JLB

Editorial

Editorial: **Heat shock proteins: Darwinistic immune modulation on dangerous grounds**

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RECEIVED APRIL 24, 2010; REVISED MAY 25, 2010; ACCEPTED MAY 29, 2010. DOI: 10.1189/jlb.0410236

▶ SEE CORRESPONDING ARTICLE ON PAGE 445

Introduction

HSPs are ubiquitously expressed intracellular molecular chaperones and protein-folding catalysts. They are often referred to as "cell stress proteins", as they are up-regulated in response to a variety of physiological and environmental insults and allow the cells to survive stress conditions by protecting protein substrates. HSPs are highly conserved in evolution, leading to striking similarities in structure and composition between mammalian HSPs and their homologues in microorganisms and even plants. Next to this remarkable conservation—HSP70 is the most conserved protein ever described—HSPs are dominant targets of adaptive immunity [1], as during an infection, the initial immune response targets HSPs of the invading microorganism. This triad of these properties—evolutionary conservation, immune dominance, and up-regulation during cell stress—is at first sight dangerous, especially when taking into account that HSPs may also trigger innate immune responses directly. As a consequence, in theory, any bacterial infection with tissue damage can put the host at risk of developing autoimmunity through antigenic mimicry, which again seems illogical given the evolutionary conservation of HSPs [2]. This conundrum has led to intense scientific dispute over the last decades, especially about the role of HSPs as extracellular signaling molecules. The debate is clouded by the possibility that contamination with endotoxin has influenced some of the data that underlie the concept of HSPs as molecular regulators of immunity. The *Journal of Leukocyte Biology* has aired both sides of the discussion, first, by publishing a 2009 review that proposed that the in vitro-observed, cytokine-like functions of HSPs are a result of molecules bound to or chaperoned by HSPs but not a direct effect of HSPs themselves [3]. The review of Henderson and Pockley [4] in this issue of the *Journal of Leukocyte Biology* completes this cycle. They give an extensive overview of compelling scientific evidence that HSPs, up-regulated intracellularly during cell stress and released in the extracellular space, act as pleiotropic signaling molecules for a variety of cells, especially cells from the immune system.

So why is this issue so heavily debated, and what are directions for resolution of this topic?

HSPS

As mentioned above, microbial HSPs are dominant immunogens in infections, and thus, because of the strong homology between species in the early 1900s of the last century, HSPs were proposed as a potential cause of autoimmunity through antigen mimicry. This concept, however, implied a built-in mistake of evolution, which obviously is highly unlikely. Indeed, although adaptive immunity toward self- and microbial HSPs is associated with a variety of autoimmune diseases, immunity to HSPs is protective rather than autoimmune-inducing [2]. For example, in the experimental model of adjuvant arthritis, the induction of self-HSP60-specific T cells protects against arthritis. Also, in patients with juvenile idiopathic arthritis, the presence of (self) HSP60-reactive T cells is associated with a favorable prognosis [5]. Also, these data were disputed initially because of potential endotoxin contamination but could be confirmed in later studies, which included precautions to avoid such contaminations (see below) [6]. This massive amount of work has led to successful proof of principle clinical trials with HSP peptides in type I diabetes and rheumatoid arthritis [7, 8].

Besides being major antigens recognized by the adaptive immune system of the host, microbial HSPs have been shown to activate macrophages and other innate immune cells directly. The innate immune system is able to recognize conserved motifs of microbial origin or PAMPs through membrane and cytosolic PRRs such as TLRs. Bacterial HSPs are thought to activate innate cells through PRRs, including TLR2, TLR4, and CD14. Although there is still con-

Abbreviations: DAMP=damage-associated molecular patterns, HSP=heat-shock protein

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troversy about the way microbial HSPs activate the immune system, the most intense debate is focused on endogenous or "self" HSPs and their role in immune activation.

THE DEBATE

The key issue in this debate is the allegation that the PAMP-like effects on innate immune cells can be explained by endotoxin contamination of the HSP preparations used in experiments [3]. In **Fig. 1**, a summary of the discussion is depicted. This discussion has been especially passionate with regard to the role of endogenous HSPs as DAMPs. Self-HSPs have been shown to bind and activate, among others, TLR4 and TLR2, but technical concerns argued against a direct role for self-stress proteins in activation of innate immune cells trough PRRs, the most common one being contamination of recombinant proteins with traces of microbial ligands. Indeed, most recombinant proteins, especially when they are expressed in *Escherichia coli*, will contain LPS or other PAMPs, unless specific precautions are taken. It is undeniable that contamination is an important reason for some of the inconsistencies observed in literature. As both sides of the discussion recently have been reviewed extensively [3, 4, 9] (see Fig. 1), we will refrain from discussing this in detail here. For now, it is sufficient to mention that straightforward solutions proposed by others and us should be sufficient to avoid major experimental flaws. It has to be stressed that such experimental approaches to avoid contamination are (and should) by now routine procedures in most laboratories working with these proteins [10]. All of these measures and more have been undertaken and show that endotoxin-free HSP preparations are capable stimulants of innate immunity, although specific receptors and signaling pathways remain to be identified. Indeed, activation of innate immunity and induction of a protective, proinflamma-

Are endogenous or "self" HSP modulators of the innate immune system?

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• HSP (over)-expressed on the cell surface induce maturation of and cytokine production by APC. • HSP purified from eukaryotic cells were shown to induce DC maturation and macrophage activation • HSP-peptide complexes released extracellularly or administered as a vaccine enhance cross-presentation by APC • Human T cell adhesion responses via TLR2 can be 1000-fold more sensitive to mammalian HSP60 than they are to LPS. • Mouse B cell responses via TLR4 can be produced by mammalian HSP60, but not by microbial HSP • Exosomal host Hsp70 induces a pro-inflammatory response in response to infection • Endotoxin-free recombinant human Hsp72 produced in insect cells induces potent calcium flux in human monocytic cell line • Endogenous HSP seem to have anti-inflammatory effects • Some effects of mammalian HSPs on TLRs of B cells and T cells are not induced by bacterial HSPs • Synthetic peptide epitopes of HSP molecules are known to function as ligands for TLRs	• HSP of various molecular masses share similar cytokine effects • The PRR involved and reported effects are similar to those of PAMP such as LPS. Most recombinant HSP is produced by bacteria, which makes contamination with bacterial products likely • Due to their hydrophobic nature HSP easily complex with other molecules including PAMP • Unclear how TLR would distinguish HSP from other proteins • Using highly purified eukaryotic HSP several papers demonstrate that previously reported cytokine effects were due to contaminating bacterial products including LPS and flagellin. • Gene expression arrays showed no effect of highly purified HSP on 96 common cytokine genes in macrophages or lymphocytes. • Purified human HSP70 does not bind to cells that have been stably induced to express CD14, CD40, TLR2 and TLR 4 contradicting previous reports that human HSP70 does bind these receptors on human monocytes.
Experimental difficulties and shortcomings • Failure to use highly purified HSP • Difficulties in identifying the precise origin and nature of the preparations used • Failure to consider other contaminants than LPS • Failure to recognize that the cytokine-inducing effect of LPS is heat sensitive (boiling is often used as a control to show that the observed effects are not due to LPS contamination).	

Figure 1. Are endogenous or self-HSPs modulators of the innate immune system? Overview of the pros and cons, as summarized in refs. [3, 4, 9].

tory response by microbial HSPs, which are always accompanied by or may even be associated with a range of other microbe-associated danger signals, may not be surprising. However, why would abundantly present self-HSPs activate the innate immune system and trigger inflammatory processes?

THE DANGER FROM WITHIN

When discussing the role of HSPs in the context of stress, innate immune activation, and inflammation, it is important to realize that inflammation is a physiological process that is activated by injury and in general, leads to healing. Well-regulated and timed inflammation maintains and restores tissue homeostasis upon tissue damage, and HSPs may play an important role in inducing these processes.

A typical inflammatory response consists of four phases: induction, sensing, release of mediators, and the target tissue response. The danger model, as first postulated by Matzinger [11], suggested that the innate immune system senses and responds to endogenous danger/alarm signals (DAMPS), actively or passively released by damaged or stressed cells. Five years ago, Oppenheim and Yang [12] introduced the term alarmins for such endogenous danger signals. Alarmins can be produced by a variety of host cells. They are part of the first line host defense, as they have important activating effects on inate and adaptive immunity [13]. Notably, they are also suggested to promote the restoration of host homeostasis. In homology, with exogenous PAMPs, they are thought to interact with PRRs. The stressors that can induce extracellular release of HSPs include TLR ligands, including LPS and cytokines. From that perspective, HSPs participate in host responses to danger signals and behave more like alarmins than like danger signals.

The identity and characteristics of these alarmins are not yet well defined and can vary greatly depending on the type of cell or injured tissues (reviewed by Bianchi [13] and Medzhitov [14]). Although the list of endogenous inducers of inflammation is growing, the scientific literature on this subject contains discrepancies. One fundamental issue is the apparent promiscuity of TLRs. How would TLR recognize PAMPs and structurally unrelated, endogenous DAMPs? Are the triggered signals expected to be equivalent?

AN ANCIENT SYSTEM OF ACTIVATION AND MODULATION

So, is the extracellular activity of stress proteins a mystery that cannot be solved? In this respect, it is helpful to reconsider the known biological features and functions of HSPs. As molecular chaperones, they are abundantly present intracellularly, and any kind of cell stress leads to a clear up-regulation of HSPs and release in the extracllular space. Moreover, there is evidence that endogenous HSPs are ligands for TLRs on T cells and B cells and directly affect the functional behavior of these cells. This is underscored by studies that show direct effects of endogenous HSPs on TLRs of B cells and T cells that are not produced by bacterial HSPs, arguing against bacterial contamination [15].

As HSPs are highly conserved, it is likely that they are part of an almost ancient evolutionary system that is set to help the body respond to injury. The question is now whether it is likely that a HSP released into the extracellular space merely acts as an extracellular chaperone or that it can activate other immune cells directly by itself. This may be a purely theoretical discussion, as in vivo, these chaperones may couple to as-yet unidentified partners, accounting for their stimulatory effects.

Thus, the evolutionary conservation, their omnipresence, and the up-regulation during stress and binding to PRRs make it sensible that stress proteins can act directly as danger signals for the immune system. In addition, HSPs are highly hydrophobic, and hydrophobicity has been proposed as an ancient alarmin that initiates immune responses [16]. An interesting issue pointed out by the Henderson and Pockley review [4] is that some HSPs are proinflammatory, and others suppress inflammatory immune responses. Although this difference may also be tissue- and trigger-dependent, both types presumably aim to restore homeostasis. Thus, in theory, HSPs are perfect candidates to act as alarmins and modulators of the immune system. The challenge is to gather solid evidence by addressing some crucial fundamental issues. For example, in contrast to other DAMPs, such as high mobility group protein 1 and ATP, the exact receptors for HSPs remain unidentified. At least 1 dozen surface receptors has been described, but the promiscuous binding characteristics of HSPs complicate the basic questions of receptor-ligand interactions and signaling pathways involved. It will further be necessary to investigate whether HSPs are passively released from damaged cells or secreted via a noncanonical pathway. In addition, release of HSPs as endogenous stress signals, active or by leakage from damaged cells or tissues, is presumably accompanied by a plethora of other stress factors, including ROS, ATP, and hypoxia [17]. These conditions are seldom mimicked in in vitro cell cultures, which hampers drawing conclusions from in vitro experiments. The major outcomes of inflammatory responses are tissue repair, adaptation to stress, and restoration of physiological conditions, and it is important to realize that inflammatory responses are organ-, tissue-, and condition-specific. Therefore, it is highly unlikely that HSPs will have the same effects in different locations and environments.

CONCLUSION

Millions of years of evolution have led to a flexible immune system that can quickly adapt to external and internal stressors. HSPs have evolved to deal with the danger that inevitably comes with immunity. This is underscored by the capacity of HSPs to modulate immunity, i.e., to enhance or inhibit an ongoing immune response through their interaction with innate and adaptive immune receptors.

The chaperone functions of HSPs and technical problems have hindered the interpretation of the in vitro data of HSPs and clouded the discussion. Still, the seemingly contradictory, biological features contributing to them make sense when taking into account the different phases of an immune response and the requirements of the local environment, in which an inflammatory insult takes place. To better comprehend the complex role of HSPs, it is crucial to develop in vitro

culture systems that bypass the technical difficulties and better mimic the in vivo environment. This is a major challenge but certainly worth the effort, as a better understanding of the multifaceted role of HSPs in inflammation could help to develop new diagnostic or therapeutic targets in human inflammatory conditions.

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KEY WORDS:
monocytes/macrophages · T cells · T reg · HSP · danger · TLR · LPS · alarmins · PAMP