Article

Modulated Electro-Hyperthermia Enhances Tumor Cell Susceptibility to Gamma Delta T Cell-Mediated Cytotoxicity and Tumor Infiltration

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Simple Summary: In the fight against cancer, the emergence of immunotherapy has shifted the treatment paradigm. Immune therapy utilizing gamma delta T, whereby the cells are expanded *ex vivo,* then adoptively transferred, have shown the potential to induce durable responses, while being well-tolerated, with limited adverse effects. However, as efficacy varies across tumor types, only a subset of patients show response to treatment. To overcome these obstacles, one segment of research has been to enhance the ability of these cells to infiltrate tumor sites. Hyperthermia is an age-old form of therapy by which malignant tissue is subjected to heat, locally or systematically. Modulated electro-hyperthermia is one clinically-approved technique, whereby cancer cells are selectively targeted with energy-transmission from an electromagnetic field to induce anti-tumor effects. This method has shown proclivity to induce immunological responses. The study explores the effects of combining these two modalities *in vitro* and in animal study.

Abstract: γδT have functions of innate and adaptive immunity, with the potential to induce durable responses while being well-tolerated, with limited adverse effects, making it attractive as a tool for immunotherapy. γδT faces challenges as a frontline tool in clinical oncology, with limited response rates due to difficulties in reaching tumor sites with consistent cytotoxic activity and strength. Modulated Electro-hyperthermia (mEHT) is a loco-regional treatment, whereby energy-transmission from an electromagnetic field selectively targets the plasma membrane of tumor cells, inducing apoptosis and activating immune cells. We hypothesized that mEHT could enhance therapeutic effects by drawing γδT to tumor cells, while also rendering tumor cells to be more susceptible to cytotoxic effects. In this study, NOD/SCID mice harboring subcutaneous human HepG2

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tumors were treated with intravenous injections of γδT after mEHT treatment. This method increased infiltration of γδT into the tumor site, significantly inhibiting tumor growth as compared to monotherapy with either modality. These data suggest that γδT could mediate a potent anti-tumor effect when combined with mEHT, and provide a strong rationale for combining these modalities in clinical application for cancer treatment.

Keywords: γδT, gamma delta T, tumor-infiltrating lymphocytes, TIL, cell therapy, cancer immunotherapy, hyperthermia, modulated electro-hyperthermia

1. Introduction

In the span of a few years, immunotherapy has become the most promising approach to the treatment of cancer [1]. Utilized as a second-line therapy, add-on immunotherapy is a vital tool for end-stage and refractory cancer [2]. Immunotherapy has also shown the ability to effectively prevent tumor recurrence after surgery or radiotherapy [3]. Roughly 1-5% of T-cells in human peripheral fluids express γδ receptors instead of conventional αβ receptors [4]. These cells, termed as γδT, have functions of innate and adaptive immunity, making them an attractive option for use as an immunotherapy agent [5]. γδT can produce inflammatory cytokines, directly lyse infected or malignant cells, and build remembrance to attack pathogens upon repeat exposure. The potential of γδT in tumor immunotherapy is promising and has been applied to Phosphoanigen (PAgs) or aminbisphosphonates (N-BP) plus interleukin-2 in vitro, or after adoptive transfer in vitro expansion culture (ex vivo) [6]. Numerous clinical trials have applied adoptive transfer γδT to various cancer types [7], including bone marrow cancer [8], advanced kidney cancer [9], advanced prostate cancer [10], etc. Results have been mixed, although patients have been shown to be stable, including many cases of remission. These trials have also shown γδT to be safe, and side effects to be rare. While promising, γδT still faces challenges as a frontline tool in the fight against cancer, facing difficulties in reaching tumor sites as well as consistency of γδT strength and activity.

It has been reported that the percentage of γδT infiltrating the inflammation site is particularly high, and recent studies have found γδT attracted to heat shock proteins (HSP) of inflammation sites begin to generate immune responses [11]. In cancer patients, there were also reports that HSP produced by tumor cells can trigger T-cell receptor (TCR) recombination of γδT, and increase the anti-tumor effect of γδT [12]. Regional hyperthermia is an effective method to induce heat shock proteins production by tumors [13]. Thus far, no literature has explored the potential of combining hyperthermia and γδT therapy.

Hyperthermia (HT), in modern clinical practice for cancer treatment, is used mainly in combination with radiotherapy or chemotherapy [14, 15]. To achieve fever-like temperature (≤ 42°C) at the tumor site, various HT techniques have been used [16, 17]. Radiofrequency (RF) is the most common method to heat deep-seated tumors, using a pair of capacitive electrodes placed on opposite sides of the patient's body. Modulated electro-hyperthermia (mEHT, trade name Oncothermia) is a loco-regional electromagnetic system, utilizing capacitive-impedance coupled 13.56MHz radiofrequency current (RF) to selectively destroy malignant cells [18-20]. mEHT has been in clinical use in Europe for more than two decades [21-23]. The energy of the RF is selectively absorbed by tumor tissues due to its relatively higher ionic concentration around the tumor milieu, which in turn causes massive apoptotic cell death, even when below cytotoxic temperature range [19, 20, 24, 25]. In previous studies, we showed that mEHT could induce heated tumors to release more Hsp70 into the extracellular environment than other forms of hyperther-

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mia [13, 26]. The released Hsp70 serves as a danger signal that alters the tumor microenvironment (TME) into a more immunological-responsive milieu, including one for immune cell infiltration [26].

In this study, we hypothesized that mEHT could enhance the infiltration of γδT into tumor sites, enabling greater efficacy for killing cancer cells, while maintaining the safety of γδT monotherapy. Such results would provide strong rationale for combining these modalities in the treatment of cancers.

2. Materials and Methods

Cancer cell line culture

HepG2, Huh7.5.1, and A549 cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO2(v/v) in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific Inc., Waltham, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100μg/mL streptomycin (Thermo Fisher Scientific). HepG2 (BCRC 60025) and A549 (BCRC 60074) were purchased from BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan). Huh7.5.1 was a kind gift from Taipei General Veterans Hospital, originally taken from a liver tumor of a 57-year-old Japanese male.

Isolation of PBMC and γδT cell culture

Informed consent was obtained for the collection of peripheral blood (PB) from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood of healthy donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). PBMC was stimulated with 5μM zoledronate (Novartis, Basel, Switzerland) in AIM-V medium (Gibco, Grand Island, NY, USA), supplemented with 1000 IU/mL human recombinant IL-2 (Proleukin, Novartis Pharmaceuticals, Canada) at 37°C, in an atmosphere supplemented with 5% CO2. Fresh medium containing IL-2 (1000 IU/mL) was added every two to three days, and the cultures were transferred into new flasks or culture bags as necessitated by the degree of cell growth. After stimulation for 12 days, ex vivo expanded γδT were harvested and screened for the percentage of CD3+TCRVγ9+T cells.

Cytotoxicity assay

Cytotoxicity assays were performed using the CytoTox 96 non-radioactive cytotoxicity assay (Promega Corp, Madison WI, USA), according to the manufacturer's protocol. Briefly, a total $5x10^3$ target cells were seeded in a 96-well flat plate overnight. The *ex vivo* expanded γδT as effector were distributed in triplicate at effector: target (E:T) cell ratios of 10:1, 5:1 and 2.5:1. After a 16h incubation at 37°C in an atmosphere supplemented with 5% CO2, the supernatant in each well was harvested and transferred to a new plate. The fluorescence emitted by the samples was measured using a microplate reader (Multiskan FC, Thermo). The absorbances were measured at λ = 490nm. The percentage cytotoxicity for each E/T was calculated as (Experimental culture medium background) - (Effector cell spontaneous release - culture medium background) - (Target spontaneous release - culture medium background) / (Target maximum release - volume correction control - Target spontaneous release - culture medium background) × 100.

Real-time digital bio-imaging cytotoxic assay

The target cells ($2x10^4$ per well) were incubated in each well of a 96-well flat plate overnight. The *ex vivo* expanded γδT ($4x10^4$ per well) were seeded in the well. The plate was spun before being placed in the cell imaging multi-mode plate read-er Cytation™ 3 (Biotek, Winooski, VT). The assay was performed at 37°C, in an atmosphere supplemented with 5% CO2. Images of each well was collected every 5 min over 16h. Cell images were processed using Gen5TM software (BioTek).

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Transwell migration assay

For analysis of γδT migration, 1.5 x10^5 HepG2 cells were seeded into the lower chamber of transwell plates (Costar, Cambridge, MA) overnight. Next day, 1.5x10^5 *ex vivo* expanded γδT were transferred into the upper chamber of 5μm pore size transwell plates and allowed to migrate for 4h at 37°C. Each condition was set up in duplicate transwells. Migrated γδT was then collected from the bottom wells and the cell numbers were quantified by CD3+TCRVγ9+ cell population using a flow cytometer (AccuriC6, BD Biosciences).

Modulated electro-hyperthermia treatment (mEHT)

Electromagnetic heating was conducted using capacitive-coupling with an amplitude-modulated 13.56-MHz radio-frequency (LabEHY, Oncotherm, Germany). The mEHT technical details of the method is readily available [29]. An *in vitro* heating model was established in an electrode chamber (LabEHY *in vitro* applicator), which was heated to 42°C for 30 min, at a mean power of 8~9 W. The cells were placed in a chamber with culture medium at 42°C for 30 min. Tumor implants in the right femoral area of NOD/SCID mice were placed in the parallel electric condenser of the heating circuit, as described elsewhere [28]. The treatment groups were given a single shot of mEHT for 30 min, at a mean power of 1.5W, under 100mg/kg Ketamine and 10mg/kg Xylazine anesthesia. Intratumoral temperature was maintained at ~42°C on the treated side of each mouse, as measured using optical sensors (Luxtron FOT Lab Kit, LumaSense Technologies, Inc., California, USA). The subcutaneous temperature underneath the electrode was maintained at 38°C or 42°C.

Animal study

Six to eight weeks old NOD/SCID mice were purchased from BioLASCO (BioLASCO Taiwan Co.) and 4 x 10^5 HepG2 cells were injected subcutaneously (s.c.) in the right femoral areas. When the tumors reached 200mm^3, mice were separated to each group for treatment as day 0. The mice of combined treatment group received twice treatment on day 1 and day 2, with local mEHT treatment (as described above), and intravenous (i.v.) injection of 5×10^6 γδT in 50uL on the following day. The mice of mEHT and γδT alone group were also received twice treatment on day 1 and day 2. Each group comprised of four to six mice. Tumor volume (mm^3) of each group was recorded with length (L) and width (W) every other day for three weeks and calculated by following the equation: Length X $(Width)^2/2$.

3. Results

3.1. Characterization γδT cell that culture from human peripheral blood

The purity and culture efficiency of γδT was confirmed by surface markers. T-cell surface marker CD3 and T-cell receptor Vg9 were used to identified the γδT by flow cytometry. After 12 days of in vitro culture in medium containing IL-2 and Zoledronic acid, CD3 and Vγ9 double positive cells increased significantly, from 2.1±1.65% to 85.1±6.33% (Figure 1a and b).

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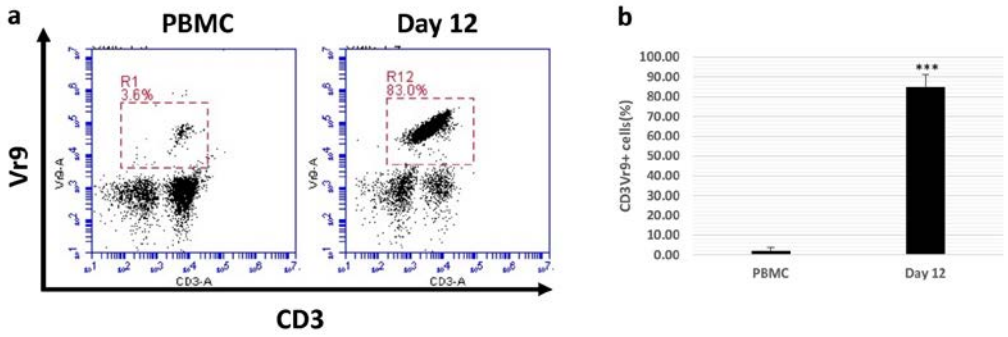

Figure 1. The phenotype of zoledronate-expanded γδT were investigated by flow cytometric analysis. Fresh peripheral blood mononuclear cells were cultured for 12 days and were stained with anti-CD3 and TCRVγ9 mAb. (a) Represented plots. (b) The number of CD3+ and TCRVγ9+ double positive cells. The data are the mean +/- SD for three independent experiments. *** indicates $p<0.0005$.

3.2. The cytotoxicity of γδT cells on mEHT treated tumor cells

We hypothesized that cancer cells treated with mEHT would be more sensitive to immune cells, enabling γδT to play a more proficient role in cytotoxicity to cancer cells. In order to observe the ability of mEHT to weaken cancer cells and stimulate γδT cytotoxicity under different temperatures, we divided the treatment of mEHT and water bath control into 38°C and 42°C. The results showed that the cytotoxicity of γδT against cancer cells after mEHT treatment was significantly increased (Figure 2). This phenomenon was observed at both of 38°C and 42°C. The lung cancer cell line, A549, was more sensitive to the difference in temperature, and the virulence of 38°C is lower (Fig. 2a), while the lethality of 42°C is higher (Fig. 2b). However, in hepatoma cell lines, Huh7 and HepG2, both 38°C and 42°C displayed increased cytotoxicity of γδT to cancer cells after mEHT treatment (Fig. 2c-f) versus water bath control at 38°C or 42°C.

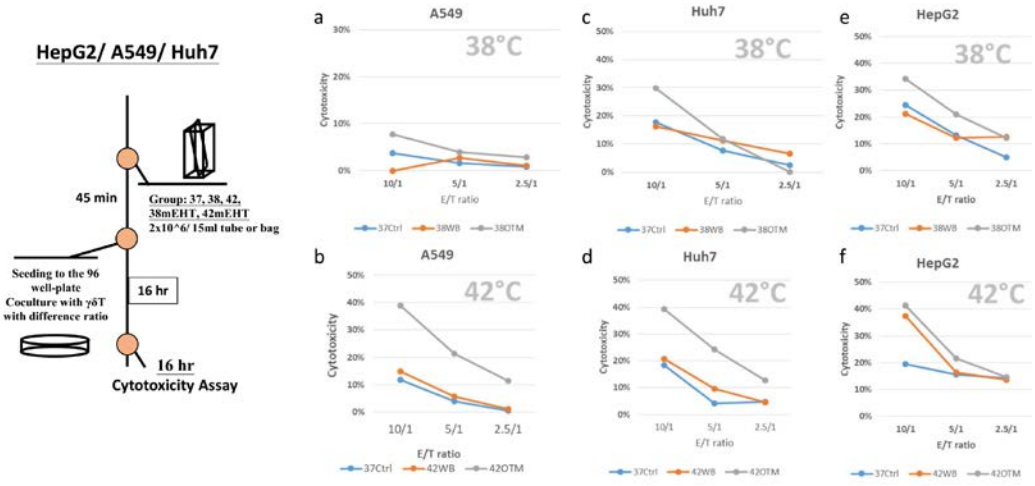

Figure 2. In vitro cytotoxicity assay in various human cancer cell lines after mEHT treatment. The experimental process was displayed in the left column. The percentages of specific lysis by γδT cells against A549 (a–b), Huh7 (c–d) and HepG2 (e–f) pre-treated with mEHT (OTM) or water bath (WB) at 38°C (a-c) or 42°C (d-f) and untreated (Ctrl) were evaluated in a 16 hr CytoTox 96 nonradioactive cytotoxicity assay. Data are shown as mean for three experiments.

3.3. mEHT enhanced γδT cell recognition and cytotoxicity to tumor cells observed by imaging

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This phenomenon of mEHT enhancing γδT recognition and cytotoxicity to tumor cells was further confirmed by living cell microscopic imaging. Owing to the lower cytotoxicity of A549 cells in 38°C of mEHT treatment, A549 was used in this assay to verify the killing effect. Video of γδT co-cultured with mEHT-treated A549 over time is available as Video S1. The enhanced cytotoxicity of γδT to A549 by 38°C of mEHT was directly observed by living cell microscopic imaging system after 10 hours co-culture (Fig. 3m-o). Untreated co-culture of A549 and γδT (37°C control, Fig. 3a-e) and conventional water bath control at 38°C (Fig. 3f-j) did not show any sign of lysis.

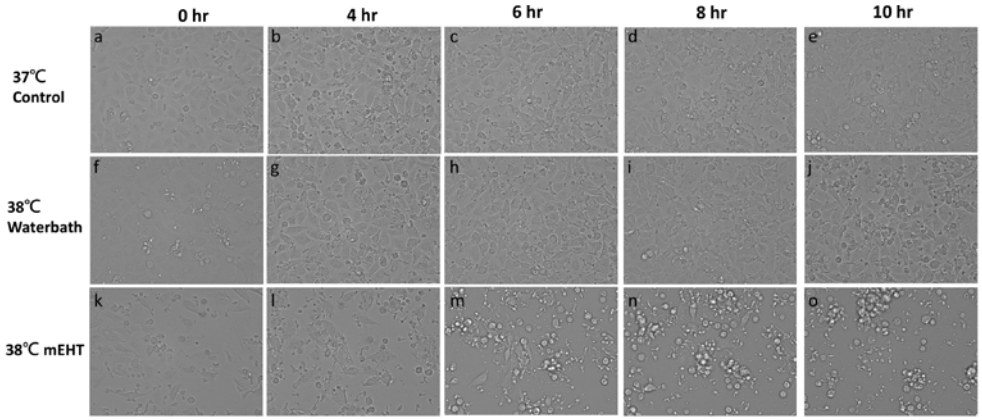

Figure 3. The cytotoxicity of γδT cells on mEHT treated tumor cells observed by living cell microscopic imaging. The CellTracker dye-labeled A549 cells were imaged by living cell microscopic imaging system after pre-treatment with 37°C control (a-e), water bath at 38°C (f-j), or mEHT (k-o), then co-cultured with purified human primary γδT treated at the indicated time point. The fluorescent area of A549 were recorded and further analyzed using Gen5™ software.

3.4. The migration ability of γδT cells induced by mEHT treated tumor cells

Next, we used transwell method to analyze whether γδT could be attracted by mEHT treated tumor cells. In this experiment, variously treated tumor cells were seeded in the bottom layer, while γδT was placed in the upper chamber. After four hours of co-culture, the number of CD3+Vγ9+γδT cells was analyzed by flow cytometry (Fig. 4a). In the mEHT groups, both at 38°C and 42°C, the migration of γδT was elevated as compared with 37°C control group or water bath treatment groups of same temperature (Fig. 4b and c). Interestingly, the 42°C water bath treatment group also showed elevated migration versus control (Fig. 4e).

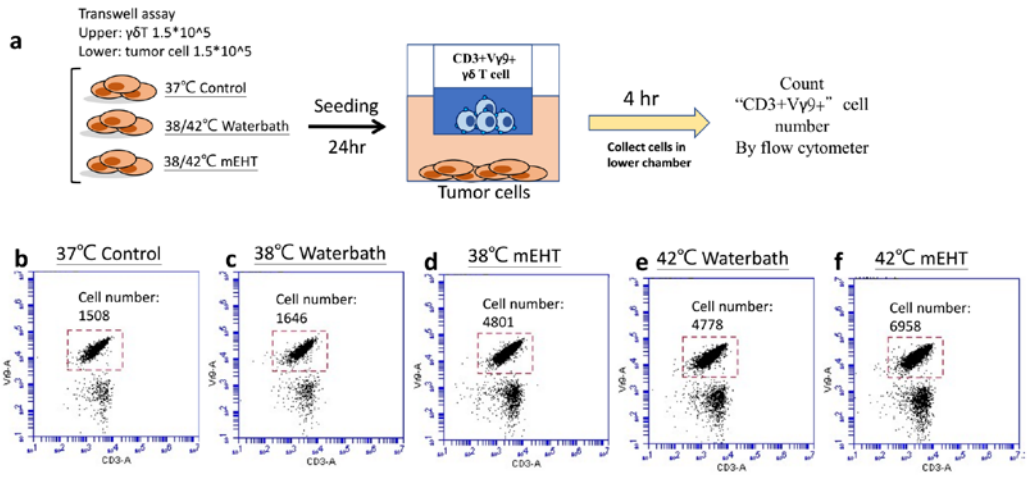

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Figure 4. Quantitative and qualitative analysis of γδT cells migration assessed by in vitro transwell assay. (a) Experimental design scheme of transwell migration assay. γδT cells were seeded on the upper side of the transwell membrane. In the lower compartment, various treatments HepG2 cells was added as a chemoattractant. (b-f) Flow cytometry images of CD3+Vγ9+ staining and cell number counting were analyzed at 4h after.

3.5. mEHT induced anti-tumor effect of γδT cell therapy in vivo

mEHT was administered to mice before γδT injection. Tumors were treated to determine the direct therapeutic effect of mEHT-γδT therapy with γδT injected into the tumor site 24h after mEHT treatment. The mice were measured for tumor size every 2 to 3 days until death (tumor size > 3000 mm^3). mEHT-γδT therapy delayed local tumor growth significantly (Figure 5). mEHT treatment alone also resulted in significant growth delay compared to control or γδT alone ($P < 0.05$).

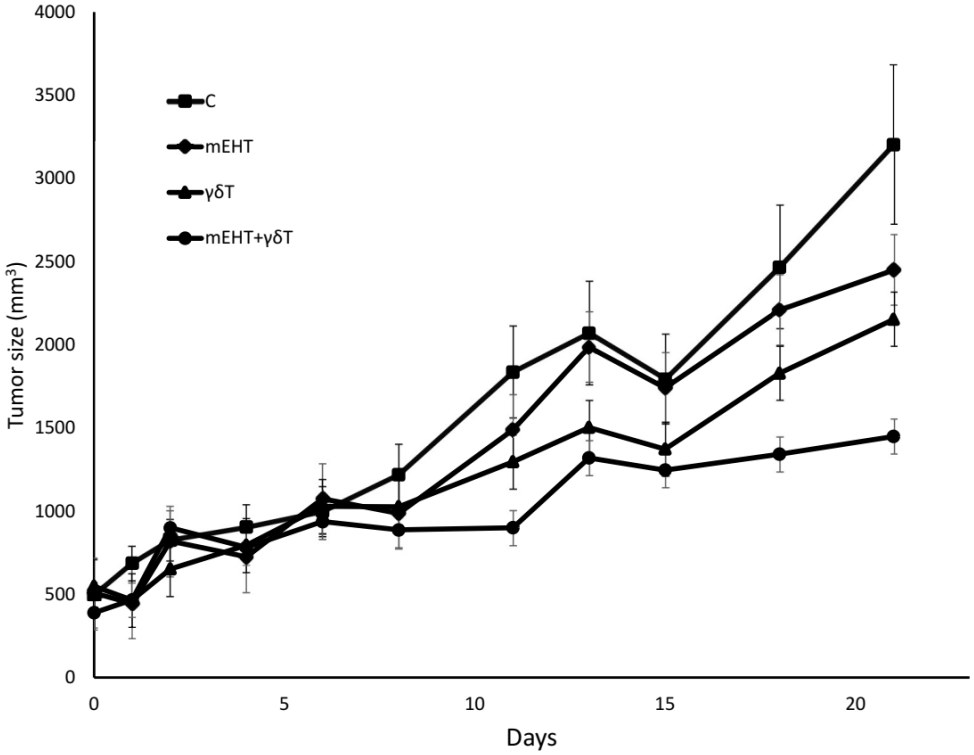

Figure 5. Tumor growth inhibition. Mice were injected with 5×10^5 HepG2 tumor cells s.c. in the right femoral area and treated with mEHT in mEHT alone and mEHT+ γδT group, after 24h followed by γδT cells i.v. injection in γδT alone and mEHT+ γδT groups. The treatments were repeated for two times with five days interval.

3.6. mEHT-γδT therapy increases tumor infiltrating leukocytes

In order to confirm the γδT migration generated following treatment with mEHT, tumors from treated and control mice were extracted and processed for immunohistochemical analysis 2 days after treatment. Tumor infiltrating human CD3+ leukocytes were identified (Fig. 6a-c). The presence of tumor infiltrating CD3+ leukocytes significantly increased in mice treated with mEHT-γδT, compared to those treated with mEHT alone, γδT treatment alone, or control (Fig. 6g). The results indicated that mEHT could recruit γδT into the tumor tissue and induce anti-tumor response.

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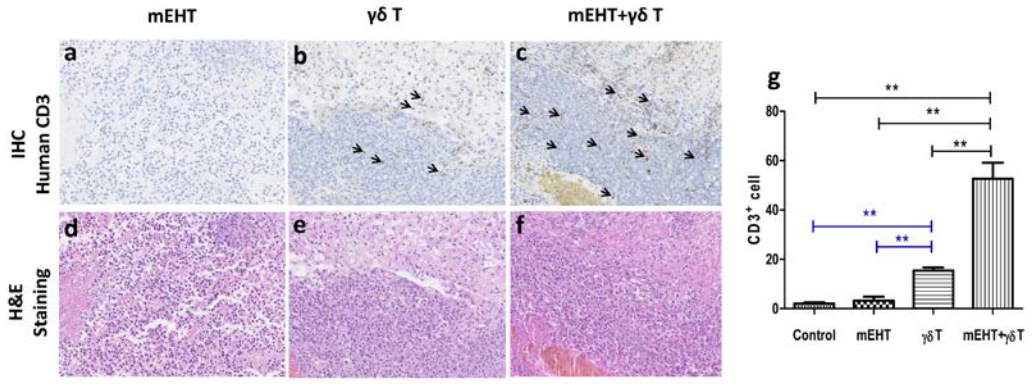

Figure 6. Immune cell infiltration of tumor sites after mEHT and γδT treatment. (a-c) Representative pictures of immunohistochemical analysis showed that human CD3 increased in tumors treated with γδT and mEHT either alone or in combination. (d-f) H&E staining. (g) Counting of infiltrated CD3+ cells in various treatments. The positive cells were calculated from one field and randomly selected from 10 fields. Error bars represent standard errors. (***) $P < 0.001$ (t-test) compared with the control.

4. Discussion

While γδT infusion *ex vivo* is generally considered safe, clinical experience has shown the benefits to be limited. This study explored whether treatment with mEHT could increase the therapeutic efficacy of γδT treatment. The principle is (1) mEHT can weaken cancer cells and increase the cytotoxic ability of γδT towards cancer cells. (2) γδT is attracted to heat shock proteins, and mEHT treatment leads to large amounts of heat shock proteins in the tumor microenvironment, thereby increasing the number of tumor-infiltrating γδT.

Recent studies on the clinical application of γδT have focused on allogeneic γδT transfer, bispecific γδT engagers (bsTCEs), and chimeric antigen receptor (CAR) γδT [27]. However, the pathway explored in these studies require further clinical trials for efficacy and safety, meaning at the very least, numerous years from practical clinical application. Alternatively, both un-adulterated γδT and mEHT therapies have been approved for clinically use, with proven track records for safety.

In this study, we tested the difference in the cytotoxicity of γδT combined with mEHT treatment. Analyzing the therapeutic effects of 38°C and 42°C heating treatment is an unique way to analyze mEHT [19]. Studies have shown mEHT can play a therapeutic effect at 38°C, without the need to reach 42°C [28]. The mild fever range of 38°C is less likely to cause systemic discomfort to the patient, which makes it easier for the patient to complete the treatment course. This study specifically added a comparison between 38°C and 42°C, hoping to confirm that mEHT at 38°C would also increase the therapeutic effect of γδT. The results of this study seem to corroborate this rationale that the cytotoxicity of γδT is enhanced with 38°C mEHT, and can be applied to clinical applications.

It has been reported that the expression of Hsp70 on the plasma membrane can induce immunogenic cell death pathway and initiate innate and adaptive immune reactions. Our previous studies have shown mEHT to induce tumors to release Hsp70 into the extracellular environment [13, 26]. The released Hsp70 serves as a danger signal, which alters the tumor microenvironment to a more immunological-responsive milieu, inducing dendritic cell differentiation for antigen presentation. This could trigger cytotoxic T-cell response. In another study, we found a combination of mEHT and intra-tumoral injection of DC not only elicited anti-tumor responses locally, but also induced a systemic immune response, including tumor-specific T-cells [26]. The ability of mEHT to compel a high percentage of tumor cell apoptosis, and increase the release of Hsp70, were key factors to induce tumor-specific immune response. Both Andocs et al. and our lab have compared mEHT heating at 38°C and 42°C to other heating methods at 42°C.

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These studies revealed the existence of thermal and nonthermal effects occurring simultaneously during treatment by mEHT [13, 29, 30]. In the presence of high non-thermal doses, powerful apoptotic forces overcome the protective facilities of HSPs and disrupt cellular integrity [31]. The HSPs could translocate to the plasma membrane, to become mHSPs [32]. The membrane expression of the major HSPs included Hsp25, Hsp60, Hsp70 and Hsp90 [33]. The function of these HSPs were seen as double-edged, either protecting the cell from apoptosis [34] or acting as a danger signal thereby stimulating immune response [35]. This duality has led to uncertainties about the prognostic value of HSPs on malignant cells [36]. When HSP is viewed as a stimulant of immune response, cancer cell-released Hsp70 can induce activation of nature killer cells (NK) [37]. mHsp70, mainly present in cholesterol-rich microdomains of cell membranes [38], is shown to be induced by mEHT, attracting NK migration to the heated tumor site [39]. These observations consist with our results for γδT, which we believe to be comparably more sensitive to HSPs, and should offer clinical benefit in combination with mEHT treatment.

The mechanism by which γδT identify tumor cells and trigger cytotoxicity is not clear. It may be related to the ability of γδT to recognize autophosphorylated antigens (PAgs), such as isopentenyl pyrophosphate (IPP), accumulated in tumor cells and F1-ATPase expressed on the surface of tumor cells. γδT can recognize some inducing molecules, such as MICA, MICB, ULBP and RAET1, Apoliportein A1, and Toll-like receptor that are abnormally expressed in tumors [40]. γδT recognizes tumor cells through NKG2D receptors in an independent manner of MHC, akin to NK, meaning even when tumor cells do not express MHC molecules, they can still be recognized and killed by γδT. Current studies suggest that γδT infiltrates the tumor site through Chemokine receptors such as CCR1, CXCR3, and CCR5, while indicating this migration ability is specific [41]. Previous studies have also shown that mTOR manifestations, mitochondrial metabolism, and increased Mevalonate metabolic pathways have resulted in the release of a large amount of Phospho-antigen (pAg) from within the cell, directly activating γδT [42]. Our current research also found that mEHT can promote the metabolism of the mitochondria, which in turn increases the metabolic pathway of Mevalonate (unpublished data).

5. Conclusions

In conclusion, using mEHT to enhance the effect of γδT in treating cancer has major advantages: 1) mEHT can selectively heat the tumor area; 2) mEHT can selectively generate heat spots on the cell membrane; 3) thermal activation caused by cell membranes can induce apoptosis in tumor cells, while accelerating the movement of lymph and other cells that are not tightly adhered to one another; 4) in various immune cells, γδT are relatively more sensitive to HSP; 5) mEHT may increase the mevalonate metabolic pathway by promoting mitochondrial metabolism, thereby releasing more phospho-antigen (pAg) from within the cell to directly activate γδT. Immune cell infiltration and tumor micro-environment are currently hot topics in oncology. Numerous studies have shown electro-hyperthermia to promote immune cell infiltration into the tumor-site. Our study demonstrates that cell therapy combined with electro-hyperthermia is a viable multi-modality option for the treatment of cancer.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Video S1: Video of γδT co-cultured with mEHT-treated A549 cells.

Author Contributions: Conceptualization, M.C.T., L.S.L. and S.K.H.; methodology, G.H.H.; validation, K.L.L., Y.W.T, S.W.L. and P.W.S.; formal analysis, Y.K.C.; investigation, Y.C.C.; data curation, Y.C.C.; writing-original draft preparation, Y.C.C. and Y.S.W.; writing-review and editing, Y.S.W. and Y.C.C.; visualization, H.J.C.; supervision, Y.C.; project administration, Y.S.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Tungs' Taichung Metro Harbor Hospital, Taichung, Taiwan, ROC (Approval Number, #110043). All in vivo experiments were approved by the Institutional Animal Care and Use Committee, National Yang Ming Chiao Tung University (IACUC No. 1090414).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: the beginning of the end of cancer? *BMC Medicine*. **2016**;14: 73.
2. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature Reviews Immunology*. **2020**;20: 651-668.
3. Yu S, Wang Y, He P, et al. Effective Combinations of Immunotherapy and Radiotherapy for Cancer Treatment. *Front Oncol*. **2022**;12: 809304.
4. Zheng J, Liu Y, Lau Y-L, Tu W. γδ-T cells: an unpolished sword in human anti-infection immunity. *Cell Mol Immunol*. **2013**;10: 50-57.
5. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol*. **2000**;18: 975-1026.
6. Hoeres T, Smetak M, Pretscher D, Wilhelm M. Improving the Efficiency of Vgamma9Vdelta2 T-Cell Immunotherapy in Cancer. *Front Immunol*. **2018**;9: 800.
7. Nussbaumer O, Koslowski M. The emerging role of γδT cells in cancer immunotherapy. *Immuno-Oncology and Technology*. **2019**;1: 3-10.
8. Knight A, Mackinnon S, Lowdell MW. Human Vdelta1 gamma-delta T cells exert potent specific cytotoxicity against primary multiple myeloma cells. *Cytotherapy*. **2012**;14: 1110-1118.
9. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K. Phase I/II study of adoptive transfer of gammadelta T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunol Immunother*. **2011**;60: 1075-1084.
10. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res*. **2007**;67: 7450-7457.
11. Hirsh MI, Junger WG. Roles of heat shock proteins and gamma delta T cells in inflammation. *Am J Respir Cell Mol Biol*. **2008**;39: 509-513.
12. Wadia P, Atre N, Pradhan T, Mistry R, Chiplunkar S. Heat shock protein induced TCR gammadelta gene rearrangements in patients with oral cancer. *Oral Oncol*. **2005**;41: 175-182.
13. Yang KL, Huang CC, Chi MS, et al. In vitro comparison of conventional hyperthermia and modulated electro-hyperthermia. *Oncotarget*. **2016**;7: 84082-84092.
14. Fotopoulou C, Cho CH, Kraetschell R, et al. Regional abdominal hyperthermia combined with systemic chemotherapy for the treatment of patients with ovarian cancer relapse: Results of a pilot study. *Int J Hyperthermia*. **2010**;26: 118-126.
15. Wust P, Hildebrandt B, Sreenivasa G, et al. Hyperthermia in combined treatment of cancer. *Lancet Oncology*. **2002**;3: 487-497.
16. Masunaga S-I, Nishimura Y, Hiraoka M, Abe M, Takahashi M, Ono K. Efficacy of Mild Temperature Hyperthermia in Combined Treatments for Cancer Therapy. *Thermal Medicine*. **2007**;23: 103-112.
17. Fiorentini G, Szasz A. Hyperthermia today: electric energy, a new opportunity in cancer treatment. *J Cancer Res Ther*. **2006**;2: 41-46.
18. Szasz A, Vincze G, Szasz O, Szasz N. An Energy Analysis of Extracellular Hyperthermia. *Electromagnetic Biology and Medicine*. **2003**;22: 103-115.
19. Andocs G, Szasz O, Szasz A. Oncothermia treatment of cancer: from the laboratory to clinic. *Electromagnetic Biology and Medicine*. **2009**;28: 148-165.
20. Szasz A, Szasz N, Szasz O. Oncothermia: A New Kind of Oncologic Hyperthermia. Oncothermia: Principles and Practices: *Springer Netherlands*, 2010:173-392.
21. Feyerabend T, Wiedemann GJ, Jager B, Vesely H, Mahlmann B, Richter E. Local hyperthermia, radiation, and chemotherapy in recurrent breast cancer is feasible and effective except for inflammatory disease. *Int J Radiat Oncol Biol Phys*. **2001**;49: 1317-1325.
22. Fiorentini G, Giovanis P, Rossi S, et al. A phase II clinical study on relapsed malignant gliomas treated with electro-hyperthermia. *In Vivo*. **2006**;20: 721-724.
23. Mole RH. Whole body irradiation; radiobiology or medicine? *Br J Radiol*. **1953**;26: 234-241.

24. Szasz A, Vincze G. Dose concept of oncological hyperthermia: heat-equation considering the cell destruction. *J Cancer Res Ther*. **2006**;2: 171-181.
25. Hegyi G, Szigeti GP, Szasz A. Hyperthermia versus Oncothermia: Cellular Effects in Complementary Cancer Therapy. *Evid Based Complement Alternat Med*. **2013**;2013: 672873.
26. Tsang YW, Huang CC, Yang KL, et al. Improving immunological tumor microenvironment using electro-hyperthermia followed by dendritic cell immunotherapy. *BMC Cancer*. **2015**;15: 708.
27. Saura-Esteller J, de Jong M, King LA, et al. Gamma Delta T-Cell Based Cancer Immunotherapy: Past-Present-Future. *Front Immunol*. **2022**;13: 915837.
28. Andocs G, Renner H, Balogh L, Fonyad L, Jakab C, Szasz A. Strong synergy of heat and modulated electromagnetic field in tumor cell killing. *Strahlenther Onkol*. **2009**;185: 120-126.
29. Andocs G, Meggyeshazi N, Balogh L, et al. Upregulation of heat shock proteins and the promotion of damage-associated molecular pattern signals in a colorectal cancer model by modulated electrohyperthermia. *Cell Stress Chaperones*. **2015**;20: 37-46.
30. Andocs G, Rehman MU, Zhao QL, Tabuchi Y, Kanamori M, Kondo T. Comparison of biological effects of modulated electrohyperthermia and conventional heat treatment in human lymphoma U937 cells. *Cell Death Discovery*. **2016**;2: 16039.
31. Danics L, Schvarcz CA, Viana P, et al. Exhaustion of Protective Heat Shock Response Induces Significant Tumor Damage by Apoptosis after Modulated Electro-Hyperthermia Treatment of Triple Negative Breast Cancer Isografts in Mice. *Cancers*. **2020**;12: 2581.
32. Vega VL, Rodriguez-Silva M, Frey T, et al. Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *J Immunol*. **2008**;180: 4299-4307.
33. Sapozhnikov AM, Ponomarev ED, Tarasenko TN, Telford WG. Spontaneous apoptosis and expression of cell surface heat-shock proteins in cultured EL-4 lymphoma cells. *Cell Proliferation*. **1999**;32: 363-378.
34. Horváth I, Vígh L. Stability in times of stress. *Nature*. **2010**;463: 436-438.
35. Hildebrandt B, Wust P, Ahlers O, et al. The cellular and molecular basis of hyperthermia. *Critical Reviews in Oncology/Hematology*. **2002**;43: 33-56.
36. Gehrmann M, Radons J, Molls M, Multhoff G. The therapeutic implications of clinically applied modifiers of heat shock protein 70 (Hsp70) expression by tumor cells. *Cell Stress and Chaperones*. **2008**;13: 1-10.
37. Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels R. Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. *J Immunol*. **1997**;158: 4341-4350.
38. Gehrmann M, Liebisch G, Schmitz G, et al. Tumor-Specific Hsp70 Plasma Membrane Localization Is Enabled by the Glycosphingolipid Gb3. *PLoS ONE*. **2008**;3: e1925.
39. Meggyeshazi N, Andocs G, Balogh L, et al. DNA fragmentation and caspase-independent programmed cell death by modulated electrohyperthermia. *Strahlentherapie und Onkologie*. **2014**: 1-8.
40. Caccamo N, Dieli F, Meraviglia S, Guggino G, Salerno A. Gammadelta T cell modulation in anticancer treatment. *Curr Cancer Drug Targets*. **2010**;10: 27-36.
41. Nicol AJ, Tokuyama H, Mattarollo SR, et al. Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. *Br J Cancer*. **2011**;105: 778-786.
42. Gruenbacher G, Thurnher M. Mevalonate Metabolism in Immuno-Oncology. *Frontiers in Immunology*. **2017**;8.