Cross-talk between ERK and p38 MAPK Mediates Selective Suppression of Pro-inflammatory Cytokines by Transforming Growth Factor-β*

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Phagocytosis of apoptotic cells by macrophages results in the production of transforming growth factor-β (TGF-β), which plays an important role in induction of an anti-inflammatory phenotype and resolution of inflammation. In this study, we show that TGF-β prevents pro-inflammatory cytokine production through inhibition of p38 mitogen-activated protein kinase (MAPK) and NF-κB. Blockade of extracellular signal-regulated kinase (ERK) signaling by the MEK-1/2 inhibitor PD 98059 reversed the inhibitory effects of TGF-β, suggesting that cross-talk between MAPKs is essential for this response. Further investigation indicated that TGF-β activated ERK, which in turn up-regulated MAPK phosphatase-1, thereby inactivating p38 MAPK. On the other hand, TGF-β maintained or slightly increased production of the CC chemokine MCP-1, which is regulated predominantly by AP-1. Although SB 203580, an inhibitor of p38 MAPK, and dominant-negative p38 MAPK both increased AP-1 transcription, lack of effect of TGF-β on lipopolysaccharide-stimulated SAPK/JNK phosphorylation along with a demonstrated inhibition of TGF-β-induced AP-1 activation by dominant-negative Smad3 suggest that TGF-β-stimulated AP-1 activation was not caused by inhibition of p38 MAPK but rather through the activation of Smads. Our data provide evidence that TGF-β selectively inhibits inflammatory cytokine production through cross-talk between MAPKs.

CC chemokine MCP-1 (1, 2) either unchanged or slightly increased. We have previously reported that the uptake of apoptotic cells by macrophages can be mediated by a phosphatidylserine receptor and that triggering this receptor is responsible for the increased production of TGF-β. The findings that anti-TGF-β-neutralizing antibodies largely reversed the inhibitory effect of apoptotic cell uptake and that exogenous TGF-β down-regulated the synthesis of the same pro-inflammatory mediators indicated that TGF-β plays an important role in the suppressive effect of interaction between apoptotic cells and macrophages (3).

TGF-β is a multifunctional cytokine that regulates numerous physiological processes, including cell growth, differentiation, apoptosis, adhesion, early embryonic development, and extracellular matrix protein synthesis (4–6). TGF-β exerts its effects through heteromeric receptor complex consisting of type I and type II transmembrane serine/threonine kinase receptors. Upon ligand binding, the type II receptor transphosphorylates the type I receptor within its GS domain, enabling it to transmit signals from TGF-β (7, 8). Smad family members (7, 9, 10), and MAPKs have been implicated in the signaling by TGF-β in regulation of growth, apoptosis, and gene expression (11–16). Although TGF-β has been shown to regulate several MAPK pathways, the mode of activation appears to be highly variable and cell type-dependent (13, 17–23).

p38 MAPK is activated by pro-inflammatory cytokines, osmotic stress, and UV irradiation (24). Following activation, p38 MAPK is capable of modulating functional responses through phosphorylation of transcription factors, such as ATF-2, and activation of other kinases. It has been reported that p38 MAPK is involved in the transcriptional regulation of interleukin-1-β, interleukin-6, and interleukin-8 expression (25–27). Furthermore, translational regulation of cytokine mRNA by p38 MAPK has also been suggested (28, 29). Most of the pro-inflammatory cytokines are regulated by NF-κB, and recently, it has been found that p38 MAPK regulates NF-κB-driven gene expression, in part, by increasing the association of the basal transcriptional factor, TATA-binding protein, with the C terminus of p65 subunit of NF-κB and increasing the binding of TATA-binding protein to the TATA box (30).

Previously we have demonstrated that the suppression of inflammatory mediator production (through the induction of TGF-β) in macrophages that have ingested apoptotic cells is a general phenomenon that does not depend on the nature of apoptotic target, the macrophage activation state, or the type of stimulus used (2). In the present study, we employed an LPS-stimulated macrophage cell line, RAW 264.7, to investigate the signaling pathway used by TGF-β for inhibition of p38 MAPK and regulation of NF-κB- or AP-1-dependent transcription and
cells (3.0 × 10^5 cells/well) were plated in each well of a 12-well tissue culture plate and incubated overnight. After stimulation, the cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.5% Triton X-100, and 1 μg Protease Inhibitor Mixture Set 1), resolved on 10% SDS-PAGE, and blotted to nitrocellulose membrane. The membranes were incubated with phospho-specific antibodies at 4 °C overnight and incubated with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature. The proteins were visualized by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer’s instructions. To confirm equal loading of proteins in each lane, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 7.8, 100 mM β-mercaptoethanol, 2% SDS) for 30 min at 50 °C and reprobed with corresponding antibodies against the native proteins. 
p38 MAPK Immunoprecipitation and Kinase Activity Assay—p38 MAPK activity measurements were performed essentially as described (24). Briefly, RAW 264.7 cells (1 × 10^5 cells/well) were plated in each well of a 12-well tissue culture plate and incubated overnight. After stimulation, the cells were lysed in 500 μl of RIPA buffer supplemented with protease inhibitors. p38 MAPK was immunoprecipitated with 2 μg/ml anti-p38 (ε-20) antibody and protein A-Sepharose beads. The immunoprecipitates were resuspended in 50 μl of kinase reaction mix containing 20 mM HEPES (pH 7.6), 200 μM MgCl2, 20 μM ATP, 20 μCi of [γ-32P]ATP, 2 mM dithiothreitol, 100 μM sodium orthovanadate, 25 μM β-glycerophosphate, and 500 ng of ATF-2(1–96) for 30 min at 30 °C. The reactions were terminated with 4× Laemmli buffer, and the proteins were separated by 10% SDS-PAGE and blotted to nitrocellulose membrane. p38 MAPK activity was visualized as the phosphorylation of the ATF-2 fragment by autoradiography.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**TGF-β1 was from R & D Systems. LPS (Escherichia coli 0111:B4) was from List Biological Laboratories. SB 203580, PD 98059, Ro 31-8220, Dapi, and 1 μM Protease Inhibitor Mixture Set 1 were from Calbiochem-Novabiochem. LipofectAMINE Plus reagent was from Invitrogen. Anti-p38 MAPK, phospho-specific antibody was from Calbiochem-Novabiochem. Phospho-ERK (E-20), ERK-2 (K 23), p38 (ε-20), MEK-3 (N-20), MEK-6 (N-19) (K-19), MKP-1 (ε-15) antibodies, and recombinant fragment of activated transcription factor III/II (ATF-2-(1–96)) were from Cell Signaling. Purified anti-mouse CD16/CD32 (Fcγ III/II Receptor) (2.4G2) was from Pharmingen.

**Cell Culture and Stimulation—**RAW 264.7 (obtained from the American Type Culture Collection) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated endotoxin-free fetal bovine serum, 2 mM t-glutamine, 100 μM/ml streptomycin and 100 units/ml penicillin under a humidified 5% CO₂ atmosphere at 37 °C. The drugs were dissolved in dimethyl sulfoxide. An aliquot of each drug solution was added to the medium, and the final concentration of the vehicle in the medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle. "Immunoblotting Analysis—"Immunoblotting analysis was carried out as described previously with some modification (24). Briefly, RAW 264.7 cells were incubated with LPS alone (100 ng/ml) for 60 min in the presence or absence of SB 203580 (10 μM) and subsequently cultured in the presence or absence of LPS (1 ng/ml) for 12 h. Culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. To confirm equal loading of proteins in each lane, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 7.8, 100 mM β-mercaptoethanol, 2% SDS) for 30 min at 50 °C and reprobed with corresponding antibodies against the native proteins. Phospho-specific antibodies, and recombinant fragment of activated transcription factor III/II (ATF-2-(1–96)) were described previously (31). RAW 264.7 cells (3.0 × 10^5 cells/well) were plated in each well of a 12-well tissue culture plate and incubated overnight. After stimulation, the cells were lysed in 500 μl of RIPA buffer supplemented with protease inhibitors. p38 MAPK was immunoprecipitated with 2 μg/ml anti-p38 (ε-20) antibody and protein A-Sepharose beads. The immunoprecipitates were resuspended in 50 μl of kinase reaction mix containing 20 mM HEPES (pH 7.6), 200 μM MgCl₂, 20 μM ATP, 20 μCi of [γ-32P]ATP, 2 mM dithiothreitol, 100 μM sodium orthovanadate, 25 μM β-glycerophosphate, and 500 ng of ATF-2(1–96) for 30 min at 30 °C. The reactions were terminated with 4× Laemmli buffer, and the proteins were separated by 10% SDS-PAGE and blotted to nitrocellulose membrane. p38 MAPK activity was visualized as the phosphorylation of the ATF-2 fragment by autoradiography.

**Transient Cell Transfection and Reporter Gene Assays—**pNF-κB-Luc (κB4, 6x, CLONTECH) and pAP-1-Luc (AP-1, 7x, Stratagene) luciferase reporter gene constructs were kindly provided by Dr. Annemiek Van Linden. cDNA for p38α was amplified by PCR from an HL60 cDNA library (Stratagene) and cloned into the BamHI/XhoI sites of pBCKS+ (Stratagene). The K287M mutation was made using the Altered Sites II kit in vitro mutagenesis system (Promega) to produce a kinase-dead form of p38α. The resulting BamHI/XhoI fragments were inserted into pcDNA3 downstream of a FLAG epitope tag to yield dominant-negative pcDNA3-FLAG-p38α(K287M) plasmid. The wild-type pXL-Smad3 and dominant-negative pXL-Smad3A were described previously (31). RAW 264.7 cells (3.0 × 10^5 cells/well) were plated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum were plated in each well of a 12-well tissue culture plate and cultured overnight. Transfection was carried out using LipofectAMINE Plus reagents according to the manufacturer’s instructions. pSV-β-galactosidase vector (Promega) was co-transfected as internal control to measure differences in transfection efficiency. Luciferase and β-galactosidase activities were measured 4 h after LPS stimulation using a luciferase assay system (Promega) and Glacoto-Light (Tropix), respectively.

**Immunostaining of Nuclear Accumulation of MKP-1**—RAW 264.7 cells were plated at a density of 3 × 10^5 cells/well in a 24-well tissue culture plate on glass coverslips and incubated overnight. The cells were incubated in the presence or absence of PD 98059 (10 μM) for 60 min, followed by TGF-β (10 ng/ml) for 60 min. The cells were then stimulated with LPS (100 ng/ml) for 15 min and fixed in phosphate-buffered saline (PBS) containing 4% glutaraldehyde and 0.2% saponin. The fixed cells were incubated in blocking buffer (PBS containing 10% bovine serum albumin, and 0.2% Triton X-100) for 30 min at room temperature, washed once with PBS, and incubated for additional 30 min in PBS containing 2% bovine serum albumin, 0.2% saponin, and 1:100 dilution of anti-MKP-1. The cells were then washed three times with PBS and incubated for 30 min in PBS containing 2% bovine serum albumin, 0.2% saponin, and 1:100 dilution of anti-MKP-1. The cells were then rinsed three times in PBS, and the coverslips were mounted onto the slides. The mounted RAW cells were viewed with a fluorescence microscope using a 63x Zeiss water objective. Confocal images were achieved using Slidebook v.3.0.4.5 (Intelligent Imaging Innovations, Inc.).

**RESULTS**

Effects of TGF-β on LPS-induced NF-κB- and AP-1-driven Gene Transcription in RAW 264.7 Cells—Previously, we demonstrated that in human macrophages or mouse J774 macrophages, apoptotic cell binding and ingestion results in the early release of TGF-β and that this cytokine contributes to the
decreased production of TNF-α and several chemokines (1, 2). Here we show that blockade of p38 MAPK by the p38 inhibitor SB 203580 also inhibited the LPS-stimulated generation of TNF-α/H9251 and MIP-2, whereas once again the CC chemokine MCP-1 was increased (Fig. 1). Because of the facts that most of the pro-inflammatory mediators are regulated by the transcription factor NF-κB and that MCP-1 is known to be regulated through AP-1 (32–34), we then examined the role of TGF-β in regulating NF-κB- and AP-1-driven gene transcription. RAW 264.7 cells were transiently co-transfected with pNF-κB-luc, together with either an empty vector or the dominant-negative p38 MAPK expression vector. After 48 h, the cells were stimulated with LPS (100 ng/ml) for 4 h and harvested. All the luciferase assays, which were normalized to β-galactosidase, are expressed as fold increase from control. The values are the means ± S.E. from three separate experiments.
TGF-β Regulates Inflammatory Cytokines

The expression of the transfected p38 MAPK was evaluated using Western blot analysis based on the different molecular weights of the transfected FLAG-tagged p38 MAPK and endogenous p38 MAPK (Fig. 2C). As shown in Fig. 2D, LPS significantly increased luciferase activity for NF-κB in cells co-transfected with pNF-κB-luc and an empty vector. The luciferase activity for NF-κB was markedly suppressed in cells transfected with dominant-negative p38 MAPK. These results indicated that p38 MAPK inhibition induces a pattern of pro-inflammatory mediator regulation remarkably similar to the effect of TGF-β in macrophages. From these observations, it seemed likely that TGF-β might act in part by inhibiting p38 MAPK activation.

**Effect of TGF-β on LPS-stimulated MAPK Activation**—Initially, the time course of LPS-stimulated macrophage activation was determined (Fig. 3A). RAW 264.7 cells were stimulated with LPS (100 ng/ml) at various time points before measurement of ERK, p38 MAPK, and JNK phosphorylation. ERK phosphorylation was observed at 5 min, reached maximum at 15 min, and declined at 60 min. Phosphorylation of p38 MAPK was detectable at 5 min, reached maximum at 15 min, and declined rapidly from 30 min to control level at 60 min. Phosphorylation of JNK, however, was only observed at 15 and 30 min. Pretreatment of RAW 264.7 cells with TGF-β for 1 h dose-dependently inhibited LPS-stimulated p38 MAPK phosphorylation (Fig. 3B, upper panel), and this effect was confirmed in p38 MAPK activity assays (Fig. 3B, lower panel).

In contrast, treatment of RAW 264.7 cells with TGF-β induced phosphorylation of ERK even in the absence of LPS. This reached a maximum at 60 min and declined at 120 min (Fig. 3C). No detectable phosphorylation of p38 MAPK or JNK was seen with TGF-β alone (data not shown).

**Cross-talk between MAPKs in TGF-β Inhibition of p38 MAPK**—Blockade of ERK activation by PD 98059, a specific inhibitor of its upstream activator MEK-1/2, increased the LPS-stimulated p38 phosphorylation (Fig. 4A), suggesting that cross-talk between ERK and p38 MAPK might contribute to the TGF-β effects. In addition, inhibition of LPS-stimulated p38 MAPK by SB 203580 resulted in a reciprocal increase of ERK and JNK phosphorylation, showing the potential for cross-inhibition in the other direction (from p38 to ERK or JNK) as well (Fig. 4A).

We next examined whether TGF-β inhibits p38 MAPK by activating ERK. As shown in Fig. 4B, the TGF-β-stimulated ERK phosphorylation was blocked by PD 98059, confirming that TGF-β stimulates ERK activation through MEK/ERK pathway. Importantly, preincubation of RAW 264.7 cells with PD 98059 for 60 min before treatment of the cells with TGF-β resulted in reversal of the TGF-β-mediated suppression of p38
MAPK activity (Fig. 4C). These findings suggested that TGF-β inhibits p38 MAPK by activating ERK.

**TGF-β Inhibits LPS-induced NF-κB-driven Gene Transcription through ERK-driven Suppression of p38 MAPK—**To determine whether TGF-β inhibits LPS-induced NF-κB-driven gene transcription through effects on ERK, we transfected RAW 264.7 cells with a pNF-κB-luc plasmid and pretreated the transfected cells with PD 98059 for 60 min before TGF-β and LPS treatment. As shown in Fig. 5, PD 98059 pretreatment in the absence of TGF-β had no effect on LPS-induced luciferase activity. Consistent with the finding that PD 98059 reversed the inhibitory effect of TGF-β on p38 MAPK activation, the inhibitory effect of TGF-β on LPS-induced luciferase activity for NF-κB was completely abrogated by PD 98059 pretreatment. Moreover, the inhibitory effect of TGF-β on LPS-stimulated MIP-2 or TNF-α production was completely abrogated by PD 98059 pretreatment (data not shown).

**TGF-β Inhibition of LPS-stimulated p38 MAPK Is Not at the Level of MKK-3 or -6—**It has been demonstrated that MKK kinases and MKKs are the upstream activators for p38 MAPK phosphorylation (35). Because both MKK-3 and MKK-6 activate p38 MAPK, we performed Western blots using a phospho-MKK-3,6 antibody to see whether the suppression of p38 MAPK by TGF-β is through its upstream activators. As shown in Fig. 6A, LPS-stimulated phosphorylation of MKK-3 and MKK-6 (seen as a single band) reached a maximum at 15 min. To determine which of the MKKs is responsible for the LPS-stimulated phosphorylation, RAW 264.7 cells were lysed and immunoprecipitated with MKK-3 or MKK-6 antibody before blotting with phospho-MKK-3,6 antibody. A detectable increase in phosphorylation of MKK-3 was observed in cells exposed to LPS when compared with unstimulated cells, whereas no phosphorylation of MKK-6 was detected (Fig. 6B). In addition, Western blot performed on the whole cell lysates using three different MKK-6-specific antibodies (see “Antibodies and Reagents”) failed to detect the protein (data not shown), suggesting that MKK-6 is not constitutively expressed in macrophages. We next evaluated whether TGF-β also inhibits LPS-stimulated MKK-3 phosphorylation. No inhibition was seen (Fig. 6C). To our surprise, both TGF-β and SB 203580 caused an increase in LPS-stimulated phosphorylation of MKK-3 (Fig. 6C). These findings suggested that the blockade of p38 MAPK by TGF-β is at the level of p38 MAPK itself, e.g. not through inhibition of its upstream protein kinases. Moreover, it raises the possibility of a negative feedback loop to regulate the upstream activator MKK-3 by p38 MAPK itself.

**TGF-β Inhibits p38 MAPK through Up-regulation of MAPK Phosphatase-1—**ERK, p38 MAPK, and JNK activation requires dual phosphorylation of threonine and tyrosine residues within the motifs TGY, TEY, and TPY, respectively (36). Dephosphorylation of either residue results in the complete loss of their activity. Inhibition of MAPKs is principally mediated in vivo by members of a family of dual specificity phosphatases. Among them, MPK-1 is up-regulated by ERK and preferentially inac-
MKP-1 after TGF-β/H9252 loading of proteins in each lane was confirmed by reprobing the same lysates were immunoblotted with phospho-MKK-3,6 antibody. Equal loading of proteins in each lane was confirmed by densitometric analysis relative to the level of LPS alone as the means ± S.E. from three separate experiments. The results shown are representative of three experiments.

MKP-1, which in turn was correlated with reversal of TGF-β-mediated inhibition of p38 MAPK.

It has been reported that the selective protein kinase C inhibitor, Ro 31-8220, inhibits MKP-1 expression (39). To further support the involvement of MKP-1 in inactivation of p38 MAPK by TGF-β, we pretreated RAW 264.7 cells with Ro 31-8220 for 30 min to see whether blocking expression of MKP-1 could reverse the TGF-β-mediated inhibition of p38 MAPK. As shown in Fig. 7D, Ro 31-8220 blocked the production of MKP-1, which in turn was correlated with reversal of TGF-β-mediated inhibition of p38 MAPK.

FIG. 7. Increases LPS-induced AP-1-driven Gene Transcription Independent of p38 MAPK Suppression—Because the combination of LPS and TGF-β resulted in a significant increase of luciferase activity for AP-1 (Fig. 2B), we sought to determine whether TGF-β increases AP-1 activation through suppression of p38 MAPK activation. We co-transfected the pAP-1-luc plasmid either with an empty vector or with the dominant-negative p38 MAPK expression vector. Stimulation of RAW 264.7 cells with LPS results in phosphorylation of JNK, which can phosphorylate c-Jun and thus increase AP-1-driven gene transcription. Inhibition of p38 MAPK by SB 203580 resulted in increase of JNK phosphorylation (Fig. 4A), indicating the presence of a suppressive signal from p38 MAPK to JNK. Therefore, inhibition of p38 MAPK might account for the effect of dominant-negative p38 MAPK in causing an increase of AP-1 activation. However, although TGF-β and SB 203580 were shown to inhibit p38 MAPK, TGF-β had no effect on JNK activation (Fig. 4A). Because the TGF-β/Smad signal pathway has been implicated in AP-1-driven gene transcription as well (40), we co-transfected the pAP-1-luc reporter construct either with wild-type or dominant-negative Smad3 plasmid into RAW 264.7 cells. As shown in Fig. 9, co-transfection of wild-type Smad3 with the reporter construct resulted in no change for AP-1 transcription compared with that in Fig. 2B. However, when a dominant-negative Smad3 was co-transfected with the reporter gene, the AP-1 activation induced by TGF-β alone or in combination with LPS was significantly inhibited. As control, the dominant-negative Smad3 had no effect on LPS-induced AP-1 activation alone. Notably, lack of inhibition of TGF-β-induced or TGF-β plus LPS-induced AP-1 activation by PD 98059 (data not shown) indicated that TGF-β-induced AP-1 activation is independent of ERK in macrophages. Taken together, we suggest that TGF-β-induced AP-1-driven gene transcription is via the Smad complex but not through p38 MAPK suppression.
DISCUSSION

In this study, we provide evidence that TGF-β/H9252 blocks inflammatory cytokine production from macrophages through inhibition of p38 MAPK and NF-κB. In contrast, the CC chemokine MCP-1, regulated by AP-1 and the Smad complex, demonstrated a potential for enhanced production after exposure to TGF-β/H9252, implying a selective effect of TGF-β/H9252 on NF-κB-mediated transcription. The key point of action for the suppression is suggested to be at the level of MAPKs and, more importantly, by ERK-dependent inhibition of p38 MAPK.

Even without the addition of TGF-β, the time course of LPS-stimulated MAPK phosphorylation showed a decline in phosphorylation of p38 MAPK as ERK activation increased. Inhibition of ERK (by blocking the activity of the upstream kinases MEK1/2) led to increased phosphorylation of p38 MAPK. TGF-β alone stimulated ERK phosphorylation but had no direct effects on p38 MAPK and JNK. However, when TGF-β was combined with LPS stimulation, ERK activation was enhanced over either stimulus alone and was accompanied by a concomitant decrease in phosphorylation and activation of p38 MAPK. This allowed examination of the possible effects of TGF-β-activated ERK on the degree or extent of p38 MAPK activation, as well as the extent of cross-talk between the different classes of MAPKs.

Thus, blockade of ERK activation using the MEK-1/2 inhibitor PD 98059 resulted in complete prevention of TGF-β-mediated suppression of p38 MAPK activation. On the other hand, blocking p38 MAPK by SB 203580 resulted in a reciprocal...
increase in phosphorylation of ERK and JNK, which suggests cross-inhibition from p38 to ERK as well. In addition, even although SB 203580 is an inhibitor of p38 MAPK activity, it was also seen to reduce p38 MAPK phosphorylation. Although unexplained at this point, the observation has been made previously (41). These inhibitor experiments do support the now widely recognized concept that considerable cross-talk occurs between the different MAPKs, including interactions between inflammatory/stress-activated signal pathways and hormone/growth factor-activated signal pathways (42).

The induction of inflammatory mediators, as well as activity of the NF-κB luciferase reporter construct in response to macrophage stimulation by LPS was shown to be dependent on p38 MAPK activity based on blockade by inhibitor of p38 MAPK and, in addition, by transfection of the cells with a dominant-negative p38 MAPK construct. Although p38 MAPK has been suggested to act at various points in NF-κB activation, nuclear translocation, and transcriptional regulation of inflammatory mediators (30, 43), at issue here is the potential regulation of p38 MAPK activity itself and the role of TGF-β-stimulated ERK activity in this process. Thus, TGF-β inhibition of both p38 MAPK activation and NF-κB luciferase reporter expression was reversed by pretreatment of the cells with PD 98059 to prevent ERK activation. The observations are consistent with MEK/ERK inhibition of NF-κB-driven transcription via suppression of p38 MAPK (44). On the other hand these observations do not support the suggestion that pretreatment of mouse macrophages with TGF-β for 24 h inhibits LPS-stimulated expression of inflammatory cytokines through down-regulation of AP-1 and CD14 receptor expression (45). In macrophages that have ingested apoptotic cells, TGF-β production is rapid (within 60 min) (46). In the present study, LPS-stimulated phosphorylation of ERK and JNK were not inhibited by TGF-β. Moreover, TGF-β combined with LPS synergistically increased luciferase activity for AP-1, indicating intact signaling for LPS via TLR4 and, putatively, CD14. We treated both human monocyte-derived macrophages and J774 cells with TGF-β and then measured CD14 expression. It is not up-regulated nor is it down-regulated (data not shown). Therefore, it seems unlikely that the inhibition of CD14 expression accounts for these early inhibitory effects of TGF-β.

In neutrophils, we have shown that LPS stimulation results in activation of MKK-3, which in turn activates p38 MAPK (47). The same upstream kinase was implicated herein for p38 MAPK activation in RAW 264.7 cells, i.e., LPS-stimulated activation of p38 MAPK preceded by activation of MKK-3. On the other hand, we show that TGF-β had no effect on MKK-3 phosphorylation, suggesting that its effect was on p38 MAPK itself rather than on the upstream activators. Interestingly, blockade of p38 with either TGF-β stimulation or the p38 MAPK inhibitor seemed to enhance MKK-3 phosphorylation. This may imply that p38 MAPK by itself can serve as a negative regulator of its own activation in macrophages. Although the mechanism and physiological significance of the negative feedback is not clear, it could play a role in shut-off and/or control of the inflammatory signal after some period of inflammation.

The most likely explanation for the inhibition of p38 MAPK is by the action of protein phosphatases. Regulation of MAPK by dual specificity MAPK phosphatases have been widely studied and discussed (37, 38, 48, 49). Among nine distinct mammalian MAPK phosphatase family members, MKP-1 (CL 100/3C134), MKP-2, and PAC-1 (phosphatase of activated cells-1) are localized primarily in the nuclear compartment, encoded by immediate-early genes, which are rapidly and highly inducible by many of the stimuli that activate MAPKs. They have distinct patterns of substrate specificity. MKP-1 is known to be up-regulated by ERK and preferentially inactivates p38 MAPK and SAPK/JNK. PAC-1 inactivates p38 MAPK but not JNK. However, PAC-1 was undetectable in RAW 264.7 cells (data not shown). MKP-2 was not considered in this study, because it does not inactivate p38 MAPK (37, 38, 50). That led us to focus on the role of MKP-1 in the p38 MAPK inhibition.

Accordingly, we present evidence to show that TGF-β can induce up-regulation of MKP-1 and that this depended on MEK and ERK. Importantly, LPS stimulation alone did induce a increase in MKP-1 levels, in keeping with the suggested balance between activation and inhibition of p38 activity discussed above. The addition of TGF-β, however, led to increased and prolonged up-regulation and nuclear localization of the phosphatase. TGF-β alone (no LPS) increased up-regulation (Fig 7A, top panel) and nuclear localization of MKP-1 very similar to that of TGF-β plus LPS (Fig 7C); this might explain why TGF-β activates ERK but not p38 MAPK and SAPK/JNK.
in macrophages. As expected, TGF-β/H9252-induced up-regulation of MKP-1 was prevented by the inhibitor of the MEK/ERK pathway. We took two approaches to definitively demonstrate that MKP-1 was responsible for the decreased p38 MAPK phosphorylation. Unfortunately, attempts to use MKP-1 antisense constructs were not successful in reducing the levels of this protein and were therefore not useful. On the other hand, Ro 318220, an inhibitor of protein kinase C that has been reported to inhibit MKP-1 expression independent of protein kinase C inhibition (39), was found to inhibit TGF-β/H9252-induced up-regulation of MKP-1. Taken together, the data support a pathway in which TGF-β activates (and/or enhances the activation of) ERK with subsequent up-regulation of MKP-1. This in turn dephosphorylates and thence limits the amount and extent of p38 MAPK activation with subsequent reduction of NF-κB-dependent inflammatory mediator production (Fig. 10). Although not addressed herein, reduced p38 MAPK activity would also be expected to alter additional p38 MAPK-dependent effects within the cell, including known roles in control of protein translation.

Production of the CC chemokine MCP-1 is known to be predominantly controlled by AP-1 rather than NF-κB (32–34), and as noted earlier, TGF-β was not effective in blocking production of this chemokine. We have suggested that this observation may be important in the sequelae of apoptotic cell clearance during inflammation because, as a monocyte chemoattractant, MCP-1 may be needed to maintain monocyte emigration into resolving lesions to complete the clearance process (2). This would be in keeping with the synergistic activation of AP-1 reporter activity seen with TGF-β and LPS co-stimulation (Fig. 2B). Because JNK-induced phosphorylation of c-Jun can lead to activation of AP-1 (51) and inhibition of p38 MAPK by SB 203580 caused an increase in the phosphorylation of JNK, the TGF-β-induced enhancement of AP-1 might proceed via this mechanism. We found a similar enhancement of AP-1 reporter activity after transfection with dominant-negative p38 MAPK. However, when TGF-β was examined, despite its increased effects on AP-1, no alteration was seen in LPS-stimulated JNK phosphorylation at 15 min (Fig. 3D) and 30 min (data not shown). This suggests that TGF-β effects on AP-1 activation in the presence of LPS are not proceeding through cross inhibition of JNK by p38 MAPK. We suggest that the reason for the inability of TGF-β to mimic the effect of SB 203580 to increase JNK activation might be due to the ERK-induced increase in MKP-1, which is known to inactivate SAPK/JNK as well as p38 MAPK.

Although many studies show functional interaction between members of the Smad family and MAPK signaling pathways (52–55), we did not find any effects of PD 98059 on TGF-β- or TGF-β plus LPS-induced AP-1 activation. It has been reported that c-Jun homodimers and c-Jun-c-Fos heterodimers activate transcription through their ability to interact directly with the AP-1-binding site and that the same is true with the complex of Smad2/Smad3 and Smad4 (40). Furthermore, following TGF-β stimulation, the Smad complex itself binds to AP-1 and results in transcription from the AP-1 binding site (10). Our results with dominant-negative Smad3 support such an interaction in macrophages. Thus, LPS and TGF-β added separately would activate AP-1 via the JNK and Smad pathway, respectively. The synergistic activation of AP-1 by TGF-β and LPS together might then be caused by the combined effects of three signaling processes: LPS/JNK/AP-1, TGF-β/Smads, and the complex of AP-1 with Smads (Fig. 10).

In conclusion, our results support the concept that TGF-β...
inhibits inflammatory cytokine production through the cross-talk between MAPKs, specifically ERK-dependent inhibition of p38 MAPK caused by up-regulation of MKP-1. The ability of apoptotic cells to initiate TGF-β production during their recognition and uptake through a specific receptor for phosphatidylserine has been shown to suppress inflammatory mediators in vitro (3) and in vivo (46) with resultant resolution of the inflammatory process (46). Presumably, TGF-β could be acting via these signaling pathways not only to enhance resolution of inflammation but also perhaps to prevent its occurrence in the first place during removal of apoptotic cells in development and tissue remodeling.

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