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

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### Abstract

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

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**Data and materials availability:** All microarray gene data is 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.

**Methods:** 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  $\alpha$ CTLA-4 and  $\alpha$ PD-L1 to study its effect on the immune response and long-term survival.

**Results:** Combining HIFU with  $\alpha$ CTLA-4 and  $\alpha$ PD-L1 significantly enhances anti-tumor response, improving survival from 0 to 62.5%. HIFU alone causes upregulation of splenic and lymph node NK cells and circulating IL-2, IFN- $\gamma$ , and DAMPs, whereas immune regulators like CD4<sup>+</sup>Foxp3<sup>+</sup>, IL-10, and VEGF-A are significantly reduced. HIFU combined with checkpoint inhibitors induced significant increases in intratumoral CD4<sup>+</sup>, CD8 $\alpha$ <sup>+</sup>, and CD8 $\alpha$ <sup>+</sup>CD11c<sup>+</sup> cells, CD11c<sup>+</sup> in regional lymph nodes, and decrease in circulating IL-10 compared to untreated group. We also report significant abscopal effect following unilateral treatment of mice with large, established bilateral tumors using HIFU and checkpoint inhibitors compared to tumors treated with HIFU or checkpoint inhibitors alone (61.1% survival,  $p < 0.0001$ ). This combination treatment significantly also induces CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>low</sup> and, CD8 $\alpha$ <sup>+</sup>CD44<sup>hi</sup>CD62L<sup>low</sup> population and are adoptively transferable imparting immunity, slowing subsequent *de novo* tumor engraftment.

**Conclusion:** Mechanical fractionation of tumors using HIFU can effectively induce immune sensitization in a previously unresponsive murine neuroblastoma model, and promises a novel yet efficacious immuno-adjuvant 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 multi-modal 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 over 50% of patients still do not survive despite intensive therapy (7). Neuroblastoma cells evade the innate and adaptive immune system by downregulation of human leucocyte antigen (HLA) -class I & 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,  $\gamma\delta$  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 TGF- $\beta$  and IL-10, and downregulation 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 (DCs), T-cell proliferation, and the phenotype of the cellular and humoral immune response to neuroblastoma tumor cells is strikingly similar in human and murine (Neuro2a) hosts (19,20).

Sensitizing and changing the tumor microenvironment is shown to improve the efficacy of checkpoint inhibitor therapy, resulting in systemic tumor regression (21). Minimally invasive treatments such as radiofrequency (RFA) and cryo-ablation 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 anti-tumor 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, 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) (36). We report that (1) partial mechanical fractionation of tumor using HIFU in combination with checkpoint inhibitors ( $\alpha$ CTLA-4 +  $\alpha$ PD-L1) significantly prolongs survival in a previously refractory unilateral and bilateral neuroblastoma tumor model; (2) 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, IL-10, TGF- $\beta$ , and VEGF-A; and (3) HIFU-based tumor mechanical fractionation elicits systemic effector memory that is adoptively transferable.

## MATERIALS AND METHODS

### Mouse Neuroblastoma Cell Culture & Checkpoint Inhibitor Antibodies

The murine neuroblastoma cell line Neuro2a is derived from an aggressive and metastatic sub-clone of the C1300 neuroblastoma cell line that was cultured from a spontaneous tumor in the spinal cord of A/J mice (ATCC, Manassas, VA). Neuro2a cells were maintained in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Gemini Bioproducts, Sacramento, CA). Cells were grown at 37°C under 5% CO<sub>2</sub>. Anti-mouse checkpoint inhibitors  $\alpha$ CTLA-4 (clone 9D9) and  $\alpha$ PD-L1 (clone 10F.9G2) were obtained from commercially available source (BioXCell, West Lebanon, NH).

## 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 (IACUC) 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 +  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 (N = 16), a combination of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 (N = 10), HIFU only (N = 10), combinations of HIFU with only either  $\alpha$ CTLA-4 or  $\alpha$ PD-L1 (HIFU +  $\alpha$ CTLA-4, N = 10); (HIFU +  $\alpha$ PD-L1, N = 10), and untreated (N = 10). Mice in these groups had large, established unilateral neuroblastoma tumors. In addition, we evaluated HIFU +  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 (N = 18) in mice with established and large bilateral tumors. Mice in all groups were subcutaneously injected with  $1 \times 10^6$  neuroblastoma (Neuro2a) cells in the flank, and were randomized once the tumors reached a volume of 1200–1750 mm<sup>3</sup>. 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, >40% mortality by day 100 was previously demonstrated for starting tumor volumes less than 300 mm<sup>3</sup> (28,37–39). Combination of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 (100  $\mu$ g/antibody/mouse/time-point) was administered intraperitoneally on ~ days 1, 4 and, 7 after HIFU (Fig. 1A). Mice were euthanized if tumor volumes exceeded 4000 mm<sup>3</sup>. Mice surviving HIFU alone or combinational treatment were re-challenged with 2x initial tumor burden ( $2 \times 10^6$  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  $\sim 8 \times 10^6$  cells were adoptively transferred into naïve mice with de novo established ( $\sim 600$ – $900$  mm<sup>3</sup> 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<sup>®</sup> V2 MR-HIFU system, Profound Medical Inc., Mississauga, ON, Canada) (32). The focal size of our HIFU transducer was 1.5×1.5×7 mm at –6dB 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, Rochester, NY) 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, Leutenbach, Germany). 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, non-overlapping foci using a computer controlled 3-axis linear-stage (Velmex, Bloomfield, NY) covering ~ 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, Fall River, MA) and remotely controlled (Galil motion controller, Rocklin, CA). A diagnostic ultrasound transducer (S12–4, Philips CX50, Philips,

Cambridge, MA) was placed co-axially with the HIFU transducer for real-time b-mode image guidance to target the tumor along the axial plane (Figure 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., La Jolla, CA) 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 +  $\alpha$ CTLA-4 +  $\alpha$ PD-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 post-treatment 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  $\pm$  standard error). 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 probe-wise microarray gene data and compute t-statistics, F-statistic, and log-odds of differential expression.

## RESULTS

### HIFU in combination with $\alpha$ CTLA-4 + $\alpha$ PD-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). Additionally, treatment with both  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 (without HIFU) resulted in marginal survival (10%, Fig. 1D, red line). In contrast, HIFU combined with  $\alpha$ CTLA-4 +  $\alpha$ PD-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 +  $\alpha$ CTLA-4 or HIFU +  $\alpha$ PD-L1) to 62.5% (Fig. 1D, green line). Tumor volumes compared 28 days after treatment demonstrate that tumors treated with HIFU +  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 were significantly smaller when compared to tumors treated with  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 alone ( $p = 0.0241$ ) (fig. S1). These results demonstrate that HIFU induces a strong synergistic effect when combined with  $\alpha$ CTLA-4 +  $\alpha$ PD-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

Since HIFU was applied locally to fractionate tumors, we first sought to measure any loco-regional 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<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> cells, although some resident macrophages (CD68<sup>+</sup>) were detected (fig. S2). Significant infiltration of both CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> cells was observed in the treated tumor at 24-hours after HIFU alone (fig. S2,  $p = 0.0001$  and  $0.033$ , respectively). This was accompanied by a significant increase in CD68<sup>+</sup> cells at 48-hours, compared to untreated tumors (fig. S2,  $p = 0.0377$ ). Significant increase in NK cells (DX5<sup>+</sup>) 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), with a non-significant increase observed in the contralateral lymph nodes (fig. S3). At this time-point, DC (CD11c<sup>+</sup>) population also moderately increased in spleen, tumor draining lymph nodes, and contralateral lymph nodes (Fig. 2A & fig. S3), while CD8 $\alpha$ <sup>+</sup> DCs (CD8 $\alpha$ <sup>+</sup>CD11c<sup>+</sup>) 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 post treatment, HIFU also caused a significant reduction in CD4<sup>+</sup> subset of regulatory T-cells (CD4<sup>+</sup>Foxp3<sup>+</sup>) in the tumor draining lymph nodes ( $p = 0.0200$ ). Likewise, at 72-hours, CD4<sup>+</sup>Foxp3<sup>+</sup> population was significantly lower in the spleen and tumor draining lymph nodes ( $p = 3.5e-9$  and  $p = 0.0001$ , respectively, Fig. 2A). CD4<sup>+</sup>Foxp3<sup>+</sup> population remained unchanged in the contralateral lymph nodes at all time-points after HIFU (fig. S3). Given that a higher cytotoxic to regulatory T-cell ratio (CD8 $\alpha$ <sup>+</sup>/Foxp3<sup>+</sup>) has been previously shown to signify favorable outcomes in several cancer types (40,41), we measured this cell ratio post-HIFU. CD8 $\alpha$ <sup>+</sup>/Foxp3<sup>+</sup> 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<sup>high</sup> 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.

**Intra-tumoral 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 to 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 (fig. S2). Thus anti-PD-L1 treatment is critical for countering this effect and enhancing anti-tumor 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 interleukin-2 (IL-2) ( $p = 0.0148$ ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) ( $p = 0.0454$ ), plus a significant decrease in vascular endothelial growth factor-A (VEGF-A) ( $p = 0.0072$ ). At 48-hours post-HIFU interleukin-6 (IL-6) ( $p = 0.0298$ ) was significantly upregulated, whilst significantly lower concentrations in interleukin-10 (IL-10) ( $p = 0.0227$ ) were measured. Further, at 72-hours post-HIFU, IL-10 continued to significantly decrease ( $p = 0.0314$ ), and in contrast interferon- $\gamma$  (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- $\alpha$ , IL-12p70, or IL-4 after HIFU treatment (fig. S4).

**Gene expression within HIFU-treated tumors**—We further characterized intra-tumor 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, while heat shock protein family A member 1B (Hsp70) and Cd72 expression were significantly overexpressed at 24 and 48-hours post-HIFU, and high mobility group box 1 (Hmgb1) expression did not change post-HIFU.

In summary, our collective evidence from cellular changes, cytokine, and genetic signatures suggest that HIFU treatment of previously refractory neuroblastoma results in early immune cell presence in the tumor, lymph nodes and spleen, converting a non-immunogenic ‘cold’ tumor to an immunogenic ‘hot’ tumor.

### **Systemic immune effects are sustained after HIFU in combination with $\alpha$ CTLA-4 + $\alpha$ PD-L1 and results in systemic ‘abscopal’ effect and, prolonged survival**

Similar to studying immune effects of HIFU only above, we analyzed circulating cytokine and systemic cellular changes in these mice at 24, 48, and 72-hours after the last dose of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1. We observed significant intratumoral infiltration of helper T-cells (CD4+), cytotoxic T-cells (CD8 $\alpha$ +), as well as CD8 $\alpha$ + dendritic cells (CD8 $\alpha$ +CD11c+) at 48-hours after the last dose of checkpoint inhibitors (Fig. 3A). In addition, significant CD8 $\alpha$ +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 were observed after the last dose of checkpoint inhibitors (Fig. 3A).

IFN- $\gamma$  was significantly elevated at 24-hours after the last dose of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 regimen ( $p = 0.0043$ ). These IFN- $\gamma$  levels in circulating blood are similar in mice at 72-hours post-HIFU only, suggesting a sustained IFN- $\gamma$  elevation at day 8 after treating tumors with HIFU. In contrast, mice receiving just  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 and not supplemented by HIFU did not demonstrate any changes in IFN- $\gamma$  concentration systemically (fig. S5). In addition, IL-10 significantly reduced by at least 10-fold at 24, 48, and 72-hours after the last dose of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 compared to untreated mice ( $p = 8.9e-10$ ,  $3.5e-11$ , and  $2.7e-10$ , respectively). IL-6 was significantly lower at 72-hours after the last dose ( $p = 0.0221$ ), while GM-CSF was significantly elevated at 72-hours after the last dose ( $p = 0.0119$ ). This downward trend in systemic IL-10 concentration is similarly observed in HIFU only treatment of mice with unilateral tumors, suggesting a sustained effect at 10 days after HIFU. Population of dendritic cells (CD11c+) in tumor draining lymph nodes significantly increased at 48-hours compared to 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 capability. Also, the frequency of both CD4+ and CD8 $\alpha$ + cells were found to be elevated in tumor at 48-hours after the last dosage of  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 compared to untreated mice ( $p = 0.0363$ ), and 24-hours after the last

dose of checkpoint inhibitors ( $p = 0.0070$ ).  $CD8\alpha+CD11c+$  cells were significantly elevated intratumorally, in the spleen and in draining lymph node at 48-hours after the last dose of  $\alpha CTLA-4 + \alpha PD-L1$  ( $p = 0.0054, 0.0001, \text{ and } 0.0010$ , respectively).  $CD11b^{low}$  in the spleen was significantly elevated at 24-hours after the last dose of  $\alpha CTLA-4 + \alpha PD-L1$  ( $p = 0.0165$ ). In addition,  $CD11b^{high}$  population in the spleen was also significantly elevated at 24-hours post final  $\alpha CTLA-4 + \alpha PD-L1$  dose compared to untreated mice ( $p = 0.0318$ ). In contrast, mice treated with HIFU only did not show any changes in  $CD11b^{low}$ . The  $CD4+Foxp3+$  cell population remained unchanged in both tumor draining and contralateral lymph nodes after the last dose of  $\alpha CTLA-4 + \alpha PD-L1$ , although they significantly reduced in mice treated with HIFU alone.

Given the local and systemic immunologic changes with HIFU alone and in combination with  $\alpha CTLA-4 + \alpha PD-L1$ , we hypothesize that local unilateral tumor fractionation using HIFU in combination with  $\alpha CTLA-4 + \alpha PD-L1$  induces an abscopal effect in mice with established bilateral tumors ( $>500 \text{ mm}^3$  on each side,  $>1000 \text{ mm}^3$  total tumor burden) (Fig. 3B). We locally treated one side only with HIFU, and followed up with a regimen of systemic  $\alpha CTLA-4 + \alpha PD-L1$  (similar to Fig. 1A). Interestingly, combining  $\alpha CTLA-4 + \alpha PD-L1$  with one-sided HIFU tumor fractionation resulted in complete bilateral tumor regression (Fig. 3B). By day 20, 75% of mice had no tumors, bilaterally (fig. S6). There was a significant improvement in survival ( $p = 0.0001$ ) compared to 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  $\alpha CTLA-4 + \alpha PD-L1$  cause a sustained systemic immune-adjunct effect and abscopal effect.

### **HIFU mediated tumor fractionation combined with checkpoint inhibitor using $\alpha CTLA-4 + \alpha PD-L1$ 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 re-challenged all surviving mice with twice the number of tumor cells ( $2 \times 10^6$  cells) compared to the initial inoculation ( $1 \times 10^6$  cells). Intriguingly, all mice survived this re-challenge for at least 75 days, without any evidence of tumor formation (Fig. 4A), except for two mice that demonstrated transient tumor formation, which eventually regressed by day 21 (fig. S7A). To determine the cellular mediator of this systemic effector memory response, we measured  $CD4+CD44^{hi}CD62L^{low}$  and  $CD8\alpha+CD44^{hi}CD62L^{low}$  cells in the spleen. The percentage of  $CD4+$  and  $CD8\alpha+$  cells expressing effector memory markers in mice that were re-challenged were 3 and 17 folds greater than in naïve mice ( $p = 0.0001$ , Fig. 4B & 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:  $600\text{--}900 \text{ mm}^3$ ). We found tumor growth retardation and prolonged survival in mice that received adoptive T-cells compared to mice that did not receive adoptive T-cells ( $p = 0.0002$ ) (fig. S7B). Combining HIFU with  $\alpha\text{-}CTLA-4 + \alpha\text{-}PD-L1$  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.

## DISCUSSION

In this study, we demonstrate that partial tumor fractionation using HIFU in combination with  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 results in an abscopal effect and significantly prolongs survival in previously untreatable, 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 serving as an adjuvant to checkpoint inhibitor therapy. We also reveal that this systemic anti-tumor effect is mediated by upregulation of dendritic cells, tumor infiltrating T-cells, proinflammatory cytokines, and DAMPs, as well as concurrent downregulation of pro-tumor regulators such as Foxp3, IL-10 and VEGF-A. Furthermore, this combination therapy elicits a systemic effector memory response that results in rejection of tumor re-challenge and can be adoptively transferred.

One of the most important findings in our study is that combining HIFU with  $\alpha$ CTLA-4 +  $\alpha$ PD-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 1200–1750 mm<sup>3</sup>, which is significantly larger than prior reported work in similar or same tumor models (27,28,38,43). The HIFU foci were calculated to cover ~2% of total tumor volume, which may be clinically relevant in treating tumors in patients (44), where complete margin treatment may not be possible. Unlike HIFU-mediated thermal ablation, BH (modality of HIFU used in this study) is capable of mechanically fractionating tissues at high spatial precision and relatively low temperature elevation. In addition, BH's two-prong approach of debulking tumor tissue via mechanical fractionation while keeping major blood vessels intact (30,45), and simultaneously producing significant sterile inflammation (45), 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) (1,46). Mechanically fractionating <10% of tumor volume with HIFU compared to thermally ablating more than 50% tumor volume, potentially reduces heat convective-associated iatrogenic effects, allows improved blood supply into the tumor and immune sensitization (37). These factors suggest mechanical fractionation using BH may lead to improved immune sensitization against tumor with little or no side effects compared to 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 the present study, we found three key mechanistic changes. First, HIFU-mediated mechanical fractionation of tumor tissue elevated concentrations of IFN- $\gamma$ , while lowering concentrations of IL-10, and sustaining IL-4, TGF- $\beta$ 1, and TGF- $\beta$ 2. These cytokine changes help improve antigen presentation capability (47,48) 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 since they are known to bind to pattern recognition receptors

(PRR) on the surface of innate immune cells, such as TLRs (49). These genes are also known to activate the S100a8/a9–NK cell axis via the receptor for advanced glycation end products (RAGE) pathway (50), facilitating dendritic cell maturation, and promoting immunogenic cell death (ICD) (51). HIFU tumor fractionation increased HSP27 and HSP70 that may act as a chemoattractant to dendritic cells via the RAGE pathway (as observed in both preclinical and clinical studies) (35,52). We also mechanistically demonstrate the upregulation of CD72 gene, which is linked in inhibition of IFN- $\gamma$  production by NK cells, although NK cell concentration significantly increased at 24-hrs. CD72 may have inhibited IFN- $\gamma$  production by NK cells (without interfering with NK cell's cytotoxic abilities), leading to suppression of IFN- $\gamma$  until 72-hours post-HIFU (53). We therefore conclude that 24–48 hours post-HIFU, a significant increase in macrophages, CD8 $\alpha$ + DCs, IFN- $\gamma$ , T-cells, NK cells, and dendritic cells have converted a 'cold', non-immunogenic tumor to a 'hot', immunogenic tumor, but not adequate to improve overall survival.

Second, when HIFU tumor fractionation is combined with  $\alpha$ CTLA-4 +  $\alpha$ PD-L1, sustained systemic increase in CD11c+, CD8 $\alpha$ +CD11c+, CD4+, and CD8 $\alpha$ + populations, while inhibited IL-10 and CD4+Foxp3+ is observed, leading to abscopal effect and long-term survival in over 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 suggests a more efficient T-cell priming and activation. Also, CD8 $\alpha$ +CD11c+ in the draining lymph nodes directly presents antigens to CD8 $\alpha$ + cells, leading to efficient priming and activation of CD8 $\alpha$ + cells. Combining HIFU tumor-fractionation with  $\alpha$ PD-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  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 improves long-term memory responses, and rejected tumor cell re-challenge with a strong adaptive immune response. Nineteen out of twenty-one mice rejected tumor re-challenge, while two presented transient tumor growth (1500–1800 mm<sup>3</sup>) before completely regressing by day 21 (fig. S7A). Overall, 100% mice initially treated with the HIFU +  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 combination eventually survived the re-challenge sans further therapy. Since long-term memory markers CD4+CD44<sup>hi</sup>CD62L<sup>low</sup> and CD8 $\alpha$ +CD44<sup>hi</sup>CD62L<sup>low</sup> were found to be significantly elevated in re-challenged mice compared to 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 to 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  $\alpha$ PD-L1, and may have prevented complete tumor regression and long-term survival.

Some important limitations remain unaddressed. Firstly, it would be valuable to assess the role of priming the immune system with  $\alpha$ CTLA-4 prior to HIFU mechanical fractionation, followed by  $\alpha$ PD-L1 in evaluating the role of T-cell priming and activation while reducing the potential effects of co-toxicity. Secondly, 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’ non-immunogenic tumor to a ‘hot’ immunogenic one. Combining this mode of HIFU with  $\alpha$ CTLA-4 +  $\alpha$ PD-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 (54,55) 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Translational Relevance**

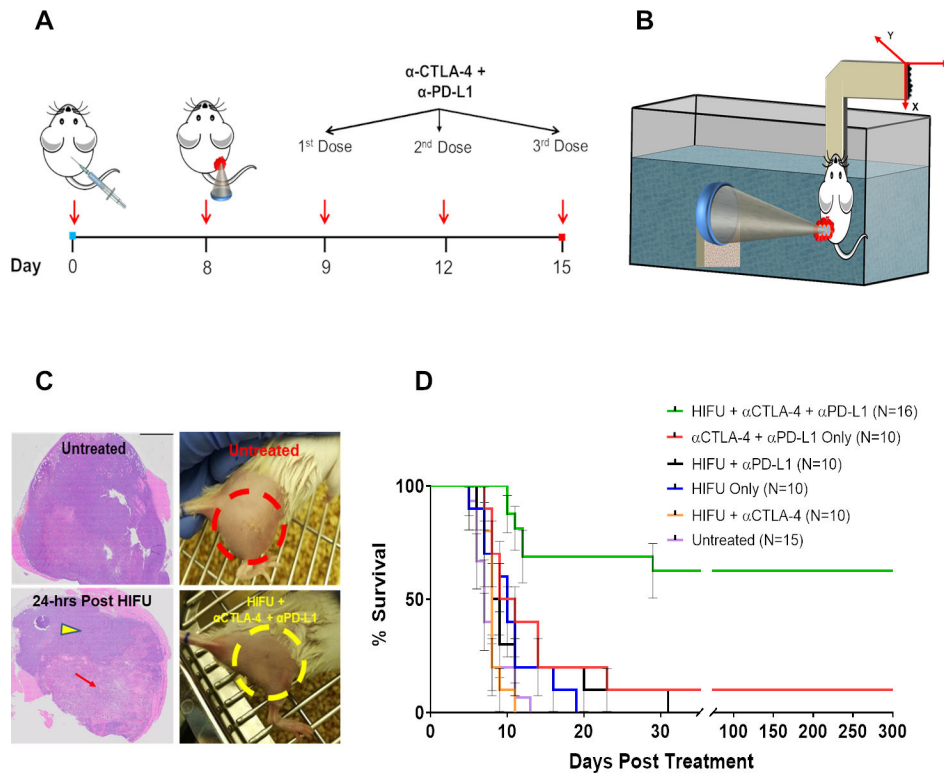
Immunotherapy promises unprecedented benefits to cancer patients. However, the majority of cancer types, including high-risk neuroblastoma remain immunologically unresponsive. High intensity focused ultrasound (HIFU) is a non-invasive technique that can mechanically fractionate tumors with high spatial precision, potentially transforming immunologically ‘cold’ tumors into responsive ‘hot’ tumors. Herein, we demonstrate that a combination of HIFU mechanical fractionation and checkpoint inhibitors significantly enhance systemic anti-tumor response and survival in previously unresponsive, large refractory murine neuroblastoma tumors. We report 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.

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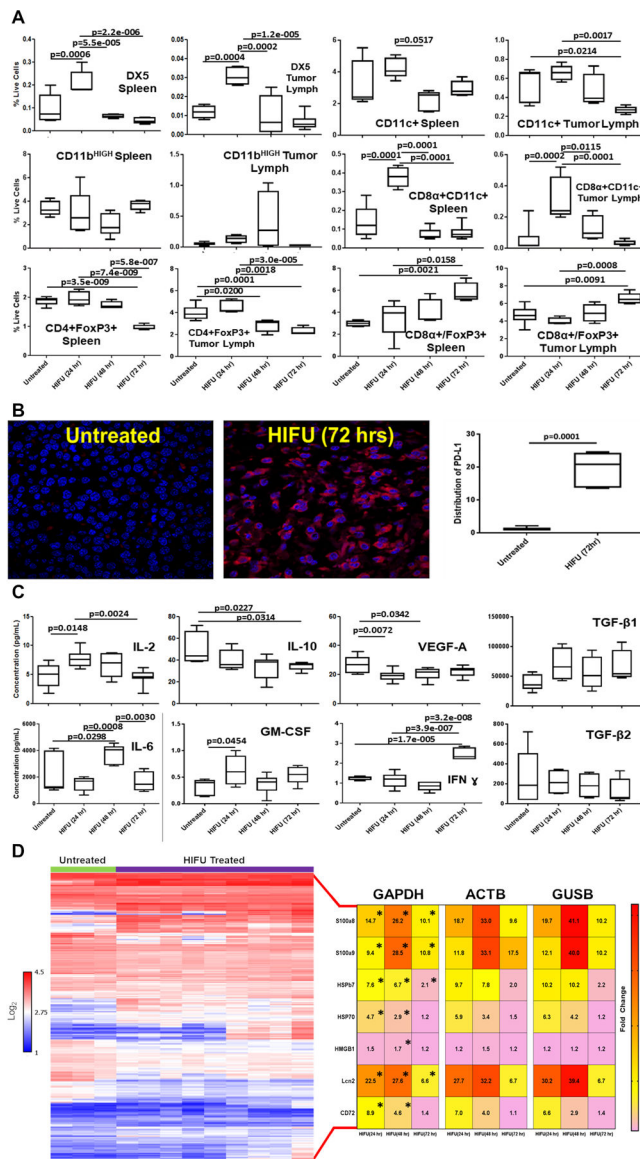
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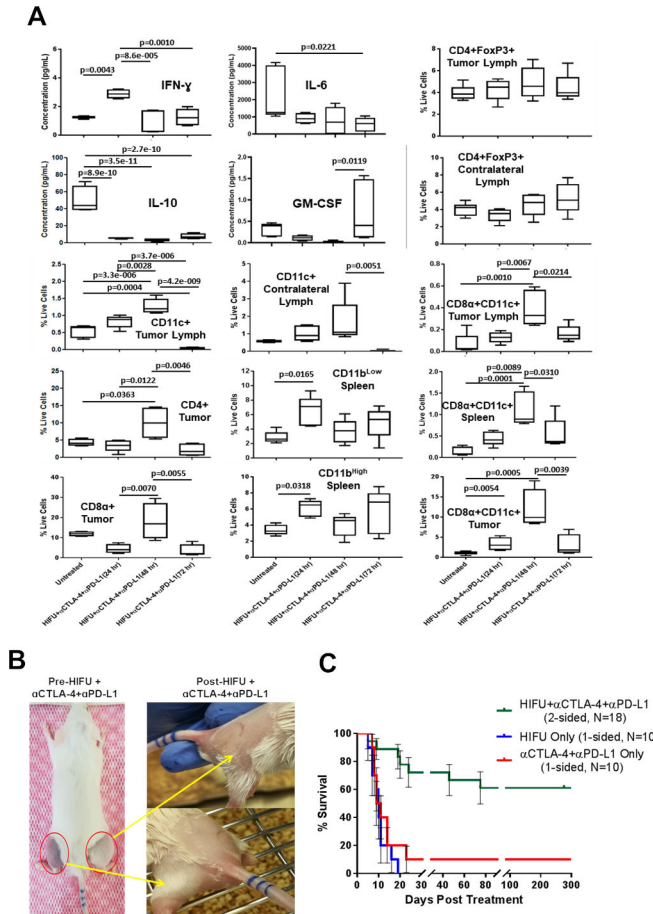
**Figure 1. Adding HIFU to  $\alpha$ -CTLA-4 +  $\alpha$ -PD-L1 significantly increases survival in mice.** (A) Experimental timeline for treating mice with large, established unilateral neuroblastoma tumors using combination of HIFU and  $\alpha$ -CTLA-4 +  $\alpha$ -PD-L1. Mice were inoculated on day 0, tumors treated with HIFU on day 8, followed by a regimen of  $\alpha$ -CTLA-4 +  $\alpha$ -PD-L1. (B) Mice are held using an arm connected to a computer-controlled 3D positioning system, and the lower half of the mice's body is submerged into a water bath filled with circulating degassed water at  $\sim 33^{\circ}\text{C}$ . Three adjacent, non-overlapping HIFU foci ( $1.5 \times 1.5 \times 7$  mm each) are positioned in the middle of the tumor. (C) H&E –stained histological sections obtained 24-hours after HIFU, demonstrating tumor fractionation and necrosis (red arrow), adjacent to intact tumor (yellow arrowhead). Representative photographs of mice with and without tumors. Red dashed-circle shows typical tumors, measuring  $\sim 1200$ – $1750$   $\text{mm}^3$  that were treated in this study, while yellow dashed-circle shows a completely tumor-free mouse after HIFU +  $\alpha$ -CTLA-4 +  $\alpha$ -PD-L1 treatment, with no evidence of iatrogenic effects from HIFU treatment. (D) Kaplan-Meier plots of six groups of mice with unilateral established tumors treated with either individual or combinational approaches. HIFU +  $\alpha$ -CTLA-4 +  $\alpha$ -PD-L1 was the only group to show significantly higher survival compared to all other groups, with more than 62% of mice surviving beyond 300 days post- treatment.



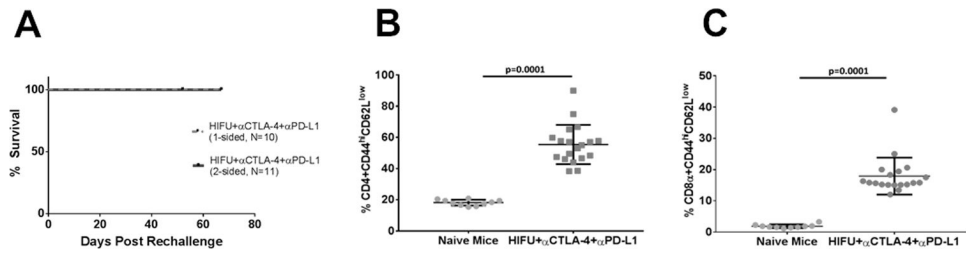


**Figure 2. Systemic cellular, cytokine and genetic signature caused by HIFU mechanical fractionation alone of neuroblastoma tumor.** (A) Box plots presenting cellular changes as percent live cells in the spleen and tumor-draining lymph nodes of mice at 24, 48, and 72-hours following HIFU were compared against cellular changes in untreated mice. Percentage of CD4+Foxp3+, CD11c+, DX5+ (NK cells), CD11b<sup>high</sup>, ratio of CD8+/CD4+Foxp3+, and CD8α+CD11c+ were evaluated using flow cytometry. (B) Representative fluorescent microscopy images of tumor tissues stained with DAPI (blue stain) + PD-L1 (red stain) obtained from untreated mice and from mice at 72-hours post-HIFU. (C) Box plots presenting circulating cytokines in mice at 24, 48, and 72-hours post-HIFU and untreated mice in pg/mL (IL-2, VEGF-A, IL-10, TGF-β1, GM-CSF, IL-6, IFN-γ, and TGF-β2) measured using ELISA from terminal cardiac blood draw. (D) Microarray data from untreated, 24, 48, and 72-hours post-HIFU, represented as log<sub>2</sub> change. This was followed-up by qRT-PCR analysis of seven key genes repeated for three different housekeeping genes (GAPDH, ACTB, and GUSB). N = 8 tumors per

time-point for each housekeeping gene were used. Asterisks in GAPDH columns represent significance from untreated tumors ( $p < 0.05$ ), and was significantly high in gene expression for ACTB and GUSB housekeeping genes as well. Significance values for all data analysis were calculated using ANOVA with Bonferroni correction ( $p < 0.05$ ).



**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-hrs 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 compared to mice with treated with HIFU only or  $\alpha$ CTLA-4 +  $\alpha$ PD-L1 only, beyond day 300.



**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 re-challenged with 2x the initial tumor inoculation ( $2 \times 10^6$  vs  $1 \times 10^6$ ). Mice from both unilateral and bilateral tumor groups were re-challenged and all mice survived. (B) Box plot showing significantly higher effector memory on CD4<sup>+</sup> cells in mice treated with HIFU + αCTLA-4 + αPD-L1 compared to naïve mice (C) Box plot presenting significantly higher effector memory on CD8<sup>+</sup> cells in mice treated with HIFU + αCTLA-4 + αPD-L1 compared to naïve mice. P-values were determined using unpaired Welch's t-test.