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Hsp72 translocation and secretion in in vivo and in vitro models

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Chapter 2

Materials and Methods

2.1 Consumables

- 0.2 ml PCR tubes Starlab (UK) Ltd. Cat. n° B1402-4300
- 0.6 ml Micro centrifuge tubes Thermo Fisher Scientific Inc. Cat. n° TUL-918-010X
- 1.2 ml Micro dilution tubes Starlab (UK) Ltd. Cat. n° E1730-9000
- 1.5 ml Micro centrifuge tubes Thermo Fisher Scientific Inc. Cat. n° TUL-918-014G
- 12-well Cell Culture Plates Thermo Fisher Scientific Inc. Cat. n° TKT-520-070H
- 25 cm² Cell Culture Flasks Thermo Fisher Scientific Inc. Cat. n° 136196
- 48-well Cell Culture Plates Thermo Fisher Scientific Inc. Cat. n° TKT-522-070S
- 96-well Cell Culture Plates Thermo Fisher Scientific Inc. Cat. n° DPS-130-010N
- 96-well TC-treated Black Clear Bottomed Plates Thermo Fisher Scientific Inc. Cat. n° DPS 130-020K
- 384-well polypropylene black assay plate, Corning, Cat. n°07-200-765
- BD Eclipse Blood Collection Needle, 21g Southern Syringe Services Ltd. Cat. n° 368609
- BD Vacutainer One Use Holder Southern Syringe Services Ltd. Cat. n° 364815
- BD Vacutainer[®] Whole Blood Tube, K₂EDTA, 6 ml Southern Syringe Services Ltd. Cat. n° 367873
- Cover slips, Fisher Scientific, Cat.n° 22-037-169
- Extra Thick Blot Paper Bio-Rad Laboratories Inc. Cat. n° 170-3965
- Glass slides, fisher Scientific, Cat n°12-550-15
- Nitrocellulose Membrane, 0.45 µm Bio-Rad Laboratories Inc. Cat. n° 162-0115
- Non-binding 96-well plates Thermo Fisher Scientific Inc. Cat. n° FB56426
- Nunc Immuno Maxisorp 96-well plates Thermo Fisher Scientific Inc. Cat. n° DIS-971-030J
- Superfrost slides, Fisher Scientific Cat. n°22-034-985

2.2 Equipment

ABI Prism 7700 Sequence Detection System Applied Bioscience

Bio-Rad ChemiDoc XRS Molecular Imaging System Bio-Rad Laboratories Ltd
Cat. n° 170-8070

Bio-Rad Radiance 2100 AGR-3Q AOFT confocal microscope (Argon 488,
Green He/Ne 500, Red Diode 535) with following lasers: Argon
(514,488,476,457), Green (543), Red (637) Blue (405)

Bio-Tek Synergy™ HT Multi-Detection Microplate Reader Labtech
International Ltd. Cat. n° SIAFR

Bright-Line™ Haemocytometer Sigma-Aldrich Ltd. Cat. n° Z359629

Circulating Water Bath Wolf Laboratories Ltd. Cat. n° GD100-P5

Dynex Ultrawash PLUS™ Plate Washer Jencons (Scientific) Ltd. Cat. n° 701-
005

E100 Binocular Microscope Jencons (Scientific) Ltd. Cat. n° 450-951

Eclipse TE2000-U Basic Unit Cat. n° MEA51010, Eposcopic Fluorescence
Attachment (Hg) Cat. n° MEE54000CFI Plan Fluor ELWD Objectives
Cat. n° MRH38220/MRH38420/MRH18620

Hamamatsu Orca – 285 Digital CCD Camera Cat. n° 1HMOC285

Coolpix Digital Colour Camera Cat. n° 85400RUK

IPLAB Suite Software Cat. n° 1SCSUITE

FACSCanto dual laser Flow cytometer BD Biosciences

Hermle Z323K Refrigerated Centrifuge VWR International Ltd. Cat. n° 521-
0221 including: swing-out rotor (8 X 15 ml) Cat. n° 521-0189, fixed-
angle Rotor (24 X 1.5 ml) Cat. n° 521-0201, fixed-angle Rotor (8 X 50
ml) Cat. n° 521-0194, swing-out rotor (2x microtiter plate) Cat. n°521-
0410

High Speed Mini Orbital Shaker Wolf Laboratories Ltd. Cat. n° SSM5

Inverted TE2000-U Microscope System Nikon Corporation Ltd

Homogenizers Glass 5ml, Jencons, Cat. n° 361-044

LMS Series 1 Cooled Incubator Wolf Laboratories Ltd. Cat. n° 305

Mini Trans-Blot Electrophoretic Transfer Cell Bio-Rad Laboratories Inc. Cat.
n° 170-3930

Mini-PROTEAN® II Electrophoresis Cell Bio-Rad Laboratories Inc. Cat. n°
165-2940

PowerPac™ 3000 Power Supply Bio-Rad Laboratories Inc. Cat. n° 165-5057

Sigma A 1- 14 microcentrifuge Sigma Aldrich, Cat.n°12084
Techne Dri-Block DB-3 Thermo Fisher Scientific Inc. Cat. n° BLD-715-010G
Temperature-controlled, stirred water bath, GD100-S5 Thermo Fisher
Scientific Inc. Cat. n° BLE- 650-010G Temperature freezing microtome,
Myneurolab
Vortex mixer, mini Thermo Fisher Scientific Inc. Cat. n° GBI-900-010E

2.3 Chemicals and Reagents

Acetic Acid, Glacial Sigma-Aldrich Ltd. Cat. n° 320099-2.5L
Acrylamide VWR International Ltd. Cat. n° 442994J
Ac-VDVAD-CHO Caspase-2 inhibitor, Biomol Ltd., Cat. n° P463-0001
Ac-DEVD-CHO Caspase-3 inhibitor, Biomol Ltd. Cat n° P410-0001
 ϵ -Amino-n-caproic acid Sigma-Aldrich Ltd. Cat. n° A-7824
Ammonium Chloride Sigma-Aldrich Ltd. Cat. n° A-9434
Ammonium Persulphate Sigma-Aldrich Ltd. Cat. n° A-3678
Annexin-V- FITC conjugated apoptosis detection kit, BD Biosciences Cat.
n°556419
Annexin-V-PE conjugated apoptosis detection kit, BD Biosciences Cat.
n°556422
Antibiotic/Antimycotic Solution (100X) Sigma-Aldrich Ltd. Cat. n° A-5955
Anti-golgin-97 human CDF4-mouse monoclonal antibody, Molecular Probes,
Cat. n°A21270
Anti-Mouse IgG (whole molecule)-Cy3 conjugated antibody Sigma-Aldrich Ltd.
Cat. n°C2181
Anti-Mouse IgG (whole molecule)-HRP conjugated antibody Sigma-Aldrich
Ltd. Cat. n° A-5278
Anti-Sheep IgG (whole molecule)–Peroxidase antibody Sigma-Aldrich Ltd.
Cat. n° A-3415
Apo-Logix Carboxyfluorescein Caspase-2 Detection Kit, Bachem, Cat.
n°9083.0025
Aspirin pain relief 300mg tablets, Superdrug
Benzamidine Sigma-Aldrich Ltd. Cat. n° B-6506
Benzyl Alcohol (BA), Sigma-Aldrich Ltd. Cat. n°30519-7
Bovine Serum Albumin (BSA) Sigma-Aldrich Ltd. Cat. n° A-7906
1-Bromo-3-chloropropane (BCP), Helena Biosciences Cat. n°BP151

Brij-98, Sigma-Aldrich Ltd. Cat. n°436240

Bromophenol Blue Thermo Fisher Scientific Inc. B/4630/44

Calcium Chloride, Sigma-Aldrich Ltd. Cat. n°C3306

Camptothecin Sigma-Aldrich Ltd. Cat. n° C9911

Caspase-3 rabbit anti active Caspase-3 antibody-FITC conjugated, BD Biosciences, Cat. n°559341 CD3-PE conjugated surface marker BD Biosciences Cat. n°555340

CD4-PE-Cy7 conjugated surface marker BD Biosciences Cat. n°557852

CD5-APC conjugated surface marker, BD Biosciences Cat, n°555355

CD8-APC-Cy7 conjugated surface marker, BD Biosciences Cat. n°557834

CD14-PE-Cy7 conjugated surface marker, BD Biosciences Cat. n°557742

CD15-APC conjugated surface marker, BD Biosciences Cat. n°551376

CD19-PE conjugated surface marker, BD Biosciences Cat. n°555413

CD34-PE-Cy5.5 conjugated surface marker, BD Biosciences Cat. n°555823

CD45-PE-Cy5.5 conjugated surface marker, BD Biosciences Cat. n°555484

CD56-APC conjugated surface marker, BD Biosciences Cat. n°555518

CellTiter® Aqueous One Solution Cell Proliferation Assay Promega UK Ltd. Cat. n° G3580

Coomassie (Bradford) Protein Assay Kit Pierce Biotechnology Inc. Cat. n° 23200

Cytofix/ Cytoperm fixation and permeabilization solution, BD Biosciences, Cat. n°554722

4,4'-Diisothiocyanatostilbene-2-2'-disulfonic acid disodium salt hydrate (DIDS), Sigma-Aldrich Ltd. Cat. n°D3514

DC Protein assay kit, Bio-Rad Laboratories Inc., Cat. n°500-0116

Diethyl pyrocarbonate (DEPC), Sigma-Aldrich Ltd. Cat. n°D-5758

Dimethyl Sulfoxide Sigma-Aldrich Ltd. Cat. n° D-2650

DL-Dithiothreitol Sigma-Aldrich Ltd. Cat. n° D-0632

Dulbecco-Phosphate Buffered Saline (D-PBS) with Ca⁺⁺ and Mg⁺⁺ Lonza, Cat n° BE17-513F

E6.1, Human Leukemic T cell lymphoblast Cell Line, European Collection of Cell Cultures ECACC Cat. n°88042803

Edelfosine SnEt-18-OCH₃, AG Scientific Inc. Cat. n°E-1027

Enzolyte™ Rh110 Caspase-3 Assay Kit Anaspec Inc. Cat. n° 71141

Ethanol, Sigma-Aldrich Ltd. Cat. n°459836

Epinephrine, Sigma-Aldrich Ltd. Cat. n°E-4250

Ethylenediaminetetraacetic acid, anhydrous (EDTA) Sigma-Aldrich Ltd. Cat. n° E-6758

Extravidin- R-Phycoerithrin (R-PE) conjugated Sigma-Aldrich Ltd. Cat. n°Z.25455

First-Strand c-DNA Synthesis Kit, GE Healthcare, Cat n°27-9261-02

Foetal Bovine Serum (FBS) Cambrex Corporation. Cat. n° 14-810F

Glybenclamide Sigma-Aldrich Ltd. Cat. n° G0639

Glycine VWR International Ltd. Cat. n° 101196X

HABA/Avidin Reagent Sigma-Aldrich Ltd. Cat. n° H-2153

Histopaque[®]-1077 Hybri-max Solution Sigma-Aldrich Ltd. Cat. n° H-8889

Hsp72 mouse monoclonal antibody, Assay design Ltd, Cat. n°SPA810

Hsp72 monoclonal antibody against TKD peptide, purchased from Prof. G. Multhoff, Multiimmune Labs Munich, Germany

Hsp72 primers and probes for pig samples purchased from MWG; sequences has been kindly provided by Dr. Y.F. Young at al Aarhus university, Denmark

Human IL-6 (Interleukin-6) ELISA Ready-SET-Go Kit eBioscience Inc. Cat. n° 88-7066

Human IL-10 (Interleukin-10) ELISA Ready-SET-Go! Kit eBioscience Inc. Cat. n° 88-7106

Human TNF- α (Tumor Necrosis Factor alpha) ELISA Ready-SET Go! Kit eBioscience Inc. Cat. n° 88-7346

Hydrochloric Acid, 37% Sigma-Aldrich Ltd. Cat. n° 320331

Ibuprofen pain relief 200mg tablets, Superdrug

IM54- 2-(1H-Indol-3-yl)-3-pentylamino-maleimide, Calbiochem, Cat. n°480060

Imperial Protein Stain Pierce Biotechnology Inc. Cat. n° 24615

Interferon gamma (IFN- γ),Sigma-Aldrich Ltd. Cat. n°I3265

Interleukin-6 (IL-6), Sigma-Aldrich Ltd. Cat. n°I1395

Isopropyl Alcohol, Sigma-Aldrich Ltd. Cat. n° W-292971

KNK423, Heat shock protein inhibitor II, Calbiochem, Cat.n°373265

β -Mercaptoethanol Thermo Fisher Scientific Inc. Cat. n° M/P200/05

Methanol VWR International Ltd. Cat. n° 152506X

Microamp[™] optical 96 well reaction plate with barcode and optical adhesive films, Applied Biosystems Cat.n°4314320

Mouse anti-Hsp72 generous gift from Dr T. Kristensen fro Aarhus University

Mouse monoclonal Anti-LAMP-1 antibody, AB Cam Cat.n° Ab25630-100

N,N,N',N'-Tetramethylethylenediamine (TEMED) Sigma-Aldrich Ltd. Cat. n° T-9281

N'N'-Methylenebisacrylamide VWR International Ltd. Cat. n° 4433003N

Norepinephrine (NE), Sigma-Aldrich Ltd. Cat. n° A-7257

Phenethyl Alcohol (PhA), Sigma-Aldrich Ltd. Cat. n°WZ8581-I-K

Phenylmethylsulfonyl fluoride (PMSF) Sigma-Aldrich Inc. Cat. n° P-7626

Phorbol 12-Myristate 13-Acetate (PMA) Sigma-Aldrich Ltd. Cat. n° P-1585

Potassium Chloride Sigma-Aldrich Ltd. Cat. n° Cat. n° P-5405

Potassium Dihydrogen Orthophosphate VWR International Ltd. Cat. n° 102034B

Prazosin hydrochloride Sigma-Aldrich Ltd. Cat. n°81515

Precision Plus[®] Protein Dual Colour Standards Bio-Rad Laboratories Inc. Cat. n° 161-0374

Precision Plus[®] Protein Unstained Standards Bio-Rad Laboratories Inc. Cat. n° 161-0363

ProLong[®] gold antifade reagent with DAPI, Invitrogen, Cat.n°P-36931

Propidium Iodide (PI), Sigma-Aldrich Ltd, Cat. n°P4170

Protease Inhibitor Cocktail 100X Sigma-Aldrich Ltd. Part no. P-8340

QuickExtract DNA extraction solution, Epicentre Cat. n°QE09050

Quick prep micro mRNA purification kit, GE Healthcare, Cat. n°27-9255-01

Quinidine Sigma-Aldrich Ltd. Cat. n°Q3625

pd(N)₆ random hexamers primers, GE healthcare, Cat. n° 27216601

Recombinant Human Hsp72 (Hsp72) Protein, baculovirus expressed, Stressmarq Biosciences Inc.Cat. n° SPR-115

Recombinant Human Hsp72 (Hsp72) Protein, Assay Designs Inc. Cat. n° ESP-555

(R)- (-)- Phenylephrine hydrochloride, Sigma-Aldrich Ltd. Cat. n°P6126

RPMI-1640 Medium, Cambrex Corporation, Cat. n° BE12-702F

Sheep polyclonal anti Hsp72 antibody in home produced and affinity purified by Elyse Ireland

Sodium Acetate, trihydrate VWR International Ltd. Cat. n° 27652.232

Sodium Bicarbonate Sigma-Aldrich Ltd. Cat. n° S-6297

Sodium Carbonate Sigma-Aldrich Ltd. Cat. n° S-7795

Sodium Chloride Sigma-Aldrich Ltd. Cat. n° S-7653

Sodium Deoxycholate Sigma-Aldrich Ltd. Cat. n° D-6750

Sodium Dihydrogen Orthophosphate 1-hydrate VWR International Ltd. Cat. n° 102454R

Sodium Dodecyl Sulphate (SDS) VWR International Ltd. Cat. n° 442444H

Sodium orthovanadate Sigma-Aldrich Ltd. Cat. n°S6508

Sucrose Sigma-Aldrich Ltd. Cat. n° S-7903

SuperSignal West Pico chemiluminescent substrate Pierce Biotechnology Inc.Cat. n° 30477

TaqMan® Ribosomal RNA control reagent, Applied Biosystems, Cat. n°4308329

TaqMan® Hsp72 gene expression assay Applied Biosystems, Cat. n°HS_00359163_s1

TaqMan® Hsp90-β gene expression assay Applied Biosystems, Cat. n°HS_00607336_gH

TaqMan® Hsp27 gene expression assay Applied Biosystems, Cat. n°HS_00356629_g1

TaqMan® Universal PCR Master Mix, No AmpErase® UNG, Applied Biosystems, Cat. n°4326614

3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Cheshire Sciences (UK) Ltd.Cat. n° UP664781

Thimerosal Sigma-Aldrich Ltd. Cat. n° T-8784

TriReagent, RNA, DNA, Protein extraction solution, Helena Biosciences, Cat n°TR11850

Tris (hydroxymethyl)methylamine VWR International Ltd. Cat. n° 443866G

Triton® X-100 Sigma-Aldrich Ltd. Cat. n° T-8787

Trypan Blue Solution (0.4%) Sigma-Aldrich Ltd. Cat. n° T-8154

Tumor necrosis factor alpha (TNF-α), Sigma-Aldrich Ltd. Cat. n°T6674

Tween® 20 Sigma-Aldrich Ltd. Cat. n° P-1379

U937 Human Caucasian histiocytic lymphoma Cell Line, European Collection of Cell Cultures. Cat. n° 85011440

Verapamil Hydrochloride Sigma-Aldrich Ltd. Cat. n°V4629

Vibrant Lipid raft labelling kit, Invitrogen, Cat. n°V34404

Z-VAD-FMK General Caspases inhibitor, Biomol Ltd. Cat n° P416-0001

2.4 Buffers and solutions

2.4.1 pH solutions

1M HCl (v/v)

A volume of 9.8 ml of 37% hydrochloric acid (HCl) was mixed with dH₂O to obtain up to 100 ml of solution, that was stirred until dissolved.

1M NaOH (w/v)

4 g of sodium hydroxide (NaOH) was added to ~ 80 ml of dH₂O and stirred until dissolved. The solution was then made up to 100 ml with dH₂O.

2.4.2 General purpose solutions

0.15M Phosphate buffered saline, pH 7.4 (PBS) (w/v)

Sodium chloride (NaCl, 8.0g), potassium chloride (KCl, 0.2g), potassium dihydrogen orthophosphate (KH₂PO₄, 0.24 g) and sodium dihydrogen orthophosphate (Na₂HPO₄, 1.44 g) were added to 1 l of dH₂O and pH adjusted to 7.2.

Paraformaldehyde (PFA) 4 % (w/v)

Paraformaldehyde (PFA, 2 g) and 100 µl of 5 M sodium hydroxide (NaOH) were diluted in 40 ml of phosphate buffered saline (PBS); the solution was stirred and heated at 56°C in a water bath until PFA was completely dissolved; the solution's pH was adjusted with approximately 100 µl 5M HCl to 7.4, filled up to 50 ml final volume with PBS and stored at 4°C for maximum a week.

Antibody wash buffer (v/v)

5% foetal bovine serum (FBS) was diluted in PBS

Red blood cells Lysing Buffer 10X solution (w/v)

Ammonium Chloride (NH₄Cl, 8.02 g), sodium carbonate (NaHCO₃, 84 g) and EDTA disodium (0.37 g) were dissolved in up to 50 ml of dH₂O; the 1X working dilution was obtained diluting 1/10 the concentrated solution with dH₂O.

DEPC-treated dH₂O (v/v)

The water to be used in molecular biology needs to be free of any RNAses. The diethylpyrocarbonate (DEPC) inactivates any of those enzymes and the use of DEPC treated water prevents any sample contamination or damage. A 0.01% concentration of DEPC was added to the distilled H₂O in a fume cabinet. The solution was allowed to stand at 25°C overnight and then was

autoclaved. The autoclave inactivates the DEPC, which is otherwise very toxic, and that can also damage the RNA by carboxymethylation. DEPC-treated dH₂O solution was stored at -20°C into single use aliquots.

Propidium Iodide (PI) stock solution (1mg/ml) (w/v)

Propidium iodide (PI) 0.001 g was weighed and then dissolved in 1 ml D-PBS. 100 µg/ml stock solution was prepared and stored at -20°C into single use aliquots.

MTS Working Solution (w/v)

MTS Stock solution was prepared dissolving 0.042 g MTS in 21 ml DPBS; pH was adjusted to 6.5 with 1M HCl; Phenazyl-Etho-Sulfate (PES) stock solution was prepared dissolving 0.0092 g of the powder in 10 ml D-PBS. MTS working solution was obtained adding 1ml of PES stock solution in each 20 ml of MTS Stock solution; the solution was stored at -20°C as single use aliquots.

Tissue culture media (v/v)

10% FBS was added to RPMI 1640 media and stored at -20°C.

Heat shock serum tissue culture media (v/v)

FBS was heat-inactivated to inactivate heat sensitive components such as growth factors and complement proteins which may adversely affect experimental results. FBS was allowed to thaw at 37°C before being placed in a 56°C water bath for 30 min after which was removed and allowed to cool on ice. 10% of this heat shocked FBS was added to RPMI 1640 media, used to culture cells and stored at -20°C.

Freeze media solution (v/v)

10 ml of DMSO was diluted into 40 ml of FBS; the solution was kept cold at least at 4°C during the whole freezing process.

75% Ethanol RNAses free (v/v)

75% ethanol was diluted into DEPC-treated dH₂O; typically 75 ml of ethanol was mixed with 25 ml of DEPC-treated dH₂O; the solution was kept at -20°C and used chilled.

2.4.3 Hsp72 ELISA buffers

0.1 M Sodium bicarbonate buffer, pH 9.6 (w/v)

Sodium carbonate (10.6 g) and sodium bicarbonate (8.4 g) were added to 1 l of dH₂O and pH adjusted to 9.6.

0.15 M Phosphate buffered saline, pH 7.2 (PBS) (w/v)

Sodium chloride (8.0 g), potassium chloride (0.2 g), potassium dihydrogen orthophosphate (0.24 g) and sodium dihydrogen orthophosphate (1.44 g) were added to 1 l of dH₂O and pH adjusted to 7.2.

Blocking buffer (w/v)

0.5 % (w/v) bovine serum albumin (BSA) was dissolved in PBS.

Wash Buffer (w/v)

Phosphate buffered saline, pH 7.2 plus 0.05 % (v/v) Tween[®]20 and 0.01 % (w/v) thimerosal.

Detector antibody (w/v)

The detector antibody, a mouse monoclonal antibody raised against Bovine Hsp72 (H-9776, Sigma-Aldrich Ltd.) which is specific for Hsp72, was a kind gift from Dr Torsten Nygård, Faculty of Agricultural Sciences, Aarhus University, Denmark. And the antibody was used at a concentration of 1 mg/ml in PBS + 0.01 % (w/v) thimerosal.

1 M Phosphoric acid (w/v)

Orthophosphoric acid (6.8 ml) was diluted with dH₂O up to 100 ml.

2.4.4 Cell extraction buffer (w/v)

Tris base (0.315 g), EDTA (0.004 g) and DL-dithiothreitol (0.01 g) was made up to 100 ml with dH₂O and pH adjusted to 7.4. This was then supplemented with phenylmethylsulfonyl flouride (0.035 g), ε-amino-n-caproic acid (0.065 g), benzamidine (0.016 g) and 0.1% (v/v) Triton X-100. This was prepared up to one month in advance and stored at 4°C.

2.4.5 SDS-PAGE buffers1.5 M Tris-HCl, pH 8.8 (w/v)

Tris base (18.5 g) was added to 40 ml of dH₂O and pH adjusted to 8.8 then made up to 100 ml with dH₂O.

0.5 M Tris-HCl, pH 6.8 (w/v)

Tris base (3.0 g) was added to 40 ml of dH₂O and pH adjusted to 8.8 then made up to 100 ml with dH₂O.

Acrylamide-bis (w/v)

Acrylamide (30.0 g) and N'N' bismethylene acrylamide (0.8 g) was added to 100 ml of dH₂O, stirred thoroughly then filtered through Whatman #1 filter paper.

10 % SDS solution (w/v)

SDS (10.0 g) was added to 100 ml of dH₂O.

10 % Ammonium persulphate (w/v)

Ammonium persulphate (0.1 g) was added to 1 ml of dH₂O. This was freshly prepared when required.

0.05 % Bromophenol blue (w/v)

Bromophenol blue (0.01 g) was added to 20 ml of dH₂O.

Reducing sample buffer (w/v)

Sucrose (2.4 g), 0.5 M Tris-HCl pH 6.8 (2.0 ml), 10 % SDS solution (2.0 ml), 0.05 % bromophenol blue (0.4 ml), DL-dithiothreitol (0.02 g) and b-Mercaptoethanol (0.2 ml) was added to 9.1 ml of dH₂O and mixed thoroughly.

Electrode buffer, pH 8.3 (w/v)

Tris base (5.4 g), glycine (25.92 g) and 10 % SDS solution (9.0 ml) was added to 900 ml of dH₂O and stirred thoroughly. This was prepared a day prior to use and stored at 4°C.

2.4.6 Western blotting buffers

Transfer buffer (w/v)

Tris base (3.03 g), glycine (14.4 g) and 200 ml methanol was made up to 1 l with dH₂O. This was prepared a day prior to use and stored at 4°C.

Tris buffered saline (TBS) (w/v)

Tris base (2.42 g) and NaCl (29.22 g) was added to 750 ml of dH₂O, pH adjusted to 7.5 then made up to 1 l with dH₂O.

Washing solution (TTBS) (w/v)

Tween[®]20 (0.05 % v/v) was added to 500 ml of TBS and stirred thoroughly.

Blocking solution (w/v)

BSA (1 % w/v) was added to 50 ml of TBS and stirred thoroughly.

Antibody buffer (w/v)

BSA (1 % w/v) was added to 100 ml of TTBS and stirred thoroughly.

2.5 Tissue culture

2.5.1 Jurkat and U937 resuscitation, culture and freezing

The cell lines that were used in these studies were both derived from blood malignancies: the E6.1 Jurkat (Cat n° 88042803) is a human leukemic T-cell lymphoblast cell line and the U937 (Cat n°85011440) is a human Caucasian histiocytic lymphoma cell line. Both are suspension cells, needing the same cell culture media, therefore they possess similar procedures for resuscitation and culture. Briefly the cells were purchased frozen and, at the moment of culture, the cryovials containing the cells were removed from the cryostat, soaked with alcohol and quickly placed in a 37°C waterbath. The vial was quickly opened and the content was placed in a 25cm² flask containing 5ml of pre-warmed RPMI 1940 media with 10% serum. The cells were then counted with the haemocytometer and cell density was adjusted to 3-9x10⁵cells/ml for E6.1 Jurkat cells and 2-9x10⁵cells/ml for U937 cells. Cells were kept at 37°C in the incubator with 5% CO₂. A cell viability test was performed after 24 hours using trypan blue assay on haemocytometer. Both cell lines were passaged every 3 days and viability was always tested by trypan blue assay.

Both cell lines were frozen according to similar procedures: cell suspension was kept at low density, between 3-5x10⁵cells/ml for E6-1 and 2-5x10⁵ cells/ml for U937 cells. At this stage the cells were in the log phase of the growth curve therefore they were actively growing. Cell suspension was centrifuged at low speed of 400g for 3 min at 25°C to minimise any damage and chilled freeze media, prepared as described above, was added to each cell pellet, transferred in cryovials and slowly frozen in liquid nitrogen vapour phase for about 2 hours. The liquid nitrogen vapour phase provides a gentle freezing of 1 to 3°C/min. The cryovials were then placed in the cryostat, stored in liquid nitrogen until needed.

2.5.2 U937 transformation into macrophages

U937 cells is a monocytic suspension cell line, however phorbol 12-myristate 13-acetate (PMA) treatment activates and transforms U937 cells into macrophages. Cells within 48 hours gradually have a cell cycle arrest, begin to adhere to the bottom of the flask, increase their size and present granules in the inside of the cells, some can also present protrusions of the cytoplasm. Transformation of U937 into macrophages was achieved using a

suspension of cells actively growing in the log phase of growth. Cells were counted; viability was tested by trypan blue, and then centrifuged at 500g for 3 min at 25°C. Cell pellet was resuspended at concentration of 5×10^5 cells/ml in RPMI medium with 10% heat-inactivated FBS, containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA). Cells were plated out in 12-well cell culture plates at 1 ml/well and incubated for 48 hours to enable differentiation of cells. After 48 hours the media was removed and cells rinsed twice with 10 % heat-inactivated-FBS RPMI media then 1 ml/well fresh heat-inactivated media was added. U937 macrophages were then ready for experimental treatments.

2.5.3 Cell preparation for experiments

The day before each treatment E6-1 and U937 cells were counted and plated into 12, 48, 96 well plates depending on the experiment design. Cells were always plated at concentration of 5×10^5 cells/ml. Regarding the activated macrophages, cells were usually plated 48 hours before into 12-well cell culture plates at concentration of 5×10^5 cells/ml (1 ml/well); prior to treatments, once the activation had occurred, cells were washed once with 10 % heat-inactivated-RPMI taking care not to resuspend the attached activated macrophages, then 1 ml 10 % heat-inactivated-RPMI was added together with the treatments, and incubated for the desired time.

2.5.4 Primary culture of blood

Whole blood was washed with two volumes of D-PBS and centrifuged at 500g for 5 min at 25°C. 1x lysing solution was prepared fresh diluting with 10x lysing solution, prepared as described above, with dH₂O. Supernatant from centrifuged whole blood was discarded and red blood cells were lysed using the 1x lysing solution. Lysing of the red blood cells took place when the opaque suspension became translucent. The lysed whole blood was then centrifuged at 500g for 5 min at 25°C and the supernatant discarded. The cell pellet was washed twice with D-PBS and the cells were counted using the trypan blue exclusion method on haemocytometer. The experiments were performed when the percentage of dead cells was <1%. Concentration of the viable cells was adjusted to 1×10^6 cells/ml and the cells were centrifuged at 500g for 5 min at 25°C. Supernatant was discarded and cells were resuspended with 10 ml of fresh media supplemented with 10% serum and

1/100 Antibiotic Antimycotic solution. The cell suspension was kept overnight and the experiment was performed within the next 24 hours. As an additional precaution a cell viability test was always performed before each experiment using trypan blue assay on a haemocytometer.

2.6 Blood cells separation

2.6.1 Whole blood collection

The blood provided for these studies came from either leukemic patients involved in a medical study or from healthy volunteers. Local research ethics committee approval was obtained for these studies, and consent forms were completed by each patient or volunteer. Blood samples were collected by venepuncture in 7ml K₂EDTA vacutainers.

2.6.2 Whole blood method

Whole blood was washed with two volumes of D-PBS and centrifuged at 500g for 5 min at 25°C. The supernatant was discarded and the red blood cells were lysed using 1x lysing buffer. The lysed whole blood was then centrifuged at 500g for 5 min and the supernatant discarded. The cell pellet was washed with D-PBS and the cells counted using the trypan Blue exclusion method on a haemocytometer. The experiments were performed when the percentage of dead cells was <1%. Concentration of the viable cells was adjusted to 1x10⁶ cells/ml and the cells were centrifuged at 500g for 5 min. Supernatant was discarded and cells were resuspended with 1 ml of wash buffer (D-PBS 5% FCS) and kept on ice for flow cytometry analysis.

2.6.3 PBMC purification with Histopaque

3 ml of whole blood was transferred in new 15 ml tube and 3 ml of D-PBS was added to the whole blood. 3 ml of Histopaque 1077 was added carefully using a glass pipette to the bottom of the tube, underneath the blood-D-PBS layer and then placed to centrifuge at 400g for 30 min at 25°C in a centrifuge with swinging rotor. After the centrifugation, different layer of cells were present in the tube, each of them formed by different cell types which were separated by their density: the top layer was represented by the plasma which was carefully discarded; the opaque layer represented the mononuclear cells, therefore it was all collected with a clean pasteur pipette, kept in a fresh tube and washed

with 10 ml of D-PBS. The bottom layer of the tube contained the hystopaque, the red blood cells with the granulocytes were discarded. The purified mononuclear cells were subjected to 3 serial washing steps with D-PBS followed by centrifugation at 250g for 10 min at 25°C. At the final washing, the cells were resuspended in 1ml of D-PBS and counted using the trypan blue exclusion method on a haemocytometer. Cell concentration was adjusted to 1×10^6 cells/ml with wash buffer (5% FCS in D-PBS) and kept on ice for flow cytometry analysis.

2.7 Cell viability and apoptosis determination

2.7.1 Microscopy analysis

Visualization of cells under a microscope allowed to make a qualitative and preliminary analysis of the samples and highlighted the macro changes occurred in cells. From cell morphology it was possible to discriminate between viable cells, necrotic cells with signs of shrinking and nuclei condensation and apoptotic cells that could be identified for their distinctive blebbings also called as apoptotic bodies.

2.7.2 Trypan blue exclusion assay

Trypan Blue is a non-permeable cell membrane DNA dye that was used to discriminate between viable and dead cells and also help the visualization of cell morphology. Viable cells do not take up the dye because of their membrane integrity, whereas necrotic cells have a loss of membrane integrity that allows the dye to stain the DNA inside the cells: therefore viable cells would appear clear white whereas dead cells would show up blue. Haemocytometer was prepared and cover-slip was put in place; 500 μ l of the cell suspension was mixed to 500 μ l of trypan blue solution, then 50 μ l of the suspension mixture was loaded to both chambers of the haemocytometer carefully touching the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Cells were counted in the 0.04 mm centre square and four 0.04 mm corner squares, as highlighted in the Figure 2.1, and a separate count of viable and non-viable cells was performed. Cell number/ml were obtained using the following formula:

$$\text{Cells/ml} = \text{cell count} * 5 * \text{dilution factor} * 10000$$

Cell batches that showed a percentage of dead cells >5% were discarded and not used for the experiments.

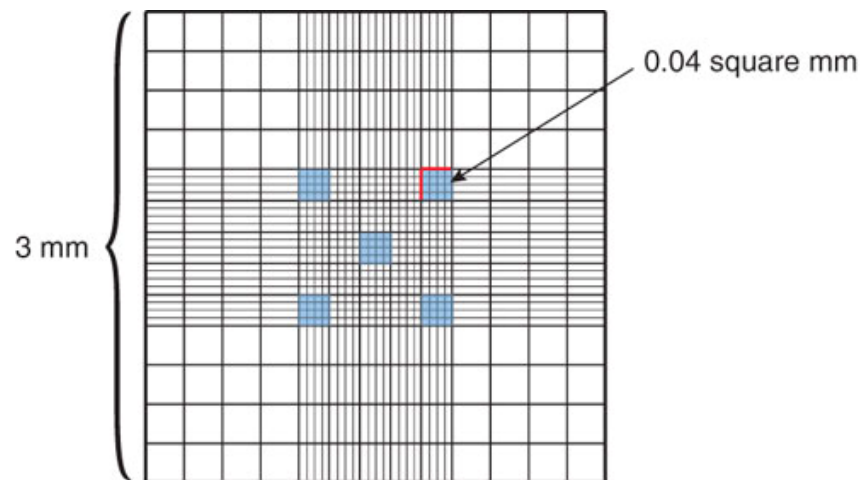


Figure 2.1: Example of an haemocytometer grid (Adapted from Nature protocols)

2.7.3 Viability assay MTS

The number of viable cells in proliferation was tested using a colorimetric method from Promega: CellTiter® MTS Aqueous solution. The solution was composed of a tetrazolium compound called MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling agent PES (Phenazyl-Etho-Sulphate). MTS solution is bio-reduced by dehydrogenases enzymes, which are specific in metabolically active cells, into formazan which is soluble and coloured in tissue culture. Therefore absorbance at 490 nm can be read directly into a 96 well plate and the quantity of formazan produced is directly proportional to the number of actively living cells in culture. Briefly MTS working solution which is composed of MTS and PES, was prepared as above described and kept at -20°C. The solution was thawed and 20 µl was added to the 96 well plates where 100 µl of cells were seeded the day before at cell density as previously described. Cells were undergone the desired treatment and, after the required times, MTS working solution was added. Cells were kept at 37°C with 5% CO₂ for the required time (each cell type requires different incubation times: 2.5 hours for Jurkat and 1 hour for U937 cells). Absorbance at 490 nm was recorded and comparison with control was performed: usually several controls were

included such as media control, positive control with viable cells and negative control with dead cells (killed by microwave irradiation for 10 sec).

2.7.4 Necrosis detection with Propidium Iodide (PI) fluorescent staining

PI binds to the DNA by intercalating between bases with a stoichiometry of one dye per 4-5 base pair. PI is membrane impermeable and is generally excluded from viable cells, for instance PI is commonly used to identify necrotic dead cells. Our laboratory has developed a fluorescence plate reader format using the dye. The cells were cultured, and treated in a 96 well plate at concentration of $0.2-0.7 \times 10^6$ cells/ml. The volume of cell used for the assay is 100 μ l per well for a 96 well plate and 30 μ l per well for a 384 well plate. The propidium iodide stock solution was kept at -20°C at 100 $\mu\text{g/ml}$ concentration and a working solution of 5 $\mu\text{g/ml}$ in PBS was prepared before each analysis. The working solution was then added to the cell suspension: 100 μ l per well for a 96 well plate and 30 μ l per well for a 384 well plate. The solution was mixed and incubated at room temperature for 20 min in the dark. The PI fluorescence of the cell suspension was read at excitation 535nm emission 617nm. Suitable controls were added in each experiment: a positive control of necrotic cells obtained irradiating the cell with microwaves for 15 sec, negative control of media only and viable cells.

2.7.5 Caspase-3 fluorimetric assay

Caspase-3 is an effector caspase that plays a key role in several apoptosis pathways, and it has been shown to cleave poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), topoisomerases, and protein kinase C. The kit chosen for the analysis, from Anaspec, uses the inhibitor $(\text{Z-DEVD})_2$ which is conjugated with the fluorogenic indicator Rh110. When caspase-3 is cleaved it binds to the $(\text{Z-DEVD})_2$ -Rh110 that at this condition generates the Rh110 (Rhodamine 110), a fluorophore that can be detected at excitation/emission=496 nm/520 nm. Briefly cell suspension with the specific treatment was incubated for the needed amount of time and 60 μ l of cells were transferred into a 384 well black plate. All the reagent needed were thawed at 25°C and a working dilution of the substrate was prepared diluting 1ml assay buffer with 40 μ l DTT and 10 μ l caspase-3 substrate

solution. 20 μ l of the solution was then distributed to the cells and the plates incubated for 1 hour at 37°C. Fluorescence at ex 496nm/em 520nm was recorded and relative quantitation compared to the no treated control was performed. Usually a positive control of cells treated with camptothecin was included.

2.7.6 Caspase-3 Flow Cytometric assay

Active caspase-3 was also tested using a polyclonal antibody-FITC conjugated directed against the active cleaved caspase-3. Cells were harvested and washed with wash buffer, prepared as above described, centrifuge at 500g for 5 min at 25°C and supernatant discarded. Cell pellet was then carefully resuspended in 70 μ l of fix/perm solution to allow the cells to fix and permeabilize; the solution was left for 20 min at 4°C. The cell suspension was then washed with wash buffer, centrifuged at 500g for 5 min at 25°C and the supernatant removed. Cell pellet was incubated with 20 μ l/1x10⁶cells/ml caspase-3-FITC conjugated antibody and incubated for 40 min at 4°C in dark. The unbound antibody was washed with wash buffer, centrifuged at 500g for 5 min at 25°C, the supernatant removed and resuspended with fresh D-PBS. Cells were analysed on the FACS Canto dual laser flow cytometer in the FITC channel and gating of the caspase-3 positive population was performed in each analysis.

2.7.7 Annexin-V FC assay

Phosphatidylserine (PS) externalization from the inside of the plasma membrane to the outside of the cell is one of the early event that characterizes apoptotic cell death. Annexin V is a protein that binds to the PS and therefore is a marker for early apoptotic events. The detection of early stage of apoptosis was performed by flow cytometry using the Annexin V/PI kit by BD-Bioscience. Cells were harvested, washed with D-PBS and centrifuged at 500g for 5 min at 25°C; supernatant was discarded and cell pellet was resuspended with 1x binding buffer, cells counted and concentration adjusted to be all 1x10⁵ cells/ml. Cell suspension was incubated with 5 μ l of Annexin V solution for 15 min at 25°C in the dark. Prior to the analysis 5 μ l of PI 5 μ g/ml solution was added to the cell suspension and samples were straight analysed by flow cytometry: Annexin V was detected at

ex/em=490/520nm and PI was detected using a >520nm longpass filter; 10000 events were recorded for each samples and compensation controls were applied for each experiment.

2.7.8 Caspase-2 FC assay

Active caspase-2 detection was performed using the fluorescent labelled caspase-2 inhibitor carboxyfluorescein-labelled-fluoromethyl-ketone-peptide (FAM-VDVAD-FMK). The inhibitor is cell permeable and non-cytotoxic and binds covalently to the active cleaved caspase-2 that is undergone to proteolytic maturation; the fluorescent molecule bound to the Caspase-2 can be then detected by flow cytometry together with the necrosis markers PI and detected respectively at ex/em=490/520nm for the active Caspase-2 detection and at ex/em=535/617nm for the PI; commercially available kits from Bachem were used.

The FAM-peptide-FMK was first reconstituted from powder by adding 50 μ l of sterile DMSO; this gave a final stock concentration of 150x.; the content was mixed at 25°C and then stored at -20° C protected from the light into single use aliquots. A 1x working dilution of wash buffer was obtained by diluting the 10x wash buffer with dH₂O. At time of the analysis an aliquot of the stock solution was then diluted 1/5 with PBS pH 7.4 to obtain a 30x working dilution; the solution was always prepared fresh and constantly protected from the light. 300 μ l of cells per well were seeded the day before at treatment concentration of 5×10^5 cells*ml⁻¹. When the 30x FAM-peptide-FMK working solution was ready 10 μ l was added directly to the cell suspension taking care of mixing the cells by flicking the plate. The plate was left incubated for 1 hour at 37°C supplemented with 5% CO₂. The cell suspension was washed twice with 1x working dilution of wash buffer and centrifuged at 400g for 5 min at 25°C and supernatant was discarded. After the second wash cell pellet was resuspended in 400 μ l of 1x working dilution wash buffer and 5 μ l of propidium iodide solution was added into the cell suspension prior the analysis. Samples were kept at 4°C and analysed at the flow cytometer taking care to include compensation controls in the samples.

2.8 Molecular techniques and Gene expression

2.8.1 mRNA extractions by affinity purification

Purification of the messenger RNA (m-RNA) from the tissue culture cells and whole blood was performed using the GE Healthcare kit "Quick-prep micro mRNA purification kit". The kit consist of an affinity purification that uses a cellulose support conjugated with oligo d-Thymidine tails: the thymidine tails binds specifically to the poly-Adenosine tails present at the 3' end of the messenger RNA only. The m-RNA bound to the thymidine tail is then eluted with an heat elution step from the column and collected for further analysis. First, total RNA was extracted from cell culture samples: cells were collected at concentration of 5×10^5 cells/ml, cell culture media was removed by centrifuging cells at 500g for 5 min at 25°C by aspirating the supernatant; extraction buffer (400 μ l) and elution buffer (800 μ l) was added to each sample in 1.5 ml tubes for purification from 80 μ l whole blood. Samples were mixed vigorously with vortex for 5 min. Meantime 1 ml of Oligo(dT) cellulose column was prepared for each sample and packaging buffer in each tube was removed by centrifugation for 1 min at 12000g; supernatant was discarded and tubes with the ready cellulose column were set aside. In order to obtain a clear homogenate of the cell culture or whole blood samples, vortexed tubes, were centrifuged at 12000g for 1 min at 25°C and 1 ml of the supernatant was carefully transferred into the tubes containing the pelleted Oligo(dt)-Cellulose. The solution was gently resuspended by tube inversion for 3 min to allow the binding between the poly-A tail of the m-RNA and the T-bases of Oligo(dT)-Cellulose. The column was then washed five times with a high concentration of salt buffer first and then 3 times with a low concentration of salt buffer, in order to remove any unreacting substances and purify the m-RNA samples: between each wash the column was resuspended and later centrifuged at 12000g for 1 min at 25°C, and supernatant was discarded. The pelleted cellulose solution was resuspended int low salt buffer and transferred in a microspin column, where two additional washes took place. Elution between the purified m-RNA and the oligo-(dT) cellulose was obtained by heat separation, by adding 500 μ l of 65°C elution buffer, followed by a centrifugation at 12000g for 1 min. The cellulose column was blocked by the filter in the spin column, while the eluted m-RNA was collected in the tube underneath. mRNA product is very unstable and subject to easy degradation

for the very abundant presence of RNAses in the environment, therefore no long term storage was planned, instead a c-DNA synthesis was performed straight away.

2.8.2 mRNA isolation with TRIReagent®

Total RNA was isolated from tissue sections by homogenization in a guanidine thiocyanate solution followed by an extraction with phenol. The guanidine thiocyanate temporarily inactivates and denaturises the endogenous RNAses, which are then removed by the phenol extraction together with other proteins and the DNA. Finally, the RNA is precipitated to remove low molecular weight molecules and the guanidine thiocyanate. The glass homogenizers were previously treated with diethylpyrocarbonate DEPC treatment in order to inactivate RNAses: the treatment was performed the day before the extraction and glass homogenisers were immersed in a 0.01% solution of DEPC in dH₂O. The solution with the homogenizers was allowed to stand at 25° under a fume cabinet overnight. The following day DEPC-dH₂O was poured out from the beaker containing the homogenizers and both the beaker containing the water and the homogenizers were sealed and autoclaved. Beakers containing the homogenizers were only opened in sterile condition to avoid any possible RNAses contamination that could affect the results.

The frozen tissue sample was transferred directly into the 5 ml homogenizer tube containing 1ml TriReagent for approximately 30 mg of tissue. The tissue was homogenized immediately by repetitive and sharp movements of the pestle inside the homogenizer; when a clear homogenate was obtained samples were left aside for 7 min at 25°C; samples were carefully transferred to a 1.5 ml tube and 100 µl 1-bromo-3-chloropropane (BCP) was then added to the homogenate: the whole solution was vortexed for 15 sec and left aside for 8 min at 25°C. Samples were then centrifuged at 12000g for 15 min at 4°C. Followed the centrifugation the mixture separated into an upper aqueous phase containing the RNA, an interphase containing the DNA and a red lower organic phase containing the proteins, including the RNAses. Most of the upper phase, about 400 µl was carefully transferred into a fresh 1.5 ml tube: great care has been taken to avoid the interphase and lower phase. Although the interphase and organic phase could have been saved at -80°C for later

purification of DNA and protein, the analysis concentrated on the RNA purification only, therefore the rest was discarded. 400 μ l of isopropanol was added to the aqueous phase previously collected in order to precipitate the RNA. The solution was mixed carefully and incubated for 8 min at 25°C, then centrifuged at 12000g for 8 min at 25°C. Supernatant was carefully removed and 1 ml 75 % ethanol (RNase-free) was added to the tube, mixed and centrifuged at 7500g for 5 min at 25°C and the supernatant was discarded; this step was repeated twice. The RNA pellet was left to air-dry for 5 min taking care not to completely dry the pellet and finally the RNA pellet was dissolved in 30 μ l RNase-free DEPC treated dH₂O and used straight away to perform c-DNA synthesis.

2.8.3 c-DNA Synthesis

The c-DNA first strand synthesis was performed using the GE Healthcare kit "First-Strand c-DNA Synthesis Kit". The kit contains separated tubes with dried beads inside which have everything needed for the DNA first strand synthesis: the Moloney Murine Leukaemia Virus (M-MuLV) a reverse transcriptase enzyme that can perform the reverse transcription from RNA to DNA, DTT which helps stabilize the synthesis reaction, nucleotides and PCR buffer. Conditions of reaction had been optimized to allow a full length transcription of 7 kb or more of RNA. The reaction required the addition of the RNA previously purified and primer of choice, in this case it has been chosen random hexamers primers (P(n)₆ random primers) to allow the amplification of the whole mRNA extract and minimise any risk of losing the sequence of interest that would be amplified anyway by PCR in the next step. The heteroduplex formed cDNA/RNA was then directly amplified by PCR. The amount of RNA required for each synthesis can have a range between 20 ng- 2 μ g which is within the range of the m-RNA extraction kit. Beads were resuspended in 30 μ l of m-RNA purified previously and heated at 65°C for 10 min. The solution was then allowed to chill for 2 min at 4°C and 1 μ l of the P(n)₆ random primers at 0.2 μ g/ μ l was added to each sample tube. Samples were mixed carefully by vortexing, placed at room temperature for 1 min and then incubated for 1 hour at 37°C. The c-DNA obtained was stored at -20°C with the addition of 200 μ l 95% chilled ethanol until further analysis. When ready to use in the PCR reaction samples needed ethanol removal, therefore

the tubes were centrifuged at 12000g for 30 min at 4°C. The supernatant was discarded and samples were left air dry for 5 min leaving the lids open under a laminar flow cabinet until all the ethanol is evaporated. c-DNA pellet was resuspended in 50 µl of DEPC treated d-H₂O.

2.8.4 Primer design and choice

Primers for human Hsp72, Hsp90-β, and Hsp27 were purchased from Applied Biosystems:

Hsp72 from *HspA1A* gene located in Chromosome 6, from 31891316 to 31893698; the sample amplified is from 2272 and is 124 bp long.

Hsp90 from *Hsp90AB1* gene located in Chromosome 6 from 44328760 to 44329596; the sample amplified is from 2142 and is 155 bp long.

Hsp27 from *Hsp B1* gene located in Chromosome 7 from 75769859 to 75771118; the sample amplified from 473 and is 66 bp long.

Pig primers were purchased from MWG and the sequence designed by Dr J.F.Young from Aarhus University (Denmark). The sequences were:

Hsp72_for: 5'-GGC AAG GCC AAC AAG ATC AC-3'

Hsp72_rev: 5'-TCC TCA GCC TCC TGC ACC AT -3'

Hsp72_ probe: 5'- FAM ACA AGG GCC GCC TGA GCA AGG - BLACKHOLE QUENCHER-3'

2.8.5 Real time PCR

Background

The amplification of target genes from the newly synthesised c-DNA was performed with ABIPRISM 7700 SDS real time PCR machine. Real-time Polymerase Chain Reaction (PCR) monitor the progress of the PCR as it occurs, in real time. In real-time PCR, reactions are characterized by the point during time when amplification of a target gene is first detected (Ct, threshold cycle): the higher amount of c-DNA of the target gene, the sooner in time an increase in fluorescence is observed. TaqMan® chemistry consist in a oligonucleotide designed within the primer amplifying sequence containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end that reduce fluorescence of the dye by FRET (fluorescence resonance energy transfer). When the target sequence is present, the probe anneals and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is

extended. The cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal in each cycle. The increased intensity in each cycle is proportional to the amount of amplicon produced. The probe used in all the experiments were MGB probes which contain a non-fluorescent quencher at the 3' end and also a minor groove binder at the 3' end that increases the melting temperature (T_m) of probes, allowing the user to choose shorter probes and giving more specificity for the design of the probes.

Preparation of reaction

In each PCR reaction plate it was included standard and unknown samples. Standard samples, used to construct standard curves for each target gene, Hsp72, Hsp27, Hsp90 and gene control, the housekeeping gene ribosome 18S; genomic DNA was used to prepare the standard curve for each gene and it was extracted from Jurkat cells according with the procedure described in the section 2.8.3. The primers and probes used in these specific amplifications could detect genomic DNA as the sequences they amplify are located within exons and are not extended across different exons. Serial dilution of the standard was performed: the dilution factor is dependent on the level of gene expression for each gene type, therefore dilution curves were tested before run the samples and for each gene in this study 1/5 dilutions was chosen to be the more adequate. Genomic DNA was diluted with DEPC treated dH_2O . Unknown samples were added to each well and a negative control was always added in order to check the PCR quality. Each sample was run always at least in duplicates. The c-DNA quantity to use in the PCR can vary, however the suggested quantity is a range between 1 ng and 100 ng of the original mRNA when DNA comes from a retro-transcription. c-DNA samples were prepared according with the method previously described in section 2.8.3. The volume of reaction was set up to be 20 μ l. Target genes were usually labelled with FAM dye, while gene controls were usually labelled with VIC dye. The PCR master mix, containing the enzymes, the nucleotides and the primers was prepared using prepared according the following recipe:

PCR Components	20 μ l
Universal PCR master mix	10 μ l
Primer and Probe solution	1 μ l
C DNA	X μ l
PCR Water	20 (11+X)

The 11 μ l of the mix was first loaded into the 96 well plates followed by the prepared standard and unknown samples by pipetting carefully several times trying to avoid bubbling inside the wells. A plastic adherent cover was placed on top of the well and the PCR reaction was started. The primer and probes purchased from applied biosystems were already optimized to be used with the default PCR settings, while the pig primers and probes were used at 5 pM/20 μ l reaction for primers and 1 pM/20 μ l reaction. PCR settings followed the default machine guidelines. Similar results are obtained at the end of the cycles:

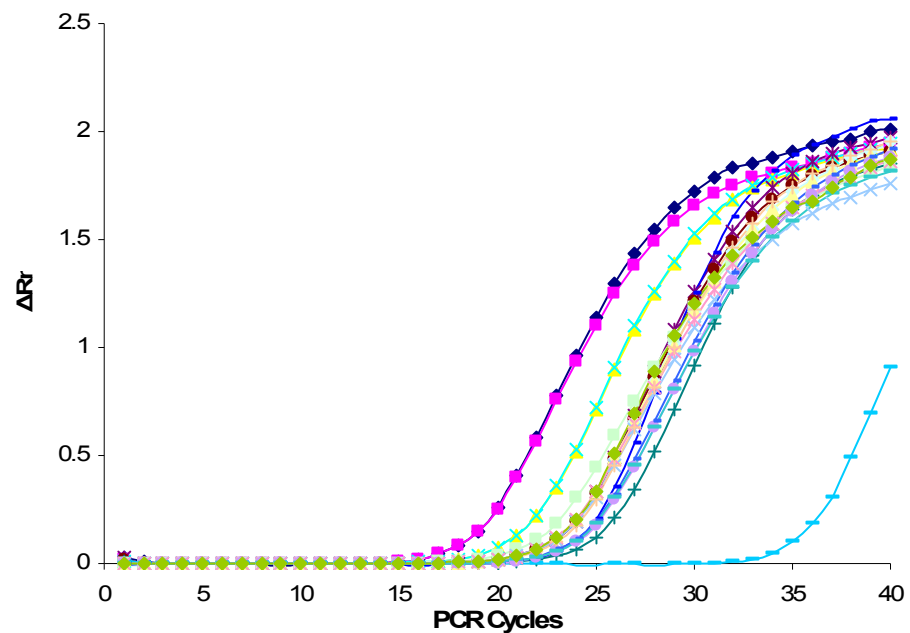


Figure 2.2: Example of a typical amplification plot obtained following a real time PCR analysis.

Baseline was adjusted in order to discard any background signal, and threshold Cycle (Ct) which is the cycle number at which the fluorescence generated within a reaction crosses the threshold, was set up; the Ct data were collected for the analysis which was performed by using the relative

quantitation method. The method analyses the changes in gene expression in a given sample relative to another reference sample, such as an untreated control sample also called calibrator sample. For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1× sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. For quantitation normalized to an housekeeping gene control, standard curves were prepared for both the target and the endogenous reference, then, the target amount is divided by the endogenous reference amount to obtain a normalized target value, then each of the normalized target values is divided by the calibrator normalized target value to generate the relative expression levels. The final result gives a relative number that is called “fold expression” which is an indication of how many times the target gene was amplified compared to the calibrator sample.

Table 2.1: Calculation related to the relative quantisation method

Sample Name	Target Gene	Control Gene	Normalized Gene	Fold expression
No Treatment	A1	B1	$A1/B1=C1$	$C1/C1$
Treatment 1	A2	B2	$A2/B2=C2$	$C2/C1$
Treatment 2	A3	B3	$A3/B3=C3$	$C3/C1$

2.8.6 Genomic DNA extraction

Genomic DNA has been extracted using a commercially available kit from Epicentre: quick extract DNA extraction solution. The protocol provides a range of 2-14 ng/ml of DNA extracted in each extraction and suggests using 5 µl in 50 µl reaction or in the case of high C-G base pair sequence use up to 15 µl in 50 µl of reaction. Briefly 1×10^6 cells/ml of cell culture was prepared and cell culture media was removed by centrifugation at 500g for 5 min followed by a supernatant discard. 500 µl of quick extract solution was added to the cell pellet and the tube was vortex for 10 sec. Samples were then incubated at 65°C for 1 min, followed by a vortex for 15 sec and an incubation at 98°C

for 2 min. Samples were vortexed for 15 sec and stored at -20°C into single use aliquots.

2.9 Detection of surface and intracellular Hsp72 protein in cells

2.9.1 Surface labelling with leukocytes markers

When analysing the entire blood cell populations it was necessary to distinguish between leukocytes subtypes to check the Hsp72 expression in each of them. For this purpose several surface CD markers have been used in conjunction with Hsp72 antibody labelling. The choice of fluorescent markers had to consider the spectra overlap and the capabilities of the FACSCanto instrument. The antibodies used and their principal characteristics are listed in Table 2.2

Table 2.2: Lymphocytes subset classification using CD Markers.

The quantities (v) are expressed in µl/test per 1×10^6 cells/ml

Normal Lymphocytes Subset		
CD3-PE	T-lymphocytes	20 µl
CD4-PE-Cy7	T-Helper lymphocytes	5 µl
CD8-APC-Cy7	T-Cytotoxic lymphocytes	20 µl
CD56-APC	NK lymphocytes	20 µl
CD45-PE-Cy5.5	General leukocytes marker	20 µl
CD19-PE	B-lymphocytes	20 µl
CD14-PE-Cy7	Monocytes	5 µl
CD15-APC	Neutrophils	20 µl
Diseased Lymphocytes Subset		
CD34-PE-Cy5.5	Malignant myeloid cells or T-cells	20 µl
CD5-APC	B-malignant cells when CD19+	20 µl

The CD labelling has been used in fresh not fixed purified white blood cells and PBMCs, after cell count adjustment to 1×10^6 cells+ml⁻¹; cell has been pelleted down by centrifugation at 500g for 5 min, resuspended with the antibody mix and incubated with the antibodies cocktail for 40 min at 4°C in

the presence of dark. This labelling is usually combined with either surface Hsp72 or intracellular Hsp72 labelling as described below in the following sections.

2.9.2 Surface Hsp72 detection by flow cytometry

Surface anti-Hsp72 cmHsp72.1 was specially produced and purchased from Dr G. Multhoff laboratories of Multimune, Munich, Germany. The use of the cmHsp72.1 antibody in this study insured that surface expression of Hsp72 is membrane embedded and not just receptor attached. This unique antibody specifically recognises the TKD peptide region, a 14-mer sequence, situated in the C-terminal region of the Hsp72 protein (aa 450–463; peptide sequence: TKDNNLLGRFELSG) and is therefore detecting protein which is embedded in the membrane. Literature evidence showed by western blots that the cmHsp72.1 antibody allows recognition of the TKD epitope of Hsp72 even when Hsp72 C-terminus is associated with the membrane which hides epitopes recognised by other antibodies (Multhoff, Pfister et al. 2001). This epitope has been demonstrated to be presented outside the cell membrane when the protein is embedded and is the target for natural killer (NK) cell anti-tumour responses (Multhoff, Pfister et al. 2001). Although the epitope for the Stressgen antibody overlaps the TKD region, it contains more amino acid residues (location between the amino acid residues 436 and 503), that become hidden in the membrane when the protein is embedded, making binding by the Stressgen antibody impossible. Tissue culture cells, white blood cells purified from whole blood and PBMCs were labelled with the surface Hsp72 antibody, sometimes in conjunction with surface markers if leukocytes subtypes analysis was needed and depending on the experiment performed; in this case both labelling happen in the same time. Cells were purified as described above, and concentration of cells was determined and adjusted to $1 \times 10^5 \text{ cells} \cdot \text{ml}^{-1}$ prior to the labelling. The cell pellet was washed with wash buffer (5% FBS in PBS), prepared as described above, centrifuged at 500g for 5 min and supernatant was discarded. The pellet was then resuspended in 3 μl antibody solution previously prepared using 1 μl of concentrated antibody per sample and 2 μl of wash buffer; samples were incubated for 40 min at 4°C in the dark. The incubation time was followed by a wash with 1 ml of wash buffer, centrifugation at 500g for 5 min followed by a

discard of the supernatant. In order to discard in the analysis any necrotic cells, 2.5 μ l of 100 μ g/ml propidium iodide was added to the cell pellet and left for 5 min at 4°C. The cell pellet was then resuspended in 20 μ l of wash buffer and analysed straight away to the flow cytometer. Cells negative to PI, therefore viable were the only gated, and the analysis of surface Hsp72 was restricted to them only.

2.9.3 Intracellular Hsp72 detection by flow cytometry

Tissue culture cells, were labelled only with the Hsp72 antibody, while white blood cells purified from whole blood and PBMCs were previously labelled with the surface CD markers, as above explained. Cells were purified as described above, and concentration of cells was determined and adjusted to 1×10^6 cells/ml prior to the labelling. The cell pellet, either previously surface labelled or not, was washed with wash buffer (5% FBS in PBS), prepared as described above, centrifuged at 500g for 5 min and supernatant was discarded. Cells were then fixed and permeabilised with 50 μ l of Fix/Perm solution (BD biosciences): the cell pellet was resuspended by pipetting carefully and the solution was incubated for 20 min at 25°C in the dark. Cells were washed by adding 1ml wash buffer, centrifuged 500g for 5 min and supernatant removed. Cells were then labelled for the intracellular Hsp72 antibody (assay design) 1 μ l+49 μ l wash buffer/test for 1×10^6 cells/ml, mixed and incubated for 40 min at 25°C in the presence of dark.. The incubation time was followed by a wash with 1 ml of wash buffer, centrifugation at 500g for 5 min followed by a discard of the supernatant. Cell pellet was resuspended in 20 μ l of wash buffer and analysed at the flow cytometer. Compensation controls for each experiment panel was run in the first experiment and settings same settings were applied throughout all the study.

2.9.4 Protein extraction from cells for ELISA

Extraction buffer was prepared according with the recipe above described. Cells culture or white blood cells or PBMCs were collected at concentration of 1×10^6 cells/ml; sample pellet was homogenized by adding in the tube 500 μ l of extraction buffer; samples were mixed vigorously for 5 min and left at 25°C for 5 min followed by a centrifugation at 13500g for 20 min. Supernatant was

collected and decanted into a clean tube, while the tube containing pellet was discarded. Samples were finally stored in -80°C freezer until required.

The frozen tissue samples, approximately 30 mg, were transferred directly into the 5 ml homogenizer tube containing 1 ml extraction buffer. The tissue was homogenized immediately by repetitive and sharp movements of the pestle inside the homogenizer; when a clear homogenate was obtained samples were left aside for 5 min at 25°C ; samples were carefully transferred to a 1.5 ml tube using the same procedure as described above.

2.9.5 Protein assay and DC protein assay DC Protein Assay

In order to calculate the amount of total protein present in each sample and to adjust the concentration of total protein used in the ELISA later on, samples extracted with the extraction buffer a protein assay has been performed

Standards were made using a known amount of bovine serum albumin (BSA) diluted in extraction buffer. The dilutions used were: 1.5-0.75-0.375-0.1875 mg/ml. The kit by Promega is composed of 3 components. In order to make a working solution A 20 μl of reagent S has been added for each ml of reagent A. A 96 microtiter plate was used and 5 μl of standard and samples were distributed in the plate. 25 μl of the working solution A and 200 μl of reagent B has been pipetted into each well the solution has been mixed for 5 sec and incubated for 15 min at 25°C after the incubation time absorbance at 750 nm was collected, standard curve of the known standard was created and concentration of the samples was worked out from the standards.

2.9.6 SDS Page electrophoresis

Protein cell extract was analysed by sodium dodecyl sulphate – polyacrylamide electrophoresis (SDS-PAGE). Solutions for the SDS electrophoresis were prepared in advance following the recipes above described. Bio-Rad mini-protean II electrophoresis apparatus was assembled and 1mm thick gel was chosen for the experiments Acrylamide separating gel was prepared according the following recipes: 6.05ml dH_2O , 3.75 ml 1,5M Tris-HCl pH 8.8, 150 μl 10% SDS, 5 ml Acrylamide-Bis were mixed together and degassed for 10 min; then 50 μl 10% Ammonium Persulphate and 7.5 μl TEMED was added to the solution to start the polymerization and gel was pipetted carefully underneath the glass slides until a certain level, then 1ml

water was added up to the top; the gel was left to polymerise for 1 hour, or at least until when the line between the gel and the water was visible. Once the gel has set the water was poured out from the top and the staking gel was prepared according with the following recipe; 3.15 ml dH₂O, 1.25 ml 0,5M Tris-HCl pH 6,8, 50 µl 10% SDS, 500 µl Acrylamide-Bis, were mixed together and degassed for 10 min, then the last two ingredients 50 µl 10% Ammonium Persulphate and 5 µl TEMED were added.

Combs were placed on top of the separating gel and the staking gel solution was pipetted carefully to the top of the separating gel taking care of not creating any bubbles. The gel was left to polymerise for about 40 min. In the mean time samples were prepared and eventually diluted in extraction buffer in order to have the same amount of total protein in each well. Samples and standard which is usually the pure protein of interest were as well diluted 1:1 with sample buffer and were heated at 85°C for 10 min to allow denaturation. After the heat treatments they were allow cooling for 10 min and then loaded in the well not before removing the combs from the staking gel and the whole electrophoresis apparatus assembled. Electrode buffer was poured into the middle of the assembly to the top and in the outer assembly until buffer covered the bottom centimetre of the middle assembly. Only then all samples and pure protein controls were loaded into the gels with Precision Plus[®] standards as a molecular weight reference. The loading quantities followed the following scheme:

N°wells	15	10	9	5
µl to add	26 µl	44 µl	44 µl	105 µl

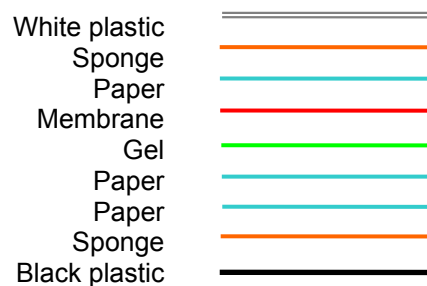
The apparatus was assembled and electricity was applied to the chamber: gel run at 150 V for most of the electrophoresis separation and at 200V for the last centimetre to obtain a better separation

If a western blot was performed straight away the gel was loaded in a western chamber as fully described below. If the gel was used to highlight all the protein it was applied to the gel a protein stain such as imperial stain which binds to amino acid residues resulting in colour formation where the proteins are: the gel was first washed three times with dH₂O and then 20 ml of the imperial stain was poured carefully over the gel and left incubating for 1 hour

in an orbital shaker. Gel was washed three times with dH₂O and was ready to be visualized and recorded with the Bio-Rad ChemiDoc imaging system

2.9.7 Western blot

Bio-Rad mini-trans blot apparatus Run the SDS gel using pre-stained or colour protein molecular weight reference standard (10 µl). While the gel is running, sponges, 3 pieces of chromatography paper and nitrocellulose membrane were soaked in transfer buffer. Once the gel was ready the electrophoresis apparatus was opened and the stacking gel was removed with a razor blade. Western chamber was assembled according with the following order:



Western chamber was assembled by putting an Ice pack inside and by placing the sandwiches into the chamber with black sides to all the left, next to the ice pack, . Transfer buffer was poured in the chamber filled up to the level. 100 V electricity was applied for 60 min. Once the run is finished the nitrocellulose paper was removed from the chamber and placed in a container with blocking solution for 60 min. The blot was then incubated with the specific biotinylated-antibody at the appropriate dilution in 50 ml of the antibody buffer overnight at 4°C. Blot was then washed 3 times with wash buffer and an appropriate dilution of peroxidase avidin-conjugated was added to the blot in 50 ml antibody buffer. The blot was incubated for 1 hour in orbital shaker, then washed three times with wash buffer then incubated with 2 ml working solution of Supersignal West Pico chemiluminescent substrate (1:1 dilution of

the two solutions) for 5 min with a gentle shaking, then visualised and recorded using the Bio-Rad ChemiDoc imaging system

2.9.8 Intracellular Hsp72 detection: Cells extract ELISA

The method was developed in house and the two antibodies were raised and purified by other researchers.

Nunc binding plates were coated the with 100 μ l solution/well of 2 μ g/ml of polyclonal affinity purified sheep anti-Hsp72 which was raised against the SIGMA Hsp72; the antibody was diluted in carbonate buffer (the recipe is above in the section 2.4.3) and incubated overnight at 4°C. Plate was washed 3 times by adding 300 μ l of wash buffer, using the plate washer, then carefully dried. Plate was then blocked with 300 μ l of 0.5% BSA in PBS and incubated for 1 hour at 25°C, followed by three washes with 300 μ l of wash buffer. The plate was dried and 100 μ l of the samples and the standards were added to each well. Samples and standard were diluted in extraction buffer; the top standard was set to be 100 ng/ml and standard curve was prepared in doubling dilutions; the plate was incubated for 2 hours at 37°C. Samples and standard were washed 6 times with 20 sec soak in between washes using the plate washer and dried. 100 μ l of the diluted detector antibody (1/5000) mouse monoclonal anti Hsp72 in 0.5%BSA in PBS solution was added to the wells and incubated for 1hour at 37°C. The plate was washed 6 times with the 20 sec soak in between wash using the plate washer and carefully dried. 100 μ l of the diluted antimouse IgG HRP (1/2500) in 0.5% BSA in wash buffer was added to each well and incubated for 1hour at 37°C. The plate was washed 6 times with the method described above and 100 μ l of TMB substrate was added and incubated at 25°C in a plate shaker for about 30 min being careful to check the colour development in between. The reaction was stopped with 1M orthophosphoric acid and the absorbance at 450 nm was recorded.

2.9.9 Extracellular Hsp72 detection: supernatant, serum and plasma ELISA

The method was developed in house and the two antibodies were raised and purified by other researchers. The method follows the protocol described in the previous section 2.9.8; the antibodies pair dilution changed with 1/1000 dilution for the capture antibody and 1/1250 for the secondary antibody.

2.10 Fluorescence Microscopy methods

Cells cultures were labelled with the several antibodies in order to find co-localization of surface and intracellular Hsp72 with cellular and membrane markers. The fluorescence slides produced were analysed with the Nikon Eclipse TE2000-U fluorescence microscopy using IPLAB Suite Software.

2.10.1 Detection of surface Hsp72 and the lysosome marker LAMP-1

LAMP-1 protein is present in lysosome but it has been previously found to be present on the surface of cells in certain conditions; protocol was tested to see if it was on the surface or on the intracellular environment; therefore two protocols have been developed in order to check the presence in both of the locations. Cells at concentration of 1×10^6 cells/ml were prepared, by washing with PBS, centrifuging at 500g 5 min and removing the supernatant. Cell pellets were blocked with wash buffer (FBS 5% in PBS) for 30 min at 25°C, then centrifuged at 500g for 5 min at 25°C and the supernatant removed. Cell pellet was labelled with 100 μ l of anti-LAMP-1 and anti-Hsp72 mixed at appropriate concentrations diluted in buffer for 1 hour at 25°C in the dark; cells were washed as described above and antimouse IgG-Cy3 conjugated was added at appropriate concentration for 1 hour at 25°C in the presence of dark. Unbound antibody was washed away and cells were fixed with PFA 4% for 30 min. Cells were then washed and resuspended in 20 μ l of PBS; the whole cell solution was added carefully on top of a glass slide; the slide was mounted by dropping antifade solution with DAPI on top of the slides and by placing the cover slip on top taking care of not create any bubbles. Slides were left overnight to cure and fluorescence analysis was performed the following day.

2.10.2 Detection of Hsp72 and Golgi apparatus marker, Golgin 97

Golgin 97 is an antibody that labels Golgi apparatus. The staining protocol was made in conjunction with anti-Hsp72 in order to test co localization of Golgi with Hsp72 protein

Cells at concentration of 1×10^6 cells/ml were prepared, by washing with PBS, centrifuging at 500g 5min and removing the supernatant. Cell pellets were blocked with wash buffer (FBS 5% in PBS) for 30 min at 25°C, then washed by centrifuging at 500g for 5 min at 25°C, supernatant removed. The cell

pellet was fixed and permeabilised by adding 100 µl of fix/perm solution (BD Biosciences) for 20 min at 25°C. Cells were washed as described above and 100ul of Golgin-97 and anti-Hsp72 was added at appropriate concentrations diluted in buffer for 1 hour at 25°C in the dark; cells were washed as described above and antimouse IgG-Cy3 conjugated was added at appropriate concentration for 1 hour at 25°C in the dark. Unbound antibody was washed away as previously described and cells resuspended in 20 µl of PBS; the whole cell solution was added carefully on top of a glass slide; the slide was mounted with DAPI on slide and covered with a cover slip as described above. Slides were left to cure overnight and fluorescence analysis was performed the following day.

2.10.3 Detection of surface Hsp72 and the lipid raft marker GM-1

Lipid rafts are detergent insoluble, sphingofosfolipid and cholesterol-rich membrane micro domains that assemble in the plasma membrane. Live cells were first labelled with, cholera toxin subunit B (CT-B)-alexa fluor 555 conjugate which binds to the pentasaccharide chain of plasma membrane ganglioside (GM-1) a component of lipid rafts. An antibody that specifically recognizes CT-B is then used to crosslink the CT-B labeled lipid rafts into distinct patches on the plasma membrane, which were visualized by fluorescent microscopy. The lipid raft labelling was coupled with anti-Hsp72 in order to obtain an analysis of co-localization of the protein with these membrane structures. Cells at concentration of 1×10^6 cells/ml were prepared, by washing with PBS, by centrifugation at 500g 5min and removing the supernatant. Cell pellets were blocked with wash buffer (FBS 5% in PBS) for 30 min at 25°C, then washed by centrifugation at 500g for 5 min at 25°C, supernatant removed. CT-B conjugate was added at appropriate concentrations for 30 min at 4°C. Cells were washed by centrifugation at 500 g for 5 min at 25°C and anti CT-B together with Hsp72 antibody was added to the cell pellet at appropriate concentrations for 1 hour at 4°C in the dark. Cells were washed as described above and fixed with 100 µl PFA 4% for 30 min at 4°C in the dark. The cell suspension was washed and resuspended in 20 µl of PBS. The slide was mounted with DAPI on slide and covered with a cover slip as described above. Slides were left to cure overnight and fluorescence analysis was performed the following day.

2.11 Confocal microscopy detecting Hsp72 and lipid raft

2.11.1 Lipid rafts and Hsp72 detection in tissue cryosections for confocal microscopy analysis

A piece of tissue was embedded in cold isopentane, snap frozen in liquid nitrogen and stored at -80°C until needed; $\sim 50\ \mu\text{m}$ frozen tissues sections were cut and dried for 2 hour at 25°C before the labelling. Slides were rinsed in PBS and fixed by adding a drop of 2% formaldehyde (diluted in PBS); slides were incubated for 30 min at 25°C . Slides were rinsed with PBS 3 or 4 times for one minute and blocked with PBS/BSA 2% buffer for 30 min at 25°C with a sufficient volume to cover each cover slip or section. Sections were rinsed with PBS and 1 ml CT-B-conjugate at appropriate concentration was added to the sections and incubated for 1 hour at 37°C . Slides were washed as described before and the anti CT-B conjugated together with the Hsp72 antibody at appropriate concentrations was added and incubated overnight at 4°C protected from light. Slides were rinsed with PBS and mounted with one drop of ProLong® Gold antifade reagent. Slides were left overnight to cure in a flat surface and protected from the light before the microscopy analysis; on the day of the analysis slides were sealed with nail varnish all around the edge of the cover slips. Confocal analysis was performed using the green laser to detect Hsp72 which has a FITC molecule conjugated and the red laser to detect lipid rafts which has the alexa fluor 555 conjugated.