HSP60 and the regulation of inflammation: Physiological and pathological

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Introduction

This chapter positions HSP60 at the center of inflammation and body maintenance. We shall discuss the following topics:
- Inflammation: Physiological
- Inflammation: Pathological
- HSP60: Autoimmune target
- HSP60: Regulator signal
- HSP60: Innate ligand
- HSP60 model
- Signal fidelity and HSP60

Inflammation: Physiological

Inflammation has come to have a bad name. We talk about inflammatory diseases – diseases apparently caused by the inflammatory process. The pharmaceutical industry abets inflammation's ill repute and works hard to develop "anti-inflammatory" drugs, which are widely prescribed by physicians and even sold over the counter to the public.

But inflammation has not always been disparaged. The bio-medical scientists who developed the concept of inflammation through the first half of the 20th Century were aware of the beneficial aspects of inflammation [1]. In his book *General Pathology* [2], Lord Florey defines inflammation by citing Ebert: "Inflammation is a process which begins following a sub-lethal injury and ends with complete healing" [3].

Defined so, inflammation is physiological. From the moment of birth, the body must be maintained in the face of constant exposure to sub-lethal injury; the response to injury is inflammation and repair. The physiological system responsible for regulating inflammation is the immune system. The cytokines, chemokines,

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adhesion molecules, and other molecules produced by the immune system's adaptive and innate agents are required for angiogenesis, wound healing, tissue remodeling and regeneration, connective tissue formation, phagocytosis, apoptosis, and other processes needed for body maintenance. Even recognition of specific antigens is involved in the regulation of inflammation. A telling example is the phenomenon of neuroprotection: It appears that the preservation and recovery of function following trauma to the central nervous system is enhanced by activated autoimmune T-cells that recognize myelin antigens [4]. The point is that the adaptive arm of the immune system also takes part in the physiology of inflammation: antibodies, B-cells and T-cells. We shall discuss below how T-cells that recognize heat shock protein 60 (HSP60) aid the regulation of inflammation. HSP60, as a ligand for innate Toll-like receptors (TLR), helps connect innate and adaptive immunity into one integrated system. Defense against infectious agents is just one aspect of the immune maintenance of a healthy body; here too, both the innate and the adaptive arms of the immune system play critical roles [5].

To maintain the body, the immune system has to diagnose the need for inflammation at any particular site and at all times, and to respond dynamically with the exact mix of inflammatory molecules, in the degree needed to repair the damage. The inflammatory response needs to be turned on, fine tuned, and turned off dynamically as the healing process progresses [6]. The physiological regulation of inflammation by the immune system involves a dynamic dialog between the immune cells and the damaged tissue. The immune system responds to molecules from the tissue that signals the state of the tissue. As we shall discuss below, the expression of HSP60 is a reliable signal. The immune system, in turn, produces molecules (cytokines, chemokines, angiogenic factors, growth factors, apoptotic factors, and so forth) that induce changes in the target tissue that, properly orchestrated, lead to healing.

Inflammation: Pathological

If the inflammatory process is not properly regulated, or not terminated, or activated at the wrong place, at the wrong time, or to an inappropriate degree, then the inflammatory process itself can become the cause of significant damage [7]. Indeed, infectious agents bent on damaging the host, usually do so by triggering inappropriate inflammation through their toxins; the host is made sick by his or her own inflammatory reaction to pathogenic stimuli that trigger TNF-α, IFN-γ and other strong pro-inflammatory mediators [8]. Autoimmune diseases are the classic example of inappropriate inflammation. Chronic inflammation plays a role in diseases such as atherosclerosis, which bear autoimmune stigmata [9]. Allergies too are the expression of inappropriate inflammation [10]. Even agents of chemical or biological warfare have been designed to activate pathological inflammation [11].

Clearly, pathological inflammation is a feature of many diseases. However, pathologic inflammation is only physiologic inflammation gone wrong. The pathophysiology of inflammation emerges from the physiology of inflammation. The immune system normally deploys the inflammatory reaction so that it maintains and repairs the body; occasionally, however, the inflammatory process runs wild and can become a pathologic reaction [12]. Indeed, inappropriate healing can be as damaging as inappropriate destruction: the pannus of rheumatoid arthritis is scar tissue [13]; scleroderma too is caused by the unregulated formation of connective tissue [14]; angiogenesis in the retina is a major cause of blindness [15, 16]. The immune system needs to receive reliable signals if it is to dispense beneficial inflammation while avoiding pathological inflammation.

HSP60: Autoimmune target

HSP60 was first discovered to function as a molecular chaperone inside cells. The HSP60 molecule is required to assist the folding of polypeptides into mature proteins in routine protein synthesis, in normal transport of proteins across membranes and in response to protein denaturation during cell stress [17, 18]. It could be said that HSP60, like the other stress proteins, performs an important function in intracellular maintenance. Intra-cellular maintenance was a subject for biochemists.

Later, and in parallel, HSP60 was unknowingly being studied as a dominant antigen in the host response to infectious bacteria. It was noted that the immune response to different bacteria tended to focus on a "common bacterial antigen" [19]. This common antigen was discovered to be the variants of HSP60 expressed by different bacteria [20–23]. The fact that HSP60 was such a dominant antigen was not explained by these studies.

The dominance of HSP60 as a T-cell antigen came to light in the study of adjuvant arthritis (AA), an autoimmune disease inducible in rats by immunization to killed mycobacteria [24]. It was discovered that a T-cell clone cross-reactive with cartilage and mycobacteria could mediate arthritis in irradiated rats [25, 26]. The mycobacterial antigen was later identified to be the HSP60 (HSP65) molecule [27]. The idea was that mycobacterial HSP65 bore a peptide epitope cross-reactive with a self-epitope in the rat joint [26]. This seminal finding aroused interest in HSP60 as a target in an autoimmune disease, albeit in an autoimmune disease induced by bacterial immunization.

The connection of HSP60 to autoimmune disease was confirmed in another system when it was discovered that HSP60, both mouse and human, was a target antigen in the Type 1 diabetes developing spontaneously in NOD mice [28, 29]. HSP60 autoimmunity was functional: immunization to human HSP60 could accelerate or abort the diabetes, an anti-HSP60 T-cell clone could mediate disease in NOD mice [30], and immunization with an HSP60 target peptide (p277) could activate tran-

sient insulitis and hyperglycemia in standard strains of mice, provided that the peptide was conjugated to an immunogenic carrier (ovalbumin) [31]. Open questions were how autoimmunity to HSP60 could be involved in diverse diseases such as AA and NOD diabetes, and how might a ubiquitous molecule like HSP60 be a tissue-specific target [32]. Autoimmunity to HSP60 was soon discovered to characterize a variety of inflammatory and autoimmune conditions such as human type 1 diabetes [28], atherosclerosis [33], Bechet's disease [34], lupus [35], and others. These findings only compounded the questions of the role of HSP60 autoimmunity in inflammation.

HSP60: Regulator signal

In direct contrast to HSP60 autoimmunity as a target in pathologic inflammation, HSP60 was also noted to down-regulate pathological inflammation. Mycobacterial HSP65 or its 180–188 peptide were found early on to vaccinate rats against adjuvant arthritis [27, 36, 37]. Work with HSP60 as a regulator of Type 1 diabetes followed.

Vaccination of NOD mice with the p277 peptide of HSP60 arrested the development of diabetes [30] and even induced remission of overt hyperglycemia [38]. Successful p277 treatment was associated with the down-regulation of spontaneous T-cell reactivity to p277 and with the induction of antibodies to p277 displaying Th2-like isotypes IgG1 and IgG2b [39]. Other peptides of HSP60 could also inhibit the development of spontaneous diabetes in NOD mice [40].

NOD mice can also develop a more robust form of diabetes induced by the administration of cyclophosphamide – cyclophosphamide-accelerated diabetes (CAD) [41]. We used DNA vaccination with constructs encoding human HSP60 (pHSP60) or mycobacterial HSP65 (pHSP65) to explore the regulatory role of HSP65 [42]. Vaccination with pHSP60 protected NOD mice from CAD. In contrast, vaccination with pHSP65, with an empty vector or with a CpG-positive oligonucleotide was not effective, suggesting that the efficacy of the pHSP60 construct might be based on regulatory HSP60 epitopes not shared with its mycobacterial counterpart, HSP65. Vaccination with pHSP60 modulated the T-cell responses to HSP60, and also to the glutamic acid decarboxilase (GAD) and insulin autoantigens: T-cell proliferative responses were significantly reduced and the pattern of cytokine secretion to HSP60, GAD and insulin showed an increase in IL-10 and IL-5 secretion and a decrease in IFN-γ secretion, compatible with a shift from a Th1-like towards a Th2-like autoimmune response. Thus, immunoregulatory networks activated by vaccination with pHSP60 or p277 can spread to other β-cell antigens like insulin and GAD and control NOD diabetes. To understand the role of HSP60 in immune signaling, we shall have to understand how HSP60 can affect autoimmunity to other molecules.

Type 1 diabetes in humans was also found to be susceptible to immunomodulation by p277 therapy. A double-blind, Phase II clinical trial was designed to study the effects of p277 therapy on newly diagnosed patients [43]. The administration of p277 after the onset of clinical diabetes preserved the endogenous levels of C-peptide (which fell in the placebo group) and was associated with lower requirements for exogenous insulin, revealing the arrest of inflammatory β-cell destruction. Treatment with p277 was associated with an enhanced Th2 response to HSP60 and p277. Taken together, these results suggest that treatment with HSP60 or its p277 peptide can lead to the induction of HSP60-specific regulators that can control the collective of pathogenic reactivities involved in the progression of autoimmune diabetes.

The administration of HSP60 or some of its peptides could also prolong the survival of skin allografts in mice [44]. Thus the regulatory effects of HSP60 were not limited to autoimmune disease.

HSP60 can also regulate AA. Vaccination of rats with HSP65 or some of its T-cell epitopes was found to prevent AA [27, 36, 37, 45]. The mechanism of protection was thought to involve cross-reactivity with the self-60 KDa heat shock protein (HSP60) [46]. We studied the roles of HSP60 and HSP65 in modulating AA [47], and identified regulatory epitopes within the HSP60 protein using DNA vaccines (Quintana et al., submitted). Susceptible rats were immunized with DNA vaccines encoding human HSP60 (pHSP60) or HSP65 (pHSP65) and AA was induced. Both pHSP60 and pHSP65 protected against AA. However pHSP60 was significantly more effective. We identified immunoregulatory regions within HSP60 using HSP60 DNA fragments and HSP60-derived overlapping peptides. A regulatory HSP60 peptide (Hu3, aa 31-50) that was specifically recognized by the T-cells of rats protected from AA by DNA-vaccination. Vaccination with Hu3, or transfer of splenocytes from Hu3-vaccinated rats, prevented the development of AA. Vaccination with the mycobacterial homologue of Hu3 had no effect. Effective DNA or peptide vaccination was associated with enhanced T-cell proliferation to a variety of disease-associated antigens, along with a Th2/3-like shift (down-regulation of IFN-γ secretion and concomitant enhanced production of IL-10 and TGF-β1) in the response to peptide Mt176-190 (the 180–188 epitope of HSP65). The regulatory response to HSP60 or its Hu3 epitope included both Th1 (IFN-γ and Th2/3 (IL10/TGF-β1) secretors. These results showed that HSP60-specific regulation can control AA and be activated by immunization with relevant HSP60-derived epitopes, administered as peptides or as DNA vaccines.

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HSP60: Innate immune signal

Innate immune system receptors have been shown to control the cytokine phenotype of the adaptive immune response [48]. Headway in unraveling the pleiotropic effects of HSP60 has been the discovery that HSP60 can signal macrophages and other cells through an innate signaling pathway, dependent on functional CD14 plus TLR-4 and/or TLR-2 [49–51]. The TLR-4 molecule does not seem to bind HSP60 directly, but TLR-4 is required to transduce the signal [50, 52]. Binding to the cell surface may be mediated by more than one receptor, as HSP60 molecules derived from various sources do not all compete for binding of labeled human HSP60 [53]. Macrophages exposed to soluble HSP60 secrete pro-inflammatory mediators such as TNF-α, IL-6, IL-12, and nitric oxide [49, 50, 54]. The pro-inflammatory effects of HSP60 can explain how HSP60 can be associated with up-regulation of inflammation in so many conditions. Early on, we observed that soluble HSP60 was present in the circulation of NOD mice developing type 1 diabetes and peaked before the onset of disease [55]; this increased HSP60 might accelerate islet inflammation through TLR-4 signaling. However, how can the same HSP60 molecule block inflammation?

Some explanation may be found in the discovery that HSP60 can directly activate anti-inflammatory effects in T-cells by way of an innate receptor. We find that HSP60 and its fragments can regulate the physiology of inflammation itself by acting as ligands for TLR-2 in T-cells [56]. HSP60 activated human T-cell adhesion to fibronectin, to a degree similar to other activators: IL-2, SDF-1α and RANTES. T-cell type and state of activation was important; non-activated $CD45RA^+$ and IL-2-activated $CD45RO^+$ T-cells responded optimally at low concentrations (0.1–1 ng/ml), but non-activated $CD45RO^+$ T-cells required higher concentrations (> 1 μg/ml) of HSP60. T-cell HSP60 signaling was inhibited specifically by a mAb to TLR-2, but not by a mAb to TLR-4. The human T-cell response to soluble HSP60 depended on PI-3 kinase and PKC signaling, and involved the phosphorylation of Pyk-2. Soluble HSP60 also inhibited actin polymerization and T-cell chemotaxis through ECM-like gels towards the chemokines SDF-1α or ELC. Exposure to HSP60 could also down-regulate the expression of chemokine receptors CXCR4 and CCR7. Most importantly, HSP60 prevented the secretion of IFN-γ by activated T-cells (unpublished observations). These results suggest that soluble HSP60 (and its fragments), through TLR-2-dependent interactions, can down-regulate T-cell behavior and control inflammation.

To examine further the contribution of innate immune signaling to autoimmunity, we inserted a TLR-4 mutation into NOD mice. Mutated TLR-4 appears to markedly increase susceptibility to autoimmune Type I diabetes (unpublished observations). Apparently TLR-4 signaling, whether by endogenous ligands such as HSP60 or foreign ligands such as LPS, can educate the immune system to avoid pathogenic autoimmunity. Further studies will examine the requirement of TLR-4

signaling for the therapeutic effects of HSP60- and HSP60-based therapies. The importance of innate receptor signaling for the down-regulation of inflammation was also confirmed in studies showing that CpG, a ligand for TLR-9 [57], can inhibit the spontaneous development of Type I diabetes in NOD mice [58]. It would appear that CpG can actually up-regulate the expression of HSP60 and enhance HSP60 regulators (unpublished observations).

Thus, HSP60 can have both pro-inflammatory and anti-inflammatory effects on various cell types. HSP60 works as a ligand both for antigen receptors on T-cells and B-cells (and auto-antibodies) and for innate receptors TLR-4 and TLR-2 on various cells types.

HSP60 model

Figure 1 summarizes our current views of inflammation (physiologic and pathologic), body maintenance and HSP60. Infection, trauma and noxious agents cause stress (damage) to cells and tissues. Unless repaired, stress can be lethal. Stress of any kind induces up-regulation of HSP60 and other stress molecules. The chaperone function of HSP60 and its allies inside stressed cells protects the cells as a type of intra-cellular maintenance. However, there is also an extra-cellular maintenance system – the immune system. The HSP60 molecule, as it performs its chaperone function in the stressed cells, also functions as a molecular signal to the wandering cells of the immune system. Macrophages, dendritic cells, endothelial cells, and others recognize HSP60 epitopes *via* innate receptors. T-cells and B-cells recognize HSP60 both *via* their adaptive antigen receptors and their innate receptors. Healthy individuals are born with a high frequency of T-cells that have been positively selected to see HSP60 epitopes; HSP60 is a member of the set of self-molecules for which there exists natural autoimmunity [6, 59].

The fine balance of the amounts of HSP60 and other molecules expressed by the damaged/healing tissues and the responding immune cells is integrated into the dynamic process we call physiologic inflammation. Physiologic inflammation results in beneficial immune maintenance. Thus the processes set into motion by HSP60 and the other stress molecules responding to cellular damage lead to both intra-cellular maintenance (chaperone function) and extra-cellular immune maintenance (signal function).

Malfunction of the inflammatory response, however, can produce pathologic inflammation and, rather than heal, compound the damage.

This model, at our present state of knowledge, is mostly words. HSP60 research has to fill in the picture in a precise and quantitative way. Which cells recognize HSP60? Which cells and which conditions lead to the secretion of soluble HSP60? What are the functional receptors and epitopes? What are the varied responses? How do the amounts and concentrations of various molecules orchestrate the

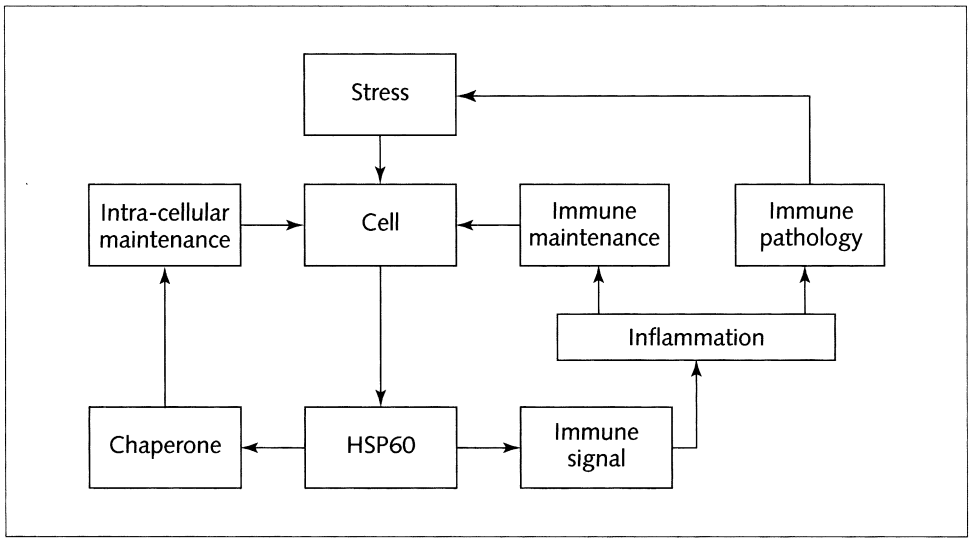

Figure 1
HSP60, as a chaperone, mediates intra-cellular maintenance; as an immune signal, HSP60 regulates inflammation. See text.

inflammatory response? How does healing occur? How does pathologic inflammation emerge? How can we control it and restore health? The program is very heavy, but HSP60 has given us a powerful tool for manipulating the inflammatory response and not only for exploring it.

Signal fidelity and HSP60

In closing, let us take note of the central position of HSP60. Why should the same HSP60 molecule function intra-cellularly as a chaperone and extracellularly as a signal? Would it not be more efficient to divide the functions and have two different molecules do the job? A chaperone should be a chaperone and a signal molecule should specialize in signaling. Is it not confusing for the system to load one HSP60 molecule with more than one important function?

The paradox of pleiotropism tells us something fundamental about signaling and about evolution. An important aspect of signaling is the fidelity of the signal. A reliable signal is a signal that never tells a lie [60]. What signal could be a more faithful sign of stress than a stress protein with a chaperone function? HSP60 signaling is foolproof.

The pleiotropism of HSP60 is yet another example of the way evolution uses old information for new purposes. HSP60 was invented at the onset of cellular life – at least in prokaryotes [61]. Moreover, the homologues of the TLR molecules involved in the transduction of the HSP60 signal appeared with multicellular organisms, but associated with development and not only with innate immunity [62]. However, like other ancient molecules, HSP60 gained a new function in higher multicellular organisms and immune systems. That's the way it goes; it takes old information to make new information [63].

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