Lipopolysaccharide-free Heat Shock Protein 60 Activates T Cells*

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A possible function of eukaryotic heat shock protein 60 (Hsp60) as endogenous danger signal has been controversially discussed in the past. Hsp60 was shown to induce the secretion of proinflammatory cytokines in professional antigen-presenting cells and to enhance the activation of T cells in primary stimulation. However, in vitro activation of macrophages by Hsp60 was attributed to contaminating endotoxin in the recombinant Hsp60 protein preparations. Here, we employ low endotoxin recombinant human Hsp60 and murine Hsp60 expressed by eukaryotic cell lines to dissect the Hsp60 protein-mediated effects from biologic effects that are mediated by prokaryotic contaminants in the Hsp60 protein preparation. The induction of tumor necrosis factor-α secretion in mouse macrophages is lost after endotoxin removal and is not mediated by Hsp60 expressed in eukaryotic systems. In contrast, the Hsp60-mediated enhancement of antigen-specific T cell activation does not correlate with endotoxin contamination. Moreover, Hsp60 that is expressed on the surface of different eukaryotic cell lines increases the activation of T cells in primary stimulation. Taken together, we provide evidence that endogenous Hsp60, which is thought to be released from dying infected cells in vivo, has a biological function that is not due to contaminating pathogen-associated molecules.

Pattern recognition receptors represent an ancient family of receptors on professional antigen-presenting cells (APC),1 sensing pathogenic infection by the presence of conserved prokaryotic molecules, the so-called pathogen-associated molecular pattern (PAMP) (1). It has been suggested that the immune system also detects the destruction of tissue that is associated with infection, leading to the expression and release of endogenous danger signals (2), e.g. molecules or super-molecular structures that are usually hidden within healthy cells (3). In support of this hypothesis, urea acid that is released from injured cells has been identified recently as such an endogenous signal, inducing APC activation and displaying adjuvant activity in vivo (4).

Several members of the conserved heat shock protein (HSP) family have long been suspected to act as danger signals since they are up-regulated and released in response to cellular stress and stimulate immune responses in vivo and in vitro (5–8). HSPs were initially described as chaperones of nascent or aberrantly folded proteins (9). In eukaryotes, Hsp60 is located within the mitochondria, where it plays an essential role in folding of imported proteins (10, 11). It has been shown, however, that extracellular eukaryotic Hsp60 also induces the up-regulation of co-stimulatory molecules on murine and human APC and the release of proinflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α (12–16). The Hsp60 signaling is thought to be mediated by CD14 (14), Toll-like receptor (TLR)-4 (17), and TLR-2 (17, 18). This receptor system also mediates lipopolysaccharide (LPS) and lipoprotein signaling (19–21).

Since the recombinant Hsp60 protein employed was expressed in *Escherichia coli*, it may be contaminated with LPS and other prokaryotic molecules. Therefore, it has been crucial to rule out that the biologic activity of these commercially available Hsp60 preparations was due to prokaryotic contaminants. In the past, this question was addressed with several controls, including heat sensitivity and polymyxin B insensitivity of Hsp60 versus LPS (6). Recent studies have cast doubt on the validity of these controls (22–24). It was demonstrated that LPS in threshold concentrations does induce TNF-α in the murine macrophage cell line RAW 264.7 and is sensitive to heat denaturation. Moreover, Hsp60 and Hsp70 protein preparations obtained from StressGen Biotechnologies (Victoria, Canada), once they had been purified from LPS by immobilized polymyxin B, did not induce TNF-α while being fully active as ATPase or chaperones, respectively.

This finding led us to reconsider our previous work with recombinant Hsp60. We have shown that the addition of Hsp60 to cultures containing peritoneal exudate macrophages (PEC) and *ex vivo* purified T cells expressing transgenic MHC class I (25) or MHC class II (26) restricted TCRs specifically enhanced and accelerated the release of antigen-specific IFN-γ. Here, we employ LPS-depleted recombinant human Hsp60 to differentiate between Hsp60- and LPS-mediated stimulation. Moreover, murine Hsp60 expressed on the surface of different eukaryotic cell lines allows us to investigate the biological activity of Hsp60 independent of not only LPS but of any other prokaryotic molecule. We reproduce the published correlation of LPS contamination in the Hsp60 protein preparations and TNF-α induction in RAW 264.7 cells (23). However, we also demonstrate that Hsp60 still leads to an enhancement of antigen-specific T cell activation in the absence of LPS or any other PAMP.

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1 The abbreviations used are: APC, antigen-presenting cells; HSP, heat shock protein; PAMP, pattern-associated molecular pattern; PEC, peritoneal exudate cell; TLR, Toll-like receptor; II, interleukin; IFN, interferon; OVA, ovalbumin; LAL, Limulus amoebocyte lysate; TNF, tumor necrosis factor; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TCR, T cell receptor; FACS, fluorescence-activated cell sorter; EU, endotoxic units.
EXPERIMENTAL PROCEDURES

Cell Culture—8–10-week-old female DO11.10 TCR transgenic mice specific for OVA25–33-2-3H-2-3A*-27 (27) and BALB/c mice were obtained from the Bundesamt fuer Risikobewertung, Berlin, Germany. RAW 264.7 cells and COS cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and L-glutamine.

To induce Pristane BALB/c mice were injected with 500 μl of pristane (Sigma) intraperitoneally and sacrificed 5 days later. PEC were harvested by peritoneal lavage. T cells from DO11.10 transgenic mice (termed DO11.10 T cells hereafter) were purified from spleens via depletion of MHC-II-positive cells by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol.

HLA Typing—E. coli-derived LPS was obtained from Sigma (strain 055:B5; order number L4005). Recombinant murine Hsp60 was obtained from StressGen Biotechnologies, either of unpurified quality (order number SPF 741, lot number 104424) or of “low endotoxin” quality (order number ESP 741, lot number 105402). Recombinant human Hsp60 was obtained from Loke Diagnostics Aps (Risskov, Denmark) (lot number 2003 NT) and termed high endotoxin Hsp60 hereafter. High endotoxin human Hsp60 protein preparations were further purified by EndoTrap endotoxin removal columns according to the manufacturer’s instructions (profs, Regensburg, Germany) to generate low endotoxin human Hsp60. Quantification of endotoxin in protein and LPS preparations was performed with the Limulus amoebocyte lysate kit (QCL-1000, BioWhittaker, Walkersville, MD). Quantification of protein was performed by Bradford assay and verified by silver stain and western blot with specific anti-Hsp60 monoclonal antibody (StressGen Biotechnologies order number SPA-810).

Vector Construction—The Hsp60 cDNA encoding for the mature protein missing the mitochondrial leader sequence was amplified from EL-4 DNA using the 5-'CGGGCGCTATGCGAAGAATGTT-3' sense and 5'-TGGACATTAGCAGCTTAAACCA-3' antisense primer and subcloned into the pCR2.1 vector (Invitrogen). The Hsp60 cDNA was amplified from this vector using the 5'-GCGACTGCGAAGAACATGCTT-3' and 5'-AATTGGTTAGGGC-3' BglII-sense and 5'-AGCTGGCAGATAGAAGTTAAGCCCCTCATCTATCT-3' HindIII antisense primer and cloned into the pCR2.1 vector (Invitrogen). The Hsp60 cDNA was cloned into the pCR2.1 vector (Invitrogen) that fuses the IgTAA stop codon. The BglII- and SalI-digested PCR product was cloned and amplified from this vector using the 5'-CGGACTGCGAAGAACATGCTT-3' and 5'-CTTACTGGCTTATCGAAATTAATAC-3' XbaI sense or 5'-ATGGCTGAGCTTAAACTGACC-3' XbaI antisense primer and cloned into the pCR2.1 vector (Invitrogen) (28) and the pFM92 vector (β-actin promter) (29).

Transfection of Eukaryotic Cell Lines—Transient transfection of COS cells was performed using the FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. In brief, 5 μg of DNA and 6 μl of FuGENE reagent were incubated in 100 μl of serum-free RPMI medium at room temperature for 45 min. COS cells were seeded the day before into 6-well culture dishes. The culture medium was exchanged against 2 ml of fresh RPMI medium containing 10% fetal calf serum, and the transfection mix was added for 24 h. The cells were then analyzed by FACS staining using myc epitope or Hsp60-specific antibodies and western blots of cell lysates. Stable transfection of X63 cells was performed by electroporation at 250 mV and 960 microfarad (Bio-Rad) using 1 × 107 cells and 10 μg of Scal-linearized pFM92 or pFM92-M Pam3CSK4 vector DNA. The cells were washed twice in serum-free RPMI medium and then incubated for 10 min at room temperature in 350 μl of serum-free RPMI medium in the presence of vector DNA before electroporation. After transfection, 450 μl of culture medium (RPMI 10% fetal calf serum) was added for 10 min. The cells were then seeded into four 24-well plates. Stably transfected cells were selected by adding G418 to the culture medium (1.2 μg/ml) the following day. Murine Hsp60 expressing cell clones were detected in a Hsp60-specific FACS staining.

FACS Staining—1–2 × 106 cells were stained with specific antibodies against the myc epitope of the recombinant protein (clone 9E10, Upstate Biotechnology, Milton Keynes, UK) or against Hsp60 (SPA806, StressGen Biotechnologies). FC receptors of cells were blocked with 30 μl of Cohn II fraction (10 mg/ml, Sigma), and 1 μg of specific antibody was added. After a 30-min incubation on ice, cells were washed, and a phycoerythrin-labeled murine IgG-specific antibody (Dianova, Hamburg, Germany) was added.

Cellular Assays and Cytokine Quantification—All assays were performed in RPMI 1640 medium supplemented with 10% fetal calf serum, HEPES, and L-glutamine. For APC activation, RAW 264.7 cells or pristane-induced BALB/c-derived PEC (1 × 105/well) were incubated with the indicated amount of Hsp60 or LPS for 24 h at 37 °C. Alternatively, the indicated amounts of COS cells or X63 cells either transfected with Hsp60 or mock-transfected with the corresponding empty vector were added to the APC culture. TNF-α in the supernatant was detected by standard sandwich ELISA. 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 8 μg/ml anti-TNF-α (clone MP6XT-2) in 10 mM NaHCO3, pH 9.6, at 4 °C for 24 h. Plates were blocked with phosphate-buffered saline containing 1% bovine serum albumin for 2 h at 37 °C and washed three times with phosphate-buffered saline containing 0.05% Tween 20. Culture supernatants were added to the coated plates and incubated at 4 °C for 24 h. After six washes, 1 μg/ml biotinylated anti-TNF-α clone MP6-XT3 was added as detection antibody and incubated at 37 °C for 1 h. Following six washes, a 1:10,000 dilution of streptavidin/peroxidase (Amersham Biosciences) in phosphate-buffered saline containing 0.1% bovine serum albumin was added for 30 min at 37 °C. Plates were washed six times and developed with 300 μg/ml tetramethylbenzidin, diluted in 0.1 M NaHPO4, pH 5.5, containing 0.003% H2O2. The reaction was stopped by the addition of 25 μl of 2 M H2SO4, and OD at 450 nm was measured immediately. All antibodies and recombinant TNF-α were obtained from Pharmingen.

MHC-II-depleted spleen cells from DO11.10 mice (5 × 105/well) were incubated with syngeneic pristane-induced LPS (1 × 105/well) to the number of 1 μg/ml ovomucoid, 323–330 K active albumin peptide, 323–330 K active albumin peptide, and 1000–2000 K active albumin peptide, respectively. Either the indicated Hsp60 species or LPS or Hsp60- or mock-transfected cell lines were added to this culture. Supernatants from the cultures were collected after 24 h and analyzed for IFN-γ content by ELISA employing IFN-γ DuoSet ELISA development system according to the manufacturer’s recommendation (R&D Systems, Minneapolis, MN).

RESULTS

No Induction of TNF-α Release from Murine Macrophages by Low Endotoxin Hsp60—The human recombinant Hsp60 protein used in the experiments contained an endotoxic activity of 22 EU/mg, as determined by the Limulus amoebocyte lysate (LAL) assay. This high endotoxin preparation was further purified by EndoTrap endotoxin removal columns, thus generating low endotoxin Hsp60 containing 1.6 EU/mg of protein (Fig. 1A). Incubation with LPS or with 10 μg/ml high endotoxin Hsp60 induced TNF-α release in the murine macrophage line RAW 264.7, whereas the same amount of low endotoxin Hsp60 did not induce elevated TNF-α levels (Fig. 1B). Moreover, the comparison of the commercially available recombinant murine Hsp60 from StressGen Biotechnologies revealed that only the endotoxin-contaminated (48 EU/mg) Hsp60 preparation induced TNF-α release in RAW 264.7 cells, whereas the low endotoxin (<3 EU/mg) preparation was biologically inactive (Fig. 1C). The E. coli-derived LPS that we employed (E. coli strain 055:B5) induced TNF-α secretion in RAW 264.7 cells at threshold concentrations between 100 pg/ml and 1 ng/ml (Fig. 1C). Since the endotoxin activity of this LPS preparation was determined to be 1 EU/μg of LPS (data not shown), the threshold THLD for TNF-α induction in RAW 264.7 cells by LPS, being 0.1–1 ng/ml, was equivalent to 0.1 and 1 EU/ml, respectively. Consequently, the TNF-α response to 10 μg/ml high endotoxin human Hsp60, still containing 0.22 EU/ml, can be explained as a response to the contaminating endotoxin. In contrast to cell lines, freshly prepared PEC responded to LPS in concentrations between 10 and 100 ng/ml and were not activated by either high or low endotoxin Hsp60 in concentrations up to 30 μg/ml (data not shown). These results in accordance with previous studies (23) strongly suggest that the LPS that is present in the recombinant human and murine Hsp60 preparations induces the observed TNF-α secretion in RAW 264.7 cells.
E. coli-derived LPS. TNF-α peptide OVA323–339. As shown in Fig. 2, interaction of the cells in the presence of the chicken ovalbumin (OVA)-derived Hsp60 preparations to induce TNF-α and IFN-γ secretion did not correlate with the endotoxin contamination of the Hsp60 protein preparation or with the potency of the Hsp60 preparations to induce TNF-α in RAW 264.7 cells (Fig. 1B). The addition of 100 ng/ml (i.e. 100 EU/ml) of E. coli-derived LPS induced the same increase in IFN-γ secretion by DO11.10 T cells as the addition of Hsp60. In contrast, LPS amounts containing approximately the same endotoxic activity as 10 μg/ml high and low endotoxin Hsp60 preparations (i.e. 0.22 and 0.016/EU ml, respectively) did not induce a significant increase in IFN-γ secretion (1.3-fold and no increase, respectively). Moreover, Fig. 2B shows that in an independent experiment, a comparable increase in IFN-γ secretion of T cells was induced by either the commercially available low endotoxin preparation of recombinant murine Hsp60 (StressGen Biotechnologies, 3.7-fold increase) or the low endotoxin human Hsp60 (Loke Diagnostics, 3.8-fold increase), whereas in the same experiment, 100 pg/ml LPS only led to a 1.5-fold increase of the IFN-γ response. These results suggest that the Hsp60-mediated increase in IFN-γ secretion of naive T cells is not due to contaminating LPS alone and does not correlate with TNF-α induction. However, it is still possible that other PAMPs, which escape recognition in the LAL assay and do not induce TNF-α secretion, contribute to the increased IFN-γ secretion by DO11.10 T cells. To rule out any contribution of prokaryotic contaminants in the Hsp60-expressing constructs, driven by different promoters (Fig. 3A). Murine Hsp60 cDNA was cloned as a fusion protein flanked with two model epitopes and containing the transmembrane domain of the platelet-derived growth factor receptor. COS cells were transiently transfected with two different constructs, driven by different promoters (Fig. 3A). Surface expression of Hsp60 was determined by extracellular FACS analysis, detecting both Hsp60 and the inserted model epitope myc (Fig. 3B). Fig. 4 shows that neither Hsp60-positive nor control COS cells induced TNF-α in RAW 264.7 cells (Fig. 4A) or in PEC (data not shown). The addition of Hsp60-expressing constructs and low endotoxin Hsp60 increased the antigen-specific IFN-γ secretion of naive T cells. The addition of both recombinant human high endotoxin Hsp60 and low endotoxin Hsp60 increased the antigen-specific IFN-γ secretion (1.3-fold and no increase, respectively). Hsp60 Increases Antigen-specific T Cell Activation Irrespective of the Endotoxin Contamination—We have previously shown that eukaryotic Hsp60 induces the acceleration and enhancement of antigen-specific IFN-γ secretion in naive CD8-positive (25) or CD4-positive (26) TCR transgenic T cells. To compare the different Hsp60 preparations in this system, we incubated BALB/c-derived PEC together with T cells that had been purified from the DO11.10 TCR transgenic mice (DO11.10 T cells) in the presence of the chicken ovalbumin (OVA)-derived peptide OVA323–339. As shown in Fig. 2, interaction of the DO11.10 TCR and OVA323–339/MHC-II complexes on the surface of PEC induced IFN-γ secretion in T cells. Since ex vivo-prepared DO11.10 T cells displayed a naive phenotype (26) and encountered the first stimulation, the IFN-γ response in the absence of co-stimulating agents was low (Fig. 2A, medium). The addition of both recombinant human high endotoxin Hsp60 and low endotoxin Hsp60 increased the antigen-specific IFN-γ release 2-fold (Fig. 2A). This Hsp60-mediated increase of IFN-γ secretion did not correlate with the endotoxin contamination of the Hsp60 protein preparation or with the potency of the Hsp60 preparations to induce TNF-α in RAW 264.7 cells (Fig. 1B).
LPS-free Hsp60 Activates T Cells

The question of whether eukaryotic heat shock proteins themselves function as stimulators of the innate immune system or whether contaminating endotoxin is responsible for the immune stimulatory effects has been discussed controversially in the past (6). The data presented in this study suggest (i) that the TNF-α secretion in macrophages, often employed as read-out for Hsp60 stimulation, is due to endotoxin contamination, as suggested by Gao and Tsan (23), and (ii) that the Hsp60-mediated increase in antigen-specific IFN-γ secretion by naive T cells is not due to LPS or other PAMPs.

We draw this conclusion from the following evidence. We reproduced previous findings (23) that recombinant Hsp60 failed to stimulate murine macrophages to secrete TNF-α once the contaminating LPS has been depleted below 10 EU/mg of protein. In contrast, human and murine Hsp60 preparations that contained less than 10 EU LPS/mg of protein and did not induce TNF-α release in RAW 264.7 cells or in PEC still increased the IFN-γ secretion in DO11.10 T cells undergoing primary stimulation. An endotoxin contamination below 10 EU/mg of protein will lead to a final amount of 0.1 EU/ml cell culture if the protein is used at 10 μg/ml as done in this study.

Although an endotoxin contamination of 0.1 EU/ml has been described as biologically inert to RAW 264.7 cells or dendritic cells by others (22–24, 30), we cannot rule out an effect on T cell activation. We addressed this problem by demonstrating that the same amount of LPS that was present in the different Hsp60 preparations failed to show a biologic effect on T cells.
the Hsp60-transfected clone X63 pFM92-mHsp60#12 (ration employed. The...in the LAL assay. Therefore, it is still possible that these
poproteins (21) or CpG-rich DNA (31), which are not recognized
activate APC through the TLR/IL-1R signaling, such as li-
Besides LPS, there are other PAMPs to consider that may
mating the LPS amount containing the biologically relevant
ployed in this study displays an endotoxic activity of 1 EU/ng of
3-fold higher endotoxic activity. Thus, the comparison of con-
LPS, whereas Gao and Tsan (23, 24) describe a highly purified
peptide. Titrated numbers (as indicated on the
content by ELISA. Results are shown as means of triplicates, and
Error bars represent the surface staining with anti-mouse Hsp60 mono-
togram
expression on the surface of X63 cells was determined by FACS staining
left panel, X63-mock) or the Hsp60-transfected clone X63 pFM92-mHsp60#12 (right panel). The
shaded histogram represents the background staining, and the open his-
togram represents the surface staining with anti-mouse Hsp60 mono-
clonal antibody. B, LPS (100 ng/ml) or mock-transfected X63 cells or X63 pFM92-mHsp60#12 (5 \times 10^5) were cultured with RAW 264.7 (1 \times 10^5)
cells for 24 h. TNF-\(\alpha\) in the supernatant was determined by ELISA. Error bars show S.D. of triplicates; the result is representative for two inde-
dependent experiments; C, 5 \times 10^5 DO11.10 T cells were cultured with 1 \times 10^5 BALB/c-derived PEC in the presence of 0.1 \mu g/ml OVA 323–339 peptide. Titrated numbers (as indicated on the x axis) of X63 pFM92 cells (open bars) or X63 pFM92-mHsp60#12 cells (black bars) were added to the
culture. Supernatant was harvested 24 h later and analyzed for IFN-\(\gamma\) content by ELISA. Results are shown as means of triplicates, and error bars indicate S.D. Asterisks indicate statistically significant increase of IFN-\(\gamma\) secretion when compared with mock-transfected cells employing Student’s t test (*, \(p < 0.05\); ***, \(p < 0.005\)). The experiment shown is representative for three independent experiments.
However, the endotoxic activity of E. coli-derived LPS may vary with the E. coli strain and with the purity of the LPS prepara-
tion employed. The E. coli strain B055:B5-derived LPS em-
ployed in this study displays an endotoxic activity of 1 EU/\(\mu g\) of
LPS, whereas Gao and Tsan (23, 24) describe a highly purified
protein-free LPS derived from JM83 E. coli K12, displaying a 3-fold higher endotoxic activity. Thus, the comparison of con-
taminating LPS in protein preparations with the same amount of purified LPS bears considerable risk of over- or underesti-
mating the LPS amount containing the biologically relevant endotoxic activity corresponding to the protein preparation.
Besides LPS, there are other PAMPs to consider that may activate APC through the TLR/IL-1R signaling, such as li-
poproteins (21) or CpG-rich DNA (31), which are not recognized in the LAL assay. Therefore, it is still possible that these
“non-LPS”-PAMPs and not the Hsp60 protein itself mediate T
cell activation.
To obtain a Hsp60 protein source that is completely free of
prokaryotic molecules, we expressed Hsp60 on the surface of
eukaryotic cell lines. We did not attempt to purify the protein
out of an eukaryotic expression system since that again may
lead to contamination with endotoxin or other prokaryotic con-
taminants. Instead, we chose to employ the eukaryotic cell
lines COS and X63 as mostly inert carriers for our target
protein. In accordance with the results presented earlier, mu-
rine Hsp60 expressed on the surface of both cell lines induced
an increase in the antigen-specific IFN-\(\gamma\) response of DO11.10
T cells but did not induce the release of TNF-\(\alpha\) in RAW 264.7
cells. We recognize that, within our experimental system, one
still cannot distinguish whether the Hsp60 protein itself is the
stimulating agent or whether it functions as a chaperone for
other intracellular molecules that mediate the final increase in
antigen-specific IFN-\(\gamma\) secretion of T cells. If Hsp60 chaperones
other agents for activation, the expression of Hsp60 in a eu-
karyotic system proves that these molecules must be of eukary-
otic origin, and thus, stimulate T cell responses in the absence of PAMPs.
Regarding the mechanism of this Hsp60-mediated T cell
activation, our results suggest that Hsp60 enhances T cell
activation independently of TNF-\(\alpha\) induction in APC, at least
above the threshold of a standard ELISA. Moreover, a recent
study employing gene expression array analysis of RAW 264.7
cells stimulated with recombinant low endotoxin Hsp60 and
Hsp70 did not induce the up-regulation of 96 common cytokine
genes (30). This does not exclude a role for these cytokines in
the Hsp60-mediated T cell activation since we use freshly pre-
pared peritoneal macrophages as APC in this system. The
macrophage cell line RAW 264.7, although responding to much
lower doses of LPS and LPS-contaminated Hsp60 preparations
with TNF-\(\alpha\) release, did not induce the Hsp60-mediated T cell
activation (data not shown). Employing the LPS-free murine
Hsp60, we will now reevaluate the role of cytokines, co-stimu-
I pointers involved in the Hsp60-mediated
T cell activation.
Endotoxin as a possible source of biologic activity, falsely at-
tributed to HSP, has moved into the focus of the scientific com-
munity lately. Regarding other HSP families, it has been dem-
strated that Gp96 displays immune stimulatory capacity in
the absence of PAMPs since transgenic mice expressing Gp96 on
the cell surface develop spontaneous autoimmune diseases (32).
A murine tumor cell line that was secreting autologous Hsp70
due to forced overexpression proved to be more immunogenic in
vivo than the parental cell line (33). Here, we provide evidence
that murine Hsp60 expressed as a transmembrane protein on the
surface of two different eukaryotic cell lines increases the T cell
activation in the absence of LPS or PAMPs in vitro. In accordance
with our data, it has been shown that skin allografts derived from
mice transgenic for autologous Hsp60 displayed accelerated re-
jection when compared with skin grafts expressing the usual
level of endogenous Hsp60 (34). Therefore, it is again possible to
speculate that Hsp60, among other HSPs, is released from dying
cells in vivo and may function as an activator of the innate
immune system.

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