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Glucocorticoids and mitogen- and stress-activated protein kinase 1 inhibitors: Possible partners in the combat against inflammation

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ABSTRACT

In the combat against inflammation, glucocorticoids (GCs) are a widespread therapeutic. These ligands of the glucocorticoid receptor (GR) inhibit the transactivation of various transcription factors, including nuclear factor-κB (NF-κB), and alter the composition of the pro-inflammatory enhanceosome, culminating in the repression of pro-inflammatory gene expression. However, pharmacological usage of GCs in long-term treatment is burdened with a detrimental side-effect profile. Recently, we discovered that GCs can lower NF-κB transactivation and pro-inflammatory gene expression by abolishing the recruitment of mitogen- and stress-activated protein kinase 1 (MSK1) (EC 2.7.11.1) to pro-inflammatory gene promoters and displacing a significant fraction of MSK1 to the cytoplasm. In our current investigation in L929sA fibroblasts, upon combining GCs and MSK1 inhibitors, we discovered a dose-dependent additive repression of pro-inflammatory gene expression, most likely due to diverse and multilayered repression mechanisms employed by GCs and MSK1 inhibitors. Therefore, the combined application of GCs and MSK1 inhibitors enabled a similar level of repression of pro-inflammatory gene expression, using actually a lower concentration of GCs and MSK1 inhibitors combined than would be necessary when using these inhibitors separately. Although H89 can inhibit both MSK1 and PKA, TNF does not activate PKA (EC 2.7.11.11) and as such PKA inhibition does not mediate H89-instigated repression of TNF-stimulated gene expression. Furthermore, the additional repressive effects of liganded GR and inhibition of MSK1, are not mediated via GR transactivation mechanisms. In conclusion, these results could entail a new therapeutic strategy using lower drug concentrations, potentially leading to a more beneficial side-effect profile.

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Abbreviations: AP-1, activator protein-1; ATF, activating transcription factor; β-gal, β-galactosidase; CBP, CREB-binding protein; COPD, chronic obstructive pulmonary disease; CREB, cAMP response element-binding protein; CpdA, Compound A; DEX, dexamethasone; DUSP1, dual-specificity phosphatase 1; ERK, extracellular signal-regulated kinase; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, GC response element; HDAC, histone deacetylase; HMGN1, high mobility group nucleosomal binding protein 1; IκB, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; luc, luciferase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MSK, mitogen- and stress-activated protein kinase; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cell; PKA, protein kinase A; PKB, protein kinase B; P-TEFb, positive transcription elongation factor b; ROCK, Rho-dependent protein kinase; RSK, 90 kDa ribosomal S6 kinase; S6K, 70 kDa ribosomal protein S6 kinase; TNF, tumor necrosis factor.

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1. Introduction

Inflammation is defined as an advantageous response to an extracellular challenge or intracellular damage, evoking the activation of various inflammatory mediators with the purpose to re-establish tissue function and structure. Although inflammation can be beneficial, return to homeostasis is of the utmost importance to prevent disadvantageous chronic inflammation [1]. As inflammation continues to be a burden on general health and health care services, researchers strive to resolve chronic inflammation. Since the discovery of glucocorticoids (GCs), this drug has been the prevalent drug in anti-inflammatory therapy. However, long-term usage of GCs for chronic inflammatory afflictions poses a problem of constitutive or acquired GC resistance, in which the patient no longer responds to GC therapy [2], but also brings about a detrimental side-effect profile [3]. Although to this date GCs remain the most beneficial choice of treatment for acute inflammation, research has put its focus on developing new anti-inflammatory, pharmacological strategies with a more advantageous benefit-to-risk ratio.

On a molecular level, pro-inflammatory signals, like tumor necrosis factor-α (TNF) and lipopolysaccharide, culminate in the activation of the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). In turn, both NF-κB and AP-1 stimulate the expression of pro-inflammatory cytokines, chemokines and adhesion molecules propagating cellular inflammation [4]. The NF-κB family comprises five members, of which NF-κB p65 is most commonly studied. The unactivated transcription factor NF-κB p65-p50 dimer is held in the cytoplasm by association with the inhibitory, NF-κB-binding protein IκB, masking the NF-κB nuclear localization motif. When cells are challenged with a pro-inflammatory signal, the IκB kinase complex (EC 2.7.11.10) phosphorylates IκBα [5] leading to IκB ubiquitination and ultimately IκB degradation by the 26S proteasome [6]. Consequently, NF-κB is released from its restraint and free to translocate into the nucleus, where it activates expression of multiple pro-inflammatory genes via the occupation of an NF-κB-specific promoter recognition site [7]. However, the transactivation of NF-κB is also fine-tuned by phosphorylation and other posttranslational modifications [8]. In particular, the TNF-mediated phosphorylation of p65 at S276 by mitogen- and stress-activated protein kinase 1 (MSK1) (EC 2.7.11.1) or protein kinase A (PKA) (EC 2.7.11.11) is pivotal for proper initiation of gene expression [9,10]. TNF-stimulated extracellular signal-regulated kinase (ERK1/2) and p38α mitogen-activated protein kinases (MAPKs) (EC 2.7.11.24) can both activate MSK1 [11–13], which in turn can phosphorylate NF-κB S276 and histone H3 S10 [10,14,15]. Phosphorylation of p65 S276 facilitates association of p65 with the activating cofactor CREB-binding protein (CBP) (EC 2.3.1.48) [16,17] and the positive transcription elongation factor b (P-TEFb) (EC 2.7.11.22, EC 2.7.11.23) [9] and is a necessary step in TNF-mediated promoter activation of IL6 [10,18].

The glucocorticoid receptor (GR), also know as NR3C1, is a GC-activated member of the nuclear receptor superfamily of transcription factors [19]. This receptor mediates the immunosuppressive and anti-inflammatory activity of GCs in multiple physiological systems. In an uninduced state, GR resides predominately in the cell cytoplasm in association with a multimeric chaperoning complex, keeping the ligand-binding pocket receptive to high affinity hormone binding and masking the nuclear localization signal [20]. Ligand binding of GCs to GR elicits a conformational change in the receptor. Consequently, GR dissociates from its chaperone complex and translocates into the nucleus, where it can act to modulate transactivation of mostly glucocorticoid response element (GRE)-containing promoters or transrepression of targeted genes mostly via crosstalk with kinases, cofactors or transcription factors such as NF-κB [21,22]. Furthermore, GR can negatively affect pro-inflammatory MAPK signalling via direct association or via an indirect enhancement of nuclear dual-specificity phosphatase (DUSP1) (EC 3.1.3.48, EC 3.1.3.16) expression [23–26]. However, GR activity itself is also subject to kinase modulation. Indeed, pro-inflammatory activated MAPKs can inhibit GR functionality via phosphorylation causing the receptor to return to the cytoplasm [27–31]. Recently, we discovered that GCs can also interfere with pro-inflammatory MSK1 signalling, without affecting MSK1 kinase activity. Because GCs interfere with the recruitment of MSK1 to pro-inflammatory gene promoters and drive MSK1 out of the nucleus via a GR- and CRM1-dependent mechanism, TNF-stimulated NF-κB phosphorylation and promoter-bound H3 S10 phosphorylation – a marker for transcription-prone chromatin – is reduced [32]. Similarly, MSK1 inhibitors, such as H89 and the upstream ERK1/2 and p38 MAPK inhibitors U0126 and SB203580, inhibit NF-κB transactivation, inflammatory gene promoter-bound H3 S10 phosphorylation and inflammatory gene promoter MSK1 occupancy [10].

As long-term therapeutic use of current GCs evokes metabolic problems/side-effects, recent research into combating inflammatory diseases is directed towards the combination of different NF-κB inhibitors [33,34] (Bougarne N and Van Cleemput M, unpublished data). By inhibiting different NF-κB activation pathways simultaneously, it is expected that the concentrations of the separate drugs can be lowered, thus eliciting a more desirable side-effect profile, while upholding the anti-inflammatory potential to an efficacious level. Here, we investigated the anti-inflammatory potential of a combined administration of GCs and MSK1 inhibitors and found that application of GCs together with MSK1 or MAPK inhibitors appears to be more efficient than the usage of either of these anti-inflammatory drugs alone. These additive anti-inflammatory effects are not mediated via GR transactivation mechanisms. Additionally, we could demonstrate that PKA is not involved in the pro-inflammatory stimulation of NF-κB-driven gene expression in fibroblasts.

2. Materials and methods

2.1. Cell culture

L929sA mouse fibroblasts were cultured in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 5% newborn calf serum and 5% fetal calf serum (Greiner bio-one, Frickenhausen, Germany), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA).

2.2. Cytokines and reagents

Dexamethasone (DEX) was obtained from Sigma–Aldrich (St. Louis, MO, USA). SB203580, U0126 and H89 were purchased from Alexis Biochemicals (Lausen, Switzerland), Promega Biotech (Madison, WI, USA) and Calbiochem (San Diego, CA, USA), respectively. Compound A (CpdA) or 2-(4-acetoxyphenyl)-2-chloro-Nmethyl-ethylammonium chloride was synthesized as described [35] (Alexis Biochemicals, Lausen, Switzerland). Recombinant murine TNF was produced and purified in our laboratory [36].

2.3. Plasmids

The plasmids $p(IL6\kappa B)_3$50hu.IL6P-luc+, p1481.IL8P-luc+, pE-selectin-luc and $p(GRE)_2$50-luc+ were described previously [37–39].

2.4. Reporter gene analysis

L929sA mouse fibroblast cells were stably transfected with various reporter gene constructs using the calcium phosphate precipitation protocol, as described [37]. The indicated inductions were performed at least in triplicate. All conditions were solvent-controlled. Subsequently, cells were washed in PBS and lysed using TROPIX lysis buffer. Ensuing, cellular luciferase (luc) (EC 1.13.12.7) levels were determined, as described [37]. Normalization of the results was performed using β-galactosidase (β-gal) (3.2.1.23) measurements, as assayed via a Galacto-Light kit (TROPIX, Bedford, MA, USA). Statistical analysis of reporter gene activity data was performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test on a representative experiment.

2.5. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

After starvation for 48 h in DMEM without serum, cells were induced. All conditions were solvent-controlled. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Concentrations of samples were determined and 500 ng RNA was used in a RT-step with MMLV reverse transcriptase (EC 2.7.7.49) (Promega, Madison, WI, USA) to produce the respective cDNA. Subsequently, the obtained cDNA was assayed in triplicate for IL6 and β-actin levels via qPCR in an ICycler (BioRad, Hercules, CA, USA) using BioRad reagents. Respective primer sequences are available upon request. The condition solvent/TNF was set to 100 and all other conditions were recalculated accordingly to allow ratio comparisons. Statistical analysis of RT-qPCR data was performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test on a representative experiment.

2.6. cAMP assay

L929sA cells were induced as indicated for 30′, after which cells were detached from the plate and counted. Per condition, 6000 cells were analyzed in triplicate using the LANCE cAMP 384 kit (PerkinElmer, Turku, Finland), according to the manufacturer's instructions. Quantification of signal was performed on a VICTOR3 multiplate reader (PerkinElmer, Turku, Finland). Statistical analysis of cAMP quantification data was performed with a paired *t*-test on a representative experiment.

2.7. PKA activity assay

L929sA cells, starved in DMEM devoid of serum for 24 h, were induced with solvent (Solv) or TNF (2000 IU/ml) for 30 min. Subsequently, cells were washed, lysed in Promega extraction buffer and 5 μg cell lysate was analyzed via the PepTag PKA assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. The PKA-specific peptide substrate, PepTag A1 Peptide, is LRRASLG (Kemptide). Samples were separated on a 0.8% agarose gel, in which negatively charged phosphorylated A1 peptide substrate migrated to the positive pole, and positively charged non-phosphorylated A1 peptide migrated to the negative pole. In control samples, the cell lysate was replaced with either deionized water (negative control) or 2 μg of recombinant active PKA (positive control). Quantity of separated phosphorylated and non-phosphorylated PKA A1 peptide substrate was determined by band densitometric analysis via Image J. The amount of A1 peptide substrate that is phosphorylated is presented relative to the total amount of A1 peptide per condition. Values for phosphorylated A1 peptide directly relate to the amount of active PKA in the cell lysates.

2.8. siRNA transfection

L929sA cells were seeded and the following day transiently transfected using Lipofectamine/Plus reagents, as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). As indicated in the figure legends, cells were either transfected with siRNA Control or a combination of siRNA MSK1 and MSK2 (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA). Cells were incubated with the respective transfection mixtures overnight (16 h). The following morning, medium was replaced with DMEM, supplemented with 5% newborn calf serum and 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, and cells were left to rest for 8 h. Ensuing, cells were starved for 16 h in DMEM without serum. Subsequent to the indicated inductions, total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were analyzed via RT-qPCR as described above. Control protein samples were analyzed via Western blotting, as described below. To control for siRNA knockdown efficiencies, we collected total protein cell lysates (using 1× SDS Sample buffer) of extra control wells.

2.9. Western blot analysis

For Western blotting, total cell lysates were prepared using 1× SDS sample buffer (50 mM Tris pH 6.8; 2% SDS; 10% glycerol; bromophenol blue and 100 mM freshly added DTT). Samples were incubated at 95 °C for 5 min and separated on a SDS-PAGE gel and subsequently blotted onto a Nitrocellulose membrane (Whatman, Dassel, Germany). Western blot analysis was performed according to the standard protocol of Santa Cruz (Santa Cruz, CA, USA). Imaging of antibody-tagged

protein signal was obtained via Western Lightning (PerkinElmer, Waltham, MA, USA). The primary antibody directed against MSK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was recognized by the secondary antibody HRP-linked donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibody directed against tubulin (Sigma, St. Louis, MO, USA), was recognized by the secondary antibody HRP-linked goat anti-mouse (Cell Signaling Technology, Danvers, MA, USA). To quantify the bands obtained via Western blot analysis, we applied band densitometric analysis via ImageJ software (http://rsb.info.nih.gov/ij/). The area under curve (AUC) of the specific signal of MSK1 was corrected for the AUC of the tubulin loading control. The value for the siRNA Control condition was set at 100 and the siRNA MSK1/2 condition was recalculated correspondingly.

3. Results

3.1. *Combined administration of H89 and DEX enhances repression of NF-κB-driven gene expression*

It has already been shown that the synthetic GC dexamethasone (DEX) as well as H89, a known MSK1 inhibitor, can repress inflammatory gene expression [10,40,41]. Therefore, we wondered how a combination of both drugs would affect the anti-inflammatory profile. Hereto, we used the recombinant p(IL6κB)$_3$50hu.IL6P-luc+ reporter gene construct, of which the promoter contains three NF-κB binding elements fused to the 50 bp minimal promoter of IL6. Additionally, we assayed full length IL8 (p1481.IL8P-luc+) and E-selectin (pE-selectin-luc) reporter gene constructs, of which the promoters contain one and three NF-κB binding elements, respectively. L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, p1481.IL8P-luc+ or pE-selectin-luc reporter gene constructs were treated with combinations of various drug concentrations of DEX and H89. Fig. 1A shows that addition of H89 dose-dependently increases the repressive effect of GCs on TNF-stimulated promoter activation in p(IL6κB)$_3$50hu.IL6P-luc+ reporter gene assays. A comparable level of repression can be obtained by either DEX (1 μM) or by combining DEX (0.1 μM) with H89 (0.4 μM) (Fig. 1A: lane 5 vs. lane 8). Furthermore, p1481.IL8P-luc+ and pE-selectin-luc reporter gene analyses corroborated that simultaneous administration of H89 and DEX gives a more intense repression of TNF-stimulated promoter activity as compared to the use of DEX alone (Fig. 1B and C). The level of repression obtained by DEX (1 μM) could also be reached by the administration of DEX (1 nM)

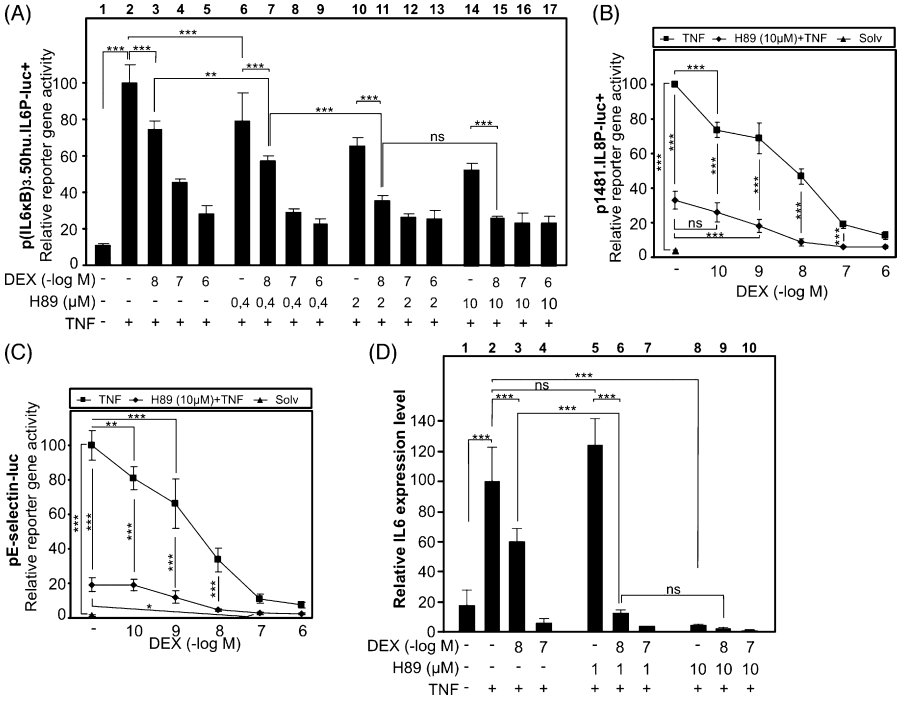

Fig. 1 – Combined administration of H89 and DEX enhances repression of NF-κB-driven gene expression. L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+ (A), p1481.IL8P-luc+ (B) or pE-selectin-luc (C) reporter gene constructs, were pretreated with either DEX or H89 alone or combinations of DEX and H89 for 2 h with the concentrations indicated in the figure. Where indicated, cells were induced with TNF (2000 IU/ml) for 5 h. Results were normalized for β-gal expression and presented as relative reporter gene activity with the condition solvent/TNF set at 100. (D) L929sA cells, starved for 48 h in DMEM devoid of serum, were induced as in (A). Total RNA was isolated and subjected to RT-qPCR assaying cellular IL6 and β-actin mRNA levels. Specific signal for IL6 was normalized to the β-actin signal. The condition solvent/TNF was set as 100 and all other conditions were recalculated accordingly to allow ratio comparisons. (A–D) Total solvent concentration was kept similar in all conditions. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant difference for selected pair-wise comparisons (ns not significant; *P < 0.05; **P < 0.01; ***P < 0.001). The data shown are representative for either 2 (B) (C) or 4 (A) (D) independent experiments.

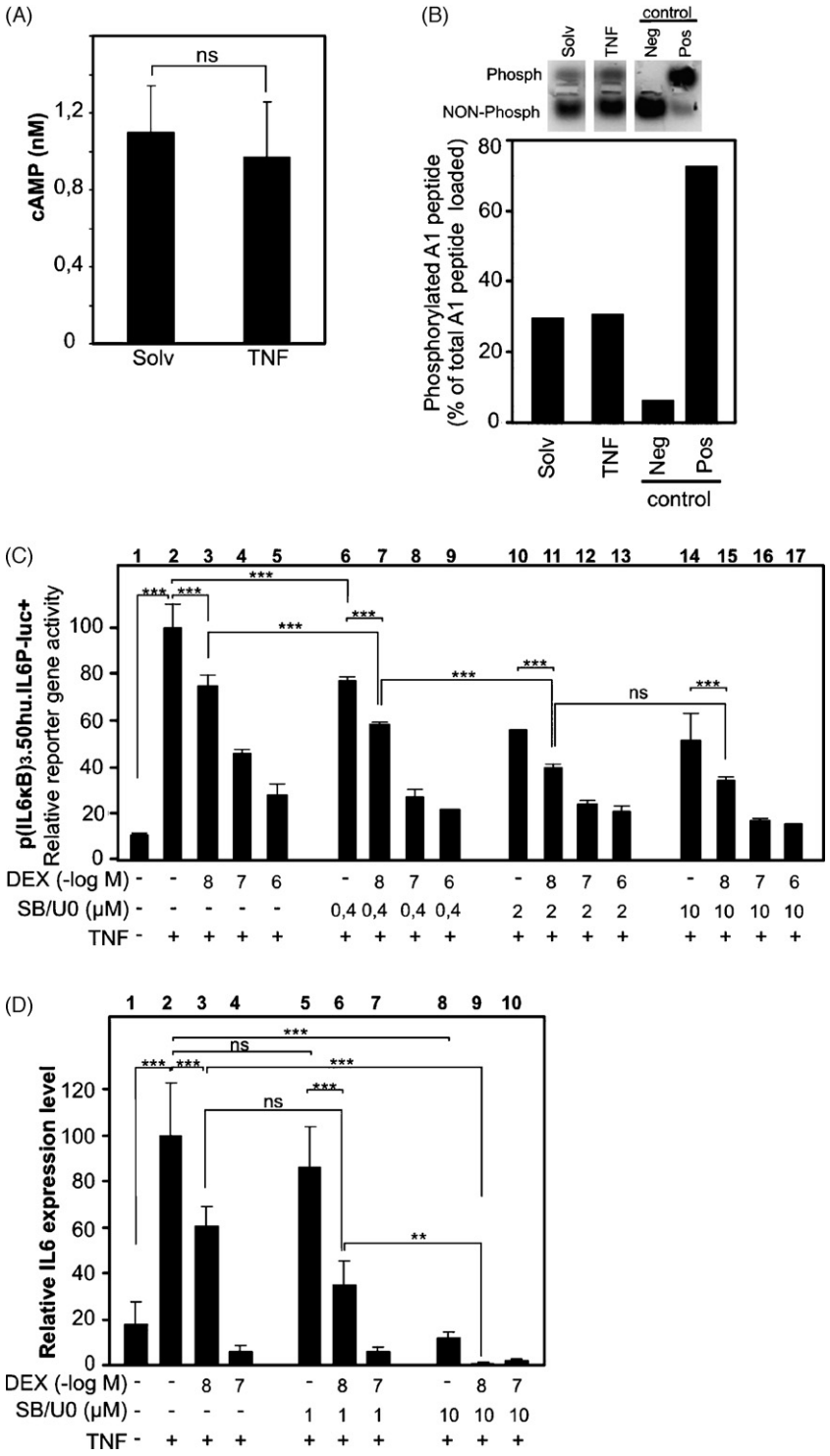

Fig. 2 – The anti-inflammatory effect of H89 does not involve PKA in fibroblasts. (A) L929sA cells were treated with solvent (Solv) or TNF (2000 IU/ml) for 30′ after which the cellular cAMP content was analyzed via a LANCE cAMP kit. Statistical analysis (paired *t*-test, df = 2, P = 0.1205) was performed to show significance of the difference (ns not significant). (B) L929sA cells, starved in DMEM devoid of serum for 24 h, were treated with Solv or TNF (2000 IU/ml) for 30′. Subsequently, cells were lysed and PKA activity was analyzed via A1 peptide PKA substrate phosphorylation in a PepTag PKA assay. In control samples, the cell lysate was replaced with either deionized water (negative control) or 2 μg of recombinant active PKA (positive control). The quantity of separated phosphorylated and non-phosphorylated PKA A1 peptide substrate was determined via Image J band densitometric analysis. The amount of A1 peptide substrate that is phosphorylated is presented relative to the total amount of A1 peptide per condition in the panel below. The bands shown were derived from the same gel. (C) L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were pretreated with either DEX and/or a combination of SB203580 and U0126 (SB/U0) for 2 h with the concentrations indicated in the figure. Where indicated, cells

combined with H89 (10 μM) (Fig. 1B and C). Additionally, similar data were obtained using L929sA cells stably transfected with p98.IL8P-luc+, a shortened form of the p1481.IL8P-luc+ reporter gene construct encompassing the NF-κB response element in its promoter (data not shown).

As inductions with DEX and H89 in Fig. 1A, B and C were performed simultaneously, the importance of the order of addition to the additional anti-inflammatory effect upon combining DEX and H89 was assessed. We could show that in L929sA cells, stably transfected with $p(IL6\kappa B)_3$50hu.IL6P-luc+ reporter gene construct, 5 h TNF treatment preceded by either simultaneous pre-inductions of DEX and H89 for 120′, or a pre-induction of 30′ of DEX before a 90′ pre-induction with H89, or vice versa, led to a similar reduction in $p(IL6\kappa B)_3$50hu.IL6P-luc+ promoter activity for combinations of equal concentrations of DEX and H89 (data not shown). In short, order of addition does not affect the anti-inflammatory potential of the combined administration of DEX and H89.

Furthermore, the effects of combined administration of the GC DEX and H89 was analyzed at the level of cytokine mRNA expression. To that end, we isolated total RNA from L929sA cells and subjected its respective cDNA to qPCR analysis assaying IL6 mRNA levels. Fig. 1D demonstrates that at the gene expression level the administration of H89 can additionally repress the GC-instigated inhibition of IL6 gene expression. Similar data were obtained for RANTES in L929sA cells and IL6 in A549 cells (data not shown), suggesting that this phenomenon occurs cell type-independent. In all analyses shown in Fig. 1, neither DEX nor H89 significantly affected basal promoter activity (data not shown). Of note, the sole addition of H89 (10 μM) could inhibit TNF-stimulated IL6 mRNA signal to basal levels (Fig. 1D), whereas H89 (10 μM) resulted in a 50% repression grade in $p(IL6\kappa B)_3$50hu.IL6P-luc+ reporter gene assays (Fig. 1A).

In short, in all reporter gene assays featuring pro-inflammatory gene promoters or recombinant promoter constructs and qPCR experiments investigating levels of pro-inflammatory gene mRNA, the addition of H89 enhances the anti-inflammatory effect of GCs in a dose-dependent manner.

3.2. *The anti-inflammatory effect of H89 does not involve PKA*

As H89 is not solely an MSK1 inhibitor, but can also target the protein kinase A (PKA) [42] and as PKA has been reported to phosphorylate NF-κB S276 in other cell lines [43,44], we wondered whether the repression of NF-κB-driven gene expression by H89 is mediated via either MSK1 or PKA. Because PKA is mostly activated in a cyclic adenosine monophosphate (cAMP)-dependent manner [45], we first evaluated the effects of TNF on the cellular cAMP content. We could show that TNF did not significantly elevate the cAMP content in L929sA cells (Fig. 2A). Similar results were obtained in A549 cells (data not shown). Next, we assessed endogenous PKA activity towards a PKA-specific A1 peptide substrate. As shown in Fig. 2B, TNF does not enhance the levels of phosphorylated A1 peptide and thus does not stimulate PKA activity, supporting the results in Fig. 2A. Similar results were obtained in A549 cells (data not shown). To sum up, the cellular cAMP content and PKA activity is not raised in L929sA or A549 cells in response to TNF.

Furthermore, we researched whether the inhibitors SB203580 and U0126, which respectively block p38 MAPK and ERK1/2 MAPK activity and thus MSK1 activation, without affecting PKA kinase activity [42], could exert similar effects as H89. In L929sA cells, stably transfected with the $p(IL6\kappa B)_3$50-hu.IL6P-luc+ reporter gene construct, we could show that the simultaneous addition of GCs together with a combination of SB203580 and U0126, yielded a significantly higher level of repression of NF-κB-driven promoter activity than either inhibitor could elicit on its own (Fig. 2C). The level of repression obtained by DEX (1 μM) could also be reached by the administration of DEX (0.1 μM) combined with SB203580 and U0126 (0.4 μM) (Fig. 2C). Additionally, we demonstrate in Fig. 2D that similar results could be obtained via analysis of IL6 gene expression levels. For Fig. 2C and D, we could show that neither DEX nor the combination of SB203580 and U0126 significantly affected basal promoter activity (data not shown). Of note, the combined administration of SB203580 and U0126 resulted in approximately 50% repression in $p(IL6\kappa B)_3$50-hu.IL6P-luc+ reporter gene assays, whereas the same compounds repressed TNF-stimulated IL6 mRNA to basal levels (Fig. 2C and D).

In addition to the above-mentioned pharmacological strategy, we used siRNA transfections of L929sA cells to knock down the protein levels of MSK1 and MSK2. We targeted both MSK1 and MSK2 because of the high similarity in structure and sequence between these kinases and because of the fact that it is often not well defined which of the substrates are targeted by MSK1 or MSK2 or both kinases [50]. To exclude possible compensatory mechanisms or residual effects of MSK2, we performed a simultaneous knockdown of MSK1 and MSK2. First, we controlled the efficiency of the siRNA-instigated knockdown via Western blot analysis and found a 63% decline in MSK1 protein upon comparing siRNA Control vs. combined siRNA MSK1 and siRNA MSK2 targeted samples (Fig. 3A). In this experiment, we also assayed the IL6 gene expression levels of DEX- and/or H89-treated cells. As expected, the siRNA Control-transfected samples displayed an additional repres-

were induced with TNF (2000 IU/ml) for 5 h. Results were normalized for β-gal expression and were presented as relative reporter gene activity with the condition solvent/TNF set at 100. (D) L929sA cells, starved for 48 h in DMEM devoid of serum, were induced as in (C). Total RNA was isolated and subjected to RT-qPCR assaying cellular IL6 and β-actin mRNA levels. The specific signal for IL6 was normalized to the β-actin signal. The condition solvent/TNF was set as 100 and all other conditions were recalculated accordingly, to allow ratio comparisons. (C and D) Total solvent concentration was kept similar in all conditions. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant differences for selected pair-wise comparisons (ns not significant; $^{**}P < 0.01$; $^{***}P < 0.001$). The data shown are representative for at least 2 independent experiments.

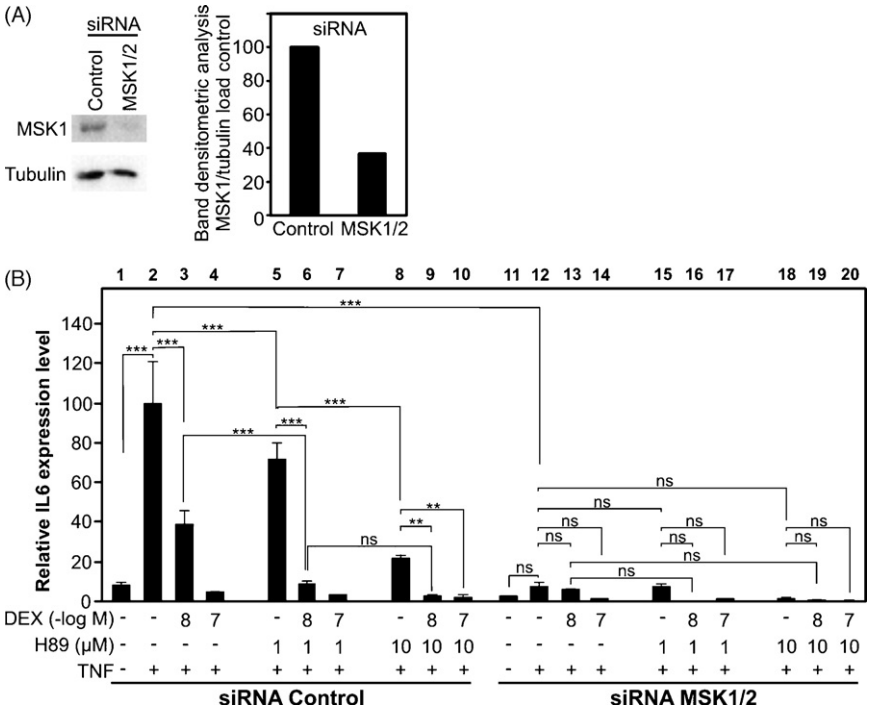

Fig. 3 – MSK1/2 is pivotal for TNF-mediated induction of IL6 gene expression. L929sA cells were transfected with siRNA Control or siRNA targeting MSK1 and MSK2. Protein and RNA extracts were prepared 48 h post-transfection. (A) After 16 h of starvation, total protein extracts of uninduced control wells were prepared. These cell lysates were subjected to Western blot analysis to detect MSK1 protein (left panel). Detection of tubulin served as a loading control. The bands visualized via Western blot analysis were subjected to band densitometric analysis (Image J) (right panel). The amount of specific signal for MSK1 was corrected to the respective tubulin loading control. The condition siRNA Control was set at 100 and the siRNA MSK1/2 condition was recalculated accordingly. (B) After 16 h of starvation, cells were pre-incubated with DEX, H89 or combinations of DEX and H89 for 2 h using concentrations as indicated in the figure. Total solvent concentration was kept similar in all conditions. Where indicated, cells were induced with TNF (2000 IU/ml) for 5 h. Total RNA was isolated and subjected to RT-qPCR assaying cellular IL6 and β-actin mRNA levels. Specific signal for IL6 was normalized to the β-actin signal. The condition solvent/TNF of the siRNA Control condition was set at 100 and all other conditions were recalculated accordingly to allow ratio comparisons. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant difference for selected pair-wise comparisons (ns not significant; $^{**}P < 0.01$; $^{***}P < 0.001$). The data shown are representative for 2 independent experiments.

sive effect upon combining DEX and H89 (Fig. 3B: lane 1–10). However, knocking down MSK1 and MSK2 protein levels resulted in the inability of TNF to stimulate the IL6 gene promoter (Fig. 3B: lane 11–20), indicating the importance of MSK for IL6 gene expression. Under these conditions, where TNF can no longer significantly induce IL6 gene expression, it is not surprising that the supplementary administration of DEX, H89 or a combination hereof does not result in any further significant effects on IL6 gene expression (Fig. 3B: lane 12–20).

To sum up, TNF does not elevate cAMP levels or stimulate PKA activity in L929sA or A549 cells. Consequently, TNF-stimulated NF-κB S276 phosphorylation is not mediated by PKA in these cells. Therefore, MSK1 would be the H89-targeted kinase. Moreover, in support, the combination of SB203580 and U0126 can, similar to H89, cooperate with GCs to repress NF-κB-driven gene expression. Finally, knockdown of MSK1/2 confirms the necessary presence and pivotal role of MSK for mediating an efficient IL6 gene promoter activation by TNF.

3.3. *Combined administration of MSK1 or MAPK inhibitors and Compound A enhances repression of NF-κB-driven gene expression*

Compound A (CpdA) is a selective GR modulator, driving GR to a monomer formation supporting NF-κB transrepression. As GRE-regulated transactivation is favoured by GR dimers, CpdA does not elevate dimeric GR-driven promoter activity [35,46]. In support, we show here in Fig. 4A that promoter activity of $p(IL6\kappa B)_3$50hu.IL6P-luc+ stably transfected in L929sA cells can be dose-dependently repressed by CpdA, whereas CpdA does not affect $p(GRE)_2$50-luc+ stably transfected in L929sA cells (Fig. 4B). Since this dissociated GR modulator inhibits NF-κB-mediated gene expression in a GR-dependent fashion, we wondered whether the dissociative mechanism of CpdA allows for the additive anti-inflammatory effect of MSK1 inhibitors. Hereto, we assayed combined effects of CpdA and H89 and CpdA and SB203580 and U0126 on the promoter activity of the $p(IL6\kappa B)_3$50hu.IL6P-luc+ reporter gene construct, stably transfected in L929sA cells and on IL6 gene expression

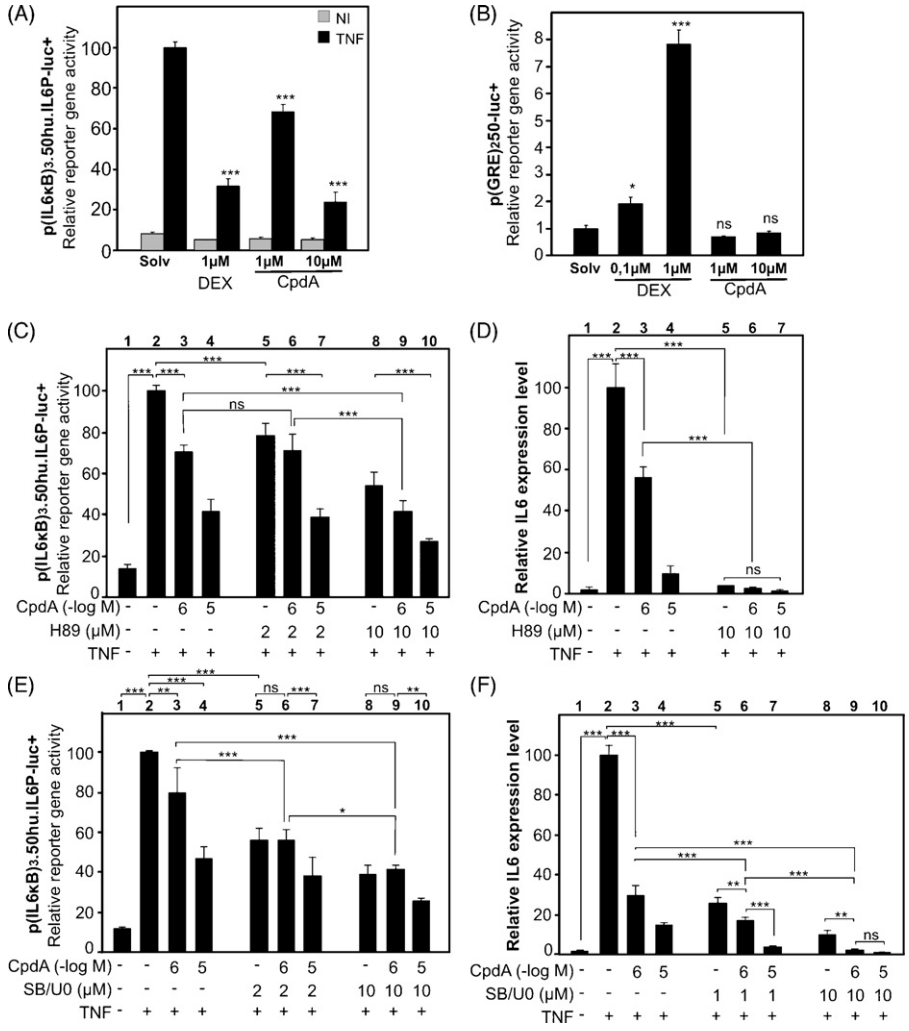

Fig. 4 – Combined administration of MSK1 or MAPK inhibitors and Compound A enhances repression of NF-κB-driven gene expression. (A) L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were treated with solvent (Solv), DEX (1 μM) or CpdA (1 μM or 10 μM) for 2 h. Subsequently, cells were induced with TNF (2000 IU/ml) for 6 h, where indicated. Results were normalized for β-gal expression and were presented as relative reporter gene activity with the condition Solv/TNF induction set at 100. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant difference with the TNF condition (***P < 0.001). (B) L929sA cells, stably transfected with p(GRE)$_2$50-luc+, were treated with Solv, DEX (0.1 μM or 1 μM) or CpdA (1 μM or 10 μM) for 8 h. Results were normalized to β-gal expression and were presented as relative reporter gene activity with the Solv condition set at 1. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant difference with the Solv condition (ns not significant; *P < 0.05; ***P < 0.001). (C) L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were pretreated with CpdA and/or H89 for 2 h with the concentrations indicated in the figure. Where indicated, cells were induced with TNF (2000 IU/ml) for 5 h. Results were normalized to β-gal expression and were presented as relative reporter gene activity with the condition solvent/TNF set at 100. (D) L929sA cells, starved for 48 h in DMEM devoid of serum, were induced as in (C). Total RNA was isolated and subjected to RT-qPCR assaying cellular IL6 and β-actin mRNA levels. The specific signal for IL6 was normalized to the β-actin signal. The condition solvent/TNF was set as 100 and all other conditions were recalculated accordingly, to allow ratio comparisons. (E) L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were pretreated with either CpdA or a combination of SB203580 and U0126 (SB/U0) or combinations of CpdA and SB/U0 for 2 h with the concentrations indicated in the figure. Where indicated, cells were induced with TNF (2000 IU/ml) for 5 h. Analysis of reporter gene activity was performed as in (C). (F) L929sA cells, starved for 48 h in DMEM devoid of serum, were induced as in (E). Analysis of IL6 mRNA was performed as in (D). For (C–F) Total solvent concentrations were kept similar in all conditions. Statistical analysis (ANOVA with Tukey's multiple comparison post-test) was performed to show significant difference for selected pair-wise comparisons (ns not significant; *P < 0.05; **P < 0.01; ***P < 0.001). Figures in (A), (B) and (C) are representative for 3 independent experiments. Figures in (D) and (E) are representative for 2 independent experiments.

levels in L929sA cells. We could show that the combination of CpdA with H89 elicits a significant additional repression of NF-κB-driven promoter activity (Fig. 4C). Similar results were obtained using L929sA cells, stably transfected with a p1481.IL8P-luc+ reporter gene construct (data not shown). Moreover, significant additive anti-inflammatory effects of CpdA and H89 could be demonstrated for IL6 mRNA levels (Fig. 4D). Moreover, the combined administration of CpdA and the inhibitors SB203580 and U0126 ensured a dose-dependent and significant additional level of inhibition of NF-κB-mediated reporter gene activity, in comparison to the use of either inhibitor alone (Fig. 4E). As expected, these results could be corroborated at the level of IL6 gene expression (Fig. 4F).

In short, combined administration of CpdA with MSK1 inhibitors evokes, similar to classical GCs, an additional level of repression towards TNF-stimulated, NF-κB-mediated gene expression. Therefore, these data suggest that the additive repression of GCs and H89 or GCs and the combination of SB203580 and U0126, is not mediated by GRE-regulated transactivation mechanisms.

4. Discussion

The underlying rationale of the combined administration of diverse inhibitors postulates that superposing the function of diverse agents on different signalling pathways could lead to an increased efficacy of therapy or would allow for lower dosage while upholding therapy potential. The latter reasoning would also implicate a decreased risk of toxicity and possible drug resistance problems [47]. In the current work, we showed that a dose-dependent additive anti-inflammatory effect is elicited upon combining GCs with MSK1 or MAPK inhibitors and thus demonstrated that a combination of NF-κB-targeting agents can enhance the overall anti-inflammatory potential of therapy. Furthermore, also a selective GR modulator, which only supports GR transrepression mechanisms, similarly displays an additive anti-inflammatory effect upon combination with either MSK1 or MAPK inhibitors.

In detail, the combined administration of H89 and the GC DEX yields an enhanced anti-inflammatory effect over the use of either drug alone in p(IL6κB)$_3$50hu.IL6P-luc+, p1481.IL8P-luc+, p98.IL8P-luc+ or pE-selectin-luc reporter gene assays and upon IL6 mRNA analysis (Fig. 1). However, as H89 does not merely target MSK1, but also PKA, possible mediation by PKA was taken into consideration. Since PKA has been reported to associate with and mediate NF-κB p65 S276 phosphorylation and thus NF-κB activation in response to LPS in 70Z/3 pre-B cell and Jurkat cells [44] and TNF, via ROS production, could activate PKA in U937 monocyte lymphoma cells [43], we wondered whether TNF could activate PKA in our cell system, the L929sA fibroblasts. However, we could show in L929sA cells that TNF cannot elevate cAMP levels and cannot activate PKA (Fig. 2A and B), suggesting PKA does not play a role in TNF-stimulated inflammatory processes in L929sA fibroblasts. An absolute prerequisite for MSK1/2 in mediating TNF-induced IL6 gene expression was confirmed via knockdown of MSK1/2 in L929sA cells. Because of this pivotal role of MSK1/2, a further administration of GCs and/or H89 to TNF-stimulated cells with knocked down MSK1/2 did not lead to significant interpretable changes in IL6 gene expression levels (Fig. 3). In further support of these data, it was previously demonstrated that TNF stimulation of IL6 gene transcription and NF-κB p65 S276 phosphorylation is critically impaired in MSK1/MSK2$^{-/-}$ mouse embryonic fibroblast (MEF) cells [10,48], evenso excluding a role for PKA in fibroblasts.

With regard to the additive repression of GCs and the inhibitor H89, we additionally noticed that similar effects could be obtained by using the p38 and ERK1/2 MAPK inhibitor cocktail SB203580 and U0126, which does not target PKA [42] (Fig. 2C and D). All arguments combined strongly suggest that PKA does not mediate TNF-instigated transactivation of NF-κB p65 or stimulation of pro-inflammatory gene expression in L929sA cells.

Since H89, besides MSK1 and PKA, has also been reported to repress the activity of S6K1 (EC 2.7.11.1) and ROCK-II (EC 2.7.11.1) [42,49], we cannot exclude a role for these kinases. However, since ROCK-II or S6K have never been reported to mediate TNF-stimulated NF-κB phosphorylation in inflammation, it seems unlikely that these kinases would mediate the NF-κB-targeting anti-inflammatory effects of H89 in fibroblasts. Furthermore, H89 can additionally inhibit RSK2 (EC 2.7.11.1) and PKBα (EC 2.7.11.1), albeit with a 20-fold higher IC50 than for the inhibition of MSK1 [42]. Therefore, and given the pivotal role of MSK1/2 for TNF-mediated stimulation of IL6 gene expression in L929sA fibroblasts (Fig. 3), MSK1 is most likely to be regarded as the main H89-targeted kinase in L929sA fibroblasts.

It is a widespread belief that GCs implement their anti-inflammatory effect via a multifactorial process, involving genomic and non-genomic effects. Although GCs, the MSK1 inhibitor H89 and the upstream ERK1/2 and p38 MAPK inhibitors SB203580 and U0126, share some anti-inflammatory features, also several divergent mechanisms have been described. The anti-inflammatory mechanisms of GCs, H89 and the combination of SB203580 and U0126 share the ability of abolishing pro-inflammatory gene promoter MSK1 occupancy and H3 S10 phosphorylation, a marker for an 'open' transcription-facilitating chromatin structure [10,32]. Although GCs can affect the subcellular localization of MSK1 and prevent its binding to pro-inflammatory gene promoters, GCs leave the MSK1 kinase activity intact [32]. Furthermore, GCs can partially inhibit NF-κB S276 and CREB S133 phosphorylation and thus activation of these transcription factors in fibroblasts [10,32] (De Bosscher K, unpublished data). In contrast, H89 and the combination of SB203580 and U0126 render MSK1 inactive [10,42] and thus abrogate phosphorylation of all MSK1's targets: CREB, ATF1, histone H3 (S10 and S28), NF-κB p65 and HMGN1/HMG14 [50]. Furthermore GCs can upregulate a number of anti-inflammatory genes, among which DUSP1, a dual-specificity phosphatase which targets p38, ERK1/2 and JNK (EC 2.7.11.24) MAPKs [51,52]. However, although in L929sA fibroblasts the DUSP1 promoter does become activated in response to GCs (Beck IME, unpublished data) this only results in JNK dephosphorylation and not in ERK1/2 or p38 MAPK dephosphorylation [53]. Naturally, SB203580 and U0126 do inhibit ERK1/2 and p38 MAPK activity, but not JNK activity [42]. Of note, in PBMCs the combined use of GCs and the 38 MAPK inhibitor SB203580 was previously reported to enhance the

GC-mediated anti-inflammatory potential via the inhibition of p38 MAPK-mediated inhibitory GR phosphorylation [27]. Additional anti-inflammatory mechanisms of GCs include direct crosstalk of GCs with NF-κB p65 [54,55], interference of GCs with the recruitment of the Ser 2 RNA polymerase II kinase complex P-TEFb [56,57], GC-instigated recruitment of HDAC2 to pro-inflammatory gene promoters [58] and destabilization of mRNA from NF-κB-driven genes [59–61]. In short, whereas GCs and MSK1 inhibitors share the ability to abolish H3 S10 phosphorylation and MSK1 recruitment at pro-inflammatory gene promoters, GCs and MSK1 inhibitors also display a wide array of unshared anti-inflammatory mechanisms, which can be deployed for the development of novel anti-inflammatory strategies in the clinic.

Compound A is a dissociative GR modulator that mediates NF-κB transrepression without activating GRE-regulated gene transcription (Fig. 4A and B) [35,46]. As the mainstay of long-term GC therapy-associated side-effects are linked to GR transactivation mechanisms [3], this dissociative compound could prove to be a promising future therapeutic in the combat against inflammation. Indeed CpdA, unlike classical GCs, does not elicit hyperinsulinemia or elevate blood glucose levels [35,46]. Here, we could show that, like classical GCs, the NF-κB-targeting anti-inflammatory potential of CpdA could be enhanced by the addition of either the MSK1 inhibitor H89 (Fig. 4C and D) or the p38 and ERK1/2 MAPK inhibitors SB203580 and U0126 (Fig. 4E and F). As CpdA does not support GRE-regulated transcription, these data suggest that the additive anti-inflammatory effects of liganded GR and MSK1 or MAPK inhibitors are not mediated via GR transactivation mechanisms. Mechanistically, CpdA can diminish NF-κB p65 recruitment to its endogenous binding elements in the promoters of pro-inflammatory genes [35]. Furthermore, we could recently show that CpdA diminishes the TNF-stimulated IκBα degradation, and subsequent translocation of NF-κB p65 to the cytoplasm in L929sA, A549 and FLS cells (Beck IME, De Bosscher K and Gossye V, unpublished data). Although CpdA does not enhance DUSP1 promoter activity (Beck IME, unpublished data), we could observe CpdA-mediated inhibition of ERK1/2 MAPK phosphorylation in L929sA fibroblasts. Even though CpdA does not affect JNK and p38 MAPK phosphorylation, a decline in ERK1/2 MAPK phosphorylation or direct effects result in the inhibition of MSK1 T581, NF-κB p65 S276 and CREB S133 phosphorylation (De Bosscher K, unpublished data). As combined administration of CpdA with MSK1 inhibitors, would allow lower dosages for a similar anti-inflammatory efficacy with the surplus of lowered or absent side-effects due to GRE-regulated gene transcription, this therapeutic strategy possibly holds great promise for the future.

Whereas GCs are already widely used in clinic, new and more specific MSK1 and MAPK inhibitors should be developed to prevent off target effects. Although new MSK1 inhibitors have been reported: cheilanthane sesterterpenoids [62] and recently imidazo (4,5-c)pyridines [63,64], intensive research into the specificity of these compounds is still necessary. Alternatively, additional specificity of treatment could be gained by using topical drug application strategies, such as inhalers for asthma and ointments for eczema. Currently, a number of JNK and p38 MAPK inhibitors are in clinical trial for the treatment of psoriasis, rheumatoid arthritis, Crohn's disease, COPD and multiple myeloma [65]. Future research, however, remains necessary to warrant the development and characterization of new MAPK and MSK inhibitors.

In conclusion, combinatorial therapy with GR ligands and MSK1 or MAPK inhibitors gives rise to additional repression of inflammatory gene expression. Because in combination therapy lower doses of GCs can be used to reach an efficacious anti-inflammatory stimulus, this strategy opens perspectives to lower detrimental side-effects of chronically inflamed patients who are obliged to undergo long-term GC therapies.

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