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Ultrasound in Tumor Immunotherapy: Current Status and Future Developments

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Abstract

Immunotherapy has considerable potential in eliminating cancers by activating the host's own immune system, while the thermal and mechanical effects of ultrasound have various applications in tumor therapy. Hyperthermia, ablation, histotripsy, and microbubble stable/inertial cavitation can alter the tumor microenvironment to enhance immunoactivation to inhibit tumor growth. Microbubble cavitation can increase vessel permeability and thereby improve the delivery of immune cells, cytokines, antigens, and antibodies to tumors. Violent microbubble cavitation can disrupt tumor cells and efficiently expose them to numerous antigens so as to promote the maturity of antigen-presenting cells and subsequent adaptive immune-cell activation. This review provides an overview and compares the mechanisms of ultrasound-induced immune modulation for peripheral and brain tumor therapy, even degenerative brain diseases therapy. The possibility of reversing tumors to an immunoactive microenvironment by utilizing the cavitation of microbubbles loaded with therapeutic gases is also proposed as another potential pathway for immunotherapy. Finally, we disuss the challenges and opportunities of ultrasound in immunotherapy for future development.

Keywords: immunotherapy, ultrasound, microbubbles, tumor microenvironment

1. Cancer treatment: a general understanding

Cancer is a highly heterogeneous and complex disease that causes the highest mortality rates globally [1, 2]. The increasing morbidity and mortality of cancer are prompting global approaches for its effective control and treatment. Cancers are usually initiated via the loss of control of optimal growth processes such as cell proliferation, differentiation, and cell death [3]. Tumors influence their neighboring normal cells and the surrounding blood vessels to build a tumor microenvironment (TME), which is a special area characterized by innutrition, ischemia, hypoxia (<10 mmHg), acidity (pH 6.5–6.8), and inflammation [4]. The innutritional and ischemic states in the TME mean that tumor needs new blood vessels for growth (i.e., angiogenesis). The newly formed blood vessels are leaky and highly irregular, and are usually characterized by a low blood flow [5]. In addition, the permeability around a tumor is lower than that of its hypoxic core, leading to an increased interstitial fluid pressure (IFP) in the tumor [5, 6]. Hypoxia is a trigger for tumor progression and metastasis to occur via the key mediators: hypoxia-inducible factors (HIFs) [7]. Cancers show specific biological properties, including sustaining proliferative signals, silencing growth suppressors, inhibiting cell death, reprogramming metabolism, promoting angiogenesis, inducing invasion/metastasis, and escaping the immune system [8].

Many cancer treatment methods are based on the molecular mechanism of cancer pathogenesis for improving or eliminating these tumor-physiological factors. The traditional therapeutic modalities for cancer comprise surgery, radiation, and chemotherapy. Surgical removal is the first option for eradicating solid tumors, but its use is limited to only a proportion of patients who are in the early stage of certain cancers. Radiation involves the use of high-energy radiation (electrons or protons) or

high-energy electromagnetic waves (X-rays) to induce cell death. However, radiation therapy not only kills cancer cells but also damages nearby healthy cells. Chemotherapy involves systemic treatment with cytotoxicity drugs and usually causes undesirable or even toxic side effects. Moreover, these traditional cancer treatments often fail or result in relapse due to the therapeutic agents being insufficient to properly treat the tumors, while the TME may also compromise the efficacy of these therapeutic agents [9, 10].

Fortunately, recent advances in immunology provide powerful approaches for achieving cancer suppression. Cancer immunotherapy can powerfully target and fight cancers by manipulating the patient's own immune system [11, 12]. Immunotherapy also results in the immune system learning to track cancer cells via immune memory, thereby reducing the likelihood of cancer recurrence.

1.1. Immunosuppressive TME

Cancers can cleverly circumvent the normal immune system and escape further immunosurveillance via different processes, such as the dysregulation of antigen-presenting-cell (APC) subsets, the disturbance of co-stimulatory/co-inhibitory molecules, and the alteration of effector/suppressor T-cell ratios [13]. The normal immune system consists of two subsystems: innate and adaptive. The innate immune system includes dendritic cells (DCs), macrophages, natural killer (NK) cells, and neutrophils. This system represents the first line of defense, but it is a nonspecific mechanism. The adaptive immune system, which is composed of T and B cells, is involved in antigen-specific immune responses, and exhibits immune memory that makes the subsequent response against a specific antigen more effective.

The latent cancer cells are usually recognized by effector cells of the immune system, such as DCs, macrophages, NK cells, neutrophils, cytotoxic T lymphocytes (CTLs), and helper T (Th) cells. These cells secret inflammatory cytokines, including interleukin (IL)-12 and interferon (IFN)-γ, and then eliminate the immunogenic cancer cells [14]. Macrophages are described as having two main phenotypes: classically activated (M1) and alternatively activated (M2). During the early stage of cancer, DCs and M1 macrophages rapidly act as APCs to present the major histocompatibility complex (MHC) proteins activating effector immune cells such as CTLs and type 1 Th (Th1) cells [15].

Unfortunately, some cancer cells evolve to escape these immune defense mechanisms. During the carcinogenesis process, cancer cells produce anti-inflammatory cytokines (IL-10 and tumor growth factor-β) to suppress APCs. Meanwhile, M2 macrophages and type 17 Th (Th17) cells that have been shown to be induced in the TME can facilitate cancer survival, exhibit inflammatory consequences, and help the evasion of the immune system [14]. In addition, the TME also elicits and re-educates macrophages toward an M2-like polarization; that is, tumor-associated macrophages (TAMs) [16, 17]. Cancers are able to further recruit various immunosuppressive cells including regulatory T cells (Tregs), M2 TAMs, and myeloid-derived suppressor cells (MDSCs) to reduce immune properties in the TME [17, 18]. According to the understanding of the abnormal immune system caused by cancers, increasing numbers of immune-related therapeutic strategies have recently been developed that have shown encouraging clinical responses. There is increasing evidence that immunotherapy could be a highly specific, effective, and durable treatment strategy for treating or even curing cancer patients.

1.2. Cancer immunotherapy

Successfully inducing immune responses for cancer immunotherapy requires the concurrent triggering of two immunal phases [19]. Initially, to activate the immune response, the MHC molecules of APCs need to be triggered and bind to T-cell receptor (TCR) complexes of CD4+ or CD8+ T lymphocytes. Meanwhile, this also requires other co-receptor molecules to together promote the cascade immune response. The primary MHC molecules and TCR interactions without concurrent co-receptor interactions (e.g., CTLA-4 co-inhibitory signaling or CD28 co-stimulatory signaling) would fail to elicit a cascaded anticancer immune response due to the interruption of the T-cell activation differentiation to direct the antitumor response or cellular apoptosis [20].

There are several categories of cancer immunotherapies, including immune checkpoint inhibitors, chimeric antigen receptor (CAR) T cells and other cellular therapies, and cancer vaccines [22]. The immune checkpoint inhibitors, including anti-cytotoxic T-lymphocyte-associated protein (CTLA-4) and anti-programmed cell death protein 1/programmed cell death protein ligand 1 (PD-1/PD-L1) antibodies, have been shown to be clinically effective for cancer treatments. The adoptive transfer of engineered CAR T cells has also been shown to produce clinically effective responses, particularly when treating hematological malignancies [21]. Cancer vaccines have been developed and approved to treat specific cancers such as those of the cervix and the head/neck regions [22]. The clinicaltrials.gov database currently includes data from >5,200 active trials that are testing immunotherapeutic pipeline drugs [23].

Recent researches have shown that most cancer immunotherapy strategies have important limitations and challenges, including safety, target effects, off-target side effects, inflammation and autoimmune reactions, and extremely high costs. How to address these problems of immunotherapy is an urgent unmet need for their clinical application. The current trend is to study a combination therapy that enhances anticancer immune responses by combining immunotherapy and other physical fields. Several physical modalities such as radiosurgery, photodynamic therapy, and ultrasound (US)-related therapy have been shown to be useful for improving cancer therapies [24]. Radiotherapy can induce the death of cancer cells, release cancer-associated antigens (neoantigens), and then recruit DCs and activate the immune system. However, radiotherapy also recruits immunosuppressive factors such as Tregs, anti-inflammatory cytokines, and inhibitory signals on cancer cells and immune cells [25]. Photodynamic therapy could induce the production of neoantigens, the expression of heat shock proteins (HSPs), and the invasion and infiltration of leukocytes into the TME; paradoxically, it also induces various forms of immmunosuppression [26]. Thus, radiotherapy and photodynamic therapy might be insufficient to trigger effective anticancer immune responses owing to the limitations of lymphocyte subtypes and the existence of immunosuppressive factors.

2. Physical mechanism of US-mediated TME regulation

There is a long history of applying US to elicit various therapeutic bioeffects in medical applications via the noninvasive energy delivery to a region of interest in the living body without disrupting surrounding tissue. Several recent clinical and preclinical researches have demonstrated the potential immunoactive effects of US for use in antitumor applications. The basic principle behind US-related immunotherapy

primarily involves the following mechanisms: (1) thermal effects via the continuous deposition of US energy in a small region; (2) mechanically fractionating tissue with short-duration bursts of high-amplitude US waves, which is called boiling histotripsy; and (3) damaging tissue via microbubble (MB)-assisted cavitation effects. The schemes and related bioeffects of US-induced antitumor immunity are described in detail in this section (Fig. 1).

2.1. US-induced thermal effect

US can be used to produce hyperthermia in tumors without causing ablation damage, such as by maintaining a temperature of ~43°C for 30–60 min. This condition will trigger several antitumor immune responses. Bandyopadhyay et al. demonstrated that treating B16 melanomas with US hyperthermia activated DCs and then increased CD4+ T-cell activation, thereby hindering tumor-induced T-cell tolerance [27]. Heating treatment also induced overexpression of glucose-regulated protein-75 & 78 and HSP-72 & 73 in prostatic cancer cells [28, 29]. Meanwhile, US hyperthermia can promote the release of Th1 cytokines (IL-2, tumor necrosis factor (TNF)-α, and IFN-γ from tumor cells, but down-regulates the number of Th2 cytokines (IL-4, IL-5, and IL-10) released from tumor-infiltrating T lymphocytes [28]. A study using the choroidal melanomas model showed that US hyperthermia can induce inverted CD4+/CD8+ T-cell populations, resulting in a normalization of the T-cell subset ratios [30].

Further increasing the US energy using high-pressure continuous waves can induce the rapid production of viscosity-generated heat to further increase the temperature. This allows US to be utilized as a thermal ablation tool at temperatures of >60°C to yield local or systemic antitumor immunity via different biological pathways. For instance, the number of CD4+ lymphocytes and the ratio of

CD4+/CD8+ lymphocytes within the circulation are increased after US ablation [31]. In the B16F10 melanoma model, US ablation was found to inhibit the expression of CD86 on B16F10 cells (down-regulation of miR-134), leading to increases in IFN-γ and TNF-α within the circulation. The intravascular B16F10 cells and metastatic pulmonary nodules were decreased by these effects, thereby prolonging mouse survival [32]. Moreover, US ablation was also found to induce IFN-γ and TNF-α secretion in an H22 hepatic tumor model [32]. It has additionally been found that CTLs were activated concurrently by US treatment, thus inducing antitumor cellular immune responses [33]. Using the same model with similar acoustic parameters, another study proved that the number of mature DCs and the secretion of IL-12 and IFN-γ could be significantly increased after US ablation treatment [34], continuningly tirggers upregulation of CTLs.

The US-ablated tumor debris could also facilitate vaccine delivery conferring specific protective immunity. Bone-marrow-derived immature DCs primed with US-ablated tumor debris produced an obvious increase in the number of mature DCs and the secretion of IL-12 and IFN-γ by CTLs [34]. Nonetheless, the up-regulated expressions of MHC-II, CD80, and CD86 were observed, suggesting that US-ablation-generated vaccines could improve tumor immunogenicity [35].

2.2. US-induced mechanical destruction effect

In recent years, several groups have investigated the use of US to mechanically destroy tissue without causing coagulative thermal damage. This so-called histotripsy technique uses US waves in short bursts (lasting from micro- to milliseconds) at high pressures (>15 MPa) and with a low duty cycle (<5%) that induce mechanical effects at the focal point to fractionate the target tissue into its subcellular components [36]. The mechanical effects of US include (1) boiling the target tissue to produce

millimeter-sized vapor bubbles within several milliseconds, with the subsequent bubble oscillation and collapse to disrupt tissues by mechanical fractionation [37], and (2) the production of a dense cloud of vapor bubbles whose interaction with US will induce a shock wave that can mechanically disrupt cells into a homogenate of subcellular debris [38]. Compared with thermal ablation, histotripsy can provide more-precise targeting of the tumor region and sparing of the surrounding normal healthy tissue by avoiding thermal diffusion to surrounding tissue and blood-flow-induced heat perfusion [39]. Furthermore, the tissue debris induced by histotripsy is likely to be absorbed due the physiological healing response, in contrast to thermal-ablation-induced lesions becoming fibrous scar tissue [40]. These advantages of histotripsy have led to increasing interest in its use in antitumor immunity applications.

Schade et al. demonstrated that histotripsy can immediately activate immunological responses that last for up to 48 h when treating renal cell carcinoma in the Eker rat model [41]. Those authors found the near-immediate and transient release of the damage-associated molecular pattern high mobility group box (HMGB)-1 into the plasma, which was attributed to triggering an inflammatory cascade. The increased infiltration of CD8+ T cells could also be observed at 48 h post-treatment, indicating the initiation of a systemic adaptive immune response. In a model of human breast adenocarcinoma cells, histotripsy stimulated the immunogenic cell death of cancer cells via a TNF-induced necrosis signaling pathway [42]. This immunogenic cell death promoted the secretion of damage-associated molecular patterns (calreticulin, HSP-70, and HMGB-1), pro-inflammatory cytokines (IFN-γ, IL-1α, IL-1β, and IL-18), and chemokines (IL-8) that are associated with the activation of M1 macrophages. In addition, the enhancement of these signaling proteins shows directly proportional to the severity of damage induced by histotripsy. Some studies

have suggested that US-induced mechanical effects induce stronger immune responses due to the absence of denatured antigenic proteins at the US focus an *in situ*, which can further enhance immune reactions [39]. Hu et al. found that histotripsy could increase CD11c+ cells by 1.3-fold and DCs by 2-fold in draining lymph nodes compared to in thermal-US groups [43]. These results demonstrate the feasibility of applying histotripsy-mediated immunostimulation against tumors.

2.3. US–MB interactions to induce cavitation

US may also be applied to generate mechanical bioeffects by co-administering MBs to trigger acoustic cavitation effects [35, 44]. The US pulsing method in MBs cavitation is different than in histotripsy reported, where the pulse duration are about 1000 times shorter as well as acoustic pressures are about 2 folds higher. The high compressibility and acoustic impedance of MBs causes their volume to oscillate periodically (stable cavitation) or violently collapse (inertial cavitation) during the oscillatory positive and negative pressures of US (Fig. 2) [45]. In stable cavitation, the repetitive contraction and expansion of MBs induced by US will induce the flow of liquid around the MBs. This so-called microstreaming applies shear stress to cells, resulting in the transient permeabilization of cell membranes (i.e., sonoporation) [46]. In inertial cavitation, the excessive US pressure causes MBs to collapse and produces strong mechanical stresses, shock waves, and micro-jets [46], leading to irreversible cellular injury or tissue destruction [47].

Using US with MBs (US–MBs) has recently been investigated for the noninvasive, local, and transient enhancement of blood-tissue drug delivery for therapeutic applications [48, 49]. In a K1735 model of melanoma, disruption of the tumor vasculature by US–MBs could generate direct cytotoxicity via hemorrhagic necrosis to include ischemia-mediated cytotoxicity, increasing the infiltration of

CD45+ and CD3+ cells into tumors [50]. In the murine CT26 colon carcinoma model, the increased tumor permeability resulting from the stable cavitation of MBs would improve the infiltration of non-Tregs and CD8+ CTLs, with tumor growth also being inhibited by the enhanced antitumor immunological response [51]. These unique characteristics have been utilized to pharmacologically modulate tumor permeability and deliver immunorelated bioactives for immunotherapies.

3. Immunotherapy assisted by US–MBs

3.1. US-stimulated MB destruction for immunoactivation

US-stimulated MB destruction (USMD) induces the inertial cavitation of MBs to produce violent mechanical forces that damage endothelial cells, resulting in effective antivascular therapy (Fig. 2) [52, 53]. Such antivascular therapy disrupts the fragile vessels of tumor and produces a large amount of cellular debris to be tumor antigens. The therapy-induced inflammation increases the availability of tumor antigens for activating immune cells that assist the tumor therapy. The antivascular agent DMXAA (5,6-dimethylxanthenone-4-acetic acid) blocks tumor perfusion to induce high levels of TNF-α, which in turn activates the release of immunostimulatory cytokines and chemokines by M1 TAMs to promote the infiltration of CD8+ T cells [54, 55]. The physical antivascular therapy produced by USMD also reduces vascular endothelial growth factor (VEGF) and increases TNF-α expression, thereby providing the possibility of regulating intratumoral activation of the immune system. Hunt et al. evaluated tumor perfusion and intratumoral immune system activation after USMD in a murine melanoma model [50], and found that the antivascular effect generated direct cytotoxicity from hemorrhagic necrosis to increase the infiltration of CD45+ and CD3+ T cells for activating the immune system.

The physical antivascular effects of USMD can not only cause tissue necrosis to activate immune responses, but also produce numerous antigens to induce the maturity of APCs. Zhang et al. investigated the *in vitro* activation and suppression of DCs after USMD [56]. Murine prostate cancer cells were disrupted by USMD and co-cultured with DCs. In the VEGF-inhibited TME, the migration ability of tumor cells was inhibited and the proliferation of DCs and CTLs was increased. The antigens produced by USMD can promote the maturity of DCs to activate CTLs for immunotherapy. Bulner et al. combined antivascular USMD treatment with the anti-PD-1 checkpoint inhibitor for immunotherapy in murine colon cell carcinoma [57]. The combined therapy produced more tumor necrosis and growth inhibition than when USMD or anti-PD-1 was applied alone. Although the counts of CTLs and Th cells from the tumor-draining lymph nodes did not increase significantly, the enhanced IFN-γ expression improved the activation of T cells. These observations indicate that the T-cell-dependent mechanisms induced by antivascular USMD should be further evaluated with the aim of enhancing antitumor immunity.

3.2. US–MBs for enhancing delivery

3.2.1. Facilitating monoclonal antibody permeation for tumor vascular normalization

Monoclonal antibody therapy is one of the key types of immunotherapy for treating tumors. A monoclonal antibody can specifically bind to target cell ligands and trigger the host immune response, and this approach has been applied to various tumors. However, antibody-based immunotherapy is rarely curative in solid tumors due to the obstacles of the TME (e.g., high IFP, large separation between vessels and tumor cells, and high complexity of the extracellular matrix) preventing antibodies being delivered from the blood to tumor cells [58-60]. US–MBs has been shown to change the vascular

integrity in a way that facilitates the delivery of therapeutic agents into the vascular walls of tumors [61]. The use of US–MBs has recently been investigated for improving the delivery of antibody in oncology [62]. In a head and neck squamous cell carcinoma model, US–MBs could increase the intracellular uptake of cetuximab (a monoclonal antibody of epidermal growth factor receptor [EGFR]) by 30%, and decrease the tumor size by the same amount compared to the cetuximab-alone group [63]. The combination of an anti-PD-1 antibody and US–MBs treatment has the potential to significantly enhance antitumor effects compared to control treatment in a colorectal cancer cell model [57]. However, the specific mechanisms underlying the antitumor enhancement effects remain unclear.

Since the HIF-1α/VEGF pathway contributes to immune suppression in the TME, antiangiogenic monoclonal antibodies might activate antitumor immunoactivity for suppressing tumor growth. Over the past 2 decades, the concept of tumor vascular normalization (VN) has been proposed for changing abnormal tumor vessels to the normal phenotype during antiangiogenic therapy [64-67]. The normalized tumor vessels with a mature and functional morphology will facilitate the repair of the malignant TME by enhancing blood perfusion and oxygen (O_2) delivery while reducing IFP, hypoxia, and metastasis [68-70]. Since tumor VN improves oxygenation so as to prevent hypoxia, impairment of the HIF-1α/VEGF pathway will reverse the immunosuppressive TME (i.e., "cold" tumor) into an immunoactive TME (i.e., "hot" tumor). Shrimali et al. disrupted VEGF/VEGFR-2 (VEGF receptor 2) signaling to significantly improve the transfer of activated T cells within B16 tumors via VN [71]. The normalized tumor vessels enhanced blood perfusion to assist the extravasation of T cells into tumors and improve the inhibition of tumor growth by immunotherapy. Huang et al. induced VN in murine breast tumors using an anti-VEGFR-2 antibody [72]. The normalized tumor vessels improved O_2 delivery to

reduce hypoxia, which could polarize TAMs from an M2- to an M1-like phenotype, and then facilitated the infiltration of Th cells and CTLs for immunoactivation. Chen et al. used erlotinib (an inhibitor of EGFR) to induce VN in 4T1 murine breast tumors, CT26 colorectal tumors, and SCC7 squamous cell carcinoma tumors, and then investigated the changes in the immunosuppressive TME [73]. The enhanced tumor oxygenation during VN reduced IL-10 but increased IL-12 secretion to demonstrate the polarization of M2 to M1 TAMs. Combining erlotinib with anti-PD-L1 for immunotherapy resulted in tumors exhibiting significant increases in the infiltration of CTLs and the levels of the cytokines IL-12p40, IFN-γ and TNF-α.

These findings indicate that tumor VN can enhance blood perfusion and reduce IFP to promote the delivery, penetration, and accumulation of O_2, drugs, and immune cells. The inhibition of angiogenesis and hypoxia can reduce the immunosuppressive cells (M2 TAMs, MDSCs, and Tregs) and activate antitumor immune cells (Th cells, CTLs, and M1 TAMs), and thereby reprogram tumors into an immunoactive TME for assisting immunotherapy [74, 75].

3.2.2. Facilitating cytokines encoding pDNA expression

Cytokine gene therapy is an attractive type of cancer treatment because the cytokine would be continually secreted from the transfected cells for initiating several antitumor immune responses [76, 77]. Such a cancer gene therapy approach requires the ability to transfer genes into tumor cells via easy, safe, and noninvasive routes. MBs-mediated permeabilization of cell membranes is expected to be useful for developing noninvasive and nonviral gene delivery systems. MBs have previously been used to transport the cytokine IL-27 encoding pDNA in three different murine models of prostate cancer: RM1, TRAMP-C1, and TRAMP-C2 [78]. The cytokine pDNA and MBs were co-injected intravenously, and then US was performed. Three

types of umors were treated three times with an interval of 2 days, and showed a significant inhibition of tumor growth. Moreover, this treatment also activated the immune system, as evident from the improved infiltration of CD3+ and CD8+ cells within the tumor.

In a hepatocellular carcinoma model, combining IFN-β pDNA, MBs, and US improved IFN-β expression and clearly reduced the cell viability [79]. The *in vivo* results showed a significant decrease in tumor growth after treatment. Suzuki et al. successful applied a novel type of liposomal bubble to transfect IL-12 encoding pDNA with US in an animal model of OV-HM tumors [80]. The local production of IL-12 would activate the invasion of CD4+ and CD8+ T cells, finally suppressing the tumor growth.

3.2.3. Modulating the expression of antigens and adaptive immune cells

DC-based immunotherapy has emerged as a potent antitumor strategy because the cells are able to prime and activate CTL and Th cell responses [81]. DCs are also suitable as vaccine carriers for cancer immunotherapy [82]. The tumor-specific CTL response elicited by DCs could be further improved by abundantly presenting tumor-associated antigens within DCs. Suzuki et al. directly transferred the tumor-associated antigen ovalbumin (OVA) into DCs using combined treatment with bubble liposomes, US, and antigen [83]. Their results demonstrated that exogenous antigens can still be recognized as endogenous antigens. Immunization with these DCs could also efficiently induce OVA-specific CTLs and act against E.G7-OVA tumors. The melanoma-derived antigen could be delivered into DCs using a similar technique [84]. The immunotherapeutic potential of these antigen-loaded DCs was also verified in an *in vivo* murine model of lung cancer metastasis.

Genetic vaccination using tumor-specific antigen-coding genes has emerged as a potent antitumor strategy [85]. However, effective genetic vaccine therapies require genes to be transferred selectively and efficiently into APCs. Un et al. developed mannose-modified bubble lipoplexes as gene carriers for transfecting targeted genes into APCs with transdermal US [86]. They found that the luciferase expression in splenic CD11c+ cells and non-parenchymal liver cells could be increased by from 500- to 800-fold using this method. Using pDNA-encoding OVA as a model antigen, they also showed that three immunizations produced a large amount of IFN-γ, enhancing the differentiation of Th cells into Th1 cells. This led to CTL activation with highly specific antitumor activity against OVA-expressing cells. In tumor models of E.G7-OVA and EL4 cells, the tumor volume decreased 4.5-fold after treatment and the antitumor effects could be maintained for at least 80 days. This method of DNA vaccination also exerted positive effects in a relapsed murine B16BL6 melanoma model [87]. Un et al. found that the CTL activities and the secretion of Th1 cytokines (i.e., IFN-γ and TNF-α) were improved after immunization by bubble lipoplexes that had been loaded with melanoma antigens encoding pDNA (i.e., gp100 and tyrosinase-related protein 2) using US.

Temmerman et al. demonstrated that combining mRNA-lipoplex-loaded MBs with US was efficient for transfecting mRNA encoding luciferase in DCs [88]. The luciferase activity within DCs could be detected at 8 h after transfection, and gradually declined with time. Although those authors did not apply this tool for antitumor treatment, they observed that the cell viability and cell maturation capacities did not change after transfection, suggesting that it could be used in immunotherapy applications. Loading both antigen mRNA and immunomodulating TriMix mRNA onto MBs can also be used for the US-triggered transfection of DCs [89]. *In vivo* experiments with *in vitro* sonoporated DCs showed the effective

induction of antigen-specific T cells, resulting in the specific lysis of APCs. In addition, complete tumor regression was observed in 30% of the animals vaccinated with the antigen and TriMix DCs, which also displayed a long-term antigen-specific immunological memory. These results indicate that DC sonoporation using MBs loaded with antigen and TriMix mRNA can elicit powerful immune responses, and might be a useful tool for further *in vivo* DC-vaccination applications.

3.2.4. Promoting tumor reoxgyenation and VN

O_2 is one of the most important gases for maintaining the survival of organisms, and it is widely used for normal tissue repair and TME regulation [90-92]. The presence of immature and dysfunctional vessels in tumors will reduce the efficiency of O_2 transport, inducing hypoxia [93, 94]. Activation of the HIF-1α/VEGF pathway contributes to immune suppression by increasing the recruitment of Tregs, MDSCs, and TAMs within tumors [95]. Hyperbaric O_2 therapy is the most-common clinical gas-based therapy for wound healing, ischemic tissue necrosis, and hypoxic tumors [96]. The enhancement of tumor oxygenation by hyperbaric O_2 therapy repairs the hypoxic immunosuppression and then modulates the maturity and function of immune cells [97].

MBs are composed of a biocompatible shell and inner gas core, and hence they represent a suitable structure for carrying specific therapeutic gases such as O_2 [91, 98]. The release of gas inside MBs can be triggered by US, thereby increasing the efficiency of local gas therapies. O_2-loaded MBs (O_2-MBs) have been demonstrated to enhance tumor oxygenation and improve the efficacy of radiotherapy by inhibiting hypoxia [99-101]. Eisenbrey et al. used US–O_2-MBs to increase the O_2 partial pressure in tumors by 19.7±9.1 mmHg (mean±SD) and prolong animal survival by a mean of 30 days after radiotherapy [99]. Khan et al. demonstrated the *in vitro*

degradation of HIF-1α by lipid-shelled O_2 nanobubbles [102]. Moreover, Ho et al. proposed that US–O_2-MBs can induce tumor VN by inhibiting the HIF-1α/VEGF pathway [103]. Tumor oxygenation, perfusion, vessel maturity, and drug penetration were all enhanced by tumor VN that occurred after US–O_2-MBs. Since enhanced tumor oxygenation can reverse an immunosuppressive TME into an immunoactive TME, US–O_2-MBs-induced tumor VN provides a potential pathway for immunomodulation.

3.2.5 Potential for other gases in tumor immunomodulation

US with O_2-MBs can locally deliver O_2 to prevent tumor hypoxia and induce tumor VN, which provides a potential way to regulate the TME for immunotherapy. Moreover, other therapeutic gases including nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO) can regulate cellular morphology and metabolism to change the TME and hence also immune responses. NO is an endothelium-derived relaxant that can induce vessel dilation to enhance tumor oxygenation by increasing blood perfusion. High levels of NO can activate M1 TAMs to significantly reduce tumor metastasis and improve the efficacy of immunotherapy [104]. H_2S is a redox regulator with physiological and pathophysiological functions. The immunosuppression of MDSCs could be reduced by H_2S treatment, with T-cell proliferation then being restored to enhance the efficacy of melanoma immunotherapy [105, 106]. CO is an inducer of mitochondrial ROS, which could regulate the biological mechanisms of cancer cells and macrophages. Nemeth et al. showed that CO can regulate the polarization of TAMs in the TME [107]. A low dose of CO (100 ppm) increased the number of M1 TAMs to activate T and NK cells for antitumor immunity, whereas a high dose of CO (250 ppm) increased the infiltration of M2 TAMs for immunosuppression. Since US–MBs provides a simple strategy for local

gas release that will improve and regulate the concentration of therapeutic gases within target regions, this potentially represents a worthwhile strategy for improving the activation of antitumor immunity.

4. US–MBs for brain barrier opening in immune regulation

Glioblastoma multiforme (GBM) is the primary malignant brain tumor in adults. Although various treatment modalities are applied to GBM, treatment outcomes are typically unsatisfactory, with an overall survival time of less than 2 years [108, 109]. While immunotherapy has recently become a key approach in anticancer therapies, so far it has not been fully utilized against GBM [110]. Two critical immune responses—the local cellular and systemic humoral immune mechanisms—can both be hampered by GBM, thereby invalidating the immune response and allowing the disease to progress. In this section we discuss the physical mechanism underlying the US–MBs-mediated permeation of the blood–brain barrier (BBB) or blood–brain tumor barrier (BTB), US–MBs triggered immune system activation via barrier opening, enhancement of monoclonal antibody delivery to the central nervous system (CNS) via US–MBs-induced barrier opening, and cytokine as well as immune-cell delivery via US–MBs-induced barrier opening.

4.1. BBB and BTB opening

GBM has long been understood to have a heterogeneous environment, with the tumor easily infiltrating and being expressed from the primary site as well as progressing. Unlike normal brain tissue, a brain tumor has a highly heterogeneous vascular distribution, with some regions also having a compromised BBB. Brain tumors have been observed to be highly permeable, with the BBB being compromised in the tumor core but retaining a normal function in the tumor periphery [111]. In

addition, brain tumors can reportedly infiltrate normal tissues and seed and migrate to distant regions and continue their progression [112]. Brain tumors are also known to exhibit active efflux effects that prevent therapeutic drugs from penetrating into brain tumor tissues, with this BTB greatly hampering the therapeutic efficacy.

Based on the existence of BBB and BTB heterogeneity in brain TMEs, the following mechanisms also assist GBM blockage or to evade immune responses. The existence of these two barriers hampers the penetration of effective T cells or antibodies into the tumor infiltrating parenchyma. Also, tumors presenting antigens and MHCs are also unlikely to leak into the circulation to trigger the local inflammatory response and consequently trigger an immune response, mainly due to blockage by the BBB or the hypoxia/diffusion-imbalance-related obstacle caused by the BTB [110]. Finally, the antitumor reaction function of CTLs and Th1 cells infiltrating into the brain tumor should also be maintained, with the concurrent cytokine-present environment providing supportive co-inhibitory signaling in the immunotherapy process [113]. GBM has also been reported to express abnormal MHC decoy molecules (termed HLA-G, which are structurally similar to normal MHC molecules) that prevent the priming of CTLs and the immune response [114].

Combined with administering MBs at clinical doses, the pulsed delivery of focused US (FUS) can reportedly locally and transiently open the BBB [115]. The presence of MBs can significantly increase acoustic cavitation under targeted US exposure, to open the BBB via the large biophysical effect of transient tight-junction disruption of the CNS endothelial lining [115]. The integrity of the BBB typically recovers within several hours, but this depends on the US exposure level [116]. Since FUS can transcranially produce a sufficient exposure level at the target position, it is greatly advantageous in permeating targeted brain tissue in a noninvasive manner, and is very attractive when clinically attempting to delivering therapeutic agents into the

deep brain tissues without damaging the intervening normal CNS tissues. FUS-induced BBB/BTB opening not only provides the opportunity of delivering therapeutics into the CNS, but is also potentially useful for modulating brain TMEs in beneficial GBM immunotherapy.

4.2. Immune-related cell activation via barrier opening

Immune-related cell activation via FUS–MBs has received a considerable amount of attention. It is already known that delivering high-intensity FUS to thermally or mechanically disrupt tumor tissue causes tissue necrosis or debris in the localized regions where the energy is deposited [43]. This typically induces proinflammatory molecules and chemokines, and triggers inflammatory responses and hence recruits microglia activation in the brain, which act as macrophages outside the brain [40].

Liu et al. used the SPIO labeling of systematically circulating macrophages to demonstrate that exposure to excessive FUS energy not only opened the BBB but also induced local erythrocyte extravasation. Due to the secretion of chemokines into the blood circulation from BBB opening sites, we were able to observe macrophage aggregation at the FUS exposure location due to local inflammatory signaling with the aid of SPIO-labeled macrophages detected by MRI [117]. The macrophage aggregation was observed to be temporary, lasting 24–48 h depending on the exposure level.

It is also interesting to know that whether FUS-induced BBB opening can trigger CTL activation. It is known that the immune therapy relies on tumor tissue constituting a relatively immune-environment rich (i.e., hot tumor) and hence also that CTL can be activated. Therefore, the approach of blocking the immune system using CTLA-4, PD-1, or PD-L1 anticancer treatment can be both effective and efficient. It

has previously been demonstrated that the ratio of CD8+ cells and Tregs in brain tumors can increase significantly after US–MBs treatment [118]. The CD8+ cell/Tregs ratio typically serves as a critical sign for evaluating the TME (with a higher ratio typically considered to be beneficiary for anticancer immunotherapy), which implies that the TME was regulated by the MB-present FUS pulsation intervention.

4.3. Monoclonal antibody delivery via barrier opening

It has been previously been demonstrated that US–MBs can successfully deliver alkylating-type small chemicals such as carmustine (216 Da) and temozolomide (196 Da) into xenograft glioma models, with promising therapeutic outcomes [119-121]. Although these alkylating agents can already penetrate the BBB, US–MBs treatment can improve drug penetration into the CNS to enhance their therapeutic effects. However, monoclonal antibodies are much larger molecular structures (typically 100 kDa or larger). Kinoshita et al. first presented the concept of using FUS-induced BBB opening to deliver HER-2 antibody into animal brains [122]. A subsequent study continued the concept of delivering HER-2 targeting antibodies by using trastuzumab for treatment in an HER-2-positive breast tumor brain metastasis model [123]. It was found that not all FUS-treated animals responded to trastuzumab, resulting in the FUS-treated group generally not showing the control of tumor progression. However, for the subgroup of animals that did respond to trastuzumab, there was a significant tumor suppression effect when compared with the untreated animals [123].

Another monoclonal antibody, bevacizumab, has also been investigated for FUS-induced BBB opening by targeted delivery to the brain of a glioma cell xenograft murine model [124]. Bevacizumab is an anti-VEGF-A monoclonal antibody that

specifically targets endothelial-cell VEGF-A ligands, and is considered to inhibit angiogenesis. GBM patients initially respond to bevacizumab due this antiangiogenic and VN effect, but its long-term administration did not produce any improvement in the progression-free survival. This is due to the VN effect preventing bevacizumab being continually supplied to the tumor bed, with this antiangiogenesis effect stopping the expected tumor-starving effect. It was previously shown that the weekly administration of bevacizumab combined with FUS-induced BBB opening for a total of 6 weeks significantly improved the glioma progression control and the median survival time [124].

4.4. Cytokine and adoptive immune-cell delivery via barrier opening

IL-12 is a critical element in the immune system that can drive anticancer immune responses, and is typically secreted from B cells, macrophages, and microglia. The expression of IL-12 reportedly benefits the proliferation of T cells [125, 126], with activated T cells also up-regulating IFN-γ to promote IL-12 secretion [127]. Zeng et al. reported that IL-12 directly triggered T-cell-related immune responses to suppress tumor progression in a subcutaneous tumor model [128].

Chen et al. attempted to combine FUS-induced BBB/BTB opening in a xenograft brain tumor model with the intraperitoneally administration of IL-12 to enhance the penetration of this cytokine at the tumor site [118]. The induction of local BBB/BTB opening did not significantly change the local Th, CD8+ cytotoxic cell, or Tregs populations; however, the ratio of the CD8+ cells and Tregs did change significantly after applying pulsed FUS. The preconditioning provided by the i.p. administration of low-dose IL-12 resulted in FUS and IL-12 exerting a synergetic tumor-suppressing effect, with a median improvement in the survival rate of the 50%.

On the other hand, NK cells are phagocytes that present with tumor-specific antigens and can target tumors through the specific binding of the HER-2 antigen. A previous preclinical study using NK cells for treatment in a brain tumor model did not produce a positive outcome, which at the time was attributed to the permeation of NK cells into the CNS being significantly restricted by the BBB/BTB [129]. Alkins et al. attempted to culture SPIO-laden NK-92 human cell lines and adoptively transfer them into an HER-2-positive breast cancer brain metastasis murine model [130]. In FUS-induced BBB/BTB opening groups those authors observed an increase in SPIO-laden NK-cell aggregation of more than 10-fold at the target tumor site in *in vivo* T2-weighted MRI observations and tracking when comparing to NK cells in the adoptively transferred group. The US-treated group also demonstrated better tumor progression than that in the control group.

5. Summary and future development of US in immunotherapy

5.1. Comparison of mechanisms of US-induced immunomodulation

US-induced immunotherapy can generate thermal or mechanical effects to activate immune cells, secrete immunoactive cytokines, or express proteins for reversing an immunosuppressive TME (Table 1). US provides a thermal effect of either hyperthermia or ablation, depending on the heating temperature. Hyperthermia enhances the permeability of vessels to promote the penetration of immune cells within tumors. Well-permeabilized cell membranes under hyperthermia can accelerate antigens presenting on DCs for activating CTLs and Th cells. The increased expression of cytokines (IL-2, IL-12, IFN-γ, and TNF-α) and HSP-70, HSP-72, and HSP-73 explain the immune system activation in the TME after US-induced hyperthermia. On the other hand, US-induced ablation directly disrupts tumor tissue to produce abundant cell debris, and then promotes the maturity of APCs to activate

subsequent adaptive immune responses. The increased temperature during US ablation therapy also enhances blood perfusion to promote the circulation of immune cells and their penetration in the target regions. Silvestrini et al. combined US thermoablation, PD-1 antibody, and Toll-like receptor agonist to accomplish immunotherapy in murine adenocarcinoma [131]. Activated immune cells after primed thermoablative immunotherapy can inhibit the growth of untreated tumors via the abscopal effect. However, tumor ablation causes vessel disruption and tissue necrosis, which inhibits subsequent drug penetration. Cell death due to overheating prevents effective antigens. Moreover, the inflammatory responses due to tumor necrosis increased the number of MDSCs and M2 TAMs to inhibit antitumor immunotherapy. Thus, the time points of US ablation and the delivery of drugs, antibodies, or cytokines for immunotherapy should be arranged to produce a suitable TME for immunoactivation.

The mechanical effect of US is associated with vapor bubble or MB cavitation. The mechanical destruction produced by histotripsy involves high acoustic pressures mechanically fractionating cells and producing gaseous components in tissues without causing thermal damage. The intertissue vapor bubble cavitation damages tumor cells to generate efficient antigens for DC activation and subsequent CTL and Th cell infiltration. Unlike US ablation, the nonthermal effects during histotripsy can maintain the function of cellular debris as efficient antigens for immunoactivation. The elevated TNF-α induces cell death to significantly enhance the secretion of HSP-70, IFN-γ, and IL-18.

On the other hand, the addition of US using MBs as a contrast agent can also generate mechanical effects when the acoustic pressures are significantly lower than those in histotripsy for triggering immune system activation. MB inertial cavitation generates violent mechanical forces to disrupt cells and vessels, producing a large

amount of cellular debris. The maturity of DCs is increased to promote CTLs and Th-cell activation and the release of IFN-γ and TNF-α. Since DCs can be matured by tumor cell debris produced by USMD *in vitro*, there is another way for delivering adaptive immune cells.

While utilizing a low US energy with MBs to produce stable cavitation and induce transient vasodilation and permeability enhancement, the enhanced vessel permeability allows monoclonal antibodies to penetrate into tumor tissue to target tumor growth factors such as EGFR, VEGF, and HER-2 for TME regulation. The improvement in the delivery of cytokines (IL-12, IL-27, and IFN-β) and immune cells (APCs and NK cells) directly activates the invasion of CTLs and Th cells to produce an efficient tumor-suppressing effect. Moreover, the stable cavitation of MBs transiently enhances the permeability of cell membranes via sonoporation, which allows antigens to directly penetrate into the cytosol of APCs. The subsequent administration of mature APCs can induce *in vivo* CTL and Th-cell activation to release cytokines (IFN-γ and TNF-α) for tumor immunotherapy. The promotion of antigens presenting on APCs via sonoporation means that US–MBs provides an efficient way to mature the APCs *in vitro* for adaptive immune-cell delivery.

5.2. US–MBs-induced immunotherapy for other CNS diseases

Besides triggering anticancer immune responses, other CNS diseases may also benefit from the US–MBs treatment strategy. Astrocytes and miroglias in the CNS are crucial regulators of immune responses, and their activities may exacerbate inflammatory reactions or promote immunosuppression, depending on the stimuli [132, 133]. Fortunately, the acoustic parameters for activating astrocytes to release beneficial neurotrophic facts (i.e., BDNF, GDNF, VEGF, and GLUT1) were identified in a rat vascular dementia model and ischemic stroke model [134-136]. In

addition, a number of previous studies have already demonstrated that during FUS-induced BBB opening, the FUS exposure also results in high glial fibrillary acidic protein (GFAP) activity in brain tissue, showing that glial cells have been activated.

It was found that not only GFAP but also Iba1 (ionized calcium-binding adaptor molecule 1) were highly expressed, showing activation of not only astrocytes or glial cells, but also microglia cells [137, 138]. In a transgenic Alzheimer's disease murine model, it was found that this microglia activation triggers β-amyloid internalization [139]. This microglia activation was also confirmed to not be related to neuroinflammation, since inflammation in the CNS is typically attributed to neurodegenerative disease progression such as Alzheimer's disease. Besides, US would induce shear stresses on the endothelial cells of vessels, thereby enhancing HSP-90 expression for inhibiting the aggregation of amyloid plaque and enhancing endothelial nitric oxide synthase activation. Such low-intensity US-mediated immunomodulation represents a new therapeutic approach for Alzheimer's disease.

5.3. US–MBs treatment triggers preinflammatory immune responses

A recent study found that FUS-induced BBB opening would induce a sterile inflammatory response (SIR) in the brain parenchyma via the NFκB pathway, as indicated by elevations of damage-associated molecular patterns (i.e., HSP-70, IL-1, IL-18, and TNF-α) [140]. In addition, proinflammatory, anti-inflammatory, and trophic factors along with neurotrophic and neurogenesis factors were also increased for 24 h. Histological evaluations showed increased albumin, TUNEL+ neurons, astrocytes, microglia, and CD68+ macrophages after treatment. Those authors therefore concluded that the FUS-induced BBB opening induces an SIR, which was similar to ischemia or mild traumatic brain injury. However, McMahon and Hynynen

reported that the occurrence of an SIR was greatly influenced by the MB dose [141]. No up-regulation of the NFκB signaling pathway gene expression was found when administering a clinical safety dose of MBs (for US contrast imaging), whereas the inflammatory response was significantly observed at high dose of MBs. These observation indicate that further investigations are needed (i.e., for optimization of the US and MB parameters) to make sure the biosafety of FUS-induced BBB opening before transfering to clinical trials.

5.4. Clinical trials of US techniques

The novel applications of US–MBs including therapeutics and diagnostics have been tested clinically for the past decades. For example, in a continuing diagnostic clinical attempts, the contrast enhancement of MBs under sonography provides blood perfusion information to trace the treatment outcome of hepatocellular carcinoma after radioembolization (clinicaltrials.gov: NCT03199274). In therapeutic US applications with MBs, tumor ablation induced by high-intensity FUS has been attempted to clinically evaluate breast cancer treatment efficacy (NCT03342625). US hyperthermia enhances tissue permeability which has been applied to combine with chemotherapy to promote drug penetration in breast cancer (NCT03749850). The combined therapy of clinically available sonographic device with MBs and chemotherapy enhanced the treatment efficacy of pancreatic adenocarcinoma and prolonged survival in patients without additional toxicities [142].

On the other hand, since MBs stable cavitation can enhance the permeability of cells and vessels, a number of clinical trials have been initiated recently in utilizing US–MBs interactions to enhance chemotherapeutic drugs/ monoclonal antibodies permeation for cancer therapy (NCT03477019, NCT03458975, and NCT04021277). In addition, the use of low-pressure burst US with the presence of MBs can

temporally open the BBB in CNS, and the clinical feasibility and its potential benefits are under evaluation. The safety, drug delivery, and treatment outcome of BBB opening by FUS-MBs in brain cancers, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis are investigated (NCT03321487, NCT03322813, NCT03671889, NCT03626896, NCT03714243, NCT03739905, NCT03671889, and NCT03608553). The first clinical trials of FUS-MBs mediated BBB opening in human brain diseases (Alzheimer's disease and glioma) were recently completed, with no detectable adverse effects [143, 144]. In reviewing recent clinical trials relating to US-MB, Snipstad et al. summarized recent clinical trials for brain, pancreatic, liver, and breast cancers [145]. Similarly, Chen et al. summarized FUS–MBs for BBB opening to explore the physical mechanisms, existing preclinical findings, and current ongoing clinical trials [146]. More and more clinical trials is accumulating, gaining the knowledge toward the understanding of utilizing US–MBs for cancer therapy and other therapeutic applications, and provides information toward translating US-induced immunoactivation into clinical immunotherapy.

6. Conclusion

US is widely utilized in radiation-free, good-penetration, and low-cost methods for clinical diagnostic examinations and therapies. The thermal and mechanical effects induced by US can regulate the TME via physical stimulation, and then reverse immunosuppresion to immunoactivation for tumor therapy. US ablation, histotripsy, and USMD can directly generate cell debris to promote the maturity of APCs and immunoactive cytokine secretion for increasing the subsequent infiltration of immune cells into the TME. US-stimulated stable cavitation of MBs enhances vessel permeability to improve the delivery of cytokines, antigens, and antibodies for activating antitumor immunity. Moreover, MBs can also carry various therapeutic

gases that can be released at the tumor site, and then regulate the TME and activate antitumor immunotherapy.

This review has shown that the development of US applications in immunotherapy could provide various pathways to accomplish immunoactivation in the TME. The mechanisms of US-induced immunoactivation should be investigated further, especially in TME modulation. US could offer greater clinical advantages including further enhanced antitumor immune responses, reduced side effects, improved treatment efficiency, and enhanced local delivery of immunotherapeutic drugs for clinical immunotherapy.

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Table 1. Immunoactivation and immunosuppression of US immunotherapy.

Immune responses			US only			US–MBs							
			Hyperthermia	Ablation	Histotripsy	USMD	Enhancing delivery			Therapeutic gas			
							Antibody	Cytokine	Antigen	O_2	NO	H_2S	CO
Immunoactivation	Immune cells	CTL (CD3+, CD8+)	↑	↑	↑	↑	↑	↑	↑	↑		↑	
		Th (CD3+, CD4+)	↑	↑		↑	↑	↑	↑				
		Mature DC (CD11c+, MHC II)	↑	↑	↑	↑	↑		↑			↑	
		Leukocytes (CD45+)				↑				↑			↑
		NK (CD11c+, CD3-)							↑	↑			↑
		M1 TAM (CD11b+, CD86+)		↑	↑		↑			↑	↑		↑
		Th1	↑						↑				
	Cytokines	IL-2	↑										
		IL-12	↑	↑			↑	↑		↑	↑		
		IL-18			↑								
		IL-27						↑					
		IFN-γ	↑	↑	↑	↑	↑	↑	↑	↑			
		IFN-β											
		TNF-α	↑	↑	↑	↑	↑		↑	↑			
	Others	HSP 70, 72, 73	↑		↑								
Immunosuppression	Immune cells	Treg (CD25+, CD4+)								↓			
		M2 TAM (CD11b+, CD206+)					↓			↓	↓		↓
		MDSC (CD11b+, Gr-1+)										↓	
		Th2	↓										
	Cytokines	IL-10	↓				↓			↓			
		TGF-B								↓			
		IL-4, 5	↓										
		VEGF				↓	↓			↓			
	Others	HIF-1α					↓			↓			
		EGFR					↓						
		HER-2					↓						
		CTLA-4								↓			
		PD-1, PD-L1				↓	↓						

Figure captions

Figure 1. The immunosuppressive and immunoactive TMEs regulated by US. The thermal and mechanical effects of US promote the activation and infiltration of immune cells for antitumor immunotherapy.

Figure 2. Illustration of immunotherapy assisted by US–MBs: stable cavitation for enhancing delivery or inertial cavitation for immune system activation.

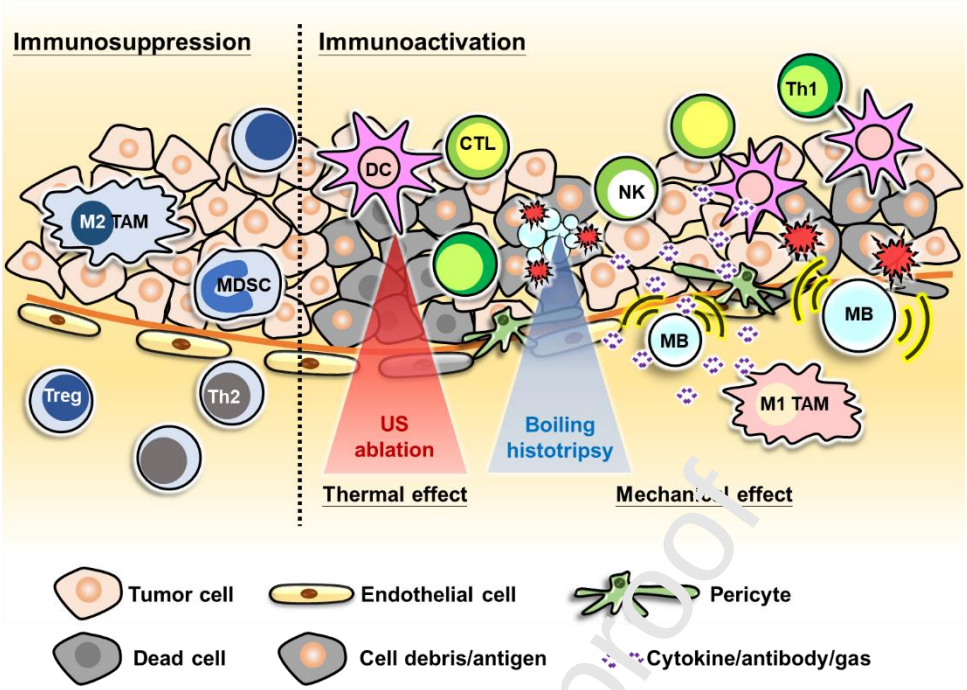

Figure 1

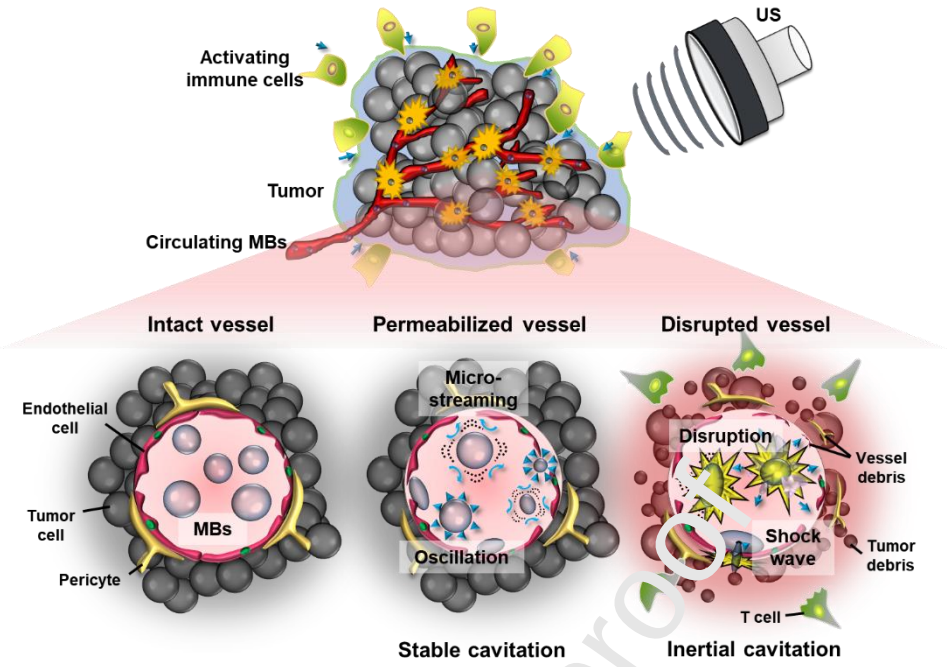

Figure 2

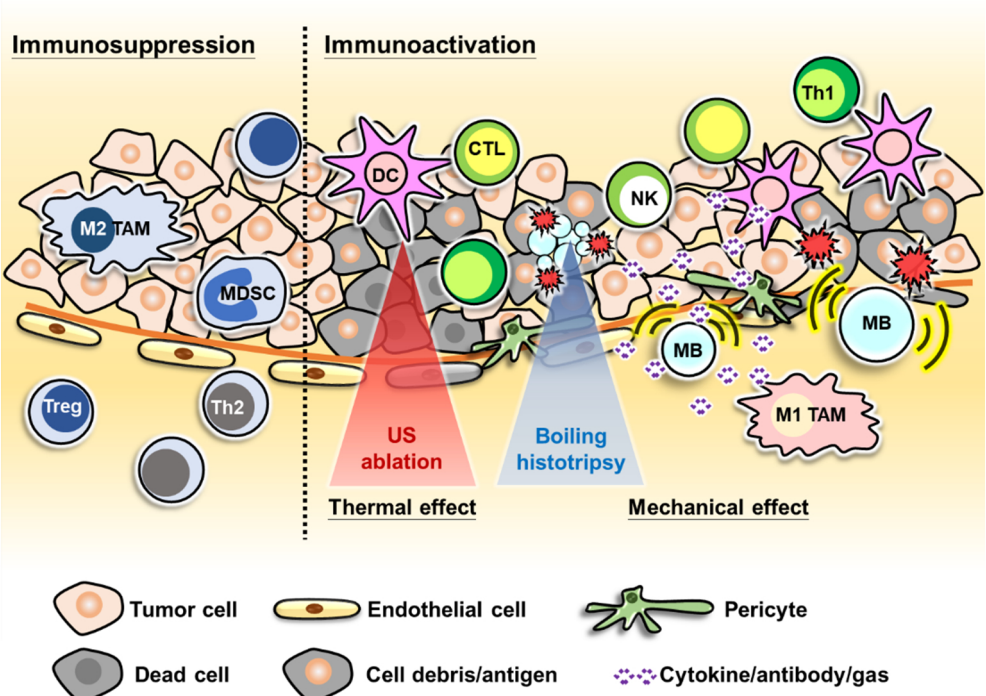
Immunosuppression
Immunoactivation
M2 TAM
MDSC
Treg
Th2
DC
CTL
NK
Th1
MB
MB
M1 TAM
US ablation
Boiling histotripsy
Thermal effect
Mechanical effect
Tumor cell
Endothelial cell
Pericyte
Dead cell
Cell debris/antigen
Cytokine/antibody/gas

Figure 1

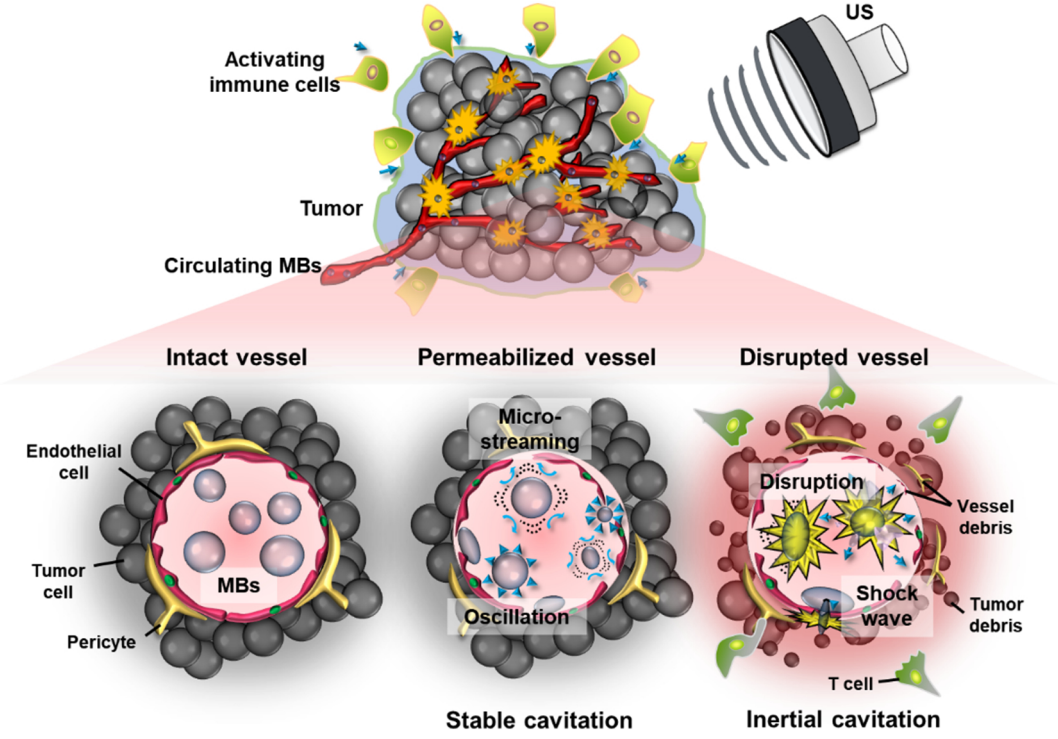
US
Activating immune cells
Tumor
Circulating MBs
Intact vessel
Permeabilized vessel
Disrupted vessel
Endothelial cell
Tumor cell
Pericyte
MBs
Micro-streaming
Oscillation
Disruption
Vessel debris
Shock wave
Tumor debris
T cell
Stable cavitation
Inertial cavitation

Figure 2