

Focused Ultrasound and Cancer Immunotherapy Workshop

18–19 July 2019

UVA Darden Sands Family Grounds
Arlington, Virginia

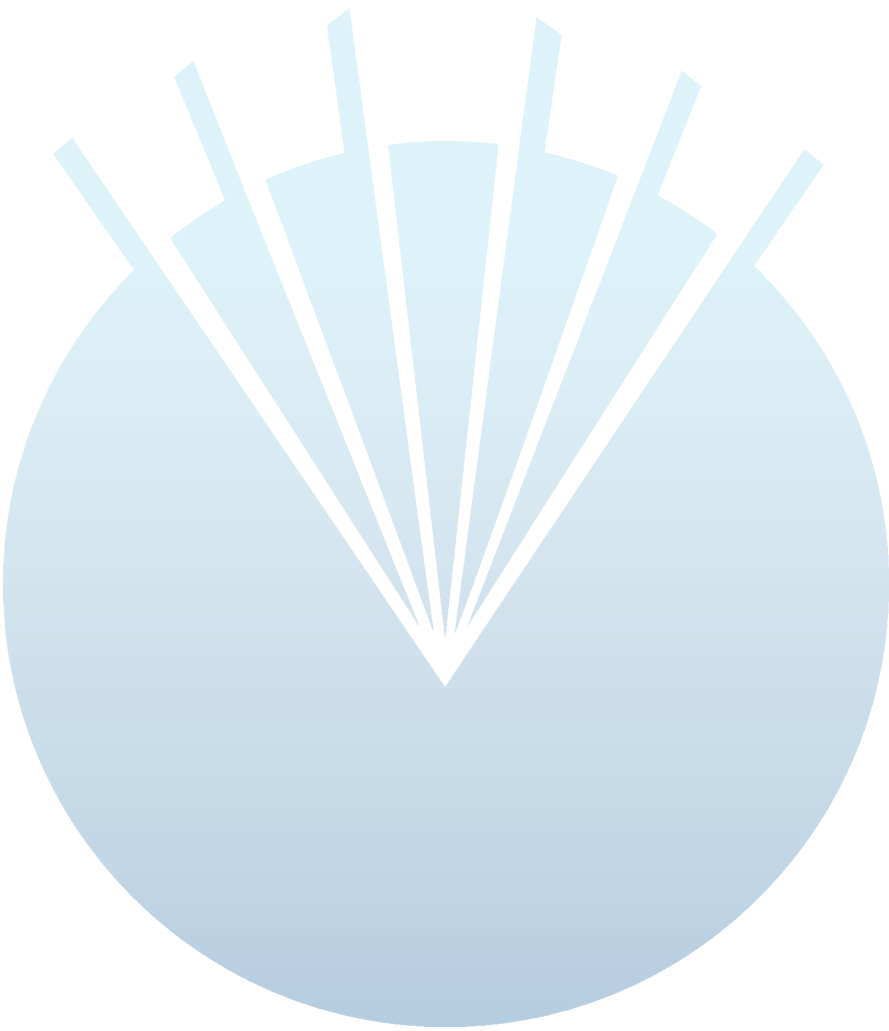
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Contents

2 **Executive Summary**

4 **Overview of Tumor Immunity and Focused Ultrasound**

• • • • •

7 **Workshop Presentations**

- 7 1 | What signaling pathways/molecules are stimulated by FUS?
 - 7 Mechanisms and Signaling Pathways
- 8 2 | Does the innate or the adaptive immune system play the largest role in responding to FUS? Does this vary by mechanism or disease?
 - 8 Innate and Adaptive Responses
 - 9 Discussion from Presentations
- 9 3 | What FUS mode is best at activating an immune response? Does this vary by tumor type? Does this vary by mechanism or disease?
 - 10 Thermal Ablation
 - 10 Hyperthermia
 - 10 Histotripsy
 - 11 Pulsed FUS
 - 11 BBB Opening
 - 12 Microvasculature Ablation/BBB Opening
 - 12 Discussion from Presentations
- 13 4 | What drugs/other therapies can be combined with FUS to enhance the response?
 - 13 Innate and Adaptive Responses
 - 14 Discussion from Presentation
- 14 5 | What clinical disease targets are ideal for FUS plus immunotherapy combinations?
 - 14 Breast Cancer Trial
 - 14 Glioblastoma Clinical Trial
 - 15 Pancreatic Cancer Trial
 - 15 Combination FUS with PD-1 Inhibitor Trial: Challenges and Lessons for FDA Applications of Combination Therapies
 - 16 Discussion from Presentations
 - 16 Discussion on Burning Questions

• • • • •

19 **Parker Institute for Cancer Immunotherapy Toolkit**

20 **Preclinical Roadmap**

22 **Clinical Roadmap**

24 **Outcomes and Next Steps**

25 **Updated Burning Questions**

• • • • •

26 **References**

28 **Abbreviations**

29 **Workshop Participants**

Workshop Summary

Focused Ultrasound and Cancer Immunotherapy Workshop

July 18–19, 2019

Executive Summary

Focused ultrasound (FUS) is an early-stage, noninvasive therapeutic technology with the potential to improve the lives of millions of patients with a variety of medical disorders by providing either an alternative or complement to existing therapies. A growing body of research has demonstrated that FUS can initiate a powerful anti-tumor immune response either alone or in combination with other immunotherapies such as checkpoint inhibitors.

On July 18–19, the Focused Ultrasound Foundation and the Cancer Research Institute (CRI) hosted a Focused Ultrasound and Cancer Immunotherapy Workshop in Arlington, Virginia, the third workshop on this topic. The meeting convened more than 50 of the world's leading experts in the fields of therapeutic ultrasound and cancer immunotherapy from 27 organizations across academia, industry, government, and advocacy.

The ultimate goal of the Focused Ultrasound Foundation's Cancer Immunotherapy Program is to reduce the time it takes for FUS and immunotherapy combination treatments to reach clinical adoption. The workshop was another step towards accomplishing this goal, by critically evaluating the current body of evidence, assessing the value of ongoing work, and creating a roadmap of projects that will address any remaining gaps and burning questions. Bringing together all critical stakeholders – researchers, clinicians, industry, government, and others—in an environment that encouraged free dissemination of information and ideas fostered a collaborative spirit that will greatly benefit the advancement of this field.

The meeting began with presentations on cancer immunology and areas of potential synergy for FUS and immunotherapy for cancer treatment. There were also presentations on potential mechanisms of action for synergy of FUS with immunotherapy for different FUS modalities including mechanical or thermal mechanisms as well as FUS-induced opening of the blood-brain barrier (BBB). Presentations were followed by moderated discussion on potential ideas for future research and essential burning questions that should be answered to move the field forward. Participants agreed that the effects of different modalities (see Table 1) of FUS on the immune system itself need to be understood in order to optimally design future combinations with immunotherapy. A roadmap of preclinical and clinical projects as well as standards development were formed as next steps for the community (see Table 3). Key tasks in this roadmap include:

- Develop guidelines for immune analysis during FUS studies, including protocols for optimal collection and storage of tissue samples and prioritized immune assays;
- Develop a data repository to track progress and foster collaboration;
- Designate a set of core laboratories that could provide immunological analysis;
- Facilitate the development of standardized methods to measure key FUS parameters (e.g., thermal dose, cavitation) across the various FUS systems.

The attendees were encouraged to continue thinking and collaborating on these issues, and to share

Table 1

Overview of focused ultrasound mechanisms that may affect the immunological response to tumors.

| Mechanism/Modality | Description | Potential Bioeffects |
|--------------------------------|---|---|
| Thermal ablation | Rapid heating designed to ablate tissues (target temperature often greater than 55°) | Coagulative necrosis, protein denaturation, release of heat-shock proteins, etc. |
| Hyperthermia | Prolonged low-level heating of tissues (e.g., 20 min at 42°C). | Release of heat-shock proteins, increased perfusion, etc. |
| Histotripsy | High-intensity pulses to fractionate soft tissue | Release of intact cellular proteins, tissue liquefaction, etc. |
| Pulsed focused ultrasound | Low to mid-pressure pulsed FUS with or without microbubbles to induce mechanically mediated tissue effects. | Release of DAMPs, various cytokines, chemokines, cellular adhesion molecules, etc. |
| Blood-brain barrier disruption | Low-pressure pulsed FUS administered with microbubbles, designed to reversibly disrupt the blood-brain barrier within the brain microvasculature and/or tumor microenvironment. | Enhanced permeability, activation of mild wound healing response, upregulation of cellular adhesion molecules, etc. |

Overview of Tumor Immunity and Focused Ultrasound

Timothy Bullock from the University of Virginia set the stage for the meeting by providing background on current concepts in cancer immunology and how FUS might integrate into this field (see Figure 1). For example, in metastatic melanoma, immune-cell (CD8+ T cell) infiltration correlated with improved survival. In general, T cells recognize degraded proteins presented at the surface of cells by major histocompatibility complex (MHC) molecules. Cytotoxic T cells are then able to respond and recognize the tumor as targets. FUS applied to tumors has many potential mechanisms to affect immune responses including the ability to enhance the infiltration of T cells that subsequently target the tumor.

Tumors with a high mutational burden provide targets for T cells. Neoantigens have recently become a major focus of interest as targets for immunotherapy.² Neoantigens are a summation of mutations occurring in the genome and contain amino acid sequences that are recognized by T cells. Mutations often provide molecular targets that allow for cancer cells to be recognized and targeted. Certain cancers, such as melanoma and lung cancer, have a disproportionately large propensity for mutations.

There are a number of extrinsic and intrinsic factors in the tumor microenvironment (TME) that help tumor cells evade the immune system.³ For example, in a study of non-small cell lung cancer (NSCLC) immune checkpoint blockade with an anti-programmed cell death (PD)-1 inhibitor resulted in a long-term durable overall survival advantage over standard chemotherapy. However, not all patients responded or had durable responses to immunotherapy. One of the main goals in the field is to determine how to create durable responses for a larger volume of patients.

Cancers can be subdivided into four major “inflammatory” categories that reflect their immunogenicity.⁴ These include type I (adaptive immune resistance), type II (immunological ignorance), type III (intrinsic induction), and type IV (tolerance). Many types of cancers have low T cell infiltration, such as breast cancer, ovarian cancer, pancreatic cancer, colorectal cancer, and glioblastoma (GBM). Various approaches have increased T cell presence/function within tumors that were dependent upon tumor classification. These include checkpoint inhibition, depletion or re-education of immunosuppressive cells, and blocking or disrupting the expression of immunosuppressive cytokines.

Strategies to improve tumor immunity were also discussed. These include cancer vaccines (shared antigens, neoantigens, and other various delivery systems); irradiation/chemotherapy with autovaccination (damage to the tumor microenvironment) through FUS, cryoablation, laser, or radiofrequency; oncolytic viruses; and adoptive transfer therapies (TCR transgenesis or chimeric antigen receptor T cell therapy (CAR-T).

SFUS increased cell membrane disruption and release of tumor cell antigen that drives the initiation of the innate immune response, which may allow the adaptive immune system to recognize tumor cells.¹ FUS also improved dendritic cell maturation via enhanced expression of DAMPS, increased antigen flow to lymph nodes, and less restricted intra-tumor T cell migration as a result of mechanical disruption of stroma, and altered cytokine production, which may

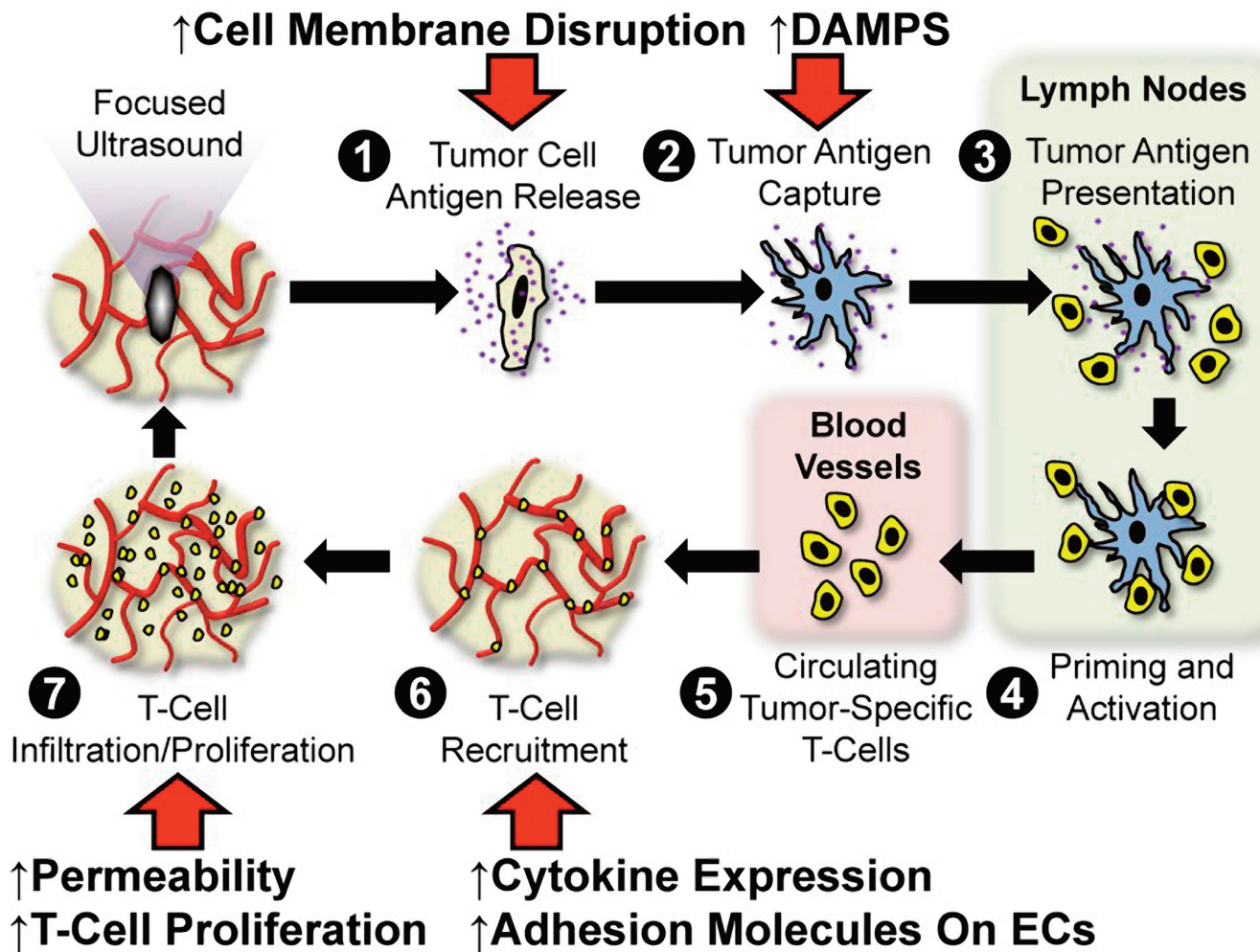


Figure 1

Hypothesized points of intersection between focused ultrasound and the cancer immunity cycle. In the cancer immunity cycle, antigens (purple) released from tumor cells (tan; 1) are captured by dendritic cells (blue; 2) and presented to T cells (yellow 3) in lymph nodes (light green), leading to priming and activation of effector T cells (4). Activated effector T cells then pass into the systemic circulation (light pink; 5) and are trafficked to the tumor via adhesion to tumor endothelium (6). T cells recruited from the circulation then infiltrate the tumor (7), where they specifically recognize and subsequently kill tumor cells. Tumor cell killing serves to release more antigen (1), allowing the cycle to continue. We hypothesize that focused ultrasound can trigger and/or boost anti-cancer immunity by intersecting at several points (red arrows) in this cycle. These include (i) enhanced tumor antigen release by cell membrane disruption, (ii) improved dendritic cell maturation via enhanced expression of damage-associated molecular patterns (DAMPs), (iii) greater antigen flow to lymph nodes and less restricted intra-tumoral T cell migration as a result of mechanical disruption of stroma, and (iv) altered cytokine production, which may lead to augmented endothelial adhesion molecule expression and/or proliferation of intra-tumoral T cells. Figure from Curley et al. 2017.¹

lead to augmented endothelial adhesion molecule expression and/or proliferation of intra-tumor T cells.¹ There were different types of FUS, such as thermal versus mechanical ablation, that likely result in different effects on the immune response. A great deal of work remains to understand the FUS-induced anti-tumor immune response such as the release of tumor antigen via tissue destruction, release of biomolecules (DAMPs) that activate antigen-presenting cells, recruitment of immune cells to tissue, and increased accessibility for immunomodulatory antibodies and drugs.

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Workshop Presentations

Immunotherapy may be used across all cancer types and is adaptable, durable, systemic, and synergistic. While immunotherapy has been successful in a subset of patients, future work remains to identify treatment strategies to enable a robust and durable response in all patients. Several presentations discussed the potential synergy of FUS with immunotherapy. These presentations were grouped by “burning questions” that were essential to understanding the combination of FUS and immunotherapy.

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1. What signaling pathways/molecules are stimulated by FUS?

Joe Frank from the National Institutes of Health described mechanisms and signaling pathways. The tumor microenvironment is heterogenous with high interstitial pressures, a dense extracellular matrix, and exosomes with microvessels. The goal is to change this from a chronic inflammatory state that suppresses T cells into an anti-tumor effect.

In brief, cancer results with the help of pro-inflammatory mediators such as NF- κ B and STAT-3.⁵ Immune components such as T cells, natural killer cells, macrophages, and neutrophils either inhibit or enhance tumor initiation depending on the type of tumor and immune cells involved. Inflammation can lead to tumorigenesis.⁶ Earlier studies showed that patients with colon cancer and higher levels of infiltrating immune cells had better clinical outcomes.⁷ Recently, hot tumors (massive infiltration of cytotoxic T cells and other immune cells) and cold tumors (little infiltration) have been defined using an immune score.⁸ Confirmatory studies suggested that patients with an inflammatory response in the tumor had better outcomes.⁹

Potentially, FUS can act as neoadjuvant to shift the tumor microenvironment from cold to hot. In prior studies, fractionated radiotherapy upregulated the immune system for 24 hours post-treatment.¹⁰ However, if too much of the tumor was treated with radiation, there was not enough tumor left to stimulate an immune response. Finding the optimal use for FUS, as an alternative to radiotherapy, to elicit an immune response is under investigation. Preclinical studies have demonstrated that high-intensity focused ultrasound (HIFU) ablation causes release of DAMPs and initiation of an inflammatory immune response.¹¹ Preclinical studies of boiling histotripsy ablation of renal cell carcinoma in vitro promoted a systemic inflammatory response (DAMPs and circulating cytokines).¹² The cellular supernatant was able to convert M1 macrophages into M2 macrophages. HIFU ablation of breast cancer tumors in humans prior to mastectomy resulted in increased infiltration of activated tumor-infiltrating lymphocytes.¹³

Preclinical studies of FUS at different peak negative pressures in B16 melanoma and 4T1 breast cancer cells created different proteomic heat maps (data not yet published). In the B16 melanoma cells, TGF β was suppressed. DNA damage was also induced in both tumor types at 6 MPa of pressure. Proteomic analysis over time suggested increased TGF β and inflammatory cytokines in the sonicated tumor when compared with an untreated control. Inflammatory changes last for 24

hours after tumor sonication. Sampling on days 1, 3, and 5 demonstrated that on day 3 there was a large influx of cytotoxic cells, and by day 5 there were cytotoxic cells in the tumor for B16 melanoma. In general, HIFU generated a systemic immune stimulatory effect.¹⁴ An interesting application for FUS and immunotherapy going forward will be to use FUS to stimulate an inflammatory response to boost immunotherapy.

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2. Does the innate or the adaptive immune system play the largest role in responding to FUS? Does this vary by mechanism or disease?

Innate and Adaptive Responses

Brett Fite from Stanford University discussed combining focal therapies with immunotherapies. Recent preliminary data in patients with pancreatic cancer suggested promising results for the combination of gemcitabine, nab-paclitaxel, and a CD40 agonist with or without nivolumab (anti-PD-1) that produced a myeloid (macrophage infiltration) rather than a T cell response.¹⁵ CD40 can directly activate dendritic cells and macrophages, and to a smaller degree it can bind to tumor cells and initiate apoptosis.¹⁶ Many solid tumors do not typically respond to immunotherapy. The goal was to create a T cell response through a combination of focal therapy, agonists, and checkpoint modulation. The innate immune response occurred early, within 12 hours, and the adaptive immune response occurred on the order of days.

There have been many clinical trials for the combination of agonists with checkpoint inhibitors and radiation therapy. Preclinical work looked at the potential for FUS combination treatments. The combination of thermal ablation with agonists may produce a systemic anti-cancer response. Preclinical mouse studies combining intra-tumoral injection of agonists (toll-like receptor (TLR)-9 and anti-PD-1) plus HIFU resulted in an increase in the number of T cells and T cell activation markers. Both thermal ablation and mechanical HIFU have been used in preclinical models. MRI guidance and thermometry were used in all experiments. For the pancreatic tumor ablation model, the aim was to ablate only part of the tumor so that there was non-damaged tissue left behind. Both mechanical and thermal ablation resulted in increased vascular permeability around the tumor.

After thermal ablation, burn scarring prevented reinvasion of that area, but that effect was not observed after mechanical ablation. HIFU treatment enhanced accumulation of therapy (agonists or antibodies) surrounding the lesion. Thermal ablation combined with immunotherapy released tumor antigen^{11,17} and enhanced local innate immune responses, mostly macrophages. It also increased distant T cell markers.¹⁸ Within 6 hours after treatment, there was a systemic type I interferon (IFN) response locally and in blood; within 48 hours there was an initial myeloid response and activated T cells at the local site; and within 7 days, there was a dense myeloid response at the local site, activated T cells at distant sites, and T cell clonality in the peripheral blood and tumor. In summary, the innate immune response occurred locally, and the adaptive immune response occurred distantly. There is a potential role for FUS in tumor debulking. It can be repeated without some of the side effects associated with chemotherapy and radiation therapy. However, the signaling between the treatment and distant sites still remain elusive.

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Discussion from Presentations

- There was a question on what happens when pulsed FUS (pFUS) heats normal tissue instead of tumor tissue.
 - Below 6 MPa there was only a mechanical effect, tissue edema was observed but resolved within 24 hours. pFUS only applies a small amount of energy and the effect was comparable to the effects mediated by strenuous exercise.
- Participants discussed the potential for systemic administration of agonists for use with FUS. There were concerns about systemic toxicity of immunotherapy agents with a preference for local delivery.

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3. What FUS mode is best at activating an immune response? Does this vary by tumor type? Does this vary by mechanism or disease?

The Focused Ultrasound Foundation launched a multi-center consortium to study the effects of FUS on the immune response to glioblastoma (GBM). Each member of the 6-site consortium used the same mouse model for glioblastoma (GL261-luc2),¹⁹ but used different modes of FUS to study the immune system response. Preliminary results of this work were presented and summarized below in Table 2.

Table 2

Summary of preliminary results from the multi-modality GBM consortium project

| GBM Consortium Results | Thermal Ablation | Hyperthermia | Histotripsy | Pulsed FUS | BBBD+PDL1 | BBBD |
|------------------------|------------------|-------------------------|--|-------------------------------|-------------------|---|
| Tumor | No change | Increased CD8, NK, MDSC | Decreased MDSC, increased IFN γ | Increased CD4 and CD8 (IHC) | Increased T cells | Increased antigen-experience d PD-1+ CD8 T cells |
| DLN | No change | Not reported | Not reported | Increased MDSC, decreased CD8 | Not reported | Increases DC maturity and repertoire, Granzyme-B, TIGIT |
| Spleen | No change | Not reported | Not reported | Increased Treg | Increased TIGIT | Not reported |

Thermal Ablation

Kullervo Hynynen from Sunnybrook Health Sciences Centre discussed tumor ablation with MRgFUS in a preclinical model of GBM (GL261-luc2). The sonication parameters (15 s, continuous wave; 2.45 W acoustic power, and $f_5 = 5.51$ MHz, $f\#$ 0.8, 25 mm diameter, 4 mm center opening for hydrophone) were optimized to heat brain tissue. Skull bone had to be removed to prevent overheating effects. The goal was to ablate a portion of the tumor in the GL261 intracranial glioma mouse model. Real-time thermometry was used for temperature mapping of the lesion, a thermal dose of 240 CEM43 was used as the threshold for lesion formation. Thermal ablation slowed tumor growth at 21 days post-treatment. Immunohistochemistry showed no difference in the number of CD4+, CD8+, or the CD4+/CD8+ T cell ratio after thermal ablation.

Hyperthermia

Costas Arvanitis from the Georgia Institute of Technology described immunomodulation with FUS hyperthermia in murine glioma model (GL261-luc2). GBM TME(g.,"). The study examined whether FUS hyperthermia could increase immune cell infiltration in the tumor microenvironment. GL261 cells were intracranially injected, FUS hyperthermia was induced on day 14. Brain tumor, spleen, and cervical lymph nodes were harvested after 21 days.

A MRgFUS closed-loop transcranial hyperthermia system (1 MHz, $f\#$:0.75) was created for the experiments. Heating was limited to regions near the skull, which may lead to suboptimal tumor targeting, particularly for tumors located deep within the brain. Optimizing the system showed that 1.7 MHz provided the maximum brain to skull intensity ratio. The maximal temperature achieved, without overheating the skull and causing tissue damage, was 41.5°C for heating up to 12 mins. FUS-induced hyperthermia caused changes in immune-cell trafficking in brain tumors. Their analysis revealed that FUS-induced mild hyperthermia (Mean \pm SEM (°C): 40.5 \pm 0.9 for 10 min) caused a significant infiltration of activated NK cells ($P < 0.01$), effector CD8 cells ($P < 0.05$) and MDSC ($P < 0.001$) in the brain tumor microenvironment of the FUS-treated cohort of mice as compared to the untreated one. There were no changes observed in immune-cell populations outside the brain. In summary, combinatorial approaches of FUS with immunotherapy might lead to anti-tumor immunity.

Histotripsy

Zhen Xu from the University of Michigan presented on histotripsy-mediated immunomodulation. Histotripsy results in tissue liquefaction generated by internal cavitation via microsecond-length pulses at low duty cycle (<1%). Histotripsy is a mechanical ablation modality based on acoustic cavitation without MR-guidance. Histotripsy-mediated immunomodulation was studied in the mouse glioma (GL261-luc2) model. Histotripsy was used to partially ablate the tumor (10-20%) 14 days after implantation. Myeloid-derived suppressor cells (MDSCs) were significantly reduced in treated tumor, and an increase in IFN γ was observed. The group has also studied histotripsy-mediated immunomodulation in a mouse melanoma (B16P33) and liver cancer (Hepa 1-6) mouse model (50-75% tumor ablation). In the melanoma model, there was also an increase in IFN γ .

along with an increase in CD8+ T cell infiltration and HMGB1 concentration. They also observed an abscopal effect in untreated tumors from the same animal and in lung metastases with histotripsy only treatment. Histotripsy enhances checkpoint inhibition in contralateral untreated tumors in both melanoma and liver cancer models.

Pulsed FUS

Graeme Woodworth from the University of Maryland discussed activating an immune response in GBM using pFUS in the GL261-luc2 murine GBM model. Previous research in ex vivo brain tissue demonstrated that pFUS expands the extracellular and perivascular spaces.²⁴ In vivo work using nanoparticles as probes demonstrated a significant increase in interstitial spaces.²⁵ Previous research suggested that pFUS facilitated the recruitment of mesenchymal stem cells.²⁶ Post pFUS-treatment, there was an increase in regulatory T cells in the spleen, suggesting a redistribution in this cell population. In the draining lymph nodes, increases in MDSCs and a decrease in CD8+ T cells were observed. In the tumor, there were no observed differences in immune-cell populations 7 days post-pFUS with fluorescence-activated cell sorting (FACS) analysis. However, immunohistochemistry of tumor tissue showed increased CD4+ and CD8+ T cell populations. In summary, there were inflammatory cell changes up to one week after pFUS.

BBB Opening

Natasha Sheybani from the University of Virginia discussed MRgFUS BBB opening for immunotherapy in a mouse model of glioblastoma (GL261-luc2). Fourteen days after tumor implantation, MRgFUS plus microbubbles at two different acoustic pressures (0.4 MPa and 0.6 MPa) was used to open the BBB in the tumor and tissue was harvested for analysis 7 days later. Acoustic emissions were detected by passive cavitation, there were differences in mechanical energy deposition across these two different pressures. The combination of FUS plus microbubbles increased dendritic cell maturity in the tumor, tumor-draining lymph nodes, and meninges. The combination also increased antigen-experienced PD-1-expressing CD8+ T cells in the tumor, but there were no changes in TIGIT or TIM3 expression. The combination also increased the percentage of CD8+ T cells that were producing Granzyme-B in the superficial draining lymph nodes, and there were increases in the number of T cells expressing TIGIT. The dendritic cells in the superficial draining lymph nodes had increased expression of PD-L1 and CD155, and this was also found in the tumor microenvironment in stromal cells. This data suggests that MRgFUS BBB opening can shift local and systemic immune signatures in gliomas and sets the stage for future approaches of combinatorial drug and gene delivery strategies aimed at achieving immunological control of gliomas with FUS.

Immuno-PET imaging for spatiotemporal mapping of monoclonal antibody transport following FUS BBB disruption is also underway. Preliminary imaging demonstrated that FUS BBB opening increased the permeability of the tumor. The number of PD-L1+ immune cells increased within the brain 24 hours after treatment.

Microvasculature Ablation/BBB Opening

Nathan McDannold from Brigham and Women's Hospital presented data from a pilot study of microbubble enhanced FUS combined with checkpoint inhibitors in a mouse glioblastoma model (GL261-luc2). Anti-PD-1 antibody was administered in combination with FUS at two different acoustic pressures (255 kPa and 300 kPa). Animals were treated starting at 14 days post-implantation. There were no effects observed at the lower FUS level. The combination of a PD-1 inhibitor and FUS showed little difference to PD-1 inhibitor alone for adaptive immunity. However, the combination increased activated M1 microglia and PD-L1+ microglia. In the spleen and cervical lymph nodes, there was an increase in T cell immunoglobulin and ITIM domain (TIGIT) receptor expression on CD3+ and CD4+ T cells only with the combination of FUS and a checkpoint inhibitor.

Microvasculature Ablation/BBB Opening

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Discussion from Presentations

- There was a comment on the tumor model used in the experiments. In preclinical models, GL261 was immunogenic, but in clinical trials it was a “cold” tumor. Human GBM is heterogeneous consisting of different cell types, unlike a cell-line model.
- There was a suggestion to use CT2A in future GBM studies as it was less immunogenic and less prone to spontaneous regression.
- There was remaining tissue from these experiments that can be analyzed. Participants suggested looking for spatial differences with histology.

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4. What drugs/other therapies can be combined with FUS to enhance the response?

Innate and Adaptive Responses

Chandan Guha from Montefiore Medical Center presented on the use of FUS for immune modulation. Restored immune surveillance was likely the best mechanism to truly cure cancer. Guha discussed what was known about the radiation-immunity cycle to illustrate how FUS might boost the immune system. In a preclinical mouse model of metastatic lung cancer the sequencing of radiation therapy followed by a Flt3 inhibitor reduced metastasis and increased survival.²⁷ In an ongoing early phase clinical trial using ablative stereotactic radiotherapy (SBRT) at different doses followed by 5 days of Flt3 inhibitor, in patients with advanced NSCLC, several patients have experienced abscopal effects.

Sparse-scan HIFU was also studied for anti-tumor immunity. A low-intensity (energy) FUS (LOFU) has been developed to look at T cell responses in the spleen. LOFU was used to prime the tumor, followed by ablative radiation as an in-situ vaccine. By priming with LOFU, lower concentrations of drugs were used to induce a synthetic-lethal response. If CD4+ T cells in the draining lymph node become inactive (anergy) there will be no effect of an in-situ cancer vaccine. By applying LOFU at the primary tumor, not the draining lymph node, IL-2 production increases suggesting a reversal of the pathway that gives rise to anergy.

Tim Bullock presented on FUS-immunotherapy combinations. Immune checkpoint blockade has had limited success in breast cancer. A preclinical model of triple-negative breast cancer (TNBC) was treated with partial thermal ablation (10-20%) and FUS treatment. FUS alone had little effect on immune-cell counts at 7 days post-treatment. However, there was an increase in the number of dendritic cells in the tumor-draining lymph nodes, but there were no increases of CD8+ T cells in the tumor microenvironment. FUS (partial ablation) combined with gemcitabine at 14 days post-implantation followed by 2 additional doses of gemcitabine at days 21 and 28 led to tumor control and increased survival. There was also an abscopal effect on lung metastases. The experiment was repeated in RAG1 deficient mice (they have no T cells or B cells), which showed that the synergism between FUS and gemcitabine was dependent on the adaptive immune system.

A melanoma model (B16F10) treated with partial ablation was analyzed with RNAseq. In this experiment, FUS induces gene signatures of inflammasomes/pyroptosis. However, 24 hours later these signals disappeared and there was a strong suppression of the inflammatory response. Future research will investigate the mechanisms through which gemcitabine was synergizing with FUS. Partial ablation with FUS will also be combined with other chemotherapies in addition to gemcitabine. The UVa team will also investigate whether MDSC modification with drugs creates a similar effect as gemcitabine. Future studies will also look at whether sequential treatments with FUS change outcomes and the impact of ablation fraction/pattern.

Bullock also discussed the potential for FUS to increase T cell access to intracranial tumors. FUS combined with microbubbles increased CD8+ T cells in mouse models of glioblastoma. Several chemokines increased after FUS. When mice were given activated donor T cells in combination with FUS and microbubbles, there was no difference in the accumulation of T cells. FUS did not

enhance elements of the vasculature. Further research to examine the immunological aspects of the brain tumor vasculature and how to promote T cell infiltration into sonicated brain tumors was ongoing.

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Discussion from Presentation

- There was a comment that for the 4T1 breast cancer model and FUS, it would be interesting to know where the drug penetrated the tumor and how much gemcitabine was delivered to the tumor.
- A participant noted that there was only one data readout time point for these studies at 7 days, and there should be more work at varying time points to look for effects.

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5. What clinical disease targets are ideal for FUS plus immunotherapy combinations?

Breast Cancer Trials

David Brenin from the University of Virginia discussed preliminary data from an ongoing trial of immunotherapy (pembrolizumab, anti-PD-1) plus FUS ablation (NCT032375722) in patients with metastatic breast cancer. Breast cancer is immune privileged and typically evades immune surveillance. Pembrolizumab (anti-PD-1) was effective for the treatment of TNBC, which is estrogen receptor negative, progesterone receptor negative, and HER2 negative. Pembrolizumab was given either before or after FUS for thermal ablation with the Echopulse device (Theraclion, France). The primary objective was to assess adverse events of the combination in patients with metastatic breast cancer and to determine whether the addition of pembrolizumab to FUS increases the proportion of CD8+ tumor-infiltrating lymphocytes (CD8+/CD4+ ratio) in the primary ablation zone. The tumor volume targeted for ablation was 50%, up to 3 cm³, so that there was enough residual tumor remaining to stimulate an immune response. Biopsies were performed at days 22 and 64 post-treatment. There was an increase in CD8+ T cells after treatment. This study was ongoing and has enrolled 7 patients, with a final enrollment of 12. There have been no adverse safety signals or dose-limiting toxicities. There were no complete or partial responses, but 2 patients received additional therapies and had durable partial responses and one patient has stable disease with no additional treatments.

Glioblastoma Clinical Trial

Graeme Woodworth from the University of Maryland discussed a preliminary combination trial concept of immunotherapy plus FUS. Surgical resection works well for the initial tumor, but tumors typically recur after one year. A major challenge in GBM treatment is whether to select treatments based on the resected or residual tumor. In early discussion with the Food and Drug Administration (FDA) there were concerns around combination trials for device plus drug(s). These concerns led to the design of a non-therapeutic application for FUS and BBB opening in patients with brain tumors prior to planned surgery. Pre-surgical MRIs are used were for navigation

during resection, but once the surgery begins, brain tissue shifts, and these images are no longer accurate. Therefore, it is difficult to visualize normal brain tissue versus tumor tissue. To address this challenge, a trial was designed using BBB opening for planned brain tumor surgery in combination with a contrast-enhancing dye (e.g. fluorescein) to visualize tumor tissue during brain surgery (NCT03322813). For the first patients treated in this ongoing trial, the FUS BBB opening was performed immediately prior to surgery, and the dye injected at the initiation of the surgical procedure. The dye allowed the visualization of tumor regions with new contrast enhancement that were previously less discernable. Histopathology found no differences between the tissue with (FUS) and without (no FUS) fluorescein. The use of phase 0 and phase 0/1 trials in neurosurgical oncology that “match” the tumor to investigative treatments can enable precision medicine for some patients.²⁸ There will be a great deal of information that can be gathered from blood and cerebral spinal fluid collected in these kind of early-stage studies of FUS and immunotherapy combinations. Graeme Woodworth stated that leveraging the operating room as a portal for discovery is an important consideration going forward, particularly for testing new therapeutic and device combinations.

Pancreatic Cancer Trial

Brett Fite presented on behalf of Kathy Ferrara, Pejman Ghanouni, Joo Ha Hwang, and Kim Butts Pauly, regarding an upcoming clinical trial of focused ultrasound for treatment of pancreatic cancer. Ten patients with painful and locally advanced or metastatic pancreatic cancer will be enrolled. An endoscopic ultrasound-guided biopsy and blood sample at baseline and 1 week after treatment will be used for RNAseq analysis and immunohistochemistry. MRgFUS thermal ablation will be used to treat the pancreatic mass. Patients will receive gemcitabine plus palbociclib as chemotherapy while on the trial. The trial has received approval from the Stanford Cancer Institute, and investigational device exemption (IDE) and institutional review board (IRB) submissions were underway. They hope to begin recruiting patients in the next few weeks. This was the first step towards initiating combination trials of focused ultrasound and immunotherapy in pancreatic cancer.

Combination FUS with PD-1 Inhibitor Trial: Challenges and Lessons for FDA Applications of Combination Therapies

Craig Slingluff from the University of Virginia discussed challenges and lessons from an FDA application for a clinical combination trial of FUS plus immunotherapy. The trial proposed was an evaluation of FUS ablation, PD-1 inhibitor, and a TLR7 agonist in advanced solid tumors. The rationale came from studies showing that FUS can induce favorable changes in the tumor microenvironment.^{1,29} Preclinical mouse studies demonstrated that FUS following immunotherapy (PD-1 inhibitor) induced complete tumor control in 80% of mice bearing an epithelial tumor.¹¹ The TLR agonist imiquimod enhanced T cell infiltration and Th1 immune signatures in melanoma and other cancers,³⁰⁻³³ and can support tumor control.³⁴ Patients treated with HIFU ablation of pancreatic primary tumors had abscopal responses in distant lymph metastases.³⁵ Patient characteristics include progressive disease after other treatments and stable disease after 12 weeks of PD-1 inhibitor therapy. The proposed trial will use partial FUS ablation in patients with advanced solid tumors. There will be two cohorts: one that remains on PD-1 inhibitor plus FUS and imiquimod and the other will be patients that receive only FUS. Both groups will have the option to crossover into an FUS plus imiquimod treatment. Primary endpoints will be safety and immunologic effect (proportion of patients with increased CD8+ T cell infiltration) of FUS-treated metastasis.

The trial was submitted to the FDA as an IDE after a lengthy process. Most of the currently FDA-approved FUS clinical trials, have been for treating benign central nervous system disease (e.g., tremor). At the time of the workshop there were 18 active trials on clinicaltrials.gov for FUS treatment as a cancer therapy. Of these, prostate was the dominant type, with a few in liver and brain. There were no clinical trials in melanoma or other cancers for which a PD-1 inhibitor was FDA-approved. The FDA advised submission to the Office of Combination Products (OCP), but the OCP requested that in order to designate the appropriate regulatory route, the final nature of the product had to be defined. However, the purpose of the trial was to obtain pilot data to guide future FUS combinations and the researchers were unable to determine the final product. Therefore, the trial was then submitted as an investigational new drug (IND) based on a previously successful application for FUS plus PD-1 inhibitor in patients with breast cancer. Ultimately, after further discussion with the FDA, it was deemed more appropriate to submit as an IDE. The investigators hope to start recruiting patients later this summer or early fall.

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Discussion from Presentations

- There was a question on the types of cells that infiltrate the tumor in the breast cancer trial and this analysis was ongoing, but MDSCs have been observed.
- From the FDA perspective, MR-guided versus ultrasound-guided FUS is only a question of safety and being able to confidently treat the target tissue without any off-target effects.
- There was some discussion on novel combinations and the FDA application success. It was important to look at how previous studies were put together and keep the focus of the application only on safety and efficacy. The hypothesis should be backed by strong arguments on why there was a benefit to the field, as well as working with the FDA prior to submission to determine the optimal submission package that places the least burden on both parties. There was a recommendation that the FUS community should share their FDA submissions to simplify the process for future FUS applications.
- There was discussion regarding the fact that FUS was already approved for treating certain cancers, and interesting to measure immune responses to FUS treatment in those conditions.

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Discussion on Burning Questions

1. What signaling pathways/molecules are stimulated by FUS?

- Determine the differences resulting from various FUS modalities, different tumor types, and the effect on different cellular types in the immune system..
- Determine whether FUS can influence IFN production.
- Adhesion molecules should be studied as they were important to the function of T cells and the overall immune response.
- Study the immune response after sonication of the draining lymph node.
- Establish which parts of the immune system are involved in tumor control.
- Determine the best way to use FUS to synergize with immunotherapy, for example antigen presentation or other mechanisms.

- Identifying promising areas of combination for FUS and immunotherapy based on what has been learned from other immunotherapy clinical trials (e.g. pancreatic cancer trial supported by CRI and Parker Institute for Cancer Immunotherapy (PICI)) may help to move this into clinical trials faster.
- Look for the rate-limiting factors of immunotherapy, and whether FUS could be used to overcome some of those obstacles.³⁷
- Looking into the mechanisms associated with radiation therapy may provide some insights for potential FUS mechanisms.
- Anti-CD47 would be interesting in combination with FUS. A post-surgical gel treatment was developed that increases phagocytosis of cancer cells by macrophages.³⁶ This target could help stimulate the immune response after post-surgical resection.

2. Does the innate or adaptive immune system play the largest role in focused ultrasound treatment? Does this vary by mechanism or disease?

- Several research projects were underway to address the role of the immune system with FUS, and differences in mechanism or disease.
- Need to determine whether the innate or adaptive immune system was more important for boosting immunotherapy.
- Both the innate and adaptive immune system will play a role, but the issue was **what** role will they play?

3. What FUS mode is best at activating an immune response? Does this vary by tumor type? Is partial tumor treatment preferred over total treatment?

- A participant mentioned that typically the whole tumor was resected for GBM, but the remaining disease was a major concern. Which of these modalities would be the best modality for treating this? The goal was to determine how to stimulate the immune system to treat GBM. The group debated whole versus partial tumor ablation. There was agreement that partial ablation was likely the most interesting strategy to see if this could stimulate the immune system.
- It will be important to look at T cell trafficking to the tumor microenvironment versus T cell priming in several of these regimens to understand how the response could be used to supplement debulking surgery.
- In radiation therapy, partial treatments (i.e. lattice therapy) were often performed. Attendees debated the merits of this kind of FUS therapy for GBM.
 - Attendees suggested performing preclinical experiments to look at this.
 - There was a comment that FUS ablation on its own was unlikely to produce a meaningful response but likely needs to be combined with other therapeutics.
 - However, there was some preclinical data suggesting that partial ablation may stimulate an immune response in tumors (like melanoma) that already had some degree of immune infiltration.

- There was a comment that perhaps other tumor types might be more responsive to partial ablation with FUS.
- Another participant mentioned that when selecting a tumor type to use with FUS, it depends on the goal of therapy. If you want to boost the effects of immunotherapy, it might be best to choose tumor types like melanoma and lung cancer that already respond to immunotherapy. However, FUS could also be investigated in tumors, like pancreatic cancer, that show little response to immunotherapy to see if FUS can stimulate an effect.
- FUS can be used for both non-ablative thermal and noninvasive destruction. FUS has the advantage of being able to be used at multiple time points, and it could perhaps be used at recurrence to help prime the immune system.
- A participant raised the concern that partial ablation of a tumor may not be appealing to patients.
- There was a comment that endothelial cells were very sensitive to radiation, and they were likely also sensitive to FUS.
- In the GBM model, there seemed to be a MDSC response after all the FUS modalities.

4. What drugs/other therapies can be combined with FUS to enhance the response?

- Anti-CD40 would be interesting. It can have strong systemic effects in patients, so it has been difficult to bring this target to clinical trials. In mouse models, anti-CD40 can activate dendritic cells and expand cytotoxic T cell responses.
- There was a suggestion that researchers work with clinicians to come up with the best way for FUS to be used in the clinic and to gain more widespread acceptance.
- There was also a comment that cautioned against using two experimental modalities, from both a cost and regulatory perspective, and that researchers should consider approved therapies such as checkpoint inhibitors.
 - There was a consensus that the US FDA was hesitant to approve trials for two experimental therapeutics.
 - Another hurdle was that very little human data exists for the efficacy of FUS in treating cancer in humans.
- There was also a suggestion to perform more phase 0 trials, or canine (comparative oncology) trials, instead of mouse trials to try to move the field forward faster.

Parker Institute for Cancer Immunotherapy Toolkit

Theresa LaVallee from the Parker Institute for Cancer Immunotherapy (PICI) discussed biomarker testing through minimally invasive approaches. The mission of PICI is to bring people together through collaboration with the technology and resources to cure cancer through immunotherapy. There has been success in the clinic with matching a drug to a genetic alteration. Multi-parameter predictive biomarkers are a complex concept that require a change of mindset. Several factors will determine who to treat and with what specific combinations. Many considerations contribute to PD-1 resistance.

PICI has 7 institutions working together as one team. Together, they design clinical trials from a hypothesis and translate to the clinic. During the development phase, standardization is achieved through proof-of-principal exploratory studies. These are used to harmonize tissue sample collections. There are harmonized methods of collection and processing with data analysis at a central biorepository. Tissue biopsy quality and availability varies widely with these methods, so blood and stool are also collected. Another method uses unbiased clustering analysis of cellular population dynamics to see if there are changes in subsets of T cells.

Multiple parameters should be considered when assessing the immune response for treating cancer. MDSCs prior to treatment are under investigation as a predictive marker. Circulating free DNA (cfDNA) is also under investigation for looking at the circulating tumor profile as a surrogate for efficacy or applying an algorithm to look for epigenetic changes in relation to immunotherapy. There has been early data that these circulating markers can provide a lot of information about the state of the disease. There are relationships between the gut microbiome profile, immune fitness, and disease. Early data has suggested differences in the gut microbiome of anti-PD-1 responders versus non-responders. The consensus in the field has been that a “hot” tumor has many T cells; however, it is not known whether this is a predictive factor. Studies to answer this question are underway. There is also active investigation of a CD8+ PET tracer in phase I trials.

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Discussion from Presentation

- An attendee asked how individual institutions could gain access to bioinformatics services. PICI is open to collaboration with the goal to make everything public. The bioinformatics division is working to make tools publicly available. Participants were encouraged to reach out to PICI for potential collaborations.

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Preclinical Roadmap

Moderator Jessica Foley asked the participants to discuss preclinical studies for FUS and immunotherapy that will help move the field forward. The conversation centered around determining the clinical responses that FUS treatment could potentially provide, followed by preclinical research to support these possibilities.

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Clinical Opportunities

- The participants proposed that it was important to define areas of clinical need that might be met by FUS.

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Mechanisms of Resistance

- PD-1 has failed in several cancer types. Understanding mechanisms of resistance for immunotherapy in these cancer types might be an important first step prior to using FUS. Once the barriers have been identified, this knowledge could be applied to selecting the best FUS modality (i.e. mechanical or thermal) that might help to overcome these barriers.
- Several attendees suggested that preclinical work with FUS could be studied in cell lines/ preclinical models that were already known to be resistant to PD-1 and CTLA-4 inhibitors to see if there was a systemic immune effect of FUS in this setting.
- Participants suggested taking what has been learned, strengths and weaknesses of the various FUS protocols, from the experiments done by the GBM consortium and then adding anti-PD-1 into those models.

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Potential Models for Mechanistic Questions of FUS and the Immune System

- Hepatocellular carcinoma is known to be PD-1 inhibitor resistant, and there is a large unmet clinical need for these patients.
- There was a suggestion to use models for cancers that were well-established in response to immunotherapy such as melanoma and colorectal cancer. Adding a new modality such as FUS to models where there is a lot of immunological understanding might be helpful.
- The preclinical model will depend on the goal of FUS. For studying whether it is an immune modulator, immunogenic models such as CD26 or MC-38 should be used.
- A participant noted that preclinical trials should be run in parallel to clinical trials for FUS to better inform study design going forward. It is also important to design preclinical endpoints that coincide with endpoints that predict success and can also be used in clinical trials.
- Tim Bullock noted that the effect of FUS on the immune system in general, such as dendritic cell migration and antigen acquisition, should be determined.

- A participant mentioned that timing of FUS would be important. FUS might be best as a neoadjuvant prior to surgery, but this is an important factor to consider looking at with preclinical models. Experiments will need to look at administering FUS both before and after surgery.
- Jill O'Donnell-Tormey proposed an experiment that builds on the recent work in pancreatic cancer with a CD40 agonist, gemcitabine, nab-paclitaxel with or without anti-PD-1.¹⁵ Pancreatic cancer has a strong stroma, and there could be an opportunity to use FUS for stromal destruction. The mouse model has been immunologically defined for previous studies, so it could be straightforward to repeat the experiments with the addition of FUS. There is also a defined pathway to the clinic. Participants were encouraged to think about additional opportunities like this from the published literature.

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Next Steps for the GBM Consortium

- Tim Bullock mentioned using multi-spectral immunohistochemistry to look for responses in signals of tumor outgrowth such as cytotoxic T cells and MDSCs. The consortium should also consider moving to other GBM models that are more clinically relevant.
- There was a discussion on creating a core laboratory, or standard operating procedures, that could analyze tissue samples from different laboratories in order to standardize results and compare across FUS modalities.
 - Kullervo Hynynen pointed out that FUS modalities between laboratories are greatly varied, and the measurements for some aspects of standardization, such as cavitation and thermal dose, have not yet been developed.
- A biorepository for the study should also be set up going forward.
- Participants agreed that a deep dive on the immune data collected from the preclinical trials was necessary. Comparisons in immune responses between different FUS modalities can be done with the tissue samples that were collected. One of the first questions that can be asked is whether there was an increase in CD8+ T cell infiltration in the tumor. Based on this data, further studies could be designed.
- The next steps that were currently underway included an immune pathologist, blinded to study condition, analyzing the slides for CD8+ and CD4+ T cells.
- Verification in other GBM models that were less immunogenic would add support to the findings.

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Clinical Roadmap

The participants discussed how to move forward with FUS and immunotherapy.

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- Participants discussed tissue collection standardization. Formalin-fixed tissue allows for a great deal of analysis over a longer period of time.
- Create a set of guidelines to prioritize immune analysis in both clinical and preclinical studies. There was discussion on the immune readouts that would help best inform clinical trials of FUS and immunotherapy. Immunohistochemistry can be used as a snapshot at specific time points to describe the immune landscape as a function of treatment. There were several comments that there was often not enough material for flow cytometry and alternative methods will be needed.
- There was a suggestion that NanoString analysis could be used in place of immunohistochemistry or flow cytometry if those methodologies were unavailable. It was also a good tool when there were limited amounts or poor-quality tissue.
- Participants agreed that having a core facility for immune analysis together with standardized collection methods would greatly increase the reproducibility of preclinical experiments.
- There was a comment that because FUS encompasses many different modalities and parameters, it would be interesting to test the different types of FUS in a single well-established model that was known to work with immunotherapy to see how FUS works in a well-studied model.

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Parameters of FUS

- Given the variety of FUS modalities and systems, it is important to measure key FUS parameters such as thermal dose and cavitation to correlate with the following tumor response. Methodology of delivering energy was varied. There was a lack of standardization and methodology to measure parameters such as thermal dose and cavitation, and a standardized way to measure this is needed.
- Consideration should be given to how various FUS modalities and parameters could affect the immune system. There was a proposal to identify a biomarker in serum that would allow correlation of biological effect. A careful analysis of FUS treatment fraction, temperature, and pressure and subsequent effects on immune parameters in the blood (i.e. IL-1, IFN γ , and HMGB1) known to be involved in the subsequent immune response was proposed. Such a study could be used to look for correlative effects such as a link between pressure and IL-1 expression, etc. at relatively short time points as potential biomarkers. This could also be looked at across tumor types in order to observe the variation of effects.

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FUS as a therapeutic delivery mechanism

- It was vital to have preclinical models predictive of the human condition. It will also be necessary to be able to measure biological effects of FUS along with a biomarker that has reproducibility across laboratories.
- There was a question on whether there was a way to quantify the amount of drug delivered to the tumor, and whether these agents were acting at the site of the tumor or distant metastasis. There was no consensus in the literature and which specific aspect to explore with FUS was still under consideration.
- Using FUS as a local delivery agent for BBB opening or direct delivery to other kinds of tumors was not the focus of the meeting but was an exciting possibility for the technology. Another aspect to consider was whether FUS delivery of agents to a local site could reduce the systemic burden and associated toxicities of immunotherapy.

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As a result of these discussions the following recommendations for preclinical and clinical projects as well as standards development were formed as next steps for the community (see Table 3 page 24).

Table 3
Action items

| Category | Project Description | Timeline | Notes |
|-------------|---|----------|--|
| Clinical | Add-on: blood sample and/or biopsy analysis | Ongoing | Take pre/post-biopsy and/or blood samples, store until markers identified for analysis, use flow chart to mandate analysis. Dependent on standards. |
| Clinical | GBM BBBD: use FUS to deliver IO | Unknown | |
| Clinical | Oxford liver metastasis follow up: use FUS to deliver IO | Unknown | |
| Preclinical | GBM consortium follow up: blinded histology analysis | 6 months | In progress, look at invasive boundary, CD4+/CD8+. |
| Preclinical | GBM consortium follow up: multiplex staining, immune scoring | 1 year | Dependent on results from IHC analysis |
| Preclinical | Consortium: test FUS modes (thermal ablation, histotripsy, mechanical ablation (with/without bubbles), hyperthermia) in well-characterized model (melanoma) | 1 year+ | Include: reversal of PD-1 resistance, PET imaging to capture immune response over time, analysis of vasculature/lymphatics, partial treatment, and/or metastatic model to replicate clinical paradigm. Consider testing repeatability across labs. |
| Preclinical | Consortium: PanCan trial follow up: can FUS (histotripsy, mechanical ablation, partial thermal ablation) disrupt stroma to improve results. | 1 year+ | Use pancreatic cancer model + clinical drug combo + FUS. Consider testing repeatability across labs. |
| Standards | Develop guidelines for immunoanalysis (clinical and preclinical) | 6 months | Types of tissue to collect (biopsy, blood, etc.), timepoints, prioritized immune assays with process flow, core lab, data repository |
| Standards | Develop core lab for immune analysis | 6 months | Input from Parker/CRI: develop standard protocols, offer verification based on small sample set, offer training |
| Standards | Create preclinical data repository | 6 months | FUS parameters, immune data, biological data, imaging parameters, experimental timeline, protocol |
| Standards | Develop guidelines for FUS treatments | 6 months | Parameters to record/measure to permit standardization |
| Standards | Determine biological index for FUS treatment | 1 year+ | Way to correlate FUS treatment with biological change (DAMPs in blood, etc.) |
| Standards | FDA pathway for FUS/IO combination clinical trials | 6 months | White paper and potential guidance to submit to FDA |

Outcomes and Next Steps

Participants were encouraged to reach out to the Foundation with any research ideas or project proposals in this area. The FUS Foundation will continue engagement with this community to advance the field as quickly as possible.

Updated Burning Questions

Following discussions with participants as well as a meeting with the Scientific Advisory Board, the Foundation created an updated list of burning questions to guide future conversations around the use of focused ultrasound in cancer immunotherapy applications.

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- 1. What are the comparative immune effects induced by different FUS modes? How do these compare to other therapies (i.e. radiation, cryoablation, RF ablation)?**
- 2. How do FUS immune effects vary by tumor type?**
- 3. What clinical disease targets are ideal for FUS + immunotherapy combinations?**
- 4. How can we improve/optimize FUS treatments for immunomodulation (i.e. drugs combinations, partial vs. total tumor treatment, timing of treatments)?**
- 5. What metrics can be used to predict clinical success? (T cell ratios, etc) Can blood samples in the absence of biopsies reliably predict response?**

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Abbreviations

| | | | |
|-------------|--------------------------------------|---------------|---|
| BBB | Blood-brain barrier | IRB | Institutional review board |
| DAMP | Damage-associated molecular patterns | LOFU | Low-intensity focused ultrasound |
| FACS | Fluorescence-activated cell sorting | MDSC | Myeloid-derived suppressor cells |
| FDA | U.S. Food and Drug Administration | MHC | Major histocompatibility complex |
| FUS | Focused ultrasound | NNSCLC | Non-small cell lung cancer |
| HIFU | High-intensity focused ultrasound | OCP | Office of Combination Products |
| IDE | Investigational device exemption | PICI | Parker Institute for Cancer Immunotherapy |
| IFN | Interferon | TLR | Toll-like receptor |
| IND | Investigational new drug | TNBC | Triple-negative breast cancer |

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