

## **Old and new facts about hyperthermia-induced modulations of the immune system**

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## **Old and new facts about hyperthermia-induced modulations of the immune system**

### ***Abstract***

Hyperthermia (HT) is a potent sensitiser for radiotherapy (RT) and chemotherapy (CT) and has been proven to modulate directly or indirectly cells of the innate and adaptive immune system. We will focus in this article on how anti-tumour immunity can be induced by HT. In contrast to some *in vitro* assays, *in vivo* examinations showed that natural killer cells and phagocytes like granulocytes are directly activated against the tumour by HT. Since heat also activates dendritic cells (DCs), HT should be combined with further death stimuli (RT, CT or immune therapy) to allocate tumour antigen, derived from, for example, necrotic tumour cells, for uptake by DCs. We will outline that induction of immunogenic tumour cells and direct tumour cell killing by HT in combination with other therapies contributes to immune activation against the tumour. Studies will be presented showing that non-beneficial effects of HT on immune cells are mostly timely restricted. A special focus is set on immune activation mediated by extracellular present heat shock proteins (HSPs) carrying tumour antigens and further danger signals released by dying tumour cells. Local HT treatment in addition to further stress stimuli exerts abscopal effects and might be considered as *in situ* tumour vaccination. An increased natural killer (NK) cell activity, lymphocyte infiltration and HSP-mediated induction of immunogenic tumour cells have been observed in patients. Treatments with the addition of HT therefore can be considered as a personalised cancer treatment approach by specifically activating the immune system against the individual unique tumour.

### ***Keywords***

Hyperthermia, heat shock proteins, dendritic cells, immunogenic tumour cell death, abscopal effects

### ***Introduction***

Hyperthermia (HT) is a clinical treatment for malignant diseases, in which tumour tissue should be heated to minimum temperatures of 40-41 C for a sufficiently long period of time. HT has been proven to modify blood circulation thereby delivering oxygen into the tumour tissue, to result in increased metabolism leading to reduced ATP levels and increased anaerobic metabolites, to foster protein aggregation aggravating DNA repair, to sensitise tumour cells for radio- and chemotherapy, and to modulate the immune system (summarised in Schildkopf et al.). However, talking as radiation immune biologists about HT nearly always evokes divided reactions. This is mainly due to contradictory published results ranging from immune activation to immune suppression induced by HT. The inconsistent outcomes after HT are often due to different temperatures and exposure times used for the experiments. We will outline in this review old and new facts about how HT modulates the immune system and will carve out factors responsible for immune activatory modes of action of HT treatment. As hour of birth of immune therapy is often regarded the treatment of cancer patients with Coley's toxin, developed by Sir William Bradley Coley at the end of the 19th century. Killed bacterial cultures were directly injected into the tumour or the blood stream evoking strong fever with resultant spontaneous tumour regressions

observed in some patients. Those findings merit a place in history, but also in the present and the future. We will outline that 'historic results' have many implications for the future of HT as an additional element for anti-cancer therapy by inducing, besides many other effects, anti-tumour immunity, most pronounced when combined with radiotherapy (RT) and/or chemotherapy (CT) or further immune therapies (IT).

In the beginning of the 20th century many studies on the pathogenesis of fever were carried out and the linkage to cells of the innate immune system was made. It was discovered that fever in response to exogenous agents is mediated by a host phagocyte product called endogenous pyrogen (EP). Pyrogens are released by phagocytosing granulocytes and stimulate immune responses. EP was later found to be identical to interleukin (IL)-1, being a powerful immune stimulator. Such findings suggest a strong relationship between fever, heat and immune responses. HT was further found to affect metabolism and membrane potential of innate immune cells (granulocytes). Rosen suggested that increased body temperatures after HT lead to modifications of cellular membranes; the tumour cell loses its 'random properties'. He concluded that HT increases the efficacy of the immune system. The latter might be the main mediator of the abscopal effects observed after distinct settings of HT treatment. Before summarising the literature on immune modulations induced by whole body HT (WBHT) and local HT (LHT) application, we will briefly introduce the immune system.

### ***Basics about innate and adaptive immunity***

An immune response can be divided into two main phases, namely an initial non-specific phase (innate immunity) mediated by granulocytes, monocytes, immature dendritic cells (DCs), natural killer (NK) cells, and soluble factors such as cytokines and a later specific phase (adaptive immunity) mediated by cellular (T lymphocytes) and humoral (B lymphocytes) immune reactions. The initial innate immune response uses germline genes to recognise foreign substances or damaged tissue. The adaptive one utilises somatically rearranged genes to generate multiple structural specificities that allow the induction of responses specific to individual invading organisms and damaged cells (modified from Colaco). It has become clear that these two responses are integrated in the response to an infectious organism and to 'danger' in the body. DCs were discovered by the Nobel Prize in Physiology or Medicine 2011 laureate Ralph Steinman, being the antigen presenting cells (APCs) as the site of this integration.

### ***The innate immune system***

Cells of the innate immune system act as first line defence against invading pathogens. Via pattern recognition receptors (PPR) on the surface of phagocytes (monocytes, macrophages, granulocytes, immature DCs), pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide (LPS) are recognised. After binding to receptors, such as Toll-like receptors (TLR), the pathogens are engulfed by phagocytes. The latter secrete cytokines initiating inflammation and the induction of adaptive immune responses. Like PAMP, damage-associated molecular patterns (DAMP) are capable of active immune responses in a similar way (Figure 1.). DAMP result from damaged cells, for example, after exposure of tumour cells to RT and HT.

Besides phagocytes, NK cells belong to the cellular innate immune defence system. They possess activating and inhibitory receptors and recognise virus infected or tumour cells often displaying a reduced MHC class I surface expression.

### ***The adaptive immune system***

In contrast to non-MHC restricted tumour cell recognition by NK cells, the triggering of adaptive immune responses requires uptake and presentation of antigen via MHC molecules by APCs. DCs are immune cells connecting innate and adaptive immunity and belong to the group of APCs. After

recognition, uptake and processing of tumour antigen (Ag) resulting, for example, from damaged tumour cells, DCs mature and migrate into the lymph nodes (LN) to present peptides of tumour-associated Ag on their MHC complexes, express costimulatory receptors and secrete cytokines to prime naive CD4+ and CD8+ T-lymphocytes against the presented Ag (Figure 1.).

Antigens derived from intracellular sources such as viruses are presented on MHC class I to CD8+ T cells whereas extracellular-derived phagocytosed material such as tumour peptides/Ag are usually presented on MHC class II to CD4+ T cells. However, the latter mentioned material can also be cross-presented on MHC class I molecules, a prerequisite for priming of CD8+ cytotoxic T cells (CTL). CTL expand and traffic from the LN to the tumour where they exert their killing function. Importantly, efficient cross-presentation of tumour Ag requires both Ag uptake and a maturation signal for DCs resulting from, for example, necrotic tumour cells. Heat shock proteins (HSPs) comprise both functions by delivering tumour Ag and fostering maturation of DCs. DCs pulsed with heat shocked tumour cells mediated a significant enhanced CD8+ cellular cytotoxicity response against the tumour cells compared to pulsed DCs with lysates of non-heat shocked cells. It has to be stressed that adaptive and innate immune responses complement each other. When tumour cells shed MHC class I molecules to escape killing by CTL, NK cells are activated against the tumour since inhibitory receptors are no longer triggered by MHC I molecules. Furthermore, a cross-talk between NK cells and DCs exists. As example, NK cells were shown to foster the maturation of DCs and DC-NK cell contact promotes NK cell proliferation. Since hyperthermia in febrile (38-41 °C) or tumour therapeutic (41 °C) ranges is known to modulate interactions between the host immune and the tumour cells (summarised in Tomasovic and Klostergaard), we focus in the following sections on how WBHT or LHT modulate innate and adaptive immune responses.

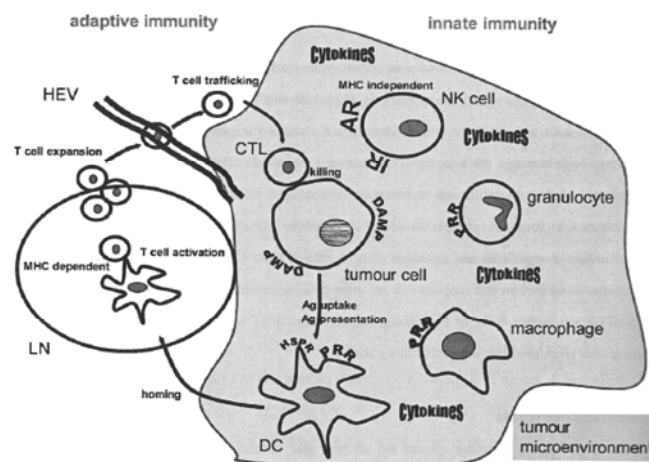


Figure 1. Hyperthermia modulates innate and adaptive immune responses

A general scheme of the main cells of the innate and adaptive immune systems that are involved in anti-tumour responses and can be modulated by hyperthermia is displayed. Heat damaged tumour cells expose damage-associated molecular patterns (DAMP) and can be recognised by innate immune cells via pattern recognition receptors (PRR). Natural killer (NK) cells are activated against the tumour via triggering activating receptors (AR) and inhibiting inhibitory receptors (IR) via MHC loss of the tumour cells. A beneficial cytokine milieu is created in the tumour microenvironment when innate immune cells are exposed to heat. Dendritic cells (DC) link the innate and adaptive immune response and take up heat shock protein/tumour antigen (Ag) complexes by way of heat shock protein receptors (HSPR). DCs present the tumour Ag to T cells and heat induces maturation and migration of DCs to lymph nodes (LN). There, T cells are activated in a MHC-dependent manner. They expand and traffick to the tumour cells by passing through high endothelial venules (HEV). Finally, the tumour cells are attacked and killed by the

activated CD8<sup>+</sup>T cells (cytotoxic T lymphocytes, CTL). As outlined in detail in the text, hyperthermia fosters in vivo all of the presented immune mechanisms against the tumour cells.

### ***General effects of HT on innate and adaptive immune responses***

Many studies have been carried out analysing the effect of WBHT on immune cell subsets. Of note is that local tumour treatments might also directly affect immune cells, since mononuclear leukocytes are commonly present in a heated tumour or in the surrounding tissues. For this reason, researchers started to examine the effects of HT on macro-phages. The response of macrophages to phytohae-magglutinin (PHA) measured by tritiated thymidine incorporation was enhanced when fever-range heat (38.5°C) was added while influenza virus depressed the macrophages' responsiveness. An enhanced production of virus-induced interferon by HT might be the cause of the beneficial effect of HT on responsiveness of influenza virus-exposed monocytes. Also lymphocyte transformation, mitogenesis, in response to PHA is temperature dependently influenced. Furthermore, IL-1 induced T cell proliferation was found to be increased by HT. This systemic activation of the immune system might contribute to target metastatic tumour cells by HT (summarized in von Ardenne). Another hint for the induction of anti-tumour immunity by heat is given by the fact that migration of Langerhans cells (DCs of the skin) is influenced by WBHT and that DCs primed with Toll-like receptor-agonists respond to HT (39.5 °C) with significant increased IL-12p70 secretion. This is the main cytokine that drives TH1 polarisation. In general, heat induces an up-regulation of TLR4 on DCs and macrophages. In addition, HT in the fever range (38-41 °C) resulted in in vitro experiments in a highly significant increase in L-selectin-dependent adhesion of these cells to LN high endothelial venules (HEV) and stimulates lymphocyte homing to secondary lymphoid tissues. This is mediated by an increased expression of L-selectin and alpha4beta7 integrin on circulating lymphocytes. They then interact specifically with HEV.

### ***Modulation of NK cells by HT***

The modulation of NK cells by WBHT is a double-edged sword. NK cell activity was found to be decreased at higher temperatures in several in vitro assays. However, NK cell activity was observed to be enhanced by HT in vivo indicating that additional factors such as interferons resulting from latent infections contribute to the immune stimulating activity of heat. WBHT with 40.5°C resulted in decreased burden of lung metastases in mice, and NK cells were found to be involved in those anti-tumour effects induced by HT. In xenogeneic tumour models with SCID mice, host NK cells and injected human NK cells were enriched at the tumour site after HT treatment. NK cell depletion led to highly reduced amounts of dead tumour cells after HT. However, WBHT also suppresses the granzyme B/perforin NK cell mediated killing of tumour cells. Nevertheless, combination of WBHT with stimulation of NK cells by alpha-galactosylceramide ligand significantly retarded tumour progression and enhanced survival of the mice. A summary of how HT modulates NK cell activities was published some years ago by Repasky and colleagues.

### ***Modulation of granulocytes by HT***

Several studies prove that even locally applied HT activates the innate immune system. HT increases the amount of neutrophilic granulocytes. Giving granulocyte colony stimulating factor (GCSF) in addition, a significant enhanced anti-tumour activity mediated via active oxygen species generation by neutrophils was observed in preclinical mouse models. Granulocytes displaying anti-tumour activity were enhanced in tumours after HT. This recruitment into the tumour could be also enhanced by addition of GCSF. Furthermore, an increase of the bactericidal

capacity of granulocytes at 40°C and 42°C relative to 37°C was observed for many, but not all bacteria. However, higher temperature did not influence, at least in the applied in vitro systems, bactericidal capacity of macrophages. The authors concluded that HT enhances certain host defence mechanisms. Furthermore, peritoneal macrophages were not functionally suppressed or injured by microwave hyperthermia in vivo.

### ***Modulation of T and B cells by HT***

In a similar manner to NK cells, beneficial and non-beneficial effects of heat on T cells were observed. HT resulted in a reduced in vitro cytolytic activity of CTL. Besides T cells, B cells are modulated as cells of the adaptive immune system by HT. In vivo, B lymphocytes are more susceptible to heat damage compared to T cells. However, this is only observed during the treatment, and many in vitro experiments were carried out just giving heat directly to the immune cells. In vivo, immune cells can recover and may interact with the immunogenic cells induced by HT. Heat induced transient lymphopenia in serum can even result in elevated levels of T and B cells in the spleen. T cells might migrate from the blood into tissue and come in contact with Ag. Experiments with immune deficient and silica (known to suppress macrophage function) injected mice and rats revealed that an activated macrophage-antigen-T cell processing is necessary for complete tumour cell destruction and systemic tumour control by local HT application. HT further enhances Fas-ligand mediated T cell cytotoxicity via over-expression of heat shock factor-1 (HSF-1). Fas-L is a type II transmembrane protein able to trigger cell death by binding to its Fas receptor (CD95). The expression of CD95 by tumour cells may enable their killing by T cells via CD95/Fas-L-dependent mechanisms. Figure 1 schematically summarises the plethora of cells of the innate and adaptive immune system that are directly and/or indirectly activated by HT. In general, starting from fever-like conditions (39°C), the immune cell activity of cancer patients is increased during heat application. High temperature short duration WBHT (41.8°C) enhanced the amount of lymphocytes, monocytes and granulocytes significantly shortly after treatment compared to low temperature, long duration WBHT (40°C). In addition to the duration of heat application, timing of WBHT application is crucial. Since heat fosters migration of Langerhans cells out of the skin, it should be applied after another stimulus. In this scenario, migrating DCs are capable of delivering Ag to the LN. The beneficial effects of WBHT accentuate the additional mechanism of killing of cancer cells directly by HT, as outlined below, and contribute to anti-tumour efficacy.

### ***Direct tumour cell killing by HT contributes to immune activation***

Overgaard's summary of the literature until 1972 indicated that tumour cells can be directly killed by HT with higher temperatures. Cavaliere and colleagues previously used temperatures of 42°C in their studies. Nowadays it has become clear that heat-mediated cell killing may result in immune stimulation. Early studies already revealed that the immunogenicity of the tumour cells can be increased by HT. Repeated local HT treatment of tumours resulted in significant reduction in the weight of retroperitoneal metastases in a mamma carcinoma model in rats. On the day of the HT treatment the tumours displayed necrotic areas and cellular injury. Some in vitro models have shown that Ag shedding of the tumour cells was fostered by HT. This again highlights that multiple tumour Ag should be accessible after treatment of tumours with HT, a scenario that can be achieved by induction of necrotic tumour cells with, for example, RT plus HT. HT further resulted in increased transcription of several tumour-associated Ag.

### ***Non-beneficial effects of HT on immune cells are timely restricted***

HT may also impair functions and counts of immune cells such as NK cells and monocytes. This effect is mostly time restricted (recovery usually after 48 h) and can be avoided by targeted

application of HT. Analyses of mRNA level on the effect of HT on immune cells within the tumour showed that the suppression in gene expression of a range of immune cells is only a transient phenomenon. Localised application of HT in a preclinical model with sheep resulted in an increased lymph flow and most importantly lymphocyte trafficking.

Local HT application on legs of mice resulted primarily in the suppression of NK cell activity. However, after 2 days the activity was partially recovered and even enhanced after another 5 days. Furthermore, the amount and density of Langerhans cells is increased after local application of HT on tongues of rats. A plethora of differences in beneficial and non-beneficial effects of HT are mostly due to varying heating times and stress conditions. It will be of great importance to focus in the future on distinct temperatures and heating times as well as efficient temperature controls. The thermal dose is mandatory (summarised in Milani and Noessner) and more complete time and temperature analyses in vitro and in vivo for various types of immune cells and end points are needed.

In the following we will go into detail how a local HT treatment finally acts systemically. One way this could happen is that HT-induced denaturation of proteins results in the unfolded protein response (UPR), in release of processed Ag bound to HSP, and finally to activation of T cells by those Ag presented by DCs (Figure 2.).

### ***HT-induced HSP-dependent immune activation can dominate over thermo-tolerance***

Before the 1980s HSPs were only known to chaper-one intracellular proteins after cell stress. Hsp70 may act as cell survival protein by inhibiting the permeabilisation of lysosomal membranes. It also protects tumour cells from monocyte cytotoxicity mediated by TNF. HSPs are highly conserved constituents of all kinds of pro- and eukaryotic cells and appeared for a long period of time only to be connected with thermotolerance. In addition, a simplified view that HT may lead to immune suppression via induction of thermotolerance in tumour cells and RT via destroying immune cells has been in the mind of clinicians, scientists and the public for many years. However, HSPs have to be regarded as double-edged swords.

Nowadays it becomes more and more evident that besides a general and timely restricted immune suppression, treatments with HT and/or RT may result in specific activation of the immune system by induction of distinct modifications of the tumour cell surface and distinct forms of cell death.

Extracellular HSPs are immunogenic 'New facts' about HSP-mediated immune effects arose with the key findings in the 1980s and 1990s that intracellular and inducible HSPs may become immunogenic when complexed with tumour peptides and that HSPs are also found outside the cells as well as located at the tumour cell surface. Viable tumour cells of a plethora of tumour entities expose Hsp70 on their surface. This represents a unique feature to discriminate them from non-cancerous cells. RT has been found to further increase the amount of Hsp70 on the surface of the tumour cells. Heat treatment also induces exposure of Hsp72 on the tumour cell surface, thereby rendering the cell susceptible to lysis mediated by NK effector cells. Of note is that HSPs are not only increased at the tumour cell's surface after RT, but can also additionally be released.

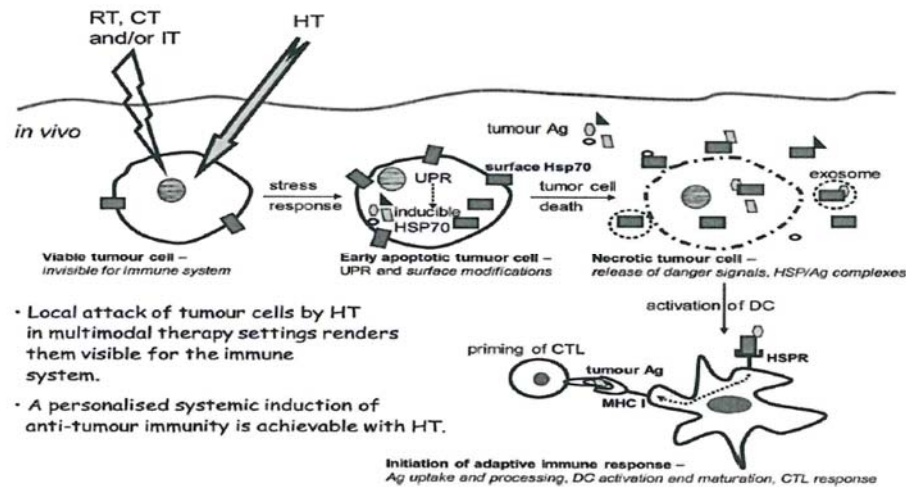


Figure 2. Hyperthermia modified tumour cells are rendered immunogenic and should be regarded as *in situ* tumour vaccine

When a tumour cell is heated, protein aggregation and denaturation induces a stress response in the cell, the so called unfolded protein response (UPR). Consequently, the transcription of inducible heat shock protein 70 (Hsp70) is increased and tumour cells expose even more Hsp70 on their surface. Furthermore, hyperthermia (HT) results in enhanced levels of tumour antigens (Ag) inside the cell. A second stress stimulus for the tumour cells such as ionising irradiation (radiotherapy, RT), chemotherapy (CT) or immune therapy (IT) together with HT fosters the induction of necrotic tumour cell death forms and modifies the tumour cell surface. Since necrotic cells have lost their membrane integrity, HSPs acting as danger signals and HSP/tumour Ag complexes are released. In addition, HSPs and tumour Ag containing exosomes can be discharged from apoptotic and necrotic tumour cells. Hsp70 containing exosomes derived from heat stressed tumour cells as well as HSP/tumour Ag complexes activate and attract dendritic cells (DC). The latter take up tumour Ag, present it with co-stimulation to CD8<sup>+</sup>T cells and thereby induce cellular anti-tumour immunity by priming cytotoxic T lymphocytes (CTL).

Increased serum levels of Hsp72 were detected in patients with prostate cancer. In addition, the expression of inducible Hsp70 correlates with increased tumour cell immunogenic properties when complexed with tumour antigens (discussed and summarised by Srivastava) by mediating antigen cross-presentation via MHC class I molecules. APCs utilise the uptake of HSP-chaperoned peptides for the loading of MHC class I molecules and thus stimulate a specific T cell response (Figure 2). Under oxidative stress, inducible Hsp70 was shown to be much more efficient in discriminating intracellular non-self-peptides than constitutive expressed Hsc70. The UPR occurs as a stress response towards heat and results in activation of the transcription factor HSF1 which regulates the transcription of Hsp70. The UPR was found to be responsible for the generation of new antigenic peptides since oncoproteins have unique characteristics and are processed via the proteasome.

Besides direct effects, activation of the immune system by HT also involves indirect effects mediated through release of HSP and HSP/peptide complexes. The mechanisms of Hsp70-mediated and DC-dependent induction of specific tumour cell killing by cytotoxic CD8<sup>+</sup> T cells are summarised in Calderwood et al. Active and passive release pathways of Hsp70 have been described. Passive release by necrotic cells and the consecutive uptake of HSP/peptide complexes by DCs can lead to efficient T cell priming. The current knowledge about the release of HSP by cells with intact membrane is based on the fact that intracellular Hsp70 is located in cholesterol-rich micro-domains and binds to globotriaosylceramide (Gb3). It might be shuffled to the outside of the cellular membrane via regular flip-flop mechanisms.



### ***Released Hsp70 is often associated with exosomes***

Hsp70-containing exosomes derived from heat stressed tumour cells have just recently been discovered to activate and attract DCs and T cells also via the chemokines CCL2, CCL3, CCL4, CCL5, and CCL20. HSPs interact with multiple surface receptors of APCs, such as CD91, LOX1, CD40, and TLR, thereby inducing the secretion of immune activatory cytokines. Released HSPs are therefore regarded as a danger signal that stimulate many steps in the induction of innate and adaptive immune reactions from DCs up to CTL activation (Figure 2.). Members of the Hsp70-related Hsp 110 family cooperate with Hsp70 in protein folding in the cytosol.

Heat-inducible proteins can be rendered more immunogenic by pre-treatment with heat, while heat-insensitive proteins were not modified in their vaccination efficacy. Hsp 110 prepared from mice treated with WBHT were significantly better vaccines compared to those proteins isolated from non-heat treated animals. The authors concluded that Ag presentation pathways might be influenced by HT.

HSPs are induced in target/tumour cells as well as in APCs. HSPs are part of intracellular aggregates with cytoskeletal proteins such as spectrin in immune cells. An exposure to mild HT (40°C) has the same effect on localisation changes of those aggregates within the cells as other stimuli leading to lymphocyte activation. DCs exposed to heat up-regulated Hsp70 and concomitantly the co-stimulatory markers CD80, CD83, and CD86. In addition, a significant increased capacity of heat stressed DCs to prime CD8<sup>+</sup>T cells was observed. Fever-like temperature further leads to increased expression of Hsp90 in immune cells and thereby induces maturation of DCs. Since HSPs are strongly induced by thermal stress and their expression is only weakly modulated by electromagnetic fields, the current notion is that heat acts as main stimulator of HSP expression and immune stimulation.

The exposure of Hsp70 on the tumour cell surface serves as a recognition signal for activated NK cells. SCID/beige mice are deficient for T and B cells and lack additionally functional NK cells. In those mice the growth of Hsp70-over-expressing tumours was not reduced compared with control tumours. In contrast, in SCID mice (having functional NK cells) bearing Hsp70-over-expressing tumours, NK cells were activated and killed tumour cells *ex vivo* that expressed NKG2D ligands. The migratory and killing activity of NK cells was found to be stimulated by Hsp70 positive exosomes derived from tumour cells. In general, the activity of NK cells can be increased by up-regulation of activating receptors and down-regulation or blocking of inhibitory ones. Heat also up-regulates Hsp60 that binds to HLA-E MHC molecules. This complex is no longer recognised by CD94/NKG2A inhibitory receptors.

Taken together, cells of the innate (NK cells and DCs) and of the adaptive (CTL) immune system become activated by heat stress induced Hsp70. Importantly, the necessity of a cross-talk between NK cells and DCs for the induction of specific anti tumour immune responses becomes more and more clear and Hsp70 is one mediator of this cell-to-cell contact-dependent interaction. For example, Hsp70 induced the expression of the NKG2D ligand MICA (the MHC class I chain-related protein A) on DCs.

### ***Immunogenic tumour cell death induced by combinatory treatments with HT***

Many additive and even supra-additive anti-tumour effects have been described when combining RT with HT. As one example, HT increases the blood flow and thereby improves tumour tissue oxygenation leading to a better outcome of RT. The complementary modes of action of RT and HT are summarised in Schildkopf et al. and van der Zee. One major factor of the enhanced

radiosensitivity of tumour cells after heat application is the inhibition of the repolymerisation step in the repair of base damages induced by RT. Just recently it was demonstrated that HT induces Breast Cancer 2 susceptibility protein (BRCA2) degradation and thereby inhibits homologous recombination. Besides radiosensitisation based on interference with cellular DNA, combinatory treatments of RT and HT may result in immune activation against tumour cells that have been modified by the therapy.

### ***Combination of RT with HT induce immunogenic necrotic tumour cells***

Modification of the tumour cell surface or the release of danger signals via damaged tumour cell membranes may render the tumour cells as visible targets for immune attack. Heated cells modify their surface in many ways, like that antibodies bind differently. HT treatment (43.5°C) enhanced cytotoxicity by antibodies mono-specific to a certain tumour Ag, suggesting that HT is capable of augmenting specific immune reactions against tumour-associated cell membrane Ag. Fresh spleen cells of mice inoculated with heat-treated tumour cells (42°C for 30 min) showed a clear tumour-neutralising activity. Tumour cells that are exposed to two stress stimuli, such as RT and HT, die of apoptosis and necrosis. Heat-induced apoptosis is mediated via caspase-9. Besides apoptotic cells, forms of programmed necrosis can also be induced in tumour cells after HT, RT or HT plus RT. Therefore, some effects of HT lie in killing of tumour cells and consecutive activation of the immune system. Already in the 1970s, Muckle and colleagues suggested that mode and form of tumour cell death and consecutive uptake of necrotic tumour cell material following treatment could be important in enabling the host to deal with metastatic cells. This indicates that local RT and HT treatment can induce systemic immune-mediated effects. Induction of tumour cell necrosis by local HT with 42°C resulted in complete disappearance of the tumour in one half of the mice. A long disease-free survival of the mice was observed (up to 18 months) suggesting that HT led to systemic tumour control. Of note, the non-surviving animals had defective immune systems. At temperatures below 41°C no necrosis occurred, whereas at temperatures between 42 and 45°C an increased rate of necrosis is observed. Necrotic cell death with release of danger signals can be induced by HT and finally leads to cross-priming of tumour Ag by DCs. In contrast, a chronic enhanced level of HMGB1 fosters inflammation, angiogenesis, evasion of cell death, and metastases. Of note is that even a resistance to tumour growth can be stimulated by pre-treatment of mice with two stimuli (heat plus X-ray) before tumour inoculation. In this model, the resistance was independent of T cells, as revealed by experiments with nude mice. One has not to conceal that WBHT with lower temperatures was not effective in inducing anti-tumour immunity and even led to compensation of anti-tumour immune responses triggered by whole tumour cell vaccines.

### ***Combinations of cytokines with HT induce immune activation***

Researchers and clinicians became even more aware in the 1980s that cancer is a complex disease not only related to the tumour cells themselves. To treat the very heterogeneous neoplastic diseases, a multimodal approach should be followed consisting of surgery, radiotherapy, chemotherapy, immunotherapy and/or HT. How HT enhances the cytotoxicity of chemotherapeutic drugs at multiple levels is excellently summarised by Issels.

The most prominent anti-tumour effect in mice bearing Lewis lung carcinoma was observed using IL-2 therapy combined with local HT. A beneficial targeting of metastatic tumours was also achieved with combinations of IL7, being important for B and T cell development, and HT. HT actually abrogated the inflammatory and thereby anti-immunotherapeutic effect of IL-8. Application of HT with a further trigger results in increased TNF-alpha levels secreted by macrophages. Distinct sequences for macrophage triggering or treatment of tumour cells with tumour necrosis factor plus application of HT is capable of augmenting the cytotoxic actions of

macrophages against the tumour cells. HT leads to fast, significantly increased, but timely restricted secretion of cytokines such as TNF-alpha and IL-1-beta fostering early activation of host defence immune mechanisms. This timely restricted pulsing of the immune system induced by HT is comparable to the new facts of action of HT where HMGB1 is released from tumour cells after RT plus HT. Combination therapies such as adding TNF-alpha to HT also resulted in massive tumour cell necrosis. As mentioned before, the pulsatile release of danger signals such as HMGB1 by necrotic cells fosters cross-presentation of Ag by DCs.

Taken together, especially combinations of HT with further stimuli may lead to efficient anti-tumour immune responses. Sublethal damages induced by CT or RT are rendered lethal by additional application of HT. This is again another important reason for the recommended combined cancer treatment with HT plus RT and/or CT. In preclinical models it has been shown that HT added to RT increases local tumour control rates from 25% up to nearly 90%, but also systemic tumour control is improved by combination of HT plus RT, as proven by longer survival rates of the patients.

### ***Combination of HT with further immune therapies induce immune activation***

Very effective anti-tumour responses were observed in preclinical models when HT was combined with further immune therapy, namely injection of DCs. DCs were activated and matured by Hsp70. In addition, HSPs fostered an increased CTL and NK cell activity. DCs loaded with melanoma cells that were heated to 42°C before killing were more efficient in priming of naive CD8<sup>+</sup>T cells than DCs loaded with unheated melanoma cells, indicating that HT fostered cross-priming of tumour Ag by DCs. In addition, heat shocked DCs themselves were potent stimulators of cytotoxic T cell responses against thyroid carcinoma. Treatment with Flt3L (which induced proliferation of DCs) and local RT led to abscopal anti-tumour effects and to eradication of small lung metastases. The induction of abscopal anti-tumour immunity and immunogenic tumour cell death by RT and further immune stimulation was recently summarised by Frey et al.

### ***DC activation after combined HT therapies as key inductor of anti-tumour immune responses***

In conclusion, local HT can lead to local, but also to systemic tumour control. A prerequisite for the latter is the migration of DCs that have taken up tumour Ag to LN (homing). Increased expression of CCR7 and decreased expression of CCR6 on DCs are required for their functional migration to regional LN. Exactly this expression profile was observed on mRNA level for Langerhans cells after local HT and also on protein level after contact of DCs with supernatants of tumour cells that have been exposed to HT plus RT. The release of Hsp70 is significantly enhanced when HT is added to RT and extracellular Hsp70 is one main player in inducing DC maturation and homing. We have to stress again that the release of immune activatory proteins such as Hsp70 and danger signals such as HMGB1, which are normally located inside the cells, after local treatment with HT plus RT mediate abscopal anti-tumour effects (see below and Figure 2). Radiotherapy regimens and certain chemotherapeutic agents trigger forms of cancer cell death that stimulate an active immune response against the tumour. RT plus HT stimulates the DC-mediated CTL response against the tumour, but also increases the expression of activating NKG2D ligands for NK cells on tumour cells thereby activating innate immune defence mechanisms. The number of cytotoxic T cells and NK cells is significantly increased after local HT treatment of melanoma in mice. In addition, increased amounts of activated monocytes (CD11b<sup>+</sup> CD69<sup>+</sup>) were present in the tumour microenvironment after HT.

### ***Local HT applications result in systemic immune activation***

First hints for an activation of the immune system after local tumour treatments were the improved survival times of patients with melanoma. A combination of IL-2 and local HT was beneficial in preclinical models for certain metastatic tumours. Tumour cells with high metastatic potential were more sensitive to HT treatment and the survival rate of mice bearing metastatic B16-F10 tumours was increased after HT. Infiltration of NK cells and macrophages into the tumour, containing necrotic melanoma cells induced by HT, takes place after local HT treatment. Notably, the addition of rIFN-beta to HT had no effect on NK cell infiltration but significantly increased the amount of T cells in the tumour. In another preclinical study using the MCA-105 sarcoma metastatic cell model, WBHT in adjunct to immune therapy had no significant effect on tumour growth.

However, the combination of LHT and immune therapy with lymphokine-activated killer cells significantly decreased the number of pulmonary metastases. LHT in addition to further stress stimuli might be considered as an autologous in situ tumour vaccination, as it is also true for RT combined with immune activators such as AnxA5. Zhang and colleagues recently summarised in detail how LHT alone and more importantly in combination with further immune therapy or RT is capable of rendering tumours immunogenic in situ. Immune defence mechanisms have always to be considered for the treatment of small tumour masses, recurrent tumours, metastases and micrometastases (being not displayed by current imaging techniques). Combinations of RT, CT or surgery with HT kill two birds with one stone: the primary tumour is reduced in size by the RT, CT or surgery, and residual tumour masses and metastases are killed via immune activation with the addition of HT (Figure 2.).

### ***HT activates systemic anti-tumour immune responses in cancer patients***

HT and immunity fit together. The clinical effectiveness of HT treatment in multimodal settings is described in detail in other papers of this special issue. Many randomized clinical trials have proven the effectiveness of HT when combined with RT, RCT, or CT. Recently, a randomized phase III multi-centre study has demonstrated that regional HT significantly increases the benefit of CT in adult cancer patients with high-risk soft-tissue sarcoma. In the following we briefly mention some further studies clearly indicating that application of LHT and WBHT mostly in addition to RT and/or CT contribute to systemic tumour immune defence mechanisms. Patients with advanced adenocarcinoma of the prostate receiving local HT treatment displayed a significant NK cell cytotoxic activity when compared to the pretreatment status. An increased NK cell activity after local HT application was even observed in patients with liver cancer, being a tumour hard to treat with HT because of its high perfusion. Preoperative radio-chemotherapy combined with local HT led to an increased lymphocyte infiltration and increased survival rate of patients with oesophageal cancer compared to radio-chemotherapy treatment alone. A prolonged T cell activation was observed after WBHT at higher temperatures (41.8 °C) in addition to CT in patients with metastatic colorectal carcinoma. Currently, combinations of HT with DC-based immune therapy are tested for therapy of squamous cell carcinoma. Without RT, a three-step therapy setting (HT with 43 °C and 41 °C and DC vaccination) seems to be most beneficial in inducing anti-tumour immunity.

In cervical cancer, a study comparing RT with RT plus HT revealed that the percentage of patients with continuing pelvic control developing metastatic disease was significantly lower in the group with combined treatment. This might be due to the HSP-mediated induction of immunogenic tumour cell death. Clinical trials using HSP/ peptide complexes have been carried out. Patients showed a longer disease-free survival indicating that systemic anti-tumour immune responses were induced. HSPs become hyperabundant under stressful conditions. We emphasise that HSPs

induced by HT may contribute to anti-tumour immunity. Smaller tumour masses in early stage diseases or metastases are attacked by those immune-mediated mechanisms.

A phase III trial showed that M1a and M1b stage IV melanoma patients receiving larger numbers of immunisation with autologous HSP gp96-peptide complexes survived longer compared to patients receiving fewer treatments. The clinical results are consistent with observations in mouse models examining the 'immunological power' of HSP-based vaccines. Another randomised phase III trial with adjuvant treatment in renal cell carcinoma revealed that patients in early stage disease have a hazard ratio for recurrence of disease of 0.57 when receiving the HSP/peptide vaccine. Of note, both trials failed to meet their end points with respect to the intention-to-treat population (receiving the HSP/peptide vaccine). Nevertheless, retrospective subgroup analyses clearly showed that distinct groups of patients receiving multiple vaccinations significantly profited from the immune therapeutic 'vaccitherapy'.

### ***Short outlook***

Repeated in situ induction of HSP tumour peptide complexes by HT treatment in combination with strong death stimuli for the tumour cells such as ionising radiation might result in great clinical benefit in the future. The in situ induction or vaccination with autologous HSP tumour material applies the strong immunogenic potential of the unique tumour composition of each individual patient as therapeutic option. A personalized cancer treatment approach that takes into account the individually unique tumour composition is feasible by combining HT with further standard and immune therapies (Figure 2.). The temporarily restricted mode of modifying the tumour microenvironment by HT likely prevents the development of immunological tolerance. Hence, repeated HT treatment cycles included in multimodal therapy settings should be arranged. HT and induction of local and systemic anti-tumour immunity are things that have and still fit together even closer.

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