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Review Article

Extracellular Heat Shock Proteins as a Natural Protector of Cells and Organisms: A Review of Recent Studies

Nishizawa K* and Nishizawa M
School of Medical Technology, Teikyo University, Japan

Abstract

The heat shock protein (HSP) family consists of molecular chaperones that are evolutionary preserved from prokaryotes and mammals. The extracellular HSPs are now known to be involved in numerous processes including would healing, tissue regeneration, tumor progression and innate and adaptive immunity. In immunity, HSPs exhibit functional dichotomy in both innate and adaptive immunity, triggering or suppressing immune responses, but the majority of studies highlight their cytoprotective and immunoregulatory roles. Especially for several autoimmune diseases including rheumatic arthritis (RA) and systemic lupus erythematosus (SLE), for which specific causative self-antigens remain elusive, the therapeutic expansion of regulatory T cells specific to HSPs is considered a promising approach. In this review, we discuss several articles extracted from diverse biological and medical frontiers of extracellular HSP research.

Keywords: Xenopus; MHC class Ib; Glycosaminoglycans; Dermatan sulphate; BiP (immunoglobulin-binding protein)

Introduction

Heat shock proteins (HSPs) belong to molecular chaperons that are involved in quality control, folding of proteins, which become important after episodes of cell stress such as thermal stress [1,2]. The expression of HSPs is upregulated during cell stress, with dying cells expressing HSPs at elevated levels [3]. Numerous researches unveiled cytoprotective and immunoregulatory roles of HSPs and thus contributed to diagnosis and treatment of many diseases [4-6].

Upon cell stress, relocation to the cell surface and eventually release into the extracellular space occur for many HSPs [7]. Recent important developments of this field involve the studies by Srivastava and coworkers that showed that HSP-peptide complexes prepared from tumors can enter antigen presenting cells (APCs) for re-presentation on both MHC class I and class II, and induce antitumor immunity [8,9]. Pathophysiologically, tumor cells exhibit increased tumor immunogenicity upon heat stress and this effect was mediated by increased expression of some HSPs of tumor cells themselves [10,11].

Another influential finding was the ability of HSPs to induce immunological tolerance. Accumulating evidence shows that many HSPs involving Hsp60 (chaperonin), Hsp70 and certain peptides derived from the HSPs can induce anti-inflammatory IL10-producing T cells in many different models of autoimmune disease [12]. Thus, HSPs are now recognized, unlike simple damage-associated molecular-pattern (DAMP) molecule that trigger innate immunity responses, but rather as pro-survival molecules that protect host organisms through inducing cytolytic T cell responses against tumors/infectious organisms and, in normal conditions, generally suppressing immune reactions, and through removing cell debris without incurring inflammation. It should be noted that HSPs, although able to transport antigenic peptides as chaperone vaccines, do not on their own induce significant APC maturation [3].

It is of importance to consider the typical cellular localization and functions of HSPs. The HSPs called glucose regulated protein (Grp), such as Grp78 (a member of Hsp70 family), Grp94/gp96 (Hsp90 family) and Grp170 (Hsp100 family) resided in ER and participate in quality control of protein folding in ER [7]. Broadly, Hsp70 an Hsp90 normally participate in antigen processing and presentation and in T cell polarization (the 'relay line model' proposed by Srivastava and coworkers) [13-15]. Hsp70, in combination with Hsp90, also mediate the targeting of proteins to the lysosome during chaperone-mediated autophagy, the process recycle degraded proteins and eliminate damaged proteins [16]. Hsp60 is mitochondria proteins phylogenetically related to bacterial GroEL, and assists protein folding in cooperation with Hsp10. Besides, Hsp60 exhibits pro-survival or pro-apoptotic effects depending on the type and condition of the cell [17-19].

Extracellular HSPs, in particular Hsp70 and gp96 (Grp94) have been well studied for their aforementioned ability to transfer chaperoned protein-cargo to APCs for cross-presentation and induce antigen/tumor-reactive immune response [20]. Such activity is also known for high molecular weight HSPs, such as Hsp110 and Grp170 [5,21], but not for Hsp60.

Hsp60, Hsp70 Hsp90, Hsp110 and Grp170, have been shown in some settings to alert the innate immune system to induce or assist APC maturation and cytokine secretion [22-26]. Hsp110 and gp170 have large sizes and are considered to have superior capacity to hold and target protein antigens for DC-mediated cross-presentation [5]. Small HSPs such as

Hsp27 and Hsp20 contribute to stress tolerance and have anti-apoptotic activities [6] and exert anti-aggregation functions in association with Hsp70 [27].

Although this article does not cover, neuroprotective functions of HSPs are of medical importance [28]. Transfer of macromolecules from glia to neuron is likely to occur via extracellular vesicles, such as exosomes. The key function of this phenomenon in the neural system is considered neuroprotection by HSP-containing exosomes [28].

HSPs as cytokines: proinflammatory *vs.* anti-inflammatory signaling

HSPs are often categorized into 'chaperokines'. As this term represents, extracellular HSPs serve not only as a chaperones that facilitates antigen presentation to T cells, but also as cytokines that activate APCs and initiate innate and adaptive immunity. Both pro-inflammatory and anti-inflammatory effects of direct HSPs binding to cell surface receptors have been reported. Thus, the antigen-independent effects of HSPs regulate the activation and maturation of APCs, which in turn becomes an important determinant for the balance between the regulatory and the cytolytic T cells.

Prior to discussing the receptors, let us consider the issue of the contaminating bacterial components in the recombinant HSPs used in early studies, which confounded some discussions. Using the detoxified recombinant human Hsp70, Bausinger et al. showed that the ability of 1-3 μg/ml of the recombinant Hsp70 to induce the maturation of human monocyte-derived dendritic cells (DCs) was abrogated in the presence of the polymyxin B (LPS antagonist) or when the recombinant Hsp70 contained <60 IU/mg endotoxin [29]. Gao and Tsan showed that a highly purified recombinant human Hsp60 preparation with low endotoxin activity did not induce TNF-α release from murine macrophages at concentrations of up to 10 μg/ml [30]. A low-endotoxin sample of Grp94/gp96 also abrogated NF-κB signaling and nitric oxide production which were observed with the unpurified sample [31]. LPS-free Hsp60 stimulated T cells, but this effect was independent of TLR4 [32]. Using ClearColi BL21(DE3) bacteria that was genetically engineered to express a modified LPS incapable of triggering TLR4-dependent response, Planesse et al. showed that recombinant Hsp60 by itself was not able to induce the NF-κB-dependent signaling pathway in THP1 monocyte cell line [33]. These and other findings have led to a consensus that none of prokaryotic or mammalian HSPs has LPS-like ability to trigger the innate immune responses.

On the other hand, Hsp60 can synergize with LPS. Hsp60 can bind to LPS upon bacterial infection, and synergistically enhanced IL-12 production and IFN-γ release in antigen–dependent T cell activation [24]. An analysis conducted with care to remove contaminating LPS showed that human Hsp60, but not mycobacterial Hsp65, activated B cells via TLR4 pathway to produce IL-10 and IL-6 and to upregulate MHC class II and some accessory molecules [34]. Zanin-Zhorov et al. also showed that T cells responds to Hsp60 via TLR2, downregulating IFN-γ and TNF-α and enhancing IL-10 [35]. Thus, although its binding may be indirect one, HSP binding to TLRs can trigger responses, which are often anti-inflammatory. In some settings pro-inflammatory effects were seen, but such effects typically are weak when the doses of HSPs are physiological. This is not surprising because, while true DAMPs, such as high mobility group 1 (HMGB1), are normally located intracellularly, HSPs, such as Hsp60 and Hsp70, are present in the extracellular space as well [7].

It should also be reminded that recurrent TLR2 and/or TLR4 stimulations cause tolerance induction ('endotoxin tolerance'); a low-level injection of bacterial endotoxin shows protective effects from subsequent lethal dose [36]. Kilmartin et al. showed tolerance induction by a prior exposure to autologous Hsp60 [37]. Stimulation of peripheral blood mono-nuclear cells (PBMC) with Hsp60 induced TNF-α expression but pre-treatment of Hsp60 for 18 h abolished this effect. This pre-treatment was also protective against LPS stimulation. The Hsp60 priming of monocytes caused down-regulation of HLA-DR, CD86 and TLR4 expression, similar to LPS priming effects. As TLR2 and TLR4 are both implicated in Hsp60 effects on the innate immunity system, it is possible that HSPs secreted from cancer keep innate immune cells in a tolerant state through this mechanism, presenting a clinical concern.

In Theriault et al., Hsp70 was shown to bind to lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) but not to CD91, CD40, TLR2, TLR4 or DC-SIGN [38]. Thus, HSPs binding to TLR2 or TLR4 may have low affinity or dependent on an indirect mechanisms requiring other primary receptor [39]. Wang et al. showed that mycobacterial, but not human, Hsp70 binds to CD40, causing the release of several CC chemokines [40]. Mammalian Hsp70 requires CD40 in pathways of antigen presentation [41] although direct binding was insignificant or with very low affinity [38]. Floto et al. showed mycobacterial Hsp70 binds to chemokine receptor CCR5 [42]. The significance of Hsp70 binding to scavenger receptors (SR) family including LOX-1, SREC-1 and FEEL/CLEVER-1 in internalization of Hsp70 has been discussed in recent papers [39,43].

Overall, multiplicity of receptors for each HSP is evident, but subtle differences in affinity to receptors are known to exist among related members of the same HSP family. This feature may be linked to the fact that HSPs had evolved earlier than the cell surface receptors. It seems that, rather than adapting to a limited number of signals, HSPs had formed and provided an environment in which all modern cellular machineries/molecules can adapt to HSPs and, when advantageous in the evolution, amplify the cytoprotective and pro-survival effects of HSPs. On the other hand, from a mechanistic perspective, further studies are necessary to understand the structural basis enabling diverse HSPs that have sequence dissimilarity to bind to similar families of receptors [43].

HSPs can exert immunosuppressive or anti-inflammatory effects and this effect is associated with the regulation of the maturation state of DCs. Motta et al. showed that LPS-free *Mycobacterium tuberculosis* Hsp70 inhibits murine DC maturation in vitro and induced IL-10 secretion by DCs, while LPS-contaminated Hsp70 induced DC maturation

[44]. In general, immature DCs are known to be adept at phagocytosis/endocytosis of antigens and exhibit tolerative effects. Hsp70 also showed an immunosuppressive effect in a proteoglycan-induced arthritis (PGIA) model mouse model [45]. Compared to mycobacterial Hsp70, mouse Hsp70-treated bone marrow-derived dendritic cell (BMDC) showed more stable tolerogenic phenotype. In an analysis focusing on DnaK, a major bacterial chaperone belonging to cytoplasmic Hsp70, macrophages treated with DnaK behaved like M2 macrophages and showed tumour promoting potential [46]. Of note, unlike classical macrophages (M1 macrophages) that produce inflammatory cytokines and help Th1 differentiation, M2 macrophages are mainly anti-inflammatory and secrete IL-10, TGF-β, VEGF, and EGF and considered serving for removal of cell debris, tissue regeneration and immune regulation [47]. It is likely that the direct binding of HSPs to receptors as well as specific recognition of epitopes of HSPs by regulatory T cells act in concert to induce immunosuppression as we discuss below.

Antigen re-presentation and priming of T cells

MHC class I antigen presentation requires the transport of antigen peptides from cytoplasm to endoplasmic reticulum, and intracellular HSPs are normally serving for this process. Once released to extracellular space, a variety of chaperones, including Hsp90, Hsp70, grp94 and calreticulin, function as vehicles to internalize associated peptides into professional APCs and facilitate re-presentation of the associated antigen peptides [48,49]. This primes antigen-specific T cells that are primarily $CD8^+$ T Cells, but $CD4^+$ T cells are also known to be activated. Hsp60 is considered to lack this ability [50] likely due to its lack of the high affinity toward CD91. Large HSPs appear to have greater such ability [51]. The relationship between the HSP-peptide affinity and immunogenicity has been analyzed. For example, Flechtner et al. designed a peptide named Javelin that can bind to Hsp70, and observed that high affinity Hsp70:Javelin-hybrid peptide complexes are better at cross-presentation causing induction of stronger $CD8^+$ T cell immune responses [52]. Thus, targeting of antigens to APCs through Hsp70 is a useful strategy for efficient cross-presentation, which is important in cancer immunotherapy. In addition to directing peptide/protein antigens toward the class I pathway, HSPs can direct them toward the class II pathway of antigen presentation [53].

The studies by Srivastava and coworkers promoted our understanding on cancer immunosurveillance and provided valuable approaches to antitumor immunity [9,54]. Later, the internalization of the HSP-peptide complexes was shown to be mediated by HSP binding to a cell surface receptor CD91 [50,55]. Following the binding and subsequent endocytosis, HSP-peptide complexes undergo processing for MHC class I or class II presentation of the APC. The CD91 signaling in the APC triggered by HSP-peptide complexes induces cytokine secretion and costimulation of T cells in favor of Th1 responses and cytotoxicity against tumors [9,56]. gp96, Hsp70 and calreticulin (CRT) induce NF-κB activation though the CD91 signaling pathway and causes classical DCs (cDCs) upregulation of CD86 and CD40. This leads to the cytokine profile and the tyrosine phosphorylation site pattern in CD91 distinct among the HSPs. CRT, but not gp96 and Hsp70, primed Th17 cells in TGF-β secreting tumor environment [56].

Besides CD91, several molecules serve as receptors for HSPs, although the following list of HSPs/receptors is not exhaustive. Hsp70 binds to CD40 [40,57]. Hsp110 and Grp170 (both belong to Hsp100 family) bind to scavenger receptor class A [58]. Hsp60 and Hsp70 bind to LOX-1, a scavenger receptor originally identified as a receptor for oxidized LDL [59,60]. Hsp90 is internalized by SREC-I (scavenger receptor expressed by endothelial cells) [61,62] Hsp70 and Hsp90 are internalized by the mannose receptor [63,64].

Gp96 is an endoplasmic resident HSP and known for its high ability to assist re-presentation of peptides for T cell responses. Gp96 is internalized with the aid of CD91. The CD91 signaling pathways cause partial maturation conferring classical DCs (cDCs) the ability to prime Th1 and cytotoxic T cells responses [56]. Paradoxically, in the experiment of CTL response induction toward hepatitis B virus, 10-20 μg/mice of gp96 induced the highest CTL response, but the immunization dose of 50-100 μg induced compromised levels of CTL response [65]. Thus, strikingly, dose of HSPs is a critical factor and may become an important axis to control the balance between CTL activation and Treg activation. Importantly, Binder and coworkers observed an association between distinct populations of DCs with such dichotomous T cell responses (i.e., priming of regulatory *vs.* cytotoxic T cells) [66]. DCs can be classified into two groups: cDCs and plasmacytoid DCs (pDCs). Compared with cDCs, pDCs are known to express relatively low levels of costimulatory molecule CD80, and after maturation, can induce Th2 cells and promote differentiation of regulatory T cells from $CD8^+$ T cells, or of $Foxp3^+$ Treg from $CD4^+$ T cells in mucous, thereby exhibiting immunosuppressive property [67]. Introducing exogenous gp96 into the mice that had been vaccinated to acquire antitumor immunity, Kinner-Bibeau et al. showed that pDC and cDC showed distinct responses to low and high doses of gp96 through CD91. That is, the low dose gp96 stimulated cDC, which in turn primed cytolytic T cells, whereas the high dose gp96 stimulated pDC, which primed Treg cells. Their whole-genome sequencing of methylated DNA showed that, compared with the low dose gp96, the high dose gp96 upregulates neuropilin-1 in pDCs to enable the long term contact of pDCs with Treg cells. As the authors discuss, high dose gp96 could be necessary to diffuse and reach pDCs, as pDCs are normally reside within the T-cell zone of lymph nodes. Or alternatively, pDCs could be less sensitive to gp96, requiring higher dose for activation [66]. CD91 is known as α2–macrogobulin receptor, and of note, C1q and the collectins enhance the uptake of apoptotic cells by macrophages through interaction with CD91 [68].

Compared to mature DCs, immature DCs are considered to patrol the periphery and engulf large quantities of protein for antigen acquisition and preservation. Immature DCs have low levels of expression of CD80, CD83 and CD86 and have limited capacity of T cell stimulation, but have expression of

adhesion markers allowing iDCs to patrol and remain resident in the periphery. Immature DCs show greater levels of expression of pattern recognition receptors [69,70].

It is of interest to address the question whether such HSP-induced antitumor immune responses are evolutionarily conserved. In a series of studies using a tumor immunity model of African clawed frog *Xenopus laevis*, Robert and coworkers showed that immunization of *Xenopus* with gp96 and cytoplasmic Hsp70 can potentiate antitumor protective T-cell responses [71]. Such antitumor effects were dependent on HSP-tumor peptide complex and no effect was observed with gp96 from normal tissues [72,73]. In general, such antigenic peptides include peptides from tumors, virus-infected cells, and minor histocompatibility antigens. The authors initially observed that, between cloned frogs named LG15 and LG6 that have difference in minor histocompatibility antigen (named H-antigen), HSPs can promote generation of immunological memory to the minor H-antigen leading to accelerated rejection of skin graft. Of note, in their 2002 study, analyses on larvae (naturally class I-deficient but immunocompetent) also showed the effect of gp96 in antitumor immunity, raising the possibility that MHC class Ia-independent antigen presentation elicited by gp96. In support of this idea, in analyses using a *Xenopus* tumor cell line (named 15/0) that does not express class Ia, they showed that both NK cells and unconventional (class Ia-unrestricted) $CD8^+$ T cells can kill 15/0 cells. Thus, despite the absence of class Ia presentation, the HSP facilitated the antitumor immune responses [74]. They further showed that although Hsp73 is as potent as Hsp72 in eliciting class Ia-restricted T cells responses (skin graft rejection), it is less efficient than Hsp72 in inducing class-Ia unrestricted antitumor T-cell responses.

MHC class Ib can present diverse molecules including glycoconjugates [75]. Future studies may focus on non-protein molecules that can bind to HSPs. Intriguingly, Hsp90-CpG complexes showed an ability to enhance TLR9 stimulation compared to CpG [76]. In patients with SLE, Hsp90-self DNA complexes may be formed within the nucleus and then released into extracellular space [76]. However, structural details about this Hsp90-CpG complexes remain to be determined.

HSPs, extracellular matrix proteins and glycosaminoglycans

Particularly under certain pathological conditions or in response to cellular stress, a number of HSP members can be found in extracellular space, as a free soluble protein, or as a part of extracellular matrix (ECM) [7]. It is likely that HSPs, which lack an N-terminal hydrophobic signal sequence, employ several non-canonical secretory pathways, including secretory lysosomes [77]. Merendino et al. showed that tumor cells release Hsp60 and Hsp70, not due to cell death, but through an active secretion mechanism [78]. Their analysis using 5,5-(N-N-Dimetyl)-amiloride hydrochloride (DMA), an exosomal inhibitor, and methyl-β-cyclodextrin (MBC), a lipid-raft pathway inhibitor suggested the requirement of exosome and lipid raft for the secretion, although for Hsp70 the DMA inhibitory effect was rather limited. Of note, Hsp72 – the stress-inducible member of the Hsp70 family, in blood has been shown to increase following a bout of moderate-intensity exercise. Importantly, this increase was observed in the absence of any overt tissue damage and suggested that exercise stress may stimulate the active release of certain HSP from intracellular locales [79].

ECM consists of a fibrillar meshwork of proteins, proteoglycans and glycosaminoglycans (GAG) [80]. Maintenance and remodeling of ECM is important in wound healing [81,82] and inflammation [83]. Extracellular HSPs are considered important in regulation of ECM. The mechanisms for this regulation have much to do with cancer cell invasiveness. We here nominate a few examples showing HSP-ECM interactions. Hunter and colleagues showed that binding of extracellular Hsp90 to fibronectin promotes fibronectin network formation [84]. Sims et al. showed that Hsp90 forms complex with chaperones including Hsp70 and activates matrix metalloproteinase-2 (MMP2) [85,86]. It has well been documented that extracellular Hsp90α acts motility of fibroblasts and neurons as well as migration, invasion and metastasis of melanoma by a mechanism mediated by MMP-2 [85,86]. McCready et al. showed that Hsp90α is secreted from invasive cancer cells via exosome, and aids in the conversion of plasminogen to plasmin, activate the precursors of proteases and assist cancer cell migration [87]. In the case of Hsp70, although not much is known about the function in ECM, Ravindran et al. showed that Grp78 (glucose regulated protein 78) functions in mineralized matrix formation [88]. BiP (immunoglobulin-binding protein) interacts with integrins and urokinase plasminogen activator receptor (uPAR) at the cell surface of colorectal cancer cells. Li et al. proposed that, BiP, through facilitating degradation of ECM, assists cancer cell invasion [89]. With respect to Hsp40, a study by Lin et al. showed that a Hsp40 family member MRJ (DNAJB6) acts as an uPAR-specific adaptor that links uPAR to Hsp70. Hsp40 MRJ (DNAJB6), together with Hsp70, stabilizes uPAR, thereby supporting adhesion of uPAR–mediated cell to vitronectin and promoting cell migration [90]. Their data also showed that Hsp70 and MRJ act to increase upregulation of genes of MMPs. It is well known that uPAR–dependent cell adhesion to vitronectin in the ECM is important in wound healing and tissue remodeling [91].

GAGs have important roles to regulate many cellular activities. Heparan sulfate (HS) permits growth factor and modifies enzyme functions [92]. Chondroitin sulfate (CS) and dermatan sulfate (DS) also regulate growth factor activity and cell migration especially of immune cells. Hyaluronic acid (HA) also participates in immune cell recruitment [92]. Thus, GAGs bind to, enrich and enhance the efficacy of growth factors. Of interest, it is likely that extracellular dynamics of HSPs are regulated at least in part by GAGs. Harada et al. showed that Hsp70 directly interacts with acidic glycans with sulfated Gal and GlcNAc residues, such as heparin, HS and DS. In particular, Hsp70 forms a large complex with heparin [93]. Harada et al. also showed that sulfatide stabilizes Hsp70 oligomer and obtained a result suggesting that Hsp70-sulfatide interaction may fine-tune the dynamics, and specifically, the time length during which the peptide binding

domain stays open for unfolded protein binding [93]. These findings corroborate with Mamelak and Lingwood that showed sulfatide binding to Hsp70 and with sea urchin sperm-binding protein SBP (Hsp70 member) binding to disilalylated gangliosides in a sialic acid-dependent manner [94]. These also provide mechanistic insight into enrichment of Hsp70 on the surface of lipid rafts. HSP-lipid raft association is well documented by, for example, Chen et al. [95]. Disruption of lipid raft structure or HSP70-lipid raft interaction abrogated the HSP-mediated increase in phagocytosis in macrophages [96].

Importantly, DS may have unique and important role in binding and enriching the molecules from dead cells [97]. Wang et al. addressed a question of, among a large pool of self-molecules, why only a small subset of molecules (<1%) become targets in autoimmune diseases [98]. A proteome analysis by Wang et al. showed that DS binds to and increases concentrations (enriches) of autoantigens released from dead cells and such DS-autoantigen complexes selectively stimulate B-1a cells [99]. In physical association with DS were apoptotic cells, including small apoptotic bodies, and fragmented nuclear material and several member of the Hsp70, Hsp90 and Hsp60. Interestingly this B-cell-activating effects is specific to DS as other GAGs did not show significant B-cell proliferative effects. Spleen cells cultured with DS produced significant amounts of IgM specific for nuclear autoantigens including ssDNA, dsDNA and histones. The authors also used a more controllable system and showed that DS-biotin non-covalently complexed with streptavidin (SA) induced high levels of SA-specific IgG, but that DS does not have any adjuvant effect on responses to physically unassociated antigens. Given such B-cell-stimulating ability of DS, it seems relevant to ask how the tolerance towards such DS-binding molecules is maintained in healthy humans. Many molecules including HSPs are localized at DS, but autoantibodies against HSPs are normally maintained at low levels. This may reflect the B cell-suppressing effects of the regulatory T cells recognizing HSP epitopes.

It seems important to know how HSPs are distributed and what molecules are directly associated with HSPs in the extracellular milieu. Binding of HSPs to DS or, sulfated lipids may be of importance, as it will raise the local concentrations of HSPs. In the light that prokaryotes HSPs have high similarity, such spatial proximity might be a hidden factor that may be a key to the epitope spreading and molecular mimicry.

HSPs as self-antigens in autoimmunity

To prevent autoimmune diseases, T cells responses to self-antigens are controlled by central tolerance (negative thymic selection) and peripheral tolerance as well. In the thymus, Hsp70 epitopes are presented by DCs [100]. Such expression of HSPs in thymus may be assisting the central tolerance [101]. It is likely that, in the periphery and in particular tolerizing gut environment, HSP-recognizing T cells are maintained through recognition of cross-reactive microbiota HSPs [101].

In general, immunosuppression by regulatory T cells is an important mechanism that controls immune responses to self-antigens [102]. For example, Legoux et al. showed that $CD4^+$ T cell tolerance toward tissue-restricted self-antigens (i.e. antigens that cannot be presented in thymus) is mediated by antigen-specific regulatory T cells rather than deletion of such clones [103]. For HSPs, it is likely that central tolerance needs to be complemented by peripheral tolerance through recurrent exposure to HSPs epitopes, which reinforces the state of HSPs-specific T cells as an immunosuppressor. As early as 1991, using the word 'homunculus', Cohen proposed the idea that responses to a vested group of self-antigens is important for maintaining self-tolerance [104]. Partly due to continuous exposure to the components of gut bacteria, proteins that are evolutionary highly conserved, such as Hsp60, are likely to be considered as important regulator of immune responses. Aalberse et al. showed that Hsp60-specific self-reactive T cells are present at birth (cord blood), and stimulation with self-Hsp60 induced production of cytokines and Foxp3 expression [105]. They also showed that Hsp60 leads to the induction of IL-10 and IFN-γ, suggesting the upregulation of Tr1 cells.

HSP60-recognizing autoantibodies are found in healthy infants and adults [106]. Normal cord blood also contain autoantibodies against double-stranded DNA, which is known for the association with autoimmune diseases. The authors suggested that, while such autoantibodies become the basis for diseases in later life, such inborn autoimmunity to self-antigens may serve to protect against autoimmune disease.

It should be noted that not all immnogenic peptides from HSPs are immunosuppressive upon recognition by T cells. Despite the high evolutionary conservation, Hsp60 of infectious organisms have been shown to trigger or suppress autoimmune responses. As is known in general T cell biology, such dichotomous outcome is influenced by many factors involving the condition and the developmental state of APCs [70,107], the genotype of MHC molecules, and the exact sequence/structure of the presented peptide.

In the following we discuss only a few examples of the HSP epitopes that showed dichotomous effects. Important roles for both B cells and T cells have been shown in models of atherosclerosis [108,109]. Although a protective role of antibodies against, for example, oxidized LDL (oxLDL) has been shown in several mouse models, analyses using transfer of T cells generally suggested causative roles of T cells including Th1 $CD4^+$ T cells and $CD8^+$ T cells. Clonal expansion of T cells suggestive of antigen-specific reactions from lesions from humans and model mice has been reported [110,111]. Hsp60, as well as LDL, has been implicated in pathogenesis of atherosclerosis. In some setting, immunization promotes formation, but oral tolerization to Hsp60 leads to atheroprotective immunity. For example, Harats et al. showed that oral tolerance to mycobacterial Hsp65 (mbHsp65) attenuated atherogenesis in the LDL receptor-deficient mice [112]. Grundtman et al. identified and characterized novel atherogenic and atheroprotective mbHsp65 epitopes [113]. Another case for whole protein-based immunosuppression is an Hsp70 family member, BiP (immunoglobulin-binding protein) [114]. In the latter phase I/II trial, an intravenous injection of BiP in patients with RA

showed safety of BiP (⩽15 mg) and some patients had clinical and biological improvements in RA activity.

An epitope-dependent dichotomous outcome is seen in periodontitis-associated atherosclerosis. Infection with periodontitis pathogens is known to show a strong association with development of atherosclerosis. Among Hsp60 peptides from *Porphyromonas gingivalis* (Pg) a peptide named peptide 19 forms an epitope that has been consistently recognized in serum of patients with periodontitis-associated autoimmune diseases including atherosclerosis [115]. While immunization with another peptide (peptide 14) induced polarization to $CD4^+$ regulatory T cells cells, immunization with the peptide 19 caused polarization to pro-atherogenic Th1 cells [116]. Recently, Kwon et al. showed that high ability of peptide 19 to induce epitope spreading [117].

A well-studied example for Treg-inducing peptides is the conserved peptide named B29 derived from Hsp70 [118]. This peptide was found through MHC class II ligandome analysis. B29-induced $CD4^+CD25^+Foxp3^+$ T cells were shown to suppress established proteoglycan-induced arthritis (PGIA) in mice [118]. Nasal application of the peptide also suppressed PGIA in mice. Another example is Hsp60 peptide P277 (DiaPep277) [119] which showed better preservation of beta cells in phase II trial of type I diabetes [120]. We suggest review articles by van Eden and coworkers for more detail [121]. A recent discussion on HSPs as bystander antigens can be found in [122].

How about the responses of B cells toward HSPs? It is generally difficult to interpret the significance of the presence of anti-HSP antibodies in patients and models. The initiation of immune response to HSPs may be a reflection of a normal preventive immune reaction that ensures removal of dying and damaged cells [123]. Or alternatively, such response is a main cause for pathology of autoimmune diseases. Presence of antibodies to HSPs has been shown in many diseases [124], and anti-HSP antibodies themselves could play, to some extent, causative roles in most autoimmune diseases. However, it is also possible that this could represent the consequence of the ill-balanced T cell regulation, not representing the causative roles of anti-HSP antibodies. Several other technical issues are mentioned by Wu et al., including variability of results among laboratories, sensitivity and variance, non-specific referring [124]. Hsp60-recognizing autoantibodies are found in healthy infants and adults [106,125]. Nonetheless, as discussed in Quintana et al. [126], anti-Hsp60 antibodies likely play pathogenic roles in multiple sclerosis (MS) as shown by the finding that pattern II MS (antibody/complement-associated demyelination) but not pattern I multiple sclerosis (T cell/macrophage-mediated demyelination) was associated with high IgG to Hsp60.

Rheumatic arthritis (RA) is an autoimmune disease with multifactorial etiology. Several autoantibodies, which can vary among patients, likely play pathological roles and rituximab, a B cells-depleting anti-CD20 antibody has been successfully used to treat RA [127]. In particular, anti-citrullinated peptide antibodies (ACPAs) are considered to play pivotal roles in the pathogenesis of RA. However, subclinical synovial inflammation usually does not coincide with the appearance of serum autoantibodies during preclinical RA [128]. Autoimmune responses to some HSPs including BiP and mycobacterial (Myc) Hsp65 have been reported for a substantial number of RA cases (~30% for anti-BiP). Although we discuss here nothing but one recent paper, in Shoda et al. [129], both anti-MycHsp70 antibody and anti-human BiP antibody titers were higher in patients with RA than in healthy donors. This and previous reports showed no increase of Hsp60 in RA patients. Anti-MycHsp70 antibody titers showed clear correlation with anti-citrullinated BiP antibody titers. They further identified MycHsp70-287-306 as an immunogenic HLA-DR4 effector epitope in patients. Mouse immunization analysis showed that immune response to MycHsp70 caused loss of self-tolerance to BiP. Oral administration of the MycHsp70 287-306 peptide induced tolerance to MycHsp70 and ameliorated in collagen-induced arthritis (CIA) mouse model. This can be regarded as an example for molecular mimicry, but as the authors suggest, epitope spreading may also be involved. It is possible that inflammation triggered by MycHsp70-specific T cells may increase the expression of BiP leading to increased presentation of antigenic BiP peptides and activation of BiP-specific T cell. This study is suggestive of the association between subclinical exposure to mycobacteria and the state of tolerance towards self-HSPs. Although the role of autoantibodies to HSPs in the RA pathogenesis is difficult to define and, in some cases, they may exacerbate the prognosis, such findings rationalize the utilization of microbial HSPs and their peptides to maintain and expand regulatory T cells recognizing self-HSPs. This idea is supported by the finding that an injection of BiP in patients with RA induced showed clinical and biological improvements [114].

Conclusion and Perspectives

As Gammazza et al. discuss [130], the immune system evolved later with respect to the chaperoning system, and the chaperoning system and the immune system probably complemented each other and interacted to ensure organismal homeostasis and survival. As we have considered above, in the immune system, HSPs appear to generally exhibit anti-inflammatory and immunosuppressive effects, but modulate or enhance inflammatory signals and assist antigen-specific immune responses. To state simply, HSPs are doing a variety of jobs to protect the host organism.

Although we did not stress in the above, some researchers are utilizing HSPs as an adjuvant in vaccination. For example, a chimera protein tandem repeat of M2 proteins fused to Hsp70 showed that, relative to the tandem repeat of M2, the fusion showed elevated humoral and cellular immune responses and improved survival in influenza infection mouse model [131]. An Hsp60 peptide p458 has been exploited in the development of anti-microbial vaccines [22]. The utilization of HSPs as adjuvants may gain more future attention.

It is generally considered that central tolerance is not sufficient and peripheral tolerance checkpoints outside the thymus are necessary to secure self-tolerance [132]. As we have seen above, the innate and adaptive immune responses to extracellular HSPs appear to contribute to maintenance of

tolerogenic environment. Then one may ask whether HSPs as antigens can be useful in clinics to expand regulatory T cells. A notable concept in this area is the induction of tolerogenic DCs [101]. Recent findings include Mansilla et al. that showed that NF-kB activity-blocked DCs, when loaded with myelin oligodendrocyte glycoprotein autoantigen, can reduce disease by the induction of Treg in experimental autoimmune encephalomyelitis (EAE) mouse model [133]. At the forefront of antigen-specific approach, the nanoparticles (NPs)-based approaches are also currently explored as carriers of self-antigens and tolerogenic signals. Such approaches enable delivery targeted at DCs. To cite only one paper, Tostanoski et al. showed that intra-lymph node injection of polymer particles encapsulating myelin self-antigen and rapamycin (immunosuppressant) reversed paralysis and induced systemic expansion of regulatory T cells and reduction of T cell infiltration into the CNS in the EAE mice [134]. Rapamycin significantly potentiated the tolerance-inducing effect of self-antigen. As the authors discuss, further understanding of local reprogramming, effect of local controlled release.

Besides the immunological relevance, roles of HSPs in wound healing is an important area. For example, topical application of the full-length or the peptide named fragment-5 of Hsp90α accelerated closure of excision, burn, and diabetic wounds in animal models [135]. Bhatia et al. further used a mouse model that expresses truncated Hsp90α (Hsp90α-Δ) that lacks intracellular chaperoning ability and showed that selective inhibition of the extracellular Hsp90α-Δprotein function by a monoclonal antibody disrupted normal wound closure in mice model [136]. Future researches may promote our understanding of mechanisms of topical application effects of HSPs in wound healing.

Broad substrate specificity of HSPs has become discussed, including interactions between HSPs such as GroEL and RNA species [137]. However much remains poorly understood, including structural details of the Hsp90-DNA complexes. If such binding to non-protein substrates is enabled by hydrophobic interactions, which are generally weak, it would become of relevance to understand how GAG and other components of ECM influence the HSP binding to substrates. It would also be interesting to understand how the GAG-mediated enrichment of protein and non-protein molecules influence the HSP association with substrates.

In the above we considered the studies by Robert and coworkers that showed HSP-mediated antigen re-presentation by non-classical MHC, which is categorized as MHC class Ib [75,138]. Specific molecules re-presented by this HSP-mediated mechanism are unknown. MHC-like fold can bind non-peptides. For example, CD1 molecules, a well-studied category of class Ib, can recognize foreign glycoconjugates and present them for recognition by $CD4^-$ $CD8^-$ double negative and γδT cells. α-galacosylceramide-presenting CD1d stimulates natural killer T (NKT) cells carrying invariant T cell receptor [139]. These findings also lead us to the question of how HSPs can propagate non-protein molecules for re-presentation as antigens.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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***Corresponding author:** Kazuhisa Nishizawa, Teikyo University School of Medical Technology, Kaga, Itabashi, Tokyo, 173-8605 Japan, Tel: +81-3-3964-1211, Fax: +81-3-5944-3354; Email: kazunet@med.teikyo-u.ac.jp

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