This is a postprint of an article published in
Osterloh, A., Kalinke, U., Weiss, S., Fleischer, B., Breloer, M.
Synergistic and differential modulation of immune responses by Hsp60 and lipopolysaccharide
(2007) Journal of Biological Chemistry, 282 (7), pp. 4669-4680.

SYNERGISTIC AND DIFFERENTIAL MODULATION OF IMMUNE RESPONSES BY HSP60 AND LPS

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Running title: Immune stimulation by Hsp60, LPS and complexed Hsp60/LPS

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[2] The Abbreviations used are: HSP: heat shock protein, LPS: lipopolysaccharide, PAMP: pathogen-associated molecular pattern, PRR: pattern recognition receptor, TLR: toll-like receptor

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 LPS. We have demonstrated earlier that both, Hsp60 and LPS, increase antigen-specific IFNγ release in T cells. Here we show that in contrast to LPS Hsp60 induces IFNα production 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: Besides its function as a classical danger signal indicating unplanned tissue destruction to the innate immune system, in the incident of bacterial infection extracellular Hsp60 may bind LPS and facilitate microbe recognition by lowering the threshold of PAMP detection and enhancing TLR signaling.

INTRODUCTION

Activation of antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages is a critical step in the initiation of innate as well as adaptive immune responses and is known to be induced by pathogen-associated molecular pattern (PAMP) molecules such as bacterial lipopolysaccharide (LPS) and other endotoxins. These molecules are recognized by pattern recognition receptors (PRR) like members of the conserved toll-like receptor (TLR) family (1,2). In the last years, several members of the heat shock protein (HSP) family including Hsp60 have been described to modulate APC functions and to stimulate immune responses *in vitro* and *in vivo* (3-6). 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 chaperons of nascent or aberrantly folded proteins in different cellular compartmens (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 playing an essential role in the folding of imported mitochondrial proteins has been observed (14-17). Extracellular Hsp60 has been shown to induce the maturation of human and murine DC and macrophages indicated by an up-regulation of co-stimulatory cell surface molecules and 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 GPI-anchored CD14 co-receptor and the TLR4 signaling receptor is known to mediate LPS signaling (25) while TLR2 is a receptor for bacterial lipoproteins and lipoteichoic acid (26-28). The Hsp60 preparations, however, that have been used in earlier studies were expressed in *E. 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 demonstrating 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 IFNαβ 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 $OVA_{323-339}$/H2-A^d (37), C57BL/6, BALB/c mice and BALB/c-IL-12p40$^{-/-}$ mice (38) were bread in the animal facilities of the Bernhard-Nocht-Institute for Tropical Medicine and the Universitaets-Klinikum Eppendorf in Hamburg, Germany. IFNαβR$^{-/-}$ (39)and IFNβ$^{-/-}$ (40) mice were generated on Sv129 and backcrossed to

C57BL/6 (41). IFNαβR$^{-/-}$ and IFNβ$^{-/-}$ 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 IFNαβR$^{-/-}$, MHC ll^{+} 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 ll depletion kit and the Pan B cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturers 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 i. p. injection of 500 µl thioglycolate into BALB/c mice and isolated by peritoneal lavage after five 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 GM-CSF and harvested after 9 days of culture.

Transfection of COS1 – Eukaryotic COS1 cells were transiently transfected using the FuGENE6 transfection reagent (Roche Applied Science, Germany) according to the manufacturers protocol. In brief, COS1 cells were plated into 6 well culture plates. 5 µg of pFM92 or pFM92-mHsp60 (42) vector DNA and 6 µl FuGENE6 reagent were added to 100 µl RPMI1640 without FCS and incubated for 30 min. Cell culture medium was replaced by 2 ml RPMI1640/10% FCS and the transfection mix was added for 24h. 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 No. B02-141205) and contained < 2 endotoxic units (EU) LPS/mg protein as determined by LAL assay (BioWhittaker, USA). For binding studies hHsp60 and BSA control protein (Sigma, Germany) were labeled with fluoresceine isothyocyanate (FITC) or Alexa647 using the protein labeling kits from Molecular Probes/Invitrogen (Germany). Proteins were labeled according to the manufacturers protocol. FITC-labeled choleratoxin B subunit (CTB), unlabeled *E. coli* lipopolysaccharide (LPS) (strain 055:B5) and Alexa488-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$_{323\text{-}339}$ peptide was synthesized by JPT (Germany).

Antibodies – Hsp60-specific antibodies clone LK-1 and clone 4B9 were obtained from Stressgen (Canada; No. SPA806) and Dianova (Germany; No. MA3-012) as was mouse IgG2a isotype antibody. Rat anti-mouse CD14 was purchased from PharMingen BD (Germany) 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 (Germany). Alexa488-conjugated rabbit anti-FITC antibody was obtained from Molecular Probes/Invitrogen (Germany) and DAPI was purchased from Sigma (Germany). Neutralizing polyclonal rabbit anti-IFNα (Order No. 32100-1) and anti-IFNβ serum (Order No. 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^{4} 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 culture medium in 96 well plate for 45 min. To test for specificity the binding of ^{3H}LPS was blocked by the 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. Afterwards cells were harvested and cell-bound radioactivity was detected. For the binding of Hsp60 to PEC and bmDC, 1×10^{6} BALB/c-derived PEC or bmDC were incubated on ice with either 30 µg/ml FITC-labeled or unlabeled hHsp60. Afterwards, cells that obtained unlabeled hHsp60 were treated with 30 µl Cohn ll fraction (Sigma, Germany) 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% PFA, centrifuged onto glass-slides and covered with antiFADE solution (BiomedDia, Germany). In addition, BALB/c PEC that had been incubated with 15 µg/ml hHsp60 were centrifuged onto glass-slides before staining. After overnight drying cells were fixed with acetone for five minutes and dried again for 1 h. After Fc block with 50 µl Cohn ll 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 Alexa488-labeled anti-FITC (1:200). In addition, cells were stained with DAPI (1:1000) and finally covered with antiFADE solution. Furthermore, BALB/c-derived PEC were incubated in chamber slides (Nunc, Wiesbaden, Germany). After adherence overnight at 37°C dead cells were washed out and cells were incubated alone or with 15 μg/ml hHsp60 for 45 min at 37°C. Afterwards 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 Cohn ll fraction /PBS/1%BSA (1:1) for 20 min. Alexa488-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 2×10^6 cells with 0.4, 2, 10, 40 or 200 μg/ml hHsp60-Alexa647 for 30 min on ice in 50 μl culture medium. To identify cell populations in spleen that bind Hsp60 2×10^6 BALB/c spleen cells were incubated 30 min on ice either alone, with 10 μg/ml BSA-Alexa647 or 10 μg/ml hHsp60-Alexa647 in 100 μl culture medium. Binding of Hsp60 was competed by incubating cells with 20, 200 or 400 μg/ml unlabeled hHsp60 30 min prior to addition of hHsp60-Alexa647. For further stainings, Fc receptors were blocked by addition of 30 μl Cohn ll 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). 6×10^5 cells were detected.

Cellular assays – All assays were performed in RPMI1640 supplemented with 10% FCS, HEPES and L-glutamine. For stimulation of APC 1×10^5 BALB/c-derived PEC or bmDC were incubated in 96 well round-bottom plates either alone, with 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 pre-incubated with LPS for 2 h at 37°C to allow complexation. For T cell stimulation 1×10^5 purified T cells from DO11.10 mice were co-cultured with 5×10^4 BALB/c-derived PEC or purified BALB/c B cells and activated with 0.5-1 μg/ml $OVA_{323-339}$ 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 isotype antibody (mouse IgG2a) was used. IFNα and IFNβ were neutralized by the addition of 2.3 kU polyclonal rabbit anti-IFNα or anti-IFNβ serum. Control cultures received 2 μg/ml isotype antibodies or serum.
Alternatively, instead of soluble recombinant hHsp60 2×10^3 or 1×10^4 transfected COS1 cells expressing cell surface Hsp60 were used. Thereby, the indicated amounts of COS1 cells were added alone or pre-incubated with LPS for 2h at 37°C before addition of 1×10^5 DO11.10 T cells, 5×10^4 PEC and OVA peptide.

Cytokine quantification – Cytokines were detected after 24h or 48h of culture. IL-12p40 was quantified by standard sandwich ELISA. 96-well Maxisorb plates (Nunc, Roskilde, DK) were coated with 2 μg/ml anti-IL-12p40/70 (clone C15.6) at 4°C for 24h. Plates were blocked with PBS containing 1% BSA for 2 h at 37°C and washed three times with PBS containing 0.05% Tween20. 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 RT for 1 h. Following 6 washes, a 1:10000 dilution of peroxidase-conjugated streptavidin (Amersham, UK) in PBS/0.1% BSA was added for 30 min at RT. Plates were washed 6 times and developed with 300 μg/ml tetramethylbenzidine, diluted in 0.1M NaH_2PO_4 pH 5.5 containing 0.003% H_2O_2. The reaction was stopped by addition of 25 μl 2M H_2SO_4, and OD at 450 nm was measured immediately. All antibodies and recombinant cytokine standards were obtained from PharMingen BD (Germany). 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 (Germany) according to the manufacturers 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 Alexa647-labeled human Hsp60 (hHsp60-Alexa647) or BSA (BSA-Alexa647) as a control. Cells were subsequently stained against different cellular marker molecules and analyzed by flow cytometry (FACS). Figure 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-Alexa647 while the control protein BSA-Alexa647 did not bind to these cells. Furthermore, figure 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 the same results (data not shown). Binding of hHsp60-Alexa647 could be inhibited by pre-incubation of spleen cells with unlabeled hHsp60. The addition of 20 μg/ml hHsp60, a 2-fold excess, already reduced the amount of hHsp60-Alexa647 binding spleen cells from 18% to 7% and binding could be further inhibited using higher concentrations (20-fold and 40-fold excess) of unlabeled hHsp60 (Fig. 1B, left and middle). As shown before, the subpopulation of 18% of Hsp60-binding spleen cells can already be detected with 10 μg/ml Hsp60-Alexa647 (Fig. 1B, left) and does not increase using higher concentrations of labeled Hsp60 (data not shown). However, the mean fluorescence intensity (MFI) of this Hsp60-binding spleen cell fraction reaches a plateau when using more than 40 μg/ml hHsp60-Alexa647 (Fig. 1B, right). These results show that binding of Hsp60 to spleen cells is specific.
In addition, we analyzed binding of Hsp60 to peritoneal macrophages (PEC) and bone-marrow-derived DC (bmDC) by FACS (data not shown) and fluorescent microscopic analysis of cytospins. Figure 2 shows that hHsp60 binds to the cell surface of bmDC as well as PEC (Fig. 2A, B). Interestingly, Hsp60 appears to concentrate in distinct membrane regions which can not be ascribed to antibody induced aggregation of the protein since directly labeled Hsp60 shows the same binding pattern. Taken together, these results reveal that Hsp60 does not bind to murine T and B lymhocytes but almost exclusively interacts with macrophages and DC.

Hsp60 binds LPS and binding of Hsp60 to APC colocalizes with CD14 – In order 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 PDGF 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. Figure 3A shows that binding of ^{3H}LPS was significantly enhanced on mHsp60-expressing COS1 cells. Furthermore, binding of ^{3H}LPS could be blocked by pre-incubation 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 Alexa488-labeled LPS (LPS-Alexa488) to PEC that had been incubated with recombinant human Hsp60. Figure 3B (upper rows) shows that the binding of LPS-Alexa488 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.
CD14 and TLR4, both part of the LPS receptor complex, have been described to be involved in Hsp60-mediated immune stimulation (18,22). Therefore, we analyzed whether binding of Hsp60 colocalizes with the CD14 receptor. PEC were incubated with hHsp60 and stained with Hsp60-specific antibody as described before. In addition, cells were stained with CD14-specific antibody. Figure 3B shows that the binding of Hsp60 indeed colocalizes with the CD14 receptor in distinct membrane areas (Fig. 3B, below).
Taken together, these findings not only show that Hsp60 interacts with bacterial LPS but also indicate that Hsp60 might associate with the LPS receptor component CD14.

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. In order 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 hHsp60 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 cytokine secretion in order 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 neglectible (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 simultanously or LPS and hHsp60 were pre-incubated before addition to the cell culture to allow complexation of both molecules. Figure 4B (left) shows that the simultanous addition of 1 ng/ml LPS and 10 or 5 μg/ml hHsp60 just lead to an additive enhancement in IL-12p40 secretion compared to stimulation with the same amounts of LPS or hHsp60 alone. Strikingly, IL-12p40 production in response to pre-incubated and thus complexed hHsp60/LPS significantly exceeds cytokine release induced by simultanous 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 (4B, right). IFNγ production induced by complexed hHsp60/LPS was significantly increased compared to stimulation with LPS or hHsp60 alone as well as simultanous addition of both molecules.

In another approach we employed mHsp60-expressing COS1 cells instead of soluble recombinant human Hsp60. Figure 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 asses whether the presence of LPS-binding cell surface Hsp60 enhances LPS-mediated stimulation compared to 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 pre-incubated with 0.5 ng/ml LPS for 2h at 37°C before addition. Figure 4D shows that the presence of mock transfected COS1 control cells pre-incubated with LPS did not lead to the release of higher amounts of IFNγ compared to 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 activation employing both, recombinant human Hsp60 expressed in *E. coli* as well as murine Hsp60 expressed on the cell surface of eukaryotic cells.

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. 5A, 5B). In another approach, anti-Hsp60 antibody (clone 4B9) that has been shown to inhibit binding of LPS to Hsp60 ((36) and Fig. 3A) was added to the test system during the hHsp60/LPS pre-incubation 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. While 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 to 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.

Differential induction of type I interferons by Hsp60 and LPS – Besides secretion of cytokines like IL-12 and TNFα the production of type I interferons IFNα and IFNβ is an early event in the activation of innate immune responses and is also known to be induced by TLR4 (43-46). Therefore, we analyzed IFNα production induced by hHsp60 and LPS in PEC and bmDC from BALB/c mice. IFNα secretion was significantly increased in cultures containing 10 μg/ml hHsp60 while as much as 1 μg/ml LPS did not lead to the release of higher amounts of IFNα compared to the control cultures (Fig. 7A). In addition, we analyzed whether IFNα production in macrophages can be enhanced by complexed hHsp60/LPS (Fig. 7B). The presence of hHsp60 alone lead to an enhanced production of IFNα compared to unstimulated control cultures and cultures containing LPS alone.

Interestingly, IFNα release was not further enhanced by complexed hHsp60/LPS compared to hHsp60 alone (Fig. 7B) indicating that IFNα induction in APC is mediated by Hsp60 itself independent of associated LPS.

In addition, T cell stimulation experiments were performed whereby neutralizing antisera against IFNα or IFNβ were added (Fig. 7C-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 knockout mice ($IFN\beta^{-/-}$) and IFNα/β receptor knockout mice ($IFN\alpha\beta R^{-/-}$) 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 wildtype C57BL/6 spleen cells, IFNγ production in response to LPS-free mHsp60 was significantly reduced in $IFN\alpha\beta R^{-/-}$ cells. IFNγ secretion was also reduced when using $IFN\beta^{-/-}$ 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 wildtype C57BL/6 or $IFN\alpha\beta R^{-/-}$ mice were activated by addition of anti-CD3 in the presence of recombinant hHsp60 (Fig. 7G). Compared to stimulation of wildtype APC and T cells, IFNγ production in response to hHsp60 was slightly reduced when $IFN\alpha\beta R^{-/-}$ T cells were activated. A comparable reduction of IFNγ release was observed when wildtype T cells were stimulated in the presence of $IFN\alpha\beta R^{-/-}$ APC and IFNγ production was further decreased in combinations of $IFN\alpha\beta R^{-/-}$ 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 while 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 same amounts of either PEC or purified B cells that contained > 97% $B220^+$ cells (data not shown) and indicated amounts of LPS or hHsp60 were added alone or pre-incubated together before addition (Fig. 8). Compared to 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 while 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 play a role in TLR signaling by binding bacterial PAMPs such as LPS (25,33-36). Here we analyze a possible function of Hsp60 in LPS signaling and we dissect immunological functions of Hsp60, LPS and Hsp60/LPS complexes.

We show that hHsp60 bound to the cell surface of macrophages colocalizes with LPS binding sites and the LPS co-receptor CD14. Thereby, Hsp60 clusters in distinct membrane regions that might represent lipid raft-like membrane regions. It has been described that CD14 containing receptor clusters exist in lipid raft microdomains of resting monocytes and that stimulation of monocytes by LPS leads to a co-assembly of additional receptors including TLR4 (47). Thus, Hsp60 might bind to membrane regions where LPS signaling receptors are located suggesting that Hsp60 may interact with these receptors and thereby influence LPS stimulation. Our results, indeed, demonstrate that Hsp60 and LPS act on APC in a synergistic manner. We not only confirm that hHsp60 specifically binds LPS as described earlier (36) but show that the addition of LPS and recombinant hHsp60, pre-incubated to allow complexation of both molecules, significantly enhanced IL-12p40 production in naïve murine macrophages. Similar observations were obtained when mHsp60-expressing COS1 cells that had been pre-incubated with LPS were employed (data not shown). These findings are in line with the observation that Hsp60 enhances LPS-mediated TNFα production in the macrophage cell line J774 (36). Moreover, we show that Hsp60 and LPS synergistically enhance IFNγ release in antigen-dependent T cell activation 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 naïve 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 pre-incubation because the simultanous 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 to 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 IFNαβR$^{-/-}$ 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 IFNαβ receptor. Thereby, 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 implicates 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 difference in responsiveness of naïve and effector T cells to Hsp60 has been described before (4). In addition, contaminating bacterial structures in the recombinant Hsp60 preparation may have contributed to the observed effects since 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. Thereby, Hsp60 may operate similar to the LPS binding protein LBP 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 appropriate anti-bacterial immune response by amplifying LPS-mediated stimulation.

Acknowledgements – We thank Thomas Jakobs for critical reading of the manuscript.

Footnotes - A. Osterloh is funded by the Mildred-Scheel-Stiftung fuer Krebsforschung, Germany.

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FIGURE LEGENDS

Fig. 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-Alexa647 (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-Alexa647 (gray) and hHsp60-Alexa647 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 (1A). 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-Alexa647 (10 µg/ml). The histogram shows the inhibition of hHsp60-Alexa647 binding (gray) by pre-incubation of the cells with 20 µg/ml unlabeled hHsp60 (black line) (1B, left). Besides cells were pre-incubated with increasing amounts of unlabeled hHsp60 as indicated on the x-axis before addition of hHsp60-Alexa647 and the percentage of hHsp60-Alexa647 binding cells (y-axis) is shown (1B, 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-Alexa647 (x-axis). The figure shows the mean fluorescence intensity (MFI; y-axis) of the Hsp60-binding cell population (1B, right). Spleen cells were gated on R2.

Fig. 2: Hsp60 binds to distinct membrane regions on macrophages and bmDC. 1×10^6 BALB/c-derived bmDC (2A) or PEC (2B) were incubated with either 30 µg/ml FITC-labeled hHsp60 (2A, upper row; green) or unlabeled hHsp60 stained with Hsp60-specific antibody clone LK-1 and TRITC-labeled goat anti-mouse secondary antibody (2A, lower row, and 2B; red). In addition, all cells were stained with DAPI (blue). After staining, cells were fixed in PBS/1% PFA and centrifuged onto glass-slides. The right panel shows an overlay of DAPI and Hsp60 stainings.

Fig. 3: Hsp60 binds LPS and colocalizes with CD14 on the cell surface of macrophages. 3×10^4 pFM92-mHsp60 transfected COS1 cells expressing the murine Hsp60 protein as a cell surface molecule or mock transfected COS1 cells were incubated with 500 ng/ml ^{3H}LPS in 96 well plates for 45 min. Binding of ^{3H}LPS was blocked by the addition of either 15 µg/ml anti-Hsp60 antibody (clone 4B/9) or 5 µg/ml unlabeled LPS for 45 min before adding ^{3H}LPS. Afterwards cells were harvested and radioactivity was detected. The figure shows the mean and SEM of triplicates. The result is representative for three individual experiments (3A). 1×10^6 BALB/c PEC were incubated either alone (-Hsp60) or with 15 µg/ml hHsp60 (+Hsp60) in chamber slides. Afterwards, Alexa488-labeled LPS was added (green) and cells were stained with anti-Hsp60 (clone LK-1), TRITC-labeled goat anti-mouse antibody (red) and DAPI (blue) (3B, upper panels). 1×10^6 BALB/c PEC were incubated alone (-Hsp60) or with 15 µg/ml hHsp60 (+Hsp60). Further stainings were performed on cells in suspension. Hsp60 was detected as described before using anti-Hsp60 (clone LK-1) and TRITC-labeled goat anti-mouse antibody (red). In addition, cells were stained with DAPI (blue) and the CD14 receptor was detected with rat anti-CD14, FITC-labeled goat anti-rat and Alexa488-labeled anti-FITC antibodies (green) (3B, lower panels).

Fig. 4: 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 24h IL-12p40 was detected in the supernatants by specific ELISA (y-axis). Bars represent the mean of triplicates and SEM are shown (4A). 1×10^5 BALB/c PEC were incubated with 1 ng/ml LPS, 1 or 5 µg/ml hHsp60, a combination of both or a combination of hHsp60 and LPS that had been pre-incubated for 2h at 37°C to allow complexation. IL-12p40 was detected in the supernatants after 24h (4B, left). 1×10^5 DO11.10 T cells were stimulated with 0.5 µg/ml $OVA_{323-339}$ peptide antigen in the presence of 5×10^4 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 24h. The results are

representative for three independent experiments (4B, right). As depicted in Fig. 4C, instead of soluble Hsp60 either mock transfected COS1 cells or membrane-bound murine Hsp60 (mHsp60)-expressing COS1 cells were added to the test cultures (4C). 1×10^5 DO11.10 T cells were stimulated with 0.5 μg/ml OVA peptide in the presence of indicated amounts of LPS, COS1 cells alone or COS1 cells that had been pre-incubated with LPS for 2h at 37°C. IFNγ was detected after 24h (4D). The mean of triplicates and SEM are shown. (** $p<0.005$; students T test, unpaired, two-tailed)

Fig. 5: Synergistic immune stimulation by Hsp60 and LPS is Hsp60-specific. 5×10^4 DO11.10 T cells were stimulated with 0.5 μg/ml $OVA_{323-339}$ antigen in the presence of 2×10^4 BALB/c PEC and indicated amounts of LPS, hHsp60 (above) or BSA (below) or a combination of hHsp60 or BSA and LPS that had been pre-incubated for 2h at 37°C. IFNγ was quantified after 24h (y-axis) (5A). 1×10^5 BALB/c PEC were treated with indicated amounts of LPS, hHsp60 (above) or BSA (below) or a pre-incubated combination of these proteins and LPS as indicated on the x-axis. IL-12p40 was detected in the supernatants after 24h (y-axis) (5B). The mean of triplicates and SEM are shown. The results are representative for three individual experiments.

Fig. 6: Synergistic immune stimulation by Hsp60 and LPS is dependent on specific binding of LPS to Hsp60. 1×10^5 DO11.10 T cells were stimulated with 0.5 μg/ml $OVA_{323-339}$ peptide in the presence of 5×10^4 BALB/c PEC and indicated amounts (y-axis) of LPS, hHsp60 or a combination of hHsp60 and LPS pre-incubated 2h at 37°C. During the pre-incubation period either 2 μg/ml murine IgG2a isotype antibody (above) or anti-Hsp60 antibody (clone 4B9; below) were added. IFNγ was quantified after 24h (y-axis) (6A). 1×10^5 BALB/c PEC were treated in a similar way with LPS, hHsp60 or pre-incubated combinations of Hsp60 and LPS as indicated on the x-axis whereby 2 μg/ml of isotype antibody or anti-Hsp60 antibody (clone 4B9) were added. IL-12p40 was detected after 24h (6B). Figures show the mean and SEM of triplicates.

Fig. 7: Hsp60 but not LPS enhances IFNα production in professional APC. 1×10^5 PEC or bmDC from BALB/c mice were stimulated with either 10 μg/ml recombinant hHsp60 (gray bars) or 1 μg/ml LPS (black bars). Control cells were cultured alone (white bars). After 24h IFNα (y-axis) was detected in the supernatants by specific ELISA (7A). 2×10^4 PEC from BALB/c mice were incubated alone, with 0.5 ng/ml LPS, indicated amounts of hHsp60 or pre-incubated combinations of LPS and hHsp60 (2h, 37°C) . IFNα was detected after 24h (7B). 1×10^5 DO11.10 T cells were incubated with 3×10^4 BALB/c PEC, 10 μg/ml hHsp60 (7C) or 1 μg/ml LPS (7D) and stimulated with 1 μg/ml OVA peptide. IFNα and IFNβ were neutralized by addition of 2.5 kU/ml polyclonal anti-IFNα or anti-IFNβ while control cultures received 5 μg/ml rabbit IgG (Isotype) (7C, D). 1×10^5 BALB/c T cells were stimulated with 0.3 μg/ml anti-CD3 antibody in the presence of 2×10^3 MHC II^+ BALB/c spleen cells, 1×10^4 mock transfected COS1 cells (white bars) or mHsp60 expressing COS1 cells (black bars). IFNα and IFNβ were neutralized by addition of 2.3 kU/ml polyclonal anti-IFNα or anti-IFNβ while control cultures received 2 μg/ml rabbit IgG (Isotype). IFNγ was detected in the supernatant after 24h (7E). 2×10^5 spleen cells from C57BL/6 wildtype mice (wt), $IFN\alpha\beta R^{-/-}$ or $IFN\beta^{-/-}$ mice were stimulated with 0.5 μg/ml anti-CD3 in the presence of 1×10^4 mock transfected COS1 cells (white bars) or mHsp60-expressing COS1 cells (black bars) and IFNγ was detected after 24h (7F). 5×10^4 PEC and 1×10^5 T cells from C57BL/6 wildtype (wt) mice and $IFN\alpha\beta R^{-/-}$ mice were combined as indicated on the x-axis and stimulated with 0.3 μg/ml anti-CD3 in the absence (white bars) or presence of hHsp60 (gray bars). IFNγ was detected after 24h (7G). Figures show the mean and SEM of triplicates. (* $p<0.05$; ** $p<0.005$; *** $p<0.0005$; students T test, unpaired, two-tailed).

Fig. 8: B cells do not mediate stimulation of T cells by Hsp60/LPS. 1×10^5 DO11.10 T cells were stimulated with 0.5 μg/ml OVA323-339 peptide and indicated amounts of LPS, hHsp60 or pre-incubated combinations (2h, 37°C) of both in the presence of either 5×10^4 PEC (left) or B cells (right). IFNγ was quantified after 24h (y-axis). The mean and SEM of triplicates are shown. The result is representative for two independent experiments.

Figure 1

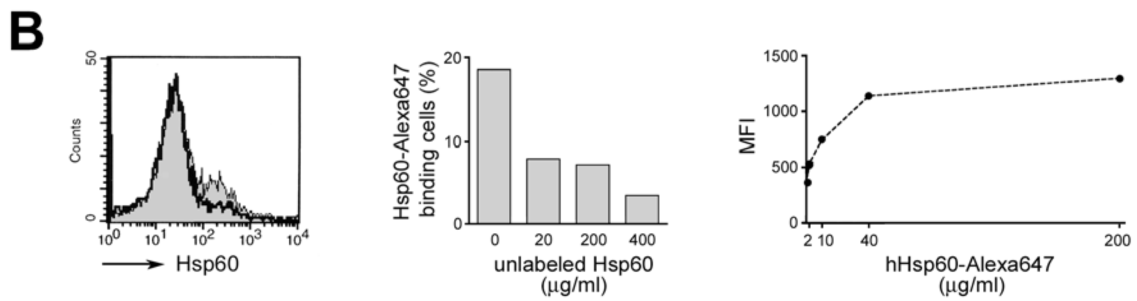


Figure 2

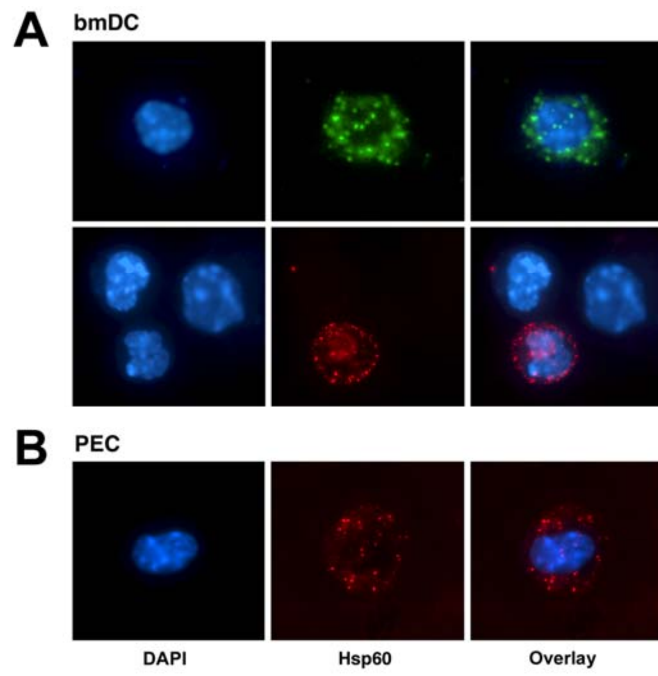
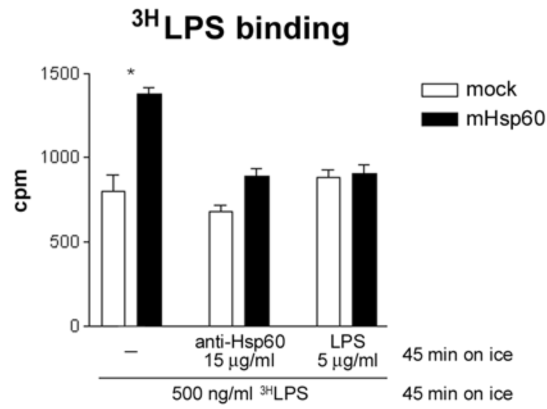


Figure 3



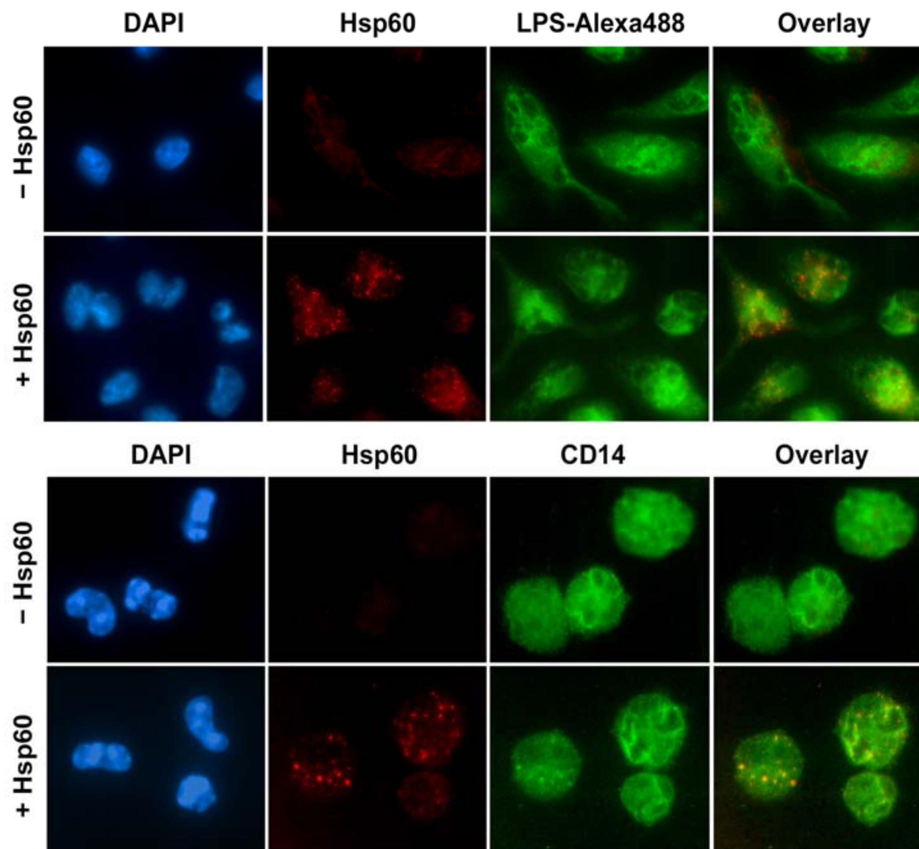


Figure 4

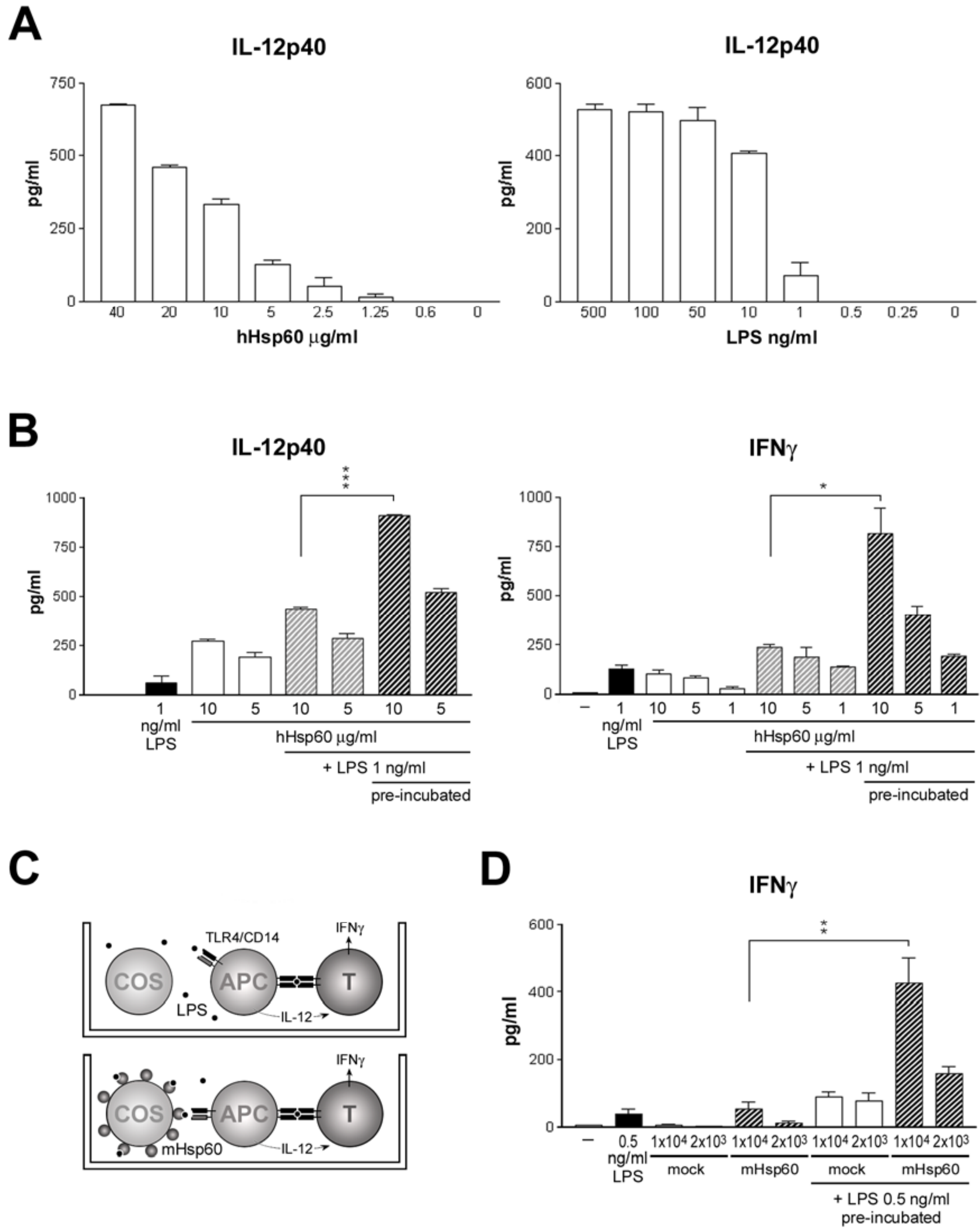


Figure 5

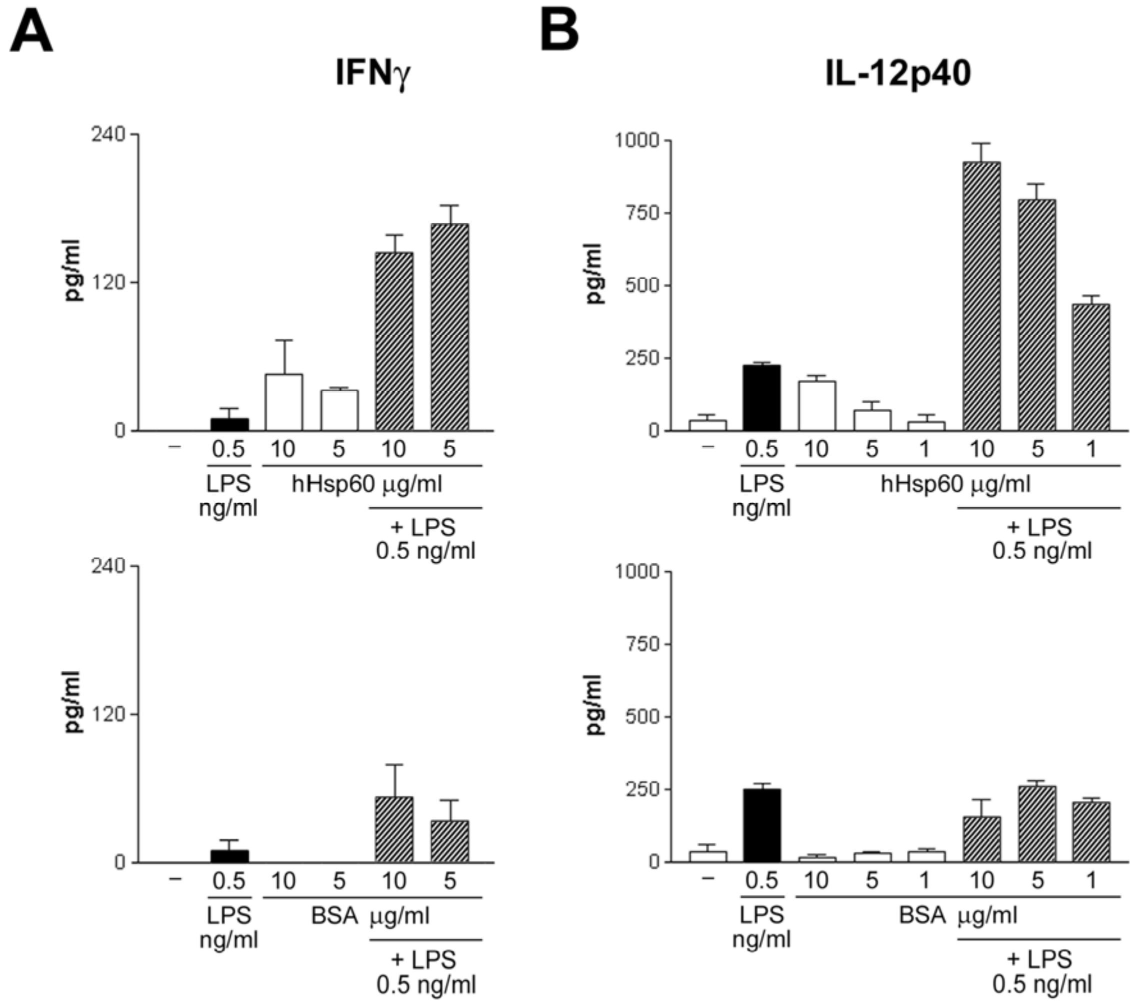


Figure 6

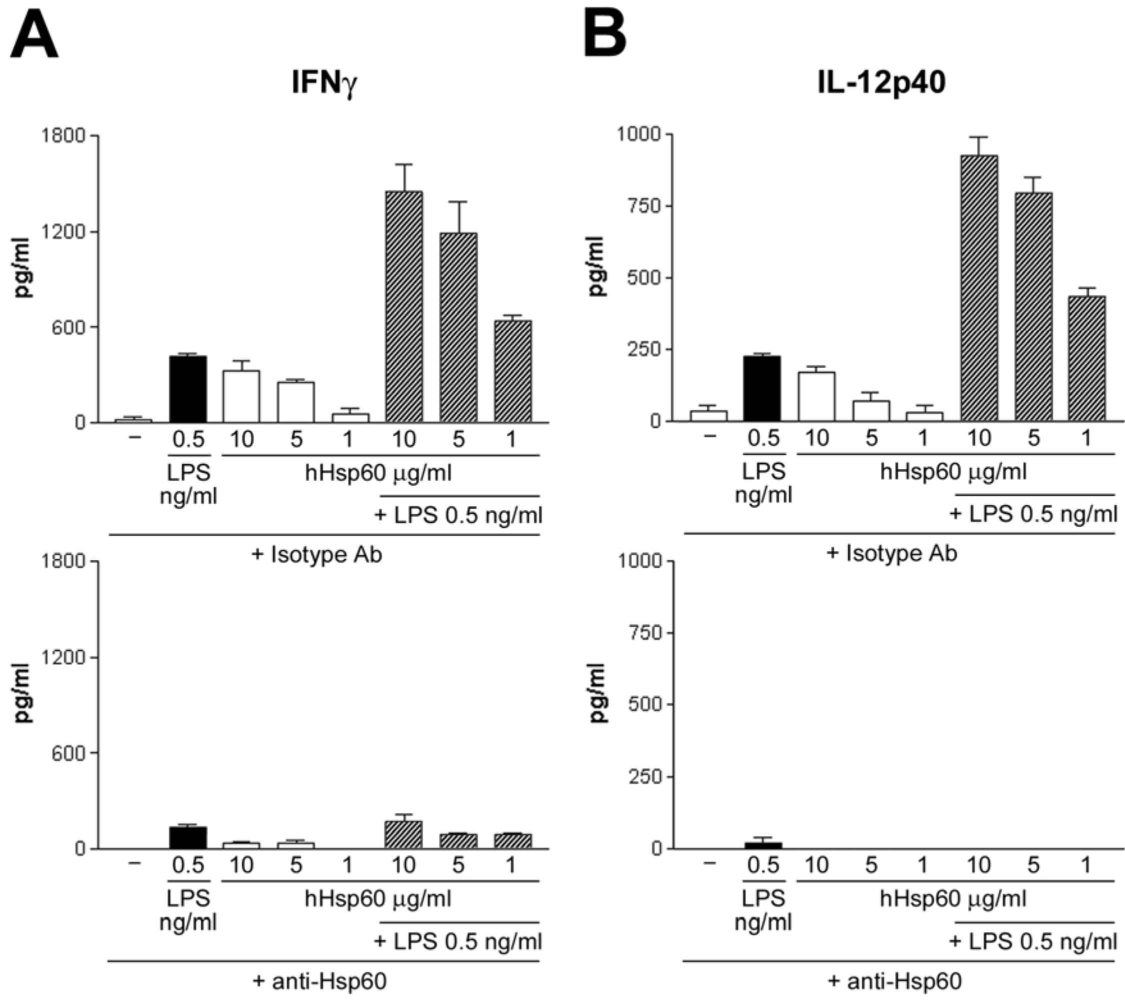


Figure 7

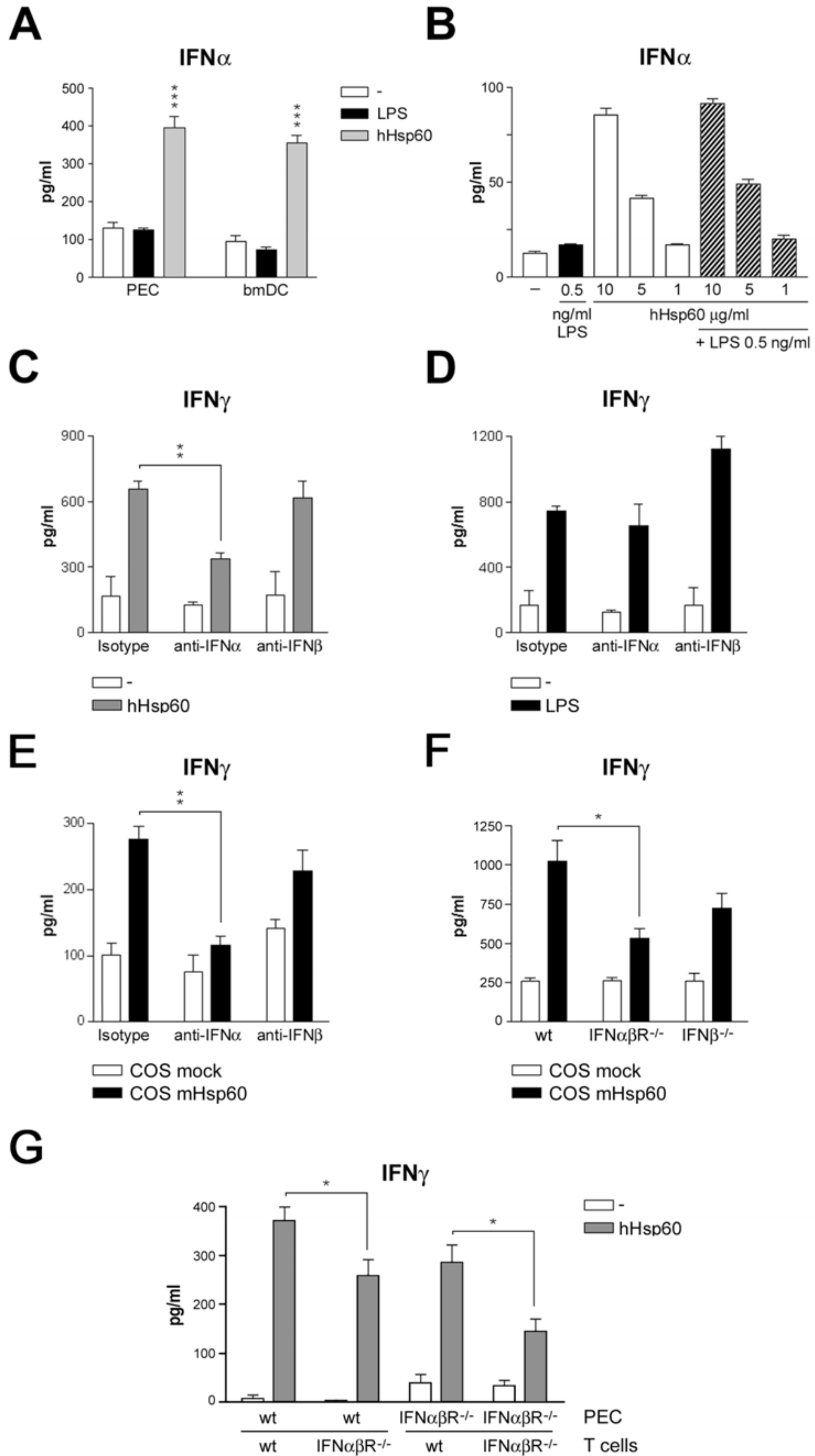


Figure 8