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1,8-Cineol inhibits nuclear translocation of NF-κB p65 and NF-κB-dependent transcriptional activity

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ABSTRACT

Natural plant-derived products are commonly applied to treat a broad range of human diseases, including cancer as well as chronic and acute airway inflammation. In this regard, the monoterpene oxide 1,8-cineol, the active ingredient of the clinically approved drug Soledum®, is well-established for the therapy of airway diseases, such as chronic sinusitis and bronchitis, chronic obstructive pulmonary disease and bronchial asthma. Although clinical trials underline the beneficial effects of 1,8-cineol in treating inflammatory diseases, the molecular mode of action still remains unclear.

Here, we demonstrate for the first time a 1,8-cineol-depending reduction of NF-κB-activity in human cell lines U373 and HeLa upon stimulation using lipopolysaccharides (LPS). Immunocytochemistry further revealed a reduced nuclear translocation of NF-κB p65, while qPCR and western blot analyses showed strongly attenuated expression of NF-κB target genes. Treatment with 1,8-cineol further led to increased protein levels of IκBα in an IKK-independent matter, while FRET-analyses showed restoring of LPS-associated loss of interaction between NF-κB p65 and IκBα. We likewise observed reduced amounts of phosphorylated c-Jun N-terminal kinase 1/2 protein in U373 cells after exposure to 1,8-cineol. In addition, 1,8-cineol led to decreased amount of nuclear NF-κB p65 and reduction of its target gene IκBα at protein level in human peripheral blood mononuclear cells.

Our findings suggest a novel mode of action of 1,8-cineol through inhibition of nuclear NF-κB p65 translocation via IκBα resulting in decreased levels of proinflammatory NF-κB target genes and may therefore broaden the field of clinical application of this natural drug for treating inflammatory diseases.

1. Introduction

Natural plant-derived products are widely applied to treat a broad range of human diseases, particularly including cancer [1] as well as chronic and acute airway inflammation [2,3]. In this regard, especially terpenoids, like the monoterpene oxide 1,8-cineol (Fig. 1A), are of eminent importance for the therapy of upper and lower airway diseases, such as chronic sinusitis and bronchitis, chronic obstructive pulmonary disease (COPD) and bronchial asthma [2,4–6].

Firstly described by Cloez in 1870, 1,8-cineol (or "eucalyptol") is known as the major constituent of the essential oil of *Eucalyptus globulus* leafs [7], while later studies also demonstrated the presence of 1,8-cineol in other plant species, such as rosemary [8] and *Psidium pohlianum* [9]. Besides its isolation from *E. globulus* oil by fractional distillation, 1,8-cineol can also be synthesized by isomerization of α-terpineol [10].

Interestingly, in addition to its utility in treating bronchitis and sinusitis [3,4], 1,8-cineol is also known to exhibit antinociceptive properties. As described by Santos and colleagues in 2000, 1,8-cineol showed an antinociceptive effect in male Swiss mice and Wistar rats, thereby indicating a potential calmative action on the central nervous system [11]. Moreover, the orally administered essential oil of *Croton nepetaefolius*, which contains 31.5% 1,8-cineol, significantly increased the latency of mice in an hot-plate test. In addition, paw licking was significantly reduced in the second phase of a formalin test, underlining the antinociceptive effect [12]. 1,8-Cineol is also well-known to exhibit direct protective effects within the rat and murine system, as in the case of ethanol-induced gastric mucosal damage [13] and liver failure in an *in vivo* model of endotoxemic shock [14].

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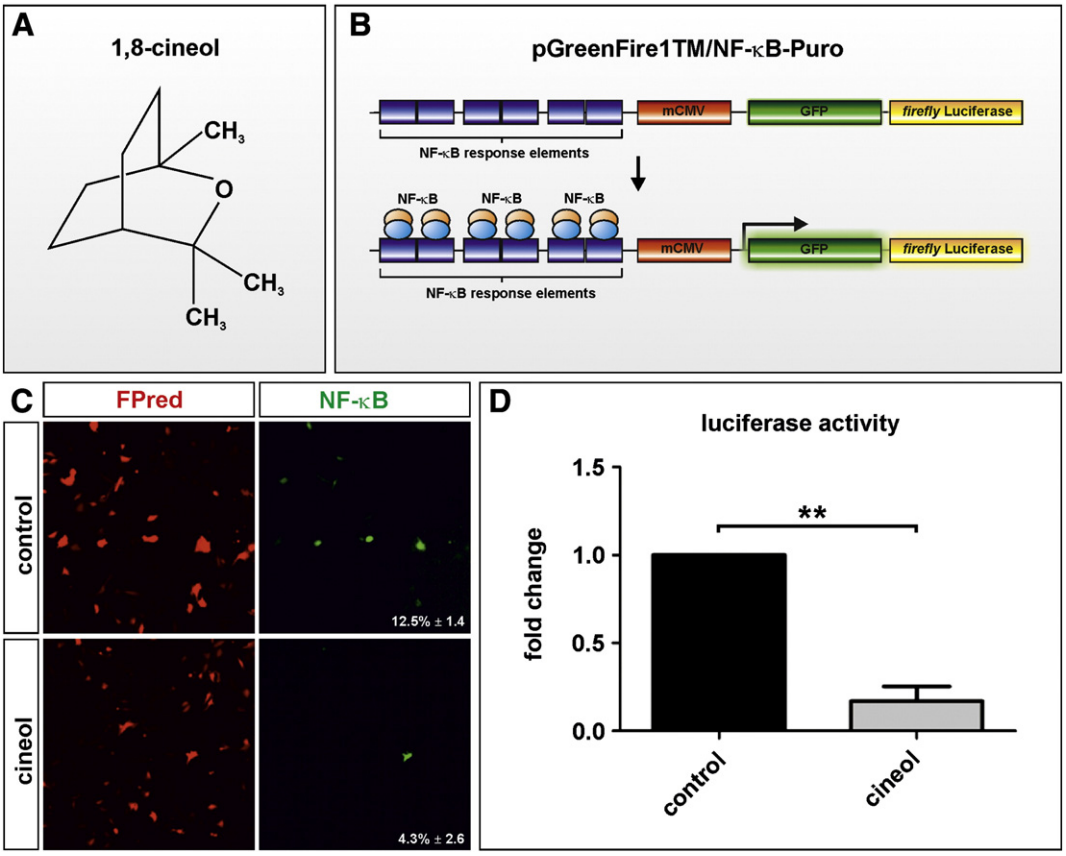

Fig. 1. *1,8-cineol reduces the activity of NF-κB in human U373 cells in a pGreenFire1TM-NF-κB-Puro reporter gene assay*. A. Structural formula of the monoterpene oxide 1,8-cineol (molecular formula $C_{10}H_{18}O$). B. Schematic view on pGreenFire1TM-NF-κB-Puro-vector containing NF-κB response elements, a minimal CMV promotor as well as GFP and *firefly* luciferase cassettes, which served as reporter gene construct to determine the NF-κB-activity. C. Confocal laser scanning microscopy revealed decreased amount of GFP-expressing (pGreenFire1TM-NF-κB-Puro-vector-transfected) U373 cells in the cineol-treated approach (4.3% ± 2, lower panels) in comparison to non-treated control (12.5 ± 1.4, upper panels). D. Transfected U373 showed significantly decreased levels of luciferase activity, when exposed to 1,8-cineol. Merged data from biological triplicate, $^{**}p < 0.01$ was considered significant (paired *t*-test, two-tailed, confidence interval: 95%).

Linking the well-described protective and antinociceptive effects of 1,8-cineol to inflammation, Santos and coworkers not only demonstrated an antinociceptive, but also an anti-inflammatory effect in both mice and rats [11]. Moreover, in 2004 the same group described a reduced colonic damage in rats on acute trinitrobenzene sulfonic acid (TNBS)-induced colitis, further confirming the anti-inflammatory action of 1,8-cineol [15]. Likewise, Inoue et al. demonstrated a partial prevention of allergic airway inflammation in a murine model of allergic asthma induced by house dust mites [16]. Besides its properly characterized direct anti-bacterial activity [17,18], 1,8-cineol was impressively shown to reduce inflammation in clinical trials. In a double-blind placebo-controlled trial with thirty-two patients, Juergens and coworkers evidenced an anti-inflammatory activity of 1,8-cineol in steroid-dependent bronchial asthma. In particular, twelve of 16 cineol patients achieved a reduction of oral steroids, while only four out of 16 placebos led to significant changes in steroid reduction [2]. Moreover, concomitant therapy with cineol was shown to reduce exacerbations as well as dyspnea and improve lung function in a placebo-controlled double-blind trial with 242 COPD patients [6].

Although these clinical trials and medical applications of 1,8-cineol as a drug in therapy of inflammatory diseases are highly promising, the detailed mode of action of 1,8-cineol still remains unknown. Suggesting a mechanism of action by cytokine inhibition, Juergens and colleagues demonstrated a significantly inhibited production of TNF-α and IL-1β human monocytes after treatment with 1,8-cineol *in vitro* [19]. This is in accordance to *in vivo* data demonstrating reduced levels of TNFα and IL-1β in airways of ovalbumin-challenged Guinea pig treated with 1,8-cineol inhalation [20].

Interestingly, several monoterpenoids are also known to directly inhibit the signaling via NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) (reviewed in [21]), a key regulator in the pathogenesis of inflammatory diseases. With regards to these well-described inhibitory effects of monoterpenoids [22–24], the aim of the present study was to determine a potential effect of 1,8-cineol on NF-κB, thereby suggesting its possible mode of action.

Here, we describe for the first time a significantly reduced activity of NF-κB after exposure to 1,8-cineol in the human cancer cell lines U373 and HeLa, even after lipopolysacharid (LPS)-depending stimulation of NF-κB-activity. Strongly reduced translocation of NF-κB p65 into the nucleus was evidenced by immunocytochemistry, while qPCR and western blot analyses revealed decreased gene expression of NF-κB target genes after their LPS-driven induction. Increased protein levels of IκBα were observed in an IKK-independent matter, accompanied by restoring of LPS-associated impairment of interaction between NF-κB p65 and IκBα shown by FRET-analyses. 1,8-Cineol was also found to reduce protein amounts of phosphorylated c-Jun N-terminal kinase 1/2 in U373 cells in comparison to LPS-treated cells. We additionally observed 1,8-cineol-induced decrease of nuclear NF-κB p65 localization and reduction of its target gene IκBα at protein level in human peripheral blood mononuclear cells.

Our findings suggest a novel mode of action of 1,8-cineol through inhibition of nuclear NF-κB p65 translocation via IκBα, thereby likely broadening its uses in the treatment of inflammatory diseases.

2. Materials and methods

2.1. Materials

1,8-Cineol was used from Soledum® capsules (Klosterfrau Healthcare Group, Cassella-med GmbH & Co. KG, Cologne, Germany). Native extract (0.6 mg/μl 600 mg/ml 1,8-cineol) was stored at 4 °C, while stock solution was prepared by solving native extract in ethanol (100 mg/ml) followed by final diluting with DMEM High Glucose (Biochrom, Berlin, Germany; 1 mg/ml).

2.2. Cell culture

Human U373 glioblastoma and human HeLa cells were cultured in a humidified incubator (Binder, Tuttlingen, Germany) at 37 °C and 5% CO_2 in tissue culture dish 100 (TPP Techno Plastic Products, Trasadingen, Austria) and DMEM High Glucose (Biochrom) containing 200 mM L-Glutamine (Sigma-Aldrich, Taufkirchen, Germany), Amphotericin B (1 ml (0.25 mg)/100 ml; PAA, Pasching, Austria), and Penicillin/Streptomycin (1 ml (2 mg)/100 ml; PAA) and 10% Fetal calf serum (lot:126 K3398, Sigma-Aldrich). Hereinafter, the described composition of medium is referred to as "standard medium". Medium change was performed every two to three days, while passaging was done after at least one week. For passaging, adherently growing cells were digested using trypsin (Trypsin-EDTA, 0.5 mg/mL; PAA) for 10 min at 37 °C followed by harvesting via centrifugation and replating in standard medium.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs) from human whole blood via density gradient centrifugation

PBMCs were isolated using *Easycoll* (Ficoll, Biochrom AG, Berlin, Germany) by density gradient centrifugation according to manufacturer's guidelines (Biochrom AG). Briefly, human whole blood samples were obtained from healthy donors after informed consent according to local and international guidelines (Bezirksregierung Detmold/Münster). Heparinized human whole blood was mixed 1:2 with PBS in a 50 ml Tube (Greiner Bio-One GmbH, Frickenhausen, Germany) and 28 ml were layered on top of 12 ml *Easycoll* followed by centrifugation for 30 min at 400 × *g* and RT. PBMCs were located in a clearly discernible white, cloudy layer in the interphase between the *Easycoll*-separation solution (lower phase) and serum (supernatant), while erythrocytes and granulocytes remained within the pellet. Collection of PBMCs by pipetting into a 50 ml Tube (Greiner) was followed by twice times washing using PBS. Isolated PBMCs were resuspended in RPMI medium (PAA, Pasching, Austria) with 10% human serum gained from supernatant of density gradient centrifugation procedure and further cultivated in Tissue Culture Dish (Waldeck GmbH & Co KG, Münster, Germany) at 37 °C and 5% CO_2 using a humidified incubator (Binder, Tuttlingen, Germany).

2.4. Transient transfection of human cell lines and gene reporter assays

For transient transfection, U373- and HeLa cells cultivated in standard medium as described above were treated with trypsin (Trypsin-EDTA, 0.5 mg/mL; PAA) for 10 min followed by harvesting via centrifugation. Afterwards, 3×10^6 cells were transfected with pGreenFire1™-NF-κB-Puro-vector (1 μg, System Biosciences), NF-AT luciferase reporter vector (Addgene Plasmid No. 10959, [25]) or cotransfected with TK(NF-κB)$_6$LUC vector (1 μg, [26]) and pRLcmv vector or pRL-TK vector (2 μg, Promega Corporation, Mannheim, Germany), using the Nucleofector II device (Lonza Group, Basel, Switzerland) and the rat NSC Nucleofector Kit (Lonza Group) according to manufacturer's guidelines. FPred (Lonza Group) or pmaxGFP (Lonza Group) served as transfection controls. Transiently transfected cells were cultivated in standard medium for 24 h, followed by addition of respective combinations of 1,8-cineol (Klosterfrau Healthcare Group), LPS (100 ng/ml, rough strains from *Salmonella enterica* Re 595, cat. no. L9764, Sigma-Adrich) and NF-κB-inhibiting factors (1 μM dexamethasone (Sigma-Aldrich), 2 μM insulin (Sigma-Aldrich), 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 200 μM indomethacin (Sigma-Aldrich)). Analysis of gene reporter activity was performed 24 h after exposure to respective substances by confocal laser scanning microscopy (LSM 510 with ZEN software, Carl Zeiss, Jena, Germany) or measurement of luciferase activity. All assays were performed at least as triplicates.

For assessment of luciferase activity, transfected cells were harvested using Cell scrapper S (TPP) and lysed in 1× Passive Lysis buffer (Promega Corporation). Dual-Luciferase® Reporter Assay System (Promega Corporation, cat. no. E1960) was applied for measuring luciferase activity according to manufacturer's guidelines, while Graph Pad Prism software (GraphPad Software, La Jolla, CA, USA) was applied for subsequent statistical analysis. In the case of dual luciferase reporter assay, signals were normalized as a ratio of *firefly* luciferase activity to *Renilla* luciferase activity.

Optimal working concentration of 1,8-cineol was investigated within preliminary assays, leading to the application of 10^{-4} M 1,8-cineol within all assays (Fig. S1).

2.5. Proliferation assay

To investigate the effects of cineol on proliferation of HeLa and U373 cells, 1×10^4 cells were plated in standard medium comprising respective concentrations (10^{-3} M–10^{-9} M) of 1,8-cineol (Klosterfrau Healthcare Group) and cultivated as described above. Cell numbers were determined after 8 days via trypsinisation (Trypsin-EDTA, 0.5 mg/mL; PAA) and subsequent cell number determination using the Cellometer Auto T4 device (Peqlab Biotechnology, Erlangen, Germany). Proliferation assay was performed as biological triplicate, statistical analysis was performed using Graph Pad Prism software (GraphPad Software).

2.6. Immunocytochemistry

For immunocytochemistry, HeLa cells, U373 cells and PBMCs were cultivated under exposure to respective combinations of 1,8-cineol (10^{-4} M, (Klosterfrau Healthcare Group) and LPS (100 ng/ml, rough strains from *S. enterica* Re 595, cat. no. L9764, Sigma-Adrich) for 24 h. Afterwards, cells were fixed using phosphate buffered 4% PFA and permeabilized with 0.02% Triton X-100 for 30 min at RT. Blocking was performed using 5% of appropriate normal serum, followed by addition of the primary anti p65-antibody (NF-κB-p65 SC-372, Santa Cruz Biotechnology) for 2 h at RT. Secondary fluorochrome-conjugated antibody (goat anti rabbit conjugated with Alexa 555; Molecular Probes, Göttingen, Germany) was subsequently incubated for 1 h at RT. For nuclear counterstaining, SYTOX green (1:20,000, Molecular Probes, Göttingen, Germany) was applied after RNAse treatment (>500 U/mL, Fermentas), while fluorescence imaging was performed using confocal laser scanning microscopy (LSM 510, Carl Zeiss). Quantification was performed using Image J [27] by defining a region of interest according to the localization of the respective nucleus and measurement of fluorescence intensity in the same region.

2.7. Real time PCR

Total RNA was isolated from U373 cells using RNeasy Mini Kit (QIAGEN, Hilden, Germany) followed by cDNA synthesis by First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to

manufacturer's guidelines. qPCR reactions were performed as triplicate using Platinum SYBR Green qPCR Super-Mix UDG (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) according to manufacturer's guidelines and assayed with a Rotor Gene 6000 (QIAGEN, Hilden, Germany). Primer sequences were CAGAGGGCCTGTACCTCATC (forward) and GGAAGACCCCTCCCAGATAG (reverse) for TNFα, GTGCAGTTTTGCCA AGGAGT (forward) and CTCTGCACCCAGTTTTCCTT (reverse) for IL-8 as wells as AGACCTGGCCTTCCTCAACT (forward) and GTCTCGGAGCTCA GGATCAC (reverse) for IκBα.

2.8. Western blot

0 min, 10 min, 30 min, 60 min, 90 min, 120 min and 24 h after exposure to respective combinations of 1,8-cineol (10^{-4}, Klosterfrau Healthcare Group) and LPS (100 ng/ml, rough strains from *S. enterica* Re 595, cat. no. L9764, Sigma-Aldrich), cells were lysed on ice using 200 μl of lysis buffer (10 mM Tris, 1% SDS, 3 mM EDTA) and homogenized with ultrasonics. After determination of protein concentration with BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) the protein amounts of each sample were adjusted to 20 ng/ml and mixed with 5 × loading buffer followed by heating at 94 °C for 5 min. Samples were subjected to electrophoresis on 10% denaturing SDS polyacrylamide gels and transferred with a semi-dry blotter to a nitrocellulose membrane (Carl Roth GmbH, Karlsruhe, Germany). Blocking of the blots using 5% milk powder (Carl Roth GmbH, Karlsruhe, Germany) in PBS was followed by incubation with the first antibody against IκBα (Enzo Life Sciences, Lörrach, Germany), phospho-IκBα (Cell Signaling Technology, Cambridge, UK), JNK1/JNK2 (BD Pharmingen), phospho-JNK1/2 (Invitrogen), ERK (Cell Signaling Technology) and phospho-ERK (Cell Signaling Technology) overnight. HRP-linked secondary antibody was applied for 1 h at RT. Visualization was performed via enhanced chemiluminescence, while GAPDH (Santa Cruz Biotechnology, Heidelberg, Germany) served as loading control.

2.9. FRET-measurements

U373-cells cultivated in standard medium as described above were treated with trypsin (Trypsin-EDTA, 0.5 mg/mL; PAA) for 10 min followed by harvesting via centrifugation. 8×10^5 cells were cotransfected with NF-κB p65-EYFP and IκBα-mCherry [28] using TurboFect Transfection Reagent (Thermo Fisher Scientific Inc) according to manufacturer's guidelines. For determination of correction factors during the analyses, single transfections were performed using NF-κB p65-EYFP, IκBα-mCherry and CMV-EYFP-mCherry [28]. Transiently transfected cells were cultivated in standard medium for 24 h, followed by addition of respective combinations of 1,8-cineol (Klosterfrau Healthcare Group) and LPS (100 ng/ml, rough strains from *S. enterica* Re 595, cat. no. L9764, Sigma-Adrich).

Analyses were performed by confocal laser scanning microscopy applying a Zeiss LSM780 imaging system. The donor EYFP was detected by PMT1 in the range of 499–550 nm and excited with the 488 nm laser line of an argonion-laser, in parallel the FRET-signal was recorded with PMT2 in the range of 569–650 nm. The same range of PMT2 was additionally applied in another sequence for detecting the acceptor emission with excitation at 561 nm by a DPSS-laser. Sequences were recorded line by line and the main beam splitter MBS488/561 was applied. A water-immersion LD-objective was used (Zeiss LCI Plan-Neofluar 63×/1.3 Imm. Korr. DIC M27), pixel dwell time has been 6.3 μs, image size 512 × 512 pixel and intensity resolution 12-bit/pixel. Frames were obtained every five minutes for a time span of 120 min at three positions for each condition. Data was evaluated based on single cell ROIs of five cells per frame.

FRET-calculation and required determination of correction factors were performed as described before [28].

3. Results

3.1. Exposure to 1,8-cineol leads to significantly decreased levels of NF-κB-activity in human U373 cells in a pGreenFire1TM-NF-κB-Puro reporter gene assay

Potential effects of 1,8-cineol on the NF-κB-activity in human U373 cells were investigated by transient transfection of a pGreenFire1TM-NF-κB-Puro-vector containing a GFP and luciferase reporter cassette (Fig. 1B). Confocal laser scanning microscopy revealed successful transfection of U373 cells, as indicated by strong expression of FPred control vector (Fig. 1C, left panels). To initially determine the activity of NF-κB, the percentage of GFP-positive to FPred-expressing cells was assessed leading to a percentage of 12.5% ± 1.4 GFP-expressing cells in the non-treated control-approach (Fig. 1C, upper panels). Notably, only 4.3% ± 2.6 cineol-treated U373 showed expression of GFP (Fig. 1C, lower panels), thereby indicating a decrease of NF-κB-activity. On account of its higher sensitivity, luciferase activity was subsequently determined in both cineol- and non-treated U373 cells. Here, significant reduction of luciferase activity was observed in the cineol approach (Fig. 1D).

3.2. 1,8-Cineol significantly reduces the NF-κB-activity in human U373 and HeLa cells

With regards to the initially demonstrated reduction of NF-κB-activity by 1,8-cineol in U373 cells, we investigated this matter in more detail using U373 and HeLa cells transiently transfected with a dual NF-κB-reporter system, which consisted of a TK(NF-κB)$_6$LUC vector (*firefly* luciferase) and *Renilla* luciferase expression vector under the control of a constitutive promotor (Fig. 2A). *Renilla* luciferase served for normalization of the *firefly* luciferase activity of the TK(NF-κB)$_6$LUC-reporter construct. Thereby allowing a more profound analyses of the NF-κB-activity, luciferase activity was measured in non-treated and cineol-exposed U373 and HeLa cells after co-transfection of TK(NF-κB)$_6$LUC vector and *Renilla* luciferase vector. As shown in Fig. 3B, significantly reduced levels of luciferase activity were observable in cineol-treated approaches in both U373 (Fig. 2B, left panel) and HeLa cells (Fig. 2B, right panel). Notably, compared to standard NF-κB-inhibiting factors (including dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin), 1,8-cineol-treatment led to a similar level of reduced NF-κB-activity in U373 cells (Fig. 2B, left panel), while an even stronger reduction of NF-κB-activity was observable in HeLa cells (Fig. 2B, right panel).

3.3. 1,8-Cineol-treatment impairs proliferation capability of U373 and HeLa cells

Given the significantly reduced NF-κB-activity in 1,8-cineol-treated U373 and HeLa cells, we additionally investigated potential effects of 1,8-cineol on proliferation. When exposed to concentrations of 10^{-4} M, 10^{-5} M, 9×10^{-6}, 7×10^{-6}, 5×10^{-6} M, 10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M 1,8-cineol for 8 days, U373 cells showed significantly decreased cell numbers (Fig. 3A), while no significant differences were observed in cells treated with 10^{-3} M and 10^{-6} M cineol. Respective concentrations of 1,8-cineol led to even more significant decreases in total cells numbers of Hela cells after 8 days of culture, except for 10^{-7} M 1,8 cineol (Fig. 3B).

3.4. 1,8-Cineol significantly attenuates the NF-κB-activity after LPS-induced stimulation accompanied by strongly reduced nuclear translocation of NF-κB p65

Regarding the use of 1,8-cineol as an anti-inflammatory drug, we investigated its potential effects after LPS-induced activation of NF-κB in U373 and HeLa cells. As shown in Fig. 4A, exposure to LPS led to highly

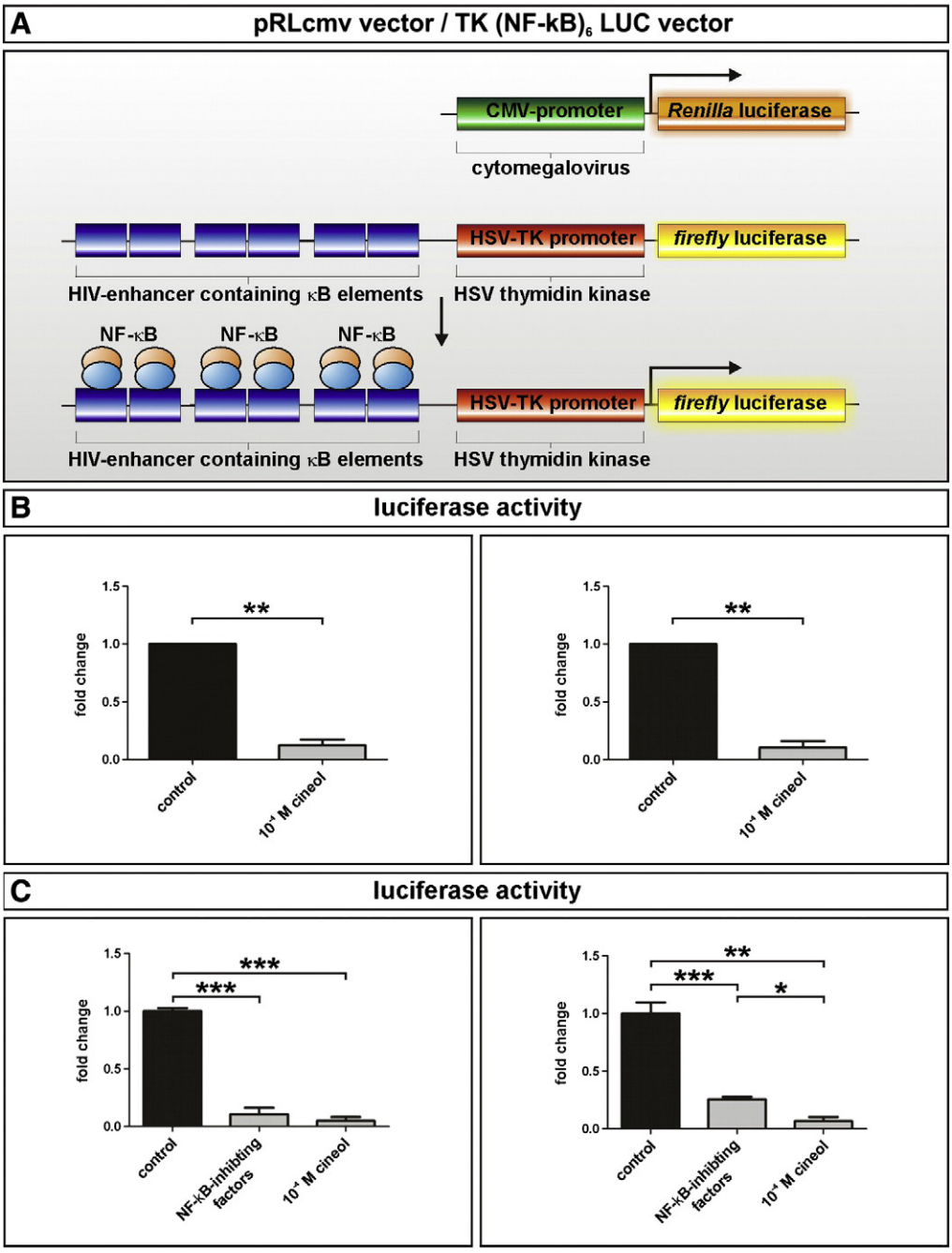

Fig. 2. *A dual NF-κB-reporter system revealed a 1,8-cineol-dependent reduction of NF-κB-activity in human U373 and HeLa cells without strongly affecting proliferation capabilities.* A. Schematic view on dual NF-κB-reporter system containing TK(NF-κB)$_6$LUC vector for detecting NF-κB-activity and a vector constitutively expressing *Renilla* luciferase for normalization. B. Significantly reduced levels of luciferase activity were observed in co-transfected U373- (left panel) and HeLa cells (right panel) after cineol-treatment in comparison to untreated control. Merged data from biological triplicate. $^{**}p < 0.01$ was considered significant (paired *t*-test, two-tailed, confidence interval: 95%). C. Compared to standard NF-κB-inhibiting factors (including dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin), 1,8-cineol-treatment led to a similar (left panel) or even stronger (right panel) reduced NF-κB-activity in co-transfected U373- (left panel) and HeLa cells (right panel). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were considered significant (unpaired *t*-test, two-tailed, confidence interval: 95%).

increased levels of luciferase activity in U373 cells. Notably, a significantly reduced luciferase activity was observed when U373 cells were exposed to both LPS and 1,8-cineol. Likewise, LPS-treated HeLa cells revealed increased levels of luciferase activity, which significantly decreased by cineol-treatment (Fig. 4B, left panel). Moreover, confocal scanning microscopy of pGreenFire1TM-NF-κB-Puro-vector transfected HeLa cells showed an increased amount of GFP-positive cells (36.3% ± 10.1) compared to control (17.0% ± 3.0). Importantly, the number of GFP-expressing cells declined even below control level, when HeLa cells were exposed to both LPS and 1,8-cineol (9.3% ± 1.0, Fig. 4B, right panel).

Considering the reduced activity of NF-κB after exposure to 1,8-cineol, even after LPS-treatment, we further determined the localization of p65-protein, a major subunit of NF-κB [29], via immunocytochemistry. In comparison to the control approach (Fig. 4C, right panel, arrowheads), we observed translocation of p65 into the nucleus of HeLa cells after LPS-stimulation (Fig. 4C, middle panel, arrows). Importantly, HeLa cells treated with LPS and 1,8-cineol showed highly

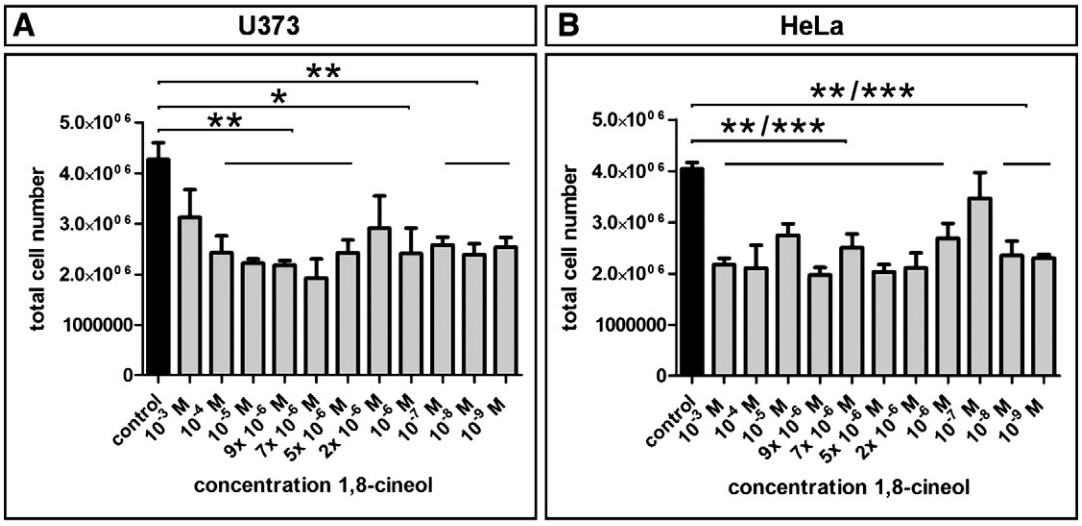

Fig. 3. *1,8-cineol-treatment impaired proliferation capability of U373 and HeLa cells.* A. Significantly decreased cell numbers were observed in U373 cells after exposure to respective concentrations of 1,8-cineol for 8 days, except for 10^{-3} M and 10^{-6} M cineol. B. 1,8-cineol-treatment led to significant decreases in total cells numbers of Hela cells after 8 days of culture, except for 10^{-7} M 1,8 cineol. *p < 0.05, **p < 0.01 and ***p < 0.001 (unpaired *t*-test, one-tailed, confidence interval: 95%) were considered significant.

decreased amounts of nuclear p65-protein, thereby indicating reduced translocation of p65 into the nucleus (Fig. 4C, right panel, arrowheads). Quantification of data gained from three independent experiments verified our observations by showing significantly increased levels of nuclear p65-protein after exposure to LPS, while cineol-treatment led to significantly reduced levels (Fig. 4E).

3.5. Decreased nuclear translocation of NF-κB p65 in 1,8-cineol-treated U373 cells is associated with reduced expression of NF-κB target genes

Substantiating our findings gained using HeLa cells, representative immunocytochemical analysis demonstrated localization of p65 within the nucleus of U373 cells after LPS-stimulation (Fig. 5A, middle panel, arrows), while exposure to LPS and 1,8-cineol led to decreased levels of nuclear p65 (Fig. 5A, right panel, arrowheads) comparable to control (Fig. 5A, left panel, arrowheads). Notably, at least some U373 cells showed low amounts of nuclear p65 even after exposure to LPS (Fig. 6A, middle panel, arrowheads). Importantly, further quantification depicted significantly increased amount of nuclear p65 in response to LPS-stimulation, while significant reduction of nuclear p65 was observed in cineol-treated U373 cells (Fig. 7B).

In order to investigate a potential correlation between nuclear translocation of NF-κB p65 and potential alterations in expression of NF-κB target genes, we further investigated expression levels of TNFα and IL-8 via real time PCR. Here, LPS-treated U373 cells showed increased gene expression levels of TNFα and IL-8 in comparison to control (Fig. 5B), while co-treatment with 1,8-cineol led to strongly attenuated gene expression. Western blot analyses demonstrated an increased protein amount of NF-κB target gene IκBα after 24 h in LPS-treated U373 cells, which was strongly reduced by co-treatment with 1,8-cineol (Fig. 5C).

3.6. Treatment with 1,8-cineol leads to increased levels of IκBα in U373 cells in an IκB kinase-independent matter

With regard to the strongly reduced levels of NF-κB target genes, we determined the potential influence of 1,8-cineol on IκBα. Western blot analyses over a short range of time (0–120 min) revealed highly decreasing protein amounts of IκBα in LPS-treated U373 cells in comparison to control (Fig. 5E, upper and middle panels, IκBα). On the contrary to the observations made in the LPS approach, the protein amount of IκBα was found to be increasing in U373 cells exposed to LPS and 1,8-cineol after 90 min (Fig. 5E, lower panels, IκBα). Since changes in IκBα-protein amount are broadly known to be associated with its proteasomal degradation mediated through IκB kinase (IKK)-dependent phosphorylation, we investigated phosphorylation of IκBα using western blot. After 10 min of LPS-treatment, U373 cells showed increased amounts of phosphorylated IκBα (Fig. 5E, middle panels, pIκBα). We observed increased amounts of phosphorylated IκBα after 10 min also in U373 cells treated with LPS and 1,8-cineol (Fig. 5E, lower panels, pIκBα), a finding suggesting that 1,8-cineol may lead to increased IκBα-protein amounts in an IKK-independent matter. Remarkably, the protein amounts of unphosphorylated and phosphorylated IκBα were likewise highly increased after 120 min in LPS and 1,8-cineol treated U373 cells compared to the LPS-approach.

3.7. LPS-induced loss of interaction between NF-κB p65 and IκBα is restored by 1,8-cineol

Considering the reduced nuclear translocation of NF-κB p65 and decreased levels of NF-κB target genes, the potential influence of 1,8-cineol on the interaction between NF-κB p65 and IκBα was investigated using Förster resonance energy transfer (FRET) analyses. We transiently transfected U373 cells with vectors containing a NF-κB p65 linked to EYFP as well as a mCherry-coupled IκBα (Fig. 6A). Transfected cells were treated with LPS or LPS and 1,8-cineol and analyzed for 120 min, while untreated cells were used as control. As representatively shown for one cell in Fig. 6B, we observed no decline in FRET efficiency in the control approach (left upper panel). Importantly, LPS-treatment led to strongly impaired FRET efficiency (Fig. 6B, right upper panel), which was restored in U373 cells treated with LPS and 1,8-cineol (Fig. 6B, left lower panel). In accordance to the increasing IκBα protein amounts observed during exposure to LPS and 1,8-cineol using western blot analyzes, FRET efficiency was particularly found to decrease in the first place up to 60 min, and increased afterwards up to control level. Analyzing slopes of regression lines of graphs depicting FRET efficiency of representative cells in three different measurement areas per approach verified our observations by showing significant reduction of FRET efficiency over time in LPS-treated cells compared to control. Treatment with LPS and cineol led to significantly increased slope of FRET efficiency within the complete timespan (Fig. 6B, right lower panel), suggesting a restored interaction between IκBα and NF-κB p65 compared to the LPS-approach.

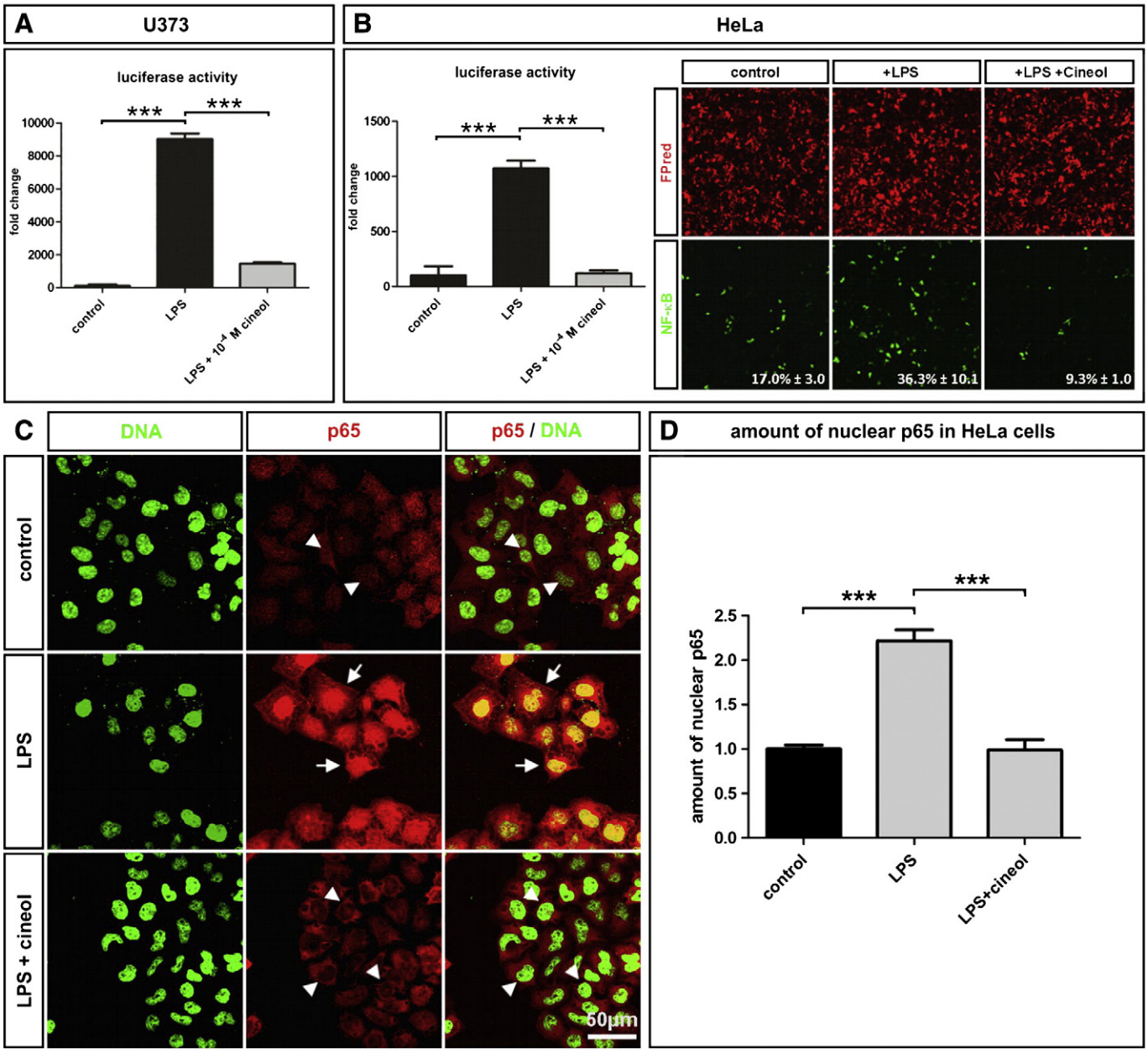

Fig. 4. *1,8-cineol significantly attenuated the NF-κB-activity after LPS-induced stimulation accompanied by strongly reduced nuclear translocation of NF-κB p65.* A. LPS-treated U373 cells showed highly increased levels of luciferase activity, which significantly decreased when U373 cells were exposed to both LPS and 1,8-cineol. Representative data from biological triplicate. Luciferase activity was normalized by detection of constitutively expressed *Renilla* luciferase. ***$p < 0.001$ was considered significant (unpaired *t*-test, two-tailed, confidence interval: 95%). B. Luciferase activity significantly increased in HeLa cells after LPS-treatment, while exposure to LPS and 1,8-cineol led to significantly reduced levels of luciferase activity (left panel). Representative data from biological triplicate. ***$p < 0.001$ was considered significant (unpaired *t*-test, two-tailed, confidence interval: 95%). Confocal scanning microscopy of pGreenFire1TM-NF-κB-Puro-vector-transfected HeLa cells demonstrated an increased amount of GFP-positive cells (36.3% ± 10.1) after LPS-treatment compared to control approach (17.0% ± 3.0). Importantly, the number of GFP-expressing cells declined (9.3% ± 1.0), when HeLa cells were exposed to both LPS and 1,8-cineol (right panel). C. Representative immunocytochemical analysis showing localization of p65 within the nucleus of HeLa cells after LPS-stimulation (middle panel, arrows), while exposure to LPS and 1,8-cineol led to decreased levels of nuclear p65 (lower panel, arrowheads). D. Quantification of immunocytochemical analyses from three independent experiments depicted significantly increased amount of nuclear p65 in LPS-stimulated HeLa cells, while cineol-treatment led to significant reduction of nuclear p65. ***$p < 0.001$ was considered significant (unpaired *t*-test, two-tailed, confidence interval: 95%).

3.8. 1,8-Cineol-treatment did not affect IκBα on mRNA level, but is also associated with reduced amounts of phosphorylated c-Jun N-terminal kinase 1/2 protein

To further investigate the potential influence of 1,8-cineol on IκBα, we analyzed IκBα-expression on mRNA-level using real time PCR. U373-cells treated with 1,8-cineol and LPS as well as LPS alone revealed no changes of IκBα-expression above threshold level (Fig. 7A), suggesting that glucocorticoid signaling is not likely to be involved in the 1,8-cineol-associated decrease of NF-κB target gene expression.

Extending our investigations on potential effects of 1,8-cineol on the Mitogen-activated protein 1 kinase (MAP1K)-pathways, we assessed phosphorylation of c-Jun N-terminal kinase 1/2 (JNK1/2) as well as extracellular signal-regulated kinase (ERK) via western blot analyses. LPS and 1,8-cineol treated U373-cells showed highly reduced amounts of phosphorylated JNK1/2 after 10 min in comparison to cells exposed to LPS only (Fig. 7B). Remarkably, the total protein amounts of JNK1/2 were

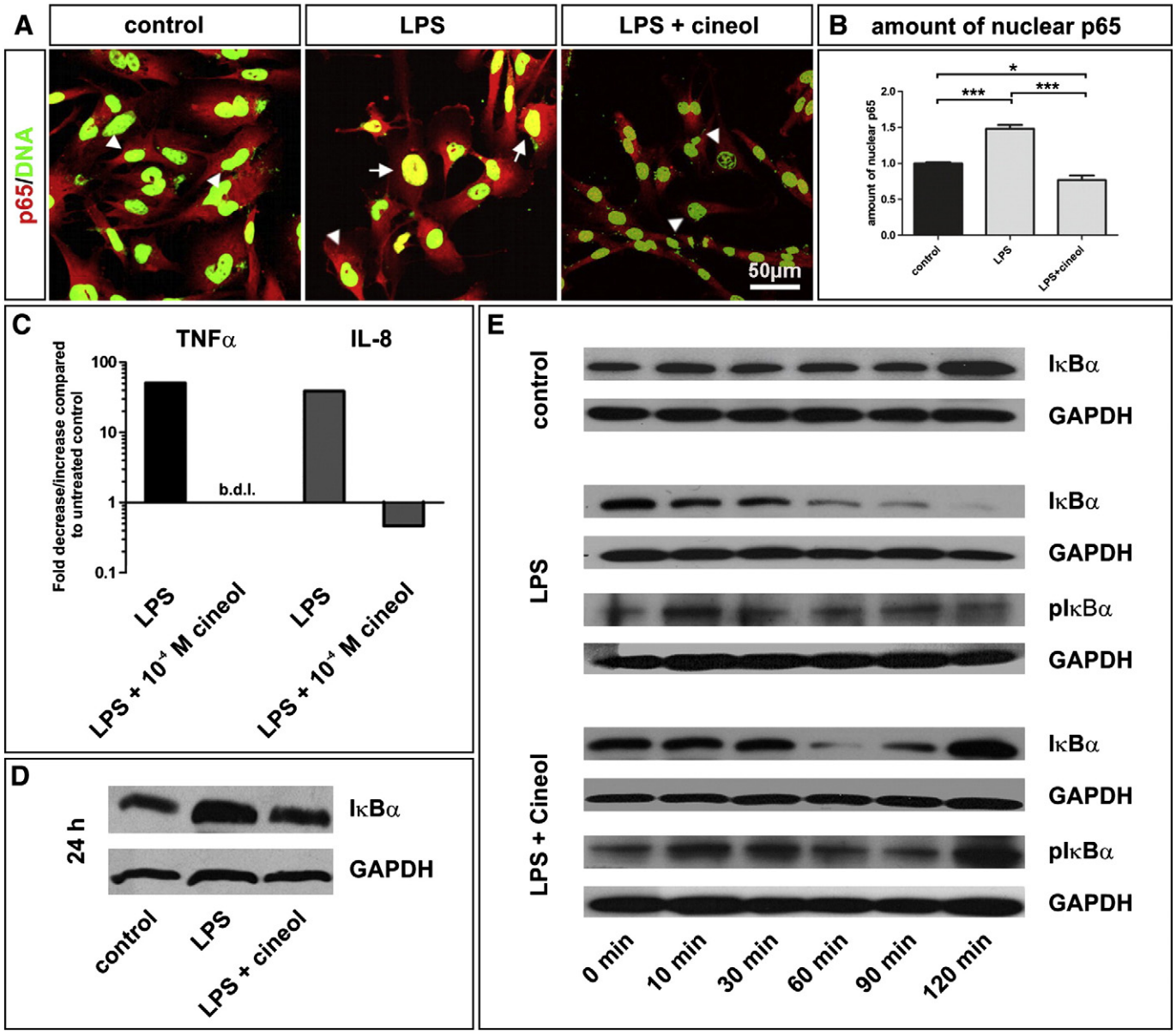

Fig. 5. *Treatment with 1,8-cineol resulted in significantly decreased nuclear translocation of NF-κB p65 in U373 cells and reduced expression of NF-κB target genes, while protein amount of* IκBα *was increased.* A. Representative immunocytochemical analysis showing translocation of p65 into the nucleus of U373 cells after LPS-stimulation (middle panel, arrows), while exposure to LPS and 1,8-cineol led to decreased levels of nuclear p65 (right panel, arrowheads). B. Quantification of immunocytochemical analyses from three independent experiments depicted significantly increased amount of nuclear p65 in LPS-stimulated U373 cells, while cineol-treatment led to significant reduction of nuclear p65. *$p < 0.05$ and ***$p < 0.001$ were considered significant (unpaired *t*-test, two-tailed, confidence interval: 95%). C. Real time PCR analyses revealed elevated gene expression levels of NF-κB target genes TNFα and IL-8 after 24 h in LPS-treated U373 cells, which were strongly reduced by co-treatment with 1,8-cineol. b.d.l.: below detection limit. D. Protein amounts of NF-κB target gene Iκbα were analyzed after 24 h via western blot revealing an LPS-induced increase, which was attenuated by 1,8-cineol. E. Western blot analyses over a short range of time (0-120 min) showed highly decreasing protein amounts of IκBα in LPS-treated U373 cells, but increasing amounts in U373 cells exposed to LPS and 1,8-cineol. Protein amount of phosphorylated IκBα (p IκBα) were augmented after 10 min in LPS as well as in LPS and 1,8-cineol treated U373 cells. U373 cells treated with LPS and 1,8-cineol showed highly increased amounts of unphosphorylated and phosphorylated IκBα after 120 min.

observed to be strongly augmented after 120 min in LPS and 1,8-cineol treated U373 cells compared to the LPS-approach. On the contrary, amounts of ERK or phosphorylated ERK were not affected by 1,8-cineol (Fig. 7C).

We also observed no significant changes in NF-AT-activity in U373 cells treated with LPS and 1,8-cineol compared to LPS- and control-approaches using a NF-AT- luciferase reporter gene assay [25] (Fig. S2).

3.9. 1,8-Cineol strongly decreases nuclear NF-κB p65 and protein amount of its target gene IκBα in human peripheral blood mononuclear cells (PBMCs)

Substantiating the inhibitory effect of 1,8-cineol on NF-κB, we investigated its potential role in human peripheral blood mononuclear cells, a well-established model in terms of inflammatory diseases. Using immunocytochemistry, a LPS-induced nuclear translocation of NF-κB p65 was observed in PBMCs (Fig. 8A, middle panels) in comparison to the control approach (Fig. 8A, left panels). Treatment with LPS and 1,8-cineol resulted in reduced nuclear NF-κB p65 (Fig. 8A, right panels). Respective Western blots showed an increased amount of the NF-κB target gene IκBα at protein level in LPS-treated PBMCs, which was strongly attenuated in PBMS exposed to LPS and 1,8-cineol (Fig. 8B).

4. Discussion

This study describes for the first time that 1,8-cineol, the active ingredient of the drug Soledum®, significantly reduces the activity of

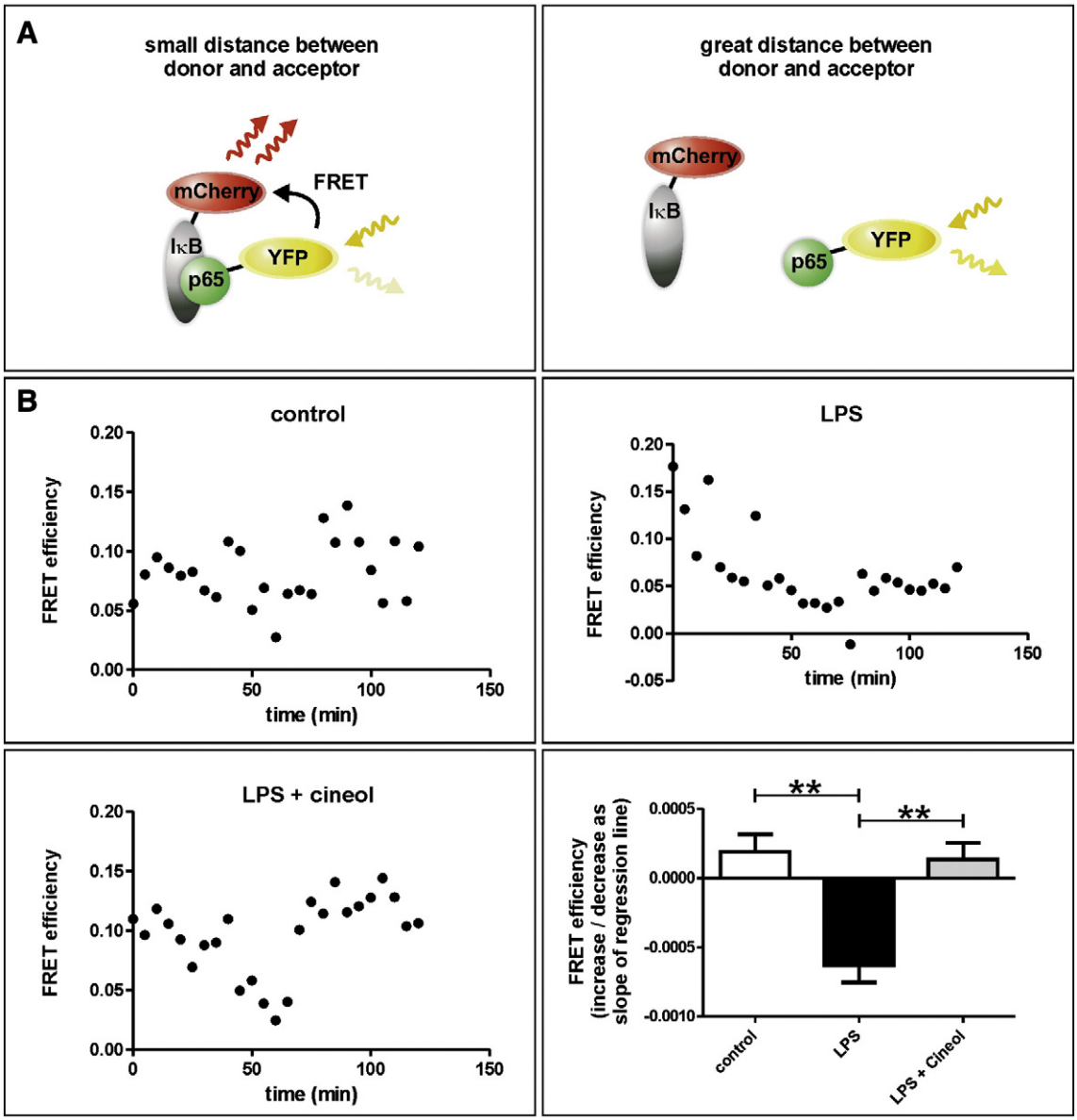

Fig. 6. *Loss of interaction between NF-κB p65 and IκBα during LPS-treatment was restored by 1,8-cineol.* A. Schematic view of Förster resonance energy transfer (FRET) between EYFP linked to NF-κB p65 and mCherry coupled to IκBα. B. FRET efficiency over time. Representative U373 cells cotransfected with NF-κB p65-YFP and IκBα-mCherry showed no decline in FRET efficiency over time in the control approach, while LPS-treatment led to strongly impaired FRET efficiency, which was restored by 1,8-cineol. Slopes of regression lines of graphs depicting FRET efficiency over time. Representative cells in three different measurement areas per approach showed significantly reduced FRET efficiency in LPS-treated cells compared to control. Treatment with LPS and cineol led to significantly increased levels of FRET efficiency. $^{**}p < 0.01$ was considered significant (unpaired *t*-test, one-tailed, confidence interval: 95%).

NF-κB in U373- and HeLa cells as well as peripheral blood mononuclear cells.

Closely linked to a broad variety of inflammatory human diseases (such as asthma, rheumatoid arthritis or septic shock), the transcription factor NF-κB is well-described to be activated in response to proinflammatory cytokines, viral infection or exposure to bacterial lipopolysaccharides [30–33]. Initiating a signaling cascade to activate NF-κB, lipopolysaccharides bind to the TLR-4 (toll-like receptor-4) [34], leading to recruitment of MyoD88 followed by phosphorylation of IKKs, in turn leading to the phosphorylation, polyubiquitylation and 26S-proteasome-mediated degradation of IκBs. Hence IκBs mask the nuclear translocation signal region (NLS) of NF-κB, this process is followed by translocation of NF-κB into the nucleus, where downstream genes are activated via binding to cognate κB-sites [30,31,35]). This is in accordance to the here presented data, since we demonstrate reduced protein amounts of IκBα, impaired interaction between IκBα and NF-κB p65, translocation of NF-κB p65 into the nucleus, the active binding to κB-sites as well as an increased gene expression of NF-κB target genes after exposure to bacterial LPS.

Most pro-inflammatory conditions are considered to activate NF-κB in various cell types [36–42]. In the present study, we investigated the molecular mechanism of the anti-inflammatory effect of 1,8-cineol using the human cancer cell lines HeLa and U373 as well as peripheral blood mononuclear cells. Particularly PBMCs represent a well-established and LPS-inducible model for investigating inflammatory diseases, as reported in terms of COPD [43] and asthma [44]. Notably, HeLa- and U373 cell lines are commonly applied to study NF-κB-depending inflammatory responses and signaling *in vitro* [45–49], also providing a direct link to the molecular mode of action. Regarding the LPS-induced stimulation of NF-κB, U373 cells have been shown to be LPS/sCD14-responsive and TLR4 expressing [50,51], while HeLa cells express TLR4 [52,53] but seem to lack MD-2-

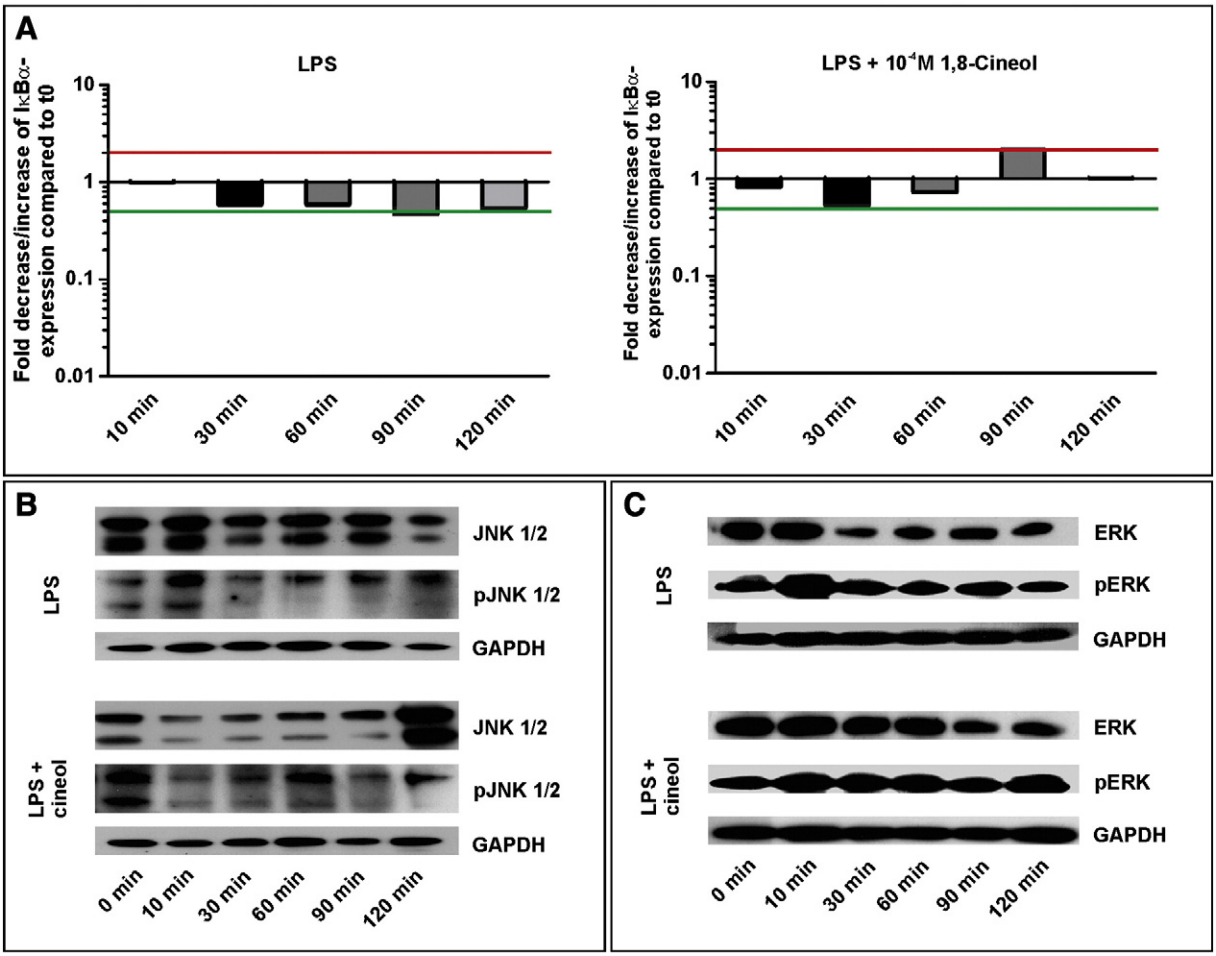

Fig. 7. *1,8-cineol-treatment did not affect expression of IκBα on mRNA level, but was associated with reduced amounts of phosphorylated c-Jun N-terminal kinase 1/2 protein.* A. Real time PCR analyses revealing no changes of IκBα-expression above threshold level. B. Protein amounts of c-Jun N-terminal kinase 1/2 (JNK1/2) as well as phosphorylated JNK1/2 (pJNK1/2) showed increased amounts of pJNK1/2 (30 min, 60 min) in U373 cells treated with LPS and 1,8-cineol in comparison to cells exposed to LPS only. C. Protein amounts of extracellular signal-regulated kinase (ERK) as well as phosphorylated ERK revealed no differences between U373 cells treated with LPS and 1,8-cineol and LPS only.

expression [54]. However, it has been suggested that LPS-induced TLR4 signaling can also occur in MD-2 independent manner ([55], although this matter is controversially discussed [56]. Since in our hands, HeLa cells can activate NF-κB after LPS-treatment, we suggest that the initial signaling step including receptor dimerization may be mediated by an alternative, unknown mechanism. Demonstrably, Yin and coworkers reported aspirin-mediated prevention of NF-κB-activation by inhibition of IKK-β in an *in vitro* model using HeLa

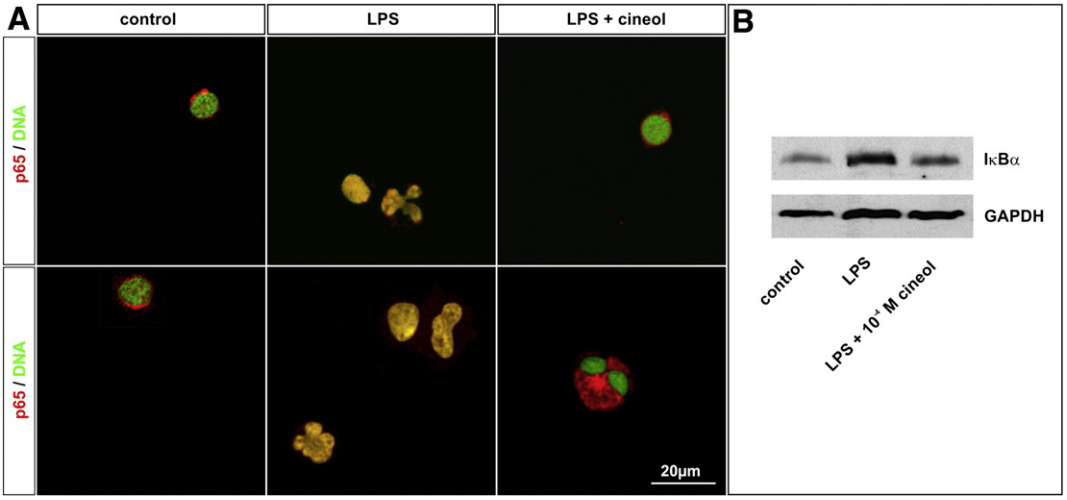

Fig. 8. *1,8-cineol decreased nuclear NF-κB p65 and protein amount of its target gene IκBα in human peripheral blood mononuclear cells (PBMCs)* A. Immunocytochemical analysis revealed localization of p65 within the nucleus of LPS-stimulated PBMCs (middle panel), while decreased levels of nuclear p65 were observed in PBMCs treated with LPS and 1,8-cineol (lower panel, arrowheads). B. Western blot analyzes showed an increased amount of the NF-κB target gene Iκbα at protein level in LPS-treated PBMCs, which was strongly attenuated by 1,8-cineol.

cells, underlining their applicability for studying NF-κB-depending inflammatory responses [47]. Besides such NF-κB-inhibiting effects of prominent drugs [47,57], a broad range of natural products like sesquiterpene lactones [58], hypericin [59], *Plagius flosculosus* extract [60], incensole acetate isolated from Boswellia resin [61], plant extracts from *Urtica dioica* [62] or monoterpenoids [21,23,63] are well-known to reduce NF-κB-activity. Interestingly, several of said NF-κB-inhibiting monoterpenoids are already applied in treating inflammation and cancer, as in the case of Aucubin [22,64], Genipin [24,64], Perillyl alcohol [65] and α-Pinene [63]. Thereby, monoterpenoids either inhibit IκB-degradation [22,24], translocation of the p65-protein [63] or change the DNA binding activity of the translocated p65 [21,65].

Although closely related to the monoterpenoid Limonene, which is inhibiting NF-κB by changing its DNA binding activity [65], 1,8-cineol (also known as Limonen-1,8-monoxid) was reported to have no effects on nuclear NF-κB in THP-1 cells [5]. However, even in 1998 Juergens and colleagues evidenced a significantly inhibited production of TNF-α and IL-1β after treatment with 1,8-cineol in human blood monocytes *in vitro* [19]. Moreover, the same group substantiated the role of 1,8-cineol as a strong inhibitor of TNF-α and IL-1β in both human monocytes and lymphocytes [66], suggesting a link between the mode of action of 1,8-cineol and NF-κB-inhibition. This putative mode of action was further supported by two studies which demonstrated eucalyptol ameliorating bacterial vaginosis and cerulein-induced acute pancreatitis by inhibition of NF-κB activation in the mice system [67,68].

The present study extended these findings by depicting increasing protein amounts of IκBα, restored interaction between IκBα and NF-κB p65, a lack of p65-translocation into the nucleus, significantly decreased binding to κB-sites as well as attenuated expression levels of NF-κB target genes in response to 1,8-cineol-treatment after stimulation with LPS. Our data suggest IκBα as well as JNK as potential targets of 1,8-cineol (see Fig. 9 for schematic overview), provided that JNK may act directly on NF-κB target gene expression via AP-1. Lack of IκBα-degradation seems to be phosphorylation-independent, suggesting the potential involvement of changed kinetics in an IKK-independent manner.

In addition to its well-described anti-inflammatory activity, Moteki and colleagues demonstrated a 1,8-cineol-dependent induction of apoptosis in human leukemia Molt 4B and HL-60 cells [69]. Given these findings, we investigated the potential effects of 1,8-cineol on the proliferation of human cell lines HeLa and U373, also used in the present study to investigate the NF-κB-activity *in vitro*. Here, 1,8-cineol-treatment was shown to impair the proliferation capability of U373 and HeLa cells, a matter closely linked to reduced activity of NF-κB [41,70–73]. Although strongly impaired by 1,8-cineol-treatment, we observed no total blockade of proliferation, suggesting a link to the also not totally blocked activity of NF-κB.

5. Conclusion

In summary, this study demonstrates that 1,8-cineol, a natural component clinically applied as the active ingredient of Soledum®, reduces the activity of NF-κB *in vitro*. We propose a novel mode of action through an inhibition of nuclear translocation of NF-κB p65 as well as a decrease in proinflammatory NF-κB target gene expression, suggesting IκBα as well as JNK as potential targets of 1,8-cineol.

The transcription factor NF-κB is well-described to be involved in a broad variety of inflammatory human diseases, including those closely linked to the airway such as asthma, but also systemic infections like rheumatoid arthritis or septic shock. Our findings may therefore broaden the range of 1,8-cineol in treating inflammatory diseases or improve its already described beneficial impact on the therapy of chronic sinusitis and bronchitis, chronic obstructive pulmonary disease and bronchial asthma.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.07.001.

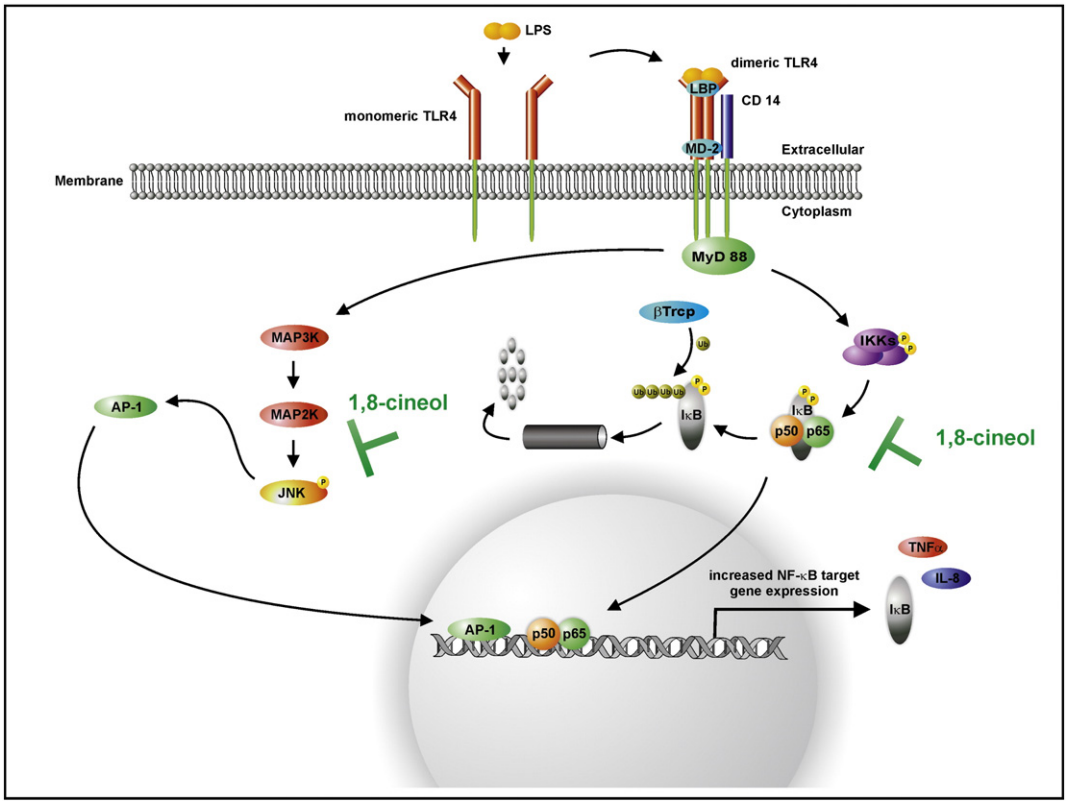

Fig. 9. *Potential mode of action of 1,8-cineol.* Schematic view on LPS-stimulated cell. 1,8-cineol inhibits nuclear translocation of NF-κB p65 and NF-κB target gene expression in a IκBα- and JNK-dependent manner.

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