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Research Article **Inflammation**

Chondrocytes are the only cells in cartilage, and their death by apoptosis contributes to cartilage loss in inflammatory joint diseases, such as rheumatoid arthritis (RA). A putative therapeutic intervention for RA is the inhibition of apoptosis-mediated cartilage degradation. The hormone prolactin (PRL) frequently increases in the circulation of patients with RA, but the role of hyperprolactinemia in disease activity is unclear. Here, we demonstrate that PRL inhibits the apoptosis of cultured chondrocytes in response to a mixture of proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ) by preventing the induction of p53 and decreasing the BAX/BCL-2 ratio through a NO-independent, JAK2/STAT3–dependent pathway. Local treatment with PRL or increasing PRL circulating levels also prevented chondrocyte apoptosis evoked by injecting cytokines into the knee joints of rats, whereas the proapoptotic effect of cytokines was enhanced in PRL receptor–null ($Prlr^{-/-}$) mice. Moreover, eliciting hyperprolactinemia in rats before or after inducing the adjuvant model of inflammatory arthritis reduced chondrocyte apoptosis, proinflammatory cytokine expression, pannus formation, bone erosion, joint swelling, and pain. These results reveal the protective effect of PRL against inflammation-induced chondrocyte apoptosis and the therapeutic potential of hyperprolactinemia to reduce permanent joint damage and inflammation in RA.

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Chondrocytes are the only cells in cartilage, and their death by apoptosis contributes to cartilage loss in inflammatory joint diseases, such as rheumatoid arthritis (RA). A putative therapeutic intervention for RA is the inhibition of apoptosis-mediated cartilage degradation. The hormone prolactin (PRL) frequently increases in the circulation of patients with RA, but the role of hyperprolactinemia in disease activity is unclear. Here, we demonstrate that PRL inhibits the apoptosis of cultured chondrocytes in response to a mixture of proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ) by preventing the induction of p53 and decreasing the BAX/BCL-2 ratio through a NO-independent, JAK2/STAT3–dependent pathway. Local treatment with PRL or increasing PRL circulating levels also prevented chondrocyte apoptosis evoked by injecting cytokines into the knee joints of rats, whereas the proapoptotic effect of cytokines was enhanced in PRL receptor–null (*Prlr*$^{-/-}$) mice. Moreover, eliciting hyperprolactinemia in rats before or after inducing the adjuvant model of inflammatory arthritis reduced chondrocyte apoptosis, proinflammatory cytokine expression, pannus formation, bone erosion, joint swelling, and pain. These results reveal the protective effect of PRL against inflammation-induced chondrocyte apoptosis and the therapeutic potential of hyperprolactinemia to reduce permanent joint damage and inflammation in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory disease with a worldwide prevalence of 1% to 2%. Autoimmunity followed by the articular infiltration of leukocytes and hyperplasia of synovial cells lead to the development of an invasive inflammatory pannus that destroys the adjacent cartilage and bone. Locally produced cytokines are crucial for initiating the inflammatory process and destroying articular tissue (1). Among these cytokines, TNF-α, IL-1β, and IFN-γ stimulate both chondrocyte apoptosis and cartilage extracellular matrix degradation, and their inhibition ameliorates joint destruction (1–4). Transgenic mice expressing TNF-α, a model of polyarthritis (5), display chondrocyte apoptosis before the onset of full arthritis, suggesting that cytokine-induced chondrocyte apoptosis is a primary cause of, rather than an event secondary to, cartilage matrix breakdown (6). Thus, factors able to counteract chondrocyte apoptosis under inflammatory conditions are relevant for the treatment of RA (7–11). One such factor is prolactin (PRL).

PRL acts both as a circulating hormone and a cytokine to regulate the function of a wide variety of tissues, including cartilage. PRL and the PRL receptor are expressed in chondrocytes (12, 13), where this hormone can promote differentiation and survival. PRL stimulates the synthesis of proteoglycans and type II collagen by bone marrow–derived chondrocytic mesenchymal cells (14), and it inhibits the apoptosis of articular chondrocytes induced by serum deprivation (13). The action of PRL on chondrocyte survival may be relevant in RA. PRL is present in RA synovial fluid (14, 15), is produced by RA synovial cells (16), and can influence cartilage survival by exerting immunoregulatory effects. The PRL receptor is a member of the hematopoietin/cytokine receptor superfamily and is expressed in a variety of immune cells, in which this hormone can be proinflammatory or antiinflammatory by regulating proliferation, survival, and the release of inflammatory mediators (17).

Given that cytokine-induced chondrocyte apoptosis contributes to cartilage destruction in RA (1, 2, 6, 9), we investigated the survival effect of PRL on chondrocytes treated in vitro or in vivo with a mixture of TNF-α, IL-1β, and IFN-γ (Cyt) and whether this effect protects against cartilage destruction in the adjuvant-induced model of inflammatory arthritis in rats. We demonstrate that PRL treatment inhibits, and PRL receptor deficiency enhances, Cyt-induced cartilage apoptosis and that the PRL effect on survival occurs in chondrocytes via a NO-independent, JAK2/STAT3–dependent pathway. We also show that hyperprolactinemia promotes the survival of arthritic cartilage by blocking the expression of proinflammatory cytokines and their proapoptotic effect on chondrocytes and that PRL delays the onset and ameliorates the severity of inflammatory arthritis. We conclude that current medications able to increase prolactinemia constitute novel potential therapies to control inflammation-driven cartilage degradation and joint damage in RA.

Authorship note: Norma Adán, Jessica Guzmán-Morales, and Maria G. Ledesma-Colunga contributed equally to this work. Sonia I. Perales-Canales is deceased.

Conflict of interest: The authors have declared that no conflict of interest exists.

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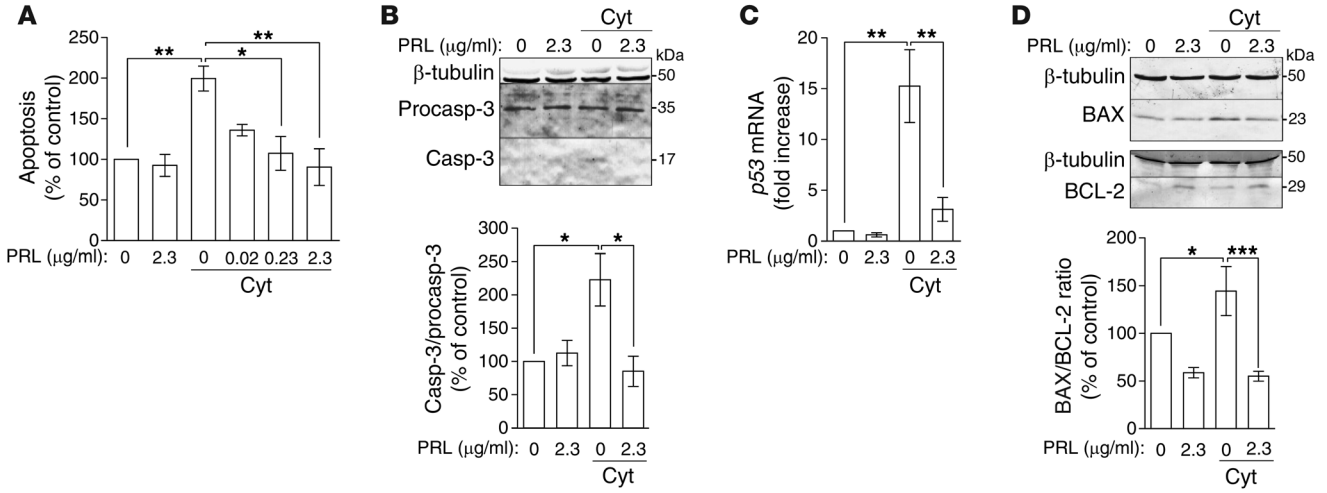

Figure 1

PRL inhibits Cyt-induced apoptosis of chondrocytes in culture. (**A**) Primary cultures of rat chondrocytes were challenged with Cyt, combined or not with different concentrations of PRL, and apoptosis was evaluated at 24 hours by ELISA (n = 3–6). (**B**) Representative Western blot of procaspase-3 and active caspase-3 (Procasp-3 and Casp-3, respectively) in lysates of chondrocytes incubated or not with Cyt in the absence or presence of PRL for 6 hours. The graph shows the quantification of active caspase-3 by densitometry after normalization to procaspase-3 (n = 3). (**C**) qRT-PCR–based quantification of *p53* mRNA levels (n = 3) in chondrocytes incubated or not with Cyt in the absence or presence of PRL for 24 hours. (**D**) Representative Western blot of BAX and BCL-2 in chondrocytes incubated or not with Cyt in the absence or presence of PRL for 4 hours. The graph shows the quantification of BAX/BCL-2 by densitometry (n = 3). Values are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

PRL blocks Cyt-induced apoptosis of chondrocytes in culture by a NO-independent, JAK2/STAT3–dependent pathway. We first studied whether PRL can act on chondrocytes to inhibit the proapoptotic effect of Cyt using primary cultures of rat articular chondrocytes. Cyt induced a 2-fold increase in chondrocyte apoptosis, as determined by chondrocyte internucleosomal DNA fragmentation measured by ELISA, and this increase was blocked in a dose-dependent manner by the coadministration of PRL (Figure 1A). The antiapoptotic effect of PRL was confirmed by Western blot analysis of procaspase-3 cleavage to the caspase-3 active form (Figure 1B). Cyt treatment enhanced the levels of active caspase-3 as compared with those after no treatment, and PRL blocked the Cyt-induced increase of active caspase-3. The graph in Figure 1B shows quantification of active caspase-3 after normalization for the amount of total procaspase-3 on the blot. We also investigated the expression of proteins that regulate apoptosis. Upon treatment with Cyt, there was a 15-fold increase in the mRNA expression of proapoptotic *p53*, as determined by qRT-PCR, and this increase was significantly reduced by PRL (Figure 1C). Also, Western blot analysis showed that PRL prevented the increase in the levels of proapoptotic BAX induced by Cyt and increased the levels of antiapoptotic BCL-2, resulting in a significant reduction in the BAX/BCL-2 ratio (Figure 1D).

Because NO produced by iNOS is a main factor mediating the effect of TNF-α, IL-1β, and IFN-γ on chondrocyte apoptosis (3, 4, 18), we tested whether the inhibition of Cyt-induced iNOS protein expression/NO production mediates the survival effect of PRL. Similar to PRL, addition of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (19) prevented Cyt-induced chondrocyte apoptosis (Figure 2A). However, PRL had no apparent effect on Cyt-induced upregulation of iNOS protein measured by Western blot (Figure 2B) or of the NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), evaluated by the Griess reaction (Figure 2C) in chondrocyte lysates or conditioned media, respectively. This indicates that inhibition of Cyt-induced apoptosis by PRL occurs through a NO-independent pathway. We next examined activation of JAK2/STAT3, a known PRL signaling pathway (20) that is implicated in chondrocyte survival (21). In the absence and presence of Cyt, addition of PRL to cultured chondrocytes stimulated the phosphorylation/activation of JAK2, as indicated by Western blotting (Figure 2D), and the activation of STAT3, as measured by its nuclear translocation (Figure 2E). STAT3 immunoreactivity was predominantly distributed throughout the cytoplasm, and treatment with PRL increased the localization of STAT3 immunostaining in the cell nucleus, indicative of STAT3 activation. Because incubation of chondrocytes with the STAT3 inhibitor S31-201 (22) resulted in chondrocyte apoptosis (Figure 2F), it is possible that activation of the JAK2/STAT3 pathway by PRL mediates its inhibitory effect on Cyt-induced chondrocyte apoptosis.

PRL inhibits the apoptosis of chondrocytes induced by the intra-articular injection of Cyt. To assess the survival action of PRL in vivo, Cyt with or without PRL were injected into the intra-articular space of knee joints of normoprolactinemic rats. Also, Cyt were injected in rats rendered hyperprolactinemic by placing 2 anterior pituitary glands (APs) under a kidney capsule for 15 days (23). After 48 hours, Cyt-injected knees showed a positive TUNEL signal on the outer border of the articular cartilage, visualized as a continuous fluorescent line, which was absent in the vehicle-injected controls (Figure 3A). The TUNEL-positive signal was located in chondrocytes (Figure 3A, inset), in which apoptosis was confirmed by active caspase-3 immunostaining and DAPI-DNA labeling (Figure 3B). There was no positive TUNEL reaction in the articular cartilage of knees coinjected with Cyt and PRL (Figure 3C) or in AP-grafted rats injected with Cyt (Figure 3E). Inhibition of the Cyt effect by PRL and AP grafts was statistically significant after quantifying

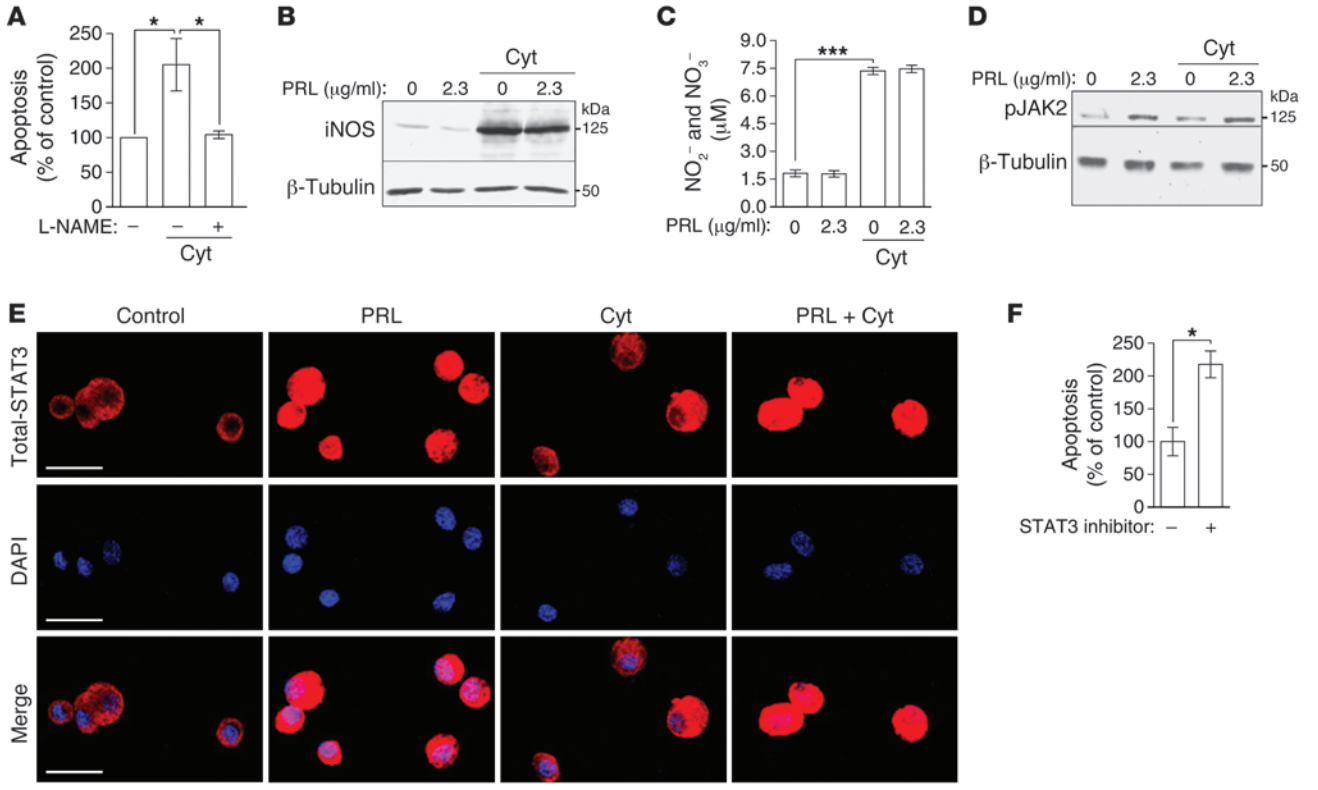

Figure 2

PRL inhibits Cyt-induced chondrocyte apoptosis by a NO-independent, JAK2/STAT3–dependent pathway. (**A**) Apoptosis evaluated by ELISA in chondrocytes incubated with Cyt in the presence or absence of the NO inhibitor L-NAME for 24 hours (n = 3–6). (**B**) Western blot analysis of iNOS (n = 3) and (**C**) NO_2^- and NO_3^- concentrations (n = 7) after incubating or not incubating chondrocytes with Cyt in the absence or presence of PRL for 6 and 24 hours, respectively. (**D**) Representative Western blot of phosphorylated JAK2 (pJAK2) in chondrocytes incubated with the various treatments for 30 minutes (n = 3). (**E**) Representative immunostaining for total STAT3 and DAPI in cultured chondrocytes treated with or without (control) PRL (2.3 μg/ml), Cyt, or PRL and Cyt (PRL + Cyt) for 1 hour (n = 3). Scale bar: 25 μm. (**F**) Apoptosis evaluated by ELISA in chondrocytes incubated in the absence or presence of 100 nM STAT3 inhibitor S31-201 for 24 hours (n = 3–4). Bars represent mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$.

the TUNEL signal (Figure 3, D and F). AP transplants resulted in a significant increase in circulating PRL levels (Figure 3G). These higher PRL levels were responsible for the reduction of Cyt-induced chondrocyte apoptosis, because this reduction was abrogated (Figure 3, E and F) by lowering circulating PRL with CB154 (Figure 3G), a dopamine D2 receptor agonist that inhibits AP PRL release (24). Therefore, intra-articular treatment with PRL or induction of high prolactinemia inhibits Cyt-induced chondrocyte apoptosis.

Cyt-induced chondrocyte apoptosis is enhanced in PRL receptor–null mice. In order to explore whether endogenous PRL helps maintain chondrocyte survival under inflammatory conditions, mice null for the PRL receptor ($Prlr^{-/-}$) were injected or not with Cyt in one knee and, after 48 hours, both the injected and noninjected knees were evaluated by TUNEL. In the absence of Cyt, there was no apparent histological alteration (data not shown) or positive TUNEL signal in the articular cartilage of $Prlr^{-/-}$ mice (Figure 4, A and B), indicating that PRL is not required for the survival of articular chondrocytes under normal conditions. After Cyt treatment, $Prlr^{-/-}$ mice showed TUNEL staining in the articular cartilage similar to that in wild-type counterparts (Figure 4, A and B). However, in $Prlr^{-/-}$ mice, but not in $Prlr^{+/+}$ mice, the noninjected knee, i.e., contralateral to the knee injected with Cyt, also showed a positive TUNEL reaction (Figure 4, C and D). These findings suggest that normal levels of PRL inhibit the proapoptotic effect of Cyt but that this action is only detected in response to lower levels of Cyt, such as those reaching a knee after contralateral intra-articular injection.

PRL prevents and reduces chondrocyte apoptosis in the adjuvant-induced model of inflammatory arthritis. Since PRL protects against Cyt-induced chondrocyte apoptosis, and Cyt can cause apoptosis-mediated cartilage loss in RA (1, 2, 6–9), we investigated whether PRL reduces chondrocyte apoptosis in the adjuvant-induced model of inflammatory arthritis in rats. Osmotic minipumps delivering PRL or subcutaneous tablets releasing haloperidol (Hal), a dopamine D2 receptor antagonist leading to hyperprolactinemia (25), were implanted 3 days before the injection of CFA (Figure 5A). On the day of CFA injection (day 0), infusion of PRL or Hal treatment elevated circulating PRL levels by 7 fold or 8 fold, respectively (Figure 5B). The hyperprolactinemic effect of PRL infusion was maintained and that of Hal decreased with time and resulted, at the end of the experiment (day 21 after CFA) (Figure 5B), in a 6-fold and 2-fold increase in serum PRL, respectively.

Consistent with cartilage destruction being a feature of adjuvant-induced arthritis (26), CFA treatment resulted in chondrocyte apoptosis, as revealed by TUNEL- and active caspase-3–

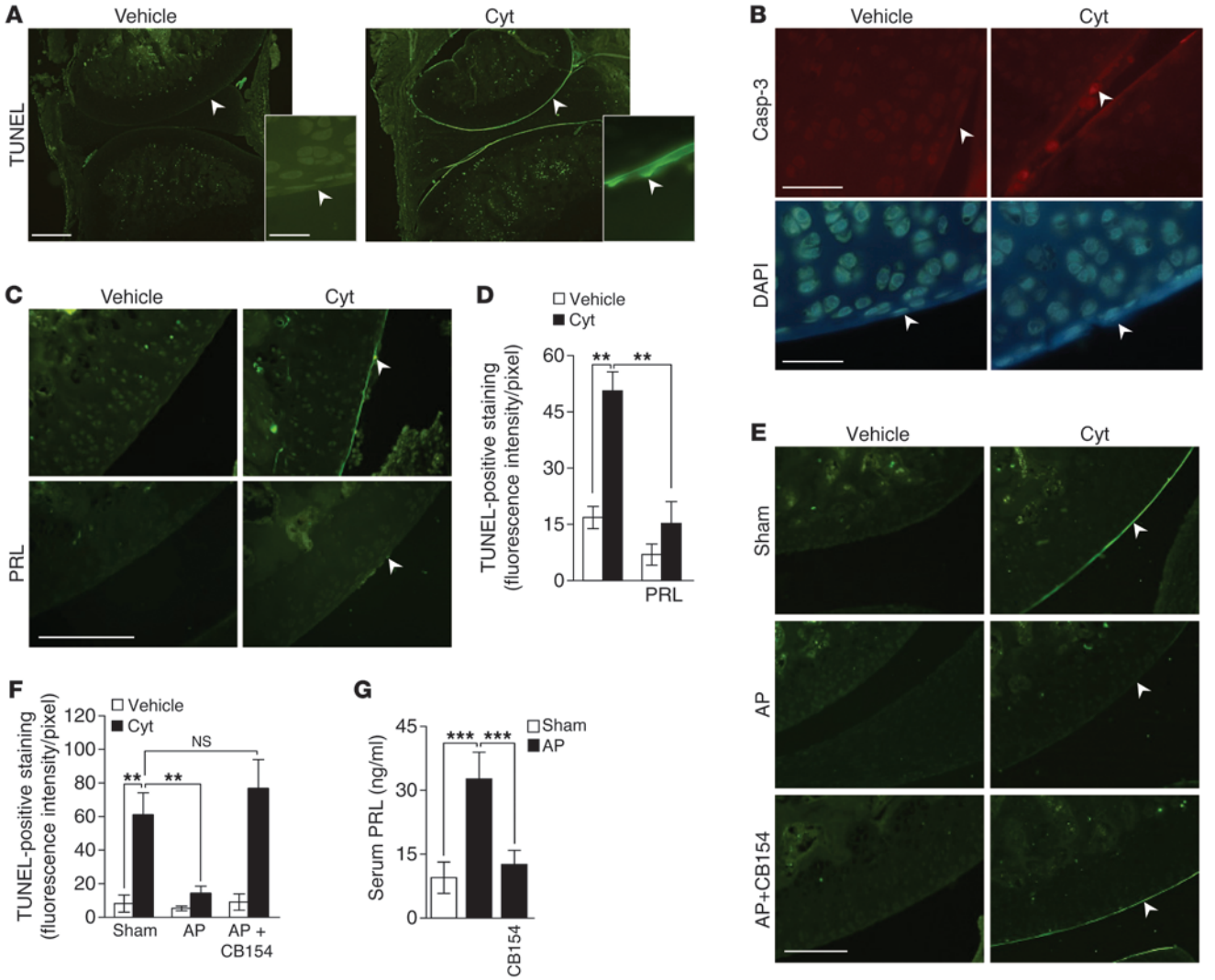

Figure 3

PRL inhibits the apoptosis of chondrocytes induced by the intra-articular injection of Cyt. (**A** and **B**) Apoptosis was assessed in rat knees injected with vehicle or Cyt by TUNEL and active caspase-3 staining. The bottom right images in **A** show enlarged views of knee cartilage. Scale bar: 500 μm (top); 25 μm (bottom). (**B**) Nucleic acids in chondrocytes were stained by DAPI. Scale bar: 50 μm. (**C** and **D**) TUNEL-positive staining and quantification in outer border cartilage of rat knees coinjected with Cyt and PRL (n = 4). Scale bar: 100 μm. (**E** and **F**) TUNEL-positive staining and quantification in outer border cartilages of rat knees from nontransplanted (sham) and AP-transplanted rats exposed to vehicle or Cyt, in the presence or absence of dopamine D2 receptor antagonist, CB154 (n = 4–6). (**A**–**C** and **E**) White arrowheads indicate cartilage outer border. Scale bar: 250 μm. (**G**) Serum PRL levels in sham or AP-transplanted rats treated or not with CB154 (n = 5–10). Bars represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

positive cells in the cartilage of knee joints on day 21 after CFA (Figure 5C), i.e., when joint swelling is at its peak, as seen below. At this time, CFA also produced a significant increase in the mRNA expression of *Casp3*, *Bax*, and *p53* in ankle joints (Figure 5D). Treatment with PRL or Hal lowered CFA-TUNEL and active caspase-3 staining and expression of proapoptotic mediators, indicating that this hormone prevents chondrocyte apoptosis in arthritic joints. We then investigated curative properties of PRL by placing osmotic minipumps delivering PRL 15 days after the injection of CFA (Figure 6A), i.e., when joint swelling is evident, as seen below. On day 21, PRL infusion had elevated serum PRL by 5 fold and 2 fold in control and CFA-treated animals, respectively (Figure 6B). Higher PRL levels correlated with reduced chondrocyte apoptosis (Figure 6C) and lower expression of proapoptotic mediators (Figure 6D) in the CFA-injected rats. These findings suggest that high prolactinemia prevents and reduces chondrocyte apoptosis in inflammatory arthritis.

PRL prevents and reduces adjuvant-induced arthritis. Because PRL has immunoregulatory properties (17), it may also promote cartilage survival in RA by attenuating joint inflammation. Early studies reported that AP-induced hyperprolactinemia reduces CFA-induced arthritis (27) and that Hal chronically suppresses inflammation in patients with RA (28, 29). Here, we show that PRL infusion, initiated 3 days before CFA injection (Figure 7A), delayed the onset and ameliorated the severity of joint inflammation, as indicated by a reduction in hind paw swelling (ankle circumference)

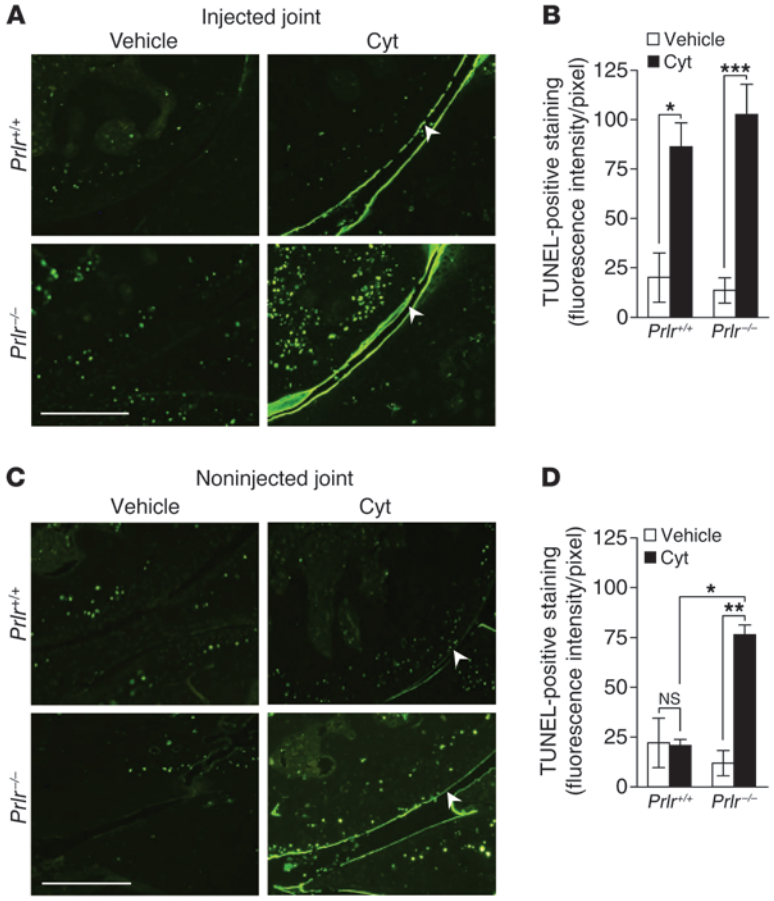

Figure 4
Cyt-induced chondrocyte apoptosis is enhanced in *Prlr*$^{-/-}$ mice. Apoptosis was assessed by TUNEL staining in knees of *Prlr*$^{-/-}$ and *Prlr*$^{+/+}$ mice intra-articularly injected or not with Cyt. (**A** and **B**) Both injected and (**C** and **D**) noninjected knee joints, i.e., ipsilateral and contralateral to the injection site, respectively, were analyzed. White arrowheads indicate cartilage outer border. Scale bar: 250 μm. Bars represent mean ± SEM. (n = 3–5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Figure 7, B and C) and the lower mRNA expression of proinflammatory mediators (*Ifng*, *Il6*, *iNos*, *Il1b*, and *Tnfa*) in the ankle joint at day 21 after CFA (Figure 7E). Also, Hal treatment 3 days before CFA (Figure 7A) counteracted inflammation even more effectively than PRL infusion. Hal suppressed ankle swelling (Figure 7F) and reduced ankle pain (Figure 7G) and proinflammatory mediator expression (Figure 7H). Consistent with these findings, the histopathological examination of knee sections stained by hematoxylin and eosin showed that PRL infusion, and to a lesser extent Hal treatment, reduced the progression of inflammatory arthritis, as revealed by the absence of pannus formation, and the thinning and destruction of bone trabeculae that occur in normoprolactinemic, adjuvant-injected rats (Figure 8).

Notably, even when started after inflammatory onset (day 15 after CFA) (Figure 9A), PRL treatment mitigated ankle swelling, pain, and expression of proinflammatory mediators (Figure 9, B–D). These findings support the antiinflammatory action of PRL and its therapeutic value for the prevention and reduction of joint destruction in inflammatory arthritis.

Discussion

Chondrocytes are responsible for the production and maintenance of the articular cartilage extracellular matrix, which largely determines the biomechanical properties of joints (30). Adult articular cartilage is postmitotic and cannot compensate for loss of chondrocytes occurring in aging (31) and in arthropathies such as osteoarthritis (3) and RA (7). In these diseases, abnormal exposure to cytokines produced by resident cells and infiltrating inflammatory cells leads to chondrocyte apoptosis and matrix degradation (1, 2, 6–9). Natural chondrocyte survival factors have the potential to be developed for therapeutic application. This study demonstrates for the first time that PRL inhibits cytokine- and arthritis-driven chondrocyte apoptosis. This effect involves the reduced expression of proinflammatory cytokines in joint tissue and the blockage of their proapoptotic effect at the chondrocyte level. Moreover, raising circulating PRL levels reduces joint swelling, pain, pannus formation, and bone erosion in the arthritic joint.

Consistent with previous studies (2, 32, 33), here we show that a combination of TNF-α, IL-1β, and IFN-γ stimulated the in vitro apoptosis of chondrocytes, as evaluated by enhanced mRNA expression of *p53*, increased BAX/BCL-2 ratio, activated caspase-3, and increased DNA fragmentation. Cyt concentrations were similar to those (1–10 ng/ml) found in synovial fluid of patients with RA with severe disease activity (34, 35) or produced by activated chondrocytes (36). PRL opposed the Cyt proapoptotic effect in a dose-dependent manner at concentrations (0.2–2.3 μg/ml) higher than those reported in RA synovial fluid (0.007–0.02 μg/ml) (14, 15) but similar to those (0.2–0.3 μg/ml) circulating in pregnancy and lactation (37). Also, PRL may be higher in cartilage than in synovial fluid due to its local synthesis in chondrocytes (12). Previous findings showed that PRL attenuates the stimulatory effect of Cyt on the expression of iNOS and the production of NO in cultured fibroblasts (38) and NO is a major mediator of Cyt-induced chondrocyte apoptosis (ref. 3 and present data). In chondrocytes, however, PRL did not inhibit Cyt-induced iNOS protein expression/NO production, indicating that its survival effect is independent of NO. On the other hand, PRL activated JAK2 and STAT3, which signal to inhibit chondrocyte apoptosis. STAT3 activation promotes the survival of growth plate chondrocytes by inducing the transcription of BCL-2 (21), and we found that the pharmacological inhibition of STAT3 leads to the apoptosis of articular chondrocytes. It is possible that STAT3-independent, JAK2-dependent mechanisms also contribute to the antiapoptotic effect of PRL. Activation of JAK2 by PRL stimulates PI3K/Akt to promote the survival of various cells (39–42), and activation of Akt inhibits the apoptosis of chondrocytes induced by endoplasmic reticulum stress or the osteoarthritic condition (43, 44).

The fact that PRL activates molecular mechanisms in chondrocytes to counteract the proapoptotic effect of Cyt argues in favor of its prosurvival effect on cartilage under inflammatory

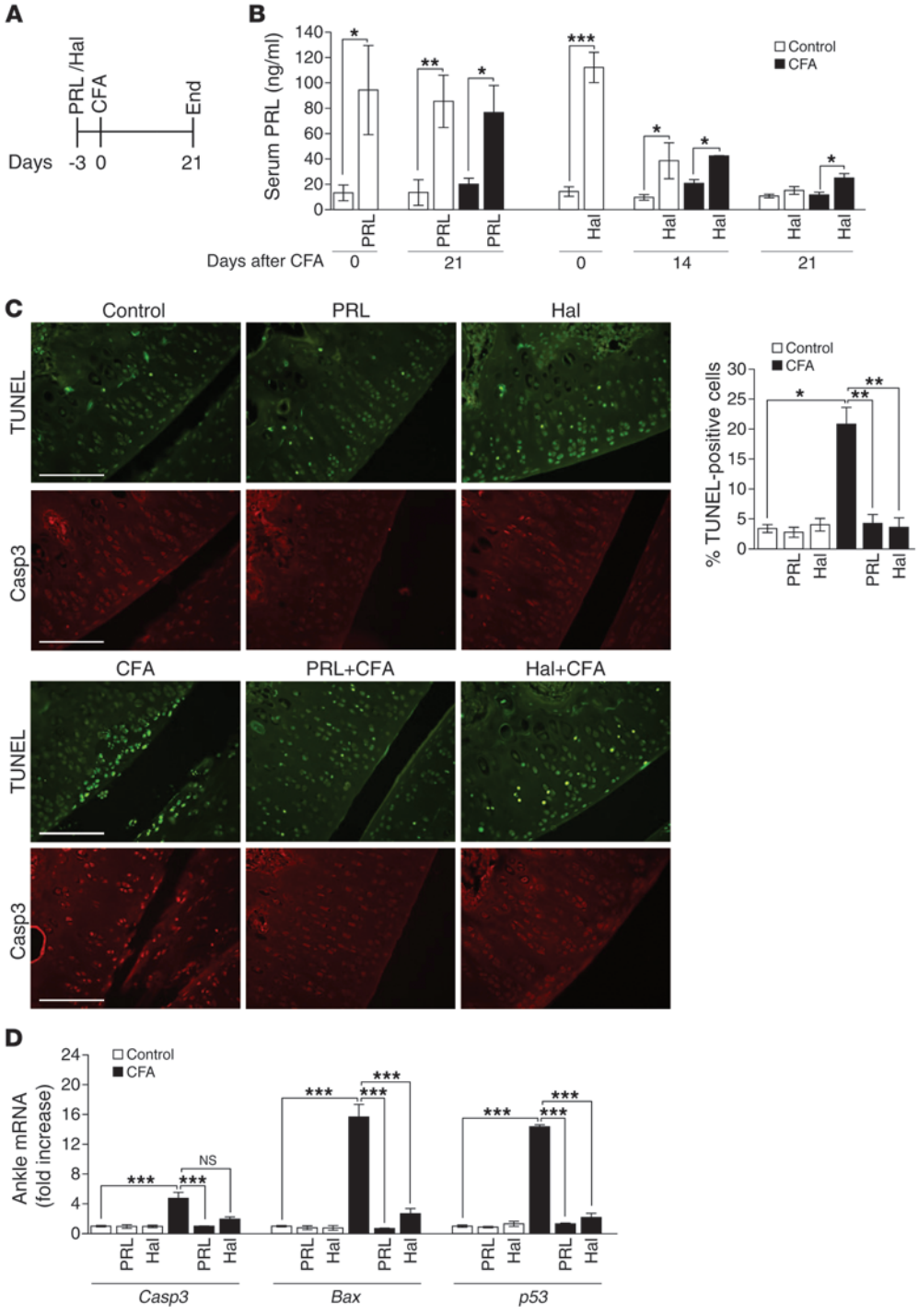

Figure 5

PRL and Hal prevent chondrocyte apoptosis in adjuvant-induced arthritis. (**A**) Experimental design diagram: osmotic minipumps delivering PRL or subcutaneous tablets releasing Hal were placed 3 days before injecting the rats with CFA, and the experiment ended on day 21 after CFA. (**B**) Serum PRL levels on days 0 and 21 after CFA in PRL-treated rats (n = 3–8) and on days 0, 14, and 21 after CFA in Hal-treated rats (n = 4–8). (**C**) TUNEL and active caspase-3 staining of articular cartilage of knees from rats treated or not with PRL or Hal under control and CFA-injected conditions on day 21 after CFA. Scale bar: 100 μm. The graph shows the quantification of TUNEL-positive cells in articular cartilage (n = 4–8). (**D**) qRT-PCR–based quantification of *Casp3*, *Bax*, and *p53* mRNA levels in ankle joints from PRL- and Hal-treated rats on day 21 after CFA (n = 5–14). Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

conditions. To investigate this concept, we extended the in vitro observations to the articular cartilage in situ. To our knowledge, this is the first report showing that the intra-articular delivery of Cyt induces the apoptosis of chondrocytes. Cyt were used at pharmacological concentrations, since, in contrast to cell-culture conditions, their intra-articular delivery results in a much shorter contact with chondrocytes (45). Apoptosis occurred at the outer surface of articular cartilage, which is the most exposed and susceptible area of the tissue. Superficial articular chondrocytes display higher numbers of IL-1 binding sites than cells in deep cartilage (46), and enhanced iNOS expression (47) and large numbers of apoptotic chondrocytes have been reported in the superficial and middle zones of osteoarthritic (48) and RA (8) cartilage. Cotreatment with a pharmacological concentration of PRL (60 μg/ml) or increasing serum PRL to levels similar to those (0.03 μg/ml) found in the circulation of patients with RA (49) blunted Cyt-induced chondrocyte apoptosis. These findings demonstrate the survival effect of PRL on articular cartilage in vivo and suggest that systemic PRL can enter the joint to protect against chondrocyte apoptosis in RA.

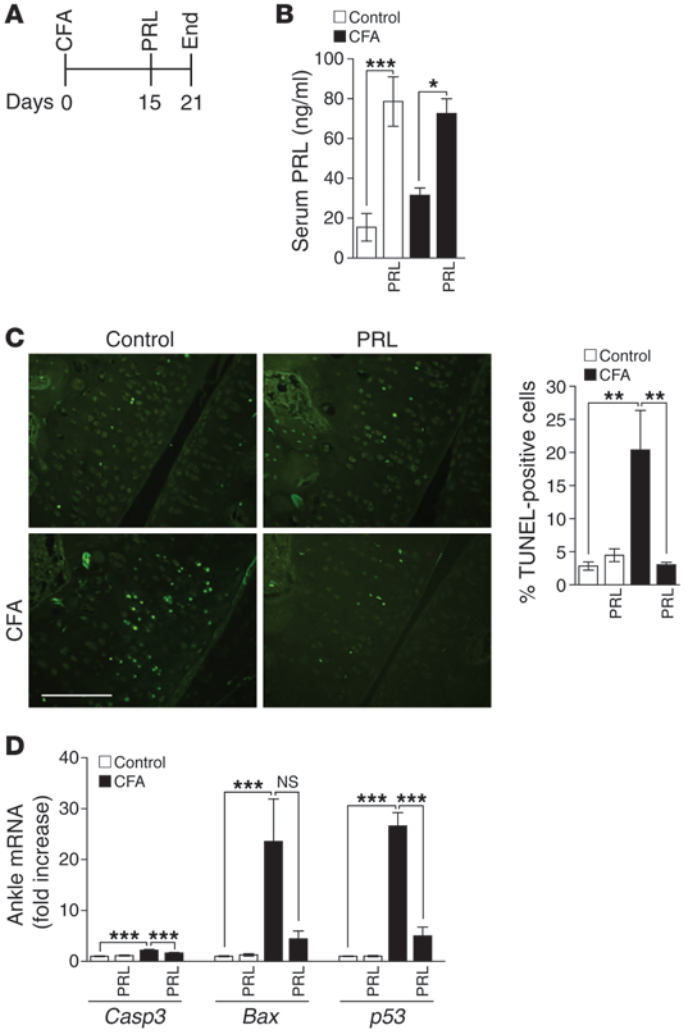

Figure 6

PRL reduces chondrocyte apoptosis in already arthritic rats. (**A**) Experimental design diagram: osmotic minipumps delivering PRL were placed 15 days after the injection of CFA in rats, and the experiment ended on day 21 after CFA. (**B**) Serum PRL levels on day 21 after CFA in PRL-treated and nontreated rats (n = 4–8). (**C**) TUNEL and active caspase-3 staining of articular cartilage of knees from rats treated or not with PRL under control and CFA-injected conditions on day 21 after CFA. Scale bar: 100 μm. The graph shows the quantification of TUNEL-positive cells in articular cartilage (n = 5–8). (**D**) qRT-PCR–based quantification of *Casp3*, *Bax*, and *p53* mRNA levels in ankle joints from PRL-treated and nontreated rats on day 21 after CFA (n = 3–8). Bars represent mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

PRL is not essential for cartilage survival under normal conditions. Targeted disruption of the PRL receptor gene has no phenotype in endochondral bone formation (50), a process involving the apoptosis of growth plate chondrocytes, and it causes no apparent alteration indicative of a defect in articular cartilage survival (present study). However, Cyt-induced chondrocyte apoptosis was enhanced in the absence of the PRL receptor, indicating that the survival effect of PRL becomes apparent in the context of inflammation. The fact that in *Prlr*$^{-/-}$ mice enhanced apoptosis was also seen in the knee contralateral to the one injected with the Cyt, suggests that the antiapoptotic effect of PRL depends on Cyt levels and thus, that higher values of PRL are needed to promote cartilage survival under highly inflammatory conditions.

Here, we show that increasing systemic PRL levels prevents and reduces chondrocyte apoptosis in CFA-induced arthritis. This model is well documented for the induction of inflammation within joint tissues and for having cartilage and bone destruction similar to that in RA (26, 51). Consistent with a previous study (52), we found that CFA-induced arthritis enhances the expression of apoptotic mediators in joints and showed for the first time that apoptosis occurs in large numbers of articular chondrocytes. Thus, in CFA-induced arthritis as in other models of inflammatory arthritis (53) and in RA (8), chondrocyte apoptosis is associated with joint destruction. Increasing prolactinemia, either by PRL infusion or Hal treatment, before or after inducing arthritis, reduced chondrocyte apoptosis and Cyt expression in joints. Also, PRL and Hal ameliorated the severity of arthritis, as evaluated by joint swelling, pain, pannus formation, and bone erosion. The effect of Hal on joint swelling and pain was stronger than that of PRL but weaker on pannus formation and bone erosion. These differences may reflect the fact that, in addition to blocking D2 receptors on the AP, which causes the release of PRL, Hal also blocks dopamine D2 receptors on immune cells, thereby modifying both cytokine release and action (29, 54). Indeed, Cyt are key mediators of CFA-induced arthritis. Their concentration and expression are significantly elevated in serum (26) and joint tissues (present results) of CFA-injected rats, respectively, and IL-1 antagonists and TNF-α–neutralizing antibodies reduce disease severity in these animals (51, 52). We propose that PRL protects against CFA-induced inflammatory arthritis by reducing Cyt levels and counteracting their proapoptotic and proinflammatory actions on synovial cells, cartilage, and bone. However, contrary to these findings, PRL enhances proliferation of cultured RA synovial cells and their release of proinflammatory cytokines and MMP (16). While the in vitro condition may contribute to this discrepancy, in vivo evidence supporting our proposal shows that AP-induced hyperprolactinemia ameliorates CFA-induced inflammation by increasing the circulating levels of corticosterone (23, 27). Because glucocorticoids and inhibitors of TNF-α and IL-1β are current treatments for RA (55), sustained PRL administration offers promise for mitigating susceptibility to the onset or flare-up of RA and disease severity, and current medications known to increase prolactinemia constitute therapeutic options in RA, as indicated by clinical studies using Hal (28, 29).

The idea of inducing high prolactinemia to help control the progression of joint damage in RA is novel and unexpected. A large body of literature has focused on PRL having a pathogenic role in RA and also in other autoimmune diseases, like SLE, Sjögren's syndrome, Hashimoto's thyroiditis, celiac disease, MS, etc. Its pathogenic role is largely based on the preponderance of autoimmune diseases in women (56) and on PRL being a sex-linked hormone, on the higher levels of circulating PRL detected in some patients (6%–45%, depending on the disease and specific study), on the therapeutic effects of the dopamine agonist bromocriptine, and on the immunoenhancing properties of PRL (57–61). However, in RA, as in the other autoimmune diseases, treatment with bromocriptine is not always effective and the association between PRL levels

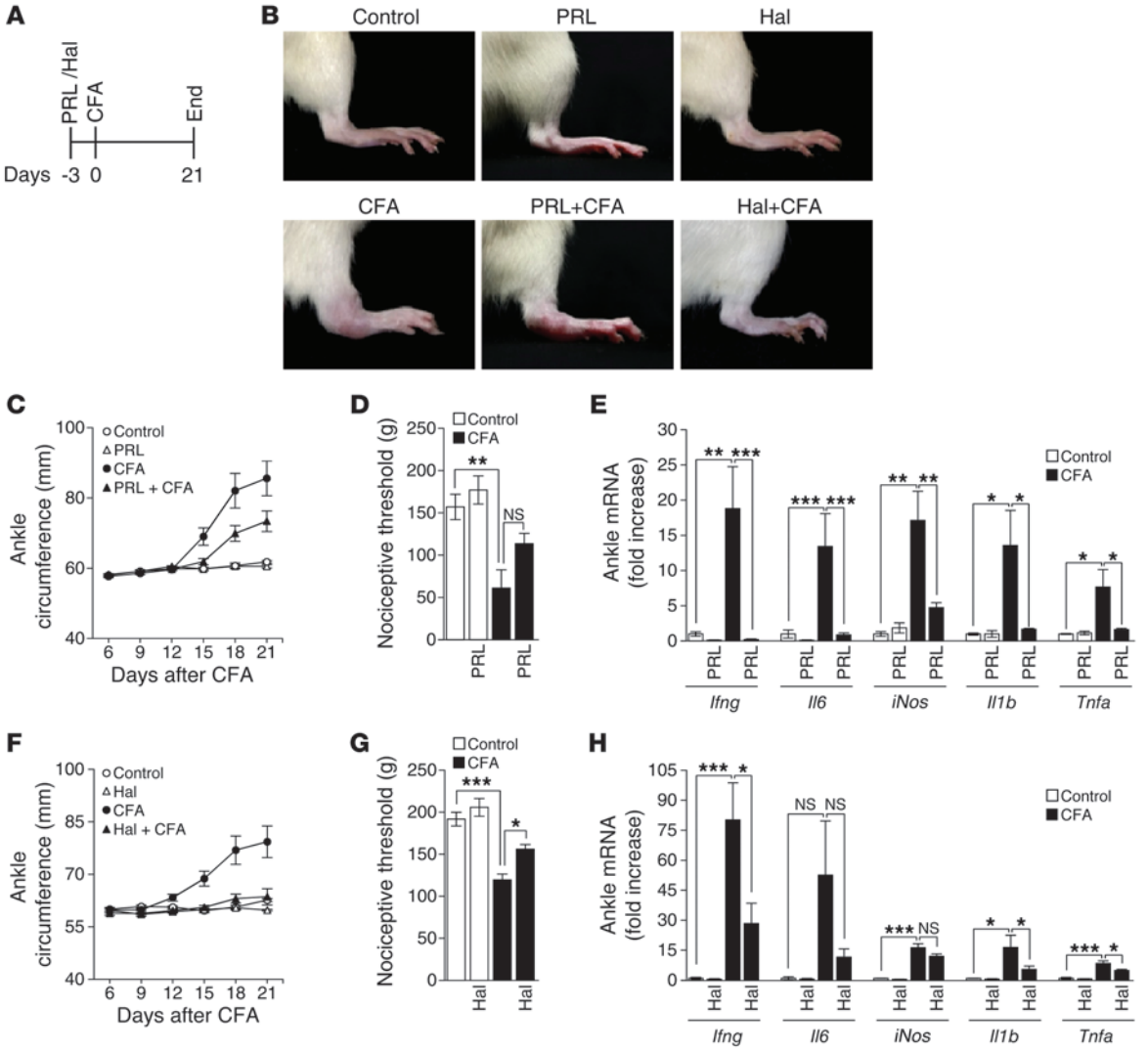

Figure 7

PRL and Hal prevent joint inflammation in adjuvant-induced arthritis. (**A**) Experimental design diagram: osmotic minipumps delivering PRL or subcutaneous tablets releasing Hal were implanted 3 days before the injection of CFA in rats. (**B**) Representative photographs of hind paws from groups injected or not with CFA. (**C** and **F**) Time course of ankle circumference in groups infused with PRL (n = 10) or treated with Hal (n = 16) under control and CFA-injected conditions. (**C**) Days 15, 18, and 21, $P < 0.001$, CFA vs. control. Days 18 and 21, $P < 0.001$, PRL vs. PRL plus CFA. (**F**) Days 15 and 18, $P < 0.001$, CFA vs. control. Days 12, 15, 18, and 21, $P < 0.001$, CFA vs. Hal plus CFA. (**D** and **G**) Nociceptive threshold in groups infused with PRL (n = 5–9) or treated with Hal (n = 5–9). (**E** and **H**) qRT-PCR–based quantification of *Infg*, *Il6*, *iNos*, *Il1b*, and *Tnfa* mRNA levels in ankle joints from rats treated with PRL (n = 3–10) or with Hal (n = 3–10) under control and CFA-injected conditions on day 21 after CFA. Bars are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and disease activity has been inconsistent (58–62). Generalizations are confounded by the contribution of PRL synthesized locally by cells like chondrocytes (12), synoviocytes and immune cells (16), and endothelial cells (63), which can potentiate the action of systemic PRL. Moreover, PRL has the ability to exert immunostimulatory or immunosuppressive effects, depending on its level and the pathophysiological conditions (17). For example, physiological concentrations of PRL (<0.02 μg/ml) are more effective than high PRL levels (0.1 μg/ml) in stimulating antibody production by SLE lymphocytes (64), low PRL levels stimulate and high levels inhibit NK cell proliferation (65), and hyperprolactinemic patients (mean serum PRL of 0.98 μg/ml or 0.20 μg/ml) show reduced NK cell numbers (66) and function (67). Also, hyperprolactinemia protects against inflammatory arthritis in rats (present study), and treatment with a high, but not a low, dose of PRL exacerbates experimental MS (61). In the latter, however, the low dose of PRL is beneficial when combined with IFN-β, and *Prlr*$^{-/-}$ mice display a significantly worse outcome than wild-type mice (61). The variability of the relationship between PRL and autoimmune diseases is further highlighted under physiological hyperprolactinemia. During pregnancy, when PRL levels are high, SLE flare-ups occur, but RA and MS go into remission (59, 68). Breastfeeding, a stimulus elevating circulating PRL, exacerbates SLE in humans (69), but it is protective in the B/W mouse model of SLE (70). Breastfeeding also

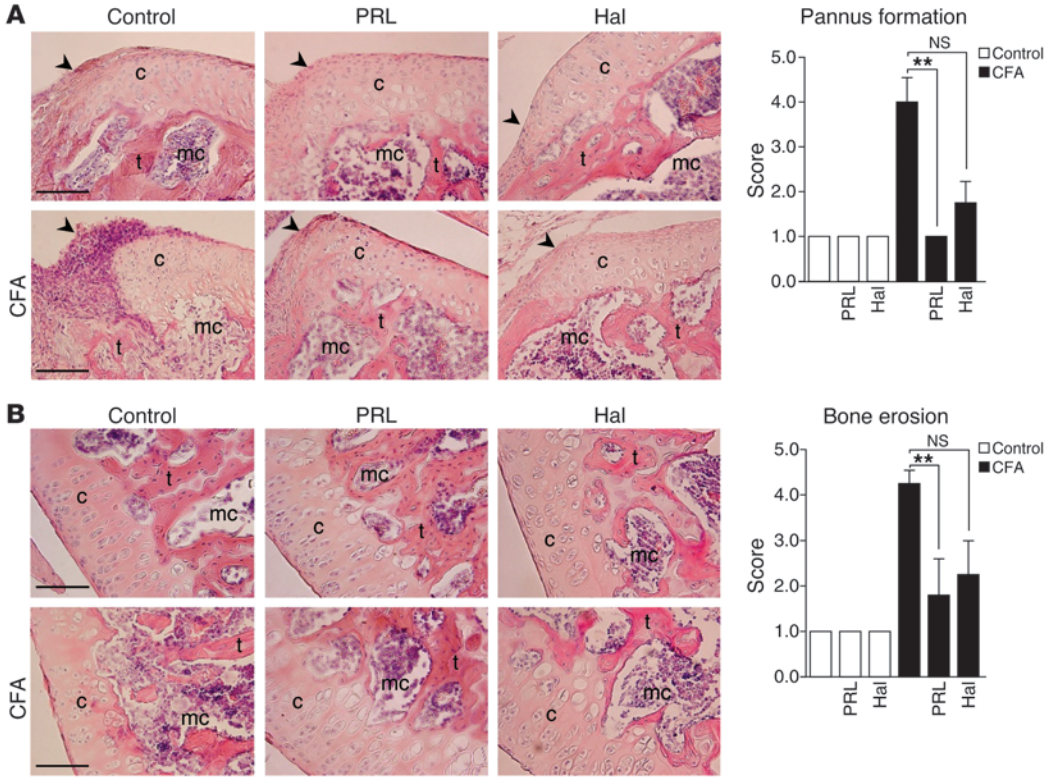

Figure 8

PRL reduces pannus formation and bone erosion in adjuvant-induced arthritis. Histological evaluation of (**A**) pannus formation and (**B**) bone erosion in sections of knee joints stained by hematoxylin and eosin from nonimplanted rats (control) or rats implanted with osmotic minipumps delivering PRL or with subcutaneous tablets releasing Hal 3 days before injecting or not injecting CFA; the histological evaluation was carried out on day 21 after CFA (n = 3–8). Pannus-associated regions in each group are indicated (arrows). c, cartilage; t, bone trabeculae; mc, bone marrow cavity. Scale bar: 100 μm. Graphs show histological scores for (**A**) pannus formation (synovial membrane hyperplasia and infiltration of leukocytes) and (**B**) bone erosion (thinning and destruction of bone trabeculae). Values are mean ± SEM. $^{**}P < 0.01$.

worsens RA (71) but protects against postpartum MS relapse (72). These contrasting observations indicate that PRL exerts opposing influences on immune function that depend on complex immune and hormonal interactions.

Although the role of endogenous PRL in autoimmune diseases has generated controversies (57–62, 73), our study reveals that elevating serum PRL levels significantly attenuates cartilage death and joint inflammation in inflammatory arthritis. This strategy may be comparable to the well-established use of glucocorticoids in patients with RA, in which levels of the endogenous hormones appear insufficient to control the disease (74). While PRL is not essential for normal immune system development and function (75, 76), it is a major stress-related hormone (77), balancing immune system homeostasis in the context of stress, trauma, and inflammation (17, 78). Studies clarifying how circulating and local PRL levels are being regulated in the proinflammatory milieu of pathological situations will help establish appropriate PRL levels for controlling ongoing inflammation and the better use of PRL for therapeutic purposes in RA and other inflammatory-related disorders.

Methods

Reagents. Recombinant human TNF-α, IL-1β, and IFN-γ were purchased from R&D Systems. Rat PRL and rat PRL radioimmunoassay reagents were obtained from A.F. Parlow (National Hormone and Pituitary Program, Los Angeles, California, USA). Ovine PRL and L-NAME were purchased from Sigma-Aldrich, and the STAT-3 inhibitor S31-201 and anti-BAX (Sc-493) and anti–BCL-2 (Sc-492) antibodies from Santa Cruz Biotechnology Inc. Antibodies anti–caspase-3 (9662) and anti-pJAK2 (Tyr1007/1008, 3771) were from Cell Signaling Technology Inc., anti-iNOS (06-573) was from Upstate, and CFA was from Difco.

Animals. Male Lewis and Sprague-Dawley rats (200–250 g), Wistar rats (130–150 g), and *Prlr*$^{-/-}$ mice (6–8 months, 129Sv/J background) were housed under standard laboratory conditions (22°C; 12-hour/12-hour light/dark cycle, free access to food and water). Animals were anesthetized with 70% ketamine and 30% xylazine (1 μl/g body weight, i.p.) for surgeries and intra-articular injections, and all procedures were performed between 9:00 and 12:00 AM. To avoid stress-induced alterations, animals were handled daily for 7 days before euthanization by carbon dioxide inhalation and decapitation.

Chondrocyte culture. Articular chondrocytes were isolated from femoral epiphyseal cartilage of male Wistar rats as described previously (12). Cells were seeded at 2 × 10^5 cells per cm^2 and incubated in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C for 24 hours. Nonadherent cells were removed, and adherent cells were incubated for 24 hours in either fresh medium or medium containing Cyt (25 ng/ml TNF-α, 10 ng/ml IL-1β, and 10 ng/ml IFN-γ) combined or not with different concentrations of rat PRL or the NOS inhibitor L-NAME (1 mM). Other cell cultures were incubated with 100 nM of the STAT3 inhibitor, S31-201. All

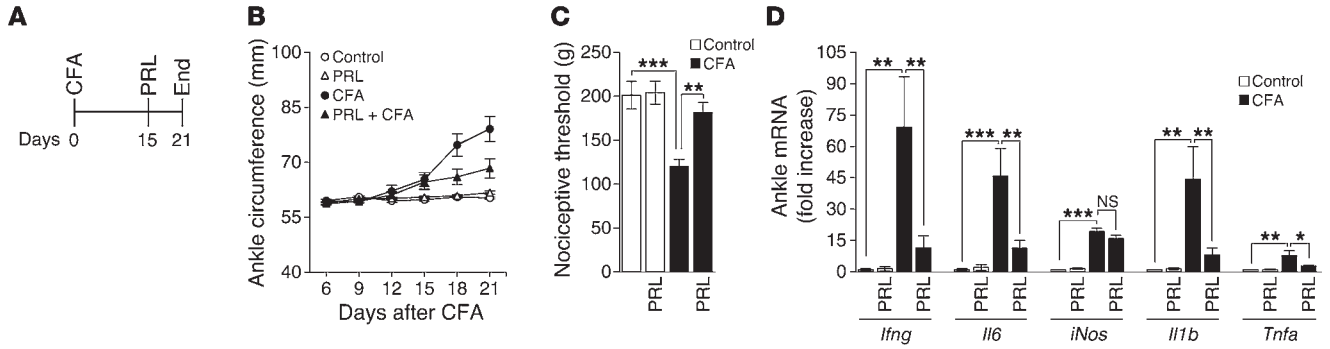

Figure 9

PRL reduces joint inflammation in already arthritic rats. (**A**) Experimental design diagram: osmotic minipumps delivering PRL were placed 15 days after the injection of CFA in rats, and the experiment ended on day 21 after CFA. (**B**) Time course of ankle circumference (n = 10–15) (days 18 and 21, $P < 0.001$, CFA vs. control), (**C**) evaluation of ankle joint nociceptive threshold (n = 5–8), and (**D**) qRT-PCR–based quantification of *Ifng*, *Il6*, *iNos*, *Il1b*, and *Tnfa* mRNA levels (n = 5–8) in ankle joints under control and CFA-injected conditions on day 21 after CFA. Bars are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

experiments were performed in the first passage of culture. Both detached and adherent chondrocytes were assayed for apoptosis by measuring fragmented nucleosomal DNA using the Cell Death Detection ELISA Kit (Roche Diagnostics). To investigate PRL-induced nuclear translocation of STAT3, chondrocytes were seeded on glass coverslips and treated with or without Cyt in the presence or absence of PRL for 1 hour. Cells were then fixed in 4% PFA at room temperature (RT) for 1 hour, washed, incubated 1 hour at RT with 5% normal goat serum in 0.05% Triton-PBS, and then incubated overnight at 4°C with a 1:100 dilution of anti-total STAT3. Cells were then washed and incubated at RT with the second antibody, Alexa Fluor 546 (1:500; Invitrogen), for 2 hours, and their nuclei were counterstained with DAPI (1 μg/ml; Sigma-Aldrich).

Western blot. Chondrocytes incubated with or without Cyt in the absence or presence of PRL were analyzed for JAK2 phosphorylation after 30 minutes, for BAX and BCL-2 after 4 hours, and for total caspase-3 (procaspase-3 and active caspase-3) and iNOS after 6 hours of treatment. Cells were resuspended in lysis buffer (0.5% Igepal, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 μg/ml aprotinin, and 100 μg/ml PMSF, pH 7.0) and subjected to SDS/PAGE, and total protein (40 μg) was blotted and probed overnight with a 1:1,000 dilution of anti–caspase-3, anti-iNOS, or anti-pJAK2; a 1:300 dilution of anti-BAX; or a 1:200 dilution of anti–BCL-2. Secondary antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc.) were used. Densitometric analysis was performed using the Quantity One 1-D image analysis software (Bio-Rad Laboratories Inc.).

The Griess colorimetric assay was used to measure the NO_2^- and NO_3^- concentrations in conditioned media of chondrocytes treated or not with Cyt with or without PRL for 24 hours.

Intra-articular injection of Cyt. Lewis rats and *Prlr*$^{-/-}$ mice were injected in the articular space of right knee joints with Cyt in a final volume of 60 μl (375 ng TNF-α, 150 ng IL-1β, and 150 ng IFN-γ) and 10 μl (62.5 ng TNF-α, 25 ng IL-1β, and 25 ng IFN-γ), respectively. A group of rats was coinjected with the Cyt and 8 μg rat PRL. As the estimated volume of rat synovial fluid is 130 μl, the intra-articular concentration of PRL was 60 μg/ml. Thirteen days before Cyt injection, other groups of rats were implanted with 2 AP grafts under the kidney capsule in order to induce hyperprolactinemia (23), and half of them were injected i.p. with 1.25 mg/d of CB154 (Parlodel, Novartis), starting 5 days before Cyt injection. Endotoxin-free water (60 μl and 10 μl for rats and mice, respectively) was used as a control vehicle. Forty-eight hours after vehicle or Cyt injection, animals were euthanized to evaluate apoptosis in knee cartilage and assess serum PRL levels by radioimmunoassay.

Adjuvant-induced arthritis. Sprague-Dawley rats were immunized intradermally at the base of the tail with 0.2 ml CFA (10 mg heat-killed *Mycobacterium tuberculosis* H37Ra 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 Alzet osmotic minipump (Alza) containing 1.6 mg ovine PRL or a tablet releasing 35 mg Hal over the 60 days of treatment (Innovative Research of America). Other rats were implanted 15 days after CFA injection with 14-day Alzet osmotic minipumps containing 0.8 mg ovine PRL. Arthritis development was evaluated every 3 days by the hind ankle circumference determined by measuring 2 perpendicular diameters, the laterolateral diameter (a) and the anteroposterior diameter (b), with a digital caliper and using the following formula: circumference = $2\pi(\sqrt{(a^2+b^2)}/2)$. On day 21 after CFA, hind paw pain was assessed using an Analgesy-Meter (Ugo Basile S.R.L.), and knee joints and hind paws were dissected to evaluate in situ apoptosis and expression of proapoptotic and proinflammatory markers, respectively. In addition, adjuvant-induced arthritis was evaluated histologically. Knee joints were fixed, decalcified, and dehydrated for paraffin embedding. Knee sections (7 μm) stained by hematoxylin and eosin were scored as reported previously (79), with the following modifications: 1 (no detectable change), 2 (slight change), 3 (moderate change), 4 (remarkable change), and 5 (severe change) for pannus formation (synovial membrane hyperplasia and infiltration of leukocytes) and bone erosion (thinning and destruction of bone trabeculae). Histological parameters were scored by 4 single-blind, independent observers (N. Adán, M.G. Ledesma-Colunga, S. Thebault, and C. Clapp).

In situ apoptosis. Seven-μm knee sections were deparaffinized, rehydrated, and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 7 minutes followed by incubation in sodium citrate buffer (10 mM sodium citrate, 0.5% Tween 20, pH 6.0) for 1 minute at 86°C. Apoptosis was detected by the TUNEL method using the In Situ Cell Death Detection Kit (Roche Diagnostics) and by immunohistochemistry using a polyclonal antibody against active caspase-3 (1:25; Millipore) and Alexa Fluor 546 secondary antibody (1:500) as well as by staining the condensed/fragmented DNA with DAPI (1 μg/ml, Sigma-Aldrich). Apoptotic signals were visualized under fluorescence microscopy (Microscope BX60F5, Olympus Optical Co. LTD) and quantified by the image analysis system software Pro-Plus (Media Cybernetics Inc.).

qRT-PCR. Frozen whole ankle joints were pulverized in liquid nitrogen using a mortar and pestle. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems). PCR products were detected and quantified with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) in a 10 μl final reaction volume containing template and 0.5 μM of each of the primer pairs for *Casp3*, forward 5′-GAAAGCCGAAACTCTTCATCA-3′, reverse 5′-ATAGTAACCGGGTGCGGTAT-3′; *Bax*, forward 5′-ACTGGACAACAACATGGTGC-3′, reverse 5′-ATCAGCTCGGGCACTTTAGT-3′; *p53*, forward 5′-AGAGACCCAGCAACTACCAACA-3′, reverse 5′-CTCAGACTGACAGCCTCTGCAT-3′; *Ifng*, forward 5′-AGCACAAAGCTGTCAATGAA-3′, reverse 5′-TTCTTCTTATTGGGCACACTC-3′; *Il6*, forward 5′-TCCAACTCATCTTGAAAGCA-3′, reverse 5′-TTCATATTGCCAGTTCTTCG-3′; *Tnfa*, forward 5′-GGGCTTGTCACTCGAGTTTT-3′, reverse 5′-TGCCTCAGCCTCTTCTCATT-3′; *iNos*, forward 5′-TTTTAGAGACGCTTCTGAGG-3′, reverse 5′-GTCCTTTTCCTCTTTCAGGT-3′; *Il1b*, forward 5′-AAAAGCGGTTTGTCTTCAAC-3′, reverse 5′-GGAATAGTGCAGCCATCTTT-3′; and *Ubc*, forward 5′-CTGACAGGGTGCGGCCATCTT-3′, reverse 5′-ACTGCAGCCAACACCGCTGAC-3′. Amplification performed in the CFX96 real-time PCR detection system (Bio-Rad) included a denaturation step of 10 minutes at 95°C, followed by 40 cycles of amplification (10 seconds at 95°C, 30 seconds at the primer pair–specific annealing temperature, and 30 seconds at 72°C). The PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method, and cycle thresholds normalized to the housekeeping gene *Ubc* were used to calculate the mRNA levels of interest.

Serum PRL. Rat PRL was measured in serum by conventional radioimmunoassay, and infused ovine PRL was measured by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to PRL, carried out as described previously (80).

Statistics. All data were replicated in 3 or more independent experiments. The statistical analyses were performed using the Sigma Stat 7.0 (Sigma Stat 7.0, Systat Software Inc.) and the GraphPad Prism (GraphPad Software Inc.) software. Data distribution and equality of variances were determined by D'Agostino-Pearson omnibus and Levene's tests. In case of data with normal distribution and/or equal variances, statistical differences between 2 and more than 3 groups were determined by 2-tailed Student's *t* test and 1-way ANOVA followed by Bonferroni's post-hoc comparison test, respectively. In case of data with nonparametric distribution, statistical differences between 2 and more than 3 groups were determined by Mann Whitney's and Kruskal-Wallis followed by Dunn's post-hoc comparison tests, respectively. The threshold for significance was set at $P < 0.05$.

Study approval. All experiments were approved by the Bioethics Committee of the Institute of Neurobiology of the National University of Mexico and comply with the US National Research Council's Guide for the Care and Use of Laboratory Animals (Eighth Edition, National Academy Press, Washington, DC, USA).

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