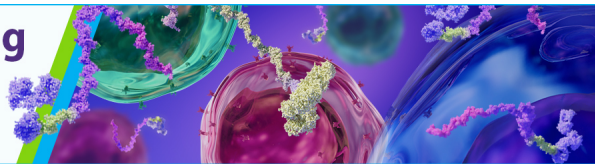


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Cutting Edge: Heat Shock Protein (HSP) 60 Activates the Innate Immune Response: CD14 Is an Essential Receptor for HSP60 Activation of Mononuclear Cells¹

Amir Kol,* Andrew H. Lichtman,[†] Robert W. Finberg,[‡] Peter Libby,*[†] and Evelyn A. Kurt-Jones^{2‡}

Heat shock proteins (HSP), highly conserved across species, are generally viewed as intracellular proteins thought to serve protective functions against infection and cellular stress. Recently, we have reported the surprising finding that human and chlamydial HSP60, both present in human atheroma, can activate vascular cells and macrophages. However, the transmembrane signaling pathways by which extracellular HSP60 may activate cells remains unclear. CD14, the monocyte receptor for LPS, binds numerous microbial products and can mediate activation of monocytes/macrophages and endothelial cells, thus promoting the innate immune response. We show here that human HSP60 activates human PBMC and monocyte-derived macrophages through CD14 signaling and p38 mitogen-activated protein kinase, sharing this pathway with bacterial LPS. These findings provide further insight into the molecular mechanisms by which extracellular HSP may participate in atherosclerosis and other inflammatory disorders by activating the innate immune system. *The Journal of Immunology*, 2000, 164: 13–17.

There is increasing interest in the role of nontraditional mediators of inflammation in atherosclerosis (1). Recent studies from our laboratory have shown that chlamydial and human heat shock protein 60 (HSP60)³ colocalize in human

atheroma (2), and either HSP60 induces adhesion molecule and cytokine production by human vascular cells and macrophages, in a pattern similar to that induced by *Escherichia coli* LPS (3, 4). These results suggested that HSP60 and LPS might share similar signaling mechanisms. CD14 is the major high-affinity receptor for bacterial LPS on the cell membrane of mononuclear cells and macrophages (5, 6). In addition to LPS, CD14 functions as a signaling receptor for other microbial products, including peptidoglycan from Gram-positive bacteria and mycobacterial lipoarabinomann (7, 8). CD14 is considered a pattern recognition receptor for microbial Ags and, with Toll-like receptor (TLR) proteins, an important mediator of innate immune responses to infection (9–14). We have examined the role of CD14 in the response of human monocytes and macrophages to HSP60.

Materials and Methods

Reagents

Recombinant human HSP60 was purchased from StressGen Biotechnologies Corporation (Victoria, BC, Canada). *E. coli* LPS, PMA, and polymyxin B were purchased from Sigma (St. Louis, MO). CD14-blocking mAb (clone MY4) was purchased from Coulter Immunology (Hialeah, FL). Recombinant chlamydial HSP60 was purified as previously described (3) and passed over a commercial endotoxin removal cartridge (END-X; Associates of Cape Cod, Wood's Hole, MA) following the manufacturer directions. Endotoxin levels were determined using a BioWhittaker *Limulus* amoebocyte lysate assay kit (Walkersville, MD) and resistance to heat-treatment (100°C, 20 min). (Different preparations of purified chlamydial HSP60 varied in their endotoxin content and heat resistance. In our previous studies, the macrophage-stimulating activity of the purified chlamydial HSP60 was completely heat labile (3). The preparation used in these studies was partially heat-resistant before removing residual endotoxin.)

Cell culture and analysis of conditioned medium

PBMC were isolated from leukopacs of healthy donors by gradient centrifugation, and human macrophages were subsequently isolated by adherence and culture in 2% human serum for 10 days, as previously described (3). Human astrocytoma cells (U373 MG) stably transfected with human CD14 cDNA (U373-CD14) and control U373 cells were provided by Dr. Douglas Golenbock (Boston University Medical Center) (15). Culture supernatants were harvested 18–24 h after stimulation, and IL-6 secretion was determined by sandwich ELISA (Endogen, Cambridge, MA). Data are expressed as the mean \pm SD of duplicate determination and are representative of 8–10 separate experiments for each cell type.

p38 mitogen-activated protein kinase (MAPK) assay

The activation of p38 MAPK was determined using an assay kit (New England Biolabs, Beverly, MA). Briefly, dually phosphorylated p38 MAPK was immunoprecipitated from cell lysates and subjected to in vitro

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³Abbreviations used in this paper: HSP, heat shock protein; TLR, toll-like receptor; MAPK, mitogen-activated protein kinase; ATF, activating transcription factor; CHO, Chinese hamster ovary.

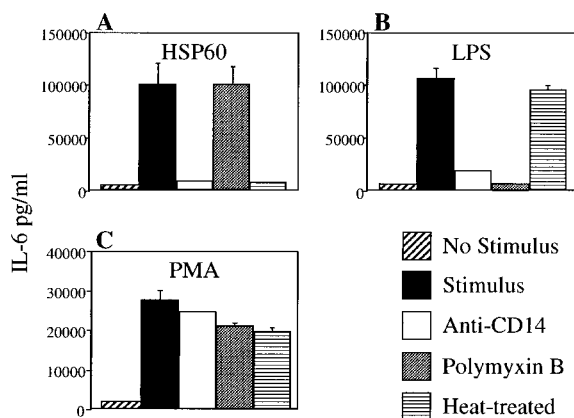


FIGURE 1. HSP60 induces IL-6 production by human PBMC in a CD14-dependent manner. PBMC were isolated from leukopacs of healthy donors. To define the optimal concentrations of HSP60 (StressGen) and *E. coli* LPS (Sigma), we tested a range of concentrations (0.1–3 $\mu\text{g/ml}$ for HSP60 and 1 ng/ml to 1 $\mu\text{g/ml}$ for *E. coli* LPS, optimal concentrations chosen for this study are shown). PBMC were incubated with medium only (unstimulated control), human HSP60 (1 $\mu\text{g/ml}$), *E. coli* LPS (10 ng/ml), or PMA (Sigma) (100 ng/ml). Supernatants were harvested 18 h later and analyzed for IL-6 by sandwich ELISA (Endogen). Where indicated, PBMC were treated with anti-CD14 Ab (10 $\mu\text{g/ml}$) (MY4 clone; Coulter Immunology) or polymyxin B (10 $\mu\text{g/ml}$) (Sigma) for 1 h before the addition of stimulant. In the indicated cultures, HSP60, LPS, and PMA were heat treated (100°C, 20 min) before addition to PBMC.

kinase assay with activating transcription factor 2 (ATF-2) as a kinase substrate. ATF-2 phosphorylation was determined by SDS-PAGE and Western blot analysis of the *in vitro* kinase reaction using a phospho-ATF-2-specific Ab.

NF- κ B activation in transfected hamster cells

Chinese hamster ovary (CHO) cells stably transfected with human CD14 and a reporter plasmid (NF- κ B response element of the E-selectin promoter fused to the coding region of the human CD25 gene) were provided by Dr. Douglas Golenbock (16). NF- κ B activation was measured by flow cytometric analysis of cells stained with anti-CD25 mAb (Coulter Immunology) or an isotype control (MOPC21; Sigma) followed by PE-labeled goat anti-mouse IgG antiserum (Sigma). LPS responsiveness in these cells is dependent on CD14 expression (17). Data are representative of five separate experiments.

Results

Human HSP60 stimulation of cytokine synthesis is CD14 dependent and not due to endotoxin

We tested the hypothesis that HSP60 activates mononuclear cells and macrophages through CD14 signaling. As a measure of cellular activation, we assessed the cellular production of IL-6, an important proinflammatory cytokine and mediator of the acute-phase response (18).

Human HSP60 induced the synthesis of IL-6 by PBMC (Fig. 1A). HSP60 induced IL-6 similarly to optimal concentrations of *E. coli* LPS (Fig. 1B). Preincubation of PBMC with an anti-CD14 Ab blocked IL-6 production in response to either HSP60 or *E. coli* LPS. PMA also induced IL-6 production, but the anti-CD14 Ab did not inhibit this response (Fig. 1C).

The recombinant HSP60 used in this study was purified from a fusion protein expressed in *E. coli* (19). Endotoxin levels were measured by *Limulus* amoebocyte lysate assay (BioWhittaker). No endotoxin was detectable in the recombinant human HSP60. To further exclude possible endotoxin contamination, HSP60 and *E. coli* LPS samples were heat treated (100°C, 20 min) before incu-

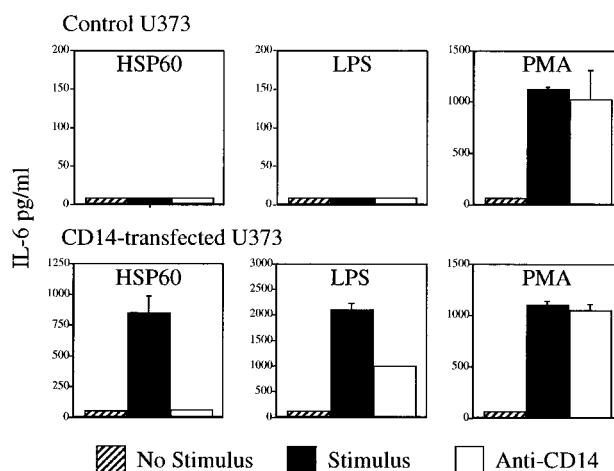


FIGURE 2. U373 cells transfected with human CD14 produce IL-6 in response to HSP60. Human astrocytoma cells (U373 MG; American Type Culture Collection, Manassas, VA), stably transfected with human CD14 (U373-CD14), were incubated with medium only (unstimulated control), human HSP60 (1 $\mu\text{g/ml}$), *E. coli* LPS (100 ng/ml), or PMA (100 ng/ml). Culture supernatants were harvested 18h later and analyzed for IL-6 by sandwich ELISA. Preincubation of U373-CD14 cells for 1 h with anti-CD14 Ab (MY4 clone, 10 $\mu\text{g/ml}$) inhibited IL-6 production in response to either HSP60 or *E. coli* LPS, but not to PMA (*lower panels*). Control, nontransfected cells responded to PMA only (*upper panels*).

bation with cells. Heat treatment abolished the ability of HSP60 to induce IL-6 (Fig. 1A), but, as expected, it did not affect the stimulation of IL-6 production by LPS (Fig. 1B), thus suggesting that the observed activity of the HSP60 was not due to endotoxin contamination. This result was verified by stimulating PBMC with HSP60 or *E. coli* LPS in the presence of polymyxin B, which blocks LPS-induced cellular activation (20). Polymyxin B abolished the PBMC response to LPS (Fig. 1B), but had no effect on the response of PBMC to HSP60 (Fig. 1A). Quantitatively and qualitatively similar results were obtained when we examined the response of monocyte-derived macrophages (not shown).

The results obtained with blocking studies suggest that CD14 is critical to the activation of human monocyte/macrophages by HSP60. To confirm this interpretation, we examined the role of CD14 in the response to HSP60 using a human astrocytoma cell line, U373. U373 cells constitutively lack responsiveness to LPS, but acquire this property when stably transfected with a human CD14 cDNA (U373-CD14) (15) or when cultured in the presence of recombinant soluble CD14 (7, 21). Nontransfected U373 responded to neither HSP60 nor *E. coli* LPS, but were activated by PMA (Fig. 2, *upper panels*). U373 cells transfected with CD14 acquired the ability to produce IL-6 when exposed to either HSP60 or *E. coli* LPS (Fig. 2, *lower panels*). The anti-CD14 Ab inhibited only the responses to HSP60 and to *E. coli* LPS, but not to PMA (Fig. 2, *upper and lower panels*), supporting the role of CD14 as a crucial receptor for HSP60 on mononuclear phagocytes.

Human HSP60 stimulates p38 MAPK activation

We further examined the intracellular activation pathways used by HSP60. The CD14-dependent activation of human monocytes induced by LPS involves the dual phosphorylation and activation of p38 MAPK (22). PBMC were treated with either HSP60 or *E. coli* LPS, and p38 MAPK activation was determined by immunoprecipitation of dual-phosphorylated p38 and *in vitro* kinase assay measuring phosphorylation of the p38 substrate, ATF-2. Kinetic

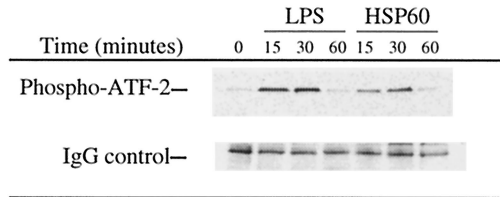


FIGURE 3. HSP60 activates p38 MAPK in PBMC. PBMC were incubated with human HSP60 (1 $\mu\text{g/ml}$) or *E. coli* LPS (10 ng/ml) for the indicated times and lysed in buffer containing Nonidet P-40 and phosphatase inhibitors. p38 MAPK activation was determined using a New England Biolabs assay kit. Briefly, dually phosphorylated p38 MAPK was immunoprecipitated from cell lysates and subjected to in vitro kinase assay with ATF-2 as a kinase substrate. ATF-2 phosphorylation was determined by SDS-PAGE and Western blot analysis of the in vitro kinase reaction and detected using a phospho-ATF-2-specific Ab (Phospho-ATF-2). Western blot analysis of Igs in the immunoprecipitates was performed to ensure equal protein loading (IgG control).

analysis showed that HSP60 and *E. coli* LPS induced similar patterns of p38 MAPK activation, with maximal activation of p38 observed within 15–30 min and a return to basal levels by 60 min (Fig. 3). In other experiments, pretreatment of PBMC with SB202190, a selective p38 MAPK antagonist (23), inhibited IL-6 production in response to either HSP60 or *E. coli* LPS by >90% (data not shown).

Chlamydial HSP60 stimulation of cytokine secretion is not due to endotoxin

We have also examined the ability of chlamydial HSP60 to stimulate monocytes. Recombinant chlamydial HSP60 was purified from *E. coli* as described (3). The initial preparation of chlamydial HSP60 contained 10 EU/ μg of endotoxin in the *Limulus* amoebocyte lysate assay. After passage through an endotoxin removal cartridge (END-X; Associates of Cape Cod), the endotoxin level was reduced to <1.5 EU/ μg . The endotoxin-depleted chlamydial HSP60 stimulated U373-CD14 cytokine secretion (Fig. 4A). Before endotoxin depletion, there was a heat-resistant activity in the purified chlamydial HSP60. This component was removed by passage over an endotoxin depletion column. The remaining cytokine-stimulating activity was entirely heat labile (Fig. 4A). Thus, chlamydial HSP60 directly stimulates cytokine secretion in U373-CD14 cells in the absence of endotoxin. Human PBMC also responded to a heat-labile component of chlamydial HSP60, and the response was blocked by anti-CD14 pretreatment (Fig. 4B).

Neither human nor chlamydial HSP60 activated NF- κ B in a hamster reporter cell line: CD14 is necessary but not sufficient for HSP60 signaling

We examined the ability of human and chlamydial HSP60 to activate NF- κ B in hamster cells transfected with human CD14 and an NF- κ B reporter gene. Transfection with human CD14 is sufficient to confer LPS responsiveness on CHO cells detected by increased CD25 expression measured by flow cytometry (Fig. 5) (16, 17). Human HSP60 and chlamydial HSP60 did not induce CD25 expression in the reporter cell line, although the cells were responsive to LPS (Fig. 5). This is in contrast to our results with transfected U373 cells (Fig. 3), where transfection with CD14 is sufficient to confer responsiveness to HSP60. Thus, CD14 expression in a human (U373) but not a rodent (CHO) cell line is sufficient to confer HSP60 responsiveness, suggesting that additional coreceptor proteins are required for HSP60 signaling in hamster cells. These experiments also demonstrate that the requirements for re-

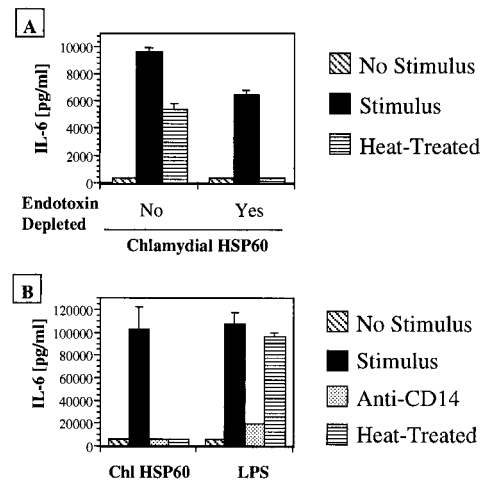


FIGURE 4. Chlamydial HSP60 induces IL-6 production. *A*, Recombinant chlamydial HSP60 was purified from *E. coli*. Purified chlamydial HSP60 was depleted of endotoxin by passage over an END-X (Associates of Cape Cod) endotoxin removal cartridge. Endotoxin levels in the initial and endotoxin-depleted chlamydial HSP60 preparation were determined by *Limulus* amoebocyte lysate assay (BioWhittaker). The initial chlamydial HSP60 preparation contained 10 EU/ μg endotoxin. After endotoxin depletion, the purified HSP60 contained <1.5 EU/ μg endotoxin. U373-CD14 cells were incubated with medium only (unstimulated control) or chlamydial HSP60 (3 $\mu\text{g/ml}$). Supernatants were harvested 18 h later and analyzed for IL-6 by sandwich ELISA (Endogen). Where indicated, chlamydial HSP60 preparations were heat treated (100°C, 20 min) before addition to cells. *B*, Human PBMC were stimulated with chlamydial HSP60, as described in Fig. 1. IL-6 secretion was measured by ELISA 18 h later. Where indicated, PBMC were preincubated with MY4 anti-CD14 Ab before the addition of chlamydial HSP60 (1 $\mu\text{g/ml}$) or LPS (10 ng/ml).

sponses to LPS and HSP60 are distinct and that the activity of the recombinant HSP60 proteins is not due to endotoxin.

Discussion

Recent studies from our laboratories and others have shown that HSP stimulate the production of cytokines from mononuclear cells (2, 3, 24–27). Both mammalian and bacterial HSP induced secretion of TNF- α and IL-6 (2, 3, 24, 25, 27). Our results show that CD14 signaling plays a fundamental role in mediating the activation of mononuclear cells and macrophages in response to human and chlamydial HSP60. (In contrast, *E. coli* groEL and *Mycobacterium tuberculosis* hsp65 stimulate monocytes in a CD14-independent manner (24, 28).) CD14 is a GPI-anchored membrane protein and, as such, it lacks a cytoplasmic domain (29). The mechanisms by which the signal mediated by CD14 is transduced across the cell membrane are not completely clear at present. Recent studies from our laboratory have suggested that the GPI-anchored CD14 is physically and functionally linked to heterotrimeric G proteins and src kinases in specialized glycolipid membrane microdomains (15).

Our studies with human CD14-transfected U373 (human) and CHO (hamster) cells demonstrate that CD14 is necessary but not sufficient for cellular responsiveness to HSP60, suggesting that human but not hamster cells express an important coreceptor for HSP60 signaling. TLRs have recently been shown to be important components of the CD14 signaling complex. TLRs are transmembrane proteins that contain large extracellular leucine-rich domains and intracellular domains that are homologous to the intracellular domain of the IL-1 receptor (12, 30). TLR2 and TLR4 have both been shown to contribute to the LPS response of human cells (9,

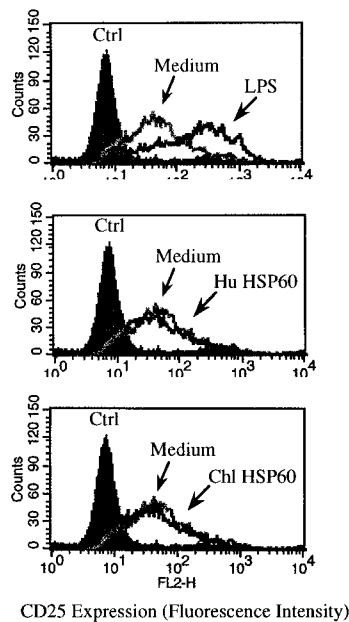


FIGURE 5. LPS activates NF- κ B in CHO cells, but human and chlamydial HSP60 do not activate NF- κ B. NF- κ B activation was measured using a CHO cell reporter line. These CHO cells are stably transfected with human CD14 and a NF- κ B reporter gene consisting of an E-selectin NF- κ B promoter sequence fused to the coding region of the human CD25 gene. The CHO-CD14 cells were incubated in medium alone or stimulated with LPS (10 ng/ml; *top panel*), human HSP60 (10 μ g/ml; *middle panel*), or chlamydial HSP60 (3 μ g/ml; *bottom panel*) for 18 h. After stimulation, the CHO cells were harvested with EDTA and incubated with monoclonal anti-CD25 Ab or isotype control Ab (CTRL), followed by PE-labeled goat anti-mouse antiserum. CD25 expression was determined by flow cytometry.

10, 31). Hamsters have recently been shown to have a null mutation in their TLR2 gene and therefore fail to express TLR2 protein on their cells (32). Despite the lack of TLR2, hamster cells are LPS responsive, suggesting that LPS responses in rodents may be primarily transduced through TLR4 (11, 32–34). In contrast, hamster cell responses to other CD14 ligands are TLR2 dependent, i.e., CHO cells are unresponsive to peptidoglycan and Gram-positive bacteria but acquire responsiveness when transfected with human TLR2 (and CD14) (35). We are currently investigating the role of Toll-like proteins in the response of cells to HSP60.

HSP may play a central role in the innate immune response to microbial infections. Because both microbes and stressed or injured host cells produce abundant HSP (36), and dying cells likely release these proteins, it is conceivable that HSP furnish signals that inform the innate immune system of the presence of infection and cell damage. The findings reported here, that human HSP60 induces IL-6 production by mononuclear cells and macrophages via the CD14, supports this hypothesis, suggesting that human HSP60 may act together with LPS or other microbial products to provoke innate immune responses.

Inflammation and immunity can contribute to the pathogenesis and complications of atherosclerosis (37). Moreover, the search for novel risk factors for atherosclerosis has revived the concept that microbial products might substantially contribute to the inflammatory reaction in the atheromatous vessel wall (38, 39). We have shown that chlamydial HSP60 colocalizes with human HSP60 in the macrophages of human atheroma (2). Therefore, bacterial and human HSP60, released from dying or injured cells during atherogenesis (40) or myocardial injury (41), may further promote local inflammation and possibly activate the innate immune system. Pre-

vious reports that immunization with mycobacterial HSP65 enhances atheroma formation in rabbits (42), have suggested an important role for HSPs in atherogenesis, particularly because the high degree of homology between HSPs of the same m.w. among different species might stimulate autoimmunity (43).

In conclusion, our findings, that CD14 mediates cellular activation induced by human HSP60 provide further insight into the molecular mechanisms by which HSP may activate the innate immune system and participate in atherogenesis and other inflammatory disorders.

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