## Chapter 7 – Discussion.

The overall aims of this thesis were to investigate the effect of Hsps on the RANKL/OPG system of bone cell regulation, to investigate whether Hsps could be actively released from cells, to determine if this release could be up regulated by factors such as immune cell activation and treatment with other Hsps such as GroEL and LPS and finally to relate this to the role of Hsps as danger signals.

For Hsps to affect bone resorption via the RANKL/OPG system or to act as danger signals to the innate immune system they would need to be released from cells. The initial part of this discussion will therefore focus on the results of Chapter 4, which aimed to determine whether Hsps could be actively released from a range of immune cells. Following the initial discussion of Hsp release, this discussion will focus on two hypotheses and the evidence both from the thesis and literature that relates to them; the first hypothesis will be that Hsps cause increased bone resorption by acting on the RANKL/OPG system, and the second will be that Hsps act as danger signals to the innate immune system.

### 7.1 Release of Hsps from Immune cells

It is known that Hsps are present in human serum (Pockley *et al.*, 1998., Pockley *et al.*, 1999) and that the levels of these Hsps become elevated during certain diseases; this leads to the question of how the Hsps end up in the extracellular environment, and whether their presence is due to release following cell necrosis or active release. Release of Hsps had been previously reported from several different cell types including rat embryo cells (Hightower and Guidon., 1989), glial cells (Guzhova *et al.*, 2001), and human carcinoma cell lines (Broquet *et al.*, 2003., Evdonin *et al.*, 2004., Wang *et al.*, 2004); there is therefore a growing body of evidence suggesting that Hsps are released from cells despite the lack of a peptide leader sequence that would target the proteins for secretion. The fact that an increasing number of secreted proteins that lack the classical peptide leader sequence are being identified does make the possibility of Hsp release more feasible (Nickel., 2003).

In this thesis it was demonstrated that it is possible for a range of immune cells and cell lines including PBMCs, Jurkat, U937, T and B cells to actively release Hsp60 and Hsp70 both in normal conditions and in response to heat stress. Western blotting confirmed the release of complete 60kDa and 70kDa proteins that were quantified by ELISA. The majority of the observed release at 37°C was not due to cellular damage, and this was confirmed by cell viability counting (<0.1% cell death) and the use of the intracellular enzyme LDH as a control. A range of protein release inhibitors were tested with the aim of determining the mechanism by which Hsps were actively released from the cells; Monensin and Brefeldin A were found to inhibit Hsp60 release, and Monensin, Methyl-β-cyclodextrin, and Methylamine were found to inhibit Hsp70 release. The fact that Hsp60 or Hsp70 proteins do not have a peptide leader sequence targeting them for secretion suggested that secretion may not be *via* the conventional ER/Golgi route (Nickel., 2003).

Monensin and Brefeldin A are classical inhibitors of ER/Golgi protein transport; Brefeldin A blocks transport of polypeptides from the ER to the Golgi, and Monensin is a $Na^+$ ionophore and so collapses intra-cellular $Na^+$ and $H^+$ gradients within the Golgi. As both Monensin and Brefeldin A inhibited Hsp60 secretion it would suggest that release occurs via a classical secretion route, though this does contradict the lack of peptide leader sequence. Unlike Hsp60, Hsp70 was inhibited by one of the classical protein secretion inhibitors tested (Monensin but not Brefeldin) and two non-classical inhibitors (Methyl-β-cyclodextrin, and Methylamine). There are several possibilities for these contrasting results. The first is that Hsp70 secretion does not involve the ER, but does involve the Golgi. The second is that the inhibition of Hsp70 secretion by monensin is due to collapsing of $Na^+$ and $H^+$ gradients across other membranes such as the plasma membrane. Methylamine inhibits endocytosis, and has been used to demonstrate protein secretion *via* lysosomes (Nickel., 2003). Methyl-β-cyclodextrin disrupts membrane rafts, that have been implicated previously in Hsp70 secretion (Broquet *et al.*, 2003). Membrane rafts are lipid microdomains that form within the exoplasmic leaflet of the Golgi (Brown and London., 2000), it is therefore feasible for monensin to inhibit proteins secreted *via* this route. Hsp70 has been shown to interact with membrane structures (Gross *et al.*, 2003., Gastpar *et al.*, 2004), therefore a secretory route consistent with the data presented here is that Hsp70 is transported *via*

the Golgi into lysosomal lipid rafts prior to exocytosis. One remaining possibility for the results is that Monensin is causing a reduction in release because of its ability to cause cell cycle arrest at relatively low concentrations (Park *et al.*, 2002). Steps were taken to determine non-lethal concentrations of the inhibitors prior to the final experiments and MTS and cell viability staining did not indicate that any cell damage was occurring. However, with any substance that is a general inhibitor of protein synthesis or transport there is a risk of non-specific effects on the cells.

The results of the initial work on release provided strong evidence for active release of both Hsp60 and Hsp70 from immune cells, and this in turn provides a foundation and mechanism for the theories related to how Hsps could affect bone resorption, and also how they could function as danger signals to the innate immune system.

## 7.2 Hypothesis 1 – Hsps cause increased bone resorption by acting on the RANKL/OPG system.

Studies carried out prior to the work in this thesis had identified that Hsps could be possible promotors of bone resorption; Hsps had been previously found to increase both osteoclast formation and bone resorption *in vitro* including GroEL (Reddi *et al.*, 1998), Hsp10 from *M. tuberculosis* (Meghji et al., 1997), GroES and DnaK from *E.coli*, and mammalian Hsp27, Hsp70 and Hsp90; mammalian Hsp60 was not tested (Nair et al, 1999). Although it was clear that Hsps were having a significant effect, no further studies have been carried out to investigate the mechanisms involved. Within the last 10 years one of the most important mechanisms involved in the regulation of bone remodelling has been discovered; the RANKL/OPG system (Simonet et al., 1997., Tsuda et al., 1997., Lacey et al., 1998., Wong et al., 1997). Prior to the discovery of this system many factors that influenced osteoclastogenesis had been discovered including M-CSF, IL-1, TGFβ, TNFα, IL-6, $1,25(OH)_2D_3$, $PGE_2$ and PTH, however genetic knock-out experiments showed that none of these factors were essential for osteoclast formation *in vivo*; it was therefore important to identify the essential factors, which turned out to be those of the RANKL/OPG system (Theill *et al.*, 2002). As the OPG/RANKL system was the most important with regard to osteoclast formation, it

was decided that it would be the initial focus when looking for the mechanism through which Hsps could affect bone resorption.

The results of Chapter 3 identified that Hsps can have a significant effect on both the OPG/RANKL system and osteoclast formation. Hsp60 and Hsp70 had a significant inhibitory effect on OPG production and release from osteoblast-like cells. LPS also had a significant effect similar to that of the Hsps. GroEL had no apparent effect on OPG release, and actually resulted in a slight increase in intracellular levels in GCT cells; it is thought that this effect was due to an increase in metabolic activity or cell proliferation caused by GroEL that became apparent following an MTS assay and which had also been observed previously in another cell line (Goulhen *et al.*, 1998). RANKL expression was upregulated by treatment with Hsp60, Hsp70, GroEL and LPS in MG63 cells, and by treatment with GroEL and LPS in GCT cells.

This treatment of osteoblasts with Hsps and LPS was found to translate to an increased effect on osteoclast formation (measured by the Trap assay) when culture medium from treated osteoblasts was added to osteoclast precursors in the presence of M-CSF. Hsp60, 70 or GroEL added alone to osteoclast precursors in non-conditioned media or in conditioned media in the absence of M-CSF had no effect on Trap activity; this suggests that the Hsps are acting by increasing RANKL production and/or decreasing OPG production by osteoblasts rather than having a direct effect on osteoclasts, as only the conditioned media had an effect.

Unlike Hsps, LPS did have an effect on the osteoblast precursors when added alone (non-conditioned media) both in the absence and presence of M-CSF; in the absence of M-CSF the LPS resulted in an increased proliferation of the osteoclast precursors, and in the presence of M-CSF LPS resulted in increased Trap activity. This suggests that LPS is capable of increasing osteoclast formation through both RANKL dependent and independent mechanisms. LPS has been reported to cause an up-regulation in RANKL gene expression and a down-regulation in OPG expression in a study carried out by Suda *et al.*, (2004) and to act directly on osteoclast formation and promote survival and fusion of osteoclasts independently of RANKL (Jiang *et al.*, 2003., Suda *et al.*, 2002).

It is apparent from the results that Hsps could potentially stimulate bone resorption by affecting the OPG/RANKL system, yet for this to have significance *in vivo* the mechanisms by which cells could be exposed to the Hsps would need to be determined. It is clear that osteoblasts may be exposed to LPS and GroEL during bacterial infection, however, with the self-hsps the pathway through which they could influence the osteoblasts is less obvious. This question was partly answered in Chapter 4 where it was demonstrated that Hsps could be actively released from immune cells, as the fact that self Hsps are released from cells provides a way by which they could come into contact with osteoblasts and influence the RANKL/OPG system. However, for this release to have relevance to diseases in which bone loss is increased it would need to be demonstrated that Hsp release could increase in response to factors causing or caused by the disease in question.

In Chapters 5 and 6 of the thesis the effects of PHA, corticosteroids, LPS and Hsps on Hsp release were investigated. It was previously known that both $CD4^+$ and $CD8^+$ T-cells could stimulate osteoclast formation when activated by PHA, and also that this happened through both RANKL dependent and independent mechanisms (Weitzmann *et al.*, 2001). It was also known that steroids result in increased bone resorption, and that this can occur via the RANKL/OPG system (Theill *et al.*, 2002., Hirayama et al., 2003). It was therefore important to know the effect that T-cell activation following PHA treatment, and exposure to steroids would have on Hsp release, thus determining whether Hsps were involved in these mechanisms. PHA treatment of PBMCs resulted in increased expression of both Hsp60 and Hsp70 following 24 h incubation. This increase did translate to increased release of Hsp60 from PBMCs, although Hsp70 release from PBMCs did not increase following PHA activation. The release of Hsp60 could potentially translate to an effect on osteoblasts. One other factor PHA affected that could have an effect on bone resorption was the increase in TNFα expression as TNFα is a known stimulator of bone resorption. Treatment with anti-TNFα antibodies or OPG has been successfully used to reduce arthritis related bone loss in mice (Saidenberg-Kermanac'h *et al.*, 2004).

Steroid treatment of PBMCs generally resulted in decreased Hsp release, particularly in the cast of prednisolone, which inhibited Hsp70 release at both 8 and 24 hours. The fact

that Hsp release was inhibited by steroids suggested that Hsps were not implicated in steroid-induced bone loss and that the steroids were most likely acting directly on the OPG/RANKL system to cause bone loss as previously described (Chung *et al.*, 2001). Though it appeared unlikely that Hsps were implicated in steroid-induced bone loss one other factor was identified in the thesis, namely the down regulation of IFN-β following steroid treatment. IFN-β has been shown to inhibit the differentiation of osteoclast precursors by interfering with RANKL-induced expression of c-fos, which is an essential transription factor for osteoclast formation (Takayanagi *et al.*, 2002) and it is thought that this is a negative feedback mechanism as IFN-β expression is induced by RANKL (Hayashi *et al.*, 2002). It may be possible that normal immune cell production of IFN-β acts to down regulate osteoclast formation as has been demonstrated for T-cell production of IFN-γ (Takayanagi *et al.*, 2000), and that steroid treatment affects this mechanism.

Following the investigation of the potential effect of PHA and steroids on Hsp-related bone resorption, the effect of human and bacterial Hsps and LPS on Hsp release was studied. The results provided evidence for several mechanisms by which Hsps could affect the RANKL/OPG system. It was found that treatment of both immune and osteoblast cells with Hsps, GroEL and LPS resulted in an up-regulation of Hsp release; treatment of PBMCs and GCT cells with GroEL resulted in a significant up-regulation of Hsp70 release, and treatment of PBMCs and GCT cells with Hsp70 resulted in a significant increase in Hsp60 release. LPS caused an increase in Hsp60 release from PBMCs, and Hsp70 release from PBMCs, Jurkat and GCT cells. These results indicate that any bacterial infection or disease that up-regulates self-hsps could trigger a cascade-like increase in released heat shock proteins. Figure 7.1 illustrates mechanisms identified in this thesis by which Hsps could affect the RANKL/OPG system of bone remodelling, pushing the balance towards increased osteoclast formation, and bone resorption. Table 7.1 summarises the factors identified during the experimental work of the thesis that provide evidence for the role of Hsps and LPS in promoting bone resorption.

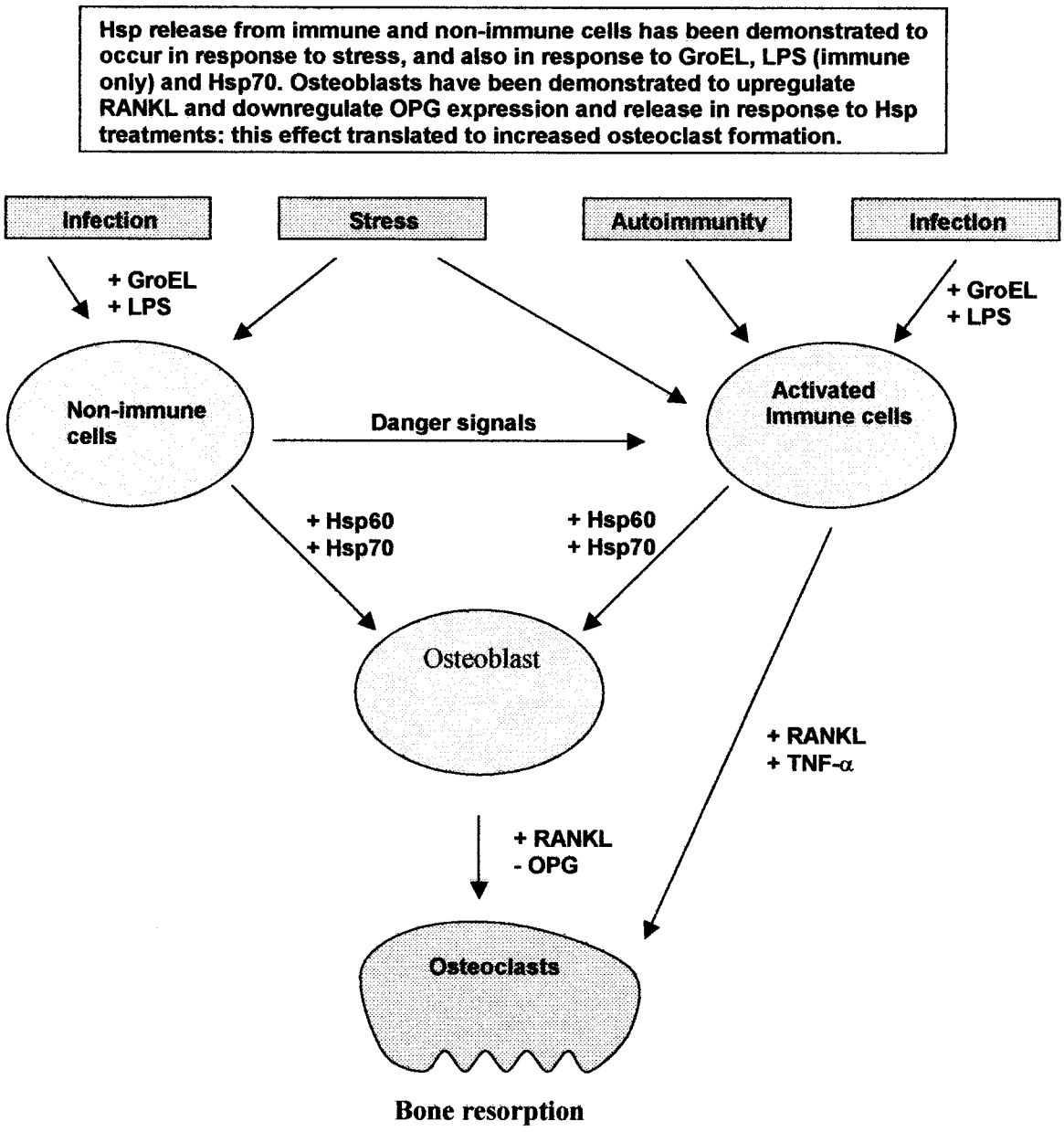


Figure 7.1. Summary of the hypothesis that Hsps lead to increased bone resorption.

Table 7.1 Summary of factors identified in experimental work from this thesis that provide a link between Hsps, LPS and increased bone resorption.

| | **Factors identified that could lead to increased bone resorption** |
|---|---|
| **Hsp60** | - Decreased OPG production and secretion by osteoblasts.<br>- Increased RANKL expression by osteoblasts (MG63).<br>- Increased TRAP activity when osteoclast precursors are incubated with media from Hsp60 treated osteoblasts.<br>- Increased release of Hsp60 following PHA activation of PBMCs (relevant to infection/autoimmunity). |
| **Hsp70** | - Decreased OPG production and secretion by osteoblasts.<br>- Increased RANKL expression by osteoblasts (MG63).<br>- Stimulation of Hsp60 release from PBMCs and osteoblasts.<br>- Increased TRAP activity when osteoclast precursors are incubated with media from Hsp70 treated osteoblasts. |
| **GroEL** | - Increased RANKL expression by osteoblasts.<br>- Stimulation of Hsp70 release from PBMCs and osteoblasts.<br>- Increased TRAP activity when osteoclast precursors are incubated with media from GroEL treated osteoblasts. |
| **LPS** | - Decrease in OPG secretion by osteoblasts.<br>- Increase in RANKL expression by osteoblasts.<br>- Proliferation of osteoclast precursors.<br>- Stimulation of Hsp60 and Hsp70 release from immune cells.<br>- Increased proliferation of osteoclast precursors when incubated with LPS alone.<br>- Increased TRAP activity when osteoclast precursors are incubated with media from LPS treated osteoblasts. |

The results of this thesis have demonstrated that Hsps can increase osteoclast formation and bone resorption by acting on the OPG/RANKL system of bone cell regulation in

osteoblasts, and the mechanism through which the cells could be exposed to Hsps has been identified. There are a range of different diseases for which the effect of Hsps on the RANKL/OPG system could have implications; In the situation of bacterial infection where GroEL and LPS would be present there would also be an up regulation of self-hsps, and as all act to promote bone resorption by the RANKL/OPG pathway the net effect could be quite significant. Also the activation of immune responses by Hsps (discussed in section 7.3) would result in increased RANKL production by activated T-cells. This would also be likely to cause bone loss in autoimmune diseases such as arthritis where Hsps and RANKL would be elevated in the affected joints.

### 7.3 Hypothesis 2 – Hsps act as a danger signal to the innate immune system

Danger signals consist of molecules or molecular structures that are released by stressed or dying cells and are then perceived by resting antigen-presenting cells (APCs) that then become activated and offer co-stimulatory signals to initiate immune responses. APCs can consist of dentritic cells, which stimulate naïve T-cells and and initiate primary immune responses, or B-cells and macrophages which can re-stimulate memory T-cells and initiate secondary responses (Gallucci and Matzinger., 2001).

There is an increasing amount of evidence that Hsps have cytokine-like functions and may act as danger signals to the innate immune system (Maguire *et al.*, 2003., Pockley., 2003., Prohaszka and Fust, 2004., van Eden *et al.*, 2003). They have been demonstrated to be capable of activating monocytes, macrophages and dendritic cells (Somersan *et al.*, 2001., Bethke *et al.*, 2002., Kol *et al.*, 2000) and also of inducing secretion of a wide range of cytokines including the induction of IL-6, TNF-α, IL-12 and IL-15 by mammalian Hsp60 (Chen *et al.*, 1999., Kol *et al.*, 1999., Ohashi et al., 2000) and TNF-α, IL-1 β and IL-6 following treatment with mammalian Hsp70 (Asea *et al.*, 2000). The main receptors that have been demonstrated to be involved in the activation of APCs by Hsps are the CD14 and TLR4/TLR2 receptors (Asea *et al.*, 2000., Kol et al., 2000., Ohashi et al., 2000., Vabulas *et al.*, 2001., Bulut *et al*, 2002), though CD14-independent pathways have been identified for Hsp70 (Asea *et al.*, 2002). It has also been demonstrated that the binding sites on macrophages for mammalian Hsp60, bacterial Hsp60 and Hsp70 may be different (Habich *et al.*, 2003).

Hsps are released from cells following death by necrosis, and it is therefore possible that this could be the mechanism by which they act as a danger signal, however, it would seem that from the evidence of their cytokine functions that for them act efficiently as a danger signal they would need to be released in a controlled manner prior to cell death. It was therefore important to demonstrate whether Hsps could be actively released from cells, and whether this release could be regulated in response to factors present in situations such as infection, that would require them to function as a danger signal.
As discussed in section 7.1, in Chapter 4 of this thesis it was demonstrated that both Hsp60 and Hsp70 could be actively released from immune cells, yet simply for Hsps to be released from cells was not sufficient evidence for them to act as danger signals as a danger signal would imply that the release could be up-regulated following exposure to a particular stress or cytokine that could be encountered *in vivo*. The up-regulation in response to heat stress did provide an indication that this was possible, though due to the fact that heat is likely to damage the membrane integrity of the cell it is far from the ideal test to provide good evidence for the danger theory.

Results in Chapter 3 had indicated that Hsps, GroEL and LPS all acted to up regulate the expression of Hsps in osteoblast-like cells, it was therefore decided to investigate the effect that these factors had on Hsp release from both immune and bone cells; the results provided significant evidence for the danger signal theory. The initial aim of using LPS was as a control for LPS contamination of the other Hsps, however the results gave it a more important relevance. Both GroEL and LPS stimulated Hsp70 release from PBMCs, and GroEL stimulated Hsp70 release from GCT cells. Hsp70 stimulated Hsp60 release from both PBMCs and GCT cells. As the response of Hsp70 release in response to GroEL and Hsp60 response to Hsp70 was seen in both the PBMCs and the non-immune GCT cells it indicates that these responses may be signals that are common to many cell types following exposure to bacterial Hsps. The only response that was not common between the two cell types was that to LPS which was only present in PBMCs. The results implied a signalling cascade in which GroEL or LPS stimulated Hsp70 release, which in turn would result in elevated Hsp60 release. This could be a danger signal cascade that would apply following a bacterial infection; Figure 7.3 summarises this proposed danger signal cascade leading to activation of the innate immune system. Following secretion the Hsp60 and Hsp70 could activate APCs

by binding to either the CD14/TLR4 or TLR2 complex as previously demonstrated (Asea *et al.*, 2000., Kol et al., 2000., Ohashi et al., 2000., Vabulas *et al.*, 2001., Bulut *et al.*, 2002) or the alternative pathways that have been proposed (Asea *et al.*, 2002). This activation of the innate immune response would then initiate cytokine release from the APCs which would cause activation of naïve T-cells (signal from dendritic cells) or memory T-cells (signal from macrophage/B cells). Though this particular model relates to infection much of it could apply to other forms of stress that would induce Hsp70 and Hsp60 release from cells.

The patterns of Hsp release observed also suggest that there are different receptors and /or responses to human and bacterial Hsp60. GroEL stimulated significant release of Hsp70 from both PBMCs and GCT cells (immune and non-immune), yet Hsp60 caused no stimulation of Hsp70 release, and actually caused an inhibition in the case of GCT cells. This corresponds with evidence from a study by Habich *et al* (2003) in which microbial and mammalian Hsp60 were demonstrated to compete for different binding sites. This difference is logical in terms of the danger signal process. It may even apply that in some cell types released self-Hsp60 acts to down regulate or maintain the initial danger response at a certain level by inhibiting further release of Hsp70 in a negative feedback role.

The responses observed could have important relevance to autoimmunity, particularly autoimmunity that is thought to be triggered by bacterial infection. Hsps have been demonstrated to have both causative and protective roles in situations of autoimmunity. In a recent review Prohaszka and Fust (2004) proposed that regular conditioning of innate immunity by pathogens/microbes mediated by LPS and Hsp dependent mechanisms is a specific means of maintaining the immune system on a level of basic activation, and that changes in this balance such as those caused by use of antibiotics and a more hygienic lifestyle can create imbalance in these mechanisms. The responses observed in this thesis demonstrated that bacterial Hsps (GroEL) and LPS cause an up regulation of self-Hsp release, which subsequently could result in activation of the innate immune system; presumably *in vivo* this would occur both in normal conditions

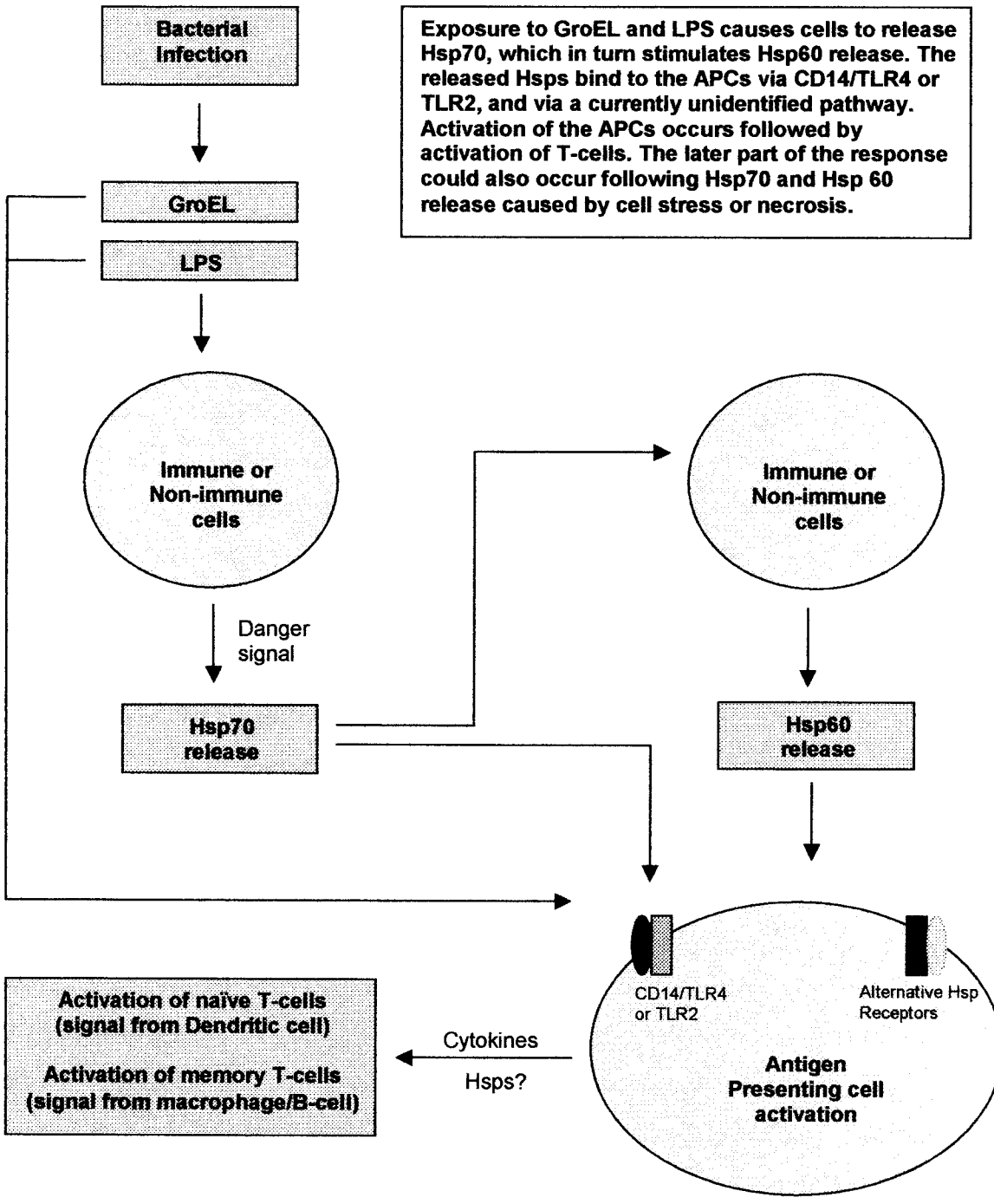


Figure 7.3 Diagram showing an Hsp signalling cascade following cell exposure to bacterial infection.

in response to commensal micro-organisms, and to a greater extent in response to bacterial infection.

Due to the homology between human and bacterial Hsps there is a chance that any bacterial infection could result in the generation of cross-reactive lymphocytes and the corresponding antibody responses (Zugel and Kaufmann., 1999., Lamb *et al.*, 2003), and this cross-reactivity has been suggested as the cause of certain diseases such as arthritis (Cohen *et al.*, 2003), Crohn's disease (Szewczuk and Depew., 1992) and Type 1 diabetes (Albulatia-Lapid *et al.*, 1999). However, increasing evidence suggests that Hsps may have a protective mechanism against autoimmunity, possibly due to the way they mediate T-cell responses (van Eden *et al.*, 2003); The release of Hsp60 demonstrated in this thesis would be particularly important for the protective mechanisms that have been proposed as much of the current work and theories are based on the assumption Hsp60 can act as an immunoregulator, and therefore that controlled release in *vivo* is a possibilty. In a study by Van Roon *et al* (1997) it was demonstrated that human but not bacterial Hsp60 reactive synovial fluid T-cells from RA patients proliferate in the presence of IL-4, and also that T-cells stimulated with human Hsp60 secrete less IFN-gamma and more IL-4 than T-cells stimulated by bacterial Hsp60; this demonstrates that self-hsp60 produces increased Th2 responses compared to bacterial Hsp60 and could therefore have an protective anti-inflammatory effect. Another series of studies that provides evidence for Hsp60 acting as a down-regulatory signal involved the vaccination of NOD (non-obese diabetic) mice with the p277 peptide of Hsp60 (Elias and Cohen., 1994); the peptide was found to arrest the development of diabetes. DNA vaccination with constructs encoding human hsp60 was also found to be effective against cyclophosphamide induced diabetes in NOD mice (Quintana *et al*, 2002), and T cell proliferative responses to Hsp60 and to insulin auto - antigens were significantly reduced following vaccination with the hsp60 construct. The pattern of cytokine secretion following vaccination with the hsp60 construct resulted in increased IL-10 and IL-5 secretion (Th2 response) and decreased IFN-γ secretion (Th1 response) (Quintana *et al.*, 2002). The trials with mice were found to translate to similar results in humans, with a study revealing the p277 Hsp60 peptide was able to arrest β-cell destruction and result in lower insulin requirements (Raz *et al.*, 2001).

These studies clearly demonstrate Hsp60 has the potential to act as a regulatory signal to the immune system, and fit with the observations of this thesis in which Hsp60 can be released from cells, and is elevated by Hsp70 (danger signal) and perhaps has the potential to act in a negative feedback role. It is known that microbial infection and innate signalling via LPS can also inhibit the spontaneous development of diabetes in the NOD mouse (Quintana *et al.*, 2002), and interestingly the results in this thesis showed that both of these (GroEL/LPS) had the potential to lead to increased levels of Hsp60 release (by causing increased Hsp70 release which in turn leads to increased Hsp60 release). As the role of Hsp60 release becomes better understood, potential treatments of autoimmunity could consist of looking at ways to elevate Hsp60 release *in vivo* as a possible alternative to treating directly with Hsp60 peptides.

One of the main questions to arise regarding Hsp60 is how can it be both a cause/indicator of autoimmunity and a down-regulator of the autoimmune response? Most of the work to this point has involved studies that either focus on levels of Hsp60/anti-Hsp60 *in vivo* associated to a particular disease, or therapy with Hsp60 for disease models. It is very likely that Hsp60 is having a different role in each of the autoimmune diseases studied, rather than acting as either antigen or down regulator in all of them, and it is important to remain open minded about the causes and mechanisms in each case at this point. For example, it is normally assumed that autoimmunity to Hsp60 would cause damage by resulting in an immune reaction due to the presence of self-hsp60 expressed on the cells, yet in light of release it is possible that increased auto-antibodies to Hsp60 may not cause damage by resulting in an attack on cells due to the presence of self-hsp60, but rather by causing decreased available Hsp60 (the down-regulator) in the extracellular environment and therefore a trend towards Th1 pro-inflammatory responses. In order to further understand the disease processes we need to establish the role of Hsp60 as an immunoregulatory molecule under normal conditions, including answering further questions about how/when it is released, what the release is stimulated by, and the signal transduction pathways involved. This is also important for determining the implications of using Hsp60 vaccination as a treatment in autoimmunity.

The responses of Hsps release observed in this thesis could have more than one function depending on the Hsps involved. On one level they could act as a danger signal to the

innate immune system (primarily Hsp70), and on another level they could serve as immunoregulators and protectors against negative consequences of cross-reactivity or bacterial infection by stimulating T-cells towards a Th2 response (primarily Hsp60). It is also possible that the release of Hsps could protect against damage by a background level of cross-reactive antibodies simply by the fact that they are present as targets and are remote from cells that could be damaged by the response. In relation to the increase in autoimmune disease that occurs in developed countries where exposure to bacteria is low there may also be connections with the responses observed if constant exposure to bacteria is required for LPS and GroEL to stimulate self-hsps, which in turn may promote Th2 responses (possibly involving increased Hsp70 and in turn increased Hsp60 release) and offer protection against damage caused by autoimmunity.

## 7.4 Conclusion

The results of this thesis support the increasing amount of evidence suggesting that Hsps have many important roles other than their traditional chaperone functions. It has demonstrated that Hsps can be actively released from mammalian cells in response to heat stress, immune cell activation, LPS and bacterial and human Hsps, and also that some of the responses observed could form part of a signalling cascade; these findings combined with existing literature make a strong case for the function of Hsps as danger signals to the innate immune system, as well as having potential relevance to autoimmunity and the role of Hsps as regulators of immune responses. It has also been demonstrated that Hsps and LPS can stimulate bone resorption via the RANKL/OPG system; the Hsp release responses observed identified how this could occur and have relevance *in vivo* and these findings could have important implications for the bone loss that can occur in response to infection and autoimmunity.

With regard to future work there are many directions that could be followed including further classification of the cell types that release Hsps and studies on the receptors and cytokines involved in the signalling pathways; this work is particularly important for establishing the role of heat shock proteins in autoimmunity causes and treatments. Many questions remain regarding the signalling and immunoregulatory roles of Hsps, and the evidence to this point suggests that the potential benefit of understanding these mechanisms justifies further research.