Evaluation of Focused Ultrasound for the Induction of Immunogenic Cell Death

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Evaluation of Focused Ultrasound for the Induction of Immunogenic Cell Death

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Abstract

Triple negative breast cancer remains a life-threatening disease throughout the world and has no cure. Immunotherapy is a promising treatment but is not effective for all patients. Focused ultrasound (FUS) may have the capacity to improve the immunogenicity of triple negative breast cancer cells (TNBCs). A platform was established to evaluate the capacity of FUS thermal ablation to induce immunogenic cell death (ICD) using *in vitro* assays of the damage associated molecular patterns (DAMPs) associated with ICD and *in vivo* vaccination studies. Immunofluorescent microscopy (IFM) protocols were established to observe localization of calreticulin (CRT) to the outer leaflet of the membrane in conjunction with a phosphatidylserine (PS) stain to confirm the membrane has not degraded. A bioluminescent assay was validated to measure ATP release from cells. The media conditions required for this test to effectively detect ATP were further established. Western blots were determined to be insufficient for quantitative measurement of HMGB1 release into the supernatant from cells. Furthermore, we found that mice primed with subcutaneous injection of thermally ablated 4T1 cells prior to challenge with 4T1 tumor implantation saw significantly improved overall survival compared with mice receiving saline injections prior to 4T1 tumor challenge. FUS-treated cell injectates did not lead to restriction in growth of primary 4T1 tumors. Through this work, a method for evaluating the ability of FUS to induce ICD was established. The potential for FUS treatment to improve the immunogenicity of cells has been suggested here but further work will be necessary for confirmation of mechanisms underpinning the anti-tumor immune response to FUS-treated cells.

Keywords: breast cancer; focused ultrasound; immunogenic cell death; damage associated molecular pattern; thermal ablation

Introduction

Every year, 170,000 women worldwide are diagnosed with triple negative breast cancer (TNBC)[1,2]. TNBC presents numerous clinical problems for treatment, particularly aggressive metastases and an immunosuppressive tumor microenvironment (TME). Most deaths from TNBC come from the metastatic burden rather than from the primary tumor as it readily metastasizes to the lungs and brain[2]. These metastatic locations present several major complications, including (i) challenges to treating those tumors without damaging the surrounding tissue and (ii) impaired function of the native tissue due to cancer invasion. Furthermore, TNBC has the capacity to evade native immune response due to mechanisms of tumor immune escape as well as the immense burden of myeloid derived suppressor cells (MDSCs) that reside in the TME. These MDSCs inhibit antigen presenting cell (APC) recruitment and activation to the tumor bed, which in turn prevents the development of a lymphocyte driven anti-tumor immune response. However, new research suggests that it may be possible to overcome these barriers to APC activation when cancer cells undergo immunogenic cell death (ICD)[3].

Historically, the field of immunology has considered apoptotic cell death to be non-immunogenic and necrotic cell death to be immunogenic[4]. Recently, however, there has been an accumulation of evidence that ICD is a distinct mode of cell death that stimulates immune response against the antigens released by dead cells, and can be

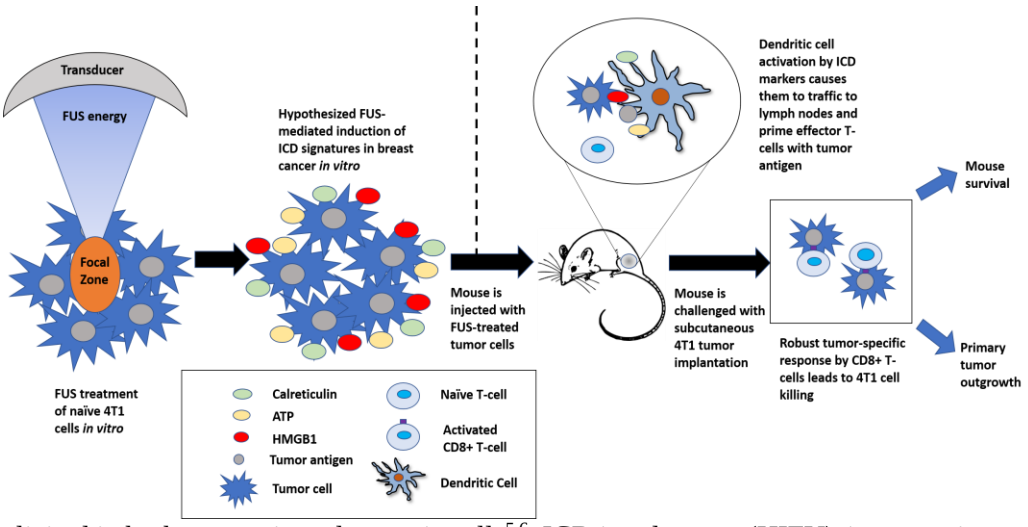

Figure 1. Experimental ICD Schematic: The experiments to be performed for this project follow the traditional methods of confirming ICD. All experiments begin with 4T1 cells treated in vitro with FUS. The cells are hypothesized to express signatures of ICD which can be measured in vitro. Following FUS treatment, these cells can be implanted into a mouse to prime the immune system by permitting APCs to be activated by the hypothesized DAMPs and initiate a robust anti-tumor T-cell response. Response will be measured through mouse survival and tumor outgrowth.

elicited in both apoptotic and necrotic cells[5,6]. ICD involves the unique expression of damage associated molecular patterns (DAMPs) that recruit and stimulate the maturation of APCs[7]. The first DAMP expressed during ICD following a stress on the cell is the exposure of calreticulin (CRT) on the extracellular surface of the cell membrane without a breakdown of the membrane. In ICD, CRT is often exposed concomitantly with other proteins of the endoplasmic reticulum (ER) such as heat shock proteins (HSPs) - which also serve as DAMPs. CRT is detected by CD91+ APCs (primarily macrophages and dendritic cells), and appears to lead to the priming of a Th17 response which increases the activity of macrophages and neutrophils at the site of the tumor [8]. The next major marker of ICD is the release of ATP in blebs from the cell. ATP is detected by P2Y purinergic receptors which activate macrophages and lead to the increased recruitment of dendritic cells [9]. The third indicator of ICD is the presence of high mobility group box 1 (HMGB1) protein, which is bound by TLR2/4 on phagocytic cells. Dying cells induce an antigen specific immune response when HMGB1 is released, but in the absence of HMGB1, they induce tolerance [10]. These DAMPs recruit and mobilize APCs which are important for priming and activating CD8+ (cytotoxic) and CD4+ (helper) effector T-cells, which are the key actors in tumor eradication.

One technique that has shown an increase in the activity of effector T-cells in the breast cancer TME is focused ultrasound (FUS)[11]. FUS is a safe, clinically available technique for non-invasive, non-ionizing tumor destruction that has demonstrated efficacy in the treatment of breast cancer [12–19]. FUS produces thermal and mechanical bioeffects in tumor cells by focusing sound waves into a small, targeted volume (Figure 1). FUS is capable of nearly instantaneously heating tissues with a sub-mm precision to produce stresses on cells. FUS can be used at high intensity (HIFU) in a continuous wave regimen to induce thermal ablation that results in coagulative necrosis at the focal zone. Around the focal zone, hyperthermic temperatures are achieved which may not induce necrotic cell death but do result in heat-mediated damage to cells. Additionally, FUS can be used at low intensity (LOFU) to induce an immediate, sub-lethal rise in temperature at the focal zone.

Thermally-based FUS treatments have been shown to produce the markers of ICD in individual studies across multiple solid tumor models [20]. Studies using HIFU to thermally ablate murine solid tumors (C1300 neuroblastoma, MC38 colon cancer, H22 liver cancer) have also shown resistance to re-challenge with the same tumor model subsequent to FUS exposure, suggesting that there is an immunologically driven abscopal effect imparted by FUS treatment [20–2220–22]. Furthermore, it has been shown that priming a mouse immune system with dendritic cells pulsed with HIFU-treated antigen imparts a more robust cytotoxic T-cell response against that tumor model than dendritic cells pulsed with untreated tumor lysate[22,23]. HMGB1 release in a neu deletion breast cancer model has been shown *in vitro* to be increased with increasing temperature in a water bath[24]. However, no comprehensive investigation has yet been reported to rigorously characterize the ability of FUS to induce ICD based upon the above-mentioned markers of ICD.

We hypothesize that FUS thermal ablation induces ICD in breast cancer. In this project, we sought to establish a platform to characterize the capacity of FUS thermal ablation regimens to induce ICD in 4T1, a murine mammary carcinoma model of TNBC, as well as to determine whether FUS treatment augments the immune response against treated 4T1 cells. A general schematic of the experimental

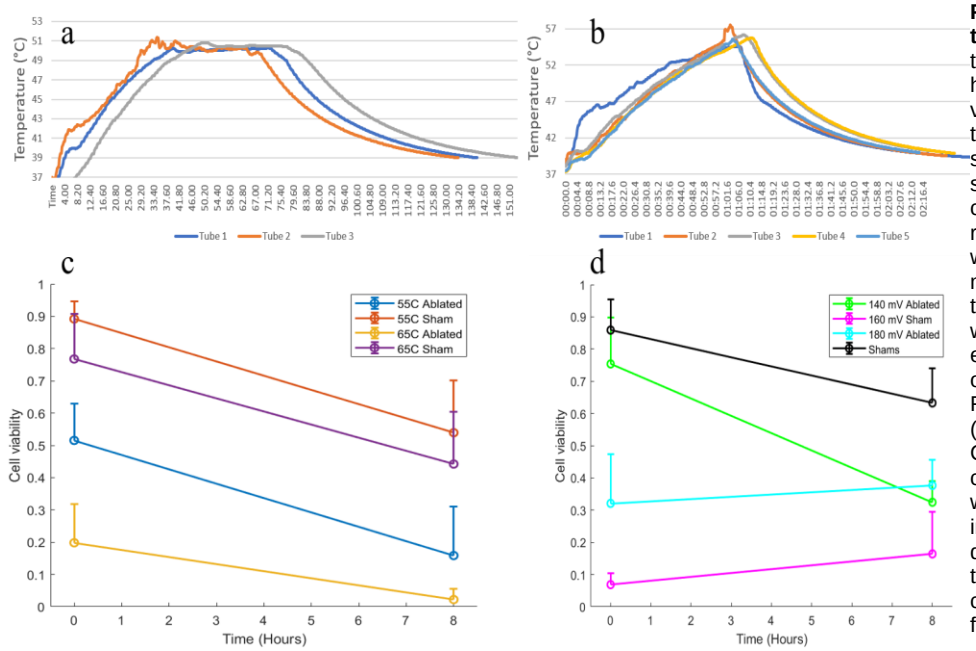

Figure 2. Cell viability responds to FUS treatment technique: a. A constant-temperature sonication produces consistent heat curves within the tube but there is some variability due to increased user interaction with the system. A 50°C target temperature curve is shown for each tube. **b.** The constant-power sonications result in more consistent heating curves with very little variability in the heat response over time. A constant input of 140 mV was used for each tube shown. **c.** Cell viability measured for 4T1 cells after constant-temperature treatments at either 55°C or 65°C with shams that were treated identically with the exception that the transducer was never turned on. Cell viability decreased initially for each FUS-treated group and declined over 8 hours (n=7 for FUS groups and n=4 for shams). **d.** Cell viability was measured as in (c) for three constant-power treatments. At 140 mV, there was no significant decrease in viability immediately after treatment but viability decreased over 8 hours. The other FUS treatments caused instantaneous decreases in cell viability that did not continue over time (n=3 for all groups).

goal is shown in Figure 1. 4T1 cells were treated with FUS thermal ablation regimens. Methods were optimized for measuring CRT translocation, ATP release, and HMGB1 secretion into the supernatant following FUS treatment. 4T1 cells treated with FUS *in vitro* were used to prime the immune systems of wild-type, syngeneic mice in a vaccination study. This study found a significant improvement in mouse survival after immune priming with FUS-treated 4T1 cells compared to priming with saline alone.

Results

FUS protocols

FUS protocols were optimized in order to develop consistent techniques that could be applied to exploring the ability of FUS to induce ICD. Two protocols for FUS thermal ablation of cells *in vitro* were validated for use in *in vitro* and *in vivo* studies. Both techniques used a single 1.1 MHz spherical transducer in a single, continuous pulse. The first protocol (constant-temperature) maintained a constant treatment temperature within the sample tube as measured by a thermocouple placed inside of the tube. The temperature in the tube was held constant for 30 seconds through manual control of the output power of the FUS system with live recordings from the thermocouple used for real-time feedback. The second experimental setup (constant-power) used a constant output power for a single, 60 second sonication and required no user input once the sonication began.

The temperature that cells were exposed to during FUS treatment was determined by the input voltage of the system. This input voltage was transformed within the system to an output power with higher inputs resulting in higher output. Cells exposed to higher temperatures should logically endure higher levels of cellular stresses. Control of the temperatures allows different samples to be exposed to stresses ranging from hyperthermia (sub-lethal) to ablation. For these studies, thermal ablation was selected as the treatment modality because it was expected to subject cells near the focal point of the transducer to lethal temperatures while raising the temperature to significantly stress surrounding cells without causing instant necrosis.

Both treatment techniques showed a high degree of consistency within experiments (Figure 2a&b). Each technique produced consistent heating over time within tubes as recorded by the thermocouple placed inside the measured PCR tube. The constant-temperature treatment was able to maintain a steady target temperature for 30 seconds consistently (Figure 2a). The constant-power technique was capable of producing highly similar curves over the 60 second duration of the sonication (Figure 2b). As both techniques appeared to produce unique, but internally consistent heating curves, both appeared to be effective potential regimens.

Cell death is a function of FUS thermal ablation temperature and time

ICD requires the release of DAMPs over a period of several hours, so in order to measure the capacity of FUS to induce ICD, the chosen treatment must not instantly kill

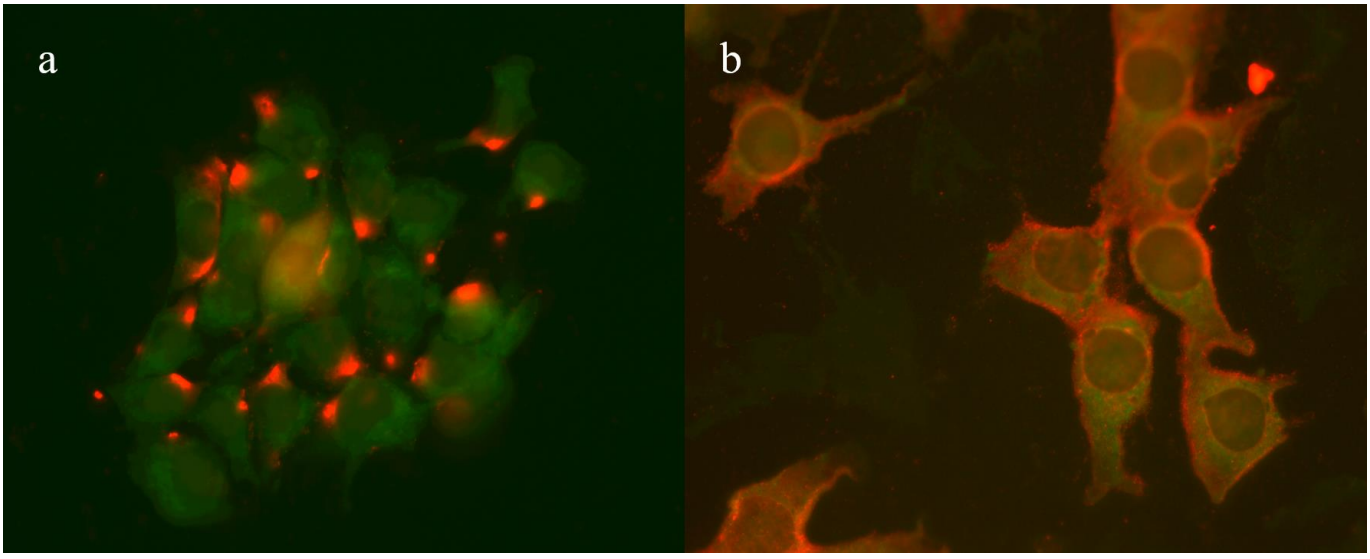

Figure 3. IFM shows that UV treatment results in a diffuse expression of CRT across the membrane of 4T1 cells: a. Cells incubated in media for 20 minutes immediately prior to fixation and staining show little CRT (red) on their membranes. CRT appears to be localized to cell junctions. PS (green) staining is present at low levels across the cells. **b.** 4T1 cells exposed to UV light for 20 minutes prior to fixation and staining express CRT across the outer leaflet of the membrane with low levels of PS staining comparable to that seen in media-incubated cells.

all cells present. Thus, the viability of cells was measured following FUS treatment at a range of temperatures or powers for the constant-temperature and constant-power techniques, respectively. We assayed for cell viability both immediately after FUS treatment and 8 hours later using Trypan blue. When treated using the constant-temperature technique, both FUS-treated groups showed significant decreases in cell viability over 8 hours ($p < 0.01$ for both groups) (Figure 2c). The 55°C sham group showed a significant decrease in viability over time ($p = 0.0476$) while the 65°C sham group did not show a significant decrease in viability from 0 to 8 hours ($p=0.1051$). FUS-treated groups had significantly lower viability than their corresponding sham for all groups at both time points. However, when treated with the constant-power technique, only the cells treated at 140 mV showed a significant decrease in cell viability over the 8 hours studied ($p=0.0154$) (Figure 2d). Both the 160 mV and 180 mV groups increased in cell viability during the incubation period. Based on this data, the constant-temperature technique appeared to be more effective for establishing a treatment that consistently produces cell death across a range of treatment levels during the incubation period.

In vitro markers

Calreticulin

Translocation of CRT from the ER to the outer leaflet of the membrane is an indicator of ICD which suggested that this could be measured using immunofluorescent microscopy (IFM). However, CRT exposure is only indicative of ICD when pre-apoptotic, so a secondary stain was necessary to establish whether cells are undergoing ICD or apoptosis[6]. Phosphatidylserine (PS) is a commonly used marker of apoptosis because PS is normally found on the cytoplasmic leaflet of the plasma membrane but becomes exposed during apoptotic membrane degradation. Anti-CRT antibodies and Alexa Fluor®488 annexin V were acquired to perform immunofluorescent co-staining for CRT and PS in cells. Cells were not permeabilized during fixation or staining to ensure that no stains reached an intracellular target.

Once IFM had been selected to measure CRT translocation, a positive inducer of ICD had to be selected to validate the stains. The first tests were performed with H_2O_2 which has the potential to induce oxidative stress which is closely linked to ICD[25,26]. However, H_2O_2 resulted in cells being lost during wash steps for staining, presumably because they killed cells and caused them to lose adherence to the plate. This revealed the need for alternative methods of cell killing that could stress cells sufficiently to initiate cell death pathways without immediate death or membrane permeabilization so that they could be fixed and appropriately stained. Ultraviolet (UV) light was later selected because it is also associated with ICD but the dose could kept low to minimize rapid cell death[27]. UV treatment of cells resulted in a diffuse pattern of CRT (red) expression across the membrane of cells without an accompanying increase in PS (green) expression compared to untreated controls (Figure 3). Thus, translocation of CRT to the outer leaflet of the cell without a breakdown of the membrane was measurable through IFM.

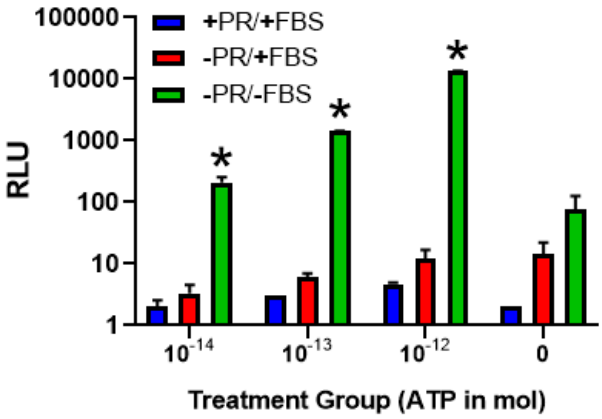

Figure 4. PR and FBS reduce the sensitivity of the ATP assay: The ATP bioluminescent assay kit was used to detect ATP in solutions buffered with media containing PR and FBS (+PR/+FBS), FBS only (-PR/+FBS), or neither PR nor FBS (-PR/-FBS). When buffered with -PR/-FBS media, the luminescence detected for equivalent amounts of ATP was significantly greater than either other media condition for all concentrations containing ATP.

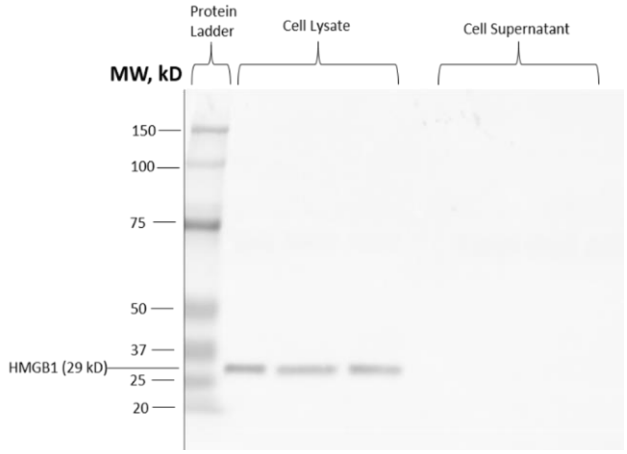

Figure 5. Western blotting detects HMGB1 in lysate but not supernatant: Supernatant from 4T1 cells was collected and cells were subsequently lysed. Western blotting was performed on the cell lysate and the supernatant. HMGB1 bands at 29 kD were detected in the cell lysate but not the cell supernatant. Bands in the supernatant only became visible after processing supernatant in protein concentration columns prior to blotting.

Bioluminescent ATP readouts are affected by media content

While CRT translocation in ICD is measured on the outer leaflet of the cell membrane, ATP blebbing must be measured from cell-free supernatants. The presence of ATP in the media was tested using a bioluminescent assay. Initial assays showed ATP readouts for duplicate samples that appeared to vary by orders of magnitude in negative controls (data not shown). Those assays also showed very little difference in ATP values after treatment with varying doses of ICD inducing chemotherapies. These unusual results prompted a review of potential interfering agents in the assay which suggested phenol red (PR) and fetal bovine serum (FBS) may attenuate ATP readouts when using luciferin/luciferase assays[28].

Testing the ATP bioluminescence kit revealed that the media used to culture cells can significantly impact the intensity of bioluminescence readouts. The growth and experimental media initially used was RPMI 1640 media containing phenol red (PR) and supplemented with 10% fetal bovine serum (FBS). The presence of PR and FBS in media decreased the luminescence measured from the bioluminescent assay by more than an order of magnitude compared to RPMI 1640 without PR or FBS (Figure 4). This observation explains the unusual standard curve observed earlier. Since ATP was being experimentally measured in the media, that media was used as the sample tested by the assay. In the standard curves, clear ATP solution at 10^{-7} M was loaded into the assay and diluted using media. Thus, high concentration standards had relatively little media while low concentration standards had far more media, leading to the exponential increase in measured luminescence with concentration. These findings led us to modify subsequent experiments by replacing the growth medium with RPMI 1640 with no PR or FBS immediately before treatments. Following this media exchange, cells were kept for only 8 hours and we have grown 4T1 cells successfully for several passages in FBS-free media so this media switch is not expected to change cell behavior during experiments. With this media exchange, ATP in the supernatant can be effectively measured to explore the capacity of FUS to induce ICD.

HMGB1

Western blots were selected initially for HMGB1 analysis in the media because they can probe a single protein of interest and are a lower cost per kit than enzyme linked immunosorbent assays (ELISAs). Ultimately, ELISAs were deemed to be the most effective method of HMGB1 quantification due to several limitations found in the Western blots including challenges with establishing total protein loaded into a well and with protein concentration causing unknown degree of sample loss.

Early attempts to perform protein quantification with BCA analysis were confounded by the presence of FBS in the sampled media. The 10% FBS supplement resulted in total protein concentrations that approximated the stock concentration available in the BCA kit. As a result, creation of an effective standard curve was not possible. Due to these challenges associated with quantifying total protein, steps were taken to ensure that the samples treated in these studies contained consistent numbers of 4T1 cells and were treated identically to maximize the consistency of the Western blot measurements.

Western blots were sufficient to identify the presence of HMGB1 in cell lysates but not in the supernatant (Figure 5). HMGB1 is constitutively present in the cell nucleus, so cell lysates were used as a positive control to validate the Western blot itself. Performing a Western blot on either lysed 4T1 cells or the culture media of those cells showed the presence of HMGB1 in cells but not in the media (Figure 5). Later Western blots did not detect the presence of HMGB1 in the supernatant of cells treated with mitoxantrone, a positive inducer of ICD (data not shown). HMGB1 in supernatant samples only became observable with Western blots after supernatant samples were concentrated using ultra-centrifugal protein concentrators (data not shown). However, protein concentration involves an unknown loss of protein so further work will be done to establish alternative approaches.

In vivo

The *in vitro* platforms were established to be able to probe for molecular markers of ICD, but confirming that cells undergo bona fide ICD requires *in vivo* experiments, as well[7]. Thus, we performed a vaccination experiment by priming mice with FUS-treated 4T1 cells. Cells were treated (at either 55°C or 65°C) using the constant-temperature FUS regimen established previously and subsequently incubated for 8 hours. Mice were injected subcutaneously with either FUS-treated cells resuspended in sterile saline or sterile saline alone. 7 days later, mice were injected in the contralateral flank with naïve 4T1 cells and monitored. With this vaccination approach, we sought to investigate the ability of the mouse immune system to respond to FUS-ablated 4T1 cells.

There was no difference in the outgrowth of the primary tumors between the mice primed with FUS-treated cells or sterile saline (Figure 6a). Measurements of subcutaneous flank tumors were performed according to the IACUC-approved protocol with digital calipers. The growth of tumors across the groups showed a great deal of similarity with no significant divergences across the length of the study. This lack of primary tumor control suggests that any survival-enhancing effects of FUS may require additional intervention in order to confer a robust anti-tumor response.

Despite the lack of primary tumor control, injection with FUS-treated cells improved the survival of mice following challenge with naïve 4T1 cells (Figure 6b). Both groups of mice primed with FUS-treated cells showed significantly longer survival compared to mice primed with saline only (Figure 6c-d). There was no difference observed in the mouse survival between the two groups primed with FUS-treated cells, suggesting that the difference resulting from priming with cells ablated at 55°C or 65°C is small, suggesting that a 10°C difference may be insufficient to cause differential consequences. The enhanced survival does not appear to be mediated by control of the primary tumor but may come from improved control of metastases following priming with FUS-treated cells. This was corroborated by our qualitative observation that mice primed with FUS-treated cells displayed less severe difficulty breathing, the most evident physical symptom of 4T1 pulmonary metastatic burden (data not shown]).

Discussion

These experiments established the foundation for an “in vitro to in vivo” pipeline for testing the capacity of FUS to induce ICD. Two FUS thermal ablation regimes

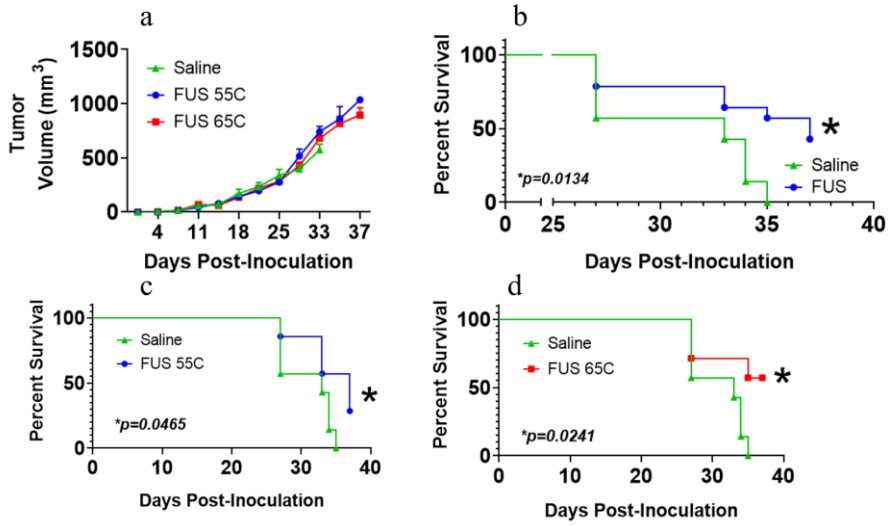

Figure 6. Priming with FUS-treated cells improves mouse survival but does not control primary tumors: Mice were primed with sterile saline or 4T1 cells treated with FUS at 55°C or 65°C (n=7 for each group). 7 days later, mice were inoculated with live 4T1 cells in the contralateral flank. All data presented come from a single experiment. **a.** Following inoculation, there was no difference in the size of primary tumors between mice that received an initial priming with saline alone or FUS-treated cells. **b.** Mice primed with FUS-treated cells survived for significantly longer than mice primed with saline alone (p=0.0134). There was no significant difference in survival between FUS groups so they are pooled into a single FUS group for this panel (n=14 for FUS group). **c. and d.** show the individual groups of mice primed with FUS-treated cells compared to saline. **c.** Mice primed with cells treated with FUS at 55°C showed significantly improved survival compared to mice primed with saline only (p=0.0465). **d.** Mice primed with cells treated with FUS at 65°C showed significantly improved survival compared to mice primed with saline only (p=0.0241).

have been established and validated as repeatable, high throughput techniques for performing tumor cell ablations *in vitro*. Though FUS ablation is considered to occur instantaneously, the consequence of cell death within the immediate focal zone is thought to be instantaneous as well. However, we demonstrate that within certain power ranges, it is feasible to immediately destroy only a fraction of the total population of cells, thereby leaving a small subset of cells that have been subjected to immunologically favorable effects without fully compromised viability. The remaining cells in the population were exposed to stresses that may cause cell death in a slower manner, permitting ICD markers to be measured if FUS is capable of inducing ICD. *In vitro* assays for the three primary molecular markers of ICD have been established and modified to be compatible with the FUS treatments. CRT localization to the outer leaflet of the plasma membrane can be recorded using IFM with a PS co-stain. ATP released through blebbing can be recorded using bioluminescent assays of the supernatant in the appropriate media conditions. HMGB1 can be quantitatively measured using ELISAs given the limitations of Western blots. With these techniques developed and documented, the measurement of *in vitro* markers of ICD following FUS treatments can be performed.

In addition to the establishment of *in vitro* assays, the experiments described here demonstrate the potential of FUS-treated 4T1 cells to act as a vaccine against naïve 4T1 cells. The data from the vaccination experiment suggested that priming mice with FUS treated cells improves their survival following tumor challenge when compared to mice primed with only saline prior to the tumor challenge. Despite the improvements in survival, there was no difference in the size of the flank tumors that developed in the mice primed with FUS-treated cells compared to mice primed with saline alone. This suggests that the improvements in survival are not due to control of the primary tumor. However, the mice primed with FUS-treated cells did not display symptoms of severe lung metastases as rapidly as mice primed with saline alone. 4T1 cells recapitulate the physiology of TNBC in that they rapidly metastasize to the lungs. In mice, this pulmonary metastatic burden is readily evidenced by the onset of lethargy, labored breathing, and ruffled or hunched physical appearance. The mice primed with saline tended to display labored breathing earlier in their timeline as compared with mice primed with FUS-treated cells; these mice tended to reach a humane endpoint earlier as a result. Lungs were harvested from mice in each group for histological staining and quantification of lung metastases, but they could not be sectioned and stained prior to lab shutdowns resulting from COVID-19. Thus, available lung metastasis data is currently qualitative and further analysis will be required to draw a more definitive conclusion about any connection between overall survival and pulmonary metastatic burden.

The *in vivo* experiments performed here used the constant-temperature FUS treatment technique of holding the temperature constant because that was the first FUS technique validated for these experiments. This was initially developed because it is designed to expose cells to a known temperature and would have allowed the expression of ICD-related DAMPs to be correlated to ablation at a particular temperature for 30 seconds. However, this technique had several limitations that led us to explore alternative techniques. The most immediate concern involved the placement of the thermocouple inside the PCR tube. This process required the tubes to be opened to the air during the FUS treatments, reducing the sterility of the cell material inside. This reduction in sterility combined with the addition of a semi-sterile thermocouple to the tube led to concerns about the potential introduction of pyrogenic material to the samples. The addition of pyrogenic material has a risk of generating an immune response in the mice that is not due solely to the FUS-treated tumor cells that could confound the results of these experiments. Furthermore, the constant-power method required less user input. Rather than a user observing the live temperature readout to adjust the treatment power multiple times throughout the experiment, the constant-power treatment technique could be fully automated once the FUS system was turned on. This would permit a more consistent FUS treatment between samples that is also faster. Despite these limitations, cell viability assays ultimately showed the constant-temperature technique to more consistently produce measurable decreases in cell viability over the 8-hour incubation. Due to its reproducible effect, this technique was utilized for all experiments performed herein. Further optimization of the constant-power technique may ultimately improve its treatment consistency. Should that occur, future FUS/ICD experiments ought to explore use of the constant-power treatment technique.

Both FUS techniques have been used to treat 4T1 cells for *in vitro* assays of ICD-related DAMPs. The supernatant samples collected from those experiments were processed as described in Materials and Methods in preparation for ATP and HMGB1 assays before being stored at -80°C. It was expected that those samples would be assayed over the following days, but lab closures from COVID-19 prevented us from performing those assays. The samples are still available for assays once labs reopen, but there is a risk of sample degradation over the subsequent months. Assays run on these samples may be valuable to confirm that the assays described herein will work as

anticipated for the analysis of ICD-related DAMPs, but if results are inconclusive it will be difficult to identify whether the failure comes from the assays or the samples. It is likely to be more efficient to treat 4T1 cells again to generate fresh samples for assaying.

Future experiments will be necessary to determine whether 4T1 cells display ICD-related DAMPs after FUS treatment and whether FUS-treatment of cells confers an immunological benefit. The mice primed with FUS-treated cells in the vaccine experiment showed improved survival compared to the saline controls, but the experiment performed does not control for the baseline effect of injecting 4T1 cellular material into an animal. Determining this effect would require injecting mice with dead tumor cells that have been killed in an alternative manner. An ideal method of performing this experiment would be to compare the survival benefit of animals primed with FUS-treated cells against animals primed with cells treated in a way known to induce ICD and cells treated in a way known not to induce ICD. One promising set of comparators would be the platinum-based chemotherapeutics oxaliplatin and carboplatin. Despite being derived from the same compound, oxaliplatin induces ICD in cancer cells while carboplatin does not[29]. In order to confirm that any survival benefit from injection with FUS-treated cells comes from an enhanced adaptive immune response, these experiments would need to be repeated in immunodeficient mice. A lack of protection against cancer in these immunodeficient mice following injection with FUS-treated would provide strong evidence for FUS conferring immunological benefits. Furthermore, while the increase in mouse survival following priming with FUS-treated cells was significant, all mice did develop tumors after the naïve 4T1 cell inoculation. This stands in contrast to several other inducers of ICD that have conferred enough immunological resistance at appropriate doses to cancer to prevent subsequent tumor take[6,27]. This difference in tumor take may be driven by the immunosuppressive microenvironment of 4T1 tumors or by a lower immunological benefit from FUS thermal ablation alone. Future studies can investigate the impact of each of these elements by combining adjuvants - such as the CD40 agonist FGK or the TLR3 agonist poly(I:C) which increase the activation of dendritic cells – with FUS treatments. The addition of adjuvant concomitantly with FUS-cell priming may enhance the efficacy of priming while subsequent adjuvant interventions may help the mouse immune system to overcome the barriers to anti-cancer immunity imposed by the immunosuppressive microenvironment.

Through this work, we have laid the groundwork for the evaluation of FUS as an inducer of ICD. The ability to test the capacity of FUS to induce ICD is particularly valuable because much work in the FUS community has emphasized the effects of FUS on the adaptive immune response but the physiological mechanisms that drive these effects is still unclear[24,30]. With the platform established here, it has become possible to systematically investigate whether adaptive immune responses are borne out of ICD or if another mechanism is involved. If FUS proves capable of inducing ICD, many new methods of using FUS to augment the immune system will become available. The most exciting of these would be the potential to use FUS treatment of a primary tumor as an autovaccine against a patient's own cancer. Even if FUS does not induce ICD in cancer cells, it still has potential to act as a primary therapy and augment the efficacy of immunotherapies used in conjunction.

Materials and Methods

Cells

4T1 murine mammary carcinoma cells were obtained from ATCC (Manassas, VA). These cells were cultured in RPMI 1640 Media containing PR and supplemented with 10% FBS (Gibco, Gaithersburg, MD). Cells were incubated at 37°C and 5% CO_2 in T-75 culture flasks (Corning Incorporated, Corning, NY). Cells were passaged once they reached 80% confluence with a DPBS (Gibco, Gaithersburg, MD) wash and chemically digested with Trypsin-EDTA (ATCC, Manassas, VA) to detach from the flask before being reseeded into fresh T-75 flasks. Immediately before FUS treatments, 4T1 cells were passaged and resuspended in RPMI 1640 Media containing no PR or FBS before being diluted into 1.5 mL PCR tubes (Eppendorf, Hamburg, Germany).

FUS treatments

All FUS treatments were performed on 4T1 cells *in vitro* The FUS system used was a custom, in-house FUS system with a 1.1 MHz, spherical transducer modified to permit in vitro treatments (Figure 7). PCR tubes containing 4T1 cells were mounted on the sample holder of the FUS system before being lowered into a degassed water bath maintained at 37°C so that the center of the tube was aligned with the transducer's focal point. Each FUS treatment was performed using a single, continuous sonication to deposit thermal energy and heat the cells. Identical voltages did not always produce identical temperature responses between days. To match treatments between experiments, voltages were measured empirically at the beginning of each

experiment by sonicating an additional sample to match temperature response curves.

The constant-temperature treatment technique described required a thermocouple to be inserted into each PCR in order to measure the temperature in real time. The temperature was raised to a target temperature using a high input voltage (between 150 mV and 200 mV) to quickly achieve ablative temperatures. Upon achieving the target temperature, the input voltage to the system was immediately decreased by 20 mV so that the temperature would be kept constant at the target for 30 seconds. After 30 seconds, the FUS system was turned off.

The constant-power treatment technique described did not require a thermocouple to be placed inside each PCR tube. In this technique, an input voltage was sent to the system that corresponds to the desired level of ablation. Once the FUS system was activated, it was allowed to run with no additional input for 60 seconds before turning off.

Cell Viability

Following FUS treatment, 25 μL from each sample were collected and diluted 1:1 in Trypan blue (Gibco, Gaithersburg, MD) and counted on an automated hemocytometer to obtain live/dead ratios with reduced bias. The remainder of the treated sample was incubated for 8 hours at 37°C and 5% CO_2 in the PCR tube. At the end of the incubation, cells were resuspended in the PCR tube by gently pipetting up and down. 25 μL was again collected from each sample and counted as before. Statistics were performed using a one-tailed Student's T test.

Immunofluorescence

Rabbit Anti-CRT antibodies were acquired from Abcam (Cambridge, UK), IRDye 800 CW Goat anti-Rabbit antibodies were acquired from Licor (Lincoln, NE), and Annexin V Alexa Fluor 488 conjugates were acquired from ThermoFisher (Waltham, MA). After treatment, 4T1 cells were plated on Ibidi 24 well-plates (Grafelfing, Germay) at a density of 3-5E4 cells/well. 18 hours after seeding, cells were treated with H_2O_2, UV light, or culture media. Cells were fixed with formalin and washed with PBS. They were then stained with anti-CRT antibodies and Annexin V Alexa Fluor 488 and allowed to incubate overnight at 4°C. Cells were washed again and stained with goat anti-rabbit antibodies. Imaging was done with a confocal microscope.

Bioluminescent Assay

The Promega Enliten ATP Assay System Bioluminescence Detection Kit (Madison, WI) was used to detect ATP in cell supernatants. After treatment and cell viability assays, the PCR tubes used were centrifuged at 1000 RPM for 5 minutes at room temperature to pellet cells. The supernatant was aspirated and placed in an Amicon Ultra 0.5 mL Filter with 10 kD cutoff (EMD Millipore, Burlington, MA). The supernatant was then centrifuged at 10,000 x g for 10 minutes. The run-off material was collected and used for ATP analysis (concentrated protein from the supernatant was used for Western blots during concentrated protein measurements). Supernatant samples were placed in a 96 well-plate (Corning Incorporated, Corning, NY) and brought to a volume of 100 μL with media free of PR and FBS. Standards were prepared according to the assay protocol using media free of PR and FBS as buffer solution. A SpectraMax iD3 (Molecular Devices, Austria) was used to administer the luciferin/luciferase reaction mix to each well and measure flash luminescence immediately afterwards.

Western blot

Western blots were performed on samples in PCR tubes following treatment and cell viability assays. After centrifugation in the Amicon Ultra 0.5 mL Filters described in *Bioluminescent Assay*, the concentrated protein was collected and mixed 1:1 with a 2x dilution of Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and boiled at 100°C for 5 minutes. 20 μL of this mixture was placed in wells of a 10% polyacrylamide gel (Bio-Rad, Hercules, CA). The gel was run at 200V for 60 minutes or until the protein ladder had fully separated the 37 kD, 25 kD, and 20 kD bands. The gel was transferred to a pre-activated PVDF membrane (ThermoFisher, Waltham, MA) with an iBlot dry transfer system (Invitrogen, Carlsbad, CA). After transfer, the membrane was washed and then blocked with 5% milk protein in 1X TBST for 60 minutes. The blocking solution was rinsed with TBST and a solution of 1 ug/mL of anti-HMGB1 antibody (Abcam, Cambridge, UK) with 5% milk protein and 1X TBST. The membrane was incubated in anti-HMGB1 antibody solution for 60 minutes on a rocker at room temperature. After incubation, the membrane was rinsed with TBST again. A solution of 1:20,000 IRDye 800CW Goat anti-Rabbit antibody (Licor, Lincoln, NE) with 3% milk protein in 1X TBST was applied to the membrane. The membrane incubated in solution for 20 minutes on a rocker at room temperature. The antibody solution was rinsed with TBST. The membrane was then imaged on a Licor Odyssey IR imaging system (Lincoln, NE).

Animals

8-week female BALB/c mice were acquired from Jackson Laboratory (Bar Harbor, ME). In vaccination experiments, mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg; Zoetis) and dexdomitor (0.25 mg/kg; Pfizer) in sterilized 0.9% saline. Flanks were depilated via shaving. Each mouse was then injected subcutaneously in the left flank with 200 µL of sterile saline containing either 4E5 FUS-treated 4T1 cells or no cells. 7 days later, mice were anesthetized once again and injected subcutaneously in the contralateral flank with 200 µL of sterile saline containing 2E5 naïve 4T1 cells. Size measurements of the primary tumors were obtained using digital calipers. Mice were euthanized at humane endpoints according to the IACUC protocol. Upon reaching the humane endpoint, primary tumors and lungs were harvested and preserved in 10% neutral buffered formalin for future use. Animal studies were prospectively reviewed and approved by the UVA Animal Care and Use Committee.

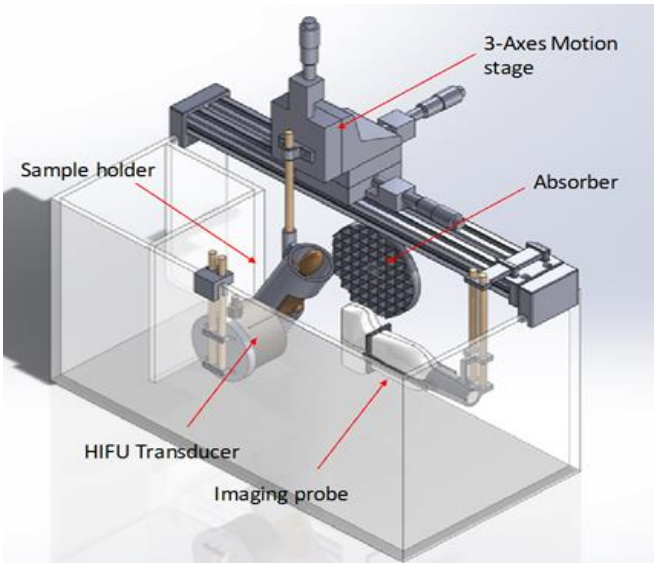

Figure 7. Schematic of FUS system used: The FUS system employed here used a tank of degassed water maintained at 37°C. A 1 MHz high intensity focused ultrasound (HIFU) transducer was positioned to treat PCR tubes positioned in the sample holder. The 3-axes motion stage permitted movement of the sample holder so that tubes could be precisely positioned and held steady in the focal point of the transducer. The imaging probe and absorber were not used in these experiments.

End Matter

Author Contributions and Notes

R.A.C. designed research; R.A.C. and N.D.S. performed research; R.A.C. analyzed data; and R.A.C. wrote the paper.

The authors declare no conflict of interest.

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