Different Mitogen-activated Protein Kinase Signaling Pathways Cooperate to Regulate Tumor Necrosis Factor α Gene Expression in T Lymphocytes*

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Tumor necrosis factor α (TNF-α) is a potent proinflammatory cytokine and plays a crucial role in early events of inflammation. TNF-α is primarily produced by monocytes and T lymphocytes. In particular, T-cell-derived TNF-α plays a critical role in autoimmune inflammation and superantigen-induced septic shock. However, little is known about the intracellular signaling pathways that regulate TNF expression in T cells. Here we show that extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38-mitogen-activated protein kinase (MAPK) pathways control the transcription and synthesis of TNF-α in A3.01 T cells that produce the cytokine upon T cell activation by costimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin. Selective activation of each of the distinct MAPK pathways by expression of constitutively active kinases is sufficient for TNF-α promoter induction. Furthermore, blockage of all three pathways almost abolishes TPA/ionomycin-induced transcriptional activation of the TNF-α promoter. Selective inhibition of one or more MAPK pathways impairs TNF-α induction by TPA/ionomycin, indicating a cooperation between these signal transduction pathways. Our approach revealed that the MAPK kinase 6 (MKK6)/p38 pathway is involved in both transcriptional and posttranscriptional regulation of TNF expression. Moreover, analysis of the progressive 5′ deletion mutants of the TNF-α promoter indicates that distinct promoter regions are targeted by either ERK, JNK, or p38-activating pathways. Thus, unlike what has been reported for other TNF-α-producing cells, all three MAPK pathways are critical and cooperate to regulate transcription of the TNF-α gene in T lymphocytes, suggesting a T cell-specific regulation of the cytokine.

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The abbreviations used are: TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; MKK6, MAPK kinase 6; MLK3, mixed-lineage kinase 3; TPA, 12-O-tetradecanoylphorbol-13-acetate; CsA, cyclosporin A; JIP-1, JNK-interacting protein 1; CRE, cAMP responsive element; LPS, lipopolysaccharide; HA, hemagglutinin; GST, glutathione S-transferase; MBP, myelin basic protein.
phosphorylation by Raf (19). JNK is activated by SAPK/ERK kinase (SEK, also known as MKK4) as well as by the recently identified kinase MKK7 (20–22). The activation of JNK is further controlled by a putative scaffold protein, JNK-interacting protein 1 (JIP-1), which binds to JNK and several other components of the JNK pathway (23). Overexpression of JIP-1 or the JNK-binding domain of JIP-1 leads to the cytoplasmic retention of JNK and the inhibition of JNK-dependent gene expression (16). One of the SAPK/ERK kinase activators (reviewed by Fanger et al., Ref. 20) is the mixed lineage kinase 3 (MLK3) also known as the SH3 domain-containing proline-rich kinase (SPRK) (24). MAPK kinase 6 (MKK6) functions as an activating kinase for all known p38 isoforms (25–27), whereas MKK3 predominantly activates the isoform p38γ (28). Until this time, a specific physiological activator of MKK6 has not been identified.

Whereas MAPK-activating pathways have been implicated in LPS-induced TNF-α expression by monocytes and macrophages at diverse control levels (10–12), and two reports show that the JNK and ERK pathways play a role in TNF-α expression by mast cells (2, 9), the contribution of MAPKs to the regulation of TNF-α expression in T lymphocytes is still unclear.

Two findings suggest a cell type-specific involvement of intracellular signaling pathways inducing TNF-α expression: (a) in lymphocytes versus monocytes, different sets of transcription factors are recruited to the promoter of the TNF-α gene (29, 30), and (b) extracellular stimuli with a cell type-specific function trigger TNF-α expression in different cells, such as LPS in monocytes, FceRI receptor aggregation in mast cells, or activation of the antigenic receptor in T lymphocytes. Antigenic activation of T lymphocytes, which can be mimicked by costimulation with a phorbol ester such as TPA and a calcium ionophore such as ionomycin (for a review, see Ref. 31), leads to a rapid induction of TNF-α transcription that does not require new protein biosynthesis (32).

To investigate T-cell-specific regulation of the TNF-α gene, we analyzed the involvement of distinct MAPK pathways in TNF-α transcription and biosynthesis upon activation of the human T-cell line A3.01. We demonstrate that ERK, JNK, and p38 pathways that are activated upon stimulation with TPA and ionomycin (TPA/ionomycin) are critical for and cooperatively contribute to the induction of TNF-α expression in these T cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies—**A3.01 human T lymphoma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum to a density of 8 × 10^5 cells/ml. Cells were incubated at 37 °C in humidified air with 7% CO_2. Antibodies raised against ERK2 (sc-154), JNK1 (sc-474), and p38 (sc-535) were purchased from Santa Cruz Biotechnology, Inc. The monoclonal antibodies against the HA tag (12CA5) were produced and purified according to a standard protocol (33). The eukaryotic expression vector for HA-SAPKα (PharMingen) was used at a concentration of 2–20 μg/ml TPA/ ionomycin (Sigma) for up to 24 h. The MEK-specific inhibitor PD098059 (Calbiochem) was used in a 20 μM concentration of a 20 μM stock solution in DMSO. Actinomycin D (Sigma) was used at a concentration of 2 μg/ml of a 0.4 mg/ml stock solution in 10% ethanol, and cyclosporin A (CSA) (Sigma) was used at a concentration of 200 μg/ml of a 10 mg/ml stock solution in DMSO. Cells were preincubated with these inhibitors 30 min before stimulation.

**TNF-specific Flow Cytometry Analysis—**To determine the expression of TNF-α, an intracellular immunostaining procedure and subsequent flow cytometry analysis were applied. A3.01 T cells were split 24 h before stimulation. The stimulation was carried out in the presence of 2 μM monensin (Sigma), which prohibits the secretion of proteins, thereby leading to intracellular retention of the produced protein. Treatment of monensin did not affect the basal or induced MAPK activity (data not shown). After a stimulation time of 2 or 10 h, cells were harvested, washed once in phosphate-buffered saline, fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline at 4 °C for 20 min, and subject to the incubation and washing steps described below in permeabilization buffer containing 1% fetal calf serum and 0.1% (w/v) saponin in phosphate-buffered saline. According to the manufacturer’s instructions (PharMingen), cells were then incubated with the primary antibody in permeabilization buffer supplemented with 2% goat serum. A mouse IgG1 antiserum (Dako) was used as an isotype-specific control. The monoclonal mouse anti-human TNF-α antibody of isotype IgG1 (PharMingen) was used at a concentration of 2–20 μg/ml of a 100 μg/ml stock solution in DMSO. Cells were preincubated with these inhibitors 30 min before stimulation.

**Immunoprecipitation, Kinase Assay, and Immunoblotting—**Cells were lysed in radioimmunoprecipitation buffer (25 mM Tris-HCl, pH 8, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml calf thymus extract). Lysates were cleared by centrifugation, and 50 μl of precleared cell lysate according to a standard protocol (38). Ten microliters of the precleared cell lysate was then subjected to immunoprecipitation, kinase assay, and immunoblotting. The eukaryotic expression vector for HA-SAPKα and the prokaryotic expression vector pGEX-KG-c-Jun(1–135) were gifts from J. Kyriakis and P. Godowski (34). Raf-BXB-CX (constitutively active Raf) lacks the N-terminal negative regulatory domain and contains the C-terminal active kinase domain of c-Raf I (18, 35). MKK6(EE) is a constitutively active mutant of MKK6 with two serines involved in the activation of the kinase replaced by glutamic acid (26). The interfering mutants of ERK2, SAPKβ, MKK6, Raf-BBX-CX, and MLK3 are ATP-binding site mutants generated by the replacement of lysine with arginine (ERK2/B3), alanine (MKK5) or glutamic acid (MKK6(EE) and MLK3 K144A) (26, 34, 36, 37). JIP-1 is a cytoplasmic protein that was identified as a putative scaffold protein that binds to several components of the JNK pathway and regulates JNK activity (23). Overexpression of JIP-1 or the JNK-binding domain of JIP-1 inhibits JNK activity by causing cytoplasmic retention of JNK that leads to the subsequent inhibition of JNK-regulated gene expression (16). The JIP-1 cDNA was used in this study consists of the JNK-binding domain fused to a Flag-Tag and was kindly provided by R. Davies. All cDNAs were subcloned in the pRSPA vector.
Regulation of TNF-α Expression by MAPKs in T Cells

Selective Activation of ERK, JNK, or p38 Signaling Pathways Stimulates TNF-α Promoter-dependent Transcription—To investigate the role of the ERK, JNK, or p38 signaling pathways as mediators of induced TNF-α transcription, we performed transient cotransfection experiments and measured the human TNF-α promoter activity as promoter-dependent luciferase expression in human T-cell line A3.01. Previously, we have established an approach to selectively activate ERK, JNK, or p38 in A3.01 T cells by expressing constitutively active versions of corresponding upstream kinases (18). Briefly, a constitutively active kinase mutant of Raf (Raf-BXB-CX) serves as a specific ERK activator. Overexpression of MLK3 results in a strong activation of JNK without affecting ERK and p38 activities. Finally, an active mutant of MKK6 (MKK6(EE)) is a specific activator of p38 (18).

Expression of each of these kinases in A3.01 cells is sufficient to induce strong TNF-α promoter activity in a concentration-dependent manner (Fig. 1A–C, see also Fig. 6), although they exert no effect on a nonspecific thyminidine kinase minimal promoter (data not shown). The corresponding catalytically inactive kinase versions showed no significant effect on the TNF-α promoter, even at the highest input (Fig. 1, A–C). Moreover, combining MKK6(EE) with either Raf-BXB-CX or MLK3 synergistically enhanced the promoter activity (Fig. 1D), suggesting cooperation between these signaling pathways in the regulation of TNF-α-specific transcription.

We next investigated the role of MAPK pathways in TNF-α gene expression of T cells activated by TPA/ionomycin in more detail.

Expression of TNF-α by Activated A3.01 T Cells—To characterize the regulation of TNF-α expression, we stimulated A3.01 T cells with TPA, ionomycin, or a combination of both. The inducibility of TNF-α expression was determined at both the translational and transcriptional levels by TNF-α-specific flow cytometry analysis and a TNF-α promoter reporter gene assay, respectively. Reporter gene analysis allows for the assessment of TNF-α promoter activity independent of TNF-α mRNA stability, another control level of expression. Unstimulated A3.01 cells do not produce any detectable amount of TNF-α (Fig. 5A). Stimulation of these cells with TPA results in a weak induction of TNF-α transcription (Fig. 2B) and synthesis (Fig. 2A). A high induction of both transcription and protein synthesis was observed by cotreatment with TPA and ionomycin (Fig. 2). TPA/ionomycin-induced TNF-α production is sensitive to cyclo-

RESULTS

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sporin A, whereas TPA-induced protein synthesis is unaffected (Fig. 2A). TNF-α protein synthesis was detectable as early as 2 h after TPA/ionomycin stimulation only in the absence of the transcriptional inhibitor actinomycin D (Fig. 2A), indicating that TPA/ionomycin-induced TNF-α synthesis requires de novo transcription of the TNF-α gene.

Therefore, we analyzed the time dependence of TNF-α transcription compared with TNF-α production. TNF-α promoter activity as well as protein synthesis is induced after 2 h of TPA/ionomycin stimulation and reaches maximal induction levels after 6 and 8 h, respectively (Fig. 3, A and B). These results indicate that the regulation of TNF-α expression in A3.01 cells is similar to that of primary T cells and other T-cell lines (reviewed in Ref. 1).

As shown previously (17, 18), TPA/ionomycin treatment leads to a strong activation of the MAPK family members ERK, JNK, and p38 in T cells, whereas TPA stimulation leads solely to a maximal activation of ERK. To correlate the kinetics of MAPK activation with the level of TNF-α transcription, we determined the kinase activities of ERK, JNK, and p38 in time course experiments. ERK activity is maximally induced within 5 min of TPA/ionomycin treatment, whereas JNK and p38 activation reach maximal induction after 15 min. After 60 min, MAPK activities drop to levels that are still detectable after 4 h. This rapid stimulation of MAPK activity preceding the induction of TNF-α transcription suggests a functional connection of both processes.

Selective Inhibition of Distinct MAPKs in A3.01 T Cells—To test whether MAPK pathways are functionally involved in TPA/ionomycin-induced TNF-α expression, we established conditions to selectively inhibit the activation of each of the three MAPKs. For this purpose, specific kinase inhibitors and negative interfering kinase mutants were tested for efficiency and specificity.

The PD98059 compound has been described as a specific inhibitor of MEK activation (39). Indeed, titration experiments in A3.01 cells (data not shown) revealed maximal inhibitory effects of this inhibitor at a concentration of 20 μM, at which it acts specifically on ERK without affecting JNK or p38 activity (Fig. 4). Preincubation of A3.01 cells with this inhibitor resulted in a 90% inhibition of TPA/ionomycin-induced ERK activation as determined by immunocomplex kinase assays (Fig. 4A, top panel). In contrast, JNK and p38 activity remained unaffected by PD98059 (Fig. 4A, middle and bottom panels).

Because TNF-α production (Fig. 2A) as well as TNF-α transcription...
promoter-dependent transcription (data not shown) is sensitive to cyclosporin A, we measured the effects of this inhibitor on the MAPK activities. Interestingly, JNK as well as p38 activities were significantly inhibited, whereas ERK was not affected (Fig. 4A). JNK inhibition by CsA has been observed previously in Jurkat T cells (17); however, p38 was not included in those studies.

Specific inhibition of JNK/SAPK activity was achieved by overexpression of the protein JIP-1 (Ref. 16; see “Experimental Procedures” for details). To determine the inhibitory efficiency of JIP-1 in transiently transfected A3.01 T cells, we coexpressed JIP-1 with HA-SAPKβ and MLK3 and/or JIP-1 and stimulated with TPA/ionomycin as indicated. HA-SAPKβ was immunoprecipitated from each sample using a HA-specific monoclonal antibody, and its activity was determined in immunocomplex kinase assays with GST-c-Jun(1–135) as a substrate. Equal protein load was verified by immunoblotting. C, the 4x AP-1/Ets promoter construct was used to determine the specificity of PD98059 and SB203580. This reporter construct is inducible by transfection of either Raf-BXB-CX, MLK3, or MKK6(EE) by 95–400- or 250-fold, respectively (data not shown). A3.01 cells were cotransfected with the 4x AP-1/Ets promoter construct and either Raf-BXB-CX, MLK3, or MKK6(EE). Cells were left untreated or treated immediately after transfection with either DMSO (solvent control), PD98059 (PD), or increasing amounts of SB203580 as indicated. Promoter activities are expressed as the percentage of relative luciferase activity based on that of untreated controls (w/o) of each kinase. The figure shows the mean of three independent experiments.

FIG. 4. PD98059, SB203580, and JIP-1 are selective inhibitors for ERK, p38, and JNK, respectively, in A3.01 T cells. A, cells were left untreated (c, w/o) or were treated with the solvent DMSO, PD98059, or CsA before stimulation with TPA/ionomycin. Kinase activities of ERK (A, top panel), JNK (A, middle panel), and p38 (A, bottom panel) were determined as described above. Numbers in bold in the autoradiograms indicate kinase activation in fold compared with the unstimulated control (c). Corresponding immunoblots verify equal amounts of immunoprecipitated kinases. B, cells were cotransfected with HA-SAPKβ and MLK3 and/or JIP-1 and stimulated with TPA/ionomycin as indicated. HA-SAPKβ was immunoprecipitated from each sample using a HA-specific monoclonal antibody, and its activity was determined in immunocomplex kinase assays with GST-c-Jun(1–135) as a substrate. Equal protein load was verified by immunoblotting. C, the 4x AP-1/Ets promoter construct was used to determine the specificity of PD98059 and SB203580. This reporter construct is inducible by transfection of either Raf-BXB-CX, MLK3, or MKK6(EE) by 95–400- or 250-fold, respectively (data not shown). A3.01 cells were cotransfected with the 4x AP-1/Ets promoter construct and either Raf-BXB-CX, MLK3, or MKK6(EE). Cells were left untreated or treated immediately after transfection with either DMSO (solvent control), PD98059 (PD), or increasing amounts of SB203580 as indicated. Promoter activities are expressed as the percentage of relative luciferase activity based on that of untreated controls (w/o) of each kinase. The figure shows the mean of three independent experiments.
ERK2 (ERK2(B3)), SAPKβ (SAPKβ(K-R)), and MKK6 (MKK6(A)) was confirmed in our previous studies by kinase assays and reporter gene analysis in different cell systems including A3.01 T cells (18, 43). These studies established ERK2(B3), SAPKβ(K-R), and MKK6(A) as efficient dominant negative mutants selective for the ERK, SAPK, and p38 signaling pathways, respectively.

ERK-, JNK-, and p38-activating Pathways Critically Contribute to TPA/ionomycin-induced TNF-α Expression—After establishing tools for the selective disruption of signaling through specific MAPK cascades, we tested the effects of these inhibitors on the TPA/ionomycin-induced TNF-α promoter activity (Fig. 5B) and TNF-α biosynthesis measured by TNF-α-specific fluorescence (Fig. 5A). Blockage of ERK and p38 activation by PD98059 and SB203580, respectively, results in a significant decrease in TNF-α specific fluorescence (Fig. 5A), indicating the crucial role of ERK and p38 pathways in TNF-α biosynthesis in A3.01 T cells.

Inhibition of ERK signaling by ERK2(B3) overexpression or pretreatment with PD98059 also impaired the TNF-α promoter activity (Fig. 5B). Moreover, we observed a partial reduction of induced promoter activity when JNK/SAPK activation is blocked by the expression of dominant negative SAPKβ(K-R) or the inhibitory protein JIP-1. In contrast, blockage of signaling through p38 by the expression of MKK6(A) or incubation with up to 4 μM SB203580 did not impair TPA/ionomycin-induced TNF-α promoter activity. The induction of TNF-α promoter is almost abolished (Fig. 5B) using higher concentrations of SB203580 (up to 20 μM), at which ERK and JNK activities are also inhibited.

These data point to a cooperation of MAPK signaling pathways in the regulation of the TNF-α promoter. To prove this assumption, we blocked p38 with specific concentrations of SB203580 (4 μM) combined with expression of JIP-1 and/or PD98059 treatment to block two or all three MAPK pathways. Although treatment with SB203580 alone did not exert any effect on the induced promoter activity, the compound enhances the inhibitory effects of JIP-1 and PD98059 (Fig. 5B). Blockage of all three pathways by the combined action of JIP-1, SB203580, and PD98059 almost abolished the TNF-α promoter induction by TPA/ionomycin (Fig. 5B).

These data suggest that all three MAPK signaling pathways cooperate in the regulation of the TNF-α promoter during T-cell activation. Whereas the p38-activating pathway appears to be indispensable for posttranscriptional events in TNF-α biosynthesis, as demonstrated by intracellular TNF-α staining in stimulated cells pretreated with SB203580 (Fig. 5A), it can be
substituted by another signaling pathway in transcriptional processes induced by TPA/ionomycin. ERK and JNK pathways, however, are necessary for maximal induction of the promoter activity.

Distinct Regions of the TNF-α Promoter Are Responsive to Each MAPK Activating Cascade—To assess whether the contribution of each MAPK pathway is connected to distinct promoter elements, a series of 5′ deletion mutants of the TNF-α promoter (Fig. 6A) were cloned in front of the luciferase cDNA in the pGL3 vector. These reporter constructs were used in cotransfection experiments with the constitutively active kinase activators. The region between nucleotides -1057 to -600 (TP-1057) resulted in a slight decrease in induction by active Raf and a strong decrease if cells were transfected with MKK6(EE) or MLK3. Whereas there is a drop in Raf-induced promoter activity by the deletion of the region up to nucleotide -120 (TP-120) (Fig. 6B), no such decline is observed for MKK6(EE) or MLK3. The basic empty luciferase vector pGL3 served as a negative control and showed no significant induction by the kinase activators.

The intact promoter up to nucleotide -1057 (TP-1057) relative to the transcriptional start site showed high inducibility by all three activators (Fig. 6B). A deletion from nucleotide -1057 to -600 (TP-600) resulted in a slight decrease in induction by active Raf and a strong decrease if cells were transfected with MKK6(EE) or MLK3. Whereas there is a drop in Raf-induced promoter activity by the deletion of the region up to nucleotide -120 (TP-120) (Fig. 6B), no such decline is observed for MKK6(EE) and MLK3-induced transcription. However, the MKK6(EE) and MLK3 inducibility of the promoter is almost abolished when the deletion is extended to nucleotide -105 (TP-105).

These data indicate that there are overlapping but distinct regions of the TNF-α promoter targeted by the different pathway activators. The region between -1057 and -600 is responsible for induction by all three pathways; Raf also targets elements within nucleotides -200 to -120. MLK3 and MKK6(EE) overlap in their responsive regions, which require nucleotides -120 to -100.

**DISCUSSION**

In this report, we show for the first time that selective activation of ERK, JNK, or p38 signaling pathways is sufficient for rapid transcriptional activation of the TNF-α promoter and that these pathways are critical and act in concert to mediate TPA/Ionomycin-induced TNF-α transcription and production in A3.01 T-cells. This indicates that in contrast to other TNF-producing cell lines, all three MAPK pathways play a pivotal role in the induction of TNF-α transcription in activated T cells.

According to our data, the first regulatory step of induced TNF-α expression in activated T cells is transcriptional initiation, because the onset of TNF-α synthesis is abolished by treatment with the transcriptional inhibitor actinomycin D (Fig. 2A). Therefore, the transcriptional regulation of TNF-α expression may be the most critical step. This assumption is supported by earlier observations in a monocytic cell line, where a large increase in secreted TNF-α levels is primarily due to transcriptional activation of the TNF-α gene by LPS (44).

Our findings demonstrate that TNF-α promoter activity is regulated by all three MAPK signaling pathways in T cells. A critical role for the ERK pathway is illustrated by the observation that selective activation (Figs. 1A and 6) or inhibition of this pathway (Fig. 5) positively or negatively interferes with the induction of TNF-α expression, respectively. In macrophages the Raf/MEK/ERK pathway has also been reported to be critical for LPS-induced TNF-α transcription (11). However, in contrast to our data in this study, constitutively active Raf alone was not sufficient to transactivate the promoter, as was the case in a mast cell line (9). In mast cells, the role of the ERK pathway in TNF production is still unclear, because one report favors the involvement of ERK (2), whereas others suggest that the Raf/MEK/ERK pathway is not required (9). This difference may be accounted for by the use of two different mast cell lines in these studies.

The JNK pathway also appears to be of critical importance.
for TNF-α transcription in A3.01 T cells because both selective activation (Figs. 1B and 6) or inhibition of JNK signaling (Fig. 5B) reduces or blocks the induced promoter activity, respectively. Similar observations have been made in a mast cell line (9) where TNF-α promoter-dependent reporter gene activity was induced by expression of the JNK activator MEK kinase (MEKK), and this induced activity was partially blocked by a dominant negative mutant of JNK. In macrophages, the involvement of the JNK pathway in the regulation of LPS-induced TNF-α biosynthesis was reported to be on the translational rather than the transcriptional level (12), because a dominant negative JNK mutant blocked the translation of TNF-α mRNA but not the LPS-induced transcription of the TNF-α promoter.

Our study also defined a functional role of the p38 pathway in the transcriptional induction of TNF-α promoter in T cells for the first time. Selective activation of this pathway readily induces transcription (Figs. 1C and 6); however, blockage of p38 activity by SB203580 only exerts inhibiting effects on the induced promoter activity in the absence of either JNK or ERK signaling (Fig. 5B). A plausible mechanism might be that the signal is at least partially mediated by SB203580-insensitive p38 isoforms such as p38δ, which is expressed in T cells (45). Nevertheless, SB203580-sensitive p38 isoforms are also involved, because the inhibitor clearly shows synergistic effects if either ERK or JNK signaling is blocked. This may be due to a dosage effect with regard to the number of MAPKs involved. The blockage of some p38 isoforms may be overcome by other unaffected MAPKs. However, if the number of active MAPKs is further reduced by other inhibitors, such as PD98059 or JIP-1, the blockage of p38 activity could not be bypassed anymore.

The role of the p38 pathway in transcription of the TNF-α gene has not been elucidated in macrophages; however, similar to our observation in T cells, p38 regulates posttranscriptional processes (10). In contrast, no critical role for the p38 pathway in TNF-α transcription and biosynthesis has been observed in mast cells (2, 9).

Although selective activation of either pathway is sufficient to induce TNF-α promoter activity (Fig. 1), the function of each MAPK pathway appears not to be redundant. Several findings support a cooperation between the MAPK pathways: (a) TPA stimulation that maximally induces ERK but not JNK or p38 activity (18) is not sufficient to achieve full promoter activity (Fig. 2B), (b) synergistic activation of the promoter is observed by the coexpression of active MKK6 with either active Raf or MLK3 (Fig. 1D), and (c) promoter induction is reduced when either JNK or ERK activation was inhibited and is almost abolished after a blockage of all three pathways (Fig. 5B). While preparing this article, a report has been published showing that the serine/threonine kinase Cot up-regulates TNF-α promoter-driven expression in Jurkat T cells (46). Cot activates both ERK and JNK via MEK and SEK kinase, respectively (47, 48). Cot-induced TNF-α promoter activity was partially inhibited by the MEK inhibitor PD98059, which is consistent with our finding that the ERK signaling pathway is involved in the regulation of TNF-α gene transcription. According to our data, the remaining promoter activity might be due to the JNK signal, which is not blocked by PD98059.

It was reported earlier that CsA affects JNK but not ERK activity in activated T cells (17). Here we show that p38 activity is also partially inhibited by CsA (Fig. 4A), suggesting a similar activation route for p38 and JNK (49). Because CsA only inhibits TNF-α production if cells are cooperatively treated with both TPA and ionomycin (Fig. 2A), it is most likely that the inhibiting effect of CsA is mediated by an inhibition of p38 and JNK, which are strongly activated in the presence of the co-stimulus ionomycin (Fig. 3, D and E) (18).

The different observations concerning the contribution of MAPK pathways to the transcriptional regulation of TNF-α in mast cells, monocytes, and lymphocytes are probably due to cell lineage specificities. A cell type-specific regulation of the cytokine has been suggested earlier when comparing TNF-α synthesis in a dendritic cell line versus a mast cell line (50). T-cell specificity is postulated to be due to an involvement of the T-cell-specific transcription factor NFATp controlling the TNF-α promoter activity (30, 51). NFATp bound to the k factor binding site 3 (k3) acts cooperatively with the CRE binding factors ATF-2/Jun. The combined CRE/k3 site is located at nucleotide −106 to −88 relative to the transcriptional start site and is required for T-cell stimulation by the antigen receptor (30, 51). Because ATF-2 is a substrate for p38, and both c-Jun and ATF-2 are JNK substrates (15, 52–54), it is most likely that the JNK and p38 signaling pathways contribute to the regulation of the CRE/k3 element. Supporting this assumption, a promoter construct without an intact CRE site (TP-105) is much less inducible by MLK3 or MKK6(EE) compared with a promoter construct with an intact CRE/k3 site (TP-120) (Fig. 6), suggesting that the CRE/k3 site is targeted by JNK- and p38-activating pathways. In contrast, a Raf-responsive region appears to be located between nucleotides −200 and −120 (Fig. 6). Thus far, binding sites for Krox-24/Egr-1 (55), SP-1, and NFAT (30, 51) have been identified in this region, and a promoter element containing the Egr-1 site was found to be essential for TPA-induced promoter activity in T cells (56).

Deletion of the promoter from nucleotide −600 to −200 does not result in remarkable changes of promoter activity, which is consistent with an earlier report (32). Interestingly, there is a significant reduction in the inducibility of the TNF-α promoter by active Raf, MLK3, or MKK6 when the region between −1057 and −600 is deleted (Fig. 6). These data indicate that there are one or more responsive sites to these kinases in T cells. Two κ factor binding sites (k1 and k2) are located within this region at nucleotides −650 and −610. These are extremely conserved and are important for LPS responsiveness in monocytes of several species (57). These sites may also contribute to induced TNF expression in activated T cells. Furthermore, there might be other regulatory regions upstream of −600 within the human TNF-α promoter that have yet to be identified.

In conclusion, our data indicate that TNF-α expression in T cells is regulated by several distinct MAPK pathways that functionally cooperate and are critical for transcriptional as well as for posttranscriptional processes. The involvement of ERK, JNK, and p38 pathways in transcriptional regulation of the TNF-α gene suggests a T-cell-specific regulation. These data might be helpful with regard to cell type-specific therapeutical modulation of the TNF-α expression in a beneficial way.

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