High-Intensity Focused Ultrasound (HIFU) Triggers Immune Sensitization of Refractory Murine Neuroblastoma to Checkpoint Inhibitor Therapy

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ABSTRACT

Purpose: Immunotherapy promises unprecedented benefits to patients with cancer. However, the majority of cancer types, including high-risk neuroblastoma, remain immunologically unresponsive. High-intensity focused ultrasound (HIFU) is a noninvasive technique that can mechanically fractionate tumors, transforming immunologically “cold” tumors into responsive “hot” tumors.

Experimental Design: We treated <2% of tumor volume in previously unresponsive, large, refractory murine neuroblastoma tumors with mechanical HIFU and assessed systemic immune response using flow cytometry, ELISA, and gene sequencing. In addition, we combined this treatment with anti-CTLA-4 and anti-PD-L1 to study its effect on the immune response and long-term survival.

Results: Combining HIFU with anti-CTLA-4 and anti-PD-L1 significantly enhances antitumor response, improving survival from 0% to 62.5%. HIFU alone causes upregulation of splenic and lymph node NK cells and circulating IL2, IFNγ, and DAMPs, whereas immune regulators like CD4+Foxp3+, IL10, and VEGF-A are significantly reduced. HIFU combined with checkpoint inhibitors induced significant increases in intratumoral CD4+, CD8α+, and CD8α+CD11c+ cells, CD11c+ in regional lymph nodes, and decrease in circulating IL10 compared with untreated group. We also report significant abscopal effect following unilateral treatment of mice with large, established bilateral tumors using HIFU and checkpoint inhibitors compared with tumors treated with HIFU or checkpoint inhibitors alone (61.1% survival, P < 0.0001). This combination treatment significantly also induces CD4+CD44hiCD62L-low and CD8α+CD44hiCD62L-low population and is adoptively transferable, imparting immunity, slowing subsequent de novo tumor engrafment.

Conclusions: Mechanical fractionation of tumors using HIFU can effectively induce immune sensitization in a previously unresponsive murine neuroblastoma model and promises a novel yet efficacious immunoadjuvant modality to overcome therapeutic resistance.

Introduction

Despite the unprecedented potential of cancer immunotherapy, many patients with cancer do not respond to immunotherapy (1, 2). Even among those who initially respond, many relapse after some period due to inadequate T-cell recognition resulting from loss of tumor antigen presentation by tumor cells (3, 4). Both local and systemic strategies are required to mitigate therapeutic resistance to immunotherapy and transform immunologically “cold” tumors into responsive “hot” tumors.

Neuroblastoma is the third most common childhood cancer and arises from the developing sympathetic nerve ganglia in the abdomen, chest or, neck (5, 6). Survival for pediatric patients with high-risk neuroblastoma has improved in recent years with the addition of multimodal therapy including high-dose chemotherapy, radiation, autologous stem cell transplantation, and immunotherapy (7). The costs of therapy associated with acute and late side effects are high and more than 50% of patients still do not survive despite intensive therapy (7). Neuroblastoma cells evade the innate and adaptive immune system by downregulation of human leukocyte antigen classes I and II (8, 9) and are likely to be ignored by the host T-cell compartment (8, 10, 11). Various efforts to facilitate immunotherapy-based strategies including engineered T cells specific to disialoganglioside (GD2), monoclonal antibodies directly targeting GD2, γδ T cells, and vaccine therapies have changed neuroblastoma treatment perspective (12–15). Immune checkpoint inhibitor therapy is a recent advance in cancer therapy for several adult tumors, but similar responses have not been appreciated in pediatric solid tumor malignancies (1, 16, 17). The lack of therapy effectiveness in pediatric neuroblastoma is due to upregulation of TGFβ and IL10 and down-regulation of ligands that activate receptors expressed on NK and T cells (8, 18). The natural inhibition of hemopoietic stem cell differentiation, generation of dendritic cells (DC), T-cell proliferation, and the phenotype of the cellular and humoral immune response to neuroblastoma tumor cells are strikingly similar in human and murine (Neuro2a) hosts (19, 20).
Sensitizing and changing the tumor microenvironment are shown to improve the efficacy of checkpoint inhibitor therapy, resulting in systemic tumor regression (21). Minimally invasive treatments such as radiofrequency (RFA) and cryoablation have been used to perform tumor ablation in the clinic that result in an inflammatory response (22–24). High-intensity focused ultrasound (HIFU) is a completely noninvasive ablation therapy that is used in the clinic to thermally ablate solid tumors (25, 26). Thermal ablation using RFA and HIFU, however, could be unfavorable immunologically due to heat-associated tumor fixation, resulting in poor tumor permeability to immune cells and antigen release deficiency (27, 28). In addition to thermal ablation, HIFU can also be used to mechanically fractionate tumors, with minimal thermal effects, referred to as histotripsy (29–31), which may improve antitumor immune sensitivity. Together with our collaborators, we have previously characterized this modality of HIFU, boiling histotripsy (BH, which will hereon be referred to as “HIFU”), a technique capable of mechanically fractionating tumors with high spatial precision using a clinical HIFU system for ablation (32–34). HIFU-mediated tumor fractionation may cause immunogenic cell death (ICD) and create an in situ tumor debris depot within the treated zone, increasing inflammation and, potentially leading to immune sensitization (28, 35), which is unlikely to occur in HIFU ablation due to lack of tumor permeability (27).

Herein, we report the role for HIFU in inducing a significant immune response in a previously refractory, large subcutaneous murine neuroblastoma tumor model (Neuro2a; ref. 36). We report that (i) partial mechanical fractionation of tumor using HIFU in combination with checkpoint inhibitors (αCTLA-4 + αPD-L1) significantly prolongs survival in a previously refractory unilateral and bilateral neuroblastoma tumor model; (ii) HIFU induces a systemic immune activation of DCs, tumor-infiltrating T cells, proinflammatory cytokine changes, and damage-associated molecular patterns (DAMPs) changes, while downregulating regulatory T cells, IL10, TGFβ, and VEGF-A; and (iii) HIFU-based tumor mechanical fractionation elicits systemic effector memory that is adoptively transferable.

Translational Relevance

Immunotherapy promises unprecedented benefits to patients with cancer. However, the majority of cancer types, including high-risk neuroblastoma, remain immunologically unresponsive. High-intensity focused ultrasound (HIFU) is a noninvasive technique that can mechanically fractionate tumors with high spatial precision, potentially transforming immunologically “cold” tumors into responsive “hot” tumors. Herein, we demonstrate that a combination of HIFU mechanical fractionation and checkpoint inhibitors significantly enhances systemic antitumor response and survival in previously unresponsive, large refractory murine neuroblastoma tumors. We report that a significant abscopal effect was induced following unilateral treatment of large, established bilateral tumors. Furthermore, this immune response is adoptively transferable. Mechanical HIFU opens a favorable time window for immunotherapy that eventually leads to therapeutic responsiveness in previously immunologically “cold” tumors. This HIFU approach can be executed on clinical HIFU systems, expediting clinical translation of this novel combination therapy.

Materials and Methods

Mouse neuroblastoma cell culture and checkpoint inhibitor antibodies

The murine neuroblastoma cell line Neuro2a is derived from an aggressive and metastatic subclone of the C1300 neuroblastoma cell line that was cultured from a spontaneous tumor in the spinal cord of A/J mice (ATCC). Neuro2a cells were maintained in DMEM supplemented with 1% penicillin–streptomycin (Invitrogen) and 10% fetal bovine serum (Gemini Bioproducts). Cells were grown at 37°C under 5% CO₂. Antimouse checkpoint inhibitors αCTLA-4 (clone 9D2) and αPD-L1 (clone 10F.9G2) were obtained from commercially available source (BioXCell).

Study Design

This study was designed to evaluate the role and efficacy of HIFU-based tumor fractionation on treatment of murine neuroblastoma tumors. Experiments were performed on a protocol (IRB# 30499) approved by Institutional Animal Care and Use Committee at Children’s National Medical Center, Washington, DC. A total of 150 A/J mice with subcutaneous tumors were used in this study (105 unilateral and 45 bilateral). Mice were assigned randomly to six treatment groups: HIFU + αCTLA-4 + αPD-L1 (N = 16), a combination of αCTLA-4 + αPD-L1 (N = 10), HIFU only (N = 10), combinations of HIFU with only either αCTLA-4 or αPD-L1 (HIFU + αCTLA-4, N = 10; HIFU + αPD-L1, N = 10), and untreated (N = 10). Mice in these groups had large, established unilateral neuroblastoma tumors. In addition, we evaluated HIFU + αCTLA-4 + αPD-L1 (N = 18) in mice with established and large bilateral tumors. Mice in all groups were subcutaneously injected with 1 × 10⁶ neuroblastoma (Neuro2a) cells in the flank and were randomized once the tumors reached a volume of 1200 to 1750 mm³. This model was desired over an orthotopic model to reduce variability and to avoid introduction of technical targeting challenges introduced by alternate models. In this and other murine models, survival by days 100 was previously demonstrated for starting tumor volumes less than 300 mm³ (28, 37–39). Combination of αCTLA-4 + αPD-L1 (100 μg/antibody/mouse/time point) was administered intraperitoneally on approximately days 1, 4, and 7 after HIFU (Fig. 1A). Mice were euthanized if tumor volumes exceeded 4,000 mm³. Mice surviving HIFU alone or combinational treatment were rechallenged with 2 × initial tumor burden (2 × 10⁶ cells) >300 days after the first tumor challenge to test long-term immune memory effect. T cells were isolated from spleens of surviving mice, and approximately 8 × 10⁵ cells were adaptively transferred into naive mice with de novo established (approximately 600–900 mm³ tumor volume) neuroblastoma tumors. Animal caretakers, as well as investigators who analyzed data were blinded to the mice groups.

Ultrasound-guided HIFU tumor fractionation

The HIFU system consists of a transducer that is capable of producing equivalent acoustic pressures to a clinical HIFU transducer (Sonalleve V2 MR-HIFU system, Profound Medical Inc.; ref. 32). The focal size of our HIFU transducer was 1.5 × 1.5 × 7 mm at −6 dB level, with a transmit frequency of 1.5 MHz, focal length of 56 mm, and transducer aperture diameter of 75 mm. A commercially available amplifier (1240L, E&I) was used to power the HIFU transducer. The HIFU transducer produced a peak positive pressure of 85 MPa and peak negative pressure of 14 MPa (shock amplitude of 80 MPa), measured in water using a fiber optic...
probe hydrophone (Rp Acoustics). A 13.33-ms long pulse at 1 Hz pulse repetition frequency, as previously described, was used for in vivo HIFU sonications (32). The HIFU focus was sequentially moved across three adjacent, nonoverlapping foci using a computer controlled 3-axis linear stage (Velmex) covering approximately 2% tumor volume (acoustically equivalent). The pulsing protocol was applied for 15 seconds/focus (Fig. 1B). Mice were anesthetized using a ketamine–xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine), attached to a custom-built holder, and positioned in the water tank to align the HIFU focus within the tumor. The linear stages were connected to stepper motors (Maxon motors) and remotely controlled (Galil motion controller). A diagnostic ultrasound transducer (S12-4, Philips CX50, Philips) was placed coaxially with the HIFU transducer for real-time b-mode image guidance to target the tumor along the axial plane (Fig. 1B). This setup enabled a robust and repeatable platform to treat murine tumors. Immediately after HIFU, mice were administered buprenorphine (0.3 mg/mL of buprenorphine diluted to 1:10 with PBS) subcutaneously. Additional doses of buprenorphine were administered at 12, 24, and 48 hours post-HIFU treatment to alleviate any pain caused by tumor fractionation.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc) version 7.0. Sample sizes were determined *a priori* using a two-sided log-rank test with an overall sample size of 30 mice (15 in HIFU + αCTLA-4 + αPD-L1 and 15 in untreated), which achieved a power of 76% at 0.05 significance level to detect a difference of survival proportion. Tumor growth curves are presented as tumor volume over time. Kaplan–Meier plots were populated for all groups posttreatment to demonstrate mice survival. Significance was assessed by log-rank test (Mantel–Cox test). Earlier time points were assessed using Gehan–Breslow–Wilcoxon test and were together presented as percent survival (mean ± SE). One-way nonparametric ANOVA, followed by Bonferroni correction was used to compare all experimental groups in both ELISA and flow cytometry data. Confidence interval of 95% was used for all tests. Empirical Bayes approach was used to analyze probewise microarray gene data and compute t statistics, F statistic, and log-odds of differential expression.

**Data and materials availability**

All microarray gene data are publicly made available on gene expression omnibus (GEO ID# GSE137124). Remaining data
associated with this study are available in the main text or the Supplementary Materials.

**Results**

**HIFU in combination with αCTLA-4 + αPD-L1 cures refractory, unilateral neuroblastoma tumors, leading to significant long-term survival**

To assess the role of HIFU mechanical fractionation in survival efficacy, we partially treated large, refractory Neuro2a tumors in mice (Fig. 1C). Treatment using HIFU alone or in combination with single checkpoint inhibitors alone was not effective and had no survival benefit (Fig. 1D, blue, black, and orange lines). In addition, treatment with both αCTLA-4 + αPD-L1 (without HIFU) resulted in marginal survival (10%, Fig. 1D, red line). In contrast, HIFU combined with αCTLA-4 + αPD-L1 resulted in complete tumor regression at the primary site, with no distant metastases (Fig. 1C) and significantly increased long-term survival (>300 days, \( P < 0.0001 \)) from 0% (untreated, HIFU only, HIFU + αCTLA-4, or HIFU + αPD-L1) to 62.5% (Fig. 1D, green line). Tumor volumes compared 28 days after treatment demonstrate that tumors treated with HIFU + αCTLA-4 + αPD-L1 were significantly smaller than tumors treated with αCTLA-4 + αPD-L1 alone (\( P = 0.0241 \); Supplementary Fig. S1). These results demonstrate that HIFU induces a strong synergistic effect when combined with αCTLA-4 + αPD-L1 and leads to long-term survival in a previously untreatable, refractory neuroblastoma tumor model.

**Local HIFU treatment alone induces systemic cellular, cytokine, and gene response**

Because HIFU was applied locally to fractionate tumors, we first sought to measure any locoregional and systemic immune responses at 24, 48, and 72 hours after HIFU alone (Fig. 2A–D).

**Temporal evolution of cellular response in tumor, lymph nodes, and spleen**

Untreated tumors did not have any infiltration of CD4\(^+\) and CD8\(^+\) T cells, although some resident macrophages (CD68\(^+\)) were detected (Supplementary Fig. S2). Significant infiltration of both CD4\(^+\) and CD8\(^+\) T cells was observed in the treated tumor at 24 hours after HIFU alone (Supplementary Fig. S2, \( P = 0.0001 \) and 0.033, respectively). This was accompanied by a significant increase in CD68\(^+\) cells at 48 hours, compared with untreated tumors (Supplementary Fig. S2, \( P = 0.0377 \)). Significant increase in NK cells (DX5\(^+\)) was measured in both spleen- and tumor-draining lymph nodes at 24 hours post-HIFU (\( P = 0.0006 \) and \( P = 0.0004 \), respectively, Fig. 2A), which demonstrates a nonsignificant increase observed in the contralateral lymph nodes (Supplementary Fig. S3). At this time point, DC (CD11c\(^+\)) population also moderately increased in spleen, tumor-draining lymph nodes, and contralateral lymph nodes (Fig. 2A; Supplementary Fig. S3), whereas CD8\(^+\) DCs (CD8\(^{\text{ex}}\)CD11c\(^+\)) subset significantly increased at 24 hours post-HIFU in both spleen- and tumor-draining lymph nodes (\( P = 0.0001 \) and 0.0002, respectively). At 48 hours posttreatment, HIFU also caused a significant reduction in CD4\(^+\) subset of regulatory T cells (CD4\(^+\)Foxp3\(^+\)) in the tumor-draining lymph nodes (\( P = 0.0220 \)). Likewise, at 72 hours, CD4\(^+\)Foxp3\(^+\) population was significantly lower in the spleen- and tumor-draining lymph nodes (\( P = 3.5 \times 10^{-9} \) and \( P = 0.0001 \), respectively, Fig. 2A). CD4\(^+\)Foxp3\(^+\) population remained unchanged in the contralateral lymph nodes at all time points after HIFU (Supplementary Fig. S3). Given that a higher cytotoxic to regulatory T-cell ratio (CD8\(^{\text{ex}}\)/Foxp3\(^+\)) has been previously shown to signify favorable outcomes in several cancer types (40, 41), we measured this cell ratio post-HIFU, CD8\(^{\text{ex}}\)/Foxp3\(^+\) cell ratios were significantly elevated in both spleen- and tumor-draining lymph nodes at 72 hours post-HIFU treatment (\( P = 0.0021 \) and 0.0091, respectively). We observed no significant changes in CD11b\(^{\text{ex}}\) in spleen- and tumor-draining lymph nodes. These results show an early and marked increase in local immune cell infiltration of the tumor after HIFU.

**Intratumoral expression of PD-L1 post-HIFU**

We further investigated the role of HIFU alone in altering the PD-L1–PD-1 axis by measuring intratumoral PD-L1 expression. Untreated neuroblastoma tumors presented no PD-L1 expression (Fig. 2B). In contrast, there was a significant increase in PD-L1 expression on most tumor cells at 72 hours after HIFU treatment compared with untreated tumor (Fig. 2B, \( P = 0.0001 \)). This acute increase in PD-L1 expression at 72 hours following HIFU suggests adaptive tumor immune suppression and loss of T-cell population (Supplementary Fig. S2). Thus anti-PD-L1 treatment is critical for countering this effect and enhancing antitumor immune responses (42).

**Temporal progression of systemic inflammatory cytokines after HIFU**

We then evaluated circulating inflammatory cytokine changes at 24, 48, and 72 hours post-HIFU alone (Fig. 2C). At 24 hours post-HIFU, we observed a significant increase in IL2 (\( P = 0.0148 \)) and GM-CSF (\( P = 0.0454 \)), plus a significant decrease in VEGF-A (\( P = 0.0072 \)). At 48 hours post-HIFU, IL6 (\( P = 0.0298 \)) was significantly upregulated, while significantly lower concentrations in IL10 (\( P = 0.0227 \)) were measured. Furthermore, at 72 hours post-HIFU, IL10 continued to significantly decrease (\( P = 0.0314 \), and in contrast, IFN\(\gamma \) was significantly higher at 72 hours (\( P = 1.7e-5 \)). We measured no significant changes in TGF\(\beta\)1, TGF\(\beta\)2 (Fig. 2C), TNF\(\alpha\), IL12p70, or IL4 after HIFU treatment (Supplementary Fig. S4).

**Gene expression within HIFU-treated tumors**

We further characterized intratumor genetic patterns 24, 48, and 72 hours following HIFU treatment (Fig. 2D). S100 calcium-binding protein A8 (S100a8), S100 calcium-binding protein A9 (S100a9), heat shock protein family, member 7 (Hspb7), and lipocalin 2 (Lcn2) were overexpressed significantly at all three time points, whereas heat shock protein family A member 1B (Hspb7) and CD72 expression were significantly overexpressed at 24 and 48 hours post-HIFU, and high mobility group box 1 (Hmg1b) expression did not change post-HIFU. In summary, our collective evidence from cellular changes, cytokine, and genetic signatures suggests that HIFU treatment of previously refractory neuroblastoma results in early immune cell presence in the tumor, lymph nodes, and spleen, converting a nonimmunogenic “cold” tumor to an immunogenic “hot” tumor.

**Systemic immune effects are sustained after HIFU in combination with αCTLA-4 + αPD-L1 and result in systemic “abscopal” effect and prolonged survival**

Similar to studying immune effects of HIFU only as discussed previously, we analyzed circulating cytokine and systemic cellular changes in these mice at 24, 48, and 72 hours after the last dose of αCTLA-4 + αPD-L1. We observed significant intratumoral infiltration of helper T cells (CD4\(^+\)), cytotoxic T cells (CD8\(^+\)), and CD8\(^{\text{ex}}\) DCs (CD8\(^{\text{ex}}\)CD11c\(^+\)) at 48 hours after the last dose of checkpoint inhibitors (Fig. 3A). In addition, significant CD8\(^{\text{ex}}\)CD11c\(^+\)
infiltration into the draining lymph nodes and spleen was measured after the last dose of checkpoint inhibitors (Fig. 3A). CD11c+ infiltration into draining and contralateral lymph nodes, as well as systemic cytokine changes, was observed after the last dose of checkpoint inhibitors (Fig. 3A).

IFNγ was significantly elevated at 24 hours after the last dose of αCTLA-4 + αPD-L1 regimen (P = 0.0043). These IFNγ levels in circulating blood are similar in mice at 72 hours post-HIFU only, suggesting a sustained IFNγ elevation at day 8 after treating tumors with HIFU. In contrast, mice receiving just αCTLA-4 + αPD-L1 and not supplemented by HIFU did not demonstrate any changes in IFNγ concentration systemically (Supplementary Fig. S5). In addition, IL10 significantly reduced by at least 10-fold at 24, 48, and 72 hours post-HIFU compared with untreated mice (P = 8.9e-10, 3.5e-11, and 2.7e-10, respectively). IL6 was significantly lower at 72 hours after the last dose (P = 0.0221), whereas GM-CSF was significantly elevated at 72 hours after the last dose (P = 0.0119). This downward trend in systemic IL10 concentration is similarly observed.
HIFU only treatment of mice with unilateral tumors, suggesting a sustained effect at 10 days after HIFU. Population of DCs (CD11c^+\textsuperscript{+}) in tumor-draining lymph nodes significantly increased at 48 hours compared with untreated mice ($P = 3.3e-6$), noticeably increasing in contralateral lymph nodes at 48 hours post-final dose of $\alpha$CTLA-4 + $\alpha$PD-L1, suggesting systemic antigen presenting.

Figure 3. 
HIFU + $\alpha$CTLA-4 + $\alpha$PD-L1 treatment of large, established bilateral tumors leads to abscopal effect. A, Box plots showing cytokine concentration (pg/mL) and cellular changes (percent live cells), quantified at 24, 48, and 72 hours after HIFU and the last dose of $\alpha$CTLA-4 + $\alpha$PD-L1, and compared with untreated mice. Significance values for all data analysis were calculated using ANOVA ($P < 0.05$). B, Representative photographs of mouse with established and large bilateral neuroblastoma tumors (red circles). After unilateral treatment with HIFU + $\alpha$CTLA-4 + $\alpha$PD-L1, both tumors completely regressed, signifying strong abscopal effect. C, Kaplan-Meier plot showing survival of mice with bilateral neuroblastoma tumors treated with HIFU + $\alpha$CTLA-4 + $\alpha$PD-L1 ($N = 18$). 61.1% of mice with bilateral tumors treated with HIFU + $\alpha$CTLA-4 + $\alpha$PD-L1 displayed significantly higher survival than mice treated with HIFU only or $\alpha$CTLA-4 + $\alpha$PD-L1 only, beyond day 300.
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Figure 4.
Mice surviving HIFU + αCTLA-4 + αPD-L1 treatment possess significantly high effector memory against neuroblastoma tumors. A, Kaplan–Meier plot shows 100% survival in mice rechallenged with 2× the initial tumor inoculation (2× 10^6 vs. 1× 10^6). Mice from both unilateral and bilateral tumor groups were rechallenged and all mice survived. B, Box plot showing significantly higher effector memory on CD4^+ cells in mice treated with HIFU + αCTLA-4 + αPD-L1 compared with naive mice. C, Box plot presenting significantly higher effector memory on CD8^+ cells in mice treated with HIFU + αCTLA-4 + αPD-L1 compared with naive mice. P values were determined using unpaired Welch’s t test.

Discussion

In this study, we demonstrate that partial tumor fractionation using HIFU in combination with αCTLA-4 + αPD-L1 results in an abscopal effect and significantly prolongs survival in previously untreated, large unilateral and bilateral murine neuroblastoma tumor models. Our results also provide a proof-of-concept for using HIFU to sensitize the systemic immune system and serve as an adjuvant to checkpoint inhibitor therapy. We also reveal that this systemic antitumor effect is mediated by upregulation of DCs, tumor-infiltrating T cells, proinflammatory cytokines, and DAMPs, as well as concurrent down-regulation of protumor regulators such as Foxp3, IL10, and VEGF-A. Furthermore, this combination therapy elicits a systemic effector memory response that results in rejection of tumor rechallenge and can be adoptively transferred.

One of the most important findings in our study is that combining HIFU with αCTLA-4 + αPD-L1 significantly improves long-term survival of mice with large and established otherwise immunologically “cold” neuroblastoma tumors. Tumors in mice were treated when the volume reached 1200 to 1750 mm^3, which is significantly larger than prior reported work in similar or same tumor models (27, 28, 38, 39). The HIFU foci were calculated to cover approximately 2% of total tumor volume, which may be clinically relevant in treating tumors in patients (43), where complete margin treatment may not be possible. Unlike HIFU-mediated thermal ablation, BH (modality of HIFU used

capability. Also, the frequency of both CD4^+ and CD8^+ cells was found to be elevated in tumor at 48 hours after the last dosage of αCTLA-4 + αPD-L1 compared with untreated mice (P = 0.0363) and 24 hours after the last dose of checkpoint inhibitors (P = 0.0070). CD8^+ CD11c^hi cells were significantly elevated intratumorally in the spleen and in draining lymph node at 48 hours after the last dose of αCTLA-4 + αPD-L1 (P = 0.0054, 0.0001, and 0.0010, respectively). CD11b^hi population in the spleen was also significantly elevated at 24 hours after the last dose of αCTLA-4 + αPD-L1 (P = 0.0165). In addition, CD11b^hi population in the spleen was also significantly elevated at 24 hours post-final αCTLA-4 + αPD-L1 dose compared with untreated mice (P = 0.0318). In contrast, mice treated with HIFU only did not show any changes in CD11b^hi. The CD4^+ Foxp3^hi cell population remained unchanged in both tumor draining and contralateral lymph nodes after the last dose of αCTLA-4 + αPD-L1, although they significantly reduced in mice treated with HIFU alone.

Given the local and systemic immunologic changes with HIFU alone and in combination with αCTLA-4 + αPD-L1, we hypothesize that local unilateral tumor fractionation using HIFU in combination with αCTLA-4 + αPD-L1 induces an abscopal effect in mice with established bilateral tumors (>500 mm^3 on each side, >1,000 mm^3 total tumor burden; Fig. 3B). We locally treated one side only with HIFU and followed up with a regimen of systemic αCTLA-4 + αPD-L1 (similar to Fig. 1A). Interestingly, combining αCTLA-4 + αPD-L1 with one-sided HIFU tumor fractionation resulted in complete bilateral tumor regression (Fig. 3B). By day 20, 75% of mice had no tumors, bilaterally (Supplementary Fig. S6). There was a significant improvement in survival (P = 0.0001) compared with mice with unilateral tumors treated with checkpoint inhibitors or HIFU only (Fig. 3C). Overall, 61.1% of mice survived longer than 300 days in this group, with no evidence of local or systemic tumor recurrence. These results provide vital evidence of local treatment of tumor with HIFU in combination with αCTLA-4 + αPD-L1 that causes a sustained systemic immune-adjuvant effect and abscopal effect.

HIFU-mediated tumor fractionation combined with checkpoint inhibitor using αCTLA-4 + αPD-L1 induces effector memory cells

In view of the fact that HIFU-treated tumors continued to regress well beyond the treatment period and the abscopal effect, we rechallenged all surviving mice with twice the number of tumor cells (2× 10^6 cells) compared with the initial inoculation (1× 10^6). Intriguingly, all mice survived this rechallenge for at least 75 days, without any evidence of tumor formation (Fig. 4A), except for 2 mice that demonstrated transient tumor formation, which eventually regressed by day 21 (Supplementary Fig. S7A). To determine the cellular mediator of this systemic effector memory response, we measured CD4^+ CD64^hi CD161^hi PD-L1 and CD8^+ CD44^hi PD-L1 cells in the spleen. The percentage of CD4^+ and CD8^+ cells expressing effector memory markers in mice that were rechallenged was 3-fold and 17-fold greater than that in naive mice (P = 0.0001, Fig. 4B and C).

To further support the induction of long-term memory response following HIFU and checkpoint inhibitor treatment, we adoptively transferred the isolated T cells from spleens in mice surviving combination therapy into mice with de novo established tumors (volume: 600–900 mm^3). We found tumor growth retardation and prolonged survival in mice that received adoptive T cells compared with mice that did not receive adoptive T cells (P = 0.0002; Supplementary Fig. S7B). Combing HIFU with αCTLA-4 + αPD-L1 in previously untreatable and refractory neuroblastoma tumor not only effectively induced tumor regression and systemic abscopal effect but also induced a long-term immune memory response.
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in this study) is capable of mechanically fractionating tissues at high spatial precision and relatively low temperature elevation. In addition, BH1's two-prong approach of debulking tumor tissue via mechanical fractionation while keeping major blood vessels intact (30, 44), and simultaneously producing significant sterile inflammation (44), potentially improves efficiency of immune checkpoint inhibitors. The checkpoint inhibitors used in this study are clinically approved for multiple indications and are currently in trials for treating neuroblastoma (NCT02304458; refs. 1, 45). Mechanically fractionating <10% of tumor volume with HIFU compared with thermally ablating more than 50% tumor volume potentially reduces heat convective-associated iatrogenic effects and allows improved blood supply into the tumor and immune sensitization (37). These factors suggest that mechanical fractionation using BH may lead to improved immune sensitization against tumor with little or no side effects compared with thermal ablation. This is potentially impactful in clinical translation of this drug-device combination therapy for patients suffering from high-risk or relapsed neuroblastoma, without other effective therapeutic options.

In this study, we found three key mechanistic changes. First, HIFU-mediated mechanical fractionation of tumor tissue elevated concentrations of IFNγ, while lowering concentrations of IL10 and sustaining IL4, TGFβ1, and TGFβ2. These cytokine changes help improve antigen presentation capability (46, 47) after HIFU-mediated mechanical fractionation of tumors. Tumor fractionation using HIFU also demonstrates remarkable DAMP changes. Key DAMPs such as S100a8/a9 and HSPs are upregulated post-HIFU and are of key interest because they are known to bind to pattern recognition receptors on the surface of innate immune cells, such as Toll-like receptors (48). These genes are also known to activate the S100a8/a9–NK cell axis via the receptor for advanced glycation end products (RAGE) pathway (49), facilitating DC maturation and promoting ICD (ref. 50). HIFU tumor fractionation increased HSP27 and HSP70 that may act as a chemoattractant to DCs via the RAGE pathway (as observed in both preclinical and clinical studies; refs. 35, 51). We also mechanistically demonstrate the upregulation of CD72 gene, which is linked in inhibition of IFNγ production by NK cells, although NK cell concentration significantly increased at 24 hours. CD72 may have inhibited IFNγ production by NK cells (without interfering with NK cell's cytotoxic abilities), leading to suppression of IFNγ until 72 hours post-HIFU (52). We therefore conclude that 24 to 48 hours post-HIFU, a significant increase in macrophages, CD8+ DCs, IFNγ, T cells, NK cells, and DCs has converted a “cold,” nonimmunogenic tumor to a “hot,” immunogenic tumor but not adequate to improve overall survival.

Second, when HIFU-mediated tumor fractionation is combined with αCTLA-4 + αPD-L1, sustained systemic increase in CD11c+ CD8α+ CD11c+ CD4+, and CD8α+ CD4+ Foxp3+ is observed, leading to abscopal effect and long-term survival in more than 61.1% of mice. The presence of significantly high levels of CD11c+ in the draining lymph nodes after tumor fractionation and downregulating CTLA-4 and PD-L1 suggest a more efficient T-cell priming and activation. Also, CD8α+ CD11c+ in the draining lymph nodes directly presents antigens to CD8α+ cells, leading to efficient priming and activation of CD8α+ cells. Combining HIFU tumor fractionation with αPD-L1 improves T-cell based tumor targeting efficiency. This synergy between HIFU and checkpoint inhibitor therapy causing systemic cellular, cytokine, and DAMP release may have facilitated this effect, leading to significantly improved overall survival.

Third, HIFU in combination with αCTLA-4 + αPD-L1 improves long-term memory responses and rejected tumor cell rechallenge with a strong adaptive immune response. Nineteen of 21 mice rejected tumor rechallenge, whereas 2 presented transient tumor growth (1,500–1,800 mm3) before completely regressing by day 21 (Supplementary Fig. S7A). Overall, 100% mice initially treated with the HIFU + αCTLA-4 + αPD-L1 combination eventually survived the rechallenge sans further therapy. Long-term memory markers CD4+ CD44hiCD62L–low and CD8α+ CD44hiCD62L–low were found to be significantly elevated in rechallenged mice compared with untreated mice, suggesting HIFU’s ability to improve T-cell memory. Adoptively transferring T cells from surviving mice into naïve mice with established neuroblastoma tumor resulted in improved survival compared with mice that did not receive adoptive T cells. This suggests a role of long-term memory T-cell response in slowing down established tumor growth. Alternatively, the increase in tumor-infiltrating lymphocytes in the adoptive transfer group may have induced upregulation of PD-L1, compounded by the lack of αPD-L1, and may have prevented complete tumor regression and long-term survival.

Some important limitations remain unaddressed. First, it would be valuable to assess the role of priming the immune system with αCTLA-4 prior to HIFU mechanical fractionation, followed by αPD-L1 in evaluating the role of T-cell priming and activation while reducing the potential effects of cotoxicity. Second, assessing the role of specific T-cell phenotypes after HIFU + checkpoint inhibitors, which may help support outcomes observed in this study. Finally, it may be interesting to assess the effect of treated tumor volumes in survival outcomes and establish an “exposure–response” relationship. In conclusion, partial tumor fractionation of refractory neuroblastoma tumors in mice using HIFU enhances innate and adaptive cellular immunity, converting a “cold” nonimmunogenic tumor to a “hot” immunogenic one. Combining this mode of HIFU with αCTLA-4 + αPD-L1 induces potent systemic immunity and cures majority of mice with large, established unilateral and bilateral neuroblastoma tumors. In addition, HIFU treatment of these tumors leads to long-term immune memory. Our group has clinical experience in using HIFU for thermal tumor ablation in patients (53, 54) and we have previously demonstrated preclinical feasibility of performing tissue mechanical fractionation on the same clinical MR-HIFU system (32). Thus, combining this technology with checkpoint inhibition is a step closer to clinical translation for patients with previously unresponsive primary neuroblastoma tumor with or without metastatic burden.

Disclosure of Potential Conflicts of Interest
B.J. Wood reports receiving commercial research grants from Philips, Siemens, Celsion Corp, Profound Medical, Exact Robotics, NVDIA, Biocompatibles BTG, Boston Scientific, and Exact Imaging, and other commercial research support from Philips. P.C.W. Kim is an employee/paid consultant for Activa Surgical Inc. No potential conflicts of interest were disclosed by the other authors.

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