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Editors

Heat Shock Proteins Volume1

Series Editors: Alexzander A.A. Asea · Stuart K. Calderwood

Heat Shock Proteins: Potent Mediators of Inflammation and Immunity

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HEAT SHOCK PROTEINS

Volume 1

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This book is dedicated to our children Ana-Cristina, Alexzander Jr., Edwina and Vanessa

TABLE OF CONTENTS

PREFACE

From their original description as primarily intracellular molecular chaperones involved in cell survival and protection against potentially harmful stimuli, heat shock proteins (HSP) have now been shown to be exit cells and exert profound effects on the host's response to several human diseases as dissimilar as cancer, cardiovascular disease, aging and autoimmunity, and in response to previously unknown stressors like physical exercise and psychological stress including predator fear, confinement and social exclusion. This book reviews the contemporary knowledge on the role of heat shock proteins as mediators of inflammation and immunity. Using an integrative approach to understanding heat shock protein immunobiology, the contributors provide a synopsis of novel mechanisms by which HSP are released from cells, specific binding and resultant receptor-mediated signaling, the process of antigen processing and presentation and finally how HSP stimulate immune responses.

Section I reviews recently discovered mechanisms by which HSP gain access to the extracellular milieu. Classical and unique stressors that stimulate HSP release, as well as pathways by which HSP are delivered to the extracellular milieu are discussed.

Following release of HSP from cells, Section II reviews our recent knowledge of HSP specific binding to cells of the immune system. In addition, the growing number of HSP receptors and the resultant receptor-mediated signaling that occurs is comprehensively reviewed.

In Section III, immune responses elicited by exogenous HSP are reviewed. An up-to-date account of the ability of HSP to act as a danger signal and thereby augment host defense against various diseases or induce devastating autoimmune responses is also discussed in this section.

Finally, in Section IV, the role of HSP in antigen processing, presentation and its effect on inflammation and disease are reviewed. Specifically, the role of HSP-peptide complexes, controlling the inflammatory process and regulatory T cells are comprehensively reviewed.

Heat Shock Proteins: Potent Mediators of Inflammation and Immunity provides the most up-to-date and exciting insights into how heat shock proteins (HSP) modulates the host's immune response. Written by leaders in the field of heat shock protein immunobiology, the chapters systematically and in a step-wise fashion

takes the reader through the fascinating sequence of events by which heat shock proteins activate immune responses and provides answers as to its biological significance to the host. The book takes the reader systematically and in a step-wise fashion, mechanisms of release, to specific binding and receptor-mediated signaling, activation of host defense or initiation of devastating autoimmunity and finally to antigen processing and presentation and its effect on human diseases. This book is a must read for graduate and postgraduates in the field of Biology (plant and mammal), Biochemistry (pro- and eukaryotic), Immunology, Microbiology, Exercise Medicine, Physiology, Inflammatory diseases, Autoimmunity, Pharmacology and Pathology.

Alexzander A.A. Asea and Antonio De Maio

PART I

MECHANISMS OF HEAT SHOCK PROTEIN RELEASE

CHAPTER 1

RELEASE OF HEAT SHOCK PROTEINS: PASSIVE VERSUS ACTIVE RELEASE MECHANISMS

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Abstract: There is now no doubt that heat shock proteins have a profound immunoregulatory effect on the host's immune system. This knowledge has successfully been harnessed to generate a number of important clinical trails. However, one intriguing question that remains to be answered is how heat shock proteins (HSP) which do not have peptide leader sequence targeting secretion can gain access to the extracellular milieu. This chapter will discuss the most recent findings in the area of HSP release and attempts to broadly categorize these findings into two basic mechanisms; the passive and active mechanisms

Keywords: Chaperokine; exosomes; heat shock proteins; inflammation; lipid rafts; protein transport; stress

Abbreviations: eHsp72, extracellular Hsp72; ER, endoplasmic reticulum; Hsp, heat shock proteins; Hsc70; constitutively expressed seventy-kilo Dalton heat shock protein; Hsp72, stress inducible seventy-kilo Dalton heat shock protein; HSF-1, heat shock factor-1; IFN-γ, interferon-gamma; IL, interleukin; LDH, lactate dehydrogenase; MßD, methyl ß-cyclodextrin

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PASSIVE RELEASE MECHANISM: NECROSIS, INFECTION AND TRAUMA

Necrosis

Necrotic cell death is an obvious mechanism by which heat shock proteins escape from cells. However, experimental conditions that conclusively demonstrate necrotic cell killing and the biological significance of the released HSP have been difficult to prove. However, Melcher and co-workers demonstrated that non-apoptotic cell killing induces increased levels of HSP concomitant with enhanced immunogenicity, whereas cells killed predominantly by apoptosis showed low levels of HSP expression and were less immunogenic (Melcher et al., 1998). Inhibition of apoptotic cell death by overexpression of bcl-2 induced increased levels of HSP. Interestingly, stable transfection of B16 and CMT93 cells with cDNA encoding Hsp70 significantly augmented the immunogenicity of both tumors (Melcher et al., 1998). These results were later independently supported by experiments performed by Basu and colleagues who reported that heat shock proteins including gp96, calreticulin, Hsp90 and Hsp72 are released from cells undergoing necrotic but not apoptotic cell death (Basu et al., 2000). These authors demonstrated that necrosis induced by freeze thaw, but not apoptosis induced by irradiation, resulted in the release of HSP into the culture supernatant, respectively. It was further demonstrated that the released HSP stimulates macrophages to secrete cytokines, and induces the expression of co-stimulatory molecules and enhanced antigen presentation by dendritic cells (Basu et al., 2000) a process known as the chaperokine activity of HSP which describes the ability of HSP to act as both chaperone and cytokine (Asea, 2003, 2005; Asea et al., 2000b). The chaperokine activity of HSP has been described in cancer (Facciponte et al., 2005; Facciponte et al., 2006; Gross et al., 2003a; Gross et al., 2003b; Gross et al., 2003c), stem cells (Son et al., 2005), complement activity (Prohaszka et al., 2002), transplantation and allograft injury (Land, 2005), and septic shock (Wheeler et al., 2005), as a trigger for autoimmune reactions (Yokota et al., 2006) and exercise immunophysiology (Fleshner and Johnson, 2005).

Infection

Infection of cells with a variety of microorganisms could result in cell death by apoptosis or necrosis (Fischetti, 2005; Gruenberg and van der Goot, 2006; Mathis et al., 2005; Thorne et al., 2005a; Thorne et al., 2005b). Lytic viruses are known to induce necrotic cell death (Brinkmann and Schulz, 2006; O'Shea , 2005; O'Shea et al., 2005). Infection of SK29-Mel-1 with the lytic parvovirus H1 occurs in the absence of HLA class I or costimulatory molecule upregulation (Moehler et al., 2003). In addition, infection is accompanied by a strong release of the inducible Hsp72, but not the constitutively expressed Hsc73. When compared with the classical non-lethal heat-shock treatment, a known inducer of HSP release (Bausero et al., 2005; Broquet et al., 2003; Gastpar et al., 2005; Lancaster and

Febbraio, 2005), the Hsp72 release is demonstrated to be higher and of longer duration (Moehler et al., 2003). Admixing parvovirus-mediated tumor cell lysate with antigen presenting cells including human dendritic cells (DC) and monocytes resulted in potent chaperokine activity. Further studies by the same group demonstrated that parvovirus-mediated cell killing enhances tumor immunogenicity by Hsp72 release and contributes to the anti-tumor effect of parvoviruses (Moehler et al., 2005). Although these authors did not directly demonstrate that H1-induced cell killing and its associated Hsp72 release promotes the loading and maturation of antigen presenting cells and by extension triggers tumor specific immune responses. One can speculate that the release of Hsp72 can facilitate priming of T cells specific for viral antigens in a similar fashion to that described in autoimmune diabetes and encephalomyelitis (Chandawarkar et al., 2004), and HIV infection (SenGupta et al., 2004).

Other kinds of infection known to stimulate innate and adaptive immune responses might also result in necrotic cell death; namely atherosclerosis. Atherosclerosis is a disease in which the immune response plays a very important role in its pathogenesis (for review see (Hansson and Libby, 2006)). The Wick laboratory was the first to provide evidence that the first stages of atherosclerosis is an autoimmune response against Hsp60 that is expressed by endothelial cells in areas that are subject to increased haemodynamic stress (Wick et al., 1995a; Wick et al., 1995b). Antibody-mediated and T-cell-mediated immune responses against Hsp60 have both been demonstrated early in arthrogenesis (for review see (Wick et al., 1995b)).

Why would the hosts own immune system turn against it in such a fashion? The complete answer has not yet been elucidated. However, there is an indication that the answer might in part be due to molecular mimicry, (for review see (Binder et al., 2002; Rose, 2000; Rose and Mackay, 2000)). Since Chlamydial heat shock proteins are potent antigenic stimuli able to induce specific cell-mediated and humoral immune responses, several studies have proposed a link between Chlamydia pneumoniae and pathologies associated with atherosclerosis and coronary heart disease (CHD) (Ausiello et al., 2005; Hoshida et al., 2005). In addition, Chlamydial heat shock proteins have been suggested to increase the risk of secondary cardiovascular events in patients with coronary heart disease with diabetes (Guech-Ongey et al., 2006).

Trauma

Severe trauma is a clear example by which intracellular Hsp72 gains free and unfettered access to the extracellular milieu. Trauma due to surgery after coronary artery bypass grafting has been shown to result in increased systemic Hsp72 levels (Dybdahl et al., 2004; Dybdahl et al., 2002). In a study designed to determine a correlation between serum levels of Hsp72 with survival of trauma patients and/or the severity of the postinjury inflammatory response, Pittet and colleagues demonstrated a significant upregulation in circulating serum Hsp72 in severely

traumatized patients as early as 30 minutes after injury (Pittet et al., 2002). In this study, patients with high initial serum levels of Hsp72 (serum levels $> 15\,ng/mL$) survived, whereas patients with low Hsp72 serum levels died from their traumatic injuries. However, in this study there was no correlation between the initial serum Hsp72 values and the severity of organ dysfunction or clinical indicators of the inflammatory response (Pittet et al., 2002). Hsp72 has been found to be significantly higher in the pulmonary edema fluid of patients with acute lung injury (ALI), and it is suggested that extracellular Hsp72 may serve as a marker of stress protein response activation in the distal air spaces of patients with ALI (Ganter et al., 2006).

However, it must be noted that in all these conditions although trauma is proposed as the mechanism of HSP release, conclusive experimental data is still lacking. Taken together, the passive release mechanism is clearly an important mechanism by which Hsp72 is released into the circulation. Undoubtedly, further studies are required to conclusively determine the fate of released HSP, its target cell/organ and its biological significance to the host.

ACTIVE RELEASE MECHANISM: INFLAMMATION, EXERCISE, PSYCHOLOGICAL STRESS, BRAIN AND CSF

Currently, the alternative to passive release of HSP is the active release mechanism. However, this mechanism initially did not get much traction because HSP do not have a peptide leader sequence targeting secretion. Therefore any study describing the release of HSP from cells via an active release mechanism was looked upon with skepticism. One such example was the initial study by Hightower and Guidon demonstrating that a small groups of HSP namely Hsp110, Hsp71, Hsc73 and Hsp72 are released from mammalian cells (Hightower and Guidon, 1989). In this study, supernatant from cultured rat embryo cells exposed to control conditions ($37\,°C$) or heat stressed ($45\,°C$) for 10 minutes and allowed to recover for 2h at $37\,°C$. At control conditions, Hsp110, Hsp71 and Hsc73 were shown to be released and heat stress additionally induced Hsp72. Pre-treatment of cells with inhibitors of the classical protein secretory pathways including monensin (known to disrupts the Golgi apparatus and vesicular transport) and colchicine (an inhibitor of microtubule assembly) did not abrogate stress-induced HSP release (Hightower and Guidon, 1989). Although, these experiments were the first indication that HSP could be released by an active mechanism, the exact mechanism of release was not yet addressed. Further studies by Guzhova and colleagues demonstrated that Hsp70 is released by glia cells in the absence of necrotic cell death (Guzhova et al., 2001) and more recently, Hsp70 has been shown to be released by B cells (Clayton et al., 2005), peripheral blood mononuclear cells (Hunter-Lavin et al., 2004) and tumors (Bausero et al., 2005; Broquet et al., 2003; Gastpar et al., 2005; Lancaster and Febbraio, 2005), under non necrotic conditions.

Inflammation

After admixing eHsp70 to APCs, specific signal transduction pathways are activated that result in the stimulation of an inflammatory response (for review see (Asea, 2005)). eHsp72 induces a plethora of immune responses, and the list continues to grow. Our group demonstrated that as early as 2-4 hours post exposure of APC to exogenous eHsp70 there is significant release of cytokines including TNF-α, IL-1β, IL-6 and IL-12 (Asea et al., 2000b; Asea et al., 2002) and GM-CSF (Srivastava, 2002); nitric oxide, a potent apotogenic mediator (Panjwani et al., 2002); chemokines, including MIP-1, MCP-1, and RANTES (Lehner et al., 2000; Panjwani et al., 2002). Earlier, we demonstrated that both peptide-bearing and non peptide-bearing eHsp70 are capable of inducing pro-inflammatory cytokine production by APCs (Asea et al., 2000a). eHsp72 induces the DC maturation by augmenting the surface expression of CD40, CD83, CD86 and MHC class II molecules on DC (Asea et al., 2000b; Basu and Srivastava, 2000; Noessner et al., 2002; Singh-Jasuja et al., 2000a; Singh-Jasuja et al., 2000b) and migration of DC (Binder et al., 2000) and NK cells (Gastpar et al., 2005).

Using conditions which will not induce significant cell death, our group was the first to demonstrate that IFN-γ and IL-10, pro-inflammatory cytokines, known to be found in high concentrations in inflammatory foci, induce the active release of constitutive Hsc73 from tumors (Barreto et al., 2003). However, these initial studies did not address the mechanism of release. Recently, our group (Bausero et al., 2005; Gastpar et al., 2005) and others (Lancaster and Febbraio, 2005) have begun to elucidate the mechanism of active release of Hsp70 from viable cells. In our study, we showed that certain cytokines normally found in high concentrations within inflammatory foci, including IFN-γ, IL-10 but not TGF-β1, mediate the active release of Hsp72. We further showed that whereas some Hsp72 could be found as free Hsp72, a proportion of Hsp72 was released within exosomes (Bausero et al., 2005; Gastpar et al., 2005). Exosomes are internal vesicles of multi-vesicular bodies (MVB) that are released into the extracellular milieu upon fusion of MVB with the cell surface (Raposo et al., 1996; Zitvogel et al., 1999; Zitvogel et al., 1998). In addition to containing Hsp72 (Bausero et al., 2005; Gastpar et al., 2005), exosomes are highly packed with immunostimulatory mediators including MHC class I and II (Raposo et al., 1996; Zitvogel et al., 1999; Zitvogel et al., 1998) and costimulatory molecules (Escola et al., 1998). This seems to help explain the potent effect observed by HSP-based immunotherapy against certain cancers (Srivastava, 2000, 2005). Our group recently demonstrated that Hsp72 was released by a non classical protein transport pathway and that intact surface membrane lipid rafts were required for efficient stress-induced Hsp72 release (Bausero et al., 2005; Gastpar et al., 2005). Similar findings were demonstrated in B cells (Clayton et al., 2005). Studies by Lancaster & Febbraio recently demonstrated that exosomes provide the major pathway for secretory vesicular release of Hsp72 (Lancaster and Febbraio, 2005). However, using methyl-β-cyclodextrin (the cholesterol-depleting agent) to disrupt lipid raft function, these authors were unable to confirm a role for lipid rafts in stress-induced Hsp72 release from human peripheral blood mononuclear cells

(PBMC) (Lancaster and Febbraio, 2005). In order to address the cellular location of Hsp72 after stress, a recent study demonstrated that newly synthesized Hsp72 protein localizes within the Golgi region of HELA cells and also concentrates on the surface of the plasma membrane and in the ruffled zone of migrating cells (Schneider et al., 2002).

However, not all interactions between HSP and cells of the immune system result in pro-inflammatory immune reactions. A recent review by Van Eden and colleagues details how in various experimental disease models, HSPs can prevent or arrest inflammatory damage. Data from clinical trials in patients with type 1 diabetes and rheumatoid arthritis show that treatment with HSP-derived peptides promote a switch from a pro-inflammatory cytokine-secretion profile of T lymphocytes to a regulatory anti-inflammatory cytokine-secretion profile (van Eden, 2006; van Eden et al., 2005a; van Eden et al., 1985; van Eden et al., 2005b; van Eden et al., 2000).

T regulatory (Treg) cells display $CD4^+CD25^+$ phenotype, a down-regulation of the CD4, CD3 and TCRβ transcripts and plasma membrane antigenic expression similar to activated T lymphocytes (Bosco et al., 2006; Gavin et al., 2002; Kasow et al., 2004). Treg represent a unique population of T lymphocytes capable of potent suppression of immune responses in a wide variety of clinically relevant diseases and disorders including transplantation, allergy, cancer, and microbial diseases (Wing et al., 2006). Initially described as a subpopulation of $CD5^{high}CD4^+$ cells with the ability to protect mice from autoimmune disease caused by neonatal thymectomy (Sakaguchi et al., 1982a, 1982b). Further studies demonstrated that during mouse ontogeny, Treg appeared shortly after birth with a $CD25^+$ phenotype (Fontenot et al., 2005b; Fontenot et al., 2005c; Fontenot and Rudensky, 2005; Sakaguchi et al., 1995). The transcription factor FoxP3 has recently been shown to play an important role in Treg function (Hori et al., 2003). This was supported by studies in knock-in transgenic mice containing a GFP-FoxP3 construct which demonstrated that FoxP3 can be used as a lineage marker for Treg (Fontenot et al., 2005a; Fontenot et al., 2005b; Fontenot et al., 2005c; Fontenot and Rudensky, 2005). *In vitro*, Treg cells have been shown to inhibit the proliferation of naïve responder T cells (Suri-Payer et al., 1998; Thornton and Shevach, 1998). *In vivo*, Treg cells appear to prevent the activation of activated T lymphocytes (Asano et al., 1996; Sakaguchi, 2004; Sakaguchi et al., 1996). Treg-mediated inhibition of T lymphocyte proliferation is advantageous in situations of autoimmunity; however, is disastrous for the host in situations of lymphopenia-induced proliferation or anti-tumor immunity (Dolnikov et al., 2003; Nomura and Sakaguchi, 2005).

In addition to T regulatory cells, natural killer (NK) cells have been shown to respond to HSP. In a study by Galazka and colleagues, Hsp70-peptide complexes (pc) isolated from brains of mice with proteolipid protein 139–151-induced experimental autoimmune encephalomyelitis (EAE) prevented the onset of EAE clinically and pathologically when administered before proteolipid protein 139–151 immunization. The transfer of NK cells from Hsp70-pc-immunized mice to recipients sensitized for EAE abolished the development of the disease (Galazka et al., 2006). Recent studies have shown that the cytolytic activity of Hsp70 can also

be greatly augmented by specific fragments of the Hsp70 protein. Both the full-length Hsp70 protein and the C-terminal domain of Hsp70 stimulates the cytolytic activity of naive NK cells against Hsp70-positive tumor target cells (Gross et al., 2003a). In addition, tumor growth in mice with severe combined immunodeficiency was shown to be naive inhibited by Hsp70-peptide-activated, CD94-positive NK cells (Moser et al., 2002). Recent work from the Multhoff laboratory demonstrates that a 14 amino acid sequence of the Hsp70 protein, termed TKD (TKDNNLL-GRFELSG, $aa_{450–463}$) is the extracellular recognition site for NK cells (Multhoff et al., 2001). These authors demonstrate that granzyme B specifically binds to portions of the Hsp70 expressed on the plasma surface of tumors but not normal cells (Gross et al., 2003b). Thus, demonstrating a hitherto unknown mechanism by which cytolytic effector cells eliminate Hsp70 expressing tumors in a perforin-independent, granzyme B-dependent manner. These studies are in agreement with recent findings that immunization of the peptide binding C-terminal portion of Hsp70 ($aa_{359–610}$) ($Hsp70_{359–610}$) is responsible for stimulating Th1-polarizing cytokine (IL-12 and TNF-α), C-C chemokine release, and acts as an adjuvant (Wang et al., 2002). Immunization of nonhuman primates with $Hsp70_{359–610}$ induced the production of RANTES and IL-12, and acted as an adjuvant when loaded with CC5-peptide (Wang et al., 2002), suggesting a possible alternative vaccine strategy for HIV infection (Bergmeier and Lehner, 2006; Lehner and Anton, 2002; Lehner and Shearer, 2002; Whittall et al., 2006a; Whittall et al., 2006b).

In addition to Hsp70, the sixty kilo-Dalton heat shock protein (Hsp60) also plays a significant role in regulating immune responses. Recent studies from the Cohen lab have demonstrated that Hsp60 suppresses Th1-mediated hepatitis by down regulating T-bet, NF-kappa-B, and NFATp and upregulating GATA-3, thereby resulting in the abrogation of TNF-α and IFN-γ secretion and a concomitant increase in secretion of IL-10 via a TLR2-dependent mechanism (Zanin-Zhorov et al., 2005a). These authors have recently demonstrated that the peptide p277 of Hsp60 inhibits T cell chemotaxis (Zanin-Zhorov et al., 2005b) and activates B cells via the TLR4-MyD88 pathway (Cohen-Sfady et al., 2005).

The pathophysiology of sickle cell disease is initiated by sickle hemoglobin (HbS) polymerization, sickling of the red blood cell under hypoxic conditions and vasoocclusive disease (VOC). Pro-inflammatory mediators including superoxide, hydrogen peroxide, peroxynitrite and the hydroxyl radical may contribute to sickle acute chest syndrome (Hammerman et al., 1999; Klings and Farber, 2001). Inflammation plays an important role in the pathophysiology of sickle cell disease (SCD) (for review see (Adewoye and McMahon, 2005; Steinberg and Adewoye, 2006)). We recently demonstrated a significant increase in circulating serum Hsp70 levels in SCD during vasoocclusive crisis (VOC) as compared with baseline steady state levels, and a significant increase in Hsp70 levels in SCD at baseline compared with normal controls, suggesting that circulating serum Hsp70 might be a marker for VOC in SCD (Adewoye et al., 2005).

Exercise

Exercise induces the release of HSP into the circulation from an as yet unknown organ. Studies by Salo and colleagues demonstrated that Hsp70 expression is induced in skeletal muscle, heart, and liver during exercise (Salo et al., 1991). Febbraio and colleagues later demonstrated that exercise induced the increase in circulating serum Hsp72 (Febbraio et al., 2002). By catheterization of the brachial artery and hepatic vein, hepatosplanchnic tissues release Hsp72 during exercise was determined these authors demonstrated that is among the organs that contribute enhanced circulating serum Hsp72 during exercise. In contrast, the arterial-venous balance over contracting musculature demonstrated that although the Hsp72 expression within the contracting skeletal muscle increased, Hsp72 was not released from the contracting skeletal muscle into circulation (Febbraio et al., 2002).

We have previously demonstrated that chronic voluntary exercise improves innate immune responses (Jonsdottir et al., 1996a; Jonsdottir et al., 1996b; Jonsdottir et al., 1997). Using spontaneously hypertensive rat models which were allowed to voluntarily run in wheels 5 weeks resulted in enhanced clearance of YAC-1 cells *in vivo* as compared with sedentary controls. The total number of mononuclear cells in the spleen was significantly decreased in runners compared with controls. Analysis of splenic lymphocyte phenotypes revealed a significantly increased fraction of $OX52^{+}/CD5^{-}$ natural killer (NK) cells in runners compared with sedentary controls. In contrast to changes in natural immunity, immunoglobulins G and M levels in serum, the antibody response to antigen *in vivo*, and the proliferation of splenic T cells *in vitro* were unchanged. We concluded that chronic voluntary exercise augments natural cytotoxicity mechanisms *in vivo*, whereas splenic T-cell proliferation and the antibody-mediated immune response remain unchanged (Jonsdottir et al., 1996a; Jonsdottir et al., 1996b; Jonsdottir et al., 1997). Physically active rats were shown to respond more efficiently than sedentary rats when challenged with *Escherichia coli (E. coli)*. In addition, these authors demonstrated that peripheral immune challenge augmented the expression of Hsp72 in the brain (Nickerson et al., 2005).

Recently, it was suggested that in addition to the well-known and reliable assays for exercise-induced oxidative stress including consumption of plasma reduced ascorbic acid (RAA), increased plasma concentration of thiobarbituric acid reactive substances (TBARS), increased erythrocyte reduced glutathione (GSH) antioxidants and changes in plasma total antioxidant status (TAS), it may be useful to clinicians to measure plasma Hsp70 levels to better assess and evaluate the benefits of training and/or supplementation programs (Banfi et al., 2006).

Psychological Stress

Its been more than a decade since psychological stress had been demonstrated to induce tissue injuries in a variety of animal models, in which server damage is seen in the cerebral cortex and hippocampus of the brain (Fukudo et al., 1995, 1997; Fukudo et al., 1999; Mizoguchi et al., 1992) and gastro-intestinal tract

(Fukudo et al., 1995, 1997; Fukudo et al., 1999; Fukudo et al., 1993). Indeed the Whitehall cohort studies of British civil servants identified an association between Hsp60 and sociodemographic, psychosocial, and biological risk factors for coronary heart disease (CHD). In these studies plasma Hsp60 levels, TNF-α, C-reactive protein, von Willebrand factor, high density lipoprotein (HDL), total cholesterol, and total/HDL ratio were measured. A positive association between plasma Hsp60 and TNF-α, and a negative association with von Willebrand factor was found. In addition, a significant association between elevated plasma Hsp60 levels, low socioeconomic status, social isolation, and psychological distress in women was demonstrated (Lewthwaite et al., 2002).

More recently psychological stress directly has been shown to result in an increase in the systemic Hsp72 concentration (Campisi et al., 2003; Fleshner et al., 2004; Fleshner et al., 2003; Gazda et al., 2003). In a study by Fleshner and colleagues, exposure of Sprague Dawley rats to a cat resulted in significant increases in circulating serum Hsp72 when measured 2 hours post exposure. No physical contact between the animals occurred thereby negating the possibility that necrotic cell death could account for the increase in systemic Hsp72 (Fleshner et al., 2004). A recent study reports that alpha1-adrenergic receptor-mediated signaling pathways mediate the release of Hsp70 into the circulation (Johnson et al., 2005a; Johnson et al., 2005b; Johnson and Fleshner, 2006). These authors demonstrated that activation of alpha1-adrenoceptors results in a rapid increase in circulating Hsp72 and inhibition of alpha1-adrenoceptors prevents the stress-induced rise in circulating Hsp72 (Johnson et al., 2005a; Johnson et al., 2005b; Johnson and Fleshner, 2006). Although the physiological function of the increased systemic HSP remains incompletely known, mounting evidence suggests that systemic Hsp72 facilitates immunological responses (Johnson et al., 2005a; Johnson et al., 2005b; Johnson and Fleshner, 2006).

Brain and Cerebral Spinal Fluid (CSF)

Guzhova and colleagues demonstrated that glial cells release Hsp72 in response to stimulation (Guzhova et al., 2001). These authors demonstrated that neuronal cells have the ability to take up extracellular Hsp70 and thereby increase their stress tolerance. Human T98G glioma cells released Hsp70 into the culture medium under both basal and heat-stressed conditions. The amount of glial Hsp70 released ranged from 5 to 15 pg/10^6 cells/day, a Albeit exposure of cells to heat shock induced an even grater release of Hsp70. LA-N-5 neuroblastoma cells were shown to take up biotinylated Hsc/Hsp70 from the culture medium. Interestingly, this uptake of extracellular Hsp70 made the cells more resistant to heat shock treatment (44 °C) and staurosporine-induced apoptosis (Guzhova et al., 2001). More recently, experiments were carried out to determine whether the human brain is capable of releasing Hsp72 in response to exercise. Hsp72 release was determined on the basis of the internal jugular venous to arterial balance and results show the human brain releases Hsp72 in response to exercise (Lancaster et al, 2004). In a recent report,

physically active rats were shown to respond more efficiently than sedentary rats when challenged with *E. coli* (Nickerson et al., 2005). Interestingly, peripheral immune challenge resulted in increased expression of Hsp72 in the brain. In this study, adult male Fischer 344 rats were housed with either a mobile or locked running wheel. Six weeks later, rats were injected intraperitoneally with saline or *E. coli* and sacrificed. Physically active rats demonstrated a greater reduction in endotoxin and *E. coli* CFUs and lower levels of circulating endotoxin and cytokines compared with sedentary rats. *E. coli* challenges elicited significantly greater time-dependent increases of both Hsp72 and IL-1beta in hypothalamus, pituitary, and dorsal vagal complex of physically active animals but not sedentary animals (Nickerson et al., 2005). A recent study to determine if pools of circulating Hsp72 in the plasma and CSF mixed, demonstrated that at rest, the concentration of Hsp72 in the CSF was 3-fold higher than in the plasma (Steensberg et al., 2006). During exercise, with and without carbohydrate ingestion, plasma Hsp72 levels increased 5-fold. However, the concentration of Hsp72 in the CSF did not change with exercise, suggesting that the CSF pool of circulating Hsp72 is segregated from that in blood (Steensberg et al., 2006).

CONCLUDING REMARKS AND SUMMARY

From its original description as an intracellular molecular chaperone of naïve, aberrantly folded or mutated proteins and primarily involved in cytoprotection in response to stressful stimuli, mounting evidence clearly demonstrates that (at least) two mechanisms play an important role by which heat shock proteins gain access to the systemic circulation; passive and active release mechanisms. Much work yet remains to determine the exact role of extracellular Hsp72 and conclusively pin point the organ(s) responsible for its release. However, it is clear from numerous clinical trials that modulating heat shock responses are of considerable therapeutic benefit in a wide range of diseases and disorders (Soti et al., 2005; Tytell and Hooper, 2001). Our working hypothesis is summarized in Figure 1. Thus far there are two mechanisms of HSP release; the passive and active release mechanisms. The passive release mechanism occurs when cells are infected (e.g., with lytic viruses) or during trauma (e.g., blunt force trauma and during surgery) or necrosis, resulting in the release of HSP into systemic circulation. We hypothesize that passive release of HSP results in high levels of free HSP and low levels of HSP contained within exosomes (Figure 1). The active release mechanism occurs in response to psychological stress (e.g., fear, immobilization, social isolation) or following stressful stimuli (e.g., heat, UV radiation, heavy metals) or in response to inflammatory mediators (e.g., IFN-γ, TNF-α, IL-1β, IL-10, IL-12) or receptor mediated events including ligation of HSP to its receptor(s) (e.g., Toll-like receptors 2 and 4 with their cofactor CD14, the scavenger receptor CD36, the low-density lipoprotein receptor-related protein CD91, the C-type lectin receptor LOX-1, and another member of the scavenger superfamily SR-A plus the co-stimulatory molecule, CD40. The active release mechanism is mediated by stimulation of the stress response which

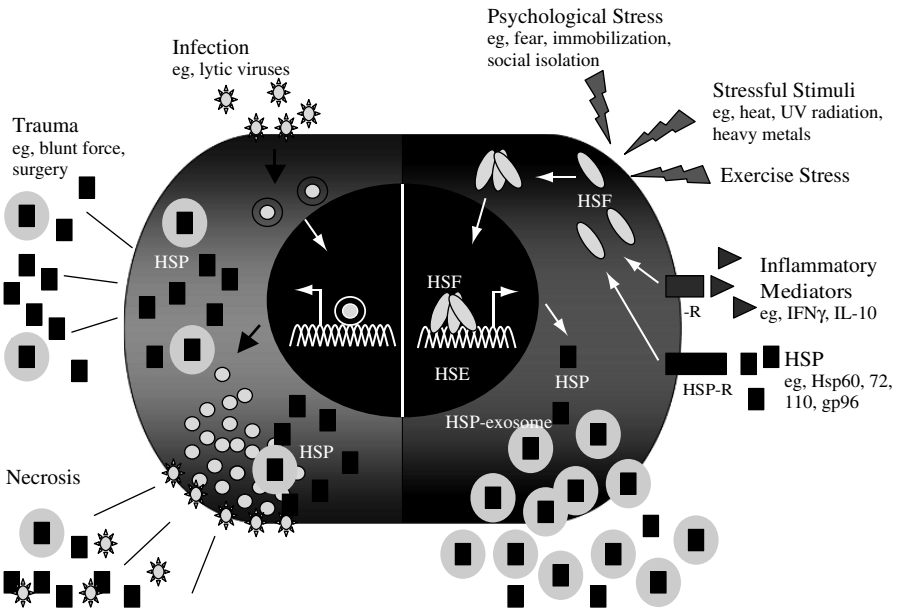

Figure 1. Schematic representation of mechanisms by which HSP is released in circulation. Currently, there are two mechanisms of HSP released; passive (left grey half of the cell) and active (right half of the cell). The passive release mechanism occurs when cells are infected (e.g., with lytic viruses (stars)) or trauma (e.g., blunt force trauma and during surgery) or necrosis, resulting in the release of HSP into systemic circulation. The active release mechanism occurs in response to psychological stress (e.g., fear, immobilization, social isolation) or following stressful stimuli (e.g., heat, UV radiation, heavy metals) or in response to inflammatory mediators (e.g., IFN-γ, TNF-α, IL-1β, IL-10, IL-12) or receptor mediated events including ligation of HSP to its receptor(s) (e.g., Toll-like receptors 2 and 4 with their cofactor CD14, the scavenger receptor CD36, the low-density lipoprotein receptor-related protein CD91, the C-type lectin receptor LOX-1, and another member of the scavenger superfamily SR-A plus the co-stimulatory molecule, CD40. The active release mechanism is mediated by stimulation of the stress response which induces monomeric heat shock factor (HSF) which is in the cytosol to trimerize and translocate to the nucleus, where it binds to the heat shock element (HSE) and activates the synthesis of HSP (filled squares). HSP are released into the circulation as free HSP (filled squares) and within exosomes (circles, with filled squares) to act as a danger signal

induces monomeric heat shock factor (HSF) which is in the cytosol to trimerize and translocate to the nucleus, where it binds to the heat shock element (HSE) and activates the synthesis of HSP (Figure 1). HSP released into the circulation as free HSP and within exosomes act as a danger signal.

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CHAPTER 2

HSP70 PEPTIDE ACTING AS A DANGER SIGNAL FOR NATURAL KILLER (NK) CELLS

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Abstract: Similar to full length Hsp70 protein the 14-mer Hsp70-peptide T-K-D-N-N-L-L-G-R-F-E-L-S-G (TKD), representing part of the C-terminal domain, could be identified as a danger signal for human natural killer (NK) cells. Following incubation with TKD plus low dose IL-2 the cytolytic activity of resting NK cells against Hsp70 membrane-positive tumors was initiated. Concomitantly the cell surface density of activatory receptors including the C-type lectin receptor CD94 was found to be up-regulated. In contrast to normal tissues, tumors frequently present Hsp70 on their cell surface as a tumor-specific recognition structure for IL-2/TKD-activated NK cells. The adoptive transfer of *ex vivo* IL-2/TKD–activated NK cells into tumor-bearing mice resulted in the control of primary tumors, in prevention of distant metastases, and an improved survival. Based on these results tolerability, feasibility, and safety of an adoptive transfer of IL-2/TKD-activated NK cells was tested in a clinical phase I trial in patients with progressive tumor diseases which were refractory to standard therapy. Apart from an excellent safety profile, the cytolytic activity of patient-derived NK cells could be stimulated in 10 of 12 patients by IL-2/TKD. Furthermore, two of five patients receiving more than four treatment cycles showed clinical responses

Keywords: Heat shock protein 70 peptide, NK cells, tumor, immunostimulation, cell-based immunotherapy

Abbreviations: APC, antigen presenting cell; HLA, human leukocyte antigen; HSP heat shock proteins; IL-2, interleukin-2; ILT, immunoglobulin-like transcripts; KIR, killer cell immunoglobulin receptor; MHC, major histocompatibility complex; NK cell, natural killer cell; NCR, natural cytotoxicity receptor; TKD, Hsp70 peptide T-K-D-N-N-L-L-G-R-F-E-L-S-G

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INTRODUCTION

Heat shock proteins (HSP) are highly conserved proteins inhabiting nearly all subcellular compartments. Already under physiological conditions they are overexpressed in a broad range of human tumors and play crucial roles in tumor invasion, metastasis, cell proliferation, differentiation and cell death (Ciocca and Calderwood, 2005). Depending on their localization HSP either exert immune activation or protection against environmental stress. Our laboratory identified membrane-bound Hsp70, the major stress-inducible heat shock protein, as a tumor-specific recognition structure for natural killer (NK) cells (Radons and Multhoff, 2005a, 2005b). In the cytosol, Hsp70 functions as a molecular chaperone supporting folding and transport of a great variety of polypeptides and proteins under both, physiological conditions and following chemical or physical stress stimuli (DeNagel and Pierce, 1992; Hartl, 1996; Lindquist and Craig, 1988). Hsp70 was also found to protect cells from apoptosis by an inhibition of the permeabilization of lysosomal membranes (Jaattela et al., 1998; Nylandsted et al., 2004; Nylandsted et al., 2000). In contrast, extracellular HSP elicit a potent anti-cancer immune response mediated either by the adaptive or innate immune system (Gehrmann et al., 2005). This review will focus on the immunological role of membrane-bound Hsp70 serving as a tumor-specific recognition structure for NK cells. We further present data on the immunostimulatory capacity of an Hsp70-derived peptide, termed TKD, which initiates an immune response against Hsp70 membrane-positive tumor cells in human NK cells. In a phase I clinical trial the adoptive transfer of *ex vivo* IL-2/TKD-activated, autologous NK cells into patients with progressive colorectal and non-small cell lung carcinoma, refractory to standard therapy, was found to be safe, feasible and well tolerated. The encouraging immune and clinical responses of patient-derived NK cells warrant future clinical trials.

NK CELLS AND TUMOR CELL KILLING

Apart from their intracellular chaperoning functions, HSP have been found to play key roles in tumor immunity. Most immunotherapeutic approaches exploit the carrier function of HSP for tumor-derived peptides. Following cross-presentation of HSP-chaperoned peptides on MHC class I molecules (Arnold-Schild et al., 1999; Basu et al., 2001; Binder et al., 2000; Binder et al., 2004; Sondermann et al., 2000) an antigen-specific CD8+ T cell response is thus initiated (Binder et al., 2001; Doody et al., 2004; Schild et al., 1999; Srivastava et al., 1998). However, even in the absence of immunogenic peptides, HSP serve as danger signals for the host's immune system (Asea et al., 2000). Exosomes have recently been described as export vehicles for Hsp70 from the endosomal compartment into the extracellular milieu (Bausero et al., 2005; Gastpar et al., 2005; Lancaster and Febbraio, 2005).

NK cells comprising 5–20% of peripheral blood lymphocytes (PBL) are important players of the innate immune system that control bacterial, virus infections and mediate protection against cancer (Trinchieri, 1989). The low affinity Fcγ receptor

CD16 which is responsible for antibody-dependent cellular cytotoxicity (ADCC) (Lanier et al., 1988) and the homophilic adhesion molecule CD56 are frequently expressed on NK cells. More recently, the molecular nature of a number of killer cell inhibitory and activating receptors was identified that control the activation status of NK cells. These receptors either belong to the killer cell immunoglobulin receptor (KIR), the immunoglobulin-like transcript (ILT), C-type lectin receptor families (Lanier et al., 1998), or belong to the natural cytotoxicity receptors (NCR) (Moretta et al., 2001). Depending on their intracellular immunoreceptor tyrosine-based inhibitory or activating motifs (ITAM/ITIM) these receptors mediate either activating or inhibiting signals (Long, 1999; Moretta et al., 2001). A variety of different MHC class I allele groups including HLA-C were determined as natural ligands for NK cell receptors. According to the "missing self" theory (Ljunggren and Karre, 1990) tumor cells with altered or lacking MHC class I expression pattern provide ideal targets for the cytolytic attack of NK cells because the inhibitory signal of self MHC molecules is missing. However, evidence is accumulating that apart from "missing self" additional activating ligands are necessary for an efficient NK cell response. For a group of NCRs including NKp30, NKp44, NKp46 and NKp80 (Moretta et al., 2001) yet undefined tumor-specific ligands are presently discussed. For the homomeric C-type lectin receptor NKG2D, non-classical stress-inducible MHC class I-related chain MICA and MICB glycoproteins, the glycosylphosphatidylinositol-linked UL-16 binding proteins, the retinoic acid early inducible-1 (RAE-1) protein and HA60, a minor histocompatibility antigen, serve as target structures (Bauer et al., 1999; Cosman et al., 2001; Lanier et al., 1998).

Under physiological conditions non-classical HLA-E molecules presenting leader peptides of HLA-A,-B,and-Calleles provide ligands for the inhibitory heteromeric receptor complex CD94/NKG2A. Following stress, an Hsp60-associated signaling peptide competes with HLA-A,-Band-Cleader peptides for binding to HLA-E. HLA-E/Hsp60-peptide complexes are no longer recognized by the inhibitory receptor complex CD94/NKG2A (Hickman-Miller and Hildebrand, 2004; Michaelsson et al., 2002). These data imply that environmental stress modulates the activation status of NK cells.

In line with these findings, we identified Hsp70 as a trigger factor for CD94-positive NK cells (Gross et al., 2003a; Gross et al., 2003c). Mapping of the Hsp70 sequence revealed that the 14-mer peptide T-K-D-N-N-L-L-G-R-F-E-L-S-G (aa 450–463) derived from the C-terminal substrate-binding domain is exposed to the extracellular milieu of tumor but not of normal cells. The localization of this 14-mer peptide TKD within the Hsp70 sequence is illustrated schematically in Figure 1.

Furthermore this TKD peptide in combination with low dose IL-2 was found to exhibit an identical immunostimulatory capacity on NK cells like full length Hsp70 protein or the C-terminal domain Hsp70-C (Botzler et al., 1998; Multhoff et al., 1999). Incubation of NK cells with the cytokine IL-2 plus soluble Hsp70 protein, Hsp70-C or TKD peptide enhances the cell surface density of a number of activating NK cell receptors including CD94 (Gross et al., 2003a).

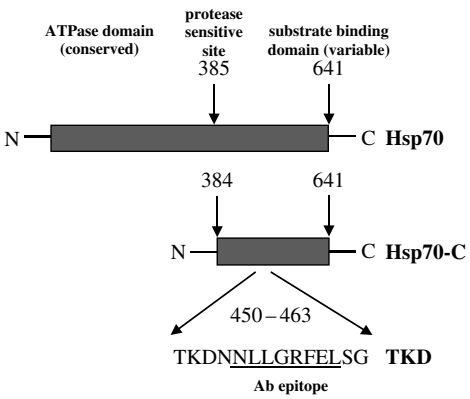

Figure 1. Localization of the TKD sequence within the C-terminal domain of Hsp70

Binding studies also suggested an important role of CD94 in the interaction of NK cells with Hsp70 (Gross et al., 2003c). Concomitant with an up-regulated CD94 cell surface density the cytolytic and migratory capacity of NK cells against Hsp70 membrane-positive tumors was initiated (Gastpar et al., 2005).

A broad screening program of human tumors revealed that Hsp70, the major stress-inducible member of the Hsp70 group, is present on the plasma membrane of a variety of human tumors including colon, lung, pancreas, mammary, head and neck and metastases derived thereof (Chen et al., 2002; Multhoff et al., 1995a; Multhoff et al., 1995b). Also bone marrow-derived leukemic blasts are frequently Hsp70 membrane-positive (Gehrmann et al., 2003). Interestingly, metastases and relapsed tumors revealed an enhanced cell surface density of Hsp70 (unpublished observations). Since the corresponding normal tissues and bone marrow cells of healthy individuals were always found to be Hsp70 membrane-negative, membrane bound Hsp70 can be considered as a tumor-selective recognition structure (Figure 2).

The cell surface density of Hsp70 on tumors could be further enhanced by clinically applied reagents and procedures including membrane-interactive alkyl-lysophospholipids (Botzler et al., 1996), cytostatic drugs including taxoides and vincristin-sulfate (Gehrmann et al., 2002), cyclooxygenase (COX-1/2) inhibitors, acetyl salicyl acid, insulin sensitizers (Gehrmann et al., 2004), hyperthermia (Milani and Noessner, 2006), and photodynamic therapy (Korbelik et al., 2005). This increased Hsp70 membrane expression renders them more sensitive to the cytolytic attack mediated by NK cells. Although a variety of other chaperones were found to be present on the plasma membrane (Shin et al., 2003), predominantly cell surface-expressed members of the Hsp70 and Hsp90 group are able to stimulate the innate immune system (Chen et al., 2002; Multhoff et al., 1995b; Pilla et al., 2005).

The mechanism of lysis of Hsp70 membrane-positive tumors was characterized as a granzyme B-mediated but perforin-independent apoptosis (Gross et al., 2003b). Apoptosis is induced by the interaction of granzyme B with surface-bound Hsp70

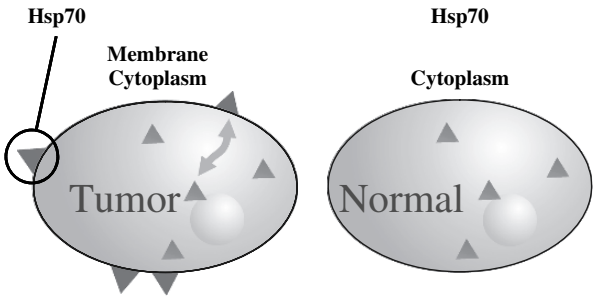

Figure 2. The cytosolic Hsp70 protein is selectively expressed on the plasma membrane of tumor cells but not on normal tissues

or the 14-mer Hsp70 peptide TKD, since both of them have the capacity to bind granzyme B (Beresford et al., 1998). This hypothesis is supported by the finding that the enhanced cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells could be blocked by the Hsp70-specific antibody. The epitope of this antibody is part of the 14-mer TKD peptide sequence as illustrated in Figure 2.

FROM BENCH TO BEDSIDE

Previous reports on different animal models revealed that IL-2-activated NK cells are able to induce regression of established lung and liver tumors and metastases of different origin (Schwarz et al., 1989; Vujanovic et al., 1995; Whiteside et al., 1998; Yasumura et al., 1994). The control of tumors and metastases corresponded with an extended life expectancy. In contrast, the injection of IL-2 alone was significantly less efficient compared to an adoptive transfer of IL-2-activated NK cells. These data indicated that cytokine-stimulated NK cells exert beneficial effects on the control of tumors and distant metastases in immunocompetent and immunocompromised animals (Basse et al., 2001; Koda et al., 2003; Kondo et al., 2003).

As mentioned earlier, screening of primary human tumors and metastases derived there of revealed that Hsp70 is frequently expressed on the cell surface of malignant cell types. In contrast, the corresponding normal tissues were always found to be Hsp70 membrane-negative. Therefore, we hypothesized that membrane-bound Hsp70 might act as a tumor-specific recognition structure for the immune system. Since we observed that the cytolytic activity of NK cells *in vitro* could be further enhanced by incubation with IL-2 plus Hsp70 peptide TKD, we asked the question as to whether these NK cells might be superior in the eradication of tumors compared to NK cells that had been stimulated with IL-2 alone. In two independent xenograft tumor mouse models we studied the immunological effects of *ex vivo* IL-2/TKDactivated NK cells (Moser et al., 2002). A single intravenous (i.v.) injection of IL-2/TKD-activated NK cells resulted in a significant regression of colon tumors in SCID/beige mice. In the absence of TKD, these effector cells were less efficient in the suppression of the growth of Hsp70 membrane-positive tumors (Multhoff

et al., 2000). We next studied the efficacy of IL-2/TKD-activated NK cells in the eradication of pancreatic tumors and metastases derived thereof (Stangl et al., 2006). Pancreatic carcinoma is the fifth leading cause of cancer related death in humans and is highly refractory to standard therapy. Phenotypic analysis revealed that Hsp70 is frequently present on the plasma membrane of pancreatic carcinomas including our model cell line Colo357. An orthotopic (o.t.) injection of Colo357 cells resulted in rapidly growing primary pancreatic tumors and in metastastic dissemination into the liver. In line with *in vitro* migration assays, IL-2/TKD-activated human NK cells had the capacity to infiltrate pancreatic tumors and liver metastases in tumor-bearing mice. These data are in line with data of the group of Yang who also showed the presence of cytokine-activated NK cells in lung metastases of immunocompetent mice (Yang et al., 2003).

We additionally analyzed life expectancy of tumor-bearing mice after a single i.v. injection of pre-activated effector cells. As summarized in Figure 3, immunodeficient control mice showed first signs of tumor disease from day 18 onwards; the maximum survival time was 35 days. Adoptive transfer of pre-activated T cells only marginally improved life expectancy with all animals dying from progressive tumor disease on day 37. In contrast, a single injection of IL-2/TKD-activated NK cells significantly prolonged the survival of the mice, with more than 60% of the mice still alive on day 72. Thus, our *in vivo* mouse data imply that IL-2/TKDactivated NK cells might provide a novel therapeutic strategy for the treatment of therapy-refractory, Hsp70-positive pancreatic tumors.

As mentioned before, a tumor-selective cell surface localization of Hsp70, the major heat-inducible member of the Hsp70 group, could be correlated with an increased sensitivity to lysis mediated by IL-2/TKD-activated human NK cells,

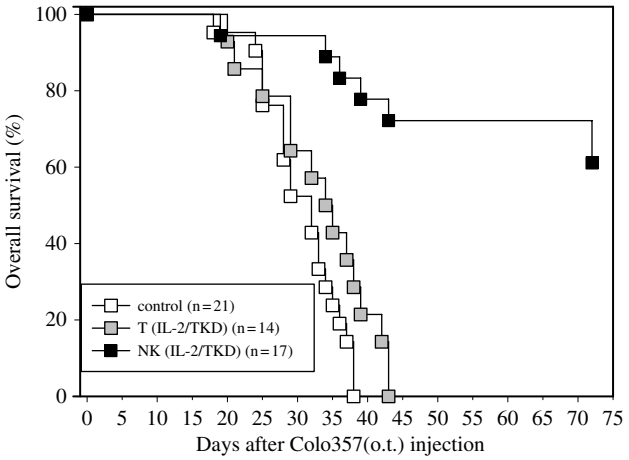

Figure 3. Overall survival of tumor-bearing SCID/beige mice treated either with PBS (control) or with IL-2/TKD-activated T or NK cells. Mice were immunoreconstituted by i.v. injection into the tail vein on day 15 after tumor cell inoculation

and therefore might be of clinical relevance. We also demonstrated that NK cells stimulated with IL-2/TKD have the capacity to eradicate Hsp70 membrane-positive tumors *in vitro* and in tumor mouse models. These findings encouraged us to test the efficacy of an adoptive transfer of *ex vivo* IL-2/TKD-activated NK cells in a clinical phase I trial. Patients with multiple metastasized colorectal and non-small cell lung carcinomas who failed standard therapy were enrolled into the study (Krause et al., 2004).

As already seen for healthy donors also patient-derived NK cells showed a significant increase in the membrane expression of CD94 following stimulation with IL-2/TKD. Concomitantly, the cytolytic activity against Hsp70 membrane-positive tumors was augmented *in vitro*. Concerning tumor response, one patient was in stable disease during therapy by formal staging and another patient showed partial response in a pulmonal metastasis following four repeated treatment cycles. This finding was not expected since all patients were in a progressive tumor stage and refractory to standard chemotherapy before entering the study. Taken together, the adoptive transfer of IL-2/TKD-activated NK cells was feasible, safe, and very well tolerated even after repeated treatment cycles. Immunological results and clinical responses in two of five therapy-refractory, multiple metastasized patients warrant additional studies in patients with lower tumor burden and an established Hsp70 membrane-positive phenotype.

CONCLUSION

Recent studies implied an important role of extracellularly localized and membrane bound HSP in mediating a cellular immune response against cancer. Our *in vivo* mouse data encouraged us to test tolerability, feasibility, and safety of *ex vivo* IL-2/TKD-activated, autologous NK cells in the therapy of patients with locally advanced, metastasized colorectal and non-small cell lung carcinomas. These data indicated that repeated treatments with IL-2/TKD-activated NK cells were well tolerated, feasible, and safe. Biological responses imply that even heavily pre-treated patients with Hsp70 membrane-positive tumors might profit form an adoptive transfer of IL-2/TKD-activated NK cells. Thus, our data might have future clinical implications with respect to the development of a cellular immunological approach based on *ex vivo* stimulated NK cells as an adjuvant therapy in patients with progressive tumor disease and a high risk for metastatic dissemination.

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CHAPTER 3

MECHANISMS OF STRESS-INDUCED CELLULAR HSP72 RELEASE

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Summary: The heat shock proteins are a family of highly conserved proteins with critical roles in maintaining cellular homeostasis and in protecting the cell from stressful conditions. While the critical intracellular roles of heat shock proteins are undisputed, evidence suggests that the cell possess the necessary machinery to actively secrete specific heat shock proteins both basally and in response to cellular stress. This chapter will discuss the secretory mechanisms identified to date that allows cells to release specific heat shock proteins. Importantly, several studies have established that this release is the result of an active secretory process, as opposed to non-specific processes such as cell lysis. Importantly, while the classical protein secretory pathway via the endoplasmic reticulum and Golgi apparatus does not seem to be involved in the stress-induced release of heat shock proteins, we discuss the evidence that lipid-rafts and exosomes are important mediators of both basal and stress-induced heat shock protein release

Keywords: Heat shock proteins, HSP release, cell stress, exosomes

INTRODUCTION

The heat shock proteins (HSP) are a family of highly evolutionary conserved proteins found in all eukaryotes and prokaryotes. Members of the HSP family are primarily classified according to their molecular size, e.g. HSP110, HSP90, HSP70 and HSP40, and contain both constitutive and stress-inducible members. HSP are quintessential intracellular proteins whose primary function is to interact with naïve and denatured proteins to prevent the aggregation of aberrantly folded proteins, facilitate the folding of naïve proteins, facilitate the refolding of denatured proteins, and to aid intracellular protein trafficking (Gething and Sambrook, 1992). One of the most fascinating aspects of HSP biology is that induction of the heat shock response confers cytoprotection and thermotolerance to subsequent and otherwise

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lethal cellular stressors. These aspects of HSP biology underscore the critical role of HSP within the intracellular environment; however, a growing body of literature is providing evidence that specific HSP can be actively released from cells.

While this idea may seem counterintuitive, extracellular HSP can exert important biological functions. Extracellular HSP have garnered particular interest within the field of immunology. Several studies have demonstrated that HSP60 and HSP70 are ligands for a family of receptors, the toll-like receptors, which are critical to the induction of both innate and adaptive immune responses (Asea et al, 2000; Asea et al, 2002; Vabulas et al, 2001; Vabulas et al, 2002). Binding of HSP to specific TLR family members results in activation of NF-kB, a transcription factor critical to the up-regulation of a number of genes central to the generation of effective immune responses. However, these studies were conducted by treating cells *in vitro* with recombinant HSP, and whether HSP play an important role in the generation of immune responses *in vivo*, and whether the release of these HSP occurs via an active secretory process or by the killing of host cells by the invading pathogen and subsequent 'leaking' of HSP into the extracellular environment, has yet to be resolved.

RELEASE OF HEAT SHOCK PROTEINS IN VIVO

Intriguingly, *in vivo* evidence does indeed suggest that in response to various physiological stimuli, HSP can be actively released and subsequently accumulate in the systemic circulation. Several years ago, we demonstrated that the circulating concentration of a specific member of the HSP family, HSP72 – the stress-inducible member of the HSP70 family, was significantly increased following a bout of moderate-intensity exercise (Walsh et al, 2001). 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, to the extracellular environment.

While an increase in temperature was the first stimulus identified that promoted the induction of HSP (and hence why they are called heat shock proteins), numerous other stimuli promote the induction of HSP. Interestingly, skeletal muscle contraction is associated with a number of cellular stresses that may induce the heat shock response, e.g. increases in muscle temperature, changes in muscle pH, oxidative stress, mechanical stress and substrate depletion. On this basis, we reasoned that the skeletal muscle may be the source of the increase in the systemic HSP72 concentration. However, determination of the arterial-venous balance over the contracting musculature showed that while the intracellular HSP72 content of the contracting skeletal muscle increased, HSP72 was not released from the contracting skeletal muscle (Febbraio et al, 2002).

It has been shown that exercise induces the expression of numerous HSP, including HSP72, in the liver of exercised rodents (Kregel and Moseley, 1996; Salo et al, 1991). To examine whether liver HSP72 release contributed to the exercise-induced increase in the systemic HSP72 concentration, we (Febbraio et al, 2002) tested the hypothesis that hepatosplanchnic tissues release HSP72 during

exercise. The arterial-venous balance of HSP72 was determined via catheterisation of the brachial artery and hepatic vein. The results of this study demonstrate that the hepatosplanchnic tissues release HSP72 during exercise and that this release contributes, in part, to the exercise-induced increase in the systemic HSP72 concentration. Importantly, a full blood analysis revealed no signs of liver damage or dysfunction as a result of the experimental protocol. Therefore, it was concluded that exercise induces the release of HSP72 from liver via a specific exocytotic pathway, as opposed to non-specific processes such as cell lysis.

It is well documented that specific cells within the brain can synthesise HSP72 in response to various cellular stresses, and, importantly, it has been shown that glial cells actively release HSP72 following stimulation (Guzhova et al, 2001). Therefore, we conducted a study to examine whether the human brain was capable of releasing HSP72 in response to exercise. HSP72 release was determined on the basis of the internal jugular venous to arterial balance and we were able to demonstrate that indeed the human brain is capable of releasing HSP72 in response to exercise (Lancaster et al, 2004).

In addition to exercise stress, it has recently been demonstrated that psychological stress results in an increase in the systemic HSP72 concentration. In an interesting study, Fleshner and colleagues exposed Sprague Dawley rats to a cat for 2h. Importantly, no physical contact between the animals occurred. Cat exposure resulted in a marked increase in the extracellular HSP72 concentration and this increase was ameliorated by adrenalectomy, suggesting that adrenal hormones are crucial to the psychological stress-induced increase in extracellular HSP72 (Fleshner et al, 2004).

Collectively, these data demonstrate that both physiological and psychological stress stimulate the release of HSP72 from intracellular locales into the extracellular environment. Importantly, the data strongly argue against cell necrosis being a key mediator of HSP72 release. We did not observe any cell necrosis in our exercise studies and the increase in extracellular HSP72 induced by cat exposure observed by Fleshner and colleagues is highly unlikely to have been mediated by cell necrosis. Thus, it appears that in response to stressful conditions, the cell contains the necessary means to efficiently export specific HSP. In the subsequent section we will discuss some of the recent studies that have examined the mechanistic basis by which cells are able to release HSP in response to stressful cellular conditions.

CELLULAR STRESS INDUCES THE RELEASE OF SPECIFIC HSP FROM MAMMALIAN CELLS: MECHANISMS OF ACTION

The discovery that mammalian cells have the capacity to actively release specific HSP was originally made over 15 years ago. In 1989, it was demonstrated that mammalian cells possessed the capacity to release selective HSP in both the basal and stress-induced state (Hightower and Guidon, 1989). In this study, culture medium from rat embryo cells, incubated at either 37 or 45 °C for 10 minutes followed by a 2h recovery period, was collected and subjected to two-dimensional polyacrylamide gel electrophoresis. It was shown that cultured cells released a

selective panel of proteins in the basal state, and in response to heat shock HSP72 was readily detectable in the cell culture medium. To address the cellular mechanism by which these proteins were released cells were treated with pharmacological inhibitors of the common secretory pathway. Intriguingly, neither monensin (a Na^{2+} ionophore that disrupts the structure of the Golgi apparatus and inhibits vesicular transport) nor colchicine (an inhibitor of microtubule assembly) had any effect on basal, or stress-induced, HSP release. Importantly, further experiments provided strong evidence that the observed HSP release is indeed an actively regulated process as opposed to a non-specific release mechanism such as cell lysis (Hightower and Guidon, 1989). While these data convincingly demonstrate that mammalian cells are indeed capable of releasing stress proteins, the cellular mechanism/s facilitating this transport remained, until recently, unknown.

Cells are able to secrete proteins via either classical or non-classical secretory pathways. Protein transport through the classical pathway occurs via the targeting of newly synthesised proteins to the endoplasmic reticulum and subsequent transfer to the Golgi apparatus where the protein may undergo modification before being packaged into secretory vesicles. These vesicles then fuse with the plasma membrane thus allowing the protein to exit the cell and interact with the extracellular environment. Evidence from several independent laboratories supports the notion that stress-induced HSP72 release from cells is not dependent upon the classical pathway, as is evidenced by the inability of inhibitors of the classical pathway to block stress-induced HSP72 release (Broquet et al, 2003; Hightower and Guidon, 1989; Lancaster and Febbraio, 2005).

Several proteins are secreted through non-classical secretory pathways, e.g. interleukin-(IL)-1β, macrophage inhibitory factor, fibroblast growth factor-2 and members of galactin family (Nickel, 2005). Recently, it was demonstrated that plasma membrane-associated microdomains, also termed lipid-rafts, expressed HSP72, and that heat shock resulted in a marked elevation in lipid-raft HSP72 content (Broquet et al, 2003); this effect was insensitive to treatment with Brefeldin A (an inhibitor of the classical transport pathway). To examine the role of lipid rafts in mediating stress-induced cellular HSP72 release, cells were treated with the cholesterol depleting agent methyl-β-cyclodextrin (cholesterol is an integral component of lipid rafts and its removal from the cell severely compromises raft integrity). Crucially, cellular stress-induced (heat shock for 1h at 43 °C) HSP72 release, compared to control (37 °C for 1h), was markedly inhibited by methyl-β-cyclodextrin treatment, providing evidence that lipid rafts play an important role in mediating the stress-induced release of HSP.

EVIDENCE THAT EXOSOMES PLAY A ROLE IN STRESS-INDUCED HSP72 RELEASE

In our laboratory, we sought to determine whether heat shock at physiological/pathophysiological temperatures, *i.e.* during febrile states, stimulated the release of HSPs, and whether stress-induced HSP72 release displayed cell specificity. Initially, we confirmed that stress-induced HSP72 release occurred

independently of the classical secretory pathway (based on the inability of Brefeldin A treatment to inhibit stress-induced HSP72 release), and that non-specific processes such as cell lysis could not account for stress-induced release of HSP72 (Lancaster and Febbraio, 2005). However, and in contrast to the results of Broquet and co-workers, we were unable to confirm a role for lipid rafts in stress-induced HSP72 release. Using methyl-β-cyclodextrin (the cholesterol depleting agent) to disrupt lipid raft function, our results demonstrate that lipid rafts do not play a role in stress-induced HSP72 release from human peripheral blood mononuclear cells (PBMCs) (Lancaster and Febbraio, 2005). Importantly, our data demonstrate that methyl-β-cyclodextrin highly effectively depleted intracellular cholesterol levels in a dose-dependent manner, confirming that our methyl-β-cyclodextrin treatment regimen was efficacious.

Almost 10 years ago (Multhoff and Hightower, 1996) it was hypothesised that small vesicles, termed 'exosomes' (Fevrier and Raposo, 2004), secreted following the fusion of multivesicular bodies (MVBs) with the plasma membrane may provide a secretory pathway allowing cells to actively release specific HSPs. In support of this notion, Johnstone and colleagues, using an antibody against the constitutive (HSC70) and inducible (HSP72) forms of HSP70, provided evidence that exosomes contain members of the 70kDa family of HSP (Mathew et al, 1995). Furthermore, recent work has identified HSP90, HSP90α and HSC70 in exosomes derived from tumour cells (Hegmans et al, 2004). These studies led us to investigate the possibility that, in response to stressful cellular conditions, cells may release HSP72 via an exosomal pathway. Cells secrete exosomes in the basal state, and their rate of release is affected by changes in intracellular calcium levels (Savina et al, 2003). Therefore, we investigated whether heat shock *per se* increased exosome secretory rate, thus facilitating an increase in cellular HSP72 release. However, heat shock (1h at either 40 or 43 °C followed by 4h recovery at 37 °C) had no effect on exosomal secretory rate compared to control conditions (5h at 37 °C) (Lancaster and Febbraio, 2005). Next, we examined whether heat shock increased exosomal HSP72 content. Importantly, heat shock resulted in a marked increase in the level of HSP72 within isolated exosomes (Lancaster and Febbraio, 2005). These data demonstrate that exosomes do indeed provide a secretory vesicle facilitating the release of HSP72 in response to cell stress. Further support for the idea that exosomes facilitate HSP72 release in response to cellular stress was recently provided by Clayton and colleagues (Clayton et al, 2005), who provide evidence that in response to heat shock, exosomes derived from a variety of B-cell lines have a markedly elevated HSP content (HSP72, HSC70, HSP27 and HSP90). In contrast with our data, Clayton and colleagues demonstrate that heat shock results in a small increase in exosomal secretory rate. Thus, an increase in both exosomal HSP content and secretory rate contribute to the cellular release of HSP.

While the classical protein transport pathway via the ER-Golgi has been considered not to be involved in mediating stress-induced HSP release (see above), a very recent study has identified a role for the classical vesicular transport pathway in HSP70 exocytosis (Evdonin et al, 2006). These data demonstrate that in A431

squamous carcinoma cells and human HaCaT keratinocytes, the early HSP70 release (within 30 minutes following heat shock) is mediated by the classical protein transport pathway, as demonstrated by the sensitivity of this process to brefeldin A and monensin, inhibitors of classical protein transport pathway.

Collectively, the current data demonstrate that exosomes provide a secretory vesicle facilitating the exocytosis of HSP in response to stressful cellular conditions. This effect is primarily mediated via a heat shock-induced increase in exosomal HSP content, although an increase in exosomal secretory rate (Clayton et al, 2005) may also contribute to increases in cellular HSP72 release. Furthermore, the work of Broquet and colleagues clearly demonstrates that lipid rafts play an important role in HSP72 export from heat shocked epithelial cells (Broquet et al, 2003). Exosomes are secreted from several hematopoietic cells, *e.g*. T- and B-lymphocytes, dendritic cells, macrophages, and platelets (Denzer et al, 2000), and it is possible that stress-induced HSP release via exosomes may be specific to these cell types.

CONCLUSION

The results of the studies described above clearly demonstrate that the exocytosis of HSP is mediated via a number of distinct mechanisms and it appears that different cell types utilise distinct mechanisms of release. Despite these cell type differences in the mechanism of release, HSP exocytosis, both basally and in response to cellular stress, is a highly conserved response. Future studies will be required to confirm the involvement of specific exocytotic pathways in HSP release from different cell types. Furthermore, and perhaps most importantly, given the highly conserved nature of HSP release from cells, what function does HSP secretion serve?

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CHAPTER 4

ROLES OF EXTRACELLULAR HEAT SHOCK PROTEINS: A NEW SENSE OF DANGER

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Abstract: The Heat shock proteins (HSP) were considered for many years to be intracellular proteins that were upregulated in response to physiological stress. Intracellular HSP have many important functions: as protein-folding machines, or chaperones; the protection of cells in response to stress; and the protection of cells against apoptosis. HSP have since been found to be present outside of the cell, and much research also now focuses on the importance of extracellular HSP and their effects on immune responses. This review will explore the potential roles of extracellular HSP and their significance in disease processes.

Keywords: Cytokines; heat shock proteins; inflammation; receptors

EXTRACELLULAR HSPS

HSP, in this case Hsp60, were first reported to be outside the cell in a glial cell culture system (Tytell et al., 1986). Subsequently there have been numerous studies demonstrating that HSP are present on the surface of cells (Multhoff et al., 1997; Xu et al., 1994), released from viable cells (Bausero et al., 2005; Guzhova et al., 2001; Hightower and Guidon, 1989; Hunter-Lavin et al., 2004a) and present in serum (Pockley et al., 1999; Pockley et al., 1998). The importance of these extracellular HSP is clear from three basic observations:

HSP such as Hsp72 and Hsp60 are very immunogenic, inducing activation of dendritic cells and complement, and the production and secretion of cytokines (Pockley, 2003; Prohaszka and Fust, 2004; Prohaszka et al., 2002; van Eden et al., 2003).

Antibodies to HSP can be found in the majority of the population (Lewthwaite et al., 2002; Xu et al., 1994; Xu et al., 1993)

Serum concentrations of HSP respond to a number of stressors – disease (Hunter-Lavin et al., 2004b; Wright et al., 2000b; Xu et al., 2000b), exercise (Bacelar et al., 2006 In Press; Milne and Noble, 2002; Walsh et al., 2001) and psychological stress (Campisi and Fleshner, 2003).

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HSPS IN SERUM

Several different HSP have been identified in serum (Table 1). The most studied are Hsp72 and Hsp60. Currently serum Hsp10 and Grp94 seem to be restricted to pregnancy and type 1 diabetes respectively (Cavanagh, 1996; Pagetta et al., 2003) with serum Hsp27 reported in only one study showing presence in controls and increased levels in breast cancer patients (Fanelli et al., 1998). Hsp72 and Hsp60 are present in the serum of normal humans (Lewthwaite et al., 2002; Pockley et al., 1999; Xu et al., 2000b) and are elevated by many disease states (Hunter-Lavin et al., 2004b; Pockley et al., 2003; Pockley et al., 2000; Wright et al., 2000b; Xu et al., 2000b).

Oxidative stress is an important factor in vascular disease and diabetes. Hsp72 is induced by oxidative stress (Marini et al., 1996; Maron et al., 2002), and is increased in the serum of patients with peripheral and renal vascular disease (Wright et al., 2000b). Type 2 diabetes patients can have elevated homocysteine, suggested to be both a cause and effect of oxidative stress (Doshi et al., 2002). Treatment of these patients with folate to reduce homocysteine levels and improve vascular function resulted in a decrease in serum Hsp72 levels (Hunter-Lavin et al., 2004b). Similar data has been reported for Type 1 diabetes patients with ketoacidosis: patients had elevated serum Hsp72 on presentation at hospital, which was reduced within 24 hours of treatment (Oglesbee et al., 2005). Data such as these would seem to suggest that Hsp72 and Hsp60 are indicative of disease and that a reduction in the stressor will reduce the HSP level. Although most studies show increased HSP in disease, others have reported exactly the opposite – an inverse relationship between the HSP and disease state (Pockley et al., 2003). Non-disease related physiological stresses also affect HSP. Intense exercise results in an increase in serum Hsp72 (Bacelar

Table 1. Heat Shock Proteins found in serum

HSP	Patient group	References
Hsp10	Pregnancy	(Cavanagh 1996)
Hsp27	Normal Breast cancer patients	(Fanelli et al. 1998)
Hsp60	Normal Cardiovascular Disease Atherosclerosis	(Pockley et al. 1999; Schett et al. 1999; Pockley et al. 2000; Xu et al. 2000; Lewthwaite et al. 2002; Pockley et al. 2002)
Hsp72	Normal Peripheral and renal vascular disease Atherosclerosis Type 1 and type 2 diabetes Surgery	(Pockley et al. 1998; Pockley et al. 2000; Wright et al. 2000; Rea et al. 2001; Walsh et al. 2001; Pockley et al. 2002; Hunter-Lavin et al. 2004; Oglesbee et al. 2005)
Grp94	Type 1 diabetes	(Pagetta et al. 2003)

et al., 2006 In Press; Walsh et al., 2001) which is intensity dependant (Fehrenbach et al., 2005; Milne and Noble, 2002).

The potential sources of serum HSP, and therefore the locations to look for mechanisms by which changes in serum concentrations occur, are necrotic cells (Basu et al., 2000; Saito et al., 2005), damaged cells or active release from viable cells (Bausero et al., 2005; Broquet et al., 2003; Guzhova et al., 2001; Hunter-Lavin et al., 2004a). HSP do not have a peptide leader sequence targeting secretion and it has been predicted that necrosis would be the mechanism by which they are released into the extracellular matrix (Johnson and Fleshner, 2006b; Matzinger, 1994; Saito et al., 2005). However, the increase in serum Hsp72 resulting from the imposition of a psychological stress (Campisi and Fleshner, 2003) suggests that cellular necrosis and tissue damage cannot be the only source.

SOURCES OF EXTRACELLULAR HSPS

How HSP interact with other cells may well be determined by the way in which they are delivered/presented into the extracellular environment. The opportunities for an interaction between HSP and other cells, including cells of the immune system, result from: cell surface expressed HSP, release of HSP from damaged and necrotic cells, and active secretion.

Cell Surface Expression of HSPS

Expression of HSP on tumor cell surfaces is important in directing immune responses against tumors. Cell surface Hsp72 induces appropriate NK and CTL responses to tumors (Botzler et al., 1998; Chen et al., 2002; Gastpar et al., 2005; Gehrmann et al., 2005). The identification of the Hsp72 peptide sequence involved in, and the mechanism of, the activation of NK cells is fully described in Chapter 2 of this volume. Hsp60 is expressed on the surface of endothelial cells under stress conditions and plays an important role in the induction of inflammation early in the development of atherosclerosis (Chen et al., 1999; Perschinka et al., 2003; Xu et al., 1994). Hsp72 surface expression seems to be limited to tumor cells (Multhoff et al., 1997), whereas Hsp60 is expressed at the endothelial cell surface after stress (Xu et al., 1994). Surface expressed HSP, therefore, have a role in stimulating both the innate and adaptive system. Because the interaction must require cell-cell contact the cell surface expressed HSP can produce a very localized response.

HSP Release from Damaged and Necrotic Cells

Necrotic cells release their contents into the extracellular environment so it is not surprising that HSP can be released in this manner. As would be expected in an unregulated release of cellular contents a range of HSP are found in the extracellular matrix during and after necrosis (Basu et al., 2000; Berwin et al., 2001; Saito et al., 2005). Necrotic cells, but not apoptotic cells, release Hsp72, Hsp90, grp94,

and calreticulin (Basu et al., 2000). The increased release of HSP from necrotic rather than apoptotic cells has been used to support their roles as danger signals, as apoptosis is a controlled process involved in cell homeostasis and there would normally be no reason to alert the immune system to cells undergoing apoptosis (Johnson and Fleshner, 2006a; Matzinger, 1994; Todryk et al., 2003). Release from necrotic cells, however, is not only due to simple coincidental loss of HSP resulting from damage to the cell. Necrotically dying cells were recently shown to increase HSP expression prior to death by activation of HSF1 (Saito et al., 2005), showing a stress response. Hsp90, Hsp72, Hsp73, Hsp60, Hsp47, Hsp40, and Hsp27 were released from the dying cells in response to acrylamide treatment, leading to the term 'dying messages' being suggested by the authors as well as 'danger signals' (Saito et al., 2005).

HSP Release from Viable Cells

Release of HSP from viable cells was recognized in the 1980s from glial and rat embryo cells (Hightower and Guidon, 1989; Tytell et al., 1986). Using inhibitors the HSP release was shown to be *via* an alternative route to the traditional/classical secretion pathway (Hightower and Guidon, 1989). This early work was paid little attention for many years, despite the development of the danger hypothesis and the proposed role of HSP as danger signals. However, relatively recently several workers have demonstrated the release of HSP from a variety of cell types, including numerous tumor cell lines, peripheral blood mononuclear cells (PBMCs), and B and T-lymphocytes (Table 2) (Barreto et al., 2003; Bausero et al., 2005; Broquet et al., 2003; Davies et al., 2006; Gastpar et al., 2005; Hunter-Lavin et al., 2004a; Lancaster and Febbraio, 2005; Schett et al., 1999; Wang et al., 2004). As the majority of work has been on Hsp72 release we will concentrate discussion on this protein.

Although many different cell types have been shown to release HSP, some common themes can be found between the studies. Hsp72 is released from many cells under normal culture conditions (Davies et al., 2006; Guzhova et al., 2001; Hunter-Lavin et al., 2004a; Jean-Pierre et al., 2006), and this release can be stimulated by elevated temperature (Broquet et al., 2003; Guzhova et al., 2001; Hightower and Guidon, 1989; Hunter-Lavin et al., 2004a; Lancaster and Febbraio, 2005).

Hsp72 has no peptide leader sequence targeting secretion and inhibitors of the classical secretory pathway fail to inhibit release (Bausero et al., 2005; Broquet et al., 2003; Hightower and Guidon, 1989; Hunter-Lavin et al., 2004a; Lancaster and Febbraio, 2005).

Hsp72 is known to associate with membranes (Gehrmann et al., 2005; Triantafilou et al., 2001) and disruption of lipid rafts resulted in a inhibition of release (Bausero et al., 2005; Broquet et al., 2003; Hunter-Lavin et al., 2004a). The data suggest that Hsp72 release operates through a non-classical pathway involving lipid rafts. The mechanism of release is further complicated by the observation that Hsp72 is released in exosomes (Bausero et al., 2005; Clayton et al., 2005; de Gassart et al., 2003; Lancaster and Febbraio, 2005) and that the exosomal Hsp72 can be either internal or

Table 2. HSP release from different cells

Cell type	Hsps	Reference
Rat embryo cells	Hsp71, Hsp73, Hsp110	(Hightower and Guidon 1989)
Glia	Hsp72, Hsp60	(Tytell et al. 1986; Bassan et al. 1998; Guzhova et al. 2001)
Tumour cells	Hsp73, Hsp72, Hsp60	(Barreto et al. 2003; Broquet et al. 2003; Wang et al. 2004; Bausero et al. 2005; Gastpar et al. 2005; Lancaster and Febbraio 2005; Davies et al. 2006)
PBMCs	Hsp72, Hsp60	(Hunter-Lavin et al. 2004; Lancaster and Febbraio 2005; Davies et al. 2006)
B-lymphocytes	Hsp72	(Hunter-Lavin et al. 2004)
T-lymphocytes	Hsp72	(Hunter-Lavin et al. 2004)
Amniotic fluid cells	Hsp72	(Jean-Pierre et al. 2006)
Vascular smooth muscle cells	Hsp90	(Liao et al. 2000)

expressed on the exosomal membrane (Bausero et al., 2005). Exosomal membrane Hsp72 expression is probably restricted to tumor cell lines, in common with cell membrane expression of Hsp72 (Bausero et al., 2005). Exosomes from B-lymphocytes carry only internal Hsp72 (Clayton et al., 2005). The data published to date suggest that the proportion of Hsp72 released *via* exosomes varies from 20–80% of total release from tumor cells depending on the treatment (Bausero et al., 2005), and is probably significantly lower in PBMCs (Lancaster and Febbraio, 2005). Factors that stimulate Hsp72 release include IFN-γ (Bausero et al., 2005), LPS (Davies et al., 2006), and GroEL (Davies et al., 2006): all of which are well known stimulators of the immune response to infection. The only immunologically relevant treatment demonstrated to inhibit Hsp72 release has been Hsp60 (Davies et al., 2006).

ACTIVITY OF EXTRACELLULAR HSPS

HSP are highly immunogenic and many studies of extracellular HSP focus on their effects on immune responses. HSP have been shown to induce a wide range of immune responses, including cell activation and upregulation and secretion of cytokines.

The LPS Problem

A common criticism of *in vitro* studies of immune responses to HSP is the use of recombinant HSP and LPS contamination. LPS-free Hsp72 and Hsp60 were not

able to stimulate cytokine production in murine macrophages or splenocytes (Gao and Tsan, 2003a, 2003b, 2004; Wang et al., 2005a). Other studies have, however, shown immune responses to low endotoxin contaminated HSP (Manjili et al., 2005; Osterloh et al., 2004). The solution is to use authentic purified HSP, tested for LPS contamination. A number of studies have used low endotoxin recombinant preparations, but LPS binds strongly to Hsp60 (Habich et al., 2006) and there is always the possibility that LPS has a higher affinity for receptors when bound to HSP. However, there is now sufficient evidence from LPS free systems to support the biological activities described below.

HSP Receptors

The first step in any of the effects of HSP on cells must be the binding of the HSP to a receptor molecule. A number of putative receptors have been identified for the binding of HSP to cell surfaces, including CD14 for Hsp72 (Asea et al., 2000b) and Hsp60 (Kol et al., 2000), and CD91 for Hsp72, Hsp90, and grp94 (Basu et al., 2001), though murine Hsp60 cannot bind human CD14 (Breloer et al., 2002). The binding of Hsp72 to CD40 results in activation of dendritic cells (Millar et al., 2003) and is essential to internalization of the protein-peptide complexes (Becker et al., 2002). The human Hsp60 epitope that binds to macrophages has also been identified (Habich et al., 2004) as has the epitope of Hsp72 that binds to the NK receptor CD94 (Gross et al., 2003). The Toll-like receptors TLR2 and TLR4 are important for signaling as a result of extracellular Hsp72 (Asea et al., 2002; Vabulas et al., 2002) and TLR4 is important for Hsp60 signaling (Vabulas et al., 2001). A recent study has demonstrated that LOX-1 shows higher affinity for Hsp72 than previously identified receptors (Theriault et al., 2005). This study also showed Hsp72 binds to epithelial and endothelial cell lines as well as the well-studied antigen presenting cells (Theriault et al., 2005).

HSPS and Cytokines

HSP have been shown to activate many cells of the immune system including monocytes (Asea et al., 2000b; Guzhova et al., 1998; Radsak et al., 2003), macrophages (Basu et al., 2000), dendritic cells (Basu et al., 2000; Flohe et al., 2003; Wang et al., 2005b), neutrophils (Radsak et al., 2003) and mast cells (Mortaz et al., 2006). Grp94 binds and activates neutrophils and monocytes, increasing phagocytosis (Radsak et al., 2003). Hsp72 and Hsp60 have effects both on cytokine production from and maturation of dendritic cells (Flohe et al., 2003; Wang et al., 2005b).

HSP induce a range of cytokine responses *in vitro* including TNF-α, IL-12, and IL-1β release from macrophages (Basu et al., 2000). Hsp72 can bind to human monocytes, resulting in the upregulation of TNF-α, IL-6, and IL-1β (Asea et al., 2000b). Released Hsp72 shows danger signalling properties by upregulating CD83

expression and IL-12 release from naïve dendritic cells (Bausero et al., 2005). Extracellular Hsp72 was suggested to mediate the induction of IL-1β and IL-12 secretion in response to oxLDL as this cytokine production and release could be inhibited by blocking Hsp70 transcription or using neutralizing anti-Hsp72 antibodies (Svensson et al., 2006). The Hsp70 family member Hsp110 has also been suggested to act as a danger signal, inducing many cytokines previously seen for Hsp72 (Manjili et al., 2005). Hsp110 induces increased expression of MHC class II, CD40, and CD86 on dendritic cells, and also their secretion of the cytokines IL-6, IL-12, and TNF-α (Manjili et al., 2005), showing many responses to danger signaling from Hsp110. Hsp110 also induces increased IL-6 and IL-12 secretion from a mouse mammary carcinoma cell line and increased CD40 expression, which the authors suggest shows danger signaling mechanisms important in anti-tumor responses (Manjili et al., 2005).

Cytokines have been known to affect HSP production since the demonstration that IL-6 induced Hsp90 expression (Stephanou et al., 1997). Pro-inflammatory cytokines IL-1β, TNF-α, IL-4, IL-6, and IL-10 upregulate Hsp60 in primary human astrocytes (Bajramovic et al., 2000) and IL-10 increases Hsp72 expression in adrenocarcinoma and erythroleukemic cells (Bausero et al., 2005). Differences have been found between the cytokines that induce different HSP, showing Hsp27 expression to be upregulated by IL-4, IL-6, and TGF-β and not by IL-1β and TNF-α except when IFN-γ was also added to a cytokine mixture (Bajramovic et al., 2000). Interestingly, IFN-γ also did not upregulate Hsp27 when added alone (Bajramovic et al., 2000), suggesting some effects of cytokines on HSP are extremely specific to a type of stress or infection and require more than one signal (Breloer et al., 2002). IFN-γ, important in immune responses to viral infection and showing anti-tumor properties, increases expression, cell surface expression, and active release of Hsp72 in tumor cell lines (Bausero et al., 2005). Hsp72 may be an important part of the mechanism targeting an immune response to IFN-γ in tumor eradication.

In vivo studies are also now supporting the *in vitro* work showing a relationship between HSP and cytokines have relevance in the body. Links between IL-6 and Hsp72 have been shown in disease (Kimura et al., 2004), following surgery (Dybdahl et al., 2002), and during exercise (Bacelar et al., 2006 In Press; Febbraio and Pedersen, 2002). The addition of IL-6 to the femoral artery *in vivo* activates Hsp72 gene expression in skeletal muscle in humans (Febbraio et al., 2002). Hsp72 is present in amniotic fluid taken from women undergoing amniocentesis testing, and correlates with the presence of TNF-α and *M. hominis* in the fluid (Jean-Pierre et al., 2006). In this study, no correlation of Hsp72 with IL-1β or IL-6 was seen (Jean-Pierre et al., 2006). Hsp72 release was increased when the fluid was cultured in media with peptidoglycan (a TLR2 ligand), and increased TNF-α release was shown in response to addition of Hsp72 (Jean-Pierre et al., 2006). Again, these studies support that *in vitro* investigations showing relationships between HSP and cytokines have biological significance *in vivo*.

Uptake of HSPS into Cells

Although most studies of extracellular HSP focus on their roles in the immune response, it is also important to remember that HSP were first identified as protective chaperones. Extracellular HSP may be important in the protection of cells, especially those cells that produce low levels of HSP. HSP can bind to cell surfaces and enter cells (Becker et al., 2002; Guzhova et al., 2001; Guzhova et al., 1998). Glial cells release Hsp72, and this release is increased in response to heat shock (Guzhova et al., 2001). Neuroblastoma cells, sensitive to heat shock, can take up Hsp72 from media, utilizing this to improve their tolerance to heat shock (Guzhova et al., 2001). Monocytes are also able to take up Hsp72 and as a result are more resistant to apoptosis and necrosis (Guzhova et al., 1998). It is possible that extracellular HSP *in vivo* is important in the protection of some cell types less able to synthesize their own HSP, and that these cells depend on uptake of extracellular HSP for their survival. Hsp72 production varies between cell types throughout the body. For example, *in vitro* (Dressel and Gunther, 1999) and *in vivo* (Oehler et al., 2001) heat shock results in varied ability between white blood cell types to produce Hsp72.

Decreased HSP levels in some tissues have detrimental consequences, and uptake of extracellular HSP may be a mechanism whereby cells with low expression can obtain HSP required for survival. Type 1 diabetes patients with polyneuropathy have very low levels of Hsp72 in blood leucocytes (Strokov et al., 2000). Administration of the anti-oxidant α-lipoic acid, which improves nerve conductance and blood supply to nervous tissue, increased Hsp72 leukocyte levels in these patients accompanied by improvement in nerve damage (Strokov et al., 2000). Uptake of extracellular HSP may have positive effects on other cells with low HSP.

A NEW SENSE OF DANGER

A complicated picture is emerging in extracellular HSP biology (Figure 1). Despite this complexity we have to consider further that extracellular HSP arise from a range of different sources, potentially carrying different peptides, and which have the potential to interact with numerous cell types with varying consequences.

The nature of the peptides carried by released HSP are likely to greatly influence their biological activity. Therefore, the biological activity of HSP released by damaged and necrotic cells is likely to be different to that of surface expressed or released from viable cells. The activity and form of extracellular HSP may also depend on its originating cell type. Further, while developing a model for the role(s) of extracellular HSP there has to be consideration of the continuous presence of Hsp72 and Hsp60 in serum. Figure 1 is, therefore, an oversimplification as there is a baseline level of serum Hsp72 and Hsp60 in most people, and not just the increased level in response to stress/disease. We will discuss the implication of HSP in health and disease issues through consideration of the positive and negative aspects of HSP as danger signals.

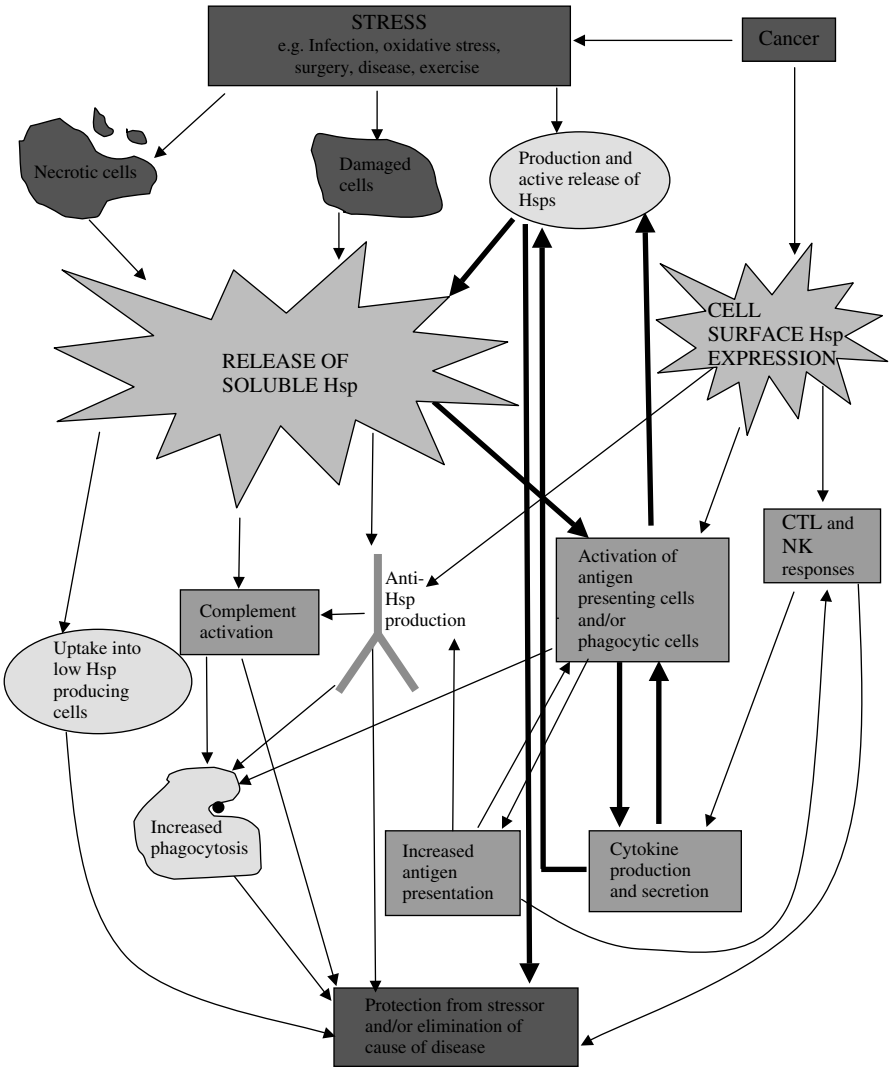

Figure 1. Overview of potential roles of extracellular Hsps. Hsps have a range of effects on immune responses and are able to initiate and respond to other immune signals. Bold arrows highlight a hypothetical cycle of events involving Hsp release, cell activation, and cytokine secretion. Uptake of Hsps into cells is another possible protective mechanism involving extracellular Hsps

The Negative Side of Danger

HSP probably act as danger signals as a warning when they have been released from damaged or necrotic cells (Johnson and Fleshner, 2006b; Matzinger, 1994, 1998;

Prohaszka and Fust, 2004). Situations where this is probably relevant are damage or necrosis due to mechanical or oxidative stress, bacterial or viral infection.

Mechanical or oxidative stresses are likely to result in the release of HSP bound to a variety of peptides. The consequences of this are likely to be promotion of the inflammatory response with the added potential problem of molecular mimicry resulting in an autoimmune reaction. Atherosclerosis, in common with Rheumatoid arthritis and type 1 diabetes, is considered an autoimmune disease – possibly resulting from molecular mimicry (Lamb et al., 2003; Wick, 2000). Both mechanical and oxidative stress result in the externalization of Hsp60 in endothelial cells leading to stimulation of inflammation and later atherosclerosis (Schett et al., 1999; Xu et al., 2000a; Xu et al. 1994). Hsp60, or peptides derived from it, can be used to induce, or immunize against, atherosclerosis, or type 1 diabetes, according to the timing and protocol used (Harkonen et al., 2003; Koffeman et al., 2005; Maron et al., 2002; Raz et al., 2005; Xu et al., 1992). Oxidized low density lipoprotein (oxLDL) has also been suggested to have important roles in atherosclerosis, and incubation of macrophages with oxLDL induces Hsp72 release as well as increased expression of Hsp70 genes (Svensson et al., 2006).

Hsp60 is present in the urine of mice, with low levels in healthy controls and increased levels in mice with nephrotoxic nephritis as a result of increased release from the kidney (Lang et al., 2005). Injection of Hsp60 into mice at the early stages of the disease along with nephrotoxic sheep serum (that induces glomerulonephritis) resulted in T-cell dependent aggravation of the disease, showing increased proteinuria and leukocyturia (Lang et al., 2005).

Each of the examples described here show a negative side to the release of HSP into the serum. All probably involve release from damaged or necrotic cells.

The Positive Side of Danger

Bacterial or viral infections are challenges to the immune system and the body needs to recognize and respond to the danger. Both LPS and bacterial HSP are important recognition signals for the immune system. Bacterial HSP were first shown to initiate immune responses to infection due to their release from bacterial cells, stimulating T cell proliferation (Estes and Teale, 1991) and inducing the expression and secretion of cytokines, such as TNF-alpha, IL-1, and IL-6 (Friedland et al., 1993; Marcatili et al., 1997; Retzlaff et al., 1994). Human and bacterial HSP may well act in conjunction with LPS *in vivo* with increased levels of HSP in response to LPS being important for its effects on immune responses to infection. GroEL (the *E.coli* version of cpn60) has been shown to stimulate cytokine production in monocytes (Tabona et al., 1998) and Hsp72 release from PBMCs and giant cell tumors (Davies et al., 2006). Despite the highly conserved nature of the different HSP families, human Hsp60 inhibits Hsp72 release from PBMCs (Davies et al., 2006) and subtle differences in the cpn60 sequence between bacterial species result in a loss of the cytokine stimulating activity (Maguire et al., 2002).

The Good and the Bad

The relationship between intracellular, cell surface, and released HSP is clearly a complex issue in cancer cell survival due to the roles of HSP in protection against apoptosis yet being immune targets on the cell surface (Gehrmann et al., 2005), and factors affecting the levels of HSP in each of these situations need further attention.

Although most studies on extracellular HSP in cancer concentrate on cell surface expression, HSP release from tumor cells has also been shown to reduce tumor cell survival (Wang et al., 2004). Hsp72 has important roles in the protection of cells against apoptosis, an undesirable property in tumor cells (Gehrmann et al., 2005; Thomas et al., 2005; Wang et al., 2004). High levels of Hsp72 in cancer cells protect the cells against radiotherapy and chemotherapy and are therefore detrimental to the patient (Gehrmann et al., 2005; Thomas et al., 2005; Wang et al., 2004). Release of Hsp72 from tumors may be important in two ways; Firstly by lowering intracellular Hsp72 and therefore reducing protection against apoptosis, and secondly by increasing extracellular HSP that may be important in immune responses against tumors (Wang et al., 2004). Hsp72 release can be induced in human prostate cell lines (Wang et al., 2004) and an epidermoid carcinoma cell line (Evdonin et al., 2004), suggesting induced release as a possible focus for cancer treatment. IFN-γ has also been shown to induce active release of the constitutive Hsp70, Hsp73 (Barreto et al., 2003) and Hsp72 (Bausero et al., 2005).

The immunogenic properties of HSP have led to many clinical trials combining this property with their chaperoning properties. HSP bind and chaperone peptides, and HSP-peptide complexes with tumor-derived peptides are being trialed in tumor immunotherapy, showing a role for HSP in antigen presentation (Basu and Srivastava, 2000; Gullo and Teoh, 2004; Todryk et al., 2003).

Extracellular HSPS as Insurance

Hsp72 and Hsp60 are present in normal healthy individuals. Hsp72 is increased by exercise (Bacelar et al., 2006 In Press; Fehrenbach et al., 2005; Milne and Noble, 2002; Walsh et al., 2001) and psychological stress (Campisi and Fleshner, 2003). It is possible that the released of Hsp72 into serum during exercise provides protection to damaged cells. Both the response to exercise and psychological stress may be an insurance against the perception of a threat. A big difference between the increases in serum Hsp72 following exercise and psychological stress is that they are transient – remove the stressor and the serum Hsp72 returns to normal concentrations (Bacelar et al., 2007 *in press*; Campisi and Fleshner, 2003). In many disease situations the elevated serum Hsp72 and Hsp60 concentrations are maintained, and therefore have greater potential to cause inappropriate interactions – an interesting concept compared to inside the cell where the molecular chaperones prevent inappropriate interactions.

In normal healthy individuals the presence of low serum concentrations of Hsp72 and Hsp60 may have housekeeping roles – e.g. binding and removal of partially

unfolded proteins. They may also serve to protect cells with low HSP concentrations, by being imported, and/or to maintain the immune system in a state of readiness.

HSPS AS BIOMARKERS

Many studies are showing serum HSP levels to be affected by several disease processes, leading to the suggestion that they may be useful clinical markers (Hunter-Lavin et al., 2004b; Oglesbee et al., 2005; Pockley, 2003; Wright et al., 2000b; Xu et al., 2000b). As well as being simply elevated in patients compared to controls, they are also affected by the extent of disease and complications in some patients (Hunter-Lavin et al., 2004b; Oglesbee et al., 2005). Lowering physiological stress by supplementation with folic acid lowers serum Hsp72 in non-insulin treated type 2 diabetes patients (Hunter-Lavin et al., 2004b), and increased physiological stress in the form of ketoacidosis in type 1 diabetes patients is associated with high serum Hsp72 that falls following treatment (Oglesbee et al., 2005). Correlations between Hsp72 and IL-6 and post-operative infection (Kimura et al., 2004) also show a stress response, providing increased Hsp72 levels that may be useful indicators of a patient's condition. A problem with using serum HSP as clinical markers is their upregulation by a range of stresses, including non-disease related physiological (Bacelar et al., 2006 In Press; Fehrenbach et al., 2005; Walsh et al., 2001) and psychological (Campisi and Fleshner, 2003) stresses. Therefore, as in other species (Bierkens, 2000; Elyse Ireland et al., 2004), HSP are unlikely to be useful as sole clinical markers, but they may nevertheless be useful sensitive early markers of stress or infection when used in conjunction with other more specific indicators, not affected by other stresses, or in production of a molecular fingerprint.

HSP stimulate a wide range of innate and adaptive immune responses, and it is therefore not surprising that antibodies to both human (Child et al., 2006; Hunter-Lavin et al., 2004b; Pockley et al., 1999; Pockley et al., 1998) and bacterial (Figueredo et al., 1996) HSP have been detected in human serum, and in cerebrospinal fluid (Chiba et al., 2006). It is unknown whether antibodies to HSP are the result of cell surface expression or released HSP or to a combination of both sources of extracellular HSP. Whether antibodies are bound to HSP in the blood *in vivo* also requires further investigation.

The significance of anti-HSP in the body is unclear, though, like the proteins themselves, anti-HSP levels are affected by disease (Chiba et al., 2006; Child et al., 2006; Pockley et al., 2000; Schett et al., 1995; Wright et al., 2000a; Xu et al., 1993). Like HSP, anti-HSP also have the potential for use as clinical markers. We recently published findings of increased anti-Hsp72 antibodies in pregnant women who gave birth to babies with birth defects, suggesting a prior increase in Hsp72 due to a stressful event (Child et al., 2006). Hsp72 may have buffering roles in evolution as shown for Hsp90 (Queitsch et al., 2002) and this may be a mechanism whereby stress in pregnancy exposes undesirable phenotypes due to Hsp72 being relocated in response to stress (Child et al., 2006).

CONCLUSION

Further work is required to determine the biological activity of released HSP, and indeed how they exist *in vivo*. It is unknown whether HSP in serum are present alone or bound to peptides or antibodies and this is extremely relevant to how they function in the body (Figure 2). Peptide-bound HSP, for example, may well increase immune responses to peptides they are chaperoning, acting as 'chaperokines' (Asea et al., 2000a; Maguire et al., 2002), whilst HSP/anti-HSP complexes may be important in complement activation as shown for Hsp60 (Prohaszka et al., 2002). Further work is also needed to determine which cells release which HSP, and whether differences exist between different cell types in terms of the form of HSP being released (ie peptide bound or not), particularly when comparing antigen presenting cells with other cell types. It also needs to be determined whether HSP exist in serum in monomeric or polymeric forms. Hsp60, for example, exists in its native form as two heptameric stacked ring structures, comprised of sub-units of approximately 60kDa, resulting in a native size of approximately 800kDa (Hartl, 1996). It is difficult to imagine a protein that size would exist in its native form in serum, and the determination of the form of HSP in serum is important for assessing their biological activity.

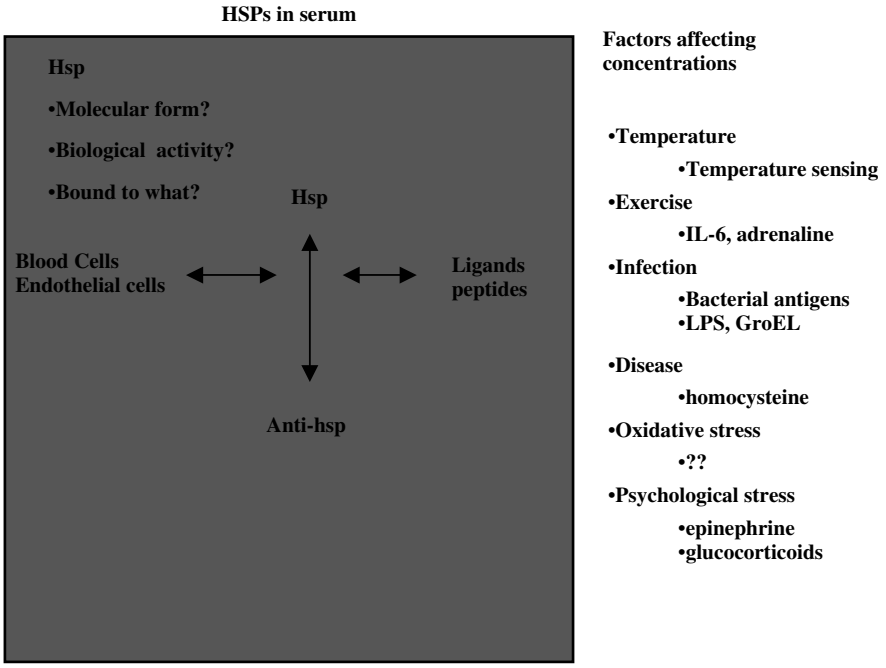

Figure 2. Hsps in serum: overview of interactions with antibodies and peptides and cells

HSP have a role as danger signals, in certain cases these signals are protective and positive for the organism. Extracellular HSP also have the potential to be internalized and offer protection to cells through this additional mechanism. However, the danger signal can be negative when there is misinterpretation of the message, and the result is an autoimmune reaction.

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PART II

HEAT SHOCK PROTEIN BINDING AND RECEPTOR-MEDIATED SIGNALING

CHAPTER 5

MACROPHAGES AND THE STRESS RESPONSE

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Abstract: Macrophages (MΦs) are key players in the immune and inflammatory responses. They become rapidly activated by the presence of pathogens, apoptotic cells and debris, which is followed by the digestion of the respective particle. In addition, MΦs are involved in the initiation of the immune response and healing process. Mediators synthesized and secreted by activated MΦs also control the function of different cell types either in close proximity or remote locations. Recently, heat shock proteins (Hsp) have been found to modulate MΦ function. In addition, Hsp that appear in circulation after stress as the result of cell lysis or secretion activate the response of MΦs. A better understanding of the role of Hsp in MΦ function may provide new directions in the development of therapeutic approaches for the treatment of inflammation, injury and other immune diseases. In this review, we attempt to provide an overview of the role of Hsp on MΦ. function and possible applications

Keywords: Heat shock proteins, phagocytosis, inflammation, injury

INTRODUCTION

More than a century ago, Metchnikoff first termed large phagocytic cells as "macrophages" (MΦs). He described their presence in all invertebrates and vertebrates (Metchnikoff, 1892). Afterward, Aschoff (1924) introduced the concept of the *reticulo-endothelial system* (RES) as a collection of cells localized in different organs and tissues that have the property of positive staining with lithium carmine, also named *vital staining* (Aschoff, 1924). More than 50 years later, van Furth et al. (1972) proposed that circulating monocytes and resident MΦs formed the *mononuclear phagocyte system*. Today, MΦs have been recognized as key players in both innate and adaptive immunity as well as in the inflammatory response. MΦs actively participate in the clearance of extracellular and intracellular pathogens, tumor cells, necrotic debris and apoptotic cells. Moreover, an alteration of MΦ function has

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been associated with the development of several diseases, such as atherosclerosis, pulmonary emphysema, Gaucher disease, HIV, granulomas formation, asbestosis and smoking-related illnesses.

MΦs are highly differentiated cells of heterogeneous morphology and function. The number of resident MΦs in a specific organ or tissue is constant, and their life span ranges from months to years. These cells get activated when they encounter different stimuli, including damaged cells, pathogens and chemicals. In addition, circulating monocytes – attracted by chemiotaxic signals – infiltrate injured tissues where they differentiate into MΦs. MΦs constitutively express a great variety of receptors, allowing them to respond to almost every single molecule produced during the response to stress. Thus, MΦs are extremely efficient at recognizing, binding and internalizing potentially harmful factors through a process called phagocytosis. In an attempt to destroy ingested pathogens, phagocytosis is accompanied by cellular activation that is characterized by a dramatic increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS) production. In addition, the inflammatory process is mediated by factors released by MΦs, such as lipid mediators and cytokines. The paradox is that substances produced by activated MΦs that are directed at destroying pathogens can also trigger the *stress response*.

The stress response is a well-orchestrated mechanism developed by cells to ameliorate the consequences of several stresses and to protect against subsequent insults. This response is characterized by an increase in heat shock protein (Hsp) expression. Hsp are ubiquitous and highly-conserved proteins, which are vital components of the response to an array of stressors (De Maio, 1999). During the last decade, a great body of evidence has pointed out the close relationship between MΦs and Hsp. One of the most surprising findings on this subject was the capacity of extracellular Hsp to activate MΦs and to modulate their immune functions (e.g., antigen presentation). Recently, Hsp have been proposed to act as "alert" or "danger" signals indicating that damage has occurred on surrounding or distant tissues.

The Phagocytic Event: Role of Hsp

Phagocytosis involves the recognition and internalization of large foreign particulates (0.5–5.0 μmΦ). Only professional phagocytes (monocytes, MΦs, neutrophils and dendritic cells) exhibit a higher phagocytic capacity and killing efficiency. As the sentinels of the innate immune system, professional phagocytes successfully recognize over a thousand pathogen-associated molecular patterns. This recognition depends on the expression of cell surface receptors that allow discerning between infectious agents and self antigens. Some of the cell surface molecules include toll-like, mannose, complement, scavenger and Fc receptors (Figure 1). Different pathogen components are recognized by various receptors, encouraging cross-talking and synergism. These ligands are engulfed in a plasma-membrane-derived vesicle called a phagosome, which undergoes a series of rapid and extensive changes

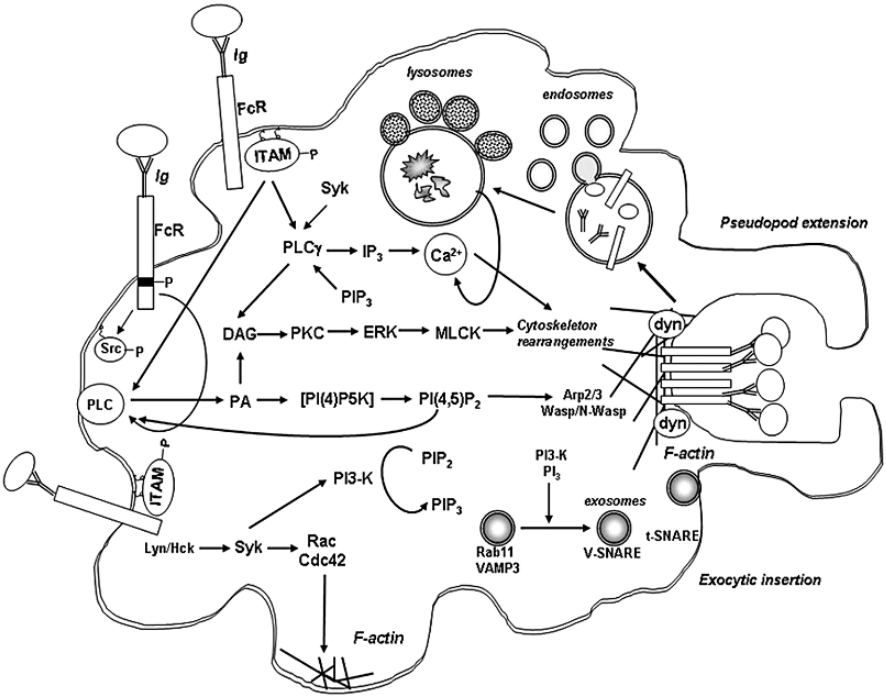

Figure 1. Signal transduction pathways trigger during phagocytosis of IgG-opsonized particles. There are three types of FcγRs: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). In humans, FcγRIIA contains two *YXXL* motif or ITAM (immunoglobulin gene family tyrosine activation motif) on the cytosolic tail. FcγRI and FcγRIIIA do not have an ITAM, and, therefore, they are provided by associated accessory trans-membrane proteins known as γ-subunit (FcγRI and FcγRIIIA) and $\tilde{\zeta}$ subunit (FcγRIIIA). FcγR signaling triggers tyrosine phosphorylation of ITAM and several other kinases (such as Src, Syc, Lyn, Hck and ERK), arachidonic acid release and activation of signaling enzymes (e.g., phosphoinositol 3-kinase (PI3-K), PKC, phospholipases (PL) C, D and A_2. FcγRs phagocytosis depends on F-actin formation, which is formed at early stages during the engulfing and phagosome formation. Increased intracellular calcium levels are also associated with FcγR-mediated phagocytosis. DAG: diacylglycerol. Dyn: dynamin. MLCK: myosin light chain kinase

known as phagosomal maturation. This process is characterized by sequential fusion with sorting endosomes (early endosomes), late endosomes and lysosomes (Desjardins et al. 1994; Scott et al., 2003). Desjardins (1995) postulated a "kiss and run" model for the phagosomal maturation, which is characterized by a transient and partial fusion (the "kiss") with endocytic organelles. This fusion allows for the transfer of selected membrane and luminal contents between phagosomes and endosomes, which is followed by a rapid separation (the "run"). This process prevents the complete intermixing of different compartments and allows the phagosome to gain full killing/destruction capacity.

The phagocytic process also triggers an increase in ROS and RNS levels as well as an increase in pro-inflammatory mediators. These substances are responsible

for the activation of the stress response in phagocytes, pathogens and surrounding cells (Barazzone et al., 1996). For a long time it was thought that the main role of this Hsp induction was to protect phagocytes (as well as other host cells) against harmful substances produced upon MΦ activation, such as ROS and RNS. Moreover, induction of the stress response was observed to block the production of pro-inflammatory mediators (Housby et al., 1999; Grossman et al., 2002; Schell et al., 2005), but increased the resistance to TNF-α cytotoxicity (Ribeiro et al., 1996). Today, we realize that the role of Hsp on MΦ function is far more complicated than just cellular protection. Hsp have been involved in both the modulation of the immune response and the inflammatory process. Recently, extracellular Hsp have been found to activate MΦs promoting local inflammation. The induction of Hsp expression has also been shown to enhance phagocytosis of platelets (Lang et al., 2002), erythrocytes (Clerget et al., 1990) and IgG-opsonized bacteria as well as non-opsonic particles (Vega and De Maio, 2005). Hsp have also been shown to increase the phagocytic capacity of microglial cells after ischemia or hemorrhagic stroke, probably in an attempt to clear cell debris after the insult (Gong et al., 2004). Studies from our laboratory showed that the induction of Hsp, either by geldanamycin (GA) treatment or by heat shock, increases phagocytosis independently of the type of ligand (Vega and De Maio, 2005) (Figure 2A and 2B). Expression of Hsp also triggers F-actin formation (Figure 2C), even in the absence of phagocytic ligands, suggesting that Hsp could prime MΦs for faster internalization of phagocytic particles (Vega and De Maio, 2005). This increase in actin polymerization correlates with faster phagosomal maturation and higher intracellular calcium levels (Vega and De Maio, unpublished data). Actin polymerization has also been observed in close association with the membranes of early-stage phagosomes (Damiani and Colombo, 2003). In addition, phagosomes are surrounded by actin-binding proteins, such as annexins, β-actinin and the ezrin/radixin/moesin proteins (Defacque et al., 2000; Diakonova et al., 1997; Desjardins et al., 1994), actin assembly network markers such as Neuronal Wiscott–Aldrich Syndrome Protein and actin-related protein complex (May and Machesky, 2001). Similarly, it has been reported that incubation of MΦs with latex beads results in recruitment of Hsc70 from the cytosol into the phagosomes (Deshaies et al., 1988; Chiang et al., 1989). This mobilization of Hsc70 is associated with calcium-dependant focal exocytosis, which is necessary for plasma membrane expansion (Zinsmaier et al., 1994). Inhibition of Hsc70 expression blocked phagocytosis of zymozan particles by alveolar MΦs (Perry et al., 1999) and caused defects in vesicle transport between the trans-Golgi, endosomes and plasma membrane. Thus, Hsc70 may play a role in vesicle trafficking (Holstein et al., 1996). The mechanisms by which Hsc70 modulates vesicle trafficking are unknown, and so far there is not much information about the Hsc70 domains involved in this process. For example, it is unknown if Hsc70 interacts directly or indirectly with vesicles or whether Hsc70 is localized in a cytosolic or luminal orientation. Although both Hsc70 and Hsp70 have been found to be involved in phagocytosis, their roles are not necessarily identical. For example, Hsp70 and Hsc70 are both associated with translating

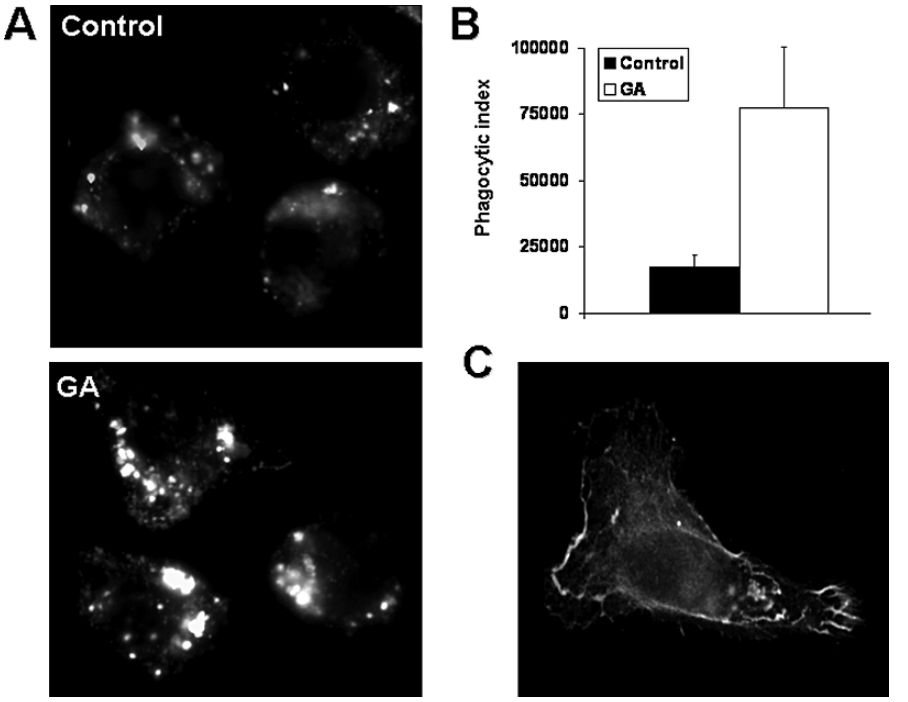

Figure 2. GA treatment increases IgG-opsonized latex beads phagocytosis by murine MΦ (A and B). J774 cells were incubated with GA (1 μg/ml) for 3 h and then incubated with FITC-conjugated IgG-coated latex beads (0.5 μm Φ) particles for 2 h at 37 °C. Phagocytosis was visualized by fluorescent microscopy (A) and quantified using a fluorometer (B). Results are expressed as phagocytic index (intensity of the signal/MTT). F-actin formation is visible after GA treatment (C). Polymerized actin was detected by incubation with Alexa Fluor 532-conjugated phalloid toxin after treatment with GA as described for (A) (unpublished data, Vega and De Maio)

ribosomes. However, Hsc70 interacts with nascent polypeptides (Beck and De Maio, 1994) whereas Hsp70 is bound to the 40S ribosomal subunit (Beck and De Maio, 1994; Cornivelli et al. 2003). Hsp70 has been localized in close proximity to biological membranes, particularly in association with lipid raft domains (Kurucz et al., 1999; Welch and Suhan, 1985; Botzler et al., 1998). Similarly, Arispe et al. (2002) showed that although both proteins interact with liposomes, inducing their aggregation, the characteristics of the liposome aggregation process are different between Hsp70 and Hsc70 (Arispe et al., 2002). This interaction of Hsc70/Hsp70 with membranes emerges as a potential mechanism by which these proteins could modulate the phagocytic process. Even more, we speculate that the interaction of Hsp70 with the plasma membrane at the site of engulfing serves as an anchor for cytoskeleton rearrangement, resulting in a more rapid internalization. The signals and the mechanisms involved in the targeting of Hsp70 to the plasma membrane are also unclear. Reports have shown the participation of Hsp70 in the assem-

bling of intermediate filaments (Liao et al., 1995), tubulin and microtubules (Liang et al., 1997). The interaction between Hsp70 and Tau proteins was proposed as the potential mechanism for this phenomenon, although the exact relevance of this observation remains unknown (Dou et al., 2003). Hsp90 also participates in cytoskeleton rearrangement activity, such as actin polymerization (Kellermayer and Csermely, 1995) and tubulin and intermediary filaments assembly (Czar and Pratt, 1996). Recently, Hsp90 has been shown to bind to LIM kinase-1 (LIMK-1), preventing the trans-phosphorylation and, therefore, the activation of this kinase, which results in the destabilization of actin filaments (Li et al., 2006). These observations are consistent with the increase in F-actin formation observed after treatment with Hsp90 inhibitors such as GA (Kellermayer and Csermely, 1995; Vega and De Maio, 2005).

Hsp as MΦ Activators

One of the major side effects during inflammation is the destruction of host cells by phagocyte-derived products. Cell destruction releases a large number of intracellular host-derived molecules that may act as alert signals for the immune system. Hsp are one of the most abundant intracellular proteins, reaching concentrations of 10^7–10^8 molecules/cell, only comparable with the cellular amount of actin (Cornivelli et al., 2003). Thus, a massive release of Hsp into circulation is likely to occur after cell lysis. It has been estimated that one gram of tissue could yield microgram amounts of gp96, Hsp70 and Hsp90 (Binder and Srivastava, 2004). During inflammation, circulating Hsp levels could be derived from host cells as well as pathogens. Unfortunately, the immune system is not able to differentiate between host- and pathogen-derived Hsp (Basu, 2000; Breloer, et al. 2001; Forsdyke, 1999). Consequently, Hsp, regardless of their origin, activate the immune response in the same form (Breloer et al., 1999; Chen et al., 1999; Multhoff et al., 1999; Asea et al., 2000). The effect of extracellular Hsp on MΦ activation has triggered a controversy regarding the potential contamination of recombinant proteins with endotoxin. Extensive studies have disregarded the possible artifact of endotoxin contamination. Thus, MΦ activation that occurred in the presence of recombinant Hsp has been demonstrated as having lower endotoxin concentrations. Moreover, co-incubation with polymixin B did not abolish Hsp-induced MΦ activation, and incubation of MΦs with Hsp in serum-free medium finally ruled out the possible effect of endotoxin contamination. Incubation of MΦs with extracellular Hsp results in up-regulation of several cell surface receptors, including those involved in phagocytosis (Binder and Srivastava, 2000; Basu et al., 2001). Extracellular Hsp70 promotes phagocytosis of Gram-negative and positive bacteria as well as fungi and inert particles (Wang et al., 2006). Rat microglial cells respond to recombinant Hsp90, Hsp70 or Hsp32 with a significant increase in amyloid-β peptide phagocytosis as well as with pro-inflammatory cytokine, IL-6 and TNF-α production (Kakimura et al., 2002). Recently, it has been proposed that the source of extracellular Hsp during inflammation is not due

only to cell lysis, but rather to an active secretory mechanism. Under special pro-inflammatory circumstances (e.g., presence of oxidized LDL), paracrine secretion of Hsp70 occurred in MΦs. This secretion reinforces cellular activation, resulting in faster and higher levels of cytokines and probably in a faster internalization of harmful substances such as oxidized LDL (Svensson et al., 2006). The mechanism involved in the secretion of Hsp70 is still unknown, but it is most likely associated with lipid rafts or other lipid environment (Broquet et al., 2003; Hightower and Guidon, 1989; Dybdahl et al., 2002). Another function of extracellular Hsp is to modulate tissue repair processes after inflammation. In general, it has been shown that most of the factors involved in healing are intracellular or intracellular-derived products (e.g., Hsp or Hsp-peptides). Extracellular Hsp actively promoted tissue repair in murine models of wound healing by promoting phagocytosis of cellular debris or damaged tissue and infiltrated cells and pathogens (Kovalchin et al., 2006). It has been suggested that the delay in wound healing observed during glucocorticoid treatment is associated in part to the inhibition of Hsp expression (Gordon et al., 1994). Similarly, chronic unhealed wounds (in the absence of infection) are associated with reduced induction of Hsp70 (Oberringer et al., 1995). It is possible Hsp can be used as compensative treatment to improve impaired wound healing in pathologies such as diabetes, sepsis, burns and in patients undergoing chemo or radiotherapy.

Phagocytosis of Apoptotic Cells

Apoptosis is an active energy-dependent process that results in cell death. In general, the cell membranes of apoptotic cells remain intact until unique surface markers are expressed on the cell surface (e.g., phosphatidylserine). These markers are rapidly recognized by phagocyte receptors (such as SR-A, CD14 and CD36), resulting in the clearance of apoptotic cells. Phagocytosis of apoptotic cells strongly differs from the phagocytosis induced by pathogens. Basically, phagocytosis of apoptotic cells results in an increase in survival as well as a decrease in cytokine production by MΦs (Figure 3) (Fadok et al., 2001; Savill et al., 2002; Weigert et al., 2006). Removal of certain apoptotic cells also causes production of anti-inflammatory mediators, resulting in the inhibition of antigen presentation or the promotion of growth of intracellular parasites within MΦs (Savill et al., 2002; Lopes et al., 2000). MΦs are able to discriminate (via an unknown mechanism) the cause of apoptosis. For example, phagocytosis of *Mycobacterium* or bacteria-infected apoptotic neutrophils — but not uninfected neutrophils — triggers a pro-inflammatory response characterized by an increase in TNF-α levels and cell surface expression of Fcγ RI (Perskvist et al., 2002; Zheng et al., 2004). In contrast, the uptake of apoptotic (uninfected) neutrophils by MΦs results in inhibition of pro-inflammatory cytokines and increases the levels of anti-inflammatory mediators such as TGF-β (Fadok et al., 1998; Huynh et al., 2002). It has been suggested that Hsp expressed on the cell surface of apoptotic neutrophils

may constitute one of the signals required to generate a more efficient and selective immune response (Asea et al., 2000; Srivastava, 2002; Wallin et al., 2002; Zheng et al., 2004).

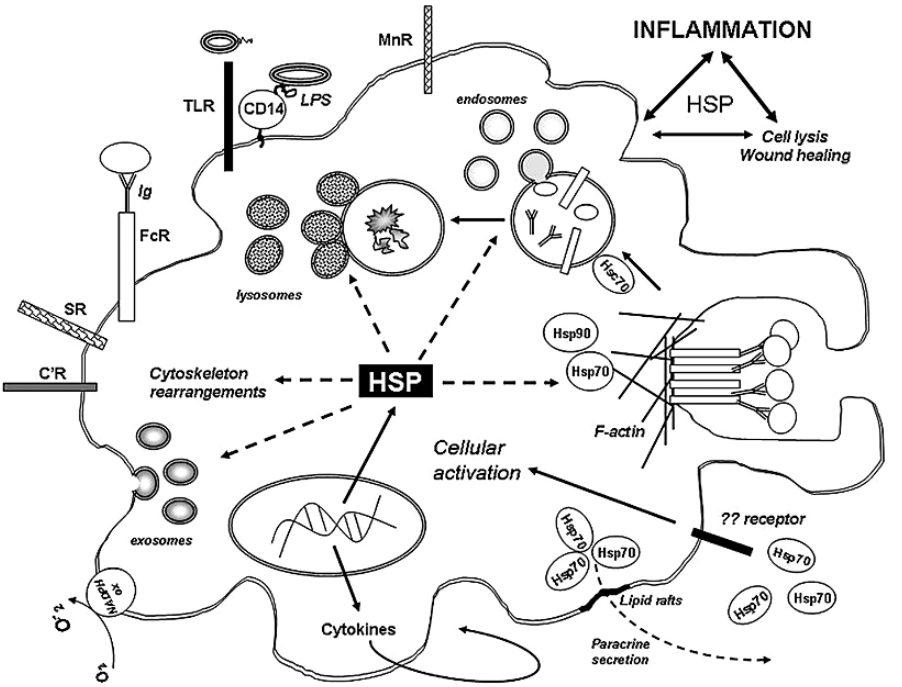

Figure 3. Hsp can modulate different components involved in phagocytosis by MΦs. It has been shown that induction of Hsp during inflammation results in an increase in phagocytosis. Extracellular Hsp also activate MΦs and promote wound healing

Bypassing Phagosomal Destruction

Antigen presenting cells or professional phagocytes are responsible for the clearance and destruction of invading microorganisms as well as antigen presenting processes. The successful destruction of these microorganisms and the development of immunity require a proper phagocytosis and phagolysosome formation. Pathogen lysis releases proteins and peptides that reinforce MΦ activation or that can be presented to T cells to reach immunity. A typical example is the induction of TNF-α, IL-1 and IL-6 secretion by extracellular bacteria-derived Hsp (Retzlaff et al., 1994). Regardless of all the strategies developed to kill and eliminate pathogens, some of them have the capacity to escape from this "pathway of destruction," resulting in persistent infections and a lack of immunity. For example, *Shigella*

flexneri and *Rickettsia* escape from the phagosome into the cytoplasm prior to fusion with the lysosomes. *Legionella pneumophilia* and *Mycobacterium* spp force phagocytic cells to place them into a special phagocytic vacuole where they are protected from ROS. For years, scientists have been trying to find a successful approach to killing and destroying the well-named "super pathogens." Surprisingly, some of these super pathogens have developed the ability to block Hsp induction in MΦs, resulting in a lower killing capacity and phagosomal maturation. It has been proposed that this blocking of host-derived Hsp induction prevents phagolysosome formation or its acidification (Rouquette et al., 1998). Non-virulent strains of *Salmonella choleras* largely induced Hsp60 and Hsp70 in MΦs resulting in their destruction (Nishimura et al., 1997). On the contrary, infection with virulent strains failed to trigger host Hsp expression, but largely induced pathogen-derived Hsp, protecting *Salmonella* from MΦ-derived harmful products. Similar results were observed in *Shigella flexneri* (Mantis and Sansonetti, 1996) and *Toxoplasma gondii*, an obligate intracellular protozoan parasite (Lyons and Johnson, 1995; Butcher et al., 2001). The mechanisms by which these virulent strains selectively over-express their own Hsp are still unknown and require further study, as do the mechanisms by which virulent pathogen-derived Hsp interfere with the host immune response. Regarding this last point, it will be important to understand whether or not pathogen Hsp require access to the host cytosol and/or the nucleus to block MΦ activation. Most likely, different pathogens develop different mechanisms to bypass or inhibit the host inflammatory response and selectively activate their own stress response to obtain parasite survival. For example, molecular genetic studies using *T. gondii* strains have shown that virulent and non-virulent strains only have a single copy of *Hsp70*, but they differ in the number of repetitions of a seven residues (GGMPGGM) sequence at the 3'-end of the gene (Lyons and Johnson, 1995). The importance of these repetitions and their relation with virulence are still under investigation (Sibley et al., 1999).

CONCLUSIONS

The host response to injury is a complex and well-orchestrated process that results in the release of inflammatory mediators and increased clearance of damaged cells and pathogens. As part of this process, immune system cells express Hsp, which help protect against the host's own machinery for eliminating harmful components. In addition, Hsp could be released into circulation after cell lysis secondary to the development of necrosis. These Hsp in circulation have been proposed as a prognosis marker for the incidence of injury (Pittet et al., 2002). Moreover, these extracellular Hsp could also activate MΦs and other immune cells. Consequently, they have been coined "danger" or "alert" signals. Recently, Hsp have been shown to accelerate the clearance of bacteria by MΦs which may be fundamental for a rapid recovery after injury.

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CHAPTER 6

HEAT SHOCK PROTEINS AND SCAVENGER RECEPTORS

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Abstract: Cytosolic heat shock proteins and endoplasmic reticulum resident chaperones (collectively referred as to Heat Shock Proteins or HSPs) control the folding and prevent the aggregation of proteins. Tumor-derived HSPs, released by dying cells or purified from tumor cells, induce protective anti-tumoral immune responses. This property of HSPs is related to their ability to chaperone tumor-derived peptides and to be internalized, in a receptor-dependent manner, by antigen-presenting cells. Studies were thus focused on identifying HSP-binding elements. Several members of the scavenger receptor family, including CD91, LOX-1, SREC-I, SR-A and CD36, were shown to bind different HSPs and to mediate their internalization. Among these receptors, CD91 and LOX-1 were also demonstrated involved in antigen-processing and MHC I presentation. HSPs activate immune cells but SR do not appear involved in this process. In conclusion, due to their ability to target APC and to cross-present exogenous antigens and to their adjuvant properties, HSPs are considered as ideal vaccine vehicles to generate anti-tumor and anti-viral protective immune responses

Keywords: Heat shock proteins, Anti-tumor immunotherapy, vaccination, antigen-presenting cells, scavenger receptors

INTRODUCTION

Professional antigen presenting cells, i.e. macrophages and dendritic cells (DC) are at the interface between innate and adaptive immunity (Banchereau et al., 2000). Upon contact with microbes, they are activated, produce pro-inflammatory mediators (Matzinger, 2002) and present an upregulation of costimulatory molecules, allowing them to efficiently stimulate T cells. Macrophages take up as many bacteria as possible via endocytic receptors (such as scavenger, complement and Fc receptors) before to kill them intracellularly; macrophages are less efficient

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than DC in activating T cells. In contrast, DC limit bacterial ingestion by entering a maturation process that renders them able to prime naive T cells (Banchereau et al., 2000). Dendritic cells are a sparely distributed migratory group of bone marrow-derived cells specialized in the uptake, transport and presentation of antigens to T cells (Banchereau et al., 2000). In the periphery, immature DC are sentinels that continuously sample the environment (Palucka and Banchereau, 2002; Pulendran et al., 2001). To sense danger in the form of microbes or tissue damages, immature DC express endocytic and signaling innate receptors. Upon contact with inflammatory stimuli (such as microbial products), DC undergo a maturation process and migrate to the draining lymph nodes. During the migration, DC up-regulate costimulatory molecule expression (B7 molecules), acquire the expression of the maturation marker CD83, lose the expression of receptors for pro-inflammatory chemokines and upregulate the expression of the receptors for constitutive chemokines (CCR7 and CXCR4) (Banchereau et al., 2000). In the T cell area of the lymph nodes, mature DC prime antigen-specific naive T cells, initiate the acquired-immune response and control T cell polarization (Banchereau et al., 2000).

Cytosolic heat shock proteins and endoplasmic reticulum resident chaperones (collectively referred as to Heat Shock Proteins or HSP) control the folding and prevent the aggregation of protein (Feldman and Frydman, 2000). Tumor-derived HSP induce antigen specific and protective anti-tumor immune responses *in vivo* (Tamura et al., 1997). The immune properties of HSP are linked to their ability to chaperone tumor-derived peptides and to be selectively and efficiently recognized and internalized by APCs (Blachere et al., 1997; Suto and Srivastava, 1995; Udono and Srivastava, 1993). The process of presentation of an exogenous antigen in the context of class I major histocompatibility complex (MHC I) molecules is called cross presentation (Heath and Carbone, 2001; Yewdell et al., 1999) and requires, to be efficient *in vivo*, that antigen are internalized in a receptor-dependent manner (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000; Sondermann et al., 2000; Wassenberg et al., 1999). Studies were thus focused on identifying the nature of the receptors involved in HSP internalization by APCs and their potential involvement in HSP-mediated antigen cross-presentation. Members of the scavenger receptor (SR) family were identified, among other molecules, as the main HSP-binding elements expressed by APCs. This chapter will review the different SR identified.

HEAT SHOCK PROTEINS AS VACCINE VEHICLES

T cell epitopes derived from exogenous antigens and endocytosed by APCs are mainly loaded into the MHC II molecules for recognition by CD4+ T cells. In contrast, endogenous antigens (i.e. auto-antigens and viral proteins) are presented in the context of MHC class I molecules to CD8+ T cells. Antigen-primed CD4+ and CD8+ T cells then differentiate into effector T helper (Th) and cytotoxic T cells, respectively. Depending on their polarization (determined on the panel of cytokines they produce), Th1 *versus* Th2 cells control the outcome of the immune response and regulate the humoral immune response. Cytotoxic T cells are involved

in the killing, in an antigen-specific manner, of tumor cells and virus-infected cells. This dichotomy in antigen presentation theoretically excluded the presentation of an exogenous antigen in the MHC I molecules. However, in some conditions, APC are able to present exogenous antigen in the context of the MHC I molecules to CD8+ T cells, a process called antigen cross-presentation (Heath and Carbone, 2001; Yewdell et al., 1999). It is thought that antigens have to be taken up via endocytic receptors to be efficiently cross-presented *in vivo* (Arnold-Schild et al., 1999; Castellino et al., 2000; Singh-Jasuja et al., 2000; Wassenberg et al., 1999). This process allowed to propose new vaccine strategies based on the targeting of endocytic receptors selectively expressed by DC. Several studies were focused on identifying vector proteins exhibiting DC-targeting properties and able to mediate cross-presentation of vaccine antigens associated or coupled to the vehicle. The outer membrane protein A of *Klebsiella pneumoniae* (Jeannin et al., 2000) and bacteria-derived toxins, such as the *Bordetella pertussis* adenylate cyclase (Fayolle et al., 1999) and *Pseudomonas aeruginosa* exotoxin A (Donnelly et al., 1993), are efficiently captured and internalized by professional APC and mediate the cross-presentation of the associated antigens.

Heat shock proteins (HSP) are intracellular chaperones, either cytosolic or resident within the endoplasmic reticulum. HSP control the folding and prevent the aggregation of proteins (Feldman and Frydman, 2000). With the aim to identify new anti-tumor vaccine strategies, Tamura et al showed that tumor-derived gp96/GRP94 (an endoplasmic reticulum resident HSP) generate a protective antitumor immune response *in vivo* (Tamura et al., 1997). Recently, tumor-derived HSP have been shown to generate tumor-specific T cells in human and clinical responses (Castellino et al., 2000; Mazzaferro et al., 2003; Rivoltini et al., 2003). The capacity to mediate antigen cross-presentation was reported for other HSP including Hsp70, Hsp90 and calreticulin (Basu et al., 2001; Delneste et al., 2002; Noessner et al., 2002; Udono and Srivastava, 1993).

The cellular and molecular mechanisms involved have been partially identified: HSP non covalently bind tumor Ag-derived peptides (Blachere et al., 1997; Suto and Srivastava, 1995; Udono and Srivastava, 1993) and bind to DC and macrophages (Arnold-Schild et al., 1999; Todryk et al., 1999; Wassenberg et al., 1999) before to be internalized in a receptor-dependent manner (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000; Sondermann et al., 2000; Todryk et al., 1999). HSP then colocalize with MHC class I molecules in the early and late multivesicular endosomal structures (Arnold-Schild et al., 1999; Sondermann et al., 2000; Todryk et al., 1999; Wassenberg et al., 1999). HSP-binding structures have characteristics of receptors: (i) saturation of the binding of gp96 to CD11c+ cells and of Hsp70 to the ANA macrophage cell line (Binder et al., 2000; Sondermann et al., 2000) and, (ii) competition and specificity: the binding of gp96 is inhibited by gp96 but not by Hsp70 and conversely, the binding of Hsp70 is inhibited by Hsp70 but not by gp96 (19). Several groups thus aimed to characterize HSP-binding elements expressed by APCs and different members of the scavenger receptor family were identified as HSP receptors.

SCAVENGER RECEPTORS: CLASSIFICATION AND BIOLOGICAL FUNCTIONS

The term scavenger receptor was first used in 1979 by Goldstein (Goldstein et al., 1979) to functionally describe receptors on their ability to bind modified low-density lipoproteins (mLDL), such as oxidized LDL (OxLDL), acetylated LDL (AcLDL), but not native LDL. Since this first description, a large family of structurally unrelated but functionally related molecules was identified (Murphy et al., 2005). Most of the SR are cell surface glycoproteins that mediate the uptake of mLDL, a large panel of polyanionic ligands, non self (microorganisms), altered self (such as apoptotic/senescent cells) and self components (such as extracellular matrix molecules). Scavenger receptors can thus be viewed as pattern recognition receptors (PRR); PRR are innate immunity receptors that act as immune "sensors" which recognize conserved patterns unique to microorganisms called pathogen-associated molecular patterns or PAMPs (Medzhitov and Janeway, 1999) and modified self.

The expression of SR is restricted to selected cell types, including mainly endothelial cell subtypes, macrophages and DC. Their expression can be modulated or induced on other cell types (such as fibroblasts and smooth muscle cells) in a pro-inflammatory context. Recognition of the SR ligands is followed by their efficient internalization, resulting either in accumulation (generation of foam cells) (de Villiers and Smart, 1999) or destruction (fusion of endosomes with lysosomes, leading to the destruction of microorganisms) (Thomas et al., 2000). Detailed reviews on the structure/biology of SR were recently published (Greaves and Gordon, 2005; Mukhopadhyay and Gordon, 2004; Murphy et al., 2005; Rigotti, 2000). We will thus focus on the main characteristics of the different classes of SR. Scavenger receptors are classified on a gene structure basis; 8 classes of SR (class A-H) have been defined.

a. Class A Scavenger Receptors

Three genes belong to the class A: (i) the SR-A gene that encodes three proteins (SR-AI, SR-AII, and SR-AIII) generated by alternative splicing of the SR-A transcript (Freeman et al., 1990), (ii) the macrophage receptor with a collagenous structure (MARCO) (Elomaa et al., 1995) and (iii) the scavenger receptor with a C-type lectin domain (SRCL) (Nakamura et al., 2001a; Nakamura et al., 2001b). These three class A SR genes share a common collagen-like domain that contains the ligand binding domain (Doi et al., 1993). These molecules are type II membrane glycoproteins that can form homotrimers. SR-AIII is retained within the endoplasmic reticulum (Gough et al., 1998). SR-AI and SR-AII bind OxLDL, lipid A moiety of lipopolysaccharide (LPS) (Hampton et al., 1991), Gram positive bacteria (Dunne et al., 1994), bacterial CpG sequences (Zhu et al., 2001) and certain carbohydrate-based ligands. MARCO is structurally similar to SR-AI-III, but lacks the α-helical coiled coil domain and contains a larger collagen-like domain than SR-A and SRCL.

MARCO is expressed by a subpopulation of macrophages (Elomaa et al., 1995) and induced on different types of macrophages upon stimulation by several ligands including LPS, bacteria or dead/apoptotic cells (Grolleau et al., 2003). MARCO binds mLDL, Gram positive and Gram negative bacteria, environmental particles but not yeast (Grolleau et al., 2003). Compared to SR-A and MARCO, SRCL has an extra Ser/Thr-rich domain and a carboxy terminal C-type lectin domain. SRCL binds OxLDL, Gram positive and Gram negative bacteria and the yeast *Saccharomices cerevisiae* (Coombs et al., 2005; Nakamura et al., 2001a; Nakamura et al., 2001b; Ohtani et al., 2001). SRCL is expressed on several cell types (Nakamura et al., 2001a; Nakamura et al., 2001b).

b. Class B Scavenger Receptors

The class B SR subfamily contains the CD36, SR-B and lysosomal integral membrane proteins 2 (LIMP-2) genes. The SR-B gene encodes two proteins resulting from alternative splicing of the transcript, SR-BI (CLA-1) and SR-BII (Acton et al., 1994; Murao et al., 1997; Webb et al., 1997). Class B SR are type III (multiple transmembrane domains) membrane glycoproteins. CD36 and SR-BI can form dimers and multimers (Reaven et al., 2004; Thorne et al., 1997). A leucine-isoleucine motif within the C-terminal cytoplasmic tail determines lysosomal localization of LIMP-2. CD36 is expressed on monocytes, macrophages, endothelial cells and platelets. SR-BI expression is restricted to liver, adipocytes, macrophages and DC. Class B SR recognize several non altered and altered self ligands such as native LDL, OxLDL, high density lipoproteins and very low density lipoproteins, collagen, thrombospondin and apoptotic cells (Acton et al., 1996; Calvo et al., 1998; Puente Navazo et al., 1996; Rigotti et al., 1995; Silver et al., 2002). CD36 also binds the *Plasmodium falciparum* erythrocyte membrane protein I (Baruch et al., 1996) and SR-BI binds the HCV-E2 glycoprotein (Maillard et al., 2006).

c. Class C Scavenger Receptors

The class C subfamily contains only gene (dSR-CI) from the fruit fly *Drosophila Melanogaster* (Pearson et al., 1995). No counterpart has been identified in eukaryotes.

d. Class D Scavenger Receptors

The class D subfamily contains the CD68 (which mouse orthologous is macrosialin) and the lysosomal membrane glycoprotein (LAMP) 1–3 genes (Carlsson and Fukuda, 1989; Febbraio and Silverstein, 1990; Fukuda et al., 1988; Sawada et al., 1993). Class D SR are type I membrane glycoproteins associated to the lysosomal/endosomal associated membrane glycoprotein family. CD68/macrosialin is expressed on macrophages, Langherans cells, DC and osteoclasts (Shimaoka

et al., 2004b). LAMP1 and 2 are widely expressed while LAMP3 expression is increased during DC maturation (de Saint-Vis et al., 1998). CD68 binds minimally and highly oxidized forms of LDL (Holness and Simmons, 1993; Ramprasad et al., 1996) while ligands of LAMPs remain undetermined.

e. Class E Scavenger Receptor

The class E contains only a lectin-like gene product called lectin-like oxidized LDL receptor-1 (LOX-1) (Sawamura et al., 1997). The gene of LOX-1 is located within a NK gene cluster that contains other C-type lectins such as CLEC-1/2 and Dectin-1 that exhibit immune functions (Sobanov et al., 2001). LOX-1 is a type II membrane glycoprotein sharing three intra chain disulfide bonds (Chen et al., 2002). In human, LOX-1 forms covalently linked dimers or multimers (Xie et al., 2004). LOX-1 is expressed by endothelial cells, monocytes, macrophages, DC and smooth muscle vascular cells. A soluble form of LOX-1, generated by shedding of the membrane form, has been reported but its function remains undetermined (Murase et al., 2000). LOX-1 binds OX-LDL, advanced glycation end-products (AGE), aged and apoptotic cells, and Gram positive and Gram negative bacteria (Moriwaki et al., 1998; Oka et al., 1998; Shimaoka et al., 2001).

f. Class F Scavenger Receptor

The class F subfamily contains two related genes: the scavenger receptor expressed by endothelial cells (SREC)-I and SREC-II (Adachi and Tsujimoto, 2002a; Ishii et al., 2002). SREC are type I membrane glycoproteins which amino terminal domain contains 10 Cys-rich repeats, some of these repeats forming EGF-like domains. EGF-like domains are involved in protein-protein interactions as well as receptor oligomerisation of SREC-I/SREC-II (Ishii et al., 2002). Alternative mRNA splicing of SREC-I generated five membrane and soluble forms (Adachi and Tsujimoto, 2002a). SREC-I is expressed on endothelial cells and monocytes/macrophages. SREC-II is predominantly expressed in human heart, lung, ovary, and placenta (Ishii et al., 2002).

SREC-I binds OxLDL, AcLDL, polyinosinic acid and advillin (an actin-binding molecule) (Shibata et al., 2004; Tamura et al., 2003). In contrast, SREC-II does not bind mLDL. Interestingly, SREC-I contains putative phosphorylation sites within its intracellular domain, suggesting that ligand binding may induce signalization (Adachi et al., 1997; Ishii et al., 2002).

g. Class G Scavenger Receptor

The class G also contains a unique member called scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX) (Shimaoka et al., 2000). This molecule was independently cloned by an other group that identified the

new molecule as a membrane-associated chemokine (Matloubian et al., 2000). SR-PSOX/CXCL16 is a type I membrane protein. Its extracellular domain contains a chemokine like domain and a glycosylated mucin-like region (Matloubian et al., 2000; Shimaoka et al., 2000). Membrane SR-PSOX/CXCL16 is expressed by monocytes/macrophages, DC, endothelial cells and smooth muscle cells (Hofnagel et al., 2002; Tabata et al., 2005). SR-PSOX binds OxLDL, phosphatidylserine and bacteria (Minami et al., 2001; Shimaoka et al., 2003). Recognition of membrane SR-PSOX/CXCL16 with CXCR6 (the CXCL16 receptor) allows interaction between SR-PSOX/CXCL16-expressing cells and T lymphocytes (Shimaoka et al., 2004a). A soluble form of SR-PSOX, generated by shedding of the membrane form, has been described (Gough et al., 2004) which retains scavenging properties (Shimaoka et al., 2004b).

h. Class H Scavenger Receptor

The class H scavenger receptor contains two genes: FEEL-1 (fasciclin, EGFlike, laminin-type EGF-like and link domain-containing SR-1) and the paralogous FEEL-2 (Adachi and Tsujimoto, 2002b; Politz et al., 2002). FEEL-1 and FEEL-2 are type 1 membrane glycoproteins that bind Gram positive and Gram negative bacteria and advanced glycation end-products (Adachi and Tsujimoto, 2002b; Politz et al., 2002; Tamura et al., 2003). FEEL-1 also internalizes AcLDL (Prevo et al., 2004). FEEL-1 and FEEL-2 are expressed in several tissues and expressed highly in the spleen and lymph nodes (Adachi and Tsujimoto, 2002b; Politz et al., 2002; Tamura et al., 2003). Only FEEL-1 is expressed by monocytes/macrophages (Adachi and Tsujimoto, 2002b) and is located in early and recycling endosomes and the trans-Golgi network. FEEL-2 is expressed at the membrane and within early endosomes. FEEL-1 has also a role in cell adhesion and lymphocyte homing (Irjala et al., 2003a; Irjala et al., 2003b).

Collectively, these data underline the crucial role played by SR in tissue homeostasis, innate immunity and lipid metabolism. The expression and the cellular localization of the SR are tightly regulated by environmental factors, including mLDL and numerous cytokines (personal unpublished data). Consequently, the functionality of these receptors may vary, depending on the cell subtype and on the environment.

SCAVENGER RECEPTORS AS HSP-BINDING ELEMENTS

Tamura *et al* demonstrated that tumor-derived HSP initiate protective and tumor specific cytotoxic responses (Tamura et al., 1997). Tumor antigens either associated (Blachere et al., 1997), coupled (Delneste et al., 2002) or fused (Wu et al., 2005) to HSP also initiate protective anti-tumor immune responses, demonstrating that HSP act as vaccine vehicles with adjuvant properties. Looking for HSP-binding structures allowed identifying different scavenger receptors.

a. CD91

CD91 (also called α2 macroglobulin receptor) was the first HSP receptor identified (Binder et al., 2000). Although CD91 is not a scavenger receptor *per se* (as it does not bind mLDL), this molecule recognizes α2 macroglobulin (α2M) associated to several ligands (activated α2M or α2M*). Consequently, CD91 exhibits scavenging properties (Herz and Strickland, 2001). CD91 was first described as a specific gp96 receptor (Binder et al., 2000). Additional experiments showed that CD91 is also a receptor for most of the HSP, such as Hsp70, Hsp90, Hsp110 and calreticulin (Basu et al., 2001). Binding of the different HSP on APCs was inhibited by α2M and corresponding unlabeled HSP. Binding was followed by internalization of the peptide-chaperone complex. *In vitro* experiments showed that CD91 mediates the re-presentation of HSP-associated peptides by the murine macrophage cell line RAW264.7 (Binder et al., 2000; Srivastava, 2002). This re-presentation was totally inhibited by anti-CD91 antibody (Ab) but only at 50% by α2M, suggesting the existence of HSP-binding structures other than CD91 on APCs (Binder et al., 2001). Moreover, anti-CD91 Ab prevented *in vivo* the generation of a HSP-mediated anti-tumor immune response (Binder et al., 2004), confirming the *in vivo* relevance of this interaction Nevertheless, other authors suggested that CD91 is not a receptor for HSP. This hypothesis was essentially based on the fact that the receptor-associated protein (RAP), an antagonist of all known CD91 ligands, did not inhibit the binding, internalization and peptide-representation by gp96 (Berwin et al., 2002). Recently, the use of anti-CD91 short interfering RNAs confirmed the role of CD91 in gp96-mediated peptide re-presentation (Binder and Srivastava, 2004). The involvement of CD91 in the binding and internalization of HSP to APC was also reported in other studies (Ogden et al., 2001; Tobian et al., 2004).

b. LOX-1

Studies by the group of PK Srivastava were essentially done with the murine macrophage cell line RAW264.7. Our experiments showed that the CD91 ligand α2M inhibits the binding of human recombinant Hsp70 on human macrophages but not on DC, the professional APCs responsible for T cell priming, suggesting Hsp70-binding structure(s) other than CD91 exist on human DC (Delneste, 2004; Delneste et al., 2002). Surprisingly, maleylated bovine serum albumin A (mBSA), a ligand for numerous scavenger receptors (Abraham et al., 1995), but not BSA, totally prevented the binding of Hsp70 to immature DC and, in a lower extent, to macrophages. Modified LDL (OxLDL, AcLDL) and the polyanionic ligand poly[IC] also prevented the binding of Hsp70 to human APCs (Delneste et al., 2002). These results demonstrated that SR are the main Hsp70-binding structures on human DC. Using CHO cell lines stably expressing recombinant human SR, we demonstrated that human Hsp70 binds selectively to the scavenger receptor LOX-1 but not to the other SR tested, CD36, SRA-1, MARCO and CLA-1. A neutralizing anti-LOX-1 Ab partly inhibited while, in contrast, mBSA totally

prevented the binding and internalization of Hsp70 in human APCs, thereby suggesting that other SR are involved in this process. To evaluate the role of LOX-1 in Hsp70-mediated antigen presentation, we used the ovalbumin (OVA) assay. The Ova-specific CD8+ T cell hybridoma B3Z is activated when the immunodominant Ova peptide SIINFEKL is presented in the MHC I molecules. The protein Ova, and not the Ova peptide, was chemically coupled to Hsp70; this model allows to directly demonstrate that the antigen Ova was processed through the MHC I pathway and that B3Z activation does not only result from peptide re-presentation. *In vitro* experiments showed that a neutralizing anti-LOX-1 Ab prevented Hsp70-mediated antigen cross-presentation. Moreover, targeting *in vivo* a tumor antigen to LOX-1 using an anti-LOX-1 Ab elicited a therapeutic and protective immune response against antigen-expressing tumor cells. However, the role of LOX-1 in HSP-chaperoned peptide re-presentation was not evaluated in this model.

c. SR-A1

Berwin et al demonstrated that the binding of gp96 and calreticulin to macrophages was inhibited by the polyanionic SR-A ligands fucoidin and carrageenan (Berwin et al., 2003). Calreticulin and gp96 bind to and are internalized by SR-A-expressing HEK 293 cells but not mock-transfected cells. Moreover, the binding and internalization of both gp96 and calreticulin was reduced by 50% on macrophages from SRA-deficient mice compared to wild type mice. Interestingly, fucoidin inhibited Ova peptide re-presentation by macrophages, using the B3Z assay (Binder and Srivastava, 2004). However, the binding of calreticulin to macrophages was inhibited by fucoidin but not by calreticulin and APCs from SR-A deficient mice are able to present gp96-associated peptide (Berwin et al., 2003).

d. SREC-I

The identification of SREC-I as an HSP-binding element by Berwin et al follows the study on SR-A. The authors observed that fucoidin, as previously reported (Berwin et al., 2003; Radsak et al., 2003), and AcLDL were able to inhibit the residual binding of gp96 and calreticulin to macrophages from SR-A-deficient mice. Using a panel of SR-expressing CHO cells, a binding of calreticulin and gp96 was observed, as expected, on SR-A but also on SREC-I-expressing cells (Berwin et al., 2004). No binding of calreticulin and gp96 was observed on LOX-1, CLA-1, SR-PSOX and CD36. Binding to SREC-I was associated to an increase of internalization. The internalization of calreticulin was increased in SREC-I-transfected RAW264.7 macrophages. However, the role of SREC-I in HSP-mediated peptide re-presentation or antigen cross-presentation was not evaluated.

e. CD36

A higher binding of gp96 was observed on macrophages isolated from CD36 transgenic mice and, in contrast to the study by Berwin et al (Berwin et al., 2004), a 50% reduction of gp96 binding was observed on macrophages derived from CD36 deficient mice compared to wild type mice (Panjwani et al., 2002). These observations suggested that CD36 could act as a HSP-binding structure. However, the binding of gp96 to macrophages was not prevented by neutralizing anti-CD36 Ab and mLDL, suggesting that the binding site of gp96 to CD36 map to other domain(s) of CD36. These results were obtained using mouse cells; in contrast, we did not detect any binding of HSP to human CD36-expressing CHO cells (Delneste et al., 2002). Collectively, these studies identified SR as the main HSP-binding elements expressed by APCs. These results are in agreement with previous studies showing that antigen maleylation, allowing their targeting to SR by adding negatively charges, induces presentation into both MHC I and MHC II molecules and that SR ligands blocked antigen presentation (Bansal et al., 1999; Shakushiro et al., 2004; Singh et al., 1998). It is interesting to underline the selectivity of HSP binding on the different SR identified: CD91 binds most of the HSP while, in contrast, gp96 and calreticulin, but not Hsp70, bind to SR-A. The molecular basis responsible for the ligand specificity is not understood. Moreover, even though most of the studies clearly reported the binding and internalization of HSP to the identified SR, the involvement of these receptors in peptide representation and/or antigen cross-presentation was not elucidated for all of these receptors HSP-binding structures. Further studies will be required to elucidate this point.

HSPs AND CELL ACTIVATION

HSP have been shown to efficiently bind to and to be internalized in a receptor dependent manner by APCs. Studies also reported that HSP may activate APCs. The term "chaperokine" was proposed to define this particular property of HSP (Asea, 2003). Several studies reported the induction of pro-inflammatory cytokines and IL-12 by APCs stimulated with HSP as well as DC maturation (Panjwani et al., 2002; Todryk et al., 1999; Wan et al., 2004; Wang et al., 2002).

Wang *et al* demonstrated that mycobacteria Hsp70, but not mammalian Hsp70, activates APCs via CD40 (Wang et al., 2001) and polarizes immune response towards a Th1 phenotype (Wang et al., 2002). In a model of immune tolerance (LCMV transgenic mice), the Hsp70-LCMV peptide complex breaks tolerance; this property is abrogated in CD40-deficient mice. Moreover, the production of IL-12 induced by Hsp70 is not observed in CD40-deficient mice. The role of CD40 in HSP-mediated antigen cross presentation was not evaluated in this study (Wu et al., 2005) and contradictory results were evoked by others, showing that the ability of Hsp70 to mediate peptide re-presentation are similar in $CD40^{-/-}$ and wild type mice (Binder et al., 2004). Thus, the precise role of CD40 in HSP-mediated peptide re-presentation or antigen cross-presentation remains discussed.

Toll-like receptors (TLR) are innate immune receptors that recognize microbial moieties highly conserved during the evolution and called pathogen-associated molecular pattern (PAMPs) (Takeda et al., 2003). TLR are signaling receptors: their recruitment by PAMPs induces the production of several cytokines and modulates the expression of costimulatory molecules (Kaisho and Akira, 2004; Kawai and Akira, 2006). TLR can thus be viewed as molecular interfaces between innate and adaptive immunity. The group of Asea demonstrated the involvement of TLR2 and TLR4 in HSP-induced cytokine production (Asea et al., 2000; Asea et al., 2002). These results were confirmed by independent groups (Liu et al., 2003; Ohashi et al., 2000; Vabulas et al., 2001; Vabulas et al., 2002a; Vabulas et al., 2002b). CD14 is suspected to potentiate HSP-induced cell signaling but is not a binding receptor; we confirmed that neutralizing anti-CD14 Ab do not inhibit the binding of Hsp70 to human APCs (Delneste et al., 2002).

In addition to activate innate antigen-presenting cells, HSP also activate cells of the adaptive immunity, B and T lymphocytes. An initial study showed that T lymphocytes potentiate the HSP-induced activation of APCs, as evidenced by an increase of IL-2 and IFN-γ production and proliferation (Breloer et al., 2001; More et al., 2001). HSP have been shown to directly activate T lymphocytes in a TLR4-independent and TLR2-dependent manner (Osterloh et al., 2004; Zanin-Zhorov et al., 2003). This observation is in agreement with the fact that T cells can be activated by TLR agonists, mainly microbial moieties (Caron et al., 2005; Gelman et al., 2004; Komai-Koma et al., 2004), in the presence of costimuli such as IL-2 or anti-CD3 antibody. Moreover, human Hsp60 activates B cells via a recruitment of TLR4 but in a LPS-independent manner (induction of IL-6 and increase of expression of the activation membrane molecules CD40, CD69 and CD86) (Cohen-Sfady et al., 2005). Whether CD40 participates in Hsp60-induced B cell activation was not evaluated. Interestingly, both B and T lymphocytes do not express SR, suggesting that HSP-mediated cell activation may occur independently of these molecules. The role and the consequences of HSP-mediated T and B cell stimulation on the initiation/development of an antigen-specific immune response remain unclear.

The complex CD14/TLR4, associated to the accessory molecules MD2 and LBP, is the receptor for LPS (Miyake, 2004), leading to suspect that some of the immunostimulatory properties of HSP may be related to contaminating LPS (Bausinger et al., 2002; Gao and Tsan, 2003). Different studies reported that contaminating endotoxin are responsible for the DC activation by HSP. Soluble molecules, such as soluble CD14 or LBP, can potentiate the HSP immunostimulatory properties. The role of RP105, a member of the LPS receptor complex expressed by B cells (Miyake, 2004), in HSP-mediated B cell activation, was also not evaluated. Further, in contrast to LPS, maleylated BSA does not induce IL-12 production by macrophages (Singh et al., 1998). Recently, LPS-low Hsp110 was reported to activate APC, as evidenced by an increase of MHC class II, CD40 and CD86 molecules, and the induction of IL-6, IL-12 and TNF-α secretion (Manjili et al., 2005). Whether TLR participate to the immunostimulatory properties of HSP remains debated. These data underline

that the presence of contaminating endotoxin and of other TLR agonists has to be carefully excluded before to conclude on the immunostimulatory properties of HSP.

Initiation of an efficient and protective immune response requires antigen internalization and optimal activation of APCs. Both endocytic (i.e. SR, mannose receptors, C-type lectins) and signaling receptors (TLRs) are efficient to mediate antigen presentation (Bonifaz et al., 2004; Jeannin et al., 2000). The current concept is that antigen internalization in the absence of stimulation leads to tolerance (Bonifaz et al., 2004). Theoretically, DC activation (mediated or not by HSP) concomitant to HSP-mediated Ag capture may lead to autoimmunity response. Studies reporting that HSP do not activate APCs (Bausinger et al., 2002; Gao and Tsan, 2003) are in agreement with the hypothesis that HSP must be recognized as auto-antigens by the immune system and do not induce immune response to avoid auto-immunity. We have shown that targeting an antigen to LOX-1 *in vivo* in the absence of adjuvant is not sufficient to induce a specific immune response and that addition of a danger signal (i.e. incomplete Freund's adjuvant) is required to induce a protective anti-tumor response (Delneste et al., 2002). Moreover, the SR ligand maleylated BSA does not activate APC (Singh et al., 1998) and cross-linking of the mannose receptor induces an anti-inflammatory immunosuppressive immune program (Chieppa et al., 2003). In contrast, Millar et al reported that Hsp70 can convert tolerance to auto-immunity *in vivo* (Millar et al., 2003).

Collectively, contradictory results are reported on the potential immunostimulatory properties of HSP. It is important to keep in mind that an activation of APC by HSP-bearing auto-antigen may theoretically result in the activation of autoreactive T cells. Additional studies are thus required to determine the role and/or the cooperation established between the different HSP-binding elements in antigen processing and cell activation.

CONCLUSION

Scavenger receptors are involved in the recognition and internalization of non self (microorganisms), altered self (apoptotic/senescent cells) and some self components. Moreover, SR-mediated internalization in APCs allows the antigens to gain access to the MHC I and II presentation pathways. SR are thus at the interface between innate and adaptive immunity. HSP that mediate antigen cross-presentation are strictly intracellular chaperones, and their extracellular location can be viewed as a "danger signal" by the immune system (Gallucci and Matzinger, 2001; Matzinger, 2002; Todryk et al., 1999). Thus, the observation that most of the HSP-binding structures identified so far are members of the SR family or exhibit potent scavenging properties is in agreement with the hypothesis that innate cells have evolved to recognize a broad spectrum of danger signals with a restricted number of structures. The binding and internalization of "extracellular" HSP via scavenger receptors makes sense, as it reflects recognition of a cellular damage that can be recognized as a potential danger that must be retrieved from the extracellular milieu.

In conclusion, HSP are excellent vaccine vehicles to generate anti-tumor and anti-viral protective immune response and further studies are required to analyze at the molecular level, the mechanism responsible for the initiation of an antigen specific immune responses by HSP.

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CHAPTER 7

THE INSIDE STORY: ANTI-INFLAMMATORY ROLES OF HSF1 AND HEAT SHOCK PROTEINS

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Abstract: The heat shock response (HSR) and the acute inflammatory response (APR) are both key homeostatic mechanisms for resisting extracellular insult. There is evolving understanding regarding the relationship between these two responses. Activation of the HSR exerts some pro-inflammatory effects when HSP are released during cell insult and such extracellular HSP induce cytokine release in inflammatory and immune modulating cells. However, the intracellular mediators of the HSR including the transcription factor heat shock factor 1 (HSF1) and the HSP have profoundly anti-inflammatory effects. HSF1 can be induced by the elevated temperatures encountered in inflamed tissues and in fever as well as by anti-inflammatory prostaglandins. Such activated HSF1 represses cytokine release both directly by inhibiting nuclear factor of interleukin 6 (NF-IL6) and indirectly when elevated HSP inhibit the potent inflammatory factor NF-kB. Reciprocal effects are observed on activation of the APR which leads to inhibition of HSF1 through stimulation of inactivating phosphorylation events involving the mitogen activated kinase (MAPK) pathways. Activation of the HSR thus constitutes a feedback regulatory mechanism for the APR and limits the lethal over stimulation of cytokine release which may occur during infection. However, in order for rapid activation of the APR, mechanisms also exist for HSF1 repression, permitting controlled activation of the response during infection

Keywords: Autoimmunity, HLA-DR, Hsp70, CD4+ T cells, Hsp70:peptide complexes, MHC class II

Abbreviations: HSR; heat shock response; APR, acute inflammatory response; HSP, heat shock protein; HSF; heat shock factor; IL-1β; interleukin-1β; NF-IL6; nuclear factor of interleukin 6; NF-κB; nuclear factor kB; ERK; extracellular signal regulated protein kinase; GSK3; glycogen synthase kinase 3.

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INTRODUCTION

The heat shock response is a potent mechanism for resisting stress whose induction can convert a cell population normally killed down to 1 cell in ten thousand to a resistant population can survive the identical stress intact (Li and Hahn, 1981). It is particularly intriguing in that many of the proteins that mediate the response are conserved throughout cellular history (Georgopolis and Welch, 1993; Lindquist and Craig, 1988). The effector proteins of the response, heat shock proteins (HSP) are remarkably conserved in *archaea*, prokaryotes and eukaryotes (Georgopolis and Welch, 1993; Lindquist and Craig, 1988). The regulatory proteins that control HSP transcription, heat shock factor (HSF) are likewise conserved in the eukaryotes (Wu, 1995). In the past this has led to the notion that the heat shock response behaves as a "molecular fossil," controlled in isolation from the surrounding regulatory networks and functioning independently as an autonomous sentinel guarding the cellular proteome. This notion is however dissipating as evidence increases that indicates that the stress protein response is integrated into cell and tissue-wide regulatory matrices.

Regulatory Intermediates of the Stress Protein Response in Mammalian Cells

In mammalian cells, a cohort of molecular chaperone proteins is induced by stress, including "small HSP" typified by HSP27 as well as HSP40, HSP60, HSP70, HSP90 and HSP110 (Georgopolis and Welch, 1993; Lindquist and Craig, 1988). Some of the proteins, including the HSP70 family are encoded by more than one gene. In addition, mammalian cells contain 3 HSF family members including HSF1, HSF2 and HSF4 (Nakai et al., 1997; Rabindran et al., 1991; Wu, 1995). No gene corresponding to avian *hsf3* has so far been observed however (Nakai and Morimoto, 1993). Transcriptional regulation during protein stress involves mainly HSF1, with HSF2 and HSF4 carrying out auxiliary or tissue specific roles (He et al., 2003; Nakai et al., 1997).

Transcriptional activation of the heat shock response by HSF1 involves release from intramolecular associations that mask the trimerization domains permitting trimerization, nuclear localization and binding to the heat shock elements in HSP promoters (Rabindran et al., 1993; Westwood and Wu, 1993; Zuo et al., 1995). These events involve major unfolding reactions, reflected in the hydrodynamic properties of HSF1 (Rabindran et al., 1993; Westwood and Wu, 1993) and HSF1 unfolding is necessary for interactions with other molecules including protein kinases and transcription factors that mediate cross talk with other responses in the cell (Soncin et al., 2003; Xie et al., 2002a). For full transcriptional activation, a further stress-induced step is required that is dependent on the activities of upstream tyrosine kinases and results in the hyperphosphorylation of HSF1 largely on serine residues (Hensold et al., 1990; Price and Calderwood, 1991; Sarge et al., 1993). The primary regulator of HSF1 in the cell is one of its products, HSP90 and

HSP90 maintains HSF1 in its inactive, compacted form in the cell (Zou et al., 1998). The central role of HSP90 in this regard is indicated by that fact that HSP90 inhibitors such as geldanomycin can activate all steps of the stress protein response (Zou et al., 1998). HSF1 is an unusual HSP90 client protein in that, while other HSP 90 associated proteins become destabilized and destroyed by proteolysis by HSP90 dissociation, HSF1 is activated and leads to abundant HSP expression (Zou et al., 1998). HSF1 appears to be repressed by a number of other pathways largely mediated by phosphorylation. These include repression mediated by a double phosphorylation at serines 307 and 303 by the ERK and GSK3 pathways, phosphorylation at serine 363 by protein kinase C and by others (Chu et al., 1996; Chu et al., 1998; Klein and Melton, 1996; Knauf et al., 1996).

Stress Protein Response in Physiology and Pathology

In addition to their functions as molecular chaperones, HSP play a number of roles in cell and tissue physiology. The HSP protect the proteome through their molecular chaperone function that permits them to recognize damaged proteins and either channel such proteins into repair/refolding pathways or to proteolysis. In terms of cell survival, these properties of the HSP family permit cells to respond to damage at source and immediately begin the processes required to resolve the cellular insult (Kampinga et al., 1995; Kampinga et al., 1994). In addition, HSP play more generic roles in cell survival and are implicated as inhibitors of programmed cell death, and block both the intrinsic and extrinsic pathways of caspase dependent apoptosis-reviewed (Beere, 2004). The HSP may thus have been co-opted from their ancient molecular chaperone roles to play a part in other processes that require cells to negotiate stressful periods. Thus the molecular chaperone properties of the 70 and 90 kD HSP families have permitted them to play a role in cell regulation often as stabilizing inhibitory components of transcription factor or protein kinase complexes (Nollen and Morimoto, 2002). Another feature of the stress protein response is the power of the gene expression system involved and the high abundance of HSP expression in stressed cells (Wu, 1995). This appears to have led to a further elaboration of the functions of stress proteins in the immune system (Srivastava, 2002; Srivastava and Amato, 2001). Dying cells often undergo the stress protein response, leading to lysis and release of HSP into the extracellular space (Shi and Rock, 2002). Such extracellular HSP appears to lead to a danger response that can activate the inflammatory response as well as the innate and adaptive immune response (Asea et al., 2000; Asea et al., 2002; Srivastava, 2002). The pro-immune effects of extracellular HSP appear to be countered by the intracellular stress protein response and both HSF1 and the HSP70 are able to inhibit the expression of proinflammatory cytokines and mediated the extremes of the acute phase response (Cahill et al., 1996; Xie et al., 2003).

Interactions Between the Stress Protein Response and Immune and Inflammatory Responses

(a) Heat Shock Response and Pro-Inflammatory Signaling/Gene Expression

Recently, it has been found that pretreatments with heat stress and the induction of multiple HSP correlate with improved survival in several rodent models exposed to endotoxin challenge (Chu et al., 1997; Hotchkiss et al., 1993; Ribeiro et al., 1994). In various models of sepsis, induction of HSR protected against sepsis-induced mortality, organ injury, cardiovascular dysfunction and apoptosis. The mechanisms by which HSR protects against sepsis-induced injury are currently under investigation. One potential mechanism involving the effect of HSR is to inhibit proinflammatory responses. HSR can inhibit expression of proinflammatory genes such as TNF-a, IL-1, IL-12, IL-18, ICAM-1, iNOS and promote expression of anti-inflammatory genes IL-10 (De et al., 2000; Kohn et al., 2002; Snyder et al., 1992; Wang et al., 2001; Wang et al., 2002). But the mechanism for the inhibitory effect of heat shock on expression of inflammatory mediators is not clear.

Upon activation of inflammatory cells by LPS or the binding of cytokines to their own receptors, the NF-kB/IκB or MAPK (p38, ERK, and JNK) pathways are activated (Guha and Mackman, 2001). Once activated, these kinases further activate transcription factors such as NF- kB and NF-IL6 that are responsible for cytokine gene transcription (Arbabi and Maier, 2002; Senftleben and Karin, 2002). Many studies have confirmed that the inactivation of NF-κB/IκB pathway plays a pivotal role in HSR mediated suppression in the expression of pro-inflammatory genes. The mechanism by which the HSR inhibited activation of NF-κB might be associated with preservation of Ik-Bα and a decrease in LPS-mediated phosphorylation of Ik-Bα that correlated to inhibition of Ik-B kinase (IKK) activity (Grossman et al., 2002b; Shanley et al., 2000). Another potential mechanism is that HSR activated Ik-Bα promoter and increases the transcription of Ik-Bα mRNA (Wong et al., 1999). In addition, our studies and those of others have shown that HSR can inhibit nuclear translocation of NF-κB and degradation of IκB, although we found minimal effect of HSR or HSP70 over-expression on the up-regulation of MAPKs activities induced by LPS (Shi et al., (in press)).

Some controversial results have also been reported regarding the effect of HSR on MAPK pathways. Wang *et al* reported that HSR inhibited IL-18 expression through the JNK pathway, while others argued that HSR did not inhibited endotoxin-mediated activation of JNK in murine macrophage model (Wang et al., 2002). Moreover, Ferlito *et al* showed that HSR exerts different effects on LPS-mediated inflammation in accordance with the magnitude of the stress, the recovery time, and the differentiation stage of the cell (Ferlito and De Maio, 2005). In addition to early inflammatory mediators, we also found that heat shock pretreatment significantly inhibits LPS-induced release of high-mobility group box 1 protein (HMGB1) which was identified as a novel inflammatory cytokine and a late mediator of endotoxin lethality, and inhibited the translocation of HMGB1 from the nucleus to the cytoplasm in RAW 264.7 mouse macrophages (Tang et al., 2005).

(b) HSP and Pro-Inflammatory Signaling/Gene Expression

Heat shock proteins, as the main products of HSR, are up-regulated and thought to play a pivotal role in protecting cells against inflammatory responses (Kindas-Mugge et al., 1993; Kindas-Mugge et al., 1996; Mizzen, 1998; van Eden et al., 1998). Exogenous hsp27 is demonstrated as a potent activator of anti-inflammatory IL-10 production in human monocytes(De et al., 2000). In addition, over-expression of HSP-70 significantly inhibited LPS-induced increases in production of TNF-α, IL-1β, IL-12 and release of HMGB1 (Ding et al., 2001; Shi et al., (in press); Tang et al., 2005).

Recent data has demonstrated that elevated HSP70 suppressed the activation and nuclear translocation of NF-κB although its exact molecular basis is still enigmatic (Ran et al., 2004). Because the activity of NF-κB depends on the phosphorylation and degradation of IκBα that is related to IKK, and the activity of IKK is counter-balanced by intracellular phosphatase activity, intracellular phosphatase activity is being increasingly recognized as playing a central role in signal transduction pathways related to inflammation (Shanley, 2002). Grossman *et al* demonstrated that inhibition of intracellular phosphatase activity leads to degradation of IκBα (Grossman et al., 2002a). It may be significant that Mivechi et al found that purified mammalian HSP70 activated phosphoprotein phosphatases *in vitro* while others showed that overexpression of HSP70 inhibited the phosphorylation of transcription factor by activating protein phosphatase (Ding et al., 1998; Mivechi et al., 1993). Our recent study argued that HSP70 overexpression up-regulated the activity of intracellular phosphatase (Shi et al., (in press)). HSP70 may thus inhibit LPS-induced phosphorylation and degradation of IκBα as well as subsequent activation and nuclear translocation of NF-κB through up-regulating phosphatase activity.

Because HSF1 is the main regulator of HSR, we assumed that inactivation of HSF1, which abrogates the heat shock response *in vivo*, would reduce stress tolerance during inflammatory challenge. We administered LPS intraperitoneally and monitored survival rates in wild-type and $HSF1^{-/-}$ mice. The survival rate of $HSF1^{-/-}$mice was significantly lower correlated with increased production of TNF-α, supporting the role of HSF1 *in vivo* as an anti-inflammatory regulator (Xiao et al., 1999).

(c) Direct Repression of Cytokine Genes by HSF1

Suspicions that there may be a link between the heat shock and acute inflammatory response date back many years. In fact an early treatment for cancer involved the use of bacterial toxins, potent activators of cytokine cascades that lead to a rise in body temperature thought to be involved in tumor regression (Coley, 1893; Rhodenburg, 1918). One of the key mediators of the pyrogenic response to bacterial endotoxins is interleukin 1b and we first investigated the link between the protein stress response and *IL1B* expression (Cahill et al., 1996). Our findings were that increasing temperature exerts a reciprocal effect on the two responses, with HSP mRNA increased and interleukin 1bmRNA levels decreased (Cahill et al., 1997; Cahill et al., 1996). As the heat inducible HSF1 is the transcriptional activator

of HSP70, we investigated whether it might regulate the inhibition of interleukin 1b expression. Indeed we found that this was the case and that induction of the proximal IL1B promoter by the endotoxin *E coli* LPS was directly repressed by HSF1 overexpressed in cells or induced by heat shock, both of which led to HSF1 binding to a repressor site in the *IL1B* promoter (Cahill et al., 1996). These experiments therefore suggested for the first time that HSF1 can act as a gene repressor and might function in a feedback loop to regulate the release of pyrogenic cytokines such as interleukin 1b(Cahill et al., 1996; Xie et al., 2002a)

Figure 1 depicts the signal transduction pathways leading to gene expression in monocytes/macrophages exposed to proinflammatory stimulus such as E. *coli* lipopolysaccharide (LPS) (Li, 2004; Takeda et al., 2003). HSF1 acts at the promoter level to repress *IL1B* (0 In addition, more recent studies show, *tnfa* and c-*fms* (the

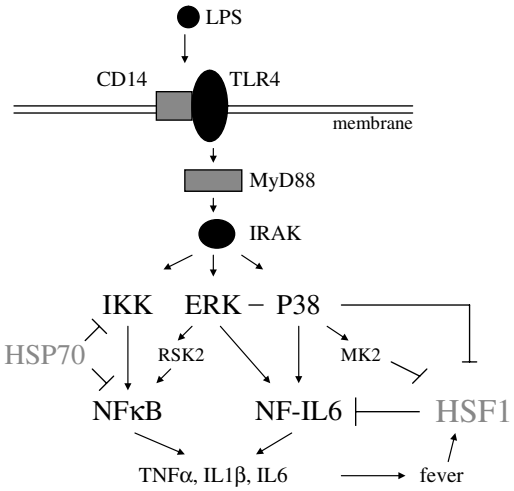

Figure 1. Interaction between the acute inflammatory response and the stress protein response (Akira and Sato, 2003; Li, 2004). Inflammatory mediators such as bacterial lipopolysaccharides (LPS) interact with monocytes and macrophages through CD14 and toll-like receptor4 (TLR4). This gives rise to transmembrane signaling events including the recruitment of adaptor proteins such as MyD888b as shown here although a number of others such as TIRAP, TRIF and TOLLIP can also be involved. These adaptor molecules subsequently associate with kinases of the interleukin 1 receptor kinase (IRAK) family including IRAK1, 2, M and 4. Activation of a number of downstream kinases including the IKK, ERK and p38/MAPK families then lead to activation of a number of transcription factors including NF-κB, NF-IL6 (shown here) as well as AP-1 and IRF family members. Combinatorial association of these factors on the promoters of cytokine genes such as *IL1B, IL6* and *TNFa* (as well as chemokines, adhesion molecules and co stimulatory molecules) lead to induction of the acute phase response, including inflammatory and febrile changes. Activation of the stress protein response leads to induction of HSF1 and expression of HSP70 and these changes are inhibitory leading to repression of NF-IL6 mediated and NFkB mediated transcription(Xie and Calderwood, 2001; Xie et al., 2003). Activation of the acute phase response may counter the effects of HSF1/HSP70 by inhibiting HSF1 through ERK, p38, PKC, GSK3 or other kinase activation and subsequent phosphorylation of HSF1 on inhibitory serine residues (Stevenson et al., 1999)

gene encoding the receptor for the CSF1 receptor, responsible for myeloid cell differentiation) are directly repressed by HSF1 (Singh et al., 2000; Singh et al., 2002; Xie et al., 2002b; Xie et al., 2003). One common factor in both *IL1B* and c-*fms* regulation is nuclear factor of interleukin 6 (NF-IL6), an essential factor for transcription in these and many genes in cells of myeloid origin (Akira et al., 1990; Cahill et al., 1996; Tenen et al., 1997; Xie et al., 1999; Xie and Calderwood, 2001; Xie et al., 2002b). We therefore examined the potential role of NF-IL6 as a target for HSF1 in myeloid cells and found that *IL1B* promoter constructs with a mutation in the NF-IL6 binding domain, although of reduced transcriptional activity in response to LPS, were immune to HSF1 repression (Cahill et al., 1996). In addition, in co-transfection experiments, overexpression of NF-IL6 could reverse the repressive effects of HSF1 overexpression on the c-*fms* promoter or artificial promoters containing only NF-IL6 elements (Xie et al., 2002b). We subsequently discovered that HSF1 can directly bind to NF-IL6 in macrophages (Xie et al., 2002a). Repression of the *IL1B* promoter involved blocking the ability of NF-IL6 to interact with the tissue specific transcription factor PU-1, an interaction essential for induction of the *IL1B* promoter (Xie et al., 2002a). Our subsequent domain mapping studies of HSF1 have demonstrated an NF-IL6 binding domain on HSF1 (Xie et al., 2003). Interestingly, we found two independent repression domains on HSF1 only one of which interacts with NF-IL6, suggesting the existence of other cooperative pathways of repression (Xie et al., 2003). As can be seen from Figure 1, NF-IL6 is activated by LPS through 1 or 2 of the branches of the TLR4/MyD88 pathway involving the ERK and p38 pathways (Takeda and Akira, 2004).

(d) HSF1 and Gene Repression

The first suspicion that HSF1 may possess functions in addition to activating HSP gene transcription came in studies in *Drosophila* which showed HSF association with non heat shock loci (Westwood et al., 1991). Such *loci* included developmental genes undergoing repression during heat shock (Westwood et al., 1991). We subsequently demonstrated that HSF1 can act as a direct repressor of non-heat shock genes (Cahill et al., 1996; Chen et al., 1997; Singh et al., 2000; Singh et al., 2002; Xie and Calderwood, 2001; Xie et al., 2002a; Xie et al., 2002b). We have subsequently dissected many of the steps involved in the repression of the prointerleukin 1b gene (Cahill et al., 1997; Cahill et al., 1996; Dinarello et al., 1986; Xie et al., 2002a; Xie et al., 2003). In order to repress its targets HSF1 must be converted from the inert monomer to the trimer form (Xie and Calderwood, 2001; Xie et al., 2002b). Activation of HSF1 then inhibits the transcription of many inducible genes involved in macrophage activation and the inflammatory response, including *IL1B, tnfa*, and c-*fms* (Cahill et al., 1997; Cahill et al., 1996; Singh et al., 2000; Singh et al., 2002; Xie et al., 2002a; Xie et al., 2002b). In addition, other inducible genes not involved in the specialized function of macrophages, including the immediate early genes c-*fos* and urokinase plasminogen activator (*uPA*) are repressed by heat shock and HSF1 (Chen et al., 1997). Indeed repression by HSF1 may be a conserved property in eukaryotes as evidenced by the *Drosophila* studies(Westwood et al.,

1991). This capacity for gene repression is specific for HSF1 within the HSF family in mammalian cells and is not a property of HSF2 (Chen et al., 1997; Xie et al., 2002a).

(e) Mechanisms of Repression

The understanding of mechanisms of repression has evolved considerably in recent years. Early hypotheses current when our study began included (1) the ability of repressing proteins to block access of activating proteins to sites on DNA in target promoters and (2) inhibitory interactions of the repressor with activators or members of the general transcription machinery (Hanna-Rose and Hansen, 1996; Herschbach and Johnson, 1993). We examined such interactions in the IL-1b gene and found that although HSF1 did not displace activators from the promoter, the IL-1b was repressed through HSF1 binding to and quenching activating effects of Nf-IL6 (Cahill et al., 1996; Xie et al., 2002a; Xie et al., 2002b; Yang et al., 2000). The interaction involves the leucine zipper domain of NF-IL6 and the leucine zipper trimerization domain of HSF1. The HSF1/NF-IL6 interaction may constitute a molecular mechanism involved in the multiple levels of cross talk between the heat shock response and the inflammatory response in myeloid cells (Asea et al., 2000; Asea et al., 2002; Xie et al., 2002a). Indeed, NF-IL6 activation caused either by overexpression of the protein from transfected expression plasmid or by bacterial endotoxin stimulation of endogenous NF-IL6 leads to the repression of the HSP70 promoter through a mechanism that appears to involve directly HSF1-NF-IL6 binding (Xie et al., 2002b). We propose that other members of the bZIP family including C/EBP, AP-1 binding or ATF/CREB family proteins may interact with HSF1 (McKnight, 1992; Xie and Calderwood, 2001). Similar interactions between HSF1 and other transcription factors may also be involved in the repression of genes such as c-*fos* or *tnfa* in which NF-IL6 does not play a major role in transcriptional activation (Xie and Calderwood, 2001).

We have further examined the mechanisms of repression by mutational analysis finding that gene repression is conferred by sequences in the N-terminal domain of HSF1 and that the C-terminus, which contains the *trans*-activation domains, does not play a major role in repression. We showed two key repressor domains in the N-terminus of HSF1 including (1) a region a sequence (amino acids 106–205) essential for NF-IL6 binding and (2) a second domain (REP-amino acids 229–279) essential for repression but not required for NF-IL6 binding. While carrying out these studies, we showed that HSF1 repression of *IL1B* is inhibited by the histone deacetylase inhibitor trichostatin A, suggesting a role for histone deacetylases (HDACs) in the repressor functions of HSF1 and the possibility that the REP domain may govern such interactions (Xie et al., 2003).

(f) Activation of Anti-inflammatory Mediators by HSF1

Because that HSR could promote LPS- induced expression of anti-inflammatory genes IL-10, one hypothesis is that HSF1 may play an important role in

HSR-mediated augment of IL-10[6]. Work from the Xiao lab showed that over-expression of HSF1 increased the expression of IL-10 and the activity of *luciferase* reporter gene which was driven by murine IL-10 promoter. Three HSEs, arranged with perfect inverted dyad nGAAnnTTCn, were located in the murine *IL-10* promoter by bioinformatics analysis. Only the promoter region (−387/ − 360nt) was identified as a high affinity binding site for HSF1 by EMSA. These data suggest that HSF1 could act as an activator of anti-inflammatory mediator IL-10

Glucocorticoids are among the most widely prescribed anti-inflammatory drugs. They act by binding to the glucocorticoid receptor (GR) that, upon activation, translocates to the nucleus and either stimulates or inhibits gene expression. In the absence of hormone, the GR is known to exist in a complex with several members of the heat shock protein family. GR inhibition of many proinflammatory response genes occurs through induction of the synthesis of anti-inflammatory proteins as well as through repression of proinflammatory transcription factors, such as NF-kB or activator protein-1 (AP-1)(Smoak and Cidlowski, 2004). Recent finding that glucocorticoids can suppress the heat shock response in cells by inhibiting binding of HSF1 to HSP70 promoter is intriguing (Wadekar et al., 2004). In addition to suppression of HSF1 activity by GR, reciprocal control of steroid receptor responses by stress can also occur. Most studies report that heat shock and other forms of cellular stress will cause an increase in steroid receptor transcriptional activity. The mechanism by which heat shock and other forms of stress cause elevation of GR function most likely requires activation of HSF1. Constitutively active HSF1 can enhance the expression of GR-mediated gene through increase of GR transactivity, not through alterations of GR protein levels or changes in GR hormone binding capacity (Jones et al., 2004).

(g) HSF1 and Histone Modification

Most current hypotheses for short-term gene repression stress the role of modification of histones and nucleosome structure on target promoters (Davis and Brackmann, 2003). The modification of residues in the tails of histones H3 and H4 histones by acetylation, methylation and phosphorylation influences transcription by two main mechanisms: (1) altering histone association with DNA (acetylation being associated with a relaxation in DNA binding, open chromatin conformation and active transcription) and (2) the pattern of histone modification gives rise to protein binding sites and is known as the histone code (Davis and Brackmann, 2003; Marmorstein, 2001). In addition, short range changes in nucleosome structure that affect transcription are carried out by ATP-dependent chromatin remodeling proteins (often associated with transcription factors) that permit RNA polymerase complexes to move processively along nucleosome containing DNA (Vignali et al., 2000). We have searched for potential co-repressor molecules involved in HSF1 dependent repression and in a proteomic screen were able to find tight association with the gene co-repressor MTA1 (Khaleque et al, in preparation: see Preliminary Data). MTA1 belongs to an unique co-repressor complex that contains both the ATP-dependent chromatin remodeling protein Mi2 and histone deacetylases 1 and

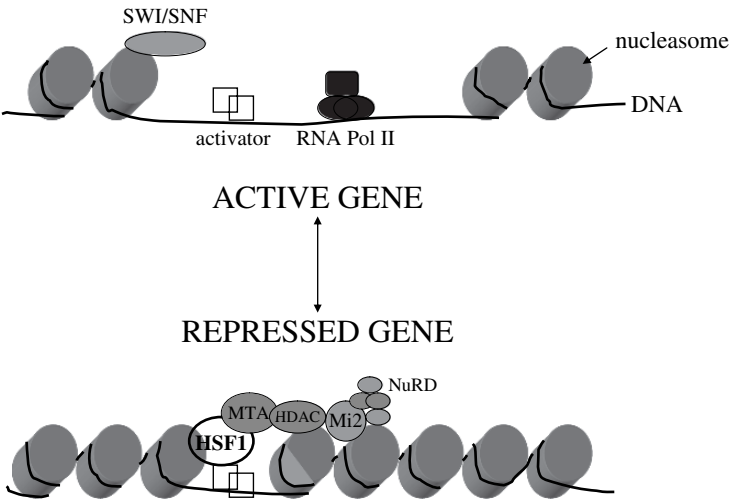

Figure 2. Model for gene repression by the HSF1/MTA1/NuRD complex. In the ACTIVE state, DNA is in an open conformation due to recruitment of HATs and ATP dependent remodeling complexes (SWI/SNF), and RNA polymerase II is able to transcribe the sequence of the structural gene. HSF1 recruitment by, for instance exposure to heregulin brings associated MTA1 and HDACs to the promoter, which deacetylates histones, recruit nucleosomes and lead to a compacted chromatin conformation that contributes to gene repression. We show HSF1/NuRD complexes interacting with the activating protein still associated with DNA This is in line with our experiments that indicate that repression involves HSF1 association with other activators such as NF-IL6. In addition however, MTA1 can itself bind directly to ER and recruitment to promoters may involve this interaction

2 (Bowen et al., 2004; Mishra et al., 2003; Xue et al., 1998). Repression of target gene may involve the mechanism described in (Figure 2).

Role of HSF1 and Heat Shock Proteins in Regulation of the NF-κB Pathway

Another branch leads to the NFkB pathway, the major transcriptional pathway in inflammation that regulates the expression of TNFa, adhesion factor molecules, chemokines and T cell co-stimulatory molecules (Baldwin, 1996) (Figure 1). We examined this phenomenon in a transcriptional study of the HIV1 long terminal repeat, an enhancer that is regulated by NF-IL6, NFkB and other elements (Y. Xie and S. K> calderwood, unpublished). We found that the native HIV1 LTR is repressed by heat shock and HSF1 overexpression; mutagenizing the NF-IL6 sites strongly reduced repression by HSF1 while mutating the NFkB site, although weakening the promoter, was less effective in inhibiting repression by HSF1 (Y. Xie & SK Calderwood, unpublished). These studies on the HIV1 LTR and other work on the effects of heat shock on the NFkB pathway suggest that HSF1 may not be a major target for HSF1 in inhibition of the acute inflammatory response (Kohn et al., 2002; Malhotra et al., 2002). However, recent studies carried out on lung

inflammation in vivo show that hsf1 inactivation inhibits the anti-inflammatory effects of heat shock and enhances NFkB activation (Wirth et al., 2004). The studies indicate that the cytokine GM-CSF is repressed by HSF1 and that decline in GM-CSF after HSF1 repression mediate the inhibition of NF-kB (Wirth et al., 2004). It is notable that GM-(Xie et al., 2002b)CSF transcription depends on NF-IL6, a factor which we have shown repeatedly to be target for HSF1 in repression of myeloid transcription (Xie et al., 2002b). A number of other studies have shown that heat shock leads to NFkB inhibition and have suggested a role for a downstream product of HSF1 such as one of the HSP or other heat-induced effects (reviewed above). One potential target for HSP70 in the NFkB pathway could be another of the heat shock proteins, HSP90 which appears to play an essential role in transmitting pro-inflammatory signals immediately down stream of MyD88 and upstream of IKK activation in the NFkB pathway (Chen et al., 2002). HSP70 and HSP90 often play antagonistic roles in cell regulation: when HSP90 displaces HSP70 from multi-chaperone complexes, this change is associated with maturation of regulatory complexes while HSP70 is associated with inactive complexes (Nollen and Morimoto, 2002). Engagement of the stress protein response, such as might occur in febrile conditions or during oxidative stress, may thus lead to inactivation of the NFkB arm of the pro-inflammatory cascade through indirect sequela of HSF1 activation including a block to monocyte maturation (Wirth et al., 2004; Xie et al., 2002b) or molecular chaperone competition reactions (Ding et al., 2001) and the blocking of a major branch in pro-inflammatory signaling (Figure 1).

Overriding the Heat Shock Response by Pro-Inflammatory Signaling

In order for activation of the inflammatory response whose prompt induction is at the physiological level is a t least as important as the stress protein response at the cellular level, it is necessary for the activity of HSF1 and HSP expression to be inhibited. That this phenomenon does occur is indicated by the fact that LPS pre-treatment of monocytes effectively blocks HSF1 activation by heat and other agents(Soncin and Calderwood, 1996). The mechanisms involved in LPS antagonism of the heat shock response are, however not clear. One significant finding that might suggest an answer to this question is that many agents that repress NFkB coordinately activate HSF1 binding to DNA with a remarkable convergence in terms of dose response and kinetics (Ianaro et al., 2003; Kopp and Ghosh, 1994; Rossi et al., 1997; Soncin and Calderwood, 1996). These studies suggest that either the factors interact directly or that they are controled reciprocally by a common upstream regulator immediately proximal to each factor. Proinflammatory agents such as LPS would thus induce a regulatory molecule involved in NFkB activity that coordinately represses DNA binding of HSF1. We have examined potential upstream kinases that are induced downstream of LPS as HSF1 inhibitors (Figure 1). These potential HSF1 inhibitors could include ERK or p38 MAP kinases. In fact, ERK1 is an effective HSF1 inhibitor, directly phosphorylates HSF1 and mediates

its repression (Chu et al., 1996; Wang et al., 2003). However, ERK is not inhibited by NSAIDs at concentrations that induce HSF1 binding to DNA. p38 is activated rather than inhibited by the NSAIDs (Stevenson et al., 1999). Two protein kinases that do phosphorylate HSF1 and are inhibited by NSAIDs at the concentrations that trigger HSF1 are the related kinases RSK2 and MK2 (Stevenson et al., 1999; Wang et al., 2000). However, while RSK2 phosphorylates IkBa, MK2 does not and while MK2 seems to repress HSF1, RSK2 is ineffective in this activity (Stevenson et al., 1999; Wang et al., 2000). Thus neither candidate perfectly fits the role of coordinate HSF1 and NFkB regulator. Other candidates of course are IKKa and IKKb whose role in HSF1 regulation is less well known. In fact IKKa activity is inhibited by the NSAIDs and heat shock, all though little is known about its potential role in HSF1 regulation (Palayoor et al., 1999)(Y. Xie & S.K. Calderwood, in preparation).

We have carefully examined the role of protein phosphorylation in the activation and inhibition of HSF1 function (Figure 3). A number of potential regulatory sites are shown in (Figure 3). Of most potential significance in regulation of repression is serine 121 (Figure 3). We aimed to determine signaling pathways through which

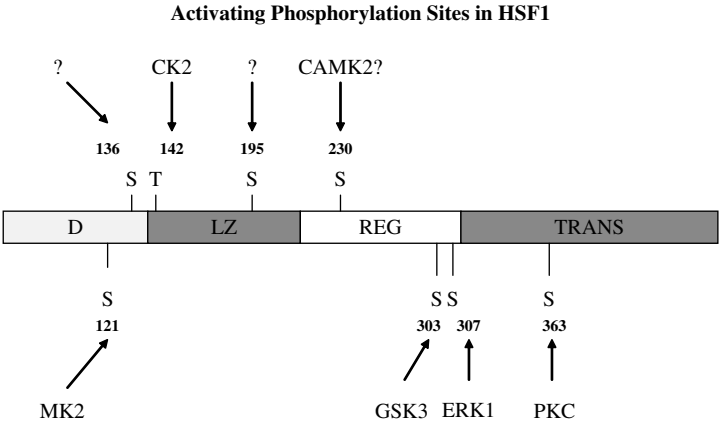

Figure 3. Phosphorylation map for HSF1. Phosphorylation sites as well as the identity of kinases that phosphorylate these sites is shown. We have identified activating sites as well as phosphorylation sites that repress HSF1(Chu et al., 1996; Chu et al., 1998; Soncin et al., 2003; Wang et al., 2005)When tested on ability of HSF1 to repress c-fos, phosphorylation events shown to activate transcription including phosphorylation at threonine 142 and serine 195 were also essential for gene repression {Y. Xie & S. K. Calderwood, in preparation(Soncin et al., 2003). These sites appear to play generic roles in HSF1 uncoiling from its inactive form during activation, to expose domains involved in both gene activation and repression (Figure 4). Serine phosphorylation that inhibit HSF1 function at serines 303, 307 and 363 also inhibited ability of HSF1 to repress c-*fos* and alanine substitutions at each of these sites increase the ability of the transfected HSF1 to repress the promoter (Xie et al., (submitted)). These phosphorylation sites function to repress HSF1 by causing nuclear export by associating with the phosphoserine binding adaptor protein 14-3-3 as well as other proteins (He et al., 1998; Wang et al., 2003; Wang et al., 2004)

HSF1 could be regulated during inflammation. Our hypothesis was that in order for effective activation of the cytokines TNFa and IL1b during the acute phase of inflammation, HSF1 must be inactivated. We had shown previously that HSF1 can be induced by non-steroidal anti-inflammatory drugs (NSAIDs) and that NSAIDs can thus repress the promoters of inflammatory cytokines (Calderwood, 2005a; Housby et al., 1999; Soncin and Calderwood, 1996). We therefore searched for protein kinases induced in inflammation that can phosphorylate HSF1 and can be inhibited by NSAIDs in the concentration range that induces HSF1. Recent studies indicate that HSF1 is regulated by the pro-inflammatory protein kinase MAPKAP kinase 2 (MK2) and that this kinase is inhibited by NSAIDs *in vitro* and *in vivo* (Wang et al., 2006). MK2 directly phosphorylates HSF1 and inhibits activity by decreasing its ability to bind the heat shock elements (HSE) found in the promoters of target genes encoding the HSP molecular chaperones and cytokine genes (Wang et al., 2006). MK2 overexpression inhibits both activation of the *hsp70* promoter as well as the repression of *IL1B*. activation of HSF1 to bind HSE in *hsp* promoters is inhibited through the phosphorylation of a specific residue, serine 121 by MK2 (Wang et al., 2006). A novel mechanism for MK2-induced HSF1 inactivation is suggested by the findings that phosphorylation of serine 121 enhances HSF1 binding to HSP90, an intracellular inhibitor of HSF1. Dephosphorylation of serine 121 in cells exposed to non-steroidal anti-inflammatory drugs leads to HSP90 dissociation from HSF1 which then forms active DNA binding trimers. These experiments indicate a novel mechanism for the regulation of HSF1 by pro-inflammatory signaling and may permit HSF1 to respond rapidly to extracellular events, permitting optimal physiological regulation (Wang et al., 2006).

CONCLUSIONS

Activation of the HSR exerts some pro-inflammatory effects when HSP are released during cell insult and such extracellular HSP induce cytokine release in inflammatory and immune modulating cells. However, the intracellular mediators of the HSR including the transcription factor heat shock factor 1 (HSF1) and the HSP have profoundly anti-inflammatory effects. HSF1 can be induced by the elevated temperatures encountered in inflamed tissues and in fever as well as by anti-inflammatory prostaglandins. Such activated HSF1 represses cytokine release both directly by inhibiting nuclear factor of interleukin 6 (NF-IL6) and indirectly when elevated HSP inhibit the potent inflammatory factor NF-kB. Reciprocal effects are observed on activation of the APR which leads to inhibition of HSF1 through stimulation of inactivating phosphorylation events involving the mitogen activated kinase (MAPK) pathways. Activation of the HSR thus constitutes a feedback regulatory mechanism for the APR and limits the lethal over stimulation of cytokine release which may occur during infection (Figure 4). However, in order for rapid activation of the APR, mechanisms also exist for HSF1 repression, permitting controlled activation of the response during infection (Figure 4).

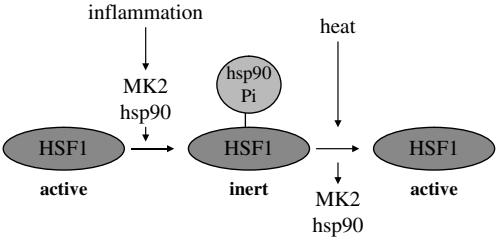

Figure 4. Cycling of HSF1 through active and inactive forms during the inflammatory response. Our experiments indicate that HSF1 can be repressed during the onset of the acute inflammatory response by activation of MK2 and formation of inert HSF1 through its increased association with HSP90 (Wang et al., 2005). Cytokines are repressed directly by HSF1 as well as indirectly through hsp70 antagonisms of NfkB (Calderwood, 2005a). During the resolution of the response when fever is triggered, the resulting heat shock overrides the effects of MK2 on HSF1, causing dephosphorylation of serine 121 and dissociation of HSP90 and MK2 and renewed active repression of cytokine genes (Wang et al., 2005)

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CHAPTER 8

INTERACTION OF HEAT SHOCK PROTEIN 60 WITH INNATE IMMUNE CELLS

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Abstract: Human heat shock protein 60 (Hsp60) elicits a pro-inflammatory response in innate immune cells. This response includes the release of inflammatory mediators like tumor necrosis factor α, interleukin (IL-)1β, IL-6 and nitric oxide. Hsp60 also has been found to induce the gene expression of the T helper (Th)1-phenotype promoting cytokines IL-12 and IL-15. Detailed studies identified specific receptor structures for the interaction of Hsp60 with innate immune cells. Accumulating evidence points to the presence of different receptor structures, which are involved in Hsp60-binding and in the Hsp60-mediated initiation of a pro-inflammatory response. These findings indicate that the interaction of Hsp60 with innate immune cells is a highly complex process. Recently, the epitopes of the Hsp60 molecule responsible for binding to innate immune cells and for the activation of these cells have been characterized. In a cell-type-specific manner, the region aa481–500 and the regions aa241–260, aa391–410 and aa461–480 were identified to account for Hsp60-binding to innate immune cells. A completely different region of Hsp60, aa354–365 was found to be involved in specific LPS-binding, thereby mediating the immunostimulatory effects of Hsp60 on innate immune cells. Because of the immunomodulatory properties of Hsp60 it has been proposed to act as an intercellular danger signal, regulating innate and adaptive immune reactions, thereby contributing to the induction and progression of inflammatory diseases

Keywords: Hsp60 – Innate immune cells – Receptor structures – Hsp60 epitopes – LPS

INTRODUCTION

Heat shock proteins (HSP) are ubiquitously expressed proteins, which are phylogenetically highly conserved across species. According to their molecular weight, HSP are classified into families including small HSP, HSP60, HSP70, HSP90 and HSP110. HSP serve as molecular chaperones with many important functions

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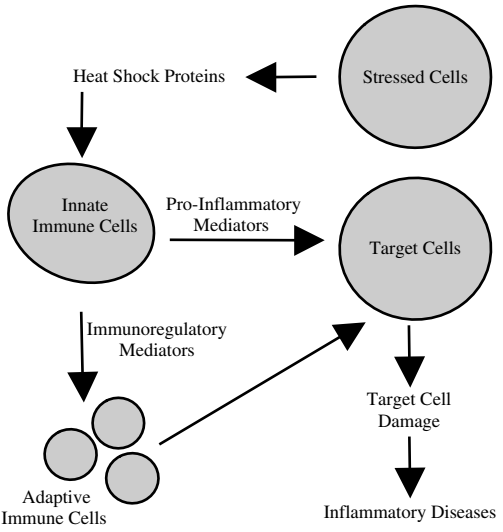

Figure 1. Immunoregulatory role of heat shock proteins

in folding, transport and degradation of proteins. In addition to being constitutively expressed, under stress conditions (e.g. high temperature, radiation, viral and bacterial infection) synthesis of HSP is markedly upregulated (Fink, 1999; Hartl, 1996). In response to stress changes in the intracellular localization of HSP and expression on the cell surface have been observed (Brudzynski et al., 1992; Brudzynski, 1993; Soltys et al., 1997; Wand-Württenberger et al., 1991). Although HSP are classically regarded as being intracellular proteins, it is now evident that they can be released into the extracellular space by stressed cells (Barreto et al., 2003; Hightower et al., 1989; Liao et al., 2000), Figure 1. Extracellular HSP have been proposed to act as intercellular immunoregulatory signalling molecules, thereby influencing a wide range of immune reactions (Chen et al., 1999; Wallin et al., 2002). This function is underlined by several studies reporting an immunoregulatory role of HSP in the development of inflammatory disorders, such as rheumatoid arthritis, type 1 diabetes or arteriosclerosis (Pockley, 2001; van Eden et al., 2005), Figure 1.

REGULATORY EFFECTS OF HEAT SHOCK PROTEIN 60 ON INNATE IMMUNE CELLS

Recently, HSP60, HSP70 and HSP90 subfamilies came into the focus of immunological research interests because of their potential immunoregulatory role in various inflammatory diseases. HSP have been described as immunodominant molecules in various diseases and HSP-directed immune responses have been implicated to contribute to the pathogenesis of autoimmune disorders and vascular diseases.

Because of the high sequence homology between mammalian and microbial HSP it has been suggested that HSP might provide a link between infection and autoimmunity, either by recognition of conserved epitopes or by immunological cross-reactivity (Lamb et al., 1989; Pockley, 2001). For Hsp60 a potential immunoregulatory role in the development of tissue-specific autoimmune disorders such as rheumatoid arthritis and type 1 diabetes and in vascular diseases like arteriosclerosis has been assumed (Abulafia-Lapid et al., 1999; Feige et al., 1991; Kamphuis et al., 2005; Xu et al., 2000).

As mentioned before, it is now evident that HSP are released into the extracellular compartment in response of cells to stressful situations, e.g. during inflammation. Hsp60 has been found to be present in the circulation of normal individuals (Pockley et al., 1999; Xu et al., 2000), and increased serum levels have been observed in a number of pathological conditions like hypertension (Pockley et al., 2000; Pockley et al., 2002), arteriosclerosis (Wick et al., 2004; Xu et al., 2000) and renal vascular diseases (Wright et al., 2000). Emerging evidence coming from several studies suggest that extracellular HSP function as intercellular signalling proteins, which serve as danger signals to the innate immune system, thereby mediating and modulating a number of inflammatory reactions (Chen et al., 1999; Wallin et al., 2002). We and others have shown that human Hsp60 stimulates pro-inflammatory reactivity in innate immune cells, such as macrophages, dendritic cells (DC) and endothelial cells (EC) (Chen et al., 1999; Flohé et al., 2003; Kol et al., 1999; Vabulas et al., 2001), (unpublished data). Hsp60 was found to induce the release of pro-inflammatory mediators like the cytokines tumor necrosis factor α (TNF-α, interleukin (IL-)1β and IL-6, the C-C chemokines RANTES, MCP-1 and MIP-1α and the short-lived radical nitric oxide (NO) from these cells. In addition, Hsp60 stimulates the production of the T helper (Th)1 cell promoting cytokines IL-12 and IL-15 in innate immune cells. And finally, Hsp60 provokes the maturation of DC demonstrated by the up-regulation of MHC molecules, CD40, CD54 and CD86. These findings indicate that Hsp60 represents an important danger signal for the innate immune system.

HEAT SHOCK PROTEIN 60 RECEPTOR STRUCTURES ON INNATE IMMUNE CELLS

Recent studies focused on the identification of specific receptor structures for Hsp60 on innate immune cells. A flow cytometry-based binding assay with human Hsp60, labelled with the fluorescent dye Alexa Fluor 488, was established. By the use of this assay it was possible to analyse and quantify the binding of Hsp60 to the surface of innate immune cells. This approach allowed us for the first time to demonstrate specific Hsp60-binding to macrophages (Habich et al., 2002), Figure 2.

Binding of human Hsp60 to macrophages showed the classic characteristics of a typical ligand-receptor interaction, i.e. Hsp60-binding occurred at submicromolar concentrations, was saturable and could be competed only with the unlabelled Hsp60 ($K_d \sim 300$ nM), but not by unrelated control proteins. Further analyses demonstrated

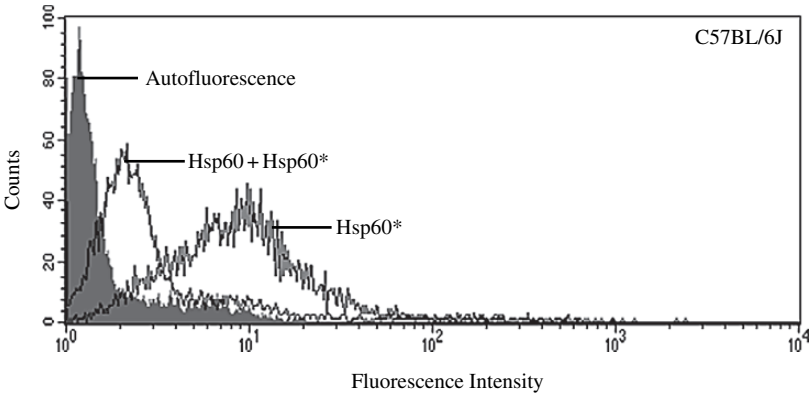

Figure 2. Specific Hsp60-binding to macrophages. Primary macrophages from C57BL/6J mice were incubated in the absence (autofluorescence) or presence of unlabelled human Hsp60, followed by the addition of Alexa Fluor 488-labelled human Hsp60 (Hsp60*). Fluorescence intensities were plotted against cell counts

that binding of Hsp60 to the cell surface was followed by rapid endocytosis at physiological temperatures. Specific Hsp60-binding was further confirmed by the use of confocal microscopy (Habich et al., 2002). Meanwhile, specific binding of Hsp60 to DC and EC could also be demonstrated, showing similar characteristics as Hsp60-binding to macrophages (unpublished data).

Further studies identified the β_2-macroglobulin receptor, also known as CD91, as a common binding receptor for Hsp70, Hsp90 and gp96 on innate immune cells (Basu et al., 2001; Binder et al., 2000). Consequently, it was tested, whether CD91 is also a binding receptor for Hsp60. The potential inhibitory effect of Hsp70, Hsp90, gp96 and β_2-macroglobulin on Hsp60-binding to macrophages was analysed in further binding studies. The results showed that CD91 is not involved in Hsp60-binding (Habich et al., 2002).

In view of the potential cross-reactivity between prokaryotic and eukaryotic Hsp60, further studies focused on the question, whether prokaryotic and eukaryotic Hsp60 species are recognized by the same binding receptor structures on innate immune cells. Therefore, the effects of different prokaryotic and eukaryotic Hsp60 preparations on human Hsp60-binding to macrophages were studied in comparative analyses (Habich et al., 2003; Habich et al., 2006). None of the analysed prokaryotic Hsp60 preparations (*Mycobacterium bovis* Hsp65, *Escherichia coli* GroEL, *Chlamydia pneumoniae* Hsp60) interfered with binding of human Hsp60 to macrophages. By contrast, all investigated eukaryotic Hsp60 proteins (human, mouse, rat and hamster Hsp60 and Hsp60 from *Histoplasma capsulatum*) inhibited human Hsp60-binding in a similar range, i.e. around 70% (Habich et al., 2006), Figure 3. The results of this study indicate that eukaryotic Hsp60 species use the same binding sites on macrophages, whereas Hsp60 species of prokaryotic origin use different receptor structures. This conclusion is supported by sequence comparisons revealing that human Hsp60 and

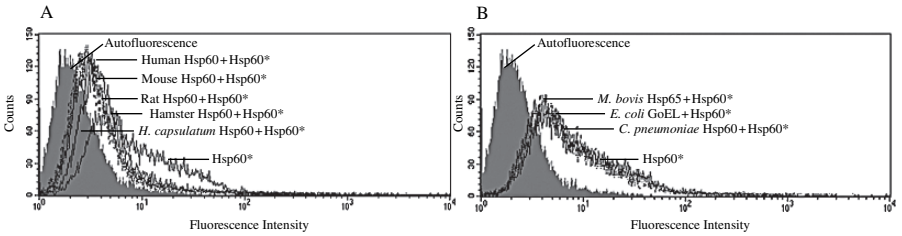

Figure 3. Differential recognition of eukaryotic (A) versus prokaryotic (B) Hsp60 species by macrophages from C57BL/6J mice. Cells were incubated in the absence (autofluorescence) or presence of unlabelled Hsp60 from different species, followed by the addition of Alexa Fluor 488-labelled human Hsp60 (Hsp60*). The analysis was performed by flow cytometry. Fluorescence intensities are plotted against cell counts

Hsp60 from mouse, rat and hamster show an amino acid identity above 95%, whereas the homology between human and prokaryotic Hsp60 species is only around 50%. For innate immune cells it might be fundamental to distinguish between endogenous (self) and infectious (non-self) Hsp60, because of the important role of Hsp60 in immunoregulation and the abundance of Hsp60 in eukaryotic and prokaryotic cells. Similar observations have been made for Hsp70. It has been reported that human Hsp70 does not share the binding site on innate immune cells with mycobacterial Hsp70, although binding sites reside on the same membrane protein (Becker et al., 2002; Wang et al., 2001).

Further approaches to identify receptor structures for human Hsp60 on innate immune cells have shown that toll-like receptor (TLR)4 and CD14, both involved in LPS-binding and -signalling (Poltorak et al., 1998), are responsible for the

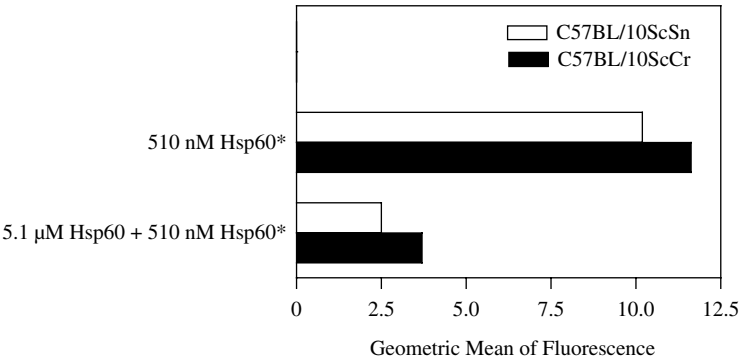

Figure 4. Hsp60-binding is TLR4-independent. Macrophages from C57BL/10ScSn mice (wild-type TLR4 expression) and from C57BL/10ScCr mice (deficient TLR4 expression) were incubated in the absence or presence of unlabelled human Hsp60, followed by the addition of Alexa Fluor 488-labelled human Hsp60 (Hsp60*). Analysis was done by flow cytometry, data represent the geometric mean of the fluorescence intensity of the cells

immunostimulatory effects of Hsp60 (Kol et al., 2000; Ohashi et al., 2000; Vabulas et al., 2001). In contrast, Hsp60-binding was found to be TLR4- and CD14-independent (Habich et al., 2002), (unpublished data), Figure 4.

These observations indicate that the interaction of Hsp60 with innate immune cells represents a highly complex process, including the contact of Hsp60 with cell surface structures involved in binding and in the initiation of pro-inflammatory responses.

EPITOPES OF THE HEAT SHOCK PROTEIN 60 MOLECULE INVOLVED IN THE INTERACTION WITH INNATE IMMUNE CELLS

In parallel to the characterization of receptor structures for Hsp60 on innate immune cells, recent studies focused on the identification of epitope regions of the human Hsp60 molecule responsible for the interaction with cell surface structures on these cells. As mentioned before, the contact of Hsp60 with innate immune cells is a highly complex process, involving different receptor structures for Hsp60-binding and for Hsp60-mediated cell activation. Therefore, it appeared reasonable to assume that also different regions of the Hsp60 molecule are involved in the contact to the appropriate receptor structures.

Epitopes of the Hsp60 Molecule Involved in Receptor Binding

Initially, analysis to identify epitope regions of the human Hsp60 molecule responsible for binding to receptor structures on innate immune cells was performed with the macrophage line J774A.1. In these studies we tested the capacity of defined oligopeptides of different length, covering the complete Hsp60 sequence and of selected Hsp60 deletion mutants to inhibit the binding of Alexa Fluor 488-labelled human Hsp60 to these permanent macrophages. A C-terminal region of the Hsp60 molecule, including aa481–500, was characterized to account for Hsp60-binding to the cells of this macrophage line (Habich et al., 2004). Surprisingly, the analysis of the same oligopeptides and deletion mutants on Hsp60-binding to primary bone marrow-derived macrophages from C57BL/6J mice resulted in the identification of three completely different epitope regions of the Hsp60 molecule, i.e. aa241–260, aa391–410 and aa461–480 (Habich et al., 2006), (Table 1).

These findings indicate that cell type-dependent different regions of the Hsp60 molecule are responsible for the interaction with appropriate receptor structures on innate immune cells, such as macrophages. Hence, also different, cell-type-specific receptor structures are proposed to be involved in recognizing the adequate epitope regions of Hsp60. These observations are consistent with studies reporting different, cell-type-specific receptors for other HSP. For Hsp70 several binding receptors have been described on different immune cells, i.e. CD91 on macrophages, CD40 on macrophages and DC, the class E scavenger receptor LOX-1 on DC and CD94 on NK cells (Basu et al., 2001; Becker et al., 2002; Delneste et al., 2002; Gross et al., 2003; Thériault et al., 2005).

Table 1. Effect of Hsp60 peptides on Hsp60-binding to permanent and primary macrophages. Permanent macrophages of the line J774A.1 or primary bone marrow-derived macrophages from C57BL/6J mice were incubated with different Hsp60 peptides, followed by the addition of Alexa Fluor 488-labelled human Hsp60. The analysis was performed by flow cytometry. Inhibition of binding of Alexa Fluor 488-labelled Hsp60 is indicated as %. Inhibitory peptides are shown in bold

Peptides	Sequences	Inhibition of Hsp60-binding(%)	
		Permanent macrophages	Primary macrophages
pep1–20	MLRLPTVFRQMRPVSRVLAP	1	8
pep11–30	MRPVSRVLAPHLTRAYAKDV	–	–
pep21–40	HLTRAYAKDVKFGADARALM	16	–
pep31–50	KFGADARALMLQGVDLLADA	–	–
pep41–60	LQGVDLLADAVAVTMGPKGR	–	–
pep51–70	VAVTMGPKGRTVIIEQSWGS	–	–
pep61–80	TVIIEQSWGSPKVTKDGVTV	–	–
pep71–90	PKVTKDGVTVAKSIDLKDKY	4	9
pep81–100	AKSIDLKDKYKNIGAKLVQD	4	–
pep91–110	KNIGAKLVQDVANNTNEEAG	–	–
pep101–120	VANNTNEEAGDGTTTATVLA	6	–
pep111–130	DGTTTATVLARSIAKEGFEK	26	–
pep121–140	RSIAKEGFEKISKGANPVEI	–	–
pep131–150	ISKGANPVEIRRGVMLAVDA	–	–
pep141–160	RRGVMLAVDAVIAELKKQSK	–	–
pep151–170	VIAELKKQSKPVTTPEEIAQ	–	–
pep161–180	PVTTPEEIAQVATISANGDK	–	–
pep171–190	VATISANGDKEIGNIISDAM	–	–
pep181–200	EIGNIISDAMKKVGRKGVIT	11	–
pep191–210	KKVGRKGVITVKDGKTLNDE	2	–
pep201–220	VKDGKTLNDELEIIEGMKFD	6	–
pep211–230	LEIIEGMKFDRGYISPYFIN	23	8
pep221–240	RGYISPYFINTSKGQKCEFQ	10	13
pep231–250	TSKGQKCEFQDAYVLLSEKK	10	–
pep241–260	**DAYVLLSEKKISSIQSIVPA**	**–**	**48**
pep251–270	ISSIQSIVPALEIANAHRKP	9	–
pep261–280	LEIANAHRKPLVIIAEDVDG	10	24
pep271–290	LVIIAEDVDGEALSTLVLNR	–	–
pep281–300	EALSTLVLNRLKVGLQVVAV	–	–
pep291–310	LKVGLQVVAVKAPGFGDNRK	–	–
pep301–320	KAPGFGDNRKNQLKDMAIAT	9	4
pep311–330	NQLKDMAIATGGAVFGEEGL	–	–
pep321–340	GGAVFGEEGLTLNLEDVQPH	1	5
pep331–350	TLNLEDVQPHDLGKVGEVIV	17	10
pep341–360	DLGKVGEVIVTKDDAMLLKG	4	7
pep351–370	TKDDAMLLKGKGDKAQIEKR	6	1
pep361–380	KGDKAQIEKRIQEIIEQLDV	–	–
pep371–390	IQEIIEQLDVTTSEYEKEKL	–	–
pep381–400	TTSEYEKEKLNERLAKLSDG	–	–
pep391–410	**NERLAKLSDGVAVLKVGGTS**	**15**	**57**
pep401–420	VAVLKVGGTSDVEVNEKKDR	10	–

(*Continued*)

Table 1. (*Continued*)

Peptides	Sequences	Inhibition of Hsp60-binding (%)	
		Permanent macrophages	Primary macrophages
pep411–430	DVEVNEKKDRVTDALNATRA	5	1
pep421–440	VTDALNATRAAVEEGIVLGG	2	16
pep431–450	AVEEGIVLGGGCALLRCIPA	5	17
pep441–460	GCALLRCIPALDSLTPANED	2	19
pep451–470	LDSLTPANEDQKIGIEIIKR	1	–
pep461–480	**QKIGIEIIKRTLKIPAMTIA**	**5**	**57**
pep471–490	TLKIPAMTIAKNAGVEGSLI	2	1
pep481–500	**KNAGVEGSLIVEKIMQSSSE**	**86**	**14**
pep491–510	VEKIMQSSSEVGYDAMAGDF	4	–
pep501–520	VGYDAMAGDFVNMVEKGIID	7	15
pep511–530	VNMVEKGIIDPTKVVRTALL	6	5
pep521–540	PTKVVRTALLDAAGVASLLT	–	–
pep531–550	DAAGVASLLTTAEVVVTEIP	5	17
pep541–560	TAEVVVTEIPKEEKDPGMGA	3	16

Epitopes of the Hsp60 Molecule Involved in Activation of Innate Immune Cells

In numerous studies, parallels between the immunostimulatory effects of HSP and LPS on innate immune cells have been observed. In addition, it has been shown that the same receptor complex, including TLR4 and CD14, is involved in the induction of a pro-inflammatory response by HSP and LPS (Flohé et al., 2003; Ohashi et al., 2000; Poltorak et al., 1998; Vabulas et al., 2001; Vabulas et al., 2002). These findings raised the question, whether endotoxin contaminations of the recombinant HSP preparations, used in these studies, are responsible for the observed stimulatory effects on innate immune cells (Gao et al., 2003; Gao et al., 2004; Tsan et al., 2004; Wallin et al., 2002). For that reason, further intensive efforts were made to clarify the role of LPS versus human Hsp60 in the stimulation of innate immune cells (Habich et al., 2005). In exhaustive control experiments the effect of protease and heat treatment on the immunostimulatory properties of Hsp60 preparations and LPS was analysed. The immunostimulatory capacity of Hsp60 was found to be protease- and heat-sensitive, whereas the immunostimulatory capacity of LPS was not. Moreover, addition of a defined amount of LPS to Hsp60 resulted in an increase of the stimulatory effects of Hsp60 on innate immune cells. The increment of the immunostimulatory activity became protease- and heat-resistant, reflecting the amount of LPS admixed. Similar results were observed for the potent LPS inhibitory peptide polymyxin B (PmB). The immunostimulatory effects of Hsp60 were PmB-resistant, whereas the increased immunostimulatory capacity, observed after the addition of a defined LPS amount, was PmB-sensitive. In a parallel analysis a further LPS-binding peptide, the defensin magainin II amide

also showed strong interference with the immunostimulatory activity of Hsp60 and of LPS (Habich et al., 2005; Matsuzaki et al., 1997; Vorland et al., 1999). These findings indicated that the activation of innate immune cells mediated by Hsp60 preparations is not caused by free contaminating LPS, but by LPS or structurally related molecules tightly bound to Hsp60. Based on these findings the capacity of Hsp60 to bind LPS was investigated in a binding assay with radioactive-labelled LPS (Habich et al., 2005). This approach allowed us for the first time to demonstrate that Hsp60 is a specific LPS-binding protein (Table 2). Incubation of Hsp60 with radioactive-labelled LPS resulted in a significant increase in bound radioactive-labelled LPS over background control. By contrast, binding of radioactive-labelled LPS to ovalbumin (OVA) or transferrin was in the range of the background control. As expected, the highly effective LPS-binding protein LBP used as positive control, also bound a significant amount of radioactive-labelled LPS (Table 2).

Subsequent analyses, using defined monoclonal anti-Hsp60-antibodies and selected oligopeptides of the human Hsp60 sequence, identified the HSP60 epitope region aa354–365 involved in specific LPS-binding (Habich et al., 2005). This region includes the central sequence motif LKGK, which was found to be critical for LPS-binding. In view of these results it was concluded that human Hsp60 mediates the activation of innate immune cells, i.e. the induction of an inflammatory response, by specifically bound LPS. The results of these studies were supported by other reports describing that mammalian Hsp70 and Hsp90 act as receptors for LPS (Byrd et al., 1999; Triantafilou et al., 2001; Triantafilou et al., 2005). In the circulation of human individuals soluble autologous Hsp60 and Hsp70 have been detected in concentration ranges up to mg/ml (Pockley et al., 1998; Pockley et al., 1999). Further studies report on LPS concentrations in humans, which should be sufficiently high to enable loading of extracellular Hsp60 with LPS under physiological conditions, in the absence of a bacterial infection (Knolle et al., 1999; Lumsden et al., 1988).

Based on these considerations, the property of mammalian Hsp60 to act as a danger signal (Chen et al., 1999; Wallin et al., 2002) includes the element to serve as a sensor for danger signals, such as LPS, and to present such structures via

Table 2. Hsp60 specifically binds LPS. Radioactive-labelled LPS was incubated in the absence (background control) or presence of human Hsp60, ovalbumin (OVA), transferrin or LPS-binding protein (LBP). Bound radioactive-labelled LPS was separated from free radioactive-labelled LPS by vacuum filtration. Data represent the mean values ± S.D. in cpm of bound radioactive-labelled LPS of three independent experiments. Significant differences to bound radioactive-labelled LPS in the absence of protein (background control) are indicated as ***, $p < 0.001$

	Bound LPS (cpm, mean ± S.D.)
Background control	401 ± 170
Hsp60	5912 ± 529***
OVA	527 ± 153
Transferrin	399 ± 131
LBP	71940 ± 9339***

the TLR4/CD14 receptor complex to innate immune cells. The concept of Hsp60 acting as a sensor for microbial structures may also be extended to other mammalian chaperones. Hsp70 and gp96 also have been observed to bind LPS in a specific manner (Bausinger et al., 2002; Reed et al., 2003). Furthermore, Hsp90 has been suggested to act as primary receptor for immunostimulatory bacterial CpG DNA and to deliver these ligands to the TLR9 complex (Bandholtz et al., 2003). These findings point to an important biological function of mammalian chaperones, i.e. the improvement of the efficient recognition of microbial structures by innate immune cells.

Localization of Epitopes Involved in Hsp60-Binding and Hsp60-Mediated Activation of Innate Immune Cells

In its physiologically active form Hsp60 consists of two stacked rings with seven identical subunits (Hsp60 monomers) per ring (Braig et al., 1994). This

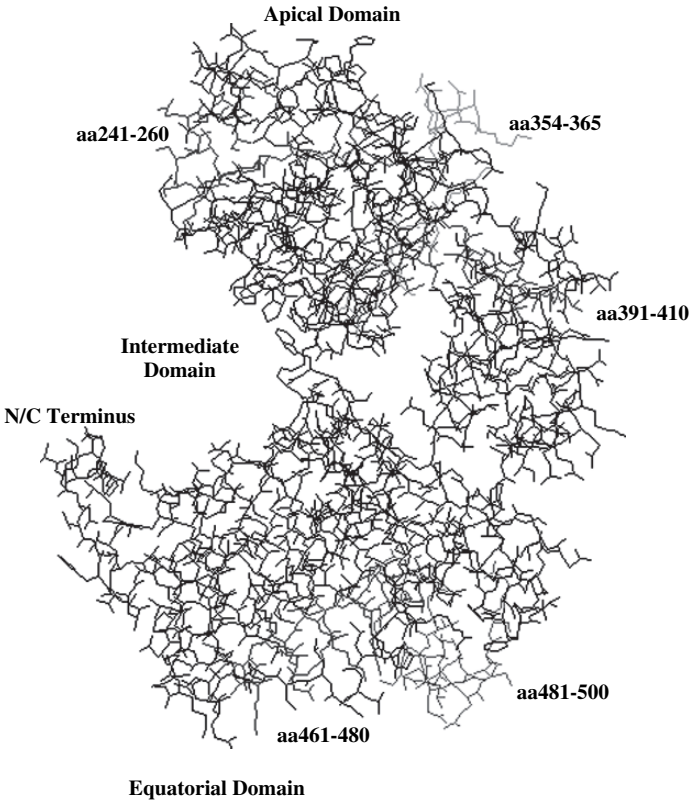

Figure 5. Human Hsp60 monomer. Localization of Hsp60 epitopes involved in receptor binding to permanent (green, aa481-500) and primary (red, aa241-260, aa391-410, aa461-480) macrophages, and in specific LPS-binding (yellow, aa354-365) in the 3-D model of the human Hsp60 monomer

polymeric structure exists in a dynamic balance between monomers, heptamers and tetradecamers (Tsan et al., 2004). In a 3-D model of the human Hsp60 molecule, deduced from *Escherichia coli* GroEL (swissmodel.expasy.org/repository), the analog of human Hsp60, the identified binding epitope regions can be located in the apical (aa241–260), the small intermediate (aa391–410) and in the large equatorial (aa461–480 and 481–500) domains, Figure 5. The specific LPS-binding region aa354–365 is located in the apical domain. The positions of the epitope regions indicate that the contributing amino acids are neither involved in intramolecular contacts nor in contacts to adjacent monomers. Hence, these regions are accessible to appropriate receptor structures and to LPS in the monomeric as well as in the oligomeric conformation of the Hsp60 molecule.

CONCLUSIONS

Our current knowledge allows the conclusion that the initial interaction of Hsp60 with innate immune cells represents a multifaceted process with important consequences for the activation of innate and adaptive immune reactions. Accumulating evidence indicates that Hsp60 is released by damaged or stressed cells, Figure 6. Specific, but yet unknown binding receptor structures for Hsp60 have been described on innate immune cells. Other cell surface structures (e.g. TLRs)

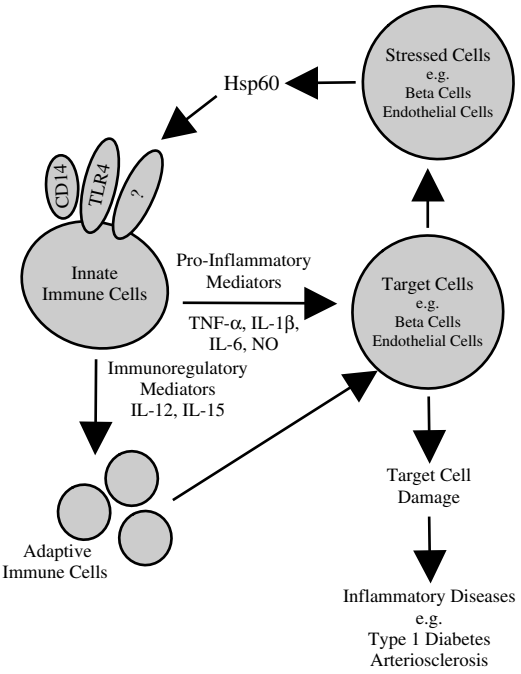

Figure 6. Immunoregulatory role of Hsp60

have been found to contribute to Hsp60-mediated activation of innate immune cells. The characterization of epitope regions of the human Hsp60 molecule has shown that in a cell-type-specific manner different regions are involved in Hsp60-binding to receptor structures on innate immune cells. Moreover, it could be demonstrated that Hsp60 mediates its immunostimulatory effects by specifically bound LPS. The interaction of Hsp60 with innate immune cells has been found to result in the release of pro-inflammatory and immunoregulatory mediators. These mediators may in turn affect target cells directly and/or have an effect on adaptive immune cells, thereby contributing to the progression of inflammatory processes such as beta cell destruction in type 1 diabetes or vascular damage in arteriosclerosis. Because of its immunoregulatory properties, Hsp60 has been proposed to act as an intercellular danger signal, performing essential biological tasks, i.e. function as a sensor for microbial structures such as LPS, thereby contributing to the improvement of the efficient recognition of such structures by innate immune cells.

ACKNOWLEDGEMENT

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PART III

IMMUNE RESPONSES ELICITED BY HEAT SHOCK PROTEINS

CHAPTER 9

HSP-APC INTERACTIONS: INITIATION OF IMMUNE RESPONSES

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Abstract: The immunogenicity of HSPs is exquisitely dependent on its interaction with professional antigen presenting cells. The interaction is specific and occurs through surface receptors on the APC such as CD91. Other molecules such as CD40, LOX-1, CD36, Toll-like receptor-2 & 4, SR-A and SREC-I have also been proposed to be HSP receptors and are discussed. The physiological situations where the HSP-APC interaction is necessary such as cross-priming of antigens and maturation of the APCs and the implication of these events for the priming of immune responses against infectious agents and tumors and for maintenance of tolerance against self antigens are deliberated

Keywords: HSPs, receptors, CD91, cross-priming

INTRODUCTION

The immunological properties of heat shock proteins (HSP) were discovered as a result of their ability to elicit anti-tumor immunity. First described for gp96 (Srivastava and Das, 1984; Srivastava et al., 1986), these properties are also intrinsic of hsp70 (Udono and Srivastava, 1993), hsp90 (Udono and Srivastava, 1994), calreticulin (Basu and Srivastava, 1999), hsp170 (Wang et al., 2001a) and hsp110 (Wang et al., 2001a). The specificity of the immune responses primed with HSP is derived from the peptides that they chaperone and not from the HSP molecule itself. These observations are entrenched in a large number of immunological and structural Studies, see (Breloer et al., 1998; Nieland et al., 1996). The mechanism by which HSP prime immune responses has been studied in some detail and at the center of this mechanism is the interaction of HSP with antigen presenting cells (APCs); where the response begins.

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The requirement for APCs was first described in 1994 where it was shown that elimination of cells with phagocytic function in mice abrogates the ability of HSP to prime immune responses (Udono et al., 1994). Studies around that time also demonstrated that in priming anti-tumor immune responses, only minute amounts (on the order of a few micrograms) of HSP were required. (The actual antigenic moiety in these preparations is on the order of femtograms). Because of this super-efficiency of antigenic economy, HSP receptors on APCs were proposed to exist (Srivastava et al., 1994). Several experiments *in vitro* including extensive binding and competition experiments with the HSP, gp96, Hsp90 and Hsp70 solidified the proposal (Binder et al., 2000c). Schild *et al*., showed that gold-tagged gp96 could specifically interact with macrophages and that after interaction with the cell surface are internalized into membrane enclosed vesicles (Arnold-Schild et al., 1999). Using fluorescent tagged gp96, the internalization process was shown to be very rapid, on the order of minutes (Binder et al., 2000c). Even though these experiments demonstrated that an HSP receptor did exist, its identity remained unknown until 2000 when CD91 was first described as an HSP receptor (Binder et al., 2000b). Over a span of 3 years several other molecules (LOX-1, CD40, TLR, CD14, SR-A and SREC-I) were proposed to be HSP receptors. The evidence for and against these proposals are examined here. Regardless of what the receptor is, the outcome of the interaction between HSP and APCs is the conditioning of the APC and the priming of a robust T, B and NK cell responses. These events are also discussed.

The HSP Receptors

CD91

The HSP receptor first received a molecular definition by chromatographic and cross-linking experiments. Membrane proteins isolated from macrophages and applied to a gp96-affinity column revealed that an 80kDa fragment of CD91 did specifically bind to this column. In addition gp96 could be cross-linked to this fragment specifically on the surface of macrophages (Binder et al., 2000b). These experiments provided evidence for a direct and specific interaction of CD91 and gp96- an essential requirement for a receptor-ligand interaction. The CD91-gp96 interaction has received independent confirmation by several groups. (i) A previously known CD91 ligand alpha2-macrogobulin (α_2M) can compete with gp96 for binding to CD91 as can gp96 itself (Habich et al., 2002). (ii) A population of T helper cells expressing CD91 can bind gp96 and such binding can be inhibited specifically by incubation with anti-CD91 antibodies (Banerjee et al., 2002). (iii) In the human system, the interaction of HSP with CD91 is critical for priming T cells responses in patients with Kaposi's sarcoma (Stebbing et al., 2003b). In this system sequestration of HSP in lysates of virus infected cells using HSP-selective inhibitors or blockade of CD91 using antibodies prevented priming of T cells. These data are consistent with the observations in mice. Despite the overwhelming evidence for the interaction of HSP with CD91 a report showed a CD91-independent binding of HSP to APCs (Berwin et al., 2002a). However a subsequent study showed that

this observation was a result of experimental lacunae and that once these lacunae were addressed CD91 played an essential role in the interaction of HSP with APCs (Binder and Srivastava, 2004).

Functional studies have heavily supported the role of CD91. Re-presentation assays which monitor the internalization of HSP-peptide complexes and the presentation of the processed peptide on MHC molecules on the cell surface can be inhibited by co-incubation with anti-CD91antibodies or other competitor ligands of CD91 such as RAP or α_2M (Basu et al., 2001; Binder et al., 2000b; Binder and Srivastava, 2004). CD91 non-expressing cells are incapable of re-presenting HSP-chaperoned peptides. In genetic studies, a knock down of CD91 expression in APCs using siRNA abrogates the ability of the APCs to re-present, and reversal of this treatment by removal of the siRNA and recovery of expression of CD91 restored the re-presentation capacity (Binder and Srivastava, 2004).

CD91 has also been described as receptor for other HSP. Hsp70, Hsp90 and calreticulin all bind to CD91 and compete with α_2M for binding and representation (Basu et al., 2001). This observation has also been independently corroborated. Ogden and colleagues have demonstrated through structural and functional studies the CD91-calreticulin interaction (Ogden et al., 2001; Vandivier et al., 2002). Delneste *et al*., showed a clear interaction of Hsp70 with CD91 on human macrophages and a weaker interaction on myeloid dendritic cells (DCs). This interaction was inhibited by α_2M (Delneste et al., 2002). Similarly Martin *et al*., inhibited the interaction of Hsp70 with PBMC by introducing α_2M into the reaction (Martin et al., 2003). These studies have been extended to bacterial HSP. Both murine Hsp70 and its bacterial homologue dnaK depend on CD91 for re-presentation of peptides that they chaperone (Tobian et al., 2004a). The HSP that depend on CD91 for internalization are classified in two different families- the Hsp70 and Hsp90 families. These HSP families have little sequence homology and almost no structural similarity. It might therefore be surprising that they interact with the same receptor; however CD91 is a large multi-domain protein with several (at least 4) ligand binding sites. Indeed as many as 32 other ligands with no apparent similarity to each other bind to CD91 (Herz and Strickland, 2001). Structural analysis of these interactions will shed light on the dynamics of HSP binding to CD91.

The *in vivo* relevance of the HSP-CD91 interaction is coming to light. Administration of anti-CD91 antibodies together with a preparation of tumor derived gp96 prevents the ability of this preparation to mount an anti-tumor immune response (Binder and Srivastava, 2004) and mice succumb to tumors. In control groups, mice rejected their tumors when they were immunized with tumor-derived gp96 with control anti-sera. In correlative studies, Stebbing *et al*., have showed that long term non-progressors with HIV infection expressed significantly higher levels of CD91 on APCs than infected HIV persons that progressed to AIDS (Stebbing et al., 2003a). The argument here is that higher level of CD91 expression leads to more efficient presentation of HSP-associated epitopes by APCs and thus an enhanced cellular immune response to these epitopes and less viral loads. The same authors have shown that PBMCs from Kaposi sarcoma patients could be stimulated with

APCs pulsed with infected cell lysates and this stimulation could be blocked with anti-CD91 antibodies (Stebbing et al., 2003b).

These data have solidified CD91 as a receptor for HSP. Ongoing studies of the physical interaction between the receptor and these HSP ligands with regards to the specific receptor domains and residues necessary for HSP binding will further shed light in this area.

LOX-1

LOX-1 has been identified as an endocytic receptor for Hsp70 on human DCs. Binding and competition studies revealed that while α_2M competed with Hsp70 for binding to macrophages strongly it did so only weakly on DCs (Delneste et al., 2002). In this study on human cells, anti-LOX-1 antibodies and acetylated albumin, another ligand for LOX-1, competed effectively with Hsp70 on DCs. While LOX-1 and CD91 both belong to the scavenger receptor superfamily the differences of receptor utilization in mice versus human or macrophages versus DCs reflects the heterogeneity of the pathways of HSP internalization. However cell biological and structural data for the LOX-1-Hsp70 interaction are lacking and further light needs to be shed on this cell surface molecule as a receptor.

CD40

CD40 is a signaling receptor which can be triggered by its ligand gp39/CD40L (van Kooten and Banchereau, 2000). It is not known to be an endocytosing receptor because it lacks the ability to recruit clathrin and other proteins necessary for endocytosis. Wang *et al*., detected binding of mycobacterial but not mammalian Hsp70 to CD40 with the help of anti-CD40 antibodies. A result of this binding was the secretion of cytokines by the CD40 expressing macrophages fulfilling its role as a signaling receptor (Wang et al., 2001b). No internalization was reported. In contrast Becker *et al*., reported an enhancement of internalization of recombinant GST-tagged mammalian Hsp70 after treatment of APCs with LPS which incidentally increases the expression of CD40 among other things (Becker et al., 2002). Internalization of peptides with CD40 was facilitated by Hsp70. The difference between these observations and the data of Wang *et al*., was attributed to the purification strategy of using ATP affinity chromatography and not ADP thus resulting in conformation differences in the Hsp70 molecule. The role of CD40 as a receptor for Hsp70 draws indirect support from two other studies. Millar *et al*., reported that immunization with rHsp70-LCMV derived peptides can break tolerance to the antigenic peptide expressed as a self antigen in transgenic mice and that the tolerance breaking activity of Hsp70 is not seen in $CD40^{-/-}$ mice (Millar et al., 2003). $CD40^{-/-}$ mice unlike $CD40L^{-/-}$ mice succumb to *M. tuberculosis* infection suggesting that there is an alternative ligand for CD40 (Lazarevic et al., 2003). *M. tuberculosis* Hsp70 causes the release of IL-12 from DCs similar to that seen during infection, a response absent in CD40 $\subseteq$ / $\subseteq$ mice. The conclusion from this report was that Hsp70 was an alternative ligand for CD40.

Several issues have to be addressed as far as the role of CD40 as a receptor for Hsp70 is concerned. The idea advanced by Becker *et al*., that conformational changes between ADP and ATP-purified Hsp70 to explain the differences between their data (binding of murine Hsp70 to APCs) and those of Wang *et al*., (the lack of such binding) needs to be tested. The regions of Hsp70 (murine or mycobacterial) that interact with CD40 have to be defined to validate the Hsp70-CD40 interaction. Experiments by Becker *et al*., showing uptake of peptides by APCs is not a demonstration of re-presentation of Hsp70-chaperoned peptides and thus functional data for this receptor is lacking. This is particularly important since CD40 is not an endocytic receptor but a signaling receptor (as reported by Wang *et al*.). $CD40^{-/-}$ mice are able to prime CD8+ responses as efficiently as the wild type counterpart after Hsp70 inoculation (Binder and Srivastava, unpublished data) suggesting a CD40 independent pathway. Indeed the interaction between Hsp70 and APCs can be mediated by CD91 and completely abolished by competing CD91 ligands.

*Toll-like receptors (TLR*s*)*

The TLR 2 and 4 have been implicated in HSP-mediated signaling and activation of APCs but not in the re-presentation of HSP-chaperoned peptides. This has been examined by stimulation of cells transfected with TLR genes and examining the outcome of reporter genes. Asea *et al*., reported rHsp70 could signal through TLR2 and 4 with a role for the LPS receptor CD14 (Asea et al., 2002). Vabulas *et al*., observed that Hsp70 stimulated IL-12p40 production by the macrophage cell line RAW264.7 and this effect is partially abrogated by introducing a double negative construct for MyD88 and TRAF6, downstream molecules for signaling through TLR4 (Vabulas et al., 2002a). In these results, transfection of non-APC 293T fibroblasts with TLR4 alone did not render them responsive to Hsp70 but transfection with TLR2 alone or with TLR4 and its co-receptor myeloid differentiation-2 (MD-2) led to an increase in NF-κB signaling. While $TLR2^{-/-}$ DCs produced normal amounts of IL-12p40 and TNF-α, $TLR4^{-/-}$ DCs did not produce either cytokine. Similar conclusions have been arrived at by Dybdahl *et al*., based on their studies with PECs from C3H/HeN which produced TNF-α when cultured with Hsp70 in contrast to PECs from C3H/HeJ mice which did not (Dybdahl et al., 2002). Also in this study, the secretion of IL-6 by human monocytes when cultured with Hsp70 could be blocked by anti-CD14 or anti-TLR4 antibodies.

Using the transfection and reporter gene tools described earlier, Vabulas *et al*., described TLR4 to be a receptor for the ER HSP gp96 (Vabulas et al., 2002b). Importantly this study used tissue-derived, non-recombinant gp96 and showed that DCs release cytokines in response to it, a process involving MyD88. In an independent approach Liu *et al*., forced expression of gp96 onto the cell surface in addition to its normal ER expression and showed that surface gp96-expressing cells are able to engage DCs, causing their maturation through a MyD88 dependent pathway (Liu et al., 2003). This observation is consistent with a role for TLR4 although the study does not directly identify a cell surface receptor.

The interaction of Hsp60 with APCs has also been suggested to occur through TLR2 or 4. With the same tools used for gp96 and Hsp70, Vabulas *et al*., showed that human Hsp60 caused the activation of NF-κB and TNF-α production by 293T cells transfected with either TLR2 or TLR4 and MD-2 (Vabulas et al., 2001). In addition the signaling molecules JNK and IKK were activated in RAW264.7 macrophages in response to culture with Hsp60. Ohashi *et al*., showed that human Hsp60 stimulated TNF-α and NO release by murine macrophages in a TLR4 dependent mechanism (Ohashi et al., 2000). While these reports from several labs make a collective case for the role of TLR2/4 a strong note of caution is raised since these TLR are well documented receptors for the bacterial ligand LPS which is a contaminant in nearly everything that exists in our present universe. Indeed these serious concerns have been studied in depth by Gao and Tsan (Gao and Tsan, 2003) who compared the effects of rHSP preparations before and after LPS was carefully removed using polymyxin-B. Hsp70 preparations with 'low' or 'very low' contamination levels of LPS showed no ability of Hsp70 to mature DCs as measured by CD83 expression. In addition while low-LPS Hsp70 caused DCs to produce IL-6, an effect greatly enhanced by the LPS receptor CD14, the very low LPS Hsp70 preparations did not do so regardless of the presence or absence of CD14. This in contrast to Hsp70 preparations contaminated with LPS (as obtained from most manufacturers). Upstream events were also examined. MAPK activation was not observed with very low-LPS Hsp70. Bausinger *et al*., demonstrated that regardless of the LPS contamination, Hsp70 could mediate the representation of an extended ovalbumin-derived peptide (Bausinger et al., 2002). Gao and Tsan similarly demonstrated that removal of LPS from Hsp70 abrogated the effects of cytokine release from macrophage cell line RAW264.7, an effect seen robustly with LPScontaminated Hsp70. To address the LPS contamination a procedure of boiling Hsp70 preparations has been used as controls, which apparently removes the innate effects of Hsp70 seen on APCs. However Gao and Tsan again demonstrated that this is an abysmal control, in that boiling of low amounts of LPS in the presence of Hsp70 removes the innate effects of LPS itself. This effect is not seen when LPS is boiled in the absence of protein, even though activity is slightly decreased in *in vitro* studies on RAW264.7 macrophages. The results from Gao and Tsan and Bausinger *et al*., strongly suggest that great care must be taken in imputing TLR as HSP receptors, unless that possibility has been effectively ruled out by real controls (other than boiling for example). This is particularly important where as little as a few nanograms of rHsp70 was able to mediate the activation of APCs or in studies where the effects of HSP were dependent on the presence of fetal calf serum in the incubation medium, long shown to be able to augment the effects of LPS through the LPS binding protein (LBP) in serum. LBP facilitates the activity of LPS especially at very low concentrations. This problem is also very important when recombinant HSP is used which is generally more heavily contaminated with LPS than endogenously derived HSP purified under LPS-free conditions. Cell biological studies and structural analysis of the interaction between TLR and HSP is lacking and will go a long way to satisfy the concerns raised.

CD14

Asea *et al*., proposed CD14 as an Hsp70 receptor. In their studies, treatment of U373 human astrocytoma cells with Hsp70 induced the release of TNF-α and this effect was enhanced by transfection of the cells with CD14 (Asea et al., 2000b). Also NF-κB transcription, as read by a luciferase reporter was enhanced in HEK293 cells treated with Hsp70. This effect was also enhanced after transfection of the cells with CD14. Thus CD14 was not essential but rather augmented the effects of Hsp70. Using U373 cells and human PBMC, Habich *et al*., reported IL-6 release after treatment with Hsp70. This effect was blocked by antibodies against CD14 or by boiling the Hsp60 (an attempt to control for LPS; see above) but not by polymyxin B (Habich et al., 2002). A similar observation was made by Kol *et al*., (Kol et al., 2000). Considering that CD14 is a bona fide receptor for LPS (Wright et al., 1990) the concerns raised in the previous section are especially pertinent regarding the possible contamination of the HSP preparations with LPS and the designation of CD14 as an HSP receptor. Several observations are pertinent in this regard: (i) Asea *et al*., reported that rHsp70 binds CD14 on monocytes but not on DCs which are known to be CD14 negative. However they also report that mammalian HSP can bind DCs and macrophages that do not express CD14 (Noessner et al., 2002). It remains unresolved where the differences lie but could be due to differences in levels of LPS contamination. (ii) Delnest *et al*., demonstrated that anti-CD14 antibodies *did not* inhibit binding of Hsp70 to immature DCs and macrophages (Delneste et al., 2002). (iii) Hsp70 and Hsp60 are internalized efficiently by DCs without the expression of CD14 or TLR4 (Lipsker et al., 2002). Thus CD14 as an HSP receptor needs a much more robust examination.

Scavenger receptor-A (SR-A)

SR-A was recently added to the list of HSP (gp96 and calreticulin) receptors due to the reported ability of the SR-A ligand fucoidin to inhibit binding of gp96 to macrophages (Berwin et al., 2003). This is interesting because the expression of SR-A on mouse or human DCs has not previously been reported. However LPS is a ligand for SR-A and thus the concerns raised in the previous sections for TLR and CD14 are pertinent here as well (Haworth et al., 1997). This study did not address the levels of LPS contamination in the HSP preparations. Surprisingly in the binding studies performed here, calreticulin could not compete with labeled calreticulin for binding suggesting that the observed inhibition with fucoidin could be due to general non-specific inhibitory effects of the inhibitor on the cell rather than the specific receptor. The relative contribution of SR-A needs to be studied much more since SR-A mice are capable of re-presenting gp96-chaperoned peptides and binding gp96. Structural analysis of gp96-SR-A or calreticulin-SR-A interaction or lack thereof, will be helpful in deciphering these data.

Scavenger receptor expressed by endothelial cell-I (SREC-I)

Yet another scavenger receptor molecule described by Berwin *et al*., to be associated with the internalization of calreticulin (Berwin et al., 2004). The studies here include

binding of calreticulin to Chinese hamster ovarian cells transfected with SREC-I and competition with various known scavenger receptor ligands. Functional data for the relevance of this interaction is lacking as is the structural nature of the interaction between these two molecules.

The Outcome of HSP-APC Interactions

The results of receptor engagement by HSP are two fold. Firstly, the HSP with the chaperoned peptide is internalized into membrane enclosed vesicles (Arnold-Schild et al., 1999; Berwin et al., 2002b; Binder et al., 2000c). Through an as yet unidentified pathway the peptide enters the cytosol where it is processed by proteasomes, transported into the ER by TAP and loaded onto MHC I molecules for trafficking to the cell surface (Basu et al., 2001). Here the peptide is presented to CD8+ T cells (Doody et al., 2004; Suto and Srivastava, 1995). In addition peptides chaperoned by HSP are presented by MHC II after endosomal processing (Doody et al., 2004; SenGupta et al., 2004; Tobian et al., 2004b). The fate of the internalized HSP itself is unclear. Secondly, HSP engage receptors and transduce signals through signaling cascades (yet to be identified) to stimulate the activation of NF-κB). Activated NF-κB translocates into the nucleus where it binds to several DNA sequences to turn on genes. On a cellular level the result is that APCs secrete cytokines (IL-1β, IL-6, IL-12, TNF-α and GM-CSF (Asea et al., 2000a; Basu et al., 2000; Chen et al., 1999; Kol et al., 1999; Moroi et al., 2000; Singh-Jasuja et al., 2000), chemokines (MCP-1, MIP-1 and RANTES) (Lehner et al., 2000; Panjwani et al., 2002) and nitric oxide (Chen et al., 1999; Panjwani et al., 2002). The expression on the cell surface of several co-stimulatory molecules (B7-1, B7-2, CD40 and MHC II) is up-regulated (Basu et al., 2000).

These events comprise the maturation signatures of APCs which are necessary for priming naïve T cells into effectors. In vivo, in addition to these presentation and maturation events, the APCs will migrate to the lymph node after engagement of HSP, where they will encounter the naïve T cells (Binder et al., 2000a). In immunotherapy of murine tumors with HSP, NK cell activity contributes to the overall regression of tumors (Tamura et al., 1997). B cells and antibodies have also been suggested to play a role in the clearance of bovine herpesvirus 1 in murine models of the disease (Navaratnam et al., 2001). Thus the stimulation of APCs by HSP leads to a broad immunological outcome comprising both the innate and (multiple arms of) the adaptive responses.

Cross-Priming and Matters of Antigen Economy

Because naïve T cells need to encounter two signals (the peptide/MHC complex and co-stimulation) priming can happen only when APCs, which have co-stimulation, are presenting the antigenic peptide (Banchereau and Steinman, 1998). Thus there is the necessity for antigens to be transferred from the antigen bearing (through infection, mutations etc) cell to the APC. This phenomenon is called cross-priming

and was first described by Bevan in 1976 (Bevan, 1976). Cross-priming is therefore necessary for host animals to mount T cell immune responses against viral and bacterial infections, tumors and other pathology. Not only in pathology but cross-priming must occur for maintenance of tolerance to self (Steinman et al., 2003).

Antigens can be transferred between cells in either of two forms; whole soluble protein or as peptides (not free but bound to chaperones). An analysis of the amount of soluble protein antigen required for priming an immune response has been examined (Binder and Srivastava, 2005; Li et al., 2001). Without adjuvants, including the ubiquitous LPS, tens of micrograms of protein are required. Presumably without efficient up-take of this 'naked' antigen through receptor mediated pathways and without maturation signals provided to the APCs, most of this antigen is lost and leads to a nonevent (as far as immune responses are considered). It appears therefore that cells simply do not have anywhere near sufficient intact antigen in order to prime T cell responses yet immunization with cell-associated antigen rapidly elicits a strong immune response. The answer to this paradox lies in utilization of receptors (Lanzavecchia, 1985). Receptors are nature's most oft-exploited tool in order to obtain economies. The use of chaperone-peptide complexes and their up-take by DCs through HSP receptors such as CD91 (Arnold-Schild et al., 1999), LOX1 (Delneste et al., 2002) and perhaps other receptors provides a highly efficient and economical means to achieve indirect presentation under conditions of antigen limitation which are perhaps the overwhelming physiological condition (Princiotta et al., 2003). This has indeed been shown to be the case. By utilization of necrotic cell lysates, it has been demonstrated that antigen in context of cell lysate is 5 orders of magnitude more efficient at priming immune responses than free soluble antigen (Binder and Srivastava, 2005; Li et al., 2001) and that HSP (Binder and Srivastava, 2005) play a sufficient and necessary role in the transfer of the antigen in the form of peptides (Serna et al., 2003). Thus HSP-chaperoned peptides-receptor internalization pathway plays an essential role in cross-priming. This pathway can be exploited in prophylaxis and therapy of cancer and infectious diseases by immunization of animals with HSP-peptide complexes which effectively primes robust immune responses (Srivastava, 2000). By extrapolation it is envisaged that antigen in context of apoptotic cells would point towards uptake of apoptotic cells through receptors on DCs (Skoberne et al., 2005) and down-stream channeling of HSP-peptide complexes into the antigen presentation pathway. Under conditions where the quantity of antigen is not limited, involvement of a receptor may be un-necessary and in such rare circumstances, intact antigen may indeed be the vehicle of cross-priming (Basta et al., 2005; Norbury et al., 2004; Shen and Rock, 2004).

Immune Regulation

The HSP-APC interaction provides a unique point in the elicitation of an immune response where the response can be regulated. The presence of several alternative ligands for HSP receptors that compete for receptor binding and occupancy is a

way in which the response could be dampened (higher presence of competitor) or exaggerated (low competitor levels). A provocative example of such a competitor is the extracellular protein α_2M which competes with HSP for binding to CD91. The competition for binding to CD91 may regulate the amount of antigen that is being cross-presented. It might be worth mentioning that the regulation of α_2M levels itself is not very well understood but there have been several reports documenting a significant increase in α_2M levels in tumor bearing mice and patients (Kanoh et al., 2001). What correlations exist between α_2M levels and tumor growth or the strength of immune responses remains largely unexplored. A second point of immune regulation could be at the receptor expression level on the APC. Events that effect the APC to down- or up-regulate receptor levels will control the amount of signaling and antigen cross-presentation (Hilf et al., 2002; Stebbing et al., 2003a). In one study Hilf *et al*., demonstrated that platelet cells, activated with thrombin, are capable of binding 10-fold more gp96 than un-activated platelets (Hilf et al., 2002). Since the binding was specific this study suggests that the gp96 receptor levels on these cells are up-regulated upon activation. Other examples for HSP receptors may yet come forth.

Concluding Remarks

As can be sensed from the discussion here there is a general lack of structural data on interaction of HSP with nearly all of the receptor candidates. In addition, there is a special burden of proof on molecules suggested to be HSP receptors which are also known to be LPS-binders, e.g. TLR2, TLR4, CD14 and SR-A since HSP preparations are routinely contaminated with LPS.

The idea of heterogeneity of receptors has yet to be fully examined in any detail and this heterogeneity can be at a cellular level (do different APCs such as monocytes, macrophage, immature and mature DCs bind specific HSP?) or at a protein level (does the same APC bind different HSP through different receptors?) or at the consequential level (are there distinct consequences of binding of an APC by different HSP?). In addition the intracellular pathways of signaling by HSP and of endocytosis of HSP-peptide complexes are completely unknown. Are these pathways susceptible to changes in the physiological status of the APCs, i.e. in presence of a cytokine? The function of HSP receptors on non-professional APCs (which do not express co-stimulatory molecules) as shown by Hilf *et al*., (Hilf et al., 2002) and Multhoff *et al*., (Multhoff et al., 1999) is provocative. A systematic screening of non-APCs for binding individual HSP and the functional consequences thereof is likely be fruitful.

Finally, while the focus of this chapter has been on the immunological side of the HSP-APC or HSP-non-APC interaction, one should not forget there may be other outcomes of a non-immunological nature of striking importance to the host. After all, HSP-receptor interactions were selected for in a different mileu than the immunological, at least as we understand that term today.

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CHAPTER 10

EXTRACELLULAR FUNCTIONS FOR AN INTRACELLULAR PROTEIN: GRP94/GP96 INTERACTIONS WITH THE MAMMALIAN IMMUNE SYSTEM

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Abstract: GRP94/gp96, the endoplasmic reticulum (ER) paralog of Hsp90, elicits anti-tumor immune responses, and in murine model systems, can suppress metastatic tumor progression. Extending from early studies into the identity(s) of tumor-specific antigens, the landmark discovery of an immunogenic function for GRP94/gp96 prompted the hypothesis that GRP94/gp96 functions as a cross-presentation antigen and, by virtue of a peptide binding activity, bears the immunological identity of its tissue of origin. Coincident with this discovery was the observation that GRP94/gp96 directly activates cells of the innate immune system (macrophages and dendritic cells). The paucity of direct evidence in support of an *in vivo* peptide binding function for GRP94/gp96 has catalyzed further research into the molecular basis for GRP94/gp96 interactions with the mammalian immune system. At present, the *in vivo* basis for GRP94/gp96 function in tumor immunity remains controversial; nonetheless, these activities identify potential clinical roles for GRP94/gp96 as a cancer immunotherapeutic and potential physiological roles of the protein as a "danger" signal/biological adjuvant. The endogenous cellular function of GRP94/gp96, as an ER-resident molecular chaperone and stress-response protein, also suggests potential roles in tumor biology. The GRP94/gp96 -interacting proteome includes proteins critical for cell-cell interaction and cell signaling, such as members of the integrin family and a subset of Toll-like receptors. As these same processes contribute to tumor growth and/or metastasis, GRP94/gp96 may prove to be a novel target for anti-neoplastic drug discovery

Keywords: GRP94, gp96, molecular chaperone, adjuvant, dendritic cell, innate immunity

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THE ORIGIN AND CELLULAR FUNCTIONS OF GRP94

GRP94, glucose-regulated protein 94, was first identified as a protein up-regulated during glucose deprivation and has subsequently been rediscovered multiple times (endoplasmin, tumor rejection antigen 1 (TRA1), glycoprotein 96 (gp96)) (Koch et al., 1985; Lewis et al., 1985; Shiu et al., 1977; Srivastava et al., 1986). For simplicities sake, we defer to the historical nomenclature and will hereafter refer to GRP94/gp96 as GRP94. GRP94 bears a very high sequence similarity to cytosolic Hsp90 and so is accepted to be the endoplasmic reticulum (ER) paralog of Hps90.

Though GRP94 is the paralog of Hsp90, available evidence indicates that the evolutionarily ancient gene duplication event yielding GRP94 has also resulted in distinct and divergent regulatory elements. Notably, while Hsp90 is up-regulated during heat shock, per its name, as well as other stresses, GRP94 is not. This difference is reflected in the promoter structure; whereas Hsp90 has a heat-shock response element, GRP94 has two ER-stress response elements (ERSE) that are most significantly up-regulated during the unfolded protein response, triggered by nutrient deprivation and ER stress, among others (Chang et al., 1989; Lee, 2001). At the sequence level, GRP94 and Hsp90 diverge at key regions of the N and C-terminal domains. In the N-terminal domain, GRP94 has a conserved 5 amino acid insertion in the adenine nucleotide/geldanamycin binding pocket (Soldano et al., 2003). This insertion confers conformational response to adenine nucleotide binding that differs substantially from that reported for Hsp90 (Immormino et al., 2004; Pearl and Prodromou, 2001), Hsp90 is a well-established ATPase, whose enzymatic cycle mirrors the split ATPases of the MutL and DNA gyrase families of DNA repair enzymes. In contrast, GRP94 has an exceptionally low ATPase activity, displaying turnover rates of approximately $2\,hr^{-1}$ (Nicchitta, unpublished observations). Due to this extremely low specific activity, alternative functions for its interaction with adenine nucleotides have been proposed, including its role as a stress sensor. In this view, ATP/ADP binding modulates the activity level of GRP94, rather than the enzymatic basis for client protein binding and release (Rosser et al., 2004). GRP94 and Hsp90 diverge as well at a key region of the C-terminus – whereas Hsp90 proteins contain a TPR motif, which serves in the assembly of a subset of co-chaperones and accessory factors (Hip, Hop, Hsp70), GRP94 contains a KDEL ER retention/retrieval motif and available data indicate that GRP94 functions in the absence of an Hsp90-like cohort of co-chaperones and accessory proteins (C. Nicchitta, unpublished observations).

There are currently two models of the origin and existence of GRP94 in the phylogenetic tree. It is apparent that GRP94 displays a very limited phylogenetic distribution, being found only in the Kingdoms Metazoa (eukaryotic, multicellular animals) and Plantae. In contrast, Hsp90 family members are ubiquitous throughout phylogeny, from the simplest prokaryotes to plants. From such a distribution, it would appear that GRP94 is required for the establishment of multicellularity/tissue organization. Though there are a limited number of apparent exceptions to this rule, such as the case of some amoeboid and parasite genomes that contain

aGRP94homolog, these exceptions are limited to organisms whose life cycles either include a multicellular state and/or who are obligate parasites of mammalian hosts (Gupta, 1995; Stechmann and Cavalier-Smith, 2004). In one model, GRP94 arose through an ancient gene duplication event and bears greatest phylogenetic similarity to cytosolic Hsp90 (Gupta, 1995). In an alternative view, GRP94 evolved from a prokaryotic Hsp90; indeed, in this model, all organellar Hsp90 paralogs evolved independent of Hsp90 (endoplasmic reticular, mitochondrial, and chloroplast) (Stechmann and Cavalier-Smith, 2004). Both models support the current data implicating divergent structural functions forGRP94in the cell relative to Hsp90, including the lack of an ATPase activity and the ability to chaperone without multiple accessory proteins.

In metazoans, GRP94 is necessary for survival; the phenotype of the GRP94 knock-out mouse is embryonic lethal at day 7, and fail to develop mesoderm, a primitive streak and the proaminiotic cavity (Yair Argon, personal communication). Nonetheless, the loss of GRP94 in tissue culture systems of mammalian cells has no apparent viability phenotype (Randow and Seed, 2001). This points to a pivotal role for GRP94 in either the establishment of cell fates and/or the ability to critically influence morphogenesis and cell migration; which one, or both, is still an open question. The necessity for GRP94 in multicellular, and not single-celled, organisms, and its role as a chaperone protein, leads to the hypothesis that the client proteins of the "GRP94-interacting proteome" will be a subset of the cell surface proteins and secreted molecules functioning in the signaling and cell movements/morphogenesis events that are responsible for the establishment of tissue structure. An important key to understanding the endogenous role of GRP94 in metazoan and plant biology is to identify the client proteins which require GRP94 for functional expression. However, and though this has been attempted for decades, there is a paucity of known clients (Yang and Li, 2005). This deficit is in large part a result of the relative inability to answer this question through established protocols. Common experimental approaches to assessing chaperone-client protein interactions, such as indirect immunoprecipitation, have yielded few results with GRP94. Indeed, the capture of GRP94-client protein complexes frequently requires chemical cross linking agents (Ho et al., 2000). The identification of GRP94 clients has also occurred serendipitously. In a screen to identify gene products functioning in the B cell LPS signaling pathway, Randow and Seed identified a clone that did not activate in response to LPS (Randow and Seed, 2001). cDNA complementation studies revealed that the mutation was in GRP94 and in the absence of GRP94 function, TLR-2 and -4 trafficking to the cell surface was prevented. When GRP94 was ectopically expressed in the line, TLR-2 and -4 reappeared on the cell surface and LPS sensitivity was restored. In another study, an RNAi screen for genes functioning in cardiogenesis in the developing Drosophila embryo, GRP94 was identified as an essential gene (Kim et al., 2004).

While the limited discoveries of client proteins have been informative, a detailed listing of the full repertoire of GRP94 clients is needed for a complete understanding of the endogenous role of GRP94 in metazoan life.

THE EXTRACELLULAR FUNCTIONS OF GRP94

In a very limited number of experimentally-induced models of cell death, GRP94 is released during necrotic, but not apoptotic, cell death (Basu et al., 2000; Berwin et al., 2001). These findings are of particular interest to the "Danger Model" (Matzinger, 2002), and the molecular identification of pathological cell death-derived molecular signals, such as uric acid and lysophosphatidyl choline (lyso-PC) (Lauber et al., 2003; Shi et al., 2003). Uric acid and lyso-PC have been shown to stimulate macrophage migration and the activation of macrophage pro-inflammatory responses (Lauber et al., 2003; Shi et al., 2003). However, there is controversy as to whether apoptotic cells themselves, though through different signals, are also capable of eliciting an inflammatory response (Gallucci and Matzinger, 2001; Ip and Lau, 2004).

With the discoveries that GRP94 can elicit macrophage activation and undergo release from necrotic cells came the hypothesis that GRP94 functions *in vivo* as a signal to differentiate between pathologic and apoptotic cell death. This hypothesis, though as yet unproven, provides an intriguing physiological rationale for the long – standing observations that GRP94 can function as a tumor rejection antigen.

GRP94 has been extensively studied over the past three decades for its role as a tumor-specific antigen (Srivastava, 2006; Srivastava et al., 1994). In this view, GRP94 functions as a cross presentation antigen; it binds the antigenic peptide repertoire of its host cell and upon release can be processed by professional antigen presenting cells to yield host tissue-directed CTL responses (Ramirez et al., 2005; Wang et al., 2005). More recently, a tumor vaccine function for GRP94 has undergone evaluation in human clinical trials (Mazzaferro et al., 2003). The reader is referred to the following review articles for discussions of these data (Parmiani et al., 2004; Srivastava, 2005). On the whole, but with notable exceptions, this approach has yielded data that support the cross-presentation antigen hypothesis. Validation of this hypothesis requires, however, that the bound peptide pool be identified and its predicted diversity and host cell restriction confirmed. Despite the passage of considerable time since the original proposal, a single peer-reviewed study has been published on this topic (Demine and Walden, 2005). In this study, a very limited number of peptides were identified by liquid chromatography/mass spectrometric analyses of acid extracts of GRP94 (Demine and Walden, 2005). These authors reported that the peptides were varied in length and sequence, and yielded no identifiable consensus sequence for recognition by GRP94. Significantly, the very low representation of peptides (determined to represent 0.25% of the GRP94 pool), led the authors to conclude that GRP94 was unlikely to serve the peptide reservoir function proposed in the original models.

This concern notwithstanding, the Srivastava laboratory has recently reported that, indeed, Grp94 is necessary and sufficient for cross-presentation (Binder and Srivastava, 2005). Why different approaches have yielded such disparate conclusions remains to be determined. As noted in a recent commentary, what is missing to date are consistent findings between laboratories regarding the cross-presentation function of GRP94 (Melief, 2005).

It has been well established and widely agreed that GRP94 can be loaded with synthetic peptides *in vitro* and used to elicit antigen-specific responses *in vivo* (Abbas et al., 2000; Gidalevitz et al., 2004; Li et al., 2005; SenGupta et al., 2004; Suto and Srivastava, 1995; Vogen et al., 2002). Indeed, the utility of GRP94 as a peptide carrier for use in therapeutic applications is not in question, though the efficacy of this treatment protocol is still under investigation. Even so, it has remains to be demonstrated that peptide shuttling is an endogenous function for GRP94 in tumor biology. Along side these studies, other groups have examined the potential role of chaperone proteins as cross-presentation antigens (Norbury et al., 2004; Shen and Rock, 2004). Though the nature of the cross-presentation antigen been extensively studied, as yet, the exact nature of the antigen form(s) is unclear. What has been concluded is that cross-presentation is based on the transfer of proteasome substrates (whole proteins, or large fragments of such), rather than peptides (Shen and Rock, 2004). In studies of direct relevance to those reported in Binder et al., 2005, in their use of ovalbumin as a model antigen, crude cell lysates immunodepleted of ovalbumin were unable to stimulate cytotoxic T cell responses. Of particular interest, a number of heat shock/chaperone proteins, which are thought to function as cross-presentation antigens, were readily separated from the antigenic cell fraction, leading these researchers to conclude that cellular proteins, and not chaperone-peptide complexes, function as cross-presentation antigens *in vivo* (Shen and Rock, 2004). The topic remains one of considerable controversy and a disarming incongruence in conclusions. As such, there is an obvious need for further studies and in particular, an unequivocal assessment of whether GRP94 can complex with peptides *in vivo* and whether such complexes serve physiological roles in mammals.

SIGNALING PATHWAYS FOR GRP94

Further controversy in the GRP94 field surrounds the identity of its receptor(s) and coincident downstream signaling pathways - to elicit bound-peptide independent immune responses, cell signaling must be occurring at some level (Basu et al., 2000; Suto and Srivastava, 1995; Vabulas et al., 2002). The first report of a GRP94 receptor was for that of CD91 (LRP, low-density lipoprotein receptor-related protein) (Binder et al., 2000). In these studies, CD91 was isolated by affinity chromatography of detergent extracts of RAW264.7 membranes on a GRP94 column. Data identified, surprisingly, a fragment from the transmembrane, rather than the extracellular, domain. Nonetheless, identity was further established in antibody blocking experiments using the commercially available 5A6 mAb (Basu et al., 2001). However, it has also been shown that there are cell lines that express CD91 but bind GRP94 poorly, and also those that do not express CD91 yet bind GRP94 (Berwin et al., 2002). It therefore follows that, in all likelihood, multiple cell surface proteins capable of binding GRP94 exist and so CD91 does not serve uniquely in such a function.

A second potential receptor has been shown to be the scavenger receptor class A (SR-A) (Berwin et al., 2003). In these studies, a reporter cell line was identified that

lacked SR-A expression and also failed to bind GRP94. Upon ectopic expression of SR-A in that cell line, GRP94 binding was established. Further, this binding is completely blocked by fucoidin, a polysaccharide known to bind SR-A. However, it has also been shown that in cell lines that bind GRP94, fucoidin is only capable of partial inhibition of binding, implying the existence of at least one other receptor for GRP94. Recent studies by Berwin and colleagues have in fact confirmed a role for scavenger receptor EC-I, a type F scavenger receptor, in chaperone internalization (Berwin et al., 2004). Whether or not these families of receptors serve a GRP94 signaling function in antigen presenting cells is not yet known, and it is possible that other, and as-yet-unidentified receptors, are responsible for the downstream signaling occurring upon binding of GRP94 to the APC cell surface. Other receptors that have been proposed to serve as GRP94 receptors, including Toll-like receptors 2 and 4 (Vabulas et al., 2002). Considerable caution has been recently been brought to these identification, though, as GRP94 was recently demonstrated to display a significant affinity for the TLR-4 ligand, bacterial lipopolysaccharide (LPS) (Reed et al., 2003). Further, most attempts to depyrogenate GRP94 through depletion of LPS also lead to depletion of GRP94, as its affinity for LPS is quite high (Reed et al., 2002).

A PROINFLAMMATORY FUNCTION FOR GRP94

GRP94 was reported to be a pro-inflammatory molecule following the demonstration of TNF-α release, along with other pro-inflammatory cytokines, from macrophages upon exposure to GRP94 (Basu et al., 2000). To date, NF-κB signaling and NO release have also been attributed to stimulation with GRP94, as well as dendritic cell maturation (Panjwani et al., 2002). Signaling pathways implicated include the MAPK, ERK, and JNK pathways (Vabulas et al., 2002). All have been described to be upstream of antigen-specific T cell responses. In parallel fields, similar findings have been described with Hsp70, Hsp90, and bacterial Hsp60. In fact, all four chaperones have been reported to share similar receptors and signaling pathways as those functioning in LPS recognition (Tsan and Gao, 2004a). These findings have raised additional concerns regarding the LPS content of biochemically-purified HSP (Tsan and Gao, 2004a). Indeed, Reed, et al., demonstrated that if purified from endogenous sources using detergent-based depyrogenation techniques, it was possible to stimulate macrophages with GRP94, as assayed by ERK activation, without apparent activation of MAPK, JNK, NF-kB, or release of NO (Reed et al., 2003). This finding, too, has been paralleled in particular in the Hsp60 and Hsp70 fields. For Hsp70, Wallin, et al., were able to purify the protein away from all the signaling effects. However, if extremely small amounts of LPS were present, the potency and signaling were restored (Wallin et al., 2002). As well, for Hsp60, essentially all the signaling pathways originally attributed to it were in fact due to LPS contamination (Beg, 2002; Ohashi et al., 2000). The reader is referred to Tsan and Gao (2004a,b) for a detailed discussion of these concerns. Interestingly, the lag in recognition of potential LPS-derived

artifacts was in part due to the assumptions made regarding LPS. For example, it is known that LPS is heat-sensitive. However, innate immune effector cells are exquisitely sensitive to LPS and so chemically minute levels can confer significant physiological responses. As well, it's been demonstrated that non-LPS contaminants (such as bacterial lipoproteins) are also a significant source of artifactual signaling and thus if an investigator used polymyxin B to remove LPS, a common practice, these other TLR-4 ligands would remain (Tsan and Gao, 2004b).

In light of more recent studies regarding the contribution of bacterial components to GRP94 signaling, it is difficult to interpret past tumor rejection data in a parsimonious manner. Indeed, the ground-breaking experiments were performed at a time when little information regarding common contaminants of heat shock/chaperone proteins were known (Srivastava et al., 1986). It is of interest to note, for example, that in studies of the GRP94 peptide-carrier hypothesis, conducted by comparison of the tumor antigen function of tumor- versus normal liver-derived protein, it was found that the normal liver-derived GRP94 did itself afford significant protection from tumor growth (Tamura et al., 1997). Due in part to the elegance of this hypothesis, as well as its aesthetic appeal, the majority of experiments conducted to date mirror in design and context the original experimental protocol, and have yielded generally similar results (Srivastava, 2005). When, however, assumptions regarding the absolute biochemical purity of the GRP94 used in such experiments were examined, the results were surprising. Using the original purification protocols (which, with minor modification, are used in studies today), it is possible to detect aminopeptidase, protein kinase, ATPase, and several other enzymatic activities in apparently highly enriched GRP94 fraction. These activities are removed upon further purification, and serve as immunological "canaries" – their presence alerts us to be duly cautious regarding the supposition that biochemical and immunological criteria for protein purity are identical (Reed et al., 2002).

In light of this data, Baker-LePain used an alternative experimental approach to study the "tumor-restricted" basis of GRP94 function (Baker-LePain et al., 2002). They used a cell-based delivery system as the source of GRP94, to eliminate biochemical purification artifacts, and the 4T1 mammary carcinoma model and the K-BALB fibroblast cell line, both syngeneic to the BALB/c strain of mice as the tumor model. GRP94 delivery was accomplished by transfection of 4T1 and K-BALB cells with a secretory form of GRP94 (lacking the ER retention signal). Subsequently the cells were γ-irradiated to arrest proliferation and administered to naive mice. Mice were subsequently challenged with live 4T1 tumor cells and a significant reduction in tumor growth was seen in both the control (K-BALB) and experimental (4T1) mice. Further, they found equivalent levels of tumor suppression when the GRP94 construct contained only the N-terminal domain (Baker-LePain et al., 2002). To date, this domain has no known peptide-binding motifs and attempts to crystallize this domain in complex with synthetic peptides have failed (Dan Gewirth, personal communication). This leads to the conclusion that, at least in the 4T1 model, GRP94 does not need to come from a tumor source to elicit tumor

suppression and so in this system, tumor suppression derived from activation of innate effector cell function and/or bystander activity of adaptive cell function.

In an alternative and intriguing test of the GRP94 cross-presentation hypothesis, the Li laboratory created a transgenic mouse expressing a cell surface form of GRP94 (Liu et al., 2003). These mice were shown to display lupus-like, systemic inflammatory symptoms that correlated with the constitutive activation of dendritic cell function. Interestingly, if dendritic cells from the cell surface GRP94-transgenic mice were adoptively transferred to a mouse of the parent strain, they then display symptoms of chronic systemic inflammation. However, when such dendritic cells were adoptively transferred to MyD88 knock-out mice, no response was seen. Conversely, when $MyD88^{-/-}$ dendritic cells were adoptively transferred to the cell surface GRP94-transgenic mice, autoimmune symptoms were observed to resolve (Liu et al., 2003). These data favor the argument that GRP94 is acting principally as an immunological adjuvant, rather than as a cross-presentation antigen and implicate potential signaling pathways in the response to adjuvants.

Relating to the observations noted above, the Kaufman laboratory has reported an unexpected link between danger signals (i.e. adjuvants), inflammation, and activation of the unfolded protein response signaling pathway (Zhang et al., 2006). It is well established that the accumulation of unfolded proteins in the ER triggers the unfolded protein response (UPR), which leads to the activation of a transcriptional program yielding a pronounced up-regulation of ER chaperone synthesis. Kaufman's group has found that pro-inflammatory cytokines (IL-1β and IL-6), and more significantly LPS, also activate the UPR signaling pathway both *in vivo* and *in vitro*. The exact mechanism leading to UPR signaling after detection of LPS or pro-inflammatory cytokines is not yet known; these findings nonetheless provide a glimpse into the signaling pathways operating in response to adjuvant administration in the animal. This research points to new avenues of research that can be pursued in an effort to understand the initial systemic mechanism of action of adjuvants. These data may also portend potential signaling pathways operating in the cell surface GRP94-expressing transgenic mouse. If, for example, GRP94 has a role as a pro-inflammatory "danger" signal, the observation of systemic autoimmunity may reflect an end-point of the UPR pathway link to systemic inflammation, as discovered by Kaufman and colleagues (Zhang et al., 2006). In this view, the noted requirement for MyD88 signaling in the GRP94 transgenic model would primarily reflect the signaling pathways yielding hyperactivation of the dendritic cells. Regardless, the GRP94 transgenic mouse model provides a persuasive argument for an extracellular function for GRP94.

FUTURE DIRECTIONS

All facets of GRP94 biology are presently in a state of controversy and discovery: its evolutionary origins and existence throughout the phylogenetic tree; its endogenous roles as a chaperone protein; and its immunological role, as either a cross presentation antigen or adjuvant. The data to date lend themselves more to the model that

GRP94 is acting an innate immune adjuvant than as a cross-presentation antigen. This conclusion is, however, far from established and is, as noted, controversial. An open question that remains is whether or not GRP94 elicits this activation alone or only in concert with other danger signals. Available data demonstrate that tumor-derived peptides are not per se required for function; however, in these experiments, GRP94 was provided to the mouse in the presence of irradiated, i.e., dying cells. As such, cells would be expected to release many of the well characterized danger signals, including uric acid and lysophosphatidyl choline. The precise molecular basis for immune activation remains to be defined.

Currently, it is not known whether GRP94 is capable of acting alone, or if it requires other concurrent danger signals, to stimulate an enhanced response to "danger" signals. This is a deficiency in the field that needs to be addressed. As well, it is critical to find a larger subset of the client proteins that require GRP94 for surface expression or secretion. The most difficult question remaining is the nature and context of the signaling in response to GRP94 – when and where it occurs, and under what biological circumstances. Though GRP94 has been studied in an immunological context for the past thirty years, there is still great controversy over its mechanism of action as well as a large number of important unknowns yet to be answered. Furthermore, its utility and efficacy in treatment of human cancers and well as a vaccine adjuvant is just beginning to be examined. The unanswered questions surrounding GRP94, combined with a well-demonstrated potential, imbue this field with vigor and excitement.

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CHAPTER 11

HSP-INDUCED STIMULATION OF IMMUNE RESPONSES

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Abstract: Heat shock proteins have a profound effect on the immune response. Extracellular uptake of antigen-bound HSP can be presented by both MHC class II and class I. Maturation of DC is stimulated by HSP, upregulating the costimulatory molecules CD40, CD80 and CD86, as well as MHC class II, all of which enhance immune responses. The state of maturity of DC may be significant in the balance between immunity and tolerance. Innate immunity is elicited by the production of CCL3, CCL4 and CCL5, IL-1β, IL-6, IL-12 and TNF-α. These extracellular chemokines attract the entire repertoire of immune cells, and the interleukins modulate the cellular responses resulting in effective immunity. Human lymphoid cell activation by microbial Hsp70 appears to be mediated by CD40, expressed mostly by DC and macrophages, and CCR5 in T cells and immature DC. The CD40 molecule is part of the CD40-CD40L costimulatory pathway and the emerging alternative CD40-Hsp70 pathway might prove to be useful in rectifying CD40L deficient states. The CCR5 chemokine receptor is also bound and stimulated by Hsp70 to produce chemokines and cytokines. Thus, Hsp70 may function both as a systemic and mucosal adjuvant in vaccination and as a designer adjuvant in HIV-1 immunization

Keywords: Innate immunity, vaccination, CCR5, CD40, HIV stimulating and inhibitory epitopes

Abbreviations: CD40L, CD40 ligand; CTL, cytotoxic T lymphocytes; DC, dendritic cells; hu, human; HSP, heat shock protein; m, microbial; NO, nitric oxide; TLR, toll-like receptors

INTRODUCTION

The highly conserved HSP (heat shock proteins) are intracellular chaperones binding unfolded polypeptides to prevent misfolding and aggregation. They bind peptides with a hydrophobic motif by non-covalent linkage. A 7-residue peptide with the

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NRLLLTG sequence was identified and the crystal structure of the substrate binding domain of DnaK (from Escherichia coli) has been determined (Zhang et al., 1993). The specificity of binding is principally determined by a 5-residue core (RLLLT) centred on Leu4 by an interaction of this residue with the hydrophobic pocket of DnaK.

The chaperone function of HSP has an important effect on the intracellular antigen processing and presentation which are crucial in adaptive immunity. Hsp70 and Hsp90 deliver exogenous antigen into the MHC class I as well as class II pathways, playing an important role in cross-priming free extracellular antigen. Hsp70 can present external proteins not only to HLA class II but also by cross presentation to HLA class I pathway (Castellino et al., 2000; Singh-Jasuja et al., 2000). This has been recently demonstrated by Hsp70-mediated presentation of HLA class II and cross-presentation of HLA class I dependent peptides (SenGupta et al., 2004).

Extensive homology between microbial and mammalian HSP has led to the concept of cross-reactivity being associated with a number of autoimmune diseases (Van Eden and Young, 1996). Although HSP are found widely in Gram-positive and –negative organisms, in animals and the human gut, comparative study of the amino acid sequences of microbial and human HSP showed significant differences in homology varying from 15 to 80% along the HSP molecule (Hunt and Morimoto, 1985). This section will deal exclusively with immune responses stimulated directly or indirectly, with special emphasis on Hsp70.

Microbial HSP Stimulate Production of CCL3, CCL4 and CCL5

The CC chemokines CCL3, CCL4 and CCL5, also known as MIP-1α, MIP-1β and RANTES are stimulated by Hsp70 and Hsp65 (Lehner et al., 2000a; Wang et al., 2002). Although DC are the most potent producers of CC chemokines (Sallusto et al., 1999), monocytes, CD4+ and CD8+ T cells (Babaahmady et al., 2002), as well as endothelial and epithelial cells (Baggiolini, 1998; Ward et al., 1998) are capable of generating chemokines. These responses are greatly enhanced if mononuclear cells are used from Hsp70 primed animals. Indeed, increased stimulation of production of CC chemokines with Hsp70 or Hsp65 has been demonstrated in mononuclear cells of macaques immunized either by the systemic or mucosal route (Lehner et al., 2000a). Furthermore, TCRαβ and γδT-cells produce CCL3, CCL4 and CCL5 when stimulated by microbial Hsp70 (Lehner et al., 2000a; Lehner et al., 2000b; Wang et al., 1999). An increase in production of these CC chemokines has been demonstrated on repeated administration of Hsp70 in macaques (Wang et al., 1999), suggesting a memory-type of response, that is dependent on sensitized T-cells.

Microbial HSP Stimulate Production of Cytokines

Stimulation of human or murine monocyte-macrophage cells with mHsp70 or mHsp65 generates IL-12, TNF-α, IL-1β, IL-6 or GMCSF (Fuss et al., 1996; Galdiero et al., 1997; Peetermans et al., 1995; Zhang et al., 1993). An investigation

of mHsp70 revealed that whilst the wild type of Hsp70 stimulates minimal production of IL-12, TNF-α or NO by human monocytes, the C-terminal portion of Hsp70 (aa359–610) stimulates about 10fold higher concentration of IL-12 or TNF-α (Wang et al., 2002). Similar results were obtained by stimulating immature DC with the C-terminal portion of Hsp70 (Wang et al., 2002). Hsp70 may undergo lysosomal digestion which breaks up the molecule to a functional C-terminal fragment, which may be responsible for the TH1-like polarizing effect of Hsp70. In addition, the pro-inflammatory cytokines play an important role in innate immunity and inflammatory reactions.

The Effect of Hsp70 on DC Maturation

Immature DC are generated from haematopoetic stem cells within the bone marrow and FLT-3 and GM-CSF are growth factors for their differentiation from hematopoetic stem cells. Immature DC reside in epithelia and can terminally differentiate into mature DC by various stimuli, such as microbial pathogens, inflammatory cytokines, or other danger signals. Maturation of DC appears to be a critical process for DC function, particularly for priming naive T cells (Mellman and Steinman, 2001). DC are commonly generated *in vitro* by culturing PBMC-derived monocytes in GM-CSF and IL4 conditioned medium for 5–7 days (Sallusto and Lanzavecchia, 1999). Under these culture conditions immature DC develop and express the following phenotypic markers: low levels of CD80, CD86, MHC class II and CCR5, but little or no CD83 and CCR7 maturation markers. Human Hsp60, Hsp70, gp90 and Hsp90 released from necrotic cells, or recombinant human Hsp70 stimulate DC maturation by up-regulation of costimulatory molecules and production of IL-12 and TNF-α (Basu et al., 2000; Kuppner et al., 2001; Somersan et al., 2001).

Microbial Hsp70 applied to immature DC cultures for 2 days, induces dramatic changes in the cell-surface expression of DC maturation markers, similar to those elicited by CD40 ligand. There is an increase in MHC class II molecules, the costimulatory molecules CD80, CD86, CD40, as well as CD83 and CCR7 but a decrease in CCR5 (Wang et al., 2002). The Th1-polarizing cytokines IL-12 and TNF-α are also produced (Wang et al., 2002), but the C-terminal portion of microbial Hsp70 (aa359–609) is much more potent than the full length Hsp70 in stimulating these cytokines and in DC maturation. In contrast, the N-terminal ATPase domain of microbial Hsp70 fails to stimulate expression of any of these DC phenotypes or production of cytokines. Thus, the C-terminal peptide binding domain of Hsp70 stimulates innate activity, delivers peptides into the MHC class I pathway and induces CD8+ cytotoxic T lymphocytes.

The mechanism that underlies the process of maturation from immature DC is not fully understood. Signaling through CD40 by engagement of the trimerized CD40 ligand is one of the best defined pathways driving DC maturation (Lee et al., 1999). Other receptors, such as TLR and TNF-α receptors may also be involved in the process. TLR2 and TLR4 can interact with human Hsp60, Hsp70 and gp96 and therefore may mediate HSP stimulated DC maturation (Asea et al., 2002; Vabulas

et al., 2001; Vabulas et al., 2002a; Vabulas et al., 2002b). By the same token, CD40 may be involved in microbial and human Hsp70 stimulation of DC maturation (Becker et al., 2002; Wang et al., 2001). It is not clear whether CD91 can mediate DC maturation stimulated by Hsp70, Hsp90 or gp96. However, CD91 mediated endocytosis of HSP may play a role in the endocytosis-dependent pathway of HSP activation of TLR (Binder et al., 2000).

Mechanism of Action of Microbial Hsp70

Human Hsp70 has been found to interact with 5 receptors: CD 14 (Asea et al., 2000), CD91 (Binder et al., 2000), CD40 (Becker et al., 2002), TLR 2 and 4 (Asea et al., 2002; Vabulas et al., 2002a) and LOX-1 scavenger receptor (Delneste et al., 2002). In contrast, only CD40 and recently TLR2, 4 and CCR5 have been identified to mediate microbial Hsp70 interactions (Bulut et al., 2005; Wang et al., 2001; Wang et al., 2002; Wang et al., 2005; Whittall et al., 2006b) and these three receptors will be discussed further. CD40 is a 40–5OkDa glycoprotein, a member of the tumor necrosis factor (TNF) receptor superfamily, and is primarily expressed on B lymphocytes, monocytes and DCs (van Kooten and Banchereau, 1997). CD40 can also be found on epithelial cells, some cancer cells and activated CD8+ T cells (Bourgeois et al., 2002; Young et al., 1998) and plays an important role in T cell-mediated immune responses. Hsp70 stimulates CD40+ DC to produce CC chemokines, as described above, it is effective in maturation of monocyte-derived DC (Wang et al., 2002) and can deliver peptides from outside to the MHC class I pathway, referred to as cross-presentation (Castellino et al., 2000; SenGupta et al., 2004). Furthermore, Hsp70 elicits via CD40 stimulation TH1 polarizing cytokines (IL-12, IFN-γ, TNF-α), as well as IL-6, IL-1β and NO (MacAry et al., 2004; Wang et al., 2002; Wang et al., 2005). The signaling pathway activated by the Hsp70-CD40 interaction involves PKC (protein kinase C), and downstream MAPK (mitogen activated protein kinase) which includes p38 (Becker et al., 2002; Wang et al., 2005).

An Alternative CD40-Hsp70 Costimulatory Pathway

Comparison of CD40L and Hsp70 activation of CD40 molecules on DC suggest that they have similar functions in stimulating the production of CC chemokines, TH-1 cytokines and DC maturation, as described above. These observations suggest an emerging CD40-Hsp70 costimulatory pathway which mimics the CD40-CD40L functions. The significance of the alternative CD40-Hsp70 costimulatory pathway has been elegantly demonstrated in the induction of protective immunity against tuberculosis in mice (Lazarevic et al., 2003). $CD40^{-/-}$ mice were susceptible to infection with low doses of *M. tuberculosis*, due to deficient IL-12 production that leads to impaired IFN-γ responses. In contrast $CD40L^{-/-}$ mice were resistant to *M. tuberculosis* and this discrepancy in the two components of the CD40-CD40L costimulatory pathway was accounted for by mycobacterial Hsp70 which served

as an alternative ligand for CD40 in stimulating DC to produce IL-12 (Lazarevic et al., 2003). Impaired IL-12 production leads to deficient IFN-γ and impaired TH-1 response in $CD40^{-/-}$ mice. It is noteworthy that overexpression of Hsp70 in *M. tuberculosis* (Stewart et al., 2001) might be an alternative mechanism in prevention of *M. tuberculosis* infection, by an increased and prolonged Hsp70 stimulation of CD40+ DC.

A Potential Role of CD40-Hsp70 Costimulatory Pathway in Immunity and Tolerance

The state of maturity of DC appears to be a pivotal point in the balance between immunity and tolerance (Steinman et al., 2003). One of the determining factors appears to be CD40-CD40L costimulatory pathway. Interaction between CD40 and CD40L leads to immunity, whereas interference in this interaction may result in tolerance (Quezada et al., 2004). Necrotic cells can induce maturation of DC and immune stimulation, by releasing antigens, including HSP, which are taken up by DC (Basu et al., 2000; Sauter et al., 2000). In contrast, apoptotic cells captured by DC may induce a state of tolerance (Ferguson et al., 2002; Steinman et al., 2003), but the mechanism is not clear. Indeed, infection of immature DC in humans may lead to inhibition of effector T cell function (Dhodapkar et al., 2001) and an intriguing suggestion has been made that chronic infection, such as that with HIV, may result when persistent microorganisms are taken up by DC which fail to mature, and these DC induce tolerance either by deleting T cells or by inducing T regulatory cells (Steinman and Nussenzweig, 2002). The effect of Hsp70 on maturation of DC may, in the presence of specific antigen, influence the critical decision between induction of tolerance and immunity. This is supported by the observation that co-administration of a tolerogenic LCMV peptide with recombinant human Hsp70 converted T cell tolerance to autoimmune diabetes in a LCMV-GP (glycoprotein) transgenic model, expressing LCMV-GP in pancreatic islet B-cells and TCR in most CD8+ T cells (Millar et al., 2003). Hsp70 alone did not induce diabetes in these mice; GP alone induced diabetes only in $< 20\%$ of mice, whereas Hsp70 with GP induced diabetes in $> 88\%$ of the transgenic mice. GP-Hsp70 induced diabetes was dependent on DC expressing CD40 but was independent of costimulation through the CD28 pathway.

The C-terminal Fragment of Hsp70 Expresses Intramolecular Stimulating and Inhibitory Epitopes

Examination of peptides within the C-terminal fragment of Hsp70 (aa359–609) revealed two immunomodulating epitopes (Wang et al., 2005). Peptide 407–426 activates the CD40 molecule on DC, enhancing cytokine production and maturation of DC stimulated by Hsp70 or CD40L. The epitope is located at the base of the peptide-binding groove of Hsp70 and has five critical residues. Surprisingly, an inhibitory epitope (p457–496) was identified downstream from the peptide-binding

groove, which inhibits TNF-α, IL-12 and CCL-5 production and maturation of DC stimulated by Hsp70 or CD40L. P38 MAP kinase phosphorylation is critical in the alternative CD40-Hsp70 pathway and is inhibited by p457–496 but enhanced by p407–426.

The beneficial effect of anti-TNF-α antibodies in patients with CD is well recognized (Rutgeerts et al., 2004; Targan et al., 1997) and TNF-α appears to be critical in the control of Crohn's disease, rheumatoid arthritis and other autoimmune disease (Feldman et al., 1998). However, it might be more desirable to prevent rather than neutralize the production of TNF-α, in order to control the immunopathogenesis of CD. Indeed, the Hsp70 inhibitory peptide (aa457–496) induced significant inhibition of TNF-α production by DC from PBMC or cells eluted from intestinal mucosa of Crohn's disease (Whittall et al., 2006a). These inhibitory peptides target the emerging CD40-Hsp70 co-stimulatory pathway and offer a novel strategy to prevent excessive production of TNF-α in Crohn's disease and probably other autoimmune diseases.

The CCR5 Chemokine Receptor Mediates Hsp70 Activation

CCR5 is a seven-transmembrane G-protein-coupled receptor expressed on TH1 and TH0 cells, macrophages and immature DC (Ostrowski et al., 1998; Sallusto et al., 1998a; Sallusto et al., 1998b). The receptor plays an important role in inflammatory processes and autoimmunity, binding CCL3, CCL4 and CCL5 chemokines, as well as acting as a major co-receptor for the R5 strains of HIV-1 (Whittall et al., 2006b). Recently, evidence has been gathered that Hsp70 binds and activates CCR5 expressed in primary human CCR5+ DC and transfected HEK 293 cells (Whittall et al., 2006b). This was based on direct binding of three extracellular peptides of CCR5 to Hsp70, demonstrated by surface plasmon resonance. Significant CCR5-mediated calcium mobilization was stimulated by Hsp70 and inhibited with TAK 779. Hsp70-mediated activation of the p38 MAP kinase phosphorylation signaling pathway was also demonstrated in CCR5 transfected HEK 293 cells. Functional evidence of interaction between Hsp70 and CCR5 was shown by enhanced production of CCL5 by HEK 293 cells transfected with CCR5. Primary monocyte-derived immature DC stimulated with Hsp70 produced IL-12 p40 which showed dose-dependent inhibition > 90% on treatment with both TAK 779 and CD40 MAb, but only partial inhibition with either TAK 779 or CD40 MAb. The co-expression of CD40 and CCR5 molecules on DC, which are bound and activated by mHsp70 is of considerable significance both in innate and adaptive immunity (Figure 1).

TLR2 and TLR4 Mediate HSP Activation

TLR are pattern recognition molecules which activate innate immune responses against a wide variety of microorganisms (Iwasaki and Medzhitov, 2004). TLR4 is expressed by DC and macrophages and is activated by human Hsp70 (Asea et al., 2000; Vabulas et al., 2002b). Microbial Hsp70 may stimulate TLR2 and

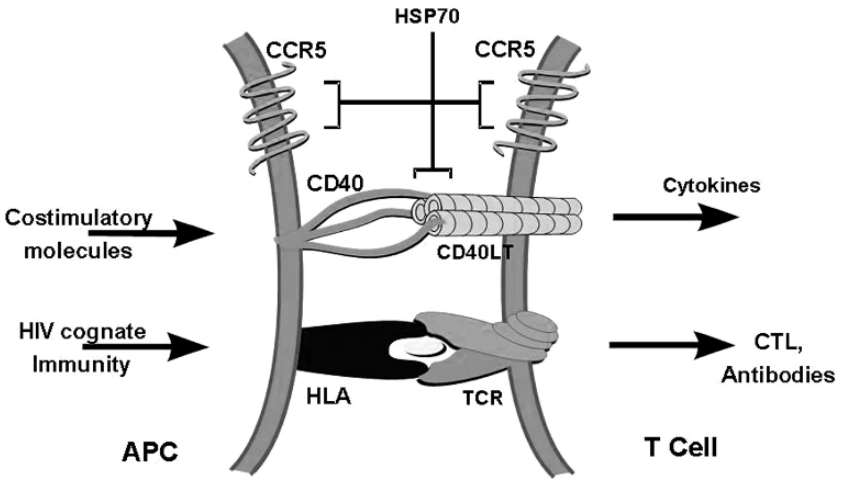

Figure 1. Schematic representation of antigen presenting cell-T lymphocyte interactions. Antigen presenting cells (APC) like dendritic cells (DC) express the costimulatory molecule CD40 and the coreceptor for HIV-1 on its plasma surface, which are activated by mHsp70 and results in innate and cognate immunity to HIV-1 by stimulating the CTL, antibody and cytokine release by T cells

TLR4, but so far this has been demonstrated only with murine macrophages, an human endothelial cell line and HEK 293 cells, though not with human lymphoid cells (Bulut et al., 2005). If mHsp70 were to stimulate TLR2 and TLR4, these are expressed by human monocyte-derived DC (Iwasaki and Medzhitov, 2004). Signalling through these TLR induces NF-κB activation, producing TNF-α and IL-6. The interplay between CD40, CCR5 and TLR4 on DC following stimulation with Hsp70 needs further investigation.

Adjuvanticity of HSPs

The adjuvanticity of microbial Hsp70 and Hsp65 have been demonstrated in systemic immunization (Barrios et al., 1992; Lussow et al., 1991; Perraut et al., 1993), as well as by mucosal administration in nonhuman primates (Lehner et al., 2000a). Both, systemic and mucosal adjuvanticity is dependent on stimulating production of 3 CC chemokines – CCL3, CCL4 and CCL5. CCL5 is a potent chemoattractant of monocytes, CD4 cells and activated CD8 cells (Kim et al., 1998; Meurer et al., 1993; Murphy et al., 1994; Schall et al., 1990). CCL3 and CCL4 attract CD4+ T and B cells (Schall et al., 1993) and the 3 chemokines attract immature DC (Dieu et al., 1998). The adjuvanticity of Hsp70 is also greatly enhanced by its propensity to take up exogenous antigen which is processed and peptides are presented by the MHC class II and class I pathways.

Furthermore, significant immunomodulating effect is exerted by Hsp70 stimulating production of IL-12, IFN-γ, TNF-α and IL-6 and there is compelling evidence

that TH1 responses are generated by DC stimulated with IL-12 (Monteleone et al., 1997; Parronchi et al., 1997; Trinchieri, 1994), IFN-γ (Fuss et al., 1996; Kakazu et al., 1999) and TNF-α (Breese et al., 1994; Kollias et al., 1999; Reinecker et al., 1993; Woywodt et al., 1994). DC take up antigen which is processed in the cell, they undergo maturation and migrate to the regional lymph nodes, where they present the processed antigen to T and B cells, which elicit cellular and antibody responses.

The Role of Hsp70 in Innate and Adaptive Immunity with Reference to Vaccination

Systemic and mucosal adjuvanticity of Hsp70 described above were studied in non-human primates. These were immunized by the vaginal mucosal or targeted lymph node route with Hsp70 linked to HIVgp120 and peptides derived from the extracellular part of the CCR5 molecule (Bogers et al., 2004). Significant protection against SHIV 89.6P was associated with specific serum and secretory antibodies, IL-2 and IFN-γ stimulated by the vaccine components. A raised concentration of CC-chemokines was inversely correlated with the proportion of CCR5+ cells (Bogers et al., 2004). CD8+ CTL can be generated by loading LCMV peptides on to human Hsp70 which elicits protective anti-viral immunity in mice (Ciupitu et al., 1998). Recently, human anti-influenza CTL were generated by pulsing DC with mHsp70 loaded with peptides from influenza virus; the CTL response was significantly greater than pulsing DC with peptides alone (MacAry et al., 2004).

The innate arm of immunity is also activated by Hsp70. Natural killer (NK) cells can be stimulated to proliferate by human Hsp70 and this function resides in the C terminal portion of Hsp70 (Multhoff et al., 1999). Cell surface Hsp70 found on some tumor cells may induce migration of and cytolysis by CD56+CD94+ NK cells (Gastpar et al., 2004). A peptide (aa 450–463) was identified within the sequence of huHsp70 which enhances NK cell activity. mHsp70 and Hsp65 upregulate $\gamma\delta^+$ T cells, both *in vitro* and *in vivo* in non-human primates and they generate CD8-suppressor factors and CC chemokines (Lehner et al., 2000b). Indeed, a significant increase in $\gamma\delta^+$ T cells was found in rectal mucosal tissue and the draining lymph nodes in macaques immunized with SIVgp120 and p27 and protected from rectal mucosal challenge by SIV (Lehner et al., 2000b).

Excluding LPS Contamination from HSP Preparations

Since some of the cytokine and chemokine stimulating activities of Hsp70 are similar to those of LPS, it is essential to exclude the possibility that the observed effects are elicited by contaminating LPS (Bausinger et al., 2002; Gao and Tsan, 2003). Currently it is difficult to prepare HSP entirely free of contamination with LPS, especially in those expressed in E coli. Most of the LPS contamination (95–99%) can be removed by using polymixin B immobilized on agarose affinity

column (Asea et al., 2000; Wang et al., 2001). Other reagents, such as RSLP derived from Rhodopseudomonas spheroids and lipid IVa also inhibit LPS activity and have no effect on HSP stimulation (Asea et al., 2000; Panjwani et al., 2002). As the stimulating activity of Hsp70 is calcium dependent, the intracellular calcium chelator BAPTA-AM is used to differentiate between LPS and Hsp70 functions (Asea et al., 2000; MacAry et al., 2004; Wang et al., 2001). Furthermore, treatment with proteinase K also inihibits Hsp70, but not LPS stimulating activity (MacAry et al., 2004). HSP stimulation is reduced by heat denaturation (Asea et al., 2000; Panjwani et al., 2002). The C3H/HeJ and C57BL/IOScCr inbred mouse strains are homozygous for a mutant Lps allele ($Lps^{d/d}$), which confers hyporesponsiveness to *LPS* challenge (Coutinho and Meo, 1978) and provides a model to study immunological functions of HSP. In studies with monocytes and DC using mHsp70, inhibition with antibodies to CD40 but not to CD 14 should discriminate between HSP and LPS (Wang et al., 2001; Wang et al., 2002). Perhaps the most conclusive evidence that LPS contamination can not account for the functions of HSP is the effect of Hsp70-derived stimulating peptide (aa407–426), which is devoid of any LPS and elicits comparable functions to that of the wild type Hsp70 (Wang et al., 2005).

Potential Therapeutic Applications of the Emerging CD40-Hsp70 Costimulatory Pathway and CCR5-Mediated Activation

The emerging concept that CD40 is activated by Hsp70, with comparable effect to that elicited by the CD40-CD40L costimulatory pathway and that Hsp70 binds and activates CCR5, may have opened novel immuno-therapeutic strategies.

a) **TH1 polarizing adjuvanticity** elicited by mHsp70 activating CD40 on DC and monocytes to produce CC chemokines, IL-12, TNF-α, and induce maturation of DC, has been applied in systemic and mucosal immunization (Lehner et al., 2000a; Wang et al., 2002). Hsp70 has specific anti-HIV-1 functions as it binds CCR5 (Whittall et al., 2006b), the major co-receptor for HIV-1 in mucosal infection, as well as generating CC chemokines which further block and downmodulate CCR5. These immunological properties and specific anti-HIV-1 functions suggest that Hsp70 may act as a designer adjuvant for HIV-1. Covalently linked antigens to Hsp70 or fusion proteins may be used in preventive vaccination against other microbial infections (Bogers et al., 2004; Harmala et al., 2002).
b) **Substitution of CD40L function by Hsp70** might be of therapeutic significance in conditions with a reduced number of CD4+ T cells, as is found in HIV infection, or in mutation of CD40L resulting in decreased cell surface expression of CD40L, as is found in the hyper-IgM syndrome. It is possible that in these conditions the deficiency might be rectified by administration of Hsp70.
c) **Immunomodulating properties of Hsp70 peptides** might be utilized in upregulating immunity in immunodeficient states and downregulating immunity in autoimmune conditions or in transplantation, by treatment with the stimulating or inhibitory peptide, respectively. Further experiments will have to be carried out

to find out if linking antigens to these peptides will elicit specific immunomodulation. However, inhibition of TNF-α and IL-12 might have important applications in a number of diseases, such as Crohn's disease, rheumatoid arthritis and other conditions.

d) **Loading viral CTL epitopes** on to Hsp70 and pulsing DC in treating viral infections, as demonstrated *in vitro* with influenza virus is highly efficient in eliciting CD8+ CTL (MacAry et al., 2004). This approach has to be demonstrated in *in vivo* studies.

e) **Therapeutic vaccination with tumor-derived Hsp70** prepared by ADP column separation, so as to retain tumor specific peptides has been extensively investigated (Srivastava, 2006).

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CHAPTER 12

THE ROLE OF HEAT SHOCK PROTEINS IN THE ELICITATION OF IMMUNE RESPONSES

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Abstract: Heat Shock Proteins (HSP), well known for their protein/polpypetide chaperone activities, display a remarkable ability to elicit peptide-based immune responses. The exact manner in which they do so, their physiological role in this process, and their distinct ability to promote adaptive immune response is the subject of this chapter. The first part of the chapter will deal with the general known antigenic stimulation properties of heat shock proteins, the second will deal with the separation of innate and adaptive immune responses as learned from studies with GP96, Hsp70, and Mycobacterium Hsp70 and finally the third will deal with the subject of chaperone rich cell lysates and means of isolating these HSP immunogenic complexes

Keywords: Adjuvanticity, antigen simulation, HSP, MHC

HEAT SHOCK PROTEINS (HSP) AND ANTIGENIC STIMULATION

General Immunogenic Properties of Heat Shock Proteins

The recent recognition that the most effective way to develop a therapeutic vaccine for established cancers via a dominant long-lasting protective response is through the use of epitopes derived from randomly mutated proteins from individual tumors (Srivastava, 2006) has led to creative searches for methods to present these unique antigens. The problem arises when one tries to attempt to characterize and isolate these novel tumor antigens from each individual. A task made impractical if not virtually impossible due to the unlimited potential numbers of unique epitopes, although some attempts have been made to do so (Echchakir et al., 2001;

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Hogan et al., 2004). The majority of in vivo generated polypeptides made by the cell are ignored or never presented due to the fact that presentation of peptide antigens by Major Histocompatability Complex (MHC) to the adaptive immune system is a highly inefficient process (Castellino et al., 2000). Proteins that chaperone nascent polypeptides to protect them from degradation, assist them in cellular transport, alter their substrate activity, and allow appropriate mulitmerization, known as Heat Shock Proteins (HSP) (Barbouche et al., 2003; Gamer et al., 1996; Richter and Buchner, 2001) indeed may provide an alternative antigen recognition system in the cell. The physiological nature behind this *in vitro* observed HSP-dependent antigenic recognition is still controversial, as are the cellular mechanisms behind the exchange of peptides from the HSP to MHC Class I. This chapter illustrates that although it is still not known if HSP naturally play a large role in presenting antigens to the immune system, HSP-peptide complexes can be certainly used in the laboratory to develop antigen dependent vaccine strategies.

Studies in the mid 1980's by Srivastava and Ullrich and colleagues, investigating the identification of tumor transplantation antigens of cancers induced in rodents, introduced a novel concept that suggested heat shock proteins were themselves strongly immunogenic (Barbouche et al., 2003; Srivastava and Das, 1984; Srivastava et al., 1986; Ullrich et al., 1986). These findings, focusing first on HSP GRP94 and then later on GRP70 and others strongly indicated that the HSP bind peptides that are immunogenic only from tumor cells while those from non tumors fail to induce strong responses (Udono and Srivastava, 1993).

These early finding suggested the following about the immunogenic properties of HSP:

1) Peptides carried by the HSP but not the HSP themselves are primarily responsible for the immunogenic effect.
2) Antigen presentation cells (APC) and receptor-dependent uptake of HSP and their immunogenic peptides are essential for this process.
3) HSP could be used in already established cancers as well as in therapeutic settings to prevent cancer development or even viral infection.
4) The process was dependent primarily on CD8+ and MHC class I dependent process (although some studies now show CD4+ T cells response can be elicited).
5) HSP mediated immune recognition was not restricted on a single cancer type or viral infection.
6) Peptide presented to MHC class I and therefore elicit CD8+ CTL can be bound and internalized by HPS via an exogenous pathway and thus results in 'cross presentation' (Suto and Srivastava, 1995).
7) Peptides from HSP from one MHC haplotype (e.g. H-2^{d}) can be processed and presented to an MHC from another haplotype (e.g. H-2^{b}), known as 'cross priming' (Arnold et al., 1995; Matzinger and Bevan, 1977).

Controversial Issues and Problems with HSP Induced Adaptive Immune Responses

However, a number of very important questions were raised by these initial observations, some of which remain unanswered today (Gullo and Teoh, 2004; Nicchitta, 2003). One important question about this newly found mechanism of HSP-dependent antigenic presentation process was that of *in vivo* relevance. (**A**) It was speculated that perhaps the *in vitro* isolation of heat shock proteins might introduce low level non-relevant contaminants (e.g. endotoxins or proteins) (Nicchitta and Reed, 2000; Reed et al., 2002) that could result in strong but nonspecific T cell activation. (**B**) Furthermore, a number of studies have shown that HSP themselves can elicit strong innate immune responses (Basu et al., 2000; Singh-Jasuja et al., 2000). This point will be discussed further in the next section below, but many experiments have recently teased out the differences between the adaptive and innate properties of HSP. (**C**) Even though a number of studies have found that the peptides found bound to HSP can be the same as those found on MHC class I (Ishii et al., 1999; Nieland et al., 1996), they do display a wider diversity of peptide binding and they exhibit low stoichiometric and irreversible binding properties (at least with GRP94) that are not consistent with the role of at least GRP94 as acceptor of peptides for antigen presentation to MHC (Blachere et al., 1997). (**D**) The mechanism behind peptide release by HSP and their transfer to the antigen processing and presentation pathway in the cell is still largely unknown, although a number of advances in our understanding have been made. For example, it seems as if HSP peptide presentation to MHC can occur through a (TAP)-dependent and independent (Arnold et al., 1995) process. Another study suggests that the HSP interact with APC in a specific receptor–ligand and saturable process that after internalization can present peptides to MHC via a cytoslic or endocytic pathway (Castellino et al., 2000). Thus, the lack of appropriate in vivo models to address some of these key issues (e.g. knock out mice or mutant cell lines) up until recently has made it difficult to address some of these still unresolved issues (Randow and Seed, 2001).

Novel Issues Surrounding HSP Immunobiology

Now that there is a large body of evidence that links the adaptive immune response to cancer and the HSP-dependent cellular heat stress response a new area of immunostimulation by inducing hypothermia to produce increased anti-viral or anti-tumor responses has evolved. This has also been expanded to autoimmune diseases; for example HSP complexes from inflamed brain tissue in rodents may help to prevent or to slow the development of experimental autoimmune encephalomyelitis (Galazka et al., 2006). Other examples include the use of HSP isolated from virus infected cells that provide both adaptive and innate immune protection form measles virus infection in mice (Oglesbee et al., 2002; Weidinger et al., 2000). In both of these cases, stress due to viral infection and/or inflammation is the driving force behind the HSP-dependent immunological effects.

Another important novel area of interest is the identification of novel receptors for the internalization of HSP-peptide complexes from infected or tumor cells. With the identification of CD91 as a receptor on a variety of cells for GRP94, Hsp70 and Hsp90 other receptors appear to be involved as well (Basu et al., 2001). In other cells however, CD40 was shown to bind and internalize Hsp70 (Becker et al., 2002). Other work with less direct but intriguing evidence suggests that Toll Like receptors might play a role in the re-presentation of HSP-peptide complexes on dendritic cells (e.g. TLR2/4) (Vabulas et al., 2002a; Vabulas et al., 2002b). Other receptors such as those on NK cells (e.g. NKG2D) are also likely to play some role (Gross et al., 2003; Kleinjung et al., 2003) in HSP capture and internalization.

PEPTIDE BINDING VERSUS ADJUVANTICITY

The conserved heat shock protein (HSP) families are a group of molecular chaperones expressed in all prokaryotic and eukaryotic cells. HSP are the most abundant constitutively expressed proteins in cells. The expression of many HSP is also induced during cellular stress responses such as those caused by exposure to toxins, glucose starvation, anoxia, irradiation and heat (Hassen et al., 2005; Maytin, 1992; Spence et al., 1990; Zimmerman et al., 1991). The major functions ascribed to HSP are in the assembly and disassembly of proteins, the prevention of aggregation and their ability to bind the hydrophobic regions of nascent polypeptide chains (Gething and Sambrook, 1992; Richarme and Kohiyama, 1993). As discussed above, this latter function has generated immense interest amongst immunologists, as HSP-derived from tumor cells have been shown to confer tumor specific immunity (Richarme and Kohiyama, 1993; Srivastava, 2002a). This has led to the identification of several HSP with immunological activity. Whilst the two most widely used are gp96 and Hsp70, it is also clear that Hsp90, Hsp65 and calreticulin can also elicit anti-tumor immune responses (Anthony et al., 1999; Basu and Srivastava, 1999; Udono and Srivastava, 1994). The association of HSP with tumor specific cellular peptides explains the induction of protective tumor specific immunity and the generation of CTL specific for the tumor cells. Following internalization of HSP-peptide complexes by professional antigen presenting cells (pAPC) such as Dendritic cells (DC), the tumor specific peptides are released from the HSP and gain access to the MHC class-I pathway in a process termed cross-presentation (Li et al., 2002). This observation has been expanded from tumor-derived proteins to antigens derived from infectious agents and minor histocompatibility antigens (Arnold et al., 1995; Ciupitu et al., 1998).

Hence, the ability to bind and chaperone antigenic peptides to the immune system is thought to be a critical aspect of HSP activity for the elicitation of adaptive tumor specific responses. For the rational design of HSP based immunotherapeutics, it is important to understand how specific peptide substrates are bound and how much bound antigenic peptide is required to elicit protective responses.

Peptide Binding Properties of GP96

For gp96, Sastry and colleagues utilized a photoreactive azido tagged peptide from vesicular stomatitis virus capsid combined with specific proteolysis to map the substrate-binding site to amino acids 624–630. This site is situated adjacent to a proposed dimerization domain and tri-dimensional model of peptide binding was constructed based on the known crystallographic structure of major histocompatibility complex class I molecule bound to a similar peptide. This gp96-peptide model predicts that the peptide ligand is held in a groove formed by α-helices and lies on a hydrophobic surface consisting of anti-parallel β-sheets (Linderoth et al., 2001). The same group has also reported that gp96 can exist as higher order structures or aggregates from dimers to octamers and retain the potential to bind antigenic peptides (Linderoth et al., 2001). Whilst this model is extremely interesting, it remains speculative due the use of chemical cross-linking to bind the peptide substrates to gp96. This is because the experimental conditions required to generate a more physiological or non-covalent form of peptide binding to gp96 have not been defined. There is also a lack of corroborative crystal structure data on gp96-peptide complexes.

Peptide Binding Properties of Hsp70

These are two areas where studies on Hsp70/peptide interactions are significantly advanced in comparison to gp96. There is a 60% amino acid sequence identity between eukaryotic Hsp70, and a 40% identity between eukaryotic and the *E. coli* Hsp70-equivalent DnaK (Robert et al., 2001). Hsp70 contains an N-terminal ATPase subunit (44kD) and a C-terminal peptide binding domain (27kD). The structural features of the Hsp70 substrate binding and the crystal structure of the bacterial Hsp70 homologue DnaK, have been resolved. Analysis of DnaK has demonstrated the presence of a hydrophobic binding channel (Zhu et al., 1996). Access of nascent hydrophobic polypeptide chains to this domain is controlled by ATP binding and hydrolysis. Binding of ATP to the nucleotide-binding domain induces a conformational change that results in the opening of a helical lid allowing peptides access to the substrate binding domain. Following hydrolysis of ATP to ADP, the helical lid is closed, preventing access to the cavity and resulting in slow 'on' and 'off' rates for bound substrates (Boice and Hightower, 1997; Popp et al., 2005).

The nature of the polypeptides, which preferentially bind to Hsp70, has been studied. Gething and colleagues tested a large panel (Srivastava, 2002b) of synthesized peptides for their binding activity. They found that the peptides which bound with the highest affinities were made up of at least 7 residues and included large hydrophobic and basic amino acids with few or none acidic residues. By comparing several HspP70 family members (Hsc70, DnaK and BiP), they were also able to demonstrate significant differences in the peptide binding specificities of these 3 closely related proteins, perhaps reflecting some notable differences in

the sequence of their peptide binding domains. These are important considerations for the design of linkers and antigenic epitopes for generation of peptide/HSP complexes (Fourie et al., 1994). Hence, NRLLLTG or HWDFAWPWGS linker sequences are often fused to the amino or carboxy terminals of antigenic peptides to facilitate HSP binding in studies of Hsp70 (Castellino et al., 2000; Javid et al., 2004).

Mycobacterial Hsp70

The importance of peptide binding for Hsp70 immune activity was formally demonstrated using peptide-binding mutants of Mycobacterial Hsp70. The design of these mutants was based on earlier studies of DnaK (Mayer et al., 2000) that demonstrated the importance of a conserved Valine residue in the peptide-binding domain. By substituting Valine 310 for Phenylalanine in Mycobacterial Hsp70, we were able to severely impair peptide binding and also compromised the ability of the HSP to induce virus specific T-lymphocytes, even in the presence of excess viral peptides. Moreover, by utilizing biophysical approaches such as fluorescence anisotropy, we have also shown that as little as 120pM antigenic peptide when delivered to Dendritic Cells in association with Hsp70 is sufficient to elicit T-lymphocyte responses (MacAry et al., 2004).

Innate Immune Activation via HSP

For HSP mediated cross-presentation to occur, professional antigen presenting cells of the immune system, such as immature dendritic cells (iDC) need to bind and internalizes HSP-peptide complexes (Singh-Jasuja et al., 2000; Wassenberg et al., 1999). A further requirement is for the DC to be modified and/or stimulated by the HSP in a way that facilitates their maturation into cells that can process and present the HSP chaperoned peptides efficiently (Kuppner et al., 2001). These cells must also be capable of migrating to local lymphoid tissues to expose themselves to circulating T-cells and be able to form direct physical interactions with other immune cells to allow for the formation of immunological synapses (Benvenuti et al., 2004; Kupiec-Weglinski et al., 1988).

While the specificity of HSP can be attributed to peptide occupancy of the substrate-binding domain, the effectiveness of HSP also relies on their pro-inflammatory activity. However, the ability of HSP to directly stimulate inflammatory responses in DC remains controversial with a large divergence in the stimulatory activity reported between the different HSP expressed by cells and in the species from which the HSP is derived (Tsan and Gao, 2004). An additional complicating factor has been the absence of suitable controls and safeguards for contaminating endotoxin activity with several studies intimating that this is responsible for the pro-inflammatory effects observed (Gao and Tsan, 2003; Reed et al., 2003). It is also clear that in studies where poor pro-inflammatory activity has been

observed, the HSP are still found to be proficient in the induction of peptide specific CTL (Bausinger et al., 2002).

Controversy aside, It has been shown that different HSP can activate innate immune cells both *in vivo* and *in vitro* (Srivastava, 2002b). However, the route of activation of innate immune cells has been poorly defined. Specific HSP binding to the cell surface of both murine and human dendritic cells and monocytes has been demonstrated and a number of potential HSP receptors described, including CD91, SR-A, CD14, CD40, TLR4 and Lox-1 (Asea et al., 2000; Berwin et al., 2003; Binder et al., 2000; Delneste et al., 2002; Vabulas et al., 2002b; Wang et al., 2001). However, a clear consensus has not emerged regarding the role that these individual receptors play in uptake and/or pro-inflammatory signaling for the different species of HSP.

HSP Immunoregulation

The Van Eden laboratory has proposed that some HSP can play an important role in regulating immune responses by inducing a regulatory phenotype in T-lymphocytes which helps to control potentially damaging autoimmune responses (Wendling et al., 2000). The potentiation of immune responses to chaperoned peptides may reflect a balance between the generation of IL-10 secreting regulatory T-cells specific for HSP epitopes versus effector cells specific for the chaperoned peptide (van Eden et al., 2003). In a previous study, a role for Mycobacterial Hsp70 in the DC mediated cross-presentation of viral peptides derived from Influenza A (Inf A) and human Cytomegalovirus (huCMV) was shown to be dependent on the induction of an intracellular calcium signaling cascade within the DC, and not on the direct stimulation of the cells to produce cytokines and chemokines. However, the provision of a second signal mediated by effector T-cell/DC interactions, greatly augmented the HSP effect on the DC resulting in the increased secretion of pro-inflammatory cytokines and chemokines (MacAry et al., 2004). Hence, Mycobacterial Hsp70 can be said to have a 'licensing' effect on human DC by lowering their threshold of activation, thus making them more responsive to additional pro-inflammatory signals. Given that the immune system will usually encounter HSP in association with the release of proteins from damaged tissues caused by pathological events (necrosis), this represents a powerful model, which potentially explains many of the conflicting observations in the Mycobacterial Hsp70 literature. The extension of this model to other species of HSP requires further investigation.

BIOCHEMICAL ISOLATION OF HSP PEPTIDE COMPLEXES

Many attempts have been made over the years at making protein based anti-tumor preparations that can serve as potential anti-cancer vaccines. One means of maintaining peptide antigen content while eschewing the need for purity of the vaccine components is to use whole (usually dead/irradiated) tumor cells or tumor

lysate as immunogens. Compared to protein purification from an autologous tumor sample, vaccines of whole cell/lysate nature may be somewhat easier to prepare, and their overall immunogenic content (protein, peptide, carbohydrate, lipid, etc), would predict that such vaccines provide all the necessary components to generate a potent anti-tumor response. However, the amount of unique or tumor related antigens in the preparations may be masked by the abundance of normal non-immunogenic proteins that are contained in the preparations. The relative undefined nature of the ensuing preparation is somewhat unsatisfying.

Standard Chaperone Protein Complex Isolation

Chaperone protein vaccinations have been demonstrated to be more effective than these whole tumor cell preparations (Graner et al., 2000a; Graner et al., 2003; Janetzki et al., 1998; Udono and Srivastava, 1994; Yedavelli et al., 1999; Zeng et al., 2003). It is very likely that there are inhibitory substances present in tumor cell lysates that could actually suppress the anti-tumor immune response (Graner et al., 2004; Schui et al., 2002). Thus, despite the relative ease of preparation of tumor cell lysates, and the expectation of high-level antigen representation, tumor cell lysates may not be the optimal antigen source for generation anti-tumor immunity. Therefore, isolation of a more defined cellular protein pool (such as heat shock proteins) may be more efficacious. Unfortunately, the preparation of chaperone protein vaccines from a tumor source can be laborious, inefficient, and difficult to standardize since there is no such thing as a "standard" tumor. The methods generally rely on chromatographic procedures followed by analysis of fractions by SDS-PAGE and Western immunoblotting to verify the identity of the proteins throughout the purification process. Specifically, some of these preparative methods include affinity chromatography followed by high performance liquid chromatography (HPLC) (Graner et al., 2000b). Others include ADP-affinity chromatography followed by resolution via fast protein liquid chromatography (FPLC) (Peng et al., 1997). Such purification methods have been utilized for clinical grade preparations of GRP94/gp96 and Hsp70 (Gordon and Clark, 2004; Menoret, 2004; Zabrecky and Sawlivich, 2004). Some success has been achieved using these methods in animal vaccine models and in some human clinical trials although the overall efficacy is still too early to judge (Hoos et al., 2004; Minev, 2003).

Whole-cell and lysate vaccines do possess the entire spectrum of antigenic chaperone proteins, which begged the question, if a single chaperone vaccine is good, could a multi-chaperone vaccine be better? Previous attempts to isolate multiple chaperones from a single tumor source have been published (Menoret, 2004) including those demonstrating vaccine activity for each of the separate chaperones (Graner et al., 2000b). However, these protocols have undoubtedly proven difficult and unruly for clinical specimens, and there do not appear to be any literature references pertaining to the use of combined purified chaperones as anti-tumor vaccines.

The Chaperone Rich Cell Lysate

There is one methodology that seeks to unite the impact of multiple chaperones with the antigen package of tumor lysate, while potentially eliminating immune inhibitory elements within lysate vaccines. One of these is referred to as "chaperone-rich cell lysate", or CRCL, and it is produced by a free-solution isoelectric focusing (FSIEF) technique utilizing a Rotofor™ device from Bio Rad (Bio Rad Laboratories, Inc. Hercules, CA, USA) (Figure 1) (Chen et al., 2006; Graner et al., 2000a; Graner et al., 2003). This procedure uses clarified tumor lysate, subjected it to FS-IEF in a solution of 6M urea, ampholyte/pH carrier molecules, and between 0.1% and 0.5% detergents at 15W power for 5 hours, and analyzes the resulting fractions for chaperone content with particular emphasis on GRP94, Hsp90 and Hsp70, as well as calreticulin. The chaperones did not separate according to their predicted or published isoelectric points (taken from two-dimensional gel electrophoresis), but instead clustered in fractions with a pH range of ~5.0–6.0. Numerous other proteins were found in these fractions as well, so this can only be considered as enrichment for chaperones, and certainly not purification per say. Despite the potentially denaturing conditions employed during FS-IEF preparation of CRCL vaccines, the vaccines were at least

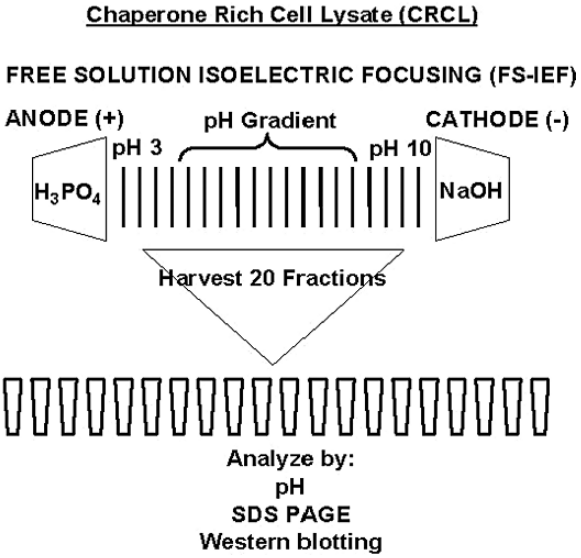

Figure 1. Diagram of Rotofor™ Device for Free Solution-Isoelectric Focusing (FS-IEF) to Generate Chaperone-Rich Cell Lysate (CRCL). The Bio Rad Rotofor™ consists of acid and base compartments at opposite ends of a focusing chamber, which establishes the extremes of the pH gradient. Lysate is loaded into the focusing chamber, which consists of a core of 19 polyester membranes (10 micron porosity) that serves to separate the compartment into 20 fractions. As the pH gradient is established, the membranes serve as barriers to diffusion for large macromolecules once those molecules have reached their isoelectric point under the force of the applied voltage. Once the separation is complete the 20 fractions are harvested into tubes for downstream analyses

as effective (and generally more so) as individually purified chaperones from the same tumor (Chen et al., 2006; Graner et al., 2000a; Graner et al., 2003; Zeng et al., 2003).

In addition, one could obtain between 6–50 times more vaccine material from a unit amount of tumor (1–2 mg CRCL from 1 g tumor) compared to single, or even multiple chaperone purifications, and it could be done in one day. If CRCL was pulsed onto DCs and utilized as a cellular vaccine, the anti-tumor activity was extended to mice bearing pre-existing tumors (Graner and Bigner, 2005; Graner et al., 2003), along with memory responses upon tumor re-challenge. The immunity generated by the vaccine relied on both $CD4^+$ and $CD8^+$ T cells, as determined by antibody depletion of effector cells (Zeng et al., 2003), and CRCL stimulated potent anti-tumor responses in a model bone-marrow transplant setting (Chen et al., 2006).

There is an extensive peptide component in CRCL vaccines; when derived from the 12B1 murine leukemia model, CRCL peptides have been analyzed immunologically for the oncogenic p210 BCR-ABL peptide GFKQSSKAL (Zeng et al., 2005) and with mass spectrometry techniques that putatively identified almost 200 peptides (Graner et al, unpublished data). Among those of potentially high interest in oncology were thymosin β4, transthyretin, glutathione transferase, triosephosphate isomerase, calreticulin, Hsp70, serum albumin, interferon-inducible protein p78, hypothetical protein DJ1186C01.1 (ATM-like), and laminin α−1. "Hits" for these (poly) peptides had high X-correlation values, were singly and multiply charged, and frequently had both b and y ions matched.

While tumor-derived CRCL contains numerous endogenous peptides, recent evidence indicates that exogenous antigenic peptides can be incorporated directly into CRCL (Kislin et al., personal communications). Either by adding peptide to the FS-IEF mixture, or even by simple mixing with "pre-formed" CRCL, this effectively "embeds" the peptides in a stable manner, but in such a way that the peptides are presentable by DCs when the peptide-incorporated CRCL is pulsed onto the APCs. This leads to the stimulation of antigen-specific T cells, and in a vaccine setting, enhanced anti-tumor immunity. Other data (Graner and Davis, personal communications) indicate that whole proteins can be incorporated into the chaperone-containing fractions of CRCL following FS-IEF. The implication is that CRCL may be able to have both an autologous and "designer" repertoire of antigens associated with it, and also raises the possibility of preparing CRCL vaccine in a setting where probable tumor antigens may be known, but there is little or no access to the tumor itself. This would require the preparation of CRCL from either tumor cells grown in culture, or from a "normal" source such as placenta. The CRCL vaccines would then be supplemented with tumor antigens likely to be present in that particular tumor type. CRCL may become a more valuable delivery system as our knowledge grows concerning tumor antigen identification for various cancers.

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CHAPTER 13

HSP70 FAMILY MEMBERS, DANGER SIGNALS AND AUTOIMMUNITY

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Abstract: The 70 kiloDalton family of heat shock proteins (Hsp70) are known to stimulate immune responses and have been increasingly implicated in autoimmune conditions. Hsp70 proteins are present in pathogens as well as in healthy cells. They can be expressed constitutively or elevated in response to heat or other cellular stress. Immune responses stimulated by Hsp70 family members include triggering of innate inflammatory responses, enhancing antigen presentation to self-reactive T cells, and cross priming of chaperoned tumor and other self antigens. In this chapter, we present an overview of immunomodulatory activities described for Hsp70 proteins and review the evidence implicating Hsp70 activity in autoimmunity. The ability of Hsp70 to stimulate anti-self tumor immunity and the prospect of using Hsp70 in vaccines or as adjuvants for cancer immunotherapy will be examined. Finally we discuss potential mechanisms by which Hsp70 proteins act as danger signals and regulatory molecules to the immune system

Keywords: Antigen presenting cells, chaperones, cytotoxic T lymphocytes, innate immunity, immunotherapy

INTRODUCTION

Cells from bacteria to higher mammals respond to various forms of stress by increasing expression of several families of stress-induced proteins (Lindquist and Craig, 1988). Thermal stress, or heat shock, is associated with a well characterized increase in production of the so-called heat shock proteins (HSP). Members of the 70 kiloDalton (kDa) family of HSP include homologous proteins in all forms of bacteria, protozoa, parasites, plants, and animals. This expansive family comprises

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constitutively expressed/slightly stress inducible, as well as regulated/highly stress inducible members, located in several intracellular compartments and exhibiting varying degrees of enzymatic and protein interaction properties.

The eponymous member of the family, Hsp70, has classically been viewed as a molecular "chaperone", binding to exposed hydrophobic segments of thermally denatured proteins, and assisting in protein dis-aggregation and re-folding via hydrolysis of ATP (Georgopoulos and Welch, 1993). However, conservation of these molecules from microbes to man, and the recently identified immunological activities of microbial HSP, purified tumor HSP, and extracellular mammalian Hsp70, has renewed interest in these proteins and their potential role in regulating immune responses and immune pathology (Todryk et al., 2003).

The innate immune system acts as a first line of defense against infections. Innate immunity involves recognition of conserved determinants of pathogens (pathogen associated molecular patterns (PAMPs)) and the initiation of non-specific microbial engulfment, elimination, inflammation, and stimulation of adaptive immune responses (Janeway and Medzhitov, 2002). The recognition of PAMPs occurs via pattern recognition receptors (PRRs) which include members of the Toll-like receptor (TLR) family (Akira et al., 2006). The ability of the innate immune system to identify pathogens via their constituent molecules that differ from the mammalian host is thought to impart the distinction between infectious non-self, requiring elimination, and unresponsiveness to noninfectious self. Failure to restrict adaptive immune activation to foreign antigens due to excessive T cell stimulation, self-antigen cross-reactivity, pathogen mimicry, or breakdown of immunosuppressive tolerance mechanisms, results in self tissue destruction and autoimmune disease (Ohashi and DeFranco, 2002).

Matzinger proposed the "Danger Hypothesis" to account for both autoimmune activation and tolerance to harmless foreign antigens (Matzinger, 1994, 2002). This hypothesis proposes that detection of alarm signals associated with tissue damage or injury should initiate adaptive immune responses. These signals could be components of pathogens themselves, i.e. exogenous danger signals, or signals appearing on host cells in responses to pathogen detection or pathogen-induced host tissue injury, i.e. endogenous danger signals (Gallucci and Matzinger, 2001).

Having striking similarity with bacterial products, increased expression under conditions of cellular stress, and interactions with cell surface receptors, Hsp70 molecules are attractive as multifunctional endogenous danger molecules. The ability of these proteins to associate with peptide substrates, and their exposure to innate and immune effector cells following extracellular release during cellular necrosis, provide robust mechanisms to alert the immune system to threatening environmental conditions. By both triggering innate inflammatory signals, and altering processing of exogenous antigen for cross presentation and stimulation of effector cytotoxic T lymphocytes (CTLs), Hsp70 can act to promote both innate and adaptive immune responses (Srivastava, 2002).

Here we examine the relationship between the Hsp70 family members, describe their immunological properties as danger signals, and review the potential mechanisms by which they can influence autoimmunity and tumor immunity.

Structural Comparison of Members of the Hsp70 Protein Super-Family

The domain arrangement and homology among ten Hsp70 super-family members is shown diagrammatically in Figure 1. The overall domain structure of Hsp70 includes an N-terminal ATPase domain of approximately 385 amino acids (residues 1–384 in Hsp70), a 10 amino acid flexible linker region (residues 385–394 in Hsp70), a peptide binding domain (PBD) of about 115 amino acids (residues 395–510 in Hsp70) an α-helical so-called "lid" region of about 100 amino acids (residues 511–614 in Hsp70), and a flexible C-terminal region of about 30 amino acids (residues 615–642 in Hsp70) containing intracellular retention sequence elements or other protein-protein interaction motifs. Hsp70 members are defined by signature sequence motifs in the ATPase domain, including two regions involved in phosphate ion coordination (DXG sequence) at residues 7–21 and 195–209 in murine Hsp70, and an adenine nucleotide binding motif (residues 333–347 in Hsp70).

Based on X-ray crystal structures (Flaherty et al., 1990; Zhu et al., 1996), including a recently reported structure of the intact "full length" protein, lacking only the extreme 10kDa C-terminus (Jiang et al., 2005), the PBD and ATPase domains are capable of independent folding and are connected via the flexible linker sequence. The orientation of the PBD relative to the ATPase domain is dynamic, with the C-terminal α-helical lid region able to block access to the peptide binding

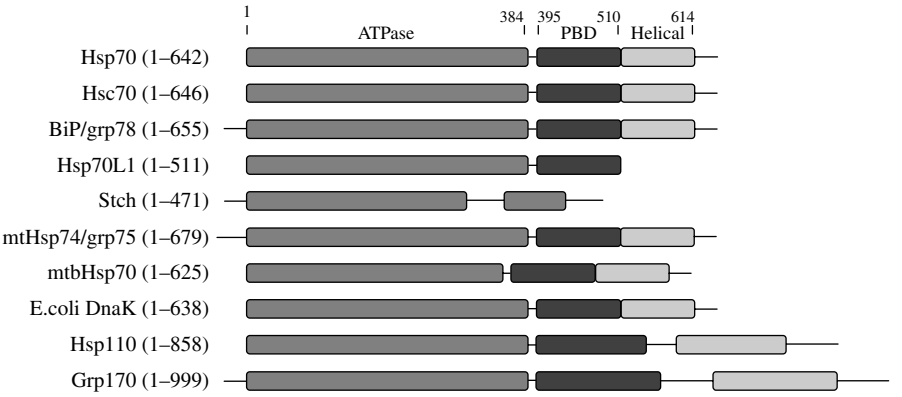

Figure 1. Schematic domain structural alignment of Hsp70 super-family members. Sequence alignment of mouse Hsp70 (Uniprot accession number P17879), Hsc70 (P63017), BiP (P20029), Hsp70L1 (Q60637), stch (Q8BM72), mtHsp74 (P38647), mtbHsp70 (P0A5B9), E. coli DnaK (P0A6Y8), Hsp110 (Q61699), and grp170 (Q9JKR6) was performed to identify conserved domains corresponding to those defined for Hsp70. Details of homologous regions including the ATPase, PBD, and helical regions, are described in the text. PBD, peptide binding domain

groove, and inter-domain communication occurring during rounds of ATP binding, hydrolysis, and ADP release, to alter substrate binding ability (Jiang et al., 2005).

In mice, Hsp70 and Hsc70 share 85% overall identity at the amino acid level, with the immunoglobulin binding protein, BiP/grp78, displaying the next highest homology (60% identical residues). Mitochondrial (mt) Hsp74/grp75 is more closely related to the bacterial DnaK proteins, for example *E. coli* DnaK and *Mycobacterium tuberculosis* (mtb) Hsp70, each sharing approximately 50% sequence identity with Hsp70. More distantly related are a recently reported Hsp70-like protein, Hsp70L1 (Wan et al., 2004), and a microsomal protein termed Stch (Otterson et al., 1994), which each share about 30% identical amino acids with Hsp70. The large molecular weight family members, Hsp110 and grp170, which contain extended domain elements and additional stretches of inserted sequences, nevertheless also display about 30% amino acid identity, when allowing for gaps.

The mammalian Hsp70s are localized to various intracellular compartments. Hsp70 and Hsc70 are mainly cytosolic but can also be associated with intracellular membranes. Hsc70 is the clathrin uncoating ATPase found at the plasma membrane and in coated vesicles, and is also involved in lysosomal protein transport (Terlecky et al., 1992). It is found to shuttle between the cytosol and nucleus and accumulates in the nucleus following heat shock (Tsukahara and Maru, 2004). Endoplasmic reticulum (ER)-targeting leader sequences are present at the N-termini of BiP, grp170 and Stch. BiP and grp170 are retained in the ER lumen by their C-terminal KDEL or NDEL sequences, while Stch lacks such a retention signal and localizes to distal compartments in the secretory pathway (Otterson et al., 1994). MtHsp74/grp75 also contains a pre-sequence which targets it to the mitochondrial matrix.

The novel Hsp70 homologue, Hsp70L1, appears to have small deletions of several stretches within its ATPase domain and lacks the C-terminal helical region (Figure 1). Hsp70L1 contains a D $\rightarrow$ H substitution in the consensus DXG motif of the first phosphate signature region, but retains high sequence similarity in the other signatures, including adenosine binding, second phosphate region, domain connecting and linker regions.

The sequence, structure, and functional relationship between Hsp70 and the large chaperones Hsp110 and grp170 have recently been extensively discussed by Easton and Subjeck (Easton et al., 2000). These family members contain conserved Hsp70 signature motifs, but with altered ATPase and peptide binding activities. Grp170 is stress-induced and glucose regulated and Hsp110 is heat-inducible and primarily localized in the nucleus.

Bacterial Hsp70 family members, including *E. coli* DnaK and *M. tuberculosis* mtbHsp70, also share conserved motifs, functional domains, and strong amino acid similarity with the mammalian proteins. Homology is highest in the N-terminal ATPase domain, with extended and divergent regions in the PBD and lid domains. MtbHsp70 contains a deletion in the ATPase domain, outside the highly conserved signature regions.

Hsp70 Family Members as Danger Signals Implicated in Autoimmunity

Hsp70

Initially, Hsp70 was demonstrated to be a potent chaperone for tumor rejection antigens, capable of activating immune responses against tumors (Udono and Srivastava, 1993). Expression of Hsp70 by tumors was found to confer enhanced immunogenicity and promote tumor rejection (Melcher et al., 1998; Menoret et al., 1995). Chaperoning of tumor associated antigens into the endogenous pathway of MHC class I peptide processing for cross presentation was demonstrated to provide the mechanism by which tumor-derived Hsp70 could promote CTL-mediated anti-tumor immunity (Suto and Srivastava, 1995; Ueda et al., 2004).

Cross presentation of exogenously acquired antigenic epitopes to MHC class I-restricted CD8+ T cells to stimulate CTL effector function is thought to be an important mechanism for generating anti-viral and anti-tumor immunity (Heath et al., 2004). An essential role for HSP, including Hsp70, for promoting cross-priming of CD8+ T cells against cellular antigens was recently identified (Binder and Srivastava, 2005). This study showed cross presentation of epitopes from antigen-expressing cell lines could be prevented by depletion of HSP from the cell lysates. In another study, antigen within cells which contained markedly reduced Hsp70 and Hsp90 (obtained from heat shock factor-1 (HSF-1)-deficient mice), was inefficiently cross presented (Zheng and Li, 2004). These studies suggest that cellular protein interaction with Hsp70, as well as with other HSP, promotes the transfer or processing of antigen into an efficient cross presentation pathway.

Danger Signal Activity of Hsp70

In addition to the ability of Hsp70 to enhance presentation of peptide antigens, subsequent studies have demonstrated that Hsp70 also has the ability to influence the innate immune system by promoting antigen presenting cell (APC) maturation. Danger signal-like activity, including receptor-mediated signaling in APCs, and adjuvant function for eliciting immune responses against non-chaperoned peptides, were reported (Asea et al., 2000a; Breloer et al., 1999; MacAry et al., 2004). Danger signal activity of released HSP were initially proposed by Matzinger as alarm signals that would originate from damaged cells (Matzinger, 1994).

Necrotic release of Hsp70 from tumor cells was shown to enhance cytokine production, APC function, and anti-tumor responses (Melcher et al., 1998; Todryk et al., 1999) and necrotic cell lysates containing Hsp70 were shown to mature dendritic cell (DC) APCs (Basu et al., 2000; Somersan et al., 2001).

The ability of Hsp70 to promote the function of cells of the innate immune system, including stimulation of pro-inflammatory cytokine and chemokine release and non-specific defense responses, have now been widely reported. Purified mouse Hsp70 has been found to stimulate cytokine production from mouse splenocytes

(Asea et al., 2000a) and bone marrow-derived (BM) DCs (Basu et al., 2000; Millar et al., 2003; Moroi et al., 2000). Recombinant human (rh) Hsp70 was also found to activate human monocytes and macrophages (Asea et al., 2000b; Zheng et al., 2004) and to enhance maturation of human monocyte-derived DCs (Kuppner et al., 2001). Both endotoxin-free rhHsp70 and Hsp70/Hsc70 purified from mouse tissues were found to stimulate mouse BMDCs, however with a restricted profile of cytokine production and little changes in surface molecule expression (Millar et al., 2003). The activity of Hsp70 from different species may display considerably different activities on different preparations of APCs i.e. on human monocytes, monocyte-derived DCs, or macrophage cell lines, versus mouse monocyte/macrophage cell lines, peritoneal macrophages, splenic DCs or BMDCs. Significant functional differences between Hsp70 from various sources and their ability to interact with different APC types should be addressed in future studies.

Currently, many potential cell surface molecules have been identified as Hsp70-binding and/or signaling receptors. These include CD14, TLR2, TLR4, CD91, CD40, CD36, LOX-1, and CD94 (reviewed in (Binder et al., 2004)). Participation of PRRs including CD14, TLR2, and TLR4 in mediating Hsp70 signaling provides further compelling evidence for its role as a conserved danger signal (Asea et al., 2002; Mortaz et al., 2006; Vabulas et al., 2002). Furthermore, potential interaction with co-stimulatory receptors, such as CD40 (Becker et al., 2002), scavenger receptors such as LOX-1 (Delneste et al., 2002), and collectins such as CD91 (Basu et al., 2001), suggest additional potential means by which Hsp70 might regulate immune responses to endogenous products.

Some controversy still surrounds the question of whether Hsp70 represents a *bona fide* danger signal capable of innate immune activation via PRRs such as the LPS receptors (CD14/TLR4), or whether these results are artifacts of impure preparations of recombinant Hsp70 containing LPS or other bacterial cell wall products (reviewed in (Bausinger et al., 2002a; Tsan and Gao, 2004)). While many of the studies cited above have employed endotoxin removal methods, controls for protein-dependence (heat or protease-sensitivity), LPS-inactivation (polymyxin B addition), or use of Hsp70 of mammalian origin to confirm intrinsic Hsp70 activity, others have found that pure preparations of Hsp70 fail to elicit cytokine production from APCs (Bausinger et al., 2002b; Gao and Tsan, 2003, 2004). This leaves open the possibility that contaminants, additional co-factors, or other unknown features of the originating HSP or cell types used may be responsible for the observed "danger" activity of Hsp70.

Nevertheless, the properties of Hsp70 to promote or maintain pro-inflammatory innate immune signals and to enhance antigen cross presentation represent the crucial elements that may provide a link between innate and adaptive immunity. Steady state presentation of self antigens in a non-inflamed environment normally results in immunological unresponsiveness or active suppression that maintains self tolerance. Signals that modulate the immuno-stimulatory state of APCs, can determine whether adaptive autoimmune responses are produced (Ehl et al., 1998;

Garza et al., 2000; Waldner et al., 2004). Such an effect of exogenous Hsp70 has been shown to alter anti-self antigen recognition, stimulate T cell activation and trigger autoimmunity. We demonstrated increased inflammatory responses and APC function following administration of Hsp70 to transgenic animals which, together with intravenous soluble self peptide, led to self antigen-specific T cell activation and autoimmune destruction of the antigen expressing tissue (Millar et al., 2003). The finding that Hsp70 stimulated DC cytokine production and enhanced antigen-dependent T cell proliferation suggests that modification of APC function by Hsp70 can convert self antigen presentation into anti-self immunity.

Effects of Hsp70 on Anti-Tumor Immunity

Autoimmune promoting activity of Hsp70 has also been demonstrated to enhance anti-tumor immune responses. Hsp70 released from engineered tumor cells undergoing HSV-tk-mediated cell death, lead to enhanced tumor rejection (Melcher et al., 1998). This enhanced tumor immunogenicity was similarly produced by transfected tumor cells which expressed Hsp70 on the cell surface. Furthermore, local exogenous Hsp70 administered via expression plasmid DNA injection at the site of HSV-tk-induced tumor cell death, produced inflammation and enhanced anti-tumor T cell activation (Daniels et al., 2004). Removal of regulatory T cells (Tregs) further improved anti-tumor immunity and produced autoimmune responses reminiscent of vitiligo (pigment lightening), possibly in response to normal skin/hair cell destruction. This indicates that suppressor T cell-mediated tolerance to self antigen(s) acts to limit both autoimmune and anti-tumor responses. Ren and co-workers (Ren et al., 2004), showed that intra-tumorally-expressed Hsp70, released by suicide-gene-mediated cell death, also stimulated anti-tumor responses mediated by CD4+ and CD8+ T cells, with isolated DCs capable of priming anti-tumor CTLs. These studies indicate that T cell-mediated anti-self tumor immunity can be generated by providing local release of Hsp70 in the milieu of self tumor antigens.

In other studies, Hsp70 purified from chaperone-rich cell lysates (CRCLs) of normal tissue stimulated anti-tumor immunity when co-injected with tumor cell vaccines (Feng et al., 2003). CRCLs from tumor cells were also reported to stimulate anti-tumor immunity (Zeng et al., 2003). CRCLs or purified Hsp70 enhanced CD40 and MHC class II expression on DCs, and activated DC immunostimulatory function. The adjuvant activity of CRCL-derived Hsp70 provides further indication of danger signal activity and ability of Hsp70 to enhance anti-self tumor responses (Zeng et al., 2006).

Recently, secreted murine Hsp70 was shown to exert adjuvant activity for generating class I restricted CTL responses against a tumor associated antigen (TAA) which resulted in a therapeutic decrease in tumor metastases, in vivo (Massa et al., 2005). The effects of secreted Hsp70 included DC maturation and co-stimulatory function, increased apoptotic tumor cell internalization, and

enhanced peptide cross presentation, and resulted in generation of functional CTL against tumor targets. These activities were apparent when the Hsp70 was secreted from tumor cells containing the target TAA, as well as when Hsp70 was secreted from bystander TAA-negative cells. These experiments demonstrated the adjuvant activity of released endogenous Hsp70 for CTL induction, independent of chaperoned antigenic peptide. In this study, the authors demonstrated that enhanced CTL and anti-tumor responses induced by Hsp70 secreted from antigen-expressing tumor cells (chaperone function) was NK cell independent. However, the adjuvant activity of bystander secreted Hsp70, presumably not associated with tumor antigen peptides, required NK cells. Thus, danger activity of released Hsp70 inducing DC maturation may not be sufficient for stimulation of immune responses, but may require further amplification via stimulation of NK cell responses.

Immunization with tumor antigens and Hsp70, either administered together, combined as non-covalently-associated mixtures, as covalently attached fusion proteins, or in DNA vaccines, enhances anti-tumor immune responses and tumor rejection. Srivastava and co-workers (Blachere et al., 1997) elegantly demonstrated the potent ability of Hsp70 to enhance stimulation of CTL responses, in vivo, following reconstitution with an MHC class I epitope peptide. A viral peptide non-covalently complexed with Hsp70 stimulated protective anti-viral immunity when administered without additional adjuvant (Ciupitu et al., 1998). Similar results were found using a novel Hsp70-binding linker peptide system to allow "chaperoning" of non-Hsp70-interacting MHC class I epitopes (Moroi et al., 2000). A DNA vaccine encoding secreted human Hsp70 fused to a papilloma virus-tumor epitope stimulated class I presentation, and was shown to induce antigen-specific antibodies, CTL responses, and confer a high degree of protection from tumor challenge in animals (Hauser et al., 2004).

Recently, Hsp70 and tumor-peptide vaccination has been combined with reagents that block immune suppressive signals to further boost anti-tumor immunity and inhibit cancer progression (Geng et al., 2006). A DNA plasmid expressing soluble PD-1 was used to block interaction with its co-inhibitory binding partner B7-H1. Hsp70-peptide vaccination was greatly augmented, leading to anti-tumor CTLs, and a significant decrease in lung metastases. The potential that other forms of combination therapies may enhance the adjuvant effects of Hsp70 holds promise for the development of more effective cancer immunotherapy.

The interaction between Hsp70 and NK cells via CD94 has been suggested to allow innate activation of the cytolytic function of these cells, and target lysis of tumors expressing Hsp70 on the cell surface (Multhoff et al., 1999). A short 14 amino acid sequence within the PBD of Hsp70 beginning at position 450, termed TKD, was found to exhibit NK cell activating properties (Multhoff et al., 2001). As critical arginine and serine residues in the TKD sequence are not conserved among any of the other Hsp70 family members, this activity appears to be unique to Hsp70. More recently, the presence of surface Hsp70 in tumor-derived exosomes was demonstrated to activate NK cells and stimulate tumor cell killing

(Gastpar et al., 2005). How this additional innate danger activity of Hsp70 might contribute to adaptive immunity against self tumors is not yet clear.

Hsp70 in Autoimmune Disease

The ability of Hsp70 to promote inflammation and the generation of adaptive immunity against cellular, soluble, or HSP-associated self antigens, suggests that aberrant induction or release of Hsp70 may be involved in the initiation or aggravation of autoimmune conditions.

Evidence also suggests that inappropriate HSP expression may promote chronic inflammatory conditions. The synovial fluid of rheumatoid arthritis (RA)-affected joints, has been reported to contain cells which express elevated Hsp70 and exhibited increased HSF activity (Martin et al., 2003). DCs from synovial fluid of RA patients expressed surface Hsp70 and both RA and normal DCs were shown to bind synovial fluid Hsp70 and purified Hsp70 via multiple receptors. Combined with previous studies demonstrating mature and immunostimuatory DCs in the RA synovium (Santiago-Schwarz et al., 2001), this suggests that cell surface Hsp70 in the RA joint may be involved in APC activation, self antigen presentation, and cross priming of auto-reactive T cells associated with chronic joint inflammation.

Hsp70 release from stressed melanocytes has recently been suggested as a contributing factor in progressive vitiligo (Kroll et al., 2005). Melanocytes exposed to 4-tertiary butyl phenol (4-TBP) as a stressor, increased Hsp70 expression and extracellular release. 4-TBP induced TRAIL-R1 and -R2 expression on melanocyes, and HSP-matured DCs expressed TRAIL. 4-TBP-stressed melanocytes were sensitive to activated DC-mediated lysis, and TRAIL-expressing DCs were identified in perilesional vitiligo skin. This study provides novel data on innate DC effector function stimulated by Hsp70, that may contribute to autoimmune melanocyte destruction.

In a murine skin graft model of psoriasis, activated APCs expressing CD91 were identified, along with increased Hsp70 expression in adjacent keratinocytes (Boyman et al., 2005). While highly circumstantial, evidence from this report suggests that this potential pro-inflammatory pathway may be active in psoriatic lesions, and thus may allow targeted therapeutic intervention.

Enhanced MHC class II-restricted presentation of myelin basic protein (MBP), thought to be a target auto-antigen involved in multiple sclerosis (MS), was observed in APCs over-expressing Hsp70 (Mycko et al., 2004). Intracellular Hsp70 associated with MBP and promoted enhanced presentation of MBP-epitopes which stimulated MBP-specific T cell proliferation. The ability of APCs to present MBP peptides was not altered, suggesting that Hsp70 interaction with full length MBP altered processing and generation of antigenic peptides, rather than MHC molecule surface expression. Antisense-mediated Hsp70 depletion markedly diminished MBP presentation. The authors speculated that elevated Hsp70 expression in APCs, promoting processing of immunogenic epitopes from the self antigen, might increase antigen presentation and stimulate auto-reactive T cell immunity in MS. However, it

was subsequently reported that presentation of Hsp70-associated MBP-peptides lead to NK cell-mediated tolerance and protection from experimentally-induced autoimmune encephalitis (Galazka et al., 2006). Thus, enhanced antigen presentation due to Hsp70 activities might not always play an immune stimulating role, but might require additional breakdown of tolerance pathways to lead to autoimmunity.

Hsc70

Heat shock cognate protein, Hsc70, is the constitutively expressed member of the Hsp70 family. Its expression is not induced by the same stress conditions which trigger Hsp70 production, however, other cellular stimuli can increase Hsc70 expression, including hemin, ethanol, and butanol (Fishelson et al., 2001). Despite the high degree of sequence identity between Hsp70 and Hsc70, the minor residue differences appear to impart several strikingly different properties. These include heparin agarose affinity (Menoret and Bell, 2000), temperature- and ATP-dependent aggregation (Angelidis et al., 1999), peptide acquisition under oxidative stress (Callahan et al., 2002) and lipid interactions (Arispe et al., 2002). Differences in the effects of Hsc70 versus Hsp70 on DCs have also been reported (Milani et al., 2002).

Bovine and murine Hsc70 were found to bind to macrophages in a saturable, receptor-mediated manner (Arnold-Schild et al., 1999; Sondermann et al., 2000). Hsc70 binding to APCs could be competed by Hsp70 but not gp96, suggesting that receptors other than the common HSP receptor, CD91 (Basu et al., 2001), interact with 70kDa HSP family members.

In a study of immune rejection of colon carcinoma-derived clones selected under various conditions, Menoret and co-workers observed that expression of Hsc70 did not correlate with tumor immunogenicity, whereas inducible Hsp70 conferred decreased tumorigenicity and increased susceptibility to immune rejection (Menoret et al., 1995). Also, while Hsc70 is a commonly identified component of exosomes (Fevrier and Raposo, 2004; Thery et al., 1999), only the additional presence of surface Hsp70 was described to promote NK cell activation for tumor cell lysis (Gastpar et al., 2005). Immunogenicity of mast cell-derived exosomal antigens occured via CD91-mediated uptake by DCs which were concomitantly matured (Skokos et al., 2003). This is in contrast to a recent report by Clayton et al. (Clayton et al., 2005), who found that B cell-derived exosomes, despite containing lumenal Hsc70, did not trigger DC maturation, even after heat stress induced an increase in Hsp70 content. Clearly additional studies examining the role of Hsp70-members in exosomes will be required to determine the functional capacity of these proteins within the context of extracellular vesicles.

Despite some differences with Hsp70, Hsc70 has been shown to enhance immune responses. Hsc70 fused with a class I epitope stimulated potent CTL responses (Udono et al., 2001). The region 280–385, lacking most of both the ATPase domain and the PBD, was sufficient for this antigen delivery/CTL adjuvant effect. Kammerer and coworkers (Kammerer et al., 2002), demonstrated that association of tumor antigen with Hsc70 promoted acquisition and cross presentation by DC,

resulting in enhanced CTL responses. CD8+ T cell-dependent tumor rejection was observed following challenge with tumor cells containing the Hsc70-associated antigen.

We have found that Hsc70, purified from liver of mice lacking HSF-1 (and thus unable to produce inducible Hsp70), can stimulate partial maturation of mouse BMDCs, including cytokine and surface molecule expression (DG Millar, AR Elford, Z Li, and PS Ohashi, unpublished observation). Also, purified recombinant bovine Hsc70, free of endotoxin, can stimulate IL-12p40 production from murine BMDC (DG Millar unpublished).

Together these findings suggest that Hsc70, abundant in most cells and immediately released by cell damage, may be a functional danger signal to the innate immune system. Determining the full extent of danger signal activity versus antigen delivery function of Hsc70 will require further investigation.

Hsp70-L1

An Hsp70 family member of 55kDa was recently cloned from a human DC cDNA library and designated Hsp70L1 (Wan et al., 2004). Hsp70L1 is 511 amino acids long in mice and highly homologous to Hsp70 in the ATPase domain (residues 1–384), but diverges considerably in the C-terminal region and truncates at the end of the peptide binding β-sandwich domain (Figure 1). Intriguingly, expression of Hsp70L1 is found to be ubiquitous but particularly abundant in lymphoid organs and immune cells. Expression was shown to be inducible by heat shock, as well as increased in DCs following treatment with maturation stimuli such as LPS or CpG DNA. Hsp70L1 was found to bind to DCs, which was only partially competed by Hsp70 and α-2-macroglobulin, suggesting interaction with additional receptors other than CD91. Interaction with TLR2 and TLR4 was suggested by co-precipitation and NFκB-reporter signaling in TLR2 or TLR4/CD14/MD-2 transfected cell lines. DC maturation and Th1 polarization were observed using recombinant Hsp70L1, with increased IL-12p70 production and IL-1β and TNF-α levels similar to those induced with Hsp70.

Adjuvant activity of Hsp70L1 was demonstrated, including co-stimulatory surface molecule up-regulation, inflammatory cytokine and chemokine production from treated DCs, and enhanced T cell stimulation in an MLR assay. Using an Hsp70L1-OVA peptide-fusion protein, enhanced antigen specific CTL responses, and protection from challenge with tumors expressing OVA were generated (Wan et al., 2004). Similar results were subsequently obtained using an Hsp70L1-CEA epitope fusion protein, with immunization in a transgenic mouse system expressing human HLA-A2.2 (Wu et al., 2005). These reports demonstrate both a potent innate danger function of Hsp70L1, contributing to inflammation and APC stimulatory function, together with potential antigenic peptide delivery function, presumably via danger signaling receptor-mediated interaction with DC. The authors postulated that the potent innate DC maturation ability of Hsp70L1, with subsequent Th1 polarization, was responsible for the adjuvant activity of Hsp70L1.

However, a carrier/delivery function of Hsp70L1 was essential, as peptide fusion to Hsp70L1 was critical while administration of epitope peptide mixed with Hsp70L1 was not able to enhance antigen specific immunity or tumor protection.

The sequence determinants within Hsp70L1 that confer this elevated innate and adaptive immune adjuvant function over Hsp70, including the possibility that lack of suppressive elements in the C-terminal region allow increased danger signal activity to be become apparent (see below for mtbHsp70), remain unknown. Clearly, further studies are required to confirm a role for this protein in promoting immunity and anti-tumor immunity, and to examine its potential contribution to autoimmune conditions.

MtbHsp70 and E. coli DnaK

Bacterial Hsp70 homologues, named DnaK in E. coli, have been recognized as multifunctional chaperones and foreign immunogens (Kaufmann, 1990). While the crystal structures of microbial and mammalian Hsp70 PBD and ATPase domains display three-dimensional similarity, most antibodies against Hsp70 do not cross react between eukaryotic and prokaryotic species. However, T cell epitope cross reactivity between bacterial and rodent Hsp70 has been reported (Wendling et al., 2000).

MtbHsp70 appears to function as a conserved molecular pattern to the innate immune system, recognized via TLR2 and TLR4, eliciting NFκB signaling pathways, and leading to release of pro-inflammatory mediators from endothelial cells and professional APCs (Bulut et al., 2005).

The ability of mtbHsp70 to promote antigen presentation and immune responses has been shown by several groups. MtbHsp70 fusion proteins can generate functional CTL responses to model and tumor-associated antigens (Huang et al., 2000; Suzue et al., 1997; Tobian et al., 2004a). The antigen delivery function stimulating CD8+ T cell activation was found to be contained in the distal region of the ATPase domain and was CD4+ T cell independent (Huang et al., 2000). The ability of these constructs to promote innate inflammatory responses was not reported.

A functional interaction between mtbHsp70 and CD40 has been extensively characterized. CD40-dependent triggering of cytokine and chemokine secretion by mtbHsp70 was demonstrated (Wang et al., 2001b), and shown to be mediated by residues 359–610, i.e. via the C-terminal PBD and helical regions of the molecule (Wang et al., 2002b). Subsequently, a minimal stimulating domain was identified within residues 380–499 (MacAry et al., 2004). Using overlapping peptides, a sequence from position 407–426 was found to promote CD40-dependent cytokine production, whereas the sequence from position 457–496 was inhibitory towards full-length mtbHsp70 stimulatory function (Wang et al., 2005). This suggested that a competing peptide region within the protein itself could antagonize the innate signaling activity of mtbHsp70. A single point mutation, V410F, prevented the peptide chaperone function of mtbHsp70, while the cytokine stimulating ability was retained, demonstrating that the signaling function is distinct from peptide binding ability.

While it is not known whether mammalian Hsp70 members contain similar functional sequence stretches within the homologous regions of the PBD, it is interesting to note that Wieland and co-workers (Becker et al., 2002), found that mammalian Hsp70 bound to CD40 in a peptide- and ADP-dependent manner, and that the 44kDa domain alone bound to CD40 in a peptide-independent manner. The presence of extracellular Hsp70-ADP with bound peptide might then be able to provide a CD40-mediated danger signal. Similarly, it will be informative to determine if the naturally occurring Hsp70 family members that lack C-terminal regions, or consist only of the ATPase core i.e. Hsp70L1 and Stch, respectively, also display CD40 binding activity.

E. coli DnaK, was reported to provide a potential innate pro-inflammatory signal during caecal puncture sepsis (Nolan et al., 2004). Enhanced IL-12 production was observed in response to DnaK in a CD40-dependent manner, and mice lacking CD40 had attenuated fatal septic shock. No other reports have examined the activity of E. coli DnaK as a potential ligand of PRRs or TLRs. Despite significant sequence similarity between mtbHsp70 and E. coli DnaK, it is currently unknown whether or not stimulatory and inhibitory sequences have been conserved, or if these proteins share similar danger signal activities, receptor interactions, or antigen-delivery enhancing effects on immune responses.

Hsp110 and Grp170

Hsp110 has also been shown to potentiate the function of the innate and adaptive immune systems in various models. Subjeck and co-workers (Manjili et al., 2005) demonstrated that murine Hsp110 could act as an innate danger signal, stimulating IL-12, IL-6 and TNFα secretion from BMDC, as well as from tumor cell lines. DC maturation, including increased surface CD40 and CD86, was also observed. The participation of CD91 in Hsp110 binding to macrophages provides further confirmation of Hsp110 similarity to the Hsp70 family and indicates that common receptor interaction motifs conserved in this molecule may impart similar adjuvant activity (Facciponte et al., 2005).

Hsp110 purified from tumors was shown to be an effective anti-cancer vaccine in a tumor cell challenge model (Wang et al., 2001a). Furthermore, over-expression of Hsp110 in tumor cells enhanced the immunogenicity and rejection of the tumors (Wang et al., 2002a). Both CD8+ T cells and NK cells were required for anti-tumor responses.

A potent adjuvant activity and ability of Hsp110 to stimulate adaptive autoimmune responses in a transgenic model of spontaneous mammary tumors (FVB-neu), was subsequently reported (Manjili et al., 2003). Hsp110-HER-2/neu complex immunization abrogated tolerance to the Her-2/neu transgene, and prevented tumor development. This ability required CD8+ T cells and was partially dependent on CD4+ cells.

Glucose regulated protein of 170kDa (Grp170), also known as Orp150 due to its regulation by oxygen/hypoxic stress, is also distantly related to the Hsp70

family (Easton et al., 2000). Grp170 purified from tumors was also shown to be an effective anti-cancer vaccine in a tumor cell challenge model (Wang et al., 2001a). Danger signal activity from murine Grp170 was also demonstrated (Manjili et al., 2006). Grp70 bound to BMDCs and up-regulated CD86 and MHC class II on BMDC and increased CD40 expression on both BMDC and a melanoma cell line. Purified endotoxin-free Grp170 stimulated IL-12, IL-6 and TNFα secretion from both BMDC and tumor cell lines. Anti-Orp150 (Grp170) auto-antibodies were reported to be elevated in individuals with type 1 diabetes (Nakatani et al., 2006). A causative or consequential relationship between the auto-antigenicity of this protein and development of diabetes is not yet known.

BiP/Grp78

BiP (immunoglobulin Binding Protein) is an ER-resident protein that functions to bind and chaperone secretory proteins and promote dis-aggregation and proper protein folding and assembly. BiP may also regulate the ER-associated degradation (ERAD) pathway by associating with ER resident sensors which respond to unfolded protein stress by stimulating expression of HSP chaperones and proteolysis of mis-folded proteins (Schroder and Kaufman, 2005). As yet, no danger signaling activity of BiP has been reported.

An association of grp78/BiP with autoimmune disease has been reported. BiP is proposed to be a target auto-antigen in both RA (Bodman-Smith et al., 2004; Corrigall et al., 2001) and auto-antigen spreading associated with anti-Ro(SS-A) autoimmunity (Purcell et al., 2003). In this light, it is interesting that exogenously administered BiP promotes anti-inflammatory cytokines, and regulatory T cell responses (Bodman-Smith et al., 2003; Brownlie et al., 2006; Corrigall et al., 2004). Thus, cross-reactivity or association with pathogenic auto-antigens, or alteration of regulatory responses normally stimulated by BiP, is suggested to be involved in autoimmune disease initiation or progression (Panayi et al., 2004).

HspA12A and HspA12B

Finally we note that two novel distantly related Hsp70 family members, named HspA12A and HspA12B have recently been found to be over-expressed in macrophages in chronically inflamed atherosclerotic lesions, but not in normal blood vessels (Han et al., 2003). Clearly, investigation of cell type-specific Hsp70-related proteins, and their expression in auto-inflammatory situations, may provide further insight into the potential immunoregulatory roles of these proteins.

MECHANISMS OF ALTERED ANTI-SELF IMMUNITY INFLUENCED BY HSP70 PROTEINS

The previously highlighted conserved structural and sequence features of bacterial and mammalian Hsp70 proteins are highly suggestive of potentially overlapping functional properties as innate pathogen-like danger signals or cross-reactive

immunogens. This might have evolved to allow recognition of both foreign Hsp70 as well as extracellularly released endogenous Hsp70 as conditions warranting innate and adaptive immune responses. Recognition could occur via PRRs, such as CD14, TLR2, TLR4, scavenger receptors (CD91, LOX-1), or via other immune signaling receptors, such as CD40.

Recognition of microbial antigens by TLRs was recently shown to promote MHC class II peptide presentation of internalized antigen for activation of CD4+ T cells (Blander and Medzhitov, 2006). The lack of exposed TLR ligands on phagocytosed apoptotic cells lead to the failure of cellular antigens to be processed for epitope presentation. This provides a rationale for how cell surface bound Hsp70 or exosomal Hsp70 may act to adjuvant anti-self immune responses.

Bacterial HSP including Hsp70 are highly immunogenic. Antibody responses can be elicited in serum following soluble Hsp70 injection, even in the absence of other adjuvants (Del Giudice, 1994). Since anti-self Hsp70 antibodies are found in healthy individuals, the relevance of HSP as target auto-antigens has been questioned (Pockley, 2003). While generation of anti-Hsp70 auto-antibodies may not necessarily lead to disease pathogenesis, antibody-Hsp70 interactions may regulate the activity of other immunological functions. Recently, a synergistic effect of Hsp70 and anti-Hsp70 antibody interaction was reported to enhance cytokine release from differentiated monocytic cell lines and adherent PBMCs (Yokota et al., 2006). This effect was shown to be due to Hsp70 cross-linking, since monovalent Fab fragments lacked stimulatory activity. Interestingly, dominant negative TLR4 was found to prevent cytokine responses to both Hsp70 alone, as well as to Hsp70 plus anti-Hsp70. This study demonstrates the potential effects of auto-antibody on Hsp70 activity, and further implicates cross linked Hsp70 structural features which may promote innate signals via the TLR4 pathway.

Recognition of bacterial HSP by the host may be advantageous for several reasons including (a) internalization and processing of HSP for stimulating Th cells, B cells, and anti-HSP neutralising/protective antibodies; (b) stimulating innate inflammatory and non-specific pathogen killing mechanisms (such as phagocyte recruitment, pathogen engulfment, and generation of reactive oxygen species); or (c) enhancing cross presentation of internalized pathogen epitopes for stimulation of CD8+ CTLs and eliminating intracellular bacteria. While all these responses would not be appropriate if directed against self antigens, maintaining regulatory mechanisms toward self and commensal bacterial HSP may ensure that only pathogen-derived HSP provoke an inflammatory milieu, or enhance antigen specific responses.

A shared epitope of Hsp70 and mtbHsp70 was shown to stimulate T cells that produced IL-10. In adjuvant-induced arthritis, a model of RA which targets HSP in inflamed joints, prior intra-nasal immunization with the shared epitope of Hsp70 attenuated arthritis induction (van Eden et al., 2000; Wendling et al., 2000). Thus, immunosuppressive anti-HSP responses that arise during mucosal exposure to microbial flora may normally act to prevent anti-HSP directed autoimmunity,

and prevent HSP danger signal-induced chronic inflammation and pathology. When mechanisms of mucosal tolerance break down, the immunogenic potential of Hsp70 and Hsp70-associated molecules may emerge.

Concluding Remarks

In summary, the extracellular release of members of the Hsp70 family can promote autoimmunity. Multiple effects on immune cell function, either via innate signals provided by Hsp70 family members, or by adaptive responses induced against HSP-associated peptides or directed towards Hsp70 members themselves have been described. The putative consequences of damage-induced release of tissue Hsp70s on chronic inflammation, self-antigen-specific immunity, and autoimmune disease induction are depicted in Figure 2.

Via interaction with PRRs or other danger signal receptors, Hsp70 family members can stimulate inflammation at sites of tissue stress or injury and initiate innate immune responses, possibly in conjunction with other released endogenous danger signals (Figure 2a). Self antigens released in the presence of extracellular Hsp70 may be subject to enhanced APC uptake, processing, and presentation (Wang et al., 2006). Chaperoned antigenic self peptides associated with Hsp70 could bind to APCs via Hsp70 receptors, leading to increased concentrations of peptide presented to self reactive T cells.

The consequences of danger signals and inflammatory mediators would be to stimulate self-antigen presentation by matured DCs with enhanced APC function

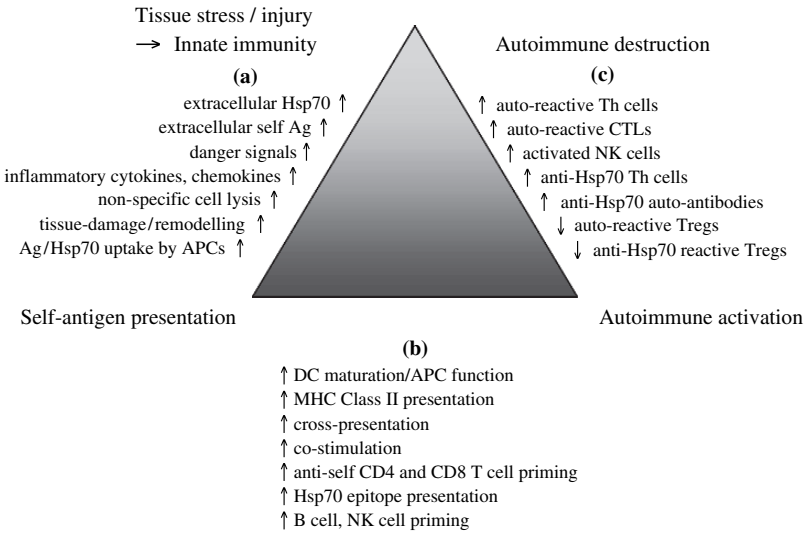

Figure 2. Putative effects of extracellular Hsp70 on innate immune responses, self antigen presentation, and autoimmunity. See text for details. Ag, antigen; Th, helper T cell; ↑ increased; ↓ decreased

and co-stimulation and, if not countered by regulatory mechanisms, this could activate auto-reactive T cells (Figure 2b). Chaperoned antigen could be trafficked into a pathway of enhanced cross presentation e.g. delivered into the cytosol, or to a "presentosome" (Srivastava, 2002), allowing stimulation of both CD4 + and CD8+ T cells. Immunogenicity of Hsp70, combined with other danger signals, may alter non-inflammatory responses to itself, and stimulate T cell and B cell responses directed at the stress proteins.

Released Hsp70 may prevent the anti-self suppressive function of regulatory T cells, either by the inflammatory danger signals produced (Pasare and Medzhitov, 2003) or by promoting self antigen presentation that activates auto-reactive helper T cells (Haug et al., 2005; Tobian et al., 2004b), resulting in auto-reactive CTL effector cells (Figure 2c). Autoimmune activation may be further enhanced by immune complexes containing anti-Hsp70 auto-antibodies. The presence of extracellular Hsp70 might also block anti-inflammatory clearance and cross tolerance of apoptotic cellular debris, via interaction with lipids, lipid-binding proteins, or other receptors. Furthermore, tissue-expressed or extracellular Hsp70 could become a target at sites of initial injury for anti-Hsp70-reactive T cells or auto-antibodies, perpetuating chronic inflammation and encouraging autoimmune destruction.

It can be seen that Hsp70 molecules may be involved at multiple stages of an auto-inflammatory loop. Identifying the range of immuno-modulatory properties of each member of the Hsp70 family of molecules will hopefully shed more light on the regulation of anti-self immunity and the triggers of autoimmune disease.

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CHAPTER 14

THE IMMUNE RESPONSE UNDER STRESS: CLASS I HLA PRESENTATION OF HOST-DERIVED PEPTIDES

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Abstract: Major histocompatibility complex (MHC) class I molecules are found at the surface of all nucleated cells. Class I molecules function as immune sentries by scanning the intracellular proteome and then reflecting the proteome at the cell surface. Through class I presented peptides, T lymphocytes and other immune effector cells can continuously survey the intracellular proteome. Viral infection and cancerous transformation results in the presentation of peptides not found on healthy cells. Class I presented peptides therefore act to distinguish infected and cancerous cells in the eyes of the immune response. Here, we review how class I molecules reflect host cell stress resulting from infection and cancerous transformation. Class I molecules display peptides derived from heat shock proteins on both cancerous and virus-infected cells, and these peptides are clearly recognized by the immune response. The class I of diseased cells also reveal less obvious stress-related signals: Peptide fragments of proteins involved in cell homeostasis act to distinguish infected or cancerous cells. Finally, peptides derived from particular host proteins act as broad indicators of cellular stress, distinguishing both cancerous and virus-infected cells. These class I presented peptides are positioned to influence adaptive and innate immune responses to cellular stress

Keywords: HLA class I molecule; MHC class I molecule; stress; virus; cancer; heat shock protein

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INTRODUCTION

The Role of MHC Class I in Immune Responses

The major histocompatibility complex (MHC) class I molecule is a membrane-bound glycoprotein located on the surface of all nucleated cells. The MHC class I molecule in humans, termed HLA (human leukocyte antigen), is a heterotrimer consisting of a 45kDa heavy chain (α-chain) that is anchored to the plasma membrane. The second component of this complex is a 12kDa light chain (β_2-microglobulin) that is noncovalently associated with the α-chain. When associated with the β-chain, the class I HLA α-chain forms a peptide-binding groove comprised of two alpha helices and eight beta sheets. This groove then accommodates the third component of the heterotrimeric complex; endogenously loaded peptides of 8–12 amino acids in length (Terhorst et al., 1976).

Assembly of the HLA class I trimeric complex in the endoplasmic reticulum (ER) begins with the co-translational insertion and association of the α-chain with the chaperone calnexin. Non-covalent binding of the light chain (β_2m) to the α-chain releases calnexin and the α : β_2m complex further associates with a complex of chaperone proteins (calreticulin, Erp57) and the transporter associated with processing (TAP) via tapasin (Shastri et al., 2002). The α : β_2m complex is now prepared to associate with peptide-precursors that are made available by several mechanisms. The majority of class I associated peptides result from cytosolic degradation of ubiquitin-tagged peptide-precursors by the cytosolic proteasome, ATP dependent transport into the ER, and N-terminal peptide trimming by an ER-resident aminopeptidase (ERAP) (Saric et al., 2002; Serwold et al., 2002). Peptide loaded trimeric class I complexes then traffic through the golgi to the cell surface where they are inspected by T lymphocytes.

Inspection of cell surface class I trimeric complexes by T lymphocytes takes place through the T-cell receptor (TCR) of thymus derived CD8+ cytotoxic T lymphocytes (CTL). The T-cell receptor of a CTL is formed by an α-chain and a β-chain, each containing a variable domain with three complementary determining regions (CDR) and a constant domain (Chothia et al., 1988). The variable regions of the TCR determine the receptor specificity for class I HLA peptide complexes. The CDR1 and CDR2 loops of the TCR α-chain closely contact the β-helices of the MHC class I molecule near the amino terminus of the bound peptide while the CDR1 and CDR2 loops of the TCR β-chain associate with the c-terminus of the bound peptide where it contacts with the MHC. The CDR3 loops of the TCR α-chain and β-chain contact the central portion of the bound peptide (Garcia et al., 1998). During an adaptive immune response, CTL recognize target cells expressing a specific peptide:MHC class I complex and then destroy targeted cells with pre-formed granzymes and secreted cytokines (IFN-γ, and TNF-α) (Krahenbuhl et al., 1988; Sandberg et al., 2001).

Class I Molecules – Nature's Proteome Scanning Chips

Class I MHC molecules fill a unique immunologic niche – they provide CTL with a proteome status report in regards to intracellular fitness. The class I molecules

of a healthy cell are loaded with peptides indicative of a normal, or "healthy", proteome. However, cancerous transformation or infection by an intracellular pathogen changes the proteome of the host cell, and class I molecules reflect such "unhealthy" changes through constant sampling and presentation of endogenously derived intracellular peptides (Figure 1). Nature has therefore placed class I proteome scanning chips on all nucleated cells, and T lymphocytes scan these chips to detect, and then eliminate, intracellular pathogens and cancerous cells.

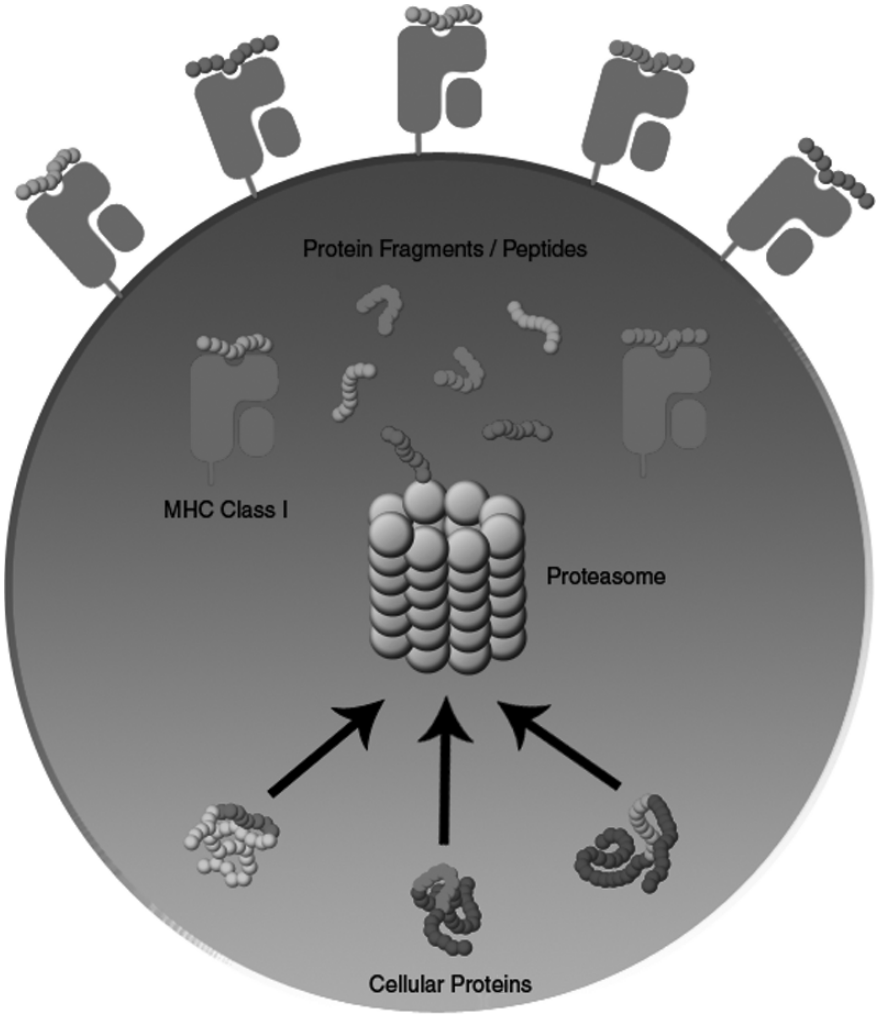

Figure 1. MHC class I molecules present endogenously loaded peptides on the surface of all nucleated cells. Changes in the host proteome are mimicked by the class I molecule and convey intracellular fitness to surveying immune cells. The unique nature class I peptide presentation posits the MHC class I molecule as a broad indicator of cell stress

A solid understanding of the peptides that distinguish the infected or cancerous cell from the healthy cell is positioned to guide the development of diagnostics and therapeutics specific for unhealthy cells. However, methods for visualizing the peptides that mark a cell as cancerous or infected are predominantly indirect and provide an incomplete view of class I peptides. For example, immune response assays demonstrate that CTL recognize virus-encoded peptides in the class I of infected cells. Such *in vitro* immune assays detect peptide-class I complexes using T cells specific for a given peptide, yet these assays do not indicate the breadth and nature of epitopes available for immune targeting. More direct assessments of the nature and number of class I presented peptides unique to cancerous and infected cells are therefore situated to reveal how class I molecules indicate stress through proteome scanning.

Class I molecules are proteome scanning chips, and the empiric challenge in directly identifying the peptides unique to unhealthy cells is one of protein quantity and purification. It is experimentally challenging to obtain sufficient, native, class I protein and peptide for downstream proteomics analyses. To enable such analyses, we have previously described methods whereby milligram quantities of class I can be gathered from virus infected cell lines, and we continue to utilize this approach to characterize class I peptide epitopes unique to cancerous and infected cells. Here we report on how class I molecules reflect the stress of virus infections and cancerous transformation through their proteome scan and subsequent presentation of peptides.

VIRUS INDUCED CHANGES IN MHC CLASS I PRESENTATION OF HOST-DERIVED PEPTIDES

The MHC Class I Molecule and Virus Associated Stress

Viral infection imposes stress upon infected cells resulting in up-regulation of host anti-viral factors and modification of host proteins involved in successful viral replication. Up-regulation and interaction with host-derived proteins during viral infection translates into an altered repertoire of host-derived ligands displayed by the MHC class I molecules on the surface of infected cells. Infection with HIV-1, measles virus, and influenza virus are all known to alter the presentation of host peptides by the class I molecules of infected cells. Host-derived peptides altered during infection have roles in the cell stress response (i.e. heat shock proteins), viral replication, and many as yet unknown cellular and viral functions. While it is recognized that altered MHC self-peptides are recognized by the immune system during periods of stress, the immunologic role of class I presented stress peptides remains largely unexplored. We will therefore focus our discussion of virus induced changes in class I self-derived peptide presentation on (1) the nature and breadth of the altered peptides and (2) the known and putative immunologic impact of these stress induced changes.

Acknowledged Stress Related Changes: The Heat Shock Proteins

Heat shock proteins (HSP), the most abundant intracellular proteins, increase in concentration from 5% to over 15% of total intracellular protein during cell stress (Srivastava, 2002a). They have several diverse physiologic functions including protein content folding, unfolding, degradation, trafficking, and assembly of polypeptide complexes (Frydman, 2001; Gradi et al., 1998; Srivastava, 2002b). Several immune response roles are also attributed to HSP. Heat shock proteins including Hsp70, Hsp90, and gp96 have been shown to bind and shuttle antigenic peptides to the MHC class I presentation pathway in professional antigen-presenting cells (APC), evoking potent anti-viral and anti-tumor responses (Srivastava, 2002b). In addition to peptide chaperoning, HSP found in the extracellular milieu signal danger and activate APC to release inflammatory cytokines (TNF-α, IL-1β, IL-12, and GM-CSF) and chemokines (MCP-1, MCP-2, and RANTES) which promote dendritic cell maturation. Possible receptors for HSP on the surface of APC include CD91, TLR4, CD36, and CD40 (Srivastava, 2002a). Heat shock proteins such as Hsp70 can also be recognized by CTL and natural killer (NK) cells as whole antigens on the surface of stressed cells (Bauer et al., 1999; Botzler et al., 1998; Multhoff and Botzler, 1998; Steinle et al., 2001).

In addition to the aforementioned roles of heat shock proteins during the immune response, HSP serve not only as molecular chaperones of MHC class I molecule presented peptides but can become peptide cargo themselves. Twenty-five HSP-derived peptides presented by four HLA-A, six HLA-B, and one HLA-C molecule have been identified through a variety of techniques illustrated in Table 1. No class I molecule or locus is solely responsible for HSP peptide presentation and HSP-derived peptides are not limited to a single cell line. The peptides identified have been directly isolated from at least eight different immortalized cell lines. The peptides are derived from seven different HSP precursor proteins and the two most often presented HSP are Hsp60 and Hsp90, each encoding eight HLA class I ligands. Class I MHC molecules consistently indicate stress through the presentation of HSP-derived peptides.

Heat shock protein production is amplified during a variety of cellular stresses including viral infection. Therefore, an increase in intracellular HSP concentration may lead to increased MHC class I presentation of HSP-derived peptides. Infection of immortalized human T cells expressing HLA B*0702 with HIV increases intracellular levels of Hsp27. These increased intracellular levels of Hsp27 lead to increased presentation of the Hsp27-derived peptide APAYSRAL by the HLA B*0702 class I molecule (Hickman et al., 2003). The Hsp90-derived HLA B*1801 ligand EEVETFAF has also been observed following HIV infection (H.D. Hickman-Miller and W.H. Hildebrand, unpublished). Increased class I presentation of HSP-derived peptides is not limited to cells stressed through HIV infection. Hsp90β production is increased in HLA A*0201 homozygous cells following Epstein-Barr virus transformation while the class I HLA of melanoma cell line MelJuSo also present Hsp90β peptides when infected with measles virus. Increased Hsp90β production in measles infected cells results in increased presentation of the Hsp90β-derived

Table 1. MHC Class I Presentation of Heat Shock Protein-Derived Peptides

Source protein	Peptide	HLA molecule	Cell line identified on	Identification method	Ref
HSP27	APAYSRAL	B*0702	Sup-T1	Mass Spectrometry	[43]
HSP27	NEITIPVTF	B*1801	721.221	Mass Spectrometry	[44]
HSP27	AAPAYSRAL	Cw*0102	721.221	Mass Spectrometry	[6]
HSP27-like	GEDIVADSV	B*1801	721.221	Mass Spectrometry	[44]
HSP47	DEKEKLQLV	B18	HP-62	Mass Spectrometry	[67]
HSP60	DELEIIEGM	B*1801	721.221	Mass Spectrometry	[44]
HSP60	DELEIIEGMKF	B*1801	721.221	Mass Spectrometry	[44]
HSP60	IRRGVMLAV	B*27	Not applicable	Synthetic Peptide	[67]
HSP60	KRIQEIIEQ	B*27	Not applicable	Synthetic Peptide	[67]
HSP60	KRTLKIPAM	B*27	Not applicable	Synthetic Peptide	[67]
HSP60	QMRPVSRVL	E*01033	Not applicable	Synthetic Peptide	[60]
HSP70	VFDAKRLIGR	Aw68	LG-2	Amino acid analysis	[40]
HSP70	EYGETKSF	B*4601	Not applicable	Synthetic Peptide	[5]
HSP70	FDNRMVNHF	B*4601	Not applicable	Synthetic Peptide	[5]
HSP70	KMKEIAEAY	B*4601	721.221	Mass Spectrometry	[6]
HSP70	LLDVAPLSL	A*0201	Not applicable	Synthetic Peptide	[25]
HSP70	LLLLDVAPL	A*0201	Not applicable	Synthetic Peptide	[25]
HSP90-α	RRIKEIVKK	B27	LG-2	Amino acid analysis	[48]
HSP90-β	ILDKKVEKV	A*0201	BLCL BSM	Mass Spectrometry	[42]
HSP90-β	KEKYIDQEEL	A*2902	BLCL Sweig	Mass Spectrometry	[11]
HSP90-β	IHEDSTNRRRL	B*1501	721.221	Mass Spectrometry	[69]
HSP90-β	EEVETFAF	B*1801	721.221; Sup-T1	Mass Spectrometry	[44]
HSP90-β	RRVKEVVKK	B27	LG-2	Amino acid analysis	[48]
HSP90	AVPDEIPPL	A*0205	C1R	Mass Spectrometry	[7]
HSP90	AEDKENYKKF	B*4403	C1R	Mass Spectrometry	[30]
HSP110/105	EEDLEDKNNF	B*1801	721.221	Mass Spectrometry	[44]

HLA A*0201 ligand ILDKKVEKV (Hsp90β570–578. In addition, Hsp90β570–578 specific CD8+ T cells have been identified in HLA A*0201 positive patients infected with measles virus (Herberts et al., 2003). These studies indicate that class I molecules present HSP derived ligands, and such presentation can be increased upon virus-associated cell stress.

The data referenced above show that the class I HLA presentation of HSP derived peptide epitopes is positioned to enhance immune responses to intracellular pathogens in a variety of ways. It will be interesting to learn whether HSP derived peptides primarily influence the adaptive recognition of virus-derived and cancer specific targets, or whether HSP peptides themselves tend to serve as adaptive immune targets. Will HSP derived peptide epitopes consistently fall at one point in the innate-to-adaptive immune spectrum? If HSP-derived peptides represent adaptive immune targets, it is plausible that altered class I presentation of these peptides during viral infection, combined with co-stimulatory signals, can break the tolerance of autoreactive CTL specific for HSP-derived ligands and lead to autoimmunity.

Additional Stress Related Changes

In addition to acknowledged stress related changes (i.e. heat shock proteins), viral infection induces many non-classical stress related changes in host proteins which translates into an altered self proteome. Characterization of up-regulated and novel host-derived MHC class I peptides eluted from HIV-1 and Influenza A infected cells has provided a unique window into virus-host interactions during infection. In addition to the classical stress-induced response, a virus infected cell can initiate non-specific (IFN-α and IFN-β) and specific viral defense mechanisms to counter viral replication. Increased presentation by the MHC class I molecule of peptides derived from anti-viral proteins is observed during HIV-1 infection and is primarily due to increased protein degradation. During infection, viral proteins may directly influence the presentation of host-derived MHC class I ligands by manipulating and/or attacking normal cellular processes to obtain successful replication (genome duplication, transcription and translation of viral proteins, and assembly and release of new progeny virions). Disruptions in cellular transcription, translation, protein regulation, motility and adhesion, and growth and differentiation during HIV-1 and Influenza A infection are reflected by the repertoire of host-derived peptides presented by the MHC class I molecule during infection.

Virus associated changes in the host cell reflected by class I HLA fall into several categories (Table 2). Many host-derived peptides identified as up-regulated by class I during viral infection are involved in transcription and translation. As two examples of these stress related changes, we will focus on eIF4G, a component of the cap-dependent translation pathway and often manipulated during viral infection, and TAR binding protein, an inhibitor of HIV-1 TAT mediated transcription.

Elongation Initiation Factor 4G

Inside the infected cell, viral mRNA must compete with host mRNA for access to ribosomes and subsequent translation of viral protein products. During 5' cap dependent initiation of protein translation, mRNA forms a "translation complex" with elongation initiation factors, eIF4A, eIF4E, eIF4F, eIF4G, eIF3, and ribosomal subunits. eIF4G acts as a scaffold during the initiation of translation and contains binding sites for eIF4A, eIF4E, and eIF3 (Gradi et al., 1998). Viruses have developed various strategies to promote viral protein production by inhibiting host mRNA translation including: dephosphorylation and subsequent inactivation of eIF4E (Adenovirus and Influenza virus) (Feigenblum and Schneider, 1996; Zhang et al., 1994), dephosphorylation of 4E-bp1 facilitating tight binding to and sequestering of eIF4E (Poliovirus and Encephalomyocarditis virus) (Feigenblum and Schneider, 1996; Gingras and Sonenberg, 1997; Gingras et al., 1996), cleavage of eIF4G by viral proteases (HIV, Poliovirus, and Foot-and-mouth disease virus) (Gradi et al., 1998; Haghighat et al., 1996; Ohlmann et al., 1996; Ventoso et al., 2001), and binding/sequestering eIF4G (Influenza virus) (Aragon et al., 2000). Given our understanding of class I molecule peptide presentation, proteins dramatically

Table 2. Non-Classical Stress Related Changes During Viral Infection

Function	Peptide-derived host protein	Virus associated	Ref
	RNA polymerase II	HIV-1	[43]
	Polypyrimidine tract binding protein 1	HIV-1	[43]
	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1	HIV-1	[43]
	snRNP polypeptide G	HIV-1	*
Transcription	hnRNP C1/C2	HIV-1	*
	STAT-1	HIV-1	*
	TAR DNA binding protein 43	HIV-1	*
	PI-3 kinase related kinase	Influenza A	**
	TATA binding protein interacting protein	Influenza A	**
	Splicing factor 3B subunit 3	Influenza A	**
	eIF4G (elongation initiation factor 4G)	HIV-1, Influenza A	[43], **
	Nuclear pore complex protein NUP133	HIV-1	*
Translation	poly(rc) binding protein 2	HIV-1	*
	TAP (Tip-associated protein)	HIV-1	*
	hnRPL	Influenza A	**
	RPL8 protein	Influenza A	**
	Ubiquitin specific protease 3	HIV-1	[43]
	Ubiquitin specific protease 22	HIV-1	*
	E6 Binding Protein	HIV-1	[43]
Protein Regulation	Ubiquitin conjugating enzyme 1	Influenza A	**
	BAG family molecular chaperone regulator 3	Influenza A	**
	Tripeptidyl peptidase II	Influenza A	**
	TCP-1 (T complex polypeptide 1)	HIV-1	[43]
	Talin	HIV-1	*
	Vinculin	HIV-1	[24]
Cell Motility and Adhesion	Dynein	HIV-1, Influenza A	*, **
	Spark-like protein	HIV-1	[43]
	Tenascin C	HIV-1	[43]
	Set binding factor	HIV-1	[43]
Cell Growth and Differentiation	MgcRacGap	HIV-1	[43]
	p68 RNA helicase	HIV-1	*

* (H.D. Hickman-Miller and W.H. Hildebrand, unpublished)
** (A. Wahl and W.H. Hildebrand, unpublished)

modified (i.e. cleaved) during viral infection should lead to their increased presentation as peptides by the class I molecule.

During HIV-1 infection, the HIV protease (PR) cleaves eIF4G into three fragments creating an N-terminal domain (140 kDa), a central domain (102 kDa), and a C-terminal domain (57 kDa). Cleavage inhibits cap-dependent protein translation of host mRNA while viral mRNA is selectively translated by its internal ribosome entry site (IRES) (Ventoso et al., 2001). Two eIF4G-derived peptides have now been eluted directly from the class I of a HIV-1 infected immortalized human T cell line. The first is a ten amino acid HLA-B*0702 ligand (AARPATSTL) (Hickman et al., 2003) and the second a nine amino acid HLAA*0201 ligand (VLMTEDIKL) (H.D Hickman-Miller and W.H. Hildebrand unpublished).

The influenza virus non-structural (NS) protein inhibits cap-dependent translation of host mRNA and promotes viral protein synthesis. The NS protein binds residues 157–550 of eIF4G and the 5' untranslated region (UTR) of viral mRNA supporting the model that influenza NS protein recruits eIF4G to the 5' UTR of viral mRNA resulting in preferential translation of viral proteins (Aragon et al., 2000). One HLA-B*0702 eIF4G-derived ligand up-regulated on the surface of Influenza A PR8 infected HeLa cells has been identified (A Wahl and W.H. Hildebrand unpublished) and sequenced by mass spectrometry and is located just c-terminally from the NS protein binding site. Although the influenza virus lacks a viral encoded protease, modification of eIF4G by NS protein binding may contribute to the increased presentation of eIF4G-derived peptides by class I molecules by an unknown mechanism.

During viral infection, both HIV and influenza virus modify eIF4G to gain selective translation of viral mRNA at the expense of host protein synthesis. Alterations in eIF4G give rise to up-regulation of eIF4G-derived peptides by the class I molecule suggesting its increased presentation during infection with other viruses that modify eIF4G (poliovirus and foot and mouth disease virus). We posit that class I presentation of eIF4G derived peptides is a broad immune indicator of virus induced stress.

TAR-DNA Binding Protein

Virus infected cells can counter virus replication with non-specific mechanisms such as IFN-α and IFN-β production and through host derived virus-specific proteins. Type I interferons IFN-α and IFN-β shut down cap-dependent and cap-independent protein translation by inducing gene expression of protein kinase, RNA-activated (Pkr). Pkr phosphorylates and subsequently inhibits eIF2α upon binding viral dsRNA, halting translation of cellular and viral mRNA. In addition, some viruses can be inhibited directly by specific anti-viral host-derived proteins. One such protein, TAR DNA binding protein 43Kda, (TDP-43) binds pyrimidine rich elements in the TAR region of HIV-1 proviral DNA and decreases viral gene expression (Ou et al., 1995). HIV-1 transcription elongation is regulated by the virally encoded protein Tat which binds the regulatory sequence TAR located in the 5' long terminal repeat region of the HIV-1 genome. TAR RNA forms a stable stem-loop structure that

serves as the site of initiation of HIV-1 proviral genome transcription by RNA polymerase II. TDP-43, a host-derived protein, binds TAR DNA and is positioned to inhibit initiation HIV-1 proviral DNA transcription creating a steric block through which RNA polymerase II and general transcription factors such as TATA binding protein (TBP) cannot proceed (Figure 2). TDP-43 decreases HIV-1 gene expression 20-fold and represents a novel cellular protein that is able to inhibit the replication cycle of a specific virus (Ou et al., 1995).

It has not been determined if HIV-1 infection influences TDP-43 gene expression or if HIV-1 counters inhibition of viral DNA transcription by modulating TDP-43. The nature of class I peptide presentation dictates that increased gene expression and/or modulation of TDP-43 should result in increased MHC class I peptide presentation. Mass spectrometric analysis of class I molecules collected from HIV-1 infected immortalized T-cells has identified increased presentation of a TDP-43 derived HLA A*0201 ligand (DLIIKGISV) during HIV-1 infection of immortalized T-cells (H.D Hickman-Miller and W.H. Hildebrand unpublished). It is possible that increased class I presentation of TDP-43 is the result of increased TDP-43 gene expression during HIV-1 infection and hence increased degradation by the proteasome or the consequence of it's cleavage by the HIV-1 protease.

During an active replication cycle, viruses often hijack and manipulate normal cell processes to quickly produce and release progeny virions before surveying CTL can recognize and destroy infected cells. The stress of viral replication upon the host cell elicits both classical and non-classical stress induced changes in host

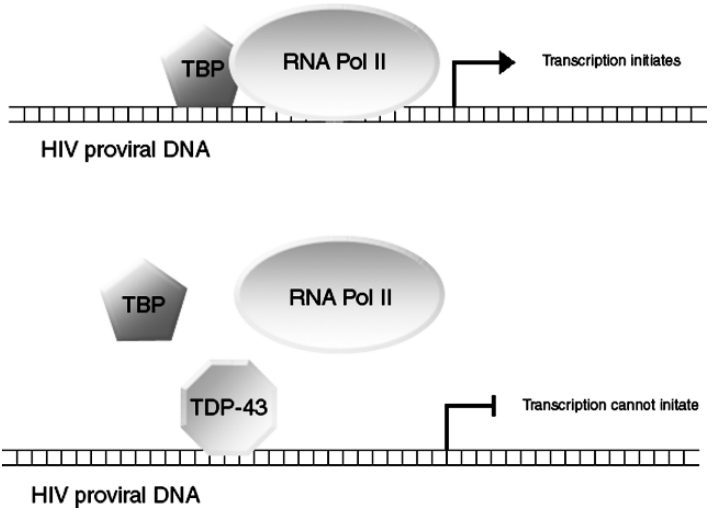

Figure 2. TAR-DNA binding protein 43Kda (TDP-43) inhibition of HIV-1 proviral DNA transcription. A. In absence of TDP-43 initiators of HIV-1 transciption, TATA binding protein (TBP) and RNA polymerase II, are able to interact with proviral HIV-1 DNA. B. Binding of TDP-43 to pyrimidine rich regions in proviral HIV-1 DNA sterically hinder binding of TBP and RNA polymerase II resulting in decreased transcription

proteins resulting in an altered self proteome that is reflected on the cell surface by the MHC class I molecule. It is plausible that increased presentation of self-derived MHC class I ligands during viral infection, combined with increased co-stimulation could stimulate autoreactive CTL leading to autoimmunity. In support of this model, increased presentation of a Hsp90β-derived peptide during measles infection by HLA-A*0201 triggers activation of Hsp90β autoreactive CTL (Herberts et al., 2003), and may account for infection's association with autoimmune diseases including systemic lupus erythematosus (Alekberova et al., 1975) and multiple sclerosis (ter Meulen and Liebert, 1993). Therefore, it is conceivable that increased presentation of self-peptides during viral infection may harm the host through initiation of autoimmunity.

TUMOR INDUCED CHANGES IN MHC CLASS I PRESENTATION OF HOST-DERIVED PEPTIDES

The MHC Class I Molecule and Tumor Associated Stress

Tumor cells experience a number of cellular stresses that translate into genetic mutations and altered gene expression and protein processing. Mutations in proteins involved in cell signaling, tumor suppression and DNA repair can lead to constitutive signaling, abnormal cell proliferation, and accumulation of numerous mutations resulting in genome wide instability. Genetic instability is detrimental to any cell but particular levels of genetic instability seem to impart a selective advantage to tumor cells (Cahill et al., 1999). In addition tumors often experience nutrient deprivation, hypoxia and oxidative stresses as they grow larger. To counteract these stresses, cancerous cells upregulate the production of proteins that induce angiogenesis, such as Her2/neu (Konecny et al., 2004), protect the cells from reactive oxygen species and inhibit apoptosis, such as the inducible heme oxygenase-1 (Mayerhofer et al., 2004).

Additionally, tumors display classical or universal stress responses that are easily identifiable with other disease states, such as viral infection. Indeed, a number of cancers are induced by viral infection by such agents as Epstein Barr Virus and Human Papilloma Viruses. Universal stress induced responses identified in tumor cells include up-regulation of MHC class I, Hsp72, and the NK cell ligands MHC-like MICA and MICB. Interestingly, these common stress induced genes are located within the MHC class I loci of chromosome 6 (Collins, 2004). In addition, many transcriptional regulators and translational regulators are over-expressed by both tumors and virally infected cells, including Stat-1, p68 and eIF4G (Abbas et al., 2000; Bates et al., 2005; Clemens, 2004).

Tumor cells can exhibit a number of stress induced changes in their genetic code, gene expression, and processing of proteins. These changes within a tumor cell can create novel and unrealized MHC class I ligands to CTL that survey surrounding tissue for "unhealthy" cells. Stress-induced class I ligands have been associated with a variety of tumors and their role in the immune response against

tumors is being actively explored. In addition, many stress related changes in class I presentation of self peptides observed in tumors cells are mirrored by virus infected cells. We will therefore focus our discussion of tumor induced changes in MHC class I presentation on characterizing (1) tumor-derived class I peptides associated with mutation and alterations in gene expression and (2) universal stress induced class I ligands found in both cancer and viral infections.

Mutation Induced Stress Related Changes

Unique tumor antigens, or neo-antigens, are presented when point mutations occur in gene coding regions of ubiquitously expressed proteins, which may or may not be directly involved in cellular transformation. These mutations may alter proteasomal processing, resulting in differential cleavage of novel and unique peptides bound for MHC class I presentation. Alternatively, these mutations can alter the primary amino acid sequence of an established class I peptide, creating a new epitope for CTL recognition. These unique antigens are tumor-specific and, although they play a significant role in the individual host's anti-tumor response (Lennerz et al., 2005), as of yet, they are not into immunotherapeutic targets. Nonetheless, common cell cycle neo-antigens (Lennerz et al., 2005), heat shock proteins (Gaudin et al., 1999), and apoptosis regulators (Mandruzzato et al., 1997) continue to be explored as stress-related, class I presented, immunotherapeutic targets. As an example of a mutation induced tumor change we will focus on caspase 8, a component of Fas and TNF mediated apoptosis pathway.

Caspase 8

Caspase 8 is a cysteine protease required for the initiation of Fas and TNF mediated apoptosis. Frame-shift deletion mutations, resulting in loss of pro-apoptotic function, are common in hepatocellular carcinomas (Soung et al., 2005). An HLA-B*3503 restricted CTL epitope derived from a loss of function caspase 8 mutant was identified from a squamous cell carcinoma of the head and neck by the generation of a tumor specific CTL clone stimulated by the autologous tumor. The mutation affected the stop codon, resulting in the extension of the protein by 88 amino acids (Mandruzzato et al., 1997). The B*3503 CTL epitope includes five amino acids of the extension site. In this manner, the MHC class I can act as a reporter of mutations in critical, transforming elements in the development of tumors.

It is important to note the possibility of changes in post-translational modifications, such as glycosylation and phosphorylation, altering the class I peptide repertoire of cancerous cells. Mutations in the molecular chaperone Cosmc affects the proper folding and activity of the core 1 beta3-galactosyltransferase which results in alterations in normal O-glycosylation (Ju and Cummings, 2002). For example, the Muc-1 antigen is an under-glycosylated mucin expressed by a number of tumor types, and mouse CTL have been generated against this glycopeptide (Xu et al., 2004). In addition, the constitutive activation of receptor tyrosine

kinases and cyclin dependent kinases result in increased phosphorylation of cellular targets. Mouse CTL have been generated against phosphorylated class I restricted peptides (Andersen et al., 1999). CTL have the ability to recognize phosphorylated and glycosylated variants of peptide epitopes. Class I molecules are able to indirectly reflect tumor stress through modifications in proteins and their constituent peptides.

Gene Expression Induced Stress Related Changes: Reactivation of Repressed Genes and Over-expression

Alterations in gene expression can induce changes in peptide presentation, including the expression of germ cell genes, often referred to as cancer-testis (CT) genes. Normally, these genes are expressed in the trophoblast and in testicular germ cells, which do not express MHC class I (Van Der Bruggen et al., 2002). Peptides novel to the mature immune system are presented on the MHC class I upon processing of the protein products of these genes. The function of most CT gene products is unknown but they are putatively involved in spermatogenesis. Their activation is likely due to genome wide demethylation seen in many tumors (Loriot et al., 2006). This extensive demethylation may be due to the loss or downregulation of DNA methyltransferases, as a result of transcriptional disregulation or mutation. These events open up the promoter regions of numerous genes that are normally transcriptionally repressed. The MAGE, BAGE, and GAGE families of CT genes were first identified in melanoma but have since been recognized in numerous tumor types (Van Der Bruggen et al., 2002). Such antigens represent ideal targets for immunotherapy, as they are common to many tumor types and are tumor specific. The up-regulation of host genes during stress can also result in changes in MHC class I presentation. A low level of class I presentation of a particular peptide may be seen ubiquitously or may be tissue specific. Over-expression of these proteins may result in significant changes in the density of these peptides presented on the cell surface, which may facilitate CTL recognition. In cancer, commonly over-expressed proteins include cell cycle regulators, such as cyclin D1 (Dengjel et al., 2004), receptor tyrosine kinases such as Her-2/neu (Fisk et al., 1995), and apoptosis regulators, such as p53 (Ropke et al., 1996). It has been difficult to discern which of these over-expressed proteins are presented in greater density, if at all, on the cell surface. Therefore, it is difficult to predict whether these epitopes are sufficiently or overly immunogenic for immunotherapeutic targeting. For example, human telomerase reverse transcriptase (hTERT) is over-expressed in a number of tumors, yet, fails to produce an anti-tumor response in hTERT peptide vaccinated patients. Although hTERT is over-expressed, it has now been determined that the telomerase derived peptides are not presented on the MHC class I molecule of telomerase expressing tumors (Parkhurst et al., 2004). Thus, while it is likely that class I present stress related over-expressed peptides, such presentation must be carefully validated. As examples of gene expression induced changes in MHC class I presentation, we will focus on peptides derived from MAGE-A1, a germ cell gene

reactivated in many melanoma tumors, and Heme Oxygenase-1, a stress induced enzyme up-regulated in several tumor types.

MAGE-A1

MAGE-A1 is one of 12 CT gene products that have been identified in numerous tumor types. MAGE-A1 interacts with the SKIP adaptor protein and histone deacetylases to effectively repress the transcriptional transactivation activity of Notch1-IC, which is involved in cell fate decisions (Laduron et al., 2004). As a transcriptional regulator MAGEA1 may play a significant role in tumorigenesis by altering gene expression patterns associated with a number of signaling pathways.

The first MAGE-A1 MHC class I associated epitope was sought when a melanoma tumor stimulated autologous CTL in a murine model. In order to identify the gene encoding the class I peptide epitope, mouse P1.HTR cells were co-transfected with tumor cDNA libraries and HLA-A*0101, and CTL stimulation was then utilized to identify the gene encoding the CTL epitope. Overlapping peptide libraries subsequently identified the MAGE-A1 peptide epitope. Additional MAGE-A1 epitopes, as well as peptide epitopes in other MAGE family members, have been identified using this (or a similar) method (Van Der Bruggen et al., 2002).

Heme Oxygenase-1

Heme oxygenase-1 (HO-1) is a stress induced enzyme which catalyzes the degradation of heme to carbon monoxide (CO), biliverdin, bilirubin and iron. Overexpression of this enzyme is associated with inhibition of apoptosis and has been recognized in several tumor types, including chronic myelogenous leukemia (CML) and renal cell carcinoma. In CML, HO-1 transcription is induced by the BCR/ABL oncoprotein, a constitutively active tyrosine kinase (Mayerhofer et al., 2004). Although the mechanism by which HO-1 inhibits apoptosis is not completely clear, CO mediates up-regulation of Hsp72 via the p38MAPK pathway (Kim et al., 2005). An HLA-B*0801 restricted peptide derived from HO-1 was differentially identified by mass spectrometry of eluted peptides from renal cell carcinoma and normal renal tissues. CTL recognizing this epitope were cloned from a healthy, HLA-B*0801 donor (Flad et al., 2006) demonstrating the functional immune recognition of cancer stress as conveyed by class I MHC.

Universal Stress Induced Changes

As described in the earlier portions of this chapter, transcriptional and translational regulators are commonly over-expressed and/or modified both in the course of cancerous transformation and during viral infections. It is therefore conceivable that tumors and virus-infected cells display similar stress-related changes on their MHC class I proteome scanning chips. For example, the most obvious shared immune diagnosis of cancerous or virus-induced stress would be through the up-regulation and class I presentation of heat shock proteins and peptides, respectively. The observation that the HSP are positioned to be broad (i.e. cancer and virus) class I indicators of stress begs the question: Are numerous proteins involved in cell cycle control, growth, and

apoptosis broad class I indicators of stress? The following section reviews emerging literature and indicates that class I molecules indeed act as broad indicators of cellular stress through their interaction with a variety of host proteins.

Hsp72

Inducible Hsp72 is over-expressed in 40–90% of carcinomas including breast, cervical, lung, renal and colorectal carcinomas. Generally, this protein is considered to be indirectly immunogenic, shuttling tumor derived peptides to APC and the MHC class I (Calderwood et al., 2005). However, human CTL directed toward multiple HLA-A*0201 restricted Hsp72 epitopes have been identified. These CTL are capable of killing Hsp72 bearing tumor cells in a manner dependent upon Hsp72 expression levels: MCF-7 breast cancer cells were lysed with greater efficiency compared to M113 melanoma cells that express barely detectable levels of Hsp72 (Faure et al., 2004). Therefore, as observed with virus-infected cells and Hsp90β, adaptive immune mechanisms recognize and respond to HSP derived peptides presented by MHC class I on tumors, as well.

Elongation Initiation Factor 4G

The translational regulator, eIF4G, is often over-expressed in squamous cell lung carcinomas and serum antibodies against eIF4G are detected in the sera of these patients (Bauer et al., 2001). Over-expression of eIF4G alone results in cellular transformation (Fukuchi-Shimogori et al., 1997), possibly due to an increased efficiency in translation of structurally complex mRNAs. Some cell cycle associated mRNAs, such as lck, cyclin D1, c-Myc and vascular endothelial growth factor (VEGF), have complex 5' untranslated regions containing multiple AUG start sites, IRES, or secondary hairpin structures between the cap and AUG which can inhibit translation (Carter et al., 1999; Chung et al., 2002; Kozak, 1991; Marth et al., 1988; Tan et al., 2000). As with other genes/proteins that are up-regulated in cancerous cells, over-expression of eIF4G is positioned to result in increased levels of class I MHC presentation on stressed cells. As an example, we have found a fragment of eIF4G on cells infected with both influenza and HIV, Table 2. In order to verify this finding, we produced a mAb specific for the fragment of eIF4G in A*0201; this antibody (or TCR mimic –TCRm) is specific for the A*0201 class I molecule containing eIF4G peptide VLMTEDIKL. We have verified that the class I A*0201 of HIV infected cells express additional eIF4G, and in Figure 3 we show that the class I A*0201 of cancerous cells also present elevated levels of eFI4G. Class I HLA presentation of eIF4G derived peptides following both cancerous transformation and viral infection represents a broad immune indicator of stress.

DEAD Box Protein p68

RNA helicases are enzymes that generally modulate RNA structure but are implicated as transcriptional, splicing and translational regulators (Abdelhaleem, 2005).

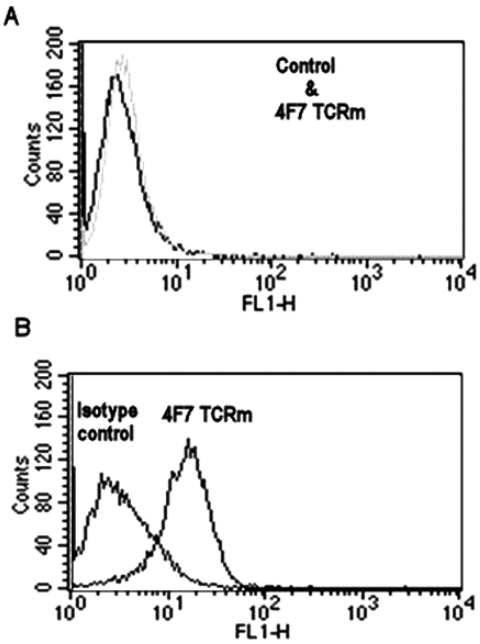

Figure 3. Detection of the eIF4G peptide$_{(720–728aa)}$ -HLA-A*0201 Epitope on HLA-A2+ tumor cells but not normal cells. A. Normal Human Mammary Epithelial Cells (184A1) stained with 4F7 TCRm followed by GAM-FITC. B. Human Breast Carcinoma Cell Line (MDA-MB-231) stained with 4F7 TCRm followed by GAM-FITC

The DEAD box protein p68 is an RNA helicase that acts as a transcriptional co-activator with the tumor suppressor p53. Expression of p68 is required for p53 target gene expression, such as p21, and apoptosis (Bates et al., 2005). The p68/p72 complex binds histone deacetylase 1 (HDAC1) to act as a promoter specific repressor (Wilson et al., 2004). In several cancer cell types, but not in normal tissues, p68 is phosphorylated (Yang et al., 2005), over-expressed and polyubiquitylated (Causevic et al., 2001). Phosphorylation of p68 may be a mechanism by which p53 target gene expression and apoptosis are inhibited and would explain why p53 can be unmutated and over-expressed with no apparent activity. Polyubiquitilation and/or proteasomal targeting of p68 is an alternative mechanism by which p53 activity may be abolished. Given the role of p68 in cancerous transformation, and the cellular changes in p68 during transformation, MHC class I reflection of proteome changes in p68 is a possibility.

While studying class I reported changes during HIV infection, we identified an increase in p68 peptide presentation by A*0201 following infection. We next generated a mAb/TCRm to the A*0201-p68 peptide complex. Staining of cancerous cells shows that class I molecules present high levels of p68, Figure 4. While this preliminary data needs to be extended to other cancerous and non-cancerous cells

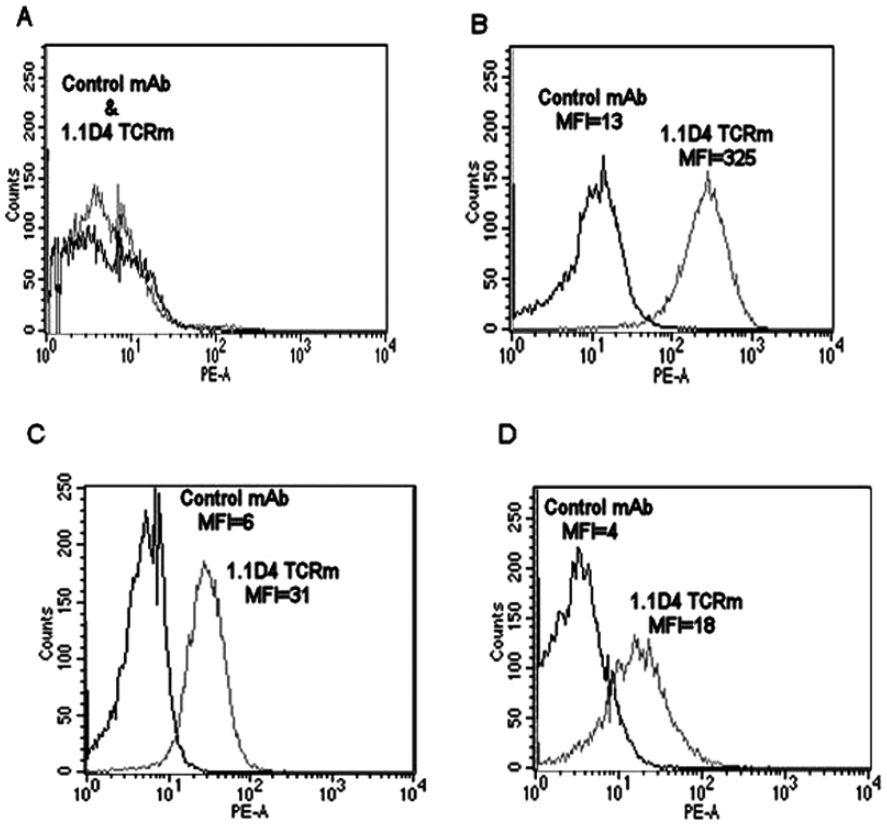

Figure 4. Detection of the p68-HLA-A*0201 Epitope on HLA-A2+ tumor cells with 1.1D4 TCRm. A. MDA-MB-268 breast carcinoma line (HLA-A2neg) B. MDA-MB-231 breast carcinoma line (HLA-A2pos) C. Colo205 colorectal carcinoma line (HLA-A2pos) D. SW620 colorectal carcinoma line (HLA-A2pos). 1.1D4 TCRm specifically recognizes endogenous p68 peptide presented by HLA-A2 molecules on tumor cells

and tissues, it indicates that class I molecules reflects stress in a universal way. The class I molecules of cancerous and virus-infected cells present like signals of stress.

Summary

MHC class I molecules continually relay each cell's intracellular fitness by surveying and presenting the proteome to immune surveillance mechanisms. Alterations in a cell's proteome due to cell stresses such as viral infection and tumorigenesis result in an altered repertoire of endogenously loaded peptides presented by the class I molecule on the cell surface. Immune effector mechanisms sense these danger signals and respond. The current paradigm is that infection leads to the class I presentation of viral peptides that are then targeted by CTL. Here we report

that virus infected cells display a collection of host-derived peptides indicative of stress; increases in gene expression, translation, and the degradation of host proteins leads to the unique presentation of peptides on virally stressed cells. Such virus-induced changes in the endogenous host proteins are represented by the class I molecules of infected cells. While it is clear that the immune system recognizes these stress related peptides, the contribution of these class I peptides to various innate and adaptive anti-viral immune responses continues to emerge. As we learn how the immune system sees and react to these class I stress peptides we will better understand how these epitopes might contribute to the induction of virus-specific and virus-associated autoimmune diseases.

Working under the paradigm that virus-derived peptides will mark the infected cell, one might not expect the class I of cancerous cells to exhibit the same changes as the infected cell. However, given the realization that host encoded stress peptides act to distinguish infected cells, together with the tight association of virus infections and cancer (i.e. hepatocellular carcinomas and cervical cancers), it seemed logical that peptides derived from particular cellular components (HSP, p68, eIF4G) would serve as broad immune indicators of stress. Indeed, it is now emerging that both virus-infected and cancerous cells are marked by like epitopes derived from proteins involved in maintaining cell homeostasis and in controlling stress. We hypothesize such broad stress-related epitopes are recognized by immune "effectors" which function to bridge the innate and adaptive immune responses following infection and cancerous transformation.

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CHAPTER 15

EXTRACELLULAR HSP 72: A DOUBLE-EDGED SWORD FOR HOST DEFENSE

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Abstract: Environmental or emotional challenge triggers a cascading series of physiological responses which are collectively termed the "stress response". The stress response can be assessed at the behavioral, neural, hormonal, immunological and single cell, levels and evolved to benefit an organism's chance of survival during times of acute challenge. The stress response has been studied for many years, however, its impact on specifically immune function has only recently been appreciated. Acute activation of the stress response has both inhibitory and stimulatory effects on immunity. The focus of this chapter is on a novel mechanism for the immunostimulatory effects of stress. Specifically, we propose that an endogenous, ubiquitous cellular stress protein, heat shock protein 72, when found in the extracellular environment may contribute to stress-induced potentiation of innate immunity. We develop the hypothesis that the release of extracellular heat shock protein 72 (eHsp72) is a normal feature of the acute stress response that can have either positive or negative consequences for host defense depending on several factors, including the nature of the eHsp72 (naked versus antigen-associated), and host health status (absence or presence of pre-existing inflammatory disease). Thus, stress-induced eHsp72 release may be a double-edged sword for host defense

Keywords: Danger signals, inflammation, stress, inflammatory bowel disease, sympathetic nervous system, α1adrenergic receptors

STRESS AND HEAT SHOCK PROTEINS

Intracellular Hsp72

Heat-shock proteins (Hsp) are highly conserved cellular proteins that can be categorized into several families and play a role in a number of important cellular functions (Morimoto 1994). The first observations demonstrating the induction of intracellular heat shock proteins were reported in 1962 when Ritossa and colleagues noted that

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Drosophila exposed to temperature shock demonstrated an unusual gene expression profile (Ritossa 1962). In 1974, the term heat shock protein was first coined (Tissieres et al., 1974). The focus of the current review is on one member of the 70-kDa Hsp (Hsp70) family of proteins, Hsp72. The Hsp70 family of proteins includes the constitutive 73-kDa protein (HSC73) protein and a highly stress-inducible 72-kDa protein (Hsp72) (Morimoto 1994; Hartl 1996). Intracellular Hsp72 is found in nearly every cell of the body and can be up-regulated after exposure to a variety of cellular and whole organism stressors (Morimoto 1994; Hartl 1996). Although basal concentrations of Hsp72 are low in most tissues, high concentrations of intracellular Hsp72 can be found in the absence of stressors in some tissues such as the frontal cortex (Heneka et al. 2003), pituitary (Campisi et al., 2003), adrenal (Campisi et al., 2003), and brown fat (Matz et al., 1996; Matz et al., 1996). Intracellular Hsp72 induction has been reported after exposure to a variety of whole organism physical stressors including heat (Kregel 2002), tail shock stress (Campisi et al., 2003; Nickerson et al., 2006), and restraint (Udelsman 1991; Udelsman et al., 1993). Both males and females demonstrate robust Hsp72 responses after acute stressor exposure (Fleshner et al., 2006). Interestingly, induction of intracellular Hsp72 is not restricted to physical stressors. We reported that the experience of predatory fear, exposing rats to a cat with no physical contact, increases intracellular Hsp72 in the brain (Fleshner et al., 2004).

The induction signals and synthesis of intracellular Hsp72 protein have been well-characterized. The gene for Hsp72 contains at least two regulatory elements that interact with heat shock transcription factors (HSFs) (Wu et al., 1986; Wu 1995). Specifically, the induction of Hsp72 protein requires HSF1 binding to the heat shock element (HSE) in the promoter region of the Hsp70 gene (Pirkkala et al., 2001). Hsp70 mRNA is transcribed, resulting in the synthesis and accumulation of cytosolic Hsp72 protein (Kregel 2002). Many factors induce transcription and translation of intracellular Hsp72 protein and vary depending on the tissue examined. Stress-induced signals reported to increase intracellular Hsp72 concentrations include the following: 1) adrenocorticotropin hormone (ACTH, (Blake et al., 1991; Blake 1994)), 2) corticosterone (CORT, (Sun et al., 2000; Valen et al., 2000; Cvoro and Matic, 2002)), 3) glycogen deprivation (Santoro 2000; Febbraio et al., 2002), 4) norepinephrine (NE) or epinephrine (E) (Udelsman et al., 1994; Udelsman et al., 1994; Matz et al., 1996; Matz et al., 1996; Paroo and Noble, 1999; Maloyan and Horowitz, 2002; Heneka et al., 2003), and 5) heat or hyperthermia (Kregel and Moseley 1996; Redaelli et al., 2001; Cvoro and Matic 2002; King et al., 2002; Thomas et al., 2002). Clearly, a wide-array physiological signals are capable of stimulating intracellular Hsp72 synthesis, making this a ubiquitous response to cellular stress.

Stress & Extracellular Hsp72

A great deal is understood about intracellular Hsp72. The focus of the current review, however, is on the potential immunomodulatory function(s) of endogenous *extracellular* Hsp72 (eHsp72). The first reports that eHsp72 was detectable in the circulation of humans were published by Pockley and colleagues in 2000. This

group reported that people suffering from a variety of disease states such as renal disease (Wright et al., 2000), hypertension (Pockley et al., 2002), and atherosclerosis (Pockley et al., 2003) have chronically elevated basal levels of eHsp72 relative to healthy aged-matched controls. In addition to elevated basal eHsp72 associated with disease pathology, Dybdahl et al. (2002) reported patients with coronary artery disease have an acute increase in eHsp72 in response to the stress of coronary bypass surgery. The role of eHsp72 in disease states has recently been reviewed (Ciocca and Calderwood 2005). Not long after these reports, we (Campisi and Fleshner 2003; Campisi et al., 2003; Fleshner et al., 2003) and Febbraio et al., (Walsh et al., 2001; Febbraio et al., 2002) reported that organisms in the *absence of clinical disease states* also rapidly increase the concentration of eHsp72 in blood after exposure to acute physical and/or psychological stressors. These papers were the first to demonstrate that an increase of eHsp72 in the blood occurs in healthy organisms after exposure to acute stressors.

The current review will focus on endogenous eHsp72 because very little is understood about its unique mechanism(s) of induction/release and its powerful *in vivo* immunological function(s). Understanding more about the stress-induced eHsp72 response is important because the function of *in vivo* endogenous eHsp72 is likely context dependent, such that eHsp72 facilitates immunity when released in a healthy organisms; whereas eHsp72 may exacerbate chronic inflammatory disease states (e.g., atherosclerosis, Alzheimer's, inflammatory bowel disease).

Releasing Signals and Secretory Pathways of Extracellular Hsp72

It was first suggested that eHsp72 is only released as a result of necrotic/lytic cell death (Gallucci et al., 1999), and while it is true that necrotic cell death can cause the release of eHsp72 (Basu et al., 2000; Sauter et al., 2000; Berwin et al., 2001), it is now recognized that elevated eHsp72 may be found in the absence of necosis. In fact, glial cells (Guzhova et al., 2001), B cells (Clayton et al., 2005) and tumor cells (Gastpar et al., 2005) have been shown to exocytotically release eHsp72 in a non-classical protein transport pathway (Hightower and Guidon 1989; Broquet et al., 2003; Gastpar et al., 2005; Lancaster and Febbraio 2005) that may involve exosomal packaging of Hsp72 (Guzhova et al., 2001; Clayton et al., 2005; Lancaster and Febbraio 2005). Furthermore, numerous whole organism stressors have been observed to elevate circulating eHsp72 within 10–25 minutes of stressor onset (Campisi et al., 2003), a speed that suggests the classic protein induction/necrosis release pathway is not likely, and in response to stressors (e.g. conditioned contextual fear, predatory stress, exercise stress) that are unlikely to result in large necrotic cell death (Fleshner et al., 2006). Thus, while necrotic cell death can result in the extracellular release on cytoplasmic Hsp72, there are accumulating data to suggest other factors such as exosomal release and hormonal receptor-mediated exocytotic pathways also exist.

It has recently been reported that Hsp72 may be released within exosomes. Exosomes are membrane vesicles that form within multivesicular bodies (MVB) and are secreted from cells when the membrane of the MVB fuses with the plasma membrane and the internal vesicles become extracellular. Since the primary role of membrane vesicles within MVB are to sort and distribute their constituents to the appropriate destination, exosomes contain various signaling molecules that have been designated for release. These molecules are already known to commonly include various immunomodulating substances such as major histocompatibility complex (MHC) class I & II, CD80 (B7.1), CD86 (B7.2), and CD54 (ICAM-1), and now several researchers have demonstrated that Hsp72 are also contained within exosomes. It should be noted that membrane vesicles within MVB could have other fates such as fusion with a lysosome that results in the degradation of proteins, or fusion with the plasma membrane resulting in the expression of proteins on the cell surface. The surface expression of Hsp72 is thought to be important in cell-to-cell interactions and the recognition of tumors (Bausero et al., 2005; Gastpar et al., 2005). Although this is an exciting feature of Hsp72-immune interactions, cell surface Hsp72 function is beyond the scope of the current chapter.

The association of Hsp72 with exosomes has been reported in a variety of cell types including B-cells, tumors, and human peripheral blood mononuclear cells (PBMC), although there appears to be some cellular specificity of Hsp72 expression and release within exosomes. For example, Hsp72 was only detected within the lumen of exosomes in B-cells, but was expressed on the exosome surface and lumen in tumor cells. In addition, interferon-gamma induced excretion of Hsp72-containing exosomes in tumor cells could be prevented by disruption of lipid rafts, but the same disruption in PBMC had no effect on the heat-stress induced release of Hsp-containing exosomes. Thus, the necessity of lipid rafts may be either cell type or stimulus dependent. Furthermore, heat-stress increased the quantity of exosomes released from B-lymphoblastoid cells, while only increasing Hsp72 levels within exosomes and not number of exosomes in PBMC. This suggests that cells can use one of two mechanisms (or both) to increase eHsp72 levels via exosomal release; 1) increase the rate of exosomal release, 2) increase the concentration of Hsp72 within each released exosome.

Currently, it is unknown whether stress-induced elevations in circulating eHsp72 are contained within or expressed on the surface of exosomes. Recent data from our laboratory, however, have determined that α1-adrenergic receptors (ADR) play a critical role in triggering the elevation of circulating eHsp72 during times of stress (Johnson et al., 2005). Johnson et al. (2005) reported that prazosin (an α1-ADR antagonist) blocked the stress-induced increase in circulating eHsp72 concentration in laboratory rats. This is particularly interesting because α1-ADR activation is known to induce a Ca^{2+} influx, an intracellular signal that has been observed to trigger the release of exosomes from many cell types. In addition, since α1-ADR stimulation is known to increase intracellular Hsp72 levels in many cell types, activation of α1-ADR may increase the amount of Hsp72 stored within each exosome, similar to that observed after heat-stress in PBMC. Since norepinephrine

binds with higher affinity than epinephrine to α1-ADRs (Hardman and Limbird 2001), and adrenalectomy, which depletes ~95–99% of epinephrine (Hessman et al., 1976; Vollmer et al., 1995) has been shown to have no effect on stress-induced eHsp72 release after stress (Johnson et al., 2005), we hypothesize that the increase in circulating eHsp72 during stressor exposure is due to sympathetic nervous system activation and the release of norepinephrine that acts at α1-ADR to increase the concentration of Hsp72 within exosomes and stimulate their release.

STRESS, EHSP72 AND HOST DEFENSE: ADAPTIVE CONSEQUENCES

Hsp72 and the "Danger Theory"

Endogenous eHsp72 released during times of stress can stimulate innate immunity leading to improvement in acquired immunity. In fact it has been proposed that eHsp72 released during stress may function as a "messenger of stress" or "danger signal" to the immune system. Matzinger (Matzinger 1994, 1998) first proposed the hypothesis that the body may release endogenous danger signals capable of stimulating immunity. In brief, the danger theory states that immune activation involves danger/non-danger molecular recognition schemas. The danger theory postulates that innate immune cells are activated by danger/alarm signals that are derived from stressed or damaged self. Although the danger theory is controversial when viewed as exclusionary, the ideas suggested are intriguing when viewed as complementary to others. Clearly innate immunity has evolved several strategies of activation. Consequently, it is reasonable to propose that innate immune cells can be activated by both pathogen-associated antigens such as lipopolysaccharide (LPS) binding to a limited number of germ-line encoded receptors (i.e., CD14, or Toll-like 4 receptors (TLR4, (Janeway and Medzhitov 2002)), and by endogenous molecules that are released during times of cellular stress or danger. One important and unresolved issue for the danger theory is what molecules serve as "danger signals" to the immune system. We (Fleshner et al., 2002; Campisi and Fleshner 2003) and others (Colaco 1998; Moseley 1998; Chen et al., 1999; Asea et al., 2000; Ohashi et al., 2000; Todryk et al., 2000; Breloer et al., 2001; Bethke et al., 2002; Habich et al., 2002; Vabulas et al., 2002) have suggested that eHsps may serve this function. Although Hsps fit the theoretical framework proposed by Matzinger, there is currently little supporting *in vivo* experimental evidence. Pittet et al. (Pittet et al., 2002) reported that humans who experienced trauma had increased serum levels of eHsp72 and that higher levels of eHsp72 correlated with improved survival. More recently this same group reported that higher concentrations of eHsp72 in pulmonary edema fluid was predictive of preserved alveolar epithelial clearance and potentially better survival following acute lung injury (Ganter et al., 2006). Based on the danger theory it would follow that if a danger signal serves to facilitate or target immune function, and eHsp72 acts as a danger signal, then organisms with increased eHsp72 should have improved immune responses and facilitated host defense to some types of pathogenic challenges.

eHsp72 and Immune Function: In Vitro Evidence and Potential Controversy

Little is known about the function of eHsp72, especially *in vivo*. While intracellular Hsp72 induction decreases cytokine production (Xiao et al., 1999; Ding et al., 2001; Ianaro et al., 2001; Xie et al., 2002; Xie et al., 2002), extracellular Hsp72 can robustly stimulate inflammatory cytokine production and other innate immune responses (Multhoff et al., 1999; Asea et al., 2000; Breloer et al., 2001). We and others have recently reported that eHsp72 *in vitro* stimulates inducible NO synthase (Panjwani et al., 2002), NO (Campisi and Fleshner 2003), TNFα (Asea et al., 2000; Campisi and Fleshner 2003), IL1β (Asea et al., 2000; Campisi and Fleshner 2003), and IL6 (Asea et al., 2000; Campisi and Fleshner 2003) production from macrophages and neutrophils. Hsp72 also stimulates DC cytokines and chemokine production as well as DC maturation (Lehner et al., 2000; Wang et al., 2002; Lehner et al., 2004; Wang et al., 2005). Interestingly, eHsp72 can also stimulate anti-inflammatory cytokines (Pockley 2003; van Eden et al., 2005; Wang et al., 2005).

In a series of elegant studies by Thomas Lehner and colleagues, unique immunological consequences of Hsp72 can be localized to specific domains of the Hsp72 molecule. For example, the C-terminal portion of Hsp72 (aa359–610) stimulates production of chemokines, IL12, TNFα, and NO; induces Th1 polarization and stimulates the maturation of DC. The N-terminal ATPase portion (aa 1–358) largely lacks these functions (Lehner et al., 2000; Wang et al., 2002; Lehner et al., 2004; Wang et al., 2005). The C-terminal portion of several species of Hsp72 (microbial and human) binds to CD14, TLR4 and CD40 on antigen presenting cells (Wang et al., 2001). CD40-CD40L interactions made between antigen presenting cells (APC) and T cells serves an important co-stimulatory role. Thus Hsp72 may function to both stimulate innate immunity via CD14 and TLRs and facilitate T cell responses via activation of CD40+ APCs. This makes Hsp72 a molecule positioned to play an important role at the interface between innate and adaptive immunity (Wang et al., 2005).

The search for the eHsp72 receptor is a topic of intense investigation and debate. There is evidence of a cell surface receptor for Hsp70 on macrophages/neutrophils (Asea et al., 2000; Reed and Nicchitta 2000; Sondermann et al., 2000; Asea et al., 2002), B cells (Arnold-Schild et al., 1999) and NK cells (Multhoff et al., 2001; Gross et al., 2003). A number of cell-surface binding proteins for eHsp have been implicated. Most research to date, however, suggests that eHsp72 transduces an inflammatory signal to innate immune cells (macrophages/dendritic/neutrophils) by binding to either Toll-like receptor-2 (TLR2) and/or TLR4 in a CD14 dependent fashion (Asea et al., 2000; Visintin et al., 2001; Asea et al., 2002; Vabulas et al., 2002).

Mammalian Toll-like receptors are transmembrane proteins that are evolutionarily conserved between very primitive organisms (such as insects) and humans (Akira et al., 2001). It has been suggested that just as released eHsps may function as "danger signals" or "messenger of stress" to the immune system, the TLRs

may function as surveillance receptors for those signals (Johnson et al., 2003). In addition, exposure to prior injury stress was recently reported to produce a long-term (1–7 days) potentiation of TLR2 and TLR4-induced IL1β, IL6 and TNFα production by spleen cells (Paterson et al., 2003), and chronic social stress (Avitsur et al., 2003) modulates TLR4-mediated responses. These data support the hypothesis that stress-induced modulation of innate immune function may involve TLR2 and TLR4. Extracellular Hsp72 exerts it effects on innate immune cells by stimulating the inflammatory MyD88/IRAK/NF-kappaβ signal transduction pathway (Vabulas et al., 2002). A rapid intracellular Ca^{2+} flux ensues within 10 seconds of eHsp72 binding with high affinity to monocytes or macrophages (Asea et al., 2000). This is important because it distinguishes eHsp72 signaling from LPS signaling, that does not induce Ca^{2+} flux (McLeish et al., 1989). Based on work by Asea and colleagues (Asea et al., 2000; Asea et al., 2000; Asea et al., 2002), eHsp72-induction of NF-kappaβ and inflammatory cytokines requires the expression of CD14, in addition to TLR2 and TLR4. Asea and colleagues have proposed that CD14 could function as a co-receptor for eHsp72 (Asea et al., 2000).

One implication of these results is that eHsp72 released into the blood after exposure to psychological and/or physical stressors may result in optimal stimulation of the inflammatory cascade only in the presence of CD14 activation. Interestingly, binding CD14 plus either TLR2 and/or TLR4 with selective receptor agonists (Pam3Cys binds TLR2 or Taxol binds TLR4) resulted in synergistic increases in NF-kappaβ (Asea et al., 2002). Thus, facilitation of innate immune responses by eHsp72 after exposure to stress may be restricted to cells that express CD14 and/or are binding bacteria, LPS, or eHsp72 bound to endogenous LPS released from the gut microfluora. In many instances, therefore, we hypothesize that acute-stress induced release of eHsp72 may have little or no effect on innate immune cell production of nitric oxide (NO) and/or inflammatory cytokines. If, however, the host is challenged with a pathogen (e.g., bacteria) or already suffers from chronic inflammatory disease (e.g., atherosclerosis, Alzheimer's disease, inflammatory bowl disease), eHsp72 could extravasate from the blood into the inflamed tissues and bind to macrophages and/or neutrophils. Cells that are binding bacteria (LPS) and eHsp72 via CD14 and/or TLR2 or TLR4 could have potentiated NO and/or cytokine response, resulting in facilitated bacterial killing and/or exacerbation of chronic inflammatory disease.

One issue that remains a source of debate is whether macrophage activation and/or inflammatory cytokine stimulation induced by Hsp72 reported in earlier *in vitro* studies is actually due to Hsp72 or is instead a result of LPS contamination inherent in the recombinant protein used. Many studies have carefully demonstrated that 1) stimulation of inflammatory cytokines and NO by eHsp72 *in vitro* is specific to eHsp72 and is not due to non-specific effects of endotoxin contamination in the recombinant Hsp72 protein, and 2) that the intracellular signaling pathways used by lipopolysaccharide (LPS) versus Hsp72 are unique and distinguishable, (Asea et al., 2000; Panjwani et al., 2002; Campisi and Fleshner 2003). Nonetheless this issue deserves discussion.

Gao and Tsuan (Gao and Tsan, 2003, 2003; Tsan and Gao 2004) have published several articles warning that researchers must consider the contribution of LPS contamination inherent in recombinant heat shock protein when testing the immune function of these proteins *in vitro*. One observation that is reported to support this idea is that endotoxin free recombinant human Hsp72 (rhHsp72) does not stimulate inflammatory cytokines from murine macrophages *in vitro* (Gao and Tsan 2003). We have replicated these observations (Johnson and Fleshner 2006) and agree with the conclusion that *in vitro* testing of the immunological function of rhHsp72 must be conducted with caution. These results, however, do not diminish our hypothesis that endogenous Hsp72 released into the blood during an acute stress response has *in vivo* immunomodulatory functions.

In fact, these findings have stimulated a new series of studies investigating which peptides are associated with eHsp72 released into the circulation and if those peptides work in concert with the Hsp72 to stimulate immune function. It seems clear that endogenous Hsp72 released into the blood is likely not "naked". In fact it has been previously suggested that considering the complex forming ability and chaperoning function of Hsp72, *in vivo* 'endotoxin-free' Hsp is probably non-existent (Prohaszka and Fust 2004). This observation and the fact that exposure to a variety of physical and psychological stressors stimulates commensal bacterial translocation from the gut (Deitch and Berg, 1987; Horton, 1994; Katafuchi et al., 2003; Nettelbladt et al., 2003; Nazli et al., 2004; Velin et al., 2004), have led us to speculate that perhaps some or all of the endogenous eHsp72 released by acute stress into the blood may be, in fact, associated with LPS released from endogenous gut bacterial flora. Perhaps it is this Hsp72/LPS complex that extravasates across the leaky vasculature or is transported from the blood into the inflamed tissues that facilitates host antimicrobial defenses.

Interestingly, Quintana and Cohen (Quintana and Cohen 2005) recently reviewed this issue and concluded that the *in vitro* work by Gao and Tsan does not negate the evidence that endogenous Hsps manifest innate immune activities. In fact they suggest that endogenous eHsps may perform diverse functions via two modes of inflammation: sterile inflammation that is triggered by endogenous Hsps and is necessary for body homeostasis, wound healing, angiogenesis, regeneration and immunoregulation, and septic inflammation that is triggered by the release of microbial Hsps. Quintana and Cohen (Quintana and Cohen 2005) also suggest that eHsp72 may bind to LPS and augment immune responses by facilitating the transfer of LPS to the TLR4-MD2 leading to improved signal transduction and inflammatory cytokines responses. Our work supports several aspects of these ideas. Specifically, we present evidence that activation of the acute stress responses results in the release of endogenous eHsp72 and that eHsp72 is not "naked" and instead is found in the blood bound to LPS. We suggest that indeed endogenous eHsp72/LPS may function to facilitate antimicrobial responses, restrict the development of bacterial inflammation and facilitate bacterial inflammation resolution and hence a restoration of body homeostasis.

Here we present preliminary data in support the hypothesis that eHsp72 in found in the blood after exposure to an acute stressor may indeed be associated with endogenous LPS. The approach taken to initially explore this idea was the following: if stress-induced eHsp72 was indeed bound to endogenous LPS, then removal of LPS from plasma samples of stressed rats but not non-stressed rats, should remove Hsp72/LPS complexes, resulting in a reduction in the concentration of eHsp72. If, in contrast, the eHsp72 found in the plasma after stress is not bound to LPS, then removal of LPS should not change eHsp72 concentrations in any plasma sample tested. Thus we conducted the following pilot study.

Specific pathogen free F344 adult male F344 rats either remained in their home cages (No Stress) or were placed in Plexiglas restraining tubes (23.4 cm long and 7.0 cm in diameter) and exposed to 100, 5 s, 1.6 mA inescapable tail shocks (Stress). The tail shocks were delivered through contact beams on the tail with a randomized average intertrial interval of 60 s. This stress procedure stimulates an robust acute behavioral (Greenwood et al., 2003), neural (Greenwood et al., 2003), hormonal (Fleshner 1995), immunological (Fleshner et al., 1998; Kennedy et al., 2005) and cellular (Nickerson et al., 2006) stress response and does not result in tissue damage to the animal. Immediately after stressor termination, blood samples were collected using cardiac punctures in order to obtain an aseptic blood sample. Cardiac punctures were performed using 18 gauge needles and 10ml syringes (BD, Franklin Lakes, NJ) on isoflurane anesthetized rats. Blood was collected in 10ml EDTA-vacutainer tubes (BD PharMingen, Franklin Lakes, NJ, USA) and stored on ice until completion of the study. Plasma was collected and stored at −20 °C until time of assay. The 1ml Detoxi-Gel Columns (Pierce, Rockford, IL) were regenerated prior to use following the manufacturer's instructions. Plasma samples from non-stressed animals and animals that received the acute stress protocol were applied to a 1ml Detoxi-Gel Column (Pierce, Rockford, IL) after the column was equilibrated with five column volumes of 0.9% pyrogen free saline (Abbott Laboratories, Chicago, IL). Samples were collected from the column and HSP70 protein levels were compared to pre-column samples using an HSP70 ELISA as per manufacturer's instructions (Stressgen, San Diego, CA).

As depicted in Figure 1, exposure to stress led to a 3–4 fold increase in plasma concentrations of eHsp72 above basal, non-stressed levels, replicating our previous reports (Campisi et al., 2003; Johnson et al., 2005; Fleshner et al., 2006). Removal of LPS reduced the stress-induced but not basal, plasma Hsp72 concentrations, suggesting that the majority of the eHsp72 found in the plasma after stress may indeed be bound to endogeneous LPS perhaps due to bacterial translocation of commensal bacteria. This idea will be further developed later in the chapter.

eHsp72 and Innate Immune Function: *In vivo* Evidence

We have previously reported that exposure to acute stress prior to bacterial challenge improves host defense to an *in vivo* bacterial challenge, and this effect is likely due to potentiation of innate immunity (Campisi et al., 2002; Campisi and Fleshner

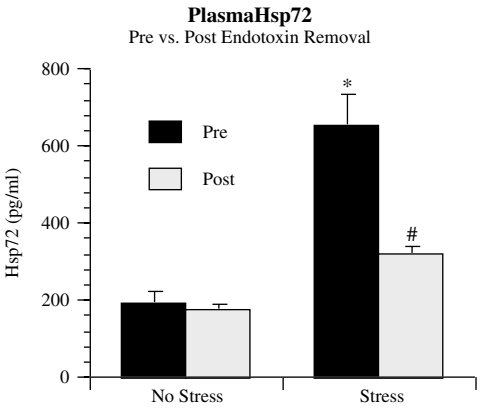

Figure 1. Exposure to tail shock stress (Stress) significantly increased circulating levels of extracellular Hsp72 (eHsp72) above non-stressed controls (No Stress). If plasma samples are depleted of circulating endotoxin (LPS) by Detoxi-Gel Column, the concentration of eHsp72 was also reduced but only in the stressed samples, suggesting that eHsp72 may be bound to LPS in the blood of stressed rats. Sterile blood samples were collected via cardiac puncture and plasma Hsp72 was measured by ELISA. Bars represent group means ($n = 3–4$) & SEM. * denotes $p < 0.05$ compared to non-stressed samples. #denotes $p < 0.05$ compared to samples not passed over the column

2003; Campisi et al., 2003). In summary we have reported that rats exposed to the identical tail shock stress procedure described above, and subcutaneously injected immediately after stressor termination with $\sim 10^8$ colony forming units (CFU) of freshly grown *E. coli*, resolve their inflammation 10–14 days faster (Campisi et al., 2002; Campisi and Fleshner, 2003; Campisi et al., 2003), experience less bacterial-induced body weight loss (Campisi et al., 2002), and release 300% more NO at the inflammatory site compared to bacterially injected, non-stressed controls (Campisi et al., 2003). The stress-induced potentiation of NO is one important in antimicrobial response contributing to the beneficial effect of stress on *in vivo* host defense. This is supported by the observation that inhibition of NO at the inflammatory site with L-NIO (NOS inhibitor) reduces the effect of stress on facilitation of recovery from bacterial challenge (Campisi et al., 2003). NO contributes to almost every stage of inflammation by impacting leukocyte migration, adherence, antimicrobial activities and phagocytic ability and, in fact, can act to restrict the development of inflammation (Ali et al., 1997). One beneficial effect of stress on recovery from bacterial challenge could be greater NO-mediated bacterial killing. Consistent with this idea is that rats challenged with *E.coli* after stress have fewer *E.coli* colony forming units (CFU) retrieved from the inflammatory site 2, 4, and 6 hours after challenge compared to non-stressed *E.coli* challenged controls (Campisi et al., 2003). The enhanced release of NO appears to be an important mediator; however, the mechanisms involved in stress-induced facilitation of NO and recovery from bacterial inflammation remain unknown.

We propose that eHsp72, possibly associated with LPS, binds to CD14 and/or TLR4 on the surface of macrophages and results in a greater and more rapid antibacterial response at the site of bacterial inflammation. The release of eHsp72 in response to a global stressor such as tail shock, therefore, facilitates innate immune function only in the presence of pathogenic challenge. There are several studies that lend support to the hypothesis that stress-induced increases in eHsp72 functions to facilitate innate immunity in the presence of pathogenic challenge (*E.coli*). We have reported, for example, that rats exposed to an acute ($\sim$ 120 min) laboratory stressor and challenged with subcutaneous *E.coli* have an increase in eHsp72 both in the blood and at the site of inflammation. eHsp72 administered to the site of inflammation in the absence of stress, improved recovery from bacterial challenge (Campisi et al., 2003), and immunoneutralization of eHsp72 by anti-Hsp70-Ab46 at the site of inflammation prevented the beneficial effect of tail shock stress on bacterial inflammation resolution. We believe that the eHsp72 and/or eHsp72/LPS complex gains access to tissue via vascular leaking at the site of inflammation due to histamine release from mast cells. Thus eHsp72 released after stressor exposure may extravasate from the blood vessels into the tissue. This is supported by the findings that injection of a selective histamine type1 receptor antagonist that prevents vascular leaking (Martin and Roemer 1977; Yokoyama et al., 1993) prior to stressor exposure, blocks the stress-induced increase of eHsp72 at the inflammatory site but not in the blood, and prevents the stress-induced decrease in bacterial load (Campisi et al., 2003; Fleshner et al., 2006). These data support our hypothesis that eHsp72 and/or eHsp72/LPS can extravasate from the blood into inflamed tissue via a type1 histamine receptor dependent mechanism, and suggest that eHsp72 may participate in improved antimicrobial responses associated with acute activation of the stress response. Thus stress-induced eHsp72 may only impact host immunity in the presence of a pathogen or inflammatory disease states. This idea is consistent with previous literature on the priming effects of stress on innate immunity (Johnson et al., 2002; Johnson et al., 2002; Johnson et al., 2002)

Additional evidence to support the supposition that innate immunity is the initial target of the stimulatory function of eHsp72 and/or eHsp72/LPS complex can be found in some recent work by our laboratory (Campisi et al., 2005). Here we report that the initial beneficial effect of stress on recovery from bacterial challenge is not dependent on T cells or immunoglobulin (Campisi et al., 2005). In this series of studies we examined the effect of tail shock stress on *E.coli*-induced inflammation in both *rnu/rnu* (athymic) nude rats (no α:β T cells) and thymic-intact stressed controls (*rnu*/+). The results of this work were that *rnu/rnu* (athymic) nude rats (no α:β T cells) have equal or even slightly greater restrictions in inflammation diameter as compared to thymic-intact stressed controls (*rnu*/+). This restriction in inflammation diameter development produced by tail shock is most likely due to a rapid improvement in bacterial killing/clearance that results in a reduction in bacterial load (0–6 hrs after stressor termination + *E.coli* challenge). Thus the effect of acute stress on *E.coli*-induced inflammation is not dependent on α:β T cell function.

The athymic nude (*rnu/rnu*) rats do have B cells and normal levels of basal immunoglobulin against bacterial antigens since such responses do not require T cell help (Rolstad 2001). One host response against bacterial challenge that is dependent in part on B cell function via immunoglobulin is opsonization-phagocytosis (Tuazon et al., 1981; Stenfors and Raisanen 1993). Opsonization is the binding of proteins such as immunoglobulin to the surface of bacteria. Once the bacteria are coated or opsonized with immunoglobulin, phagocytic cells (i.e. neutrophil and macrophages) efficiently engulf or phagocytized the bacteria. It is possible, therefore, that stress is facilitating the immunoglobulin response leading to potentiated host defense. In fact there is evidence in the literature that some types of acute stressors can stimulate immunoglobulin responses to some doses of antigen (Moynihan 1994; Moynihan et al., 2000). If acute stressor exposure facilitates host defense by stimulating the opsonization-phagocytosis pathway of bacterial killing and clearance, then rats challenged with opsonization-phagocytosis resistant *E.coli* should fail to show the positive effects of acute stressor exposure. To test this hypothesis, the effect of tail shock on two different strains of *E.coli* that differ in their sensitivity to opsonization-phagocytosis mechanism of killing was tested. K-encapsulated *E.coli* strains are resistant to the opsonization-phagocytosis killing pathway (Kim et al., 1992; Cross et al., 1995). In contrast, the K-negative or non-encapsulated (i.e., ATCC15746) *E.coli* strains are sensitive to the opsonization-phagocytosis killing pathway (Kim et al., 1992; Cross et al., 1995). Thus we examined the effect of inescapable tail shock stress on *E.coli*-induced inflammation produced by either a K-encapsulated *E.coli* strain (Bort, kindly provided by Dr. Alan Cross, Walter Reed) or not K-encapsulated *E.coli* strain (ATCC15746). The results presented in Campisi et al., (Campisi et al., 2005) were that exposure to acute stress prior to subcutaneous challenge of either strain of bacteria resulted in an equal effect of stress. Thus the effect of inescapable tail shock is *not dependent* on the opsonization-phagocytosis-killing pathway. These data support to the hypothesis that the early effect of stress on primary host defense against *E.coli* challenge is due to stress-induced potentiation of innate immune processes and not to changes in T cell or immunoglobulin opsonization responses.

Figure 2 depicts our hypothesis for the adaptive effects of eHsp72. Stress-induced release of eHsp72 acts in concert with other aspects of the APR (neutrophilia, NO/O_2^-, complement activation, etc.) to facilitate host defense. Specifically, we propose that one previously unrecognized and adaptive feature of the acute stress response is the release of endogenous eHsp72 into the circulation. If an organisms is subsequently challenged with a pathogen such as *E.coli*, eHsp72 possibly bound to LPS from intestinal bacteria, extravasates from the blood into the subcutaneous space across blood vessels that are rendered leaky due to bacterial-stimulated histamine release from mast cells (Ali et al., 1997). Extracellular Hsp72 and or Hsp72/LPS complexes accumulate at the inflammatory site, bind to TLR4 on macrophages, neutrophils and or dendritic cells. Macrophages and/or neutrophils that have received a stimulatory signal via CD14 binding to LPS will mount potentiated innate immune responses (i.e., NO, TNF, IL1, IL6) that result in an immediate optimal bacterial killing.

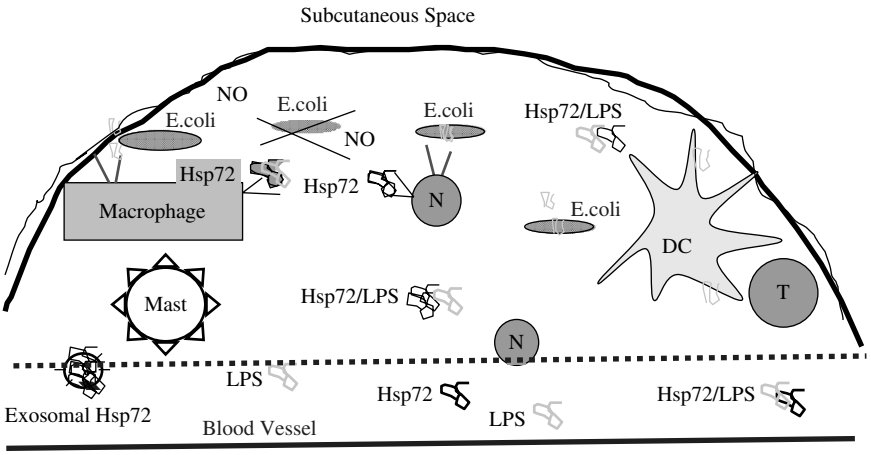

Figure 2. Depicted in Figure 2 is the positive edge of the double-edged sword of extracellular Hsp72 on host defense. We hypothesize that activation of the acute stress response and sympathetic nervous system release of norepinephrine induces the release of eHsp72 and/or eHsp72/LPS into the blood via an α1 adrenergic receptor mediated mechanism. If an organism is challenged subcutaneously with a pathogen (*E.coli* bacteria), and an inflammatory response is initiated in part due to mast cell histamine release, eHsp72 and/or eHsp72/LPS can extravasate across the leaky blood vessels and accumulate in the inflamed tissues. The eHsp72 and/or eHsp72/LPS complex can then interact with CD14 and/or TLR4 receptors expressed on macrophages, dendritic cells (DC), neutrophils (N) leading to potentiated antimicrobial responses (such as nitric oxide release (NO), and the generation of pathogen-specific T cell responses)

STRESS, HEAT SHOCK PROTEINS AND HOST DEFENSE: MALADAPTIVE CONSEQUENCES

Stress, Bacterial Translocation and the eHsp72/LPS Complex

We propose that the majority of the eHsp72 in the blood after exposure to an intense acute stressor may be bound to LPS derived from communal enteric bacterial translocation. This hypothesis is plausible given the evidence that activation of the stress responses can induce translocation of enteric bacterial. Movement of enteric bacteria from the gastrointestinal lumen through the intestinal mucosal epithelial barrier is referred to as bacterial translocation. This process can occur in two ways, either via transcellular passage, movement of bacteria through epithelial cells, or via paracellular passage, movement of bacteria between adjacent epithelial cells. During normal physiological conditions, adjacent epithelial cells are connected by tight junctions that prevent the passage of bacteria between cells thus only transcellular passage of bacteria occurs and this process is mediated by specialized mucosa epithelial cells called microfold or M cells. M cells are interspersed in the follicle-associated epithelia, part of the epithelia that is association with gut-associated lymphoid tissue (GALT) (i.e. Peyer's patchs) (Gebert et al., 1996). The apical surface of M cells have specialized receptors that enhance their interaction

with luminal bacteria and mediate the transcytosis of bacteria to immune cells contained with the underlying GALT (Giannasca et al., 1999; Neal et al., 2006; Tyrer et al., 2006). The immune response within GALT is predominantly mediated by the release of transforming growth factor-beta that favors B cell class switching to IgA (Cazac and Roes 2000) and promotes immune tolerance by suppressing inflammatory responses (McGuirk and Mills 2002).

Exposure to a wide number of stressors such as, restraint (Ando et al., 2000; Dunn et al., 2003), social disruption (Bailey et al., 2006), water avoidance stress (Velin et al., 2004), and sleep deprivation (Bergmann et al., 1996), increase bacterial translocation across the mucosal epithelial barrier. One consequence of translocation is that live, replicating bacteria can often be cultured from tissue homogenates of mesenteric lymph nodes, spleen, and liver. In fact, bacterial translocation is thought to be a critical factor involved in morbidity and mortality following some stressors such as burn injury (Sittig and Deitch, 1988; Gianotti et al., 1996), heat stroke (Gathiram et al., 1987, 1988), and ischemia-reperfusion injury (Gathiram et al., 1988). As described above, bacteria translocation can occur either via transcellular or paracellular passage and during times of stress it appears bacteria translocation occurs via both routes. Some data suggest transcellular permeability may be more sensitive to disruption by stressor exposure compared to paracellular permeability. This is based on the findings that acute mild stressor exposure increases the passage of HRP molecules through epithelial cells but the epithelial layer remain impermeable to ^{51}Cr-EDTA (a paracellular marker molecule), while chronic mild stressor exposure increases the passage of both HRP and ^{51}Cr-EDTA molecules (Ferrier et al., 2003; Velin et al., 2004). The mechanisms by which stressor exposure results in increased permeability of mucosal epithelial are still being investigated.

Currently, there is good evidence to suggest that mast cells, corticotropin-releasing hormone (CRH), and neural activation are all critically involved in the increased permeability of the mucosal barrier to bacterial translocation during stressor exposure. Exactly how all these factors interact to result in mucosal barrier damage is unknown. One hypothesis is that exposure to a stressor results in activation of the sympathetic nervous system and the release of CRH from postganglionic neurons stimulates mast cell degranulation resulting in an inflammatory cascade and damage to epithelial cells (Webster et al., 1998; Soderholm and Perdue 2001; Soderholm et al., 2002). Studies have shown that rats deficient in mast cells are protected from stress-induced barrier dysfunction and increased mucosal permeability (Castagliuolo et al., 1998; Santos et al., 2000). Mast cells are often found in close proximity to neurons (Heine and Forster 1975; Stead et al., 1990) and administration of CRH to laboratory animals results in mast cell degranulation (Theoharides et al., 1995) and increased colonic ion secretion and permeability similar to that observed in animals exposed to a laboratory stressor (Santos et al., 1999). Furthermore, blockade of CRH receptors or inhibiting adrenergic nerve excitability prevents stress-induced mucosal dysfunction and increased permeability (Santos et al., 1999; Gareau et al., 2006; Yang et al., 2006). The idea that CRH may initiate inflammatory responses during times of stress is not unique

to the gastrointestinal tract, but has been proposed as a critical mediator in many autoimmune/inflammatory phenomena (Crofford et al., 1992; Kawahito et al., 1995; Webster et al., 1998).

Recent studies from our laboratory suggest that tail shock stress also results in bacterial translocation as indicated by a time-dependant increase in circulating endotoxin levels. In these studies, adult, male Fisher rats either remained in their home cage as non-stressed controls (No Stress) or were exposed to intermittent tail shock stress previously described (Stress). Animals were anesthetized with isofluorane 0, 15, 25, or 100 min after the onset of the stressor or 60 min post the termination of 100 min of stress and cardiac punctures were used to obtain asceptic blood samples, as previously described. Endotoxin levels were measured in plasma using a LAL assay while Hsp72 was measured by ELISA. As observed in Figure 3A, stressor exposure resulted in a time-dependant increase in endotoxin levels that reached significance 100 min following the onset of stress and remained elevated for at least 60 min following stressor termination. As previously reported, tail shock stress also significantly elevates circulating levels of eHsp72 and in this study tail shock stress significantly increased plasma Hsp72 by 25 min of stressor exposure, was maximal after 100 min, and started to decline by 60 min following stressor termination (Figure 3B). Extracellular Hsp72 increases were detectable prior to the elevation of circulating endotoxin and began to decline prior to endotoxin levels returning to normal suggesting that eHsp72 may contribute endotoxin release. In addition, there is also considerable length of time when both eHsp72 and endotoxin are elevated in blood making it feasible to propose that eHsp72 may indeed bind endogenous endotoxin, in these circumstances.

The mechanism(s) for stress-induced bacterial translocation and endogenous LPS release, as well the nature of the potential eHsp72/LPS association remain unknown. It is possible that the increase in LPS and eHsp72 concentrations in the blood after stress occurs via independent releasing mechanisms and from different cell types or tissues. It this were so, then the two independently released proteins would enter the circulation separately and associate with each other in the blood. This is possible given that Hsp72 has been reported to readily directly bind to LPS (Byrd et al., 1999; Triantafilou et al., 2001, 2001; Triantafilou et al., 2001; Reed et al., 2003). A second possibility is that eHsp72 directly participates in the changes in gut permeability, thus mechanistically contributing to bacterial translocation. There is evidence for this idea as well. Neal et al., (Neal et al., 2006) recently reported that activation of TLR4 on enterocytes is both necessary and sufficient to induce both the phagocytosis and translocation of gram negative bacteria across the intestinal barrier. In addition, exposure to stressors has been shown to stimulate intestinal epithelia bacterial uptake (Velin et al., 2004). Thus, the early wave eHsp72 released into the blood could bind to TLR4 and initiate bacterial translocation, leading to the later elevation in LPS in the blood. The eHsp72/LPS complexes, then could form in the blood after the intestinal enterocytes have been stimulated. A third possibility is that the enterocytes themselves are the cellular source of the eHsp72 released after stress and that the LPS is complexed to Hsp72 within the enterocyte,

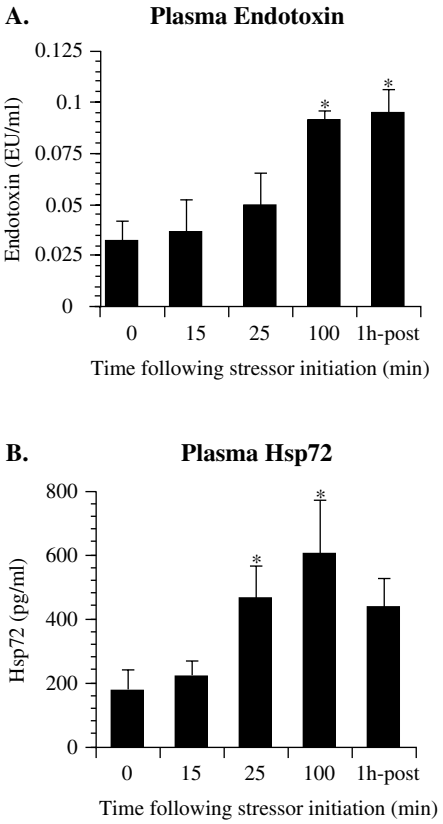

Figure 3. Exposure to tail shock stress significantly increased circulating levels of endotoxin and extracellular Hsp72 in a time-dependant fashion. Fisher 344, male rats were anesthetized with isofluorane following 0, 15, 25, or 100 min of intermittent tail shocks or 60 min post the termination of 100 min of tail shock stress. Sterile blood samples were collected via cardiac puncture and plasma endotoxin (A) was measured by LAL assay and plasma Hsp72 (B) was measured by ELISA. Bars represent group means ($n = 6–7$) & SEM. * denotes $p < 0.05$ compared to 0 min.

prior to release. Thus the enterocytes release the eHsp72/LPS complex directly either via exocytosis or necrotic cell death. A final possibility is that the eHsp72 and LPS are derived from and released from independent sources, but that the eHsp72/LPS association occurs in the mesenteric lymph nodes. We have previously reported that tail shock stress rapidly elevates Hsp72 in the mesenteric lymph nodes (Campisi et al., 2003; Fleshner et al., 2006; Nickerson et al., 2006). In addition, LPS concentrations also increase in the mesenteric lymph nodes after tail shock stress. Thus it is possible that activation of the intense stress response could trigger the up-regulation of mesenteric lymph node eHsp72 and bacterial translocation. Consequently LPS could accumulates in the mesenteric lymph nodes where it comes

in contact with high concentrations of Hsp72, binds the LPS, and the eHsp72/LPS complex is released into the blood.

Each of these scenarios is possible, but remain purely speculative at this time. What is clear, however, is that stress increases both plasma eHsp72 and LPS and that removing LPS from the plasma of stressed rats, greatly reduces the concentration of eHsp72. These data suggest that the eHsp72 in the blood may indeed be bound to endogenous LPS, and that perhaps eHsp72 may function as an adjuvant to enhance stimulation of inflammatory cytokines and the innate immune response. The stimulation of innate immunity is adaptive for host defense when the host is healthy and acutely challenged with bacteria. In contrast, however, this same response can be maladaptive if the host is suffering from chronic inflammatory disease. Hence the stress-induced release of eHsp72 can indeed function as a double-edged sword in host defense. The final section this review further develop our ideas concerning the impact of stress and eHsp72 release, on inflammatory disease. To this end, we will focus on the negative effect of activation of the stress response in the relapse or recurrence of specifically inflammatory bowel disease.

Stress and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a relapsing and remitting inflammatory syndrome that is characterized by the presence of chronic inflammation of the bowel. The nature of the immunological disorder in IBD differs depending on the disease sub-type, that is Crohn's disease and ulcerative colitis. Although both disorders share many clinical and pathological characteristics, they differ in their immunological process. Crohn's disease is characterized by an inflammatory Th1 dominated disease, whereas ulcerative colitis is characterized by inflammatory Th2 dominated allergic-like disease (Bouma and Strober 2003; Macdonald and Monteleone 2005; Hibi and Ogata 2006). Although advances in our understanding continue, the etiology of IBD remains unknown. One hypothesis that has gained support in the literature is that IBD is the consequence of an inappropriate immune response to normal gut microflora (McKay 1999; Guarner and Malagelada 2003; Nazli et al., 2004; Macdonald and Monteleone 2005; Hibi and Ogata 2006). This is supported by several lines of evidence. First, the presence of enteric bacteria is necessary to trigger nearly all animal models of gut inflammation (Taurog et al., 1994; Danese et al., 2004; Nazli et al., 2004). Second, alterations in gut microflora with antibiotics (Pimentel 2002; Cuoco and Salvagnini 2006; Quigley 2006), probiotics (Campieri and Gionchetti 1999; Shiba et al., 2003) and/or diet (Zar and Kumar 2002) offers some relief to IBD patients. And third. genetic analysis of patients with Crohn's disease has identified a mutation in the NOD2 gene. NOD2 protein is found in macrophages that may be an intracellular receptor or sensor for bacterial LPS (Strober et al., 2006). Thus, the mechanism(s) for the "inappropriate" immune response to normal gut microflora remain a mystery, however, alterations in intestinal barrier function and bacterial translocation likely play a role (Macdonald and Monteleone 2005; Hibi and Ogata 2006).

There are numerous reports in the literature supporting a prominent role of stress in the pathophysiology (Milne et al., 1986; Mayer et al., 2001; Bernstein et al., 2006), and clinical presentation of IBD (Mayer 2000; Mayer et al., 2001; Mayer et al., 2001; Longstreth et al., 2005). In humans, disease relapse is often coincident with elevated stressful life events and people with IBD stimulate a greater stress response to a laboratory stressor (Farhadi et al., 2005). Several animal models of acute and chronic stress also have been reported to facilitate the development, exacerbate symptoms, and/or trigger the relapse of, IBD (Collins 2001; Colon et al., 2004). One way that stress may exacerbate IBD is via the well-documented impact of the stress response on intestinal barrier function and bacterial translocation described previously. Investigation into the physiological mechanisms for the effect of stress on intestinal barrier function has intensified with our growing knowledge of the regulation of the enteric nervous system and the power of the brain-gut interactions (Soderholm and Perdue 2001). There are several neurotransmitters of neuropeptides released by stress that can directly impact both immune cells implicated in IBD (mast cells, macrophages, neutrophils, T cells, B cells) and intestinal epithelial cells. As previously described, for example, both CRH and Ach may directly impact mast cells and granulocytes leading to a breakdown in barrier function and potentially triggering an inappropriate response to gut microflora and eventually IBD.

In addition to possible neural-immune interactions of stress on intestinal barrier function, stress could also contribute to IBD pathology via direct effects on intestinal epithelial cells. It has been reported, for example, that intestinal epithelia experiencing metabolic stress perceive commensal bacteria as threatening or "dangerous" to the body, leading to loss of barrier function, increased bacterial translocation, and increased chemokine synthesis by the intestinal epithelial cells (Nazli et al., 2004). As depicted in Figure 4, we have proposed that eHsp72 could be a signal to relay cellular stress or "danger" to the body. Perhaps eHsp72 released after exposure to stress binds to TLR4 receptors expressed on epithelia cells. This signal consequently triggers bacterial translocation of endogenous bacteria, increased phagocytosis, and an up-regulation of co-stimulatory molecules, and these changes contribute to an inappropriate response to harmless commensal bacteria and the development and exacerbation of IBD. Another way that eHsp72 could contribute to the inappropriate response to commensal bacteria is by stimulating chemokines, IL12, TNFα, and NO, and Th1 polarization (Lehner et al., 2000; Wang et al., 2001; Wang et al., 2002; Lehner et al., 2004; Wang et al., 2005). In addition, stress-induced eHsp72 release could exacerbate IBD by altering normal bacterial tolerance mechanisms. Intestinal epithelial cells express both CD14 and TLR4 (Hornef et al., 2002). These cells are initially highly responsive to LPS and can recognize intracellular LPS (Hornef et al., 2003), however, they normally develop tolerance after chronic exposure and no longer respond to commensal bacteria. The precise mechanism for the development of the tolerant intestinal epithelial phenotype is not known, however, TLR4 does not down-regulate cellular expression. One way that stress might trigger the onset or relapse of inflammatory bowel is that the released

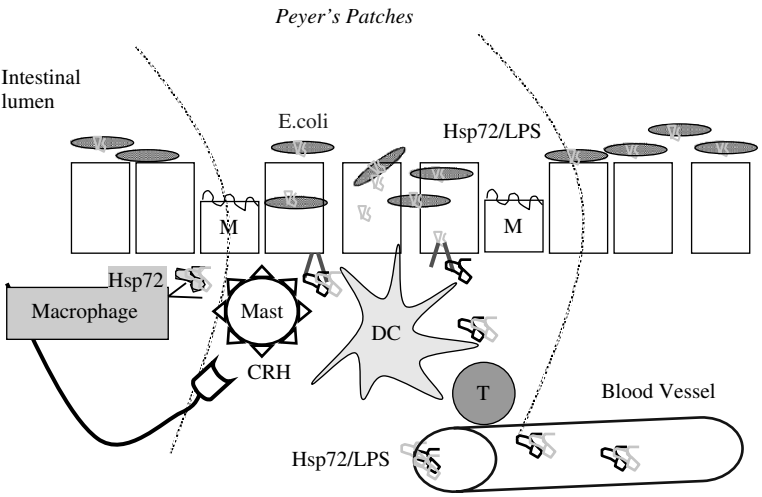

Figure 4. Depicted in Figure 4 is the negative edge of the double-edged sword of extracellular Hsp72 on host defense in inflammatory bowel disease (IBD). We hypothesize that activation of the acute stress response and sympathetic nervous system release of norepinephrine induces the release of eHsp72 and/or eHsp72/LPS into the blood via an α1 adrenergic receptor mediated mechanism. Acute activation of the stress-response also stimulates the release of corticotropin releasing hormone (CRH) from nerve terminals in many tissues including the intestine. This can lead to bacterial translocation of endogenous enteric *E.coli* in part due to mast cell histamine release. Bacterial translocation in a susceptible host (possible due to genetic alterations in Nod2) can lead to an "inappropriate" host responses to enteric bacteria and IBD. Stress-induced release of eHsp72 and/or eHsp72/LPS complex could contribute to both the development and relapse of IBD by accumulating in Peyer's Patches, and then interacting with CD14 and/or TLR4 receptors expressed on gut epithelia cells, macrophages, and dendritic cells (DC), contributing to bacterial translocation as well as the development/recurrence or inappropriate inflammatory and pathogen-specific Th1 cell responses to endogenous communal bacteria

eHsp72/LPS complex bypasses the normal tolerance by triggering a unique TLR4-MD2 pathway. This would result in an inappropriate immune response to gut microflora. And finally, it has recently been suggested that bacterial translocation could contribute to IBS by disrupting the healthy balance of gut microflora leading to a breakdown in mucosal protection and a susceptibility to infection with adherent-invasive pathogenic *E.coli* (Boudeau et al., 2003; Darfeuille-Michaud et al., 2004; Hibi and Ogata 2006).

CONCLUSIONS AND IMPLICATIONS

Thus the mechanisms for the negative effect of activating of the stress response on IBD remain unknown, however, the release of eHsp72 and/or eHsp72/LPS could play a role. If this hypothesis is correct, then these results could lead to new pharmacological treatments for IBD. Recall that exposure to acute tail shock (Campisi et al., 2003) and psychological (Fleshner et al., 2004) stress triggers the

release of eHsp72 into the blood. The release of this protein is medicated via activation of the α1-ADR (Johnson et al., 2005; Johnson and Fleshner 2006) and prazocin (α1-ADR antagonist) blocks the effect. We don't yet know what impact, if any, prazocin might have on stress-induced bacterial translocation, LPS release or eHsp72/LPS complex release. Nonetheless, the results of this research could lead to the use of prazocin or to the development of other α1-ADR antagonists to reduce the negative impact that stress has on the exacerbation and relapse of IBD.

The acute stress response includes a complex cascade of behavioral, neural, endocrine, immunological, and cellular responses that are designed to facilitate fight/flight responses and host survival. Our work has revealed that the release of endogenous Hsp72 is an additional feature of the acute stress response that has both positive and negative implications for host defense. One positive example of this response is as follows: if a gazelle were running across the savanna being chased by a lion, the acute stress response would be activated to facilitate escape. If that gazelle manages a successful escape but is wounded during the attack, perhaps the released eHsp72 and/or eHsp72/LPS in the circulation would accumulate in the wound and possibly infected tissue and facilitate a faster immune response that also improves the gazelle's chances of survival. If, in contrast, you were suffering from IBD and you experienced the stress of divorce. Activation of the stress response would also occur and possible the subsequent release of eHsp72 and/or eHsp72/LPS could re-ignite your bowel inflammation. Thus, more work needs to be done to fully characterize and appreciate the function of this newly recognized immunomodulatory endogenous protein and its double-edged impact on host defense.

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CHAPTER 16

HSP60: A PLEIOTROPIC IMMUNE SIGNAL

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Abstract: Heat shock protein (HSP) 60 functions as a key signal to the immune system: its expression is up-regulated under inflammation and HSP60-reactive T and B cells were observed in almost all inflammatory diseases. Moreover, HSP60 induces pro-inflammatory phenotype in innate immune cells via Toll-like receptors (TLRs). Accordingly, HSP60 has been considered a pro-inflammatory "danger signal". However, HSP60 have immunoregulatory potential and could arrest inflammatory damage. In this chapter we discuss recent findings indicating that T and B cells may directly respond to HSP60 via TLR-2 and TLR4 respectively. HSP60 inhibits T-cell chemotaxis, shift the cytokine secretion balance towards a Th2 phenotype, and activates the suppression ability of Treg. B cells responding to HSP60 secreted also high levels of IL-10. Then, these innate effects of HSP60 on adaptive immune system lead to resolution of inflammation. Thus, HSP60, which is up-regulated by stress and inflammation, can innately resolve it

Keywords: Inflammation; Adaptive; Innate; Toll-like receptors; T cell; B cell; Th1/Th2 responses

ADAPTIVE VS INNATE IMMUNE SYSTEMS

The immune system is an organization of cells and molecules with specialized roles in defending against infection. There are two fundamentally different types of responses to invading microbes. Innate (natural) responses occur to the same extent however many times the infectious agent is encountered, whereas acquired (adaptive) responses improve on repeated exposure to a given infection. The innate responses use phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines. Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the antigen receptors of these cells bind to antigen (Delves and Roitt 2000).

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Adaptive immune responses are decisively controlled by the innate immune system: antigen-specific B or T cells recognize their peptide epitopes bound to innate MHC molecules expressed on innate antigen-presenting cells (APCs). Moreover, costimulatory molecules (CD80/CD86) and different sets of cytokines (IL-12 or IL-4) secreted by APCs determine the final destination of T-cell differentiation and the nature of the adaptive response (Dustin et al. 2001; Lanzavecchia and Sallusto 2002; Chambers and Allison 1997).

HEAT SHOCK PROTEINS AND THE ADAPTIVE IMMUNE SYSTEM

Heat shock proteins (HSP) are highly conserved proteins induced in response to cellular stress, such as heat shock or nutrient deprivation (Lindquist and Craig 1988) (Ferm et al. 1992). HSP assist the folding of newly synthesized proteins, participate in protein transport across membranes and refold proteins denatured during cell stress (Jindal et al. 1989). However, HSPs, and in particular, HSP60 interests immunologists because, in addition to serving as a chaperone inside the cell, HSP60 also functions as an antigen in host defense. It was discovered that variants of HSP60 were "common bacterial antigens" dominant in the immune response to many infectious agents (Young 1992). Later, HSP60 became a subject for autoimmunity research by the finding that a peptide epitope of Mycobacterial HSP60 was recognized by a clone of T cells capable of mediating arthritis in the adjuvant arthritis model (Cohen 1991; van Eden et al. 1988). Soon afterwards, mammalian HSP60 was found to be a target of T cells involved in the pathogenesis of type 1 diabetes in the NOD mouse model (Elias et al. 1990). HSP60 has been reported to be an important target for immune responses during chronic inflammation such as atherosclerosis (Wick 2000), Behcet's disease (Direskeneli et al. 2000), lupus (Yokota et al. 2000), and uveitis (Hu et al. 1998). Autoimmunity to self-HSP60, moreover, did not necessarily cause disease. The cord blood of newborn humans, like the peripheral blood of adults, manifests a relatively high frequency of T cells that can recognize to HSP60 (Fischer et al. 1992), and healthy adults manifest T-cell reactivity to HSP60 (Abulafia-Lapid et al. 1999). Thus, adaptive immune cells (T and B) respond to HSP60 via "adaptive" immune receptors (TCR and BCR, respectively).

HSP60 AND THE INNATE IMMUNE SYSTEM

A partial answer to the dominance of HSP60 as a target of autoimmune attack was provided by the discovery that the HSP60 could directly activate APCs, including macrophages and dendritic cells (Kol et al. 2000; Flohe et al. 2003), probably through binding to cell-surface receptors such as CD14, CD40, Toll-like receptors (TLRs) and the scavenger receptors CD36 and CD91. The observation that human HSP60 interact with TLRs (Ohashi et al. 2000; Vabulas et al. 2001) is intriguing, as it refutes the idea of TLRs as 'pattern recognition receptors' reserved for pathogens only (Cohen 2000a; Medzhitov and Janeway 2000). However, it was argued that

many of the reported pro-inflammatory effects that result from exposure of cells to HSP60 are actually mediated through LPS or other microbial compounds contaminating the HSP60 (Gao and Tsan 2003; Tsan and Gao 2004). In spite of the controversy surrounding the involvement of TLRs in the effects of HSP60 on the immune system, it became clear that HSP60 could play an important role in controlling adaptive immune responses through its effects on APCs and function as a powerful accessory signal in the induction of Th1-like responses (Flohe et al. 2003).

ADAPTIVE CELLS EXPRESS FUNCTIONAL INNATE RECEPTORS

The fact that B and T cells also express TLRs on their surface raised the question about the direct function of these receptors on the adaptive immune system. It was recently reported that TLR2 is expressed on the surface of activated and memory T cells. Furthermore, TLR2 functions as a co-stimulator receptor molecule for T-cell activation and helps to maintain T cell memory (Komai-Koma et al. 2004). Although TLR4 is also expressed on T cells, LPS did not affect T-cell function (Xu et al. 2005). Moreover, it was shown that TLR2 and TLR8 directly control function of regulatory T cells, a small subset of $CD4^+$ T cells with the ability to suppress the immune response (Sutmuller et al. 2006; Peng et al. 2005; Liu et al. 2006).

B cells are a principal component of the adaptive immune system, but also serve various innate immune functions (Tzehoval et al. 1983). Mature B cells express TLR4 and TLR2 and respond to innate stimuli, such as LPS and peptidoglycan (Han et al. 2003; Babu and Zeiger 1983).

HSP60 ACTIVATES B CELLS VIA TLR4

Recent studies in our laboratory demonstrated that human HSP60 induced naïve B cells to proliferate and to secrete IL-10 and IL-6 (Cohen-Sfady et al. 2005). In addition, the HSP60-treated B cells up-regulated their expression of MHC class II and accessory molecules CD69, CD40, and B7-2 (Cohen-Sfady et al. 2005). The effects of HSP60 on B cells were found to be largely dependent on TLR4 and MyD88 signaling, and not likely to be due to bacterial contamination. Moreover, treatment with HSP60 up-regulates the ability of B cells to activate an allogeneic T cell response, expressed in enhanced secretion of both IL-10 and IFN-γ by the responding T cells (Cohen-Sfady et al. 2005). Thus, HSP60 exerts innate immune effects on B-cell physiology directly via TLR4 signaling.

HSP60 DIRECTLY REGULATES T-CELL FUNCTIONS VIA TLR2

Human and mouse T cells treated with soluble HSP60 or HSP60-derived peptide p277 undergo a signal transduction cascade, activate integrin receptors, adhere specifically to fibronectin, down-regulate specific chemokine receptors (CXCR4 and CCR7) and show inhibited migration toward specific chemokines (SDF-1α and ELC) (Zanin-Zhorov et al. 2003). One of the possible mechanisms of the

inhibitory effects of HSP60 on T-cell chemotaxis is the ability of HSP60 to induce activation of the JAK/STAT pathway leading to up-regulation of the suppressor of cytokine signaling (SOCS)3 (Zanin-Zhorov et al. 2005a). In fact, up-regulation of SOCS3 was shown to lead to its interaction with chemokine receptor CXCR4 and impair cellular responses to SDF-1α, such as cell migration (Soriano et al. 2002). Moreover, specific inhibition of SOCS3 by siRNA completely prevented HSP60-mediated inhibition of T-cell chemotaxis (Zanin-Zhorov et al. 2005a).

The finding that HSP60 inhibits inflammatory diseases, such as adjuvant arthritis and type I diabetes by shifting specific immune reactions to a Th2-like response (Elias et al. 1997; Raz et al. 2001; Quintana et al. 2002) suggested a possible direct effect of the molecule on the cytokine secretion profile of T cells. Indeed, HSP60 was shown to modulate the expression of Th1/Th2 transcription factors: HSP60 down-regulates expression of the Th1-cell-promoting transcription factor

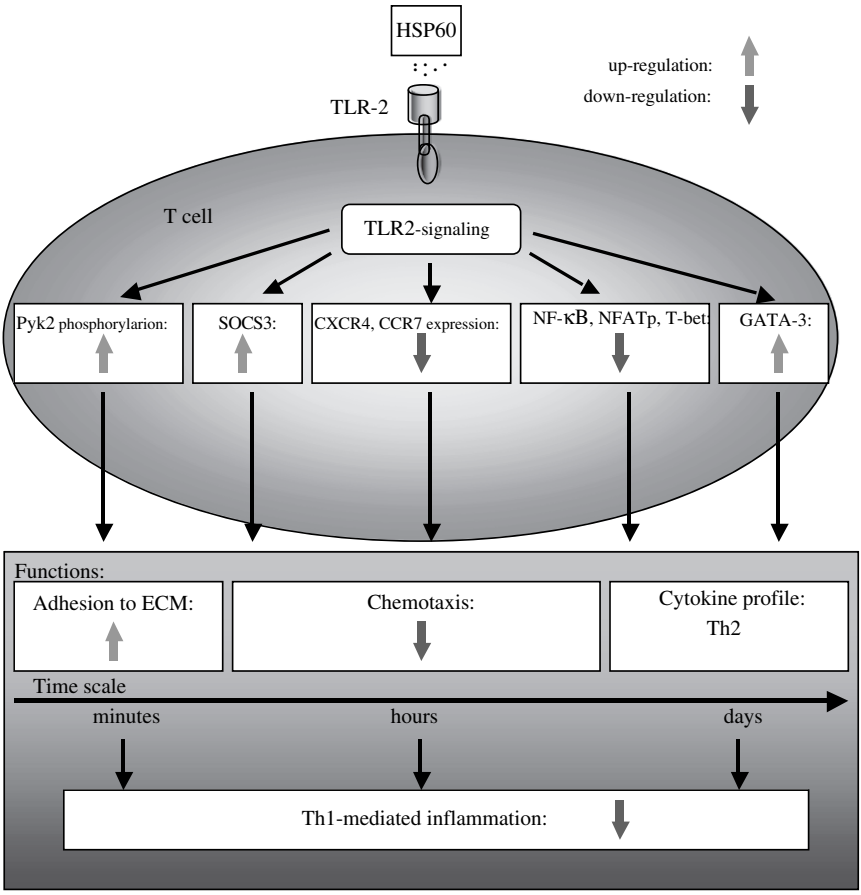

Figure 1. TLR2-dependent innate effects of HSP60 on T-cell mediated inflammation

T-bet, the transcription factor NF-κB, and the intracellular-signaling molecule NFATp; HSP60, in contrast, up-regulates the expression of the Th2-cell-promoting transcription factor GATA-3. This leads, in turn, to decreased secretion of TNFα and IFNγ and enhanced secretion of IL-10 (Zanin-Zhorov et al. 2005b). The innate effects of HSP60 on T cells were dependent on TLR2 signaling, and not on TLR4 (Zanin-Zhorov et al. 2003; Zanin-Zhorov et al. 2005a; Zanin-Zhorov et al. 2005b). Although a direct interaction between HSP60 and TLR2 on human T cells has not yet been shown, our findings indicate that TLR2 is involved in transducing HSP60 signaling (Figure 1).

HSP60 ENHANCES $CD4^+CD25^+$ REGULATORY T-CELL FUNCTION

A key issue in immunology is understanding how the immune system is able to discriminate between self and non-self, inhibiting autoimmune responses, but allowing effective immune responses against microbial antigens. One of the mechanisms evolved by the immune system is generation of $CD4^+CD25^+$ regulatory T cells, which actively suppress the activation and population expansion of potentially pathogenic self-reactive T cells (Shevach 2002; Sakaguchi et al. 2001). The involvement of $CD4^+CD25^+$ regulatory T cells in HSP60-mediated suppression of T-cell responses seems to be an attractive explanation for the protection effect of the molecule *in vivo* as was mentioned above. Indeed, the HSP60 molecule can function as a co-stimulator of $CD4^+CD25^+$ Tregs by way of an innate signaling pathway that involves TLR-2 (Zanin-Zhorov et al. 2006). Treatment of Tregs with HSP60, or its peptide p277, before anti-CD3 activation significantly enhanced the ability of relatively low concentrations of the Tregs to down-regulate $CD4^+CD25^-$ or $CD8^+$ target T cells, detected as inhibition of target T-cell proliferation and IFN-γ and TNF-α secretion. The enhancing effects of HSP60 co-stimulation on Tregs involved innate signaling via TLR-2, led to activation of PKC, PI-3 kinase, and p38, and were further enhanced by inhibiting ERK. HSP60-treated Tregs suppressed target T cells both by cell-to-cell contact and by secretion of TGF-β and IL-10. The down-regulated target T cells manifested inhibited ERK, NF-κB and T-bet (Zanin-Zhorov et al. 2006). Interestingly, known exogenous agonist of TLR2, Pam_3Cys too could augment Treg inhibition of cytokine secretion by $CD4^+CD25^-$ T cells. Thus, HSP60 via TLR2 acts as a co-stimulator of Treg function.

HSP60: A PLEIOTROPIC IMMUNE SIGNAL

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 (Cohen 2000b). If the inflammatory process is not properly regulated and balanced, then it itself can become the cause of significant damage.

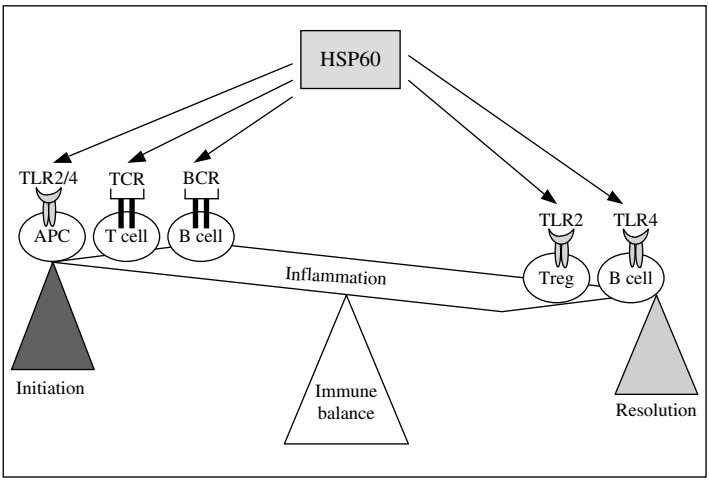

Figure 2. HSP60 has a balanced effect on immune inflammation. HSP60 can initiate an inflammatory response by activating APCs innately through TLR4 or by activating antigen-specific T cells and B cells by way of their antigen receptors. However, HSP60 can also resolve inflammation by inducing Treg function through TLR2. HSP60 can also inhibit the inflammatory response by its effects on B cells through TLR4

The HSP60 molecule, as it performs its chaperone function in stressed cells, also functions as a regulatory signal molecule to the wandering cells of the immune system on the look out for trouble (Figure 2). HSP60 is present in the blood during inflammation, and has been found to be a target of autoantibodies and autoimmune T cells. Moreover, HSP60 induces pro-inflammatory responses in innate immune cells. However, the administration of HSP60 was found to arrest the destructive inflammation responsible for various models of autoimmune diseases. Some explanation for this fact may be found in our recent discovery that HSP60 can directly regulate T and B-cell responses, via TLR2 and TLR4 respectively, leading also to the resolution of inflammation. The full clarification of dynamics of the crosstalk between the two arms of responses induced by HSP60 need to be worked out; the results will give us a new perspective in understanding of the role of HSP60 in inflammatory response and how we may manipulate it to our advantage.

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PART IV

ANTIGEN PROCESSING, PRESENTATION AND EFFECT ON INFLAMMATION AND DISEASE

CHAPTER 17

IMPACT OF HSP-CHAPERONED PEPTIDES ON THE MHC CLASS II-DEPENDENT PRESENTATION AND ACTIVATION OF CD4$^+$ T CELLS IN REGARD OF ALLO- AND AUTOANTIGENS

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Abstract: HSP70:peptide complexes have multiple impact on APCs and consequently on the stimulation of T cells. HSPs activate APCs (innate response) and facilitate presentation of chaperoned peptides via MHC class I and II molecules (adaptive response) followed by effective CTL and CD4$^+$ T cell responses. Cross-presentation of MHC class I-restricted epitopes is well investigated, the role in MHC class II-restricted antigen-presentation remains less clear but it becomes more and more obvious that peptides chaperoned by eukaryotic as well as prokaryotic HSPs particularly of the 70 kDa family are involved in MHC II-restricted presentation. HSP70 molecules bind a wide variety of self- and foreign peptides in an ATP- and pH-dependent manner and resulting HSP:peptide complexes can access MHC class II loading compartments via different possible mechanisms. Pro- and eukaryotic HSP70 chaperones have been found to facilitate processing and presentation of MHC class II-restricted antigens resulting in enhanced CD4$^+$ T cell activation in response to allo- and auto-antigens. Further investigating the role of HSP70 in MHC II-restricted antigen-presentation will be of particular interest for a better understanding of the pathogenesis of autoimmune disorders and CD4$^+$ T helper cell mediated tumour recognition

Keywords: MHC class II, HLA-DR, Hsp70, CD4$^+$ T cells, Hsp70:peptide complexes, autoimmunity

PEPTIDE-BINDING BY HSP70 MOLECULES

The highly conserved 70 kDa HSPs are chaperones that assist in many different intracellular processes including folding of newly synthesized proteins, refolding of misfolded, aggregated or partially folded proteins, degradation of proteins as well as translocation of proteins across membrane barriers (Bukau *et al*. 2000;

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Chiang *et al*. 1989; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005; Pilon and Schekman 1999). All of these housekeeping functions are based on the property of HSP70 molecules to interact in an ATP-dependent manner with hydrophobic peptide sequences.

Members of the HSP70 family consist of an N-terminal ATPase unit of 45 kDa and a C-terminal substrate binding domain of 25 kDa which is further subdivided into a β-sandwich subunit of 15 kDa and a C-terminal alpha-helical subdomain. The ATPase cycle of HSP70 proteins is involved in binding and release of substrates and consists of an interplay between the ATP state with low affinity and fast exchange of substrates and the ADP state with high affinity and slow exchange rates for substrates. The molecular mechanism of the ATPase and substrate binding cycle shows some differences between HSP70 homologues and has been characterized only for a few HSP70 proteins in detail so far, including *E.coli* DnaK and bovine stress-inducible Hsc70 (Flaherty *et al*. 1990; Mayer *et al*. 2000; Mayer and Bukau 2005; Rudiger *et al*. 1997a). Binding specificities for 3'-sulfogalactolipids (Mamelak and Lingwood 2001) as well as O-linked N-acetylglucosaminylated proteins (Lefebvre *et al*. 2001) have been described for the ATPase domain of HSP70 molecules.

Besides nucleotide exchange, peptide binding is influenced by the interaction of HSP70 with co-chaperones of the Hsp40 family. DnaJ and GrpE have been identified as co-chaperones of *E.coli* DnaK (Liberek *et al*. 1991). They play a key-function in stimulating the ATPase activity and activating the substrate binding of DnaK (Rudiger *et al*. 2001; Suh *et al*. 1998). In eukaryotes Hip (HSP70-interacting protein), Hop (HSP70-HSP90-organizing protein) and CHIP (carboxy terminus of HSP70 interacting protein) have been identified as HSP70 co-chaperones so far (reviewed in (McClellan and Frydman 2001)). In view of the complex and not yet completely understood mechanism of HSP70-interaction with substrates, it is surprising that HSP70 molecules can bind and release peptides in the absence of ADP/ATP and co-chaperones in vitro, and that even truncated DnaK lacking the ATPase domain binds peptides (MacAry *et al*. 2004; Zhu *et al*. 1996).

The substrate binding domain is highly conserved among members of the HSP70 family. Most structural information on this domain is provided for *E.coli* DnaK, for which the X-ray structure of the substrate binding domain was identified in complex with a peptide substrate by Zhu *et al*. (Zhu *et al*. 1996). The architectural requirements for binding substrates to the substrate binding cavity of DnaK deduced from the X-ray structure were found to be in good accordance with data determined by binding assays (Gragerov *et al*. 1994; Richarme and Kohiyama 1993; Rudiger *et al*. 1997a; Rudiger *et al*. 1997b): The binding motif of DnaK was found to consist of a core sequence of four or five amino acids (aa) enriched in hydrophobic residues and two flanking regions enriched in basic residues. Acidic aa residues were excluded from the core region and disfavoured in flanking regions. DnaK-affinity to the positively charged aa arginine and lysine was reported, particularly in the context with hydrophobic residues (Gragerov *et al*. 1994). Binding sites in proteins were estimated to occur in proteins statistically every 36 aa residues (Rudiger *et al*.

1997b). Similar binding motifs were determined for other HSP70 proteins (Gragerov and Gottesman 1994) and constituively expressed Hsc70 showed enhanced affinity to hydrophobic peptides enriched in the positively charged sequences KK, KR, RR (Takenaka *et al*. 1995).

INTERACTION OF HSP70 MOLECULES WITH ANTIGENIC PEPTIDES

As mentioned afore, HSP70 molecules interact with a wide variety of preferentially hydrophobic peptide sequences. It is known that HSP-preparations (HSP70, HSP90, gp96, calreticulin, HSP110 and GRP170) from tumour cells elicit tumour-specific immunity, whereas HSP-preparations from normal tissue did not (reviewed in (Srivastava *et al*. 1998)). This immunogenicity resulted from peptides generated by protein degradation in the tumour cells from which the HSPs were purified. Similar to MHC molecules, HSP molecules are able to bind a variety of peptides from different proteins existing in the cell from which they originate which can be termed the peptide fingerprint of the cell. HSPs bind and protect these peptides from further proteasomal degradation and subsequently make them accessible for presentation via MHC molecules. This scenario implicates a role for HSP molecules as scanner and carrier for antigenic peptides that are presented afterwards via MHC molecules to T cells, eliciting antigen-specific T cell responses.

In regard to binding of HSP70 to viral and auto-antigens, HSP70-interaction with antigenic peptides from common antigens like tetanus-toxin C-fragment and influenza hemagglutinin was found (Haug *et al*. 2005; MacAry *et al*. 2004). For mycobacterial HSP70 molecule K_D-values for the affinity to antigenic peptides were determined in the low micromolar range (MacAry *et al*. 2004) and are thus of relatively low affinity. Affinities decreased when ATP was added to the binding buffer. Furthermore stress-inducible Hsp70 but not Hsp90 has been shown to co-precipitate with the putative autoantigens myelin basic protein (MBP) and myelin proteolipid protein (PLP) in brain tissue from multiple sclerosis (MS) patients (Cwiklinska *et al*. 2003). Immunodominant epitopes from MBP were shown to interact selectively with Hsp70 in an ATP- and pH-dependent manner (Mycko *et al*. 2004).

Beside the notion that HSP70 molecules are involved in the onset of multiple sclerosis, they seem to play a role in the pathogenesis of other autoimmune disorders like rheumatoid arthritis. Mammalian and bacterial HSP70 molecules have been shown to interact with peptide fragments from a highly variable region of HLA-DR molecules (Auger *et al*. 1996). Interestingly a peptide fragment that is exclusively found in HLA-DR molecules mediating strong protection in a proposed rheumatoid arthritis protection model (Zanelli *et al*. 1995) did not interact with HSP70 (Maier *et al*. 2002). Speculating about the relevance of this finding for the pathogenesis of rheumatoid arthritis, a scenario is conceivable where non-binding of the peptide fragment from RA-protective HLA-DR molecules could admit these peptides to proteolysis, whereas complete proteolysis of other HLA-DR peptide fragments is

prevented by binding to HSP70 proteins. Some of the protected peptide sequences particularly from HLA-DR molecules highly associated with rheumatoid arthritis could fit into other associated MHC molecules like HLA-DQ and subsequently be presented to T cells, thus inducing an autoimmune reaction, e. g. by molecular mimicry. Natural processing and presentation of endogenous peptide fragments covering this extremely variable region of HLA-DR has been demonstrated (Chicz *et al*. 1993).

INNATE AND ADAPTIVE EFFECTS OF HSP ON APC

Upon cell stress and cell death HSP:peptide complexes are released (Basu *et al*. 2000; Bausero *et al*. 2005) and subsequently interact with APCs with different consequences. HSPs are able to induce cytokine release and the maturation and activation of APCs such as dendritic cells (Asea *et al*. 2000; Singh-Jasuja *et al*. 2000a; Vabulas *et al*. 2002). The receptors mediating these innate HSP-effects on APCs are not yet definitely identified; TLR2, TLR4, CD36, CD40 and CD91 are some receptors that are discussed so far.

On the other hand extracellular HSP:peptide complexes have adaptive (antigen-specific) effects on APCs: the re-presentation of the HSP-chaperoned peptides via MHC class I and II molecules. For this effect the extracellular HSP:peptide complexes are endocytosed e.g. via CD91 in a receptor-mediated process (Basu *et al*. 2001; Becker *et al*. 2002; Binder *et al*. 2000; Binder and Srivastava 2004; Vabulas *et al*. 2002), enabling them to re-present CTL- and Th cell-epitopes.

The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime $CD8^+$ cell effector responses has been well established (reviewed in (Srivastava 2002). Mammalian HSPs such as the human cytosolic 70 kDa HSP (Hsp70) or gp96 are able to form highly immunogenic HSP:peptide complexes and to elicit antigen-specific $CD8^+$ T cell responses to the chaperoned peptides (Singh-Jasuja *et al*. 2000b; Udono and Srivastava 1993). This effect can be attributed to the peptide-binding domain of HSP70: Truncated mycobacterial HSP70 consisting only of the minimal HSP70 peptide binding domain was found to bind antigenic peptides and to elicit CTL-responses against chaperoned peptides as effective as wild-type HSP70 (MacAry *et al*. 2004).

In contrast, the role for HSP:peptide complexes in the MHC class II-pathway is less clear, although several findings indicate that HSP:peptide complexes also facilitate the presentation of MHC class II-restricted epitopes. The comprehension of the role of HSP70 molecules in MHC class II-restricted T cell responses is of particular interest for a better understanding of the pathogenesis of autoimmune disorders and $CD4^+$ T cell help in tumour recognition.

UNVEILING THE FUNCTIONS OF HSP:PEPTIDE-COMPLEXES IN MHC CLASS II-RESTRICTED ANTIGEN PRESENTATION

First evidence for an involvement of members of the HSP70 family in the MHC class II processing and presentation pathway was implicated by DeNagel and Pierce in the early 1990s (DeNagel and Pierce 1992) suggesting that HSP70 molecules

might be involved in facilitating the assembly process of MHC class II molecules and antigenic peptides. However these findings did not provide a mechanism by which HSP70 molecules could be able to access the late MHC class II loading compartments. This deficit has meanwhile been abrogated by other studies as mentioned below.

Another hint for a role of endogenous HSP70 molecules in MHC class II peptide presentation was the observation by Hoeger *et al*. (Hoeger *et al*. 1994) that the irreversible HSP70 inhibitor 15-desoxyspergualin (DSG) inhibited MHC class II-restricted antigen presentation. Treatment of monocytes with DSG inhibited the T cell response to tetanus and diphtheria allo-antigens, but not to non-specific T cell mitogens like phytohemagglutinin (PHA).

Some years later Panjwani *et al*. (Panjwani *et al*. 1999) demonstrated that over-expression of the constitutively expressed Hsc73 in a murine macrophage cell line led to an increased presentation of exogenous antigen via MHC class II. Exogenous antigen was found to associate with Hsc73 within the macrophages, the association was found to be sensitive to ATP and the HSP70 inhibitor DSG. The authors concluded that Hsc73 plays a role in protecting peptides from degradation and facilitates the peptide transfer onto MHC class II molecules.

In a murine model, gp96:peptide complexes facilitated the *in vivo* presentation of a MHC class I and II-restricted epitopes from hemagglutinin (Doody *et al*. 2004). Immunization of mice with gp96:peptide complexes induced proliferation of naïve $CD4^+$ T cells but interestingly neither the secretion of the effector cytokines IL-4 and IFN-γ nor induced tolerance were detected. This work with gp96, an endoplasmatic reticulum-member of the 90 kDa HSP family, demonstrates that HSP-mediated effects on MHC class II antigen presentation is not exclusively restricted to HSPs of the 70 kDa family. Furthermore it has been shown that $CD4^+$ and $CD8^+$ T cell epitopes are electively processed and presented from an exogenously administered HIV-1 peptide complexed to gp96 (SenGupta *et al*. 2004).

A role in MHC class II-restricted antigen presentation is not exclusive to mammalian HSP. Also bacterial HSP70:peptide complexes were found to enhance immunogenicity of HSP-chaperoned peptides. Enhanced processing and presentation of exogenous full-length recombinant human acetylcholine receptor alpha subunit to a specific $CD4^+$ T cell clone was caused by *E.coli* DnaK (Roth *et al*. 2002). Interestingly the enhancement was found to be dependent on the HLA-DR haplotype of the APCs. Tobian *et al*. (Tobian *et al*. 2004b; Tobian *et al*. 2004a) demonstrated enhanced MHC class I and II-restricted presentation and processing of an extended OVA-peptide in murine macrophages and DCs when the peptide was complexed to *E.coli* DnaK or *Mycobacterial tuberculosis* HSP70. Enhanced MHC class I-restricted presentation with prokaryotic HSP70 was dependent on receptor-mediated uptake via CD91 (Tobian *et al*. 2004a), whereas the effect on MHC class II-restricted presentation was found to be independent of CD91 (Tobian *et al*. 2004b). It was hypothesized that differences in MHC class I- and MHC class II-pathway and intracellular trafficking of CD91 could explain these observations. These findings also implicate that receptors other than CD91 are involved

in HSP70-enhanced MHC class II-restricted antigen-presentation. The same study showed that uptake and processing of the extended OVA-peptide was required for an enhanced antigen-presentation with bacterial HSP70. Bacterial HSPs facilitated delivery and binding of chaperoned peptides to MHC class II molecules under acidic but not neutral conditions. Therefore it might be speculated that the transfer of HSP70-chaperoned peptides onto MHC class II-molecules involves direct HSP70:HLA-DR interaction.

SEPARATING HSP70-EFFECTS FROM EFFECTS OF MICROBIAL CONTAMINANTS

Most of the above-mentioned studies use recombinant HSP70-preparations purified from bacterial source. Therefore, it is most important to confirm that the observed HSP70-effects can be clearly attributed to HSP70 molecules and are not artifacts due to microbial contaminations in HSP-preparations. Recombinant proteins purified from gram-negative bacteria can contain endotoxins like LPS which are well known to have an activating effect on APCs on their own via TLR-signalling.

Therefore low-endotoxin HSP70-preparations have to be used when testing the effect of HSP70 on activation of $CD4^+$ T cells and LPS should be used in appropriate control settings. Tobian *et al*. therefore demonstrated that HSP70-enhanced presentation is intact in knock-out cells lacking most TLR-signalling (Tobian *et al*. 2004b) and other studies showed that pre-incubation of antigenic peptide did not result in an enhanced activation of antigen-specific $CD4^+$ T cells (Haug *et al*. 2005). These results are supported by experiments where adding peptide and HSP70 without pre-incubation (no complex-formation) had no effect on $CD4^+$ T cell activation (Haug *et al*. 2005; Tobian *et al*. 2004b) thus ruling out an effect of contaminating bacterial substances like LPS.

POSSIBLE MECHANISMS OF IMMUNOGENICITY

MHC class II molecules are loaded in specialized vesicles, the MHC class II loading compartments (MIIC) (Neefjes 1999). To participate in MHC class II antigen presentation, HSP:peptide-complexes must access these acidic compartments. Mechanisms for extra- and intracellular HSP:peptide complexes may be hypothesized.

HSP:peptide complexes are normally located within the cell, but can be released for example by necrotic cells (Basu *et al*. 2000; Bausero *et al*. 2005). These extracellular HSP:peptide complexes can be endocytosed by APCs via CD91 in a receptor-mediated process (Basu *et al*. 2001; Becker *et al*. 2002; Binder *et al*. 2000; Binder and Srivastava 2004; Vabulas *et al*. 2002). Other receptors may be involved in the uptake, particularly of prokaryotic HSP:peptide complexes (Tobian *et al*. 2004b). It has been shown that internalized HSP:peptide-complexes can then enter endocytic compartments enriched in MHC class I and II molecules (Lipsker *et al*. 2002).

Autophagic processes might also be involved as it was recently demonstrated that autophagic compartments are enriched in Hsp70 (Dengjel *et al*. 2005). Thus cytosolic Hsp70:peptide complexes may reach the endosomal pathway.

A direct HSP70:HLA-DR interaction has been discussed since several years. For example co-precipitation of HLA-DR molecules with HSP70 molecules was found in immune-precipitation experiments, where differences between the allelic variants of HLA-DR were detected (Auger *et al*. 1996; Auger *et al*. 2002). Particularly highly rheumatoid arthritis susceptible HLA-DR molecules carrying the so-called shared-epitope (Gregersen *et al*. 1987) were found to interact with Hsc70. The possibility of a direct and allele-specific interaction of HSP70 with certain HLA-DR molecules would be of great significance towards a better understanding of the HLA-association of certain auto-immune disorders.

However, the findings by Auger *et al*. have been discussed controversial. Rich *et al*. (Rich *et al*. 1998) did show a non-specific co-precipitation of Hsc70 and MHC class I and II molecules. Furthermore allele-specific differences in processing and presentation of human acetylcholine receptor alpha subunit mediated by DnaK (Roth *et al*. 2002) cannot be explained by the findings of Auger *et al*. (Auger *et al*. 1996; Auger *et al*. 2002) as both HLA-DR haplotypes used as APCs in these experiments did carry the shared-epitope sequence. In a quantitative binding assay a specific interaction between HLA-DR molecules and constitutively expressed and stress-inducible mammalian HSP70 could be detected (Haug *et al*. 2007). This interaction was independent of the allelic variant of the HLA-DR used and no interaction between HSP70 and MHC class I molecules or control proteins was observed; affinities were found to increase at lowered pH, whereas peptide-affinity of HSP70 decreased at acidic pH-values. Thus, a scenario is conceivable, in which Hsp70 interacts with HLA-DR at the acidic pH of vascular compartments and directly transfers chaperoned peptide onto MHC class II molecules in a ternary complex.

This mechanism is speculative so far and the role of other molecules like HLA-DM and MHC class II invariant chain (li) have to be addressed. Interestingly constitutively expressed Hsc70 has been identified as cytosolic protein capable of interacting with li (Lagaudriere-Gesbert *et al*. 2002).

IMPLICATIONS FOR HSP INVOLVEMENT IN CD4$^+$ T CELL ACTIVATION: ALLO- AND AUTOIMMUNITY, TUMOUR IMMUNOLOGY

Most papers addressing the role of HSP:peptide complexes in processing and presentation of MHC class II-presented antigens have investigated CD4$^+$ T cell response in the context with allo-antigens derived for example from tetanus-toxin, diphtheria toxin, influenza hemagglutinin or HI-virus (Doody *et al*. 2004; Haug *et al*. 2005; Hoeger *et al*. 1994). In regard to autoimmunity and tumour immunology,

HSP-impact on the presentation of antigens possibly involved in autoimmune processes is of particular interest as well as $CD4^+$ T cell help in tumour immunology.

As outlined above, HSP molecules bind to antigenic peptides discussed to be involved in autoantigenic processes as well as to tumour antigens. An involvement of inducible Hsp70 in MHC class II-dependent processing and presentation of autoantigen was reported by Mycko *et al*. (Mycko *et al*. 2004). Hsp70 was found to co-localize with MBP, a major putative autoantigen in multiple sclerosis (Steinman 1996), in the endocytic pathway of APCs. Over-expression of Hsp70 yielded to enhanced responses of a MBP-specific T cell hybridoma. The peptides $MBP_{80–99}$ and $MBP_{85–99}$, both interacting selectively with Hsp70, were used for T cell stimulation. But only with the longer peptide Hsp70-enhanced responses were detected. Therefore it was suggested that Hsp70 rather contributes to antigen-processing than presentation.

Two more examples for an involvement of HSPs in other autoimmune diseases, as already mentioned above, are the *E.coli* DnaK-enhanced processing of exogenous human acetylcholine receptor alpha subunit (Roth *et al*. 2002), a protein targeted in myasthenia gravis and the finding that DnaK binds to HLA-DR peptide fragments with the exception of a peptide-fragment mediating protection from rheumatoid arthritis (Maier *et al*. 2002).

However, most of these studies showing an enhanced processing and presentation of HSP-chaperoned peptides via MHC class II molecules were performed in murine systems or with human T cell clones. $CD4^+$ T cells only recognize peptide antigens in the context with distinct HLA-DR molecules. It is difficult to analyze specificity of these cells in the human system. Traditionally, functional assays such as cytokine secretion or proliferation in response to antigen were used to infer from qualitative or semi-quantitative data to T cell specificity. Alternatively T cell clones or hybridomas have been used to identify specificity of $CD4^+$ T cells. This problem has been solved by the development of HLA class II tetramers, a tool that allows direct visualization of human antigen-specific $CD4^+$ T cells (Novak *et al*. 1999; Buckner *et al*. 2002; Danke and Kwok 2003; Nepom *et al*. 2002). Using HLA class II tetramers antigen-specific $CD4^+$ T cells can be distinguished from all other $CD4^+$ T cells, allowing the visualization of antigen-specific T cells in peripheral blood at a frequency as low as 0,003% (Danke and Kwok 2003). HLA class II tetramers are multimers of recombinant HLA molecules associated with bound peptide antigens, cross-linked by fluorescence-labeled streptavidin. The tetramers provide multiple ligands for interaction with antigen-specific T cell receptors. This multiplies overall avidity of the molecule, allowing the analysis of low-affinity interactions of T cell receptors with HLA-molecules (Davis *et al*. 1998).

Using HLA-DR tetramer technology it could recently be shown in a human antigen-specific setup that cytosolic Hsp70 enhances proliferation of $CD4^+$ memory T cells (Haug *et al*. 2005). Proliferation of human peripheral $CD4^+$ T cell was investigated in response to peptide epitopes from tetanus toxin C-fragment and influenza hemagglutinin that have been described as highly immunogenic in the context with certain HLA-DR molecules. Antigen-specificity of proliferated human $CD4^+$ T cells

was confirmed using HLA-DR tetramers. With this setup it could be demonstrated that Hsp70:peptide-complexes amplify the proliferation of human antigen-specific $CD4^+$ memory T cells in healthy individuals in response to allo-antigens from tetanus-toxin and the influenza-virus. Complex-formation of the antigenic peptide with the Hsp70 molecule was crucial for an enhancement in T cell activation, peptide and Hsp70 added to T cells without complex formation did not result in an enhanced antigen-specific $CD4^+$ T cell proliferation. Interestingly stimulation with Hsp70:peptide complexes was found to be most superior at limiting conditions, such as extremely low concentration of antigen or reduced number of APCs. These findings confirm the results from the murine system and with T cell clones or hybridomas and extend them to a human antigen-specific setting demonstrating an enhanced immunogenicity of Hsp70-chaperoned alloantigenic peptides with T cells from the peripheral blood of healthy donors.

Concerning tumour immunology, increasing attention is attracted to the $CD4^+$ help in CTL-activation. Tumour antigens are not only recognized by T cells directly on the surface of tumour cells but also after cross-priming via APCs. An immune response against MHC-negative tumours as well as maintenance of an effective immune response may be elicited by this mechanism (Kalams and Walker 1998). Little is known about an impact of HSP molecules in this mechanism but it has been shown that IFN-γ mediates enhanced surface expression and active release of Hsp70 from tumour cells (Bausero *et al*. 2005). Hsp70 can be detected on the surface of tumour but not normal cells and has been shown as tumor-specific recognition structure for NK cells (Multhoff *et al*. 1997). Furthermore it was reported that the gp90 antigen of the murine CT26 colon cancer interacts with the endoplasmatic reticulum chaperone calreticulin (CRT) (Golgher *et al*. 2001). The immunogenicity of gp90 correlated well with CRT-binding.

OUTLOOK

A whole series of MHC II-restricted putative tumour and autoantigens have been identified meanwhile. However few is known about mechanisms why for example autoimmune disorders occur in some individuals and others not, even if carrying the same MHC class II haplotype.

$CD4^+$ T cells directed against autoantigens as well as antigens expressed on tumour surfaces can be detected in both, patients and healthy individuals. T cells directed against the diabetes-associated antigen glutamic acid decarboxylase 65 (GAD65), the melanoma and vitiligo-associated antigen tyrosinase as well as the cancer and testis antigen NY-ESO1 have been detected in healthy individuals (Danke *et al*. 2004). Analysis of GAD 65-reactive $CD4^+$ T cells in type 1 diabetes patients and healthy subjects suggested that there is no difference in thymic selection amongst type 1 diabetes patients and healthy individuals, although GAD 65-reactive T cells were found to be preferentially activated in patients (Danke *et al*. 2005). These findings indicate that deficiencies in thymic depletion

of autoreactive T cells can not be charged as the unique factor in onset of autoimmune disorders. Other results propose that suppression by $CD4^+CD25^+$ T regulatory (T_{reg}) is defective in patients with type 1 diabetes. This poor understanding of onset of autoimmune diseases as well as $CD4^+$ T cell help in tumour recognition draws the interest to further investigate the role of HSPs in these processes.

With regard to the onset of autoimmune disorders the enhanced processing and presentation of HSP-chaperoned MHC class II-restricted antigens make a scenario conceivable, where the induction of HSP by certain stress factors facilitates an immune response to (auto-) antigenic peptides. Peptides chaperoned by HSP could decrease the threshold of T cell activation resulting in an enhanced number of (auto-) antigen-specific $CD4^+$ T cells. Thus the increased presence of HSP in "stressed" areas can alter MHC II-restricted antigen presentation and activate $CD4^+$ T cells that would not have been activated in the absence of HSP. This could explain why for example infections seem to be involved in the onset of some autoimmune disorders and why autoimmunity occurs in some individuals with the same HLA haplotype but not in others.

Different associations of HSP molecules and autoimmunity have been implicated (Millar *et al*. 2003; Mycko *et al*. 2004; Pockley 2003; van Eden *et al*. 2005) and are discussed to some extent in other parts of this book. From the data obtained with HSP-chaperoned peptides the hypothesis may be postulated, that the presence of HSP, e.g. after cell death, might be involved in the initiation of autoimmunity by facilitating an immune reaction to a possible auto-antigenic peptide at concentrations where peptide without HSP would be not immunogenic at all. Another aspect of HSP-function that has to be considered concerning onset of autoimmune disorders is for example the feature that HSPs are capable to activate APC and to alter cytokine secretion of different cell types (innate HSP effects) and thus may affect $CD4^+$ T cell activation. Furthermore there are multiple studies focussing on T cell reactivity against HSP-derived epitopes themselves. For example in patients with juvenile idiopathic arthritis that had a favorable prognosis, increased reactivity towards Hsp60 was found (De-Graeff-Meeder *et al*. 1995). This increased Hsp60-reactivity might contribute to immunoregulation and remission of disease, possible Hsp60 T cell epitopes were recently identified (Kamphuis *et al*. 2005). Other studies show that induction of self-Hsp60 and self-Hsp70 T cell reactivity can control pro-inflammatory responses by production of regulatory Th2-type cytokine responses and $CD4^+CD25^+$ T_{regs} (reviewed in (Pockley 2003; van Eden *et al*. 2005)).

This induction of regulatory T cell responses by T cells reactive to self-HSP has to be distinguished from the enhanced reactivity to peptides chaperoned by HSPs. But increased reactivity of T cells against peptides chaperoned by HSP versus reactivity against HSP itself inducing immunoregulation are different aspects explaining possible mechanisms of an involvement of HSP in the pathogenesis of autoimmune diseases. The balance between these two phenomenons may play an important role in regard of initiation and remission of these diseases.

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CHAPTER 18

HEAT SHOCK PROTEINS ARE TARGETS FOR T CELL REGULATION: HOW MICROBIAL HSP INDUCE IL10 PRODUCING ANTI-INFLAMMATORY T CELLS

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Abstract: Mechanisms of anti-inflammatory T cell regulation are studied very actively, especially since the (re-)discovery of T cell subpopulations with specialized regulatory activities. This is expected to lead to the development of novel immunotherapeutic approaches, especially in chronic autoimmune diseases. HSP are possible targets for regulatory T cells due to their enhanced expression in inflamed (stressed) tissues and the collected evidence that HSP immunizations induced anti-inflammatory immunoregulatory T cell responses. First evidence for an immuno-regulatory role of heat shock proteins (HSP) in autoimmunity was obtained through the analysis of T cell responses in the rat model of adjuvant arthritis and the findings that HSP immunisations protected against the induction of various forms of autoimmune arthritis in rat and mouse models. Since then, immune reactivity to HSP was found to result from inflammation in various disease models and in a variety of human inflammatory conditions. Now, also in the light of a growing interest in T cell regulation, it is of interest to further explore the mechanisms through which HSP have the potential to trigger immune regulatory pathways, capable of suppressing inflammatory diseases. And this even more, since in initial clinical trials in patients with RA and type 1 diabetes, HSP-derived peptides have been seen to promote the production of anti-inflammatory cytokines. In addition, the growing concern over the rise of some autoimmune conditions and allergies in Western countries, possibly related to changed life style, has generated interest in immune responsiveness to particular microbial antigens, such as HSPs, which are known to be dominantly immunogenic and to have a disease preventive/therapeutic effect in models of autoimmune diseases and allergic asthma. Heat shock proteins have received attention by immunologists since their discovery and especially since it was observed that under various conditions immune responses to HSP were readily developing. Through their evolutionary conservation, their role in maintaining integrity of cellular proteins and their stress inducibility, their potential impact

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on the organization of immune reactivity in mammals is considered to be broad and multi-facetted. Their role as chaperones for intracellular proteins such as tumor antigens, their role as proteins to deliver protein antigens for cross-presentation to CD8+ T cells and some other aspects have been the subject of various reviews (Morimoto, 1998; Nichitta, 2003). The current chapter will concentrate on immune responses induced by HSP as antigens, and the potential of such responses to control inflammation

Keywords: Arthritis, autoimmunity, HSP, inflammation, MHC, tolerance

MICROBIAL HSP SUPPRESS DISEASE IN EXPERIMENTAL MODELS OF AUTOIMMUNE DISEASES AND OTHER INFLAMMATORY CONDITIONS

We obtained initial evidence for a role of HSP in autoimmunity when working with the adjuvant arthritis model. This model was coincidentally discovered by Pearson in the 1950's, when he immunized rats using heat killed mycobacteria in oil as an adjuvant. On the basis of the histology of affected joints, adjuvant arthritis was characterized as a model for chronic rheumatoid arthritis in humans and its nature as a T cell mediated autoimmune condition was established through the successful transfer of disease into naïve recipient rats with T cells obtained from diseased animals (Whitehouse et al., 1969). These transfer studies were refined later by Holoshitz et al. when they succeeded in transferring disease into irradiated Lewis rats with a T cell clone, called A2b, obtained from mycobacteria immunized animals and having specificity for an unknown M. tuberculosis antigen (Holoshitz et al., 1983). Having available, herewith, a T cell clone with a single antigen specificity and having the potential of transferring arthritis, a search was started to uncover the hypothetical self antigen in joints recognized by T cell A2b. On the basis of proliferative responses in the presence of crude cartilage preparations, the self-antigen involved could be determined to be related to an antigen in the proteoglycan fraction of joint cartilage (van Eden et al., 1985). Thus, although the exact antigen in mycobacteria that induced disease producing T cells was unknown, adjuvant arthritis seemed to be narrowed down to a case of antigenic mimicry on the basis of T cell cross-reactivity between an antigen of cartilage proteoglycan and mycobacteria.

Mycobacterial Hsp60 Discovered as a Critical Antigen in Adjuvant Arthritis

The exact nature of the arthritogenic mycobacterial antigen was discovered, when we tested a 60kDa recombinant mycobacterial antigen, cloned by van Embden. T cell A2b showed a vigorous proliferative response in the presence of this antigen, which even exceeded the level of responsiveness towards its original antigen, crude mycobacteria (van Eden et al., 1988). Analysis of the gene sequence coding for this antigen revealed its nature: it was a member of the Hsp60 family of bacterial heat shock proteins. This finding of a conserved bacterial HSP as the antigen for arthritis producing T cells in the AA model, triggered a wide search for HSP immune

reactivity in other disease models and in human autoimmune diseases. For obvious reasons these studies were initiated with the idea that self-HSP were possibly the long sought autoantigens with a broad significance to autoimmunity in general. And indeed, autoreactivity to HSP has been found in almost every human autoimmune condition studied, as we will discuss later. The actual disease causing T cell epitope for A2b in mycobacterial Hsp60, the sequence 180–188 of mycobacterial Hsp60, was however not conserved. And, as therefore expected, disease producing T cell A2b did not respond to rat Hsp60. Thus, the T cell that had defined HSP as an antigen critical to disease did not have the self homologous HSP as a target autoantigen (van Eden et al., 1988).

And in addition to this, despite the fact that Hsp60 was the mycobacterial antigen that stimulated the arthritis producing capacity of T cell A2b in AA, when the mycobacterial Hsp60 molecule was isolated from the context of the entire mycobacterium and used to immunize, no disease developed. Interestingly however, immunization was seen to lead to resistance to subsequent disease induction instead (van Eden et al., 1988). This was also found in a similar model in Lewis rats, where disease was induced with a fully synthetic non-antigenic compound known as avridine or CP20961 and which does not contain any microbial constituents (Billingham et al., 1990; Kingston et al., 1996). And herewith, it became clear that the protective effect of mycobacterial Hsp60 in AA was not dependent on antigenic relationships between the disease inducing and the protective antigen.

Mycobacterial Hsp60 and Other HSP Induce Protection in Mouse and Rat Arthritis Models

Subsequent experiments carried out in various experimental models have now further substantiated the arthritis inhibitory effect of mycobacterial Hsp60 immunisation. This was the case for streptococcal cell wall induced arthritis in rats (van den Broek et al., 1989), adjuvant arthritis in rats (Billingham et al., 1990), pristane induced arthritis in mice (Thompson et al., 1998) and to some extend collagen induced arthritis in rats (Kingston et al., 1996). Ragno et al. have shown the protective effect of mycobacterial Hsp60 as a naked DNA vaccine in adjuvant arthritis (Ragno et al., 1997).

Several studies now also have shown the protective effects of mycobacterial Hsp60 derived peptides in adjuvant arthritis (Anderton et al., 1995; Moudgil et al., 1997; Ulmansky et al., 2002). These studies have included successful attempts to produce disease suppressive tolerance for the arthritogenic mycobacterial Hsp60 180–188 peptide in mycobacteria induced adjuvant arthritis (Feige and van Eden, 1996; Prakken et al., 1997). Interestingly the nasal administration of the latter peptide also suppressed avridine arthritis. In other words, the mimicry relationship of 180–188 with cartilage proteoglycans, was possibly targeting a form of by-stander suppression to the joint, irrespective of the actual trigger inducing disease (Prakken et al., 1997). Also altered peptide ligands based on the 180–188 peptides have been developed and found to suppress adjuvant arthritis. An alanine substitution

on position 183 led to a peptide, A183, which upon nasal administration prevented disease and also suppressed existing disease. This disease suppression was transferable with activated T cells from such nasally exposed animals. In addition, the disease suppressive effect of A183, appeared to be mediated by IL10 producing regulatory T cells at the site of inflammation (Prakken et al., 2002).

Besides nasal administration of peptide, oral administration of mycobacterial Hsp60 has been successful (Haque et al., 1996). Studies on the potential of repetitive oral administration of mycobacterial Hsp60 to suppress adjuvant arthritis, have revealed that in the presence of soy-been trypsin inhibitor very low dosages of Hsp60 (30 μg) can suppress disease. The oral administration, by gastric gavage, was also effectively suppressing disease, when the procedure was started at the time of already clinically overt disease (Cobelens et al., 2000). In the absence of soy-been trypsin inhibitor the disease suppressive effect could be potentiated by co-administering orally the beta2-adrenergic agonist Salbutamol (Cobelens et al., 2002). Also other HSP family members were tested and found to inhibit experimental arthritis. In rat adjuvant arthritis this was done with Hsp10, and the protective effect was seen for mycobacterial Hsp10 and not for the *E.coli* Hsp10 (GroES) or the rat Hsp10 (Ragno et al., 1996). For mycobacterial Hsp70, disease prevention by prior HSP immunization was seen in rat adjuvant arthritis, avridine arthritis and to a lower extent collagen II arthritis (Kingston et al., 1996; Prakken et al., 2001). Recently, we also have seen strong disease inhibition in a mouse (Balb/c) aggrecan induced arthritis model (Berlo et al., 2005) using pre-immunisation with mycobacterial Hsp70 in DDA as an adjuvant (Berlo et al. in prep.). When analyzing an *E.coli* derived bacterial extract called OM-89, which is used as an oral slow-acting anti-rheumatic drug in humans, it appeared that one of its major protein constituents was Hsp70 and some lower amounts of Hsp60. Upon oral administration in adjuvant arthritis rats prior to disease induction, OM-89 induced T cell reactivity to Hsp60 and Hsp70 and had a profound disease suppressive activity (Bloemendal et al., 1997), indicating that also GroEL and DnaK of *E.coli* have disease suppressive activity.

In an elegant study Tanaka et al. showed that *Listeria monocytogenes* infections in Lewis rats activated T cells directed against the Hsp70 234–252 sequence, conserved between mycobaterial and Listeria Hsp70. The T cells responding to this peptide suppressed inflammatory responses against listerial infection and produced IL10. The findings made were compatible with a scenario where T cells recognizing this Hsp70 epitope were involved in terminating Th1 mediated excessive inflammation after 'the battle against *L. monocytogenes* has been won'. The same (mycobacterial) Hsp70 peptide, prevented the development of adjuvant arthritis and this effect was inhibited by the administration of anti-IL10 antibodies (Tanaka et al., 1999). Wendling defined several other T cell epitopes in mycobacterial Hsp70 in Lewis rats. One of these peptides, Hsp70 111–125, was found to stimulate production of IL10 in responding T cells. Upon nasal administration the 111–125 peptide was found to prevent the subsequent induction of adjuvant arthritis.

Given the fact that the various HSP families are antigenically unrelated and that they have seemingly equal capacities to inhibit arthritis, we asked ourselves whether or not other non-HSP antigens, sharing immune characteristics with HSP, would also have arthritis inhibitory effects. Therefore, we have tested other bacterial immunogens of a conserved nature, for their protective qualities in experimental arthritis. For this we selected the antigens superoxide dismutase (SOD) of *E.coli*, glyceraldehyde-3-phosphate dehydrogenase of Bacillus (G3PDH) and aldolase of Staphylococcus. These antigens were found to be immunogenic, as they induced proliferative T cell responses and delayed type hypersensitivity reactions. All three antigens were relatively conserved, having homologues present in mammalian cells. Clearly enough, upon immunization none of these antigens was seen to affect arthritis to any extent and this was apparent in both adjuvant and avridine induced arthritis (Prakken et al., 2001). Therefore, the evidence collected sofar, is strongly indicative of the induction of arthritis protective mechanisms as a shared characteristic of microbial heat shock proteins, which is absent in other immunogenic and conserved bacterial antigens.

Microbial HSP and Disease Suppression in Other Chronic Inflammatory Disease Models

In NOD diabetes the effects of mycobacterial Hsp60 have been found to vary with the form of their administration (Elias et al., 1991). In PBS, mycobacterial Hsp60 was found to inhibit disease development and in IFA it induced more rapid diabetes. The spontaneous onset of beta cell destruction went together with the development of anti-mycobacterial Hsp60 T cells, the release of self-Hsp60 in the blood and the subsequent production of anti-Hsp60 antibodies. These studies have led to a further analysis of the mouse Hsp60 and especially the p277 peptide of Hsp60 in NOD diabetes (Bockova et al., 1997). Other antigens, such as mycobacterial Hsp70 were not seen to have any measurable effect on the development of diabetes. Thus, in contrast to what has been seen in arthritis, in the NOD the effects of mycobacterial Hsp60 cannot be explained as a mere result of immunization to any bacterial HSP. From this analysis in NOD mice it seems that Hsp60 may a unique function in beta cells and in the destructive process of type I diabetes (Brudzynski et al., 1992).

In agreement with this were our findings in NOD and in genetically protected NOD-asp mice. The development of insulitis was characterized by the *in vivo* priming of mycobacterial or human Hsp60 responsive T cells that produced IL10 upon *in vitro* restimulation with Hsp60. This was not seen in control mice without developing insulitis. Apparently, expression of endogenous Hsp60 in insulitis was associated with the regulation of insulitis (van Halteren et al., 2000). When administered at a high dose, also the non-microbial HSP gp96 was found to suppress diabetes in NOD mice (Chandawarkar et al., 2004). Other studies have indicated that, intracellularly, HSP may play a role in protecting beta-cells. For Hsp70 a role as a molecule involved in cellular repair in diabetes was postulated, when it was

shown that (heat stress) induction of endogenous Hsp70 in islets was impaired in diabetes prone BB rats at a young age but also at the older diabetes sensitive age (Wachlin et al., 2002).

A recent study has indicated the potential of Hsp70 not to protect against autoimmunity but to promote autoimmunity instead (Millar et al., 2003). In the RIP-GP transgenic model, where an LCMV-GP is expressed in the islet beta cells and where the majority of CD8+ T cells express the LCMV-GP specific P14 T cell receptor, immunization with the GP peptide in the presence of Hsp70 was seen to lead to induction of diabetes. This finding showed that Hsp70 stimulated DC function and converted the default tolerogenic response to the peptide into activation of autoimmunity. In this case the Hsp70 was mouse Hsp70 and the effect was probably mediated through triggering of TLR, a mode of action supposedly inherent to the proposed qualities of HSP as molecules signaling danger to the cells of the innate immune system. Similar activities to trigger receptors in cells of the innate immune system have also been reported for microbial HSP, such for Hsp60 (Habich et al., 2002; Kol et al., 2000). Although there is controversy in this area, and in some cases LPS contaminating the HSP preparations have been responsible for the effects observed (Tsan and Gao, 2004), the existing evidence available now is convincingly impressive (Vabulas et al., 2001).

The possible role of microbial Hsp60 in atherosclerosis has received more and more attention over recent years. Initially, Hsp60 was seen as antigen with a disease initiating or perpetuating role. This was based on evidence in humans that the presence of antibodies to Chlamydia Hsp60 was associated with developing atherosclerosis and that in rabbits immunizations with mycobacterial Hsp60 induced disease (Wick et al., 2004; Wick et al., 2001; Xu et al., 1992). Now however, various studies have shown the potential of microbial HSP to suppress disease. Maron et al. showed in atherosclerosis prone LDL receptor knock-out mice, which were fed with high-cholesterol diet, that nasal administration of mycobacterial Hsp60 caused a decrease of atherosclerotic plaques in the aortic arch (Maron et al., 2002). The reduction of the size of the plaques was accompanied by a reduction of macrophage-positive areas and increased expression of IL10 in the plaques. In another experimental atherosclerosis model, Harats et al. studied oral tolerance induced with mycobacterial Hsp60. In this case early atherosclerosis was attenuated and the effect seemed mediated by IL4 (Harats et al., 2002). Very similar results were obtained by van Puyvelde et al. in a model of atherosclerosis in LDL receptor knock-out mice on a high-cholesterol diet, where a collar is placed around the carotid artery. In this model low dose oral mycobacterial Hsp60 caused a dramatic drop in the size of plaques developed. A very similar effect was seen after the oral administration of a mycobacterial Hsp60 derived peptide that comprised a previously mapped T cell epitope, indicating that the effect on plaque sizes was T cell mediated (van Puyvelde, in prep.).

The critical role of inflammation in atherosclerosis is now widely accepted. Apart from oxidized LDL, microbial Hsp60 is now one of the major antigens supposed to be implicated in the inflammatory process of atherosclerosis. Having such an

antigen, vaccination against atherosclerosis may have become an appealing option (Hansson et al., 2002). In the rat model of MBP induced experimental autoimmune encephalomyelitis (EAE), a 12 kDa PPD derived protein with sequence homologies to Hsp10 was found to suppress disease (Ben-Nun et al., 1995). In a mouse EAE study the reduced EAE susceptibility of conventionally reared animals as compared to SPF animals, was associated with skewing of Hsp60 specific T cell cytokines toward a Th2 pattern. These findings were indicative of the fact that frequent exposure to infectious agents leads to a Th2 skewing of immune responses to HSP and that this is associated with milder and less frequent relapses of EAE (Birnbaum et al., 1998). Immunization with non-microbial HSP gp96 suppressed MBP induced EAE in SJL mice (Chandawarkar et al., 2004). Rha et al. (Rha et al., 2002) reported a distinctive quality of *M. leprae* Hsp60, not found for other microbial HSP. *M. leprae* Hsp60 suppressed disease in a murine model of allergic airway inflammation and airway hyperresponsiveness. In the bronchoalveolar lavage fluid, IL4 and IL5 production were found to be suppressed, while IL10 and IFN-γ production were increased.

MAMMALIAN HSP ARE TARGETS OF IMMUNE RESPONSES ELICITED (OR INDUCED) BY STRESSED CELLS

HSP Immune Responses and Lack of Self-Tolerance

Besides their immunodominance as microbial antigens, under various circumstances HSP do elicit immune responses also when (over-)expressed as self antigens by cells or tissues. And this seems to be a peculiar feature of HSP, especially because in many cases immune responses to this self antigen are not associated with pathogenic autoimmunity. Evidently, healthy individuals have a broad repertoire of T and B cells with specificity for mammalian or self HSP. Self HSP specific immunity has been seen to exist in mice, rats, humans and other species studied so far. Apparently, thymic selection ensures the selection of a repertoire of cells with cognate receptors that can recognize such proteins, despite the fact that they are omni-present in almost every cell of our body.

The mechanism of the lack of tolerance for self HSP is unclear. A low level of constitutive expression or a restricted tissue representation would be an obvious explanation. However, in the normal thymus HSP are relative abundantly expressed in the medullary epithelium (Anderson et al., 2002; Birk et al., 1996; Ostberg et al., 2002) and yet fail to induce tolerance by negative selection. And also the transgenic overexpression of mouse Hsp60 in the thymic cortical epithelium and bone marrow derived cells of NOD mice, through the mouse class II Eα promoter, did not eliminate T cell responses to a panel of self Hsp60 peptides, although an epitope shift with disease suppressing potential was noted (Birk et al., 1996). Possibly, a peculiar subcellular localization characteristic for HSP creates a threshold effect, somehow allowing for an efficient expansion of cells with low affinity receptors that thereby escape deletion.

Whatever the mechanisms behind this lack of tolerance may be, it needs to be compensated efficiently by immune regulation in the periphery in order to control self HSP specific immunity. And indeed, as we will discuss later, immune regulatory mechanisms have been shown to be a prominent feature of HSP specific immune responses. And here we seem to have arrived at one of the most intriguing aspects of immunity for HSP. Their evolutionary conservation, their omni-presence in cells and tissues and their inducible nature seems to have made HSP into very dependable self-antigens for the immune system to target regulation. For the same reason Cohen has mentioned HSP as prime examples of his so-called homuncular antigens (Cohen et al., 2003). And along similar lines of reasoning, the findings that HSP such as HSP90 are part of a subset of highly conserved and immunodominant self antigens that are target for natural autoantibodies in normal human IgG also indicate that HSP may be essential for selection of natural B and T cells repertoires and for the maintenance of self-tolerance (Pashov et al., 2002).

Cell Stress Leads to HSP Expression and Induction of Immune Responses

The inducible nature of HSP is particularly evident in inflammation. Many features of inflammation, such as production of inflammatory mediators, cytokines such as TNF-α and IL1, cell debris, reactive oxygen intermediates and raised temperature are perfect inducers of heat shock protein expression. In fact raised temperature due to fever and inflammation are key components of virtually all immune responses. The effects of raised temperature in the form of experimental hyperthermia on HSP expression and its facilitation of many distinct forms of specific immunity, has been the subject of many studies already (Repasky and Issels, 2002).

HSP have been claimed to become expressed on the cell surface, serving as a target for NK cells and for antibodies leading to ADCC (Multhoff and Hightower, 1996), despite the fact that these proteins are lacking the molecular features of cell surface antigens. In patients with active RA, and not during remission, peripheral blood and synovial fluid derived T cells were shown to present cell surface HSP (Sato et al., 1996). Expression of HSP in T cells has been reported earlier and it seems that HSP are expressed in T cells at lower levels of stress as compared to other cells (such as B cells), which may indicate that T cells do need overexpressed HSP in order to resist the harsh environment of the site of inflammation (Gothard et al., 2003). Also rheumatoid joint DCs were shown to have cell surface expression of Hsp70 (Martin et al., 2003).

Class I Restricted T Cell Responses

HSP are intracellular cytosolic proteins. Therefore HSP fragment are presented in the context of MHC class I molecules, and induction of HSP specific Class I restricted CTL responses has been documented in many different situations of cell stress.

One of the first observations was made with CTL raised against mycobacterial Hsp65, as these CD8+ class I restricted T cells recognized macrophages subjected to various forms of cells stress. This was a first demonstration of the fact that HSP are processed in stressed host cells and can be presented in the context of class I molecules (Koga et al., 1989).

Hsp60 peptide specific CTLs were found to lyse IFN-γ stressed macrophages and such lysis was specifically inhibited by Hsp60-specific antisense oligonucleotides (Zugel et al., 1995). In measles virus-infected patients CTLs were detected with specificity for a Hsp90 derived peptide in the context of HLA-A2 (Herberts et al., 2003). In cancer patients, tumor infiltrating lymphocytes were found to harbor CTLs with specificity for Hsp70 derived peptides (Azuma et al., 2003; Faure et al., 2004). In other words, CTL perceive the altered levels of HSP peptides in the class I molecules after infection and tumorigenesis.

Class II Restricted Responses

Besides class I, also class II molecules are loaded with HSP peptides. In fact, Hsp70 peptides were prominently represented in the RP-HPLC profile of the content of class II molecules of a human lymphoblastoid cell line (Newcomb and Cresswell, 1993). Many studies have shown the production of HSP specific antibodies, as a reflection of MHC II driven antigen presentation of stress induced HSP. Especially in chronic inflammatory diseases the presence of HSP specific autoantibodies has been widely documented (see hereunder). Also in infectious diseases HSP antibodies have been shown, such as higher titers of IgG antibodies specific for Hsp70 in sera of patients with HIV infection (Kocsis et al., 2003). In experimental heart transplantation in the rat, chronic rejection of the cardiac allograft was seen to go along with graft-infiltrating auto-reactive T cells reactive to HSP (Duquesnoy et al., 1999). In human renal transplant patients an increasing percentage of IL10 producing Hsp60 specific T cells was documented in the late post-transplant period (Caldas et al., 2004). Also the production of IL4 by Hsp60 specific T cells was seen to be associated with absence of rejection (Granja et al., 2004). Birk et al. showed in a Hsp60 transgenic mouse skin transplant model that the stress of rejection led to Hsp60 over-expression in the graft and that anti-graft responses were modulated with Hsp60 antibodies or peptides (Birk et al., 1999). In the low dose streptozotocin model of insulin dependent diabetes in the mouse, it was shown that the beta-cell toxin led to cell stress leading to the production of increased levels of antibodies and T cells reactive to Hsp60 (Elias et al., 1994).

Class Ib Restricted Responses

Besides the adaptive immune responses to HSP also innate responses are involved in the recognition of over-expressed HSP. And this makes sense, HSP being an evolutionary ancient collection of proteins that has been into existence before the

philogenetic development of the mediators of adaptive immunity. Most likely, HSP have fulfilled and still fulfill a primitive task in signaling cellular stress and therefore there is logic in the recognition of these proteins by cells of the innate immune system.

Molecules presenting the stress signals of cells are non-classical MHC class I molecules, known as Qa-1b in the mouse and HLA-E in humans. It had been shown that Qa-1b was up-regulated during cellular stress (Imani and Soloski, 1991). Now, Michaelsson et al., have shown that HLA-E molecules on human cell lines become loaded with a Hsp60 signal peptide under cell stress, which replaces other peptides in the cleft of HLA-E, which normally are leader sequences of other class I MHC molecules. The introduction of the Hsp60 signal peptide into HLA-E was shown to lead to interference with CD94/NKG2A recognition, which is an inhibitory NK receptor. In this manner stress would enable NK cells to become activated by the detection of stressed cells (Michaelsson et al., 2002); (Soderstrom, 2003).

In an earlier study a Hsp60 peptide in Qa-1b was reported to be a target for CD8+ CTL. These CTL were induced by *Salmonella typhimurium* infected macrophages, were found to be specific for a Hsp65 specific peptide and to cross-recognize IFN-γ or LPS stressed macrophages (Lo et al., 2000). Most likely, a similar phenomenon has been underlying earlier studies where MHC-unrestricted recognition was claimed to exist for human CD8+ CTL that killed infected target cells and not heat-killed bacteria loaded target cells (Hermann et al., 1992). A recent study has shown that the Hsp60 peptide 216–224 (homologous to the 192–200 sequence in bacterial Hsp65), in the absence of Qdm, the leader sequence of many class 1a molecules, is the dominant peptide bound by Qa-1b (Davies et al., 2003). These findings are especially intriguing with respect to the resurrected interest in suppressor T cells (Chess and Jiang, 2004). Originally, after its serological detection, Qa-1 was found to be expressed on lymphocytes that preferentially induced CD8+ suppressor T cells. More recently, Hu at al. showed in an *in vivo* transgenic model that Qa-1 restricted CD8+ T cells do control CD4+ T cell responses, thereby suppressing the generation of experimental EAE. In the absence of Qa-1 no such suppressive effect was mounted (Hu et al., 2004). Along similar lines, a critical role for Qa-1 was claimed in T cell vaccination. Originally T cell vaccination was described as the phenomenon that attenuated auto-reactive T cells conferred resistance to subsequent active disease induction (Ben-Nun et al., 1981). Now in two murine models of autoimmunity it was shown that T cell vaccination depended on the activation of suppressive CD8+ T cells that specifically recognize Qa-1 and self peptides expressed by activated autoreactive CD4+ T cells (Panoutsakopoulou et al., 2004). As HSP, like Qa-1, are up-regulated in activated T cells, and as we now know that HSP sequences are major antigens for presentation through Qa-1, it is possible that herewith we see one of the mechanistic pathways through which HSP immunizations lead to regulatory activities. That such a pathway may be clinically relevant is suggested by the data that in humans HLA-E is needed for CD8+ T cells to differentiate into regulatory cells (Li et al., 2001).

Self HSP Immunization Produces Immune Responses to Self-HSP and Stressed Cells

Immunization with mammalian Hsp60 was seen to lead to production of both self HSP specific antibodies and T cells. Using a recombinant vaccinia virus as carrier this immunization was seen to modulate experimental arthritis (Lopez-Guerrero et al., 1993). Using a naked DNA vaccination procedure, the production of regulatory cytokines IL10 and TGF-β was observed and again this led to suppression of disease (Quintana et al., 2004). These observations suggested that the experimental priming of responses to self-HSP led to recognition of self-HSP as expressed at the site of inflammation. However, no formal proof was given.

First evidence that stressed antigen presenting cells were recognized by CD4+ T cells responding to self-HSP, was obtained with T cells generated against a conserved sequence of mycobacterial Hsp65 in the rat adjuvant arthritis model (Anderton et al., 1995). T cells obtained from Lewis rats immunized with Hsp65, were repetitively re-stimulated with a peptide representing the Hsp65 sequence at positions 256–265. The resulting T cell line was responding to the rat Hsp60 and also responded to splenocytes that had been exposed to 41°C for 30 minutes. Similarly, when we used the mostly conserved core sequence of this rat T cell epitope to generate a T cell line, we were able to obtain, after several rounds of re-stimulation, a T cell that responded spontaneously in the presence of non-stressed antigen presenting cells (Paul et al., 2000). The autoproliferation of this T cell line was fully inhibitable by blocking anti-MHCII antibodies. Upon transfer these T cells were shown to protect against AA induction. At the message level the autoproliferative T cells were found to produce IFN-γ, IL4 and IL10. In a different series of experiments, T cells obtained from Lewis rats immunized with this conserved Hsp65 peptide were studied in co-culture with antigen specific responder T cells. When the in vivo primed T cells were re-stimulated in vitro with heat stressed APC, the resulting T cells did suppress the antigen specific responder T cells in the co-culture (Eden, 2003). Altogether these findings have illustrated the recognition of self HSP sequences in the context of MHCII by CD4+ T cells. And in addition, that such T cells have the capacity to regulate.

MECHANISMS OF IMMUNO-REGULATORY T CELL INDUCTION

Adaptive immune responses to HSP, as for other antigen specific immune responses, will depend for both their initiation and the development of particular effector mechanisms, on the signals provided by the innate immune system (Iwasaki and Medzhitov, 2004). In the paragraph on the non-classical molecules of antigen presentation we have already discussed the innate recognition of stress. In the perspective of the uncertainties described here above, given the variety of cells involved in innate immune responses and the variety of innate HSP receptors discovered sofar, one may conclude that innate responses to HSP exist and prepare the ground for finely tuned adaptive responses to these ubiquitous proteins. In other

words, likely enough the quality of the adaptive response will be determined by the circumstances, as noticed by the elements of the innate immune system. Teleologically arguing, the regulation mediated through HSP specific adaptive responses in most cases of chronic inflammation, must be fostered by the innate response. Of interest in this respect are the claims that both TLR2 and TLR4 signaling, is not only associated with production of Th1 cytokines in DC, but also the induction of IL10 (Higgins et al., 2003; Netea et al., 2004). Chlamydia Hsp60 has been shown to induce IFN-γ and IL10 in lymphocytes obtained from women that have experienced chlamydia infections (Kinnunen et al., 2003). Also the heat shock response in cells has been noted to produce LPS induced IL10 production and to suppress LPS induced IL12 production (Wang et al., 2001). This latter finding is in accordance with the suggestion that IL10 is in fact a stress cytokine IL-10 is a stress cytokine produced not only in response to microbial pathogens but also to cellular injuries of diverse origins (Stordeur and Goldman, 1998). Ulmansky et al. have observed that in some cases protection in adjuvant arthritis is mediated by protective mycobacterial Hsp60 specific antibodies (Ulmansky et al., 2002). These antibodies induced the production of IL10 in murine and human mononuclear cells. And in addition, as HSP mediated adaptive responses do not cause general immunosuppression, it is attractive to suppose that the local circumstances of innate triggering do secure mechanisms of adaptive regulation to remain localized in areas of inflammation. And this may be true, especially in the case of inflammation which is not dominated by an abundant presence of LPS and other truly pro-inflammatory mediators of infection. In such cases IL10 and other Th2 associated cytokines seem to dominate the pattern of cytokines produced, as a consequence of immune exposure to heat shock proteins. Also related to this, can be the earlier proposed idea that Th2 cytokines may inhibit epitope spreading and help to focus immune responsiveness to certain epitopes (Elson et al., 1995).

Microbial HSP Trigger Self-HSP Cross-Reactive Regulatory T Cell Responses

Anderton has analyzed the protection as seen in the Lewis rat adjuvant arthritis model that resulted from immunization with mycobacterial Hsp60 (van Eden et al., 1988). He tested lymphocytes obtained from such protected rats and monitored T cell responses to an overlapping set of 15-mer peptides spanning the complete mycobacterial Hsp60 sequence. Nine distinct dominant T cell epitopes were discovered. In extensive adoptive transfer studies, using T cell lines generated to all epitopes, he revealed that only T cells directed to a very conserved 256–265 sequence transferred protection into naïve animals (Anderton et al., 1995). The latter T cells were shown to recognize the tissue or rat Hsp60 homologous peptides and also heat-shocked autologous spleen cells. Furthermore, active immunization with the conserved mycobacterial peptide protected against the induction of both mycobacteria induced adjuvant arthritis and avridine induced arthritis. All other peptides encomprizing Lewis rat Hsp60 T cell epitopes failed to produce such protection.

In other words, of all epitopes analysed, only the epitope that was conserved to the extent that it induced self HSP cross-reactive T cells, was able to fully protect.

This mechanism of mycobacterial Hsp60 mediated suppression of arthritis through a conserved microbial HSP sequence, has been reproduced very similarly for mycobacterial Hsp70 by others (Moudgil et al., 1997; Tanaka et al., 1999) and ourselves (Wendling et al., 2000). Also, in these latter cases T cells recognizing the very conserved mycobacterial Hsp70 peptides were found to produce protection, and the other non-conserved peptides were not. Microbial HSP have been seen to induce protective adaptive immune responses in a multitude of models of experimental autoimmunity, involving a wide variety of disease triggering substances or antigens. Although, in classical adjuvant arthritis there exists an antigenic relationship between the inciting antigen, whole mycobacteria and the protection inducing protein, mycobacterial Hsp60, in most other models where single molecules such as mycobacterial Hsp60 and 70 were found to be protective, no such relationship existed. Therefore, the induction by microbial HSP of regulatory self-HSP cross-reactive T cell responses seems an attractive explanation for the protection seen. In all cases, the stress inflicted on cells in the vicinity of the inflammatory process will lead to upregulation of endogenous HSP, and hereby the cross-reactive T cells will target their regulatory effects to the actual site of inflammation.

In various studies the cytokines produced by T cells defined to cross-respond to self-HSP were analyzed. Self Hsp70 cross-reactive T cells were found to produce inhibitory cytokines, notably IL10 (Tanaka et al., 1999; Wendling et al., 2000). Also, the T cell line generated to the conserved core of a mycobacterial Hsp60 Lewis rat T cell epitope, which was identical with self (rat) Hsp60 and which was auto-proliferative due to a MHC II restricted recognition of the self-Hsp60 epitope, produced regulatory cytokines such as IL-4, IL-10 and IFN-γ (Paul et al., 2000).

In pristane arthritis the mycobacterial Hsp60 epitope that was defined as the antigen for protective T cells was, however, not conserved (Thompson et al., 1998). Despite the fact that in this case there was no evidence for cross-recognition of self Hsp60; the protective T cells produced inhibitory cytokines IL4, IL5 and IL10. In this case, the mechanism behind the capacity of this peptide to produce regulatory cytokines is unclear. The observation that E.coli Hsp60 and mycobacterial Hsp70 are driving Th2 responses in the pristane model, but do not protect, suggests that there is a very specific and unique relationship between the defined Hsp60 epitope and the disease inhibition of pristane arthritis (Beech et al., 1997).

Also the protection mediated by mycobacterial Hsp70 in aggrecan induced arthritis in Balb/c mice seemed mediated through production of regulatory cytokines in T cells. Interestingly, not only Hsp70 specific T cells but also the aggrecan specific T cells were seen to produce IL10 in HSP protected animals (Berlo et al. submitted).

The Hsp60 peptide that had a protective effect in NOD diabetes was derived from mouse Hsp60 and not from microbial Hsp60. When tested in patients with early type I diabetes, this peptide was able to switch the production of inflammatory cytokines into the production of regulatory cytokines IL10 and IL13 (Raz et al.,

2001). When we tested the conserved and immunogenic bacterial proteins SOD, G3PDH and Aldolase for their capacity to protect in arthritis, as discussed above, we also tested whether they were capable of inducing the production of IL10 and other Th2 cytokines. And this was done in comparison with Hsp70. The mycobacterial Hsp70 and also the conserved mycobacterial Hsp70 derived peptide p111 and its rat homologue induced the production of IL-10 in T cells (Wendling et al., 2000). The other bacterial antigens did not induce the production of significant amounts of IL-10 and induced IFN-γ and TNF-α instead. Intracytoplasmatic staining for cytokines showed that these cytokines were produced by the antigen specific T cells (Prakken et al., 2001).

The fact that bacterial HSP have been effective in inducing protection against experimental autoimmunity, further suggests that HSP mediated immune regulation may also be one of the underlying mechanisms through which environmental flora can contribute to disease resistance. There are many experimental findings that have indicated that bacterial recontamination of germ-free animals induced resistance against immune disorders. In a more wider perspective, one may conclude that HSP may constitute a molecular factor in the mechanisms underlying the hygiene hypothesis (Guarner et al., 2006).

TRANSLATION OF THIS CONCEPT INTO CLINICAL INTERVENTIONS

As discussed above, a likely factor driving the immunodominance of HSP is repeated contact with environmental bacteria, not only during infection but also by continuing contact with commensal flora. Through the wide variety of bacteria that is encountered, the immune system is confronted with a variety of microbial HSP. Obviously, the more frequently encountered HSP sequences are those that are conserved between different bacteria. Herewith, the immune system sets a focus on conserved areas of HSP, which in many cases have striking homologies with self HSP. The frequent or possibly continuous exposure to the microbial HSP in the commensal flora contacting the mucosa may impose a form of mucosal tolerance on this repertoire of self HSP reactive lymphocytes. Also in diabetes, the exposure to bacteria has been seen to reduce disease (Feillet and Bach, 2004). Rook and Stanford (Rook and Stanford, 1998) have argued that exposure to microbial agents is an essential factor in resistance against chronic immune mediated disorders. In the perspective of this, the exposure to HSP could well be promoted by oral HSP vaccines, and possibly also by probiotic strains of bacteria known to express HSP.

The dramatic disease suppressive effects of the oral administration of tiny amounts of mycobacterial HSP in arthritis models, give again support to the idea that mucosal exposure to HSP is a powerful means of imposing a regulatory mode on the HSP specific repertoire of the adaptive immune system. Some of the HSP derived peptides have already shown their capacity to drive regulatory T cell responses in clinical trials. This, for instance, was found for peptide 277 of mammalian Hsp60

in type I diabetes. The immunomodulatory effect was accompanied by a reduced need for insulin administration in the treatment group (Beech et al., 1997).

Oral administration of *E.coli* derived DnaJ (a smaller subunit of the Hsp70 complex) peptide in patients with rheumatoid arthritis was also seen to lead to immune deviation of pro-inflammatory T cells. In this study no clinical effects were studied (Prakken et al., 2004). A selection of mycobacterial Hsp60 peptides that have shown *in vitro* similar immune deviating (IL-10 producing) potential has been made by Kamphuis et al. (Kamphuis et al., 2005). The latter selection is expected to offer us interesting candidate peptides for immunotherapy in children with juvenile rheumatoid arthritis.

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CHAPTER 19

THE PRO- AND ANTI-INFLAMMATORY PROPERTIES OF THE STRESS PROTEIN GP96

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Abstract: Although the stress protein gp96 is commonly perceived as being a universal activator of antigen presenting cells and an inducer of tumour-specific immunity, at high doses it can inhibit the induction of tumour-specific immunity and experimental autoimmune disease by a mechanism which appears to involve immunoregulatory $CD4^+$ T cells. Studies have shown that gp96 can also delay the rejection of allogeneic skin and cardiac transplants. This chapter summarises the work which has attributed pro- and anti-inflammatory properties to gp96 and highlights the potential mechanisms that might mediate the dual functionality of this molecule

Keywords: gp96, inflammation, tumour immunity, anti-inflammatory, immunoregulation

INTRODUCTION

The search for tumour-specific antigens has led to the identification of heat shock (stress) proteins as being potential inducers and/or mediators of tumour immunity. This is, in part at least, due to their ability to bind peptide (Gething and Sambrook 1992) and to interact with a number of receptors on professional antigen presenting cells (APCs) such as dendritic cells (DCs), as well as macrophages (Asea et al., 2000, Binder, Vatner and Srivastava 2004), T cells and natural killer (NK) cells (Wells and Malkovsky 2000). It has been known for some time that certain stress proteins bind peptide and chaperone a large number of peptides that are derived from the cells from which they are isolated – the so-called 'antigenic repertoire' or 'fingerprint' of that cell (Udono, Levey and Srivastava 1994). The ability of tumour-derived gp96 to induce protective tumour-specific immunity has been attributed to its capacity to form stable complexes with antigenic peptides and deliver these into

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the antigen processing machinery of APCs for subsequent presentation to specific effector T cell populations following internalisation of the stress protein by APCs (Srivastava 2002). The antigen re-presentation occurs concomitant with a triggering of pro-inflammatory cytokine production and results in the activation of naïve and/or memory effector T cells and the induction of tumour-specific, $CD8^+$ T cell-mediated immunity (Baker-LePain, Sarzotti, Fields, Li and Nicchitta 2002, Basu, Binder, Suto, Anderson and Srivastava 2000, Reed, Berwin, Baker and Nicchitta 2003, Schild, Arnold-Schild, Lammert and Rammensee 1999, Singh-Jasuja et al., 2000b, Tamura, Peng, Liu, Daou and Srivastava 1997, Zheng, Dai, Stoilova and Li 2001). The capacity of stress proteins to induce specific immunity to a spectrum of antigens has made them attractive vehicles for the delivery of antigenic peptides into the antigen presentation pathway for the subsequent induction of protective immunity to tumours and pathogenic organisms. One such stress protein is gp96, and it is on the multiple immunological properties of this protein that this chapter focuses.

THE PRO-INFLAMMATORY PROPERTIES OF GP96

Gp96, also known as glucose-regulated protein (grp) 94 (Lee 1981), endoplasmin (Koch, Smith, Macer, Webster and Mortara 1986) and 99 kDa endoplasmic reticulum protein Erp99 (Lewis, Mazzarella and Green 1985) is a 94–96 kDa member of the Hsp90 family of molecular chaperones/stress proteins which resides within the lumen of the endoplasmic reticulum (ER) (Csermely, Schnaider, Soti, Prohászka and Nardai 1998, Welch, Garrels, Thomas, Lin and Feramisco 1983). The immunological properties of gp96 first became apparent in studies by Pramod Srivastava and colleagues in the 1980s which demonstrated that the administration of a 96 kDa protein fractionated from a murine tumour cell lysate (which was subsequently shown to be gp96) to mice induced resistance to the same tumour from which the protein had been originally isolated (Srivastava, DeLeo and Old 1986). It was proposed that the specificity of the response was defined by tumour-derived peptides that were associated with the administered gp96 (Chandawarkar, Wagh and Srivastava 1999, Srivastava et al., 1986, Udono et al., 1994).

Gp96 and Tumour Immunity

Broadly speaking there are two opinions regarding the mechanisms by which gp96 induces tumour-specific immunity. The first is based on the pioneering studies by Srivastava and colleagues, from which the concepts that stress proteins function as carriers of tumour-specific antigenic peptides, and that the specificity of the immune responses which are induced following the administration of gp96 is defined by the profile of peptides that characterise the tissue from which the protein is isolated have developed (Srivastava 2002, Srivastava 1994, Srivastava 1997). APCs such as DCs have been reported to spontaneously internalise gp96 by receptor-mediated endocytosis (Arnold-Schild et al., 1999, Castellino et al., 2000, Singh-Jasuja et al.,

2000a) via a number of receptors including the α_2-macroglobulin receptor (CD91 molecule) (Binder, Han and Srivastava 2000, Binder and Srivastava 2004), the LPS receptor (Vabulas, Wagner and Schild 2002b) and members of the Toll-like receptor family (Ramirez et al., 2005) (TLR-2, TLR-4), the latter either alone or in combination with CD14 (Vabulas et al., 2002b). Gp96 internalisation by human APCs has also been found to take place via scavenger receptor A (Berwin et al., 2003). Receptor-mediated endocytosis of gp96 leads to the transfer of chaperoned peptides into the intracellular pathways for MHC class I-restricted presentation to $CD8^+$ T cells (Arnold-Schild et al., 1999, Berwin, Rosser, Brinker and Nicchitta 2002, Binder et al., 2000, Binder, Kumar and Srivastava 2002, Castellino et al., 2000, Singh-Jasuja et al., 2000c) and MHC class II-restricted presentation to $CD4^+$ T cells (Doody, Kovalchin, Mihalyo, Hagymasi, Drake and Adler 2004, SenGupta et al., 2004). Further details on the receptors used by gp96 and the consequences of these interactions are provided elsewhere in this book.

For some time, the '2-signal model' has proposed that antigen delivered to T cells by DCs (signal 1) in the absence of essential co-stimulatory molecules and appropriate immunostimulatory cytokines (signal 2) induces T cell tolerance, whereas antigen presented to T cells by DCs in the presence of signal 2 results in antigen-specific immunity (Mellman and Steinman 2001). Although the interaction of MHC-peptide complexes expressed on APCs such as DCs function as 'signal 1', for naïve T cells to be fully primed and activated, DCs must undergo maturation and provide 'signal 2' by expressing essential co-stimulatory molecules such as CD80 and CD86 and secreting the bioactive IL-12_{p70} heterodimer. IL-12_{p70} is a potent driver of pro-inflammatory Th1 cell differentiation, and T cells responding to IL-12_{p70}-secreting DCs typically assume a pro-inflammatory Th1 phenotype (Trinchieri 2003). Gp96 has been shown to induce the maturation of DCs and other APCs (Baker-LePain et al., 2002, Basu et al., 2000, Reed et al., 2003, Singh-Jasuja et al., 2000b, Zheng et al., 2001), and their potential to concomitantly deliver 'signal 1' (via the cross-presentation of chaperoned peptides) and 'signal 2' to responding T cell populations (Figure 1) has led to the proposition that stress proteins such as gp96 can break peripheral tolerance against tumour-associated antigens (Liu, Ewing and DeFilippo 2004). This proposition has been supported by a number of observations. For example, immunisation of tumour-bearing hosts (in which tumour-specific tolerance has likely been induced) with stress proteins such as gp96 elicits tumour protection in a T cell-dependent manner (Tamura et al., 1997). The fact that the priming of tumour-protective $CD8^+$ T cell-mediated tumour immunity by gp96 can be independent of $CD4^+$ T cells (Udono et al., 1994) highlights the ability of gp96 to serve as both a peptide chaperone and an immunological adjuvant. It also indicates that tumour peptides associated with gp96 are delivered to APCs for direct cross-presentation to $CD8^+$ T cells (Singh-Jasuja et al., 2000c).

Many studies support the widely-accepted view that tumour peptides are associated with tumour-derived gp96 and that its administration leads to the induction of tumour-specific immunity via a mechanism involving receptor-mediated uptake into APCs and cross-presentation (Banerjee and Li 2005).

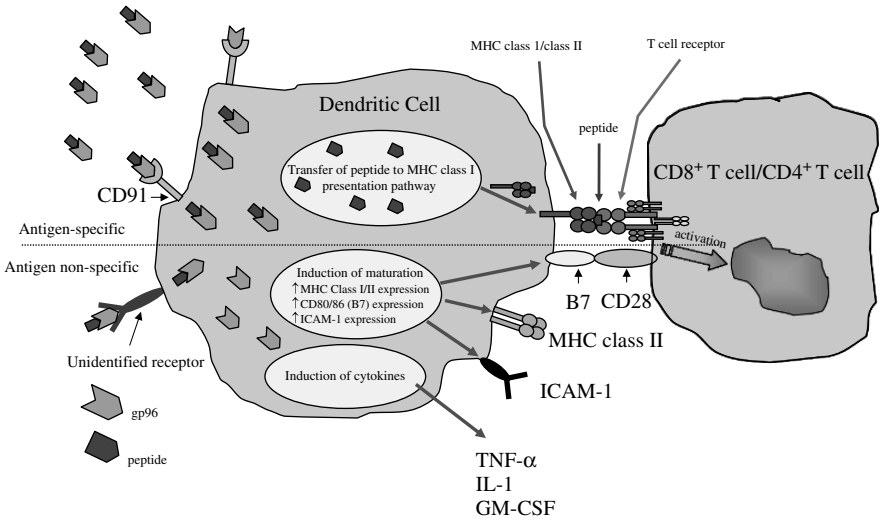

Figure 1. Effects of gp96 on DCs, and the induction of peptide-specific immunity. Gp96 is internalised by DCs via the CD91 molecule (2-macroglobulin receptor), and/or an as yet unidentified receptor. Chaperoned peptides are delivered into the MHC class I presentation pathway for subsequent presentation to MHC class I-restricted $CD8^+$ T cells or into the MHC class II presentation pathway for subsequent presentation to MHC class II-restricted $CD4^+$ T cells. Gp96 has been shown to concomitantly induce DC maturation, as reflected by the induction of MHC class II, B7 and ICAM-1 molecule expression, and their release of TNF-α, IL-1 and granulocyte-macrophage colony stimulating factor GM-CSF

However, Liu and colleagues have reported problems with identifying peptides that are associated with gp96 (Liu et al., 2004). In addition, the observation that the yield of peptides extracted from gp96 is highly sub-stoichiometric, with an estimated occupancy of between 0.1% and 0.4%, appears to argue against gp96 having a regular role as a peptide chaperone in antigen processing (Demine and Walden 2005). Nicchitta and colleagues believe that gp96 does not interact with tumour peptides, but instead with polypeptides (Baker-LePain, Reed and Nicchitta 2003, Nicchitta 2003, Nicchitta, Carrick and Baker-Lepain 2004). In the absence of tumour-derived peptides, another peptide-binding stress protein (Hsp70) has been shown to stimulate the secretion of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α by APCs via a TLR and CD14-associated pathway (Asea et al., 2000). The outcome of this is a non-specific stimulation of the innate immune system and an alternative mechanism relating to the primary involvement of innate (non antigen-specific) immune activation in the capacity of gp96 to induce tumour immunity has been proposed.

In essence, this suggests that gp96-induced tumour rejection is not dependent on bound peptides, rather that it is mediated initially via non-specific interactions with the innate immune system (Baker-LePain et al., 2003, Nicchitta 2003). This alternative model suggests that the secretion of IFN-γ, IL-12 and other cytokines by gp96-stimulated DCs activates NK and/or NKT cells, and that the gp96-induced

cross-talk between DCs and NK cells enhances the cytolytic activity of NK cells and the production of IFN-γ. This leads to the generation of cytotoxic mediators such as nitric oxide, TNF-α, and IL-1β, the induction of tumour cell death and consequently the release of tumour antigens into the extracellular milieu. Released tumour antigens can then be internalised and processed by DCs, thereby leading to the presentation of tumour-derived peptides and activation of tumour-specific $CD8^+$ T cells. Most importantly, in this model, gp96-mediated activation of innate immune responses provides an immunological environment which is suitable for the induction of adaptive immune responses (Nicchitta 2003). In the absence of peptide, both the induction of stress proteins and their cell surface expression or secretion might conceivably induce a non-specific inflammatory response which ultimately results in tissue damage and the induction of specific immunity.

Gp96 as an Effective Therapeutic Vaccine?

Should the mechanism by which gp96 induces tumour immunity indeed involve the cross-presentation of tumour-derived antigenic peptides then the use of gp96 as a tumour vaccine would exhibit two distinct advantages over other strategies that are aimed at 'immunising' against cancer. Firstly, cancer vaccines that are based on stress protein-peptide complexes would bypass the requirement to identify relevant tumour-specific peptides. Secondly, this approach delivers multiple antigenic peptides and therefore will induce multi-specific immune responses. Irrespective of the precise mechanism that might be involved, the experimentally-demonstrated potential of gp96 to induce tumour immunity has made it an attractive candidate as a cancer vaccines and the potential therapeutic value of this stress protein has attracted a significant amount of interest and attention (Banerjee and Li 2005).

The practical implications of gp96 as a tumour therapeutic and its efficacy are now being examined by a number of well-designed clinical trials. Unlike traditional cancer treatments, stress protein-peptide based vaccines are tailored towards an individual tumour of an individual patient. This is based on the concept that tumours are antigenically unique to each another, most likely as a result of random DNA mutations in the transformed cells which lead to differences in the peptide pools amongst different tumours (Liu et al., 2004). Should the cross-presentation of tumour-peptides be the mechanism via which tumour-specific immunity is induced by gp96 then an effective approach requires that the gp96 is isolated from the patient's tumour. Gp96-based vaccines have been tested in early phase clinical trials in solid tumours, as well as lymphoma and leukemias; all of which showed minimal toxicity and potential efficacy (Belli et al., 2002, Janetzki, Palla, Rosenhauer, Lochs, Lewis and Srivastava 2000, Mazzaferro et al., 2003). Patients with metastatic melanoma patients vaccinated with patient-derived autologous gp96 developed MHC class I-restricted tumour-specific T cell immunity, clinical responses and long-term disease stabilisation (Belli et al., 2002). Interestingly, when the same investigators performed a second trial in which autologous gp96 was combined

with immunostimulatory agents (GM-CSF and IFN-α), the clinical and immunological responses were no better than those that were induced using autologous gp96 alone (Pilla et al., 2006). Phase III clinical trials using tumour-derived gp96 for the treatment of melanoma and renal cell carcinoma are ongoing (Li 1997, Oki and Younes 2004). Data from these studies will provide further insight into the potential therapeutic value of gp96 as an anti-cancer vaccine.

THE ANTI-INFLAMMATORY PROPERTIES OF GP96

Although gp96 has been reported to have pro-inflammatory effects and to induce peptide-specific protective immunity (summarised above), anti-tumour immunity is not apparent when high doses of the protein are administered. Indeed, it has been demonstrated that high-dose gp96 can attenuate inflammatory disease and delay skin transplant rejection (Chandawarkar, Wagh, Kovalchin and Srivastava 2004, Chandawarkar et al., 1999, Kovalchin, Mendonca, Wagh, Wang and Chandawarkar 2006). The anti-inflammatory properties of gp96 were originally identified in murine studies which demonstrated that the induction of immunity to methylcholanthrene-induced (Meth A) fibrosarcoma by the administration of gp96 purified from Meth A fibrosarcoma cells was dose-dependent, in that 2 intra-dermal injections of 1 μg protect whereas 2 injections of 10 μg do not (Chandawarkar et al., 1999). The lack of tumour immunity appeared to result from the induction and/or activation of an immunoregulatory $CD4^+$ T cell population, as the induction of tumour immunity in animals that had been treated with low-dose gp96 could be inhibited by the adoptive transfer of $CD4^+$ T cells from animals that have been treated with high-dose gp96 (Chandawarkar et al., 1999). In this study, the authors suggested that the suppressive activity which was observed was source-specific, i.e. that for the suppression of tumour immunity to occur, the gp96 must be purified from that tumour (Chandawarkar et al., 1999). However, subsequent work from the same laboratory indicated that that the administration of high-dose gp96 ($2 \times 100\,\mu$g subcutaneously) purified from normal liver tissue was equally able to suppress tumour immunity in mice, the onset of diabetes in non-obese diabetic mice and the induction of autoimmune encephalomyelitis (EAE) by myelin basic protein and proteolipid protein in SJL mice, again via a mechanism which appears to involve the induction and/or activation of an immunoregulatory $CD4^+$ T cell population (Chandawarkar et al., 2004). This latter study indicated that the timing of the high-dose gp96 administration is important, as the immunoregulatory activity was only apparent if the gp96 is administered at the time of, or shortly after encounter with the antigenic stimulus (Chandawarkar et al., 2004). This suggests that the primary targets of the suppressive activity are recently-activated rather than memory T cells (Chandawarkar et al., 2004). For reasons that are remain unclear, administration of gp96 via the intradermal route is 10-fold more efficient at inducing its biological effects than administration via the subcutaneous route (Chandawarkar et al., 1999).

High-dose gp96 administration has also been shown to prolong the survival of murine skin transplants exhibiting minor and major antigenic disparity (Kovalchin

et al., 2006). In this study, the capacity of gp96 to prolong graft survival was not dependent on the source of the tissue from which it was isolated (Kovalchin et al., 2006), thereby confirming that the immunoregulatory activities of high-dose gp96 does not appear to be source-specific, unlike the influence of low-dose gp96 on tumour immunity (Chandawarkar et al., 2004, Chandawarkar et al., 1999). We have recently demonstrated that high-dose gp96 can modestly, but significantly prolong the survival of rat cardiac allografts (from a median of 8 days to a median of 14 days). However, in our studies the immunoregulatory effect was specific, as the prolongation of transplant survival required the administration of donor tissue-derived gp96; no prolongation of transplant survival was observed when recipients were treated with recipient tissue-derived gp96 (Slack et al, 2007).

Should the effects of gp96 administration indeed be donor tissue-specific, then the specificity of the immunoregulatory response will be defined by alloantigenic peptides that are chaperoned by the administered gp96. In this case, the specificity of the response will be to antigen presented via the indirect (donor alloantigen presented by recipient APCs) rather than the direct (donor alloantigen presented by donor APCs) presentation pathway. This would explain the observed inability of gp96 to have a more pronounced effect in the early stages of rejection, as this phase of the rejection process is primarily driven by allogeneic responses that are directed towards, and are specific for directly presented antigen. The basis to these apparently discrepant results has yet to be elucidated.

THE MECHANISTIC BASIS TO THE ANTI-INFLAMMATORY PROPERTIES OF STRESS PROTEINS?

The precise mechanism underlying the anti-inflammatory properties of high-dose gp96 is currently uncertain, however there are a number of different stages in the development of an immune response at which gp96 might influence the qualitative nature of that response. Firstly, it might influence the phenotype (antigen expression, cytokine secretion profile) of APCs, particularly DCs, and their capacity to activate and influence the functional phenotype of responding T cell populations. Secondly, stress proteins might directly interact with, and influence the functionality of different T cell populations.

Influence of Gp96 on the Phenotype and Function of Antigen Presenting Cells?

DCs must be mature and express essential co-stimulatory molecules such as CD80 and CD86 in order to fully activate T cells, and in this regard gp96 has been reported to induce the maturation of, and cytokine secretion from these and other APCs (Baker-LePain et al., 2002, Basu et al., 2000, Reed et al., 2003, Singh-Jasuja et al., 2000b, Zheng et al., 2001) (Figure 1). Although the view that gp96 is a universal activator of DCs is commonly-held, data from our own laboratory demonstrates that this might not necessarily be the case, as in our hands, gp96 has no effect on the

expression of surface antigens by rat bone marrow-derived DCs (BMDCs) or their secretion of cytokines (Mirza et al, 2006). Nor have we been able to demonstrate receptor-mediated binding of gp96 to DCs (Mirza et al, 2006). These findings confirm previous work by others which has found gp96 to have no effect on the phenotype of human peripheral blood monocyte-derived DCs (Bethke et al., 2002). The inability of gp96 to influence the phenotype of DCs in these studies therefore questions the widely held view that gp96 is a universal ligand for, and activator of DCs.

An interesting observation has been that although we have been unable to demonstrate receptor-mediated binding of gp96 to a population of BMDCs, gp96 is internalised by pinocytosis (Mirza et al, 2006). It is known that DCs can subsequently present antigenic peptides derived from pinocytosed exogenous material to T cells (reviewed in (Norbury 2006)) and these findings suggest that antigenic peptides derived from pinocytosed gp96 might be presented to appropriate T cell populations by immature DCs in the absence of essential co-stimulatory signals (as we have shown gp96 to have no effect on the maturational status of rat BMDCs). This could result in the generation/recruitment of a population of immunoregulatory T cells which has the capacity to influence and control inflammatory responses. Further studies are underway in order to test this and associated hypotheses regarding the anti-inflammatory effects of gp96.

Influence of Gp96 on the Phenotype and Function of T Cells?

As indicated earlier, high-dose gp96 administration appears to induce and/or activate immunoregulatory $CD4^+$ T cell populations, as the inhibition of tumour immunity and protection from diabetes and EAE can be achieved by the adoptive transfer of $CD4^+$ T cells from animals that have been treated with high-dose gp96 (Chandawarkar et al., 2004, Chandawarkar et al., 1999). The anti-inflammatory activity of $CD4^+$ T cells has been shown in a number of well-characterised model systems to partition, in part at least, into a naturally-occurring $CD25^+$ subset (Gavin and Rudensky 2003, Lee et al., 2004, Shevach 2002, Waldmann, Graca, Cobbold, Adams, Tone and Tone 2004, Wood and Sakaguchi 2003), and it might be expected that gp96 specifically influences the presence and/or functional activities of such cells. This is especially the case given that gp96 has been reported to be a ligand for TLRs (Binder et al., 2004, Vabulas et al., 2002a, Vabulas et al., 2002b) which are expressed on such regulatory T cells (Caramalho, Lopes-Carvalho, Ostler, Zelenay, Haury and Demengeot 2003). Furthermore, the triggering of TLRs on $CD4^+CD25^+$ T cells by LPS has been shown to induce a 10-fold increase in their suppressive activity (Caramalho et al., 2003). However, the suppressive effect of high-dose gp96 has been shown not to partition with the $CD4^+CD25^+$ or $CD4^+CD25^-$ phenotypes (Chandawarkar et al., 2004).

To date there have been no definitive studies into the effects of gp96 on the induction or activities of specific regulatory T cell populations, nor is the mechanism by which high-dose gp96 can down-regulate inflammatory events known. However,

it appears that high-dose gp96 administration can result in a state of peripheral $CD3^+$ T cell hyporesponsiveness, as indicated by a reduced secretion of cytokines when cells from animals that have been treated with high-dose gp96 are polyclonally stimulated via CD3 in vitro (Slack et al, 2007). It is also possible that gp96 has direct effects on T cells and preferentially induces and/or recruits T cells exhibiting an immunoregulatory phenotype, as we (Mirza et al, 2006) and others (Banerjee et al., 2002) have demonstrated that it can act as a co-stimulatory molecule for T cells and promote the secretion of an immunoregulatory Th2-like (IL-4, IL-10) cytokine profile.

CONCLUDING STATEMENT

It is clear from a number of studies that the administration of gp96 can have both inflammatory and anti-inflammatory consequences. The mechanisms underlying these opposing effects are currently unclear and it is important that the dose-dependent effects of gp96 administration are better understood as this dual capacity has significant implications for the use of this stress protein as an immunotherapeutic agent.

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CHAPTER 20

ANTI-TUMOR RESPONSE AND HEAT SHOCK PROTEINS (HSP): A FRIEND OR FOE RELATIONSHIP?

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Abstract: Heat shock proteins (HSP), particularly inducible HSP72 have a role in generating an effective antitumoral response as immunogenic peptide carriers or as immunostimulants; inducing activation and maturation of dendritic cells (DC). Their basic function is as molecular chaperones, ATP dependant; increasing cell survival under any type of stress. Chaperone function is natural to protein family HSP70 structure, having a C-terminal domain that binds unfolded proteins and peptides and a N-terminal ATPase domain that controls peptide binding pocket opening and closing. Their immunostimulant role might antagonized with their protective activity against cell death induced by stress or cytotoxic agents. Inducible HSP70 is implicated in carrying out these two functions; purpose of the present review. Furthermore, is possible other members of HSP70 protein family to be implicated, but in different ways; by inducing immune response or as tumoral growth promoters inhibiting apoptosis. Comprehension of mechanisms that regulate both activities, is crucial in developing an effective antitumoral therapy through the search of substances that preserving their immunogenic potential do not increase tumor resistance to classical antitumoral therapy

Keywords: HSP70, immunostimulation, exosomes, apoptosis, antitumoral therapy

HSP AS IMMUNOSTIMULANTS

HSP as molecular chaperone assist in folding and transportation under normal physiological conditions and following stress stimuli of a variety of polypeptides and proteins. Inducible HSP70 was describe as an antigen associated with rejection of chemically induced murine sarcoma tumors. (Udono et al., 1994). The prevailing idea is that the tumor derived HSPs can carry peptides with antigenic potential to

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favor "cross-priming"; phenomenon consisting in extracellular antigen presentation through the endogenous pathway and cytotoxic T lymphocytes prime by HSP70 chaperoned peptides and presented by Histocompatibility Major Complex HMC I (Blachere et al., 1997; Srivastava PK, 1994). When antigen presenting cells (APC) capture HSP/peptide complexes through HSP receptors (CD91, CD36), presentation through endogenous pathway is efficient giving the peptide protection from cytoplasmic proteases degradation (Basu et al., 2001; Binder et al., 2001). A clear demonstration of HSP role in antigen transference, was perform using Hsf1 gen knock out mice, a main transcription factor of inducible HSP70 synthesis. Cross-priming of antigens expressed only in keratinocytes, are clearly diminish in these animals (Zheng et al., 2004).

HSP per se, through Toll 2, Toll 4 and CD14 receptors can activate APC by inducing co-stimulatory molecules and pro-inflammatory cytokines that regulate the adaptive immune response, reason why they have been named chaperokines (Somersan et al., 2001; Asea et al., 2000, Asea et al., 2002; Basu et al., 2000). Several researchers have proposed that abnormal cell surface expression of HSPs on tumor cells could help recognition by natural killer cells (NK cells) (Fujieda et al., 1995; Multhoff et al., 1997) or LT γδ (Tamura et al., 1993). This cytotoxic mechanism might involve an increase in granzyme B uptake, through its HSP70 union (Gross et al., 2003), explaining previous observations where the increase in activity of cytotoxic cells is mediated by HSP70 (Dressel et al., 2000). Furthermore, HSP70 could also increase apoptosis mediated by TCR interaction in direct association with caspase activated DNase (CAD), enhancing its activity (Liu et al., 2003).

RELEASE HSP AND IMMUNOMODULATION

Since HSP immunomodulatory function is extracellular depends upon cell liberation. It has been recently reported, active liberation of HSP70 proteins by peripheral blood mononuclear cells without jeopardizing cell viability (Hunter-Lavin et al., 2004). The latter explains the detectable levels of HSP70 found in apparently normal individual sera, patients with inflammatory processes or chronic infections; (Pittet et al., 2002; Njemini et al., 2003; Njemini et al., 2004), even in mononuclear cells of individuals with Hodgkin lymphoma (Quijano et al., 2003) or with other tumors. In addition, we have observed constitutive forms of HSP, the HSC70 be released by tumor cells stress by heat or by cytokines as IFN γ or IL-10. In this case HSP70 is mobilized to the plasma membrane, and then perhaps secreted (Barreto et al., 2003) as reported in other models (Broquet et al., 2003). HSP70 mobilization is cytokine-dependant and even more HSP70 secretion in response to stimulus make us suppose that immunoregulator function exists in normal and pathological conditions as a second messenger. Evidence of the latter is shown by macrophage mediated phagocytosis stimulate by HSP70 allowing to speed up tissue healing in case of tissue injury (Kovalchin et al., 2006).

Treatments with monesin, brefeldin A or methyl-β-cyclodextrin has revealed that HSP release is through a non classic exocytic pathway, Ca^{++} dependent and in

some cases associated the lipid rafts (Hunter-Lavin et al., 2004; Broquet et al., 2003). Alternatively, the HSP70 increase in cell supernatants treated with detergents has lead to propose an important pathway for HSP liberation in response to heat, IFN γ and IL-10 treatments. Is it association to a membrane vesicular structure call exosome (Lancaster et al., 2005; Gastpar et al., 2005; Bausero et al., 2005). These observations suggest that soluble HSP70 might have immunomodulatory function by activating immune response though DC, acting as a "danger signal" and on the contrary, HSP70 in the exosomes might be a Trojan horse transporting tumor antigens to be presented by cross priming. These apparently excluding functions might be closely bound.

EXOSOMES AS IMMUNE SYSTEM REGULATORS

Exosomes are complex structures to which immunomodulatory activity has been attributed specially in antitumor immunity models (Thery et al., 2002). Exosomes can be derived from diverse cells types as; tumor cells, intestinal epithelial cells, mast cells, virus infected cells and DCs (Andre et al., 2002a; Andre et al., 2002b; Wolfers et al., 2001; Nguyen et al., 2003; Van Niel et al., 2003; Skokos et al., 2003; Thery et al., 1999; Thery et al., 2002). Exosome formation is associated with multivesicular bodies and endocytic pathway (Raposo et al., 1996); as well as to lipid rafts which are structures holding an important role in the selection of proteins during exosome formation, at least for MHC II molecules. Additionally presence of HSP70 and HSC 70 in exosomes has been clearly demonstrated (Lancaster et al., 2005; Thery et al., 1999) and because their chaperone role could regulate protein or peptide selection in this structures.

The role of DC derivate exosomes in the induction of antitumoral immune response have been establish (Zitvogel et al., 1998) and presence of molecules associated with antigen presentation (MHC Class I and II) and with cell adherence (some integrins ICAM-1) (Review in Thery et al., 2002), facilitates the induction of this immune response. The role of tumor or infected cells derived exosomes *in vivo* have not been completely assess. In fact, exosome secretion is normally made by somatic cells as normal cell waste discarding mechanism (Johnstone et al., 1987) and is possible that secretion and capture of exosomes by DC will maintain suppressor immune response homeostasis against self antigens. Is also feasible that in inflammatory and infectious processes, exosomes will be carriers of target antigens that are to be transported into the extracellular milieu and presented by APCs. Effective activation of DC, and the type of peptide contained in exosomes, could depend upon the cytokine present in extracelular milieu. Indeed, exosomes might be released in response to IL-10 or IFN-γ, (Kim et al., 2005; Bausero et al., 2005), but the differences between the peptides released after treatment with one or other cytokine are unknown. It is not surprising to find that each peptide load will be different due to cytokine action in the activity over the proteasome and immune proteasome (Loukissa et al., 2000) and that the immunodominant or subdominat peptides representation in each exosome be the difference. It can

be hypothesized that exosome release in response to IL-10, induce activation of regulatory T lymphocyte clones, while the ones release in response to IFN-γ, will induce an effector response.

Immunization efficiency with exosomes derived from dendritic cells, has began human evaluation (Escudier et al., 2005; Morse et al., 2005) and although a decrease in tumor has been observed owe to antitumoral response induction, is clear that obtaining exosomes from autologous DC is a complicated and impractical process. In contrast, the use of exosomes derived from tumor cells has proof to be useful choice to induce an effective antitumoral response in melanoma and murine plasmocitoma (Altieri et al., 2004; and personal observations). It might be a good alternative then for antitumoral induction if selective in vivo secretion of tumoral exosomes may be induced. In fact, exosomes derived from heat stress tumor cells contains higher concentrations of HSP70, induce a better priming of cytotoxic T lymphocytes (Dai et al., 2005), suggesting that presence of HSP70 is not only important for transport of immunogenic peptides through exosome structures but also during death of tumoral cells. Therefore, induction of HSP70 within the target cell before death caused by cytotoxic agents might be a good alternative to increase immunogenicity of tumors and to direct a specific immune response.

This alternative must be well evaluated, so inoculation of exosomes derived from tumor cells some times, have adverse effects, favoring tumor growth (Liu et al., 2006). The latter makes us take into consideration existence of additional mechanisms allowing activation of DC, for example: a good inflammatory environment; could allow these cells to activate effector cells after the capture of antigens transported by exosomes. The absence of this environment, would lead to T cells anergization. The environment might be generated by exosomes, but seems that exosomes are antigen carriers, not activating structures, probably because the absence of HSP70 on their surface as recently shown (Clayton et al., 2005). It is comprehensible then that the coexistence of both secretion ways of HSP70 to be important.

HSP RELEASE DURING CELL DEATH

When the release of HSP70 is passive, it can exert immunostimulant activity, specially in necrotic cell death, although apoptotic cell death may also have such activity (Todryk et al., 1999; Chen et al., 2001; Kokhaei et al., 2004). However, death by necrosis is better immunostimulant than death by apoptosis. Tumor cell treatment with doxorubicin or epirubicin, enhances HSP70 expression, induces necrosis delay, allows better antigen transference to DC and consequently efficient cross-priming of T lymphocytes, class I restricted (Buttiglieri et al., 2003). We recently demonstrated that DC loaded with necrotic but not apoptotic bodies, from T cell lymphoid leukemia cells, being poorly immunogenic, induce antitumoral response that can be increased in absence of regulatory cells T cells $CD4^{+}CD25^{+}CTLA4^{+}$; allowing complete tumor elimination and appearance of protective immune memory (Fiorentino et al., 2006).

Many factors had been postulate in order to explain differences. The presence of an inflammatory environment generated by tissue destruction is one of them (Crittenden et al., 2003). Additionally, formation of HSP70-peptide complexes and possibly their immunogenicity might be enhanced in conditions of stress as recently suggested (Callahan et al., 2002). In fact we suppose that for a good immune response the subsequent kinetics should be follow. Initial HSP liberation by the target cell, can stimulate NK activity and IFNγ synthesis independent of chaperone activity (Multoff et al., 1999). The IFNγ produce by NK cells, allows the later activation of the inmunoproteasome that will process inmunogenic proteins producing different immunodominant peptides from subdominant produced by constitutive proteasome (Basler et al., 2004; Rodriguez et al., 2002). At the same time, IFN-γ will increase exosome secretion favoring a specific antitumor response induction.

The latter, could mean that cell death through mechanisms that allows an effective processing of tumor antigens, efficient transference to DC and adequate presentation by enough activated cells, are key elements for induction of protective immune response. Now days, is clear the decisive role that Hsp70 plays.

HSP70 REGULATION IN TUMOR CELLS IN APOPTOTIC AND NON-APOPTOTIC CELL DEATH

Cell death resistance, particularly apoptotic death, is an important aspect in the development of anticancer drug resistance. However, most tumor cells retain the ability to sustain permanent growth arrest or undergo non-apoptotic types of cell death, such as necrosis, autophagy, senescence and mitotic catastrophe. Therefore, it might be possible to exploit these properties to induce tumour-cell death by non-apoptotic means and to confound the survival of drug-resistant clones. It has been well documented the HSP70 contribution to increase cell survival above apoptotic death. However, its participation in different types of death are least known. It is now accepted that apoptosis or necrosis death is negatively regulated by HSP being an interesting field to develop effective antitumoral therapy. Cellular integrity maintenance is a classical function of HSP70, protecting the cell against natural or induced death (Gabai et al., 2005; Gibbons et al., 2000); regulating negatively many pathways implied in apoptosis (Beere et al., 2004b; Beere et al., 2005c; Sreedhar et al., 2004). Antitumor conventional therapy induce HSP synthesis, and although it can participate in transference of tumor antigens, it can also favor tumor accumulation mutations, by preventing apoptosis and facilitating the progression into more aggressive tumors (Csermely, 2001).

ROLE OF HSP70 IN APOPTOSIS: INITIATION PHASE

Apoptotic cell death is mediated by caspases (cystein proteases) that cleave aspartate residues inhibiting or activating target substrates (Wolf et al., 1999). The sequence of events that ends at caspase activation can be divide into three phases, i) initiation phase (*signaling phase)*, phase where death domains participate with surface

receptors, particularly the members of Tumor Necrosis Factor family, and the mitochondrial pathway which can be activate through this receptors or by other stimulus; ii) transduction phase (preparation phase) where activation of initiator caspases, certain kinases and phophatases occurs iii) execution phase (death phase) where the effector capases are activated (Sreedhar et al., 2004).

Initiation phase can be trigger when a specific ligand encounters one of several cell surface death receptors present in LT lymphocytes or NK cells (Screaton et al., 2000). Tumor Necrosis Factor Receptor 1 (TNFR-1) and Fas receptors, both contain death domains (DD), that can recruit molecules containing adapter molecules associated to DD as the death domain associated to TNFR1 (TRADD) and the death domain associated to Fas (FADD). Homotypic interactions between Fas DD and FADD induce recruitment and auto cleavage of caspase 8 (Chinnaiyan et al., 1995). In TNF signaling, TRADD is recruited by FADD as a consequence of formation and release of the TNFR1 complex (Chinnaiyan et al., 1996; Hsu et al., 1996a; Hsu et al., 1996b; Micheau et al., 2003) to initiate pro-caspase activation which in turns is autocleaved, allowing the effector caspases 3, 6 and 7 to activate (Chang et al., 2000). The receptor for apoptotic inductor ligand related with TNF (TRAIL)-R1 (death receptor 4) or TRAIL-R2 (death receptor 5) also recruits and activates procaspase 8 (MacFarlane et al., 1997; Pan et al., 1997; Walczak et al., 1997) in a FADD dependent manner (Schneider et al.,1997). Although Fas induced apoptosis normally implies FADD union and caspase 8 activation, an alternative path exists. In this case, the recruitment of an alternative adaptor molecule, Daxx leads to MAPKKK kinase activation, which in turn is regulated by apoptotic signals (ASK-1) (Chang et al., 1998) in order to activate SAPK/JNK and consequently induce apoptosis (Yang et al., 1997b).

HSP70 or HSP90 expression can significantly increase cell death susceptibility, induced by TNF with cycloheximide and Fas or to the union of TCR/CD3 (Galea-Lauri et al., 1996; Liossis et al., 1997). However, it has been demonstrated that HSP70 among the HSPs, modulate signaling coming from Fas death receptors, TNF and TRAIL activation (Liossis et al., 1997; Mehlen et al., 1996b). HSP70 and HSP27 can suppress apoptosis when link to Daxx and ASK-1, inhibiting their function (Charette et al., 2000; Park et al., 2002). Both proteins can also inhibit apoptosis induced by TRAIL and TNF in several cell types (Ozoren et al., 2003; Jaattela et al., 1993; Jaattela et al., 1992; Mehlen et al., 1995a; Mehlen et al., 1995b; Mehlen et al., 1996b). In other words, while HSP70 leads to cytotoxic T cells activation by favoring cross priming, it also can inhibit induced apoptosis through death receptors as Fas and TNF.

HSP ROLE IN DEATH INDUCE THROUGH MITOCHONDRIAL PATHWAY

Mitochondria contributes to both extrinsic and intrinsic apoptosis inducing pathways. Furthermore, the mitochondria can integrate and spread intrinsic pathway signals from inside the cell like oxidative stress. Apoptotic inducing conditions

involves mitochondrial membrane permeability disruption, leading to the eventual rupture of the outer mitochondrial membrane, with the release of pro-apoptotic proteins into the cytoplasm. The aperture of the permeability transition pore (PTP) is believe to produce dissipation of the mitochondria membrane potential, impairing respiratory chain function and allowing entrance of solutes, water, and consequently inducing matrix swelling and rupture of the outer membrane releasing the caspase-activating proteins. PTP is believed to be form by association of adenine nucleotide translocase (ANT) localized in the mitochondrial inner membrane, the voltage-dependent ion channel (VDAC) localized in the outer membrane, the mitochondrial peripheral benzodiazepine receptor (PBR) and the peptidyl-prolyl isomerase cyclophilin D (Cyp-D).

The mitochondria cell death pathway can be activated by stress, cytotoxic drugs and different signals that can trigger proapoptotic members of the Bcl-2 family to translocate the mitochondrial membrane and release pro-apoptotic factors into the cytosol such as; cytochrome c (Kluck et al., 1997; Yang et al., 1997a), Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), AIF (Susin et al., 1999), EndoG (Li et al., 2001) and HtrA2/Omi (Suzuki et al., 2001). The precise mechanism by which cytochrome c is release is still not clear, but is known to be regulated by the antagonist activity of the Bcl-2 protein family (Green et al., 1998a; Willis et al., 2003); which includes pro-apoptotic members such as; Bax, Bak, Bok, Bik, Blk, Hrk, BNIP3, Bim, Bad, Bid and DIVA, and anti-apoptotic proteins closely related with Bcl-2 as Bcl-xl, Mcl-1, A1 y Boo (Green et al., 1998b; Gross et al., 1999, Creagh et al., 2000). A subclass of pro-apoptotic members containing exclusively the homology domain 3 (BH3) mediates the association homo/hetero dimers between family members including Bim, Bid and Bad, (Bouillet et al., 2002). Such proteins facilitate Bax and Bak pro-apoptotic activities (Desagher et al., 1999; Eskes et al., 1998) and are the targets of pro-survival proteins as Bcl-2 and Bcl-xl (Cheng et al., 2001; Vieira et al., 2002). Bid is cleave by caspase 8 to generate a truncated Bid (tBid), allowing the release of mitochondrial proapoptotic factors in a Bax dependent manner (Li et al., 1998; Luo et al., 1998, Gross et al., 1999).

Mitochondrial pathway can be inhibited by HSP. In fact, Bid cleavage induced by TNF is prevented by HSP70 (Gabai et al., 2002). Is probable that the inhibiting activity is related to the activation of antiapoptotic factors NF-κB dependent (Beere et al., 2005c). Is also possible that HSP70 interacts directly or indirectly with members of the Bcl-2 protein family. The Bcl-2 union protein, BAG-1 associates with survival proteins such as Raf-1, growth factor receptors and the inducible and constitutive HSP70 proteins (Takayama et al., 1998). The latter suggests that BAG-1 might be an intermediary protein that associates between HSP and Bcl-2 causing apoptosis inhibition. It has been shown that HSP70 has capability to interact directly with Bax, avoiding its mitochondrial translocation, in case ATP levels are low and finally inhibiting the release of AIF (Ruchalski et al., 2006). Alternatively, it has been shown that HSP27 and HSP70 exhibit important roles in the apoptotic pathway which is Fas dependent, due to their capacity to inhibit Bid. It has been establish, in the case of HSP27, that the protein avoids mitochondrial Bid translocation and

therefore the release of proapoptotic factors dependent of Bax. Recently, it has been implied a cooperative role between HSP70 and its co-chaperones HSP40 (Hdj-1) or HSDJ (Hdj-2) in inhibiting the mitochondrial Bax translocation, in Nitric Oxide induce apoptosis (Gotoh et al., 2004).

HSP ROLE IN POST-MITOCHONDRIAL PATHWAY

Cytochrome c once in the cytosol bounds to Apaf-1, a cytoplasmic protein containing a caspase recruit domain (CARD), a nucleotide union domain and multiple WD-40 repetitions. This union is ATP/dATP independent, generating an increase in affinity of Apaf-1 for this nucleotide. This complex is known as the apoptosome (Zou et al., 1997; Zou et al., 1999), which recruits and activates caspase 9 (Srinivasula et al., 1998). The union between CARD domain at the N-terminal of the Apaf-1, facilitates the cleavage and downstream activation of caspases 3, 6, and 7 (Chang et al., 2000; Gross et al., 1999). This caspase activation is responsible for the morphologic changes characteristic of apoptosis; as chromatin condensation, nucleosomal DNA fragmentation, rupture of nuclear membrane, externalization of phosphatidyl serine and formation of apoptotic bodies (Wang et al., 2001).

It has been shown recently that HSP70 has capacity to associate with Apaf-1 through the ATPase domain (Sreedhar et al., 2004), inhibiting oligomerization (Saleh et al., 2000) or maintaining the oligomers in an incompatible conformation with its biological activity and preventing CARD dominium exposure (Beere et al., 2000a). Any of these later actions will prevent apoptosome formation and consequently recruitment of caspase 9 and the activation of the apoptotic machinery (Beere et al., 2000a; Saleh et al., 2000). In addition, HSP70 could inhibit induced nitric oxide apoptosis (the union of NO with the superoxides forms nitrogen peroxide which is good apoptotic initiator), thus contributing to the maintenance of the mitochondrial membrane (Sreedhar et al., 2004).

The apoptosis inducing factor (AIF) is another protein released into the cytosol exhibiting a mitochondrial localization sequence (MLS). The mature form of the protein is produce by cleavage of the MLS and under adequate stimuli could induce apoptosis through chromatin condensation and DNA fragmentation (Wang et al., 2001). It has also been shown that HSP70 could prevent independent caspase cell death, due to the absence of APAF-1 protein. This mechanism of cell death can be present in some tumoral cells aiding the development of new survival ways. However, this pathway can be also regulated by HSP70 through association with AIF, recruiting it in the cytoplasm or avoiding the release from the mitochondria (Belmokhtar et al., 2003; Ruchalski et al., 2006; Parcellier et al., 2003; Gurbuxani et al., 2001; Stankiewicz et al., 2005).

Currently, is well known that concerted action of apoptotic and non apoptotic programs is required to achieve tumor reduction. In fact, study of anticancer drug responses in transgenic lymphomagenesis mice model, shows that acute response of lymphoma to anticancer agents is mostly due to apoptosis. However, the remaining lymphoma cells subsequently go into a senescence–like state that depends on the

integral functions of p53 and INK4A. In order for anticancer therapy to be effective in these transgenic animals, drug induced apoptosis and drug induce senescence must occur (Schmitt et al., 2002). Co-expression of HSP70 and p53, in esophageal squamous cell carcinoma is a bad response predictor factor (Miyazaki et al., 2005), suggesting that HSP70 can diminish cell death through apoptosis or senescence regulating p53 localization as previously proposed (Zylicz et al., 2001).

Complexity of HSP70 family could explain functional diversity of HSP70 proteins. In fact a recent work shows that HSP70-2, a protein essential for spermatogenesis, is an important regulator of cancer cell growth, (Rohde et al., 2005; Daugaard et al., 2005), but its role as an immunostimulant protein is not known.

CONCLUSIONS AND PERSPECTIVES

In summary, soluble HSP70 could have immunomodulator function by activating immune response through DC, serving as "danger signal" and in the exosomes, transporting tumor antigens or antigens use for cross-priming. These apparently excluding functions, could nevertheless be closely bound and occur spontaneously in vivo. However, there is still a need to more clearly define the role of each of the HSP family members. In searching for new anti-tumoral agents, it is also need to know the mechanisms regulating HSP70 synthesis or mobilization and the ones regulating anti-apoptotic and immunostimulant activities. Knowing the overall basic behavior of these proteins it may guide us to develop new methodologies for analyzing potential anti-tumoral effect of the new compounds. Possible candidates to exert this function are natural products used in traditional medicine and known for their antitumor activity. Both perspectives are at the present, the study aim of our laboratory.

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CHAPTER 21

HEAT SHOCK PROTEINS AND THE RESOLUTION OF INFLAMMATION BY LYMPHOCYTES

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Abstract: Depletion of phagocytes that infiltrate host organs like the lungs reduces inflammatory damage to tissues. Understanding the mechanisms by which this process occurs could lead to new therapeutic approaches to limit the detrimental effects of inflammation. The lungs, gastrointestinal tract, and skin are particularly prone to infection. Specialized immune cells protect these organs from tissue damage by eliminating phagocytes from inflamed tissues by recognizing signals produced by the phagocytes. One such signal is heat shock proteins (HSP) expressed on the cell surface of phagocytes. These HSP closely resemble their microbial equivalents, and therefore phagocytes that are labeled by HSP are recognized as target cells. T lymphocytes bearing γδT cell receptor (TCR) elicit fast responses to invading pathogens. Since the γδTCR has limited germline-encoded diversity, HSP are an ideal target for recognition by these cells. γδT cells exert cytotoxic actions towards macrophages and neutrophils that express Hsp60 or Hsp70, respectively, on their cell surface. Through the recognition of HSP on the cell surface of inflamed cells, γδT cells eliminate phagocytes from inflammatory sites, thereby preventing host tissue damage

Keywords: γδTCR, T lymphocytes, macrophages, neutrophils, inflammatory tissue damage, immunoregulation

INTRODUCTION

Trauma, hemorrhagic shock, and sepsis result in a systemic inflammatory response syndrome (SIRS) that impairs the function of host organs by an uncontrolled and destructive immune response that results in severe tissue damage (Rangel-Frausto et al., 1995). Inflammatory damage to critical organ systems (e.g., vasculature, lungs, and liver) is a major cause of post-traumatic morbidity and mortality of intensive care patients. Despite extensive scientific efforts, the mechanisms involved in the initiation, development, and resolution of inflammatory host organ damage are not completely understood. This is one of the reasons that limit the successful

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treatment of trauma casualties and patients with sepsis, despite of the broad range of therapeutic modalities available today (Abraham, 1997). Although the specific events leading to the loss of regulation of inflammatory reactions are not known, it is clear that acute lung injury (ALI) is associated with an accumulation and activation of various inflammatory cells within the lungs (Downey et al., 1999). Neutrophils (PMN) play a prominent role in lung injury. The uncontrolled and self-perpetuating pulmonary inflammation seen in ALI can result in full-blown acute respiratory distress syndrome (ARDS). Most patients with ARDS require mechanical ventilation and at least 50% of these patients die in the intensive care unit, usually due to multiple organ system failure (MOSF), nosocomial infections of the injured lungs, and severe sepsis (Haslett et al., 2000). The mechanisms by which the host down-regulates inflammatory reactions to limit the risk of ALI and ARDS are not fully understood. Moreover, the control systems that coordinate the activities of the various immune cell types within inflamed tissue are incompletely characterized. Recent advances in our understanding of these mechanisms may lead to the development of novel therapeutic approaches to prevent acute inflammatory organ injury in trauma casualties and in sepsis patients.

RESOLUTION OF THE ACUTE INFLAMMATORY PROCESS

Although the rapid initiation of the protective immune response to invading pathogens is critically important to protect the host from infectious agents, it is equally important to terminate this immune response in order to protect host tissues from the harmful effects of prolonged exposure to the toxic mediators released from inflammatory cells. Clear evidence for the importance of this balanced immune response can be observed during the course of pulmonary inflammation secondary to trauma or sepsis. The initial moderate inflammatory reactions seen in the lungs can derail, become self-destructive, and can ultimately develop into lethal ARDS. Similarly, other clinical conditions such as tuberculosis, asthma, and glomerulonephritis are associated with a failure of the cellular immune response to terminate its inflammatory cascades, ultimately leading to chronic disease characterized by extensive tissue damage and scaring that can seriously impair organ system functions (Carding and Egan, 2000; Haslett, 1995, 1999). The resolution of the inflammatory immune response coincides with the normalization of vascular tone and permeability, drainage of edema fluid, and the clearance of activated immune cells recruited to affected tissues (Haslett, 1995, 1999). Since vascular components of inflammation, such as increased vascular permeability and edema are primarily modulated by inflammatory mediators (i.e., amines, eicosanoides, nitric oxide, cytokines, etc.), which are of cellular origin, it is clear that the pivotal mechanism leading to the resolution of acute inflammation is based on the inactivation and elimination of inflammatory cells sequestered to inflamed tissues.

Macrophages (MΦ) are able to effectively eliminate inflammatory cells by phagocytic ingestion, once they recognize target cells through appropriate signals (Melley et al., 2005; Savill, 1997). Target cells may provide such signals through functional

or structural mechanisms such as the release of chemical mediators or the expression of cell surface markers. One such mechanism is based on changes of the cellular phenotype, for example due to the termination of specific cellular functions or due to programmed cell death, which facilitate phagocytosis of target cells by MΦ. Such mechanisms have been demonstrated in several systems that drive the elimination of PMN or lymphocytes from inflamed tissues (Cox et al., 1994; Haslett, 1999; Savill, 1997). The involvement of activation-induced apoptosis in the clearance of immune cells was primarily described for the inactivation of T lymphocytes as a result of Fas/Fas ligand interplay (Nagata, 1997). In a similar manner, ligation of Fas or TNF-α receptors on the cell surface was shown to induce apoptosis of PMN (Matsumoto et al., 1995; Murray et al., 1997).

Phagocytosis of apoptotic immune cells by MΦ is not the universal mechanism by which inflammatory cells are cleared from inflamed tissue. Many inflammatory mediators (e.g., LPS, chemotactic peptides, prostaglandins) are known to inhibit PMN apoptosis, which would prevent subsequent clearance of PMN through phagocytosis by MΦ (Lee et al., 1993; Rossi et al., 1995; Whyte et al., 1993). Moreover, it is not clear how MΦ themselves would be eliminated upon completion of their task. The mechanisms responsible for the down-regulation and final elimination of MΦ are poorly understood. Although, similar to PMN, MΦ have been shown to undergo apoptosis in response to various stimuli *in vitro* (Khelef et al., 1993; Mangan and Wahl, 1991), it is not clear if this process also occurs *in vivo* (Bellingan et al., 1996), and if so, how it is regulated. However, such mechanisms must exist, since the enormous influx of activated PMN, lymphocytes, and MΦ in response to microbial or other inflammatory challenges is usually cleared from affected tissue without residual tissue damage. Conversely continued accumulation of large amounts of MΦ is a hallmark feature of chronic microbial and autoimmune inflammatory diseases (Egan and Carding, 2000). Given the complexity of the issue, there is an assumption that inflammatory cells express signals alternative to apoptosis inducing non-phagocytic mechanisms that lead to their inactivation and elimination. One possible signaling pattern is the externalization of stress proteins, which are exhibited upon abnormal cellular homeostasis, and thereby appeal to the host's immune surveillance. An example of this mechanism is the recent observation that surface-expressed heat shock proteins (HSP) are recognized by natural killer (NK) cells (Multhoff et al., 1997).

HEAT SHOCK PROTEINS AS STRESS RESPONSE MARKERS

Heat shock protein (HSP) molecules comprise a family of cellular proteins that perform important housekeeping functions (Zugel and Kaufmann, 1999; Zwierska et al., 2006). Under normal conditions, cells constitutively produce several different HSP family members that contribute to the regulation of cell homeostasis. The major chaperone functions of intracellular HSP are related to protein folding, transport, and repair (Hartl, 1996; Schlesinger, 1990). HSP are also involved in intracellular signal transduction (Pratt and Toft, 1997). Under various pathologic conditions both

prokaryotic and eukaryotic cells increase intracellular expression of HSP in order to protect the cells from stress, primarily by preventing uncontrolled protein unfolding induced by stressful agents (Parsell and Lindquist, 1993; Zugel and Kaufmann, 1999). Members of the Hsp70 family, for example, have been shown to protect cells from oxidative injury and metabolic stress like ethanol intoxication (Dastoor and Dreyer, 2000; Su et al., 1998). Transgenic mice expressing high levels of Hsp70 revealed increased resistance to ischemic myocardial injury (Radford et al., 1996), while overexpression of Hsp70 in transfected cells conferred resistance to TNF-α (Jaattela et al., 1992). HSP are typical cytosolic proteins that lack the specific leader sequence usually required for expression on the cell surface. Nevertheless, several studies have demonstrated cell surface expression of HSP. Hsp60 was detected on the surface of Daudi cells, a human B cell lymphoma cell line (Fisch et al., 1990; Kaur et al., 1993). Hsp70 was found on Daudi cells and H9 cells, a T cell lymphoma cell line (Di Cesare et al., 1992), while Hsp90 was identified on the cell surface of cells in human solid tumors (Ferrarini et al., 1992). Other studies identified HSP on non-transformed cells: surface expressed Hsp60 was identified on MΦ (Wand-Wurttenberger et al., 1991) and endothelial cells (Xu and Wick, 1993), while Hsp70 and Hsp90 products were found on B cells (Vanbuskirk et al., 1989), monocytes (Erkeller-Yuksel et al., 1992), and PMN (Hashiguchi et al., 2001). The mechanisms involved in the translocation of HSP to the cell surface are poorly understood. In several reports, this phenomenon was attributed to apoptotic transformation of the cells (Di Cesare et al., 1992; Poccia et al., 1993). Immuno-precipitation experiments revealed that HSP molecules expressed on the cell surface contain their entire amino acid structures (Ferrarini et al., 1992; Kaur et al., 1993), however crucial questions as to whether these HSP expression requires *de novo* synthesis or not remain to be addressed. Since HSP are known to have strong protein-binding capacity, it is reasonable to suggest that some HSP members may be transported to the cell surface and bound to membrane proteins such MHC products (DeNagel and Pierce, 1992). Alternatively, lipid anchorage of HSP molecules to the cell surface may be an additional mechanism that cannot be ruled out.

In contrast to the well defined roles of intracellular HSP, the functions of HSP proteins expressed on the surface of normal (Erkeller-Yuksel et al., 1992; Ishiyama et al., 1996), infected (Di Cesare et al., 1992), transformed (Ferrarini et al., 1992; Hantschel et al., 2000; Multhoff et al., 1997; Multhoff and Hightower, 1996), and apoptotic cells (Poccia et al., 1996) are poorly understood. Several investigators have indicated that surface HSP can serve as markers of a cell's commitment to undergo elimination by the innate immune surveillance system (Carding and Egan, 2000; Egan and Carding, 2000; Hirsh et al., 2006; Multhoff et al., 1997; Multhoff and Hightower, 1996; Roigas et al., 1998). The mechanisms that lead to the translocation of HSP to the cell surface may be related to the ability of HSP to stabilize plasma membranes (Torok et al., 1997). Cells may use HSP to reinforce membranes destabilized by toxins and stress-related metabolites the cells encounter during episodes of infection or inflammation. The functions of membrane-associated HSP may not be restricted to membrane and protein stabilization. Rather, cell surface

localization of HSP may represent an intermediate step in the secretion of HSP into the extracellular space (Hightower and Guidon, 1989; Multhoff and Hightower, 1996). However, the release of HSP in the extracellular environment can also be the result of cell destruction and the release intracellular content from damaged cells (Basu et al., 2000). Although several HSP members have been detected in the extracellular milieu, the detailed mechanisms responsible for the secretion of HSP and the exact functions of extracellular HSP have not been defined.

HEAT SHOCK PROTEINS AS TARGETS FOR IMMUNE CELLS

Once HSP appear on the cell surface, they are accessible to the immune surveillance system. HSP expressed on cell surfaces can elicit strong immune responses, as these molecules contain several highly conserved epitope sequences with strong immunogenic properties (Kaufmann and Schoel, 1994; Shinnick, 1991; Zugel and Kaufmann, 1999). On the other hand, an overzealous immune response to HSP can have several undesirable effects. Due to high degree of phylogenic conservation, HSP species of microbial origin and HSP molecules produced by stressed host cells have similar immunogenic properties (Zugel and Kaufmann, 1999). Moreover, abundantly expressed HSP undergo processing by antigen presenting cells, and cells expressing HSP alone or presenting them in the context of MHC molecules are recognized by immune cells as potential targets of self-reactive antibodies or lymphocytes with specificity for HSP (Kaufmann and Schoel, 1994; Zugel and Kaufmann, 1999). In a number of autoimmune disorders in humans and animal models, HSP expression by affected cells has been observed, supporting the concept that HSP expression contributes to immunopathologic changes associated with autoimmune diseases. For example, a significant proportion of patients with systemic lupus erythematosus (SLE) expressed Hsp90 on lymphocytes and monocytes (Erkeller-Yuksel et al., 1992). Similarly, in MRL/lpr mice, a strain used in animal models of SLE, increased surface Hsp90 localization and antibodies against Hsp90 have been found (Faulds et al., 1994; Latchman and Isenberg, 1994). In these two systems, the overexpression of Hsp90 and the presence of antibodies provide tentative evidence that this molecule serves as an autoantigen, possibly causing the autoimmune alterations in SLE. Another example of HSP cell surface localization has been described for lesions in chronic experimental autoimmune encephalomyelitis (Gao et al., 1995).

γδT LYMPHOCYTES – MAJOR RESPONDERS TO HEAT SHOCK PROTEINS

Upon recognition of HSP expressed on the cell surface, the immune system proceeds to eliminate the pathogen-associated antigen by the destruction of the HSP bearing cell. This mechanism may be relevant for the clearance of infected or transformed cells as well as for the elimination of inflammatory cells, which may be a key event in the termination of acute inflammation.

T lymphocytes bearing the γδ heterodimer of the T cell receptor (γδT cells) have been implicated in the regulation of the host defense against microbial invaders and in the response to inflammatory challenges (Born et al., 1999; Cai and Tucker, 2001). The regulatory function of γδT cells is mediated through the production of cytokines such as IFN-γ, IL-10, and TNF-α as well as through direct cytotoxicity towards target cells (Cai and Tucker, 2001; Calandra and Heumann, 2000). Accumulation of γδT cells is seen at sites of inflammation associated with intracellular bacterial, viral, and parasitic infections. Moreover, in experiments with mice that lack γδT cells, bacterial infections culminate in an abnormally exaggerated and prolonged pro-inflammatory response and in increased mortality (D'Souza et al., 1997; Saunders et al., 1998; Tam et al., 2001). These findings suggest that γδT cells protect host tissues from inflammatory injury (Hirsh et al., 2004b).

Like conventional αβT lymphocytes, γδT cells can recognize antigen in the context of MHC molecules, but the majority of γδT cells employ a strategy of recognition of multiple surface molecules that present antigens such as classical MHC gene products (Schild et al., 1994; Weintraub et al., 1994) or alternative constitutive and stress-induced MHC class I-related molecules (Groh et al., 1998; Kim et al., 1995; Porcelli et al., 1989; Soloski et al., 1995). In addition, γδT cells appear to directly recognize antigens expressed on the surface, as this recognition is independent of antigen processing and presentation by MHC molecules (Li et al., 1998). These specialized mechanisms allow γδT cells to rapidly respond to invading pathogens. However, due to a limited germline-encoded diversity of the γδTCR (Hayday, 2000), this response is not very specific, and thus, conserved ligands such as HSP seem to be ideal targets for recognition by γδT cells. In fact, peptides derived from bacterial HSP are ligands well-characterized for their ability to activate γδT cells (O'Brien et al., 1992). Experimental listeriosis, for example, specifically activates γδT cells that are reactive for Hsp60 and Hsp70, and these cells may have a protective role in the immunity against infection (Hiromatsu et al., 1992; Kimura et al., 1996; Kimura et al., 1998). Similarly, in the mouse model of malaria, Hsp60 reactive γδT cells adoptively transferred into recipients conferred partial protection against the parasites (Tsuji et al., 1994). Hsp70 reactivity among γδT cells has been described as well (Beagley et al., 1993). In summary, HSP expressed on the cell surface is recognized by the immune system as a pathogen-associated molecular pattern that requires the induction of a cytotoxic immune response (Janeway, 1992; Zugel and Kaufmann, 1999).

This cytotoxic immune response is more widely utilized than to just eliminate cells infected with intracellular pathogens. It may also be directed at cells that exhibit HSP following transformation into cancer cells. Carcinogenesis usually includes several genetic and phenotypic changes, which render cancer cells sensitive to an immune attack. A well known mechanism of tumor surveillance involves NK cells that kill tumor cells without the need for antigenic specificity (Lanier, 1997). The cytotoxic activity of NK cells depends on antibodies bound to surface structures of the target cell or on the recognition of abnormal MHC class I molecules (Kos and Engleman, 1996; Lanier, 1997). HSP molecules have also been shown to be

involved in NK cell cytolytic activity. Specifically Hsp70 has been revealed as a targeting molecule of NK cells (Gastpar et al., 2004; Multhoff et al., 1997) that stimulates both cytotoxicity and migration in a concentration-dependent, highly selective, and chemokine-independent manner (Gastpar et al., 2004). Hsp60 is also involved in the regulation of NK cell cytotoxicity by interfering with the inhibitory CD94/NKG2A complex (Michaelsson et al., 2002). It has been demonstrated that human, murine, and rat γδT cells transcribe NK-associated genes and possess NK-like cytolytic activities (Dyugovskaya et al., 2003; Fahrer et al., 2001; Hirsh et al., 2004b; Laad et al., 1999). Hence, although freshly isolated γδT cells usually do not express NK cell surface markers (Born et al., 1999), these cells possess NK-like cytotoxic activity.

It was recently found that γδT cells exert cytotoxicity against activated macrophages, suggesting the involvement of γδT cells in terminating inflammation and in facilitating the recovery from infections (Carding and Egan, 2000; Egan and Carding, 2000). Infectious challenges strongly induce the expression of Hsp60 on the external cell membrane of MΦ (Belles et al., 1999), which significantly increases the susceptibility of MΦ to an attack by γδT cells (Carding and Egan, 2000; Egan and Carding, 2000). It has also been suggested that HSP-specific γδT cells interact directly with Hsp60 expressed on the cell surface, which is similar to the interaction of γδT cells with cancer cells (Fisch et al., 1990; Kaur et al., 1993; Laad et al., 1999). The recognition of cells expressing Hsp60 could be significantly inhibited in these reports by pretreatment of the target cells with Hsp60-specific antibodies (Fisch et al., 1990; Kaur et al., 1993). Thus, Hsp60 plays a significant role in the recognition and destruction of MΦ after their exposure to pathogens. However, to date no data are available on the role of other HSP family members in this process that results in the clearance of activated MΦ and perhaps other inflammatory cells.

SURFACE EXPRESSION OF HSP FACILITATES KILLING OF INFLAMMATORY PMN BY γδT CELLS

Activated PMN play a central role in the damage of lung tissue in sepsis (Downey et al., 1999; Hirsh et al., 2004a; Windsor et al., 1993). However, the mechanisms that control the lethal actions of PMN and diminish their damaging effects on lung tissues in individuals who recover from sepsis are unknown. We recently demonstrated that γδT cells can recognize and eliminate PMN and that the recognition of inflammatory PMN activated by lipopolysaccharides depends on the expression of Hsp70 on the cell surface of the PMN (Hirsh et al., 2006).

LPS Induces Hsp60 and Hsp70 Expression in PMN

Gram-negative bacteria are the main cause of sepsis in the animal model used in our study. LPS (lipopolysaccharides) are products of gram-negative bacteria that are involved in the initiation of the inflammatory response to infection (Bone, 1991).

The mammalian immune system recognizes LPS as a danger signal that activates MΦ and PMN (Janeway, 1992). LPS has been shown to induce the expression of HSP by MΦ (Kim et al., 1999). Because of the central role of PMN in the development of lung tissue damage in sepsis, we studied whether LPS can also induce HSP expression by PMN. We isolated human PMN from healthy volunteers, exposed them to LPS, and measured the expression of Hsp60 and Hsp70 on the cell surface of the PMN (Figure 1A, B).

LPS exposure resulted in the rapid and dose-dependent expression of Hsp70 that reached a maximum within 30 min after the stimulation. By contrast, the expression

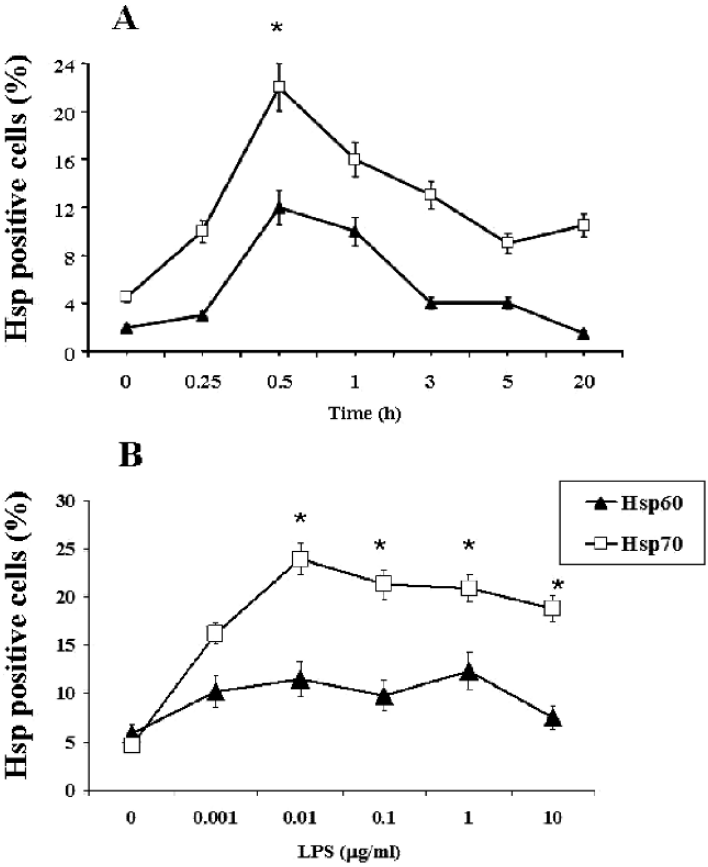

Figure 1. Heat shock protein expression by PMN. A. The time course of cell surface expression of Hsp60 and Hsp70 was determined at different time points after the stimulation of PMN with 1 μg/ml LPS. The data shown are aggregate average of 3 experiments with cells from different donors (mean ± SD). B. Isolated PMN were exposed to the indicated LPS concentrations and surface Hsp expression was determined after 60 min (data are aggregate averages of cells from 3 different donors; mean ± SD). *$p<0.02$ compared to untreated cell (0)

of Hsp60 on the cell surface seemed delayed and less pronounced than that of Hsp70. Maximum surface expression of Hsp70 was more than twice as high as that of Hsp60. After overnight incubation with LPS, surface expression of Hsp70 but not of Hsp60 was still significantly elevated compared to untreated controls (Figure 1A). These findings show that the inflammatory stress induced by LPS causes rapid and sustained Hsp70 expression on the cell surface of PMN.

Sepsis Induces Hsp70 Expression by PMN Sequestered into the Lungs

Using a mouse model of cecal ligation and puncture (CLP) to induced sepsis (Hirsh et al., 2004a; Hirsh et al., 2004b; Hirsh et al., 2006), we tested if sepsis induces Hsp70 surface expression *in vivo*. Mice were subjected to CLP and surface Hsp70 on the PMN sequestered to the lungs and on PMN in the peripheral blood was determined after 24 h with flow cytometry. We found that $17.2^{\pm}2.9\%$ of the PMN in lung tissues of septic mice were Hsp70-positive, while only $5.6^{\pm}1.5\%$ of the PMN of sham-operated control animals without sepsis expressed Hsp70 ($p < 0.002$). Interestingly, Hsp70 expression of PMN in the peripheral blood of septic animals was negligible and did not differ from that of sham controls ($0.4^{\pm}0.1$ *vs*. $0.5^{\pm}0.2\%$). These findings were consistent with the assumption that γδT cells in the lungs of septic animals may be able to eliminate inflammatory PMN through interactions through Hsp70 expressed on the cell surface of the PMN.

Hsp70 Surface Expression Requires *de novo* Protein Synthesis

Comparison of the time courses of intracellular *vs*. extracellular HSP expression of human PMN stimulated with LPS suggested that *de novo* protein expression and protein translocation to the cell membrane is required for the expression of Hsp60 and Hsp70 on the cell surface. In order to determine if HSP expression on the cell surface of PMN involves protein transcription, translation, and protein transport, we treated isolated human PMN with inhibitors that interfere with these different processes. The inhibitors of transcription (actinomycin D), translation (cycloheximide), and protein transport (brefeldin A) caused a significant suppression of Hsp70 expression on the cell surface of LPS-stimulated PMN (Figure 2). The same inhibitors were less effective in preventing the expression of Hsp60 and only brefeldin A was able to significantly reduce Hsp60 expression by LPS-stimulated PMN. These data indicate that LPS-stimulation of Hsp70 expression on the cell surface of PMN requires *de novo* protein synthesis.

γδT Cells Kill LPS-treated PMN by Direct Cell-to-Cell Contact that Involves Hsp70

The data shown above indicated that LPS induces rapid expression predominantly of Hsp70 on the cell surface of PMN. Together with our recent report (Hirsh et al., 2006), our data suggest that Hsp70 is involved in the interaction of γδT cells

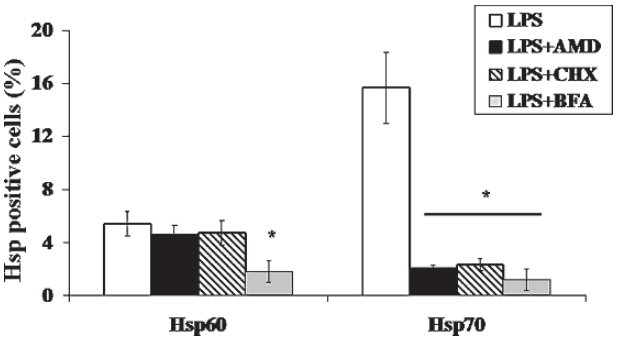

Figure 2. Up-regulation of Hsp70 cell surface expression by LPS depends on de novo protein synthesis. PMN were incubated for 1 h with LPS (1 μg/ml) in the presence or absence of actinomycin D (AMD; 2 μg/ml), cycloheximide (CHX; 5 μg/ml), or brefeldin A (BFA; 2 μg/ml) and cell surface expression of Hsp60 were determined by flow cytometry and expression of Hsp60 and Hsp70 were determined by flow cytometry and expressed as the percentage of Hsp-positive PMN. *$p<0.05$ vs. LPS alone

with LPS-stimulated PMN. To test if γδT cells can kill LPS-stimulated PMN, we co-cultured isolated human PMN with autologous γδT cells and measured PMN killing using propidium iodide uptake. Co-cultures with αβT cells or PMN alone served as controls.

As shown in Figure 3A, co-culture with αβT cells over a period of 20 h did not increased the death of PMN. After incubation overnight, the killing of PMN in the presence of γδT cells was significantly enhanced when compared to the αβT controls ($p < 0.021$) or to controls in the absence of T cells, representing spontaneous cell death of PMN ($p < 0.009$). The exposure of cultured cells to LPS significantly ($p = 0.038$) increased γδT cell-mediated PMN killing (Figure 3A). The majority of target cells were killed by γδT cells within the first 2 h after co-culture (Figure 3B).

These findings show that γδT cells but not αβT cells efficiently kill LPS stimulated PMN, suggesting that γδT cells may serve to eliminate inflamed PMN from inflammatory sites such as the lungs during sepsis. In order to gain more insight into the cellular mechanisms by which γδT cells target LPS-stimulated PMN, we performed real-time imaging experiments of human PMN in co-culture with autologous γδT cells. These cells were placed in the stage micro-incubator of an inverted microscope and time-lapse images were taken after the addition of 1 μg/ml LPS. We observed a number of highly mobile γδT cells that interacted with multiple groups of sedentary PMN through filopodia- and pseudopodia-like protrusion produced by the γδT cells. Shortly after the direct cell-to-cell contact between the PMN and these protrusions of the γδT cells, the PMN swelled and the targeted PMN disintegrated within 10 min. These observations demonstrate that γδT cells are highly effective in locating and killing LPS-stimulated PMN through direct cell contact between the effector and target cells. This process is rapid and the number of PMN killed closely corresponded to the percentage of PMN that express Hsp70 on the cell surface after LPS-stimulation. As discussed above, HSP

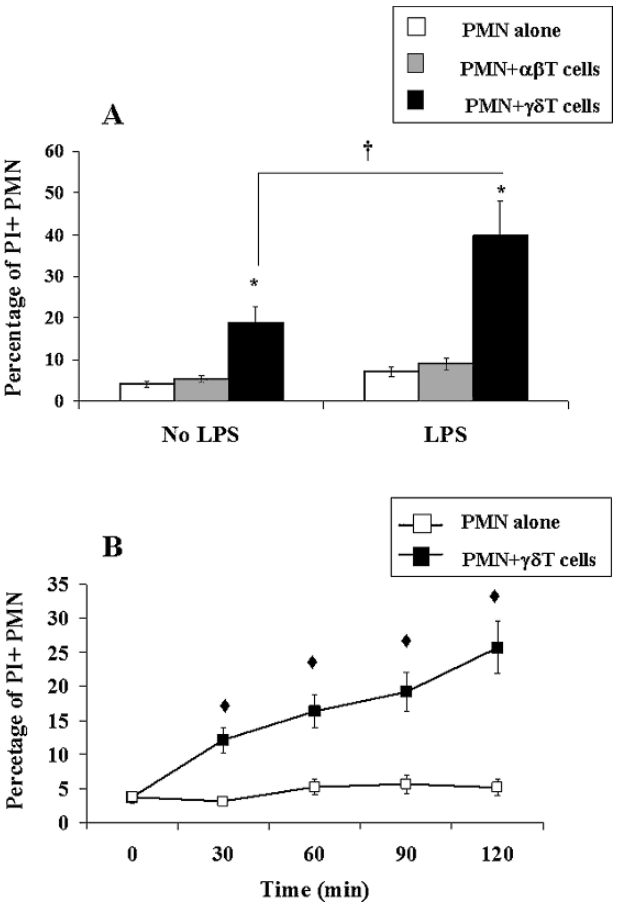

Figure 3. Killing of PMN by αβT and γδT lymphocytes. Autologous PMN co-cultured in the absence or presence of 1 μg/ml LPS with αβT or γδT lymphocytes for 20 h (A) or for the indicated time periods (B). PMN killing was determined as percentage of propidium iodide (PI)-positive PMN using flow cytometry (n=5), and compared with the rate of spontaneous cell death. *p<0.025 compared to αβT cells; †p=0.038 compared to untreated cells; ♦ p<0.05 compared PMN alone

are known ligands of γδT cell receptors, suggesting that Hsp70 on the cell surface of PMN could serve to facilitate the targeting and killing of LPS-stimulated PMN by γδT cells. To test this possibility, we treated PMN with neutralizing antibodies to Hsp70 or with an unrelated isotypic control immunoglobulin preparation. Then the cells were co-cultured overnight with γδT cells in the presence of 1 μg/ml LPS and PMN killing was assessed using the PI uptake method described above. We found that treatment with Hsp70 antibodies but not with isotypic control IgG caused a significant reduction of PMN killing from $16^{\pm}3\%$ to $5^{\pm}2\%$ ($p = 0.012$).

These data show that Hsp70 displayed on the cell surface of LPS-stimulated PMN is required for γδT cell-mediated killing of inflammatory PMN.

CONCLUDING REMARKS

Due to specialized patterns of antigen recognition and activation (Hayday, 2000), γδT cells are capable of fast and vigorous responses to various pathogens. Conventional αβT lymphocytes need 5–7 days to manifest their cytotoxic activity (Born et al., 1999; Cai and Tucker, 2001) and thus it is not surprising that self-tolerant clones of circulating αβT lymphocytes do not recognize autologous PMN as targets. The cytotoxic activity of γδT cells towards activated macrophages has been recognized as an important feature leading to the down-regulation of inflammatory reactions (Carding and Egan, 2000; Egan and Carding, 2000). Several clones of γδT cells have been shown to be able to kill activated macrophages that express HSP on their cell surface (Egan and Carding, 2000). Hence, the LPS-induced expression of Hsp70 on the cell surface of PMN suggests that γδT cells utilize a similar mechanism to recognize and eliminate inflammatory PMN for example from lungs of patients during recovery from a septic episode. Increased PMN cell surface expression of Hsp70 has been reported in critically ill patients who suffer from trauma, endotoxemia, and sepsis (Hashiguchi et al., 2001; Kindas-Mugge et al., 1993). Moreover, we found that surface Hsp70 expression on PMN sequestered into the lungs of mice after experimental sepsis was increased. Although there are clear differences in the phenotypes of the γδT cell subpopulations in human blood and the lungs of mice, our previous works (Hirsh et al., 2004b; Hirsh et al., 2006) and the data shown by other investigators (Wang et al., 2001) suggest that γδT cell populations in both species may have functional similarities in their abilities to recognize and kill Hsp70-bearing PMN. However, future studies will be needed to determine if the killing of PMN by γδT cells *in vivo* indeed requires target cell recognition through surface Hsp70 expression.

LPS-induced expression of Hsp60 and Hsp70 in PMN differed with regard to the timing of cell surface expression (Figure 1). The relatively slow externalization of Hsp60 and its sensitivity to brefeldin A, an inhibitor of protein processing and transport in the Golgi complex, suggest posttranslational modifications of the existing protein before the expression on the cell surface (Ripley et al., 1993). Under identical conditions, LPS induced rapid Hsp70 expression in the cytoplasm and on the cell membrane of PMN. This process was blocked by a broad range of inhibitors suggesting *de novo* synthesis of Hsp70. Similarly rapid appearance of newly synthesized Hsp70 also has been demonstrated in LPS-stimulated macrophages (Kim et al., 1999).

The data suggest that Hsp70 could serve as an important regulatory signal that prompts immunological attack by γδT cells of Hsp70–bearing PMN (Figure 4). We could confirm the direct involvement of cell surface expression of Hsp70 in γδT cell-mediated killing of LPS-treated PMN with the use of neutralizing anti-Hsp70 antibodies. While antibodies to Hsp70 blocked γδT cell-mediated PMN killing, no

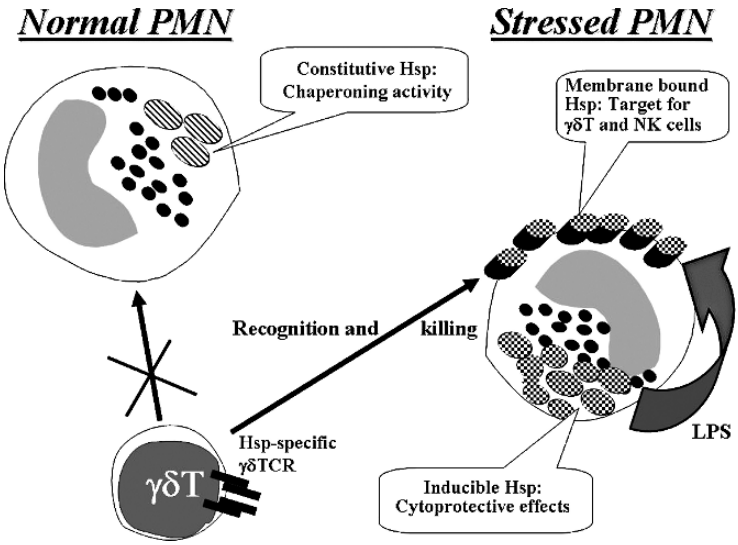

Figure 4. Under normal conditions Hsp70 synthesized by PMN are located in the cytoplasm and not recognized by γδT cells with Hsp70-specific TCR. Under stress conditions, e.g. during inflammation, PMN express increased levels of Hsp70 that rapidly translocates to the cell

such blocking effect was seen with antibodies to Hsp60. These findings suggest that Hsp60 plays only a minor, if any role in mediating the killing of LPS-stimulated PMN, although Hsp60 appears to have a more significant role in the clearance of other target cells, i.e. MΦ by γδT cells (Egan and Carding, 2000; Laad et al., 1999).

Inhibition of the recruitment of inflammatory cells to host organs such as the lungs, gut, and liver can successfully control the detrimental consequences of inflammatory responses due to host tissue damage. Another effective protective mechanism is the inactivation and depletion of inflammatory cells that are sequestered to these host tissues. The results of our study complement previous reports on the important protective functions of γδT cells in host protection. Increased γδT cell levels in the lungs of animals with sepsis were paralleled by decreased PMN counts, diminished tissue damage, and improved survival (Hirsh et al., 2004b; Hirsh et al., 2006). The majority of the γδT cells in the lungs were of the Vδ4-subtype, suggesting intestinal origin (Cai and Tucker, 2001) while the portion of other γδT cell subpopulations usually found in healthy lungs or lymphoid organs (Vγ2Vδ5/6 and Vγ1.2Vδ5/6) was comparatively minor. These findings are consistent with reports suggesting that mice deficient of γδT cells exhibit abnormally strong inflammatory responses to various infectious insults. These animals show extensive tissue necrosis, delayed resolution of inflammatory infiltrates, and increased overall mortality (Carding and Egan, 2000; D'Souza et al., 1997; Griffin et al., 1991; Tam et al., 2001). Taken together, these findings suggest that the presence of γδT cells at inflammatory sites is essential to control the inflammatory response and to protect host tissues from collateral damage caused during episodes of fierce immune defense.

Besides their ability to eliminate inflammatory cells, γδT cells possess additional mechanisms that allow them to protect host tissues and organ integrity. For example, γδT cells produce anti-inflammatory cytokines such as IL-10 (Hirsh et al., 2004b; Schaible et al., 1999) that inhibit the production of pro-inflammatory mediators including IL-1, TNF-α, and IL-8 generated by inflammatory cells within affected tissues. Through such indirect mechanisms, γδT cells may reduce the recruitment of additional PMN to sites of inflammation, suppress oxidative burst and degranulation, and thereby further diminish the risk of PMN-mediated tissue injury (Calandra and Heumann, 2000; Capsoni et al., 1997). These additional mechanisms of action in combination with the ability of γδT cells to kill inflammatory Hsp70-positive PMN (Figure 4) may explain the powerful role of γδT cells in limiting inflammatory processes and preventing host tissue damage.

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INDEX