

Hsp72 modulation of inflammatory immune responses

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Chapter 1

Introduction

1.1 Rationale for Study

The immune system mounts a response to exogenous molecules, such as bacterial products, and it has been shown that auto-antibodies can be generated, which are implicated in autoimmune diseases, such as diabetes (Brudzynski, 1993; Brudzynski, Martinez & Gupta, 1992). This demonstrates that it also responds to endogenous molecules which have recently been identified as danger signals that are released by damaged cells and able to activate the antigen-presenting cells (APCs) (Gallucci & Matzinger, 2001; Matzinger, 1994; Matzinger, 1998; Matzinger, 2002).

Heat shock proteins (Hsps), particularly Hsp72, have been reported as being danger signals to the innate immune system when found in the extra-cellular milieu (Bianchi, 2007; Campisi & Fleshner, 2003; Campisi, Leem & Fleshner, 2003; Matzinger, 2002; Williams & Ireland, 2008). The presence and levels of extra-cellular Hsp72 in particular has been studied extensively in many stressful situations such as *in vitro* and *in vivo* heat stress (Aït-Aïssa *et al.*, 2003; Currie, Moyes & Tufts, 2000; Guzhova *et al.*, 2001), infection (Njemini *et al.*, 2007; Njemini *et al.*, 2003b), exercise (Fehrenbach *et al.*, 2000; Madden *et al.*, 2008; Walsh *et al.*, 2001) and fever (Oehler *et al.*, 2001).

Another protein which has been recently termed a danger signal is high mobility group box-1 (HMGB-1) which is normally found bound to chromatin within the nucleus but can be released under stressful conditions such as sepsis (Wang *et al.*, 2001a; Yang *et al.*, 2007), arthritis (Kokkola *et al.*, 2002; Taniguchi *et al.*, 2003) and lung inflammation (Abraham *et al.*, 2000; Li *et al.*, 2003), and functions extra-cellularly as a late mediator of the pro-inflammatory response (Andersson *et al.*, 2000; Chen *et al.*, 2004; Harris & Raucchi, 2006; O'Connor *et al.*, 2003; Qin *et al.*, 2006; Williams & Ireland, 2008).

In relation to the innate immune response, Hsp72 is thought to bind to certain receptors on cells which can then trigger an inflammatory response resulting in the release of cytokines, and that Hsp72 itself can act as a chemokine (Gouwy *et al.*, 2005; Lehner *et al.*, 2004; Panjwani, Popova & Srivastava, 2000; Wang *et al.*, 2001b). The stimulatory immune function of extra-cellular Hsp72 has been questioned recently as being due to LPS contamination of recombinant proteins

during *in vitro* experiments (Bausinger *et al.*, 2002; Gao & Tsan, 2003a; Gao & Tsan, 2004; Tsan & Gao, 2004a; Tsan & Gao, 2004b).

HMGB-1 also stimulates the production of pro-inflammatory cytokines and under normal stress conditions such as tissue injury or trauma, functions in a regulatory protective manner by stimulating the immune system to repair the damage. However, when the immune system is stimulated by HMGB-1 and lipopolysaccharide (LPS), increased amounts of HMGB-1 are released in an autocrine manner in response to up-regulated TNF- α secretion which stimulates a solely pro-inflammatory response. If this response is not controlled it can lead to sepsis, and eventually death (Chen *et al.*, 2004; Gaini *et al.*, 2007a; Gaini *et al.*, 2007b; Qin *et al.*, 2006; Wang *et al.*, 2001a).

The measurement of extra-cellular Hsp72 by enzyme linked immuno-sorbent assay (ELISA) has been reported in various matrices such as serum, plasma and tissue culture supernatant, but the levels reported vary widely (Njemini, Demanet & Mets, 2003a; Njemini *et al.*, 2003b; Pockley, Shepherd & Corton, 1998; Wright *et al.*, 2000), although all rely upon the same components. Part of this thesis therefore was to develop a method to accurately quantify Hsp72 in various sample types.

The work in this thesis demonstrates that extra-cellular Hsp72 is able to stimulate the innate immune system independently of LPS contamination and that Hsp72 is able to modulate the immune response by binding to various cell surface receptors involved in cytokine signalling. Also, it is postulated that Hsp72 may help to regulate the pro-inflammatory actions of HMGB-1.

1.2 The Danger model

The immune system has recently been suggested to be able to be activated by endogenous molecules, now described as danger signals which has lead to a new theory regarding how the immune system responds (Matzinger, 1994).

Originally, in 1959, Burnet suggested a response known as the 'self- non-self' (SNS) model describing that lymphocytes were able to recognise a foreign body which stimulates the cell to generate cell surface receptors and proposed that the immune system acted to distinguish between 'self and non-self'. However, it was later discovered that B cells were able to generate an autoimmune response in conjunction with T cells in the presence of a 'signal'. This 'signal' was found to be antigen which was presented by APCs. This potentially meant that the immune system could be directed against the 'self', resulting in auto-immunity and therefore

questioning the SNS model. In 1989, Janeway described APCs as being dormant until activated through pattern recognition receptors (PRRs) which recognise pathogen-associated molecular patterns (PAMPs) from bacteria, and then up-regulate co-stimulatory signals and then present the bacterial antigen to T cells. Therefore, it was the PRRs that allowed the immune system to discriminate between 'infectious-nonself', and, 'non-infectious-self' (Janeway, 1989; Janeway Jr *et al.*, 2005b). The SNS model could still not explain certain aspects of the immune response such as responses to viruses, auto-immunity or transplant rejection.

In 1994, Matzinger proposed a new model of how the immune responses are activated. It was suggested that APCs are activated by danger signals from damaged cells. These signals appear in the extra-cellular environment by necrosis following a stressful event. This event would up-regulate the intra-cellular production of certain endogenous molecules that when presented in the extra-cellular environment are recognised by PRRs as being a result of an abnormal cell death. These signals would not be present when cells are healthy or die *via* apoptosis (Adams, 2003; Kaczorowski *et al.*, 2008; Matzinger, 1994; Matzinger, 1998; Matzinger, 2002; Williams & Ireland, 2008) (Figure 1.1).

The Danger model provides an explanation regarding a number of issues about how the immune system responds. For example, transplants and fetuses are 'non-self' but are not associated with bacterial products so should not stimulate an immune response. However, transplants often stimulate a response and are rejected whereas fetuses are generally not. As they are not 'self', using the SNS model, both should stimulate a response, but using the Danger model it can be explained in terms that a fetus does not send 'danger signals' but transplants often do. With regards to auto-immunity, in which the immune system responds to the 'self', the Danger model simply suggests that this occurs in some cases, due to a genetic mutation, resulting in an inadequate clearance of apoptotic cells, such as Lupus (Kaplan *et al.*, 2002), or by incorrectly recognising 'self' as being 'non-self' following cellular damage caused by a pathogen, such as type 1 diabetes (Abulafia-Lapid *et al.*, 1999; Reines, 2001). In both cases, the immune system is simply responding to 'danger signals' which would ordinarily be cleared. Necrosis and inadequate clearance of apoptotic cells can lead to the accumulation of endogenous danger signals. These endogenous molecules mediate the innate immune response by stimulating APCs to secrete cytokines and other inflammatory signals, leading to the recruitment of immune cells to the site of infection and the activation of the adaptive immune response (Dybdahl *et al.*, 2005; Gastpar *et al.*, 2004; Wang *et al.*, 2006a; Yang *et al.*, 2006).

1.2.1 The innate immune response

The innate immune system provides an immediate defence against pathogens and other endogenous molecules and involves the recruitment of cells such as macrophages, natural killer (NK) cells, dendritic cells (DCs) and several other lymphocyte populations (Janeway Jr *et al.*, 2005b; Janeway Jr *et al.*, 2005c). Both exogenous and endogenous mediators are readily recognised by the innate immune system by conserved small motifs known as PAMPs for pathogens, or damage-associated molecular patterns (DAMPs) (Bianchi, 2007; Prohászka & Füst, 2004). These PAMPs include lipopolysaccharide (LPS), flagellin, lipoteichoic acid, and DAMPs include Hsp60, Hsp72 and HMGB-1 (Gutsmann *et al.*, 2001; Nilsen *et al.*, 2008; Smith *et al.*, 2003; Sugawara *et al.*, 1999; Ye & Gan, 2007; Asea *et al.*, 2000b; Dybdahl *et al.*, 2005; Fiuza *et al.*, 2003; Habich *et al.*, 2002; Kol *et al.*, 2000; Rouhiainen *et al.*, 2004). These molecules are recognised by PRRs, which include cell membrane receptors, for example toll-like receptors (TLRs) and cluster of differentiation (CD) markers, which are able to recognise microbial products and other endogenous molecules (Asea *et al.*, 2002; Binder, Han & Srivastava, 2000; Bosco *et al.*, 1997; O'Connell *et al.*, 2004; Takeda, Kaisho & Akira, 2003; Triantafilou & Triantafilou, 2002; Wang *et al.*, 2006b). Interaction between PAMPs and PRRs/TLRs leads to the activation of an inflammatory response, including the production of feedback signals, such as cytokines, and/or release of mediators, such as Hsp72 or HMGB-1, leading to the recruitment of immune cells to the site of inflammation and the activation of the adaptive immune response (Ding *et al.*, 2001; Peetermans *et al.*, 1994; Wang *et al.*, 2005).

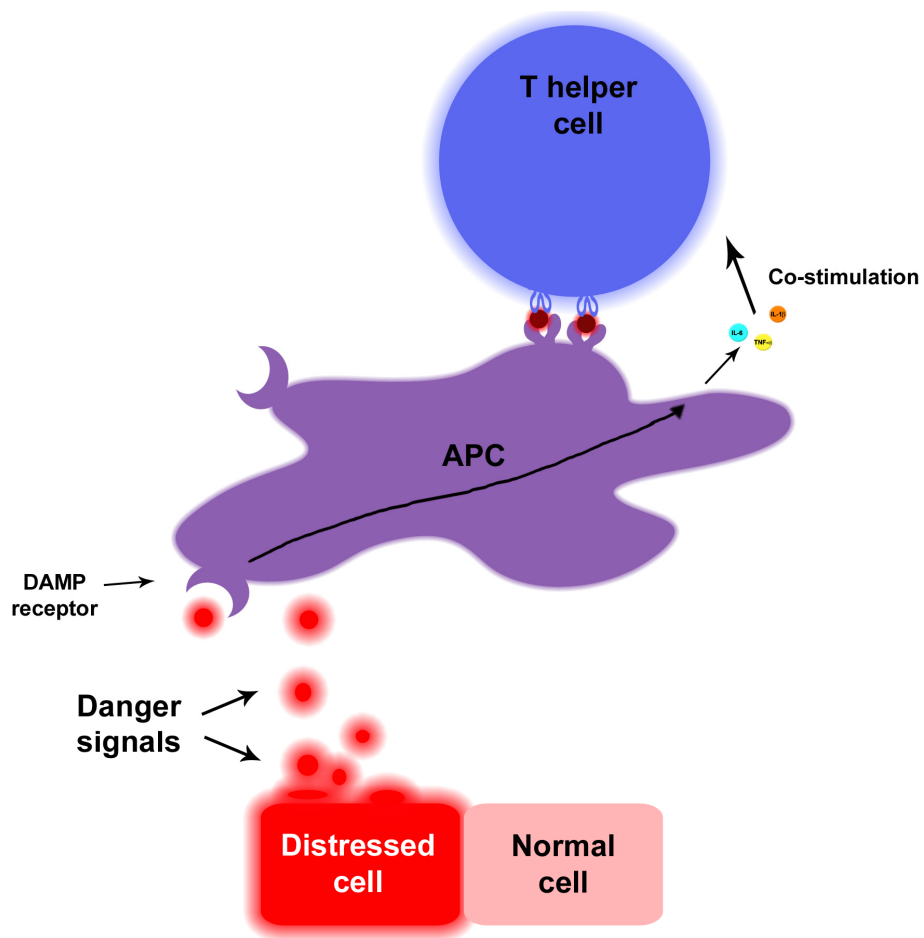


Figure 1.1: The Danger model of immune stimulation.

APCs are activated by DAMPs from distressed cells and either through internalisation and re-presentation, or by co-stimulation, stimulate the adaptive immune response.

(Adapted from Matzinger, 2002; Bianchi, 2007).

1.2.2 The adaptive immune response

The adaptive immune response is initiated when antigens are bound by APCs, such as macrophages and DCs, and are presented to T cells (Janeway Jr *et al.*, 2005b). Adaptive immunity was originally characterised by four fundamental elements:

- being antigen specific and allowing the immune system to be able to differentiate between two highly conserved proteins;
- being highly diverse in the ability to recognise a multitude of different proteins and molecules;
- being able to generate memory B and T cells which are able to mount a stronger and quicker immune response when the same antigen is encountered;
- ability to differentiate between 'self' and 'non-self' molecules and responding only to the non-self molecules.

(Janeway Jr *et al.*, 2005b).

Being able to differentiate between 'self' and 'non-self' molecules is obviously now debatable with the Danger model proposal that the activation of the adaptive immune system by APCs can also be activated by DAMPs which are released by cells undergoing stress and damage (Matzinger, 1998), such as infection leading to necrosis (Basu *et al.*, 2000; El Mezayen *et al.*, 2007; Mambula & Calderwood, 2006a; Saito, Dai & Ohtsuka, 2005; Scaffidi, Misteli & Bianchi, 2002).

Two of these proposed DAMPs, or endogenous molecules will be discussed further: Hsp72 and HMGB-1.

1.3 Heat shock proteins

The term heat shock response was first used by Ritossa *et al.* (1962) to describe an increase in the expression of certain genes when *Drosophila melanogaster* larval salivary gland cells were exposed to a 30°C heat stimulus. These genes were subsequently found to lead to an increase in the expression of proteins with molecular weights of 27 and 70 kDa (Tissieres, Mitchell & Tracy, 1974), and these proteins were named heat shock proteins (Hsps). Many studies following this discovery found that Hsps were expressed by the majority of prokaryotes and eukaryotes, and were found to be highly conserved (Boorstein, Ziegelhoffer & Craig, 1994; Hartl, 1996).

In most plant and animal systems, Hsps are synthesised following a variety of stresses such as heavy metal exposure, oxidative stress and *in vitro* serum deprivation (Ireland *et al.*, 2004; Lang *et al.*, 2000; Ménoret *et al.*, 2002). Within the cell, Hsps act as molecular chaperones by assisting the correct folding of nascent proteins, prevention of inappropriate protein aggregation, and mediating transport of proteins across intra-cellular membranes (Bukau & Horwich, 1998; Mayer & Bukau, 1998). Hsps are able to bind to hydrophobic surfaces of unfolded polypeptides which expose hydrophobic amino acid side chains that can result in aggregation. This binding results in the stabilisation and correct folding of polypeptides (Hartl, 1996; Tavaría *et al.*, 1996).

Hsps are named based on their molecular weight in kDa and gene sequence homology. The main Hsp families are small Hsps (10 – 40 kDa), Hsp60, Hsp70, Hsp90 and Hsp100 (Table 1.1).

Table 1.1: Classification of Hsps, their localisation and function.

Name	Molecular weight (kDa)	Localisation	Function
Small Hsps	15-40	Cytosol, Nucleus	Cytoskeletal stabilisation
Hsp60	58-65	Cytosol, Mitochondria	Protein folding Prevention of aggregation
Hsp70	66-78	Cytosol, Mitochondria, Endoplasmic reticulum	Protein refolding Protection against stress Downregulation of HSF1 activity
Hsp90	82-90	Cytosol, Endoplasmic reticulum	Prevention of aggregation Maintenance of HSF1
Hsp100	97-120	Cytosol, Nucleus, Mitochondria	Thermotolerance Protein refolding

Adapted from (Pockley, 2003) and (Srivastava, 2002)

1.3.1 Regulation of the heat shock response

The heat shock response is regulated by the activation and translocation from the cytosol to the nucleus of heat shock transcription factor 1 (HSF1), which upon heat shock, trimerises and binds to heat shock elements (HSEs) that are located within promoter regions of Hsp genes (Morimoto, 1993; Sarge, Murphy & Morimoto, 1993) which result in the transcriptional activation and synthesis of Hsps (Cotto, Kline & Morimoto, 1996). As Hsp synthesis increases, Hsp72 and other Hsps (such as Hsp90) re-localise to the nucleus where they bind to the HSF1 trans-activation domain and repress transcription of Hsp genes (Shi, Mosser & Morimoto, 1998; Wu, 1995). Under unstressed conditions, HSF1 is present within the cytosol as a monomer which is bound to Hsps and other chaperones, and is deficient in translational activity (Shi *et al.*, 1998).

1.4 The Hsp70 family

The Hsp70 family of proteins is among the most widely studied of the heat shock proteins and are the major chaperones involved in assisting the correct and biologically active folding of nascent polypeptides. This process is ATP-dependent in eukaryotic cells (Gebauer, Zeiner & Gehring, 1997; Mayer & Bukau, 1998). In mammalian systems, the most well known members of the family include the constitutive cytosolic Hsc70 (Hsp73), the stress-induced cytosolic form (Hsp72), Grp78 (BiP), which is found within the endoplasmic reticulum (ER), and the mitochondrial Grp75 (Gebauer *et al.*, 1997; Liu *et al.*, 2005). The main form of Hsp72 in *E. coli* is DnaK (Krska, Elthon & Blum, 1993).

Proteins from this family contain two domains: the N-terminal domain, and the C-terminal domain (Mallouk *et al.*, 1999; Sriram *et al.*, 1997; Tavaría *et al.*, 1996). The N-terminal domain is highly conserved and is also known as the ATPase domain. It is able to hydrolyse ATP to ADP which leads to conformational changes in the other domain. The C-terminal domain contains a variable region known as the substrate binding domain. The substrate binding domain contains a groove to which hydrophobic peptides bind. The C-terminal domain also contains a hinge region which allows it to form a lid for the substrate binding domain (Gething & Sambrook, 1992; Hartl, 1996; Hartl & Hayer-Hartl, 2002; Ruchalski *et al.*, 2006; Rudiger, Buchberger & Bukau, 1997) (Figure 1.2).

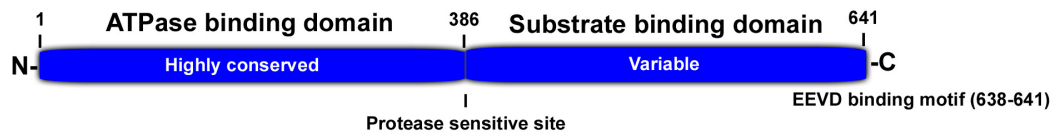


Figure 1.2: The structure of Hsp72 protein.

(Adapted from Gething & Sambrook, 1992; Rudiger *et al.*, 1997; Mallouk, *et al.*, 1999; Ruchalski, *et al.*, 2006).

1.5 Intra-cellular functions of Hsp72

Within the cell, Hsp72 has a clearly defined role. Under normal conditions, its main role is to facilitate folding and assembly of nascent proteins. It does this by binding nascent proteins in an ATP-dependent manner (Figure 1.3). When the N-terminal is ATP bound, the lid is open and unfolded protein is bound and released rapidly. Following recognition and binding of the unfolded protein (by extended hydrophobic regions), hydrolysis of ATP occurs leading to Hsp72 being ADP bound. In this substrate bound state, the lid is closed, and the protein is tightly bound within the groove of the substrate binding domain. In order for Hsp72 to release the protein, the co-chaperones Hsp40 and Hsp90 bind to the N-terminal of Hsp72 resulting in a conformational change leading to the release of ADP. This then allows ATP to be bound which leads to the release of the substrate protein, which is then either completely folded into the correct conformation either by being rebound by Hsp72, or being transferred to another co-chaperone, such as Hsp60 (Bukau & Horwich, 1998; Mallouk *et al.*, 1999).

Hsp72 is also involved with translocation of proteins across membranes *via* ATP hydrolysis, to within the mitochondria (Neupert & Brunner, 2002) and the ER (De los Rios *et al.*, 2006; Rapaport *et al.*, 1998). Elevated levels of intra-cellular Hsp72 have also been shown to inhibit apoptosis, or programmed cell death (Garrido *et al.*, 2001). When cells are undergoing a stressful event, NF- κ B signalling pathway is up-regulated, and this in turn up-regulates HSF1 and Hsp72, which in turn acts to suppress NF- κ B signalling. This suggests that a negative feedback loop associated with Hsp72 could provide an anti-inflammatory response (Hamilton *et al.*, 2004). This response is thought to involve inhibition of I κ B kinase resulting in the protection of I κ K (Ran *et al.*, 2004), or prevention of I κ B degradation which would interfere with the release and translocation of NF- κ B to the nuclear compartment, thus preventing the up-regulation of pro-inflammatory cytokines (Weiss *et al.*, 2007; Yoo *et al.*, 2000). It has been demonstrated that Hsp72 can bind to tumour necrosis

factor receptor-associated factor 6 (TRAF6) preventing NF- κ B activation which may be useful in helping cells survive tissue injury (Chen *et al.*, 2006).

In relation to HMGB-1, elevated levels of intra-cellular Hsp72 attenuate HMGB-1 secretion by transfected RAW264.7 cells, by interfering with the CRM1 translocation export pathway. Following stimulation by LPS, or TNF- α (24 h), levels of HMGB-1 secretion into the cell culture media was reduced in transfected cells. HMGB-1 remained within cell extracts in transfected cells (Tang *et al.*, 2007a). Hsp72 is also found in the extra-cellular milieu. The mechanisms of release and its actions outside the cell are still being debated and are discussed further here.

1.6 Mechanisms of Hsp72 release from cells

Release of Hsp72 was first reported in a study on cultured rat embryo cells following heat treatment (Hightower & Guidon Jr, 1989). Following this a number of studies have reported the release of Hsp72 from a variety of cells (Baretto *et al.*, 2003; Broquet *et al.*, 2003; Evdonin *et al.*, 2006b; Guzhova *et al.*, 2001; Hunter-Lavin *et al.*, 2004).

The mechanisms by which Hsp72 is released are unclear. It has been shown that necrosis leads to Hsp72 release in a passive manner (Basu *et al.*, 2000; Mambula & Calderwood, 2006a; Saito *et al.*, 2005), which may be the case for the majority of disease states. However, active secretion is now thought to be a possible mechanism, but how Hsp72 is secreted from the cell still remains unclear. The classical pathway of secretion (ER/Golgi-dependent secretory pathway) allows for soluble proteins containing an N-terminal signal (leader sequence) to be transported to the translocation apparatus of the ER. From here they are transported to the Golgi, undergoing post-translational modifications such as N-glycosylation, and are enclosed within secretory vesicles which are able to fuse with the cell membrane, leading to the release of proteins (Nickel, 2003). However, Hsp72 lacks the N-terminal signal required for this form of transport, and the use of inhibitors of the classical secretion pathway, such as brefeldin A and monensin, have been found to have no effect on the release of Hsp72 (Broquet *et al.*, 2003; Hightower & Guidon Jr, 1989; Hunter-Lavin *et al.*, 2004) (Boyce & Yuan, 2006).

Mambula *et al.* (2007) have postulated that there are likely three possible mechanisms of Hsp72 release. Firstly, Hsp72 is passively released from necrotic cells following a severe heat shock (Mambula & Calderwood, 2006a) and correlates highly with other markers of myocardial necrosis (Dybdahl *et al.*, 2005). Secondly, it can be released within vesicles in a similar way to IL-1 α (Arai & Kuwajima, 2000), or IL-1 β , which are subsequently lysed in the extra-cellular environment

(MacKenzie *et al.*, 2001). Thirdly, a mechanism has been described which involves secretion through endolysosomes that fuse with the cell membrane and release Hsp72, as it has been found to be expressed with cathepsin D, a lysosome marker. The heat shock which resulted in the release of Hsp72 also correlated with an increased expression in cell surface LAMP-1, a lysosomal-associated membrane protein (Mambula & Calderwood, 2006b).

Other studies have also demonstrated that Hsp72 may be released by exosomes which are lipid-bound (Lancaster & Febbraio, 2005; Tytell, 2005), or may be released through specialised membrane domains known as lipid rafts. Lipid rafts are specialised sphingolipid and cholesterol rich areas within the cell membrane which allow for transport of proteins, both intra- and extra- cellular (Pike, 2004). Transport of proteins across lipid rafts can be disrupted by methyl- β -cyclodextrin, and Hsp72 release has been found to be disrupted in the cell line, Caco-2 (Broquet *et al.*, 2003), PBMCs (Hunter-Lavin *et al.*, 2004), and Hsp72 has been shown to integrate within an artificial lipid bi-layer (Arispe, Doh & De Maio, 2002; Vega *et al.*, 2008).

It is therefore likely that there are at least four mechanisms by which Hsp72 is released from cells (Figure 1.4).

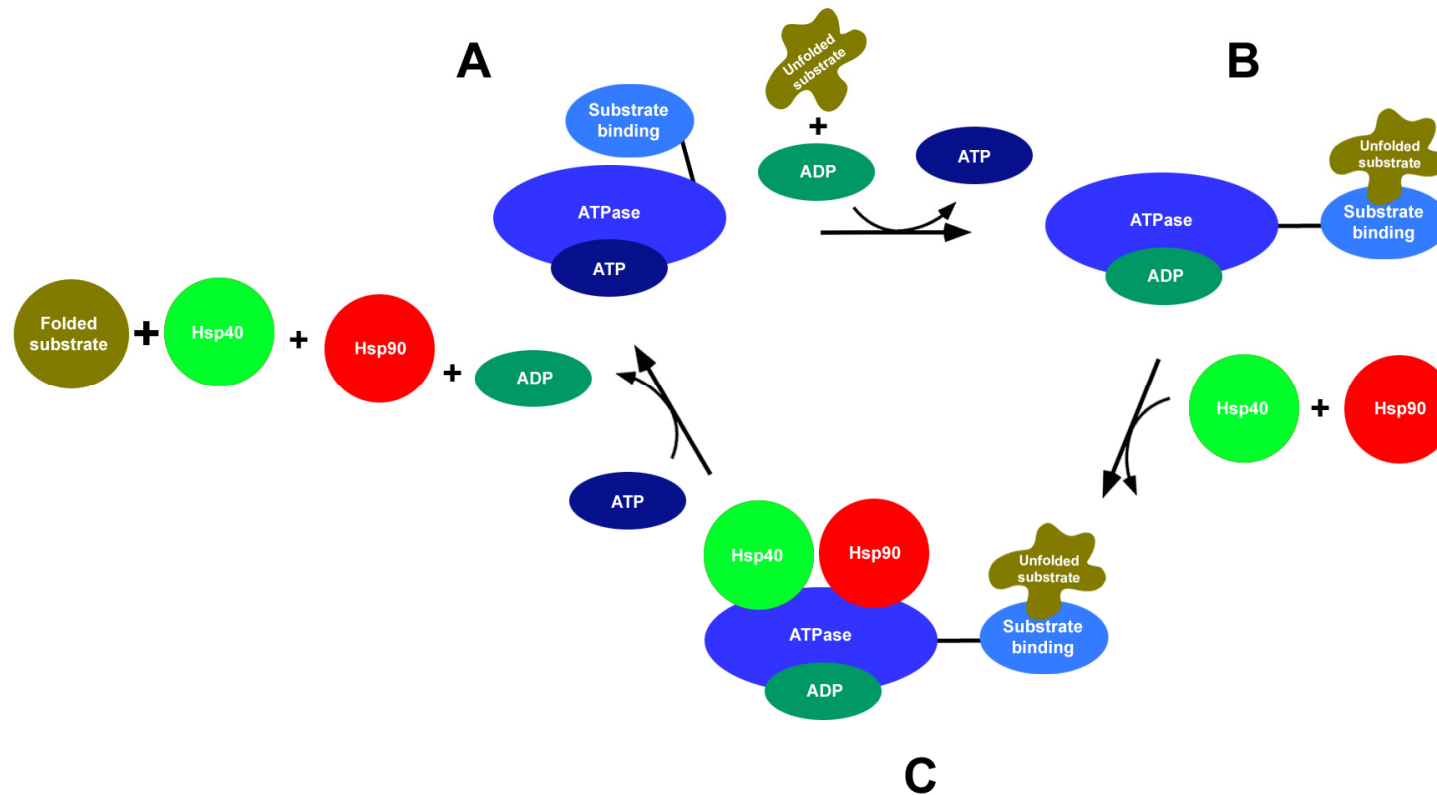


Figure 1.3: The facilitation of protein folding by intra-cellular Hsp72.

(A) Hsp72 is found in an ATP bound state with the substrate-binding lid open. (B) Following recognition and binding of the unfolded protein hydrolysis of ATP occurs leading to Hsp72 being ADP bound. In this substrate bound state, the lid is closed, and the protein is tightly bound within the groove of the substrate binding domain. (C) The co-chaperones Hsp40 and Hsp90 then bind to the N-terminal of Hsp72 resulting in a conformational change leading to the release of ADP. This then allows ATP to be bound which leads to the release of the substrate protein, Hsp40 and Hsp90. Hsp72 is then ready to facilitate protein folding again.

(Adapted from Bukau *et al.* 1998; Mallouk *et al.*, 1999; Hartl and Hayer-Hartl, 2002; De los Rios *et al.*, 2006).

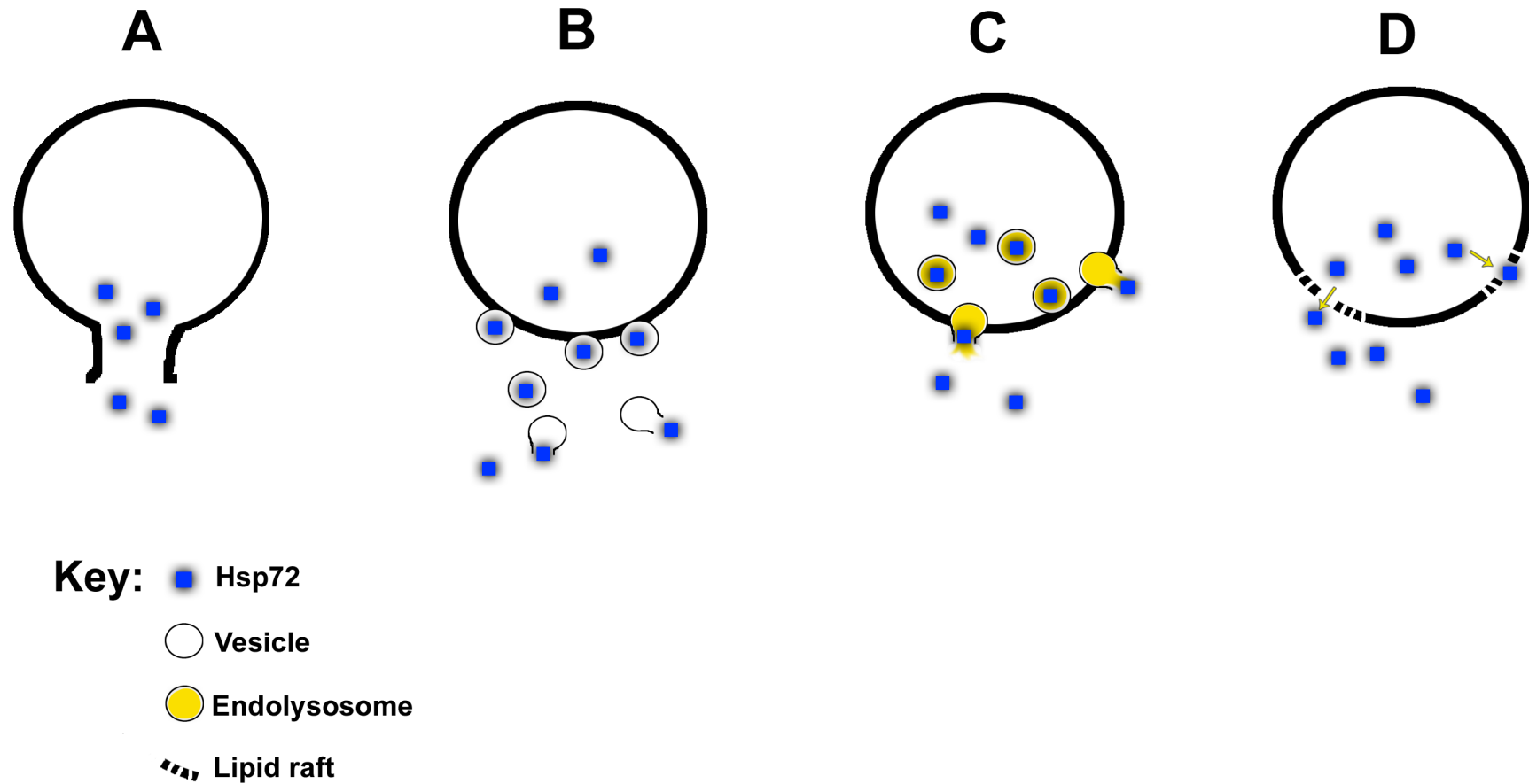


Figure 1.4: Proposed mechanisms of Hsp72 release from cells.

(A) Hsp72 released by necrosis; (B) release within vesicles followed by lysis; (C) release by endolysosomes which fuse with the cell membrane; (D) transported through lipid rafts directly into the extra-cellular milieu.

(Adapted from Broquet *et al.*, 2003; Hunter-Lavin *et al.*, 2004; Mambula *et al.*, 2007).

1.7 Extra-cellular functions of Hsp72

The presence of Hsp72 in the extra-cellular milieu has been discovered fairly recently and has been found to have a variety of potential roles. It has been found on the cell surface of human tumour cells and has been shown to stimulate the immune system by causing NK cells to attack tumour cells which have part of the Hsp72 C-terminal domain expressed on the extra-cellular surface (Botzler *et al.*, 1998).

Hsp72 has also been found to be present in the peripheral circulation of healthy individuals (Njemini *et al.*, 2003b; Pockley *et al.*, 1998; Wright *et al.*, 2000), and can be found at increased levels in those with certain diseases (Njemini *et al.*, 2007; Wright *et al.*, 2000), and also following exercise (Walsh *et al.*, 2001). Hsp72 appears to be especially prevalent in the circulation of patients with peripheral and renal vascular disease (Wright *et al.*, 2000). Levels of Hsp72 are also found to be elevated during infection and fever (Njemini *et al.*, 2003b; Stewart & Young, 2004), and could be due to cells actively releasing Hsp72, necrosis or apoptosis. Hsp72 has also been demonstrated to stimulate the production of inflammatory cytokines through activation of the NF- κ B signalling pathway (Asea *et al.*, 2000b; Hall, 1994). This function of extra-cellular Hsp72 appears to contrast with its intra-cellular function of suppressing NF- κ B (Chen *et al.*, 2006; Weiss *et al.*, 2007). This may indicate that extra-cellular Hsp72 acts as a danger signal to nearby viable cells by stimulating NF- κ B, leading to up-regulation of intra-cellular Hsp72 and anti-inflammatory signals, and an improved chance of survival of the viable cells during a stressful insult.

Conversely, one study has shown that LPS-free Hsp72 from *Mycobacterium tuberculosis* (*M. tb*) inhibits the maturation of murine DCs and induces pro-inflammatory IL-10 secretion, whereas Hsp72 contaminated with LPS does induce maturation and the secretion of TNF- α . Thus Hsp72 may have anti-inflammatory properties (Motta *et al.*, 2007). Immunization of rats with *M. tb* Hsp72 also resulted in the induction of IL-10 (Prakken *et al.*, 2001), and immunization of rats with an *M. tb* Hsp72 peptide (aa 234-252) suppressed the progression of arthritis. T cells derived from rats which were pre-treated with this peptide prior to stimulation with *M. tb* secreted excessive IL-10. *In vivo* administration of an anti-IL-10 antibody partly reduced the effect of the Hsp72 peptide against arthritis progression (Tanaka *et al.*, 1999). A similar study by Wendling *et al.* (2000) also revealed that T cells from rats which were immunised with specific *M. tb* peptides which corresponded to rat Hsp72 (GenBank accession no. Q07439) produced large amounts of IL-10

when re-stimulated with specific peptides *in vivo*. This evidence demonstrates that Hsp72 has anti-inflammatory activity, especially in the absence of LPS.

More recently, another study has demonstrated that cytokine stimulation by Hsp72, could be reduced using anti-Hsp72 antibodies in THP-1 and U937 cell lines (El Mezayen *et al.*, 2007). This study did not use recombinant protein to stimulate, but instead used pre-stimulated necrotic cell lysate (NCL) which contained cell derived Hsp72. NCL was applied directly to cell cultures, or pre-incubated with antibodies to Hsp72 then applied to cells in conjunction with LPS. They showed that non-recombinant Hsp72 induced cytokine production could be reduced using these antibodies.

The possible mechanisms of extra-cellular Hsp72 are discussed further in relation to the immune response.

1.8 The role of Hsp72 within the immune system

Since Hsp72 is found in the extra-cellular matrix, and is implicated as being a significant marker of some disease states, such as infection (Njemini *et al.*, 2003b), atherosclerosis (Lamb, El-Sankary & Ferns, 2002; Svensson *et al.*, 2006), Alzheimer's disease (Hamos *et al.*, 1991; Yoo *et al.*, 2001), Huntington's disease (Hay *et al.*, 2004), sickle cell anaemia (Adewoye *et al.*, 2005), and vascular disease (Wright *et al.*, 2000), it might have a functional role. The functional role of Hsp72 could be as an inflammatory danger signal to the immune system (Campisi *et al.*, 2003; Vega *et al.*, 2008; Williams & Ireland, 2008).

1.9 Recognition of Hsp72 by the immune system

The Danger model allows for recognition of endogenous self molecules as markers of tissue damage or cellular stress which activate the immune system. These have now been termed damage-associated molecular patterns (DAMPs) (Bianchi, 2007). Hsp72 is a candidate for one of these danger signals. In the innate response, Hsp72 is able to bind PRRs and TLRs to stimulate NF- κ B activation resulting in the release of cytokines and nitric oxide (NO) and up-regulating the expression of co-stimulatory molecules and MHC II (Asea *et al.*, 2000b; Basu *et al.*, 2000; Basu & Matsutake, 2004; Binder, Vatner & Srivastava, 2004; Knowlton, 2006; Panjwani, Popova & Srivastava, 2002). In the adaptive response, Hsp-peptide complexes are readily taken up by APCs and internalised. The peptides are presented to MHC class I and MHC class II molecules and transported for recognition by T cells (Basu & Matsutake, 2004; Park *et al.*, 2006a). Hsp72 has also been found in a membrane-bound form which externalises a specific epitope (TKD) found only on

tumour cells which acts as a receptor for NK cells by binding to NK cells, possibly *via* Fc receptors on NK cells. The TKD peptide was found to bind to NK cells resulting in the up-regulation of CD94 which is associated with lytic activity against tumours expressing membrane-bound Hsp72, and stimulates migration of NK cells (Botzler *et al.*, 1998; Gastpar *et al.*, 2004; Multhoff, 2007; Vega *et al.*, 2008).

Therefore, Hsps such as Hsp72 appear to have a dual role in activating the immune system (Figure 1.5).

1.10 Stimulation of the innate immune response by Hsp72

Hsp72 is known to strongly activate the immune system, resulting in the secretion of cytokines. It is thought they are able to do this by binding to receptors on the cell surface, much in the same way as PAMPs are recognised by PRRs (Calderwood *et al.*, 2007; Takeda *et al.*, 2003; Triantafilou & Triantafilou, 2002). These receptors may recognise Hsp72, Hsp72-peptide complexes, or just the peptide which is bound to Hsp72 (Basu *et al.*, 2001; Binder *et al.*, 2004). These PRRs are thought to include the following described here (Figure 1.6).

1.10.1 CD91

CD91 is an oxidized low density lipid (oxLDL) binding protein found on the surface of membranes of APCs involved in receptor-mediated endocytosis. It was first described as a common receptor to Hsp 60, 72, Gp96 and calreticulin by Binder *et al.* (2000). It does appear to have an important role in antigen presentation by Hsp, as it has been shown that *in vitro* blocking experiments which utilised a CD91 receptor associated protein (RAP), there was a strong inhibition of Hsp72 to human macrophages, and a weak inhibition of binding to DCs (Delneste *et al.*, 2002). RAP has also been reported to inhibit binding of Hsp72 to peripheral blood mononuclear cells (PBMCs) (Martin *et al.*, 2003). Other studies have doubted the binding ability of CD91 with Hsp72, as one study demonstrated that Hsp72 which was free in solution showed little difference in binding ability between cells with or without CD91 expression (Thériault *et al.*, 2005).

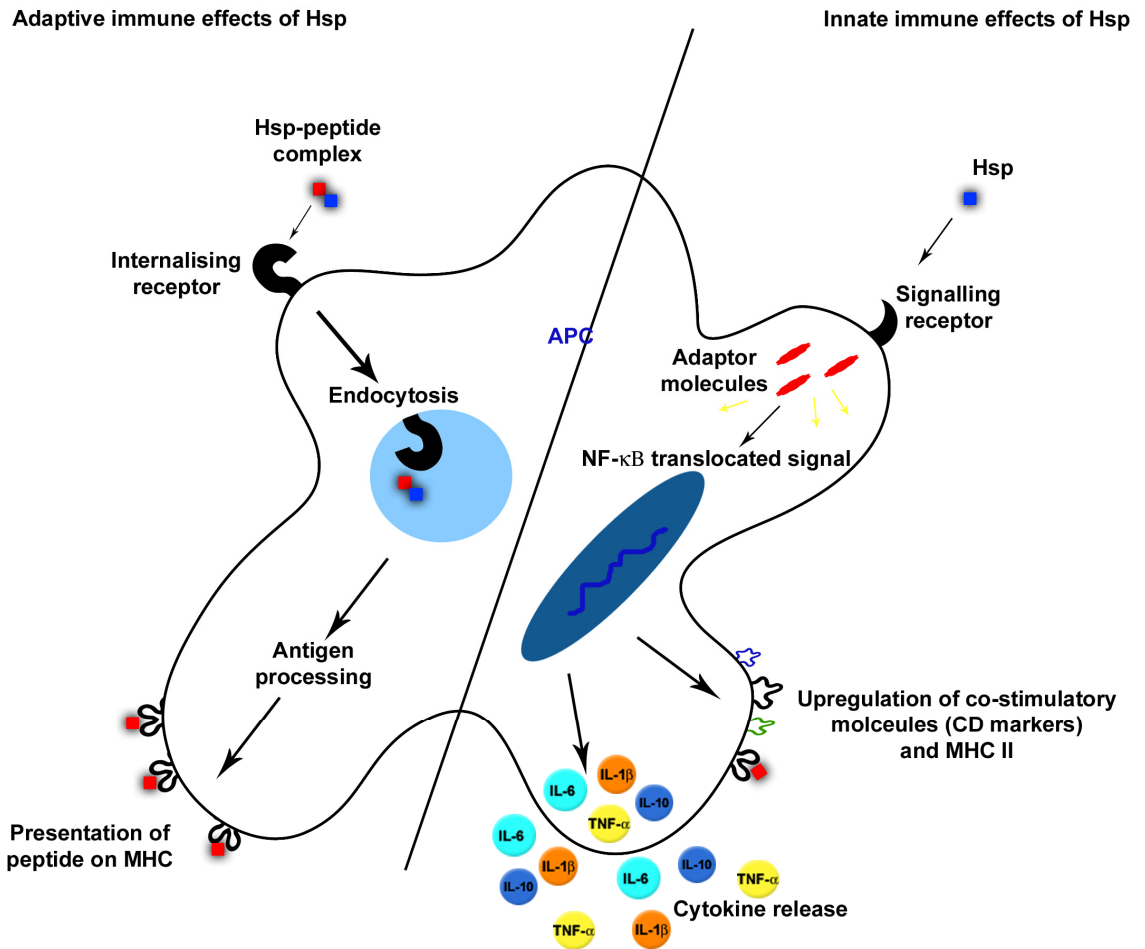


Figure 1.5: Interaction of Hsps with receptors of the adaptive and innate immune system.

In the adaptive immune response, Hsp-peptide complexes are internalised by endocytosis and are processed and presented on MHC molecules of APCs. In the innate immune response, Hsps bind to signalling receptors which activate NF- κ B and result in release of cytokines, chemokines and nitric oxide (NO), and expression of CD markers and MHC II.

(Adapted from Binder *et al.*, 2004; Motta *et al.*, 2007; El Mezayen *et al.*, 2007).

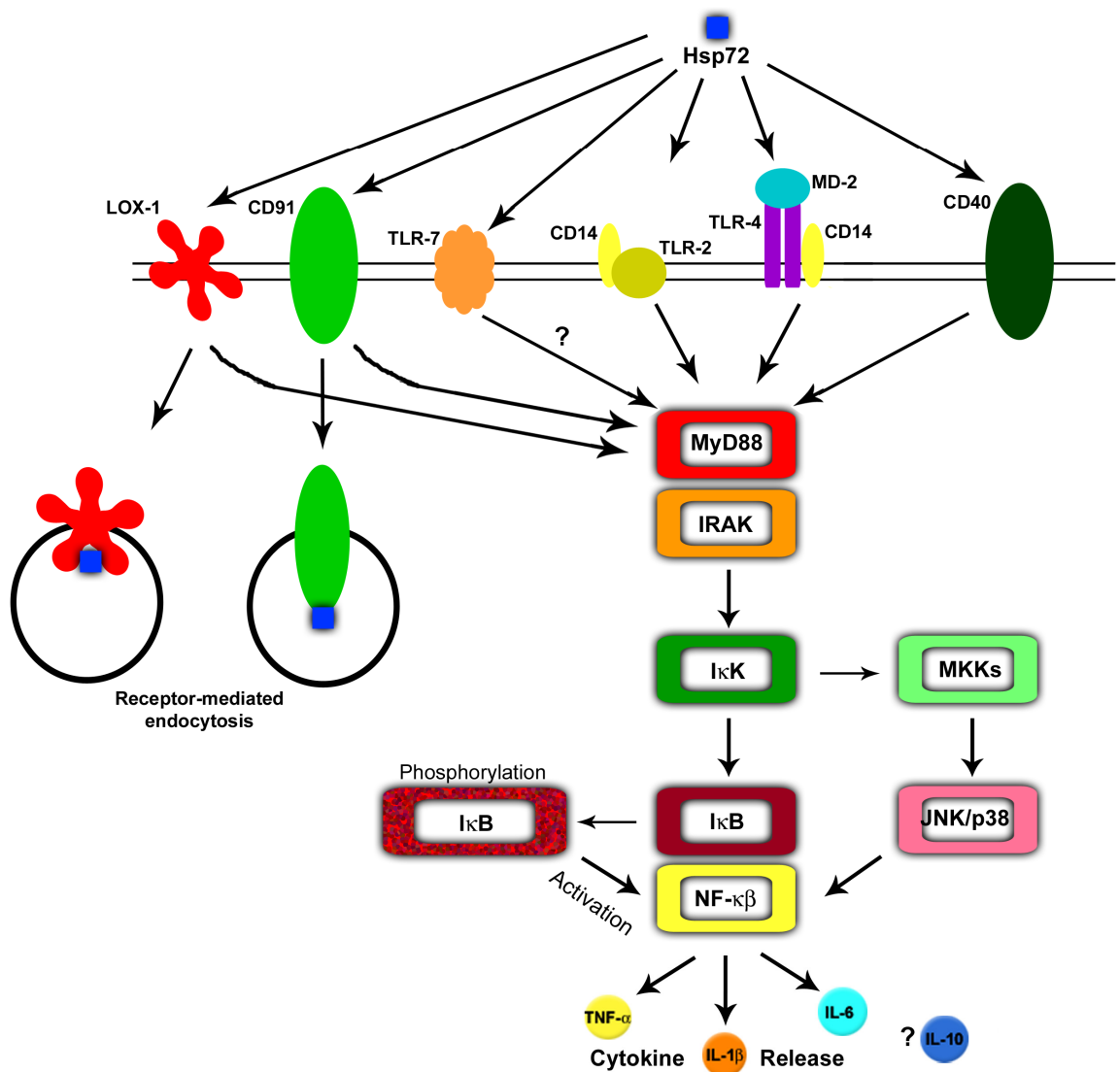


Figure 1.6: Cell surface receptors involved in the immune functions of extra-cellular Hsp72.

The scavenger receptors LOX-1 and CD91 are shown to either stimulate activation of NF- κ B or internalise *via* endocytosis. Activation through TLR-7 is as yet unknown. All other receptors are thought to exclusively mediate cell signalling and activation of NF- κ B.

(Adapted from Binder *et al.*, 2000; Pockley *et al.*, 2003; Janeway Jr. *et al.*, 2005; Pockley *et al.*, 2007; Calderwood *et al.*, 2007; Wang *et al.*, 2006; Motta *et al.*, 2007).

1.10.2 CD40

CD40 is a co-stimulatory protein found on APCs which is a member of the TNF- α receptor family. When stimulated it binds to CD40L on activated T cells, which leads to an increase in expression of CD40 on APCs (O'Sullivan & Thomas, 2003). CD40 was initially identified as a receptor for mycobacterial Hsp72 through use of competitive binding studies with anti-CD40 antibodies (Wang *et al.*, 2001b). Becker *et al.* (2002) demonstrated that murine Hsp72 could also bind to recombinant CD40 in cell lysates. The binding of mammalian Hsp72 to CD40 was demonstrated by Millar *et al.* (2003) by showing that bone marrow-derived DCs require CD40 for the Hsp72-induced secretion of IL-12. Bone marrow-derived DCs from chimeric mice which lacked CD40 did not produce IL-12 when challenged with Hsp72, whereas CD40 expressing DCs from control mice (C57BL/6J) did (Millar *et al.*, 2003).

1.10.3 CD14

CD14 is a receptor found on the surface of macrophages, and other APCs, and can also be found in a soluble form. Its primary function is to bind LPS in conjunction with TLR-4 and MD-2 as part of an LPS signalling receptor cluster, but only in the presence of lps binding protein (Heidenreich, 1999). It was originally postulated as a receptor for Hsp72 by Asea *et al.* (2000b) who demonstrated that Hsp72 binding to CD14 resulted in an up-regulation of TNF- α , IL-1 β and IL-6. However, they also found another Hsp72 signalling pathway which was independent of CD14 and led to an increase in TNF- α only. Another study by the same group showed that NF- κ B activation was enhanced in response to Hsp72, and that the enhancement was increased following transfection of HEK293 cells with CD14 (Asea *et al.*, 2002a). In contrast, it has been demonstrated that Hsp72 did not bind to CD14 in transfected CHO cells using flow cytometry analysis (Delneste *et al.* 2002).

1.10.4 CD36

CD36 is a member of the class B scavenger receptor family, and also binds oxLDL, like CD91 and LOX-1 (Pluddemann, Neyen & Gordon, 2007). On macrophages, CD36 has been reported to form part of a receptor complex (CD36- α V β 3 complex) which is involved in the stimulation of phagocytosis (Bottcher *et al.*, 2006). It has yet to be demonstrated as a receptor for Hsp72, but it has been shown to bind gp96 (Binder *et al.*, 2004). Delneste *et al.* (Delneste *et al.*, 2002) did not show any binding of Hsp72 to CD36 in transfected CHO cells using flow cytometry analysis.

1.10.5 TLR-2/TLR-4

TLR-2 and TLR-4 are members of the TLR family of receptor proteins which act as PRRs recognising PAMPs, leading to downstream activation of NF- κ B and interference response factor (IRF) signalling pathways, and the production of cytokines and adhesion molecules which mediate innate and adaptive immunity in response to inflammation (Takeda *et al.*, 2003). TLR-2 and/or TLR-4 have been reported by several groups to be receptors for Hsp60, Hsp72, Gp96 and HMGB-1 (Ohashi *et al.*, 2000). TLR-2/TLR-4 is involved in the CD14 dependent stimulation by Hsp72 described by Asea *et al.* (2002). Dybdahl *et al.* (2002) demonstrated that Hsp72 could stimulate the production of TNF- α from macrophages derived from C3H/HeN mice but not C3H/HeJ. When Hsp72 was pre-incubated with antibody to TLR-4, the production of TNF- α and IL-6 from human adherent monocytes was abrogated. Antibody to TLR-2 had no effect.

1.10.6 TLR-7

TLR-7 is known to recognise single-stranded RNA (ssRNA) within endosomes as part of the innate immune response to viruses (Krieg, 2007; Triantafilou *et al.*, 2005). A recent study by Wang *et al.* (2006) demonstrated that the phagocytic response to Hsp72 in wild-type murine macrophages was significantly increased when compared to TLR-7 deficient mice (C57BL/6). They also demonstrated that when RAW264.7 cells were transfected with small interfering RNA (siRNA) for TLR-7, phagocytic activity and TNF- α release was reduced compared to RAW264.7 cells transfected with an empty vector (control). Presence of TLR-7 and Hsp72 within the lipid raft region of macrophages membranes was verified by western blot. As Hsp72 in this study was able to stimulate phagocytosis through TLR-7, it may indicate that when Hsp72 is released from necrotic cells, it is able to act as a chemokine (Asea, 2006; Asea *et al.*, 2000a; Asea *et al.*, 2000b) when receptors from APCs, such as TLR-7, are bound by it.

1.10.7 LOX-1

LOX-1 is a Class E scavenger receptor which binds oxLDL, but has been found to bind with high affinity to Hsp72 (Binder *et al.*, 2004; Delneste *et al.*, 2002; Theriault, Adachi & Calderwood, 2006; Thériault *et al.*, 2005). LOX-1 is known to be involved in cross-presentation of antigens and Hsp72 is thought to bind LOX-1, possibly in a peptide-bearing manner, and is internalised by endocytosis leading to stimulation of

the adaptive immune response *via* MHC I (Binder *et al.*, 2004; Calderwood *et al.*, 2007; Delneste *et al.*, 2002; Inoue & Sawamura, 2007).

1.11 High mobility group box protein-1 (HMGB-1)

The Danger model postulates that damaged cells release their contents into the extra-cellular milieu which contain endogenous danger signals that are capable of stimulating an immune response (Bianchi, 2007; Palumbo *et al.*, 2007; Williams & Ireland, 2008). Hsp72 has already been discussed as a candidate signal but there are likely others, one of which is HMGB-1.

HMGB-1 is a 25 kDa nuclear protein consisting of two DNA-binding domains, known as HMG box A and B, and an acidic tail which interacts with the HMG boxes, possibly regulating their molecular interactions (Andersson *et al.*, 2002; Bianchi & Manfredi, 2007; Knapp *et al.*, 2004; Thomas, 2001; Wang *et al.*, 2007) (Figure 1.7). HMGB-1 proteins are highly conserved, and in mammals are virtually identical. It is generally found within the nucleus but can also be found in the cytosol, external membranes and within the extra-cellular milieu (Bonaldi *et al.*, 2002; El Gazzar, 2007; Thomas, 2001; Zetterström *et al.*, 2002). Within the box A region there is a heparin binding domain (aa 6-12) which is similar in sequence to a number of heparin binding proteins (Cardin & Weintraub, 1989), and a region which functions to stabilise amyloid fibrils (Takata *et al.*, 2003). The complete box A region is capable of being a RAGE antagonist (Bianchi & Manfredi, 2007). The box B region contains the pro-inflammatory region of HMGB-1 (aa 89-108), and part of box B along with part of the connecting segment before the acidic tail is able to bind RAGE (aa 150-183) (Huttunen *et al.*, 2002; Huttunen & Rauvala, 2004; Li *et al.*, 2003).

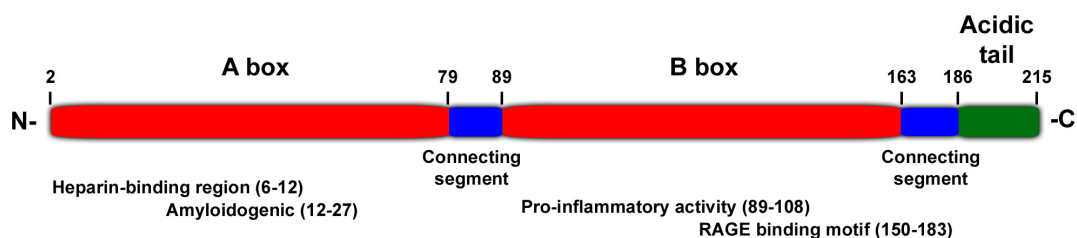


Figure 1.7: Structure of HMGB-1 protein.

(Adapted from Thomas, 2001; Bianchi & Manfredi, 2007; Takata *et al.*, 2003; Huttunen *et al.*, 2002; Li *et al.*, 2003).

1.12 Regulation of HMGB-1

The regulation of HMGB-1 is not very well understood. Only very recently has it been demonstrated that expression of HMGB-1 may be activated by the JAK/STAT pathway (Liu *et al.*, 2007). This study showed that when murine macrophages were stimulated with LPS, HMGB-1 expression was increased and this correlated with the activation of JAK/STAT. This activation was suppressed when specific inhibitors of JAK2, STAT1 or STAT3 were applied, and HMGB-1 expression was reduced. To determine whether this mechanism was responsible for the secretion of TNF- α following challenge with HMGB-1, HMGB-1 was applied to macrophages and induced secretion of TNF- α . Using the JAK/STAT inhibitors prior to HMGB-1 treatment resulted in a significant decrease in TNF- α expression (Liu *et al.*, 2007). TNF- α , which is secreted by activated immune cells also leads to the up-regulation of HMGB-1 (Wang *et al.*, 1999).

1.13 Intra-cellular functions of HMGB-1

Within the cell HMGB-1 is transiently bound to chromatin and functions as a DNA chaperone by promoting DNA-protein interactions (Bonaldi *et al.*, 2002). It regulates transcription by bending DNA to enable binding of transcription factors (Thomas, 2001). HMGB-1 also binds non-specifically to the minor groove within DNA to induce these changes. These structural changes allow DNA to bind and interact with various proteins, such as p53, NF- κ B, steroid hormone receptors and other homeobox-containing proteins (Bianchi, 2007).

HMGB-1 is also found at the periphery of the cell membrane in cells that are motile or can be activated, such as macrophages (Rouhiainen *et al.*, 2004). It is thought that HMGB-1 is translocated into the cytoplasm by lysosomal exocytosis prior to release into the extra-cellular milieu (Gardella *et al.*, 2002).

1.14 Mechanisms of HMGB-1 release from cells

HMGB-1 is released from necrotic cells in a passive manner (Scaffidi *et al.*, 2002), and, like Hsp72 has been found to be released *via* a non-classical secretory pathway, despite lacking a peptide leader sequence (Bell *et al.*, 2006; Gardella *et al.*, 2002; Rouhiainen *et al.*, 2004) (Figure 1.8).

The release of HMGB-1 from necrotic cells has been demonstrated to induce the inflammatory response, including release of pro-inflammatory cytokines and

migration of immune cells to sites of inflammation (Degryse *et al.*, 2001; Palumbo *et al.*, 2007; Yang *et al.*, 2006). Scaffidi *et al.* (2002) demonstrated that macrophages incubated with necrotic cells induced NF- κ B activation and release of TNF- α . Incubation with apoptotic cells did not, and it was found that HMGB-1 in apoptotic cells is tightly bound to chromatin due to hypoacetylation of histones (Scaffidi *et al.*, 2002). The release of HMGB-1 from necrotic cells therefore leads to inflammation and tissue regeneration by attracting dendritic cells (DCs) and neutrophils, and the activation of stem cells (Palumbo *et al.*, 2007).

HMGB-1 has also been found to be actively secreted from immune cells in a non-classical manner by lysosomes (Gardella *et al.*, 2002). This mechanism of HMGB-1 release is associated with the late onset of chronic inflammation. This study showed that HMGB-1 relocates to the periphery of the cell membrane in response to 18 h incubation with LPS, and is secreted following a short co-incubation (10 min) with lysophosphatidylcholine (LPC). LPC is a bioactive lipid which is generated by inflamed cells (Gilon & Henquin, 2001).

One report has implicated that HMGB-1 is also released during apoptosis (Bell *et al.*, 2006). Jurkat cells were treated with apoptosis inducers and HMGB-1 release was determined by western blot. HMGB-1 was found to be present in supernatants after 30 h following treatment with all apoptosis inducers tested. Another study reported that RAW264.7 cells could release HMGB-1 through apoptosis when stimulated with LPS for 20 h, and that when stimulated with polyinosinic-polycytidylic acid (poly(I:C)), release from apoptotic cells was dependent on IFN- α . Anti-IFN- α antibodies prevented HMGB-1 release (Jiang, Bell & Pisetsky, 2007). Late-stage apoptosis is also termed as secondary necrosis, whereby apoptotic bodies become permeable and release their contents. Apoptotic bodies are known to retain fragments of chromatin and it is likely that HMGB-1 bound to this chromatin is released following permeabilisation (Widlak *et al.*, 2002).

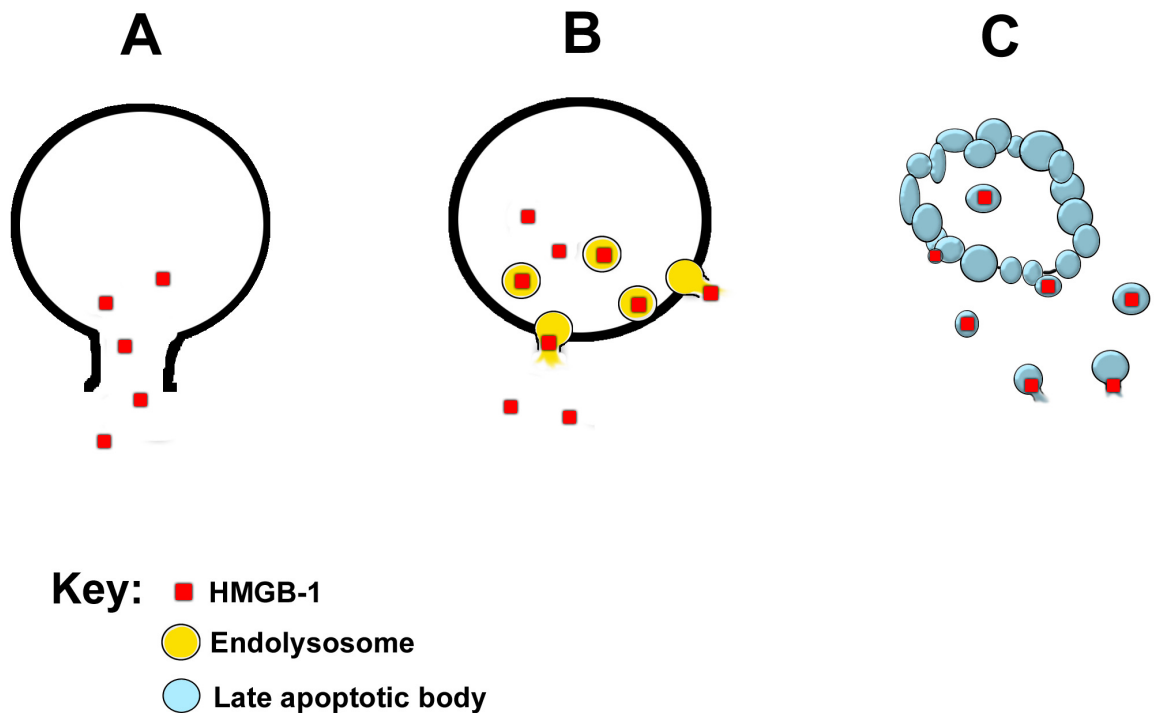


Figure 1.8: Proposed mechanisms of HMGB-1 release from cells.

(A) HMGB-1 released by necrosis; (B) release by endolysosomes which fuse with the cell membrane; (C) release by apoptotic bodies during late stage apoptosis. (Adapted from Scaffidi *et al.*, 2002; Gardella *et al.*, 2002; Bell *et al.*, 2006).

1.15 Extra-cellular functions of HMGB-1

Extra-cellular HMGB-1 acts as a late inflammatory mediator of endotoxin lethality leading to sepsis (Qin *et al.*, 2006; Wang *et al.*, 1999) and is found at increased levels in serum from patients with sepsis (Gaini *et al.*, 2007a; Gaini *et al.*, 2007b), and pancreatitis (Sawa *et al.*, 2006; Yamada *et al.*, 2003). Studies have shown that extra-cellular HMGB-1 functions to stimulate the release of pro-inflammatory cytokines such as TNF- α and IL-1- β , but suppress mRNA transcription of anti-inflammatory cytokines IL-10 and TGF- β (El Gazzar, 2007). TGF- β has many functions in controlling cell cycle, survival and proliferation, and is a known inducer of apoptosis (Schuster & Krieglstein, 2002). IL-10 is able to suppress cytokine release from activated macrophages (Gazzinelli *et al.*, 1996; Janeway Jr *et al.*, 2005a; Malefyt *et al.*, 1991). This suggests that increasing levels of HMGB-1 actively down-regulate anti-inflammatory, and, encourage pro-inflammatory responses, leading to systemic inflammation (Wang *et al.*, 2001a).

In Balb/C mice, elevated serum levels of HMGB-1 are associated with lethality following LPS infection. This effect could be suppressed and mortality reduced following injection with anti-HMGB-1 antibodies (Wang *et al.*, 1999). Similar studies

have also shown the attenuating effects of anti-HMGB-1 antibodies on cytokine secretion in other models (El Gazzar, 2007; Sawa *et al.*, 2006). Sawa *et al.*, (2006) induced pancreatitis in C3H/HeN mice and following administration of anti-HMGB-1 found that anti-HMGB-1 attenuated the onset of pancreatitis and associated organ dysfunction. An *in vitro* study by El Mezayen *et al.* (2007) demonstrated that cytokine stimulation by HMGB-1 could be reduced using anti-HMGB-1 antibodies in THP-1 and U937 cell lines which were stimulated with NCL containing HMGB-1 in conjunction with LPS. They showed that HMGB-1 induced cytokine production could be reduced using these antibodies.

Migration of some immune cells in response to HMGB-1 has been reported in some cells types. In DCs, HMGB-1 has been found to stimulate the expression of the chemokine receptors, CCR7 and CXCR4. When DCs were stimulated with LPS in the presence of anti-HMGB-1 antibodies, the expression of these receptors was reduced resulting in the reduced migration of DCs to the receptor ligands CCL19 and CXCL12 (Dumitriu *et al.*, 2007). Migration of fibroblasts to HMGB-1 was demonstrated by Palumbo *et al.* (2007) to be dependent on activation of NF- κ B by HMGB-1 which could be blocked by pre-incubating fibroblasts with ribobenzimidazole (DRB), an inhibitor of transcription.

Chemotaxis of DCs in response to HMGB-1 was demonstrated by Yang *et al.* (2007) who showed that migration of DCs could be induced by HMGB-1 in a dose dependent manner and that this migration is receptor mediated by the binding of HMGB-1 to the receptor of advanced glycation end products (RAGE).

In relation to intra-cellular Hsp72, IL-1 β and TNF- α expression and release was reduced in RAW264.7 cells which were given a non-lethal heat shock (42.5°C, 1.5 h) followed by 12 h recovery (37°C) prior to stimulation with HMGB-1. In RAW264.7 cells which were transfected to over express Hsp72, cytokine secretion was also reduced following stimulation with HMGB-1. Elevated intra-cellular levels of Hsp72 are known to inhibit NF- κ B activation as a feedback response to a stressful insult which would lead to the release of HMGB-1 (Tang *et al.*, 2007a; Weiss *et al.*, 2007).

1.16 The role of HMGB-1 within the immune system

As HMGB-1 is implicated as being a mediator in the late inflammatory response and in many disease states, such as sepsis (Gaini *et al.*, 2007b; Wang *et al.*, 2001a), arthritis (Kokkola *et al.*, 2001), atherosclerosis (Kalinina *et al.*, 2004), pancreatitis (Yasuda *et al.*, 2007), and lung inflammation (Abraham *et al.*, 2000), HMGB-1 must play a functional role in the immune response, and when found in

the extra-cellular milieu is now widely regarded as a 'danger signal' (Bianchi, 2007; Harris & Raucci, 2006; Williams & Ireland, 2008).

1.17 Recognition of HMGB-1 by the immune system

HMGB-1 recognition by the immune system is similar to that previously described for Hsp72. In the innate immune response, HMGB-1 is released following cell damage, stimulation with LPS, and oxidative stress (Chen *et al.*, 2004; Scaffidi *et al.*, 2002; Tang *et al.*, 2007b). It is able to bind PRRs and TLRs leading to the stimulation of NF- κ B activation and consequent up-regulation of cytokines (Andersson *et al.*, 2000; O'Connor *et al.*, 2003; Taniguchi *et al.*, 2003; Wang *et al.*, 2001a), leading to further secretion of HMGB-1 (Kalinina *et al.*, 2004). The up-regulation and expression of CD markers following HMGB-1 stimulation leads to an adaptive response, whereby macrophages are activated (Fiuza *et al.*, 2003; Rouhiainen *et al.*, 2004) and DCs mature and migrate towards HMGB-1 (Dumitriu *et al.*, 2007).

1.18 Stimulation of the innate immune response by HMGB-1

HMGB-1 is able to stimulate the immune response by binding certain cell receptors, leading to the up-regulation of cytokines. There are only three identified receptors for HMGB-1 to date: TLR-2, TLR-4 and RAGE (Huttunen *et al.*, 2002; Park *et al.*, 2004). More recent studies suggest that TLR-9 (Tian *et al.*, 2007) may be linked to HMGB-1 in association with RAGE, although direct binding has not been elucidated. The effects of binding to the three known receptors are discussed here.

1.18.1 RAGE

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor belonging to the immunoglobulin family. It has been shown to bind s100 proteins, amyloid- β protein and HMGB-1 (Chavakis, Bierhaus & Nawroth, 2004; Huttunen *et al.*, 2002; Schmidt *et al.*, 2001). RAGE is found within the cell membrane but can also be found in a soluble form which blocks the action of RAGE ligands, such as HMGB-1 (Rong *et al.*, 2004; Taniguchi *et al.*, 2003).

When HMGB-1 binds to membrane-bound RAGE, it signals partly through the MAPK signalling pathway resulting in the secretion of more HMGB-1, and autocrine stimulation of HMGB-1 leads to signalling *via* NF- κ B signalling pathway, resulting in the release of pro-inflammatory cytokines (Lotze & Tracey, 2005; Park *et al.*, 2003; RiuZZi, Sorci & Donato, 2006) (Figure 1.9).

1.18.2 TLR-2/TLR-4

As described before, TLR-2 and TLR-4 are PRRs which when stimulated lead to downstream activation of NF- κ B and interference response factor (IRF) signalling pathways, and the production of cytokines and adhesion molecules which mediate innate and adaptive immunity in response to inflammation (Takeda *et al.*, 2003).

HMGB-1 binds rapidly to TLR-2 and TLR-4 expressed on RAW264.7 cells when assessed by FRET analysis, and NF- κ B activation is up-regulated in HEK-293 cells, through TLR-2 in a manner similar to the TLR-2-specific stimulus, Pam₃CSK₄, and through TLR-4, similar to LPS (Park *et al.*, 2006b; Park *et al.*, 2004) (Figure 1.9).

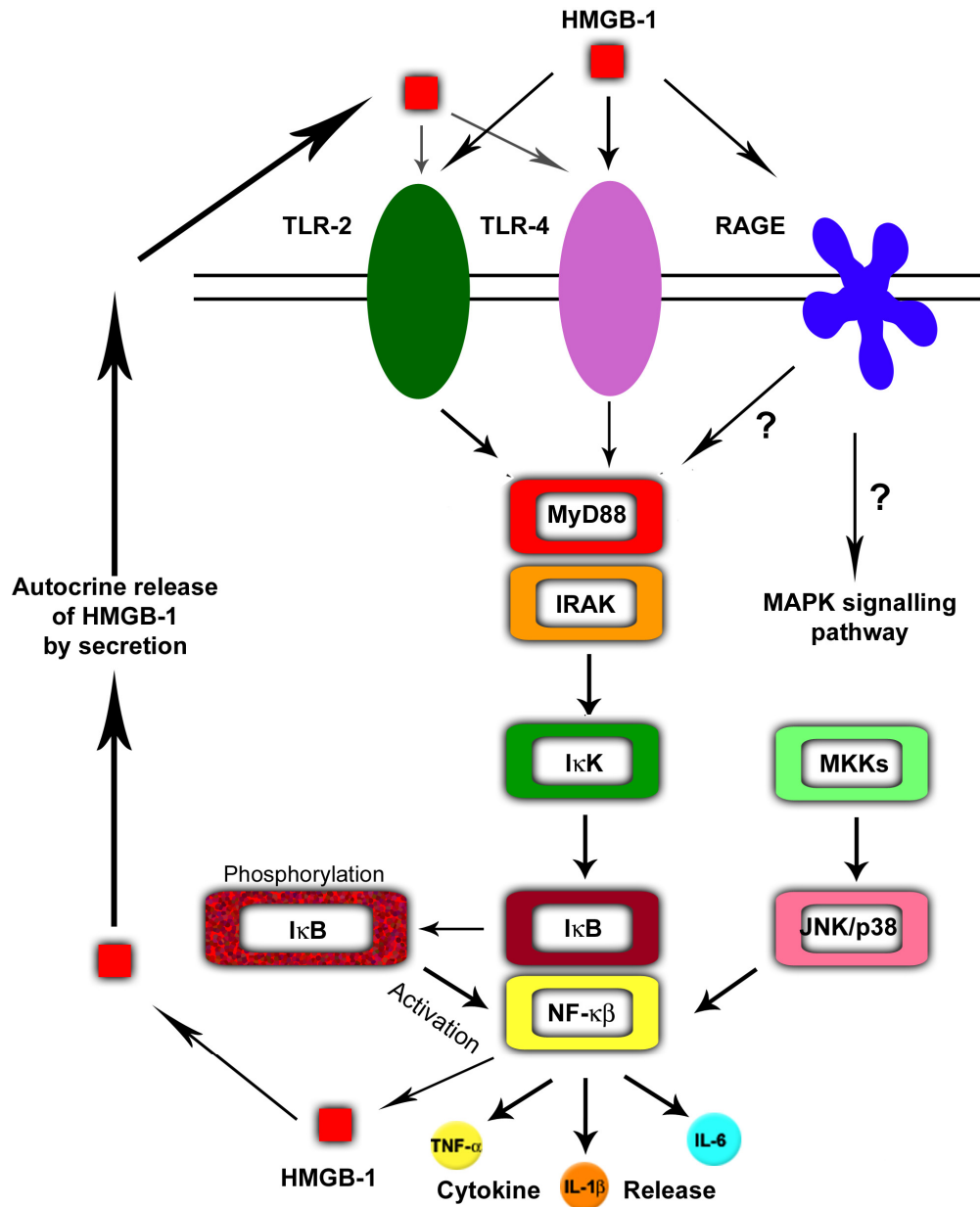


Figure 1.9: Cell surface receptors involved in the immune functions of HMGB-1.

Extra-cellular HMGB-1 binds to TLR2 and TLR-4 and activates the MyD88-dependent NF-κβ pathway leading to the release of pro-inflammatory responses. Binding through RAGE also follows the MyD88-dependent pathway but MAPK is also implicated whereby MAPK leads to NF-κβ activation and the autocrine up-regulation of HMGB-1 which is in turn secreted externally and binds TLR-2, TLR-4 and/or RAGE.

(Adapted from Lotze & Tracey, 2005; Park *et al.*, 2006; Takeda *et al.*, 2003; Huttunen *et al.*, 2002; Taneguchi *et al.*, 2003).

1.19 Stimulation of the innate immune response by LPS

As previously described Hsp72 and HMGB-1 are able to stimulate the immune response through PRRs which include CD14, TLR-2 and TLR-4. This stimulation is very similar, if not identical to the immune response to LPS or other bacterial products.

LPS stimulates the production of cytokines and activation of the adaptive immune response by being presented to a complex receptor cluster on the surface of APCs by a lipopolysaccharide-binding protein (LBP) (Mathison *et al.*, 1992; Triantafilou *et al.*, 2001; Triantafilou & Triantafilou, 2002). LPS from Gram negative and lipoteichoic acid (LTA) from Gram positive bacteria are able to bind *via* this cluster which consists of CD14 and TLR-4 in association with MD-2. LPS binds directly to CD14 and is cross-linked specifically to TLR4 and MD-2, but only when co-expressed with CD14 (Dauphinee & Karsan, 2006; Triantafilou & Triantafilou, 2002). When bound, LPS activates NF- κ B signalling pathway through MyD88, leading to the production of various cytokines, including IL-1 β , IL-6 and TNF- α (Dauphinee & Karsan, 2006; Latz *et al.*, 2002; Triantafilou & Triantafilou, 2002) (Figure 1.10).

TLR-2 binds Gram negative LPS, Gram positive LTA and peptidoglycans, and also binds LPS-associated lipopeptides (Kurt-Jones *et al.*, 2002; Means, Golenbock & Fenton, 2000; Zhang & Ghosh, 2001). Again, binding is associated with CD14 and activates NF- κ B signalling pathway downstream of TLR-2, and is the same as for TLR-4 (Means *et al.*, 2000) (Figure 1.10).

The immune system can also be stimulated by flagellin, the major component of flagellum of Gram negative bacteria which enables motility, such as in *E. coli*, (Hayashi *et al.*, 2001). Flagellin is known to bind TLR-5 and stimulates the activation of the NF- κ B pathway (Hayashi *et al.*, 2001; Smith *et al.*, 2003; Ye & Gan, 2007).

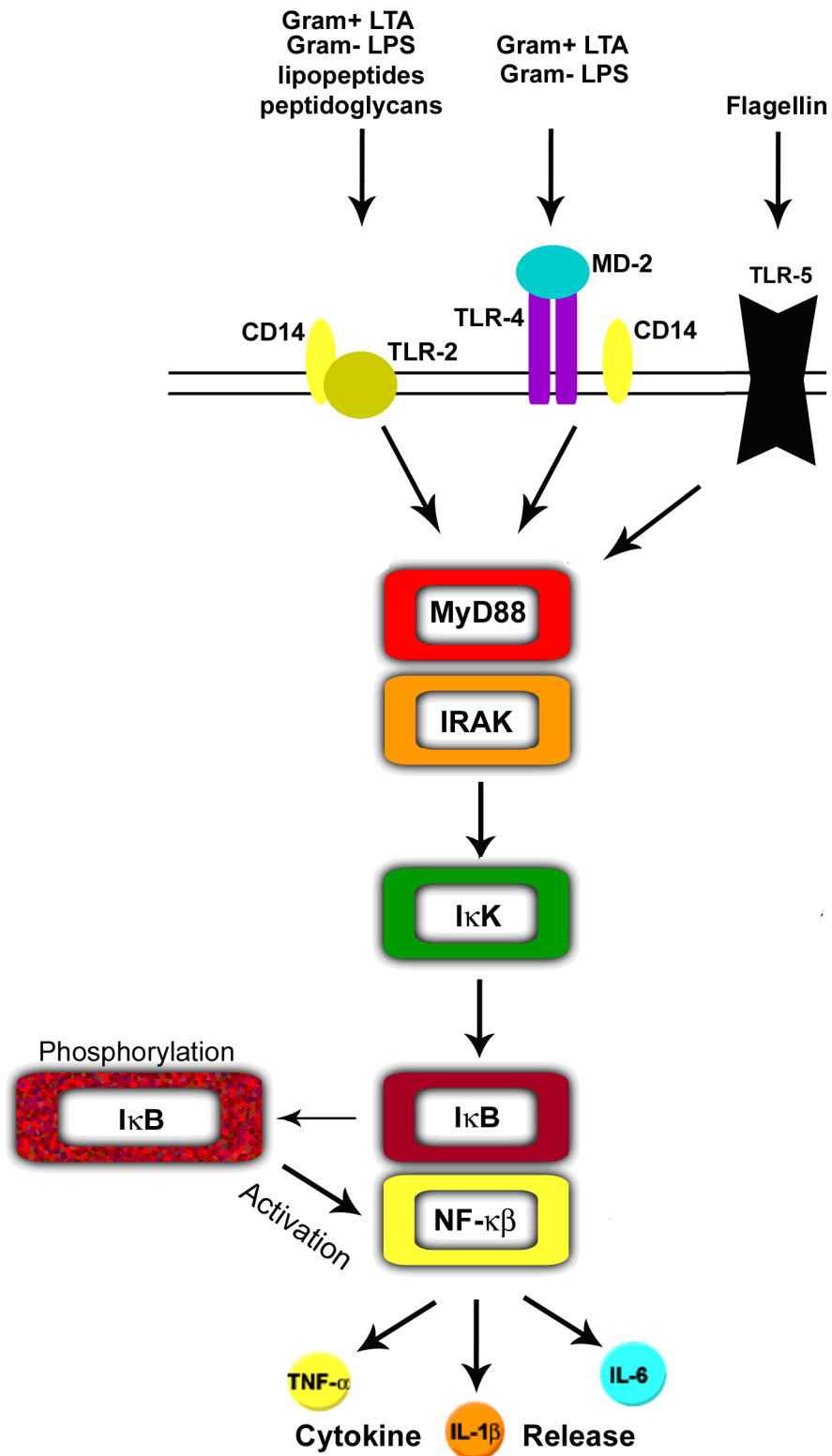


Figure 1.10: Stimulation of the immune response by various bacterial components.

(Adapted from Means *et al.*, 2000; Hayashi *et al.*, 2001; Zhang *et al.*, 2001; Kurt-Jones *et al.*, 2002; Smith *et al.*, 2003).

1.20 Contamination of recombinant proteins by LPS

Many reports have suggested that Hsps, including Hsp72, and HMGB-1 can interact with receptors such as CD40, CD14 and TLR-2 and TLR-4 (Asea *et al.*, 2000b; Dybdahl *et al.*, 2005; Park *et al.*, 2004; Wang *et al.*, 2001b; Yu *et al.*, 2006). As these are known receptors for bacterial products, it has been suggested by others that the effects on the immune response by recombinant Hsps are the result of contamination with bacterial products (Bausinger *et al.*, 2002; Gao & Tsan, 2003a; Gao & Tsan, 2003b; Tsan & Gao, 2004a). This is potentially an issue with studies which have demonstrated an effect by Hsp72 which are recombinantly produced from *E. coli* (Asea *et al.*, 2000a; Ménoret, 2004; Valentis *et al.*, 2008).

Recombinant protein production usually involves transfecting *E. coli* with plasmid DNA containing the desired gene for the protein of interest. The gene also has a polyhistidine-tag (His-tag) codon encoded to allow downstream purification of the recombinant proteins. Following sufficient replication of *E. coli*, cells are harvested, centrifuged and lysed (usually with detergent or enzymes) before being applied to an immobilised metal affinity column. This affinity column is bound with either nickel or cobalt ions to which the His-tag binds and any unbound proteins are removed by washing with phosphate buffer. The recombinant protein of interest is then eluted with imidazole.

Bausinger *et al.* (2002) originally suggested that maturation of DCs was the result of LPS contamination in recombinant Hsp72 preparations. They showed that when LPS was reduced from these preparations by polymyxin B agarose, cytokine production was not induced, even in the presence of soluble CD14. Gao and Tsan (2003a) confirmed that removal of LPS by polymyxin B agarose abrogated any effect of Hsp72 on TNF- α secretion by RAW264.7 cells and that pre-incubation of cells with polymyxin B solution prior to challenge with Hsp72 gave similar results. Their study also indicated that the effect of boiling Hsp72 on TNF- α abrogation was due to the low level of LPS contaminant being heat sensitive. Similar experiments on recombinant Hsp60 demonstrated the same effect (Gao & Tsan, 2003b). In 2004, Gao and Tsan published more evidence disputing the effects of Hsps on the immune response, through use of gene expression arrays on 96 cytokine genes in murine macrophages. Hsp72 and Hsp60 (5 $\mu\text{g}/\text{mL}$) were found to have no enhanced effect on gene expression over PBS treated cells, but LPS (1 ng/mL) enhanced expression markedly.

There are many studies which oppose this theory that contamination by bacterial products is solely responsible for activation of the immune response by Hsps. In 2000, Kol *et al.* reported that pre-incubation of PBMCs with polymyxin B did not

affect the production of IL-6 but pre-incubation with anti-CD14 antibodies or heat treatment did. They further demonstrated that Hsp60 could stimulate a CD14 transfected human cell line (U373) but not a transfected rodent cell line (CHO). This suggests that another co-receptor may be involved in cytokine activation within rodent systems. It has been demonstrated that pre-incubating Hsps with anti-Hsp antibodies markedly reduces cytokine expression in a variety of systems. Retzlaff *et al.* (1994) showed that stimulation of IL-1 β from BALB/c mice macrophages was significantly reduced when various Hsps were pre-incubated with neutralising antibodies prior to application on macrophages. Dybdahl *et al.* (2002) used antibodies to CD14 and TLR-4 on adherent monocytes which were able to inhibit TNF- α and IL-6 secretion in response to recombinant Hsp72. The use of antibodies in this way has been criticised as it is an indirect way of demonstrating binding of Hsps, as antibodies to receptors may just be blocking binding of LPS, and anti-Hsp antibodies (especially Hsp60) may interfere with Hsp-bound LPS to certain receptor complexes (Tsan & Gao, 2007). This is possible as there is growing evidence that some Hsps, including Hsp72 can bind LPS (Habich *et al.*, 2005; Triantafilou *et al.*, 2001).

However, Svensson *et al.* (2006) applied Hsp72-rich supernatant from macrophages stimulated with oxLDL, to naïve macrophages. IL-1 β and IL-12 secretion were increased from macrophages treated with this supernatant and the effect on secretion could be significantly reduced when the oxLDL supernatant was pre-incubated with neutralising anti-Hsp72 antibodies. Also reported is the effect of Hsp72 release into supernatant from transfected U937 cells, which was then applied to U937 cell cultures (Lee *et al.*, 2006). Hsp72-rich supernatant was able to significantly increase expression of matrix metalloproteinase-9 (MMP-9) gene which plays a major role in migration of cells (Watanabe *et al.*, 1993). This led to an increase in the migration of U937 cells compared to treatment with supernatant from mock transfected cells. Pre-incubation of Hsp72-rich supernatant with anti-Hsp72 reduced the expression of MMP-9 and also the migration of U937 cells (Lee *et al.*, 2006). More recently, another study has demonstrated that cytokine stimulation by Hsp72, or HMGB-1 could be reduced using anti- Hsp72 or HMGB-1 antibodies in THP-1 and U937 cell lines (El Mezayen *et al.*, 2007). This study did not use recombinant protein to stimulate, but instead used pre-stimulated necrotic cell lysate (NCL) which contained cell derived Hsp72 and HMGB-1. NCL was applied directly to cell cultures or pre-incubated with antibodies to Hsp72 or HMGB-1, then applied to cells in conjunction with LPS. They showed that non-

recombinant Hsp72 and HMGB-1 induced cytokine production could be reduced using these antibodies.

Studies on the interaction between HMGB-1 and TLR-2/4 used either cell line expressed or purified porcine HMGB-1 (Park *et al.*, 2004; Yu *et al.*, 2006). Yu *et al.* (2006) added polymyxin B to the culture medium of cells prior to experiments and still demonstrated that HMGB-1 utilises TLR-2 and TLR-4 to induce TNF- α release which could be inhibited in a dose dependent manner with anti- TLR2 and TLR-4 antibodies. Interestingly, they found that HMGB-1 acted through TLR-4 in human whole blood and primary macrophages, and through TLR-2 in RAW264.7 macrophages and CHO cells over-expressing both TLRs. The reasons for this are not clear. Also, the study by El Mezayen *et al.* (2007) mentioned previously demonstrated that antibodies to HMGB-1 could reduce the stimulation of cytokines from human cell lines which were incubated with pre-stimulated NCL and LPS, thus showing that HMGB-1 itself does stimulate the innate immune response.

These data provide strong evidence that Hsp72 and HMGB-1 do play an important role within the immune response.

1.21 Measuring Hsp72

Hsp72 is widely regarded as a biomarker for stress in a variety of plant and animal systems (de Pomerai, 1996; Grosvik & Goksoyr, 1996; Ireland *et al.*, 2004; Krasko *et al.*, 1997; La Porte, 2005; Lewis *et al.*, 1999; Nadeau *et al.*, 2001; Pyza *et al.*, 1997; Schröder *et al.*, 2000; Scofield, Bowyer & Duffy, 1999; Terry *et al.*, 2006). There is growing evidence demonstrating that Hsp72 could be used as a marker of disease and disease progression (Adewoye *et al.*, 2005; Dybdahl *et al.*, 2005; Hay *et al.*, 2004; Martin *et al.*, 2003; Wright *et al.*, 2000; Yasuda *et al.*, 2006; Zhu *et al.*, 2003), and tissue damage following exercise (Madden *et al.*, 2008). This is due to the presence of Hsp72 in the peripheral circulation and that it is found elevated in some disease states (Njemini *et al.*, 2003b; Wright *et al.*, 2000). It has also been demonstrated *in vitro* to be released under stress compared to controls (Guzhova *et al.*, 2001; Luo *et al.*, 2008; Mambula & Calderwood, 2006a).

The measurement of intra-cellular Hsp72 from tissue and cell extracts, and extra-cellular Hsp72 in supernatants, plasma and serum have been reported using a variety of methods, such as densitometry, flow cytometry and ELISAs (Bachelet *et al.*, 1998; Davies *et al.*, 2006; Dybdahl *et al.*, 2005; Fehrenbach *et al.*, 2000; Lang *et al.*, 2000; Njemini *et al.*, 2003a; Njemini, Demanet & Mets, 2005; Theodorakis, Drujan & De Maio, 1999; Tong & Luo, 2000; Walsh *et al.*, 2001). Densitometry is generally not a valid method of measurement of any protein as it measures

differences in optical density between samples of interest, rather than measurement against known standards, and it is difficult to compare between film/blot due to inherent differences between exposures (Theodorakis *et al.*, 1999). Also, densitometry measurements using western blots for Hsp72 in complex matrices do not account for the blocking of Hsp72 binding to nitrocellulose by other proteins of a similar molecular weight, such as albumin in serum and tissue culture supernatants (Mambula & Calderwood, 2006b; Park, Yeo & Park, 2006c). The same applies when using densitometry to distinguish between mRNA expression (Theodorakis *et al.*, 1999; Tong & Luo, 2000). Therefore, densitometry should only be used as a qualitative measure of differences in Hsp72 levels between samples. Hsp72 ELISAs are reported to accurately measure Hsp72 protein levels in a variety of matrices (Njemini *et al.*, 2005; Njemini *et al.*, 2003b; Walsh *et al.*, 2001; Wright *et al.*, 2000). The commercial Hsp72 ELISAs are also reportedly extremely sensitive at quantifying Hsp72 in various matrices (Pockley *et al.*, 1999; Walsh *et al.*, 2001). They are indirect sandwich ELISAs which use a monoclonal capture antibody immobilised to the plastic surface of a treated ELISA plate. Antigen in the form of standards and unknown samples are then applied to the plate and any Hsp72 binds to the capture antibody. The bound Hsp72 is then detected by a primary rabbit anti-Hsp72 antibody which is subsequently bound by a horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody. The addition of an enzyme substrate enables the colorimetric detection of bound Hsp72 (Fukushima *et al.*, 2005; Walsh *et al.*, 2001; Zhu *et al.*, 2003).

The commercially available ELISAs are costly and have recently been found to be unreliable in the determination of levels of Hsp72 in cell culture supernatants that contain serum, and serum or plasma samples. It is likely that in the studies quantifying Hsp72 in serum or tissue culture supernatants there are some non-specific matrix problems, such as lipids or other proteins which either prevent Hsp72 binding to the bound antibody, or that Hsp72 is already peptide or antibody bound (Asea *et al.*, 2000a; Child *et al.*, 2006; Murray *et al.*, 2001; Pockley *et al.*, 1998; Urbonaviciute *et al.*, 2007). As a result, many studies have employed their own in-house ELISAs, although they are mainly based on those commercially available (Njemini *et al.*, 2005; Njemini *et al.*, 2003b; Pockley *et al.*, 1998; Rea, McNerlan & Pockley, 2001). The reported levels of Hsp72 found in serum samples with these commercial or in-house methods are extremely varied. All the lower Hsp72 levels reported utilised a commercial Hsp72 ELISA (Stressgen Inc., EKS-700). Interestingly, the studies reporting high levels of Hsp72 in serum all perform ELISAs using the same components utilised by the commercially available ELISA.

Therefore it cannot be assumed that measurements of Hsp72 in serum and cell culture supernatants which have been reported are accurate, although supporting the specificity of these antibodies are western blots that have been used to detect intra-cellular Hsp72 in cell extracts (Davies *et al.*, 2006; Downs *et al.*, 2002; Tavarina *et al.*, 1996; Tsuchiya *et al.*, 2003).

Also, other studies measuring Hsp72 by ELISA in tissue culture supernatants do not use serum supplemented media when performing experiments which may result in inaccurate effects due to extra stress on cells (Mambula & Calderwood, 2006b).

1.22 Aims and objectives

The role of extra-cellular Hsp72 is still not fully understood.

This thesis aims to examine aspects of the role for Hsp72 in being a danger signal to the innate immune system.

The objectives of this thesis are:

- To develop a method of measuring Hsp72, both intracellular and extracellular, from cell extracts and cell culture supernatants.
- To determine whether LPS contamination is responsible for the reported stimulation by recombinant Hsps.
- To determine the relative contributions of Hsp72 and LPS to the immune response.
- To determine the cell surface receptors Hsp72 can bind leading to stimulation of the immune system.
- To determine whether cell derived Hsp72 can stimulate an immune response in macrophages.
- To assess how Hsp72 may interact with other elements of the Danger model.