Prolactin blocks the expression of receptor activator of nuclear factor κB ligand and reduces osteoclastogenesis and bone loss in murine inflammatory arthritis

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Abstract

**Background:** Prolactin (PRL) reduces joint inflammation, pannus formation, and bone destruction in rats with polyarticular adjuvant-induced arthritis (AIA). Here, we investigate the mechanism of PRL protection against bone loss in AIA and in monoarticular AIA (MAIA).

**Methods:** Joint inflammation, trabecular bone loss, and osteoclastogenesis were evaluated in rats with AIA treated with PRL (via osmotic minipumps) and in mice with MAIA that were null (Prlr−/−) or not (Prlr+/+) for the PRL receptor. To help define target cells, synovial fibroblasts from Prlr+/+ mice were treated or not with proinflammatory cytokines (Cyt), including TNFα, IL-1β, and interferon (IFN)γ with or without PRL, and these synovial cells were co-cultured or not with bone marrow osteoclast progenitors from Prlr+/+ or Prlr−/− mice.

**Results:** In AIA, PRL treatment reduced joint swelling, increased trabecular bone area, lowered osteoclast density, and reduced mRNA levels of osteoclast-associated genes (tartrate-resistant acid phosphatase (Trap)), cathepsin K (Ctsk), matrix metalloproteinase 9 (Mmp9), and receptor activator of nuclear factor κB or RANK (Tnfrsf11a), of genes encoding cytokines with osteoclastogenic activity (Tnfa, Il1b, Il6, and receptor activator of nuclear factor κB ligand or RANKL (Tnfrsf11)), and of genes encoding for transcription factors and cytokines related to T helper (Th)17 cells (Rora, Rorc, Il17a, Il21, Il22) and to regulatory T cells (Foxp3, Ebi3, Il12a, Tgfb1, Il10). Prlr−/− mice with MAIA showed enhanced joint swelling, reduced trabecular bone area, increased osteoclast density, and elevated expression of Tnfa, Il1b, Il6, Trap, Tnfrsf11a, Tnfrsf11, Il17a, Il21, Il22, T123, Foxp3, and Il10. The expression of the long PRL receptor form increased in arthritic joints, and in synovial membranes and cultured synovial fibroblasts treated with Cyt. PRL induced the phosphorylation/activation of signal transducer and activator of transcription-3 (STAT3) and inhibited the Cyt-induced expression of Il1b, Il6, and Tnfrsf11 in synovial fibroblast cultures. The STAT3 inhibitor S31-201 blocked inhibition of Tnfrsf11 by PRL. Finally, PRL acted on both synovial fibroblasts and osteoclast precursor cells to downregulate Cyt-induced osteoclast differentiation.

**Conclusion:** PRL protects against osteoclastogenesis and bone loss in inflammatory arthritis by inhibiting cytokine-induced expression of RANKL in joints and synovial fibroblasts via its canonical STAT3 signaling pathway.

**Keywords:** Hormones, Bone, Inflammation, Experimental arthritis, Osteoclasts, Synovial fibroblasts, Prolactin receptor, RANKL, RANK

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**Background**

Rheumatoid arthritis (RA) is an immune-mediated type of inflammatory arthritis that causes massive bone destruction by increasing osteoclast development (osteoclastogenesis) and activity. Receptor activator of nuclear factor κB ligand (RANKL), its cellular receptor, receptor activator of nuclear factor κB (RANK), and the decoy soluble RANKL receptor osteoprotegerin (OPG) are key regulators of osteoclastogenesis in RA [1–3]. RANKL is produced by osteoblastic lineage cells, activated T cells, synovial fibroblasts, and other stromal cells, and it binds to RANK on osteoclast precursors to stimulate their differentiation, activity, and survival [4–8]. The effects of RANKL are neutralized by OPG which, by binding to RANKL, prevents it from activating RANK. RANKL and OPG are pivotal downstream signals upon which many cytokines, growth factors, and steroid and peptide hormones converge to regulate bone homeostasis [7, 8]. Shifting the RANKL-to-OPG ratio in favor of bone protection is a promising therapeutic strategy against joint destruction in RA. Prolactin (PRL), the hormone essential for mammary gland development and milk production, may have this protective effect.

The female preponderance and the influence of reproductive states in RA, together with PRL immune-enhancing actions, have long linked this disease to a detrimental effect of PRL [9–11]. However, accumulating evidence has challenged this view by showing that PRL is also immunosuppressive [12–14], and that hyperprolactinemia occurring during pregnancy and lactation [15, 16] or induced pharmacologically by the dopamine D2 receptor antagonist haloperidol [17], is associated with a reduction in the severity and risk of RA. Moreover, increasing prolactinemia by PRL infusion or treatment with haloperidol ameliorates inflammation and joint destruction in AIA as revealed by reduced joint swelling, lower local expression of proinflammatory cytokines TNFα, IL-1β, IFNγ, and IL-6, and inhibition of chondrocyte apoptosis, pannus formation, and bone erosion [18].

PRL protection against bone loss in inflammatory arthritis is not unexpected. PRL-receptor-null mice are osteopenic [19] and PRL treatment increases bone formation during growth by decreasing the RANKL/OPG ratio in osteoblasts [20]. Moreover, PRL stimulates the proliferation of pancreatic β cells by promoting OPG-mediated inhibition of RANKL [21], and RANKL is under the control of PRL to promote mammary gland lobulo-alveolar development during pregnancy [22].

Here, we investigated whether PRL, either by its exogenous administration or by the genetic deletion of the its receptor, reduces trabecular bone loss and osteoclastogenesis by modifying proinflammatory cytokine-induced upregulation of the RANKL/RANK/OPG system in rodent polyarticular AIA and monoarticular AIA (MAIA), and whether these actions involve a direct effect of PRL on synovial fibroblasts and osteoclast progenitor cells.

**Methods**

**Animals**

Male Sprague-Dawley rats (200–250 g) and female C57BL6 mice, wild type (Prlr+/+) or null for the PRL receptor (Prlr−/−) (8 weeks old, 20–25 g), were housed under standard laboratory conditions (22 °C; 12-hour/12-hour light/dark cycle; free access to food and water).

**Induction of AIA**

AIA was induced in rats as described [18], by a single intradermal injection at the base of the tail of 0.2 ml complete Freund’s adjuvant (CFA, Difco Laboratories, Detroit, MI, USA; 10 mg heat-killed Mycobacterium tuberculosis per 1 ml of Freund’s adjuvant). Three days before CFA injection some rats were rendered hyperprolactinemic by the subcutaneous implantation of a 28-day osmotic minipump (Alza, Palo Alto, CA, USA) containing 1.6 mg of ovine PRL (Sigma Aldrich, St. Louis, MO, USA). Ankle swelling, monitored as described [18], indicated that the onset of arthritis appeared on day 12 after CFA injection and was maximal by day 21, when the animals were euthanized, serum samples collected for PRL and cytokine determinations, and ankle joints processed for histological examination, quantitative (q)PCR, and western blot.

**Induction of MAIA**

Monoarticular AIA was induced and assessed in mice as described [23]. Briefly, mice were injected into the articular space of the right knee joint with CFA (5 μg in 10 μl) once every 7 days for 18 days. The diameter across the knee joint (left and right) was measured twice a week with a micro-caliper. Mice were euthanized 18 days after the initial CFA injection, and knee joints were removed and processed as above.

**Intra-articular injection of TNFα, IL-1β, and IFNγ (Cyt)**

Mice were injected in the articular space of the knee joints with Cyt in a final volume of 20 μl (62.5 ng TNFα, 25 ng IL-1β, and 25 ng IFNγ; R&D Systems, Minneapolis, MN, USA) or with endotoxin-free water as vehicle. Forty-eight hours after Cyt or vehicle injection, mice were euthanized and synovial membranes were extracted and processed for quantitative real-time (qRT)-PCR evaluation.

**Serum measurements**

Infused ovine PRL was measured in serum by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to PRL, carried out as described [18]. The serum levels of C-reactive protein...
and TNFα were quantified using ELISA kits from BD Biosciences (San Jose, CA, USA) and R&D systems (Minneapolis, MN, USA), respectively.

**Histological examination and image analysis**

Ankle and knee joints were fixed, decalcified, and dehydrated for paraffin embedding. Four 7-μm-thick sections spaced 380 μm or 126 μm apart, per each rat tibia/tarsal joint or mouse femur/tibia joint, respectively, were stained with Harris's hematoxylin-eosin solution for measurement of trabecular bone area and with tartrate-resistant acid phosphatase (TRAP) for evaluation of osteoclast number. For the latter, deparaffinized sections were washed, and incubated for 3 hours at 37 °C in TRAP activity staining mix (Fast Red Violet LB Salt (80 mg; Sigma Aldrich), Naphthol AS-MX (40 mg; Sigma Aldrich), formamide (4 ml; Invitrogen, Carlsbad, CA, USA), 0.04 M sodium acetate (0.656 g), 0.2 M disodium dihydrogen phosphate (9.2 g), and distilled water (200 ml)). The pH was adjusted to 5.0 and pre-incubated to 37 °C before use. After incubation, sections were rinsed with distilled water, counterstained with Mayer's hematoxylin (Sigma Aldrich), and covered with mounting medium (Entellan, Merck Millipore Corporation, Billerica, MA, USA). Hematoxylin-eosin-stained and TRAP-stained tissue sections were visualized by light microscopy (Olympus BX60F5, Olympus Tokyo, Japan). Trabecular bone surface and number of TRAP-stained purple spots (osteoclasts) were quantified (Image-Pro Plus analysis software; Media Cybernetics, Silver Spring, MD, USA) and divided by total bone area to obtain trabecular bone area and osteoclast density. Two independent observers, blind to the experiments, performed the measurements.

**qRT-PCR**

Frozen whole ankle and knee joints were pulverized in liquid nitrogen using a mortar and pestle. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR products were detected and quantified using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a final reaction of 10 μl containing template and 0.5 μM of each of the primer pairs for different genes (see Additional file 1: Table S1). Amplification performed in the CFX96 real-time PCR detection system (Bio-Rad, Richmond, CA, USA) included a 10-minute denaturation step at 95 °C, followed by 35 cycles of amplification (10 sec at 95 °C, 30 sec at the primer pair-specific annealing temperature, and 30 sec at 72 °C). The PCR data were analyzed by the 2-ΔΔCT method, and cycle thresholds (CT) normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt) were used to calculate the mRNA levels of interest.

**Isolation and culture of synovial fibroblasts**

Synovial fibroblasts were isolated from the hind limbs of wild-type mice separated at the femur/fibula/tibia junctions, as previously described [24]. Briefly, limbs were washed in Hanks' balanced salt solution (HBSS), and dissected while immersed in high glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin; Invitrogen) to remove all soft tissues from bones. The joint space was opened with a scalpel to expose the synovial tissues and incubated in culture medium containing 1 mg/ml collagenase type IV (Difco Laboratories) and 0.1 mg/ml of deoxynucleoside I (Sigma Aldrich) in a shaking bath for 3 hours at 37 °C. Tissues were then vortexed vigorously to release cells. The supernatant was passed through an 80-μm filter and centrifuged; cells were re-suspended in fresh culture media and grown to confluence. Cells were used for experiments after 2–4 passages, when the cultures showed >98% synovial fibroblast phenotype (CD90.2+, VCAM1+, and ICAM-1+) as described [24].

Synovial fibroblasts were seeded at 10⁶ cells/well in 6-well plates and incubated in 2 ml of culture medium for 16 hours with or without PRL (100 nM, recombinant human PRL provided by Michael E. Hodsdon, Yale University, New Haven, CT, USA). Cyt (0.25 ng/ml TNFα, 0.05 ng/ml IL-1β, and 0.05 ng/ml IFNγ) were then added or not to the culture for a period of 24 hours. Other cell cultures were pre-incubated for 2 hours with or without the STAT inhibitor, S31-201 at 50 nM (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated or not with 100 nM PRL for 16 hours, followed by the treatment with or without Cyt for 24 hours. To evaluate PRL effects on phosphorylation/activation of STAT3, synovial fibroblasts were seeded in complete medium for 24 hours, cultured in serum-free medium for another 24 hours, and then pre-treated for 6 hours with the Cyt followed by a 30-minute incubation in the presence or absence of Cyt with or without 100 nM PRL.

**Co-culture of synovial fibroblasts and osteoclast progenitors**

Synovial fibroblasts (passage 4) were seeded at 5 x 10⁵ cells in 48-well plates and after 12 hours the cells were treated or not with PRL for 16 hours. Cyt were then added or not to the culture, and after 24 hours, bone marrow cells (2 x 10⁶) containing osteoclast progenitors, harvested from the tibias and femurs of 8-week-old C57BL/6 mice, wild type (Prlr+/+) or null for the PRL receptor (Prlr−/−), were delivered in culture medium with 1, 25 dihydroxy-vitamin D3 (10⁻⁸ M, Sigma-Aldrich). Co-cultures were incubated for 10 days changing for
new medium (containing 1, 25 dihydroxy-vitamin D3, +/− Cyt, +/− PRL) every 2 days. Co-cultures were fixed with 3.7% formaldehyde for 10 minutes at room temperature, washed twice with PBS, air-dried, and incubated for 20 minutes at 37 °C with TRAP activity staining mix. Osteoclastogenesis was assessed by counting the number of enlarged TRAP-positive (TRAP+) cells.

Isolation of chondrocytes, osteoblasts, and osteoclast-like cells

To identify other joint cells expressing the PRL receptor, chondrocytes, osteoblasts, and osteoclast-like cells were obtained from Prlr+/+ and Prlr−/− mice. Synovial fibroblasts obtained from both mouse groups served as positive controls. Articular chondrocytes were isolated from femoral epiphyseal cartilage as described previously [25]. Murine bone marrow stromal cells were obtained by flushing the bone marrow from femur and tibia with PBS. Cells were maintained in DMEM with 20% FBS and antibiotics until 70% confluence, when they were differentiated into osteoblasts by treatment with 100 μM ascorbate phosphate and 5 mM β-glycerol phosphate for 10 days and medium replacement every 2 days [26]. Other bone marrow cells were differentiated into osteoclasts by their culture in α-MEM (Sigma-Aldrich) supplemented with 10% FBS and antibiotics, 25 ng/ml recombinant macrophage colony stimulating factor and 50 ng/ml RANKL (PreproTech, Rocky Hill, NJ, USA) for 10 days and medium replacement every 2 days [27]. All cells were processed for PCR.

The purity of the different cell types has been documented [27–29] and was confirmed by the mRNA expression of specific markers: collagen type II (chondrocytes), runt related transcription factor 2, alkaline phosphatase, and osteocalcin (osteoblasts), and RANK, calcitonin receptor, and TRAP (osteoclast-like cells). Total RNA was isolated, reverse transcribed, and used for the PCR amplification of a 139-bp fragment from exon 5 of the PRL receptor gene (exon 5 is deleted in Prlr−/− mice) in a final reaction mixture (10 μl) containing template, 0.02 U/μl of Phusion DNA Polymerase (Thermo Fisher, Waltham, MA, USA), 2 μl of 5X Phusion Buffer HF (Thermo Fisher), 0.4 mM of deoxyribonucleotide triphosphates (dNTPs) and 0.5 μM of each of the primer pairs for PRLr (forward 5′-CAC ATA AAG TGG ATC CGA GGT A-3′; reverse 5′-TGA ATG TCC AGA CTA CAA AAC CA -3′), using Hprt as loading control (Additional file 1: Table S1). The amplification in the Eppendorf Mastercycler ep Gradient S equipment (Eppendorf, Hamburg, Germany) included denaturation for 2 minutes at 95 °C, followed by 36 cycles of 15 sec at 95 °C, 15 sec at 56 °C, and 30 sec at 72 °C, followed by one cycle of 5 minutes at 72 °C. PCR products were resolved on a 1.2% agarose gel. The specificity of the reaction was confirmed by the lack of amplification products in samples from Prlr−/− mice.

Western blot

Pulverized ankle joints or synovial fibroblasts were resuspended in lysis buffer (0.1 M Tris-HCl, 0.2 M EGTA, 0.2 M EDTA, 100 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM sodium acid pyrophosphate, 250 mM sucrose, pH 7.5) and total protein (60 μg), subjected to SDS/PAGE, blotted, and probed overnight with 1:1000 anti-PRL receptor (sc-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:250 anti-phospho-STAT3 (Tyrosine 705) (9131; Cell Signaling, Beverly, MA, USA), 1:250 anti STAT3 (sc-483; Santa Cruz Biotechnology), or 1:1000 anti-β tubulin (ab6046; Abcam, Cambridge, MA, USA) primary antibodies. Blots were washed in Tris-buffered saline/Tween-20 and detection was performed using goat anti-rabbit conjugated to alkaline phosphatase (1:5000) or to horseradish peroxidase (1:10,000) as secondary antibodies (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Protein densities were quantified with Quantity One software (Bio-Rad, Richmond, CA, USA).

Statistical analysis

The Sigma Stat 7.0 software (Systat Software, San Jose, CA, USA) was used. Data distribution and equality of variances were determined by the D’Agostino-Pearson test. When the distribution was normal and variances were equal, the t test was used to evaluate differences between two groups and one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used to compare differences between more than three groups. In the case of data with a non-parametric distribution, statistical differences between two groups and more than three groups were determined by the Mann-Whitney U test and Kruskal-Wallis test followed by Dunn’s post-hoc correction, respectively. The threshold for significance was set at P < 0.05.

Results

The long PRL receptor isoform increases in the joints and PRL reduces the systemic levels of C-reactive protein and TNFα and the expression of transcription factors and cytokines related to Th17 cells and regulatory T cells in the joints of rats subjected to AIA

Confirming our previous findings [18], osmotic mini-pumps delivering PRL, implanted 3 days before the injection of CFA, elevated serum PRL fivefold (Fig. 1a), reduced ankle joint swelling (ankle circumference, Fig. 1b), and lowered joint expression of the proinflammatory cytokine genes, Tnfa, Il1b, Il6, and Ifng (Fig. 1c) at day 21 after inducing AIA with CFA in rats. At this
The long prolactin (PRL) receptor isoform increases in the joints and PRL reduces the systemic levels of C-reactive protein and TNFα and the expression of transcription factors and cytokines related to T helper 17 (Th17) cells and regulatory T cells in the joints of rats subjected to polyarticular adjuvant-induced arthritis (AIA). Osmotic minipumps delivering PRL were positioned (or not positioned) 3 days before the intradermal injection of complete Freund’s adjuvant (CFA) to induce AIA in rats. All evaluations were performed 21 days after injection of CFA.

- **a** Serum PRL levels.
- **b** Ankle circumference.
- **c** Quantitative real-time PCR (qRT-PCR) quantification of Tnfa, Il1b, Il6, and Ifng mRNA levels in ankle joints.
- **d** qRT-PCR and western blot evaluation of PRL receptor (Prlr) long (Long) and short (Short) isoform mRNA and long PRL receptor protein (PRLR Long), respectively. Bars show the quantification of PRLR Long/β-Tubulin by densitometry (n = 3).
- **e** C-reactive protein (CRP) and **f** TNFα levels in serum.
- **g** qRT-PCR quantification of the mRNA levels of the Th17-cell-related transcription factors, Rora and Rorc, and cytokine genes Il17a, Il21, Il22, and Il23 in the ankle joints.
- **h** qRT-PCR quantification of the mRNA levels of the regulatory-T-cell-related transcription factor Foxp3 and cytokine genes Ebi3, Il12a, Tgfb1, and Il10 in the ankle joints. Values are means ± SEM (n = 5–12). *P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant.
time, the levels of the long form of the PRL receptor mRNA and protein increased in the AIA joints, whereas no changes in the transcript of the short PRL receptor isoform were detected (Fig. 1d). PRL infusion in AIA was associated with reduced serum levels of C-reactive protein (CRP, Fig. 1e) and TNFα (Fig. 1f), two biomarkers of systemic inflammation in arthritis. Moreover, the imbalance between Th17 and T-regulatory lymphocytes influences inflammation and osteoclastogenesis in arthritis [30], and PRL blocked the AIA-induced increase in the joint expression of the genes Rorc and Rora encoding for retinoic acid orphan nuclear receptors RORγt and RORA, respectively, which are transcription factors specific for Th17 cells, and the expression of the Th17 cell cytokine genes, Il17a, Il21, and Il22 (Fig. 1g). Also, PRL inhibited the AIA-induced expression of the gene encoding for forkhead box protein 3 (Foxp3), a transcription factor specific for regulatory T cells, and the AIA-induced expression of the regulatory-T-cell-related genes encoding for the cytokines IL-35 (a heterodimer composed of IL12a and Ebi3 subunits), TGFβ-1, and IL-10 (Fig. 1h). PRL had no effects in non-arthritic, control animals (Fig. 1a-c and e-g).

PRL reduces loss of trabecular bone and osteoclast density in AIA
Because systemic and local CRP, TNFα, IL-1β, IL-6, and IL-17A trigger osteoclastogenesis by upregulating RANKL in the arthritic joint [2, 31, 32], we evaluated the effect of PRL infusion on trabecular bone loss and osteoclastogenesis in rats with AIA. PRL reduced the loss of trabecular bone area (Fig. 2a) and blocked the increase in osteoclast density in arthritic joints (Fig. 2b). Moreover, PRL decreased osteoclastogenesis as revealed by the reduction in the mRNA levels of the osteoclast-associated genes: Trap, Mmp9, Ctsk, and the gene encoding for RANK (Tnfrsf11a) (Fig. 2c). Also, PRL blocked the AIA-induced expression in the mRNA levels of the gene encoding for RANKL (Tnfrsf11) (Fig. 2d). The expression of the gene encoding for OPG (Tnfrsf11b) was not modified by AIA or by PRL, but this hormone reduced the Tnfrsf11/Tnfrsf11b mRNA ratio in the arthritic joint (Fig. 2d). PRL had no effects on trabecular bone area and osteoclastogenesis in the joints of control rats.

MAIA-induced joint inflammation and expression of proinflammatory cytokines and that of transcription factors and cytokines related to Th17 cells and regulatory T cells are enhanced in PRL-receptor-null mice
To further explore the protective effect of PRL against inflammation and osteoclastogenesis in arthritis, mice null (Prlr/-) or not (Prlr+/+) for the PRL receptor were subjected to MAIA, as this is a highly efficient model for inducing unilateral joint inflammation and bone erosion in the genetic background (C57BL6) of these mice [23]. Inflammation was greater (Fig. 3a) and the expression of the proinflammatory cytokine genes, Tnfa, Il1b, Il6, Infg (Fig. 3b), the Th17 cell-related genes, Il17a, Il21, Il22, Il23 (Fig. 3c); and the regulatory-T-cell-associated genes, Foxp3 and Il10, were significantly elevated (Fig. 3d) in the arthritic knee-joint of Prlr/-/- mice compared to Prlr+/+ mice. The expression of Il12a was increased in MAIA joints but that of Rora, Rorc, and Tgfb1 was not, and these four genes remained unchanged in the absence of PRL receptors (Fig. 3c and d).

Trabecular bone area is reduced and osteoclast density is increased in PRL receptor-null mice subjected to MAIA
Confirming that PRL-receptor-null mice are osteopenic [19], the loss of trabecular bone area was greater in control non-arthritic Prlr/-/- mice relative to Prlr+/- mice (Fig. 4a). Moreover, the trabecular bone area was reduced (Fig. 4a), whereas the density of osteoclasts (Fig. 4b) and the mRNA levels of Trap and Tnfrsf11 (Fig. 4c, d) were higher in the arthritic joints of Prlr/-/- compared to wild-type mice. There were no differences in the expression levels of Mmp9, Ctsk, Tnfrsf11a, and Tnfrsf11b between Prlr/-/- and Prlr+/- mice, but the Tnfrsf11/Tnfrsf11b ratio was higher in Prlr/-/- mice. Non-arthritic, control Prlr/-/- mice showed elevated, albeit non-significant, osteoclastogenesis parameters relative to non-arthritic, control Prlr+/- mice (Fig. 4b, c, d). These findings indicate that bone loss and osteoclast development are potentiated in arthritic joints in the absence of PRL signaling and that this hormone influences the maintenance of bone mass under normal conditions.

TNFα, IL-1β, and IFNγ induce the expression of the long PRL receptor in synovial membranes and synovial fibroblasts
To further investigate the role of PRL receptor signaling in the arthritic joint, a combination of proinflammatory cytokines (Cyt: TNFα, IL-1β, and IFNγ) was injected into the intra-articular space of knee joints of mice in an attempt to mimic the AIA inflammatory condition. The intra-articular Cyt injection elevated the levels of the long form of the PRL receptor mRNA in whole synovial membranes 48 hours after injection (Fig. 5a). Likewise, Cyt treatment for 24 hours stimulated the expression of the long PRL receptor mRNA and protein in primary cultures of mouse synovial fibroblasts (Fig. 5b), implying that these cells become relevant targets of PRL under inflammatory conditions.
Fig. 2 (See legend on next page.)
Fig. 2 Prolactin (PRL) reduces loss of trabecular bone area and osteoclastogenesis in polyarticular adjuvant-induced arthritis (AIA). Osmotic minipumps delivering PRL were positioned (or were not positioned) 3 days before the intradermal injection of complete Freund’s adjuvant (CFA) to induce AIA in rats. The experiments ended on day 21 after injection of CFA, when all evaluations were performed. a Representative images of hematoxylin-eosin-stained tibiotarsal joint sections. Scale bar 500 μm. Ep epiphyseal plate, Bm bone marrow, Ct cortical bone, Tb trabecular bone, ep cartilage, sm synovial membrane. Graph indicates the values of trabecular bone area (trabecular surface area divided by the total bone area). b Representative images of tartrate-resistant alkaline phosphatase (TRAP)-stained tibiotarsal joint sections where TRAP-positive purple spots (multinucleated cells [osteoclasts]) below the growth plate are indicated (arrows). Scale bar 200 μm. Graph shows the number of osteoclasts per bone surface (NOc/BS). c Quantification by quantitative real-time PCR (qRT-PCR) of the mRNA levels of the osteoclast gene markers: Trap, Mmp9, Csk, and Tnfrsf11a in the ankle joints. d qRT-PCR quantification of the mRNA levels of Tnfrsf11 and Tnfrsf11b and of the Tnfrsf11/Tnfrsf11b mRNA ratio in the ankle joints. Values are means ± SEM (n = 5–8). *P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant.

Fig. 3 Joint inflammation and expression of proinflammatory cytokines and of transcription factors and cytokines related to T helper 17 (Th17) cells and regulatory T cells are enhanced in prolactin (PRL)-receptor-null mice with monoarticular adjuvant-induced arthritis (MAIA). Mice null (Prlr−/−) or not (Prlr+/+) for PRL receptors were injected with complete Freund’s adjuvant (CFA) into the right knee joint once every 7 days to induce MAIA. The experiments ended on day 18 after the initial CFA injection, when all evaluations were performed. a Time course of knee circumference in control and MAIA groups. b Quantification by quantitative real-time PCR (qRT-PCR) of Tnfa, Il1b, Il6, and Ifng mRNA levels in the knee joints. c qRT-PCR quantification of the mRNA levels of the Th17-cell-related transcription factors, Rora and Rorc, and cytokine genes Il17a, Il21, Il22, and Il23 in the knee joints. d qRT-PCR quantification of the mRNA levels of the regulatory-T-cell-related transcription factor Foxp3 and cytokine genes Ebi3, Il12a, Tgfb1, and Il10 in the knee joints. Values are means ± SEM (n = 8–12). *P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant.
Fig. 4 (See legend on next page.)
Trabecular bone area is reduced and osteoclastogenesis is enhanced in prolactin (PRL)-receptor-null mice subjected to monoarticular adjuvant-induced arthritis (MAIA). Mice null (Prlr-/-) or not (Prlr+/+) for the PRL receptor were injected with complete Freund’s adjuvant (CFA) into the right knee joint once every 7 days to induce MAIA. The experiments ended on day 18 after the initial CFA injection, when all evaluations were performed. Representative images of hematoxylin-eosin-stained tibiofemoral joint sections. Scale bar 500 μm. Ep epiphyseal plate, Bm bone marrow, Ct cortical bone, Tb trabecular bone, c cartilage, sm synovial membrane. Graph indicates the values of trabecular bone area (trabecular surface area divided by the total bone area).

**a** Representative images of tartrate-resistance alkaline phosphatase (TRAP)-stained tibiofemoral joint sections where TRAP-positive purple spots (multinucleated cells (osteoclasts)) below the growth plate are indicated (arrows). Scale bar 200 μm. Graph shows the number of osteoclast per bone surface (N.Oc./BS). c Quantification by quantitative real-time PCR (qRT-PCR) of the mRNA levels of the osteoclast gene markers: Trap, Mmp9, Ctsk, and Tnfrsf11a in the knee joints. d qRT-PCR-based quantification of the mRNA levels of Tnfrsf11 and Tnfrsf11b and of the Tnfrsf11b:Tnfrsf11 mRNA ratio in the knee joints. Values are means ± SEM (n = 5–12). *P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant.
PRL blocks Cyt-induced Tnfrsf11 expression in synovial fibroblasts by a STAT3-dependent pathway

We next studied the contribution of synovial fibroblasts to the anti-osteoclastogenic effects of PRL by culturing synovial fibroblasts with Cyt in the absence or presence of PRL. Cyt induced the expression of the osteoclastogenesis-inducing cytokines Il1b, Il6, and Tnfrsf11, and this effect was blocked by PRL (Fig. 5c). Cyt and PRL did not alter the expression of Tnfa or Tbfrsf11b (Fig. 5c). Because PRL signals through STAT3 in synovial fibroblasts, PRL stimulated the expression of Stat3 mRNA in synovial fibroblasts in culture (Fig. 5d) and promoted the phosphorylation/activation of STAT3 when the cells were cultured in the presence of Cyt (Fig. 5e). Moreover, incubation of synovial fibroblasts with the STAT3 inhibitor S31-201 [34] blocked PRL inhibition of Cyt-induced Tnfrsf11 expression (Fig. 5f), thereby suggesting that PRL signals through STAT3 to inhibit osteoclastogenesis in joints under inflammatory conditions.

PRL reduces Cyt-induced osteoclast formation in co-cultures of synovial fibroblasts and osteoclast progenitor cells

To investigate synovial fibroblasts and osteoclast progenitor cells as targets of PRL inhibition of osteoclastogenesis, we evaluated the effect of PRL on synovial fibroblasts co-cultured with bone-marrow-derived osteoclast progenitors in the presence of Cyt, which act on synovial fibroblasts to induce the secretion of RANKL necessary for osteoclast differentiation [6] (Fig. 6a). The time course of the co-culture was as follows: 12 hours after being seeded, synovial fibroblasts were treated or not with PRL for 16 hours. Cyt were then added or not to the culture and after 24 hours osteoclast progenitors were delivered in medium containing 1, 25-dihydroxy-vitamin D3. Co-cultures were incubated for 10 days, replacing with new medium (containing +/- Cyt, +/- PRL, and 1, 25-dihydroxy-vitamin D3) every 2 days. Cyt treatment promoted the differentiation of osteoclast precursors into TRAP+, enlarged, multinucleated cells (mature osteoclasts), and this effect was significantly reduced by PRL (Fig. 6a). PRL can act on synovial fibroblasts to inhibit osteoclastogenesis (Fig. 5). To investigate osteoclast progenitors as PRL targets, synovial fibroblasts derived from Prlr +/+ mice were co-cultured with osteoclast progenitors from either Prlr+/- mice or Prlr-/- mice. PRL had no significant effect on the number of mature osteoclasts when osteoclast progenitors were derived from Prlr-/- mice. However, the number of mature osteoclasts was similar between the co-cultures treated with Cyt and PRL, whether or not osteoclast progenitors were derived from PRL-receptor-null mice (Fig. 6b). These findings support a direct effect of PRL on both synovial fibroblasts and osteoclast progenitors to reduce osteoclastogenesis.

Expression of PRL receptor transcripts in chondrocytes, osteoblasts, and osteoclast-like cells

In addition to synovial fibroblasts, other cells in joint tissues express PRL receptors. RT-PCR performed on chondrocytes, osteoblasts, osteoclast-like cells, and synovial fibroblasts (positive controls) from Prlr+/+ mice revealed a single band of 139 bp, the size of which corresponded to the region amplified from exon 5 of the Prlr gene (Fig. 6c). Specificity of the reaction was confirmed by the absence of such a transcript in cells obtained from Prlr-/- mice. In these knockout mice, exon 5 of the PRL receptor has been deleted, which results in a very short peptide unable to bind to PRL.

Discussion

Articular bone loss and increased fracture risk in arthritis are indicative of an imbalance between bone resorption and formation. Bone resorption depends on the development of bone resorbing osteoclasts (osteoclastogenesis) in response to systemic and local signals that converge to regulate the RANKL/RANK/OPG system [2, 3, 7, 8]. Here, we showed that PRL reduces the systemic levels and the joint production of cytokines with osteoclastogenic activity, lowers the joint expression of the genes encoding for RANKL and RANK, decreases osteoclast density, and reduces trabecular bone area in two murine models of inflammatory arthritis. Moreover, synovial fibroblasts are an important source for RANKL-induced bone loss in arthritis [6], and we showed that PRL downregulates cytokine-induced expression of the RANKL gene in cultured synovial fibroblasts via a STAT3-dependent pathway, and that this hormone reduces osteoclastogenesis in co-cultures of synovial fibroblasts and osteoclast progenitor cells.

Chronic inflammation determines local and systemic bone loss in RA [2, 3] and may be attenuated by PRL. PRL treatment reduces joint swelling, proinflammatory cytokine expression (TNFa, IL-1β, IL-6, and IFNγ), pan-nus formation, and the destruction of bone trabeculae in rats with AIA [18], a model of inflammatory arthritis. Here, we have extended these findings by showing that increasing serum PRL to levels similar to those (40 ng/ml) found in the circulation of some patients with RA [35] reduced systemic levels of CRP and TNFα, two osteoclastogenic cytokines [2, 3, 31]. The serum levels of CRP and TNFα in RA patients correlate with their levels in the
synovial fluid and reflect systemic and joint inflammatory responses [31, 36]. Moreover, PRL treatment reduced the expression of transcription factors and cytokines associated with Th17 and T regulatory cells in arthritic joints. Th17 cells promote inflammation, osteoclastogenesis, and bone erosion in arthritis [32, 37], whereas T regulatory cells suppress immune responses [38], and the imbalance of the two T lymphocyte subpopulations has been identified as a key event in the pathogenesis of rheumatoid arthritis [30]. It remains to be determined how PRL actions, particularly the downregulation of T regulatory cells, would contribute to PRL protection against inflammation and osteoclastogenesis in arthritis. The matter is complex as the differentiation and function of Th17 and T regulatory cells are closely related and vary in the presence of strong inflammatory conditions [39, 40]. Both T cell populations require transforming growth factor (TGF)β for differentiation [41], and in vivo studies have identified a subset of T cells that dually express elements of both the T regulatory and Th17 phenotypes [40, 42] with potent inflammatory and osteoclastogenic effects in autoimmune arthritis [43].

In support of PRL acting locally on inflamed joint tissues, we show that the long PRL receptor isoform is upregulated in the joints of rats with AIA and in the synovial membranes and synovial fibroblasts of mice treated with the proinflammatory cytokines, TNFα, IL-1β, and IFNy, which contribute to AIA inflammatory lesions [44]. PRL receptors exist in various molecular forms that differ primarily in the sequence and length of their cytoplasmic domain and are classified as long,
intermediate, and short [45]. The long PRL receptor is considered the major isoform signaling all PRL actions, the intermediate isoform transmits cell proliferation and survival signals, and the short PRL receptor exerts dominant-negative effects on signals by the long form [46]. The long form of the PRL receptor appears to predominate in osteoblasts [19] and chondrocytes [25], where PRL promotes bone development and cartilage survival. Also, fibroblasts and T cells in the synovium of patients with RA express PRL receptors [47], and proinflammatory cytokines induce the expression of the long form of the PRL receptor in lung fibroblasts to mediate anti-inflammatory effects of PRL in the airways [48]. Therefore, these findings could imply that the long form of the PRL receptor mediates the protective effects of PRL in arthritis.

Supporting the anti-inflammatory role of PRL in arthritis, PRL-receptor-null mice exhibited increased joint swelling and higher joint expression of the genes encoding for TNFα, IL-1β, IL-6, IFNγ, IL-17A, IL-21, IL-22, IL-23, and IL-10 when subjected to monoarticular AIA (MAIA). These findings are consistent with PRL treatment inhibiting the expression of the same cytokines in the joints of rats with AIA [18] and with previous reports showing that targeted disruption of PRL [13] and of PRL receptors [49] enhances immune responses and mortality under stress-related conditions. Besides their crucial effects on joint inflammation, TNFα, IL-1β, IL-6, and IL-17A also drive bone loss. They promote osteoclastic bone resorption largely by stimulating the expression and function of RANKL and RANK [2, 32]. Accordingly, we next investigated whether PRL downregulates osteoclast development and bone loss in AIA and MAIA.

Increasing systemic PRL levels in rats with AIA and blocking PRL receptor signaling in Prlr−/− mice with MAIA reduced and increased, respectively, the loss of trabecular bone area, osteoclast density, the accumulation of phenotypic markers identifying osteoclasts (the gene for RANKL cognate receptor RANK and the genes encoding for acid (TRAP and cathepsin K) and neutral (MMP-9) proteases used by osteoclasts to degrade the organic matrix of bone [3]). Moreover, PRL and lack of PRL signaling reduced and increased, respectively, the expression of RANKL gene (Tnfrsf11) in the arthritic joint, which resulted in a lower and higher Tnfrsf11/Tnfrsf11b mRNA ratio for each condition, respectively. Tnfrsf11b encodes for OPG, a soluble decoy receptor that neutralizes RANKL and prevents bone erosion in AIA [4]. A high RANKL/OPG ratio occurs in patients with RA and is associated with increased bone resorption [50].

Our findings indicate that inhibition of RANKL-induced osteoclast development is a component of PRL protection against bone loss in inflammatory arthritis. It is unclear whether PRL signals directly on osteoclasts or indirectly through other cell types responsible for bone loss. Arguing against osteoclasts being direct targets of PRL is the finding that bone marrow cells differentiated into osteoclasts are devoid of the PRL receptor [19]. On the other hand, chondrocytes, osteoblasts, and synovial fibroblasts express PRL receptors and are major sources of osteoclastogenic cytokines including RANKL [2, 5, 6, 51]. Here, we support direct effects of PRL on chondrocytes, synovial fibroblasts, and osteoblasts, but also in osteoclasts by showing the PCR-mediated amplification of a PRL receptor transcript in the various cell types obtained from Prlr+/+ mice but not in those obtained from Prlr−/− mice. While differences in osteoclast differentiation protocols may contribute to our contrasting finding [19], the presence of the PRL receptor in osteoclasts reinforces the role of PRL on bone remodeling.

Consistent with synovial fibroblasts being cellular targets of PRL-induced anti-osteoclastogenesis, the incubation of primary cultures of synovial fibroblasts with TNFα, IL-1β, and IFNγ (Cyt) elevated the expression of RANKL and RANK [2, 32]. These findings support the anti-osteoclastogenic role of PRL in arthritis.

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joints. Reduction of RANK levels may contribute to PRL inhibitory effects on osteoclastogenesis and bone loss at the level of osteoclast progenitors and mature osteoclasts.

PRL regulation of the RANKL/RANK/OPG system for physiological bone remodeling during growth and reproduction is well-substantiated [58]. PRL decreases the RANKL/OPG ratio by downregulating RANKL and upregulating OPG in osteoblasts to promote bone formation early in life [20], whereas in pregnancy and lactation, PRL accelerates bone turnover by raising the RANKL/OPG ratio in osteoblasts [59, 60] in order to supply calcium for fetal growth and milk production. The opposing effects of PRL on bone remodeling indicate that the outcome of its action depends on complex age-related and hormone-related interactions.

In experimental inflammatory arthritis, PRL appears to be beneficial; however, the role of PRL in RA is controversial (as reviewed [58]). PRL increases in the circulation of some patients with RA, but it is not clear whether systemic PRL levels correlate with disease severity. Confounding factors include the contribution of PRL synthesized locally by joint tissues like chondrocytes [61], endothelial cells [62], synoviocytes and immune cells [47]; and the ability of PRL to exert immunostimulatory or immunosuppressive effects, depending on its level and that of other cytokines and hormones [58]. Hyperprolactinemia in the context of reproduction [59] and non-inflammatory pathology (prolactinoma) [63] promotes bone loss, whereas high circulating PRL levels can be anti-inflammatory and reduce bone loss under inflammatory conditions [18, 64] (and present findings). The mechanisms governing the effects of PRL on bone remodeling are challenging but a better understanding of them could lead to the use of hyperprolactinemia-inducing drugs as therapeutic agents in the clinic.

**Conclusion**

We demonstrated in this study that PRL protects against bone loss and osteoclastogenesis in inflammatory arthritis by inhibiting cytokine-induced activation of RANKL in joints and synovial fibroblasts via its canonical STAT3 signaling pathway.

**Additional file**

Additional file 1: Table S1. Primers used for qRT-PCR. Primers and their annealing temperatures used in quantitative RT-PCR studies. (DOCX 24 kb)

**Abbreviations**

AIA: Adjuvant-induced arthritis; bp: Base pairs; CD90.2: Cluster of differentiation 90; CFA: Complete Freund’s adjuvant; CRP: C-reactive protein; Ctsk: Cathepsin K gene; Cyt: Combination of the proinflammatory cytokines TNFa, IL-1β, and interferon-γ; DMEM: Dulbecco’s modified Eagle’s medium; Ebi3: Gene encoding for Epstein-Barr virus induced 3; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; Foxp3: Gene encoding for forkhead box protein 3; Hprt: Hypoxanthine-guanine phosphoribosyltransferase; ICAM-1: Intercellular adhesion molecule 1; IFNγ: Interferon γ; IL: Interleukin; iNOS: Gene encoding for interleukin 10; iNOS2: Gene encoding for interleukin 12A; JAK-2: Janus kinase-2; MAIA: Monoarticular adjuvant-induced arthritis; MMP9: Matrix metalloproteinase 9; OPG: Osteoprotegerin; PBS: phosphate-buffered saline; PRL: Prolactin; PRLR: Prolactin receptor; qPCR: Quantitative polymerase chain reaction; qRT-PCR: Quantitative real-time polymerase chain reaction; RA: Rheumatoid arthritis; RANK: Receptor activator of nuclear factor κB; RANKL: Receptor activator of nuclear factor κB ligand; ROSr: Gene encoding for retinoic acid orphan nuclear receptors RORα; RORc: Gene encoding for retinoic acid orphan nuclear receptors RORγt; STAT3: Signal transducer and activator of transcription 3; Tgfβ1: Gene encoding for transforming growth factor β 1; Th: T helper; Tnfrsf11: Gene encoding for RANKL; Tnfrsf11a: Gene encoding for RANK; Tnfrsf11b: Gene encoding for OPG; TNFa: Tumor necrosis factor alpha; TRAP: Tartrate-resistant acid phosphatase; VCAM1: Vascular cell adhesion molecule 1

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**Availability of data and materials**

Our study did not yield datasets suitable to include in online repositories and no biological material was left to include in a biobank.

**Authors’ contributions**

Conception and design: MGL-C, CC. Acquisition of data: NA, GO, MS-G, FL-B. Analysis and interpretation of data: MGL-C, GME, CC. Drafting of the manuscript: MGL-C, CC. All authors were involved in critical revision of the manuscript for important intellectual content, and all authors approved the final version of the manuscript and take responsibility for appropriate portions of the content, accuracy and integrity.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval**

Experiments were conducted in accord with institutional guidelines and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The Bioethics Committee of the Institute of Neurobiology of the National University of Mexico (UNAM) approved all animal experiments.

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