Inducible Heat Shock Protein 70 Reduces T Cell Responses and Stimulatory Capacity of Monocyte-derived Dendritic Cells*3

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Background: An increased presence of Hsp70 has been observed in many clinical conditions; however, its role remains elusive.

Results: Hsp70 reduces the stimulatory capacity of dendritic cells and T cell responses in an in vitro experimental setup.

Conclusion: Hsp70 might moderate inflammatory reactions mediated by the immune system.

Significance: Provides additional insight into the role of Hsp70 in autoimmune diseases, transplantation, and cancer.

Heat shock protein 70 (Hsp70) has gained a lot of attention in the past decade due to its potential immunoregulatory functions. Some of the described proinflammatory functions of Hsp70 became controversial as they were based on recombinant Hsp70 proteins specimens, which were later shown to be endotoxin-contaminated. In this study we used low endotoxin inducible Hsp70 (also known as Hsp72, HSPA1A), and we observed that after a 24-h incubation of monocyte-derived immature dendritic cells (mo-iDCs) with 20 μg/ml of low endotoxin Hsp70, their ability to stimulate allogenic T cells was reduced. Interestingly, low endotoxin Hsp70 also significantly reduced T cell responses when they were simulated with either IL-2 or phytohemagglutinin, therefore showing that Hsp70 could alter T cell responses when they were simulated with either IL-2 or phytohemagglutinin, therefore showing that Hsp70 could alter T cell responses when they were simulated with either IL-2 or phytohemagglutinin. Additionally, low endotoxin Hsp70 also significantly reduced T cell responses when they were simulated with either IL-2 or phytohemagglutinin. Taken together, our observations strongly suggest that Hsp70 might dampen, rather than provoke, T cell-mediated inflammatory reactions in many clinical conditions where up-regulation of Hsp70 is observed.

Heat shock proteins (HSPs)3 have been studied as potential antigenic molecules that can induce a specific antitumor immune response. In 1993 Srivastava and colleagues showed that the two most extensively studied HSPs, Hsp70 and gp96, both acted as antigenic peptide carriers but were not antigenic themselves (1, 2). These HSPs were shown to be potent antigenic peptide carriers (3, 4), which when isolated from a tumor can deliver transported antigenic peptides to professional antigen-presenting cells (APCs) and prime CD8+ T cells against the same tumor from which the HSPs were purified (5). The peptide delivery by Hsp70 to APCs was described as being mediated by CD91 or/and LOX-1 receptors (6–8).

Controversially, in addition to its role in the antigen cross-presentation process, Hsp70 at a concentration as low as 0.5 μg/ml has been described to be a potent proinflammatory cytokine and to be involved in the activation of innate immunity (9). The Hsp70 receptors responsible for this activation have been proposed to be CD14 (9) and Toll-like receptors (TLRs) (10, 11), which also mediate activation of innate immunity caused by microbial endotoxins. The shared activation pathway as well as the similar activation profile for endotoxins and Hsp70 led to speculation that the observed cytokine-like property of Hsp70 may have been incorrectly identified. Accordingly, it was shown that the endotoxin contamination in recombinant Hsp70 is common and exclusively responsible for the described proinflammatory properties, including activation of APCs (12–16). It was also shown that endotoxin-free Hsp70 did not induce APC activation while still effectively delivered antigenic peptides for cross-presentation (17). In a different study, Hsp70 failed to bind to null cells overexpressing either CD14, TLR2, or TLR4 which had previously been associated with the Hsp70-mediated activation of innate immunity (8). Hsp70 was also shown to be involved in lipopolysaccharide (LPS) signal transduction (18, 19), suggesting that even a negligible amount of microbial endotoxin in combination with Hsp70 might potentially be amplified and subsequently responsible for the activation of innate immunity.

Conversely, in some studies where Hsp70 was purified from a nonbacterial origin, excluding the possibility of microbial contamination, it was shown that Hsp70 could actually stimulate macrophages to secrete nitric oxide (20, 21). Hsp70 purified from a nonbacterial origin at concentrations as high as 400 μg/ml was shown to have very moderate and only partial stim-
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ulmonary capability on dendritic cells (DCs), which did not resemble that of endotoxins (20). Recently low endotoxin Hsp70 was shown to protect neuroblastoma cells against heat stress, stimulated intracellular calcium flux in monocytes, and moderately induced mouse splenocyte cytokine secretion and the selective proliferation of particular subpopulations of CD4 and CD11, but not CD8 cells (22). In a different study Hsp70 was shown to induce immune responses of CD4 and CD8 cells upon extended incubation with Hsp70 (23).

In this study, we examined the properties of low endotoxin Hsp70 on monocyte-derived immature DCs (mo-iDCs) as well as purified T cell populations. Interestingly, we observed that in our in vitro experimental model low endotoxin specimen of Hsp70 reduced the stimulatory capacity of mo-iDCs and abrogated T cell proliferation independently from used stimuli, which included either mo-iDCs, phytohemagglutinin (PHA), or IL-2. This novel property of Hsp70 may advance our understanding of immunological mechanisms associated with inflammation and tumor development, which are directly associated with the up-regulation of Hsp70.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Cells were cultured in RPMI 1640 medium (Invitrogen) containing 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 2 mM l-glutamine (Invitrogen) supplemented with 10% human AB serum (PAA Laboratories) unless otherwise indicated. All cultures were incubated at 37 °C in a humidified 5% CO2 in air incubator (IR 1500; Flow Laboratories). Before collecting human blood samples, informed consent was obtained, and the local ethics committee approved the project.

Generation of Monocyte-derived Immature DCs—Peripheral Blood Mononuclear Cells (PBMCs) were prepared from the blood of healthy donors by density gradient centrifugation on Lymphoprep (Axis-Shield). Monocyte-derived DCs (mo-DCs) were prepared as previously described (24). MACS technology (Miltenyi Biotec) was used to isolate monocytes from PBMCs. Purified monocytes were then grown in 24-well plates (Greiner Bio-One) in the serum-free medium, CellGro DC (CellGenix), at a concentration of 5 × 105/well (1 ml). To differentiate the monocytes into mo-DCs, 50 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Immunotools) and 50 ng/ml IL-4 (Immunotools) were added to the culture. After 3 days the medium and cytokines were refreshed and cultured for additional 3 days; these cells were then referred to as immature mo-DCs (mo-iDCs). The level of endotoxin contamination in the cytokines according to the manufacturer: GM-CSF <0.1 EU/µg; IL-4 <0.1 EU/µg.

mo-iDC Phenotype and Morphology—Hsp70 from three different sources was tested for its influence on maturation of mo-iDCs. Two Hsp70 samples produced in Escherichia coli were acquired from Assay Design (previously StressGen, currently Enzo Life Sciences) which according to the manufacturer had an endotoxin content of either >500 EU/mg or <50 EU/mg, catalogue number NSP-555 or ESP-555, respectively. Hsp70 with a low endotoxin contamination was purified and kindly provided by Britta Eiz-Vesper (23). As described previously, a low endotoxin Hsp70 sample was purified from the HEK293 human cell line and without any further endotoxin removal tested for endotoxin contamination using Limulus amebocyte lysate assay (Lonza) indicating endotoxin content of ~0.32 EU/mg (23). These Hsp70 samples were incubated at different concentrations with 2 × 105 mo-iDCs/well in 96-well, round-bottomed plates for 24 h. The cells were in the serum-free medium, CellGro DC (200 µl/well). As a positive control, LPS (Sigma-Aldrich) at a concentration of 1 µg/ml was used. After 24-h incubation the cell morphology was acquired using light microscopy as well as flow cytometry, FACSCalibur (BD Biosciences). Nonviable cells were represented by the characteristic change in their size and granularity visible using forward and side scatter plots. Flow cytometry was also used to test the expression of CD14 (BD Biosciences, clone rmC5–3), CD1a (Dako, clone NA1/34), CD80 (BD Biosciences, clone L307.4), CD86 (BD Biosciences, clone 2331:FUN-1), CD83 (BD Biosciences, clone HB15e), and HLA-DR (BD Biosciences, clone L243). Isotype control was mouse IgG FITC- or phycoerythrin-conjugated (BD Biosciences, clone X40). For apoptosis detection the TACS Annexin-V FITC detection kit (R&D Systems) was used according to the manufacturer’s instructions. For each test 10,000 events were acquired, and data were analyzed using FlowJo software (Tree Star).

Generation of Activated T Cells—Healthy donors were serologically typed for HLA-A*03 (Abcam, clone 4i153); T cells from HLA-A*03-positive donors were isolated using a T cell enrichment mixture (Stemcell Technologies). For stimulation of T cells, either an influenza antigen (ILRGSVAHK) or Pmel-17/gp100 (ALLAVGATK) specific to HLA-A*03 was used and commercially synthesized at a purity of >95% (GenScript). Autologous mo-iDCs were mixed with the T cells at a 1:10 ratio in the presence of 10 µM peptide. The cells were plated at a concentration of 0.7 × 106/ml in 6-well plates with addition of 0.5 IU/ml IL-2 (Roche Applied Science). After 2 days of incubation 20 IU/ml IL-2 and 5 µM peptide were added to the culture and further incubated for 8 days. The cells were then restimulated every 7 days with feeder cells (autologous PBMCs primed with 10 µM peptide overnight and then irradiated with 20 grays). The T cells were mixed with feeder cells at a 1:1 ratio in the presence of 10 µM peptide and 20 IU/ml IL-2. After 7 weeks the cells were tested using flow cytometry for the expression of CD25 (BD Biosciences, clone 2A3) in either CD8 (BD Biosciences, clone SK1) or CD4 (BD Biosciences, clone SK3) population. The activated T cell expression profile was compared with a freshly enriched T cell population, and for each test 100,000 events were acquired.

T Cell Proliferation and IFNγ Production—Either freshly isolated T cells (nonactivated T cells) or activated T cells were stimulated with mo-iDCs at a ratio of 10:1 (0.5 × 10^6 T cells versus 0.5 × 10^6 mo-iDCs). CD3, CD8, or CD4 cells were enriched from whole blood using CD3, CD8, or CD4 enrichment cocktails by negative selection (Stemcell Technologies). Where indicated mo-iDCs were preincubated with the low endotoxin Hsp70 at different concentrations for 24 h before being used in the proliferation assay. The preincubation of 2 × 10^5 mo-iDC/well in 96-well, round-bottomed plates was performed in the serum-free medium, CellGro DCs (200 µl/well), after preincubation the cells were washed with an excess of PBS.
Where no preincubation was performed, Hsp70 at different concentrations was added upon setup of the proliferation assay. Alternatively, T cell stimulation was performed with 5 μg/ml PHA (Remel) or 40 IU/ml IL-2. The proliferation assay was performed on 96-well round-bottomed plates (200 μl/well). When mo-iDCs were used for stimulation, the assay lasted for either 1 or 4 days, for activated or nonactivated T cells, respectively; when PHA or IL-2 was used for stimulation, the incubation for either activated or nonactivated T cells was 1 day only (in these assays the activated T cells used were 7 days after their last restimulation). Then 50 μl of the medium was collected for IFN-γ analysis before being pulsed with [3H]thymidine (TRA310; GE Healthcare) at a concentration of 0.037 MBq/ml. The cells were further incubated for 16 h, and [3H]thymidine incorporation of proliferating cells was determined using a β-counter (Matrix 9600; Packard Instrument). Each experiment was performed in triplicate. IFN-γ secretion was analyzed using a cytometric bead array (BD Biosciences) and analyzed using the BD-FCAP Array software (BD Biosciences).

**Statistical Analysis**—Statistical significance was assessed with the two-tailed, paired Student t test. At least three repeats of each experiment were performed, and a p value < 0.05 was considered significant. For the statistical analysis SPSS version 14.0 was used (SPSS).

**RESULTS**

**Endotoxin Contamination in Hsp70 Samples Is Responsible for Maturation of mo-iDCs**—The mo-iDC generation protocol used in this study produced a cell population that was shown to be >90% both positive for CD1a and negative for CD14 (supplemental Fig. 1A). mo-iDCs are known to respond to endotoxin contaminants inducing their maturation. Their maturation can be monitored by measuring the expression of certain molecules such as CD80, CD86, CD83, or HLA class II. Accordingly, mo-iDCs can be used as an indicative tool for the detection of endotoxins in protein samples. Three Hsp70 samples with different endotoxin content were incubated with mo-iDCs for 24 h and tested for their expression of CD80 (A), CD86 (B), HLA-DR (C), and CD83 (D) molecules, which were used as maturation markers. LPS was used as a positive control at a concentration of 1 μg/ml. Data are presented as fold increase in mean fluorescence compared with untreated control ± S.D. (error bars). n = 3 for 0, 5, 10, and 20 μg/ml concentrations of Hsp70 samples, and n = 1 for LPS and the remaining concentrations of Hsp70.

**FIGURE 1.** Adjuvant properties of Hsp70 depend on endotoxin contamination level. Hsp70 samples with different endotoxin contents of 0.32, <50, or >500 EU/mg were tested for their activatory potential. mo-iDCs were incubated with the different samples of Hsp70 for 24 h and then monitored for their expression of CD80 (A), CD86 (B), HLA-DR (C), and CD83 (D) molecules, which were used as maturation markers. LPS was used as a positive control at a concentration of 1 μg/ml. Data are presented as fold increase in mean fluorescence compared with untreated control ± S.D. (error bars). n = 3 for 0, 5, 10, and 20 μg/ml concentrations of Hsp70 samples, and n = 1 for LPS and the remaining concentrations of Hsp70.
Low Endotoxin Hsp70 Influences Morphology of mo-iDCs—Although the expression of tested molecules of mo-iDCs after low endotoxin Hsp70 treatment was only marginally abrogated, the morphology of the mo-iDCs incubated with the low endotoxin Hsp70 was visibly affected (Fig. 2). An incubation of Hsp70 with the cells resulted in a visibly lower number of clusters in the culture compared with the untreated control (Fig. 2C). The Hsp70-treated mo-iDCs were distributed evenly at the bottom of the U-shaped well whereas the spread of the control cells was distinctly different and created cell clusters. We observed that mo-DCs clustering was related to the maturation level; the more mature the cells become, the more clusters were observed in the culture. Newly generated mo-iDCs, before being used for the experiments, showed a small amount of clusters which were increasing in number with time, even when unstimulated (data not shown). Addition of Hsp70 to the culture seemed to reduce this spontaneous maturation process consequently affecting the cell morphology, by decreased cluster formation, as well as the expression profile of maturation markers (Figs. 1 and 2). However, mo-iDCs incubated with the commercially available Hsp70, containing medium or high endotoxin levels, had an opposite effect on the maturation marker expression (Fig. 1) and the morphology of the cells (data not shown). Low endotoxin Hsp70-treated mo-iDCs were observed to be more viable than the untreated cells as assessed using flow cytometry (Fig. 2, A and B). Forward and side scatter were used to determine the morphology of the cells, and the observed changes in size and granularity were characteristic of nonviable cells as assessed with Annexin-V staining (supplemental Fig. 1C). The survival advantage of the Hsp70-treated cells was ~20% (Fig. 2, A and B). In contrast, LPS treated mo-iDCs showed a decreased number of viable cells by >15% compared with the untreated control and by >25% compared with Hsp70-treated cells (Fig. 2, A and B).

Low Endotoxin Hsp70 Reduces Stimulatory Capacity of mo-iDCs—To assess the effect of low endotoxin Hsp70 on mo-iDC functional characteristics we investigated whether Hsp70-treated mo-iDCs would show altered stimulatory capacity of either activated or freshly isolated nonactivated T cells. Activated T cells were generated using an enriched population of CD3 cells, which were primed against an antigen. The flow cytometric analysis showed that the activated T cells increased in size and in cytoplasmic granularity compared with nonactivated T cells (supplemental Fig. 2). The population of either activated or nonactivated T cells contained CD8 and CD4 cells which were tested for their expression of the activation marker, CD25. Activated T cell populations contained 97% of CD8 cells positive for CD25, whereas nonactivated T cells contained only CD8 cells negative for CD25. When the population of CD4 cells was analyzed for the expression of CD25, it revealed that 69.5% of nonactivated T cells were negative for CD25 and 30.3% were positive. The activated T cell population of CD4 cells was 98.2% positive for CD25 (supplemental Fig. 2).

In further experiments either activated or nonactivated T cells were used and tested for their response against untreated or Hsp70 preincubated mo-iDCs. After preincubation with Hsp70, mo-iDCs were washed with PBS to remove the Hsp70 remaining in the medium; the control cells were treated the
same way as those preincubated with Hsp70. Preincubation of mo-iDCs with Hsp70 for 24 h altered their stimulatory capacity as observed by analyzing the activated T cell proliferative response and IFNγ secretion (Fig. 3, A and B). The effect was shown to be concentration-dependent; proliferation and IFNγ secretion decreased by ~20% at a concentration of 20 μg/ml Hsp70 (Fig. 3, A and B). However, nonactivated T cells (CD3+ or purified subpopulations, either CD8+ or CD4+, did not show altered proliferative responses to mo-iDCs preincubated with 20 μg/ml Hsp70 (Fig. 3C); however, a significant down-regulatory effect was observed for CD3+, CD8+, and CD4+ cells in the IFNγ secretion assay (Fig. 3D).

Low Endotoxin Hsp70 Abrogates T Cell Responses—Alternatively designed experiments, in which Hsp70 was added to the culture medium and remained present during the T cell response, were shown to have a cumulative down-regulatory effect. The altered activated T cell response was dependent on Hsp70 concentration and showed that Hsp70 at a concentration as low as 1 μg/ml effectively reduced proliferation and IFNγ secretion in the activated T cell population. Furthermore, activated T cell proliferation and IFNγ secretion decreased by >50% at a protein concentration of 20 μg/ml (Fig. 4, A and B). Nonactivated CD3+, CD8+, or CD4+ cells showed ~20% decrease of proliferation and ~50% decrease of IFNγ secretion when Hsp70 at a concentration of 20 μg/ml, was present in the culture medium (Fig. 4, C and D).

In the light of the obtained results we hypothesized that Hsp70 may not only have an effect on mo-iDC stimulatory capacity but also modulate T cell responses independently, in
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FIGURE 5. Low endotoxin Hsp70 reduces T cell response upon IL-2 or PHA stimulation. A and B, activated T cell (A) or nonactivated T cell proliferation (B), either with or without IL-2 at a concentration of 40 IU/ml; Hsp70 was added to the cells at concentrations of 1, 10, or 20 μg/ml. C and D, nonactivated CD3, CD8, or CD4 T cell proliferation (C) and IFNγ secretion (D) upon stimulation with 5 μg/ml PHA in the absence or presence of Hsp70 at a concentration of 20 μg/ml. Data are presented as mean percentage change relative to the untreated control ± S.D. (error bars). *, statistical significance of the results (n = 5, p < 0.02, t test).

In this study we used Hsp70 containing very low level of endotoxin that failed to activate maturation of mo-iDCs within the tested concentration range. We also observed very reproducible changes in morphology of mo-iDCs that occurred after incubation with Hsp70. However, it has to be emphasized that these effects were only observed when DC serum-free medium was used. We noticed that mo-iDCs were very sensitive to human and fetal calf serum, which induced their spontaneous maturation that was reduced when mo-iDCs were incubated in the serum-free medium (data not shown). Therefore, we speculate that the addition of Hsp70 to mo-iDCs might have decreased the spontaneous maturation process that was further translated to their reduced stimulatory capacity toward T cells.

We noticed that when Hsp70 was present in the culture medium during the T cell response assays it was able to reduce activated and nonactivated T cell response upon stimulation with allogenic mo-iDCs. The decrease in T cell response was higher than when Hsp70 was washed out after preincubation with mo-iDCs, thus suggesting that Hsp70 may have an influence on proliferating T cells independently from its effect on mo-iDCs. To test this hypothesis, T cell responses were assessed when stimulated by either IL-2 or PHA. Only activated T cells responded to the addition of IL-2, and that response was down-regulated by the presence of Hsp70. PHA stimulated activated and nonactivated T cells, and that activity was reduced by Hsp70. Therefore, Hsp70 was able to reduce both activated and nonactivated T cell responses independently from its modulatory effect on mo-iDCs. We also did not observe any difference in response to Hsp70 when either CD4 or CD8 subpopulations were used; however, this experiment was only performed with nonactivated T cells because the majority of the activated T cells co-expressed both T cell markers.

Controversially, an extended incubation of T cells with low endotoxin Hsp70 has recently been reported to increase T cell proliferation and cytokine responses against target cells (23). Because Figueiredo et al. and we used the same specimen of Hsp70, leading to contradictory conclusions, it is important to critically appraise used methodology. We speculate that in the Figueiredo et al. study the extended (7 days) incubation of T cells with Hsp70 would have improved the viability of cells, thus improving their proliferation and cytokine secretion but as a function of cell viability rather than stimulation. Accordingly,
Acknowledgment—We thank Prof. Britta Eiz-Vesper for providing toxin-free response of murine splenocytes was down-regulated by endotoxin-free *Mycobacterium tuberculosis* Hsp70 (15).

Although it has been shown repeatedly that Hsp70 expression generally correlated with the grade of inflammation, it remains unclear whether the up-regulation of Hsp70 during the immune response is potentiating, antagonizing, or remains a bystander in this process. However, it was observed that Hsp70 was up-regulated in the late stages, rather than during the onset of an immune reaction, and by the affected tissue and not immune cells (26–29). Consequently, one might speculate that the overexpression of Hsp70 is a protective mechanism that is activated by the targeted cells to counteract immune cytotoxicity. Furthermore, active secretion as well as an abundant surface expression of Hsp70 is common to tumor cells (30, 31). Surface expression of Hsp70 was shown to be specific to tumor cells and associated with poorer survival in cancer patients (32, 33). In the light of our results, it can therefore be speculated that Hsp70 provides a general mechanism that allows affected cells to counteract the immune response and was adapted by cancer cells as an immune evasive mechanism.

Our results exhibit a novel role for Hsp70 in the regulation of the immune response. Hsp70 decreased T cell proliferation and cytokine secretion regardless of stimuli used and affected the stimulatory capacity of mo-iDCs. These observations might explain the role of Hsp70 as an immune modulator in several clinical scenarios where overexpression of Hsp70 is observed.

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