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Review Article

A Review of Ultrasound-Mediated Checkpoint Inhibitor Immunotherapy

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Over the past decade, immunotherapy has emerged as a major modality in cancer medicine. However, despite its unprecedented success, immunotherapy currently benefits only a subgroup of patients, may induce responses of limited duration and is associated with potentially treatment-limiting side effects. In addition, responses to immunotherapeutics are sometimes diminished by the emergence of a complex array of resistance mechanisms. The efficacy of immunotherapy depends on dynamic interactions between tumour cells and the immune landscape in the tumour microenvironment. Ultrasound, especially in conjunction with cavitation-promoting agents such as microbubbles, can assist in the uptake and/or local release of immunotherapeutic agents at specific target sites, thereby increasing treatment efficacy and reducing systemic toxicity. There is also increasing evidence that ultrasound and/or cavitation may themselves directly stimulate a beneficial immune response. In this review, we summarize the latest developments in the use of ultrasound and cavitation agents to promote checkpoint inhibitor immunotherapy.

Introduction

Cancer immunotherapy

Although there is evidence of the exploitation of the immune system in treating diseases in medical texts dating back to the Ancient World, the use of immunotherapy in oncology was not widely explored until the 18th century [1]. In 1863, Rudolf Virchow reported a connection between tumours and inflammation after observing that neoplastic tissues are often surrounded by leukocytes. Since then, deeper understanding of the inflammatory microenvironment of solid tumours has supported Virchow's hypothesis, and the correlation between malignant tissue and inflammation has spurred the development of cancer immunotherapy [2–5]—the harnessing of the body's natural defences to treat and prevent tumour growth. The most widely reported example of early cancer immunotherapy was the experiment by William B. Coley in 1891, in which he attempted to treat cancer patients with bacteria-derived products [1,6]. Coley [7] reported remarkable effects in certain cancer patients, but his lack of systematic reporting, inconsistent success rates and competition from the contemporaneous development of radiotherapy meant that his work remained largely ignored and indeed discredited for most of the 20th century. Decades later, interest in the immune system re-emerged following advances in immunology and cancer research, including the discovery of interferon and dendritic cells [8,9], understanding of the crucial role of T cells in immunity [10] and development of the first vaccine based on a single purified surface antigen [11,12]. Over the past 40 years the field of immunology has emerged as a major influence in cancer research and treatment. In 2018, James P. Allison and Tasuku Honjo were jointly awarded the Nobel Prize in Physiology or Medicine "for their discovery of cancer therapy by inhibition of negative immune regulation" [13].

Immunotherapeutics

In cancer immunotherapy, agents are utilized to either activate or boost the immune system to attack cancer cells through natural mechanisms, which otherwise are evaded, are repressed or never develop at all enabling uninterrupted disease progression [14] (Figs. 1 and 2).

In recent years, a wide variety of immunotherapeutic products have been approved by the U.S. Food and Drug Administration (FDA), including checkpoint inhibitors, recombinant cytokines, adoptive cell therapies and cancer vaccines (Fig. 2). To date, immune checkpoint inhibitors represent the most thoroughly investigated class of immunotherapeutics. Current mainstream drugs that block checkpoint proteins target CTLA-4 (cytotoxic T lymphocyte-associated protein 4), PD-1

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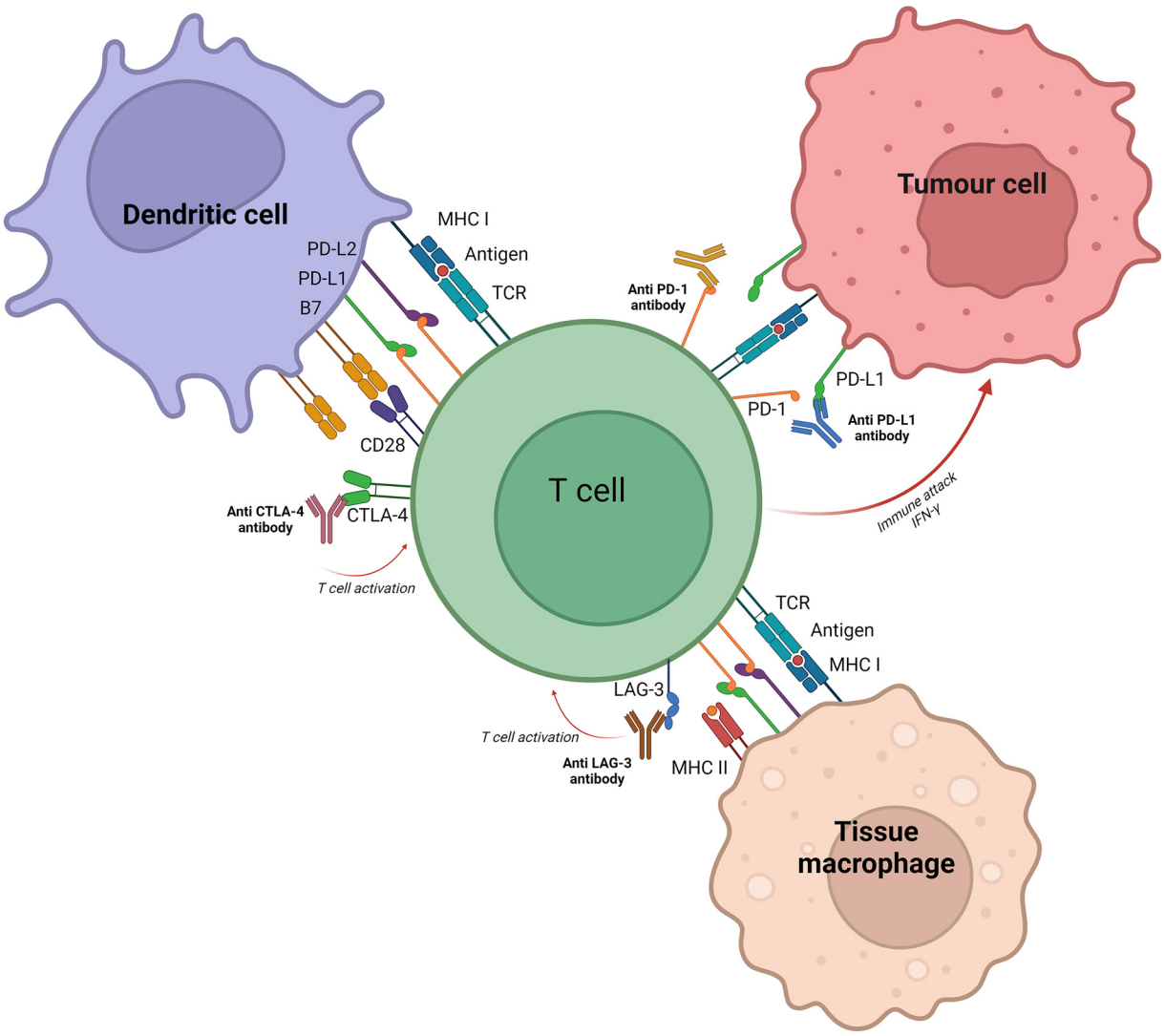

Figure 1. Effects of immune checkpoint inhibitors. Monoclonal antibodies that block the regulatory immune targets CTLA-4, PD-1 and PD-L1 are the mainstream drugs currently applied in the clinic. A deeper understanding of tumour immunology has led to an increasing number of immunotherapies, including the first recent U.S. Food and Drug Administration approval of anti-LAG-3. For tumour-directed T cells to become activated, two signals are required. The first signal corresponds to the binding of specific tumour peptides (TAA) to TCR on antigen-specific T cells. The second signal is typically mediated between the interaction of co-stimulatory molecules on antigen-presenting cells and T-cell receptors. Blocking immune checkpoint proteins, including PD-1, PD-L1, CTLA-4 and LAG-3, with monoclonal antibodies promotes increased T-cell activation, proliferation and enhanced effector function. After the monoclonal antibody binds to the immune targets, the tumour-specific killing ability of T cells is then activated (causing an immune attack) [15,16]. MHC, major histocompatibility complex; TAA, tumour-associated antigen; TCR, tumour cell receptor; IFN-γ, interferon-γ. Created in Biorender.com.

(programmed cell death protein 1) and PD-L1 (programmed cell death ligand 1) [6,19,20]. Whilst CTLA-4 and PD-1 are found on T cells, PD-L1 is found mainly on cancer cells [19]. The physiological role of immune checkpoints is to maintain appropriate immune responses and protect healthy tissues from auto-immune attack (Fig. 1). Over the past decade, the most impactful class of novel anti-tumour drugs comprises those that inhibit PD-1/PD-L1 [21–28]. These drugs block the binding of inhibitory molecules to their receptor ligand on tumour or cytotoxic T cells [29–31]. As of December 2021, the number of active interventional clinical trials testing the effectiveness of anti-PD-1/PD-L1 increased from 1 in 2006 to 5683 as monotherapy or in combination with other treatments [5,32]. In 2016, the first clinical trial of a PD-1-targeted monoclonal antibody, nivolumab, was reported (NCT01454102). Since then, at least seven monoclonal antibodies targeting PD-1 or its ligand PD-L1 have been approved by the FDA alone or in combination with other therapies for the treatment of more than 14 cancer types (Table 1). In March 2022, the FDA approved a combination of nivolumab and relatlimab, the first immune checkpoint inhibitor targeting LAG-3 (lymphocyte-activation gene 3), for patients with untreated unresectable or metastatic melanoma [33].

Similar to Coley's toxins, however, the clinical success of immunotherapy has been variable, with only 20%–30% of patients responding to monotherapy, and some only temporarily. Solid tumours present a particular challenge, with the efficacy of anti-PD-1/PD-L1 inhibitors fluctuating between 10% and 30% [37,38]. The tumour microenvironment (TME) not only presents a physical barrier to drug uptake and accumulation; it also contributes directly to the suppression of a normal immune response through a number of mechanisms such as production of immunosuppressive cytokines and induction of regulatory T cells [39,40]. If a therapeutic concentration of an immunotherapeutic cannot be achieved, then there will be insufficient restoration of the anti-tumour immune response [41], development of resistance, tumour

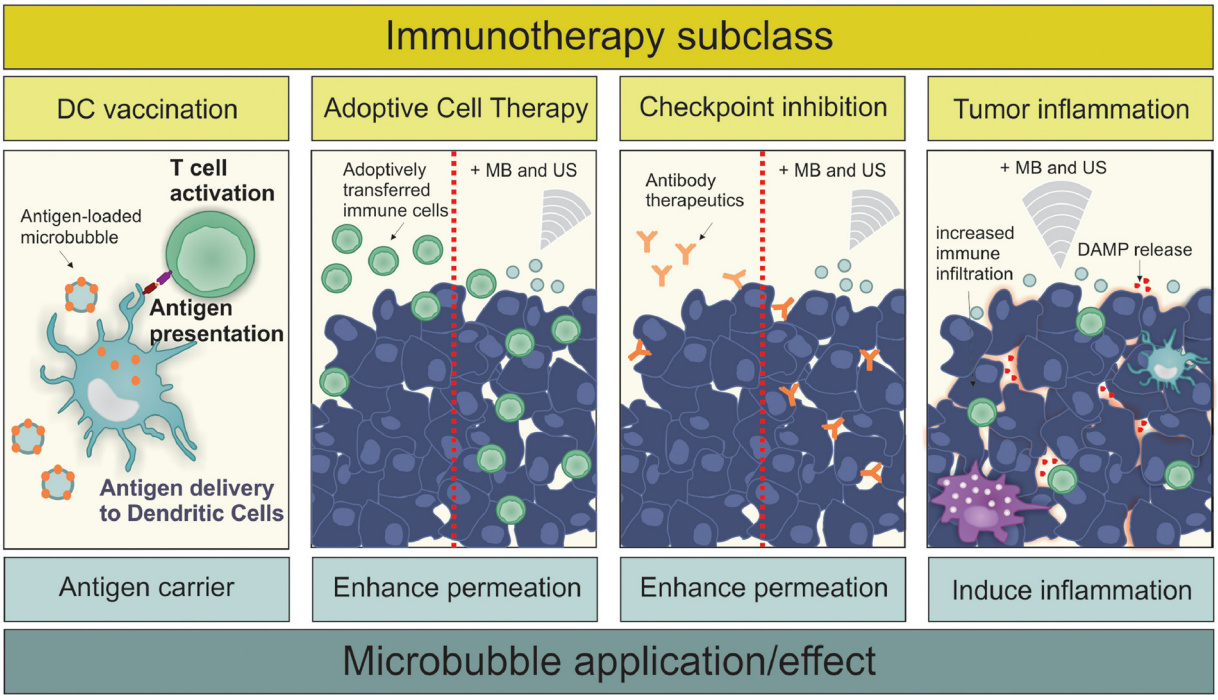

Figure 2. Effects of microbubbles and ultrasound in enhancement of different classes of cancer immunotherapy. Immunomodulatory agents or immunotherapeutics can be categorised as follows. (i) Agents that promote the activity of dendritic cells, antigen-presenting cells (APCs) and/or T lymphocytes, to identify tumour cells as "foreign." (ii) Agents that inhibit immunosuppressive responses in tumours: Such agents may inhibit myeloid-derived suppressor cells (MSDCs) or regulatory T cells (Tregs) or may inhibit the suppressive function of immune checkpoint molecules, including CTLA-4 (ipilimumab), PD-1 (pembrolizumab and nivolumab) and PD-L1 (atezolizumab, avelumab, durvalumab) [17]. (iii) Cellular therapies including chimeric antigen receptor (CAR) T cells, engineered T cell receptor (TCR) therapy, adoptive transfer of tumour-infiltrating lymphocytes (TILs) and cell-based vaccines. This illustration represents distinct categories of immunotherapy and how the effects of microbubbles and ultrasound could enhance each immunotherapy subclass. Reprinted, with permission, from Kooiman et al. [18].

Table 1
CTLA-4 and PD-1/PD-L1 targeted drugs (inhibitors of CTLA-4 and PD-1/PD-L1) approved by the U.S. FDA

Drug name (brand name, manufacturer)	Type of cancer approved to treat	First FDA approval date	Type of checkpoint inhibitor
Ipilimumab (Yervoy, BristolMeyersSquibb)	Melanoma, RCC, colorectal, HCC, NSCLC, malignant pleural mesothelioma	2011	Anti-CTLA-4
Pembrolizumab (Keytruda, Merck&Co)	Melanoma, NSCLC, RCC, Hodgkins lymphoma, HNSCC, Merkel cell carcinoma, MSI-H or dMMR markers, colorectal cancer, gastric cancer, HCC, cervical cancer, PMBCL, SCLC, cutaneous squamous, bladder cancer, breast cancer, endometrial cancer, esophageal cancer, TMB-high cancer	2014	Anti-PD-1
Nivolumab (Opdivo, Bristol-Myers Squibb)	Melanoma, NSCLC, RCC, Hodgkin's lymphoma, HNSCC, colorectal cancer, gastric cancer, HCC, SCLC, bladder cancer, esophagus, malignant mesothelioma	2014	Anti-PD-1
Atezolizumab (Tecentriq, Roche)	Melanoma, NSCLC, HCC, SCLC, bladder cancer, breast cancer	2016	Anti-PD-L1
Durvalumab (Imfinzi, AstraZeneca)	NSCLC, SCLC, bladder cancer, biliary track	2017	Anti-PD-L1
Avelumab (Bavencio, EMD Serono)	RCC, Merkel cell carcinoma, bladder cancer	2017	Anti-PD-L1
Nivolumab + ipilumab (Opdivo + Yerboy, Bristol-Meyers Squibb)	Malignant pleural mesothelioma, NSCLC	2020	Anti-CTLA-4 and anti-PD-1
Tremelimumab + durvalumab (Imjudo + Imfinzi, Pfizer/AstraZeneca Pharmaceuticals)	Metastatic NSCLC, no sensitizing EGFR mutation, anaplastic lymphoma kinase genomic tumour aberrations	2022	Anti-CTLA-4
Relatlimab-rmbw + nivolumab (Opdualag, Bristol-Meyers Squibb)	Unresectable or metastatic melanoma	2022	Anti-LAG-3 and anti-PD-1
Cemiplimab-rwlc (Libtayo, Regeneron)	NSCLC, cutaneous squamous-cell carcinoma	2022	Anti-PD-1
Dostarlimab-gxly (Jemperli, GlaxoSmithKline)	dMMR solid cancers, endometrial carcinoma	2023	Anti-PD-1

In addition to the FDA-approved drugs, a few more drugs have been approved by agencies of the European Union, Japan and China: camrelizumab, sintilimab, tislelizumab and toripalimab [32,34–36].
dMMR, DNA mismatch repair; EGFR, epidermal growth factor receptor; FDA, U.S. Food and Drug Administration; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell cancer; MSI-H, high microsatellite instability; NSCLC, non-small cell lung cancer; PMBCL, primary mediastinal B-cell lymphoma; RCC, renal cell carcinoma; SCLC, small cell lung cancer; TMB, tumour mutational burden.

progression and, in some cases, treatment-limiting side effects [42]. Therefore, a method that can either enable greater delivery efficiency and/or shift the TME from immunosuppressive to immunopermissive would have a major impact for many cancer patients who would otherwise fail immunotherapy [43]. The aim of this review is to discuss the potential application of ultrasound in this context to overcome the limitations of checkpoint inhibitor immunotherapy.

Ultrasound therapy and immunostimulation

High-intensity focused ultrasound

High-intensity focused ultrasound (HIFU, *i.e.,* ultrasound with sufficient intensity to promote tissue ablation) has been utilized in the treatment of multiple diseases, including essential tremor [44,45], uterine fibroids [46] and cancer [47–51]. Several studies have suggested that HIFU can also increase the effectiveness of immunotherapeutic drugs through enhanced delivery and/or by amplification of the natural anti-tumour immune response [52,53]. HIFU enables the local ablation of tumour tissue and can promote other beneficial effects in the TME [54] including a systemic or *abscopal* immune response [55]. For example, improved T lymphocyte-mediated function, measured by a marked increase in the CD4+/CD8+ ratio in peripheral blood of cancer patients with posterior choroidal melanoma, has been observed following hyperthermia generated by HIFU (frequency = 0.8 MHz, acoustic focal peak intensities ranging from 5000 to 20000 W/cm^2, duration ranging from 2.5 to 8 h) [41,56–58]. Currently, there is a growing number of ongoing pre-clinical studies supporting the rationale for combining immunotherapy with focused ultrasound [59]. Silvestrini et al. [60] combined ablative focused ultrasound with anti-PD-1 and CpG, a toll-like receptor (TLR) agonist, in murine B16 melanoma to induce an abscopal effect, resulting in a complete response to therapy in 80% of treated mice with bilateral disease at day 90. The underlying mechanisms have yet to be fully explained and are likely multifactorial, for example, enabling antigen release, promoting vascular alteration, chemokine, or cytokine alteration or dampened immune resistance. Interestingly, other physical cancer treatments such as surgery, microwave and external beam radiotherapy have also been reported to stimulate abscopal effects in pre-clinical and clinical studies [41,61–64], although similarly the underlying mechanisms have never been fully explained.

Ultrasound-mediated cavitation

High-intensity focused ultrasound can induce both thermal and mechanical effects. Of the latter, cavitation—the generation and subsequent oscillation of gas/vapour-filled bubbles—has been reported to be especially important in cancer therapy from accelerating ablative processes to facilitating uptake of chemotherapy [65,18]. In addition, anti-tumour immune responses may be activated by cavitation through potentiation of immunostimulatory or chemotactic factors or through their delivery into cells [52,66–69]. The presence of cavitation bubbles during the application of ultrasound may also lead to bio-effects in tumours such as (i) an increase in blood vessel permeability, (ii) alterations in the molecular composition of the TME, (iii) recruitment and penetration of tumour-infiltrating lymphocytes into tumour and (iv) enhanced extravasation of particles or therapeutics into the interstitial space (Fig. 2) [18,70]. It is also possible that cavitation promotes the release of structurally unaltered tumour-associated peptides, allowing presentation of tumour neoantigens [71].

Pulsed mode high-intensity focused ultrasound (pHIFU) in combination with checkpoint inhibitor immunotherapy has also recently been determined to improve anti-tumour effects compared with control subjects and with the treatments alone. In pulsed mode, exposures last milliseconds or shorter and use high peak negative pressure amplitudes (10–20 MPa) similar to the acoustic parameters used in histotripsy studies [72,73]. In a murine orthotopic model of pancreatic cancer, pHIFU in combination with antibody immunotherapy shifted the TME from an immunosuppressive to a pro-inflammatory microenvironment by an increased ratio of $CD8^+IFN\gamma^+$ T cells to $CD4^+$ T cells, regulatory T cells and MDSCs (peak negative pressure = 17 MPa, frequency = 1.5 MHz, duty cycle = 1%, 1 pulse/s, duration = 25 s) [74]. Acoustic cavitation and physical disruption of the tumours were also reported, and it was suggested that focused ultrasound in combination with checkpoint inhibitor immunotherapy may be able to enhance anti-tumour effects in patients with dense tumours, such as pancreatic cancer [74].

Localised drug delivery

Two key advantages of HIFU are that it can be applied extracorporeally, and the ultrasound beam can be tightly focused to localise tissue damage to the tumour site. Cavitation agents, including gas microbubbles, liquid droplets and solid particles [18], can be used to enable predictable generation of cavitation at the ultrasound focus and thus limit the risk of off-target effects. They can also be used to enable therapy to be delivered at lower, non-ablative ultrasound intensities. Microbubbles have been extensively used as image contrast agents for several decades, and more recently, clinical studies have reported their utility in restoring blood flow after myocardial infarction [75], disrupting the blood–brain barrier [76] and identifying liver lesions [77,78]. Clinical trials have also illustrated the safety and efficacy of ultrasound-triggered microbubble destruction combined with other cancer therapies, such as transarterial radioembolization [79,80].

Cavitation agents offer a means of further increasing treatment localisation by conjugating drug molecules to them to enable their release and/or tissue uptake at the target site. Microbubbles, in particular, can be readily engineered for disease-specific targeting and/or loaded with therapeutic agents to enable controlled drug release by low-intensity focused ultrasound (LIFU, *i.e.,* focused ultrasound with pulse intensity similar to that of diagnostic ultrasound) guided by imaging. Microbubble oscillations induced by LIFU can generate mechanical forces that cause vascular disruption and promote extravasation [70,81,82]. This is particularly important for cancer therapy as, unlike the vessels found in healthy tissue, tumour blood vessels are structurally disorganised [83], and low response rates to immunotherapy may be at least partly attributed to this aberrant vasculature [84]. Li et al. [82] designed and developed a multifunctional microbubble system that incorporated dual loading with a chemotherapeutic agent (Docetaxel) inside the lipid shell, and the anti-PD-L1 monoclonal antibody conjugated to the surface. This group suggested that the system exhibited improved delivery to the tumour site through three combined effects: (i) anti-PD-L1 conjugated to the microbubble surface allowed tumour targeting; (ii) low-intensity focused ultrasound collapsed the microbubbles and released the encapsulated chemotherapeutic agent within the tumour (acoustic focal peak intensity = 2.0 W/cm^2, frequency = 1 MHz, duty cycle = 50%, duration = 5 min); and (iii) cavitation increased tumour vessel permeability and enhanced drug penetration across the tumour vessel walls and interstitium [81]. Anti-PD-1 has been coupled to microbubbles for deployment by low-intensity ultrasound. This combination therapy amplified immune-tumour responses and increased survival compared with conventional delivery of free drug [31].

Immune adjuvants, including TLR agonists, have been used to enhance the therapeutic efficiency of immunotherapy [60,85]. Immune adjuvants are molecules that elicit activation of innate and adaptive immune responses by promoting recruitment of antigen-specific CD8+ T cells to tumour sites [85–87]. The mechanisms underlying immunostimulatory adjuvants for cancer immunotherapy are comprehensively detailed in other reviews and so are not specifically discussed here [88–91]. Despite the benefits of immunostimulatory adjuvants in cancer immunotherapy, however, the systemic administration of TLR agonists inevitably leads to an uncontrolled immune response and damage to healthy tissue [85,92]. Zheng et al. [85] recently reported a study indicating local administration of immune adjuvants by a drug-loaded

microbubble delivery system enhances checkpoint inhibitory immunotherapy (acoustic focal peak intensity = 1.5 W/cm^2, frequency = 1.5 MHz, duty cycle = 50%, duration = 3 min). In this study, docetaxel and the immune adjuvant R837 were loaded into microbubbles and co-injected with the immune checkpoint inhibitor, anti-PD-L1. The tumour inhibition rate for primary and distant tumours reached 88.28 ± 1.04% and 89.86 ± 5.76%, respectively, in the group receiving docetaxel, R837 and systemic delivery of anti-PD-L1 [85].

Anti-vascular effect

The combination of ultrasound and microbubbles has also been used as an "anti-vascular" agent to disrupt blood vessels with several downstream direct and indirect effects when combined with immunomodulators. These include potentially increased trafficking or penetration of immune checkpoint inhibitors (or resulting T cells) into tumour tissues or even enhanced processes for antigen-presenting cell maturation and differentiation. In a colorectal cancer murine model, ultrasound-mediated microbubble therapy induced necrosis via an instant shutdown of blood flow within the tumour tissue [31]. Another study reported cytokine recruitment of immune cells to both local and distant tumour sites in a Her2+ (NDL) murine breast cancer mouse model. This system produced anti-tumour effects via three pathways: (i) carriage of an anti-CD326 antibody to target tumour cells, (ii) sonoporation-enhanced transduction of a non-viral gene encoding IFN-β and (iii) tumour shrinkage by direct mechanical forces. Tumour transfection with IFN-β enhanced the damage-associated profile resulting from the destructive application of ultrasound to targeted microbubbles (frequency = 250 kHz, peak negative pressure = 500 kPa, burst length = 4 ms, pulse repetition frequency = 30 Hz, total duration = 3 min). The combination of microbubbles with checkpoint inhibition reduced tumour growth in both the directly treated and distant tumours, inducing local and systemic immune effects [93].

Future perspectives

Clinical evidence has revealed that the combination of a PD-1/PD-L1 checkpoint inhibitor with a chemotherapeutic agent may be synergistic in specific clinical settings [82,94,95], and indeed, 90% of the new trials that started in 2020 involved combination strategies [96]. However, this combination approach has led to some quite severe side effects, including cardiotoxicity, hematotoxicity, hepatotoxicity and neurotoxicity. To address this challenge, the studies referred to in the previous section suggest that ultrasound-mediated cavitation may enhance the delivery of immunotherapeutic and chemotherapeutic agents while reducing toxicity compared with the free drug combination. Clinical studies are now ongoing to determine the efficacy of combining focused ultrasound with immunotherapy. A clinical trial evaluating the combination of focused ultrasound with pembrolizumab in solid tumours (AM-003/NCT04116320) is currently underway at the University of Virginia [59,97]. Another clinical trial also from the University of Virginia and investigating the combination of focused ultrasound with pembrolizumab in metastatic breast cancer patients (Breast-48/NCT03237572) was completed on 17 June 2022 but no results have been yet reported. Aside from these trials, there are others listed, recruiting or completed clinical studies worldwide exploring the use of focused ultrasound in combination with checkpoint inhibitors.

Combinations of anti-PD1/PD-L1 agents with anti-angiogenic or anti-vascular agents are also currently being tested in >80 ongoing clinical trials [98], and again, pre-clinical studies have determined a potential

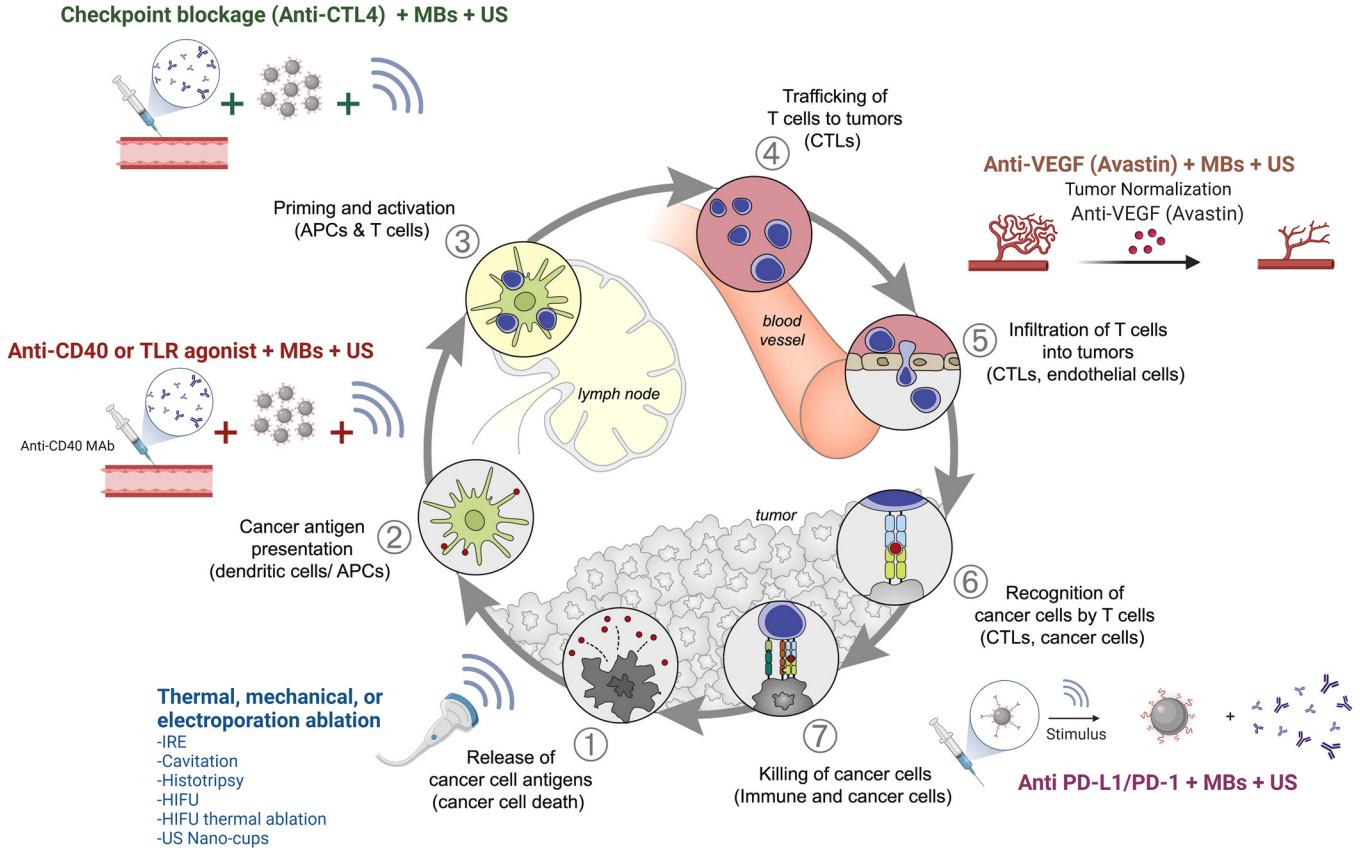

Figure 3. Therapeutic applications of ultrasound and cavitation nuclei in the cancer immunity cycle. The generation of immunity to cancer is a cyclic process characterized by inhibitory factors that lead to immune regulatory feedback mechanisms. This cycle can be divided into seven major steps, starting from the release of cancer cell antigens and ending with the killing of cancer cells. The goal of cancer immunotherapy is to enable amplification and propagation of a self-sustaining cycle of cancer immunity *but* avoid a generation of unrestrained autoimmune deleterious inflammatory responses. The illustration simplifies the temporal pathways and interactions between immune compartments during the immune response to cancer. Retrieved and adapted for ultrasound and cavitation nuclei from Chen and Mellman [19]. Created in Biorender.com.

role for ultrasound here too, through cavitation-enhanced delivery or direct ablation of the vasculature. Many questions remain, however, regarding the most effective combination of therapeutic modalities, including the optimization and choice of targeted therapies to enhance antigen presentation and cytotoxic T-cell priming in specific histology, indications or clinical scenarios [99]. A wide variety of checkpoint inhibitors and monoclonal antibodies are currently available in the clinic for cancer treatment. It is therefore of great importance to have a comprehensive scientific understanding of the mechanism(s) underlying the combination of ultrasound therapy and immunomodulation.

The cancer immunity cycle (Fig. 3) is a further key consideration to determine the appropriate timing of therapy delivery [19]. The different classes of immunotherapies face differing delivery challenges, and in many cases, their success relies mainly on the interaction with the targeted protein. These targets may focus on different time points in the linear evolution of a dynamic immune response or enhanced tumour immunity. In a syngeneic model of epithelial cancer [60], focal ultrasound therapy following immunotherapy resulted in increased number of leukocytes and CD8+ T cells, as compared with all control groups. Other studies have similarly conclued that, because of mechanical and immunological changes in the TME following local ablation, there may be a optimal window of opportunity for employing immunotherapy [31].

Two further important questions are whether a robust systemic immune response may be maintained without continued systemic exposure over time to an immunotherapeutic and whether there are any long-term adverse effects. Emerging evidence indicates that clinicians should be aware of the risk of chronic immune-related adverse events (irAEs) and their long-term outcomes, which are currently understudied in clinical trials [100].

Conclusions

Despite major advances in cancer immunotherapy, its widespread clinical success has been hindered by multiple factors, including cost, poor delivery efficiency and severe adverse effects in non-target organs. As a non-invasive and well-established clinical modality, ultrasound offers a potentially attractive means of addressing these challenges. Ultrasound ablation has been found to promote a systemic (abscopal) immune response and to enable potent anti-vascular effects. Ultrasound-mediated cavitation has similarly been found to promote a range of immunostimulatory effects and to both localise and enhance delivery efficiency of immunotherapeutics and/or other anti-cancer drugs. In recent years there have been numerous pre-clinical studies demonstrating therapeutic efficacy in a range of tumour models. The underpinning mechanisms, however, remain poorly understood and hence further work is required to identify optimal treatment regimens.

Conflict of interest

The authors declare no competing interests.

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