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Heat Shock Protein 60: Specific Binding of Lipopolysaccharide[1]

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Human heat shock protein 60 (HSP60) has been shown to bind to the surface of innate immune cells and to elicit a proinflammatory response. In this study we demonstrate that the macrophage stimulatory property of recombinant human HSP60 is tightly linked to the HSP60 molecule and is lost after protease treatment. However, inhibition of macrophage stimulation was reached by the LPS-binding peptide magainin II amide. Indeed, HSP60 specifically bound [^{3}H]LPS. [^{3}H]LPS binding to HSP60 was saturable and competable by the unlabeled ligand. To identify the epitope region of the HSP60 molecule responsible for specific LPS binding, we analyzed the effect of several anti-HSP60 mAbs on HSP60-induced production of inflammatory mediators from macrophages. We identified only one mAb, clone 4B9/89, which blocked the macrophage stimulatory activity of the chaperone. The epitope specificity of this mAb points to the region aa 335–366 of HSP60. Clone 4B9/89 also strongly inhibited [^{3}H]LPS binding to HSP60. A more detailed analysis was performed by screening with selected overlapping 20-mer peptides of the HSP60 sequence, covering the region aa 331–380. Only one peptide blocked LPS binding to HSP60, thereby restricting the potential LPS-binding region to aa 351–370 of HSP60. Finally, analysis of selected 15-mer peptides and a 13-mer peptide of the HSP60 sequence revealed that most of the LPS-binding region was accounted for by aa 354–365 of HSP60, with the motif LKGK being critical for binding. Our studies identified a defined region of HSP60 involved in LPS binding, thereby implicating a physiological role of human HSP60 as LPS-binding protein. ***The Journal of Immunology,*** **2005, 174: 1298–1305.**

Heat shock proteins (HSPs)[3] are highly conserved intracellular proteins expressed in all pro- and eukaryotic cells, both constitutively and under stress conditions. Because of their important role in correct folding of newly synthesized proteins and subunit assembly, they are termed molecular chaperones. Furthermore, HSPs exert cytoprotective functions by preventing the aggregation of denatured proteins, initiating their refolding or proteolytic degradation (1–4).

Because of their potential roles in immunologically relevant processes, HSP60, HSP70, and HSP90 subfamilies have attracted increasing attention over the past years. Several studies have identified HSPs as targets of immune responses during microbial infections (5, 6). Because microbial HSPs and endogenous HSPs derived from damaged or stressed tissue display high sequence homology, immunological cross-reactivity to microbial HSPs has been suggested as a cause of the development of autoimmune disorders, including rheumatoid arthritis and diabetes (7–9).

For human HSP60, a strong capacity to stimulate proinflammatory reactivity has been described in human and murine cells of the innate immune system, such as macrophages and dendritic cells (DC) (10–13). This response includes the release of the inflammatory mediators TNF-α, NO, and IL-6. Additionally, HSP60 was found to induce gene expression of the cytokines IL-12(p70) and IL-15, which are involved in the promotion of a Th1 phenotype (10, 13). These findings suggest a role for HSP60 as a danger signal for the innate immune system (10, 14).

Recently, we characterized the binding receptor for human HSP60 on macrophages (15). Binding of fluorescence-labeled HSP60 was found to be specific, saturable, and competable by the unlabeled ligand. Additional approaches to identify the potential HSP60 binding receptor have shown that HSP60 binding to macrophages is independent of TLR4 (15), which is involved in LPS signaling (16). In contrast, we and others have found that the presence of a functionally active TLR4 protein is essential for the inflammatory signaling of HSP60 (12, 13, 17). These observations indicate that the contact between HSP60 and macrophages represents a highly complex process, including the interaction of HSP60 with cell surface structures involved in binding and in the initiation of a proinflammatory response.

Moreover, the striking similarities between the macrophage stimulatory properties of HSP60 and LPS have raised a concern that the recombinant HSP60 preparations might be contaminated with endotoxin, which, in turn, could be responsible for the observed inflammatory effects of HSP60 (14, 18, 19). Recent biochemical studies of recombinant HSP60 preparations lend additional support to this idea (20–22).

The present study was designed to identify the role of HSP60 vs LPS in macrophage stimulation. Our results indicate that HSP60 mediates its macrophage stimulatory effect by specifically bound LPS. Furthermore, we identified the HSP60 epitope region aa 354–365, including the motif LKGK, as being involved in specific LPS binding. This region is clearly different from the epitope of

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[3] Abbreviations used in this paper: HSP, heat shock protein; BMM, bone marrow-derived macrophage; DC, dendritic cell; EU, endotoxin unit; LBP, LPS-binding protein; MALP-2, macrophage-activating lipopeptide; PmB, polymyxin B.

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HSP60 responsible for binding to a surface receptor on macrophages, recently identified in the C-terminal region (aa 481–500) of the chaperone (23).

Materials and Methods

Cell lines and mice

The mouse macrophage cell line J774A.1 was purchased from the German Collection of Microorganisms and Cell Culture. J774A.1 cells were cultured in RPMI 1640 medium (PAA Laboratories) supplemented with 10% FBS (Invitrogen Life Technologies), ampicillin (25 mg/l), penicillin (120 mg/l), streptomycin (270 mg/l), 1 mM sodium pyruvate, 2 mM glutamine, nonessential amino acids (10 ml/l, 100×), 24 mM $NaHCO_3$, and 10 mM HEPES. C57BL/6J mice were purchased from The Jackson Laboratory. 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 were incubated in tissue culture dishes in 10 ml of Pluznik medium containing 5% horse serum, 15% FBS, 15% L929 cell-conditioned medium, and 65% RPMI 1640, supplemented as described above (17). After 6–7 days, adherent bone marrow-derived macrophages (BMM) were detached by Accutase (PAA Laboratories), followed by washing with RPMI 1640 (500 × *g*, 5 min), and were used for additional studies.

Reagents

Recombinant human HSP60 was obtained from Peptor. Low endotoxin recombinant human HSP60 (<0.05 endotoxin unit (EU)/μg protein) was purchased from StressGen Biotechnologies. Selected overlapping peptides of 20 aa, covering the region 331–380 of the sequence of the unprocessed precursor of human HSP60 (24), were obtained from Leiden University Medical Center (Leiden, The Netherlands). Selected overlapping 15-mer peptides, covering the region aa 346–370 of the human HSP60 sequence, were synthesized by automated simultaneous multiple peptide synthesis as described previously (25). Synthetic peptides of 13 aa, peptide 354–365 (corresponding to $_{354}$DAMLLKGKGDKA$_{365}$ of the human HSP60 molecule, N-terminally one amino acid was added for technical reasons) and an unrelated control peptide, were obtained from Eurogentec Deutschland. *Escherichia coli* O26:B6 LPS, *Tritirachium album* proteinase K, polymyxin B (PmB), magainin I, magainin II, magainin II amide, and OVA were purchased from Sigma-Aldrich. Transferrin was obtained from Molecular Probes. Recombinant human LPS-binding protein (LBP) was purchased from R&D Systems. *E. coli* K12 LCD25 [^{3}H]LPS and unlabeled LPS were obtained from List Biological Laboratories. Macrophage-activating lipopeptide-2 (MALP-2) was provided by Dr. P. Mühlradt (German Research Center for Biotechnology, Immunobiology Research Group, Braunschweig, Germany) (26). Murine anti-human HSP60 mAbs were obtained from StressGen Biotechnologies (clone LK1), Dianova (clone 4B9/89), and BD Transduction Laboratories (clone 24). Goat anti-mouse IgG Ab was used as an isotype control for all murine mAbs (Sigma-Aldrich). The recombinant human HSP60(Peptor) used in the macrophage-stimulating assays was tested for its endotoxin content by quantitative *Limulus* amebocyte lysate assay (BioWhittaker) and was found to contain <1 EU/μg protein. The LPS batch used in these assays gave a reactivity of 0.01 EU/pg. All other substances used in macrophage-stimulating assays showed no reactivity in the *Limulus* amebocyte lysate assay.

Stimulation of macrophages for TNF-α production

For stimulation of TNF-α production, J774A.1 cells and BMM from C57BL/6J mice 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 incubation for 18 h (37°C, 5% CO_2), LPS, recombinant human HSP60 (Peptor), or MALP-2 was added to the macrophage cultures. After another 6 h of incubation, supernatants were collected and stored at −20°C until analysis. To test their effects on the macrophage stimulatory properties of LPS, HSP60, or MALP-2, the different magainins were preincubated with the cells (30 min, 37°C). When analyzing the effect of anti-HSP60 mAbs, HSP60 was preincubated with the mAbs for 30 min at room temperature and then added to the cells. Heat treatment of LPS or HSP60 was performed by boiling the substances for 10 min before addition to the cells. For protease or PmB treatment, LPS or HSP60 was incubated with proteinase K (1 h, 37°C) or PmB (1 h, 4°C) before addition to the cells. The amounts of TNF-α in culture supernatants were quantified by sandwich ELISA using an OptEIA mouse TNF-α Set (BD Pharmingen) as described previously (10, 15). The TNF-α content was calculated by using a standard curve obtained with recombinant mouse TNF-α with substrate solution as a blank.

Radioactive [^{3}H]LPS binding assay

[^{3}H]LPS (sp. act., 0.695 μCi/μg LPS) binding was determined by incubating low endotoxin recombinant human HSP60 (StressGen Biotechnologies; 1 or 5 μg/ml, corresponding to 17 and 83 nM, respectively) or other control proteins with 500 nM [^{3}H]LPS for 30 min at 4°C in a final volume of 50 μl of 50 mM Tris (pH 7.0). When analyzing the effects of unlabeled LPS or Abs on [^{3}H]LPS binding to HSP60, these substances were preincubated (30 min, 4°C and room temperature, respectively) with HSP60 before [^{3}H]LPS was added. When analyzing the effects of different peptides on [^{3}H]LPS binding to HSP60, peptides were preincubated (30 min, 4°C) with [^{3}H]LPS before HSP60 was added. Bound vs free [^{3}H]LPS was assayed by vacuum filtration of the binding reactions on Protran nitrocellulose membranes (Schleicher & Schuell). Vacuum filtration was performed with a Bio-Dot microfiltration apparatus (Bio-Rad). Filters were rapidly washed with a total volume of 4.5 ml of ice-cold 50 mM Tris (pH 7.0), placed in 1 ml of scintillation fluid (Ultima Gold; PerkinElmer), and mixed, and the retained radioactivity was quantified by liquid scintillation counting. All binding reactions were corrected by subtraction of background values (membrane-bound [^{3}H]LPS in the absence of protein).

Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis was performed using Student's two-tailed *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

Characterization of the macrophage stimulatory property of HSP60

In the first series of experiments we tested whether the immunostimulatory principle of HSP60 is tightly associated with the intact chaperone molecule. Recombinant human HSP60 was digested by

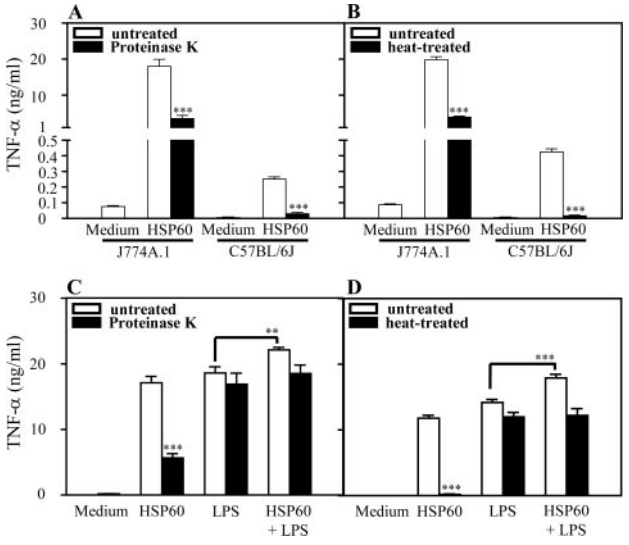

FIGURE 1. Effects of treatment with proteinase K and heat treatment on HSP60-stimulated TNF-α production from J774A.1 macrophages and BMM from C57BL/6J mice. *A*, Cells were incubated with medium or with 10 μg/ml untreated or proteinase K-treated (10 μg/ml, 1 h, 37°C) HSP60. *B*, Cells were incubated with medium or with 3 μg/ml untreated or heat-treated (boiling for 10 min) HSP60. *C*, J774A.1 macrophages were incubated with medium, 10 μg/ml untreated or proteinase K-treated HSP60, 1 ng/ml untreated or proteinase K-treated LPS, or 10 μg/ml HSP60 plus 1 ng/ml LPS (untreated or proteinase K-treated). *D*, J774A.1 macrophages were incubated with medium, 3 μg/ml untreated or heat-treated HSP60, 1 ng/ml untreated or heat-treated LPS, or 3 μg/ml HSP60 plus 1 ng/ml LPS (untreated or heat-treated). After 6 h of incubation at 37°C, TNF-α concentrations in the cell culture supernatants were measured by sandwich ELISA. Data represent the mean TNF-α concentration (nanograms per milliliter) ± SD of two independent experiments, each performed in triplicate. Significant differences from the corresponding control group are indicated: **, $p < 0.01$; ***, $p < 0.001$.

treatment with proteinase K (1 h, 37°C), a serine endopeptidase, which cleaves proteins with a very low specificity for amino acid sequences (27). As shown in Fig. 1*A* for J774A.1 macrophages and primary BMM from C57BL/6J mice, the HSP60 preparation lost most of its macrophage stimulatory activity after exposure to the protease. Next, HSP60 was denatured by boiling for 10 min (Fig. 1*B*), which led to a significant reduction of TNF-α production from J774A.1 macrophages as well as from BMM of C57BL/6J mice. These findings indicate that the stimulatory property of HSP60 on cells of a macrophage cell line as well as on freshly isolated primary BMM depends on the integrity of the HSP60 molecule. In contrast, when testing the stimulatory activity of LPS, we found that protease treatment did not alter the capacity of 1 ng/ml LPS to stimulate TNF-α production in J774A.1 macrophages (Fig. 1*C*). As an additional control, artificial mixtures of HSP60 and LPS were prepared and treated with proteinase K. Cells incubated with an untreated combination of HSP60 and LPS secreted significantly higher amounts of TNF-α (22.11 $\pm$ 0.39 ng/ml) than macrophages incubated with untreated LPS alone (18.59 $\pm$ 0.95 ng/ml; $p <$ 0.01). After proteinase K treatment of the mixture, TNF-α production dropped to a level similar to that induced with proteinase K-treated LPS alone. When incubating J774A.1 cells with untreated or heat-treated LPS (1 ng/ml), the cells produced comparable high levels of TNF-α (14.17 $\pm$ 0.46 and 11.99 $\pm$ 0.69 ng/ml, respectively; Fig. 1*D*). The experiment was repeated with a mixture of HSP60 and LPS. Cells exposed to the untreated mixture of HSP60 and LPS released significantly higher amounts of TNF-α (17.90 $\pm$ 0.58 ng/ml) than those from macrophages exposed to untreated LPS alone (14.17 $\pm$ 0.46 ng/ml; $p < 0.001$). After heat treatment of the mixture, TNF-α production dropped to almost the same level as that induced with heat-treated LPS alone. These experiments demonstrate that the macrophage stimulatory property of HSP60 cannot be accounted for by contaminating free LPS, but is tightly linked to the intact HSP60 molecule.

In addition, we analyzed the inhibitory capacity of several compounds known to bind LPS or LPS-like structures. First, we revisited the effects of the LPS inhibitor PmB (28) by studying dose dependency and using artificial mixtures of HSP60 and LPS. As shown in Fig. 2*A*, preincubation of LPS with PmB dose-dependently decreased TNF-α release from J774A.1 macrophages, almost reaching background levels at 10 μg/ml PmB. In contrast, preincubation with 0.1–10 μg/ml PmB did not suppress the capacity of HSP60 to stimulate TNF-α production. As an additional control, macrophages were stimulated with a mixture of HSP60 and LPS (Fig. 2*B*). Cells exposed to the untreated mixture of HSP60 and LPS released significantly higher amounts of TNF-α (6.93 $\pm$ 0.42 ng/ml) than those from macrophages exposed to untreated HSP60 alone (3.51 $\pm$ 0.09 ng/ml; $p < 0.001$). After preincubation of the mixture with PmB, TNF-α production dropped to a level similar to that induced by PmB-treated HSP60 alone. Although we observed interexperimental variations in TNF-α production, the results clearly demonstrate that PmB inhibits only the macrophage stimulatory activity of LPS, not that of HSP60. Additional analyses were performed with three closely related LPS-binding defensins of the magainin family, magainin I, magainin II, and magainin II amide (29, 30). As shown in Fig. 2 (*C* and *D*), increasing concentrations of magainin I or magainin II (0, 1, 3, and 10 μg/ml) failed to suppress HSP60- or LPS-induced TNF-α production from J774A.1 cells. The addition of magainin II amide resulted in a dose-dependent decrease in TNF-α production (Fig. 2*E*). A significant ($p < 0.01$) decrease in HSP60-stimulated TNF-α production was observed at a concentration of 1 μg/ml

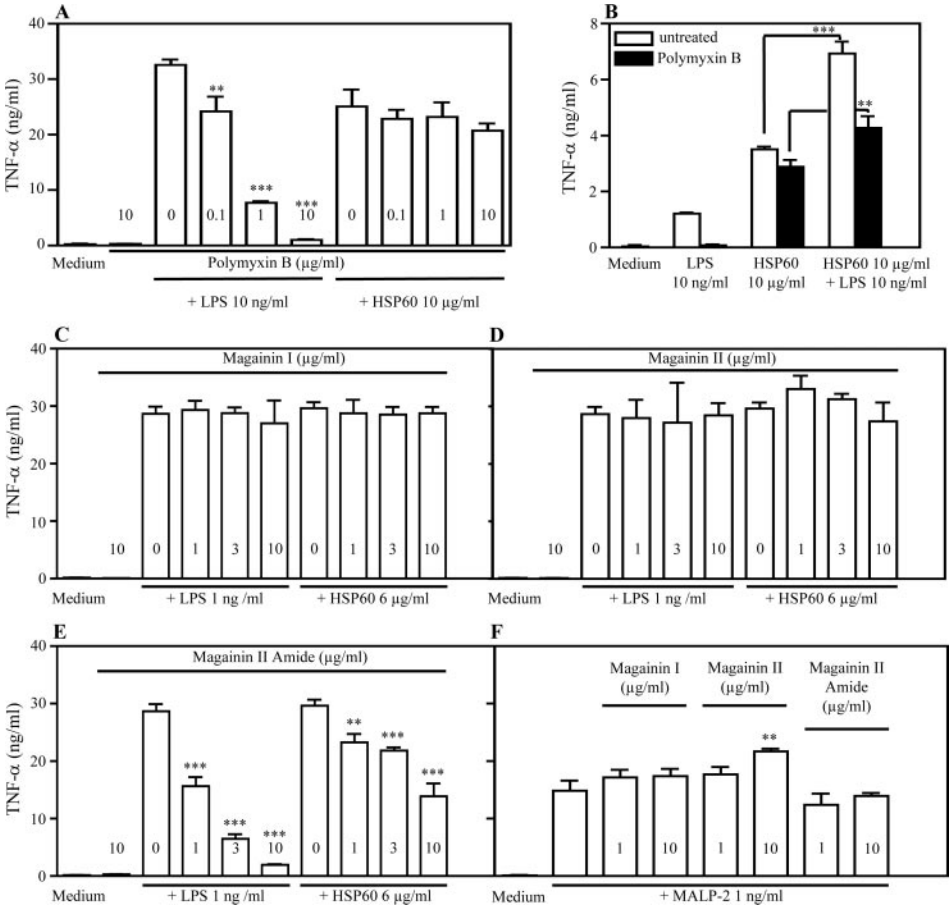

FIGURE 2. Effects of PmB, magainin I, magainin II, and magainin II amide on HSP60-stimulated TNF-α production from J774A.1 macrophages. *A*, Cells were incubated with medium, LPS (10 ng/ml), or HSP60 (10 μg/ml) pretreated with various concentrations of PmB (1 h, 4°C). *B*, Cells were incubated with medium, 10 μg/ml untreated or PmB-treated (10 μg/ml) HSP60, 10 ng/ml untreated or PmB-treated LPS, or 10 μg/ml HSP60 plus 10 ng/ml LPS (untreated or PmB-treated). J774A.1 cells were preincubated with increasing concentrations of magainin I (*C* and *F*), magainin II (*D* and *F*), or magainin II amide (*E* and *F*) for 30 min at 37°C. Subsequently, medium, 1 ng/ml LPS (*C–E*), 6 μg/ml HSP60 (*C–E*), or 1 ng/ml MALP-2 (*F*) was added. After 6 h of incubation at 37°C, TNF-α concentrations in the culture supernatants were measured by sandwich ELISA. Data represent the mean TNF-α concentration (nanograms per milliliter) $\pm$ SD of two to four independent experiments, each performed in triplicate. Significant differences from TNF-α production induced by LPS, HSP60, or MALP-2 alone are indicated: **, $p < 0.01$; ***, $p < 0.001$.

magainin II amide (23.26 ± 1.46 ng/ml). At concentrations of 3 and 10 μg/ml magainin II amide, the TNF-α response to HSP60 was reduced to 21.83 ± 0.54 and 13.87 ± 2.23 ng/ml, respectively ($p < 0.001$), corresponding to inhibitory effects of 26% and 53%. The stimulatory effect of LPS could also be inhibited dose-dependently by magainin II amide. Preincubation of J774A.1 cells with increasing doses of magainin II amide resulted in a reduction in TNF-α production to 1.96 ± 0.11 ng/ml at a concentration of 10 μg/ml magainin II amide. Essentially the same findings were observed with NO as a parameter of macrophage activation (data not shown). To exclude a nonspecific inhibitory activity of magainins, MALP-2 was used as a TLR4-independent stimulus of TNF-α secretion (31). None of the different magainins was found to inhibit MALP-2-induced TNF-α production from J774A.1 cells (Fig. 2*F*). Interestingly, preincubation of the cells with 10 μg/ml magainin II resulted in enhanced MALP-2-induced TNF-α production. These findings suggest that either the macrophage stimulatory epitope of HSP60 is an LPS-like molecule binding to HSP60, or the immunostimulatory epitope of HSP60 consists of negatively charged side chains of amino acids of the HSP60 peptide chain with a conformation mimicking the charge pattern of LPS.

HSP60 specifically binds LPS

Based on these findings, we analyzed the capacity of HSP60 to bind LPS by a radioactive binding assay. In this assay, [^{3}H]LPS and low endotoxin recombinant human HSP60 were mixed in solution, and free vs HSP60-bound [^{3}H]LPS was separated by vacuum filtration on a nitrocellulose membrane. As shown in Fig. 3, binding of [^{3}H]LPS to HSP60 could be demonstrated. Incubation of 83 nM HSP60 with 500 nM [^{3}H]LPS resulted in a significant ($p < 0.001$) increase in bound [^{3}H]LPS from 401 ± 170 cpm (background control, membrane-bound [^{3}H]LPS in the absence of protein) to 5912 ± 529 cpm in the presence of HSP60. By contrast, binding of [^{3}H]LPS to the same amounts of OVA or transferrin was in the range of the background control. LBP, which is known to be a highly effective LPS-binding protein (32, 33), was used as a positive control. As expected, LBP (83 nM) bound a significant amount of [^{3}H]LPS (71,940 ± 9,339 cpm). Binding of LPS to HSP60 (17 nM) dose-dependently increased up to 1 μM [^{3}H]LPS (2,874 ± 1494 cpm), where saturation of LPS binding was reached (Fig. 4*A*). At concentrations higher than 5 μM [^{3}H]LPS, we observed an additional considerable increase in LPS retention on the membrane, which was most likely caused by self-aggregation of LPS molecules independent of direct HSP60-LPS interactions. Next, we investigated the inhibition of [^{3}H]LPS binding to HSP60

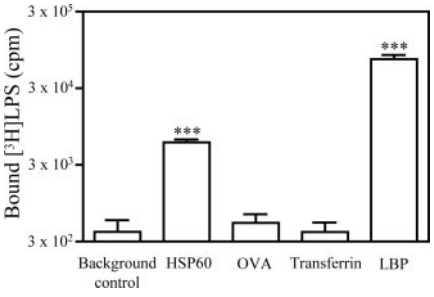

FIGURE 3. Binding of [^{3}H]LPS to various proteins. [^{3}H]LPS (500 nM) was incubated in the absence (background control) or the presence of 83 nM HSP60, OVA, transferrin, or LBP for 30 min at 4°C. Bound [^{3}H]LPS was separated from free [^{3}H]LPS by vacuum filtration. Data represent the mean ± SD cpm of bound [^{3}H]LPS in three independent experiments. Significant differences from bound [^{3}H]LPS in the absence of protein (background control) are indicated: ***, $p < 0.001$.

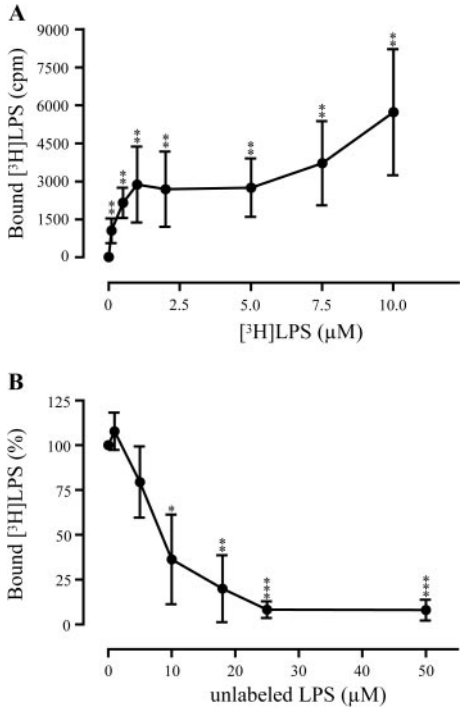

FIGURE 4. Dose dependence and inhibition of [^{3}H]LPS binding to HSP60. *A*, Low endotoxin HSP60 (17 nM) was incubated with increasing concentrations of [^{3}H]LPS for 30 min at 4°C as indicated. Bound [^{3}H]LPS was separated from free ligand by vacuum filtration. *B*, Low endotoxin HSP60 (83 nM) was preincubated with increasing concentrations of unlabeled LPS for 30 min at 4°C as indicated. Subsequently 500 nM [^{3}H]LPS was added, and the incubation was continued for another 30 min at 4°C. Bound [^{3}H]LPS was separated from free ligand by vacuum filtration. HSP60-bound [^{3}H]LPS in the absence of unlabeled LPS was set at 100%. Data represent the mean ± SD in cpm (*A*) or as a percentage (*B*) of bound [^{3}H]LPS minus background (bound [^{3}H]LPS alone) of two or three independent experiments. Significant differences from HSP60 in the absence of [^{3}H]LPS (*A*) or from HSP60-bound [^{3}H]LPS in the absence of unlabeled LPS (*B*) are indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

by unlabeled LPS as an additional proof of the specificity of this interaction (Fig. 4*B*). Binding of [^{3}H]LPS (500 nM) to HSP60 (83 nM) in the absence of unlabeled LPS was set at 100%. Preincubation of HSP60 with increasing doses of unlabeled LPS in the range of 1–18 μM (2- to 36-fold molar excess) resulted in an increased inhibition of [^{3}H]LPS binding to HSP60 up to 80%. At concentrations >18 μM unlabeled LPS, the inhibitory effect remained at ~92%. Taken together, the results of these experiments demonstrate direct and specific binding of LPS to the HSP60 molecule.

Identification of the LPS-binding site on the HSP60 molecule

Consequently, we attempted to identify the potential LPS-binding region on the HSP60 molecule. In the first approach we investigated the effects of several commercially available mAbs, directed against different epitopes of the native human HSP60 protein, i.e., clone 24 (aa 1–200), clone 4B9/89 (aa 335–366), and clone LK1 (aa 383–447), on HSP60-induced TNF-α production from J774A.1 cells (Table I). When analyzing these Abs in three different concentrations (1, 8, and 20 μg/ml), preincubation of HSP60 with mAb clone 4B9/89 resulted in a significant ($p < 0.01$) and dose-dependent decrease in TNF-α production to 42 ± 7% of the TNF-α production induced by HSP60 alone (set at 100%). None of the other tested mAbs to HSP60 or the IgG isotype control inhibited HSP60-induced TNF-α release from J774A.1 cells. These

Table I. *Effect of anti-HSP60 mAbs on HSP60-induced TNF-α production from macrophages*[a]

Anti-HSP60 mAb			
Clone	Recognized Epitope	Concentration (μg/ml)	Production of TNF-α (%)
—	—	—	100
4B9/89	aa 335–366	1	89 ± 10
		8	59 ± 6[b]
		20	42 ± 7[b]
Clone 24	aa 1–200	1	113 ± 20
		8	129 ± 10[c]
		20	122 ± 9[c]
LK1	aa 383–447	1	98 ± 20
		8	91 ± 10
		20	102 ± 15
IgG isotype control		1	114 ± 8
		8	101 ± 13
		20	111 ± 13

[a] HSP60 (3 μg/ml) was preincubated with various mAbs to HSP60 (30 min, room temperature) and then added to J774A.1 cells. TNF-α production was analyzed by ELISA, and TNF-α production induced by HSP60 alone was set at 100%. The data represent the mean percentage TNF-α ± SD of three independent experiments, each performed in triplicate. Significant differences from HSP60-induced TNF-α production are indicated.

[b] $p < 0.01$.

[c] $p < 0.05$

findings indicated that the region aa 335–366 of the HSP60 molecule is involved in the interaction with LPS. As a more direct proof, we investigated the effect of mAb clone 4B9/89 on the binding of [^{3}H]LPS to HSP60 in the radioactive [^{3}H]LPS binding assay (Fig. 5). Preincubation of low endotoxin human HSP60 (83 nM) with various concentrations of the mAb (1, 10, and 25 μg/ml) resulted in a significant ($p < 0.001$) decrease in LPS binding to HSP60 to 9 ± 5%. In contrast, mAb clone LK1 used as control did not inhibit LPS binding to HSP60.

In a more detailed analysis, we screened individual 20-mer peptides with an overlap of 10 aa, covering the human HSP60 region aa 331–380, which includes the region recognized by the mAb clone 4B9/89, for inhibiting [^{3}H]LPS binding to HSP60 (Table II). Preincubation of 75 μM peptide 351–370 with 500 nM [^{3}H]LPS resulted in a significant ($p < 0.001$) decrease in LPS binding to low endotoxin HSP60, i.e., to 54 ± 17%. In contrast, none of the other peptides interfered with LPS binding to HSP60. This result further restricted the region potentially involved in LPS binding to aa 351–370 of the HSP60 molecule.

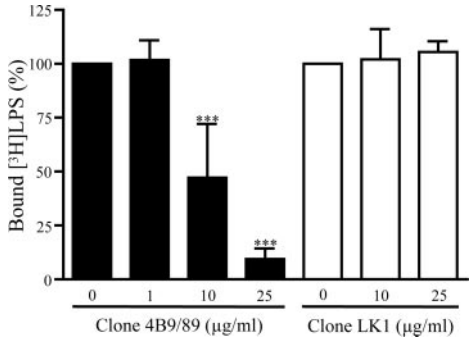

FIGURE 5. Effect of anti-HSP60 mAbs on [^{3}H]LPS binding to HSP60. Low endotoxin HSP60 (83 nM) was preincubated with various concentrations of mAbs clone 4B9/89 or clone LK1 for 30 min at room temperature as indicated. Subsequently, 500 nM [^{3}H]LPS was added, and the incubation was continued for another 30 min at 4°C. Bound [^{3}H]LPS was separated from free ligand by vacuum filtration. HSP60-bound [^{3}H]LPS was set at 100%. Data represent the mean ± SD percentage of bound [^{3}H]LPS minus mAb-bound [^{3}H]LPS in the absence of HSP60 in four independent experiments. Significant differences from HSP60-bound [^{3}H]LPS in the absence of mAbs are indicated: ***, $p < 0.001$.

In addition, we analyzed the effect of selected 15-mer peptides with a different overlap, covering the human HSP60 region aa 346–370 on [^{3}H]LPS binding to HSP60 (Table III). Preincubation of 75 μM peptide 351–365 with 500 nM [^{3}H]LPS resulted in a significant ($p < 0.001$) decrease in LPS binding to low endotoxin HSP60, i.e., to 69 ± 7%. This inhibitory effect was almost completely lost after preincubation with peptide 356–370. Peptide 346–360 had no inhibitory effect on LPS binding to low endotoxin HSP60. These findings indicated that the region aa 351–365 of the HSP60 molecule is potentially involved in LPS binding.

Finally, we analyzed the effect of a 13-mer peptide covering the region aa 354–365 of the human HSP60 molecule (Fig. 6). Preincubation of 500 nM [^{3}H]LPS with increasing concentrations of peptide 354–365 in the range of 0.5–15 μM resulted in a dose-dependent decrease in LPS binding to low endotoxin HSP60 from 100% (HSP60 alone) to 32 ± 5% ($p < 0.05$). At higher concentrations (35–100 μM) the inhibitory effect remained stable at ~65%. By contrast, an unrelated control peptide did not interfere with [^{3}H]LPS binding to HSP60.

To exclude the possibility that the observed inhibitory effects of peptide 351–370 and peptide 354–365 were due to competition of the peptides for LPS binding to HSP60, we analyzed the data of [^{3}H]LPS binding to the peptides. The mean values of peptide-bound [^{3}H]LPS were in the range of 18–23% (Table II and Fig. 6), and the values of the inhibitory peptides were in the same range, i.e., 21 ± 6% (peptide 351–370) and 17 ± 4% (peptide 354–365), thus showing no significant difference.

Taken together, these findings indicate that the region aa 354–365 of the HSP60 molecule is involved in specific binding of LPS.

Discussion

Our study demonstrates that the capacity of human HSP60 to activate innate immune cells depends on LPS specifically bound to the chaperone. Previous investigations have shown that the induction of a proinflammatory response by HSP60 in macrophages and DC is mediated via the TLR4/CD14 receptor complex, which is also involved in LPS signaling (13, 16, 17). Because of the similarity of the immunostimulatory properties of HSP60 and LPS, it had been suggested that endotoxin contaminations might be responsible for the observed inflammatory effects of the stress protein preparations (14, 18–22).

Our experiments revealed that the immunostimulatory property of human HSP60 is sensitive to protease treatment or heat denaturation, but cannot be inhibited by the potent LPS-binding peptide PmB. More importantly, when small amounts of LPS were added to HSP60 as defined contamination, the immunostimulatory properties became protease resistant, heat resistant, and PmB sensitive, reflecting the amount of LPS admixed. Furthermore, from studies with LPS-binding peptides other than PmB, we identified magainin II amide (29, 30), which interfered with the immunostimulatory property of human HSP60 and also of LPS. These findings indicate that macrophage stimulation by human HSP60 is not due to free contaminating LPS, but to LPS or structurally related molecules tightly bound to the intact HSP60 molecule.

This assumption was confirmed by analysis of the binding of radioactive-labeled LPS to low endotoxin human HSP60. For the first time we demonstrated a direct binding of LPS to HSP60,

Table II. *Effect of selected 20-mer peptides on [^{3}H]LPS binding to HSP60*[a]

Peptides	Binding of [^{3}H]LPS to HSP60 (%)
$_{335}$EDVQPHDLGKVGEVIVTKDDAMLLKGKGDKAQ[b]$_{366}$	
$_{331}$TLNLEDVQPHDLGKVGEVIV$_{350}$	91 ± 19
$_{341}$DLGKVGEVIVTKDDAMLLKG$_{360}$	107 ± 11
$_{351}$TKDDAMLLKGKGDKAQIEKR$_{370}$	54 ± 17[c]
$_{361}$KGDKAQIEKRIQEIIEQLDV$_{380}$	113 ± 6

[a] [^{3}H]LPS (500 nM) was incubated with 75 μM of the various 20-mer peptides for 30 min at 4°C. Subsequently, 83 nM HSP60 was added, and the incubation was continued for 30 min at 4°C. HSP60-bound [^{3}H]LPS in the absence of peptides was set at 100%. Data represent the mean ± SD percentage of bound [^{3}H]LPS minus background (bound [^{3}H]LPS alone) of two to six independent experiments. Significant differences from HSP60-bound [^{3}H]LPS in the absence of peptides are indicated. The mean value of peptide-bound [^{3}H]LPS was in the range of 23 ± 17%.

[b] HSP60 epitope recognized by mAb clone 4B9/89.

[c] $p < 0.001$.

which was specific, saturable, and competable by unlabeled LPS. Saturable binding of LPS to HSP60 was reached at 1 μM LPS, with a K_d of ~300 nM. At concentrations >5 μM LPS, we noted an additional increase in LPS retention on the membrane. This observation could be explained by increased aggregation of LPS molecules at higher concentrations. In solution, amphipathic molecules such as LPS usually occur as monomers. However, at higher concentrations of LPS, changes in physical properties may result in the formation of organized aggregates, i.e., micelles or lamellar structures (34). Another explanation for the finding could be the existence of additional low affinity binding sites on HSP60 for LPS. By analysis of our results we determined an occupancy ratio of ~2 mol LPS:1 mol HSP60.

Interestingly, two other chaperones, mammalian HSP70 and HSP90, have been reported to act as receptors for LPS (35, 36). In addition, it has been shown that HSP70 binds to other bioactive lipids and that the recognition of HSP70 by a CD14-containing receptor cluster is dependent on HSP70-lipid association (37). Although mammalian HSPs are typically regarded as being located intracellularly, they can be expressed on the surface of mononuclear cells and endothelial cells (38, 39). Moreover, soluble autologous HSP60 and HSP70 have been identified in the circulation of human individuals, where serum concentrations can reach the microgram level (40–42). Furthermore, LPS concentrations in blood range from 20–100 pg in the periphery to 1 ng close to the gut (43, 44) and therefore are sufficiently high to allow loading of extracellular HSP60 with LPS, even in the absence of bacterial infections, under physiological conditions. In view of these findings, it seems possible that the ability of HSP60 to bind LPS and present it to the TLR4/CD14 receptor complex represents a meaningful physiological property.

Table III. *Effects of selected 15-mer peptides on [^{3}H]LPS binding to HSP60*[a]

Peptides	Binding of [^{3}H]LPS to HSP60 (%)
$_{351}$TKDDAMLLKGKGDKAQIEKR[b]$_{370}$	
$_{346}$GEVIVTKDDAMLLKG$_{360}$	103 ± 17
$_{351}$TKDDAMLLKGKGDKA$_{365}$	69 ± 7[c]
$_{356}$MLLKGKGDKAQIEKR$_{370}$	78 ± 4

[a] [^{3}H]LPS (500 nM) was incubated with 75 μM of the various 15-mer peptides for 30 min at 4°C. Subsequently, 83 nM HSP60 was added, and the incubation was continued for 30 min at 4°C. HSP60-bound [^{3}H]LPS in the absence of peptides was set at 100%. Data represent the mean ± SD percentage of bound [^{3}H]LPS minus background (bound [^{3}H]LPS alone) of two independent experiments. Significant differences from HSP60-bound [^{3}H]LPS in the absence of peptides are indicated. The mean value of peptide-bound [^{3}H]LPS was in the range of 14 ± 29%.

[b] Sequence of the inhibitory 20-mer peptide (Table II).

[c] $p < 0.001$.

After these considerations, the previously described property of mammalian HSP60 to act as a danger Ag (10, 14) includes the element to serve as a sensor for danger signals and to present such structures to the innate immune system. The concept of HSP60 acting as a sensor for microbial structures may also be extended to other mammalian chaperones. HSP70 has been observed to bind LPS in a tight and PmB-resistant manner (45). High affinity and specific binding of LPS to gp96 has been shown in a recent study (46). Furthermore, HSP90 has been suggested to act as a primary receptor for immunostimulatory bacterial CpG DNA and to deliver these ligands to the TLR9 complex (47). An important biological function of mammalian chaperones seems possible, i.e., improvement of recognition of microbial structures by innate immune cells.

Subsequently, we tried to identify the epitope region of the human HSP60 molecule responsible for LPS binding. The first indication came from inhibition studies with mAbs directed against different epitopes of the native human HSP60 molecule. Only one mAb, clone 4B9/89, caused a dose-dependent inhibition of HSP60-stimulated TNF-α production from J774A.1 macrophages. This mAb was reported to bind to region aa 335–366 of the human HSP60 sequence (23). In addition to its capacity to interfere with the stimulatory activity of HSP60, this mAb strongly inhibited [^{3}H]LPS binding to HSP60 by >90%, thereby indicating that the

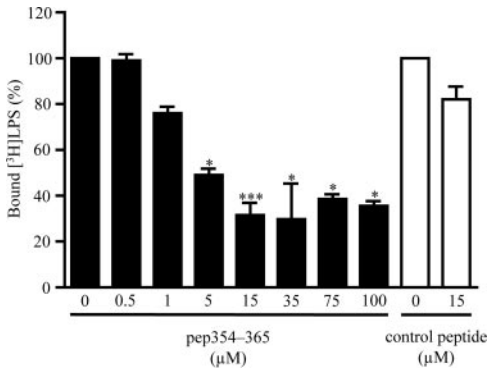

FIGURE 6. Effect of peptide 354–365 on [^{3}H]LPS binding to HSP60. [^{3}H]LPS (500 nM) was incubated with various concentrations of peptide 354–365 or an unrelated 13-mer control peptide for 30 min at 4°C as indicated. Subsequently, 83 nM HSP60 was added, and the incubation was continued for 30 min at 4°C. HSP60-bound [^{3}H]LPS was set at 100%. Data represent the mean ± SD percentage of bound [^{3}H]LPS minus peptide-bound [^{3}H]LPS in the absence of HSP60 in two independent experiments. Significant differences from HSP60-bound [^{3}H]LPS are indicated: *, $p < 0.05$; **, $p < 0.01$. The mean value of peptide-bound [^{3}H]LPS was in the range of 18 ± 14%.

region aa 335–366 of the HSP60 molecule is involved in LPS binding. By screening selected 20-mer peptides covering the region aa 331–380 of human HSP60 in the [^{3}H]LPS binding assay, we could further restrict the region of the LPS-binding epitope. Only peptide 351–370 strongly inhibited LPS binding to HSP60, whereas none of the adjacent 20-mer peptides did. To confirm these results, we screened the region aa 346–370 of the human HSP60 using a set of 15-mer peptides with a different overlap. In this approach we identified the peptide 351–365, which inhibited LPS binding to HSP60, but to a lesser extent than the 20-mer peptide 351–370. Comparison of the two inhibitory peptides revealed that both possess a common sequence motif, LKGK. Disruption of this motif abolishes the inhibitory capacity of the peptide. Peptides 341–360 and 346–360 possessing the incomplete motif (i.e., LKG) at their C terminus had no inhibitory effect on LPS binding to HSP60. The relative position of the LKGK motif within the peptide seems to be of critical importance. In the case of the 20-mer peptide 351–370, which showed the strongest inhibitory effect on LPS binding to HSP60, the LKGK motif is located in the central region of the peptide. By contrast, in the 15-mer peptide 351–365, which was less inhibitory, the motif LKGK is shifted C-terminally. Furthermore, in the 15-mer peptide 356–370, which exhibits almost no inhibitory effect on LPS binding, the LKGK motif is shifted N-terminally. Additional evidence for the position of the LPS-binding epitope came from the analysis of a 13-mer peptide covering the region aa 354–365 of the human HSP60 sequence. This peptide, with a centrally located LKGK motif, dose-dependently decreased [^{3}H]LPS binding to HSP60 to ~30% when tested in a range of 0.5–15 μM. These findings strongly indicate that the region aa 354–365 of the HSP60 molecule is involved in specific binding of LPS.

By searching for sequence homologies between the human HSP60 region aa 354–365 and other LPS-binding proteins, we identified factor C, present in the lysate of amebocytes of the horseshoe crab *Limulus polyphemus* (48). Factor C is a serine protease that initiates the coagulation cascade in the hemolymph of *Limulus* after contact with LPS. Recognition of LPS by factor C obviously depends on the presence of several high affinity LPS-binding regions on the protease, so-called sushi domains (49). By analysis of peptides derived from the sequence of sushi domains 1 and 3, a modification of sushi domain 1-derived peptide S1 (replacement of two amino acids with lysine residues, termed SΔ1) was found to improve the LPS-neutralizing capacity and LPS binding affinity compared with those of the wild-type S1 peptide (48). By sequence alignments of the LPS-binding region aa 354–365 of HSP60 with peptides S1 and SΔ1, we identified the amino acid motifs LKG and LKGK, respectively, displaying 100% identity. This finding also supports the relevance of the sequence motif LKGK in LPS binding to HSP60, as shown by our observations.

Based on the three-dimensional model of *E. coli* GroEL, the analog of mammalian HSP60, the identified LPS-binding region can be located in the apical domain of the HSP60 molecule. The contributing amino acids are involved in neither intramolecular contacts nor contacts to adjacent monomers (50, 51), indicating that the binding site is accessible to LPS in the monomeric as well as the oligomeric state of HSP60.

An additional search for sequence homologies in human LPS-binding proteins did not result in the identification of sequence identities. However, amino acid sequence homology does not necessarily allow us to deduce the LPS-binding capacity. For example, LBP and bactericidal/permeability-increasing protein, which are both members of the bactericidal/permeability-increasing protein family (52), are known to share only 45% primary structural identity, but their tertiary structures form closely similar regions, which are suggested to be involved in LPS binding (53).

Taken together, our present study identified a specific binding site for LPS on the human HSP60 molecule, region aa 354–365, including the critical sequence motif LKGK, which is suggested to be involved in macrophage stimulation. Furthermore, our data demonstrate that this region of the HSP60 molecule is different from the epitope responsible for binding to the surface of macrophages, previously identified as region aa 481–500 on the HSP60 molecule (23). In mammals, proteins such as HSP60, which is specialized for the recognition and binding of microbial structures, may serve important biological functions, i.e., the improvement of the efficiency of LPS recognition by innate immune cells.

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