Inhibition of IL-6 signaling by a p38-dependent pathway occurs in the absence of new protein synthesis

Simi T. Ahmed,* Alexander Mayer,† Jong-Dae Ji,† and Lionel B. Ivashkiv*,†

*Graduate Program in Immunology, Weill Graduate School of Medical Sciences of Cornell University, and †Department of Medicine, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, New York

Abstract: Negative regulation of cytokine signaling is important for limiting the intensity and duration of cytokine action and for maintaining homeostasis. Several constitutive mechanisms for suppressing cytokine Jak-STAT signaling have been described. Inducible or regulated inhibition of cytokine signaling is equally important, and much attention has been focused on inhibition mediated through the induction of expression of suppressors of cytokine signaling (SOCS proteins). We have previously reported IL-1-induced inhibition of IL-6 signaling in monocytes, and herein we use inhibitors of protein synthesis to demonstrate that inhibition of IL-6 signaling can occur in the absence of new protein synthesis. Surprisingly, some protein synthesis inhibitors themselves inhibited IL-6 signaling rapidly, strengthening the conclusion that IL-6 signaling can be inhibited in the absence of protein synthesis. Inhibition of IL-6 signaling by IL-1 and protein synthesis inhibitors was dependent on the p38 stress kinase, and activation of p38 secondary to inducible expression of MKK6 was sufficient to inhibit IL-6 signaling. Inhibition was specific for IL-6, as induction of STAT activation by IFN-γ, IFN-α, and vanadate was not inhibited. IL-1-induced inhibition of IL-6 signaling was not mediated by the activation of tyrosine phosphatases or by p38-dependent activation of phospholipase A_2 or cyclooxygenases, which could lead to indirect inhibition via production of prostaglandins. These results identify an inducible mechanism of inhibition of IL-6 signaling that is direct and independent of induction of negative regulators such as SOCS proteins. A role for p38 in mediating inhibition suggests that multiple cytokines and stress agents that activate p38 pathways in monocytes, such as IL-1, TNF, Toll-like receptors, and Fc receptors, can modulate Jak-STAT signaling by pleiotropic cytokines such as IL-6. *J. Leukoc. Biol.* 72: 154–162; 2002.

Key Words: *cytokines · signal transduction · monocytes/macrophages*

INTRODUCTION

Many, if not most, cytokines important for immune responses use the Jak-STAT (signal transducer and activator of transcription) signaling pathway. Jaks are receptor-associated protein tyrosine kinases, and STATs are latent cytoplasmic transcription factors expressed in the cytoplasm of most cells (reviewed in ref. [1]). Binding of cytokines to their receptors leads to activation of Jaks, phosphorylation of tyrosine motifs in receptor cytoplasmic domains, and recruitment of signaling molecules, including STATs, to the receptor complex. STATs are then tyrosine-phosphorylated rapidly, dimerize, and translocate to the nucleus, where they bind to gene promoters and activate transcription. Tyrosine phosphorylation of STATs is essential for activation, dimerization, and DNA binding. STATs play critical, nonredundant roles in mediating cellular transcriptional responses to cytokines and in cell activation, survival, and proliferation [2].

Ligation of cytokine receptors typically results in a transient activation of Jaks, transient phosphorylation of receptor tyrosine motifs, and transient activation of STATs. Often STAT activity peaks 5–30 min after cytokine stimulation, followed by a decay back to baseline over the subsequent 1–4 h. These observations suggest that mechanisms for down-regulating cytokine Jak-STAT signaling must exist, and recently multiple inhibitory mechanisms have been described. Several inhibitory pathways appear to be constitutively active in cells and are not currently known to be regulated. These include proteolysis of Stat1 and Stat5; dephosphorylation of receptors, Jaks, or STATs; and interaction of STATs with protein inhibitors of activated STATs that block STAT-DNA binding [3–10].

It has become clear that inducible inhibitory mechanisms also exist and that signaling by the Jak-STAT pathway can be suppressed after preincubation with antagonistic cytokines. Several regulated or inducible inhibitory mechanisms have been identified, mediated by down-regulation of receptor expression [11–14], via induction of inhibitory molecules termed suppressor of cytokine signaling (SOCS)/JAB/SSI/CIS proteins [15–20], by rapid mitogen-activated protein kinase (MAPK)- or protein kinase C-dependent modification of pre-existing signaling components [21–26], and by proteosome-dependent degradation of signaling proteins [27]. Much attention has been focused on inhibition mediated by SOCS proteins [15–20, 28],

Correspondence: Dr. Lionel B. Ivashkiv, Department of Medicine, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail: ivashkivl@HSS.edu

Received June 4, 2001; revised December 31, 2001; accepted January 14, 2002.

eight of which contain SH2 domains and thus can potentially interact with phosphotyrosine motifs on cytokine receptors or Jaks. To date, CIS (the original family member to be cloned), SOCS1, and SOCS3 have been shown to inhibit cytokine signaling. SOCS1 and SOCS3 interact with Jaks via an SH2-phosphotyrosine interaction, and SOCS1 is an effective inhibitor of Jak catalytic activity [19]. SOCS3 is much less effective at inhibiting Jaks directly and has been shown recently to interact with tyrosine 759 in the gp130 signaling chain of the interleukin (IL)-6 receptor and that this interaction is necessary for effective inhibition of signaling to occur [29, 30]. CIS interacts with the IL-3 receptor and inhibits signaling, possibly by competing for and blocking phosphotyrosine docking sites important for signal transduction. SOCS proteins are short-lived and are ubiquitinated, and it is possible that they also work by targeting receptors for rapid degradation. Except for a few tissues such as thymus and fetal liver [31, 32], SOCS are not expressed at baseline and are induced rapidly as immediate early genes in response to cytokines that themselves activate the Jak-STAT pathway or inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF) [19, 20, 28]. Thus, SOCS may play an important role in feedback inhibition and in cytokine antagonism.

IL-1 inhibits the expression of a subgroup of IL-6-inducible genes in monocytes and hepatocytes, and this inhibition plays a role in the physiology of macrophage differentiation and the acute phase response [25, 33, 34]. IL-6 is a member of a family of cytokines that share the gp130 signaling receptor chain and activates Stat3 and, in a dose-dependent and tissue-specific manner, Stat1. Several laboratories, including ours, have demonstrated the inhibition of IL-6 activation of STATs by IL-1 [25, 35, 36], thus identifying a molecular basis for the inhibition of IL-6-induced gene expression by IL-1. In hepatocytes, inhibition is selective for Stat1 and may be mediated by a tyrosine phosphatase [36]. In myeloid cells, there is agreement that inhibition of IL-6 signaling by IL-1 is mediated by the p38 group of MAPKs [25, 35]. Graeve and colleagues [35] have demonstrated that IL-1 activates SOCS3 expression in a p38-dependent manner and that inhibition of p38 blocks SOCS3 expression and reverses inhibition of IL-6 signaling. Taken together with the demonstration that overexpressed SOCS3 binds to gp130 and inhibits IL-6 signaling [29, 30], these findings have led to the proposal that IL-1 inhibition of IL-6 is dependent on SOCS3. In contrast, our group has found that IL-1 inhibits IL-6 signaling by a p38-dependent mechanism even when SOCS3 is not expressed and when de novo gene expression is blocked by the use of actinomycin D (Act. D) [25]. This has led to the proposal of an alternative mechanism by which p38 inhibits IL-6 signaling independent of new gene expression. Additional support for rapid and direct inhibitory pathways of inhibition of Jak-STAT signaling is provided by a study demonstrating that IL-1-mediated inhibition of interferon (IFN)-α signaling in hepatocytes does not depend on de novo gene expression [27]. Our previous results did not exclude the possibility that IL-1 inhibits IL-6 signaling indirectly via induction of p38-dependent increases in stability or translation of constitutively expressed mRNAs (see **Fig. 1**). We now address the important issue of resolving whether IL-1 inhibition of IL-6 signaling is direct or occurs via induction of inhibitory molecules.

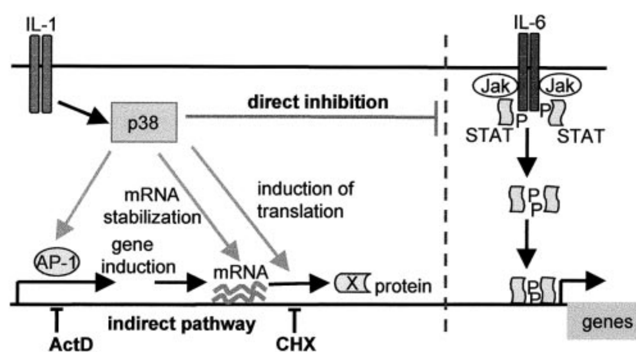

Fig. 1. Direct and indirect mechanisms of p38-dependent inhibition of IL-6 signaling. p38 may act indirectly by inducing expression of new proteins, by activating de novo gene transcription (blocked by Act. D), by stabilizing constitutively expressed mRNAs, or by inducing translation. All such indirect mechanisms are blocked by CHX. A direct inhibitory mechanism involves post-translational modification of constitutively expressed IL-6 signaling proteins and would not be blocked by CHX.

MATERIALS AND METHODS

Cell isolation and tissue culture

Monocytes were obtained from peripheral blood mononuclear cells using anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA), and were >92% pure as verified using flow cytometry as previously decribed [37]. Monocytes were used after 1–2 days of culture in RPMI-1640 medium supplemented with 10% fetal bovine serum and 10–20 ng/ml macrophage colony-stimulating factor. Previously, we had found that IL-6 signaling was inhibited effectively in monocytes cultured under several different conditions [25].

Electrophoretic mobility shift assays (EMSAs)

Cell extracts were prepared as previously decribed [38]. Extracts corresponding to 3.3×10^5 cells (approximately 12 μg protein) were incubated for 15 min at room temperature with 0.5 ng ^{32}P-labeled, double-stranded hSIE oligonucleotide [22] in a 15 μl binding reaction containing 40 mM NaCl and 2 μg poly-dI-dC (Pharmacia, Piscataway, NJ), as previously described [38], and complexes were resolved on nondenaturing, 4.5% polyacrylamide gels.

Immunoblotting

Cell lysates were fractionated on 7.5% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with phospho-specific (tyr 705) Stat3 antibody, phospho-specific (thr180/tyr182) p38 antibody (New England Biolabs, Beverly, MA), monoclonal Stat1, Stat3, Jak1, and Tyk2 antibodies (Transduction Laboratories, Lexington, KY), and p38 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Enhanced chemiluminescence was used for detection.

Flow cytometry

Flow cytometry was performed as previously described [37]. The binding of IL-6 to cell surface IL-6Rs was measured using a Fluorokine kit according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

Transient transfections

293TetOn cells were purchased from Clontech (Palo Alto, CA). These cells express the rTetR protein stably, which, upon binding tetracycline, is able to bind the pTRE promoter element on a second plasmid (introduced into the cells through transfection) and turn on the expression of genes downstream

from that site. The sequence encoding a FLAG-tagged, constitutively active mutant of MAPK kinase 6 {MKK6(CA) obtained from Dr. R. Davis, see ref. [39], was cloned into the pTRE2 vector and transiently transfected into the 293TetOn cells along with a plasmid encoding a TrkC-gp130 fusion protein (Tg) using the calcium phosphate coprecipitation technique as previously described [25]. Transfection efficiency was normalized using a cotransfected internal control plasmid encoding β-galactosidase.

RESULTS

Rapid inhibition of IL-6 signaling in the absence of de novo protein synthesis

Our laboratory and others have established that IL-1 inhibits IL-6 signaling by a p38-dependent pathway [25, 35]. p38 is a serine/threonine kinase that plays a role in the activation of transcription factors and de novo transcription [40], stabilization of mRNA [41, 42], induction of translation [43–46], and post-translational modification of numerous proteins [40]. Thus, IL-1-induced, p38-dependent inhibition of IL-6 signaling may occur via de novo induction of gene expression (of inhibitory proteins such as SOCS), increased levels of inhibitory proteins secondary to increased message stability or increased translation of constitutively expressed mRNAs, or post-translational modification of pre-existing IL-6 signaling components (**Fig. 1**). We have previously shown that inhibition can occur in the absence of de novo mRNA transcription [25].

To exclude the possibility that IL-1 inhibited IL-6 signaling by increasing synthesis of new proteins in the absence of new gene expression, we performed experiments using the protein synthesis inhibitor cycloheximide (CHX; **Fig. 2**). Purified human blood monocytes were preincubated with Act. D, an inhibitor of transcription, or with CHX, treated with IL-1, and then stimulated with IL-6. Similar to our previous results [25], IL-6 activated STAT-DNA binding activity, and this activation was rapidly inhibited by IL-1 in a fashion that was not reversed by Act. D; thus, inhibition was not dependent on new transcription (Fig. 2A, left panel). We have previously confirmed that Act. D essentially blocked transcription completely in these experiments [25]. Surprisingly, treatment with CHX alone for only 15 min almost completely blocked activation of STAT-DNA binding (Fig. 2A, right panel, lane 4). Inhibition occurred at the level of tyrosine phosphorylation, and Stat3 protein levels did not change, indicating that inhibition by CHX was not secondary to lowering levels of STAT proteins (Fig. 2A, right panel; see also Fig. 4). The level of inhibition by CHX was comparable with inhibition when IL-1 was used (lane 3). We and others have previously demonstrated that this level of inhibition of signaling has physiologic significance in terms of suppression of gene activation [25, 27, 33].

Inhibition of IL-6 signaling by CHX in most experiments was sufficiently strong that it was difficult to assess any additional inhibition mediated by IL-1 (Fig. 2A, right panel, lanes

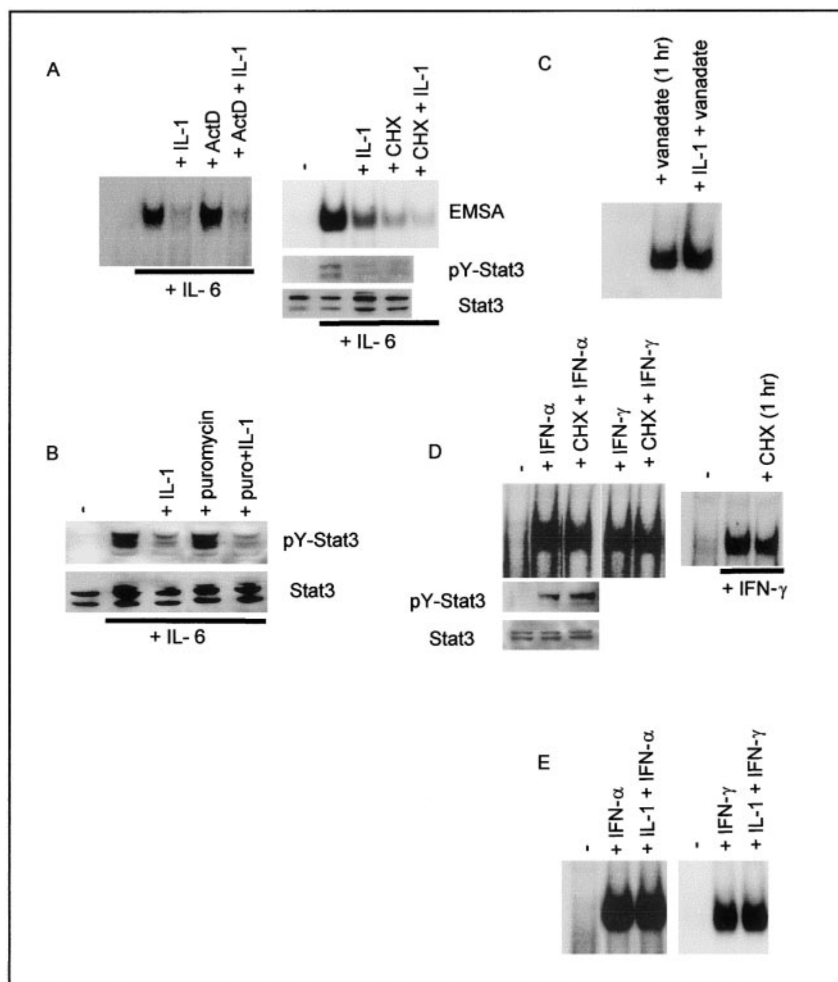

Fig. 2. Rapid inhibition of IL-6-, but not vanadate- or IFN-mediated STAT activation by IL-1, CHX, and other protein synthesis inhibitors. (A) Purified monocytes were treated with Act. D (5 μg/ml), CHX (15 μg/ml), or IL-1 (50 ng/ml) and were then stimulated with IL-6 (50 ng/ml). Cells were preincubated with Act. D for 20 min, followed by a 15-min stimulation with IL-1 (left panel). CHX was added for 15 min and IL-1, for 5 min, before adding IL-6 (right panel). Cell extracts were analyzed using EMSA with the hSIE oligonucleotide as described [22], and some extracts were also analyzed using sequential immunoblotting of the same membrane with antibodies against tyrosine-phosphorylated Stat3 and Stat3. (B) Monocytes were treated with puromycin (10 μM) for 10 min followed by IL-1 pretreatment for 20 min and IL-6 stimulation for 10 min. (C) Monocytes were treated with 5 mM sodium orthovanadate for 1 h. IL-1 was added 20 min prior to vanadate. Cell extracts were assayed by EMSA with the hSIE oligonucleotide. (D, E) The effect of CHX (D) and IL-1 (E) on IFN-α and IFN-γ signaling was tested. IFNs were used at 8 ng/ml. In the upper left panel, CHX pretreatment was for 20 min and for 60 min on the right. IL-1 pretreatments were for 20 min. pY, Phosphorylated tyrosine.

3–5). To further address whether IL-1 could inhibit IL-6 signaling when protein synthesis was blocked, we tested a panel of protein synthesis inhibitors, all of which inhibit the peptidyltransferase activity of the ribosomal 60S subunit, at concentrations that result in >98% inhibition of protein synthesis [47]. The protein synthesis inhibitor puromycin had no detectable effect on activation of Stat3 by IL-6 (Fig. 2B, lanes 2 and 4; no change in band intensity was detected using densitometry). IL-1 inhibited IL-6-induced Stat3 activation in the presence of puromycin to a comparable extent as in its absence (Fig. 2B, lanes 3 and 5; densitometry revealed that the band intensity in lane 3 was 57% lower than in lane 2, and the band intensity in lane 5 was 58% lower than in lane 4). This indicates that IL-1 is capable of inhibiting IL-6 signaling in the absence of new protein synthesis. Addition of IL-1 resulted in inhibition of IL-6 signaling in the presence of several additional protein synthesis inhibitors, but these results are difficult to interpret, as the protein synthesis inhibitors themselves suppressed IL-6 signaling partially (data not shown). The additive inhibition of IL-6 signaling observed when protein synthesis inhibitors and IL-1 were used together may be explained by reinforcement of a common inhibitory pathway or induction of different inhibitory pathways.

The specificity of inhibition was investigated by examining the effects of IL-1 and CHX on activation of Jak-STAT signaling by vanadate, IFN-α, and IFN-γ. Treatment of cells for 1 h with vanadate, a tyrosine-phosphatase inhibitor, resulted in activation of the Jak-STAT pathway even in the absence of addition of any exogenous cytokine (Fig. 2C, lane 2), and this activation was not suppressed by IL-1 (lane 3). This result indicates that IL-1 did not block Jak-STAT signaling globally. IFN-α and IFN-γ use the same Jaks and STATs as does IL-6 (Jak1, Jak2, Tyk2, Stat1, and Stat3). Activation of STATs by IFN-α or IFN-γ was not affected by IL-1 or CHX (Fig. 2, D and E). These results demonstrate specificity of inhibition for IL-6 signaling and suggest that Jaks and STATs are not direct targets for the IL-1- and CHX-induced inhibitory pathways. Taken together with our previous results that gp130-activated Jaks are inhibited by p38 in B cells [25], these results suggest that inhibition occurs at a proximal step in signal transduction and targets the IL-6R or IL-6R-associated molecules.

CHX and IL-1 inhibit IL-6 signaling via a p38-dependent pathway

The rapid inhibition of IL-6 signaling in the absence of de novo protein synthesis suggested the possibility of a direct mechanism, such as activation of an inhibitory kinase or phosphatase. Because protein synthesis inhibitors have been shown to activate stress kinases (refs. [47, 48], and references therein), and p38 plays a role in inhibition of IL-6 signaling [25, 35], we tested whether CHX may inhibit IL-6 signaling in monocytes by activating p38. CHX activated p38 rapidly in monocytes, and the level of activation was comparable with that achieved using IL-1 (**Fig. 3A**). Inhibition of p38 activity using SB203580, at several doses where this compound is believed to be a specific inhibitor of p38, reversed CHX- and IL-1-induced inhibition of IL-6 signaling (Fig. 3B, and data not shown). Thus, inhibition of IL-6 signaling by CHX, similar to IL-1, is dependent on p38 [25, 35, 49]. The strength of inhibition of

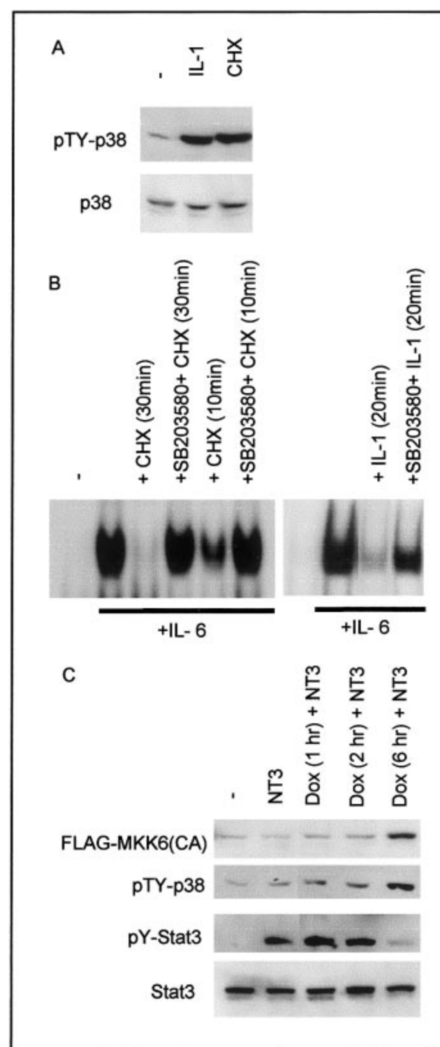

Fig. 3. Inhibition of IL-6 signaling is dependent on p38. (A) Rapid activation of p38 by CHX. Monocytes were treated for 10 min with CHX or IL-1, and cell extracts were analyzed by immunoblotting. pTY-p38, activated form of p38 that is phosphorylated on threonine and tyrosine. (B) Reversal of CHX and IL-1 inhibition of IL-6 signaling by the p38 kinase inhibitor SB203580. Monocytes were preincubated with 10 μM SB203580 for 30 min before adding CHX or IL-1. Similar results were obtained using 5 μM and 2 μM SB203580. (C) 293Tet-ON cells were transiently transfected via the calcium phosphate coprecipitation method with plasmids encoding a Tg that is ligated and activated by neurotropin 3 (NT3), β-galactosidase, and pTRE2-MKK6(CA). Cells were treated with 200 ng/ml doxycycline (DOX) for the periods indicated to induce expression of MKK6(CA) prior to a 10-min stimulation with 50 ng/ml NT3. Cell extracts were analyzed by immunoblotting.

IL-6 signaling by IL-1 and several different protein synthesis inhibitors correlated with the level of p38 activation, but of the agents tested, only anisomycin induced detectable levels of activated JNK in our system (data not shown). Although JNK may be capable of inhibiting IL-6 signaling, these results suggest that JNK is unlikely to play an important role in our system. We wished to investigate whether activation of p38 was sufficient to inhibit IL-6 signaling. p38 was activated by inducing expression of MKK6(CA), a kinase that activates p38, but not JNK or ERK (refs [25, 39], and data not shown), by adding doxycycline to cells that had been transfected with MKK6(CA) under the control of an inducible promoter. Low-

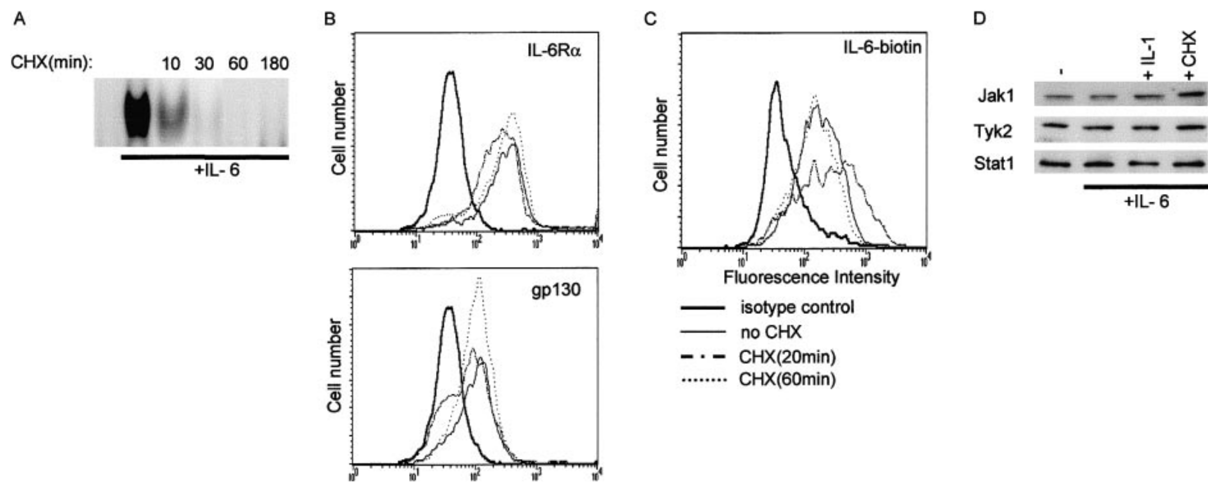

Fig. 4. CHX inhibits IL-6 signaling rapidly and does not alter expression of IL-6 signaling proteins during this time frame. (A) Cell extracts were prepared after various times of CHX treatment and were analyzed by EMSA. (B, C) Monocytes were harvested after 20 or 60 min of incubation with CHX (15 μg/ml) and were analyzed using flow cytometry with antibodies against IL-6R α and gp130 subunits (B) or with biotinylated IL-6 followed by streptavidin-fluorescein isothiocyanate (C). (D) Monocytes were incubated for 30 min with CHX or IL-1, and extracts were analyzed using immunoblotting.

level expression of MKK6(CA) and low-level p38 phosphorylation were observed in the absence of doxycycline (Fig. 3C) and may affect the responsiveness of these cells relative to nontransfected cells. However, activation of p38 over baseline after the addition of doxycycline effectively suppressed activation of Stat3 by a fusion receptor containing the gp130 cytoplasmic domain (Fig. 3C). Thus, activation of p38 is sufficient for inhibition of IL-6 signaling. However, it remains possible that the additive effects of IL-1 and protein synthesis on inhibition of IL-6 signaling (Fig. 2A) may be secondary to activation of additional inhibitory pathways. Activation of p38 by overexpression of MKK6(CA) was unable to suppress activation of Jaks, which occurs independently of receptors, after overexpression [50] (data not shown). Taken together with the results that IL-1 did not inhibit signaling by other cytokines that use Jak1 (Fig. 2E), these results provide further support for the notion that the inhibitory pathway targets the IL-6R or IL-6R-associated molecules.

It is possible that inhibition of IL-6 signaling after incubation with CHX was secondary to decreased levels of a necessary signaling protein that is rapidly turned over. However, CHX inhibited IL-6 signaling strongly when added just 10 min prior to IL-6 (**Fig. 4A**), suggesting that this putative signaling protein is extremely labile or that CHX acts by a different mechanism. The most labile proteins in the IL-6-Jak-STAT pathway are the α and gp130 chains of the IL-6 receptor, which can also undergo ligand-mediated internalization [51]. The effects of CHX on IL-6R expression were assessed using flow cytometry. Treatment with CHX for 20 min or 1 h did not result in decreased cell surface expression of either IL-6R subunit (Fig. 4B) or decreased binding of IL-6 to IL-6Rs (Fig. 4C), indicating that inhibition of signaling by CHX was not mediated by changes in IL-6R expression. CHX also did not affect the levels of expression of Jak1, Tyk2, Stat1, and Stat3, the Jak-STAT proteins that are used by IL-6 (Figs. 2A and 4D). The normal expression and function of these proteins after CHX treatment are supported further by the lack of CHX-mediated inhibition of signaling by IFN-α and IFN-γ (Fig. 2E), which use these proteins as well. Taken together, these results demonstrate that inhibition of IL-6 signaling by CHX does not occur secondary to decreased expression of known IL-6 signaling proteins but instead suggest that CHX acts directly via p38.

Although CHX most likely activates p38 by triggering ribotoxic stress pathways [47], we wished to address whether lipopolysaccharide (LPS) contamination of CHX could contribute to p38 activation. Four different preparations of CHX from different manufacturers inhibited IL-6 signaling at similar doses, suggesting LPS contamination was not likely (data not shown). More importantly, CHX blocked IL-6 signaling effectively under serum-free conditions where LPS is not active, secondary to absence of LPS-binding protein (**Fig. 5**). Thus, inhibition of IL-6 signaling by CHX cannot be attributed to LPS contamination.

Rapid p38-dependent inhibition of IL-6 signaling is not mediated by a tyrosine phosphatase or prostaglandins (PGs)

Using IL-1 and protein synthesis inhibitors, our results suggested that inhibition of IL-6 signaling is mediated by a p38-

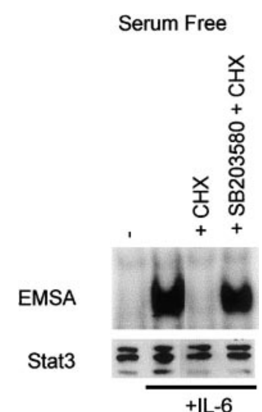

Fig. 5. CHX inhibition of IL-6 signaling is not secondary to LPS contamination. CHX was added to monocytes 20 min prior to IL-6 under serum-free conditions.

dependent, post-translational modification of a pre-existing signaling component (see Fig. 1). One possibility is p38-dependent activation of an enzyme, such as a tyrosine phosphatase, which inactivates IL-6 signaling by dephosphorylating receptors, Jaks, or STATs, or activation of an enzyme that produces an inhibitor of Jak-STAT signaling. Vanadate, an inhibitor of tyrosine phosphatases, was used to determine if inhibition of IL-6 signaling by IL-1 was dependent on a tyrosine phosphatase. In 10 independent experiments, vanadate had a variable effect on basal STAT activity, which appeared to be dependent on variability among blood donors and the length of time of incubation with vanadate (data not shown). A representative experiment is shown in **Figure 6A**; in all cases, IL-1 strongly inhibited the IL-6-inducible component of STAT activation in the presence of vanadate (Fig. 6A and data not shown). The weaker activation of STATs by vanadate alone in Figure 6A relative to Figure 2C is most likely secondary to shorter incubation with vanadate (15 vs. 60 min). These results indicate that inhibition of IL-6 signaling by IL-1 cannot be mediated solely by the activation of a tyrosine phosphatase. p38 also activates phospholipase A_2, which leads to release of arachidonic acid and production of PGs by cyclooxygenases. Because prostaglandin E triggers the cyclic adenosine monophosphate signaling pathway that has been implicated in the inhibition of Jak-STAT signaling [22, 52], we tested whether inhibition of IL-6 signaling by IL-1 may be mediated by PGs. Inhibition of phospholipase A_2 using AACOCF3 and of cyclooxygenase by indomethacin had no effect on IL-6 signaling or on IL-1-mediated inhibition of IL-6 signaling (Fig. 6B, C), indicating that small molecule mediators produced by these enzymes have no role in inhibition.

DISCUSSION

The major finding of this study is the conclusive demonstration that inhibition of IL-6 Jak-STAT signaling can occur independent of the synthesis of inhibitory proteins, such as SOCS proteins. This inhibitory mechanism is dependent on p38 ([25] and Fig. 3) and can be triggered by physiologic ligands such as IL-1 and by inhibitors of protein synthesis themselves. Such a direct inhibitory pathway would act by modifying IL-6 signaling proteins that are already expressed in the cell. Pathways activated by IL-1 that can modify proteins include ubiquitination and proteolytic destruction [27], activation of caspase 1 [53], production of PGs, and phosphorylation of proteins. Experiments using phospholipase A_2, COX, proteosome, and caspase inhibitors did not reverse inhibition (Fig. 6 and S.A., unpublished data), and thus we favor the possibility that IL-6 signaling is inhibited by p38-dependent phosphorylation of an IL-6-signaling molecule.

An important question is the identity of the molecular target for the IL-1-induced, p38-dependent inhibitory pathway. Several lines of evidence suggest that the most likely target is the IL-6R itself, specifically the gp130 subunit. First, if the inhibitory pathway targeted the STATs or Jaks directly, one would predict that signaling induced by IFN-α and IFN-γ should also be suppressed, as these cytokines use the same Jaks and STATs as does IL-6. Activation of STATs by vanadate alone would also be suppressed. In contrast to these predictions, STAT activation by IFN-α, IFN-γ, and vanadate was not affected by IL-1 or protein synthesis inhibitors. Secondly, overexpression of Jaks leads to autoactivation in the cytoplasm, and this activation was not blocked by p38 (S.A., unpublished data). In contrast, activation of gp130-associated Jaks was blocked [25]. Last, activation of p38 led to inhibition of signaling by a fusion receptor that contains only gp130-derived cytoplasmic sequences. These results lead us to propose that the most likely target for IL-1-mediated inhibition is the gp130 cytoplasmic domain. This idea is supported by the fact that the gp130 cytoplasmic domain contains a perfect MAPK phosphorylation site PHTP^{671}, gp130 has been shown to be phosphorylated on threonine residues [54], and a mutation of this putative MAPK site leads to a nonsignaling receptor (S.A., unpublished data). We have been unable to directly assess gp130 phosphorylation status secondary to low-level expression in monocytes and inefficient immunoprecipitation using several different antibodies. Future work will use mutational analysis to further analyze whether gp130 serves as the target of IL-1-mediated inhibition.

It is becoming clear that there are several different inducible mechanisms of inhibition of cytokine Jak-STAT signaling. One important difference among these mechanisms is the kinetics of induction. Direct inhibition of signaling, as described herein and in [23–25, 27, and 48], allows rapid modulation of cytokine signaling and might prevent the immediate and early cellular responses to a cytokine. In contrast, indirect inhibition via induction of SOCS expression will take longer to become established and will reach a peak after 1–4 h if inhibition by SOCS proteins is inferred to correlate with expression levels [19, 20, 28]. Expression of SOCS proteins can be transient and wane after several hours or can be sustained for longer time

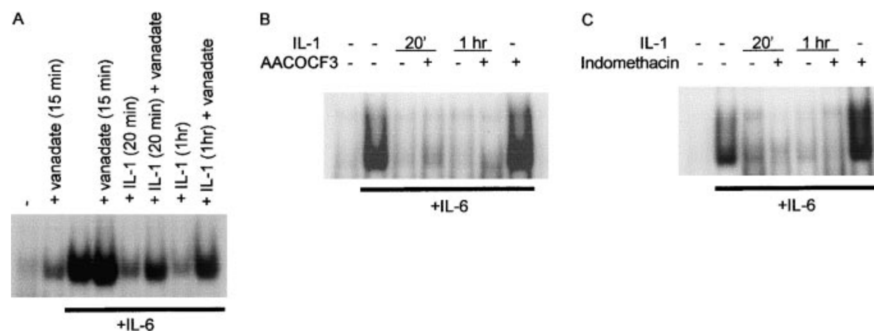

Fig. 6. Inhibition of IL-6 signaling by IL-1 is not dependent on protein tyrosine phosphatases or on PG production. (A) Monocytes were treated with IL-1, and sodium orthovanadate (5 mM) was added 5 min before adding IL-6 for 10 min. Vanadate was added at the same time to controls that did not receive IL-1 or IL-6. (B, C) Inhibition of IL-6 signaling by IL-1 is not dependent on PG production. The phospholipase A_2 inhibitor AACOCF3 (20 μM) or the cyclooxygenase inhibitor indomethacin (10 μM) was added 20 min before adding IL-1.

periods, and thus the time frame during which SOCS-mediated inhibition plays an important role can be variable. Another important inducible mechanism of inhibition of cytokine signaling is regulation of receptor expression, which often takes one or several days to become apparent [11–14]. Thus, cytokine signaling appears to be subject to negative regulation by different mechanisms that come into play at different time points and could potentially work together.

p38 is activated by a many stress stimuli, inflammatory cytokines, and by transforming growth factor (TGF)-β. Of these, several stress stimuli, IL-1, TNF, and TGF-β, have been shown to inhibit Jak-STAT signaling in a rapid manner [25, 55], and it is likely that p38 plays a role in mediating inhibition by many stimuli other than IL-1 and CHX. Within the innate and acquired immune systems, p38 is activated by receptors that recognize foreign antigens, such as T and B cell antigen receptors, Fc, complement, and Toll-like receptors. Thus, p38 may play an important role in modulation of cytokine Jak-STAT signaling by a large variety of stimuli that regulate immune cell activation and differentiation. p38 can affect cytokine signaling negatively or positively by several different mechanisms. On the negative side, signaling by IL-6 is suppressed by a direct mechanism and by induction of expression of SOCS3 and possibly other SOCS proteins. On the positive side, p38 may increase expression of cytokine receptors and may potentiate transcriptional responses to cytokines such as IFN-γ or IL-12 by serine phosphorylation of STATs, which increases their transcriptional potency [56]. Thus, p38 will affect cytokine Jak-STAT signaling in a complex and balanced way, similar to its positive and negative regulation of nuclear factor-κB activity [57].

In terms of the physiology of an inflammatory response, IL-1, which is predominantly proinflammatory, induces expression of, and is coexpressed with, IL-6, which is more pleiotropic and has pro- and anti-inflammatory actions. In this context, inhibition of IL-6 signaling by IL-1 could serve two purposes. First, IL-1 suppression of additional IL-6 induction of proinflammatory genes could serve a homeostatic function to limit the extent of inflammation. We have previously shown p38-mediated inhibition of IL-6 induction of expression of IFN regulatory factor 1, a transcription factor important in T_H1 responses [25, 58], and others have shown IL-1 suppression of IL-6 induction of the type II acute-phase response genes thiostatin and fibrinogen [33]. Second, IL-1 blockade of IL-6 anti-inflammatory effects could serve to help maintain an early inflammatory response. One possible scenario where this may play an important role is peritoneal inflammation, where IL-6 attenuates expression of IL-8 and growth-related oncogene α and suppresses neutrophil influx [59]. The overall outcome of the interactions of IL-1 and IL-6 signaling in physiology and inflammation will likely be determined by the relative strength and timing of the opposing signals. Modulation of IL-6 signaling by stress pathways may also play a role in regulation of the growth of IL-6-dependent cells. We have described inhibition of IL-6 signaling in U266 myeloma cells by agents that activate p38 [25], and this inhibition correlated with growth arrest and apoptosis (S.A., unpublished data). It is interesting that certain chemotherapeutic agents, such as etoposide and vinblastine, activate the stress kinases [60, 61].

Overall, consistent with its proposed proinflammatory role, p38 appears to suppress or modulate signaling by pleiotropic or anti-inflammatory cytokines such as IL-6 or IL-10 ([25] and Fig. 3) and to potentiate Jak-STAT signaling by a proinflammatory cytokine such as IFN-γ or IL-12 [56, 62, 63]. The final effects of p38 on cytokine action during an immune or inflammatory response will be determined by the balance between the positive and negative effects of p38, which will likely vary with different cytokines and cell types, and may possibly be regulated according to the physiologic state of the cell.

ACKNOWLEDGMENTS

We thank Paul Anderson and Philip King for helpful discussions and Xiaoyu Hu and Tapas Sengupta for critical review of the manuscript. We thank N. Stahl, G. Yancopoulous, R. Davis, and J. N. Ihle for providing plasmids. This work was supported by grants from the National Institutes of Health (NIH) and the American Chemical Society. S. T. A. was supported partially by a predoctoral fellowship from the Cancer Research Institute and by a T32 predoctoral training grant from the NIH.

REFERENCES

1. Schindler, C., Darnell Jr., J. E. (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. 64, 621–651.
2. Ivashkiv, L. (2000) Jak-STAT signaling pathways in cells of the immune system. Rev. Immunogenet. 2, 220–230.
3. Azam, M., Lee, C., Strehlow, I., Schindler, C. (1997) Functionally distinct isoforms of STAT5 are generated by protein processing. Immunity 6, 691–701.
4. Kim, T. K., Maniatis, T. (1996) Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. Science 273, 1717–1719.
5. Haspel, R. L., Salditt-Georgieff, M., Darnell Jr., J. E. (1996) The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase. EMBO J. 15, 6262–6268.
6. Shuai, K., Liao, J., Song, M. M. (1996) Enhancement of antiproliferative activity of gamma interferon by the specific inhibition of tyrosine dephosphorylation of Stat1. Mol. Cell. Biol. 16, 4932–4941.
7. Aragane, Y., Kulms, D., Luger, T. A., Schwarz, T. (1997) Down-regulation of interferon gamma-activated STAT1 by UV light. Proc. Natl. Acad. Sci. USA 94, 11490–11495.
8. Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., Shuai, K. (1997) Specific inhibition of stat3 signal transduction by PIAS3. Science 278, 1803–1805.
9. Wang, D., Moriggl, R., Stravopodis, D., Carpino, N., Marine, J. C., Teglund, S., Feng, J., Ihle, J. N. (2000) A small amphipathic alpha-helical region is required for transcriptional activities and proteasome-dependent turnover of the tyrosine-phosphorylated Stat5. EMBO J. 19, 392–399.
10. Yu, C. L., Jin, Y. J., Burakoff, S. J. (2000) Cytosolic tyrosine dephosphorylation of STAT5. Potential role of SHP-2 in STAT5 regulation. J. Biol. Chem. 275, 599–604.
11. Pernis, A., Gupta, S., Gollob, K. J., Garfein, E., Coffman, R. L., Schindler, C., Rothman, P. (1995) Lack of interferon gamma receptor beta chain and the prevention of interferon gamma signaling in TH1 cells. Science 269, 245–247.
12. Bach, E. A., Szabo, S. J., Dighe, A. S., Ashkenazi, A., Aguet, M., Murphy, K. M., Schreiber, R. D. (1995) Ligand-induced autoregulation of IFN-gamma receptor beta chain expression in T helper cell subsets. Science 270, 1215–1218.
13. Bianchi, M., Meng, C., Ivashkiv, L. B. (2000) Inhibition of IL-2-induced Jak-STAT signaling by glucocorticoids. Proc. Natl. Acad. Sci. USA 97, 9573–9578.

14. Wu, C. Y., Wang, K., McDyer, J. F., Seder, R. A. (1998) Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. J. Immunol. 161, 2723–2730.
15. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., Yoshimura, A. (1997) A new protein containing an SH2 domain that inhibits JAK kinases. Nature 387, 921–924.
16. Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., Kishimoto, T. (1997) Structure and function of a new STAT-induced STAT inhibitor. Nature 387, 924–929.
17. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., Hilton, D. J. (1997) A family of cytokine-inducible inhibitors of signalling. Nature 387, 917–921.
18. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., Miyajima, A. (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, 2816–2826.
19. Yasukawa, H., Sasaki, A., Yoshimura, A. (2000) Negative regulation of cytokine signaling pathways. Annu. Rev. Immunol. 18, 143–164.
20. Chen, X. P., Losman, J. A., Rothman, P. (2000) SOCS proteins, regulators of intracellular signaling. Immunity 13, 287–290.
21. Sengupta, T. K., Talbot, E. S., Scherle, P. A., Ivashkiv, L. B. (1998) Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. Proc. Natl. Acad. Sci. USA 95, 11107–11112.
22. Sengupta, T. K., Schmitt, E. M., Ivashkiv, L. B. (1996) Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. Proc. Natl. Acad. Sci. USA 93, 9499–9504.
23. Lee, I. H., Li, W. P., Hisert, K. B., Ivashkiv, L. B. (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, 1263–1274.
24. Zhu, J., Huang, H., Guo, L., Stonehouse, T., Watson, C. J., Hu-Li, J., Paul, W. E. (2000) Transient inhibition of interleukin 4 signaling by T cell receptor ligation. J. Exp. Med. 192, 1125–1134.
25. Ahmed, S. T., Ivashkiv, L. B. (2000) Inhibition of IL-6 and IL-10 signaling and stat activation by inflammatory and stress pathways. J. Immunol. 165, 5227–5237.
26. Kovanen, P. E., Junttila, I., Takaluoma, K., Saharinen, P., Valmu, L., Li, W., Silvennoinen, O. (2000) Regulation of jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells. Blood 95, 1626–1632.
27. Tian, Z., Shen, X., Feng, H., Gao, B. (2000) IL-1beta attenuates IFN-alphabeta-induced antiviral activity and STAT1 activation in the liver: involvement of proteasome-dependent pathway. J. Immunol. 165, 3959–3965.
28. Alexander, W. S., Starr, R., Metcalf, D., Nicholson, S. E., Farley, A., Elefanty, A. G., Brysha, M., Kile, B. T., Richardson, R., Baca, M., Zhang, J. G., Willson, T. A., Viney, E. M., Sprigg, N. S., Rakar, S., Corbin, J., Mifsud, S., DiRago, L., Cary, D., Nicola, N. A., Hilton, D. J. (1999) Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. J. Leukoc. Biol. 66, 588–592.
29. Schmitz, J., Weissenbach, M., Haan, S., Heinrich, P. C., Schaper, F. (2000) SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. J. Biol. Chem. 275, 12848–12856.
30. Nicholson, S. E., De Souza, D., Fabri, L. J., Corbin, J., Willson, T. A., Zhang, J. G., Silva, A., Asimakis, M., Farley, A., Nash, A. D., Metcalf, D., Hilton, D. J., Nicola, N. A., Baca, M. (2000) Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. Proc. Natl. Acad. Sci. USA 97, 6493–6498.
31. Marine, J. C., McKay, C., Wang, D., Topham, D. J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., Ihle, J. N. (1999) SOCS3 is essential in the regulation of fetal liver erythropoiesis. Cell 98, 617–627.
32. Marine, J. C., Topham, D. J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., Ihle, J. N. (1999) SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. Cell 98, 609–616.
33. Baumann, H., Prowse, K. R., Marinkovic, S., Won, K. A., Jahreis, G. P. (1989) Stimulation of hepatic acute phase response by cytokines and glucocorticoids. Ann. N. Y. Acad. Sci. 557, 280–295, discussion 295–296.
34. Chomarat, P., Banchereau, J., Davoust, J., Palucka, A. K. (2000) IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat. Immunol. 1, 510–514.
35. Bode, J. G., Nimmesgern, A., Schmitz, J., Schaper, F., Schmitt, M., Frisch, W., Haussinger, D., Heinrich, P. C., Graeve, L. (1999) LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. FEBS Lett. 463, 365–370.
36. Shen, X., Tian, Z., Holtzman, M. J., Gao, B. (2000) Cross-talk between interleukin 1beta (IL-1beta) and IL-6 signalling pathways: IL-1beta selectively inhibits IL-6-activated signal transducer and activator of transcription factor 1 (STAT1) by a proteasome-dependent mechanism. Biochem. J. 352, 913–919.
37. Castro, A., Sengupta, T. K., Ruiz, D. C., Yang, E., Ivashkiv, L. B. (1999) IL-4 selectively inhibits IL-2-triggered Stat5 activation, but not proliferation, in human T cells. J. Immunol. 162, 1261–1269.
38. Sengupta, T. K., Chen, A., Zhong, Z., Darnell Jr., J. E., Ivashkiv, L. B. (1995) Activation of monocyte effector genes and STAT family transcription factors by inflammatory synovial fluid is independent of interferon gamma. J. Exp. Med. 181, 1015–1025.
39. Whitmarsh, A. J., Yang, S. H., Su, M. S., Sharrocks, A. D., Davis, R. J. (1997) Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. Mol. Cell. Biol. 17, 2360–2371.
40. Karin, M. (1998) Mitogen-activated protein kinase cascades as regulators of stress responses. Ann. N. Y. Acad. Sci. 851, 139–146.
41. Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., Clark, A. R. (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. Mol. Cell. Biol. 20, 4265–4274.
42. Brook, M., Sully, G., Clark, A. R., Saklatvala, J. (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. FEBS Lett. 483, 57–61.
43. Cohen, P. S., Nakshatri, H., Dennis, J., Caragine, T., Bianchi, M., Cerami, A., Tracey, K. J. (1996) CNI-1493 inhibits monocyte/macrophage tumor necrosis factor by suppression of translation efficiency. Proc. Natl. Acad. Sci. USA 93, 3967–3971.
44. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., Kollias, G. (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 10, 387–398.
45. Prichett, W., Hand, A., Sheilds, J., Dunnington, D. (1995) Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor alpha. J. Inflamm. 45, 97–105.
46. Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L., Vries, R. G., Baines, I. A., Cooper, J. A., Proud, C. G. (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J. Biol. Chem. 273, 9373–9377.
47. Shifrin, V. I., Anderson, P. (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J. Biol. Chem. 274, 13985–13992.
48. Fuchs, S. Y., Tappin, I., Ronai, Z. (2000) Stability of the ATF2 transcription factor is regulated by phosphorylation and dephosphorylation. J. Biol. Chem. 275, 12560–12564.
49. Bode, J. G., Gatsios, P., Ludwig, S., Rapp, U. R., Haussinger, D., Heinrich, P. C., Graeve, L. (1999) The mitogen-activated protein (MAP) kinase p38 and its upstream activator MAP kinase kinase 6 are involved in the activation of signal transducer and activator of transcription by hyperosmolarity. J. Biol. Chem. 274, 30222–30227.
50. Quelle, F. W., Thierfelder, W., Witthuhn, B. A., Tang, B., Cohen, S., Ihle, J. N. (1995) Phosphorylation and activation of the DNA binding activity of purified Stat1 by the Janus protein-tyrosine kinases and the epidermal growth factor receptor. J. Biol. Chem. 270, 20775–20780.
51. Taga, T., Kishimoto, T. (1997) Gp130 and the interleukin-6 family of cytokines. Annu. Rev. Immunol. 15, 797–819.
52. Delgado, M., Ganea, D. (2000) Inhibition of IFN-gamma-induced janus kinase-1-STAT1 activation in macrophages by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. J. Immunol. 165, 3051–3057.
53. Seki, E., Tsutsui, H., Nakano, H., Tsuji, N., Hoshino, K., Adachi, O., Adachi, K., Futatsugi, S., Kuida, K., Takeuchi, O., Okamura, H., Fujimoto, J., Akira, S., Nakanishi, K. (2001) Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta. J. Immunol. 166, 2651–2657.
54. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., Kishimoto, T. (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 58, 573–581.

55. Bright, J. J., Kerr, L. D., Sriram, S. (1997) TGF-beta inhibits IL-2-induced tyrosine phosphorylation and activation of Jak-1 and Stat 5 in T lymphocytes. J. Immunol. 159, 175–183.
56. Decker, T., Kovarik, P. (2000) Serine phosphorylation of STATs. Oncogene 19, 2628–2637.
57. Bowie, A. G., O'Neill, L. A. (2000) Vitamin C inhibits NF-kappaB activation by TNF via the activation of p38 mitogen-activated protein kinase. J. Immunol. 165, 7180–7188.
58. Lohoff, M., Ferrick, D., Mittrucker, H. W., Duncan, G. S., Bischof, S., Rollinghoff, M., Mak, T. W. (1997) Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. Immunity 6, 681–689.
59. Hurst, S. M., Wilkinson, T. S., McLoughlin, R. M., Jones, S., Horiuchi, S., Yamamoto, N., Rose-John, S., Fuller, G. M., Topley, N., Jones, S. A. (2001) IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity 14, 705–714.
60. Osborn, M. T., Chambers, T. C. (1996) Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. J. Biol. Chem. 271, 30950–30955.
61. Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., Wimalasena, J. (1998) Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J. Biol. Chem. 273, 4928–4936.
62. Gollob, J. A., Schnipper, C. P., Murphy, E. A., Ritz, J., Frank, D. A. (1999) The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. J. Immunol. 162, 4472–4481.
63. Kovarik, P., Stoiber, D., Novy, M., Decker, T. (1998) Stat1 combines signals derived from IFN-gamma and LPS receptors during macrophage activation. EMBO J. 17, 3660–3668.