

## Chapter 4: Methodological Developments in the Measurement of Stress Protein Synthesis in Leukocytes

### 4.1 Introduction

There are numerous difficulties that arise when attempting to conduct research into stress protein expression in human leukocytes using the current methodologies. Firstly, many potential study participants are reluctant to donate the volume of blood required (20 – 30 ml), especially when multiple samples are necessary. The processes of separating the leukocytes from the blood, applying the appropriate stress treatments, then extracting the protein from the leukocytes are extremely time- and labour-intensive preventing the preparation of more than a few samples at the one time. Another issue of concern is that the multiple steps involved in these processes may somehow change the way that the cells behave, thus not providing a true indication of how the cells behave *in vivo*.

Despite many years of research conducted into stress protein synthesis, there are still many questions remaining regarding appropriate and alternate methodologies for their detection. Some of these questions include:

- Whether stress protein synthesis can be measured in cryopreserved cells
- What happens to stress protein synthesis after cells are rested
- What is the minimum volume of whole blood required to measure stress protein synthesis by autoradiography and Western immunoblot
- Is it possible to heat shock whole blood and observe the heat shock response in leukocyte hsp's and/or hsp's measured in plasma
- Are there alternate methodologies to autoradiography, Western immunoblot and ELISA for the measurement of hsp's

The aim of the studies presented in this Chapter was to further develop current methodologies for the measurement of stress protein synthesis in human leukocytes.

## 4.2 Study Design and Methodologies

### 4.2.1 Expression of stress proteins in cryopreserved cells

Heparinized blood samples of approximately 25 ml were collected from 5 HIV-positive patients (4 male and 1 female) at the Albion Street Centre, Surry Hills, Sydney. These patients ranged in age from 25 – 59 years, were clinically stable with CD4+ counts between 70 and 940 and log<sub>10</sub> viral loads ranging from <1.9 to 4.9 copies/ml.

#### **Cell cryopreservation:**

Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque density centrifugation (Chapter 2). Cells were washed twice at room temperature with methionine-free RPMI. Cell density was counted using a haemocytometer (Chapter 2). Cells were resuspended, in a drop-wise manner, at between  $5 \times 10^6$  and  $10 \times 10^6$  cells/ml in chilled freezing solution (90% FCS + 10% DMSO Dimethyl sulfoxide). Cryovials containing 1 ml aliquots of cell suspension were placed in a “Mr Frosty” freezing vessel containing 100% isopropanol. The freezing vessel was immediately placed in a  $-70^\circ\text{C}$  freezer. The cells were left undisturbed in  $-70^\circ\text{C}$  freezer for between 4 h and 24 h. The frozen vials were removed from the freezing vessel and placed in liquid nitrogen tank for long term storage.

#### **Thawing cells**

For thawing, the cryovials were transferred from the liquid nitrogen tank to a  $37^\circ\text{C}$  water bath. Cryovials were held at the surface of the water bath with an occasional gentle flick during thawing to allow mixing and maintaining even temperature in the vial. When a small ice crystal remained in the cryovials, they were transferred to a biosafety cabinet. The cells were transferred from the cryovials to 15 ml Falcon centrifuge tubes. Approximately 10 ml chilled complete media (RPMI +) was added in a drop-wise manner over a 2 min period. Cells were washed twice with complete media by centrifugation ( $300 \text{ g} \times 10 \text{ min}$ ), with gentle resuspending of the cells between washes. Cell number and viability were determined using a haemocytometer (Chapter 2).

Following thawing, cells were resuspended in RPMI medium without methionine and supplemented with 10% foetal calf serum (FCS). Stress treatment, analysis of protein synthesis and Western immunoblotting were carried out according to the procedures outlined in Chapter 2.

#### **4.2.2 Minimum volume of blood required to measure stress protein expression**

A heparinized blood sample of approximately 20 ml was collected from one male volunteer, by staff at the Armidale and New England Hospital Pathology Laboratory. Heparinized, whole blood volumes of 5 ml and 10 ml were used for the analysis of hsp70 expression by Western immunoblotting as per the techniques described in Chapter 2.

#### **4.2.3 Comparison of hsp70 expression in plasma and serum**

Blood samples were collected by staff at the pathology department of the Armidale and New England Hospital into one 5 ml blood collection tube containing an anticoagulant (lithium heparin) and one 5 ml tube without anticoagulant from one male and one female volunteer. Samples were centrifuged at 400 x *g* for 10 min after which plasma and serum were removed from the tubes and analyzed for hsp70 by ELISA (Chapter 2). Statistical analysis was conducted using the Student's t-test (paired).

#### **4.2.4 Kinetics of expression of plasma hsp70 after heat shock**

Heparinized blood samples of approximately 9 ml were collected from one male and one female volunteer by staff at the pathology department of the Armidale and New England Hospital. The blood samples were divided into 2 x 10 ml centrifuge tubes per sample and placed in water baths for standard heat shock protocol (Chapter 2). One ml aliquots were removed at 1 h (after rest), 2 h (after heat shock), 3 h (1 h after heat shock), 4 h (2 h after heat shock), 5 h (3 h after heat shock). Samples were centrifuged at 400 x *g* for 10 min, then frozen at -80°C for later analysis of hsp70 levels by ELISA (Chapter 2). Statistical analysis was conducted using the Student's t-test (paired).

#### **4.2.5 Flow cytometric analysis of hsp70 expression in leukocytes**

Heparinized blood samples of approximately 30 ml were collected from one male and one female volunteer by staff at the Armidale and New England Hospital Pathology Laboratory.

#### **Cell preparation**

Leukocytes were isolated from a 25 ml portion of the whole blood samples by Ficoll-Paque density gradient centrifugation and cells were counted using the Trypan Blue Exclusion Method as per Chapter 2. Cells were resuspended in RPMI culture media

supplemented with 10 % heat inactivated FCS, to a concentration of  $1 \times 10^6$ /ml. The remaining 5 ml whole blood samples were aliquoted into centrifuge tubes.

### **Heat shock**

All cell and whole blood samples were allowed to rest for 1 h in a 37°C water bath. Heat shock' samples were exposed to a 42.5°C thermal challenge for 1 h by floating microfuge tubes in a thermostatically controlled water bath. 'Control' samples were placed into a 37°C water bath during this time. All samples were allowed to recover for 3 h at 37°C in a water bath. Cells (but not whole blood) were washed in PBS by centrifugation ( $400 \times g$  for 3 min), then transferred to 5 ml disposable polypropylene tubes.

### **Fixation**

Cells were resuspended in 100  $\mu$ l 0.3 % w/v paraformaldehyde solution. Similarly, 100  $\mu$ l 0.3 % w/v paraformaldehyde solution was added to 200  $\mu$ l whole blood samples. Cells and whole blood were incubated for 10 min at room temperature. Samples were washed by centrifugation ( $470 \times g$  for 3 min) in 1 ml PBS supplemented with 1 % BSA or FCS.

### **Permeabilization, hsp70 detection and staining FITC- anti-hsp70**

Samples were resuspended in 50  $\mu$ l of 0.6% v/v saponin in PBS and 50  $\mu$ l Fluorescein-(FITC)-anti-Hsp70 antibody diluted to 1:100 in PBS-BSA/FCS and incubated for 10 min at room temperature. Samples were washed in 1 ml PBS-BSA/FCS centrifuging for 3 min at  $400 \times g$ . Samples were resuspended in 50  $\mu$ l saponin solution and incubated for a further 10 min at room temperature, in the dark. Fifty  $\mu$ l anti-CD14- Phycoerythrin-(PE)-conjugated antibody (diluted to 1:100 in PBS-BSA/FCS) was added and samples were incubated for 10 min at 4°C. For isolated leukocytes, cells were washed in PBS-BSA, centrifuged for 3 min at  $400 \times g$ , then resuspended in 1 ml PBS-BSA/FCS and kept at 4°C in the dark until flow cytometry analysis.

### **Erythrocyte lysis**

Formalin in PBS (100  $\mu$ l, 8 % w/v) was added to each tube of whole blood. Tubes were flicked to mix and left to sit for 1-2 min. Warm (37°C ) ddH<sub>2</sub>O (1 ml ) was added to each tube which was flicked to mix then placed in a 37°C water bath for 3-4 min.

PBS (2 ml) was added, then tubes were centrifuged at 1400-1800 rpm at 4°C for 5 min. The supernatant was removed and 2 ml PBS added to each tube. Samples were centrifuged at 1400-1800 rpm at 4°C for 5 min. The supernatant was removed and 200 µl PBS added to each tube. Samples were covered and store at 4°C until analysis was completed using a FACSVantage flow cytometer (Becton Dickinson) with CELL Quest software.

#### 4.2.6 Multicolour flow cytometric analysis of hsp70 expression in leukocyte subpopulations

A 6 ml heparinized blood sample was collected from a female staff member of the Garvan Institute for Medical Research, Sydney.

**Table 4.1: Antibodies to extracellular leukocyte markers**

| TUBE     | ANTIBODIES USED |           |             |            |          |            |
|----------|-----------------|-----------|-------------|------------|----------|------------|
|          | COLOUR 1        | COLOUR 2  | COLOUR 3    | COLOUR 4   | COLOUR 5 | COLOUR 6   |
| <b>A</b> | PerCP-CD3       | PECy7-CD4 | APCCy7- CD8 | PE- CD19   | APC-CD56 | FITC-hsp70 |
| <b>B</b> | PerCP- HLA-DR   | PECy7-CD4 | PE-CD36     | FITC-hsp70 | -        | -          |

**Fluorochrome key:** APC: Allophycocyanin, APCCy7: Allophycocyanin-Cy7, FITC: Fluorescein, PE: Phycoerythrin, PECy7: Phycoerythrin-Cy7, PerCP: Peridinin Chlorophyll Protein.

Antibodies to leukocyte extracellular markers conjugated to various fluorochromes were aliquoted into FACS tubes in the order listed in Table 4.1. (Eight tubes labeled 37A, 37A control, 37B, 37B control, 42.5A, 42.5A control, 42.5, 42.5B control). Whole blood (100 µl) was added to each tube and tubes were gently vortexed. Samples were incubated at room temperature for 12 – 15 min. FACSLyse (2 ml) was added then tubes were gently vortexed. Samples were incubated at room temperature for 10 min. Tubes were centrifuged at 1500 rpm for 7 min. The supernatant was discarded and 500 µl FACSPerm was added, tubes were gently vortexed and samples incubated at room temperature for a further 10 min. Two ml PBA was added and tubes were centrifuged at 1500 rpm for 7 min. The supernatant was discarded and human immunoglobulin (expired IV INTRAGAM {IgG}) was added. The isotype control (20 µl IgG-FITC) was added to control tubes and the intracellular antibody (50 µl anti hsp70-FITC diluted 1:100) was added to test tubes. Samples were incubated for 30 min in the dark, at room temperature. PBA (2 ml) was added and tubes were centrifuged at 1700 rpm for 7 min. The supernatant was discarded and 500 µl, 0.5 %

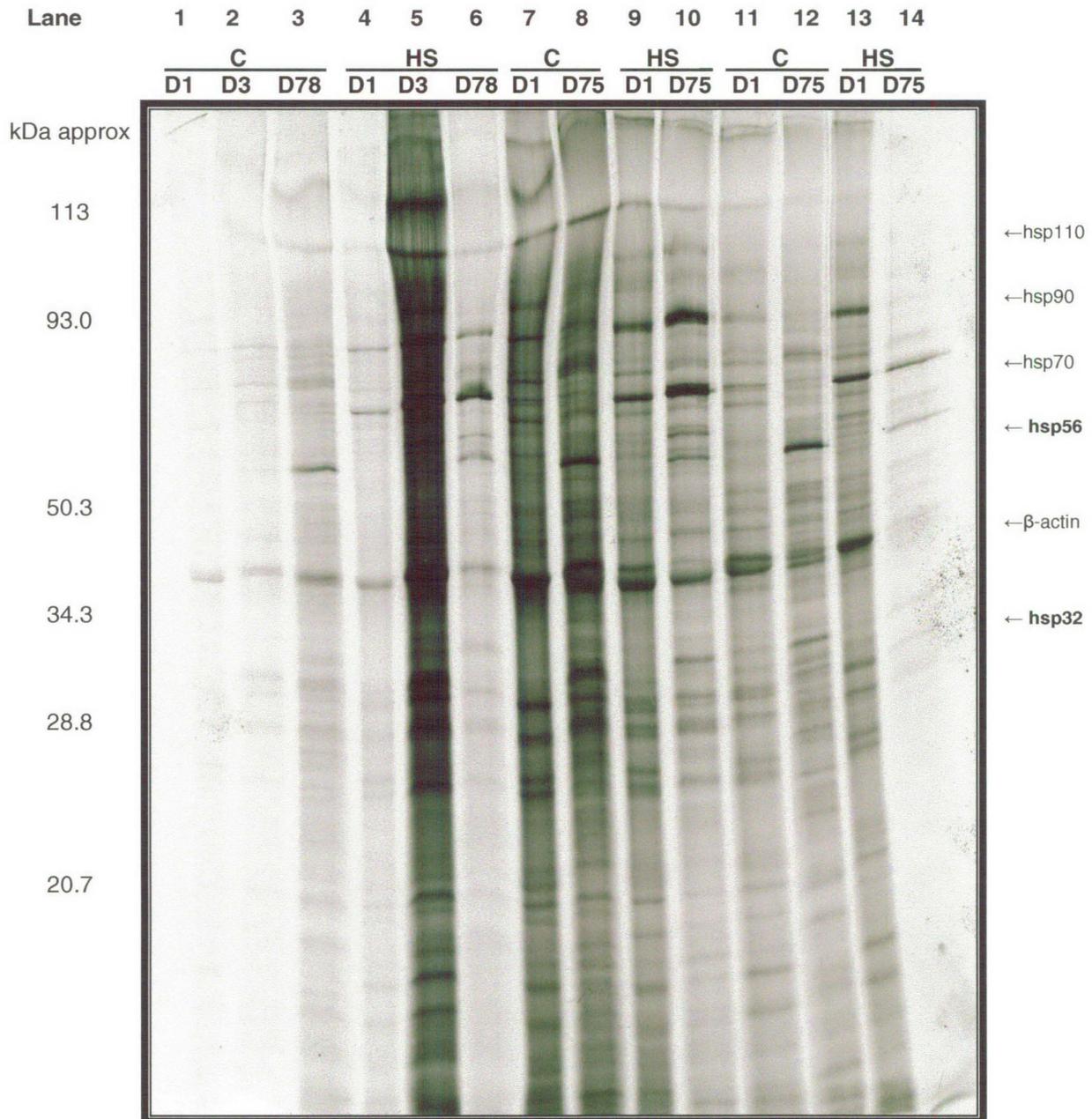
(v/v) formaldehyde in PBS was added. Analysis was performed on a LSR 2 flow cytometer (Becton Dickinson) with FACSDiva software.

## **4.3 Results**

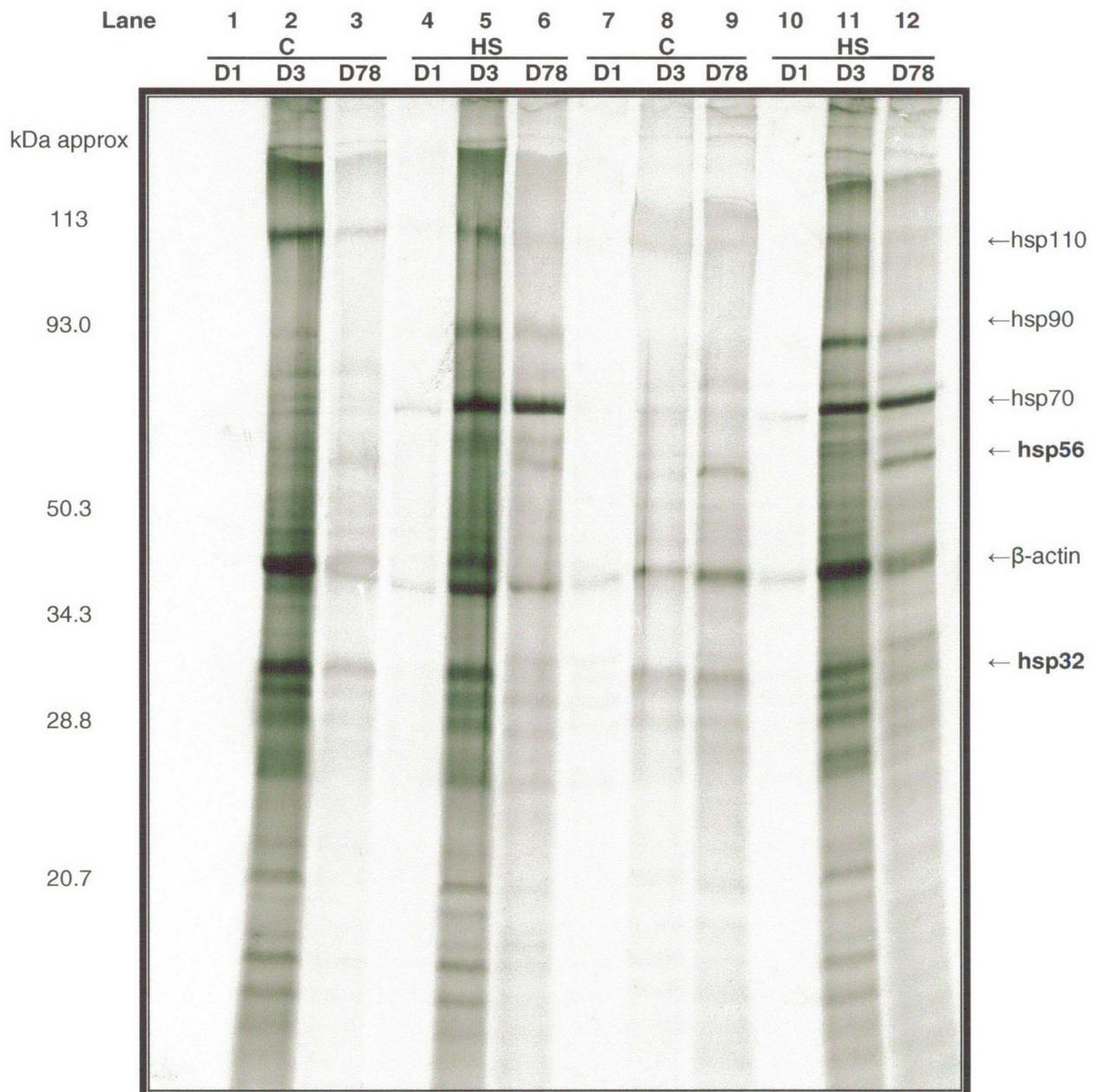
### **4.3.1 Expression of stress proteins in cryopreserved cells**

#### **Autoradiograms**

<sup>35</sup>S labeled proteins (10 µg) extracted from lymphocytes were run on 1D SDS-PAGE to determine the effect of cryopreservation on induction of hsp's in leukocytes. Figures 4.1 and 4.2 demonstrated that lymphocytes are able to synthesize new proteins after cryopreservation. Fresh (D1) and cryopreserved (D3, D75, D78) samples from each subject were run side by side in each gel. Control (37°C) and heat shock (42.5°C) samples from each subject were similarly grouped (Figures 4.1 and 4.2).



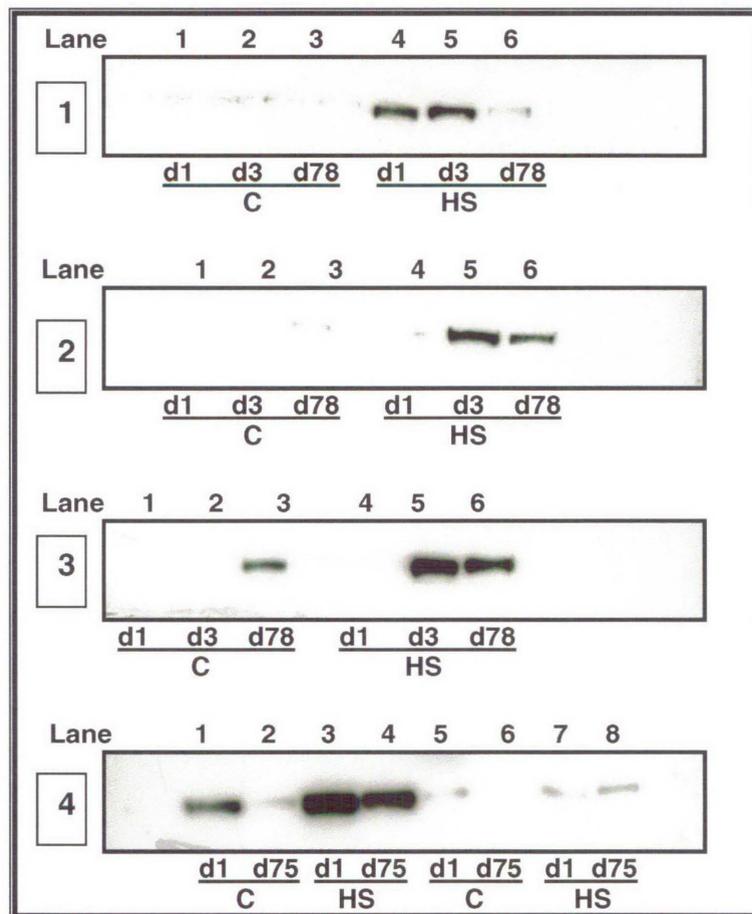
**Figure 4.1: The effect of cryopreservation on *de novo* protein synthesis.** Autoradiogram of SDS-PAGE gel of lymphocytes obtained from three HIV-positive individuals. C = control samples incubated at 37°C for 4 h. HS = Heat shock samples incubated at 42.5°C for 1 h then at 37°C for 3 h. D1 = protein extracted from lymphocytes on the same day as blood collected. D3 = sample frozen in liquid nitrogen for 3 days then protein extracted from lymphocytes. D75 = sample frozen in liquid nitrogen for 75 days then protein extracted from lymphocytes. D78 = sample frozen in liquid nitrogen for 78 days then protein extracted from lymphocytes. Lanes 1 – 6 = individual 1, Lanes 7 – 10 = individual 2, Lanes 11 – 14 = individual 3.



**Figure 4.2: The effect of cryopreservation on *de novo* protein synthesis.** Autoradiogram of SDS-PAGE gel of lymphocytes obtained from two HIV-positive individuals. C = control samples incubated at 37°C for 4 h. HS = Heat shock samples incubated at 42.5°C for 1 h then at 37°C for 3 h. D1 = protein extracted from lymphocytes on the same day as blood collected. D3 = sample frozen in liquid nitrogen for 3 days then protein extracted from lymphocytes. D78 = sample frozen in liquid nitrogen for 78 days then protein extracted from lymphocytes. Lanes 1 – 6 = individual 4, Lanes 7 – 12 = individual 5.

### Western immunoblots

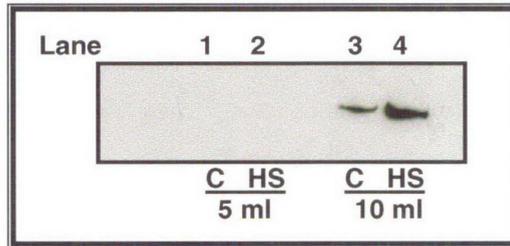
Lymphocyte protein extracts were examined by Western immunoblots to determine the effect of cryopreservation on total lymphocyte hsp70 levels. Fresh (D1) and cryopreserved (D3, D75, D78) samples were run side by side in each gel. Control (37°C) and heat shock (42.5°C) samples from each subject were similarly grouped (Figure 4.3).



**Figure 4.3: Western immunoblots of hsp70 expression in cryopreserved lymphocytes.** C = control samples incubated at 37°C for 4 h. HS = Heat shock samples incubated at 42.5°C for 1 h then at 37°C for 3 h. D1 = protein extracted from lymphocytes on the same day as blood collected. D3 = sample frozen in liquid nitrogen for 3 days then protein extracted from lymphocytes. D75 = sample frozen in liquid nitrogen for 75 days then protein extracted from lymphocytes. D78 = sample frozen in liquid nitrogen for 78 days then protein extracted from lymphocytes.

### 4.3.2 Minimum volume of blood required to measure stress protein expression

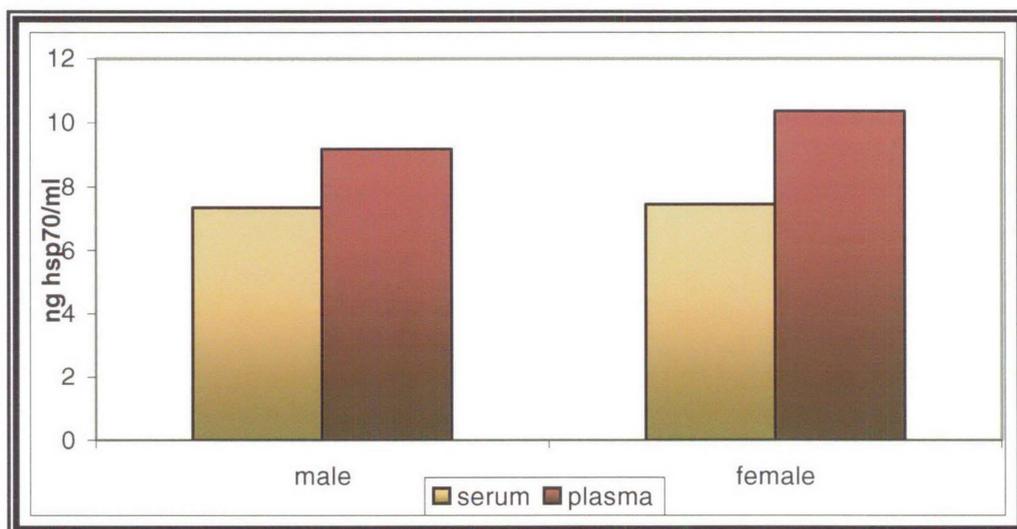
The results of the current study clearly demonstrate that it is possible to measure hsp70 expression by Western immunoblot from a 10 ml sample of whole blood. The 5 ml sample of whole blood did not yield adequate protein, therefore there were no visible bands for either the control or heat shock treated cells (Figure 4.4).



**Figure 4.4: Western immunoblot of hsp70 expression in lymphocytes.** Lane 1: protein extracted from 5 ml whole blood. C = control samples incubated at 37°C for 4 h. Lane 2: protein extracted from 5 ml whole blood. HS = heat shock samples incubated at 42.5°C for 1 h then at 37°C for 3 h. Lane 3: protein extracted from 10 ml whole blood. C = control samples incubated at 37°C for 4 h. Lane 4: protein extracted from 10 ml whole blood. HS = heat shock samples incubated at 42.5°C for 1 h then at 37°C for 3 h.

### 4.3.3 Comparison of hsp70 expression in plasma and serum

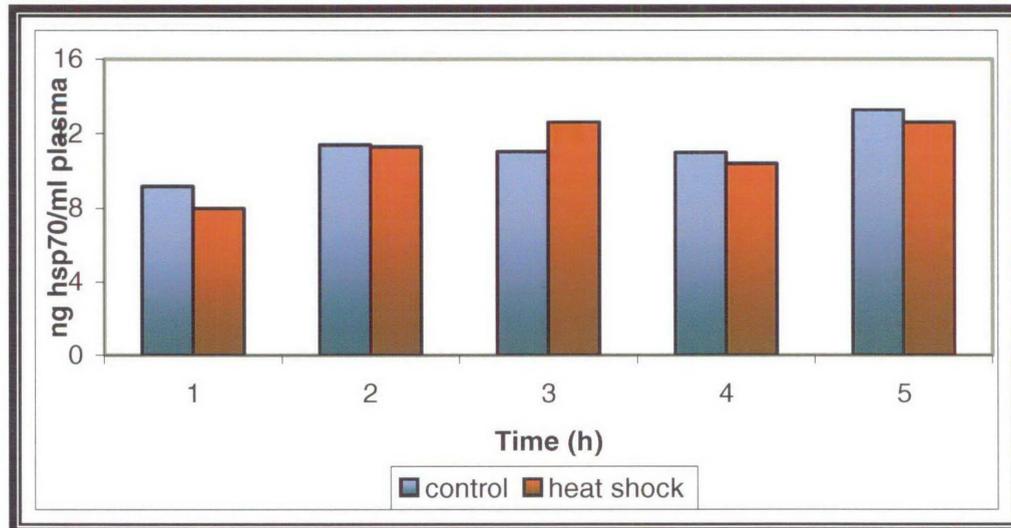
The results of experiments comparing the expression of hsp70 in plasma and serum are illustrated in Figure 4.5. Whilst visually it appeared that hsp70 expression was greater in plasma samples than in serum samples for the two individuals tested, the differences were not statistically significant according to a paired t-test ( $p > .05$ ).



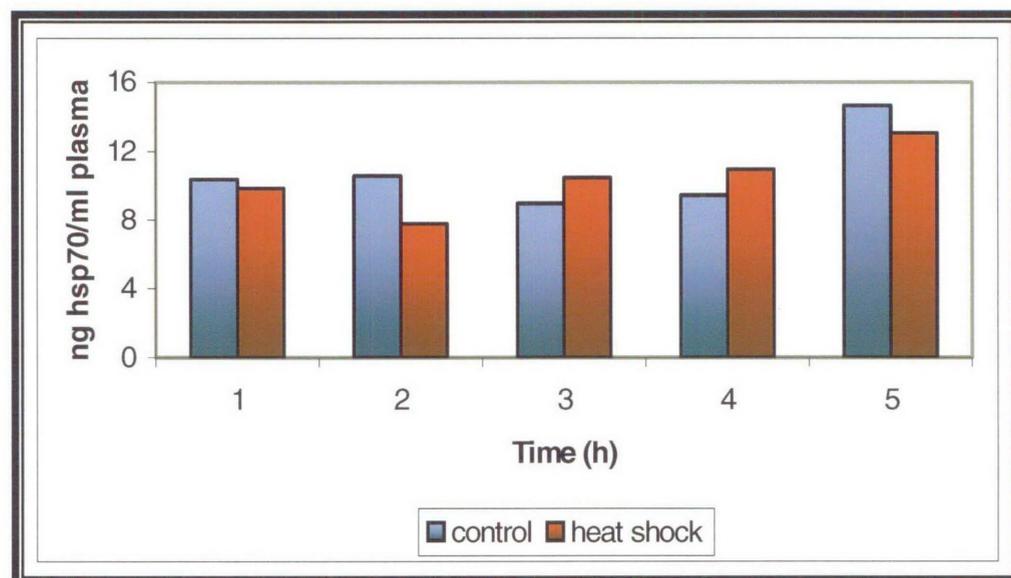
**Figure 4.5: Comparison of hsp70 levels in plasma and serum.** ELISA analysis of hsp70 expression in plasma and serum collected from one male and one female volunteer. Male serum 7.35 ng/ml; plasma 9.18 ng/ml ( $p = 0.28$ ). Female serum 7.46 ng/ml; plasma 10.36 ng/ml ( $p = 0.24$ ).

#### 4.3.4 Kinetics of expression of plasma hsp70 after heat shock

The results of experiments to determine the kinetics of hsp70 expression in plasma following a mild, non-lethal heat shock (42.5°C) are presented in Figures 4.6 and 4.7. Paired t-tests revealed there was no difference between control or heat shock plasma hsp70 levels in either the male ( $p = 0.70$ ) or the female ( $p = 0.67$ ) samples, over the 5 h period of measurement.



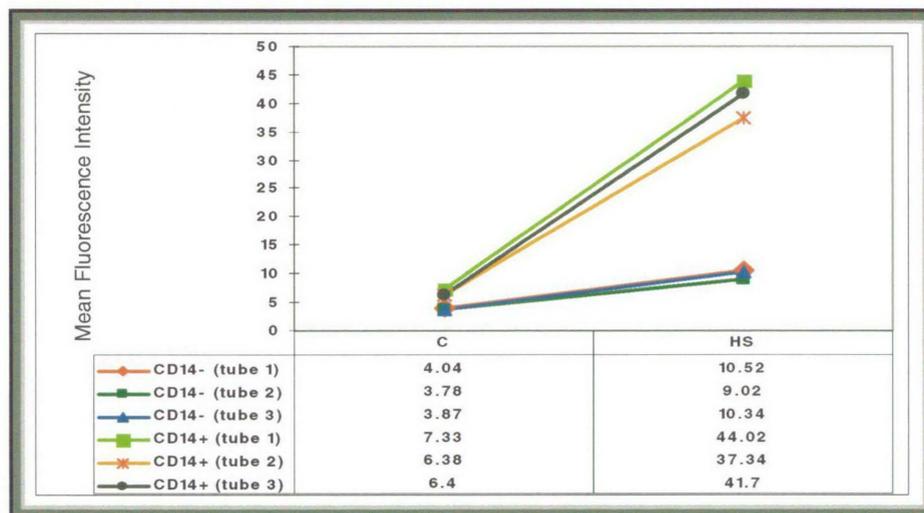
**Figure 4.6: Male hsp70 plasma kinetics.** Expression of hsp70 in plasma over a 5 h time period as measured by ELISA. Control samples were incubated at 37°C. Heat shock samples were incubated at 42.5°C.



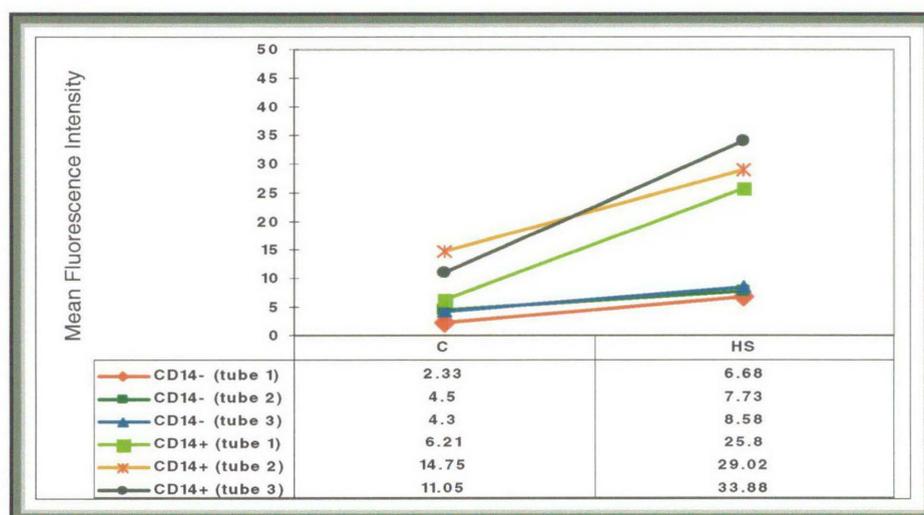
**Figure 4.7: Female hsp70 plasma kinetics.** Expression of hsp70 in plasma over a 5 h time period as measured by ELISA. Control samples were incubated at 37°C. Heat shock samples were incubated at 42.5°C.

### 4.3.5 Flow cytometric analysis of hsp70 expression in leukocytes

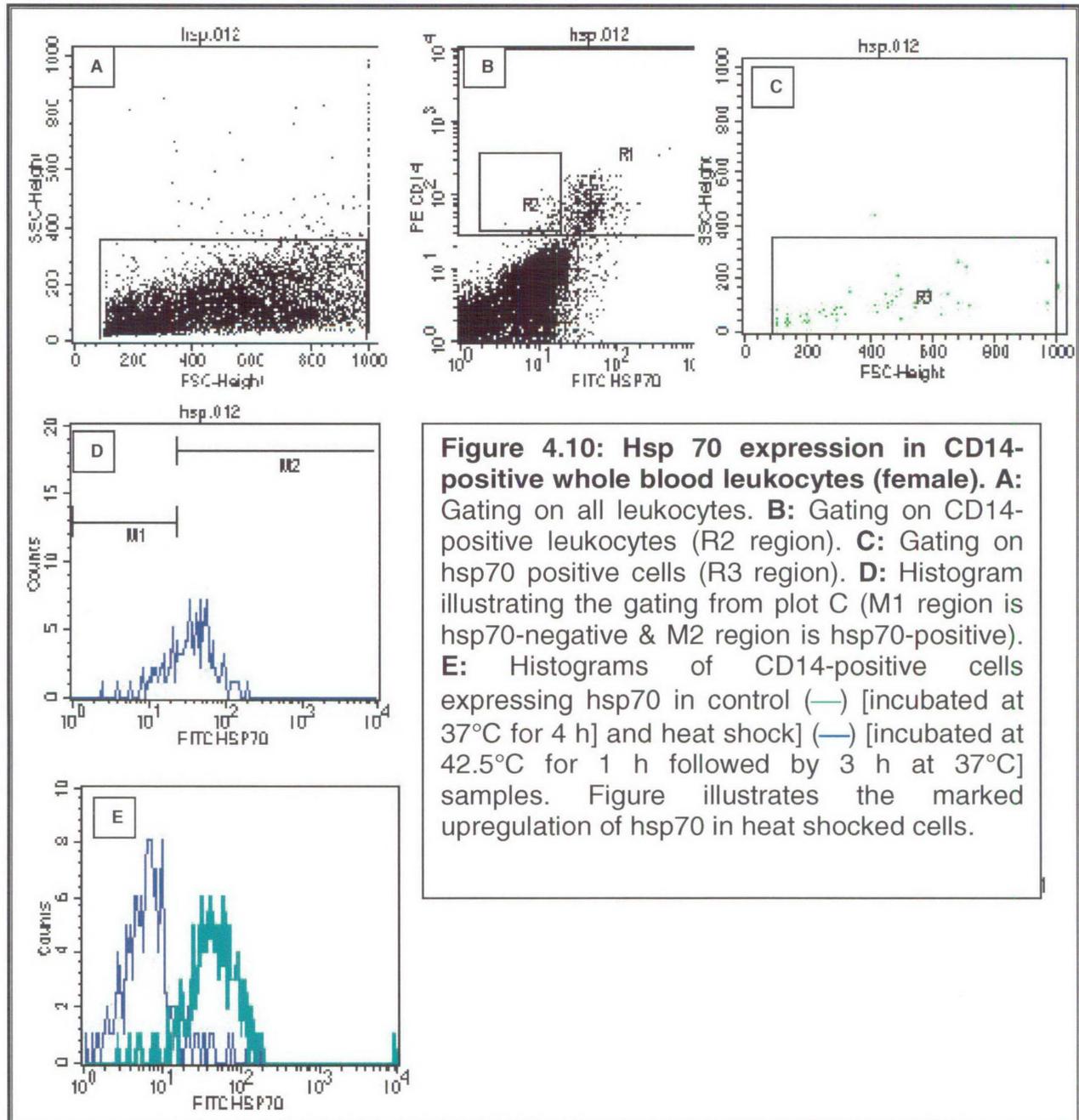
The staining of CD14-positive cells from the leukocytes harvested by Ficoll-Paque gradient centrifugation was variable and minimal. The hsp70 staining, however, was consistent. Subsequently, there was very little dual CD14-hsp70 staining (results not shown). The whole blood staining was markedly more successful with good CD14, hsp70 and subsequent dual staining with consistent results across triplicate samples. There was a significant upregulation in hsp70 expression in the heat shocked whole blood samples. Differences were also noted in the expression of hsp70 in CD14-positive (Figure 4.8) and CD14-negative (Figure 4.9) leukocytes in both control and heat shock samples.

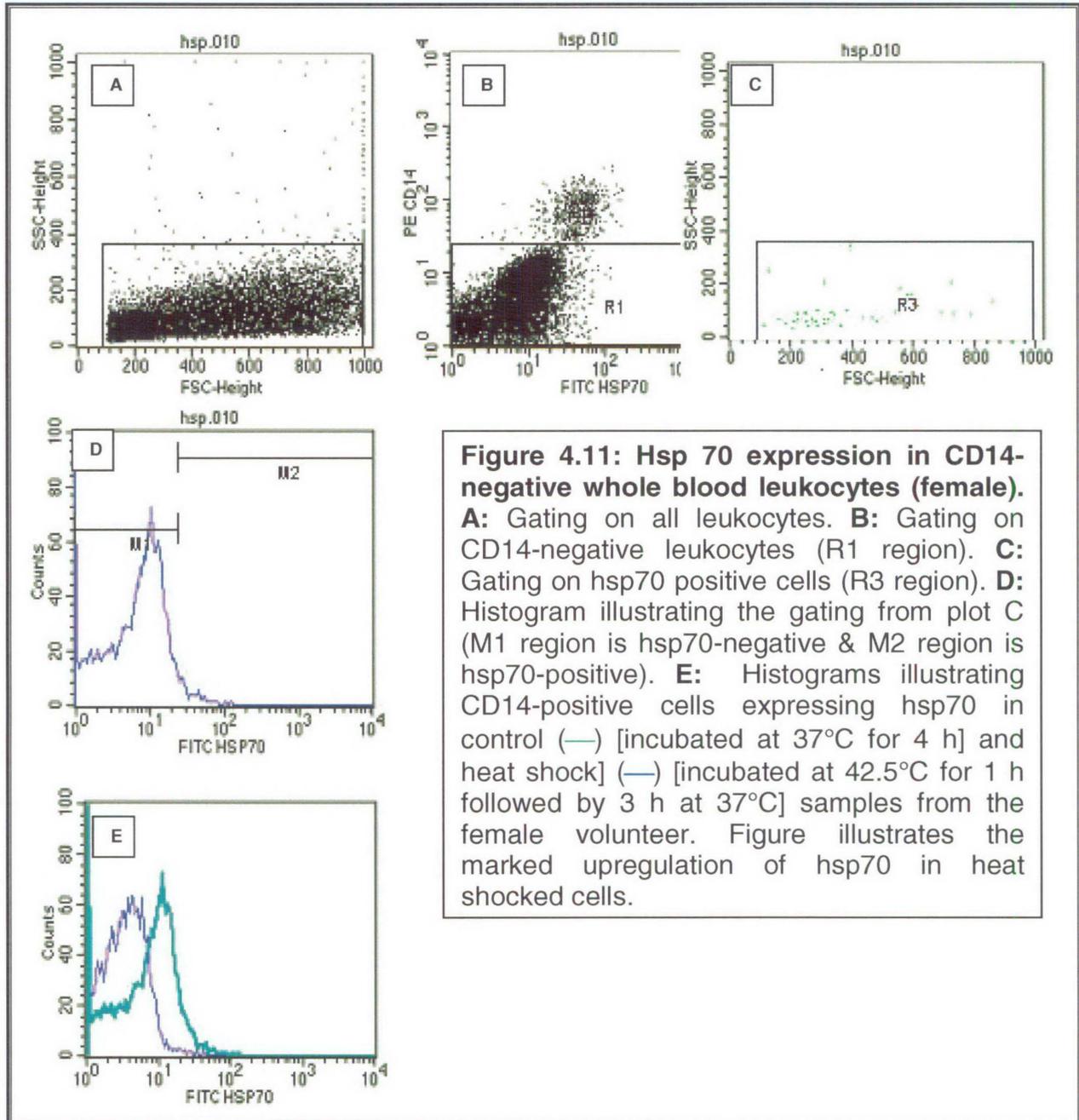


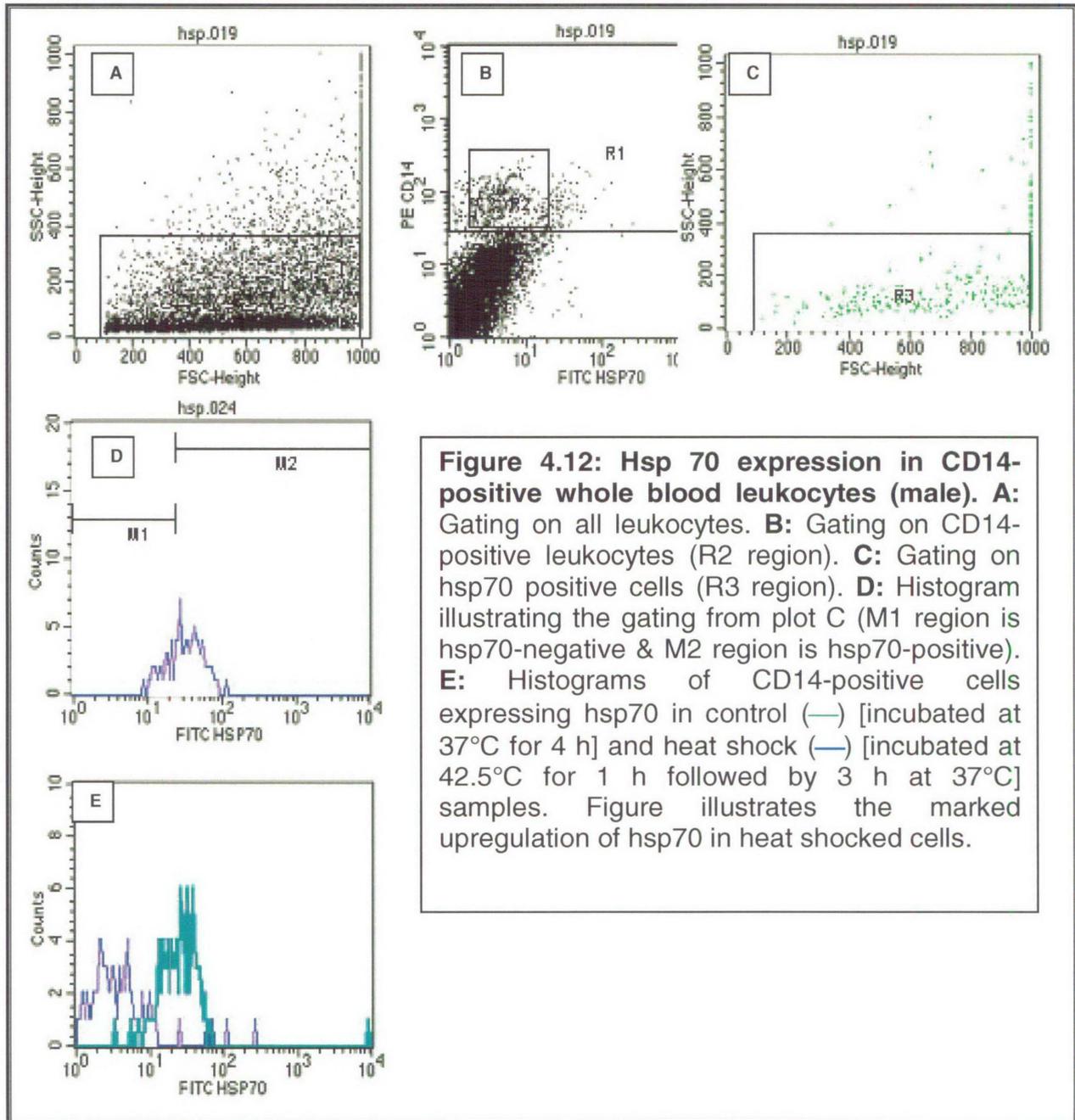
**Figure 4.8: Comparison of hsp70 expression CD14-positive and CD14-negative cells from the female volunteer.** C = control, HS = heat shock. Values are for three replicate samples from whole blood.

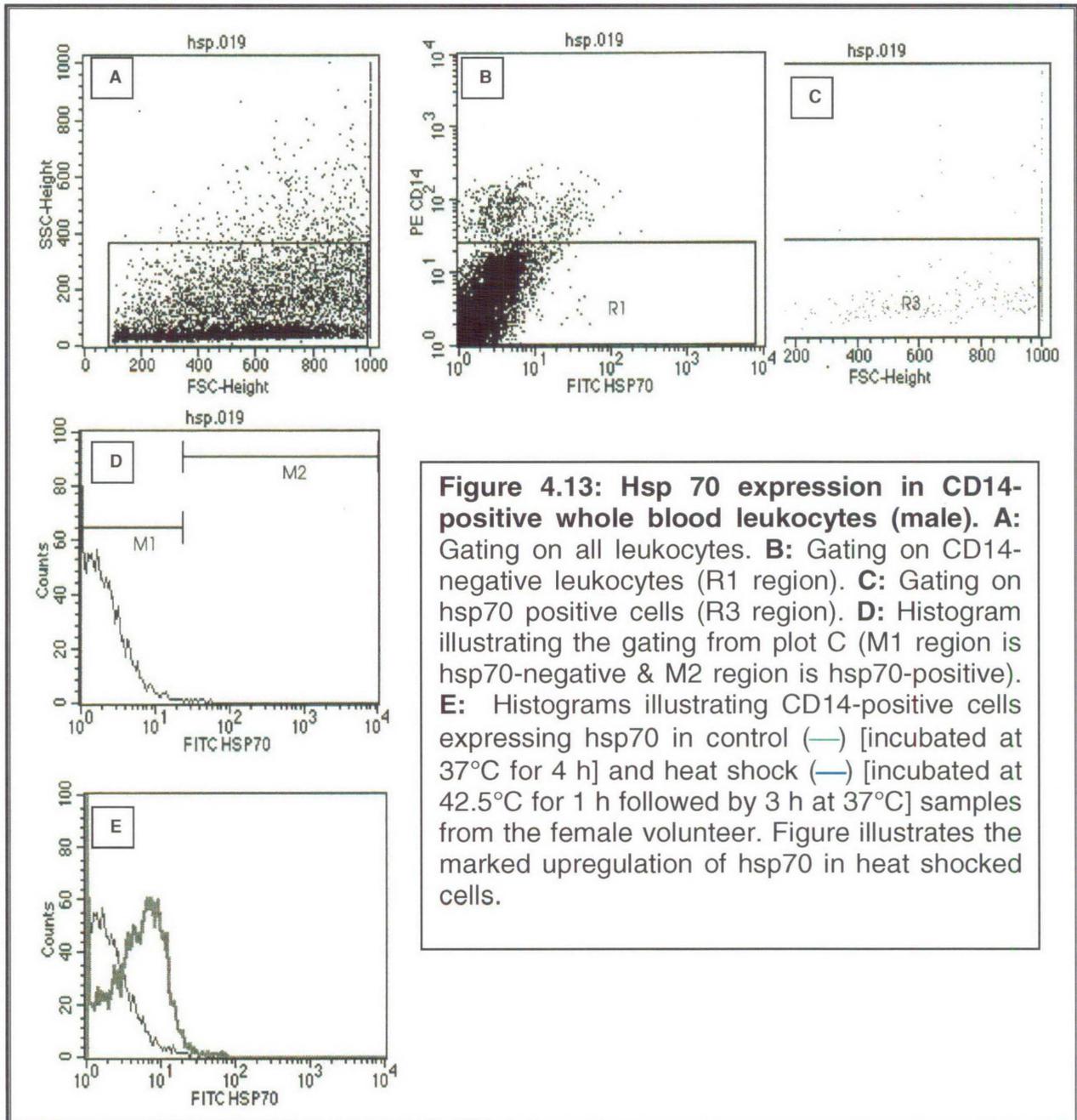


**Figure 4.9: Comparison of hsp70 expression CD14-positive and CD14-negative cells from the male volunteer.** C = control, HS = heat shock. Values are for three replicate samples from whole blood.



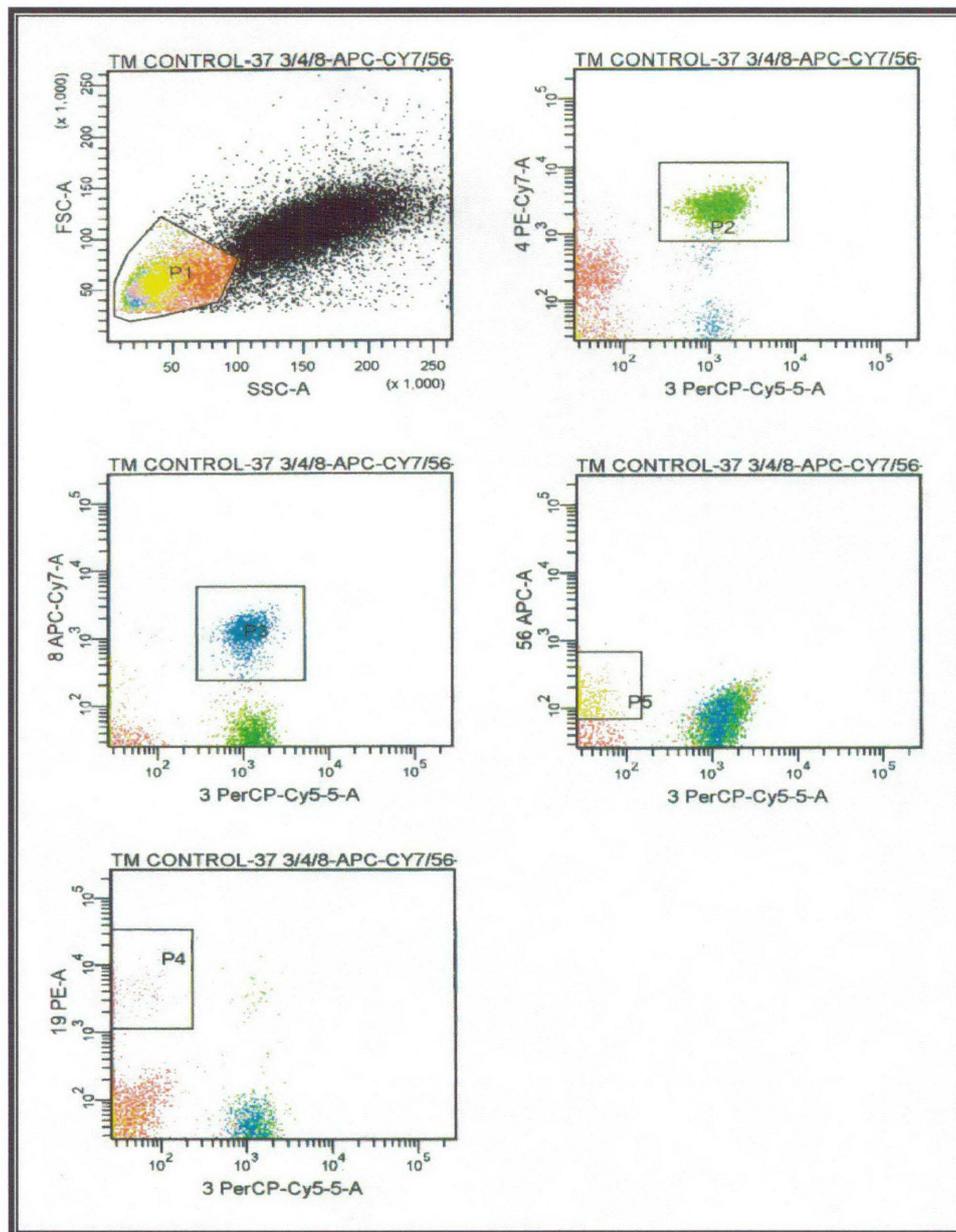




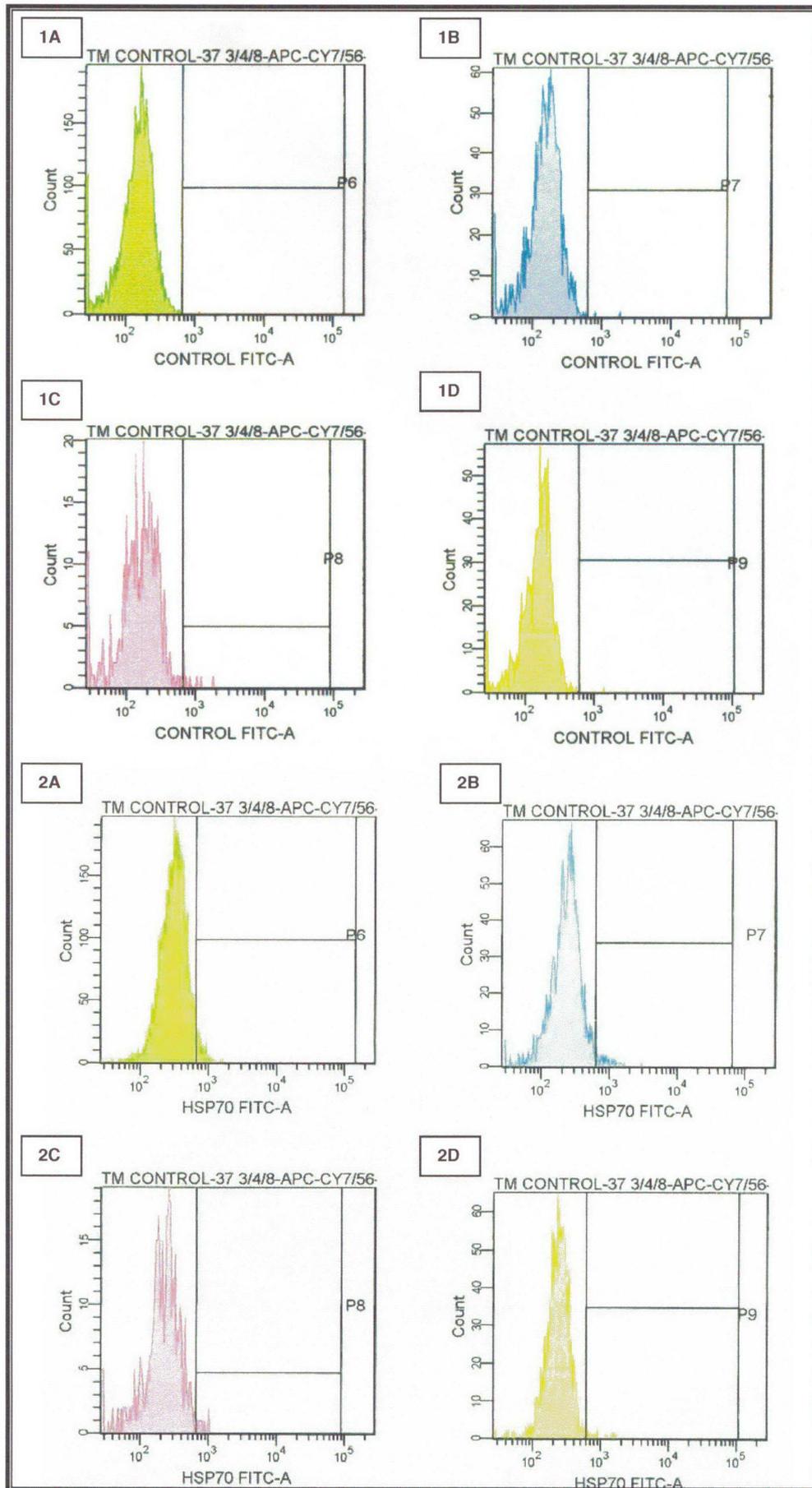


#### 4.2.6 Multi-colour flow cytometric analysis of leukocyte hsp70 expression

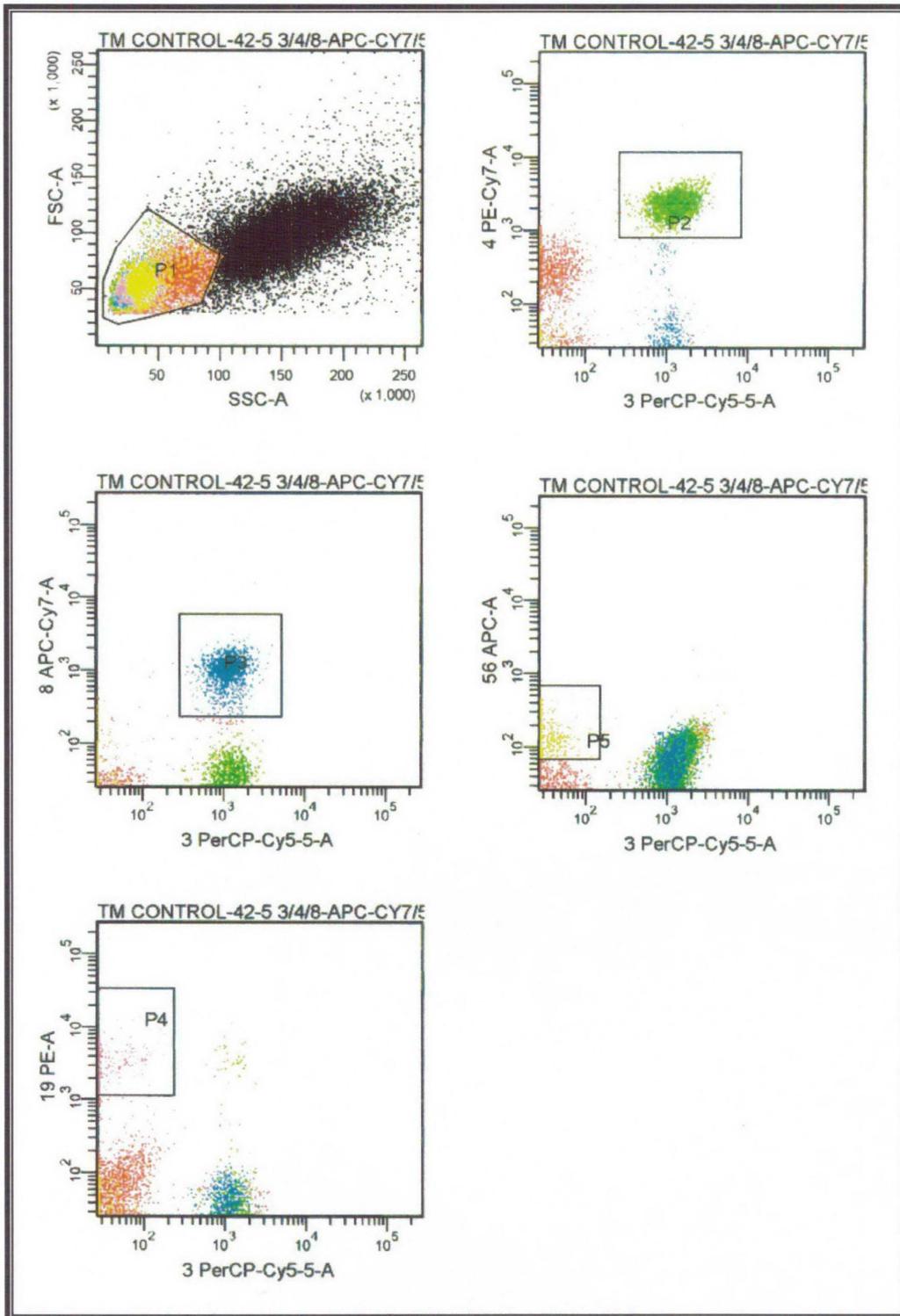
Leukocytes examined by flow cytometry displayed differential patterns of hsp70 expression at both basal and heat shock levels (Figure 4.22). All of the leukocyte cell types examined (helper T lymphocytes, cytotoxic T lymphocytes, B lymphocytes, Natural Killer cells and monocytes) displayed a significant upregulation in hsp70 after a mild, non-lethal heat shock. Gating on specific cell types is illustrated in Figures 4.14, 4.16, 4.18 and 4.20. Histograms of control isotype staining and hsp70 expression at basal and heat shock levels are presented in Figures 4.15, 4.17, 4.19 and 4.21.



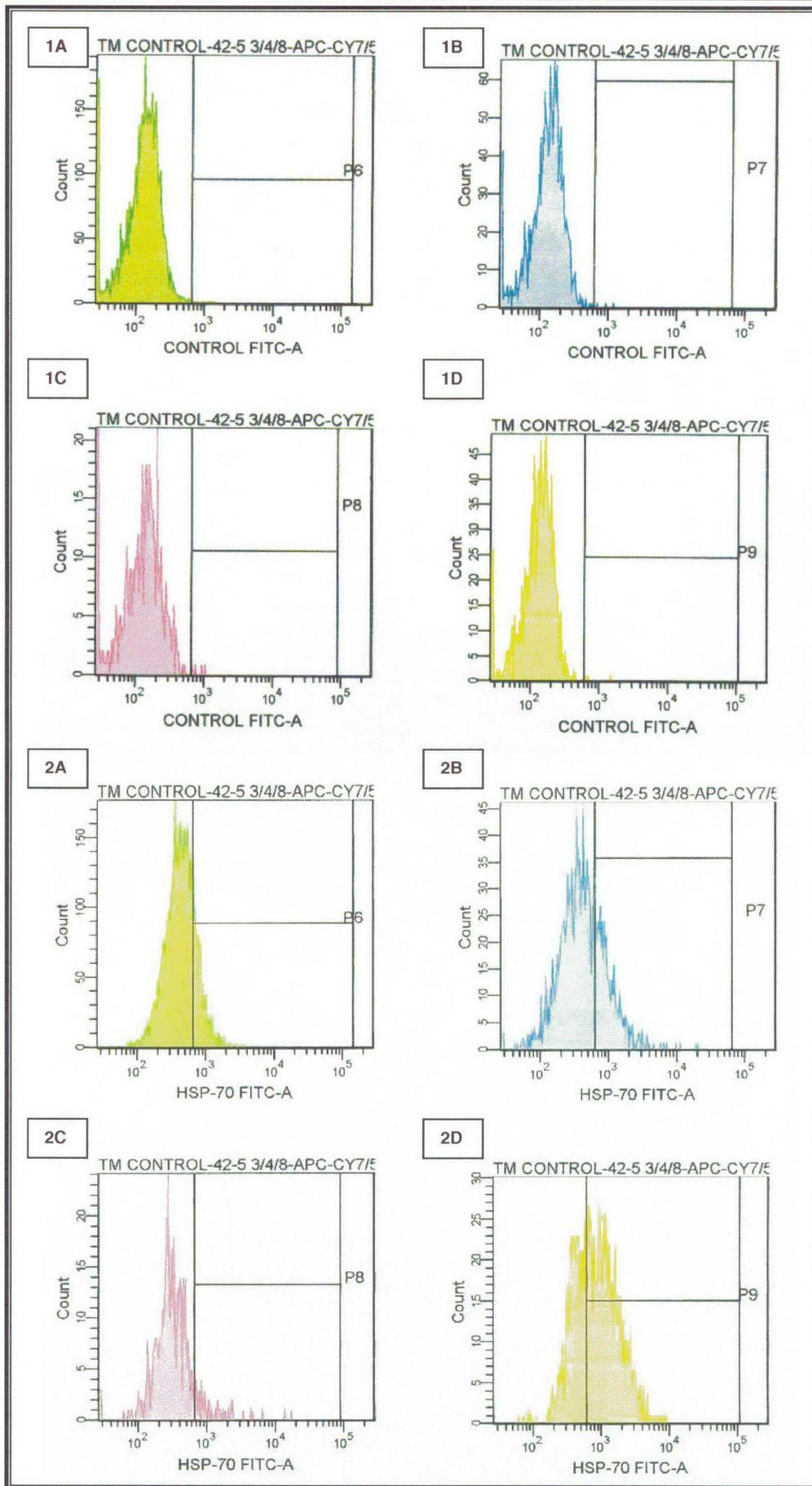
**Figure 4.14: Gating on leukocyte sub-populations at 37°C.** P1 = gating on all CD3+ leukocytes. P2 = gating on CD3+ cells that were also CD4-positive. P3 = gating on CD3+ leukocytes that were also CD8-positive, P4 = gating on CD3+ cells that were also CD19-positive. P5 = gating on CD3+ cells that were also CD56-positive.



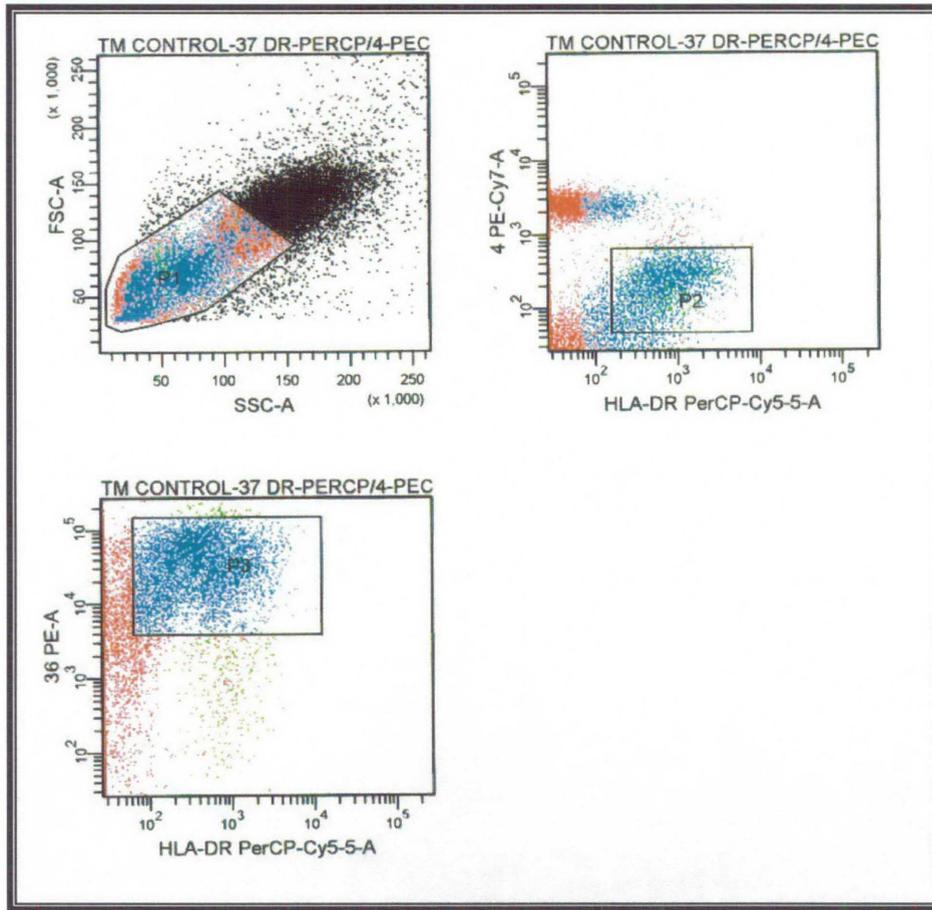
**Figure 4.15: Histograms depicting leukocyte hsp70 expression at 37°C.** 1 = IgG1 isotype control staining. 2 = Hsp70 staining. A = CD4+ T lymphocytes. B = CD8+ T lymphocytes. C = CD19+ B lymphocytes. D = CD56+ Natural killer cells.



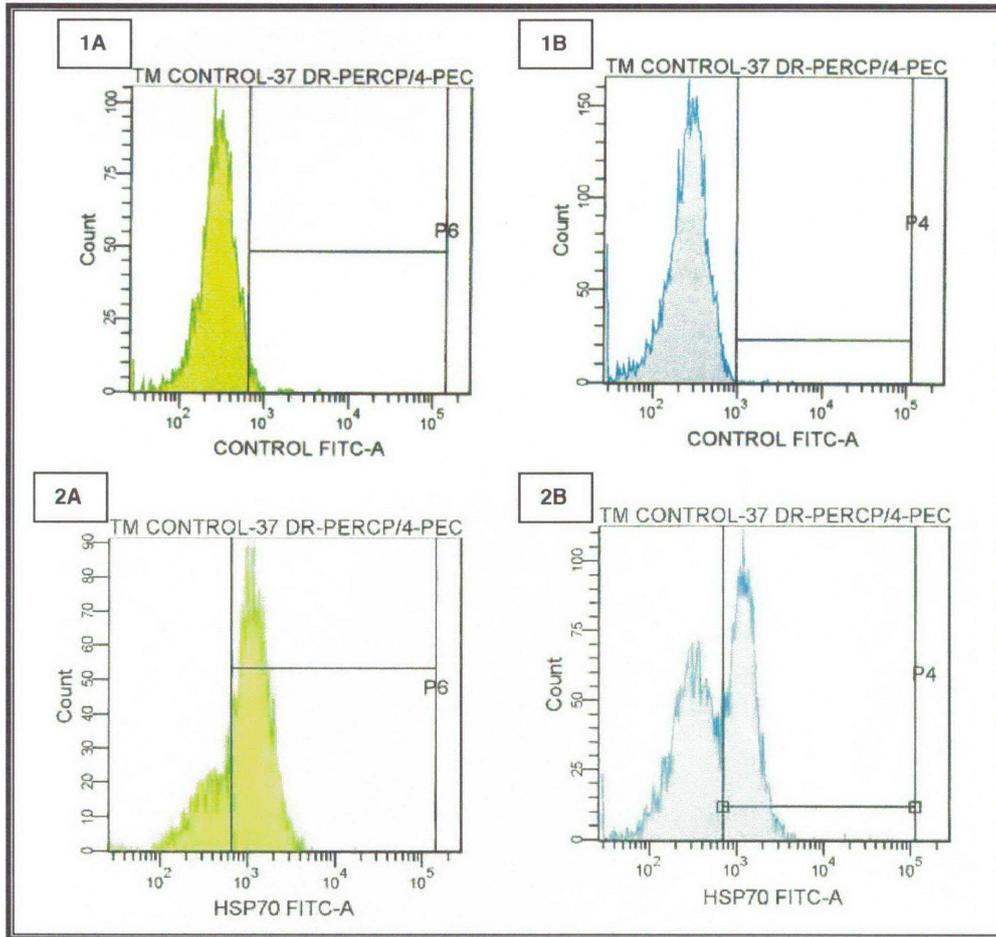
**Figure 4.16: Gating on leukocyte sub-populations at 42.5°C.** P1 = gating on all CD3+ leukocytes. P2 = gating on CD3+ cells that were also CD4-positive. P3 = gating on CD3+ leukocytes that were also CD8-positive, P4 = gating on CD3+ cells that were also CD19-positive. P5 = gating on CD3+ cells that were also CD56-positive



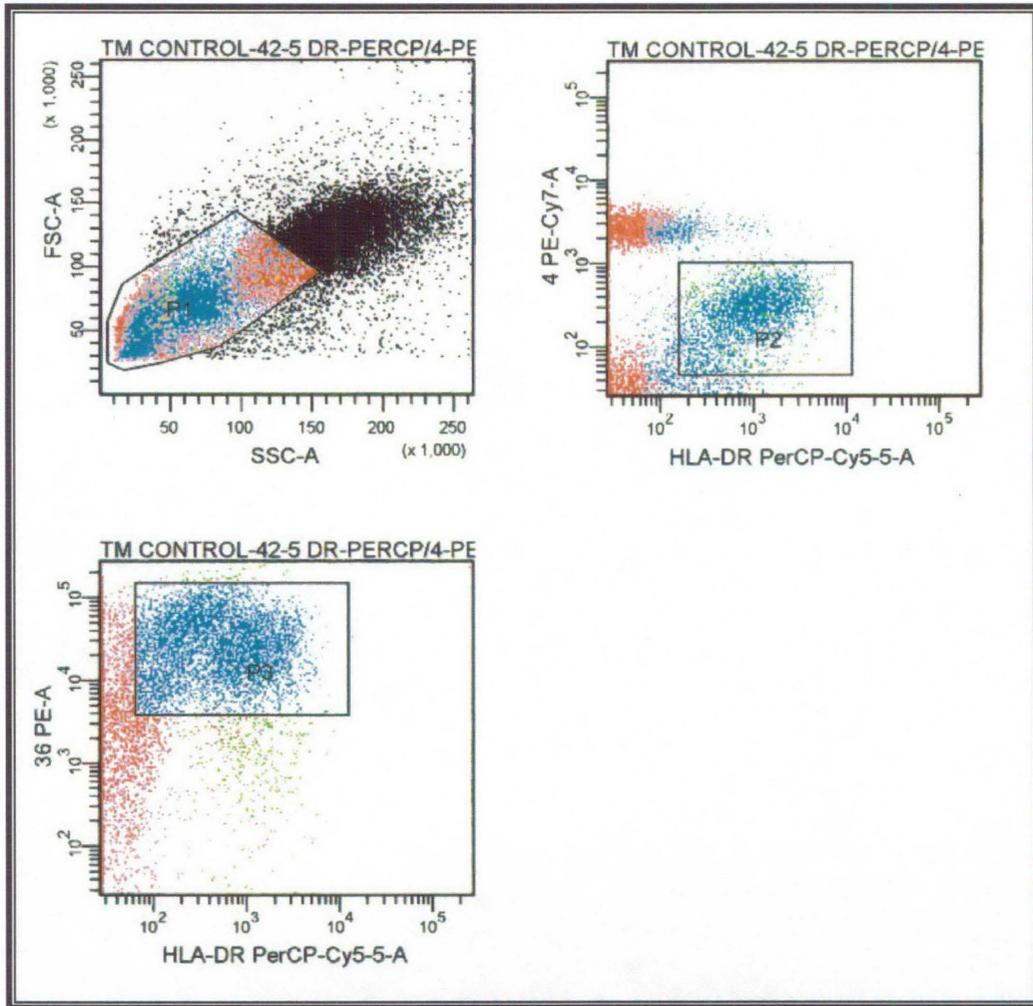
**Figure 4.17: Histograms depicting leukocyte hsp70 expression at 42.5°C.** 1 = IgG1 isotype control staining. 2 = Hsp70 staining. A = CD4+ T lymphocytes. B = CD8+ T lymphocytes. C = CD19+ B lymphocytes. D = CD56+ Natural killer cells.



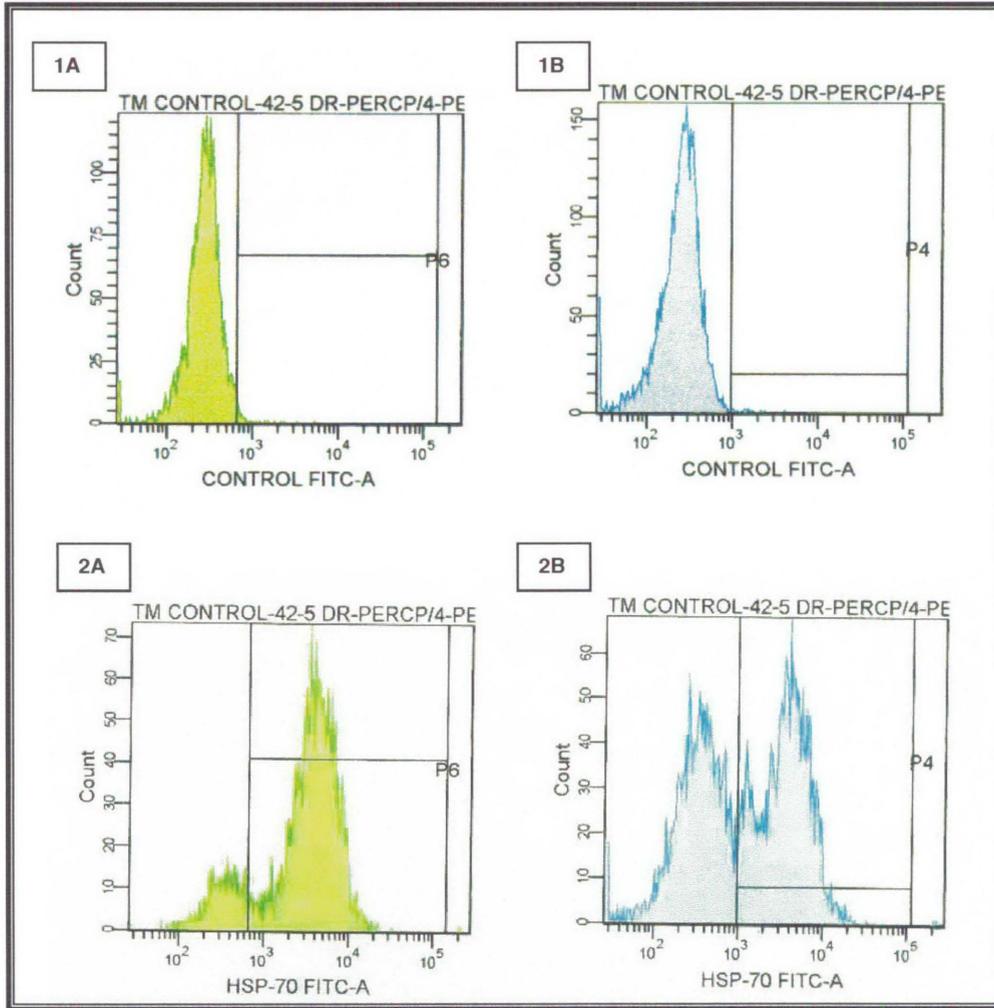
**Figure 4.18: Gating on leukocyte sub-populations at 37°C.** P1 = gating on all CD3+ leukocytes. P2 = gating on CD3+ cells that were also CD4-positive and HLA-DR-positive. P3 = gating on CD3+ leukocytes that were also CD36-positive and HLA-DR-positive.



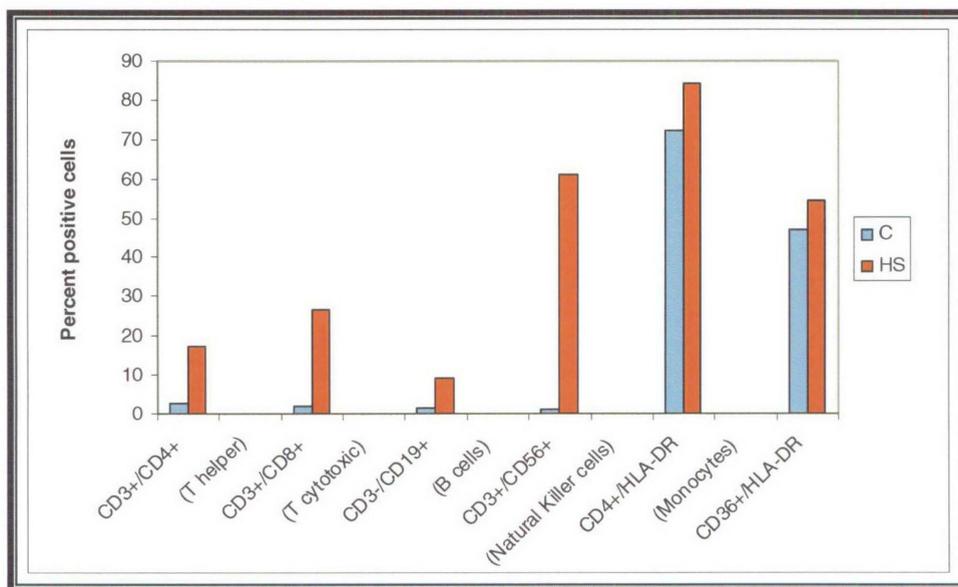
**Figure 4.19: Histograms depicting leukocyte hsp70 expression at 37°C.** 1 = IgG1 isotype control staining. 2 = Hsp70 staining. A = CD4+/HLA-DR+ monocytes. B = CD36+/HLA-DR+ monocytes.



**Figure 4.20: Gating on leukocyte sub-populations at 42.5°C.** P1 = gating on all CD3+ leukocytes. P2 = gating on CD3+ cells that were also CD4-positive and HLA-DR-positive. P3 = gating on CD3+ leukocytes that were also CD36-positive and HLA-DR-positive.



**Figure 4.21: Histograms depicting leukocyte hsp70 expression at 42.5°C.** 1 = IgG1 isotype control staining. 2 = Hsp70 staining. A = CD4+/HLA-DR+ monocytes. B = CD36+/HLA-DR+ monocytes.



**Figure 4.22: Flow cytometric analysis of hsp70 expression in leukocyte subtypes.** C = cells incubated at 37°C for 4 h. HS = cells incubated at 42.5°C for 1 h followed by 3 h at 37°C. Values are expressed as percentage of cells expressing hsp70.

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## 4.4 Discussion

### 4.4.1 Expression of stress proteins in cryopreserved cells

Cryopreservation is the process by which cells are stored in liquid nitrogen for extended periods of time. This process makes it possible to use stored cells for a range of assays. Cryopreservation is a particularly useful laboratory technique given that there are many situations where it is impossible or inconvenient to assay cells immediately after collection. Studies have shown that cryopreserved peripheral blood mononuclear cells (PBMC) may be stored for at least 12 years with no general tendency toward cell death or loss of viability over time (Kleeberger *et al.*, 1999). However, a consistent set of functional and immunophenotypic changes have been reported in cryopreserved cells from both healthy and HIV-positive donors (Costantini *et al.*, 2003). On the other hand, studies conducted by Reimann *et al.* (2000) reported only minor changes in the expression of lymphocyte extracellular markers after cryopreservation, leading to the conclusion that freezing PBMC is feasible for immunophenotyping and functional assays. The researchers did note, however, that the small changes that occur in lymphocyte subsets should be measured and accounted for in the design of each study.

Reports that PBMC can be stimulated to proliferate, secrete cytokines and exhibit antigen specific or non-specific lytic activity after cryopreservation (Reviewed by Reimann *et al.*, 2000), support the hypothesis that stored PBMCs may also be able to synthesise hsps. It has also been reported that cryopreservation of fibroblasts grown in three-dimensional culture induced a specific cellular stress response, including growth factors (Liu *et al.*, 2003).

The ability to measure hsp expression in cryopreserved leukocytes is of particular interest to this project since there are a number of collections of stored cells from HIV-positive patients. It would also enable the collection and subsequent preparation of blood samples at a variety of given times and locations and the ability to include the measurement of hsp expression as a parameter of immune response in some of the large, multi-centre studies on HIV-infection.

The results of the current study revealed that not only was hsp70 expression measurable in cryopreserved lymphocytes, but also that these cells retained the ability to synthesise new proteins. This study also demonstrated that after cryopreservation,

*de novo* lymphocyte expression of some hsp's was altered. These differences were most notable after longer periods of storage (75/78 days) and included the up-regulation of a protein of approximately 56 kDa and 32 kDa in both control (37°C) and heat shock (42.5°C) samples. It is likely that these proteins are hsp56, also known as FK506 binding protein 59 or FK506 binding protein 52 (FKBP59/FKBP52), and hsp32 also known as heme-oxygenase (HO-1). The upregulation of the latter proteins may be due to oxidative stress induction during cryopreservation.

These findings were consistent with the results of the study conducted by Liu *et al.* (2003), who observed an induction of the stress response in cryopreserved fibroblasts. Specifically, they noted transient increases in cellular hsp27 and hsp90 in suspension and three dimensional culture, and increases in hsp70 in suspension only. After a 24 h culture, results showed decreases in hsp56, hsp70, hsp90 and GRP78 in three-dimensional culture and in hsp90 in suspension. Of particular interest was their finding that after only 4 h of culture, *de novo* synthesis of hsp47 was induced together with synthesis of hsp90, Grp78 and other unidentified proteins.

Dimethyl sulfoxide (DMSO) is the most commonly used cryoprotectant as it reduces the electrolyte content of the solution in which the cells are suspended (Lovelock & Bishop, 1959), is permeable to the cell membrane and has a relatively low toxicity (McGann & Watterson, 1987). In addition, DMSO has a range of pharmacological actions including antioxidant properties (Jacob & Herschler, 1986). Studies on yeast cells have demonstrated that cryopreservation causes major injury to cells as a result of the formation of superoxide radicals in the cytoplasm from leakage of oxygen and electrons from the mitochondrial electron transport chain (Park *et al.*, 1998). Considerable changes have also been observed in the mitochondria and plasma membranes of cryopreserved lymphocytes that include an increase in the respiration and oxidative phosphorylation rates of cells frozen in DMSO (Tsutsaeva & Gordienko, 1982). It is likely, therefore, that the concentration of DMSO used (10% v/v) during the cryopreservation process in the current studies was insufficient to counteract the oxidative burst that occurred, thus resulting in the upregulation of the stress proteins.

#### **4.4.2 Minimum volume of blood required to measure stress protein expression**

The requirement for relatively large volumes of blood (approx 30 ml) from participants in studies of hsp expression as measured by the traditional methods of autoradiography and Western immunoblot is a major drawback to conducting these studies. This is

particularly evident when blood samples are required from individuals who are unwell, frail, elderly or very young. It can also be a limiting factor in facilitating clinical trials as these studies often require participants to donate multiple blood samples. Consequently, the development of techniques for measuring hsp expression that require less blood could broaden the spectrum of potential participants in such studies.

In the current studies, smaller volumes were tested to determine the optimum blood volume required for conventional autoradiography and Western immunoblotting measurements. These studies revealed that the minimum blood volume required to produce adequate lymphocyte protein extracts is 10 ml.

#### **4.4.3 Comparison of hsp70 expression in plasma and serum**

Some researchers measure circulating hsps in plasma whilst others use serum. The essential difference between plasma and serum is the presence of fibrinogen, a plasma protein responsible for clotting. As there are no clear indications in the literature as to which of these fluids is preferable, the current study sought to determine whether the presence of fibrinogen (in plasma) affects the analysis of hsp70, by comparing hsp70 measurements in plasma and serum.

Although numerically, it appeared that in both male and female samples examined, plasma hsp70 concentrations were greater than serum concentrations of hsp70, statistically there was no significant difference. These results suggest that there may not be any difference between plasma hsp70 concentrations and serum hsp70 concentrations. However, there is simply insufficient published data to verify this conclusion which awaits confirmation or otherwise from studies on a much larger number of individuals.

#### **4.4.4 Kinetics of expression of plasma hsp70 after heat shock**

Some time ago, researchers reported the presence of hsp70 in the plasma of healthy individuals (Pockley *et al.*, 1998). It has been suggested that the release of hsp70 from PBMC is rapid and occurs by active secretion, via a non-classical pathway that may involve lysosomal lipid rafts, rather than as a result of cell damage (Hunter-Lavin *et al.*, 2004). The results of the current study as illustrated in Figures 4.6 and 4.7 showed an increase in the amount of plasma hsp70 (male) from 9.18 to 13.27 ng/ml and (female) from 10.36 to 14.66 ng/ml in the 4 h testing period suggesting that the release of hsp70 from PBMC may occur in a time-dependent manner.

The results of the current study also demonstrated that the hsp70 released from cells into circulation was not heat shock inducible and was, therefore, likely to be the constitutive form of the protein hsc70.

#### 4.4.5 Flow cytometric analysis of hsp70 expression in leukocytes

Flow cytometry is a method of measuring particular physical and chemical characteristics of cells as they travel in suspension, one by one, past a sensing point. Forward and side scatter of light by cells provides information on cell size and cytoplasmic granularity. The staining of cells with specific antibodies tagged with different fluorochromes facilitates detection of cell surface antigens and intracellular molecules. Flow cytometry is a rapid, quantitative method of determining intracellular hsp70 expression in individual cells from a heterogeneous population such as peripheral blood mononuclear cells (PBMC) (Bachelet *et al.*, 1998). Quantitative comparisons of both the constitutive and inducible forms of hsp70 as measured by Western immunoblot and flow cytometry have shown excellent agreement, demonstrating the accuracy of flow cytometric analysis of hsp expression (He & Fox, 1996). Flow cytometric methods have demonstrated that hsp70 is constitutively expressed in human leukocytes but that the level of expression varies considerably between different cell types (Oehler *et al.*, 2001). This method holds several advantages over the more traditional methods of determining levels of heat shock protein expression, such as Western immunoblot and 1D-SDS-PAGE. These advantages include the specific information provided by this technique as to which cells are expressing the protein, and the significantly smaller volume of blood required to conduct the flow cytometric analysis.

The current study used an antibody to the CD14 leukocyte extra-cellular marker. The CD14 antigen is expressed primarily by monocytes, macrophages and activated granulocytes (Govert *et al.*, 1988). It has been previously reported that hsp70 expression is variable in leukocyte subtypes and is preferentially expressed by monocytes (Bachelet *et al.*, 1998). It should be noted that the techniques employed by the current study to compare leukocyte and whole blood methods, identified particular problems associated with the isolation of leukocytes prior to flow cytometry. It is likely that the minimal and variable results obtained for CD14 staining in these samples was a result of loss of CD14-positive cells during the cell harvesting process. As CD14 is a monocyte extra-cellular marker and it is well documented that freshly isolated monocytes are notoriously sticky cells that readily adhere to plastic surfaces (Kelzer *et*

*al.*, 1987), a characteristic that is exploited for the harvest of these cells for some assays (Rubenstein & Ballow, 1989). It is possible, therefore, that the loss of cells may have been due to adherence of the CD14-positive monocytes to the microfuge tube during the separation and incubation processes. Hence it can be concluded that using whole blood for the analysis of hsp70 expression in CD14-positive cells is a much more robust method.

The study of heat shock protein expression in human peripheral blood has traditionally involved a requirement for relatively large volumes of blood (at least 30 ml) from study participants and a lengthy process of separating and preparing leukocyte fractions for subsequent analysis. In many cases, participants are reluctant to provide such a volume of blood. This is particularly apparent when subjects are already undergoing regular blood collections for routine monitoring of disease progression, patients who are unwell or frail, elderly or very young. It would therefore be ideal to develop a method to examine hsp expression in leukocytes using a smaller volume of peripheral blood.

Using traditional methods, cells need to be separated from whole blood using Ficoll-Paque gradient centrifugation (Chapter 2) prior to a heat shock treatment. The application of a transient, non-lethal heat shock has long been used to measure the stress response of cells (Rodenhiser *et al.*, 1985). The results of the current study clearly demonstrated that heat shocking whole blood produced consistent and reliable upregulation of hsp70, as illustrated by the shift to the right of histogram curves comparing control and heat shock samples in Figures 4.9 – 4.12. This finding is highly significant as it enables the measurement of the stress response in leukocytes by flow cytometric methods using whole blood instead of in separated leukocytes.

#### **4.4.6 Multicolour flow cytometric analysis of hsp70 expression in leukocytes**

Multicolour flow cytometric analysis enables examination of complex cellular interactions in mixed cell populations. The sensitivity of this technique allows simultaneous analysis of cell surface markers and intracellular molecules at the single cell level. Since cell surface molecules are involved in mediating immune responses, flow cytometry may provide important information regarding a number of related parameters including cell lineage, activation status, adhesion, migration, ability to respond to stimuli & interaction with other cells.

The ability to stain cells for multiple extracellular markers in a single tube has a range of benefits for the researcher. This method uses less blood, eliminates potential errors and allows for the comparison of dual-positive cell types as compared to multiple tubes and a flow cytometer with fewer lasers.

The results of the current study confirm and extend the findings of the previous section of this Chapter. These results demonstrate the differential nature of hsp70 expression in leukocyte subpopulations, namely, helper and cytotoxic T lymphocytes, B lymphocytes, Natural killer cells and monocytes. The results of the current study support previous findings that hsp70 expression is variable in leukocyte subtypes and is preferentially expressed by monocytes (Bachelet *et al.*, 1998). Furthermore, the benefit of using an isotype control antibody reduces the risk of false positive results, an issue of particular importance when examining cell subsets expressing small amounts of hsp70.

### **Conclusions**

Although lymphocytes are able to synthesize new proteins after cryopreservation, the finding that the process of cryopreservation itself induces hsp synthesis renders this technique unsuitable for the measurement of stress protein expression. The measurement of hsp expression by autoradiography and Western immunoblot requires a minimum of 10 ml whole blood to obtain adequate protein yields. Hsp70 is detectable in both serum and plasma and the current studies indicate that there is no difference in these measurements, however, further studies with a larger cohort are recommended before a definitive statement can be made. Furthermore, it was demonstrated that hsp70 release from leukocytes into the plasma occurred in a time-dependant manner.

This Chapter has reported the process of development of a new, cutting-edge methodology for the measurement of leukocyte stress protein expression in whole blood samples. This technique has been further developed and applied to larger scale studies as detailed in the Chapters that follow.