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Functions of Heat Shock Proteins in Pathways of the Innate and Adaptive Immune System

Robert Julian Binder

For more than 50 years, heat shock proteins (HSPs) have been studied for their role in protecting cells from elevated temperature and other forms of stress. More recently, several roles have been ascribed to HSPs in the immune system. These include intracellular roles in Ag presentation and expression of innate receptors, as well as extracellular roles in tumor immunosurveillance and autoimmunity. Exogenously administered HSPs can elicit a variety of immune responses that have been used in immunotherapy of cancer, infectious diseases, and autoimmune disease. The Journal of Immunology, 2014, 193: 5765–5771.

Heat shock proteins (HSPs) were inadvertently discovered in 1962 when Drosophila salivary glands were grown overnight at elevated temperatures and were found to be highly upregulated (1). Several HSPs have been discovered since then, and they are grouped into families based on m.w. (e.g., hsp10, hsp40, hsp60, hsp70, hsp90), although family members may be structurally unrelated (2). The ability of HSPs to chaperone nascent polypeptides for proper folding and expression is their best known function (2); however, a significant role for HSPs in the immune system has emerged in the past three decades and is the focus of this review. The usefulness of HSP-mediated immune responses for immunotherapy of disease is mentioned. The immunological roles for HSPs, in situ or in the extracellular environment, rely fully or partially on the fundamental property of a select few HSPs to act as a chaperone of peptides, in addition to whole proteins.

Peptide-binding properties of select HSPs

The intracellular association of HSPs with peptides has been demonstrated on structural, biochemical, and immunological levels. The bacterial homolog of mammalian hsp70, DnaK, was crystallized in 1996, together with its associated peptide occupying a peptide-binding groove (3, 4). Peptide binding sites have been identified for calreticulin (5) and hsp90 (6), as well as its endoplasmic reticulum (ER) parologue gp96 (7). Peptides associate with each of these HSPs with low affinity and no or low specificity (8–10). The peptides are generally 10–30 aa in length and are generated during normal protein turnover in cells (8–10). Because the peptide binding sites for each HSP are different, the case can be made for distinct associated peptide repertoires. It is important to note that the association of HSPs with peptides is not an artifact of cell lysis (11). In many immunological systems tested, peptides derived from antigenic proteins were shown to be associated with cytosolic (hsp90 and hsp70) and ER (calreticulin and gp96) HSPs. These antigenic peptides include those derived from viral (12–18) or bacterial (19) proteins from infected cells, tumor Ags from malignant cells (20–22), model Ags from transfected cells (23–27), and alloantigens from MHC-mismatched cells (28, 29). Immunologically, peptides chaperoned by HSPs reflect the antigenic fingerprint of a cell. Finally, the association of HSPs with peptides appears to be an evolutionarily conserved property, because HSPs from bacteria (3), amphibians (30), rodents (12–14, 17–19, 21–25, 31), and primates (16, 20) can bind peptides. The HSPs and MHC are the two prominent families of peptide-binding proteins in immunology.

In situ HSPs and the immune system

HSPs and the MHC class I Ag-presentation pathway. The classical view of the pathway that leads to productive peptide presentation by MHC class I (MHC I) begins with protein degradation by the cytosolic proteasome. Although the source of proteasome substrates is under intense debate (32, 33), extended polypeptides are cleaved to small peptides with the correct C-termini, but extended N-termini, for occupying MHC I. For several years these peptides were inexplicably assumed to diffuse from the proteasome to the TAP for transportation into the ER (34). ER peptides are loaded onto nascent MHC I H chains with the assistance of the peptide loading complex (PLC) comprising TAP, tapasin, ERp57, and calreticulin. During loading onto MHC I, peptides simultaneously undergo a final round of N-terminal trimming by ER-associated protease (34). Peptide-loaded MHC I H chains associate with β2M, and the trimeric complex is transported through the Golgi and ultimately expressed on the cell surface, where it can be recognized by T cells. Studies from several laboratories argue strongly for the involvement of HSPs in several steps of MHC I loading. For example, intact HSP70 has been shown to associate with nascent MHC I(H-2Kd) and stimulate N-terminus trimming of peptides (35). Studies from several laboratories argue strongly for the involvement of HSPs in several steps of MHC I loading.

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Abbreviations used in this article: DC, dendritic cell; ER, endoplasmic reticulum; HSP, heat shock protein; MHC I, MHC class I; PLC, peptide-loading complex; RA, rheumatoid arthritis; Treg, regulatory T cell.

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modifications to this oversimplistic classical pathway. First, hydrophobic amino acids present in short peptides (which cannot assume higher-ordered structures) cannot exist in the aqueous intracellular environment without being shielded (35), a role provided by HSPs (Fig. 1). Second, diffusion as a transport mechanism is too uneconomical to satisfy the calculated efficiency of production of peptide/MHC I on the cell surface (36, 37). This efficient process can be satisfied by factoring in the HSPs. hsp70, hsp90, gp96, and calreticulin were proposed, in 1994, to form a relay line for efficient transport of peptides from the proteasome to MHC I (38). The following evidence strongly supports this proposal. 1) Disruption of peptide binding to hsp70 or hsp90 with deoxyergpergin or tansipemin, respectively, abrogates peptide presentation by MHC I (39, 40). Knock down of hsp90α with small interfering RNA produces a similar phenotype (41). 2) When peptides are eluted from highly purified, apparently homogenous HSPs, MHC-binding peptides and their precursors (intermediates of the processing events) are found (8–10, 12–29). 3) Transfer of progressively trimmed peptides between HSPs and MHC I has been observed in the ER (42). 4) Naturally occurring free peptides cannot be detected in cells even after the most rigorous of experimental exploration (11). Once cells or their derived lysates are treated with ATP or mild acid, agents known to release peptides from chaperones, peptides can be readily detected (11). 5) Peptides transported by TAP associate with gp96 and other ER chaperones, including protein disulfide isomerase (43).

These HSPs adhere to certain specifications to satisfactorily transfer peptides for MHC I presentation. The ubiquitous expression of hsp70, hsp90, calreticulin, and gp96 in cells essentially ensures that the MHC I–presentation pathway remains functional in all nucleated cells. IFN-γ inducibility of a majority of the components of the MHC I–presentation pathway also applies to HSPs involved in the pathway (44, 45). The receipt and delivery of peptides by HSPs suggest a physical association of those HSPs with other components of the pathway. hsp90 was shown to physically associate with the proteasome and, in certain instances, replace the PA28 subunits (46, 47), situating hsp90 in an optimal position to receive peptides being generated by the proteasome. hsp70 and hsp90 form heteromultimeric complexes in the cytosol with other chaperones (48). Although this complex is essential for client protein–refolding roles, peptide transfer within this complex has yet to be tested. hsp70 also were shown to be in physical proximity to TAP (49), an association that would allow delivery of HSP-chaperoned peptides to TAP for onward translocation into the ER. This suggests that the proteasomes, hsp70, hsp90, and TAP could exist as a single cytosolic multimeric unit akin to the PLC. Within the ER, the PLC has been well defined, with calreticulin playing a peptide-binding role. The transfer of peptides among calreticulin, gp96, and MHC I in the ER has been documented (42). The apparent lack of association of gp96 (and ER-associated protease) with other PLC members may reflect a weaker or transient interaction with this complex, considering the other significant roles for gp96 in ER biology. Other unidentified chaperones may be involved in this pathway.

Cytosolic HSPs in endosome–cytosol trafficking of cross-presented Ags. APCs capture Ags from the extracellular space and internalize them into vesicular bodies. Following processing of the Ags, resultant peptides can be presented by MHC I, a process referred to as cross-presentation (34). There is debate regarding the cellular compartment in which Ags are processed for cross-presentation (34, 50). Vesicular bodies into which Ag is internalized, such as endosomes or phagosomes, may become specialized peptide-processing and MHC I–loading compartments through recruitment of selective ER proteins that perform these roles. MHC I–peptide complexes in these vesicles recycle to the cell surface (50). A prevalent second pathway involves trans-endosomal membrane transport of the Ag to the cytosol where it enters the classical MHC I Ag-presentation pathway (34). In support of this, the proteasome dependence for cross-presentation of Ags by APCs was shown (34). hsp90 and hsp70 are intimately involved in the extrusion of Ags from the endosomes to the cytosol through a putative pore structure (51, 52). This mechanism involves the unfolding and linearization of the protein Ags by hsp90 prior to extrusion. Pharmacological inhibition of hsp90 or its deletion in cross-presenting APCs or Ag rendered structurally inflexible through fixation abrogated Ag cross-presentation (51). Interestingly, hsp70 was shown to negatively regulate Ag extrusion, arguing for cooperative regulation of this process by two cytosolic HSPs. These specific roles for hsp70 and hsp90 may account, at least in part, for the extraordinary efficiency of extracellular HSPs at cross-presenting Ags that are chaperoned by them (as described below). Endogenous HSPs, through functionality or expression levels, appear to be key regulators of Ag cross-presentation. Coincidentally, hsp90 and hsp70 are induced in response to stress and IFNs, the precise situations during which cross-presentation is required.

gp96 as a chaperone of innate immune receptors. gp96 has a well-described function in the ER for folding nascent polypeptides into their final conformation (2). These client proteins are wholly dependent on gp96 for proper expression. All TLRs, with the exception of TLR3, are client proteins of gp96 (53). Folding of TLRs is dependent on dimerization of gp96 and its chaperone CNPY3 also present in the ER lumen. Following gp96 depletion in APCs, TLRs are rapidly removed from the cell surface and intracellular vesicles, rendering APCs functionally unresponsive to corresponding TLR ligands, as measured by a lack of NF-κB activation and secretion of cytokines. Structural studies on gp96 identified the TLR-binding domains, which are distinct from ATP-binding domains (54). Several other client proteins, such as α and β integrins, known for their roles in influencing immune responses, bind to the same domain. Thus, mice deficient in gp96 expression are severely susceptible to bacterial infection (55). TLRs are the critical sensors for recognition of microorganisms, and their expression patterns are closely related to function. Upstream processes that determine TLR expression are expected to regulate immunity to pathogens; thus, gp96 is a master chaperone that can control immune responses with fine specificity based on selectivity and titratability in client binding.

Extracellular HSPs and influence of adaptive immunity Collectively, HSPs are the most abundant proteins in cells, and their liberation into the extracellular environment is a key immunological indicator of loss of cellular integrity. HSPs in the extracellular environment are a product of pathologic cell
FIGURE 1. (AA) hsp90 and hsp70 are intricately involved in shuttling peptides from the proteasome to TAP in the cytosol. In the ER, gp96 and calreticulin (CRT) sequentially transfer peptides from TAP to MHC. (AB) hsp90 and hsp70 regulate the extrusion of Ags from endosomes to the cytosol through a putative pore during Ag cross-presentation. (AC) gp96 folds and controls the expression of TLRs and other client proteins. HSPs released from tumor cells through a variety of mechanisms (BA) are endocytosed by CD91 expressed on APCs (BB). HSPs and the chaperoned peptides are processed within the cell (BC) before presentation of the peptide on MHC molecules to CD4 or CD8 T cells (BD). (BE) CD91 also serves as a signaling receptor, controlling the expression of cytokines and costimulatory molecules. (BF) Thus, HSPs can prime diverse Th cell responses.
death, abnormal active secretory mechanisms, or nonconventional cellular expression on the membrane (56–59). A select group of HSPs, when extracellular, is recognized by cellular immune sentinels. Such recognition allows HSPs to be critical mediators of immune responses, initiating specific immune responses against cancers or pathogen-infected cells, exacerbating pre-existing inflammatory conditions, or suppressing ongoing immunity. The surprising discovery of HSP receptors on APCs afforded a molecular description of these immunological mechanisms (60–63) (Fig. 1B).

**Extracellular HSPs are necessary for immunosurveillance of cancer.** The ability of the immune system to recognize aberrant, premalignant cells and eliminate them before they become pathological is the hallmark of tumor immunosurveillance (64). Immunosurveillance and resulting immunoediting include an elimination, equilibrium, and escape phase (64). T cell responses, initiated during the elimination phase, are primarily responsible for rejection of the tumorigenic cells (65). T cells receive signal 1 through TCR recognition of Ag presented by MHC. Recognition of costimulatory ligands on APCs by corresponding receptors on T cells provides signal 2. These signals are provided in the context of a cytokine milieu. In a majority of cases, these signals are sufficient for T cell priming, and all abnormal premalignant cells are eliminated. A failure of the immune system to completely eliminate the abnormal cells sets the stage for an equilibrium phase, a state that can persist for many years. Cancers then develop when they escape immune control.

The conventional mechanism of priming T cells has been revised to include an elimination, equilibrium, and escape phase (64). Immunosurveillance and resulting immunoediting in vertebrates are specific to the CD91+ APC and the HSP receptor(s) on immune cells (57–59, 80). Therefore, immunologically dangerous situations can be classified quantitatively and qualitatively according to the potential for release of HSPs, or more generally, intracellular content, which provide signals that may complement, supersede, or antagonize those originating from other pathways (e.g., pathogen-associated molecular pattern–pattern recognition receptor) or, in the absence of other apparent signals, elicit exclusive responses.

1) Tumor Ags have been grouped into neoantigens resulting from mutated, overexpressed, or abnormally expressed proteins (67), although the former are ubiquitous and appear to be the most immunogenic (67). Priming of tumor-specific T cells requires that these tumor Ags are transferred from the Ag-expressing (tumor) cell to APCs for cross-presentation (68). Mutated self proteins, expressed by a few abnormal cells, set the limitations for the amount of material available for cross-presentation during tumorigenesis. These limits have been quantified and are in the picogram range (69, 70). However, cross-presentation of soluble protein Ags, as measured in several model Ag and pathogen experimental systems, requires ~10 to hundreds of micrograms [e.g., (66, 71)]. The five orders of disparity between these two quantities are satisfied when HSP-peptide complexes are considered as the mediators of Ag transfer (69). Peptides chaperoned by HSPs are efficiently cross-presented by APCs, and the efficiency is largely attributed to the presence of an endocytic receptor CD91 (60–62, 72). The mechanisms associated with cross-presentation of HSP-chaperoned peptides have been quantified and detailed in a large body of work (60–63, 73). Importantly, femtogram to picogram levels of a specific peptide complexed to 10 μg of gp96, hsp70, or calreticulin are sufficient for cross-presentation (74). My group proposed that the HSP–peptide–CD91 mode of Ag transfer must be used during tumorigenesis given limiting Ag levels. Mice deficient in CD91 expression on APCs failed to mount immune responses to growing tumors (75), a phenotype previously seen in IFN-γ- or rag-deficient mice (64). CD91-deficient mice or tumors expressing endogenous inhibitors of CD91 fail to adequately cross-present Ag and stimulate T cell responses. These observations point to an indispensable role for endogenous tumor-derived HSPs and their receptor CD91 in establishing immune responses to a developing tumor. In other pathological situations in which Ag load is considerably higher, other modes of Ag transfer may apply (76).

2) Tumors, being of self origin, lack expression of pathogen-associated molecular patterns, which typically elicit costimulation. We do not know the origin of costimulation during tumorigenesis. Purified gp96, hsp70, and calreticulin were shown to stimulate and mature APCs, which are associated with cytokine release and upregulation of costimulatory molecules (77, 78). HSP-stimulated dendritic cells (DCs) are perfectly suited for priming T cells. Incidentally, CD91 was shown to be a signaling receptor for immunogenic HSPs activating p38 MAPK and NF-κB (78). The repertoire of cytokines released and costimulatory molecules expressed are specific to the CD91+ APC and the HSP used, but routinely includes IL-1β, TNF-α, IL-6, IL-12, and GM-CSF (78). HSP-stimulated APCs upregulate CD80, CD86, CD40, and MHC class II (77). In a tumor microenvironment, with the release of multiple HSPs and with APCs in this locale, the response is of the Th1 type, capable of rejecting the tumor.

As a single entity, the HSP–peptide complexes are capable of priming T cell responses. These responses are considered in juxtaposition with other intracellular molecules capable of stimulating APCs, such as HMGB1, dsDNA, and uric acid (79).

**Cellular necrosis, which is a near-universal occurrence in cancer, appears to be the most rational mechanism for extracellular HSP release (56).** However, there are other mechanisms through which HSPs are accessible to their receptor(s) on immune cells (57–59, 80). Therefore, immunologically dangerous situations can be classified quantitatively and qualitatively according to the potential for release of HSPs, or more generally, intracellular content, which provide signals that may complement, supersede, or antagonize those originating from other pathways (e.g., pathogen-associated molecular pattern–pattern recognition receptor) or, in the absence of other apparent signals, elicit exclusive responses.

**Extracellular HSPs and etiology of autoimmunity.** During homeostasis, it is necessary that HSPs are largely inaccessible to their receptors. Sustained release of HSPs into the extracellular environment would be expected to trigger autoimmunity based on the observed proinflammatory conditions elicited by some HSPs. Although there is no formal experimental demonstration of this expectation, there is some suggestion of it in various pathological conditions.
The etiology of rheumatoid arthritis (RA) remains largely unresolved and is likely to be a combination of genetic, environmental, and immunological factors. However, a case can be made for extracellular HSPs as a factor contributing to the initiation and/or progression of RA. Elevated levels of hsp70 and gp96 are observed in synovial fluids and fibroblasts from inflamed joints of RA patients (81–83). This increase is not observed in nonarthritic joints of the same patient. Macrophages lining the synovial tissue of RA joints are highly responsive to extracellular HSPs and produce cytokines, including IL-1β, IL-6, and TNF-α, which exacerbate the inflammatory conditions (83). Already, therapy targeting HSPs with Abs is being envisaged. A therapeutic focus on the upstream molecules that initiate inflammation is expected to be more successful than the current anti–TNF-α therapy. A majority of the peptides chaperoned by HSPs are self peptides. In the event that autoreactive T cells are not thymically deleted, they can be primed following HSP-mediated cross-presentation of these self peptides (84), further contributing to cellular destruction of arthritic joints. In some other instances, these self peptides may even be derived from the HSP molecule itself and, thus, are a target for cellular immunity or are able to induce protective immunity (85–87).

In experimental systems, transgenic mice with enforced cell surface expression of gp96 develop spontaneous lupus-like autoimmune disease as a result of chronic stimulation of DCs (88). Although there were no apparent gross abnormalities, these mice displayed severe glomerulonephritis by 20 wk of age. DCs in these mice were found to be hyperactive and capable of priming self-reactive T cells, and of breaking down peripheral tolerance. These studies were able to demonstrate the direct proinflammatory properties of constitutive extracellular gp96 in mice, a condition similar to immunization with tumor cells secreting HSPs (89).

Exogenously administered HSPs and modulation of immune responses. Immunogenicity of HSPs was first shown in 1986 during a search of the immunogenic entity of tumor cells (90). Srivastava et al. biochemically fractionated tumor cells and tested each fraction for its ability to elicit tumor rejection in mice. Apparently homogenous preparations of gp96 were shown to do so. Thus, when mice are immunized with gp96 isolated from a tumor, they become resistant to a subsequent challenge of that specific tumor. The specificity of the immune responses was against peptides chaperoned by gp96. These observations are true for other peptide-chaperoning HSPs, including hsp70 (91, 92), hsp90 (91), calreticulin (93), hsp110 (94), and gp170 (94), and are effective in many disease models in rodents (90–94), frogs (30), and humans (see below). Following on those pioneering studies, depending on the dose and experimental setting, HSPs were shown to prime not only specific antigen-tumor immunity (90–94), but also regulatory T cell (Treg) responses [hsp70 (85), hsp60 (95, 96), gp96 (97–99)], Ab responses [hsp60 (95), gp96 (13)], or Th17 responses (78) (calreticulin, gp96). The natural extension of these observations was therapeutic experimentation in various diseases. First, in rodent models, HSPs were shown to regress established tumors and prevent metastasis [gp96 (100), hsp70 (100), hsp110 (94), gp170 (94)], prevent or delay autoimmunity [hsp60 (95), gp96 (97–99)], and reduce pathogen load [gp96 (13, 18, 101)]. There is an extensive list of clinical trials in patients with a variety of cancers [with gp96, hsp70 (see Ref. 102)], in patients with autoimmune diabetes [with hsp60 (95, 103)], and in virally infected patients [with hsp70 (104)].

The plasticity of HSP-mediated immunity (i.e., priming Th1, Th2, Th17, and/or Treg) depends on the ability of HSPs to engage local APCs through cell surface receptors, thereby stimulating diverse costimulatory signals (77, 78, 95). CD91 is one such prominent receptor. When HSPs are introduced at higher doses, we speculate that additional APCs and/or (lower-affinity) cell surface receptors (63) could be targeted. The 24mer peptide of hsp60 offers a clear example: it was shown to bind TLR2 and TLR4 mediating anti- and proinflammatory conditions, respectively (95). The repertoires of cytokines released by bone marrow–derived dendritic cells, peritoneal exudate cells, and lymph node DCs stimulated with HSPs are overlapping, but distinct, and dependent on receptor usage (78). The overall signal received by the cell from engagement of multiple signaling receptors, as well as the resulting costimulation, determines the Th cell response that is primed. The recent demonstration that blocking Treg generation allowed gp96 to mediate stronger peptide-specific CTL responses in BALB/c mice provides further evidence for this dynamic interplay, as do the studies on hsp60 (95). Although many of the experimental systems are necessarily minimalistic, it is important to consider the sum of these signals during pathology in conjunction with signals emanating from other engaged (pattern recognition) receptors.

Conclusions
It has been 30 years since Srivastava and colleagues first described the immunogenicity of HSPs. The HSPs (and the receptor CD91) are evolutionarily ancient; thus, it is unsurprising that the immune system has evolved to use HSPs in conserved pathways. As we continue to better understand their functions, other roles for HSPs in innate and adaptive immunity may be uncovered, not only in the diseased state but also during homeostasis. With the recent appreciation of various commensals in regulating immune responses, for example, we are yet to appreciate the role of commensal-derived HSPs in generating signals that may affect those emanating from PRRs.

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