP activity is represented as OD_{540}, relative to the US condition. (D—F) show expression of IL-4 receptor subunits (D), osteoclast differentiation-associated genes (E), and osteoclast activation-associated genes (F), as measured by quantitative PCR analysis of relative mRNA expression, in US or + IL-4 osteoclasts. (G-I) Dose response of osteoclast to stimulation with recombinant IL-4, as measured by supernatant TRAP activity (G), osteoclast differentiation-associated gene expression (H), and osteoclast activation-associated gene expression (I). (J-K) Representative low-magnification (upper) and high-magnification (lower) immunohistochemical staining of osteoclasts markers CD68 (J) and cathepsin K (K) in the femoro-tibial joint from wild type (WT) and IL-4 deficient ($IL\text{-}4^{-/-}$) mice subjected to DMM. Scale bars in the images, 200 μm. Gene expression is reported as fold change over the unstimulated condition. For 2 group comparisons, *P* values were calculated using two-tailed, unpaired Student's t-test; $^{*}P < 0.05$, $^{**}P < 0.01$. For multiple group comparisons, *P* values were calculated using one-way ANOVA and *post hoc* comparison with Tukey correction; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

diseased joint. Further, IL-4 stimulates M2 polarization of macrophages *via* the type I receptor, even in the presence of cartilage debris that would otherwise be an M1 stimulus. Though the exact polarization state of macrophages in an osteoarthritis joint remains unclear, the effects of IL-4 on macrophages may be essential for maintaining tissue homeostasis and in the damage response in synovial joints, similar to its known role in other tissue types [48].

IL-4 stimulation of osteoclasts confirmed previous findings that IL-4 potently inhibits osteoclast formation. We extended these findings by using receptor subunit-deficient osteoclasts to demonstrate that IL-4 uses the type I receptor to downregulate expression of osteoclast differentiation and activation genes. Given the known role of osteoclast overactivation in osteoarthritis pathogenesis [20–23], these data suggest that, in a healthy joint, IL-4 can prevent pathogenetic differentiation and activation of osteoclasts. Further, the finding that IL-4 acts through the same receptor complex in both macrophages and osteoclasts highlights the potential role of the type I receptor in mediating joint-protective functions through a common mechanism in both cell types.

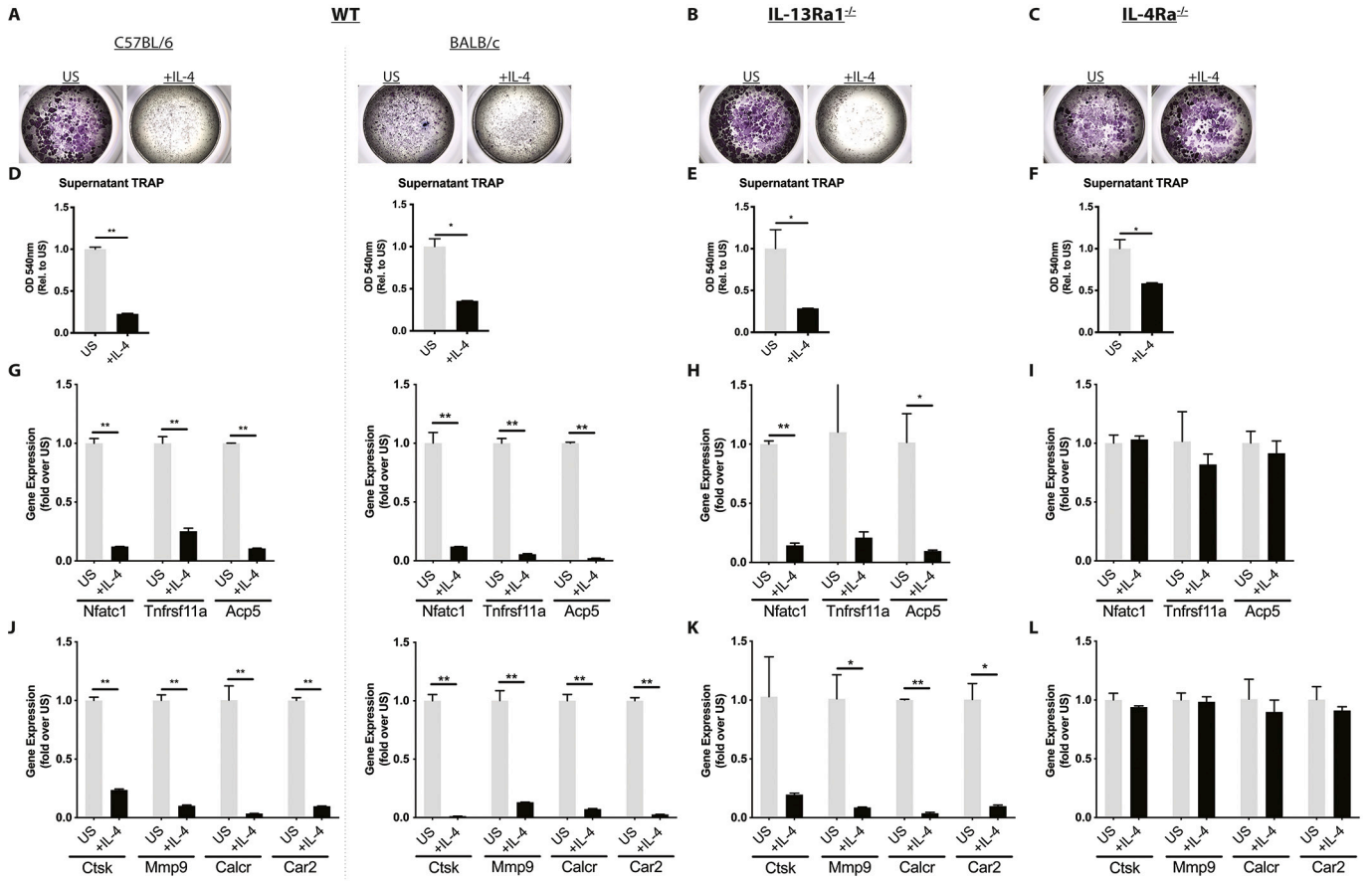

Fig. 5. IL-4 inhibits murine osteoclast formation *via* the Type 1 receptor.
(A-C) TRAP staining of unstimulated (US) and IL-4 stimulated (+IL-4) osteoclasts from both C57BL/6 and BALB/c wild-type (WT; A), IL-13Ra1-deficient (IL-13Ra1$^{-/-}$; B), and IL-4Ra-deficient (IL-4Ra1$^{-/-}$; C) mice. (D—F) Quantification of supernatant TRAP activity from US and + IL-4 osteoclasts from both C57BL/6 and BALB/c WT (D), IL-13Ra1$^{-/-}$ (E), and IL-4Ra1$^{-/-}$ (F) mice. TRAP activity is represented as OD_{540} relative to the US condition. (G-I) Expression of osteoclast differentiation-associated genes, as measured by quantitative PCR, in US, or + IL-4 stimulated osteoclasts from both C57BL/6 and BALB/c WT (G), IL-13Ra1$^{-/-}$ (H), and IL-4Ra1$^{-/-}$ (I) mice. (J-L) Expression of osteoclast activation-associated genes, as measured by quantitative PCR, in US or + IL-4 stimulated osteoclasts from both C57BL/6 and BALB/c WT (J), IL-13Ra1$^{-/-}$ (K), and IL-4Ra1$^{-/-}$ (L) mice. Gene expression is reported as fold change over the unstimulated condition. P values were calculated using two-tailed, unpaired Student's t-test; $*P < 0.05$, $**P < 0.01$.

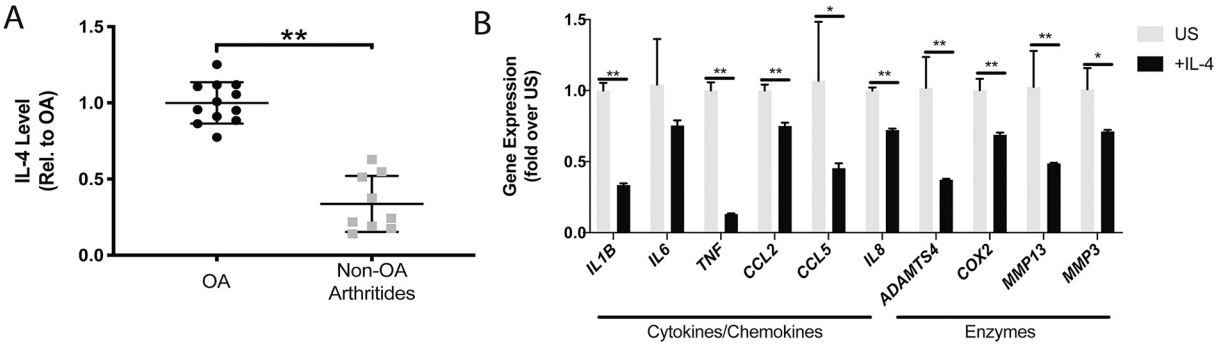

Fig. 6. IL-4 pathway components are dysregulated in human osteoarthritis.
(A) IL-4 levels in synovial fluid from patients with osteoarthritis ($n = 12$) and from patients with other, non-OA arthritides ($n = 9$). These data are represented as relative to average IL-4 level (pg/mL) in osteoarthritis synovial fluid. Data represent both individual values and means ± SD for each disease group. (B) Gene expression of osteoarthritis-associated genes, as measured by quantitative PCR analysis of relative mRNA expression, in human osteoarthritis-synovial explant stimulated with (+IL-4) or without (US) recombinant IL-4 for 24 h. Expression is reported as fold change over the unstimulated condition. *P* values were calculated using two-tailed, unpaired Student's t-test; $**P < 0.01$.

The implication of osteoclast dysregulation as one mechanism by which IL-4 modules the joint environment highlights this osteoimmunologic axis as a potentially relevant target for pharmacologic intervention, either at the level of the osteoclast or the IL-4 cytokine. Many studies to date have investigated the effects of osteoclast-specific pharmacologics including bisphosphonates, calcitonin, cathepsin K inhibitors, and estrogen replacement therapy on osteoarthritis development [21,24]. While select animal models have shown small

beneficial effects with early osteoclast inhibition with bisphosphonates, multiple animal model studies and human trails have largely failed to show significant benefit of bisphosphonates in the prevention or treatment of osteoarthritis [24]. The lack of success with these osteoclast-specific agents likely highlights the multifactorial nature of osteoarthritis pathogenesis and, thus, the need to target multiple pathologic cell types, potentially using IL-4.

Though more often studied in models of atopic and other T-cell mediated disease than in osteoarthritis-specific models, pharmacologic targeting of IL-4 has been investigated with both anti-cytokine [49] and cytokine enhancement [50] strategies. Recombinant IL-4 has been found to be protective in models of inflammatory arthritis [51] and peri-implant osteolysis [52]. In models of osteoarthritis, intra-articular injection of IL-4 in rats [53] and systemic treatment with IL-4-10 fusion protein in canines [54] protect against cartilage damage, highlighting the therapeutic potential of this cytokine. Recent advances in IL-4 therapeutics have developed chemically modified IL-4 'superkines', with enhanced cytokine affinity and receptor specificity, that may further enable its safe and effective therapeutic use [50].

Taken in the context of these previously published pharmacologic studies that have demonstrated chondroprotective effects of IL-4, our findings present additional mechanisms by which IL-4 may protect against joint disease. Further investigation of osteoclast-modulating pharmacologics such as bisphosphonate in combination with IL-4-modulating pharmacologics such as superkines in a model of post-traumatic osteoarthritis could provide a deeper understanding of both the pathobiology that underlies our observed effect and the potential for effective pharmacologic intervention at this osteoimmunologic junction.

Limitations of this study include lack of comparison to healthy human joint tissues and synovial fluids, due to difficulty in obtaining samples matched for age and other factors. This limitation complicates interpretation of our findings of elevated IL-4 in OA synovial fluids relative to non-OA arthridites. Without an understanding of IL-4 levels in healthy synovial fluids, it is difficult to determine the clinical significance of the observed levels in OA synovial fluids. We hypothesize that the presence of IL-4 in OA synovial fluids is indicative of the presence of a protective mechanism (namely, IL-4) which is not sufficient to overcome the pathogenic forces driving disease. Perhaps in healthy and early stage OA individuals, the levels of IL-4 are sufficient to overcome the drivers of disease, but not so in late stage disease. Further studies are needed to investigate how IL-4 levels differ throughout OA progression and to further characterize the potential role of IL-4 in human OA. The analysis of human data was also limited by the small number of biological samples and limited information on age, gender, or other donor characteristics available for *in vitro* analysis of macrophage function. Additionally, in the cartilage debris phagocytosis experiments performed, the macrophage polarization was characterized by gene expression while phagocytic function was characterized by cell counts, and as a result the differences observed may be related to increased number of M2 macrophages.

Another limitation of this study is that determining the source of IL-4 in human joint tissue remains elusive. Identifying the source of protective cytokines is fundamental to understanding the mechanisms that underlie protection against osteoarthritis. IL-4 has been shown to be produced by both non-immune cell types including fibroblasts, chondrocytes, synoviocytes, osteoblasts, osteoclasts, and adipocytes, and more classic immune cell types such as macrophages, mast cells, eosinophils and T cells [49]. Interestingly, depending on the disease state, IL-4 can be pathogenic, such as in asthma and atopic disease, or protective, such as in wound repair [48]. Further investigation is needed to identify and characterize which cell types in healthy and OA joints modulate the production of IL-4.

The use of multiple genetic knockout models for *in vitro* and *in vivo* experiments necessitated the use of mouse strains generated in different backgrounds, making interpretation of the relative magnitude of effects observed between different strains difficult. While the differing genetic backgrounds may complicate comparisons between different knockout strains, for all *in vitro* and *in vivo* experiments appropriate age-, sex-, and background-matched controls were used enabling clear interpretation of strain-specific results. Finally, while $LysM^{Cre}$ conditional knockout system has been widely used for study of myeloid cell types and is generally accepted as a reliable tool for study of endogenous myelomonocytic cells, there have been reports that LysM may be expressed in certain other cell types including type II lung alveolar cells [55]. As such, it is important to consider the possibility that other joint-resident LysM expressing cells may contribute to the *in vivo* findings observed.

The observations presented here suggest a potential, novel therapeutic strategy of using recombinant IL-4 or activators of the IL-4R signaling pathway to treat osteoarthritis. Currently, therapeutic recombinant IL-4 is being investigated for the prevention of periprosthetic osteolysis [52]. Given the type I receptor specificity reported here, the use of type I receptor-targeted engineered IL-4 superkines, as described by Junttila et al. [50], might also prove useful in developing the first disease-modifying treatment for osteoarthritis. Further studies will be needed to assess the effects of supraphysiologic levels of IL-4 in the joint and to investigate other approaches to promote this protective mechanism in osteoarthritis.

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Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2021.108784.

References

[1] Z. Huang, C. Ding, T. Li, S.P. Yu, Current status and future prospects for disease modification in osteoarthritis, Rheumatology (Oxford) 57 (suppl_4) (2018) iv108-iv23.
[2] D.J. Hunter, S. Bierma-Zeinstra, Osteoarthritis, Lancet 393 (10182) (2019) 1745–1759.
[3] Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. 2016. p. 580–92.
[4] C.R. Scanzello, Role of low-grade inflammation in osteoarthritis, Curr. Opin. Rheumatol. 29 (1) (2017) 79–85.
[5] M. Vargiolu, Z.A.T. Silvestri, E. Bonora, P. Dolzani, L. Pulsatelli, O. Addimanda, et al., Interleukin-4/interleukin-4 receptor gene polymorphisms in hand osteoarthritis, Osteoarthr. Cartil. 18 (2010) 810–816.
[6] O.C. Rogoveanu, D. Calina, M.G. Cucu, F. Burada, A.O. Docea, S. Sosoi, et al., Association of cytokine gene polymorphisms with osteoarthritis susceptibility, Exp Ther Med. 16 (3) (2018) 2659–2664.
[7] T. Forster, K. Chapman, J. Loughlin, Common variants within the interleukin 4 receptor ? Gene (IL4R) are associated with susceptibility to osteoarthritis, Hum. Genet. 114 (4) (2004) 391–395.
[8] S. Yigit, A. Inanir, A. Tekcan, E. Tural, G.T. Ozturk, G. Kismali, et al., Significant association of interleukin-4 gene intron 3 VNTR polymorphism with susceptibility to knee osteoarthritis, Gene. 537 (1) (2014) 6–9.
[9] T. Silvestri, L. Pulsatelli, P. Dolzani, A. Facchini, R. Meliconi, Elevated serum levels of soluble interleukin-4 receptor in osteoarthritis, Osteoarthr. Cartil. 14 (7) (2006) 717–719.

[10] H. Raghu, C.M. Lepus, Q. Wang, H.H. Wong, N. Lingampalli, F. Oliviero, et al., CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis, Ann. Rheum. Dis. 76 (5) (2016) 914–922, annrheumdis-2016–210426.
[11] M. Rahmati, A. Mobasheri, M. Mozafari, Inflammatory mediators in osteoarthritis: a critical review of the state-of-the-art, current prospects, and future challenges, Bone. 85 (2016) 81–90.
[12] F. Berenbaum, Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!), Osteoarthr. Cartil. 21 (1) (2013) 16–21.
[13] V.B. Kraus, G. McDaniel, J.L. Huebner, T.V. Stabler, C.F. Pieper, S.W. Shipes, et al., Direct in vivo evidence of activated macrophages in human osteoarthritis, Osteoarthr. Cartil. (2015) 1–9.
[14] A. Bertuglia, M. Lacourt, C. Girard, G. Beauchamp, H. Richard, S. Laverty, Osteoclasts are recruited to the subchondral bone in naturally occurring post-traumatic equine carpal osteoarthritis and may contribute to cartilage degradation, Osteoarthr. Cartil. 24 (3) (2016) 555–566.
[15] M. Siebelt, J.H. Waarsing, H.C. Groen, C. Muller, S.J. Koelewijn, E. de Blois, et al., Inhibited osteoclastic bone resorption through alendronate treatment in rats reduces severe osteoarthritis progression, Bone. 66 (2014) 163–170.
[16] G. Mohan, E. Perilli, I.H. Parkinson, J.M. Humphries, N.L. Fazzalari, J.S. Kuliwaba, Pre-emptive, early, and delayed alendronate treatment in a rat model of knee osteoarthritis: effect on subchondral trabecular bone microarchitecture and cartilage degradation of the tibia, bone/cartilage turnover, and joint discomfort, Osteoarthr. Cartil. 21 (10) (2013) 1595–1604.
[17] J.P. Pelletier, C. Boileau, J. Brunet, M. Boily, D. Lajeunesse, P. Reboul, et al., The inhibition of subchondral bone resorption in the early phase of experimental dog osteoarthritis by licofelone is associated with a reduction in the synthesis of MMP-13 and cathepsin K, Bone. 34 (3) (2004) 527–538.
[18] S.M. Botter, G.J.V.M. Van Osch, S. Clockaerts, J.H. Waarsing, H. Weinans, J.P.T. M. Van Leeuwen, Osteoarthritis induction leads to early and temporal Subchondral plate porosity in the Tibial plateau of mice an in vivo microfocal computed tomography study, ARTHRITIS & RHEUMATISM. 63 (9) (2011) 2690–2699.
[19] H. Fang, L. Huang, I. Welch, C. Norley, D.W. Holdsworth, F. Beier, et al., Early changes of articular cartilage and subchondral bone in the dmm mouse model of osteoarthritis, Sci. Rep. 8 (1) (2018) 2855.
[20] I. Reimann, H.J. Mankin, C. Trahan, Quantitative histologic analyses of articular cartilage and subchondral bone from osteoarthritic and normal human hips, Acta Orthop. Scand. 48 (1) (1977) 63–73.
[21] T. Neogi, S. Li, C. Peloquin, D. Misra, Y. Zhang, Effect of bisphosphonates on knee replacement surgery, Ann. Rheum. Dis. 77 (1) (2018) 92–97.
[22] A. Shibakawa, K. Yudoh, K. Masuko-Hongo, T. Kato, K. Nishioka, H. Nakamura, The role of subchondral bone resorption pits in osteoarthritis: MMP production by cells derived from bone marrow, Osteoarthr. Cartil. 13 (8) (2005) 679–687.
[23] M. Durand, S.V. Komarova, A. Bhargava, D.P. Trebec-Reynolds, K. Li, C. Fiorino, et al., Monocytes from patients with osteoarthritis display increased osteoclastogenesis and bone resorption: the in vitro osteoclast differentiation in arthritis study, Arthritis Rheum. 65 (1) (2013) 148–158.
[24] M.A. Karsdal, A.C. Bay-Jensen, R.J. Lories, S. Abramson, T. Spector, P. Pastoureau, et al., The coupling of bone and cartilage turnover in osteoarthritis: opportunities for bone antiresorptives and anabolics as potential treatments? Ann. Rheum. Dis. 73 (2) (2014) 336–348.
[25] S. Boonen, E. Rosenberg, F. Claessens, D. Vanderschueren, S. Papapoulos, Inhibition of cathepsin K for treatment of osteoporosis, Curr Osteoporos Rep. 10 (1) (2012) 73–79.
[26] S.M. McCormick, N.M. Heller, Commentary: IL-4 and IL-13 Receptors and Signaling, 2015.
[27] A. Saradna, D.C. Do, S. Kumar, Q.L. Fu, P. Gao, Macrophage polarization and allergic asthma, Transl. Res. 191 (2018) 1–14.
[28] P. Miossec, P. Chomarat, J. Dechanet, J.F. Moreau, J.P. Roux, P. Delmas, et al., Interleukin-4 inhibits bone resorption through an effect on osteoclasts and proinflammatory cytokines in an ex vivo model of bone resorption in rheumatoid arthritis, Arthritis Rheum. 37 (12) (1994) 1715–1722.
[29] K. Watanabe, Y. Tanaka, I. Morimoto, K. Yahata, K. Zeki, T. Fujihira, et al., Interleukin-4 as a potent inhibitor of bone resorption, Biochem. Biophys. Res. Commun. 172 (3) (1990) 1035–1041.
[30] J.A. Riancho, M.T. Zarrabeitia, J. Gonzalez-Macias, Interleukin-4 modulates osteoclast differentiation and inhibits the formation of resorption pits in mouse osteoclast cultures, Biochem. Biophys. Res. Commun. 196 (2) (1993) 678–685.
[31] J. Xue, V. Sharma, M.H. Hsieh, A. Chawla, R. Murali, S.J. Pandol, et al., Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis, Nat. Commun. 6 (May) (2015) 7158.
[32] Q. Wang, A.L. Rozelle, C.M. Lepus, C.R. Scanzello, J.J. Song, D.M. Larsen, et al., Identification of a central role for complement in osteoarthritis, Nat. Med. 17 (12) (2011) 1674–1679.
[33] S. Kamekura, K. Hoshi, T. Shimoaka, U. Chung, H. Chikuda, T. Yamada, et al., Osteoarthritis development in novel experimental mouse models induced by knee joint instability, Osteoarthr. Cartil. 13 (7) (2005) 632–641.
[34] S.S. Glasson, T.J. Blanchet, E.A. Morris, The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse, Osteoarthr. Cartil. 15 (9) (2007) 1061–1069.
[35] A.A. Barkal, R.E. Brewer, M. Markovic, M. Kowarsky, S.A. Barkal, B.W. Zaro, et al., CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy, Nature. 1 (2019).
[36] G. Ramaswamy, H. Kim, D. Zhang, V. Lounev, J.Y. Wu, Y. Choi, et al., Gsalpha controls cortical bone quality by regulating osteoclast differentiation via cAMP/PKA and beta-catenin pathways, Sci. Rep. 7 (2017) 45140.
[37] S.-Y. Hwang, J.W. Putney, Orai1-mediated calcium entry plays a critical role in osteoclast differentiation and function by regulating activation of the transcription factor NFATc1, FASEB J. 26 (4) (2012) 1484–1492.
[38] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.
[39] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, Trends Immunol. 25 (12) (2004) 677–686.
[40] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (12) (2008) 958–969.
[41] D.T. Felson, Arthroscopy as a treatment for knee osteoarthritis, Best Pract. Res. Clin. Rheumatol. 24 (1) (2010) 47.
[42] C. Zhou, H. Zheng, J.A. Buckwalter, J.A. Martin, Enhanced phagocytic capacity endows chondrogenic progenitor cells with a novel scavenger function within injured cartilage, Osteoarthr. Cartil. 24 (9) (2016) 1648–1655.
[43] M. Hm Berkelaar, N.M. Korthagen, G. Jansen, W. Evert Van Spil, Journal of rheumatic diseases and treatment synovial macrophages: potential key modulators of cartilage dam-age, osteophyte formation and pain in knee osteoarthritis, J Rheum Dis Treat 4 (2018) 59.
[44] A. Shapouri-Moghaddam, S. Mohammadian, H. Vazini, M. Taghadosi, S. A. Esmaeili, F. Mardani, et al., Macrophage plasticity, polarization, and function in health and disease, J. Cell. Physiol. 233 (9) (2018) 6425–6440.
[45] M. Kapoor, J. Martel-Pelletier, D. Lajeunesse, J.-P. Pelletier, H. Fahmi, Role of Proinflammatory cytokines in the pathophysiology of osteoarthritis, Nat. Publ. Group 7 (1) (2010) 33–42.
[46] N. Torabinia, S.M. Razavi, Z. Shokrolahi, A comparative immunohistochemical evaluation of CD68 and TRAP protein expression in central and peripheral giant cell granulomas of the jaws, J Oral Pathol Med. 40 (4) (2011) 334–337.
[47] H. Liu, W. Feng, Yimin, cui J, lv S, Hasegawa T, et al. histological evidence of increased osteoclast cell number and asymmetric bone Resorption activity in the tibiae of Interleukin-6-deficient mice, J. Histochem. Cytochem. 62 (8) (2014) 556–564.
[48] R.L. Gieseck, M.S. Wilson, T.A. Wynn, Type 2 immunity in tissue repair and fibrosis, Nat. Publ. Group 18 (2017).
[49] R.D. May, M. Fung, Strategies targeting the IL-4/IL-13 axes in disease, Cytokine. 75 (1) (2015) 89–116.
[50] I.S. Junttila, R.J. Creusot, I. Moraga, D.L. Bates, M.T. Wong, M.N. Alonso, et al., Redirecting cell-type specific cytokine responses with engineered interleukin-4 superkines, Nat. Chem. Biol. 8 (12) (2012) 990–998.
[51] L.A. Joosten, Lubberts, E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction, Arthritis Rheum. 40 (2) (1997) 249–260.
[52] Y. Wang, N.-N. Wu, Y.-Q. Mou, L. Chen, Z.-L. Deng, Inhibitory effects of recombinant IL-4 and recombinant IL-13 on UHMWPE-induced bone destruction in the murine air pouch model, J. Surg. Res. 180 (2) (2013) e73–e81.
[53] M. Yorimitsu, K. Nishida, A. Shimizu, H. Doi, S. Miyazawa, T. Komiyama, et al., Intra-articular injection of interleukin-4 decreases nitric oxide production by chondrocytes and ameliorates subsequent destruction of cartilage in instability-induced osteoarthritis in rat knee joints, Osteoarthr. Cartil. 16 (7) (2008) 764–771.
[54] E.M. van Helvoort, J. Popov-Celeketic, N. Eijkelkamp, K. Coeleveld, M. A. Tryfonidou, C.D. Wijne, et al., Canine IL4-10 fusion protein provides disease modifying activity in a canine model of OA; an exploratory study, PLoS One 14 (7) (2019), e0219587.
[55] J. Shi, L. Hua, D. Harmer, P. Li, G. Ren, Cre driver mice targeting macrophages, Methods Mol. Biol. 2018 (1784) 263–275.