ARTHRITIS & RHEUMATISM
Vol. 62, No. 3, March 2010, pp 722–731
DOI 10.1002/art.27269
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Blocking ERK-1/2 Reduces Tumor Necrosis Factor α–Induced Interleukin-18 Bioactivity in Rheumatoid Arthritis Synovial Fibroblasts by Induction of Interleukin-18 Binding Protein a

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Objective. **To examine the mechanism of regulation of interleukin-18 (IL-18) bioactivity by IL-18 binding protein (IL-18BP) induction.**

Methods. **Levels of IL-18 and IL-18BPa in synovial fluid samples from patients with osteoarthritis (OA) or rheumatoid arthritis (RA) were determined by enzyme-linked immunosorbent assays (ELISAs), followed by calculation of free IL-18. IL-18 and IL-18BPa synthesis in RA synovial fibroblasts that had been treated with proinflammatory and antiinflammatory cytokines were assessed by quantitative real-time polymerase chain reaction and ELISA, respectively, followed by IL-18 bioactivity determination using KG-1 cells. Chemical signaling inhibitors were used for determination of the signal transduction pathways involved in IL-18BPa/IL-18 regulation. Tumor necrosis factor α (TNFα)–induced caspase 1 activity was determined by a colorimetric assay.**

Results. **IL-18BPa was lower in RA synovial fluid than in OA synovial fluid ($P < 0.05$; n = 8), and free IL-18 was higher in RA synovial fluid than in OA synovial fluid. TNFα induced RA synovial fibroblast IL-18BPa and IL-18 in a time-dependent manner ($P < 0.05$). Evaluation of signaling pathways suggested that TNFα induced IL-18 production through the ERK-1/2, protein kinase Cδ (PKCδ), and Src pathways, whereas IL-18BPa synthesis was mediated through the NFκB, PKC, Src, and JNK pathways. Furthermore, addition of exogenous IL-18BPa-Fc reduced the RA synovial fibroblast phosphorylation of ERK-1/2 induced by TNFα.**

Conclusion. **These results suggest that IL-18BPa reduces IL-18 bioactivity induced by TNFα, by regulating the ERK-1/2 pathway in RA synovial fibroblasts. Targeting IL-18 bioactivity by induction or addition of IL-18BPa may provide another therapeutic option in the management of RA.**

Rheumatoid arthritis (RA) and osteoarthritis (OA) are two common chronic joint disorders whose etiology remains unknown. The RA synovium is characterized by angiogenesis, or new blood vessel growth, and leukocyte infiltration that lead to tissue invasion and joint destruction (1). OA is considered mainly a noninflammatory disease, in which mild to moderate inflammatory changes at certain stages of the disease correlate with disease progression (2). However, proinflammatory cytokines play an important role in the pathophysiology of both diseases (3).

Interleukin-1 (IL-1) family members play a key part in the pathogenesis of both RA and OA (4). Among this family, IL-18 plays an important role in inducing the Th1 immune response through the induction of interferon-γ (IFNγ) in T cells and natural killer cells (5) and has both a local and a systemic effect on angiogenesis (6,7). IL-18 plays an important role in the pathophysiology of RA and OA (8,9). Various sources of IL-18 have been identified, including Kupffer cells, dendritic cells, keratinocytes, articular chondrocytes, osteoblasts, and synovial fibroblasts (8,10–12).

Dr. Marotte's work was supported by the French Society of Rheumatology, the Lavoisier Foundation, and the Philippe Foundation. Dr. Ahmed's work was supported by the NIH (grants AT-003633 and AR-055741). Dr. Ruth's work was supported by the NIH (grants AR-049907 and AR-048310). Dr. Koch's work was supported by the NIH (grants AI-40987 and AR-48267), the Frederick G. L. Huetwell and William D. Robinson, MD, Professorship in Rheumatology, and the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

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Submitted for publication June 10, 2009; accepted in revised form November 3, 2009.

IL-18 is produced as a precursor molecule (pro–IL-18), then is processed by IL-1β–converting enzyme (caspase 1) to obtain the mature form of IL-18, which is biologically active (5). The importance of IL-18 has also been shown in an animal model of arthritis (13). To control some of the potentially deleterious properties of IL-18, IL-18 binding protein (IL-18BP) has been identified as a specific endogenous inhibitor of IL-18 bioactivity (14,15). Four isoforms of IL-18BP are described in humans as isoforms a, b, c, and d, which are produced as a result of an alternative splicing. IL-18BPa is the major splicing variant with the highest binding affinity, 400 p*M*, for IL-18 (14). Furthermore, the potentially beneficial role of IL-18BP therapy has been demonstrated; the administration of IL-18BP led to the resolution of rodent arthritis (15,16).

The mechanism by which IL-18BPa may control IL-18 bioactivity in RA synovial fibroblasts is not yet known. In the present study, we demonstrated that free IL-18 is higher in RA synovial fluid than in OA synovial fluid due to a paucity of IL-18BPa expression in RA synovial fluid. Tumor necrosis factor α (TNFα) was found to be a powerful inducer of IL-18BPa through the Src, protein kinase C (PKC), JNK-2, and NF-κB pathways. TNFα also induced IL-18 and caspase 1 expression and activity in the same manner, but TNFα induced IL-18 through the Src, PKCδ, and ERK-1/2 pathways. Exogenous IL-18BPa-Fc significantly reduced TNFα–induced phosphorylation of ERK-1/2. Our results indicate that in RA, a disease in which TNFα plays a key role, blocking the ERK-1/2 pathway reduces IL-18 bioactivity by the reduction of IL-18 production and the concomitant increase in IL-18BPa production.

MATERIALS AND METHODS

Cytokines and culture of human RA synovial fibroblasts. TNFα, IL-1β, IL-13, IL-17, IL-18, IL-18BPa-Fc, IgG-Fc, and mouse monoclonal anti-human IL-18 were purchased from R&D Systems (Minneapolis, MN). IFNγ, IL-4, and IL-10 were purchased from PeproTech (Rocky Hill, NJ).

Fibroblasts were isolated from synovium obtained from RA patients who had undergone total joint replacement or synovectomy according to an institutional review board–approved protocol and were processed as described previously (17,18). RA synovial fibroblasts were grown in RPMI 1640 with 10% fetal bovine serum supplementation. All of the experiments were performed in serum-free media.

Enzyme-linked immunosorbent assay (ELISA) for IL-18 and IL-18BPa. Synovial fluid samples were obtained from patients with RA or OA with joint effusions and were then stored at −80°C until IL-18BPa determination. To avoid any possible confounding effects of rheumatoid factor on the assays, rheumatoid factor was immunodepleted from synovial fluid using anti-IgM antibodies coupled to agarose beads (Sigma, St. Louis, MO) as previously described (19). RA synovial fibroblasts (2×10^5/well in 6-well plates) were stimulated with cytokines for 2–48 hours in serum-free media. Upon termination, conditioned medium was collected and concentrated 10-fold using Amicon Ultra 10,000 mW concentrators from Millipore (Bedford, MA). IL-18 and IL-18BPa levels in synovial fluid and concentrated conditioned medium were determined using ELISA kits from Bender MedSystems (Burlingame, CA) and R&D Systems, respectively.

Calculation of free IL-18. Free IL-18 was determined according to the law of mass action and by assessments of both total IL-18 and total IL-18BPa by ELISA. The calculation of free IL-18 was based on a 1:1 stoichiometry in the complex of IL-18 and IL-18BPa with a dissociation constant of 0.4 n*M* (14).

Cytokine profiling. To study the effect of several proinflammatory and antiinflammatory cytokines on IL-18BPa production, RA synovial fibroblasts were treated with IL-1β (10 ng/ml), TNFα (20 ng/ml), IL-17 (50 ng/ml), IL-18 (180 ng/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml), or IL-13 (50 ng/ml) for 24 hours. IL-18BPa expression was assessed at the messenger RNA (mRNA) level and at the protein level.

RNA extraction and quantitative real-time polymerase chain reaction (PCR). RNA was isolated using RNeasy Mini kits in conjunction with QIAshredders, according to the recommendations of the manufacturer (Qiagen, Valencia, CA), as described previously (17). Following isolation, RNA was quantified and checked for purity by spectrophotometry (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) was then prepared using a Reverse-IT MAX first-strand synthesis kit (ABgene, Rochester, NY), as described previously (17). Quantitative PCR was performed using Platinum SYBR Green quantitative PCR Supermix-UDG according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA), using the following specific primer sequences: for human IL-18, forward 5′-GCTTGAATCTAAATTATCAGTC-3′ and reverse 5′-GAAGATTCAAATTGCATCTTAT-3′ (20); for IL-18BPa, forward 5′-ACCTCCCAGGCCGACTG-3′ and reverse 5′-CCTTGCACAGCTGCGTACC-3′ (21); for caspase 1, forward 5′-CAAGGGTGCTGAACAAGG-3′ and reverse 5′-GGGCATAGCTGGGTTGTC-3′ (22); and for β-actin, forward 3′-GTCAGGCAGCTCGTAGCTCT-5′ and reverse 5′-GCCATGTACGTTGCTATCCA-3′. Diluted cDNA was mixed with Platinum SYBR Green quantitative PCR Supermix-UDG and forward and reverse primers specific for each gene (0.2 μM final concentrations) and was incubated under the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 30 seconds using an Eppendorf Mastercycler ep realplex thermal cycler (Eppendorf, Hamburg, Germany). All samples were run in duplicate and analyzed using Eppendorf software. For quantification, the relative abundance of each gene was normalized to β-actin.

Treatment of RA synovial fibroblasts with TNFα. To study the signaling mechanism of IL-18BPa production by TNFα, RA synovial fibroblasts were incubated with MAPK inhibitors (ERK-1/2 [PD98059], p38 [SB202190], and JNK-2 [SP600125]), PKC inhibitors (PKCα/β [Gö6976] and PKCδ [Rottlerin]), an Src inhibitor (PP2), an NF-κB inhibitor (pyr-

rolidine dithiocarbamate [PDTC]), or a JAK-2 inhibitor (AG-490) for 1 hour, followed by stimulation with TNFα (20 ng/ml) for 48 hours. Supernatants were processed for estimation of IL-18BPa production. Cells were pretreated for 1 hour with 10 μ*M* each inhibitor (except for PDTC [200 μ*M*]) before stimulation with TNFα (20 ng/ml). The concentration of the inhibitors was based on our previous studies (17,23). All inhibitors were purchased from Calbiochem (San Diego, CA).

Caspase 1 activity assay. RA synovial fibroblasts (2×10^6/well) were treated with TNFα (20 ng/ml), for 8, 24, or 48 hours in serum-free RPMI 1640. Cells were washed and then lysed with the lysis buffer from the caspase 1 activity assay kit. Cell lysates were centrifuged, and the supernatant was used as the cell extract. Caspase 1 activity in cell extract was determined using a colorimetric caspase 1 activity assay kit (R&D Systems).

IL-18 bioactivity. The biologic activity of IL-18 was measured by using human myelomonocytic KG-1 cells, as previously described (24). KG-1 cells (3×10^6 cells/ml; 100 μl), with or without mouse monoclonal anti–IL-18 antibody at 1 μg/ml (R&D Systems), were dispensed into the wells of 96-well microtiter plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). Then, 100 μl of samples or recombinant human IL-18 standards was added to each well. The plates were incubated, and culture supernatants were harvested 24 hours later. The IFNγ concentration in the media was determined by ELISA (Invitrogen). IL-18 bioactivity was determined by the difference in IFNγ levels between the cultures with and those without mouse monoclonal anti–IL-18 antibody.

Cell lysis and Western blotting. To study the effect of IL-18BPa on TNFα–induced ERK-1/2, RA synovial fibroblasts were incubated with or without TNFα (20 ng/ml) after 1 hour of preincubation with IgG-Fc or IL-18BPa-Fc (25 ng/ml and 50 ng/ml) in serum-free RPMI 1640 for 20 or 30 minutes. Cells were lysed in cell lysis buffer containing 1 m*M* phenylmethylsulfonyl fluoride, and protease inhibitors (Thermo Scientific, Rockford, IL) as previously described (18). Protein was measured using a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein (15 μg) were loaded, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA). Nitrocellulose membranes were blocked with 5% nonfat milk in Tris buffered saline–Tween 20 (TBST) for 60 minutes at room temperature. Blots were incubated overnight at 4°C with optimally diluted specific primary antibody in TBST containing 5% nonfat milk. Phosphorylation state–specific antibodies to ERK-1/2, JNK-2, PKCδ, c-Jun, or NF-κB (Cell Signaling Technology, Beverly, MA) were used as the primary antibody. Blots were washed 3 times and incubated in horseradish peroxidase–conjugated antibody (1:1,000 dilutions) for 1 hour at room temperature. Protein bands were detected using enhanced chemiluminescence according to the recommendations of the manufacturer (GE Healthcare, Piscataway, NJ). After stripping, blots were probed again for β-actin using a rabbit polyclonal anti–β-actin antibody. Blots were scanned and analyzed for band intensities using Un-Scan-It software, version 5.1 (Silk Scientific, Orem, UT).

RESULTS

Higher levels of IL-18 and lower levels of IL-18BPa in RA synovial fluid samples than in OA synovial fluid samples. Synovial fluid samples from RA patients contained significantly lower levels of IL-18BPa than did

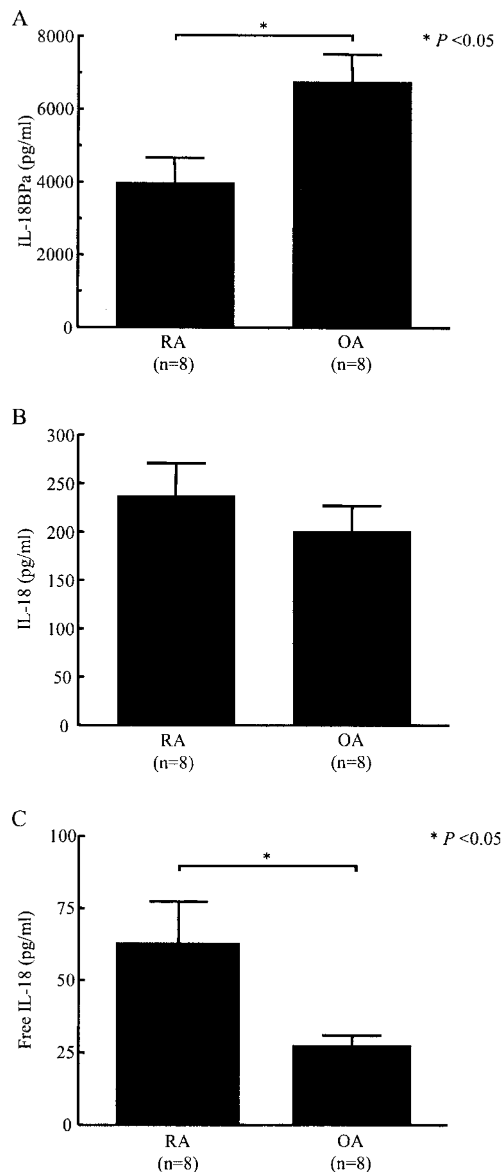

Figure 1. High levels of free interleukin-18 (IL-18) in rheumatoid arthritis (RA) synovial fluid samples due to decreased expression of IL-18 binding protein a (IL-18BPa). **A** and **B**, Levels of IL-18BPa (**A**) and IL-18 (**B**), as determined by enzyme-linked immunosorbent assay of synovial fluid samples from patients with RA and patients with osteoarthritis (OA). **C**, Levels of free IL-18, as determined according to total IL-18 and IL-18BPa levels and the dissociation constant. Bars show the mean and SEM.

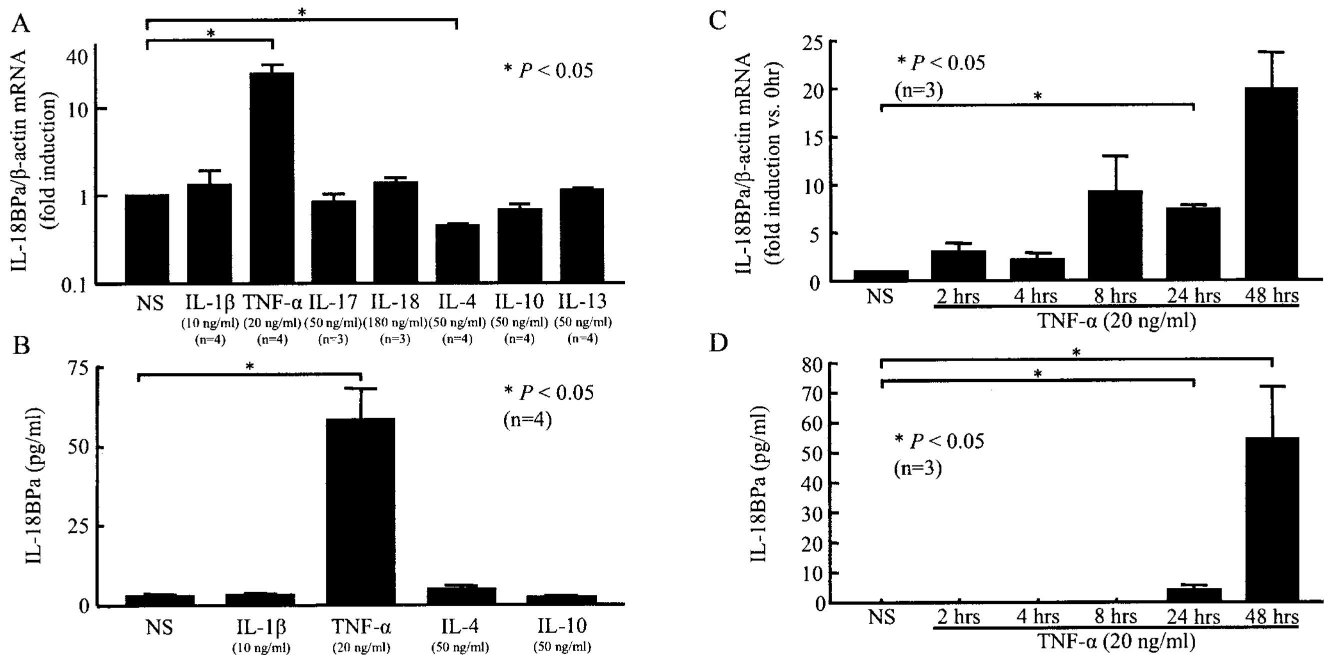

Figure 2. Up-regulation of IL-18BPa in RA synovial fibroblasts exposed to tumor necrosis factor α (TNFα). **A** and **B**, RA synovial fibroblasts (2×10^5/well; 2 ml/well) were stimulated with proinflammatory cytokines (IL-1β, TNFα, IL-17, and IL-18) or antiinflammatory cytokines (IL-4, IL-10, and IL-13) for 24 hours in serum-free media. IL-18BPa expression was assessed at the mRNA level (**A**) and at the protein level (**B**). **C** and **D**, RA synovial fibroblasts (2×10^5/well; 2 ml/well) were stimulated with TNFα (20 ng/ml) for different time periods (2, 4, 8, 24, or 48 hours) in serum-free media. IL-18BPa expression was assessed at the mRNA level (**C**) and at the protein level (**D**). Bars show the mean and SEM. NS = nonstimulated; n = number of donors and independent experiments (see Figure 1 for other definitions).

synovial fluid samples from OA patients (mean ± SEM 3,995 ± 660 pg/ml versus 6,765 ± 761 pg/ml [$P < 0.05$]; n = 8 patients per group) (Figure 1A). In the same samples, IL-18 levels were similar in RA and OA synovial fluid (237 ± 33 pg/ml versus 201 ± 26 pg/ml; n = 8 patients per group) (Figure 1B). However, levels of free IL-18, calculated according to the dissociation constant, were higher in RA than in OA synovial fluid samples (63 ± 14 pg/ml versus 27 ± 4 pg/ml [$P < 0.05$]; n = 8 per group). This suggests that the high level of free IL-18 in RA synovial fluid is the result of decreased IL-18BPa expression.

Cytokine profiling for IL-18BPa production in RA synovial fibroblasts. TNFα (20 ng/ml) stimulation resulted in a 24-fold induction in IL-18BPa transcription in RA synovial fibroblasts ($P < 0.05$) (Figure 2A). Other proinflammatory cytokines, such as IL-1β (10 ng/ml), IL-17 (50 ng/ml), and IL-18 (180 ng/ml), showed no regulation of IL-18BPa in RA synovial fibroblasts (Figure 2A). Among the battery of antiinflammatory cytokines used for stimulation, including IL-4 (50 ng/ml), IL-10 (50 ng/ml), and IL-13 (50 ng/ml), only IL-4 down-regulated IL-18BPa expression at the transcriptional level. We then confirmed IL-18BPa induction by TNFα at the protein level ($P < 0.05$) (Figure 2B). However, IL-4 had no effect on IL-18BPa production at the protein level. Of all of the cytokines profiled, only TNFα induced IL-18BPa in RA synovial fibroblasts.

TNFα induction of IL-18BP in a time-dependent manner in RA synovial fibroblasts. TNFα (20 ng/ml) stimulation of RA synovial fibroblasts was performed for the following time periods: 2, 4, 8, 24, and 48 hours. IL-18BPa was induced by TNFα in a time-dependent manner, with a maximal effect at 48 hours at the mRNA and protein levels ($P < 0.05$) (Figures 2C and D). Accordingly, we then used the 48-hour time point for further studies.

Role of JNK-2, PKCα/β, PKCδ, Src, and NF-κB pathways in TNFα-induced IL-18BPa. To identify the signaling events that are critical for TNFα-induced IL-18BPa, RA synovial fibroblasts were incubated with chemical signaling inhibitors for 1 hour, followed by stimulation with TNFα (20 ng/ml) for 48 hours. The results of this study showed that the inhibitors of the JNK-2 (SP600125), PKCα/β (Gö6976), PKCδ (Rottlerin), Src (PP2), and NF-κB (PDTC) pathways signif-

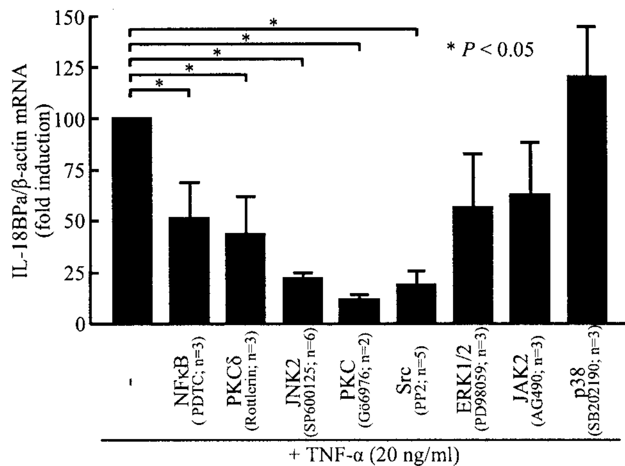

Figure 3. Effect of signaling inhibition on tumor necrosis factor α (TNFα)–induced IL-18BPa levels in RA synovial fibroblasts. RA synovial fibroblasts (2×10^5/well; 2 ml/well) were preincubated with the indicated inhibitors for 2 hours, followed by stimulation with TNFα (20 ng/ml) for 48 hours. Bars show the mean and SEM expression of mRNA for IL-18BPa. PDTC = pyrrolidine dithiocarbamate; PKCδ = protein kinase Cδ; n = number of donors and independent experiments (see Figure 1 for other definitions).

icantly inhibited TNFα-induced IL-18BPa transcription (by 78%, 88%, 56%, 72%, and 49%, respectively, from the levels observed with TNFα stimulation alone) in RA synovial fibroblasts ($P < 0.05$) (Figure 3). In contrast, inhibitors of ERK-1/2 (PD98059), p38 (SB202190), and JAK-2 (AG-490) did not significantly inhibit TNFα-induced IL-18BPa transcription. These results indicate that TNFα induction of IL-18BPa in RA synovial fibroblasts occurs via the JNK-2, PKC, and NF-κB pathways, but not via the ERK-1/2 pathway.

TNFα induction of IL-18 and caspase 1 in a time-dependent manner in RA synovial fibroblasts. To determine whether the production of IL-18BPa has any regulatory effect on free IL-18 induced by TNFα, we examined the effect of TNFα on IL-18 expression at the mRNA level. Our study showed that TNFα also induced IL-18 expression in a time-dependent manner (Figure 4A). Furthermore, the ratio of IL-18 to IL-18BPa showed no significant variation and remained in the range of 0.5–1.6 over time (Figure 4B), suggesting a similar induction of IL-18 and IL-18BP by TNFα. Since pro–IL-18 needs to be cleaved by caspase 1 to be

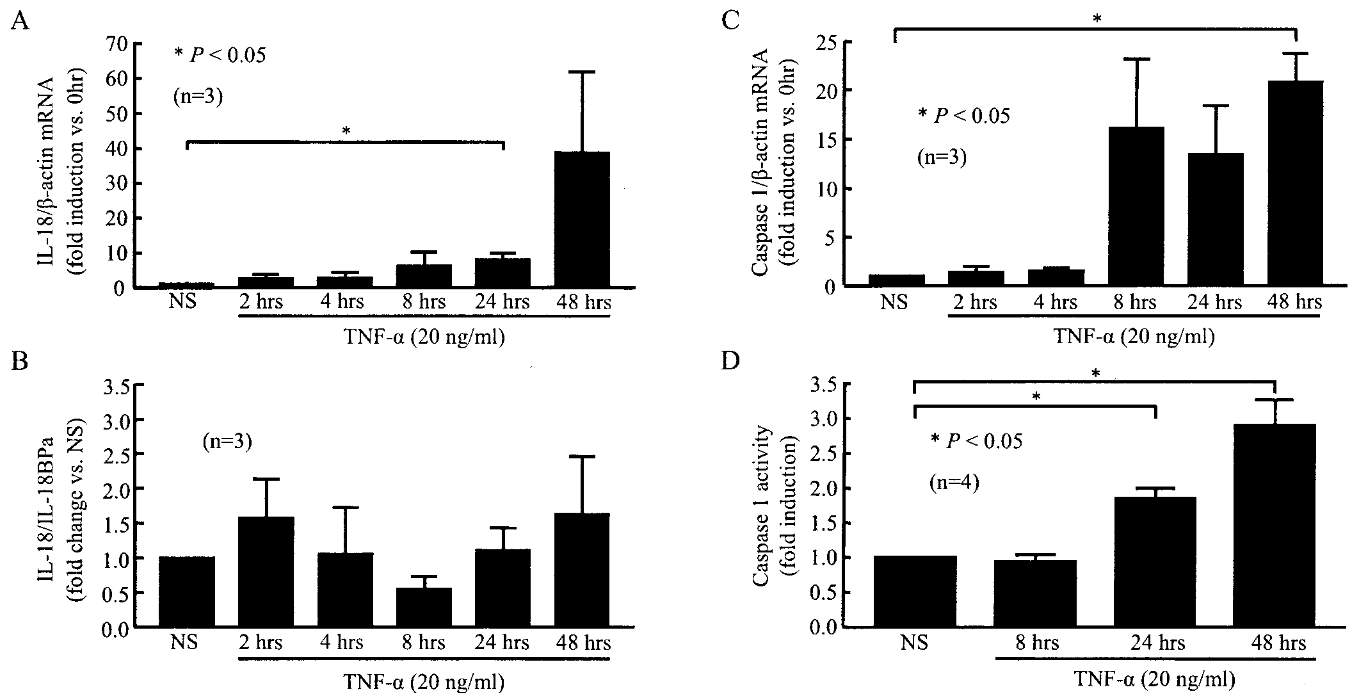

Figure 4. A–C, RA synovial fibroblasts (2×10^5/well; 2 ml/well) were treated with tumor necrosis factor α (TNFα; 20 ng/ml) for up to 48 hours to examine IL-18 and caspase 1 expression. Expression of mRNA for IL-18 (**A**), the ratio of IL-18 to IL-18BPa (**B**), and expression of mRNA for caspase 1 (**C**) over time after TNFα stimulation were determined. **D**, RA synovial fibroblasts (2×10^6/well) were treated with TNFα (20 ng/ml) for 8, 24, or 48 hours in serum-free RPMI 1640. Cells were washed in ice-cold phosphate buffered saline, scraped, centrifuged, and then lysed with lysis buffer. Caspase 1 activity in cell lysates was determined using a colorimetric assay. Bars show the mean and SEM. NS = nonstimulated; n = number of donors and independent experiments (see Figure 1 for other definitions).

activated, we then investigated TNFα-induced caspase 1 expression at the mRNA and functional levels. We observed that TNFα induced caspase 1 at both the transcriptional level (Figure 4C) and the functional level (Figure 4D). Since TNFα induces both IL-18 and caspase 1 at the transcriptional level and induces functional caspase 1, TNFα may be able to induce bioactive IL-18 in RA synovial fibroblasts.

Significance of ERK-1/2, PKCδ, and Src pathways in TNFα-induced IL-18 in RA synovial fibroblasts. We next investigated whether the same signaling events found to be critical for TNFα induction of IL-18BPa are also required for TNFα induction of IL-18. We found that the inhibitors of ERK-1/2 (PD98059), PKCδ (Rottlerin), and Src (PP2) significantly inhibited TNFα-induced IL-18 production (by 73%, 83%, and 75%, respectively) in RA synovial fibroblasts ($P < 0.05$) (Figure 5A). In contrast, the inhibitors of NF-κB (PDTC), p38 (SB202190), JNK-2 (SP600125), JAK-2 (AG-490), and PKCα/β (Gö6976) did not significantly inhibit TNFα-induced IL-18. These results indicate that TNFα induction of IL-18 by RA synovial fibroblasts occurs via ERK-1/2, PKCδ, and Src, but not via NF-κB.

Reduction of TNFα-induced IL-18 bioactivity in RA after blocking ERK-1/2. Synovial fibroblasts were preincubated with or without PD98059 for 2 hours before TNFα stimulation (20 ng/ml) for 48 hours. IL-18 bioactivity was then determined in the conditioned medium using KG-1 cells. TNFα induced IL-18 bioactivity, and this induction was reduced by 53% when the ERK-1/2 pathway was blocked by chemical inhibitor ($P < 0.05$; n = 7) (Figure 5B). These results indicate a crucial role of the ERK-1/2 pathway in regulating TNFα-induced IL-18 bioactivity.

Inhibition of TNFα-induced ERK-1/2 phosphorylation by exogenous IL-18BPa-Fc. RA synovial fibroblasts were preincubated with IL-18BPa-Fc or IgG-Fc 1 hour prior to stimulation with TNFα. Phospho–ERK-1/2 was then detected in cell lysates after 20 minutes. As expected, TNFα induced ~11-fold phosphorylation of ERK-1/2 ($P < 0.05$; n = 3) (Figure 5C), while treatment with IL-18BPa-Fc at 25 ng/ml and 50 ng/ml reduced this phosphorylation by 42% and 68%, respectively ($P < 0.05$ for 50 ng/ml; n = 3). Preincubation with IgG-Fc had no observable effect on ERK-1/2 phosphorylation. These results suggest an important effect of IL-18BPa-Fc on TNFα-induced ERK-1/2 phosphorylation.

Absence of cross-talk between TNFα-mediated signaling pathways. TNFα induces phosphorylation of Src, ERK-1/2, JNK-2, and PKCδ in RA synovial fibroblasts in a time-dependent manner, with the maximal

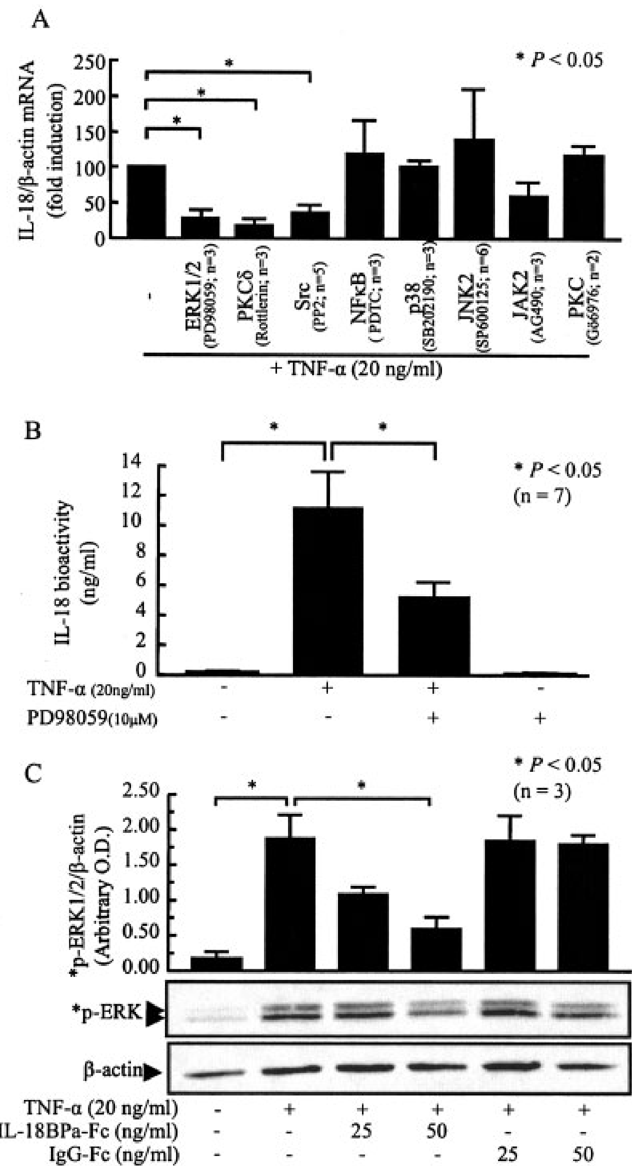

Figure 5. Effect of signaling inhibition on tumor necrosis factor α (TNFα)–induced IL-18 levels and ERK-1/2 phosphorylation in RA synovial fibroblasts. **A**, Expression of mRNA for IL-18 in RA synovial fibroblasts preincubated with chemical signaling inhibitors for 2 hours, followed by stimulation with TNFα for 48 hours. Bars show the mean and SEM. PKCδ = protein kinase Cδ; PDTC = pyrrolidine dithiocarbamate. **B**, IL-18 bioactivity in conditioned medium, assessed using KG-1 cells. Bars show the mean and SEM. **C**, Phosphorylation of ERK-1/2 in RA synovial fibroblasts treated with IL-18BPa-Fc or IgG-Fc (25 ng/ml or 50 ng/ml) for 1 hour, followed by stimulation with TNFα (20 ng/ml) for 20 minutes. Phosphorylation of ERK-1/2 in 15 μg of each sample was determined by Western blotting. A representative blot is shown. Equal loading of protein was verified by reprobing blots for β-actin. The intensity of the bands was quantified using Un-Scan-It software. Bars show the mean and SEM of 3 independent donors examined under similar conditions. OD = optical density; n = number of donors and independent experiments (see Figure 1 for other definitions).

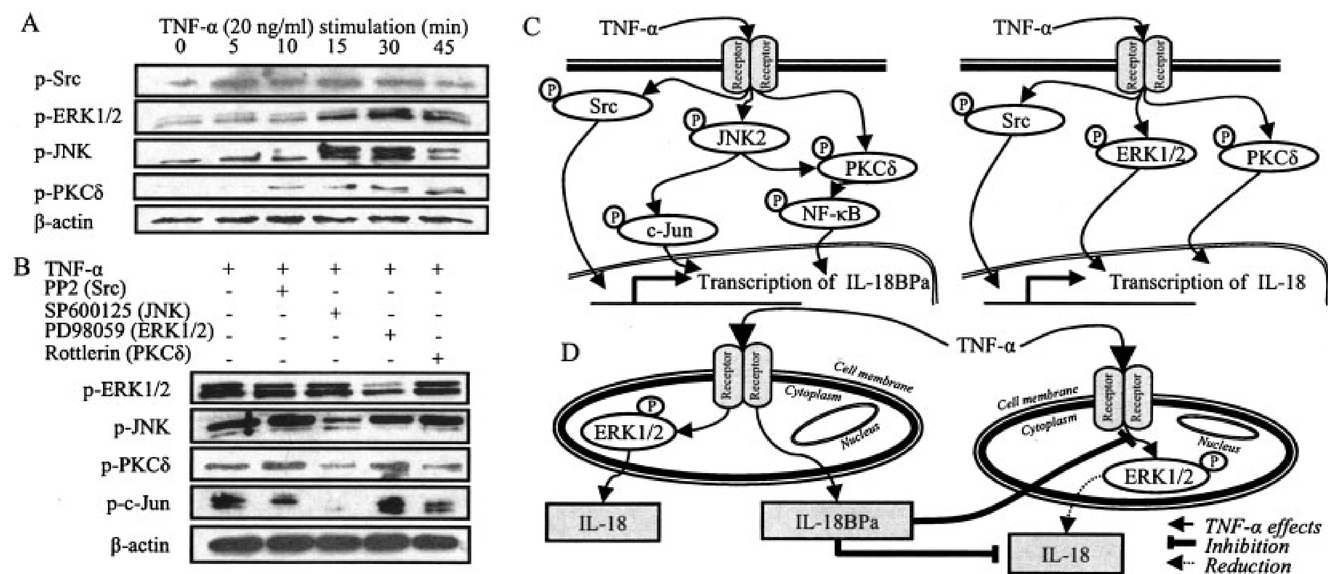

Figure 6. A, Western blot analysis showing time-dependence of tumor necrosis factor α (TNFα) stimulation of phosphorylation of signaling intermediates. RA synovial fibroblasts were stimulated with TNFα (20 ng/ml) for the indicated periods of time. Phosphorylation of ERK-1/2, JNK-2, and protein kinase Cδ (PKCδ) in 15 μg of each sample was determined by Western blotting. To verify equal loading, the blot membranes were stripped and reprobed with β-actin. Results are representative of at least 3 experiments. **B**, Western blot analysis showing evidence of activation of multiple pathways of TNFα phosphorylation of signaling intermediates. RA synovial fibroblasts were preincubated with signaling inhibitors before stimulation with TNFα (20 ng/ml) for 30 minutes. Phosphorylation of ERK-1/2, JNK-2, and PKCδ in 15 μg of each sample was determined by Western blotting. Phosphorylation of PKCδ was inhibited by inhibitors of JNK-2 and PKCδ. Blot membranes were stripped and reprobed with β-actin. Results are representative of at least 3 experiments. **C**, Schematic representation of the mechanism of TNFα induction of IL-18 and IL-18BPa through different pathways. TNFα induces IL-18 through the ERK-1/2, PKCδ, and Src pathways, while TNFα induces IL-18BPa through the NF-κB, PKC, Src, and JNK-2 pathways. **D**, Schematic representation of the effect of IL-18BPa. IL-18BPa reduces IL-18 bioactivity by binding to IL-18 and also reduces phosphorylation of ERK-1/2 induced by TNFα. See Figure 1 for other definitions.

response at 15–30 minutes (Figure 6A). Western blotting was performed to determine which of the phosphorylated kinases might be upstream or downstream of the others. The phosphorylation of PKCδ and c-Jun were reduced by the JNK-2 inhibitor (Figures 6B), demonstrating that PKCδ and c-Jun are downstream of JNK-2.

Summary of signaling induced by TNFα on RA synovial fibroblasts. The results from this inhibitor study showed that TNFα enlists differential pathways in RA synovial fibroblasts for IL-18 and IL-18BPa expression. These signaling pathways are depicted in Figure 6C, and the effects of IL-18BPa are represented in Figure 6D.

DISCUSSION

Recently, higher levels of IL-18 were observed in the synovial fluid, synovial tissue, and sera of RA patients as compared with that of OA patients (8,25,26). Previous studies have shown that RA synovial tissue expressed higher levels of IL-18 mRNA and spontaneously released larger amounts of IL-18 protein than did OA tissue (25). Our findings confirm that IL-18BPa levels in RA synovial fluid are lower than those in OA synovial fluid (26). Furthermore, higher levels of free or bioactive IL-18 were present in RA synovial fluid than in OA synovial fluid, and this was related to a lower level of IL-18BPa in RA synovial fluid (26). Since IL-18BPa regulates IL-18 bioactivity, IL-18BPa needs to be assessed for free IL-18 or IL-18 bioactivity, as suggested previously by the discordance between IL-18 bioactivity and the level of IL-18 protein in RA synovial fluid. In fact, IL-18 bioactivity was ~17-fold lower than its total protein level (25). Similar modulation by a natural inhibitor was recently reported for IL-1β and its natural inhibitor, IL-1 receptor antagonist (IL-1Ra). Despite a higher level of IL-1Ra in RA synovial fluid than in OA synovial fluid, the ratio of IL-1Ra to IL-1 was higher in OA synovial fluid than in RA synovial fluid (27), and explained in part the mild to moderate inflammation seen in OA versus RA. Taken together, these data suggest that inhibition of IL-18 bioactivity can be attained by increasing the level of IL-18BPa.

RA synovial fibroblasts constitutively produced IL-18BPa. IFNγ is an important regulator of IL-18BPa

expression (28). Since IFNγ levels are low in the RA joint (29), we focused on TNFα, a cytokine known to be important in RA. To our knowledge, this is the first study to investigate the mechanism of TNFα-induced IL-18BPa synthesis. Few other cytokines that up-regulate IL-18BPa production have been described, such as IL-12 in RA synovial tissue cells or activated peripheral blood mononuclear cells after 7 days of stimulation (28). IL-1Ra in combination with IFNγ has also been shown to induce IL-18BPa in a human epithelial cell line after 17 hours of stimulation (30). The combination of TNFα, IL-1β, and IFNγ appeared to induce higher levels of IL-18BPa in RA synovial fibroblasts than did IFNγ alone after 48 hours of stimulation (21). We also confirmed that IL-18 has an effect on IL-18BPa production (28). Thus, the delayed effect of TNFα on IL-18BPa production is consistent with the findings of previous studies using other stimuli.

TNFα transduces its signal by binding to TNF receptors, which can be divided into 2 subtypes (1 and 2) in RA synovial fibroblasts (31). Previously, we described a bioassay to assess circulating TNFα bioactivity in RA patients, using the ability of RA synovial fibroblasts to produce IL-6 in response to TNFα (32,33). After 48 hours, TNFα induces IL-6 production in a dose-dependent manner (32), suggesting that at this time point, all TNFα (at 20 ng/ml) is likely bound and therefore consumed in the culture supernatant.

Despite the induction of IL-18BPa by TNFα, TNFα also induced IL-18 in RA synovial fibroblasts, as previously described in such different cell types as human adipocytes (34), rat intestinal epithelial cells (35), and rat cardiomyocytes (36). In addition, the ratio of IL-18 to IL-18BPa remained equal over 48 hours, with a few nonsignificant variations. Caspase 1 activity is necessary to cleave pro–IL-18 to its active IL-18 form (5). Previously, TNFα was also shown to induce caspase 1 transcription in a human lung carcinoma cell line (37). This study is the first to show that TNFα induces caspase 1 transcription and activity in RA synovial fibroblasts. Our data suggest that TNFα induced IL-18 and caspase 1 in the same proportion. Therefore, IL-18 induced by TNFα is bioactive, as confirmed by KG-1 cells.

The signaling mechanisms involved in TNFα-induced IL-18BPa production in RA synovial fibroblasts have not been examined previously. In the present study, we found that TNFα-induced IL-18BPa production in RA synovial fibroblasts is dependent on JNK-2, PKC, Src, and NF-κB. TNFα is known to activate JNK-2, PKC, ERK-1/2, and NF-κB in RA synovial fibroblasts (18,38–40). However, JAK-2 and ERK-1/2 inhibitors did not significantly inhibit TNFα-induced IL-18BPa production. Conversely, TNFα-induced IL-18 secretion is dependent on ERK-1/2, PKCδ, and Src. We showed that blocking ERK-1/2 altered the IL-18:IL-18BPa ratio by suppressing IL-18 and enhancing IL-18BPa expression, and so reduced IL-18 bioactivity. The ERK pathway, which is 1 of the 3 most well-characterized mammalian MAP kinase pathways (along with the JNK and p38 pathways), enhances the production of a variety of proinflammatory cytokines, such as TNFα (41). Moreover, the ERK pathway is a survival pathway that was found to be activated in the RA synovium (42,43). Furthermore, TNFα induces activation of the ERK pathway in human RA synovial fibroblasts (39). TNFα has also been shown to stimulate ERK activity in synovial tissue in vivo in the TNFα-transgenic mouse (44), an effect inhibited by blockade of TNFα. Thus, those studies provide additional data suggesting that the ERK-1/2 pathway is a central pathway for IL-18 activity induced by TNFα.

After finding that the ERK-1/2 pathway was a key pathway controlling TNFα-induced IL-18 bioactivity, we investigated the putative effect of IL-18BPa-Fc on ERK-1/2 phosphorylation induced by TNFα. We observed that IL-18BPa-Fc reduced the activation of the ERK-1/2 pathway that was induced by TNFα. These data suggest the presence of a feedback loop for controlling free IL-18 and for extending IL-18 bioactivity. IL-18BPa has been shown to down-regulate IFNγ expression induced by IL-18, defining a different feedback loop (45,46) in blood and in RA synovial fibroblasts (21). IL-18 induces TNFα in RA synovial fibroblasts (8,25) and human monocytes (47).

With regard to TNFα signaling in RA synovial fibroblasts, we did not identify cross-talk between Src, ERK-1/2, and NF-κB. The finding that the JNK-2 inhibitor reduced the TNFα-induced activation of PKCδ and NF-κB in RA synovial fibroblasts suggests that JNK-2 is upstream of PKCδ and NF-κB.

As described herein, TNFα activates PKCδ, ERK-1/2, and JNK-2 in RA synovial fibroblasts. These kinases are reported to regulate production of various proinflammatory mediators in RA synovial fibroblasts (23,38,42,48,49). With specific regard to the expression of IL-18 and IL-18BPa, we have demonstrated a critical role of the Src, ERK-1/2, and PKCδ pathways for IL-18 and of the Src, PKC, JNK-2, and NF-κB pathways for IL-18BPa. In the present study, we found that PKCδ was regulated by JNK-2, an observation that was consistent with the findings of a previous study that used IL-18 as stimulus in RA synovial fibroblasts (23).

We propose that the role of IL-18 in RA is as

follows. TNFα induces both pro–IL-18 and caspase 1, which cleaves pro–IL-18 to active IL-18. However, TNFα also induces IL-18BPa. Furthermore, IL-18 is known to induce TNFα in RA synovial fibroblasts. This is thus a positive feedback loop that could explain the cytokine predominance in RA. The main known function of IL-18BPa is to reduce IL-18 bioactivity. We observed that blocking the ERK pathway reduced TNFα-induced IL-18 expression without interfering with TNFα-induced IL-18BPa expression. Therefore, blocking the ERK pathway reduced TNFα-induced IL-18 bioactivity. Furthermore, IL-18BPa itself reduces TNFα-induced phosphorylated ERK-1/2. This suggests that there may be a negative feedback loop, with synthesis of IL-18BPa down-regulating TNFα-induced IL-18, in a manner similar to that described for IFNγ. Thus, like other genes encoding cytokine inhibitors (soluble receptors, receptor antagonists, and binding proteins), the cytokine itself or a related cytokine induces its own negative regulator in a feedback loop. Furthermore, this feedback loop was suggested by the results of a previous in vivo study (50). Recombinant human IL-18BPa reduced the level of TNFα colon homogenates in a mouse model of Crohn's disease, which is an inflammatory disorder in which IL-18 plays a crucial role (50).

Overall, the findings of the present study suggest that the presence of IL-18BPa, either by endogenous induction or exogenous addition, may contribute to the regulation of TNFα-induced IL-18 bioactivity in RA synovial fibroblasts. The results further suggest that IL-18BPa may be considered as a potential therapeutic strategy for RA.

ACKNOWLEDGMENTS

The authors thank the National Disease Research Interchange for providing RA synovial tissue and Dr. K. W. Janczak for providing the KG-1 cell line.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Koch had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Marotte, Ahmed, Koch.
Acquisition of data. Marotte, Ruth.
Analysis and interpretation of data. Marotte, Ahmed.

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