Review

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Nano-ablative immunotherapy for cancer treatment

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Abstract: Immunotherapy has provided a new avenue to treat metastatic cancers, which result in ~90% of cancer related deaths. However, current immunotherapies, such as immune checkpoint therapy (ICT), have met with limited success, primarily due to tumor intrinsic and extrinsic factors that inhibit antitumor immune responses. To overcome the immune suppression of the tumor microenvironment (TME) and enhance the tumoricidal activity of ICT, phototherapy, particularly photothermal therapy (PTT), combined with nanomedicine has become a viable option. PTT disrupts target tumor homeostasis, releasing tumor associated antigens (TAAs), tumor specific antigens (TSAs), danger associated molecular patterns (DAMPs), and scarce nutrients required to "feed" activated antitumor immune cells. While nanoparticles localize and specify the phototherapeutic effect, they can also be loaded with immune stimulants, TME modulators, and/or chemotherapeutic agents to greatly enhance immune stimulation and tumor killing. Combining these three technologies, which we term nano-ablative immunotherapy (NAIT), with ICT can greatly enhance their therapeutic effects. In this review, we will discuss the successes and limitations of NAIT + ICT. Specifically, we will discuss how the TME limits tumoricidal activity and what should be considered to overcome these limitations.

Keywords: photonics; nanomedicine; cancer immunotherpay; immune checkpoint therapy; nano-ablative immunotherapy; tumor microenvironment.

1 Introduction

Nanoparticles, photonics, immunology, and immunotherapy are seemingly modern terminologies, yet the science and applications of these technologies have been with us for thousands of years. From colorful paintings on ancient church windows to other glasses and metallic devices, metal nanoparticles have been used to bring technological ideas into our daily life. However, only recently have these nanoparticles been incorporated in to medicine and emerged as a new field of study, nanomedicine.

For the human body, light is both beneficial and harmful. Too much exposure can result in DNA damage, premature aging, and skin cancer formation [1–4]. Conversely, light can prevent vitamin D deficiency, promote wound healing, and treat/cure cutaneous infections and other skin conditions [4–7]. With the advent of lasers, light has emerged as a useful medical tool for harnessing the biomedical effects of photothermal, photomechanical, and photochemical interactions to treat specific conditions including cancer.

Immunotherapy has become a major branch of modern medicine and cancer therapy [8–11]. Exploiting the immune system to attack cancer cells is highly favorable because immune cells travel throughout the body, providing constant immune surveillance, and help prevent relapse [11–13]. Unfortunately, not all tumors are created equal, and most do not respond to current immunotherapies for a variety of reasons [9, 14–16].

Combining different technologies has time and again produced new, exciting innovations to overcome current technological and medicinal limitations. Synergizing nanomedicine with phototherapy and immunotherapy is

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a viable option to overcome the current limitations of cancer therapeutics and has been the subject of several recent reviews [17–21]. In this review, we will discuss the types of tumor microenvironment (TME) that lead to failure of immune checkpoint therapy (ICT) in human patients, and the competing "arms race" that continually evolves between cancer and the immune system. This will provide a unique angle to review the current research in this field and to lay the foundation for designing cancer therapeutic approaches combining nano-, photo-, and immuno-treatments. Furthermore, we specifically highlight preclinical and clinical studies that employed compound nanoparticles that successfully combined all three approaches, which we term nano-ablative immunotherapy (NAIT). We also highlight the fact that NAIT could turn ICT resistant tumors into responders. Lastly, we will discuss the current limitations of NAIT + ICT due to tumor intrinsic and extrinsic factors and provide guidance for incorporating TME modulators with NAIT + ICT to potentially surmount these obstacles to enhance abscopal effect and promote long-term cancer control, to effectively treat metastatic cancers and to prevent cancer recurrence.

2 Cancer immunology and cancer immunotherapy

With the complexity of life, the immune system has evolved ways to monitor "self" cells and remove "altered" or "non-self" cells to prevent abnormal cell growth and promote organismal survival. The key immune cells involved in this process are natural killer (NK) cells and $CD4^+$ and $CD8^+$ T cells [22–24]. Cancer arises when these cells fail to eliminate and/or control abnormal cell growth. At some point in cancer development, an equilibrium is reached in which the immune system inadvertently selects and tolerates tumor cells better capable of evading immune attack, and then these tumor cells establish a TME that further alludes antitumor immune responses and promotes unimpeded growth. This process of cancer development was coined "cancer immunoediting" by Dunn and Schreiber [25] in that the TME co-evolves in response to its interactions with its surrounding tissues and immune cells. This hypothesis is given further credence by the fact that essentially all solid tumors are infiltrated with a diverse array of myeloid and lymphoid leukocytes [26]. Importantly, the level of immune infiltration, activation, and composition are dependent on cancer type and malignancy stage [27]. Moreover, the type of infiltrating immune cells correlates with prognosis of patients with similar cancer types. For example, patients with large numbers of immature myeloid cells, tumor associated macrophages (TAMs), plasmacytoid dendritic cells (pDCs), and/or neutrophils are associated with treatment resistance and poor prognosis [28–33]. In contrast, cancer patients with high concentrations of $CD8^+$ and memory Th1 $CD4^+$ T cells, B cells, and NK cells in the TME have significantly greater disease-free survival (DFS) and overall survival (OS) following various cancer treatments [33–38]. To unleash the antitumor capability of the tumor infiltrating T cells, cancer immunotherapy holds high promise to treat cancer through immunological means.

Several immunotherapies have been used to treat cancer patients, such as bacterial or viral inoculation, cytokine infusion, cancer vaccines, and CAR-T therapies [39–41]. Unfortunately, each of these therapies is limited to enhancing the existing immune response or identifying tumor specific antigens (TSAs) for efficient vaccination. To overcome these limitations, other targets are currently being pursued to specifically target the immune suppressive cytokines, metabolites, and immune suppressive cells within the TME [42–46].

Among the immunotherapies, ICT is the most used immunotherapy to date. While it also relies on the existing immune response, it has shown increased efficacy when combined with other therapies [47–50], and such combinations will be the focus of this section. The most used ICT to date targets the immune inhibiting ligands CTLA-4 and PD-1 on the surface of T cells via blocking antibodies. While there are several other inhibiting ligands [51, 52], we will focus on these two ligands in this review. CTLA-4 and PD-1 are expressed on activated T cells and under normal conditions are required to maintain immune homeostasis and prevent the development of autoimmunity [53]. However, in cancer, these molecules are highly expressed in the TME and severely dampen antitumor immune responses and prevent tumor killing. Unfortunately, across a broad range of cancers, the response rate to ICT is only ~10–20%, far below an acceptable rate [15]. There are several factors contributing to this failure, and clinically patients are grouped into three categories based on their failure to respond to ICT: Primary Resistance, Adaptive Resistance, and Acquired Resistance (Figure 1) [54–57]. Primary resisters do not have a response to ICT, and it is hypothesized that a lack of sufficient T cell responses, tumor mutational burden, tumor peptide presentation on the tumor surface, and/or an unfavorable TME actively prevents sufficient immune responses. Adaptive immune resisters are thought to have sufficient antitumor T cell responses, but the TME prevents sufficient tumor killing through a variety of

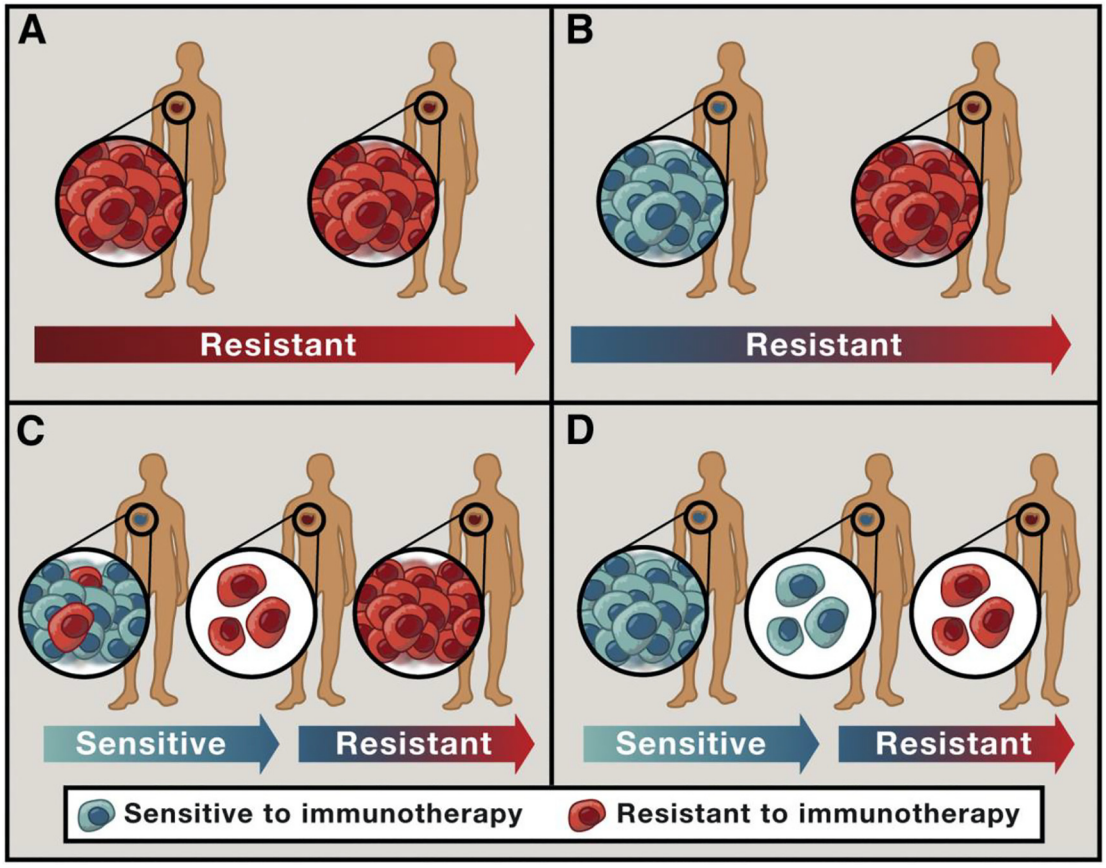

Figure 1: Resistance against immune checkpoint therapy.
(A) Patient's tumor is resistant to immunotherapy with no active immune response. **(B)** Patient's tumor is resistant to immunotherapy with active anti-tumor immune response but turned off by checkpoints or other adaptive resistance mechanisms. **(C)** Patient has an initial response to immunotherapy but later progressed; heterogeneous population and selection of resistant clones were present before treatment started. **(D)** Patient has an initial response to immunotherapy but later progressed, a true acquired resistance during the immunotherapy. Reprinted with permission from reference [54]. Figure obtained from https://doi.org/10.1016/j.cell.2017.01.017.

mechanisms. There are also patients who initially respond to ICT but then relapse and the cancer progresses, resulting in acquired resistance. Based on these clinical presentations, the immune system and the TME are enemies at war. The immune system is trying to attack and control tumor growth while the TME is continually evolving to find an avenue to stymie immune attack via tumor intrinsic or extrinsic factors.

Each TME is unique as it is actively shaped by its surrounding tissue environment and immune responses as it evolves [58]. It is for this reason that a one-size-fits-all cancer approach has had little success. Despite these differences, there are several key hallmarks of solid TMEs which favor tumor progression and inhibit antitumor immune functions. This includes hypoxia, amino acid, glucose, glutamine, and lipid metabolism, lactate buildup, reactive oxygen species (ROS) dependence, acidification due to hypoxia and lactate accumulation, dysregulated angiogenesis, and high interstitial fluid pressure (IFP) [59–65]. These hallmark signatures are generated through coordinated interactions between tumor cells, immune cells, and stromal cells at the behest of the tumor cells.

Hypoxia is a characteristic of ~50% of solid tumors and plays a critical role in driving tumor progression and shaping the antitumor immune response through a variety of pathways (Figure 2) [65–68]. In tumors, hypoxia is one of the strongest angiogenesis signals driving blood vessel formation to "feed" the growing tumor. Hypoxia also upregulates the glucose uptake machinery, to compensate for the inefficiency of glycolysis for energy production, while simultaneously inhibiting mitochondrial respiration [69]. By exclusively using glycolysis, lactate accumulates and drives acidification of the TME. Additionally, hypoxia induces tumor cells to secrete anti-inflammatory cytokines TGF-β, IL-10, and chemokine ligands (CCLs), which recruit immunosuppressive cells such as tumor associated

macrophages (TAMs), tumor associated neutrophils (TANs), and myeloid derived suppressor cells (MDSCs), which further promote tumor growth and immune repression (Figure 2) [70–72]. TGF-β plays a critical role in increasing the IFP as it transforms fibroblasts into cancer-associated fibroblasts (CAF) and induces collagen deposition [64, 73–75]. CAFs contribute to tumor growth and metastases by releasing extracellular matrix (ECM) proteins that promote survival, inhibit immune activation, promote oxidative phosphorylation, and drive epithelial to mesenchymal transition (EMT), as shown in Figure 2 [76].

TAMs are the largest portion of myeloid cells residing in solid tumors [77]. With the use of animal studies, it is suggested that a majority of the TAMs residing in the TME originates as monocytes that are recruited into the TME, via cytokines and chemokines [78, 79], where they encounter an immune suppressive environment [80–82]. Monocytes that differentiate into macrophages are incredibly dynamic and diverse, and they adapt to their microenvironment based on the environmental cues they receive [83, 84]. In an established TME, monocytes will encounter factors such as hypoxia, low pH, metabolic and environmental stress signals, and anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β, all of which drive the differentiation of monocytes into macrophages with pro-tumor, immune inhibiting functions [78, 79, 85–87]. With tumor progression, TAMs display a more pro-tumor phenotype, and can produce an abundance of immune-modulatory chemokines and cytokines that prevent efficient immune mediated tumor killing (Figure 2). Additionally, TAMs can directly interact with T cells to physically prevent them from associating with tumor cells. To the benefit of the tumor, these TAMs also secrete several molecules that contribute to tumor angiogenesis, inhibit tumor apoptosis, and promote metastasis [84]. For this reason, large numbers of TAMs with these properties residing within the TME lead to treatment resistance and poor patient prognosis [84, 88, 89].

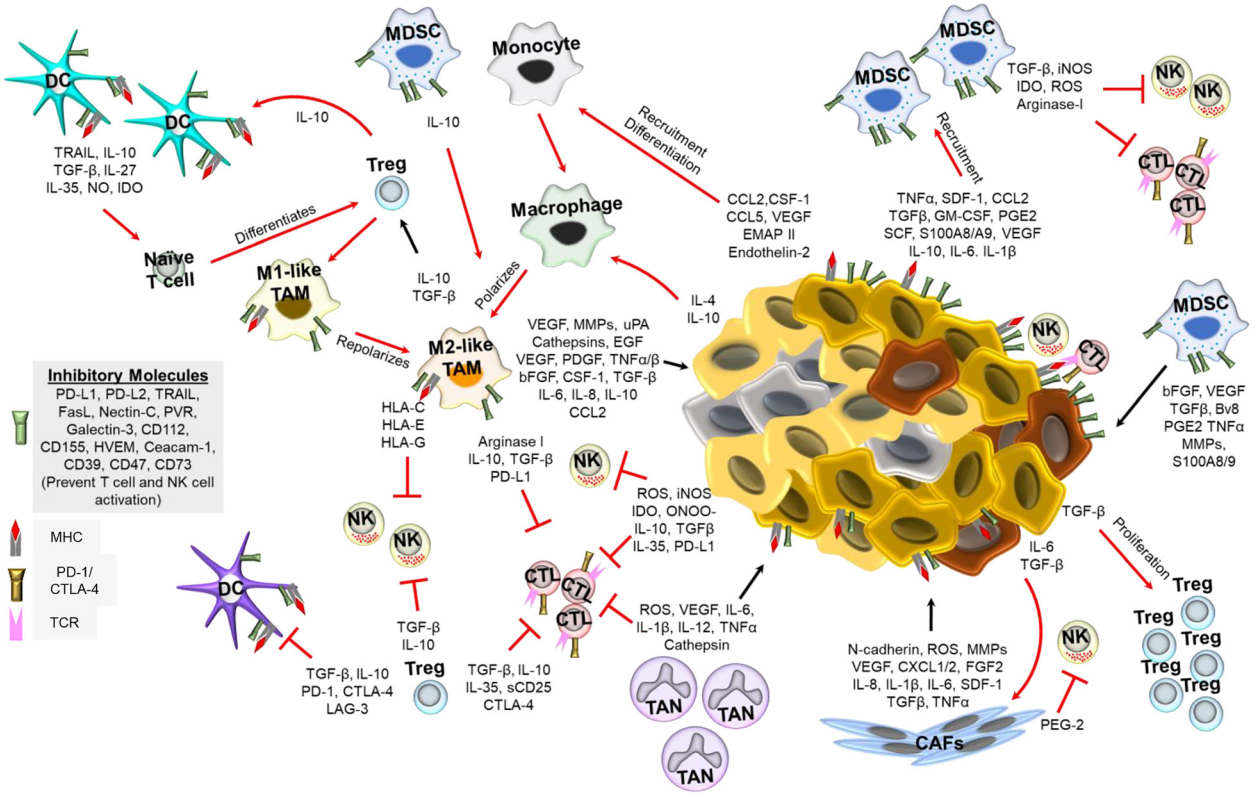

Figure 2: The interconnected web of the tumor microenvironment (TME). In the TME, tumor cells and immune cells are in constant communication. The TME is acidic, nutrient deficient, and hypoxic, which promotes an immunosuppressive environment. Additionally, the tumor cells and cancer associated fibroblasts (CAFs) secrete factors that promote tumor growth, inhibit immune cell activation, and recruit immunosuppressive immune cells. The immunosuppressive cells, such as M2-like macrophages (M2-like TAMs), myeloid derived suppressor cells (MDSCs), T regulatory cells (Treg), and tumor associated neutrophils (TANs), not only suppress antitumor immunity directly (via expression of inhibitory receptors) or indirectly (cytokines, chemokines, etc.), but also promote tumor growth and metastasis through various mechanisms such as inhibiting dendritic cell (DC), natural killer (NK) cells, and cytotoxic T cell (CTL) functions.

Like TAMs, MDSCs have a dual functionality in promoting tumor progression and inhibiting antitumor immunity (Figure 2) [90, 91]. MDSCs secrete several growth factors and matrix metalloproteases that promote tumor angiogenesis, growth, and metastases [92, 93]. While MDSCs have global tumor promoting and immunosuppressive functions, their primary targets are T cells through the production of arginase-1, iNOS, TGF-β, IL-10, COX2, indoleamine 2,3-dioxygenase (IDO), sequestration of cysteine, etc. [90, 91, 94]. More importantly, MDSCs are capable of driving antigen specific Treg formation, effectively tolerizing T cells to the tumor, hence preventing tumor killing [90]. Tregs are highly suppressive T cells capable of inhibiting cytotoxic T cell (CTL) function either directly or indirectly through killing the cells, induction of checkpoint signaling, anti-inflammatory cytokines, sequestration of IL-2, inhibition of dendritic cell function, and/or metabolic modulation of adenosine and tryptophan (Figure 2) [95–97]. Due to their various abilities to suppress CTL responses, high infiltration of Tregs into tumors is associated with poor prognosis [98–100].

As briefly described above, the TME generates a localized ecosystem composed of many inter-connected players that contribute to cancer survival. For this reason, removing and/or activating one key player will likely have little effect as many of the other players can easily fill the void, allowing the tumor to evolve and overcome the treatment regimen (Figure 2). This is reflected in the fact that cancers develop resistance and require alternative treatment therapies. Furthermore, late-stage tumors are more resistant to current cancer treatments, with single treatments having little benefit [101, 102]. Thus, a multimodal therapeutic strategy is required to surmount the resistance mechanisms and plasticity inherent to well established tumors. In the following sections, we will describe how photo-ablative therapy combined with nanomedicine can address and overcome some of these obstacles and what is required to further enhance their efficacy.

3 Phototherapy

3.1 Photodynamic therapy

Photodynamic therapy (PDT) is a minimally invasive, well established treatment modality that drives the production of ROS to induce cellular toxicity and apoptosis [103–106]. PDT combines non-ionizing light at a wavelength of 400–700 nm [107], a photosensitizer (PS), and oxygen [108, 109]. PS are generally aromatic molecules that absorb light at specific wavelengths and generate ROS in the presence of oxygen and light [110]. ROS results in tissue damage and immunogenic cell death (ICD) that is capable of stimulating an immune response [108]. Specifically, the PDT-induced ICD releases danger associated molecular patterns (DAMPs), leading to $CD8^+$ T cell proliferation, cytotoxic cytokine secretion, and other immune responses, which can trigger or potentiate antitumor immunity [111–113]. Unfortunately, in hypoxic TMEs, PDT is severely hindered due to lack of oxygen, which is required to generate ROS [114], and PS are generally hydrophobic which limits their solubility and ability to enter target tissues [115–119]. Furthermore, PDT-induced immune responses cannot achieve satisfactory therapeutic effects alone, particularly against metastatic tumors. Another limitation of PDT is the tissue penetration of light [120]. To overcome these limitations PDT is being combined with nanotechnology to improve PS delivery to target tissues, overcome hypoxic TMEs [114, 121–125], and enhance the light absorption for deep-seated tumors. The use of nanoparticles and PDT for cancer therapy has been extensively reviewed elsewhere [116–119, 122, 125–127].

3.2 Photothermal therapy

Photothermal therapy (PTT) is a treatment modality that commonly employs near-infrared (NIR) light energy and locally placed light-absorbing agents to generate heat and destroy cancer cells. PTT is minimally invasive and can be used topically or interstitially for tumor treatment with minimal toxicity to surrounding tissues. Temperature elevation has a direct impact on the tissue biological responses. Above 100 °C, tissue experiences carbonization and evaporation, which have minimum contribution to immune responses. Cytotoxic temperature (46–100 °C) usually leads to cell death and release of antigens and DAMPs. Below the cytotoxic temperature, cells are under thermal distress, but usually remain viable. Even though each tumor and its TME are unique, there is a consensus that effective PTT requires a sustained tumor temperature of ~50–60 °C [128–131] for optimal tumor cell killing and immune stimulation via ICD. This is largely due to the type of cell death that is required to stimulate an immune response.

To date, three main types of programmed cell death have been described, apoptosis, pyroptosis, and necroptosis [132, 133]. Apoptosis is largely an anti-inflammatory form of cell death that does not release cellular antigens or DAMPs capable of stimulating immune responses [134].

Necrosis, in contrast to necroptosis, is an uncontrolled form of ICD, in which cellular contents and DAMPs are released into the cytosol from rupturing cells [135, 136]. Therefore, the goal of PTT should be to initiate ICD to release tumor associated antigens (TAAs), TSAs, and DAMPs to stimulate antitumor immunity, and disrupt overall tumor homeostasis to allow for immune infiltration and tumor killing [128–131, 137, 138].

To protect the surrounding healthy tissue, combining PTT with nanoparticles is desirable. Nanoparticles make it possible to specifically increase target tumor temperature at much lower power densities, resulting in negligible temperature changes in the surrounding nanoparticle-free tissues [139, 140]. Several nanoparticles with varying heat generating mechanisms and morphologies have been developed [141]. These include rare earth ion nanocrystals, carbon nanoparticles, metallic nanoparticles, organic nanoparticles, and organic biodegradable nanoparticles, which have been extensively reviewed elsewhere [140–144].

Due to the immunosuppressive nature of the TME, the release of DAMPs, TSAs, and TAAs, as well as disruption of tumor homeostasis by PTT alone are not sufficient to stimulate satisfactory systemic antitumor immunity. We will highlight a few approaches that have combined nano-mediated PTT with immune stimulating agents, which we will refer to as NAIT. Furthermore, we would like to emphasize that stimulating immunity alone may not be enough and that combining NAIT with TME modulating molecules may be required to further improve the systemic effects to treat a broad range of metastatic cancers.

4 Nano-ablative immunotherapy

In the United States, ~44% of cancer patients are eligible for ICT, but only 13% of these patients are estimated to respond favorably [15]. There are many factors that contribute to low response rates, and clinically patients who resist ICT are grouped into three categories, as listed above, due to tumor intrinsic and extrinsic factors. To overcome the TME obstacles and allow for better responses to ICT, NAIT is becoming a viable option. PTT disrupts target tumor homeostasis and releases TAA, TSA, and DAMPs, allowing for the influx of immune cells. The nanoparticles used to enhance the photo-ablative effect can also be used to deliver immune stimulating molecules and chemotherapeutic drugs capable of enhancing existing immune responses and possibly driving *de novo* antitumor T cell responses. Through its T cell activation, NAIT has great potential to synergize with ICT to enhance tumor killing. In the following sections, evidence from clinical and preclinical models illustrating this effect will be presented.

4.1 Clinical case reports

In a case report, Chen and colleagues treated a stage IV aggressive metastatic melanoma patient with a combination of PTT, immune stimulation via imiquimod, and ipilimumab (anti-CTLA-4) ICT [145]. This patient had failed other traditional treatment modalities and had epidermal metastases on the neck and head and deep metastases in the lungs. The cutaneous lesions were treated with PTT and topical imiquimod for three courses. Three months after the final course, the cutaneous recurrences were completely cleared, but the lung metastases remained so the patients began anti-CTLA-4 ICT. Within 9 months, the lung metastases were cleared (Figure 3), and the patient remained tumor free at the time of this report, 7 years after the treatment.

For a clinical pilot study, Halas and colleagues used AuroShells (gold-silica nanoshells) and laser to specifically ablate prostate tumors for patients in stage IIa or below [146]. Within 3 months, 10/16 patients were tumor free while at 12 months 14/16 were tumor free in the ablated region. The nanoparticles were well tolerated with little to no side effects reported, giving further credence to the use of nanoparticles with phototherapy for cancer treatment.

4.2 Experimental NAIT

While very few clinical studies have been performed using nano-based ablation and immune stimulation, several preclinical studies have been performed with varying degrees of success. In this section, we will specifically highlight studies that generated multifunctional nanoparticles, which combined immune stimulants into the nanoparticle, to increase the efficacy of PTT and/or their ability to synergize with ICT.

Using a photothermally activated polymeric nanoagonist (APNA), Pu and colleagues generated an NP to specifically target deep seeded tumors via PTT. APNA consists of a semiconducting polymer backbone, which absorbs NIR-II light and is conjugated to the immune stimulant resiquimod (R848), via a thermolabile linker. Upon PTT, R848 is cleaved and able to stimulate tumor-infiltrating DCs and promote TAA and TSA uptake. This leads to significantly delayed 4T1 tumor growth and higher overall survival in the PTT + APNA treated animals [147].

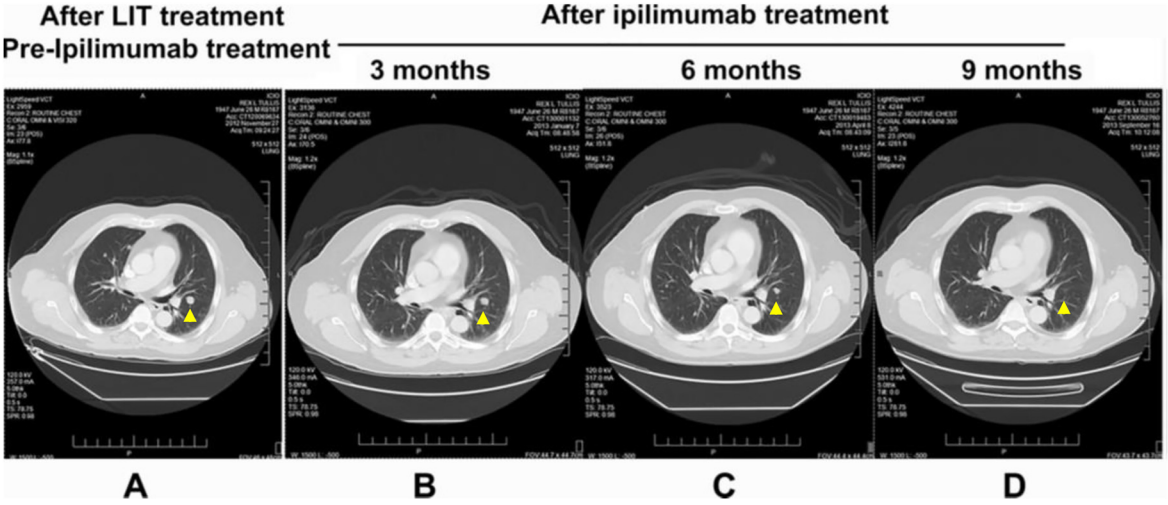

Figure 3: CT images of lung metastases of a patient treated with PTT and topical imiquimod for three courses, followed by anti-CTLA-4 (ipilimumab) ICT, showing the same level in the thorax.
(A) The image was taken before ipilimumab treatment (2 months following LIT) and demonstrates the size and location of the pulmonary metastases (yellow arrow). **(B, C)** The image shows shrinkage of the pulmonary metastases 3 months **(B)** and 6 months **(C)** after completion of the combination of LIT and ipilimumab. **(D)** The image shows that pulmonary metastases were completely resolved 9 months after completion of the combination of LIT and ipilimumab. Reprinted with permission from reference [145]. Figure obtained from doi:10.1002/jbio.201600271.

Similarly, using manganese ferrite ($MnFe_2O_4$), generated from iron oxide and manganese oxide, Chen and colleagues developed a compound NP containing R837, a TLR-7 agonist, and coated with ovalbumin. The $MnFe_2O_4$ NPs alone were able to stimulate bone marrow derived dendritic cells (BMDCs), enhanced by the addition of R837 [148]. *In vivo*, this combinatorial NP, when combined with PTT, reduced tumor cell growth and lung metastases, prolonging the survival of 4T1 inoculated mice. These experiments demonstrate that combining immune stimulation with PTT enhances antitumor immunity presumably through T cell activation, but these therapies were not combined with ICT to assess synergy.

Combining upconversion nanoparticles with indocyanine green (ICG), rose bengal (RB), and a lipid molecule (DSPE-PEG-maleimide, mal) as an antigen capturing agent, Chen and colleagues generated an NIR-triggered antigen capturing nanoplatform [149]. UCNP/ICG/RB-mal was able to directly stimulate BMDCs *in vitro*, suggesting that this type of NP could not only capture antigen but also enhance DC activation upon antigen delivery. Using the 4T1 orthotopic breast tumor model, PTT combined with UCNP/ICG/RB-mal resulted in ~70% long-term animal survival. Combining PTT with the NP and anti-CTLA-4 ICT further improved survival to ~80%. Most interestingly, upon 4T1 rechallenge of the cured survivors, only some of the anti-CTLA-4 ICT receiving mice were capable of completely preventing tumor growth. This demonstrates that despite successful tumor elimination upon treatment, other components, in particular the checkpoint inhibition, are required to generate long-term antitumor immune memory.

Fe_3O_4 nanoparticles have been widely used in cancer diagnosis and are deemed safe for clinical use. However, their molar extinction coefficient (MEC) is low, making the unmodified version unsuitable for PTT applications. Yang and colleagues generated Fe_3O_4 superparticles (SP) by using Fe_3O_4 as the core and polydopamine (Fe_3O_4@PDA) as the shell to greatly increase their MEC, biocompatibility, and physiological stability [150]. They further combined this SP with poly(ethylene glycol)-*block*-poly(lactic-co-glycolic acid) copolymer (mPEG-PGLA) and R837 to generate a nanodrug carrier that localizes within the TME via magnetic attraction and releases R837 upon PTT. 4T1 breast tumors respond very well to Fe_3O_4-R837 SP, with the PTT treated tumors regressing significantly after treatment. However, the abscopal response had little effect until it was combined with anti-PD-1 ICT [151]. Fe_3O_4-R837 SP in combination with anti-PD-1 effectively impeded untreated tumor growth for up to 24 days but was unable to eliminate the tumor. This response highlights the fact that immune stimulation alone is not sufficient in eliminating “cold” tumors, which are characterized by poor immune infiltration.

Similarly, Wong and colleagues developed FePSe3 chitosan coated nanoparticles conjugated with the anti-PD-1 peptide (APP) to block PD-1/PD-L1 signaling. It was further coated with cancer cell membrane (CCM) from

CT26, the tumor being treated, to camouflage the NP from the immune system and allow trafficking to the targeted tumor [152]. The NP generated in this study could modestly activate dendritic cells (DCs) and induce the production of IL-12 and IFNγ. Following PTT, FePSe3@APP@CCM NP was able to significantly control CT26 tumor growth and prolong survival in ~50% of the animals. Memory and abscopal responses were not measured in this study. It would be interesting to learn if the therapeutic effect of this NP could be enhanced by the addition of a strong immune stimulant.

Single-walled carbon nanotubes (SWNTs) are toxic at high concentrations, but they do result in tumor shrinkage. If locally administered, they can selectively enhance the photoabsorbance within the targeted tumor. Chen and colleagues generated SWNTs coated with a novel immune stimulant, glycated chitosan (GC), to enhance the anti-tumor immune response following PTT [153]. Using the 4T1 tumor model, this combinatorial NP was able to extend survival and reduce tumor metastases in the lungs. 4T1 tumors are refractory to anti-CTLA-4 ICT. However, when anti-CTLA-4 ICT was combined with PTT and GC-SWNTs, the efficacy was greatly enhanced resulting in long-term survival of ~50% of the treated animals. This study highlights the fact that PTT + immune stimulation can make ICT resistant tumors responsive, supporting the hypothesis that NAIT synergizes with ICT.

4.3 NAIT combined with TME modulators

In all the examples detailed above, immune stimulating NP + PTT therapy was able to control treated tumor growth and untreated metastasis, but abscopal effects and the formation of immune memory varied greatly. Additionally, the treatments were rarely 100% effective even with the addition of ICT, likely due to heterogeneity of the TME, and varied immune activation responses among individual animals.

To expand on this hypothesis, Hou and colleagues developed a nanocomposite drug delivery system composed of MXene Ti_3C_2 to treat MDA-MB-231 xenographs in nude mice. The Ti_3C_2 was etched, intercalated, and delaminated to form sheets, in which metformin (Met) and compound polysaccharide (CP) were added through layer-by-layer adsorption [154]. The resulting nanoparticle (Ti_3C_2@Met@CP) was able to target the tumor via a three-pronged approach. First was the disruption of tumor homeostasis via PTT. Second was the activation of the innate immune system through CP, which is a composite of three immune stimulating polysaccharides: lentinan, pachymaran, and tremella. Last is the metformin, which activates AMPK via p53, which in turn ceases cell cycle progression and leads to autophagy and cell death [155]. Thus, any tumor cells not undergoing ICD because of thermal ablation were further eliminated through chemical means either directly or indirectly via the enhanced infiltration and activation of antitumor immune cells (Figure 4). While these experiments were done in nude mice, which have NK cells but lack T cells, this approach shows promise. Future experiments in immune competent mice are required to ascertain whether this therapy can effectively generate long-term antitumor immunity and prevents tumor growth upon rechallenge.

Using a poly(lactic-co-glycolic) acid (PLGA) nanoparticle shell, Liu and colleagues generated nanoparticles containing R837 and water-soluble catalase to break down tumor generated H_2O_2 into O_2 [156]. When combined with radiotherapy, this combinatory therapy dramatically reduced and/or eliminated tumor growth and prevented tumor growth upon rechallenged with CT26 tumors (Figure 5). While this combination therapy used radiotherapy as the form of ablation, it further expands on the idea that NAIT + TME modulators are a viable means to overcome a highly dynamic TME and generate effective anti-tumor immune memory.

A good example to date of a multimodal therapeutic approach to the generation of a robust antitumor immune memory comes from Chen and colleagues (Figure 6). They generated a multimodal NP from reduced graphene oxide (rGO), loaded with mitoxantrone (MTX) and SB-431542 (SB) [157]. rGO is a dual nanoparticle in that it absorbs NIR light, but also stimulates the production of proinflammatory cytokines, such as TNFα, IL-6, and IL1β [158]. MTX functions by disrupting DNA synthesis and repair [159], while SB prevents TGF-β responses by binding to TGF-β receptors ALK5, ALK4, and ALK7 [160]. This combination worked well in treating 4T1 breast tumors, as 70% of the PTT + rGO/MTX/SB treated animals went on to clear the tumors. Furthermore, when the PTT + rGO/MTX/SB cured survivors were rechallenged with 4T1 tumors, 100% of the initially cured animals rejected tumors, demonstrating that this four-tier approach successfully generated a robust anti-tumor immune memory response. To measure the abscopal affect, a bilateral 4T1 tumor model was employed, and T cell infiltration and tumor regression was measured on the untreated tumors. These experiments revealed that rGO + PTT could significantly increase the percentage of tumor-infiltrating $CD8^+$ T cells within the untreated tumor and is slightly enhanced with the addition of MTX and SB to the rGO. Twenty days after treatment, the PTT + rGO/MTX/SB treated animals were able to control the growth of the

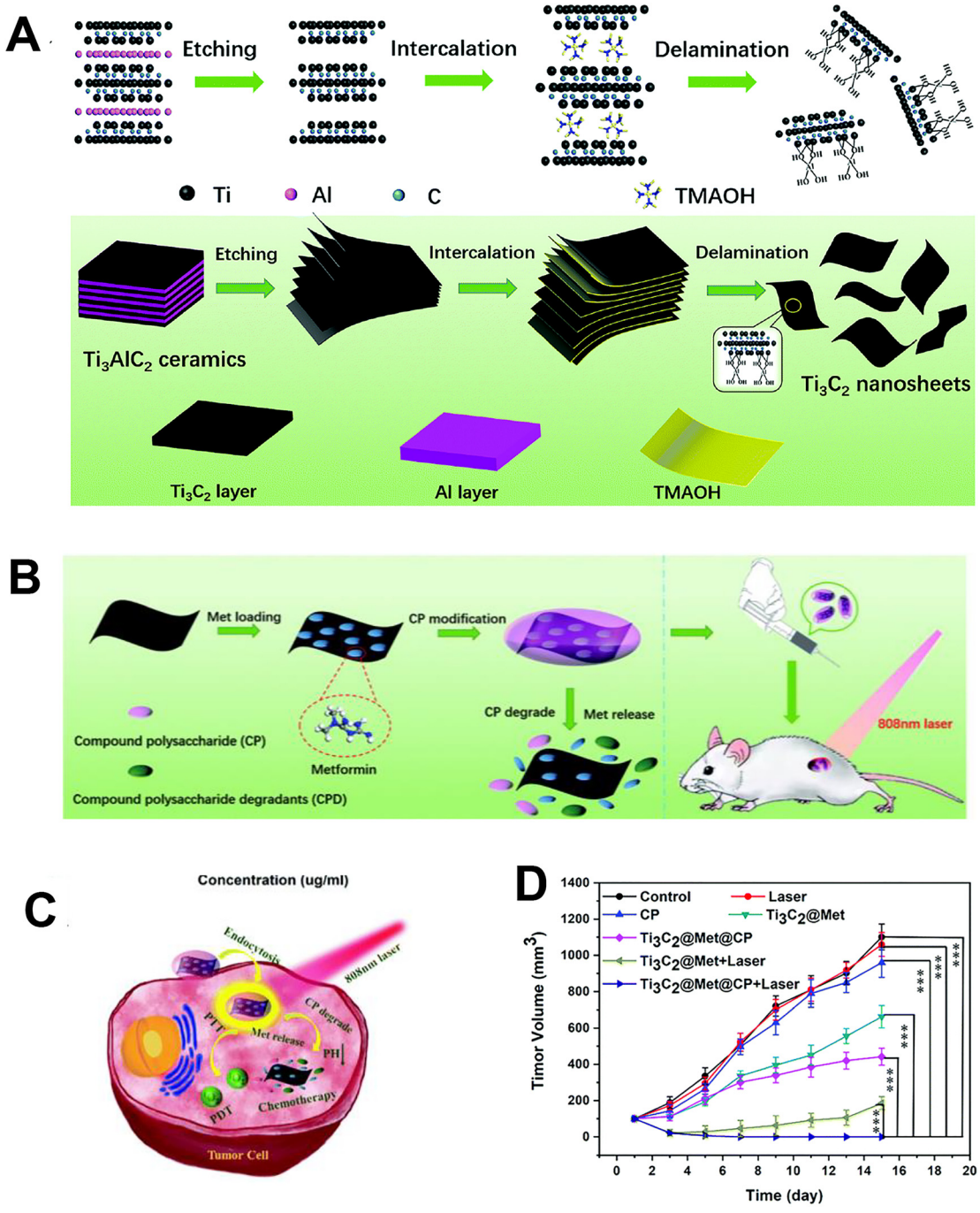

Figure 4: Nanocomposite drug delivery system composed of MXene Ti_3C_2 for treatment of MDA-MB-231 xenographs in nude mice. **(A)** Schematic of the synthetic process of the (AlOH)4-functionalized Ti_3C_2 nanosheets by two-step exfoliation, including a ball-and-stick model (top) and layer model (below). **(B)** Schematic of drug loading of Ti_3C_2 nanosheets, their further surface modification by CP, drug release, and CP degradation. **(C)** Schematic of the synergetic therapy effect of PTT, PDT and chemotherapy based on Ti_3C_2 nanosheets. **(D)** Time-dependent tumor growth curves after the different treatments. Reprinted with permission from reference [154]. Figure obtained from https://doi.org/10.1039/D0TB01084G.

untreated tumors, while PTT + rGO and PTT + rGO/MTX treated animals did not. This is an important finding, as it further supports the notion that tumor ICD and immune stimulation are not enough. It reveals that TME modulators are required for immune control of untreated tumors. The other important finding is that PTT + rGO/MTX/SB treated animals were able to control the growth of the untreated tumor but not eliminate it. This suggests that other factors

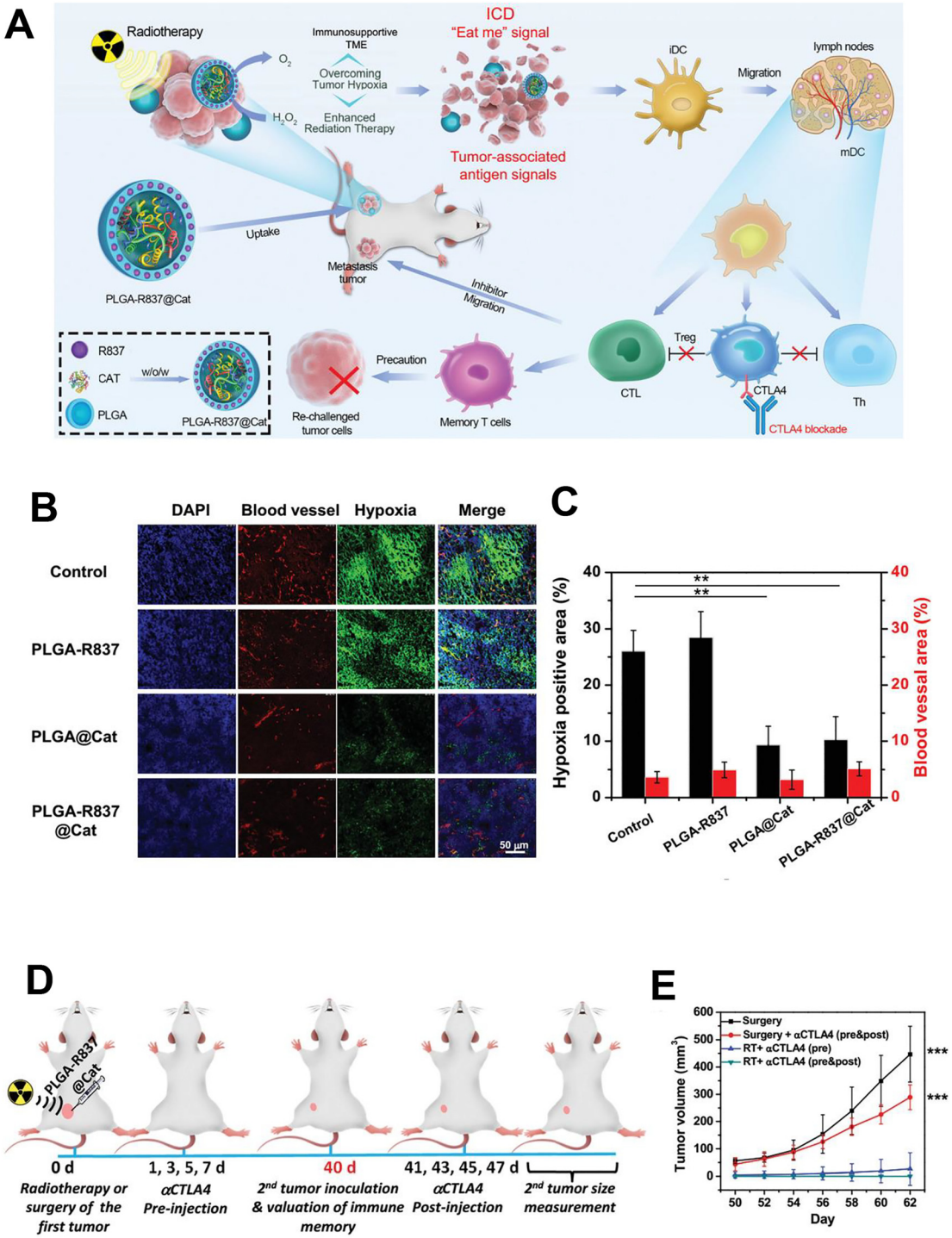

Figure 5: A poly(lactic-co-glycolic) acid (PLGA) nanoparticle shell, combined with radiotherapy, for treatment of CT26 tumors. **(A)** The schematic illustration for mechanism of antitumor immune responses induced by PLGA-R837@Cat-based radiotherapy in combination with checkpoint-blockade to inhibit cancer metastases and recurrence. **(B)** Modulation of tumor microenvironment after intratumoral injection of PLGA-R837@Cat. Representative immunofluorescence images of tumor slices after hypoxia staining. The hypoxia areas and blood vessels were stained by anti-pimonidazole antibody (green) and anti-CD31 antibody (red), respectively. **(C)** The relative hypoxia positive areas and blood vessel densities as recorded from more than 10 images for each sample using the ImageJ software. **(D)** Schematic illustration of PLGA-R837@Cat-based radiotherapy (abbreviated at RT) and αCTLA4 combination therapy to inhibit cancer relapse. **(E)** Tumor growth curves of rechallenged tumors inoculated 40 days post elimination of their first tumors (eight mice per group) by surgery or RT plus αCTLA4. Reprinted with permission from reference [156]. Figure obtained from https://doi.org/10.1002/adma.201802228.

are required such as another dose of the three-tier NP, or another TME modulator to disrupt the immunosuppressive function of the established TME for complete immune-mediated tumor removal. Furthermore, the type of immune stimulant used to enhance antitumor immunity needs to be carefully considered. Type I IFNs are critical for antitumor immune responses and controlling tumor growth [161–163]. Combining this type of multimodal NPs with a strong type I IFN stimulant may lead to greater abscopal effects and tumor elimination.

The pre-clinical and clinical studies described above are summarized in Table 1.

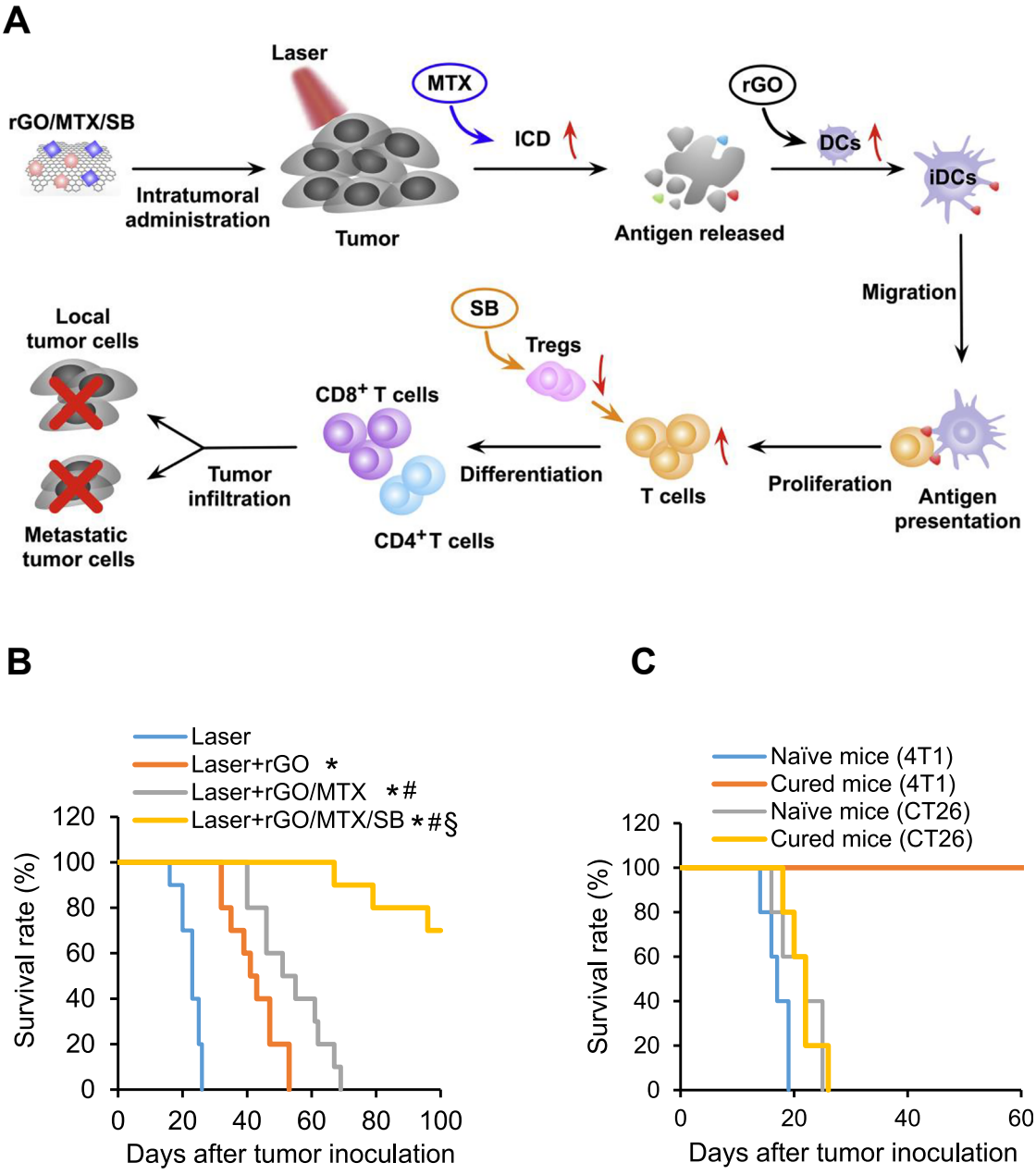

Figure 6: Reduced graphene oxide (rGO), loaded with mitoxantrone (MTX) and SB-431542 (SB), for multimodal therapy treatment of 4T1 tumor. **(A)** Schematic of laser + rGO/MTX/SB treatment of 4T1 tumors in mice and the mechanism of induced antitumor immune response. **(B)** Survival rates of tumor-bearing mice in the indicated treatment groups (with laser irradiation) up to 100 days after the initial tumor inoculation. ($n = 10$, *$p < 0.001$ vs. Laser; #$p < 0.05$ vs. Laser + rGO; §$p < 0.005$ vs. Laser + rGO/MTX). **(C)** Survival rates of rechallenged mice, after successfully treatment by rGO/MTX/SB based PTT. ($n = 5$). 4T1 tumor-bearing mice 'cured' by rGO/MTX/SB based PTT were rechallenged with 2×10^5 viable 4T1 or CT26 tumor cells 100 days after the initial challenge. Naïve mice of the same age were used as controls. Reprinted with permission from reference [157]. Figure obtained from https://doi.org/10.1016/j.biomaterials.2020.120421.

Table 1: Nano-ablative immunotherapy strategies for cancer treatment highlighted in this review paper.

Treatment approach	Tumor model species	Parameters	Major results	Ref.
Imiquimod + Ipilimumab (anti-CTLA-4) + PTT	Metastatic melanoma, human	PTT 805 nm 1 W/cm^2 10 min	Clearance of cutaneous recurrences 3 months after treatment. Clearance of lung metastases 9 months after treatment. Patient remains tumor free 7 years after treatment.	[145]
AuroShells (gold-silica nanoshells) + PTT	Prostate cancer (stage IIa or below), human	PTT 810 ± 10 nm 4.5–6.5 W 3 min	10/16 patients: tumor-free in ablation zone within 3 months 14/16 patients: tumor-free in ablation zone within 12 months. Little to no side effects reported due to nanoparticles.	[146]
APNA + PTT	4T1, BALB/c	PTT 1064 nm 1 W/cm^2 10 min	Significant delay of primary and metastatic tumor growth. Higher overall survival in treated animals.	[147]
R837-OVA-PEG-$MnFe_2O_4$ NPs + PTT	4T1, BALB/c	PTT 805 nm 1.2 W/cm^2 10 min	Inhibition of tumor growth and prevention of lung metastasis. Improvement of survival rate.	[148]
UCNP/ICG/RB-mal + PTT	4T1, BALB/c	PTT 805 nm 0.75 W/cm^2 10 min	Survival rate: 67%. Combination with anti-CTLA-4 ICT: achievement of long-term survival in 84% of mice & development of tumor-specific immunity in 34% of mice. 4T1 rechallenge on surviving mice: complete rejection of tumor growth was only observed in some UCNP/ICG/RB-mal + PTT + anti-CTLA-4 ICT receiving mice.	[149]
Fe3O4@PDA SPs + PTT	Hela, BALB/c-nude	PTT 808 nm 0.33 W/cm^2 10 min	Complete elimination of tumor after 4 days in intratumorally injected group and 6 days in intravenously injected group. No observed recurrence after 16 days.	[150]
Fe_3O_4 SPs-R837 + anti-PD-L1 + PTT	4T1, BALB/c	PTT 808 nm 0.33 W/cm^2 30 min	Elimination of primary tumor and prevention of metastases to lungs/liver. Control (but not elimination) of untreated tumor growth for up to 24 days.	[151]
FePSe3@APP@CCM + PTT	CT26, C57BL/6J	PTT 808 nm 1.5 W/cm^2 10 min	Control of CT26 tumor growth. Prolongation of survival in approximately 50% of animals.	[152]
SWNT-GC + PTT	4T1, BALB/c	PTT 1064 nm 1 W/cm^2 10 min	Inhibition of lung metastasis and extension of survival time. Combination with anti-CTLA-4: enhancement of efficacy leading to long-term survival of approximately 50% of treated animals.	[153]
Ti_3C_2@Met@CP + PTT	MDA-MB-231, BALB/c-nude	PTT 808 nm 1 W/cm^2 10 min	Complete eradication of tumor. Inhibition of tumor recurrence and metastasis.	[154]
PLGA-R837@Cat + RT	CT26, BALB/c	RT X-ray dose: 8 Gy	Reduction and/or elimination of tumor growth. Prevention of tumor growth after rechallenge. Combination with anti-CTLA-4: 60% long-term survival after treatment.	[156]
rGO/MTX/SB + PTT	4T1, BALB/c	PTT 805 nm 0.75 W/cm^2 5 min	Long-term survival rate: 70%. Rechallenge: rejection of tumor growth in 100% of animals. 20 days after treatment: control (but not elimination) of untreated tumor growth.	[157]

APNA, activated polymeric nanoagonist; APANP, anti-PD-1 peptide; Cat, catalase; CCM, cancer cell membrane; CP, compound polysaccharide; CT26, murine colorectal carcinoma; GC, glycated chitosan; Hela, human cervix epithelioid carcinoma; ICG, indocyanine green; Met, metformin; MDA-MB-231: human breast adenocarcinoma; MTX, mitoxantrone; OVA, ovalbumin; PEG, poly(ethylene-glycol); PLGA, poly(lactic-co-glycolic) acid; PTT, photothermal therapy; RB, rose bengal; RT, radiotherapy; rGO, reduced graphene oxide; SB, SB-431542; 4T1, murine mammary carcinoma; SP, superparticle; SWNT, single-walled carbon nanotube; UCNP, upconversion nanoparticle.

5 Impact of TME on cancer treatment

The TME is a complex ecosystem consisting of many different types of cells that influence immune cell activation and responses to cancer therapeutics (Figure 2). The goal of NAIT is to alter the TME to promote immune-mediated killing of the treated tumor and untreated metastases. From the experimental models described above, NAIT combined with ICT was often not enough to treat certain tumors, and/or generate effective immune memory that targets metastases. Most of the studies highlighted in this review used "cold" tumor models with little immune infiltration, which explains why ICT does not have considerable abscopal affects following NAIT. To circumvent this limitation, the type of TME the tumor establishes needs to be considered (Figure 2). Based on responses to ICT, the TME can generally be divided into four different types (Figure 7), based on tumor mutational burden (TMB) and inflammatory gene signatures (IGS) [14]. **Type 1** tumors have a high TMB and IGS, making these tumors the prime responders to ICT. **Type 2** tumors have a low TMB and IGS. Several factors contribute to this, including lack of chemokine production to recruit immune cells into the tumor, and/or the production of immunosuppressive cytokines to prevent immune activation via TAMs, MDSC, CAFs, and/or tumor cells. Small molecules specifically inhibiting the immunosuppressive cytokines or immunosuppressive cells residing within these TMEs should be combined with NAIT to improve the abscopal effect of tumor infiltrating T and NK cells. Other factors, not fully appreciated, are high IFP and the rigidity of the solid tumor, which are known barriers, preventing diffusion of chemotherapeutic drug and nanoparticles [164, 165]. These physical barriers are also likely preventing tumor immune cell infiltration and limiting the abscopal effect of NAIT. For this reason, using a combination of ablative approaches and nanomedicine may be necessary.

Type 3 tumors exhibit a high TMB but lack a substantial IGS. This TME is characterized by the expression of immunosuppressive metabolites, cytokines, and immune modulating molecules like PD-L1. Furthermore, chemokines that recruit inflammatory cells are reduced in expression, preventing the intratumoral recruitment of tumoricidal T cells. ICD of the tumor cells will initiate the production of chemokines that recruit proinflammatory immune cells [166, 167]. However, if the immunosuppressive cells, cytokines, and/or metabolites are not altered, the infiltrating tumoricidal cells will have limited effect. For these types of tumors, NAIT should be combined with NPs containing chemotherapeutic drugs and immunosuppressive metabolite inhibitors to enhance abscopal effects. The idea being that the multifunctional NP delivers the chemotherapy drug(s) that initiate tumor ICD, while simultaneously blocking the functions of the immunosuppressive metabolites, resulting in satisfactory immune infiltration, stimulation, and tumor killing. Thus, chemotherapies combined with metabolite modulators that inhibit IDO, arginase I, ROS, etc., or inhibitory cytokines (Figure 2) will help transform the TME to make it more amenable for tumoricidal activity and enhance the abscopal effect of NAIT. Another factor that may contribute to the lack of ISG is IFP and rigidity of the tumor as mentioned for type 2 TMEs. To circumvent these barriers, combination ablative approaches may be required to enhance abscopal affects.

Type 4 tumors present a low TMB but a high IGS. Immune escape is primarily contributed to the infiltration of immunosuppressive TAMs, MDSCs, and Tregs. These cells produce copious amounts of immune inhibiting molecules but can also directly interact with tumoricidal NK and T cells to prevent tumor killing. Additionally, the high production of anti-inflammatory chemokines prevents the recruitment of antitumor immune cells. Combining chemotherapeutic drugs with cytokine and MDSC/TAM/Treg inhibitors with NAIT may lead to greater abscopal effects and potential long-term control of secondary tumors. The rationale is as follows. In the untreated metastasis, the NP can deliver the chemotherapeutic drug(s) to initiate ICD. When combined with anti-inflammatory cytokine blockers or MDSC/TAM/Treg inhibitors that either block their recruitment and/or function, proinflammatory immune cells can infiltrate the tumor and maintain their tumor killing functions. Another factor to consider when dealing with any of the four types of TMEs listed above is that the TME is constantly evolving and selecting more "fit" tumor cells, capable of resisting cancer therapy. Additionally, the metastatic tumors can have different TMEs compared to the primary tumor and/or NAIT treated tumors. Thus, several doses of multimodal NPs will likely be required for a curative effect, and/or the treatments will likely evolve along with the tumors to maintain therapeutic efficacy.

6 Further considerations and conclusions

Over the past several decades, considerable advances have been made in nano-, photo-, and immuno-mediated therapies. Separately, these technologies have provided diagnostic and therapeutic tools for many different diseases.

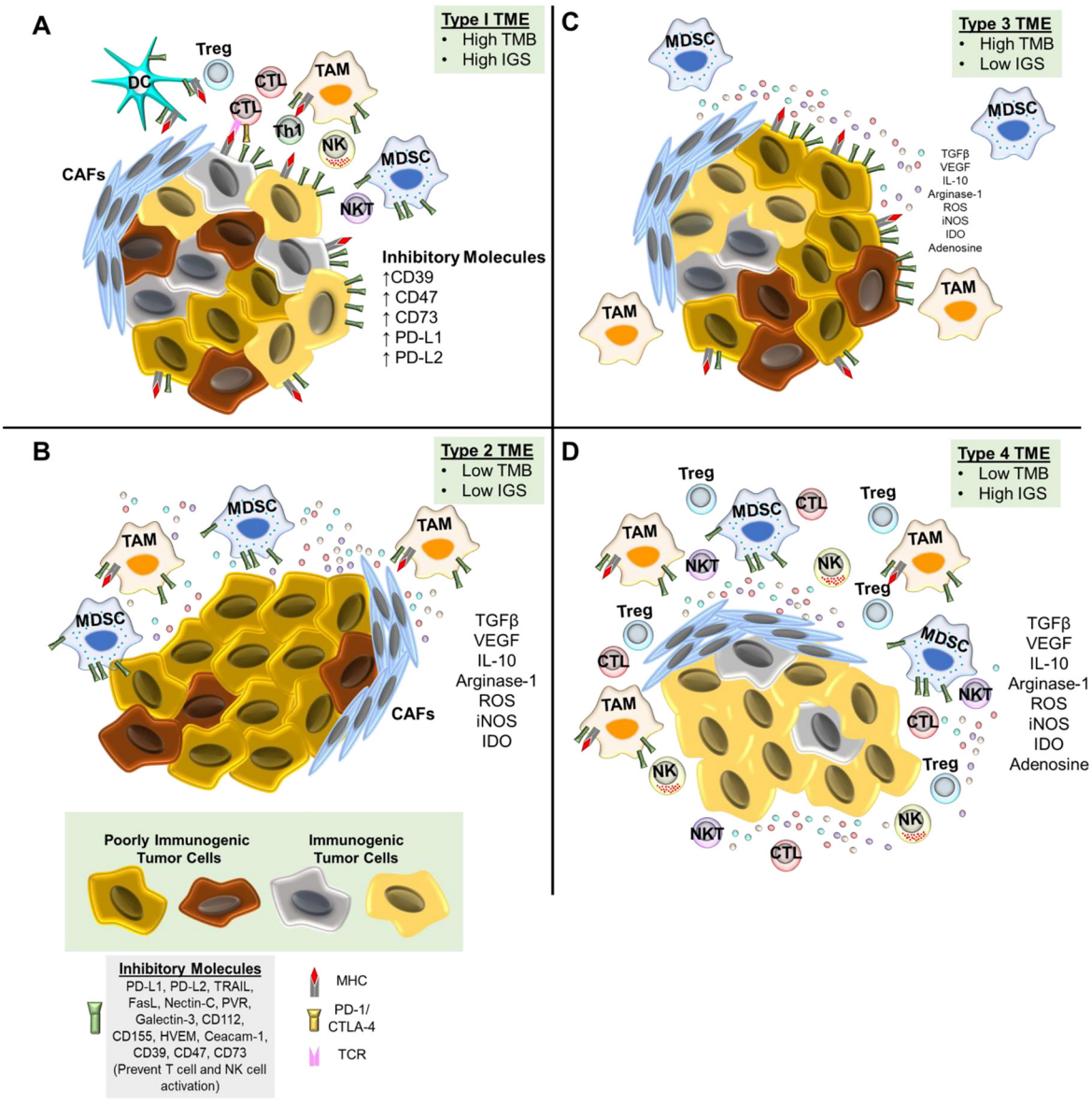

Figure 7: The tumor microenvironment can be divided into four groups based on tumor mutational burden (TMB) and inflammatory gene signatures (IGS) such as type II IFN response genes. **(A)** Type 1 tumors with a high TMB and IGS and high T cell infiltration. **(B)** Type 2 tumors with a low TMB and IGS, and low T cell infiltration. **(C)** Type 3 tumors with a high TMB but a low IGS and low T cell infiltration. **(D)** Type 4 tumors with a low TMB but a high IGS and high T cell infiltration.

However, NAIT synergizes all three to provide a cancer therapeutic approach capable of overcoming the complex TME and promoting tumor regression. Great progress has been made in this area of research, leading to novel combination therapies that enhance current immunotherapies such as ICT. As discussed above, PTT combined with immune stimulation allows for ICT-resistant tumors to respond to ICT and undergo regression in both preclinical and clinical studies. However, as the above studies also revealed, stimulating the immune system alone is not enough to overcome highly immunosuppressive TMEs of nontreated metastases. For this reason, combining immune stimulation with TME modulators may be required for activated immune cells to enter the highly immunosuppressive TMEs and maintain their tumor killing activity. Chen and colleagues combined PTT with rGO that initiates proinflammatory cytokine production (IL-1β, IL-6, and TNFα), a TGFβ inhibitor, and a chemotherapeutic drug MTX to initiate immune mediated tumor killing [157]. This worked well on small immunologically "cold" 4T1 tumors, but it had limited abscopal effects on untreated distant tumors. This is largely due to the TME, which is a major

factor limiting successful cancer therapy, especially on cancers that cannot be treated with PTT to disrupt tumor homeostasis in order to enhance tumor killing. The type of TMEs impacts the efficacy of ICT and other immunotherapies. Different approaches could be combined to possibly overcome these limitations.

NAIT provides a powerful approach to deliver combinatorial therapeutics to treat cancer through multiple pathways. The personalization of NAIT makes it incredibly durable in unleashing the full potential of antitumor immunity, not only to eliminate the treated primary tumor, but also to eliminate distant metastases. Future work combining TME modulators with NAIT offers a bright future for cancer treatment, particularly for eliminating distant metastases with unique TME immune signatures and stimulating effective antitumor memory.

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