Different heat shock protein 60 species share pro-inflammatory activity but not binding sites on macrophages

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Abstract In a study of seven different hsp60 species, we found that all mammalian and microbial proteins shared the property of eliciting an inflammatory response in mouse macrophages. In all cases, TNFα production was induced by 0.1 μM concentrations of hsp60. However, the different hsp60 preparations did not compete for the same binding site. The binding of fluorescence-labeled human hsp60 was inhibited by excess unlabeled human, rat or mouse hsp60, but not hamster, *Escherichia coli*, *Chlamydia pneumoniae* or *Mycobacterium bovis* hsp60. We conclude that phylogenetically separate hsp60 species interact with innate immune cells via different recognition pathways.

Key words: Heat shock protein 60; Innate immunity; Macrophage receptor; Tumor necrosis factor α

1. Introduction

Human heat shock protein 60 (hsp60) and several microbial homologs have been reported to elicit a pro-inflammatory response in cells of the innate immune system. This response includes the release of inflammatory mediators such as IL-6, TNFα and NO and the induction of the T helper cells type 1 promoting cytokines IL-12 and IL-15 [1–3]. These findings suggest a role of hsp60 as a danger signal for the innate immune system [2]. Recently, we have demonstrated the presence of a specific binding receptor for human hsp60 on macrophages [4]. Binding of fluorescence-labeled human hsp60 was found to be saturable, occurred with submicromolar affinity and could be competed by unlabeled human hsp60. Furthermore, our data indicate that the binding receptor for human hsp60 is different from a common receptor for human hsp70, hsp90 and gp96, recently identified as the α_2-macroglobulin receptor [4,5,6].

Hsp60 proteins are highly conserved throughout phylogeny, as are other stress proteins, probably reflecting their crucial and uniform chaperone function in pro- and eukaryotic cells [7]. However, it has not been determined whether all hsp60 species share the ability to activate innate immune cells, nor whether they would employ the same recognition pathway. We report here that all four mammalian and three microbial hsp60 preparations are similarly potent in stimulating TNFα production in mouse macrophages. However, only human, rat and mouse hsp60 were found to compete for the same binding site on mouse macrophages, suggesting differential recognition of phylogenetically hsp60 species.

2. Materials and methods

2.1. Cell cultures

The mouse macrophage cell lines J774A.1 (German Collection of Microorganism and Cell Culture, Braunschweig, Germany) and RAW264.7 (American Type Culture Collection, Manassas, VA, USA) were cultured as previously described [4].

C3H/HeJ mice, expressing a functionally defective toll-like receptor 4 (TLR4) molecule and mice of the control strain C3H/HeN with the complete functionally active TLR4 protein [8] were purchased from Charles River (Wilmington, MA, USA). Bone marrow cells were obtained by flushing femurs and tibias with ice-cold phosphate-buffered saline (PBS). The cell suspension was washed by centrifugation ($500\times g$, 5 min), and a total of 3.5×10^6 bone marrow cells was incubated in tissue culture dishes with Pluznik medium [9]. After 6–7 days, adherent bone marrow-derived macrophages (BMM) were detached by incubation with ice-cold Ca^{2+}-, Mg^{2+}-free HBSS (Hank's balanced salt solution) for 10 min, followed by washing with HBSS ($500\times g$, 5 min), and were used for further studies.

2.2. Reagents

Recombinant human hsp60 was obtained from Peptor Ltd. (Rehovot, Israel) or StressGen Biotechnologies (Victoria, BC, Canada). Recombinant *Chlamydia pneumoniae* hsp60 was kindly provided by Dr. T. Miethke (Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany). Recombinant mouse hsp60 and *Mycobacterium bovis* hsp65 were obtained from the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands [10]. Recombinant rat and *Escherichia coli* GroEL were purchased from StressGen Biotechnologies. Recombinant hamster hsp60 was obtained from IMMPACT Biotechnologies (Hamburg, Germany). *E. coli* O26:B6 lipopolysaccharide (LPS), bovine serum albumin (BSA) and ovalbumin (OVA) were from Sigma (Deisenhofen, Germany). Endotoxin contents of the various substances were determined by quantitative limulus amebocyte lysate assay (BioWhittaker, Verviers, Belgium). In human (Peptor Ltd. and StressGen Biotechnologies) and hamster hsp60 endotoxin contents were ≤ 1 EU/μg protein and in hsp60 proteins from rat, mouse, *C. pneumoniae*, *E. coli* and *M. bovis* endotoxin contents were in the range of 0.008–0.1 EU/μg protein corresponding to 0.84–12 pg LPS equivalents/μg protein. LPS contained 0.01 EU/pg. The endotoxin content of hsp60 preparations did not correlate with bioactivity. For the batches of human and chlamydial hsp60 used here, controls using polymyxin B or heat treat-

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Abbreviations: hsp60, heat shock protein 60; LPS, lipopolysaccharide; BSA, bovine serum albumin; OVA, ovalbumin; TLR, toll-like receptor

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ment excluded that an endotoxin contamination accounts for bioactivity [2,4,9,11]. The immunostimulatory oligonucleotide (ODN)1668 [9], containing a CpG motif, was purchased from Life Technologies (Karlsruhe, Germany).

2.3. Protein labeling

Labeling of human hsp60 (Peptor Ltd.) with the fluorescence dye Alexa488 was performed as previously described [4] using the Alexa Fluor 488 Protein Labeling kit (Molecular Probes, Leiden, The Netherlands).

2.4. Hsp60 binding and competition

Binding and competition assays were performed as previously described [4]. Briefly, after 2 days of continuous culture J774A.1 and RAW264.7 cells were centrifuged (500×*g*, 5 min, 4°C) and resuspended in PBS/1% BSA (4°C). Macrophages (1×10^6 cells/ml) were incubated with 350 nM of the Alexa488-labeled human hsp60 for 45 min on ice for the binding studies. For the competition assays the macrophages were preincubated with a 10-fold molar excess of the unlabeled ligand for 30 min on ice. Then hsp60-Alexa488 was added and the incubation was continued for another 45 min on ice. Subsequently, cells were washed with PBS/1% BSA and resuspended in PBS/1% paraformaldehyde. The fluorescence signals of the samples were quantified using a FACSCalibur flow cytometer (BD Bioscience, Rockville, CA, USA). Binding of Alexa488-labeled hsp60 was calculated using the geometric mean fluorescence value after subtracting the autofluorescence of the cells.

2.5. Stimulation of macrophages for TNFα and NO production

For the stimulation of TNFα or NO production, mouse macrophage cell lines and BMM were seeded (1×10^6 cells/ml) in the wells of flat-bottom 96-well plates (200 µl/well). After incubation for 18 h (37°C, 5% CO_2), LPS, different hsp60 preparations or ODN1668 were added to the macrophage cultures. After another 6 h (TNFα) or 24 h (NO) of incubation, supernatants were collected. The amounts of TNFα in culture supernatants were quantified by sandwich ELISA

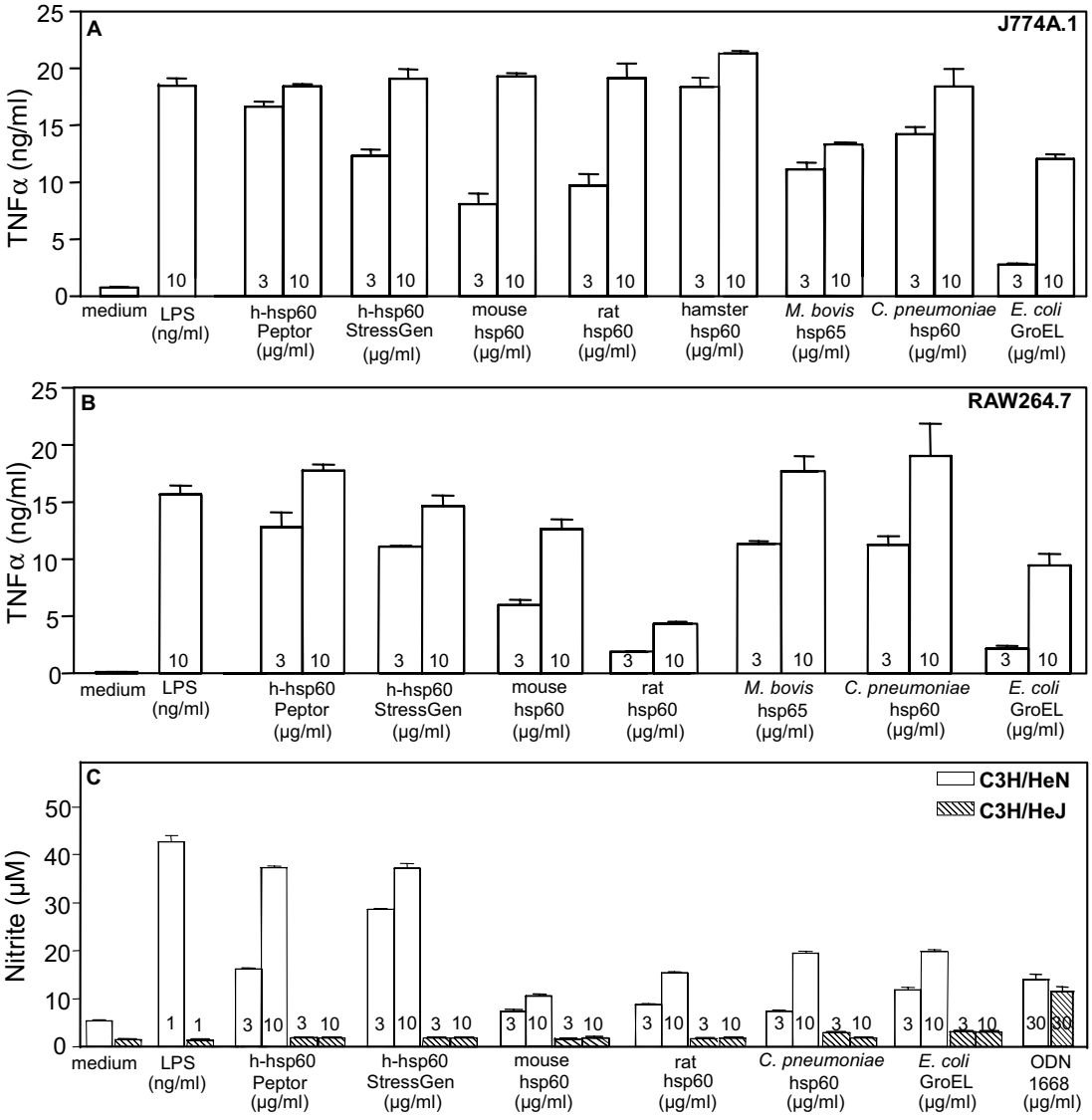

Fig. 1. Pro-inflammatory response in J774A.1 (A), RAW264.7 (B) or bone marrow-derived macrophages (C) stimulated with different mammalian and microbial hsp60 proteins. A,B: cells were incubated with medium alone or with medium supplemented with LPS (10 ng/ml) or with different hsp60 proteins (3 and 10 µg/ml, corresponding to approximately 0.05 and 0.15 µM) as indicated. After 6 h of cultivation, the TNFα concentrations in the cell supernatants were determined by ELISA. C: BMM from C3H/HeN (open bars) and C3H/HeJ (hatched bars) mice were incubated with medium alone or with medium supplemented with LPS (1 ng/ml), different hsp60 proteins (3 and 10 µg/ml) or ODN1668 (30 µg/ml) as indicated. After 24 h of cultivation, the nitrite concentrations in the cell supernatants were determined by the Griess reaction. The data represent the mean concentrations of TNFα+S.D. and of nitrite+S.D. of three independent experiments, each performed in triplicate. h-hsp60, human hsp60; m-hsp60, mouse hsp60; r-hsp60, rat hsp60.

(enzyme-linked immunosorbent assay; OptEIA mouse TNFα Set, BD PharMingen, San Diego, CA, USA). The TNFα content was calculated using a standard curve obtained with recombinant mouse TNFα [2]. NO released by macrophages was assessed by the determination of accumulated nitrite in the culture supernatants by the colorimetric Griess reaction [2].

3. Results

3.1. Macrophage stimulatory activity of different mammalian and microbial hsp60 proteins

A prominent feature of macrophage activation by hsp60 is the release of substantial amounts of TNFα within 4–6 h. We therefore compared the production of TNFα after exposure of the macrophages to the different hsp60 species at concentrations of 3 and 10 μg/ml for 6 h at 37°C. LPS (10 ng/ml) was used as positive control. Each of the four mammalian hsp60 species was found to elicit a TNFα response. When J774A.1 cells were exposed to human (Peptor Ltd. or StressGen Biotechnologies), mouse, rat or hamster hsp60 comparable amounts of TNFα (8.1–21.2 ng/ml) were induced in a dose-dependent manner (Fig. 1A). TNFα secretion in response to *M. bovis* hsp65, *C. pneumoniae* hsp60 or *E. coli* GroEL was also dose-dependent and in the range of 2.8–18.4 ng/ml. In parallel, we determined TNFα production in RAW264.7 cells (Fig. 1B) after incubation with the different hsp60 proteins. Again, all mammalian hsp60 species tested induced a dose-dependent release of TNFα, with the lowest levels seen for rat hsp60. Incubation with *M. bovis* hsp65, *C. pneumoniae* hsp60 or *E. coli* GroEL also induced the release of TNFα (2.2–19.0 ng/ml) in a dose-dependent manner. These results show that rat, mouse and hamster hsp60 share pro-inflammatory activity with human hsp60. Furthermore, dose dependence and amounts of TNFα released appear to be quite similar for mammalian and microbial hsp60 proteins.

Most hsp60 species were also analyzed for the induction of NO production. In all cases, dose-dependent NO production, measured as nitrite, was observed. There was a stronger response to human than to mouse, rat or microbial hsp60 species (Fig. 1C). In the same experiment macrophages with defective TLR4 receptor were analyzed. These cells were unresponsive to each of the different hsp60 species, but not to a TLR4-independent stimulus (Fig. 1C). We conclude that all of the hsp60 species tested require an intact TLR4 for inducing a pro-inflammatory response.

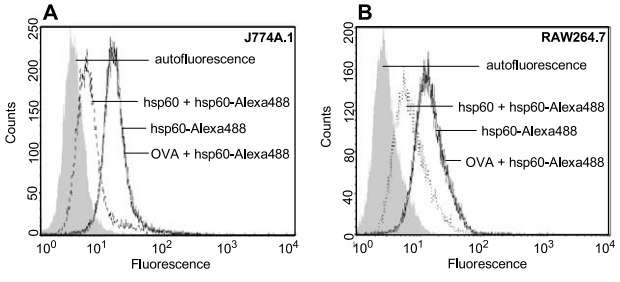

Fig. 2. Specific binding of human hsp60-Alexa488 to J774A.1 (A) or RAW264.7 (B). Cells of the macrophage lines (1×10^6 cells/ml) were preincubated for 30 min in the absence or presence of 3.5 μM unlabeled human hsp60 or 3.5 μM OVA, followed by the addition of 350 nM human hsp60-Alexa488 for 45 min on ice. Fluorescence intensities (*x*-axis) were plotted against cell counts.

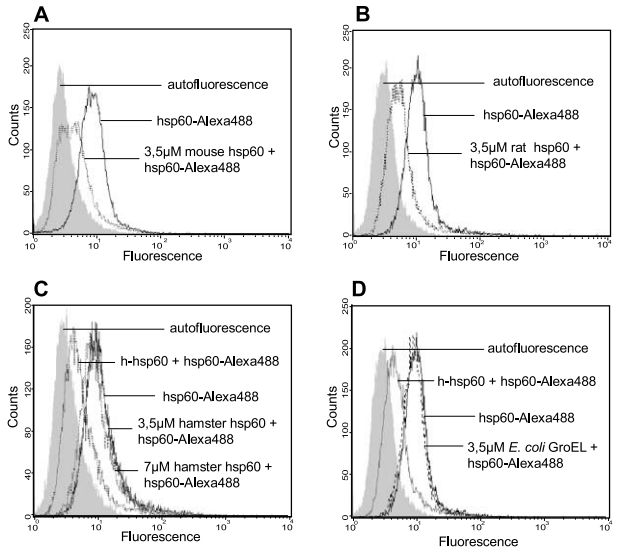

Fig. 3. Differential recognition of mammalian hsp60 and microbial hsp60 by J774A.1 cells. Macrophages (1×10^6 cells/ml) were preincubated for 30 min in the absence or presence of 3.5μM mouse hsp60 (A), 3.5 μM rat hsp60 (B), 3.5 μM and 7 μM hamster hsp60 (C), 3.5 μM *E. coli* GroEL (D) or 3.5 μM unlabeled human hsp60 (h-hsp60, panels C and D), followed by the addition of 350 nM human hsp60-Alexa488 for 45 min on ice. Fluorescence intensities (*x*-axis) were plotted against cell counts.

3.2. Different hsp60 species do not share binding sites

The specificity of binding of the different hsp60 proteins to the macrophage cell surface was analyzed by quantitative competition experiments. Binding of fluorescence-labeled human hsp60 occurred to a similar degree in both mouse macrophage lines, J774A.1 and RAW264.7 (Fig. 2). A 10-fold molar excess of OVA did not diminish binding, whereas an unlabeled human hsp60 strongly competed for the binding site. A similar extent of inhibition was observed for either of the two macrophage lines. Mouse hsp60 was found to be a similarly potent inhibitor as unlabeled human hsp60 (Fig. 3A). This indicates that the binding site for human hsp60 on mouse macrophages is identical with the receptor for self hsp60. Rat hsp60 also competed with human hsp60 for binding (Fig. 3B). By contrast, hamster hsp60 did not exhibit any cross-reactivity. Even at a 20-fold molar excess (7 μmol/l) hamster hsp60 did not displace human hsp60 from the macrophage receptor (Fig. 3C). Finally, *E. coli* GroEL as an example of microbial hsp60 was also unable to inhibit the binding of human hsp60 (Fig. 3D).

A comparison of all hsp60 proteins analyzed is presented in Fig. 4. In the experiments using J774A.1 macrophages (Fig. 4A), a different preparation of human hsp60 (from StressGen Biotechnologies) caused inhibition of binding to a similar degree as the original hsp60 protein (84% versus 82% inhibition). Similar degrees of inhibition were also observed with mouse hsp60 (78% inhibition) or rat hsp60 (70% inhibition), but not with hamster hsp60 (15% inhibition). Also, preincubation of J774A.1 cells with the same 10-fold molar excess of unlabeled *C. pneumoniae* hsp60, *M. bovis* hsp65 or *E. coli* GroEL did not interfere with the binding of human hsp60 (Fig. 4A). The series of experiments was repeated with a second mouse macrophage line, RAW264.7 (Fig. 4B). Essentially the same results were obtained, i.e. inhibition of the binding of Alexa488-labeled human hsp60 was seen after preincubation of

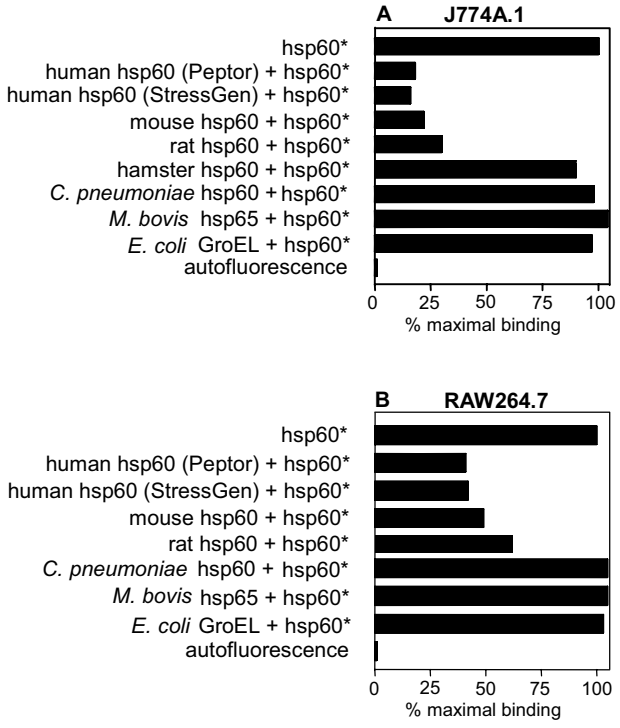

Fig. 4. Differential recognition of mammalian hsp60 versus microbial hsp60 by J774A.1 (A) and RAW264.7 (B). Cells of the macrophage lines (1×10^6 cells/ml) were preincubated for 30 min in the absence or presence of different unlabeled hsp60 proteins (3.5 μM), followed by the addition of 350 nM human hsp60-Alexa488 (hsp60*) for 45 min on ice. The analysis was performed by flow cytometry and the binding of hsp60* in the absence of any inhibitory protein was set 100%.

RAW264.7 cells with excess unlabeled human hsp60 from Peptor Ltd. (59% inhibition), human hsp60 from StressGen Biotechnologies (58% inhibition), mouse hsp60 (51% inhibition) or rat hsp60 (39% inhibition). Hamster hsp60 was not available for this study. As with J77A.1 cells, excess unlabeled *C. pneumoniae* hsp60, *M. bovis* hsp65 or *E. coli* GroEL did not compete with hsp60-Alexa488 binding.

4. Discussion

The current study represents a first comparative analysis of a large number of hsp60 species for their interaction with innate immune cells. A first important observation was that three different mammalian hsp60 proteins, from rat, mouse and hamster, shared with human hsp60 the ability to elicit a pro-inflammatory response in macrophages, as determined by the secretion of TNFα. The same outcome was noted for two different mouse macrophage lines. We therefore conclude that the ability to stimulate macrophage activity is a common property of mammalian hsp60 species.

Interestingly, similar concentrations of the different mammalian hsp60 proteins were required to induce a TNFα response, and this applied also to the three microbial hsp60 species tested. In general, the extent of macrophage activation, as measured by the amounts of TNFα secreted, was comparable between mammalian and microbial hsp60 proteins. Most of the hsp60 preparations were also tested for the induction of NO formation. Here, the response was also dose-dependent, but human hsp60 was a better inductor than mouse, rat or microbial hsp60. All hsp60 species tested required a functional TLR4 on macrophages for pro-inflammatory activity. This observation extends similar findings reported previously for human and chlamydial hsp60 [9,11–15].

In the second part of this comparative study binding properties of hsp60 to the macrophage membrane were analyzed. These studies are based on our previous findings which showed that binding of fluorescence-labeled human hsp60 to mouse macrophages is concentration-dependent, saturable and can be competed by submicromolar concentrations of non-labeled human hsp60 [4]. Furthermore, binding of hsp60 occurred in the absence of TLR4, which suggests that TLR4 is essential for a pro-inflammatory response but not for initial binding [4].

We find here that mouse hsp60 is an equally potent inhibitor of binding to mouse macrophages as human hsp60. This indicates that the receptor for self hsp60 on mouse macrophages recognizes with similar avidity mouse and human hsp60. Rat hsp60 also showed cross-competition, and hence, all three hsp60 species are recognized by the macrophage receptor for self hsp60.

By contrast, neither hamster hsp60 nor any of the three microbial hsp60 were able to displace human hsp60 from its receptor, despite a 10- or 20-fold molar excess of the inhibitor. These data demonstrate some species restriction of the macrophage receptor for self hsp60. It seems probable that hamster and microbial hsp60 proteins employ other sites of binding, because all hsp60 species exhibit similar potency of inducing a TNFα response, at similar concentrations. The homology of amino acid sequences between mouse and microbial hsp60 species is around 50% whereas the homology between mouse and other mammalian species is above 95%. Therefore, relatively small differences between mouse and hamster hsp60 must account for the lack of cross-reactivity.

Our findings are supported by recent reports that the recognition of hsp70 by innate immune cells follows similar rules, i.e. human hsp70 does not share the binding site on innate immune cells with mycobacterial hsp70 [16,17] although binding sites reside on the same membrane protein [18]. Because of an important role of hsp60 in immunoregulation and the abundance of hsp60 proteins in both, mammalian and microbial cells, it may be mandatory for innate immune cells to distinguish extracellular or cell surface hsp60 by their endogenous or infectious origin. Hsp60 has been suggested to function as a danger antigen to the innate immune system, when released during cell necrosis at sites of inflammation or when released by microbial organisms [1,2,19]. In addition, hsp60 has been reported to deliver bound peptides to MHC presentation pathways upon endocytosis [20]. Taken together, these findings suggest that hsp60 may provide a link between innate and adaptive immunity, by facilitating peptide presentation and inducing co-stimulatory signals at the same time. In this context it should be noted that it is currently unknown whether the co-stimulatory activity of hsp60 is an endogenous property or whether immunostimulatory compounds such as LPS or lipopeptides are tightly bound by the chaperone and 'presented' to the innate immune system. Peptides presented by hsp60 include endogenous sequences of the chaperone molecule itself. The latter appear to be primary targets in several autoimmune diseases and have been found to induce disease-regulatory cells [21–23]. The important role of hsp60 epitopes in regulating self-tolerance and inflamma-

tory responses may render it advantageous that antigen presenting cells are able to distinguish between self and microbial hsp60. Indeed, it has been reported repeatedly that immunization with self versus microbial hsp60 or its peptides exerts quite opposite effects in animal models of autoimmune disease [21,24–27].

Taken together, our data indicate similar pro-inflammatory potency of different mammalian and microbial hsp60 but no common recognition mechanism. The latter may enable macrophages to distinguish between self and potentially pathogen-associated hsp60 proteins.

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