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Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex[1]

Koji Ohashi, Volker Burkart, Stefanie Flohé, and Hubert Kolb[2]

Human heat shock protein 60 (hsp60) elicits a potent proinflammatory response in cells of the innate immune system and therefore has been proposed as a danger signal of stressed or damaged cells. We report here that macrophages of C3H/HeJ mice, carrying a mutant Toll-like-receptor (Tlr) 4 are nonresponsive to hsp60. Both the induction of TNF-α and NO formation were found dependent on a functional Tlr4 whereas stimulation of macrophages by CpG DNA was Tlr4 independent. We conclude that Tlr4 mediates hsp60 signaling. This is the first report of a putative endogenous ligand of the Tlr4 complex. ***The Journal of Immunology*****, 2000, 164: 558–561.**

Recent studies have suggested that autologous heat shock protein 60 (hsp60)[3] serves as a danger signal to the innate immune system. Mouse or human macrophages, as well as endothelial or smooth muscle cells, were found to elicit a proinflammatory response when incubated with recombinant human hsp60 (1, 2). The response included the up-regulation of adhesion molecule expression and the release of inflammatory mediators such as IL-6 and TNF-α. In addition, human hsp60 induced gene expression of IL-12 and IL-15 (2). These two cytokines are essential in driving the Th1 response. Since autologous hsp60 may be aberrantly expressed on the cell surface in response to stress (3, 4) and will be set free from the cell interior during necrosis, these findings point to a role of hsp60 in initiating or sustaining Th1-dependent tissue inflammation (2). Interestingly, microbial hsp60/65 also induces a proinflammatory response in innate immune cells (5–7), suggesting that damaged autologous cells and microbial pathogens may alert innate immunity via the same recognition system.

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[3] Abbreviations used in this paper: hsp, heat shock protein; Tlr, Toll-like receptor; ODN, oligodeoxynucleotide.

In search for a possible receptor for hsp60 on the macrophage cell surface, we were led by the striking similarity of innate immune responses to hsp60 and LPS although a role of contaminations of hsp60 with endotoxin could be excluded (1, 2). Since the stimulatory action of mycobacterial hsp65 was not inhibited by an Ab to CD14 (5), we tested for a role of Toll-like receptor 4 (Tlr4). The latter has recently been identified as product of the *lps* gene and to mediate LPS signaling in mouse cells (8–10). In human cells, Tlr2 rather than Tlr4 appears to be important in LPS binding and signaling, but the situation is less clear (11–13).

Toll-like receptors are the human homologue of the *Drosophila* Toll protein. They belong to the IL-1 receptor family containing repeated leucine-rich motifs in their extracellular portion and are linked to a signaling pathway that involves the IL-1-receptor-associated kinase and NF-κB (14, 15).

Materials and Methods

Reagents

Recombinant human hsp60 was obtained from StressGen Biotechnologies (Victoria, Canada). The two immunostimulatory oligonucleotides 5′-ACC GAT AAC GTT GCC GGT GAC G-3′ (Pal^+) (16) and 5′-TCC ATG ACG TTC CTG ATG CT-3′ (ODN1668) (17, 18) containing a CpG motif and the corresponding nonstimulatory oligonucleotide 5′-TCC ATG AGC TTC CTG ATG CT-3′ (ODN1720) (17, 18) lacking a CpG motif were purchased from Life Technologies (Karlsruhe, Germany). LPS from *Escherichia coli* B 0.26 was obtained from Sigma (Deisenhofen, Germany).

Mouse bone marrow-derived macrophages

C3H/HeN and C3H/HeJ mice were purchased from Charles River (Sulzfeld, Germany), and C57BL/6JBom mice were from Breeding & Research Center A/S (Bomholtgård, Ry, Denmark). Bone marrow cells were obtained by flushing femurs and tibias of 8- to 12-wk-old mice with ice-cold PBS. After washing, 2.5×10^6 bone marrow cells were incubated (37°C, 5% CO_2) in tissue culture dishes with 10 ml of Pluznik medium containing 5% heat-inactivated horse serum, 15% FCS (Life Technologies), 15% L929 cell-conditioned medium (19), and 65% RPMI 1640 supplemented with ampicillin (25 mg/L), penicillin (120 mg/L), streptomycin (270 mg/L), 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids (10 ml/L, 100×), 24 mM $NaHCO_3$, and 10 mM HEPES. After 7 days of cultivation, adherent bone marrow-derived macrophages were detached by incubation with ice-cold Ca^{2+}-, Mg^{2+}-free HBSS (Life Technologies) for 10 min followed by two washes with HBSS (500 g, 5 min). By nonspecific esterase stain (20), >98% of cells exhibited macrophage characteristics.

Stimulation of macrophages

Cells were seeded in 96-well flat-bottom microtiter plates (Falcon/Becton Dickinson, Franklin Lakes, NJ) (2×10^5 cells in 200 μl per well). After 24 h of preincubation (37°C, 5% CO_2), various concentrations of hsp60, LPS, and oligonucleotides were added to the cultures, and the incubation was continued for different time intervals.

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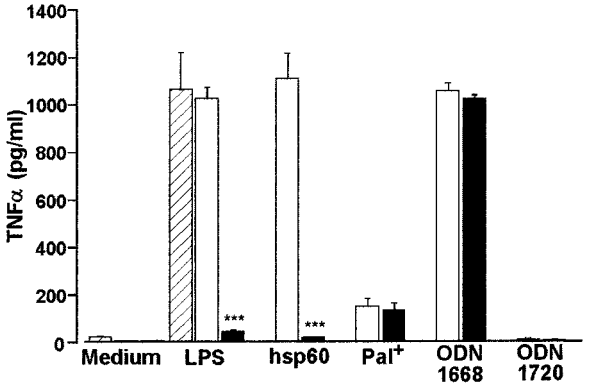

FIGURE 1. Differential effects of human hsp60 on TNF-α production from bone marrow-derived macrophages with wild-type or mutant Tlr4. Bone marrow-derived macrophages of C57BL/6 (▨), C3H/HeN (□), and C3H/HeJ mice (■) were incubated with medium, LPS (10 ng/ml), hsp60 (10 μg/ml), Pal$^+$ (30 μg/ml), ODN1668 (30 μg/ml), or ODN1720 (30 μg/ml). After 6 h, the TNF-α concentration in the culture supernatant was determined by ELISA. The data represent means ± SD of three to four experiments performed in quadruplicates. Significant differences to C3H/HeN (and C57BL/6) macrophages are indicated as ***, $p < 0.001$.

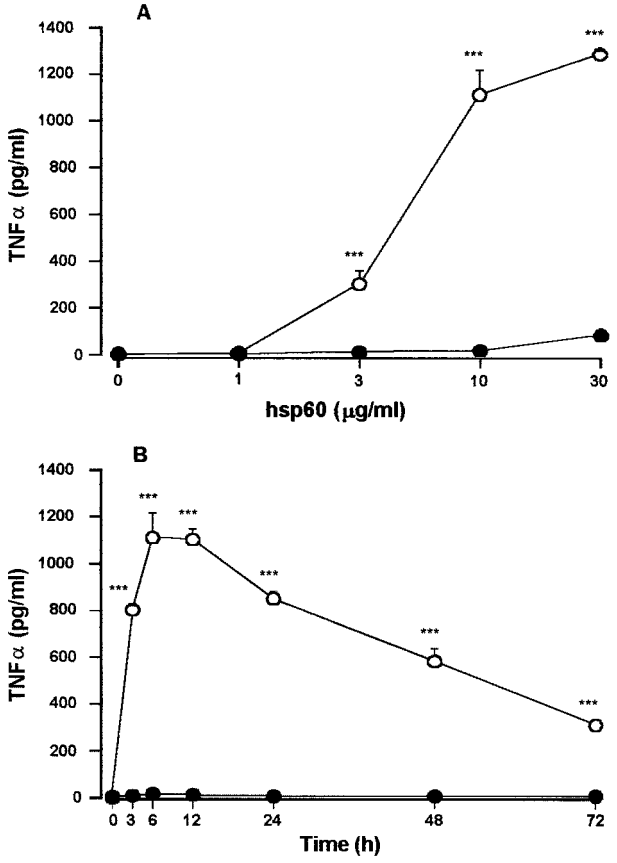

FIGURE 2. Dose dependency and time course of hsp60-induced TNF-α production. Bone marrow-derived macrophages of C3H/HeN (○) and C3H/HeJ mice (●) were incubated for 6 h with increasing concentrations of hsp60 (*A*) or they were incubated for different time intervals at a dose of 10 μg/ml hsp60 (*B*). The TNF-α concentrations in the culture supernatant were determined by ELISA. The data represent means ± SD from three experiments performed in triplicate. Significant differences to C3H/HeJ-derived macrophages are indicated as ***, $p < 0.001$.

TNF-α measurements

The amounts of TNF-α released from the macrophages were quantified by sandwich ELISA (2). The TNF-α was quantified using a standard curve obtained with the recombinant cytokine (Genzyme, Kent, U.K.) vs medium alone as blank. The results were expressed as pg TNF-α per ml.

Measurement of nitrite production

The amount of NO released by macrophages was assessed by determining the concentration of nitrite (NO_2^-) accumulated in the culture supernatant using the colorimetric Griess reaction as described previously (2). The results show micromoles of NO_2^- per milliliter.

Statistical analysis

Data were expressed as mean + SD. Statistical analysis was performed using the Student *t* test, two-sided. Differences were considered statistically significant with $p < 0.05$.

Results

Induction of TNF-α in macrophages by human hsp60 is Tlr4 dependent

Bone marrow-derived macrophages of mouse strains C57BL/6 and C3H/HeN responded to LPS or human hsp60 with rapid secretion of large amounts of TNF-α (Fig. 1). A parallel study of macrophages from C3H/HeJ mice showed no response to either LPS or hsp60. However, these cells were not completely refractory to inflammatory stimuli. An oligodeoxynucleotide derived from mycobacterial sequences (ODN1668) and containing a potent immunostimulatory CpG motif induced a strong TNF-α response in the LPS nonresponder strain. A second CpG containing oligonucleotide (Pal$^+$) was less stimulatory whereas a CpG-deficient oligonucleotide (ODN1720) did not provoke a response (Fig. 1).

The dose dependence of the response to hsp60 was analyzed with macrophages from the two C3H strains. A significant TNF-α response was obtained in C3H/HeN macrophages with 3 μg/ml hsp60 (0.05 μmol/L) whereas no such was response was seen in C3H/HeJ macrophages even at ten times higher hsp60 levels (Fig. 2*A*). An analysis of the kinetics revealed peak levels of secreted TNF-α between 6 and 12 h in C3H/HeN macrophages. In the Tlr4-defective macrophages, TNF-α production was absent throughout the observation period of 72 h (Fig. 2*B*).

Involvement of Tlr4 signaling in the induction of NO formation

Macrophage cultures were analyzed for their ability to respond with the production of NO to the various stimuli, by measuring the stable end product nitrite in supernatants. Macrophages of C57BL/6, C3H/HeN but not of C3H/HeJ mice responded to LPS challenge with NO production, which indicates that endotoxin induced NO formation is Tlr4 dependent (Fig. 3). The same outcome was obtained when hsp60 was taken as stimulus, with a complete lack of a NO response in Tlr4-defective macrophages. In contrast, the two macrophage types showed a very similar NO response when the strongly stimulatory CpG DNA ODN1668 was used as stimulus whereas the less stimulatory CpG oligonucleotide Pal$^+$ failed to induce NO formation, as was the case for the CpG deficient ODN1720 (Fig. 3).

Similar concentrations of hsp60 (3 μg/ml) were required for eliciting NO compared with TNF-α production. Increasing the hsp60 concentration 10-fold yielded about five times higher nitrite levels in C3H/HeN macrophages whereas Tlr4-defective macrophages remained completely refractory (Fig. 4*A*). Nitrite accumulation in the supernatant reached maximum levels between 48 and 72 h of culture. There was no indication of NO production in C3H/HeJ macrophages throughout this period (Fig. 4*B*).

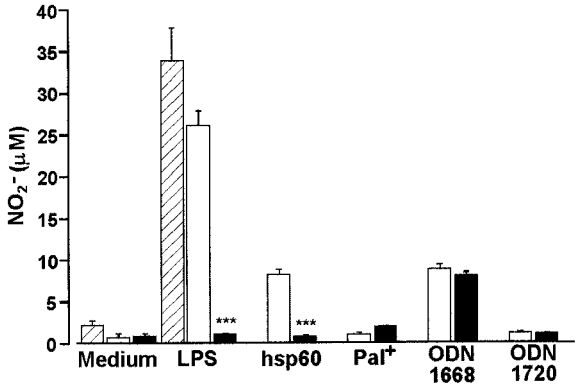

FIGURE 3. Differential effects of human hsp60 on NO production from bone marrow-derived macrophages with wild-type or mutant Tlr4. Bone marrow-derived macrophages of C57BL/6 (▨), C3H/HeN (□), and C3H/HeJ mice (■) were incubated with medium, LPS (10 ng/ml), hsp60 (10 μg/ml), Pal$^+$ (30 μg/ml), ODN1668 (30 μg/ml), or ODN1720 (30 μg/ml). After 24 h, the concentration of nitrite accumulated in the culture supernatant was determined by the Griess reaction. The data represent means ± SD of three to four experiments performed in quadruplicate. Significant differences to C3H/HeN (and C57BL/6) macrophages are indicated as ***, $p < 0.001$.

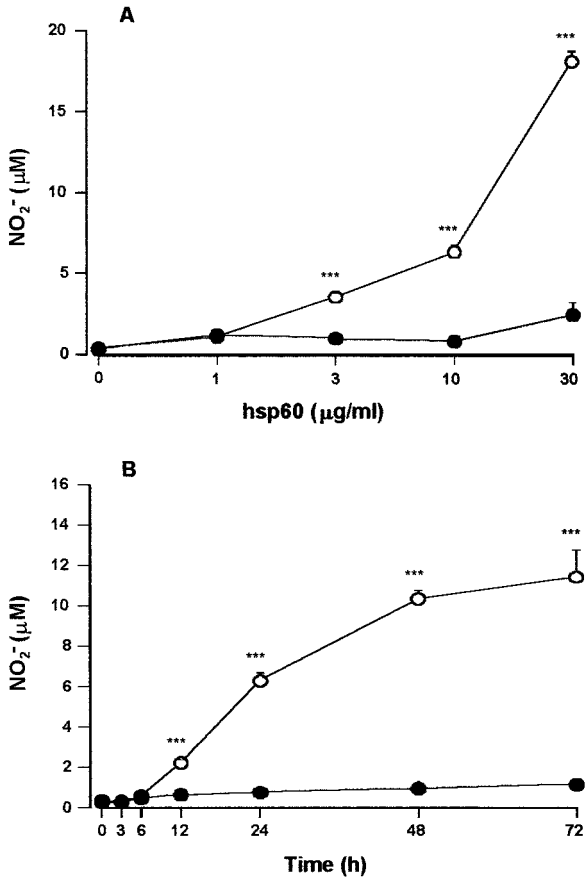

FIGURE 4. Dose dependency and time course of the hsp60-induced NO production. Bone marrow-derived macrophages of C3H/HeN (●) and C3H/HeJ mice (○) were incubated for 24 h with increasing concentrations of hsp60 (*A*), or they were incubated for different time intervals at a dose of 10 μg/ml hsp60 (*B*). The concentrations of nitrite accumulated in the culture supernatants were determined by the Griess reaction. The data represent means ± SD of three experiments performed in triplicate. Significant differences to C3H/HeJ-derived macrophages are indicated as ***, $p < 0.001$.

Discussion

The data presented suggest that extracellular hsp60 is an endogenous ligand of Tlr4. We compared bone marrow-derived macrophages of two closely related strains, C3H/HeN and C3H/HeJ, differing in the ability to respond to endotoxin with an inflammatory response (21, 22). This functional difference was recently identified as being due to a functionally defective Tlr4 membrane protein in C3H/HeJ mice. In the latter strain, the C-terminal part of Tlr4 contains a mutation at codon 712 that interferes with LPS-induced signaling (*lps*^{d}) (8–10). Interestingly, Tlr4-defective macrophages are not completely nonresponsive to LPS because endoxin is still able to stimulate the expression of metalloproteinase-9 (23).

Indeed, macrophages from C3H/HeJ (*lps*^{d}) mice were found clearly refractory to LPS-induced TNF-α production. Interestingly, inducible NO formation was also found strictly dependent on a functional Tlr4 although signaling requirements differ from that of TNF-α (24, 25). Exposure of *lps*^{d} macrophages to immunostimulatory CpG DNA did stimulate both TNF-α and NO production, which shows that Tlr4-dependent and -independent pathways for stimulating innate immune responses coexist in macrophages. Also, these data demonstrate that CpG DNA signaling does not occur via Tlr4.

Taken together, stimulation of TNF-α or NO response by human hsp60 was found here as fully dependent on the presence of a functional Tlr4 membrane protein. Thus, the same transmembrane signaling receptor appears to mediate the innate immune response to hsp60 and LPS. Extensive controls were performed in two previous reports of the immunostimulatory activity of extracellular mammalian hsp60 to exclude a role of endotoxin contamination (1, 2). These controls included the use of polymyxin B for neutralization of LPS, or denaturing of protein by heat treatment, which suppressed hsp60 but not LPS activity. Also, we were able to repeat the essential findings with an endotoxin-free preparation from another source (Peptor, Rehovot, Israel; less than 0.1 μEU of endotoxin contamination per μg hsp60; D. Elias, personal communication). Finally, endotoxin-free bacterial lipoproteins have been recently reported to mediate an innate immune response via the Tlr-2 pathway (26–28). These observations underscore that Tlr4 and T1r2 not only function as receptors of LPS (8, 12, 28) but are also involved in the recognition of protein ligands.

The previously described ligands for Toll-like receptors in mammalian cells are of microbial origin, which is in line with a function of these receptors in innate immune responses. We report here for the first time on a putative endogenous ligand of Toll-like receptors in mammals, the chaperone hsp60. This finding suggests that Toll-like receptors not only may have a function in innate immune defense against microbial pathogens but also may serve physiological functions by interacting with endogenous ligands. This is reminiscent of the situation in *Drosophila* where Toll controls dorsal-ventral patterning with S*pätzle* serving as endogenous ligand (29), whereas in adult insects Toll controls the antifungal and antibacterial response (30).

It is noteworthy that both Toll-like receptors and hsp60 are found early in phylogeny and both are of remarkably conserved structure. This indicates that their interaction is relevant and may also occur in more primitive organisms. Mammalian hsp60 usually is sequestered to the cell interior, in accordance with its ability to function as chaperone. However, hsp60 becomes accessible when it is set free during necrosis of tissue cells during inflammation or when hsp60 is partially translocated to the plasma membrane in

response to diverse types of stress (3, 4). We therefore have proposed that autologous hsp60 may serve as danger Ag to the innate immune system (2).

The exact mechanism of interaction between mammalian hsp60 and the Tlr4 complex remains to be elucidated. With CD14 and MD-2, two members of the Tlr4 complex have been identified, both of which strongly potentiate LPS responsiveness of Tlr4 (31, 32). LPS appears to bind to Tlr4 via CD14 (31) as well as independent of CD14 (32). For human T1r2, direct binding to LPS was demonstrated in vitro (11), and efficient signaling appears to require serum CD14 (12). Similarly, the mechanism of interaction between bacterial lipoproteins and T1r2 has not been determined (26–28).

In summary, the proinflammatory signaling of human hsp60 was found dependent on a functional Tlr4. This finding suggests the existence of endogenous ligands of the Tlr4 complex, and a role of Toll-like receptors in innate immune discrimination of normal vs stressed or damaged tissue cells.

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