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Preclinical Verification of Modulated Electro-Hyperthermia

—Part III. Immunogenic Effects

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Abstract

The modulated electro-hyperthermia (mEHT) method is a unique approach that utilizes all the essential apoptotic pathways through an external radio-frequency (RF) signal. The high-frequency RF is amplitude-modulated and coupled capacitively to the target. The provided energy triggers the death receptors and FAS-FADD complexes in the malignant cells. Multi-pathway apoptosis produces immunogenic cell death (ICD). This ICD provides intracellular information about cancer cells by releasing damage-associated molecular patterns (DAMP), including membrane expression of calreticulin (CRT) and extracellular ATP, HMGB1, and HSP70, executing tumor-specific antigen presentation. The antigen-presenting cells (APCs) play a crucial role in reestablishing immune surveillance and hampering the tumor cells' ability to hide, thereby evading immune attacks. The matured DCs (generally APCs) produce tumor-specific killer and helper T-cells, which have the potential to be active in distant metastases from the treated location. This unique mechanism of action underscores its potential in cancer treatment and extends the local mEHT treatment to the whole body anticancer therapy with an abscopal effect.

Keywords

mEHT, Cancer, Thermal, Nonthermal, Immunogenic, ICD, DAMP, Tumor-Specific Immune

1. Introduction

Hyperthermia in oncology has its roots in ancient medicine. Heat is regarded as the overall "healer," expecting unspecific effects of the body's natural corrective systems for different symptoms, expecting normalized homeostatic regulation,

including immune surveillance.

Hyperthermia is an external disturbance that induces the contra effects of homeostasis, which is the body's natural tendency to maintain a stable internal environment despite changes in external conditions. Thermal homeostatic regulation acts to cool down the stressfully overheated volume. The extreme heat triggers thermal homeostasis to correct the irregularity, extending the therapy task to consider the regulating counteractions.

Hyperthermia must find a way to synergize its cooperation with homeostatic regulation. It must support normal homeostatic processes and avoid provoking physiological regulations like intensive blood perfusion in the target, which may increase the risk of tumor supply and dissemination of malignant cells. The critical components of homeostasis (receptors, neural control, and effectors) must be smoothly tuned to fix the homeostasis at a new, physiologically accepted equilibrium below the thermal limit (42˚C in all regions). Focusing on the signaling (social signals) of cells [1] and the pathways of cell communication [2] are the key processes that ensure the harmony of hyperthermia with homeostasis.

Oncological hyperthermia faces many technical and medical challenges that are far from a final solution. The complexity of electromagnetic interaction and the physiological feedback mechanisms need extended explanations. Incomplete knowledge of electromagnetic biological and medical processes and the lack of precise measurement of the applied dose hinder the widespread use of hyperthermia in oncology.

Oncological hyperthermia needs to rethink its strategy in the light of immuno-oncology. The strategy to eliminate the tumor must use the well-known military consideration: attack the weakest side of cancer, avert a direct fight with its most potent forces, and stop trying directly to inhibit the most robust property of the malignant development [3]. Cancer's main strength is its proliferation, which makes these cells adaptable and forms the tumor immortal. However, their weakest side is their autonomy, their evading ability of immune surveillance. Cancer cells fight against all healthy and fellow malignant cells, competing for the energy sources to proliferate. The lack of healthy networking creates weak cooperation abilities during malignant development [4]. The weak networking and the "loneliness" behavior make the malignant cells vulnerable. The weak network otherwise helps the proliferative development due to the easy motility and micro and macro metastases forming.

The hyperthermic strategy must be revised to attack the weak side of the malignancy with an alliance of the host system itself. The goal is to provide tumor-specific information to the immune surveillance activity to recognize the malignant cells despite their hidden "identity." The active tumor-specific immune process and the thermally increased enzymatic activity offer a reliable tool to fulfill our final aim of eliminating cancer cells all over the body.

Optimal curative heating helps the natural control to reverse the irregularities caused by cancer and supports the immune processes against the malignant ef-

fects. The modulated electro-hyperthermia (mEHT) uses thermal and nonthermal effects synergically, increasing the impact of only heat. The rapidly developed method changed its focus over time [5]. Electromagnetic energy absorption has a double effect: heating for optimal conditions and acting nonthermally to modify the chemical processes [6] [7]. The synergy of these effects supports the homeostatic control with the heterogenic actions: selects the tumor cells and forces energy absorption in microregions of their cell membrane (membrane rafts), heats them high, and excites multiple apoptotic pathways [6] [7], promoting the lost apoptotic surveillance of the homeostasis. Furthermore, the heated microdots (the rafts) heat as secondary heat conduction the cells and the complete tumor, which on this heat transport remains in mild temperature, which does not induce extensive counteraction of the homeostasis. Furthermore, this article aims to present the immunogenic effects that restore the immunosurveillance of the homeostatic control.

The mEHT became a reliable alternative to conventional hyperthermia, concentrating on the molecular and physiological processes directed to immunogenic effects, heading to immuno-oncology [8] [9].

2. Methods

The primary effect of mEHT works in harmony with natural homeostasis. The method uses physiological processes and a modulated radiofrequency (RF) current, which allows the precise targeting and destruction of malignant cells in four steps. These steps involve the use of thermal and electric heterogeneities caused by malignant proliferation.

1) Malignant cells, which are characterized by their high metabolic rate, produce a high ionic concentration in the tumor microenvironment (TME). The amplitude-modulated 13.56 MHz RF current chooses the highly conductive paths of the TME, automatically selecting these tumors.

2) The missing healthy network makes the TME significantly disordered (high dielectric constant), which allows the applied RF to recognize the malignant cell environment.

3) The broken bonds of the network produce multiple transmembrane proteins and their clusters (rafts). The rafts are good energy absorbers from the RF current [10].

4) The autonomic cancer cells are unsynchronized, which can be recognized by modulation [11].

The relatively low average temperature accompanies a high thermal effect of mEHT on the transmembrane proteins concentrated primarily in the membrane rafts (**Figure 2**). This solution ensures a high temperature on the membrane of malignant cells, which are selected based on the high concentration of membrane rafts [12]. At the same time, whole tissue temperature is only moderately increased, which increases blood flow sufficiently to support radiation therapy (RT) [13] and drug delivery [14]. However, it does not promote cell dissemina-

tion and has no thermal toxicity. In this way, mEHT providessafeand effective hyperthermia (HT).

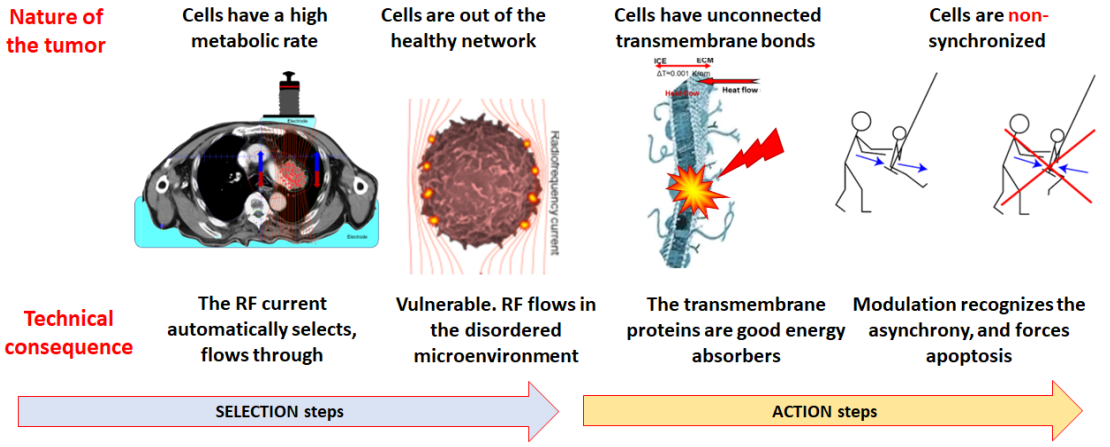

Figure 1. The steps of mEHT: 1) find the tumor with its high metabolic rate, 2) find the cells with their disordered TME, 3) absorb energy and excite transmembrane proteins, 4) Make harmony with homeostatic control.

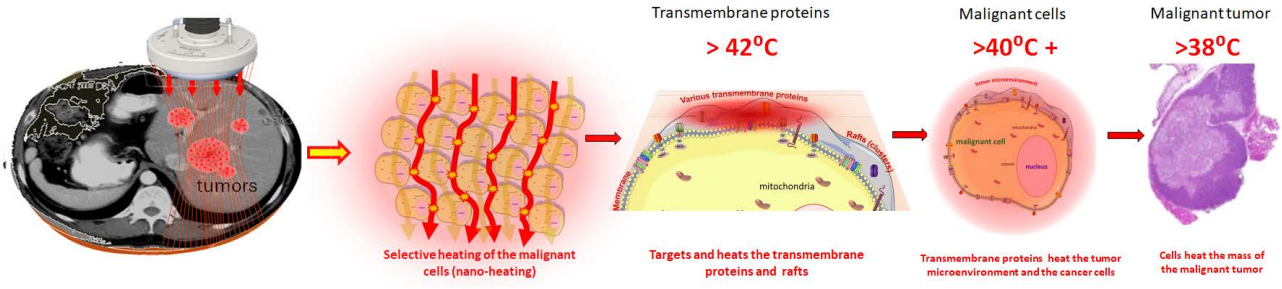

Figure 2. Temperature development with mEHT action: 1) RF current goes through the body part and selects the tumor, as shown in **Figure 1**. 2) The RF current mostly flows between the cells because of the electric isolation of cell membranes, 3) the membrane rafts absorb energy and are heated up at high temperatures, 4) The heated rafts heat the entire cell with conductive and convective actions, 5) the hot cells heat the entire tumor to mild temperatures.

The mEHT (modulated electro-hyperthermia) technique diverges from the traditional homogeneous (isothermal) heating approach [13], utilizing bioelectromagnetic properties to deliver nonthermal energy, too. This energy stimulates death receptors and other molecules, initiating apoptotic signals within cancer cells [15]. Unlike inflammatory necrosis, which can induce cancer due to the inflammation it causes, mEHT promotes controlled programmed cell death (apoptosis and necroptosis) [6] [7] [16]. This method avoids uncontrolled thermal necrosis, which involves membrane rupture and the release of cellular contents that can harm healthy tissue and trigger inflammation.

Necrosis triggers inflammatory immune reactions, whereas apoptosis allows the immune system to clean up without causing inflammation. Apoptosis is an immunogenic (immune response-inducing) form of cell death, provoking a robust immune response. Immunogenic cell death (ICD) is the pathway that activates the immune system against the dying cells. While apoptosis is a common

pathway for ICD, some recent research has identified another immunogenic pathway: necroptosis. Necroptosis is a regulated, programmed form of cell death with some necrotic characteristics.

Effective immunogenic cancer elimination requires tumor-specific information to prepare the immune system for innate and adaptive responses, targeting malignant cells throughout the body via the bloodstream. Antigen-presenting cells (APCs) play a crucial role in this process. Various cells can function as APCs, including professional APCs like dendritic cells (DCs), macrophages, and B cells, as well as nonprofessional APCs like endothelial cells, fibroblasts, and epithelial cells, which can present antigens to T cells under certain conditions.

The controlled apoptosis does not randomly destroy the cell membrane, allowing the cell to fragment into apoptotic bodies. This process releases damage-associated molecular patterns (DAMPs), which further signal and prepare the immune system.

Calreticulin (CRT) is expressed in the cell membrane in the set of DAMP molecules. The ER stress response forces the CRT [17], which has an "eat me" signal for phagocytosis [18] [19]. Apoptosis expresses the cell membrane exposure of CRT, while necrosis does not. Necroptotic processes may do it in some peculiar conditions. The CRT-controlled strong Ca^{2+} influx [20] is a significant factor in apoptosis caused by mEHT [21]. The other member of the DAMP set is the released HMGB1, which represents a "danger signal" [22]. The HMGB1 contributes to immune activation, but contrary, its oxidized form makes the inflammatory responses [23]. Furthermore, the oxidized HMGB1 participates in immune tolerance [24] and may boost the immune checkpoint molecule PD-L1 expression, limiting anticancer immunity [25]. Therefore, the oxidation status of HMGB1 determines the role of HMGB1 in DAMP [26]. The mild thermal process of mEHT favors conditions that do not support oxidization. HMGB1 is a valuable set of DAMP in mEHT processes. Furthermore, ATP is released to the TME as a stress response in the apoptotic phase [27]. The released ATP functions as a "find me" signal [28] and mostly follows an autophagy-dependent pathway [29]. The complex thermal and electric (nonthermal) stress induces heat shock proteins (HSPs) in the cell, functioning as an antiapoptotic chaperon. When the intracellular HSPs (iHSPs) are overloaded in the cell, these start translocation to the malignant cell's membrane [30], forming membrane HSPs (mHSPs) [31]. The mHSP expression may subsequently alert the innate immune reaction, attracting the NK cells to the mHSP location [32] and trying to eliminate the malignant cell. In the further process, the mHSPs are released extracellularly. Extracellular HSP (eHSP) appears as an "info signal" [33], carries tumor-specific antigen [34], and may build up a tumor immunity [35]. The delivered eHSP peptides can be identified by the innate and adaptive immune systems [36] [37]. In the above set of DAMP molecules (CRT, HMGB1, ATP, and HSP), the eHSPs are crucial components [38]. The spatiotemporal development of DAMP activates APCs to express high levels of MHC class II molecules, essential for presenting antigens to T cells. The mEHT method gently kills tumor

cells with programmed cell death, releasing damage-associated molecular patterns (DAMP) [39] and delivering information about malignancy hidden in the intact cancer cell (Figure 3).

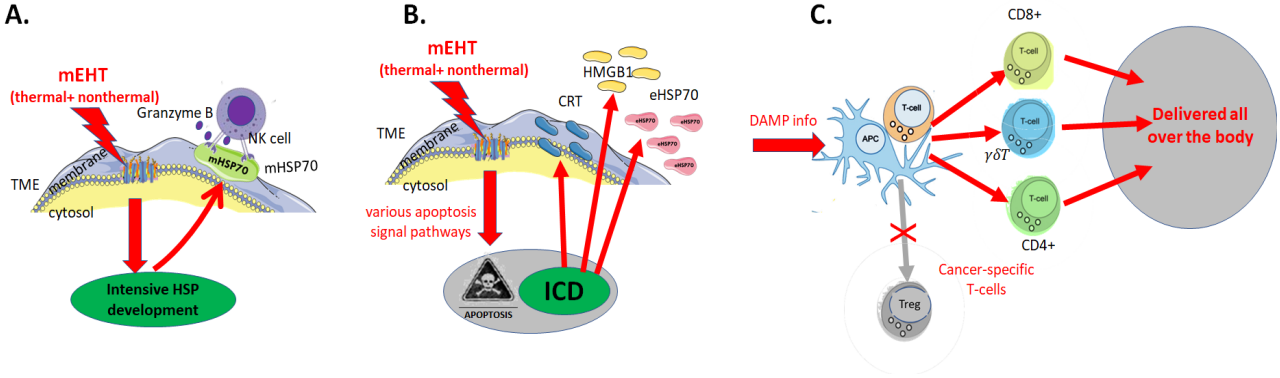

Figure 3. The immunogenic processes. (A.) The broken immune evasion starts with the expression of HSP70 on the membrane, which attracts the NK cells to destroy the cell with released Granzyme B. (B.) The ICD develops DAMP in optimal spatiotemporal order. (C.) The APC cells produce T cells with tumor-specific information to recognize the cancer cells.

Conventional hyperthermia has a relatively high average temperature in the tumor mass (> 40˚C) which inactivates the immune cells in the volume [40], and the uncontrolled production of DAMP cannot activate the antigen-presenting APC processes. The mEHT focuses on apoptotic effects hindering the uncontrolled necrotic processes. The "gentle" apoptotic cell death as apoptosis produced by mEHT in moderate average temperature allows obtaining the undeformed spatiotemporal order of the DAMP set of molecules with complete genetic information orchestrating the APC activity for tumor-specific antigen presentation. The relatively mild hyperthermia process keeps the average temperature below 40˚C, to ensure the activity of the immune cells, including the APC "professionals".

Choosing the model animal for various experimental setups is crucial for immunologic studies. Immunocompetent or deficient (immunocompromised) model animals express different reactions in immune modulatory experiments. Immunocompromised mice were chosen when two independent distant tumors were measured, with the untreated tumor as the control on the same animal. The two distant tumors may be connected in immunocompetent mice and model a distant metastasis.

The mEHT method uses an amplitude-modulated 13.56 MHz carrier frequency. The modulation has a special frequency distribution to harmonize with the homeostatic regulation. The experimental device was LabEHY (Oncotherm Kft. Budaors, Hungary). In the earlier parts of this series, we described the *in vitro* [6] and *in vivo* [7] experimental setups.

3. Results

Numerous studies were devoted to investigating the immunogenic effect of

mEHT. The immunogenic effects were proven in multiple *in vitro* and *in vivo* model systems. The leading publications are listed in Table 1.

Table 1. Leading publications on immunogenic preclinical research with mEHT.

Tumor cells	Method	Result	Conclusion	Ref.
HT29 xenograft model. BALB/c nude mice treated with mEHT.	mEHT 42˚C, 30 min, 4 W, time-course study with Immuno-histochemistry, microarray, apoptosis array.	Measured damage-associated molecular patterns (HSP70, CRT, HMGB1 and HSP90).	mEHT induces a spatiotemporal sequence of DAMP signals HT29 cancer, and it can be a potential local inducer of immunogenic cell death.	[41]
C26 allografts of immunocompetent (BALB/c) mice plus immunostimulant MTE.	mEHT 42˚C, 30 min, 1 - 3 W, Immunohistochemistry, TUNEL assay apoptosis analysis, comparison of the treated and nontreated tumors of the same mouse. Extra immune stimulant (MTE) is applied.	The damage-associated molecular pattern was measured (CRT, HSP70, HMGB1). Ki67 was suppressed. Dendritic cells were matured, and antigen-presenting showed tumor-specific killer T-cells and their invasion into the tumor.	The mice with immune stimulants had an abscopal effect, showing the same development in the untreated tumor as in the treated one. ICD formed tumor-specific immune reactions.	[42]
CT26 allografts of immunocompetent (BALB/c) mice plus dendritic cell immunostimulant.	mEHT 42˚C, 30 min, DC cells inoculated intratumorally to the femoral area, cytotoxic T lymphocyte assay, ELISPOT, Immunohistochemistry.	Significant HSP70 release was measured in comparison to wHT with the same temperature. The DC-combined mEHT inhibited cell growth, and the considerable appearance of CD45+, F4/80, and Eosinophil leukocytes was measured.	mEHT can create a favorable tumor microenvironment for an immunological chain reaction. Rechallenging the same tumor was ineffective. The DC-combined mEHT worked as an antitumor-vaccination.	[43]
A2058 human melanoma cell line and NK cell immunotherapy combined with mEHT.	mEHT 42˚C, 30 min, NK cells or IL-2 independent NK-92MI injected subcutaneously to right and left femoral region. Only the right side was treated.	Intensive hsp70 expression followed by significant upregulation of cC3 and p53. NK cell accumulation was in the treated tumor but not in the untreated side. Upregulation of CXCL11 and MMP2 was observed.	mEHT ultimately lead to the complete eradication of melanoma and appears to be a good combination with NK immunotherapy.	[44]
C3H/He mice inoculated with SCCVII to the left femoral and chest region. Dendritic cell immune stimulation was applied.	mEHT 42˚C, 30 min, Flow cytometry, immunohistochemistry, CD3+, CD4+ and CD8+ immune-cell detection.	The CD8+ and S100 were more strongly expressed in the DC plus mEHT treatment group than in control or stand-alone therapies, although Foxp3 expression was much higher in the control group.	A significant abscopal effect was measured between the treated femoral and untreated chest regions of the mice. The local treatment also became a whole-body immune attack on the metastatic lesions.	[45]
CT26/BALB/c mice tumor model mEHT with Cur and Res combined treatment.	mEHT 42˚C, 30 min, 10 - 12 W, tumor growth, and immune cell infiltration were measured with cell viability, apoptosis, Western blot, and immunohistochemistry comparison with wHT.	mEHT with Cur and Res combination inhibited the growth of CT26 cancer by inducing apoptosis and HSP70 expression of tumor cells while recruiting CD3+ T-cells and F4/80+ macrophages.	Cur and Res combined with mEHT synergistically increased HSP release and immune response, showing enhanced anti-tumor efficacy.	[46]

Continued

B16F10 allograft cells inoculated to female C57Bl/6 mice (offspring of C57Bl/6 colonies) in the right inguinal area.	mEHT 42˚C, 30 min, measured tumor-size, HSP70, PUMA, γH2AX, DAMP signaling was measured.	Upregulation of cytoplasmic and cell membrane HSP70 Increased PUMA and AIF1 and rise of cC3 without significant apoptosis. But, γH2AX indicated DNA double-strand breaks, which upregulated p53 protein and downstream p21waf1 and p27kip. mEHT promoted the release of DAMP, it reduced MHC-I levels in tumor cells.	mEHT-related tumor shrinkage was primarily mediated by p53, upregulating the cyclin-dependent kinase inhibitors. Reduced cytotoxic T-cell response was observed despite increased DAMP signaling. Decreased tumor antigen and MHC-I levels suggest that NK cells and macrophages were the major contributors to tumor eradication.	[47]
Effect of repeated mEHT treatment in 4T07 TNBC cells inoculated orthotopically in female BALB/c mice.	mEHT 42˚C, 30 min, skin temperature just over the tumor 40˚C (3 W). The repeated treatments were studied by tumor size, cC3, HSP70, immune profile (CD3+, CTLA4, PD-1, PD-L1), p21, and Ki67.	The five-treatment repetition shows a significant positive change in all measured parameters in 4T07 TNBC.	Repeated treatment improves the effect of mEHT and is well applicable for TNBC tumors, suggesting positive clinical performance.	[48], [49], [50]
BALB/c mice were treated with mEHT, curcumin, and resveratrol in the CT26 allograft model.	mEHT 42˚C, 30 min, dose dependence is measured by proliferation assay, apoptosis, cell viability, Western Blot, FACS, CD3+, F4/80 and HSP70 were measured.	Immunogenic cell death was observed with intensive DAMP at the combined treatment.	Resveratrol and curcumin significantly increased the immunogenic effect of mEHT treatment.	[46]
mEHT treatment studied *in vitro* and *in vivo* with HepG2 cell line.	mEHT (42˚C, 30 min) was used combined with γδT cells 4 h after treatment, and *in vivo* in SCID mice comparing the combined treatment with the standalone therapies.	The mEHT treatment caused γδT ll migration to tumor cells even at 38˚C, where wHT had no effect. *In vivo*, a considerable inhibition of tumor growth was observed.	The γδT cells combined with mEHT significantly destroy tumor cells *in vitro* and *in vivo*.	[51] [52]
mEHT for B16F10 melanoma pulmonary metastases. Repeated treatment of lung.	mEHT 30 min, 42˚C (lung), 40˚C (pharyngeal) and 38.5˚C (rectal temperatures). Measured immunohistochemistry, flow cytometry, and apoptotic and necrotic cell death. 6 times repeated treatment.	mEHT induced significant anti-tumor effects and the downregulation of Ki67 expression, as well as made significant DNA double-strand breaks, significantly increased CD3+, CD8+ T-lymphocytes, and F4/80+CD11b+ macrophage density in the whole lung.	mEHT inhibits tumor growth and spontaneous proliferation of B16F10 melanoma in a mouse pulmonary metastasis model. mEHT is a complementary therapeutic option to chemo- and/or radiotherapy. The massive infiltration of tumors by CD3+ and CD8+ T-lymphocytes and by F4/80 CD11b-positive macrophages indicates the ability of mEHT to mobilize the immune response in treated animals.	[53]

Numerous furtherpublications dealt with the immunogenic effect of modulated electro-hyperthermia and presented its systemic tumor-specific immune effect using only a local treatment [54]-[56]. Thermal and nonthermal effects synergy became a useful complementary tool for radiotherapy, forming dynamic and well-regulated tumor-specific immune reactions following abscopal effects [57] [58]. The abscopal outcome creates a new perspective for the local treatment, transforming to systemic immune impact on the malignant cells in the entire body, targeting both the micro and macro metastases [59]-[61]. The immunogenic processes may also be gained by oncolytic viral treatment enhancing the immune reactions of the therapy [62] [63].

The developed released HSPs by the stresses of various treatments show the considerable addition of the nonthermal component after 24 h to the standard thermal one *in vitro* measurements [43] [64] (**Figure 4(A)**). Due to the intensive stress with mEHT, the results show considerable HSP release from the tumor cells *in vitro* (Hep-G2 cell line), which makes it possible to transfer the tumor-specific information from the cancer cell. The high iHSP content exhausts the cellular protection, as was shown *in vivo* in 4T1 TNBC isograft experiments [48], and makes it possible to express the membrane and release the molecules to TME. A massive intracellular presence and moderate membrane and extracellular expression were observed in a study of a B16F10 melanoma tumor *in vivo* [47] (**Figure 4(B)**). The eHSP70 proteins appear almost immediately posttreatment *in vitro* [64], while *in vivo*, it takes more time (2 days) (**Figure 4(C)**) having iHSP70 in the first 48 h period [41]. Due to the suppressed chaperone activity, inhibiting HSP synthesis in the B16F10 melanoma cell line with Quercetin *in vivo* suppresses tumor growth [65].

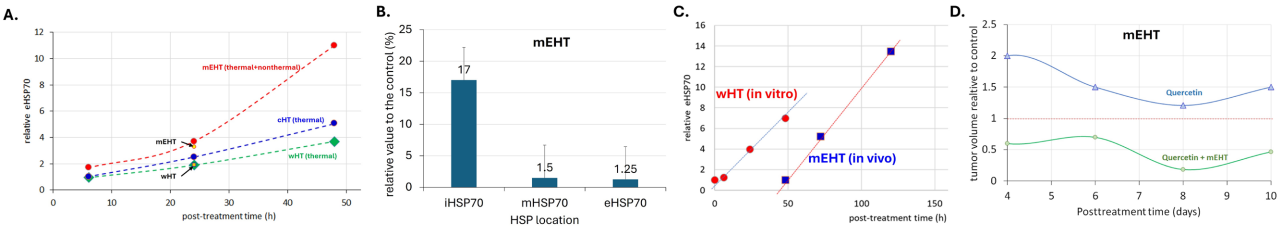

Figure 4. Development of HSP70 by mEHT. (A.) The relative expression of HSP70 in the extracellular matrix [43]. (B.) The relative values of HSP70 proteins in various locations [47]. (C.) The development of the eHSP70 *in vitro* [64] and *in vivo* [41]. The inhibition of iHSP synthesis also inhibits the tumor growth [65].

As we had proved in the previous two parts of the present series [6] [7], the mEHT triggers massive apoptosis. The model animals were BALB/c nu/nu immunocompromised mice with two distant tumor inoculations of the HT-29 human colon cancer cell line. The right tumors were treated, and the left untreated tumors were used as a control (**Figure 5(A)**). The first experiments investigated how independent the distant untreated tumor is from the treated one, observing the tumor growth for a longer time after mEHT treatment. The untreated liaison in the xenograft of immunocompromised mice changes in the standard way,

with some necrotic nodules inside. The dead tissue in the treated tumor of the same animal grows over time and develops a leukocyte invasion ring 72 h post-treatment (**Figure 5(B)**). Intensive apoptosis appears in the ring in which cCas3 and late apoptotic detection can follow [66]. Late apoptosis showed the opposite development in treated and untreated tumors [67] (**Figure 5(C)**), the apoptosis continues in the treated and is inhibited in the untreated tumor. The experiments proved that the untreated tumor can be used as a reference in the double tumor model in immunocompromised mice.

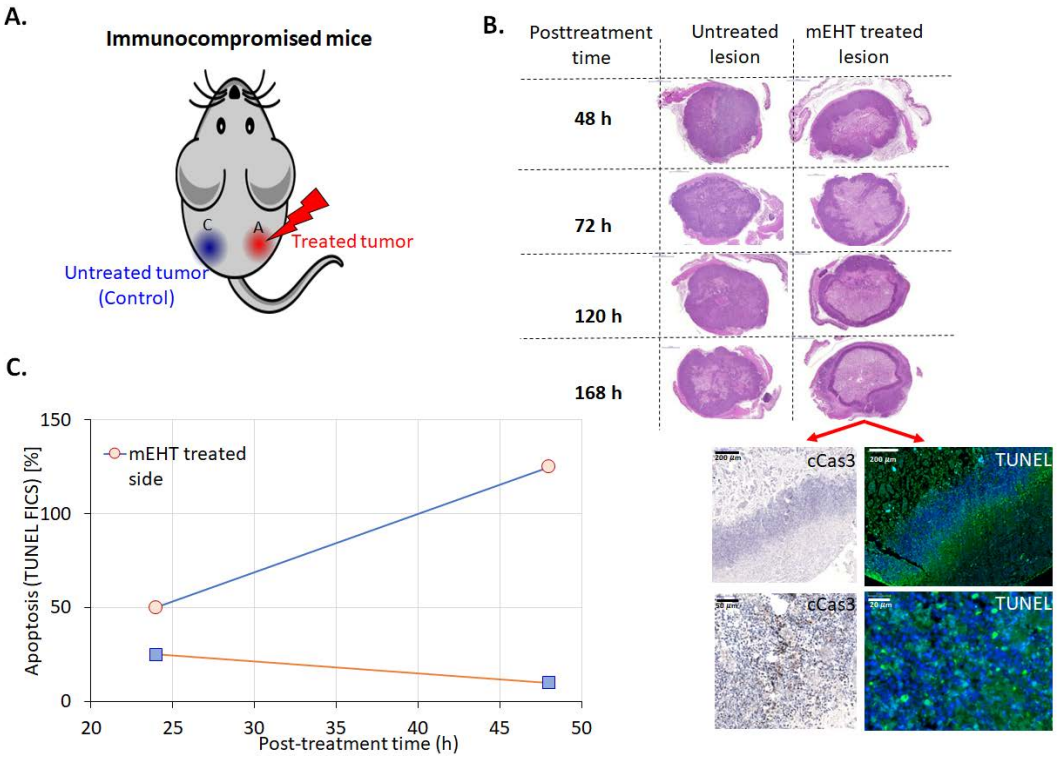

Figure 5. The double tumor connection in immunocompromised mice [66]. (A.) The two tumors in distant sites: "C" is the untreated control tumor, and "A" is the actively treated tumor. (B.) The time development of the treated and untreated tumors. The changes in the "C" site are not significant. (C.) Development of the apoptosis by elapsed time from the mEHT treatment [67].

The development of HSPs in xenograft model HT-29 cancer shows a peculiar feature. The HSP70 has a definite, significant minimum at 48 h, showing a return to the baseline. The HSP90 returned earlier, at 14 h [68] (**Figure 6**) and was observable intracellularly at 24 h posttreatment and measured extracellularly at 168 h. The immunohistochemical immunofluorescent staining of HSP70 shows a significant expression of these proteins on the cell membrane (mHSP), triggering an innate immune attack by NK cells [32]. The most likely explanation for the double peak of HSP70 is that the sizeable intracellular concentration could not inhibit the apoptosis, which was executed within two days. After this time, the release of HSP70 to the ECM became dominant, which is well observable with immunohistochemical staining at 72 h timepoint [68].

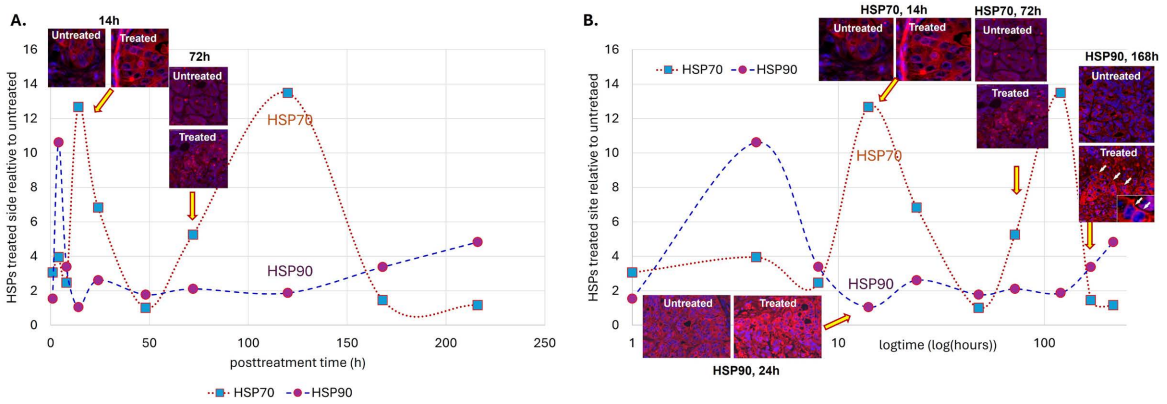

Figure 6. The relative development of HSP70 and HSP90 proteins after the mEHT treatment [68]. (A.) The double peak of HSP70 is probably due to its relocalization from the cytosol. (B.) The same is true for (A.) on a logarithmic scale for a more explicit demonstration of the expression shift of two HSPs.

Both extracellular HSP70 and HSP90 play essential roles in immunogenic processes. These chaperones are intracellularly antiapoptotic and help fold and stabilize proteins within cells. However, the game changes under extreme stress, releasing these HSPs to TME. Because they are proapoptotic, they represent an essential part of the DAMP molecular set. In this way, both proteins activate the immune responses, for example, they can bind to Toll-like receptors (TLRs) or CD91 on dendritic cells and macrophages, activating downstream signaling pathways involved in antigen presentation facilitated by these HSPs. In cancer, extracellular HSP70 and HSP90 released by tumor cells can modulate the TME and influence immune responses against the tumor. Depending on the context, their presence in the TME can induce tumor-specific immune reactions and affect cancer immunotherapy's efficacy. Their multifaceted roles in immunogenic processes act as danger and info signals, modulate immune responses, and influence the interaction between the immune system and cancer. The immunogenic action of HSP70 and HSP90 shares similarities but also exhibits some differences, particularly in their downstream effects on immune responses. HSP70 and HSP90 can activate innate immune responses by binding to cell membrane receptors on immune cells (mHSP70 and mHSP90). They can interact with receptors like Toll-like receptors (TLRs) and CD91, triggering signaling pathways that lead to the production of pro-inflammatory cytokines and chemokines. However, the specific receptors and signaling pathways activated by mHSP70 and mHSP90 may vary, leading to differences in the magnitude and nature of the immune response. The extracellularly released HSPs (eHSP70 and eHSP90) can enhance antigen presentation by promoting the uptake and processing of antigens by antigen-presenting cells. This can lead to the activation of T cells and the adaptive immune response against tumor cells. However, the mechanisms by which eHSP70 and eHSP90 promote antigen presentation may differ, potentially influencing the quality and specificity of the adaptive immune response.

In a short time after treatment, the development of DAMP molecules expresses calreticulin (CRT) 4 h posttreatment with mEHT (**Figure 7**). Notably,

the wHT and cHT treatments do not express CRT (*in vitro* Hep-G2 cell experiments, [64]), but mEHT significantly produces these membrane-located molecules both *in vitro* [64], and *in vivo* (HT-29 xenograft experiments [41]) (**Figure 7**). CRT on the membrane (mCRT) marks the cancer cells for recognition and subsequent engulfment by the immune cells, promoting ICD. The mCRT potentially slows tumor growth and aids the natural defense mechanisms against cancer.

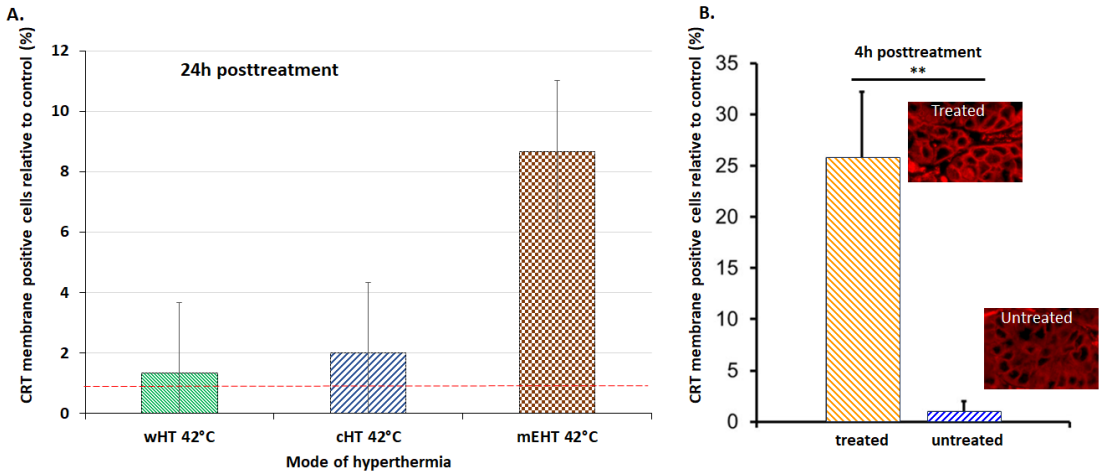

Figure 7. Development of calreticulin after mEHT. A dashed line shows the reference level. (A.) The wHT and cHT have significantly less CRT expression than the mEHT-treated HepG2 cells *in vitro* [64] 24 h after treatment. The relative expression of CRT as early as 4 h after mEHT treatment shows significant differences between the two tumors. The inserts show the membrane localization of CRT in mEHT-treated tumors, while it is not the case in the control [68].

The other immunogenic protein, the HMGB1 in the ICD process is freed from the cytosol, and the cell becomes practically empty from these molecules over 50 h posttreatment in HT29 tumors of immunocompromised mice [41] (**Figure 8**). The distant untreated tumor of the same animal constantly expresses intracellularly HMGB1. The released HSP70 and free ATP appear together with HMGB1, completing the DAMP molecular set in immunocompetent mice with B16F10 melanoma tumors [47].

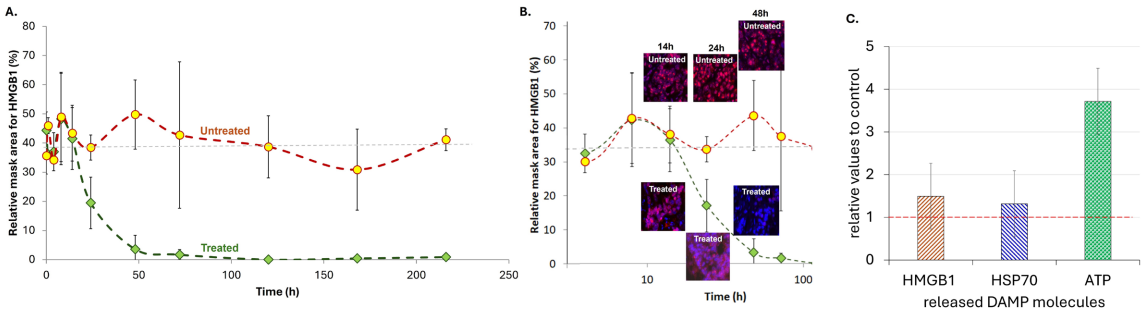

Figure 8. The development of HMGB1 proteins in time course after treatment. A dashed line shows the reference level. (A.) The treated tumor releases the HMGB1 to the extracellular milieu so its expression in the cytosol rapidly decreases, while the control cells keep these proteins [41]. (B.) The logarithmic scale shows that the first 12 h develops parallel in both tumors. The release starts only afterward. (C.) The relative expression of the leading DAMP molecules [47].

The CD3+ T cells play a crucial role in cancer immune defense. CD3+ T cells can recognize cancer cells as foreign or abnormal by recognizing Major Histocompatibility Complex type 1 (MHC I) presentation on the surface of cancer cells. CD3+ cells are T cells that express the CD3 protein on their surface. CD3 is a complex of proteins essential for the signal transduction process in T cells, particularly in activating T cell receptors upon binding to APCs. The CD3+-related cytotoxic T cell-mediated killing is crucial for eliminating cancer cells and controlling tumor growth. The cytokines the CD3+ T cells produce can also exert direct anti-proliferative effects on cancer cells and contribute to their elimination. CD3+ T cells can activate the memory response of adaptive immune surveillance, capable of a rapid and robust immune response upon re-challenging to the same tumor antigens, working like a tumor vaccination, protecting against tumor recurrence. The CD3+ expression does not show deviation until 72 h posttreatment. After a transition period, CD3+ has a maximum at 168 h posttreatment, showing the relatively long time to develop the contribution to the adaptive immune activity, while until 72 h posttreatment, it remains at baseline (Figure 9).

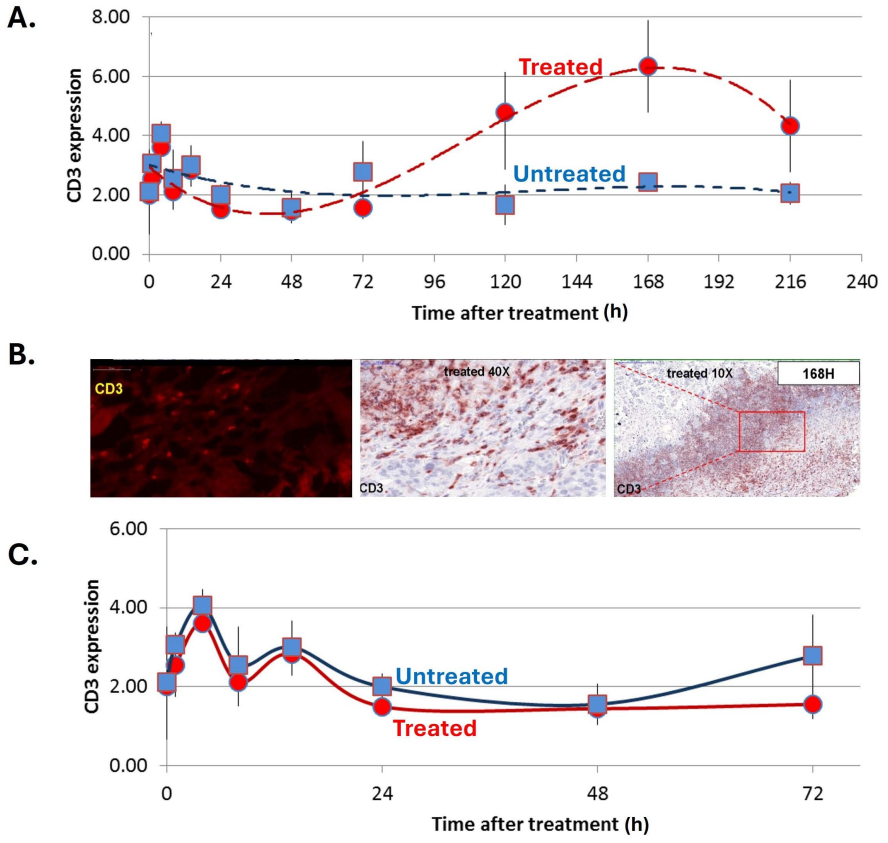

Figure 9. The time course of CD3 expression [68]. (A.) The CD3+ expression significantly develops 72 h after treatment. (B.) The immunohistochemical patterns of CD3+ expression at 168 h after treatment. (C.) The first two days have no deviation of the CD3+ from the control, and it starts being substantially developed after a transition period from 48 h to 72 h.

In a longer timescale after 72 h posttreatment, the neutrophilic activity increase was detected by the development of the enzyme myeloperoxidase (MPO) [68] (**Figure 10**). MPO participates in immune defense and anticancer processes as well. MPO generates reactive oxygen species (ROS), which can induce oxidative stress in cancer cells, leading to DNA damage, lipid peroxidation, and ultimately cell death. The oxidative stress helps in killing cancer cells or making them more susceptible to other treatments like chemotherapy or radiotherapy. MPO can modulate the inflammatory TME by regulating inflammatory mediators and immune cell recruitment, which may contribute to controlling tumor growth and metastasis. MPO has been implicated in regulating immune responses, including activation of T and dendritic cells, facilitating the recognition and elimination of cancer cells, and contributing to anticancer immunity. Like all molecular processes in a complex system, MPO is a double-edged sword, its action is context-dependent. While it may have anticancer effects in specific contexts, it could also promote tumor growth and metastasis under different conditions. The mEHT activates the MPO's anticancer behavior, showing a parallel CD3+ development. CD3+ T cells produce various cytokines, including interferon-gamma (IFN-γ) [52] (**Figure 10(B)**). IFN-γ is primarily produced by activated T cells and NK cells, promoting an anti-tumor immune response to the activation and differentiation of T cells, especially CD8+ cytotoxic T cells and CD4+ T helper 1 (Th1) cells.

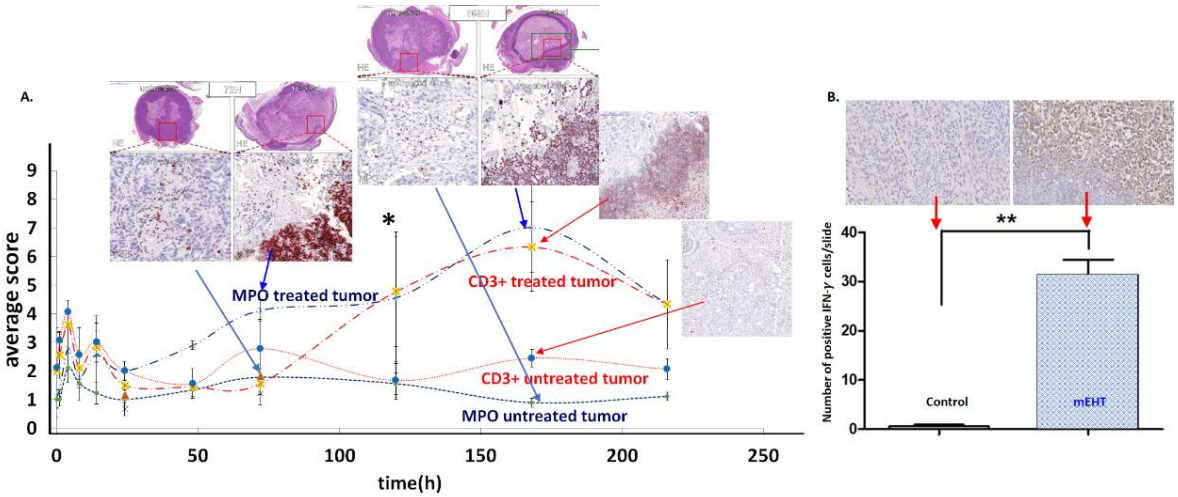

Figure 10. The time course of immune effects. (A.) Development of myeloperoxidase in HT-29 allograft tumor model [68]. (B.) The IFN-γ, compared to the control after mEHT treatment [52].

A melanoma pulmonary metastasis research was conducted with B16F10 injected tumors systemically to the tail vein of female C57BL/6 mice [53] (**Figure 11(A)**). A significant decrease in the metastatic tumor mass was observed in the mEHT-treated tumors compared to the untreated control. The treatment followed a reduced number of pulmonary metastatic nodules accompanied by significant DNA damage (increased γH2AX), downregulation of Ki67 proliferation marker, enhanced immune cell infiltration, and upregulation of p21waf1 expres-

sion. The tumor and the whole lung had significantly increased CD3+, CD8+ T-lymphocytes, and F4/80+CD11b+ macrophage density (**Figure 11(B)**), which proves the immunogenic activity of the mEHT treatment.

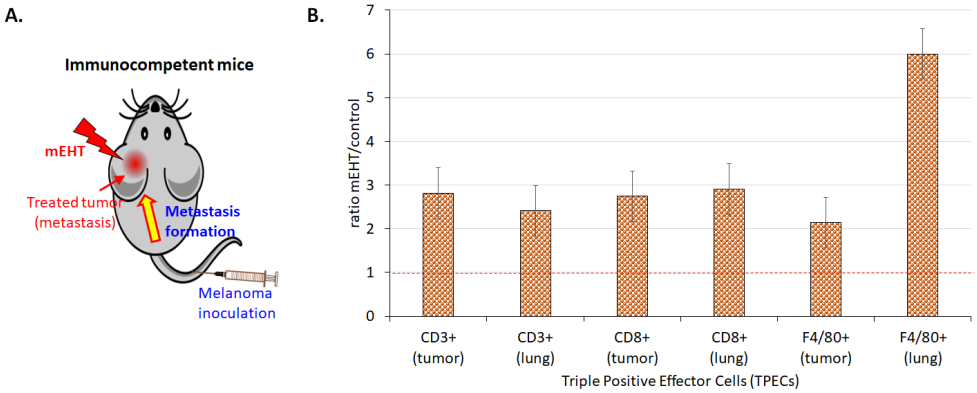

Figure 11. The treatment of lung metastases. (A.) The model. (B.) The gained pulmonary metastasis in immunocompetent mice intensively develops immunogenic cells in the tumor and its vicinity in the healthy tissue of the lung [53]. A dashed line shows the reference level.

Another immune activation with naturopathic remedies (Curcumin and Resveratrol) was applied in an extended study of *ex vivo* (serum from a treated SD rat) for pharmacokinetic research and *in vivo* (allograft BALB/c mice model) for treatment study using CT26 tumor cell subcutaneous inoculation. Curcumin and Resveratrol are natural antioxidants, and both may inhibit the proliferation of some malignant tumors [69] [70]. The mEHT treatment was concomitantly applied with subcutaneously inoculated micro-cumin and micro-resveratrol suspension (curcumin 200 μg and resveratrol 105 μg). For comparison, groups of animals were used as control, and two other groups had Curcumin and Resveratrol or mEHT standalone. The measured immunogenic effect was significant [46] (**Figure 12**).

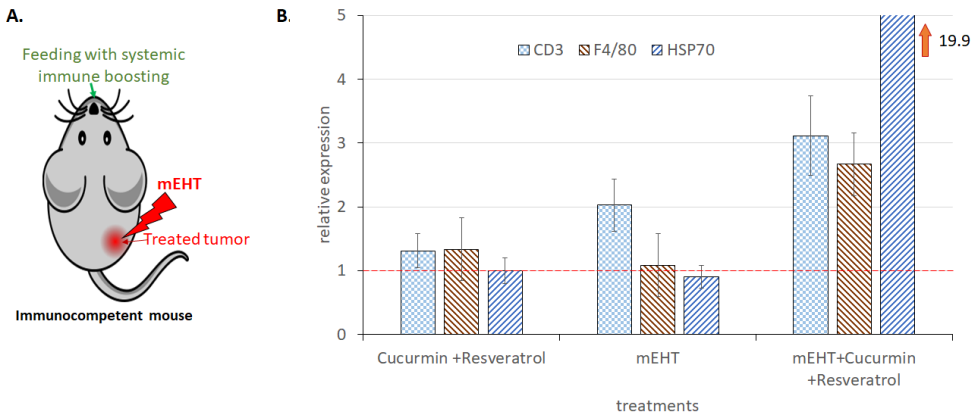

Figure 12. Combined mEHT treatment with curcumin and resveratrol [46]. (A.) The mice were fed curcumin and resveratrol. (B.) The standalone curcumin and resveratrol or mEHT therapies had significantly less DC and macrophage penetration to the tumor than their combined treatment. The prominent HSP expression is due to the different localizations of this protein not being selected. A dashed line shows the reference level.

For checking the attraction of the NK cells by mHSP70 on the membrane of tumor cells produced by mEHT, the tumor infiltration of NK cells was measured and demonstrated the effect [44]. A2058 melanoma cells were inoculated to an immunocompromised xenograft mouse model in both flanks of the animal and mEHT-treated on the right side, while the other was used as control. Substantial NK accumulation happened in the mEHT-treated tumor, while the untreated side did not show changes. Primary human NK or the IL-2 independent NK-92MI cells were distantly injected, far from the tumors, into the research animals after the mEHT treatment. The p53-driven apoptosis was ignited by mEHT monotherapy, supporting the attraction of the distantly injected NK cells. The chemoattractant CXCL11 metalloproteinase MMP2 induced upregulation was observed only in the treated tumor, supporting its growth inhibition and destruction. The destruction was more effective with NK-92MI cell injection than with primary NK, but both NK additions show a higher destruction rate than mEHT alone (**Figure 13**).

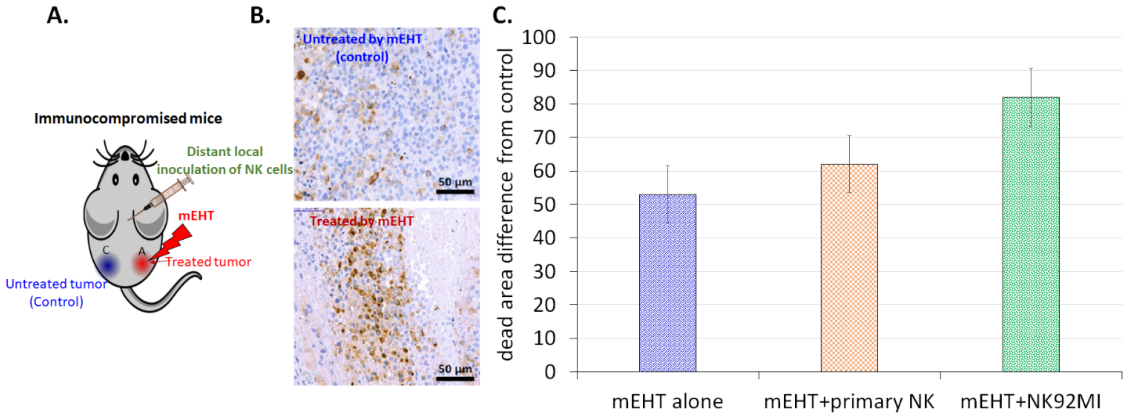

Figure 13. NK cell enrichment by mEHT treatment [44] (A.) In two tumor model experiments, NK cells were injected in a distant location into immunocompromised mice. (B.) The difference between the treated and untreated tumors is shown with immunohistochemical staining. (C.) The effect of NK cells increases the dead tumor area when the mEHT is combined with NK cells.

Another study on inhibiting tumor growth with immune stimulation and mEHT showed the tumor's destructive processes and the effect of immune memory. The immunostimulant was an intratumorally injected DC to the CT26 tumor. Relative to the DC or mEHT alone, the combined therapy significantly inhibited tumor growth [43] [71] (**Figure 14(B)**). The CD45 and F4/80 immunohistochemical studies show a significant elevation of the leucocytes and macrophages in the tumor treated with the combined therapy (**Figure 14(C)**). Notably, the significant increase in the eosinophils in tumors with combined treatment shows the potential role of these cells in immune combat, [72] [73], having substantial changes in the tumor microenvironment which could participate in tumor regression [74], and believed that it is a predictive biomarker for better prognosis [75]. Notably, the rechallenging of the same tumor in the

mEHT + DC-treated mice was rejected in **Figure 14(D)**, proving the tumor-specific memory of the immune activity of the animal.

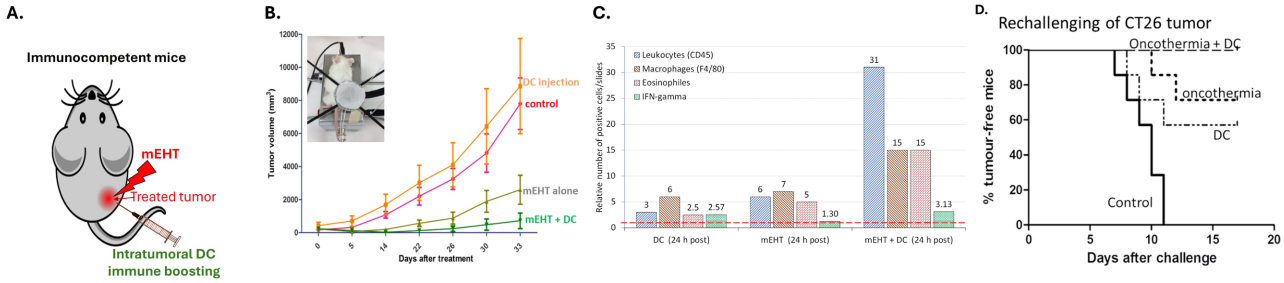

Figure 14. Combined treatment of mEHT with DC intratumoral injection into CT26 allograft *in vivo* model [43]. (A.) The model. (B.) The tumor volume is developed after the various treatments. (The treatment setup is in the insert.) (C.) Relative expression of some immunogenic proteins. A dashed line shows the reference level. (D.) Survival of the animals after rechallenging the tumor.

SCCVII mouse squamous cell carcinoma was inoculated to C3H/He immunocompetent mice in the femoral and chest regions of the animals [45]. This allograft model aims to check the abscopal effect with additional immune stimulation only of the treated tumor. Dendritic cells were injected directly into the femoral tumor three times every other day. The tumor volumes of both locations were measured and found a moderate inhibition of the growth of both tumors. When mEHT was applied to the femoral tumor, the tumor growth inhibition was more prominent and again in both tumors, even though only one was treated. Another group of experimental animals had direct intratumoral DC injection only into the mEHT-treated tumor posttreatment at the 9th, 11th, and 13th days. Tumor volume was measured again in the DC+mEHT treated (femoral location) and nontreated (chest location) tumors. The growth inhibition was drastic in both sites, and the tumor growth was practically arrested (**Figure 15**). The most important immune reactions were measured from the serum together with the tumor volumes. Flow cytometry detected a large expression of CD3+ and CD8+ T cells in the various groups, with the significantly highest value in the DC+mEHT treatment group (**Figure 15(D)**). The optical density measurements confirmed the intensive increase of the CD8+ T cells in combined treatment together with the S100 DC marker protein, which can modulate dendritic cell function and immune responses by promoting the maturation and activation of dendritic cells, enhancing antigen presentation, and influencing the migration of dendritic cells to lymphoid organs. At the same time, the Foxp3 expression was significantly suppressed in the DC + mEHT group of animals, showing the inactivation of Treg cells [45] [76].

The $\gamma\delta$T cells bridge the innate and adaptive immune responses [77]. The role of $\gamma\delta$T cells in tumor degradation is multifaceted. $\gamma\delta$T cells MHC-independently can directly recognize and kill tumor cells, directly exocytosis perforin and granzyme B in the shaped immune synaptic space. They recognize stress-induced molecules on the cell membrane (like mHSP70) [78], and it has excellent support for

immunotherapies [79]. Like CD3+ T cells, the γδT produces various cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) upon activation. These cytokines have anti-tumor effects by enhancing the activity of other immune cells, such as macrophages and cytotoxic T cells, and inhibiting tumor cell proliferation. γδT cells can modulate the activity of different immune cells within the tumor microenvironment [80]. The γδT cells injection to NPD-SCID mice having HepG2 hepatocellular tumor [51] [52], the mEHT treatment became more effective than without this injection (**Figure 16(B)**). Notably, the γδT cells alone do not have an antitumoral effect in this model. The *in vitro* experiments show increased tumor degradation with just thermal effect (wHT) at 42˚C, but the mEHT-treated HepG2 cell line, together with γδT cells, caused notable cell destruction at 38˚C, which is further increased in higher 42˚C temperature with mEHT (**Figure 16(C)**).

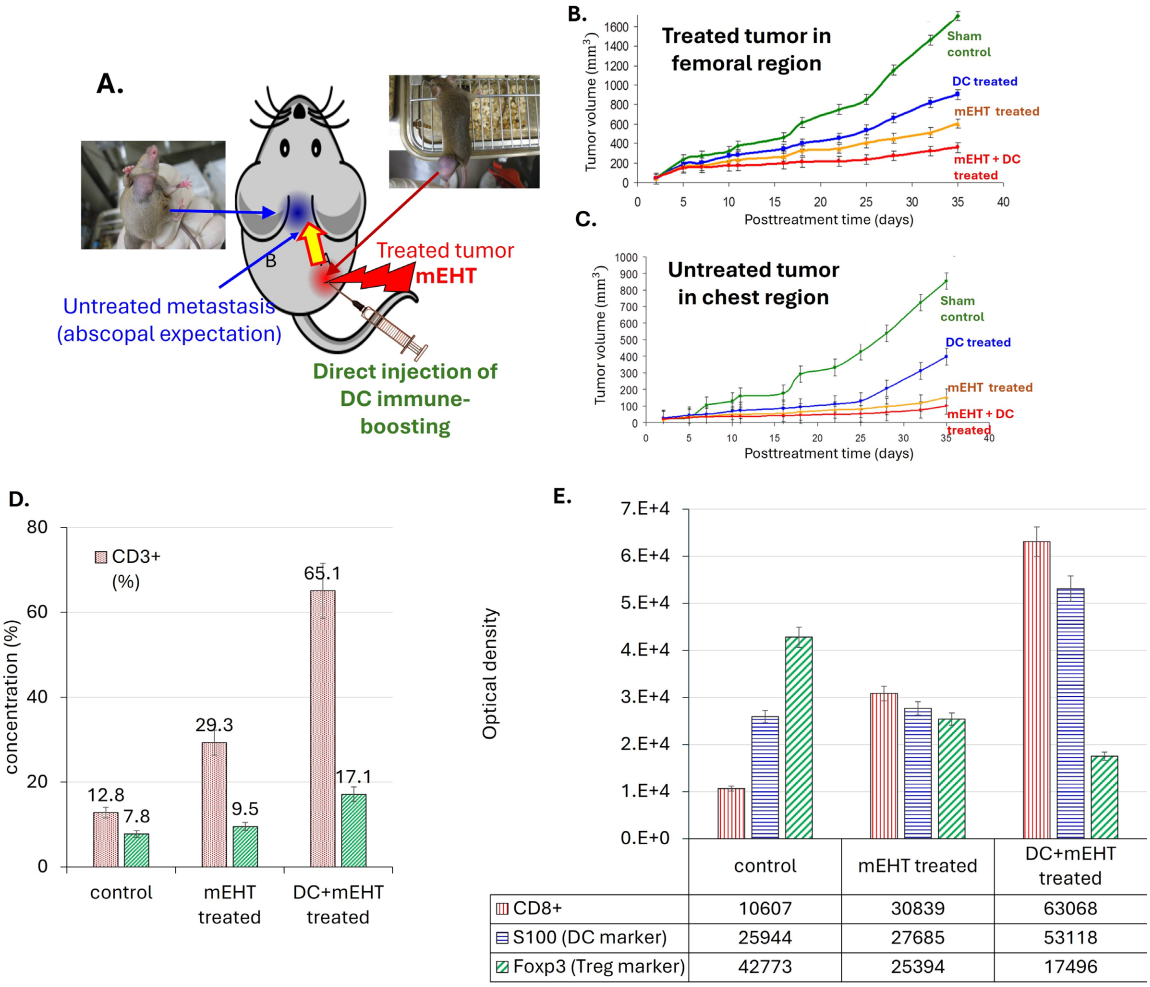

Figure 15. Model of abscopal effect [45]. (A.) The setup of the *in vivo* model shows the distant tumors in the inserts. (B.) The development of the tumor volume after the treatment in the treated (femoral region) SCCVII tumor. (C.) The development of the tumor volume after the treatment was measured in the untreated tumor in a distant location (chest region). (D.) The CD3+ and CD8+ T cells appeared systemically in the mice. (E.) The optical density measurement shows the high density of the killer T cells while the protumoral Tregis suppressed.

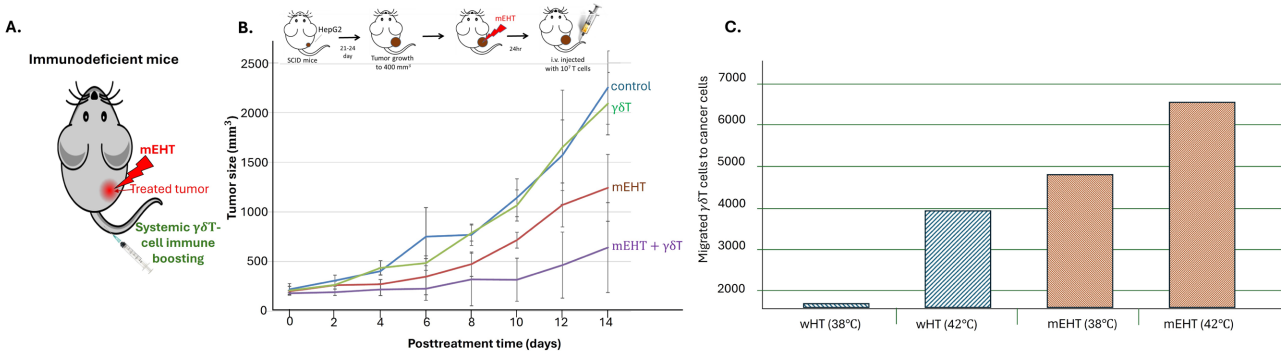

Figure 16. The effect of the $\gamma\delta$T cells in immunocompetent mice model [51] [52]. (A.) The set-up of the model. (B.) The tumor volume is developed after the different treatments. The protocol is shown in the insert above the graph. (C.) $\gamma\delta$Tmigrated to cancer cells. The migration ability of $\gamma\delta$T cells was measured after mEHT treatment HepG2 cell-line (separated → seeding 24 h → collected 4 h later → measured by flow cytometry).

The new development direction of oncotherapies targets immunogenic effects. mEHT therapy fits this trend and enhances the antitumor immune activity shown in the above results. One of the promising immunotherapies is connected to the inhibition of Programmed Cell Death Protein 1 (PD-1) and Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). PD-1 on the surface of T cells acts as a brake on the immune system by binding PD-L1 to protein PD-L1 on cancer cells, preventing the T cells from attacking the cancer cells. Anti-PD-1 (aPD-1) therapy allows T cells to attack and destroy cancer cells. CTLA-4 is another protein on the surface of T cells. It is a checkpoint inhibitor, regulating the amplitude of immune responses and hindering the immune system's ability to attack cancer cells. The anti-CTLA-4 (aCTLA-4) therapy reduces the immune evasion of the cancer cells, enabling a more robust immune response against the cancer cells. A study deals with the cooperative activity of these checkpoint inhibitors and mEHT in their complementary application [52]. The complex study compared the stand-alone mEHT, aPD-1, and aCTLA-4 therapies to the control. The therapy protocol for immunocompetent mice has standard mEHT (42˚C, 30 min) first and adding aPD1 of aCTLA-4 concomitantly to the tail vein of the mice, and the administration of the checkpoint inhibitors (CPI) was repeated two more times with 3 3-day pauses. (We denote these protocols as aPD1 (=) and aCTLA4 (=)). The action time was also studied using a protocol that administered the checkpoint inhibitors 2 days after mEHT and afterward repeated the CPI two more times with 3 3-day pauses, as was applied in the previous protocol. (We denote these protocols as aPD1 (>) and aCTLA4 (>)). Results show the advantage of mEHT in improving the effect of checkpoint inhibitors (**Figure 17**). The best results were achieved when aCTLA-4 was applied concomitantly with mEHT (aCTLA4 (=) protocol), with almost half the tumor size at the 16th day posttherapy compared to aCTLA-4 alone. The same difference was observed in aPD-1 therapy, as shown in **Figure 17(C)**.

The systemic immune activity can be measured in the double-tumor mice when the effect of the locally treated tumor on the distant other could be studied.

The first observation of the interaction was measured by injection of lipopolysaccharide (LPS) [81] into mice, triggering primarily the innate immune response of the animal, activating the toll-like receptor 4 (TLR4) signaling pathway and also activating APCs. This immune stimulation developed a distant tumor distortion (abscopal effect), which could be measured 72 h posttreatment (Figure 18). Another observation was the immune stimulation with Newcastle Disease Viruses (NDVs) [62] [63], which activates the TRL's signal pathways and creates innate and adaptive immune reactions.

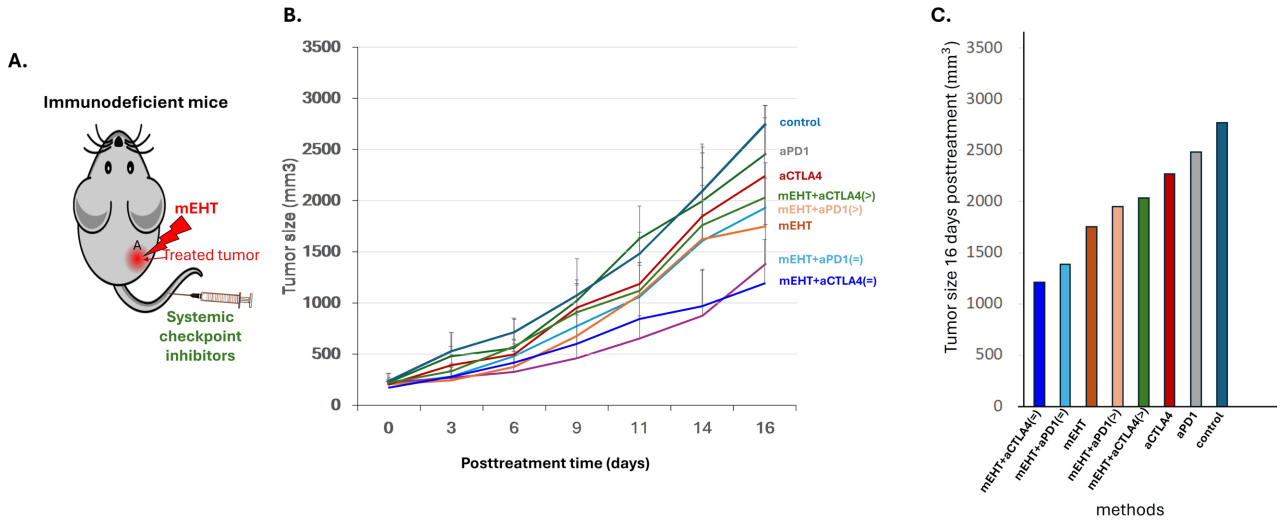

Figure 17. Check point inhibitor study with mEHT support [52]. (A.) The experimental setup NOD-SCID mice, HepG2 tumor. (B.) Growth of the tumor volume by time (days). (C.) Comparison of tumor sizes (mm^3) on the 16^{th} day. Significant differences were observed between control and mEHT ($p \cong 0.0006$), control and aPD1 (=) ($p \cong 0.00002$), control and aPD1 (>) ($p \cong 0.002$), control, and aCTLA4 ($p \cong 0.028$), control and aCTLA4 (=) ($p \cong 0.00001$), control and aCTLA4 (>) ($p \cong 0.04$).

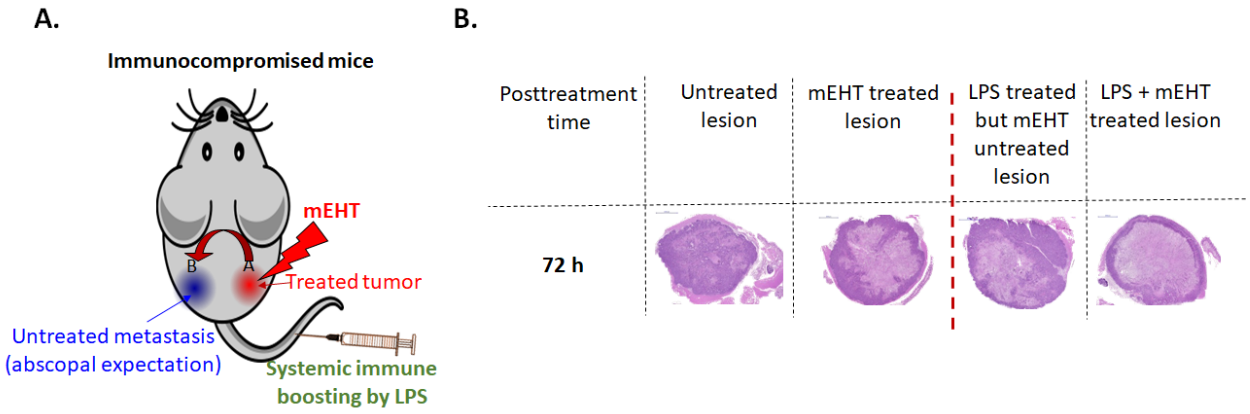

Figure 18. Experiment with lipopolysaccharide (LPS). (A.) The experimental setup. (B.) The treated and untreated tumors were 72 h after treatment.

Learning the results of the LPS experiment, a direct immunostimulant Marsdeniatenacissima (MTE) in BALB/c immune-competent double tumor-bearing mice was injected into the tail vein of the animals (Figure 19(A)). For compari-

son, the mEHT stand-alone therapy was performed first. It was observed the expected phenomena: blocks the proliferation measured with the Ki67 marker [42] and develops a significant amount of DAMP, the eHSP70, CRT, and HMGB1 proteins, which were much less in the untreated tumor (**Figure 19(B)**). The DAMP development was accompanied by significant tumor infiltration by S100 positive antigen-presenting dendritic cells and CD3+ T-cells with only negligible FoxP3 positive regulatory T-cells compared to the distant untreated tumor [42]. When direct immune stimulation with MTE was applied concomitantly with mEHT, tumor destruction was significant in untreated tumors, too. The difference between the two tumors drastically decreased typical DAMP production, and immune reactions also appeared in the distant untreated tumor. The difference between the treated and untreated tumors is drastically reduced, and the abscopal effect actively destroys the untreated tumor, too. [42] (**Figure 19(B)**)

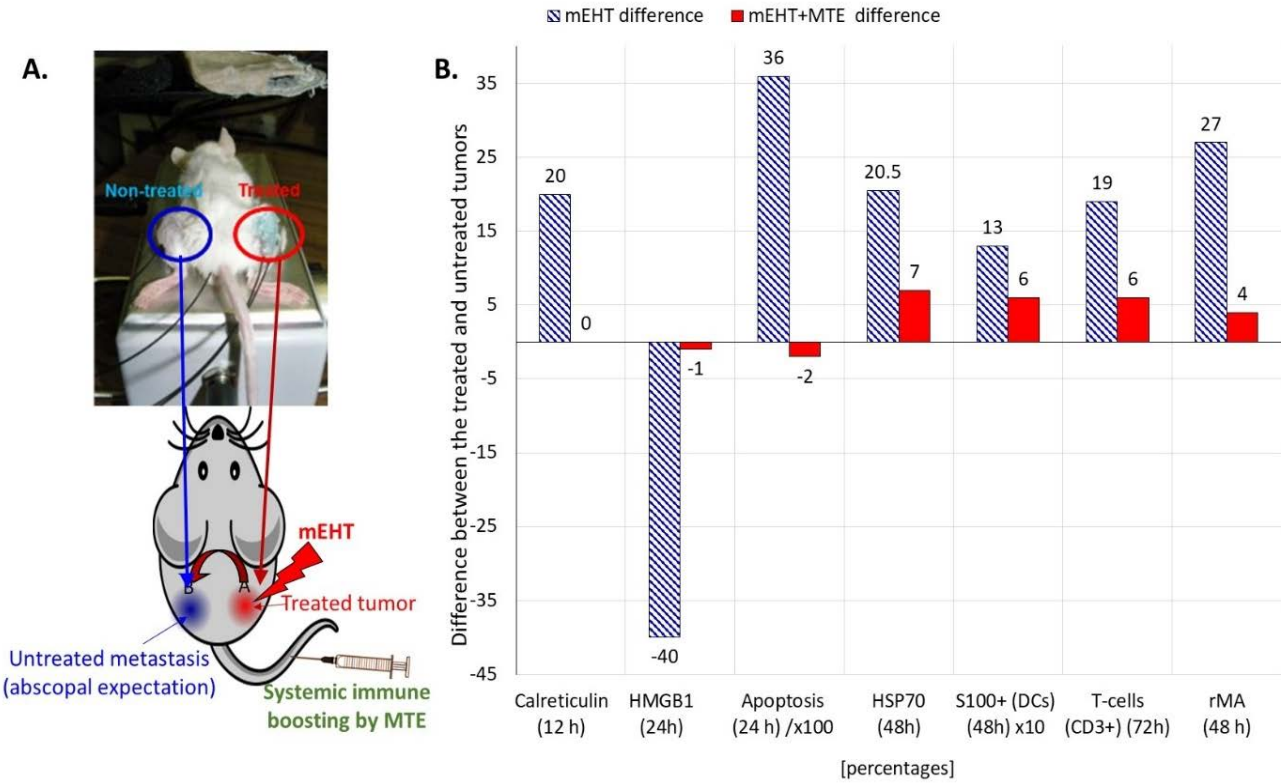

Figure 19. The abscopal research with immunostimulatory MTE in immune-competent mice with C26 murine colorectal adenocarcinoma. (A.) The experimental setup of the double tumor model. (B.) The difference of immunogenic cells of treated and untreated tumors was measured. Without immunostimulatory MTE, the difference was significantly higher in the treated tumor, while when MTE was applied concomitantly with mEHT, the difference was drastically reduced. No significant difference was observed between the treated and untreated tumors.

The immunogenic processes have a definite spatiotemporal order (**Figure 20**) producing the optimally harmonized immune action in the entire body attacking the malignant cells in the treated tumor and distant locations from the treatment site.

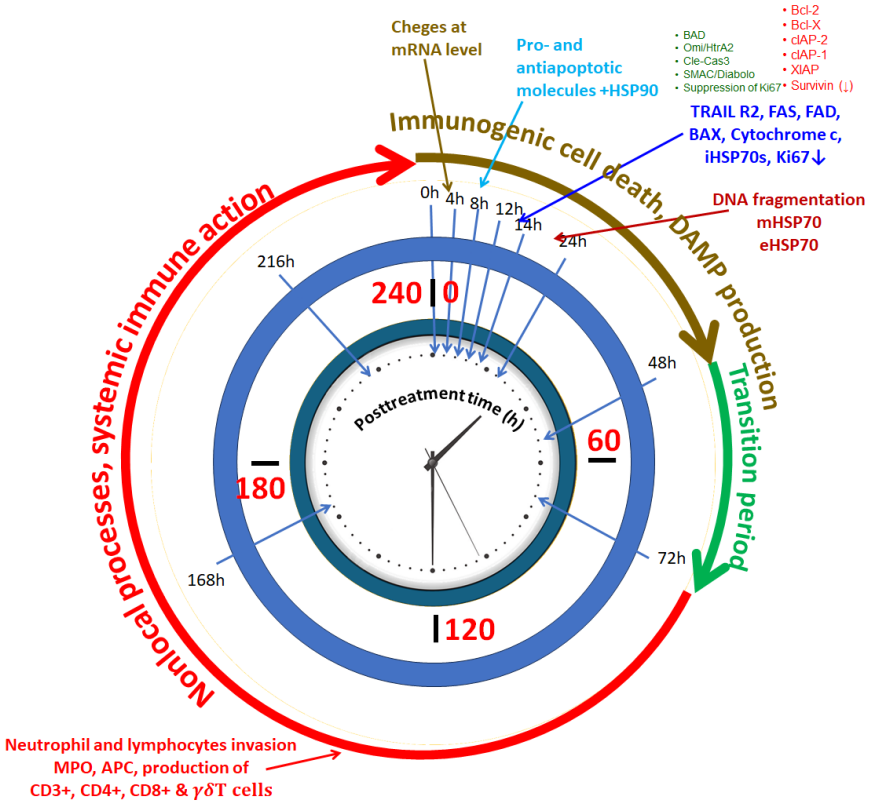

Figure 20. The observed immunogen changes and their timing in the processes induced by mEHT are a synergy of thermal and nonthermal effects.

4. Conclusions

The results described above strongly support the immunogenic activity of mEHT treatment. The ignited and controlled apoptosis releases DAMP, completing ICD. The correctly released DAMP molecules deliver various signals for the nearby antigen-presenting immune cells (like DCs and macrophages), and the APC generates tumor-specific immune activity to attack the malignant cells all over the entire body. The specialties of mEHT in this complex process, which distinguishes it from conventional (thermal alone) hyperthermia, ensure the immunogenic actions:

1) mEHT uses non-thermal effects to ignite immunogenic cell death by exciting some transmembrane proteins.

2) The thermal effect optimizes the nonthermal processes and increases the chemical reaction rates, including the critical enzymatic effects.

3) The expression of the DAMP molecules has a strict spatiotemporal order for optimal ICD processes.

4) The average temperature of the tumor mass remains under 40˚C ensure the just-in-time activity of the available immune cells in the TME.

5) The apoptotic process and the mild average temperature do not damage the info delivery from the tumor cells to the TME and APCs.

6) The mEHT transforms the local treatment into a non-local, systemic ther-

apy, avoiding metastatic proliferation and so increasing survival and quality of life.

The mEHT technology renews conventional hyperthermia. The synergy of heating with bioelectromagnetism and its harmony with homeostatic physiology opens possibilities of stable, controlled therapy with survival time and quality of life endpoints. The trends in oncotherapies and hyperthermia oncology show a common direction: the immunogenic goals [82]-[84].

The clinical studies of various medical centers in different countries provide the final validation of these preclinical data [85]-[87]. A phase III clinical trial proved the abscopal effect for advanced uterus cervix patients [88] when adding mEHT to systemic conventional radiochemotherapy significantly increased the abscopal effects. The abscopal immunogenic activity of mEHT with a local radiotherapy combination was observed in another clinical study [89]. Altogether, 150+ clinical articles show the advantages of mEHT therapy. The article to summarize the clinical studies is in progress.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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Abbreviations

APC	antigen presenting cell
ATP	adenosine triphosphate
DAMP	damage associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
ECM	extracellular matrix
HMGB1	high-mobility group protein
HSP	heat-shock protein
ICD	immunogenic cell death
mEHT	modulated electro-hyperthermia
NK cell	natural killer cell
ROS	reactive oxygen species
TLR	toll-like receptor