Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1

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1Faculty of Pharmacy, University of Sydney, New South Wales, Australia; 2Woolcock Institute of Medical Research, University of Sydney, New South Wales, Australia; and 3Centre for Translational Inflammation Research, School of Immunity and Infection, University of Birmingham, Edgbaston, United Kingdom

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Prabhala P, Bunge K, Rahman MM, Ge Q, Clark AR, Ammit AJ. Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1. Am J Physiol Lung Cell Mol Physiol 308: L973–L980, 2015. First published February 27, 2015; doi:10.1152/ajplung.00219.2014.—Cytokines drive many inflammatory diseases, including asthma. Understanding the molecular mechanisms responsible for cytokine secretion will allow us to develop novel strategies to repress inflammation in the future. Harnessing the power of endogenous anti-inflammatory proteins is one such strategy. In this study, we investigate the p38 MAPK-mediated regulatory interaction of two anti-inflammatory proteins, mitogen-activated protein kinase phosphatase 1 (MKP-1) and tristetraprolin (TTP), in the context of asthmatic inflammation. Using primary cultures of airway smooth muscle cells in vitro, we explored the temporal regulation of IL-6 cytokine mRNA expression upon stimulation with TNF-α. Intriguingly, the temporal profile of mRNA expression was biphasic. This was not due to COX-2-derived prostaglandin upregulation, increased expression of NLRP3 inflammasome components, or upregulation of the cognate receptor for TNF-α-TNFR1. Rather, the biphasic nature of TNF-α-induced IL-6 mRNA expression was regulated temporally by the RNA-stabilizing molecule, TTP. Importantly, TTP function is controlled by p38 MAPK, and our study reveals that its expression in airway smooth muscle cells is p38 MAPK-dependent and its anti-inflammatory activity is also controlled by p38 MAPK-mediated phosphorylation. MKP-1 is a MAPK deactivator; thus, by controlling p38 MAPK phosphorylation status in a temporally distinct manner, MKP-1 ensures that TTP is expressed and made functional at precisely the correct time to repress cytokine expression. Together, p38 MAPK, MKP-1, and TTP may form a regulatory network that exerts significant control on cytokine secretion in proasthmatic inflammation through precise temporal signaling.

p38 MAPK; tristetraprolin; MKP-1; inflammation; IL-6

Many chronic inflammatory diseases are a common consequence of overactive inflammatory signaling pathways. Hence, using these pathways as potential drug targets represents a way to re-establish control and attenuate the severity of such chronic inflammatory diseases. Asthma is a chronic inflammatory disease, which is characterized by reversible airway obstruction, structural remodeling, and airway hyperresponsiveness. A plethora of proinflammatory cytokines have been implicated in the pathophysiology of asthma. The common therapies for asthma include glucocorticoids and β2-agonists; however, there is still a proportion of the population for which this treatment is ineffective. Hence, a need has arisen to find alternative anti-inflammatory strategies, such as using small-molecule inhibitors of inflammatory cascades or using agents that could increase activity of anti-inflammatory proteins. To achieve this goal, a greater understanding of the regulatory networks that control cytokines are urgently required.

Our group along with others has uncovered the important role played by the anti-inflammatory protein mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) in the control of airway inflammation. MKP-1 is a MAPK deactivator with multiple modes of anti-inflammatory action: it is an endogenous protein that functions as a negative feedback effector to repress MAPK-mediated proinflammatory signaling and cytokine secretion (7, 19), and it can be upregulated by anti-asthma medicines such as β2-agonists and corticosteroids (8, 20, 21, 27, 28), serving as an important way in which these front-line asthma medicines achieve their anti-inflammatory function.

Our current studies reveal that a key member of the MAPK superfamily, p38 MAPK, is intrinsically involved in regulating the expression and activity of important anti-inflammatory molecules. Our previous studies showed that p38 MAPK activation was responsible for expression of the early-response gene MKP-1. Once made, the phosphatase activity of MKP-1 dephosphorylates p38 MAPK and acts as a negative feedback effector that limits the strength and duration of p38 MAPK signaling (19). We now add an additional player to this regulatory network and show that the dynamic interaction between p38 MAPK and MKP-1 controls the expression and activity of tristetraprolin (TTP) to repress cytokines.

TTP is an immediate early response gene that functions to destabilize mRNA of many cytokines (5), including those involved in asthma (26). TTP confers this mRNA instability and degradation by binding the conserved adenose/uridine-rich element present within the 3′-untranslated region of many mRNA transcripts (6, 15). Importantly, its function is controlled by p38 MAPK; its expression is p38 MAPK-dependent (18), and its anti-inflammatory activity is controlled by p38 MAPK-mediated phosphorylation (phosphorylated, OFF; unphosphorylated, ON) (14, 18).

The importance of TTP was first elucidated when TTP was knocked out, and the mice developed a proinflammatory phenotype due to the overexpression of the cytokine TNF-α resulting in cachexia, myeloid hyperplasia, and a host of other inflammatory conditions (29). Since that time, the role of TTP in inflammatory conditions, such as arthritis, has become firmly established, while its impact on regulation of asthmatic inflammation is relatively underexplored. We address this herein by using human airway smooth muscle (ASM), a cell

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type characterized as having an important immunomodulatory role perpetuating airway inflammation through the secretion of various proinflammatory cytokines, including IL-6 (17). We are the first to investigate the role and function of TTP in this pivotal airway cell type in asthmatic inflammation. By stimulating cells with TNF-α, we demonstrate the kinetics of TTP expression and confirm p38 MAPK dependency. Notably, by controlling p38 MAPK phosphorylation status in a temporally distinct manner, MKP-1 ensures that TTP is expressed and made functional at an exact time to repress cytokine expression. Together, p38 MAPK, MKP-1, and TTP form a regulatory network that exerts significant control on cytokine secretion in asthmatic inflammation through precise temporal signaling.

MATERIALS AND METHODS

ASM cell culture. Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified, and cultured, as previously described by Johnson et al. (13). A minimum of three different ASM primary cell lines were used for each experiment.

Chemicals. TNF-α was purchased from R&D Systems and celecoxib from Cayman Chemical. Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich.

Real-time RT-PCR. Total RNA was extracted using the RNeasy mini kit (Qiagen) and reverse transcribed using the RevertAid first strand cDNA synthesis kit (Fermentas Life Sciences). Real-time RT-PCR was performed on an ABI Prism 7500 with IL-6 (Hs00174131_m1), nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3: Hs00918082_m1), TNFR1 (TNFRSF1a: Hs00610256_g1), TTP (Zfp36: Hs001856583_m1), MKP-1 (Dusp1: Hs00610256_g1), ElisaGene Expression Assays, and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression (fold increase) quantified by delta delta cycle threshold (Ct) calculations.

Western blot analysis. Western blot analysis was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr\(^{180}\)/Tyr\(^{182}\)) and total p38 MAPK (from Cell Signalling Technology). TTP was measured by Western blot analysis using rabbit antisera against TTP (Sak21). MKP-1 was measured using a rabbit polyclonal antibody (C19: Santa Cruz Biotechnology), compared with α-tubulin as the loading control (mouse monoclonal IgG1, clone DM 1A). Primary antibodies were detected with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer).

TTP siRNA. ASM cells were transiently transfected using nucleofection with 1 µg of TTP-specific ON-Target SMART pool siRNA, consisting of a pool of four individual siRNA (Dharmacon: Thermo Fisher Scientific, Waltham, MA) or a scrambled siRNA control (ON-Target plus control nontargeting siRNA: Dharmacon), using methods established in our previous publication (27). Briefly, ASM cells were transfected with the Nucleofector (Ammaxa, Köln, Germany), using the basic kit for primary smooth muscle cells with the manufacturer’s optimized protocol of P-024. ASM cells were plated for 16 h after transfection, before being growth-arrested for a further 24 h. Cells were then stimulated with TNF-α (10 ng/ml) for the indicated times before TTP and IL-6 mRNA measurement by RT-PCR and IL-6 protein secretion measured by ELISA.

Statistical analysis. Statistical analysis was performed using regression. Student’s unpaired t-test, one-way ANOVA then Fisher’s post hoc multiple-comparison test, or two-way ANOVA then Bonferroni’s post hoc test. P values <0.05 were sufficient to reject the null hypothesis for all analyses.

RESULTS

TNF-α-induced IL-6 secretion is due to biphasic IL-6 mRNA expression. To investigate the temporal regulation of IL-6 expression by TNF-α, growth-arrested ASM cells were stimulated with TNF-α (10 ng/ml) for up to 24 h, and the temporal profiles of IL-6 gene expression and subsequent protein secretion were compared. First, we confirmed that TNF-α-induced IL-6 in ASM cells (27), with significant amounts of IL-6 protein secretion observed by 24 h (P < 0.05). We then conducted a detailed assessment of the temporal profile of IL-6 mRNA expression responsible for IL-6 secretion. Figure 1B demonstrates that TNF-α-induced IL-6 mRNA has a biphasic expression profile. The peak in the first phase of TNF-α-induced mRNA expression occurs at the 1-h time point, when a 54.5 ± 11.5-fold increase in TNF-α-induced IL-6 mRNA can be observed (P < 0.05). This initial increase in mRNA is followed by lower levels of mRNA expression until the 4-h time point when the levels of TNF-α-induced IL-6 mRNA expressed are no longer significant. Following this nadir at 4 h, a secondary phase of IL-6 mRNA expression ensues, reaching a peak of 35.1 ± 9.1-fold increase at 24 h (Fig. 2; P < 0.05).

Fig. 1. TNF-α-induced IL-6 secretion is due to biphasic IL-6 mRNA expression. Growth-arrested airway smooth muscle (ASM) cells were treated with TNF-α (10 ng/ml) for 0, 1, 2, 4, 8, and 24 h. IL-6 protein secretion was measured by ELISA, and IL-6 mRNA expression (A) was measured by RT-PCR (results expressed as fold increase compared with vehicle-treated cells at 0 h). Statistical analysis was performed using one-way ANOVA and then Fisher’s post hoc multiple-comparison test (where * denotes a significant effect of TNF-α on IL-6 expression (P < 0.05)). Data are expressed as means ± SE; values provided are from n = 12 primary ASM cell cultures.
The biphasic nature of TNF-α-induced IL-6 mRNA expression was not due to COX-2-derived prostanoids, increased expression of NLRP3 inflammasome components, or upregulation of the cognate receptor for TNF-α-TNFR1. We were intrigued to uncover the cell-signaling mechanisms responsible for the biphasic nature of TNF-α-induced IL-6 mRNA expression. First, we examined whether prostanoid products of COX-2 might be involved as TNF-α is known to induce COX-2 in this cell type (1, 4, 25), and prostanoids, including PGE2 and PG12, may increase IL-6 via cAMP-dependent COX-2 in this cell type (1, 4, 25). To investigate this possibility, growth-arrested ASM cells were pretreated with the COX-2-selective inhibitor celecoxib for 1 h, prior to stimulation with TNF-α for 24 h. Figure 2A illustrates the impact of celecoxib on TNF-α-induced mRNA expression. Consistent with our earlier results, IL-6 mRNA is augmented after 24 h of TNF-α stimulation; IL-6 mRNA levels are increased by 27.0 ± 4.9-fold, compared with vehicle alone (P < 0.05). However, celecoxib has no significant effect on IL-6 mRNA expression in the presence or absence of TNF-α. Thus, as TNF-α-induced IL-6 mRNA is unaffected by celecoxib, our results indicate that IL-6 mRNA is upregulated in a prostanoid-independent manner. We then tested whether components of the inflammasome NLRP3 might be involved. We measured the expression level of NLRP3 after 24-h stimulation with TNF-α and found no significant difference compared with vehicle-treated cells (Fig. 2B). Finally, we examined a potential autoregulatory mechanism by assessing whether TNF-α upregulates expression of its own receptor, TNFR1, to enhance IL-6 expression in a biphasic manner. As shown in Fig. 2C, the levels of TNFR1 were not significantly upregulated after 24-h stimulation with TNF-α.}

**Fig. 2.** The biphasic nature of TNF-α-induced IL-6 mRNA expression was not due to COX-2-derived prostanoids, increased expression of NLRP3 inflammasome components, or upregulation of the cognate receptor for TNF-α-TNFR1. Growth-arrested ASM cells were treated with vehicle or TNF-α (10 ng/ml) for 24 h. A: cells were pretreated with vehicle or 10 μM celecoxib for 1 h. IL-6 (A), NLRP3 (B), and TNFR1 (C) mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared with vehicle-treated cells). Statistical analysis was performed using Student’s unpaired t-test, where * denotes a significant effect of TNF-α (P < 0.05). Data are expressed as means ± SE; values are provided from n = 3 primary ASM cell cultures.

**Fig. 3.** TNF-α rapidly upregulates MKP-1 in a p38 MAPK-dependent manner. A: growth-arrested ASM cells were treated with TNF-α (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, and 24 h, and the temporal kinetics of MKP-1 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared with vehicle-treated cells at 0 h). Statistical analysis was performed using a one-way ANOVA and then Fisher’s post hoc multiple-comparison test, where * denotes a significant effect of TNF-α on MKP-1 expression (P < 0.05). Data are expressed as means ± SE values from n = 8 primary ASM cell cultures. B and C: to confirm that TNF-α-induced MKP-1 mRNA expression (B) and protein upregulation (C) was p38 MAPK-dependent, growth-arrested ASM cells were pretreated for 0.5 h with vehicle, 1 μM SB203580, or 10 μM PD98059, prior to stimulation with TNF-α. B: MKP-1 mRNA expression was quantified by real-time RT-PCR at 0.5 h (results expressed as % TNF-α-induced MKP-1 mRNA expression). Statistical analysis was performed using Student’s unpaired t-test, where § denotes significant inhibition of TNF-α-induced MKP-1 mRNA expression (P < 0.05). Data are expressed as means ± SE; values are provided from n = 8 primary ASM cell cultures. C: MKP-1 protein upregulation at 1 h was analyzed by Western blot analysis (with α-tubulin as the loading control). Results are representative Western blots (from n = 6 primary ASM cell lines).
tion of MKP-1 mRNA at the 1-h time-point ($P < 0.05$), which then rapidly returns to basal levels. MKP-1 mRNA and protein expression are augmented via the p38 MAPK-mediated, but not ERK-mediated, pathway. This is shown in Fig. 3, B and C, where growth-arrested ASM cells were pretreated for 30 min with either vehicle or pharmacological inhibitors of the p38 MAPK (SB203580) and ERK (PD98059) pathways, prior to stimulation with TNF-α. TNF-α-induced MKP-1 expression was then observed as early as 30 min and was significantly attenuated by SB203580, but not PD98059 (Fig. 3B; $P < 0.05$).

MKP-1 mRNA is rapidly translated into MKP-1 protein by 1 h (Fig. 3C) and was similarly repressed when the p38 MAPK-mediated pathway was inhibited in accordance with our previous study (19).

Temporal kinetics and p38 MAPK dependence of TTP mRNA expression induced by TNF-α. We are now the first to demonstrate the dynamic regulation of cytokine regulatory networks controlled by MKP-1 and p38 MAPK in ASM cells. This intricate regulation involves the destabilizing RNA-binding protein TTP. This is demonstrated in Fig. 4, where we show that TNF-α upregulates TTP mRNA expression in ASM cells and does so in a p38 MAPK-dependent manner. To explore the temporal kinetics of TNF-α-induced TTP mRNA expression, growth-arrested ASM cells were treated with TNF-α, and TTP mRNA was measured by RT-PCR at 0, 0.5, 1, 2, 4, 8, and 24 h. As shown in Fig. 4A, TTP mRNA is significantly and rapidly increased by TNF-α with 8.7 ± 2.0-fold at the 1-h time point ($P < 0.05$). To determine whether this augmentation is mediated via the p38 MAPK pathway, growth-arrested ASM cells were pretreated with SB203580 and PD98059 before stimulation with TNF-α for 30 min. The results show that TNF-α-induced TTP mRNA is repressed in the presence of the p38 MAPK inhibitor, while unaffected by the ERK inhibitor (Fig. 4B). Hence, this shows that TNF-α-induced TTP mRNA expression in ASM cells is p38 MAPK-dependent.

TTP activity is temporally regulated by p38 MAPK via control of p38 MAPK phosphorylation. Our previous publications have shown that p38 MAPK is rapidly activated by TNF-α (19, 27) and that upregulation of MKP-1 in a p38 MAPK-dependent manner gives rise to a negative feedback loop (19). We now show, for the first time in ASM cells, that this intricate control of p38 MAPK phosphorylation by MKP-1 regulates cytokine secretion via modulation of TTP expression and activity. Figure 5, A and B illustrates the temporal interrelationship that exists between p38 MAPK, MKP-1, and TTP. This is shown by Western blot analysis and densitometry, respectively. We observe that TNF-α rapidly induces p38 MAPK phosphorylation by 0.25 h and 0.5 h. MKP-1 protein is expressed in response to an increase in p38 MAPK phosphorylation, with a peak of protein upregulation at 1 h. However, as levels of this MAPK phosphatase increase, there is a corresponding decrease in phospho-p38 MAPK. Then as MKP-1 degrades (being subject to proteosomal degradation), the restraint on p38 MAPK is lessened and phospho-p38 MAPK builds up again as best shown in densitometric analysis (Fig. 5B). These data are in accord with our earlier studies (19) and provide the first complete analysis of temporal interrelationship between p38 MAPK phosphorylation and MKP-1.

To then explore the extent of TTP regulation conferred by phosphorylated p38 MAPK, TTP protein levels were measured in parallel to MKP-1 and phosphorylated p38 MAPK. TTP is detected using rabbit antisera against TTP (Sak21). Previous studies utilizing this antibody demonstrate two immunoreactive bands for TTP that are indicative of TTP phosphorylation status and, thus, activity; the upper immunoreactive band was identified as the phosphorylated (inactive) form, and the lower band is identified as the unphosphorylated (active) form (14, 18). As shown in Fig. 5A, TNF-α induces TTP protein expression at 0.25 h and 0.5 h. Since TTP mRNA expression is p38-MAPK-dependent and protein translation occurs during peak p38 MAPK phosphorylation levels, the phosphorylated form of TTP predominates. By 1 h, however, when p38 MAPK phosphorylation is switched off due to the action of MKP-1, the lower unphosphorylated TTP immunoreactive band appears (Fig. 5B). Thence, when MKP-1 subsides and p38 MAPK is no longer deactivated, both immunoreactive forms of TTP are present, switching to the phosphorylated, upper immunoreactive band further along the time course. Collectively, these results reveal a temporally coordinated regulatory mechanism involving phosphorylated p38 MAPK, MKP-1, and TTP.
**Figure 5. TTP activity is temporally regulated by MKP-1 via control of p38 MAPK phosphorylation.** Growth-arrested ASM cells were treated with TNF-α (10 ng/ml) for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h, and the temporal kinetics of p38 phosphorylation, MKP-1, and TTP protein upregulation was compared by Western blot analysis (with α-tubulin as the loading control). Please note two bands of immunoreactivity for TTP: bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). Results are representative Western Blots (A), while densitometric analysis of TNF-α-induced p38 MAPK phosphorylation (B) is demonstrated compared with MKP-1 protein upregulation over time. Data are expressed as means ± SE; values are presented from n = 4 primary ASM cell cultures.

**impact of TTP knockdown (by siRNA) on TNF-α-induced IL-6 mRNA expression and protein secretion.** Finally, we examined the impact of specifically knocking down TTP with siRNA on TNF-α-induced IL-6 mRNA expression and protein secretion. Primary cultures of ASM are hard to transfect with conventional transfection techniques; thus, we utilized nucleofection to knock down TTP, according to previously established methods (27). ASM cells were transiently transfected using nucleofection with scrambled control or TTP siRNA. Cultures were then treated with TNF-α (10 ng/ml) for 0, 1, 4, 8 and 24 h. As shown in Fig. 7A, the peak of TTP mRNA expression at 1 h was significantly reduced by TTP siRNA (P < 0.05) as predicted by the difficult-to-transfect nature of ASM cells, the transfection efficiencies ranged from a low of 23.2% to a high of 77.2% knockdown, with the average being 53.5 ± 5.8% (n = 6 primary ASM cell cultures). Results were expressed as a percentage of TTP mRNA in cells nucleofected with siRNA, compared with scrambled control, after 1-h stimulation with TNF-α. When we focused on the primary cell cultures with ≥50% knockdown of TTP (n = 6 primary ASM cell cultures), regression analysis revealed that there was a significant relationship between the % of TTP

TNF-α-induced IL-6 mRNA expression is biphase due to the temporal regulation of TTP activity by p38 MAPK. We hypothesize that the temporally specific control of p38 MAPK phosphorylation by MKP-1 governs cytokine secretion via regulation of TTP expression and activity. Specifically, we propose that the secondary phase of IL-6 mRNA expression occurs because MKP-1 is no longer present to restrain p38 MAPK, and, consequently, TTP, although expressed, is phosphorylated and, therefore, inactive. To test this experimentally, growth-arrested ASM cells were stimulated with TNF-α and then treated with vehicle or the p38 MAPK inhibitor (SB203580) 3.5 h after TNF-α. Cell lysates were then prepared and treated with the p38 MAPK antibody (Fig. 6, A and B) and the impact on IL-6 mRNA expression and secreted IL-6 measured by RT-PCR and ELISA, respectively (Fig. 6, C and D). Figure 6A shows the protein expression profile of TTP and phosphorylated p38 MAPK. In the absence of SB203580, TNF-α-induced p38 MAPK phosphorylation is sustained at 4–24 h, and although overall expression levels of TTP are elevated, it is mainly the upper phosphorylated (inactive) form. Notably, after SB203580 treatment, p38 MAPK phosphorylation is reduced at 4 h, and while the upper immunoreactive band is less intense, importantly, the lower unphosphorylated band prevails. As this unphosphorylated band is the active form of TTP, we can demonstrate a significant inverse relationship between p38 MAPK phosphorylation and TTP (% active) (i.e., % unphosphorylated TTP/total TTP) analyzed by densitometry (Fig. 6C; r² = 0.59; P < 0.0001). Accordingly, by reducing p38 MAPK phosphorylation at 3.5 h (with SB203580), we have increased TTP activity and significantly (P < 0.05) repressed TNF-α-induced IL-6 mRNA expression (Fig. 6C) and IL-6 secretion (Fig. 6D). In this way, we reveal the molecular mechanisms responsible for the biphasic expression of IL-6 mRNA expression and show how p38 MAPK controls TTP phosphorylation status (and, therefore, TTP activity) in a temporally specific manner to regulate cytokine secretion in ASM cells.
knockdown and the impact on % TNF-α-induced IL-6 mRNA expression at 4 h (y = 45.103 + 0.104x; r² = 0.745; P < 0.0267). Figures 7B and C show the impact of TTP knockdown (by siRNA) on TNF-α-induced IL-6 mRNA expression and protein secretion in these ASM cell cultures. The kinetics of IL-6 mRNA expression in cells transfected with TTP siRNA differed compared with those transfected with scrambled control, albeit nonsignificantly (Fig. 7B), and TTP knockdown resulted in significantly greater IL-6 secretion after TNF-α stimulation, compared with cells nucleofected with scrambled control (Fig. 7C; P < 0.05).

**DISCUSSION**

To date, research on TTP has primarily focused on its role in rheumatoid arthritis, leaving investigations into the anti-inflammatory function of TTP in airway disease relatively unexplored. Importantly, a number of critical cytokines that drive
inflammation in asthma are destabilized by TTP (16, 30). This includes IL-6 (27), and in this study, we investigate the temporal profile of TNF-α-induced IL-6 mRNA expression in ASM cells to reveal the important role played by TTP. Notably, TTP expression and activity are regulated by p38 MAPK and controlled in a temporally distinct manner by MKP-1.

Investigations into ASM cells have revealed their immunomodulatory function outside their originally proposed roles, regulating airway bronchomotor tone. This led to the finding that when primary cultures of ASM cells were treated in vitro with proinflammatory stimuli (such as TNF-α), they secreted numerous cell adhesion molecules and cytokines (17), including IL-6 (2, 22). IL-6 has many proinflammatory functions relevant to inflammation in asthma, and our in vitro investigations using primary cultures of ASM cells stimulated with TNF-α have allowed us to delineate many of the molecular mechanisms responsible for IL-6 cytokine secretion in this pivotal immunomodulatory cell type.

We had previously observed that TNF-α-induced IL-6 mRNA expression occurred in a biphasic manner (10, 27); to date, however, the cellular signaling regulatory networks responsible had not been explored. Studies had shown that the NLRP3 inflammasome and its components are able to elicit an inflammatory response through the activation of caspase-1. This caspase-1 is able to cleave proforms of IL-1β and IL-18 into bioactive cytokines that can initiate or amplify diverse downstream signaling pathways (11). Along with the inflammasome, TNF-α is also able to induce COX-2 expression and COX-2 products, including prostanoids, such as PGE₂ and PGI₂, have been shown to play a role in the pathogenesis of asthma (3, 4, 24, 25). These prostanoids are cAMP-elevating agents and can trigger transcriptional responses leading to cytokine secretion in a number of different cell types, including ASM cells (2, 20). Thus, because of their inflammatory capabilities, whether NLRP3 inflammasome and cAMP-regulating prostanoids were upregulated by TNF-α was examined in this study as the potential basis of the secondary phase of IL-6 mRNA upregulation. Moreover, we also examined upregulation of tumor necrosis factor receptor 1 (TNFR1) to determine whether TNF-α was involved in a positive feedback loop with its own receptor. However, our studies showed that TNF-α-induced IL-6 mRNA was upregulated in an inflammasome- or COX-2-independent manner. The results also indicated that the TNFR1 receptor was not upregulated as a result of TNF-α. However, as our earlier studies showed that TNF-α is able to induce IL-6 and because TNF-α is known to elicit its proinflammatory effects via the p38 MAPK pathway, we were intrigued to investigate the role of p38 MAPK in the temporal regulation of IL-6 mRNA expression. Our studies demonstrate that the dynamic interaction between p38 MAPK and MKP-1 regulates TTP expression and activity and, thus, exerts a significant impact on cytokine expression.

MKP-1 is the archetypal member of a family of dual specificities phosphatases, which can dephosphorylate, thereby deactivating, members of the MAPK family (9). Studies have supported a role for MKP-1 as a negative feedback regulator of the p38 MAPK pathway and as a repressor of proremodeling functions of ASM cells (8, 12, 19, 23, 27). MKP-1 has been widely explored; however, its potential interaction with TTP is less well studied. We confirm that TNF-α increases the level of MKP-1 via a p38 MAPK-dependent pathway (19). We now show that, like MKP-1, TNF gene expression is p38 MAPK-mediated. The upregulation of TTP mRNA has been previously shown in human monocytes treated with LPS (18) and A549 cells treated with IL-1β (14); we are the first to present these data in ASM cells. TTP activity [as indicated by upper and lower immunoreactive bands, in accordance with King et al. (14)] is also temporally regulated by p38 MAPK. We show that TNF-α increases TTP mRNA by as early as 30 min. At this time, p38 MAPK has peak levels of phosphorylation and any translated TTP protein present is phosphorylated and, therefore, inactive (upper band). However, MKP-1 mRNA is also expressed at 30 min and MKP-1 protein rapidly produced. It then restrains p38 MAPK phosphorylation to allow greater levels of the active form of TTP protein to be present (lower band). Active TTP reduces cytokine expression and, thus, the initial peak of cytokine expression subsides. But MKP-1 up-regulation after TNF-α is also transitory, and so when the restraint of MKP-1 phosphatase activity is removed, p38 MAPK levels build again over time. Correspondingly, TTP is less active and allows a secondary phase of cytokine expression.

Collectively, these studies provide evidence that the biphasic nature of the TNF-α-induced IL-6 mRNA expression profile is due to the coordinated regulation of TTP expression and activity by p38 MAPK. As p38 MAPK activity is regulated by MKP-1, these results provide further support for the important anti-inflammatory role played by MKP-1 in asthma and airway remodeling and demonstrate that targeting these molecules represent potential anti-inflammatory therapies in the future.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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