

Regulatory role of heat-shock proteins in autoimmune and inflammatory diseases

Cristina Daneri-Becerra¹ and Mario D. Galigniana^{*1,2}

¹Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Buenos Aires (C1428ADN), Argentina

²Departamento de Química Biológica de la Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires, Buenos Aires (C1428EGA), Argentina

Abstract

Classically, molecular chaperones play a pivotal role in the maintenance of cellular proteostasis and thus, in the safeguarding of the cell homeostasis while reducing the deleterious effects of extracellular and intracellular stresses. They are also active players in immunologically relevant scenarios such as the activation of innate immunity, antitumour immunity, and autoimmune diseases. It is currently accepted that misdirected immune responses may target self-antigens and generate severe inflammatory responses, a typical signature of autoimmune diseases. In addition to numerous components in immune responses, chaperone proteins are also detected in the extracellular fluids and have been implicated in autoimmune and inflammatory diseases acting as pro- and anti-inflammatory factors. In several inflammatory pathologies, chaperones are greatly induced as a direct consequence of the inflammatory stress and are released from the cell thanks to a poorly understood mechanism. These extracellular chaperones are capable to stimulate anti-inflammatory regulatory T cell responses, thereby inducing the negative feedback control of inflammation. Therefore, it has been proposed that immunization with heat-shock protein peptides could prevent the development of certain diseases. In this article we review the basics of the stress response, summarize current controversies over the role of extracellular chaperones in inflammatory reactions and autoimmunity, and discuss the cytoprotective and immunoregulatory roles of heat-shock proteins, a challenging subject that may open a new avenue for the drug discovery and treatment of diseases related to autoimmune disturbs.

Introduction to the stress response

Cells are always exposed to a number of sudden and potentially harmful variations of their biological milieu. With the purpose to protect proteins from misfolding, denaturation and/or aggregation, cells trigger a fast response characterized by a number of events able to protect them from the hostile environment, and restore a balanced and safe new steady-state of protein homeostasis commonly referred to as 'proteostasis' [1,2]. Importantly, such response builds a physiological network that shields cells and tissues from the risky challenges they may encounter, such as heat, cold, toxics, chemicals, UV light, radiation, drugs, infections, infestations, inflammation, pH variations, osmotic changes, nutrient deprivation, oxidative stress, hypoxia-ischemia, apoptotic stimuli, and stressful conditions for individuals such as psychiatric disorders and socially traumatic experiences [3]. The generation of a new proteostasis network implies an immediate role in protein synthesis, folding, disaggregation, or degradation, processes that encompass the translational machinery, molecular chaperones and their associated cochaperones, the ubiquitin-proteasome machinery, and the autophagy system.

The stress response to heat-shock was originally described in the early '60s by the Italian researcher Ferruccio Ritossa [4]. One of his colleagues accidentally switched the temperature of his incubator where *Drosophila* cells were grown, and a quite different puffing pattern was acquired by those cells. The biological consequence was an exceptional increase of transcriptional activity for a family of proteins that, for this particular reason, were generically named heat-shock proteins (HSPs). Then, it was noted that HSPs are expressed in a highly conserved manner in all organisms from bacteria to humans, to the point that many times they may be efficiently interchanged. HSPs

form oligomeric complexes constitutively expressed under normal conditions (ranging up 5-10% of the total protein). They function as molecular chaperone machineries capable to regulate the folding of all proteins, intracellular transport of soluble and particulate cargoes among the different subcellular compartments, repair or degradation of proteins, refolding of misfolded proteins, etc. [5]. In addition to being constitutively expressed, HSPs are also greatly induced by the onset of stress (up to 15%). It should be noted that the terms HSP and chaperone are frequently used as synonyms. However, while all HSPs are always chaperones, not all chaperones are induced by heat-shock.

The biological function of molecular chaperones is not limited to solve abnormal situations, but they also have an active participation in the biological function of native factors such as steroid receptors, protein-kinases, transcription factors, enzymes, oncogenes, structural proteins, etc. Actually, the 90-kDa heat-shock protein, Hsp90, has more than four hundred client proteins [6].

Correspondence to: Dr. Mario D. Galigniana, IBYME, Vuelta de Obligado 2490, Buenos Aires (C1428ADN), Argentina, **E-mail:** mgali@qb.fcen.uba.ar, mgaligniana@conicet.gov.ar

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Signal Transduction and Autoimmunity

Charles J. Malemud, PhD

Professor of Medicine

Case Western Reserve University

E-mail: cjm4@case.edu

As it was stated above, those molecular chaperones induced by heat stress are also called HSPs. However, temperature is not the sole stimulus to trigger the stress response, but also a wide variety of other stimuli (UV radiation, chemicals, toxic compounds, metals, inappropriate pH or osmotic pressure, nutrient starvation, oxidants, fever, cancer, infections, neurodegenerative diseases, etc [7]). Also, the heat-shock response can come in handy during the normal growth cycle of the cells even without the existence of stressors in the medium.

The essential role of the heat-shock factor

The HSP genes are regulated by a master transcription factor, the HSF (Heat-Shock Factor) [2]. In vertebrates four HSF members have evolved (HSF1, HSF2, HSF3, and HSF4), the HSF1 being so far the most abundant and best characterized member of the family. The inactive monomer of HSF1 is cytoplasmic and forms oligomers with the Hsp90-based heterocomplex [8]. Upon the exposure of cells to stress, HSF1 homotrimerizes, translocates to the nucleus, and acquires specific DNA-binding and transcription-enhancing activities [9,10]. How stressing situations are sensed by the cell and conveyed to HSF1 is not entirely understood. As mentioned before, a great number of stresses can trigger a heat-shock response, therefore it has been postulated that different types of molecular sensors may exist for each type of stimulus. Alternatively, it could be that a given proteotoxic environment may converge in a common signal able to initiate the biological response. The classical model postulates that under normal conditions, the association of molecular chaperones to HSF1 maintains this transcription factor inactive. When cells are subjected to stressing situations, the increased level of protein misfolding would release the Hsp90-based complex from HSF1 monomers allowing its trimerization [8,11]. Other models propose that HSF1 has itself the ability to detect proteotoxic conditions, as evidenced by the capacity of purified HSF1 to homotrimerize *in vitro* upon the onset of different types of stresses [12].

The induction of the stress response results in high levels of expression of molecular chaperones, which bind to nascent chains and other metastable soluble proteins preventing misfolding and/or aggregation. During the attenuation of stress and the subsequent recovery steps, the pool of non-native proteins dissipates and protein homeostasis is therefore restored. However the persistence of the original problem may perpetuate mechanisms responsible for proteostasis maintenance by constant activation of influencers. This is manifested with the existence of a steady-state in pathologies such as Alzheimer's disease, chronic emotional distress, psychiatric diseases, cancer, and diabetes [1].

Molecular chaperones

The molecular chaperone family comprises a very heterogeneous group of proteins that may be grouped into eight subfamilies [1,13,14]: 1)-The small HSPs/ α -crystallin group, which show molecular weight ranging from 12-kDa to 43-kDa. There are 10 mammalian sHSPs, all of them are cytosolic and most of which contain an α -crystalline domain [15]. They favour the client protein refolding by Hsp70 by forming large homo-oligomeric cages able to trap misfolded proteins and preventing the formation of undesirable intra- or intermolecular interactions. This process is ATP-independent and thought to be complementary for those commanded by other ATP-dependent chaperones. 2) The DNAJ/Hsp40 subfamily, characterized by the presence of a J domain that permits interactions with Hsp70. These chaperones can be thought of as adaptors that provide versatility to Hsp70 function [16] assisting protein refolding by presenting misfolded proteins to Hsp70

and by enhancing its ATPase activity. 3)- The Hsp60 mitochondrial chaperonin-like protein is essential for maturation and maintenance of the mitochondrial proteome and is therefore intimately linked to energy production [13]. 4)- The Hsc70/Hsp70 subfamily of chaperones with intrinsic ATPase activity that is enhanced by Hsp40. It works in a cooperative fashion with Hsp90 in several heterocomplexes, and is also recruited to newly synthesized polypeptides by the ribosome-associated complex [17]. Importantly, Hsp70 shows antiapoptotic properties [18]. 5) The Hsp90 scaffold protein is able to form large heterocomplexes with other chaperones and cochaperones. It is perhaps the most versatile protein of the entire family and certainly is the most abundant soluble protein of the cell [19]. 6)- The large HSPs/Clp subfamily of AAA+ ATPases (molecular weight of 100-kDa to 110-kDa), which have the ability to solubilise almost any protein that becomes aggregated after severe stress, but are not required under normal growth conditions [20]. They work associated with Hsp70 extracting trapped polypeptides from aggregates *via* a threading mechanism. 7)- An additional group comprises chaperones present in the endoplasmic reticulum that participates in the proper folding and eventual degradation of new born proteins, such as the glucose regulating proteins (Grp), the ubiquitin and lectin chaperones calnexin and calreticulin. 8)- Immunophilins, endogenous proteins that are characterized by the presence of a signature domain named PPIase (peptidyl-prolyl-*cis/trans*-isomerase), *i.e.*, an enzymatic activity of isomerase able to interconvert reversibly Xaa-Pro bonds. High molecular weight immunophilins are frequently bound to Hsp90 playing the role of cochaperones or forming a functional regulatory unit with the chaperone [21,22].

The Hsp90-Hsp70-Immunophilin complex

Both chaperones Hsp90 and Hsp70 are highly expressed in all types of cells, and they cooperate in most of the complexes with client proteins. The cytoplasmic pool of Hsp90 forms homodimers, each monomer unit consisting of three domains: a) The N-terminal domain contains an ATP binding site whose hydrolysis to ADP is critical in the chaperoning activity of the Hsp90 homodimer. b) The M- domain is a flexible middle region able to interact with a great numbers of client proteins and is linked to the N-terminal domain by a highly charged linker sequence. The intrinsic ATPase activity of Hsp90 requires an arginine located in the "catalytic loop" of the middle domain [23]. c) The C-terminal domain is responsible for the dimerization of the chaperone and also possesses a key pentapeptide MEEVD sequence that permits the association of TPR-domain proteins such as immunophilins, Hop, WISp39, Tom70, CHIP, Tah1, etc. [19,24,25].

Dimers of Hsp90 are in dynamic equilibrium between two states, the ADP-bound isoform and the ATP-bound isoform, which are able to form different oligomeric heterocomplexes with other chaperones (Figure 1-A). The ADP-bound Hsp90 shows higher affinity for the TPR-domain-containing cochaperone Hop. Hop brings together Hsp70 and Hsp90. Hop also prevents Hsp90 from effectively chaperoning client proteins whose binding favours a high, sustained level of ATP [26,27]. Hop binding to Hsp90 blocks ATP binding and the ATPase activity of the chaperone, which makes binding of the cochaperone p23 significantly weak or void [28,29]. This ADP-bound conformation of Hsp90 is favoured by the Hsp90 disrupting agent geldanamycin, a benzoquinone ansamycin antibiotic that shows high affinity for the nucleotide binding site of the chaperone. In this state, the associations of both p23 and TPR-domain immunophilins to the complex are impaired. On the other hand, when ADP is exchanged with ATP, the ATP-bound isoform of Hsp90 is stabilized by p23 binding, and other

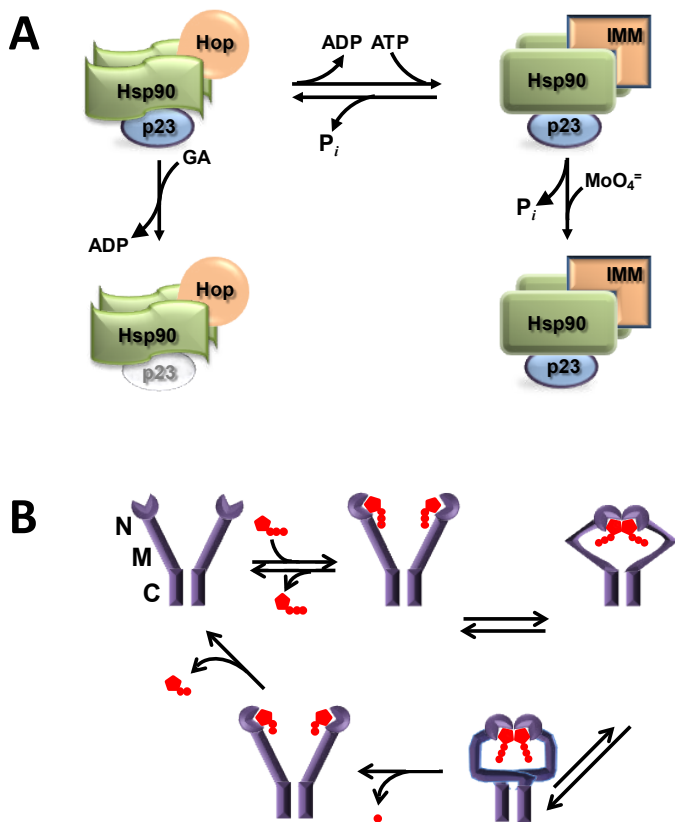


Figure 1. (A) ATP-dependent conformational states of Hsp90. Dimers of Hsp90 adopt different conformation when ATP or ADP is bound. The best chaperone activity for a given client protein is reached by the ATP form of the dimer, which is also able to interact more efficiently with TPR-domain immunophilins (IMM) and the co-chaperone p23. This optimal conformation is stabilized by molybdate, perhaps due to its conformational similarity with the hydrolyzed gamma phosphate of ATP (released by the intrinsic ATPase activity of Hsp90). On the other hand, the ADP-dependent conformation favors the association of the TPR-domain co-chaperone Hop rather than IMM binding. The ADP-dependent form of Hsp90 is stabilized by the benzoquinone ansamycin geldanamycin (GA) when the drug replaces the ADP from its pocket. The lower efficiency of this dimer to interact with client proteins compared to its ATP isoform, usually targets the destabilized client factor to degradation by the proteasome. **(B)** Conformational Cycle of Hsp90 Dimers Interacting with a Client Protein. The V-shaped open conformation of Hsp90 binds ATP at the N-terminal domain (N). The M-middle domain (M) repositions and interacts with the N-domain, and the dimer twists over itself. The intrinsic ATPase activity is favored by this close association of the M and N domains leading to the separation of the N-domains to release the products of the hydrolysis, and Hsp90 returns to the original open conformation to restart the cycle. The C-terminal domain (C) maintains Hsp90 as a dimer.

TPR proteins such as the high molecular weight immunophilins are efficiently recruited [26,27]. The ATP-bound isoform of Hsp90 is a better modulator of the biological activity of client proteins. It appears that p23 binding to Hsp90 changes the nature of ATP binding to Hsp90 and prevents the intrinsic ATPase activity of the chaperone [30], which in turn may affect the biological activity of the Hsp90-bound client factor. Hsp90 interacts with hundreds of client proteins and other signaling molecules, including steroid receptors, p53, tyrosine-kinases, oncogenes, Stat5, NOS, HSF1, Akt, Chk1, Mdm2, IκB kinases, cytoskeletal proteins, importins, nucleoporins, histones, DNA helicases, telomerase reverse transcriptase, DNA polymerases, SmyD methyltransferases, p300 acetyltransferase, HDAC6, Aha1, SGT1, and Cdc37. In other words, Hsp90 creates a broad scaffold platform for the regulation of signalling cascades, nuclear architecture and cell function [28,31,32]. The mechanism involved in client protein-Hsp90 interaction also involves the ATPase intrinsic activity of the chaperone. Thus, Hsp90 exists in a conformational equilibrium between two states

—an open (V-shaped) conformation and a close conformation (Figure 1-B), a state where ATP-hydrolysis takes place, the N-terminal domains dissociate, and Hsp90 returns to the open conformation again [33].

Perhaps one of the most remarkable biological functions of high molecular weight immunophilins associated to Hsp90/Hsp70 complexes is the ability to interact with the dynein/dynactin motor complex [34], a property used to favour the retrotransport of soluble factors in an active manner using cytoskeletal tracks. This machinery is used for the transport of proteins such as GR [35], MR [36], ecdysone receptor [37], RAC3/AIF complexes [38], NF-κB [39], etc., as well as for viral particles when cells are infected by a virus [40]. Importantly, Hsp90-based complexes have been related to microbial invasion of host cells [41,42] and increased antigenicity of pathogen soluble factors [43].

Role of heat-shock proteins in immunity

The best biological incentive to trigger the innate immunity response is the exposure to a foreign molecule in a hazardous milieu. The critical decision of an organism for responding or ignoring that stimulus is taken by innate immune recognition receptors upon their activation by PAMP (pathogen-associated molecular patterns) molecules, i.e. factors associated with groups of pathogens that are small molecular motifs conserved within a class of pathogens (for example lipopolysaccharides, bacterial CpG DNA, etc.) and recognized by cells of the innate immune system by PRRs (or Pattern Recognition Receptors) [44]. A number of these PRRs have been cloned and showed to belong to the expanding family of toll-like receptors (TLRs). An alternative pathway for the activation of innate immunity is that proposed by the ‘danger theory’ [45]. According to this hypothesis, the innate immunity can be additionally activated by endogenous substances released by the damaged or stressed tissue itself. In this way, stressed cells can messenger stress to other cells of any type. Some of the stress signals released by cells are the HSPs induced in response to the insult, such that they are potential candidates for signalling tissue damage or cellular stress. In this regard, some of them such as Hsp60 and Hsp70 have been actually found capable of signalling through CD14, TLR-2, and TLR-4 [46,47].

The first findings reported a heat-shock-like protein described as a glia-axon transfer protein of the squid giant axon [48], as well as the

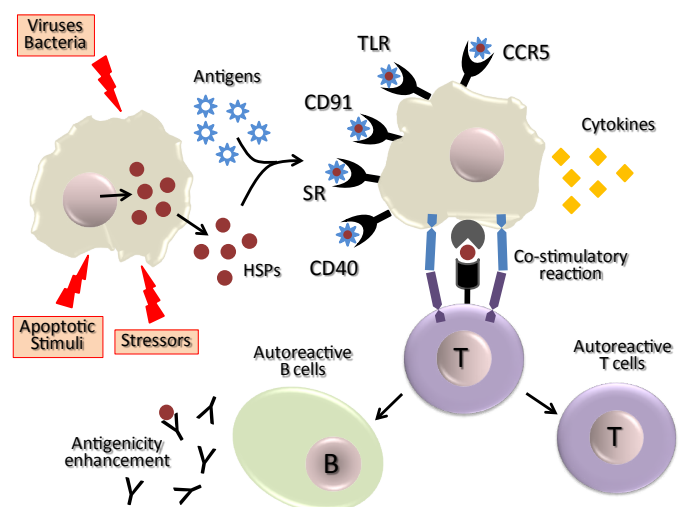


Figure 2. Schematic mechanism for the role of exported heat-shock proteins in the pathogenesis of autoimmunity.

fact that Hsp70 is released from cells by a mechanism not impaired by inhibitors of the known secretory pathways [49]. This original early observation was deemed not relevant, and the presence of HSPs in the extracellular environment was disregarded for many years until it was demonstrated that recombinant Hsp70 activates cells of the immune system [50]. These findings were disputed based on the possibility that the activation of immune cells was due to contamination [51,52]. Nonetheless, these worries were ruled out after experiments using pure recombinant Hsp70 isolated from insect cells, non-recombinant Hsp70, treatments with antibiotics, boiling treatments, incubation in serum free media, etc. [53,54]. Today, it is well established and broadly accepted that Hsp70 is responsible for the activation of macrophages, monocytes, dendritic cells, natural killer cells, hepatocytes, etc. [55-58]. Moreover, extracellular HSPs have been shown to work as potent immunostimulatory or immunosuppressive molecules depending on the circumstances by which they interact with cells [59].

In addition to Hsp70, other HSPs have also been found in the extracellular milieu, such as Hsp60 [60], Hsp90 [61-63], Grp78/BIP [64,65], and Hsp27 [66]. The biological relevance of these extracellular factors has been reinforced by the detection of Hsp70 in the serum of patients suffering from a number of pathologies such as chronic inflammation [67], myocardial infarction [68], lung injury [69], coronary artery disease [70,71], infections [67], cancer [72,73], ischemia/reperfusion events [74], diabetes [75], hypertension during pregnancy [76], etc. HSPs can also be detected in the serum of healthy individuals, although at lower levels [59]. Importantly, the presence of Hsp70 in plasma is correlated with improved survival of critically ill patients [77,78]. Other extracellular HSPs such as Hsp27 [66], Hsp60 [79], and Hsp90 [61,80] have also been linked to several diseases such as pancreatic carcinoma, coronary heart disease, cancer metastasis or systemic lupus erythematosus.

Heat-shock protein secretion

Regarding the origin of extracellular HSPs, while it was originally demonstrated a basal HSP release from healthy cells [49], others researchers proposed that the source of extracellular HSPs could be the consequence of cell lysis after necrosis [81]. Later on it was demonstrated that HSPs are released by an active mechanism independent of cell death [82]. Nevertheless, these studies cannot preclude the possibility that necrosis could be an additional source of extracellular HSPs, so two different sources of extracellular HSPs can coexist—the active pathway due to a non-conventional secretory mechanism, and a passive one secondary to cell death and subsequent lysis [55,83].

Following HSP release from external pathogens, foreign cells, autologous inflamed or necrotizing tissue (such as tumours or those target tissues of autoimmune diseases), they are recognized by receptors expressed in the surface of immune cells of the host—TLR-4, TLR-4 like, TLR-2, CD14, CD91, CD94, LOX-1, SR-A, or as an immunodominant antigens [55,84,85] 8–11. With the contribution of these released HSPs, the immune system of the host is therefore “informed” about the presence of pathologic processes (Figure 2).

The secretion mechanism of Hsp70 is perhaps the most studied, although definitive conclusions are still uncertain. An active mechanism for Hsp70 secretion has not been demonstrated to date, whereas typical inhibitors of the classic endoplasmic reticulum-Golgi pathway, such as brefeldin-A, showed no effect on the release of Hsp70 [49]. On the other hand, this chaperone does not possess a consensus peptide signal for secretion *via* the classic ER-Golgi pathway. Therefore, it is likely that the export mechanism is *via* a putative alternative pathway called the

non-classical or unconventional secretory pathway [86], which could also be shared by other factors such as some interleukins, galectins, and growth factors, although unifying common steps for this proposed mechanism remain still unrevealed [86]. Three possible scenarios for Hsp70 active secretion could be its export a)- *via* the lysosome-endosome pathway, in which Hsp70 is translocated into the lysosome lumen *via* ATP-binding cassette (ABC) transport-like system and further transported outside cells *via* the endocytic process [87]; b)- *via* secretory-like granules loaded of Hsp70 in similar manner as to those granules used by exocrine cells and the endocrine and neuroendocrine systems to release hormones and granines in secretory vesicles [88]; and c)- *via* extracellular vesicles [55] derived from the plasma membrane by membrane blebbing, ectosome vesicles, and/or by endocytosis, *i.e.*, due to the formation of multiple vesicles with the same topology as the plasma membrane (exosomes) [89]. Regardless of the origin of the vesicles, other HSPs such as Hsp90, Hsp60, Hsp27, Grp78/BIP have also been detected in the lumen and membrane of these extracellular vesicles [90]. It is thought that the HSPs in the lumen of the vesicle are the consequence of protein being trapped during the formation of the vesicle since HSPs are very abundant in the cytosol. Therefore, it is expected that vesicles should burst to release the intravesicular HSPs allowing them to interact with other proteins and cells. Alternatively, vesicles can fuse with the membranes of cells, releasing the lumen content into the cytosol as well as those HSP associated with the vesicle membrane, as reported for Hsp70 [53,91] and Hsp60 [60,92]. HSPs are highly concentrated in the vesicles (for example, in the mM order for Hsp70 [55]), which favours interactions with other factors and cell surface receptors increasing the biological activity of the chaperone. It has been reported that the specific activity of Hsp70 in these vesicles is more than 250 fold greater for activating macrophages than the soluble Hsp70 [55]. Similar to Hsp70, Hsp90 also lacks a secretory signal; therefore it has been postulated as being exported *via* exosomes [93,94] or by direct translocation across the plasma membrane [95].

Other molecular chaperones are also present in the external cell surface, such as Grp78/BIP [96], Grp94 on the plasma membrane of sarcoma cells [97], Hsp60 on the surface of human endothelial cells [98] and T cell-lines [99], as well as in mouse liver and spleen [100]. Hsp90, which was first detected on the cell surface of tumour cells [101], was subsequently found on monocytes [43], mesenchymal cells [102], and neuroblastoma cells [61,103].

Role of extracellular chaperones in immunity

Exported HSPs trapped the attention of several laboratories because of their potential action on the immune system, where which they function as signalling molecules. Accordingly, it has been stated that extracellular Hsp70 is able to activate monocytes, macrophages natural killer and dendritic cells [55]. Also, Hsp70 is a stimulating factor for autoimmunity [104,105] by a peptide-specific immune response that involves the α 2-macroglobulin receptors CD91, CD40 and OLR1/LOX-1 expressed in antigen-presenting cells [106]. The innate immunity response is triggered by recognition of toll-like receptor (TLR) signals, one of the activating signals being the exported HSPs. Thus, to evaluate if exported Hsp70 shows antigenic properties, isolated lymphocytes from rat spleens were exposed to purified Hsp70, and the result was lymphocyte proliferation [107]. Then, it was demonstrated that the immunogenic form of Hsp70 corresponds to a well-conserved 14 amino acid sequence (amino acids 139–153) [108].

Additionally, HSPs can also play a role in generating antigen-specific T cell responses [109] It is thought that complexed peptides

with Hsp70, Hsp90B1/gp96, calreticulin, etc. are delivered to antigen-presenting cells by receptor-mediated internalization of the HSPs. This could make them available for further processing and presentation to the major histocompatibility complex. Inasmuch as specific receptor-mediated mechanisms exist for binding and internalization of HSPs [110], it may be envisaged that the cross-presentation of HSP-derived antigenic determinants is a realistic mechanism for cross-priming by antigen-presenting cells. Consequently, it is logical to propose that these HSPs highly induced in several types of pathologies are indeed targets of the adaptive immune response.

It has been demonstrated that exported Hsp70 increases neutrophil chemotaxis and their microbicidal ability [111], as well as macrophage phagocytosis properties [112], and modulation of the response of monocytes to endotoxin [113]. Similarly, extracellular Hsp70 is related to both immunostimulatory and immunosuppressive activities [114]. Nonetheless, the exact molecular mechanisms responsible for such modulation of the response of cells of the immune system are still poorly understood. It was reported that during the inflammatory response generated by tumours, both IFN- γ and IL-10 induce the active release of both Hsp70 isoforms Hsc73 and Hsp72) from the malignant tissue [115]. It is still uncertain whether these secreted HSPs produce antitumour immunogenicity responses.

Taking advantage of the capability of HSPs to target a chaperoned antigen to antigen-presenting cells and elicit cross-presentation, it has been developed immunotherapy strategies using the secretable forms of HSPs. Thus, it is known that the Hsp90B1/gp96 secreted from tumour cells carries an antigenic peptide able to activate specific antitumour cytotoxic T lymphocyte responses [116]. This property is also shared by exported Hsp70 and Hsp90 [117]. The endoplasmic reticulum chaperone BiP (Binding of immunoglobulin protein) is also exported from tumour tissues and capable to elicit antigen-specific tumour immunity [118]. This secreted BiP complexed with antigenic peptides is taken up by dendritic cells and cross-presented in association with MHC class I molecules. This leads to cytotoxic T lymphocyte responses, which in turn stimulates tumour cells to produce their own endogenous "cellular vaccine". Such immunogenic strategy could be more advantageous than a peptide-generated vaccine approach because single peptide-based cancer vaccines have certain disadvantages. For example, single peptide vaccination usually induces a HLA-restricted cytotoxic T lymphocyte response, thereby allowing tumour cells to escape from lymphocyte recognition. Contrarily, because HSPs are associated to a broad-spectrum antigenic peptide repertoire, it is likely that the subsequent induction of a cytotoxic T lymphocyte response is more effective because it is directed against multiple antigens. In addition, this type of secreted HSP-based cancer vaccine may be useful for all classes of patients regardless of HLA restrictions. In short, gene modification of HSPs for secretion could be a practical strategy to provide an exceptional beneficial approach for cancer immunotherapy.

Similar to cytoplasmic chaperones, it has been proposed that Hsp90B1/gp96, an endoplasmic reticulum-resident partner of the chaperone Hsp90, might participate in mechanisms that are critical for cell growth, differentiation, and responses to endoplasmic reticulum stress [119]. Also, it is a natural adjuvant for chaperoning antigenic self peptides into the immune surveillance pathways, and may also be involved in the maintenance of morphostasis and self tolerance. Recently, it was demonstrated that high levels of up-regulation of Hsp90B1/gp96 in regenerating liver and thymus are followed by signs of transient autoimmunity, augmented apoptosis in thymus, and the presence of autoreactive NKT and regulatory T cells that might

be involved in the control of rapid liver growth induced by partial hepatectomy [120].

Hsp90 is a key regulator of innate immunity

It is accepted that exogenous antigens are processed by two main pathways [83,121]: a)- the classical MHC class I loading pathway involving the transporter-associated antigen-presenting (TAP)-dependent pathway, and b)- the endosome-recycling pathway, which involves a post-Golgi loading mechanism of MHC class I in endocytic compartments. It has been demonstrated that soluble Hsp90 is able to translocate extracellular antigens from endosome to cytosol for TAP-dependent cross-presentation [122], where antigens are processed in the endosomes by cathepsin S and other peptidases, and thereafter are loaded in endocytic compartments onto MHC class I molecules. These complexes are recycled from plasma membranes [123,124]. Hsp90 also works as an efficient route-finder for those chaperoned antigens targeted to the cross-presentation pathway [125,126]. Also, it has recently been demonstrated that Hsp90-cancer antigen peptide complexes are efficiently cross-presented by monocyte-derived dendritic cells and stimulated peptide-specific cytotoxic T lymphocyte responses [127]. Also, it was demonstrated [128] that stimulation of CD4+T cells by dendritic cells DC that had been activated by Hsp90-peptide complexes involved pathways that share many of the essentials mechanisms involved in stimulation of antigen cross presentation.

Hsp90 empowers the chaperoned ligands to activate an immune response. It is well known that unmethylated single-stranded DNA containing a cytosine-phosphate-guanine (CpG) motif binds TLR9 receptors of plasmacytoid dendritic cells (pDCs) and B cells, which results in the production of IFN- α [129]. However, these cells normally do not respond to self-DNA, a phenomenon that was explained by the fact that both bacterial and viral DNA have multiple CpG nucleotides able to bind and consequently activate TLR9 more efficiently than mammalian self-DNA contains, which contains a lower number of these motifs and are usually masked by methylation. There are situations where self-DNA might have the potential to activate TLR9, but it may not succeed since it fails to access the TLR9-containing endolysosomal compartments [130]. Synthetic CpG-DNAs have been assayed— CpG-A, able to stimulate the receptors of pDC cells and produce IFN- α , and CpG-B, which is inactive in this regard [130], but stimulates the release of IL-6 and TNF- α in these cells. While CpG-A forms large multimeric aggregates and is retained for long periods of time in early endosomes, whereas CpG-B is a monomer, cannot oligomerize, and rapidly translocate from early to late endosomes or lysosomes of pDCs [131]. Such a long-lasting retention of CpG-A results in prolonged activation of TLR9 signaling, leading to strong IFN- α production. In other words, the retention time of the CpG/TLR9 complex in endosomes is a key determinant for the TLR9-dependent signalling cascade. Inasmuch as Hsp90 binds and retains client ligands within early endosomes, studies specifically conducted to evaluate the role of such interaction demonstrated that human pDCs pulse Hsp90•CpG-A complexes inducing a larger production of IFN- α than the monomeric CpG-A [130]. In contrast to human dendritic cells, murine cells also express TLR7 receptor in addition to TLR9 [132]. In this system, the Hsp90•CpG-A complex is retained within early endosomes for longer periods leading to sustained activation of murine cells and eliciting TLR9 signaling for IFN- α production, which is indeed TLR9-mediated since TLR9 KO cells do not produce IFN- α . By contrast, CpG-A alone was transferred to late endosomes and lysosomes. Importantly, not only CpG-A•Hsp90 complexes, but also CpG-B•Hsp90 complexes are strong stimulating ligands of TLR9

resulting in high production of IFN- α .

It may be stated that extracellular Hsp90 has the capacity to chaperone CpGs in early endosomes leading to a more abundant production IFN- α . Therefore, there are two important considerations derived from that property: a)- extracellular Hsp90 (and HSPs in general) could play a crucial role in the pathogenesis of nucleic acid-mediated autoimmune diseases, for example in systemic lupus erythematosus; and b)- it may be implied that the use of extracellular HSPs in combination with CpG may enhance the biological effectiveness of cancer vaccines.

Heat-shock proteins and autoimmune diseases

The participation of HSPs in the pathogenesis of autoimmune diseases has been analyzed using two basic animal models: a)- artificially induced arthritis by the natural saturated terpenoid alkane pristane, adjuvant-induced arthritis, avridine-induced arthritis, arthritis generated by Streptococcus cell wall and collagen type II [133]; and b)- insulin-dependent diabetes mellitus model in non-obese diabetic mice (NOD)[134]. The adjuvant-induced arthritis is generated by intracutaneous injection with heat-killed *Mycobacterium tuberculosis*. Arthritis can be transferred from diseased injected animals to healthy naive animals with a single T cell clone specific for a particular sequence of Hsp60 *M. tuberculosis* isoform. Thus, the microbial agent initiates a self-perpetuating autoimmune process in a susceptible individual generating antibodies against the host's Hsp60, and perhaps an epitope of the cartilage proteoglycan [135]. Regarding the potential advantages of this phenomenon from the perspective of therapeutic applications, it was observed the induction of tolerance after immunization with the HSP. Accordingly, pristane oil-generated arthritis disease shows high levels of IL-2 and IFN- γ after *in vitro* restimulation of arthritic mice splenocytes with mycobacterial Hsp60 [136]. Therefore, mycobacterial Hsp60 is capable of preimmunize mice and protected them from pristane actions.

Similarly, anti-Hsp70 antibodies have been found in the sera of patients with malaria [137]. HSPs have been shown to play a cardinal role in antigen direct presentation and cross-presentation leading to CD8+ T cell activation [109]. There is strong evidence to support the notion that cytosolic HSPs such as Hsp70 and Hsp90, and those of the endoplasmic reticulum such as Hsp90B1/gp96, have the ability to bind antigenic peptides generated within the cells, as well as they are also part of the endogenous pathway of antigen presentation *via* the major histocompatibility complex class I molecules [138,139]. Peptides that are chaperoned by HSPs when released extracellularly, or HSP-peptide complexes assembled *in vitro*, are taken up by antigen-presenting cells such as dendritic cells *via* α 2-macroglobulin receptor (CD91)-mediated endocytosis, resulting in representation by the major histocompatibility complex molecules [138,140]. Vaccination of mice with Hsp70, Hsp90 and Hsp90B1/gp96 isolated from murine tumour cells elicits immune response sufficient for tumour rejection and suppression of metastatic tumour progression [141]. This tumour immunity was shown to result from tumour-derived peptides associated with the HSPs, rather than from the HSPs themselves [117].

Insulin-dependent diabetes mellitus is the other example of Hsp60 involvement in autoimmune diseases. The regular disease is the clinical consequence of pancreas β -cell destruction by T cells. Infection of non-obese diabetic mice with *M. avium* prevents the development of diabetes [142]. Similarly immunization of NOD mice with mycobacterial Hsp60 significantly reduces the incidence of the disease [143], suggesting that autoantibodies against Hsp60 could play a major role in the etiology of

this type of diabetes.

Systemic lupus erythematosus is other prototype of autoimmune disease characterized by the presence of plasma autoantibodies against self-nuclear structures, such as dsDNA and RNA-containing antigens, including Sm and RNP [144,145]. This disease is characterized by the presence of autoantibodies against Hsp90 [146] and enhanced expression of Hsp90 in peripheral blood mononuclear cells of patients [147]. Cytosolic Hsp90 translocates to the nuclear compartment under stressful conditions, which raises the possibility that Hsp90 may bind self-DNA in the nucleus. In a following step, self-DNA associated with endogenous Hsp90 can be released into the extracellular milieu when cells undergo necrosis, triggering the production of IFN- α by dendritic cells. Serum Hsp90 α levels has been found increased in patients with lupus [148,149], and these high concentrations are significantly decreased after treatment. Moreover, immunodepletion of extracellular Hsp90 from serum decreased IFN- α production by pDCs [148], indicating that depletion of Hsp90 might induce remission and prevent end-organ damage. Thus, control of deregulated pDC activation and IFN- α production represents an attractive treatment strategy for systemic lupus erythematosus.

Collectively, extracellular Hsp90 plays a crucial role in the disease activity of SLE and that Hsp90 inhibitors have promise for the treatment of IFN- α -mediated autoimmune diseases. In line with this postulate, it has recently been reported the evaluation of small molecule inhibitors of Hsp90 as potential therapeutic target for this disease in an autoimmune mouse model [150]. The novel resorcinolic triazolone inhibitor STA9090/ganetespib [151] was found to be equally efficacious and tolerable when compared to an effective weekly dosing regimen of the standard-of-care immunosuppressive agent cyclophosphamide. Importantly, co-treatment of ganetespib with a sub optimal, intermittent dosing schedule of cyclophosphamide resulted in superior therapeutic indices and maximal disease control. These findings highlight the potential of HSP90 inhibition as an alternative, and potentially complementary, strategy for therapeutic intervention in systemic lupus erythematosus.

HSPs in the Autoimmune Diseases of the central nervous system (CNS)

The very first reports of HSPs release from neuronal cells were related with the secretion of both Hsp70 and Hsp110 along with actin [49]. Further publications reported that Hsp70 is released from glial cells and taken up by adjacent neurons [48,152]. It has been demonstrated that many neurons have reduced expression of some HSPs due to inadequate HSP gene transcription activity [153]. However glial cells adjacent to synapses release HSPs into the extracellular medium and chaperones are captured by neuronal receptors [152,154,155]. The functions of such non-neuronal HSPs are not properly understood to date, but probably they may provide some chaperoning properties to neurons and protection from programmed cell death, which can accompany neurotransmission [155].

In view of the limited regenerative capacity of CNS, protection against immune responses requires special mechanisms of self defence. This is achieved due to singular conditions able to reduce the exposure to the immune system, referred to as '*immune privilege*' [156]. This is possible due to the existence of blood-brain barrier, the limited expression of major histocompatibility complex molecules of the brain, and neuronal expression of molecules with immunosuppressive properties, e.g. TGF- β and CD200. These characteristics contribute to

protect neurons from microglia and T-cell-induced damage. However activated immune cells can invade the CNS under specific conditions through selective receptor-ligand interactions [157], and the list of neurological diseases where T cells are involved in targeting neurons or their axons, directly or indirectly, is continuously growing [158]. The most frequent diseases where the immune system targets neurons are multiple sclerosis, the paraneoplastic cerebellar degeneration, and Parkinson's disease.

Multiple sclerosis is a multifocal inflammatory autoimmune disease affecting only the CNS in which the target is the myelin and the myelin-producing cells, oligodendrocytes. This disease may be considered a standard model of an autoimmune disease of the CNS from the perspective of the molecular mechanism of action of HSPs. Myelin is destroyed in patches, resulting in the formation of astrocytic scars (or plaques), showing high accumulation of T cells and macrophages, increased concentrations of cytokines and chemokines, and antibodies to myelin proteins [159]. Although the exact etiology of the disease is not fully understood, current evidence suggests a critical role for the immune system in its pathogenesis. The inflammatory and oxidative environment generated in the CNS of these patients favours the overexpression of most of HSPs, including Hsp70 [160,161], whose induction occurs predominantly in oligodendrocytes [162]. The biological consequences of Hsp70 induction are dual. Based on their activity as chaperone, the release of Hsp70 into the milieu act as an adjuvant that binds immunogenic peptides, which increases the inflammatory local reaction and autoimmunity. On the other hand, Hsp70 is an antiapoptotic factor [18,163,164] and, as all chaperones do, it prevents the accumulation of abnormal protein aggregates [165], a common histopathological hallmark that contributes to neuronal degeneration [166]. Therefore, inadequate induction of Hsp70 can increase the rate of protein aggregation upon the onset of stressing situations leading to apoptosis, a typical end for most neurodegenerative and autoimmune diseases such as multiple sclerosis. Accordingly, it has been reported that Hsp70 overexpression in neuronal cell cultures and also mouse models of neurodegenerative diseases shows advantageous actions to ameliorate the severity of the phenotypes by reducing the number and size of inclusion bodies and the accumulation of disease-causing factors [161].

Since Hsp70 works as a neuroprotective factor for ischemia-induced cell death [167], it may be argued that the overexpression of the chaperone in multiple sclerosis lesions might protect the CNS against the typical inflammatory environment of this disease [168]. In line with this, it has been demonstrated that the stimulating action of proinflammatory cytokines (IL-1, TNF α , IFN-, etc.) in glial cells induce Hsp70 expression, which in turn exerts favourable final effects on glial cells and neurons [162]. However, all coins have two sides. At the same time, exported Hsp70 leads to the activation of the NF- κ B transcription factor due to the activation of TLRs; this induces the production of proinflammatory cytokines (IL-12, IL-1 β , IL-6, TNF- α , and GM-CSF) [50,81,135], chemokine secretion (MCP-1, RANTES, and MIP-1 α) [169], and nitric oxide production [170] by macrophages and dendritic cells. Moreover, extracellular Hsp70 enhances the expression of CD83, CD86, CD40 and the major histocompatibility complex class II on dendritic cells, as well as the migration of these cells to draining lymph nodes [81,171]. All of these elicit priming adaptive immune responses. Therefore, it is thought that the acute stress induces the release of Hsp70, which works as a danger signal to prepare the immune system against a possible pathogen challenge.

Taken all together the above exposed observations together, it can

be concluded that the inflammatory phase occurring at the initial stages of multiple sclerosis is a sort of preconditioning stimulus for glial cells to induce Hsp70 expression and release, which in turn has a protective action on neurons in the subsequent neurodegenerative phase. This permits to envisage an obvious scenario—a failure to induce Hsp70 or the insufficient production of HSPs in the CNS is a possible determining factor for multiple sclerosis development, and conducts to postulate therapeutic strategies focusing on HSP up-regulation for different neuropathologies. This logical hypothesis, however, cannot be applied for the case of multiple sclerosis because there is an additional conflicting factor—the strong autoimmune reaction that accompanies multiple sclerosis triggers a process of neurodegenerative damage that leads to the typical clinical signs of the disease. Thus, extracellular Hsp70 is actually a harmful rather than beneficial factor. Compared to other CNS pathologies such as Alzheimer's and Parkinson's diseases, the released Hsp70 in multiple sclerosis strongly exacerbates the immune response by acting as a very efficient adjuvant for myelin peptides and as a proinflammatory cytokine [172]. These effects cannot be counterbalanced by Hsp70 beneficial actions such as the reduction of protein misfolding or antiapoptotic effects. These concepts are particularly valid for viral infections. Researchers are considering the possibility that viruses, and also bacterial infections, may cause multiple sclerosis. Viruses are known to cause inflammation and a breakdown of myelin (or demyelination). Therefore, it is highly possible that a virus could trigger the disease. Several viruses and bacteria are currently being investigated to determine if they are involved in the development of multiple sclerosis. These studies include measles virus, human herpes virus-6 (HHV-6), and Epstein-Barr virus (EBV).

All these features can also be observed in autoimmune diseases such as glaucomatous pathology, where elevated serum titers of autoantibodies to many optic nerve components [173] and retina antigens [174] are present, as well as abnormal T-cell subsets [175]. In this case the master chaperone involved in the disease is Hsp27. In addition to the intrinsic neuroprotective functions of Hsp27, like other HSPs, this chaperone also serves as an antigenic stimulus activating the innate and adaptive immune response during glaucomatous neurodegeneration [176]. By serving as a danger signal, up-regulation of Hsp27 may facilitate detection and elimination of stressed retinal ganglion cells by the immune system. Thus, uncontrolled immune activity, including T-cell mediated cytotoxicity to retinal ganglion cells and their axons, may eventually facilitate the progression of neurodegeneration. In summary, the overall summary of these biological events tells us that potential errors during the processing of the native cellular interactions are relevant for the conversion of protective immunity and self-limiting inflammatory responses into chronic neurodegeneration and autoimmune diseases.

Concluding remarks

The export of HSPs is still a poorly understood process as a consequence of our reduced understanding of the actual mechanism for the release of the chaperones and the lack of a standard action in that extracellular milieu, with biological consequences that are even opposite to the classical and broadly accepted actions of these proteins. One piece of evidence, however, is clear today after so many years of controversy: the extracellular HSPs are not artefacts. They play roles as extracellular factors ranging from ligands to activate cell surface receptors to favour antigenicity of pathogenic factors, such that autoimmune responses are developed. The overall function of these chaperones seems to be at the level of signalling or cellular communication rather than at the traditional chaperone activity. In

this regard, to date no overwhelming experimental evidence exist that allow us to affirm that extracellular HSPs play the same chaperone activity outside cells, to the point that the requirement of co-chaperones and other factors associated to them for playing their intracellular roles are unlikely not required for their extracellular functions as signalling molecules. A particularly relevant effect is the modulation of the immune system, which is visualized as a novel priming event for cellular communication under stressing situations. Ultimately, these exported molecular chaperones represent a new opportunity to explore eventual therapeutic approaches to modulate the biological response of exported HSPs in the development and progression of autoimmune diseases.

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