Synergistic and Differential Modulation of Immune Responses by Hsp60 and Lipopolysaccharide*

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Activation of professional antigen-presenting cells (APC) is a crucial step in the initiation of an efficient immune response. In this study we show that Hsp60 mediates immune stimulation by different mechanisms, dependent and independent of lipopolysaccharide (LPS). We have demonstrated earlier that both, Hsp60 and LPS, increase antigen-specific interferon (IFN) secretion in T cells. Here we show that in contrast to LPS Hsp60 induces IFNα secretion in professional APC. Neutralization of IFNα as well as the absence of functional IFNαβ receptor on APC and T cells interfered with Hsp60-mediated IFNγ secretion in antigen-dependent T cell activation, strongly suggesting that IFNα represents one factor contributing to Hsp60-specific immune stimulation. On the other hand, we show that Hsp60 bound to the cell surface of APC colocalizes with the LPS co-receptor CD14 and LPS binding sites. Hsp60 specifically binds bacterial LPS and both molecules synergistically enhanced IL-12p40 production in APC and IFNγ release in antigen-dependent T cell activation. This effect was Hsp60-specific and dependent on LPS-binding by Hsp60. Furthermore, we show that Hsp60 exclusively binds to macrophages and DC but not to T or B lymphocytes and that both, T cell stimulation by Hsp60 as well as Hsp60/LPS complexes, strictly depends on the presence of professional APC and is not mediated by B cells. Taken together, our data support an extension of the concept of Hsp60 as an endogenous danger signal to the immune system. Therefore, HSP have been suspected to function as endogenous danger signals to the immune system (4, 7–9). HSP are highly conserved and ubiquitously expressed proteins that are normally hidden within the cell and function as molecular chaperones of nascent or aberrantly folded proteins in different cellular compartments (10, 11). HSP are up-regulated and released from cells upon various cellular stresses and necrotic cell death (12, 13). Furthermore, stress-induced cell surface expression of HSP like Hsp60, which is normally localized within the mitochondria, is essential for the in vitro folding of imported mitochondrial proteins (14–17). Extracellular Hsp60 has been shown to induce the maturation of human and murine DC and macrophages by an up-regulation of co-stimulatory cell surface molecules and by the production of the proinflammatory cytokines IL-1, IL-6, IL-12, and TNFα (7, 18, 19). Moreover, Hsp60 has been shown to enhance IFNγ production in antigen-dependent T cell activation (4, 6), an effect that was mainly ascribed to the release of IL-12 by APC (20, 21). The receptors that have been proposed to be responsible for Hsp60-mediated immune effects are CD14 (18) and members of the TLR family, namely TLR4 (22, 23) and TLR2 (23, 24). The receptor complex consisting of the glycophosphatidylinositol-anchored CD14 co-receptor and the TLR4-signal receptor is known to mediate LPS signaling (25), whereas TLR2 is a receptor for bacterial lipopolysaccharides and lipoteichoic acid (26–28). The Hsp60 preparations, however, that have been used in earlier studies were expressed in Escherichia coli and, therefore, were likely to be contaminated with bacterial endotoxins. For this reason, it could not be excluded that the observed effects were due to contaminating bacterial structures, especially LPS, rather than the Hsp60 protein itself, although controls like heat sensitivity and polymyxine B insensitivity of Hsp60 versus LPS were included (8).

Employing eukaryotic cell lines expressing the murine Hsp60 as a membrane-bound cell surface protein we have shown that Hsp60 enhances IFNγ production in antigen-dependent T cell activation in an endotoxin-free environment, clearly demon-
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...strating that Hsp60 possesses an intrinsic immunostimulatory potential (6). On the other hand, this endotoxin-free Hsp60 did not induce TNFα production in APC, an effect that was described to be mediated by contaminating LPS in the recombinant *E. coli*-expressed Hsp60 preparations used in earlier studies (29, 30). In addition, also Hsp70-mediated cytokine secretion in APC has been ascribed to contaminating bacterial endotoxins (31, 32) and it was suggested that HSP such as Hsp70 and Hsp90 bind bacterial LPS and modulate LPS signaling (25, 33, 34). Recently, the stress protein gp96 was shown to bind different TLR agonists including LPS, thereby enhancing the biological effect of the associated PAMP (35). Interestingly, also Hsp60 has been shown to bind LPS and to enhance LPS-induced TNFα production in a macrophage cell line (36) indicating that Hsp60 may influence LPS signaling.

Therefore, the present study was performed to dissect the immunological functions of Hsp60, LPS, and Hsp60/LPS complexes. We show that Hsp60 exclusively binds to professional APC but not to T- or B-lymphocytes. Thereby, Hsp60 colocalizes with the CD14 receptor as well as LPS binding sites. Furthermore, we confirm that Hsp60 specifically binds bacterial LPS and show that both molecules synergistically stimulate innate and adaptive immune responses indicated by enhanced IL-12p40 production in APC and IFNγ release in antigen-dependent T cell activation. On the other hand, we observe that Hsp60 stimulates IFNα production in APC, an effect that is not induced by LPS and not further enhanced by Hsp60-LPS complexes. Furthermore, we show that IFNα release as well as expression of functional IFNaβ receptor on APC and T cells is important in Hsp60- but not LPS-mediated stimulation of T cell activation. Thus, Hsp60 and LPS differentially stimulate leukocyte functions.

Taken together, our results reveal different mechanisms by which Hsp60 can modulate immune responses in the absence or presence of LPS: (i) Hsp60 enhances antigen-dependent T cell activation in an endotoxin-free environment (6) whereby IFNα, which is released by APC upon Hsp60 stimulation, is one mediator. (ii) Hsp60 functions as a LPS carrier protein that enhances LPS-induced TLR4 signaling in APC and as a consequence augments LPS-mediated T cell activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—8–10-Week-old female DO11.10 TCR transgenic mice expressing a TCR specific for OVA<sub>323-339</sub>/H2-A<sup>d</sup> (37), C57BL/6, BALB/c mice, and BALB/c-IL-12p40<sup>−/−</sup> mice (38) were bred in the animal facilities of the Bernhard-Nocht-Institute for Tropical Medicine and the Universitaets-Klinikum Eppendorf in Hamburg, Germany. IFNaβ<sup>−/−</sup> (39) and IFNβ<sup>−/−</sup> (40) mice were generated on Sv129 and backcrossed to C57BL/6 (41). IFNaβ<sup>−/−</sup> and IFNβ<sup>−/−</sup> mice were bred at the Paul Ehrlich Institute, Langen, Germany, and the Helmholtz Center for Infection Research, Braunschweig, Germany. T cells from DO11.10 mice (DO11.10 T cells termed hereafter), C57BL/6 and IFNaβ<sup>−/−</sup>, MHC II<sup>−/−</sup> cells, and B cells from BALB/c mice were purified from spleens by magnetic cell sorting using the Pan T cell isolation kit, the MHC II depletion kit, and the Pan B cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer’s protocol. Cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), HEPES, and 10 mM L-glutamine. Peritoneal exudate cells (PEC) were induced by intraperitoneal injection of 500 μl of thioglycollate into BALB/c mice and isolated by peritoneal lavage after 5 days. Bone marrow-derived dendritic cells (bmDC) were obtained from purified BALB/c bone marrow cells that were cultured in RPMI1640/10% FCS supplemented with 20 ng/ml granulocyte macrophage colony-stimulating factor and harvested after 9 days of culture.

**Transfection of COS1**—Eukaryotic COS1 cells were transiently transfected using the FuGENE 6 transfection reagent (Roche Applied Science, Germany) according to the manufacturer’s protocol. In brief, COS1 cells were plated into 6-well culture plates. 5 μg of pFM92 or pFM92-mHsp60 (6, 42) vector DNA and 6 μl of FuGENE 6 reagent were added to 100 μl of RPMI1640 without FCS and incubated for 30 min. Cell culture medium was replaced by 2 ml of RPMI1640, 10% FCS and the transfection mixture was added for 24 h. Expression of cell surface Hsp60 was monitored by FACS staining as described earlier (6).

**Reagents**—Low-endotoxin recombinant human Hsp60 (named hHsp60 hereafter) was obtained from Loke Diagnostics APs (Denmark; batch number B02-141205) and contained <2 endotoxic units of LPS/mg of protein as determined by limulus amoebocyte lysate assay (BioWhittaker). For binding studies hHsp60 and BSA control protein (Sigma) were labeled with fluorescein isothiocyanate (FITC) or Alexa 487 using the protein labeling kits from Molecular Probes. Proteins were labeled according to the manufacturer’s protocol. Unlabeled *E. coli* lipopolysaccharide (LPS) (strain 055:B5) and Alexa 488-labeled LPS of the same strain were purchased from Sigma (Germany). 3H-LPS (*E. coli* strain K12 LCD25) was obtained from List Biological Laboratories (Canada). OVA<sub>323-339</sub> peptide was synthesized by JPT (Germany).

**Antibodies**—Hsp60-specific antibody clones LK-1 and 4B9 were obtained from Stressgen (number SPA806) and Dianova (number MA3-012) as was mouse IgG2a isotype antibody. Rat anti-mouse CD14 was purchased from BD Pharmingen and TRITC- or PE-labeled goat anti-mouse as well as FITC-labeled goat anti-rat secondary antibodies and PE- or FITC-labeled antibodies against CD11c, CD11b, B220, CD4, and CD8 were purchased from Dianova. Alexa 488-conjugated rabbit anti-FITC antibody was obtained from Molecular Probes and DAPI was purchased from Sigma. Neutralizing polyclonal rabbit anti-IFNα (number 32100-1) and anti-IFNβ serum (number 32400-1) as well as rabbit IgG control serum were obtained from R&D Systems (Germany).

**Binding Studies**—To analyze whether LPS binds to Hsp60, 3 × 10<sup>9</sup> pFM92-mHsp60-transfected COS1 cells expressing the murine Hsp60 protein as a cell surface molecule or mock-transfected COS1 cells that obtained the pFM92 control vector were incubated on ice with 500 ng/ml 3H-LPS in 200 μl of culture medium in a 96-well plate for 45 min. To test for specificity the binding of 3H-LPS was blocked by addition of either 15 μg/ml anti-Hsp60 antibody (clone 4B9) or 5 μg/ml unlabeled LPS for 45 min on ice before addition of 3H-LPS. Afterward cells were harvested and cell-bound radioactivity was detected. For binding of Hsp60 to PEC and bmDC, 1 × 10<sup>6</sup> BALB/c-derived PEC
or bmDC were incubated on ice with either 30 μg/ml FITC-labeled or unlabeled Hsp60. Afterward, cells that obtained unlabeled Hsp60 were treated with 30 μl of Cohn II fraction (Sigma) and stained with Hsp60-specific antibody (clone LK-1, 1:100 in PBS), TRITC-labeled goat anti-mouse secondary antibody (1:400 in PBS) and DAPI (1:1000 in PBS). After staining cells were fixed in PBS/1% paraformaldehyde (PFA), centrifuged onto glass slides, and covered with anti-FADE solution (Biomedia, Germany). In addition, BALB/c PEC that had been incubated with 15 μg/ml of Hsp60 were centrifuged onto glass slides before staining. After overnight drying cells were fixed with acetone for 5 min and dried again for 1 h. After Fc block with 50 μl of Cohn II fraction cells were stained with mouse anti-Hsp60 antibody (LK-1, 1:100), rat anti-CD14 antibody (1:100), TRITC-labeled goat anti-mouse (1:400), and FITC-labeled goat anti-rat (1:200) secondary antibodies. FITC staining of CD14 was further enhanced by addition of Alexa 488-labeled anti-FITC (1:200). In addition, cells were stained with DAPI (1:1000) and finally covered with anti-FADE solution. Furthermore, BALB/c-derived PEC were incubated in chamber slides (Nunc, Wiesbaden, Germany). After adhesion overnight at 37 °C dead cells were washed out and cells were incubated alone or with 15 μg/ml Hsp60 for 45 min at 37 °C. Afterward cells were washed and fixed in ice-cold acetone/methanol (1:1) at −20 °C for 10 min. After drying, cells were blocked by addition of 200 μl of Cohn II fraction with PBS, 1% BSA (1:1) for 20 min. Alexa 488-conjugated LPS (1:100) was added for 30 min and cells were stained with DAPI, anti-Hsp60 (clone LK-1) and TRITC-labeled goat anti-mouse antibody as described before.

Dose-dependent binding of Hsp60 to BALB/c spleen cells was analyzed by incubating 2 × 10⁶ cells with 0.4, 2, 10, 40, or 200 μg/ml Hsp60-Alexa 647 for 30 min on ice in 50 μl of culture medium. To identify cell populations in spleen that bind Hsp60, 2 × 10⁶ BALB/c spleen cells were incubated 30 min on ice either alone, with 10 μg/ml BSA-Alexa 647 or 10 μg/ml hHsp60/Alexa 647 in 100 μl of culture medium. Binding of Hsp60 was competed by incubating cells with 20, 200, or 400 μg/ml unlabeled Hsp60 30 min prior to addition of hHsp60-Alexa 647. For further stainings, Fc receptors were blocked by addition of 30 μl of Cohn II fraction for 20 min and PE- or FITC-labeled CD11c-, CD11b-, B220-, CD4-, or CD8-specific antibodies (1:200) were added for 30 min. Cells were analyzed by FACS whereby two different gates were used: gate R1 that mainly contains CD4⁺, CD8⁺, and B220⁺ lymphocytes, and gate R2 that contains the majority of CD11c⁺ and CD11b⁺ cells (data not shown). 6 × 10⁵ cells were detected.

Cellular Assays—All assays were performed in RPMI1640 supplemented with 10% FCS, HEPEs, and l-glutamine. For stimulation of APC, 1 × 10⁵ BALB/c-derived PEC or bmDC were incubated in 96-well round-bottom plates either alone or with the indicated amounts of hHsp60 or LPS. In addition, cells were treated with a combination of LPS, hHsp60, or BSA or a combination of the same amounts of these proteins that had been preincubated with LPS for 2 h at 37 °C to allow complexation. For T cell stimulation, 1 × 10⁵ purified T cells from DO11.10 mice were co-cultured with 5 × 10⁴ BALB/c-derived PEC or purified BALB/c B cells and activated with 0.5–1 μg/ml OVA₃₂₃–₃₉₉ peptide. In parallel experiments complexation of hHsp60 and LPS was inhibited by addition of 2 μg/ml anti-Hsp60 antibody (clone 4B9) during the pre-incubation period. As a control 2 μg/ml of isotype antibody (mouse IgG2a) was used. IFNα and IFNβ were neutralized by the addition of 2.3 kilounits of polyclonal rabbit anti-IFNα or anti-IFNβ serum. Control cultures received 2 μg/ml isotype antibodies or serum.

Alternatively, instead of soluble recombinant hHsp60, 2 × 10⁵ or 1 × 10⁴ of transfected COS1 cells expressing cell surface Hsp60 were used. Thereby, the indicated amounts of COS1 cells were added alone or preincubated with LPS for 2 h at 37 °C before addition of 1 × 10⁵ of DO11.10 T cells, 5 × 10⁴ of PEC, and OVA peptide.

Cytokine Quantification—Cytokines were detected after 24 or 48 h of culture. IL-12p40 was quantified by standard sandwich ELISA. 96-Well Maxisorb plates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml anti-IL-12p40/70 (clone C15.6) at 4 °C for 24 h. Plates were blocked with PBS containing 1% BSA for 2 h at 37 °C and washed three times with PBS containing 0.05% Tween 20. Culture supernatants and a standard of recombinant IL-12 were added to the coated plates and incubated at 4 °C for 24 h. After 6 washes, 1 μg/ml biotinylated anti-IL-12p40/70 (clone C17.8) was added as detection antibody and incubated at room temperature for 1 h. Following 6 washes, a 1:10000 dilution of peroxidase-conjugated streptavidine (Amersham Biosciences) in PBS/0.1% BSA was added for 30 min at room temperature. Plates were washed 6 times and developed with 300 μg/ml tetramethylbenzidine, diluted in 0.1 M NaH₂PO₄ pH 5.5, containing 0.003% H₂O₂. The reaction was stopped by addition of 25 μl of 2 M H₂SO₄, and OD at 450 nm was measured immediately. All antibodies and recombinant cytokine standards were obtained from BD Pharmingen. IFNγ and IFNα content in the supernatants were determined employing the IFNγ DuoSet ELISA development system and the mouse IFNα ELISA kit from R&D Systems according to the manufacturer’s protocols.

RESULTS

Hsp60 Binds to Macrophages and DC but Not to B and T Lymphocytes—It has been shown that Hsp60 modulates APC as well as T and B cell activation (4, 6, 7, 22). To mediate a stimulatory effect it has to be assumed that Hsp60 binds to the cells via specific receptors. Therefore, we identified cell populations that bind Hsp60 to elucidate which cells might be able to respond to Hsp60 in a direct way. For this purpose BALB/c spleen cells were incubated with Alexa 647-labeled human Hsp60 (hHsp60-Alexa 647) or BSA (BSA-Alexa 647) as a control. Cells were subsequently stained against different cellular marker molecules and analyzed by flow cytometry (FACS). Fig. 1A shows that Hsp60 binds to subpopulations of CD11b⁺ and CD11c⁺ cells. About 35% of the CD11c⁺ cells and 21% of the CD11b⁺ cells were positive for hHsp60-Alexa 647, whereas the control protein BSA-Alexa 647 did not bind to these cells. Furthermore, Fig. 1 shows that hHsp60 does not bind to CD4⁺ or CD8⁺ T and B220⁺ B lymphocytes. Similar binding studies were also performed using unlabeled hHsp60 whereby bound hHsp60 was detected with Hsp60-specific antibody leading to
Hsp60 binds to distinct membrane regions on macrophages and bmDC. 1 × 10^6 BALB/c-derived bmDC (A) or PEC (B) were incubated with either 30 μg/ml FITC-labeled hHsp60 (A, upper row; green) or unlabeled Hsp60 stained with Hsp60-specific antibody clone LK-1 and TRITC-labeled goat anti-mouse secondary antibody (A, lower row, and B, red). In addition, all cells were stained with DAPI (blue). After staining, cells were fixed in PBS, 1% paraformaldehyde and centrifuged onto glass slides. The right panel shows an overlay of DAPI and Hsp60 stainings.

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Hsp60 binds LPS and Binding of Hsp60 to APC Colocalizes with CD14—To confirm the finding that Hsp60 binds bacterial LPS (36), we employed eukaryotic COS1 cells that express the murine Hsp60 protein fused to the transmembrane region of the platelet-derived growth factor receptor as a membrane-bound cell surface molecule (mHsp60) (6). Hsp60-negative control cells or mHsp60-expressing COS1 cells were incubated with 3H-LPS and cell-bound radioactivity was measured. Fig. 3A shows that binding of Hsp60 to spleen cells is saturable and specific. Furthermore, binding of Hsp60 could be blocked by preincubation of the cells with unlabeled LPS as well as by the addition of anti-Hsp60 antibody clone 4B9, which has been described to specifically inhibit binding of LPS to Hsp60 (36). These findings show that binding of LPS to Hsp60 is specific. In another approach we analyzed the binding of Alexa 488-labeled LPS (LPS-Alexa 488) to PEC that had been incubated with recombinant human Hsp60. Fig. 3B (upper rows) shows that the binding of LPS-Alexa 488 occurs in distinct membrane areas distributed around the whole cell membrane. Nevertheless, both molecules seem to concentrate within the same regions. This result again argues for complex formation of Hsp60 and LPS.

In addition, we analyzed binding of Hsp60 to PEC and bmDC by FACS (data not shown) and fluorescent microscopic analysis of cytospins. Fig. 2 shows that Hsp60 binds to the cell surface of bmDC as well as PEC (Fig. 2, A and B). Interestingly, Hsp60 appears to concentrate in distinct membrane regions that cannot be ascribed to antibody-induced aggregation of the protein because directly labeled Hsp60 shows the same binding pattern. Taken together, these results reveal that Hsp60 does not bind to murine T and B lymphocytes but almost exclusively interacts with macrophages and DC.

Hsp60 and LPS Synergistically Stimulate Immune Responses—We showed that Hsp60 specifically binds bacterial LPS and colocalizes with CD14 on the cell surface of APC. To investigate a possible function of Hsp60 in LPS signaling we performed stimulation experiments using BALB/c PEC and DO11.10 T cells that were activated with OVA peptide antigen in the presence of either recombinant Hsp60 or mHsp60-expressing COS1 cells and LPS. In a first experiment, PEC were stimulated with titrated amounts of hHsp60 and LPS alone to determine the concentration of both molecules necessary to induce cyto-

FIGURE 1. Hsp60 binds to professional APC but not to T and B lymphocytes. 2 × 10^6 BALB/c spleen cells were incubated with 10 μg/ml BSA-Alexa 647 (left panel, y axis) or hHsp60-Alexa647 (middle panel, y axis) and stained with PE-labeled CD11c, CD11b, B220, CD4, or CD8-specific antibodies (x axis). 6 × 10^5 cells were detected in FACS analysis. As indicated, two different gates were used whereby R1 contains the majority of the B and T lymphocytes and CD11c- and CD11b- cells are mainly found in R2. Numbers represent the percentage of cells in each quadrant. Histogram plots (right) show an overlay of BSA-Alexa 647 (gray) and hHsp60-Alexa 647-stained cells (thick black line) of the indicated cell population. Numbers represent the percentage of Hsp60-positive cells. The result is representative for four independent experiments (A). 2 × 10^6 BALB/c spleen cells were incubated with 0, 20, 200, or 400 μg/ml unlabeled hHsp60 protein prior to binding of hHsp60-Alexa 647 (10 μg/ml). The histogram shows the inhibition of hHsp60-Alexa 647 binding (gray) by preincubation of the cells with 20 μg/ml unlabeled hHsp60 (black line) (B, left). Besides cells were preincubated with increasing amounts of unlabeled hHsp60 as indicated on the x-axis before addition of hHsp60-Alexa 647 and the percentage of hHsp60-Alexa 647 binding cells (y axis) is shown (B, middle). Dose-dependent binding of Hsp60 was analyzed by incubating 2 × 10^6 BALB/c spleen cells with 0.4, 2, 10, 40, or 200 μg/ml hHsp60-Alexa 647 (x axis). The figure shows the mean fluorescence intensity (MFI; y axis) of the Hsp60-binding cell population (B, right). Spleen cells were gated on R2.

FIGURE 2. Hsp60 binds to distinct membrane regions on macrophages and bmDC. 1 × 10^6 BALB/c-derived bmDC (A) or PEC (B) were incubated with either 30 μg/ml FITC-labeled hHsp60 (A, upper row; green) or unlabeled Hsp60 stained with Hsp60-specific antibody clone LK-1 and TRITC-labeled goat anti-mouse secondary antibody (A, lower row, and B, red). In addition, all cells were stained with DAPI (blue). After staining, cells were fixed in PBS, 1% paraformaldehyde and centrifuged onto glass slides. The right panel shows an overlay of DAPI and Hsp60 stainings.
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kine secretion to allow detection of a Hsp60-mediated amplification of cytokine secretion. At concentrations below 1 ng/ml LPS or 1 μg/ml hHsp60, respectively, IL-12p40 secretion was neglected (Fig. 4A). For further experiments, 1–10 μg/ml hHsp60 were used for stimulation of PEC and T cells in the presence or absence of 0.5 or 1 ng/ml LPS. First, PEC were stimulated with LPS or hHsp60 alone or with a combination of both. Thereby, LPS and hHsp60 were added to the cell culture simultaneously or LPS and hHsp60 were preincubated before addition to the cell culture to allow complexation of both molecules. Fig. 4B (left) shows that the simultaneous addition of 1 ng/ml LPS and 10 or 5 μg/ml hHsp60 just lead to an additive enhancement in IL-12p40 secretion compared with stimulation with the same amounts of LPS or hHsp60 alone. Strikingly, IL-12p40 production in response to preincubated and thus complexed hHsp60/LPS significantly exceeds cytokine release induced by simultaneous addition of LPS and hHsp60, indicating a synergistic activity of Hsp60 and LPS (Fig. 4B, left). The synergistic effect of Hsp60 and LPS became even more obvious when T cells were activated in the presence of hHsp60 and LPS (Fig. 4B, right). IFNγ production induced by complexed hHsp60/LPS was significantly increased compared with stimulation with LPS or hHsp60 alone as well as simultaneous addition of both molecules.

In another approach we employed mHsp60-expressing COS1 cells instead of soluble recombinant human Hsp60. Fig. 4C depicts a model of stimulation experiments using these mHsp60-expressing eukaryotic cells. Employing this in vitro test system we have previously shown that Hsp60 enhances antigen-dependent T cell activation in the absence of bacterial PAMPs (6). Now, we intended to assess whether the presence of LPS-binding cell surface Hsp60 enhances LPS-mediated stimulation compared with control cultures containing non-LPS-binding mock transfected cells. DO11.10 T cells were activated with OVA peptide antigen in the presence of PEC and transfected COS1 cells. COS1 cells were added alone or preincubated with 0.5 ng/ml LPS for 2 h at 37 °C before addition. Fig. 4D shows that the presence of mock transfected COS1 control cells preincubated with LPS did not lead to the release of higher amounts of IFNγ compared with stimulation with LPS alone, whereas the presence of cell surface mHsp60 significantly increased IFNγ production. These observations clearly show that Hsp60 and LPS synergistically act on APC and T cell activa-
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In contrast to hHsp60, the addition of BSA did not stimulate IFNγ production nor IL-12p40 release on its own nor did BSA enhance LPS-induced production of these cytokines (Fig. 5, A and B). In another approach, anti-Hsp60 antibody (clone 4B9) that has been shown to inhibit binding of LPS to Hsp60 (Ref. 36 and Fig. 3A) was added to the test system during the hHsp60/LPS preincubation period. The presence of anti-Hsp60 4B9 led to a dramatically reduced IFNγ production (Fig. 6A) and abrogated IL-12p40 release (Fig. 6B) induced by hHsp60/LPS.

Whereas IL-12p40 production in cultures containing hHsp60 alone was completely diminished when anti-Hsp60 4B9 was added, IFNγ release induced by hHsp60/LPS was comparable with stimulation with the same amount of LPS alone. These results demonstrate that the synergistic effect of Hsp60 and LPS on immune stimulation is dependent on specific binding of LPS to Hsp60.

Synergistic immune stimulation by Hsp60 and LPS. 1 × 10^5 BALB/c-derived PEC were incubated either alone or with titrated amounts of hHsp60 (left) or LPS (right) as indicated on the x axis. After 24 h IL-12p40 was detected in the supernatants by specific ELISA (y axis). Bars represent the mean of triplicates and S.E. as indicated on the x axis. After 24 h IL-12p40 was detected in the supernatants after 24 h (B, left). 1 × 10^5 DO11.10 T cells were stimulated with 0.5 ng/ml OVA peptide antigen in the presence of 5 × 10^5 BALB/c PEC and the indicated amounts of LPS, hHsp60, or combinations of both as described before and indicated on the x axis. IFNγ was quantified after 24 h. The results are representative for three independent experiments (B, right). As depicted in C, instead of soluble Hsp60 either mock transfected COS1 cells or membrane-bound murine Hsp60 (mHsp60)-expressing COS1 cells were added to the test cultures. 1 × 10^5 DO11.10 T cells were stimulated with 0.5 μg/ml OVA peptide antigen in the presence of the indicated amounts of LPS, COS1 cells alone, or COS1 cells that had been preincubated with LPS for 2 h at 37 °C IFNγ was detected after 24 h (D). The mean of triplicates and S.E. are shown. **, p < 0.005; Student’s t test, unpaired, two-tailed.

In contrast to hHsp60, the addition of BSA did not stimulate IFNγ production nor IL-12p40 release on its own nor did BSA enhance LPS-induced production of these cytokines (Fig. 5, A and B). In another approach, anti-Hsp60 antibody (clone 4B9) that has been shown to inhibit binding of LPS to Hsp60 (Ref. 36 and Fig. 3A) was added to the test system during the hHsp60/LPS preincubation period. The presence of anti-Hsp60 4B9 led to a dramatically reduced IFNγ production (Fig. 6A) and abrogated IL-12p40 release (Fig. 6B) induced by hHsp60/LPS.

Experiments were performed whereby neutralizing antisera against IFNα or IFNβ was added (Fig. 7, C–E). The presence of anti-IFNα or anti-IFNβ did not interfere with LPS-mediated IFNγ production (Fig. 7D), whereas neutralization of IFNα significantly reduced IFNγ production in response to recombinant hHsp60 (Fig. 7C) as well as cell surface-expressed mHsp60 (Fig. 7E). On the other hand, neutralization of IFNβ did not influence stimulation of IFNγ release by recombinant hHsp60 (Fig. 7C) but slightly reduced IFNγ production in response to mHsp60 expressed by COS1 cells (Fig. 7E).

To further investigate the function of type I interferons in Hsp60-mediated immune stimulation we employed spleen cells
from IFNβ knock-out mice (IFNβ−/−) and IFNα/β receptor knock-out mice (IFNαβR−/−) that are unresponsive to IFNα as well as IFNβ (39). Spleen cells were stimulated with anti-CD3 in the presence of mHsp60 expressing COS1 cells (Fig. 7F). In comparison to wild-type C57BL/6 spleen cells, IFNγ production in response to LPS-free mHsp60 was significantly reduced in IFNαβR−/− cells. IFNγ secretion was also reduced when using IFNβ−/− cells although this effect was less pronounced and not significant. Similar results were also obtained when cells were stimulated in the presence of recombinant hHsp60 (data not shown). These results argue for a function of type I interferons, especially IFNα, in Hsp60-mediated immune stimulation in the absence of bacterial PAMPs.

Next, we addressed the issue whether APC, T cells, or both need to respond to type I interferons in Hsp60-mediated stimulation. Therefore, various combinations of APC and T cells from either wild-type C57BL/6 or IFNαβR−/− mice were activated by addition of anti-CD3 in the presence of recombinant hHsp60 (Fig. 7G). Compared with stimulation of recombinant APC and T cells, IFNγ production in response to hHsp60 was slightly reduced when IFNαβR−/− T cells were activated. A comparable reduction of IFNγ release was observed when wild-type T cells were stimulated in the presence of IFNαβR−/− APC and IFNγ production was further decreased in combinations of IFNαβR−/− APC and T cells (Fig. 7G). Taken together, these results indicate that type I interferons represent important but not the only mediators in immune stimulation by PAMP-free Hsp60, acting on both, APC and T cells.

B Cells Do Not Mediate Hsp60- or Hsp60/LPS-induced IFNγ Production in T Cells—Having shown that binding of Hsp60 is restricted to professional APC such as macrophages and DC, whereas Hsp60 does not bind to B and T lymphocytes (Fig. 1), we now asked for the relevance of this finding in immune stimulation. To this end we analyzed IFNγ induction in T cell activation by hHsp60 or hHsp60/LPS in the presence of either macrophages or B cells as APC. As described before, DO11.10 T cells were activated with OVA peptide in the presence of the same amounts of either PEC or purified B cells that contained >97% B220+ cells (data not shown) and the indicated amounts of LPS or hHsp60 were added alone or preincubated together before addition (Fig. 8). Compared with the control cultures LPS as well as hHsp60 alone clearly induced IFNγ release in the presence of PEC, whereas both molecules failed to enhance IFNγ production in T cell cultures containing B cells as APC. Moreover, hHsp60 and LPS synergistically increased IFNγ production in cultures containing PEC, whereas this effect was not observed when B cells were added. These results show that T cell stimulation by Hsp60 as well as Hsp60/LPS depends on the presence of professional APC such as macrophages and DC capable to bind Hsp60 but is not mediated by B cells.

**DISCUSSION**

HSP that are released by necrotic cells have been discussed to function as endogenous danger signals indicating cellular stress and tissue damage to the immune system (4, 7–9). By activating professional APC, HSP are believed to contribute to the initiation of effective innate as well as adaptive immune responses. In the last few years, however, the immunostimulatory potential of heat shock proteins has been questioned by the finding that contaminations of the HSP preparations with bacterial structures rather than the HSP themselves were responsible for cytokine production in macrophages (29–32). Nevertheless, we demonstrated that Hsp60 enhances antigen-dependent T cell activation in the absence of not only LPS but any bacterial structures belonging to the group of PAMPs (6). On the other hand, recent findings suggest that HSP including Hsp60 might
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employing both, recombinant human Hsp60, as well as murine Hsp60 expressed as a cell surface molecule on eukaryotic COS1 cells. Taken together, these findings show for the first time that Hsp60 modulates LPS signaling in naive murine leukocytes, enhancing LPS-induced activation of innate as well as adaptive immune responses.

A prerequisite for synergistic APC and T cell stimulation by hHsp60 and LPS was the complex formation of both molecules by preincubation because the simultaneous addition of hHsp60 and LPS just lead to an additive effect regarding IL-12p40 production by macrophages as well as IFNγ release in antigen-dependent T cell activation. These results indicate that the binding of LPS to Hsp60 is necessary for the synergistic activity of both molecules. Moreover, the inhibition of the binding of LPS and Hsp60 by Hsp60-specific antibody completely abolished IL-12p40 production in APC and also led to a drastically reduced hHsp60/LPS-stimulated IFNγ release in T cell activation. These results not only show that the synergistic immune activation is Hsp60-specific as was also demonstrated by the addition of inert BSA control protein, but clearly show that it is dependent on specific binding of LPS to Hsp60. These findings are in concordance with earlier observations indicating a LPS-binding function for other HSP such as Hsp70, Hsp90 (25, 33, 34), and gp96 (35), suggesting that HSP in general may bind TLR ligands and modulate PAMP-induced innate and adaptive immune responses.

On the other hand, we observed that hHsp60 stimulates IFNα release in peritoneal macrophages and bmDC. In contrast to hHsp60, LPS did not stimulate the production of this type I interferon, which is in line with the finding that TLR4 engagement by LPS enhances IFNβ release but does not stimulate IFNα production in APC in vitro (43, 48–50). Moreover, IFNα release was not further increased by complexed hHsp60/LPS compared with hHsp60 alone, indicating that IFNα induction is a Hsp60-specific effect that is not dependent on bound LPS. These findings show that Hsp60 and LPS differentially activate APC functions and argue for the existence of additional signaling mechanisms in Hsp60-mediated immune stimulation that are independent of LPS and may not involve TLR4 engagement. This hypothesis is supported by the observation that endotoxin-free Hsp60 does not stimulate the production of the LPS-inducible cytokines IL-6, IL-12, or TNFα in APC (29, 30). Furthermore, the neutralization of IFNα led to a reduced IFNγ production in antigen-dependent T cell activation in response to recombinant hHsp60 as well as to endotoxin-free mHsp60
but did not influence LPS-mediated stimulation. Moreover, stimulation of cells from IFNαR−/− mice clearly demonstrates that a functional type I interferon system is involved in immune stimulation by endotoxin-free Hsp60 because IFNγ secretion was significantly reduced in the absence of IFNα receptor. Therefore, response of APC as well as T cells to type I interferons equally contributes to Hsp60-mediated IFNγ release.

Given the findings that endotoxin-free Hsp60 enhances IFNγ production in antigen-dependent T cell activation (6) and that Hsp60 but not LPS induces IFNα release in APC, we suggest that instead of IL-12 this type I interferon represents one mediator in Hsp60-induced T cell stimulation in an endotoxin-free environment. The fact, however, that neutralization of type I interferons as well as unresponsiveness to these cytokines did not completely abrogate Hsp60-mediated stimulation strongly suggests that other yet undescribed mediators are involved.

Finally, we show that enhancement of IFNγ production in antigen-dependent T cell activation by hHsp60 as well as complexed hHsp60/LPS is strictly dependent on the presence of professional APC such as macrophages and DC. CD11c+ and CD11b+ spleen cells as well as PEC and bmDC were shown to bind hHsp60. In contrast to these professional APC, T and B lymphocytes did not bind hHsp60 (Fig. 1). Moreover, B cells did not mediate hHsp60- or hHsp60/LPS-induced T cell stimulation. These observations not only demonstrate that Hsp60 does not directly act on B cells but also implicate that it does not affect T cell activation in a direct way. We therefore suggest that Hsp60 exclusively binds to macrophages and DC via specific receptors that are not expressed by T and B cells, and that the influence of Hsp60 as well as complexed Hsp60/LPS on T cell stimulation is a consequence of the activation of professional APC, most likely the induction of IFNα or IL-12, respectively. These findings are in contrast to earlier observations showing an influence of Hsp60 on the activation of purified B and T cells in the absence of professional APC (24, 51). In these studies, however, human T cells were investigated. These cells are at least in part not naïve and might respond to Hsp60 in a different way than naïve murine cells as analyzed in our study. Such differences in responsiveness of naïve and effector T cells to Hsp60 have been described before (4). In addition, contaminating bacterial structures in the recombinant Hsp60 preparation may have contributed to the observed effects because T and B lymphocytes themselves express certain members of the TLR family and may directly respond to TLR ligands (52–55).

Taken together, our results reveal that Hsp60 possesses different functions. The intrinsic stimulatory capacity of Hsp60 itself leads to an enhanced antigen-dependent T cell activation in the absence of bacterial endotoxins (6) whereby IFNα may represent one link between Hsp60-mediated innate and adaptive immune response. On the other hand, Hsp60 binds bacterial LPS and synergistically enhances LPS-induced innate and adaptive immune responses. Therefore, Hsp60 may operate similar to the LPS-binding protein, which is known to facilitate the binding of LPS to its CD14-TLR4 receptor complex and to enhance LPS-mediated TLR4 signaling. Such function of Hsp60 would explain the stimulation of LPS-inducible cytokines in APC by recombinant E. coli-expressed and, thus, endotoxin-contaminated Hsp60, and extend the concept of Hsp60 as an endogenous danger signal by an additional aspect. In bacterial infection in vivo Hsp60 that is released by necrotic cells in damaged tissue or expressed on the cell surface of stressed or infected cells may interact with LPS in the extracellular space. By this means Hsp60 would not only contribute to the detection of tissue damage by the immune system, but facilitate microbe recognition in early bacterial infection and help to elicit an

![Image](image-url)
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appropriate anti-bacterial immune response by amplifying LPS-mediated stimulation.

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