

Hsp72 modulation of inflammatory immune responses

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Chapter 4

The effects of recombinant heat shock proteins on cytokine secretion from peripheral blood mononuclear cells and U937 macrophages.

4.1 Introduction

Extra-cellular heat shock proteins have been reported to have a stimulatory effect on the innate immune system (Asea, 2006; Asea *et al.*, 2000b; Campisi & Fleshner, 2003; Guzhova *et al.*, 1998; Milani *et al.*, 2002; Svensson *et al.*, 2006). These effects include the secretion of many cytokines, both pro-and anti-inflammatory (Campisi *et al.*, 2003; El Mezayen *et al.*, 2007; Moré, Breloer & von Bonin, 2001; Retzlaff *et al.*, 1994; Singh-Jasuja *et al.*, 2000; Svensson *et al.*, 2006). Most of these studies employed the use of recombinant proteins expressed in transfected *E. coli*. More recently it has been reported that these innate immune responses may actually be due to contamination of these proteins by LPS, or possibly flagellin (Bausinger *et al.*, 2002; Gao & Tsan, 2003a; Gao & Tsan, 2003b; Gao & Tsan, 2004; Kol *et al.*, 2000; Tsan & Gao, 2004a; Ye & Gan, 2007).

Gao and Tsan (2004) utilised highly purified recombinant human (rh) Hsp72 and Hsp60 and subjected murine macrophages to a 4 h incubation with 5 µg/mL of either stress protein or LPS (1 ng/mL) and performed gene array expression on a number of common cytokines. They reported there was no increased expression of any of these cytokines in response to rhHsp72 or rhHsp60 in contrast to incubation with LPS. A time course experiment measuring IL-1β also showed no expression. However, Kol *et al.* (2000) stimulated cytokine production from U373 cell line with Hsp60 and the effect was completely abrogated by heat treatment which suggested no LPS contamination present as LPS is heat labile. Campisi *et al.* (2003) also demonstrated that even in the presence of polymixin B, Hsp72 could stimulate TNF-α, IL-1β and IL-6 secretion from rat splenocytes and macrophages. More recently, it has been suggested that Hsp72 can activate macrophages, especially when in a membrane bound form (Vega *et al.*, 2008).

Svensson *et al.* (2006) observed that supernatant from stimulated peripheral blood mononuclear cells (PBMCs), which contained released Hsp72, resulted in a 3-fold increase in IL-1β and IL-12 secretion in naïve macrophages that could be blocked by anti-Hsp72 antibodies.

The aim of this chapter is to determine whether recombinant heat shock proteins are able to induce cytokine expression and production, or if the effect is due to LPS contamination.

4.2 Methods

All preparations and cell culture experiments were carried out within a Class II tissue culture hood.

4.2.1 Preparation of cells for treatment.

U937 macrophages were prepared as in section 2.3.28. PBMCs were isolated and prepared for treatment as in section 2.3.29.

4.2.2 Preparation of heat shock proteins for experiments.

Heat shock proteins used for experiments were: Hsp72, low endotoxin Hsp72, DnaK, Hsp60, GroEL, Cpn10, GroES. Proteins were diluted in 10 % HI-RPMI to give 10 000, 1000 and 100 ng/mL stock solutions.

For heat-treated stress protein treatments, stock solutions were heated at 75°C for 20 min and allowed to cool prior to use.

4.2.3 Preparation of LPS for experiments.

LPS from *E. coli* O111:B4 was reconstituted in 10 % HI-RPMI at 1 mg/mL and allowed to dissolve. LPS was then further diluted in 10 % HI-RPMI to give 1 µg/mL stock solution.

4.2.4 Determination of endotoxin content of heat shock proteins.

Endotoxin content of 1000 ng/mL native or denatured heat shock proteins was determined using the Limulus Amebocyte Lysate assay as described in section 2.3.32.

4.2.5 Treatment of cells with native or denatured heat shock proteins.

Native or denatured heat shock proteins were applied to cells at 1/10 dilution (100 µL protein stock to 900 µL 10 % HI-RPMI) so that cells were treated with 1000, 100 and 10 ng/mL stress protein. Cells were then incubated for 24 h.

4.2.6 Time course experiments with native or denatured, Hsp72 or low endotoxin Hsp72.

Native or denatured Hsp72 or low endotoxin Hsp72 were applied to U937 macrophages at 1/10 dilution (100 µL protein stock to 900 µL 10 % HI-RPMI) so that cells were treated with 1000 ng/mL heat shock protein. Cells were then incubated for up to 6 h.

4.2.7 Treatment of U937 macrophages for 4 h, with Hsp72 and polymyxin B.

Hsp72 (1000 ng/mL) or LPS (100 ng/mL) was pre-incubated with or without 20 µg/mL polymyxin B before being added to U937 macrophages for 4 h.

4.2.8 Cell counts and viability tests by trypan blue exclusion.

Following treatment, cells were re-suspended into the media by repeated pipetting. Cells were then counted and assessed by trypan blue exclusion (Section 2.3.24).

4.2.9 Determination of apoptosis.

Cells were plated onto a black 96 well cell culture plate and treated with stress proteins or LPS and cells were then analysed for apoptosis by caspase 3/7 detection (Section 2.3.30).

4.2.10 Determination of necrosis.

Following treatments, necrosis was determined by propidium iodide (Section 2.3.31).

4.2.11 Measurement of secreted cytokines.

Following treatment, media was removed from the wells and clarified by centrifugation at 400 g for 5 min and transferred to a clean 1.5 mL microcentrifuge tube. Media was then either used fresh or frozen at -70°C until required. Secretion of TNF- α , IL-1 β , IL-6 and IL-10 were measured as described in sections 2.3.33 - 2.3.36.

4.3 Results

4.3.1 Endotoxin contamination of recombinant heat shock proteins determined by LAL assay.

Endotoxin contamination of recombinant heat shock proteins was determined as endotoxin units (EU) per μg of protein. All heat shock proteins, whether native or denatured had less than 0.075 EU per μg except for native and denatured, DnaK (2.344 and 2.274 EU per μg respectively) and GroEL (0.396 and 0.239 EU per μg respectively). None of the proteins tested showed any significant difference from controls (HI-RPMI) except for native and denatured, DnaK ($P < 0.001$) and GroEL ($P < 0.001$) (Figure 4.1).

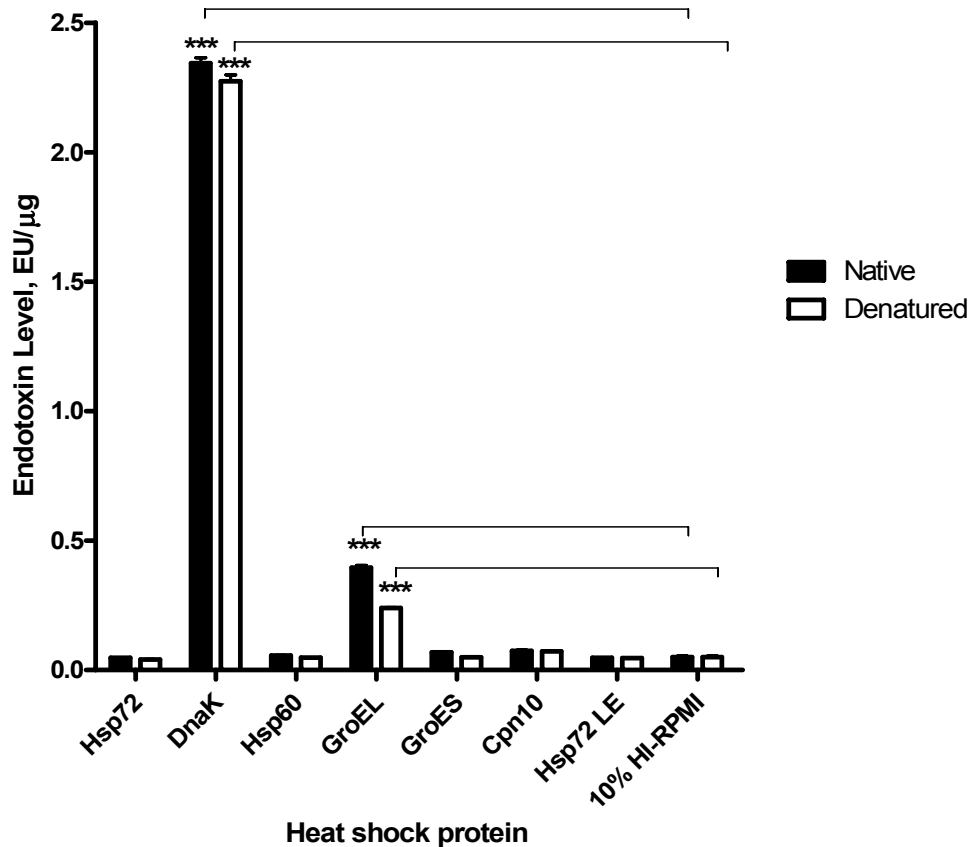


Figure 4.1: Endotoxin contamination of recombinant heat shock proteins in native and denatured forms.

Native and denatured heat shock proteins were applied to a LAL assay at 1 $\mu\text{g}/\text{mL}$ in 10% HI-RPMI. Data are presented as mean \pm SEM, $n = 4$. Significance shown as difference from 10% HI-RPMI using two-way ANOVA with Bonferroni's *post hoc* test: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

4.3.2 Cytokine secretion from PBMCs following 24 h incubation with native or denatured heat shock proteins.

Cytokine secretion from PBMCs following treatments with native or denatured heat shock proteins are presented in Figures 4.2 – 4.13 and Tables 4.1 – 4.17.

4.3.2.1 Treatment of PBMCs with native or denatured Hsp72 or DnaK.

Following incubation of PBMCs with Hsp72 for 24 h, an almost 4-fold increase ($P < 0.001$) in IL-1 β secretion was seen at 100 ng/mL native Hsp72 (2049.210 pg/mL) compared to denatured Hsp72 (601.144 pg/mL) (Figure 4.2A). A 4-fold increase ($P < 0.001$) was also observed at 1000 ng/mL native Hsp72 when compared to denatured Hsp72 (405.545 and 7.184 pg/mL respectively) (Figure 4.2A). IL-6 secretion increased almost 2-fold when incubated with 10 ng/mL native Hsp72 (31.352 pg/mL) compared to denatured Hsp72 (17.101 pg/mL) although this was not significant (Figure 4.3A). IL-6 secretion increased more than 3-fold ($P < 0.001$) when incubated with native Hsp72 compared to denatured Hsp72 at 100 ng/mL (2874.279 and 798.760 pg/mL respectively), and 3-fold ($P < 0.001$) when incubated with 1000 ng/mL (4370.564 and 1915.391 pg/mL respectively) (Figure 4.3A). TNF- α secretion increased 2-fold ($P < 0.05$) when PBMCs were incubated with 10 ng/mL native Hsp72 compared with denatured Hsp72 (97.288 and 39.732 pg/mL respectively) (Figure 4.4A). TNF- α increased over 2-fold ($P < 0.001$) when incubated with 100 ng/mL native Hsp72 (781.203 pg/mL) compared to denatured Hsp72 (362.789 pg/mL), and also increased nearly 3-fold ($P < 0.001$) when incubated with 1000 ng/mL native compared to denatured Hsp72 (2298.341 and 816.756 pg/mL respectively) (Figure 4.4A). IL-10 secretion increased over 40-fold ($P < 0.001$) when PBMCs were incubated with 100 ng/mL native Hsp72 (136.370 pg/mL) compared to denatured Hsp72 (3.024 pg/mL), and increased nearly 10-fold ($P < 0.001$) when incubated with 1000 ng/mL native Hsp72 compared to the denatured form (558.350 and 59.007 pg/mL respectively) (Figure 4.5A).

All cytokine responses were dose dependent at 1000 and 100 ng/mL native Hsp72 ($P < 0.01$ – 0.001), but only TNF- α was dose dependent at 10 ng/mL ($P < 0.05$) when compared to untreated control cells (Table 4.1). A significant correlation was seen between TNF- α and all other cytokines ($r^2 = 0.941$ – 0.996, $P < 0.01$) and between IL-1 β and IL-10 ($r^2 = 0.999$, $P < 0.01$) (Table 4.2).

Cytokine responses were dose dependent when treated with denatured 1000 ng/mL Hsp72 ($P < 0.001$), but only IL-6 and TNF- α were dose dependent when incubated with 100 ng/mL ($P < 0.001$). No significant difference was observed with 10 ng/mL treatment (Table 4.1). Significant correlations were seen between IL-1 β

and IL-10 ($r^2 = 1.000$, $P < 0.01$), and between IL-6 and TNF- α ($r^2 = 1.000$, $P < 0.01$) (Table 4.2).

When incubated with 10 ng/mL native DnaK, IL-1 β secretion increased over 20-fold ($P < 0.001$) compared to denatured DnaK (164.730 and 7.435 pg/mL respectively) (Figure 4.2B). A 15-fold increase ($P < 0.001$) was observed with 100 ng/mL native compared to denatured DnaK (1012.419 and 69.688 pg/mL respectively), and an almost 3-fold increase ($P < 0.001$) was seen when incubated with 1000 ng/mL native DnaK (2247.721 pg/mL) compared to denatured DnaK (891.625 pg/mL) (Figure 4.2B). IL-6 secretion increased nearly 40-fold ($P < 0.001$) when incubated with 10 ng/mL native DnaK compared to denatured DnaK (1066.259 and 28.298 pg/mL respectively); nearly 5-fold ($P < 0.01$) with 100 ng/mL native (2404.094 pg/mL) compared to denatured (522.136 pg/mL), but were not significantly different when incubated with 1000 ng/mL native DnaK compared to the denatured form (4617.849 and 4298.035 pg/mL) (Figure 4.3B). TNF- α secretion increased over 2-fold ($P < 0.01$) when incubated with 10 ng/mL native DnaK compared to denatured DnaK (584.657 and 263.869 pg/mL respectively) (Figure 4.4B). A 2-fold increase ($P < 0.001$) in TNF- α was observed when incubated with 100 ng/mL native compared to denatured DnaK (1036.363 and 347.935 pg/mL respectively), and there was an increase ($P < 0.001$) incubated with 1000 ng/mL native or denatured DnaK (6584.202 and 5925.299 pg/mL respectively) (Figure 4.4B). IL-10 increased 8-fold ($P < 0.05$) following incubation with 10 ng/mL native DnaK compared to denatured DnaK (42.755 and 7.345 pg/mL respectively); a 34-fold increase ($P < 0.001$) was observed following incubation with 100 ng/mL native (442.932 pg/mL) compared to the denatured form (13.996 pg/mL); a 2-fold increase ($P < 0.001$) in IL-10 secretion was seen when incubated with 1000 ng/mL native DnaK compared to denatured DnaK (750.417 and 336.107 pg/mL respectively) (Figure 4.5B).

All cytokine responses were dose dependent at all when incubated with native DnaK ($P < 0.05 - 0.001$) (Table 4.1). Significant correlations were observed between IL-1 β , IL-6 and IL-10 ($r^2 = 0.988$, $P < 0.01$) (Table 4.2).

Cytokine responses were dose dependent when treated with denatured 1000 ng/mL DnaK ($P < 0.001$), but only IL-6 and TNF- α were dose dependent when incubated with 100 ng/mL ($P < 0.01 - 0.001$). No significant differences were observed with 10 ng/mL treatment except for TNF- α ($P < 0.05$) (Table 4.1). Significant correlations were seen between all cytokines ($r^2 = 0.996 - 1.000$, $P < 0.01$) (Table 4.2).

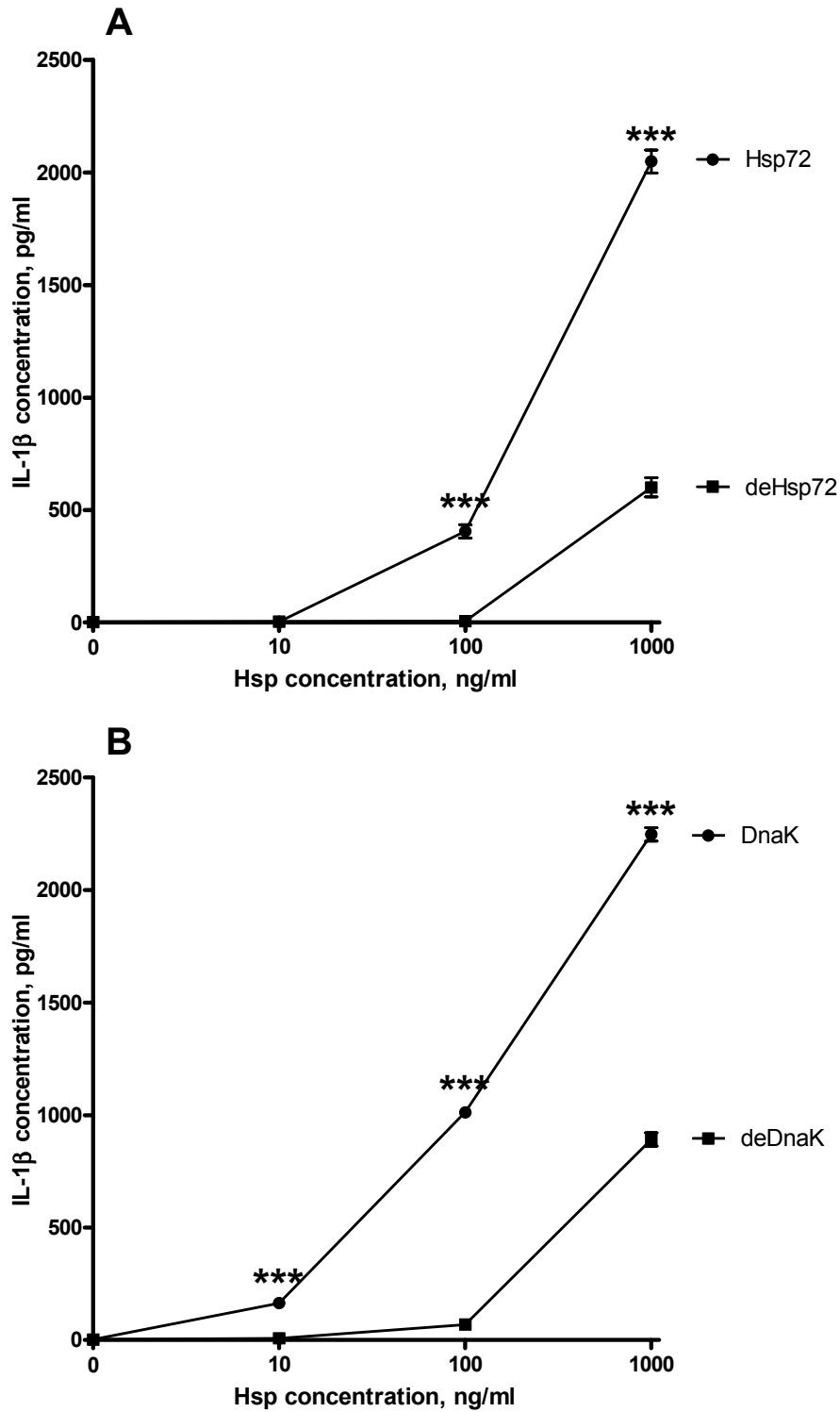


Figure 4.2: Effect on IL-1 β secretion by PBMCs when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

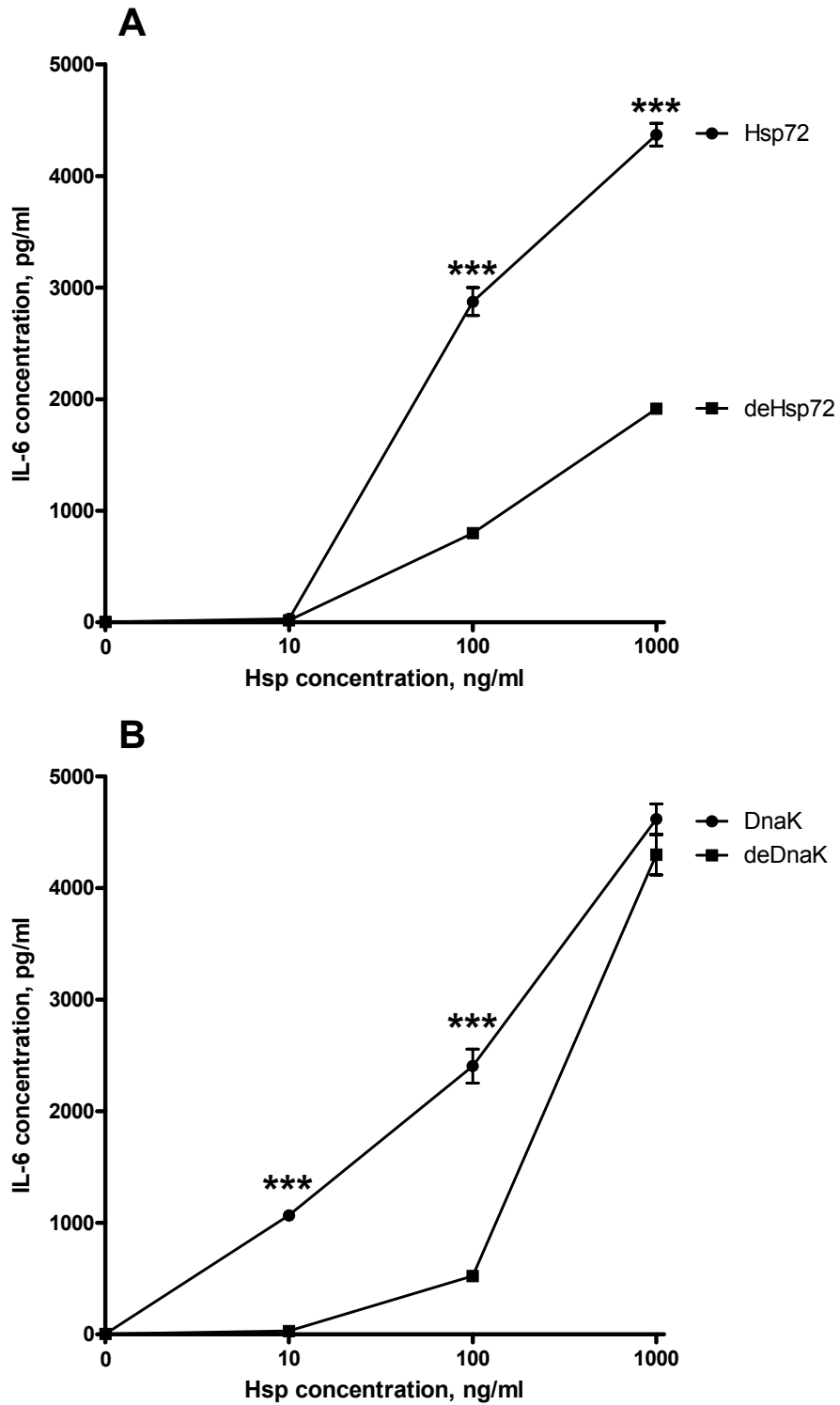


Figure 4.3: Effect on IL-6 secretion by PBMCs when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

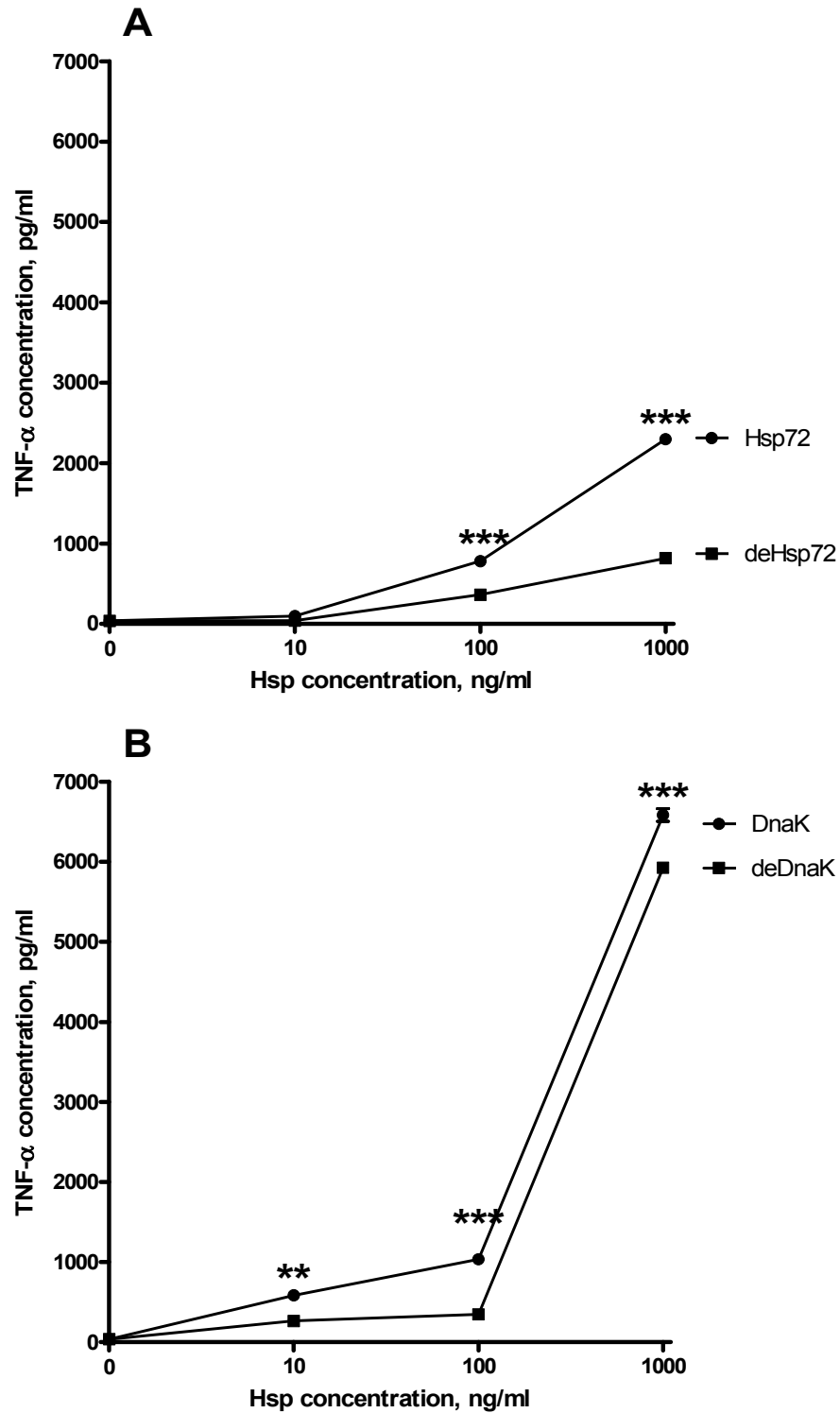


Figure 4.4: Effect on TNF- α secretion by PBMCs when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, $n = 4$. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

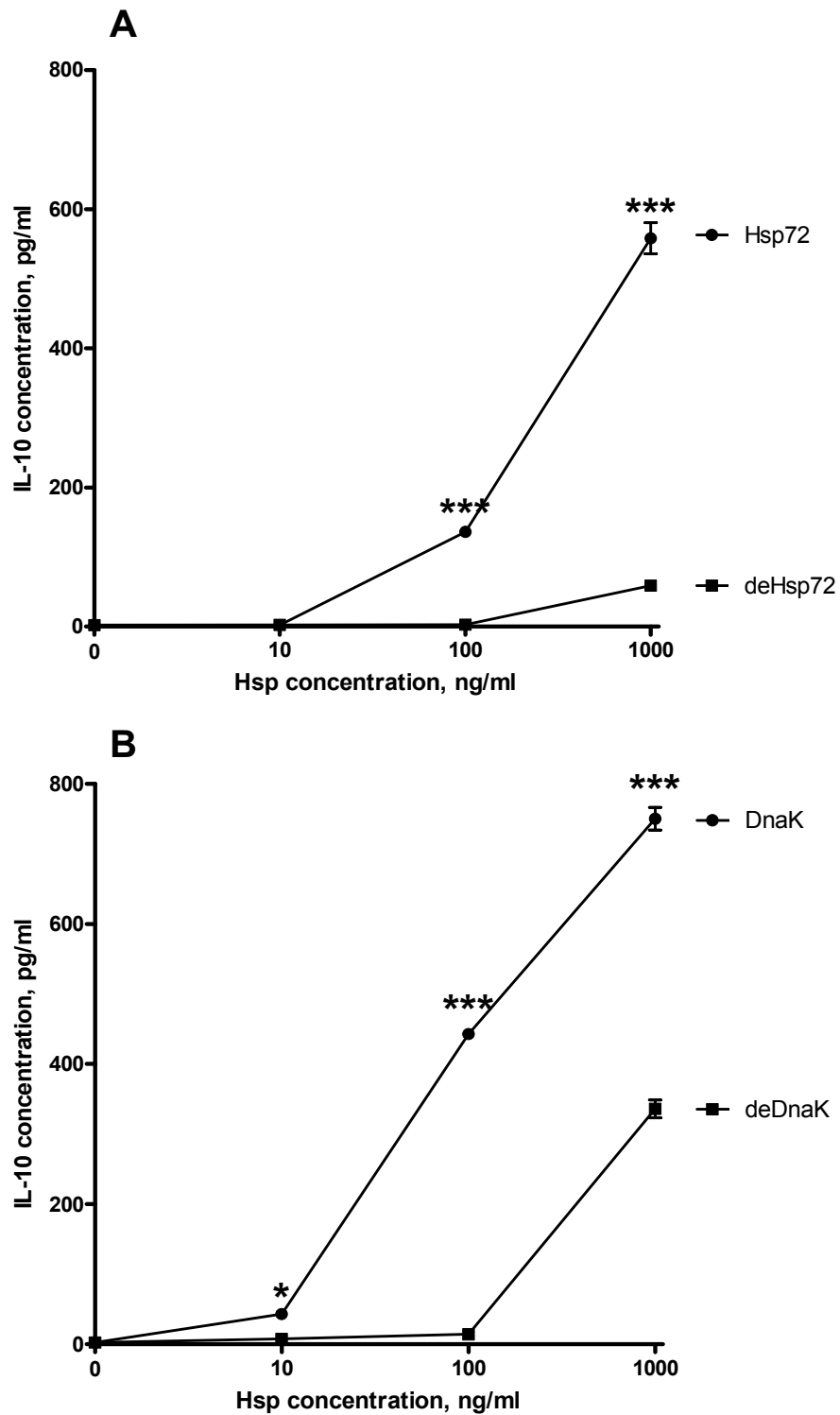


Figure 4.5: Effect on IL-10 secretion by PBMCs when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.1: Dose responses of cytokine secretion by PBMCs incubated for 24 h with native or denatured, Hsp72 or DnaK.

Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|---------------------|--------|------------|---------|--------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs. 100 | |
| Hsp72 | | | | | | |
| | Significance | | | | | |
| IL-1 β | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | * | P<0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deHsp72 | | | | | | |
| IL-1 β | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | ** | P<0.01 |
| DnaK | | | | | | |
| IL-1 β | * | P<0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-10 | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| deDnaK | | | | | | |
| IL-1 β | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| TNF- α | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | *** | P>0.001 |

Note: ns = not significant

Table 4.2: Correlation between cytokine secretion by PBMCs when incubated for 24 h with native or denatured, Hsp72 or DnaK.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Hsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.890 | 0.991** | 0.999** |
| IL-6 | 0.890 | | 0.941* | 0.910 |
| TNF-α | 0.991** | 0.941* | | 0.996** |
| IL-10 | 0.999** | 0.910 | 0.996** | |
| deHsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.910 | 0.910 | 1.000** |
| IL-6 | 0.910 | | 1.000** | 0.920 |
| TNF-α | 0.910 | 1.000** | | 0.910 |
| IL-10 | 1.000** | 0.920 | 0.910 | |
| DnaK | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.988** | 0.939 | 0.988** |
| IL-6 | 0.988** | | 0.920 | 0.978* |
| TNF-α | 0.939 | 0.920 | | 0.876 |
| IL-10 | 0.988** | 0.978* | 0.876 | |
| deDnaK | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.999** | 0.999** | 0.999** |
| IL-6 | 0.999** | | 0.996** | 0.996** |
| TNF-α | 0.999** | 0.996** | | 1.000** |
| IL-10 | 0.999** | 0.996** | 1.000** | |

4.3.2.2 Treatment of PBMCs with native or denatured Hsp60 or GroEL.

Incubation of PBMCs with 100 ng/mL native Hsp60 resulted in a 60-fold increase ($P < 0.001$) in IL-1 β secretion compared to incubation with denatured Hsp60 (188.399 and 3.158 pg/mL respectively) (Figure 4.6A). A 13-fold increase ($P < 0.001$) was also observed when incubated with 1000 ng/mL native Hsp60 (2007.328 pg/mL) compared to incubation with the denatured form (152.586 pg/mL) (Figure 4.6A). IL-6 secretion increased 70-fold ($P < 0.001$) when incubated with 100 ng/mL native Hsp60 compared to denatured Hsp60 (1488.644 and 21.173 pg/mL respectively), and increased 2-fold ($P < 0.001$) when incubated with 1000 ng/mL native (4069.630 pg/mL) compared to denatured Hsp60 (2014.229 pg/mL) (Figure 4.7A). Incubation with 100 ng/mL native Hsp60 resulted in a 10-fold increase in TNF- α ($P < 0.001$) in comparison to denatured Hsp60 (554.023 and 54.586 pg/mL respectively); TNF- α also increased nearly 3-fold ($P < 0.001$) when incubated with native Hsp60 compared to the denatured form (2525.781 and 975.481 pg/mL respectively) (Figure 4.8A). IL-10 secretion increased almost 6-fold ($P < 0.001$) following incubation with 100 ng/mL native Hsp60 (69.007 pg/mL) when compared to denatured Hsp60 (12.026 pg/mL), followed by a 44-fold increase when incubated with 1000 ng/mL Hsp60 compared to the denatured form (397.218 and 44.238 pg/mL respectively) (Figure 4.9A).

All cytokine responses were dose dependent in PBMCs that were incubated with 100 and 1000 ng/mL ($P < 0.001$) native Hsp60 but were not significant when incubated with 10 ng/mL compared to untreated cells. Significant correlations were observed amongst all cytokines ($r^2 = 0.960 - 0.999$, $P < 0.05 - 0.01$) (Table 4.4).

IL-1 β and TNF- α responses were only dose dependent when PBMCs were treated with 1000 ng/mL denatured Hsp60 ($P < 0.001$), compared to untreated cells. IL-6 and IL-10 secretion were also dose dependent at 100 ng/mL ($P < 0.05$). No significance in cytokine response was observed when PBMCs were incubated with 10 ng/mL denatured Hsp60 (Table 4.3). Significant correlations were observed amongst all cytokines ($r^2 = 0.973 - 1.000$, $P < 0.05 - 0.01$) (Table 4.4).

Following incubation of PBMCs with GroEL, IL-1 β secretion increased 2-fold when incubated with 10 ng/mL native GroEL compared to the denatured form (4.206 and 2.339 pg/mL respectively) although this was not significant; a 4-fold increase ($P < 0.001$) was observed when incubated with 100 ng/mL native compared to denatured (221.981 and 54.332 pg/mL respectively); and a nearly 4-fold increase ($P < 0.001$) was seen at 1000 ng/mL native GroEL compared to the denatured form (1839.356 and 542.800 pg/mL respectively) (Figure 4.6B). IL-6 secretion increased

2-fold following incubation with 10 ng/mL native GroEL (19.137 pg/mL) compared to denatured GroEL (8.449 pg/mL) although this was not significant; a 20-fold increase ($P < 0.001$) was observed when incubated with 100 ng/mL native compared to denatured form (2517.183 and 127.036 pg/mL respectively); an significant increase ($P < 0.001$) was also seen when incubated with 1000 ng/mL native GroEL compared to denatured (4770.412 and 4197.884 pg/mL respectively) (Figure 4.7B). TNF- α secretion from PBMCs increased 2-fold following incubation with 10 ng/mL native GroEL compared to the denatured form (74.081 and 36.948 pg/mL respectively) but this was not significant; a 3-fold increase ($P < 0.01$) was observed when incubated with 100 ng/mL native GroEL (541.955 pg/mL) compared to denatured GroEL (167.841 pg/mL); a significant increase ($P < 0.001$) was also seen when incubated with 1000 ng/mL native GroEL compared to denatured (2419.952 and 1967.859 pg/mL respectively) (Figure 4.8B). Secretion of IL-10 increased almost 2-fold ($P < 0.001$) when PBMCs were incubated with 100 ng/mL GroEL compared to denatured GroEL (75.183 and 39.655 pg/mL respectively), and increased over 2-fold ($P < 0.001$) following incubation with 1000 ng/mL native GroEL (578.758 pg/mL) compared to the denatured form (283.360 pg/mL) (Figure 4.9B). All cytokine responses were dose dependent ($P < 0.001$) when incubated with 100 or 1000 ng/mL native GroEL when compared to untreated PBMCs. No significant difference was seen when incubated with 10 ng/mL GroEL. Significant correlations were seen between TNF- α and all other cytokines ($r^2 = 0.944 - 0.996$, $P < 0.05 - 0.01$) and between IL-1 β and IL-10 ($r^2 = 1.000$, $P < 0.01$) (Table 4.4). A dose response was seen for IL-1 β secretion when incubated with denatured GroEL at 100 and 1000 ng/mL ($P < 0.001$) but not at 10 ng/mL. A dose response ($P < 0.001$) was only seen when incubated with 1000 ng/mL denatured GroEL for IL-6 and TNF- α secretion when compared to untreated PBMCs (Table 4.3). Significant correlations were seen between all cytokines ($r^2 = 0.995 - 1.000$, $P < 0.01$) (Table 4.4).

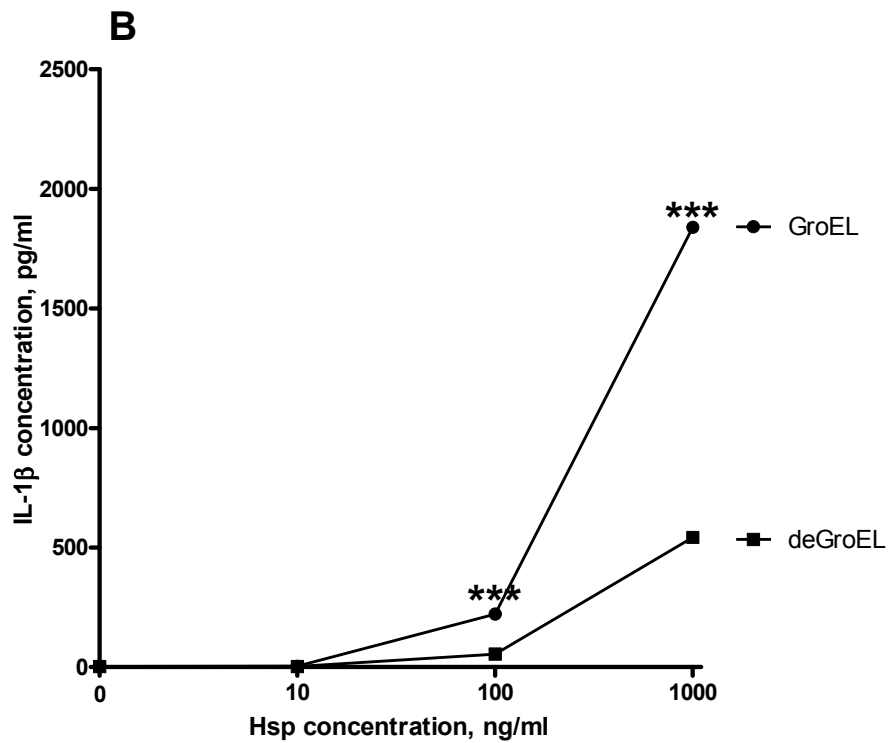
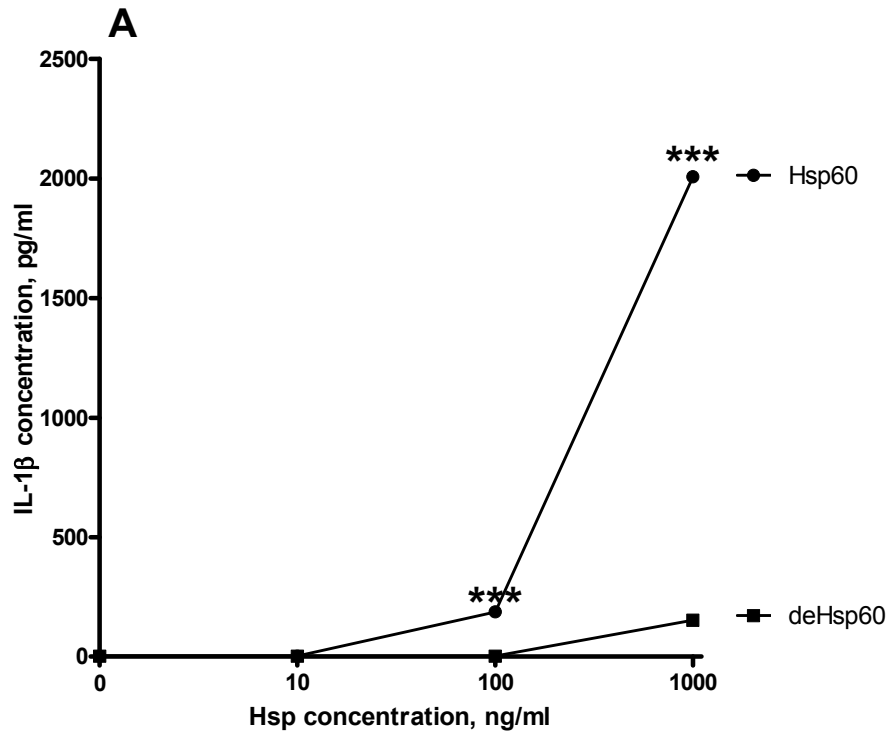


Figure 4.6: Effect on IL-1 β secretion by PBMCs when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

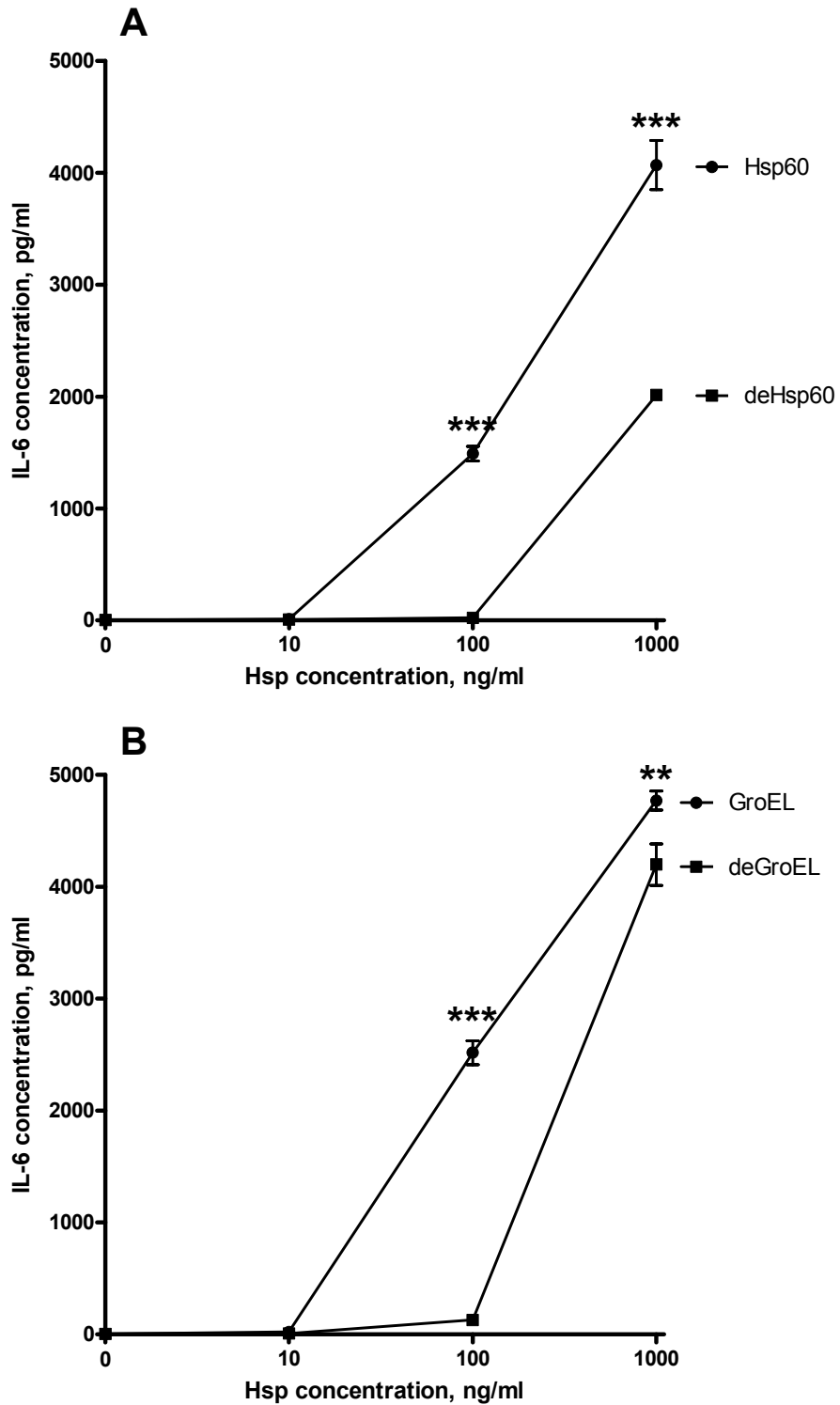


Figure 4.7: Effect on IL-6 secretion by PBMCs when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

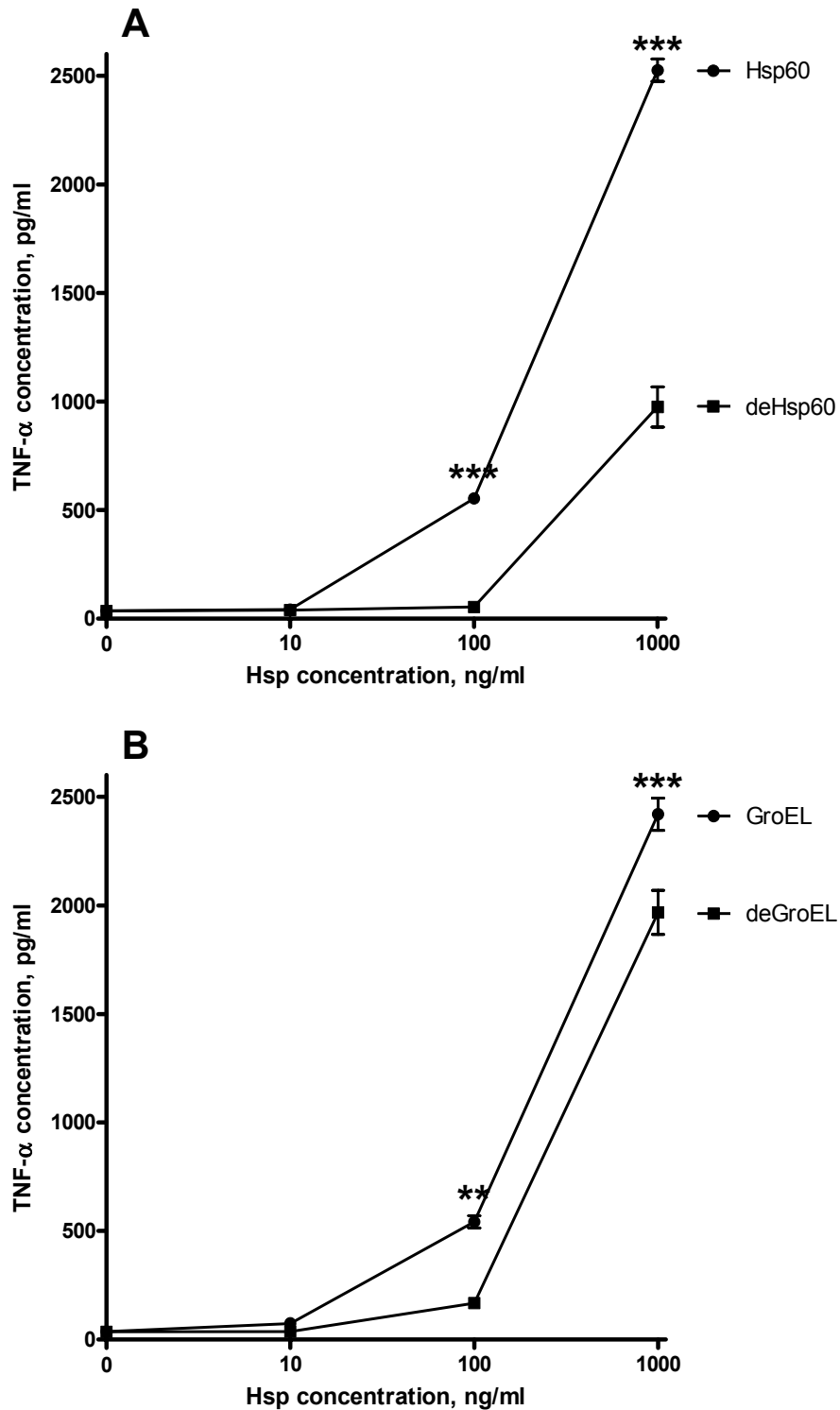


Figure 4.8: Effect on TNF- α secretion by PBMCs when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

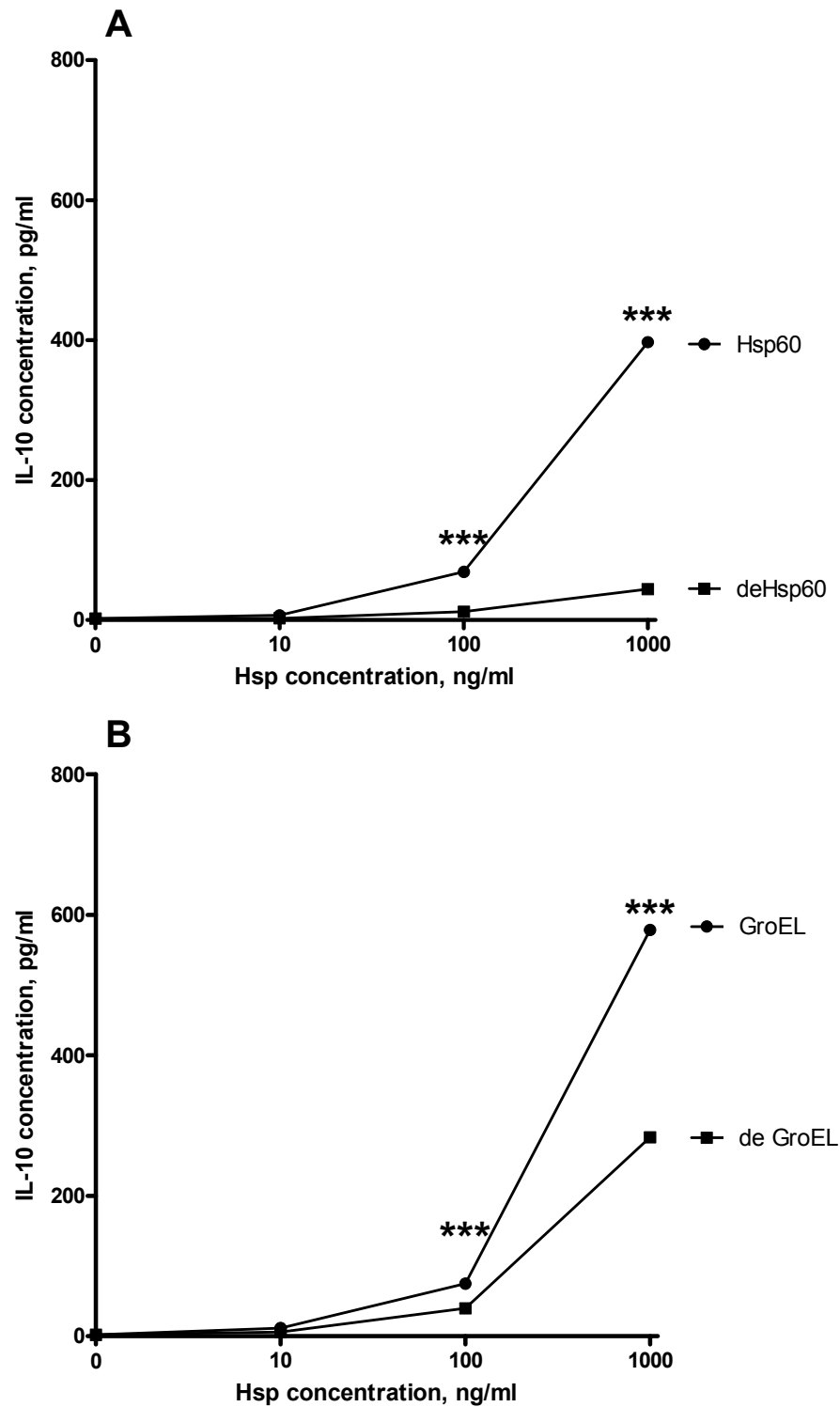


Figure 4.9: Effect on IL-10 secretion by PBMCs when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.3: Dose responses of cytokine secretion by PBMCs incubated for 24 h with native or denatured, Hsp60 or GroEL.

Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|---------------------|--------|------------|---------|--------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs. 100 | |
| Hsp60 | | | | | | |
| | Significance | | | | | |
| IL-1 β | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deHsp60 | | | | | | |
| IL-1 β | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| GroEL | | | | | | |
| IL-1 β | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-10 | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| deGroEL | | | | | | |
| IL-1 β | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |

Note: ns = not significant

Table 4.4: Correlation between cytokine secretion by PBMCs when incubated for 24 h with native or denatured, Hsp60 or GroEL.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Hsp60 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.960* | 0.993** | 0.997** |
| IL-6 | 0.960* | | 0.986* | 0.978* |
| TNF-α | 0.993** | 0.986* | | 0.999** |
| IL-10 | 0.997** | 0.978* | 0.999** | |
| deHsp60 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 1.000** | 1.000** | 0.973* |
| IL-6 | 1.000** | | 1.000** | 0.974* |
| TNF-α | 1.000** | 1.000** | | 0.976* |
| IL-10 | 0.973* | 0.974* | 0.976* | |
| GroEL | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.911 | 0.996** | 1.000** |
| IL-6 | 0.911 | | 0.944* | 0.911 |
| TNF-α | 0.996** | 0.944* | | 0.996** |
| IL-10 | 1.000** | 0.911 | 0.996** | |
| deGroEL | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.998** | 1.000** | 0.999** |
| IL-6 | 0.998** | | 0.999** | 0.995** |
| TNF-α | 1.000** | 0.999** | | 0.998** |
| IL-10 | 0.999** | 0.995** | 0.998** | |

4.3.2.3 Treatment of PBMCs with native or denatured Cpn10 or GroES.

Following incubation of PBMCs with Cpn10 for 24 h, there were no significant differences in IL-1 β secretion at any concentration of native Cpn10 compared to denatured Cpn10 (Figure 4.10A). IL-6 secretion increased when incubated with 100 ng/mL native Cpn10 compared to the denatured form (9.467 and 6.922 pg/mL respectively) although this was not significant; a 2-fold increase ($P < 0.001$) in IL-6 secretion was observed when incubated with 1000 ng/mL native Cpn10 (19.101 pg/mL) compared to denatured Cpn10 (10.485 pg/mL) (Figure 4.11A). There were no significant differences in TNF- α secretion following incubation with native Cpn10 compared to denatured Cpn10 at any concentration (Figure 4.12A). IL-10 secretion increased almost 4-fold ($P < 0.001$) following 100 ng/mL treatment with native Cpn10 compared denatured Cpn10 (9.810 and 2.715 pg/mL respectively), and increased almost 4-fold ($P < 0.001$) following incubation with 1000 ng/mL Cpn10 compared to the denatured form (11.323 and 3.266 pg/mL respectively) (Figure 4.13A).

IL-1 β , IL-6 and IL-10 secretion from cells treated with native Cpn10 was dose dependent at 100 and 1000 ng/mL when compared to untreated cells ($P < 0.05$ - $P < 0.001$). IL-1 β , IL-6 and IL-10 secretion from cells treated with denatured Cpn10 was dose dependent at 1000 ng/mL ($P < 0.05$ - $P < 0.001$). IL-1 β secretion was also significant at 100 ng/mL denatured Cpn10 ($P < 0.01$). There were no significant differences observed for TNF- α secretion at any dose for either native or denatured Cpn10 (Table 4.5). There was a significant correlation between IL-1 β and IL-10 secretion when incubated with native Cpn10 ($r^2 = 0.961$, $P < 0.05$), and denatured Cpn10 ($r^2 = 0.995$, $P < 0.01$) (Table 4.6).

PBMCs incubated with 100 ng/mL native GroES showed a significant increase ($P < 0.05$) in IL-1 β secretion when compared to denatured GroES (4.961 and 3.535 pg/mL respectively; a 3-fold increase ($P < 0.001$) was seen at 1000 ng/mL native GroES compared to denatured GroES (12.910 and 4.165 pg/mL respectively) (Figure 4.10B). IL-6 secretion increased almost 3-fold ($P < 0.001$) when incubated with 10 ng/mL native GroES (19.137 pg/mL) compared to the denatured form (7.665 pg/mL); a 2-fold increase ($P < 0.001$) was observed at 100 ng/mL native compared to denatured GroES (22.191 and 11.502 pg/mL respectively), and also at 1000 ng/mL native compared to denatured GroES (25.244 and 13.476 pg/mL respectively) ($P < 0.001$) (Figure 4.11B). TNF- α increased significantly ($P < 0.05$) when incubated with 10 ng/mL native GroES (60.156 pg/mL) compared to the denatured form (42.518 pg/mL); secretion increased at 100 ng/mL native GroES

when compared to denatured GroES (63.868 and 49.015 pg/mL respectively) although this was not significant; TNF- α increased significantly ($P < 0.05$) when incubated with 1000 ng/mL native GroES (80.723 pg/mL) compared to the denatured form (60.156 pg/mL) (Figure 4.12B). An almost 2-fold increase ($P < 0.01$) in IL-10 secretion was observed following treatment with 10 ng/mL native GroES (2.474 pg/mL) compared to denatured GroES (1.921 pg/mL); 100 ng/mL native compared to the denatured form (4.290 and 2.711 pg/mL respectively, $P < 0.001$); and with 1000 ng/mL native GroES compared to denatured GroES (7.846 and 4.220 pg/mL respectively, $P < 0.001$) (Figure 4.13B).

Cytokine secretion was dose dependent ($P < 0.05$ - $P < 0.001$) when cells were treated with native GroES compared to untreated cells, except for IL-1 β and IL-10 secretion at 10 ng/mL which were not significant (Table 4.5). Significant correlations were seen between IL-1 β and IL-10 following incubation with native GroES ($r^2 = 0.983$, $P < 0.05$), and IL-6 and TNF- α ($r^2 = 0.960$, $P < 0.05$) (Table 4.6).

When treated with denatured GroES, secretion of all cytokines were dose dependent at 1000 ng/mL ($P < 0.05$ - $P < 0.001$). IL-6 and IL-10 were also dose dependent ($P < 0.05$ - $P < 0.01$) when treated with 100 ng/mL denatured GroES (Table 4.5). Significant correlations were seen between IL-1 β , TNF- α and IL-10 following incubation with denatured GroES ($r^2 = 0.957 - 0.074$, $P < 0.05$), and IL-6 and TNF- α ($r^2 = 0.961$, $P < 0.05$) (Table 4.6).

4.3.2.4 Cell viability and cell death in PBMCs following treatment with native and denatured heat shock proteins.

There was no significant change in cell number or viability (0.91×10^6 cells/mL) when compared to seeding density (1×10^6 cells/mL) although cell counts were reduced. No significant cell death by apoptosis or necrosis was detected for any treatment when compared to untreated cells, and all were significantly different from the positive controls for either apoptosis or necrosis (Table 4.7). A decrease in cell number was observed when treated with either native or denatured DnaK, and GroEL, as well as a small increase in apoptosis and necrosis, although these were not significantly different from untreated cells (Table 4.7).

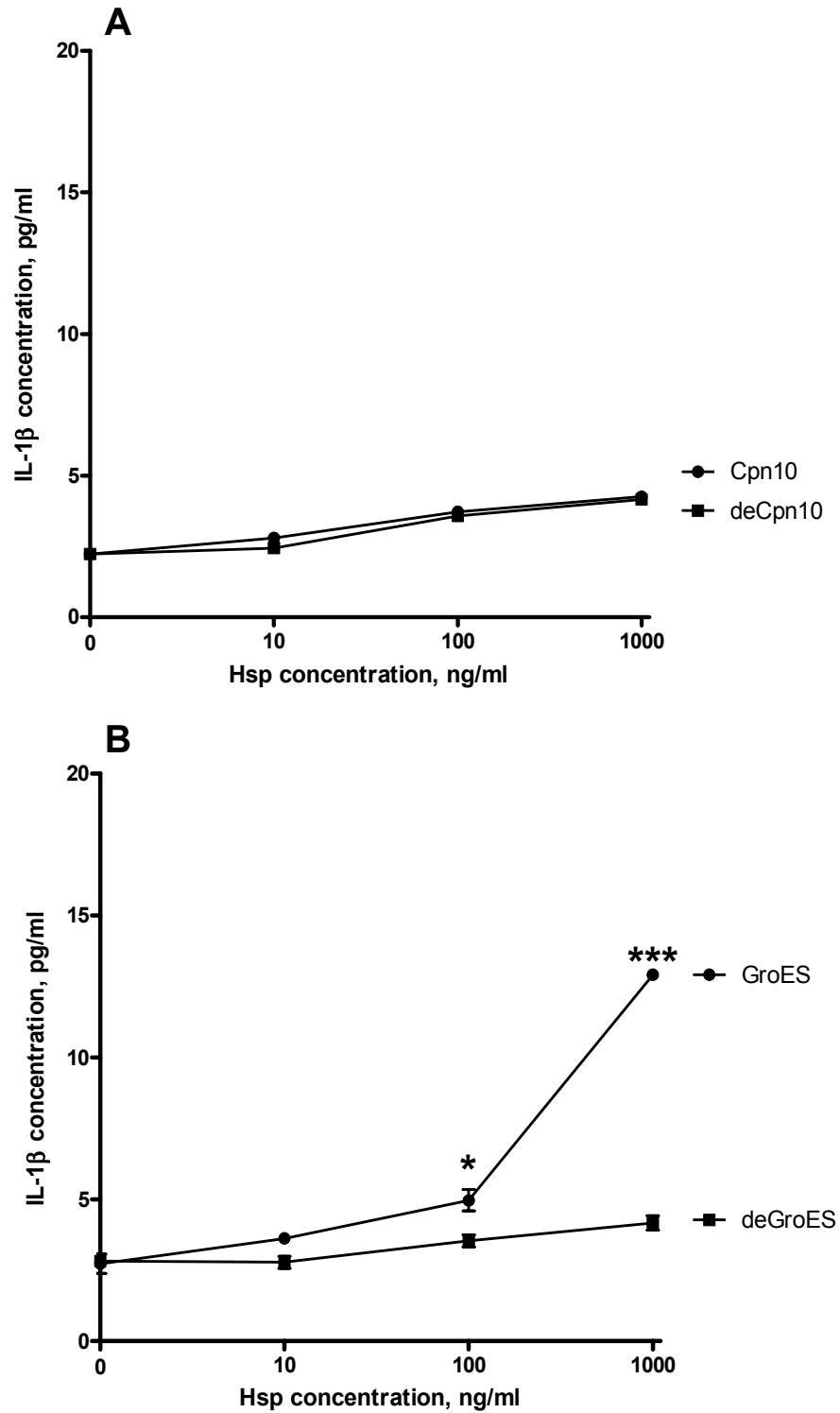


Figure 4.10: Effect on IL-1 β secretion by transformed PBMCs when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

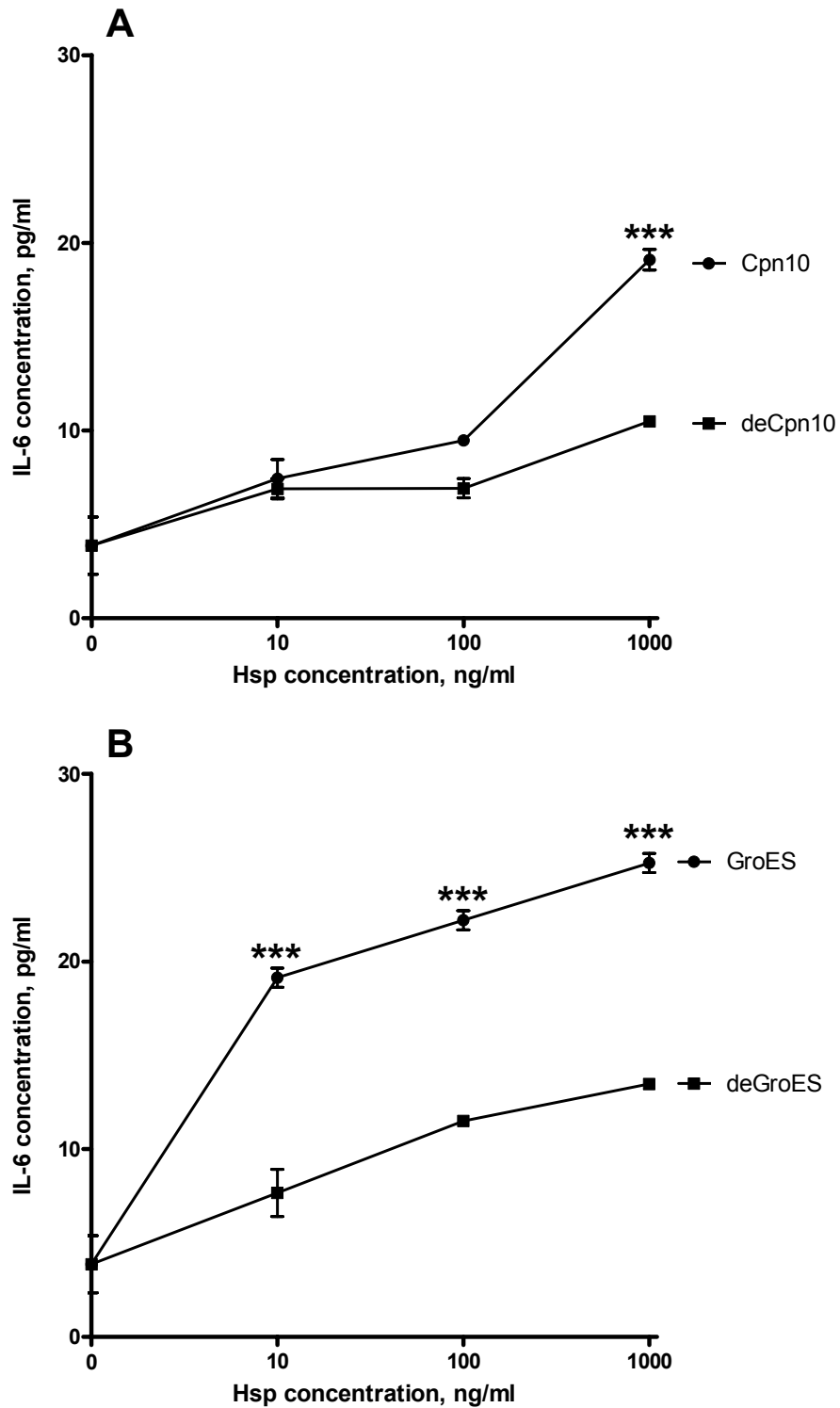


Figure 4.11: Effect on IL-6 secretion by PBMCs when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

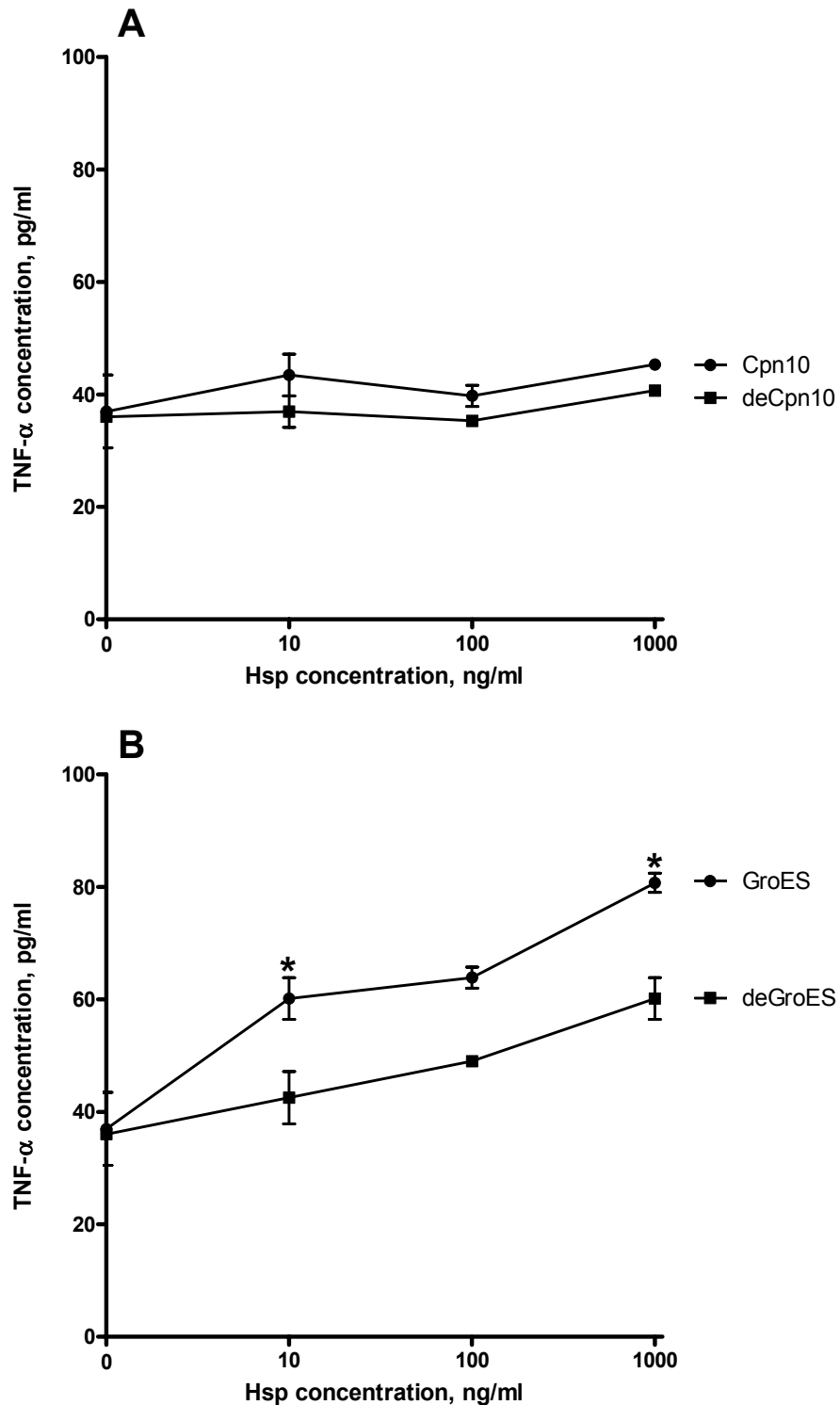


Figure 4.12: Effect on TNF- α secretion by PBMCs when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

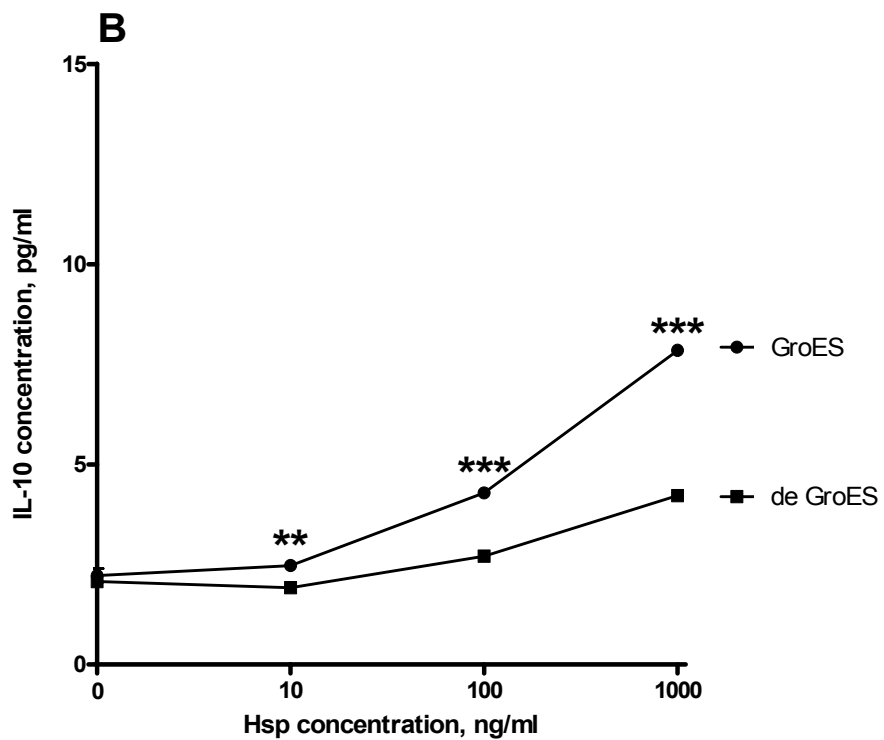
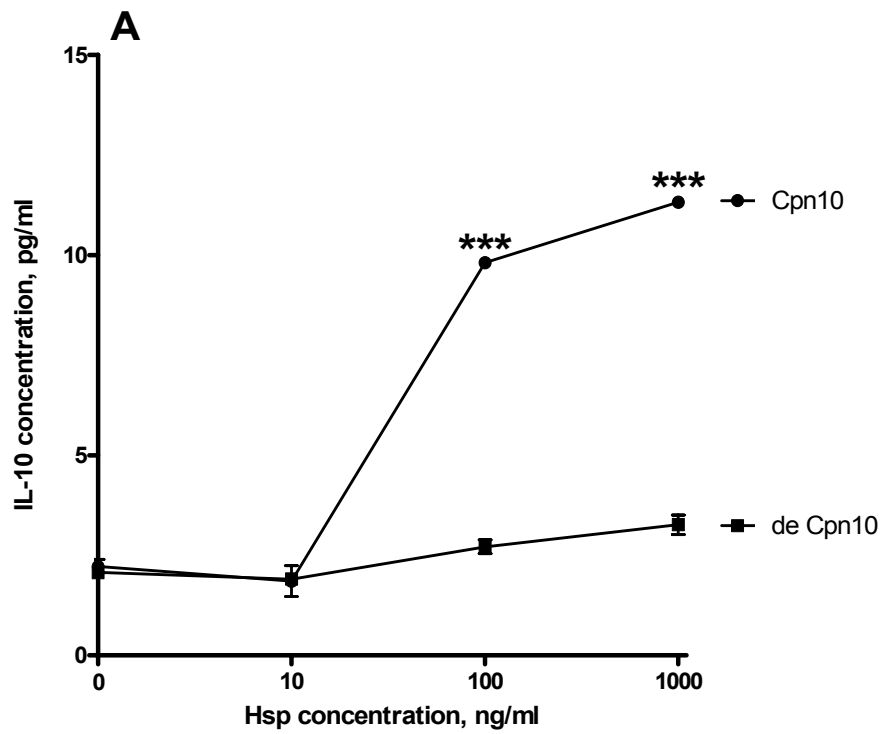


Figure 4.13: Effect on IL-10 secretion by PBMCs when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms.

Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.5: Dose responses of cytokine secretion by PBMCs incubated for 24 h with native or denatured, Cpn10 or GroES.

Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|---------------------|---------|------------|---------|-------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs.100 | |
| Cpn10 | | | | | | |
| | Significance | | | | | |
| IL-1 β | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | * | P<0.05 | ** | P<0.01 |
| TNF- α | ns | P>0.05 | ns | P>0.05 | ns | P>0.05 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deCpn10 | | | | | | |
| IL-1 β | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | ns | P>0.05 | * | P<0.05 |
| TNF- α | ns | P>0.05 | ns | P>0.05 | ns | P>0.05 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | ** | P<0.01 |
| GroES | | | | | | |
| IL-1 β | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | * | P<0.05 | * | P<0.05 | ** | P<0.01 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deGroES | | | | | | |
| IL-1 β | ns | P>0.05 | ns | P>0.05 | * | P<0.05 |
| IL-6 | ns | P>0.05 | * | P<0.05 | ** | P<0.01 |
| TNF- α | ns | P>0.05 | ns | P>0.05 | * | P<0.05 |
| IL-10 | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |

Note: ns = not significant

Table 4.6: Correlation between cytokine secretion by PBMCs when incubated for 24 h with native or denatured, Cpn10 or GroES.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Cpn10 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.942 | 0.644 | 0.961* |
| IL-6 | 0.942 | | 0.799 | 0.827 |
| TNF-α | 0.644 | 0.799 | | 0.409 |
| IL-10 | 0.961* | 0.827 | 0.409 | |
| deCpn10 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.735 | 0.592 | 0.995** |
| IL-6 | 0.735 | | 0.823 | 0.801 |
| TNF-α | 0.592 | 0.823 | | 0.650 |
| IL-10 | 0.995** | 0.801 | 0.650 | |
| GroES | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.673 | 0.849 | 0.983* |
| IL-6 | 0.673 | | 0.960* | 0.714 |
| TNF-α | 0.849 | 0.960* | | 0.863 |
| IL-10 | 0.983* | 0.714 | 0.863 | |
| deGroES | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.904 | 0.957* | 0.974* |
| IL-6 | 0.904 | | 0.961* | 0.826 |
| TNF-α | 0.957* | 0.961* | | 0.938 |
| IL-10 | 0.974* | 0.826 | 0.938 | |

Table 4.7: Cell viability, caspase-3 and necrosis measurements of PBMCs following 24 h incubation with native or denatured heat shock proteins.

| Treatment | Viable cell count ($\times 10^6$) (Trypan blue exclusion) | | | Apoptosis (Caspase-3, RFU) | | | Necrosis (Propidium iodide, RFU) | | |
|---|---|------------|-----------|----------------------------|------------|-----------|----------------------------------|------------|-----------|
| | 1000 | 100 | 10 | 1000 | 100 | 10 | 1000 | 100 | 10 |
| No Treatment | 0.91 | | | 252 | | | 97 | | |
| Positive Control | - | | | 2884 | | | 761 | | |
| Concentration of treatment (ng/mL) | 1000 | 100 | 10 | 1000 | 100 | 10 | 1000 | 100 | 10 |
| Hsp72 | 0.88 | 0.88 | 0.89 | 255 | 252 | 253 | 102 | 99 | 99 |
| Denatured Hsp72 | 0.89 | 0.90 | 0.91 | 254 | 251 | 252 | 98 | 98 | 98 |
| DnaK | 0.82 | 0.84 | 0.85 | 257 | 257 | 256 | 111 | 105 | 102 |
| Denatured DnaK | 0.84 | 0.85 | 0.84 | 255 | 256 | 254 | 107 | 105 | 103 |
| Hsp60 | 0.87 | 0.87 | 0.89 | 251 | 253 | 251 | 101 | 100 | 97 |
| Denatured Hsp60 | 0.88 | 0.89 | 0.90 | 253 | 252 | 254 | 98 | 96 | 98 |
| GroEL | 0.84 | 0.85 | 0.87 | 255 | 255 | 256 | 103 | 103 | 99 |
| Denatured GroEL | 0.84 | 0.86 | 0.88 | 256 | 253 | 253 | 103 | 101 | 98 |
| Cpn10 | 0.89 | 0.90 | 0.90 | 254 | 252 | 250 | 98 | 98 | 97 |
| Denatured Cpn10 | 0.90 | 0.91 | 0.91 | 253 | 252 | 253 | 99 | 98 | 98 |
| GroES | 0.90 | 0.91 | 0.90 | 253 | 254 | 254 | 96 | 97 | 97 |
| Denatured GroES | 0.89 | 0.90 | 0.90 | 254 | 253 | 251 | 97 | 98 | 98 |

Note: n = 4. Caspase-3 positive control = 2 μ m camptothecin; necrosis positive control = autoclaved (see section 2.3.32). Seeding density = 1×10^6 cells/mL.

4.3.3 Cytokine secretion from U937 macrophages following 24 h incubation with native or denatured stress proteins.

Cytokine secretion from U937 macrophages following treatments with stress proteins are presented in Figures 4.14 – 4.25 and Tables 4.8 – 4.14.

4.3.3.1 Treatment of U937 macrophages with native or denatured, Hsp72 or DnaK.

Following incubation of U937 macrophages with Hsp72, a 2-fold increase in IL-1 β secretion was seen with 100 ng/mL native Hsp72 (8.090 pg/mL) compared to denatured Hsp72 (4.906 pg/mL), although this was not significant. A 12-fold increase ($P < 0.001$) was seen at 1000 ng/mL native Hsp72 (76.850 pg/mL) compared with denatured Hsp72 (6.035 pg/mL) (Figure 4.14A). IL-6 levels increased 30-fold ($P < 0.001$) after 24 h when incubated with 1000 ng/mL native Hsp72 (69.062 pg/mL) compared to denatured Hsp72 (2.154 ng/mL) (Figure 4.15A). Secretion of TNF- α increased 4-fold following incubation with 10 ng/mL Hsp72 (6.455 pg/mL) compared to denatured Hsp72 (28.988 pg/mL). TNF- α levels also increased 4-fold ($P < 0.001$) when incubated with 100 ng/mL native Hsp72 compared to the denatured form (45.428 and 11.939 pg/mL respectively), and increased over 6-fold ($P < 0.001$) following treatment with 1000 ng/mL native Hsp72 (338.369 pg/mL) when compared to denatured Hsp72 (52.817 pg/mL) (Figure 4.16A). IL-10 increased 200-fold ($P < 0.001$) when incubated with native Hsp72 compared to denatured Hsp72 (129.098 and 0.640 pg/mL respectively), and increased 15-fold ($P < 0.001$) following incubation with 1000 ng/mL native (301.906 pg/mL) compared to denatured Hsp72 (21.774 pg/mL) (Figure 4.17A).

Cytokine secretion from cells treated with native Hsp72 was dose dependent at 10, 100 and 1000 ng/mL when compared to untreated cells ($P < 0.05 - 0.001$), except for IL-6 and IL-10 at 10 ng/mL which were not significant (Table 4.8). Significant correlation was seen between all cytokines ($r^2 = 0.995 - 1.000$, $P < 0.01$) except for IL-10 (Table 4.9).

No significant differences were seen in relation to dose for denatured Hsp72 except for TNF- α secretion at 100 and 1000 ng/mL ($P < 0.05$ and $P < 0.01$ respectively) (Table 4.8). There was no significant correlation between any cytokine when incubated with denatured Hsp72 (Table 4.9).

IL-1 β increased 6-fold ($P < 0.001$) following incubation with 100 ng/mL native DnaK compared to denatured DnaK (31.778 and 7.540 pg/mL respectively), and 4-fold ($P < 0.001$) at 1000 ng/mL (218.973 and 57.395 pg/mL respectively) (Figure 4.14B).

IL-6 secretion increased significantly ($P < 0.001$) following incubation with 1000 ng/mL native DnaK compared to denatured DnaK (157.922 and 143.424 pg/mL respectively) (Figure 4.15B). A significant increase ($P < 0.01$) in TNF- α secretion from cells was observed when treated 1000 ng/mL native DnaK compared to denatured DnaK (593.236 and 539.368 pg/mL respectively) (Figure 4.16B). IL-10 secretion increased 38-fold ($P < 0.001$) following incubation with 10 ng/mL native DnaK compared to denatured DnaK (38.998 and 1.179 pg/mL respectively); a 25-fold increase ($P < 0.001$) was observed when incubated with 100 ng/mL native DnaK compared to denatured form (270.009 and 10.522 pg/mL respectively); and increased 2-fold ($P < 0.001$) following incubation with 1000 ng/mL native DnaK (436.974 pg/mL) compared to denatured DnaK (277.607 pg/mL) (Figure 4.17B).

Cytokine secretion from cells treated with native or denatured DnaK was dose dependent at 10, 100 and 1000 ng/mL ($P < 0.05$ - $P < 0.001$) when compared to untreated cells, except for IL-6 secretion when treated with 10 ng/mL denatured DnaK which was not significant (Table 4.8).

Correlation was observed between TNF- α and all other cytokines ($r^2 = 0.957 - 0.979$, $P < 0.05$) when treated with native DnaK, and between IL-1 β and IL-6 ($r^2 = 0.999$, $P < 0.01$) (Table 4.9).

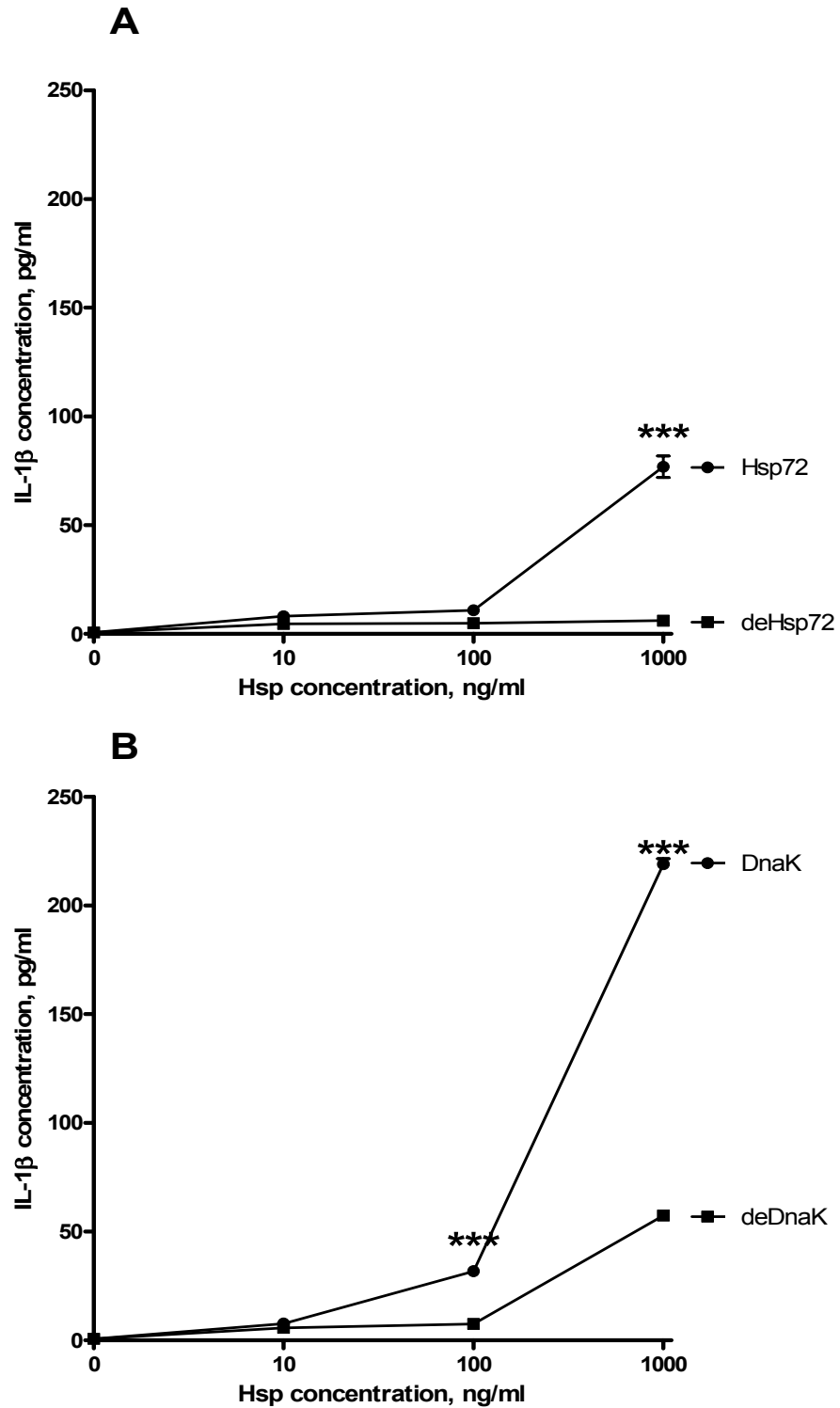


Figure 4.14: Effect on IL-1 β secretion by U937 macrophages when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

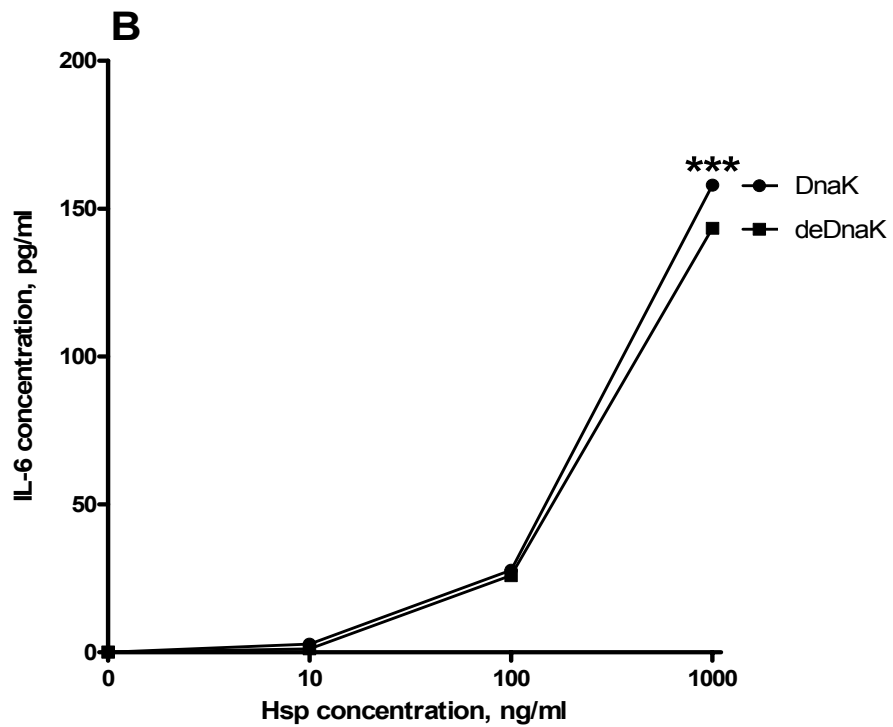
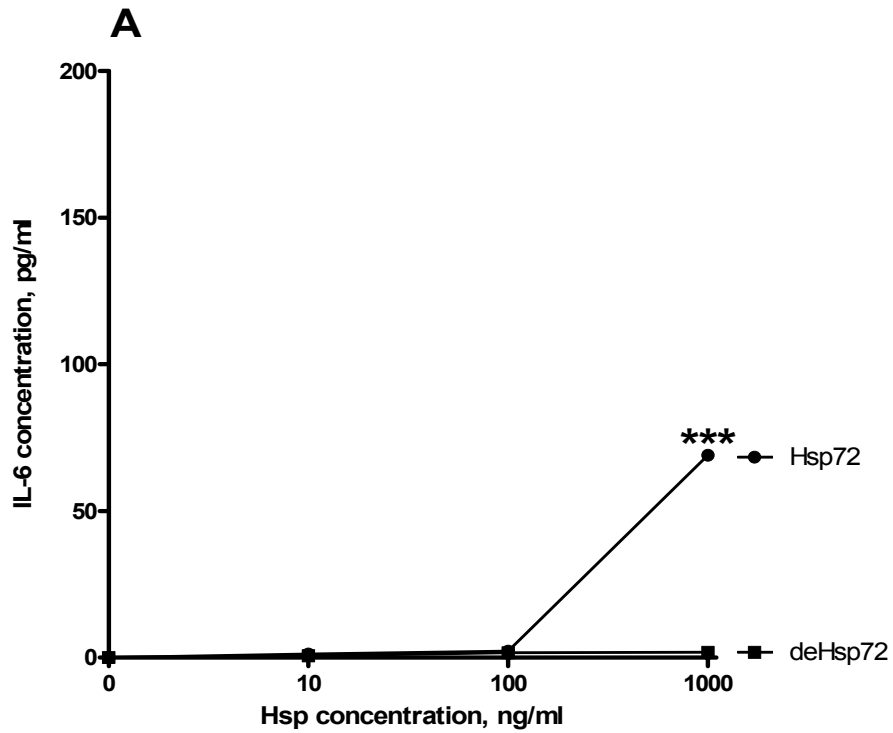


Figure 4.15: Effect on IL-6 secretion by U937 macrophages when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

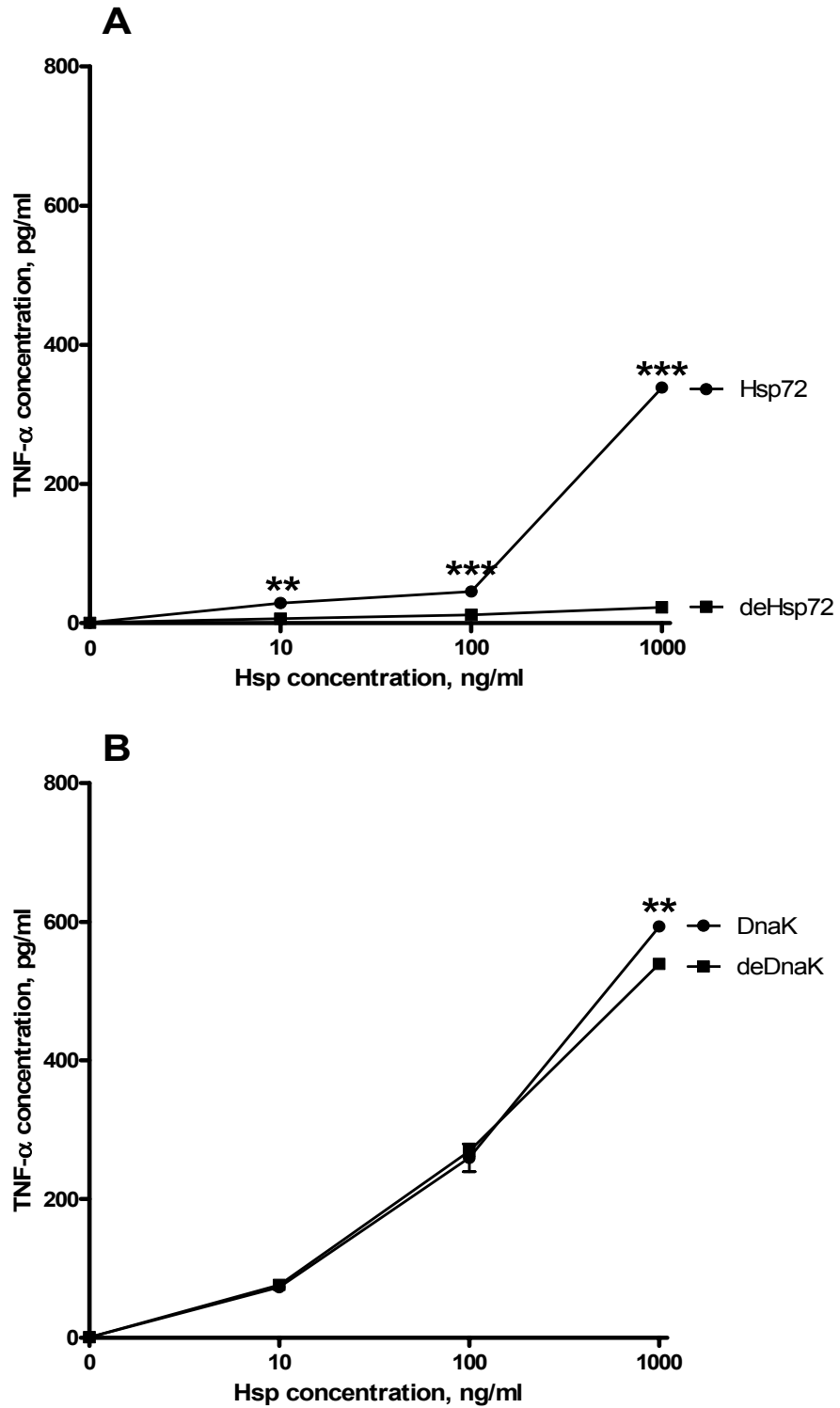


Figure 4.16: Effect on TNF- α secretion by U937 macrophages when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

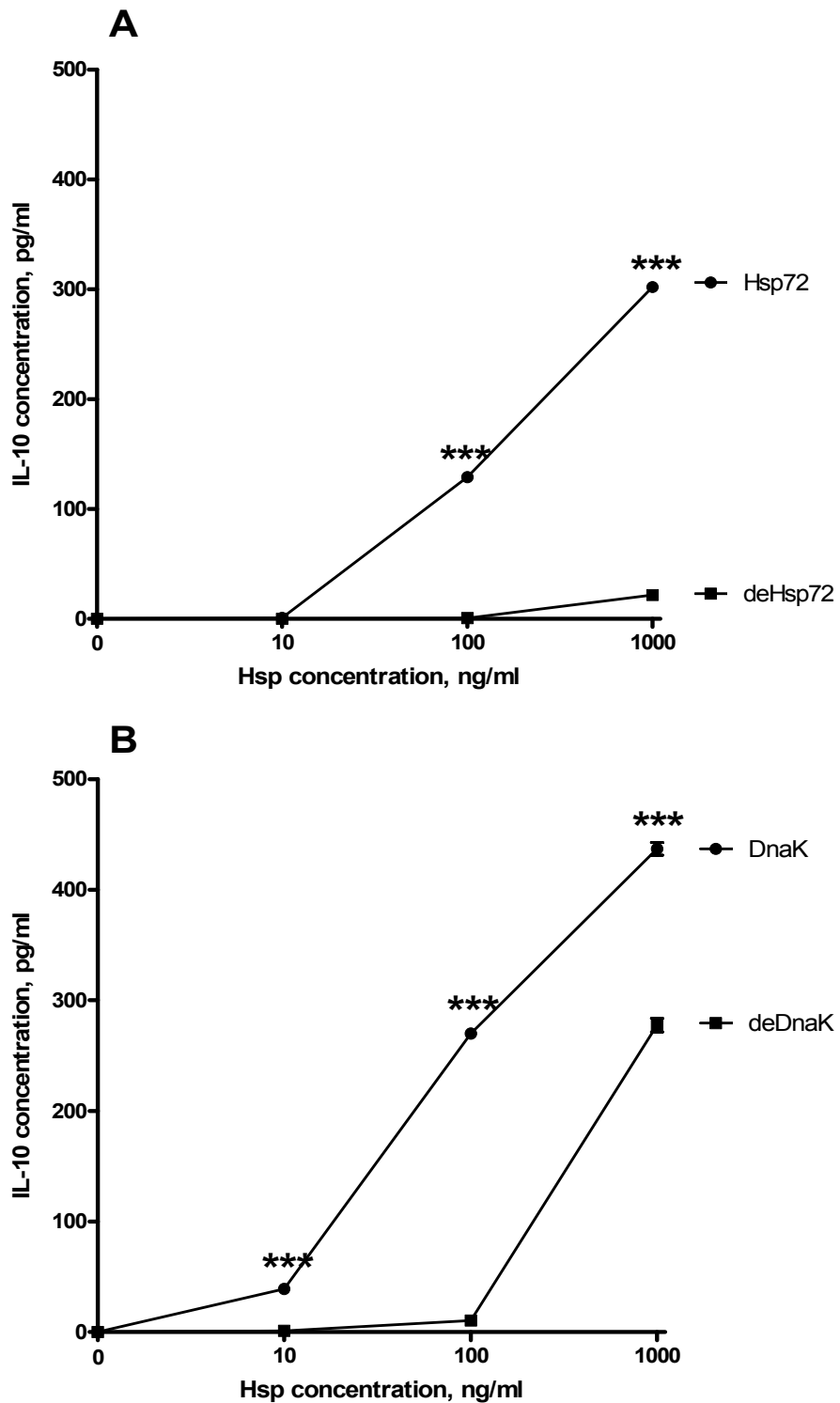


Figure 4.17: Effect on IL-10 secretion by U937 macrophages when incubated for 24 h with Hsp72 (A) or DnaK (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.8: Dose responses of cytokine secretion by U937 macrophages incubated for 24 h with native or denatured, Hsp72 or DnaK.
Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|---------------------|---------|------------|---------|--------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs. 100 | |
| Hsp72 | | | | | | |
| | Significance | | | | | |
| IL-1 β | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deHsp72 | | | | | | |
| IL-1 β | ns | P>0.05 | ns | P>0.05 | ns | P>0.05 |
| IL-6 | ns | P>0.05 | ns | P>0.05 | ns | P>0.05 |
| TNF- α | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| DnaK | | | | | | |
| IL-1 β | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | * | P<0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| deDnaK | | | | | | |
| IL-1 β | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |

Note: ns = not significant

Table 4.9: Correlation between cytokine secretion by U937 macrophages when incubated for 24 h with native or denatured, Hsp72 or DnaK.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Hsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.995** | 1.000** | 0.935 |
| IL-6 | 0.995** | | 0.996** | 0.915 |
| TNF-α | 1.000** | 0.996** | | 0.938 |
| IL-10 | 0.935 | 0.915 | 0.938 | |
| deHsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.880 | 0.850 | 0.580 |
| IL-6 | 0.880 | | 0.930 | 0.660 |
| TNF-α | 0.850 | 0.930 | | 0.890 |
| IL-10 | 0.580 | 0.660 | 0.890 | |
| DnaK | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.999** | 0.957* | 0.882 |
| IL-6 | 0.999** | | 0.966* | 0.899 |
| TNF-α | 0.957* | 0.966* | | 0.979* |
| IL-10 | 0.882 | 0.899 | 0.979* | |
| deDnaK | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.992** | 0.922 | 0.996** |
| IL-6 | 0.992** | | 0.949* | 0.990** |
| TNF-α | 0.922 | 0.949* | | 0.898 |
| IL-10 | 0.996** | 0.990** | 0.898 | |

4.3.3.2 Treatment of U937 macrophages with native or denatured Hsp60 or GroEL.

Incubation of U937 macrophages with native Hsp60 resulted in an increase of IL-1 β secretion at 100 ng/mL native Hsp60 compared to denatured Hsp60 (12.038 pg/mL and 8.299 pg/mL respectively), although this was not significant. There was a 3.5-fold increase in IL-1 β secretion at 1000 ng/mL native Hsp60 (53.053 pg/mL) compared to denatured Hsp60 (14.563 pg/mL) ($P < 0.001$) (Figure 4.18A). Secretion of IL-6 increased 3-fold ($P < 0.001$) when incubated with 10 ng/mL native Hsp60 compared to the denatured form (3.016 and 1.102 pg/mL respectively) followed by a 2-fold increase ($P < 0.001$) at 100 ng/mL native Hsp60 (5.713 pg/mL) compared to denatured Hsp60 (2.356 pg/mL) ($P < 0.05$), and increased 6-fold at 1000 ng/mL native Hsp60 compared to denatured Hsp60 (35.434 pg/mL and 6.421 pg/mL respectively, $P < 0.001$) (Figure 4.19A). TNF- α secretion increased almost 20-fold ($P < 0.01$) following incubation with 10 ng/mL native Hsp60 (19.148 pg/mL) compared to denatured Hsp60 (1.732 pg/mL), followed by a nearly 5-fold increase ($P < 0.001$) at 100 ng/mL (45.798 pg/mL native and 9.102 pg/mL denatured) and a 5-fold increase ($P < 0.001$) at 1000 ng/mL (276.411 pg/mL native and 56.049 pg/mL denatured) (Figure 4.20A). Secretion of IL-10 increased 16-fold ($P < 0.001$) after incubation with 100 ng/mL Hsp60 compared to denatured Hsp60 (59.906 and 3.043 pg/mL respectively), and a 10-fold increase ($P < 0.001$) was observed when incubated with 1000 ng/mL native compared to denatured Hsp60 (286.818 and 26.243 pg/mL respectively) (Figure 4.21A).

Cytokine secretion from cells treated with native or denatured Hsp60 were dose dependent at 10, 100 and 1000 ng/mL ($P < 0.05$ - $P < 0.001$) when compared to untreated cells, except for IL-1 β and TNF- α when treated with 10 ng/mL denatured Hsp60 which were not significant (Table 4.10).

There was a significant correlation between all cytokines when treated with native Hsp60 ($r^2 = 0.993 - 1.000$, $P < 0.01$). When treated with denatured Hsp60 there was a significant correlation between IL-6 and all other cytokines ($r^2 = 0.969 - 0.980$, $P < 0.05$), and between TNF and IL-10 ($r^2 = 0.999$, $P < 0.01$) (Table 4.11).

IL-1 β increased 5-fold ($P < 0.001$) following incubation with native GroEL compared to denatured GroEL at 1000 ng/mL (64.677 and 12.996 pg/mL respectively). No significant differences were seen at other concentrations (Figure 4.18B). IL-6 secretion increased 2-fold ($P < 0.001$) when incubated with 100 ng/mL native GroEL (11.145 pg/mL) compared to denatured GroEL (5.198 pg/mL) and also increased at 1000 ng/mL (49.230 pg/mL and 33.961 pg/mL respectively, $P < 0.001$) (Figure

4.19B). TNF- α secretion increased significantly ($P < 0.001$) from cells treated with 1000 ng/mL native GroEL compared to denatured GroEL (459.852 pg/mL and 360.298 pg/mL respectively) (Figure 4.20B). A 6-fold ($P < 0.001$) increase of IL-10 secretion was observed following incubation with 100 ng/mL GroEL (95.975 pg/mL) compared to the denatured form (16.231 pg/mL), and a 3-fold increase following incubation with 1000 ng/mL native compared to denatured GroEL (420.020 and 137.439 pg/mL respectively) (Figure 4.21B).

Cytokine secretion from cells treated with native and denatured GroEL were dose dependent at 10, 100 and 1000 ng/mL ($P < 0.05$ - $P < 0.001$) when compared to untreated cells, except for IL-6 secretion when treated with 10 ng/mL native or denatured GroEL which were not significant (Table 4.10).

Significant correlation was observed between all cytokines when treated with native GroEL ($r^2 = 0.984 - 1.000$, $P < 0.05 - 0.01$). A significant correlation was also seen between IL-6 and TNF- α ($r^2 = 0.998$, $P < 0.01$), and IL-6 and IL-10 ($r^2 = 0.999$, $P < 0.01$) and, TNF- α and IL-10 ($r^2 = 0.997$, $P < 0.01$), when treated with denatured GroEL (Table 4.11).

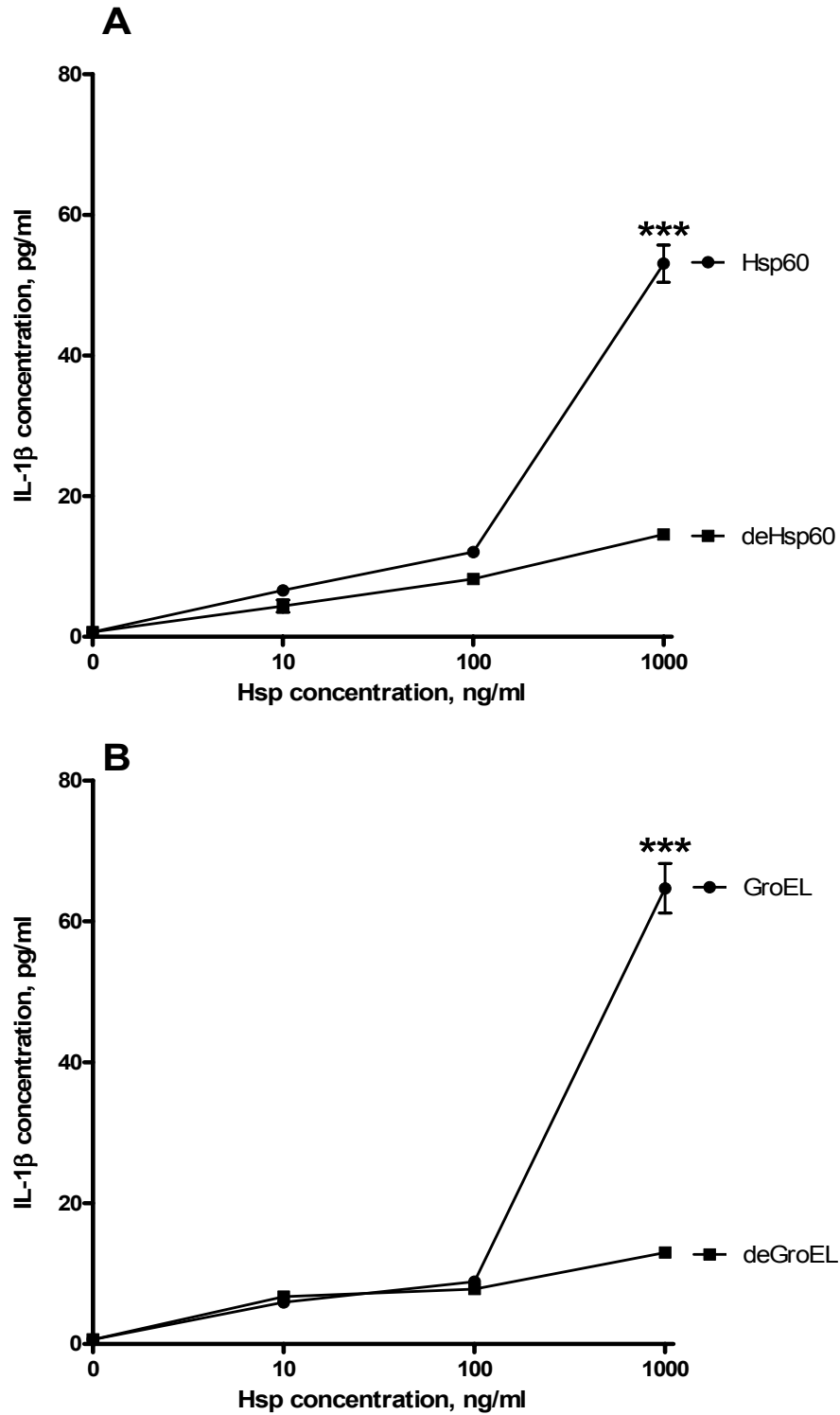


Figure 4.18: Effect on IL-1 β secretion by U937 macrophages when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

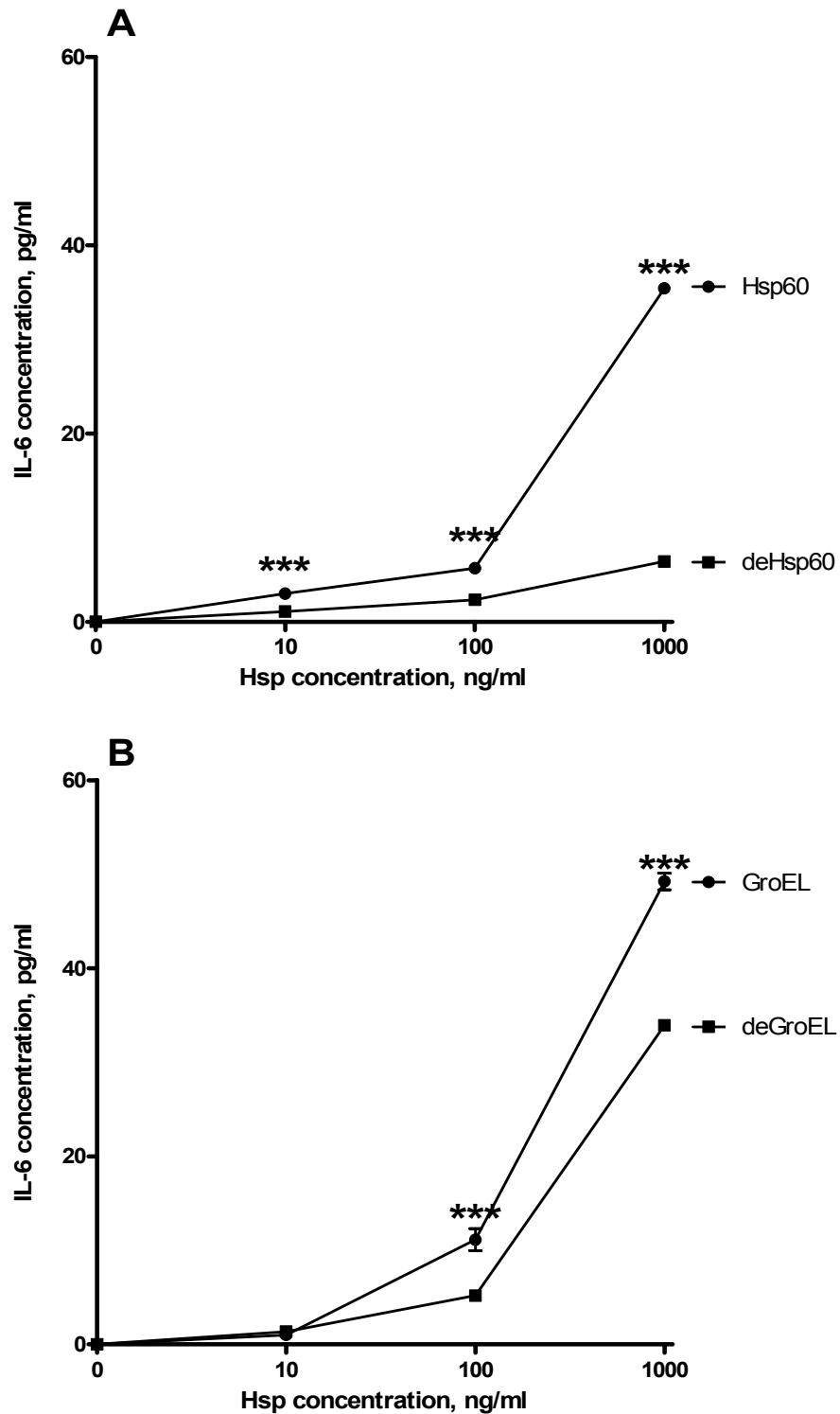


Figure 4.19: Effect on IL-6 secretion by U937 macrophages when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

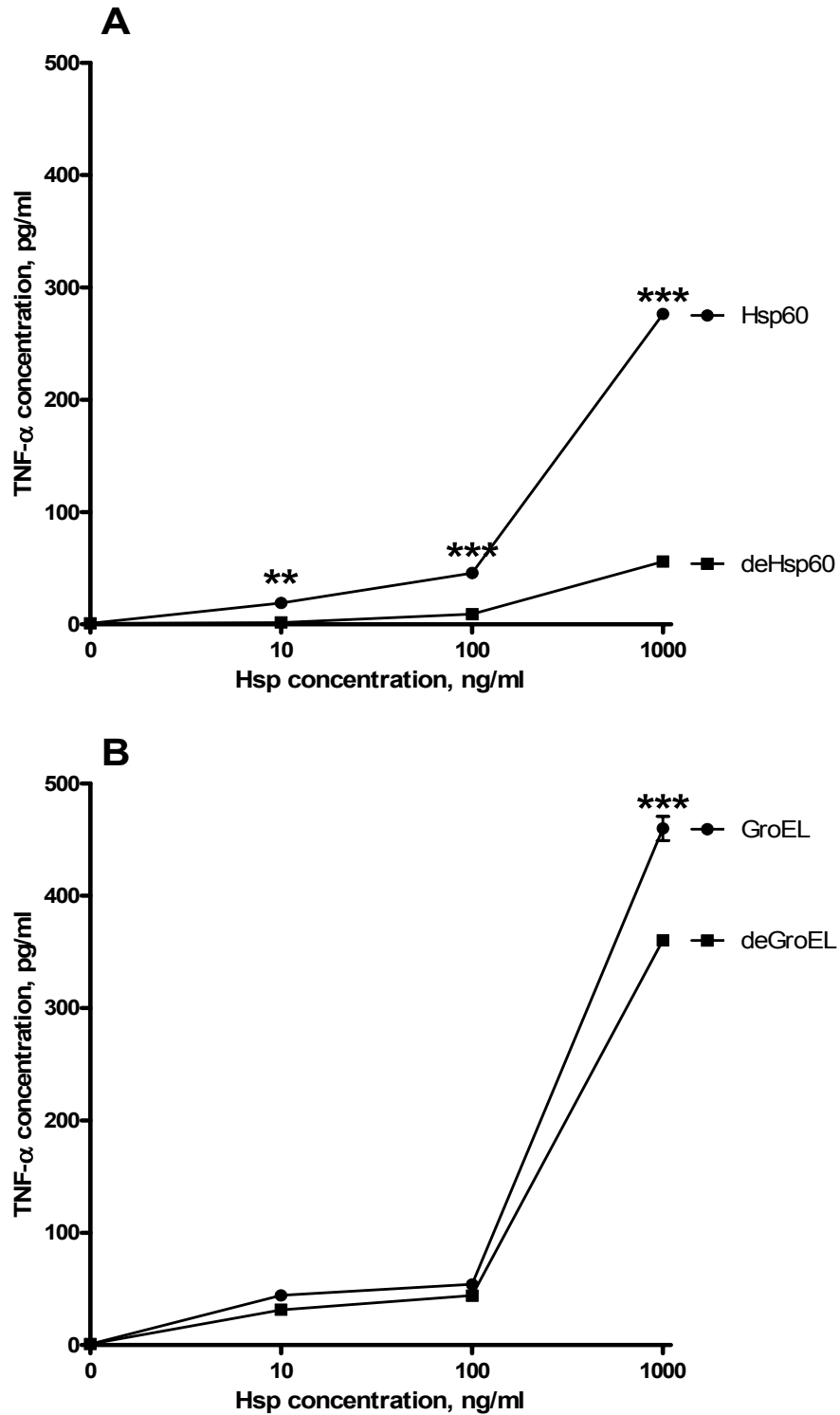


Figure 4.20: Effect on TNF- α secretion by U937 macrophages when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

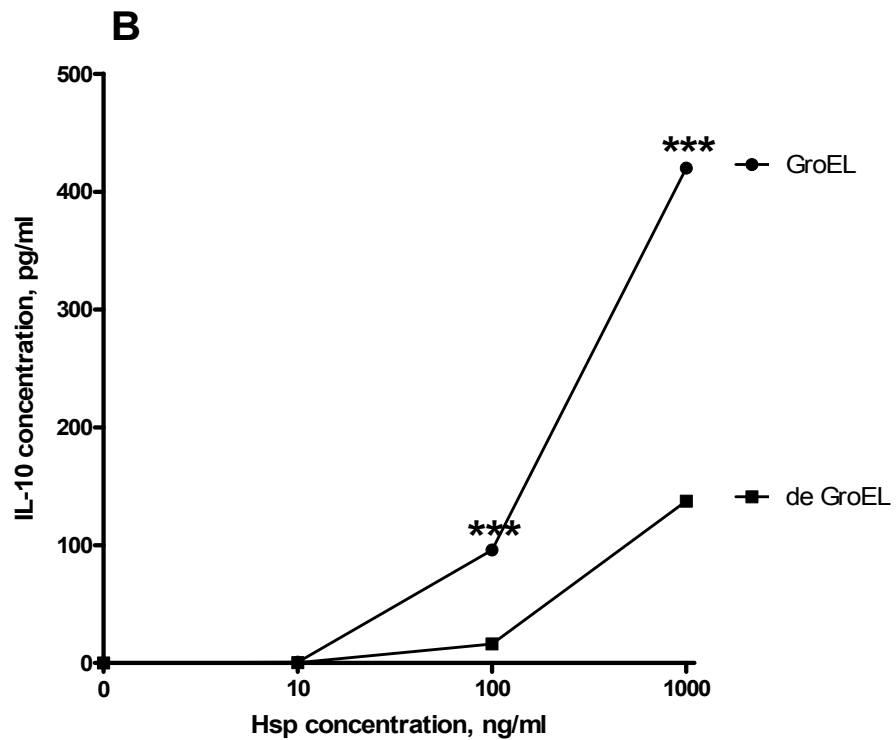
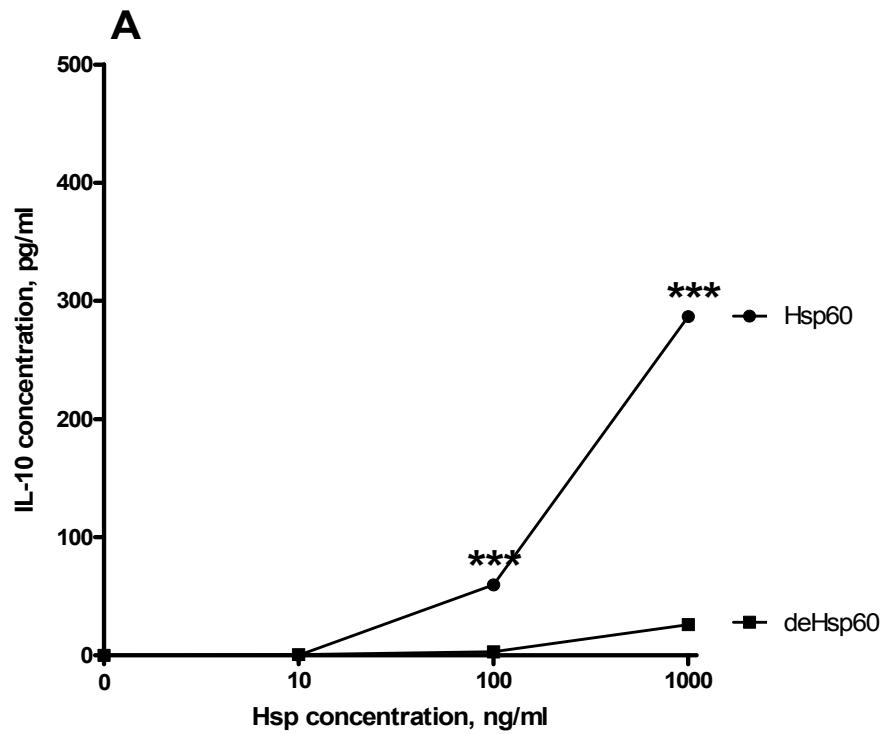


Figure 4.21: Effect on IL-10 secretion by U937 macrophages when incubated for 24 h with Hsp60 (A) or GroEL (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.10: Dose responses of cytokine secretion by U937 macrophages incubated for 24 h with native and denatured Hsp60 and GroEL.
Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|----------|---------|------------|---------|--------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs. 100 | |
| Hsp60 | | | | | | |
| Significance | | | | | | |
| IL-1 β | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deHsp60 | | | | | | |
| IL-1 β | ns | P>0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| GroEL | | | | | | |
| IL-1 β | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deGroEL | | | | | | |
| IL-1 β | * | P<0.05 | ** | P<0.01 | *** | P<0.001 |
| IL-6 | ns | P>0.05 | * | P<0.05 | *** | P<0.001 |
| TNF- α | ** | P<0.01 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |

Note: ns = not significant

Table 4.11: Correlation between cytokine secretion by U937 macrophages when incubated for 24 h with native or denatured, Hsp60 or GroEL.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Hsp60 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.999** | 0.998** | 0.994** |
| IL-6 | 0.999** | | 1.000** | 0.993** |
| TNF-α | 0.998** | 1.000** | | 0.995** |
| IL-10 | 0.994** | 0.993** | 0.995** | |
| deHsp60 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.980* | 0.912 | 0.901 |
| IL-6 | 0.980* | | 0.975* | 0.969* |
| TNF-α | 0.812 | 0.975* | | 0.999** |
| IL-10 | 0.901 | 0.969* | 0.999** | |
| GroEL | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.990** | 1.000** | 0.988* |
| IL-6 | 0.990** | | 0.987* | 1.000** |
| TNF-α | 1.000** | 0.987* | | 0.984* |
| IL-10 | 0.988* | 1.000** | 0.984* | |
| deGroEL | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.844 | 0.846 | 0.823 |
| IL-6 | 0.844 | | 0.998** | 0.999** |
| TNF-α | 0.846 | 0.998** | | 0.997** |
| IL-10 | 0.823 | 0.999** | 0.997** | |

4.3.3.3 Treatment of U937 macrophages with native or denatured Cpn10 or GroES.

When incubated with native Cpn10, there was no significant difference in IL-1 β secretion at any dose when compared to denatured Cpn10 (Figure 4.22A). IL-6 secretion increased significantly ($P < 0.01$) when U937 macrophages were incubated with 100 ng/mL native Cpn10 compared to denatured Cpn10 (4.302 and 3.303 pg/mL respectively); secretion increased significantly ($P < 0.001$) when incubated with 1000 ng/mL native (7.628 pg/mL) compared to the denatured form (4.453 pg/mL) (Figure 4.23A). TNF- α secretion increased 2-fold ($P < 0.001$) when incubated with 10 ng/mL native Cpn10 compared to denatured Cpn10 (4.573 and 2.345 pg/mL respectively), which increased 3-fold ($P < 0.001$) when treated with 100 ng/mL (8.594 and 2.600 pg/mL respectively), followed by a 4-fold increase ($P < 0.001$) in secretion when treated with 1000 ng/mL native Cpn10 compared to denatured Cpn10 (11.876 and 2.680 pg/mL respectively) (Figure 4.24A). IL-10 increased 10-fold ($P < 0.001$) following incubation with 100 ng/mL native compared to denatured Cpn10 (0.356 and 0.038 pg/mL respectively), and an almost 6-fold ($P < 0.001$) increase following 1000 ng/mL native Cpn10 (0.703 pg/mL) compared to the denatured form (0.120 pg/mL) (Figure 4.25A).

Cytokine secretion from cells treated with native and denatured Cpn10 was dose dependent at 10, 100 and 1000 ng/mL when compared to untreated cells ($P < 0.05$ - $P < 0.001$) (Table 4.12).

A significant correlation was observed between IL-1 β and IL-10 when treated with either native ($r^2 = 0.961$, $P < 0.05$) or denatured ($r^2 = 0.995$, $P < 0.01$) Cpn10 (Table 4.13).

Following incubation of U937 macrophages when treated with native GroES, IL-1 β secretion increased significantly ($P < 0.001$) compared to denatured GroES at 10 ng/mL (8.888 and 7.163 pg/mL respectively); secretion was significantly increased ($P < 0.001$) following incubation with 100 ng/mL native GroES (9.613 ng/mL) compared to the denatured form (8.104 pg/mL). No significant increase was seen when incubated with 1000 ng/mL (Figure 4.22B). IL-6 secretion increased significantly ($P < 0.01$) when incubated with 100 ng/mL native GroES (2.794 pg/mL) compared to denatured GroES (2.109 pg/mL) and was increased 0.5-fold ($P < 0.001$) when treated with 1000 ng/mL native GroES compared to denatured GroES (6.325 and 4.188 pg/mL respectively) (Figure 4.23B). There was a 2-fold increase ($P < 0.001$) of TNF- α secretion when cells were treated with 10 ng/mL native GroES compared to denatured GroES (4.410 and 1.199 pg/mL

respectively), which increased 4-fold ($P < 0.001$) when treated with 100 ng/mL (9.283 and 1.918 pg/mL respectively), followed by an 8-fold increase ($P < 0.001$) in secretion when treated with 1000 ng/mL native GroES compared to denatured GroES (16.526 and 2.352 pg/mL respectively) (Figure 4.24B). Incubation with 100 ng/mL native GroES resulted in a significant ($P < 0.001$) increase in IL-10 secretion compared to denatured GroES (0.442 and 0.000 pg/mL respectively), and a 4-fold ($P < 0.001$) increase following incubation with 1000 ng/mL native compared to denatured GroES (0.937 and 0.120 pg/mL respectively) (Figure 4.25B).

Cytokine secretion from cells treated with native and denatured GroES was dose dependent at 10, 100 and 1000 ng/mL when compared to untreated cells ($P < 0.05 - P < 0.001$), except for TNF- α secretion when treated with 10 ng/mL denatured GroES which was not significant (Table 4.12).

Significant correlation between IL-1 β and IL-10 ($r^2 = 0.983$, $P < 0.05$), and IL-6 and TNF- α ($r^2 = 0.960$, $P < 0.05$) was observed following treatment with native GroES. There was correlation between IL-1 β , TNF- α and IL-10 ($r^2 = 0.957 - 0.974$, $P < 0.05$), and between IL-6 and TNF- α ($r^2 = 0.961$, $P < 0.05$) following treatment with denatured GroES (Table 4.13).

4.3.3.4 Cell viability and cell death in U937 macrophages following treatment with native and denatured heat shock proteins.

There was no significant change in cell number or viability (4.66×10^5 cells/mL) when compared to seeding density (5×10^5 cells/mL) although cell counts were reduced. No significant cell death by apoptosis or necrosis was detected for any treatment when compared to untreated cells, and all were significantly different from the positive controls for either apoptosis or necrosis (Table 4.14).

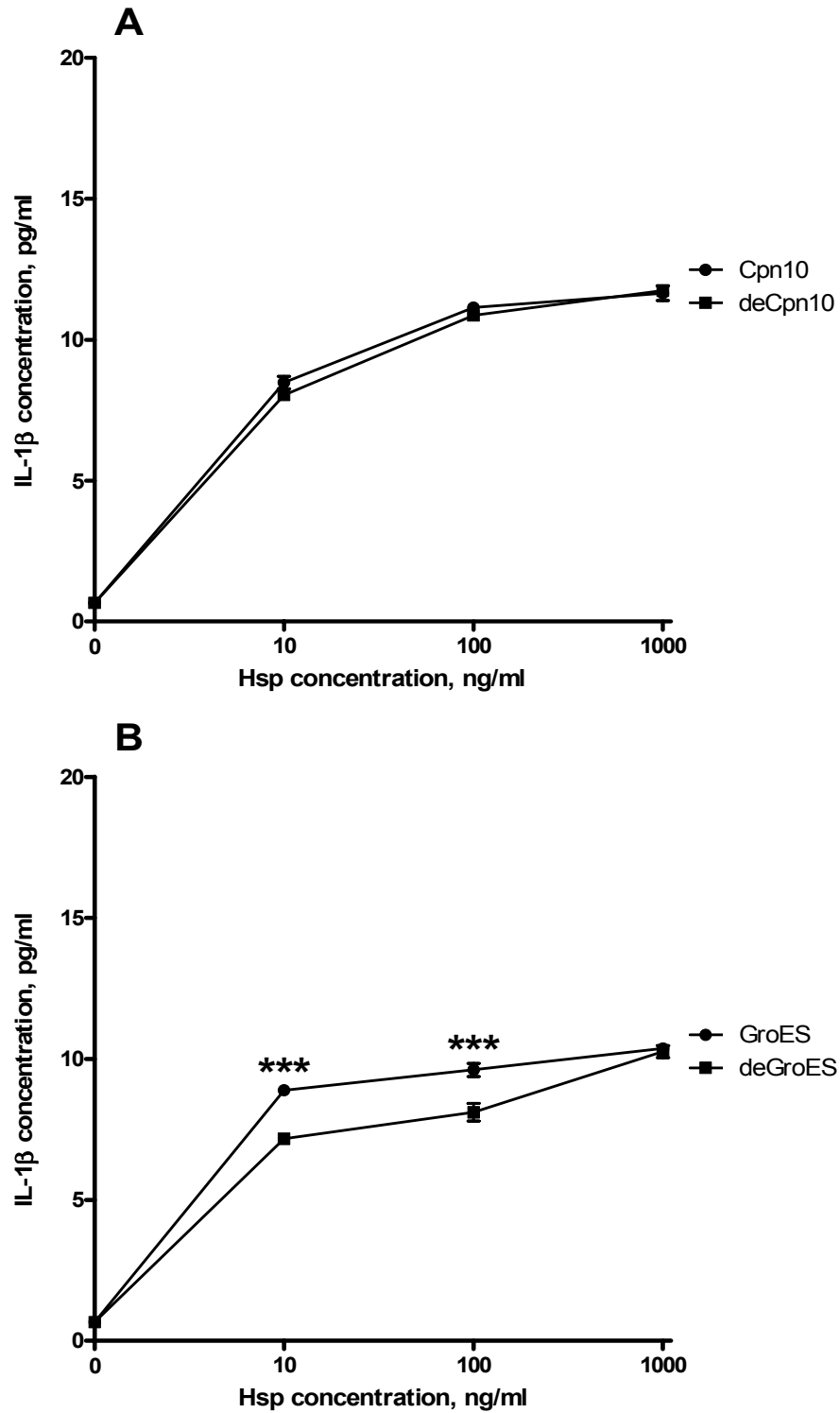


Figure 4.22: Effect on IL-1 β secretion by U937 macrophages when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

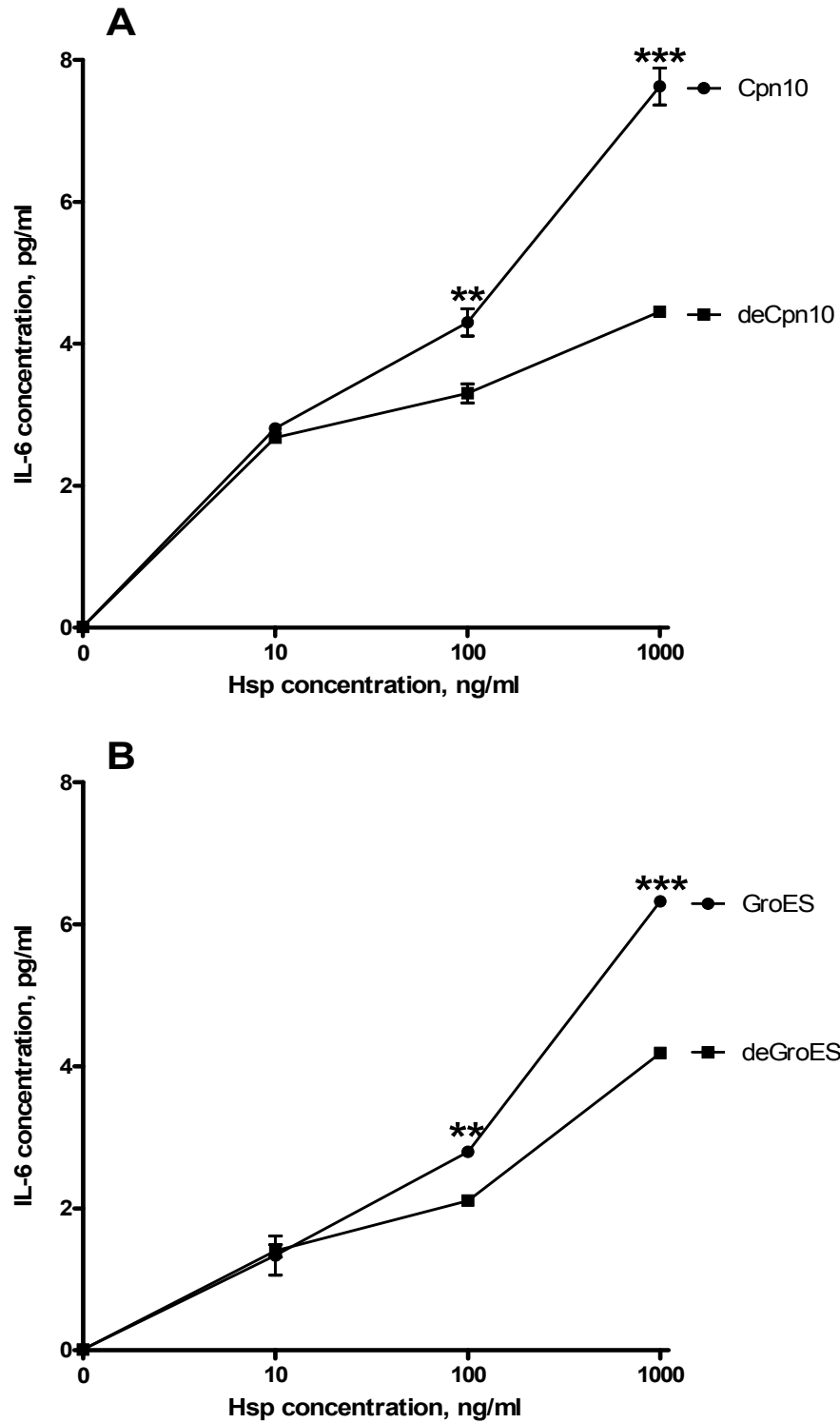


Figure 4.23: Effect on IL-6 secretion by U937 macrophages when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

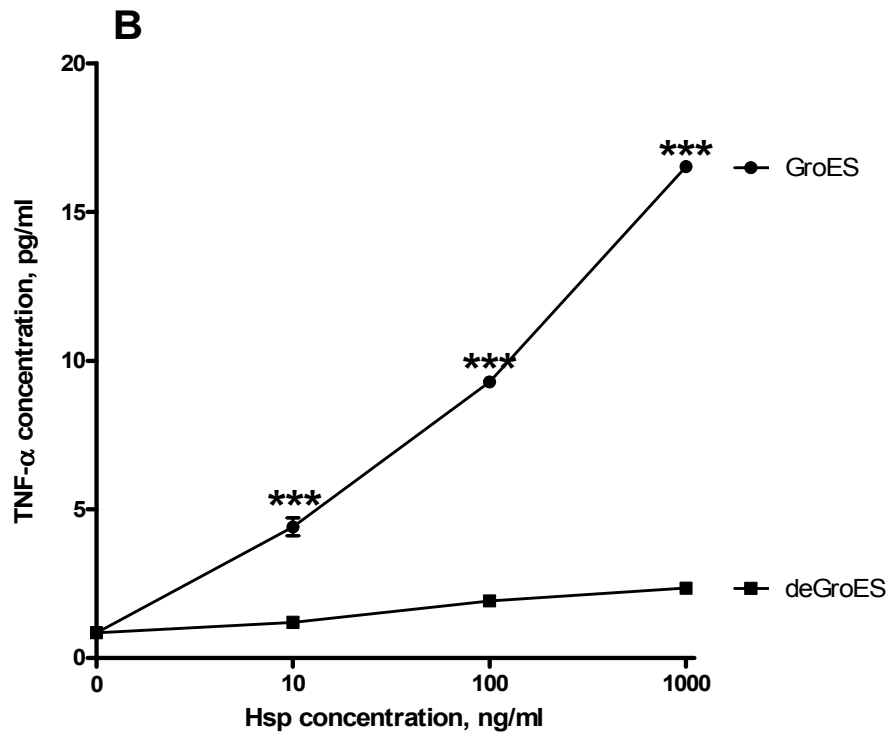
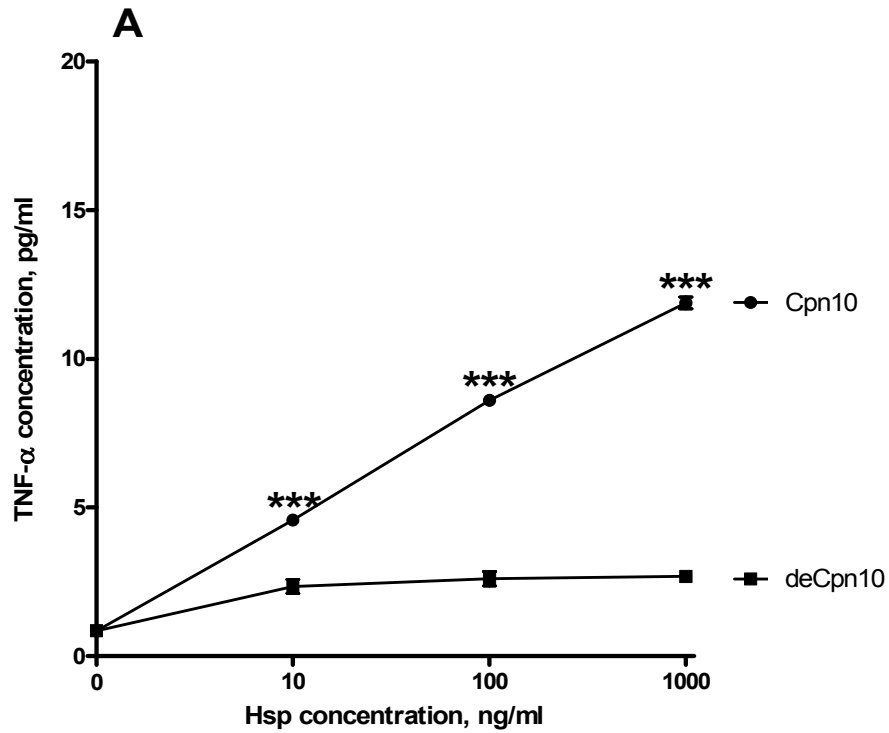


Figure 4.24: Effect on TNF- α secretion by U937 macrophages when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

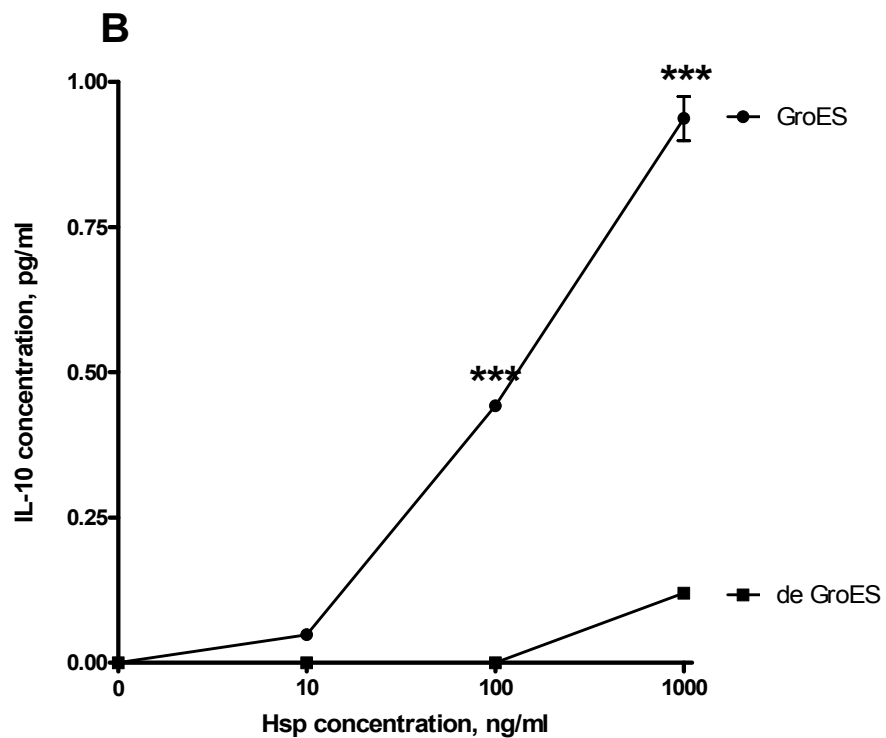
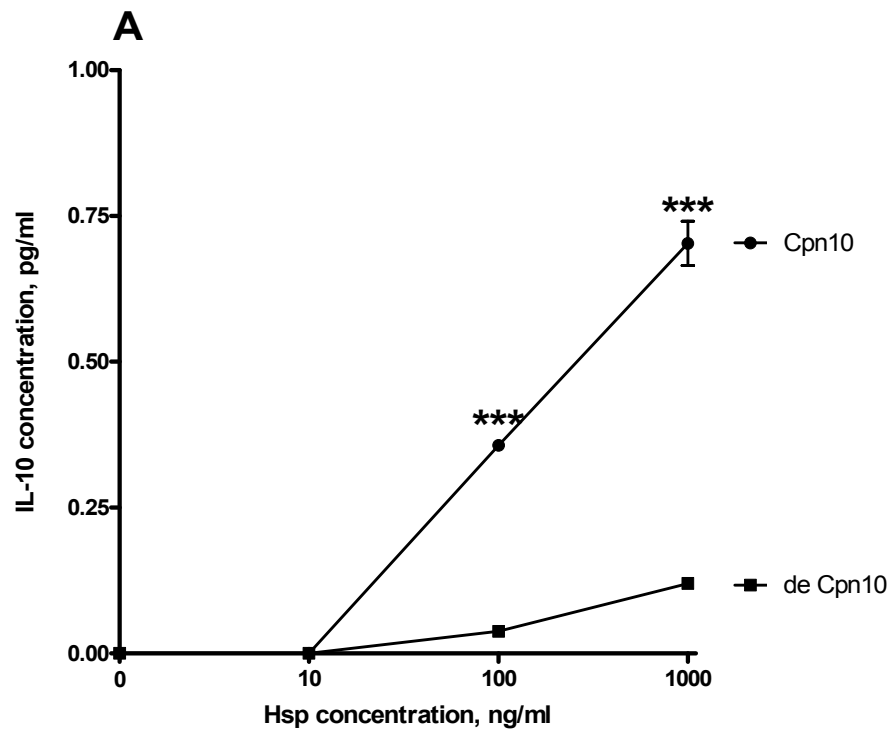


Figure 4.25: Effect on IL-10 secretion by U937 macrophages when incubated for 24 h with Cpn10 (A) or GroES (B), in native or denatured (de) forms. Data are presented as mean \pm SEM, n = 4. * Indicates significant difference between mean concentration points through use of two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.12: Dose responses of cytokine secretion by U937 macrophages incubated for 24 h with native or denatured, Cpn10 or GroES.
Significance shown as difference between treatments using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| Concentration of Treatment (ng/mL) | | | | | | |
|------------------------------------|---------------------|---------|------------|---------|--------------|---------|
| Treatment | 10 vs. 0 | | 100 vs. 10 | | 1000 vs. 100 | |
| Cpn10 | | | | | | |
| | Significance | | | | | |
| IL-1 β | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deCpn10 | | | | | | |
| IL-1 β | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |
| GroES | | | | | | |
| IL-1 β | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| deGroES | | | | | | |
| IL-1 β | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| IL-6 | *** | P<0.001 | *** | P<0.001 | *** | P<0.001 |
| TNF- α | ns | P>0.05 | *** | P<0.001 | *** | P<0.001 |
| IL-10 | ns | P>0.05 | ns | P>0.05 | *** | P<0.001 |

Note: ns = not significant

Table 4.13: Correlation between cytokine secretion by U937 macrophages when incubated for 24 h with native or denatured, Cpn10 or GroES.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Cpn10 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.888 | 0.914 | 0.722 |
| IL-6 | 0.888 | | 0.985* | 0.932 |
| TNF-α | 0.914 | 0.985* | | 0.940 |
| IL-10 | 0.722 | 0.932 | 0.940 | |
| deCpn10 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.984* | 0.989* | 0.685 |
| IL-6 | 0.984* | | 0.966* | 0.774 |
| TNF-α | 0.989* | 0.966* | | 0.588 |
| IL-10 | 0.685 | 0.774 | 0.999** | |
| GroES | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.733 | 0.771 | 0.655 |
| IL-6 | 0.733 | | 0.994** | 0.984* |
| TNF-α | 0.771 | 0.994** | | 0.985* |
| IL-10 | 0.655 | 0.984* | 0.985* | |
| deGroES | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.909 | 0.883 | 0.599 |
| IL-6 | 0.909 | | 0.958 | 0.866 |
| TNF-α | 0.883 | 0.958 | | 0.757 |
| IL-10 | 0.599 | 0.866 | 0.757 | |

Table 4.14: Cell viability, caspase-3 and necrosis measurements of U937 macrophages following 24 h incubation with native or denatured heat shock proteins.

| Treatment | Viable cell count (x 10 ⁵) (Trypan blue exclusion) | | | Apoptosis (Caspase-3, RFU) | | | Necrosis (Propidium iodide, RFU) | | |
|------------------------------------|--|------|------|----------------------------|-----|-----|----------------------------------|-----|----|
| | 1000 | 100 | 10 | 1000 | 100 | 10 | 1000 | 100 | 10 |
| No Treatment | 4.66 | | | 228 | | | 74 | | |
| Positive Control | - | | | 2031 | | | 547 | | |
| Concentration of treatment (ng/mL) | 1000 | 100 | 10 | 1000 | 100 | 10 | 1000 | 100 | 10 |
| Hsp72 | 4.55 | 4.61 | 4.62 | 231 | 227 | 229 | 81 | 76 | 73 |
| Denatured Hsp72 | 4.56 | 4.62 | 4.62 | 232 | 231 | 230 | 78 | 78 | 76 |
| DnaK | 4.49 | 4.56 | 4.59 | 234 | 228 | 226 | 86 | 79 | 76 |
| Denatured DnaK | 4.50 | 4.56 | 4.60 | 232 | 229 | 228 | 82 | 77 | 77 |
| Hsp60 | 4.52 | 4.56 | 4.60 | 231 | 231 | 230 | 79 | 79 | 75 |
| Denatured Hsp60 | 4.53 | 4.53 | 4.56 | 227 | 225 | 228 | 78 | 76 | 71 |
| GroEL | 4.52 | 4.53 | 4.57 | 228 | 228 | 229 | 76 | 76 | 73 |
| Denatured GroEL | 4.52 | 4.55 | 4.58 | 229 | 226 | 224 | 76 | 75 | 75 |
| Cpn10 | 4.61 | 4.62 | 4.62 | 226 | 227 | 226 | 77 | 72 | 73 |
| Denatured Cpn10 | 4.61 | 4.61 | 4.63 | 228 | 226 | 227 | 76 | 76 | 74 |
| GroES | 4.59 | 4.62 | 4.61 | 226 | 226 | 228 | 76 | 78 | 73 |
| Denatured GroES | 4.61 | 4.62 | 4.62 | 228 | 227 | 226 | 75 | 75 | 74 |

Note: n = 4. Caspase-3 positive control = 2 μ m camptothecin; necrosis positive control = autoclaved (see section 2.3.32). Seeding density = 5 x 10⁵ cells/mL.

4.3.4 Effects on U937 macrophages following a time course of treatments with native or denatured, Hsp72 or low endotoxin (LE) Hsp72.

Cytokine secretion from U937 macrophages following treatments are presented in Figures 4.26 – 4.29 and Table 4.15.

Following a time course, IL-1 β secretion increased in a time dependent manner from U937 macrophages when treated with either Hsp72 (12.545 pg/mL) or LE-Hsp72 (11.984 pg/mL), when compared to 0 h (4.813 pg/mL). Significant differences were observed at 3 h (11.929 and 9.242 pg/mL respectively) and 4 h (12.720 and 9.753 pg/mL respectively) between, Hsp72 or LE-Hsp72 (Figure 4.26A). There was no difference over time between either, denatured Hsp72, or denatured LE-Hsp72 (Figure 4.26B). IL-6 secretion also increased in a time dependent manner with significant differences seen between Hsp72 and LE-Hsp72 at 4 h (26.319 and 21.480 pg/mL, $P < 0.01$), 5 h (39.916 and 31.075 pg/mL, $P < 0.001$), and 6 h (44.699 and 39.900 pg/mL, $P < 0.001$) (Figure 4.27A). There was no difference at any time between denatured, Hsp72 or LE-Hsp72 at any time point (Figure 4.27B). TNF- α secretion increased in a time dependent manner reaching a peak at 6 h for both Hsp72 and LE-Hsp72 (716.870 and 604.050 pg/mL respectively). Significant differences ($P < 0.01 - 0.001$) were observed between Hsp72 and LE-Hsp72 at all time points except at 0 h (Figure 4.28A). There was an increase in TNF- α secretion over time following incubation with both denatured forms but these were not significantly different from 0 h (Figure 4.28B). IL-10 secretion increased in a time dependent manner that peaked at 6 h for both Hsp72 and LE-Hsp72 (75.775 and 66.898 pg/mL respectively). Significant differences were observed after 3 h until 6 h between Hsp72 and LE-Hsp72 ($P < 0.05 - 0.001$) (Figure 4.29A). There was no difference between 0 h and any time point following incubation with denatured forms of Hsp72 and LE-Hsp72 (Figure 4.29B).

Significant correlation was observed between all cytokines over time when incubated with Hsp72 ($r^2 = 0.830 - 1.000$, $P < 0.05 - 0.01$) and LE-Hsp72 ($r^2 = 0.900 - 1.000$, $P < 0.01$) (Table 4.15).

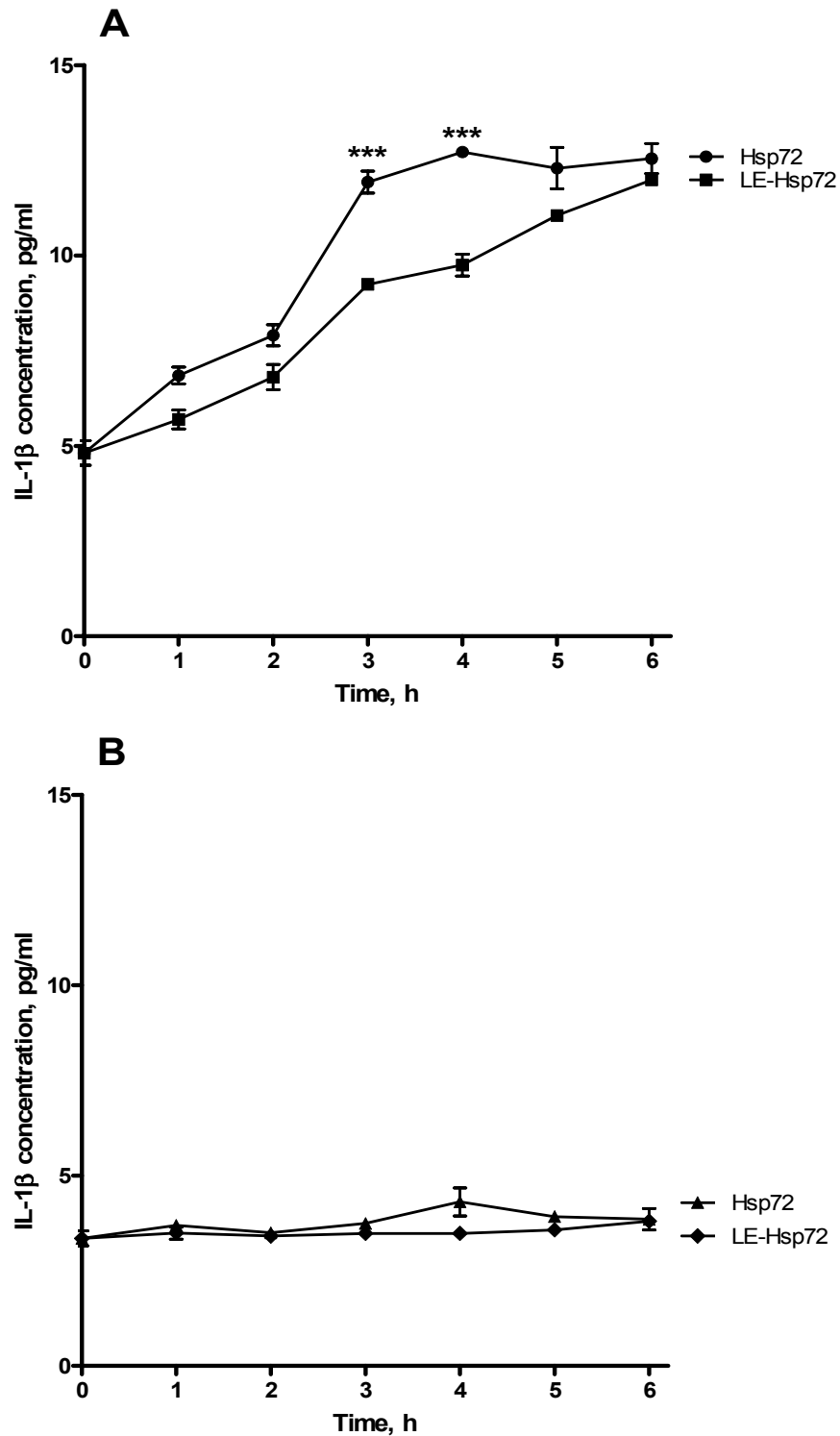


Figure 4.26: Time course of IL-1 β secretion by U937 macrophages treated with 1000 ng/mL Hsp72 or low endotoxin (LE) Hsp72 in native (A) or denatured form (B).

U937 macrophages were treated with 1000 ng/mL Hsp72 from 0-6 h. U937 macrophages were treated with 100 ng/ml LPS from 0-6 h. Data are presented as mean \pm SEM, $n = 4$. Significance shown as difference from 0 h using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

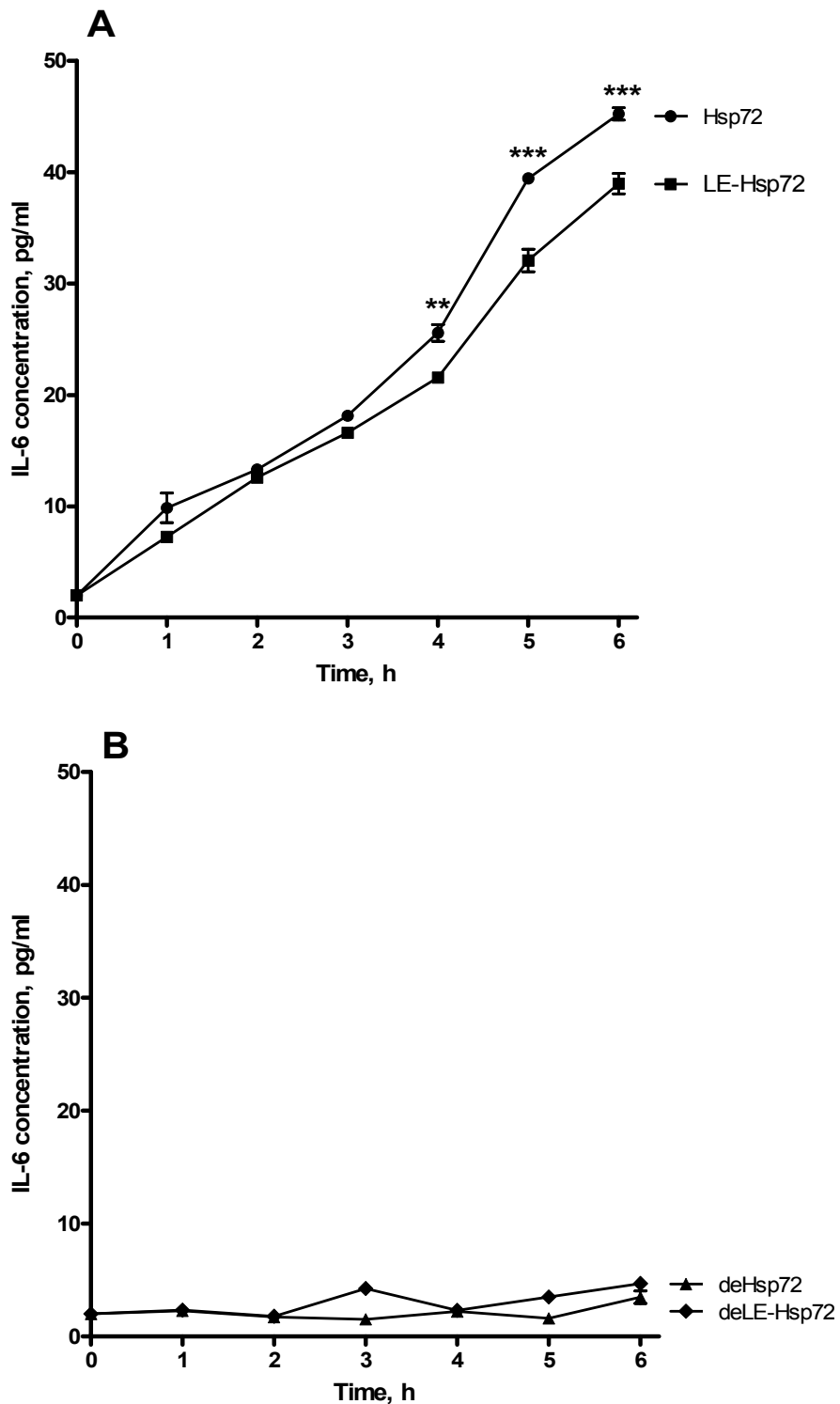


Figure 4.27: Time course of IL-6 secretion by U937 macrophages treated with 1000 ng/mL Hsp72 or low endotoxin (LE) Hsp72 in native (A) or denatured form (B).

U937 macrophages were treated with 1000 ng/ml Hsp72 from 0-6 h. Data are presented as mean \pm SEM, $n = 4$. Significance shown as difference from 0 h using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

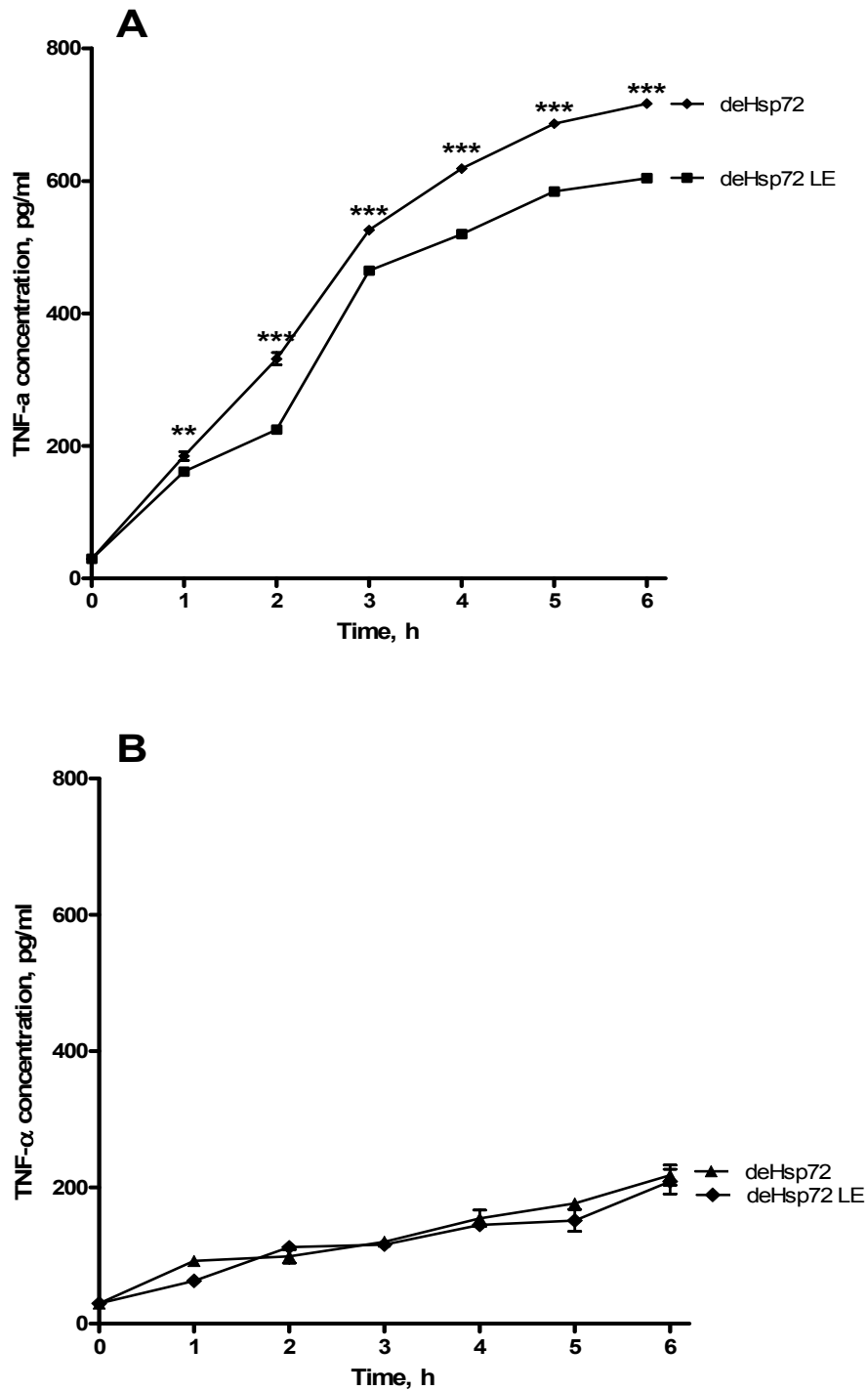


Figure 4.28: Time course of TNF- α secretion by U937 macrophages treated with 1000 ng/mL Hsp72 or low endotoxin (LE) Hsp72 in native (A) or denatured form (B).

U937 macrophages were treated with 1000 ng/ml Hsp72 from 0-6 h. Data are presented as mean \pm SEM, n = 4. Significance shown as difference from 0 h using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

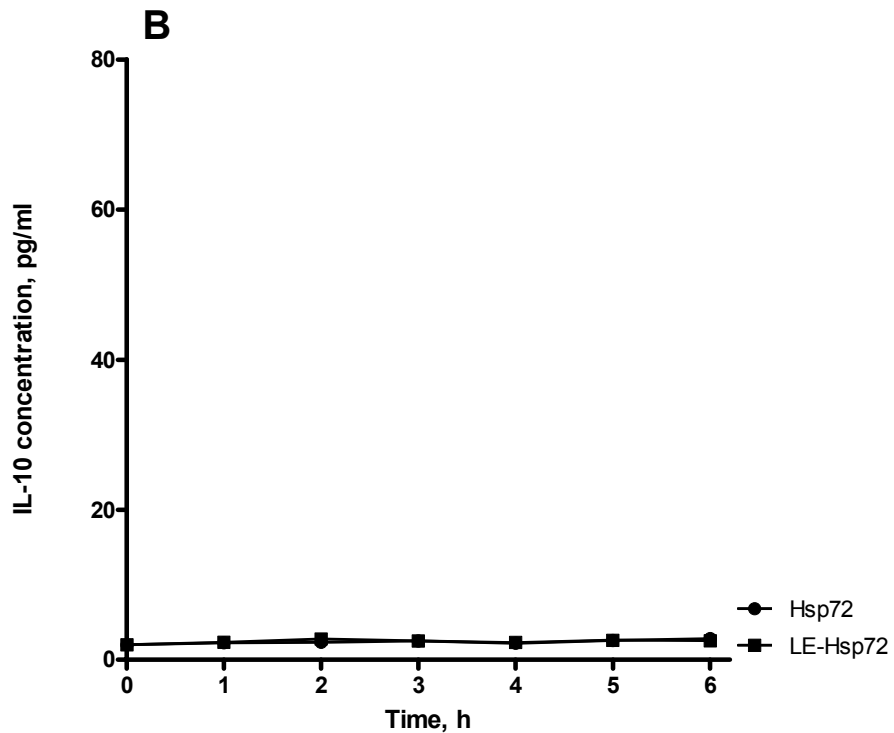
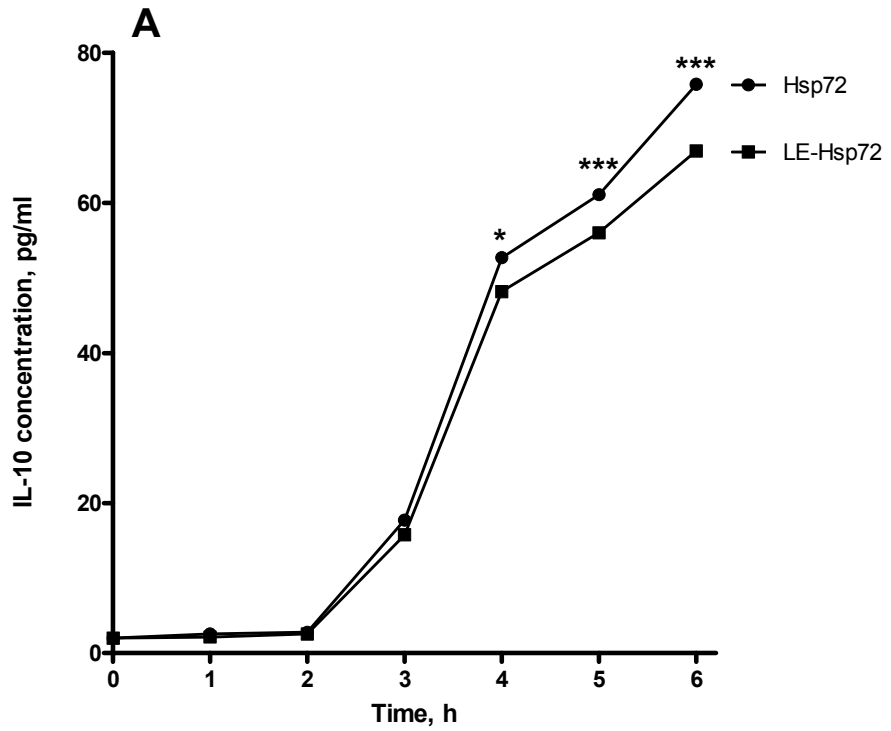


Figure 4.29: Time course of IL-10 secretion by U937 macrophages treated with 1000 ng/mL Hsp72 or low endotoxin (LE) Hsp72 in native (A) or denatured form (B).

U937 macrophages were treated with 1000 ng/ml Hsp72 from 0-6 h. Data are presented as mean \pm SEM, n = 4. Significance shown as difference from 0 h using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.15: Correlation between cytokines secreted by U937 macrophages when incubated for up to 6 h with native Hsp72.

Significance shown following Pearson correlation: * (P<0.05); ** (P<0.01)

| Hsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
|--------------------------------|-------------------------------|-------------|--------------------------------|--------------|
| IL-1β | | 0.830* | 0.980** | 0.830* |
| IL-6 | 0.830* | | 0.890** | 1.000** |
| TNF-α | 0.980** | 0.890** | | 0.890** |
| IL-10 | 0.830* | 1.000** | 0.890** | |
| LE-Hsp72 | IL-1β | IL-6 | TNF-α | IL-10 |
| IL-1β | | 0.930** | 0.990** | 0.930** |
| IL-6 | 0.930** | | 0.900** | 1.000** |
| TNF-α | 0.990** | 0.900** | | 0.900** |
| IL-10 | 0.930** | 1.000** | 0.900** | |

4.3.5 Effect on cytokine secretion from U937 macrophages following 4 h treatment with Hsp72 or LPS, pre-incubated with polymyxin B.

Cytokine secretion from U937 macrophages following treatments are presented in Figures 4.30 – 4.33 and Table 4.16.

Following incubation with Hsp72, IL-1 β , IL-6, TNF- α and IL-10 secretion were significantly decreased when pre-incubated with polymyxin B compared to no polymyxin B (IL-1 β : 11.021 and 14.939 pg/mL respectively, $P < 0.05$; IL-6: 21.945 and 26.738 pg/mL respectively, $P < 0.001$; TNF- α : 529.203 and 618.647 pg/mL respectively, $P < 0.001$; IL-10: 242.079 and 415.763 pg/mL respectively, $P < 0.001$) (Figures 4.30 – 4.33).

A significant decrease in all cytokine secretion was seen when LPS was pre-incubated with polymyxin B (IL-1 β : 10.684 and 33.070 pg/mL respectively, $P < 0.001$; IL-6: 75.188 and 1576.964 pg/mL respectively, $P < 0.001$; TNF- α : 382.900 and 9793.075 pg/mL respectively, $P < 0.001$; IL-10: 293.218 and 1381.471 pg/mL respectively, $P < 0.001$) (Figures 4.30 – 4.33).

No significant differences were observed when HI-RPMI was pre-incubated with polymyxin B.

When pre-incubated with polymyxin B, Hsp72 resulted in significant increases ($P < 0.05$ – 0.001) in cytokine secretion when compared to HI-RPMI which was pre-incubated with polymyxin B, as did LPS (Table 4.16).

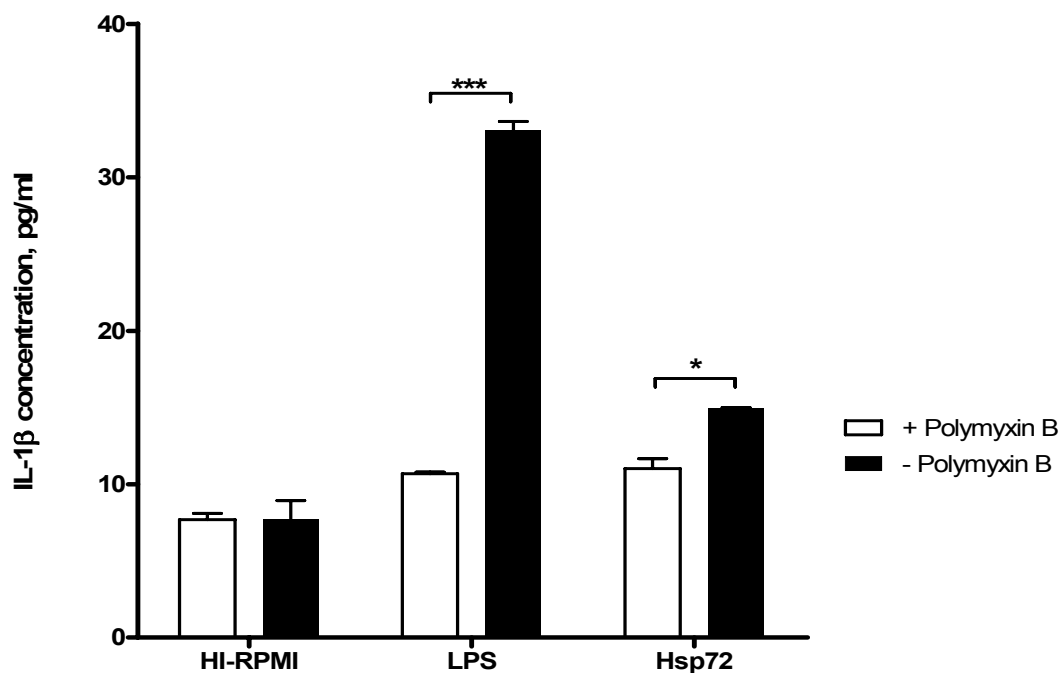


Figure 4.30: Secretion of IL-1 β by U937 macrophages treated with Hsp72 or LPS with or without polymyxin B for 4 h.

U937 macrophages were treated with 1000 ng/ml Hsp72 or 100 ng/mL LPS either with or without pre-incubation with 20 μ g/mL polymyxin B. Data are presented as mean \pm SEM, n = 4. Significance shown as difference from HI-RPMI using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

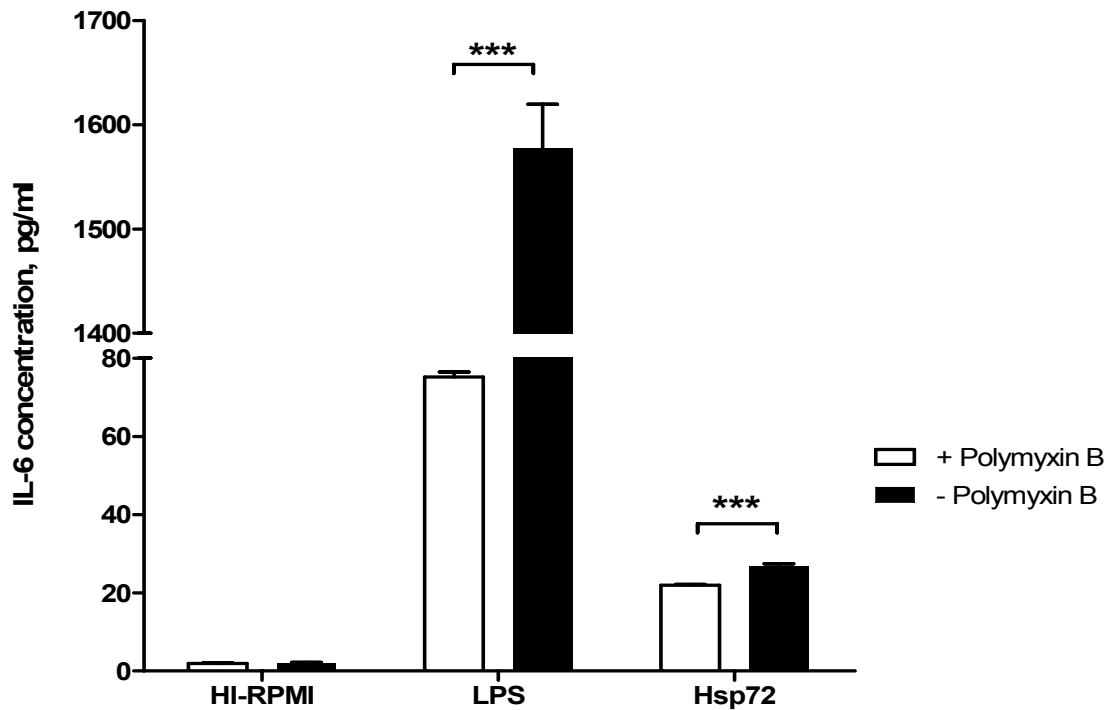


Figure 4.31: Secretion of IL-6 by U937 macrophages treated with Hsp72 or LPS with or without polymyxin B for 4 h.

U937 macrophages were treated with 1000 ng/ml Hsp72 or 100 ng/mL LPS either with or without pre-incubation with 20 μ g/mL polymyxin B. Data are presented as mean \pm SEM, n = 4. Significance shown as difference from HI-RPMI using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

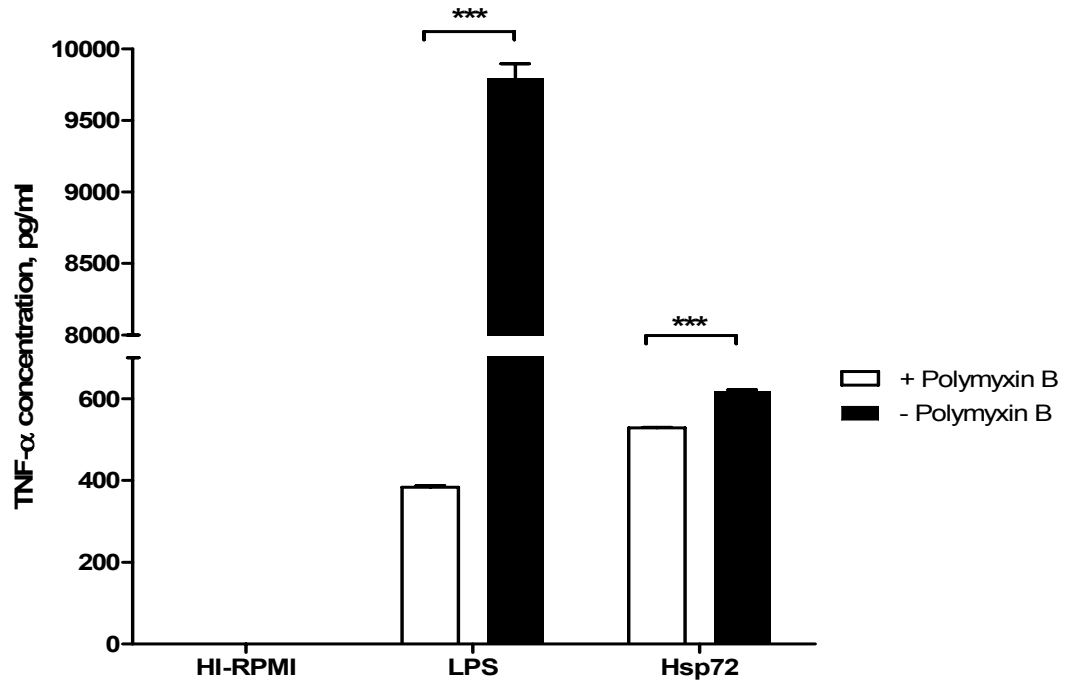


Figure 4.32: Secretion of TNF- α by U937 macrophages treated with Hsp72 or LPS with or without polymyxin B for 4 h.

U937 macrophages were treated with 1000 ng/ml Hsp72 or 100 ng/mL LPS either with or without pre-incubation with 20 μ g/mL polymyxin B. Data are presented as mean \pm SEM, n = 4. Significance shown as difference from HI-RPMI using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

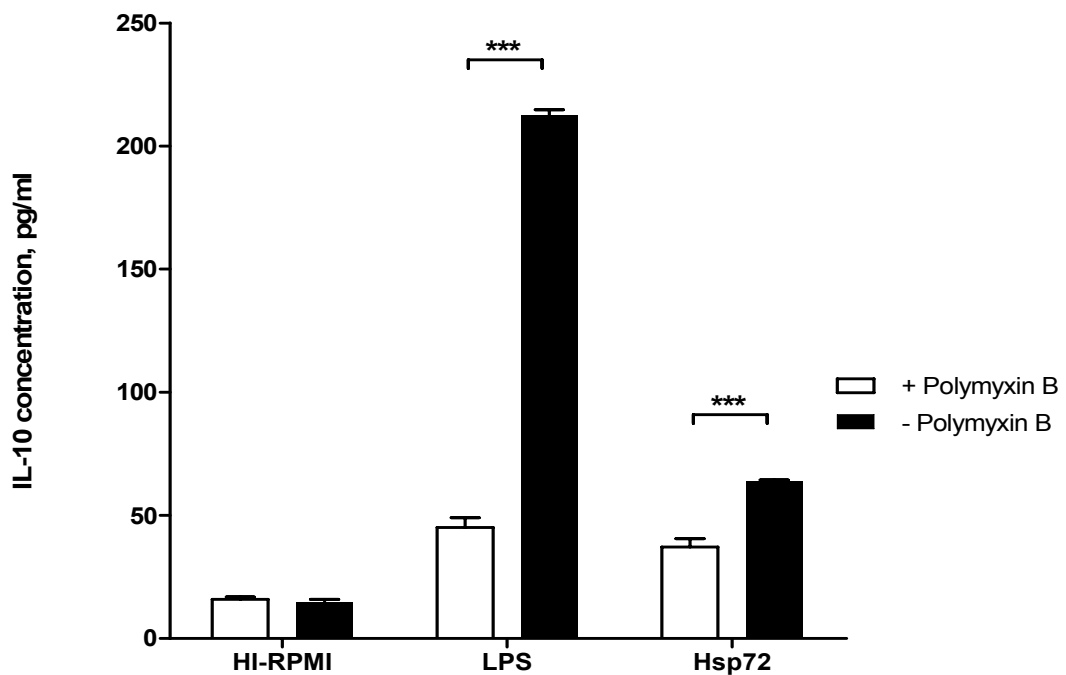


Figure 4.33: Secretion of IL-10 by U937 macrophages treated with Hsp72 or LPS with or without polymyxin B for 4 h.

U937 macrophages were treated with 1000 ng/ml Hsp72 or 100 ng/mL LPS either with or without pre-incubation with 20 µg/mL polymyxin B. Data are presented as mean ± SEM, n = 4. Significance shown as difference from HI-RPMI using two-way ANOVA with Bonferroni's multiple comparison *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

Table 4.16: Significant differences in cytokine secretion between HI-RPMI (no treatment) and Hsp72, or LPS that were pre-incubated with polymyxin B.

Significance shown as difference from HI-RPMI using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

| | IL-1β | IL-6 | TNF-α | IL-10 |
|-------|------------|--------------|--------------|------------|
| Hsp72 | * (P<0.05) | ***(P<0.001) | ***(P<0.001) | * (P<0.05) |
| LPS | * (P<0.05) | ***(P<0.001) | ***(P<0.001) | * (P<0.05) |

4.4 Discussion

The aim of this chapter was to determine whether recombinant heat shock proteins are able to induce cytokine expression and production, or if the effect is due to LPS contamination.

A number of recombinant heat shock proteins were applied to PBMCs or U937 macrophages, in native or denatured forms. It was found that all native proteins caused a dose dependent stimulation of pro- and anti- inflammatory cytokines in both cell types. When Hsp72 and Hsp60 were applied to U937 macrophages, there was a high correlation between cytokines secreted when stimulated with the native form compared to the denatured form which suggests that at least some heat shock proteins are capable of stimulating an immune response independently of LPS. This is in agreement with other studies (Kol *et al.*, 2000; Svensson *et al.*, 2006). However, as the stimulation of cytokines was not completely abrogated by denaturation, it is possible that there is an LPS component to some of the activity. Kol *et al.* (2000) demonstrated that any activity could be abrogated by boiling recombinant proteins. The experiments described here only used a relatively mild denaturation compared to boiling as proteins are able to be denatured at lower temperatures than boiling, and boiling has been demonstrated to reduce the ability of LPS to stimulate cytokine secretion by as much as 90% (Gao, Wang & Tsan, 2006). Also, as demonstrated by LAL assay, the only recombinant heat shock proteins found to contain any detectable LPS were DnaK and GroEL. When these proteins were denatured, this LPS contamination was only marginally reduced. This evidence suggests that at least some heat shock proteins are able to stimulate cytokine secretion.

When recombinant human proteins were used, there was a reduced cytokine response when compared to the equivalent bacterial protein. This is likely to be partially due to LPS contamination, but also the recognition of a foreign protein by immune cells. For example, human Hsp72 and the *E. coli* equivalent, DnaK, are both known to activate cytokine synthesis (Vabulas *et al.*, 2002; Wang *et al.*, 2005), but Hsp72 appears to be able to bind a number of receptors, including CD14 (Asea *et al.*, 2000b), TLR2/4 (Asea *et al.*, 2002), LOX-1 (Delneste, 2004) and CD40 (Becker, Hartl & Wieland, 2002) whereas DnaK only appears to bind CD40 (Nolan *et al.*, 2004).

Recombinant human Hsp60 was able to stimulate pro- and anti- inflammatory cytokines, as was GroEL, both of which are known to stimulate pro-inflammatory cytokines (Graham *et al.*, 2004) (Moré *et al.*, 2001). The response of Hsp60 could

be due to Hsp60 being bound to LPS, as it has been demonstrated that Hsp60 is able to bind LPS through a distinct region (aa 354-365) which has the motif LKGGK (Habich *et al.*, 2004; Habich *et al.*, 2005). Interestingly, Hsp72 also contains this LKGGK motif (aa 558-561) but there have been no studies reporting whether this may be an LPS-binding region. It is therefore possible that Hsp72 may act as an LPS-binding protein (LBP). Some studies have shown that the cell surface receptor for LPS involves a complex of which Hsp72 is a component which is indirect evidence that Hsp72 can bind to LPS (Triantafilou *et al.*, 2001; Triantafilou & Triantafilou, 2002).

The cytokine responses to Cpn10 and GroES were very low when compared to the other heat shock proteins, although a dose dependent increase was observed for both proteins. Again, this could be due to LPS contamination as secretion was reduced following denaturation, but it has recently been suggested that Cpn10 functions to increase the anti-inflammatory cytokine IL-10 by inhibiting the activation of NF- κ B by LPS (Johnson *et al.*, 2005). The results here are not comparative with this study as their study utilised 100 μ g/mL Cpn10 which is at least 100-fold higher than the concentrations used here.

A higher correlation between cytokines secreted was seen when U937 macrophages were incubated over a short time course with Hsp72 or LE-Hsp72, than at 24 h which demonstrated that the cytokines measured here are upregulated quickly following stimulation with Hsp72. As there was no difference between Hsp72 and LE-Hsp72, it is likely that Hsp72 itself can stimulate the cytokine response.

As Hsp72 is able to bind CD40, this leads to stimulation of the pro-inflammatory cytokine TNF- α (Becker *et al.*, 2002). Secretion of TNF- α in turn initiates the stimulation of the anti-inflammatory cytokine IL-10, which acts to inhibit the production of TNF- α , as well as IL-1 β and IL-6 (Moore *et al.*, 2001). Hsp72 is therefore likely to be involved in the pro-inflammatory modulation of the immune response, although it has recently been suggested that an LPS-free preparation of *Mycobacterial* Hsp70 does not stimulate pro-inflammatory cytokines but stimulates IL-10 (Motta *et al.*, 2007). Interestingly, when either Hsp72 or LE-Hsp72 was denatured, the only cytokine secreted was TNF- α , which is the primary immune response when stimulated with LPS. The abrogation of IL-10 secretion when Hsp72 was denatured may indicate that the primary function of extra-cellular Hsp72 is anti-inflammatory which is in agreement with the work by Motta *et al.* (2007), and has been previously suggested by others (Prakken *et al.*, 2001).

LPS contamination of recombinant Hsp72 was further investigated by pre-incubating Hsp72 with polymyxin B which is a potent inhibitor of LPS activation. When LPS (100 ng/mL) was pre-incubated with polymyxin B, there was a significant reduction in the secretion of cytokines from U937 macrophages. Pre-incubation of Hsp72 also resulted in a significant decrease in secretion. However, this reduction, although significant, did not abrogate secretion completely and was still significantly higher than untreated U937 macrophages. This again suggests that Hsp72 has the ability to stimulate cytokine secretion of immune cells independently of LPS.

The results found in this chapter conclude that many recombinant heat shock proteins, especially Hsp72, are able to stimulate a cytokine response which is likely to be independent of LPS.