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IL-8 production by macrophages is synergistically enhanced when cigarette smoke is combined with TNF-α

Hadi Sarir [a,1], Esmaeil Mortaz [a,b,c,1,*], Willem. T. Janse [a], Masoumeh E. Givi [a], Frans P. Nijkamp [a], Gert Folkerts [a]

[a] Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands
[b] Chronic Respiratory Disease Research Center and National Research Institute of Tuberculosis and Lung Disease (NRITLD), Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Basic Science, Section of Biochemistry, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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ABSTRACT

Macrophages are key inflammatory cells in chronic obstructive pulmonary disease (COPD). The pathophysiology of cigarette smoke-induced lung emphysema is complex but there is a clear role for reactive oxygen species (ROS, such as peroxynitrite), tumor necrosis factor (TNF-α) and interleukin (IL)-8. We investigated whether TNF-α or cigarette smoke medium (CSM) alone or in combination induces the production of IL-8 by human macrophages or monocyte lymphoma U937. CSM and TNF-α induce a dose- and time-dependent increase in IL-8 production. Interestingly, when sub-threshold concentrations of CSM and TNF-α were co-incubated, a 1500% increase in IL-8 production was observed compared to either of the compounds alone. Similar results were obtained with TNF-α and the peroxynitrite donor SIN-1. Moreover, the overproduction of IL-8 was associated with an enhanced increase in the translocation of NF-κB and an enhanced decrease in glutathione levels. Preincubation of the cells with antioxidants, such as N-acetyl-L-cysteine (NAC), prevented the overproduction of IL-8 and activation of NF-κB.

In conclusion, CSM exposure of macrophages up-regulates the expression and the production of IL-8 via reactive oxygen species and NF-κB activation. Moreover, CSM dramatically enhances the production of IL-8 in combination with TNF-α. Based upon the strong synergistic action, a combination therapy directed against ROS and TNF-α could be a new approach to stop the progression in lung damage during emphysema.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major global health problem with an increasing incidence and mortality [1,2]. Oxidative stress, which can be defined as an increased exposure to oxidants and/or decreased antioxidant capacities, is widely recognized as a central aspect of COPD [3–7]. Cigarette smoke, which is the major etiological factor in this condition [8] contains high concentrations of free radicals and oxidants (short/long-lived) [9–11] Free radicals activate inflammatory cells which, in turn, generate high levels of reactive oxygen and nitrogen species (ROS and RNS) and other toxic metabolites. Activation of immune cells by these radicals leads to the production of oxidants and cytokines, such as IL-8 and TNF-α [12,13].

IL-8 is a powerful chemotactic and paracrine mediator for neutrophils, and infiltration of activated neutrophils is key in pulmonary inflammation and oxidative injury [14–16]. Oxidative stress or cigarette smoke extract can induce the production of IL-8 [17–22]. In addition, peroxynitrite mediates IL-8 gene expression and release in human whole blood. High levels of TNF-α are present in peripheral blood, sputum and bronchoalveolar lavage fluid (BALF) of patients with COPD [23,24]. TNF-α induces neutrophil degranulation accompanied by the release of proteolytic enzymes and

Abbreviations: CSM, cigarette smoke medium; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; IκB, inhibitory IκB; GSH, glutathione; MDMs, monocyte-derived macrophages; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; SODs, Superoxide dismutase; SIN-1, 3-morpholino-sydnonimine hydrochloride.

* Corresponding author at: Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands.
E-mail address: emortaz@gmail.com (E. Mortaz).

[1] These authors contributed equally to this work.

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activation of macrophages to produce matrix metalloproteinases (MMPs) [25]. In addition, TNF-α causes the formation of ROS and peroxynitrite in mononuclear leukocytes [26–28].

The presence of different antioxidants in the lung lining fluid protects the lung against oxidants by neutralizing and scavenging the free radicals [29]. The major intracellular antioxidant is reduced glutathione (GSH) which inactivates ROS and RNS (reactive nitrogen species) and has a protective effect, either in vitro or in vivo, against oxidative stress [30]. N-acetyl-L-cysteine (NAC) is a proglutathione drug because cysteine is a fundamental factor for GSH synthesis [31]. Moreover, the potential direct interaction of NAC with ROS makes it a good candidate to investigate the cellular consequences of oxidative stress [31].

Either ROS or TNF-α amplify the inflammatory response by activating NF-κB [32–35] which is a critical regulator of many proinflammatory genes, including IL-8 [36–37].

It is evident that an increase in TNF-α and ROS occur concomitantly in lung emphysema patients. Surprisingly, many researchers studied either the effect of oxidants or the effects of TNF-α. In the present study however, the combination of CSM with TNF-α was investigated on the production of IL-8 by macrophages. In addition, the effect of CSM was mimicked by using SIN-1, as a peroxynitrate donor. U937 cells were used because they share phenotypic (IgG receptor expression and inducible differentiation) and functional (inducible cytokine expression) features with human monocytes [38,39]. We found that macrophages co-incubated with CSM and TNF-α resulted in a profound IL-8 release which could be prevented by antioxidants.

2. Materials and methods

2.1. Reagents

3-Morpholinosydnonimine hydrochloride (SIN-1) was obtained from Cayman chemical (Cyman, Huissen, The Netherlands). Recombinant human TNF-α was purchased from Biosource (Invitrogen, Breda, The Netherlands), and anti-human TNF-α from Biosource (Tebu-bio, Heerhugowaard, The Netherlands). N-acetyl-L-cysteine, dimethyl sulphoxide (DMSO), 2-mercaptoethanol, aprotinin and PMSF were purchased from Sigma (Sigma–Aldrich Chemie B.V. Zwijndrecht, The Netherlands). Rabbit polyclonal anti IκB-α and -p65, β-actin and Lamin (as a loading control) were obtained from Santa Cruz Biotechnology (Tebu-bio). Goat anti-rabbit secondary Abs conjugated with horseradish peroxidase (HRP) was prepared from Dako (Dako B.V. Heverlee, Belgium) and ECL reagents were purchased from Amersham (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). Nuclear and cytoplasmic extraction reagents and BCA protein assay kit were purchased from Pierce (Perbio Science, Amsterdam, The Netherlands). The ELISA kit and DCFH-DA were purchased from Invitrogen. The ELISA kit for human TNF-α was purchased from Biosource (BD Biosciences, Erembodegem-Aalst, Belgium). DCFH-DA was purchased from Invitrogen. IKK inhibitor (IKK Inhibitor III, BMS-345541) was purchased from Calbiochem (Merk groups, Darmstadt, Germany).

2.2. Cell culture

Human monocyte lymphoma cells, U937 (American Type Culture Collection N. CRL-1593.2) were grown to approximately 80–90% confluency culture at 37 °C, 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids.

2.3. Isolation of PBMC and culture of human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were separated [22] by density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) of buffy coats obtained from normal blood donors. Thereafter, neutrophils were removed by centrifugation on a Percoll density gradient. The remained cells were used for preparation of the lymphocyte fraction by centrifugation on a Percoll density gradient (purity 85%). Human blood monocytes were obtained using RosetteSep™ (Stem cell Technologies) according to manufacturer's instructions. Briefly, fresh blood was incubated with RosetteSep™ cocktail at room temperature followed by Ficoll-Paque gradient centrifugation (Life Technologies, Cergy Pontoise, France). The enriched monocytes were collected from the Ficoll:plasma interface and purity was assessed by FACS analysis using a FITC-labeled anti-CD14 mAb (95%). Macrophages were obtained by culturing monocytes for 5 days in medium containing 2.5 ng/ml GM-CSF and 25 ng/ml M-CSF (R&D), as described before [22].

2.4. Cell activation

U937 or MDM were washed with PBS and then stimulated with CSM, SIN-1, and TNF-α in 1% serum containing media for different time points. For investigation of the effects of antioxidants, anti-TNF-α and IKK inhibitor, cells were pretreated for 30 min with these compounds and then stimulated. All treatments were performed in duplicate.

2.5. Cigarette smoke medium preparation

Cigarette smoke medium (CSM) was prepared by using a smoking machine (Teague Enterprises, Davis, Ca, USA) as described previously [22]. Briefly, a smoking machine (Teague Enterprises, Davis, CA, USA) was used to direct main and side stream smoke from one cigarette through 5 ml culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically and the media were standardized to a standard curve of CS medium concentration against absorbance at 320 nm. The pH of the resultant extract was titrated to pH 7.4 and diluted with medium. This solution is considered to be 100% CSM. Solutions ranging from 0.03 to 0.06 OD were used in the present study following preliminary experiments, which indicated that these were non-toxic concentrations (viability $\geq$ 96%).

Non-toxic concentrations of CSM were detected performing different toxicological assays (LDH) and FACS analysis (annexin-V and 7-AAD staining).

2.6. Measurement of cellular GSH content

Intracellular GSH content was assessed using cell lysate according to the methods of Tietze [40].

2.7. Cytokines assay

Immunoreactive IL-8 and TNF-α were quantitated using commercially available ELISA kits (BD OptEIA™, Biosource), according to the manufacturer's instructions.

2.8. Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) levels were measured by flow cytometry in cells cultured in serum-free medium and loaded with the redox-sensitive dye DCFH-DA as described before [41]. Briefly, cells were incubated with CSM (0.06 OD), PMA

(1 nM) or TNF-α (10 ng/ml), SIN (500 μM) or in combination of TNF (10 ng/ml)/CSM (0.06 OD) or SIN (500 μM)/TNF-α (10 ng/ml), for 4 h, and ROS generation was assayed by incubation of the cells with CM-H2DCFDA (10 μmol/l) oxidation based fluorescence for 10 min at 37 °C and analyzed by FACS analysis.

2.9. Real-time quantitative PCR

Total RNA was extracted using High Pure RNA Isolation Kit (Roche Applied Science) according manufacturer's instruction. Quantity and purity of the extract were measured by Nanodrop (Nanodrop Tec, Wilmington, DE, USA) and the ratio of 260/280 nm of all the samples was higher than 2. Equal amounts of total RNA was reverse-transcribed using Transcriptor first strand cDNA synthesis kit (Roche) using oligo (dT). Real-time PCR was performed using SYBR Green PCR Master-Mix (ABGene) in 20 μl reactions with 0.5 μM primer for 40 cycles on an ABI Prism 7000 sequence detector (Applied Biosystems). PCR conditions were 50 °C for 2 min and 95 °C for 15 min, follows by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers were designed using the Primer beb3 software which are as followed:, *il-8*, Forward 5′-AACAGGTGCAGTTTTGCCAAG-3′; Reverse 5′-CGCAGTGTGGTC-CACTCTCA-3′ and gapdh, Forward 5′-CCAGGTGGTCTCCTCT-GACTTC-3′; Reverse 5′-CACCCTGTTGCTGTAGCCAAA-3′. The raw CTs from the reactions were analyzed by a modified delta-C_t method with efficiency correction using a PCR data analysis program, qBase to obtain relative quantification values.

2.10. Nuclear extract preparation

Cells were washed twice with PBS and allowed to equilibrate for 10 min in ice-cold cytoplasmic extraction reagent (Pierce) containing 1 mM PMSF and 20 μg/ml aprotinin. Cells were lysed on ice for 10 min in mentioned reagent. Following centrifugation at 20,000 × *g* for 10 min, the supernatants (cytoplasmic extracts) were collected and frozen at −70 °C. The pellets were suspended in nuclear extraction buffer containing 1 mM PMSF and 20 μg/ml aprotinin. After vigorous mixing and incubating for 40 min on ice, the solution was clarified by centrifugation at 20,000 × *g* for 10 min, and the supernatant (nuclear extract) was collected and stored at −70 °C. Protein concentrations were determined by using a BCA protein assay kit (Pierce).

2.11. Western blot analysis

After preincubation of cells with NAC and activation with CSM, TNF-α for 30 min, nuclear extract was prepared, and the protein concentrations were determined by BCA protein assay kit. 50 μg lysate was resolved on 10% acrylamide gels, and separated proteins were electroblotted on PVDF membranes (Bio-Rad). Membranes were then washed once with Tris/HCI, pH 7.4, containing 159 mM NaCI and 1% Tween 20 (TBS-T), and then blocked in super-blocking buffer (Pierce) for 1 h. and subsequently incubated with antibody directed IκB-α or -p65 at a dilution of 1:3000 in for 1 h. After three washes with TBS-T, membranes were treated for 1 h with HRP-conjugated goat anti-rabbit IgG, diluted to 1:20,000 in TBS-T. The membrane was then washed as before and immunoreactivity was detected by electrochemiluminescent (ECL) detection system (Amersham).

2.12. Viability assay

Cytotoxicity was determined by using trypan blue. After incubation, the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue, and the viable cells were counted and compared with the total amount of cells in the suspension and in all of the experiment the viability was higher than 85%.

2.13. Statistical analysis

Experimental results are expressed as mean ± S.E.M. Results were tested statistically by an unpaired two-tailed Student's *t*-test or one-way ANOVA, followed by Newman–Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (version 4). Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. CSM, SIN-1 and TNF-α dose dependently increase IL-8 production

To determine the effect of CSM, SIN-1 and TNF-α on IL-8 release, U937 cells were treated for 7 h with increasing concentrations of CSM (0.03, 0.06, 0.12 and 0.25 OD), SIN-1 (10, 100, 500 and 1000 μM) and TNF-α (0.1, 1, 10, 100 and 1000 ng/ml). CSM, SIN-1 and TNF-α caused a concentration dependent increase in IL-8 production (Fig. 1A–C). The production of IL-8 by TNF-α is far more pronounced than that caused by CSM or SIN-1. Significant IL-8 production started at concentrations of 0.03 OD for CSM, 500 μM for SIN-1 and 1 ng/ml for TNF-α. We chose concentrations of 0.06 OD of CSM, 500 μM of SIN-1 and 10 ng/ml of TNF-α for the ensuing experiments, unless otherwise stated.

3.2. TNF-α with CSM or SIN-1 synergistically induce IL-8 release

Fixed concentration of CSM (0.06 OD) and SIN-1 (500 μM) were incubated with increasing concentrations of TNF-α (0.1–100 ng/ml). As demonstrated in Fig. 2A and B, respectively, all the concentrations of TNF-α synergized with fixed concentration of CSM or SIN-1 after 7 h. In reverse, combinations of a fixed

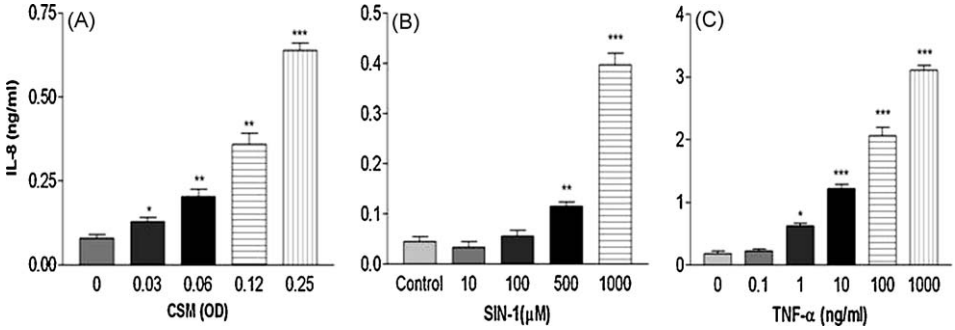

Fig. 1. CSM, SIN-1 and TNF-α elicit a dose-dependent IL-8 production. U937 cells were exposed to various concentrations of CSM (A), SIN-1 (B) and TNF-α (C) for 7 h. Supernatants were collected and for IL-8 protein was measured by ELISA. Data shown are mean ± SEM of three separate experiments. Statistically significant changes in comparison with control cells: (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

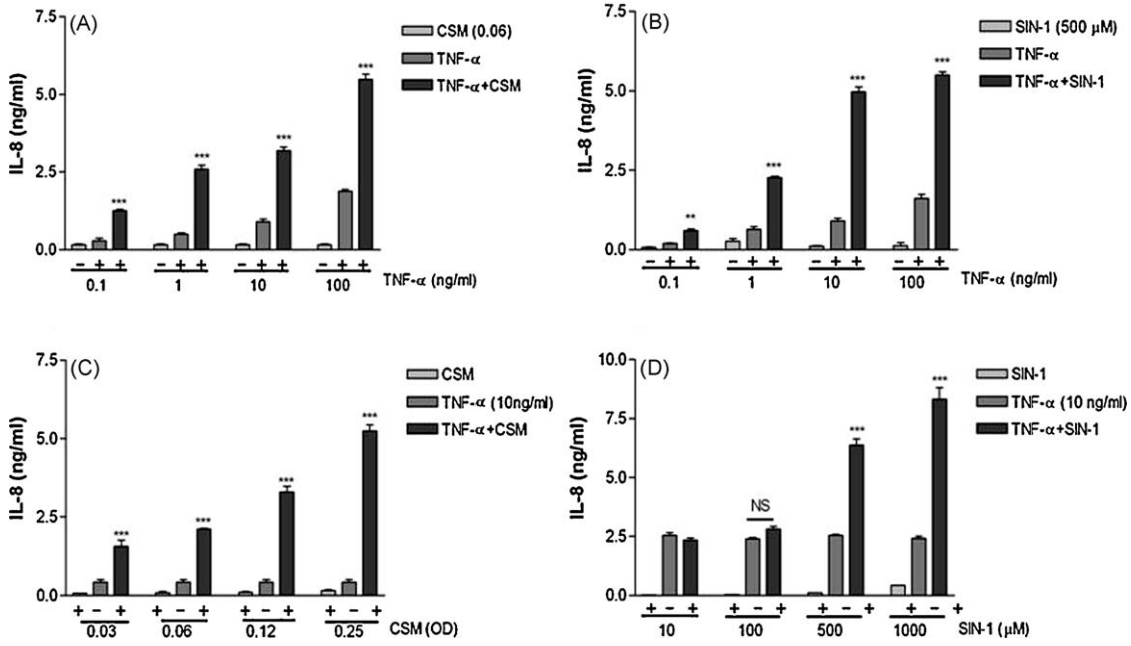

Fig. 2. Effect of different concentration of CSM, SIN-1, and TNF-α on the induction of the synergy. U937 cells were incubated with different concentration of TNF-α (0.1, 1, 10, and 100 ng/ml) and fixed concentration of (A) CSM (0.06 OD) and (B) SIN-1 (500 μM) or fixed concentration TNF-α (10 ng/ml) and different concentration of (C) CSM (0.03, 0.06, 0.12, 0.25 OD) and (D) SIN-1 (10, 100, 500, and 1000 μM) for 7 h. Supernatants were collected and tested for IL-8 protein by ELISA. Data shown are mean ± SEM of three separate experiments. Statistically significant changes in comparison with control cells (CSM, SIN-1 or TNF-α): $^{**}p < 0.01$, $^{***}p < 0.001$.

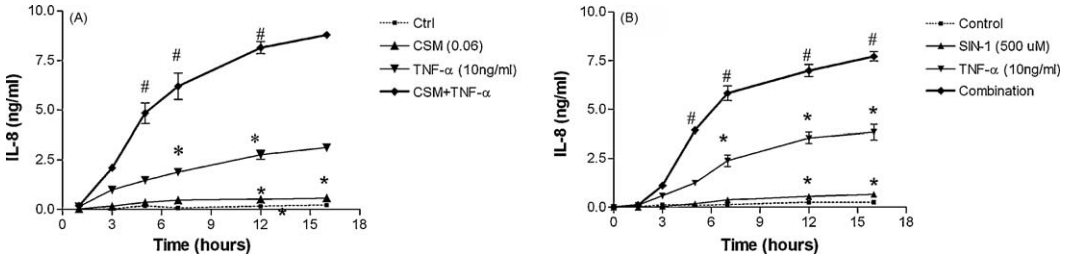

Fig. 3. Time-dependent IL-8 production by CSM and/or TNF-α (A) and SIN-1 and/or TNF-α (B). U937 cells were exposed to CSM (0.06 OD), SIN-1 (500 μM), and/or TNF-α (10 ng/ml) for various time points. Supernatants were collected and IL-8 protein was measured by ELISA. Data shown are mean ± SEM of three separate experiments. Data shown are mean ± SEM of three separate experiments. $^{*}p < 0.05$ versus unstimulated cells; $^{\#}p < 0.05$ versus cells stimulated with CSM, TNF-α or SIN-1 alone.

concentration of TNF-α (10 ng/ml) with increasing concentration of CSM (0.03–0.25 OD) or SIN-1 (10–1000 μM) were examined (Fig. 2C and D, respectively). TNF-α in combination with all the concentrations of CSM revealed synergy in IL-8 production. In contrast, SIN-1 had only synergistic effect at higher concentration.

Next, the effects of the combination of CSM (0.06 OD) and/or TNF-α (10 ng/ml) on IL-8 production at different time points were investigated in U937 cells. CSM and TNF-α alone showed a mild time-dependent increase in IL-8 production, which becomes significantly different from controls at 3 h (Fig. 3A). Combination of the two compounds showed a synergistic increase in IL-8 production which is obvious at 3 h up till 12 h was observed (Fig. 3A). Thereafter, a slight increase up till 16 h is observed. Similar results were found by SIN-1 (500 μM) and TNF-α (10 ng/ml) (Fig. 3B).

To confirm these findings in MDMs underwent a similar procedure and mRNA and protein for IL-8 were measured. A significant enhancement in IL-8 mRNA levels was detected 4 h after incubation with CSM, SIN-1 or TNF-(alone (Fig. 4A). TNF-α in combination with CSM or SIN-1 enhanced the IL-8 expression (Fig. 4A). Similar results were obtained with the U937 cell line (data not shown).

Compared to U937 cells, MDMs more potently produced IL-8. For this reason, and to find the most optimal synergy, somewhat lower concentrations for CSM (0.03 OD vs 0.06 OD) and TNF-α (1 ng/ml vs 10 ng/ml) were used. Stimulation of MDMs with CSM and SIN-1 for 7 h significantly increased IL-8 secretion and TNF-α did not have a significant effect. However, TNF-α in combination with SIN-1 synergistically enhanced IL-8 production and in the case of CSM, there was even a 1500% increase (Fig. 4B).

3.3. Anti-TNF-α antibody blocks the synergy effects of IL-8 releases

To assess the role of TNF-α on the secretion of IL-8, a polyclonal antibody against TNF-α (1 μg/ml) was used prior to stimulation of U937 with CSM and TNF-α alone or in combination. Anti-TNF-α antibodies suppressed the IL-8 production induced by TNF-α but not that induced by CSM or SIN-1 (Fig. 5). Interestingly, the synergic effects of TNF with CSM or TNF with SIN-1 was strongly suppressed by the anti-TNF-α antibody (Fig. 5).

3.4. Anti-oxidants and IKK inhibitor suppress IL-8 production

Several antioxidants were used such as NAC, DMSO and SOD to assess the participation of ROS on the synergistic effects of CSM on

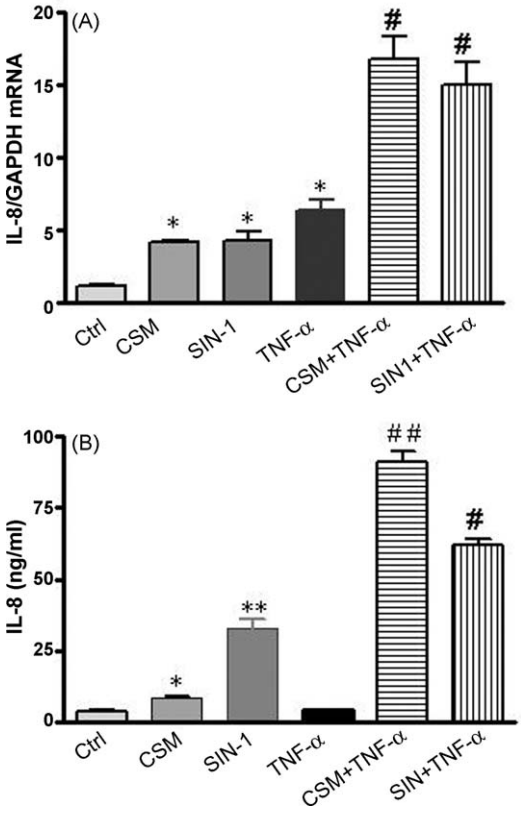

Fig. 4. Synergistic effect of CSM and TNF-α or SIN-1 and TNF-α on the expression and release of IL-8 by macrophages

MDMs were exposed to CSM (0.06 OD), SIN-1 (500 μM), and/or TNF-α (10 ng/ml) for 4 h. RNA was extracted, reverse-transcribed, and expression of IL-8 and GAPDH were determined by real-time PCR (A). Data shown are mean ± SEM of three separate experiments. $^{*}p < 0.05$ versus unstimulated cells; $^{\dagger}p < 0.05$ versus cells stimulated with CSM, SIN-1 or TNF-α only.

Cells were exposed to CSM (0.03 OD), SIN-1 (500 μM), and/or TNF-α (1 ng/ml) for 7 h. Supernatants were collected and IL-8 protein was measured by ELISA (B). Data shown are mean ± SEM of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus unstimulated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different compared to CSE/SIN-1/ TNF-α alone.

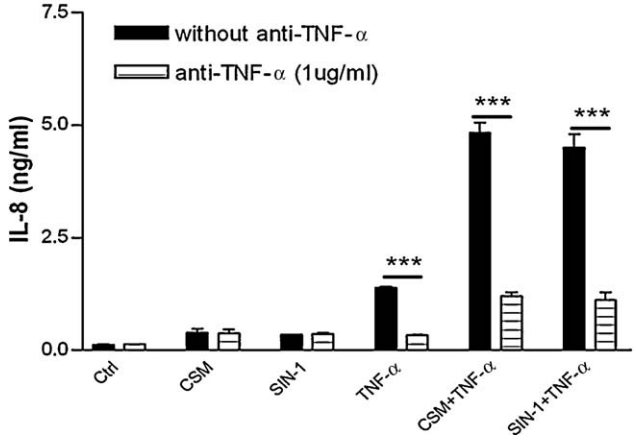

Fig. 5. Inhibitory effect on anti-TNF-α on IL-8 production after stimulation with CSM, SIN-1 or TNF-α and the combinations

U937 cells were preincubated with anti-TNF-α (1 μg/ml) for 20 min and then exposed to CSM (0.06 OD), SIN-1 (500 μM) or TNF-α (10 ng/ml), and combinations of CSM or SIN-1 and TNF-α. Supernatant were collected after 7 h and IL-8 was measured by ELISA. Data shown are mean ± SEM of three separate experiments. Statistically significant changes in comparison with medium-treated cells: $^{***}p < 0.001$.

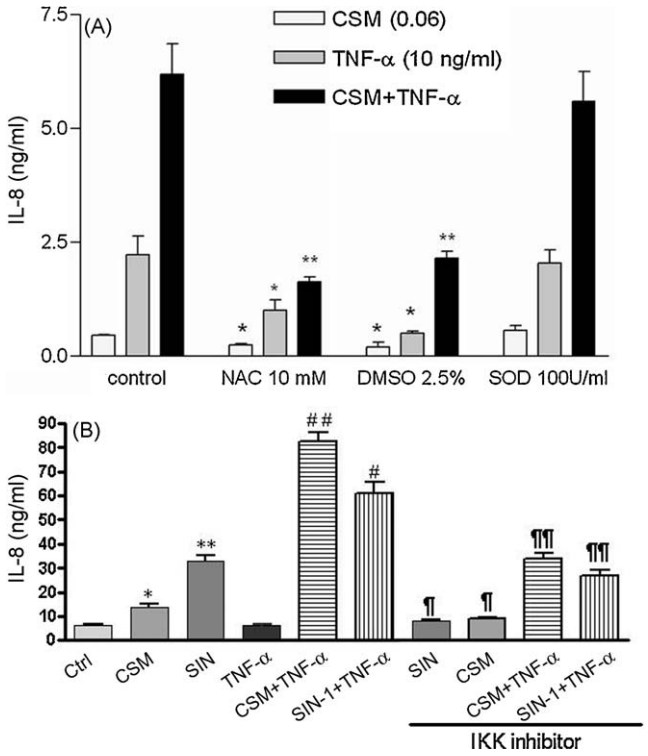

Fig. 6. CSM and/or TNF-α-induced IL-8 production is inhibited by the radical scavenger NAC and DMSO. U937 cells were incubated with NAC (10 mM), DMSO (2.5%) (A) or IKK inhibitor (B) for 30 min and then exposed to CSM (0.06 OD) and/or TNF-α (10 ng/ml) for 7 h. Supernatants were collected and tested for IL-8 protein by ELISA. Data shown are mean ± SEM of three separate experiments. Statistically significant changes in comparison with control cells: $^{*}p < 0.05$ and $^{**}p < 0.01$ versus medium-treated cells, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ significantly different compared to CSE and SIN-1 and $^{\P}p \leq 0.05$, $^{\P\P}p \leq 0.01$ significantly different compared to CSM/TNF and SIN/TNF alone.

TNF for IL-8 production. Cell membrane permeable radical scavengers such as NAC (10 mM) and DMSO (2.5%) were pretreated for 30 min before stimulation. In contrast to SOD, NAC and DMSO suppressed the IL-8 production by CSM or TNF-α (Fig. 6). Moreover, NAC and DMSO could largely prevent the synergy in IL-8 production by the incubation of CSM with TNF-α (Fig. 6A). Anti-TNF-α antbodies together with NAC abrogated IL-8 releases induced by CSM, TNF-α or in combination (data not shown).

Incubation of cells with IKK inhibitor suppressed the release of IL-8 induced by CSM and SIN-1 (Fig. 6B). Moreover, incubation of cells with IKK inhibitor and then stimulation with SIN-1/TNF-α or CSM/TNF-α substantially suppressed IL-8 production (Fig. 6B).

3.5. Combination of CSM and TNF-α substantially induced the generation of ROS

Incubation of MDM cells with PMA, CSM, TNF-α induced the generation of ROS (Fig. 7). The combination of CSM with TNF-α or SIN substantially increased the generation of ROS (Fig. 7).

3.6. CSM and/or TNF-α decreases glutathione levels

To further elucidate the role of ROS, the level of GSH as an indicator for oxidative stress, was measured after 1, 4 and 8 h with CSM (0.06 OD), TNF-α (10 ng/ml) or in combination. Exposure of U937 cells with CSM or TNF-α caused a significant decrease in GSH levels after 1 h incubation (20% and 15%, respectively). Interestingly, the combination of CSM and TNF-α enhanced the effect of depletion up to 60% (Fig. 8). Increasing the time of incubation to 4 h

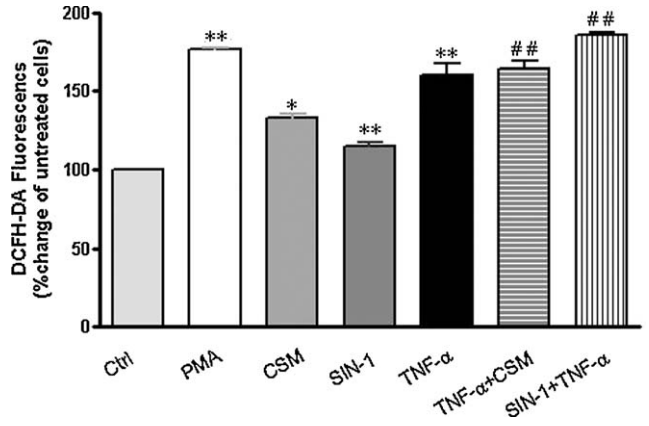

Fig. 7. Intracellular ROS generation was substantially increased by the combination of CSM/TNF-α or SIN/TNF-α by MDM. Cells (1×10^6/ml) were treated with PMA (1 nM), CSM (0.06 OD, TNF-α (10 ng/ml), SIN (500 μM), or in combination of CSM/TNF-α and SIN/TNF-α (100 ng/ml) for 4 h. Then after washing with PBS, cells loaded with DCFH-DA (10 μmol/l) for 30 min in medium without phenol red. After exposure to PMA, the number of cells exhibiting increased fluorescence of oxidized DCF was evaluated by flow cytometry. Histograms represent mean values ± S.E.M. of three independent experiments carried out in MDMs. Ctrl represents DCF fluorescence of untreated cells (100%). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. untreated condition and $^{\#\#}p < 0.01$ significantly different compared TNF-α/CSM/SIN-1 alone.

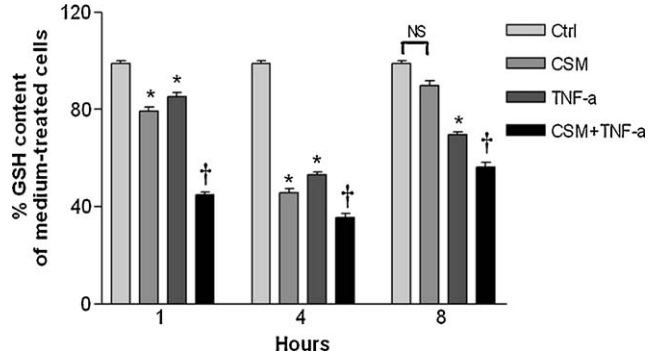

Fig. 8. Intracellular GSH was affected after exposure to CSM and/or TNF-α. U 937 cells were exposed to CSM (0.06 OD) and/or TNF-α (10 ng/ml) for different time points (1, 4, and 8 h). Intracellular GSH content was measured in cellular lysate and expressed as mean percentage ± SEM of medium-treated cells. $^{*}p < 0.05$ versus unstimulated control; $^{\dagger}p < 0.05$ versus CSM and TNF-α alone.

caused a further decrease by both CSM and TNF-α and the combination caused a decrease to 65%. A rebound effect was observed by longer incubation time (8 h) for both compounds.

3.7. CSM and TNF-α synergistically enhanced the degradation of IκB-α and translocation of p65 subunits of NF-κB

NF-κB exists as an inactive form bound to the inhibitory protein IκB-α in the cytoplasm and degradation of IκB-α must occur in order to translocate NF-κB to the nucleus.

We explored the possibility that CSM and TNF-α affects the degradation of IκB-α in activated cells. Protein levels of IκB-α were analyzed by immunoblotting of whole cell extracts with IκB-α-specific Ab's. Activation of cells with CSM, TNF-α or in combination resulted in a reduction of IκB-α levels (Fig. 9A), which was partially, prevented by NAC (1 mM, Fig. 9A, lane 5).

Translocation of p65 subunits of NF-κB into the nucleus is a crucial step in NF-κB activation and inflammatory gene transcription [42]. TNF-α (10 ng/ml) induced translocation of p65 to the nucleus (Fig. 9B). In contrast, CSM (0.06 OD) hardly induced nuclear expression of p65 in the nucleus. Combination of TNF-α

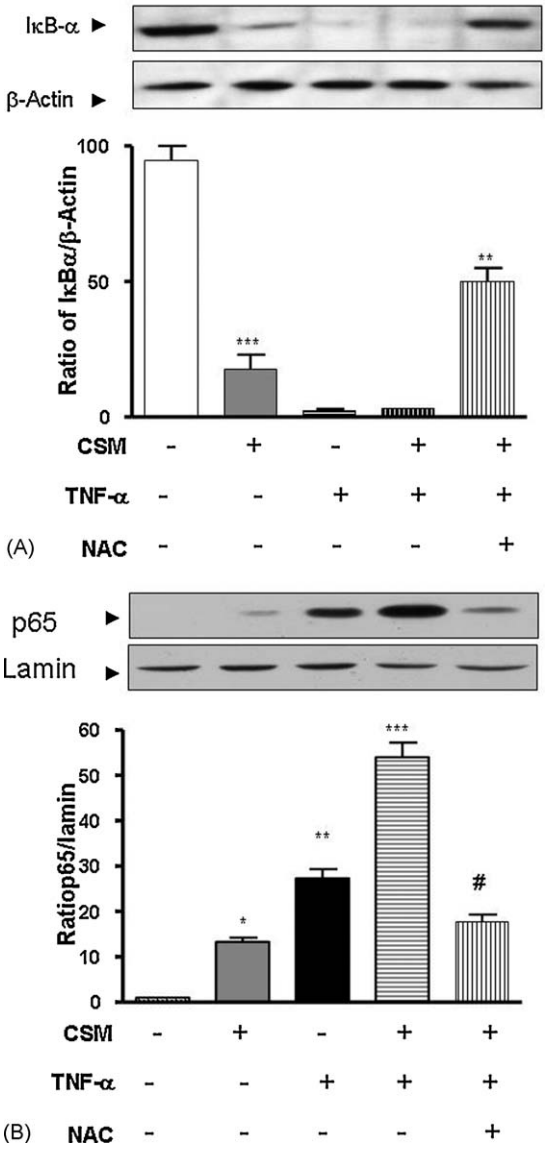

Fig. 9. Effect of CSM and TNF-α-induced degradation of IκB-α and NF-κB translocation and the inhibition by antioxidants. Cells were exposed to CSM (0.06 OD) and/or TNF-α (10 ng/ml) for 30 min in presence or absence of indicated concentrations of NAC and then nuclear extract isolated and Western blotting performed as described in Section 2 for IκB-α (A) and p65 (B). The membranes were stripped and probed with β-actin or lamin antibodies and represented as a control loading of proteins. Representative results of three independent experiments are shown. The ratios of p65 to Lamin expression from three separate gels are shown in the lower panels. Data shown are mean ± SEM of three separate experiments. The asterisks represent significant differences compared with activated cells alone ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). (#) represented significant between TNF/CEM with TNF/CEM and NAC.

and CSM increased p65 translocation. The translocation of p65 by a combination of CSM and TNF-α was effectively suppressed when cells were pretreated with NAC (10 mM) for 30 min (Fig. 9B).

4. Discussion

Cigarette smoke is a rich source of ROS and a general inducer of oxidative stress which has been associated with COPD and cardiovascular diseases [43–47]. Since exposure to cigarette smoke and TNF-α occur concomitantly in COPD patients, a combination of CSM and TNF-α was applied to macrophages which lead to a 1500% increment in the production of IL-8 by human MDM's, compared to the IL-8 production by either of the compounds alone. In the gas

phase of cigarette smoke mainly superoxide radical ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) are found which immediately react to form the highly reactive peroxynitrite ($ONOO^-$) molecule [9]. We mimicked this effect by using SIN-1 as a peroxynitrite-generating system and found that SIN-1 induces IL-8 secretion in the same manner as CSM. These findings are in agreement with previous studies which show that peroxynitrite and CSM alone increases IL-8 production [26,27,48]. The induction of IL-8 by these agents seems to be quite specific, since TNF-α levels did not change. Moreover, the combination of TNF-α and CSM/SIN-1 did not increase the TNF-α production. Further, anti-TNF-α had no effect on CSM/SIN-1-induced IL-8 production, but completely inhibited IL-8 release induced by TNF-α. IL-8 overproduction by the combinations of TNF-α with CSM or SIN-1 was also strongly suppressed by anti-TNF-α. These results indicate that TNF-α is important in the induction of the synergy.

A possible mechanism of IL-8 induction by CSM and/or TNF-α may be through reactive oxygen species. NAC and DMSO are cell membrane permeable radical scavengers that profoundly inhibited IL-8 production induced by CSM or TNF-α. The inhibition by the antioxidants was more obvious when the combination of CSM and TNF-α were used. This may indicate that the combination of CSM and TNF-α induces more ROS production (Fig. 2E). Interestingly, SOD did not inhibit the IL-8 production. In contrast to NAC and DMSO, SOD is unable to pass the cell membrane. This indicates that ROS are probably produced intracellularly.

NADPH oxidase is the main cellular source of ROS in proinflammatory cells and is involved in host defense. CSM induces $O_2^{\bullet -}$ production via NADPH oxidase in cell culture systems [49]. Furthermore, NADPH oxidase-derived H_2O_2 activates NF-κB to release cytokines and chemokines [50,51]. On the other hand, TNF-α is able to generate ROS by NADPH oxidase [52].

We cannot exclude the role of IL-1β in this study, since it has been shown that CSM and oxidative stress induced release of IL-1β which in turn could potentiate IL-8 release [53–55].

Taken together, the combination of CSM and TNF-α may account for the synergical induction of IL-8 by MDM's via the additional NADPH and ROS generation.

Furthermore, CSM and TNF-α induced a decrease in the level of GSH which was time-dependent. Short-term stimulation by the combination of CSM and TNF-α caused an additional drop of GSH. Again, these results indicate that the combination potentiates the production of ROS. The initial depletion of GSH is followed by an increase of GSH after prolonged exposure of U937 cells. This may imply that up-regulation of glutathione synthesis provides a protective or adaptive mechanism against oxidative stress. These findings are in accordance with earlier studies which showed short- and long-term effects of oxidants on GSH content [56–58]. Recent studies have demonstrated that CSM induced depletion of GSH levels is associated with an increase in ROS release [59,60]. Moreover, there is increasing evidence that TNF-α induces $O_2^{\bullet -}$ formation in mitochondria [28]. Collectively, it can be concluded that the synergy between CSM and TNF-α on IL-8 production might be mediated by the production of ROS as we demonstrated in Fig. 7.

The IL-8 gene contain cis-regulatory element for NF-κB, AP-1 and NF-IL-6 [36] and among them NF-κB plays a key role in the induced expression of IL-8 [41]. Furthermore, ROS and the cellular redox status can be involved in the activation of NF-κB which leads to the expression of proinflammatory cytokines [61,62]. Accordingly, we investigated whether CSM and/or TNF-α induce translocation of NF-κB and whether NAC inhibited this translocation. TNF-α potently induced NF-κB translocation while CSM did not cause a substantial activation of NF-κB. The combination of CSM and TNF-α potentiated the translocation of NF-κB. A similar potentiation was observed for IL-8 mRNA (Fig. 4A). Although, there was a massive synergy for IL-8 protein levels, only an additive effect was found for IL-8 mRNA and NF-κB translocation. These results may indicate that there is no direct correlation between these parameters or that the massive synergy observed at the protein level would seem to occur post-transcriptionally. The central role of oxidative stress in this process was shown by the ability of NAC to prevent NF-κB translocation. These results are in agreement with a previous report that NAC inhibited TNF-α-induced NF-κB activation [63,58]. Thus, the ability of NAC to suppress NF-κB activation indicates that an upstream signaling pathway to NF-κB is at least related to redox regulation. We believe that these results are very exciting and that this assay system will be a useful tool for exploring the effect of CSM and may lead to a greater understanding of disease pathogenesis, possible disease modifying targets and the identification of active component(s) in tobacco-smoke. In conclusion, CSM exposure of macrophages up-regulates the expression and the production of IL-8 via reactive oxygen species and NF-κB activation. Moreover, CSM dramatically enhances the production of IL-8 in combination with TNF-α. Based upon the strong synergistic action, a combination therapy directed against ROS and TNF-α could be a new approach to stop the progression in lung damage during emphysema.

Competing interests

None.

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