Endocytosed HSP60s Use Toll-like Receptor 2 (TLR2) and TLR4 to Activate the Toll/Interleukin-1 Receptor Signaling Pathway in Innate Immune Cells*

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Heat shock proteins (HSPs) require no adjuvant to confer immunogenicity to bound peptides, as if they possessed an intrinsic “danger” signature. To understand the proinflammatory nature of HSP, we analyzed signaling induced by human and chlamydial HSP60. We show that both HSP60s activate the stress-activated protein kinases p38 and JNK1/2, the mitogen-activated protein kinases ERK1/2, and the I-κB kinase (IKK). Activation of JNK and IKK proceeds via the Toll/IL-1 receptor signaling pathway involving MyD88 and TRAF6. Human fibroblasts transfected with TLR2 or TLR4 plus MD-2 gain responsiveness to HSP60, while TLR2- or TLR4-defective cells display impaired responses. Initiation of signaling requires endocytosis of HSP60 that is effectively inhibited by serum component(s). The results revealed that adjuvanticity of HSP60 operates similar to that of classical pathogen-derived ligands.

Heat shock proteins (HSPs)† represent a collective of evolutionary conserved proteins, molecular chaperones, that bind nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of nonnative states of other proteins and assist them to reach functional conformation (1, 2). In addition, HSPs act as molecular shuttles for antigens (3). For example, certain HSPs isolated from (tumor) cells are associated with a large repertoire of...
Reagents and Antibodies—Recombinant human HSP60 was purchased from StressGen Biotechnologies (Victoria, Canada). The Escherichia coli expression vector for His epitope-tagged chlamydial HSP60 was kindly provided by B. Kaltenboeck (Auburn University, AL). Recombinant chlamydial HSP60 was produced by standard protein purification procedures using a Ni²⁺-nitrilotriacetic acid column (Qiagen, Hilden, Germany) followed by size exclusion chromatography (Superdex 200; Amersham Pharmacia Biotech). The purity of produced protein was determined by SDS-polyacrylamide gel electrophoresis and silver staining. Endotoxin contamination of recombinant chlamydial HSP60 was less than 0.1 pg/µg protein as determined by LAL-Test (Acila GMN, Weiterstadt, Germany). Phosphoantigen-stabilized CpG oligonucleotides 1668 (TCC ATG ACG TTC ATG CT) and 2006 (TCG TCG TTT TGT CTT GTC GTT) were purchased from TIB MOLBIOL (Berlin, Germany). LPS from Salmonella minnesota Re 595, polymyxin B (PMB), monodansylcadaverine (MDC), and anisomycin were purchased from Sigma, poly(dI-dC) was from Amersham Pharmacia Biotech, and murine recombinant interferon-γ (IFN-γ) was from PeproTech (Rocky Hill, NJ).

Antibodies to ERK1/2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and antibodies from New England Biolabs included anti-phospho-JNK1/2 (Thr183/Tyr185), anti-JNK1/2, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-STAT1 (Tyro701), and anti-STAT1.

Cell Culture and Transfection—The murine macrophage cell line RAW264.7 was grown in VLE-RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin G, and 100 IU/ml streptomycin sulfate (all from Biochrom KG). 5–1000 RAW264.7 cells were transfected by electroporation in a 400-µl final volume (RPMI, 25% FCS) at 280 V (for JNK kinase assay) or 300 V (for IKK kinase assay) and 960 microfarads in a Gene Pulser (Bio-Rad). 5 µg of HA-JNK1 or 9 µg of HA-IKKα were used for transfection together with different amounts of specific expression vectors as indicated in the figure legends. The overall amount of plasmid DNA was held constant at 30 µg/electroporation by the addition of the appropriate empty expression vector. After electroporation, cells were washed and split into six-well plates for subsequent stimulation and lysis.

The human embryonic kidney fibroblasts 293T were cultured in Dulbecco’s modified Eagle’s medium (Biochrom KG) with the same supplements as for RAW264.7 macrophage cell cultures. For luciferase reporter assays, the 293T cells were transfected by the calcium phosphate method as described (22). Briefly, 24 h before transfection, 293T cells were plated at 10⁵ cells/well in 12-well plates in 10% FCS, Dulbecco’s modified Eagle’s medium (Biochrom KG) with the same supplements, and split into six-well plates for subsequent stimulation and lysis.

Mice, Generation of BMDDC, and Determination of TNFα—Mice deficient in TLR2 generated by gene targeting were obtained from Tularic Inc. (San Francisco, CA, Briefly, a portion of the TLR2 gene sequence encoding a portion of the extracellular and of the transmembrane domain was replaced by gene targeting in ES cells from 129Sv/Av mice with a neomycin cassette oriented in the opposite direction of the gene (Deltagen Inc., Menlo Park, CA). TLR2-deficient mice were generated by aggregation in C57BL/6 mice and intercrossing of resulting heterozygous mice. Inactivation of the TLR2 gene was confirmed by Western blotting of extracts from thioglycolate-elicited peritoneal macrophages using rabbit polyclonal serum raised against TLR2. Groups of corresponding wild-type and TLR2-deficient mice were applied. All experiments used homozygous F1 generation mice. TLR4-mutated C3H/HeJ and wild-type C3H/HeN mice were purchased from Charles River (Sulzfeld, Germany). Age-matched groups of wild-type and TLR-deficient mice were used for the experiments.

Bone marrow-derived dendritic cells (BMDDC) were prepared as described previously (23). For stimulation, nonadherent BMDDC at days 7–9 were washed and plated at 7.5⋅10⁴ in medium without FCS, and after a 4-h rest cells were stimulated in duplicates for 18 h. Unless otherwise stated, stimulations were performed in the presence of 20 µg/ml PMB, sufficient to block at least 50 ng/ml LPS. TNF-α levels in culture supernatants were determined by a commercially available enzyme-linked immunosorbent assay kit (R&D Systems) according to the instructions of the manufacturer.

Western Blotting, Immunocomplex Kinase Assays, and Luciferase Assays—For Western blotting, cells were grown and stimulated as indicated in the figure legends. After stimulation, cells were lysed in Triton X-100 lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 20 mM β-glycophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. Lysates were boiled in SDS sample buffer, sonicated, centrifuged at 10,000 g for 10 min, resolved on a 10% SDS-polyacrylamide gel electrophoresis, and blotted onto Protran nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked in 5% skim milk solution, probed with the indicated antibodies, and visualized using the chemiluminescence kit Renaissance (PerkinElmer Life Sciences).

For in vitro kinase assays, 18 h after transfection cell cultures were
deprived of FCS for 2 h and then stimulated as indicated in legend of Fig. 4. Afterward, medium was aspirated, cells were lysed in Triton lysis buffer, and lysates were precleared by centrifugation at 10,000 × g for 10 min. To precipitate HA-tagged kinases, antibodies to the HA tag (clone 12CA5, Roche Molecular Biochemicals) together with protein A-Sepharose (Amersham Pharmacia Biotech) were incubated with the lysates overnight. Precipitates were washed 3 times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and equilibrated in kinase buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM β-glycerophosphate, 0.5 mM EDTA, 0.5 mM sodium fluoride, 0.5 μM sodium orthovanadate, 20 μM ATP). Kinase assays were initiated by adding [γ-³²P]ATP (Hartmann Analytic, Braunschweig, Germany) and the number of amino acids from C-JUN (GST-c-Jun (79)) for JNK or the number of amino acids from IKB (GST-IκBα (54)) for IKK. Reactions were performed at 30 °C for 30 min for JNK or 40 min for IKK and stopped by boiling in SDS sample buffer. Probes were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and the radioactive intensity was measured using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). For loading control, membranes were probed with antibodies to HA-tag (clone 3F10; Roche Molecular Biochemicals).

For luciferase assays, transfected cells were stimulated as described in Fig. 5A and lysed, and luciferase activity in extracts was measured was measured with the Luciferase Assay System kit from Promega (Mannheim, Germany) according to manufacturer’s instructions.

Protein Labeling and Live Cell Confocal Laser-scanning Microscopy—Chlamydial HSP60 was labeled using the Alexa Fluor™546 Protein Labeling Kit (Molecular Probes, Leiden, The Netherlands) following the manufacturer’s instructions. Concentration of protein after labeling was controlled by the BCA assay kit from Pierce. To study the cellular uptake of chlamydial HSP60, 7 × 10⁵ RAW264.7 macrophages were plated 1 day before image collection in Lab-Tek II two-chambered cover glasses (Nunc International, Wiesbaden-Biebrich, Germany) in 10% FCS, RPMI medium. Two h prior to stimulation, medium was exchanged with fresh 10% or 0% FCS-containing medium. 5 μg/ml Alexa Fluor™546-labeled chlamydial HSP60 was added, and uptake of protein was observed, saving pictures every 5 min. The specimens were held at 37 °C during the experiment. The inset shows one digitally magnified cell 30 min after the addition of labeled HSP60 in the absence of FCS. One representative experiment out of three is shown.

RESULTS

HSP60s Trigger Classical Signaling Cascades—Initial attempts to correlate HSP60-induced TNFα production with activation of IKK or JNK consistently resulted in weak signals or no signals. However, if macrophage cell line RAW264.7 cultures were deprived of FCS for several hours and then stimulated with HSP60, an unequivocal activation of, for example, stress-activated protein kinase JNK1/2 ensued (Fig. 1A). This was paralleled with production of higher amounts of TNFα at each concentration of HSP60 tested (Fig. 1B). The potential of contamination with LPS could be excluded, since all experiments were performed in the presence of PMB at concentrations able to neutralize 50 ng/ml LPS (Figs. 1B and 3B). We speculated that FCS might block the contact of HSP60 with macrophages. To analyze this possibility, we compared stimulation of cells with fluorescently labeled HSP60 in the presence or absence of serum. Labeling of HSP60 did not affect its stimulatory capacity as measured by TNFα production (data not shown). Confocal laser-scanning microscopy revealed that in the absence of FCS fluorescently labeled HSP60 became rapidly endocytosed by macrophages (Fig. 2). On the other hand, FCS (10% in culture medium) effectively blocked the internalization of HSP60 (Fig. 2). Overall, these data implied that component(s) within FCS interfered with HSP60 endocytosis by macrophages and perhaps with subsequent triggering of intracellular signaling cascades.

Having established that serum-free conditions allow efficient HSP60-mediated activation of macrophages, we compared the signaling pathways induced with those triggered by bacterial CpG-DNA or LPS. In addition to JNK1/2 (Fig. 1A) HSP60 also activated stress-activated protein kinase p38, although slightly delayed when compared with CpG-DNA (oligonucleotide 1668) or LPS (Fig. 3A and data not shown). Furthermore, chlamydial HSP60 and its human homologue activated IKK (see below), caused degradation of IκB-α (Fig. 3A) and induced in macrophages NF-κB luciferase reporter activity (data not shown). Finally, both HSP60s activated the classic mitogen-activated protein kinase ERK1/2 (Fig. 3A), as shown for LPS (24) or CpG-DNA (25).

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and then stimulated in the absence of FCS for 30 min with 2 H9262 cultures were deprived of FCS. Cells were stimulated with 1 RPMI medium were plated in 24-well plates. 2 h before stimulation, cell p-p38 activated form of p38 (\(\text{p-p38}\)), for I-kB protein preparations. Cells were lysed, and Western blotting for the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Cells were lysed, and Western blotting for the activated form of p38 (\(\text{p-p38}\)), for I-kB, and for activated forms of ERK1/2 (\(\text{p-ERK1/2}\)) was performed. Additionally, the total amount of p38 and ERK1/2 in lysates was determined. One representative experiment out of three is shown.

**FIG. 3.** Signaling cascades triggered by HSP60. A, 1 day before stimulation, 2.5 \(\times 10^5\) RAW264.7 macrophages/well in 0.5 ml of 10% FCS/RPMI medium were plated in 24-well plates. 2 h before stimulation, cell cultures were deprived of FCS. Cells were transfected with 1 \(\mu\)g/ml of human or chlamydial HSP60 for 60 and 120 min and with 2 \(\mu\)M CpG oligonucleotide 1668 for 30 min. 20 \(\mu\)g/ml PMB was used throughout the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Cells were lysed, and Western blotting for the activated form of p38 (\(\text{p-p38}\)), for I-kB, and for activated forms of ERK1/2 (\(\text{p-ERK1/2}\)) was performed. Additionally, the total amount of p38 and ERK1/2 in lysates was determined. One representative experiment out of three is shown.

**B**

|          | 10% FCS | 0% FCS |
|----------|---------|--------|
| +PMB     | +PMB    | +PMB   |
| CpG-DNA  | LPS     | LPS    |
| hHSP60   | hHSP60  | hHSP60 |
| cHSP60   | cHSP60  | cHSP60 |

**TRA6**—Pathogen-derived (exogenous) ligands such as LPS or bacterial CpG-DNA activate innate immune cells via the evolutionary ancient TIR signaling pathway (26, 27). Signaling through TIR is believed to occur via sequential recruitment of the adapter molecule MyD88, the IL-1 receptor-associated kinase, and the adapter molecule TRAF6 (18, 28). To address the question of whether HSP60s share intracellular signaling cascades with classic bacterial ligands, we employed transient transfections of RAW264.7 with dominant negative forms of signaling intermediates, concentrating our analysis on downstream activation of JNK and IKK. Having established that the peak of JNK and IKK activity in RAW264.7 macrophages stimulated with 5 \(\mu\)g/ml of HSP60 was reached at 60 and 30 min, respectively (data not shown), these time points were used to “read out” the effects of dominant-negative constructs.

First, we analyzed whether HSP60-mediated activation of the intracellular signaling cascades is controlled by MyD88. To this, RAW264.7 cells were cotransfected with either HA-tagged JNK1 or IKK\(\alpha\) together with a C terminus of MyD88 (MyD-C) known to act as a dominant negative version of MyD88 (28). As shown in Fig. 4A, increasing amounts of MyD-C inhibited dose-dependently both JNK and IKK activation after stimulation with human or with chlamydial HSP60. Dose-dependent expression of the MyD-C construct was confirmed by Western blotting (data not shown). To control for unspecific effects of MyD-C overexpression, we used poly(dI-dC) as an MyD88-independent stimulus (27). Fig. 4A shows that induction of JNK and IKK activity by poly(dI-dC) was not affected by transfection of dominant negative MyD-C.

TRAF6, originally cloned as a CD40-interacting molecule, is composed of a highly conserved C-terminal TRAF domain and an N-terminal RING finger domain (29). The overexpressed C-terminal TRAF domain (TRAF-C) acts as a dominant-negative molecule in TIR- and CD40-dependent signaling (18, 30, 31). To investigate whether TRAF6 is involved in HSP60-induced signaling, we cotransfected TRAF-C together with HA-tagged JNK1 or IKK\(\alpha\) into RAW 264.7 cells, activated them with human or chlamydial HSP60, and measured induced kinase activities. As shown in Fig. 4B, TRAF-C suppressed dose-dependently both JNK and IKK activation induced by HSP60 yet did not affect JNK and IKK activity triggered by poly(dI-dC). Dose-dependent expression of TRAF-C construct was confirmed by Western blotting (data not shown). These results implied that HSP60s signal via MyD88 and TRAF6, leading to downstream activation of JNK and IKK.

**HSP60s Signal via TLR2 and TLR4**—Activation of the TIR pathway by bacterial ligands LPS, peptidoglycans, or CpG-DNA is brought about via TLR4, TLR2, or TLR9, respectively (17, 32–34). Participation of MyD88 and TRAF6 in HSP60-triggered signaling events prompted us to consider the involvement of Toll-like receptors. To test whether one of these TLR acts as a HSP60 receptor component we resorted to the system of transient reconstitution of unresponsive human embryonic kidney fibroblasts 293T. These cells were transiently transfected with luciferase reporter driven by synthetic enhancer harboring NF-\(\kappa\)B binding consensus sites. Upon cotransfection of human TLR2 expression vector but not of the empty control vector, both human and chlamydial HSP60 activated the NF-\(\kappa\)B reporter in these cells (Fig. 5A). Furthermore, transient transfection with human TLR4 was insufficient to make 293T responsive to HSP60, yet cotransfection of TLR4 plus human MD-2 conferred responsiveness (Fig. 5A). Cotransfection of TLR2 and TLR4 plus MD-2 did not yield in synergistic responsiveness (data not shown), indicating that TLR2 and TLR4 appear not to cooperate in the response to HSP60, at least in regard to NF-\(\kappa\)B activation. Finally, transfection of a human TLR9 construct selectively conferred responsiveness to CpG-DNA but not to HSP60 (Fig. 5A), implying specificity in “gain of function.” These data suggested that TLR2 or a complex of TLR4 and MD-2 represent essential receptor components for endogenous (human) and exogenous (chlamydial) HSP60.

To verify the gain of function experiments, we performed “loss of function” assays by using BMDDC from TLR2-k.o. mice and from C3H/HeJ mice known to carry the codominant point mutation Lpsd that compromises the function of TLR4 (32). Compared with wild type BMDDC, TLR2-deficient BMDDC displayed a strongly reduced capacity to respond to HSP60 as measured by TNF\(\alpha\) production (Fig. 5B). The reactivity to LPS
was, however, only slightly compromised due to contaminating lipoproteins in the commercially available LPS preparation (35). Similarly, TLR4-defective BMDDC from C3H/HeJ mice responded under serum-free conditions to HSP60, but the response was curtailed compared with control cells from C3H/HeN mice (Fig. 5B). To control for equal stimulatory conditions for both wild-type and mutant cells, we included stimulation with PMB throughout the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Afterward, cells were assayed for TNF-α in supernatants was measured. Each bar represents the mean of two stimulations, with error bars showing S.D. values. One representative experiment out of two is shown.

FIG. 4. HSP60 activates macrophages through MyD88 and TRAF6. A, RAW264.7 macrophages were cotransfected with 5 µg of HA-JNK1 or 9 µg of HA-IKKα and increasing amounts of MyD-C. 18 h after transfection, cells were deprived of FCS and 2 h later stimulated with 5 µg/ml human HSP60, 5 µg/ml chlamydial HSP60, and 100 µg/ml poly(dI:dC). Stimulation with HSP lasted 60 min for JNK kinase assay (KA) or 30 min for IKK kinase assay. Stimulation with poly(dI:dC) lasted 90 min for JNK kinase assay and 45 min for IKK kinase assay. 20 µg/ml PMB was used throughout the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Cells were lysed and lysates were assayed for JNK or IKK activities by Western blotting. The induction of kinase activities was calculated and is shown. B, RAW264.7 macrophages were cotransfected with 5 µg of HA-JNK1 or 9 µg of HA-IKKα and increasing amounts of TRAF-C. 18 h after transfection, cells were deprived of FCS, and 2 h later they were stimulated with 5 µg/ml human HSP60, 5 µg/ml chlamydial HSP60, and 100 µg/ml poly(dI:dC). Stimulation with HSP lasted 60 min for JNK kinase assay or 30 min for IKK KA. Stimulation with poly(dI:dC) lasted 90 min for JNK kinase assay and 45 min for IKK kinase assay. 20 µg/ml PMB was used throughout the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Cells were lysed and lysates were assayed for JNK or IKK activities by Western blotting. The induction of kinase activities was calculated and is shown.

Fig. 5. HSP60 activates cells through TLR2 and TLR4. A, 293T fibroblasts were transfected with the indicated constructs, deprived of FCS for 1 h and stimulated with 1 µg/ml human HSP60, 5 µg/ml chlamydial HSP60, and 1 µM CpG oligonucleotide 2006 overnight (14 h). PMB was used throughout the stimulations to exclude the effects of trace amounts of LPS in protein preparations. Afterward, cells were lysed, luciferase activity in lysates was measured, and fold induction was calculated. Each bar represents the mean of two stimulations, with error bars showing S.D. values. One representative experiment out of three is shown. B, BMDDC from TLR2-k.o or TLR4-mutant (C3H/HeJ) mice (black bars) and from respective wild-type control mice (gray bars) were stimulated in the absence of FCS with 10 µg/ml chlamydial HSP60 or 1 µM CpG oligonucleotide 1668 for 18 h. LPS stimulation (50 ng/ml for 18 h) was performed in medium containing 10% FCS in the absence of PMB. Afterward, TNF-α in supernatants was measured. Each bar represents the mean of two stimulations, with error bars showing S.D. values. One representative experiment out of two is shown.
with CpG-DNA (Fig. 5B) known to be TLR9-dependent but not TLR2- or TLR4-dependent. Based on the TLR2- and TLR4-associated gain of function and loss of function data, we concluded that both TLR2 and TLR4 confer responsiveness to HSP60.

Role of Endocytosis in HSP60-mediated Signal Triggering—Since FCS effectively impaired HSP60 endocytosis by macrophages and HSP60 triggered signaling as well (Fig. 1 and 2), we analyzed whether endocytosis of HSP60 is a condition for signal initiation or whether it reflects only scavenging of the ligand. There are at least five independent pathways known for endocytic internalization: the clathrin-dependent pathway, the caveolar pathway, a clathrin- and caveolin-independent pathway, macropinocytosis, and phagocytosis (36). In view of the known receptor-dependent uptake of certain HSP, that is HSC70, HSP70, gp96 and HSP90 (8–11), we decided to block the clathrin-dependent endocytosis known to be a major route for internalization via transmembrane receptors (37). To this end, we employed as inhibitor of membrane-bound transglutaminase the agent monodansylcadaverine (MDC) shown to inhibit receptor trafficking at a step proximal to the formation of endocytotic vesicles (38). RAW 264.7 cells preincubated with MDC and subsequently exposed to fluorescently labeled chlamydial HSP60 failed to internalize HSP60 (Fig. 6). Furthermore, HSP60-mediated JNK activation was severely compromised in MDC-treated cells (Fig. 7A). The inhibitory effect of MDC was dose-dependent (Fig. 7A). On the other hand, JNK activity induced by anisomycin used as specificity control was not suppressed by MDC. To further exclude toxicity of MDC, we included as additional control the ability of IFN-γ to activate STAT1. As shown in Fig. 7B, IFN-γ-induced STAT1 phosphorylation in RAW 264.7 was not attenuated by MDC. We thus concluded that receptor-dependent uptake of HSP60 preceded HSP60-initiated signaling via TLR2 or TLR4 plus MD-2.

DISCUSSION

HSP60s are now recognized as powerful activators of murine and human innate immune cells able to trigger production of proinflammatory cytokines TNFα, IL-6, or IL-12 (19–21). However, signaling pathways involved are poorly understood except that activation of NF-κB (20) and stress-activated protein kinase p38 (39) was noted. Here we describe that HSP60 activates macrophages and dendritic cells via the ancient TIR signaling pathway involving MyD88 and TRAF6, which ultimately leads to activation of NF-κB, of the stress-activated protein kinases JNK1/2 and p38, and of the mitogen-activated protein kinases ERK1/2. Both TLR2 and TLR4 appear to impact recognition of HSP60, which is preceded by receptor-mediated endocytosis. Endocytosis of HSP60 is strongly attenuated by a component(s) within FCS, which may explain the power of HSP60 to activate innate immune cells under serum-free conditions. Since pathogen-derived (“exogenous”) ligands and cell-borne (“endogenous”) ligands such as HSP60 display surprising similarities in the signaling pathways used, HSP60 released from necrotic cells may be a set point for inflammation similar to exogenous microbial products.

In support of a previous report on NF-κB activation in endothelial cells after HSP60 stimulation (20), we detected induction of IKK activity in macrophages stimulated by HSP60. Degradation of IκB fit well in time to IKK activity. IKK is known to phosphorylate IκB on critical serines 32 and 36 and thus initiate degradation of IκB (40). Degradation of IκB and release of NF-κB subunits from the inhibited state in cytoplasm triggers their translocation to the nucleus and binding to NF-κB-responsive sites in the enhancers of genes of numerous cytokines, acute phase response proteins, and cell adhesion molecules (41).
cells were stimulated with 500 units/ml recombinant murine IFN-

...next day, cells were deprived of FCS, and 2 h later (20 min before stimulation) 200 μg/ml PMB was used throughout the experiments to stimulate the effects of trace amounts of LPS in protein preparations. Cells were lysed, and Western blotting for activated forms of JNK1/2 (p-JNK1/2) was performed. Additionally, the total amount of JNK1/2 in lysates was determined (JNK1/2). One representative experiment out of three is shown. B, in the same manner as in A, pretreated cells were stimulated with 500 units/ml recombinant murine IFN-γ for 5 and 10 min. Cells were lysed, and Western blotting for activated forms of STAT1 (p-STAT1) was performed. Additionally, the total amount of STAT1 in lysates was determined (STAT1). One representative experiment out of three is shown.

doglycans, and bacterial CpG-DNA, utilize the TIR signaling pathway to activate innate immunity (26, 27, 42, 43). This pathway is characterized by recruitment of the adapter molecule MyD88 to the cytoplasmic TIR homology domain of the respective receptor (18, 28). Here we show that activation of murine macrophages by either human (endogenous) or chlamydial (exogenous) HSP60 is MyD88-dependent because dominant negative MyD-C dose-dependently attenuated induction of both JNK and IKK. Transient transfection and gene knock-out studies have shown that also TRAF6 is essential in IL-1 and LPS signaling (30, 44). We show in this study that activation of macrophages by both human and chlamydial HSP60 is TRAF6-dependent, since the dominant-negative TRAF-C dose-dependently attenuated induction of JNK and IKK.

Independently of our demonstration that HSP60s activate macrophages via the TIR signaling pathway, several reports strengthened our interest to analyze the role of TLR in HSP60-mediated cell activation. First, macrophages from TLR4-deficient C3H/HeJ mice were claimed to be nonresponsive to HSP60 (45). Here we describe, however, that under serum-free conditions TLR4-deficient BMDDC responded to HSP60, albeit their responsiveness is attenuated. Second, shared receptor(s) for HSP60 and LPS were suggested in human astrocytoma cell line U373 stably transfected with CD14 (39), since transfection conferred responsiveness to human and chlamydial HSP60. In addition, antibodies to CD14 blocked the reactivity of human peripheral blood mononuclear cells toward HSP60 (39). This information prompted us to choose the gain of function approach encompassing transfections of expression vectors for different human TLR into human embryonic kidney fibroblasts 293T. Transfection of TLR2 or TLR4 plus MD-2 rendered 293T cells responsive to both human and chlamydial HSP60s as read out by induction of the NF-κB-driven reporter. On the other hand, BMDDC from TLR2- and TLR4-deficient mice expressed a loss of function phenotype (Fig. 5B). Thus, gain of function by transfection of TLR2 or TLR4 plus MD-2 is mirrored by a loss of function in TLR2- and TLR4-defective BMDDC. These data allowed us to conclude that both TLR2 and TLR4 participate in HSP60 recognition, providing the first evidence that the HSP60 can engage multiple (but not any) members of the TLR family. It would be interesting to look for differences of HSP60-induced effects arising from stimulation of cells expressing different TLR combinations. The effect of different TLR combinations expressed in different cell types could contribute, for example, to the above mentioned discrepancy between the published absolute loss of HSP60 reactivity in TLR4-defective macrophages (45) and the only partial dependence described here using BMDDC (Fig. 5B).

Internalization of certain HSP by APC was recently shown to proceed via receptor-mediated endocytosis (8–11). This applies for HSP60 as well, since under serum-free conditions HSP60 become rapidly endocytosed by RAW264.7 macrophages. The observed blocking effect of FCS on uptake of HSP60 could be explained with component(s) in serum that scavenges HSP60. Another possibility might be that serum withdrawal stresses cells so that they alter the expression of membrane proteins necessary for HSP60 internalization. Alternatively, a component(s) in serum could compete with HSP60 for the putative receptor on macrophages. Since HSP gp96 is endocytosed by APC via CD91 and since serum-borne α2-macroglobulin by sharing the same receptor inhibits uptake of gp96 (11), we favor a similar mechanism to explain the inhibitory effect of serum on HSP60 internalization.

HSP70 and HSP90 family members not only shuttle immunogenic peptides (3) but also activate APC (12, 13, 46, 47) as do HSP60. Whether endocytosis of HSP is necessary for activation of APC has been unclear. Here we report that HSP60 needs to be internalized in order to stimulate the TIR signaling pathway in macrophages, since inhibition of membrane bound transglutaminase by means of MDC not only blocked uptake of HSP60 by RAW264.7 macrophages (Fig. 6) but also inhibited activation of JNK, a downstream effector kinase of the Toll/IL-1 receptor signaling pathway (Fig. 7A).

The innate immune response is considered to be the first line of defense toward invading pathogens. Chlamydial HSP60 obviously shares with other pathogen-derived ligands the ability to alert innate immunity via the TIR signaling pathway. Since, however, endogenous HSP60, potentially released from necrotic cells (48, 49), is also able to activate the TIR signaling pathway in innate immune cells, inflammation caused by injury and induction of alertness toward infection may reflect two opposing tasks of TLR-mediated signaling.

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