Novel Signal Transduction Pathway Utilized by Extracellular HSP70

ROLE OF Toll-LIKE RECEPTOR (TLR) 2 AND TLR4*

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Recent studies have initiated a paradigm shift in the understanding of the function of heat shock proteins (HSP). It is now clear that HSP can and do exit mammalian cells, interact with cells of the immune system, and exert immunoregulatory effects. We recently demonstrated that exogenously added HSP70 possesses potent cytokine activity, with the ability to bind with high affinity to the plasma membrane, elicit a rapid intracellular Ca2+ flux, activate NF-κB, and up-regulate the expression of pro-inflammatory cytokines in human monocytes. Here for the first time, we report that HSP70-induced proinflammatory cytokine production is mediated via the MyD88/IRAK/NF-κB signal transduction pathway and that HSP70 utilizes both TLR2 (receptor for Gram-positive bacteria) and TLR4 (receptor for Gram-negative bacteria) to transduce its proinflammatory signal in a CD14-dependent fashion. These studies now pave the way for the development of highly effective pharmacological or molecular tools that will either up-regulate or suppress HSP70-induced functions in conditions where HSP70 effects are desirable (cancer) or disorders where HSP70 effects are undesirable (arthritis and arteriosclerosis).

Innate immunity is the first line of host defense against infection and malignant transformation and has a profound effect on the establishment of adaptive immunity (1, 2). Cells of the innate immune system are adorned with recognition structures called pattern recognition receptors (2, 3). Pattern recognition receptors such as Toll-like receptors (TLRs),1 CD14, β2-integrins (CD11/CD18), complement receptors (CR1/CD35), and C-type lectins are expressed either as soluble proteins or plasma membrane-bound proteins that recognize invariant molecular structures called pathogen-associated molecular patterns (e.g. LPS, peptidoglycan, unmethylated CpG DNA, bacterial lipoprotein, and mannans of yeast), that are shared by numerous pathogens but are not normally expressed on host tissues (2). Recent studies on the recognition of microbial pathogen-associated molecular patterns have highlighted the central role played by one group of pattern recognition receptors, the TLR, in pathogen recognition and host defense (see review in Refs. 4–6). TLRs are similar in sequence and structure to the Drosophila Toll protein and they share a conserved extracellular leucine-rich region important for ligand binding. Both Toll and TLRs are type 1 transmembrane proteins whose intracellular signaling domains have a Toll/IL-1R homology motif (7–9). Toll was originally identified as an essential component of dorsal-ventral development in flies, and has since been linked to an immune response against fungal infection in adult flies (10, 11). The mammalian homologues of Toll also control innate immune responses through conserved signaling pathways. An adapter protein, MyD88, binds to a receptor Toll/IL-1R-domain through its own Toll/IL-1R motif, while a death domain on its C terminus recruits IL-1R-associated kinase (IRAK) to the complex. IRAK is then autophosphorylated, and released from the complex to bind TRAF6, which can then activate either the NF-κB pathway or the mitogen-activated protein kinase cascade (8). The TLR family is a fast growing family whose ligands have not all been identified. The most well characterized TLRs are TLR2 and TLR4. TLR4 initiates signaling cascades in response to lipopolysaccharide (LPS), the abundant glycolipid of the outer membrane of Gram-negative bacteria, Taxol, or HSP60, while TLR2 initiates the signal cascade in response to Gram-positive bacteria, Mycoplasma, Yeast, and Spirochetes.

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Under normal physiological conditions HSP are expressed at low levels (12). However, a wide variety of stressful stimuli including environmental (UV radiation, heat shock, heavy metals, and amino acids), pathological (viral, bacterial, parasitic infections or fever, inflammation, malignancy, or autoimmunity), or physiological stimuli (growth factors, cell differentiation, hormonal stimulation, or tissue development), induce a marked increase in intracellular HSP synthesis (13), known as the stress response. The primary function ascribed to HSP is as intracellular molecular chaperones of naïve, aberrantly folded, or mutated proteins as well as in cytoprotection following the kinds of stressful stimuli mentioned above. Recent findings showing that both HSP60 (14) and HSP70 (15, 16) can use CD14 to induce the generation of proinflammatory cytokines suggest that HSPs...
might represent a novel class of putative endogenous ligands for TLRs.

In this report, we address the signal transduction cascade stimulated by exogenous HSP70 and reveal that HSP70-induced NF-κB promoter activity is MyD88-dependent, CD14-dependent, and is transduced via both TLR2 and TLR4. In addition, we show that TLR2 and TLR4 synergize to greatly augment HSP70-induced cytokine production. The physiological relevance of extracellular HSP70s ability to synergistically activate proinflammatory cytokine production helps elucidate recent findings indicating that HSP70 is a potent adjuvant for eliciting immune responses and a powerful inducer of anti-tumor immunity.

EXPERIMENTAL PROCEDURES

Isolation and Enrichment of Human DCS—Peripheral blood mononuclear cells were isolated from freshly drawn peripheral venous blood (Kraft Family Blood Center, Dana-Farber Cancer Institute) using the Ficoll-Paque separation technique as described in detail elsewhere (17). Monocytes were enriched from the peripheral blood mononuclear cell fraction by negative depletion using magnetic beads coated with appropriate monoclonal antibodies. Briefly, using a monocye isolation kit (Miltenyi Biotec, Auburn, CA), peripheral blood mononuclear cells were treated with FcR blocking agent followed by hapten-antibody mixture containing monoclonal hapten-conjugated CD3 (mouse Ig2A), CD7 (mouse Ig2A), CD19 (mouse IgG1), CD45RA (mouse IgG1), CD56 (mouse IgG2b), and anti-IgE (mouse IgG2A) antibodies and incubated for 5 min at 4 °C. Cells were washed twice in phosphate-buffered saline and incubated in FcR blocking buffer and MACs anti-hapten microbeads. After a 5-min incubation at 4 °C, cells were washed and passed through a column attached to a magnet. Unlabeled monocyes (CD45 CD14) eluted from the column were >98% pure, as judged by flow cytometry. Cell viability was measured by Trypan blue exclusion. CD45 CD14 monocyes were cultured for 8 days in RPMI (supplemented with 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 1% autologous serum). To generate DC, cells were treated with granulocyte-macrophage colony-stimulating factor (500 units/ml) and IL-4 (800 units/ml) on day 0. On day 4 of culture, granulocyte-macrophage colony-stimulating factor was added to the cells.

Cell Lines and Tissue Culture Conditions—HEK293 cells (ATCC, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium (ATCC) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine, and 2% sodium bicarbonate. Cells were cultured at 37 °C in 5% CO2, 95% air humidified incubator. At ~70–90% confluence, cells were harvested using trypsin-EDTA, washed, and counted using a hemocytometer. Cells were grown in this fashion for no more than 10 passages.

Construction of Human Dominant Negative (DN) TLR2 and TLR4—Mutations corresponding to the dominant negative TLR4 (P712H substitution) in C3H/HeJ-mice were introduced into hemaggultinin-tagged cDNAs of human TLR2 and TLR4 (kindly provided by Felix Randow) by PCR-directed mutagenesis, sequenced, and subcloned into the expression plasmid pRC/CMV (Invitrogen).

Transient Transfection of Cells and Measurement of Luciferase Activity—Transient transfection of HEK293 (293 wild type), 293-TLR2, 293-TLR4, or 293-TLR2+4 cells was performed using FuGENE 6 (Roche Molecular Biochemicals) transfection reagent following the manufacturer’s instructions. Briefly, cells were incubated with plasmids containing 1 μg of NF-κB reporter luciferase plasmid and, to correct for differences in transfection efficiency, each group of cells was transfected with 90 ng of pSV40LACZ and co-transfected with either 1 μg of CD14 or 1 μg of TLR2DN or 1 μg of TLR4DN or 3 μg of MyD88DN or 3 μg of empty vector and incubated overnight. The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. Following the various treatment protocols, cell extracts were prepared for determination of luciferase activity, each group of cells was transduced with 90 ng of pSV40LACZ and co-transfected with either 1 μg of CD14 or 1 μg of TLR2DN or 1 μg of TLR4DN or 3 μg of MyD88DN or 3 μg of empty vector and incubated overnight. The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. Following the various treatment protocols, cell extracts were prepared for determination of luciferase activity using enhanced luciferase assay reagents (Analytical Luminescence) according to the manufacturer’s instructions. Luciferase assays were performed on a Monolight 2010 luminometer (Analytical Luminescence). Results are expressed as the ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate and repeated at least three times.

Flow Cytometric Analysis—Following treatment, 2 × 106 cells were simultaneously fixed and permeabilized using 2 μl of PermeaFix (OrthoDiagnostics, Raritan, NJ) for 40 min at room temperature in the dark, as previously described. Cells were then washed three times in phosphate-buffered saline. Nonspecific binding was inhibited by treating cells with 5.5% normal goat serum in phosphate-buffered saline for an additional 1 h at room temperature with gentle rocking. For the measurement of co-stimulatory molecule expression, cells were treated with anti-human CD68-PE or anti-human MHCII (1 μg/ml; BD PharMingen, Mountain View, CA) for 40 min at room temperature in the dark. Cells were then washed twice in phosphate-buffered saline and analyzed by flow cytometry. Flow cytometric analysis was performed on a FACScan with Lysis II software program (BD PharMingen). Individual cells were gated on the basis of forward (FSC) and orthogonal scatter (SSC). The photomultiplier for fluorescein isothiocyanate (FL1-height) or PE (FL2-height) was set on a logarithmic scale. Cell debris was excluded by raising the FSC-height PMT threshold. The flow rate was adjusted to <200 cells/s and at least 10,000 cells were analyzed for each sample.

Assay of Proliferation—To determine the amount of proliferation, following the various treatment protocols the cells were pulsed with [3H]thymidine ([3H]TdR) specific activity 2 Ci/mmol, PerkinElmer Life Science, Beverly, MA) for 8 h. When incubated with rapidly proliferating cells, [3H]TdR incorporates into the DNA. The amount of cellular incorporation of [3H]TdR is then estimated by liquid scintiography.

RESULTS

Exogenous HSP70 Signals through TLR2 and TLR4—We (15, 18) and others (16, 19) have recently shown that extracellular recombinant HSP70 (StressGen Biotechnologies; Victoria, BC, Canada) activates proinflammatory cytokine production by antigen presenting cells. Among the proinflammatory cytokines induced by HSP70 interaction with human monocytes, we identified IL-1β, IL-6, and TNF-α (15, 18). IL-1β has been shown to be produced following interaction at the surface membrane by complex formation between extracellular IL-1 and the transmembrane IL-1R type I (IL-1R1) and IL-1R accessory proteins (IL-1RαC) (reviewed in Ref. 20). The resulting intracellular signaling cascade recruits two IRAK, IRAK1 and IRAK2, and an adapter protein MyD88 (21). Interestingly, IL-1 receptors (22, 23), IL-18 receptor (24–26), and Toll-like receptors (3, 27), signal through the MyD88/IRAK-NF-κB signal transduction pathway. To test whether HSP70-induced signaling cascade occurs via a similar pathway, THP1 monocyctes were treated with various concentrations of HSP70 and NF-κB promoter activity was measured using a β-luciferase reporter construct after 2 h incubation at 37 °C (Fig. 1). HSP70 dose dependently increased NF-κB promoter activity in THP1 monocyctes (Fig. 1). Control protein OVA used at
equimolar concentrations did not up-regulate NF-κB promoter activity significantly above background levels (Fig. 1). HSP70 at a concentration of 0.1 μg/ml resulted in submaximal activation of NF-κB promoter activity, and unless otherwise stated, was the concentration we used in subsequent experiments. Luciferase activity was observed at Hsp70 concentrations as low as 0.01 μg/ml (0.14 nm).

Mechanism by Which HSP70 Activates NF-κB Promoter Activity: Role of CD14 and MyD88—To determine the mechanism by which HSP70 activates the signal cascade that results in the activation of NF-κB promoter activity, we used HEK293 cells. HEK293 cells do not express IL-1R, CD14, or TLR. For these studies, we generated HEK293 cells that stably express IL-1R (293-IL-1R), TLR2 (293-TLR2), and TLR4 (293-TLR4). LPS (0.1 μg/ml) but not Hsp70 (0.1 μg/ml) activated NF-κB promoter activity in 293-TLR4 cells (Fig. 2). However, Hsp70-induced NF-κB promoter activity was strongly restored in 293-TLR4 cells transiently transfected with CD14 to a level comparable with LPS-induced NF-κB promoter activation (Fig. 2). This is in line with our previous findings in which we demonstrated that HSP70 stimulates proinflammatory cytokine production in a CD14-dependent fashion (15). Transient transfection of 293-TLR4 cells with CD14 and the dominant negative MyD88 (MyD88DN) completely abrogated HSP70-induced and LPS-induced NF-κB promoter activity (Fig. 2). To rule out bacterial contamination of HSP70, we preincubated 293-TLR4 cells with Polymyxin B, a potent LPS inhibitor (28, 29). Preincubation of 293-TLR4 cells with Polymyxin B (10 μg/ml) 30–60 min before activation abrogated LPS-induced but not HSP70-induced NF-κB promoter activity (Fig. 2). As an additional control, boiling HSP70 at 100 °C for 1 h completely abrogated HSP70-induced, but not LPS-induced NF-κB promoter activity (data not shown). Furthermore, HSP70 but not LPS induced a rapid intracellular Ca2+ flux in monocytes (15) (data not shown). Taken together, these results demonstrate that HSP70-induced NF-κB activity is dependant on CD14 to signal through TLR4, which initiates a signal cascade that is transduced via MyD88 (Fig. 2). As an additional control to demonstrate that HSP70-induced signaling specifically occurs through TLR4 and CD14, we used HEK293 cells stably transfected with IL-1R (293-IL-1R). We hypothesized that if the HSP70 signaling cascade is specific, HSP70 will not activate NF-κB promoter activity in 293-IL-1R. This is because, although both IL-1β and TLR transduce signals via the Toll/IL-1R domain in a MyD88/IRAK/TRAF6-NF-κB signaling pathway, the external surface receptors for IL-1R and TLR are different. As expected, IL-1β but not HSP70 activated NF-κB promoter activity in 293-IL-1R cells (Fig. 3). For additional specificity, IL-1β-induced NF-κB promoter activity was abrogated in 293-IL-1R cells transiently transfected with MyD88DN (Fig. 3). Taken together these results show that the external receptor for HSP70 is not the IL-1R and that extracellular HSP70 cannot directly interact with molecules downstream of TLR or IL-1R.

Signaling Intermediates in HSP70-induced NF-κB Activation: Role of TLRs—We also examined a possible role of TLR2 in extracellular HSP70-mediated signaling. To our surprise, stimulation of 293-TLR2 cells co-transfected with CD14 with HSP70 (0.1 μg/ml) induced potent NF-κB promoter activity to a level equivalent to 293-TLR4 cells co-transfected with CD14 (Fig. 4). To confirm that HSP70 was indeed signaling through TLR2 and CD14, we pretreated 293-TLR2 cells with anti-TLR2 before stimulation with HSP70. Pretreatment of 293-TLR2 cells with anti-TLR2 completely abrogated HSP70-induced NF-κB promoter activity in HEK293-TLR2, but not HEK293-TLR4 cells (Fig. 4). We then asked the question as to what kind of signaling would occur if both TLR2 and TLR4 were expressed on the surface of HEK293 cells. Therefore, HEK293 cells were generated that stably expressed both TLR2 and TLR4 (293-TLR2+4). Treatment of 293-TLR2+4 with HSP70 resulted in the synergistic activation of NF-κB promoter activity in a CD14-dependant fashion (Fig. 5). The HSP70-induced NF-κB promoter activity was only partially inhibited by co-transfection of 293-TLR2+4 with dominant negative TLR2 (TLR2DN) or dominant negative TLR4 (TLR4DN) (Fig. 5). Co-transfection of 293-TLR2+4 with both TLR2DN and TLR4DN completely abrogated HSP70-induced CD14-dependent NF-κB promoter activity (Fig. 5). On the other hand, co-transfection of 293-TLR2+4 with CD14 plus MyD88DN only partially inhibited HSP70-induced NF-κB promoter activity (Fig. 6). It has recently been suggested that although TLR4 might act as a homodimer, TLR2 cannot form functional homodimers and acts only in combination with selected TLR proteins, forming het-
were transfected with 1 μg of NF-κB reporter luciferase plasmid alone or co-transfected with 3 μg of CD14 or empty vector. Cells pretreated with anti-TLR2 for 30 min before stimulation with 0.1 g/ml HSP70 or 0.1 μg/ml OVA and incubated for 2 h after which extracts were prepared for determination of luciferase activity. Bars represent the ratio of luciferase (luc) to β-galactosidase (β-gal) and are the mean ± S.D. of three experiments performed in triplicate. *, p < 0.05 versus respective controls (Student’s t test).

FIG. 4. HSP70-induced signal cascade utilizes both TLR2 and TLR4 to activate NF-κB activity. HEK293 wild type cells (open bars), HEK293-TLR2 cells (left hatched bars), HEK293-TLR4 cells (right hatched bars), or HEK293-TLR2+4 cells (filled bars) were transfected with 1 μg of NF-κB reporter luciferase plasmid alone or co-transfected with 3 μg of CD14 or TLR2DN or TLR4DN or TLR2DN+TLR4DN or empty vector. Cells were stimulated with 0.1 μg/ml HSP70 or OVA and incubated for 2 h after which extracts were prepared for determination of luciferase activity. Bars represent the ratio of luciferase (luc) to β-galactosidase (β-gal) and are the mean ± S.D. of four experiments performed in triplicate. *, p < 0.05 versus respective controls (Student’s t test).

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FIG. 5. Exogenous HSP70 synergistically augments NF-κB promoter activity in HEK293 cells expressing both TLR2 and TLR4. HEK293 wild type (open bars), HEK293-TLR2 cells (left hatched bars), HEK293-TLR4 cells (right hatched bars), or HEK293-TLR2+4 cells (filled bars) were transfected with 1 μg of NF-κB reporter luciferase plasmid alone or co-transfected with 3 μg of CD14 or TLR2DN or TLR4DN or TLR2DN+TLR4DN or empty vector. Cells were stimulated with 0.1 μg/ml HSP70 or 0.1 μg/ml OVA and incubated for 2 h after which extracts were prepared for determination of luciferase activity. Bars represent the ratio of luciferase (luc) to β-galactosidase (β-gal) and are the mean ± S.D. of three experiments performed in triplicate. *, p < 0.05 versus respective controls (Student’s t test).

Biological Significance of HSP70-induced NF-κB Activity—Linking Innate and Adaptive Immunity—The functional consequence of the interaction between extracellular HSP70 and human monocytes has recently been investigated. We (15, 18) and others (16) have recently demonstrated that extracellular HSP70 induces potent cytokine production in human monocytes via interaction with CD14. These findings suggest that HSPs may not only serve as a vehicle for antigenic peptides but also as a “danger signal” to the innate and specific immune system as suggested for other endogenous activators of antigen presenting cells (31). Once activated, mature antigen presenting cells display their full co-stimulatory potential and are able to communicate very effectively with naive T-helper lymphocytes and prime cytotoxic T lymphocytes to mount potent and effective adaptive immune responses. Therefore, we investigated whether HSP70 was able to deliver a maturation signal to DCs. Human DCs were generated in vitro from peripheral blood mononuclear cells obtained from freshly drawn peripheral venous blood and incubated with granulocyte macrophage colony stimulating factor as previously described (32). Treatment of human DCs with HSP70 and LPS augmented pro-inflammatory cytokine, IL-1β, IL-12, and TNF-α, production, as determined by enzyme-linked immunosorbent assay (Table I). To exclude the possibility that this effect was due to contamination of the protein preparation by endotoxins such as LPS, boiling (100 °C, 1 h) HSP70 abrogated cytokine production, whereas LPS-induced cytokine production was not significantly abrogated by boiling (Table I). The ability of antigen presenting cells to induce an immune response depends both on the regulated expression of cytokines, which act directly on T lymphocytes and on the up-regulation of co-stimulatory molecules on DCs (32, 33). In line with these findings, intact HSP70 and LPS, but not boiled HSP70, up-regulated the expression of co-stimulatory molecules CD86 (B7.2) and myosin heavy chain class II on human DCs, as determined by flow cytometric analysis (Table I). Treatment of irradiated immature DCs with HSP70 resulted in augmentation in proliferation of T lymphocytes 7–8-fold greater than in T lymphocytes incubated with irradiated immature DCs treated with either boiled HSP70 or control protein OVA, as determined after 5 days by incorporation of [3H]Thymidine (data not shown). Proliferation of mature DCs was significantly lower as compared with immature DCs in response to stimulation with intact HSP70 and LPS or boiled LPS (Table I). This is in agreement with previous data showing that activated mature DCs grow slower than non-activated immature DCs (Table I). Treatment of DCs with boiled HSP70...
required for potent HSP70-induced cytokine production, we play an important role in linking innate and adaptive immune stimulatory molecule expression on DCs suggests that it might (Table I). The ability of HSP70 to induce cytokine and co-stimulatory molecules and are representative of three independently performed experiments with similar results.

To further demonstrate that CD14, TLR2, and TLR4 are required for potent HSP70-induced cytokine production, we grew HEK293 cells on culture slides. The cells were then transfected with CD14 alone or CD14 + TLR2 or CD14 + TLR2 + TLR4 followed by stimulation with equimolar amounts (0.1 μg/ml) of HSP70 or control protein OVA for 4 h at 37 °C, stained with IL-6-fluorescein isothiocyanate, mounted with a 4,6-diamidino-2-phenylindole-based mounting media, and analyzed using fluorescence microscope (Fig. 7A). In accordance with the luciferase data, HEK293 cells transfected with CD14 + TLR2 and stimulated with HSP70 but not control protein OVA, resulted in enhanced intracellular IL-6 production (Fig. 7A). As expected, HEK293 cells transfected with CD14 + TLR2 + TLR4, when stimulated with HSP70 but not control protein OVA, resulted in potent augmentation of intracellular IL-6 production, which was far in excess to that of HSP70 stimulated HEK293 cells transfected with CD14 + TLR2 (Fig. 7A) or CD14 + TLR4 (data not shown). Extracellular HSP70 was not effective at stimulating intracellular IL-6 production from HEK293 cells transiently transfected with either TLR vector alone or CD14 alone (Fig. 7A). The phase-contrast images show the total number of cells that were present at the time of the experiment (Fig. 7B).

DISCUSSION

This report for the first time demonstrates that HSP70-induced proinflammatory cytokine production is mediated via the MyD88/NF-κB signal transduction pathway and that HSP70 utilizes both TLR2 (receptor for Gram-positive bacteria) and TLR4 (receptor for Gram-negative bacteria) to transduce its proinflammatory signal in a CD14-dependent fashion. The biological significance of these findings now paves the way for the development of highly effective pharmacological or molecular tools that will either up-regulate or suppress HSP70-induced functions in conditions where HSP70 effects are desirable or disorders where HSP70 effects are undesirable.

A major concern of these studies was to ascertain that our HSP70-induced effects were not being masked by endotoxin contamination. We show that boiling (100 °C, 1 h) abrogated HSP70 but not LPS-induced effects (Table I). In addition, pre-treatment with LPS antagonist, Polyomixin B abrogated LPS but not HSP70 effects (Fig. 2). We also performed the Limulus lysate assay (BioWhittaker) on every HSP70 sample and all reagents and culture media used in the experiments to ensure that LPS contamination was always below 5 pg/ml. Taken together, these results demonstrate that the effect of HSP70 are thus genuine properties of HSP70 rather than due to endotoxin contamination.

The requirement for CD14 in HSP70-induced signaling via TLR is in line with our previous findings that CD14 is a co-receptor for HSP70-induced proinflammatory cytokine production in human monocytes (15). It has been known that host recognition of microbial products like LPS, peptidoglycan, and bacterial lipoprotein is principally mediated by either a membrane-bound or by a soluble form of the glycoprotein, CD14 (for review, see Refs. 34 and 35). Ligation of microbial products to CD14 triggers the production of proinflammatory cytokines. However, CD14 is a glycosylphosphatidylinositol-anchored membrane protein, devoid of an intracellular domain (36). The mechanism by which CD14 transmits signals was until recently unknown. Studies of mammalian TLRs have now provided intriguing evidence that TLRs are the CD14-associated signal transducers for different classes of microbial products. Here we show that CD14 is required for optimal HSP70-induced signaling via TLR2 and TLR4 (Fig. 4). This is unique since HSP60 (14) and LPS (34) have recently been shown to utilize only TLR4 and CD14 for signaling that results in the induction of proinflammatory cytokines (14).

Ozinsky and co-workers (37) have recently shown that both TLR2 and TLR6 are required for macrophage-mediated TNF-α production in response to yeast and Gram-positive bacterial products, but not Gram-negative bacteria. These findings suggest that TLR proteins might act as dimers and in some cases as heterodimers. Indeed, it was suggested that TLR4 might behave as a homodimer, whereas TLR2 cannot form functional homodimers and acts only in combination with selected TLR proteins, like TLR1 and TLR6 (for review, see Refs. 30). In this report, we show that HSP70 can interact with both TLR2 and TLR4 in a CD14-dependent fashion, and that this combination is particularly effective resulting in the synergistic activation

| Table I | Chaperokine effect of extracellular HSP70: induction of cytokine production, co-stimulatory molecule expression and proliferation of DCs |
| --- | --- | --- |
| Cell type | Treatment | Cytokine production (ng/ml) | Expression of co-stimulatory molecules (MFI) | Proliferation assay (CPM × 10⁻³) |
| | | IL-1β | IL-12 | TNF-α | MHCII | CD86 | [³H]Tdr uptake |
| Mature DC | Control | 0.1 | 0.5 | 1.2 | 11.7 | 5.8 | 3.8 |
| | HSP70 (intact) | 45.3 | 22.4 | 35.3 | 55.3 | 38.9 | 14.7 |
| | HSP70 (boiled) | 0.4 | 0.9 | 1.5 | 13.9 | 4.9 | 3.0 |
| | LPS (intact) | 67.6 | 25.7 | 50.7 | 96.2 | 43.8 | 21.2 |
| | LPS (boiled) | 58.9 | 29.1 | 45.8 | 118.7 | 37.7 | 18.9 |
| Immature DC | Control | 1.3 | 4.3 | 3.5 | ND | ND | 5.0 |
| | HSP70 (intact) | 55.6 | 31.2 | 45.8 | ND | ND | 40.6 |
| | HSP70 (boiled) | 2.2 | 6.6 | 4.1 | ND | ND | 8.3 |
| | LPS (intact) | 69.4 | 29.9 | 50.4 | ND | ND | 37.9 |
| | LPS (boiled) | 71.3 | 30.7 | 55.7 | ND | ND | 40.4 |

* Cells were treated with 0.1 μg/ml intact HSP70, HSP70 (intact), or 0.1 μg/ml intact LPS; LPS (intact) or 0.1 μg/ml boiled (100 °C, 1 h) HSP70; HSP70 (boiled) or 0.1 μg/ml boiled (100 °C, 1 h) LPS; LPS (boiled). Controls were cells treated with 0.1 μg/ml OVA (Control).

* Following an overnight incubation at 37 °C, supernatant was recovered and analyzed for respective cytokines by enzyme-linked immunosorbent assay according to the manufactures directions (BD PharMingen, Mountain View, CA). Data represent concentration of cytokines (ng/ml) and are a representative sample of two independently performed experiments with similar results.

* After a 48-h incubation at 37 °C cells were stained for respective co-stimulatory molecules on the surface of the cells and analyzed for expression of such molecules on the surface of the cells by flow cytometry. Data represent mean fluorescence intensity (MFI) of cells expressing respective co-stimulatory molecules and are representative of three independently performed experiments with similar results.

* After a 5-day incubation cells were pulsed with [³H]Tdr for 8 h before harvesting cells. Data represent CPM × 10⁻³ (proliferation) and are representative of two independently performed experiments with similar results.
HSP70 Signals through TLR2 and TLR4

of TLR stimuli that is known to exist. Extensive insight into the complex nature in which DCs discriminate different pathogens (Escherichia coli, Candida albicans, and influenza virus) at a genetic level has recently been initiated using a novel DNA microarray method that reveals the genome-wide location of DNA-bound proteins (38, 39). In another report, it was recently shown that LPS activates cytokine production via a MyD88-dependent pathway and activates co-stimulatory molecule expression via a MyD88-independent pathway (40). Experiments showing that HSP70-induced NF-κB promoter activity is only partially inhibited by MyD88DN in HEK293 cells co-expressing both TLR2 and TLR4 (Fig. 6), suggest that under certain conditions HSP70 might signal by both a MyD88-dependent and MyD88-independent signal transduction pathway. To conclusively answer this question experiments are now underway to test HSP70-induced cytokine and co-stimulatory molecule expression in primary cultures of human DC after transient transfection with MyD88DN, TLR2DN, and/or TLR4DN. At the submission of this report, a novel adaptor protein was described known as MyD88-adaptor-like and shown to be an adaptor for TLR4 signaling (41). In addition, MyD88-adaptor-like was demonstrated to abrogate TLR4, but not IL-1 or IL-18-induced signaling (41). If confirmed, studies into the role of MyD88-adaptor-like in HSP70-induced signaling could provide useful information on how extracellular HSP70 mediates its proinflammatory and anti-tumor effects.

The biological significance and diversity of responses attributed to HSP70 is remarkable. The term chaperokine has recently been coined to better describe the ability of HSP70 to act as a chaperone and cytokine (15, 18). In addition to functioning as an antigen carrier, HSP70 coupled to peptides has recently been shown to induce cytokine production (42). Furthermore, a recombinant p24-HSP70 fusion protein elicits both humoral and cellular immune responses against p24 in mice (43), and tumor-derived HSP70 is taken up by immature DCs and causes production of TH1 cytokines such as IFN-γ, TNF-α, and IL-12 (44). In this report, we demonstrate that the effects observed in vitro are applicable to the biological system. Thus, experiments with HEK293 cells confirmed that TLR2 and TLR4 work synergistically to potently augment intracellular IL-6 production in response to HSP70 stimulation (Fig. 7A). This demonstrates the specificity of the HSP70-induced signaling pathway and its dependence on TLR and the MyD88/NF-κB signal transduction pathway.

This report demonstrates for the first time that HSP70-induced proinflammatory cytokine production is mediated via the MyD88/NF-κB signal transduction pathway and that HSP70 utilizes both TLR2 (receptor for Gram-positive bacteria) and TLR4 (receptor for Gram-negative bacteria) to transduce its proinflammatory signal in a CD14-dependent fashion. These findings now pave the way for the development of highly effective pharmacological or molecular tools that will either up-regulate or suppress HSP70-induced functions in conditions where HSP70 effects are desirable or disorders where HSP70 effects are undesirable.

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