

HSP70 as Endogenous Stimulus of the Toll/Interleukin-1 Receptor Signal Pathway*

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Human heat-shock protein (HSP)70 activates innate immune cells and hence requires no additional adjuvants to render bound peptides immunogenic. Here we tested the assumption that endogenous HSP70 activates the Toll/IL-1 receptor signal pathway similar to HSP60 and pathogen-derived molecular patterns. We show that HSP70 induces interleukin-12 (IL-12) and endothelial cell-leukocyte adhesion molecule-1 (ELAM-1) promoters in macrophages and that this is controlled by MyD88 and TRAF6. Furthermore, HSP70 causes MyD88 relocalization and MyD88-deficient dendritic cells do not respond to HSP70 with proinflammatory cytokine production. Using the system of genetic complementation with Toll-like receptors (TLR) we found that TLR2 and TLR4 confer responsiveness to HSP70 in 293T fibroblasts. The expanding list of endogenous ligands able to activate the ancient Toll/IL-1 receptor signal pathway is in line with the “danger hypothesis” proposing that the innate immune system senses danger signals even if they originate from self.

Stimuli that activate innate immune cells such as dendritic cells (DCs)¹ are at present subject of intensive studies, because of the central role of these cells in initiating and controlling adaptive immune responses. There are at least two lines of thought in regard to the nature of such stimuli. The first implies that “exogenous” pathogen-associated (hence, “foreign”) molecular patterns (PAMPs) are selectively recognized by germ line encoded Toll-like receptors (TLRs), which subsequently drive innate immune cell activation (1). In favor of this view, PAMPs such as bacterial lipopeptides, lipopolysaccharides (LPS) from Gram-negative bacteria, flagellin, and bacterial CpG-DNA are recognized by TLR2, TLR4, TLR5, and TLR9, respectively (2). Ligand-driven TLR activation causes

consecutive recruitment of the adaptor molecule MyD88, the IL-1 receptor-associated kinases and the adaptor molecule TRAF6 (3). This ancient Toll/IL-1 receptor (TIR) signal pathway ultimately leads to activation of transcription factors that switch on production of proinflammatory mediators such as IL-12 and tumor necrosis factor (TNF) α , costimulatory molecules from B7 family, and adhesion molecules, for example, endothelial cell-leukocyte adhesion molecule (ELAM-1; E-selectin). Given the importance of IL-12 in cell-mediated immunity (4), the indispensability of costimulatory molecules for full activation of lymphocytes (5), and the role of adhesion molecules in recruiting leukocytes (6) and forming the proper immunological synapses between interacting cells (7), it becomes clear why TLRs have the central position in both innate and adaptive anti-infectious responses.

Alternatively, it has been suggested that DCs act as sentinels of “endogenous” ligands released from cells undergoing unprogrammed necrotic death as opposed to “silent” programmed cell death, *i.e.* apoptosis (8, 9). According to this view not the “foreign-ness” is sensed but the danger, thus the name “danger hypothesis” (10). Identification of endogenous danger signals will help to better understand how the immune system functions. First candidates in line are family members of intracellular HSPs, which, upon necrotic cell death might be released and thus could be able to activate DCs (11, 12). For example HSP60 (13), HSP70 (14, 15), as well as gp96 (16) deliver activation signals to innate immune cells. Because induction of specific CD8 T cell responses by HSP-peptide complexes appears not to require adjuvants to confer immunogenicity to bound peptides (17) the ability of HSPs to activate DCs may not only explain the immunogenicity of HSP-peptide complexes but also it represents a strong argument in favor of the danger hypothesis.

Members of the HSP70 cytosolic group are either constitutively expressed (HSC70) or can be induced by a broad range of stress factors (HSP70). The inducible HSP70 has been recently characterized as a potent maturation stimulus for DCs (18). Driven by the quest to identify/compare the pathways through which exogenous and endogenous ligands activate innate immune cells, and encouraged by recent data on HSP60 signaling (19), we searched for further cases of dual specificity (endogenous and exogenous) of the Toll/IL-1 receptor signal pathway by examining HSP70-triggered intracellular events. Our data define HSP70 as an endogenous stimulus for the Toll/IL-1 receptor signal pathway that engages TLR2 and TLR4.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—A FLAG epitope-tagged C terminus of murine MyD88 (dnMyD88) was described previously (19). The expression vectors for the C terminus of human TRAF6 (dnTRAF6), human flag-

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¹ The abbreviations used are: DCs, dendritic cells; PAMPs, pathogen-associated molecular patterns; TLRs, Toll-like receptors; LPS, lipopolysaccharide; TIR, Toll/IL-1 receptor; TNF, tumor necrosis factor; ELAM-1, endothelial cell-leukocyte adhesion molecule; IL, interleukin; HSP, heat-shock protein; BMDc, bone marrow-derived dendritic cells; EGFP, enhanced green fluorescence protein; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; dn, dominant negative.

tagged TLR2, TLR4, and E-selectin (ELAM-1) promoter luciferase construct were gifts from Tularik, Inc. (South San Francisco, CA); murine TLR9 in pcDNA3 was a gift from S. Bauer (Technical University of Munich, Munich, Germany); the human MD-2 expression vector was kindly provided by K. Miyake (Saga Medical School, Nabeshima, Japan). The luciferase reporter driven by a synthetic enhancer harboring 6 NF- κ B binding consensus sites was a gift from P. Baeuerle (Munich, Germany). The IL-12p40 promoter (−703 to +53) luciferase construct was a gift from K. M. Murphy (Washington University, St. Louis, MO).

Recombinant human HSP70 was purchased from StressGen Biotechnologies (Victoria, Canada). To control contamination with LPS, HSP70 preparations were boiled for 30–60 min where indicated. Phosphothioate-stabilized CpG oligonucleotide 1668 (TCC ATG ACG TTC CTG ATG CT) was purchased from TIB Molbiol (Berlin, Germany). LPS from *Escherichia coli* 055:B5 and PMA were from Sigma-Aldrich (Munich, Germany).

Cell Culture, Transfection, and Luciferase Assays—The murine macrophage cell line RAW264.7 was grown in VLE-RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin G, and 100 IU/ml streptomycin sulfate (all from Biochrom KG). $5\text{--}10 \times 10^6$ RAW264.7 cells were transfected by electroporation in a 400- μ l final volume (RPMI/25% FCS) at 300 V and 960 microfarads in a Gene Pulser (Bio-Rad Laboratories, Munich, Germany). 5 μ g of reporter plasmids was used for transfection together with different amounts of specific expression vectors as indicated in the figure legends. The overall amount of plasmid DNA was held constant at 20 μ g per electroporation by addition of the appropriate empty expression vector. After electroporation, cells were washed and split into 12-well plates for subsequent stimulation and lysis.

The human embryonic kidney fibroblasts 293T were cultured in Dulbecco's modified Eagle's medium (Biochrom KG) with the same supplements as for RAW264.7 macrophage cell cultures. For luciferase reporter assays the 293T cells were transfected by electroporation similar as RAW264.7 except that 200 V were used. 10 ng of 6 $\times$ NF- κ B luciferase reporter together with 0.1 μ g of respective expression vector or empty control vector (as indicated in figure legend) per transfection were used. The overall DNA amount was held constant at 20 μ g by addition of empty vector.

For luciferase assays, transfected cells were stimulated as described in the Fig. 5 legend, lysed, and luciferase activity in extracts was measured with the Luciferase Assay System kit from Promega (Mannheim, Germany) according to manufacturer's instruction.

Mice, Generation of BMDC, and Determination of Cytokines—MyD88-deficient mice were a kind gift from S. Akira (Osaka University, Osaka, Japan) (20). Gene-targeted mice deficient for TLR2 (TLR2^{−/−}) were granted by Tularik Inc. (21). C3H/HeJ mice (TLR4^{d/d}) and C3H/HeN (TLR4^{+/+}) were purchased from Harlan (Germany) for 5-fold backcrossing of TLR2^{−/−} mice toward the C3H genetic background. Mice were genotyped for the point mutation of the TLR4 gene responsible for the amino acid exchange P712H of the intracellular domain of TLR4 and characteristic for the C3H/HeJ strain (22). F₁ generation mice of parent mice 5-fold backcrossed toward the C3H background carrying both the TLR2 and the TLR4 mutations heterozygously were used for further crossing. Offspring mice carrying one of each, the mutated or the wild type allele homozygously (TLR2^{+/+}/TLR4^{+/+}, TLR2^{−/−}/TLR4^{+/+}, TLR2^{+/+}/TLR4^{d/d}, TLR2^{−/−}/TLR4^{d/d}) were selected pairwise for further breeding. Age-matched groups of wild-type and mutant mice were used for the experiments.

Bone marrow-derived dendritic cells (BMDC) were prepared as described previously (23). For stimulation, nonadherent BMDC at days 6–7 were washed, plated at $3.8\text{--}7.5 \times 10^5$ /ml, and after a 2-h rest cells were stimulated in triplicates for 20 h. IL-12p40 and TNF α levels in culture supernatants were determined by commercially available ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the instructions of the manufacturer.

Confocal Laser Scanning Microscopy— 7×10^5 RAW264.7 macrophages stably expressing MyD88-EGFP² were plated 1 day before image collection on round glass slides in 12-well dishes in 10% FCS, VLE-RPMI medium containing 500 units/ml interferon γ . 3 h prior to stimulation, medium was exchanged with fresh 0% FCS-containing medium. Cells were stimulated as indicated in the legend of Fig. 3, washed, and formalin-fixed. Samples were viewed with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) using a Argon 488-nm

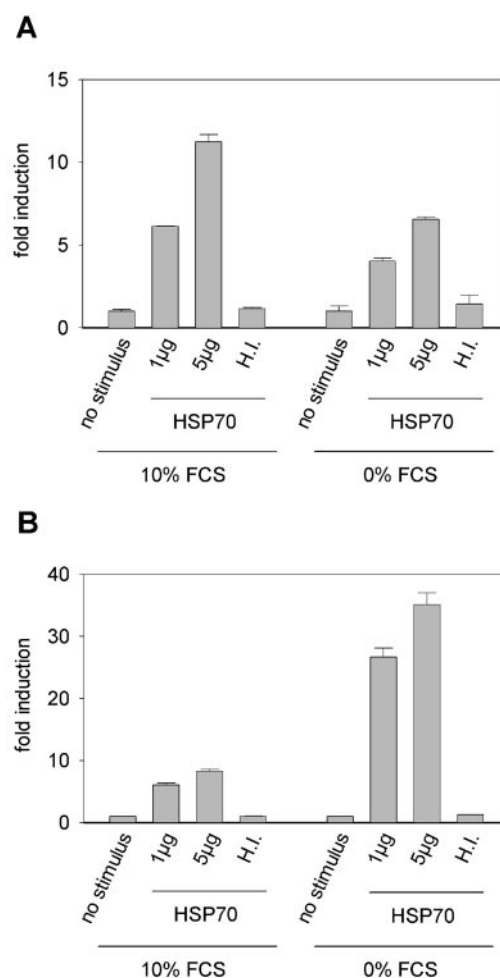


FIG. 1. Human HSP70 activates IL-12 and ELAM-1 promoters in macrophages. 16 h before stimulation RAW264.7 macrophages were transfected with IL-12p40 promoter (A)- or ELAM-1 promoter (B)-driven luciferase construct as described under "Experimental Procedures." 1 h before stimulation, medium was reduced to 0.5 ml or alternatively exchanged with 0.5 ml of 0% FCS medium. Cells were stimulated with the indicated amount of human HSP70 and with 5 μ g/ml heat-inactivated HSP70 (H.I.) for 10 h. Afterward, cells were lysed, luciferase activity was determined, and -fold induction was calculated referring to unstimulated samples. Each bar represents the mean of two stimulations with error bars showing standard deviations. One representative experiment out of three is shown.

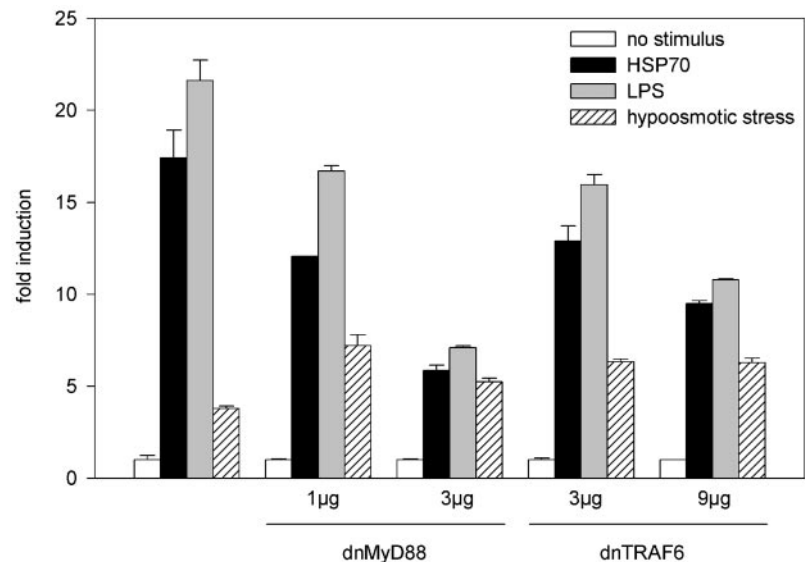
laser, a Plan-Neofluar 40 1.3 oil lens, and LSM 510 version 2.02 software. Slices 1- μ m thick were collected at a resolution of 1024×1024 pixels.

RESULTS

HSP70 Activates Macrophages via TIR Signal Pathway—Recruitment of the adaptor molecule MyD88 to the cytoplasmatic TIR domain of ligand-activated TLRs is considered as a crucial early step during initiation of the Toll/IL-1 receptor signal pathway (3). To probe whether a dominant negative (dn) version of MyD88 influences HSP70-induced signaling, we first established experimental conditions for HSP70-driven macrophage activation. The macrophage-like cell line RAW264.7 was chosen for luciferase reporter analysis. As shown in Fig. 1 human HSP70 dose-dependently caused induction of IL-12p40 (Fig. 1A) and ELAM-1 (Fig. 1B) promoters. No increase above basal expression was noted in parallel cultures after addition of heat-denatured HSP70, thus excluding potential LPS contamination. It was recently demonstrated that α_2 -macroglobulin receptor (CD91) functions as an uptake receptor for HSP70 (24). Because high amounts of α_2 -macroglobulin are present in

² P. Ahmad-Nejad, manuscript in preparation.

FIG. 2. Human HSP70 activates macrophages via the Toll/IL-1 receptor signal pathway. 16 h before stimulation, RAW264.7 macrophages were transfected with ELAM-1 promoter-driven luciferase construct and the indicated amounts of dominant negative MyD88 and TRAF6 constructs as described under "Experimental Procedures." 1 h before stimulation, medium was exchanged with 0.5 ml of 0% FCS medium. Cells were stimulated with 5 μ g/ml HSP70, 100 ng/ml LPS, or 500 μ l of water for 10 h. Afterward, cells were lysed, luciferase activity was determined, and -fold induction was calculated referring to unstimulated samples. Each bar represents mean of two stimulations with error bars showing standard deviations. One representative experiment out of three is shown.



FCS one could presume an inhibitory effect of serum on HSP70 interaction with macrophages. To probe for that possibility serum-free stimulation with HSP70 was performed. As shown in Fig. 1 this impaired IL-12p40 reporter activity but greatly enhanced ELAM-1 promoter induction by HSP70.

Having defined conditions for HSP70-mediated macrophage activation we next analyzed whether transient overexpression of dominant negative variants of MyD88 and TRAF6 in RAW264.7 cells would affect HSP70-driven activation. As shown in Fig. 2 both dnMyD88 and dnTRAF6 dose-dependently suppressed ELAM-1 promoter activation triggered by HSP70. As a well-established MyD88- and TRAF6-dependent signal inducer, LPS was included for reference. Inhibition of cell activation by dominant negative molecules from the Toll/IL-1 receptor signal pathway was significant but incomplete for both HSP70 and LPS, which could be explained by MyD88-independent signaling pathways (25, 26). Expression of dnMyD88 and dnTRAF6 in RAW264.7 was dose-dependent, as controlled by Western blotting (data not shown). To rule out unspecific or toxic effects of overexpressed dominant negative constructs, stimulation of cells by hypo-osmotic stress was used as a specificity control (Fig. 2).

Visualization of MyD88 Recruitment by HSP70—Because a dominant negative version of MyD88 suppressed HSP70-induced signaling, we next attempted to visualize HSP70-driven recruitment of MyD88 to subcellular sites where signaling is initiated. RAW264.7 macrophage clones stably expressing MyD88 fused to enhanced green fluorescent protein (EGFP) were selected and analyzed by confocal laser scanning microscopy upon activation with HSP70 (5 μ g/ml, 2-h stimulation). As shown in Fig. 3A MyD88-EGFP was homogeneously distributed within the cytoplasm in nonstimulated cells. Upon stimulation with HSP70, MyD88-EGFP became recruited to irregularly distributed endosome-like vesicular structures and partially to the cell membrane (Fig. 3B). Heat-denatured HSP70 failed to cause any changes of MyD88 distribution within the cells (Fig. 3C). These data confirmed the involvement of the Toll/IL-1 receptor signal pathway and suggested that HSP70 may initiate signaling upon endocytosis as has been described for HSP60 (19).

Verification of MyD88 Involvement in Cell Activation by HSP70—Next we used cells from MyD88-deficient mice to verify the central role of MyD88 in cell activation by HSP70. HSP70 has been shown to stimulate murine antigen-presenting cells to produce IL-12p40 and TNF α (14). We therefore

analyzed the ability of HSP70 to trigger production of aforementioned cytokines in murine DCs derived from bone marrow (BMDC) of MyD88-deficient mice and of respective control mice. HSP70 dose-dependently caused BMDC from control mice to initiate IL-12p40 (Fig. 4A) and TNF α (Fig. 4B) production, yet responsiveness of MyD88-deficient BMDC was ablated. Heat-inactivated HSP70 showed no stimulatory activity for DCs. To control that wild-type and knock-out DCs were responsive, the MyD88-independent TNF α inducer PMA was used (Fig. 4B).

Stimulation under serum-free conditions was performed synchronously, to test for potential enhancement of the stimulatory capacity of HSP70. Serum deprivation affected cytokine production at higher HSP70 concentrations (Fig. 4). This result might be explained by a negative impact on cytokine production of the stress imposed by serum deprivation. Indeed, stimulation with PMA under FCS-free conditions resulted in reduced TNF α production, supporting this explanation.

Overall, these results confirmed that signaling for HSP70-driven cytokine production occurs via the Toll/IL-1 receptor signal pathway.

HSP70 Signals via TLR2 and TLR4—The essential role of MyD88 and TRAF6 in HSP70-triggered cell activation suggested an involvement of Toll-like receptors. To analyze this possibility we first utilized genetic complementation with TLRs of the HSP70-unresponsive human embryonic kidney fibroblast cell line 293T. Cells were transiently transfected with a luciferase reporter driven by a synthetic enhancer harboring 6 NF- κ B binding consensus sites. Upon cotransfection with TLR2 the cells gained responsiveness to HSP70 (and LPS (27)) but not to heat-inactivated HSP70 (Fig. 5A). Interestingly, cotransfection of TLR4 alone was ineffective, yet cotransfection of TLR4 plus MD-2, a coreceptor of TLR4 in case of LPS signaling (28), resulted unequivocally in responsiveness (Fig. 5A). The grade of HSP70-induced NF- κ B activity was comparable to that caused by LPS, implying that stimulatory potential of endogenous ligand HSP70 is as high as that of the potent microbial stimulus LPS. Gain of function was TLR-specific, because TLR9 conferred responsiveness to bacterial CpG-DNA but not to HSP70 or LPS (Fig. 5A).

We also analyzed a potential role of serum components in this reductionist genetic complementation system. Although the absence of FCS-enhanced TLR2 mediated "gain of function" dose-dependently, TLR4 plus MD-2-mediated HSP70 responsiveness was not influenced by serum and the dose of the

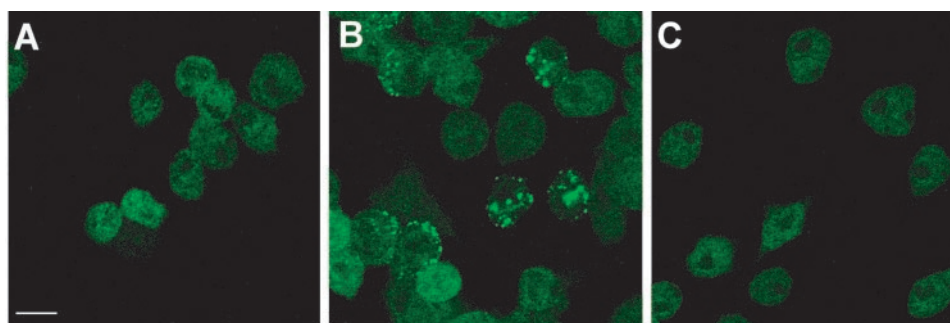


FIG. 3. **Human HSP70 recruits MyD88-EGFP.** MyD88-EGFP-expressing RAW264.7 macrophages were prepared for stimulation as detailed under "Experimental Procedures." In non-stimulated macrophages MyD88-EGFP was homogenously distributed in the cytoplasm (A). 5 µg/ml HSP70 (B) or the same amount of heat-inactivated HSP70 (C) was added for 2 h. Afterward, cells were treated and examined with confocal laser scanning microscopy as described under "Experimental Procedures." One representative experiment out of three is shown. (Bar = 10 µm.)

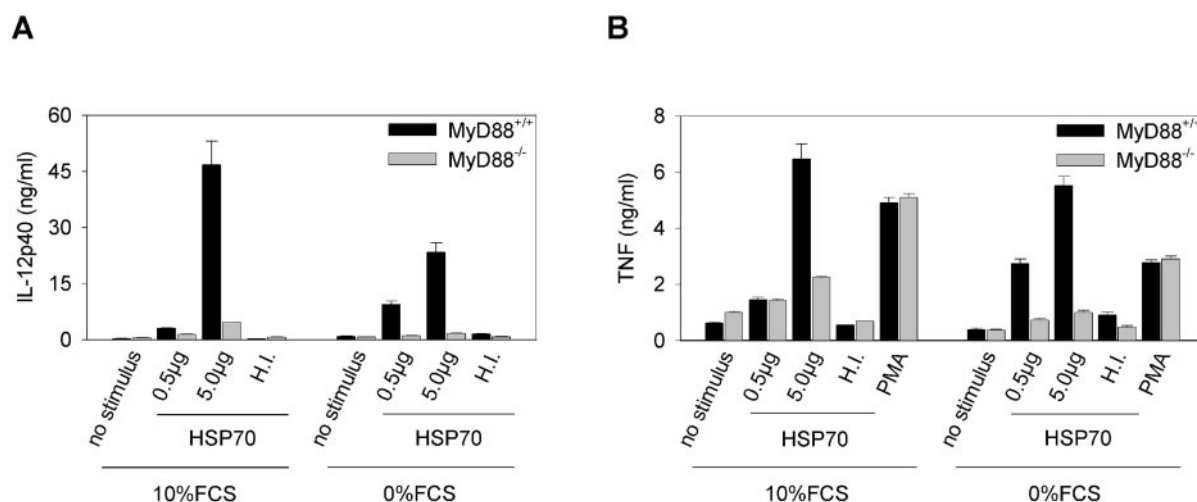


FIG. 4. **Response to HSP70 is impaired in MyD88-deficient dendritic cells.** Nonadherent BMDC from MyD88^{+/+} and MyD88^{-/-} mice at day 6 were plated at 7.5×10^5 /ml in medium containing 10% FCS or 0% FCS. After a 2-h rest, cells were stimulated with the indicated amounts of human HSP70, with 5 µg/ml heat-inactivated HSP70 (H.I.) or with 20 ng/ml PMA for 20 h. Afterward, culture supernatants were collected and IL-12p40 (A) or TNFα (B) levels were determined by ELISA. Each bar represents the mean of three stimulations with error bars showing standard deviations. One representative experiment out of three is shown.

stimulus (Fig. 5B). It is possible that TLR4/MD-2 is more sensitive to HSP70 engagement and that the concentration range we used for stimulation represents a functional plateau where positive effects of serum deprivation may disappear. On the other hand, one or more FCS components might affect primarily TLR2-mediated signaling, for example, by competing for receptor-mediated endocytosis of HSP70 (24).

Because genetic complementation with TLR2 or TLR4 plus MD-2 resulted in gain of function to HSP70, we also analyzed responsiveness to HSP70 of BMDC from TLR2-deficient (TLR2^{-/-}) and TLR4-mutant mice (TLR4^{d/d}) (Fig. 6, A and B). To control for equal stimulatory conditions, IL-12p40 production in response to CpG-DNA was measured, known to be dependent on TLR9 but not on TLR2 or TLR4 (Fig. 6C). TLR2^{-/-}/TLR4^{d/d} and TLR2^{+/+}/TLR4^{d/d} BMDC failed to produce either IL-12p40 or TNFα in response to HSP70, yet HSP70 responsiveness of TLR2^{-/-}/TLR4^{+/+} BMDC was not affected. These results raise the question, whether TLR2 requires cooperation with yet undefined cofactor that is absent in BMDC but present in 293T cells. This is, for example, the case for TLR4, which requires MD-2 for sensing LPS.

DISCUSSION

Heat-shock protein-peptide complexes do not require adjuvants to elicit peptide-specific CD8 T cell responses (17). This raises the question whether certain HSPs not only chaperonize peptides mirroring the antigenic repertoire of (tumor) cells they

originate from but also act as endogenous adjuvants able to deliver maturation signals to antigen-presenting DCs. The co-segregation of immunogenicity of cancers with higher levels of expression of HSP70 without change in the antigenic repertoire of the cancer cells is a case in point (29). We hypothesized that during evolution recognition of "dangerous" microbial molecular patterns by TLRs might have been modeled on a more ancient mechanism of responsiveness to HSPs signaling danger when released from cells undergoing unprogrammed necrotic death (8, 9). This thesis would imply dual specificities within TLR family members able to sense both exogenous PAMPs as well as endogenous HSPs. For HSP60 this prediction was recently substantiated, because HSP60 activates innate immune cells via TLR2 and TLR4 (19). Here we extended our studies to stress-inducible HSP70 known to deliver robust activation signals to macrophages and DCs (14, 15, 18). We found that inhibition of MyD88 and TRAF6 impairs the ability of HSP70 to activate RAW264.7 macrophages. Furthermore, murine MyD88-deficient BMDC failed to produce cytokines in response to HSP70. Overall, these data imply that HSP70 activates innate immune cells via the Toll/IL-1 receptor signal pathway.

The α₂-macroglobulin receptor (CD91) has been reported to function as an uptake receptor of HSP70 (24). α₂-Macroglobulin and/or other CD91 ligands present in serum may thus compete with HSP70 for CD91 and thus inhibit HSP70-APC interaction.

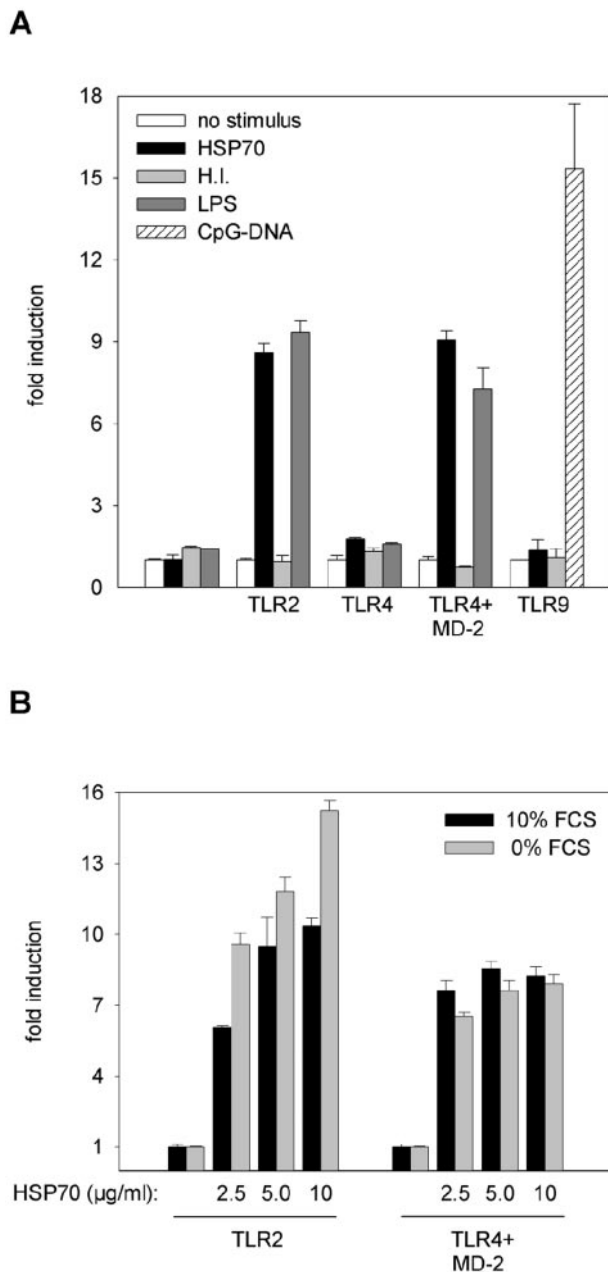


FIG. 5. TLR2 and TLR4 plus MD-2 confer responsiveness to human HSP70. A, 293T fibroblasts were transfected with NF- κ B reporter together with indicated expression constructs as detailed under "Experimental Procedures," withdrawn of FCS and stimulated with 5 μ g/ml human HSP70, 5 μ g/ml heat-inactivated HSP70 (H.I.), 100 ng/ml LPS and 1 μ M CpG oligonucleotide 1668 (CpG-DNA) overnight (14 h). Afterward, cells were lysed, luciferase activity in lysates was measured, and -fold induction was calculated referring to unstimulated samples. Each bar represents the mean of two stimulations with error bars showing standard deviations. One representative experiment out of three is shown. B, 293T fibroblasts were transfected with NF- κ B reporter together with TLR2 or TLR4 plus MD-2 expression constructs as detailed under "Experimental Procedures." Before stimulation, medium was reduced to 0.5 ml or alternatively exchanged with 0.5 ml of 0% FCS medium. Cells were stimulated with the indicated amounts of human HSP70 overnight (14 h) and lysed, luciferase activity in lysates was measured, and -fold induction was calculated referring to unstimulated samples. Each bar represents the mean of two stimulations with error bars showing standard deviations. One representative experiment out of three is shown.

Furthermore, we reported that the ability of HSP60 to activate DCs depends on endocytosis of HSP60 both being blocked by serum (19). Here we describe recruitment of MyD88-EGFP to

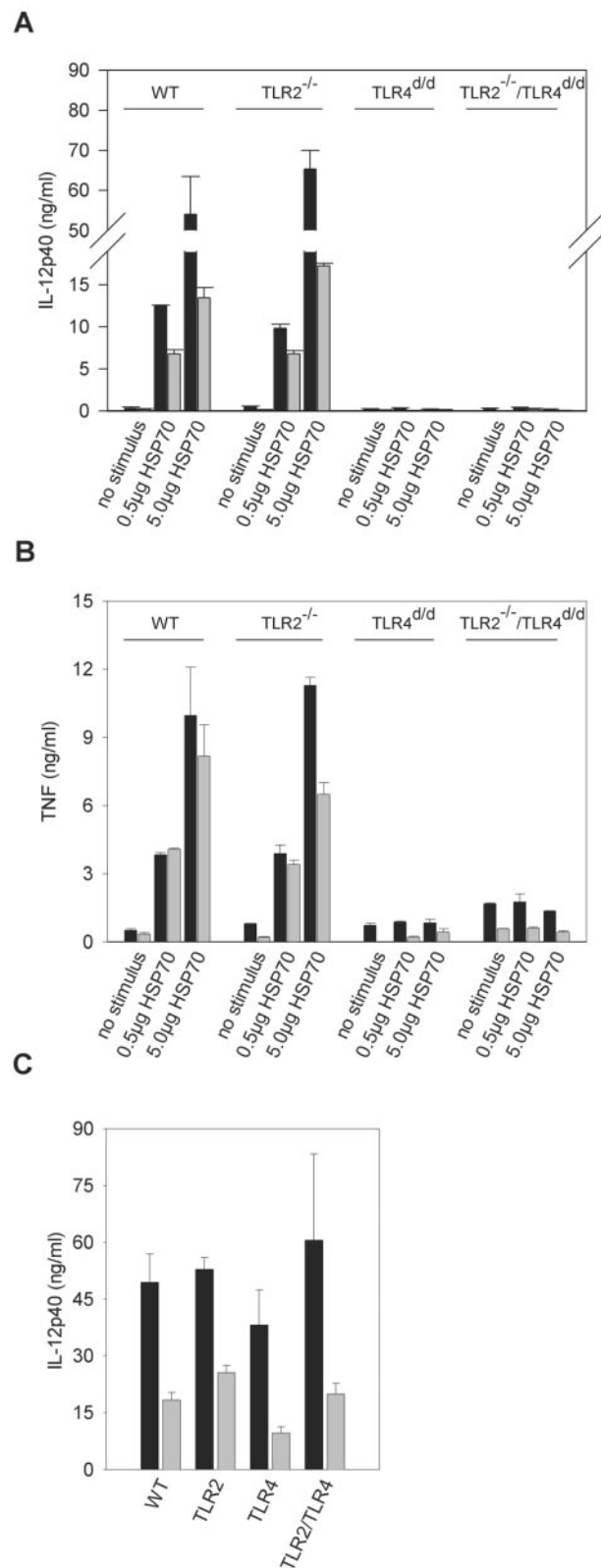


FIG. 6. TLR4 controls response of dendritic cells to human HSP70. Nonadherent BMDC from TLR2-deficient (TLR2^{-/-}), TLR4-mutant (TLR4^{d/d}), TLR2-deficient plus TLR4-mutant (TLR2^{-/-}/TLR4^{d/d}) and control (WT) mice at day 6 were plated at 7.5×10^5 /ml in 10% FCS (black bars) or 0%FCS (gray bars) containing medium. After a 2-h rest, cells were stimulated with the indicated amounts of human HSP70 (A, B) or with 1 μ M CpG-DNA oligonucleotide 1668 (C) for 20 h. Afterward, culture supernatants were collected and IL-12p40 (A, C) or TNF α (B) levels were determined by ELISA. Each bar represents the mean of three stimulations with error bars showing standard deviations. One representative experiment out of three is shown.

endosome-like vesicular structures after stimulation of macrophages with HSP70 implying that endocytosis of HSP70 may precede signaling (Fig. 3A). This conclusion bears a caveat, because TLR2-transfected fibroblasts showed a clear and dose-dependent inhibitory effect of serum on the activity of HSP70 whereas TLR4 plus MD-2-transfected cells did not (Fig. 5B). It could be that HSP70 activates TLR2 only upon endocytosis, whereas TLR4 is activated independently of internalization, as has been shown for CpG-DNA and LPS, respectively.² This question needs to be addressed by analyzing whether inhibition of endocytosis differentially affects HSP70-driven activation of TLR2-transfected *versus* TLR4 plus MD-2-transfected cells.

Experimentally, TLR2 conferred responsiveness of 293T cells to HSP70, yet TLR2-deficient BMDC responded to HSP70. Because TLR4-defective BMDC were completely unresponsive toward HSP70, we conclude that TLR2 does not autonomously function as a HSP70 receptor. Whether a coreceptor is missing or a threshold concentration for HSP70-TLR2 signaling is much higher in BMDC needs to be analyzed.

As reported for HSP60 (19, 30) we show here that HSP70 also acts as an endogenous TLR ligand. It follows that during evolution TLR family members have been selected as receptors for different HSPs released from necrotic cells. Because sequences and structures are homologous only between members of a given HSP subfamily, the question arises why different HSPs engage selectively TLR2 and TLR4. This intriguing question may only be solved by analyzing TLR-HSP interactions at the molecular level. Because of its robust inflammatory response the innate immune system can now be characterized as being highly reactive to HSPs released from dying cells. Furthermore, the ancient Toll/IL-1 receptor signal pathway originally thought to alert toward invading pathogens turns out to additionally be a sensor of endogenous ligands. In essence, our data support the danger hypothesis (10) because the immune system does not appear to care whether danger signals originate from the self or the non-self.

In addition we anticipate practical implications. Different expression of heat-shock proteins has been described in malignant *versus* normal human tissue (12). Furthermore, gene transfer of HSP70 into B16 and CMT93 melanoma cells enhanced tumor immunogenicity (29). Our finding that HSP70 activates the Toll/IL-1 receptor signal pathway classifies HSP70 as an endogenous natural adjuvant, similar to LPS. It follows that the immunogenicity of tumors, at least in part, may be linked to their ability to release the endogenous adjuvant HSP70. The notion that typing of individual tumors for their endogenous HSP70 content represents a basis for therapeutic protocols aimed at increasing HSP70 release and thus

tumor immunogenicity needs to be analyzed.

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HSP70 as Endogenous Stimulus of the Toll/Interleukin-1 Receptor Signal Pathway

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