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# Synergies between therapeutic ultrasound, gene therapy and immunotherapy in cancer treatment

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## Abstract

Due to the ease of use and excellent safety profile, ultrasound is a promising technique for both diagnosis and site-specific therapy. Ultrasound-based techniques have been developed to enhance the pharmacokinetics and efficacy of therapeutic agents in cancer treatment. In particular, transfection with exogenous nucleic acids has the potential to stimulate an immune response in the tumor microenvironment. Ultrasound-mediated gene transfection is a growing field, and recent work has incorporated this technique into cancer immunotherapy. Compared with other gene transfection methods, ultrasound-mediated gene transfection has a unique opportunity to augment the intracellular uptake of nucleic acids while safely and stably modulating the expression of immunostimulatory cytokines. The development and commercialization of therapeutic ultrasound systems further enhance the potential translation. In this Review, we introduce the underlying mechanisms and ongoing preclinical studies of ultrasound-based techniques in gene transfection for cancer immunotherapy. Furthermore, we expand on aspects of therapeutic ultrasound that impact gene therapy and immunotherapy, including tumor debulking, enhancing cytokines and chemokines and altering nanoparticle pharmacokinetics as these effects of ultrasound cannot be fully dissected from targeted gene therapy. We finally explore the outlook for this rapidly developing field.

### Keywords

Ultrasound; Cancer; Gene therapy; Immunotherapy

## I. Introduction

Ultrasound has been used as a common diagnostic tool in medicine for more than fifty years. Recently, ultrasound therapy has expanded in clinical applications. Ultrasound can be combined with other therapies to treat various diseases, including cancer [1–4]. The ability of ultrasound to simultaneously image and generate local therapeutic effects improves

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spatial selectivity and therefore can reduce systemic toxicity [5]. Here, we will examine the application of therapeutic ultrasound in cancer immunotherapy, with a particular focus on the incorporation of ultrasound-mediated transfection to alter tumor and immune cell phenotypes.

The effect of ultrasound on tissue varies with the acoustic frequency (Figure 1), and therapeutic ultrasound typically employs a center frequency ranging from 20 kHz to 5 MHz. Within the lower frequency range (20–100 kHz) sound waves can destroy kidney stones or coronary artery calcifications [6, 7] and enhance transdermal drug delivery [8, 9], allowing lidocaine, insulin or vaccines to diffuse through the permeabilized skin. With frequencies ranging from 500 kHz to 2 MHz, focused ultrasound enhances drug transport through the cornea [10] and enhances fracture healing [11]. Frequencies above 1 MHz have also been applied in mechanical and thermal high-intensity focused ultrasound (HIFU) for tumor ablation [12, 13]. Some of these treatments have been approved for clinical use, whereas most are in pre-clinical development.

Microbubbles (MBs) are used as contrast agents in diagnostic applications, and MBs have been explored recently in therapeutic protocols [4]. The combination of ultrasound and MBs has been applied for transient blood-brain barrier (BBB) opening [3, 14], thrombolysis [15], and gene transfection [16, 17]. Particularly in the presence of MBs, ultrasound can achieve localized transfection [18]. Ultrasound-mediated gene therapy (UMGT) is minimally invasive, and has been safely applied over a range of frequency (250 kHz - 2.25 MHz) and intensity ($0.1 – 4\ W/cm^2$).

UMGT is typically performed in vivo after systemic or local administration of MBs and nucleic acids. While in vivo UMGT is reported to achieve a 10 to 30-fold increase in transfection efficiency over the application of nucleic acids alone, in vitro results have shown up to several thousand-fold increase in efficacy, motivating numerous studies to further optimize preclinical ultrasound transfection parameters [19]. In comparison to other transfection approaches, UMGT may improve spatial and temporal control, since transfection can be localized, monitored by imaging and titrated as to the dose [20]. In addition, molecularly-targeted MBs can be employed to carry nucleic acids. Molecular targeting enhances UMGT by bringing MBs and genetic materials close to the tumor cells [21].

Importantly, UMGT does not require dedicated therapeutic ultrasound devices. Fully featured, affordable and practical theragnostic devices can be applied in combination with specific ultrasound intensities and protocols [22]. Still, there are limitations of diagnostic equipment in theragnostics. The center frequency, acoustic intensity and pulse repetition frequency operate within limits that were optimized for imaging rather than therapy. Further, the sequencing of pulses between lines-of-sight within an image frame is often difficult to discern and therefore quantification of the acoustic dose is challenging.

Immunotherapy has shifted the paradigm for cancer treatment, and can induce long-lasting responses in patients with a subset of cancers. Immunotherapeutic agents are often used to activate the immune system, or to block immunosuppressive checkpoints in the tumor

microenvironment (TME) [23, 24]. To date, the benefit of immunotherapy has been limited to a minority of patients with specific cancer types, with particular limitations in the treatment of solid tumors. The ability of ultrasound to debulk the TME while enhancing beneficial immune cell populations is particularly attractive. Herein, this review begins with a concise background on gene therapy, the underlying mechanisms of therapeutic ultrasound and the related bioeffects, followed by a summary of the opportunities to integrate ultrasound within gene therapy and immunotherapy protocols. We particularly focus on the intersection of ultrasound, gene therapy and immunotherapy.

## II. Gene delivery

Currently, there are various methods for the delivery of therapeutic genetic materials, including viral and non-viral delivery systems, each with advantages and limitations discussed below (Table 1).

### 2.1 Viral vectors

Viral vectors have been widely applied in clinical transfection protocols due to the fact that viruses efficiently transfer DNA or RNA into host cells. Recently, gene therapy clinical trials have relied on adenoviruses [25], lentiviruses [25, 47] and adeno-associated viruses (AAVs) [48]. Although viral vectors have substantially advanced the field of gene-based immunotherapy, disadvantages and safety issues include potential toxic immunostimulatory effects, varied tropism and difficulties in targeting tissues of interest [28]. Other concerns include limited gene payload capacity, induction of a potentially fatal inflammatory response, carcinogenesis, and insertional mutagenesis [29, 49].

### 2.2 Non-viral delivery systems

Non-viral delivery vehicles and other physical methods have been investigated to provide an alternative to viral systems. Chemical approaches include modified delivery systems that can bind or electrostatically-encapsulate condensed gene constructs to facilitate endocytosis [50]. Delivery systems include liposomes, nanoparticles, polymers and dendrimers [30], and typically incorporate cationic or ionizable lipids, peptides, or polymers to enhance uptake and nuclear localization [4, 51]. Nanoparticles can be designed with a high loading capacity, extended pharmacokinetic profile and controlled release properties to increase the therapeutic index of immunotherapeutic genes and protect nucleic acids from degradation [32]. By modifying surface ligands, nanoparticles can specifically target host cells and can be engineered to release their cargo in response to biochemical changes in the TME or external stimuli [33]. In addition, nanoparticles can be tuned to stimulate immune cells [34]. Furthermore, nanoparticle delivery systems can prevent off-target effects and systemic toxicity, when compared to viruses, reducing dose-limiting toxicity. Molecularly-targeted non-viral delivery systems can selectively activate immunotherapies at the target site [50]. Nevertheless, the transfection efficiency of nanoparticle delivery systems is less than that obtained with viruses [30, 51].

Physical methods, including microinjection [36], biolistic particle bombardment [37], phototransfection [38], electroporation [40, 41] and ultrasound-mediated transfection [22]

induce pores in cell membranes to introduce their cargo into cells. These methods often lead to transient therapeutic gene expression [36–38, 52]. Electroporation can deliver genetic materials by applying short electric pulses that can transiently generate perforations in the cell membrane [40, 41, 52]. This technology was used with cDNA in natural killer (NK) cells, to engineer primary NK cells to express chimeric antigen receptors (CARs) [39] or generate immune cytokines [53]. Stable transgene expression has been achieved with electroporation-based regimens; however, the efficacy is typically lower compared to viral transfection. In some cases, such physical methods are invasive and limited to superficial tissues; this reduces the potential applications [50]. This drawback can potentially be circumvented with ultrasound techniques.

### 2.3 UMGT

UMGT has shown the capacity for delivering genes into cells of interest and includes a variety of applications [43, 54–56]. For example, 1-MHz focused ultrasound enhanced DNA plasmid transfer with polyethyleneimine (PEI) MBs in xenograft sarcoma models [57]. Systemic administration of DNA-cationic lipid complexes followed by the localized application of ultrasound in mice increased transfection, and consequently led to a significant tumor growth reduction [45]. Similarly, in hepatocellular carcinoma models, following the transfection of pre-miR-139 and -378a plasmids, phosphoinositide 3-kinase catalytic subunit alpha (PI3K CA) expression was inhibited, resulting in tumor suppression [58].

Advantages for those entering the field include the site specificity; disadvantages include the currently limited number of preclinical studies and the technical challenges for those trained in the biological sciences. Many parameters impact the resulting efficacy of ultrasound techniques, and the nonlinear oscillation and nonlinear response to input parameters can be difficult for those lacking training in physics to appreciate. For example, while most studies have used low ultrasound frequencies and a relatively small range of parameters, transfection can also be accomplished with higher frequencies. Yoon et al. used 150-MHz (high-frequency) ultrasound to accomplish precise targeting and size-dependent macromolecular delivery with low cytotoxicity [59]. The acoustic pulses perturbed the lipid bilayer of the cell membrane of a targeted cell to induce intracellular delivery of exogenous molecules. Clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease (CRISPR-Cas9) was then used for gene editing [60].

In the following section, as summarized in Figure 2, we explore the mechanisms of therapeutic ultrasound in gene therapy.

## III. Therapeutic ultrasound and its applications in gene therapy and immunotherapy

The key to the versatility of therapeutic ultrasound results from the range of interactions and the titrated and controlled intensity of these interactions. Further development of ultrasound in gene therapy requires a better understanding of these underlying mechanisms in order to plan for future clinical applications. The TME, which not only includes tumor cells, but also

stromal fibroblasts, infiltrating immune cells, blood vessels and the extracellular matrix [61], plays an important role in cancer growth, development and progression [62]. Ultrasound-induced effects spanning each TME component are the foundation for ultrasound-mediated gene transfer. Therefore, here we begin with a brief discussion of the underlying physical mechanisms by which ultrasound results in transfection or alters the biodistribution of therapeutics. Such mechanisms include thermal and mechanical effects. Although pressure oscillations directly affect cells and tissues, secondary effects of insonation can also play an important role and are also described here.

### 3.1 Thermal effects

Ultrasound has the potential to rapidly increase the local temperature due to energy absorption. The impact of ultrasound-induced heating varies with the amplitude and duration of the temperature change. Thermal effects directly alter gene and protein expression [63–65], e.g., heat shock proteins in the TME can influence immunotherapy and cancer therapeutic efficacy [66, 67]. A commonly utilized metric to gauge these effects is the cumulative equivalent minutes at 43°C (CEM43).

$$CEM43 = \int_0^t R^{(43-T)} dt(\text{min})$$

where t represents the treatment time, T is the applied temperature at the target site during the treatment, and R is a constant. The CEM43 reflects the impact of the heat exposure [68]. For a low CEM43 as encountered in hyperthermia, temporary and reversible effects, such as vasodilation or vasoconstriction or a change in pH enhanced immune response [69, 70]. Higher CEM43 can result in permanent changes such as tissue coagulation and cellular effects such as apoptosis, necrosis, immunogenic cell death and cytokine release. A CEM43 thermal dose of 240 or above results in tissue ablation [71]. The presence of MBs or nanodroplets can significantly enhance the energy absorption and consequently the temperature elevation [72, 73]. Clinical adaptation of local hyperthermia has been limited due to the challenge of non-invasive temperature and CEM43 control. Magnetic Resonance Imaging (MRI) is frequently applied for real-time and noninvasive thermometry but the cost limits its usage.

The acoustic intensity in tissue is determined by the pulse duration, instantaneous intensity (directly related to the acoustic pressure), and pulse repetition frequency (PRF) [74]. Intensity can be reported in terms of spatial peak (SP, referring to the region in space where the intensity is maximum) or spatial average (SA, integrating the intensity field and its variations in space) and temporal peak (TP, referring to the maximum instantaneous intensity during the pulse) or temporal average (TA, integrating the intensity in time). For example, the spatial-peak temporal-average acoustic intensity ($I_{SPTA}$, commonly expressed in $W/cm^2$) is defined as the maximum intensity measured at any point in the ultrasound beam averaged over the pulse repetition period and is a good indicator of the magnitude of thermal bioeffects (e.g. a higher $I_{SPTA}$ will result in a greater temperature change). A given temporal-average intensity ($I_{TA}$) can be achieved both with a low amplitude and long pulse with a high PRF (high duty cycle) or a short and high amplitude pulse with a low PRF

(low duty cycle) (Figure 3). In many therapeutic ultrasound papers, the specific intensity measure (e.g. $I_{SPTA}$ vs $I_{SATA}$) is not clearly described and in those cases is described here as intensity or is interpreted from the referenced papers. This is a limitation of current therapeutic ultrasound research.

Energy absorption in tissue is also proportional to the ultrasound frequency, meaning that at a given $I_{TA}$, a higher frequency will yield a greater temperature elevation. The spatial characteristics of the acoustic beam (focal depth and focus size) are set by the transducer dimensions and shape (flat or focused). Focused transducers are generally employed in UMGT to limit exposure to the surrounding healthy tissue. The use of phased arrays adds the ability to move the focus in space under imaging guidance with electronic steering but requires dedicated hardware [75].

### 3.2 Mechanical force and cavitation

Mechanical effects of insonation also play an important role in ultrasound therapy. Cavitation results from the nucleation, growth and collapse of gaseous cavities induced by pressure changes from ultrasound waves [77]. Such cavities can be generated from dissolved gas in the bloodstream. The acoustic pressure induced by ultrasound waves oscillates between compression and rarefaction phases; during rarefaction phases of high amplitude, bubble nucleation can occur (Figure 4A). While high amplitude, short ultrasound bursts propagate through tissue and interact with bubbles, cavitation can be accomplished with minimal thermal elevation. A parameter used to determine the likelihood of cavitation is the mechanical index (MI) [78]. The MI is defined as the maximum value of the peak rarefactional pressure (or peak negative pressure) divided by the square root of the acoustic center frequency. In the presence of MBs, this index has been updated due to the greater effect of center frequency [79]. Injected MBs enhance and localize cavitation activity with the oscillation characterized by high-speed photography of MB oscillation (Figure 4B) [80, 81].

Cavitation regimes include stable and inertial cavitation [77]. Stable cavitation is the sustained oscillation of gas bubbles as the pressure fluctuates, which can result in adiabatic heat generation, microstreaming of surrounding fluid and localized shear stresses [82]. Such cavitation can induce cellular changes such as membrane permeabilization, referred to as sonoporation [83].

When the rate of MB collapse is enhanced by fluid inertia, the collapse velocity is enhanced and the phenomenon is termed inertial cavitation. The energy released during bubble collapse can generate violent shock waves with pressure up to 10,000 atm during a single ultrasound exposure within a duration of <1 μs [84]. Inertial cavitation can cause irreversible tissue lesions, depending on the bubble size range. Both cavitation regimes can lead to substantial physical, chemical and biological effects in surrounding tissues [85].

### 3.3 Endocytosis and therapeutic delivery via sonoporation

A common mechanism behind ultrasonic enhancement of drug or gene uptake involves cavitation-induced transient membrane perforation of target cells, e.g. endothelial cells on the BBB or tumor cells in the TME [87, 88] (Figure 5A). For example, cellular uptake of

hypoxia-inducible factor 1 alpha (HIF-1α) siRNA was increased by 90% in MDA-MB-231 human breast cancer cells following insonation [89]. The concentration of pDNA encoding for luciferase was enhanced significantly in the cytoplasm and not in endosomes as a result of ultrasound, indicating that the internalization can be diffusive and not endocytosis-based.

The MBs used in transfection protocols are commercially available and are approved for use in clinical imaging protocols. Importantly, since MBs act both as contrast agents and transfection potentiators, it is possible to increase the spatial localization of gene delivery by monitoring MB oscillation in tumors through ultrasound imaging guidance [21, 86]. MB-mediated gene delivery achieved localized transfection within the area of ultrasound exposure, and can further refine site-specificity on the cellular or even molecular level. MBs can also be functionalized by conjugating peptide ligands or antibodies on the surface [90]. MB accumulation in tumors was enhanced by surface grafting of ligands such as vascular endothelial growth factor receptor 2 (VEGFR2) or $\alpha_v\beta_3$ integrins [91–93].

The ultrasound intensity required for sonoporation typically ranges between 0.5 and 3 W/$cm^2$. The peak negative pressure (PNP) required for inertial cavitation in the presence of MBs is frequency dependent and is typically in the range of 0.2 to 1.5 MPa. For example, at a PNP of 200 kPa and frequency of 2.25 MHz, transfection was obtained in 5–10% of total cells with cell viability of 80–90% [94]. When the acoustic pressure was increased above 300 kPa, MB destruction increased, 15–25% expressed the reporter gene, and the cell viability was ~70%. At a frequency near 1.3 MHz, sonoporation has been accomplished with diagnostic ultrasound systems and intensities (0.1 to 100 mW/$cm^2$) [16, 95]. The duty cycle is an additional important parameter in UMGT [96], and typically ranges from 20 to 50% in gene therapy studies. The reported ultrasound exposure duration ranges from a few seconds to 30 minutes. The thermal effect also depends on ultrasound exposure parameters, tissue properties and beam configuration [97]. Tran et al. investigated lower-pressure conditions close to the inertial cavitation threshold of MBs, and discovered that prolonging pulse duration time enhanced gene transfer efficiency without cell damage [98].

### 3.4 Effect of ultrasound on vasculature

**3.4.1 Vasodilation and constriction—**Due to the direct reflective activation of vascular smooth muscles via temperature receptors, when the CEM43 is low, thermal effects can induce vasodilation [99], increasing blood flow in a local region. The enhanced blood volume can enhance drug or gene delivery to the target site (Figure 5A). In the context of mild hyperthermia, intratumoral pressure can be reduced and nanoparticle accumulation increased [100]. In the context of thermal ablation, nanoparticle accumulation can be enhanced in the ablated rim [13].

For mild hyperthermia, the process is largely reversible, and vessels can respond without tissue damage. However, a higher CEM43 can induce temporary or permanent vasoconstriction within the tumor core while also creating a hyperemic rim. As shown in Figure 5B, when exposed to high intensity ultrasonic pulses, blood vessels can undergo a temporary or even permanent reduction in diameter. HIFU can also be combined with MBs to achieve an anti-vascular effect to deprive nutrients to the TME [101]. Antivascular treatments result in hypoxia and enhanced immune cell recruitment [102].

#### 3.4.2 Endothelial permeability enhancement—

In addition to cell membrane permeability enhancement, cavitation can enhance the effective endothelial permeability to further enhance therapeutic delivery (Figure 5C). Physiological barriers exist between the vessel lumen and surrounding tissue, including tight junctions that limit drug delivery to the target sites [103]. In addition to the thermal effects on the vasculature described above, focused ultrasound can transiently increase vascular permeability through mechanical effects, thereby temporarily allowing therapeutic agents to diffuse into the surrounding tissue with higher efficiency [104, 105]. The systemic administration of MBs acting as cavitation nuclei particularly enhances permeability at the site of ultrasound application [80]. This phenomenon has been studied in opening the blood-brain barrier (BBB), a particularly challenging tight obstruction of capillary endothelial cells that can inhibit the invasion of exotoxins and therapeutics [106]. Using cytokine analysis and enzyme-linked immuno sorbent assay (ELISA), ultrasound-mediated BBB opening produced an immediate sterile inflammatory response (SIR) in the parenchyma including increases in heat-shock protein (HSP) 70, interleukin-1 (IL-1), IL-18, and tumor necrosis factor-alpha (TNFα) [107]. Within limited ultrasound parameter sets, the BBB opening has been shown to be reversible after several hours via MRI and histological analysis [108]. Mechanical BBB opening has been applied in UMGT for the treatment of brain tumors, such as glioma [109] or glioblastoma [110]. In a preclinical dose escalation study, increasing focused ultrasound intensity within safety limits in glioblastoma tumors induced therapeutic benefits including increasing tumor infiltrating lymphocytes (TILs) and creating an immunostimulatory TME [111]. We find that ultrasound-mediated BBB opening has been combined with either gene therapy [109, 110, 112] or immune therapy [113, 114] but not yet reported with the combination of the two.

### 3.5 Triggered release from vehicles

Ultrasound can also trigger therapeutic agent release from delivery systems (Figure 6A) such as MBs [115], nanoparticles [116], liposomes [117] and polymeric constructs [118]. For example, in [93], a plasmid encoding for herpes simplex virus thymidine kinase (pHSV-TK) was bound to VEGFR2-targeted cationic MBs to reduce the required systemic dose. The plasmids were released at the tumor site by applying ultrasound. *In vivo* studies with brain tumor-bearing rats showed that the tumor volume was reduced significantly after systemic administration of MBs-encapsulated pHSV-TK combined with focused ultrasound [93].

### 3.6 Reactive oxygen species (ROS) and sonochemical effects

Particularly when MBs are used to deliver gene therapy through enhanced membrane permeability, focused ultrasound can produce free radicals that result in chemical transformations in the surrounding environment [119]. During the cavitation of gaseous bubbles, the core temperature can instantaneously increase by ~1000 K, leading to sonochemical effects in the medium. Such effects induce the reaction of water molecules and dissolved oxygen, resulting in a higher local concentration of free radicals, such as reactive oxygen species (ROS), to enhance cytotoxicity (Figure 6B). Additionally, ultrasound can be combined with sonosensitive compounds to facilitate the generation of ROS that induces peroxidation to cell membranes and mitochondria, referred to as the sonodynamic effect [120]. Sonosensitive compounds are usually photosensitizers owning to their aromatic ring structure that facilitates effective photon energy transfer,

including hematoporphyrin, phthalocyanine, pheophorbide or erythrosine [121]. The incorporation of sonodynamic effects has been proposed as a synergistic technique for cancer immunotherapy, leading to calreticulin expression on the cell surface, an antitumor vaccination and abscopal effects [122].

### 3.7 Release of antigen

The thermal and mechanical effects generated by focused ultrasound can facilitate the release of tumor antigen into the bloodstream and the draining lymphatics [123–126]. Tumor antigen released into circulation can be used as a biomarker for cancer progression or recurrence [127]. These biomarkers are typically too dilute to be detected in the blood. However, after insonation, increased levels may be more easily detected. After tumor treatment, focused ultrasound is proposed to track therapeutic efficacy by monitoring circulating biomolecules [128]. Several groups have focused on the detection of circulating protein and nucleic acid biomarkers that are enhanced after insonation. For example, preclinically, carcinoembryonic antigen (CEA), cancer antigen 19–9 (CA19–9), miR-141 and miR-200c were enhanced by insonation of liver tumors [125]. Detection of enhanced protein markers was also established after uterine ablation in human studies [125]. Antigenic debris can recruit antigen-presenting cells (APCs), such as DCs and macrophages which prime $CD8^+$ T cells for an antigen-specific cellular response [129]. Tumor-specific antigen [130] has been detected on macrophages or DCs in the blood and spleen following immunotherapy protocols that incorporate ultrasound [123].

### 3.8 Impact of therapeutic ultrasound on tumor burden, cytokines and chemokines

In UMGT, therapeutic ultrasound can also debulk tumors [21]. The T-cell invigoration-to-tumor burden ratio has been shown to be associated with anti-PD-1 response [131]. Therefore, reducing tumor burden through UMGT may impact treatment efficacy even in the absence of an effective transfection.

Following specific focused ultrasound protocols, wound healing and inflammation in the TME upregulate danger signals, such as HSPs, damage-associated molecular patterns (DAMPs) [97] and ATP [132, 133]. In [134], a non-ablative pulsed ultrasound protocol (1.15 MHz, PNP of 6 MPa, intensity spatial average temporal peak ($I_{SATP}$) of 2683 W/$cm^2$, 10% duty cycle, 5 Hz PRF, 100 pulses per site), altered the expression of cytokines, chemokines, and cell adhesion molecules, and immune cell phenotypes shifted to an inflamed TME. Melanoma (B16 cell line) and breast cancer (4T1 cell line) growth rates decreased as a result of the protocol. Proteomic changes were observed within 24 h, and the tumor growth rate slowed over 5 days after pulsed ultrasound. However, the immune cell trafficking and activation of signaling pathways between the two tumor types differed [134].

As a result of thermal and mechanical ablative ultrasound alone or in combination with immune agonists, RNA sequencing (RNAseq) and T cell receptor sequencing (TCRseq) have been applied to define the changes in the tumor and TME [80, 135]. MRI was used to monitor the tumor temperature in each study. For thermal ablation, the goal was to achieve a local temperature of 65°C or slightly greater within the central ½ fraction of the murine tumor. For mechanical ablation, a PNP of 16.9 MPa at 3 MHz was used

to mechanically disrupt the tumor [135]. Based on this work, both mechanical HIFU and thermal ablation induced a potent inflammatory response and changed macrophage polarization compared to a no-treatment control (NTC). HIFU also upregulated innate immune receptors and related pathways. Priming with an immune agonist (CpG) attenuated the increase in inflammatory cytokines (e.g. IL-6) and further increased expression of innate immune receptors. Intra-tumoral antigen cross-presentation reached ~8% of $CD45^+$ cells with ablation alone. Ablation combined with CpG amplified cross-presentation in the same-site draining lymph node (~16% of $CD45^+$ cells) compared to ablation only (~0.1% of $CD45^+$ cells). Type I interferon (IFN) release also increased with the ablation-immunotherapy treatment as compared with ablation or immunotherapy alone. Expression of T-cell activation genes increased up to 89-fold with ablation-immunotherapy treatment as compared to the NTC. Similar CDR3 sequences arose between mice after immunotherapy, whereas TCR overlap between mice was minimal before treatment. The number of unique CDR3 rearrangements in the distant tumor increased when ablation was combined with the immunotherapy protocol.

Non-thermal HIFU ablation, specifically histotripsy [136, 137] and boiling histotripsy also induce bioeffects that complement immunotherapy. Histotripsy is defined as short (<50 msec) very high intensity ultrasound pulses that create controlled cavitation and mechanically homogenize targeted tissues. Histotripsy (50 pulses at 100 Hz PRF, estimated ~30 MPa PNP, duration from 4 to 15 min) increased both innate and adaptive immune cell populations in treated and distant tumors [136]. Boiling histotripsy is defined as a shock front with amplitude sufficient to induce vapor bubble formation in less than 20 msec, pulse lengths of 2 to 4 times longer than the time to reach boiling, and duty factors of less than 2%. In [138], the time to reach boiling was estimated as 2.7 msec (shock amplitude of 70 MPa, pulse duration of 10 msec and PRF of 1 Hz) and the protocol released 3 – 32-fold more tumor-derived miRNA than ultrasound permeabilization or mild heating. Similarly, in a renal cell carcinoma rat model, boiling histotripsy enhanced $CD8^+$ cytotoxic T cell infiltration in both treated and abscopal tumors [139]. Combining boiling histotripsy (shockwave amplitude of 80 MPa, peak positive pressure of 85 MPa, peak negative pressure of 14 MPa) with checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 resulted in an increase of systemic TILs that translated to improved survival benefits [140].

In breast cancer patients, HSP-70 expression increased in cancer cells within the central necrotic zone after insonation, while only a few positively-stained cells were observed in the periphery [141, 142]. Further, focused ultrasound can decrease the level of immunosuppressive cytokines, such as VEGF and transforming growth factor-beta (TGF-β), to alleviate tumor-induced immunosuppression and renew antitumor immunity [142, 143]. TGF-β can reduce antigen presentation to DCs, T cell differentiation, macrophage and NK cell proliferation, and immunostimulatory cytokine secretion [144]. Consequently, therapeutic ultrasound application can result in tumor regression and a reduction in metastasis [145]. These effects must also be considered when dissecting the impact of UMGT on the immune system.

## IV. Current immunotherapy approaches

New immunotherapy protocols aim to improve efficacy and safety. To date, the benefit of immunotherapy has been limited to a minority of patients with specific cancer types. Further, a considerable subset of patients who initially respond eventually relapse [146]. Conventional vaccination methods for immunotherapy can induce tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs), but have typically shown limited clinical efficacy [147].

Recombinant cytokines for immune cell activation were the first immunotherapies approved by the US Food and Drug Administration (FDA), including interferon-alpha (INF-α) for hairy cell leukemia [148] and IL-2 for metastatic renal cancer and metastatic melanoma [149]. However, unfavorable pharmacokinetics of these recombinant cytokines can cause severe adverse effects such as cytokine release syndrome [150]. Over the past several years, ipilimumab, a checkpoint inhibitor that can specifically target cytotoxic T lymphocyte associated protein 4 (CTLA4) [151] was approved for clinical treatment of advanced melanoma [152, 153]. Shortly thereafter, mAbs targeting programmed cell death 1 (PD-1) or its ligand, PD-1 ligand 1 (PD-L1) were developed for clinical use [154]. While these mAbs showed high efficacy in certain groups of patients, efficacy is greatest in cancers with elevated expression of checkpoint receptors [155, 156].

Recently, CAR-T cell therapy was developed as an alternative approach to small molecule and biologic-based therapies. Similar to adoptive cell transfer commonly seen in preclinical models, T cells isolated from the host were modified with gamma-retroviral or lentiviral vectors to express a CAR and then re-injected. CAR-T therapy has been approved to treat hematological malignancies with trials in other tumors underway [157]. Despite the remarkable efficacy in hematological cancer, it remains challenging to promote infiltration of CAR-T cells into solid tumors. Major hurdles of current immunotherapeutic interventions include inefficient tumor infiltration and changes in the T cell phenotype after migration [158]. Overcoming the aforementioned impediments will likely require innate immune activation. To introduce nucleic acids, including plasmids, small interfering RNA (siRNA), messenger RNA (mRNA) or microRNA (miRNA) [159] into target cells, the fundamental engineering challenge is to develop safe and effective techniques to deliver those nucleic acids [160]. In the next section, we describe the combination of UMGT and immunotherapy protocols.

## V. Ultrasound, gene delivery and immunotherapy

In combination with immunotherapy, UMGT can be used to treat cancer and produce an efficacious immune response in preclinical models. UMGT can be subdivided to include four approaches: tumor transfection with ultrasound only, MB-based transfection, ultrasound-sensitive delivery systems and modulation of immune cell functionality. As summarized in Table 2, the parameters in UMGT vary and the corresponding approaches are explained in the following sections.

### 5.1 Transfection with ultrasound without other components

Ultrasound alone has been observed to deliver DNA plasmids efficiently, accurately and safely without transfection reagents. In one study, an unfocused therapeutic ultrasound system (Sonitron 2000, Rich-Mar Corp., Inola, OK, USA) with an unfocused 1.13-$cm^2$ 1-MHz applicator was used to insonify a relatively large region. $I_{SATA}$ of 1.0 W/$cm^2$ at 20% duty cycle for 5 min (60 J/$cm^2$) was found to optimize transfection of reporter genes and was applied in an immunotherapy regimen [159]. Higher intensities reduced the effective transfection. In this work, naked plasmids coding for granulocyte-macrophage colony-stimulating factor (GM-CSF) were used to treat fibrosarcoma-bearing mice (Figure 7A). The efficiency was comparable to electroporation-based gene transfer. Ultrasonic shock-wave treatment combined with IL-12 plasmids has been investigated in both mouse melanoma and renal carcinoma models. A laboratory lithotripter system applied 500 shock waves at a 2-Hz rate to enhance IL-12 expression, resulting in significant tumor reduction as compared to the corresponding monotherapies [160].

### 5.2 MB-based transfection

MB-based gene delivery is performed by administration of MBs and immunotherapeutic materials prior to insonation. Many studies have utilized a transmission center frequency of 1 MHz, and the ultrasound intensity ranged from 0.15 to 2 W/$cm^2$. Lower insonation pressures can prolong pulse duration time and enhance gene transfer efficiency without cell damage [162]. For example, SonoVue MBs were used to transfer an IL-27 plasmid with a 1 MHz transducer at an $I_{SATA}$ of 1 W/$cm^2$ (SonoPore KTAC-4000, Protech International) [161]. The sonoporation strategy was studied in both fully murine models and human prostate tumor xenografts, resulting in over 60-fold increase in expression as compared with either MBs alone or naked DNA. Similarly, Sonazoid MBs combined with 1.011 MHz oscillation frequency at an $I_{SATA}$ of 0.22 W/$cm^2$ (SonoPore KTAC-4000) facilitated IFN-β plasmid delivery to treat melanoma [162]. In the above studies, MBs were simply mixed with genes, while in [35] cationic lipid MBs were used as carriers for miR34a, and were designed to treat cervical cancer by improving the efficacy in anti-PD-1 therapy.

Additionally, a protocol with a lower transmission frequency (250 kHz) and PNP of 500 kPa efficiently transfected tumor and stromal cells with DNA plasmid encoding IFN-β [21] (Figure 7B). MB wall collapse velocity was more rapid with the lower ultrasound frequency. The MB oscillation reached an expansion ratio of 35, resulting in a 150-fold increase of transfected cells observed after the 250 kHz insonation (H115, Sonic Concepts, Bothell, Washington). As reported in this study, the tumors were debulked, the plasmid transfection rates were increased, and more immune cells infiltrated the TME.

### 5.3 Ultrasound-responsive delivery systems

With systemic injection of plasmids, in vivo MB-based gene transfer is inefficient due to the different pharmacokinetic profiles of the nucleic acids and MBs. MBs have a limited circulation time (typically a few minutes), while nucleic acids can be loaded by alternative carriers for prolonged half-life and have been reported to circulate for extended periods after cutaneous injection [165]. Therefore, it is challenging to efficiently deliver both MBs and nucleic acids to the target sites simultaneously. Ultrasound-responsive

liposomes with diameters ranging from 150 to 200 nm have been used to improve delivery. In one study, liposomes encapsulating perfluoropropane gas were used to deliver the IL-12 gene with a 1-MHz center frequency and intensity of 0.7 W/cm$^2$ [164]. This approach reduced systemic adverse effects and resulted in a tumor regression rate of 80%. In other studies, mannosylated lipoplexes encapsulating perfluoropropane gas were developed to selectively target and transfect APCs to treat cancer [166]. With ultrasound exposure (f = 2.062 MHz, intensity 4.0 W/cm$^2$), the transfection efficiency of pCMV-OVA was significantly increased. Mannose-modified lipoplexes encapsulating pCMV-OVA [165] or pUb-M encoding ubiquitylated melanoma-specific antigens, gp100 and tyrosinase-related protein (TRP-2) [166] were then applied in lymphoma or melanoma therapy, respectively (Figure 7C). As a result, the immune response was enhanced and sustained after transfection.

### 5.4 Modulation of immune cell functionality

Adoptive cell therapy uses cells from the patients' own immune system to eliminate cancer [172] after isolation and expansion in vitro. Ultrasound is an ideal tool to precisely control genes to enhance immune response. Chimeric antigen receptor-expressing T (CAR-T) cells are commonly used as immune cell vaccines [173]. Piezo-1 in T cells was integrated with engineered genetic circuits in live HEK293T cells to convert ultrasound-activated Piezo1 into transcriptional activity. MB oscillation using ultrasound at a center frequency of 2 MHz and PNP of 0.6 MPa stimulated the expression of the anti-CD19-CAR, enabling T cells to recognize and kill CD19$^+$ cancer cells [167].

Heat generated by an MRI-guided focused ultrasound system composed of a 1.5 MHz 8-element annular array transducer has also been used for CAR-T control through a heat shock protein driven Cre-lox gene switch [168]. By generating heat (43°C), ultrasound induced the activation of the Cre-lox gene, and elevated the level of anti-CD19-CAR or prostate-specific membrane antigen (PSMA)-CAR expression. In both lymphoma and prostate cancer mouse models, CAR-T cells were injected locally at tumor sites and led to a reduction in tumor growth.

For DC-based vaccines, the feasibility and safety were tested with *in vitro* DCs harvested from murine bone marrow. Messenger RNA encoding GFP or luciferase was mixed with cationic lipoplexes, and MBs loaded with mRNA-lipoplexes were incubated with DCs. As a result of insonation of the complex, transfection efficiency was enhanced, with limited impact on DC viability and maturation [170]. In another study, MBs loaded with both TAA-encoding mRNA as well as immunomodulating TriMix mRNA were utilized to sonoporate using ultrasound at a frequency of 1 MHz and $I_{SPTA}$ of 2 W/cm$^2$ to load the mRNA [170]. The resulting DCs activated CD8$^+$ T cells produce antigen-specific lysis of target cells (Figure 7D), resulting in significant tumor regression. 30% of the vaccinated animals maintained long-term antigen-specific immunological memory.

Lastly, MB-based techniques have been applied to transfect regulatory T cells (Tregs) with Forkhead box P3 (Foxp3) [171]. Tregs are responsible for reducing immune response to maintain homeostasis and self-tolerance, but also inhibit anti-cancer immunity. The ultrasound parameters were optimized to result in 50% in Treg transfection without an effect

on proliferation. Once transfected with Foxp3 siRNA using ultrasound with 2.5-MHz center frequency and MI of 1.4, the Tregs were deactivated, and the immunosuppressive TME was reversed [171].

### 5.5 Integration of UMGT in combinational regimens

UMGT has been combined with other immunotherapies, such as immune checkpoint blockade, to improve the recruitment of tumor-specific T cells and their functionality [21, 174, 175]. UMGT-based immunotherapy can further modulate TME and prime immune cells by combining the technique with other non-immunological approaches, such as radiotherapy, chemotherapy [176], ablation therapy [135], photothermal therapy (PTT) [177], and photodynamic therapy (PDT) [89]. By exploiting the immunogenic cell death-inducing properties of conventional therapies, UMGT-based strategies can not only kill cancer cells but also stimulate in situ vaccination. Moreover, the gene transfection process can also be enhanced by combinatorial methods. A challenge of adenoviral methods is their high immunogenicity and host-specificity during systemic circulation. Therefore, a method using MBs and ultrasound was developed to protect human adenoviruses and deliver them to the target tumor site [178]. Transfection efficiency was enhanced without reducing activation of innate or acquired immunity.

## VI. Limitations, challenges and future application of ultrasound in immunotherapy

The combination of ultrasound with drug and gene therapy is attractive due to the unique capabilities of therapeutic ultrasound to reduce major physiological barriers for delivery. In addition, the rapid growth of our understanding of immune cell receptors and signaling has provided a wealth of opportunities to transfect tumor, immune and stromal cells to treat cancer. Thus, the convergence of therapeutic ultrasound, gene therapy and immunotherapy is a particularly timely area of study. Further, the number of clinical studies that incorporate therapeutic ultrasound is rapidly increasing. At Stanford University, successful clinical trials have now led to the incorporation of therapeutic ultrasound in clinical care. Still, the safety of therapeutic ultrasound in particular tissues and organs must be established before gene therapy or immunotherapy can be incorporated. Such safety studies are underway around the world, and we are optimistic that combinatorial studies will follow soon. A study of the combination of focused ultrasound ablation and aPD-1 is underway (NCT04116320), and it is likely that other studies combining therapeutic ultrasound with approved agonist or checkpoint antibodies will emerge soon. Ablative protocols do induce a vaccination effect and this is exploited in such studies. Similarly, the use of ultrasound and MBs to open the BBB is the focus of multiple planned and recruiting human trials [179], and therefore the opportunity to pair this work with approved immunotherapies is likely imminent. The incorporation of UMGT with nanodelivery of nucleic acids in human immunotherapy protocols will require additional study. Most optimization studies for such UMGT techniques have been limited to pre-clinical models, and have not yet been tested in primates nor humans. Studies in large models (particularly porcine and canine models) will be required steps for translation.

Importantly, we emphasize that the many biological effects of therapeutic ultrasound can be synergistic with gene and immune therapy. Therapeutic ultrasound has the potential to selectively ablate tissue deep within the body without impacting the surrounding tissue, and can be repeated as needed. This debulking effect, together with the ability to release tumor-specific protein and nucleic acids are likely the greatest strengths of the technique. However, many therapeutic ultrasound protocols do induce additional biological changes. Therefore, combinatorial protocols involving ultrasound and gene therapy or immunotherapy must carefully account for individual effects through adequate controls. In this review, we have included considerations of vasoconstriction or dilation, cell membrane perforation, triggered cargo release, ROS generation, antigen release, and cytokine and chemokine production, as each will impact the ultimate therapeutic result.

### 6.1 Parameter space

A subset of current studies has shown different outcomes and effects depending on ultrasound parameters, MB, drug and gene dosage, and administration routes of MBs and genes. It will be important for prospective studies to systematically assess the parameter space of UMGT. Consistent reporting of all parameters is also key to the repeatability and assessment of protocols. In many cases, $I_{SPTA}$ is reported; however, PNP and duty cycle or equivalent parameters must also be provided in order to replicate the study. The development of standardized methods to measure and report pressure, pulse length and associated parameters is needed. Although the American Institute of Ultrasound in Medicine (AIUM) established guidelines for safe use of ultrasound [180], there are still few standards for therapeutic ultrasound and UMGT applications.

### 6.2 Drug carriers and targeted MBs

New delivery systems to carry and release nucleic acids are needed. Many recent studies involve loading nucleic acids onto cationic MBs; however, cationic materials suffer from limited circulation time and potential toxicity [181]. Additionally, improving MB binding specificity could enhance transfection efficiency and therapeutic outcome. Strategies include the development of alternate bioconjugation methods, and the identification of additional disease-specific ligands [182].

Further, much of the previous work has emphasized DNA as the nucleic acid cargo due to its stability. However, transport across the nuclear membrane is challenging and relatively few studies have documented how this transport changes with ultrasound or MB parameters. The emergence of RNA therapeutics provides an attractive alternative for UMGT with both silencing and transfection options increasing.

### 6.3 Molecular imaging

Molecular Imaging is an evolving discipline that enables the noninvasive visualization, evaluation and quantification of specific biologic processes at the cellular levels in living subjects [183, 184] and provides a potential strategy to characterize transfection efficiency and immunotherapeutic efficacy during and after UMGT [185]. For example, CAR-T cells designed to express both herpes simplex virus type 1 thymidine kinase (HSV1-TK) as a

reporter gene and IL-13 as a therapeutic gene have been reported [186]. This concept could be applied to further improve the UMGT development in immunotherapy.

**Final summary—**Strategies to combine therapeutic ultrasound with gene and immune therapies are developing to treat cancer, and the convergence of the three fields is particularly exciting. We look forward to further advances in this promising field.

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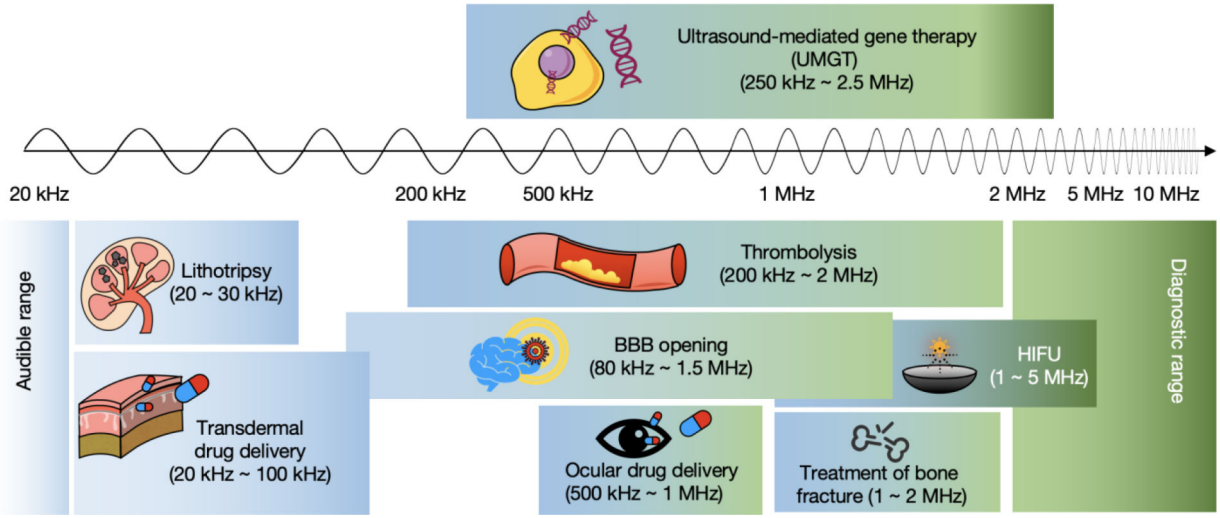


**Figure 1.**

Schematic of current research related to therapeutic ultrasound and UMGT with the corresponding frequency ranges.

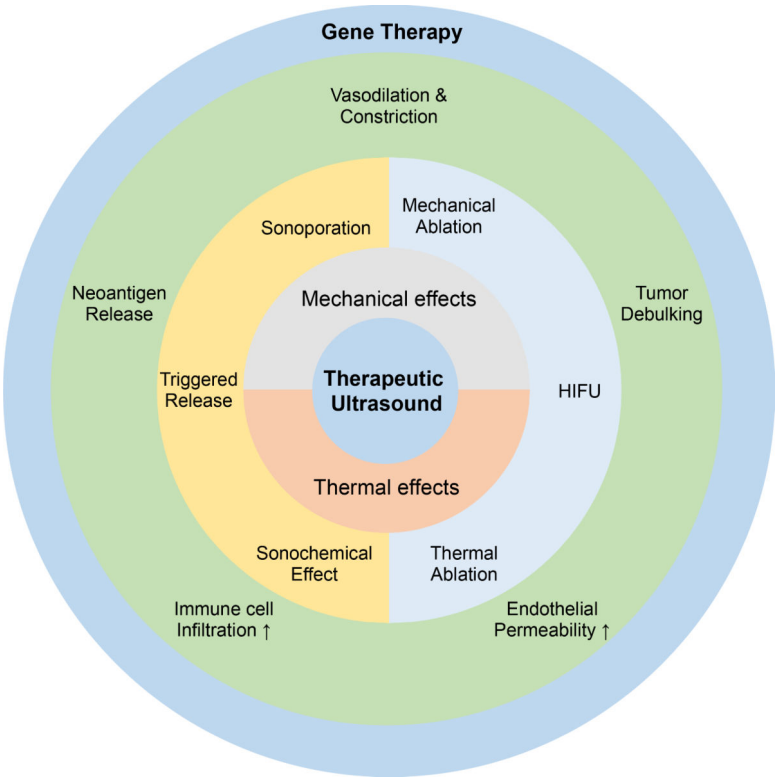


**Figure 2.**

The mechanisms of therapeutic ultrasound in gene therapy, including mechanical and thermal effects with the induced biological effect in various aspects.

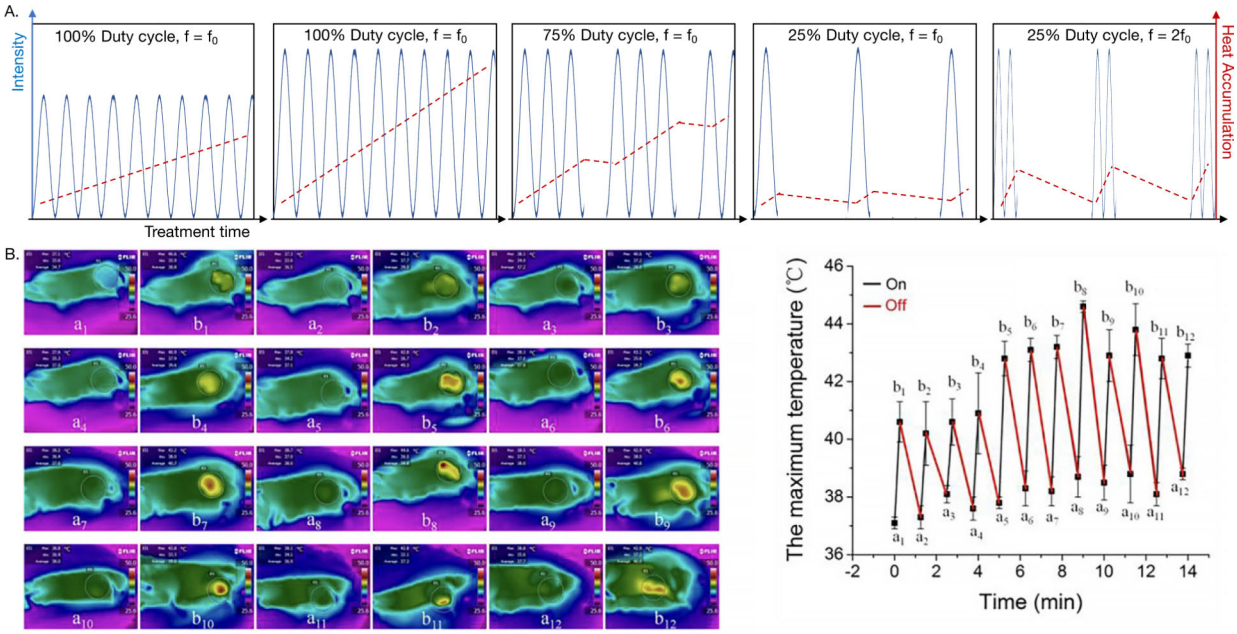


**Figure 3.**

A) Schematic of thermal effects produced by ultrasound. The temperature elevation varies with acoustic intensity, duty cycle, treatment time and ultrasound frequency. At a given duty cycle, higher intensity leads to greater heat accumulation. B) Infrared (IR) temperature maps at the corresponding time points before and after ultrasound irradiation cycles, and the measured maximum temperature at each point [76]. Adapted from [76]. Copyright from Ivyspring International Publisher.

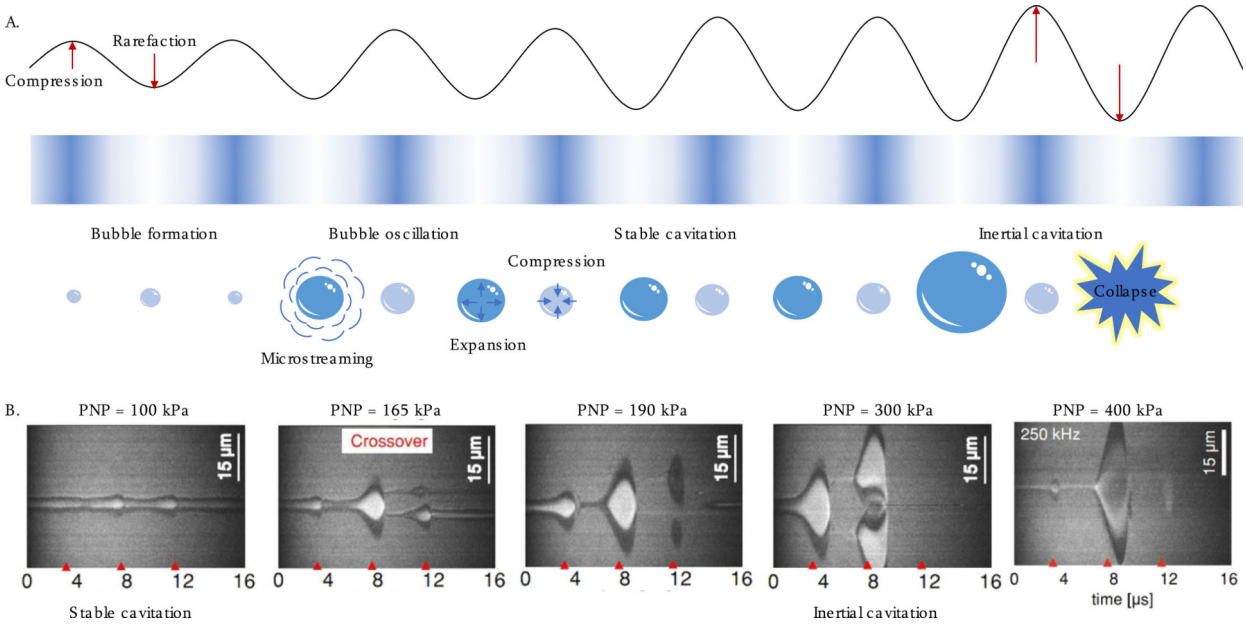


**Figure 4.**

A) Schematic of non-thermal mechanical force, and the induced acoustic cavitation effect. In an oscillating pressure field, MBs expand and contract in response to the compression and rarefaction phases of the pressure wave. Stable cavitation occurs at low pressures resulting in microstreaming around the MBs. At higher pressures, this regime transforms into inertial cavitation where the increased expansion is followed by a violent collapse generating shock waves and the MBs destruction. B) Images of oscillating MBs (initial radius = 1.5 µm) captured by ultra-high-speed camera showing the transition from stable to inertial cavitation at ultrasound frequency of 250 kHz when increasing the acoustic pressure [86].

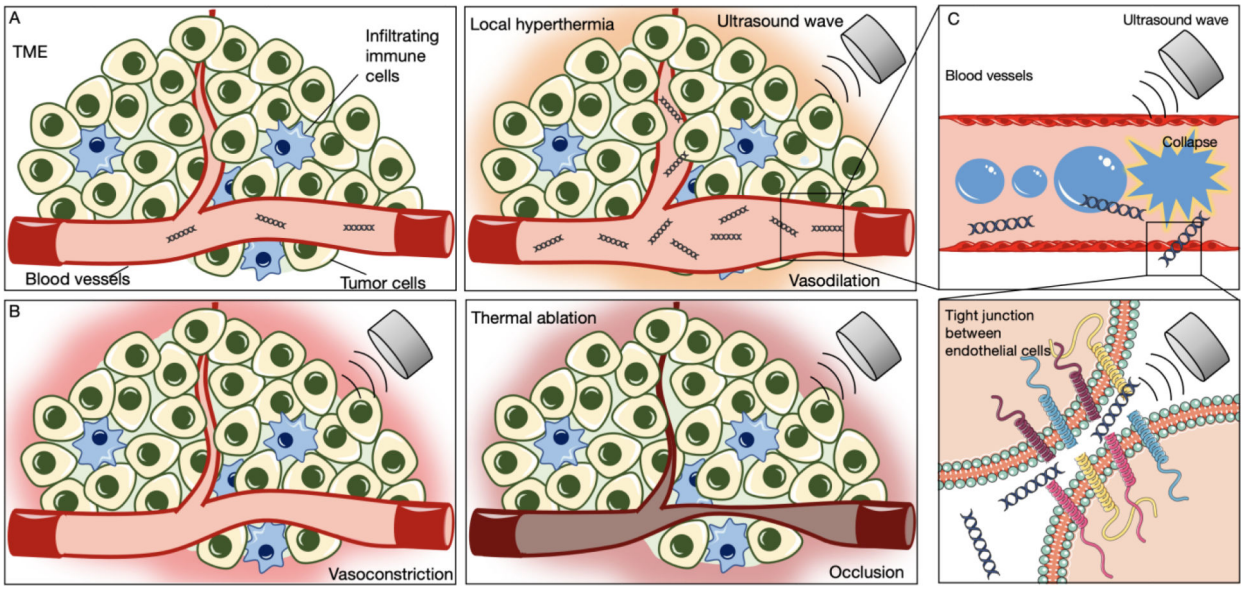


**Figure 5.**

Schematics of A) vasodilation caused by minimal local hyperthermia, B) vasoconstriction and thermal ablation with the increased energy absorption, and C) temporary enhancement of vascular permeability by reversible inhibition of tight junction between endothelial cells.

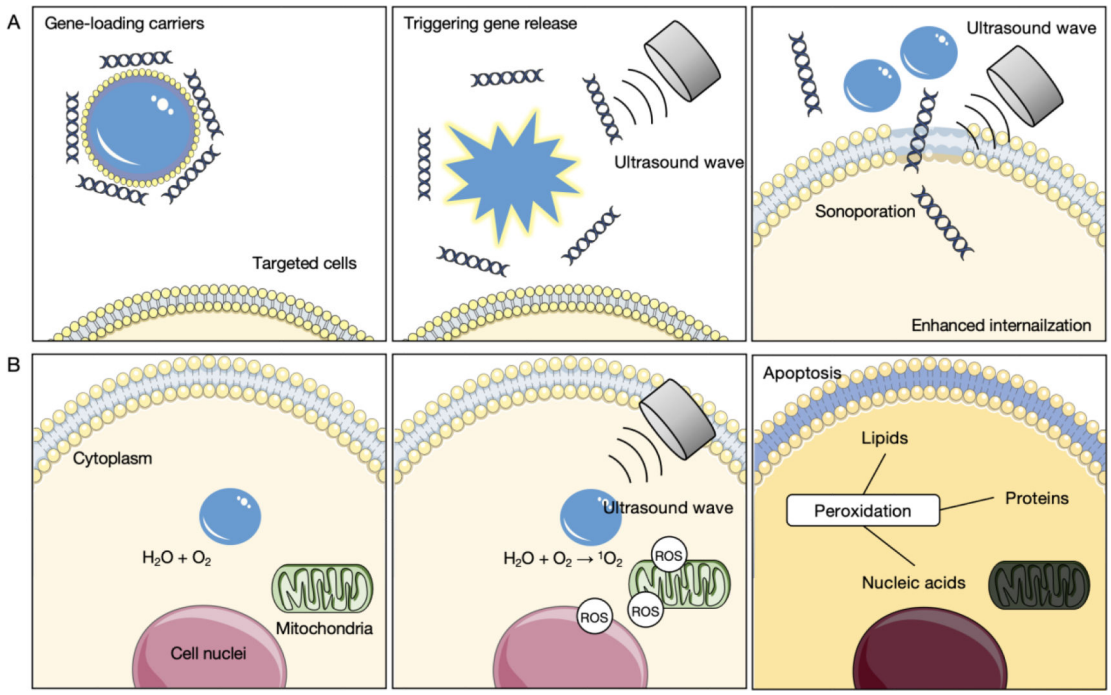


**Figure 6.**

Schematic of the effect on cells for transfection, including A) triggering gene release from gene-loading carriers and enhanced gene internalization by sonoporation, and B) generation of cytotoxic radicals by the sonochemical effect.

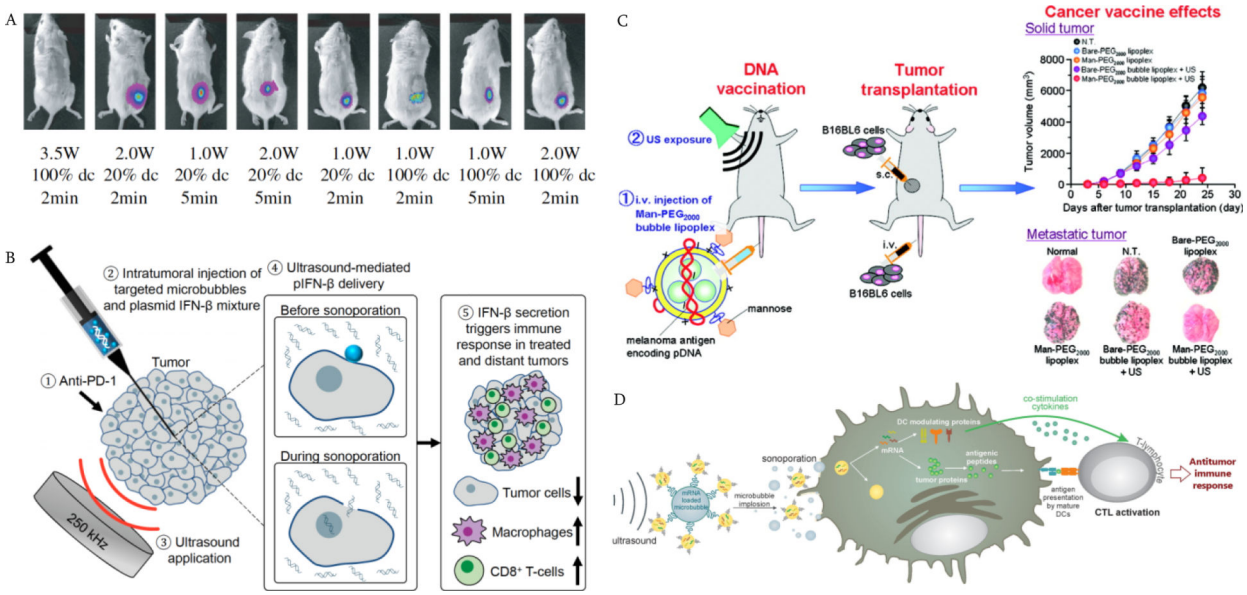


**Figure 7.**

Approaches of UMGT for cancer immunotherapy. A) Transfection with plasmids and ultrasound. The ultrasound parameters were optimized to administer an immune gene construct in murine fibrosarcoma tumor models [159]. Copyright (2010) Elsevier; B) Transfection mediated by MB oscillation. Low frequency ultrasound was applied with the mixture of IFN-β plasmids and targeted MBs, and the immune cells were recruited at both sites of treated and distant tumors [21]. Copyright (2020) National Academy of Sciences; C) Transfection by ultrasound-sensitive delivery system. The mannose-modified lipoplexes were developed as ultrasound-responsive and APC-selective carriers for tumor antigen gene delivery. Reprinted with permission from [166]. Copyright (2011) American Chemical Society; D) Modulation of immune cells using UMGT. TriMix mRNA-loaded MBs were used to transfect DCs by sonoporation to activate T cells [170] Copyright (2014) Elsevier.

**Table 1.**

Summary of the advantages and limitations of gene transfection methods.

| | Methods | Advantages | Limitations | Ref. |
|---|---|---|---|---|
| **Viral delivery** | Adenovirus<br>Retrovirus<br>Lentiviruses<br>AAVs | High transfer efficiency<br>Clinically approved<br>Varied duration of expression | Innate immune response<br>Insertional mutagenesis<br>Complexity in production and scaleup<br>Small payload in AAVs | [25–29] |
| **Non-viral vectors** | Liposomes<br>Nanoparticles<br>Polymers<br>Dendrimers | Easy to prepare<br>Inexpensive to produce<br>Targeted delivery<br>Enhanced circulation time for some constructs | Relatively low transfer efficiency<br>Burden on the liver to metabolize<br>Toxicity of cationic lipids | [30–35] |
| **Physical methods** | Microinjection<br>Biolistic particle delivery<br>Electroporation<br>Phototransfection | Relatively high transfer efficiency<br>Less dependent on cell types and conditions<br>No need for vectors and carriers | Invasive<br>Requires expensive and dedicated instrumentation<br>Low penetration of light in tissues | [36–41] |
| | UMGT | Noninvasive<br>Site-specific<br>Viral vectors and carriers enhance efficacy but are not required<br>Temporal control<br>Available devices | Insufficient preclinical and clinical research<br>Technically demanding | [21, 42–46] |

**Table 2.**

Summary of preclinical studies of UMGT for cancer immunotherapy.

| UMGT Approaches | Tumor models | Gene constructs | Ultrasound parameters | Target cell | Ref. |
|---|---|---|---|---|---|
| 5.1 Transfection with ultrasound | Fibrosarcoma, murine models | GM-CSF pDNA | f = 1 MHz, $I_{SATA}$ = 1.0 W/cm$^2$, Duty cycle = 20%, t = 5 min | Tumor cells | [159] |
| | Melanoma and renal carcinoma murine models | IL-12 pDNA | f = 200 kHz (laboratory lithotripter similar to Dornier HM-3 lithotripter), PNP = 7.4 MPa, PRF = 2 Hz, Shock Waves = 500 (Duty cycle: 0.5%) + intratumoral air bubbles | Tumor cells | [160] |
| 5.2 MB-based transfection | Prostate cancer murine models, human prostate tumor xenografts | IL-27 pDNA | f = 1 MHz, PNP = 0.12 MPa, $I_{SATA}$ = 1 W/cm$^2$, PRF = 2 Hz, Duty cycle = 50%, t = 2 min, + SonoVue MBs | Tumor cells | [161] |
| | Melanoma murine models | IFN-β pDNA | f = 1.011 MHz, $I_{SATA}$ = 0.22 W/cm$^2$, Duty cycle = 50%, PRF = 0.5 Hz, t = 3 min, + Sonazoid MBs | Tumor cells | [162] |
| | Cervical cancer murine models | miR-34a | f = 1 MHz, I = 1.5 W/cm$^2$, Duty cycle = 50%, t = 90 s, + cationic lipid MBs | Tumor cells | [163] |
| | Breast cancer, murine models | IFN-β pDNA | f = 250 kHz, PNP = 500 kPa, $I_{SPTA}$ = 1 W/cm$^2$, PRF = 30 Hz, t = 3 min, Duty Cycle = 12%, + anti-EpCAM conjugated MBs | Stromal cells, tumor cells | [21] |
| 5.3 Ultrasound-responsive delivery system | Ovarian carcinoma, murine models | IL-12 pDNA | f = 1 MHz, I = 0.7 W/cm$^2$, t = 60 s, + liposomal bubbles | Cells in TME | [164] |
| | Lymphoma murine models | OVA pDNA | f = 2.062 MHz, I = 4 W/cm$^2$, Duty cycle = 50%, t = 20 s, +mannose-modified liposomes | APCs | [165] |
| | Melanoma murine models | pUb-M cDNA | Duty cycle = 50%, PRF = 10 Hz, t = 2 min, +mannose-modified liposomes<br>f = 1.045 MHz, I = 1 W/cm$^2$ (for intravenous injection)<br>f = 2.062 MHz, I = 4 W/cm$^2$ (for intradermal and intrasplenic injection) | APCs | [166] |
| 5.4 Modulation of immune cell functionality | Lymphoma and leukemia cell models | Anti-CD19 CAR gene loaded lentiviruses | f = 2 MHz, $I_{SPTA}$ = 0.6 W/cm$^2$, PNP = 0.6 MPa, PRF = 5 Hz, Duty cycle = 5%, t = 10 min, +MBs | Jurkat T cells and PBMCs | [167] |
| | Lymphoma and prostate cancer models | Cre-lox gene with HSP promotor | f = 1.5 MHz | T cells | [168] |
| | DCs from bone marrow of C57Bl/6 mice | mRNA encoding luciferase or GFP | f = 1 MHz, I = 2 W/cm$^2$, Duty cycle = 50% t = 30 s, + lipoplexes + MBs | DCs | [169] |
| | Melanoma lung metastasis murine models | Antigen mRNA and TriMix mRNA | f = 1 MHz, $I_{SPTA}$ = 2 W/cm$^2$, PNP = 800 kPa, Duty cycle = 20%, PRF = 100 Hz, t = 30 s, lipid MBs | DCs | [170] |
| | Hepatocellular carcinoma human cells | Foxp3 siRNA | f = 2.5 MHz, MI = 1.4, t = 150 s, + SonoVue MBs | Tregs | [171] |

Abbreviation: f: frequency, I: acoustic intensity (this notation is used when the specific intensity metric cannot be determined), PNP: peak negative pressure, PRF: pulse repetition frequency, MI: mechanical index, EpCAM: Epithelial cell adhesion molecule, IFN-β: Interferon-beta, IL-27: interleukin 27, IL-12: interleukin 12, GM-CSF: granulocyte-macrophage colony-stimulating factor, OVA: ovalbumin, HSP: heat shock protein, Foxp3: Forkhead box P3, APC: antigen-presenting cells, DCs: dendritic cells, PMBCs: peripheral blood mononuclear cells, $I_{SPTA}$: Spatial peak temporal average intensity, $I_{SATA}$: Spatial average temporal average intensity