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REVIEW

Heat shock proteins: linking danger and pathogen recognition

Anke Osterloh · Minka Breloer

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Abstract Besides their central function in protein folding and transport within the cell, heat shock proteins (HSP) have been shown to modulate innate and adaptive immune response: (1) HSP mediate uptake and MHC presentation of HSP-associated peptides by antigen-presenting cells (APC). (2) HSP function as endogenous danger signals indicating cell stress and tissue damage to the immune system. (3) HSP bind pathogen-associated molecular pattern (PAMP) molecules and modulate PAMP-induced Toll-like receptor (TLR) signaling. Thus, HSP contribute to both, recognition of "danger" defined as uncontrolled tissue destruction and recognition of dangerous "nonself". In this review these different aspects of immune stimulation by HSP will be discussed.

Keywords HSP · PAMP · PRR · TLR · LPS

Introduction

Heat shock proteins (HSP) are higly conserved and ubiquitously expressed proteins that play an essential role as molecular chaperones in protein folding and transport within the cell [1–5]. HSP prevent apoptosis and cellular damage by inhibiting protein aggregation and expression of HSP is upregulated upon stress like heat [4], viral [6] or bacterial infection [7]. Apart from their intracellular chaperone activity, immunological funcions of HSP have been described. Initially, bacterial HSP, in particular Hsp60 and Hsp70, attracted attention as highly immunogenic molecules that activate T cells [8] and cross-reactivity with eukaryotic HSP has been implicated in autoimmune and inflammatory diseases [9]. Besides this well known immunogenicity of prokaryotic HSP, eukaryotic HSP have been shown to act as carriers of antigenic peptides. HSP/peptide complexes were shown to be internalized by APC such as macrophages and dendritic cells (DC) [10–12] via receptor-mediated endocytosis ([11], for a review see [13]), leading to major histocompatiblity class I (MHC I) presentation of the HSP-associated peptide antigens and the induction of cytotoxic T lymphocytes (CTL) (for a review see [14]).

In addition, HSP are discussed to function as endogenous danger signals. Thereby several HSP have been shown to activate APC by engagement of TLR [15–21]. Because the same signaling mechanisms function in pathogen-associated molecular pattern (PAMP) recognition, it was an obvious concern that some of the stimulatory effects of recombinant HSP that had been expressed in bacteria were due to PAMP contamination. Moreover, recent research provides evidence that HSP may participate in pathogen recognition by binding bacterial PAMP [22–26]. Therefore in this review both, a danger signal function and a function of HSP as modulators of PAMP signaling in immune stimulation will be discussed.

SNS, INS and the danger model of immune regulation: a short introduction

The self–nonself (SNS) theory of immunity

The widely accepted SNS model of immunity originally introduced by Burnet [27] states that an immune response is

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A. Osterloh (✉) · M. Breloer
Department of Immunology,
Bernhard-Nocht-Institute for Tropical Medicine,
20359 Hamburg, Germany
e-mail: osterloh@bni-hamburg.de

based on the discrimination of "self" and "nonself" antigens. It suggests that each lymphocyte expresses multiple copies of a unique receptor that specifically recognizes foreign antigen and initiates an immune response while self-reactive lymphoytes are deleted early in life. The SNS model was supported by Medawar et al., who showed that adult mice accept foreign skin grafts if they had been injected as babies with cells from the donors [28]. The SNS model, however, has undergone significant modifications because it is now known that recognition of "nonself" antigen alone is not sufficient to trigger T cell activation. T cell activation was proposed to depend on a second "costimulatory" signal [29], which is provided by professional antigen presenting cells (APC) such as dendritic cells (DC) [29, 30].

The infectious nonself (INS) theory of immunity

In 1989 Janeway argued that the innate immune system was the real gatekeeper that controls immune response and introduced the idea that immune response depends on discrimination between "infectious nonself" (e.g., bacteria) and "noninfectious self" by APC. He postulated that APC use ancient pattern recognition receptors (PRR) that recognize conserved structures of a pathogen, so called PAMP, and suggested that APC stay quiescent unless they get activated by PAMP/PRR interactions to produce costimulatory signals, process foreign antigens and present them to passing T cells [31]. The INS model of immune activation was supported by the discovery of evolutionary conserved Toll-like receptors (TLR). These receptors have been shown to recognize components of bacteria, fungi and viruses to activate APC and, thus, to function as PRR.

The danger theory of immunity

Both, the SNS and the INS model of immunity, do not explain several immunologial phenomenons such as autoimmunity and transplant rejection where "nonself" or "infectious nonself", respectively, is missing or the presence of pathogens without immune activation, e.g., bacteria in the gut. This question was addressed by Polly Matzinger who introduced the danger theory [32]. In contrast to Janeways concept of "infectious nonself" detection, the danger theory is based on the idea that an immune response is ultimately controlled by recognition of the damage induced by a pathogen rather than the pathogen itself. It can be viewed as an extension of immune signals by self molecules that act as endogenous danger signals. Danger signals are considered to be conserved, abundant and ubiquitously expressed self molecules that are normally hidden within the cell in healthy tissue but that can be released from abnormally distressed, infected, injured or necrotic cells. In the extracellular space danger signals are suspected to be recognized by APC via conserved receptors that mediate activation of APC to produce costimulatory signals allowing the initiation of adaptive immune response. In this way, a dangerous situation can be detected by the immune system to induce an effective immune response when it is necessary.

HSP as danger signals

Heat shock proteins, abundant and ubiquitously expressed proteins, came into focus to function as immunological danger signals because HSP expression is upregulated under various conditions of stress and HSP are released by stressed, infected, necrotic or tumor cells [33, 34]. Furthermore, cell surface expression of mitochondrial Hsp60 [35], cytosolic Hsp70 [35–37] and Hsp90 [35] has been described. Thus, HSP become accessible to cells of the immune system in the extracellular space in a dangerous situation. Indeed, several HSP have been shown to modulate innate immune response as summarized in Table 1.

Immune stimulation by Hsp60, Hsp70 and gp96 is thought to be mediated by members of the conserved TLR family. These receptors have initially been described to function as PRR that recognize conserved PAMP molecules. Exogenous PAMP ligands such as bacterial lipopolysaccharide (LPS), CpG-DNA or lipoproteins activate innate immune cells via the evolutionary ancient Toll/IL-1 receptor (TIR) signaling pathway. Signaling through TIR is transduced by sequential recruitment of the adapter molecule MyD88, the IL-1 receptor-associated kinase (IRAK), and the adapter molecule TRAF6. This signaling pathway finally leads to the activation of NFκB and the release of proinflammatory cytokines (for an overview see [38]).

HSP have not only been shown to induce the release of proinflammatory cytokines in murine and human APC (see Table 1) but to share TLR and intracellular signaling cascades with classical bacterial ligands. Hsp60, Hsp70 and gp96 were shown to induce the activation of NFκB [15, 18, 19, 21] and MyD88 and TRAF6 were found to be involved in HSP-mediated APC stimulation [15, 18, 19]. Finally, TLR2 and TLR4 were identified to be responsible for Hsp60 and Hsp70 as well as gp96-induced APC activation. Expression of TLR2 or TLR4 together with MD2, which is also part of the TLR4 receptor complex [38], in otherwise non-responsive human cell lines conferred responsiveness to Hsp60 and Hsp70 as indicated by activation of NFκB-controlled reporter genes [15, 18]. Moreover, APC derived from C3H/HeJ mice that express a nonfunctional mutant TLR4 receptor failed to respond to Hsp60 [16, 39], Hsp70 [18] and gp96 [21]. Thereby, the defective cytokine response to gp96 was even more pronounced in APC from

Table 1 Immune stimulatory properties ascribed to HSP

HSP	Cell type	Effect	Ref.
Hsp60	Monocytes/macrophages	NO, IL-6, IL-12 and TNFα production	[16, 53, 54]
	DC	IL-1β, IL-6, IL-12 and TNFα production, maturation	[39, 54]
	Vascular endothelium, smooth muscle cells	Upregulation of adhesion molecules, IL-6 production	[55]
	B cells	Proliferation, IL-6 and IL-10 production, upregulation of MHCII, CD69, CD40, CD86	[56]
		Proposed signaling receptors: TLR2, TLR4, CD14	[15–17]
Hsp70	Monocytes/macrophages	NO, IL-6, IL-12 and TNFα production	[54, 57]
	DC	Maturation; IL-1β, IL-6, IL-12 and TNFα production	[33, 54, 57, 58]
	NK	Proliferation and cytolytic activity	[59]
		Proposed signaling receptors: TLR2, TLR4, CD14	[18–20]
Hsp90	DC	Maturation; IL-1β, IL-6, IL-12 and TNFα production	[33]
Gp96	Monocytes/macrophages	Production of NO and IL-8	[57, 60]
	DC	Maturation; IL-1β, IL-6, IL-12 and TNFα production	[21, 33, 61]
		Proposed signaling receptors: TLR2, TLR4	[21]
Calreticulin	DC	Maturation; IL-1β, IL-6, IL-12 and TNFα production	[33]

C3H/HeJ/TLR2$^{-/-}$ mice indicating the involvement of TLR4 and TLR2 in gp96-mediated stimulation [21]. In addition, the glycosyl-phosphatidylinositol (GPI)-anchored TLR4 coreceptor CD14 was implicated in HSP-mediated immune stimulation because anti-CD14 antibody interfered with Hsp60-induced IL-6 production in human PBMC [17] and Hsp70-induced expression of IL-1β and IL-6 in CD14 transfected human astrocytoma cells [20]. These results strongly suggested that HSP represent potent endogenous immune stimulators that share signaling pathways with bacterial PAMP.

HSP versus bacterial TLR ligands

PAMP contamination of HSP

Stimulation of innate immune response by HSP, however, has been controversially discussed because recombinant HSP that had been expressed in *E. coli* were used. These protein preparations were likely to be contaminated with bacterial structures belonging to the group of PAMP and, therefore, an obvious major concern was that these contaminants rather than the HSP themselves were responsible for the observed effects. Thereby, special attention was paid to LPS, the bacterial ligand of the TLR4/CD14 receptor complex [38, 40], which has been proposed to be involved in HSP signaling.

To address this question, HSP preparations were purified from LPS by binding to LPS-specific polymyxine B (PmB) columns and LPS content was quantified in HSP preparations using the limulus amoebocyte lysate (LAL) assay. Nevertheless, purification of HSP from LPS was incomplete and, therefore, controls were introduced to in vitro stimulation experiments to discriminate between LPS- and HSP-induced effects. One of these was the inhibition of LPS with the LPS-specific inhibitor PmB, and the other was the heat sensitivity of HSP versus heat insensitivity of LPS. However, these controls were not sufficient to completely exclude the contribution of LPS to HSP-mediated stimulation because low amounts of LPS revealed to be heat sensitive and inhibition of LPS by PmB was not complete [41]. In fact, it has recently been shown that virtually LPS-free Hsp60 and Hsp70 did not stimulate production of TNFα, IL-12 and many other cytokines in macrophage cell lines [42, 43], spleen cell cultures [44] and DC [45]. Bacterial endotoxin was also shown to be in large part responsible for NFκB activation and NO production in macrophages induced by recombinant gp96 and calreticulin [46]. These observations indicate that many of the proinflammatory responses induced by HSP were due to contamination of the recombinant HSP preparations with bacterial endotoxin and maybe other PAMP that are not addressed by the previously mentioned controls.

Immune stimulation by PAMP-free HSP

To circumvent major PAMP contaminations, HSP purified from eukaryotic tissue were used in a few studies. For example, Hsp70, Hsp90 and gp96 purified from liver were shown to induce the maturation of DC and to activate cytokine release in macrophages [33, 47]. However, a better insight into a possible function of HSP as endogenous danger signals was obtained by employing transgenic eukaryotic cell

lines that overexpress HSP or express HSP as membrane-bound cell surface proteins. Using such systems HSP were shown to modulate immune cell functions in the absence of bacterial PAMP. Cell surface HSP including Hsp60, Hsp70, Hsp90 and gp96 were shown to induce the maturation of APC and the production of cytokines by APC leading to enhanced NK and T cell activation in vitro (for an overview see Table 2). While induction of cytokines like IL-12 and TNFα in APC by recombinant HSP was attributed to endotoxin contamination of the HSP preparations [42–45], Hsp70 and gp96 expressed on the cell surface of eukaryotic cells were shown to induce DC maturation and IL-12 and TNFα release in DC [48–50]. On the other hand, cell surface Hsp60 did not stimulate production of these cytokines but induced IFNα expression in APC, which was found to be important for enhanced T cell activation by PAMP-free Hsp60 [22, 51]. Taken together, these observations indicate an immunostimulatory potential and, thus, danger signal function of HSP independent of bacterial PAMP (Table 3).

HSP: enhancers of pathogen recognition

HSP bind PAMP and associate with PRR

Apart from a potential function as danger signals, accumulating evidence suggests that at least some HSP are able to bind PAMP molecules and to modulate PAMP-induced stimulation. The first hint that HSP interact with bacterial PAMP was obtained in 1999 when Byrd and colleagues found that members of the Hsp70 and Hsp90 family specifically bound LPS [23]. Soon after, human Hsp60 as well as murine Hsp60 expressed on the cell surface of eukaryotic cells were shown to specifically bind ^{3}H-labeled LPS [22, 26]. Thereby, binding of LPS by Hsp60 was saturable and competable by unlabeled ligand [22, 26]. A Hsp60 epitope responsible for LPS-binding was identified [26] and specific antibody against this epitope was able to inhibit LPS-binding [22, 26]. Recently, while purifying gp96, Warger and colleagues observed that gp96 containing protein fractions always contained much more LPS than gp96-negative protein fractions indicating an LPS-binding function of gp96 that they could ascribe to the N-terminal domain of gp96 [52]. These observations show that some HSP are able to specifically interact with PAMP molecules, especially with LPS.

Moreover, HSP have been shown to associate with PRR. After binding of LPS to the cell surface, Hsp70 and Hsp90 are localized in a receptor cluster in lipid rafts, including chemokine receptor 4 (CCR4), growth differentiation factor 5 (GDF5), TLR4 and CD14 [24, 25]. These observations indicated that LPS recognition is based on the recruitment of multiple signaling molecules including CD14, TLR and HSP in lipid rafts. In addition, extracellular Hsp60 was shown to bind to distinct areas on the cell surface of macrophages and DC where it colocalizes with CD14 [22]. Thus,

Table 2 Immune stimulation by PAMP-free HSP

HSP	Method	Effect	Ref.
Hsp60	Cell surface expression on murine X63 B cells and monkey COS1 kidney cells	IFNα expression in APC; enhanced IFNγ production in T cell activation in vitro independent of TLR4 and IL-12 but dependent on IFNα	[22, 51] and unpublished results
Hsp70	Purified from liver	IL-1β, TNFα and IL-12 release in macrophages; maturation of DC	[33, 47]
	Overexpression in MCA26 colon cancer and EG7 T cell lymphoma cells	DC maturation after phagocytosis of Hsp70 positive apoptotic tumor cells	[62]
	Cell surface expression on murine P815 mastoma cells	Enhanced NK cell activity and CTL induction in vivo; enhanced tumor rejection	[63]
	Cell surface of human CX colon carcinoma cells	Enhanced NK cell activity	[59, 64]
	Cell surface rexpression of Hsp70 on B16 melanoma and CMT93 colorectal tumor cells	Enhanced expression of Il-2, IFNγ, IL-12p40, TNFα and GM-CSF in Hsp70 expressing tumors in vivo; enhanced tumor rejection	[48, 65]
Hsp90	Purified from liver	IL-1β, TNFα and IL-12 release in macrophages; maturation of DC	[33]
Gp96	Purified from liver	IL-1β, TNFα and IL-12 release in macrophages; maturation of DC	[33, 47]
	Cell surface expression on murine MethA fibrosarcoma and CT-26 colon carcinoma cells	maturation of wt DCs cocultured with gp96 expressing cells in vitro (upregulation of MHC I and II, CD80, CD86 and CD40; secretion of IL-1, IL-12 and MCP-1); enhanced tumor rejection	[49]
	Cell surface expression in transgenic mouse	MyD88-dependent secretion of IL-1, IL-12 and RANTES in wt DC coincubated with gp96 transgenic cells in vitro; development of severe glomerulonephritis and systemic autoimmune disease due to enhanced IL-12 release by gp96 transgenic DC	[50]

Table 3 HSP in PAMP recognition

HSP	PAMP	Observation	Ref.
Hsp60	LPS	Hsp60 binds LPS and enhances LPS-stimulated TNFα production in macrophages	[26]
	LPS	HSP60 potentiates LPS-stimulated TNFα production in macrophages	[7]
	LPS	Hsp60 binds LPS, potentiates LPS-stimulated IL-12 production in macrophages and IFNγ release in antigen-dependent T cell activation; colocalization of Hsp60 with CD14 after binding to the cell surface of APC	[22]
Hsp70	LPS	Hsp70 binds LPS	[23]
	LPS	HSP70 potentiates LPS-stimulated TNFα production in macrophages	[7]
	LPS	Colocalization of Hsp70 and Hsp90 with CD14 and TLR4 in lipid rafts after LPS binding; anti-Hsp70 and anti-Hsp90 antibodies inhibit LPS-induced cytokine production in macrophages and monocytes	[24, 25]
Hsp90	LPS	Hsp90 binds LPS	[23]
	LPS	Colocalization of Hsp70 and Hsp90 with CD14 and TLR4 in lipid rafts after LPS binding; anti-Hsp70 and anti-Hsp90 antibodies inhibit LPS-induced cytokine production in macrophages and monocytes	[24, 25]
Gp96	LPS	gp96 enhances LPS-mediated IL-12 and IL-6 production in bmDC in a TLR4-dependent manner	[52]
	Pam_3Cys	gp96 enhances Pam_3Cys-mediated IL-12 and IL-6 production in bmDC in a TLR2-dependent manner	[52]

different HSP localize to membrane regions, where PAMP signaling receptors concentrate suggesting that HSP may interact with these receptors and thereby influence PAMP signaling.

Modulation of PAMP-signaling by HSP

Inspired by the observation that HSP interact with PAMP molecules, especially LPS, the functional relevance of this finding was analyzed. Triantafilou and colleagues showed that antibodies against Hsp70 and Hsp90 inhibited LPS-induced IL-6 production in MonoMac6 cells and human monocytes and proposed that Hsp70 and Hsp90 function as LPS receptor molecules mediating LPS-induced activation [24, 25]. Furthermore, addition of recombinant Hsp70 and Hsp60 was shown to enhance LPS-induced TNFα release in macrophages [7]. Recombinant human Hsp60 as well as murine cell surface Hsp60 enhanced LPS-induced IL-12p40 production in peritoneal macrophages and IFNγ release in antigen-dependent T cell activation in vitro in a synergistic manner [22]. These effects required pre-incubation of Hsp60 and LPS and, thus, complex formation of both molecules. In addition, synergistic stimulation by Hsp60 and LPS could be inhibited by specific antibody that interferes with LPS-binding by Hsp60 [22, 26]. Moreover, stimulation of bone marrow derived DC with gp96 and LPS revealed synergistic enhancement of CD86 and CD40 expression and IL-6 and IL-12 release which could be restricted to the N-terminal domain of gp96. This activity was lost when DC from TLR4-deficient mice were used and therefore dependent on TLR4. Moreover, the authors show that gp96 also synergistically enhances stimulation by another TLR agonist, Pam_3Cys, that acts on TLR2. Again, pre-incubation and complex formation of the HSP with these TLR ligands was needed to induce synergistic stimulation. Finally, the authors showed that bmDC that were stimulated with gp96/LPS or gp96/Pam_3Cys complexes were superior to DC stimulated with the TLR ligands or gp96 alone to induce IFNγ producing CTL [52]. Taken together, these observations indicate a function of HSP in pathogen recognition by binding bacterial PAMP and enhancing PAMP signaling in APC (Fig. 1).

Concluding remarks

Intensive study of immune functions of HSP within the last years clearly demonstrated that HSP possess immune stimulatory potential. It is well established that HSP participate in the initiation of adaptive immune response by chaperoning peptide antigens for cross-presentation. Moreover, it is now emerging that HSP bind bacterial endotoxins and modulate PAMP-induced stimulation while, on the other hand, there are several hints arguing for an intrinsic immune stimulatory function of HSP that cannot be ascribed to bacterial contaminants. Therefore, we believe that HSP function in different ways connecting “danger” and pathogen recognition as depicted in Fig. 2: (1) HSP that are released by stressed, necrotic or infected cells in bacterial infection get in contact with bacterial structures. HSP bind bacterial PAMP and enhance PAMP-induced TLR signaling. In this situation HSP may facilitate the binding of PAMP to TLR

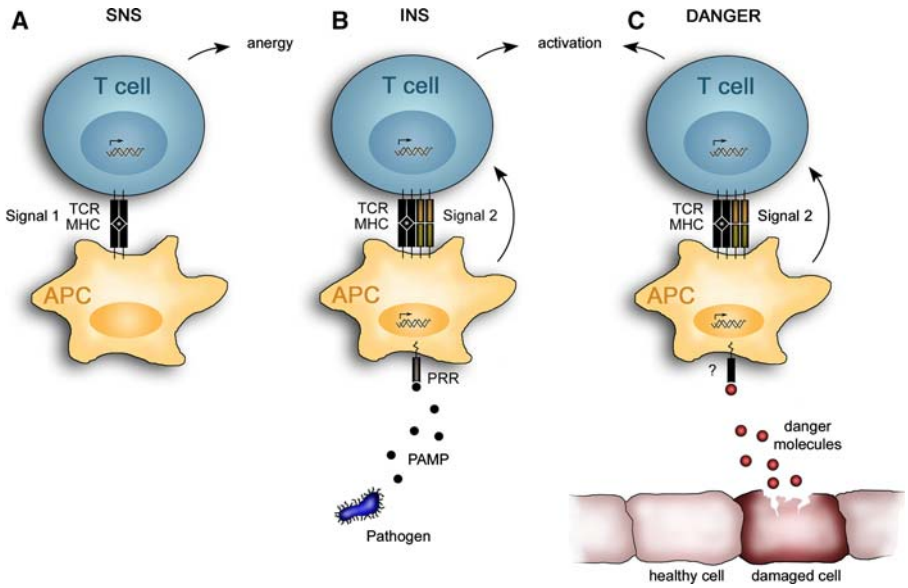

Fig. 1 The SNS, INS and danger theory of immune stimulation. **a** The SNS model of immunity suggests that adaptive immunity depends on the discrimination of "self" and "nonself". Foreign or "nonself" antigen is recognized by lymhpocytes via specific receptors (signal 1) leading to proliferation and clonal expansion of the lymphocyte. T cell activation, however, depends on a second costimulatory signal, which is provided by professional APC. In the absence of signal 2, T cells get anerg. **b** The INS model states that APC express a set of germline encoded receptors, so called pathogen recognition receptors (*PRR*) that allow APC to discriminate between "infectious-nonself" and "noninfectious-self". Recognition of conserved pathogen-associated molecular pattern (*PAMP*) activates the APC to upregulate costimulatory signals, to process antigen and present them to T cells. **c** The danger model suggests that immune activation is initiated by the damage a pathogen induces rather than the pathogen itself. Necrotic, infected or damaged cells release self molecules, so-called danger signals, that activate APC to provide costimulatory signals for the initiation of adaptive immune response. Thus, danger signals indicate a dangerous situation and allow the initiation of immune response when it is necessary

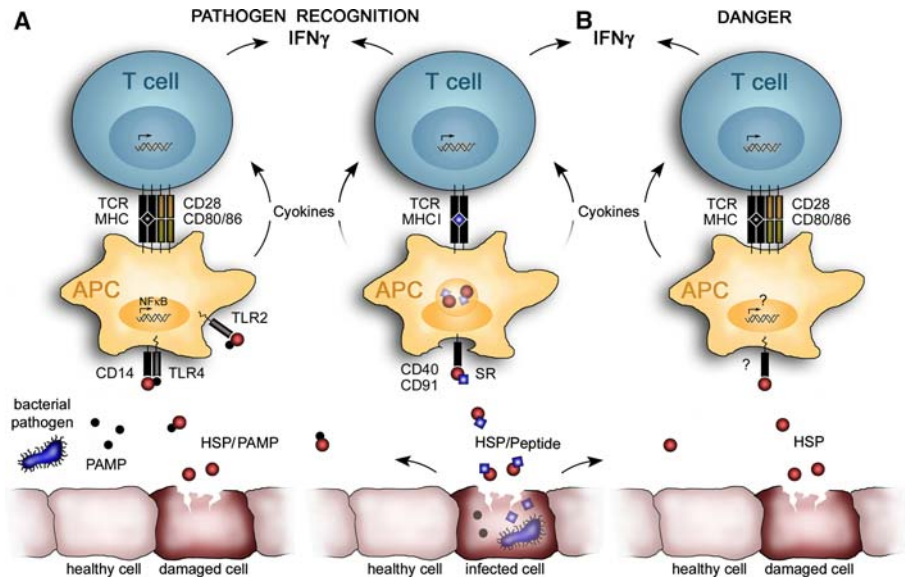

Fig. 2 Immune stimulation by HSP: Linking danger and pathogen recognition. **a** In bacterial infection HSP that are released by stressed, infected and/or necrotic cells bind bacterial PAMP and enhance PAMP-induced TLR signaling. Activation of APC leads to an upregulation of costimulatory CD80/CD86 molecules, the release of proinflammatory cytokines like IL-12 and enhanced T cell activation. Second, HSP that are released from bacteria or virus infected cells are complexed to foreign antigenic peptides. HSP/peptide complexes are taken up by APC via specific receptors (e.g., CD40, CD91, scavenger receptors (SR)) leading to MHC I presentation of HSP-associated peptides and to the induction of specific $CD8^+$ cytotoxic T cells. **b** In the absence of pathogen in sterile injury, HSP that are released by damaged cells function as classical endogenous danger signals. Thereby, recognition of PAMP-free HSP by APC might not involve TLR and not lead to the release of TLR-inducible cytokines like IL-12 but other mediators, for example type I interferons. Both, activation of APC by complexed HSP/PAMP or HSP alone, may contribute to the induction of T cell responses specific for HSP-associated antigens

and, thus, pathogen recognition by lowering the threshold of PAMP detection. Such function of HSP would explain the involvement of TLR and the production of TLR-inducible cytokines like IL-12 in HSP-mediated stimulation even when using HSP preparations containing very low amounts of PAMP that are not sufficient to stimulate these effects on their own. Second, HSP that are released by bacteria or virus-infected cells are complexed to pathogen-derived peptide antigens. These HSP/peptide complexes are taken up by APC via receptor-mediated endocytosis leading to MHC I presentation of HSP-associated peptides. (2) Under sterile conditions HSP function as classical danger signals indicating unplanned tissue destruction to the innate immune system. Stimulation of immune response by HSP in the absence of bacterial PAMP might not involve TLR and TLR-inducible cytokines like IL-12 but yet unkown receptors and mediators, for example type I interferons. Immune stimulation by HSP/PAMP complexes as well as HSP alone may contribute to the induction of adaptive immune response initiated against HSP-chaperoned peptides derived from bacteria- or virus-infected cells as well as necrotic tumor cells.

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