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Recombinant human HSP60 produced in ClearColi™ BL21(DE3) does not activate the NFκB pathway

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ABSTRACT

HSP60, an intracellular molecular chaperone has been largely described as an alarmin or damage-associated molecular pattern when released outside the cell. HSP60 has been reported as a possible ligand of TLR2 or TLR4 inducing NFκB-dependant signaling pathway leading to cytokine secretion. However, recent publications suggested that HSP60 could not act as an activator of TLR4 by itself. The observed effect could be due to the presence of endotoxin in HSP60 preparation especially LPS. In order to clarify the controversy, we produced recombinant human HSP60 in two different strains of *Escherichia coli*, standard strain for protein overproduction, BL21(DE3), and the new ClearColi BL21(DE3) strain which lacks LPS-activity through TLR4. Undoubtedly, we have shown that recombinant HSP60 by itself was not able to induce NFκB-dependant signaling pathway in a model of THP1 monocyte cell line. Our data suggest that HSP60 needs either pathogen-associated molecules, specific post-translational modification and/or other host factors to activate immune cells via NFκB activation.

1. Introduction

Heat shock proteins (HSPs) are a group of proteins highly conserved during evolution. HSPs are molecular chaperones which assist the refolding of non-native or misfolded proteins. Thus, HSPs help proteins to recover their complete function or provoke their proteolytic degradation [1,2]. Mitochondrial 60 kDa-heat shock proteins (HSP60) belong to the family of type I chaperonins. HSP60 plays a key role as a shuttle of newly translated, translocated and stress-denatured or misfolded proteins [3].

HSP60 is able to stimulate innate and adaptive immunity [4,5]. During stress in prokaryotic or eukaryotic cells, it has been shown that the synthesis of HSP60 was up-regulated. This up-regulation occurred also under viral and bacterial infections, autoimmune or metabolic disorders [6–9]. Although HSP60 localizes primarily within mitochondria, its localization can also change when its level of expression is dramatically increased. These proteins can be exposed on the cell surface or released into the extracellular space [10–14]. Since 1993, HSP60 preparations from diverse sources (bacteria, mammalian and human tissues) were shown to activate the innate immune system. Indeed, extracellular HSP60 (exHSP60) has been considered as an alarmin or a Damage-Associated Molecular Pattern (DAMP) [15–19]. Interestingly, HSP60 cytokine effect was mediated by Toll-Like Receptor (TLR) 2 (TLR2) and TLR4 signal transduction pathway which triggers the activation of Nuclear Factor *kappa* B (NFκB) in different cells types [20–22]. CD14, which was required for the interaction between lipopolysaccharide (LPS) and TLR4, was shown to be necessary for exHsp60-mediated inflammatory signaling [23].

Abbreviations: APC, antigen presenting cells; CD14, cluster of differentiation 14; DAMP, damage-associated molecular pattern; exHSP60, extracellular 60 kDa-heat shock protein; GST, glutathion-S-transferase; HSP, heat shock proteins; HSP60, 60 kDa-heat shock protein; hTNF-α, human tumor necrosis factor α; LPS, lipopolysaccharide; MD2, myeloid differentiation 2; NFκB, nuclear factor *kappa* B; PMA, phorbol 12-myristate 13-acetate; PMB, polymixin B; rhHSP60, recombinant human 60 kDa-heat shock protein; RPMI medium, Roswell Park Memorial Institute medium; SEAP, secreted alkaline phosphatase; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4.

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Most of the experiments revealing exHSP60 as an activator of the NFκB pathway in immune cells have been performed using recombinant HSP60 overexpressed and purified from *Escherichia coli*. It is well known that one HSP60 molecule can bind to two LPS molecules with high affinity, after overexpression and purification from *E. coli* bacteria LPS [24]. Between 1993 and 2002, to assess a specific HSP60 effect on NFκB activation, authors used essentially an inhibitor of LPS mediated inflammation without any decrease in HSP60 cytokine effect (polymixin B, PMB) or heat-denatured of HSP60 [4,20,25–27]. In 2003, Gao and Tsan did not observe any cytokine effect when highly purified HSP60 from *E. coli* was used [28]. Hence, the function of exHSP60 in the immune system remains disputed [24,29–31]. More recent studies using recombinant proteins still associate cytokine effects to HSP60 but without addressing the possible contribution of contaminating LPS [16,25,32].

To clarify this controversy, we have used here a new host for bacterial protein overexpression. We prepared recombinant human HSP60 (rhHSP60) using new commercially available strain of *E. coli* called ClearColi BL21(DE3). ClearColi BL21(DE3) bacteria was genetically engineered to express a modified LPS. This modified LPS did not trigger the endotoxic TLR4-dependant response in mammalian cells. Using this system, we found that our recombinant HSP60 was not able to induce NFκB dependant pathway. Thus, we suggest that HSP60 by itself has no cytokine effect in immune cells through the TLR4-NFκB pathway.

2. Materials and methods

2.1. Reagents

All reagents were from Sigma–Aldrich (Sigma–Aldrich, France), excepted when indicated.

2.2. LPS extraction

LPS extraction from standard BL21(DE3) (Invitrogen, Life Technologies, France) or ClearColi BL21(DE3) strain (Lucigen, Euromedex, France) was performed as described [33,34]. No proteins and nucleic acid contaminations were found using respectively SDS–PAGE analysis followed by silver staining or GelRed agarose gel electrophoresis. LPS were quantified through the generation of lipid chromophore in the presence of concentrated sulfuric acid and at a λ_{max} of 300 nm [35]. Briefly 20 µL of each LPS preparation was treated for 20 min at 90 °C with 100 µL of concentrated sulfuric acid (95% v/v). Reactions were stopped in ice water bath and absorption at 300 nm was recorded using an UV–visible spectrophotometer (UV mini 1240, Shimadzu, Japan). Commercially available LPS (L2630) was used to generate a standard curve.

2.3. Protein expression

The plasmids used during this study were pGEX-4T-1 (GE, Amersham, France) and pGEX-4T-1 GST-rhHSP60 from Dr. Altieri (The Wistar institute). They were transformed into two different strains of *E. coli*, standard BL21(DE3) and ClearColi BL21(DE3). Clones were grown in 50 mL LB medium with Ampicillin at 37 °C overnight at 200 rpm. The 50 mL overnight culture was put in 1L of 2YT medium with Ampicillin. The expression of human GST-rhHSP60 was induced at an OD of 0.6 with 1 mmol L^{-1} isopropyl beta-D-thiogalactoside (IPTG) and left at 37 °C during 3 h at 200 rpm. Then, bacteria suspensions were centrifuged at 3000 g during 20 min at 4 °C. The pellets were frozen at −20 °C or used directly for the following stage.

2.4. Protein purification

The pellets were suspended in 10 mL of cold Lysis Buffer 0.5% Triton X100 in PBS with 1 tablet EDTA-free protease inhibitor (Roche Diagnostic, France). Lysis of bacteria were performed by sonication (Misonix ultrasonic liquid processor, France) at 80% of amplitude for 2 min, with 10 s of burst and 10 s of pause, maintained constantly in the ice. Lysates were centrifuged at 15,000g for 10 min at 4 °C. The supernatants were loaded on 0.5 mL of Glutathione Sepharose 4B column (GE, Amersham, France). The column was washed with 10 column volumes with storage buffer (25 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl).

Then 20 units mL^{-1} thrombin in 500 µL of storage buffer were added in the recapped column. The column was incubated on a roller mixer at room temperature for 2 h incubation. After the incubation step, rhHSP60 was recovered in the flow through and GST remained bound to the column. For all experiments using rhHSP60, the negative control was obtained using the flow through of GST-expressing bacteria as starting materials.

All recombinants proteins were stored at −80 °C after a flash frozen step in liquid nitrogen. Protein purifications were monitored by SDS–PAGE followed by Coomassie blue staining. We also performed GelRed agarose gel electrophoresis to be sure that recombinant protein preparation lacks DNA or RNA. The proteins were quantified by bicinchoninic acid assay (BCA Assay). Recombinant proteins were finally analyzed by 12.5% SDS–PAGE, and proteins were visualized with Coomassie blue staining (Biorad) as before [36].

2.5. Cell line culture

THP1 (ATCC® TIB-202™) human monocyte cell lines and THP1-XBlue-CD14-MD2 cells (Invivogen) were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 completed with 10% (v/v) Foetal Bovine Serum (FBS), 2 mmol mL^{-1} glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.5 µg mL^{-1} amphotericin B (PAN Biotech, Germany) at 37 °C, 5% CO_2 in a humidified cell culture incubator.

2.6. Cells viability

THP1-XBlue-CD14-MD2 cells were platted in 24-well plates at a density of 5×10^5 cells mL^{-1} in MegaCell™ RPMI (PAN Biotech, Germany) without FBS and were treated with 5000 ng mL^{-1} rhHSP60 purified from the standard BL21(DE3) or from ClearColi BL21(DE3) strain or 1000 ng mL^{-1} LPS (L2630). After 16 h treatment, viable cells were counted using a Malassez's counting chamber and trypan blue exclusion test of cell viability.

2.7. NFκB/SEAP activity

THP1-XBlue-CD14-MD2 cells were platted in 96-well plates at a density of 5×10^5 cells mL^{-1} in MegaCell™ RPMI (PAN Biotech, Germany) without FBS and were treated during 16 h with different concentrations of GST, GST-rhHSP60 or rhHSP60 (dilution from 0 to 5000 ng mL^{-1}) or LPS (dilution from 0 to 1000 ng mL^{-1}), purified from the standard BL21(DE3) or from ClearColi BL21(DE3) bacteria. For treatment with LPS-HSP60 complex, HSP60 (10 µg mL^{-1}), LPS (2 ng mL^{-1}) or LPS (2 ng mL^{-1}) mixed with HSP60 (10 µg mL^{-1}) were incubated in PBS at 37 °C for 2 h, then these preparations were diluted ten-fold in MegaCell™ RPMI and THP1-XBlue-CD14-MD2 cells were treated with this media as above. NFκB/SEAP activity was evaluated in the supernatants using Quanti-blue as described by supplier (Invivogen, France).

2.8. TNFα quantification

THP1 cells were platted in 96-well plates at a density of 5×10^5 cells mL^{-1} and pre-treated with 10 ng mL^{-1} PMA in complete RPMI medium for 48 h. PMA-treated THP-1 cells were exposed, in MegaCell™ RPMI without FBS, to purified rhHSP60 ClearColi BL21(DE3) at 2500 ng mL^{-1} or LPS at 1000 ng mL^{-1} during 6 h. Supernatants were collected and the amount of TNF-α was measured by ELISA (Ready-SET-Go!®, e-bioscience, Austria) according to the manufacturer's protocols.

2.9. Statistical analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using ©1992–2007 GraphPad Prism 5 Software, Inc. One-way ANOVA, followed by Turkey's test were performed to compare each treatments. *P* values < 0.05 were considered significant.

3. Results

3.1. LPS extracted from ClearColi BL21(DE3) does not induce NFκB activation

We decided to use the new ClearColi BL21(DE3) strain to make sure that cytokine effect of rhHSP60 was not due to contamination with bacterial LPS. These ClearColi BL21(DE3) bacteria were genetically engineered in order to express a modified LPS that lacks two of the six acyl chains and the oligosaccharide part (Fig. 1). To our knowledge this is the first report using this strain, so we tested the capacity of the remaining lipid IV_A to activate the NFκB pathway in a THP1-XBlue-CD14-MD2 experimental model which expressed all TLRs, NOD1 and NOD2 [37]. First, using the hot phenol extraction protocol, we extracted LPS from the conventional strain of *E. coli* used for recombinant production of proteins, BL21(DE3), or from the new ClearColi BL21(DE3) strain. As the conventional Limulus Amebocyte Lysate (LAL) activity were not adapted to quantify modified lipid IV_A (Fig. S1), the LPS content were quantified using lipid chromophore formation [35]. These two sources of LPS were used to test for their capacity to activate the expression of the NFκB reporter gene, Secreted Embryonic Alkaline Phosphatase (SEAP), in THP1-XBlue-CD14-MD2 cells. LPS extracted from BL21(DE3) strain triggered NFκB activation with elevated SEAP activity and comparable to the commercially available LPS (LPS L2630). As expected, the LPS extracted from ClearColi BL21(DE3) strain displayed no significant effect on NFκB/SEAP activity (Figs. 2 and S2). This result lead us to conclude that modified LPS (Lipid IV_A) from ClearColi BL21(DE3) bacteria did not trigger NFκB response in our human monocyte cell line.

3.2. rhHSP60 purified from ClearColi BL21(DE3) lacks the capacity to induce NFκB activation

Glutathion-S-Transferase (GST) and GST-rhHSP60 were produced in the two *E. coli* strains described above. In order to discard eventual GST-tag interference with rhHSP60 specific action, we have chosen to cleave the GST-tag with thrombin. The SDS–PAGE analysis confirmed the homogeneity of the protein solution (90–95%) and the removal of GST (Fig. 3). The Western blot confirmed rhHSP60 production and purification (Fig. S3A).

To determine the effect of these proteins on NFκB signaling, we performed the NFκB/SEAP activity test on THP1-XBlue-CD14-MD2 cells. rhHSP60 produced by standard BL21(DE3) bacteria provoked a dose dependent activation of NFκB. rhHSP60 expressed in ClearColi BL21(DE3) bacteria did not activate NFκB (Fig. 3B). To

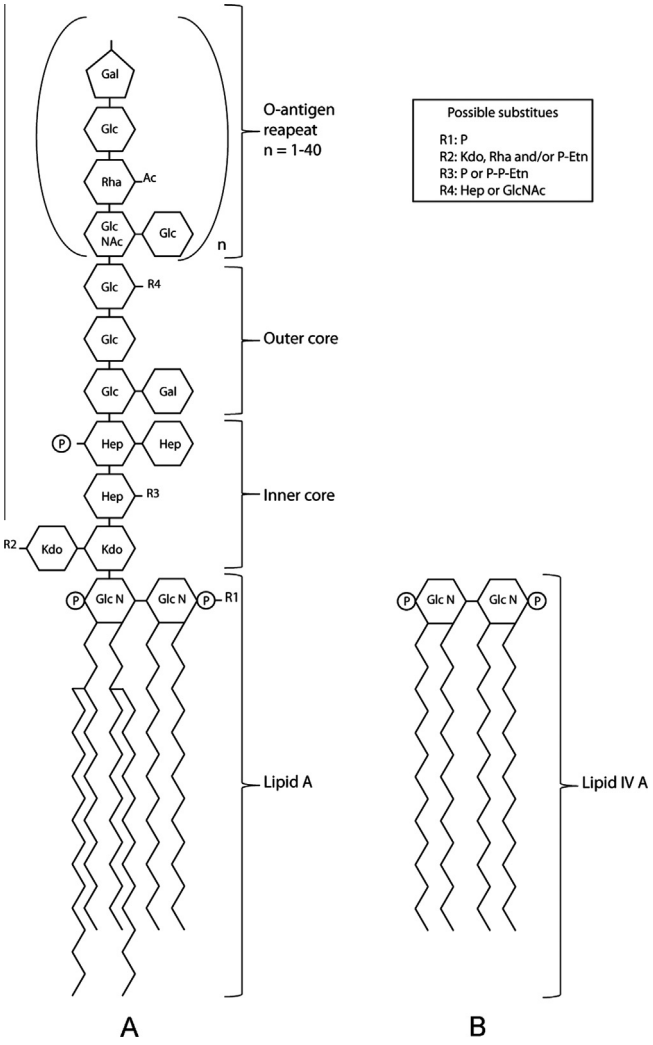

Fig. 1. ClearColi® BL21(DE3) cells express a modified LPS. (A) LPS of *E. coli* consists of a polysaccharide region and is anchored in outer bacterial membrane by a carbohydrate lipid moiety (lipid A), which contains six fatty acyl groups responsible of the LPS immunostimulatory activity. (B) ClearColi modified LPS lacks the polysaccharide part and contains an underacylated lipid A with only four fatty acids (lipid IV A).

rule out any cytotoxic effect of the recombinant proteins used we have observed good viability of THP1 cells after all treatments using the trypan blue method (Fig. S3B).

As rhHSP60 produced in ClearColi was not able to activate NFκB, we further tested the folding and biochemical properties of HSP60. We tested a native refolding for HSP60 produced in ClearColi using heat denaturation curve, ATP binding assay and chaperonin activity [38] (Fig. S4). We confirmed that HSP60 from ClearColi conserved its chaperonin property and had a native folded state.

In agreement with above observations, THP1-XBlue-CD14-MD2 cells treated with GST-rhHSP60 produce in ClearColi were not able to triggered NFκB dependant response (Fig. S5B).

Some studies indicated that HSP60 pro-inflammatory effect can be related to unidentified receptor different from TLR2 and TLR4 [39]. We used another macrophage reporter cell line (RAW-Blue™). These cells are derived from RAW 264.7 macrophages which express TLR2 and 4 and also specific receptors for HSP60 [16,39]. We observed the same results as obtained for THP1-XBlue-CD14-MD2 (Fig. S5C). All together these results showed that HSP60 produced in ClearColi BL21(DE3) strain does not by itself signal through the NFκB pathway.

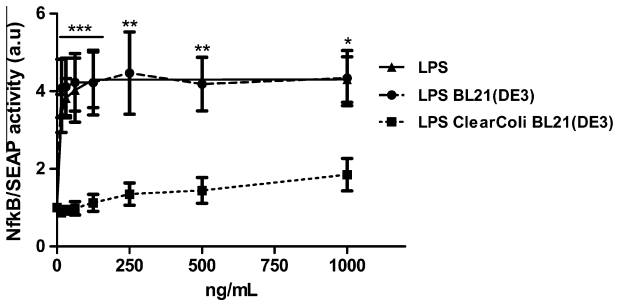

Fig. 2. LPS extracted from ClearColi BL21(DE3) strain did not activate NFκB/SEAP production in human THP1 monocyte cell line. THP1-XBlue-CD14-MD2 cells (5×10^5 cells mL^{-1}) were treated with different concentrations (from 0 to 1000 ng mL^{-1}) of LPS extracted from the standard BL21(DE3) (●) or from ClearColi BL21(DE3) strain (■) during 16 h. The results were normalized to the control (cells treated with storage buffer). The commercial LPS (▲) is used as the positive control. Results are presented as the mean ± SEM from 3 independent experiments. Differences were determined by One-way ANOVA Turkey's test: * difference ($P < 0.05$) and ** difference ($P < 0.01$) and *** difference ($P < 0.001$) between LPS from standard BL21(DE3) and LPS from ClearColi BL21(DE3) strain. No different between LPS of BL21(DE3) bacteria and LPS ($P > 0.05$).

3.3. TNFα secretion in PMA-treated THP1 stimulated by rhHSP60

Above experiments were based on NFκB/SEAP reporter gene assay. Given that TNFα is a prototypical inflammatory marker and used previously to screen for HSP60 activity on macrophages, we decided to test whether our recombinant HSP60 could affect TNFα secretion. We treated THP1 cells with phorbol 12-myristate 13-acetate (PMA) in order to optimize cytokine cell response [40]. Then, we treated these macrophage-like THP1 cells with rhHSP60 produced from ClearColi BL21(DE3) strain or the BL21(DE3) strain. LPS treatment confirmed that PMA-treated THP1 cells were able to respond to inflammation inducer and released TNFα at high levels (Fig. S6). In contrast, rhHSP60 did not induce significant TNFα secretion (Figs. 4 and S6A). We also tested another major proinflammatory marker IL6 and failed to observe that IL6 secretion was affected by HSP60 (Fig. S6B). Altogether these observations suggested that rhHSP60 itself does not have any pro-inflammatory cytokine effect.

3.4. HSP60 potentialises the LPS-dependant activation of NFκB pathway

Using highly purified HSP60, it was previously shown that HSP60 can induce NFκB activation through TLR4 only in association with LPS [24,30]. Thus, HSP60 could potent LPS action. Hence, we tested if our HSP60 produced in ClearColi was able to transduce NFκB when it was pre-bound to low dose of LPS. We pre-incubated HSP60 with LPS and then we treated THP1-XBlue-CD14-MD2 cells with the complex (Fig. 5). LPS at this concentration alone activated only weakly NFκB pathway. When LPS was prebound to HSP60 this activation was increased significantly. These observations confirmed that HSP60 potent the pro-inflammatory effect of LPS and HSP60 needs LPS to transduce NFκB pathway in THP1 cells.

4. Discussion

Since 1993, numerous studies have shown that HSP60 was involved in innate immune system activation [22,41,42], and that HSP60 was a link between the innate and adaptive immune system [4,43–45]. Indeed, HSP60 was able to activate immune cells such as, macrophages, dendritic cells, B cells, T cells and others cells such as endothelial cells and adipocytes [20,22,32,45]. Moreover, numerous studies found that HSP60 could activate TLR2 and TLR4 signaling pathway upstream NFκB inflammatory factor [20,23,46]. TLR2 and TLR4 are known to be respectively involved

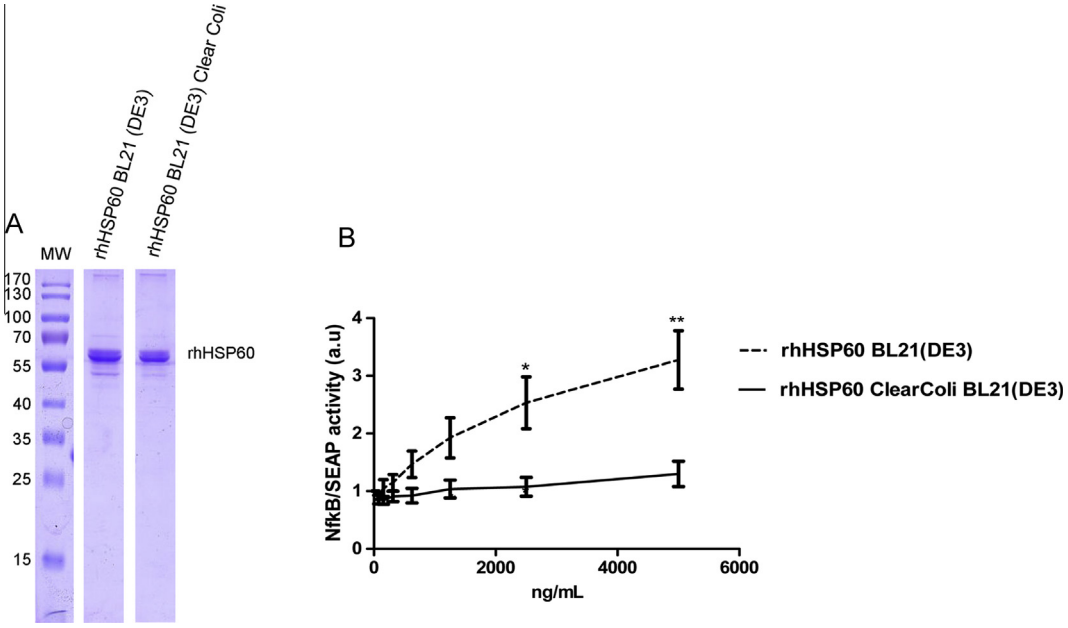

Fig. 3. Recombinant human HSP60 produced in ClearColi BL21(DE3) *E. coli* did not induce NFκB/SEAP activity in monocyte THP1 cell line. (A) Biochemical evaluation of rhHSP60 purity by SDS–PAGE followed by Coomassie blue staining. (B) THP1-XBlue-CD14-MD2 cells (5×10^5 cells mL^{-1}) were treated with different concentrations of rhHSP60 (from 0 to 5000 ng mL^{-1}) produced in the standard BL21(DE3) (dotted line) or produced in ClearColi BL21(DE3) (solid line) during 16 h. Then NFκB/SEAP activity was evaluated in the supernatants using Quanti-blue. The results were normalized to the control. Results are presented as the mean ± SEM from 3 independent experiments. Differences were determined by One-way ANOVA Turkey's test: * difference ($P < 0.05$) and ** difference ($P < 0.01$) between rhHSP60 produced in standard BL21(DE3) and rhHSP60 produced in ClearColi BL21(DE3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

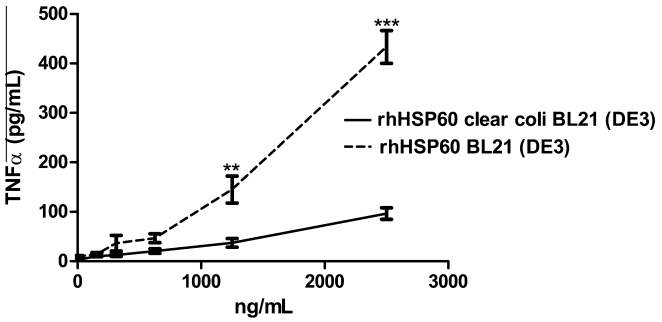

Fig. 4. rhHSP60 produced in ClearColi BL21(DE3) *E. coli* did not increase TNFα secretion from PMA-treated THP1. THP1 cells (5×10^5 cells mL^{-1}) were first treated with 10 ng mL^{-1} PMA during 48 h then they were treated with different concentrations of rhHSP60 (from 0 to 2500 ng mL^{-1}) produced in the standard BL21(DE3) (dotted line) or produced in ClearColi BL21(DE3) (solid line) during 6 h. TNFα quantification in the supernatant was performed by ELISA. Results are presented as the mean ± SEM from 3 independent experiments. Differences were determined by One-way ANOVA Turkey's test: ** difference ($P < 0.01$) and *** difference ($P < 0.001$) between rhHSP60 produced in standard BL21(DE3) and rhHSP60 produced in ClearColi BL21(DE3).

in Gram-positive bacteria (lipopeptides or peptidoglycans) and Gram-negative bacteria (lipopolysaccharides) recognitions [47,48].

In this study, we have first checked that ClearColi BL21(DE3) strain had a non-endotoxic LPS using the *in vitro* model, THP1-XBlue-CD14-MD2 (Figs. 1 and 2), as considered by the supplier. We decided to use these endotoxin-free bacteria for recombinant human HSP60 expression. rhHSP60 did not provoke any pro-inflammatory response via NFκB activation in THP1-XBlue-CD14-MD2 cells. Moreover rhHSP60 failed to modulate TNF-α secretion in PMA-treated THP1 cells (Figs. 3 and 4). However, HSP60 pre-bound to LPS was able to induce NFκB (Fig. 5).

In studies dealing with HSP60 inflammatory response, recombinant proteins from the same supplier or produced in prokaryotic host have been used. As we have shown in the standard BL21(DE3) bacteria, these recombinant proteins are very likely contaminated by traces of LPS. Even when eukaryotic cells were used for HSP60 overexpression [16], other contaminants could be involved in the observed inflammatory effects [15,49] such as molecules presenting hydrophobic domain which bind to HSP60 [15]. All these studies considered HSP60 with low endotoxin using Limulus Amebocyte Lysate (LAL) assay or LPS inhibition with PMB. Nonetheless, it has been reported that HSP60 could bind LPS with high affinity and that this interaction could interfere with LPS inhibition or that the quantification of LPS could be underestimated [24,50,51]. Some experiments have used heat-denatured HSP60 as a negative control [24,29,48], but it is known that HSP60 could act as an enhancer in the presence of very low concentration of LPS [24,29].

Of critical note, we have used *E. coli* bacteria to produced recombinant human HSP60. This bacterially-expressed protein can lack post-translational modification or eukaryotic co-factors essential to induce cytokine release. Nevertheless, and as demonstrated since 2003 [24,48,49,52,53], we propose that highly purified recombinant HSP60 from *E. coli* does not induce cytokine responses in THP1 cells.

Recent studies suggested that HSP60 induced a specific IFNα production in professional Antigen Presenting Cells (APC), in contrast to LPS which induced IFNγ production and the NFκB pathway [29,30]. Moreover, it has been shown that HSP60 induced myocyte apoptosis via TLR4 signaling pathway [54]. It would be interesting to clarify and revisit alarmin activity in immune and non-immune cells using our HSP60 protein produced in ClearColi BL21(DE3) as all of these study used prokaryotic sources of human HSP60 [20,23,26,46].

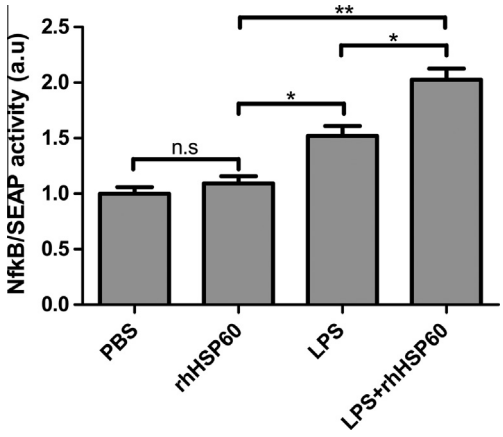

Fig. 5. rhHSP60 produced in ClearColi BL21(DE3) *E. coli* required LPS to induce NFκB/SEAP activity in monocyte THP1 cell line. THP1-XBlue-CD14-MD2 cells (5×10^5 cells mL^{-1}) were treated with LPS-HSP60 complex. HSP60 (10 μg mL^{-1}) produced in ClearColi BL21(DE3), LPS (2 ng mL^{-1}) or LPS L2630 (2 ng mL^{-1}) mixed with HSP60 (10 μg mL^{-1}) produced in ClearColi BL21(DE3), were incubated in PBS at 37 °C for 2 h, then these preparations were diluted ten-fold to cells during 16 h. NFκB/SEAP activity was evaluated in the supernatants using Quanti-blue. The results were normalized to the controls. Results are presented as the mean ± SEM from 3 independent experiments. Differences were determined by One-way ANOVA Turkey's test: * difference ($P < 0.05$) and ** difference ($P < 0.01$).

5. Conclusion

To summarize, we proposed that inflammatory effect of HSP60 previously described, was due to effects of bacterial contaminants, such as LPS. This may have strong implication in bacterial infection settings. We suggest that HSP60 binds to LPS and acts as an alarmin carrier. So during bacterial infection extracellular HSP60 released from stressed-cells binds to LPS and facilitate microbe recognition by decreasing the threshold of LPS detection and enhancing TLR4 mediated NFκB activation triggering pro-inflammatory cytokine expression such as TNFα.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2015.01.028.

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