Dimethylfumarate inhibits NF-κB function at multiple levels to limit airway smooth muscle cell cytokine secretion

Seidel P ¹,²,³, Merfort I ², Hughes JM ³, Oliver BGG ⁴, Tamm M ¹, Roth M ¹

¹ Pulmonary Cell Research, Department of Research and Pneumology, Department of Internal Medicine, University Hospital Basel, Switzerland
² Institute of Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Freiburg, Germany
³ Respiratory Research Group, Faculty of Pharmacy and
⁴ Discipline of Pharmacology, University of Sydney, Australia

authors:

1. Petra Seidel, Tel.: +41-61-2652337, Fax: +41-61-265 29 39, E-mail: petra.seidel@stud.unibas.ch
2. Irmgard Merfort, Tel.: +49 761 203 8373, Fax: +49 761 203 8383, E-mail: irmgard.merfort@pharmazie.uni-freiburg.de
3. J Margaret Hughes, Tel.: +61 2 9351 2323, Fax: +61 2 9351 4391, E-mail: margh@pharm.usyd.edu.au
4. Brian Oliver, Tel.: +61 2 9351 2315, Fax: +61 2 9351 3868, E-mail: boliver@med.usyd.edu.au
5. Michael Tamm, +41 061 265 51 84, Fax: +41 061 265 45 87, E-mail: mtamm@uhbs.ch

Correspondence to: Michael Roth
Abstract

The anti-psoriatic dimethylfumarate (DMF) has been anecdotically reported to reduce asthma symptoms and to improve quality of life of asthma patients. DMF decreases the expression of pro-inflammatory mediators by inhibiting the transcription factor nuclear factor κB (NF-κB) and might therefore be of interest for the therapy of inflammatory lung diseases. In this study we determined the effect of dimethylfumarate (DMF) on platelet derived growth factor (PDGF)-BB and tumor necrosis factor (TNF)-α induced asthma relevant cytokines and NF-κB activation by primary human asthmatic and non-asthmatic airway smooth muscle cells (ASMC). Methods: Confluent non-asthmatic and asthmatic ASMC were incubated with DMF (0.1-100 μM) and/or dexamethasone (0.0001-0.1 μM), NF-κB p65 siRNA (100 nM), the NF-κB inhibitor helenalin (1 μM) before stimulation with PDGF-BB or TNF-α (10 ng/ml). Cytokine release was measured by enzyme linked immunosorbsent assay. NF-κB, MSK-1, and CREB activation was determined by immuno-blotting and EMSA. Results: TNF-α induced eotaxin, RANTES and interleukin (IL)-6 as well as PDGF-BB induced IL-6 expression was inhibited by DMF and by dexamethasone from asthmatic and non-asthmatic ASMC, but the combination of both drugs showed no glucocorticoid sparing effect in either of the two groups. NF-κB p65
siRNA and/or the NF-κB inhibitor helenalin reduced PDGF-BB and TNF-α induced cytokine expression, suggesting the involvement of NF-κB signaling. DMF inhibited TNF-α induced NF-κB p65 phosphorylation, NF-κB nuclear entry and NF-κB -DNA complex formation, whereas PDGF-BB appeared not to activate NF-κB within 60 min. Both stimuli induced the phosphorylation of mitogen and stress activated kinase (MSK)-1, NF-κB p65 at Ser276, and CREB and all were inhibited by DMF. These data suggest that DMF down-regulates cytokine secretion not only by inhibiting NF-κB but a wider range of NF-κB linked signaling proteins which may explain its potential beneficial effect in asthma.

Keywords: MSK-1, CREB, DMF anti-inflammatory action, chronic lung inflammation, DMF steroid sparing effect.

**Introduction**

Fumaric acid esters (FAE) have been used in the treatment of psoriasis for several decades. A mixture of dimethylfumarate (DMF) with Ca^{2+}, Mg^{2+} and Zn^{2+} salts of ethylhydrogen fumarate is currently registered in Germany for the systemic treatment of severe psoriasis (32). The clinical efficacy and the long-term safety of FAE in the treatment of psoriasis have been proven in several clinical trials (19, 32). However, neither the molecular mechanisms underlying the biological action of FAE nor the active compound have been completely identified. DMF, the main ingredient of the FAE mixture, was shown to be clinically most efficacious and was therefore proposed to be the active compound.
DMF inhibited NF-κB and down-regulated NF-κB dependent mediators in psoriasis relevant cells. DMF (140 μM) inhibited NF-κB and the expression of IL-8 and IL-20 mRNA in human keratinocytes (15). DMF at 20 μM was sufficient to inhibit binding of NF-κB to DNA in human T-cells (14). ICAM-1 expression and the nuclear accumulation of the NF-κB p50 subunit were inhibited by 100 μM DMF in human dermal fibroblasts (49). At 84 μM DMF inhibited TNF-α induced tissue factor mRNA and protein expression as well as the nuclear entry of NF-κB p65 and p50 (27).

NF-κB is one of the most potent activating transcription factors of pro-inflammatory and inflammatory gene activity (48). Therefore, it is not surprising that NF-κB is an important mediator of inflammation in the pathogenesis of psoriasis and other chronic inflammatory diseases, including multiple sclerosis and poly-arthritis (7, 24). Therefore NF-κB inhibitors such as FAE are of pharmacological interest in the therapy of such diseases (24). Based on occasional reports by psoriasis patients that their asthma symptoms were reduced during DMF therapy we hypothesized that DMF may be a potential novel drug for inflammatory lung diseases.

The NF-κB family consists of five members that homo- and heterodimerize with each other to regulate gene transcription. NF-κB activity is mainly controlled by its association with the inhibitor of κB (IκB) proteins, which retain NF-κB in the cytosol and therefore prevent its nuclear entry and binding to DNA. Stimulation of the IκB kinase (IKK) leads to the phosphorylation and ubiquitination of IκB which is then degraded, resulting in the
release of active NF-κB. Posttranslational modifications such as phosphorylation or acetylation represent another level of NF-κB regulation. NF-κB p65 can be phosphorylated at several sides, which has been shown to modify the import and export of NF-κB, as well as its transactivation potential (20, 22). Many genes of pro-inflammatory factors which are up-regulated in chronic inflammatory airway diseases contain a NF-κB binding promoter site (7, 2), and thus NF-κB contributes to the pathogenesis of asthma (7).

The most frequently applied therapeutic strategy in chronic inflammatory airway diseases are inhaled glucocorticoids (GC) and long acting β2-agonists which in combination control symptoms much better than increased doses of GCs alone (30). However, both drugs control the symptoms and do not cure the disease. Furthermore, a reasonable number of patients does not respond sufficiently to GCs or only respond to high doses of systemically applied GCs (21). Then GCs have severe side effects and it is therefore necessary to define new therapeutic options that could reduce GC doses (GC sparing) or even replace GCs in asthma therapy.

Beside inflammatory cells, the airway smooth muscle cell (ASMC) plays a central role in the pathogenesis of asthma and chronic obstructive pulmonary disease (17). ASMC contribute significantly to airway remodeling and release pro-inflammatory cytokines, thereby recruiting more immune cells into the inflamed airway (6, 17, 45). In chronic inflammatory airway diseases tumor necrosis factor-α (TNF-α) is one of the most prominent cytokines and stimulates the synthesis of interleukin (IL)-6 (1), eotaxin
(CCL11) (38) and RANTES (regulated upon activation, normal T cell expressed and secreted; CCL5) (1). A second pro-inflammatory factor is the platelet derived growth factor (PDGF)-BB, which is a strong inducer of ASMC proliferation (44), as well as of IL-6 and IL-8 secretion (3, 47). Together these cytokines attract immune cells into the inflamed airway and amplify the inflammation.

The aims of this study were to determine the effects of DMF on PDGF-BB and TNF-α induced NF-κB activation and cytokine secretion (eotaxin, RANTES and IL-6) by ASMC and a possible synergism with the GC dexamethasone.

Experimental procedures

*Isolation, culture and characterization of human ASMC*

Human ASMC were isolated and grown from either endobronchial biopsies or therapeutic lung resections from donors with and without asthma as previously described (23). ASMC were grown in RPMI-1640 supplemented with 25 mM HEPES and 2mM L-glutamine (Thermo Trace, Melbourne, Australia), 5% heat - inactivated fetal bovine serum (FBS), 1x (v/v) MEM vitamin-mix, 100 U/l penicillin, 100 μg/ml streptomycin, and 0,25 μg/ml of amphotericin B (GIBCO/BRL, Australia) in a humidified atmosphere at 37°C in 5% CO2, 95% air. ASMC lines were used between passages 5-8. ASMC were characterized as described previously (23) by positive immuno-blotting for: calponin, and α-SMA; and by negative staining for fibronectin (all antibodies: Santa Cruz Biotech, Santa Cruz, CA). Results obtained in ASMC were compared to fibroblasts,
grown from the same lung tissue sample, and a representative immuno-blot is shown in figure 1 A)

*Drug preparation*

DMF (Fluka Chemie, Buchs, Switzerland), dexamethasone (Sigma, Sydney, Australia), H89 and helenalin (Sigma) were dissolved in dimethysulfoxide (DMSO; Sigma) and diluted to the required concentration in low serum medium [RPMI-1640, 0.1% heat-inactivated FBS, 1x MEM vitamin-mix, 100 U/l penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 mM HEPES, 2 mM L-glutamine].

*Cell treatment*

ASMC were seeded into 24-well culture plates and grown to confluence. The growth medium was replaced with low serum medium 24h before stimulation. DMF (0.1-100 μM), dexamethasone (0.0001-1 μM), helenalin (1 μM) and/or the vehicle DMSO (final conc. 0.1%) were added to the cells 1h prior to PDGF-BB or TNF-α stimulation (both: 10 ng/ml) (R&D Systems, Minneapolis, MN). The concentration of both stimuli had been determined as optimal on cultured ASMC in previous studies and were used under similar conditions (1, 3, 8)

To assess the effect of the DMF and dexamethasone in combination, DMF (10, 100 μM) was added together with dexamethasone (0.01, 0.0001 μM) 1h before to PDGF-BB or TNF-α stimulation (both: 10 ng/ml). After 24h cell culture medium samples were collected and eotaxin, RANTES and IL-6 protein levels were measured by enzyme-linked immunosorbent assay (ELISA Duo Set, R&D Systems).
To determine the effect of NF-κB p65 siRNA on cytokine expression, ASMC were plated into 12-well plates at ~ 70% confluence and were then transfected with either NF-κB p65 siRNA (100 nM) or a negative control siRNA according to the manufacturer’s protocol (SignalSilence NF-κB p65 siRNA kit, Cell Signaling Technology, Beverly, MA). Briefly, the transfection reagent was added to serum free medium and incubated for 5 min, the appropriate volume of siRNA or control RNA was added and incubated for 5 min. The cell culture medium was replaced with fresh growth medium and the siRNA or control RNA mixture was added. After 24h cells were serum deprived for 24h and then stimulated with TNF-α or PDGF-BB, or left un-treated. Cell culture medium and total cell lysates were collected after 24h for ELISA and protein analysis.

**LDH membrane integrity assay**

To determine cytotoxic effects of DMF in the presence or absence of the stimuli, ASMC were plated into 96-well plates, pretreated with DMF 100 µM for 1h and then stimulated with TNF-α or PDGF-BB (both: 10 ng/ml). After 24h stimulation, the LDH release was assayed with a LDH membrane integrity assay kit according to the manufactures protocol (CytoTox-OneTM homogeneous membrane integrity assay, Promega, Madison, WI). Briefly, a lysis solution was added to positive control cells to determine the maximum LDH release (100%). CytoTox-ONE™ Reagent was added to each well and was incubated for 10 min at room temperature, then the reaction was stopped and fluorescence was recorded at 560 nm excitation and at 590 nm emission. The assay was
performed in triplicates for each condition in ASMC of two different donors. Cytotoxicity of each condition was calculated as a percentage of maximal LDH release.

Transcription factor activity

The effect of DMF on TNF-α and PDGF-BB induced NF-κB, IκB-α, CREB and MSK-1 activation was determined in confluent ASMC. Cells were treated with DMF 100 μM for 1h before stimulation with PDGF-BB or TNF-α (both: 10 ng/ml). Total cell lysates were collected at various time points in Laemmli buffer (62.5 mM Tris HCl [pH 6.8], 2% SDS, 0.5% mercapto-ethanol, 10% glycerol) containing Pierce HaltTMprotease inhibitor cocktail (Thermo Scientific, Rockford, IL).

Nuclear and cytosolic protein extracts were prepared with NE-PER nuclear and cytosolic extraction kit (Pierce Biotech.) according to the manufacturer’s protocol. Briefly, ASMC were trypsinized, collected by centrifugation (3.000 rpm, 3 min) and the cell pellet was incubated on ice with 200 μl of buffer CER I. After 10 min, 11 μl of buffer CER II were added and after 1 min, the sample was centrifuged at 13.000 rpm (4°C, 5 min), and the supernatant was collected as cytosolic protein fraction. The remaining pellet was resuspended in 50 μl of buffer NER and after 40 min, the sample was centrifuged (13.000 rpm, 4°C, 10 min) and the supernatant was collected as nuclear protein fraction. Both buffer, CER I and NER, contained the Pierce HaltTMprotease inhibitor cocktail.
To assess DMF effects on active NF-κB, ASMC were first stimulated with TNF- (10 ng/ml) for 30 min before DMF (100 μM) was added and nuclear protein extracts were prepared after 0, 1, 2 and 3 hrs.

To confirm the inhibition of phosphorylation of NF-κB p65 at Ser276 by the MSK-1/PKA inhibitor H89 and to assess its effect on NF-κB nuclear entry and DNA binding, H89 (10 μM) was added to ASMC 1h prior to stimulation with TNF-α and total or nuclear proteins were extracted after 30 min.

**Immunoblotting**

Laemmli running buffer (2x) was added (1:1) to cell lysates and samples were boiled for 5 min. Equal amounts of protein were then size-fractionated by electrophoresis (140V, 60 min) in a 4-20% gradient PAGE-gel (Pierce Biotech.). The gel was then sandwiched between two nitrocellulose membranes and placed in transfer buffer (0.05 M NaCl, 2 mM Na-EDTA, 0.l mM dithiothreitol, 10 mM Tris HCl [pH 7.0]) and incubated over night at 50°C (4). Protein transfer was confirmed by Ponceau staining. Membranes were incubated with blocking buffer (5% w/v non-fat dry milk in Tris-buffered saline 0.1% Tween 20) for 1h at room temperature before being incubated with a primary antibody, as listed in table 1. Primary antibodies were detected by horseradish peroxidase-conjugated IgG antibodies diluted 1:2000- 1:40000 (anti-rabbit IgG #7074, or anti-mouse IgG #7076; Cell Signaling Tech., Beverly, MA) and protein bands were visualized by enhanced chemiluminescence (ECL, Pierce Biotech.).
**Electrophoretic mobility shift assay**

Nuclear protein extracts were prepared by NE-PER (Pierce Biotech.) according to the manufacturers protocol and the protein content was determined by Bradford assay (Biorad). Equal amounts of protein (5 μg) were mixed with 20 μg bovine serum albumin (BSA, Sigma), 2 μg poly(dI,dC) (Roche Molecular Biochemicals, Basel, Switzerland), 2 μl buffer D+ [20 mM Hepes, (pH 7.9), 20% glycerol,100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF], 4 μl buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, and 0.1% PMSF), and 100,000 cpm (Cerenkov) of [33P]-labeled oligonucleotide for NF-κB (50-AGT TGA GGG GAC TTT CCC AGG C-30, Promega, Mannheim, Germany) made up to a final volume of 20 μl with distilled water. Samples were incubated at room temperature for 25 min. NF-κB oligonucleotide was labeled using [γ33P]-dATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Freiburg, Germany) and a T4 polynucleotide kinase (New England Biolabs, Hilden, Germany). For supershift analysis antibodies against NF-κB p65 (H286) and p50 (C19) (1:3; both Santa Cruz Biotech.) were added to the binding reaction, and samples were size fractionated by electrophoresis in a nondenaturing 4% or 6% polyacrylamide gel.

**Data analysis**

All cytokine data are presented as mean ± SEM, data representing image analysis is shown as representative immuno-blots and as mean ± SEM after densitometric image analysis of at least three independent experiments. Statistical analysis was performed using one-way ANOVA or a paired/unpaired Student’s t test. The “Null-hypothesis” was: no effect of drugs and p-values <0.05 were considered significant.
Results

DMF cytotoxicity in ASMC

In ASMC treated with the vehicle 0.1% DMSO the percentage of LDH release was 21.6 ± 1.4% and this was not notably altered by 100 µM DMF (21.9 ± 5.0%) or by 100 µM DMF with TNF-α (19.5 ± 2.3%) or by PDGF-BB (20.7 ± 1.7%).

TNF-α and PDGF-BB induced cytokine release from ASMC

ASMC of asthmatic and non-asthmatic donors were stimulated with TNF-α or PDGF-BB or were left un-stimulated for 24h and cytokine levels were determined in the cell culture medium. Unstimulated ASMC released low levels of eotaxin and RANTES with no significant differences of eotaxin (p=0.6420; Fig.1 B) or RANTES (p=0.7089; Fig.1 C) comparing asthmatic to non-asthmatic ASMC. Baseline levels of IL-6 were also low in both cell groups, but there was a trend of higher IL-6 levels in asthmatic ASMC when compared to non-asthmatic ASMC (p=0.0664; Fig.1 D, E). Stimulation with TNF-α (10 ng/ml) significantly induced the secretion of eotaxin from non-asthmatic (p=0.0050; Fig.1 B) and asthmatic (p=0.0242; Fig.1 B) ASMC with no significant difference between asthmatic and non-asthmatic ASCM (p=0.1842; Fig.1 B).

Similarly, RANTES was significantly up-regulated by TNF-α in non-asthmatic (p=0.0038; Fig.1 C) and asthmatic (p=0.0462; Fig.1 C) ASMC with no difference between the two groups (p=0.5490; Fig.1 C).

TNF-α also significantly induced the release of IL-6 from non-asthmatic (p=0.0031; Fig.1 D) and asthmatic (p=0.0083; Fig.1 D) ASMC and again there was no significant
difference comparing the to groups (p=0.2028; Fig.1 D). PDGF-BB only significantly stimulated the secretion of IL-6 from non-asthmatic (p=0.0380; Fig.1 E) and asthmatic ASMC (p=0.0318; Fig.1 E) with no difference between the two groups (p=0.1769; Fig.1 E), while it did not increase the secretion of eotaxin or RANTES by ASMC.

*The inhibitory effect of DMF and/or dexamethasone on TNF-α and PDGF-BB stimulated cytokine release by ASMC*

To determine the effects of DMF and dexamethasone on TNF-α and PDGF-BB induced cytokine expression, we treated ASMC from asthmatic and non-asthmatic donors with increasing concentrations of the single drugs or the drugs combination. DMF at 50 μM and 100 μM abolished TNF-α induced secretion of eotaxin (p<0.0001; Fig.2 A) and RANTES (p<0.0001; Fig. 2 C) and DMF 10 μM reduced RANTES and eotaxin to 50-60% of the TNF-α induced amount (p≤0.0445; Fig. 2 A, C) in both asthmatic and non-asthmatic ASMC. In comparison, dexamethasone at 0.01 μM and 0.1 μM reduced eotaxin (p≤0.0183, Fig. 2 B) and RANTES (p<0.0001, Fig. 2 D) secretion to 30-50% of the TNF-α induced level in both groups. There was no significant difference in eotaxin or RANTES secretion comparing asthmatic to non-asthmatic ASMC treated with DMF or dexamethasone (Fig. 2 A-D).

In asthmatic ASMC the TNF-α induced IL-6 secretion was inhibited in a concentration dependent manner by DMF at concentrations ≥ 10 μM (p≤0.0445; Fig.2 E), while in non-asthmatic ASMC only the highest concentration of DMF 100 μM reduced TNF-α induced IL-6 secretion by 70% compared to TNF-α (p<0.0001; Fig.2 E). The inhibitory effect of DMF on IL-6 secretion was significantly different comparing asthmatic to non-
asthmatic ASMC at 10 μM (p=0.0079; Fig. 2E) and 50 μM (p=0.0492; Fig. 2E) while it reached similar effects at 100 μM (p=0.3314; Fig. 2E).

Dexamethasone also inhibited TNF-α induced IL-6 secretion at 0.01 μM (p=0.0039; Fig. 2F) and 0.1 μM (p=0.0003; Fig. 2F) with no difference comparing asthmatic to non-asthmatic ASMC (Fig. 2F). In PDGF-BB stimulated ASMC, IL-6 secretion was reduced to baseline levels by 100 μM DMF (p≤0.0110; Fig. 1G). DMF at 10 μM and 50 μM reduced IL-6 to approximately 70% of the PDGF-BB induced level (p≤0.0110; Fig. 1G) in both asthmatic and non-asthmatic ASMC. Dexamethasone at 0.01 μM and 0.1 μM reduced IL-6 to 50-70% of PDGF-BB induced levels (p≤0.0228; Fig. 2H), with no difference between the two groups. The highest concentration of DMF (100 μM) alone had no significant effect on the baseline level of any of the assessed cytokines (Fig. 2A-H).

When combined dexamethasone (0.0001 μM) with DMF (10 μM) the secretion of eotaxin (Fig. 3A, E) and RANTES (Fig. 3B, F) by ASMC was significantly reduced compared to the GC alone, but not compared to DMF alone. At other concentrations this effect occurred at a non-significant level. No such effect was observed for IL-6 secretion (Fig. 3C-H).

Effects of the specific NF-κB inhibitor helenalin on ASMC cytokine expression

In order to confirm that NF-κB is involved in PDGF-BB and TNF-α cytokine expression by ASMC we used the specific NF-κB inhibitor helenalin. Pre-incubation of ASMC with helenalin (1 μM) strongly inhibited TNF-α induced RANTES (p<0.0001; Fig. 4B) and eotaxin (p<0.0001; Fig. 4A) secretion. The expression of IL-6 induced by PDGF-BB was
significantly reduced by helenalin to 70% of the PDGF-BB control (p=0.0027; Fig.4 D). However, helenalin had no effects on TNF-α induced IL-6 (Fig.4 C) and the baseline levels of all three cytokines were not altered by helenalin (Fig.4 A-D).

Effects of NF-κB p65 siRNA on p65 expression and cytokine release from ASMC

ASMC were transfected with siRNA to NF-κB p65 to determine its role on PDGF-BB and TNF-α induced cytokine secretion. siRNA to NF-κB p65 inhibited the expression of NF-κB p65 (Fig.5 A), but had no effect on p42 expression (Fig.5 B), providing evidence that the siRNA has no non-specific effects on other transcription factors. In the presence of the negative non-targeted control siRNA NF-κB p65 or p42 were still highly expressed (Fig.5A, B). TNF-α induced RANTES (p<0.0001; Fig.5 D) and IL-6 (p=0.0002; Fig.5 E) release was inhibited by NF-κB p65 siRNA, while it had no effects on TNF-α induced eotaxin (Fig.5 C) and PDGF-BB induced IL-6 (Fig.5 F). The baseline levels of all cytokines were not altered by NF-κB p65 siRNA when compared to the unstimulated cells containing non targeted control RNA (Fig.5 C-F).

DMF inhibits NF-κB nuclear entry and DNA binding, but has no effect on IκB-α degradation

In order to determine DMF effects on NF-κB activation we assessed IκB-α degradation, NF-κB nuclear entry and NF-κB/DNA binding. TNF-α stimulation of asthmatic and non-asthmatic ASMC caused IκB-α degradation after 10 min which was slightly resynthesised after 60 min (Fig. 6 A). TNF-α also induced nuclear accumulation of the NF-κB p65 (p=0.0213; Fig.6 C,D) and p50 (p=0.0098; Fig.6 C,D) and DNA binding of the NF-κB
heterodimer p65/p50 and homodimer p50/p50 (Fig.6 E) after 15 to 60 min. DMF inhibited TNF-α induced nuclear entry of NF-κB p65 and p50 and its DNA binding, but had no effect on IκB-α degradation (Fig. 6 A, C, D, E). PDGF-BB did not significantly induce IκB-α degradation (Fig.6 B), the nuclear entry of NF-κB p65 and p50 (Fig.6 C) or binding of NF-κB to the DNA within 60 min (Fig.6 F) and this was not altered in the presence of DMF (Fig.6 B, C, F).

*DMF inhibits NF-κB/p65 phosphorylation at serine 276 and serine 468, but had no effect on serine 536 phosphorylation*

To determine possible changes of NF-κB p65 phosphorylation by DMF, ASMC were treated with the stimuli in the presence and absence of DMF. TNF-α time-dependently induced the phosphorylation of NF-κB/p65 at serine 276, serine 468 and serine 536 (Fig.7 A, B, C). It activated NFκB/p65 (Ser276) phosphorylation within 30 min, NF-κB/p65 (Ser468) between 5-10 min and NFκB/p65 (Ser536) between 5-60min, with a maximum between 5-15 min (Fig.7A-C). Preincubation with DMF (100 μM) inhibited TNF-α induced p65 phosphorylation at serine 276 and serine 468 (Fig.7 B, C), but had no effect on serine 536 phosphorylation (Fig.7 A). PDGF-BB induced NF-κB p65 phosphorylation at Ser276 between 5-30 min and this was inhibited by pre-incubation with DMF (Fig.7 E). However, PDGF-BB did not activate NF-κB p65 phosphorylation on Ser468 and Ser536 and this was not changed in the presence of DMF (data not shown). The level of total NF-κB/p65 was not altered by the cell stimuli (TNF-α or PDGF-BB), or by DMF pre-incubation (Fig.7 D, F). Equal protein loading was confirmed by re-probing the membranes for α-tubulin expression (Fig.7 A-F).
**H89 inhibits TNF-α induced NF-κB nuclear entry and DNA binding**

To assess if there is a possible link between the inhibition of NF-κB p65 Ser276 phosphorylation and inhibition of NF-κB nuclear entry, we determined the effects of MSK-1/PKA inhibitor on NF-κB nuclear entry and DNA binding. The MSK-1/PKA inhibitor H89 inhibited the phosphorylation of NF-κB p65 by ASMC (Fig.8 B) and also inhibited TNF-α induced NF-κB p65 (p=0.0189; Fig.8 C, D) and p50 (p=0.0186; Fig.8 C, E) nuclear entry and NF-κB -DNA complex formation (p=0.0111; Fig.8 F, G) by ~15%. To exclude the possibility that inhibition of IκB-α degradation by H89 might mediate the inhibitory effect on NF-κB p65 and p50 nuclear entry, we determined the effect of H89 on IκB-α and found that its degradation was not affected by H89 (Fig.8 A).

**DMF inhibits DNA binding of active NF-κB**

To determine whether DMF can also inhibit active NF-κB, we first stimulated ASMC with TNF-α for 30 min and subsequently added DMF for 1-3h. NF-κB -DNA complex formation was inhibited by DMF at every time point measured with a maximal effect already after 1h (p≤0.0019; Fig.9), with no time dependent further inhibition after 3h.

**DMF inhibits TNF-α and PDGF-BB induced MSK-1 and CREB activation**

To determine whether inhibition of NF-κB p65 phosphorylation is possibly mediated by inhibition of its up-stream kinase MSK-1 we determined DMF effects on MSK-1 and CREB, another down-stream molecule of MSK-1. Both TNF-α and PDGF-BB time-dependently stimulated the phosphorylation of MSK-1 and CREB. TNF-α stimulated
phosphorylation of MSK-1 after 10-30 min and CREB phosphorylation after 5-30 min was inhibited by DMF treatment (Fig.10 A, C). PDGF-BB induced the phosphorylation of MSK-1 after 5-15 min and CREB phosphorylation was induced after 5-60 min and both effects were inhibited by DMF (Fig.10 B, D). Total MSK-1 and CREB were not significantly affected by either stimuli or DMF treatment (Fig.10 A-D).

Discussion

In this study the effect of DMF on human asthmatic and non-asthmatic ASMC cytokine production and NF-κB activation under inflammatory conditions, mimicked by TNF-α and PDGF-BB stimulation, were determined. Our results showed that DMF inhibited: (i) secretion of all studied cytokines from asthmatic and non-asthmatic ASMC and the inhibitory effect was similar to dexamethasone with no additive or synergistic effect of the drug combination. These effects of DMF occurred most likely through inhibition of NF-κB activity as DMF inhibited (i) NF-κB p65 Ser276 and Ser468 phosphorylation, (ii) the nuclear entry of NF-κB p65 and p50, and (iii) NF-κB-DNA complex formation. Furthermore, DMF reversed the activation of NF-κB, a situation which is important since in vivo NF-κB is already activated (2). Therefore, DMF is likely to reduce airway inflammation and thus be of benefit in chronic inflammatory lung diseases.

In vitro the inhibition of NF-κB down-regulated a range of pro-inflammatory mediators released by human pulmonary epithelial cells (34) and in ASMC (8). In addition, in a mouse model of asthma the inhibition of NF-κB reduced IL-13 and eotaxin levels in bronchoalveolar lavage fluid, as well as eosinophilia in the airways, and airway
hyperresponsiveness to methacholine challenge (18). Our findings also indicate a pivotal role for NF-κB in airway inflammation and asthma, thus it might be a target for asthma therapy (33).

TNF-α is released by different cell types of the human airway and there is evidence that TNF-α levels are elevated in the asthmatic airways (5). TNF-α increased the responsiveness in human airway (51) and was also involved in the development of airway inflammation as it stimulated the secretion of cytokines by a variety of airway cells. A recent study showed that specific TNF-α antagonism led to an improvement in symptom control and systemic inflammation in asthma patients and therefore, further emphasizes the importance of TNF-α in asthma (31). In ASMC, TNF-α induced the expression of cytokines and activated NF-κB (1, 38). Furthermore, NF-κB inhibition was shown to be anti-inflammatory in ASMC (8). Here we demonstrated that in ASMC TNF-α induced: (i) the degradation of IκB-α, (ii) the nuclear entry of the NF-κB subunits p50 and p65, and (iii) NF-κB/DNA complex formation. DMF had no effect on TNF-α induced IκB-α degradation, but prevented the nuclear entry of NF-κB and its DNA binding. A similar inhibitory mechanism on NF-κB was described for dehydroxymethylepoxyquinomicin and celecoxib which both inhibited NF-κB nuclear translocation without affecting the degradation of IκB (12, 41). IκB independent inhibition of the nuclear entry of NF-κB by DMF has also been reported previously in human dermal fibroblasts and endothelial cells and it was postulated that changes in the phosphorylation pattern of NF-κB might underlie these effects (49, 27).
NF-κB p65 can be phosphorylated at several serines which was suggested to alter its transactivation potential, its nuclear import, and export (22). NF-κB p65 is phosphorylated by MSK-1 and PKAc at Ser276 (50), GSK-3β at Ser468 (16) and IKK at Ser536 (42). In renal tubular epithelial cells, inhibition of NF-κB p65 Ser468 phosphorylation led to decreased synthesis of RANTES, MCP-1 and IL-8 mRNA (16), and the inhibition of NF-κB p65 Ser276 resulted in decreased IL-6 expression in fibroblasts (50). These data provide evidence that modulation of the NF-κB p65 phosphorylation pattern can alter secretion of pro-inflammatory cytokine production. In human ASMC we found that TNF-α induced the phosphorylation of NF-κB p65 at Ser276, Ser468 and Ser536, and that DMF inhibited the phosphorylation at Ser276 and Ser468. NF-κB p65 at Ser536 can be phosphorylated by IKK (42) and the fact that DMF failed to inhibit p65 Ser536 phosphorylation and IκB-α degradation suggests that IKK is not affected by DMF.

The signal transducer MSK-1 is regarded as a nuclear kinase, but there is evidence that it is also present in the cytosol where it was suggested to control NF-κB (15). In HeLa cells phosphorylation of NF-κB p65 at Ser276 promoted its nuclear translocation and transactivation (13) and in human keratinocytes DMF inhibited MSK-1 activation without affecting its upstream mitogen activated protein kinases p38 MAP and ERK1/2 (15). In this study DMF inhibited TNF-α induced MSK-1 activation, an effect which may be responsible for the drugs inhibition of NF-κB p65 Ser276 phosphorylation and also for the inhibition of CREB activity. When ASMC were treated with H89, a PKA/MSK-1 inhibitor, which was shown to inhibit the phosphorylation of NF-κB p65 Ser276 in fibroblasts (50) we observed a reduction of NF-κB p65 Ser276 phosphorylation and also
a weak inhibition by 15% of NF-κB nuclear entry, and DNA binding. This suggests that
NF-κB p65 Ser276 phosphorylation can promote NF-κB nuclear translocation and DNA
binding in ASMC.

EMSA analysis revealed that DMF inhibited active NF-κB. This is of importance because
NF-κB was shown to be continuously activated in chronic inflammatory diseases (2). The
ability to inhibit active NF-κB also distinguishes DMF from other NF-κB inhibitors,
which only inhibit the activation but failed to inhibit active NF-κB (43). Helenalin,
another inhibitor of active NF-κB, directly alkylates NF-κB p65 (28) and DMF can form
adducts with the tripeptid glutathione by a Michael-type reaction (11). The NF-κB p65
and p50 contain a number of cysteine residues (9, 29), therefore it is possible that DMF
also suppresses the nuclear translocation of NF-κB and its DNA binding by a direct
interaction with sulfhydryl groups located in the DNA binding domain and the
nuclear localisation sequence.

In human ASMC NF-κB was identified as a key transcription factor for TNF-α induced
eotaxin secretion (35). In addition, ASMC treatment with the NF-κB inhibitors PS-1145
and ML120 down-regulated TNF-α induced RANTES and IL-6 synthesis (8). Our data
show that TNF-α induced RANTES secretion was inhibited by both NF-κB p65 siRNA
and helenalin. In contrast, TNF-α induced eotaxin secretion was strongly inhibited by
helenalin, but remained unaffected by NF-κB p65 siRNA, indicating a NF-κB subunit
specific regulation of eotaxin. A similar NF-κB subunit dependent stimulation was
described for IL-8 in Jurkat T lymphocytes (26).
TNF-α induced IL-6 from non-asthmatic ASMC was only inhibited by the highest concentration of DMF and NF-κB p65 siRNA, but remained unaffected by helenalin. This suggests that TNF-α induced IL-6 expression is less sensitive to NF-κB inhibition than eotaxin or RANTES. Interestingly, lower doses of DMF significantly reduced IL-6 secretion by asthmatic ASMC. However, the effect of DMF on IκB-α degradation, NF-κB /DNA complex formation and NF-κB phosphorylation at serine 276, were not different between ASMC of asthmatics and non-asthmatics (data not shown). Therefore, the reason for the difference in DMF efficacy on TNF-α induced IL-6 is probably not mediated via a NF-κB dependent way and remains to be determined in future studies.

PDGF-BB is another factor with pro-remodeling and pro-inflammatory properties and is a major mitogen for ASMC proliferation. It further stimulated ASMC to secrete components of the extracellular matrix thus increasing tissue remodelling (25, 44). Furthermore, PDGF-BB stimulated the secretion of IL-6 and IL-8 from human lung cells as well as it activated NF-κB in animal lung cell (3, 10, 37, 47). Interestingly the activation of NF-κB by PDGF-BB might be cell type specific, as it was induced in animal airway smooth muscle cells (10), fibroblasts (37), and human skin fibroblasts (40), but not in human vascular smooth muscle cells and fibroblasts (39). In pulmonary artery derived smooth muscle cells PDGF-BB induced NF-κB activation occurred only after 24h (36). In this study, in human primary ASMC, PDGF-BB did neither degrade IκB-α, nor stimulate NF-κB nuclear entry, or NF-κB binding to DNA. However, PDGF-BB induced the phosphorylation of NF-κB p65 Ser276 and this effect was inhibited by DMF. In addition, the PDGF-BB induced IL-6 secretion was inhibited by helenalin, however,
NF-κB p65 siRNA had no significant effect. Thus, indicating that sole inhibition of the NF-κB p65 subunit is not sufficient to inhibit PDGF-BB induced IL-6 secretion by ASMC. We here provide evidence that DMF blocks the signaling cascade at the level of MSK-1 activity since both NF-κB p65 Ser276 and CREB are inhibited. CREB is another down-stream signaling protein of MSK-1. Since the human IL-6 promoter contains also a CREB binding site, the inhibition of CREB by DMF may add to the drug’s inhibitory effect on IL-6 secretion by ASMC.

The combination of DMF with the glucocorticoid dexamethasone did not result in any significant additive or synergistic effect on cytokine secretion when compared to both drugs alone. There was an overall trend of an improved inhibitory effect of the drugs combination on some of the cytokines, which became not significant compared to 10 µM DMF alone, but was significant compared to 0.0001 µM dexamethasone. Thus, pre-incubation with DMF may have steroid saving potential at low concentrations. However, more detailed studies on the supportive effect of DMF on GC action have to be performed in order to define the optimal concentration range. It may also be possible that DMF has a clear significant effect when combined with other GC than dexamethasone. In addition, future studies will have to investigate the cell biological mechanism underlying the mechanism of action of DMF on GC.

In regard to the therapeutic long term use of NF-κB inhibitors, concerns have been raised that they might lead to immune suppression and impaired host defense (33). However, long-term use of FAE in psoriasis has not revealed any severe side effects such as
increased risk of infection or malignancy (19). This further argues for a therapeutic potential of DMF in the control of chronic inflammatory lung diseases.

This paper showed that DMF down-regulated pro-inflammatory cytokine secretion by human ASMC by inhibiting NF-κB function on multiple levels. DMFs anti-inflammatory effects in human ASMC and its known clinical efficacy and long term safety profile in psoriasis, make it an interesting drug that could help to control lung inflammation and resultant airway wall remodeling observed in people with asthma.

Acknowledgement

We thank Mr. C. T. S’ng for his help preparing this manuscript.

Grants/Disclosures

The study was sponsored by the Swiss National Foundation, grant # 320000-116022 (M. Roth), and by a unrestricted research grant to Prof. M. Tamm in 2005, by Biogen Idec. International GmbH, Zug, Switzerland.

Reference List


refractory asthma: a double blind, randomised, placebo controlled trial.

Thorax;63(7):584-91, 20081


47. Tamm, M., Bihl, M., Eickelberg, O., Stulz, P., Perruchoud, A. P., Roth, M.


Figure legends

Figure 1: Representative immunoblots for calponin, α-SMA; and fibronectin in two independent ASMC (A) and fibroblast (F) cell lines (I, II). Cytokine release in response to TNF-α (B-D) and PDGF-BB (E) by non-asthmatic and asthmatic ASMC. Asthmatic
(n=5) and non-asthmatic (n=9) ASMC were stimulated with TNF-α or PDGF-BB (10 ng/ml) or left untreated for 24h. Eotaxin, RANTES and IL-6 were measured in the culture medium by ELISA. Statistics: student’s paired t-test * p<0.05 compared to unstimulated cells.

Figure 2: Effects of DMF and dexamethasone on eotaxin (A, B), RANTES (C, D) and IL-6 (E-H) secretion by asthmatic and non-asthmatic ASMC. ASMC from asthmatic (n=4-5) or non-asthmatic (n=4-5) donors were treated with DMF or dexamethsone for 1h prior to 24 hrs stimulation with TNF-α or PDGF-BB. Triplicate experiments of each cell line were averaged and the mean ± SEM of all cell lines was calculated. Results are expressed as percentage of cells treated with TNF-α or PDGF-BB in the presence of 0.1% DSMO (drug vs stimulus, one-way ANOVA, * p<0.05), differences between asthmatic and non-asthmatic ASMC were assessed by unpaired student’s t-test (§ p<0.05 significant).

Figure 3. Effect of DMF in combination with dexamethasone on TNF-α and PDGF-BB induced eotaxin, RANTES and IL-6 secretion from non-asthmatic and asthmatic ASMC. Non-asthmatic (n=4; left panel) and asthmatic (n=4; right panel) ASMC were pre-treated with a combination of DMF (10 µM) and dexamethasone (0.01 or 0.0001 µM) for 1h and then stimulated with TNF-α or PDGF-BB (both 10 ng/ml) for 24h. Eotaxin (A, E), RANTES (B, F) and IL-6 (C, D, G, H) in the culture supernatant were measured by ELISA. Data are expressed as a percentage of cells treated with TNF-α (10 ng/ml) containing 0.1% of the drug vehicle DMSO (V; mean ± SEM). Further inhibition by the drugs in combination compared to the drug alone was determined by one-way ANOVA.
(* indicates p<0.05 and n.s indicates not significant comparing the Dexa./DMF combination to the single drugs).

Figure 4: Effects of helenalin on eotaxin (A), RANTES (B) and IL-6 (C, D) secretion by non-asthmatic ASMC. ASMC lines (n = 4) were treated with helenalin for 1h prior to 24 hrs stimulation with TNF-α or PDGF-BB. Triplicate experiments of each cell line were averaged and the mean ± SEM of all cell lines was calculated. Results are expressed as percentage of cells treated with TNF-α or PDGF-BB containing 0.1% DSMO (drug vs stimulus, one-way ANOVA, * p<0.05).

Figure 5: Effects of NF-κB p65 siRNA on p65 and p42 expression (A, B) and eotaxin (C), RANTES (D) and IL-6 (E, F) secretion by non-asthmatic ASMC. ASMC lines (n = 2) were transfected with either NF-κB p65 siRNA or a non-targeted negative control siRNA (cRNA) for 24h cells, then serum deprived (24h) and stimulated with TNF-α or PDGF-BB. NF-κB p65 and p42 protein expression was determined in total cell lysates by immunoblotting and cytokine release was measured in cell culture medium by ELISA. Results are expressed as mean ± SEM, and expressed as the percentage of cells treated with TNF-α or PDGF-BB containing cRNA (siRNA vs stimulus, unpaired student’s t-test, * p<0.05).

Figure 6: Effect of DMF on IκB-α degradation (A, B), NF-κB p65/p50 nuclear entry (C, D) and NF-κB to DNA binding (E, F) in non-asthmatic ASMC lines (n = 3). Equal protein loading was confirmed by histone H1 or α-tubulin immuno-blotting. The
formation of a NF-κB/DNA complex was determined in nuclear cell lysates by EMSA. The composition of the NF-κB/DNA complex was characterized in the presence of antibodies to NF-κB p65 or p50 as supershifts (E, F). (D) The densitometric analysis of DMF-mediated inhibition of TNF-α–induced NF-κB p50 and p65 nuclear entry at 30 minutes (n = 3 ASMC) and bars represent mean + SEM (unpaired students t-test, * p<0.05).

Figure 7: Effect of DMF on NF-κB p65 phosphorylation at serine (Ser)276 (B, E), Ser468 (C) and Ser536 (A) and total NF-κB p65 (D, F) in non-asthmatic ASMC (n=3). NF-κB p65 phosphorylation was determined by immunoblotting. To confirm equal protein loading membranes were re-probed with α-tubulin. Immunoblots are representative for three independent experiments.

Figure 8: Effect of MSK-1/PKA inhibitor H89 on IκB-α degradation (A), NF-κB p65 (Ser276) phophorylation (B), NF-κB p65 and p50 nuclear entry (C) and NF-κB/DNA binding (F) in non-asthmatic ASMC lines (n = 3). (A) IκB-α degradation at 30 minutes; (B) NF-κB p65 (Ser276) pohphorylation as determined in total cell lysate, and (C) Nuclear NF-κB p65 and p50 accumulation. Equal protein loading was confirmed by histone H1 or α-tubulin. (D) densitometric analysis of nuclear accumulation of NF-κB p65, and (E) accumulation of NF-κB p50 (n = 3, unpaired students t-test, * p<0.05). (F) Representative EMSA of the NF-κB/DNA complex formation and the effect of H89-dependent inhibition of TNF-α–induced NF-κB p65 and p50 nuclear entry at 30 min. (G)
Densitometric analysis of three independent EMSA (mean ± SEM; unpaired students t-test, * p<0.05).

Figure 9: Effect of DMF on existing NF-κB/DNA complex formation by EMSA. (A) Non-asthmatic ASMC lines (n = 3) were stimulated with TNF-α for 30 min, DMF was subsequently added and nuclear cell lysates were prepared after 1, 2, and 3 hrs and the NF-κB/DNA complex formation was determined by EMSA. (B) Densitometric analysis of three independent experiments (mean ± SEM, unpaired students t-test, * p<0.05).

Figure 10: Effects of DMF on MSK-1 and CREB activation in non-asthmatic ASMC. ASMC were pre-incubated with 100 μM DMF for 1h prior to stimulation with TNF-α or PDGF-BB and then incubated for 0, 5, 10, 15, 30, or 60 min. (A) TNF-α and (B) PDGF-BB-induced phosphorylation of MSK-1 (p-MSK-1) and total MSK-1; and of (C, D) CREB (p-CREB) and total CREB were detected by immunoblotting. Membranes were re-probed with α-tubulin to confirm equal protein loading. All immunoblot are representative for independent experiments in four ASMC lines.
Figure 1

A

<table>
<thead>
<tr>
<th>ASMC</th>
<th>fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>AI</td>
</tr>
<tr>
<td>AI</td>
<td>AI</td>
</tr>
<tr>
<td>FI</td>
<td>FII</td>
</tr>
</tbody>
</table>

- α-SMA
- calponin
- fibronectin
- GAPDH (loading control)

B

- Eotaxin (pg/ml)
- Non-asthmatic ASMC
- Asthmatic ASMC
- TNF-α (10 ng/ml) stimulation

C

- RANTES (pg/ml)
- Non-asthmatic ASMC
- Asthmatic ASMC
- PDGF-BB (10 ng/ml) stimulation

D

- IL-6 (pg/ml)
- Non-asthmatic ASMC
- Asthmatic ASMC

E

- IL-6 (pg/ml)
- Non-asthmatic ASMC
- Asthmatic ASMC

Legend:
- Un-stimulated
- TNF-α (10 ng/ml)
- PDGF-BB (10 ng/ml)
Figure 3

Non-asthmatic ASMC

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

Asthmatic ASMC

![Graph E](image)

![Graph F](image)

![Graph G](image)

![Graph H](image)
Figure 4

A

TNF-α (%TNF-α control)

V
Helenalin 1 µM
V
Helenalin 1 µM

B

RANTES (%TNF-α control)

V
Helenalin 1 µM
V
Helenalin 1 µM

C

IL-6 (%TNF-α control)

V
Helenalin 1 µM
V
Helenalin 1 µM

D

IL-6 (%PDGF-BB control)

V
Helenalin 1 µM
V
Helenalin 1 µM

TNF-α (10 ng/ml)

PDGF-BB (10 ng/ml)
Figure 5

A

B

\[+ \quad + \quad + \quad + \quad - \quad - \quad - \quad - \]
\[- \quad - \quad - \quad + \quad + \quad + \quad + \quad + \]

\[\text{cRNA} \quad \text{siRNA}_{p65} \quad \text{siRNA}_{p65}\]

\[\text{TNF-}\alpha \quad \text{TNF-}\alpha \quad \text{PDGF-BB} \quad \text{PDGF-BB}\]

C

D

E

F

\[\text{Eotaxin} \quad \text{RANTES} \quad \text{IL-6} \quad \text{IL-6}\]

\[\% \text{ TNF-}\alpha \% \text{ TNF-}\alpha \% \text{ PDGF-BB} \% \text{ PDGF-BB}\]

\[\text{cRNA} \quad \text{siRNA}_{p65} \quad \text{cRNA} \quad \text{siRNA}_{p65} \quad \text{cRNA} \quad \text{siRNA}_{p65} \quad \text{cRNA} \quad \text{siRNA}_{p65}\]

\[\text{US} \quad \text{TNF-}\alpha (10 \text{ ng/ml}) \quad \text{US} \quad \text{TNF-}\alpha (10 \text{ ng/ml}) \quad \text{US} \quad \text{PDGF-BB (10 ng/ml)} \quad \text{US} \quad \text{PDGF-BB (10 ng/ml)}\]
Figure 6

A

- + + + + + - + + + + + - + + + + +
- - - - - - - + + + + - + + + + +

TNF-α (10 ng/ml)
DMF (100 μM)
0 5 10 15 30 60 0 5 10 15 30 60

Time (min)

B

- + + + + + - + + + + + - + + + + +
- - - - - - - + + + + - + + + + +

PDGF-BB (10 ng/ml)
DMF (100 μM)
0 5 10 15 30 60 0 5 10 15 30 60

Time (min)

C

- - - - + + + + + - - - - + + + + +
- + + + - - + + + + - + + + + +

TNF-α 10 ng/ml
PDGF-BB 10 ng/ml
DMF 100 μM
0 15 30 60

Time (min)

D

% TNF-α induced p50 nuclear level
% TNF-α induced p65 nuclear level

* *

E

- - - - + + + + + - - - - + + + + +
- + + + - - + + + + - + + + + +

DMF 100 μM
TNF-α (10 ng/ml)
0 5 10 15 30 60

Time (min)

F

- - - - + + + + + - - - - + + + + +
- + + + - - + + + + - + + + + +

DMF 100 μM
PDGF-BB (10 ng/ml)
0 15 30 60

Time (min)
**Figure 7**

A. Western blot analysis showing the expression of p-p65 (Ser276) and α-Tubulin. The expression levels are measured at different time points (0, 5, 10, 15, 30, 60 minutes) with treatments of TNF-α (10 ng/ml) and DMF (100 µM).

B. Western blot analysis similar to A, but with additional treatment of PDGF-BB (10 ng/ml).

C. Western blot analysis showing the expression of p-p65 (Ser536) and α-Tubulin.

D. Western blot analysis similar to C, but with additional treatment of PDGF-BB (10 ng/ml).

E. Western blot analysis showing the expression of p-p65 (Ser276) and α-Tubulin.

F. Western blot analysis similar to E, but with additional treatment of PDGF-BB (10 ng/ml).
Figure 8

<table>
<thead>
<tr>
<th>30 min</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α  (10 ng/ml)</td>
<td>H89 (10 µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A

B

C

D

E

F

G

p65/loading control

p50/loading control

% (TNF-α induced NF-κB/DNA binding)
Figure 9

A

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>TNF-α (10 ng/ml)</th>
<th>DMF (100 µM)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td>210</td>
</tr>
</tbody>
</table>

- p65/p50
- p50/p50
- Non-specific band

Unbound oligonucleotide

B

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>TNF-α (10 ng/ml)</th>
<th>DMF (100 µM)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td>210</td>
</tr>
</tbody>
</table>

% (TNF-α induced NF-κB-DNA binding)

* * *
Figure 10

A

- + + + + + - + + + + + - + + + + +
- - - - - - + + + + + + +

TNF-α (10 ng/ml)
DMF (100 µM)

0 5 10 15 30 60 0 5 10 15 30 60 Time (min)

α-Tubulin
CREB

B

- + + + + + - + + + + + - + + + + +
- - - - - - + + + + + + +

PDGF-BB (10 ng/ml)
DMF (100 µM)

0 5 10 15 30 60 0 5 10 15 30 60 Time (min)

α-Tubulin
CREB

C

- + + + + + - + + + + + - + + + + +
- - - - - - + + + + + + +

TNF-α (10 ng/ml)
DMF (100 µM)

0 5 10 15 30 60 0 5 10 15 30 60 Time (min)

α-Tubulin
CREB

D

- + + + + + - + + + + + - + + + + +
- - - - - - + + + + + + +

PDGF-BB (10 ng/ml)
DMF (100 µM)

0 5 10 15 30 60 0 5 10 15 30 60 Time (min)

α-Tubulin
CREB
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Buffer</th>
<th>Incubation Condition</th>
<th>Time</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-phospho-NF-κB p65 (Ser276, Ser468, Ser536)</td>
<td>1:1000</td>
<td>5% BSA in TBS-Tween 20 0.1%</td>
<td>4°C</td>
<td>Over night</td>
<td>Cell Signaling Technology, Beverly, MA</td>
</tr>
<tr>
<td>p-CREB (Ser133)</td>
<td>1:1000</td>
<td>5% BSA in TBS-Tween 20 0.1%</td>
<td>4°C</td>
<td>Over night</td>
<td>Cell Signaling Technology, Beverly, MA</td>
</tr>
<tr>
<td>CREB (48H2)</td>
<td>1:1000</td>
<td>5% BSA in TBS-Tween 20 0.1%</td>
<td>4°C</td>
<td>Over night</td>
<td>Cell Signaling Technology, Beverly, MA</td>
</tr>
<tr>
<td>p-MSK-1 (Ser376)</td>
<td>1:1000</td>
<td>5% BSA in TBS-Tween 20 0.1%</td>
<td>4°C</td>
<td>Over night</td>
<td>Cell Signaling Technology, Beverly, MA</td>
</tr>
<tr>
<td>anti- NF-κB/p65 (C-20)</td>
<td>1:500</td>
<td>TBS-Tween 0.1%</td>
<td>Room Temperatur (RT)</td>
<td>1h</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti- NF-κB/p50 (H-119)</td>
<td>1:500</td>
<td>TBS-Tween 0.1%</td>
<td>RT</td>
<td>1h</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti-κB-α (C21)</td>
<td>1:500</td>
<td>TBS-Tween 0.1%</td>
<td>RT</td>
<td>1h</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti-MSK-1</td>
<td>1:2000</td>
<td>TBS-Tween 0.1%</td>
<td>RT</td>
<td>1h</td>
<td>aa50320, Abcam</td>
</tr>
<tr>
<td>anti-α-Tubulin (DM1A)</td>
<td>1:5000</td>
<td>TBS-Tween 0.1%</td>
<td>RT</td>
<td>30min</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti-Histone H1 (FL-219)</td>
<td>1:500</td>
<td>Blocking buffer</td>
<td>4°C</td>
<td>Over night</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>anti-p42 MAPK</td>
<td>1:1000</td>
<td>5% BSA in TBS-Tween 20 0.1%</td>
<td>4°C</td>
<td>Over night</td>
<td>Cell Signaling Technology, Beverly, MA</td>
</tr>
</tbody>
</table>