Supplementary Information (SI)

Combining activatable nanodelivery with immunotherapy in a murine breast cancer model

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SUMMARY: In this paper, we demonstrate that activatable delivery of immunogenic chemotherapy can synergize with immunotherapy to cure primary and metastatic solid tumors by debulking the primary tumor and releasing tumor antigens that can amplify local and systemic antitumor responses when combined with immunotherapeutic agonists. We found that administration of an immunotherapy priming protocol prior to administration of one treatment of activatable delivery of doxorubicin creates curative responses in both primary and metastatic tumors of a murine model of breast cancer.

In this supplement, we include additional methods and supplementary figures. The additional methods include the study design, doxorubicin loading, *in vivo* studies summary, details of the therapeutic ultrasound system, methods for immunohistochemistry and the full table of animal cohorts studied. We follow these methods with supplementary figures regarding the efficacy of the treatment.

1. **Study design and materials.** First, we verified that doxorubicin induces immunogenic cell death and release of type 1 interferon in various murine cancer lines. Second, cross-presentation of a model antigen by antigen-presenting cells was demonstrated after a single administration of activatable drug delivery (ADD) *in vivo*. Third, flow cytometric and histological analyses were performed after one and two complete treatments of ADD-IT. Fourth, antitumoral efficacy of chemo-immunotherapy treatment protocols was evaluated in both directly-treated and distant tumors when immunotherapy was administrated prior to and post ADD. Fifth, survival was compared among various treatment protocols. We found that administration of an immunotherapy priming protocol followed by one administration of ADD and immunotherapy maximized the treatment outcome and improved survival. Details and breakdown of in vivo study groups are provided in SI Table S1.

2. **Doxorubicin Loading.** The copper-loaded temperature-sensitive liposomes (Cu-TSL) were mixed with doxorubicin at a drug-to-lipid ratio of 0.2:1 (wt:wt) and incubated at 37°C for 1.5 h. Liposomal copper-doxorubicin (CuDox-TSL) was then separated from non-encapsulated Dox using Sephadex G-75 spin columns and eluted with saline. Total amount of Dox loaded in CuDox-TSL was quantified by disintegrating liposomes in the presence of 0.25% Triton X-100 and dissociating copper and Dox using either 30 mM ethylenediaminetetraacetic acid (EDTA) at 55°C for 1.5 h or 20 mM citrate-buffered saline, pH 4.0 at 55°C for 30 min. Dox concentration was determined by measuring fluorescence intensity using a Tecan (San Jose, CA) Infinite® M1000 Microplate Reader at excitation and emission wavelengths of 485 nm and 590 nm, respectively.

3. ***In vivo* Studies.** NDL tumor biopsies of approximately 1 mm^3 were transplanted into both mammary inguinal fat pads of 4-5-week-old FVB female mice (15-20 g, Jackson Laboratory, Bar Harbor, ME) on day 0. Treatment initiated when tumors reached ~3 to 5 mm (~ 20 - 60 mm^3) in longitudinal diameter 3 weeks after transplantation on day 21. Mice were anesthetized with 3.5% isoflurane in 0.4 L/min O_2 and maintained at 2.0-2.5% isoflurane in 0.2 L/min O_2 during injection. For treatments involving ultrasound (US), one tumor per animal was insonified for 5 min at 42°C prior to administration of drug; the tumor insonation was continued for an additional 20 min at 42°C post injection.

   Tumor progression/regression was monitored using a 2D Acuson Sequoia® 512 ultrasound imaging system (Siemens Medical Systems, Inc., Issaquah, WA) equipped with a 14-MHz high frequency linear array transducer (15L8-S, Siemens Medical Systems, Inc.). After the hair surrounding the tumor region was shaved and completely removed, the tumor was viewed in both the transverse and sagittal planes and the tumor boundary was fitted with an ellipse in each view resulting in D_1, D_2, and D_3 representing the three axes of the tumor. The volume of the tumor was then calculated using the following equation:

   $$V = \frac{\pi}{6}(D_1 \times D_2 \times D_3)$$

   Mice were euthanized when tumors reached 1.5 cm in diameter. Mice were either euthanized at certain time points of treatment or at the end of treatment to evaluate treatment efficacy, tumor immune cell profile, and tumor histology or kept after termination of treatment to assess open-ended survival.

4. **Therapeutic Ultrasound System.** Hyperthermia was performed using a programmable US system combining imaging and therapy (Vantage 256, Verasonics, Kirkland, WA). A custom 128-element 1.5 MHz therapeutic array [1] was used to heat the tumor. Heating was performed with bursts of 2.5 MPa peak negative pressure, with a pulse repetition frequency of 100 Hz and burst duration ranging from 0 to 7 ms as controlled by a proportional integral derivative (PID) controller (duty cycle ranging from 0 to 0.7) set to maintain the tumor temperature at 42°C. The tumor temperature feedback control was accomplished using a 30-gauge needle thermocouple (HYP-1, Omega Engineering, Inc., Stamford, CT), which was placed at the tumor rim and assuming a 1°C difference between the tumor center and tumor rim. Imaging was done with a commercial linear array L12-5 (Philips, WA, USA) positioned at the center of the therapeutic array. The thermocouple was interfaced to a data acquisition system controlled by LabVIEW™ (National Instruments Co., Austin, TX). A proportional-integral-derivative (PID) controller was used to maintain the tumor temperature at 42°C by controlling the duty cycle of the acoustic pulses by varying the burst duration.

The animal's core temperature was monitored using a rectal thermocouple and was maintained at ~37°C during the experiment.

5. **Histology and Immunohistochemistry.** Tumors were fixed in 10% formalin for 24 h and transferred to 70% ethanol. Paraffin sections of 4 µm thickness were cut and stained with Hematoxylin and Eosin (H&E), or with immunohistochemistry (IHC) staining of macrophages, $CD8^+$ T-cells, and regulatory T-cells using F4/80, CD8, Foxp3 antibodies at the University of California, Davis, Dept. of Pathology and Laboratory Medicine, respectively.

SI Table S1. Breakdown of study groups

Study	Mouse Strain	Tumor Model	Biological Group	Time point	Number
SIINFEKL Studies	C57BL/6	B16-F10/B16-F10	NTC		3
		B16-F10/B16-OVA	NTC	Day 14	5
			ADD		5
Flow/Immunohistochemistry	FVB/n	NDL	NTC		8
			CpG		4
			αPD-1	Day 28	4
			IT		4
			US-IT		2
			ADD-IT		7
			NTC		4
			CuDox-TSL	Day 31	4
			ADD		4
			NTC		5
			ADD-IT	Day 35	5
Immunohistochemistry	FVB/N-Tg(MMTV-PyVT)634Mul/J	PyMT	NTC	Day 10	5
			IT	Day 16	2
			ADD-IT	Day 25	5
	FVB/n	NDL	NTC		7
			IT	Day 38	5
			IT-ADD		3
Survival Studies	FVB/n	NDL	NTC		17
			CpG		8
			US-CpG		7
			IT		9
			ADD	Day 100	6
			ADD-CpG		8
			ADD-IT (3X)		4
			IT-ADD (1X)		10
			IT-ADD (3X)		4
Total NDL					139
Total C57BL/6					13
Total PyMT					12
Total					164

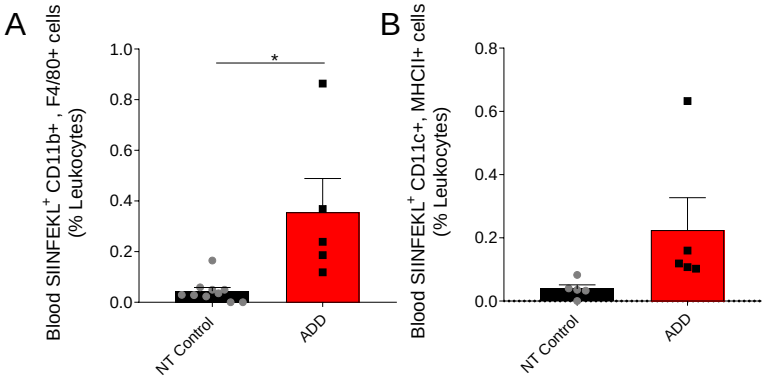

Figure S1. Tumor antigens released by CuDox+US treatment were presented by circulating immune cells. A-B) Flow cytometric analysis of blood samples collected from mice treated with CuDox+US (ADD) and no-treatment control mice 48 h post treatment. Frequency of SIINFEKL-labeled blood immune $CD11b^+$, $F4/80^+$ macrophages (A) and $CD11c^+$, $MHCII^+$ cells (B) as a percentage of total blood leukocytes. Statistical analysis was performed using an unpaired, one-tailed *t* test assuming unequal variance * $p < 0.05$.

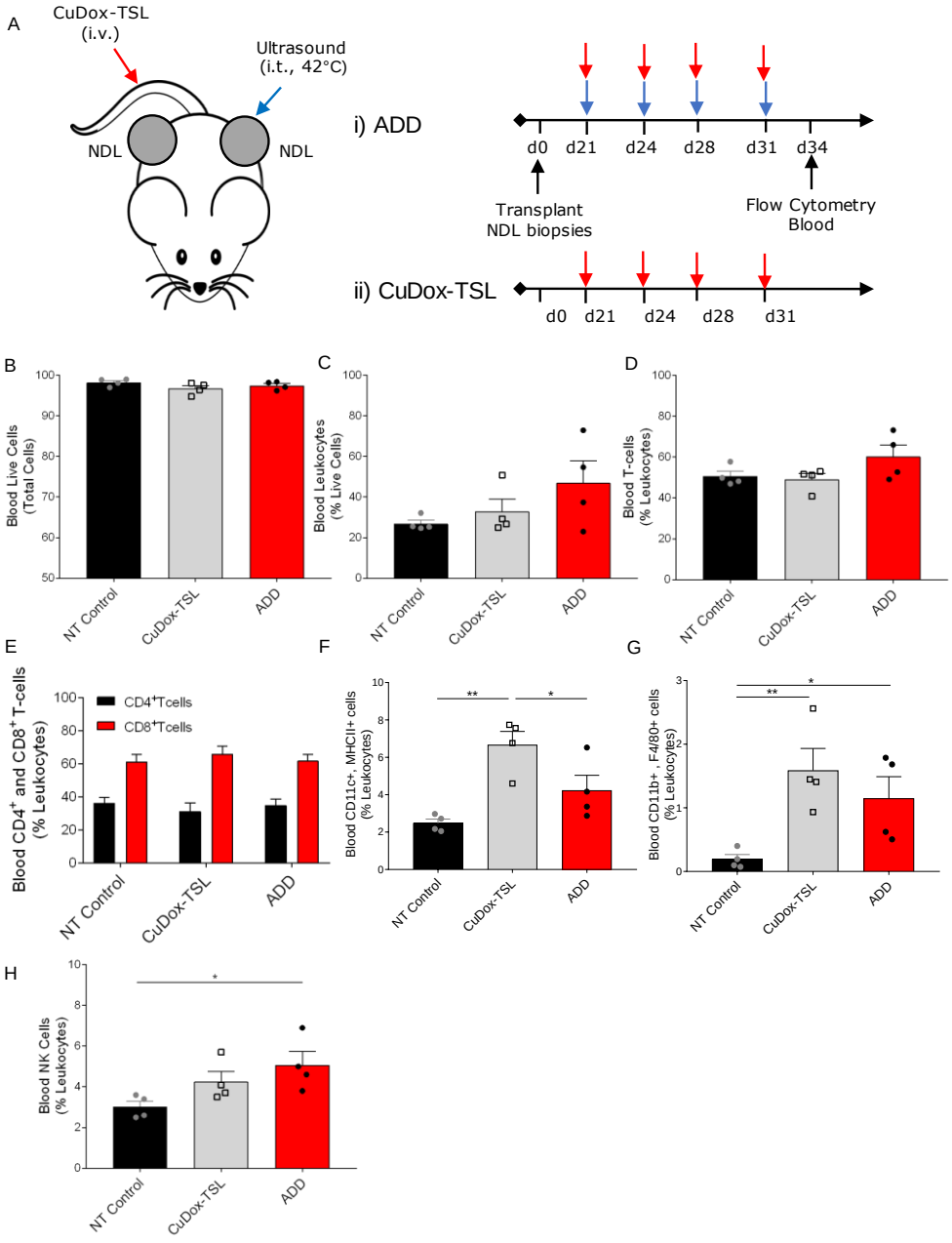

Figure S2. Repeated administration of CuDox+US did not reduce circulating immune cells. A) Representative treatment protocol. B-H) On day 34 after four complete treatments of ADD (n = 4) or CuDox-TSL (n = 4), blood collected from the treated mice was stained with the antibody cocktail as listed in the Materials and Methods, analyzed via flow cytometry and compared with untreated control tumors (n = 4). Frequency of blood live cells given as a percentage of total isolated cells (B), frequency of leukocytes ($CD45^+$ cells) as a percentage of live cells (C), frequency of all T-cells as a percentage of total leukocytes (D), $CD4^+$ and $CD8^+$ T-cell subsets as a percentage of total leukocytes (E), fraction of $CD11c^+MHCII^+$ cells as a percentage of leukocytes (F), fraction of macrophages ($CD11b^+F4/80^+$ cells) as a percentage of leukocytes (G), and fraction of natural killer (NK) cells as a percentage of leukocytes (H) across treatments. * $p < 0.05$, ** $p < 0.01$.

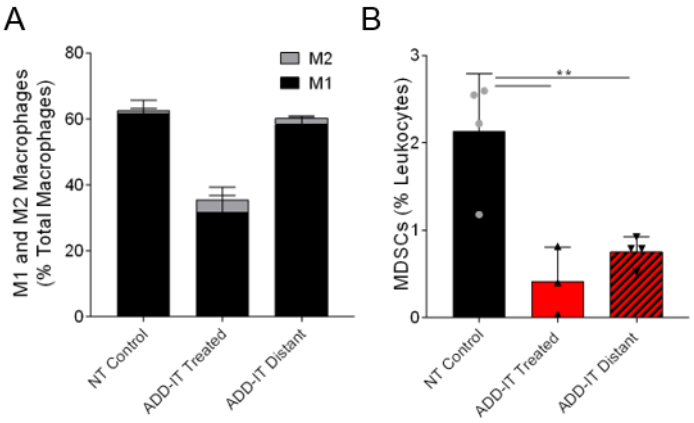

Figure S3. Two treatments of CuDox+US+CpG+αPD-1 altered macrophage phenotype and reduced MDSCs. A-B) After two sequential treatments of CuDox+US+CpG+αPD-1 (ADD-IT), the entire inguinal fat pads containing tumor and lymph node were harvested from treated and untreated control mice on day 35 and analysis of immune cells was performed using flow cytometry. Frequency of macrophages ($CD11b^+F4/80^+Gr\text{-}1^-$) with M1 and M2 phenotypes as a percentage of total macrophages (A) and fraction of myeloid derived suppressor cells (MDSCs, $CD11b^+Gr\text{-}1^+$) as a percentage of leukocytes (B). ** $p < 0.01$.

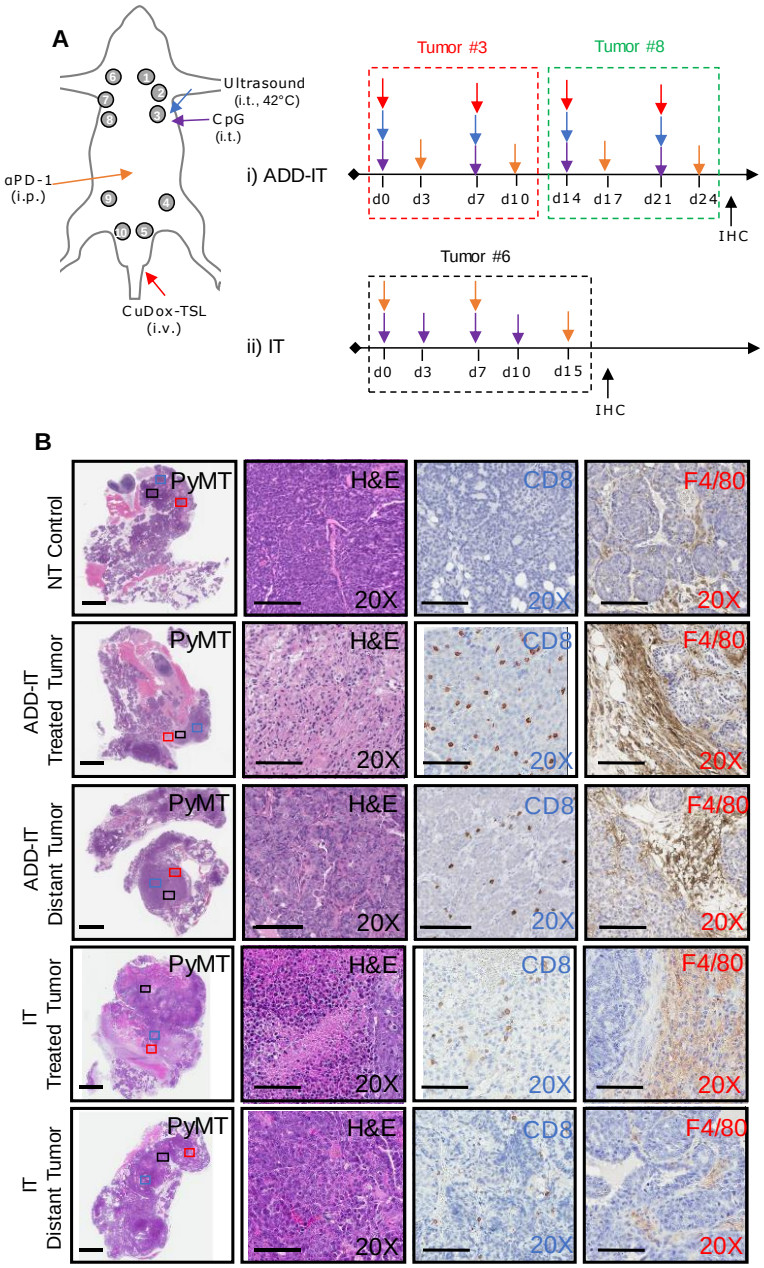

Figure S4. Combinatorial CuDox+US+CpG+αPD-1 protocol increased tumor infiltration of cytotoxic T lymphocytes and macrophages in local and distant tumors of transgenic PyMT mice. A) Treatment protocols applied to PyMT transgenic mice. i) The axillary lymph node tumor (#3) was treated with two sequential administrations of CuDox+US+CpG+αPD-1 (ADD-IT) for two weeks, then the same two-treatment protocol was applied to the axillary lymph node tumor (#8) for another 2 weeks (n = 5). ii) The cervical lymph node tumor (#6) was treated with CpG+αPD-1 (IT) for 15 days (n = 2) and all treatment groups were compared to no-treatment (NT) control mice (n = 5). Mice were euthanized and all lymph node tumors were isolated for histology and immunohistochemistry. B) histological sections of NT control tumor, local and distant tumors of mice treated with either ADD-IT or IT and stained for H&E (the left first column, whole tumor view) and (second column, magnified view), CD8 (third column, magnified view), and F4/80 (fourth column, magnified view). Whole tumor sections and the magnified views enclosed by black or colored boxes are shown. Scale bars correspond to 3 mm (whole tumor panels) and 100 μm (magnified panels).

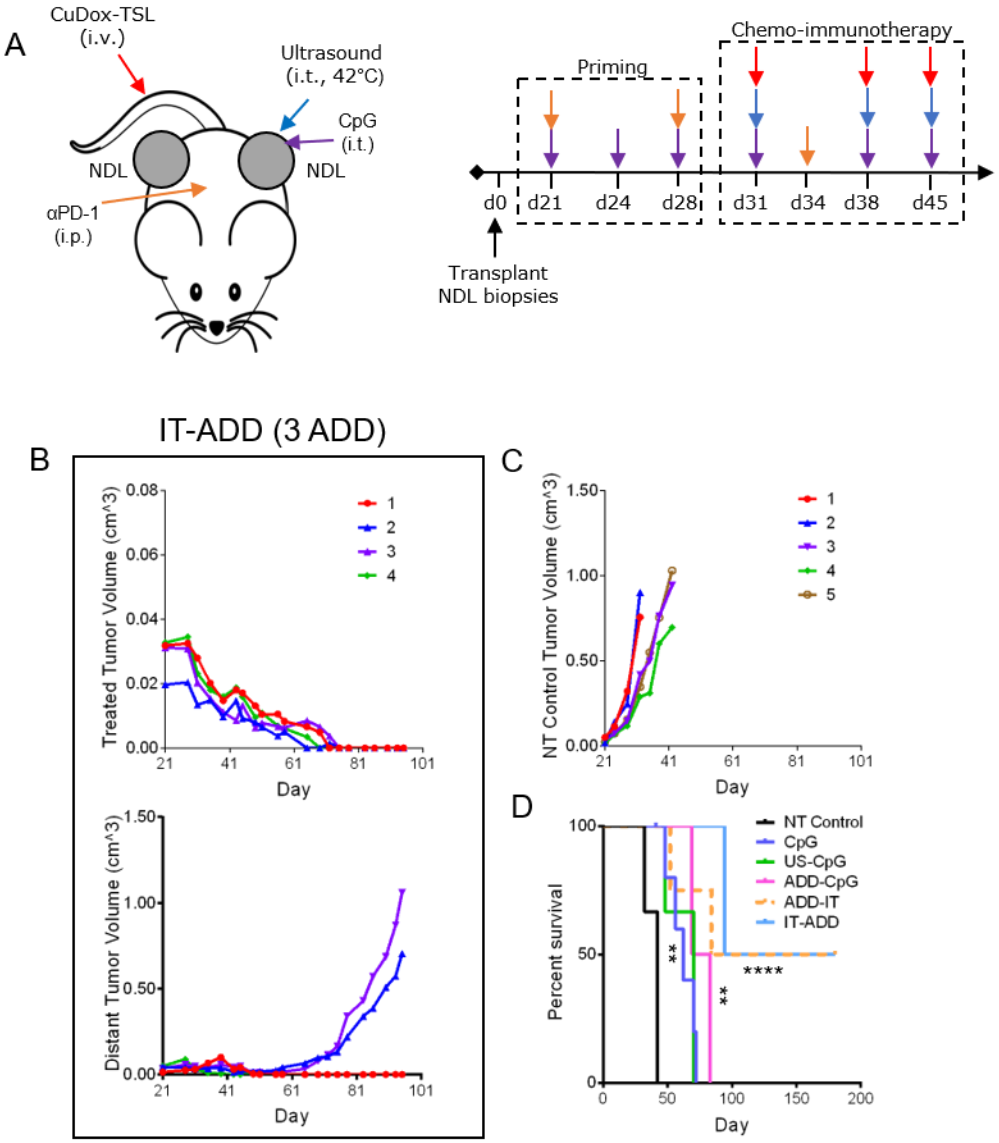

Figure S5. Repeated CuDox+US+CpG+αPD-1 protocol with immunopriming induced complete local and systemic responses with 50% long term survival. A) Treatment protocol integrating immunopriming sequence prior to chemo-immunotherapy. B-C) Growth of directly treated and distant tumors in bilateral NDL-tumor bearing mice treated with primed (IT-ADD, n = 4) (B) compared to no-treatment control (NT Control) tumors (C). D) Open-ended survival achieved with IT-ADD and ADD-IT compared with tri-combinatorial treatment of CuDox+US+CpG (ADD-CpG), bi-combinatorial treatment of US+CpG, and CpG only treatment. The survival curves for both IT-ADD and ADD-IT ($p<0.0001$), ADD-CpG ($p<0.01$), and CpG ($p<0.01$) were found statistically significant compared to the NT Control group as evaluated by Log-rank (Martel-Cox) test. ** $p< 0.01$, **** $p<0.0001$.

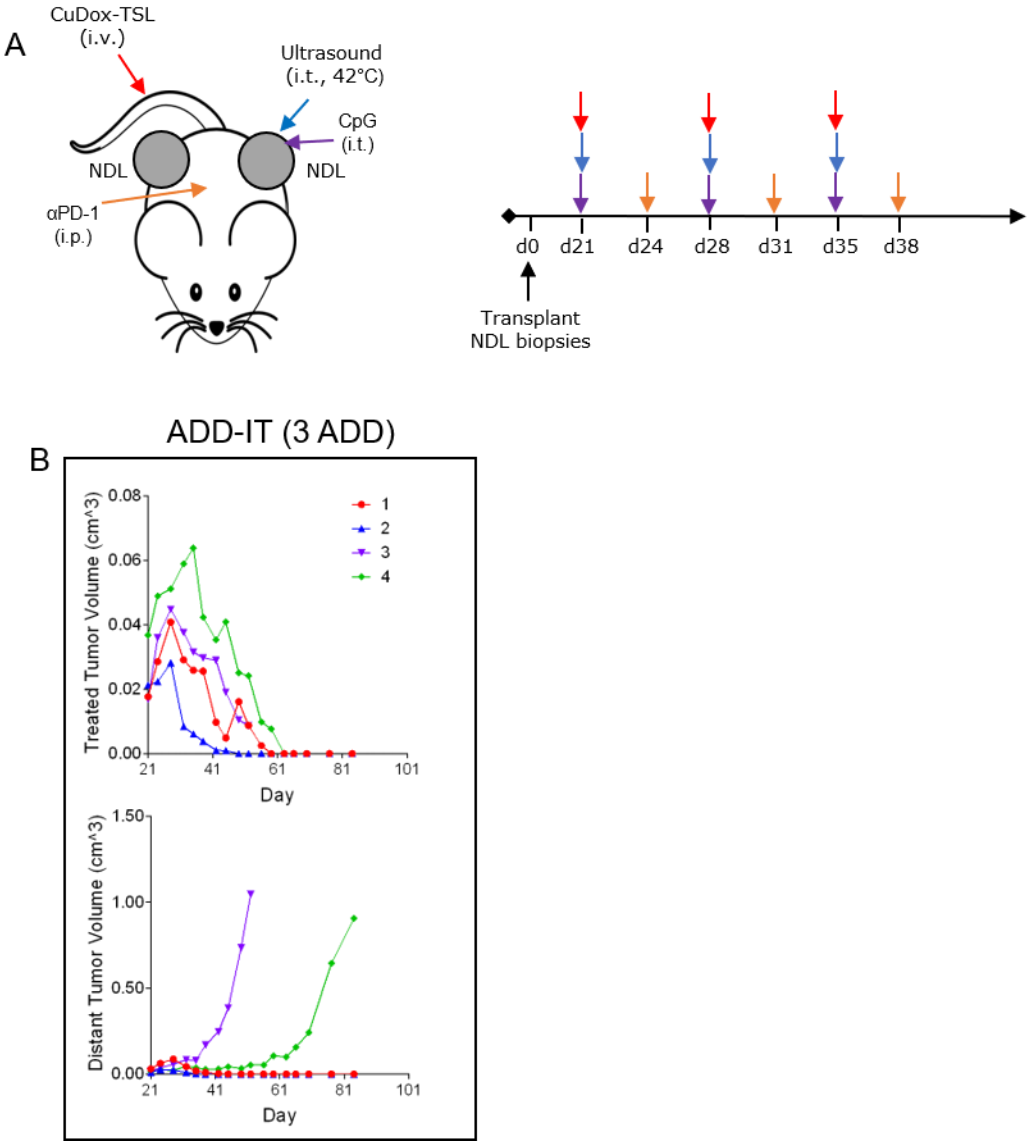

Figure S6. Three administrations of CuDox+US+CpG+αPD-1 without immunopriming achieved 50% long term survival. A) Treatment protocol starting with three cycles of ADD-IT without immunopriming. B) Growth of directly treated and distant tumors in bilateral NDL-tumor bearing mice treated with three administrations of (ADD-IT, n = 4).

Reference

[1] J.F. Liu, J. Foiret, D.N. Stephens, O. Le Baron, K.W. Ferrara, Development of a spherically focused phased array transducer for ultrasonic image-guided hyperthermia, Phys. Med. Biol. 61 (2016) 5275–5296. doi:10.1088/0031-9155/61/14/5275.