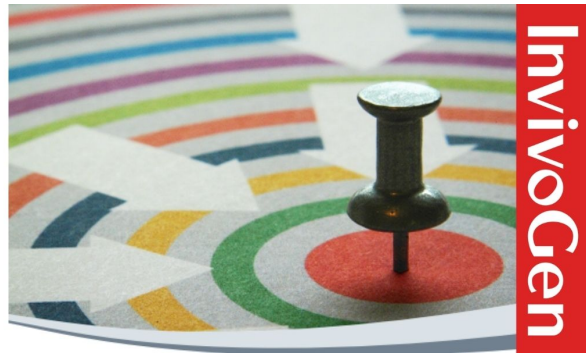


# Custom Screening & Profiling Services for immune-modulating compounds

TLR - NOD 1/NOD2 - RIG-I/MDA5 - STING  
DECTIN-1 - MINCLE



*The Journal of*  
**Immunology**

RESEARCH ARTICLE | NOVEMBER 01 2001

## Cross-Talk Between IL-1 and IL-6 Signaling Pathways in Rheumatoid Arthritis Synovial Fibroblasts<sup>1</sup> ✓

David Deon; ... et. al

*J Immunol* (2001) 167 (9): 5395–5403.

<https://doi.org/10.4049/jimmunol.167.9.5395>

### Related Content

IL-15 and the Initiation of Cell Contact-Dependent Synovial Fibroblast-T Lymphocyte Cross-Talk in Rheumatoid Arthritis: Effect of Methotrexate

*J Immunol* (July,2004)

Cross-Linking of CD44 on Rheumatoid Synovial Cells Up-Regulates VCAM-1

*J Immunol* (February,1999)

Modulation of TNF-Induced Macrophage Polarization by Synovial Fibroblasts

*J Immunol* (September,2014)

# Cross-Talk Between IL-1 and IL-6 Signaling Pathways in Rheumatoid Arthritis Synovial Fibroblasts<sup>1</sup>

David Deon,\* Simi Ahmed,<sup>†</sup> Katy Tai,\* Nicholas Scaletta,\* Carmen Herrero,\* In-Hong Lee,<sup>2\*</sup> Anja Krause,\* and Lionel B. Ivashkiv<sup>3\*†</sup>

The balance between pro- and anti-inflammatory cytokines plays an important role in determining the severity of inflammation in rheumatoid arthritis (RA). Antagonism between opposing cytokines at the level of signal transduction plays an important role in many other systems. We have begun to explore the possible contribution of signal transduction cross-talk to cytokine balance in RA by examining the effects of IL-1, a proinflammatory cytokine, on the signaling and action of IL-6, a pleiotropic cytokine that has both pro- and anti-inflammatory actions, in RA synovial fibroblasts. Pretreatment with IL-1 suppressed Janus kinase-STAT signaling by IL-6, modified patterns of gene activation, and blocked IL-6 induction of tissue inhibitor of metalloproteases 1 expression. These results suggest that proinflammatory cytokines may contribute to pathogenesis by modulating or blocking signal transduction by pleiotropic or anti-inflammatory cytokines. The mechanism of inhibition did not require de novo gene activation and did not depend upon tyrosine phosphatase activity, but, instead, was dependent on the p38 stress kinase. These results identify a molecular basis for IL-1 and IL-6 cross-talk in RA synoviocytes and suggest that, in addition to levels of cytokine expression, modulation of signal transduction also plays a role in regulating cytokine balance in RA. *The Journal of Immunology*, 2001, 167: 5395–5403.

Cytokines play an important role in the pathogenesis of a wide variety of inflammatory and autoimmune diseases, and several lines of investigation provide support for a critical role for cytokines in rheumatoid arthritis (RA)<sup>4</sup> (reviewed in Refs. 1–4). Certain proinflammatory cytokines, e.g., IL-1, IL-8, IL-15, GM-CSF, and TNF- $\alpha$ , are expressed in the majority of patients with RA. The known activity of proinflammatory cytokines detected in inflamed synovium can account for many processes important in joint destruction: production of proteases and reactive oxygen intermediates, synovial fibroblast (SF) proliferation, cartilage degradation, influx of inflammatory cells, and angiogenesis. The most compelling support for cytokines in RA pathogenesis comes from the attenuation of arthritis by therapies aimed at modulating or blocking cytokine activity (2, 5). Exciting studies have demonstrated dramatic improvement in synovial inflammation in RA patients after treatment with neutralizing anti-TNF- $\alpha$  Abs or soluble TNF receptors (5–7), and decreased joint destruction after treatment with IL-1R antagonist (IL-1RA) (Ref. 8 and references therein). Interestingly, immunosuppressive and anti-inflammatory

cytokines, including TGF $\beta$ , IL-10, and IL-1RA, are highly and consistently expressed during RA synovitis (1, 2). Production of these cytokines has been proposed to reflect the patients' attempts to contain or control inflammation and achieve homeostasis (3, 4). Treatment with TGF $\beta$ , IL-10, and IL-1RA has been successful in ameliorating disease in acute animal models of arthritis, such as collagen-induced arthritis (CIA). An interesting question is why the high endogenous levels of anti-inflammatory factors that are expressed in RA joints are unable to suppress long-term chronic synovitis in patients.

IL-6 is one member of a family of related cytokines (IL-6, IL-11, oncostatin M (OsM), LIF, and cardiotropin) that share the gp130 signaling receptor subunit. IL-6, IL-11, OsM, and LIF are highly expressed during RA synovitis (1, 9). Although these cytokines are pleiotropic and can promote immune responses, they also have clear-cut anti-inflammatory effects, especially on macrophages and fibroblasts. For example, IL-11 suppresses expression of IL-12, IFN- $\gamma$ , TNF- $\alpha$ , adhesion molecules, and proteases (10–13), inhibits cytokine production in RA synovium (9), and has been used successfully to treat CIA (14) and psoriasis (15). In contrast to IL-11, IL-6 appears to have different actions on different cell types and can act to either promote or suppress inflammatory arthritis. Consistent with its known stimulatory effects on T and B cells, IL-6 appears to be critically important in the initiation phase of CIA, which is mediated by lymphocyte responses against type II collagen (16, 17). In addition, IL-6 induces chemokine expression in endothelial cells (18). In contrast to its effects on lymphocytes and endothelial cells, IL-6 has been reported to have suppressive effects on other cell types, including macrophages and SFs, that are important in RA pathogenesis. The reported anti-inflammatory effects of IL-6 include induction of anti-inflammatory cytokines such as IL-1RA, induction of acute phase reactants that subserve anti-inflammatory functions, induction of glucocorticoid production, suppression of cytokine production (IL-1, TNF, and IL-12) and adhesion molecule

\*Department of Medicine, Hospital for Special Surgery, and <sup>†</sup>Graduate Program in Immunology, Weill Graduate School of Medical Sciences, Cornell University, New York, NY 10021

Received for publication December 6, 2000. Accepted for publication August 23, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by grants from the National Institutes of Health.

<sup>2</sup> Current address: Hanyang University, Seoul, Korea.

<sup>3</sup> Address correspondence and reprint requests to Dr. Lionel B. Ivashkiv, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail address: IvashkivL@HSS.edu

<sup>4</sup> Abbreviations used in this paper: RA, rheumatoid arthritis; OsM, oncostatin M; sIL-6R $\alpha$ , soluble IL-6R $\alpha$  subunit; CIA, collagen-induced arthritis; SF, synovial fibroblast; SOCS, suppressor of cytokine signaling; MAPK, mitogen-activated protein kinase; ERK, extracellular stimulus-regulated kinase; Jak, Janus kinase; IL-1RA, IL-1R antagonist; IRF, IFN regulatory factor; dex, dexamethasone; MIG, monokine induced by IFN- $\gamma$ .

expression, suppression of proliferation of RA SFs, inhibition of protease expression, and induction of protease inhibitors (such as TIMP-1) in RA SFs (19–28). IL-6 induction of TIMP expression in RA SFs blocks IL-1-induced collagenolytic activity and likely plays an important role in suppressing the ability of these cells to invade and destroy cartilage (28). Consistent with these anti-inflammatory effects, IL-6 ameliorates inflammation in several animal models, including inflammatory lung disease (29), and plays a chondroprotective role in zymosan-induced arthritis (30). The strongest evidence of an anti-inflammatory role for IL-6 in arthritis is the development of spontaneous arthritis in mice containing a mutation that partially abrogates IL-6 signal transduction (31). These results suggest that the role of IL-6 in RA pathogenesis will depend upon the balance between its pro- and anti-inflammatory actions on different cell types.

The idea that the balance between pro- and anti-inflammatory factors is important in regulating the rate of progression, and thus the eventual severity and morbidity, of RA has gained acceptance among RA researchers and formed one rationale for developing anti-TNF- $\alpha$  therapies (2). The current concept is that one important determinant of the balance between pro- and anti-inflammatory factors is the relative level of expression of, on the one hand, inflammatory cytokines, and, on the other hand, anti-inflammatory cytokines, receptor antagonists, and soluble receptors (2, 3). However, it is becoming increasingly clear that cytokine effects can be blocked intracellularly at the level of signal transduction. For example, the potent anti-inflammatory cytokine IL-10 blocks signaling by IFN- $\gamma$  (32), and IL-6 and IL-11 block signaling and NF- $\kappa$ B activation by LPS (33). Conversely, IFN- $\gamma$  and IL-1 block signaling by TGF $\beta$  (34, 35). Thus, the final effect of cytokines on cellular phenotype is not determined solely by the relative levels of expression of different cytokines. We hypothesized that one mechanism by which inflammatory cytokines perpetuate synovitis in the presence of anti-inflammatory factors in the synovium is through inhibition or modulation of signaling by anti-inflammatory or pleiotropic cytokines. This hypothesis was tested by analyzing the effects of IL-1 and TNF on IL-6 signaling in RA SFs, cells in which IL-6 has effects consistent with an anti-inflammatory role, such as inhibition of proliferation and induction of TIMP-1 production (27, 28). Our results show that Janus kinase (Jak)-STAT signaling by IL-6 in RA synoviocytes is inhibited by IL-1 and TNF- $\alpha$ . These results identify a novel level of cytokine cross-talk in RA synovium and suggest that cytokine antagonism at the level of signal transduction contributes to the balance of cytokine activity, and thus pathogenesis.

## Materials and Methods

### Cell isolation and tissue culture

Synovial tissues were obtained perioperatively from patients who fulfilled the revised American College of Rheumatology criteria for definite RA and were undergoing total joint replacement; the protocol was approved by the Institutional Review Board of the Hospital for Special Surgery (New York, NY). A total of 24 specimens from different donors were analyzed. Synovial cells were obtained by finely mincing freshly isolated synovial tissue, followed by treatment with collagenase A (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) for 2 h at 37°C. Fibroblasts were obtained by allowing cells to adhere to tissue culture plates, followed by removal of nonadherent cells. The initially plated adherent cells contained contaminating macrophages, and, in experiments in which cells were used before in vitro culture and fibroblast expansion, macrophages were removed using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA), as previously described (36). Similar to previous reports, most of the experiments were performed using SFs between the third and fifth passages in tissue culture. At the third passage, there were <2% contaminating lymphocytes, NK cells, or macrophages, as assessed by flow cytometry and staining with Abs

against CD3, CD14, CD16, and CD19, as previously described (36). SFs were cultured in DMEM supplemented with 10% FBS, and cells were routinely split and replated the day before an experiment.

### EMSA

Cell extracts were prepared as previously described (37) and protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (typically 10  $\mu$ g) were incubated for 15 min at room temperature with 0.5 ng of  $^{32}$ P-labeled double-stranded hSIE oligonucleotide (38) in a 15- $\mu$ l binding reaction containing 40 mM NaCl and 2  $\mu$ g of poly(dI-dC) (Pharmacia, Piscataway, NJ), as previously described (37), and complexes were resolved on nondenaturing 4.5% polyacrylamide gels.

### Immunoblotting

Cell lysates were fractionated on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with phosphospecific (Tyr<sup>705</sup>) Stat3 Ab, phosphospecific (Thr<sup>180</sup>/Tyr<sup>182</sup>) p38 Ab, phosphospecific (Thr<sup>202</sup>/Tyr<sup>204</sup>) extracellular stimulus-regulated kinase (ERK)1/2 Ab (New England Biolabs, Beverly, MA), monoclonal Stat3 and ERK1/2 Abs (BD Transduction Laboratories, Lexington, KY), and p38 Abs (Santa Cruz Biotechnology, Santa Cruz, CA). ECL was used for detection.

### ELISA

Paired TIMP-1 capture and detection Abs and TIMP-1 protein were purchased and used in a sandwich ELISA according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

### Proliferation analysis

Cells (10<sup>4</sup>) in 100  $\mu$ l of medium were seeded in triplicate in 96-well tissue culture plates, cytokines were added after 8 h, and cells were cultured for an additional 24 h. Cells were pulsed for the final 8 h of culture with 10  $\mu$ Ci/ml [<sup>3</sup>H]thymidine and harvested using an automated cell harvester (Harvester 96; Tomtec, Orange, CT), and [<sup>3</sup>H]thymidine incorporation was quantitated using a Wallac Microbeta Trilux Scintillation Counter (PerkinElmer Wallac, Gaithersburg, MD). Cell counts were performed on parallel wells in duplicate and cell viability was determined using trypan blue and propidium iodide exclusion.

### Analysis of mRNA levels

For semiquantitative RT-PCR, total cellular RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. RNA was treated with RNase-free DNase, and cDNA was obtained using Moloney murine leukemia virus reverse transcriptase (Life Technologies). A total of 2.5% of each cDNA was subjected to 22–25 cycles of PCR using conditions that result in a single specific amplification product of the correct size, as previously described (36, 39): 30 s denaturation at 94°C, 1 min annealing at 55°C, and 30 s extension at 72°C in a GeneAmp 9600 thermal cycler (PerkinElmer, Norwalk, CT). dNTPs were used at 100  $\mu$ M and 1  $\mu$ Ci of [<sup>32</sup>P] $\alpha$ -dATP was added to each reaction. No amplification products were obtained when reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA. Amplification was empirically determined to be in the linear range. For real-time, quantitative PCR, DNA-free RNA was obtained using the RNeasy Mini kit from Qiagen (Valencia, CA) with DNase treatment, and 1  $\mu$ g of total RNA was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. Real-time PCR was performed in triplicate using the iCycler iQ thermal cycler and detection system (Bio-Rad) and the PCR Core Reagents kit (Applied Biosystems, Foster City, CA) with 500-nM primers. The final Mg<sup>2+</sup> concentration was adjusted to 4 mM. Four-fold serial dilutions of cDNAs were used to generate curves of log input amount vs threshold cycle, and comparable slopes for a given primer set were obtained for the group of cDNAs being tested (signifying comparable efficiencies of amplification). Fold induction was normalized for levels of GAPDH. When reverse transcriptase was omitted, threshold cycle number increased by at least 10, signifying lack of genomic DNA contamination or nonspecific amplification, and the generation of only the correct size amplification products was confirmed using agarose gel electrophoresis. The primer sequences used were: GAPDH, 5'-CGA CGC CTG CTT CAC CAC CTT-3' and 5'-CGG GGC TCT CCA GAA CAT CAT CC-3'; IFN regulatory factor (IRF-1), 5'-CTG GCT CCT TTT CCC CTG CTT TGT-3' and 5'-CAA ATC CCG GGG CTC ATC TGG-3'; suppressor of cytokine signaling (SOCS)3, 5'-TCG CCC CCG GAG TAG ATG TAA TAG-3' and 5'-CAC TAC ATG CCG CCC CCT GGA G-3'; IP-10 5'-TCT CAC CCT TCT TTT TCA TTG TAG-3' and 5'-ATT TGC

TGC CTT ATC TTT CTG-3'; monokine induced by IFN- $\gamma$  (MIG), 5'-GCT TTT TCT TTT GGC TGA CCT GTT-3' and 5'-ATC AGC ACC AAC CAA GGG ACT ATC-3'.

## Results

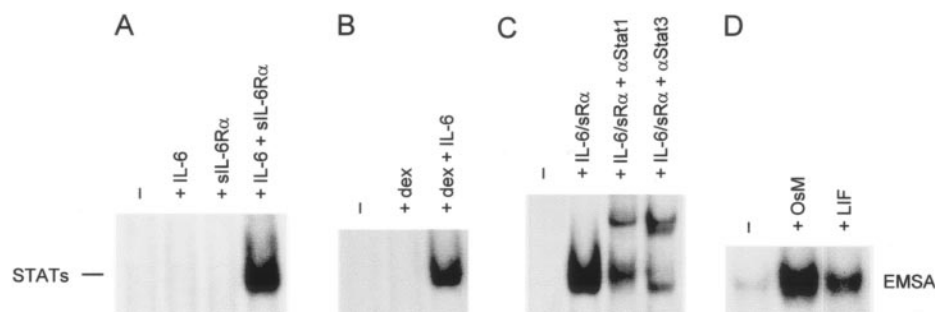
### Activation of the Jak-STAT signal transduction pathway by IL-6 and related cytokines in RA SFs

Activation of the Jak-STAT pathway by IL-6 in RA SFs was investigated by using EMSAs to detect the activation of DNA binding of STAT transcription factors. Addition of IL-6 alone did not result in any detectable binding of protein complexes to a hSIE oligonucleotide that binds Stat1, Stat3, and Stat4 (Fig. 1A, lane 2); binding activity was not detected using the IRF oligonucleotide that binds Stat5 and Stat6 (data not shown). This result is consistent with previous reports suggesting that SFs express only the gp130 signaling subunit of the IL-6R, but not the IL-6-binding  $\alpha$  subunit (27, 28). As previously described in other cells types (40), addition of extracellular soluble IL-6R $\alpha$  subunit (sIL-6R $\alpha$ ) together with IL-6 resulted in initiation of signal transduction, as determined by activation of STAT DNA-binding activity (Fig. 1A, lane 4). Use of sIL-6R $\alpha$  has clinical relevance, because this factor is elevated in RA and expressed in RA synovium at concentrations as high as 200 ng/ml (1). DNA binding was specific as determined by competition experiments with unlabeled oligonucleotide (data not shown). When SFs were pretreated with the glucocorticoid dexamethasone (dex), addition of IL-6 alone activated STAT DNA binding (Fig. 1B), consistent with previous results that cell surface expression of IL-6R $\alpha$  is induced by glucocorticoids (41). The induction of cellular responsiveness to IL-6 by the potent immunosuppressive agent dex is consistent with an anti-inflammatory action of IL-6 on SFs. IL-6 has been reported to activate Stat1 and Stat3 in other cell types, and supershift experiments with specific Abs confirmed that IL-6 plus sIL-6R $\alpha$  activated both Stat1 and Stat3 in SFs (Fig. 1C). Although the relative ratio of Stat1 and Stat3 varied among independent experiments using different donors, activation of these STATs was consistently detected in SFs that were derived from 24 different patients and used immediately (after depletion of macrophages and lymphocytes) or after three, six, or 10 passages. The effect of addition of the IL-6-related cytokines LIF, OsM, and IL-11 on STAT DNA binding activity was determined. LIF and OsM activated STAT DNA binding (Fig. 1D), but IL-11 did not (data not shown). These results are consistent with previous observations that SFs express the LIF- and OsM-specific  $\alpha$  receptor subunits, and respond to IL-11 only if soluble IL-11R $\alpha$  is provided (9). These results demonstrate that IL-6 and related cytokines activate Stat1 and Stat3 in RA SFs.

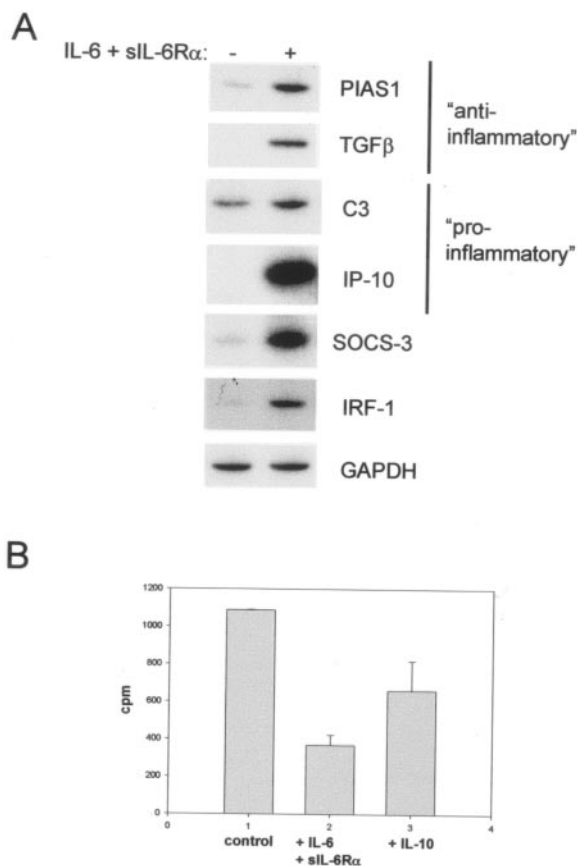
Activation of Stat1 and Stat3 would be predicted to result in induction of expression of STAT target genes, and therefore we determined the effects of IL-6/sIL-6R $\alpha$  stimulation on gene expression in SFs. Third-passage RA SFs were stimulated for 3 h with IL-6 plus sIL-6R $\alpha$ , and mRNA levels for several genes were determined (Fig. 2A). Interestingly, IL-6 induced expression both of genes that may be considered anti-inflammatory (protein inhibitor of activated Stat1, an inhibitor of IFN- $\gamma$ -Stat1 signaling, and TGF $\beta$ ), genes that are considered proinflammatory (complement component 3, and IP-10 (a chemokine)), and genes whose functions in synoviocytes are not known (SOCS3 and IRF-1, a transcription factor). This pattern is consistent with the pleiotropic nature of IL-6, as discussed above. The extent of gene activation (fold induction) varied among different patients, but a similar pattern of activation was detected in eight independent experiments; similar results were obtained when OsM was used (data not shown). SOCS3 and IRF-1 genes are directly transcriptionally activated by STATs, and thus these results demonstrate that IL-6 activation of the Jak-STAT pathway in RA SFs is coupled to transcriptional responses. Addition of IL-6 and sIL-6R $\alpha$  suppressed synoviocyte proliferation, reproducing recently reported results (27) (Fig. 2B). The level of suppression of proliferation was greater than that observed with IL-10, which has been shown to be effective in suppressing RA synoviocytes that have been implanted into SCID mice (42, 43). Suppression of synoviocyte proliferation (and induction of TIMP-1 expression; see Fig. 4B) by IL-6 further demonstrates a functional cellular response to this cytokine and is consistent with a down-regulatory effect of IL-6 on SFs.

### Inhibition of STAT activation and IL-6 signaling by IL-1 and TNF

IL-1 is a potent proinflammatory cytokine expressed in most inflamed RA joints, and the effect of IL-1 on IL-6 signaling was determined. IL-1 pretreatment for 20 min strongly blocked activation of STAT DNA binding, and inhibition was persistent, in that STAT activation was blocked when the period of incubation with IL-1 was extended to 3 h (Fig. 3A, top panel). One representative experiment of over 15 experiments using different RA synovial tissues is shown. Activation of STAT DNA binding activity is dependent upon tyrosine phosphorylation (44), and preincubation with IL-1 effectively blocked Stat3 tyrosine phosphorylation (Fig. 3A, middle panel) in parallel with inhibition of DNA binding. Stat3 levels did not change after IL-1 treatment (Fig. 3A, bottom panel), indicating that the decrease in Stat3 DNA binding was not secondary to decreased protein levels. A rapid block in STAT DNA binding and tyrosine phosphorylation suggests that inhibition may



**FIGURE 1.** Activation of STATs by IL-6 and related cytokines in RA SFs. Third-passage SFs from patients with RA (<2% contaminating macrophages or lymphocytes) were treated with cytokines for 10 min, and cell extracts were analyzed for STAT DNA binding activity using EMSA with the hSIE oligonucleotide, as previously described (37). A, IL-6 (50 ng/ml) and sIL-6R $\alpha$  (100 ng/ml) were used. B, Cells were incubated overnight with  $10^{-6}$  M dex before adding IL-6. C, Stat1 and Stat3 Abs were used under conditions where they react specifically with either Stat1 or Stat3, as previously described (37). D, OsM or LIF were used at 100 ng/ml.



**FIGURE 2.** IL-6 activates gene expression and suppresses proliferation in RA SFs. *A*, RNA was prepared 3 h after addition of IL-6 plus sIL-6R $\alpha$ , and steady state mRNA levels were determined using semiquantitative RT-PCR that had been validated using Northern blotting, as previously described (36, 39). *B*, [ $^3$ H]Thymidine incorporation was determined in triplicate, as described in *Materials and Methods*. One representative experiment of eight performed is shown.

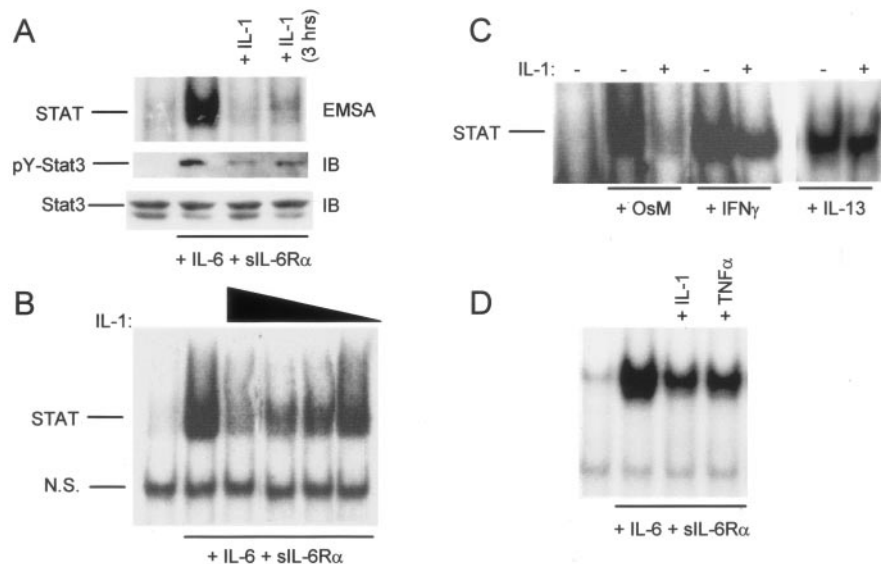
occur at a proximal step in signal transduction, before phosphorylation and activation.

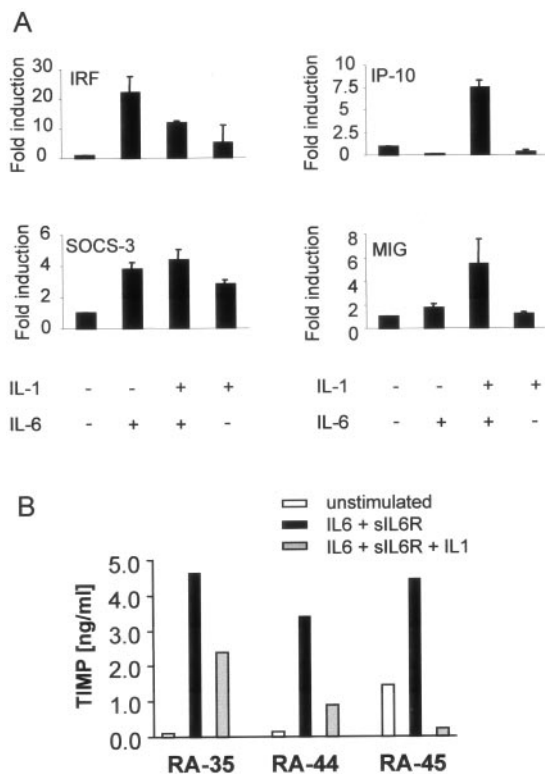
The inhibitory effect of IL-1 on IL-6 signaling was observed at a range of IL-1 concentrations and was dose-dependent (Fig. 3*B*); in some experiments >50% inhibition of IL-6 signaling was ob-

served using doses of IL-1 as low as 100 pg/ml (data not shown). Similar to inhibition of IL-6, IL-1 treatment inhibited activation of STATs by OsM and LIF (Fig. 3*C* and data not shown), indicating that IL-1 blocks signaling by members of the IL-6 family of cytokines that share similar receptors (40) and have anti-inflammatory effects on synovitis. In contrast, IL-1 had only a weak and partial inhibitory effect on activation of Stat1 by IFN- $\gamma$  (Fig. 3*C*, lanes 4 and 5); in two additional experiments no effect of IL-1 on IFN- $\gamma$  signaling was detected (data not shown). IL-1 did not inhibit activation of Stat6 by IL-13 to any appreciable extent (Fig. 3*C*). These results demonstrate that Jak-STAT signaling was not globally blocked by IL-1. Treatment of SFs with TNF- $\alpha$ , which activates similar signaling pathways to those activated by IL-1, also inhibited IL-6-induced STAT activation (Fig. 3*D*). These results, taken together, indicate that proinflammatory cytokines present during RA synovitis inhibit Jak-STAT signaling by pleiotropic cytokines of the IL-6 family more strongly than signaling by inflammatory cytokines such as IFN- $\gamma$ .

Both synergistic and antagonistic interactions of IL-1 and TNF with cytokines of the IL-6 family have been described. IL-1 and IL-6 work together in the induction of type I acute phase protein genes (21), metalloproteases (45), and HIV expression (46). In contrast, IL-1 and TNF can block other IL-6 responses, such as induction of type II acute phase response genes thiostatin and fibrinogen (21, 47) and IL-6-induced proliferation of thymocytes (48). Our results suggested that IL-1 may also block IL-6 responses in RA SFs, especially those that are dependent on the Jak-STAT signaling pathway. The consequences of IL-1 pretreatment on IL-6 induction of gene expression were investigated. Surprisingly, genes that were induced by IL-6 were also induced by IL-1, and analysis of the interaction of IL-1 and IL-6 was difficult secondary to the limitations of the semiquantitative nature of the RT-PCR assay used. Therefore, real-time quantitative PCR was used to accurately measure expression levels of four genes, IRF-1, SOCS3, IP-10, and MIG (Fig. 4*A*; one representative experiment of four performed is shown). IRF-1 mRNA levels were strongly induced by IL-6 and, to a lesser extent, by IL-1. Interestingly, preincubation with IL-1 effectively blocked any additional induction of IRF-1 expression by IL-6 (Fig. 4*A*). Because induction of IRF-1 by type I cytokines and IFNs is entirely dependent upon the Jak-STAT signaling pathway, this result demonstrates that blocking IL-6 signaling via STATs results in an effective block of IL-6

**FIGURE 3.** Pretreatment with IL-1 suppresses STAT activation by IL-6 and related cytokines in RA SFs. *A*, IL-1 (100 ng/ml) was added 20 min or 3 h before addition of IL-6 plus sIL-6R $\alpha$ , and the same cell extracts were analyzed using EMSA, or by immunoblotting, as previously described (57). *B*, Progressively lower doses of IL-1 were added 20 min before adding IL-6 plus sIL-6R $\alpha$  (10 ng/ml, 5 ng/ml, 2 ng/ml, and 1 ng/ml IL-1). *C*, OsM (100 ng/ml), IFN- $\gamma$  (100 U/ml = 8 ng/ml), and IL-13 (100 ng/ml) were used at saturating doses. *D*, IL-1 (10 ng/ml) and TNF- $\alpha$  (20 ng/ml) were added 20 min before adding IL-6 plus sIL-6R $\alpha$ .





**FIGURE 4.** Interaction between IL-1 and IL-6 in gene activation in RA SFs. *A*, After pretreatment with IL-1, IL-6 and sIL-6R $\alpha$  were added for 3 h, and mRNA levels were determined using quantitative real-time PCR, as described in *Materials and Methods*. mRNA levels were corrected for relative levels of GAPDH expression. *B*, TIMP-1 levels in culture supernatants 96 h after the addition of IL-1 and IL-6 plus sIL-6R $\alpha$  were determined in duplicate using ELISA. Cell viability was not affected by addition of cytokines.

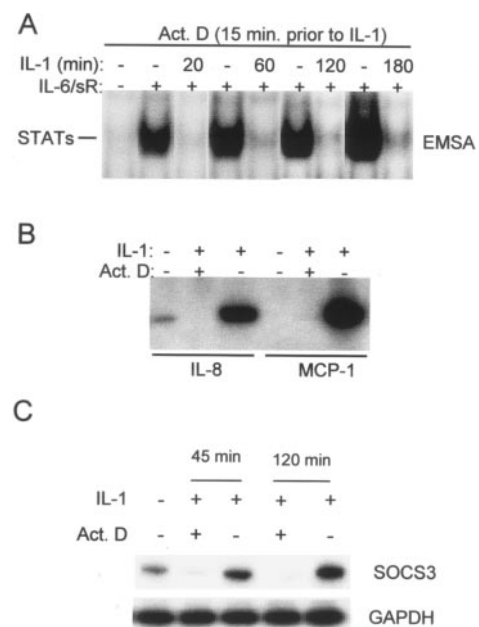
activation of a STAT-dependent gene. Although IL-1 itself activated IRF-1 expression, it also limited the levels to which IRF-1 expression was induced, and the potential physiological significance of this pattern of regulation is addressed in *Discussion*. IL-1 induced expression of SOCS3 mRNA, consistent with previous reports (49–51), and also blunted the induction of SOCS3 expression by IL-6 (the effects of IL-6 and IL-1 are clearly not additive, Fig. 4A). In contrast to IRF-1 and SOCS3, IL-1 and IL-6 synergized in the activation of the IP-10 and MIG genes (Fig. 4A; this effect was reproducible in four independent experiments, and a representative experiment is shown). These results demonstrate a functional correlate to inhibition of IL-6 activation of STATs and suggest that IL-1 modulates IL-6 action in a complex fashion.

Modest alterations in early cellular responses are often amplified in ensuing cascades of signal transduction and expression of regulatory molecules and transcription factors. Therefore, we tested whether IL-1 may have a more pronounced effect on IL-6 induction of TIMP-1, an effect that is not observed until ~4 days after addition of IL-6 and sIL-6R $\alpha$  (28). Basal levels of TIMP-1 production varied among different synovial specimens and, consistent with a previous report (28), addition of IL-6 plus sIL-6R $\alpha$  resulted in induction of TIMP-1 expression that was detected after 96 h (Fig. 4B). Induction of TIMP-1 expression by IL-6 plus sIL-6R was effectively blocked by the addition of IL-1 (Fig. 4B). Fig. 4B shows results obtained using SFs from three different RA patients; an additional three experiments yielded similar results. Although it is unlikely that Stat3 directly regulates the TIMP-1 promoter, these results show the following: 1) IL-1 can effectively inhibit IL-6

effects upon RA SF phenotype; 2) IL-1 suppresses IL-6 induction of a molecule, TIMP-1, that blocks IL-1-induced collagenolytic activity; thus, IL-1 prevents IL-6-mediated feedback inhibition of IL-1-induced collagenolytic activity; and 3) IL-1 blocks an IL-6 effect on RA SFs that is clearly anti-inflammatory in terms of suppressing tissue destruction.

*IL-6 signaling is blocked by a mechanism that does not require new RNA synthesis, is not dependent on a tyrosine phosphatase, but is dependent on MAPKs*

One widely studied mechanism of inhibition of Jak-STAT signaling is mediated through inhibitory proteins termed SOCS, JAB, SSI, or CIS proteins (referred to herein as SOCS proteins) that likely act, at least in part, by binding to and inactivating Jaks (52–55). Inhibition by SOCS proteins depends upon de novo RNA and protein synthesis, and thus is unlikely to explain inhibition of IL-6 signaling observed at early time points, such as 20 min after addition of IL-1, but could potentially contribute to inhibition observed at later time points (Fig. 3A). The dependence of IL-1-mediated inhibition on de novo RNA and protein synthesis was examined. Inhibition of protein synthesis by cycloheximide resulted in a rapid loss of IL-6 signaling (within 1 h), possibly explained by loss of IL-6R expression, as previously reported by others (56). The effect of actinomycin D, an inhibitor of RNA synthesis and de novo gene expression, was determined. As predicted, actinomycin D treatment did not affect IL-1 inhibition of IL-6 signaling when IL-1 was added 20 min before adding IL-6 (Fig. 5A, lanes 2 and 3). Surprisingly, blocking de novo RNA synthesis using actinomycin D did not affect IL-1 inhibition of IL-6-induced STAT activation when cells were preincubated with IL-1 for longer periods, including 3 h (Fig. 5A, lanes 4–9). The efficacy of actinomycin D in blocking RNA synthesis was confirmed in duplicate wells to those used to make cell extracts (Fig. 5B). Because IL-1 induction of SOCS3 expression may contribute to inhibition of IL-6 signaling, we were interested in assessing the



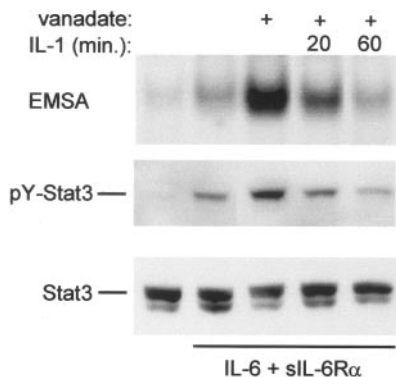
**FIGURE 5.** Inhibition of IL-6 signaling by IL-1 does not depend upon de novo gene activation. *A*, Actinomycin D (Act. D; 5  $\mu$ g/ml) was added 15 min before IL-1. The time of incubation with IL-1 before the addition of IL-6 plus sIL-6R $\alpha$  is indicated. *B*, mRNA levels were analyzed in parallel wells to those used in *A*. IL-1 treatment was for 3 h. *C*, mRNA levels were analyzed after 45 or 120 min of culture with actinomycin D and IL-1.

effects of actinomycin D treatment on SOCS3 mRNA levels. Actinomycin D not only completely blocked induction of SOCS3 mRNA levels by IL-1, but, consistent with previous reports showing that SOCS3 mRNA is highly unstable, SOCS3 mRNA levels decayed rapidly in actinomycin D-treated cells, even when IL-1 was added (Fig. 5C); similar results were obtained in the absence of IL-1 (data not shown). Although SOCS3 may regulate IL-6 signaling in the basal state and may contribute to IL-1-mediated inhibition of IL-6 signaling, this result suggests that IL-1 also triggers an inhibitory pathway that is independent of SOCS3.

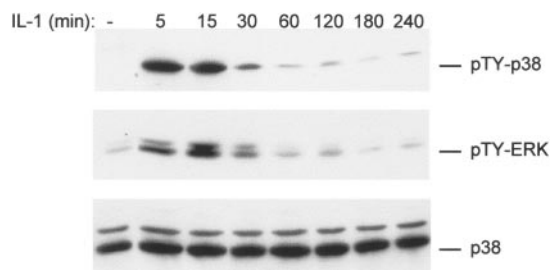
Constitutively active tyrosine phosphatases play a role in determining the basal rate of deactivation of Jak-STAT signaling and thus the kinetics of decay of STAT activity toward baseline. Although to date it appears that the activity of these phosphatases is not regulated, we considered the possibility that IL-1 may inhibit IL-6 signaling by activating a tyrosine phosphatase. As predicted, addition of vanadate, a tyrosine phosphatase inhibitor, potentiated Stat3 activation by IL-6 (Fig. 6, lanes 2 and 3). IL-1 effectively blocked IL-6-induced STAT activation even in the presence of vanadate (Fig. 6, lanes 3–5). These results indicate that inhibition of IL-6 signaling by IL-1 is not mediated by a tyrosine phosphatase.

One major signaling pathway triggered by IL-1 leads to activation of mitogen-activated protein kinases (MAPKs), which consist of three major subfamilies of kinases, termed ERKs, Jun N-terminal kinases, and p38 kinases. These kinases are active in RA synovium, and we have previously described inhibition of IL-6 signaling that is dependent upon ERKs or p38 when these kinases are activated by expression of constitutively active kinases in cell lines (49, 57). Therefore, the role of ERKs and p38 in mediating IL-1 inhibition of IL-6 signaling in SFs was investigated. Consistent with previous reports (58, 59), IL-1 activated both ERKs and p38 in SFs (Fig. 7). The time course of p38 and ERK activation was typical in that peak levels of activated kinases were detected 5–15 min after addition of IL-1, and decayed to near baseline over the next 3 h.

The effects of inhibiting ERK and p38 activation, using PD98059 (a specific inhibitor of MAP/ERK kinase, the kinase that activates ERKs) and SB203580 (a specific inhibitor, at the doses used, of p38), on IL-1 inhibition of IL-6 signaling were determined. The first set of experiments was conducted using RA SFs that were treated with IL-1 for 20 min before adding IL-6, such that IL-6 was added when p38 and ERKs were highly active (see Fig. 7). Inhibition of p38 resulted in near complete reversal of IL-1-mediated inhibition (Fig. 8A, lanes 3 and 4), whereas inhibi-

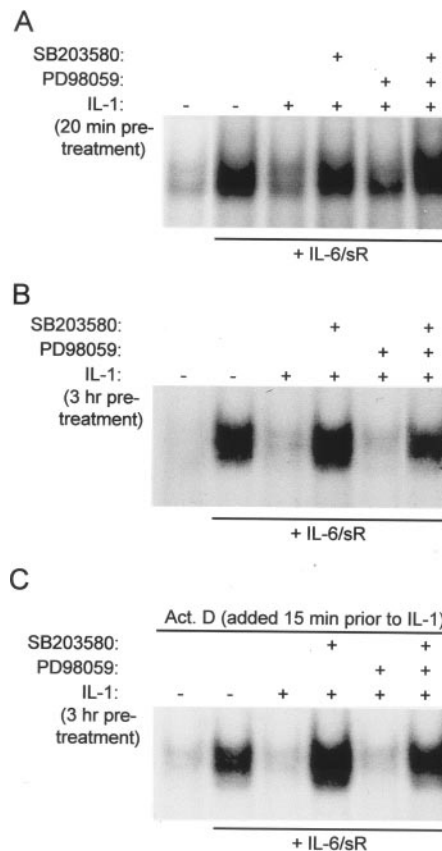


**FIGURE 6.** Inhibition of IL-6 signaling by IL-1 is not dependent upon a protein tyrosine phosphatase. IL-1 was added 20 or 60 min, and sodium orthovanadate (5 mM) was added 5 min, before the addition of IL-6 plus sIL-6R $\alpha$ . The same extracts were analyzed using EMSA and immunoblotting.



**FIGURE 7.** IL-1 activates the p38 and ERK MAPKs in RA SFs. Cell extracts were analyzed with sequential immunoblotting of the same filter.

tion of ERKs had only a small effect (Fig. 8A, lane 5). Essentially complete inhibition of ERK activation by PD98059 was verified in the same experiment (data not shown). These results suggest that rapid inhibition of IL-6 signaling by IL-1 is mediated by MAPKs that are active when IL-6 is added to cells, and that p38 plays a more important role than the ERKs in RA SFs. The effects of inhibiting p38 and ERKs on IL-6 signaling were also determined when IL-1 was added 3 h before addition of IL-6; at this time point, p38 and ERK activity had decayed close to baseline (see Fig. 7). Interestingly, inhibition of p38 resulted in complete reversal of the IL-1 effect, whereas inhibition of ERKs had a minimal effect (Fig. 8B). SB203580 has been shown to partially suppress induction of SOCS3 expression by TNF or CpG-DNA (51, 60). Therefore, these experiments were also conducted in the presence



**FIGURE 8.** Inhibition of IL-6 signaling by IL-1 is dependent upon p38 to a greater extent than on ERKs. *A* and *B*, SB203580 (5  $\mu$ M), an inhibitor of p38 activation, and PD98059 (50  $\mu$ M), an inhibitor of MAP/ERK kinase and ERK activation, were added 15 min before addition of IL-1. Similar results were obtained when 2  $\mu$ M or 1  $\mu$ M of SB203580 was used. *C*, Actinomycin D (Act. D) (5  $\mu$ g/ml) was added 15 min before adding IL-1.

of actinomycin D, and similar results were obtained (Fig. 8C), suggesting that reversal of inhibition by SB203580 is not mediated by an effect on SOCS3 expression. Taken together, these results indicate that MAPKs, predominantly p38, play an important role in IL-1 inhibition of IL-6 signaling at both early and late time points after addition of IL-1, and that induction of gene expression and de novo RNA synthesis are not required for inhibition of IL-6 activation of STATs.

## Discussion

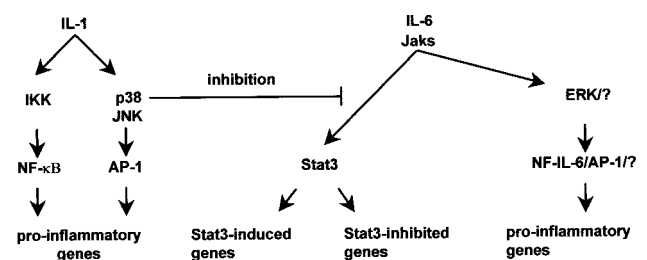
Data from several laboratories, including ours, have demonstrated that proinflammatory cytokines can suppress the activity of anti-inflammatory cytokines at the level of signal transduction (34, 35, 49). Many of these inhibitory effects on signal transduction are cell-type specific, and thus we have begun to investigate the potential role of modulation/inhibition of signal transduction in RA SFs, cells that are important for the pathogenesis of RA. The evidence presented herein demonstrates that major RA inflammatory cytokines, IL-1 and TNF, modulate activation of the Jak-STAT signaling pathway by the pleiotropic cytokines related to IL-6 that use the gp130 signaling receptor subunit. Although the need to work with human RA specimens has limited these experiments to *in vitro* observations, we believe that these results are relevant to RA pathogenesis because IL-1, IL-6, and sIL-6R $\alpha$  are expressed in RA joints at levels comparable to those used herein, and the p38 kinase is active during RA synovitis and has been proposed to represent a good therapeutic target. These results advance the concept of cytokine balance in RA to the level of signal transduction and suggest that expression levels of cytokines are not the only factor that regulates cytokine balance during RA. Manipulation of cytokine signaling to block the proinflammatory actions of pleiotropic cytokines or to potentiate the actions of anti-inflammatory cytokines that are already present in RA synovium may represent a promising novel therapeutic approach to RA.

Our studies focused upon IL-6, a pleiotropic cytokine that has pro- and anti-inflammatory effects on different cell types, but overall likely plays a suppressive role *vis a vis* SFs (27, 28, 31). These studies could not be extended to the anti-inflammatory cytokine IL-10, as activation of Jak-STAT signaling by IL-10 in SFs was not detected (D. Deon, unpublished results). IL-6 expression is induced by IL-1 and these two cytokines are coexpressed at many sites of inflammation. Our analysis of the effects of IL-1 on IL-6 action and IL-6-induced gene expression in RA synoviocytes revealed that these cytokines interact in a complex fashion. Several genes that were induced by IL-6 were also induced by IL-1, and, in the case of IRF-1 and SOCS3, IL-1 suppressed the further induction of gene expression by IL-6 (Fig. 4A). These results demonstrate that a block in signal transduction was effectively translated into a suppression of gene activation. Because IRF-1 and SOCS3 genes are regulated by STATs, our results identify inhibition of STAT activation by IL-1 as one molecular mechanism underlying suppression of IL-6-induced gene expression by IL-1. The pattern of regulation of IRF-1 and SOCS3 gene expression suggests a negative feedback loop, whereby IL-1 activates expression of IL-6 and at the same time limits the ability of IL-6 to further increase expression levels of these genes. Because IRF-1 and SOCS3 regulate cellular responses to cytokines and IFNs, we propose that tight regulation of these genes is important for RA synoviocyte physiology, but the exact role of these genes in RA inflammation remains to be elucidated.

IL-6 is a pleiotropic cytokine that activates expression of both pro- and anti-inflammatory genes. One may propose two mechanisms by which IL-1 modulation of IL-6 activity may contribute to RA pathogenesis: 1) IL-1-mediated suppression of the IL-6 induc-

tion of anti-inflammatory genes; and 2) lack of suppression or superinduction of IL-6 proinflammatory genes by IL-1. Our results identify an example of both of these mechanisms, namely suppression of IL-6 induction of TIMP-1 expression (thus suppressing the ability of IL-6 to limit tissue damage by metalloproteases), and synergistic activation of IP-10 and MIG expression (thus potentiating recruitment of inflammatory cells). These results suggest that IL-1 modulation of IL-6 signaling may contribute to RA pathogenesis.

One interesting aspect of our results is that inhibition of IL-6 STAT activation by IL-1 had opposite effects on IL-6 induction of different genes. These results are consistent with previous reports demonstrating a complex interplay between IL-1 and IL-6, including mutual antagonism as well as synergy, even in the same cell type (11, 13, 15, 19, 21, 25, 45–48). For example, IL-1 and IL-6 coactivate expression of type I acute phase response genes, but IL-1 blocks IL-6 induction of type II acute phase response genes (thiostatin, fibrinogen) in hepatocytes (21). Divergent effects of inhibition of STATs on expression of different genes is consistent with data that suggest that STATs can function as either activators or inhibitors of transcription of different genes (61, 62). An additional possible molecular basis for divergent patterns of gene expression is that IL-6 activates signaling pathways in addition to the Jak-STAT pathway (40) that may work together with IL-1 signaling pathways. These possibilities are detailed in Fig. 9. It would be of interest to test whether p38 plays a role in mediating the effects of IL-1 on expression of IL-6-inducible genes, similar to its role in modulation of IL-6 signaling. However, SB203580 alone blocked the induction of gene expression by IL-6, thus precluding this analysis (N. Scaletta, unpublished data). These results are in accord with previous descriptions of a block of Jak-STAT-inducible transcription by inhibitors of p38, which may work by disrupting interactions of STATs with transcriptional coactivators, by acting directly on STATs themselves, or by acting on as yet unknown proteins (63, 64). A more complete understanding of the consequences of IL-1 and IL-6 signal transduction cross-talk on RA synoviocyte phenotype will require delineation of the roles of Stat1 and Stat3 in RA synoviocytes, and these experiments are in progress. In contrast to synoviocytes, IL-1 and TNF did not affect IL-6 signaling in B or T cells (S. Ahmed, unpublished data), and therefore would not suppress the proimmune functions of IL-6. Thus, the complex interplay between IL-1 and IL-6 signaling also varies according to cell type, which has important implications for the ability of these two cytokines to act together, or in opposition, in an inflammatory process.



**FIGURE 9.** Model for the complex interactions of IL-1 and IL-6. IL-1 blocks Stat3 activation and thus inhibits expression of genes that are strongly dependent on Stat3 for expression. In contrast, genes that are repressed by Stat3 would be expressed to a higher level in the presence of IL-1. In addition, IL-6 signaling pathways other than the STAT pathway may not be suppressed by p38 and may work together with IL-1 signaling pathways to coactivate certain genes.



It is becoming apparent that multiple mechanisms exist for down-modulating cytokine signaling by the Jak-STAT pathway. Constitutively active inhibitory mechanisms include dephosphorylation of Jaks or STATs by tyrosine phosphatases, inhibition of DNA binding by protein inhibitor of activated Stat proteins, and, under certain circumstances, degradation of STATs by proteolysis (65). An important inducible mechanism for blocking cytokine signaling works via the induction of expression of SOCS/JAB/SSI/CIS proteins that contain SH2 domains and have been proposed to inhibit signaling by binding to Jaks or cytokine receptors and blocking function (52–55, 65). In contrast to inhibition mediated by induction of SOCS expression, several laboratories, including ours, have demonstrated rapid inhibition of Jak-STAT signaling that does not require de novo RNA or protein synthesis and presumably acts by direct modification of pre-existing signaling components (36, 66–68). In one system, inhibition of IL-6 signaling is dependent on MAPKs (57), and transfections of cell lines with constitutively active kinases have shown that either the ERK or p38 families of MAPKs (49, 57) may play an important role in inhibition. The results presented herein extend this work to a physiologic system relevant to RA pathogenesis and show that in IL-1-stimulated RA SFs p38 plays a more important role in inhibition of IL-6 signaling than do the ERKs. Experiments using actinomycin D to block de novo gene induction, including SOCS3, showed that IL-6 signaling was strongly inhibited even in the absence of induction of SOCS3 expression. However, we would like to emphasize that, similar to other cell types, IL-1 activates SOCS3 expression in RA synoviocytes, and that SOCS3 likely contributes to inhibition of IL-6 signaling in the experiments in which actinomycin D was not used. Thus, it appears that IL-1 induces at least two mechanisms of inhibition of IL-6 signaling, which allows for strong inhibition over an extended time frame. These experiments also reveal two novel aspects of the mechanism of inhibition: the lack of dependence upon induction of tyrosine phosphatase expression or activity and the persistence of inhibition over time, even when new gene expression has been blocked and p38 activity has returned to baseline. This latter observation suggests that p38 acts either by irreversibly deactivating a component of the IL-6 signaling pathway or by activating an enzyme that produces an inhibitor of Jak-STAT signaling. Experiments using pharmacological inhibitors have excluded p38-dependent activation of PLA<sub>2</sub> and cyclooxygenase-2 (69) as the mechanism of action (D. Deon, unpublished data). Additional experiments will be required to identify the molecular target of p38 inhibitory action on IL-6 signaling.

## Acknowledgments

We thank Peggy Crow and Luminita Pricop for critical review of the manuscript and the staff of the Hospital for Special Surgery (New York, NY) for providing synovial specimens. We thank Dr. J.-M. Dayer for suggesting the TIMP-1 experiments.

## References

- Ivashkiv, L. B. 1996. Cytokine expression and cell activation in inflammatory arthritis. *Adv. Immunol.* 63:337.
- Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* 14:397.
- Arend, W. P., and J. M. Dayer. 1995. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor  $\alpha$  in rheumatoid arthritis. *Arthritis Rheum.* 38:151.
- Firestein, G. S., and N. J. Zvaifler. 1997. Anticytokine therapy in rheumatoid arthritis. *N. Engl. J. Med.* 337:195.
- Koopman, W. J., and L. W. Moreland. 1998. Rheumatoid arthritis: anticytokine therapies on the horizon. *Ann. Intern. Med.* 128:231.
- Elliott, M. J., R. N. Maini, M. Feldmann, J. R. Kalden, C. Antoni, J. S. Smolen, B. Leeb, F. C. Breedveld, J. D. Macfarlane, H. Bijl, and et al. 1994. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor  $\alpha$  (cA2) versus placebo in rheumatoid arthritis. *Lancet* 344:1105.
- Moreland, L. W., S. W. Baumgartner, M. H. Schiff, E. A. Tindall, R. M. Fleischmann, A. L. Weaver, R. E. Ettlinger, S. Cohen, W. J. Koopman, K. Mohler, et al. 1997. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N. Engl. J. Med.* 337:141.
- Jiang, Y., H. K. Genant, I. Watt, M. Cobby, B. Bresnihan, R. Aitchison, and D. McCabe. 2000. A multicenter, double-blind, dose-ranging, randomized, placebo-controlled study of recombinant human interleukin-1 receptor antagonist in patients with rheumatoid arthritis: radiologic progression and correlation of Genant and Larsen scores. *Arthritis Rheum.* 43:1001.
- Hermann, J. A., M. A. Hall, R. N. Maini, M. Feldmann, and F. M. Brennan. 1998. Important immunoregulatory role of interleukin-11 in the inflammatory process in rheumatoid arthritis. *Arthritis Rheum.* 41:1388.
- Leng, S. X., and J. A. Elias. 1997. IL-11 inhibits macrophage IL-12 production. *J. Immunol.* 159:2161.
- Richards, C. D., C. Langdon, F. Botelho, T. J. Brown, and A. Agro. 1996. Oncostatin M inhibits IL-1-induced expression of IL-8 and granulocyte-macrophage colony-stimulating factor by synovial and lung fibroblasts. *J. Immunol.* 156:343.
- Trepicchio, W. L., M. Bozza, G. Pedneault, and A. J. Dorner. 1996. Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. *J. Immunol.* 157:3627.
- Trepicchio, W. L., and A. J. Dorner. 1998. Interleukin-11: a gp130 cytokine. *Ann. NY Acad. Sci.* 856:12.
- Walmsley, M., D. M. Butler, L. Marinova-Mutafchieva, and M. Feldmann. 1998. An anti-inflammatory role for interleukin-11 in established murine collagen-induced arthritis. *Immunology* 95:31.
- Trepicchio, W. L., M. Ozawa, I. B. Walters, T. Kikuchi, P. Gilleaudeau, J. L. Bliss, U. Schwertschlag, A. J. Dorner, and J. G. Krueger. 1999. Interleukin-11 therapy selectively downregulates type I cytokine proinflammatory pathways in psoriasis lesions. *J. Clin. Invest.* 104:1527.
- Alonzi, T., E. Fattori, D. Lazzaro, P. Costa, L. Probert, G. Kollias, F. De Benedetti, V. Poli, and G. Ciliberto. 1998. Interleukin 6 is required for the development of collagen-induced arthritis. *J. Exp. Med.* 187:461.
- Sasai, M., Y. Saeki, S. Ohshima, K. Nishioka, T. Mima, T. Tanaka, Y. Katada, K. Yoshizaki, M. Suemura, and T. Kishimoto. 1999. Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice. *Arthritis Rheum.* 42:1635.
- Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, et al. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* 6:315.
- Oh, J. W., N. J. Van Wagoner, S. Rose-John, and E. N. Benveniste. 1998. Role of IL-6 and the soluble IL-6 receptor in inhibition of VCAM-1 gene expression. *J. Immunol.* 161:4992.
- La Flamme, A. C., A. S. MacDonald, and E. J. Pearce. 2000. Role of IL-6 in directing the initial immune response to schistosome eggs. *J. Immunol.* 164:2419.
- Baumann, H., K. R. Prowse, S. Marinkovic, K. A. Won, and G. P. Jahreis. 1989. Stimulation of hepatic acute phase response by cytokines and glucocorticoids. *Ann. NY Acad. Sci.* 557:280.
- Papanicolaou, D. A., R. L. Wilder, S. C. Manolagas, and G. P. Chrousos. 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med.* 128:127.
- Jin, F., C. F. Nathan, D. Radzioch, and A. Ding. 1998. Lipopolysaccharide-related stimuli induce expression of the secretory leukocyte protease inhibitor, a macrophage-derived lipopolysaccharide inhibitor. *Infect. Immun.* 66:2447.
- Tilg, H., E. Trehu, M. B. Atkins, C. A. Dinarello, and J. W. Mier. 1994. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 83:113.
- Shingu, M., T. Isayama, C. Yasutake, T. Naono, M. Nobunaga, K. Tomari, K. Horie, and Y. Goto. 1994. Role of oxygen radicals and IL-6 in IL-1-dependent cartilage matrix degradation. *Inflammation* 18:613.
- Aderka, D., J. M. Le, and J. Vilcek. 1989. IL-6 inhibits lipopolysaccharide-induced TNF production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* 143:3517.
- Nishimoto, N., A. Ito, M. Ono, H. Tagoh, T. Matsumoto, T. Tomita, T. Ochi, and K. Yoshizaki. 2000. IL-6 inhibits the proliferation of fibroblastic synovial cells from rheumatoid arthritis patients in the presence of soluble IL-6 receptor. *Int. Immunol.* 12:187.
- Silacci, P., J. M. Dayer, A. Desgeorges, R. Peter, C. Manueddu, and P. A. Guerne. 1998. Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *J. Biol. Chem.* 273:13625.
- Xing, Z., J. Gauldie, G. Cox, H. Baumann, M. Jordana, X. F. Lei, and M. K. Achong. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.* 101:311.
- van de Loo, F. A., S. Kuiper, F. H. van Enckevort, O. J. Arntz, and W. B. van den Berg. 1997. Interleukin-6 reduces cartilage destruction during experimental arthritis: a study in interleukin-6-deficient mice. *Am. J. Pathol.* 151:177.
- Ernst, M., M. Inglese, P. Waring, I. K. Campbell, S. Bao, F. J. Clay, W. S. Alexander, I. P. Wicks, D. M. Tarlinton, U. Novak, et al. 2001. Defective gp130-mediated signal transducer and activator of transcription (STAT) signaling results in degenerative joint disease, gastrointestinal ulceration, and failure of uterine implantation. *J. Exp. Med.* 194:189.
- Ito, S., P. Ansari, M. Sakatsume, H. Dickensheets, N. Vazquez, R. P. Donnelly, A. C. Larner, and D. S. Finbloom. 1999. Interleukin-10 inhibits expression of

- both interferon  $\alpha$ - and interferon  $\gamma$ -induced genes by suppressing tyrosine phosphorylation of STAT1. *Blood* 93:1456.
33. Trepicchio, W. L., L. Wang, M. Bozza, and A. J. Dorner. 1997. IL-11 regulates macrophage effector function through the inhibition of NF- $\kappa$ B. *J. Immunol.* 159:5661.
  34. Ulloa, L., J. Doody, and J. Massague. 1999. Inhibition of transforming growth factor- $\beta$ /SMAD signalling by the interferon- $\gamma$ /STAT pathway. *Nature* 397:710.
  35. Bitzer, M., G. von Gersdorff, D. Liang, A. Dominguez-Rosales, A. A. Beg, M. Rojkind, and E. P. Bottinger. 2000. A mechanism of suppression of TGF- $\beta$ /SMAD signaling by NF- $\kappa$ B/RelA. *Genes Dev.* 14:187.
  36. Lee, I. H., W. P. Li, K. B. Hisert, and L. B. Ivashkiv. 1999. Inhibition of interleukin 2 signaling and signal transducer and activator of transcription (STAT)5 activation during T cell receptor-mediated feedback inhibition of T cell expansion. *J. Exp. Med.* 190:1263.
  37. Sengupta, T. K., A. Chen, Z. Zhong, J. E. Darnell, Jr., and L. B. Ivashkiv. 1995. Activation of monocyte effector genes and STAT family transcription factors by inflammatory synovial fluid is independent of interferon  $\gamma$ . *J. Exp. Med.* 181:1015.
  38. Sengupta, T. K., E. M. Schmitt, and L. B. Ivashkiv. 1996. Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. *Proc. Natl. Acad. Sci. USA* 93:9499.
  39. Castro, A., T. K. Sengupta, D. C. Ruiz, E. Yang, and L. B. Ivashkiv. 1999. IL-4 selectively inhibits IL-2-triggered Stat5 activation, but not proliferation, in human T cells. *J. Immunol.* 162:1261.
  40. Kishimoto, T., T. Taga, and S. Akira. 1994. Cytokine signal transduction. *Cell* 76:253.
  41. Snyers, L., L. De Wit, and J. Content. 1990. Glucocorticoid up-regulation of high-affinity interleukin 6 receptors on human epithelial cells. *Proc. Natl. Acad. Sci. USA* 87:2838.
  42. Muller-Ladner, U., C. H. Evans, B. N. Franklin, C. R. Roberts, R. E. Gay, P. D. Robbins, and S. Gay. 1999. Gene transfer of cytokine inhibitors into human synovial fibroblasts in the SCID mouse model. *Arthritis Rheum.* 42:490.
  43. Jorgensen, C., F. Apparailly, F. Canovas, C. Verwaerde, C. Auriault, C. Jacquet, and J. Sany. 1999. Systemic viral interleukin-10 gene delivery prevents cartilage invasion by human rheumatoid synovial tissue engrafted in SCID mice. *Arthritis Rheum.* 42:678.
  44. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415.
  45. Ito, A., Y. Itoh, Y. Sasaguri, M. Morimatsu, and Y. Mori. 1992. Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts. *Arthritis Rheum.* 35:1197.
  46. Poli, G., A. L. Kinter, and A. S. Fauci. 1994. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc. Natl. Acad. Sci. USA* 91:108.
  47. Mackiewicz, A., T. Speroff, M. K. Ganapathi, and I. Kushner. 1991. Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines. *J. Immunol.* 146:3032.
  48. Hernandez-Caselles, T., and O. Stutman. 1993. Immune functions of TNF. I. TNF induces apoptosis of mouse thymocytes and can also stimulate or inhibit IL-6-induced proliferation depending on the concentration of mitogenic costimulation. *J. Immunol.* 151:3999.
  49. Ahmed, S., and L. B. Ivashkiv. 2000. Inhibition of IL-6 and IL-10 signaling and STAT activation by inflammatory and stress pathways. *J. Immunol.* 165:5227.
  50. Boisclair, Y. R., J. Wang, J. Shi, K. R. Hurst, and G. T. Ooi. 2000. Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1 $\beta$  on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. *J. Biol. Chem.* 275:3841.
  51. Bode, J. G., A. Nimmesgern, J. Schmitz, F. Schaper, M. Schmitt, W. Frisch, D. Haussinger, P. C. Heinrich, and L. Graeve. 1999. LPS and TNF $\alpha$  induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett.* 463:365.
  52. Yoshimura, A., T. Ohkubo, T. Kiguchi, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Hara, and A. Miyajima. 1995. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* 14:2816.
  53. Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature* 387:917.
  54. Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, et al. 1997. Structure and function of a new STAT-induced STAT inhibitor. *Nature* 387:924.
  55. Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, et al. 1997. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 387:921.
  56. Kim, H., T. S. Hawley, R. G. Hawley, and H. Baumann. 1998. Protein tyrosine phosphatase 2 (SHP-2) moderates signaling by gp130 but is not required for the induction of acute-phase plasma protein genes in hepatic cells. *Mol. Cell. Biol.* 18:1525.
  57. Sengupta, T. K., E. S. Talbot, P. A. Scherle, and L. B. Ivashkiv. 1998. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. USA* 95:11107.
  58. Han, Z., D. L. Boyle, K. R. Aupperle, B. Bennett, A. M. Manning, and G. S. Firestein. 1999. Jun N-terminal kinase in rheumatoid arthritis. *J. Pharmacol. Exp. Ther.* 291:124.
  59. Miyazawa, K., A. Mori, H. Miyata, M. Akahane, Y. Ajisawa, and H. Okudaira. 1998. Regulation of interleukin-1 $\beta$ -induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J. Biol. Chem.* 273:24832.
  60. Dalpke, A. H., S. Opper, S. Zimmermann, and K. Heeg. 2001. Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J. Immunol.* 166:7082.
  61. Darnell, J. E., Jr. 1997. STATs and gene regulation. *Science* 277:1630.
  62. Ramana, C. V., M. Chatterjee-Kishore, H. Nguyen, and G. R. Stark. 2000. Complex roles of Stat1 in regulating gene expression. *Oncogene* 19:2619.
  63. Decker, T., and P. Kovarik. 2000. Serine phosphorylation of STATs. *Oncogene* 19:2628.
  64. Uddin, S., F. Lekmine, N. Sharma, B. Majchrzak, I. Mayer, P. R. Young, G. M. Bokoch, E. N. Fish, and L. C. Platanius. 2000. The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon  $\alpha$ -dependent transcriptional activation but not serine phosphorylation of Stat proteins. *J. Biol. Chem.* 275:27634.
  65. Starr, R., and D. J. Hilton. 1999. Negative regulation of the JAK/STAT pathway. *BioEssays* 21:47.
  66. Zhu, J., H. Huang, L. Guo, T. Stonehouse, C. J. Watson, J. Hu-Li, and W. E. Paul. 2000. Transient inhibition of interleukin 4 signaling by T cell receptor ligation. *J. Exp. Med.* 192:1125.
  67. Shen, X., Z. Tian, M. J. Holtzman, and B. Gao. 2000. Cross-talk between interleukin 1 $\beta$  (IL-1 $\beta$ ) and IL-6 signalling pathways: IL-1 $\beta$  selectively inhibits IL-6-activated signal transducer and activator of transcription factor 1 (STAT1) by a proteasome-dependent mechanism. *Biochem. J.* 352:913.
  68. Kovanen, P. E., I. Junttila, K. Takaluoma, P. Saharinen, L. Valmu, W. Li, and O. Silvennoinen. 2000. Regulation of Jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells. *Blood* 95:1626.
  69. Waterman, W. H., T. F. Molski, C. K. Huang, J. L. Adams, and R. I. Sha'afi. 1996. Tumour necrosis factor- $\alpha$ -induced phosphorylation and activation of cytosolic phospholipase A2 are abrogated by an inhibitor of the p38 mitogen-activated protein kinase cascade in human neutrophils. *Biochem. J.* 319:17.