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Heat shock proteins (HSPs) in the homeostasis of regulatory T cells (Tregs)

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Abstract

Heat shock proteins (HSPs) belong to the family of conservative polypeptides with a high homology of the primary structure. The uniqueness of this family lies in their ability to interact with a large number of different proteins and provide protection from cellular and environmental stress factors as molecular chaperones to keep protein homeostasis. While intracellular HSPs play a mainly protective role, extracellular or membrane-bound HSPs mediate immunological functions and immunomodulatory activity. In immune system are subsets of cells including regulatory T cells (Tregs) with suppressive functions. HSPs are implicated in the function of innate and adaptive immune systems, stimulate T lymphocyte proliferation and immunomodulatory functions, increase the effectiveness of cross-presentation of antigens, and induce the secretion of cytokines. HSPs are also important in the induction, proliferation, suppressive function, and cytokine production of Tregs, which are a subset of CD4$^+$ T cells maintaining peripheral tolerance. Together HSPs and Tregs are potential tools for future clinical interventions in autoimmune disease.

Key words: *heat shock proteins (HSPs), immune regulation, lymphocytes, regulatory T cells (Tregs).*

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Introduction

Heat shock proteins (HSPs) belong to the family of conservative polypeptides with a high homology of the primary structure in both *Procaryota* and *Eucaryota*. They are classified into five families according to their molecular weight: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), HSPD (HSP60/GroEL), and DNAJ (HSP40), as well as human chaperonin families HSPD/E (HSP60/HSP10) and CCT (TRiC), and small – HSPB [1]. HSPs are mainly intracellular, although they may also be secreted into extracellular space. The uniqueness of this family results from their ability to interact with a large number of different proteins, called substrates or clients. HSPs provide protection from cellular and environmental stress factors as molecular chaperones to keep protein homeostasis (proteostasis). HSPs constitute a high proportion (5-10%) of cellular proteins under steady state, and their intracellular concentration may rise even several times under stress conditions, when the formation of misfolded proteins or aggregates occurs. The stressors, such as heat shock, oxidation, viral infection, nitric oxide (NO), UV, ethanol, heavy metal ions, pro-inflammatory factors (TNF-α, IFN-γ), or non-steroidal anti-inflammatory drugs (e.g. ibuprofen), stimulate various HSPs, which inhibit or diminish the effects of these stress factors. In addition, HSPs play an essential role in physiological processes such as: folding of nascent and stress-accumulated protein-substrate assembly, prevention of the aggregation of these proteins, transport across membranes, and the degradation of other proteins [1-4]. While intracellular HSPs mainly play a protective role, extracellular or receptor-bound HSPs mediate immunological functions and immunomodulatory activity. This paper shows the role of HSPs in the functionality of the immune system, particularly regulatory T cells (Tregs).

Tregs constitute about 2-10% of peripheral CD4$^+$ T cells in healthy humans and are responsible for the maintenance of peripheral tolerance and suppression of exacerbated immune responses. Currently, based on their site of development, Tregs are divided into: 1) thymus derived Tregs, natural Tregs (nTregs), 2) peripherally induced pTregs, and 3) *in vitro* induced iTregs. tTregs and pTregs are characterised by expression of transcription factor Foxp3, which is called the master regulator of Tregs function and is necessary for their development and suppressive capabilities [5]. Tregs are characterised also by the constitutive expression of several other markers: the glucocorticoid-induced TNF receptor family-related protein (GITR) [6], OX40 (CD134) [7], and cytotoxic T lymphocyte antigen-4 CTLA4 (CD152) [8], but these markers are

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expressed also by other subsets. Basic suppressive mechanisms used by Tregs are:

1) secretion of anti-inflammatory cytokines such as: transforming growth factor beta (TGF-β), interleukin (IL) 10, and IL-35 [6],
2) killing of target cells in granzyme and perforin dependent fashion [9],
3) suppression of dendritic cell (DC) maturation,
4) induction of indoleamine 2,3-dioxygenase (IDO) expression in DCs in CTLA-4 (cytotoxic T lymphocyte antigen-4)-CD80/CD86-dependent fashion that suppresses both T helper (Th) and cytotoxic (Tc) conventional lymphocytes converting tryptophan into proapoptotic metabolites [8],
5) deprivation of other T cells of IL-2 due to high consumption of this cytokine (high expression of IL-2 receptor),
6) expression of CD39 and CD73 that are ecto-enzymes that dephosphorylate adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (CD39), and then into adenosine (CD73), leading to inhibition of the immune response [8, 10, 11], and
7) transfer of a potent inhibitory second messenger such as cyclic adenosine monophosphate (cAMP) via membrane gap junctions of effector T cells (Teffs) [8]. Interestingly, suppressive activity of Tregs is also controlled. For example, when effector T cells are stimulated via TCR receptor, IL-6, high doses of IL-2 and expression of GITR on Tregs surfaces render effectors resistant to Treg-mediated suppression. Tregs can be inhibited when activation of TLR4 and TLR9 on APCs occurs because it induces the secretion of inflammatory cytokines (as IL-6 and TNF-α) [12, 13].

Significance of heat shock proteins for the function and homeostasis of the immune system

HSPs are implicated in innate and adaptive immune systems. They can stimulate dendritic cells (DCs), NK cells, and macrophages [14]. Both autologous and recombinant HSPs stimulate T lymphocyte proliferation and immunomodulatory functions [15, 16]. HSPs increase the effectiveness of cross-presentation of antigens by APCs in the context of major histocompatibility complex class one (MHC I). HSP receptor CD91 is required in this process and increases T-cell-mediated responses related to Th1, Th2, and Tregs [17]. HSPs may induce the secretion of pro- or anti-inflammatory cytokines and are responsible for monitoring the immune response [18, 19]. Extracellular HSPs have cytokine-related properties necessary for immune response acting via the association with pattern recognition receptors (PRR) including toll-like receptors (TLRs) and CD14 [4, 20], and thus affect the release of cytokines such as TNF-α, IL-1β, IL-6, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Further increased levels of extracellular stress and located in the cell membrane protein HSPA induction correlates with apoptosis that precedes the activation of receptors on the surface of NK cells, and antigen presentation by GRP94/96, which initiates a response CTL/$CD8^+$ [15].

Small HSPs (HSPB)

sHSPs oligomers were found to protect cells from oxidative stress, heat, and inhibit apoptosis. One of these chaperonins is HSPB1 (HSP27), which was identified as a protein with high homology with the eye lens α-crystallin (HSPB4 and HSPB5) protein [2]. The phosphorylation of HSPB1 regulates its interaction with other proteins. In neutrophils, unphosphorylated HSPB1 creates complex with kinase AKT (protein kinase B) and MAPKAP (mitogen-activated protein kinase-activated protein) kinase 2 that prevents constitutive neutrophil apoptosis and promotes an inflammatory response. While after phosphorylation HSPB1 dissociates from AKT disrupting the signalling complex and promoting neutrophil apoptosis. HSPB1 also stimulates the production of IL-10 in monocytes and thus can suppress the immune response. Extracellular HSPB1 inhibits the differentiation of monocytes towards macrophages and dendritic cells and blocks their maturation. In addition, extracellular HSPB1 was found to induce T-cell anergy and to secrete anti-inflammatory mediators [21].

DNAJ (HSP40)

DNAJ is called a co-chaperone because it acts in a complex with HSP70. DNAJ was found to bind damaged polypeptides and then facilitate their binding with HSPA [22]. It has been shown that bacterial and human extracellular DNAJ inhibits proliferation of autoreactive T cells and induces the expression of IL-10 in peripheral blood mononuclear cells (PBMC) from patients with rheumatoid arthritis (RA) [23]. A clinical trial with a dnaJ peptide was done in RA as a pilot phase II. dnaJ was safe and well-tolerated. In this way significant reduction in the percentage of T cells producing TNF-α and increased percentage of T cells producing IL-10 were observed. This immunisation leads to immune deviation and clinical efficacy [24-28].

HSPD (HSP60)

HSPD seems to be a link between innate and adaptive immunity because it was shown to stimulate production of proinflammatory cytokines and other proteins involved in inflammation [23]. In culture of PBMC it was observed that CD14 receptor bounded HSPD and activated human PBMC, as well as monocyte-derived macrophages. It was also reported that HSPD protein acts via TLR2, TLR4, and CD40 inducing production of Th-1 cytokines and increasing adhesion to extracellular matrix. It was also shown that during cellular stress HSPD protein was presented to $CD8^+$

cells and $CD4^+$ cells, in the context of MHC class I and MHC class II receptors, respectively [24-27]. Mycobacterial HSPD (65-kD) activates monocytes to synthesise and secrete proinflammatory cytokines (IL-6, IL-8 and TNF) [28] and nitric oxide. HSPD also stimulates IL-6 [29], IL-12, and IL-15 production by antigen-presenting cells and together with IFN-γ activates macrophages. In addition, the protein was shown to enhance antigen-specific IFN-γ secretion and CD69 expression on $CD4^+$ T cells after primary stimulation [30].

Nevertheless, current observations suggest that the results of experiments where recombinant HSPs were used should be reconsidered. It was proven that production of recombinant HSPs in microbial expression systems is commonly associated with contamination with microbial products. Therefore, proinflammatory effects observed after HSP administration might have been mistakenly interpreted as biological action of HSP itself, while in fact they resulted from bacterial impurities that were also recognised by TLRs [31].

These problems deserves more attention, especially since current studies underline the immunoregulatory properties of HSPD. It has been shown that stimulation of human cord blood mononuclear cells (CBMC) with HSPD induced differentiation and proliferation of IL-10 and IFN-γ producing T CD4+ cells that expressed a Treg marker – FoxP3 phenotype. Nevertheless, it has been reported that proinflammatory activity could be caused by DAMPs (damage-associated molecular patterns) – molecules produced under stressful conditions. The effect of recombinant HSP was misinterpreted as pro-inflammatory. In fact, they were contaminated by proteins from the bacteria producing them [32].

HSPA (HSP70)

The HSPA subfamily comprises eight proteins that have the highest evolutionary stability and high affinity to adenosine triphosphate (ATP) [33]. HSPAs are present in the cytosol, nucleus, mitochondria, and endoplasmic reticulum. They can also be present in the intercellular space, where they are recognized and bound by different cell types, including NK cells, dendritic cells, macrophages, monocytes, and B cells using specific receptors (CD36, CD40) on all of these cells. Binding to these receptors, HSPA start a signal cascade leading to the synthesis and secretion of pro-inflammatory cytokines [34-36]. These proteins act also as messengers between innate and adaptive immunity [37-39]. HSPA proteins are capable of activating immune cells such as macrophages, monocytes, dendritic cells, natural killer (NK), and T cells. This activation is exerted by direct interaction of the HSPA protein with subsequent surface receptors TLR2, TLR4, CD14, CD40, and CD91. HSPA protein can also be recognised by the TCR receptors during presentation with MHC molecules [40, 41]. Bausero *et al*. have shown that many tumour cell types exhibit increased HSPA protein exosomal transport after treatment with IFN-γ. In this study, the serum of patients suffering from various cancer diseases and tumour cell cultures were found to contain high concentrations of HSPA proteins. Moreover, the results confirmed the finding that HSPA can be a membrane-bound protein in cancer cells, which was not observed in normal cells [42].

HSPC (HSP90)

So far, the best known representatives of HSPC are HSPC2 (HSP90α), HSPC3 (HSP90β), and HSPC4 (GRP94/96) [43]. Stress induces expression of HSPC2, and its level correlates positively with the tumour progression. HSPC3 is involved in the proper functioning of the cells under physiological conditions, is responsible for the development and maturation of embryos and stabilisation of the cytoskeleton [44]. While HSPAs are mainly expressed in the endoplasmic reticulum and mitochondria, HSPC family members are predominantly expressed in the cytosol and nucleus [45, 46].

HSPs in antigen presentation

Many studies have shown an active role of chaperones in the MHC class I pathway and also evidence that HSP-peptide complexes facilitate *in vivo* presentation of MHCII-restricted epitopes [47-50]. As was shown by Rajagopal *et al*., HSPC enhanced MHC class II-mediated antigen presentation because it was required for stable MHC class II heterodimer formation and persistence and for peptide loading on MHC class II. In case of MHC class I molecules the role of HSPC in antigen presentation depends on cell lineage [51].

HSPs in autoimmune diseases

HSPs are involved in autoimmune reactions, which results from the so-called molecular mimicry, a phenomenon associated with the occurrence of origin similarities between exo- and endogenous molecules. People infected with bacteria can develop an immune response against HSPs of these bacteria, which is then directed against human homologues of these proteins. A typical example is RA, which is an autoimmune, systemic disease of connective tissue and one of the most frequent rheumatic diseases [52]. The development of response to HSPs is considered an early event in the pathogenesis of the disease. The presence of HSPs in the extracellular space, and the presence of anti-HSPs antibodies, was observed in patients with RA [53-55]. Moreover, immunisation of rats with recombinant HSPD was found to protect against rheumatic disease [3, 56, 57]. T cells with regulatory functions primarily recognised HSPD epitopes in a highly conserved way. Interestingly, the transfer of these cells to the other animal

prone to rheumatic disease prevented the disease onset [28]. HSPA and HSPD have been shown to have immunomodulatory effects and stimulate anti-inflammatory Tregs when used to treat arthritis [2]. That is why there is an observed paradox in the influence of HSPs on the immune system: HSPs protect cells from inflammation (cytoprotection), but on the other hand – when the inflammation is established – they lead to cell death [58]. At this point the question arises whether the proinflammatory activity of HSPs is a part of their physiological role or is just an artefact resulting from bacterial contamination of recombinant HSPs used in the experiments *in vitro* [35].

HSPs in other diseases

Ischaemia/reperfusion-induced acute kidney injury is another condition in which HSPs play an important role. Kim *et al*. tested renoprotective effects of HSPs using a mouse model with or without heat preconditioning. When T cells from heat-preconditioned mice were adoptively transferred to T cell–deficient nu/nu mice, they failed to reconstitute post-ischaemic injury. It was also observed that the number of Tregs was increased in heat-preconditioned ischaemic kidneys. HSPA was found to have a renoprotective effect, which may be partially mediated by a direct immunomodulatory effect exerted by Tregs. When Tregs were depleted before heat preconditioning, then the renoprotective effect was abolished. Transferring Tregs in quercetin-treated (quercetin is an HSPA inhibitor) heat-preconditioned mice partially restored the effect of heat preconditioning [59].

Moreover, HSPs also play a role in atherosclerosis, which is a chronic inflammatory process ongoing in the arteries. More and more attention is being paid to the role of the immune system in the pathogenesis of atherosclerosis because $CD4^+$ T-cells and macrophages were found to be involved in its development. HSPs released from damaged cells during inflammatory response are particularly important autoantigens, which are assigned to the development of atherosclerotic plaques [60, 61]. In an animal model, the presence of specific HSP60 was demonstrated in the spleen and lymph nodes in both B and T lymphocytes, which was suggested to confirm autoimmune stimulation by HSP60 and indicate the role of HSP60 as a stimulator of the immune system in the development of atherosclerosis [62-65].

Diabetes type 1 (DM1) is a multifactorial autoimmune disease with a genetic background. Autoimmune reaction is directly responsible for the development of DM1. In type 1 diabetes the patient's immune cells destroy insulin-producing β cells of pancreatic islets [66, 67]. HSP60 is an autoantigen involved in type 1 diabetes pathogenesis, which was shown using non-obese diabetic (NOD) mice, and moreover they had autoreactive T cells specific to epitopes of HSPD, which were later identified as a β-cell target antigen [68, 69]. Therefore, an HSPD-based vaccine DiaPep277 was elaborated with the aim to protect β cells and prolong insulin secretion. The vaccine contained a dominant epitope of HSPD, present in secretory granules of β cells, which has been suggested to induce Tregs and enhance their regulatory function [70-72]. In animal studies, the peptide inhibited the destruction of β cells and preserved insulin production in NOD mice with newly diagnosed diabetes. A phase II clinical study showed that the group treated with the vaccine had a higher level of C-peptide and lower insulin requirements, suggesting a protective effect of the peptide DiaPep2077 on β-cells [73-75].

The role of heat shock proteins in the function of regulatory T cells

HSPs are necessary for the induction of T-cell phenotypes and are important in the induction, proliferation, suppressive function, and cytokine production of Tregs. HSPs induce regulatory T cells that have the capacity to suppress autoimmunity [76, 77].

DNAJ

Epitopes derived from human HSP40 can induce differentiation and/or stimulate cell proliferation of human Tregs. In addition, in patients with juvenile idiopathic arthritis (JIA), DNAJ was found to improve suppressive function of Tregs in culture. Moreover, this study has shown also that DNAJ stimulates T cells for the production of IL-10, and measured high serum levels of DNAJ correspond with milder course of the disease [76].

HSPD

These proteins act as co-stimulators of human Tregs and can downregulate adaptive immune responses by stimulation of Tregs via TLR2 signalling. This resulted in the inhibition of target T cell proliferation, IFN-γ, and TNF-α secretion, as well as upregulation of IL-10 in activated $CD4^+$ T cells. This co-stimulation led to activation of PKC, PI3K, and p38, and were further enhanced by inhibition of ERK [77]. HSPD enhanced regulatory T-cell suppression and proliferation via binding of TLR2 on the Treg surface [78, 79]. The level of CD30 expression in response to human HSPD correlated positively with the production of IL-10 and negatively with IFN-γ [80]. Immunomodulatory activity of HSPD manifests also as T-cell immobilisation through increased adhesion to fibronectin and reduced expression of chemokine receptors: CCR7 and CXCR4 [81]. HSPD also enhances the differentiation of CBMC into $CD4^+IL\text{-}10^+Foxp3^+$ Tregs [82].

HSPA

HSPA stimulates suppressive activity of Tregs [83]. Immunisation with HSPA increases IL-10 production by Tregs,

down-regulates production of inflammatory cytokines, and affects the permeability of the epithelial barrier [84, 85]. HSPA confers its activity via TLR4-signaling pathway, which may be important for Foxp3 induction and suppression of inflammatory reactions [86]. Nevertheless, it has been demonstrated that HSPA does not interact directly with Foxp3 transcription factor [87]. Animal studies have shown that oral, nasal, intraperitoneal, or intradermal administration of HSPA significantly inhibits the development of the autoimmune arthritic model [88-91]. It was suggested that suppression of autoimmune response in experimental animals (rats with adjuvant-induced arthritis) was mediated by increased expansion of Tregs specific for HSPA, and the secretion of anti-inflammatory IL-10 [89]. This also suggests the presence of antigen-specific Treg cells before immunisation, which are probably generated at the time of positive selection in the thymus. This does not preclude that also HSPA-specific Tregs were induced in response to bacterial infection and the presence of commensal bacteria [90]. Bacterial HSPA is a highly immunogenic protein with a similar linear and spatial structure to the mammalian HSPA. Therefore, T cells specific to bacterial HSPA seem to cross-react with human HSPA [52]. The immunosuppressive effect of Tregs specific to HSPA and activated in inflamed tissues was confirmed recently. It has been observed, namely, that B29 epitope of HSPA in conjunction with the murine MHC class II receptor was able to activate Tregs and led to clinical improvement in mice arthritis model [91].

HSPC

HSPC can be regulated by histone deacetylases (HDACs). HDAC6 causes decrease of Foxp3 expression via deacetylation of gene encoding this protein and incidentally Tregs function [92]. Moreover, HDAC6 or its downstream target, HSPC, can promote Treg-dependent suppression [93].

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