Molecular Immunology 111 (2019) 53–63

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Effects of extracellular Hsp70 and cigarette smoke on differentiated THP-1 cells and human monocyte-derived macrophages

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ARTICLE INFO

Keywords:
Cigarette smoke
eHsp70
Monocyte-derived macrophages
THP-1

ABSTRACT

Extracellular Hsp70 (eHsp70) can act as pro-inflammatory mediator and is elevated in blood of chronic obstructive pulmonary disease (COPD) patients. Most of those patients are smokers, and it was suggested previously that cigarette smoke might induce Hsp70 secretion from the circulating cells. Therefore, we aimed to explore inflammation-associated effects of cigarette smoke extract (CSE) and its combinations with eHsp70 in monocyte-derived macrophages (MDMs) and THP-1 cell line, used as systemic component models of COPD. We hypothesized that eHsp70 induces inflammation, but that it can also modulate cigarette smoke extract (CSE)-stimulated inflammatory responses.

We assessed IL-8 secretion, TLR2, TLR4 and Hsp70 expressions, MAPKs and NF-κB activation, and cytotoxicity after treating the cells with CSE (2.5 and 5%) and its combinations with low-endotoxin recombinant human (rh) Hsp70, used to mimic eHsp70 effects.

CSE induced IL-8 secretion from both cell types, but its combinations with rhHsp70 increased IL-8 release compared to CSE alone only from MDMs. In THP-1, combinations of rhHsp70 with 2.5% CSE induced TLR2 and TLR4 mRNA, while 5% CSE decreased TLR2 expression. In MDMs, CSE alone attenuated TLR2, while rhHsp70 increased TLR2 and lowered TLR4 gene expression. Hsp70 mRNA expression was suppressed in THP-1 with rhHsp70 and CSE; however, the same treatments increased its level in MDMs. CSE had cytotoxic effect only on MDMs, but cytotoxicity was reduced in co-treatments with rhHsp70, which also triggered apoptosis. CSE and rhHsp70 activated p38 and JNK, while ERK was activated only by rhHsp70 in MDMs. In THP-1, 2.5% CSE activated ERK, and 5% CSE activated p38. Inhibition of NF-κB and JNK in MDMs, and ERK and JNK in THP-1 cells, attenuated IL-8 release after rhHsp70 treatment.

In conclusion, rhHsp70 provoked pro-inflammatory effects and could also modulate inflammatory response to CSE on protein and gene expression levels in THP-1 cells and MDMs, which suggests that eHsp70 might be implicated in systemic inflammation induced by cigarette smoke.

1. Introduction

Heat shock proteins (Hsp) are mostly known as intracellular proteins that exert protective and anti-inflammatory effects. However, some Hsps, such as the inducible Hsp70, can be excreted into the extracellular milieu and then act as a "danger signal" for the immune system, *i.e.* as a damage-associated molecular pattern (DAMP) molecule (Asea et al., 2002). The extracellular Hsp70 (eHsp70) possesses chaperone and cytokine activity and can stimulate the immune response that leads to enhanced synthesis and secretion of pro-inflammatory cytokines. Binding of eHsp70 to Toll like receptors (TLR) 2 and 4 results in activation of nuclear factor κB (NF-κB), and mitogen-activated

Abbreviations: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; DAMP, damage-associated molecular pattern; ERK, extracellular signal-regulated kinase; eHsp70, extracellular heat shock protein 70; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MDM, monocyte-derived macrophage; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; rh, recombinant human; TLR, Toll- like receptors; TNF, tumor necrosis factor

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https://doi.org/10.1016/j.molimm.2019.04.002
Received 18 December 2018; Received in revised form 11 March 2019; Accepted 3 April 2019
Available online 11 April 2019
0161-5890/

protein kinases (MAPKs) signal transduction pathways, and consequently cytokine production. It is believed that lower concentrations of eHsp70 may be protective and be a part of normal response to infection or stress. However, above a critical threshold eHsp70 could provoke and/or potentiate dysregulated inflammatory response. It seems that its *in vivo* functions are context-dependent (Asea et al., 2002; Krause et al., 2015).

In blood of patients with chronic obstructive pulmonary disease (COPD), elevated eHsp70 levels were measured (Cui et al., 2015; Hacker et al., 2009). COPD is characterized with a progressive airflow limitation and an altered inflammatory response of the respiratory tract to different noxious particles, most commonly originating from cigarette smoke. Apart from lung manifestations of the disease, COPD has also systemic consequences characterized by chronic inflammation, oxidative stress, and immune cell activation (Agusti and Soriano, 2008).

It was previously shown that Hsp70 was elevated in lung epithelial cells of COPD patients, which could be associated to more severe disease (Dong et al., 2013). On the contrary, reduced expression of Hsp70 was found in total leukocytes and lymphocytes from COPD subjects, and this was dependent on patient's smoking status (Giuliano et al., 2011; Rumora et al., 2008).

Most of COPD patients are smokers, and it was suggested previously that cigarette smoke might induce Hsp70 secretion from the circulating cells, leading to increased eHsp70 concentrations in the blood. Therefore, in this study we explored not only single, but also combined effects of eHsp70 and cigarette smoke on human monocyte-derived macrophages (MDMs) from peripheral blood and their cell line counterpart THP-1 cells, used as systemic compartment models of COPD. We hypothesized that eHsp70 induces inflammation, but that it ccould also modulate cigarette smoke extract (CSE)-stimulated inflammatory responses. We used low-endotoxin recombinant human (rh) Hsp70, both alone and in combinations with CSE, to mimic eHsp70 effects, and we determined concentrations of pro-inflammatory cytokines secreted into cell supernatants, mRNA expressions of Hsp70, TLR2 and TLR4 as well as NF-κB and MAPKs activation.

2. Materials and methods

2.1. Cell cultures

THP-1 human monocytic cell line (American Type Culture Collection, USA) was cultured in RPMI-1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin/amphotericin B at 37 °C in a humidified 5% CO_2 atmosphere. Before the experiments, cells were seeded on 12- or 96-well plates and differentiated into macrophage-like cells with 20 nM phorbol 12-myristate 13-acetate (PMA) for 48 h.

Monocytes isolated from blood of healthy donors (Lonza Ltd., Switzerland) were seeded on 24- or 96-well plates and differentiated into macrophages with 25 ng/ml macrophage colony-stimulating factor (M-CSF) in RPMI-1640 with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin/amphotericin B and 1% non-essential amino acids for 7 days.

2.2. Treatments of cells

Cigarette smoke extract (CSE) was prepared by bubbling 2 cigarettes (Kentucky Research Cigarettes 3R4F, University of Kentucky, USA), after removing filters, through 25 ml of RPMI-1640 medium at constant rate (70 ml/min). Obtained CSE was considered as 100% and cells were treated with CSE diluted in RPMI-1640 medium as indicated. CSE was freshly prepared for each experiment and used within 1 h. Standardization of CSE was done by measuring the absorbance at 320 nm (Somborac-Bačura et al., 2018a).

Cells were treated with recombinant human (rh) Hsp70 protein, with a low endotoxin concentration (as determined by manufacturer) (Enzo Life Sciences, USA), in concentrations of 1, 3 or 10 μg/ml as well as in combination with 2.5 and 5% CSE. Equivalent amount of lipopolysaccharide (LPS) as found in rhHsp70 was used in all experiments as negative control and no difference between non-treated cells and negative controls was found.

To elucidate the signalling mechanisms involved in inflammation, cells were pre-treated with 10 μM Bay11-7082 (NF-κB inhibitor), 50 μM PD98059 (ERK inhibitor) (Calbiochem, USA), 10 μM SP600125 (JNK inhibitor) (Santa Cruz Biotechnologies, USA) or 10 μM SB202190 (p38 inhibitor) (Cell Signaling Technology, USA) for 1 h, and then 10 μg/ml rhHsp70 was added into wells and incubated for further 24 h. Three independent experiments (n = 3) were performed for all analyses.

2.3. Measurement of cytokine concentration

Cells were treated with CSE alone or in combinations with rhHsp70 for 24 h and cell-free supernatants were collected for determination of cytokine concentration using DuoSet ELISA kits (Human IL-1alpha/IL-1F1 DuoSet ELISA, Human IL-1beta/IL-1F2 DuoSet ELISA, Human IL-6 DuoSet ELISA, Human CXCL8/IL-8 DuoSet ELISA, Human TNF-alpha DuoSet ELISA; R&D Systems, USA), according to manufacturer's protocols.

2.4. Expression of TLR2, TLR4 and Hsp70 mRNA

Cells remaining after removal of cell-free supernatant for determination of cytokines (after 24 h treatment) were used for RNA isolation. Trizol/chlorophorm method (Rio et al., 2010) with Tri reagent (Applied Biosystems, USA) was used for RNA isolation. cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA), and qPCR were performed using TaqMan Gene Expression Assays (Applied Biosystems, USA): Hs02621280_s1 for tlr2; Hs00152939_m1 for tlr4; and Hs00359163_s1 for hsp70, on the 7300 Real Time PCR System (Applied Biosystems, USA). Hs99999907_m1 for β_2-microglobulin (B2M) and Hs99999904_m1 for peptidyl-prolyl isomerase A (PPIA) were used as reference genes.

2.5. Western blotting

THP-1 cells and MDMs were lysed for 20 min on ice by using lysis buffer containing 50 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium-orthovanadate, and protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche). Lysates were centrifuged at 15,000*g* for 20 min at 4 °C and protein concentration was determined by bicinchoninic acid (BCA) assay (Smith et al., 1985). Denaturation of samples was performed by boiling the samples for 5 min with 6× Laemmli sample buffer containing 0.375 M Tris-HCl pH 6.8, 12% w/v SDS, 3% v/v glycerol, 0.2% w/v bromophenol blue, 12% β-mercaptoethanol in distilled water in 5:1 ratio.

Electrophoresis of proteins (30 μg) was performed on 10% polyacrylamide gel at 100 V for 90 min, while transfer onto nitrocellulose membrane was performed at 250 mA for 90 min. Membranes were blocked with 5% skimmed milk as blocking buffer for 1 h, and then incubated with specific antibodies [anti-ERK antibody (ERK 1 (C-16), #sc-93); anti-JNK antibody (JNK (FL): #sc-571); anti-p38 antibody (p38α (C-20): #sc-53); Santa Cruz Biotechnology, USA; anti-phospho-ERK antibody (Phospho p44/42 MAPK (ERK1/2) (D13.14.4E) XP Rabbit mAb, #8544); anti-phospho-JNK antibody (Phospho-SAPK/JNK (Thr183/Tyr185), #9251); anti-phospho-p38 antibody (Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP Rabbit mAb, #4511); anti-phospho-IκBα antibody (Phospho-IκBα (Ser32/Ser36) (5A5) mouse mAb, #9246); anti-IκBα antibody (IκBα L35A5 mouse mAb, #4814); Cell Signaling Technology, USA], followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody, according to the manufacturer's protocols. Appropriate bands were detected using

chemiluminescence reagent [(5 mg luminol (Sigma-Aldrich), 1 ml 1.5 M Tris-HCl pH 8.8, 14 ml ddH_2O, 5 µl H_2O_2 and 150 µl enhancer (11 mg p-coumaric acid (Sigma-Aldrich) in 10 ml DMSO)], and photographed on Amersham AI600 Imager (GE Healthcare, USA).

2.6. Measurement of cytotoxicity

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, USA) was used for determination of metabolic activity of the cells according to manufacturer's protocol, and measured at 490 nm on a microplate reader (VICTOR3 1420 Multilabel counter, Perkin Elmer, USA).

Apoptotic caspases activities (caspase-8 and -9) were determined by the luminescent assays (Caspase-Glo 8 Assay and Caspase-Glo 9 Assay; Promega, USA).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.01 software (GraphPad Software Inc., USA). Data were tested using One-Way Analysis of Variance (ANOVA) and post hoc testing was done using Sidak method. All results are presented as the mean ± standard error of the mean (SEM). The level of $P < 0.05$ was considered statistically significant for all analyses.

For assessment of combined effects of rhHsp70 with CSE, comparison of measured values and calculated (expected) values was used (Hulina et al., 2018; Šegvić Klarić et al., 2014).

3. Results

3.1. Effects of different CSE concentrations on cytokine secretion after 24h

Smoking is recognized as being one of the most important factors in COPD development, contributing to the worsening of systemic consequences of the disease. Therefore, we first assessed the effect of CSE on secretion of pro-inflammatory cytokines. We treated THP-1 cells and MDMs with 2.5%, 5%, 10%, 15% and 20% CSE for 24 h. 2.5% and 5% CSE triggered significant IL-8 release from THP-1 cells, while 15% and 20% CSE supressed its secretion (Fig. 1a). Only 2.5% CSE had a significant impact on IL-8 concentration in MDMs (Fig. 1b). Concentrations of IL-1α, IL-1β, IL-6 and TNF-α were also measured; however, they were below detection limit for our experimental conditions in both THP-1 cells and MDMs. Therefore, 2.5% and 5% CSE were selected for further treatments of the cells in this study, and only concentrations of IL-8 were measured later.

3.2. Effects of rhHsp70 on CSE-provoked IL-8 secretion

To assess if rhHsp70 could alter CSE-induced inflammatory effect, we treated cells with combinations of CSE and 1 or 3 µg/ml rhHsp70 during 24 h, based on our preliminary results of concentration-dependent effects of rhHsp70 on secretion of IL-8 (data not shown). We found that 1 µg/ml rhHsp70 stimulated IL-8 release from THP-1 cells, while 3 µg/ml rhHsp70 stimulated IL-8 release from MDMs, compared to non-treated control cells. We assumed that it would be interesting to explore whether IL-8 stimulating and IL-8 non-stimulating rhHsp70 concentrations will modulate CSE inflammatory response differently.

In THP-1 cells, secretion of IL-8 was significantly higher after treatment with 2.5% and 5% CSE alone and in combinations of 5% CSE with rhHsp70, compared to non-treated cells. However, rhHsp70 had no significant effect in combinations compared with CSE alone (Fig. 2a). In MDMs, secretion of IL-8 was increased after treatment with 2.5% CSE alone and in combination with rhHsp70, compared to non-treated cells (Fig. 2b). Furthermore, release of IL-8 in cell medium was stimulated in 5% CSE and rhHsp70 co-treatments, compared to both non-treated cells and 5% CSE alone. Despite that, calculated combined effect for 5% CSE and 3 µg/ml rhHsp70 was antagonistic (Fig. 2c).

3.3. Effects of CSE and rhHsp70 co-treatments on cell viability

To determine possible cytotoxic effects of rhHsp70 or CSE alone and their combinations, metabolic activity of cells and mode of cell death were investigated by MTS assay and measurement of apoptotic caspase-8 and -9 activities, respectively. Cells were treated with rhHsp70, CSE or their combinations during 24 h for MTS, and during 6 and 8 h for determination of caspases' activity.

rhHsp70 and CSE alone were not cytotoxic to THP-1 cells, as assessed by MTS test, while combinations of 2.5% CSE and rhHsp70 had a slight, but significant, proliferative effect (Fig. 3a). Contrary to this, 2.5% and 5% CSE provoked strong reduction of MDM's metabolic activity. However, rhHsp70 modulated this cytotoxic effect and significantly increased cell viability compared to CSE alone (Fig. 3c). In addition, we found that calculated combined effects between 2.5% CSE and rhHsp70 were antagonistic (Fig. 3d).

In THP-1 cells, caspase-8 was significantly activated with 2.5% CSE at 6 h treatment. In addition, combinations of 2.5% CSE with 1 or 3 µg/ml rhHsp70 lead to a significant activation of caspase-8 after 6 and 8 h, compared to non-treated cells. Caspase-9 was activated with 2.5% CSE after both 6 and 8 has well as with its combinations with rhHsp70 after 6 h (Table 1). 5% CSE did not significantly affect caspase-8 and -9 activities in THP-1 cells (data not shown).

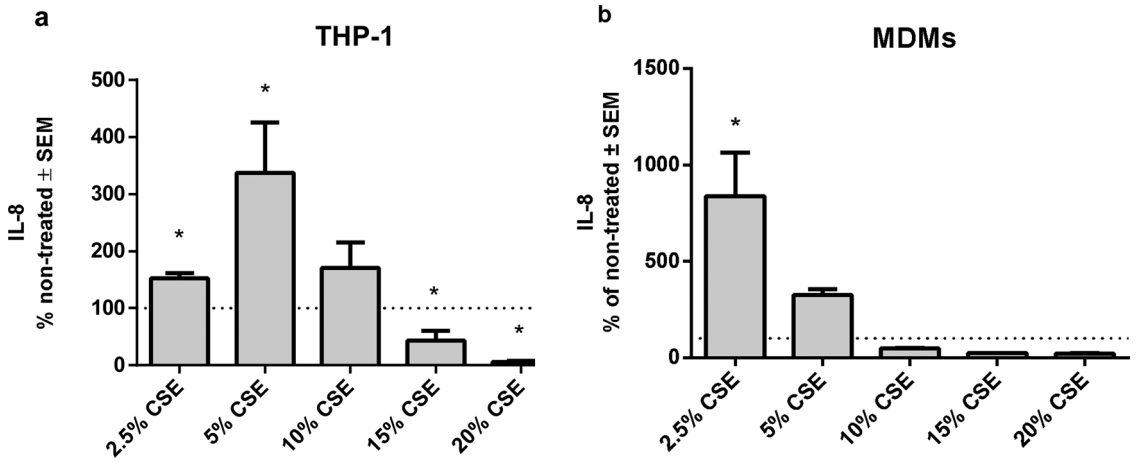

Fig. 1. Effects of different CSE concentrations on secretion of IL-8 from THP-1 cells (a) and MDMs (b). Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents IL-8 concentration secreted from non-treated THP-1 or MDM cells, set as 100%.
* statistically significant ($P < 0.05$) *vs.* non-treated cells.

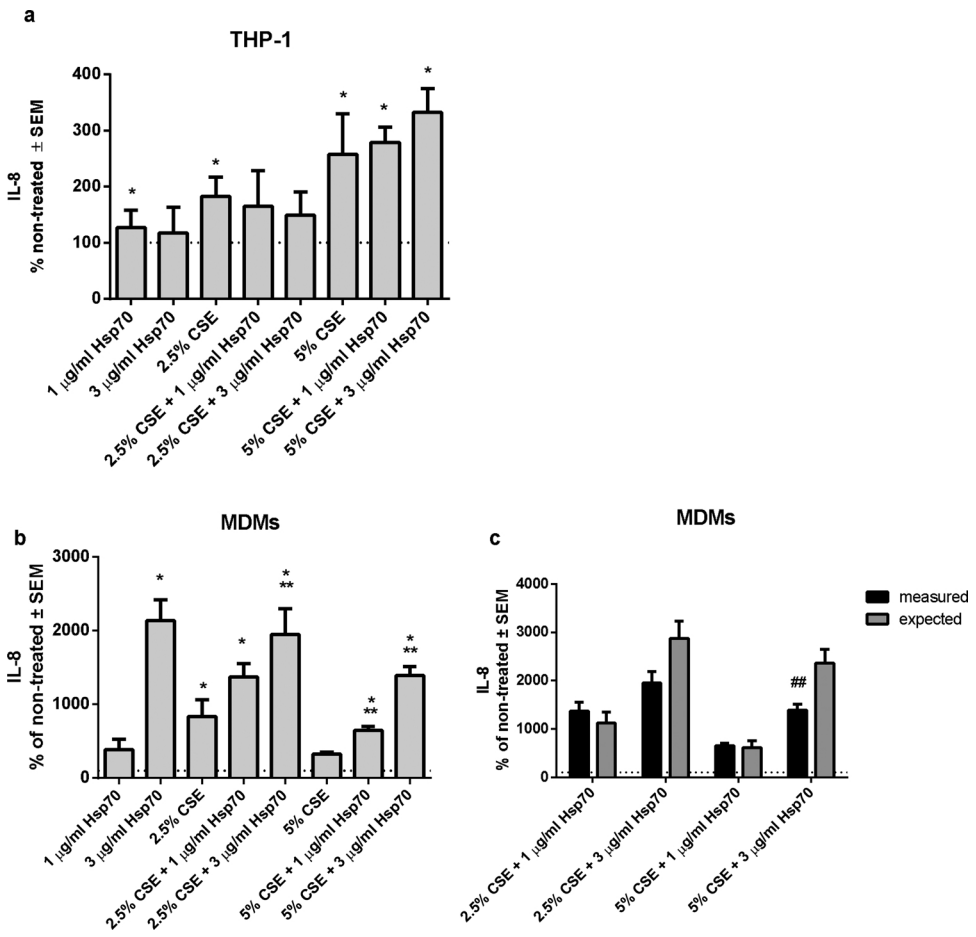

Fig. 2. Effects of rhHsp70 or CSE alone and in combinations on production of IL-8 from THP-1 cells (a) and MDMs (b). Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents IL-8 concentration secreted from non-treated THP-1 or MDM cells, set as 100%.
* statistically significant ($P < 0.05$) *vs.* non-treated cells.
** statistically significant ($P < 0.05$) *vs.* CSE alone.
rhHsp70 and CSE interactions on IL-8 secretion from MDMs (c). Dark bars represent the measured values and grey bars represent the expected (calculated) values.
statistically significant ($P < 0.05$) antagonistic effect.

In MDMs, 2.5% and 5% CSE significantly decreased caspase-9 activity after 8 h (Fig. 4c), while rhHsp70 alone did not affect its activation. However, combinations of 5% CSE and rhHsp70 significantly stimulated caspase-9 activity after 6 h, compared to non-treated cells (Fig. 4a). In addition, rhHsp70 applied together with 2.5% CSE increased caspase-9 activity at 6 and 8 h compared to 2.5% CSE alone (Fig. 4a and c), and calculated interactions were synergistic (Fig. 4b and d). Caspase-8 was not significantly activated under tested conditions in MDMs (data not shown).

3.4. Alterations in TLR2, TLR4 and Hsp70 gene expression

eHsp70 mediates its effects on cells mostly *via* receptors TLR2 and TLR4. On the other hand, intracellular Hsp70 has cytoprotective and anti-inflammatory functions, and it was shown that cell's faith might depend on a delicate balance between intracellular and extracellular Hsp70. Therefore, next we explored TLR2, TLR4 and Hsp70 gene expressions after 24 h treatments by qPCR method.

Based on our preliminary results, for gene and protein expressions as well as signalling pathways experiments, including those with specific signalling molecule inhibitors, we used the same CSE and rhHsp70 concentrations for combined treatments as for cytokine and viability investigations, but we used 3 and 10 μg/ml rhHsp70 for rhHsp70 individual treatments, as 10 μg/ml rhHsp70 induced the strongest pro-inflammatory effects (data not shown).

Compared to non-treated THP-1 cells, 2.5% CSE alone (1.66 ± 0.08 fold change) and in combinations with 1 and 3 μg/ml rhHsp70 (3.63 ± 1.52 and 3.56 ± 1.69 fold change, respectively) provoked a significant increase in TLR2 gene expression. In contrast, 5% CSE alone (0.60 ± 0.11 fold change) and in combinations with 1 and 3 μg/ml rhHsp70 (0.61 ± 0.06 and 0.58 ± 0.04 fold change, respectively) decreased TLR2 gene expression as well as 10 μg/ml rhHsp70 (0.80 ± 0.05 fold change), compared to non-treated cells (Fig. 5a).

THP-1 cells treated with 10 μg/ml rhHsp70 (0.53 ± 0.03 fold change) significantly reduced TLR4 mRNA expression, compared to non-treated cells. 2.5% CSE (3.48 ± 0.27 fold change) and combinations of 2.5% CSE with 3 μg/ml rhHsp70 (1.82 ± 0.49 fold change) as well as 5% CSE with 3 μg/ml rhHsp70 (1.38 ± 0.06 fold change) resulted in increased expression of TLR4, compared to non-treated cells. However, combinations of 2.5% CSE with rhHsp70 suppressed TLR4 gene expression compared to THP-1 cells treated only with 2.5% CSE (Fig. 5b).

Expression of Hsp70 mRNA was significantly decreased in THP-1 cells in all experimental conditions, compared to non-treated cells except when combinations of 5% CSE and rhHsp70 were applied. Combinations of 5% CSE with 1 and 3 μg/ml rhHsp70 (0.75 ± 0.06 and 0.73 ± 0.03 fold change, respectively) induced Hsp70 gene expression compared to 5% CSE alone (0.49 ± 0.04 fold change) (Fig. 6a).

In MDMs, expression of TLR2 mRNA was increased after treatments with 3 and 10 μg/ml rhHsp70 (2.59 ± 0.33 and 6.10 ± 0.50 fold change, respectively), and decreased after treatment with 2.5% and 5% CSE alone (0.31 ± 0.01 and 0.30 ± 0.12 fold change, respectively) (Fig. 5c). Combinations of 5% CSE with rhHsp70 (1.55 ± 0.21 and 1.69 ± 0.18 fold change for 1 and 3 μg/ml rhHsp70, respectively) induced expression of TLR2 compared to both non-treated cells and CSE alone, while 2.5% CSE applied together with 3 μg/ml rhHsp70 (1.33 ± 0.22 fold change) increased TLR2 expression compared to 2.5% CSE.

The level of TLR4 gene expression in MDMs was less responsive to rhHsp70 and CSE treatments (Fig. 5d). Only 10 μg/ml rhHsp70

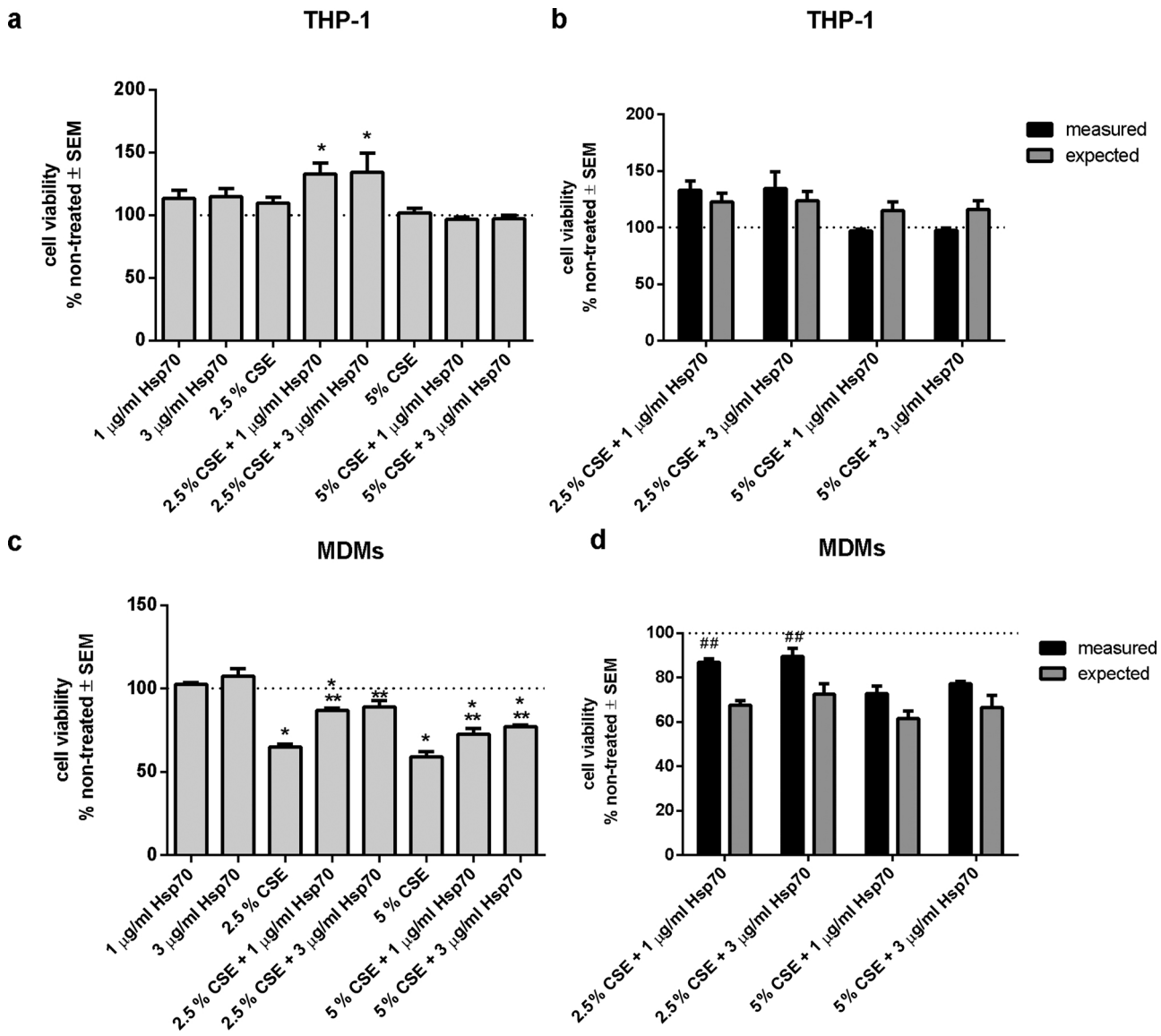

Fig. 3. Cytotoxic effects of rhHsp70 or CSE alone and in combinations on THP-1 cells (a) and MDMs (c), assessed by MTS assay. Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents viability of non-treated cells, set as 100%.
* statistically significant ($P < 0.05$) *vs*. non-treated cells.
** statistically significant ($P < 0.05$) *vs*. CSE alone.
rhHsp70 and CSE interactions on viability of THP-1 cells (b) and MDMs (d). Dark bars represent the measured values and grey bars represent the expected (calculated) values.
statistically significant ($P < 0.05$) antagonistic effect.

Table 1
Effects of rhHsp70 or 2.5% CSE alone and in combinations on caspase-8 and -9 activity in THP-1 cells treated for 6 or 8 h. Results are presented as percentage (%) of caspase activity in non-treated cells.

	caspase-8		caspase-9	
	6 h	8 h	6 h	8 h
1 μg/ml rhHsp70	125.40 ± 2.17	83.26 ± 9.46	101.60 ± 5.09	79.67 ± 7.31
3 μg/ml rhHsp70	96.67 ± 11.57	80.25 ± 7.94	96.64 ± 4.38	88.58 ± 6.74
2.5% CSE	**181.40 ± 7.29***	129.60 ± 31.93	**149.40 ± 7.63***	**124.80 ± 2.20***
+ 1 μg/ml rhHsp70	**173.20 ± 19.28***	**175.30 ± 20.80***	**162.90 ± 8.56***	123.40 ± 16.24
+ 3 μg/ml rhHsp70	**150.20 ± 2.97***	**179.50 ± 0.50***	**154.60 ± 8.59***	125.00 ± 11.24

* Statistically significant ($P < 0.05$) *vs*. non-treated cells.

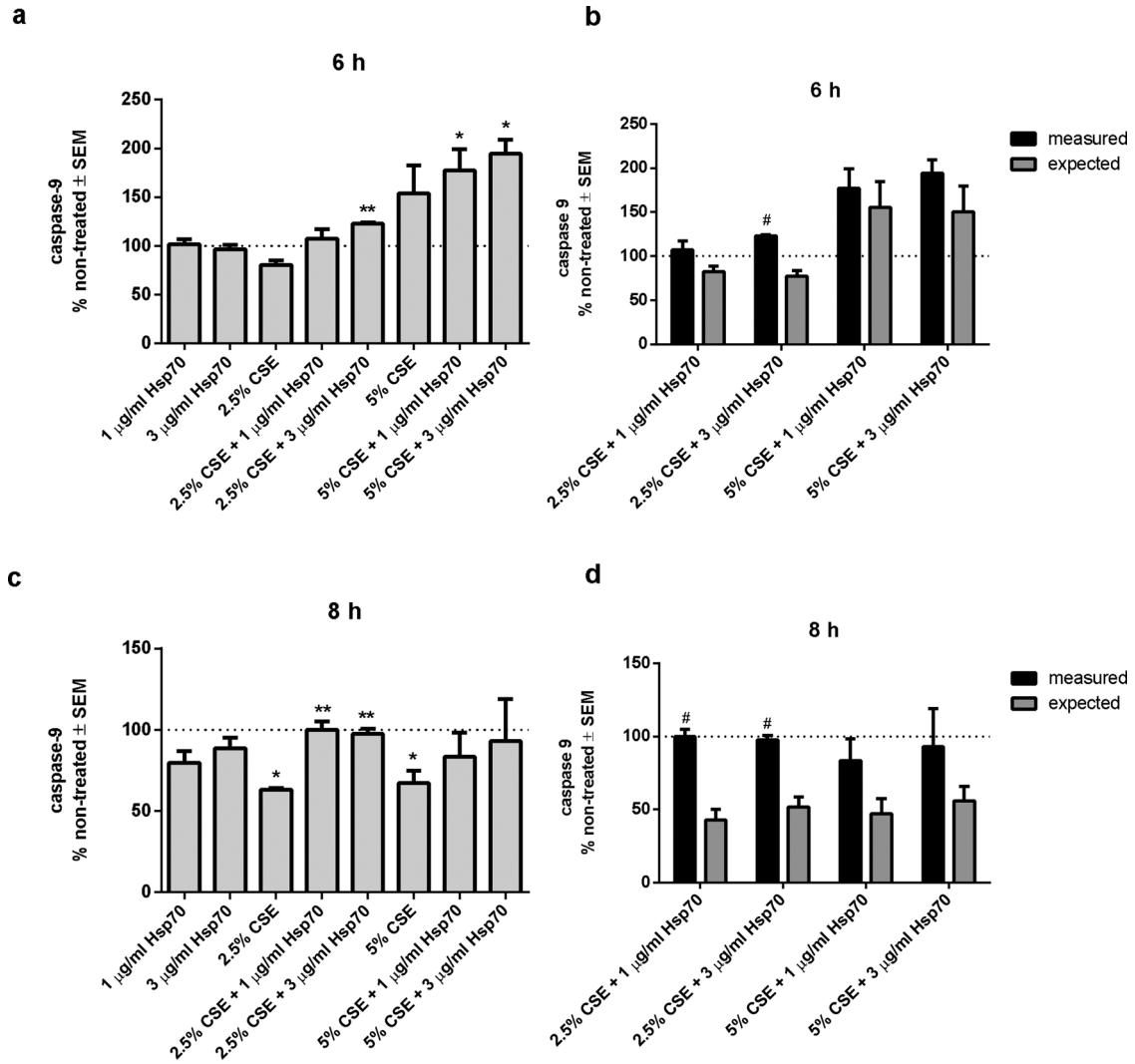

Fig. 4. Effects of rhHsp70 or CSE alone and in combinations on caspase-9 activity in MDMs treated for 6 h (a) or 8 h (c). Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents caspase-9 activity in non-treated MDMs, set as 100%.
* statistically significant ($P < 0.05$) *vs.* non-treated cells.
** statistically significant ($P < 0.05$) *vs.* CSE alone.
rhHsp70 and CSE interactions on caspase-9 activity (b, d). Dark bars represent the measured values and grey bars represent the expected (calculated) values.
statistically significant ($P < 0.05$) synergistic effect.

attenuated expression of TLR4 mRNA (0.66 ± 0.15 fold change), and combination of 5% CSE with 3 μg/ml rhHsp70 increased its expression (1.63 ± 0.31 fold change), compared to non-treated cells.

Regarding the Hsp70 gene expression in MDMs, it was strongly induced by 10 μg/ml rhHsp70 (2.73 ± 0.89 fold change), 2.5% and 5% CSE (5.63 ± 1.56 and 167.20 ± 78.67 fold change, respectively), while combinations of CSE with rhHsp70 suppressed the expression of Hsp70 mRNA compared to CSE alone (Fig. 6b).

3.5. Effects of CSE and rhHsp70 on MAPKs and NF-κB signalling pathways

Binding of eHsp70 to TLR2 and TLR4 results in the stimulation of NF-κB and MAPK signalling pathways leading to the synthesis of pro-inflammatory cytokines. Expression and activation of NF-κB (presented through detection of total IκBα and phospho-IκBα, respectively) and major MAPKs (ERK1/2, JNK1/2 and p38) were investigated by Western blot analysis.

THP-1 cells were treated with rhHsp70 and CSE alone as well as with their combinations for 30 min, 2 and 8 h. Due to a more restrictive possibilities in obtaining large number of primary cells for research purposes, we did not perform combined treatments in MDMs for Western blotting only.

In THP-1 cells, co-treatment with 2.5% CSE and rhHsp70 decreased ERK activation after 30 min, but 2.5% CSE alone and in combination with rhHsp70 activated ERK after 8 h. In addition, 2.5% CSE applied together with rhHsp70 activated p38 after 30 min (data not shown). On the other hand, 5% CSE and its combinations with 1 and 3 μg/ml rhHsp70 caused significant activation of p38 MAPK at all time points examined, compared to untreated cells (Fig. 7). We did not detect JNK and IκBα activation under our treatment conditions.

In MDMs (Fig. 8), 3 μg/ml rhHsp70 activated ERK after 8 h, p38 after 30 min and 2 h, and JNK at all time points examined. Similarly, 10 μg/ml rhHsp70 caused specific phosphorylation of ERK after 2 and 8 h, and JNK and p38 after 8 h.

On the other hand, both 2.5 and 5% CSE induced JNK activation at 2 h and 8 h, and p38 MAPK at 30 min, 2 h and 8 h. Once again, we could not detect IκBα activation.

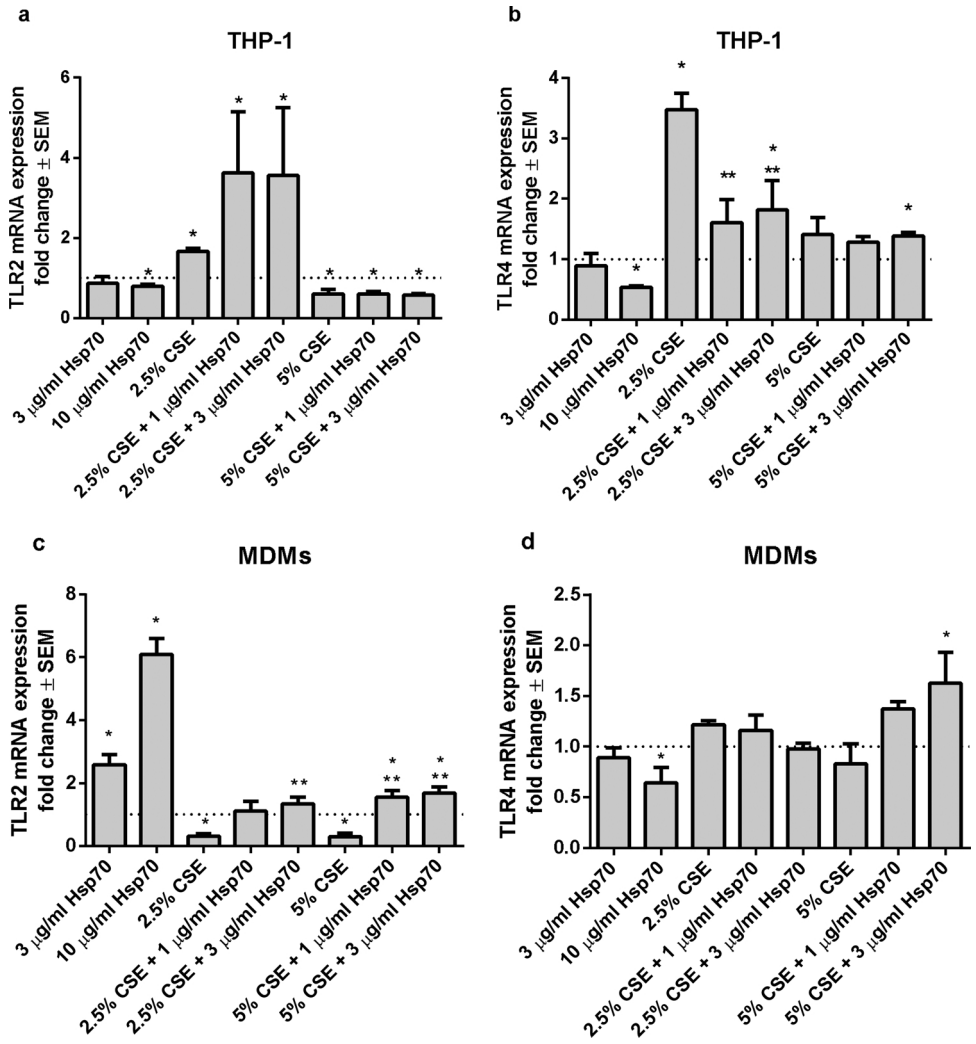

Fig. 5. TLR2 (a, c) and TLR4 (b, d) mRNA expression in THP-1 cells and MDMs treated with rhHsp70, CSE or their combinations. Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents expression of mRNA in non-treated cells, set as 1.
* statistically significant ($P < 0.05$) *vs.* non-treated cells.
** statistically significant ($P < 0.05$) *vs.* CSE alone.

3.6. Influence of MAPKs and NF-κB inhibitors on inflammatory response to rhHsp70

To investigate the mechanism underlying the pro-inflammatory effects of eHsp70 in THP-1 cells and MDMs, we used inhibitors for NF-κB (BAY11-7082) as well as for ERK (PD98059), JNK (SP600125) and p38 MAPK (SB202190). Cells were pre-treated with specific inhibitors for 1 h before adding 10 μg/ml rhHsp70 for 24 h.

In THP-1 cells, ERK and JNK inhibitors significantly suppressed IL-8 release when combined with rhHsp70, compared to treatment with rhHsp70 alone (Fig. 9a).

In MDMs, NF-κB inhibitor almost completely attenuated secretion of IL-8 and JNK inhibitor significantly reduced IL-8 secretion after 24 h incubation with rhHsp70, compared to treatment with rhHsp70 alone (Fig. 9b).

4. Discussion

Systemic component of COPD is characterized, among other things, with chronic inflammation, activation of immune cells and elevated concentration of inflammatory cytokines in the blood (Agusti et al., 2010; Agusti and Soriano, 2008). High concentrations of IL-1β, IL-6, IL-8 and TNF-α were found in the sputum of patients in stable phase of COPD (Keatings et al., 1996). In addition, increased release of pro-inflammatory cytokines IL-1, IL-8 and TNF-α as well as anti-inflammatory cytokine IL-10 from alveolar macrophages from smokers and patients with COPD was detected (Chung, 2001). "Systemic inflammome" defined as elevated concentrations of general inflammatory markers as well as IL-6, IL-8 and TNF-α was observed in 16% of COPD patients (Agusti et al., 2010; Agusti and Sin, 2014; Kelly et al., 2013). In addition, increased production of cytokines is associated with exposure of different cell lines to CSE (Dong et al., 2013; Heijink et al., 2015; Overbeek et al., 2013; Pace et al., 2014). Based on previous literature data, in this study we assumed that CSE would have inflammatory effect on THP-1 cells and MDMs, and we hypothesized that the addition of rhHsp70 would lead to a more pronounced pro-inflammatory effect of CSE on those cells.

CSE alone induced IL-8 secretion from THP-1 cells and MDMs, which is consistent with previously published studies (Kode et al., 2006; Walters et al., 2005). Furthermore, in THP-1 cells, combinations of CSE and rhHsp70 did not lead to a different production of IL-8 compared to CSE alone. Contrary to this, in MDMs, CSE applied together with rhHsp70 caused significantly higher IL-8 secretion, which could be due to increased TLR2 gene expression found in MDMs after treatment with combination of CSE and rhHsp70. However, IL-8 increase was not as high as expected and those combined effects were antagonistic. As far

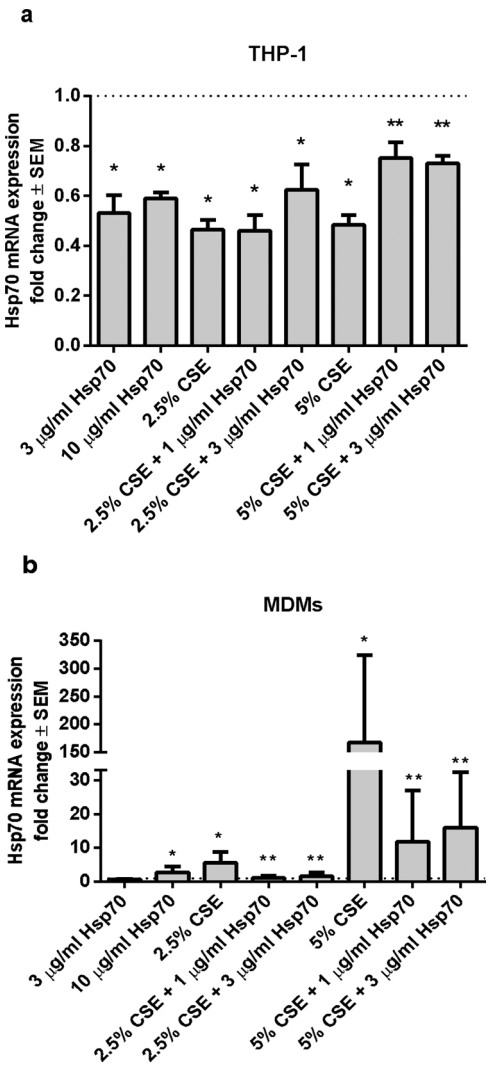

Fig. 6. Hsp70 mRNA expression in THP-1 cells (a) and MDMs (b) treated with rhHsp70, CSE or their combinations. Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents expression of mRNA in non-treated cells, set as 1.
* statistically significant ($P < 0.05$) *vs.* non-treated cells.
** statistically significant ($P < 0.05$) *vs.* CSE alone.

as we are aware, there are no published data about simultaneous application of CSE and rhHsp70 on these cells.

TLR2 and TLR4 receptors are expressed on epithelial cells of the respiratory tract, alveolar macrophages, monocytes, and neutrophils (Zuo et al., 2015). Their stimulation results in the activation of MyD88/IRAK/NF-κB and MAPK signalling pathways. Therefore, we determined gene expression of TLR2 and TLR4, and protein expression and activation of MAPKs and NF-κB after treatment of the cells with CSE, rhHsp70, and combinations of CSE with rhHsp70.

In both THP-1 cells and MDMs, TLR2 was more susceptible to CSE, whether applied alone or together with rhHsp70, but TLR2 and TLR4 expressions strongly depended on type of cells and concentrations of

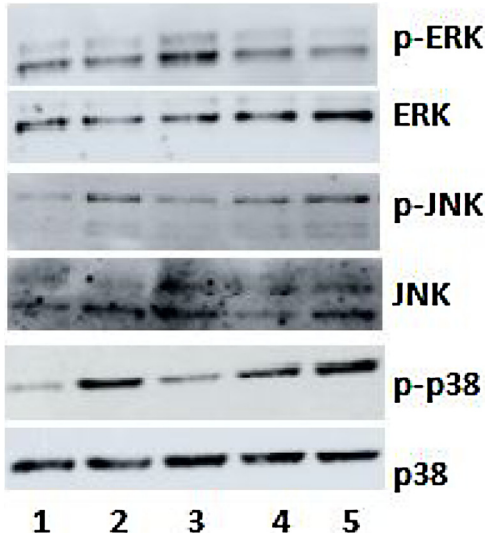

Fig. 8. Expression and activation of MAPKs in MDMs treated with rhHsp70 or CSE for 2 h.
(1) non-treated; (2) 3 μg/ml rhHsp70; (3) 10 μg/ml rhHsp70; (4) 2.5% CSE; (5) 5% CSE.

both CSE and rhHsp70. Furthermore, increase in IL-8 secretion in MDMs could be associated with increase in TLR2 expression after treatment with rhHsp70 or its combinations with CSE. However, in THP-1 those effects do not seem to be related to either TLR2 or TLR4 expressions and could be mediated through some other eHsp70 receptor (*e.g.* RAGE), which might be a point of interest in our future research.

It was previously demonstrated that exposure to cigarette smoke can activate TLR4 in mouse and human cells. Cigarette smoke could act directly on TLR4 receptors or indirectly causing damage of epithelial cells and release of DAMP molecules which subsequently bind to TLR4 (Opitz et al., 2010). It was shown that cigarette smoke exposure correlated with expression of TLR4 and TLR9 as well as with cytokine synthesis, particularly IL-8 (Dong et al., 2013; Zuo et al., 2015). Furthermore, CSE increased the expression of TLR2 and TLR4 within and on the surface of bone marrow cells (Zhou et al., 2011). In macrophages of monocytic origin, short-term exposure to CSE suppressed expression of TLR4 on cell membrane (Sarir et al., 2009). Expression of TLR4 was also reduced in nasal epithelial cells from smokers and patients with severe COPD, compared to healthy non-smokers and patients with mild COPD (MacRedmond et al., 2007). In contrast, Metcalfe et al. showed that CSE did not change the expression of TLR2 and TLR4 in alveolar macrophages isolated from COPD patients (Metcalfe et al., 2014).

Important point to consider is the cell viability after treatment with CSE and rhHsp70. It was shown previously that CSE concentration, duration of the treatment and cell type have an impact on cell death pattern caused by cigarette smoke (Kim et al., 2008; Somborac-Bačura et al., 2018b; van der Toorn et al., 2007). In our study, CSE had a significant cytotoxic effect only on MDMs, and it was reduced when rhHsp70 was added together with CSE, compared to the cytotoxicity of CSE alone. Previous research showed inconsistent results regarding the influence of CSE on cell viability. Seehasee et al. showed that lower

Fig. 7. Expression of total p38 and activated p-p38 in THP-1 cells treated with 5% CSE alone and in combinations with rhHsp70 for 30 min (a), 2 h (b) and 8 h (c).
(1) non-treated; (2) 5% CSE; (3) 5% CSE + 1 μg/ml rhHsp70; (4) 5% CSE + 3 μg/ml rhHsp70.

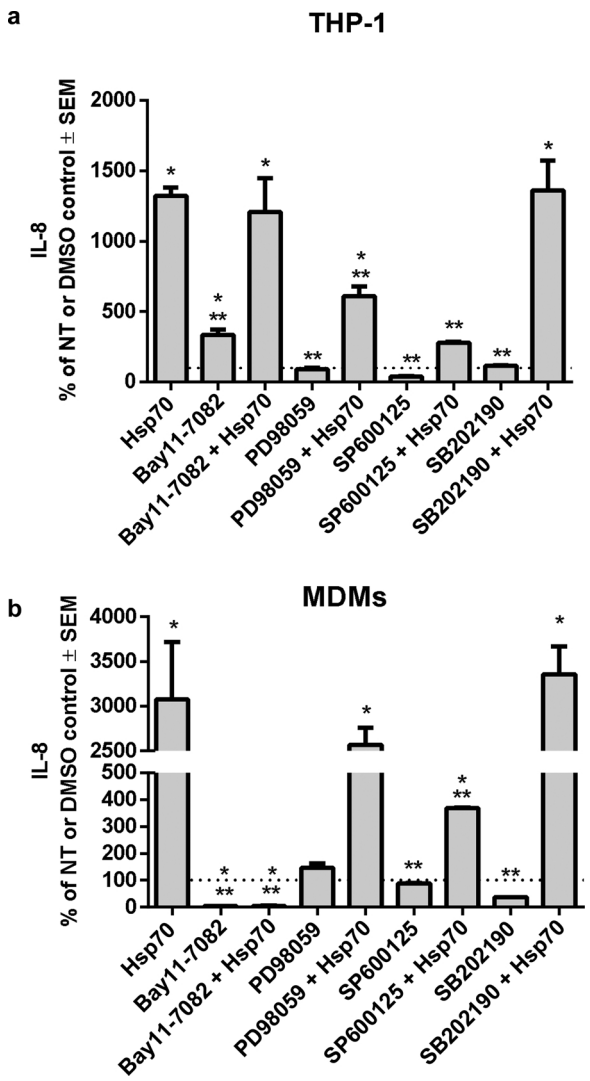

Fig. 9. Secretion of IL-8 from THP-1 cells (a) and MDMs (b) pretreated with inhibitors of NF-κB (BAY11-7082), ERK (PD98059), JNK (SP600125), or p38 MAPK (SB202190) for 1 h, and treated with 10 μg/ml rhHsp70 during 24 h. Data are presented as mean ± SEM of three independent experiments (n = 3). Dotted line represents concentration of IL-8 secreted from appropriate control cells (non-treated or treated with DMSO), set as 100%.
* statistically significant ($P < 0.05$) *vs*. non-treated cells.
** statistically significant ($P < 0.05$) *vs*. rhHsp70 alone.

concentration of CSE are cytotoxic to A549 cells (Seehase et al., 2014). However, even proliferative effect of lower CSE concentrations was observed in the same cell line (Kaushik et al., 2008), which is similar to our results with 2.5% CSE and rhHsp70 co-treatments in THP-1 cells. On the other hand, elevated concentrations of CSE and longer exposure time resulted in apoptosis of A549 cells (Kaushik et al., 2008; Li et al., 2013; Somborac-Bačura et al., 2018b). Lower concentrations of IL-8 and expression of TLR2 in MDMs treated with CSE found in our study could potentially be due to lower cell viability. Although we explored cell viability by MTS assay and measurement of apoptotic caspase-8 and -9 activities, different well format, number of cells, cell density and CSE volume were used for those analyses, which could have a significant effect on the final results. Therefore, it could be worthwhile to assess cell viability in the same wells from which IL-8 concentration and gene expressions were tested in the future experiments.

TLR2 and TLR4 activation could also trigger apoptosis, which is one of the mechanisms that can lead to disease development or progression (Vanden Berghe et al., 2014). Caspases have a central role in apoptosis and caspases' cascade could be activated through death receptor pathway (for caspases-2, -8 and -10) or mitochondrial pathway (specific for initiator caspase-9) (Elmore, 2007). In this research, 2.5% CSE alone and in combination with rhHsp70 activated both apoptotic pathways (caspases-8 and -9 activation) in THP-1 cells, while only mitochondrial pathway was activated in MDMs after co-treatments with 5% CSE and rhHsp70. Although it was shown that eHsp70 may act anti-apoptotic in human macrophage cell line U937 (Franco et al., 2016), it is possible that in human monocytic cell line THP-1 and in peripheral blood monocytes, both differentiated into macrophages, activation of the apoptotic machinery does occur.

Immunomodulatory effects of Hsp70 might be inflammatory or anti-inflammatory, which depends on total inflammatory context, concentration ratio of intracellular and extracellular Hsp70 as well as on the type of cell (Giuliano et al., 2011; Krause et al., 2015). In our study, CSE and rhHsp70 alone significantly increased (in MDMs) or decreased (in THP-1) Hsp70 mRNA. However, CSE in combination with rhHsp70 produced opposite effect. Hsp70 has protective effect on the cells, and we could speculate that cells exposed to cigarette smoke could try to compensate the damage by increasing Hsp70 production. Indeed, the largest increase in Hsp70 mRNA was observed in MDMs after treatment with 5% CSE, the same condition where cell viability was decreased.

Previously, inconsistent results were obtained about influence of cigarette smoke on intracellular Hsp70 expression. Reduced expressions of Hsp70 and Hsp27 proteins in total leukocytes of COPD smokers and ex-smokers compared to non-smokers with COPD as well as in healthy smokers compared to healthy ex-smokers or non-smokers were observed (Rumora et al., 2008). Zhao et al. showed that expression of Hsp70 protein and mRNA were decreased in lymphocytes of COPD patients compared to healthy subjects, and they suggested that it was probably due to Hsp70 gene transcription regulation disorders in COPD (Zhao et al., 2005). However, Dong et al. showed the increased expression of Hsp70 mRNA and protein in lung tissues isolated from COPD patients, and the level of Hsp70 expression correlated with the severity of the disease (Dong et al., 2013). Also, CSE induced Hsp70 protein in pulmonary fibroblasts, depending on concentration and time (Li et al., 2007).

The inflammation, together with proliferation, differentiation and cell death, is a possible consequence of activation of MAPK signalling pathways due to a cell response to a stressful stimulation (Rumora and Žanić Grubišić, 2008). In addition, NF-κB transcription factor also regulates different cellular processes like inflammation, immune system activation, cell proliferation and apoptosis. NF-κB is present in cells in its inactive form, by forming a complex with IκB inhibitory protein, and released by phosphorylation of IκBα on Ser32 and Ser36, and translocated into the nucleus (Moynagh, 2005). It was shown previously that exposure of different kind of cells, *e.g.* epithelial and endothelial airway cells, and alveolar macrophages to cigarette smoke resulted in MAPK activation, and activation of various transcription factors such as NF-κB and AP-1 (Kode et al., 2006; Manzel et al., 2011; Pace et al., 2014).

In MDMs, CSE and rhHsp70 activated both p38 and JNK, while ERK was activated only by rhHsp70. In THP-1 cells, lower concentration of CSE activated ERK, while higher CSE concentration activated p38. We did not detect differences in MAPK activation between combined treatments and treatment with CSE alone. In general, Western blot method is not sensitive enough to detect minor differences in the activation of the signalling pathways. Although we could not detect activation of IκBα in tested experimental conditions, it cannot be asserted that activation of the NF-κB pathway does not occur, *e.g.* at different time-points, as inhibition of NF-κB in MDMs completely attenuated IL-8 release after rhHsp70 treatment. Besides NF-κB signalling pathway, JNK was shown to be involved in IL-8 secretion in MDMs by the inhibition experiments, while underlying mechanism of IL-8 secretion after rhHsp70 treatment in THP-1 cells included both ERK and JNK activation. These results are consistent with some previously obtained results that showed that eHsp70 can activate MAPK and/or NF-κB

signalling pathways, depending on the cell type (Somensi et al., 2017; Zhe et al., 2016).

In conclusion, our study demonstrates that rhHsp70 provokes pro-inflammatory effects and it might also modulate the inflammatory response to CSE in THP-1 cells and human MDMs on protein and gene expression levels. Those effects are exhibited *via* MAPK and NF-κB signalling pathways. The results suggest that extracellular Hsp70 might be implicated in systemic inflammation induced by cigarette smoke. When both extracellular Hsp70 and cigarette smoke components are present in the blood of *e.g.* COPD patients, most of which are smokers, their interactions could provoke a modulation of systemic inflammation by either increasing it or trying to dampen it, which could lead to inappropriate inflammatory responses. This could be of a potential danger especially during disease exacerbations, when the immune system is confronted with some potent noxious stimuli, such as bacteria or viruses. Therefore, this confirms once again that smoking cessation is of upmost importance for COPD patients; however, eHsp70-targeted strategies might have some beneficial effects as well, and it could be interesting to explore those in the years to come.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work has been fully supported by the Croatian Science Foundation under the project number IP-2014-09-1247.

References

Agusti, A., Sin, D.D., 2014. Biomarkers in COPD. Clin. Chest Med. 35 (1), 131–141. https://doi.org/10.1016/j.ccm.2013.09.006.

Agusti, À., Soriano, J.B., 2008. COPD as a systemic disease. COPD J. Chronic Obstr. Pulm. Dis. 5 (2), 133–138. https://doi.org/10.1080/15412550801941349.

Agusti, A., Calverley, P.M., Celli, B., Coxson, H.O., Edwards, L.D., Lomas, D.A., MacNee, W., Miller, B.E., Rennard, S., Silverman, E.K., Tal-Singer, R., Wouters, E., Yates, J.C., Vestbo, J., 2010. Characterisation of COPD heterogeneity in the ECLIPSE cohort. Respir. Res. 11 (122). https://doi.org/10.1186/1465-9921-11-122.

Asea, A., Rehli, M., Kabingu, E., Boch, Ja, Baré, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K., 2002. Novel signal transduction pathway utilized by extracellular HSP70. J. Biol. Chem. 227 (17), 15028–15034. https://doi.org/10.1074/jbc.M200497200.

Chung, K.F., 2001. Cytokines in chronic obstructive pulmonary disease. Eur. Respir. J. (Suppl. 34), 50s–59s. https://doi.org/10.1183/09031936.01.00229701.

Cui, X., Xing, J., Liu, Y., Zhou, Y., Luo, X., Zhang, Z., Han, W., Wu, T., Chen, W., 2015. COPD and levels of Hsp70 (HSPA1A) and Hsp27 (HSPB1) in plasma and lymphocytes among coal workers: a case-control study. Cell Stress Chaperones 20, 473–481. https://doi.org/10.1007/s12192-015-0572-5.

Dong, J., Guo, L., Liao, Z., Zhang, M., Zhang, M., Wang, T., Chen, L., Xu, D., Feng, Y., Wen, F., 2013. Increased expression of heat shock protein 70 in chronic obstructive pulmonary disease. Int. Immunopharmacol. 17, 885–893. https://doi.org/10.1016/j.intimp.2013.09.003.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516. https://doi.org/10.1080/01926230701320337.

Franco, L., Terrinca, J., Rodríguez, A.B., Espino, J., Pariente, J.A., 2016. Extracellular heat shock proteins protect U937 cells from H2O2-induced apoptotic cell death. Mol. Cell. Biochem. 412, 19–26. https://doi.org/10.1007/s11010-015-2604-y.

Giuliano, J.S., Lahni, P.M., Wong, H.R., Wheeler, D.S., 2011. Extracellular heat shock proteins: alarmins for the host immune system. Open Inflamm. Journal 4 (Suppl 1-M6), 49–60. 1–12. https://doi.org/papers3://publication/uuid/3291970D-EF92-4616-A205-A2D831850B86.

Hacker, S., Lambers, C., Hoetzenecker, K., Pollreisz, A., Aigner, C., Lichtenauer, M., Mangold, A., Niederpold, T., Zimmermann, M., Taghavi, S., Klepetko, W., Ankersmit, H.J., Hacker, S., Lambers, C., 2009. Elevated HSP27, HSP70 and HSP90α in chronic obstructive pulmonary disease: markers for immune activation and tissue destruction. Clin. Lab. 55, 31–40.

Heijink, I.H., Pouwels, S.D., Leijendekker, C., De Bruin, H.G., Zijlstra, G.J., Van Der Vaart, H., Ten Hacken, N.H.T., Van Oosterhout, A.J.M., Nawijn, M.C., Van Der Toorn, M., 2015. Cigarette smoke-induced damage-associated molecular pattern release from necrotic neutrophils triggers proinflammatory mediator release. Am. J. Respir. Cell Mol. Biol. 52, 554–562. https://doi.org/10.1165/rcmb.2013-0505OC.

Hulina, A., Grdić Rajković, M., Jakšić Despot, D., Jelić, D., Dojder, A., Čepelak, I., Rumora, L., 2018. Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells. Cell Stress Chaperones 23, 373–384. https://doi.org/10.1007/s12192-017-0847-0.

Kaushik, G., Kaushik, T., Khanduja, S., Pathak, C.M., Khanduja, K.L., 2008. Cigarette smoke condensate promotes cell proliferation through disturbance in cellular redox homeostasis of transformed lung epithelial type-II cells. Cancer Lett. 270, 120–131. https://doi.org/10.1016/j.canlet.2008.04.039.

Keatings, V.M., Collins, P.D., Scott, D.M., Barnes, P.J., 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am. J. Respir. Crit. Care Med. 153, 530–534. https://doi.org/10.1164/ajrccm.153.2.8564092.

Kelly, E., Owen, Ca, Pinto-Plata, V., Celli, B.R., 2013. The role of systemic inflammatory biomarkers to predict mortality in chronic obstructive pulmonary disease. Expert Rev. Respir. Med. 7, 57–64. https://doi.org/10.1586/ers.12.82.

Kim, H.P., Wang, X., Chen, Z.H., Lee, S.J., Huang, M.H., Wang, Y., Ryter, S.W., Choi, A.M., 2008. Autophagic proteins regulate cigarette smoke-induced apoptosis: protective role of heme oxygenase-1. Autophagy 4, 887–895 https://doi.org/6767 [pii].

Kode, A., Yang, S.-R., Rahman, I., 2006. Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. Respir. Res. 7 (132). https://doi.org/10.1186/1465-9921-7-132.

Krause, M., Heck, T.G., Bittencourt, A., Scomazzon, S.P., Newsholme, P., Curi, R., Homem De Bittencourt, P.I., 2015. The chaperone balance hypothesis: the importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the implications for clinical management. Mediators Inflamm. 2015, 249205. https://doi.org/10.1155/2015/249205.

Li, C.-J., Ning, W., Matthay, Ma, Feghali-Bostwick, Ca, Choi, A.M.K., 2007. MAPK pathway mediates EGR-1-HSP70-dependent cigarette smoke-induced chemokine production. Am. J. Physiol. Lung Cell Mol. Physiol. 292, L1297–303. https://doi.org/10.1152/ajplung.00194.2006.

Li, X., Peng, B., Nie, C., Shang, P., Liu, H., 2013. Evaluation of the cytotoxicity of cigarette smoke total particulate matter using three in vitro assays and two types of cells. Toxicol. Mech. Methods 23, 240–246. https://doi.org/10.3109/15376516.2012.743640.

MacRedmond, R.E., Greene, C.M., Dorscheid, D.R., McElvaney, N.G., O'Neill, S.J., 2007. Epithelial expression of TLR4 is modulated in COPD and by steroids, salmeterol and cigarette smoke. Respir. Res. 8 (84). https://doi.org/10.1186/1465-9921-8-84.

Manzel, L.J., Shi, L., O'Shaughnessy, P.T., Thorne, P.S., Look, D.C., 2011. Inhibition by cigarette smoke of nuclear factor-κB-dependent response to bacteria in the airway. Am. J. Respir. Cell Mol. Biol. 44, 155–165. https://doi.org/10.1165/rcmb.2009-0454OC.

Metcalfe, H.J., Lea, S., Hughes, D., Khalaf, R., Abbott-Banner, K., Singh, D., 2014. Effects of cigarette smoke on Toll-like receptor (TLR) activation of chronic obstructive pulmonary disease (COPD) macrophages. Clin. Exp. Immunol. 176, 461–472. https://doi.org/10.1111/cei.12289.

Moynagh, P.N., 2005. The NF-kappaB pathway. J. Cell. Sci. 118, 4589–4592. https://doi.org/10.1242/jcs.02579.

Opitz, B., Van Laak, V., Eitel, J., Suttorp, N., 2010. Innate immune recognition in infectious and noninfectious diseases of the lung. Am. J. Respir. Crit. Care Med. 181 (12), 1294–1309. https://doi.org/10.1164/rccm.200909-1427SO.

Overbeek, S.A., Braber, S., Koelink, P.J., Henricks, P.A.J., Mortaz, E., LoTam Loi, A.T., Jackson, P.L., Garssen, J., Wagenaar, G.T.M., Timens, W., Koenderman, L., Blalock, J.E., Kraneveld, A.D., Folkerts, G., 2013. Cigarette smoke-induced collagen destruction; key to chronic neutrophilic airway inflammation? PLoS One 8. https://doi.org/10.1371/journal.pone.0055612.

Pace, E., Ferraro, M., Di Vincenzo, S., Gerbino, S., Bruno, A., Lanata, L., Gjomarkaj, M., 2014. Oxidative stress and innate immunity responses in cigarette smoke stimulated nasal epithelial cells. Toxicol. In Vitro 28, 292–299. https://doi.org/10.1016/j.tiv.2013.11.004.

Rio, D.C., Ares, M., Hannon, G.J., Nilsen, T.W., 2010. Purification of RNA using TRIzol (TRI Reagent). Cold Spring Harb. Protoc. 5. https://doi.org/10.1101/pdb.prot5439.

Rumora, L., Milevoj, L., Popović-Grle, S., Barišić, K., Grubišić, T.Ž., Čepelak, I., 2008. Reduction in peripheral blood leukocyte heat shock proteins 27 and 70 expression in chronic obstructive pulmonary disease. Croat. Chem. Acta 81.

Rumora, L., Žanić Grubišić, T., 2008. Kinase signalling cascades in cell proliferation and apoptosis. In: Markotić, A. (Ed.), Biochemistry and Immunology Intersections. Research Signpost, Trivandrum, pp. 151–171.

Sarir, H., Mortaz, E., Karimi, K., Kraneveld, A.D., Rahman, I., Caldenhoven, E., Nijkamp, F.P., Folkerts, G., 2009. Cigarette smoke regulates the expression of TLR4 and IL-8 production by human macrophages. J. Inflamm. (Lond) 6 (12). https://doi.org/10.1186/1476-9255-6-12.

Seehase, S., Baron-Luehr, B., Kugler, C., Vollmer, E., Goldmann, T., 2014. Alveolar epithelial cells type II show a high sensitivity to cigarette smoke extract. Pneumologie. pp. 68–A11.

Šegvić Klarić, M., Medić, N., Hulina, A., Žanić Grubišić, T., Rumora, L., 2014. Disturbed Hsp70 and Hsp27 expression and thiol redox status in porcine kidney PK15 cells provoked by individual and combined ochratoxin A and citrinin treatments. Food Chem. Toxicol. 71, 97–105. https://doi.org/10.1016/j.fct.2014.06.002.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85. https://doi.org/10.1016/0003-2697(85)90442-7.

Somborac-Bačura, A., Popović-Grle, S., Zovko, V., Žanić-Grubišić, T., 2018a. Cigarette smoke induces activation of polymorphonuclear leukocytes. Lung 196, 27–31. https://doi.org/10.1007/s00408-017-0077-3.

Somborac-Bačura, A., Rumora, L., Novak, R., Rašić, D., Dumić, J., Čepelak, I., Žanić-Grubišić, T., 2018b. Differential expression of HSPs and activation of MAPKs in A549 alveolar epithelial cells exposed to cigarette smoke extract. Exp. Physiol. 103 (12), 1666–1678. https://doi.org/10.1113/EP087038.

Somensi, N., Brum, P.O., de Miranda Ramos, V., Gasparotto, J., Zanotto-Filho, A., Rostirolla, D.C., da Silva Morrone, M., Moreira, J.C.F., Pens Gelain, D., 2017. Extracellular HSP70 activates ERK1/2, NF-kB and pro-inflammatory gene transcription through binding with RAGE in A549 human lung Cancer cells. Cell. Physiol. Biochem. 2507–2522. https://doi.org/10.1159/000480213.

van der Toorn, M., Slebos, D.-J., de Bruin, H.G., Leuvenink, H.G., Bakker, S.J.L., Gans, R.O.B., Koëter, G.H., van Oosterhout, A.J.M., Kauffman, H.F., 2007. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. Am. J. Physiol. Lung Cell Mol. Physiol. 292, L1211–1218. https://doi.org/10.1152/ajplung.00291.2006.

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., Vandenabeele, P., Berghe, T. Vanden, Linkermann, A., Jouan-Lanhouet, S., Walczak, H., Vandenabeele, P., 2014. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat. Rev. Mol. Cell Biol. 15, 135–147. https://doi.org/10.1038/nrm3737.

Walters, M.J., Paul-Clark, M.J., McMaster, S.K., Ito, K., Adcock, I.M., Mitchell, Ja, 2005. Cigarette smoke activates human monocytes by an oxidant-AP-1 signaling pathway: implications for steroid resistance. Mol. Pharmacol. 68, 1343–1353. https://doi.org/10.1124/mol.105.012591.smoke.

Zhao, J., Xie, J., Xu, Y., Zhang, Z., Zhang, N., 2005. Expression of heat stress protein 70 mRNA in patients with chronic obstructive pulmonary disease and its significance. J. Huazhong Univ. Sci. Technol. Med. Sci. 1, 20–23.

Zhe, Y., Li, Y., Liu, D., Su, D.M., Liu, J.G., Li, H.Y., 2016. Extracellular HSP70-peptide complexes promote the proliferation of hepatocellular carcinoma cells via TLR2/4/JNK1/2MAPK pathway. J. Immunother. Emphasis Tumor Immunol. 37, 13951–13959. https://doi.org/10.1007/s13277-016-5189-5.

Zhou, J., Eksioglu, E.A., Fortenbery, N.R., Chen, X., Wang, H., Epling-Burnette, P.K., Djeu, J.Y., Wei, S., 2011. Bone marrow mononuclear cells Up-Regulate Toll-Like receptor expression and produce inflammatory mediators in response to cigarette smoke extract. PLoS One 6. https://doi.org/10.1371/journal.pone.0021173.

Zuo, L., Lucas, K., Fortuna, C.A., Chuang, C.-C., Best, T.M., 2015. Molecular regulation of toll-like receptors in asthma and COPD. Front. Physiol. 6. https://doi.org/10.3389/fphys.2015.00312.