



Proteasomal inhibition upregulates the endogenous MAPK deactivator MKP-1 in human airway smooth muscle: Mechanism of action and effect on cytokine secretion

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ABSTRACT

Asthma is a chronic inflammatory condition. Inhibition of the ubiquitin–proteasome system offers promise as a anti-inflammatory strategy, being responsible for the degradation of key proteins involved in crucial cellular functions, including gene expression in inflammation (e.g. inhibitory I κ B- α and the endogenous MAPK deactivator – MKP-1). As MKP-1 inhibits MAPK-mediated pro-remodeling functions in human airway smooth muscle (ASM; a pivotal immunomodulatory cell in asthma) in this study we investigate the effect of the proteasome inhibitor MG-132 on MKP-1 and evaluate the anti-inflammatory effect of MG-132 on cytokine secretion from ASM cells. Examining the time-course of induction of MKP-1 mRNA and protein by MG-132 (10 μ M) we show that MKP-1 mRNA was first detected at 30 min, increased to significant levels by 4 h, resulting in a 12.6 ± 1.5 -fold increase in MKP-1 mRNA expression by 24 h ($P < 0.05$). MKP-1 protein levels corroborate the mRNA results. Investigating the effect of MG-132 on secretion of the cytokine IL-6 we show that while short-term pretreatment with MG-132 (30 min) partially reduced TNF α -induced IL-6 via inhibition of I κ B- α degradation and the NF- κ B pathway, longer-term proteasome inhibition (up to 24 h) robustly upregulated MKP-1 and was temporally correlated with repression of p38-mediated IL-6 secretion from ASM cells. Moreover, utilizing a cytokine array we show that MG-132 represses the secretion of multiple cytokines implicated in asthma. Taken together, our results demonstrate that MG-132 upregulates MKP-1 and represses cytokine secretion from ASM and highlight the potential of the proteasome as a therapeutic target in asthma.

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1. Introduction

Asthma is a chronic inflammatory condition that is characterized by reversible airway obstruction, airway hyper-responsiveness and airway remodeling. It is a multi-faceted condition involving numerous cells, pro-inflammatory mediators and signaling molecules. The MAPK phosphoproteins are critical signaling molecules that drive pro-inflammatory pathways in asthma [1,2].

The MAPK superfamily of signaling molecules (p38, ERK and JNK) are serine/threonine protein kinases that when activated can phosphorylate numerous downstream effectors, including transcription factors, cytoskeletal proteins and other phosphoproteins, to play a crucial role in a wide variety of cellular functions ranging from proliferation, migration and synthesis of fibrotic and inflammatory proteins (including cytokines). Thus, MAPKs have emerged as critical pathways that drive development of airway remodeling to significantly contribute to asthma pathophysiology [1,2]. Thus, inhibition of

MAPK has emerged as an attractive strategy for reversing inflammation and remodeling in a wide variety of chronic inflammatory conditions, including asthma.

Cellular function is profoundly affected by both strength and duration of MAPK activation, which must be strictly controlled to modulate functional outcome. This crucial negative feedback control is achieved by MKPs, a family of endogenous dual-specificity MAPK phosphatases (also known as dual-specificity phosphatases (DUSPs) [3,4]. We [5,6] and others [7,8] have recently uncovered the key role played by MKP-1 in the inhibition of MAPK-mediated pro-remodeling functions in airway smooth muscle (ASM) cells; a pivotal immunomodulatory cell in asthma. Thus, because MKP-1 serves a crucial negative feedback role in regulating the strength and duration of pro-inflammatory signal transduction, discovering mechanisms to regulate the protein level or enzymatic activity of this endogenous MAPK deactivator may be exploited as a novel anti-inflammatory strategy in asthma and airway remodeling.

MKP-1 is a 367 amino acid protein expressed by an immediate-early gene [9]. MKP-1 expression is controlled at multiple steps, broadly divided into three levels: transcriptional, post-transcriptional and post-translational. Diverse stimuli can induce MKP-1 transcription

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to rapidly upregulate MKP-1 protein [10]. However, the increase in MKP-1 protein levels is transient, as MKP-1 protein (expressed as a result of increased transcription and/or mRNA stability) then undergoes rapid degradation by the proteasomal machinery [11,12]. In this study, we aim to increase MKP-1 by inhibiting the ubiquitin–proteasome system with the cell-permeable, reversible proteasome inhibitor MG-132.

The ubiquitin–proteasome system is responsible for the degradation of key proteins involved in crucial cellular functions, including those responsible for gene expression in inflammation (including inhibitory $\text{I}\kappa\text{B-}\alpha$, as well as MKP-1, among others [13]); thus, proteasome inhibition has emerged as a potential pharmacotherapeutic strategy for treating inflammatory processes [13]. Since the approval of the proteasome inhibitor bortezomib in 2003 for the treatment of multiple myeloma, a number of structurally and mechanistically distinct proteasome inhibitors have been trialed in the treatment of cancer, autoimmune and inflammatory diseases, myocardial infarction, and ischemic brain injury [14,15]. However, the potential of proteasome inhibition as an anti-inflammatory strategy in asthma has been comparatively less well-studied. Intriguingly, proteasomal inhibition has been shown to reduce cyclin D1 in ASM cells *in vitro*, a critical cell cycle protein controlling ASM hyperplasia [16], eosinophil chemotaxis *in vitro* [17] and eosinophilia in an *in vivo* animal model of asthma [18]; all hallmarks of asthma. In a recent study, proteasome inhibition was shown to reverse the reduction of histone deacetylase 2 (HDAC2) abundance in cells from human airway and mouse lung [19]. As HDAC2 reduction is associated with steroid resistance in severe asthmatics and patients with chronic obstructive pulmonary disease, further studies exploring the potential of proteasome inhibition in inflammatory lung diseases, such as asthma, are warranted.

Our study is the first to examine the effect of proteasomal inhibition on the upregulation of the endogenous MAPK deactivator – MKP-1 – in a human cell with relevance in asthma. We examined the temporal kinetics of MKP-1 mRNA and protein expression by MG-132 induced by the proteasome inhibitor MG-132 and evaluated the anti-inflammatory effect of MG-132 on cytokine secretion from human ASM cells. Our results demonstrate that MG-132 upregulates MKP-1 mRNA and protein expression and repress cytokine secretion from ASM cells; underscoring the potential of the proteasome as a therapeutic target in asthma.

2. Materials and methods

2.1. Reagents

PD98059, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA). All other reagents used in this study, including MG-132, were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

2.2. Cell culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the South West Sydney Area Health Service and the Human Ethics Committee of the University of Sydney. ASM cells were dissected, purified and cultured as previously described by Johnson et al. [20]. A minimum of three different ASM primary cell lines were used for each experiment.

2.3. Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcription performed by using the RevertAid First strand cDNA Synthesis Kit

(Fermentas Life Sciences, Hanover, MD) as per the manufacturer's protocol for the use of random hexamer primers. MKP-1 mRNA levels were measured using real-time RT-PCR on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) with a MKP-1 primer set (Assays on Demand, dual-specificity phosphatase 1, Hs00610256_g1; Applied Biosystems) and the eukaryotic 18 S rRNA endogenous control probe (Applied Biosystems) and 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.

2.4. Western blotting

Western blotting was performed using rabbit polyclonal IgG antibodies against MKP-1 (M18: Santa Cruz Biotechnology, Santa Cruz, CA), $\text{I}\kappa\text{B-}\alpha$ (C-21: Santa Cruz Biotechnology), total and phosphorylated p38 (Thr¹⁸⁰/Tyr¹⁸²), total and phosphorylated ERK (Thr²⁰²/Tyr²⁰⁴), total and phosphorylated JNK (Thr¹⁸³/Tyr¹⁸⁵). All MAPK antibodies were from Cell Signaling Technology (Danvers, MA). α -tubulin was used as the loading control (mouse monoclonal IgG₁, DM1A: Santa Cruz). Primary antibodies were detected with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.5. ELISAs and cytokine arrays

Cell supernatants were collected and stored at –20 °C for later analysis by ELISA or cytokine array. IL-6 (BD Biosciences Pharmingen, Franklin Lakes, NJ: detection limit, 7.8 pg/ml) and IL-8 (BD Biosciences Pharmingen: detection limit, 15.6 pg/ml) ELISAs were performed according to the manufacturer's instructions. The human cytokine array (Proteome Profiler™ Array Human Cytokine Array Panel A, ARY005: R&D Systems, Minneapolis, MN) was used to simultaneously detect the relative levels of 36 different cytokines, chemokines, and acute phase proteins. The array was performed according to the manufacturer's instructions. Levels of proteins were visualized by chemiluminescence and within a linear range of exposure quantified by Quantity One® software (Bio-Rad, Hercules, CA). The protein levels on each array were standardized against an internal positive control on the array.

2.6. Luciferase assays

The NF- κB reporter vector, pNF- κB -Luc, was purchased from Clontech (Mountain View, CA). Transient transfection of ASM cells was performed as described previously [21] and luciferase activity assessed according to manufacturer's instructions (Promega, Madison, WI).

2.7. Statistical analysis

Statistical analysis was performed using Student's unpaired *t* test. *P* values <0.05 were considered significant. Data represent mean + SEM.

3. Results

3.1. MG-132 upregulates MKP-1 mRNA and protein

To examine the time-course of induction of MKP-1 mRNA and protein expression by MG-132, growth-arrested ASM cells were treated with vehicle or the proteasome inhibitor MG-132 (10 μM) for 0, 5, 10, 30 and 60 min and 2, 4, 8 and 24 h. The concentration of MG-132 has been used previously in ASM cells [22]. As shown in Fig. 1A, MG-132 induced a small increase in MKP-1 mRNA expression as early as 30 min after treatment with MG-132; although this increase was not significantly different from the vehicle control. However, after 4 h

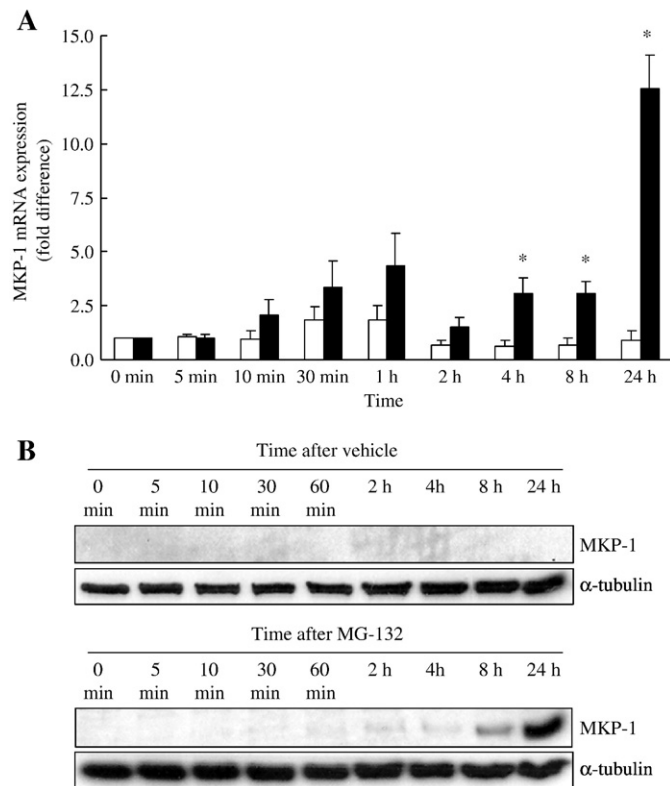


Fig. 1. MG-132 upregulates MKP-1 mRNA and protein. (A) To examine the time-course of induction of MKP-1 mRNA by MG-132, growth-arrested ASM cells were treated with vehicle (empty histograms) or MG-132 (10 μ M; filled histograms) for 0, 5, 10, 30 and 60 min and 2, 4, 8 and 24 h, and MKP-1 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase over 0 min). Statistical analysis was performed using the Student's unpaired *t* test (where * denotes significant upregulation induced by MG-132 ($P < 0.05$)). Data are mean \pm SEM values from $n = 3$ replicates. (B) To examine the upregulation of MKP-1 protein induced by MG-132 over time, growth-arrested ASM cells were treated with vehicle or MG-132 (10 μ M) for 0, 5, 10, 30 and 60 min and 2, 4, 8 and 24 h. Cells were then lysed and analyzed by Western blotting using rabbit polyclonal IgG antibodies against MKP-1. α -tubulin was used as the loading control. Results are representative Western blots from $n = 6$ primary ASM cell lines.

treatment with MG-132 MKP-1 mRNA was significantly increased ($P < 0.05$), resulting in a 12.6 ± 1.5 -fold increase in MKP-1 mRNA expression by 24 h (Fig. 1A). The levels of MKP-1 protein corroborate the mRNA results; MKP-1 was detected as early as 1–2 h after treatment with MG-132, increased over time and was robustly upregulated by 24 h (Fig. 1B).

3.2. MG-132 increases MKP-1 via an ERK-independent signaling pathway

Our results corroborate earlier studies where MG-132 was shown to upregulate MKP-1 protein via increased MKP-1 mRNA expression [23], in addition to inhibiting proteasomal degradation [11]. Interestingly, in cell types apart from ASM, MKP-1 mRNA expression has been shown to occur via an ERK-mediated pathway [24]. To investigate whether MG-132 increase MKP-1 via an ERK-dependent signaling pathway in ASM cells, we first examined the kinetics of ERK phosphorylation in cells treated with MG-132 in the absence and presence of a specific inhibitor of the ERK pathway – PD98059 (at 10 μ M). As shown in Fig. 2A, pretreatment with PD98059 inhibited the temporal activation of ERK following treatment with MG-132, measured from 0 min to 24 h (Fig. 2C), but had no effect on MKP-1 protein levels. MG-132 induced a rapid and sustained activation of ERK; ERK phosphorylation occurred within 5 min and achieved peak phosphorylation by 30 min. MG-132 does not activate JNK or p38 in

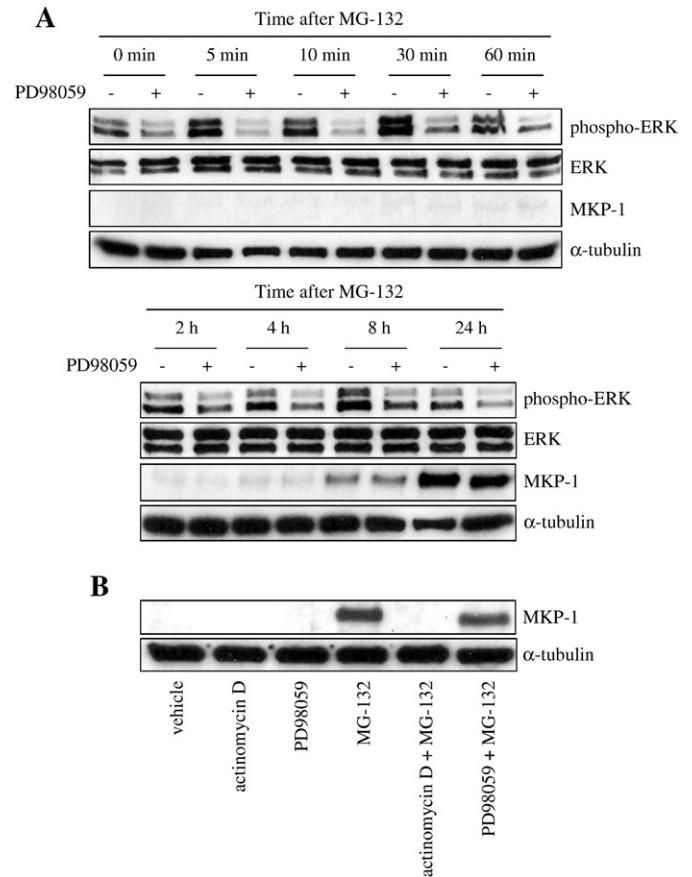


Fig. 2. MG-132 increases MKP-1 via an ERK-independent signaling pathway. (A) Growth-arrested ASM cells were pretreated with vehicle or PD98059 (10 μ M) for 30 min, then treated with MG-132 (10 μ M) for 0, 5, 10, 30 and 60 min and 2, 4, 8 and 24 h. Cells were then lysed and analyzed by Western blotting using rabbit polyclonal IgG antibodies against phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total ERK, compared to MKP-1. α -tubulin was used as the loading control. Results are representative Western blots from $n = 4$ primary ASM cell lines. (B) Growth-arrested cells were pretreated with vehicle, actinomycin D (5 μ g/ml) or PD98059 (10 μ M) for 30 min and then either left untreated, or treated with MG-132 (10 μ M) for 24 h. Cells were then lysed and protein levels of MKP-1 were determined by Western blotting. α -tubulin was used as the loading control. Results are representative Western blots from $n = 4$ primary ASM cell lines.

ASM cells (data not shown). Secondly, we pretreated ASM cells with the transcription inhibitor actinomycin D, and compared the resultant effect on MG-132-induced MKP-1 protein with cells pretreated with the ERK inhibitor PD98059. As shown in Fig. 2B, pretreatment with actinomycin D completely inhibited the upregulation of MKP-1 protein at 24 h, confirming that transcription is involved in MKP-1 mRNA and protein upregulation induced by MG-132. In comparison, PD98059 had no effect on MKP-1 protein induced by MG-132 (Fig. 2B). Collectively our results demonstrate that MG-132 induces ERK phosphorylation, however ERK activation does not contribute to the upregulation of MKP-1 by MG-132.

3.3. MG-132 inhibits TNF α -induced IL-6, but not IL-8 secretion

Because we demonstrated proteasomal inhibition was an effective way to upregulate MKP-1 in ASM cells, we now sought to examine the effect of this upregulation on cytokine secretion. We focused on IL-6 and IL-8 secretion, two important cytokines secreted from ASM cells [5,21,25]. Growth-arrested ASM cells were pretreated with vehicle or MG-132 (10 μ M) for 30 min, and then stimulated with TNF α (10 ng/ml) for 24 h. Cell supernatants were collected and IL-6 and IL-8 concentrations were determined by ELISA. Pretreatment of ASM

cells for 30 min with MG-132 significantly ($P < 0.05$) inhibited TNF α -induced IL-6 secretion (Fig. 3A), but had no significant effect on IL-8 secretion from ASM cells (Fig. 3B).

3.4. Molecular mechanisms underlying the effect of short-term pretreatment with MG-132 on cytokine secretion

We now sought to investigate the molecular mechanisms underlying the repressive effect of short-term pretreatment (i.e. 30 min) with MG-132 on cytokine secretion from ASM cells. Our previous publications have underscored the importance of the p38 MAPK signaling pathway in TNF α -induced IL-6 and IL-8 secretion from ASM cells [5,21,25]. Because the duration and intensity of MAPK signaling is sensitively controlled by the temporal induction of the endogenous MAPK deactivator – MKP-1 – we now wished to examine whether MG-132 inhibits MAPK-mediated synthetic function in ASM cells by upregulating MKP-1 and shortening the temporal phosphorylation of MAPKs. In the current study (Fig. 3A), we have shown that 30 min pretreatment with MG-132 significantly inhibited TNF-induced IL-6 secretion from ASM cells. Here (Fig. 4), we explored whether MKP-1 induced by short-term pretreatment with MG-132 was responsible for the anti-inflammatory effect.

In Fig. 4A, we examined the temporal activation of MAPKs by TNF α , in the absence and presence of MG-132 pretreatment. In corroboration of earlier work [5], we show that TNF α transiently activates MAPK family members and that TNF α alone can also induce transient upregulation of MKP-1. However, pretreatment with MG-132 did not inhibit the temporal profile of MAPK activation. The critical time point for MKP-1 to interrupt MAPK signaling and to have a repressive effect on cytokine secretion in ASM cells appears to be at 30 min after stimulation [5,7]. At this time point (see 30 min in Fig. 4A), there were no discernible differences in MKP-1 levels in cells pretreated without and with MG-132. By 60 min however, there was more MKP-1 protein present in cells treated with TNF α and MG-132

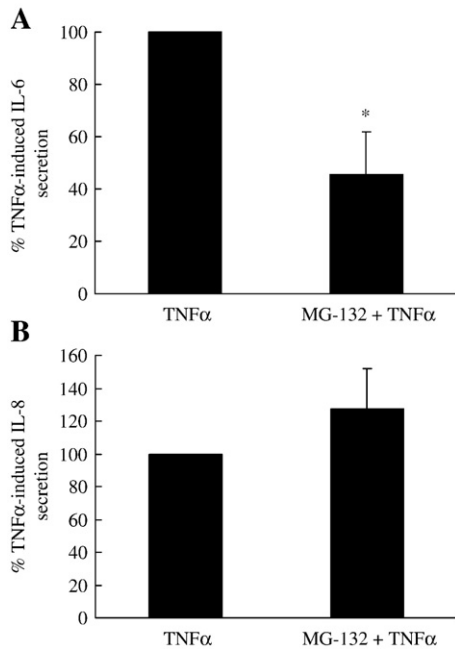


Fig. 3. MG-132 inhibits TNF α -induced IL-6, but not IL-8 secretion. Growth-arrested ASM cells were pretreated with vehicle or MG-132 (10 μ M) for 30 min and then stimulated with TNF α (10 ng/ml) for 24 h. Cell supernatants were collected and (A) IL-6 and (B) IL-8 concentrations determined by ELISA. Statistical analysis was performed using the Student's unpaired *t* test (where * denotes significant inhibition by MG-132 ($P < 0.05$)). Data are mean + SEM values from 5 replicates.

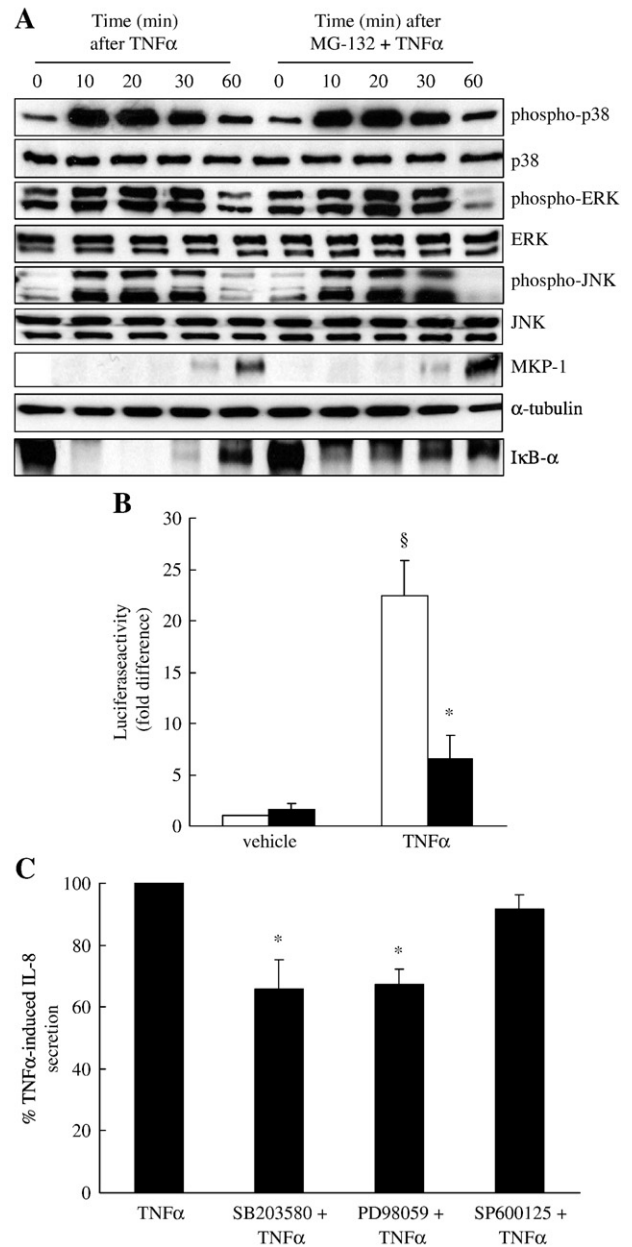


Fig. 4. Molecular mechanisms underlying the effect of short-term pretreatment with MG-132 on cytokine secretion. (A) Growth-arrested ASM cells were pretreated with vehicle or MG-132 (10 μ M) for 30 min and then stimulated with TNF α (10 ng/ml) for 0, 10, 20, 30 and 60 min. Cells were then lysed and analyzed by Western blotting using rabbit polyclonal IgG antibodies against phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total p38, phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total ERK, phosphorylated (Thr¹⁸³/Tyr¹⁸⁵) and total JNK, MKP-1, α -tubulin (loading control) and I κ B- α . Results are representative Western blots from *n* = 7 primary ASM cell lines. (B) ASM cells transfected with a NF- κ B reporter vector, pNF- κ B-Luc, were pretreated with vehicle (empty histograms) or MG-132 (10 μ M; filled histograms) for 30 min, and then treated with vehicle or TNF α (10 ng/ml) for 6 h. Cells were harvested and luciferase activities assessed. Data represent normalized luciferase activity, relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student's unpaired *t* test (where § denotes a significant effect of TNF α , and * denotes significant inhibition by MG-132 ($P < 0.05$)). Data are mean + SEM values from 4 to 9 replicates. (C) Growth-arrested ASM cells were pretreated with vehicle, SB203580 (1 μ M), PD98059 (10 μ M), or SP600125 (10 μ M) for 30 min and then stimulated with TNF α (10 ng/ml) for 24 h. Cell supernatants were collected and IL-8 concentration determined by ELISA. Statistical analysis was performed using the Student's unpaired *t* test (where * denotes significant inhibition of TNF α -induced IL-8 ($P < 0.05$)). Data are mean + SEM values from 14 to 22 replicates.

than with TNF α only, but by this time the peak of MAPK had subsided; thus, the effect of MG-132 on cytokine secretion (observed in Fig. 3A) is temporally independent of its effect on MKP-1 upregulation.

We were intrigued then to discover the molecular mechanism responsible for the repression of cytokine secretion by short-term pretreatment with MG-132. Both IL-6 and IL-8 are secreted by ASM cells in response to TNF α via an NF- κ B-dependent transcriptional pathway [21,26]. Inhibition of the ubiquitin–proteasome system by MG-132 reduces degradation of numerous key proteins, including those responsible for gene expression in inflammation, such as I κ B- α . Because I κ B- α inhibits the NF- κ B pathway by restricting its location to the cytoplasm and inhibiting its DNA binding activity, we now explored whether inhibition of the NF- κ B pathway was the molecular mechanism responsible for the effect of short-term pretreatment with MG-132 on cytokine secretion.

In Fig. 4A, we examined the degradation of I κ B- α protein in response to TNF α by Western blotting. We show that the level of I κ B- α protein was greater when ASM cells were pretreated for 30 min with the proteasome inhibitor MG-132 (Fig. 4A). By inhibiting the proteasome, MG-132 reduced the degradation of I κ B- α , demonstrated by reappearance of I κ B- α at 30 min. In parallel, we confirmed that MG-132 acts to inhibit the TNF α -induced NF- κ B pathway in ASM cells in experiments where we transiently transfected ASM cells with a commercially available NF- κ B luciferase reporter vector. In corroboration of our previous studies [21], we confirm that TNF α significantly enhances NF- κ B activity ($P < 0.05$; Fig. 4B). After pretreatment with MG-132, TNF α -induced NF- κ B activity was significantly reduced from 22.4 ± 3.5 -fold to 6.5 ± 2.3 -fold ($P < 0.05$; Fig. 4B). Taken together, these results demonstrate that TNF α -induced IL-6 secretion was repressed by short-term pretreatment with MG-132, due to an inhibitory effect on the NF- κ B pathway, and was temporally independent of its effect on MKP-1 expression.

Additionally, we wished to address why 30 min pretreatment with MG-132 inhibits TNF α -induced IL-6, but not IL-8 secretion from ASM cells. With this study we have demonstrated that MG-132 activates ERK in ASM cells (Fig. 2A). Hence, it is possible that the ability of MG-132 to inhibit IL-6, but not IL-8, reflected the involvement of the other MAPK pathways in IL-8 production. We previously published that TNF α -induced IL-6 secretion in ASM cells was independent of the ERK and JNK pathways [5]. In a series of separate studies, researchers have identified the MAPK signaling pathways underlying TNF α -induced IL-8 secretion in ASM cells. The ERK [27] and p38 [21] pathways predominate, with JNK-mediated signaling involved to a lesser extent [28]. In Fig. 4C, we compare the relative contribution of MAPK family members to TNF α -induced IL-8 secretion by employing specific inhibitors of these pathways. Growth-arrested ASM cells were pretreated with either the p38 inhibitor SB203580 (1 μ M), the ERK inhibitor PD98059 (10 μ M), or the JNK inhibitor SP600125 (10 μ M) for 30 min, and then stimulated with TNF α (10 ng/ml) for 24 h. Supernatants were collected and IL-8 secretion determined by ELISA. In corroboration of earlier work, we confirm that pretreatment with SB203580 or PD98059 inhibits IL-8 production in ASM cells ($P < 0.05$; Fig. 4C). Inhibition of the JNK pathway with SP600125 (at 10 μ M) had no significant effect on IL-8 secretion. Taken together, these results suggest that despite inhibition of NF- κ B activation by MG-132, MG-132 also enhances ERK activation; thus, these opposing effects may have led to unaffected IL-8 secretion in ASM cells.

3.5. Proteasome inhibition increases MKP-1 and reduces TNF α -induced IL-6 secretion in a time-dependent manner

Because we found that short-term pretreatment of ASM cells with MG-132 (for 30 min) had not resulted in sufficient MKP-1 upregulation at the crucial time point required to interrupt TNF α -induced MAPK signaling (30 min; Fig. 4A), we now explored pretreatment of ASM cells for up to 24 h with MG-132, and examined the resultant effect on TNF α -induced IL-6 secretion.

As shown in Fig. 5A, pretreating ASM cells with MG-132 for longer time periods before stimulation with TNF α resulted in a significant

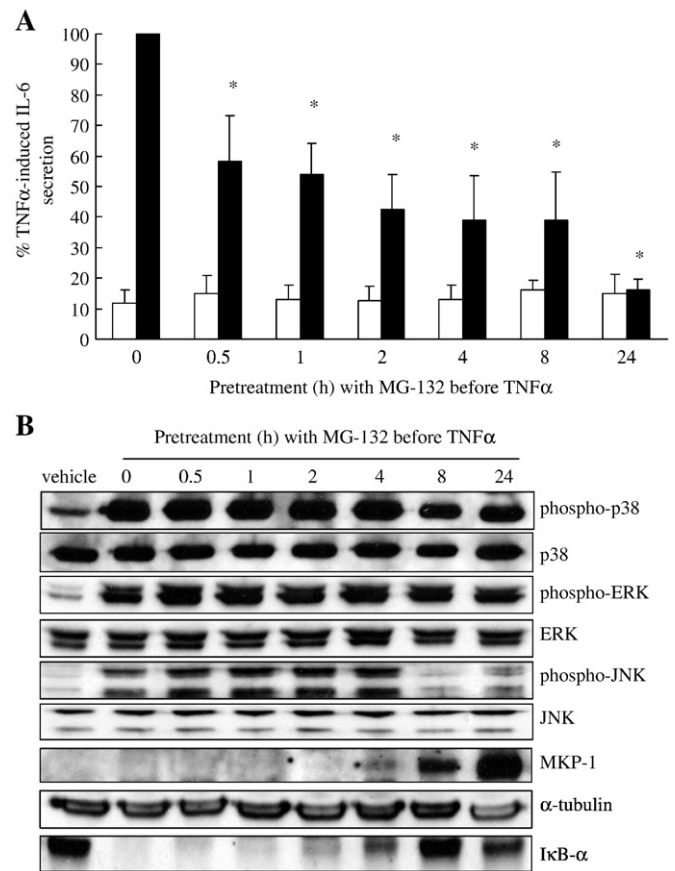


Fig. 5. Proteasome inhibition increases MKP-1 and reduces TNF α -induced IL-6 secretion in a time-dependent manner. (A) Growth-arrested ASM cells were pretreated with MG-132 (10 μ M) for 0, 0.5, 1, 2, 4, 8, and 24 h and then left without further treatment (empty histograms) or stimulated with TNF α (10 ng/ml; filled histograms) for 24 h. Cell supernatants were collected and IL-6 determined by ELISA. Statistical analysis was performed using the Student's unpaired t test (where * denotes significant inhibition by MG-132 ($P < 0.05$)). Data are mean \pm SEM values from 4 replicates. (B) Growth-arrested ASM cells were pretreated with MG-132 (10 μ M) for 0, 0.5, 1, 2, 4, 8 and 24 h and then stimulated with TNF α (10 ng/ml) for 24 h. Vehicle-treated cells served as an unstimulated control. Cells were then lysed and analyzed by Western blotting using rabbit polyclonal IgG antibodies against phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total p38, phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total ERK, phosphorylated (Thr¹⁸³/Tyr¹⁸⁵) and total JNK, MKP-1, α -tubulin (loading control) and I κ B- α . Results are representative Western blots from $n = 3$ primary ASM cell lines.

inhibition of IL-6 secretion ($P < 0.05$). The degree of inhibition of IL-6 secretion was inversely correlated with MKP-1 protein expression (Fig. 5B). Notably, when cells were pretreated for 24 h with MG-132, resulting in robust MKP-1 upregulation (Fig. 5B), IL-6 secretion was significantly repressed to control levels (Fig. 5A; $P < 0.05$). The increase in MKP-1 appeared to mirror the decrease in TNF α -induced p38 MAPK phosphorylation (especially at 8 and 24 h), demonstrating that MG-132-induced MKP-1 shortens the temporal phosphorylation of p38 MAPK in response to TNF α . JNK phosphorylation was also repressed at 8 and 24 h (and ERK to a lesser extent), however, as published previously [5], TNF α -induced IL-6 secretion in ASM cells was independent of the JNK and ERK pathways.

3.6. MG-132 represses multiple cytokines implicated in asthma

To demonstrate the potential of proteasomal inhibition as an anti-inflammatory approach in asthma we profiled the repressive effect of MG-132 utilizing a cytokine array containing multiple cytokines, chemokines and acute phase proteins (see map in Fig. 6A). Many of these proteins have been implicated in asthma. Protein expression was examined using supernatants from ASM cells (from $n = 3$ primary

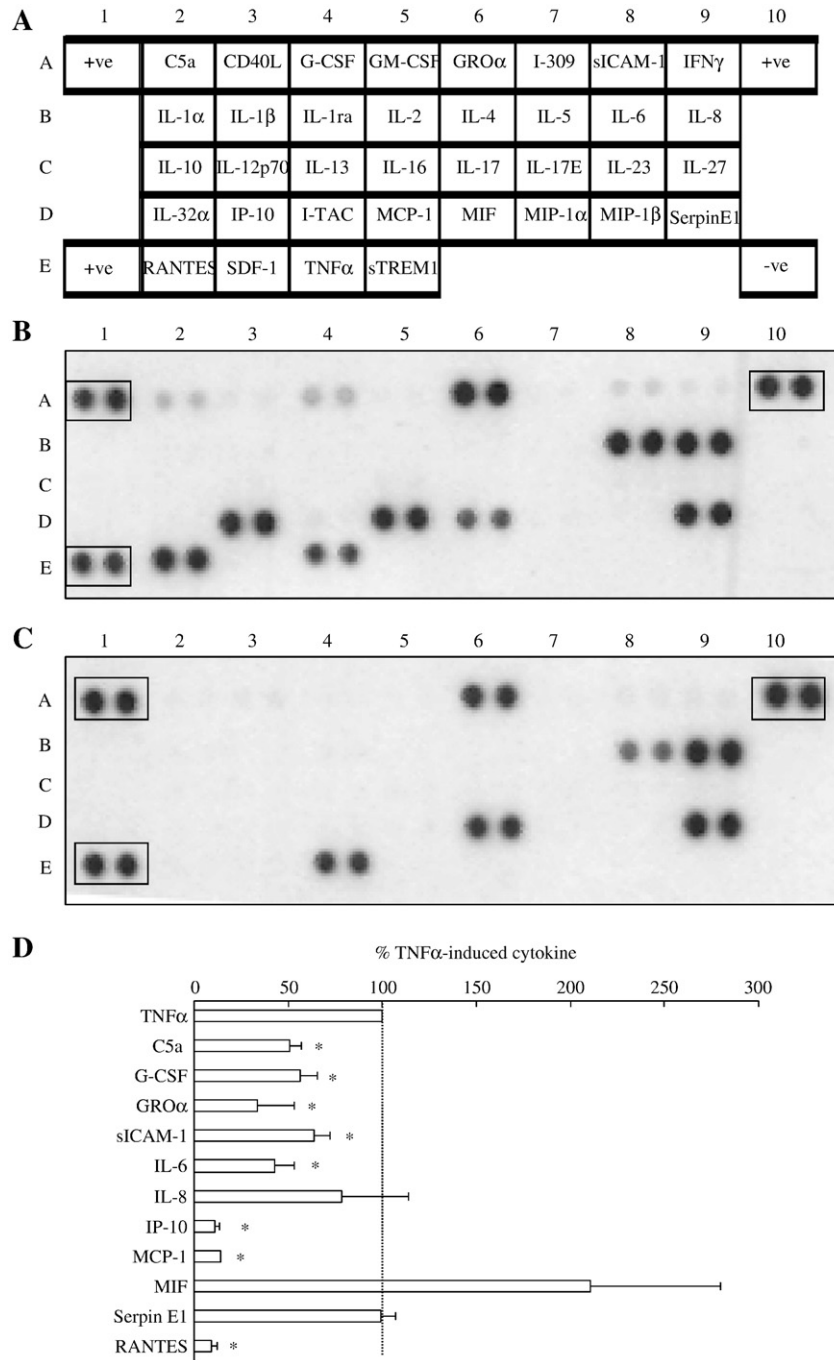


Fig. 6. MG-132 represses multiple cytokines implicated in asthma. To profile the repressive effect of MG-132 on a cytokine array (see map shown in (A)), we compared protein expression in supernatants from ASM cells stimulated with TNF α for 24 h (B), with supernatants from cells that had been pretreated for 24 h with MG-132 prior to stimulation (C). (B and C) illustrate representative arrays (from $n = 3$ primary ASM cell lines), while (D) demonstrates densitometric analysis where data are expressed as % TNF α -induced cytokine (mean \pm SEM values from $n = 3$ primary ASM cell lines) and statistical analysis performed using the Student's unpaired t test (where * denotes significant inhibition by MG-132 ($P < 0.05$)).

ASM cell lines) stimulated with TNF α for 24 h (Fig. 6B), compared to supernatants from cells that had been pretreated for 24 h with MG-132 prior to stimulation (Fig. 6C). As demonstrated in Fig. 6B, a number of proteins were found at high levels in supernatants from ASM cells stimulated with TNF α , namely GRO α , IL-6, IL-8, IP-10, MCP-1, MIF, Serpin E1 and RANTES. In comparison, C5a, G-CSF and sICAM-1 were found at relatively lower levels. Importantly, pretreatment with MG-132 substantially represses the expression of the majority of these cytokines, i.e. 8/11 (Fig. 6C). Following densitometric analysis (Fig. 6D), we demonstrate that MG-132 significantly repressed C5a, GRO α , sICAM-1, G-CSF, IL-6, IP-10, MCP-1, MIF, RANTES ($P < 0.05$).

Notably, MG-132 pretreatment reduced TNF α -induced IP-10 and RANTES by $89.1 \pm 2.7\%$ and $91.1 \pm 3.3\%$ respectively. Like IL-8, Serpin E1 and MG-132 were not significantly affected by MG-132 pretreatment. Thus, using a cytokine array we show that MG-132 represses the secretion of multiple cytokines implicated in asthma.

4. Discussion

The ubiquitin–proteasome system is a multi-subunit complex that degrades various cellular proteins and plays an important role in

regulating the expression of numerous signal transduction molecules involved in inflammation. The endogenous MAPK deactivator, MKP-1, is one such protein degraded by this system. Inhibition of the proteasome has previously been shown to substantially increase MKP-1 [11,12]. Our study is the first to demonstrate this in ASM, a pivotal cell with important roles in airway remodeling.

MKP-1 is encoded by an immediate-early gene [24,29] and in our study we show that inhibition of the proteasome with the inhibitor MG-132 in ASM cells occurs via a combination of increased MKP-1 gene expression and inhibition of protein degradation by the proteasome. In cell types apart from ASM, ERK activation can induce MKP-1 expression [24] and stabilize the protein to degradation [30,31]; however, we have shown that ERK does not contribute to the upregulation of MKP-1 by MG-132 in this cell type.

We then examined the effects on MG-132 cytokine secretion by ASM cells. Our results underscore the importance of the temporal regulation of MAPK and negative feedback control by MKP-1. We show that short-term (30 min) preincubation of ASM cells with MG-132 had not yet resulted in sufficient MKP-1 upregulation to have an inhibitory effect on MAPK activation or MAPK-mediated cellular events. This was simply due to timing, since the peak of MAPK activation had occurred before the induction and expression of MKP-1. Under these conditions, TNF α -induced IL-6 secretion was repressed by MG-132, due to an inhibitory effect on I κ B- α degradation and the NF- κ B pathway, and was temporally independent of its effect on MKP-1 expression. However, when cells were pretreated for up to 24 h with proteasome inhibitor, we show that MG-132-induced MKP-1 represses phosphorylation of p38 MAPK in response to TNF α stimulation, and reduces IL-6 secretion from ASM, in a time-dependent manner.

To underscore the potential of proteasome inhibition as an anti-inflammatory strategy to repress cytokine secretion by ASM cells we employed a cytokine array to simultaneously measure 36 cytokines, chemokines and acute phase proteins. We show that a number of proteins were found at high levels in supernatants from ASM cells stimulated with TNF α , namely GRO α , IL-6, IL-8, IP-10, MCP-1, MIF, Serpin E1 and RANTES; while C5a, G-GSF and sICAM-1 were found at relatively lower levels. Many of these proteins have been implicated in asthma (reviewed in [32,33]). Importantly, long-term treatment with MG-132 significantly repressed the secretion of the majority of these proteins. For example, MG-132 inhibited secretion of IP-10 by ~90%; IP-10 is a CXC chemokine secreted in elevated amounts from the ASM of asthmatics and responsible for the attraction of mast cells [34]. MCP-1 and RANTES, two important chemokines responsible for the chemoattraction of monocytes and eosinophils in airway inflammation in asthma [32], were also repressed by 80–90% following MG-132 treatment. MAPK-mediated pathways are responsible for secretion of these chemokines from ASM cells [25,28]([35]). Given that cytokines play a major role in perpetuating airway inflammation in asthma and that ASM is a pivotal immunomodulatory cell in the asthmatic airway, our study demonstrating that MG-132 inhibits cytokine secretion from ASM cells is among the first to highlight the potential of the proteasome as a therapeutic target in asthma.

In summary, proteasomal inhibition represents an effective means to increase protein levels of the endogenous MAPK deactivator MKP-1 in ASM cells. Given the pivotal role played by MAPKs in asthma and the critical role of MKP-1 in their regulation, further studies investigating the strategies designed to specifically upregulate MKP-1 may demonstrate the potential of MKP-1 as a novel therapeutic target in asthma.

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