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The Receptor for Heat Shock Protein 60 on Macrophages Is Saturable, Specific, and Distinct from Receptors for Other Heat Shock Proteins

Christiane Habich, Karina Baumgart, Hubert Kolb, and Volker Burkart

Previous studies have shown that human heat shock protein (hsp) 60 elicits a strong proinflammatory response in cells of the innate immune system with CD14, Toll-like receptor (TLR) 2, and TLR4 as mediators of signaling, but probably not of binding. In the present study, we directly demonstrate binding of hsp60 to the macrophage surface and find the binding receptor for hsp60 different from the previously described common receptor for several other heat shock proteins, including hsp70, hsp90, and gp96. Fluorescence-labeled human hsp60 bound to cell surfaces of the murine macrophage lines J774 A.1 and RAW264.7 and to mouse bone marrow-derived macrophages. By flow cytometry, we could demonstrate for the first time that hsp60 binding to macrophages occurred at submicromolar concentrations, is saturable, and can be competed by unlabeled hsp60, but not by unrelated proteins, thus confirming the classic characteristics of specific ligand-receptor interactions. Binding of hsp60 at 4°C was followed by endocytosis at 37°C. Hsp60 binding to macrophages could not be competed by excess hsp70, hsp90, or gp96, all of which share the \( \alpha_2 \)-macroglobulin receptor as binding site. Hsp60 binding occurred in the absence of surface TLR4. However, no cytokine response was induced by hsp60 in TLR4-deficient macrophages. We conclude that hsp60 binds to a stereo-specific receptor on macrophages, and that different surface molecules are engaged in binding and signal transduction. Furthermore, the binding site for hsp60 is separate from the common receptor for hsp70, hsp90, and gp96, which suggests an independent role of hsp60 as danger Ag and in immunoregulation.

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hsp60 involves at least two functionally different components for binding and signaling.

Materials and Methods

Cell lines

The mouse macrophage cell line J774 A.1 was purchased from the German Collection of Microorganism and Cell Culture (Braunschweig, Germany). J774 A.1 cells were cultured in RPMI 1640 medium (PAA Laboratories, Linz, Austria) supplemented with 10% (v/v) FCS (Life Technologies, Rockville, CA), ampicillin (25 mg/L), penicillin (120 mg/L), streptomycin (270 mg/L), 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids (10 mM l-glutamic acid, 100 mM NaHCO3, and 10 mM HEPES). The mouse macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM (PAA Laboratories GmbH) supplemented with 4 mM L-glutamine, 1.4 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mM sodium pyruvate, and 10% (v/v) FCS.

Mouse bone marrow-derived macrophages

C57BL/10ScCr mice, lacking the complete TLR4 protein, and mice of the normal TLR4 protein, expressing control strain C57BL/10ScSn (27), were kindly provided by M. Freudenberg (Max Planck Institute for Immunology, Freiburg, Germany). C57BL/6J mouse, purchased from the breeding and Research Center (Bomholtgarden, Ry, Denmark), were used as a further control. Bone marrow cells were obtained by flushing femurs and tibias with ice-cold PBS and washed by centrifugation (500 × g, 5 min). A total of 3.5 × 10^6 bone marrow cells was incubated in tissue culture dishes with Pluznik medium (19, 28). After 7–8 days, adherent bone marrow-derived macrophages (BMM) were detached by incubation with ice-cold Ca2+-, Mg2+-free HBSS for 10 min, followed by washing with HBSS (500 × g, 5 min), and were used for binding studies.

Reagents

Recombinant human hsp60 was obtained from Peptor (Rehovot, Israel) or StressGen Biotechnologies (Victoria, BC, Canada). Recombinant human hsp60 was obtained from Peptor (Rehovot, Israel) or StressGen Biotechnologies, and recombinant mouse gp96 from IMMATICs Biotechnologies (Tübingen, Germany). Escherichia coli O6:B6 LPS, BSA, OVA, alcohol dehydrogenase (ADH), and αM were from Sigma (Deisenhofen, Germany), and transferrin was from Molecular Probes (Leiden, The Netherlands).

All proteins used in this study were tested for their endotoxin contents by quantitative Limulus amebocyte lysate assay (BioWhittaker, Verviers, Belgium). Endotoxin contents of hsp70, hsp90, and OVA were ≤0.25 EU/μg protein. The endotoxin amount of αM was ≤0.025 EU/100 ng protein, whereas gp96 was ≤1 EU/μg protein. For the various hsp60 preparations, an endotoxin content in the range of 0.01–0.02 EU/μg protein was determined. No difference in binding or competition for binding was observed for preparations varying in LPS content.

Protein labeling

Labeling of hsp60 and ADH with fluorescent dye was performed using the Alexa Fluor 488 Protein Labeling kit (Molecular Probes), according to the manufacturer’s recommendations. Briefly, 1 mg hsp60 or ADH was incubated with Alexa Fluor 488 in 0.1 M sodium bicarbonate for 1 h at room temperature, followed by incubation for 3 h at 4°C. Unconjugated dye was removed by extensive dialysis in PBS. The number of dye molecules bound per protein molecule was determined by measuring the OD at 280 and 494 nm. It was calculated that six to nine Alexa Fluor molecules bind to each protein molecule. Alexa Fluor 488-labeled transferrin was purchased from Molecular Probes, and gp96-FITC was supplied by IMMATICs Biotechnologies.

Hsp60 binding and uptake

After 2 days of continuous culture, J774 A.1 and RAW264.7 cells were gently washed off the culture flask. BMM were generated as described above. The cells were centrifuged at 500 × g for 5 min (4°C) and resuspended in PBS with 1% BSA for the binding assay (4°C) or in RPMI medium with 10% FCS for the uptake experiments (37°C). Cells of either macrophage line (1 × 10^6 cells/ml) were incubated in a total volume of 100 μl with various concentrations of Alexa488-labeled hsp60, transferrin, ADH, or FITC-labeled gp96 for 45 min on ice for the binding studies or for 15 min on ice, and subsequently for 30 min at 37°C for the uptake studies. For the competition assays, the macrophages were preincubated with the unlabeled ligands for 30 min on ice. Then the labeled ligand 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 containing 1% parafomaldehyde. The samples were evaluated using a FACScan flow cytometer (BD Biosciences, Rockville, CA). Cell surface binding of fluorescent-labeled proteins was calculated using the geometric mean fluorescence value after subtracting the autofluorescence of the cells.

Confocal microscopy

J774 A1 macrophages were adjusted to a density of 1 × 10^6 cells/ml and seeded on glass slides in a volume of 200 μl (2 × 10^5 cells). After incubation for 24 h (37°C, 5% CO2), the adherent cells were treated with hsp60-Alexa488 or transferrin-Alexa488 in the absence or presence of the unlabeled ligands at 4°C as described above. Then the cells were washed with PBS, followed by fixation with 1% parafomaldehyde for 15 min. Finally, the cells were washed three times with PBS and resuspended in 100 μl medium. Labeled cells were visualized using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Stimulation of macrophages for TNF-α and NO production

For the stimulation of TNF-α or NO production, mouse macrophages were adjusted to a density of 1 × 10^6 cells/ml and seeded in the wells of flat-bottom 96-well plates (200 μl/well). After cultivation at 37°C and 5% CO2 for 18 h, macrophages were incubated with hsp60 (Peptor and StressGen Biotechnologies) or LPS. After 6 h (TNF-α) and 24 h (NO), culture supernatants were collected and stored at −20°C until analysis.

TNF-α measurements

The amounts of TNF-α in culture supernatants were quantified by sandwich ELISA using an OpEIA mouse TNF-α Set (BD Pharmingen, San Diego, CA). A 96-well ELISA plate (BD Falcon, San Diego, CA) was coated with a capture Ab anti-mouse TNF-α mAb diluted in coating buffer (PBS, pH 6.5) overnight at room temperature. After washing (PBS/0.05% Tween 20, pH 7.4) and blocking (PBS/10% FCS, 1 h, room temperature), binding of TNF-α was performed by incubation of 50 μl culture supernatants diluted 1/10-fold with assay diluent or serial standard dilution of mouse TNF-α for 2 h at room temperature. Subsequently, the wells were washed, and a total of 50 μl biotinylated mouse TNF-α mAb and avidin-HRP conjugate was placed in each well (1 h, room temperature). After extensive washing, the samples were incubated with 50 μl substrate solution (tetramethylbenzidine and hydrogen peroxide, 20 min, room temperature). After addition of 50 μl stop solution (2 N H2SO4), the OD was measured at 450/550 nm on a microplate reader. The TNF-α content was calculated by using a standard curve of mouse rTNF-α with substrate solution as a blank.

Measurement of NO production

The amount of NO released by macrophages was assessed by the determination of accumulated nitrite (NO2−) in cell-free supernatants detected by the colorimetric Griess reaction, as described previously (29). Briefly, 50 μl supernatant and serial dilutions of NaNO2 standard solution were placed in a 96-well microtiter plate (Greiner, Frickenhausen, Germany) and then mixed with an equal volume of Griess reagent containing 1% sulfanilamide, 0.1% naphthylethenediamide dihydrochloride, and 2.5% H3PO4. After incubation for 10 min at room temperature, the OD of the reaction products was measured at 550 nm on a microplate reader, and the amount of accumulated nitrite in the samples was quantified using the standard curve obtained with NaNO2.

Results

Specific and saturable binding of hsp60 to mouse macrophages

A flow cytometry-based assay was established to investigate the interaction of human hsp60 with cells of the mouse macrophage lines J774 A.1 and RAW264.7. Therefore, human recombinant hsp60 was labeled with Alexa488 fluorescence dye under physiological conditions in PBS, resulting in the coupling of six to nine molecules of Alexa488 per molecule of hsp60. We used different hsp60 preparations for these binding studies and obtained reproducible results. The cells were incubated in the presence of hsp60-Alexa488 or for inhibition studies, the cells were preincubated with unlabeled proteins, followed by the incubation with hsp60-Alexa488. Throughout the assay, the temperature was kept at 4°C to minimize nonspecific endocytotic events. After washing and fixation of the cells, quantitative analysis of the binding was performed by measuring the fluorescence intensity of the cells.
In a first series of experiments, the intensity and specificity of hsp60 binding to J774 A.1 and RAW264.7 were investigated (Fig. 1). Binding of hsp60 to J774 A.1 (Fig. 1A) and RAW264.7 (Fig. 1B) cells was demonstrable as shown by the increasing mean fluorescence after hsp60 binding to 10 and 15 compared with the mean autofluorescence values of 2.5 and 2. The specificity of the hsp60 binding was proved by strong inhibition of hsp60-Alexa488 binding after preincubation with a 10-fold excess of unlabeled hsp60, whereas preincubation with the same molar excess of OVA was without effect. OVA was used as control because its binding should not interfere with binding sites for hsp60. To exclude the possibility of nonspecific binding of Alexa488-labeled proteins, similar binding assays were performed with Alexa488-labeled ADH, a cytosolic enzyme that should not bind to cell surface receptors. As expected, no significant binding of ADH-Alexa488 to the investigated macrophages could be observed (data not shown).

The binding of hsp60 to J774 A.1 cells was found to be dose dependent. As shown in Fig. 2A, the mean autofluorescence of the cells was \( \sim 3.6 \), and with increasing concentrations of hsp60 up to 1.2 \( \mu \)M, we observed higher mean fluorescence intensities in the cell preparations (8.8–15.8 mean fluorescence). Saturable binding of hsp60 to J774 A.1 cells was reached at \( \sim 700 \) nM.

Next we investigated the inhibition of hsp60 binding by unlabeled hsp60 (Fig. 2B). J774 A.1 cells were preincubated with increasing doses of unlabeled hsp60, followed by the addition of 350 nM hsp60-Alexa488. The mean fluorescence after hsp60-Alexa488 binding was \( \sim 8.2 \). Preincubation with increasing doses of unlabeled hsp60 in the range of 350 nM up to 1.75 \( \mu \)M (5-fold excess) resulted in an increased inhibition of hsp60 binding (6.8–3.9 mean fluorescence). At concentrations higher than 1.75 \( \mu \)M unlabeled hsp60, the inhibition remained at a range of \( \geq 70\% \), corresponding to a mean fluorescence signal of 3.9–3.

**Binding and uptake of hsp60**

To investigate, if binding of hsp60 is followed by the uptake of the bound ligand, we performed a further series of experiments with J774 A.1 cells incubated at 4°C and 37°C. In these experiments, Alexa-labeled transferrin was used as a positive control since the transferrin receptor is known to be expressed on the surface of all cell types (30). Furthermore, receptor-mediated uptake has been described for transferrin (31). At 4°C, preincubation with a 10-fold excess of unlabeled transferrin inhibited the binding of the fluorescent-labeled transferrin by 90%, whereas the same concentration of OVA did not affect transferrin binding (Fig. 3A). As shown before, preincubation with a 10-fold excess of unlabeled hsp60 strongly inhibited the binding of hsp60-Alexa488 at 4°C (Fig. 3C). Moreover, when we tested for competition of hsp60 binding by transferrin and vice versa, no inhibitory effects could be detected (Fig. 3, A and C). This finding underlines the specificity of hsp60 and transferrin binding to J774 A.1 cells.

Incubation at physiological temperature (37°C), at which endocytosis can occur, resulted in an apparent uptake of transferrin (Fig. 3B) and hsp60 (Fig. 3D), as suggested by the shift of the mean fluorescence from 8 to 60 for transferrin and from 10 to 50.
for hsp60. The specificity of the uptake was proved by the competition of the labeled proteins with the corresponding unlabeled ligand. Inhibition of the uptake was about 80% for both proteins (Fig. 3, B and D).

**Localization of bound hsp60 by confocal microscopy**

The specific binding of hsp60 to the cell surface of J774 A.1 cells was confirmed by the use of confocal microscopy. J774 A.1 cells were incubated either with hsp60-Alexa488 or transferrin-Alexa488 at 4°C for 45 min (Fig. 4, C and D). ADH-Alexa488 served as a negative control (Fig. 4B). Cells clearly showed surface binding of hsp60 and transferrin, but no binding of ADH.

**Hsp60 receptor is different from receptors of hsp70, hsp90, and gp96**

To further characterize the receptor for hsp60, we tested for an involvement of the receptor(s) for hsp70, hsp90, or gp96. J774 A.1 cells were preincubated with a 10-fold excess of either unlabeled hsp70, hsp90, gp96, or OVA at 4°C for 30 min, followed by the addition of 350 nM hsp60-Alexa488 (Fig. 5A). Preincubation

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**FIGURE 3.** Specific binding (A and C) or uptake (B and D) of transferrin (Tf)-Alexa488 (A and C) and hsp60-Alexa488 (B and D) by J774 A.1 macrophages. Cells were incubated with 100 nM transferrin-Alexa488 (A and C, solid line) or 350 nM hsp60-Alexa488 (B and D, solid line) at 4°C (A and B) or at 37°C (C and D) in the absence or presence of the indicated unlabeled ligands (10-fold molar excess). Cells were analyzed as described in Fig. 1. A and C, OVA plus transferrin-Alexa488 (black dashed line), hsp60 plus transferrin-Alexa488 (gray dashed line). B and D, OVA plus hsp60-Alexa488 (black dashed line), transferrin plus hsp60-Alexa488 (gray dashed line).

**FIGURE 4.** Cell surface binding of hsp60-Alexa488 and transferrin-Alexa488 by J774 A.1 macrophages. Cells were incubated with PBS/1% BSA (A, autofluorescence) or 425 nM ADH-Alexa488 (B) as control. For the binding, cells were incubated with 425 nM hsp60-Alexa488 (C) or 100 nM transferrin-Alexa488 (D) for 45 min at 4°C. The fluorescent staining patterns of the cells were analyzed by confocal microscopy.
with hsp70, hsp90, gp96, or αM led to negligible inhibition of hsp60 binding. OVA, which was used as control, did not compete hsp60 binding. The specificity of hsp60 binding was confirmed again by preincubation with unlabeled hsp60, resulting in an 80% inhibition of the binding of hsp60-Alexa488.

To confirm independently these findings, we investigated the binding of FITC-labeled gp96 to J774 A.1 cells (Fig. 5B). Specific binding of gp96 to J774 A.1 cells was observed, which could be inhibited by a 10-fold excess of unlabeled gp96. Preincubation with hsp60 did not reduce gp96 binding. Gp96 binding was also not inhibited by an excess of hsp90 or hsp70, whereas effective competition for gp96 binding was observed in the presence of unlabeled αM (65% inhibition) or gp96 (56% inhibition). Taken together, these results clearly indicate that the receptor for hsp60 on macrophages is not identical to the receptors for gp96, hsp90, and hsp70.

**TLR4 as a candidate receptor for hsp60**

Since previous observations (19, 20) indicate that TLR4 may mediate the inflammatory signaling of hsp60, we investigated the role of TLR4 as a potential binding receptor for hsp60. We therefore compared the binding of hsp60 to BMM of C57BL/10ScSn mice, which express the complete functionally active TLR4 protein (Fig. 6A), with the hsp60 binding to C57BL/10ScCr-derived BMM (Fig. 6B), which completely lack the TLR4 protein (27). Our experiments showed binding of hsp60 to the primary cultures of mouse BMM in a similar range as to J774 A.1 and RAW264.7 cells, thus demonstrating that hsp60 binding is not a property restricted to cells of macrophage lines. Binding of hsp60 to BMM of C57BL/10ScSn and C57BL/10ScCr mice occurred with comparable intensity and showed similar dose dependency. Incubation of BMM of C57BL/10ScSn mice (Fig. 6A) at 4°C with hsp60-Alexa488 in the indicated concentrations resulted in an increase of the mean fluorescence values to 13 and 17 compared with the mean autofluorescence value of 6.5. The binding of hsp60 to BMM of C57BL/10ScCr (Fig. 6B) for the tested hsp60 concentrations was in a similar range (14 and 18) as the binding to BMM of C57BL/10ScSn mice. As a further control, we tested hsp60 binding to C57BL/6Jbom mouse-derived BMM and observed binding in a similar range (data not shown). These results indicate that TLR4 does not make a major contribution to the binding of hsp60-binding receptor, although TLR4 is involved in hsp60 signaling.

**Biological activity of hsp60**

To confirm that the preparations of hsp60, used for the binding assays, were functionally competent, their stimulatory activity was tested on cells of the macrophage lines J774 A.1 (Fig. 7, A and B) and RAW264.7 (Fig. 7, C and D) by measuring their TNF-α and NO production. LPS (10 ng/ml), which was used as a positive control in these assays, induced the release of 16–22 ng/ml TNF-α in both cell lines. When J774 A.1 cells were exposed to hsp60 (Peptor or StressGen Biotechnologies), substantial amounts of...
TNF-α in the range of 12–19 ng/ml were detectable (Fig. 7A). In parallel, we determined TNF-α production in RAW264.7 cells (Fig. 7C) after incubation with hsp60 (Peptor or StressGen Biotechnologies). As shown in Fig. 7C, cells treated with hsp60 released TNF-α in a range of 11–18 ng/ml. We further analyzed the level of NO release by measuring the accumulated nitrite in cell-free supernatants. LPS (10 ng/ml), which served again as a positive control in these assays, stimulated the production of 61–79 μM nitrite in both cell lines. In the supernatants of J774 A.1 cells treated with hsp60 (Peptor or StressGen Biotechnologies), nitrite levels in the range of 29–47 μM (Fig. 7B) were detectable. In parallel, we determined NO production in RAW264.7 cells (Fig. 7D) after incubation with hsp60 (Peptor or StressGen Biotechnologies). As shown in Fig. 7D, cells exposed to hsp60 accumulated nitrite concentrations in a range of 72–81 μM in their supernatants. Taken together, the results clearly indicate that the tested hsp60 preparations were competent to induce a biological response in J774 A.1 and RAW264.7 cells, as shown by the stimulation of TNF-α and NO production.

Discussion
The results of this study provide strong evidence for a specific receptor for hsp60 on the surface of macrophages. Specificity of binding, saturability of this interaction, and the ability of the ligand to compete with itself are essential features of a ligand-receptor interaction and differentiate it from nonspecific adherence or endocytosis. These characteristics were demonstrated for the binding of human hsp60 to macrophages in this study. The observation of dose-dependent hsp60 binding to macrophage cell lines and to primary cultures of macrophages derived from bone marrow indicates that the ability to bind hsp60 is a general property of macrophages. Saturation of hsp60 binding occurred in the range of 700 nM. Competition experiments with unlabeled ligand and unrelated ligands as control revealed dose-dependent and specific displacement of the labeled ligand, yielding a $K_d$ of $\sim 300$ nM. When macrophages were incubated with hsp60 at $37^\circ C$ for 30 min, about 5 times more labeled ligand was seen per cell in comparison with binding studies at $4^\circ C$. This indicates that binding of hsp60 to the macrophage cell surface is followed by rapid endocytosis. The latter process was also inhibitable by excess unlabeled hsp60, and therefore involves prior specific binding to a hsp60 receptor on the cell surface. Binding and uptake of hsp60 therefore share the characteristics of receptor-mediated endocytosis with properties described for gp96 and heat shock cognate protein 70 (32).

We therefore analyzed whether hsp60 would make use of the receptor system described for other hsps. Recently, Binder et al. (23) have identified the α2M (CD91) receptor as a receptor for gp96. In this study, it was shown that gp96 as well as the phylogenetically related hsp90 and α2M effectively compete with labeled gp96 for binding to macrophages, and a common receptor for gp96 and hsp90 has been proposed (23, 24). Specific binding of hsp70 to macrophages and monocytes has also been recently shown (25, 33). Most recently, it has been demonstrated that gp96, hsp90, as well as hsp70 utilize the α2M receptor as a common receptor (26).
Our data provide evidence that the recognition unit for hsp60 is different from that used by hsp70, hsp90, or gp96. In a first experimental approach, we studied whether excess hsp70, hsp90, or gp96 would compete with binding of hsp60 to macrophages. We did not observe any cross-reactivity. αM, a potent inhibitor of the gp96 receptor, also did not decrease hsp60 binding. A second approach was to use labeled gp96 and attempt inhibition of binding by hsp60. gp96 bound to the cell surface with similar fluorescence intensity as observed for hsp60. Furthermore, we confirmed the specificity of binding in that unlabeled gp96, but not OVA was bound by the gp96 receptor, also did not decrease hsp60 binding. A second approach was to use labeled gp96 and attempt inhibition of binding by hsp60. gp96 bound to the cell surface with similar fluorescence intensity as observed for hsp60. Interestingly, a 10-fold molar excess of hsp70 or hsp90 was not able to significantly decrease binding of gp96. This does not exclude that hsp70 or hsp90 may interact with the gp96 receptor, albeit at much lower affinity. Indeed, in the binding studies published, much higher concentrations of hsp70 were required for displacement of gp96 (24). By contrast, Sondermann et al. (25) reported that effective displacement of labeled hsp70 by unlabeled hsp70 required inhibitor concentrations in a similar range as used in this study. These findings suggest the existence of a high affinity receptor for hsp70 in addition to the gp96 receptor.

Both preparations of human hsp60 used in this study showed proinflammatory activity, as evident from the induction of TNF-α and NO release in both macrophage lines. The biological activity of hsp60 is dose dependent, and a cytokine response requires concentrations of 1–3 μg protein/ml (18, 19). These concentrations correlate well with the dose dependency of hsp60 reported in this work. Since 50% saturation of binding sites is seen at about 0.3 μM, an inflammatory response may require binding to 10–20% of hsp60 receptors.

We and others have reported previously that CD14, MD-2, TLR2, and TLR4 may mediate the inflammatory signaling of hsp60 (19, 20, 34). However, these studies were confined to functional assays and did not determine actual binding. When considering the physicochemical variety of lipids, glycoconjugates, and peptides signaling via the TLR complex (21, 22), direct interaction with all of these potential ligands, including hsp60, seems improbable. Indeed, in a preliminary experimental approach, BMM of C57BL/10ScCr mice completely lacking the TLR4 protein showed similar hsp60-binding intensity and dose dependency as BMM of a control mouse strain with normal TLR4 expression. In addition, exogenous hsp60 could not provoke a functional response in BMM of TLR4-deficient C57BL/10ScCr mice and in BMM of C3H/HeJ mice that express a mutated TLR4 molecule unable to transduce a binding signal (Ref. 19, unpublished observations). We therefore assume that the CD14-TLR4 receptor complex is not involved in binding of hsp60, but mediates the further signal transduction of hsp60 in macrophages, downstream of ligand binding.

Taken together, our results provide evidence for the existence of a specific receptor for hsp60 binding and endocytosis, which is distinct from receptors for hsp70, hsp90, or gp96. We conclude that at least two different pathways for the recognition of hsp by innate immune cells have evolved, suggesting different roles of hsp60 vs hsp70, hsp90, and gp96 as danger Ags and in immunoregulation. Moreover, our results indicate that the cell surface structure interacting with hsp60 involves separated binding and signaling components, suggesting a more complex structure of hsp receptors than hitherto supposed.

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