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# Therapeutic Ultrasound-Enhanced Immune Checkpoint Inhibitor Therapy

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Immune checkpoint inhibitors (ICIs) are designed to reinvigorate antitumor immune responses by interrupting inhibitory signaling pathways and promote the immune-mediated elimination of malignant cells. Although ICI therapy has transformed the landscape of cancer treatment, only a subset of patients achieve a complete response. Focused ultrasound (FUS) is a noninvasive, nonionizing, deep penetrating focal therapy that has great potential to improve the efficacy of ICIs in solid tumors. Five FUS modalities have been incorporated with ICIs to explore their antitumor effects in preclinical studies, namely, high-intensity focused ultrasound (HIFU) thermal ablation, HIFU hyperthermia, HIFU mechanical ablation, ultrasound-targeted microbubble destruction (UTMD), and sonodynamic therapy (SDT). The enhancement of the antitumor immune responses by these FUS modalities demonstrates the great promise of FUS as a transformative cancer treatment modality to improve ICI therapy. Here, this review summarizes these emerging applications of FUS modalities in combination with ICIs. It discusses each FUS modality, the experimental protocol for each combination strategy, the induced immune effects, and therapeutic outcomes.

**Keywords:** focused ultrasound, immune checkpoint inhibitor, antitumor immune response, combination therapy, high-intensity focused ultrasound, low-intensity focused ultrasound

## INTRODUCTION

Immune checkpoint inhibitor (ICI) therapy has revolutionized the paradigm of cancer immunotherapy. Under normal physiological conditions, immune checkpoints are crucial to maintaining immune tolerance. However, in the tumor environment, tumor cells hijack these inhibitory mechanisms to avoid antitumor immune responses. ICIs are monoclonal antibodies that disrupt the engagement of immune checkpoints, which enables tumor-reactive T cells to overcome inhibitory mechanisms and mount effective antitumor immune responses [1]. The United States Food and Drug Administration (FDA) has approved ICIs that target cytotoxic T lymphocyte-associated protein-4 (CTLA-4), programmed cell death-1 (PD-1), and programmed cell death-ligand 1 (PD-L1) for the treatment of a wide variety of cancers [2]. Despite the clinical success of ICIs, advancing clinical applications of ICIs face challenges related to both efficacy and safety. Most cancer patients are unable to derive durable remission, while >50% of cancer patients develop immune adverse events after they receive ICIs [3]. The combination of multiple ICIs with other cancer therapies has improved cancer treatment by enhancing direct tumor killing and indirect antitumor immunity [4].

The past two decades have witnessed exciting breakthroughs in the clinical translations of focused ultrasound (FUS) modalities for cancer treatment [5]. FUS concentrates extracorporeally generated ultrasound energy through the body to a tight focus with an exceptional spatial resolution (on the

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millimeter scale) and deep penetration depth. The focal point can be mechanically and electronically steered in three-dimensional space to form a sonication volume that conforms to the shape of the target. FUS therapy is often performed under the guidance of magnetic resonance imaging or ultrasound imaging [6]. As a promising therapeutic technology, FUS has the unique combined advantages of being noninvasive, nonionizing, nonpharmaceutical, spatially targeted, and deeply penetrating the body. Since 2017, five FUS modalities, including high-intensity focused ultrasound (HIFU) thermal ablation [7–11], HIFU hyperthermia [12], HIFU mechanical ablation [13–17], ultrasound-targeted microbubble destruction (UTMD) [18–20], and sonodynamic therapy (SDT) [21, 22], have been investigated in combination with ICIs for treating solid tumors in mouse models. The enhancement of antitumor immune responses by these FUS modalities demonstrated the great promise of FUS as a transformative cancer treatment modality to improve ICI therapy.

In this review, we provide a brief introduction of ICI therapy basics and discuss the challenges facing ICI therapy. We then introduce each FUS-enhanced ICI therapy and summarize the therapeutic outcomes achieved by the combination therapy. Finally, we discuss the limitations of existing studies and provide future perspectives.

## Immune Checkpoint Inhibitor Therapy Basics and Challenges

ICIs bind to immune checkpoints, including CTLA-4, PD-1, and PD-L1, and “release the brakes” on T cells, resulting in anticancer immune responses. CTLA-4 inhibits T-cell activation by attenuating T-cell receptor signaling through competing with the costimulatory molecule CD28 for binding to B7 ligands on antigen-presenting cells (APCs) [23]. PD-1 regulates T-cell activation through interaction with its ligand PD-L1. The engagement of PD-1 and PD-L1 results in a negative costimulatory signal and leads to T-cell apoptosis, anergy, and exhaustion [24]. Efficient ICI therapy requires reactivation and clonal expansion of antigen-experienced T cells present in the tumor microenvironment (TME) [25]. Initially, naive tumor-specific CD8 T cells are primed by antigen presentation by APCs (often referred to as immune priming) and activated in the presence of costimulatory pathways and cytokines. Tumor-specific CD8 T cells subsequently differentiate into effector T cells, undergo clonal expansion, traffic to the TME, and ultimately kill tumor cells. A subset of effector T cells can differentiate into memory T cells under the guidance of CD4 T cells and dendritic cells (DCs) to develop long-term immunologic memory against the tumor.

The introduction of ICI therapy in the clinic has been considered to be a paramount achievement in cancer treatment in the last decade [26]. Since 2011, the FDA has approved ICIs targeting PD-1 (pembrolizumab, nivolumab, and cemiplimab), PD-L1 (atezolizumab, durvalumab, and avelumab), and CTLA-4 (ipilimumab). They have produced remarkable results regarding tumor control in many malignancies, such as melanoma, metastatic non-small-cell

lung cancer (NSCLC), head and neck squamous cancers, urothelial carcinoma, gastric adenocarcinoma, mismatch-repair-deficient solid tumors, and classic Hodgkin lymphoma. Many clinical studies with ICIs are currently underway to test their efficacy in various other diseases.

Despite the clinical success of ICIs, ICI therapy faces challenges related to both efficacy and safety. With regard to ICI efficacy, the majority of patients do not benefit from the treatment, and some responders relapse after a period of response. Ongoing studies indicate that both tumor cell-intrinsic and tumor cell-extrinsic factors contribute to the resistance mechanisms [27]. Tumor cell-intrinsic factors include lack of tumor-associated antigens (TAAs), ineffective antigen presentation, activation of oncogenic pathways, and insufficient interferon- $\gamma$  (IFN- $\gamma$ ) signaling. Tumor cell-extrinsic factors are within the TME and include exhausted CD8 T cells, regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and other immunosuppressive cells and factors. With regard to safety, a significant number of patients on ICIs develop immune-related adverse events affecting almost every organ. Immune-related adverse events occur when ICIs result in an immune-based attack on normal tissue. These events, such as dermatitis, thyroiditis, pneumonitis, colitis, hepatitis, and nephritis, are unpredictable, heterogeneous, and in some instances life-threatening. Management of these adverse events remains a challenge [28]. These challenges call for concepts to maximize the clinical benefits of ICIs in combination with other therapies. An abundance of clinical trials are currently underway in evaluating the combination of ICIs with other immunotherapies, chemotherapy, radiotherapy, or targeted therapies. Strategies that can improve antigen presentation and immune recognition, reinforce the activity and infiltration of CD8 T cells, and reduce immunosuppression can potentially be combined with ICIs to improve the efficacy of ICI therapy [27]. Meanwhile, novel drug delivery strategies that enable the targeted delivery of ICIs within the TME have the potential to reduce the toxicities associated with ICIs [29, 30].

FUS is a promising platform technology to be combined with ICIs to improve its efficacy and safety. Various FUS therapeutic modalities have been developed, and some of them have been used in the clinic for the treatment of various diseases (**Table 1**). Among them, HIFU thermal ablation has been approved by the FDA for the treatment of prostate cancer, uterine fibroids, bone metastasis, and essential tremor and has been used worldwide for the treatment of various diseases. Although other FUS modalities, including HIFU hyperthermia, HIFU mechanical ablation, UTMD, and SDT, have not been approved for clinical use, clinical studies are currently ongoing, with multiple studies already reported. Advances in the clinical applications of these FUS techniques have encouraged new studies to combine FUS with ICIs, as summarized in **Table 2**. The effectiveness of FUS-enhanced ICI therapy is often demonstrated by increased tumor infiltrated CD8 T cells, decreased tumor volume, and prolonged survival. The systemic immune response of ICI therapy can also be demonstrated by the presence of the abscopal effect, which occurs when the treatment not only shrinks the targeted tumor but also leads to shrinkage of untreated tumors elsewhere in the

**TABLE 1** | Overview of different FUS modalities.

Modalities	Commonly used FUS parameters	Physical mechanism	Clinical applications
HIFU thermal ablation	Continuous (duty cycle = 100%) HIFU with spatial peak temporal average intensity ( $I_{SPTA}$ ) >1000 W/cm <sup>2</sup> and relative high pressure (a few MPa) to rapidly heat tumor to >60°C within a few seconds	Coagulative thermal necrosis due to tissue absorption of ultrasound energy	The only modality currently approved for use in the clinic worldwide for the treatment of a variety of solid malignant tumors (e.g., tumors in the pancreas, liver, kidney, bone, prostate, and breast), benign tumors (e.g., uterine fibroids and fibroadenomata), and non-tumor diseases (e.g., essential tremor and Parkinson's disease) [32]
HIFU hyperthermia	HIFU with a high duty cycle (up to 70%) and relative high pressure (a few MPa) to heat up tissue to 40–45°C for up to 60 min [35]	Thermally controlled drug release and increasing blood flow and oxygen	Multiple reported clinical studies of HIFU hyperthermia to enhance the delivery of thermosensitive liposomal doxorubicine in patients with liver tumors [35]
HIFU mechanical ablation	HIFU with $I_{SPTA}$ >100 W/cm <sup>2</sup> and a low duty cycle (1–2%) and extremely high pressure (>10 MPa) to induce mechanical tissue damage while minimizing heating [49]	High-amplitude HIFU pulses are used to induce bubble cavitation to fractionate tissue by mechanical effects	No reported clinical results, but clinical trials ongoing for the treatment of primary and metastatic liver tumors (ClinicalTrials.gov Identifier: NCT04572633)
UTMD	LIFU with $I_{SPTA}$ <10 W/cm <sup>2</sup> and a low duty cycle (e.g., 10%) and relative low pressure (<1 MPa) to induce mechanical effects [42]	Targeted and controlled release of therapeutics, sonoporation for drug/gene delivery, and mechanical disruption of the blood vessels and tumor tissue [50]	One reported clinical study of drug delivery using UTMD in pancreatic patients [51]. Multiple reported clinical studies on opening the blood-brain barrier for brain drug delivery in patients with glioblastoma [52, 53], Alzheimer's disease [54], and amyotrophic lateral sclerosis [55]
SDT	LIFU with $I_{SPTA}$ <10 W/cm <sup>2</sup> and a duty cycle of 10–100% to activate sonosensitizers [56]	Sensitizer-dependent sonochemical or sonophotochemical reactions in an acoustic field that lead to cytotoxicity [48]	Several reported clinical studies investigated a combination of light and ultrasound to stimulate a sensitizer in patients with a variety of cancer [57]

HIFU (high-intensity focused ultrasound), LIFU (low-intensity focused ultrasound), UTMD (ultrasound-targeted microbubble destruction, SDT (sonodynamic therapy).

body. In the following, each FUS-enhanced ICI therapy is introduced.

## High-Intensity Focused Ultrasound Thermal Ablation-Enhanced Immune Checkpoint Inhibitor Therapy

HIFU thermal ablation induces thermal coagulation by rapidly (in a few seconds) heating tissue at the focus to >60°C, often with high-intensity continuous ultrasound waves. Only tissue within the focal region is selectively ablated, while tissue in the ultrasound beam path is spared from ablation [31]. Compared with other local ablative therapies, such as ablative radiotherapy, radiofrequency ablation, and cryotherapy, HIFU thermal ablation is the only noninvasive and nonionizing ablation technique, allowing the procedure to be performed and repeated without the need for surgical implantation of applicators and concerns about radiation-induced toxicities. HIFU thermal ablation causes very few side effects to normal surrounding tissues, and patient comfort and safety are maximized [32]. HIFU thermal ablation has been widely applied for the treatment of a variety of solid tumors, as well as many other benign diseases in the clinic [32]. HIFU thermal ablation has been reported to increase the release of damage-associated molecular patterns (DAMPs) and TAAs, promote DC maturation, increase tumor-infiltrating lymphocytes, and change circulating immunosuppressive cytokine levels [33], suggesting the potential to improve ICI efficacy for tumors that do not respond well to ICIs.

The first study on therapeutic ultrasound-enhanced ICI therapy was reported in 2017 by Silvestrini et al. [7]. They

explored whether HIFU thermal ablation could be effectively incorporated with ICIs to boost antitumor immune responses in murine breast cancer models. Breast cancer is often resistant to most chemotherapies and molecular targeted therapies, including ICI therapies [7]. Half of the reported FUS-enhanced ICI studies summarized in Table 2 used murine breast cancer models. In Silvestrini's study, systemic anti-PD-1 antibody ( $\alpha$ PD-1) and local adjuvant, CpG, were administered prior to HIFU thermal ablation for immunotherapy priming. Only with initial immunotherapy priming, coincident HIFU thermal ablation and immunotherapy suppressed tumor growth in both treated and contralateral nontreated tumors and increased the survival rate [7]. The potential mechanisms for the enhanced antitumor response from this multistep protocol were proposed as follows [8]: immunotherapy priming expanded the number of tumor-infiltrating CD8<sup>+</sup> T cells and macrophages. The subsequent HIFU thermal ablation released tumor antigens, inflammatory chemokines and cytokines, increased interferon stimulating genes, and altered the local macrophage phenotype. These effects led to cross-presentation and cross-priming mediated by macrophages and DCs, resulting in an effective abscopal response.

The combination of HIFU thermal ablation with ICIs has also been explored to treat colorectal tumors since some of them are not suitable for ICIs [34]. Without immunotherapy priming, the combination of HIFU thermal ablation with ICIs and local adjuvants was able to produce therapeutic benefits in colorectal tumor-bearing mice [9]. HIFU thermal ablation was followed by direct injection of nanoadjuvants into the ablated site and intravenous injection of anti-CTLA-4 antibody ( $\alpha$ CTLA-4).

**TABLE 2** | Summary of therapeutic ultrasound-enhanced ICI therapies.

FUS modality	References	Cell/mouse (tumor model)	FUS parameters	Combined FUS and ICI protocol	Key results from combination therapy
HIFU thermal ablation	Silvestrini et al. [7]	NDL/FVB/n (orthotopic breast cancer)	3 MHz, 3.1 MPa, T > 65°C, target: circular pattern within 1–2 mm of the tumor edge	Adjuvant CpG (i.t.) injected to single tumor on days 21, 24, 28, 31, 38, 45; αPD-1 (i.p.) injected on days 21, 28, and 35; HIFU thermal ablation applied to tumor on days 31, 38, 45	Distant tumor leukocytes ↑ and CD8 <sup>+</sup> ↑; spleen IFN-γ <sup>+</sup> CD8 <sup>+</sup> ↑; treated and distant tumor volume ↓; survival ↑
	Chavez et al. [8]	NDL/FVB/n (orthotopic breast cancer) PyMT/PyVT (spontaneous breast cancer)	3 MHz, T > 60°C, target: 2–3 mm within tumor	Adjuvant CpG (i.t.) injected to single tumor on days 21, 24, 28, 31; αPD-1 (i.p.) injected on days 21, 28, 35, HIFU thermal ablation applied to tumor on day 31	Treated tumor interferon-stimulated gene expression ↑; treated tumor CD169 <sup>+</sup> DCs ↑ and CD169 <sup>+</sup> MPs ↑; distant tumor DCs ↑; spleen CD169 <sup>+</sup> MPs ↑
	Han et al. [9]	CT26/BALB/c (subcutaneous colorectal cancer)	4 MHz, 43 W, FUS on = 1 s, FUS off = 5 s for a single point, repeat 20 rounds to completely eliminate tumor	For metastatic tumor: HIFU thermal ablation performed to remove tumor on day 7; adjuvants directly injected into ablated site afterward; αCTLA-4 (i.v.) injected on days 8, 10, 12, 14 For recurrent tumor: HIFU thermal ablation performed to remove tumor on day 7, adjuvants directly injected into ablated site afterward; tumor rechallenged on day 47; αCTLA-4 (i.v.) injected on days 48, 50, 52, 54	Distant tumor CD8 <sup>+</sup> /Tregs ↑ and MDSCs ↓; spleen effector memory T cell (CD3 <sup>+</sup> CD44 <sup>hi</sup> CD62 <sup>Lo</sup> ) ↑; serum IFN-γ ↑ and TNF-α ↑; distant tumor volume ↓; survival ↑; tumor rechallenge suppressed
	Sheybani et al. [10]	4T1/BALB/c (subcutaneous breast cancer)	3 MHz, 15 W, FUS on = 10 s for each sonication, target: ~10–20% of total tumor volume	Every 3 days for a total of five doses of αPD-1 (i.p.) injected prior to or with HIFU thermal ablation with gemcitabine	Tumor volume ↓
	Fite et al. [11]	NDL/FVB/n (orthotopic breast cancer)	3 MHz, 3.1 MPa, T > 65°C, circular pattern within 1–2 mm of the tumor edge	Adjuvant CpG (i.t.) injected to single tumor on days 21, 24, 28, 30; αPD-1 (i.p.) injected on days 21, 28, 34, HIFU thermal ablation applied to tumor on day 30	Treated tumor IL-6 and IL-β mRNA ↑; treated tumor MDSCs ↑; distant tumor MDSCs ↑
HIFU hyperthermia	Kheiriloom et al. [12]	NDL/FVB/n (orthotopic breast cancer) PyMT/PyVT (spontaneous breast cancer)	1.5 MHz, PNP 2.5 MPa, PRF 100 Hz, pulse length 0–7 ms, heating at 42°C for 5 min before and 20 min post liposome injection	Copper-doxorubicin-loaded temperature-sensitive liposomes (i.v.) injected on day 31, HIFU hyperthermia applied to tumor afterward; adjuvant CpG (i.t.) injected to single tumor on days 21, 24, 28, 35, 49; αPD-1 (i.p.) injected on days 21, 28, 35	Treated and distant tumor CD8 <sup>+</sup> ↑; treated and distant tumor volume ↓; survival ↑

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**TABLE 2 |** (Continued) Summary of therapeutic ultrasound-enhanced ICI therapies.

FUS modality	References	Cell/mouse (tumor model)	FUS parameters	Combined FUS and ICI protocol	Key results from combination therapy
HIFU mechanical ablation	Wang et al. [13]	GL261/C57BL/6 (subcutaneous glioblastoma)	1.1 MHz, duty cycle 2%, treatment duration 2 min	Perfluorocarbon-filled microshells directly injected to large tumors (400–700 mm <sup>3</sup> ), HIFU mechanical ablation applied to tumor afterward; αPD-1 (i.p.) injected on days 0, 2, 4, 6, 8, 10 <sup>a</sup>	Tumor CD45 <sup>+</sup> ↑, CD3 <sup>+</sup> ↑, CD8 <sup>+</sup> ↑, and IFN-γ ↑; tumor size ↓; survival ↑; tumor rechallenge rejected
	Eranki et al. [14]	Neuro2a/C57BL/6 (subcutaneous neuroblastoma)	1.5 MHz, PNP 14 MPa, PRF 1 Hz, pulse duration 13.33 ms, 5 s/location, 3 locations, cover 2% tumor volume	Histotripsy applied to tumor on day 8; αCTLA-4 (i.p.) + αPD-L1 (i.p.) injected on days 9, 12, 15	Tumor CD4 <sup>+</sup> ↑, CD8α <sup>+</sup> ↑ and CD8α <sup>+</sup> CD11c <sup>+</sup> ↑; spleen and TDLN CD8α <sup>+</sup> CD11c <sup>+</sup> ↑; spleen CD11b <sup>low</sup> ↑; spleen effector memory T cells (CD4 <sup>+</sup> or CD8 <sup>+</sup> CD44 <sup>+</sup> hiCD62L <sup>low</sup> ) ↑; serum IFN-γ ↑, IL-6 ↑ and IL-10 ↓; complete abscopal response; survival ↑; transferred T cells suppressed tumor in recipients
	Nam et al. [15]	4T1/BALB/c (subcutaneous breast cancer); CT26/BALB/c (subcutaneous colorectal cancer)	1.5 MHz, electrical power 525 W, PRF 1 Hz, duty cycle 1%, pulse length 10 ms, 50 pulses	Histotripsy applied to tumor on days 6, 7, 8; αPD-1 (i.p.) injected on day 9, 11, 13	Tumor CD8 <sup>+</sup> ↑, CD8 <sup>+</sup> CD107a <sup>+</sup> ↑, CD8 <sup>+</sup> PD-1 <sup>+</sup> ↑ and MDSCs ↓; tumor volume ↓; tumor weight ↓
	Qu et al. [16]	B16GP33/C57BL/6 (subcutaneous melanoma) Hepa1-6/C57BL/6 (subcutaneous hepatoma)	1 MHz, PNP 30 MPa, PRF 100 Hz, 50 pulses, pulse duration 1–2 μs; total treatment duration 4–15 min depending on tumor volume	For B16GP33 tumor, histotripsy applied to tumor on day 7; αCTLA-4 (i.p.) injected at days 6, 9, 12 For Hepa1-6 tumor, histotripsy applied to tumor on day 10; αCTLA-4 (i.p.) injected on days 3, 6, 9, 12	Tumor CD8 <sup>+</sup> ↑; tumor volume ↓
	Singh et al. [17]	B16F10/C57BL/6 (subcutaneous melanoma)	1.5 MHz, acoustic power 450 W, PRF 1 Hz, duty cycle 1%, cover 40–50% of the tumor	Histotripsy applied to tumor (330–400 mm <sup>3</sup> ); single dose of αCD40 (i.t.) injected afterward (within 2 h); 3 dose of αCTLA-4 (i.p.) + αPD-L1 (i.p.) injected at 3 days interval	Tumor growth ↓; survival ↑
UTMD	Li et al. [18]	LLC/C57BL/6 (subcutaneous and orthotopic lung cancer)	1 MHz, 2.0 W/cm <sup>2</sup> , duty cycle 50%, treatment duration 5 min	Docetaxel and αPD-L1-coated microbubbles (i.v.) followed by LIFU applied to tumor on days 8, 11, 14, 17, 20 for subcutaneous tumor and on days 6, 9, 12, 15, 18 for orthotopic tumor	Tumor TUNEL ↑, Ki67 <sup>+</sup> ↓, CD4 <sup>+</sup> ↑, CD8 <sup>+</sup> ↑; tumor growth ↓; body weight loss ↓; survival ↑
	Bulner et al. [19]	CT26/BALB/c (subcutaneous colorectal cancer)	1 MHz, PNP 1.65 MPa, pulse length 0.1 ms, duty cycle 10%, wait 20 s after every 50 pulses, total treatment duration 2 min	LIFU+microbubbles two repeats with 10 min interval on day 10–12; αPD-1 (i.p.) injected on days 0, 3, 6 after LIFU+microbubbles for acute study and 0, 3, 5, 9, 12 for longitudinal study	Tumor necrosis ↑ and growth ↓; survival ↑; one survived mouse suppressed tumor rechallenge
	Ilovitsh et al. [20]	NDL/FVB/n (orthotopic breast cancer)	250 kHz, PNP 500 kPa, PRF 30 Hz, burst length 4 ms, total treatment duration 3 min	αCD326-loaded microbubbles and pIFN-β (i.t.) injected followed by LIFU applied to tumor on day 14; αPD-L1 (i.p.) injected on days 11, 16	Tumor CD8 <sup>+</sup> ↑ and F4/80 MPs ↑; treated and distant tumor growth ↓; survival ↑

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**TABLE 2 |** (Continued) Summary of therapeutic ultrasound-enhanced ICI therapies.

FUS modality	References	Cell/mouse (tumor model)	FUS parameters	Combined FUS and ICI protocol	Key results from combination therapy
SDT	Yue et al. [21]	4T1/BALB/c (breast cancer, orthotopic and intravenous injection); CT26/BALB/c (subcutaneous colorectal cancer)	1 MHz, 1.5 W/cm <sup>2</sup> , 50% duty cycle, treatment duration 5 min	Sonosensitizers and adjuvant loaded liposomes (i.v.) injected on days 7, 8, 10, 11; LIFU applied to tumor 12 and 24 h post injection; αPD-L1 (i.v.) injected on days 8, 11, 13, 15	Tumor leucocytes ↑, CD8 <sup>+</sup> ↑ and CD8 <sup>+</sup> /Tregs ↓; serum IFN-γ ↑ and TNF-α ↓; tumor volume ↓; survival ↑
	Um et al. [22]	CT26/BALB/c (colorectal cancer, subcutaneous and intravenous injection)	Acoustic power 10 W, duty cycle 20%, PRF 1 Hz, treatment duration 10 min	Sonosensitizers and gas loaded PEGylated carboxymethyl dextran (i.v.) injected and 6 hrs later LIFU applied on tumor on days 8, 11, 14; αPD-L1 (i.p.) injected on days 9, 12, 15	Tumor CD8 <sup>+</sup> ↑; tumor volume ↓; tumor weight ↓

<sup>a</sup>Days referred to treatment start date, all the other days referred to tumor implantation date. T (temperature), PNP (peak negative pressure), PRF (pulse repetition frequency), i. p. (intraperitoneally), i. v. (intravenously), i. t. (intratumorally), FUS (focused ultrasound), HIFU (high-intensity focused ultrasound), LIFU (low-intensity focused ultrasound), UTMD (ultrasound-targeted microbubble destruction), SDT (sonodynamic therapy), Tregs (regulatory T cells), MPs (macrophages), MDSCs (myeloid-derived suppressor cells), TDLN (tumor-draining lymph nodes), IFN-γ (interferon-gamma), TNF-α (tumor necrosis factor-alpha), IL-6 (interleukin 6), IL-7β (interleukin 7 beta), IL-10 (interleukin 10), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), CpG (cytosine-phosphodiester-guanine oligodeoxynucleotide).

The nanoadjuvants were formed by loading poly (lactic-co-glycolic) acid nanoparticles with either a TLR7 agonist (imiquimod, R837) or TLR4 agonist (monophosphoryl lipid A, MPLA). This combination strategy increased the intratumoral CD8 T cell/Treg ratio, reduced MDSCs within tumors, achieved complete distant tumor eradication, prolonged mouse survival, and prevented tumor recurrence, indicating the generation of sustained immune memory against colorectal tumors. In contrast, none of the mice given HIFU thermal ablation plus either nanoadjuvants or αCLTA-4 survived [9], which suggests that additional agents (e.g., adjuvants, chemotherapeutics) may be required for the success of HIFU thermal ablation and ICI combination treatment. Another study reported that initiating αPD-1 treatment prior to versus shortly after HIFU thermal ablation with chemotherapy did not bear a marked difference in primary tumor growth in a murine breast cancer model [10].

These studies suggest that the optimal protocol for HIFU thermal ablation-enhanced ICI therapy may depend on the tumor model, the type of adjuvants used, with or without immunotherapy priming, and the HIFU ablation protocol. HIFU thermal ablation as a combination therapy with ICIs has the potential limitation that excessive heat generation by HIFU thermal ablation may induce protein denaturation and inactivate antigen presentation [13]. Meanwhile, HIFU thermal ablation was also reported to increase tumor infiltrated MDSCs and Tregs at both directly treated and distant tumors, leading to inhibition of antitumor immunotherapy [11]. These negative effects highlight the complexity of combining HIFU thermal ablation with ICI therapy.

### High-Intensity Focused Ultrasound Hyperthermia-Enhanced Immune Checkpoint Inhibitor Therapy

HIFU hyperthermia raises tissue temperature within the focal region to 40–45°C for up to 60 min. It is different from thermal ablation in that hyperthermia is not intended to produce substantial cell death directly. Instead, HIFU hyperthermia is often combined with chemotherapy and radiation therapy or used for local drug release in combination with temperature-sensitive nanoparticles [35]. HIFU hyperthermia can directly promote antigen cross-presentation and tumor-reactive T cell formation and expansion [36].

Kheirrolomoom et al. investigated the combination of HIFU hyperthermia with chemotherapy, CpG, and αPD-1 [12]. HIFU hyperthermia was utilized to control the release of temperature-sensitive liposomes loaded with a chemotherapy drug, doxorubicin (Dox). The liposome carrier was designed to minimize the severe cardiac toxicity of Dox and enhance its delivery efficiency to tumors. Dox released at HIFU hyperthermia-treated tumors enhanced the presentation of tumor-specific antigens at distant tumor sites. Similar to HIFU thermal ablation [7], only with immunotherapy priming by CpG and αPD-1, the combined HIFU hyperthermia, Dox-loaded liposomes, and αPD-1 treatment increased tumor infiltrated CD8 T cells and achieved complete tumor destruction in both treated and distant tumors as well as prolonged tumor-free

survival. However, repeated Dox delivery by HIFU hyperthermia either with or without immunotherapy priming reduced the complete response rate, which was considered to be caused by rapid tumor cell death resulting from repeated Dox release that weakened the impact of local antigen and cytokine release. These findings highlighted the importance of the dosing of HIFU hyperthermia-mediated chemotherapy and the timing of immunotherapy to augment ICI efficacy for cancer treatment.

These reported studies [7, 9, 10, 12] suggest that neither HIFU thermal ablation nor HIFU hyperthermia alone is sufficient to enhance ICI efficacy in murine tumor models. Both FUS modalities were found to enhance the release of TAAs and recruitment of CD8 T cells, but in the absence of additional stimuli (e.g., adjuvants, chemotherapeutics), the recruited CD8 T cells might not have sufficient antigen cross-presentation and cross-priming mediated by DCs and macrophages [8, 11]. Future studies are needed to investigate the optimal combination therapy by HIFU thermal ablation or hyperthermia with ICIs and adjuvants/chemotherapeutics to achieve systemic, long-term effects for cancer treatment.

## High-Intensity Focused Ultrasound Mechanical Ablation-Enhanced Immune Checkpoint Inhibitor Therapy

HIFU mechanical ablation utilizes short pulse lengths (microsecond to millisecond) and low duty cycles to produce mechanical ablation of tissues while limiting tissue temperature increase. The primary physical mechanism of HIFU mechanical ablation is cavitation, which is defined as the formation, oscillation, and collapse of bubbles in the acoustic field. Cavitation can induce tissue damage by various mechanisms, including microjetting, streaming, and shear stresses [37]. The formation of cavitation in tissue by HIFU can be facilitated by the injection of exogenously made cavitation nuclei, for example, microbubbles or phase-changing materials (e.g., perfluorocarbon). Without the injection of cavitation nuclei, cavitation can be initiated using ultrasound pulses with high tensile pressure, which stretches the tissue and generates cavitation bubbles *in situ*. When extremely high tensile pressures are generated, HIFU can lead to complete liquefaction of the tumor tissue into submicron fragments, which is named histotripsy [38]. Several reports have shown that HIFU mechanical ablation can cause immunogenic cell death and release tumor debris *in situ*, promote antigen presentation, and enhance the inflammatory response [33].

The clinical applications of ICIs in brain tumors (e.g., glioblastoma and neuroblastoma) have been challenging, potentially because these tumors harbor a “cold” immune microenvironment that lacks requisite T cells and sufficient TAAs and contains high densities of immunosuppressive cell populations [39, 40]. One recent study demonstrated that HIFU mechanical ablation combined with silica microshells mechanically disrupted glioblastoma tumors and augmented the efficacy of  $\alpha$ PD-1 [13]. The combination of HIFU mechanical ablation with microshells and  $\alpha$ PD-1 increased tumor-infiltrating CD8 and IFN- $\gamma$ <sup>+</sup>CD8 T cells, prolonged

tumor-free survival and protected against tumor rechallenge, suggesting the formation of long-term immune memory against glioblastoma. In a murine neuroblastoma model, Eranki et al. demonstrated that histotripsy potentially transformed immunologically “cold” tumors into responsive “hot” tumors and provided an efficacious adjuvant to ICI therapy [14]. Histotripsy followed by systemic injection of  $\alpha$ CTLA-4 and  $\alpha$ PD-1 induced significant increases in intratumoral CD4, CD8 $\alpha$ , and CD8 $\alpha$ <sup>+</sup> DCs in regional lymph nodes and circulating IFN- $\gamma$  and decreases in circulating IL-10. Notably, the combination therapy improved long-term survival, achieved complete bilateral tumor regression, and induced an effective long-term immune memory response to suppress subsequent tumor engraftment. Other recent studies found that histotripsy stimulated more potent intratumoral CD8 T cells and antigen presentation than HIFU thermal ablation in a murine breast cancer model [15] and melanoma model [16]. One recent study showed that combining histotripsy with intratumor anti-CD40 agonist antibody,  $\alpha$ CTLA-4, and anti-PD-L1 antibody ( $\alpha$ PD-L1) significantly improved the therapeutic efficacy against ICI refractory murine melanoma [17].

These findings [13–16] suggest that HIFU mechanical ablation alone, without the need for adjuvants, is sufficient to enhance ICI therapy for the treatment of cancers that are unresponsive to ICIs. One advantage of HIFU mechanical ablation over HIFU thermal ablation is that tumor fragmentation instead of tumor coagulation may protect TAAs and DAMPs from protein denaturation by excessive heat and stimulate more effective antitumor immune responses [15, 16, 33, 41].

## Ultrasound-Targeted Microbubble Destruction-Enhanced Immune Checkpoint Inhibitor Therapy

There is no consensus regarding the definition of low-intensity focused ultrasound (LIFU). It can be regarded as FUS with pulse intensity similar to that of diagnostic ultrasound. Microbubbles are made of a phospholipid, surfactant, albumin, or synthetic polymer shell filled with a high molecular weight gas with low water solubility. These microbubbles were initially introduced into the clinic as ultrasound contrast agents to enhance ultrasound signals from the blood circulation [42]. Over the past decades, they have been developed into theranostic agents. Their shells can be used for disease-specific targeting and loaded with drugs as carriers for controlled drug release at the LIFU-targeted region. Moreover, microbubble cavitation upon LIFU sonication can generate mechanical forces on surrounding tissue and induce vascular disruption [43].

PD1/PD-L1 ICIs have been used in the clinic for the treatment of NSCLC in combination with chemotherapeutic drugs. However, the combination of these drugs leads to aggravated cardiotoxicity, hematotoxicity, hepatotoxicity, and neurotoxicity [44]. Li et al. used microbubbles as carriers of immunotherapy and chemotherapy drugs to produce antitumor effects while reducing the toxicities of the drug combination [18]. Docetaxel was loaded inside the lipid shell of the microbubbles, and  $\alpha$ PD-L1



was conjugated to the surface of the microbubbles. UTMD improved drug delivery to the tumor potentially through three combined effects:  $\alpha$ PD-L1 on the surface of the microbubbles specifically targeted the tumor cells; ultrasound sonication ruptured the microbubbles and released the carried drug at the LIFU-targeted tumor site; microbubble cavitation increased tumor permeability and promoted drug penetration across the vessel and into the tumor tissue. As a result, this therapeutic strategy inhibited tumor growth and improved the survival of mice implanted with tumor cells in the lung. It is worth to point out that lung diseases are often considered difficult to treat with FUS because the lungs are air-filled cavities. However, clinical studies have combined ultrasound and microbubbles to enhance drug delivery to the lungs of patients with pneumonia, acute respiratory distress syndrome, and NSCLC [45, 46]. It was proposed that because the diseased areas of the lung are filled with fluid, ultrasound waves could penetrate through the diseased area and leave normal air-filled areas of lung unaffected.

Microbubbles were also used as “anti-vascular” agents to disrupt blood vessels and increase the antitumor effects of ICI therapy of colorectal cancer in a study by Bulner et al. [19]. They found that UTMD alone induced an instant shutdown of blood flow within tumor tissue and resulted in tumor necrosis in a mouse model of colorectal cancer. The combination of UTMD and  $\alpha$ PD-1 treatment conferred better tumor growth constriction and a higher survival rate than USMB or  $\alpha$ PD-1 alone and rejected subsequent tumor rechallenge. However, the results did not support that the combined UTMD and  $\alpha$ PD-1 treatment shifted T-cell subpopulations to a more favorable antitumor state.

In a murine breast cancer model, UTMD produced triple antitumor effects simultaneously: carrying an anti-CD326 antibody to target tumor cells, nonviral gene transduction of IFN- $\beta$  by sonoporation, and tumor debulking by mechanical forces [20]. Such proximity of microbubbles to tumor cells using targeted microbubbles was crucial for effective sonoporation to transfect tumor cells. IFN- $\beta$  expression plus  $\alpha$ PD-1 led to a decreased tumor cell population and increased tumor-infiltrating CD8 T cells. The complete combination treatment attained greater tumor growth reduction in treated and distant tumors and prolonged survival than any partial treatments in the murine breast cancer model.

## Sonodynamic Therapy-Enhanced Immune Checkpoint Inhibitor Therapy

SDT utilizes LIFU to activate sonosensitizers and induces cytotoxicity [47, 48]. Unlike chemotherapy drugs that have massive toxicity on healthy cells, SDT induces tumor cell disruption only at the LIFU-targeted site. Preclinical studies have found that tumor cell debris generated by SDT could provide TAAs for initiating antitumor immunological effects [47, 48]. One report employed SDT using liposomes loaded with sonosensitizers and adjuvants [21]. Strikingly, SDT combined with  $\alpha$ PD-L1 eradicated the primary tumor, suppressed distant tumor growth, inhibited whole-body metastasis in murine breast cancer models and produced

sufficient immune memory responses to reject subsequent tumor rechallenge in murine breast and colorectal cancer models. The SDT-elicited antitumor effects, immune adjuvant-containing sonosensitizers, and  $\alpha$ PD-L1-mediated systemic antitumor immune response were attributed to the robust antitumor response.

Recently, Um et al. used nanobubbles loaded with a sonosensitizer (chlorin e6) for the treatment of pulmonary metastasis of colorectal cancer [22]. Upon sonication, these nanobubbles caused cell membrane disruption by cavitation, which triggers immunogenic cancer cell death and releases intact DAMPs for *in situ* cancer vaccination. The combination of  $\alpha$ PD-L1 with nanobubbles loaded with the sonosensitizer effectively suppressed primary and metastatic tumors, which suggested that physically induced tumor cell death by the nanobubbles combined with SDT can augment the efficacy of ICIs. More work is needed to determine whether this strategy can improve long-term survival and generate long-lasting immune memory responses against tumor recurrence.

## DISCUSSION

Recent publications have presented exciting and promising results that FUS modalities can improve ICI therapy. Combination therapies were reported to suppress tumor growth, achieve tumor remission, improve long-term survival, and prevent tumor recurrence for cancer types that are not readily responsive to ICI treatments. The field of FUS-enhanced IC therapy is still in its infancy, with all existing studies focused on proofing the concept. Further development of the combination strategy requires a multidisciplinary approach with a proper choice of FUS parameters for particular tumors, a complete examination of the correlation between FUS parameters and antitumor immune effects, a thorough evaluation of the biological mechanisms for therapeutic outcome, and a good understanding of the clinical challenges in cancer immunotherapy.

Although each FUS modality has the capability to improve ICI immunotherapy, it is still unknown which regimen has the greatest potential to combine with ICIs. One major challenge is the inconsistent reporting of FUS parameters and antitumor immune effects, which prevents correlating FUS parameters with antitumor immune effects. It is important to standardize reporting on FUS procedures to include all key parameters, such as ultrasound frequency, intensity, pressure, duty cycle, pulse repetition frequency, sonication target locations, and sonication duration. It is also critical to establish standards in reporting antitumor immune effects to enable comparisons across different studies. Another challenge is that the choice of the optimal FUS modality to improve ICI immunotherapy may depend on tumor type.

The biological mechanisms of each FUS-enhanced ICI therapy remain to be revealed. The reported HIFU thermal ablation-enhanced therapy required adjuvants to provide sufficient antigen cross-presentation and cross-priming for CD8 T cells. In contrast, HIFU mechanical ablation alone was sufficient to

effectively stimulate antitumor immune responses to enhance ICI therapy. There was only one report on HIFU hyperthermia-enhanced ICI therapy. UTMD has great potential to improve ICI therapy through targeted and controlled release of therapeutics, sonoporation, and mechanical disruption of the blood vessels and tumor tissue. SDT induces tumor cell disruption only at the FUS-targeted site, resulting in reduced toxicity. Further investigations are warranted to better understand the biological mechanisms of each combination therapy.

FUS-enhanced ICI therapies have already undergone early stage clinical evaluations. Currently, two clinical trials have begun to evaluate the combination of HIFU thermal ablation with pembrolizumab ( $\alpha$ PD-1) for the treatment of various advanced solid tumors, such as melanoma, breast cancer, and Merkel cell carcinoma (ClinicalTrials.gov Identifier: NCT04116320 and NCT03237572). The primary outcome will assess a change in the CD8/CD4 T cell ratio in the ablation zone, and the secondary outcome will assess adverse events. Meanwhile, one clinical trial has started evaluating the use of UTMD to enhance the permeability of the blood-brain barrier without causing vascular damage to facilitate the delivery of nivolumab ( $\alpha$ PD-1) to melanoma metastases in the brain and boost immunity in the brain (NCT04021420).

For the successful clinical translation of FUS-enhanced ICI therapy, we need strong collaboration between ultrasound

engineers and immunologists. Ultrasound engineers can optimize FUS parameters to induce the optimal biological effects that effectively induce antitumor immune responses with minimized side effects. Immunologists can better characterize the resulting antitumor immune responses and therapeutic outcomes. Through appropriate tuning of FUS exposure conditions and comprehensive immunological characterization, the prospect of unmasking the utility of FUS with ICI therapy could be attainable.

## AUTHOR CONTRIBUTIONS

HC conceived the outline of the review article and edited the manuscript. JY collected the literature, wrote the initial manuscript, and revised the manuscript. DY and SC revised the manuscript. All authors proofread and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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