Aus der Medizinischen Klinik und Poliklinik III-Großhadern
der Ludwig-Maximilians-Universität München
Vorstand: Prof. Dr. med. W. Hiddemann

Effects of intracellular and extracellular heat shock proteins on anti-tumor immune responses

Implications for the treatment of solid tumors using hyperthermia and HSP-based vaccines

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
der Ludwig-Maximilians-Universität zu München

vorgelegt von

Valeria Milani

aus
Rom

2005

Mit Genehmigung der Medizinischen Fakultät
der Universität München

1. Berichterstatter:	Prof. Dr. med. dipl. biochem. Rolf Dieter Issels
2. Berichterstatter:	Prof. Dr. A. Wagner Prof. Dr. W. Machleidt Priv. Doz. Dr. G. Hartmann
Mitbetreuung durch den promovierten Mitarbeiter:	PD Dr. rer. nat. Elfriede Nössner
Dekan:	Prof. Dr. med. Dr. h. c. K. Peter
Tag der mündlichen Prüfung:	13.01.2005

Dedicated to

Titti

and

Maria-Giulia

INDEX

ABBREVIATIONS

aa, amino acid (only with numbers)
Ab, antibody
ADP, adesonise diphosphate
Ag, antigen
APC, antigen-presenting cell
ATP, adenosine triphosphate
APS, Ammonium peroxidisulfate
A42: HLA-A2 restricted Melan-A/MART-1 peptide-specific T cell clone
bp, base pair (only with numbers)
B-LCL: B-lymphoblastoid cell line
BSA, bovine serum albumin
CCR, C-C chemokine receptor
CD, cluster of differentiation
CHAPS: 3-((3-cholamidopropyl)dimethylammonio)-1propanesulfonate;
CML, cell-mediated lysis
cpm, counts per minute
CSF, colony-stimulating factor
CTL, cytotoxic T lymphocyte
CXCR, CXC chemokine receptor
D_3, Downscale 1:3
DC, dendritic cell
DIC, digital interference contrast
DMSO, dimethylsulfoxide
DNA, deoxyribonucleic acid
DRiPs: defective ribosomal products
ds, double-stranded (as dsDNA)
ECL, enhanced chemiluminescence
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay ER, endoplasmic reticulum
E:T ratio, effector to target ratio
Fab, antigen-binding fragment of immunoglobulin
FA, Formaldehyde
FACS, fluorescence-activated cell sorter
FCS, fetal calf serum
FITC, fluorescein isothiocyanate
FPLC, Fast Protein Liquid Chromatography
g, gram (only with numbers)
GM-CSF, granulocyte-macrophage CSF
gp, glycoprotein (e.g., gp100)
h, hour (only with numbers)
HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HLA, human histocompatibility leukocyte antigens
2-ME, 2-mercaptoethanol
MFI: mean fluorescence intensity
mg, milligram (only with numbers)
MHC, major histocompatibility complex
min, minute (only with numbers)
ml, milliliter (only with numbers)
mRNA, messenger RNA
MyD88, myeloid differentiation factor 88
µg, microgram (only with numbers)
µl, microliter (only with numbers)
m.w., molecular weight
n, number in study or group
MEM, non essential amino acids
NF-kB, nuclear factor kB
NK cell, natural killer cell
NO, nitric oxide
NS, not significant
OVA, ovalbumin
PAGE, polyacrylamide gel electrophoresis
PBL, peripheral blood lymphocyte
PBMC, peripheral blood mononuclear cell
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PE, plating efficiency
PFA, paraformaldehyde
PG, prostaglandin
PHA, phytohemagglutinin
PI, propidium iodide
PMA, phorbol myristate acetate
PMSF, phenylmethylsulfonyl fluoride
r, recombinant, (e.g., rIFN-g)
rh-, recombinant human
R, receptor (e.g., IL-2R)
RCC: renal cell carcinoma
RNA, ribonucleic acid
rpm, revolutions per minute
RT-PCR, reverse transcriptase polymerase chain reaction
s.c., subcutaneous
SD, standard deviation
SDS, sodium dodecyl sulfate
SEM, standard error of the mean
SF, survival fraction
TAA, tumor associated antigens
TAP, transporter associated with antigen processing

HRP, horseradish peroxidase
HSV, herpes simplex virus
HSP: heat shock protein
HSC70: constitutively expressed heat shock protein cognate 70 (M_r 73 kD)
HSP70: inducible heat shock protein 70 (M_r 72 kD)
HSP70-PC: HSP70/HSC70 peptide complexes
HS: human serum
IFN, interferon (e.g., IFN-g)
Ig, immunoglobulin
IL, interleukin (e.g., IL-2)
IRAK, IL-1-receptor-associated kinase
kDa, kilodalton (only with numbers)
LAK-cells: Lymphokine-activated killer cells LPS, lipopolysaccharide
mAb, monoclonal antibody
MAPK, mitogen-activated protein kinase
MART-1, melanoma antigen recognized by T cell 1
TBS, Tris-buffered saline
TBST, TBS with Tween 20
TCA, trichloroacetic acid
TCR, T cell receptor
TEMED, tetramethylethylendiamine
Th cell, T helper cell
TGF, tumour growth factor
TID, thermal isoeffect dose
TIR, Toll/IL-1 receptor domain
TLR, Toll-like receptor
TMB, tetramethylbenzidine
TNF, tumor necrosis factor
TRAF6, TNF receptor-associated factor 6
Tris, tris(hydroxymethyl)aminomethane
Tyr, tyrosinase
TyrF8: HLA-A2 restricted, tyrosinase peptide-specific T cell clone
VLE, very low endotoxin

1 INTRODUCTION

1.1 THE CELLULAR ANTITUMOR IMMUNE RESPONSE: EFFECTOR CELLS AND MECHANISMS

Host defense relies on a concerted action of both non-antigen specific innate immunity and antigen-specific adaptive immunity (Fearon *et al.* 1996; Medzhitov *et al.* 1997). Key features of the mammalian innate immune system include the ability (a) to rapidly recognize pathogen and/or tissue injury and (b) to signal the presence of danger to cells to the adaptive immune response (Matzinger 1994). The innate immune system is the very first inflammatory reaction and includes phagocytic cells, natural killer cells, complement and interferons. Evolutionary pressure has led to the development of the adaptive immunity, whose key features are (a) the ability to rearrange genes of the immunoglobulin family and the T cell receptor (TCR), permitting creation of a large diversity of antigen-specific clones and (b) antigen specific memory.

1.1.1 Dendritic cells: the link between the innate and adaptive immune response

The adaptive immune system is a highly sophisticated and potent system and needs to be instructed and regulated by antigen-presenting cells (APCs). **Dendritic cells** (DC) are unique APCs because they are the only ones that are able to induce primary immune responses, thus permitting the establishment of immunological memory (Banchereau *et al.* 1998). DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells with high phagocytic capacity. Following tissue damage, immature DCs capture antigen and subsequently migrate to the lymphoid organs, where they select rare antigen-specific T cells, whose TCR matches the antigenic ligand that DC had encountered. DCs directly present Ag to CD4+ T-helper cells, which regulate immune effectors, like B cells and NK cells and to Ag-specific CD8+ cytotoxic T cells. Morever, DCs prime CD8+ T cells to differentiate into effector cells and educate effector cells to home to the site of injury. DCs constitute a complex system of cells which, under different microenvironmental conditions, can induce contrasting states such as immunity and tolerance (Banchereau *et al.* 2000).

1.1.2 Maturation of dendritic cells

Immature DCs are defined by cell surface markers that represent functional capacity. They express the chemokine receptors CCR-1, CCR-2, CCR-5 and CXCR-1, commonly thought to allow DCs to migrate in response to inflammatory chemokines expressed by inflamed tissues. Immature DCs express the molecules CD1a, CD11 and the specific DC marker DC-specific

ICAM-3-grabbing nonintegrin DC-sign (CD209). Immature DCs are phagocytic and have a high level of macropinocytosis, allowing them to take up antigens. Expression of the FcγR1 (CD64) and the mannose receptors allow efficient capture of IgG immune complexes and antigens that carry mannose or fucose residues. The high content of ER and endocytic compartments allows efficient antigen processing. If DCs receive inflammatory stimuli, DCs are remodeled. FcγR1 and mannose receptors are downregulated, resulting in a loss of endocytic activity and acidic intracellular compartments disappear. During this maturation process, the DC marker CD83 is induced, the levels of intracellular and surface MHC class II molecules, adhesion and costimulatory molecules are upregulated, and chemokine receptor expression changes. For example, the induced expression of CCR-7 on mature DCs allows them to home to T cell areas of secondary lymph nodes, where they present antigen to naive T cells and induce T cell differentiation to effector cells (Banchereau *et al.* 2000).

In vitro human DC can be generated from peripheral blood mononuclear cells (PBMN) by adding GM-CSF and IL-4; addition of CD40L, LPS and TNF-a ("danger signal") (Banchereau *et al.* 2000) (Matzinger 1994) to the culture on day 7 generates mature DCs (figure 1).

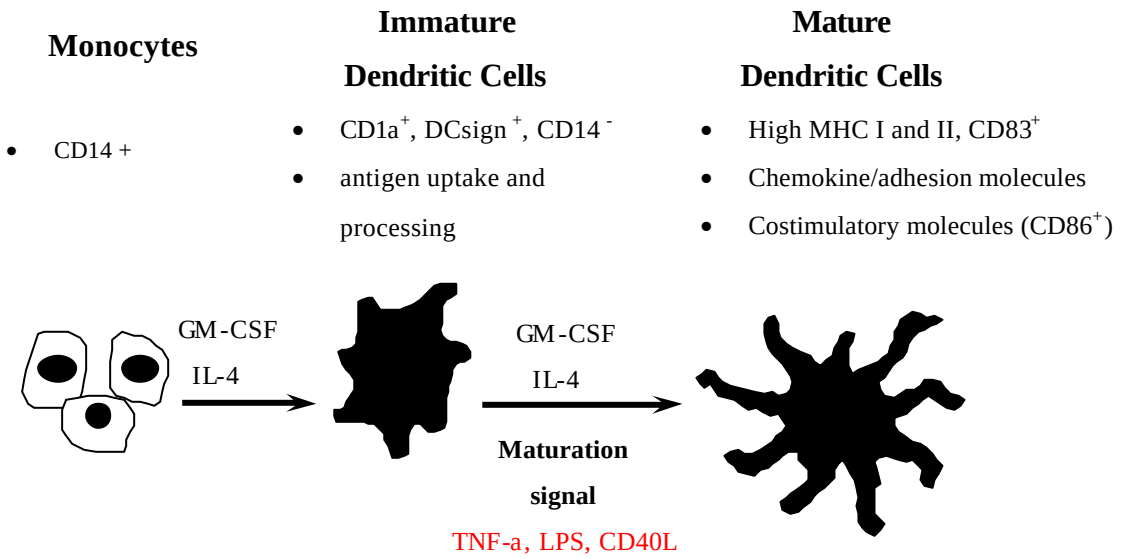


Figure 1: Generation of human DC in vitro. To obtain immature DCs, blood monocytes are cultured in medium containing GM-CSF plus IL-4 for 7 days. The addition of TNF-α or LPS induces immature DCs to mature within 1 to 2 days.

1.1.3 DC/T cell interaction

The ability to prime naïve CD4+ and CD8+ T cells constitutes a unique and critical function of DCs. This ability results from the expression of molecules unique to DCs, like the DC-specific ICAM-3-grabbing nonintegrin (DC-Sign) or from the high density of molecules involved in the DC/T cell interaction, like costimulatory and adhesion molecules. MHC-peptide complexes are 10 to 100-fold higher on DCs than on other APCs, like B cells or monocytes and macrophages.

Priming of a naïve T cell requires two signals. **Signal one** is the recognition of MHC-peptide complexes on DCs by the Ag-specific TCRs on T cells. The **second signal** is the interaction of costimulatory molecules expressed by DCs (like CD86, CD80, CD40) and their ligands, CD28 and CD40L on T cells. Signal 1 and 2 must be provided by the same cell. T cells that receive both signals become activated and produce cytokines, proliferate and differentiate into effector cells. In the absence of CD28 ligation, T cells undergo apoptosis or become anergic.

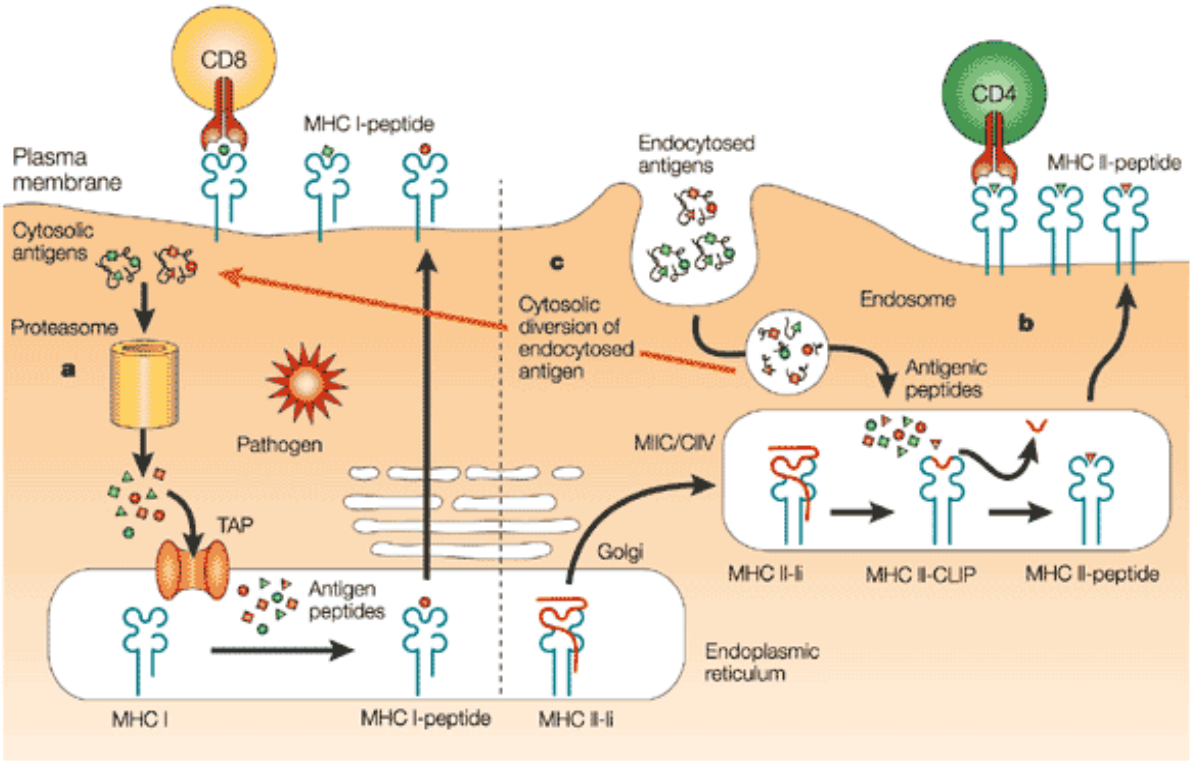
1.1.4 Antigen presentation

T cells recognize antigens exclusively as peptides presented by MHC class I or II molecules. All nucleated cells are equipped with the machinery for endogenous antigen processing, leading to presentation by MHC class I molecules. The processing for MHC class II presentation (exogenous pathway) is restricted to antigen presenting cells (APC). Among APCs, DC are best equipped for both pathways and in addition may employ a third pathway, called cross-presentation.

Soluble and particulate antigens are efficiently captured either through macropinocytosis or receptor-mediated endocytosis and they are subsequently degraded in endosomes **(exogenous pathway)**. The generated peptides are usually 13-20 amino acids long. In immature DCs MHC II molecules are retained in a special compartment and have a short half-life on the cell surface due to rapid internalization. Inflammatory stimuli lead to DC maturation, resulting in a burst of class II synthesis and translocation of the MHC II-peptide complexes to the cell surface, where they remain stable for days and are available for recognition by CD4+ T cells (Adler *et al.* 1998) (figure 2).

Antigens presented by MHC class I molecules are generally derived from intracellular proteins (self or foreign). They are degradated **i**nto peptides of 8-9 amino acids length and located into MHC class I classically via the **endogenous pathway** (Kurts *et al.* 1996) (figure 2). Processing starts in the cytosol by the proteasomes. The peptides generated are transported to the endoplasmic reticulum (ER) by TAP (= **t**ransporter **a**ssociated with antigen **p**rocessing)

proteins in a ATP-dependent manner. Several ER-resident chaperones facilitate and control the loading of peptides into MHC class I molecules. Assembled peptide-MHC I complexes then move through the Golgi apparatus to the cell surface where they can be recognized by CD8+ T cells (figure 2).



Endogenous **Exogenous**

Figure 2: Antigen-processing pathways for the MHC class I and class II molecules. A) MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self or pathogen origin. These proteins are degraded into peptides by the proteasome and then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum for loading on MHC class I molecules. **B)** By contrast, MHC class II molecules present proteins that enter the cell through the endocytic route. During maturation of MHC class II molecules, they are prevented from binding to endogenous antigens in the endoplasmic reticulum by association with the invariant chain (Ii). Invariant chain–MHC class II complexes (MHC II–Ii) move through the Golgi to the MIIC/CIIV compartment where the invariant chain is degraded to CLIP (class II-associated invariant-chain peptide). CLIP is removed from the CLIP–MHC class II (MHC–CLIP) complexes and exchanged for antigenic peptide. **C)** Dendritic cells can endocytose antigens released from other cells and cross-present them to $CD8^+$ cytotoxic T lymphocytes. The TAP-dependence of such cross-presentation, indicates that it involves diversion of the cellular antigens into the conventional MHC class I pathway (Heath *et al*. 2001).

Among APCs, DC have the unique ability to load peptides derived from exogenous antigens into MHC class I. This process is called **cross-presentation.**

The intracellular sites of peptide loading on MHC class I molecules during cross-presentation are still unclear (Yewdell *et al.* 1999; Amigorena 2003). Two main, not-mutually-exclusive models have been proposed based on *in vitro* results (figure 3). The endosomal model predicts that after antigen uptake and degradation in endosomes and lysosomes, peptide exchange allows loading on internalized MHC class I molecules, which then recycle to the cell surface (TAP-independent pathway) (Yewdell *et al.* 1999). In contrast, the cytosolic pathway model proposes that incoming antigens are delivered from endosomes to the cytosol. Once in the cytosol, antigens are shunted to the conventional MHC class I antigen–loading endoplasmic reticulum pathway dependent on transporter associated with antigen processing (TAP) and proteasomes (Yewdell *et al.* 1999 (figure 4) (figure 3). These two models can be distinguished experimentally because the former is sensitive to lysotropic agents (such as NH_4Cl) and is independent of TAP and proteasomes, whereas the latter is less sensitive to inhibitors of lysosomal functions, but is dependent on TAP and proteasomes (Yewdell *et al.* 1999). Depending on the nature of the antigens and of the antigen-presenting cells, cross-presentation is either TAP dependent or independent and can be sensitive or resistant to proteasome inhibitors at least *in vitro* (Amigorena 2003).

Just recently a model for the TAP-dependent MHC class I loading during cross-presentation based on the fusion of phagosomes with the endoplasmic reticulum in antigen-presenting cells has been suggested (Guermonprez *et al.* 2003; Houde *et al.* 2003). This fusion event generates a mixed endoplasmic reticulum–phagosome compartment, where peptide loading during cross-presentation may occur. The working model from these studies predicts that phagocytosed antigens are delivered to the cytosolic face of phagosomes, where they are ubiquitinated and degraded by phagosome-associated proteasomes. The resulting peptides are then reimported into phagosomes by TAP and loaded onto phagosomal MHC class I molecules. Thus, in the cytosolic pathway of cross-presentation, peptide loading would occur in mix endoplasmic reticulum–phagosome compartments (Guermonprez *et al.* 2003; Houde *et al.* 2003).

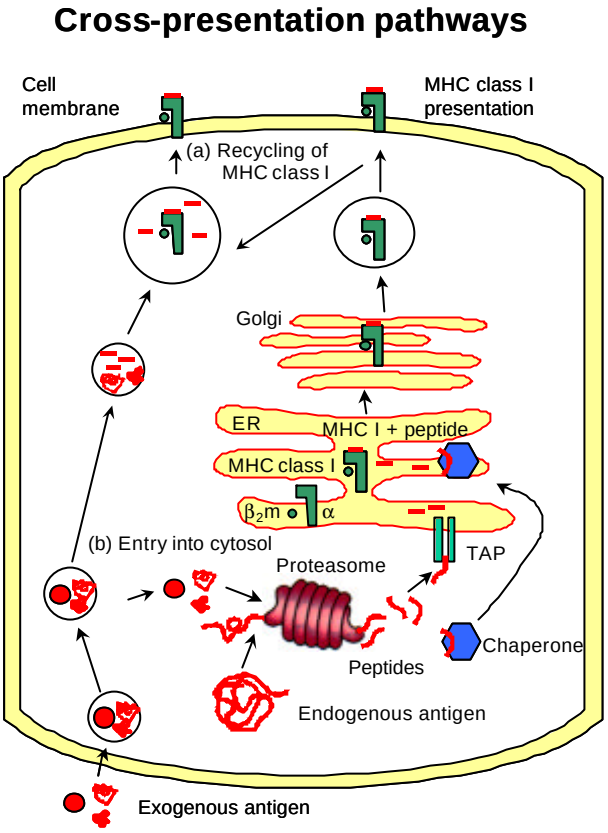


Figure 3: Cross-presentation. The cross-presentation is the process by which peptides originated extracellularly (exogenous antigens) can be presented in the context of MHC class I molecules; this is a key feature of DCs.

1.1.5 T cell mediated anti-tumor immune response

Identification of T cells that recognize tumors in vitro and the identification of tumor antigens provided clear indication that the immune system has the potential to recognize and even eliminate neoplastic cells. The fact however that tumors develop and grow indicate that control of tumor growth by the immune system often fails.

Several steps are involved in the induction of tumor immunity:

1) presentation of tumor molecules by DC precursors that involves capture of tumor cells and cross-presentation of tumor-associated-antigens (TAAs) by immature DCs
2) selection and activation of TAA-specific T cells
3) homing of TAA-specific effector T-cells to the tumor site and recognition of restriction elements leading to elimination of the tumor.

1.1.5.1 Tumor-associated-Antigens (TAA)

TAA may be classified according to their tissue distribution (Renkvist *et al.* 2001):

- Ubiquitous antigens are widely expressed on many normal and neoplastic tissue. Examples are carcino-embryonic antigen (*CEA) and HER2-neu.*

- Tumor-restricted antigens are only expressed on neoplastic cells. They can be **shared** among tumors of the same or of different origin and may also be expressed by normal spermatocytes and/or spermatogonia of testis (cancer/testis antigens). Examples include MAGE antigens, which are a family of related genes (A1-A2-A3-A4-A6-A10-A12), BAGE, GAGE, NY-ESO. Tumor-restricted antigens also include **unique** antigens, which result from point mutations or frame shifts. Example are: CDK-4, MUM-1, MUM-2, β-catenin, HLA-A2-R170I, ELF2m, myosin-m, caspase-8, KIAA0205, HSP70-2m. Unique antigens derive also from fusion proteins (fusion proteins are never found in normal tissues), like *bcr-abl* in chronic myeloid leukemia, *ETV6/AML* in acute lymphatic leukemia or *Pml/RARa* in acute myeloid leukemia.

- Differentiation antigens are expressed in normal and neoplastic cells of the same lineage such as melanocytes and melanoma or prostate and prostate carcinoma. Example are antigens of the melanocyte lineage, like tyrosinase, MART-1 and gp100, or prostate specific antigen (PSA).

1.1.6 Failure of tumor control by the immune system: tumor immune escape mechanisms

The revival of the immunosurveillance theory came with the knowledge of well-defined and structurally characterized tumor-associated antigens (TAAs) and the progress in understanding the molecular pathways required for induction and maintenance of an immune response. Advances in methodologies to generate TAA-specific cytotoxic T lymphocytes (CTLs) and monoclonal antibodies (mAbs) as immunological probes were of critical importance for the development of cancer immunotherapies and vaccines. However, contrary to the positive results obtained with TAA-specific immunotherapy in animal model systems, the clinical response in patients has been disappointing. Frequently, the immune responses do not correlate with the clinical responses. Analysising the underlying mechanisms for this dichotomy discovered the low immunogenicity of TAAs, lack of immunological markers to predict clinical outcomes, and the ability of tumors to escape immune recognition and destruction. Those hurdles are currently the challenges for the development and application of immunotherapy.

Tumors have developed mechanisms to escape immune surveillance already in the priming phase by influencing the ability of antigen-presenting cells to adequately prime T cells. In the effector phase of an immune response tumor cells block recognition by effector cells of the innate immune system (macrophages, complement and/or natural killer cells) and of the adaptive immune system (CD8+T cells and B cells) and divert effector cells by affecting T cell activity.

Known mechanisms are the loss or down-regulation of HLA class I antigens, the loss of tumor antigens and immunodominance. Loss of surface antigen expression can occur independently of the dysregulation of HLA class I expression. The phenomenon of immunodominance is the preferential immunodetection of only one or a few epitopes among many expressed on a given target (Khong *et al.* 2002).

Defect in death receptor signalling, like Fas ligand (FasL) and TRAIL play a role in immune surveillance against tumor. Defective death receptor signaling is a mechanism that may contribute to the survival and proliferation of tumor cells. Lack of costimulation is also a very well known immune escape mechanism: lack of expression of costimulatory molecules by tumor cells may lead to T cell anergy and suboptimal activation of NK cells. Activation or inhibition of T cells also depends on the presence or absence of cytokines in their immediate microenvironment. Tumor cells produce a variety of immunosuppressive cytokines (VEGF, IL-10, TGF-β) and chemokines that can negatively effect maturation and function of immune cells. *In vitro* studies show that VEGF inhibits DC differentiation and maturation. IL-10 can exert an inhibitory effect on DC differentiation from stem cell precursors. In addition, maturation and the functional status of DCs are also compromised by IL-10. This cytokine also inhibits antigen presentation, IL-12 production and induction of T helper type 1 responses *in vivo*. TGF-β inhibits the activation, proliferation and activity of lymphocytes *in vivo*.

Finally, the tumor can induce apoptosis of activated T cells, via FasL-mediated T cell death (Khong *et al.* 2002).

An immunotherapeutic strategy may be optimal if it achieves three things concurrently: provision of appropriate immune activating signals, elimination of inhibitory factors and avoidance of the emergence of immunoresistant phenotypes. The latter might be achieved when different modalities are combined to sustain a response long enough to complete tumor destruction (Khong *et al.* 2002).

Many strategies have been developed to overcome these diverse escape mechanisms. More recently interest arose from the observation that stress condition may induce a specific

immune response against the tumor. These studies started more than twenty years ago, when Srivastava discovered that HSPs isolated from tumor were able to induce an immune response in mice that protected them from subsequent tumor chanllenges (see session heat shock proteins).

The goal of this study was to investigate whether and when heat shock proteins expressed from the tumor, either constitutively or induced upon stress, are appropriate activating signals for an antitumor immune response and whether a stress-like heat-treatment affects the immunophenotype of a given tumor.

1.2 EFFECTS OF HYPERTHERMIA ON TUMOR ANTIGENICITY AND SUSCEPTIBILITY TO IMMUNE EFFECTOR MECHANISMS

Among the various treatment modalities for cancer, hyperthermia has recently taken a position as treatment for several human cancers (Wust *et al.* 2002). By acting as a chemo- and radiosensitizer and by increasing the blood flow, loco-regional hyperthermia was found to be effective in clinical trials for melanoma (Overgaard *et al.* 1995) as well as for certain other solid tumors (Falk *et al.* 2001). The initial rationale for the use of hyperthermia was based on the observation that temperatures above 43°C are directly cytotoxic to tumor cells as a function of time. However, the heterogeneous temperature distribution achieved at the tumor site ranges between 40°C and 44°C and is frequently below 43°C (Hildebrandt *et al.* 2002). Furthermore, fever-like whole body hyperthermia which ranges between 39°C and 40°C shows also efficacy in mouse tumor models and is currently being investigated in phase I clinical trials (Kraybill *et al.* 2002). These studies indicate that the biological effects of hyperthermia is most likely not only the result of direct cytotoxicity, but might include other mechanisms (Wang *et al.* 2001; Atanackovic *et al.* 2002).

Several groups are currently investigating the immunologically relevant changes in tumor cell physiology and the susceptibility to immune effector cells after heat shock treatment. Davies *et al.* published the first studies on the effect of hyperthermia on antigen expression, showing a heat-related, dose-dependent decrease of melanoma surface antigens by shedding and masking of surface antigens (Davies Cde *et al.* 1985; Davies *et al.* 1990). Following the heat shock response, further studies showed a decrease in the presentation of exogenous antigens by MHC class II (Pepin *et al.* 1996) and an abrogation of co-stimulatory functions in antigen presenting cells (Kuperberg *et al.* 1991). Some groups showed that heat shock conditions reduce the susceptibility of target cells to monocyte cytotoxicity (Jaattela *et al.* 1993), to CTL attack (Sugawara *et al.* 1990; Geginat *et al.* 1993; Jackson *et al.* 2000) and to the effect of cytokines such as tumor necrosis factor (TNF-a) (Gromkowski *et al.* 1989; Jaattela 1990;

Kusher *et al.* 1990; Jaattela 1995; Van Molle *et al.* 2002). Another aspect of hyperthermia is that it induces the heat-inducible HSP70, which has a role in protection against stress-induced apoptosis (Mosser *et al.* 2000). In general, these results suggest that heat shock induces a state of immunological resistance in tumor cells.

On the other hand, evidence has accumulated that hyperthermia and the associated heat shock response increase the immunogenicity of cancer cells (Mise *et al.* 1990; Wells *et al.* 2000). These changes include induction of MHC class II-restricted presentation of endogenous antigens (Michalek *et al.* 1992) and the enhancement of MHC class I antigen presentation via heat shock protein expression (Melcher *et al.* 1998; Clark *et al.* 2001; Ito *et al.* 2001). Expression of inducible HSP70 was also found to be associated with increased tumor immunogenicity {Menoret, 1995 #45) and with enhanced susceptibility of tumor cells to cytotoxic lymphocytes (Multhoff *et al.* 1997; Dressel *et al.* 1999; Dressel *et al.* 2000).

With regard to these heat-induced immunological effects the results reported sofar are inconsistent, claiming heat-induced immunoresistance as well as immune stimulation.

Infact it is known that tumor cells surviving a stress like heat-treatment acquire survival advantage and resistance against subsequent stress (thermotolerance). This transient resistance has been associated with the induction of heat shock proteins (HSP), that are known to exert a cytoprotective role. Intracellular HSPs are known to confer resistance to apoptosis and protection from LPS toxicity (sepsis) and ischemia/reperfusion injury. These characteristics may lead to malignant transformation, resistance to treatment and may influence the invasive phenotype. The challenge now is to elucidate when and in what way induced intracellular HSPs interferes with the immunorecognition of heated tumors.

1.3 HEAT SHOCK PROTEINS AND THEIR ROLE IN INNATE AND ADAPTIVE IMMUNE RESPONSE

Heat shock proteins (HSPs) were discovered by Ritossa in 1962, who described for the first time the heat shock response in the Drosophila salivary gland (Ritossa 1962). In the following decades many investigators showed that HSPs are phylogenetically one of the most conserved families of proteins found in all organisms, from procaryotes, yeasts and plants, to eucaryotes. In their classical function HSPs are molecular chaperones that assist in protein folding and protein translocation. They prevent misfolding and aggregation of proteins and facilitate refolding of denaturated proteins (Georgopoulos *et al.* 1993; Bukau *et al.* 1998). The induced synthesis of HSPs is, at the molecular level, the cellular response to a wide range of threatening stimuli such as heat shock, heavy metals, oxidative stress, fever, inflammation and serum deprivation. HSPs are not only induced by stress, but they are also associated with

physiological processes such as cell cycle, cell proliferation and differentiation (Milarski *et al.* 1986). HSP expression has been associated with several pathological states, in particular with cancer (Fuller *et al.* 1994); HSPs are associated with oncogenesis, e.g. through interaction with p53 and protection of tumor cells from apoptosis (Jolly *et al.* 2000). In recent years HSPs have been proposed as a tool for cancer therapy. They may function as a tumor-associated activation structure (e.g. HSP70), if detected on the surface of certain tumor cells, thereby activating the response of NK cells (Multhoff *et al.* 1997) (Multhoff *et al.* 1995; Multhoff *et al.* 1996; Multhoff *et al.* 1999); or as antigen-presenting molecules, eliciting a specific immune response by T cells through associated peptides in HSP-peptide complexes (HSP-PC) (Srivastava *et al.* 1994; Wells *et al.* 2000).

Most recently HSPs have been discovered as 'danger signals' to the innate immune system, inducing selective chemokines (CC and CXC chemokines) and their receptors, cytokines (IL-1, IL-8, interferons, TGF-β, TNFα), acute phase proteins, NK cell receptors, and molecules upstream and downstream of the Toll signaling pathways (Magor *et al.* 2001). Connecting the innate with the adaptive immune system has been the rationale for HSP-based anti-tumor vaccination (Matzinger 1994; Melcher *et al.* 1998; Srivastava *et al.* 1998; Singh-Jasuja *et al.* 2001). Table 1 gives an overview of immunologically relevant HSPs.

Table 1: Heat shock proteins with relevant functions in the immune system (Milani et al. 2002).

HSP family	**Localisation**	**Family member**	**Immunological function**	**Reference**
HSP60	Mitochondria	HSP60 (60 kDa chaperonin)	ATP-dependent chaperone function; 'Danger signal' to the innate immune system	(Bukau *et al.* 1998) (Chen *et al.* 1999; Breloer *et al.* 2001)
HSP70	Cytoplasm Cytoplasm	HSC70 (constitutive) HSP70 (inducible)	ATP-dependent chaperone function; 'Danger signal' to the innate immune system; Cross-presentation of tumour antigens as fusion protein 'Danger signal' to the innate immune system; cytokine-like function; Cross-presentation of tumour antigens	(Bukau *et al.* 1998) (Panjwani *et al.* 1999) (Somersan *et al.* 2001) (Wang *et al.* 2001) (Udono *et al.* 2001; Kammerer *et al.* 2002) (Asea *et al.* 2000; Kuppner *et al.* 2001) (Udono *et al.* 1993; Udono *et al.* 1994; Suto *et al.* 1995; Binder *et al.* 2001; Castelli *et al.* 2001)
HSP90	Cytoplasm ER Lumen	HSP90 α/β (HSP86/HSP84) Gp96 (Gp94, endoplasmic)	Steroid hormone receptors Refolds and maintains protein in vitro ATP-independent chaperone function; 'Danger signal' to the innate immune system Cross-presentation of tumour antigens	(Georgopoulos *et al.* 1993) (Singh-Jasuja *et al.* 2000; Zheng *et al.* 2001) (Udono *et al.* 1994; Suto *et al.* 1995)
HSP110	Cytoplasm	HSP110	Cross-presentation of tumour antigens	(Wang *et al.* 2001)
Grp170	ER	Grp170	Cross-presentation of tumour antigens	(Wang *et al.* 2001)

For most HSP families there is at least one family member that exerts an important function in the immune response. The functions are diverse, covering 'danger signal' (Chen *et al.* 1999; Breloer *et al.* 2001) and induction of cytokine secretion (Asea *et al.* 2000; Singh-Jasuja *et al.* 2000; Kuppner *et al.* 2001; Zheng *et al.* 2001), chaperone function (Panjwani *et al.* 1999), cross-presentation and T cell stimulation (Udono *et al.* 1993; Udono *et al.* 1994; Suto *et al.* 1995; Binder *et al.* 2001; Castelli *et al.* 2001).

1.3.1 Biochemistry of the HSP70 family

Within the HSP70 family two forms can be distinguished: the inducible form HSP70 (also named HSP72, according to their molecular weight MW= 72 kDa) and the constitutive isoform HSC70 (also HSP73, MW= 73 kDa). The members of the HSP70 family are molecular chaperones which transiently bind and stabilize unfolded and partially folded proteins. They control correct folding, oligomerization, translocation through membranes or disposal of proteins by degradation (Georgopoulos *et al.* 1993; Bukau *et al.* 1998). HSP70 recognize a wide spectrum of heptapeptide segments with a preference for hydrophobic residues (leucine oder isoleucine) with its C-terminal domain (figure 4).

HSP70 is organized in domains (figure 4): peptide recognition is performed by amino acids of the C-terminal domain. The N-terminal amino acids encode the ATPase domain, which is important for peptide binding and release. In its ATP-bound state, HSP70 binds and releases peptide rapidly, whereas after hydrolysis, in the ADP-state, bound peptide is held tightly (Flynn *et al.* 1989). If HSPs have to be isolated as complex with associated peptides, it is essential to use a method which avoids ATP hydrolysis (see methods).

Figure 4: Domain structure of HSP70. HSP70 consists of a phylogenetically conserved NH_2-terminal nucleotide-binding domain of 44 kD and a less conserved COOH-terminal domain, that binds peptide or polypeptide substrate. (*Institute of biochemistry Zurich: seminars (www.biochem.unizh.ch/seminars/)*

1.3.2 Receptors and signal mediators for heat shock proteins

In the last few years effords were directed toward the identification of receptors and mechanisms involved in the HSP-mediated activation of the innate and adaptive immune system. In 2000, CD91 was identified as a receptor for the HSP gp96. Later Basu *et al.*

proposed that CD91 might be a universal receptor for HSP70, HSP90, gp96 and calreticulin (Basu *et al.* 2001). Meanwhile other receptors have been identified, like CD40 and LOX-1, which may be involved in the HSP-mediated cross-presentation of antigens (Becker *et al.* 2002; Delneste *et al.* 2002).

Recently it has been shown that Toll-like receptors (TLR) are involved in the activation and maturation of dendritic cells by HSPs (Vabulas *et al.* 2002). Toll-like receptor proteins are important mediators/signal-transducers of the innate immune response to infection (Pugin *et al.* 1994; Medzhitov *et al.* 1997; Kirschning *et al.* 1998; Poltorak *et al.* 1998; Akira *et al.* 2000) and are involved in recognizing pathogen associated molecular patterns (PAMPs), like LPS. In an attempt to define the mechanistic events for the induction of pro-inflammatory responses by HSP it was noted that the response elicited by the different HSPs was similar to that of lipopolysaccharide (LPS) (Kol *et al.* 1999). The binding of LPS to TLR4 results in the recruitment of the adaptor molecule MyD88, IL-1 receptor associated kinases (IRAK) and the adaptor molecule TRAF6 ultimately leading to the production of pro-inflammatory cytokines (Medzhitov *et al.* 1997; Akira *et al.* 2001).

HSP60 (Vabulas *et al.* 2001), gp96 (Vabulas *et al.* 2002) and HSP70 (Asea *et al.* 2002; Vabulas *et al.* 2002) were found to bind to TLR4 and induce a cascade of events similar to that of LPS, leading to the secretion of inflammatory cytokines and maturations signals (figure 5).

Thus HSP appear to use these same highly conserved signal transduction pathways and receptors, which organisms have used for millions of years to recognize and attack pathogens.

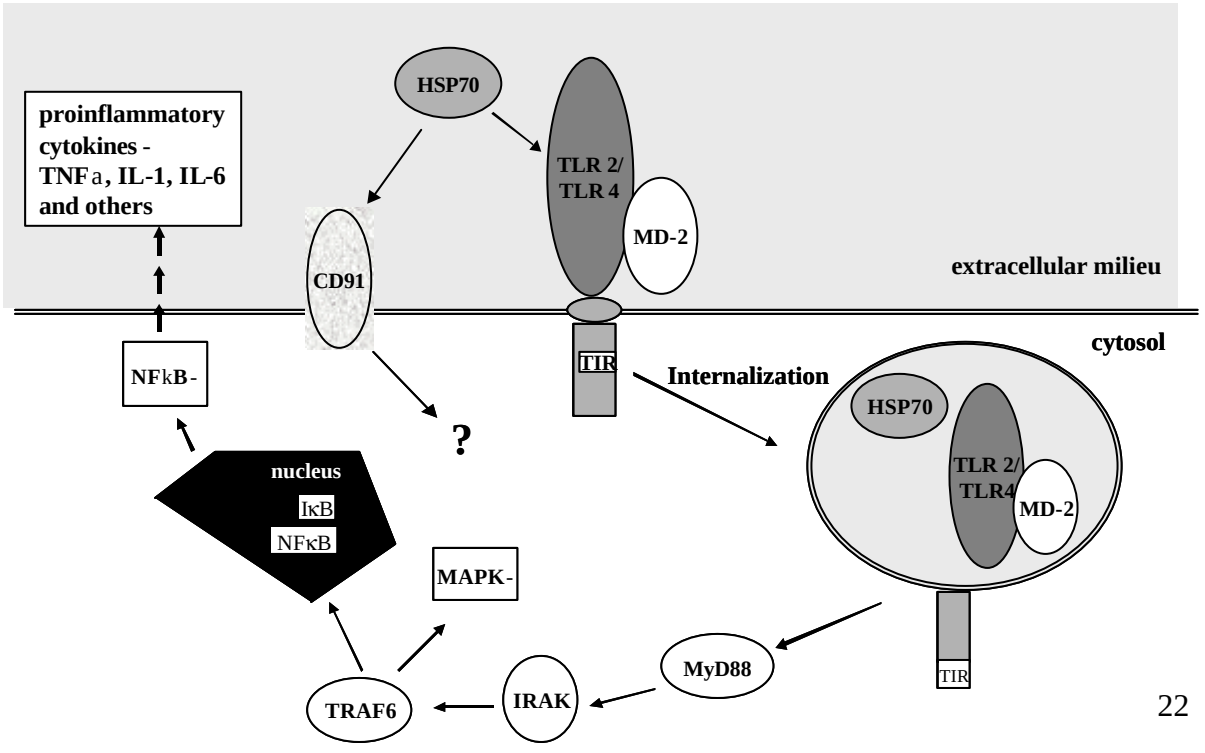


Figure 5: HSP70 delivers signals through TLR2 and TLR4 in 293T cells. Ligand activation of TLR results in the recruitment of the adaptor molecule MyD88, IRAK and the adaptor molecule TRAF6, ultimately leading to the production of pro-inflammatory cytokines. How CD91 integrates into this pathway is currently unknown (Milani *et al.* 2002).

1.3.3 Heat shock protein-peptide complexes (HSP-PC): a tool for immunization

In murine systems vaccination with heat shock proteins (HSPs) such as glucose regulated protein (gp) 96, HSP70, and HSP90 isolated from cancer tissue but not from normal tissue, induces specific immunity and cytotoxic T lymphocyte (CTL) activation (Srivastava *et al.* 1994). The specificity of the induced CTLs relies on the peptides chaperoned by these HSPs (Srivastava *et al.* 1994) (Suto *et al.* 1995). This property allows CTL activation without the need to characterize the corresponding antigens, and provides the basis for a new type of vaccine against cancer (Tamura *et al.* 1997; Janetzki *et al.* 2000; Srivastava 2000; Srivastava 2002).

Immunization with HSP-peptide complexes (HSP-PC) is exquisitely dependent on the presence of functional antigen presenting cells (APCs) in the immunized host, since depletion of such cells renders the host incapable of mounting immune responses after injection of HSP-PC preparations (Udono *et al.* 1994). Dendritic cells (DCs) are very effective activators of CTLs, a process that requires the presentation of antigen bound to MHC molecules, together with expression of adhesion and costimulatory molecules (Banchereau *et al.* 2000). In particular the ability to present exogenous antigens through "cross-presentation“ is a key feature of DCs. It became evident that gp96- and HSP70-chaperoned peptides can be presented to CTLs by DCs in the context of MHC class I molecules and uptake of gp96 or HSP70 requires receptor-mediated endocytosis (Suto *et al.* 1995) (Arnold-Schild *et al.* 1999; Singh-Jasuja *et al.* 2000). The mechanisms involved in the stimulation of T cell responses via HSP70 and gp96 were studied in murine systems using induced tumors and model antigens (Srivastava *et al.* 1986) (Udono *et al.* 1993; Udono *et al.* 1994) ovalbumin (Breloer *et al.* 1998) and viral antigens (Blachere *et al.* 1997; Ciupitu *et al.* 1998). More recently, immunization with tumor-derived HSPs has also been demonstrated for spontaneous tumors (Vanaja *et al.* 2000). These analyses provided the principle knowledge of HSP-mediated cross-presentation and the involvement of antigen presenting cells and have been the basis for the first clinical trials involving tumor-derived gp96 (Tamura *et al.* 1997; Janetzki *et al.* 2000).

2 GOAL OF THE STUDY

Since malignant cells are reliably more sensitive to heat than normal cells, raising the temperature of the tumor is one way to selectively destroy cancer cells. Upon necrosis tumor cells release HSP in the extracellular milieu where they acquire immunostimulatory properties and are able to mount an efficient immune response directed against the tumor from which they derive. However not every cell dies upon heat treatment and those which survive acquire a survival advantage. In fact cytoprotection has been correlated with expression of intracellular heat shock proteins, whose induction is the main event of the heat shock response.

To understand the function of the tumor heat shock proteins expressed under physiological and stress conditions and to dissect their role in tumor immune recognition as a function of intra- versus extracellular location was the goal of this study.

In particular it was the goal of this study to achieve a comprehensive and integrated view of a) the in vitro immunological properties of heat shock protein 70 family members, either constitutively produced by tumor cells or induced upon heat shock; b) the interrelation of heat shock induced changes in tumor physiology and immune competence, as defined by tumor cell sensitivity to immune effector cells.

2.1 THE MODEL SYSTEM

I selected the human melanoma system because it is well characterized with regards to tumor-associated antigens, like tyrosinase and Melan-A/MART-1, their epitopes and restriction elements for MHC class I and II presentation, and tumor lines with defined antigen expression as well as T cells that recognize specific epitopes are readily available.

2.1.1 The human melanoma model

Cutaneous melanoma expresses several immunogenic melanoma-associated differentiation antigens (Renkvist *et al.* 2001), whose peptides are recognized by tumor-reactive T cells of melanoma patients (Kawakami *et al.* 2000) which appear to be involved in tumor regression and autoimmune vitiligo (Ogg *et al.* 1998). Their level of expression has been correlated with CTL recognition (Rivoltini *et al.* 1995) (Cormier *et al.* 1999; Riker *et al.* 2000) and favourable disease outcome (Takeuchi *et al.* 2003). Table 2 lists some melanoma differentiation antigens, their epitopes and the HLA class I restriction elements (Renkvist *et al.* 2001).

Table 2: Melanoma-associated differentiation antigens

Antigene	HLA-restriction element	Epitope
*Melan-A/MART-1**	A2	AAGIGILTV
	A2	EAAGIGILTV
	A2	ILTVILGVL
	B45	AEEAAGIGIL
	B45	AEEAAGIGILT
Gp100	A2	KTWGQYWQV
	A2	TDQVPFSV
Tyrosinase	A1	KCDICTDEY
	A1	SSDYVIPIGTY
	A2	YMDGTMSQV
	A2	MLLAVLYCL
	A24	AFLPWHRLF
	B44	SEIWRDIDF
TRP-1 (or gp75)	A31	MSLQRQFLR
TRP-2	A2	SVYDFFVWL
	A2	TLDSQVMSL
	A31	LLGPGRPYR
	A33	LLGPGRPYR
	Cw8	ANDPIFVVL

* Two different groups simultaneously discovered this gene and gave it two different names, MART-1 and Melan-A respectively

2.1.2 Melanogenesis and antigenic profile associated with progression of malignant melanoma

Many of the melanocyte lineage-related proteins are involved in the biosynthesis of melanin (figure 6), in the maintenance of melanosome ultrastructure and in the melanocytes proliferation/cell death (i.e. TRP-1) (del Marmol *et al.* 1996).

Melanoma often becomes amelanotic as the malignancy progresses, and thereby loses expression of some of these genes. This changes the antigenic profile and increases the possibility that such cells will selectively escape immune destruction. It has been shown that there is a downward trend in the presence of these antigens as the tumor becomes amelanotic. The downregulation of tyrosinase and other melanocyte-specific gene expression is likely to be mediated by oncogenes or is due to metabolic changes, like an abnormal acidification, that induces an aberrant retention of tyrosinase in the early secretory pathways (ER) and an accelerated degradation (Halaban 2002), (Halaban *et al.* 1997).

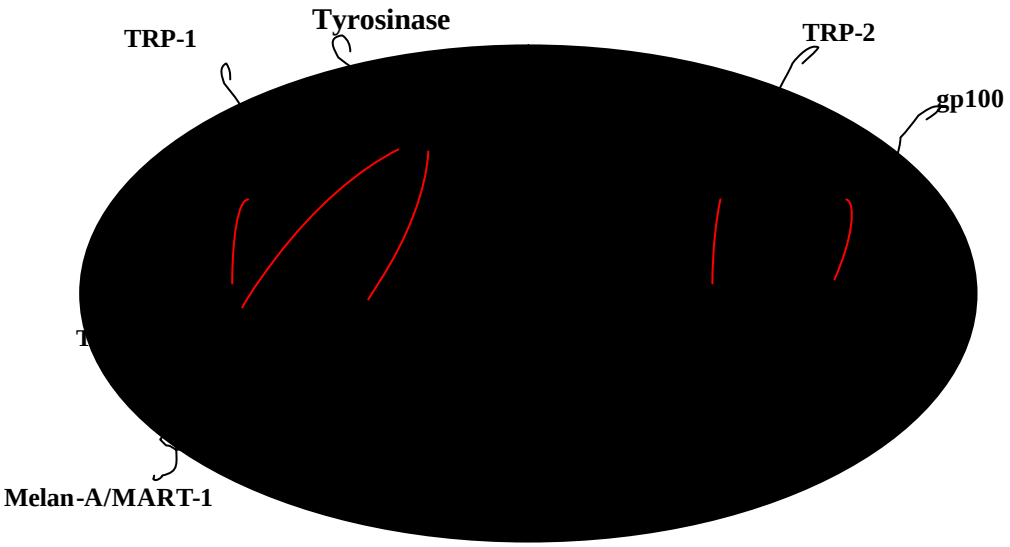


Figure 6: Melanogenesis. Melanogenesis involves the conversion of tyrosine to dopachrome, catalizised by tyrosine-related protein 1 (TRP-1) in a two-step reaction involving the rate limiting enzyme tyrosinase. Subsequent conversions are mediated by TRP-2 (defining the brown or black appearance of eumelanin) and gp100 (controlling the deposition of dihydroxi indole (DHI) into eumelanin). The role of Melan-A/MART-1 (melanoma antigen recognized by T cell 1) in melanogenesis has not been defined yet (del Marmol *et al.* 1996).

For this study I have focused on two antigens, tyrosinase and Melan-A/MART-1, expressed by the human melanoma cell lines 624.38-MEL and SK-Mel 23. The melanoma cell line A375 does not express those antigens and served as a control cell line for most of the analyses.

Differentiation antigen	***Melanoma cell lines***		
	624.38 Mel	***A375***	***SK-mel 23***
tyrosinase	+++	-	+++
Melan-A/MART-1	+++	-	+++
Melanotic	no	no	yes

2.2 DESIGN OF THE STUDY

I investigated the effects of heat shock treatment and in particular the role of heat shock proteins in the cellular anti-tumor immune response (figure 7). The effects of heat exposure and of the heat shock proteins was studied at different levels:

1. Endogenous antigen presentation and processing of tumor antigens
2. Susceptibility of tumor cells to immune effector mechanisms
3. Cross-presentation of tumor antigens by tumor derived HSP70-PC

a) In the first part of the study I focused specifically on the time-temperature dependent effects of heat exposure. Based upon the clinical experience of hyperthermia treatment of melanoma, I selected two different thermal doses (41,8°C/120 minutes and 45°C/22 minutes) that mimic the heterogeneity of the achieved temperature distribution within the tumor and determined the time-temperature dependent changes in: a) antigen expression (tyrosinase and Melan-A/MART-1) at the protein and mRNA level; b) expression of the inducible HSP70 and the constitutive HSC70; c) processing and presentation of tyrosinase and MART-1 via MHC class I; d) susceptibility of melanoma cell lines to cytotoxic T lymphocytes like CD8+ T cells, LAK and NK cells.

b) In the second part of the study, I characterized the role of members of the HSP70 family in the cross-presentation of the human tumor-associated antigen tyrosinase. Most studies investigating mechanistic events related to HSP-mediated cross-presentation involved highly immunogenic antigens either induced by mutagenesis or overexpressed by transfection, a situation that does not reflect most human cancers. Different to those studies, I investigated the ability of HSP70 to cross-present a naturally expressed human tumor antigen, tyrosinase, that is of low immunigenicity, a situation that more closely resembles the patient situation.

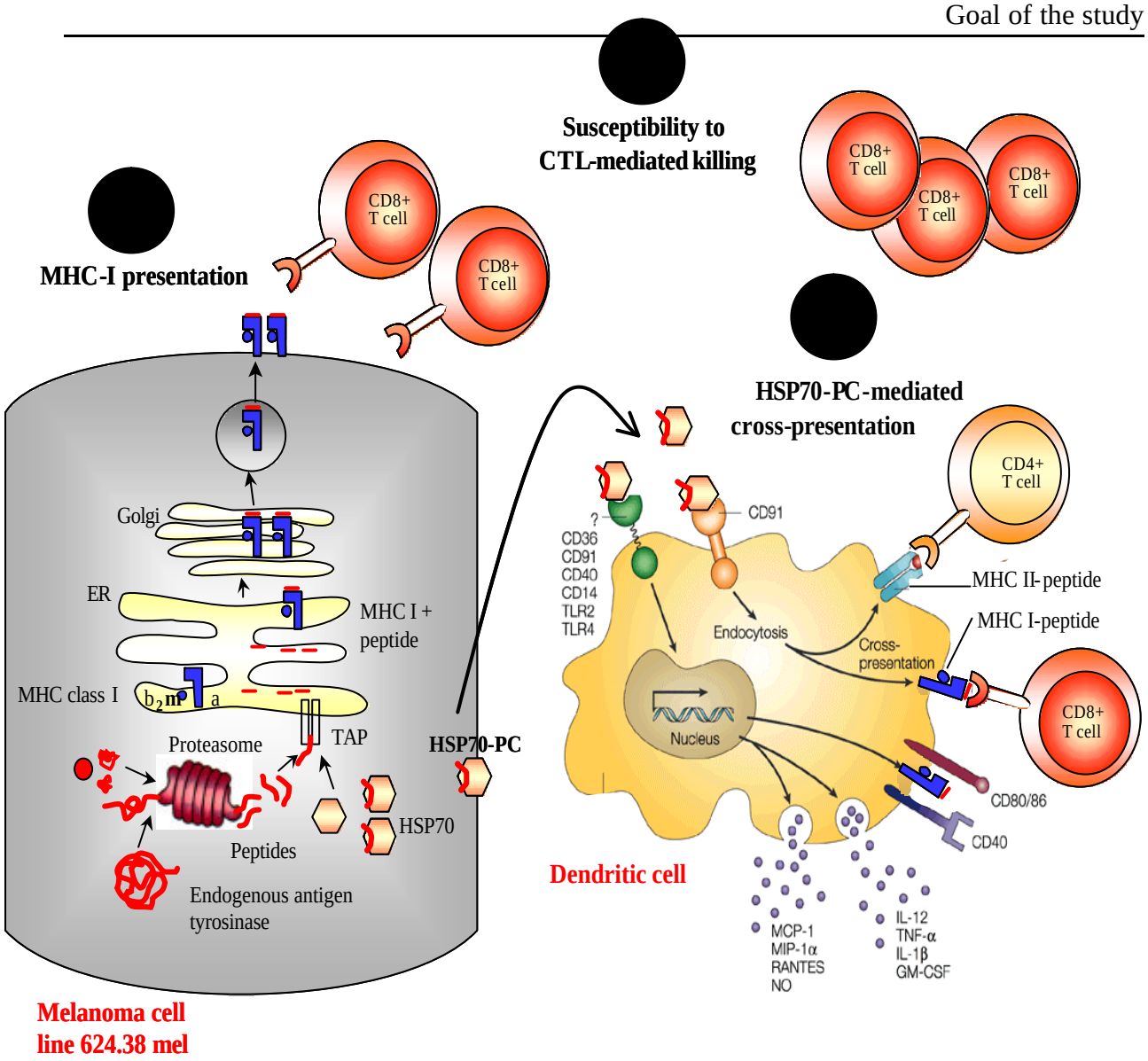


Figure 7: Design of the study. The human melanoma cell line 624.38-MEL naturally expresses, processes and presents tyrosinase and Melan-A/MART-1 in the context of MHC class I molecules and expresses both HSP70 and HSC70. Therefore it was selected as the model system. The goal of the study was to investigate at 37°C and during the heat shock response 1) antigen expression, processing and presentation; 2) the susceptibility of tumor cells to cytotoxic lymphocytes; 3) role of tumor-derived HSP70-PC in the cross-presentation of tyrosinase and activation of dendritic cells.

3 MATERIALS

3.1 CHEMICALS

* Corrosive*; ** Toxic; *** Inflammable*

Acetic acid (Merck, Darmstadt, Germany) *

Acrylamide/Bisacrylamide 40% (Roth, Karlsruhe, Germany) **

Adenosine-diphosphate (ADP) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

ADP-Agarose (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Ammonium peroxidisulfate (APS) (Bio-rad, Munich, Germany)

Antipain (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Aprotinin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Bovine serumalbumin (BSA) (Fluka, Buch, CH)

CHAPS (3-((3-Cholamidopropyl)-dimethylammonio)-1-propanesulphonate) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Chymostatin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Cloridric acid (Neolab, Migge Laborbedarf, Heidelberg, Germany)

Crystal violet (Merck, Darmstadt, Germany)

Ethanol (Merck, Darmstadt, Germany) ***

FluoroLink^{TM}Cy5 (Amersham Pharmacia Biotech, UK)

Formaldehyde (FA) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Glycerine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Glycine (Merck, Darmstadt, Germany)

G-sephadex (Amersham Pharmacia Biotech, UK)

HEPES (N-(2-Hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Hydrogen Peroxide (Pharmingen, BD,San Diego California)

Leupeptin A (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Liquid nitrogen (Linde, Munich, Germany)

Magnesiumchlorid (Merck, Darmstadt, Germany)

2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Methanol (Merck, Darmstadt, Germany) ***

Natrium Chromate Cr^{51} (PerkinElmer, Boston, MA)

Paraformaldehyde (PFA) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Pepstatin A (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Phosphoric acid (Merck, Darmstadt, Germany) ***

Ponceau S (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Q-sepharose (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Silver Nitrate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Skim milk powder (Merck, Darmstadt, Germany)

Sodium Acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Sodium Carbonate (Merk, Darmstadt, Germany)

Sodium Cloride (Merk, Darmstadt, Germany)

Sodiuhydrogencarbonate (Merk, Darmstadt, Germany)

Sodiumhydrogenphosphate (Merck, Darmstadt, Germany)

Sodium Fluoride (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Sodium Pyrophosphate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Sodium Thiosulfate Pentahydrate (Merk, Darmstadt, Germany)

Sodium Vanadate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Sodiumdodecylsulfat (SDS) (Bio-rad, Munich, Germany)

Tetramethylbenzidine (TMB) (Pharmingen, BD,San Diego California)

Tetramethylethylendiamine (TEMED) (Serva, Heidelberg, Germany)

Trypsin-EDTA (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

Tris(hydroxymethyl)aminomethane (Tris) (Merck, Darmstadt, Germany)

Trypan blue (Serva, Heidelberg, Germany) **

Tween 20 (Merk, Darmstadt, Germany)

3.2 KITS

Annexin V/Propidium Iodide (PI) kit (Alexis, Lausen, Switzerland)

Bio-rad Protein estimation kit Lowry and Bradford (Bio-rad, Munich, Germany)

Detection system ECL® (enhanced chemiluminescence) (Amersham Pharmacia, Freiburg, Germany)

FIX und Perm (Dianova, Hamburg, Germany)

Mycoplasma detection kit by fluorescent DNA staining (Biochrom, Berlin, Germany)

OPTEIA ™ Set Human IFN-γ (OptEIA, Becton Dickinson; Heidelberg, Germany)

3.3 LABORATORY EQUIPMENT, CONSUMABLES

Autoradiography film (Valmex, Augsburg, Germany)

Balance BL310 d=0,01g (Sartorius, Göttingen, Germany)

Balance Mettler Delta Range PM460 d=0,001 g (Mettler Toledo, Columbus, OH, USA)

Blottingchamber (Bio-rad, Munich, Germany)

Cell culture CO_2 incubator (Haereus, Rodenbach, Germany)

Cell culture laminar flow (Bio Flow Technik, Meckenhein, Germany)

Centrifuges ROTIXA/P (Hettich, Tuttlingen, Germany)

Developing machine M35X-OMAT Processor (Kodak AG, Stuttgart, Germany)

ELISA reader (SLT, Crailsheim, Germany)

Eppendorf ultracentrifuge 2K15 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

FACSscan (Beckton Dickison, Mountain View, CA)

Fast Protein Liquid Chromatography (FPLC) Biologic LP (Bio-rad, Munich, Germany)

Refrigerator (4°C, -20°C) (Siemens AG, Germany)

Refrigerator (-80°C) UF80-450S (Colora Messtechnik GmBH, Lorch, Germany)

Gammacell 40 (Atmonic Energy of Canada, Ottowa, Canada)

Gel electrophoresis systems (Bio-rad, Munich, Germany)

Heating block BT 130-2 (HLC, Haep Labor Consult, Bovenenden, Germany)

Liquid nitrogen tank (Cryoson, Schöllkrippen, Germany)

Magnetic stirrer (Cenco, Breda, the Netherlands)

Microscope (Carl Zeiss Jena, Germany)

Microscope, model LSM510 confocal laser-scanning (Carl Zeiss Jena, Germany)

Nitrocellulose membranes (Sartorius, Goettingen, Germany)

pH-meter 766 (VWR International, Ismaning, Germany)

Rotor Ti 75 (Beckman, Palo Alto, CA)

Shaker (Edmund Bühler, Tübingen, Germany)

Spectophotometer Smartspec ™ 3000 (Bio-rad, Munich, Germany)

Topcount NXT (Packard, Bioscence, Meriden, CT)

Ultracentrifuge L7-65 (Beckman, Palo Alto, CA)

Ultra-turrax (IKA Werke, Staufen, Germany)

VECTASHIELD (Vector Laboratories Inc., Burlingame, CA)

Vortex (Cenco, Breda, the Netherlands)

Waterbath (HAAKE, Karlsruhe, Germany)

3.4 CELLS

3.4.1 Tumor cell lines

Suspension cells

Cell type	**Description**	**Origin**
K562	human myelogenous leukemia cell line	ATCC, Rockville, MD
Daudi	Burkitt lymphoma cell line	ATCC, Rockville, MD

Adherent cells

Cell type	**Description**	**Origin**
624.38 Mel (Rivoltini *et al.* 1995)	human melanoma cell line; tyrosinase positive; HLA-A2 positive	was a kind gift from Dr. M.C. Panelli (National Institute of Health, Bethesda, Maryland, USA)
SK-Mel 23	human melanoma cell line; tyrosinase positive; HLA-A2 positive	was a kind gift from Dr. M.C. Panelli (National Institute of Health, Bethesda, Maryland, USA)
A375	human melanoma cell line; tyrosinase negative; HLA-A2 positive	ATCC, Rockville, MD
BLM	human melanoma cell line; tyrosinase negative; HLA-A2 positive	was a kind gift from Dr. Y. Vissers (Dept. of Clinical Oncology, Leiden University Hospital, NL)
A9	Mouse fibrosarcoma cell line which expresses Fas after transfection	was a kind gift of Dr. Engelmann, LMU- Munich
RCC-26 (Schendel *et al.* 1993)	renal cell carcinoma cell line; HLA-A2 positive	was established by D.J. Schendel from a primary renal cell carcinoma

3.4.2 Cytotoxic lymphocyte clones

Cell type	Description	Origin
TyrF8 (Visseren *et al.* 1995)	HLA-A*0201-restricted tyrosinase peptide $_{(aa\ 368\text{-}376;}$ YMNGTMSQV) specific cytotoxic T cell clone	was kindly provided by Dr. P. Schrier (Dept. of Clinical Oncology, Leiden University Hospital, NL)
A42 (Kawakami *et al.* 1994; Kawakami *et al.* 1994)	The HLA-A2-restricted Melan-a/MART-1 peptide (AAGIGILTV)- specific cytotoxic T-cell clone A42	was kindly provided from Dr. M.C. Panelli (National Institute of Health, Bethesda, Maryland, USA)
JB4	HLA-A2-resctricted cytotoxic T-cell clone	generated in GSF-IMI (E.N.)
LAK	Lymphokine-activated killer cells	were generated from PBMC of healthy donors (GSF-IMI)
NKL (Robertson *et al.* 1996)	Human natural killer (NK) leukemia cell line	was kindly provided by Dr. C.S. Falk (GSF-Institute of Molecular Immunology, Munich)
B-LCL LAZ388	EBV-transformed allogeneic B-LCL	Dr. A. Mackensen, Department Hematology, University Regensburg

3.4.3 Primary cells (PBMC)

Samples were obtained from leukapheresis or healthy donors, after having informed and obtained consent of the donor. Every donor was tested for HLA-A2 surface expression and entered into a database.

3.5 MATERIALS USED FOR CELL CULTURE

3.5.1 Cell culture media

3.5.1.1 Medium for tumor cell lines

RPMI 1640 (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

10% FCS (Fetal calf serum) (Biochrom AG seromed, Berlin, Germany)

1% NEM (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

1% Penicillin/streptomycin (GIBCO BRL, Kalsruhe, Germany)

1% L-glutamine (GIBCO BRL, Kalsruhe, Germany)

3.5.1.2 Medium for CD8+ T cells

RPMI 1640 (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

7,5% FCS (fetal calf serum) (Biochrom AG seromed, Berlin, Germany)

7,5% human serum (mixed from 4 healthy donors)

2mM L-glutamine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

1mM Sodium Pyruvate (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)
50 U/ml rIL-2 (Proleukin, Cetus Corp. Emeryville, CA)

Addition for LAK cells
1% Phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI)
10 ng/ml OKT3 (ATCC, Rockville, MD)

3.5.1.3 Medium for dendritic cells

VLE RPMI 1640 medium (Biochrom AG seromed, Berlin, Germany)
Human serum (mixed from 4 healthy donors)
Penicillin/streptomycin (GIBCO BRL, Kalsruhe, Germany)
L-glutamine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)
GM-CSF (Hölzel Diagnostika, Köln, Germany)
IL-4 (Biomol, Hamburg, Germany)
TNF-a (Biomol, Hamburg, Germany)

3.5.1.4 Other reagents for cell culture

Ficoll/hypaque (PAA, Linz, Austria)
Phosphate Buffer Saline (PBS) (GIBCO BRL, Kalsruhe, Germany)
Trypan Blue (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)
Trypsin-EDTA (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

3.5.2 Culture vessels and plastic ware

3.5.2.1 Culture flasks and plates and other plastic material

96-well flat/round plate (Nunc, Wiesbaden, Germany)
Centrifuge vials (15-50 ml) (Sarstedt, Nümbrecht, Germany)
Culture flasks (50-250-600 ml) (Greiner Labortechnik, Frickenhausen, Germany)
Eppendorf tubes (0,5-1 ml) (Eppendorf, Hamburg, Germany)
Freezing tubes (Nunc, Wiesbaden, Germany)
Micropipettes, Pipettes (Gilson, Langenfeld, Germany)
Parafilm M (American National Can, Greenwich, USA)

3.5.3 Solutions and antibodies for flow cytometry

3.5.3.1 FACS solutions

PBS, containing 5% FCS

1% PFA in PBS only when fixing cells.

3.5.3.2 Antibodies

Antigen Specificity	**Primary antibody**	**secondary**	**origin**
HSP70	6B3: anti-human HSP70, rat monoclonal; 1:100	anti-rat-FITC	GSF-IMI (Noessner *et al*. 2002)6)
HSC70	SPA815: anti-human HSC70, rat monoclonal; 1:1000	anti-rat-FITC	StressGen Biotechnologies, Victoria, Canada
Pan MHC-Class I	W6/32: anti-human MHC I, hybridoma cell supernatant, mouse monoclonal; 1:2	PE-conjugated goat anti-mouse IgG	ATCC HB-95 (Barnstable *et al*. 1978)7)
HLA-A2	HB-54: anti-human HLA-A2, hybridoma cell supernatant, mouse monoclonal	PE-conjugated goat anti-mouse IgG	ATCC HB-54 (McMichael *et al*. 1980)8)
Isotype IgG1	MOPC 21	PE-conjugated goat anti-mouse IgG	Immunotech (Hamburg, Germany).
Isotype IgG2a	MOPC 141	PE-conjugated goat anti-mouse IgG	Immunotech (Hamburg, Germany).
CD14	Anti-human CD14, mouse monoclonal	PE-conjugated goat anti-mouse IgG	Immunotech, Hamburg, Germany
CD40	Anti-human CD40, mouse monoclonal	PE-conjugated goat anti-mouse IgG	Pharmingen, BD, San Diego California
CD1a	Anti-human CD1a, mouse monoclonal	PE-conjugated goat anti-mouse IgG	Pharmingen, BD, San Diego California
CD83	Anti-human CD83, mouse monoclonal	PE-conjugated goat anti-mouse IgG	Immunotech, Hamburg, Germany
CD86	Anti-human CD86, mouse monoclonal	PE-conjugated goat anti-mouse IgG	Pharmingen, BD, San Diego California
Apo-1	anti-FAS mouse monoclonal	PE-conjugated goat anti-mouse IgG	was a gift of Dr. H. Engelmann (LMU-Munich)
ML-957	Antibody that cross-link the Fas receptor	-	was a gift of Dr. H. Engelmann (LMU-Munich)
ML-958	Antibody that does not cross-link the Fas receptor	-	was a gift of Dr. H. Engelmann (LMU-Munich)

PE= Phycoerythrin; FITC= Fluorescein-Isothyocyanat

Secondary antibodies PE- or FITC conjugated goat anti-mouse IgG, were purchased by DAKO, Glostrop, Denmark.

3.5.4 Fluorescence labeling kits

- FluoroLink^{TM}Cy5= cyanine dye; produces an intense signal in the far-red region of the spectrum, emission ?= 670 nm
- FITC: Fluorescein-Isothyocyanate; emission ?= 525 nm
- Conjugation buffer: carbonate-bicarbonate buffer (0,1 M; pH 9,5)
- Gel filtration column (Sephadex G-25) or 12-14 kDa dialysis membrane

3.5.5 Buffer and antibodies for ELISA

3.5.5.1 Buffers for ELISA

COATING BUFFER (stored at 4-8°C)
0,1 M carbonate, pH 9,5

ASSAY DILUENT (stored at 4-8°C)
PBS + 10% FCS

WASH BUFFER (stored at 4-8°C)
PBS + 0,05% Tween-20

SUBSTRATE SOLUTION
Tetramethylbenzidine (TMB) and Hydrogen Peroxide

STOP SOLUTION
1mM Phosphoric acid

3.5.5.2 Standard and Antibodies for IFN-γ ELISA

Standard	recombinant human IFN-γ	lyophilized	Serial dilution*
Capture antibody	anti-human IFN-γ	liquid	1:250
Detection antibody	biotinylated anti-human IFN-γ	liquid	1:250
Enzyme reagent	avidin-horseradish peroxidase conjugate	liquid	1:250

* Serial dilution of the standard after reconstitution. For every new kit, dilution is performed to obtain following final concentration: 0/41,6/83,2/166/333/1000 pg/ml

3.6 MATERIALS USED FOR BIOCHEMISTRY

3.6.1 Buffers and antibodies for Western Blot and Silver Stain

3.6.1.1 Buffers for SDS-Polyacrylamidgelelectrophoresis (PAGE)

for 2 gels	**Running gel (10%)**	**Stacking gel (10%)**
Acrylamide	6ml	1,3
Tris	(pH 8,8) 7,6 ml	(pH 6,8) 2,5 ml
10% SDS	200 µl	100 µl
APS (10% Ammonium Persulfate)	400 µl	100 µl
Temed (tetramethyl ethylene diamine)	10 µl	10 µl

SDS-PAGE GEL LOADING DYE (2X)

0,07 g Tris

1 g glycerin

0,5 g SDS

bromphenolblue

pH 6,8 in 10 ml H_2O

ELECTROPHORESIS BUFFER (10X)

144 g glycin

10 g SDS

30 g Tris

pH 8,3 in 1000 ml H_2O

3.6.2 Buffers for cell lysis and Western Blot

WHOLE CELL LYSIS BUFFER CHAPS

2% CHAPS

50 mM HEPES

200 mM NaCl

10 mM Sodium Pyrophosphate

50 mM Sodium Fluoride

0.2 mM Sodium Vanadate

PMSF 10µl/ml

Pepstatin A, Leupeptin A, Aprotinin, Antipain, chymostatin all at 1µl/ml, freshly added just before cell lysis

pH 7,5

PONCEAU SOLUTION (10X)

30 g trichloroacetic acid (TCA)

2,0 g ponceau

in 100 ml H_2O

TRANSFER BUFFER,

3,03 g TRIS

14,4 g Glycine

200 ml Methanol

pH 8,3

in 1000 ml H_2O

WESTERN BLOT STRIPPING SOLUTION

7 ml 2-Mercaptoethanol

20 g SDS

7,56 g Tris

pH 6,7 in 1000 ml H_2O

TRIS-BUFFERED SALINE (TBS) 10X

12,11 g Tris

88 g NaCl

pH 7,4 in 1000 ml H_2O

TBST (TBS PLUS TWEEN20)

500 ml TBS

200 µl Tween20

BLOCKING BUFFER

5% nonfat dry milk in TBST

PRIMARY ANTIBODY DILUTION BUFFER

5% nonfat dry milk in TBST

3.6.2.1 Antibodies for Western Blot

Antigen	**Primary antibody**	**secondary**	**company**
Tyrosinase	C-19: anti-human tyrosinase, goat polyclonal, 1:500	anti-goat HRP 1:3000	Santa Cruz Biotechnology, Santa Cruz, California, USA
HSP70	6B3: anti-human HSP70, rat monoclonal; 1:100	anti-rat HRP 1:2000	GSF-IMI (130)
HSC70	SPA815: anti-human HSC70, rat monoclonal; 1:1000	anti-rat HRP 1:2000	StressGen Biotechnologies, Victoria, Canada
Tubulin	anti-human β-tubulin; rabbit monoclonal, 1:1000	anti-rabbit HRP 1:2000	Santa Cruz Biotechnology, Santa Cruz, California, USA
MART-1/Melan A	A103: anti-human Melan-A/MART-1, mouse monoclonal, 1:100	anti-mouse HRP 1:2000	DAKO, Glostrop, Denmark
gp-100	HMB45: anti-human gp100, mouse monoclonal, 1:50	anti-mouse HRP 1:2000	DAKO, Glostrop, Denmark

3.6.2.2 Silver Stain solutions

FIXING SOLUTION

20 ml methanol

30 ml H_20

25 µl 37% formaldehyde

in 50 ml

THIOSULFATE SOLUTION

0,2g/l $Na_2S_2O_3$ in 50 ml

NITRATE SOLUTION

0,1% silver nitrate in 50 ml

DEVELOPING SOLUTION

15 g Na_2CO_3

2 mg $Na_2S_2O_3$

37% formaldehyde (freshly before use)

in 500 ml

DRYING SOLUTION

5 ml ethanol

2 ml glycerol

in 43 ml H_20

3.6.3 Buffers and column materials for purification of HSP70-PC

3.6.3.1 Buffers for protein purification

Step	Buffer	Composition
Sample extraction	Buffer A: hypotonic lysis	10 mM $NaHCO_3$ 0,5 mM PMSF pH 7,1
ADP-Affinity chromatography	Buffer B	20 mM Tris 20 mM NaCl 3 mM $MgCl_2$ 15 mM 2-Mercaptoethanol 0,5 mM PMSF pH 7,5
Anion-exchange chromatography	Buffer C 1	20 mM Na_2HPO_4 20 mM NaCl pH 7,0
	Buffer C2	20 mM Na_2HPO_4 600 mM NaCl pH 7,0
	Buffer C3	1000 mM Na_2HPO_4 600 mM NaCl pH 7,0

3.6.3.2 Column material for protein purification (Downscale 1:3= D_3)

	HiPrep 26/10 Desalting column	**ADP-Agarose**	**Q- Sepharose**
Column volume	53 ml D_3= 53 ml	5 ml D_3= 2 ml	5 ml D_3= 2 ml
Matrix	Sephadex-G 25 fine	Adenosine 5´-diphosphate - Agarose	Q-sepharose high performance
Column dimension	2,6 x 10 cm	1x10 cm	1 x 10 cm
Number	3 (D_3= 1)	1	1
Flow rate	3-5 ml/min (4 ml/min; Fractionation)	2 ml D_3= 0,5 ml/min by loading; 1 ml/min by eluting	2 ml D_3= 1 ml/min
Company	Amersham # 175087-01	Sigma-Aldrich; Taufkirchen, Germany; #A4398	Sigma-Aldrich; Taufkirchen, Germany # Q-1754

3.6.3.3 Material for protein concentration

Concentrations tubes, Membra-spin Macro 15 ml (Membrapure, Bodenheim, Germany)

Characteristics

Length of the filter	96 mm x 26 mm
Maximum Sample volume	15 ml
Maximum final concentrate volume	50 µl
Effective filtration area	314 mm^2
Tube capacity	50 ml
Operating temperature	4°C
Maximal centrifugal force	6000 g
Rotor angle	45°

3.6.4 Plastic ware

Centrifuge vials (15-50 ml) (Sarstedt, Nümbrecht, Germany)

Cuvettes (Ratiolab, Dreeich, Germany)

Eppendorf cups (0,5-1 ml) (Eppendorf, Hamburg, Germany)

Freezing tubes (Nunc, Wiesbaden, Germany)

Immuno Maxi 96-Well-Plates (NUNC, Wiesbaden, Germany)

Micropipettes, Pipettes (Gilson, Langenfeld, Germany)

Parafilm M (American National Can, Greenwich, USA)

3.7 SOFTWARE

Biorad Biologic LP (Bio-rad, Munich, Germany) for protein purification

Cellquest software (Bekton Dickison, Mountain View, CA)

Endnote

Microsoft Word, Excel, Powerpoint, Photoshop

Origin 4.1 program

Software KS 400 (Carl Zeiss Vision, Germany)

4 METHODS

4.1 CELL CULTURE METHODS

4.1.1 Cell culture techniques

4.1.1.1 Storage of cells

In order to minimize the cellular injury induced by freezing and thawing procedures (intracellular ice crystals and osmotic effects), a cryoprotective agents such as dimethyl sulphoxide (DMSO) or glycerol are added. A variable number of suspension cells between 2-10 x 10^6 are spun down and resuspended in 500 µl of 10% DMSO solution (DMSO diluted in fetal calf serum). Freezing vials are cooled before and every step is performed on ice. While short-term preservation of cell lines using mechanical freezers (-80°C) is possible, storage in liquid nitrogen (-196°C) or its vapour (-120°C) is much preferred. Rapid thawing of cell suspension is essential for optimal recovery.

4.1.1.2 Cell culture of adherent cells

Base culture medium for tumor adherent cells (624.38-Mel, A375, SK-Mel 23, BLM, A9, RCC-26) was RPMI 1640 supplemented with 10 % FCS, 1% penicillin and streptomycin + 1% L-glutamine. Melanoma cells were supplemented also with 1% NEM non-essential aminoacids.

Cells growing as monolayers on plastic surfaces are held together and to the substratum by mucoproteins and sometimes by collagens; in addition, many cell monolayers require divalent cations (Ca^{2+}, Mg^{2+}) for their integrity. Thus for releasing cells from monolayers various protease solutions are used, sometimes in association with chelating agent. Therefore Trypsin-EDTA was used for subculturing of adherent tumor cell line according to the following protocoll: when cells were seen to be confluent old medium was washed out, monolayers were washed with PBS and incubated with 3 ml of Trypsin-EDTA for 5-10 minutes in the incubator. Prolonged exposure to trypsin should be avoided as this damages the cells. To inactive trypsin, medium containing serum, which contains a natural trypsin inhibitor, was added. The cell suspension was then subcultered in a split ratio of 1:3.

Cell lines were routinely tested for mycoplasma by fluorescent DNA staining using the manufactures directions. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% humidity.

4.1.1.3 Cell culture of lymphocytes

TyrF8 and A42 cells were cultured in 24 well plates using RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 7.5% heat-inactivated FCS, 7.5% heat-inactivated pooled human serum (HS) and 50 U/ml of r-IL-2. CTLs were stimulated biweekly with irradiated (10.000 rad, using a 137Cs-source Gammacell 40) IL-2-secreting HLA-A*0201-positive and tyrosinase- and MART-1/Melan-A-positive melanoma cells, irradiated (5.000 rad) pooled allogeneic PBMNC and irradiated (15.000 rad) EBV-transformed allogeneic B-LCL (LAZ 388). Fresh medium was given every third day and TyrF8 and A42 cells were split when wells were confluent. For functional assays measuring antigen-specific induction of cytokine secretion or cell-mediated cytotoxicity TyrF8 and A42 were used between day 8 and 14 after the last stimulation.

JB4 is a T cell clone that recognizes HLA-A2 expressing cell lines independent of specific peptides and indiscriminative of the HLA-A2 molecular subtypes (HLA-A*0201, A*0205 and A*0220). The clone was generated in GSF-Institute for Molecular Immunology (E.N.) by limiting dilution cloning of an allogeneic mixed lymphocyte tumor cell culture. Briefly, PBL from the healthy donor JB (HLA-A2 negative) were used as responder cells against an in-house renal cell carcinoma line (RCC-26, HLA-A2 positive). After two rounds of stimulation, responding T lymphocytes were cloned at a density of 0.3 cells per well in a 96 well round bottom plate using irradiated (10.000 rad) RCC-26 cells and pooled allogeneic PBMC (5.000 rad) as feeder cells. The specificity of JB4 was determined in cytotoxicity and cytokine stimulation assays using various allogeneic HLA-typed cell lines and was found to be HLA-A2 specific. JB4 was propagated similarly to TyrF8 with the exception that irradiated RCC-26 cells were used instead of melanoma cells.

Lymphokine-activated killer (LAK) cells were generated from PBMC of healthy donors. The culture was started in medium containing 15% heat-inactivated FCS, 100 U/ml of recombinant IL-2 and one single inducing dose of 1% of phytohemagglutinin and 10ng/ml of mAb OKT3. PHA and anti-CD3 were omitted during expansion culture. After 3 to 4 weeks a population of activated CD4- and CD8-positive T cells was obtained. The percentage of NK cells was less than 2%. Non-MHC-restricted cytotoxicity was confirmed by testing the culture against ^{51}Cr-labelled MHC class I negative Daudi cells. The percentage of lysis was routinely between 60-75% at an effector to target ratio of 10:1. The human natural killer cell line NKL was cultured in medium containing 15% heat-inactivated FCS, and 100 U/ml rIL-2. Cell density was adjusted to $0.3x10^6$/ml the day before use. Cytotoxicity against MHC class I negative K562 was always more than 30% at an effector to target ratio of 10:1.

4.1.1.4 Generation of dendritic cells from monocytic precursors

The base medium for the generation of dendritic cells was a very low endotoxin (VLE) (< 0,01 EU/ml) RPMI 1640 supplemented with 1 % human serum, 1% glutamine and 100 U/ml penicillin and streptomycin. This special medium was chosen to avoid/reduce the interference of endotoxin on the maturation of dendritic cells. Peripheral blood mononuclear cells (PBMC) were prepared from leukapheresis samples or from venous blood samples of healthy donors by density gradient centrifugation over Ficoll/hypaque. To obtain CD14+ monocytes, 30 x 10^6 PBMC were incubated in 75 cm^2 plastic flasks for 2 hours in 15 ml base medium and the non-adherent cells were washed off. The adherent cells were then cultured for 8 days in 15 ml dendritic cell base medium, containing GM-CSF (1000 U/ml) and IL-4 (800 U/ml) were. GM-CSF was added again on day 4 of culture.

4.1.2 Heat shock treatment

Heating was performed by directly immersing the cell culture flasks sealed with parafilm in a temperature-controlled water bath. Control cells were sealed and incubated at 37°C for exactly the same time as the heat-exposed samples. After treatment the cells were returned to 37°C/5% CO_2 and allowed to recover for different time periods (up to 7 days). At defined time points of recovery, non-viable cells in the supernatant were washed out and only adherent, viable cells were trypsinized and harvested for analysis.

4.1.3 Clonogenic assay

The ability of tumor cells to form colonies after various hyperthermia exposures was assessed using the clonogenic assay as described previously (Overgaard *et al.* 1979; Issels *et al.* 1990). Exponentially growing cultured melanoma cells were exposed to different temperatures between 41,8°C and 45°C for different incubation times (10 to 150 minutes).

At defined time-points viable, adherent cells were harvested by trypsinization and seeded in duplicate T25 tissue culture flasks at 100 and 500 cells/flask and allowed to form colonies in an undisturbed, humidified, 37°C/5% CO_2 air atmosphere. After 7 days, flasks were washed with 0,9% NaCl solution and cell colonies were stained with crystal violet solution (20% ethanol, 0,8% ammoniumoxalat and 2% cristal violet). Only colonies containing at least 50 cells were considered to be viable survivors. The number of colonies was counted and colony efficiency of each treatment was scored as survival fraction (SF), according to the formula: SF= number of colonies for a defined temperature and duration/(number of plated cells x PE). PE is the plating efficiency that is determined by dividing the number of colonies of untreated cells by the number of plated cells. The mean PE in three independent experiments for the

624.38-MEL cell line was 89% (0,89 ± 0,01). The survival fractions were plotted on a logarithmic scale (y-axis) against the time of heat exposure (in minutes) on a linear scale (x-axis). The “breakpoint temperature” was defined as the critical temperature where cells above this level start to dye exponentially. The breakpoint temperature for 624.38-MEL cells was determined to be 43,5°C.

4.1.4 Thermal isoeffect doses and cell viability

Heat exposure as a function of time was performed using temperatures below (41,8 °C) and above (45°C) the breakpoint temperature (43,5°C) for 624.38-MEL cells. The duration of heat exposure was adjusted to achieve equivalent survival fractions at the specified temperatures (thermal isoeffect dose, TID) according to the classical methods (Dewey 1994). In 624.38-MEL cells isosurvival fractions of 45% were achieved when cells were heated either at 41,8°C for 120 minutes or at 45°C for 22 minutes.

4.1.5 Flow cytometry analysis

Flow cytometry is as a technology to measure properties of cells related to scatter light. In the flow cytometer, the light source is a laser. Cells in liquid suspension flow in response to air pressure past an objective lens. Filters remove the excitation light to allow the emission light to be seen or measured. Most flow cytometers can measure two kinds of light from cells: Light scatter and fluorescence (FL). Light scatter is the interaction of light and matter. All materials, including cells, will scatter light. The measurements made by the detectors are called forward light scatter (FSC), which provides some information on the *relative size* of individual cells and side light scatter (SSC), which provides some information on the *relative granularity* of individual cells. In fluorescence, dye molecules are excited by light of a characteristic wavelength (or "color"), which then produce emitted light of a longer wavelength. The only light measured originates from the dye molecules. The fluorescent dyes that I used were PE (excited by 488 nm argon laser; emission peak at 578 nm; detected in FL-2), FITC (excited by 488 nm argon laser; emission peak at 519 nm) detected in FL-1) and Cy5 (excited by dye or 633 nm He-NE lasers; emission peak at 670 nm) detected in FL-4 on dual laser instruments). The method for staining is described below.

To consider specific populations of cells within a dot plot a gate is drawn around the desired population in the FSC/SSC plot. Live cell were defined according to light scatter properties. Usually a total of 10.000 events in the live cell gate were analyzed.

4.1.5.1 Surface staining

For surface immunostaining, cells were incubated with primary antibodies for 30 minutes at 4°C, washed with PBS + 5% fetal calf serum and then incubated with PE-conjugated goat anti-mouse IgG for 30 minutes at 4°C. After washing, cells were analyzed for fluorescence staining using a FACScan and Cellquest software.

To measure overall MHC class I molecule expression, the hybridoma cell supernatant W6/32 was used in a 1:2 dilution. To detect the HLA-A2 allotype, the hybridoma supernatant HB54 was used undiluted; the antibody APO-1 recognizing the Fas receptor was used at 10 ng/ml. Levels of apoptosis and/or necrosis were tested using the Annexin V/Propidium Iodide (PI) kit. To assess DC maturation CD1a, CD40, CD86, CD14 and CD83 antibodies were used.

4.1.5.2 Intracellular staining

Intracellular staining for HSP70 and HSC70 was performed using the permeabilization kit "Fix and Perm" according to the manufacturer´s instructions. Briefly, cells were washed with PBS plus 5% fetal calf serum and permeabilized with reagent A for 15 minutes at room temperature. After washing, cells were incubated with reagent B and the primary antibody for 30 minutes at 4°C in the dark. Cells were washed with PBS plus 5% FCS and then incubated with reagent B containing the secondary antibody for 30 minutes at 4°C in the dark. After washing, cells were fixed in 1% paraformaldehyde and analyzed for fluorescence staining using a FACScan and Cellquest software. Primary antibodies included the monoclonal rat IgG1 6B3 recognizing the inducible human HSP70, the monoclonal rat SPA-815 specific for the constitutively expressed HSC70. All rat-derived primary antibodies were detected using a FITC-conjugated secondary antibody (1:50).

4.1.6 T cell stimulation assay

Adherent melanoma cells were harvested, counted and plated in triplicates in 96-well flat bottom plates at 10.000 or 5000 cells/well in 100 µl fresh base medium. CD8+ T-cell clones were added at ratios of 5:1 or 2,5:1 respectively, i.e 2000/well in 100 µl T cell medium. The T-cell clones were TyrF8, recognizing the HLA-A2/Tyr $_{368\text{-}376}$ (YMNGTMSQV)-peptide complex, A42, recognizing the HLA-A2/MART-1 (AAGIGILTV) peptide complex and JB4**,** a CD8+ T-cell clone that recognizes the HLA-A2 protein itself, independently from its bound peptide repertoire. Culture supernatants were harvested after 24 hours and the content of IFN-γ as a surrogate marker for T-cell activation was determined using a commercially available ELISA. Background values arising from T-cells or melanoma cells cultured alone were generally below 5 pg/ml and were substracted from the experimental values.

4.1.7 Cell mediated cytolysis (CML)

Cell-mediated lysis of 624.38-MEL by MHC-restricted T-cell clones TyrF8, A42 and JB4 and non-MHC-restricted lymphocytes, LAK and NKL was quantitated using the standard 4-5h ^{51}Cr-release assay (Schendel *et al.* 1979). One milion target cells were incubated with radioactive sodium chromate ($Na_2\,^{51}CrO_4$) for 1,5 hours at 37°C. After the incubation time, in which target cells uptake sodium chromate, cells were washed twice with RPMI + 15% FCS to remove sodium chromate in the supernatant. Meanwhile effector cells were counted and plated in 96-well round bottom plate in a final medium (RPMI/15% FCS) volume of 200 µl/well. Effector cells were then titrated to obtain effector to target cell ratios ranging from 10:1 to 1,25:1. Target cells were added to effector cell titrations at a concentration of 2000/100 µl/well and coincubated for 4 hours at 37°C. After 4 hours incubation, 50 µl of supernatant were collected and the radioactivity was measured by a TopCount NXT microplate scintillation and luminescence counter with TopCount NXT software. Sodium chromate in the supernatant is released by the target cells through the effector cells-mediated lysis. Spontaneous release was determined by incubating target cells alone and the total release was determined by directly counting labeled cells. The percentage of cytotoxicity was calculated as follows: % specific lysis = (experimental cpm-spontaneous cpm/ maximal cpm – spontaneous cpm) X 100. Duplicate measurements of four-step titration of effector cells were used for all experiments. K562 and Daudi cells, which lack MHC class I molecules, were used as negative control for the T cell clones and as positive control for LAK and NKL lysis.

4.1.8 Cross-presentation assay

In this assay the ability of an antigen-presenting cell to uptake, process and present an exogenous antigen in the context of MHC class I molecules to specific CTLs is tested. This mechanism is known as cross-presentation and is a key feature of dendritic cells. Pre-requisite for this assay is that APC must be viable. I hypothesized that HSP70-PC isolated from the tyrosinase-positive 624.38-MEL cell line (see biochemical methods) carry tyrosinase-derived peptides and are able to transfer them into the MHC class I presentation pathway of dendritic cells (cross-presentation of tyrosinase). In this assay DC from peripheral blood monocytes were used as APC. Immature DC were loaded with tumor-derived HSP70-PC and finally matured with a “danger signal” to reach the optimal stage to prime and/or activate CD8+ T cells. In particular, DCs derived from monocytic precursors were seeded on day 7 at a concentration of 10^4 cells per well in 96-well round bottom plates in 100 µl of DC culture medium (see materials) and HSP70-PC were added to obtain final concentration of 1-10-100-

1000 ng/ml. After 24 hours, DCs were induced to mature by addition of 200 U/ml TNF-α and incubated for additional 24 hours. As a control for the T cell stimulation capacity of the DCs tyrosinase peptide (aa 368-376; YMNGTMSQV) was added exogenously at concentrations ranging from 1 to 10 µg/ml to TNF-α-matured DCs two hours prior to addition of T-cells. TyrF8 cells were added (2 x 10^4 cells/well) in 100 µl of medium to give final concentrations of 25 U/ml IL-2, 5% FCS and 5% HS. After 24 hours, culture supernatants were harvested and the content of IFN-γ was measured by OptEIA. To rule out the possible influence of contaminating LPS, which influences the maturation of DC being itself a "danger signal", polymyxin B was added at a concentration of 1 µg/ml together with HSP70-PC. To confirm that the cross-presentation of tyrosinase-derived peptides was HLA-A2 restricted, antibody-blocking experiments were performed. Before adding the T-cells, DCs were incubated for 1 hour at 37°C with the mAb HB54 (20 µg/ml), which binds specifically to HLA-A2 molecule (see material). Moreover, to prove that the mechanism of cross-presentation can only be done by viable cells, DC were fixed with paraformaldehyde. Fixed cells are not able to uptake or process antigens, but they express MHC molecules on their cell surface and are able to bind exogenously added peptides. Immature DCs were fixed with paraformaldehyde (1%) for 10 min at room temperature, then they were washed three times with excess of VLE-medium before being used in cross-presentation assays.

4.1.9 ELISA

Measurement of IFN-γ was used to detect the activation of T cell clones, when incubated with DC or with melanoma cell lines.

Immuno Maxi 96-Well-Plates were coated with 100 µl/well of capture antibody diluted in coating buffer. The plates were sealed and incubated at 4°C over night. Wells were aspirated and washed three times with 400 µl/well wash buffer. The plates were blocked with 100 µl blocking buffer, sealed and incubated at room temperature for 1 hour. Wells were aspirated and washed three times with 400 µl/well wash buffer. 45 µl of the samples and the standard were pipetted in the wells, sealed and incubated at room temperature for 2 hour. Wash step was repeated for 5 times. Detection and avidin-horseradish peroxidase conjugate (HRP) antibodies diluted in assay diluent were given at 100 µl/well. Plates were sealed and incubated at room temperature for 1 hour. Wash step was repeated for 7 times. Substrate solution was added at 100 µl/well, plates were not sealed and incubated for 30 minutes in the dark. Stop solution was added at 50 µl to each well. Absorbance was finally read within 30 minutes at 450 nm by a standard ELISA-reader.

4.1.10 Confocal microscopy

Immature DCs were incubated with 5 µg Cy5-labeled HSP70-PC or Cy5-labeled BSA for 30 min at 4°C (surface staining) or at 37°C (uptake studies). After washing, cells were settled on poly-L-lysine coated glass slides, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and mounted with VECTASHIELD. Samples were analyzed for transmission and fluorescence using a Zeiss model LSM510 confocal laser-scanning microscope equipped with an external Helium-Neon Laser 633 nm), lens CAPO 63x/1.2 W as well as equipment for digital interference contrast (DIC). Confocal section of specific fluorescence and DIC contrast image were taken simultaneously. Fluorescence of the Cy5-labeled probe was detected using an excitation wavelength of 633 nm, and a 650 nm LP emission filter. DIC Images were treated by multiplicative shading correction using software KS 400.

4.2 BIOCHEMICAL METHODS

4.2.1 Fluorescence labeling of proteins (Cy5)

Purified HSP70-PC (from A375-MEL melanoma cells) or BSA (1 mg/ml) was incubated with FITC (50 µg) or FluoroLink^{TM}Cy5 in carbonate-bicarbonate buffer overnight at 4°C, or 5 h at room temperature for Cy5. Free unconjugated FITC was removed by passing the mixture over a gel filtration column (Sephadex G-25). For Cy5-labeling, protein was dialyzed (12-14 kDa dialysis membrane) for 12-14 h. FITC-conjugated proteins were analyzed by SDS-PAGE and immunoblotting using anti-HSP70 mAb and anti-FITC Ab. Labeled proteins were centrifuged at 100.000 x *g* before use to remove any particulate matter.

4.2.2 Biochemical analysis of protein expression

4.2.2.1 Cell lysis

Melanoma cells were lysed in 3-((3-cholamidopropyl)dimethylammonio)-1propanesulfonate (CHAPS) buffer for 30 minutes at 4°C.

4.2.2.2 SDS-Polyacrylamidgelelectrophoresis (PAGE)

To separate proteins according to their molecular weight, whole cell lysates containing 40 µg protein as determined by the Lowry method (see estimation of protein concentration) were first denatured by boiling at 95°C for 5 min in SDS gel loading dye (see material), loaded into a lane of an horizontal 10% SDS-PAGE gel and electrophoretically separated at 150 volts in running buffer. A marker with well-characterized proteins allows to identify the molecular weight of unknown proteins. Gel must be freshly prepared.

4.2.2.3 Western Blot analysis

After electrophoresis, proteins can be transferred to nitrocellulose membranes in blotting buffer for 1 hour at 75 volts and probed with appropriate Ab.

To visualize proteins on the membrane, the membrane can be stained with Ponceau staining, which binds every protein. To detect specific proteins, selected antibodies were used. Before incubating with appropriate Ab, the membrane has to be blocked in 5% dry-milk in PBST for at least 1 hour, in order to block nonspecific binding sites. The membrane is incubated with the primary antibody diluted in 5% milk solution for variable times: 1 hours at room temperature for HSP70 and HSC70, over night at 4°C for tyrosinase or Melan-A/MART-1. The membrane is washed 3 times for 15 minutes in TBST and then incubated for 1 hour at

room temperature in horseradisch peroxidase (HRP)-conjugated antibody diluted in 5% milk solution. The membrane is washed 3 times for 15 minutes. Detection of protein/antibody complexes is achieved by the ECL (Enhanced Luminol Reagent) system. This western blot chemiluminescence reagent is a non-radioactive light emitting system which detect the complex protein/antibody immobilized on a membrane by the oxidation of luminol, which results in light emission at a wavelength of 428 nm, captured by an autoradiography film.

4.2.2.4 Silver staining of proteins separated by SDS-PAGE gels

The protocol was from Bloom (Bloom *et al.* 1987). After electrophoresis, the gel is fixed for 10 minutes in 50 ml formaldehyde fixing solution; then washed twice in water for 5 minutes and soaked for 1 minute in 50 ml thiosulfate solution by agitating slowly. Then, the gel is washed for 20 seconds with water and soaked for 10 minutes in 50 ml of nitrate solution. After washing with water, the gel is soaked in 50 ml thiosulfate developing solution until band intensities reach the desired intensity (about 1 minute); after washing with water for 10 minutes, the gel is fixed in 50 ml drying solution. Gels are dried over night at room temperature between two pieces of wet dialysis membrane, clamped on the edges of special frames.

4.2.2.5 Estimation of protein concentration

To estimate the protein concentration of a sample different assays were used: the Bradford (Bradford 1976) and Lowry (Lowry *et al.* 1951) assay are colorimetric assays. The Lowry assay is based on the reaction of protein with an alkaline copper tartrate (reagent A) solution and Folin reagent (reagent B), which leads to a blue reaction with maximum absorbance at 750 nm and minimum at 405 nm. Proteins are measured at 750 nm. The Lowry method is a widely used method with high sensitivity (2-100 µg), but with a significant chemical interference (incompatible with ß-mercaptoethanol). The Bradford assay has few chemical interferences, is more sensitive (1-20 µg) and rapid. Proteins are measured at 595 nm.

4.2.3 Purification of HSP70-PC

4.2.3.1 Introduction to the purification of proteins

Proteins are purified using chromatographic purification techniques, which separate according to different properties: charge, biorecognition and size.

Protein property	Technique	Features	Sample end condition	Column matrix for HSP70-PC
Charge	ion exchange (IEX)	high resolution, capacity and speed	high ionic strength, **CONCENTRATED**	Q-sepharose
Biorecognition	affinity (AC)	high resolution, capacity and speed	elution condition, **CONCENTRATED**	ADP-Agarose
Size	gel filtration (GF)	good resolution	buffer exchanged; **DILUTED**	G-Sephadex 25

Before starting the purification it is important to define the properties of target proteins in order to check the "stability window", especially concerning pH and ionic strength, to avoid irreversible inactivation. General rules are to work rapidly at low temperature to maintain temperature stability, select the right buffers for extraction and purification and the condition for ion exchange to maintain pH stability; and add inhibitors to block protease activity.

4.2.3.2 The "3-Phase Purification" strategy

The basic scenario for protein purification is best described by a "3-Phase Purification strategy": capture, intermediate purification and polishing.

1) **Capture**: The goal is to isolate, concentrate and stabilize the target product. The technique used is affinity chromatography. Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand attached to a chromatographic matrix. It offers high selectivity, high resolution, and high capacity for the protein of interest. Our target protein, HSP70-PC is specifically and reversibly bound to a complementary binding substance (ADP-Agarose). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed away (not ADP binding proteins) and the bound target protein (HSP70-PC) is eluted by using a specific competitive ligand (ADP).
2) **Intermediate purification**: the goal is to remove most of the bulk impurities (other proteins, nucleic acids, endotoxins). The technique used is ion exchange chromatography. Ion exchange chromatography separates proteins according to charge. It has a very high-resolution with high sample loading capacity. The separation is based on the reversible interaction of a charged protein with an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. After binding, proteins are eluted differentially by using either stepwise or a continuous salt (NaCl) gradient. For the purification of HSP70-PC, proteins were eluted using a gradient from 20 to 600 mM NaCl. The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein

will bind to an anion exchanger, when below to a cation exchanger. The pI of HSP70 is below pH 7,0 (pI= 5,37-5,48), therefore a strong anion exchanger (Q) has to be used.

3) **Polishing**: the goal is to achieve high purity by removing any remaining trace impurities or closely related substances. The technique used is gel filtration, which separates proteins according to the molecular size. This is ideal for final polishing steps in a purification in which the sample complexity has been reduced.

4.2.3.3 Column packing, equilibrating and storage

Before use, all columns have to be equilibrated with appropriate buffer in order to remove storage buffer or, for flow gradients, to bring the column material at the working concentrations of ions and pH. To equilibrate a column, at least two-column-volumes (2 CV) of buffer have to be run through the column. For anion exchange chromatography, the equilibration is done using a high salt continuous gradient between 20 mM and 1000 mM NaCl. After use, columns are stored in 20% ethanol at 4°C.

4.2.3.4 Source of material for HSP70-PC purification

The sources for HSP70-PC were cell-pellets from the tyrosinase-positive human melanoma cell lines 624.38-MEL, SK-MEL 23 and the tyrosinase-negative human melanoma cell line A375. Cells were harvested by centrifugation, were resuspended in few µl of PBS and frozen at –80°C. The size of the cell-pellet for one isolation was between 5 to 10 ml. For the downscale method (1:3) D_3, the cell-pellet was reduced to 1/3. Cell-pellets of appropriate volume were thawed, resuspended and re-frozen in buffer A (hypotonic lysis containing anti-protease PMSF).

4.2.3.5 Cell lysis, centrifugation and ultracentrifugation

The goal of this step is to solubilize the cellular proteins, to remove the particulate matter like cell membrane fragments or organelles and clarify the sample before loading the chromatographic columns. 10 ml cell pellet of 624.38-MEL, A375 or SK-Mel 23 were lysed in hypotonic buffer by 3 cycles of freeze/thaw, followed by homogenization using Ultra-turrax about 2 minutes. The cellular homogenate was centrifuged for 5 minutes at 3000 rpm. Homogenization and centrifugation of the cell pellet was repeated twice. Before loading the first chromatography column, ultracentrifugation was performed to remove lipids and particulate matter (50.000 x g for 50 minutes at 4°C; Rotor Ti75).

4.2.3.6 Gel filtration

In order to remove small molecules and salts and to exchange the sample buffer from the hypotonic buffer into the appropriate buffer for affinity chromatography (buffer B: 20 mM Tris-acetate, 20 mM NaCl, 15 mM 2-ME, 3 mM $MgCl_2$, 0,5 mM PMSF, pH 7,5) after sample extraction, Sephadex G-25 gel filtration was used. In the following figure a profile example of a gel filtration is shown. Proteins are detected at 280 nm (left axis, absorbance) and salts interfere with the conductivity (right axis, msiemens/cm). Higher molecular weight molecules are eluted first.

Figure 8

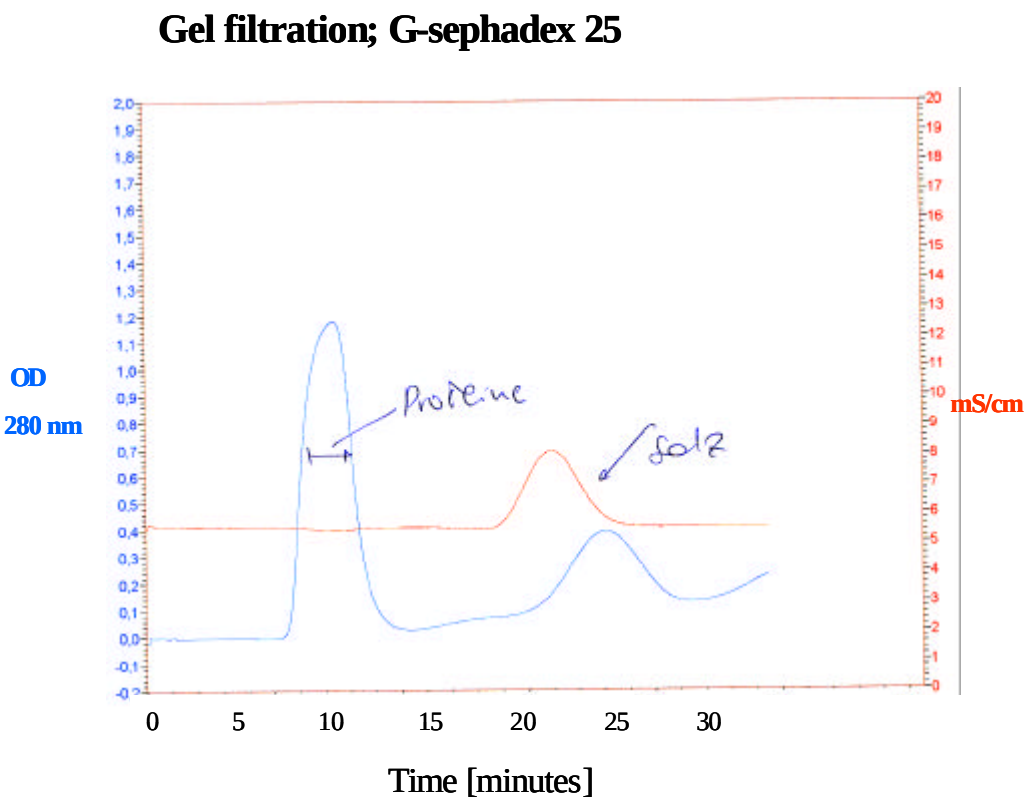


Figure 8: Example of diagram of a gel filtration (Biologic LP Software). For explanation see text.

4.2.3.7 ADP-Affinity chromatography

The sample eluted from the Sephadex G-25 was applied directly to an ADP-agarose column equilibrated with buffer B. HSP70-PC bind to ADP-agarose, while non-ADP binding proteins are immediately eluted. The column was washed extensively with buffer B until no more protein was detectable in the eluate by measuring the absorbance at 280 nm. To eluate ADP-binding proteins, the column was incubated with buffer B containing 3 mM ADP at room

temperature for 30 min and subsequently eluted with the same buffer. If the volume of the sample loaded is too high, the binding sites of ADP-agarose may be saturated and some ADP-binding proteins may be eluted together with the non-ADP-binding proteins. Therefore the eluted non-ADP binding protein fractions may be reloaded on the ADP-agarose. In the following figure an example of two consecutive ADP-affinity chromatography is shown. 2' and 2'' represents the first and second elution of non-ADP binding proteins, respectively. 3'and 3'' are the first and second elution of ADP-binding proteins, respectively, which can be eluted only after adding the competitive agent ADP.

Figure 9

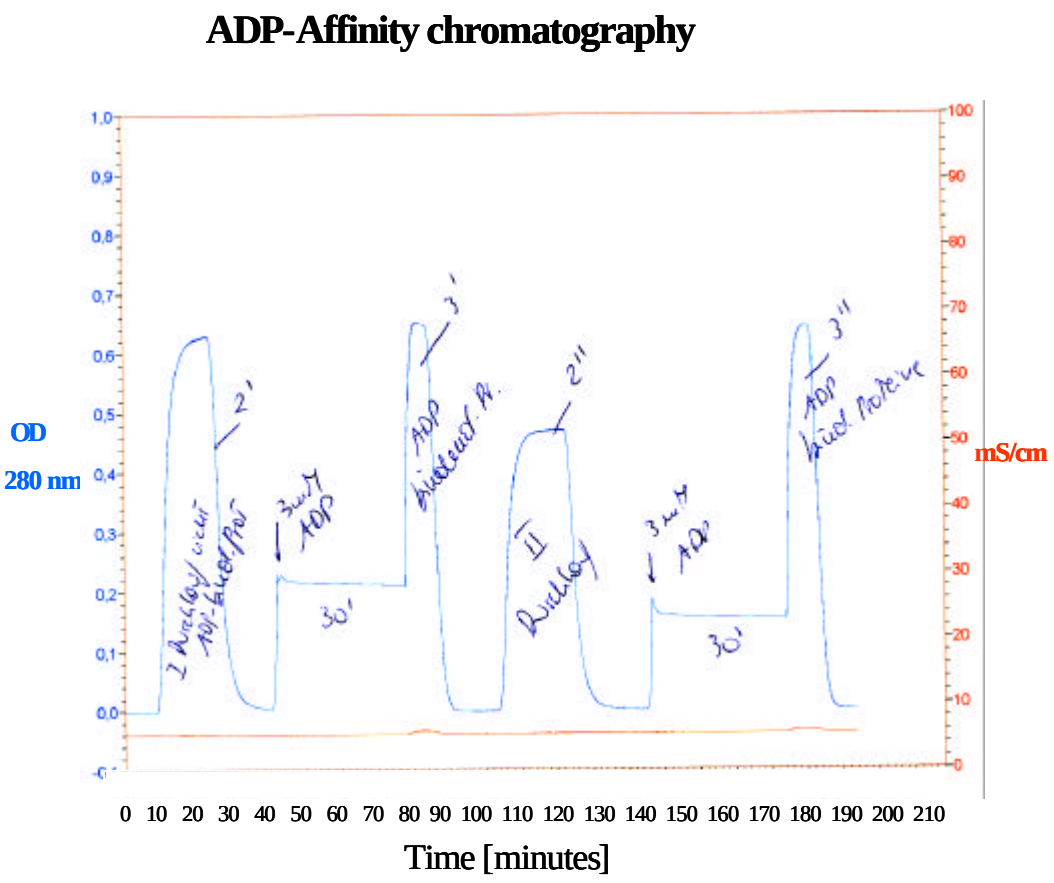


Figure 9: Example of diagram of a ADP-affinity chromatography (Biologic LP Software). For explanation see text.

4.2.3.8 Ion exchange chromatography

To separate the HSP70-PC from other ADP-binding proteins, ion exchange chromatography followed. ADP-binding proteins eluted from the ADP-agarose column were loaded into a Mono-Q-Sepharose and eluted over a 20-600 mM NaCl gradient (figure 10). The fractions

59-69 were pooled and analzed by SDS-page and western blot (Figure 10). The fractions containing HSP70 had less contaminating proteins, as determined by SDS-PAGE silver stain and by dot blot with anti-HSP70 mAb (figure 11, lane 7), than before Mono-Q-Sepharose (figure 11, lane 6).

Figure 10

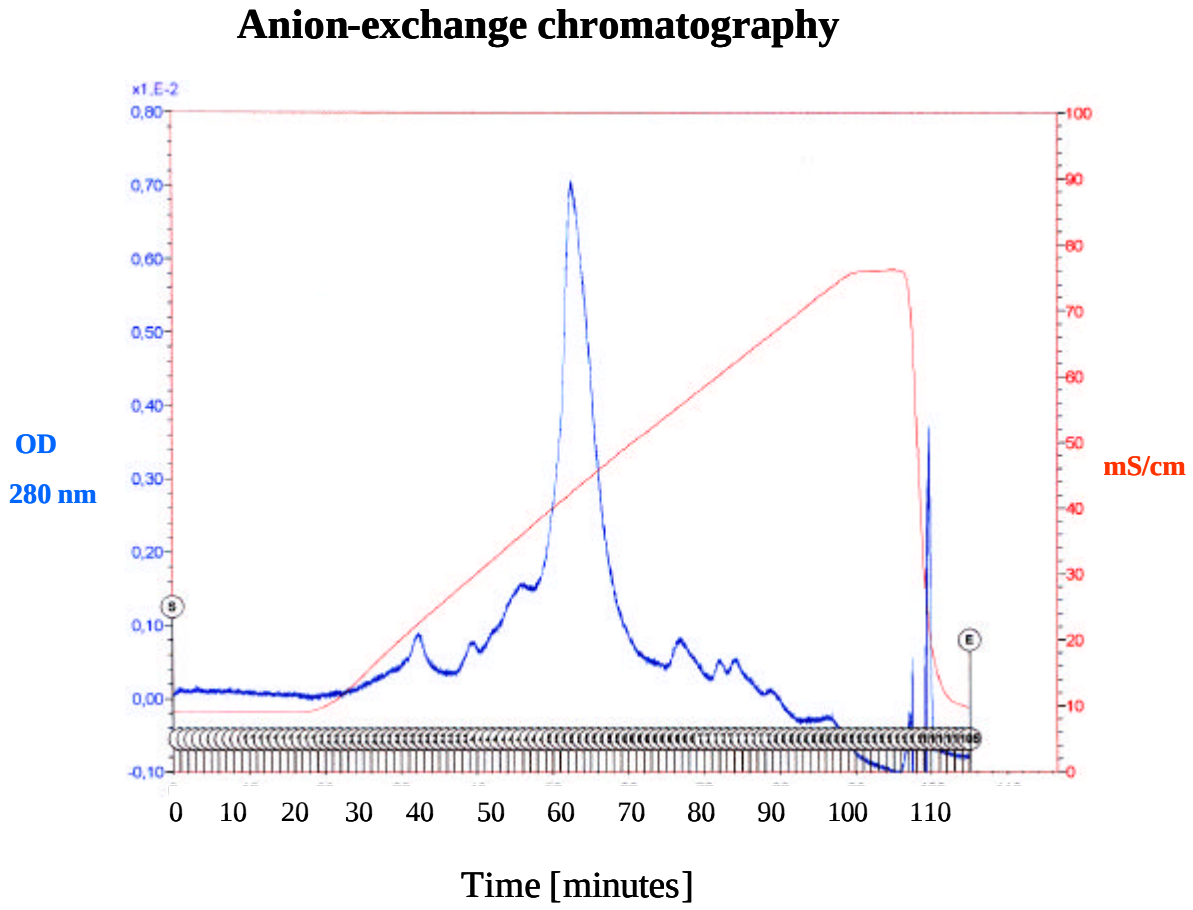


Figure 10: Example of diagram of an anion-exchange-chromatography (Biologic LP Software). For explanation see text. Fractions 59-69 were pooled.

4.2.3.9 Gel filtration

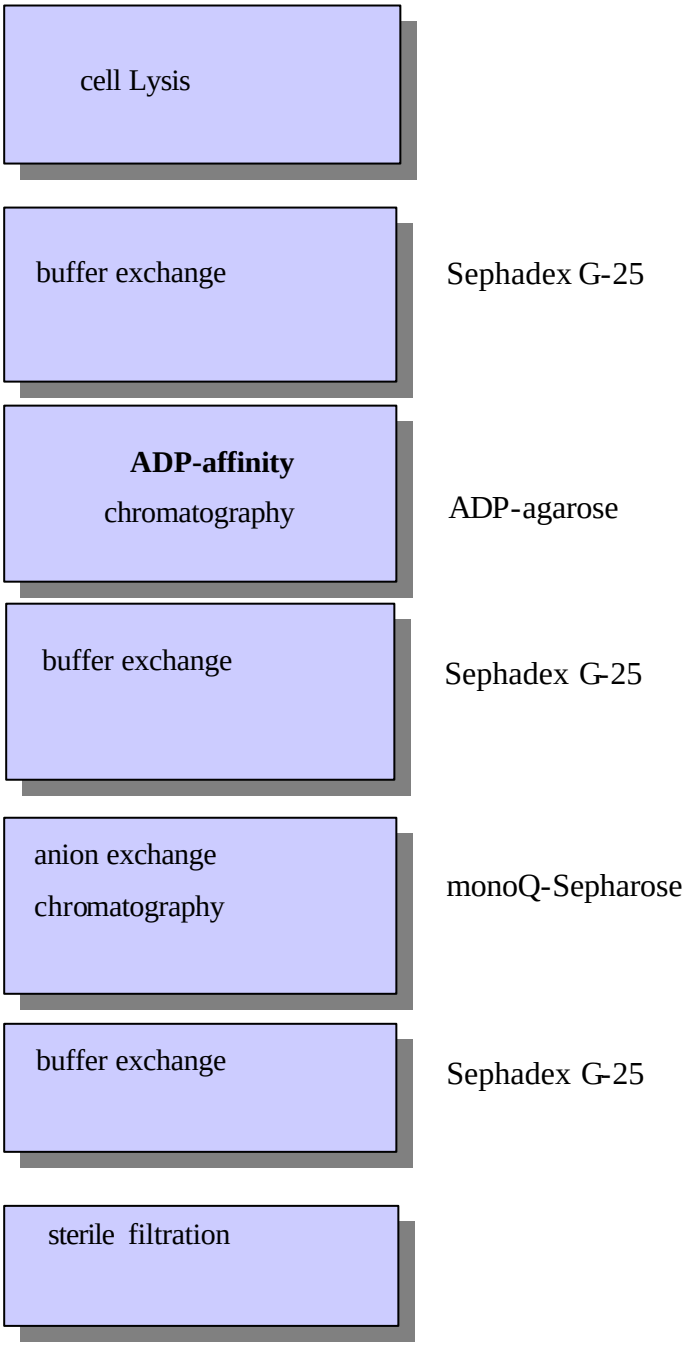
The pre-packed column PD-10 containing G-Sephadex 25 was selected for gel filtration: on a PD-10 column 2,5 ml of sample was loaded. Elution followed with sterile PBS. The first 3 ml contain the proteins; the following 10 ml contain salts. In this way, salts from the Mono-Q Sepharose column were removed and the buffer exchanged to PBS.

4.2.3.10 Sample concentration

During gel filtration the sample volume increased. Therefore, to achieve high protein concentration, samples were loaded into a membra-spin Macro concentration tube. To avoid undesiderable binding of proteins to the membrane filter, tubes were pre-incubated for 2 hours

with PBS + 5% Tween-20 solution. These tubes were then washed with PBS and filled with 3,5 ml of the sample and spun at 3000 rpm in a cooled centrifuge (4°C) for 30-60 minutes. The concentrated protein was filtered through a 2 µm filter to remove any possible bacterial contamination, aliquoted and stored at – 80°C.

4.2.3.11 Flow-chart of HSP70-PC purification *(Noessner et al. 2002)*



4.2.3.12 Final protein characterization through the steps of the HSP70-PC purification from melanoma lines

Figure 11

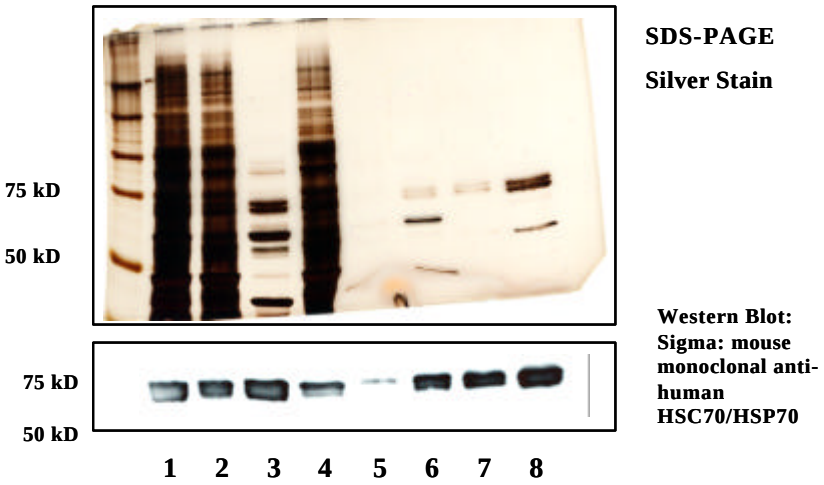


Figure 11 shows a silver stained gel and the western blot for HSP70/HSC70 of the single steps of the isolation of HSP70-PC. By silver staining the purity of a purified protein can be assessed (presence of not desired proteins) and in western blot its identity. Aliquots from each purification step were resolved by SDS-PAGE (10%) and silver stained or transferred to a membrane for western blot. Lane 1: Cell lysate after buffer exchange through Sephadex G-25; lane 2: run through the first ADP-Affinity chromatography, containing non-ADP binding proteins; lane 3: eluate from the first ADP Affinity chromatography containing ADP-agarose bound protein; lane 4: run through the second ADP-Affinity chromatography; lane 5: eluate from the second ADP Affinity chromatography; lane 6: ADP-binding proteins buffer exchange through Sephadex G-25; lane 7: Anion-exchange chromatography through mono-Q Sepharose: Pool of the pure fractions; lane 8: endproduct: HSP70-PC in PBS. A contaminating protein of molecular weight of about 60 kDa was detected in this experiment. Sequencing analysis in collaboration with Dr. Kähne, "Immunology research center", University of Magdeburg, revealed that this protein was keratin. This was not reproducible.

4.3 STATISTICAL ANALYSIS

The statistical significance of experimental values was assessed by means of Student's *t*-test. The t-Test is typically used to compare the means of two populations. Specifically, it can be used to determine whether or not the means in two sample populations are significantly different. The probability value (p-value) is compared with the significance level and, if it is smaller, the result is significant. *, **, and *** represent p values of $p<0.5$, $p<0.05$ and $p<0.005$, respectively.

For the calculation of the dose response value Y_{50}, the statistics menu of the Origin 4.1 program and the logistic non-linear curve fitting for the dose response in pharmacology and biology were used.

5 RESULTS

The first goal of this study was to investigate whether induced heat shock protein expression in tumor cells affects antigenicity and immune recognition of a given tumor.

Of particular interest was the relationship between the intensity of the initial stress and changes in tumor physiology and immune competence. Since the heat shock protein 70 family contains members that are constitutively expressed and induced upon heat shock, respectively, it was selected for the study. The cellular model was the human melanoma, because it is well characterized for antigens and immune recognition by T cells.

In the first part I have determined the effects of heat treatment on the endogenous presentation and processing of tyrosinase and Melan-A/MART-1 and the susceptibility of 624.38-MEL cells to immune effector mechanisms.

The second part of my study addressed the question of extracellular HSP70, as it may occur after stress-induced released by dying cells. Because mouse studies pointed to HSPs as inducers of tumor antigen specific immune responses (Srivastava 2000), I asked the question whether HSP70 can carry the tumor antigen tyrosinase and deliver it to dendritic cells for presentation to T-cells, following the mechanismus which is know as cross-presentation.

5.1 EFFECTS OF HYPERTHERMIA TREATMENT ON TUMOR ANTIGENICITY AND SUSCEPTIBILITY TO IMMUNE EFFECTOR MECHANISMS

624.38-MEL cells express tyrosinase and Melan-A/MART-1 antigens and are recognized by antigen-specific HLA-A2 restricted CD8+ T-cell clones TyrF8 and A42 respectively, indicating that tyrosinase and Melan-A/MART-1 are processed into peptides that bind to the class I allotype HLA-A2. The cell line was exposed to heat shock to study the influence of two selected thermal doses on antigen expression, processing and presentation via MHC class I as well as the ability to induce effector function in MHC class I-restricted T-cells and non-MHC restricted NK and LAK cells. IFN-γ secretion by immune effector cells was used to assess the antigen presentation of tyrosinase and Melan-A/MART-1. Cytotoxicity assays were performed to determine susceptibility to T-cell- and NK-cell-mediated lysis. Expression levels of MHC class I molecules, HLA-A2, tyrosinase and Melan-A/MART-1 antigen were determided by FACS analysis, western blot and quantitative RT-PCR.

5.1.1 Selection of two thermal isoeffect doses in 624.38-MEL cells

The thermal damage induced by heat is dependent on the magnitude of change in temperature and the duration of the elevated temperature. The thermal dose required to induce cell death in vitro varies from cell type to cell type and is influenced by microenvironmental factors. In

general, exposure of tumor cell lines to temperatures below 42°C leads to a dose-dependent cell cycle arrest, but is nonlethal. Higher temperatures (>43°C) for a short time may induce the same amount of thermal damage in a given cell type and result in the same survival ability (thermal isoeffect dose). Therefore for each cell type it the so-called "breakpoint temperature", which is the critical temperature where cells above this level start to dye exponentially, has to be determined.

The breakpoint temperature for 624.38-MEL cells was determined by exposing cells to different temperatures from 41,8°C to 45°C for various time periods ranging from 15 minutes to 150 minutes. After heat exposure, cells were seeded at low density and allowed to grow at 37°C/5% CO_2. After 7 days, the number of colonies was counted and expressed as the survival fraction, which is plotted against the duration of heat exposure. Figure 12 shows the clonogenic cell survival of 624.38-MEL cells in response to different temperatures. Exposure of cells to temperatures below 43,5°C had a minimal impact on their survival, whereas temperatures above 43,5°C (44°C and 45°C) exponentially reduced tumor cell survival, thus defining 43,5°C as the breakpoint temperature (figure 12). In order to compare the immunological consequences of heat treatment of different initial intensity, but with the same cytotoxic effect. i.e. the same survival ability, I defined the thermal isoeffect doses (TID) at the specified temperature of 41,8°C and of 45°C by adjusting exposure times (120 minutes and 22 minutes, respectively). These thermal doses resulted in equivalent clonogenic survival rate (isosurvival-rate of 45%).

Figure 12

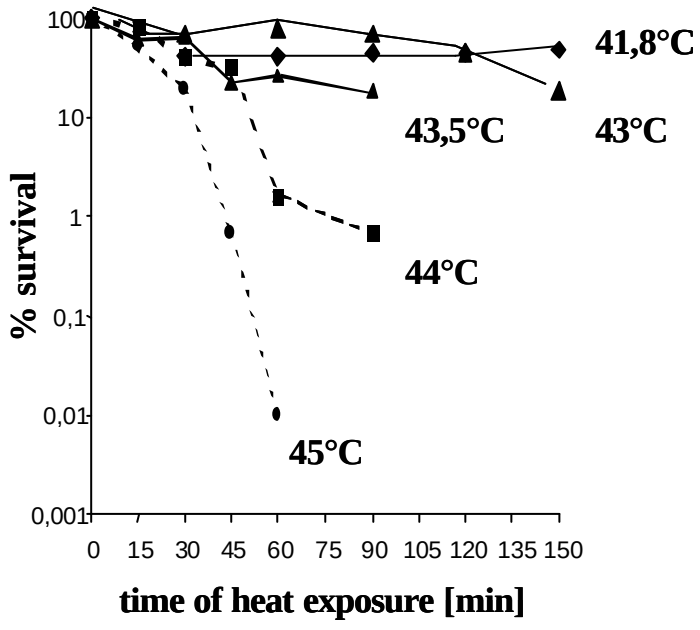


Figure 12: Clonogenic cell survival of 624.38-MEL cells after heat exposure.

624.38-MEL cells were exposed to temperatures of 41,8°C/43°C/43,5°C/44°C/45°C for various time periods up to 150 minutes. At intervals of 15 minutes, 624.38-MEL were trypsinized and plated at 100 and 500 cells/T25 flask and allowed to grow and form colonies in a 37°C/5% CO_2 environment. After 7 days cells were fixed, stained and colonies were counted. The survival fraction was determined and plotted on a logarithmic scale on the y-axis versus the time of heat exposure on the x-axis in a linear scale. 43,5°C was determined as the "breakpoint temperature", defined as the critical temperature where cells above this level start to die exponentially. One experiment representative of 3 is shown.

5.1.2 Thermal dose-related differential kinetics of HSP70 protein expression

Increased levels of misfolded or aggregated proteins induced by heat shock overwhelm the binding capacity of the basal pool of cytosolic molecular chaperones and induce the heat shock response. The heat shock response is the time period after heat exposure during which expression of heat shock proteins is transcriptionally and translationally upregulated to assist in the renaturation or degradation of misfolded proteins (Lindquist 1986) (Welch *et al.* 1986) (Georgopoulos *et al.* 1993).

To assess the characteristics of the heat shock response of the melanoma cell line 624.38-MEL, the cells were treated with the selected thermal isoeffect doses (41,8°C/120 minutes or 45°C/22 minutes), and the expression levels of HSC70 (constitutive) and HSP70 (inducible) were investigated by Western blot analysis and flow cytometry.

At the physiological growth temperature of 37°C both the constitutively expressed HSC70 and the inducible HSP70 were detected in 624.38-MEL cells (figure 13A/37°C) by Western blot. Heat exposure did not change the expression of the constitutive form of the HSP70 family (HSC70) but stimulated the expression of inducible HSP70 protein (figure 13A). Quantitating HSP70 induction using FACS analysis revealed different kinetics after the two thermal doses (figure 13B). For cells heated at 41,8°C/120 minutes, a 4-fold increase HSP70 was already detected after 4 hours after heat treatment and levels increased linearly to peak levels of a 9-fold increase (8,9 ± 0,4) at 48 hours time point (figure 13B, left panel). Cells exposed to 45°C/22 minutes showed no increase in HSP70 protein expression after 4 hours of recovery. Induction started at the 15 hours time point and reached peak levels of 15-fold induction compared to the physiological growth temperature of 37°C (14,9 ± 0,6 at 48 hours) (figure 13B, right panel).

HSP70 protein expression decreased after 48 hours but remained above the pre-heat shock level for several days after heat shock (day 7+) (data not shown).

Figure 13A

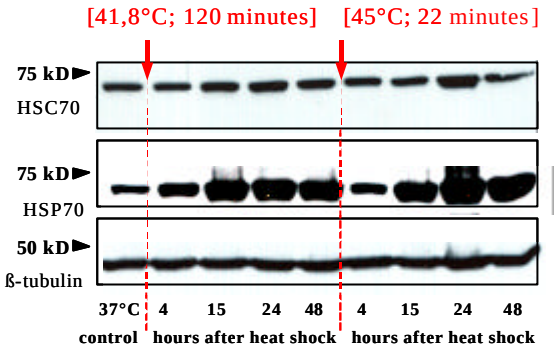
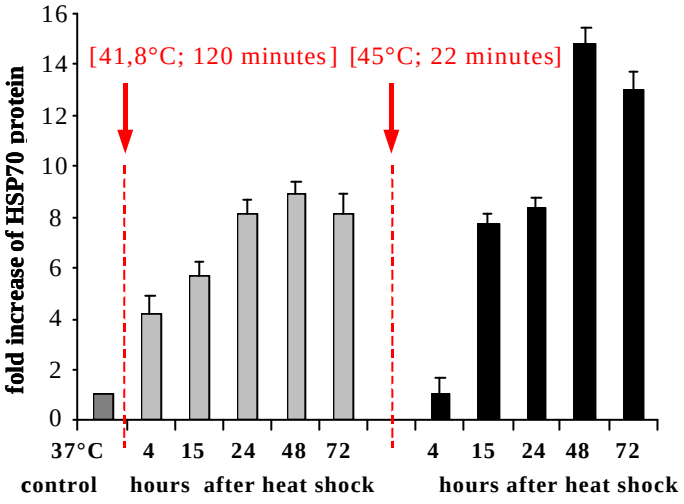


Figure 13: A) Western blot analysis of HSC70 and HSP70 expression in 624.38-MEL cells: 624.38-MEL cells were exposed to 41,8°C for 120 minutes or to 45°C for 22 minutes and then returned to 37°C for recovery. Cells were harvested 4, 15, 24 and 48 hours after heat treatment, counted and an equal amount of viable cells was lyzed and separated by 10% SDS-PAGE. After electrophoresis, proteins were blotted onto a nitrocellulose membrane and stained with antibodies against HSP70 (6B3) or HSC70 (SPA-815). To control for equal protein loading, staining for ß-tubulin was performed. The blot is representative of 5 independent experiments.

Figure 13B



B) Intracellular flow cytometry for inducible HSP70: 624.38-MEL cells were subjected to 41,8°C/120 minutes (light grey histograms) or 45°C/22 minutes (black histograms) respectively, and harvested at different time points after heat treatment. Viable cells were analyzed for intracellular protein levels of HSP70 by intracellular flow cytometry using the monoclonal antibody 6B3 and isotype control antibody. Protein expression level was calculated as the difference in mean fluorescence intensity (MFI) of 6B3 and the isotype control. Fold increase values were calculated using the value at 37°C as a reference value (1 fold, dark grey histogram; MFI of HSP70 at 37°C was 47 ± 17). Shown is the mean of 5 independent experiments (± standard deviations).

5.1.3 Thermal-dose related changes in expression levels of immunologically relevant proteins: MHC class I, HLA-A2, tyrosinase and Melan-A/MART-1 antigens during the heat shock response

624.38-MEL cells were subjected to 41,8°C for 120 minutes or to 45°C for 22 minutes and analyzed for tyrosinase and Melan-A/MART-1 expression at various time points after heat shock. Western blot analysis revealed a significant increase in tyrosinase protein levels for both thermal doses. The kinetics and expression levels however differed for both doses and were similar to HSP70 expression kinetics: tyrosinase protein levels increased linearly after exposure to 41,8°C/120 minutes reaching peak levels at 24 and 48 hours, whereas a delayed expression and subsequent strong induction was observed after 45°C/22 minutes treatment (figure 14A). Tyrosinase expression levels returned to physiological levels after 6-7 days after heat shock (data not shown). Interestingly, no significant change in Melan-A/MART-1 protein expression occurred during the heat shock response after the two different thermal doses (figure 14A).

Figure 14A

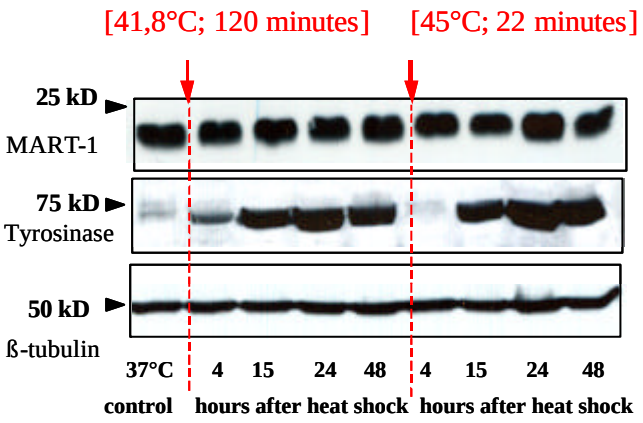


Figure 14: A) Western blot analysis of Melan-A/MART-1 and tyrosinase expression in 624.38-MEL cells. 624.38-MEL cells were exposed to 41,8°C for 120 minutes or to 45°C for 22 minutes and then returned to 37°C for recovery. Cells were harvested 4, 15, 24 and 48 hours after heat treatment, counted and an equal amount of viable cells was lyzed and separated by 10% SDS-PAGE. After electrophoresis, proteins were blotted onto a nitrocellulose membrane and stained with antibodies against tyrosinase (C-19) and Melan-A/MART-1 (A103). To control for equal protein loading, staining for ß-tubulin was performed. The blot is representative of 5 independent experiments.

To determine whether the protein levels correlated with transcript levels, quantitative RT-PCR for tyrosinase and Melan-A/MART-1 expression was performed (The RT-PCR experiments have been performed by Bernhard Frankenberger, GSF-Institute for molecular Immunology). There were no significant changes in Melan-A/MART-1 transcripts during the recovery period after the two selected thermal doses (figure 14B). As shown in figure 14C, tyrosinase transcript level also remained unchanged after treatment of 41,8°C/120 minutes, whereas after a thermal dose of 45°C/22 minutes transcript levels dropped to 10% (0,1-fold increase, $p<0,01$) and to 13% (0,13-fold increase, $p<0,01$) of control levels (37°C=1-fold) at 15 and 24 hours of recovery. Obviously, the induction in tyrosinase protein did not correlate with transcript levels.

Figure 14 B **Figure 14 C**

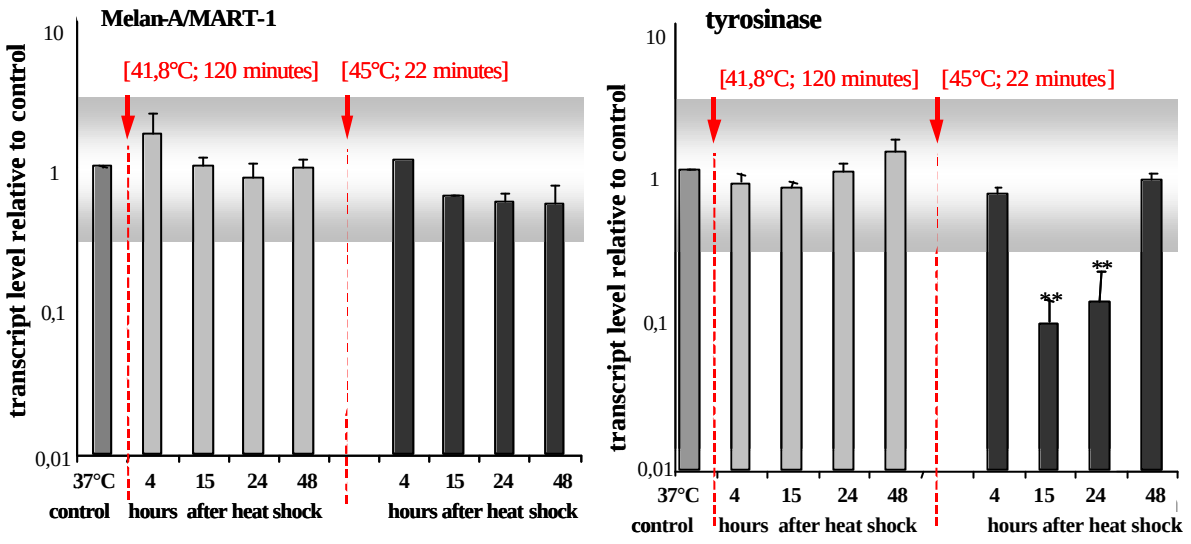


Figure 14: (B) Quantification of Melan-A/MART-1 (b) and tyrosinase (C) transcripts in 624.38-MEL cells. 624.38-MEL cells were harvested 4, 15, 24 and 48 hours after exposure to 41,8°C for 120 minutes or 45°C for 22 minutes. Equal amounts of total RNA were analyzed by quantitative real-time RT-PCR using the LightCycler system and SYBR green fluorescence. No signals were observed in the negative control (water in place of template). Amplification of the house-keeping gene α-enolase was performed in separate capillaries but in the same real-time PCR run as the tyrosinase and Melan-A/MART-1 samples to confirm that comparable amounts of total RNA were used for all samples. Blotted is the fold-change in transcript levels with the level at 37°C set to one (crossing points at 37°C were approximately 13 for Melan-A/MART-1 and 16 for tyrosinase). Values are the mean of 3 independent experiments (± SD). The grey horizontal box crossing the bars defines a confidence interval in which all values show a difference of ± 1,5 cycles compared to the reference value at 37°C. According to the manufacturers´ instructions this cut off interval was defined to discriminate between significant overexpression of transcripts from significant underexpression (0,35 – 2,8). The RT-PCR experiments have been performed by Bernhard Frankenberger, GSF-Institute for molecular Immunology).

5.1.3.1 Thermal dose-related expression of MHC class I and HLA-A2 molecules

Due to its essential role in antigen presentation for T-cell recognition the expression of MHC class I molecules by tumor cells is the prime determinant for the cellular immunogenicity. Tumor cells use the downregulation or loss of MHC class I molecules to escape recognition by the immune system (Rivoltini *et al.* 1995).

Since tyrosinase and Melan-A/MART-1 are processed into peptides presented by HLA-A2 (Rivoltini *et al.* 1995) (Kawakami *et al.* 1994) (Wolfel *et al.* 1994) the antibody HB-54 was used to specifically detect the HLA-A2 allotype.

Antibody HB54 showed reduced staining for cells 4 hours after a treatment at 41,8°C/120 minutes indicating reduced HLA-A2 surface expression. However staining intensity recovered and increased above control levels with prolonged recovery times (figure 15, left panel; MFI= 669 ± 59 at 72 hours vs 458 ± 92 at 37°C). After treatment at 45°C/22 minutes, cells had a more pronounced reduction in surface staining that lasted through 15 hours of recovery (figure 15, right panel; MFI= 367 ± 31 at 4 hours; 385 ± 10 at 15 hours vs 458 ± 92 at 37°C). After 24 hours of recovery, however, expression was increased above control levels (MFI= 578 ± 109 at 24 hours vs 458 ± 92 at 37°C) and was maintained at a high level up to 72 hours of recovery (MFI= 620 ± 22 at 72 hours).

Figure 15

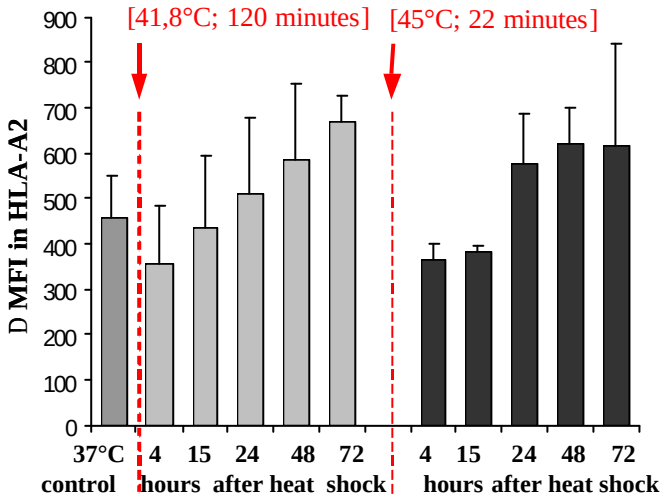


Figure 15: Flow cytometry for surface levels of HLA-A2 after heat shock. 624.38-MEL cells were cultured at 37°C and exposed to 41,8°C for 120 minutes or 45°C for 22 minutes. Flow cytometry was performed on viable cells harvested at 37°C and at different time points after treatment. Δ mean fluorescence intensity (MFI) was calculated by substracting the mean fluorescence values of the isotype control from the mean fluorescence values obtained with the specific antibodies HB54 directed against HLA-A2 molecules. The results represent the mean values and standard deviations from 4 independent experiments.

Overall class I surface expression as measured using W6/32 antibody, showed similar but less pronounced effects (table 3). HLA-A2 mRNA levels remained unchanged after by both selected heat treatments (data not shown. Bernhard Frankenberger, GSF-Institute for molecular Immunology has performed the RT-PCR experiments).

Table 3A: *Thermal isoeffect dose: 41,8°C/120 minutes and sub-sequent recovery at 37°C*

	37°C	**4h recovery**	**15h recovery**	**24h recovery**	**48h recovery**	**72h recovery**
Δ MFI [1] MHC I (W6/32)	427 ± 108	408 ± 166	602 ± 156	706 ± 272	715 ± 234	653 ± 156
Δ MFI HLA-A2 (HB-54)	458 ± 92	354 ±131	437 ± 157	511 ± 165	584 ± 169	669 ± 59

Table 3B: Thermal isoeffect dose: 45°C/22 minutes and sub-sequent recovery at 37°C

	37°C	**4h recovery**	**15h recovery**	**24h recovery**	**48h recovery**	**72h recovery**
Δ MFI [1] MHC I (W6/32)	427 ± 108	333 ± 95	275 ± 64	390 ± 177	463 ± 146	505 ± 75
Δ MFI HLA-A2 (HB-54)	458 ± 92	367 ± 31	385 ± 10	578 ± 109	622 ± 75	620 ± 220

[1] Δ mean fluorescence intensity (MFI) was calculated by substracting the mean fluorescence values of the isotype control (MOPC21) from the mean fluorescence values obtained with the specific antibodies HB54 directed against HLA-A2 molecules or W6/32 directed against MHC class I. MFI of isotype control ranged between 3 and 25. Results represent the mean values of Δ MFI and standard deviations from 4 independent experiments

In addition to flow cytometry analysis, the HLA-A2 expression level was determined by its T-cell immunostimulatory capacity. JB4 recognizes the HLA-A2 protein itself, independently from its bound peptide repertoire and is stimulated to secrete IFN-γ. The relative amount of IFN-γ produced directly correlates with the expression level of HLA-A2.

For the stimulation assays low stimulator to effector ratios were selected (5:1, 2,5:1) to ensure that the response capacity of JB4 was not saturated. Nevertheless, increased surface expression of HLA-A2 did not translate into better T-cell stimulation (compare figure 16 and 15, 48 and 72 hours after a thermal dose of 41,8°C/120 minutes and 45°C/22 minutes). However, less IFN-γ was produced when melanoma cells had reduced HLA-A2 staining (figure 16; 45°C/22 minutes 4 and 15 hours of recovery $p<0,05$, N=2; 24 hours of recovery $p<0,05$, N=6). This indicates that even a moderate decrease in HLA-A2 surface expression is immunologically relevant, at least for the T-cell clone JB4.

Figure 16

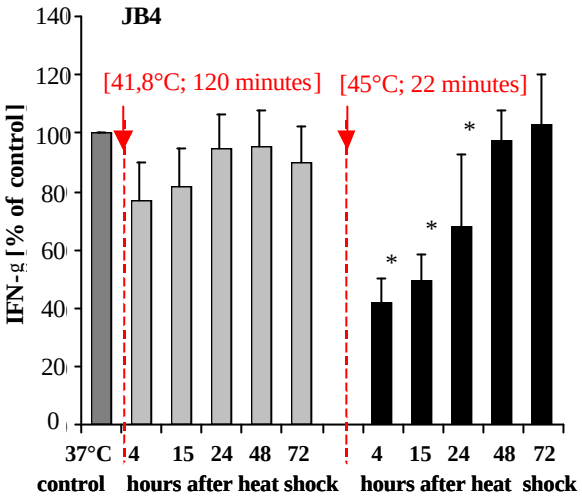


Figure 16: IFN-g secretion by JB4 induced by heat-treated melanoma cells. 624.38-MEL cells were exposed to 41,8°C/120 minutes or 45°C/22 minutes, harvested after 4, 15, 24, 48 and 72 hours of recovery at 37°C and cocultured with the CTL clone JB4. A stimulator to effector cell ratio of 5:1 is shown. Similar results were obtained at a ratio of 2,5:1 (not shown). IFN-γ secretion by JB4 is expressed as percent of IFN-γ secretion relative to control cells grown at 37°C (absolute values for IFN-γ at 37°C at a ratio 5:1 was 703 ± 69 pg/ml for JB4); values represent the mean and standard deviations of 6 independent experiments for the time points 24, 48 and 72 hours and of 2 independent experiments for the time points 4 and 15 hours of recovery at 37°C. * is $p<0,05$ and ** is $p<0,01$.

5.1.4 Thermal dose-related endogenous HLA-A2-restricted tyrosinase and Melan-A/MART-1 peptide presentation

624.38-MEL cells present the tyrosinase peptide tyr $_{368-376}$ (YMNGTMSQV) and the Melan-A/MART-1 peptide (AAGIGILTV) via HLA-A2. The tyrosinase-specific CTL clone TyrF8 and the Melan-A/MART-1-specific CTL A42 were used to test the influence of heat shock on the capacity of 624.38-MEL to process and present peptides derived from the antigen tyrosinase and Melan-A/MART-1 in the context of HLA-A*0201. This was of particular interest since significant changes in tyrosinase expression were observed after heat shock (figure 14). Melanoma cells were subjected to 41,8°C for 120 minutes or 45°C for 22 minutes and were used to stimulate the TyrF8 and A42 T-cell clones at different time points of recovery.

IFN-γ secretion by TyrF8 was decreased when using cells 4 and 15 hours after a heat treatment at 41,8°C/120 minutes ($p<0,05$, N=2). Cells that had recovered for 24 hours regained a stimulating capacity comparable to control cells (37°C) (figure 17A). Melanoma

cells that had been exposed to 45°C for 22 minutes showed a decreased stimulatory capacity for TyrF8 beyond 24 hours ($p<0,01$ for 4 and 15 hours N=2 and $p<0,01$ for 24 hours N=6). The stimulatory capacity was restored 72 hours after heat treatment (figure 17A).

IFN-γ secretion by A42 cells incubated with heat-treated melanoma cells showed a kinetic similar to that of JB4, with a moderate and transient decrease in IFN-γ secretion until 24 hours of recovery after 45°C/22 minutes ($p<0,05$ for 4 and 15 hours N=2 and $p<0,05$ for 24 hours N=6) (figure 17B). Obviously, the cells recovered earlier for stimulation of A42 and JB4 than for TyrF8.

The capacity to stimulate IFN-γ synthesis did not correlate with HSP70 protein levels (figure 13 vs 16 and 17) and did not correlate with tyrosinase or Melan-A/MART-1 protein levels. Indeed, antigen protein levels increased or stayed constant, respectively, during the entire recovery period after the two selected thermal doses (figure 14 vs 16 and 17). The observed reduction in stimulatory capacity at 4 and 15 hours correlated with diminished HLA-A2 surface expression levels as determined by FACS (figure 11 vs 16 and 17).

The IFN-γ profile of TyrF8 is determined by the combination of HLA-A2 protein and tyrosinase mRNA levels. At 4 hours of recovery after both hyperthermic protocols reduced HLA-A2 inhibits normal stimulatory capacity despite significant tyrosinase mRNA levels. After 15 hours of recovery from 45°C/22 minutes treatment HLA-A2 and tyrosinase mRNA were low resulting in low stimulatory capacity. At 24 hours after heat treatment HLA-A2 had recovered to the physiological levels. However the remaining impairment in stimulatory capacity correlated with very low tyrosinase mRNA levels (figure 14, 15 vs 17A).

Figure 17A

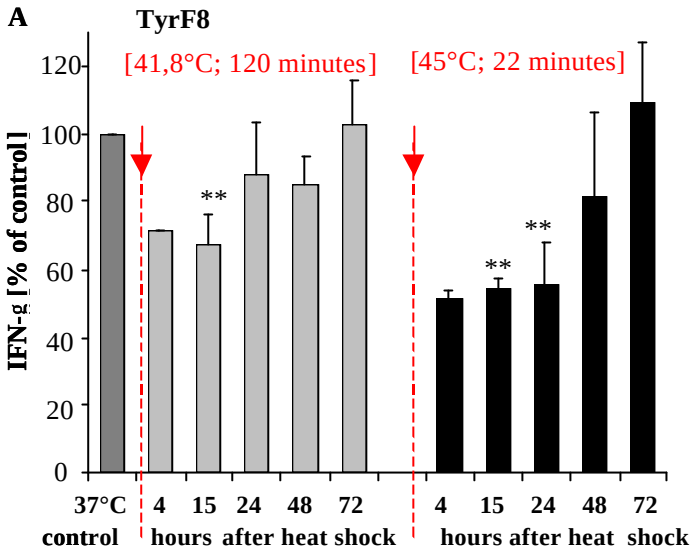


Figure 17B

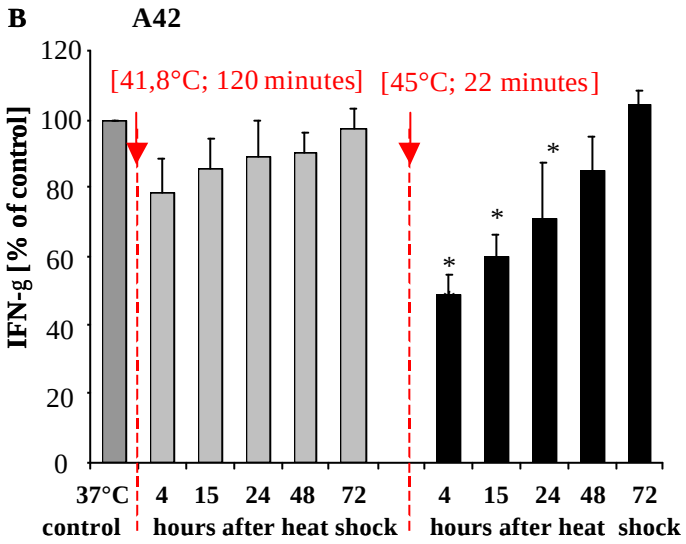


Figure 17: IFN-g secretion by TyrF8 (A) and A42 (B) induced by heat-treated melanoma cells. 624.38-MEL cells were exposed to 41,8°C/120 minutes or 45°C/22 minutes and harvested after 4, 15, 24, 48 and 72 hours of recovery at 37°C. Viable cells were cocultured with the CTL clone TyrF8 (A) and A42 (B). A stimulator to effector cell ratio of 5:1 is shown. Similar results were obtained at a ratio of 2,5:1 (not shown). IFN-γ secretion by TyrF8 and A42 are expressed as percent of IFN-γ secretion relative to control cells grown at 37°C (absolute values for IFN-γ at 37°C at a ratio 5:1 were 477 ± 50 pg/ml for TyrF8 and 1606 ± 100 pg/ml for A42); values represent the mean and standard deviations of 6 independent experiments for the time points 24, 48 and 72 hours and of 2 independent experiments for the time points 4 and 15 hours of recovery at 37°C. * is $p<0,05$ and ** is $p<0,01$.

5.1.5 Thermal dose-related susceptibility of heat treated melanoma cells to cytotoxic effector mechanisms

Using the T cell stimulation assay and measuring IFN-γ produced by antigen specific T cells it was observed that the thermal dose (45°C/22 minutes) that induced downregulation of HLA-A2 and antigen specific mRNA levels, reduced antigen specific/HLA-A2 complexes on the melanoma cell surface. To determine whether the treatment also had an impact on the tumor cell susceptibility to cytotoxic mechanisms, a standard ^{51}Cr-release assay was performed.

After a heat shock of 41,8°C/120 minutes there was no significant change in susceptibility of melanoma cells to lysis by the three T-cell clones JB4, A42 and TyrF8 over 72 hours of recovery, indicating that this thermal dose does not induce resistance to T-cell mediated cytotoxicity (figure 14A, B, C left panel).

The isoeffect thermal dose of 45°C/22 minutes induced temporary resistance to cytotoxic effector cells which lasted through 15 hours of recovery time (figure 18A, B, C right panel; $p<0,01$, N=2).

After 24 hours of recovery the susceptibility of the melanoma cells to lysis by the T cell clones JB4 and A42 was restored to control levels, which correlated with restored HLA-A2 expression in 624.38-MEL cells (figures 15 and 18A and B). For TyrF8, susceptibility to lysis after 24 hours of recovery after 45°C/22 minutes was still below control levels, in accordance with reduced HLA-A2/tyr-peptide ligand as measured by IFN-γ secretion (figure 17A and 18C). After 48 and 72 hours of recovery after heat exposure susceptibility to lysis dropped (with exception of TyrF8). This is in contrast to the stimulatory capacity, to HLA-A2 expression and antigen expression, all of which had returned to control levels by then. This indicates that cytoprotective mechanisms, independent of HLA-class I and antigen expression, occur later (after 24 hours) during the recovery from high temperature heat exposure (45°C/22 minutes), that are not induced by low temperature heat exposure (41,8°C/120 minutes).

Figure 18

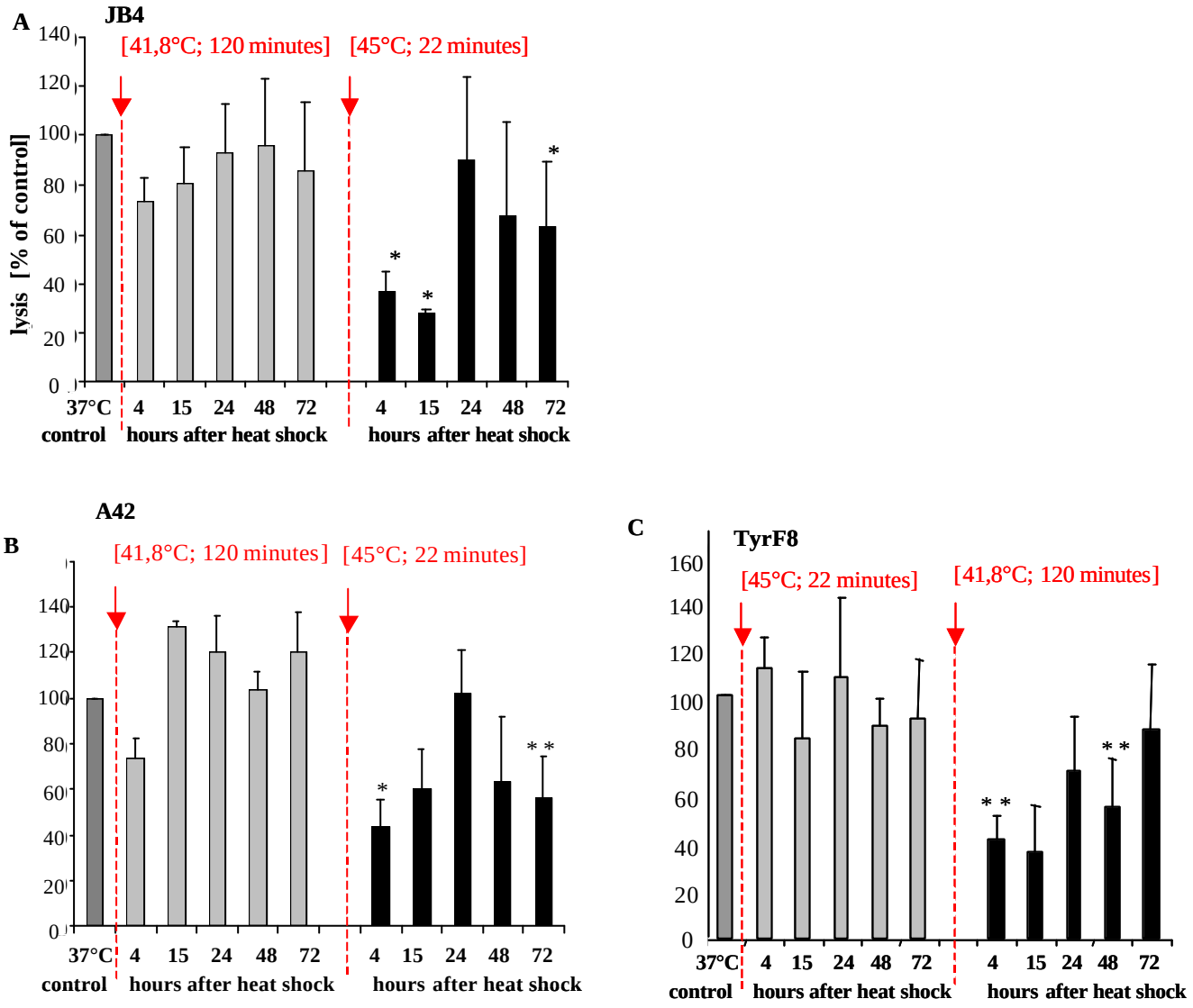


Figure 18: Lysis of heat-treated melanoma cells by the CTL clones JB4 (A), A42 (B) and TyrF8 (C). Melanoma cells treated at 41,8°C for 120 minutes or 45°C for 22 minutes were allowed to recover at 37°C for 4, 15, 24, 48 and 72 hours. At indicated time points adherent, viable cells were harvested and used in a standard 5 hour ^{51}Cr release assay at effector to target cell ratios of 10:1, 5:1 and 2,5:1. Shown are the results of E:T ratio 10:1. Spontaneous chromium release was below 10% for all time points, except at 4 and 15 hours of recovery after both thermal dosages, ranging between 15 and 25%. Lysis values (± SD) were calculated as percent of specific lysis at control temperature of 37°C (absolute value of specific lysis at 37°C was 49% ± 4 for JB4, 19% ± 5 for A42 and 34% ± 12 for TyrF8).

Susceptibility towards other immune effector mechanisms were also analyzed. These included NK cells and lymphokine-activated killer cells (LAK), which are predominantly T cells. The melanoma cell line used is not sensitive to LAK and NK-mediated killing at 37°C and treatment employing the two selected thermal dosages did not impact their susceptibility (data not shown). Fas surface expression of 624.38-MEL cells before and after heat shock was measured by flow cytometry using the antibody APO-1. The levels of Fas surface expression was significantly increased from 24 to 72 hours after a heat exposure of 41,8°C/120 minutes and 45°C/22 minutes. However, apoptosis was not induced when cells were treated with functional anti-CD95 mab (data not shown).

5.2 TUMOR-DERIVED HSP70-PEPTIDE COMPLEXES MEDIATE CROSS-PRESENTATION IN HUMAN DENDRITIC CELLS

The goal of the second part of the study was to investigate the immunological properties of the heat shock protein 70 family members. Once HSPs are released in the extracellular milieu, for example as consequence of necrosis, they acquire immunostimulatory properties.

5.2.1 Purification and characterization of HSP70 from melanoma cell lines

HSP70 was isolated from human melanoma cell lines, 624.38-MEL, SK-mel 23 and A375-MEL (see material and methods) and analyzed by silver staining and western blot (figure 11, see text in methods). It was observed that HSP70 consisted of both the constitutively expressed HSC70 (73 kDa) and the inducible HSP70 (72 kDa) proteins (figure 11, silver staining, lane 8). This composition was found to reflect the natural HSP70 expression pattern of the melanoma cell lines, 624.38-MEL, SK23-MEL and A375-MEL that were used for HSP70 isolation. As demonstrated by intracellular FACS analysis and western blot, these cell lines expressed the constitutive HSC70 and the heat inducible HSP70 isoforms at physiological growth conditions (figure 19).

Figure 19

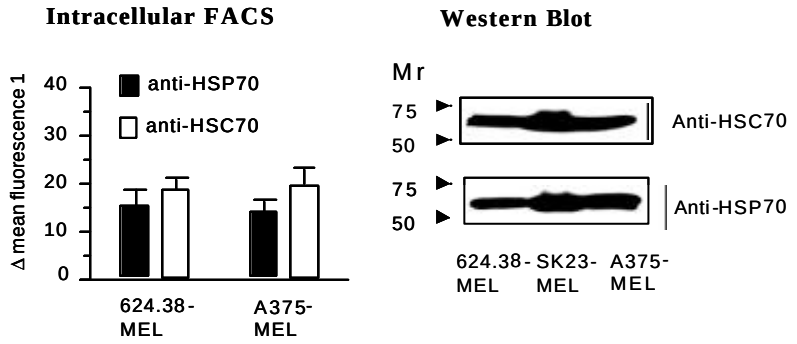


Figure 19. Natural expression (at 37°C) of HSP70, HSC70 and tyrosinase by melanoma cell lines, 624.38-MEL, SK23-MEL and A375-MEL. Intracellular FACS staining was done for 624.38-MEL and A375-MEL filled bars: mAb SPA-810, specific for HSP70; and open bars: mAb SPA-815, specific for HSC70). Rat and mouse isotype control Abs did not show significant staining for both cell lines (mean fluorescence of 1.4 and 1.1, respectively) and were used for reference settings. Depicted on the y-axis is the difference between the mean fluorescence of experimental mAb and isotype control (= mean fluorescence 1). Error bars indicate s.e.m. Expression of HSC70, HSP70 and tyrosinase was also analysed by western blotting. Gels were run in parallel and probed with Abs to HSC70 (SPA-815), HSP70 (HSP-6B3) and tyrosinase (C-19).

5.2.2 DCs bind and uptake tumor-derived HSP70-PC

The binding of HSP70 to DCs and early downstream consequences of binding were analyzed by confocal laser scanning microscopy. Immature DCs were incubated for 30 min with Cy5-conjugated HSP70 at 4°C to exclude endocytosis, or at 37°C to induce uptake. Cy5-labeled BSA was used as a negative control. After staining, cells were settled on poly-L-lysine coated glass slides, fixed and analyzed for transmission and fluorescence. BSA did not result in detectable staining of DCs (figure 20: c, d, g, h, k, l), while all DCs were stained strongly positive with HSP70 (figure 20: a, b, e, f, i, j). Different staining patterns were observed at 4°C and 37°C. At 4°C (figure 20: a, b, e, f), the fluorescence signal was localized to the cell surface. In contrast, at 37°C (figure 20: i, j) this surface staining was replaced by a vesicular staining at two distinct subcellular locations. Fluorescent signals were localized to perinuclear areas and clusters of focal staining near the cell surface, presumably early endosomes.

Figure 20

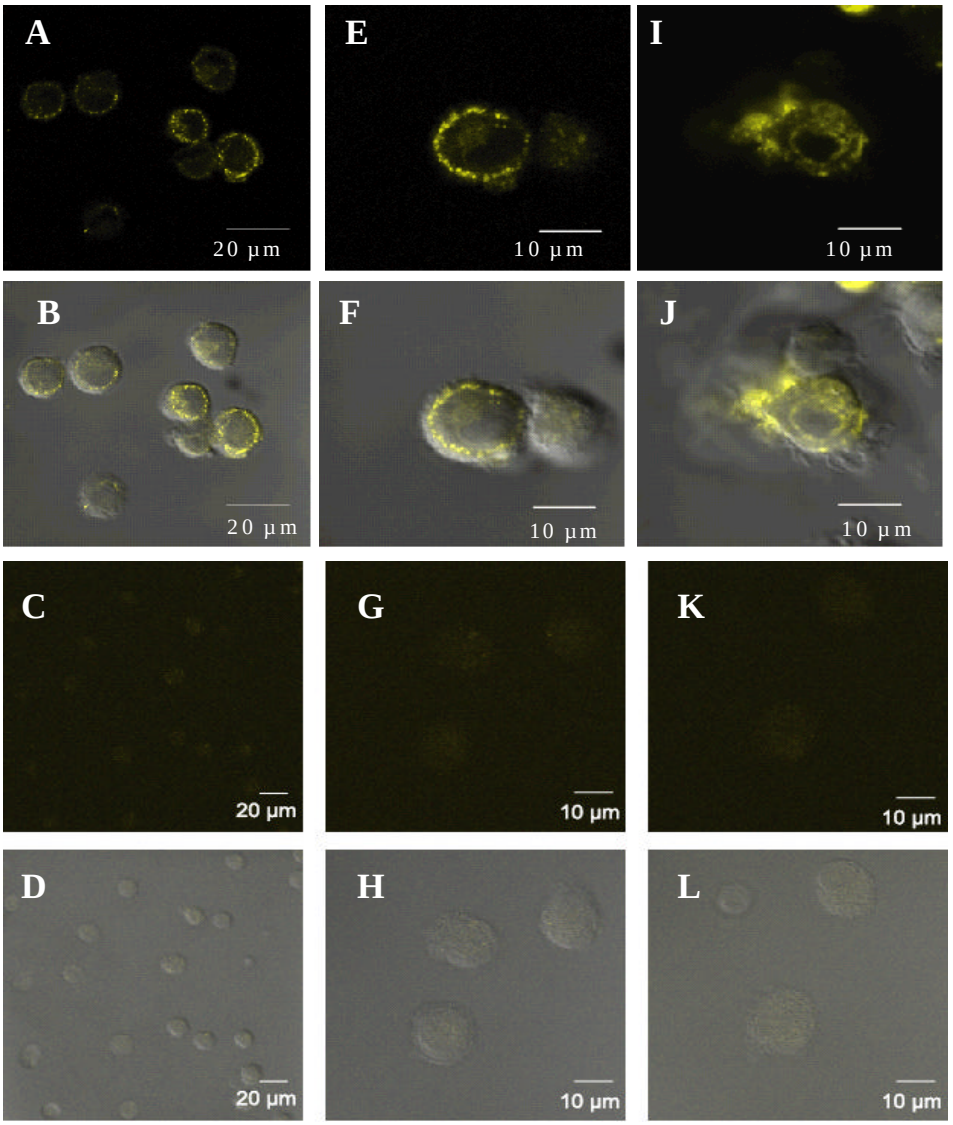


Figure 20. Receptor-dependent binding of HSP70-PC, uptake and active cell metabolism are required for T cell stimulation. (A) Confocal microscopy of immature DCs stained with Cy5-labeled HSP70-PC or Cy5-labeld BSA at 4°C (surface binding, a-h) and 37°C (uptake, i-l). Cy5-labeled BSA was used for control staining (c, d, g, h, k, l). Cells were analysed for fluorescence (shown in yellow) and transmission (shown as overlay with the fluorescence signal). Scale bars indicate respective magnifications. Panels a-d represent an overview for the staining at 4°C demonstrating that all cells stain positive for Cy5-HSP70-PC and none are positive for Cy5-BSA. For presentation purpose fluorescence images for Cy5-BSA stainings (panels c, d, g, h, k, l) are digitally enhanced twice to allow detection of residual fluorescence. Individual cells are depicted at higher magnification (panels e-l) to visualize discrete staining patterns at 4°C (surface) and 37°C (perinuclear, vesicular) (see text)

5.2.3 HSP70 from melanoma cells chaperone the tyrosinase peptide and delivers it to DCs for MHC-I-restricted cross-presentation

From physiologic properties as chaperones, HSPs are thought to exist as peptide complexes. It was postulated that these peptides lead to antigen-specific T-cell stimulation. To determine whether HSP70 isolated from melanoma cells carry melanoma-associated peptides, HSP70 isolated from two tyrosinase-positive (HSP70-PC/tyr+) and tyrosinase-negative (HSP70-PC/tyr-) melanoma cell lines, 624.38-MEL, SK23-MEL and A375-MEL, respectively (Riker *et al.* 2000) (Rivoltini *et al.* 1995) (figure 21A) were incubated with immature DCs from HLA-A*0201-positive donors *in vitro*. If HSP are HSP-PC, than it was expected that antigens bound are transferred to DC, which in turn may present them via their MHC class I. MHC I/tyr-peptide complexes which can occur on DC surface can be detected with the tyrosinase-specific CTL clone, TyrF8.

The experimental procedure was incubation of DC with HSP70-PC, treatement of DC with TNF-α to induce maturation and to switch function from antigen uptake and processing to presentation. Pulsed DCs were then incubated with TyrF8. If MHC I/Tyr-peptide complexes are present than TyrF8 is stimulated to secrete IFN-γ, which can be measured by ELISA.

The results showed that DCs pulsed with HSP70-PC/tyr+ stimulated TyrF8 to secrete IFN-γ in an HSP70-PC dose-dependent manner (figure 21A, filled squares). IFN-γ secretion reached a plateau at 600 ng/ml, with a half-maximal value (Y_{50}) of IFN-γ achieved at 74.76 ng/ml of HSP70-PC/tyr+. The amount of IFN-γ secreted was comparable to the maximum stimulation obtained using the HLA-A*0201- and tyrosinase-positive melanoma cell line 624.38-MEL and SK23-MEL (data not shown). TyrF8 activation was dependent on the HSP70 source. HSP70 purified from melanoma cells not expressing the tyrosinase antigen (HSP70-PC/tyr-) were unable to stimulate TyrF8 (figure 21A, open squares). IFN-γ secretion was further dependent on the interaction of both DCs and T cells, since neither DCs alone (figure 21B) nor T cells alone (figure 21C) incubated with HSP70 secreted IFN-γ.

In conclusion, HSP70 transfer antigen specificity of the tumor cells from which they are isolated to DC. HSP reflect the antigenic spectrum of the tumor cell since only HSP70 from tyrosinase-positive cells transfer tyrosinase to DC.

Figure 21

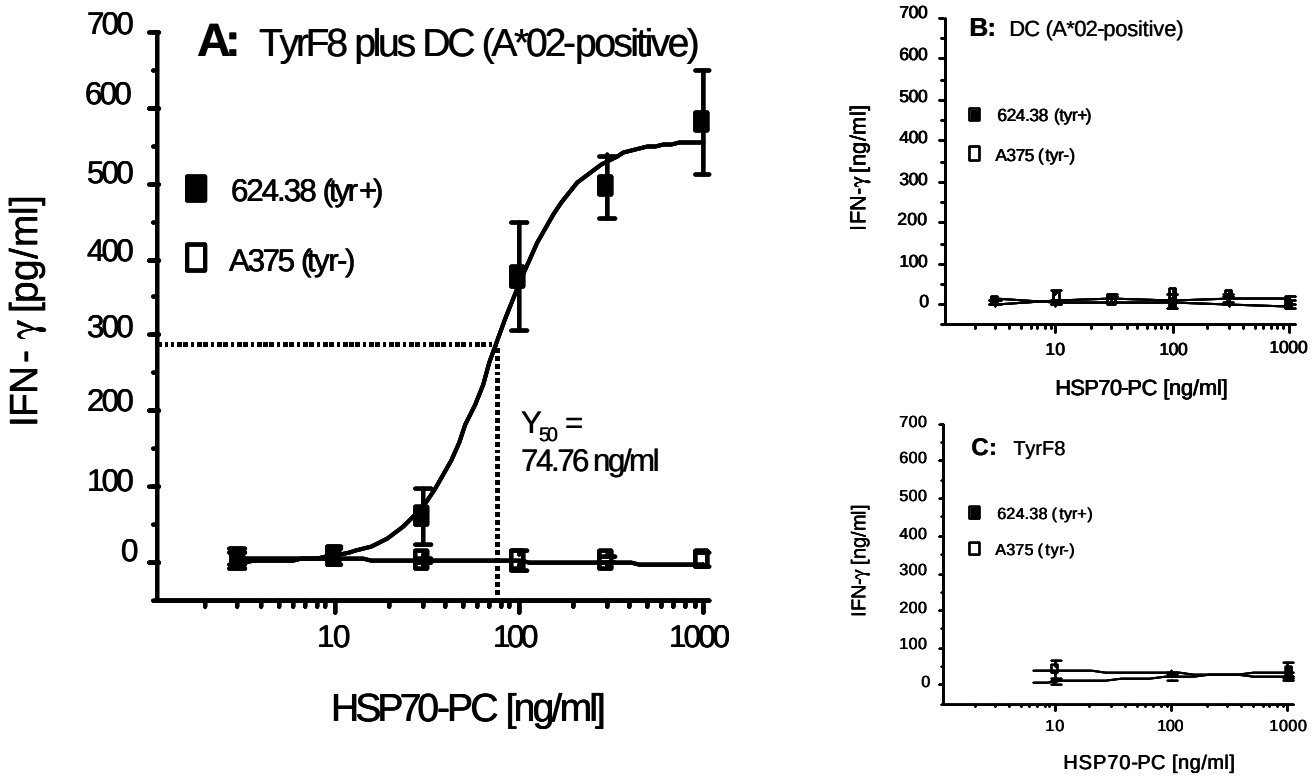


Figure 21: **HSP70 from melanoma cells chaperone the tyrosinase peptide for MHC class I-restricted cross-presentation by human DCs**. **A)** Immature DCs (HLA-A*02-positive) were incubated with indicated amounts of HSP70-PC/tyr+ (from 624.38-MEL cells, filled squares) or HSP70-PC/tyr- (A375-MEL cells, open squares), matured with exogenous TNF-α and co-cultured with the HLA-A2-restricted tyrosinase-specific T cell clone TyrF8. Antigen-specific stimulation of TyrF8 is demonstrated by the amount of IFN-γ secreted. Y_{50} indicates the amount of HSP70-PC/tyr+ (74,76 ng/ml) required for half-maximal stimulation of TyrF8. T cell stimulation requires co-culture of T cells with HSP70-PC loaded DCs. **B)** Neither DCs alone **C)** nor TyrF8 alone secrete IFN-γ after incubation with HSP70-PC/tyr+ (filled squares) or HSP70-PC/tyr- (open squares). Five independent preparations of the HSP70-PC from the tyrosinase-positive 624.38 cell line and one preparation of tyrosinase-positive cell line SK23-MEL (not shown) were tested for cross-presentation. All preparations were tested repeatedly and induced IFN-γ secretion with experimental variations ranging between 500 pg/ml and 80 pg/ml of IFN-γ at 100 ng/ml of HSP70-PC (not shown). The tyrosinase-negative cell line A375-MEL and tyrosinase-negative B-LCL were also repeatedly tested and never found to induce significant amounts of IFN-γ (not shown). Variations in dose-dependency of individual HSP70-PC preparations from tyrosinase-positive melanoma cell lines might be related to different purities of the preparations. As determined by silver staining, some HSP70-PC preparations contain other, yet undefined proteins, different from HSC70 or HSP70 (not shown).

5.2.4 The HSP70-PC mediated cross-presentation of tyrosinase peptide is MHC-I-restricted

To confirm that the stimulation of TyrF8 by DCs pulsed with HSP70-PC from tyrosinase-positive melanoma cells was indeed specific and HLA-A*02-dependent, we performed blocking experiments using the anti-HLA-A*02-specific antibody HB54 (see materials). The release of IFN-γ by TyrF8 was inhibited by pre-incubation of HSP70-PC-pulsed DCs with HB54 ($p<0.05$ at 10 ng/ml of HSP70-PC/tyr+ and $p<0.005$ at 100 ng/ml of HSP70-PC /tyr+) (figure 22).

Figure 22

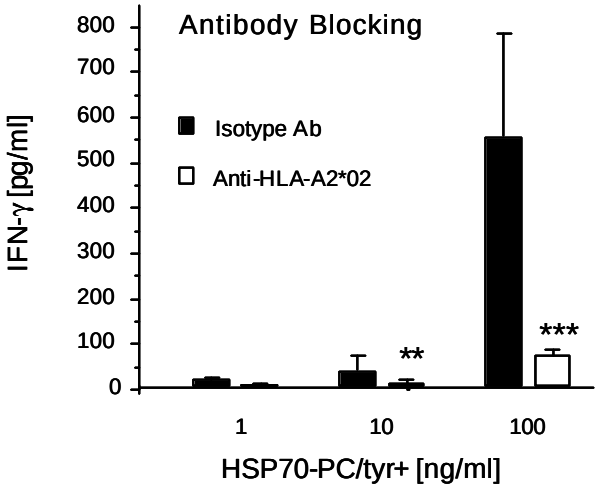


Figure 22: Stimulation of TyrF8 by HSP70-PC-loaded DCs is HLA-A*02-restricted. IFN-γ secretion of TyrF8 was measured after co-culture with HSP70-PC/tyr+ (from 624.38-MEL)-loaded DCs in the absence (filled bars) or presence (open bars) of anti-HLA-A2 antibody (HB54). p values were calculated for all data, comparing IFN-γ values in the presence of HB54 to that in the absence of HB54. ** and *** represent $p<0.05$ and $p<0.005$, respectively. Data represent the mean IFN-γ concentration in pg/ml (mean ± s.e.m.) of results from four independent experiments.

5.2.5 Active cell metabolism is required for cross-presentation of chaperoned peptides and T cell stimulation

Staining patterns of DCs incubated with HSP70 at 37°C and at 4°C suggested that HSP70-PC after binding to the cell surface was translocated into the cell interior. This suggests that the bound peptides might enter the MHC I loading pathway within the cells and not through loading on the cell surface MHC. If peptides access via uptake of HSP the MHC I intracellular pathway, fixation of DC should abolish this pathway, while peptide loading to

surface MHC is still possible. To prove that uptake is a process requiring active cell metabolism, immature DCs were fixed with paraformaldehyde (PFA) before being used in cross-presentation assays. PFA-fixation completely abrogated T cell stimulation while exogenously added tyrosinase peptide was still efficiently presented (figure 23).

Figure 23

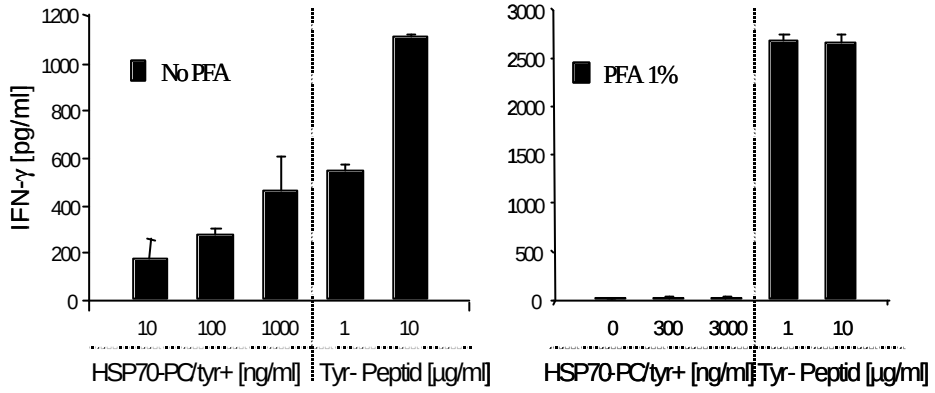


Figure 23: PFA-fixation of DCs abrogates T cell stimulation. DCs fixed with PFA (1% for 10 min at room temperature) were unable to perform HSP70-PC-mediated cross-presentation, but retained the ability to present exogenously added tyrosinase peptide (left panel). Untreated DCs are shown in the left panel.

5.2.6 HSP70-PC-dependent cross-presentation and T cell stimulation do not require additional external DC maturation signals

In general, immature DCs for cross-presentation assays were used because they demonstrated strongest binding for HSP70-PC and because they are highly efficient in antigen uptake and processing (Banchereau *et al.* 2000). Based on the rationale that for the process of T cell stimulation mature DCs are the most efficient, TNF-α was added exogenously after HSP70-PC had bound to immature DCs. In the meantime, news results indicated that rh-HSP70 stimulates secretion of inflammatory cytokines, including TNF-α, from monocytes and DCs (Asea *et al.* 2000) (Asea *et al.* 2002; Vabulas *et al.* 2002) and induces maturation of DCs (Todryk *et al.* 1999; Kuppner *et al.* 2001; Somersan *et al.* 2001). Therefore, it was reasoned that DCs through binding HSP70-PC might be stimulated to release TNF-α and induce an autocrine maturation loop. To investigate whether DCs after incubation with HSP70-PC need exogenous TNF-α for efficient cross-presentation immature DCs were incubated with HSP70-PC/tyr+ and either left them untreated (intrinsic DC maturation by HSP70-PC) or

gave TNF-α (external DC maturation) before addition of the T cells. As shown in Figure 24, cross-presentation by HSP70-PC-treated DCs without exogenously added TNF-α was even stronger than that with additional TNF-α. ($p<0,005$). Polymyxin B, a potent inhibitor of LPS, was included in the cross-presentation assay. No inhibitory effect on IFN-γ secretion by the T cells was observed ruling out the possibility that endotoxin contamination within the HSP70-PC preparations was responsible for DC maturation and their ability to efficiently stimulate the T cells. Immature DCs treated with HSP70-PC only consistently performed better in antigen-specific T cell stimulation assay.

Figure 24

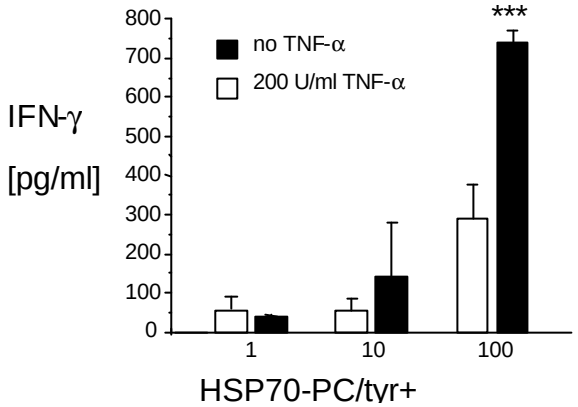


Figure 24. HSP70-PC-mediated cross-presentation and T cell stimulation do not require external (*i.e.* TNF-α-induced) DC maturation. Immature DCs were incubated with indicated amounts of HSP70-PC/tyr+ (from 624.38-MEL) and TNF-α was given (external DC maturation signal) (open squares) or was omitted (intrinsic, HSP70-mediated, DC maturation) (filled squares) before the addition of T cells. Polymyxin B was present in all reactions throughout the cross-presentation assay. TyrF8 stimulation was determined by measuring the amount of secreted IFN-γ. p values were calculated from data comparing IFN-γ values obtained with TNF-α to that without TNF-α. ***, $p<0.005$.

6 DISCUSSION

The goal of this study was to investigate the function of the tumor-expressed heat shock protein 70 family members and to dissect their role in tumor immune recognition as a function of intra- versus extracellular location. Another goal was to investigate whether heat-treatment at clinically relevant thermal doses affects the immunophenotype of a given tumor, as defined by tumor cell sensitivity to immune effector cells. In fact it has been observed that tumor cells surviving heat-treatment acquire survival advantage and resistance against subsequent stress. This transient resistance has been associated with the induction of intracellular heat shock proteins that exert a cytoprotective role. Other studies however, did not observe those effects and some even reported increased susceptibility. To understand these contradictory findings at a mechanistical level, it is necessary to elucidate when and in what way induced HSPs interfere with the immunorecognition of heated tumors.

6.1 EFFECTS OF HYPERTHERMIA TREATMENT ON TUMOR ANTIGENICITY AND SUSCEPTIBILITY TO IMMUNE EFFECTOR MECHANISMS

The first part of this study thus addresses the concern that heat shock treatment of tumor cells may reduce the presence of tumor antigens, due to heat shock-related reduced transcription, translation or protein degradation (Lindquist 1986) (Welch *et al.* 1986), enabling tumor cells to escape immune recognition and immune surveillance. It is already known that during the progression of malignant melanoma, changes in antigenic profile occur with concordant loss of multiple melanocyte melanocytic differentiation proteins (Slingluff *et al.* 2000).

Two doses were selected, which resulted in equivalent clonogenic survival rate and mimic those achieved during clinical hyperthermia treatment of tumors. The time-temperature dependent effects on tumor antigen expression, processing and presentation by MHC class I and on the susceptibility of heat treated cells to antigen specific cytotoxic T cells, NK and LAK cells was specifically emphasized.

The results of this study were that during the heat shock response 1) a dissociation between tyrosinase protein and mRNA level occured with a significant increase in tyrosinase protein and a parallel decrease in its transcripts. No changes were observed for Melan-A/MART-1. 2) T cell recognition did not correlate to changes in antigen protein expression level; 3) Melanoma cells maintained the ability to be recognized and to be killed by antigen specific CTL clones during the heat shock response after a heat shock of 41,8°C/120 minutes and this ability was only transiently decreased after an initial high temperature (45°C/22 minutes).

The kinetics and the degree of induction of HSP70 as well as the degree of dissociation between tyrosinase protein level and tyrosinase mRNA level correlated with the severity of the initial stress (results: figure 13 and 14A vs 14C), demonstrating that these two different thermal doses have differential longterm biological effects, even if they have the same clonogenic survival ability. This principle recurred in all the results.

Since tyrosinase protein levels did not correlate with its transcript levels, it was reasoned that the mechanism by which tyrosinase protein increased, was a heat-dependent decrease in the degradation rate. In amelanotic melanoma cells steady-state tyrosinase (37°C) was found to accumulate in the ER; through retrograde translocation from ER into the cytoplasm, tyrosinase becomes then a target for proteasome-dependent proteolytic degradation (Halaban *et al.* 1997; Mosse *et al.* 2001). Furthermore it is known that heat shock blocks the translocation of proteins from the ER into the cytosol. This results in ER retention and reduced degradation (VanSlyke *et al.* 2002) providing an explanation for the increased tyrosinase level that was observed in the study (figure 25).

Figure 25

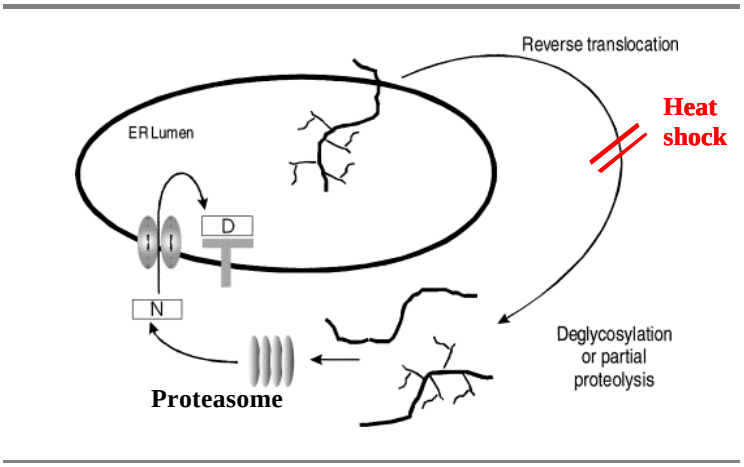


Figure 25: The ER retention model. In melanoma cells steady-state tyrosinase (37°C) accumulates in the ER as a 70-kDa high mannose glycoform and is translocated into the cytoplasm for degradation in the cytosol; it is known that heat shock blocks this reverse traslocation, resulting in a stronger ER retention (Engelhard *et al.* 2002).

At transcriptional level, it is known that severe heat shock blocks RNA splicing and represses normal transcription (Yost *et al.* 1990) providing an explanation for the observed reduction in transcript levels after 15 and 24 hours of recovery after heating melanoma cells to 45°C for 22 minutes. After a mild heat shock RNA splicing is protected and mature mRNA can

accumulate (Yost *et al.* 1990) which was reflected by the unaltered level of tyrosinase transcripts after a heat shock of 41,8°C/120 minutes.

Surprisingly, no changes in mRNA and protein levels were found for Melan-A/MART-1 (results: figure 14 B) and HLA-A2. This indicates that not all genes are equally affected. The differences may be a reflection of house-keeping genes and constitutive expressed genes which are essential for cell survival and may reflect an antigen dominance within the cell.

The heat-induced quantitative changes of surface expression of MHC class I and allotype HLA-A2 were also a function of the initial thermal dose and of the time of recovery after heat exposure. Consistent with previous observations that showed an increase in MHC class I surface expression 24 and 48 hours after a heat shock of 43°C/60 minutes (cytostatic, sub-lethal) (Ito *et al.* 2001) and after 5 days at 39°C (Aboud *et al.* 1992), this study showes an increase in surface expression of MHC class I and HLA-A2 molecules after a heat shock of 41,8°C/120 minutes. As mRNA levels of HLA-A2 remained constant over time (not shown), these changes appear to be translationally or post-translationally regulated, as already suggested by others (Aboud *et al.* 1992). The transient decrease of MHC Class I surface expression observed 4 and 15 hours after exposing melanoma cells to 45°C/22 minutes was also in agreement with other studies (Blom *et al.* 1997; Dressel *et al.* 2000). The transient nature of the decrease however has not been described sofar. Indeed surface expression is restored already at 24 hours of recovery at 37°C after heat shock (results: figure 15 and table 3).

In the B16 mouse model Wells *et al.* (Wells *et al.* 1998) correlated the augmentations of MHC class I with the level of HSP70 and they observed better antigen presentation. Our results did not show a correlation between heat-induced HSP70 overexpression and the increase in MHC class I or HLA-A2 surface expression. Neither did we observe that increase MHC class I surface expression lead to better antigen presentation. Indeed, in our system antigen presentation either remained unchanged (41,8°C/120 minutes) or was transiently decreased (45°C/22 minutes) despite an increase in HSP70 expression. One explanation could be that the basal level and regulation of both HSP70 and MHC class I are substantially different in the mouse and human system. B16 cells have very low basal levels of HSP70 and MHC class I and are not efficiently recognized by class I-restricted CTL. Induced overexpression of HSP70 after transfection may generate B16 cell clones with higher class I expression and better CTL recognition. The human melanoma cell line 624.38-MEL already expressed very high levels of MHC class I and HLA-A2 (Rivoltini *et al.* 1995) and showed detectable amounts of the inducible isoform HSP70 at 37°C (results: figure 13A). Increased

HLA-A2 surface expression did not lead to increased T cell stimulation, as it might have reached a saturation of HLA-A2/peptide complexes on the cell surface. On the contrary a slight decrease of HLA-A2 lead to a significantly reduced T cell stimulation. In studying the capability to stimulate the tyrosinase specific CTL clone it was observed that the lysability response pattern was determined by the combination of HLA-A2 and tyrosinase mRNA, supporting the notion that expression of melanoma-associated antigens is a significant co-factor in addition to MHC class I in determining recognition of melanoma targets (Cormier *et al.* 1999). The observation that mRNA levels and not protein levels of the antigen tyrosinase impacts specific T cell recognition is in accordance with recent publications (Kayser *et al.* 2003) and supports the DRiPs (defective ribosomal products) hypothesis (Yewdell *et al.* 1996; Schubert *et al.* 2000). According to Yewdell *et al*. (Yewdell *et al.* 1996) antigenic peptides may not derive from the native proteins, and are not proportional to the relative abundance of this protein.

While the role of heat shock and of HSP70 overexpression in protecting against T-cell mediated lysis is quite controversial, there is a general agreement that heat treatment can increase the susceptibility to LAK and NK cells (Jaattela 1990) (Multhoff *et al.* 1997) (Fujieda *et al.* 1995) (Kubista *et al.* 2002). Our melanoma cell line 624.38-MEL was not susceptible to LAK and NK cells and to Fas-triggered apoptosis irrespective of heat shock treatment (not shown), but was susceptible to cytotoxic CTL clones before and after heat shock. As a function of the initial thermal dose and of the time of recovery after heat treatment, the degree of susceptibility to lysis changed moderately. An early resistance to CTL lysis was observed until 24 hours after a heat shock of 45°C/22 minutes. This transient resistance was due to a failure in sufficient antigen presentation, as evident in the concordant diminished in IFN-γ stimulation. However, when HLA-A/peptide complexes were restored on the cell surface at 48 and 72 hours after heat exposure, a novel late resistance was detected. With the exception of TyrF8, this late induced resistance may be explained by the very high expression of HSP70 (almost 15-fold increase), since overexpression of HSP70 has been shown to result in resistance to monocytes, cytokines like TNF-α and apoptosis (Sugawara *et al.* 1990; Geginat *et al.* 1993; Jaattela *et al.* 1993) (Jaattela 1990; Kusher *et al.* 1990; Jaattela 1995; Van Molle *et al.* 2002).

6.1.1 Relevance of the first study

The goal of clinical hyperthermia to reach temperatures which cause cell death is usually not achieved homogeneously throughout the tumor. *In situ* an heterogenous distribution of

temperatures is observed, which ranges between 40°C and 44°C. Virtually, the major parts of the tumor tissue might be only heated at sub-lethal temperatures. Therefore, the observation that tumor cells treated with temperatures below the breakpoint temperature maintain an immunological homeostasis during the heat shock response, is of critical importance for the clinical application of hyperthermia in the treatment of such tumors. Moreover, the observation that important dominant antigens are overexpressed during the heat shock response may open a new window of heat application to potentiate antigen expression.

6.2 TUMOR-DERIVED HEAT SHOCK PROTEIN 70-PEPTIDE COMPLEXES ARE CROSS-PRESENTED BY HUMAN DENDRITIC CELLS

The second part of my study addressed the immunological competence of heat shock proteins to induce antitumor immunity. In the last years HSPs have been proposed as a tool for cancer therapy. They can function as tumor associated activation structures (*i.e.* HSP70), if detected on the surface of tumor cells, thereby activating NK cells (Multhoff *et al.* 1997) (Multhoff *et al.* 1999) or as antigen-presenting molecules, eliciting a specific T cell response through peptides associated in HSP-peptide complexes (HSP-PC) (Srivastava *et al.* 1994; Tamura *et al.* 1997; Janetzki *et al.* 2000; Srivastava 2000; Srivastava 2002).

Using HSP70-PC purified from tyrosinase-positive and tyrosinase-negative melanoma cell lines and by focusing on the nature of the APCs that mediate cross-presentation as well as the biochemical composition of the tumor-derived HSP70-PC it was possible to demonstrate that the immunogenic potential of HSP70 as a tool to induce anti-tumor immune responses can be extended to naturally expressed, non-mutated human tumor antigens of low immunogenicity (Sette *et al.* 1994). Binding of HSP70-PC to DCs and intracellular events are required for HSP70-mediated cross-presentation. Although HSP70-PC bound to immature and mature DCs with similar intensity, immature DCs were more efficient in cross-presentation than mature DCs (not shown). This makes sense from an immunological point of view, since immature DCs are better in antigen uptake than monocytes or mature DCs and have a strong capacity to process antigen (Banchereau *et al.* 2000). Antigen processing ability is potentially useful if peptides bind to HSP70 as longer precursors.

These results demonstrated further that HSP-PC-mediated cross-presentation by immature DCs does not require external maturation signals, such as TNF-α. This finding is consistent with previous observations that HSPs represent natural danger signals to the immune system. When released by stressed cells (Srivastava *et al.* 1998) (Chen *et al.* 1999) they stimulate monocytes and dendritic cells to secrete proinflammatory cytokines (TNF-α, IL-12) (Asea *et*

al. 2000) (Asea *et al.* 2002; Vabulas *et al.* 2002), and are maturation signals for immature DCs (Somersan *et al.* 2001) (Singh-Jasuja *et al.* 2000; Kuppner *et al.* 2001) (Todryk *et al.* 1999) (Binder *et al.* 2000). The results demonstrating efficient cross-presentation without external DC maturation signals indicate that the two properties – the chaperoning of antigenic peptides and the induction of DC maturation - are intimately linked within tumor-derived HSP70-preparations (figure 26).

Figure 26

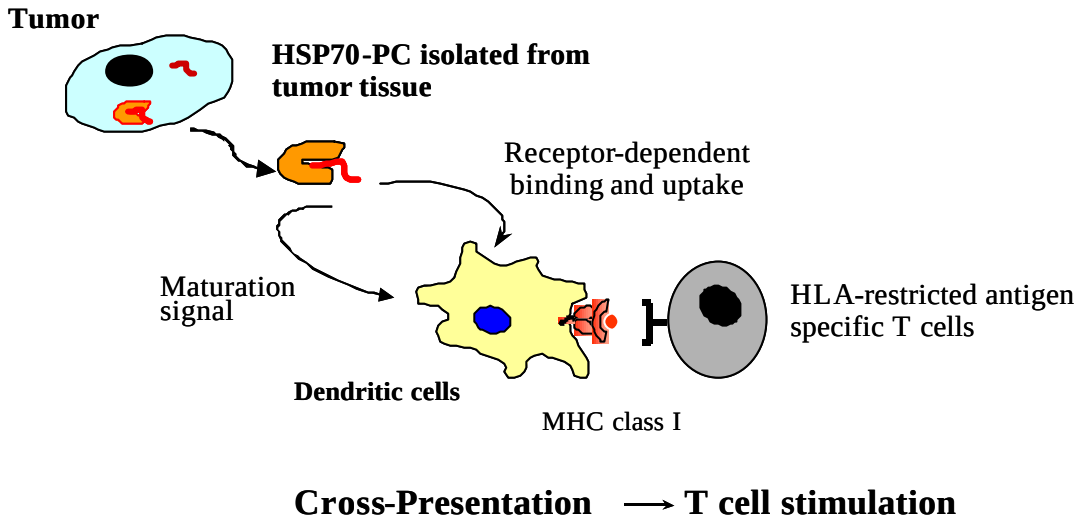


Figure 26: Dual function of HSP70 as a chaperone for tumor-derived T cell epitopes and as a signal for DC maturation. HSP70 isolated from tissues or cell lines exist as peptide complexes. By binding to DCs the peptide cargo is delivered to MHC class I molecules (cross-presentation) and presented on the cell surface for recognition by T cells. In parallel receptor-mediated binding induces DCs to mature into efficient antigen presenting cells. Therefore, T cells that recognize the MHC class I peptide-complex also receive co-stimulation and are optimally primed to evolve into effector cells. (Milani *et al.* 2002)

A similar conclusion can be reached for gp96 (Zheng *et al.* 2001). The significance for the clinical use of tumor-derived HSP-preparations in stimulating anti-tumor immune responses is discussed below.

The biochemical analysis of HSP70 preparations revealed that they consisted of both the constitutively expressed HSC70 and the inducible HSP70. This was found to reflect the natural expression pattern of HSP70 and HSC70 in the melanoma cell lines used for HSP70-PC isolation. Similar constitutive expression of HSP70 has also been described for surgical specimens of primary and metastatic human melanoma (Protti *et al.* 1994). Our previous findings that rh-HSP70 but not r-HSC70 is able to deliver the DC maturation signal (Kuppner

et al. 2001) indicate that the heterogeneous composition of tumor-derived HSP70-PC might be of functional relevance for the cross-presentation.

The ability to cross-present HSP-bound antigen has also been shown for APCs other than DCs, including monocytes (Castelli *et al.* 2001) and macrophages (Heath *et al.* 2001). Using blood monocytes, Castelli et al. (Castelli *et al.* 2001) demonstrated HSP70-mediated cross-presentation for melanoma antigens other than tyrosinase. However, in their system a much higher number of APCs and significantly more HSP70-PC were required for T cell stimulation than in our system. Possibly the poor binding of HSP70 to monocytes is one explanation for this difference. The studies presented here are of special clinical interest for HSP70-based vaccinations. *(i)* HSP70-PC-mediated antigen presentation by DCs is very efficient requiring low amounts (in the nanogram range) of HSP70-PC (results: figure 21) (Srivastava 2000). This can be explained by the receptor-mediated uptake for HSP70-PC versus fluid phase uptake and surface peptide exchange mechanisms for exogenous peptides, respectively. In addition, Binder et al. (Binder *et al.* 2001) described that HSP70 positively influences cross-presentation of chaperoned peptides by efficiently directing them to an ER/Golgi compartment where loading onto the MHC class I molecules occurs. The observation that an ER/Golgi localization is important for efficient cross-presentation is consistent with the confocal microscopic data that show a perinuclear staining of immature DCs incubated with Cy5-labeled HSP70-PC at 37°C (results: figure 20). An additional explanation for the efficacy of HSP-preparations to induce anti-tumor immune responses might be related to the dual function of HSPs delivering antigen and inducing DC maturation. Linking these two properties is one possibility to ensure that antigen presentation occurs in an environment optimal for T cell stimulation. *(ii)* Initial vaccination studies using murine tumor models demonstrated that the anti-tumor immunity achieved by vaccination with gp96 or HSP70 preparations is restricted to the tumor from which the gp96/HSP70 was isolated (Srivastava *et al.* 1994) (Srivastava 2000). HSP-based vaccination strategies are therefore considered patient-individual treatment modalities. This view is challenged by our observation, and that of Castelli et al. (Castelli *et al.* 2001), demonstrating that HSP70 isolated from human melanoma cells chaperone naturally expressed non-mutated and shared human melanoma antigens and transfer them to APCs for T cell recognition. If cross-presentation of shared human tumor antigens by HSP70-PC followed by efficient T cell stimulation is routinely achieved with HSP70-PC the clinical application of HSP70-based vaccines may be extended from a patient-individual treatment to use in an allogeneic vaccination setting. *(iii)* This novel insight into the mechanistic events responsible for HSP70-

mediated cross-presentation by DCs are of additional interest for treatments involving hyperthermia.

6.3 WORKING HYPOTHESIS OF HYPERTHERMIA

The biological rationale for the use of hyperthermia in the treatment of cancer is based on the observation that temperatures above 42°C are cytotoxic to tumor cells as a function of time. Cytotoxicity is high for radioresistant S-phase cells and for cells nutritionally deprived and acidotic (in the region of tumor with many radioresistant hypoxic cells). Heat has also been shown to be a radiosensitizer and a chemosensitizer (Stewart *et al.* 1984).

Hyperthermia was found to be effective in clinical trials if integrated in a loco-regional treatment strategy for certain solid tumors, like melanoma (Overgaard *et al.* 1995), soft tissue sarcomas and tumors of the bone, cervix and rectum, head and neck cancers as an adjuvant to radiation and chemotherapy (Falk *et al.* 2001). Besides its direct cytotoxic effect on tumor cells, immunologically relevant changes in tumor cell physiology occur after heat shock treatment. Based upon the observations that HSP70-peptide complexes chaperone antigenic peptides and deliver them to DCs in an immunogenic way resulting in efficient T-cell stimulation, and that the tumor itself remains susceptible to immune effector mechanisms during the heat shock response, the following chain of events may be proposed (figure 27).

HSP70 can be expressed on the cell surface of some tumor cells and act as an activator of NK cells (Multhoff *et al.* 1997) (Multhoff *et al.* 1995; Multhoff *et al.* 1996; Multhoff *et al.* 1999). During clinical hyperthermia peak temperatures may reach up to 42°C inducing HSP70 upregulation (Fuller *et al.* 1994). Within this temperature range local necrosis occurs resulting in the release of HSPs (Melcher *et al.* 1998) (Basu *et al.* 2000), uptake by DCs and subsequent processing and presentation of associated peptides (Todryk *et al.* 1999). Surviving tumor cells during the heat shock response maintain the ability to be recognized and killed by immune effector mechanisms. Since HSP70-mediated cross-presentation is efficient without additional DC maturation signals an environment optimal for T cell stimulation is ensured. The proposed dual role of HSP70 as a chaperone for antigenic peptides and as a signal for DC maturation then leads to efficient priming of circulating T cells. Therefore, upregulating HSP70 expression or other stress proteins (*e.g.* HSP110 and grp170) (Wang *et al.* 2001) and causing local necrosis (Melcher *et al.* 1998) by heating the tumor tissue has the potential to directly activate the immune system against tumors ("vaccination in situ").

Figure 27

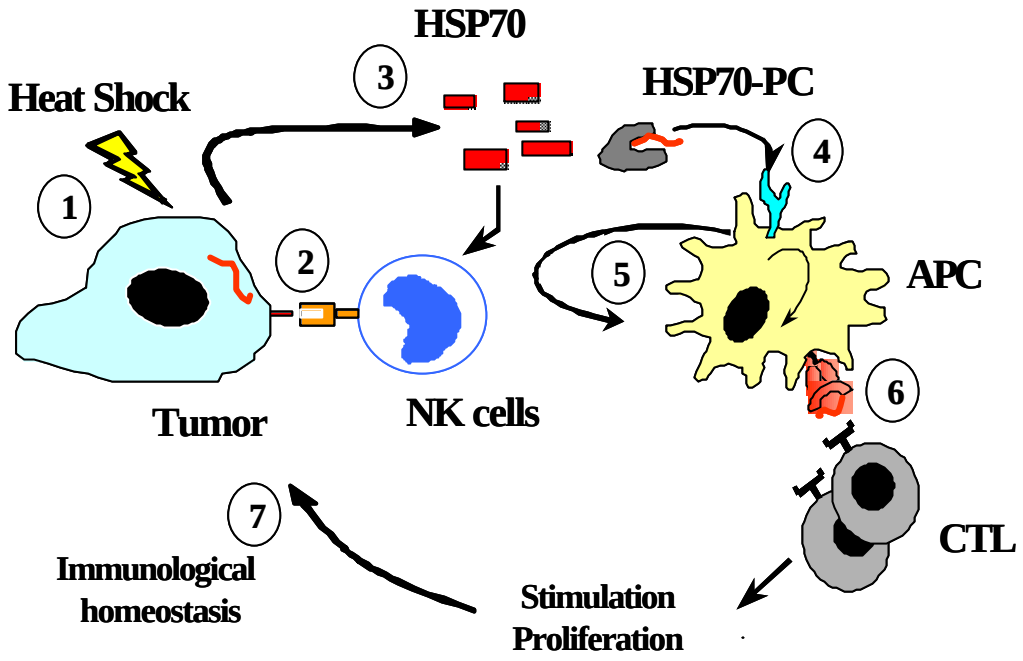


Figure 27: Working hypothesis for hyperthermia (1) Clinical hyperthermia (heat shock) upregulates HSP70 expression in tumor tissues. (2) HSP70 surface expression may occur in some tumor cells activating NK cells. (3) Due to induction of local necrosis, HSP70 and HSP70-PC can be released (4) HSP70-PC bind to APCs and induce cytokine secretion, APC activation, and in parallel deliver the peptide cargo into the cross-presentation pathway for MHC class I restricted presentation and antigen specific T cell activation (6). (7) Tumor cells remain susceptible to lysis from specific activated T cells (immunological homeostasis).

7 SUMMARY

The goal of this study was to investigate the function of the heat shock protein 70 family members, expressed in tumors under physiological and stress conditions and to dissect their role in tumor immune recognition as a function of intra- versus extracellular location. Another goal was to investigate whether heat-treatment at clinically relevant thermal doses affects the immunophenotype of a given tumor, as defined by tumor cell sensitivity to immune effector cells.

For these questions, the human melanoma system was selected because it is well characterized with regards to tumor-associated antigens, like tyrosinase and Melan-A/MART-1, their epitopes and restriction elements for MHC class I presentation.

In the first part of the study the focus specifically was on the time-temperature dependent effects of heat exposure. Two different thermal doses (41,8°C/120 minutes and 45°C/22 minutes) were selected that mimic the heterogeneity of the achieved temperature distribution within the tumor and the time-temperature dependent changes were determined in: a) antigen expression (tyrosinase and Melan-A/MART-1) at the protein and mRNA level; b) expression of the inducible HSP70 and the constitutive HSC70; c) processing and presentation of tyrosinase and MART-1 via MHC class I; d) susceptibility of melanoma cell lines to cytotoxic T lymphocytes like CD8+ T cells, LAK and NK cells. It was demonstrated that HSP70 and antigen expression display distinct expression and kinetics that reflect the thermohistory of the cells, i.e. exposure to high or low thermal doses. Immunologically, a low thermal dose did not alter immune recognition of the cells despite the fact that intracellular HSP70 and tyrosinase protein were upregulated. High thermal dose induced a pleiotropy of effects, including stronger upregulation of HSP70 and tyrosinase protein but downregulation of tyrosinase at mRNA level. Concordant with reduced HLA-A2 surface expression and tyrosinase mRNA levels, immune recognition of the heat-treated cells was initially reduced, but pretreatment levels were restored after 72 hours of recovery. The observation that tumor cells treated with temperatures below the breakpoint temperature maintain an immunological homeostasis during the heat shock response is of critical importance for the clinical application of hyperthermia in the treatment of tumors.

In the second part of the study, the ability of HSP70 to cross-present a naturally expressed human tumor antigen, tyrosinase, that is of low immunigenicity, a situation that more closely resembles the patient situation was investigated. It was demonstrated that HSP70-peptide complexes (HSP70-PC) purified from tyrosinase-positive (HSP70-PC/tyr+) but not from tyrosinase-negative (HSP70-PC/tyr-) melanoma cells deliver the tyrosinase antigen to immature DCs for MHC class I restricted T cell recognition. T cell stimulation by HSP70-

PC/tyr+ incubated with immature DCs with was very efficient even without additional DC maturation signals (*e.g*. exogenous TNF-α) demonstrating the ability of tumor-derived HSP70-PC to act as a chaperone for peptides and a signal for DC maturation. HSP70-PC in exerting both functions on DCs, delivering antigens and maturing DCs, ensures that the peptides that are delivered to the DCs are presented in an immunogenic context optimal for T cell stimulation.

In conlusion, induction of intracellular heat shock proteins (HSPs) by heat does not interfere with the tumor immune recognition and when HSPs are expressed extracellularly they acquire immunostimulatory properties. These observations open new perspectives for the application of hyperthermia in combination with HSP-based vaccine in the treatment of solid tumors.

8 ZUSAMMENFASSUNG

Das Primärziel dieser Arbeit war, die Funktion von Hitzeschockproteinen (HSP) der Familie 70, die entweder konstitutiv exprimiert oder unter Stressbedingungen induziert werden, zu untersuchen. In diesem Zusammenhang sollte vor allem ihre Rolle in der anti-tumoralen Immunantwort in Abhängigkeit von ihrer intra- oder extrazellulären Lokalisation analysiert werden. Ein weiteres Ziel war, den Einfluss einer Hyperthermiebehandlung auf den Immunophänotyp des Tumors zu untersuchen, welcher durch die Tumorsensitivität gegenüber Immuneffektorzellen definiert wird.

Um diesen Fragestellungen nachzugehen, wurde das humane Melanomsystem gewählt, da zahlreiche tumorassoziierte Antigene, wie zum Beispiel Tyrosinase und Melan-A/MART-1, sowie Epitope und Restriktionselemente bekannt sind.

Im ersten Teil wurde insbesondere auf die Zeit- und Temperatur-abhängige Wirkung von Hitzeschock fokussiert und hierfür wurden zwei unterschiedliche Hitzedosen (41,8°C/120 Minuten und 45°C/22 Minuten) gewählt, welche die intratumorale Hitzeverteilung *in vivo* nachahmen. Insbesondere wurden die Zeit- und Temperatur-abhängigen Veränderungen in a) der Antigenexpression von Tyrosinase und Melan-A/MART-1 auf mRNA- und Proteinebene; der b) der Expression des konstitutiven HSC70 und des induzierbaren HSP70; c) Antigenprozessierung und -präsentation über MHC Klasse I und d) der Suszeptibilität der Melanomzelllinien gegenüber zytotoxischen T-Zellen wie CD8+, LAK und NK Zellen, untersucht. Es zeigte sich, dass HSP70 und die Antigen-Expression eindeutig unterschiedliche Kinetiken aufweisen, welche die „Thermohistorie“ der Tumorzellen widerspiegeln. Nach immunologischem Gesichtspunkt beeinträchtigte eine niedrige Hitzedosis die Immunerkennung der Tumorzellen nicht, obwohl sowohl das intrazelluläre HSP70 als auch das Tyrosinase-Protein stark heraufreguliert wurden. Bei höherer Hitzedosis hingegen wurden verschiedenartige Effekte beobachtet, darunter eine starke Hochregulation des HSP70 und des Proteins-Tyrosinase, aber auch eine Herabregulation der Tyrosinase-mRNA. Die Immunerkennung der hitzebehandelten Tumorzellen war zunächst herabgesetzt, was durch die vorübergehende Reduktion der HLA-A2 Oberflächenexpression wie auch durch die geringere Tyrosinase-mRNA erklärt werden kann. Diese transient reduktierte Immunsensitivität war reversibel und wurde innerhalb von 72 Stunden wiederhergestellt. Die Beobachtung, dass mit niedrigen Hitzedosen behandelte Tumorzellen eine immunologische Homöostase erhalten, ist von entscheidender Bedeutung für die klinische Anwendung der Hyperthermie.

Im zweiten Teil wurde die Fähigkeit von HSP70 untersucht, Tyrosinase-Peptide in den Cross-präsentationsweg von DZ zu vermitteln. Tyrosinase ist ein natürliches, nicht-mutiertes

humanes Tumorantigen, welches wenig immunogen ist. Dies ist eine Konstellation, die der Patientensituation ähnelt. Es konnte nachgewiesen werden, dass DZ, die mit HSP70-PC aus Tyrosinase-positiven Zell-Linien (HSP70-PC/Tyr+) beladen wurden, eine konzentrationsabhängige Stimulation des Tyrosinase-spezifischen T-Zell Klons zeigten. Zur Stimulation war ein zusätzliches „Maturierungssignal“ wie z.B. TNF-α nicht nötig, was beweist, dass HSP70-PC die Eigenschaft besitzen, antigene Peptide in den Cross-präsentationsweg zu vermitteln und DZ auszureifen. In ihrer dualen Funktion, als Vermittler für Tumorantigene an DZ und als Gefahrsignal für die Ausreifung von DZ, führen HSP70-PC zu einem effizienten „Priming“ zirkulierender T-Zellen und zur Induktion einer anti-tumoralen Immunantwort.

Zusammenfassend konnte gezeigt werden, dass die Induktion von intrazellulären HSP durch Hitzeschock nicht mit der Immunerkennung des Tumors interferiert und dass HSPs, die sich im extrazellulären Milieu befinden, immunostimulatorische Eigenschaften erwerben. Diese Beobachtungen eröffnen neue Perspektiven für die Anwendung der Hyperthermie in Kombination mit HSP-basierten Vakzinen bei der Behandlung solider Tumore.

9 CURRICULUM VITAE

Personal data **Valeria Milani, MD**
italian and swiss nationality
Born 23. April 1974 in Rom
Married

Privat Address Tengstrasse 22, 80798 Munich; Telefon: + 49 089 522116

Institute CCG Hyperthermia, Medical Clinic III, Klinikum Großhadern, LMU-Munich, GSF-Hämatologikum, Marchioninistrasse 25, 81377 Munich
Telefon: 089 7099403; Fax: 089 7099450, email: milani@gsf.de

Education

10/93-07/99	University School of Medicine, Genoa, Italy. Graduation with the thesis "Autologous tandem transplant in high-grade lymphomas"	**Genoa**
1988-1993	High school for science, Genoa, Italy	**Genoa**

Clinical activity

07-08/1996	Klinikum Innenstadt, LMU; Hematology/Oncology Unit; (Prof. Dr. B. Emmerich)	**Munich**
08-09/1998	Brigham and Women's Hospital/Dana Farber Cancer Institute, „Bone marrow transplantation" Station, Boston, Massachusetts, Famulatur (Prof. K. Anderson, Prof. J. Gribben)	**Boston**
8-9/1999	Children´s Hospital, University School of Medicine, Genoa, Italy	**Genoa**
10/99-3/00	Klinikum Großhadern, LMU-Munich Kidney transplantation Unit (Prof. Dr. W. Land); Bone Marrow Transplantation Unit, Medical Clinic III (Prof. Dr. H.J. Kolb)	**Munich**
5-7/2000	German and Italian Medical approbation	
since 02/04	Physician at internal medicine III, LMU, Klinikum Großhadern (Prof. Dr. med. W. Hiddemann)	**Munich**

Scientific activity

08/00-2003 **CCG Hyperthermia, Klinikum Großhadern-LMU** **Munich**
Medical Clinic III, (Prof. Dr. med. dipl. biochem. R.D. Issels),
LMU and GSF-Hämatologikum-Institute for Molecular Immunology
(Prof. Dr. D. Schendel).
Topic „Heat shock proteins and activation of immune system"

Partecipation in projects of the **Deutsche Krebshilfe** 7023011-is/2 „Immunological Parameter by regional Hyperthermia and systemic chemotherapy" and of the **Sonderforschungsbereich 455**:"Viral Functions and Immunmodulation", LMU-Munich, DFG; SFB455/B9 (Nössner/Issels)."Heat shock proteins (HSP) as immunomodulatory mediators for dendritic cells: cytokine function and antigen presentation**"**

Cooperations

Cooperation mit Prof. Giorgio Parmiani, Director Immuntherapy Unit, Instituto Nazionale Tumori	**Milan**
Cooperation with Dr. Sutter, Group Viral vector, GSF-Institut für Molecular Virology, Klinikum RDI-TU	**Munich**
Cooperation: DFCI-Harvard Medical School und CMSR Center Molecular Stress Responses, Boston Medical Center, (Dr. Calderwood, Dr. Asea)	**Boston**

Awards and memberships

since 2001	Member of ESHO (European society of hyperthermic oncology)
June 2003	Member of „Clinical and Technical Committee“ of the European Society Hyperthermic Oncology, 04-07 Juni 2003, Munich
01/03-6/03	**ESHO 2003**: **Kim´s young investigator award** from ESHO-BSD award committee.
April 2004	**ICHO 2004: Kim´s Award** in the “outstanding young investigator symposium” t the International Congress of Hyperthermic Oncology, 19-24 April 2004, St. Louis, MO, USA
2004-2007	Nomination as **Board Membe r** for the ESHO Council; topic , „Clinical/Biology“. Member of the ESHO biological Commitee

Advanced training courses

10/2003	“One day introduction to the EORTC trials”, EORTC Headquarters, Brussels, Belgium
01/2003	International Symposium: „Selected issues in kidney transplantation: allograft injury mediated by (oxidative) stress: from evolutionary conserved proteins of drosophila to acute and chronic allograft rejection“, Pegnitz, Germany
11-12/02	“Hematological diagnostic“ (Prof. T. Haferlach, Prof. H. Diem), Klinikum Großhadern, LMU-Munich
03/2000	„Walter-Brendel-Kolleg“ for Transplantationsmedecine, Wildbad Kreuth/Tegernsee, Germany
01-03/94	CBA, Advanced Biotechnology Center, Genoa, Italy (Prof. Cancedda); Practice in the Laboratory „Cell differentiation“

Languages	**German** (Kleines Deusches Sprachdiplom, Goethe Institut, May 2002) **English** (fluent); **Italian and French** (mother languages)

Originals

Noessner E *, Gastpar R *, **Milani V** *, Brandl A, Hutzler PJ, Kuppner M, Roos M, Kremmer E, Asea A, Calderwood SK, Issels RI. „Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells".
(*) equally contributed. *J Immunol 2002;169(10):5424-32*

Kuppner M, Scharner A, **Milani V**, Hesler C, Tschöp K, Heinz O, Issels R.
Ifosfamide impairs the allostimulatory capacity of human dendritic cells by intracellular glutathione depletion.
Blood. 2003 Nov 15;102(10):3668-74

Reviews

Milani V, Noessner E, Ghose S, Kuppner M, Ahrens B, Scharner A, Gastpar R, Issels RD. "Heat shock protein 70: role in antigen presentation and immune stimulation".
Int J Hyperthermia 2002;18(6):563-575

Schlemmer M, **Milani V**, Tschöp K, Wendtner CM.
Gastrointestinale Stromatumoren.
Dtsch Med Wochenschr. 2003 Sep 26;128(39):2015-9

Milani V, Endres M , Kuppner MC , Issels RD , Noessner E.
Hitzeschockproteine, Immunkompetenz und Vakzinierung.
Dtsch Med Wochenschr. 2004 Jan 2;129(1-2):31-5.

Manuscripts submitted or in preparation

Milani V, Frankenberger B, Heinz O, Brandl A, Ruhland S., Issels RD, Noessner E.
„ Melanoma-associated antigen expression and presentation dissociate the heat shock response."
Submitted (see abstract)

Arbogast E, Arbogast S, **Milani V**, Noessner E, Fertmann J, Hoffmann JN, Issels R, Land W. „Expression of heat shock proteins in cadaveric human renal allografts-a role in activation of innate immunity?"
In preparation

Milani V, Issels, R, Noessner E.
"Effects of heat shock on the antitumor cell-mediated immunity".
In preparation (see abstract)

Talks

05/1995	„Sviluppo del sistema nervoso", scientific seminars of the institute of human anatomy (Prof. Zaccheo), Medical school, University of **Genua**
07/1999	„Autotrapianto di cellule staminali emopoietiche nella terapia die linfomi non-Hodgkin", Thesis discussion for the MD degree, University of **Genua**
5/2001	**"**Antigen uptake and presentation by dendritic cells" and "Heat shock protein 70 and dendritic cell maturation" European society of hyperthermic oncology, ESHO 2001, **Verona**, Italy
6/2001	" Role of heat shock protein 70 in antigen presention and DC maturation", Weekly seminars of the Institute for Molecular Immunlogy (GSF-IMI), Prof. D. Schendel, GSF-Hämatologikum, **Munich**
9/2001	"Heat shock proteins-their functional role in dendritic cell maturation and antigen presentation"; Symposium " Immunobiology of HSP70", Graduierter Kolleg, Immunogenetic, Prof. Günther; Georg-August-Universität, **Göttingen**
4/2002	"Tumor-derived heat shock protein peptide-complexes are cross-presented by human dendritic cells" SFB455 Retreat I, Prof. U Koszinowsky, Akademie Schloss Hohenkammer, **Munich**

5/2002 "Immunregulation through heat shock protein expression: rationale for the use of hyperthermia in different clinical application"
European society of hyperthermic oncology, ESHO 2002, **Bergen**, Norway

6/2002 "Tumor-derived heat shock protein peptide-complexes are cross-presented by human dendritic cells"
Center for Molecular Stress Response (CMRS), Boston Medical Center, Prof. Calderwood, Boston University, **Boston**, Massachusetts

7/2002 "Heat shock-induced immunogenicity is a function of time and temperature: in vitro immunokinetic studies using a human melanoma cell line" Weekly seminars of the Institute for Molecular Immunlogy (GSF-IMI), Prof. D. Schendel, GSF-Hämatologikum, **Munich**

5/2003 „Aktivierung des immunsystems durch Hyperthermie: die Rolle des Stressproteins HSP70 in der Antigenpräsentation und Immunstimulation",
61. Wissenschaftliche Jahrestagung der Gesellschaft für Pädiatrische Onkologie und Hämatologie (GPHO), **Berlin**

6/2003 "Dissociation of antigen expression and susceptibility to immune effector mechanisms during the heat shock reposne" Weekly seminars of the Institute for Molecular Immunlogy (GSF-IMI), Prof. D. Schendel, GSF-Hämatologikum, **Munich**

6/2003 „Tumor antigen presentation and heat shock response".
European society of hyperthermic oncology, ESHO 2003, **Munich**

6/2003 "Heat shock proteins as regulators of inflammtory and antitumoral immune responses".
Kim young investigator Award 2003; European society of hyperthermic oncology, ESHO 2003, **Munich**

7/2003 "Dissociation of antigen expression ans susceptibility to immune effector mechanisms durignthe heat shock response"; Fifth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LM U-Klinikum Grosshadern, **Herrsching**

9/2003 **"**Dissociation of tumor antigen expression and susceptibility to immune effector mechanisms during the heat shock response". Biological Therapy of cancer, LMU-Klinikum Grosshadern, **Munich**

4/2004 **"**Dissociation of tumor antigen expression and susceptibility to immune effector mechanisms during the heat shock response". International congress of hyperthermic oncology, ICHO 2004, **St. Louis**, MO, USA

Abstracts/Poster

Issels RD, Falk M, Schneider F, Kuppner M, **Milani V**, Wick M , Gastpar R. „Depletion of intracellular glutathione and functional impairmnet of peripheral blood lymphocytes (PBL) by high-dose Ifosfamide in vivo"; second scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, July 2000, Wildbad Kreuth (Poster)

Milani V, Kuppner M, Issels RI. „Recombinant heat shock protein 70: a potential maturation factor for AML-derived dendritic cells"; first scientific symposium of GSF-Hämatologikum (Prof. D. Schendel), January 2001, Wildbad Kreuth (Poster)

Milani V, Kuppner, Gastpar R, Noessner E, Roos M, Issels R. Heat shock proteins and dendritic cells: effects on maturation and antigen presentation**"**, third scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, July 2001, Wildbad Kreuth (Poster)

Kuppner M, **Milani V**, Gastpar R, Noessner E, Roos M, Issels R. "Heat shock proteins and dendritic cells: effects on maturation and antigen presentation**"** European Journal of Cancer 2001 (37) Suppl 3, Biological therapy of Cancer 2001, September 2001, München (Abstract)

Noessner E, Gaspar R, **Milani V**, Kuppner M, Issels R. "Cross-presentation of human shared tumor antigen by dendritic celss: dual function of HSP70 as chaperone for tumor-derived T-cell epitopes and cytokine for DC maturation" International Workshop Molecular Biology of Stress Responses; Mendoza, Argentina, Oktober 2001 (Abstract)

Noessner E, Gastpar R, **Milani V**, Kuppner M, Issels R. "Crosspresentation of human shared tumor antigen by dendritic cells: dual function of HSP70 as chaperone for tumor-derived T cell epitopes and cytokine for DC maturation". DGFI Deutsche Gesellschaft für Immunologie; Dresden September 2001 (Poster)

Noessner E, **Milani V**, Gaspar R, Endres M, Hutzler P, Issels RD. "Stimulating tumor antigen specific T lymphocytes through heat shock proteins 70-peptide complexes cross-presented by human dendritic cells". The Tegernsee Conference of Immunotherapy of solid cancer, Tegernsee, Juli 2002 (Poster)

Milani V, Noessner E, Issels RD. "Heat shock-induced immunogenicity is a function of time and temperature: in vitro immunokinetic studies using a human melanoma cell line", Fourth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, Juni 2002, Wildbad Kreuth (Poster)

Kuppner M, **Milani V**, Von Hesler C, Scharner A, Heinz O, Issels RD. „Ifosfamid decreases intracellular glutathione levels in dendritic cells: effect on immune function", Fourth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern Juni 2002, Wildbad Kreuth (Poster)

Milani V, Nössner E, Wagner H, Issels RD. „Aktivierung des Immunsystems durch hyperthermie: die Rolle des Stressproteins HSP70 in der Antigenpräsentation und Immunstimulation". Monatsschrift Kinderheilkd 2003;151:467-76 (Abstract)

Arbogast H, Arbogast S, **Milani V**, Fertmann J, Hoffmann JN, Noessner E, Issels R, Land W. „Expression of heat shock proteins (HSP) in cadaveric human renal allografts-a role in activation of innate immunity?. " European society of hyperthermic oncology, ESHO, Juni 2003, München (Abstract)

Tschoep K., Frankenberger B., Kohlmann A. , Schlemmer M., Hammer D., Kuppner M., **Milani V,** Nössner E., Issels R. **„**Kinetics of heat shock protein70 mRNA and protein expression in Ewing`s sarcoma cell line RD-ES after sublethal and lethal heat shock exposure for subsequent microarray analysis". European society of hyperthermic oncology, ESHO, Juni 2003, München (Poster)

Milani V, Frankenberger B, Heinz O, Brandl A, Tschöp K, Issels RD, Nössner E. „Dissociation of antigen expression ans susceptibility to immune effector mechanisms durign the heat shock response". Fifth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, July 2003, Herrsching (Med III, Book)

Kuppner MC, Scharner A, **Milani V**, Von Hesler C, Tschöp K., Heinz O, Issels RD. **"**Regulation of human dendritic cells functional activity by ifosfamide induced glutathione depletion", Fifth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, July 2003, Herrsching (Poster)

Tschoep K., Frankenberger B., Schlemmer M., Hammer D., Kuppner M., **Milani V.,** Nössner E, Issels R. „ Heat shock.induced gene expression in sarcoma cells", Fifth scientific symposium of the Internal medicine department III, (Prof. Dr. med. W. Hiddemann), LMU-Klinikum Grosshadern, July 2003, Herrsching (Abstract)

Milani V., Frankenberger B., Heinz O., Brandl A., Tschöp K., Issels R., Nössner E. Dissociation of tumor antigen expression and susceptibility to immune effector mechanisms during the heat shock response. Deutsche Gesellschaft für Hämatologie und Onkologie, DGHO, October 2003, Basel (Poster)

Kuppner MC, Scharner A, **Milani V**, Von Hesler C, Tschöp K., Heinz O, Issels RD. **"**Regulation of human dendritic cells functional activity by ifosfamide induced glutathione depletion"; Biological Therapy of cancer, Klinikum der Universität, LMU-Grosshadern, Munich (Poster)

Milani V, Frankenberger B, Heinz O, Issels R, Nössner E. „Dissociation of tumor antigen expression and susceptibility to immune effector mechanisms during the heat shock response",Deutsche Gesellschaft für Immunologie**,** DGfI, Berlin (Poster)

Tschoep K., Kohlmann A, Frankenberger B., Schlemmer M., Hammer D., Kuppner M., **Milani V.,** Nössner E, Haferlach T., Issels R. „ Heat-induced regulation of immunological relevant genes in sarcoma cells", Deutsche Gesellschaft für Immunologie, DGfI, Berlin (Poster)

Milani V., Frankenberger B., Heinz O., Issels R., Nössner E. „ Melanoma-associated antigen expression and presentation dissociate during the heat shock response", International congress of hyperthermic oncology, ICHO, 19-24 April 2004, St-Louis, MO, USA (Abstract)

Kuppner M., **Milani V**., Von Hesler C., Tschöp K., Heinz O., Issels RD. "Regulation of human dendritic cell functional activity by Ifosfamide induced glutathione depletion". International congress of hyperthermic oncology, ICHO, 19-24 April 2004, St-Louis, MO, USA (Poster)

Noessner E., **Milani V.**, Issels RD. "Heat shock proteins, hyperthermia and antitumor immunity"; International congress of hyperthermic oncology, ICHO, 19-24 April 2004, St-Louis, MO, USA (Abstract)

10 ACKNOWLEDGEMENTS

This work was done at the GSF Hämatologikum, KKG Hyperthermie, Internal Medicine III, Klinikum Grosshadern in the group of Prof. Dr. med. dipl. biochem. Rolf Dieter Issels under the supervision of PD Dr. Elfriede Nössner (Institute for Molecular Immunology, GSF-Hämatologikum). I owe Prof. Issels my sincere gratitude for the challenging research subject and for his constant interest in my work and career, as well as for his encouragement to participate in scientific events and communicate our results. I am particularly grateful to PD Dr. Elfriede Nössner for her constant presence and her indispensable and precious help to draw experiments and to write the results of this study, for the exciting scientific discussions and her reliable suggestions. Many thanks to her also for the human support and friendship. I owe many thanks to Dr. Robert Gastpar for teaching me the isolation of proteins and for the usefull scientific discussions. Many special thanks also to Bernhard Frankenberger (Institute for Molecular Immunology, GSF-Hämatologikum) for providing me with the RT-PCR measurements for this study. I thank the KKG Hyperthermie team, especially Dr. Kuppner, Dr. Endres, Dr. Tschöp for the collaboration and the nice working atmosphere and in particular Oksana Heinz, Miriam Roos, Christoph von Hesler and Doreen Hammer for their excellent technical assistance. My thanks also go to the members of the Institute for Molecular Immunology, GSF-Hämatologikum (Prof. D. Schendel) and in particular to Dr. Joseph Mysliwietz for his cooperativeness and support. Mrs. Martina Lahm, Mrs. Beatrice Stockheim and Mrs. Sylvia Donhauser have my sincere appreciaton for their constant willingness to help and for their friendly advice in administrative and personal matters. My thanks go to all the floor members and my italian colleagues who supported my work and made my stay here very enjoyable. I am extremely grateful to my parents and family for their ever-reliable encouragement and belief in me. Finally, I thank my husband Alexander Brühl for his patience in listening to all-day lab problems and for his constant support during the writing of this thesis and publication. The SFB455 and Deutsche Krebshilfe are gratefully acknowledged for financial support of this research.

11 LITERATURE

Aboud, M., S. Segal, E. Priel, D. G. Blair and B. O'Hara (1992). "Effect of temperature on the expression of major histocompatibility complex class-I antigens." Immunol Invest **21**(3): 219-29.

Adler, A. J., D. W. Marsh, G. S. Yochum, J. L. Guzzo, A. Nigam, W. G. Nelson and D. M. Pardoll (1998). "CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells." J Exp Med **187**(10): 1555-64.

Akira, S., K. Hoshino and T. Kaisho (2000). "The role of Toll-like receptors and MyD88 in innate immune responses." J Endotoxin Res **6**(5): 383-7.

Akira, S., K. Takeda and T. Kaisho (2001). "Toll-like receptors: critical proteins linking innate and acquired immunity." Nat Immunol **2**(8): 675-80.

Amigorena, S. (2003). "Y in X priming." Nat Immunol **4**(11): 1047-8.

Arnold-Schild, D., D. Hanau, D. Spehner, C. Schmid, H. G. Rammensee, H. de la Salle and H. Schild (1999). "Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells." J Immunol **162**(7): 3757-60.

Asea, A., S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, R. W. Finberg, G. C. Koo and S. K. Calderwood (2000). "HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine." Nat Med **6**(4): 435-42.

Asea, A., M. Rehli, E. Kabingu, J. A. Boch, O. Bare, P. E. Auron, M. A. Stevenson and S. K. Calderwood (2002). "Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4." J Biol Chem **277**(17): 15028-34.

Atanackovic, D., A. Nierhaus, M. Neumeier, D. K. Hossfeld and S. Hegewisch-Becker (2002). "41.8 degrees C whole body hyperthermia as an adjunct to chemotherapy induces prolonged T cell activation in patients with various malignant diseases." Cancer Immunol Immunother **51**(11-12): 603-13.

Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran and K. Palucka (2000). "Immunobiology of dendritic cells." Annu Rev Immunol **18**: 767-811.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-52.

Barnstable, C. J., E. A. Jones and M. J. Crumpton (1978). "Isolation, structure and genetics of HLA-A, -B, -C and -DRw (Ia) antigens." Br Med Bull **34**(3): 241-6.

Basu, S., R. J. Binder, T. Ramalingam and P. K. Srivastava (2001). "CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin." Immunity **14**(3): 303-13.

Basu, S., R. J. Binder, R. Suto, K. M. Anderson and P. K. Srivastava (2000). "Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway." Int Immunol **12**(11): 1539-46.

Becker, T., F. U. Hartl and F. Wieland (2002). "CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes." J Cell Biol **158**(7): 1277-85.

Binder, R. J., K. M. Anderson, S. Basu and P. K. Srivastava (2000). "Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells in vivo." J Immunol **165**(11): 6029-35.

Binder, R. J., N. E. Blachere and P. K. Srivastava (2001). "Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules." J Biol Chem **276**(20): 17163-71.

Blachere, N. E., Z. Li, R. Y. Chandawarkar, R. Suto, N. S. Jaikaria, S. Basu, H. Udono and P. K. Srivastava (1997). "Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity." J Exp Med **186**(8): 1315-22.

Blom, D. J., I. De Waard-Siebinga, R. S. Apte, G. P. Luyten, J. Y. Niederkorn and M. J. Jager (1997). "Effect of hyperthermia on expression of histocompatibility antigens and heat-shock protein molecules on three human ocular melanoma cell lines." Melanoma Res **7**(2): 103-9.

Bloom, H., H. Beier and H. Gross (1987). "New proved silver staining of plant proteins, RNA and DNA in polyacrylamide gels." Electrophoresis **8**: 93-99.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Breloer, M., B. Dorner, S. H. More, T. Roderian, B. Fleischer and A. von Bonin (2001). "Heat shock proteins as "danger signals": eukaryotic Hsp60 enhances and accelerates antigen-specific IFN-gamma production in T cells." Eur J Immunol **31**(7): 2051-9.

Breloer, M., T. Marti, B. Fleischer and A. von Bonin (1998). "Isolation of processed, H-2Kb-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and gp96." Eur J Immunol **28**(3): 1016-21.

Bukau, B. and A. L. Horwich (1998). "The Hsp70 and Hsp60 chaperone machines." Cell **92**(3): 351-66.

Castelli, C., A. M. Ciupitu, F. Rini, L. Rivoltini, A. Mazzocchi, R. Kiessling and G. Parmiani (2001). "Human heat shock protein 70 peptide complexes specifically activate antimelanoma T cells." Cancer Res **61**(1): 222-7.

Chen, W., U. Syldath, K. Bellmann, V. Burkart and H. Kolb (1999). "Human 60-kDa heat-shock protein: a danger signal to the innate immune system." J Immunol **162**(6): 3212-9.

Ciupitu, A. M., M. Petersson, C. L. O'Donnell, K. Williams, S. Jindal, R. Kiessling and R. M. Welsh (1998). "Immunization with a lymphocytic choriomeningitis virus peptide mixed with heat shock protein 70 results in protective antiviral immunity and specific cytotoxic T lymphocytes." J Exp Med **187**(5): 685-91.

Clark, P. R. and A. Menoret (2001). "The inducible Hsp70 as a marker of tumor immunogenicity." Cell Stress Chaperones **6**(2): 121-5.

Cormier, J. N., M. C. Panelli, J. A. Hackett, M. P. Bettinotti, A. Mixon, J. Wunderlich, L. L. Parker, N. P. Restifo, S. Ferrone and F. M. Marincola (1999). "Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an in vitro melanoma model." Int J Cancer **80**(5): 781-90.

Davies, C. D. and T. Lindmo (1990). "Hyperthermia-induced shedding and masking of melanoma-associated antigen." Int J Hyperthermia **6**(6): 1053-64.

Davies Cde, L., E. K. Rofstad and T. Lindmo (1985). "Hyperthermia-induced changes in antigen expression on human FME melanoma cells." Cancer Res **45**(9): 4109-14.

del Marmol, V. and F. Beermann (1996). "Tyrosinase and related proteins in mammalian pigmentation." FEBS Lett **381**(3): 165-8.

Delneste, Y., G. Magistrelli, J. Gauchat, J. Haeuw, J. Aubry, K. Nakamura, N. Kawakami-Honda, L. Goetsch, T. Sawamura, J. Bonnefoy and P. Jeannin (2002). "Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation." Immunity **17**(3): 353-62.

Dewey, W. C. (1994). "Arrhenius relationships from the molecule and cell to the clinic." Int J Hyperthermia **10**(4): 457-83.

Dressel, R., L. Elsner, T. Quentin, L. Walter and E. Gunther (2000). "Heat shock protein 70 is able to prevent heat shock-induced resistance of target cells to CTL." J Immunol **164**(5): 2362-71.

Dressel, R., M. Lubbers, L. Walter, W. Herr and E. Gunther (1999). "Enhanced susceptibility to cytotoxic T lymphocytes without increase of MHC class I antigen expression after

conditional overexpression of heat shock protein 70 in target cells." Eur J Immunol **29**(12): 3925-35.

Engelhard, V. H., A. G. Brickner and A. L. Zarling (2002). "Insights into antigen processing gained by direct analysis of the naturally processed class I MHC associated peptide repertoire." Mol Immunol **39**(3-4): 127-37.

Falk, M. H. and R. D. Issels (2001). "Hyperthermia in oncology." Int J Hyperthermia **17**(1): 1-18.

Fearon, D. T. and R. M. Locksley (1996). "The instructive role of innate immunity in the acquired immune response." Science **272**(5258): 50-3.

Flynn, G. C., T. G. Chappell and J. E. Rothman (1989). "Peptide binding and release by proteins implicated as catalysts of protein assembly." Science **245**(4916): 385-90.

Fujieda, S., I. Noda, H. Saito, T. Hoshino and M. Yagita (1995). "Heat shock enhances the susceptibility of tumor cells to lysis by lymphokine-activated killer cells." Arch Otolaryngol Head Neck Surg **121**(9): 1009-14.

Fuller, K. J., R. D. Issels, D. O. Slosman, J. G. Guillet, T. Soussi and B. S. Polla (1994). "Cancer and the heat shock response." Eur J Cancer **30A**(12): 1884-91.

Geginat, G., L. Heine and E. Gunther (1993). "Effect of heat shock on susceptibility of normal lymphoblasts and of a heat shock protein 70-defective tumour cell line to cytotoxic T lymphocytes in vitro." Scand J Immunol **37**(3): 314-21.

Georgopoulos, C. and W. J. Welch (1993). "Role of the major heat shock proteins as molecular chaperones." Annu Rev Cell Biol **9**: 601-34.

Gromkowski, S. H., J. Yagi and C. A. Janeway, Jr. (1989). "Elevated temperature regulates tumor necrosis factor-mediated immune killing." Eur J Immunol **19**(9): 1709-14.

Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert and S. Amigorena (2003). "ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells." Nature **425**(6956): 397-402.

Halaban, R. (2002). "Pigmentation in melanomas: changes manifesting underlying oncogenic and metabolic activities." Oncol Res **13**(1): 3-8.

Halaban, R., E. Cheng, Y. Zhang, G. Moellmann, D. Hanlon, M. Michalak, V. Setaluri and D. N. Hebert (1997). "Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells." Proc Natl Acad Sci U S A **94**(12): 6210-5.

Heath, W. R. and F. R. Carbone (2001). "Cross-presentation in viral immunity and self-tolerance." Nat Rev Immunol **1**(2): 126-34.

Hildebrandt, B., P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix and H. Riess (2002). "The cellular and molecular basis of hyperthermia." Crit Rev Oncol Hematol **43**(1): 33-56.

Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M. F. Princiotta, P. Thibault, D. Sacks and M. Desjardins (2003). "Phagosomes are competent organelles for antigen cross-presentation." Nature **425**(6956): 402-6.

Issels, R. D. and A. Nagele (1990). "Influence of thiols on thermosensitivity of mammalian cells in vitro." Methods Enzymol **186**: 696-708.

Ito, A., M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi (2001). "Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia." Cancer Immunol Immunother **50**(10): 515-22.

Jaattela, M. (1990). "Effects of heat shock on cytolysis mediated by NK cells, LAK cells, activated monocytes and TNFs alpha and beta." Scand J Immunol **31**(2): 175-82.

Jaattela, M. (1995). "Over-expression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells." Int J Cancer **60**(5): 689-93.

Jaattela, M. and D. Wissing (1993). "Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection." J Exp Med **177**(1): 231-6.

Jackson, K. M., M. DeLeon, L. Sistonen and C. R. Verret (2000). "Heat-shocked A20 lymphoma cells fail to induce degranulation of cytotoxic T lymphocytes: possible mechanism of resistance." Cell Immunol **203**(1): 12-8.

Janetzki, S., D. Palla, V. Rosenhauer, H. Lochs, J. J. Lewis and P. K. Srivastava (2000). "Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study." Int J Cancer **88**(2): 232-8.

Jolly, C. and R. I. Morimoto (2000). "Role of the heat shock response and molecular chaperones in oncogenesis and cell death." J Natl Cancer Inst **92**(19): 1564-72.

Kammerer, R., D. Stober, P. Riedl, C. Oehninger, R. Schirmbeck and J. Reimann (2002). "Noncovalent association with stress protein facilitates cross-priming of CD8+ T cells to tumor cell antigens by dendritic cells." J Immunol **168**(1): 108-17.

Kawakami, Y., N. Dang, X. Wang, J. Tupesis, P. F. Robbins, R. F. Wang, J. R. Wunderlich, J. R. Yannelli and S. A. Rosenberg (2000). "Recognition of shared melanoma antigens in association with major HLA-A alleles by tumor infiltrating T lymphocytes from 123 patients with melanoma." J Immunother **23**(1): 17-27.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki and S. A. Rosenberg (1994). "Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor." Proc Natl Acad Sci U S A **91**(9): 3515-9.

Kawakami, Y., S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Appella and S. A. Rosenberg (1994). "Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes." J Exp Med **180**(1): 347-52.

Kayser, S., I. Watermann, C. Rentzsch, T. Weinschenk, D. Wallwiener and B. Guckel (2003). "Tumor-associated antigen profiling in breast and ovarian cancer: mRNA, protein or T cell recognition?" J Cancer Res Clin Oncol.

Khong, H. T. and N. P. Restifo (2002). "Natural selection of tumor variants in the generation of "tumor escape" phenotypes." Nat Immunol **3**(11): 999-1005.

Kirschning, C. J., H. Wesche, T. Merrill Ayres and M. Rothe (1998). "Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide." J Exp Med **188**(11): 2091-7.

Kol, A., T. Bourcier, A. H. Lichtman and P. Libby (1999). "Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages." J Clin Invest **103**(4): 571-7.

Kraybill, W. G., T. Olenki, S. S. Evans, J. R. Ostberg, K. A. O'Leary, J. F. Gibbs and E. A. Repasky (2002). "A phase I study of fever-range whole body hyperthermia (FR-WBH) in patients with advanced solid tumours: correlation with mouse models." Int J Hyperthermia **18**(3): 253-66.

Kubista, B., K. Trieb, H. Blahovec, R. Kotz and M. Micksche (2002). "Hyperthermia increases the susceptibility of chondro- and osteosarcoma cells to natural killer cell-mediated lysis." Anticancer Res **22**(2A): 789-92.

Kuperberg, G., J. Ellis, J. Marcinkiewicz and B. M. Chain (1991). "Temperature-induced stress abrogates co-stimulatory function in antigen-presenting cells." Eur J Immunol **21**(11): 2791-5.

Kuppner, M. C., R. Gastpar, S. Gelwer, E. Nossner, O. Ochmann, A. Scharner and R. D. Issels (2001). "The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors." Eur J Immunol **31**(5): 1602-9.

Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller and H. Kosaka (1996). "Constitutive class I-restricted exogenous presentation of self antigens in vivo." J Exp Med **184**(3): 923-30.

Kusher, D. I., C. F. Ware and L. R. Gooding (1990). "Induction of the heat shock response protects cells from lysis by tumor necrosis factor." J Immunol **145**(9): 2925-31.
Lindquist, S. (1986). "The heat-shock response." Annu Rev Biochem **55**: 1151-91.
Lowry, O., N. Rosebrough, A. Farr and R. Randall (1951). "Protein measurements with the folin phenol reagent." J Biol Chem **193**: 265-275.
Magor, B. G. and K. E. Magor (2001). "Evolution of effectors and receptors of innate immunity." Dev Comp Immunol **25**(8-9): 651-82.
Matzinger, P. (1994). "Tolerance, danger, and the extended family." Annu Rev Immunol **12**: 991-1045.
McMichael, A. J., P. Parham, N. Rust and F. Brodsky (1980). "A monoclonal antibody that recognizes an antigenic determinant shared by HLA A2 and B17." Hum Immunol **1**(2): 121-9.
Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: the virtues of a nonclonal system of recognition." Cell **91**(3): 295-8.
Medzhitov, R., P. Preston-Hurlburt and C. A. Janeway, Jr. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." Nature **388**(6640): 394-7.
Melcher, A., S. Todryk, N. Hardwick, M. Ford, M. Jacobson and R. G. Vile (1998). "Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression." Nat Med **4**(5): 581-7.
Michalek, M. T., B. Benacerraf and K. L. Rock (1992). "The class II MHC-restricted presentation of endogenously synthesized ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is up-regulated by heat shock." J Immunol **148**(4): 1016-24.
Milani, V., E. Noessner, S. Ghose, M. Kuppner, B. Ahrens, A. Scharner, R. Gastpar and R. D. Issels (2002). "Heat shock protein 70: role in antigen presentation and immune stimulation." Int J Hyperthermia **18**(6): 563-75.
Milarski, K. L. and R. I. Morimoto (1986). "Expression of human HSP70 during the synthetic phase of the cell cycle." Proc Natl Acad Sci U S A **83**(24): 9517-21.
Mise, K., N. Kan, T. Okino, M. Nakanishi, K. Satoh, Y. Teramura, S. Yamasaki, K. Ohgaki and T. Tobe (1990). "Effect of heat treatment on tumor cells and antitumor effector cells." Cancer Res **50**(19): 6199-202.
Mosse, C. A., W. Hsu and V. H. Engelhard (2001). "Tyrosinase degradation via two pathways during reverse translocation to the cytosol." Biochem Biophys Res Commun **285**(2): 313-9.
Mosser, D. D., A. W. Caron, L. Bourget, A. B. Meriin, M. Y. Sherman, R. I. Morimoto and B. Massie (2000). "The chaperone function of hsp70 is required for protection against stress-induced apoptosis." Mol Cell Biol **20**(19): 7146-59.
Multhoff, G., C. Botzler, L. Jennen, J. Schmidt, J. Ellwart and R. Issels (1997). "Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells." J Immunol **158**(9): 4341-50.
Multhoff, G., C. Botzler, M. Wiesnet, G. Eissner and R. Issels (1995). "CD3- large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat shock protein on human sarcoma cells." Blood **86**(4): 1374-82.
Multhoff, G. and L. E. Hightower (1996). "Cell surface expression of heat shock proteins and the immune response." Cell Stress Chaperones **1**(3): 167-76.
Multhoff, G., L. Mizzen, C. C. Winchester, C. M. Milner, S. Wenk, G. Eissner, H. H. Kampinga, B. Laumbacher and J. Johnson (1999). "Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of natural killer cells." Exp Hematol **27**(11): 1627-36.

Noessner, E., R. Gastpar, V. Milani, A. Brandl, P. J. Hutzler, M. C. Kuppner, M. Roos, E. Kremmer, A. Asea, S. K. Calderwood and R. D. Issels (2002). "Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells." J Immunol **169**(10): 5424-32.

Ogg, G. S., P. Rod Dunbar, P. Romero, J. L. Chen and V. Cerundolo (1998). "High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo." J Exp Med **188**(6): 1203-8.

Overgaard, J., D. Gonzalez Gonzalez, M. C. Hulshof, G. Arcangeli, O. Dahl, O. Mella and S. M. Bentzen (1995). "Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. European Society for Hyperthermic Oncology." Lancet **345**(8949): 540-3.

Overgaard, J. and H. D. Suit (1979). "Time-temperature relationship th hyperthermic treatment of malignant and normal tissue in vivo." Cancer Res **39**(8): 3248-53.

Panjwani, N., O. Akbari, S. Garcia, M. Brazil and B. Stockinger (1999). "The HSC73 molecular chaperone: involvement in MHC class II antigen presentation." J Immunol **163**(4): 1936-42.

Pepin, E., C. L. Villiers, F. M. Gabert, V. A. Serra, P. N. Marche and M. G. Colomb (1996). "Heat shock increases antigenic peptide generation but decreases antigen presentation." Eur J Immunol **26**(12): 2939-43.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-8.

Protti, M. P., S. Heltai, M. Bellone, M. Ferrarini, A. A. Manfredi and C. Rugarli (1994). "Constitutive expression of the heat shock protein 72 kDa in human melanoma cells." Cancer Lett **85**(2): 211-6.

Pugin, J., I. D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias and R. J. Ulevitch (1994). "CD14 is a pattern recognition receptor." Immunity **1**(6): 509-16.

Renkvist, N., C. Castelli, P. F. Robbins and G. Parmiani (2001). "A listing of human tumor antigens recognized by T cells." Cancer Immunol Immunother **50**(1): 3-15.

Riker, A. I., U. S. Kammula, M. C. Panelli, E. Wang, G. A. Ohnmacht, S. M. Steinberg, S. A. Rosenberg and F. M. Marincola (2000). "Threshold levels of gene expression of the melanoma antigen gp100 correlate with tumor cell recognition by cytotoxic T lymphocytes." Int J Cancer **86**(6): 818-26.

Ritossa, F. (1962). "A new puffing pattern induced by temperature shock and DNP in Drosophila." Experentia **18**: 571-73.

Rivoltini, L., K. C. Barracchini, V. Viggiano, Y. Kawakami, A. Smith, A. Mixon, N. P. Restifo, S. L. Topalian, T. B. Simonis, S. A. Rosenberg and et al. (1995). "Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes." Cancer Res **55**(14): 3149-57.

Robertson, M. J., K. J. Cochran, C. Cameron, J. M. Le, R. Tantravahi and J. Ritz (1996). "Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia." Exp Hematol **24**(3): 406-15.

Schendel, D. J., B. Gansbacher, R. Oberneder, M. Kriegmair, A. Hofstetter, G. Riethmuller and O. G. Segurado (1993). "Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines." J Immunol **151**(8): 4209-20.

Schendel, D. J., R. Wank and B. Dupont (1979). "Standardization of the human in vitro cell-mediated lympholysis technique." Tissue Antigens **13**(2): 112-20.

Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell and J. R. Bennink (2000). "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes." Nature **404**(6779): 770-4.

Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W. M. Kast, C. J. Melief, C. Oseroff, L. Yuan, J. Ruppert and et al. (1994). "The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes." J Immunol **153**(12): 5586-92.

Singh-Jasuja, H., N. Hilf, D. Arnold-Schild and H. Schild (2001). "The role of heat shock proteins and their receptors in the activation of the immune system." Biol Chem **382**(4): 629-36.

Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H. G. Rammensee, R. E. Toes and H. Schild (2000). "The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor." Eur J Immunol **30**(8): 2211-5.

Singh-Jasuja, H., R. E. Toes, P. Spee, C. Munz, N. Hilf, S. P. Schoenberger, P. Ricciardi-Castagnoli, J. Neefjes, H. G. Rammensee, D. Arnold-Schild and H. Schild (2000). "Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis." J Exp Med **191**(11): 1965-74.

Slingluff, C. L., Jr., T. A. Colella, L. Thompson, D. D. Graham, J. C. Skipper, J. Caldwell, L. Brinckerhoff, D. J. Kittlesen, D. H. Deacon, C. Oei, N. L. Harthun, E. L. Huczko, D. F. Hunt, T. L. Darrow and V. H. Engelhard (2000). "Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens." Cancer Immunol Immunother **48**(12): 661-72.

Somersan, S., M. Larsson, J. F. Fonteneau, S. Basu, P. Srivastava and N. Bhardwaj (2001). "Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells." J Immunol **167**(9): 4844-52.

Srivastava, P. (2002). "Roles of heat-shock proteins in innate and adaptive immunity." Nat Rev Immunol **2**(3): 185-94.

Srivastava, P. K. (2000). "Immunotherapy of human cancer: lessons from mice." Nat Immunol **1**(5): 363-6.

Srivastava, P. K., A. B. DeLeo and L. J. Old (1986). "Tumor rejection antigens of chemically induced sarcomas of inbred mice." Proc Natl Acad Sci U S A **83**(10): 3407-11.

Srivastava, P. K., A. Menoret, S. Basu, R. J. Binder and K. L. McQuade (1998). "Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world." Immunity **8**(6): 657-65.

Srivastava, P. K. and H. Udono (1994). "Heat shock protein-peptide complexes in cancer immunotherapy." Curr Opin Immunol **6**(5): 728-32.

Stewart, J. R. and F. A. Gibbs, Jr. (1984). "Hyperthermia in the treatment of cancer. Perspectives on its promise and its problems." Cancer **54**(11 Suppl): 2823-30.

Sugawara, S., M. Nowicki, S. Xie, H. J. Song and G. Dennert (1990). "Effects of stress on lysability of tumor targets by cytotoxic T cells and tumor necrosis factor." J Immunol **145**(6): 1991-8.

Suto, R. and P. K. Srivastava (1995). "A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides." Science **269**(5230): 1585-8.

Takeuchi, H., C. Kuo, D. L. Morton, H. J. Wang and D. S. Hoon (2003). "Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas." Cancer Res **63**(2): 441-8.

Tamura, Y., P. Peng, K. Liu, M. Daou and P. K. Srivastava (1997). "Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations." Science **278**(5335): 117-20.

Todryk, S., A. A. Melcher, N. Hardwick, E. Linardakis, A. Bateman, M. P. Colombo, A. Stoppacciaro and R. G. Vile (1999). "Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake." J Immunol **163**(3): 1398-408.

Udono, H., D. L. Levey and P. K. Srivastava (1994). "Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo." Proc Natl Acad Sci U S A **91**(8): 3077-81.

Udono, H. and P. K. Srivastava (1993). "Heat shock protein 70-associated peptides elicit specific cancer immunity." J Exp Med **178**(4): 1391-6.

Udono, H. and P. K. Srivastava (1994). "Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70." J Immunol **152**(11): 5398-403.

Udono, H., T. Yamano, Y. Kawabata, M. Ueda and K. Yui (2001). "Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70." Int Immunol **13**(10): 1233-42.

Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker and H. Wagner (2001). "Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells." J Biol Chem **276**(33): 31332-9.

Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels and H. Wagner (2002). "HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway." J Biol Chem **277**(17): 15107-12.

Vabulas, R. M., S. Braedel, N. Hilf, H. Singh-Jasuja, S. Herter, P. Ahmad-Nejad, C. J. Kirschning, C. Da Costa, H. G. Rammensee, H. Wagner and H. Schild (2002). "The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway."J Biol Chem **277**(23): 20847-53.

Vabulas, R. M., H. Wagner and H. Schild (2002). "Heat shock proteins as ligands of toll-like receptors." Curr Top Microbiol Immunol **270**: 169-84.

Van Molle, W., B. Wielockx, T. Mahieu, M. Takada, T. Taniguchi, K. Sekikawa and C. Libert (2002). "HSP70 protects against TNF-induced lethal inflammatory shock." Immunity **16**(5): 685-95.

Vanaja, D. K., M. E. Grossmann, E. Celis and C. Y. Young (2000). "Tumor prevention and antitumor immunity with heat shock protein 70 induced by 15-deoxy-delta12,14-prostaglandin J2 in transgenic adenocarcinoma of mouse prostate cells." Cancer Res **60**(17): 4714-8.

VanSlyke, J. K. and L. S. Musil (2002). "Dislocation and degradation from the ER are regulated by cytosolic stress." J Cell Biol **157**(3): 381-94.

Visseren, M. J., A. van Elsas, E. I. van der Voort, M. E. Ressing, W. M. Kast, P. I. Schrier and C. J. Melief (1995). "CTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells." J Immunol **154**(8): 3991-8.

Wang, X. Y., L. Kazim, E. A. Repasky and J. R. Subjeck (2001). "Characterization of heat shock protein 110 and glucose-regulated protein 170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity." J Immunol **166**(1): 490-7.

Welch, W. J. and J. P. Suhan (1986). "Cellular and biochemical events in mammalian cells during and after recovery from physiological stress." J Cell Biol **103**(5): 2035-52.

Wells, A. D. and M. Malkovsky (2000). "Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view." Immunol Today **21**(3): 129-32.

Wells, A. D., S. K. Rai, M. S. Salvato, H. Band and M. Malkovsky (1998). "Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation." Int Immunol **10**(5): 609-17.

Wolfel, T., A. Van Pel, V. Brichard, J. Schneider, B. Seliger, K. H. Meyer zum Buschenfelde and T. Boon (1994). "Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes." Eur J Immunol **24**(3): 759-64.

Wust, P., B. Hildebrandt, G. Sreenivasa, B. Rau, J. Gellermann, H. Riess, R. Felix and P. M. Schlag (2002). "Hyperthermia in combined treatment of cancer." Lancet Oncol **3**(8): 487-97.

Yewdell, J. W., L. C. Anton and J. R. Bennink (1996). "Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules?" J Immunol **157**(5): 1823-6.

Yewdell, J. W., C. C. Norbury and J. R. Bennink (1999). "Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines." Adv Immunol **73**: 1-77.

Yost, H. J., R. B. Petersen and S. Lindquist (1990). "RNA metabolism: strategies for regulation in the heat shock response." Trends Genet **6**(7): 223-7.

Zheng, H., J. Dai, D. Stoilova and Z. Li (2001). "Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity." J Immunol **167**(12): 6731-5.