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REVIEW ARTICLE

# Immunological effects of nano-enabled hyperthermia for solid tumors: opportunity and challenge

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**Abstract** Compared to conventional hyperthermia that is limited by low selectivity and severe side effects, nano-enabled hyperthermia yields great potentials to tackle these limitations for cancer treatment. Another major advance is the observation of immunological responses associated with nano-enabled hyperthermia, which introduces a new avenue, allowing a potential paradigm shift from the acutely effective and cytotoxicity-centric response to the next-phase discovery, i.e., long-lasting and/or systemic anti-tumor immunity. This perspective first discusses the temperature-gradient and the spatially-structured immunological landscape in solid tumors receiving nano-enabled hyperthermia. This includes the discussion about underlying mechanism such as immunogenic cell death, which initiates a profound immunological chain reaction. In order to propagate the immune activation as a viable therapeutic principle, we further discussed the tumor type-specific complexity in the immunological tumor microenvironment, including the creative design of nano-enabled combination therapy to synergize with nano-enabled hyperthermia.

**Keywords** nano-enabled hyperthermia, immunogenic cell death, heterogeneous immunological landscape, tumor microenvironment

## 1 Introduction

The nascence of hyperthermia (HT) can trace back to a few decades ago when "percutaneous energy-based ablation" was utilized to treat different cancer types, such as breast cancer, head and neck cancer and melanoma [1–4]. The effect of HT was also investigated in other deep-seated tumors, including liver, kidney, lung and pancreatic cancer [1–4]. While heat can be generated through different means (e.g., microwave, radiofrequency, laser and ultrasound technology), a common pitfall for the traditional HT is the lack of "thermal discrimination", meaning that the energy dissipation in the normal tissue becomes a non-neglect factor. Indeed, classic HT suffers from a long list of limitations, such as safety concern, severe pain, incomplete ablation and tumor recurrence [1–4]. In recent years, various engineered nanomaterials (ENMs) have been creatively established as a multifunctional platform to fulfil the task, i.e., the local generation of high temperature, targeting and precise heat control, in studies involving a variety of cancer cell types and tumor disease models [5,6]. In terms of chemical composition and heat generation approach, nano-enabled HT (*n*-HT) can be conceptualized and divided into photothermal therapy (PTT), magnetic HT (MHT), radio-frequency ablation (RFA) and ultrasound HT, etc. [7].

The different modes of HT hold unique advantages and also have certain limitations [7]. PTT and MHT are the two most widely utilized approaches for targeted HT. PTT effect, stimulated by visible (380–650 nm) or near infrared (NIR) light (650–1100 nm), can be greatly enhanced by nanoparticles (NPs) such as gold NPs (AuNPs) and certain carbon-based nanomaterials, which significantly improve the efficiency of light absorption and energy conversion [8–10]. The major advantages of nano-PTT include high safety, efficiency and accessibility to the tumors in body cavities and lumens, while low penetration (for visible irradiation) and requirement of complex equipment (for deep-seated tumors) are the common limitation [7].

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Compared to PTT, magnetic NPs (MNPs) mediated *n*-HT can overcome the surface heating issues and is suitable for repeatable treatment with a single dose of NPs [5–7,11]. However, the specific absorption rate for most magnetic *n*-HT is relatively low, which may require high NP concentration in order to achieve effective ablation effect. Recently, nano RFA based on the thermal effects of certain NPs, such as AuNPs, single-walled carbon nanotubes (SWNTs), Pt and Si NPs, become an emerging technology [7,12–14]. The nano RFA is completely noninvasive with high light penetration and focusing capability, however, non-uniform heating and fat overheating remain to be overcome. Another emerging approach called nano-ultrasound HT (NUHT), which exhibits similar advantages while the fat overheating problem is solved [7]. Noteworthy, the thermal effects of NUHT depend on the attenuation coefficient and thermal conductivity of the NPs. It was reported that AuNPs, MNPs and nano-graphene oxide (GO) have significant sonosensitizing characteristics and can lead to high heating effect during sonication [15–17]. However, NUHT is usually limited by air cavity and has the issue of forming hot spots at bone interfaces.

With the rapid advent of different *n*-HT approaches, it has become clear that understanding the biological effects of thermal ablation is of great importance for improving the therapeutic effect and designing the next-generation of *n*-HT. The cellular response to heat is very complicated. The primary effect is based on denaturation of cytoplasmatic and membrane proteins [18]. An increase in temperature results in increased motion of all molecules, more rapid reaction rates, increased metabolism and transitions in cellular structures, such as membranes and macromolecules (e.g., protein and DNA). This jeopardizes cellular hemostasis, i.e., from an ordered native state to a more disordered state [19]. The integrated effects on protein folding and cellular stress lead to specific end points such as cell killing and sensitization to additional stresses [20]. While the alteration of cell membrane integrity and nuclear damage were originally considered to be the primary reason of *n*-HT-mediated killing effect, nanobiologists gradually realize that *n*-HT also generates profound local and systemic immunological responses, which are way beyond acute cancer cell killing. Early evidence includes the observation of the pro-inflammatory cytokine production such as interleukin-1 family (e.g., IL-1$\beta$), IL-6 and tumor necrosis factor-$\alpha$ [21]. Moreover, ample data also demonstrated the induction of heat shock protein 70 (HSP70), which plays a key role in facilitating the immunological effects in both classic HT and *n*-HT [22,23]. For the HSP proteins, while intracellular HSPs function as a protective mechanism from apoptosis, extracellular version was a proven antigen chaperone and "danger signal"; it also involved in the unique form of cell death, such as immunogenic cell death (ICD) [24,25], which will be discussed later. Against this background, it is rational to hypothesize that the immunological effect of HT could be pursued as a new strategy to augment the performance of HT, including *n*-HT. In this perspective, we outlined the recent observation of the immunological effects of nanotechnology mediated HT, followed by a critical discussion on the key barriers that require additional investigation at the immunological nano/bio interface.

## 2 What happened at tumor site receiving *n*-HT treatment modality from the immunological perspective?

Numerous studies have been focused in the context of *n*-HT for cancer treatment. For the early-stage investigations, the biological endpoints are frequently measured in its acute phase or be cell killing-centric. Take AuNPs for example, it was possible to use the photothermal properties of gold nanomaterials to convert the optical energy into heat at tumor sites [8]. Recently, it became popular to engineer the aspect ratio of AuNPs, and the resulting gold nanorods exhibited two surface plasmon resonance (SPR) bands. This includes an intense longitudinal SPR band, which is tunable from visible wavelength to NIR region (i.e., 650–900 nm) through aspect ratio fine-tuning (e.g., 2–4) [8]. Previous studies showed that NIR light was capable of tissue penetration up to several cm depth, a characteristics that cannot be easily achieved using gold nanosphere [26]. For proof-of-principle, it was showed that the photothermal effect of polyethylene glycol (PEG) coated gold nanorods in HSC-3 tumor xenograft bearing nude mice [27]. The authors demonstrated significant and equally potent anti-cancer effects 13-day post intratumoral (IT) and intravenous (IV) administration with highly significant $p$ values compared to control (i.e., $p < 0.0001$ and $p < 0.0008$, respectively). In another study, efficient anti-cancer effect was achieved using gold nano rods with targeting design, such as epidermal growth factor receptor or arginine-glycine-aspartic acid (RGD) peptide modification [26]. In addition to nano gold, other ENMs such as graphene are responsive to laser irradiation and therefore proposed as an emerging PTT option in a wide range of cancer types [28]. In these early attempts, the mechanism of *n*-HT's efficacy is usually considered as the induction of irreversible apoptosis or necrosis, evidenced by the activation of caspase signaling pathways and/or compromised cell integrity [9]. Noteworthy, considerable amount of the early studies were performed using immortal human cancer cell lines that can be used to grow subcutaneous or orthotopic tumors in immunocompromised animals — therefore the immunological effects of *n*-HT has been largely overlooked in the early era.

In addition to the direct anti-cancer effect, optimism has emerged with the advent of cancer immunotherapy, which includes the presence of T-cell immunity capable of adding

additional therapeutic benefits in solid tumors receiving *n*-HT treatment (Table 1) [29–42]. One possible mechanism is the involvement of ICD at the tumor site (Fig. 1). ICD, which is a unique form for cell death that can be triggered by certain chemotherapeutic agent such as anthracyclines and certain physical stimuli (including *n*-HT), is characterized by the surface expression of calreticulin (CRT) on the dying tumor cell surface. The resulting CRT molecule functions as a potent "eat-me" signal through a CD91-mediated dendritic cell (DC) internalization process. ICD also triggered the subsequent release of high mobility group box 1 protein (HM GB1) and adenosine triphosphate (ATP), which serve as endogenous adjuvants capable of facilitating DC activation, followed by T cell recruitment and maturation [43,44]. Generally speaking, ICD provides an attractive endogenous vaccination approach of triggering tumor infiltrating lymphocytes recruitment, and therefore generate a "hot" starter in the comparative immune deplete (a.k.a. "cold") microenvironment that represents the majority of solid tumor types [43,45]. For examples, Yu et al. demonstrated that the PTT effects of myeloid-derived suppressor cell (MDSC) membrane-coated iron oxide magnetic nanoparticle can enhance antitumor response by inducing ICD, characterized by the elevated expression of CRT and HMGB1 in B16-F10 melanoma mouse model [35]. The immunogenic effect of PTT led to reprogram the tumor infiltrating macrophages and reduce the tumor's metabolic activity (Fig. 2) [35]. In another study, Ma et al. developed self-assembly complex of liposome with the inclusion of AuNPs, which enabled PTT using irradiation from NIR(I) to NIR(II) range. It was demonstrated that NIR(II)-mediated PTT could trigger ICD in a more homogeneous and deeper fashion compared to NIR(I) and red light in a 4T1 breast cancer model [40]. The NIR(II) PTT provoked innate and adaptive immunity, leading to efficient antitumor and anti-metastasis effects when combined with a-PD-1 checkpoint blockade therapy (Fig. 3) [40]. Another example is to use melanin NPs (M NPs) with cancer cell membrane coating (M@C NPs). In a 4T1 breast cancer, M@C NPs mediated PTT effects enhanced antitumor immune response by inducing ICD, which led to impressive therapeutic effect for both primary and abscopal tumors, when combined with a metabolic checkpoint inhibitor, indoleamine 2,3-dioxygenase (IDO) inhibitor (Fig. 4) [42].

**Table 1** Representative examples of NP-mediated HT effect on immunotherapy

| HT type | Design of NP | Animal model | Administration route | Major immunological endpoints | Ref. |
|---|---|---|---|---|---|
| NIR PTT | Chitosan-coated hollow CuS NPs with immunoadjuvants oligodeoxynucleotides containing the cytosine-guanine (CpG) motifs | EMT6 breast cancer | i.t. (intratumorally injection) | The HCuSNPs-CpG-mediated photothermal immunotherapy elicits more effective systemic immune responses than immunotherapy or PTT alone, resulting in combined anticancer effects against primary treated as well as distant untreated tumors | [29] |
| NIR PTT | PEGylated SWNTs | 4T1 breast cancer | i.t. | The PEGylated SWNTs mediated photothermal tumor destruction could release tumor-associated antigens and act as an immunological adjuvant to greatly promote maturation of DCs and production of anti-tumor cytokines. The combination of SWNT-based PTT with antiCTLA-4 therapy could modulate the adaptive immune responses especially cellular immunity for the treatment of metastatic cancer | [30] |
| NIR PTT | PEG and polyethylenimine (PEI) dual-polymer-functionalized GO (GO-PEG-PEI) carrying CpG | CT26 colon cancer | i.t. | The GO-PEG-PEI mediated photothermal effect enhanced immunostimulation responses of CpG, owing to the photothermally induced local heating that accelerated intracellular trafficking of nanovectors | [31] |
| NIR PTT | Poly(lactic-co-glycolic) acid (PLGA) NPs encapsulating NIR photothermal agent indocyanine green (ICG) and toll-like-receptor-7 agonist imiquimod (R837) | 4T1 breast cancer CT26 colon cancer | s.c. (subcutaneously injection), i.v. (intravenously injection) | The photothermal ablation of primary tumours using PLGA-ICG-R837 NPs, generated tumour-associated antigens, which in the presence of R837-containing NPs as the adjuvant showed vaccine-like functions. In combination with anti-CTLA4, the generated immunological responses was able to attack remaining tumour cells in mice to inhibit metastasis. This strategy offered a strong immunological memory effect, which provided protection against tumour rechallenging post elimination of their initial tumours | [32] |
| NIR PTT | PEG coated plasmonic gold nanostar (GNS) | MB49 bladder cancer | i.t., i.v. | The GNS-mediated PTT combined with anti-PD-L1 were able to achieve complete eradication of primary treated tumors and distant untreated tumors in some mice with effective long-lasting immunity against MB49 cancer cells rechallenge | [33] |

(*Continued*)

| HT type | Design of NP | Animal model | Administration route | Major immunological endpoints | Ref. |
|---|---|---|---|---|---|
| NIR PTT | Polydopamine-coated spiky AuNPs (SGNP@PDA) | CT26 colorectal cancer; TC-1 head and neck squamous cell carcinoma | i.t. | SGNP@PDA PTT combined with a sub-therapeutic chemo dose of doxorubicin (DOX), elicits robust anti-tumor responses in both cellular ($CD8^+$ T and NK cells) and humoral compartments. Chemo-PTT eliminates residual tumor cells from locally treated tumors and exerts an abscopal effect against untreated, distant tumors, and also exhibits long-term resistance against tumor rechallenge due to the establishment of immunological memory | [34] |
| NIR PTT | MDSC membrane-coated iron oxide MNP (MNP@MDSC) | B16-F10 melanoma | i.v. | MNPs@MDSC mediated PTT effects enhanced antitumor response by inducing ICD, reprogramming the tumor infiltrating macrophages, and reducing the tumor's metabolic activity | [35] |
| NIR PTT | CpG self-crosslinked NPs-loaded IR820-conjugated hydrogel | B16 melanoma | i.t. | IR820-hydrogel mediated PTT induced tumor antigens release for enhancing the immunotherapy effect. CpG NPs serve as adjuvant to improve the immune stimulation. The combined specific antitumor immunity achieved more effective systemic therapeutic effect than PTT or immunotherapy alone | [36] |
| NIR PTT | Intracellularly generated AuNPs | B16F10 Melanoma 4T1 breast cancer | s.c. | The AuNPs were generated intracellularly and then exocytosed as nanoparticle trapped vesicles with retained original bioinformation. After further introduction to DCs, DCs-derived immunological AuNPs induced HT under NIR irradiation and provoked strong antitumor immune responses, promoting DCs maturation, multiple cytokines secretion, and T cells activation | [37] |
| Magnetic HT | PEGylated iron NPs (FeNPs) | 4T1 breast cancer CT26 colon cancer | i.t. | The combination of FeNP-based MHT with local injection of nanoadjuvant and systemic injection of anti-CTLA4 checkpoint blockade would result in systemic therapeutic responses to inhibit tumor metastasis and a robust immune memory effect to prevent tumor recurrence | [38] |
| NIR PTT | Mesoporous silica NPs decorated with AuNPs (Au@XL-MSNs) loaded with CpG-ODNs | 4T1 breast cancer | i.t. | The photothermal effect of AuNPs enhanced cancer immunotherapy by generating a cancer antigen at the tumor site, which can be processed by tumor-infiltrated DCs and induce antigen-specific adaptive immune response | [39] |
| NIR(II) PTT | Self-assembly complex of liposome with AuNPs; two-dimensional polypyrrole nanosheets | 4T1 breast cancer | i.v. | PTT effect induced by NIR(II) light could trigger ICD more homogeneously and deeper than NIR(I) and red light, and more effective than oxaliplatin in solid tumors. The NIR(II) PTT provoked innate and adaptive immunity led to efficient antitumor and anti-metastasis effects when combined with checkpoint blockade therapy | [40] |
| NIR PTT | c-RGD-functionalized conjugated polymer NPs (CP NPs) | 4T1 breast cancer | i.t. | The CP NPs mediated photothermal effect demonstrated effective activation of proinflammatory immune response, induced antitumor immunity activation and ultimately inhibited tumor growth | [41] |
| NIR PTT | M NPs coated with cancer cell membrane (M@C NPs) | 4T1 breast cancer | i.v. | M@C NPs mediated PTT effects enhanced antitumor immune response by inducing ICD, which led to good therapeutic effect for primary and abscopal tumor when combined with immunoblocking inhibitor | [42] |

Moreover, the administration route should also be considered. While i.v. and i.t. administrations were traditionally regarded as "systemic" and "local" treatment, respectively, this classification may need to be adjusted when taking the immunological effects into consideration. Take nano-PTT for example, while locally generated heating effect during administration primarily applies at tumor microenvironment, the resulting immune activation

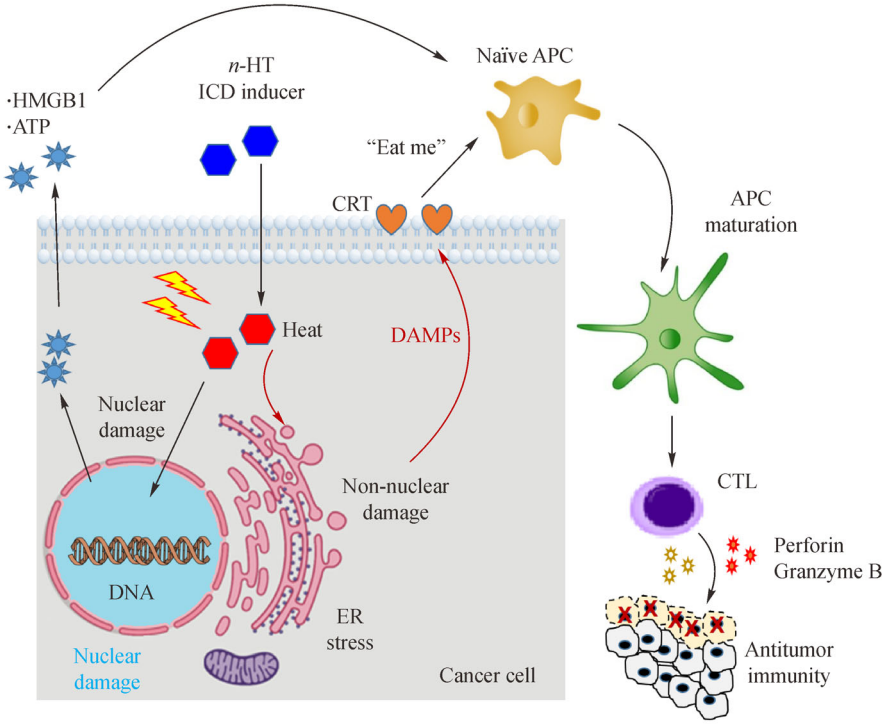


**Fig. 1** *n*-HT induced immunological genetic cell death (Schematic to illustrate the action of an inducer of ICD, achieved by *n*-HT. *n*-HT reagents induce an ICD response in which CRT expression on the dying cancer cell surface provides an "eat-me" signal for antigen presenting cells (APC). ICD response is also associated with the release of adjuvant stimuli (C), which promote APC maturation. This can further trigger the activation and recruitment of $CD8^+$ T cells capable of mediating cytotoxic cancer cell death by the release of perforin and granzyme. DAMPs, damage-associated molecular patterns).

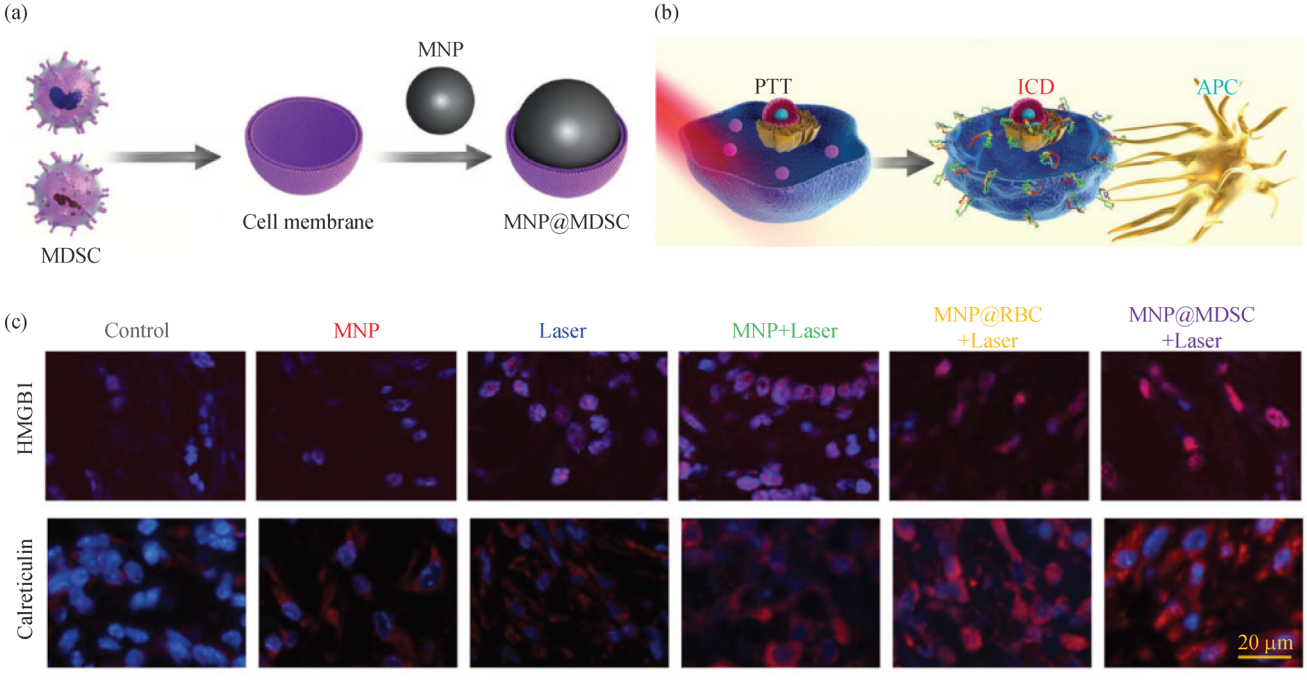


**Fig. 2** Schematic illustration of (a) the synthesis of MNP@MDSC by coating magnetic $Fe_3O_4$ nanoparticle with MDSC membrane and (b) PTT inducing ICD. (c) *In vivo* ICD induced by PTT of MNP@MDSC characterized by the elevated expression of HMGB1 and CRT, representative images of mouse tumor slices stained for HMGB1 (top) and CRT (bottom) after the indicated treatments in B16/F10 melanoma model. Reprinted with permission from ref. [35], copyright 2018, Wiley.

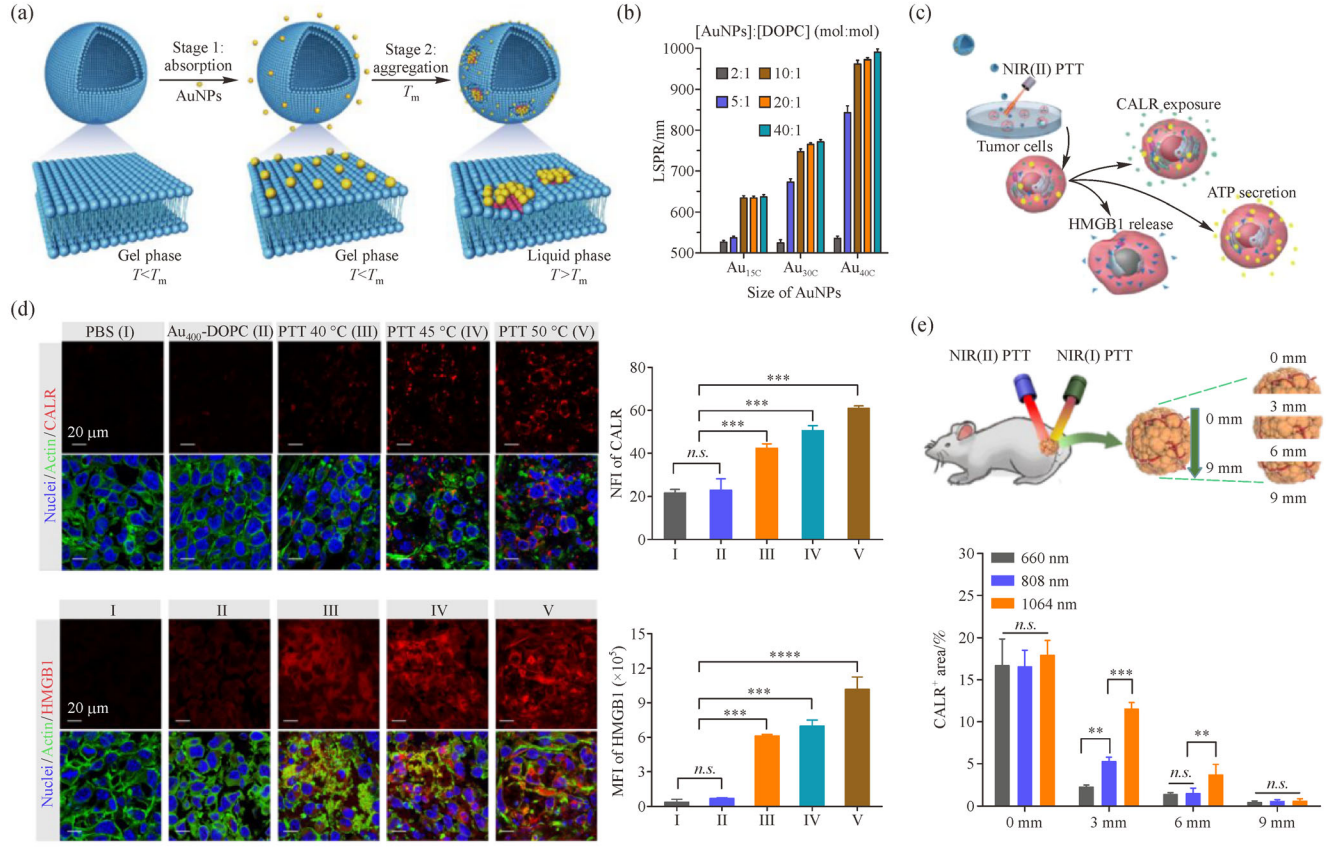


**Fig. 3** (a) Schematic showing controllable aggregation of AuNPs on fluidic liposomes; (b) localized SPRs of different sized Au15C, Au30C, and Au40C incubated with 100 nm DOPC liposomes at varied molar ratios; (c) NIR(II) PTT induced cell apoptosis and the subsequent release of DAMPs; (d) immunofluorescence staining and quantifications of CRT (upper panel) and HMGB1 release (bottom panel) in 4T1 tumors post-PTT; (e) upper panel: schematic diagram of the *in vivo* CRT exposure at different depths inside the tumor under the NIR(I) or NIR(II) laser irradiation; bottom panel: percentage of CRT positive areas of dissected tumor tissues at different depths (0, 3, 6, and 9 mm). Reprinted with permission from Ref. [40], copyright 2019 American Chemical Society.

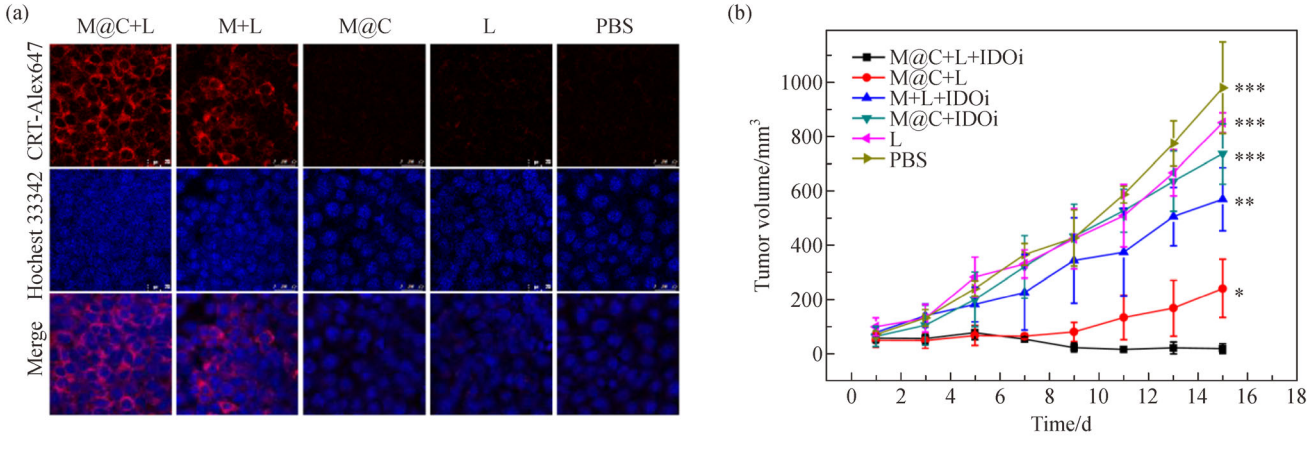


**Fig. 4** (a) *In vitro* ICD induced by PTT of M@C NPs characterized by the elevated expression of CRT on cell surface; (b) changes of tumor growth of 4T1 tumor-bearing mice with different treatment. $p$ values were calculated using the $t$-test (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). M: natural M NPs; M@C: cancer cell membrane coated M NPs; L: laser; IDOi: indoleamine IDO inhibitor. Reprinted with permission from ref. [42], copyright 2020, The Royal Society of Chemistry.

is profound and indeed engages systemic components, i.e., DC activation, T cells recruitment, and generation of memory T cells, etc. In this regard, IT-mediated PTT was associated with rejection of tumor re-challenge at a contralateral side [34], an indicative of engagement of systemic immunity. Noteworthy, while IT administration may require lesser dose compared to IV dose, it is relatively difficult to apply IT for deep-seated tumor types; systemic administration such IV could be an alternative [30].

While the immunological feature post *n*-HT treatment is likely to be case-specific, it is reasonable to discuss the temperature-gradient effect and resulting spatially-structured immunological landscape. Interestingly, this has been previously speculated in the literature when "applicator tip" was utilized (Fig. 5) [1]. While the putative "3-zone" model, i.e., "central zone" *vs.* "transitional zone" *vs.* "surrounding tissue zone" (Fig. 5(a)), needs to be fully characterized and validated by *n*-HT experimental data, many IT-administrated *n*-HT reagents may generate a biological scenario that is comparable to such a 3-zone model, evidenced by the in situ thermal imaging data that are widely available in the *n*-HT literature (including our own data [46]) (Figs. 5(b–e)). For example, post IT injection, the temperature in the "central zone" (the 1st zone) could reach up to ~50 °C where necrosis occurs, characterized by cell membrane collapse, protein denaturation, compromised enzyme activity and mitochondrial dysfunction damage [1]. The "transitional zone" (the 2nd zone) exerts a steep negative temperature-gradient, which can be exemplified by our previous experiment using B16 model receiving IT-injected copper sulfide nanocrystals (Figs. 5(b) and 5(c)) [46]. At moderately high temperature (around 41–45 °C), heat-induced injury is usually sublethal, including the robust generation of reactive oxygen species (ROS) (among other effects) that play a key role in ICD induction (Figs. 5(d) and 5(e)) [46,47]. Noteworthy, ROS production and unique cellular stress, i.e., endoplasmic reticulum (ER) stress, seem to be important mechanistic features allowing the separation of "type I" *vs.* "type II" ICD [48] (Fig. 6), which *n*-HT provisionally belongs to "type II" response, based on the limited information. While a "type I" ICD primarily targets the nucleus, with secondary impact on the ER, in a "type II" response ER stress is the primary event that secondarily leads to cell death and nuclear damage (Fig. 6) [48]. As far

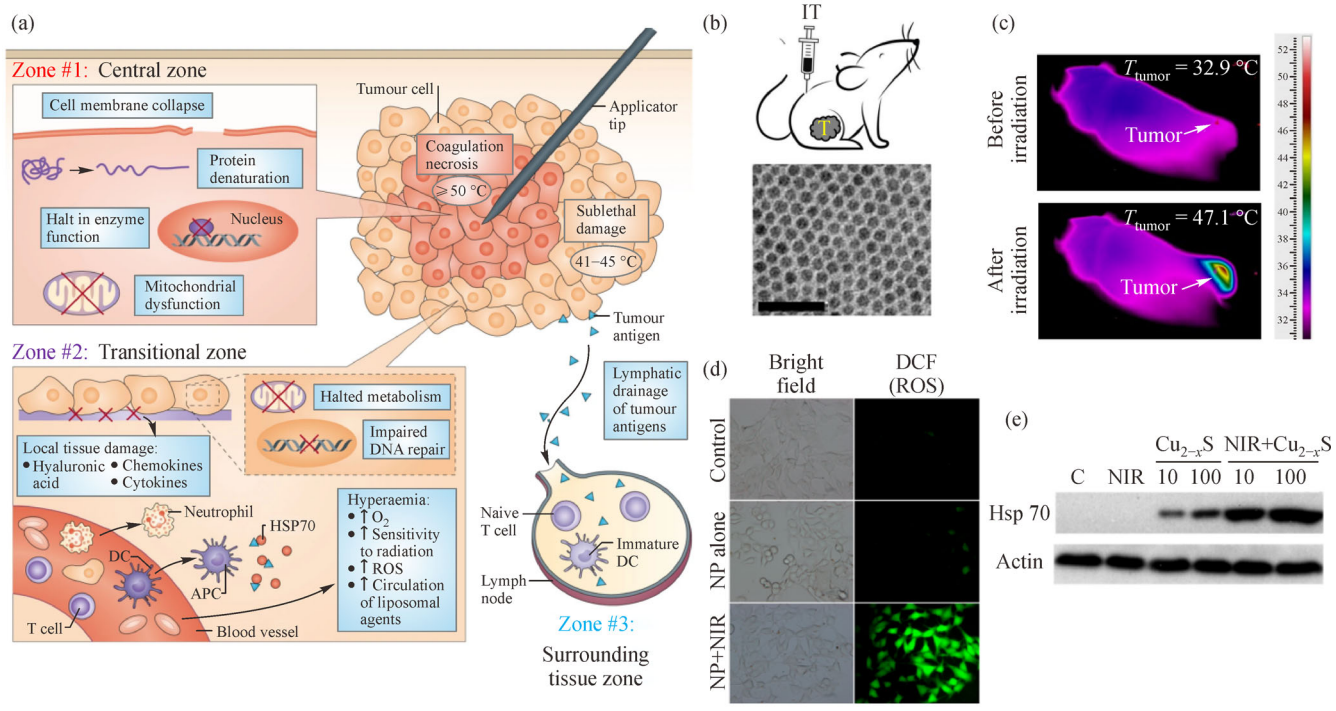


**Fig. 5** Immunological characteristics for *n*-HT mediated treatment for tumor treatment. (a) Use of 3-zone model to conceptualize the temperature-gradient effect and resulting spatially-structured immunological landscape at tumor site receiving *n*-HT. The picture obtained from literature with minor modification. Reprinted with permission from ref. [1], copyright 2014, Springer Nature. (b) B16 tumor bearing mice received IT injection of copper sulfide nanocrystals (transmission electron microscopy). (c) Body temperature was captured post the injection of copper sulfide nanocrystals w/wo NIR light. (d) *In vitro* evidence of ROS production and (e) HSP70 over-expression in response to the copper sulfide nanocrystal treatment in the presence of NIR irradiation. Reprinted with permission from ref. [46], copyright 2015, American Chemical Society.

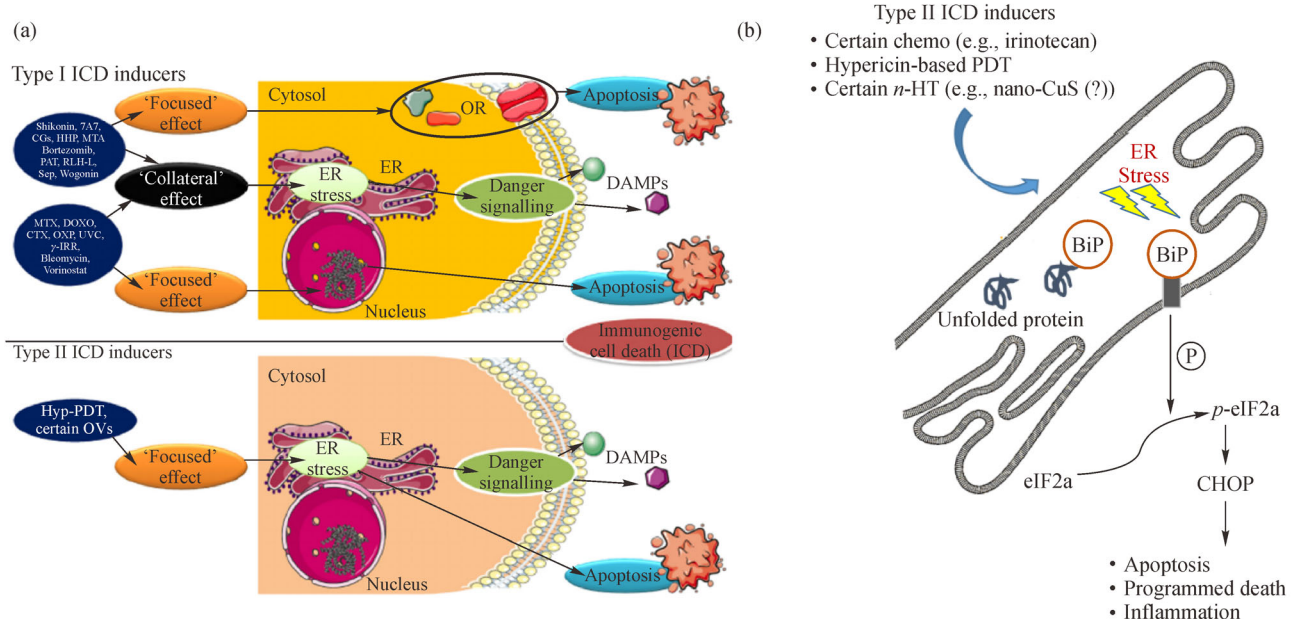


**Fig. 6** Type I *vs.* type II ICD. (a) Type I ICD inducers are modalities that induce cell death via non-ER associated targets (primary) and danger signaling via ER stress (secondary). Type II ICD inducers selectively target the ER to induce both cell death as well as danger signaling thereby causing ICD-associated immunogenicity in an ER-focused manner. Reprinted with permission from ref. [48], copyright 2013, Annual Reviews, Inc. (b) Simplified schematic to show the unfolded protein stress response in the ER during a type II ICD. The basis of the HT-induced effect on the ER has been shown to involve ROS production that leads to ER-associated proteotoxicity (i.e., an unfolded protein response). This includes the phosphorylation of the eukaryotic initiation factor (eIF2$\alpha$) that, in turn, is responsible for transcriptional activation of the CHOP protein. CHOP induces apoptotic cell death through the generation of immunological danger signals that promote antitumor immunity. Panel B was obtained from our previous publication with minor modification. Reprinted with permission from ref. [49], copyright 2021, Wiley.

as we know, only minority of chemo agents (e.g., irinotecan [49] and Pt-N-heterocyclic carbene [50]) and certain photodynamic therapy (using hypericin [51,52]) were professionally classified into "type II" ICD. At this stage, we can already envisage the unique therapeutic benefits from the perspective that "type II" inducers (including *n*-HT) are considered more robust inducers of the anti-tumor immune response [48,53,54]. Moreover, the *n*-HT mediated local stress provides the access to hyaluronic acid and markers of endothelial damage, which promote the chemokines release capable of immune cell recruitment [1,43]. It is therefore not surprising that the "transitional zone" contains the most abundant inflammatory immune cells, such as macrophages, DCs, $CD8^+$ T lymphocytes and natural killer cells [1]. Moreover, extracellular HSP70 is capable of chaperoning antigens to APC cells. For example, scientists designed an ER-targeting pardaxin peptides modified-, ICG conjugated-hollow gold nanospheres, together with an oxygen-delivering hemoglobin liposome [55]. The data demonstrated that the ER-targeting nano-complex induced robust ER stress (evidenced by CCAAT-enhancer-binding protein homologous (CHOP) upregulation), CRT overexpression and $CD8^+$ T cells recruitment under near-infrared light irradiation in a CT26 colon cancer model; similar results were shown in the B16 melanoma model [55]. In the "normal surrounding tissues" (the 3rd zone), the vasculature system serves as a "heat-sink", which dissipates the increased temperature. Moreover, tumor antigens that are released during ICD drain to the nearby lymph nodes, where DC and T cells activation occur. Collectively, this constellation of well-structured immunological response, places *n*-HT in the category of more potent ICD inducing approach (type II), which sets it apart from classic ICD inducing reagents (e.g., oxaliplatin and DOX) [48,53,54].

## 3 Pursing nano-HT mediated immunological response as a viable principle for tumor treatment

Having demonstrated that using *n*-HT to achieve immunological activation in a "cold" tumor microenvironment (TME) is possible, the key questions now become (1) whether it is possible to develop additional therapeutic paradigm to implement this unique finding, and (2) whether it is possible to design combination therapy in order to achieve more robust, long-lasting and even systemic therapeutic outcome for cancer management. Although it might be too early to formulate the detailed

blueprint of how to establish an ideal *n*-HT that could be locally effective, supplemented with long-lasting immune benefit, we are beginning to appreciate the intellectual values of below questions, some of which require additional investigations.

(1) What is the best temperature and heating procedure? Minimally, high-temperature mediated *n*-HT (thermal ablation) is useful in dealing with bulky tumor mass where relatively mild *n*-HT is likely capable of evoking the immunological response or rendering the cancer cells to be more sensitive to other anti-cancer modalities. Rather than the over-simplified data generation by looking at the acute "cooking" effect, more impactful contribution is expected to be a systematic dissection of the physicochemical properties of *n*-HT platforms including their roles in the killing and cellular stress mechanisms in the heterogeneous immune TME. Deliberate efforts need go into the iterative optimization on the *n*-HT procedures, such as ENM design, dose, on/off schedule, administration routes, etc. This differs, in our opinion, from the over-simplified experimental designs that frequently overlook these clinically relevant granularities. Rather than the overblown claims of *n*-HT acting as a magic tumor cure, we advocate to develop the next-generation of *n*-HT with long-lasting and systemic immunological benefits. This includes the exploration of anti-metastasis effect of *n*-HT, which may possibly evoke immune memory effects.

(2) Cancer-specific and case-specific considerations. It is important to consider tumor type, geometry, shape, size and different tumor location, which could largely influence the "heating" and immunological outcomes. Noteworthy, it is an essential step, for the traditional HT, to create a patient tumor imaging model, from magnetic resonance imaging or computerized tomography. With software, complex algorithms and imaging-guided HT, it is possible to proactively determine heat distribution in the region-of-interest, power absorption, and assessment on tumor burden and blood vessel networks. This prior art could be highly relevant in terms of design and implementation of the next generation *n*-HT, which per se is endowed with multifunctionality, including imaging. Moreover, it is necessary to make a strategic decision on administration route, such as IT *vs.* IV. While the former is easy to practice and suitable for superficial tumor, the latter could be a viable option for deep-seated tumors. We also surmise that the IT ENM distribution and spatially unique post-HT immunological profiling could be more complex than 3-zone model as we outlined above. During this investigation, it is helpful to consider more robust tumor models, such as using orthotopic and spontaneous cancer model in immunocompetent mice, which will have the additional benefit of a more apropos tumor microenvironment, better recapitulating the microenvironment seen in human solid tumors. Moreover, tumor infiltration of immune cell needs in-depth investigation, especially for solid tumors, some of which are stroma-rich [56–59]. Stroma is a physical and functional barrier that may generate compartmentalization of immune cells even, a.k.a. T-cell exclusion [60,61]. To experimentally probe the phenomena, a multi-factor immune profiling technology is required to look at the spatial distribution of immune cells. With that being said, *n*-HT induced ICD may be dampened owing to the inefficient infiltration in the tumor microenvironment. While the exclusion pathway has been investigated in classic cancer immunology research, it was generally an unexplored area in *n*-HT research. An obvious perspective, however, is to combine *n*-HT with small molecule inhibitor that interferes T-cell exclusion if cell infiltration becomes a concern.

(3) Creative design of *n*-HT based combinatorial therapy. Ample evidence has emerged to show the synergy in terms of the combined use HT with radiotherapy, chemotherapy and targeted therapy [4,62]. Combined use of *n*-HT is not an exception. In fact, IFN-$\gamma$ production that is made during ICD induced negative feedback loop in the TME. This triggers the transcriptional activation of the various non-redundant immunological suppressive pathways, preventing the activation of anti-tumor immunity. This is in agreement with the interesting attempts now emerging for multistage and combination nanomedicine design to provide an impact on immune checkpoint pathways, such as PD-1/PD-L1, IDO pathway, etc. [32,63,64]. With the respect to "chemo + *n*-HT", we would advocate the prioritization of ICD inducing chemo agent, such as oxaliplatin and irinotecan (proven ICD options in our recent studies) [34,49,63,65–71]. These carefully selected chemotherapeutic options may be complimentary or synergistic to *n*-HT. Similarly, it is useful to consider gene therapy to interfere the major immune checkpoint or immune editing pathways during *n*-HT [69,72–76], such as using siRNA to target PD-L1 [77].

(4) Compatibility with other standard-care. While it remains largely unexplored, it would be interesting to look at the interaction between *n*-HT with standard-care (or procedure). To name a few, what is the net outcome when combine *n*-HT with radiotherapy, which could be immunogenic [78,79]. While immune activation process (including *n*-HT mediated ICD) requires the involvement of lymph nodes, will the surgical removal or radiation of lymph nodes be overall beneficial in terms of survival outcome? It would be highly interesting to studies these questions in stringent and immunocompetent preclinical cancer models.

## 4 Conclusions

While *n*-HT has become a major therapeutic modality in cancer research, the immunological effects of this powerful treatment approach require additional investigation. This is important, in our opinion, with a view to provide a paradigm shift from an acutely effective heat response to a

long-lasting systemic immunological outcome, which the latter could be impactful, i.e., providing potential cancer cure. Mechanistically speaking, deliberate induction of ICD serves as an important basis of immune activation, which recruits immune effector cells, such as cytotoxic T cells. Since the newly recruited immune cells triggers up-regulation of multiple and non-redundant immune suppressive pathways, this highlights the necessity to introduce various immunological modulation component (s), which ensure the propagation of *n*-HT induced immune activation. Moreover, it is also helpful to look at temperature and heating procedure during *n*-HT from an immunological aspect, which could largely influence immune profiling and killing effect, including the potency when combine with cancer standard care options.

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