Ultrasound induced cancer immunotherapy

Johan Unga[a,b], Mitsuru Hashida[a,c*]

a) Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
b) Japan Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo 102-8471, Japan
c) Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida Ushinomiya-cho, Sakyo-ku, Kyoto 606-8501, Japan

* Corresponding author. Tel: +81-75-753-4545; fax: +81-75-753-9260.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

Contents

ABSTRACT

Recently, use of ultrasound (US) has been shown to have potential in cancer immunotherapy. High intensity focused US destruction of tumors may lead to immunity forming *in situ* in the body by immune cells being exposed to the tumor debris and immune stimulatory substances that are present in the tumor remains.

Another way of achieving anti-cancer immune responses is by using US in combination with microbubbles and nanobubbles to deliver genes and antigens into cells. US leads to bubble destruction and the forces released to direct delivery of the substances into the cytoplasm of the cells thus circumventing the natural barriers. In this way tumor antigens and antigen-encoding genes can be delivered to immune cells and immune response stimulating genes can be delivered to cancer cells thus enhancing immune responses. Combination of bubbles with cell-targeting ligands and US provides an even more sophisticated delivery system whereby the therapy is not only site specific but also cell specific.

In this review we describe how US has been used to achieve immunity and discuss the potential and possible obstacles in future development.

Abbreviations: AB, antibody; BL, bubble liposome; CTL cytotoxic T lymphocyte; DC, dendritic cell; FUS, focused ultrasound; HIFU, high intensity focused ultrasound; hsp, heat shock protein; IFN, interferon; IL, interleukin; MB, microbubble; NK, natural killer cell; pDNA, plasmid DNA; PFC, perfluorocarbon; TNF-α, tumor necrosis factor α; T_{reg}, regulatory T cell; US, ultrasound.

Keywords: Tumor vaccine, Immunity, HIFU, Tumor ablation, Microbubbles, Bubble liposomes, Nanobubbles

1. Introduction

Cancer is caused by the patient's own cells growing in an uncontrolled and harmful way. In general the immune response towards the cancer is weak since the immune system sees the cells as "self". Furthermore, in cancer tissues the environment often suppresses the immune response by expression of receptors on the cancer cells and secretion of various immune suppressing substances [1].

In recent years there have been a number of reports that US can be used to boost immune response towards cancer. In this review we describe both the direct effect of US on tumors that can induce immune response and the use of US-sensitive drug carriers for delivery of immune-stimulating substances.

2. Overview of cancer immunotherapy

Immunotherapies are therapies where the natural immune response of the patient is activated or enhanced so that it acts to combat the disease. In cancer this means that the immune system should be made to attack the tumor or cancer cells but leave normal, healthy cells alone. This can be done in different ways, by unspecific increase of the immune system, by using monoclonal antibodies, by adoptive cell transfer and by *in vivo* cancer vaccines [2]. Immunity can be divided into humoral immunity and cell-mediated immunity. Humoral immunity acts through antibodies (ABs) produced by B lymphocytes and cell-mediated immunity through T lymphocytes. Both types of lymphocytes can be activated by tumor antigens (TAs), which are mainly proteins and peptides from tumor cells. ABs are proteins that have affinity for a specific structure, for example a surface protein of a cancer cell. T cells are activated when the antigen is presented to them by major histocompatibility complex (MHC) molecules on cell surfaces. T helper cells (CD4+ cells) are activated by TA on MHC class II on antigen presenting cells (APCs), most importantly dendritic cells (DCs) and cytotoxic T cells (CTL, CD8+ cells) which can be activated by TA on MHC class I which is expressed on all cells [3]. CTLs can directly attack cancer cells showing the right antigens by releasing cytotoxins that lead to death of the target cell. The

T helper cells act by releasing cytokines, which is an important factor in CTL and B cell activation.

There are three steps essential for effective immune response against cancer [4]. Firstly DCs need to be exposed to TAs. The DCs also need to get a "maturation signal" that leads to immunity to the antigens instead of tolerance. Many maturation signals have been identified, such as pathogen associated molecular patterns, toll like receptor ligands, tumor necrosis factor α (TNF-α) and many more; however, the exact relationship between these signals is still not clear [5]. The second condition for immune response is T cell activation by DCs in the lymph nodes. If DCs that present antigens have not been activated by maturation signals they will instead induce tolerance in the T cells and thus counteract immune reaction [6]. The third step for effective immune action against a tumor is infiltration by the activated T cells in the tumor tissue and that they retain their activity and kill the cancer cells. The tumor microenvironment can also prevent the T cell effect in different ways, e.g. by the action of myeloid-derived suppressor cells and regulatory T cells (T_{reg}) that oppose the action of the activated immune cells and by the tumor cells down-regulating their MHC class I expression and release immune suppressing substances [1]. Immune therapies can act anywhere in this complex system but understanding the whole process will be essential for a successful outcome.

ABs are the immune modulating treatments against cancer most often clinically used today, and there are several approved products on the market [7]. Since ABs can be designed to bind to virtually any cell surface structure, they are very attractive tools for targeted treatment. In cancer therapy, ABs have been used to

achieve targeted drug and radioisotope delivery and have been employed as immune-regulating agents. In immunotherapy, ABs can have several roles: (1) as a new antigen by binding to cancer cells and be discovered as non-self by immune cells; (2) as blocking agents of receptors involved in down-regulation of the activity of CTLs; or (3) oppositely be used to stimulate receptors that enhance the activity of immune cells [8].

Cancer vaccination can be performed in different ways. The simplest is the "classic" vaccine type where whole cancer cells removed by surgery or cancer cell line cells that carry some characteristic antigens of the cancer in question have been made non-viable by, for example, freeze–thawing or ultraviolet radiation. Then the cells or cell parts are injected into the patient [9]. This makes the TAs available for detection by DCs *in vivo* and can thus trigger an immune response. Another type of vaccination is adoptive cell transfer (ACT) in which activated anti-tumor lymphocytes are infused into the patient. The T cells are taken from the tumor tissue, tested for anti-cancer activity, expanded *ex vivo* to greater numbers and then re-infused into the patient [10, 11]. DNA vaccination of tumor cells is another approach. Instead of directly potentiating the immune response towards the tumor, the tumor itself is made more immunogenic [12]. As mentioned there are several AB products on the market today. For example, Bevacizumab is an antibody that binds to vascular endothelial growth factor (VEGF) and prevents its function. VEGF is involved in the formation of new blood vessels in the tumor so blocking VEGF reduces this and thus the delivery of nutrients to the tumor [13]. Rituximab is another example, which is used in

lymphoma where it binds to CD20 lymphoma cells and causes cell lysis and apoptosis [14].

When it comes to cancer vaccines there is only one substance approved today in the USA, Sipuleucel-T (or Provenge as it is known) induces targeting by the immune system of the antigen PAP and is approved for treatment of prostate cancer [15]. Sipuleucel-T is a cell-based therapy; cells are taken from the patient, cultured *ex vivo* with PA2024, a fusion protein where PAP has been conjugated with granulocyte-macrophage colony stimulating factor (GM-CSF). The cells are then infused back into the patient, discovered by the immune system and lead to immune response towards PAP which is expressed in about 95% of prostate cancers.

3. US

US is sound waves of frequencies from about 20 kHz and above, which is higher than can be detected by the human ear [16]. An US wave is created at the US transducer and propagates as intermittent high and low pressure zones through a medium.

3.1. Biological effect of US

Since US has a long history in medical applications the effects on biological tissues are well known. US used for *in vivo* imaging is generally considered safe but it is not completely without side effects (for a review see [17]). Adverse effects come primarily from two mechanisms: thermal effects and mechanical or cavitation effects [18]. Thermal effects are due to absorption of the US energy.

The amount of heating depends on both the US and the tissue exposed. From the US side, the energy of the US source, the tissue volume irradiated (i.e. concentration of radiation) and exposure time affect the heating. The heating of a tissue depends on the molecular composition, thermal conduction and blood perfusion.

Bubble destruction due to inertial cavitation can cause direct tissue damage through heat and jet streams from the collapsing bubble but this may also lead to formation of free radicals that can then cause indirect tissue damage [19, 20].

3.2. Medical applications of US

US diagnosis can be performed with or without the use of contrast agents. US contrast agents are gas bubbles of a few micrometers that are injected and increase the contrast of blood vessels and blood perfused organs [21, 22].

Low energy US can also be used to assist healing. Therapeutic US has been used for repairing ligaments, inflamed tendons, stiffened joints, fractured bones and cartilage, wound healing, nerve stimulation and more [23]. The main cause of these beneficial effects is considered to be heating and increased blood flow but also more specific effects like increased protein expression [24] and stimulation of macrophages [25].

Another application is high intensity focused ultrasound (HIFU) for tumor destruction (ablation) in cancer. Focusing the US energy achieves a high concentration of US energy in a small volume. This can cause local necrosis of tumor tissue while doing minimal harm to adjacent tissue. HIFU will be described in more detail in section 4.

3.3. US in drug delivery

US has been used in many drug delivery applications, generally taking advantage of the possibility to "activate" drug delivery vehicles *in situ* to release the loaded drug in a specific place in the body and thereby achieve a localized effect. The drug vehicles have been various dispersions e.g. temperature sensitive liposomes [26], bubbles [27] and perfluorocarbon emulsions [28]. The procedure is generally that the US-sensitive formulation is injected intravenously or in the target area and then the US is applied from an external source. This approach has been applied to facilitate transdermal drug delivery [29], delivery to the heart [30], crossing the blood brain barrier [31], to skeletal muscle [32], gene delivery, to achieve plasma membrane poration [33] and delivery of drugs to treat cancer [34].

4. Direct US for enhanced immunotherapy

4.1. US tumor ablation and immunity

Cancer tumor ablations aim to cause cancer cell necrosis in the tumor with no or limited damage to surrounding tissue. Apart from with US, ablation can be achieved by different methods including radiofrequency [35], microwaves [36], laser irradiation [37] and cryo ablation [38]. Much of the knowledge about the connection between tumor ablation and an immune response towards the tumor comes from research on cryo ablation in which the tumor is frozen causing tissue damage and cell death. Upon freezing and thawing cells are lysed leaving debris such as cell membrane fragments and intracellular proteins. It has been shown

that cryo ablation can cause infiltration of immune cells in the tumor [39] as well as DC maturation [38]. Combining cryo ablation with immunomodulatory agents can further increase the efficacy of the treatment [40].

Tumor ablation can also be achieved using HIFU. In HIFU a transducer outside the body is used to focus the US radiation at a small point in the body so that the US wave density at that point becomes very high. Continuous irradiation leads to local temperature increase but shorter pulses can also cause damage by mechanical means due to the pressure oscillations. Temperatures above 55°C for at least 1 s lead to coagulative necrosis and immediate cell death in the targeted tissue [41].

The cell debris differs depending on the US ablation method. If the thermal effect dominates then cell death is caused by heating leaving a coagulated tissue; however, with mechanical damage the cells are torn to pieces but because the temperature increase is low coagulation does not occur [42].

At higher US intensities acoustic nonlinearities cause higher harmonics and shock waves that can lead to considerably more mechanical damage and higher temperatures at the focus point than would be expected if this nonlinearity is not taken into account [43, 44]. This can lead to tissue boiling after very short exposure times, so-called millisecond boiling. Wang and colleagues showed that with a 10-ms exposure complete tissue destruction (or emulsification as it is also called) was achieved without any heat damage, whereas if the exposure time was increased substantial thermal damage could be achieved with exposures up to 500 ms [45]. Since the volume that is irradiated with each HIFU burst is small, many single bursts are usually needed to treat the whole tumor volume. Today

HIFU has been applied in clinical studies to treat prostate cancer, breast cancer, kidney tumors, liver tumors, pancreas tumors and glioblastoma in the brain [46].

One of the early clues to the immune-stimulating potential of HIFU came from Yang and co-workers [47]. They treated mice with subcutaneous murine neuroblastoma C1300 with HIFU to ablate the tumors. HIFU alone cured 53% of the animals and after tumor ablation, the tumor growth after a second tumor challenge was significantly reduced compared to animals that had not had a tumor at all and animals that had an untreated tumor since previously. The difference between the HIFU-treated mice and those with an untreated tumor showed that this effect was induced or at least increased by the tumor ablation. Combination of mechanical HIFU and surgery has also shown a down-regulation of signal transducer and activator of transcription 3 (STAT3), which is known to be involved in tumor development [48]. Compared to only surgery the combination treatment also led to an increase in dendritic cell numbers and activity. Decrease of immuno-suppressing cytokines like VEGF and transforming growth factor β 1 and 2 in circulation in patients has also been seen after HIFU tumor ablation [49].

The immune modulating potential of HIFU ablation *in vivo* in human has been known for some time. Rosberger and colleagues demonstrated the ability of US treatment to affect the T cell helper/suppressor ratio (CD4+/CD8+) [50]. Patients with posterior chordial melanoma were treated with HIFU. Of the five patients treated, two had inverted CD4+/CD8+ ratios (CD4+<CD8+) prior to the

treatment – one week after the treatment the ratio had reverted to normal (CD4+>CD8+). Furthermore, an increase of CD4+/CD8+ ratio was noted in one of the three patients that initially had normal ratios. The change in CD4+/CD8+ ratio as well as a general increase of CD4+ lymphocytes in circulation after HIFU ablation of tumors has also been reported elsewhere [51].

Further supporting the conclusion that the ablation of the tumor and not the US itself is the cause of the immune regulation is the fact that other ablation methods have also shown an increase in immune response, such as radiofrequency ablation and cryo ablation [35, 40].
Differences between thermal and mechanical ablation were studied by Hu and colleagues [52]. Firstly, the appearance of the cell debris differed, the mechanical HIFU had completely destroyed the tumor cells whereas after thermal HIFU large lumps of coagulated cells as well as seemingly intact cells remained. The two different lysates also showed different potency to induce an immune reaction. Dendritic cells exposed to the cell debris *ex vivo* had up-regulated expression of co-stimulatory molecules and secretion, and macrophages showed an increased secretion of TNF-α compared to non-treated cells (Fig. 1). In both cases the effect was higher in cells exposed to the debris from mechanically lysed cells than those exposed to debris from thermal HIFU.

Similarly, Zhang and co-workers examined the difference between H22 hepatocellular carcinoma (HCC) cancer cell vaccines generated from HIFU-treated tumors and non-treated tumors [53]. The cytotoxicity of CTLs *in vitro* was found to be higher; and when used as a vaccine the tumor growth rate was

decreased in the HIFU group compared to non-HIFU vaccine. In a similar way as for Yang and co-workers [47], if mice first had a tumor treated with HIFU and then were once again challenged the survival rate was 100% after 48 days (20% in both vaccinated groups). Recently, transfer of T lymphocytes from mice after HIFU treatment of H22 HCC was reported. It was seen that tumor ablation led to an increase in the cytotoxicity of tumor-specific T lymphocytes in the spleen, and T lymphocytes from harvested spleen showed increased cytotoxicity and secretion of IFN-γ and TNF-α *in vitro* [54]. When the T lymphocytes were transferred to tumor cell challenged mice, the metastasis rate decreased and complete tumor regression and survival increased.

The reason for the difference between mechanical and thermal HIFU ablation is not clear. It has been suggested that the mechanical treatment leaves more cell components like proteins in their natural structure whereas heat treatment might denature them. Still, cell destruction by freeze–thawing that could be expected to be more similar to the mechanical HIFU in the preservation of the natural conformations of molecules gave lower maturation of DCs by co-culture than debris from thermal HIFU-ablated tumors [55]. Probably HIFU also has effects other than the destruction of the tissue. One factor may be expression of heat shock proteins that have been found to increase by heating such as after HIFU ablation [52-54, 56, 57] but also by MBs combined with focused US treatment, the latter probably due to the mechanical stress exerted on the tissue [58].

Different thermal HIFU methods have also shown differences in effect. A lower temperature HIFU (55°C) gave a stronger immune response seen as more DC

infiltration and *in situ* maturation than a higher temperature (80°C). Also a sparse HIFU scanning approach that left a gap of non-treated tissue between the lesions gave a stronger response than complete ablation [59]. Contributing to the immune response is that HIFU can facilitate penetration of the tumor tissue by dendritic cells [42], activated CTLs and natural killer (NK) cells [60].

Generally, all the reports show an induced or modified immune response following HIFU ablation of tumors *in vivo* and cancer cells *in vitro* (see Table 1). The transfer of immunity in the form of a vaccine has been shown to be possible, and the most promising results in transferred immunity may be transfer of live activated lymphocytes from one host to another, which resulted in higher CTL activity and increased survival [54]. HIFU treatment is not completely without risks: skin redness, edema pain and necrosis outside the target area are common complications [61] and more serious are reports of metastasis formation [62].

4.2. Sonoporation-assisted immunotherapy

If lower US energy than needed for ablation is used it can still induce temporary disruptions in cell membranes [63]. This phenomenon is known as sonoporation and has been examined as a means to improve the delivery of various drugs and other bioactive agents [33]. The exact mechanism of sonoporation of cell membranes is not yet clear but morphological changes in cell membranes have been shown [63]. US that irradiates a tissue will lead to heating and this increases the fluidity of lipid bilayers and makes passive transport (by diffusion) through the bilayers easier [64]. Compared to bubble assisted sonoporation (see section 5) higher energy is needed since the whole tissue needs to be affected

and not only the US sensitive bubbles. However, the damages caused are limited and in general completely reversible. Sonoporation can be utilized to facilitate drug transport into cells and thereby the normal barriers can be circumvented. Another effect that US can directly have on tissue is increased extravasation from the bloodstream to surrounding tissue [65].

Casey and co-workers utilized direct US sonoporation to deliver GM-CSF to solid tumors [66]. Administration of plasmids carrying the GM-CSF after US resulted in 55% survival rate (0% with no US) as well as complete resistance to repeated tumor challenge, demonstrating an anti-cancer immune response. Transfer of splenocytes from treated mice to naïve mice also resulted in complete resistance (100% survival) to tumor challenge leading to the conclusion that the immune response was cell mediated.

Other studies have also reported successful US sonoporation-assisted delivery of plasmid DNA to prostate cancer cells *in vitro* [67], prostate tumor cells *in vivo* [68] and IFNβ-loaded cationic liposomes to hepatic tumors [69]. Monoclonal antibodies have also been used in combination with non-destructive US, and showed increased cell cytotoxicity *in vitro* [70, 71] and increased tumor uptake of monoclonal antibodies with pulsed non-destructive HIFU have been demonstrated *in vivo* [72, 73].

5. Immunotherapy assisted by gas bubbles and US

In the majority of reports where US has been utilized for enhancement of immunotherapy by delivery of bioactive molecules, it has been in combination

with some sort of gas bubble in the nanometer and micrometer size range that can be made to cavitate by US at the target site.

In an US field of sufficient power, bubble destruction will occur [74, 75]. The bubbles can thus serve as targets resulting in local release of energy in the form of radiation force, micro-streaming, shock waves, free radicals, microjets and strain [16]. The destruction of MBs in a US field generally increases with decreasing frequency and increasing power [76].

The destruction of a bubble close to a cell membrane can lead to temporary formation of pores in the membrane [77, 78] but also non-destructive bubble oscillation can lead to increased cell membrane permeability [79-81].

There is in principle no difference between MBs and so-called bubble liposomes (BLs) in how they are used. For both types the fundamental idea is the same, that bubbles are delivered together with an active substance and US irradiation of the bubble facilitates the delivery of the substance. The most important difference is that the BLs are smaller (below 1 μm) and appear to consist of nanometer-sized gas bubbles enclosed in somewhat larger liposomes, whereas the MBs are gas bubbles stabilized by surface active compounds [80, 82]. Often in cancer treatment the aim is to take advantage of the so-called EPR (enhanced permeability and retention) effect by which nanoparticles can enter into tumor tissue due to blood vessels there being more leaky and then remain in the tumor tissue [83]. Although the BLs are larger than the ideal size to take advantage of the EPR effect there are reports of extravasation even for bacteria larger than 1000 nm [84] so it may be possible that the BLs could also accumulate in tumors. The size difference also has implications on such things as stability and potential

applications and therefore we have chosen to treat them separately. Reports of studies on the immune effects of bubbles in combination with ultrasound are listed in Table 2.

5.1. MBs

Gene delivery using a combination of MBs and US has been explored for some time [85]. MBs for *in vivo* use were originally developed as contrast agents for US imaging. At first only bare air bubbles were utilized but modern MBs consist of an inert gas with low water (and blood) solubility (mostly fluorinated alkanes, C_xF_{2x+2}, or sulfur hexafluoride, SF_6) stabilized by a shell of polymer, protein or surface active agents (usually phospholipids), and are in the order of 1–8 μm in diameter [82]. However, the stability is limited and most MBs have half-lives for the retention of the gas in the order of a few minutes *in vivo*. Commercial MBs are usually provided as a freeze-dried powder in a vial containing the gas. The bubbles can then be re-constituted by addition of water and some agitation.

Many of the studies on MBs for drug and gene delivery have used commercially available US imaging contrast agents such as Albunex [85], BR14 [86], SonoVue [58, 87], Optison [32, 67, 77, 88], Definity [89] or similar bubbles produced in-house. Yet others have used a similar type of bubble equipped with targeting ligands on the surface [90] or bubbles complexed with active substances [91, 92].

5.1.1. Co-injection

The simplest approach for MB delivery of bioactive substances is to use co-injection. MBs and the bioactive substance are mixed in solution *ex vivo* and the mixture is then injected and US is applied *in vivo*. This approach has the advantages that both components can be handled completely separately up until immediately before injection, and two separate injections of the components can even be done. Furthermore, this makes adjusting the treatment simple since it is possible to change the relative ratio of components and also to add or exchange one component without the other having to be taken in consideration.

Several reports have demonstrated the feasibility of this approach. Sakakima and colleagues used a lipid-stabilized perfluoropropane MB together with plasmid DNA coding for IFN-β [86]. *In vitro* tests of a combination of bubbles, IFN-β pDNA and US showed an increase in IFN-β expression and a significant decrease of cell viability. The treatment resulted in a significant decrease of tumor growth for mice *in vivo*.

In another report on pDNA delivery with MBs, SonoVue MBs were utilized to deliver the cytokine Interleukin-27 (IL-27) encoding pDNA to prostate tumors in mice [87]. The IL-27 pDNA was co-injected with the MBs intravenously and US was applied immediately. This treatment was repeated three times with 48-h intervals and resulted in a substantial reduction of tumor growth in all three tumor models tested (RM1, TRAMP-C1 and TRAMP-C2). Additionally, an increase in the infiltration of CD3+ and CD8+ cells in the tumor after treatment was detected, indicating an activation of the immune system.

Even if plasmid DNA is not actively coupled to the MB, interaction may occur. When pDNA was pre-mixed with Optison MBs (with albumin shell), the pDNA

delivery to cells *in vitro* was improved compared to MBs and pDNA separately put to the cells [77]. Microscopy and gel electrophoresis showed that the pDNA had accumulated on the MBs.

5.1.2. Conjugates of MB and drug

One potential problem with co-injection of MBs and the active substance is that they might not distribute identically in the body. Therefore MB formulations in which the MBs have been coupled to the active agents delivered have also been tested. This also gives another potentially advantageous benefit, namely that the bioactive substance will be in very close proximity to the MB.

MBs coupled to DNA/RNA lipoplexes with a biotin–avidin coupling have been shown to deliver the lipoplexes into the cytoplasm of adjacent cells when exposed to US [92]. The mechanism was deduced to be either diffusion through pores appearing in the cell membranes following MB destruction or direct "injection" of the lipoplexes by microjet streams created when the MB collapses. Once inside the cell mRNA loaded lipoplexes have the ability to induce gene transcription [91]. The mRNA–lipoplex–MB complexes were put to DCs *in vitro* and exposed to US. Using fluorescently labeled mRNA, successful delivery was seen in over 50% of the treated cells, and using mRNA coding for green fluorescent protein showed that the mRNA was active after delivery. Additionally, treatment with MB–mRNA lipoplexes and US resulted in a slight increase of DC maturation markers CD40 and CD86 compared to control [91].

Lemmon and co-workers presented an interesting method for delivery of pDNA to phagocyting mouse macrophages. Rabbit ABs were bound to MBs and were then put to the macrophages which took up the MBs by phagocytosis and finally

US was applied, causing bubble cavitation and releasing the pDNA inside the cells [93].

Other applications of MBs and US have also been tested. Adenovirus delivered immunotherapy, with GM-CSF and IL-12 genes, has also been combined with MB–US enhanced delivery of endostatin and calreticulin genes with a synergistic effect of the two treatments [94]. MBs without US exposure have also been used as an adjuvant in delivery of ovalbumin, leading to increased antibody and T cell responses *in vivo* [90].

5.1.3. MB without drug

MBs without any active agent have been shown to have potential as immune response triggers. Liu and co-workers examined the effect of SonoVue MBs in combination with focused ultrasound (FUS) on solid CT-26 tumors in mice [58]. MBs were injected intravenously followed by immediate US exposure of the tumor. The treatment resulted in decreased tumor growth compared to treatment with only US. Immune cell infiltration increased in the tumor tissue; both CD8+ CTL and CD4+ non-T_{reg} levels increased, whereas CD4+ T_{reg} levels were not affected by the MB–FUS treatment.

MBs with US have also been used to increase the permeability of the blood–brain barrier to immune cells. With MB+US exposure the NK cells present in the blood moved across the blood–brain barrier to a much higher extent than if only US was used [89].

5.2. BLs

Several reports have been made on the use of BLs, gas-containing lipid particles. BLs are prepared from liposome dispersions to which perfluoropropane (PFP) gas is added at elevated pressure, and the sealed vial is then sonicated and PFP-containing particles with diameter less than 1 μm are formed (typically 500–700 nm) [95-100]. The suggested structure of these particles are gas bubbles enclosed in a larger liposome [80].

5.2.1. Non-targeting BLs

BLs have been utilized for delivery of ovalbumin (OVA) to DCs *in vitro* [99]. DCs were treated with OVA in combination with BLs and US. After treatment, OVA fragments were presented on MHC class 1, and cells treated with BLs + US gave a significantly higher IL-2 levels when co-cultured with CD8-OVA1.3 cells (see Fig. 2). The DCs were also used to immunize mice against E.G7-OVA tumors, leading to complete rejection of the tumor, and transfer of OVA+BL+US-treated DCs led to increased survival of the mice [99]. Immunization with DCs treated with TAs with the use of BL and US has also been shown to decrease melanoma tumor metastasis rate [95].

BLs have also been employed to deliver the immune-stimulating IL-12 gene to solid tumors leading to significantly decreased tumor growth. The effect was CD8+ T lymphocyte dependent; no effect was seen in CD8+ T lymphocyte depleted mice [96].

5.2.2. Targeting BLs

BLs have also been used with active targeting to increase the efficacy and specificity of the effect. For example, enhanced gene delivery was achieved to mannose receptor expressing DCs using lipoplexes with a mannose moiety as a targeting ligand on their surface, together with BLs and US [97]. Complexes with pDNA and mannose-modified BLs have also been utilized for immunization towards cancer of mice *in vivo*, leading to substantially increased secretion of cytokines TNFα, IFN-γ, IL-4 and IL-6 and also enhanced activity of CTL [98]. The treatment also led to decreased tumor growth and increased survival (Fig. 3). Similar results of pDNA immunization have also been achieved in another tumor model (E.G7-OVA) [101].

The mechanism for gene transfection with targeting BLs has been examined in more detail by Un and co-workers [102]. They found that, firstly, the use of a targeting ligand (mannose) on the BLs increased the overall association with mannose receptor expressing macrophages. Secondly, US exposure significantly increased the internalization of the pDNA, and the internalized pDNA after US was to a high degree in the cytoplasm and not in endosomes, which was in contrast to without the use of US (Fig. 4). The involvement of transcriptional factors of the cells in overall transfection following US–BL delivery of pDNA was also studied [103]. It was found that after US exposure, transcription related to activator protein 1 (AP-1) and nuclear factor-κB (NFκB) was increased, thus giving higher protein levels, whereas other tested activators were unchanged. This means that care must be taken in the design of plasmids for gene delivery so that appropriate promoters are used [80, 83, 84]108].

6. Conclusion

It is well established that ablation of a tumor can cause specific immunity towards the cancer. An ablated tumor is very much like a vaccine, i.e. various pieces of the original tumor can serve as antigens. It has also been shown that immediate removal of the tumor after ablation leads to a lower immune protection compared to if the tumor remains are left in the body [62]. This together with the fact that a previous HIFU treatment of a tumor gives better protection than a cancer vaccine made from ablated tumor (as in [53]) shows that not only the antigens themselves are important but also the "presentation". Additionally, at the border of a HIFU-ablated volume there is a high secretion of cytokines by immune cells and high levels of heat shock proteins making it a suitable environment for maturation of CTLs [56, 57].
Furthermore, lower temperature HIFU ablation will result in a stronger immune response, probably by preserving the antigens and facilitating DC penetration [42, 52, 59]. For tumor destruction and curing the primary disease a high temperature ablation has been shown to be more efficient [42]; so using mechanical HIFU requires serious consideration of the pros and cons.

Non-destructive US with or without any bubbles has been shown to increase the delivery of active substances including ABs and immune-stimulating pDNA to tumors, without invoking any serious damage [66]. Immunity has been achieved by two different approaches, delivery of TAs to naïve DCs or delivery of immune-stimulating agents to tumors. The interaction between US and the bubbles,

leading to bubble destruction, can deliver substances into the cytoplasm of cells and thus bypass the normal obstacles [92, 102].

Different types of macromolecules can be delivered in this way, including mRNA delivery to DCs [91], pDNA to tumor cells [86, 87, 96, 98] and various cancer antigens to DCs [95, 99]. This has great possibilities since virtually any type of bioactive substance could be delivered using bubbles and US – of course it may not always be feasible due to the complexity of these delivery systems. Even more complex but also showing good potential are bubbles equipped with targeting ligands [98].

Both micrometer- and nanometer-sized bubbles have two major drawbacks for use as delivery vehicles to tumors; firstly, they are too large to effectively penetrate many tumors and secondly, the gas content and thus the ability to be triggered by US has a fairly short half-life *in vivo*. One possible solution to this problem is to instead use emulsions containing liquid that could be turned to gas at the desired site and time by US irradiation, and some groups have begun looking at this possibility [28, 104, 105]. Another potential problem is that the formulations are often complicated and require some preparation steps immediately before use, and due to the generally quite poor stability of any gas bubble it might be difficult to make a ready-to-use product that can be stored for months or years.

Overall the use of US to achieve immune response towards cancer is showing promise. US has the great advantage of being non-invasive and is possible to use on only the tumor tissue, making it attractive for both direct US treatment and

activated drug delivery. Immune responses against cancer have been achieved both with US alone and by delivering immune-stimulating substances with the assistance of US. The field is fairly young and many mechanisms are still not fully understood, both on the technical side regarding the interaction between US, delivery vehicles, bioactive molecules and target tissues and also on the biological, cancer and immune system side.

Acknowledgments

The authors would like to thank The Japan Foundation for the Promotion of Science (JSPS) for financial support.

Table 1
Summary of tumor types and immunological effects after HIFU treatment in the references of the present paper.

Table 2
Summary of experiments and immune responses after treatment with microbubbles or bubble liposomes in combination with US.

Fig. 1. Difference in TNF-α secretion from RAW264.7 macrophages after exposure to MC38 cancer cell debris from different destruction methods. (Reproduced with permission from [52]).

Fig. 2. MHC class I restricted OVA presentation after OVA delivery into DCs using a combination of BLs and US exposure. DCs were pulsed with OVA alone or OVA in conjunction with US exposure and/or BLs. After US exposure, the DCs were incubated for 1 h at 37°C and then washed with PBS. After culturing for 24 h, the DCs were co-cultured with CD8-OVA1.3 cells for 20 h. The concentration of IL-2 in the supernatants was measured. Each data point represents the mean ± S.D. for triplicate measurements. □Pb0.05 compared to the group treated with BLs or US, or without BLs and US. □ □Pb0.01 compared to the group treated with BLs or US, or without BLs and US. (Reproduced with permission from [99]).

Fig. 3. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG2000 bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments on solid tumors. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging effects of survival in tumor-transplanted mice (C) by DNA vaccination using Bare–PEG2000 lipoplexes, Man–PEG2000 lipoplexes and Bare–PEG2000 bubble lipoplexes with US exposure and Man–PEG2000 bubble lipoplexes with US exposure (50 μg of pDNA). Two weeks after the last immunization, B16BL6 cells were transplanted subcutaneously into the back of mice (n = 8–10). The tumor volume was evaluated. (Reproduced with permission from [98]).

Fig. 4. The effects of US exposure timing on cellular association and internalization of pDNA obtained by unmodified and Man–PEG2000 bubble lipoplexes with US exposure in cultured mouse macrophages. *In vitro* confocal images of cellular associated pDNA obtained by unmodified and mannose–PEG2000 lipoplexes (5 μg of pDNA) with US exposure at 60 min after the addition of bubble lipoplexes. US was exposed at 0, 3, 5, 10, 20 and 30 min after addition of bubble lipoplexes. The pDNA complexed with each bubble liposome and the endosomes of macrophages were labeled with TM–rhodamine (red) and AlexaFluor®–488 transferrin conjugates (green), respectively. Nuclei were counterstained by DAPI (blue). Scale bars, 10 μm. (Reproduced with permission from [102]).

References

[1] D. Lindau, P. Gielen, M. Kroesen, P. Wesseling, G.J. Adema, The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells, Immunology, 138 (2013) 105-115.
[2] S.L. Topalian, G.J. Weiner, D.M. Pardoll, Cancer immunotherapy comes of age, J. Clin. Oncol., 29 (2011) 4828-4836.
[3] J.M. Kirkwood, L.H. Butterfield, A.A. Tarhini, H. Zarour, P. Kalinski, S. Ferrone, Immunotherapy of cancer in 2012, CA Cancer J Clin, 62 (2012) 309-335.
[4] I. Mellman, G. Coukos, G. Dranoff, Cancer immunotherapy comes of age, Nature, 480 (2011) 480-489.
[5] S.K. Pathak, A.E. Skold, V. Mohanram, C. Persson, U. Johansson, A.L. Spetz, Activated apoptotic cells induce dendritic cell maturation via engagement of Toll-like receptor 4 (TLR4), dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), and beta2 integrins, J Biol Chem, 287 (2012) 13731-13742.
[6] C.J.M. Melief, Cancer immunotherapy by dendritic cells, Immunity, 29 (2008) 372-383.
[7] L.M. Weiner, J.C. Murray, C.W. Shuptrine, Antibody-Based Immunotherapy of Cancer, Cell, 148 (2012) 1081-1084.
[8] A.M. Scott, J.D. Wolchok, L.J. Old, Antibody therapy of cancer, Nat Rev Cancer, 12 (2012) 278-287.
[9] C.L.L. Chiang, F. Benencia, G. Coukos, Whole tumor antigen vaccines, Semin. Immunol., 22 (2010) 132-143.
[10] S.A. Rosenberg, N.P. Restifo, J.C. Yang, R.A. Morgan, M.E. Dudley, Adoptive cell transfer: a clinical path to effective cancer immunotherapy, Nat. Rev. Cancer, 8 (2008) 299-308.
[11] K. Palucka, H. Ueno, J. Banchereau, Recent Developments in Cancer Vaccines, J. Immunol., 186 (2011) 1325-1331.
[12] J. Rice, C.H. Ottensmeier, F.K. Stevenson, DNA vaccines: precision tools for activating effective immunity against cancer, Nat. Rev. Cancer, 8 (2008) 108-120.
[13] A. Sandler, R. Gray, M.C. Perry, J. Brahmer, J.H. Schiller, A. Dowlati, R. Lilenbaum, D.H. Johnson, Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer, N Engl J Med, 355 (2006) 2542-2550.
[14] R. Marcus, K. Imrie, A. Belch, D. Cunningham, E. Flores, J. Catalano, P. Solal-Celigny, F. Offner, J. Walewski, J. Raposo, A. Jack, P. Smith, CVP chemotherapy plus rituximab compared with CVP as first-line treatment for advanced follicular lymphoma, Blood, 105 (2005) 1417-1423.
[15] B.M. Olson, D.G. McNeel, Sipuleucel-T: immunotherapy for advanced prostate cancer, Open Access J Urol, 3 (2011) 49-60.
[16] W.D. O'Brien, Ultrasound-biophysics mechanisms, Prog. Biophys. Mol. Biol., 93 (2007) 212-255.
[17] H. Shankar, P.S. Pagel, Potential Adverse Ultrasound-related Biological Effects A Critical Review, Anesthesiology, 115 (2011) 1109-1124.
[18] S.B. Barnett, H.D. Rott, G.R. terHaar, M.C. Ziskin, K. Maeda, The sensitivity of biological tissue to ultrasound, Ultrasound Med. Biol., 23 (1997) 805-812.
[19] I. Rosenthal, J.Z. Sostaric, P. Riesz, Sonodynamic therapy - a review of the synergistic effects of drugs and ultrasound, Ultrasonics Sonochemistry, 11 (2004) 349-363.
[20] P. Riesz, T. Kondo, Free-radical formation induced by ultrasound and its biological implications, Free Radic. Biol. Med., 13 (1992) 247-270.

[21] J. Raja, N. Ramachandran, G. Munneke, U. Patel, Current status of transrectal ultrasound-guided prostate biopsy in the diagnosis of prostate cancer, Clin. Radiol., 61 (2006) 142-153.
[22] R. Lencioni, F. Piscaglia, L. Bolondi, Contrast-enhanced ultrasound in the diagnosis of hepatocellular carcinoma, J. Hepatol., 48 (2008) 848-857.
[23] S. Paliwal, S. Mitragotri, Therapeutic opportunities in biological responses of ultrasound, Ultrasonics, 48 (2008) 271-278.
[24] D.F. Webster, J.B. Pond, M. Dyson, W. Harvey, The role of cavitation in the in vitro stimulation of protein synthesis in human fibroblasts by ultrasound, Ultrasound Med Biol, 4 (1978) 343-351.
[25] S.X. Zhou, M.G. Bachem, T. Seufferlein, Y.P. Li, H.J. Gross, A. Schmelz, Low intensity pulsed ultrasound accelerates macrophage phagocytosis by a pathway that requires actin polymerization, Rho, and Src/MAPKs activity, Cell. Signal., 20 (2008) 695-704.
[26] H. Grull, S. Langereis, Hyperthermia-triggered drug delivery from temperature-sensitive liposomes using MRI-guided high intensity focused ultrasound, J. Control. Release, 161 (2012) 317-327.
[27] B. Geers, I. Lentacker, S. Cool, J. Demeester, S.C. De Smedt, N.N. Sanders, Ultrasound responsive doxorubicin-loaded microbubbles; towards an easy applicable drug delivery platform, J. Control. Release, 148 (2010) E59-E60.
[28] K. Shiraishi, R. Endoh, H. Furuhata, M. Nishihara, R. Suzuki, K. Maruyama, Y. Oda, J.-i. Jo, Y. Tabata, J. Yamamoto, M. Yokoyama, A facile preparation method of a PFC-containing nano-sized emulsion for theranostics of solid tumors, Int. J. Pharm., 421 (2011) 379-387.
[29] B.E. Polat, D. Hart, R. Langer, D. Blankschtein, Ultrasound-mediated transdermal drug delivery: Mechanisms, scope, and emerging trends, J. Control. Release, 152 (2011) 330-348.
[30] R.V. Shohet, S.Y. Chen, Y.T. Zhou, Z.W. Wang, R.S. Meidell, T.H. Unger, P.A. Grayburn, Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium, Circulation, 101 (2000) 2554-2556.
[31] K. Hynynen, Ultrasound for drug and gene delivery to the brain, Adv. Drug Deliv. Rev., 60 (2008) 1209-1217.
[32] S.V. Pislaru, C. Pislaru, R.R. Kinnick, R. Singh, R. Gulati, J.F. Greenleaf, R.D. Simari, Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities, Eur. Heart J., 24 (2003) 1690-1698.
[33] S. Mehier-Humbert, T. Bettinger, F. Yan, R.H. Guy, Plasma membrane poration induced by ultrasound exposure: Implication for drug delivery, J. Control. Release, 104 (2005) 213-222.
[34] S. Mo, C.C. Coussios, L. Seymour, R. Carlisle, Ultrasound-enhanced drug delivery for cancer, Expert Opin. Drug Deliv., 9 (2012) 1525-1538.
[35] S.A. Dromi, M.P. Walsh, S. Herby, B. Traughber, J.W. Xie, K.V. Sharma, K.P. Sekhar, A. Luk, D.J. Liewehr, M.R. Dreher, T.J. Fry, B.J. Wood, Radiofrequency Ablation Induces Antigen-presenting Cell Infiltration and Amplification of Weak Tumor-induced Immunity, Radiology, 251 (2009) 58-66.
[36] R.C.G. Martin, C.R. Scoggins, K.M. McMasters, Safety and Efficacy of Microwave Ablation of Hepatic Tumors: A Prospective Review of a 5-Year Experience, Ann. Surg. Oncol., 17 (2010) 171-178.

[37] S.P. Haen, P.L. Pereira, H.R. Salih, H.G. Rammensee, C. Gouttefangeas, More Than Just Tumor Destruction: Immunomodulation by Thermal Ablation of Cancer, Clin. Dev. Immunol., (2011).
[38] M.H. den Brok, R.P. Sutmuller, S. Nierkens, E.J. Bennink, C. Frielink, L.W. Toonen, O.C. Boerman, C.G. Figdor, T.J. Ruers, G.J. Adema, Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity, Br J Cancer, 95 (2006) 896-905.
[39] S.F. Matin, P. Sharma, I.S. Gill, C. Tannenbaum, M.G. Hobart, A.C. Novick, J.H. Finke, Immunological response to renal cryoablation in an in vivo orthotopic renal cell carcinoma murine model, J Urol, 183 (2010) 333-338.
[40] A. Sidana, Cancer immunotherapy using tumor cryoablation, Immunotherapy, 6 (2014) 85-93.
[41] G. Ter Haar, C. Coussios, High intensity focused ultrasound: Physical principles and devices, Int. J. Hyperthermia, 23 (2007) 89-104.
[42] Z. Hu, X.Y. Yang, Y. Liu, G.N. Sankin, E.C. Pua, M.A. Morse, H.K. Lyerly, T.M. Clay, P. Zhong, Investigation of HIFU-induced anti-tumor immunity in a murine tumor model, J. Transl. Med., 5 (2007).
[43] E.A. Filonenko, V.A. Khokhlova, Effect of acoustic nonlinearity on heating of biological tissue by high-intensity focused ultrasound, Acoust. Phys., 47 (2001) 468-475.
[44] M.S. Canney, V.A. Khokhlova, O.V. Bessonova, M.R. Bailey, L.A. Crum, Shock-Induced Heating and Millisecond Boiling in Gels and Tissue Due to High Intensity Focused Ultrasound, Ultrasound in Medicine & Biology, 36 (2010) 250-267.
[45] Y.-N. Wang, T. Khokhlova, M. Bailey, J.H. Hwang, V. Khokhlova, Histological and Biochemical Analysis of Mechanical and Thermal Bioeffects in Boiling Histotripsy Lesions Induced by High Intensity Focused Ultrasound, Ultrasound in Medicine & Biology, 39 (2013) 424-438.
[46] O. Al-Bataineh, J. Jenne, P. Huber, Clinical and future applications of high intensity focused ultrasound in cancer, Cancer Treat. Rev., 38 (2012) 346-353.
[47] R. Yang, C.R. Reilly, F.J. Rescorla, N.T. Sanghvi, F.J. Fry, T.D. Franklin, J.L. Grosfeld, Effects of high-intensity focused ultrasound in the treatment of experimental neuroblastoma, J. Pediatr. Surg., 27 (1992) 246-251.
[48] X.Y. Huang, F. Yuan, M.H. Liang, H.W. Lo, M.L. Shinohara, C. Robertson, P. Zhong, M-HIFU Inhibits Tumor Growth, Suppresses STAT3 Activity and Enhances Tumor Specific Immunity in a Transplant Tumor Model of Prostate Cancer, PLoS One, 7 (2012).
[49] Q. Zhou, X.Q. Zhu, J. Zhang, Z.L. Xu, P. Lu, F. Wu, Changes in circulating immunosuppressive cytokine levels of cancer patients after high intensity focused ultrasound treatment, Ultrasound Med. Biol., 34 (2008) 81-87.
[50] D.F. Rosberger, D.J. Coleman, R. Silverman, S. Woods, M. Rondeau, S. Cunningham-Rundles, Immunomodulation in choroidal melanoma: reversal of inverted CD4/CD8 ratios following treatment with ultrasonic hyperthermia, Biotechnol Ther, 5 (1994) 59-68.
[51] F. Wu, Z.B. Wang, P. Lu, Z.L. Xu, W.Z. Chen, H. Zhu, C.B. Jin, Activated anti-tumor immunity in cancer patients after high intensity focused ultrasound ablation, Ultrasound Med. Biol., 30 (2004) 1217-1222.
[52] Z.L. Hu, X.Y. Yang, Y.B. Liu, M.A. Morse, H.K. Lyerly, T.M. Clay, P. Zhong, Release of endogenous danger signals from HIFU-treated tumor cells and their

stimulatory effects on APCs, Biochem. Biophys. Res. Commun., 335 (2005) 124-131.
[53] Y. Zhang, J.A. Deng, J. Feng, F. Wu, Enhancement of antitumor vaccine in ablated hepatocellular carcinoma by high-intensity focused ultrasound, World J. Gastroenterol., 16 (2010) 3584-3591.
[54] J.Z. Xia, F.L. Xie, L.F. Ran, X.P. Xie, Y.M. Fan, F. Wu, High-intensity focused ultrasound tumor ablation activates autologous tumor-specific cytotoxic T lymphocytes, Ultrasound Med. Biol., 38 (2012) 1363-1371.
[55] J. Deng, Y. Zhang, J. Feng, F. Wu, Dendritic cells loaded with ultrasound-ablated tumour induce in vivo specific antitumour immune responses, Ultrasound Med. Biol., 36 (2010) 441-448.
[56] G. Kramer, G.E. Steiner, M. Grobl, K. Hrachowitz, F. Reithmayr, L. Paucz, M. Newman, S. Madersbacher, D. Gruber, M. Susani, M. Marberger, Response to sublethal heat treatment of prostatic tumor cells and of prostatic tumor infiltrating T-cells, Prostate, 58 (2004) 109-120.
[57] F. Wu, Z.B. Wang, Y.D. Cao, Q. Zhou, Y. Zhang, Z.L. Xu, X.Q. Zhu, Expression of tumor antigens and heat-shock protein 70 in breast cancer cells after high-intensity focused ultrasound ablation, Ann Surg Oncol, 14 (2007) 1237-1242.
[58] H.L. Liu, H.Y. Hsieh, L.A. Lu, C.W. Kang, M.F. Wu, C.Y. Lin, Low-pressure pulsed focused ultrasound with microbubbles promotes an anticancer immunological response, J Transl Med, 10 (2012) 1479-5876.
[59] F. Liu, Z.L. Hu, L. Qiu, C. Hui, C. Li, P. Zhong, J.P. Zhang, Boosting high-intensity focused ultrasound-induced anti-tumor immunity using a sparse-scan strategy that can more effectively promote dendritic cell maturation, J. Transl. Med., 8 (2010).
[60] P. Lu, X.Q. Zhu, Z.L. Xu, Q. Zhou, J. Zhang, F. Wu, Increased infiltration of activated tumor-infiltrating lymphocytes after high intensity focused ultrasound ablation of human breast cancer, Surgery, 145 (2009) 286-293.
[61] S.E. Jung, S.H. Cho, J.H. Jang, J.Y. Han, High-intensity focused ultrasound ablation in hepatic and pancreatic cancer: complications, Abdom. Imaging, 36 (2011) 185-195.
[62] Y.F. Xing, X.C. Lu, E.C. Pua, P. Zhong, The effect of high intensity focused ultrasound treatment on metastases in a murine melanoma model, Biochem. Biophys. Res. Commun., 375 (2008) 645-650.
[63] K. Tachibana, T. Uchida, K. Ogawa, N. Yamashita, K. Tamura, Induction of cell-membrane porosity by ultrasound, Lancet, 353 (1999) 1409-1409.
[64] J.F. Nagle, S. Tristram-Nagle, Structure of lipid bilayers, Biochim. Biophys. Acta-Rev. Biomembr., 1469 (2000) 159-195.
[65] H.A. Hancock, L.H. Smith, J. Cuesta, A.K. Durrani, M. Angstadt, M.L. Palmeri, E. Kimmel, V. Frenkel, Investigations into pulsed high-intensity focused ultrasound-enhanced delivery: Preliminary evidence for a novel mechanism, Ultrasound Med. Biol., 35 (2009) 1722-1736.
[66] G. Casey, J.P. Cashman, D. Morrissey, M.C. Whelan, J.O. Larkin, D.M. Soden, M. Tangney, G.C. O'Sullivan, Sonoporation mediated immunogene therapy of solid tumors, Ultrasound Med. Biol., 36 (2010) 430-440.
[67] V.G. Zarnitsyn, M.R. Prausnitz, Physical parameters influencing optimization of ultrasound-mediated DNA transfection, Ultrasound Med. Biol., 30 (2004) 527-538.

[68] P.E. Huber, P. Pfisterer, In vitro and in vivo transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound, Gene Ther., 7 (2000) 1516-1525.
[69] S. Hayashi, M. Mizuno, J. Yoshida, A. Nakao, Effect of sonoporation on cationic liposome-mediated IFNβ gene therapy for metastatic hepatic tumors of murine colon cancer, Cancer Gene Ther., 16 (2009) 638-643.
[70] D. Danno, M. Kanno, S. Fujimoto, L.B. Feril Jr, T. Kondo, S. Nakamura, Effects of ultrasound on apoptosis induced by anti-CD20 antibody in CD20-positive B lymphoma cells, Ultrasonics Sonochemistry, 15 (2008) 463-471.
[71] T. Masui, I. Ota, M. Kanno, K. Yane, H. Hosoi, Low-intensity ultrasound enhances the anticancer activity of cetuximab in human head and neck cancer cells, Exp Ther Med, 5 (2013) 11-16.
[72] A. Khaibullina, B.S. Jang, H. Sun, N. Lee, S. Yu, V. Frenkel, J.A. Carrasquillo, I. Pastan, K.C.P. Li, C.H. Paik, Pulsed high-intensity focused ultrasound enhances uptake of radiolabeled monoclonal antibody to human epidermoid tumor in nude mice, J. Nucl. Med., 49 (2008) 295-302.
[73] S. Wang, I.S. Shin, H. Hancock, B.S. Jang, H.S. Kim, S.M. Lee, V. Zderic, V. Frenkel, I. Pastan, C.H. Paik, M.R. Dreher, Pulsed high intensity focused ultrasound increases penetration and therapeutic efficacy of monoclonal antibodies in murine xenograft tumors, J. Control. Release, 162 (2012) 218-224.
[74] A. Bouakaz, M. Versluis, N. de Jong, High-speed optical observations of contrast agent destruction, Ultrasound Med. Biol., 31 (2005) 391-399.
[75] H.R. Villarraga, D.A. Foley, B.C. Aeschbacher, K.W. Klarich, S.L. Mulvagh, Destruction of contrast microbubbles during ultrasound imaging at conventional power output, J. Am. Soc. Echocardiogr., 10 (1997) 783-791.
[76] K. Wei, D.M. Skyba, C. Firschke, A.R. Jayaweera, J.R. Lindner, S. Kaul, Interactions between microbubbles and ultrasound: In vitro and in vivo observations, J. Am. Coll. Cardiol., 29 (1997) 1081-1088.
[77] M. Duvshani-Eshet, D. Adam, M. Machluf, The effects of albumin-coated microbubbles in DNA delivery mediated by therapeutic ultrasound, J. Control. Release, 112 (2006) 156-166.
[78] S. Ibsen, M. Benchimol, S. Esener, Fluorescent microscope system to monitor real-time interactions between focused ultrasound, echogenic drug delivery vehicles, and live cell membranes, Ultrasonics, 53 (2013) 178-184.
[79] A. van Wamel, K. Kooiman, M. Harteveld, M. Emmer, F.J. ten Cate, M. Versluis, N. de Jong, Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation, J. Control. Release, 112 (2006) 149-155.
[80] R. Suzuki, Y. Oda, N. Utoguchi, K. Maruyama, Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles, J. Controlled Release, 149 (2011) 36-41.
[81] Y. Zhou, K. Yang, J. Cui, J.Y. Ye, C.X. Deng, Controlled permeation of cell membrane by single bubble acoustic cavitation, J. Control. Release, 157 (2012) 103-111.
[82] S. Tinkov, R. Bekeredjian, G. Winter, C. Coester, Microbubbles as Ultrasound Triggered Drug Carriers, J. Pharm. Sci., 98 (2009) 1935-1961.
[83] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release, 65 (2000) 271-284.

[84] J. Fang, H. Nakamura, H. Maeda, The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Adv Drug Deliv Rev, 63 (2011) 136-151.
[85] W.J. Greenleaf, M.E. Bolander, G. Sarkar, M.B. Goldring, J.F. Greenleaf, Artificial cavitation nuclei significantly enhance acoustically induced cell transfection, Ultrasound Med. Biol., 24 (1998) 587-595.
[86] Y. Sakakima, S. Hayashi, Y. Yagi, A. Hayakawa, K. Tachibana, A. Nakao, Gene therapy for hepatocellular carcinoma using sonoporation enhanced by contrast agents, Cancer Gene Therapy, 12 (2005) 884-889.
[87] O. Zolochevska, X.Q. Xia, B.J. Williams, A. Ramsay, S.L. Li, M.L. Figueiredo, Sonoporation Delivery of Interleukin-27 Gene Therapy Efficiently Reduces Prostate Tumor Cell Growth In Vivo, Hum. Gene Ther., 22 (2011) 1537-1550.
[88] Y. Taniyama, K. Tachibana, K. Hiraoka, M. Aoki, S. Yamamoto, K. Matsumoto, T. Nakamura, T. Ogihara, Y. Kaneda, R. Morishita, Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle, Gene Ther., 9 (2002) 372-380.
[89] R.D. Alkins, A. Burgess, M. Ganguly, G. Francia, R.S. Kerbel, W.S. Wels, K. Hynynen, Focused Ultrasound Delivers Targeted Immune Cells To Metastatic Brain Tumors, Cancer Res, 9 (2013) 9.
[90] G. Bioley, A. Lassus, P. Bussat, J. Terrettaz, F. Tranquart, B. Corthesy, Gas-filled microbubble-mediated delivery of antigen and the induction of immune responses, Biomaterials, 33 (2012) 5935-5946.
[91] M.-L. De Temmerman, H. Dewitte, R.E. Vandenbroucke, B. Lucas, C. Libert, J. Demeester, S.S.C. De, I. Lentacker, J. Rejman, mRNA-Lipoplex loaded microbubble contrast agents for ultrasound-assisted transfection of dendritic cells, Biomaterials, 32 (2011) 9128-9135.
[92] I. Lentacker, N. Wang, R.E. Vandenbroucke, J. Demeester, S.C. De Smedt, N.N. Sander, Ultrasound Exposure of Lipoplex Loaded Microbubbles Facilitates Direct Cytoplasmic Entry of the Lipoplexes, Mol. Pharm., 6 (2009) 457-467.
[93] J.C.M. Lemmon, R.J. McFarland, J.M. Rybicka, D.R. Balce, K.R. McKeown, R.M. Krohn, T.O. Matsunaga, R.M. Yates, In vitro and in vivo transfection of primary phagocytes via microbubble-mediated intraphagosomal sonoporation, Journal of Immunological Methods, 371 (2011) 152-158.
[94] Z.K. Liao, K.C. Tsai, H.T. Wang, S.H. Tseng, W.P. Deng, W.S. Chen, L.H. Hwang, Sonoporation-mediated anti-angiogenic gene transfer into muscle effectively regresses distant orthotopic tumors, Cancer Gene Therapy, 19 (2012) 171-180.
[95] Y. Oda, R. Suzuki, S. Otake, N. Nishiie, K. Hirata, R. Koshima, T. Nomura, N. Utoguchi, N. Kudo, K. Tachibana, K. Maruyama, Prophylactic immunization with Bubble liposomes and ultrasound-treated dendritic cells provided a four-fold decrease in the frequency of melanoma lung metastasis, J. Control. Release, 160 (2012) 362-366.
[96] R. Suzuki, E. Namai, Y. Oda, N. Nishiie, S. Otake, R. Koshima, K. Hirata, Y. Taira, N. Utoguchi, Y. Negishi, S. Nakagawa, K. Maruyama, Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure, J. Control. Release, 142 (2010) 245-250.
[97] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Enhanced Transfection Efficiency into Macrophages and Dendritic Cells by a

Combination Method Using Mannosylated Lipoplexes and Bubble Liposomes with Ultrasound Exposure, Hum. Gene Ther., 21 (2010) 65-74.
[98] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier, Mol. Pharm., 8 (2011) 543-554.
[99] R. Suzuki, Y. Oda, N. Utoguchi, E. Namai, Y. Taira, N. Okada, N. Kadowaki, T. Kodama, K. Tachibana, K. Maruyama, A novel strategy utilizing ultrasound for antigen delivery in dendritic cell-based cancer immunotherapy, J. Controlled Release, 133 (2009) 198-205.
[100] R. Suzuki, T. Takizawa, Y. Negishi, K. Hagisawa, K. Tanaka, K. Sawamura, N. Utoguchi, T. Nishioka, K. Maruyama, Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound, J. Control. Release, 117 (2007) 130-136.
[101] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy, Biomaterials, 31 (2010) 7813-7826.
[102] K. Un, S. Kawakami, M. Yoshida, Y. Higuchi, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, The elucidation of gene transferring mechanism by ultrasound-responsive unmodified and mannose-modified lipoplexes, Biomaterials, 32 (2011) 4659-4669.
[103] K. Un, S. Kawakami, Y. Higuchi, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Involvement of activated transcriptional process in efficient gene transfection using unmodified and mannose-modified bubble lipoplexes with ultrasound exposure, J. Control. Release, 156 (2011) 355-363.
[104] M.L. Fabiilli, J.A. Lee, O.D. Kripfgans, P.L. Carson, J.B. Fowlkes, Delivery of water-soluble drugs using acoustically triggered perfluorocarbon double emulsions, Pharm Res, 27 (2010) 2753-2765.
[105] R. Singh, G.A. Husseini, W.G. Pitt, Phase transitions of nanoemulsions using ultrasound: experimental observations, Ultrason Sonochem, 19 (2012) 1120-1125.

Figure 1
Click here to download high resolution image

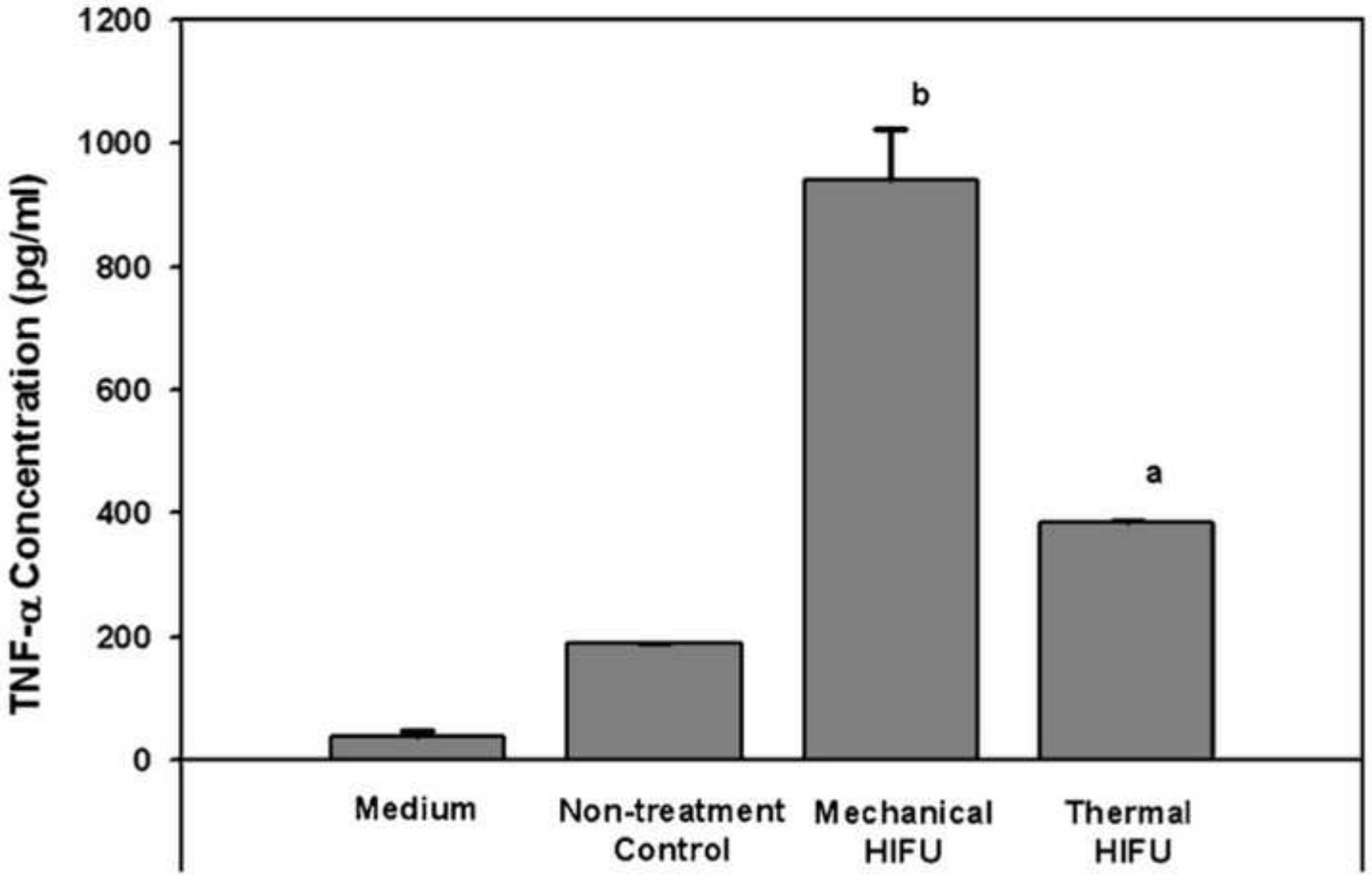

Figure 2
Click here to download high resolution image

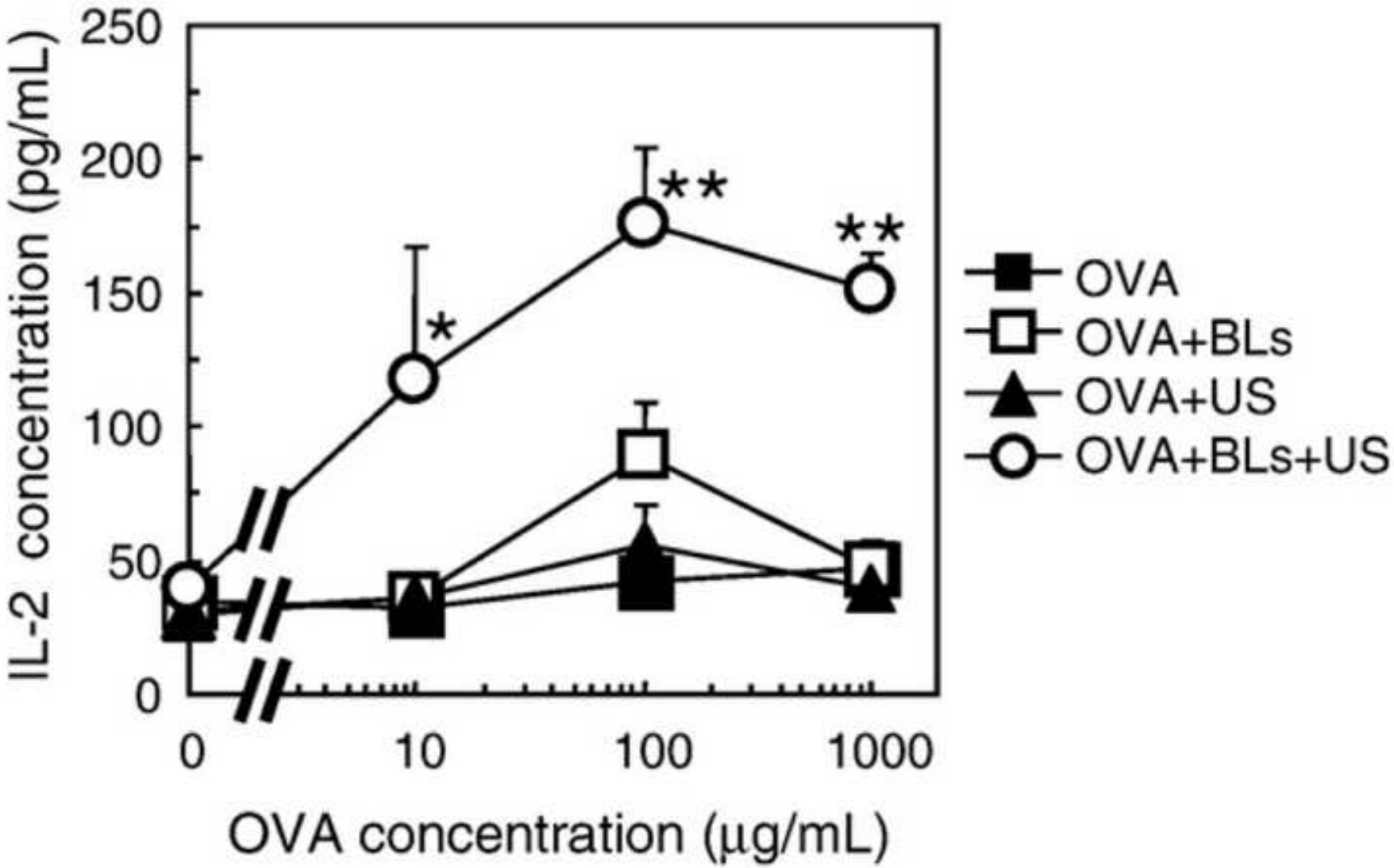

Figure 3

Click here to download high resolution image

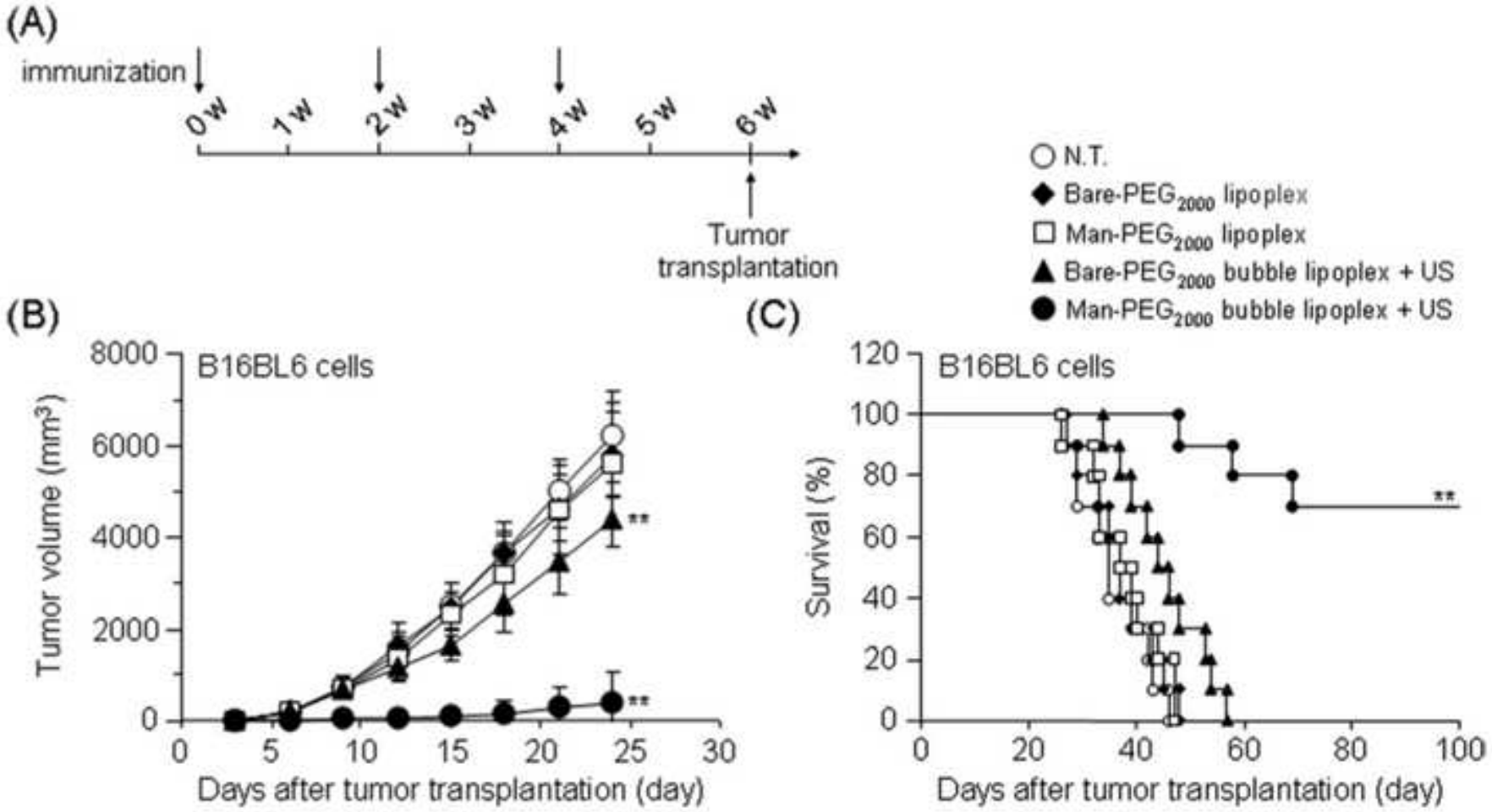

Figure 4
Click here to download high resolution image

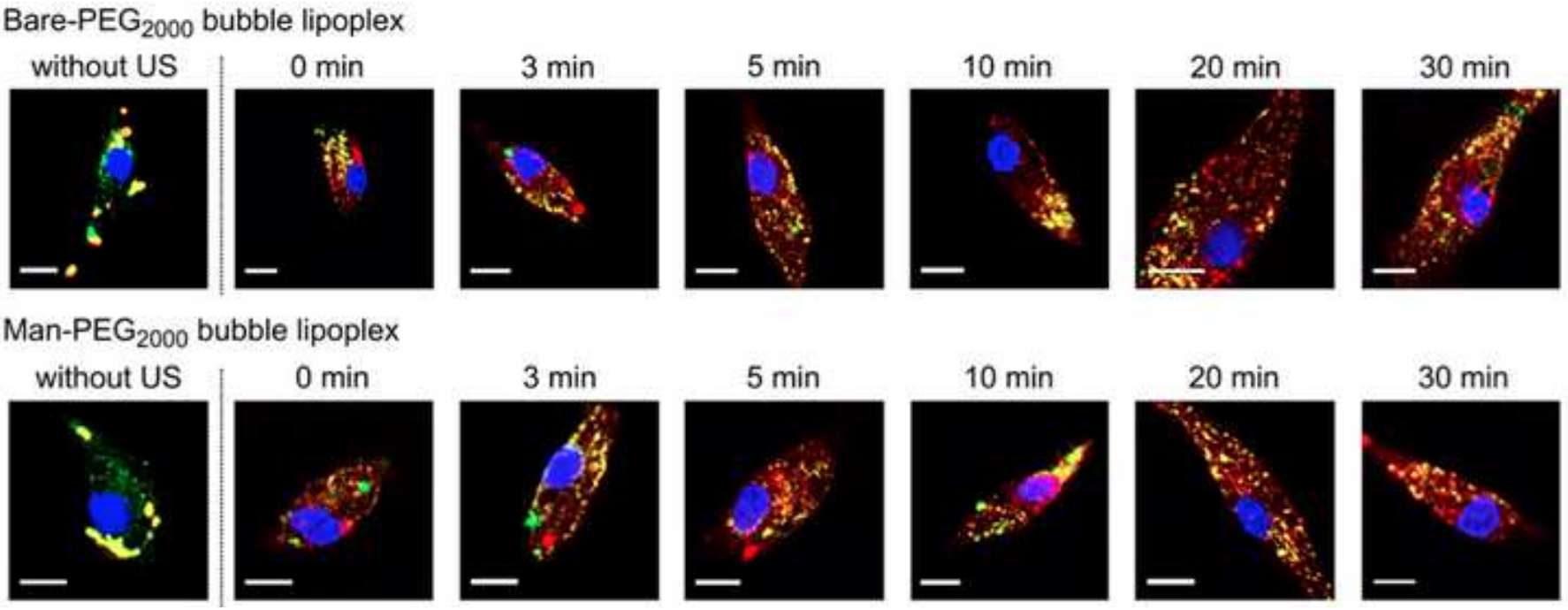

Table 1

Species	Tumour	Model	Effect of HIFU	Ref.
Mouse	Neuroblastoma	C1300	Tumour growth after re-challenge ↓ Survival ↑	[47]
Human	Melanoma		CD4+ ↑ Normalisation of CD4+/CD8+ ratio	[50]
Human	Various		CD4+ ↑ CD4+/CD8+ ratio ↑	[51]
Human	Breast cancer		HSP70 ↑ EMA increase	[57]
Mouse	Cancer cell culture	MC-38	Danger signals (ATP, hsp60) ↑ DC, macrophage activation ↑ Mech. HIFU more effective then thermal	[52]
Mouse	Hepatocarcinoma	H22	Cytotoxicity of tumour vaccine from HIFU ↑ DC maturation by HIFU vaccine ↑	[53]
Mouse	Hepatocarcinoma	H22	CTL ↑ IFN-γ, TNF-α ↑ Survival ↑	[54]
Mouse	Hepatocarcinoma	H 22	CD8 increase (spleen) Tumour growth ↓	[55]
Human	Hepatocarcinoma or osteosarcoma		Immuno-suppressing cytokines ↓	[49]
Mouse	Adenocarcinoma, Melanoma	MC-35, B16	Lower temperature ablation (55°C) gave stronger immune response than at 80°C	[59]
Mouse	Adenocarcinoma	MC-38	DC tumour infiltration ↑ CTL activity ↑	[42]
Mouse	Prostate cancer	RM-9	STAT3 ↓ DC number and activity ↑ Tumour growth after re-challenge ↓	[48]
Human	Breast cancer		CTL and NK ↑ Immune cell infiltration of tumour ↑	[60]

Table 2

Species/tumour type	Method	US-sensitive agent	Bioactive	Immune effect	Ref.
Mouse, CT-26	Treatment with MBs and US only	SonoVue	None	Immune cell infiltration ↑ Tumour growth ↓	[58]
Mouse, HCC (SK-Hep1)	Co-injection of pDNA and bubbles + US	BR14	IFNβ pDNA	IFNβ ↑ Tumour growth ↓	[86]
Mouse, TRAMP-C1, TRAMP-C2, RM1	Co-injection of pDNA and bubbles + US	SonoVue	IL-27 pDNA	Expression immune genes ↑ Immune cell infiltration ↑	[87]
Mouse, E.G7-OVA	Animal immunisation with DCs given antigen *in vitro*	Bubble liposomes	Ovalbumin	IL-2 ↑ Survival ↑	[99]
Mouse, B16BL6	Animal immunisation with DCs given antigen *in vitro*	Bubble liposomes	B16BL6 extracted antigens	Lung metastasis ↓	[95]
Mouse, OM-HM	*In vivo* gene delivery + US	Bubble liposome	IL-12 pDNA	CD8+ T cell migration ↑ Tumour growth ↓	[96]
Mouse, B16BL6 melanoma	*In vivo* gene delivery with targeting BLs + US	Bubble liposome	pDNA coding for gp100 and TRP-2.	TNFα, IFN-γ, IL-4, IL-6 ↑ Tumour growth ↓ Survival ↑	[98]
Mouse, E.G7-OVA	*In vivo* gene delivery with targeting BLs + US	Bubble liposome	Ovalbumin pDNA	IFN-γ ↑ Tumour growth ↓ Survival ↑	[101]