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Review

MAPKAP kinases MK2 and MK3 in inflammation: Complex regulation of TNF biosynthesis via expression and phosphorylation of tristetraprolin

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ABSTRACT

Downstream of mitogen-activated protein kinases (MAPKs), three structurally related MAPK-activated protein kinases (MAPKAPKs or MKs) – MK2, MK3 and MK5 – signal to diverse cellular targets. Although there is no known common function for all three MKs, MK2 and MK3 are mainly involved in regulation of gene expression at the post-transcriptional level and are implicated in inflammation and cancer. MK2 and MK3 are phosphorylated and activated by $p38^{MAPK\alpha,\beta}$ and, in turn phosphorylate various substrates involved in diverse cellular processes. In addition to forwarding of the p38-signal by MK2/3, protein complex formation between MK2/3 and p38 mutually stabilizes these enzymes and affects $p38^{MAPK}$ signaling in general. Among the substrates of MK2/3, there are mRNA-AU-rich-element (ARE)-binding proteins, such as tristetraprolin (TTP) and hnRNP A0, which regulate mRNA stability and translation in a phosphorylation-dependent manner. Phosphorylation by MK2 stabilizes TTP, releases ARE-containing mRNAs, such as TNF-mRNA, from default translational repression and inhibits their nucleolytic degradation.

Here we demonstrate that MK2/3 also contribute to the *de novo* synthesis of TTP. Whether this contribution proceeds via transcription factors directly targeted by MK2/3 or via chromatin remodeling by the reported binding of MK2/3 to the polycomb repressive complex is still open. A model is proposed, which demonstrates how this new function of transcriptional activation of TTP by MK2/3 cooperates with the role of MK2/3 in post-transcriptional gene expression to limit the inflammatory response.

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1. Introduction

Both $p38^{MAPK\alpha}$ and MK2 are elements of TLR- and cytokine-signaling and are, therefore, preferential target molecules to treat chronic inflammation involved in asthma, rheumatoid arthritis, inflammatory bowl disease, atherosclerosis, Alzheimer's disease, ischemic heart and brain diseases as well as cancer for orally available small molecules [1,2]. Inhibitors against $p38^{MAPK}$, such as the mostly used compound SB203580 and its successors, have been tested in animal models and in the clinics, block acute and chronic inflammation efficiently, but show side effects such as liver toxicity and skin rash. Unexpectedly, these "side effects" might

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result from "on target"-effects of the p38 inhibitors, since the feedback control of $p38^{MAPK\alpha}$ on TAK1 [3], an activator of $p38^{MAPK\alpha}$ and JNKs, is inhibited as well. Hence, inhibition of $p38^{MAPK\alpha}$ leads to increased JNK activity [4] which may contribute to liver toxicity [5] and tumor growth [6]. Furthermore, $p38^{MAPK\alpha}$ also phosphorylates and activates the mitogen- and stress-activated kinases MSK1 and MSK2, which were recently demonstrated to display anti-inflammatory function [7]. $p38^{MAPK\alpha}$ is a central signaling element, stimulated not only by inflammation, and its targeted deletion in mouse leads to embryonic lethality due to placental defects [8,9].

Due to these problems with the use of inhibitors of $p38^{MAPK\alpha}$, targets downstream to $p38^{MAPK\alpha}$, such as MK2 and MK3, become recently more interesting for anti-inflammatory therapy. In contrast to $p38^{MAPK\alpha}$, MK2 and MK3 do not participate in the feedback signaling loop to TAK1. Furthermore, the MK2 knockout and the MK2/3 double knockout are viable and fertile and, apart from defects in LPS-induced cytokine biosynthesis, do not display any further abnormalities [10,11]. Due to reduced TNF production, MK2-deficient mice are resistant to endotoxic shock [10] and collagen-induced arthritis [12]. Only a few potent and selective MK2/3 inhibitors are described so far, but progress has already been made [13–18]. Recently, the first orally available small molecule MK2 inhibitor of the benzothiophene type, PF-3644022, was demonstrated to be effective in a chronic streptococcal cell wall-induced arthritis model in rats [19]. This ATP-competitive inhibitor displays a K_i in the low nanomolar range and good selectivity when profiled against 200 human protein kinases. However, the biochemical efficiency of PF-3644022, the ratio of binding affinity to target versus cellular activity, is rather poor (0.03) compared to most drugs on the market. Hence, the search for further small molecule inhibitors against MK2/3 will be necessary. In this regard, it will be absolutely required to know more about the molecular mechanisms of MK2/3 action in regulation of TNF biosynthesis to find new targeting strategies for these essential molecules, their activation and substrates.

2. Molecular mechanisms of regulation of TNF biosynthesis by MK2/MK3

The role of MK2 in TNF biosynthesis became obvious from the phenotype of the MK2 knockout mice, which showed increased resistance against LPS/galactosamine-driven endotoxic shock due to strongly reduced TNF serum levels (about 10% of wild type levels) [10]. Interestingly, the TNF-mRNA level in MK2-deficient spleen cells and macrophages after LPS-induction does not show the same strong reduction, but was, at best, modestly reduced [10,20]. This indicated the intriguing possibility that MK2 contributes to TNF biosynthesis by specific post-transcriptional regulation of TNF-mRNA stability and translation [21]. The role of MK3 in TNF biosynthesis could be elucidated when comparing MK2-deficient and MK2/MK3-deficient mice: in the absence of MK2, the much lower expressed MK3 still contributes to TNF biosynthesis and its additional deletion reduces TNF production of LPS-stimulated macrophages from about 10% to 1–3% [11].

2.1. The AU-rich-element (ARE) of TNF-mRNA and the ARE-binding protein tristetraprolin (TTP) are genetically downstream to MK2/MK3

Since the AU-rich-element (ARE) of TNF-mRNA was demonstrated to be essential for post-transcriptional regulation of TNF biosynthesis [22], it was hypothesized that MK2/3 could modify substrate proteins which directly or indirectly bind to the ARE of TNF-mRNA. A prominent member of the TNF-mRNA-ARE-binding proteins is tristetraprolin (TTP). TTP-deficiency in mice leads to increased basal TNF production, cachexia and arthritis [23],

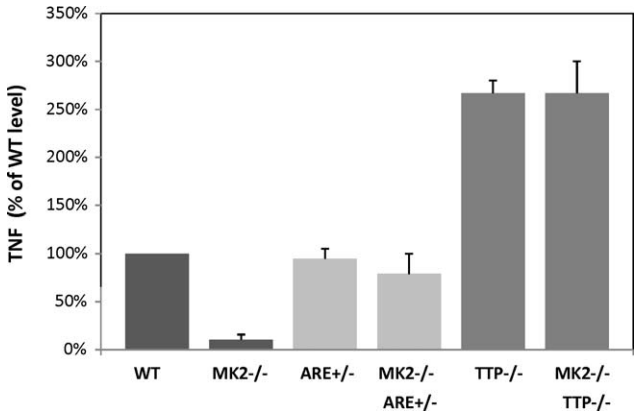

Fig. 1. LPS-induced TNF production of mouse spleen cells *in vitro* depends on the genotype. Deletion of MK2 leads to a dramatic reduction of TNF levels in the cell culture supernatant. TNF production is rescued by additional deletion of the ARE of TNF in one allele or even increased by deletion of both alleles of TTP (summarized from [20,25]).

indicating that TTP is a suppressor of TNF production at the post-transcriptional level [24]. To genetically proof whether the ARE of TNF-mRNA and the ARE-binding protein TTP are downstream to MK2/3 in the same signaling pathway, the appropriate double knockout mice were generated and LPS-induced TNF production was measured for spleen cells and macrophages of these genotypes [20,25]: as seen from Fig. 1, the deletion of the TNF-ARE from only one allele already leads to the release from the effect of MK2-deletion and to restoration of TNF production in the absence of MK2, indicating that the mRNA arising from this allele is no longer controlled in stability/translation by MK2. Obviously, the ARE-deleted allele has major impact on total TNF production, since it could escape the ARE-dependent feedback control via IL-10 [26] and since the MK2-regulated mRNA derived from the wild type allele is more efficiently suppressed because of the higher TTP/ARE-mRNA ratio. The deletion of TTP protein by mutation of both alleles also makes TNF production independent of the presence of MK2. Hence, both TTP and the ARE of TNF-mRNA are genetically downstream to MK2. The simplest biochemical scenario for their downstream action is that (i) TTP is a direct substrate of MK2/3 and that (ii) the ARE-dependent suppressor function of TTP is modified by phosphorylation.

2.2. TTP is a substrate for $p38^{MAPK\alpha}$ and MK2

Phosphorylation of TTP by MAPKs was already demonstrated before its role in TNF biosynthesis was recognized: *in vitro* serine (S) 220 of mouse TTP could be efficiently phosphorylated by the proline-directed kinase ERK1 [27]. Later, *in vitro* phosphorylation of TTP by $p38^{MAPK\alpha}$ and JNK has been demonstrated [28] and TTP has been characterized as a heavily phosphorylated protein containing various phosphorylated sites *in vivo* [29]. Of these, two sites were identified and characterized as phosphorylation sites for MK2/MK3: S52 and S178 [30,31]. Hence, TTP has been described as substrate with multiple phosphorylation sites for the $p38^{MAPK\alpha}$/MK2/3-pathway (cf. Fig. 2), and the functional consequences of these phosphorylations are of specific interest.

2.3. Functional consequences of TTP phosphorylation

The functions of the various TTP phosphorylations are so far not completely understood for all sites. However, for the two MK2/3 phosphorylation sites of TTP, it became clear that these sites are necessary for specific binding to 14-3-3 proteins [30,31] and that

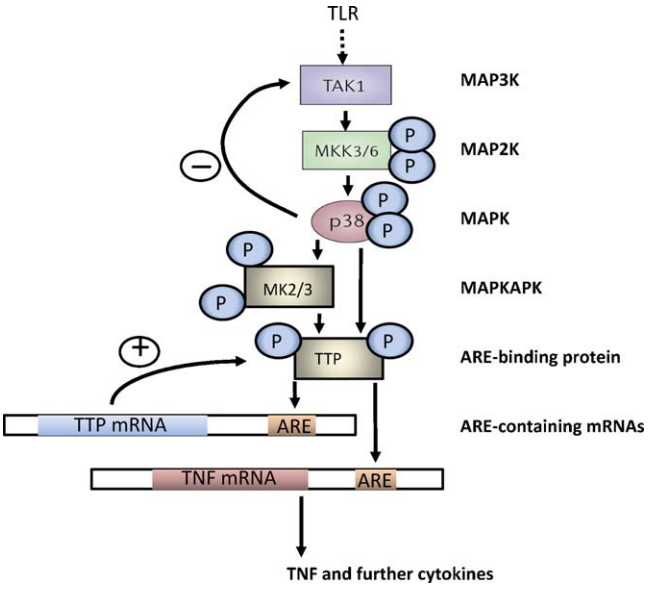

Fig. 2. Schematic representation of the regulation of TNF biosynthesis by the p38/MK2/3-signaling pathway. For details see text.

this binding protects TTP from dephosphorylation by protein phosphatase 2A [32]. In general, phosphorylation of TTP is assumed to block its function as TNF suppressor by (i) inhibiting ARE-mediated decay (AMD) of TNF-mRNA and by (ii) allowing its efficient translation via subcellular translocation of the mRNA from P-bodies, where the AMD occurs, to cytoplasmic sites with high ribosome activity [33]. The fact that the TTP-S52A-S178A mutant is a constitutive active repressor of TNF biosynthesis and cannot translocate away from P-bodies/stress granules supports this notion [31].

However, further properties of TTP are changed in response to phosphorylation. Expression of TTP is increased, since phosphorylation by MK2 stabilizes the protein [25,34] and excludes it from proteasomal degradation [35]. TTP phosphorylation leads to its cytoplasmic retention, which contributes to stabilization of TTP [34]. In addition, expression of TTP is further regulated by a feedback mechanism: TTP mRNA itself carries three AUUUA pentamers in its 3′ untranslated region which contribute to TTP-dependent regulation of this transcript [36]. Hence, as a result of TTP phosphorylation by MK2 the TTP mRNA is stabilized and increasingly translated into protein. The stabilizing influence of MK2 and MK3 on TTP is impressively seen in MK2/3-deficient macrophages, where TTP can hardly be detected in Western blot analysis [11] (Fig. 3). This "no TTP" situation is similar to the "no TTP phenotype" in the TTP knockout, but has completely different functional consequences: while in the TTP-knockout TNF production is increased due to loss of TTP suppression, in the MK2/3 double knockout TNF is strongly reduced probably due to default co-degradation of TTP and TNF-mRNA.

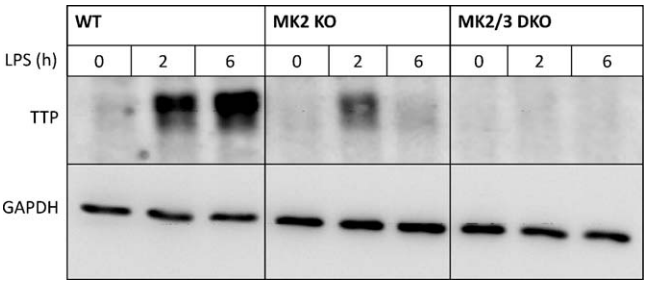

Fig. 3. LPS-induced expression of TTP protein in mouse macrophages of different genotypes. TTP expression before (0) and 2 h and 6 h after LPS-stimulation is detected by Western blot. GAPDH is detected as loading control. TTP level is reduced in MK2-deficient cells and almost undetectable in MK2/3 double-deficient cells (after [11], modified).

It is controversial whether specific phosphorylations also reduce the affinity of TTP for mRNA *in vivo* [25,28]. Finally, it is completely open how TTP phosphorylation regulates its association with the various proteins of the RNA decay machinery and of mRNA processing bodies, which were recently identified as interaction partners [37,38].

2.4. A new aspect: MK2 also stimulates de novo synthesis of TTP at the transcriptional level

So far, the influence of p38 and MK2/3 on post-transcriptional regulation of TTP was discussed. However, it is also clear that TTP underlies a stringent transcriptional regulation, since it is known for a long time that TTP mRNA transcription is rapidly induced by mitogenic stimuli and insulin, placing TTP into the group of immediate early genes [39,40]. As for other immediate early genes [41], the contribution of $p38^{MAPK}$ to the known stress-dependent activation of the TTP gene [42] is mainly believed to be due to direct phosphorylation and activation of the mitogen- and stress-activated protein kinase MSK1, which in turn phosphorylates and activates the transcription factor CREB [43].

Since MK2 and MK3 stabilize their activator $p38^{MAPK\alpha}$ by formation of binary protein complexes [44], the MK2 knockout and the MK2/3 double knockout display reduced $p38^{MAPK\alpha}$ levels and should be regarded a $p38^{MAPK\alpha}$ knockdown as well [11,45]. To distinguish between direct MK2/3 effects and indirect MK2/3 effects on p38 level and activity, we compared MK2/3-deficient cells rescued with MK2 or a catalytic-dead mutant of MK2 (K79R), which completely restores p38 level and activity but does not display kinase activity towards MK2/3 substrates (see [45,11] for rescue of MK2-deficient cells; for MK2/3-deficient cells: data not shown). The transcript level of TTP in the rescued MK2/3-deficient

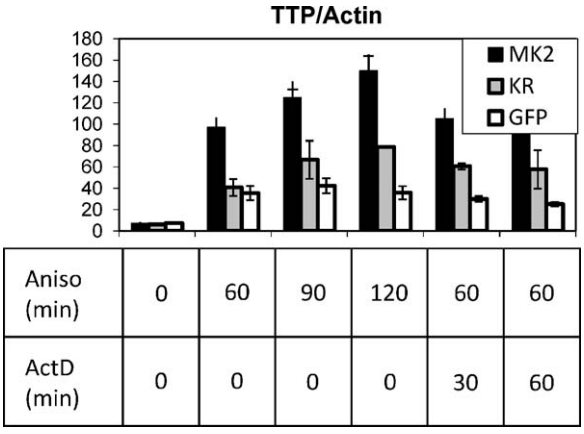

Fig. 4. Detection of TTP transcript levels in rescued MK2/3-deficient cells. MK2/MK3 double-deficient immortalized mouse embryonic fibroblasts were transduced with the bicistronic pMMP-IRES MK2 or MK2-K79R vectors, or with the empty pMMP-IRES vector (GFP) as negative control, following the previously reported method [11]. The different cell lines were sorted for comparable expression of GFP. MEFs were stimulated with 10 μg/ml anisomycin for the indicated times and RNA was extracted using the NucleoSpin RNA purification method (Machery-Nagel). RNA was reverse-transcribed (Fermentas), and Taqman assays (Applied Biosystems) were used for quantifying TTP and actin expression. PCR was performed on a Rotorgene 2000 real-time PCR instrument (Corbett). TTP transcript levels, normalized against actin mRNA were determined using the instrument software and plotted. The results shown are determined from two independent experiments with three separate PCR reactions for each condition. Note that the *p*-values of the transcript differences between MK2 and KR for 60 min, 120 min and 60 + 30 min ActD are <0.05 in the 2-tailed *t*-test and indicate significance. The difference between KR for 60 min and KR for 60 + 30 min ActD is characterized by a *p*-value >0.05 (0.083) and should be regarded as experimental variation.

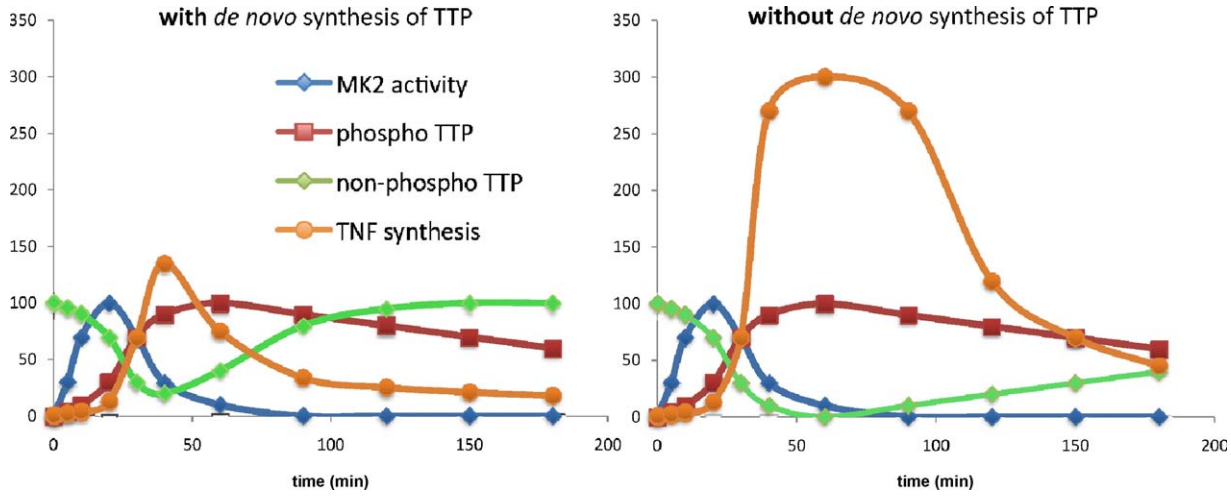

Fig. 5. A qualitative model of the action of MK2/3 after LPS-stimulation on the degree of TTP phosphorylation and TNF production. The following assumptions based on and supported by experimental data [25] were made in this model: (i) MK2/3 activity peaks 20 min after LPS-stimulation and declines in 1 h. (ii) Pre-existing non-phosphorylated TTP is completely phosphorylated by MK2/3 in about 1 h. (iii) In the absence of MK2/3 activity, phospho-TTP decreases with a half-life of about 2 h. (iv) *de novo* synthesis of TTP resulting from transcriptional activation by MK2/3 starts after about 30 min and reconstitutes the normal non-phospho-TTP level in about 2 h. (v) TNF biosynthesis is proportional to the ratio of phospho-TTP to non-phospho-TTP. Left panel: TNF production in the presence of *de novo* synthesis of TTP. Right panel: TNF production without MK2-induced *de novo* synthesis of TTP. An increase in the non-phosho-TTP level (green) can be seen in the right panel already after about 40–60 min while in the left panel this increase is slower and starts after 60–90 min. This results in differences of TNF production (yellow).

cells was analyzed by real-time RT-PCR before and different times after anisomycin treatment, a stress stimulus that activates $p38^{MAPK\alpha}$ (Fig. 4). In the MK2-rescued MK2/3-deficient cells there is a strong time-dependent induction of the TTP transcript level detected, which indicates a transcriptional activation of the TTP gene. As negative control, cells transduced only with green fluorescent protein-expressing construct (GFP) show a clearly reduced transcriptional activation, which could be due to reduced signaling of the $p38^{MAPK}$ pathway. Unexpectedly, the catalytic-dead MK2-K79R mutant (KR), which completely restores $p38^{MAPK}$ level and activity (not shown), is not able to rescue the full range of the transcriptional response of the TTP gene. Since the steady state transcript level might results from transcription and mRNA decay, we applied ActinomycinD as transcriptional inhibitor after 60 min of anisomycin treatment and followed TTP mRNA stability by real-time RT-PCR after further 30 and 60 min. Under the experimental conditions chosen, the TTP transcript is relatively stable and no significant changes in TTP transcript stability could be detected between the different variants of transduced MEFs. This does not exclude lower TTP transcript stability and TTP auto-regulation of its transcript stability as mentioned above under other experimental conditions, such as LPS-stimulation of macrophage-derived cell lines (not shown). However, in the experiments with rescued MEFs represented in Fig. 4 the difference of transcript level between the p38-rescued (KR) and the MK2-rescued cells is due to a transcriptional activation of the TTP gene by catalytic active MK2 suggesting a complex role for MK2 in regulation of TTP expression at transcriptional and post-transcriptional levels.

3. A speculative model for the role of MK2/3 in limiting the TTP-dependent inflammatory response

We showed that MK2/3 stimulate rapid transcription of the TTP. The effect of TTP on TNF biosynthesis depends on its phosphorylation state: the dephospho-isoform of TTP destabilizes TNF-mRNA [24] while the p38- and MK2/3-phosphorylated isoforms promote TNF-mRNA stability and translation [25,46]. The finding that MK2 contributes to both, expression of TNF by inactivating TTP and destabilization of TNF-mRNA by contributing to *de novo*-transcription and synthesis of active TTP, at the first glance seems controversial. However, when seen in the light of kinetics of TNF biosynthesis, this parallel action of MK2/3 may have substantial physiological importance by allowing a short pulse of TNF production and inherent feedback control of the TNF response. To make this more visible, we qualitatively modeled kinetics of TNF production, TTP synthesis, decay and phosphorylation after stimulation of MK2/3 (cf. Fig. 5): in the scenario where MK2/3-induced both, *de novo* synthesis and phosphorylation of TTP, TNF production shows a relatively sharp peak and declines rapidly. In contrast, if *de novo* synthesis of TTP is omitted from the same scenario, there is excess and sustained TNF synthesis. This model is based on several empiric assumptions (see legend to Fig. 5) and, hence, is rather speculative. However, it indicates that the dual action of MK2/3 on TTP phosphorylation and TTP transcription may represent a default mechanism to limit inflammation. In this regard, the model is also compatible with the observation that inhibition of *de novo* protein expression by cycloheximide leads to sustained and increased induction of cytokines [47]. Taken together, in addition to post-transcriptional regulation of cytokine biosynthesis via phosphorylation of ARE-binding proteins the so far unknown contribution of MKs to transcriptional activation of TTP significantly contributes to a coordinated scenario of regulation of gene expression.

4. Outlook: things are more complicated

Here, we have focused on one specific ARE-containing mRNA, namely TNF-mRNA, one specific ARE-binding protein, TTP, and a single signaling pathway. We found that, even under this reductionistic view, the understanding of regulation of cytokine biosynthesis is rather complicated. However, many cytokine mRNAs contain AREs and many ARE-binding proteins, which are targeted by different signaling pathways, are described. 100–250 different TTP-regulated ARE-containing mRNAs have been identified [48,49], including cytokine mRNAs, such as IL-2, IL-10-, GM-CSF- and IFNγ-mRNA [48–52]. For TNF-mRNA other described ARE-binding proteins are hnRNP A0 [53] and A1 [54], the AUF1-related protein CBF-A [55], the translational silencers TIA [56] and TIAR [57] and the mRNA-stabilizing protein HuR [58]. It is also clear that different signaling pathways cooperate to stimulate TNF biosynthesis. Beside the p38/MK2/3-pathway, the NFkB- [59] and Tpl2-MEK-ERK-pathway [60,61] are significantly contributing to

TNF biosynthesis. At the ARE of TNF-mRNA, at least the ERK2-MNK1-hnRNP A1- and the p38-MK2/3-hnRNP A0/TTP-pathways converge [53,54]. Hence, regulation of TNF biosynthesis is tightly controlled and more complex than reported here.

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