Review

Heat Shock Protein and Innate Immunity

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In addition to serving as molecular chaperones, heat shock proteins (HSPs) have been implicated in autoimmune diseases, antigen presentation and tumor immunity. Extensive work in the last 10 years has also suggested that HSPs such as Hsp60, Hsp70, Hsp90 and gp96, may be potent activators of the innate immune system capable of inducing the production of pro-inflammatory cytokines by the monocyte-macrophage system, and the activation and maturation of dendritic cells via the Toll-like receptor 2 and 4 signal transduction pathways. However, recent evidence suggests that the reported cytokine effects of HSPs may be a result of the contaminating bacterial cell-wall products. This concise review summarizes the current controversy over the role of HSPs in innate immunity. *Cellular & Molecular Immunology*. 2004;1(4):274-279.

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Introduction

Since the initial description of the puffing of salivary gland chromosomes in *Drosophila busckii* induced by temperature shock in 1962 by Ritossa (1) and the subsequent identification of the gene products by Tissieres et al. in 1974 (2), heat shock proteins (HSPs) have been shown to be the most phylogenetically conserved proteins present in all prokaryotes and eukaryotes. HSPs primarily function as molecular chaperones (3, 4). However, HSPs have attracted considerable interest among the immunologists in recent years, because HSPs have been implicated in the pathogenesis of a number of autoimmune diseases (5), and in antigen presentation, cross-presentation and tumor immunity (6, 7). More recently, it has also been suggested that HSPs are potent activators of the innate immune system (8, 9). The purpose of this concise review is to critically evaluate the current controversy regarding the role of HSPs in innate immunity.

Heat shock proteins

Heat shock proteins are expressed both constitutively and under stressful conditions. In addition to the heat shock, a variety of stressful situations including environmental (ultraviolet radiation or heavy metals), pathological (infections or malignancies) and physiological (growth factors or cell differentiation) stimuli, induce a marked increase in HSP synthesis, a phenomenon known as the stress response (10, 11). Traditionally, HSPs are regarded as intracellular molecules. However, upon necrotic, but not apoptotic, cell death, HSPs are released into the extracellular compartments (12). In addition, HSPs can be released extracellularly in response to a number of stressful conditions (13-15). The mechanism and the physiological significance of the HSP release independent of necrotic cell death are not clear. However, HSPs are present in circulation of normal individuals (16, 17), and their circulating levels are decreased in aging (18), and increased in a number of pathological conditions such as hypertension (19), atherosclerosis (17, 20) and after open-heart surgery (21).

The primary function of HSPs appears to serve as molecular chaperones in which they recognize and bind to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding, or as chaperonins that directly mediate protein folding (3, 4, 10, 11). The classification of HSPs is based on their related functions and sizes (molecular masses). Using the nomenclature adopted after the Cold Spring Harbor Meeting of 1996 (22), family names are written in capitals, e.g., HSP70, while members of a family are conventionally written as Hsps, e.g., Hsp70. Major classes of HSPs include the small HSPs, HSP40, 60, 70, 90 and 110 families (3, 4, 11). As HSP60, 70 and 90 families are the major HSPs implicated in autoimmune diseases, antigen presentation and innate immunity, we will briefly

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Abbreviations: ER, endoplasmic reticulum; grp, glucose-regulated protein; LPS, lipopolysaccharide; MHC, major histocompability complex; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; TIR, Toll/IL-receptor; TLR, Toll-like receptor; HSP, heat shock protein; IL, interleukin; TNF, tumor necrosis factor.

summarize these HSPs here. For a more detailed description of HSPs, we refer the reader to excellent reviews of the subject (3-5, 10, 11, 16, 17, 23).

The mammalian HSP60 (chaperonin) family consists of mitochondrial Hsp60 (mt-Hsp60) and cytosolic Hsp60 (T-complex polypeptide-1) (3, 4, 11). The mt-Hsp60 exists in a dynamic equilibrium among monomers, heptamers, and tetradecamers (3, 24). It dissociates into monomers at low concentrations and assembles into tetradecamers in the presence of ATP and mt-Hsp10, the co-factor of mt-Hsp60 (25). The cytosolic Hsp60 forms hetero-oligomeric ring structures and functions in cytosol to fold cytoskeletal proteins such as actin and tubulin (26). The HSP70 family includes the constitutive cytosolic Hsc70 (or Hsp73), the stress-induced cytosolic Hsp70 (or Hsp72), the endoplasmic reticulum (ER) Bip (or Grp78) and the mitochondrial mt-Hsp70 (3, 4, 11). The Hsp70 is composed of two major functional domains. The NH2-terminal domain containing the highly conserved ATPase, binds ADP and ATP tightly and hydrolyzes ATP, whereas the COOH-terminal domain is required for polypeptide binding (3, 4). The HSP90 family includes the cytosolic Hsp90 (α and β) and the ER form, gp96 (grp94). Glucose-regulated proteins (grp) such as grp78, grp94/gp96, are molecular chaperones in the ER that are upregulated in response to glucose starvation and other stressful stimuli that disrupt protein folding in the ER (22, 23).

HSPs and autoimmunity

Bacterial HSPs, particularly Hsp60 and Hsp70, are highly immunogenic capable of inducing antibody production and T cell activation (27). The antibodies and T cells against bacterial Hsp60 and Hsp70 also recognize mammalian Hsp60 and Hsp70 respectively, as a result of cross reactivity (28). These anti-Hsp60 and anti-Hsp70 antibodies and T cells injure tissues and cause inflammatory reactions. Thus, Hsp60 and Hsp70 have been implicated in the pathogenesis of a number of autoimmune diseases and inflammatory conditions such as Type-1 diabetes (29, 30), Crohn's disease (31), atherosclerosis (17, 32), and juvenile chronic arthritis (33, 34).

HSPs and antigen presentation

Since the original observation by Srivastava et al. (35) in 1986 that immunization of mice with tumor-derived gp96 induced anti-tumor immune responses, HSPs have been shown to play an important role in antigen direct presentation and cross-presentation leading to $CD8^+$ T cell activation (6, 7). HSPs of the cytosol such as Hsp70 and Hsp90, and of the ER such as gp96, bind antigenic peptides generated within the cells, and are part of the endogenous pathway of antigen presentation via the major histocompatibility complex (MHC) class I molecules (6, 36, 37). Peptides that are chaperoned by HSPs when released extracellularly, or HSP-peptide complexes constituted *in vitro*, are taken up by antigen-presenting cells, e.g., dendritic cells, *via* the α2-macroglobulin receptor (CD91)-mediated endocytosis, resulting in representation by the MHC molecules (6, 38, 39). Vaccination of mice with Hsp70, Hsp90 and gp96 isolated from murine tumor cells elicits immune response sufficient for tumor rejection and suppression of metastatic tumor progression (35). This tumor immunity was shown to result from tumor-derived peptides associated with the HSPs, rather than from the HSPs themselves (40). Likewise, immunization of virally transformed or viral protein-infected cells elicits viral peptide-restricted cytotoxic T lymphocytes (41). In a pilot clinical trial, 6 out of 12 patients immunized with gp96-peptide complexes prepared from their own tumors demonstrated tumor-specific $CD8^+$ T cell responses (42). The advantage of using tumor-derived HSPs as the immunogen is that identification of tumor-specific antigens is not necessary and is, thus, of great clinical potential.

HSPs and cytokine function

Since 1993, HSPs such as Hsp60, Hsp70, Hsp90 and gp96 from a variety of sources including purified preparations from bacterial (43-45) and mammalian (12, 46-49) sources as well as recombinant bacterial (50-59) and human (21, 56, 57, 60-66) products, have been shown to be potent activators of the innate immune system (8, 9). These HSP preparations are shown to induce the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-12, and the release of nitric oxide (NO) and C-C chemokines by monocytes, macrophages and dendritic cells. They also induce the maturation of dendritic cells as demonstrated by the up-regulation of MHC class I & II molecules, and co-stimulatory molecules such as CD80 and CD86 (12, 47, 48, 59, 67, 68). The Hsp60 and Hsp70 preparations purified from bacterial sources or from recombinant bacterial and human products are capable of inducing the above effects in concentrations ranging from less than 1 μg/ml to a few μg/ml, while Hsp70, Hsp90 and gp96 isolated from the mouse liver require concentrations that are 1-2 orders of magnitude higher (e.g., 10-100 μg/ml). The reason for this discrepancy is not clear. These HSP cytokine effects, as compared to their molecular chaperone and antigen presentation functions, are unique in that they require no HSP-associated peptides, no ATP hydrolysis, no co-factors and no protein complex assembly (9). Asea et al. (46, 63) designated a new term, "chaperokine", for HSPs to indicate their dual functions as molecular chaperones and cytokines.

More recently, the above observed HSP cytokine effects are shown to be mediated via the Toll-like receptor (both TLR2 & 4) signal transduction pathways leading to the activation of nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPKs), i.e. ERKs (p42 & p44 extracellular regulated kinases), JNK (c-JUN N terminal kinase) and p38 kinase (21, 49, 57, 58, 62, 64, 65). Toll-like receptors are pattern recognition receptors involved in the innate immunity for the pathogen recognition and host defense (69, 70). They are type I transmembrane proteins with an extracellular domain containing a leucine-rich repeat and a cytoplasmic domain analogous to that of the IL-1 receptor (IL-1R) family. An adapter protein, MyD88 (myeloid differentiation protein

88), binds to the Toll/IL-1R homology (TIR) motif through its own TIR motif, while a death domain on its C-terminus recruits IL-1R-associated kinase (IRAK) to the complex (71). IRAK is then autophosphorylated, and released from the complex to bind TRAF6 (TNF receptor associated factor 6), which in turn activates NFκB and MAPKs (72, 73). Together with CD14 and an accessory protein MD2, TLR4 initiates signaling cascades in response to LPS (73, 74), while TLR2 initiates the signal cascades in response to bacterial lipoproteins and peptidoglycans (73, 75).

The reported activation of the innate immune system by HSPs as described above, has been hailed as an important new function of HSPs with broad biological significance. First, the induction of pro-inflammatory cytokines by Hsp60 and Hsp70 may contribute to the pathogenesis of autoimmune diseases and chronic inflammation (5, 8, 9). Chlamydial Hsp60 frequently co-localizes with human Hsp60 in macrophages of atherosclerotic plaques (55). Induction of pro-inflammatory cytokine release from macrophages by chlamydial Hsp60 could provide a potential mechanism by which chlamydial infections may promote atherogenesis and precipitate acute ischemic events (55, 56). Second, the activation and maturation of dendritic cells by Hsp70, Hsp90 and gp96 not only prime the innate immune system to provide a favorable environment for the induction of adaptive immune response, but also activate effector natural killer (NK) cells (7, 76). Baker-LePain et al. (76) argued that gp96-induced activation of the innate immune system alone might be sufficient for the observed tumor immunity. Third, through their cytokine function, HSPs may serve as a "danger signal" to the immune system at the site of tissue injury (8, 60), and that HSPs could be the endogenous ligands for TLR2 and TLR4 (62, 64). In fact, HSPs are considered to be the prototype of endogenous ligands for Toll-like receptors (77). Thus, there is considerable interest to further explore the implications and therapeutic potential of these HSP cytokine effects (16, 78, 79).

Cytokine function: HSPs *vs* contaminants

The reported HSP cytokine effects are similar to those of LPS and bacterial lipoprotein (8, 9). Since the recombinant bacterial and human HSPs are produced by *Escherichia coli* genetically engineered to express HSP cDNAs, the final preparations may be contaminated with bacterial products. Likewise, purified HSP preparations isolated from bacteria or from murine tissues are also frequently contaminated with bacterial cell-wall products such as LPS and lipoprotein (80). The fact that Hsp60, 70 and gp96 have similar cytokine effects, and share TLR2 and TLR4 as the signal transducers, is of particular concern (9, 77). While TLR2 and TLR4 are pattern recognition receptors, they are capable of discriminating LPS from lipoprotein (74, 75, 81). It is unlikely that they failed to distinguish HSPs from LPS or lipoprotein. Investigators are cognizant of the possibility of LPS contamination and have attempted diligently to rule out the possibility of LPS contamination being responsible for the observed HSP cytokine effects. However, none have tried to rule out the possibility of lipoprotein contamination, even though the observed HSP cytokine effects were shown to be mediated *via* TLR 2 as well as TLR4. As LPS and bacterial lipoproteins show similar cytokine effects, co-contamination of LPS and lipoprotein will render the criteria for ruling out LPS contamination such as polymyxin B inhibition, invalid (9, 77).

In fact, recent studies (8, 82-87) using HSP preparations essentially free of LPS suggest that the previously reported cytokine function of HSPs may be due to contaminants. Specifically, Wallin et al. (8) noted that highly purified murine liver Hsp70 had no cytokine effects even at concentrations as high as 200-300 μg/ml. However, a LPS contaminated preparation at Hsp70 concentrations as low as 50-100 ng/ml caused cytokine effects. Wallin et al. (8) was unable to explain the observed cytokine effects of the LPS contaminated Hsp70 preparation, because they were heat-sensitive and were not inhibitable by polymyxin B. Bausinger et al. (82) reported that LPS-free recombinant human Hsp70 (rhHsp70) did not induce the activation of dendritic cells. Gao and Tsan (83, 84) demonstrated that contrary to the popular belief, LPS was heat-sensitive, and that the ability of commercially available rhHsp70 to induce TNF-α production was entirely a result of the contaminating LPS (83), while that of rhHsp60 was due to contamination by LPS as well as LPS-associated molecules (84). Reed et al. (85) reported that the activation of NFκB and the production of nitric oxide by gp96 were due to LPS contamination. Importantly, these investigators demonstrated that these highly purified, essentially LPS-free HSPs retained their normal biological functions such as molecular chaperone function and/or ATPase activity (82-85). Thus, failure of Hsp60, Hsp70 and gp96 to induce cytokine or nitric oxide production by macrophages or to activate antigen-presenting cells, was not due to defective HSPs as a result of purification (9). The reasons for previous failure to recognize the contaminant(s) being responsible for the reported HSP cytokine effects include: failure to use highly purified, low LPS preparations of HSPs, failure to recognize the heat sensitivity of LPS, and failure to consider contaminant(s) other than LPS (9).

On the other hand, Liu et al. (88) recently demonstrated that transgenic expression of cell-surface gp96 lead to the *in vivo* activation of dendritic cells and the development of lupus-like systemic autoimmune disease in mice. Likewise, Baker-Lepain et al. (89) reported the *in vivo* activation of $CD11b^+$ and $CD11c^+$ antigen presenting cells after vaccination with gp96-secreting fibroblasts in mice. These studies have avoided the potential of contamination with bacterial cell-wall products and establish that gp96 is capable of activating the innate immune system *in vivo*. However, it is not clear whether the observed *in vivo* activation of the innate immune system was a result of a direct effect of gp96 on dendritic cells or was indirectly mediated by other cellular mediators. If it is a result of the direct effect of gp96 on dendritic cells, the simultaneous presence of other co-stimulatory molecules may be necessary, since highly purified gp96 free of LPS contamination has been shown to be unable to activate dendritic cells *in vitro* (85).

Conclusion

In addition to serving as molecular chaperones, HSPs have been implicated in autoimmune diseases, antigen presentation and tumor immunity. Considerable work has also suggested that HSPs such as Hsp60, Hsp70, Hsp90 and gp96, may be potent activators of the innate immune system capable of inducing the production of pro-inflammatory cytokines by the monocyte-macrophage system, and the activation and maturation of dendritic cells *via* the TLR2- and 4-signal transduction pathways. However, recent evidence suggests that the reported cytokine effects of HSPs may be a result of the contaminating bacterial cell-wall products. Thus, it is essential that effort should be directed to conclusively determine whether the reported HSP cytokine effects are due to HSPs or a result of contaminant(s) present in the HSP preparations, before exploring further the implication and therapeutic potential of the putative cytokine function of HSPs.

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References

1. Ritossa FA. A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia. 1962;18:571-573.
2. Tissieres A, Mitchell HK, Tracy U. Protein synthesis in salvary glands of Drosophila melanpgaster: relation to chromosome puffs. J Mol Biol. 1974;84:389-398.
3. Fink AL. Chaperone-mediated protein folding. Physiol Rev. 1999;79:425-449.
4. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science. 2002;295:1852-1858.
5. Pockley AG. Heat shock proteins as regulators of the immune response. Lancet. 2003;362:469-476.
6. Li Z, Menoret A, Srivastava P. Roles of heat-shock proteins in antigen presentation and cross-presentation. Curr Opin Immunol. 2002;14:45-51.
7. Srivastava PK. Role of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol. 2002;2:185-194.
8. Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG. Heat-shock proteins as activators of the innate immune system. Trends Immunol. 2002;23:130-135.
9. Tsan MF, Gao B. Cytokine function of heat shock proteins. Am J Physiol. 2004;286:C739-C744.
10. Jaattela M. Heat shock proteins as cellular lifeguards. Ann Med. 1999;31:261-271.
11. Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631-677.
12. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. Int Immunol. 2000; 12:1539-1546.
13. Hightower LE, Guidon PT Jr. Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. J Cell Physiol. 1989;138:257-266.
14. Liao DF, Jin ZG, Baas AS, et al. Daum G, Gygi SP, Aebersold R, Berk BC. Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells. J Biol Chem. 2000;275:189-196.
15. Barreto A, Gonzalez JM, Kabingu E, Asea A, Fiorentino S. Stress-induced release of HSC70 from human tumors. Cell Immunol. 2003;222:97-104.
16. Pockley AG. Heat shock proteins in health and disease: Therapeutic targets or therapeutic agents? Expert Rev Mol Med. 2001;3:1-21.
17. Xu Q. Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol. 2002;22:1547-1559.
18. Rea IM, McNerlan S, Pockley AG. Serum heat shock protein and anti-heat shock protein antibody levels in aging. Exp Gerontol. 2001;36:341-352.
19. Pockley AG, Wu R, Lemne C, Kiessling R, de Faire U, Frostegard J. Circulating heat shock protein 60 is associated with early cardiovascular disease. Hypertension. 2000;36: 303-307.
20. Xu Q, Schett G, perschinka H, et al. Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. Circulation. 2000;102:14-20.
21. Dybdahl B, Wahba A, Lien E, et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through Toll-like receptor-4. Circulation. 2002;105: 685-690.
22. Hightower LE, Hendershot LM. Molecular chaperones and the heat shock response at Cold Spring Harbor. Cell Stress Chaperon. 1997;2:1-11.
23. Snoeckx LHEH, Cornelussen RN, Van Nieuwenhoven FA, Reneman RS, Van Der Vusse GJ. Heat shock proteins and cardiovascular pathophysiology. Physiol Rev. 2001;81:1461-1497.
24. Levy-Rimler G, Bell RE, Ben-Tal N, Azem A. Type I chaperonins: not all are created equal. FEBS Lett. 2002;529: 1-5.
25. Levy-Rimler G, Viitanen P, Weiss C, et al. The effect of nucleotides and mitochondrial chaperonin 10 on the structure and chaperone activity of mitochondrial chaperonin 60. Eur J Biochem. 2001;268:3465-3472.
26. Llorca O, Martín-Benito J, Ritco-Vonsovici M, et al. Eukaryotic chaperonin CCT stabilizes actin and tubulin folding intermediates in open quasi-native conformations. EMBO J. 2000;19:5971-5979.
27. Zugol U, Kaufmann SH. Immune response against heat-shock proteins in infectious diseases. Immunobiol. 1999;201:22-35.
28. Kissling R, Growbug A, Tvanyi J, et al. Role of hsp60 during autoimmune and bacterial inflammation. Immunol Rev. 1991;121:91-111.
29. Abulafia-Lapid R, Elias D, Raz L, Keren-Zur Y, Atlan H, Cohen IR. T cell proliferative responses of type 1 diabetes patients and healthy individuals to human hsp60 and its peptides. J Autoimmun. 1999;12:121-129.
30. Elias D, Reshef T, Birk OS, van der Zee R, Walker MD, Cohen IR. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. Proc Natl Acad Sci USA. 1991;88:3088-3091.
31. Szewezuk MR, Depew WT. Evidence for T lymphocyte reactivity to the 65 kilodalton heat-shock protein of mycobacteria in active Crohn's disease. Clin Invest Med. 1992;15:494-505.
32. Pockley AG. Heat shock proteins, inflammation, and cardiovascular disease. Circulation. 2002;105:1012-1017.
33. Pope RM, Lovis RM, Gupta RS. Activation of synovial fluid T lymphocytes by 60-kd heat shock proteins in patients with inflammatory synovitis. Arthritis Rheum. 1992;35:43-48.
34. Res PC, Schear CG, Breedveld FC, et al. Synovial fluid T cell reactivity against 65 kD heat shock protein of mycobacteria in early chronic arthritis. Lancet. 1988;2:478-480.
35. Srivastava PK, DeLeo AB, Old LJ. Tumor rejection antigens of chemically induced tumors of inbred mice. Proc Natl Acad Sci USA. 1986;83:3407-3411.
36. Ishii T, Udono H, Ohta H, et al. Isolation of MHC class

1-restricted antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. J Immunol. 1991;162:1303-1309.
37. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity. 1998;8:657-665.
38. Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity. 2001;14:303-313.
39. Srivastava PK, Udono H, Blachere NE, Li Z. Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics. 1994;39:93-98.
40. Udono H, Srivastava PK. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90 and hsp70. J Immunol. 1994;152:5398-5403.
41. Navaratnam M, Deshpande MS, Hariharan MJ, Zatechka DS Jr, Srikumaran S. Heat shock protein-peptide complexes elicit cytotoxic T-lymphocyte and antibody responses specific for bovine herpesvirus 1. Vaccine. 2001;29:1425-1434.
42. Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. Int J Cancer. 2000;88:232-238.
43. Galdiero M, DeL'ero GC, Marcatili A. Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. Infect Immun. 1997;65:699-707.
44. Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW. Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. Infect Immun. 1994;62:5689-5693.
45. Skeen MJ, Miller MA, Shinnick TM, Ziegler HK. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J Immunol. 1996;156:1196-1206.
46. Asea A, Kabingu E, Stevenson MA, Calderwood SK. HSP70 peptide-bearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperon. 2000;5:425-431.
47. Somersan S, Larsson M, Honteneau JF, Basu S, Srivastava P, Bhardwaj N. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. J Immunol. 2001;167:4844-4852.
48. Panjwani NN, Popova L, Srivastava PK. Heat shock protein gp96 and hsp70 activate the release of nitric oxide by APCs. J Immunol. 2002;168:2997-3003.
49. Vabulas RM, Braedel S, Hilf N, et al. The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. J Biol Chem. 2002;277:20847-20853.
50. Friedland JS, Shattock R, Remake DG, Griffin E. Mycobacterial 65-kd heat shock protein induces release of proinflammatory cytokines from human monocytic cells. Clin Exp Immunol. 1993;91:58-62.
51. Zhang Y, Doerfler M, Lee TC, Guillemin B, Rom WN. Mechanisms of stimulation of interleukin-1β and tumor necrosis factor α by Mycobacterium tuberculosis components. J Clin Invest. 1993;91:2076-2083.
52. Peetermans WE, Raats CJI, Langemans JAM, Van Furth R. Mycobacterial heat-shock protein 65 induces proinflammatory cytokines but does not activate human mononuclear phagocytes. Scand J Immunol. 1994;39:613-617.
53. Peetermans WE, Raats CJI, van Furth R, Langermans JAM. Mycobacterial 65-kilodalton heat shock protein induces tumor necrosis factor α, interleukin 6, reactive nitrogen intermediates and toxoplasmastatic activity in murine peritoneal macrophages. Infect Immun. 1995;63:3454-3458.
54. Retzlaff C, Yamamoto Y, Okubo S, Hoffman PS, Friedman PS, Klein TW. Legionella pneumophila heat-shock protein-induced increase of interleukin-1β mRNA involves protein kinase C signaling in macrophages. Immunol. 1996;89:281-288.
55. Kol A, Sukhova GK, Lichtman AH, Libby P. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-α and matrix metalloproteinase expression. Circulation. 1998;98:300-307.
56. Kol A, Bourcier T. Lichtman AH, Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest. 1999;103:571-577.
57. Vabulas RM, Ahmad-Nejad P, da Costa C, et al. Endocytosed HSP60s use Toll-like receptor 2 (TLR2) and TLR4 to activate the Toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem. 2001;276:31332-31339.
58. Bulut Y, Faure E, Thomas L, et al. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. J Immunol. 2002;168:1435-1440.
59. Wang Y, Kelly CG, Singh M, et al. Stimulation of Th-1 polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. J Immunol. 2002;169:2422-2429.
60. Chen W, Syldath U, Bellmann K, Burkart V, Kolb H. Human 60-kDa heat-shock protein: a danger signal to the innate immune system. J Immunol. 1999;162:3212-3219.
61. Kol A., Lichtman AH, Finberg R W, Libby P, Kurt-Jones EA. Cutting Edge: Heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. J Immunol. 2000;164:13-17.
62. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting Edge: Heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol. 2000;164:558-561.
63. Asea A, Kraeft S-K, Kurt-Jones EA, et al. Hsp70 stimulates cytokine production through a CD-14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med. 2000;6:435-442.
64. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. Hsp70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J Biol Chem. 2002;277:15107-15112.
65. Asea A, Rehli M, Kabingu E, et al. Novel signal transduction pathway utilized by extracellular hsp70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem. 2002;277:15028-15034.
66. Bethke K, Staib F, Distler M, et al. Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: Superiority of HSP60. J Immunol. 2002; 169:6141-6148.
67. Singh-Jasuja H, Scherer HU, Hilf N, et al. The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. Eur J Immunol. 2000;30:2211-2215.
68. Flohe SB, Bruggemann J, Lendemans S, et al. Human heat shock protein 60 induces maturation of dendritic cells versus a TH1-promoting phenotype. J Immunol. 2003;170:2340-2348.
69. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Dorsophila Toll. Proc Natl Acad Sci USA. 1998;95:588-593.
70. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1:135-145.
71. Medzhitov R, Preston-Hurlburt P, Kopp E, et al. MyD88 is an adapter protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell. 1998;2:253-258.
72. Muzio M, Natoli G, Saccani S, Levrero M, Mantovani A. The

human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). J Exp Med. 1998;187:2097-2101.
73. Akira S. Toll-like receptor signaling. J Biol Chem. 2003;278: 38105-38108.
74. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282:2085-2088.
75. Takeuchi O, Hishino K, Kawai T, et al. Differential role of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999;11:443-451.
76. Baker-LePain JC, Reed RC, Nicchitta CV. ISO: a critical evaluation of the role of peptides in heat shock/chaperone protein-mediated tumor rejection. Curr Opin Immunol. 2003; 15:89-94.
77. Tsan MF, Gao B. Endogenous ligands of Toll-like receptors. J Leukoc Biol. 2004; in press.
78. Audibut F. Adjuvants for vaccine. A quest. Int Immunopharmacol. 2003;3:1187-1193.
79. Xu Q. Special Commentary: Infections, heat shock proteins, and atherosclerosis. Curr Opin Cardiol. 2003;18:245-252.
80. Majde JA. Microbial cell-wall contaminants in peptides: a potential source of physiological artifacts. Peptides. 1993;14: 629-632.
81. Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-like receptor 4, but not Toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharide. J Immunol. 2000;165:5780-5787.
82. Bausinger H, Lipsker D, Ziylan U, et al. Endotoxin-free heat shock protein 70 fail to induce APC activation. Eur J Immunol. 2002;32:3708-3713.
83. Gao B, Tsan MF. Endotoxin contamination in recombinant human Hsp70 preparation is responsible for the induction of TNFα release by murine macrophages. J Biol Chem. 2003; 278:174-179.
84. Gao B, Tsan MF. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor α from murine macrophages. J Biol Chem. 2003;278:22523-22529.
85. Reed RC, Berwin B, Baker JP, Nicchitta. GRP94/gp96 elicits ERK activation in murine macrophages: A role for endotoxin contamination in NFκB activation and nitric oxide production. J Biol Chem. 2003;278:31853-31860.
86. Chen K, Lu J, Wang L, Gan Y-H. Mycobacterial heat shock protein 65 enhances antigen cross-presentation in dendritic cells independent of Toll-like receptor 4 signaling. J Leukoc Biol. 2004;75:260-266.
87. Gao B, Tsan MF. Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. Biochem Biophys Res Comm. 2004;317:1149-1154.
88. Liu B, Zheng H, Stoilova D, Sun S, Li Z. Cell surface expression of an endoplasmic reticulum resident heat shock protein gp96 triggers MyD88-dependent systemic autoimmune diseases. Proc Natl Acad Sci USA. 2003;100:15824-15829.
89. Baker-LePain JC, Sarzotti M, Nicchitta CV. Glucose-regulated protein94/glycoprotein 96 elicits bystander activetion of CD4(+) T cell Th1 cytokine production *in vivo*. J Immunol. 2004;172:4195-4203.