


Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells

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Abstract Extracellular Hsp70 (eHsp70) can act as damage-associated molecular pattern (DAMP) via Toll-like receptors TLR2 and TLR4, and stimulate immune and inflammatory responses leading to sterile inflammation and propagation of already existing inflammation. It was found elevated in the blood of patients with chronic obstructive pulmonary disease (COPD), who might suffer occasional bacterial colonizations and infections. We used a monocytic THP-1 cell line as a cellular model of systemic compartment of COPD to assess inflammatory effects of eHsp70 when present alone or together with bacterial products lipopolysaccharide (LPS) and lipoteichoic acid (LTA). THP-1 cells were differentiated into macrophage-like cells and treated with various concentrations of recombinant human Hsp70 protein (rhHsp70), LPS (TLR4 agonist), LTA (TLR2 agonist), and their combinations for 4, 12, 24, and 48 h. Concentrations of IL-1 α , IL-6, IL-8, and TNF- α were determined by ELISA. Cell viability was assessed by MTS assay, and mode of cell death by luminometric measurements of caspases-3/7, -8, and -9 activities. rhHsp70 showed cell protecting effect by suppressing caspases-3/7 activation, while LPS provoked cytotoxicity through caspases-8 and -3/7 pathway. Regarding inflammatory processes, rhHsp70 alone induced secretion of IL-1 α and IL-8, but had modulatory effects on release of all four

cytokines when applied together with LPS or LTA. Combined effect with LPS was mainly synergistic, and with LTA mainly antagonistic, although it was cytokine- and time-dependent. Our results confirmed pro-inflammatory function of extracellular Hsp70, and suggest its possible implication in COPD exacerbations caused by bacterial infection through desensitization or inappropriate activation of TLR2 and TLR4 receptors.

Keywords THP-1 cells · Extracellular Hsp70 · LPS · LTA · COPD

Introduction

Heat shock protein 70 (Hsp70) is a stress-inducible 72-kDa intracellular protein with functions in prevention of protein aggregation, facilitation of protein refolding, and chaperoning of proteins. Its expression is up-regulated in various states of physiological and environmental stresses, such as infections, inflammation, cellular injury, or heat stress (Pockley et al. 2008). In the case of elevated cellular stress, Hsp70 can also be released from cells. Different mechanisms have been proposed, including translocation across the plasma membrane and release associated with lipid vesicles, an endolysosomal pathway, and the passive release after cell death by necrosis (Johnson and Fleshner 2006). Extracellular Hsp70 (eHsp70) then acts as damage-associated molecular pattern (DAMP), “danger signal” for the immune system. eHsp70 has chaperone and cytokine regulating activity (chaperokine) and can stimulate immune and inflammatory responses leading to sterile inflammation and propagation of already existing inflammation (Asea et al. 2002; Vabulas et al. 2002; Pouwels et al. 2014). eHsp70 has been shown to activate the host innate immune and inflammatory responses by engaging TLR2 and

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TLR4 receptors, via signaling pathways dependent on myeloid differentiation factor 88 (MyD88), which in turn activate nuclear factor κ B (NF- κ B) and mitogen-activated protein kinases (MAPK), causing release of pro-inflammatory cytokines (Midwood and Piccinini 2010; Giuliano et al. 2011). People suffering from a variety of inflammatory and infective diseases have chronically elevated basal levels of eHsp70 relative to healthy, aged-matched controls (Pockley 2003). In addition, an increase of eHsp70 in the blood was reported in healthy organisms after exposure to acute stressors (Johnson and Fleshner 2006). It was suggested that the release of Hsp70 could aggravate an already active host immune response, thereby leading to poor outcome. Alternatively, eHsp70 could serve a cytoprotective function at lower levels as a normal response to infection or stress, and once a certain critical threshold is surpassed, it could potentiate the dysregulated inflammatory response (Giuliano et al. 2011). Thus, it is possible that in vivo function of eHsp70 is context-dependent, such that in a normal physiological state eHsp70 facilitates immunity, whereas in a pathophysiological state eHsp70 exacerbates inflammatory diseases (Johnson and Fleshner 2006; Pockley et al. 2008; Giuliano et al. 2011). eHsp70 was found elevated in the blood of patients with chronic obstructive pulmonary disease (COPD) and some other inflammatory diseases (Hacker et al. 2009).

COPD is one of the leading causes of morbidity and mortality in the world, with the prevalence of 12% in individuals over the age of 40 years (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017). It is characterized by progressive airflow limitation associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases, most common being cigarette smoke. Exacerbations and comorbidities contribute to the overall severity in individual patients. Patients with COPD develop abnormal inflammatory reaction, and its chronic presence can lead to destruction of lung parenchyma (emphysema) and chronic bronchitis (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017). Besides its pulmonary manifestations, COPD has also a systemic component characterized by chronic inflammation, oxidative stress, activation of immune cells, and elevated concentration of pro-inflammatory cytokines in the blood (Agusti et al. 2003). Patients with COPD are susceptible to occasional bacterial colonizations and infections which can lead to exacerbations and worsening of already active inflammation.

In this study, we used a monocytic THP-1 cell line as a cellular model of systemic compartment of COPD to assess inflammatory effects of eHsp70 in COPD and its interactions with bacterial products lipopolysaccharide (LPS), a TLR4 agonist, and lipoteichoic acid (LTA), a TLR2 agonist, that could potentially lead to the worsening of existing inflammation. The development of inflammation was assessed by measuring concentrations of pro-inflammatory cytokines

interleukin (IL)-1 α , IL-6, IL-8, and tumor necrosis factor (TNF)- α secreted into the cell culture media.

Materials and methods

Cell culture

THP-1 monocytic cell line (American Type Culture Collection, USA) was cultured in an RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, and 1% penicillin and streptomycin, at 37 °C in a humidified 5% CO₂ atmosphere. Cells were grown in 75-cm² culture flasks, and subcultured every 4 or 5 days by three to five times dilution. Before treatments, cells were seeded on 12- or 96-well plates and induced into macrophage-like cells with 20 nM phorbol 12-myristate 13-acetate (PMA) for 48 h.

Treatments of THP-1 cells

Cells were treated with low endotoxin recombinant human Hsp70 protein (rhHsp70) (Enzo Life Sciences, USA). Endotoxin concentration in rhHsp70 was 2.7 EU/mg, determined by Limulus amoebocyte lysate (LAL) test by manufacturer. Equivalent amount of LPS was used as negative control in all experiments. Cells were also treated with LPS (positive control for TLR4 activation) and LTA (positive control for TLR2 activation). LPS was isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich, USA). LTA was isolated from *Staphylococcus aureus* (Invivogen, France), and had endotoxin concentration of 10 EU/mg. Concentrations of rhHsp70 (0.3–3 μ g/ml), LPS (0.1 μ g/ml), and LTA (1 μ g/ml) were used in this study based on our preliminary results. Three independent experiments ($n = 3$) were performed for all analysis (cytokines and viability measurements).

Measurement of cytokine concentration

THP-1 cells were seeded on 12-well cell culture plates (1×10^6 cells per well) and differentiated for 48 h with 20 nM PMA. Cells were treated with rhHsp70, LPS, LTA, and their combinations for 4, 12, 24, and 48 h. Cell medium was harvested and frozen at -80 °C until analysis.

Concentrations of IL-1 α , IL-6, IL-8, and TNF- α were determined in cell supernatants by DuoSet ELISA kits (R&D Systems, USA).

A 96-well plate was coated with 100 μ l per well of cytokine specific capture antibody diluted in PBS and incubated overnight at room temperature. Each well was washed three times with 400 μ l of washing buffer. The plate was then blocked with 300 μ l of reagent diluent and incubated at room temperature for a minimum of 1 h followed by washing. Then,

100 μl of samples or standards was added and incubated at room temperature for 2 h, followed by washing. After that, 100 μl of the specific detection antibody was added and the plate was incubated for 2 h at room temperature. After washing, 100 μl of streptavidin-HRP was added, and the plate was incubated for 20 min at room temperature in the dark, followed by washing. 100 μl of substrate solution (equal volume of hydrogen peroxide and tetramethylbenzidine) was added and the plate was once again incubated for 20 min at room temperature in the dark. Reaction was stopped by adding 50 μl of stop solution (2 N sulfuric acid). The optical density was determined at 450 nm, using a microplate reader (VICTOR3 1420 Multilabel counter, Perkin Elmer).

MTS assay

THP-1 cells were seeded on 96-well cell culture plates (5×10^4 cells per well) and differentiated for 48 h with 20 nM PMA. Cells were treated with rhHsp70, LPS, LTA, and their combinations for 24 h. Afterwards, 20 μl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, USA) reagent was added, and cells were incubated for 1 h in a humidified 5% CO_2 atmosphere. Absorbance was measured at 490 nm using a microplate reader (VICTOR3 1420 Multilabel counter, Perkin Elmer, USA).

Caspases activity (caspase-9, -8, -3/7)

THP-1 cells were seeded on white 96-well cell culture plates (2×10^4 cells per well for caspase-3/7, 2.5×10^4 cells per well for caspases-8 and -9) and differentiated for 48 h with 20 nM PMA. Cells were then treated for 2, 4, 6, and 8 h. After incubation, activities of caspases-9, -8, and -3/7 were measured according to the manufactures protocol using the Caspase-Glo[®] luminescent assay kits (Promega, USA). Briefly, 100 μl of reagent for specific caspase was added to each well and the plate was gently mixed at 300–500 rpm for 30 s. the plate was then incubated at room temperature for 3 h. Luminescence of samples was measured using a microplate reader (VICTOR3 1420 Multilabel counter, Perkin Elmer).

Statistical analysis

Data are presented as the mean \pm standard error of the mean (SEM). Unpaired *t* test or analysis of variance (ANOVA) followed by post hoc testing by Sidak method were used for statistical analysis. The level of $P < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 6.01 software (GraphPad Software Inc., USA).

When assessing combined effects of rhHsp70 with LPS and LTA, we compared our measured values with the calculated (expected) values. The values were calculated as the mean value obtained after exposure to one substance alone (rhHsp70)

plus the mean value obtained after exposure to the second substance (LPS or LTA) (Šegvić Klarić et al. 2014):

mean (expected for rhHsp70 + LPS or LTA)

$$= \text{mean (rhHsp70)} + \text{mean (LPS or LTA)} - \text{mean (non - treated)}$$

SEM (expected for rhHsp70 + LPS or LTA)

$$= \left[(\text{SEM for rhHsp70})^2 + (\text{SEM for LPS or LTA})^2 \right]^{\frac{1}{2}}$$

Significant difference between expected (calculated) and measured values was determined using *t* test. The level of $P < 0.05$ was considered statistically significant.

The results were as follows: a synergistic effect was implied if the measured values were significantly above the expected values; an antagonistic effect was implied if the measured values were significantly below the expected values.

Results

Inflammatory effects of eHsp70

Production of pro-inflammatory cytokines IL-1 α , IL-6, IL-8, and TNF- α was measured in differentiated THP-1 cells treated for 24 h with different concentrations of rhHsp70 (0.3, 1, and 3 $\mu\text{g/ml}$). Cytokine concentrations in cell culture media of non-treated (control) cells are expressed as 100%, and those of treated cells as percentages of concentration of non-treated cells. All tested concentrations of rhHsp70 significantly induced IL-1 α secretion (Fig. 1a). The level of IL-8 was increased only after treatment with 3 $\mu\text{g/ml}$ rhHsp70 (Fig. 1b). However, rhHsp70 failed to induce IL-6 and TNF- α (data not shown).

Modulation of inflammatory effects of LPS and LTA with different eHsp70 concentrations

LPS and LTA are known agonists of TLR4 and TLR2 receptors, respectively. In order to mimic simultaneous bacterial infection and inflammation propagated by eHsp70, we treated cells with 0.1 $\mu\text{g/ml}$ LPS or 1 $\mu\text{g/ml}$ LTA together with various rhHsp70 concentrations (0.3, 1, and 3 $\mu\text{g/ml}$) for 24 h. Cytokines IL-1 α , IL-6, IL-8, and TNF- α were measured in cell supernatants.

Treatment of THP-1 cells with LTA provoked strong IL-1 α secretion. However, when cells were treated with combination of LTA and various concentrations of rhHsp70, release of IL-1 α was similar to non-treated cells (Fig. 2a). Interaction between LTA and all applied concentrations of rhHsp70 was antagonistic, since combined treatment stimulated lower IL-1 α secretion than LTA alone (Fig. 2b). Compared to the cells treated with LPS alone, IL-1 α secretion was not significantly different in combinations with any concentration of rhHsp70 ($P = 0.0868$), and it had no significant interactions (data not shown).

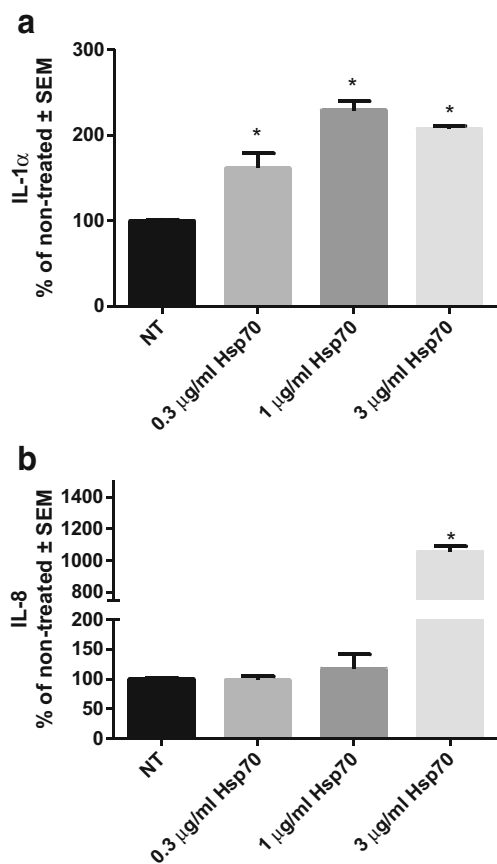


Fig. 1 Concentration-dependent effects of rhHsp70 on production of IL-1 α (a) and IL-8 (b). Data are presented as mean \pm SEM and tested by ANOVA followed by multiple comparison Sidak method. NT, non-treated cells. **a** * $P = 0.0097$, $P = 0.0034$, and $P = 0.0012$, for 0.3, 1, and 3 $\mu\text{g/ml}$ rhHsp70, respectively vs. non-treated cells. **b** * $P < 0.0001$ vs. non-treated cells

When measuring IL-8, LTA induced significant release of this cytokine as well. We observed that only 3 $\mu\text{g/ml}$ rhHsp70 in combination with LTA stimulated a significantly higher concentration of IL-8 than LTA alone (Fig. 3a). Still, the type of interaction between rhHsp70 and LTA was antagonistic (Fig. 3b) On the other hand, secretion of IL-8 did not differ significantly when combined treatment with LPS was applied as compared to LPS alone (data not shown). However, the calculated interactions between 3 $\mu\text{g/ml}$ rhHsp70 and LPS were significantly antagonistic (data not shown).

IL-6 and TNF- α concentrations were below detection limit in non-treated THP-1 cells. Therefore, we expressed the levels of those cytokines in treated cells in absolute values as pg/ml. Treatment with LPS alone significantly stimulated IL-6 secretion. A concentration of 0.3, 1, or 3 $\mu\text{g/ml}$ rhHsp70 applied together with LPS showed significantly increased IL-6 levels compared to LPS alone (Fig. 4a). Those effects were synergistic for 0.3 and 1 $\mu\text{g/ml}$ rhHsp70 combination (Fig. 4b). LTA alone or in combination with different concentrations of rhHsp70 did not induce significant changes in IL-6 secretion (data not shown).

Secretion of TNF- α was stimulated after treatment with LPS and LTA. Still, combined treatment of LPS or LTA and 0.3, 1, or 3 $\mu\text{g/ml}$ rhHsp70 did not differ significantly when compared to LPS or LTA alone after 24 h treatment (data not shown), although calculated interactions showed synergism between LPS or LTA and 3 $\mu\text{g/ml}$ rhHsp70 (data not shown).

Time-dependent interactions of LPS and LTA with eHsp70

Next, we wanted to explore if inflammatory effects of combined treatments were time-dependent. We have selected 1 $\mu\text{g/ml}$ rhHsp70 concentration, and we treated THP-1 cells for 4, 12, 24, and 48 h. IL-1 α was detected only at a 24-h time-point. However, other cytokines showed time-dependent secretion.

Secretion of IL-8 increased after treatment with LPS or LTA alone at all time-points. Compared to the cells treated with LPS alone, IL-8 secretion was significantly higher in the cells treated with rhHsp70 and LPS combination for 4 h and for 12 h while it was lower in cells treated together with LPS and rhHsp70 for 48 h (Fig. 5a). Cells treated with 1 $\mu\text{g/ml}$ rhHsp70 and LTA produced significantly less IL-8 after 4 h than the cells treated with LTA alone (Fig. 5b).

We calculated rhHsp70 and LPS or LTA interactions on IL-8 secretion (Fig. 6). Combination of rhHsp70 and LPS showed significant synergistic effect after 4 and 12 h and antagonistic effect after 48 h. On the other hand, combination of 1 $\mu\text{g/ml}$ rhHsp70 and LTA showed significant antagonistic effect after 4 h.

As for IL-6, cells treated with combination of LTA and rhHsp70 had lower IL-6 secretion compared to those treated with LTA alone after 4 and 12 h with an antagonistic type of interaction, while after 48 h significantly more IL-6 was secreted, and that effect was synergistic (Fig. 7). Combined treatment with LPS showed a synergistic type of interaction only after 24 h treatment (data not shown).

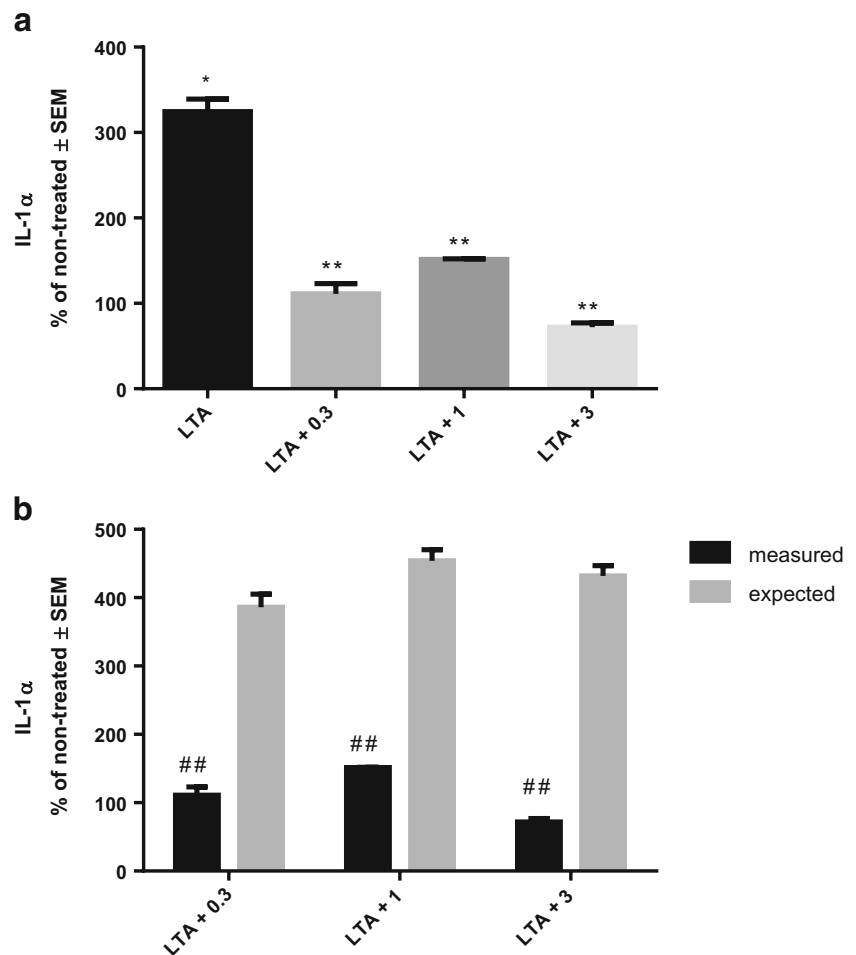
Finally, secretion of TNF- α was higher after treatment with LPS and LTA at all time-points. Compared to the cells treated with LPS alone, combination of LPS and 1 $\mu\text{g/ml}$ rhHsp70 had antagonistic type of effect on TNF- α production after 12 h. Treatment with LTA together with rhHsp70 exhibited antagonistic effects after 4, 12, and 48 h (Fig. 8).

Cytotoxicity of eHsp70, LPS, and LTA

To explore cytotoxicity of eHsp70, LPS, and LTA, cells metabolic activity and possible apoptotic cell death were assessed by MTS assay and by caspase activity measurements, respectively.

Results of the MTS assay showed that rhHsp70 did not influence cell viability, nor did the treatment with LTA alone, when compared to the control cells (expressed as 100%). However, 0.1 $\mu\text{g/ml}$ LPS caused significant cytotoxic effect

Fig. 2 Effect of rhHsp70 and LTA combination on production of IL-1 α after 24 h treatment. **a** Production of IL-1 α after 24 h treatment with 1 μ g/ml LTA alone and in combination with 0.3 (LTA + 0.3), 1 (LTA + 1), and 3 (LTA + 3) μ g/ml rhHsp70. Data are presented as mean \pm SEM and tested by ANOVA followed by multiple comparison Sidak method. * P = 0.0247 vs. non-treated cells. ** P = 0.0002, P = 0.0005, and P = 0.0001, for combination of LTA with 0.3, 1, and 3 μ g/ml rhHsp70, respectively vs. LTA alone. **b** rhHsp70 and LTA interactions on IL-1 α secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired t test. Double number signs indicate statistically significant antagonistic effect; P = 0.0067, P = 0.00029, and P = 0.0019 for combination of LTA with 0.3, 1, and 3 μ g/ml rhHsp70, respectively



(Fig. 9). On the other hand, when we compared combined treatments with LTA or LPS and different rhHsp70 concentrations with individual LPS or LTA treatments, we did not observe any significant differences in viability. To confirm those results, we calculated possible rhHsp70 and LPS or LTA cytotoxicity interactions. Combination of 0.3, 1, or 3 μ g/ml rhHsp70 and LPS or LTA showed no significant interaction (data not shown).

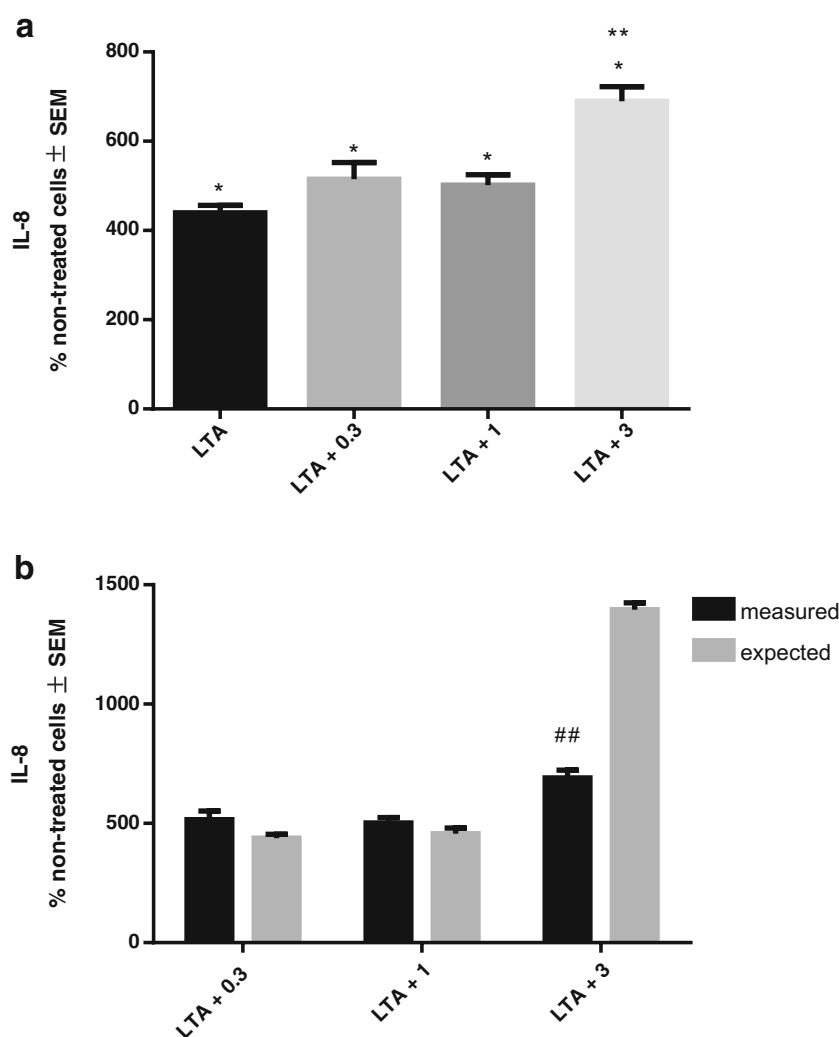
In addition, activities of caspases-9, -8, and -3/7 in THP-1 cells were measured after 2, 4, 6, and 8 h treatment with rhHsp70, LPS, or LTA. Compared to control cells, activity of caspase-9 did not differ with aforementioned treatments (data not shown). LPS provoked significant caspase-8 activation after 6 and 8 h, while LTA and rhHsp70 did not activate caspase-8 (data not shown). Furthermore, we did not observe significant difference in caspase-3/7 activity between non-treated cells and cells treated with rhHsp70 after 2, 4, or 6 h, whereas the activity of caspase-3/7 was decreased after 8 h treatment for all rhHsp70 concentrations examined. LTA failed to activate caspase-3/7 at any time-point, while LPS caused significant activation of caspase-3/7 after 4 and 6 h compared to non-treated cells (Fig. 10).

Discussion

In this study, we explored the effects of extracellular Hsp70 when presented alone or together with well-known pathogen-associated molecular pattern (PAMP) molecules, LPS, and LTA, on inflammatory response determined by the levels of secreted pro-inflammatory cytokines (IL-1 α , IL-6, IL-8, and TNF- α). We also assessed cell viability and mode of cell death by MTS assay and caspases-3/7, -8, and -9 activity measurements, respectively. We used a human monocytic cell line THP-1 as a cellular model of systemic compartment in COPD.

COPD is one of the most prevalent human diseases in the world, affecting about 12% of the population older than 40 years (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017). It is characterized by chronic inflammation which in turn induces destruction of parenchyma and small airway fibrosis leading to airflow limitation (Dong et al. 2013; Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017). Innate and adaptive immunity cells, as well as structural cells, such as epithelial cells and fibroblasts, are involved in the process of inflammation, and they secrete various mediators of inflammation (Barnes and Celli 2009; Dong et al.

Fig. 3 Effect of rhHsp70 and LTA combination on production of IL-8 after 24 h treatment. **a** Production of IL-8 after 24 h treatment with 1 $\mu\text{g/ml}$ LTA alone and in combination with 0.3 (LTA + 0.3), 1 (LTA + 1), and 3 (LTA + 3) $\mu\text{g/ml}$ rhHsp70. Data are presented as mean \pm SEM and tested by ANOVA followed by multiple comparison Sidak method. $*P = 0.0024$ for LTA alone vs. non-treated cells. $*P = 0.0002$, $P = 0.0005$, and $P = 0.0001$, for combination of LTA with 0.3, 1, and 3 $\mu\text{g/ml}$ rhHsp70, respectively vs. non-treated cells. $**P = 0.0196$ vs. LTA alone. **b** rhHsp70 and LTA interactions on IL-8 secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired *t* test. Double number signs indicate statistically significant antagonistic effect; $P = 0.0038$ for combination of LTA with 3 $\mu\text{g/ml}$ rhHsp70



2013; Rovina et al. 2013). Inflammation in COPD can be sterile (mediated by DAMPs), mediated by pathogens (PAMPs), or could be a consequence of their combined actions, as bacterial colonization can be present both in stable COPD and during its exacerbations (Wouters et al. 2007; Sethi et al. 2012).

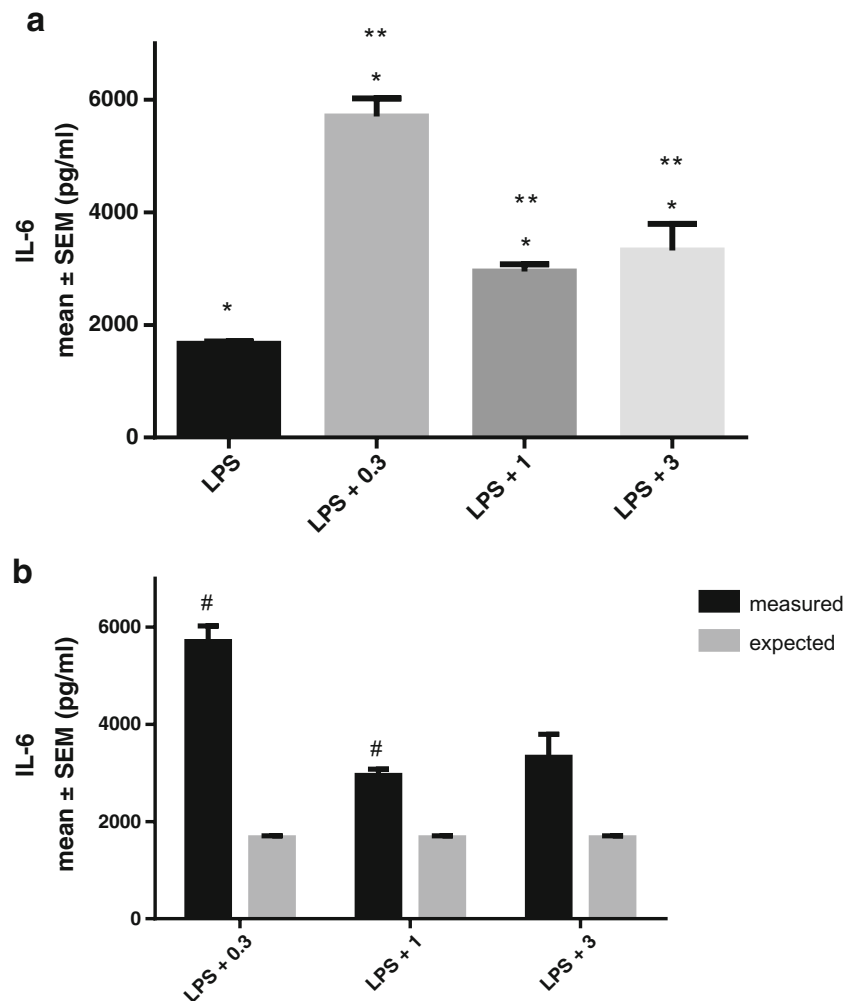
It was shown that extracellular Hsp70 is a DAMP molecule that activates monocytes and macrophages via binding to TLR2 and TLR4 receptors, causing a pro-inflammatory cytokine production through MyD88/IRAK/NF- κ B signal transduction pathway activation in a CD14-dependent fashion (Thériault et al. 2005; Ferat-Osorio et al. 2014).

In our study, rhHsp70 provoked IL-1 α and IL-8 secretion. However, IL-6 and TNF- α were not induced, which is in accordance with Ferat-Osorio et al. who even found down-regulated expression of TNF- α gene after treatment with Hsp70, albeit in primary monocytes, not THP-1 cells (Ferat-Osorio et al. 2014). In addition, eHsp70 was previously found to stimulate pro-inflammatory effect in other types of cells different from monocytic-macrophagic lineage (Chase et al. 2007; Wheeler et al. 2009; Giuliano et al. 2011; Mathur et al.

2011). Despite aforementioned evidence of eHsp70 pro-inflammatory effects, some researchers challenged these assumptions, and considered those effects to be false positive, i.e., due to endotoxin (LPS) contamination of recombinant Hsp70 protein (Tsan and Gao 2004; De Maio and Vazquez 2013). To rule out this possibility, we used low endotoxin Hsp70, with its level being 2.7 EU/mg, as determined by LAL test. Equivalent dose of LPS was included in all our experiments, and did not induce a pro-inflammatory effect in the THP-1 cell line (data not shown), and neither did so in our previous research on NCI-H292 bronchial epithelial cells (non-published results). Also, in our preliminary experiments with polymyxin B, boiling of rhHsp70, and application of specific antibodies against Hsp70, TLR2, and TLR4, we have excluded that the effects on cytokine secretion and cell viability might be from endotoxin or some other contaminants, rather than from rhHsp70 itself.

LPS is a component of gram-negative bacteria and is able to induce inflammatory response via TLR4 receptor, while LTA is a constituent of the cell wall of gram-positive bacteria

Fig. 4 Effect of rhHsp70 and LPS combination on production of IL-6 after 24 h treatment. **a** Production of IL-6 after 24 h treatment with 0.1 $\mu\text{g/ml}$ LPS alone and in combination with 0.3 (LPS + 0.3), 1 (LPS + 1), and 3 (LPS + 3) $\mu\text{g/ml}$ rhHsp70. Data are presented as mean \pm SEM and tested by ANOVA followed by multiple comparison Sidak method. * $P < 0.001$ vs. non-treated cells. ** $P = 0.0018$, $P = 0.0104$, and $P = 0.0400$, for combination of LPS with 0.3, 1, and 3 $\mu\text{g/ml}$ rhHsp70, respectively vs. LPS alone. **b** rhHsp70 and LPS interactions on IL-6 secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired t test. A number sign indicates statistically significant synergistic effect, $P = 0.0063$ and $P = 0.0103$ for combination of LPS with 0.3 and 1 $\mu\text{g/ml}$ rhHsp70, respectively



and TLR2 agonist. TLR2 and TLR4 activation can induce NF- κ B and MAPK kinase pathways, leading to the production of TNF- α , IL-1, IL-6, IL-8, and IL-10 (Hutchison et al. 2000; Dokladny et al. 2010; Vinokurov et al. 2012). Therefore, expectedly, when we treated THP-1 cells with LPS or LTA, production of cytokines was induced, with LPS having a stronger effect. In addition, effects of LPS and LTA seem to be time-dependent.

As bacterial colonization and infections are occasionally present in COPD patients, we explored a combined effect of eHsp70 and LPS or LTA. Combination of rhHsp70 and LPS had a synergistic effect on IL-8 secretion at early time-points, but it becomes antagonistic after 48 h. Effect of combination of rhHsp70 and LPS on IL-6 secretion is synergistic only after 24 h, while its effect on TNF- α secretion is antagonistic after 12 h. Dual treatment with rhHsp70 and LTA mostly has antagonistic effects: IL-1 α secretion is significantly lower after 24 h, IL-8 after 4 and 24 h, IL-6 after 4 and 12 h, and TNF- α after 4, 12, and 48 h. However, the effect is synergistic for IL-6 secretion after 48 h. It could be concluded that effects of LPS together with rhHsp70 are mainly synergistic, and those for

LTA and rhHsp70 are mainly antagonistic, although those effects are time- and cytokine-dependent. Some previous publications reported that extracellular Hsp70 preferentially interacts with TLR2, rather than TLR4 (Jean-Pierre et al. 2006; Joly et al. 2010; Mathur et al. 2011), and therefore the antagonistic effect might be a consequence of modulation of the receptor sensitivity by eHsp70 and interference with LTA binding, while eHsp70 does not interfere with LPS binding to TLR4 (Bangen et al. 2007). Explanation of different synergistic/antagonistic effects on cytokines production at different time-points might also be an alteration of TLR2 and TLR4 receptor expression by eHsp70 (Jean-Pierre et al. 2006; Tulapurkar et al. 2015). Another possible option is secretion or elevated expression of other molecules that could affect the combined effects by activating positive or negative feedback mechanisms that can in the end render receptors less responsive or unresponsive (Gupta et al. 2013; Quintana and Cohen 2005; Aneja et al. 2006). It was shown in several studies that if low eHsp70 concentrations are added before LPS, eHsp70 was able to induce endotoxin tolerance and can even act protective against LPS in sepsis in rats (Aneja et al. 2006;

Fig. 5 Production of IL-8 after 4, 12, 24, and 48 h treatment with LPS (a) and LTA (b) alone and in combination with rhHsp70. Data are presented as mean \pm SEM and tested by unpaired *t* test. **a** * $P < 0.0001$ after 4 h, $P = 0.0047$ after 12 h, $P = 0.0020$ after 24 h, and $P < 0.0001$ after 48 h of treatment with LPS alone vs. non-treated cells. ** $P = 0.0004$ after 4 h, $P = 0.0051$ after 12 h, and $P = 0.0247$ after 48 h of treatment with combination of rhHsp70 and LPS vs. LPS alone. **b** * $P < 0.0001$ after 4 h, $P = 0.0113$ after 12 h, $P = 0.0315$ after 24 h, and $P < 0.0001$ after 48 h of treatment with LTA alone vs. non-treated cells. ** $P < 0.0001$ after 4 h of treatment with combination of rhHsp70 and LTA vs. LTA alone

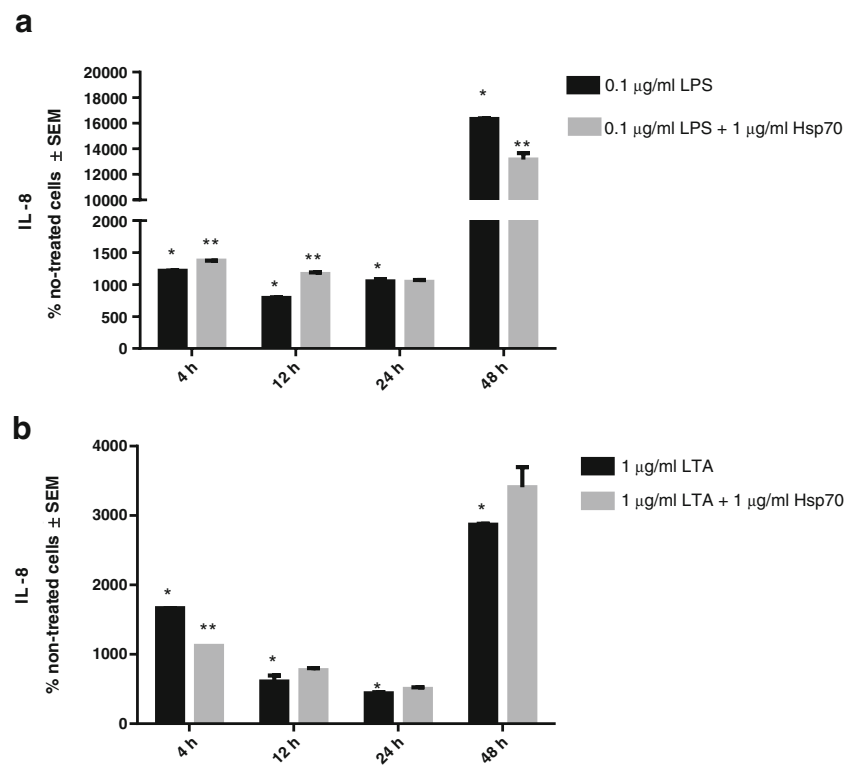


Fig. 6 rhHsp70 and LPS (a) or LTA (b) interactions on IL-8 secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired *t* test. A number sign indicates statistically significant synergistic effect; $P = 0.0006$ after 4 h and $P = 0.0054$ after 12 h for combination of LPS and rhHsp70. Double number signs indicate statistically significant antagonistic effect; $P = 0.0254$ after 48 h for combination of LPS and rhHsp70; $P < 0.0001$ after 4 h for combination of LTA and rhHsp70

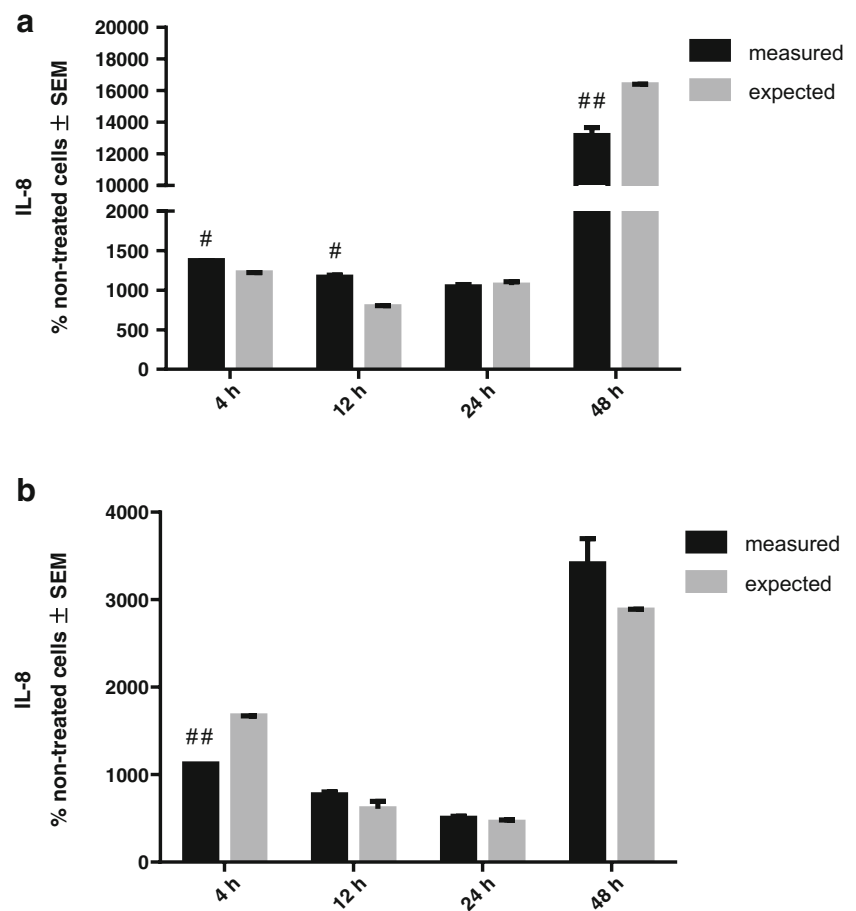


Fig. 7 Effect of rhHsp70 and LTA combination on production of IL-6 after 4, 12, 24, and 48 h treatment. **a** Production of IL-6 after 4, 12, 24, and 48 h treatment with LTA alone and in combination with rhHsp70. Data are presented as mean \pm SEM and tested by unpaired *t* test. $**P = 0.0072$ after 4 h, $P = 0.0198$ after 12 h, and $P = 0.0337$ after 48 h for combination of rhHsp70 with LTA vs. LTA alone. **b** rhHsp70 and LTA interactions on IL-6 secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired *t* test. A number sign indicates statistically significant synergistic effect; $P = 0.0337$ after 48 h. Double number signs indicate statistically significant antagonistic effect $P = 0.0072$ after 4 h and $P = 0.0198$ after 12 h

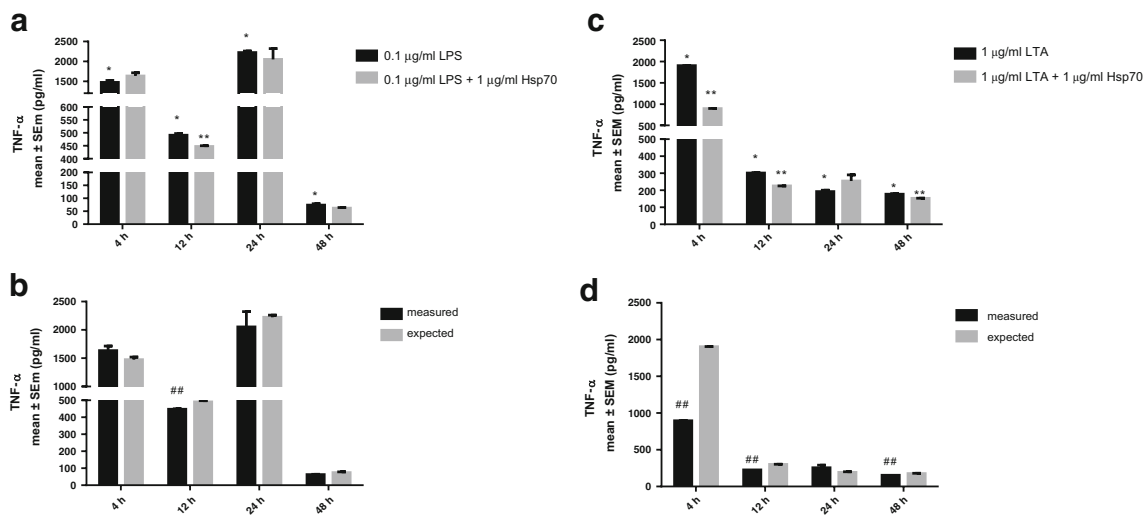
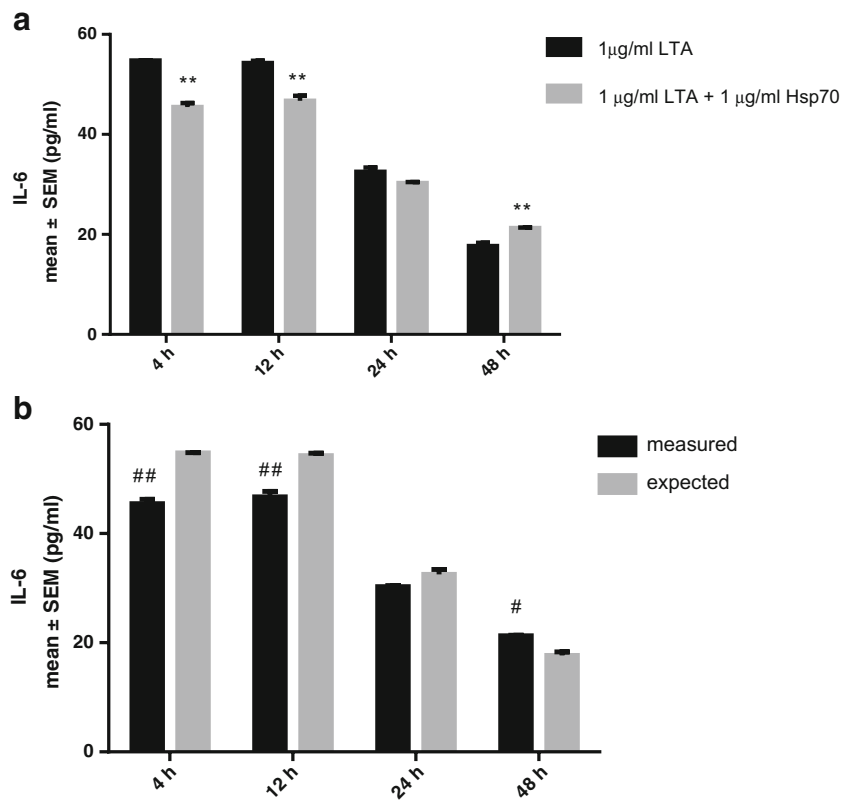


Fig. 8 Effect of rhHsp70 and LPS or LTA combination on production of TNF- α after 4, 12, 24, and 48 h treatment. Upper panel: production of TNF- α after 4, 12, 24, and 48 h treatment with LPS (a) or LTA (c) alone and in combination with rhHsp70. Data are presented as mean \pm SEM and tested by unpaired *t* test. **a** $*P < 0.0001$ after 4, 12, and 24 h, $P = 0.0020$ after 48 h of treatment with LPS vs. non-treated cells. $**P = 0.0292$ after 12 h for combination of rhHsp70 and LPS vs. LPS alone. **c** $*P < 0.0001$ after 4 and 12 h, $P = 0.0179$ after 24 h, and $P = 0.0001$ after 48 h of treatment with LTA vs. non-treated cells. $**P < 0.0001$ after 4 h,

$P = 0.0012$ after 12 h, and $P = 0.0283$ after 48 h for combination of rhHsp70 and LTA vs. LTA alone. Lower panel: rhHsp70 and LPS (b) or LTA (d) interactions on TNF- α secretion. Dark bars represent the measured values and gray bars the expected values. Data are expressed as mean \pm SEM. Tested using unpaired *t* test. Double number signs indicate statistically significant antagonistic effect $P = 0.0292$ after 12 h for combination of rhHsp70 and LPS; $P < 0.0001$ after 4 h, $P = 0.0012$ after 12 h, and $P = 0.0283$ after 48 h for combination of rhHsp70 and LTA

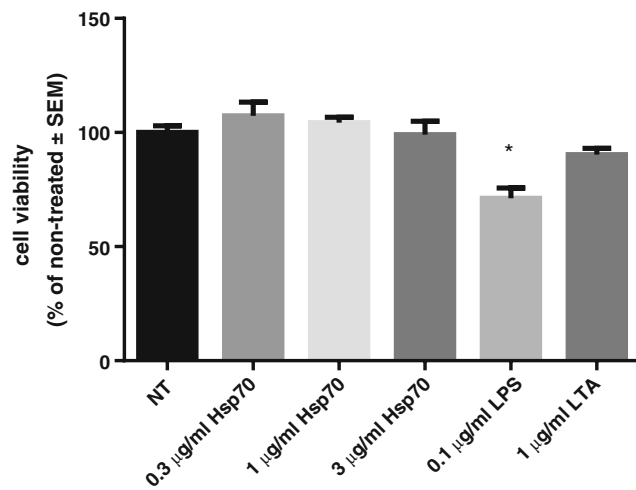


Fig. 9 Effect of treatment with rhHsp70, LPS, and LTA on cell viability. Data are presented as mean \pm SEM and tested by ANOVA followed by multiple comparison Sidak method. * $P = 0.0007$ vs. non-treated cells

Abboud et al. 2008; Garcia et al. 2013; Hsu et al. 2014). Also, besides TLR2 and TLR4, other receptors such as CD40, CD91, scavenger receptors, and c-type lectin receptor DECTIN might be implicated in eHsp70 inflammatory activity (Thériault et al. 2005). Further investigations are needed to elucidate underlying molecular pattern involved in inflammatory processes provoked by extracellular Hsp70.

Besides inflammation, another important aspect of understanding mechanisms of diseases is cell death, as activation of TLR2 and TLR4 receptors could lead to inflammation and/or programmed cell death. Inflammation could also cause necrotic cell death, leading to the leakage of intracellular content and further propagation of inflammation. Normally, necrotic cells are immunostimulatory, in contrast to apoptotic cells which are immunologically silent (Zhang et al. 2010; Ofengeim and Yuan 2013; Vanden Berghe et al. 2014). Apoptosis, or programmed cell death, is a vital part of normal function of the immune system and inappropriate apoptosis is associated with

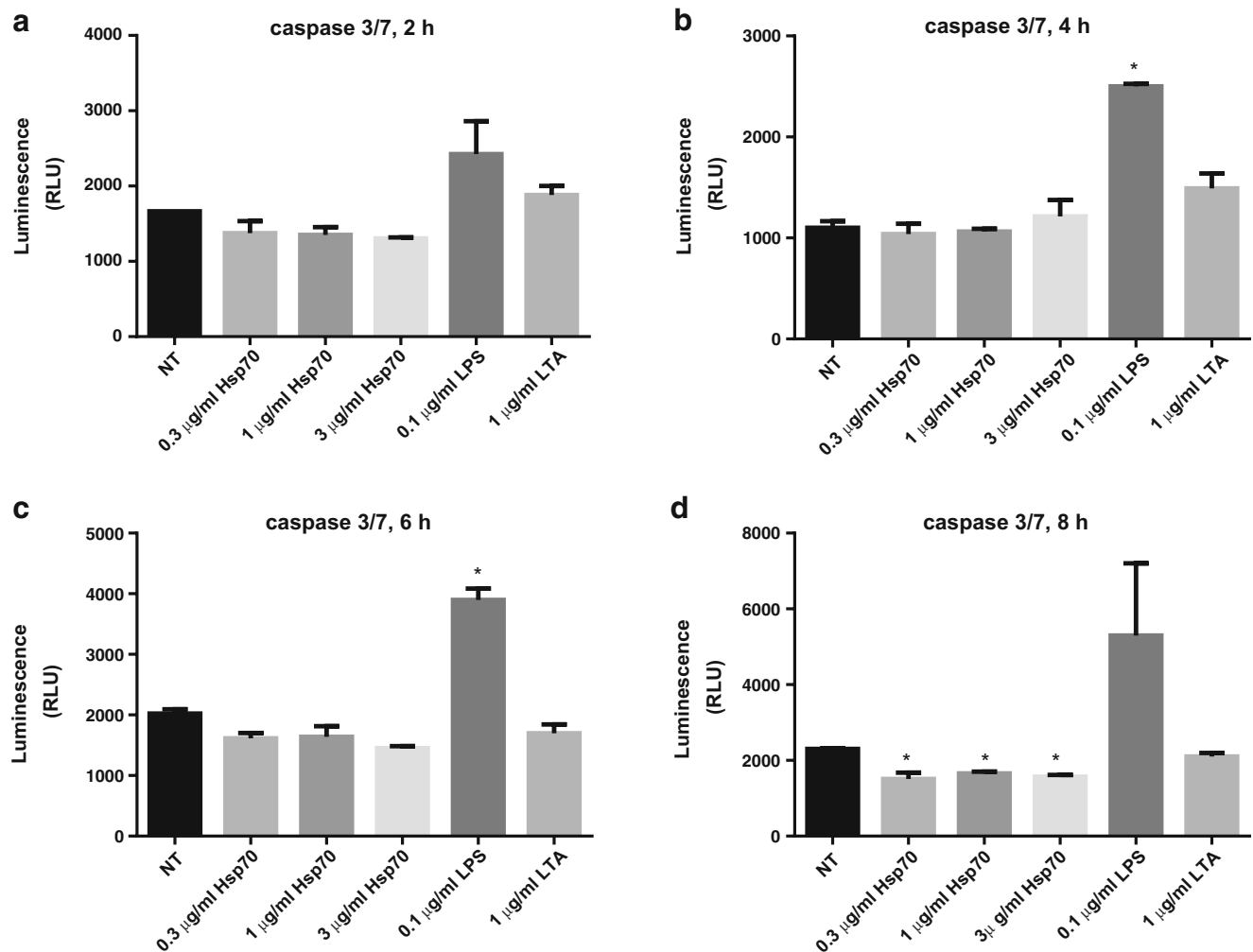


Fig. 10 Apoptotic effects of rhHsp70, LPS, and LTA on THP-1 cells after 2 (a), 4 (b), 6 (c), and 8 (d) h of treatment, as assessed by the caspase 3/7 activity. Data are represented as mean \pm SEM and tested by ANOVA followed by multiple comparison by Sidak method. $P < 0.05$ is

considered statistically significant. **b** * $P = 0.0036$ vs. non-treated. **c** * $P = 0.0052$ vs. non-treated. **d** * $P = 0.0103$, $P = 0.0221$, $P = 0.0140$ for 0.3, 1, and 3 µg/ml rhHsp70, respectively vs. non-treated cells

various diseases (Elmore 2007). Cysteiny l aspartate-specific proteases, caspases, play a central role in the apoptotic process. They can be activated by two pathways, intrinsic or mitochondrial via initiator caspase-9 activation, and extrinsic or death receptors pathway via initiator caspase-2, -8, and/or -10 activation. Both pathways converge on downstream executioner caspases-3, -6, and/or -7 (Duprez et al. 2009).

We assessed cell viability using MTS assay, and explored whether apoptotic pathways were activated by determining activities of caspases-9, -8, and -3/7. rhHsp70 did not cause a decrease in THP-1 cell viability, and neither did LTA. However, LPS caused a significant reduction in metabolic activity of the cells. It was reported that Hsp70 did not provoke cytotoxic effects when applied on HL-60 promyelocytic leukemia cells (Wheeler et al. 2009) or neutrophils (Vinokurov et al. 2012), and LTA was found to decrease viability of dermal fibroblasts only at very high doses (Choi 2010), while it did not affect viability of A549 alveolar epithelial cells (Liu et al. 2012). Contrary to our results, Huang et al. found that only doses larger than $\geq 10 \mu\text{g/ml}$ LPS affect cell viability in THP-1 cell line (Huang et al. 2012). Combinations of rhHsp70 with LPS or LTA did not show any significant interactions on cell viability. It was shown before that pre-treatment with Hsp70 can have a protective effect against cytotoxicity of various agents, including LPS and LTA (Aneja et al. 2006; Vinokurov et al. 2012; Franco et al. 2016), but we added rhHsp70 and LPS or LTA simultaneously and this could be the reason for a discrepancies of results.

In our study, treatment of THP-1 cells with rhHsp70, LTA, or LPS did not induce mitochondrial apoptotic pathway (caspase-9), nor did rhHsp70 or LTA induce death receptor apoptotic pathway (caspase-8). In contrast, LPS induced apoptosis by the way of activation of caspases-8 and -3/7, which is in accordance with some previous studies (Xaus et al. 2000; Munshi et al. 2002). In addition, we observed a significantly lower activity of executioner caspases-3/7 in THP-1 cells after 8 h treatment with rhHsp70. It is a well-established fact that intracellular Hsp70 has anti-apoptotic function (Joly et al. 2010), but anti-apoptotic functions of eHsp70 have also been demonstrated. Luo et al. reported that eHsp70 prevents activation of caspases-3 and -9 dependent on cytochrome c, and Franco et al. reported that extracellular exposure to Hsp70 is able to lower caspase-3 activation induced by hydrogen peroxide (Luo et al. 2012; Franco et al. 2016). Exact mechanisms of eHsp70 effect on cell death are not thoroughly explored and further investigation is needed.

In conclusion, we demonstrated that extracellular Hsp70 can induce inflammation (secretion of IL-1 α and IL-8) in THP-1 cells. Furthermore, when added in combination with LPS or LTA, antagonistic or synergistic interactions were observed even in situations when rhHsp70 alone did not exhibit

a significant pro-inflammatory effect. Also, it seems that eHsp70 can suppress apoptosis execution. Hsp70 was found elevated in sera of COPD patients (Hacker et al. 2009), and those patients are proven to be susceptible to bacterial colonization and infections (Wouters et al. 2007). Therefore, we suggest that extracellular Hsp70 might be implicated in inflammatory processes in a stable phase of COPD as well as during its exacerbations, as TLR2 and TLR4 desensitization or inappropriate activation might lead to the worsening of the patient's condition.

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