

The MNK-1/eIF4E pathway as a new therapeutic pathway to target inflammation and remodelling in asthma

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ABSTRACT

Therapeutic targets in asthma are reduction of airway inflammation and remodelling, the latter is not affected by available drugs. Here we present data that inhibition of MAPK-activated protein kinase (MNK)-1 reduces inflammation and remodelling. MNK-1 regulates protein expression by controlling mRNA stability, nuclear export and translation through the eukaryotic initiation factor 4E (eIF4E). Airway smooth muscle cells were derived from asthmatic and non-asthmatic donors. Cells were pre-treated with CGP57380 (MNK-1 inhibitor) or MNK-1 siRNA, before TNF- α stimulation. Cytokine and protein expression was analysed by ELISA, real time PCR and immunoblotting. Proliferation was monitored by cell counts. TNF- α activated MNK-1 phosphorylation between 15 and 30 min. and subsequently eIF4E between 15 and 60 min. eIF4E activity was inhibited by CGP57380 dose-dependently. Inhibition of MNK-1 by CGP57380 or MNK-1 siRNA significantly reduced TNF- α induced CXCL10 and eotaxin mRNA expression and secretion, but had no effect on IL-8. However, CXCL10 mRNA stability or NF- κ B activity were not affected by MNK-1 inhibition. Furthermore, eIF4E was detected in the cytosol and the nucleus, but TNF- α did not affect its export from the nucleus. Cytokine array assessment showed that in addition to eotaxin and CXCL10, asthma relevant GRO α and RANTES were down-regulated by MNK-1 inhibition. In addition, MNK-1 inhibition significantly reduced FCS and PDGF-BB induced cell proliferation. We are the first to report that MNK-1 controls chemokine secretion and proliferation in human airway smooth muscle cells. Therefore we suggest that MNK-1 inhibition may present a new target to limit inflammation and remodelling in asthmatic airways.

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1. Introduction

Asthma is characterized by chronic inflammation, hyper-responsiveness (AHR), and airway remodelling. Airway smooth muscle cells (ASMC) are important remodelling relevant cells and also secrete pro-inflammatory chemokines such as CXCL10 or eotaxin that subsequently attract immune cells into the airway to increase inflammation and remodelling in asthma [1]. Increased CXCL10 expression was reported in ASMC in vitro and in airway biopsies derived from asthma patients [2,3]. Furthermore, CXCL10 derived from ASMC induced the infiltration of mast cells into asthmatic airway smooth muscle bundles [3,4]. The activation of mast cells leads to the activation of airway smooth muscle cells and increases the secretion of further pro-inflammatory cytokines such as eotaxin [5].

Eotaxin expression correlated with asthma severity [6] and it was expressed at higher levels in ASMC derived from asthma patients [7,8]. Eotaxin is a chemoattractant for eosinophils and elevated numbers of infiltrated eosinophils in the airway is a prominent pathological feature of asthma [9,10]. Several pro-inflammatory conditions increase both CXCL10 and eotaxin through the mitogen activated protein kinases (MAPK), which is followed by the activation of MAPK-activated protein kinase (MNK)-1 in different cell types [11] but its contribution to asthma has not been investigated yet.

In regard to its stimulating role in inflammation toll-like receptor-agonist-stimulated TNF- α , monocyte chemoattractant protein (MCP)-1 or IL-6 production in macrophages, and this effect was significantly reduced by inhibition of MNK-1 [12]. Similarly, TNF- α , IL-6 and IL-1 β expression was down regulated by MNK-1 inhibition in human keratinocytes [13]. Interestingly the action of MNK-1 does not seem to be mediated via transcription, but is also regulated through translation control as it was reported in two other cell types. MNK-1 up-regulated the expression of IFN- γ and IL-4 by natural killer T cells [14] and that of CCL3 and CCL4 by neutrophils [15] via increased translation not

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transcription, both effects were reduced in the presence of the MNK-1 inhibition.

MNK-1 is activated by both p38 MAPK and ERK 1/2 MAPK [16] and directly phosphorylates the eukaryotic initiation factor 4E (eIF4E) in response to external stimuli [17]. Whereas MNK-2 mediates the constitutive phosphorylation of eIF4E, which is not further increased by activation of p38 or ERK 1/2 MAPK [17,18]. Interestingly, in MNK-1/2 double knockout mice the phosphorylation of eIF4E is absent, which suggests that MNK-1 and MNK-2 are the only kinases phosphorylating eIF4E [19]. Furthermore, eIF4E is over-expressed in many forms of human cancer and regulates protein expression through protein translation control, mRNA nuclear export or mRNA stability [20]. In asthma, eIF4E is required for ASMC hypertrophy [21]; however, its role in ASMC cytokine secretion or airway inflammation had not yet been investigated.

In this study, we investigated whether the MNK-1/eIF4E pathway is activated by TNF- α in asthmatic and non-asthmatic ASMC. Furthermore, we determined the effect of the MNK-1 inhibitor CGP57380 or MNK-1 siRNA on chemokine expression and on proliferation.

2. Material and methods

2.1. Culture of human ASMC

Primary human ASMC were isolated from airway tissue obtained by endobronchial biopsy or therapeutic lung resection, characterized and grown as previously described [22]. The use of human primary ASMC was approved by the local ethical committee of the University Hospital, Basel, Switzerland (EKB05/06) and written consent was given by each patient.

2.2. Chemokine secretion by ASMC (ELISA and human cytokine array)

Confluent ASMC were growth arrested, then pre-treated (1 h) with either CGP57380 (1.25–20 μ M; Merck, Darmstadt, Germany) or SB203580 (10 μ M), or PD98059 (30 μ M; both: Calbiochem, Luzern, Switzerland) or MNK-1 siRNA before being stimulated with TNF- α (10 ng/ml; R&D Systems, Minneapolis, USA) for 24 h. Cell supernatants were collected and CXCL10, eotaxin and IL-8 protein was measured by enzyme-linked immunosorbent assay (ELISA, Duo Set, R&D Systems).

Four human cytokine array membranes, each detecting 36 different cytokines relevant to asthma, (Proteome Profiler™ Antibody Arrays Human Cytokine Array, R&D Systems, Minneapolis, MN) following the procedure as described by the manufacturer's instructions. Conditioned cell culture medium was collected from ASMC under two conditions: 1) after stimulation with TNF- α alone or 2) from cells that were pre-treated for 72 h with MNK1 siRNA as described below.

2.3. MNK-1 siRNA treatment

For further experiments investigating the role of MNK-1, **corresponding** small interfering RNA (siRNA) was purchased from Qiagen and ASMC were transfected with MNK1 and control siRNA (20 nM) for 72 h according to manufacturer's protocols. Transfection reagent was HiPerFect Transfection Reagent (Qiagen). ASMC were then starved for 24 h before being treated with TNF- α for 3 h (RNA) or 24 h (protein secretion).

2.4. Real-time RT-PCR

Growth arrested ASMC were pre-treated with the inhibitors (1 h) and then stimulated with TNF- α . After 3 h, total RNA was extracted with a Quick-RNA MiniPrep Kit (ZymoResearch, Orange, CA) and reverse transcription (RT) was performed with an Omniscript® RT kit (Qiagen, Hombrechtikon, Switzerland). The levels of cytokine mRNA were determined by real-time PCR using a CXCL10 (Hs00171042_m1),

eotaxin (CCL11, Hs00237013_m1) or GAPDH (Hs03929097_g1) TaqMan® Gene Expression Assays (all Applied Biosystems, Foster City, CA). Samples were run at 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 cycle. mRNA expression was quantified by the delta delta Ct calculation method.

2.5. Immunoblotting

Growth arrested ASMC were treated as described above and total cell lysates were collected between 0 and 120 min, size-fractionated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes as described previously [23]. Membranes were blocked and then incubated with antibodies: phospho-MNK-1, MNK-1eIF4E, phospho-eIF4E, Lamin A/C (all Cell Signaling Technology, Beverly, MA), or to α -Tubulin (Santa Cruz Biotechnology, Santa Cruz, USA). Membranes were then incubated with horseradish peroxidase-conjugated IgG antibodies (anti-rabbit IgG, or anti-mouse IgG; Santa Cruz Biotech.) and protein bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA).

2.6. ASMC viability (MTT assay)

Growth arrested ASMC were either treated with CGP57380 (10, 20 μ M), a positive control (Triton X-100, 0.5 mg/ml, Sigma) or left untreated (negative control). After 24 h, the cells were incubated with 3-(3,4-dimethylthiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT, Sigma) for 6 h before being lysed by DMSO (Sigma) and the optical density was measured at 550 nm on a microplate reader.

2.7. mRNA stability

ASMC (growth arrested) were pre-treated with CGP57380 (10 μ M, 1 h) and then stimulated with TNF- α for 3 h. In order to stop new mRNA synthesis, ASMC were washed and actinomycin D (5 μ g/ml) in low serum medium was added. Total RNA was extracted after 0, 2, 4, 6, or 24 h, followed by reverse transcription and quantification of CXCL10 mRNA expression as described earlier.

2.8. Nuclear and cytosolic distribution of eIF4E

ASMC were stimulated with TNF- α or left un-stimulated for 30 and 60 min. ASMC were then trypsinised and incubated in low-salt buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.25% Nonidet P-40 and 1 $\times$ protease inhibitor cocktail) to extract the cytosolic protein fraction. Nuclei were re-suspended in high-salt buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 $\times$ protease inhibitor cocktail). Cytosolic and nuclear protein extracts were analysed by immunoblotting as described in Section 2.4 earlier.

2.9. NF- κ B EMSA

ASMC were pre-treated with CGP57380 (10 μ M, 1 h), stimulated with TNF- α for 30 min, and then fractionated into cytosolic and nuclear extracts as described above. NF- κ B p65/DNA binding was determined in the nuclear protein extract by using the NF- κ B p65 TransAm™ transcription factor assay kit (Active Motif, Carlsbad, CA).

2.10. ASMC proliferation

ASMC were pre-treated with CGP57380 (1–10 μ M for 1 h) and then stimulated with either FCS (5%) or PDGF-BB (10 ng/ml). After 72 h, ASMC were trypsinized and cell numbers were determined using a Beckman Coulter particle counter Z1 (Beckman, Nyon, Switzerland).

2.11. Data analysis

Duplicate results of each experiment were averaged for each primary ASMC line and the mean $\pm$ S.E.M was calculated. The statistical analysis was performed using an un-paired Student's *t*-test, *p*-values ≤ 0.05 were considered significant.

3. Results

3.1. Expression of CXCL10 mRNA and protein as well as eotaxin protein was reduced by MNK-1 inhibition, whereas eotaxin mRNA expression was hardly affected

TNF- α -induced CXCL10 protein secretion by asthmatic and non-asthmatic ASMC was dose-dependently reduced by CGP57380 at concentrations between 2.5 and 20 μ M, while the lowest concentration (1.25 μ M) had no significant effect (Fig. 1A). The basal level of CXCL10 protein secretion (ASMC treated with vehicle only) was not affected by CGP57380 (20 μ M) (Fig. 1A). Similarly, CXCL10 mRNA expression was strongly induced by TNF- α and significantly inhibited by CGP57380 at 10 μ M in both asthmatic and non-asthmatic ASMC (Fig. 1B).

Eotaxin protein secretion induced by TNF- α in asthmatic and non-asthmatic ASMC was dose-dependently inhibited by CGP57380 at all concentrations (1.25–20 μ M), while the basal eotaxin protein level was not altered by CGP57380 (20 μ M) (Fig. 1C). In contrast to protein, TNF- α induced eotaxin mRNA was inhibited by CGP57380 (10 μ M) in non-asthmatic ASMC (0.867 ± 0.05 ; $p = 0.02$) but not in asthmatic ASMC (Fig. 1D).

The protein expression of TNF- α -induced IL-8 was not inhibited by CGP57380 (1.25–20 μ M) in either asthmatic or non-asthmatic ASMC (data not shown).

3.2. MNK-1 controls eotaxin and CXCL10 de novo synthesis

Next we investigated the role of MNK-1 on the regulation of eotaxin and CXCL10 by down regulating MNK-1 with corresponding siRNA. As shown in Fig. 2A siRNA down-regulated MNK-1 expression in unstimulated, and TNF- α stimulated ASMC. TNF- α induced mRNA and protein expression of eotaxin where significantly inhibited in cells with down-regulated MNK-1 (Figs. 2B, C). A similar effect was observed for CXCL10, where MNK-1 siRNA treated cells expressed significantly less mRNA and protein (Figs. 2D, E). Neither MNK-1 siRNA nor control siRNA had any effect on eotaxin or CXCL10 expression in unstimulated cells (Figs. 2B–2E).

3.3. TNF- α induces MNK-1 and eIF4E phosphorylation, which is dose dependently inhibited by CGP57380

TNF- α induced the phosphorylation of ERK1/2 MAPK within 15 to 30 min which declined thereafter (Fig. 3A). TNF- α also induced the phosphorylation of p38 MAPK which was strongest after 15 min, and declined after 30 min (Fig. 3A). MNK-1 was phosphorylated 15 and 30 min after TNF- α stimulation (Fig. 3A). The phosphorylation of eIF4E was stimulated by TNF- α at 15 min and declined after 60 min (Fig. 3A). Importantly, TNF- α treatment had no effect on the level of total protein for any of the above investigated signalling transmitters (Fig. 3A).

As shown in Fig. 3B, the inhibition of MNK-1 by pre-treatment with CGP57380 dose-dependently reduced the TNF- α induced phosphorylation of its down-stream target eIF4E at 30 min, whereas total protein expression of eIF4E was not affected. We did not observe any difference of the MNK-1 inhibitor action comparing asthmatic to non-asthmatic ASMC (data not shown).

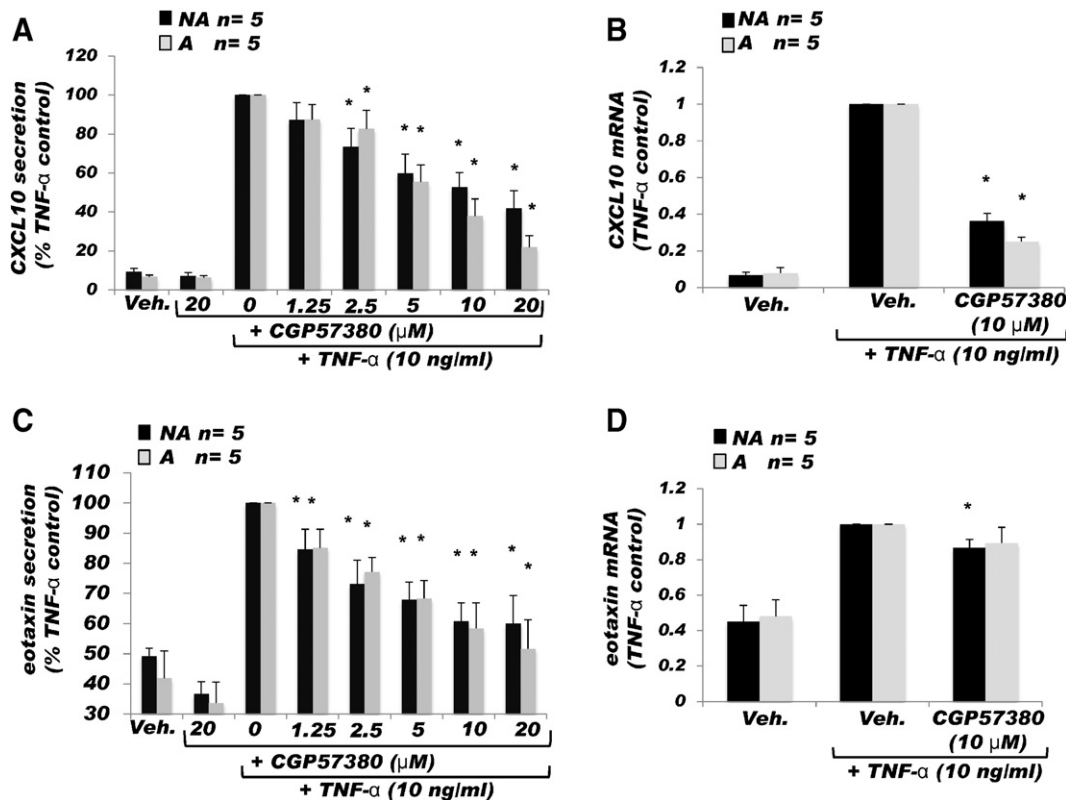


Fig. 1. ASMC derived from asthmatic (grey bars) or non-asthmatic (black bars) donors were treated with the MNK-1 inhibitor CGP57380 or vehicle DMSO (Veh.) prior to stimulation with TNF- α . CXCL10 secretion (A) or mRNA expression (B) as well as eotaxin secretion (C) or mRNA expression (D) was measured by ELISA or RT-real time PCR respectively. Data are expressed as mean $\pm$ S.E.M. and were analysed by unpaired Student's *t*-test. *Indicates $p \leq 0.05$ TNF- α vs. TNF- α + drugs.

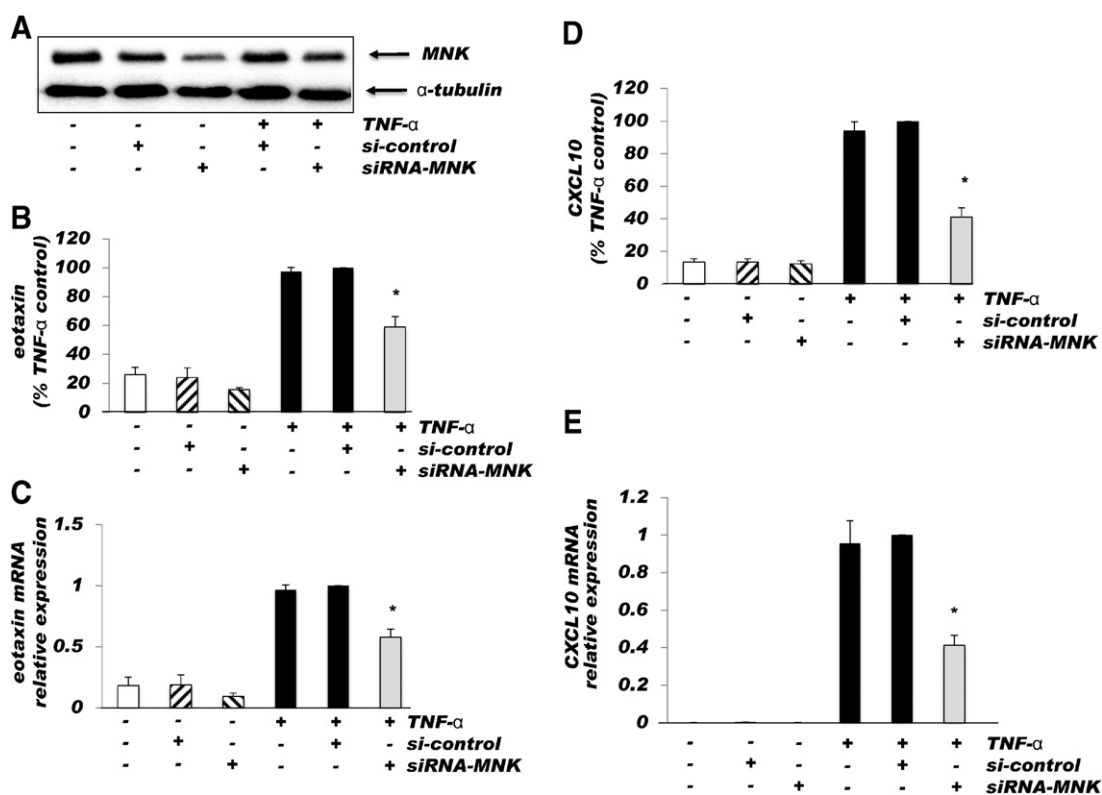


Fig. 2. MNK-1 regulates eotaxin and CXCL10 expression. (A) Representative Western-blot of MNK-1 expression in response to TNF- α and in the presence of siRNA to MNK-1 ($n = 4$). We used α -tubulin as a housekeeping control. (B) The effect of siRNA for MNK-1 on eotaxin expression in unstimulated and TNF- α stimulated ASMC ($n = 6$). (C) The effect of siRNA for MNK-1 on mRNA expression of eotaxin in unstimulated and TNF- α stimulated cells ($n = 6$). (D) The effect of siRNA for MNK-1 on CXCL10 expression in unstimulated and TNF- α stimulated ASMC ($n = 6$). (E) The effect of siRNA for MNK-1 on mRNA expression of CXCL10 in unstimulated and TNF- α stimulated cells ($n = 6$). Bars represent the mean $\pm$ S.E.M. of experiments performed in 6 ASMC lines in duplicates. *Indicates $p \leq 0.05$ compared to TNF- α treatment.

To exclude toxicity of CGP57380, non-asthmatic or asthmatic ASMC were treated with the vehicle control, positive control (Triton X-100, 0.5 mg/ml) or CGP57380 (10 and 20 μ M) for 24 h (Fig. 3C). The positive control significantly reduced cell viability in both cell types, whereas CGP57380 had no effect on cell viability when compared to vehicle treated cells (Fig. 3C).

3.4. Inhibition of the p38 MAPK reduces TNF- α induced eIF4E phosphorylation as well as CXCL10 and eotaxin protein secretion and mRNA expression

In order to investigate the role of MAPK, ASMC were pre-treated with either the ERK 1/2 MAPK inhibitor PD98059 (30 μ M) or the p38 MAPK inhibitor SB203580 (10 μ M), and then stimulated with TNF- α

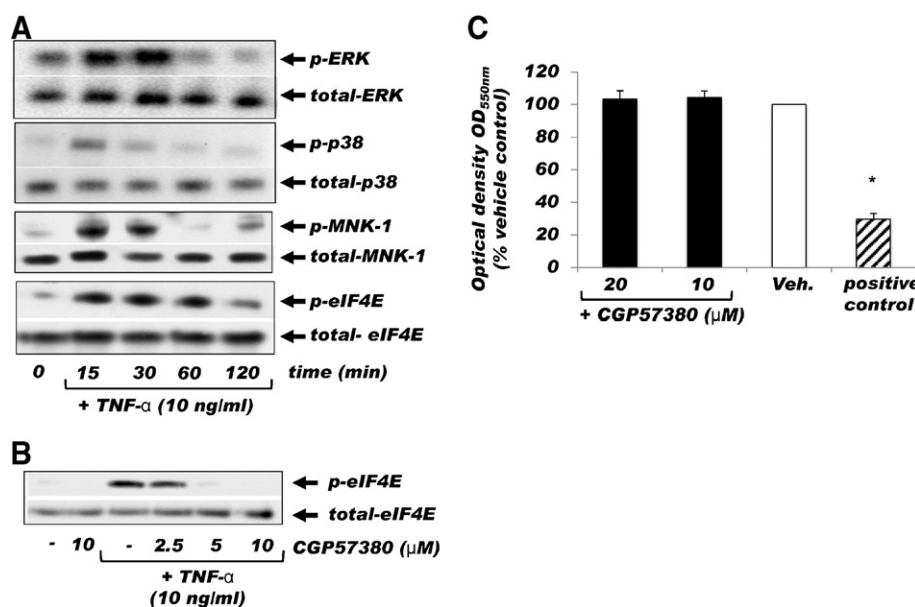


Fig. 3. (A) Kinetic of TNF- α induced phosphorylation of Erk1/2 MAPK, p32 MAPK, MNK-1 and eIF4E shown as one representative Western-blot from 6. (B) Representative Western-blot (one from six) showing the dose dependent inhibition of CGP57380 on eIF4E phosphorylation at 30 min. (C) Toxicity assay of CGP57380 on viability over 24 h in $n = 8$ ASMC lines. Bars represent the mean $\pm$ S.E.M. of 8 independent experiments, and Triton X-100 (0.5 mg/ml) was used as positive controls. *Indicates $p \leq 0.05$ from positive control.

(10 ng/ml). As shown by a representative Western-blot, the p38 MAPK inhibitor strongly down-regulated TNF- α induced phosphorylation of p-eIF4E, while the Erk1/2 MAPK inhibitor had only a weak inhibitory effect (Fig. 4A).

SB203580 significantly reduced TNF- α stimulated CXCL10 secretion (Fig. 4B), CXCL10 mRNA expression (Fig. 4C), eotaxin secretion (Fig. 4D), and eotaxin mRNA expression (Fig. 4E). In contrast, PD98059 had no significant effect on TNF- α induced CXCL10 secretion (Fig. 4B) and only weakly inhibited the secretion of eotaxin (Fig. 4D). The two MAPK inhibitors had no significant effect on CXCL10 (Fig. 4B) or eotaxin (Fig. 4D) basal protein secretion and there was no difference of the two MAPK inhibitors comparing non-asthmatic to asthmatic ASMC (data not shown).

3.5. Inhibition of TNF- α induced CXCL10 or eotaxin is independent of mRNA stability, altered eIF4E nuclear export, or NF- κ B activity

ASMC were pre-treated with CGP57380 (10 μ M) before being stimulated with TNF- α for 3 h and mRNA stability was determined in the presence of actinomycin D (5 μ g/ml) over 24 h (Fig. 5A). The CXCL10 mRNA produced after TNF- α stimulation was not significantly altered by actinomycin D treatment and CGP57380 had no significant effect on CXCL10 mRNA stability at the time points measured (Fig. 5A).

The nuclear export of eIF4E was determined after TNF- α stimulation in nuclear and cytosolic protein (Fig. 5B), showing that eIF4E is present in the cytosol and the nucleus in ASMC (Fig. 5B). TNF- α stimulation for 30 and 60 min did not change the levels of eIF4E in the nuclear or cytosolic fraction (Fig. 5B). Equal protein loading and the purity of the nuclear and cytosolic fraction was confirmed by probing the membranes with α -tubulin and laminin A/C (Fig. 5B).

TNF- α significantly induced the binding of NF- κ B p65 to its corresponding binding-DNA sequence in nuclear protein extracts isolated

from ASMC ($n = 5$) after 30 min ($p = 0.00012$), and this effect was not inhibited by pre-incubation (1 h) of the cells with the MNK-1 inhibitor CGP57380 (Fig. 5C).

3.6. MNK-1 inhibition reduces FCS- and PDGF-BB-stimulated ASMC proliferation

FCS-induced proliferation of non-asthmatic and asthmatic ASMC was inhibited by CGP57380 at 10 μ M, while the lower concentration of 1 μ M had no significant effect (Fig. 6A). CGP57380 did not alter basal ASMC proliferation since there was no difference in proliferation in ASMC treated with the vehicle or CGP57380 alone (Fig. 6A). The proliferation of non-asthmatic and asthmatic ASMC induced by PDGF-BB was also inhibited by CGP57380 at 10 μ M. CGP57380 at 5 μ M only significantly inhibited proliferation of asthmatic ASMC and the lower concentration of CGP57380 at 2.5 μ M had no significant effect on PDGF-BB-induced ASMC proliferation (Fig. 6B).

3.7. MNK-1 inhibition down-regulates TNF- α induced cytokine secretion

To further support the potential of MNK-1 inhibition as a therapeutic target in asthma we measured the inhibitory effect of MNK-1 siRNA on multiple cytokines, using a cytokine array detecting 36 different human cytokines (Fig. 7A). In medium collected from ASMC stimulated with TNF- α for 24 h the following cytokines were expressed: MIF, MCP-1, SerpinE1, RANTES, IL-6, IL-8, GRO α and CXCL10 (Fig. 7B). MNK-1 inhibition by MNK-1 siRNA strongly inhibited the TNF- α induced expression of RANTES, GRO α , CXCL10 and MCP-1, whereas it had no effect on MIF, SerpinE1, IL-6 or IL-8 (Fig. 7B).

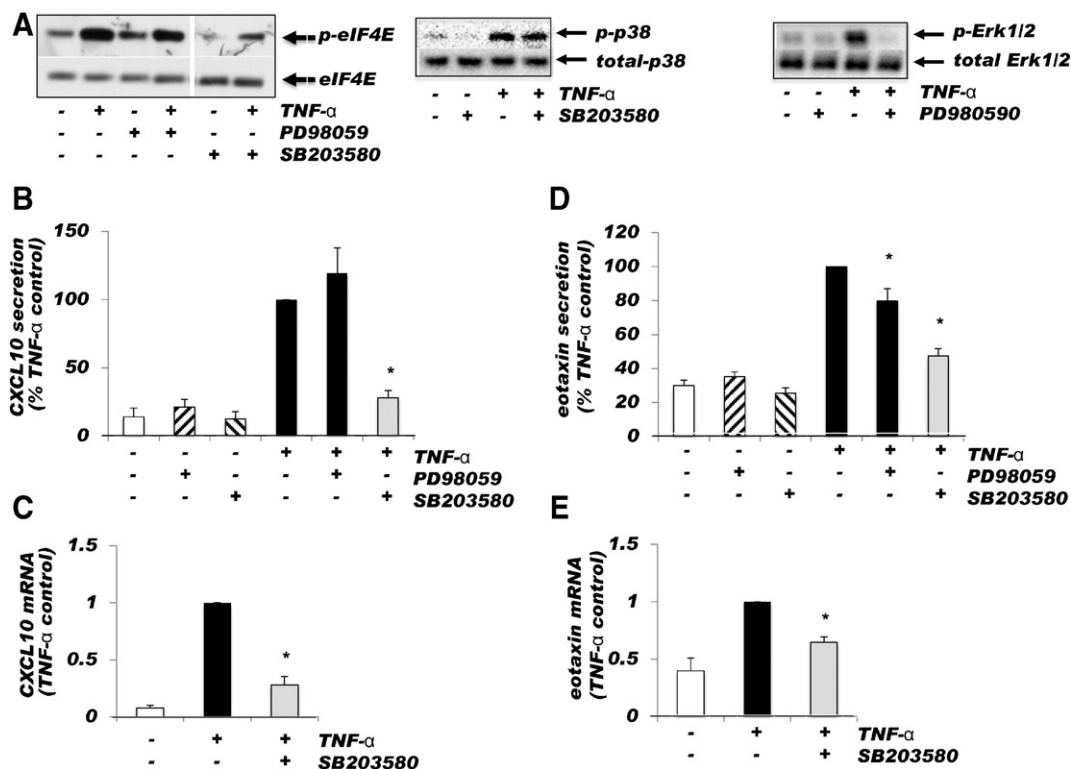


Fig. 4. The role of MNK signalling in eIF4E phosphorylation and synthesis of CXCL10 and eotaxin. (A) Representative Western blot (one from six) of eIF4E phosphorylation by TNF- α in cells pre-treated with either the Erk1/2 MAPK or the p38 MAPK inhibitor. (B) The effect of MAPK inhibitors on CXCL10 protein expression in unstimulated and TNF- α stimulated ASMC. (C) The effect of p38 inhibition on CXCL10 mRNA expression. (D) The effect of MAPK inhibitors on eotaxin protein expression in unstimulated and TNF- α stimulated ASMC. (E) The effect of p38 inhibition on eotaxin mRNA expression. Bars represent the mean $\pm$ S.E.M of 4 independent experiments. *Indicates $p \leq 0.05$ from TNF- α control.

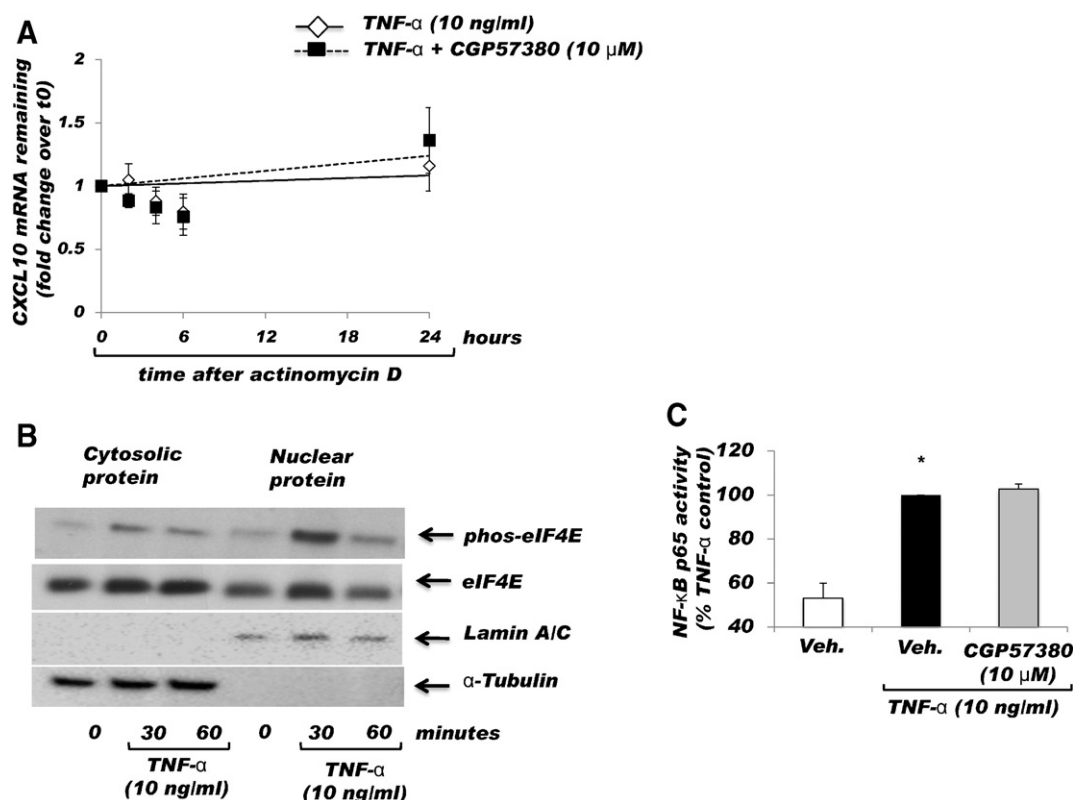


Fig. 5. (A) ASMC ($n = 4$) were pre-treated with CGP57380 or vehicle DMSO (Veh.) before to stimulation with $\text{TNF-}\alpha$. After 3 h, actinomycin D was added to the cells and mRNA expression was measured at the indicated time points and expressed as a fold change over the mRNA levels determined at time point 0 h. (B) ASMC ($n = 4$) were stimulated with $\text{TNF-}\alpha$ and cytosolic and nuclear protein was isolated between 0 and 60 min, eIF4E, Lamin A/C or α -tubulin protein were determined by immuno-blotting. (C) ASMC ($n = 5$) were pre-treated for 30 min with CGP57380 or the vehicle DMSO (Veh.) before to stimulation with $\text{TNF-}\alpha$. NF- κ B p65 DNA binding activity was determined with an NF- κ B p65 TransAm™ transcription factor assay kit and expressed as percentage of $\text{TNF-}\alpha$ positive control. *Indicates $p \leq 0.05$ for the comparison of $\text{TNF-}\alpha$.

4. Discussion

In this study, we show for the first time that $\text{TNF-}\alpha$ induces phosphorylation of MNK-1 and its down-stream target eIF4E in human primary ASMC derived from asthmatic and non-asthmatic donors. MNK-1 inhibition reduced eIF4E phosphorylation and secretion of CXCL10 and eotaxin protein as well as ASMC proliferation. The analysis of a cytokine array for 36 asthma relevant human cytokines showed that MNK-1 inhibition also reduced the levels of other asthma-relevant RANTES, GRO α , CXCL10 and MCP-1. Our results therefore suggest that MNK-1 is a potential new target for asthma therapy.

The regulation of cytokine expression on the level of transcription control has been intensively investigated, whereas the role of translation control mechanisms remains largely unknown. A key player in translational control is eIF4E, which is directly and exclusively phosphorylated by MNK-1 and 2 [19]. Active eIF4E binds to the 5'-cap structure of the mRNA to initiate protein translation [24]. In addition, eIF4E modified mediator expression by altering mRNA stability or mRNA nuclear export [20]. Phosphorylation of eIF4E can be induced by several pro-inflammatory or pro-proliferative stimuli [25] and an increased phosphorylation is thought to enhance eIF4E affinity to the 5'-cap structure of the mRNA [26] as well as its ability to export mRNAs from the nucleus into the cytosol [27].

Here we show that MNK-1 and its down-stream target eIF4E are phosphorylated in human primary ASMC in response to $\text{TNF-}\alpha$. Based on the earlier reported and here confirmed activation of p38 MAPK and ERK 1/2 MAPK by $\text{TNF-}\alpha$ in ASMC [1,22] and a report that MNK-1 activity is induced via p38 and ERK 1/2 MAPK following exogenous stimulation [16]. We further investigated the role of the two MAPK on eIF4E activation. Our data provide evidence that in human ASMC, $\text{TNF-}\alpha$ induces MNK-1 phosphorylation by activation of p38 MAPK.

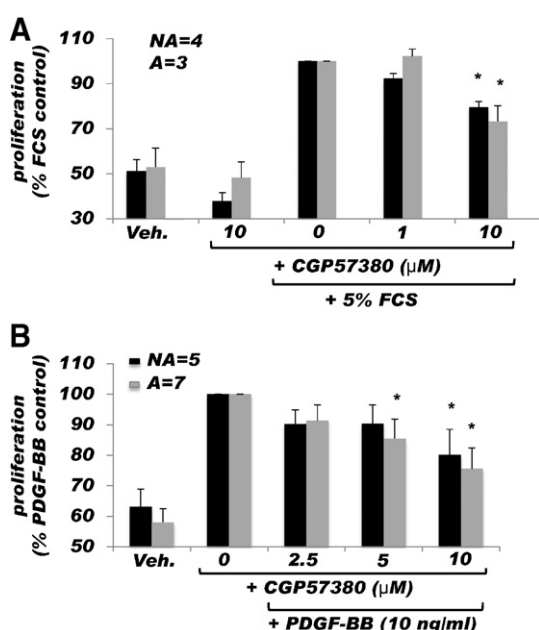


Fig. 6. ASMC from asthmatic (grey bars) or non-asthmatic (black bars) donors were pre-treated (30 min) with CGP57380 or vehicle DMSO (Veh.) prior to 72 h stimulation with FCS (A) or PDGF-BB (B). ASMC proliferation was determined by cell enumeration using a Beckman Coulter counter. Data are expressed as mean $\pm$ S.E.M. and were analysed by unpaired Student's t -test. *Indicates $p \leq 0.05$ for the comparison of $\text{TNF-}\alpha$ vs. $\text{TNF-}\alpha$ + drugs.

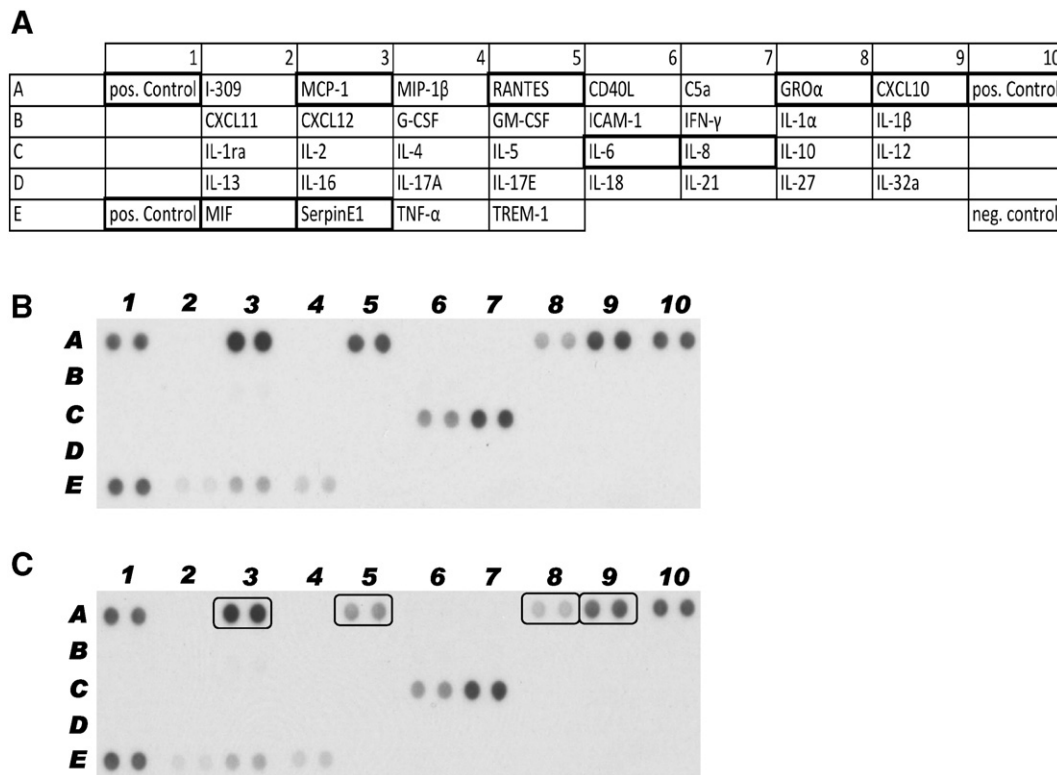


Fig. 7. The effect of MNK-1 siRNA treatment on the protein expression of asthma relevant pro-inflammatory and pro-angiogenic proteins. (A) cyto- and chemokine array. (B) representative array of TNF- α stimulated airway smooth muscle cells. (C) representative array of airway smooth muscle cells pre-treated with MNK-1 siRNA and then stimulated with TNF- α . Framed cytokines were different from TNF- α stimulated cells.

This stimulatory effect of TNF- α on MNK-1 occurred in both asthmatic and non-asthmatic ASMC with no disease specific difference. Furthermore, we show that the MNK-1 inhibitor CGP57380 dose-dependently inhibits TNF- α induced phosphorylation of eIF4E, confirming that MNK-1 mediates eIF4E activation in ASMC.

Earlier we reported that asthma might be linked to faulty translation control of cell differentiation regulating proteins [28]. However, this effect seems to be protein or mRNA specific, as we did not detect any difference of TNF- α induced MNK-1 or eIF4E phosphorylation comparing asthmatic to non-asthmatic ASMC. Furthermore, the inhibitory effect of CGP57380 on chemokine secretion or ASMC proliferation was not significantly different comparing asthmatic to non-asthmatic ASMC.

Inhibition of MNK-1 reduces protein secretion of CXCL10 and eotaxin, which is of importance to asthma as these chemokines attract mast cells and eosinophils into the asthmatic airways and perpetuate inflammation [2,5]. The observation that the MNK-1 inhibitor CGP57380 down-regulates TNF- α protein secretion, without affecting its mRNA levels in macrophages suggests that protein translation plays a role in this pathological aspect of asthma [29]. This idea is further supported by a report that CGP57380 reduced CCL3 or CCL4 protein secretion but did not alter the cytokines mRNA expression in neutrophils [15]. However, our findings in ASMC show that MNK-1 inhibition reduces eotaxin and CXCL10 RNA expression, as well as their secretion, suggesting an inhibitory effect on the level of chemokine transcription.

In regard to CXCL10, we have previously shown that CXCL10 protein secretion was inhibited by ciglitazone without affecting CXCL10 mRNA levels [30], suggesting that CXCL10 is regulated on the level of translation. However, we here show that MNK-1 inhibition reduces both CXCL10 protein and mRNA. Others have shown that CGP57380 inhibits LPS-induced TNF- α mRNA expression by decreasing TNF- α mRNA stability [31]. However, in our experiments MNK-1 inhibition did not alter the TNF- α induced CXCL10 mRNA stability in ASMC.

It was reported that eIF4E promotes the export of mRNA into the cytoplasm, thereby increasing cytoplasmic mRNA levels and subsequent

protein expression [32]. We here show that eIF4E is present in both the cytosol and the nucleus in ASMC, and that TNF- α does not alter the ratio of cytosolic-nuclear distribution of eIF4E. The data suggest that the alteration of nuclear mRNA export is not relevant to the inhibitory effect of CGP57380 on TNF- α induced CXCL10 or eotaxin expression. In addition, we detected TNF- α stimulated eIF4E phosphorylation in the cytosol and the nucleus, suggesting that phosphorylated eIF4E translocated to the nucleus. However, the question whether phosphorylation is relevant eIF4E to transcription of chemokines remains to be determined in future studies.

We and others have previously shown that TNF- α strongly activates NF- κ B in ASMC within a time window of 15–60 min and that its inhibition reduces TNF- α -induced CXCL10 and eotaxin secretion by ASMC [22,23,33]. We therefore investigated the effect of CGP57380 on NF- κ B activation and found that CGP57380 has no inhibitory effect on TNF- α -induced NF- κ B DNA binding in ASMC. The CXCL10 or eotaxin promoter include response element for many other transcription factors including interferon regulatory factor (IRF)-1, STAT-1, AP-1, or C/EBP [34]. However, whether the activation of these factors is affected by CGP57380 remains to be determined in future studies.

Besides airway inflammation, airway wall hypertrophy is an important pathology in the asthmatic airway. We and others have shown that ASMC derived from asthma patients proliferate faster than cells from non-asthmatic donors in vitro [35,36]. Elevated ASMC proliferation is thought to contribute to the increased airway smooth muscle mass observed in asthmatic airways [37,38]. Our data indicate a stimulatory role of MNK-1 in ASMC proliferation since the inhibition of MNK-1 reduces FCS and PDGF-BB induced ASMC proliferation. These findings are in line with results obtained in breast cancer and a glioma cell lines, in which CGP57380 inhibits cell proliferation by reducing the levels of phosphorylated eIF4E [39,40]. The underlying mechanism of CGP57380's anti-proliferative effect still needs to be investigated, however, our findings strengthen the hypothesis that MNK-1 presents a new therapeutic target for asthma therapy.

In summary, we demonstrate in this study that in human primary ASMC TNF- α activates the MNK-1/eIF4E pathway through p38 MAPK activation. Inhibition of MNK-1 down-regulates chemokine secretion as well as it inhibits proliferation of ASMC. Our data support the hypothesis that MNK-1 may serve as new potential target for asthma therapy, which reduces both inflammation and remodelling.

5. Conclusion

In human primary ASMC TNF- α activates the MNK-1/eIF4E pathway through p38 MAPK activation.

Inhibition of MNK-1 down-regulates CXCL10 and eotaxin secretion.

Inhibition of MNK-1 reduces proliferation of ASMC.

MNK-1 may serve as new target for asthma therapy, which reduces both inflammation and remodelling.

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