

1 **Acoustogenetic Control of CAR T Cells via Focused Ultrasound**

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14 **Abstract**

15 Optogenetics can control specific molecular events in living systems, but the penetration depth of
16 light is typically limited at hundreds of micrometers. Focused ultrasound (FUS), on the other
17 hand, can deliver energy safely and noninvasively into tissues at depths of centimeters. Here we
18 have developed an acoustogenetic approach using short-pulsed FUS to remotely and directly
19 control the genetics and cellular functions of engineered mammalian cells for therapeutic
20 purposes. We applied this acoustogenetic approach to control chimeric antigen receptor (CAR) T
21 cells with high spatiotemporal precision, aiming to mitigate the potentially lethal “on-target off-
22 tumor” effects of CAR T cell therapy. We first verified the controllability of our acoustogenetic
23 CAR T cells in recognizing and killing tumor cells *in vitro*, and then applied this approach *in*
24 *vivo* to suppress tumor growth of both lymphoma and prostate cancers. The results indicate that
25 FUS-based acoustogenetics can allow the noninvasive and remote activation, without any
26 exogenous cofactor, of different types of CAR T cells for cancer therapeutics.

27 Optogenetics enables the control of specific molecular events and cellular functions in living
28 systems with high spatiotemporal resolutions. However, optogenetics cannot reach deep tissues,
29 with the penetration depth of light typically limited at micrometer to millimeter scales (1).
30 Ultrasound can be focused to deliver mechanical energy safely and noninvasively into small
31 volumes of tissue deep inside the body up to tens of centimeters (1). The rapidly oscillating
32 pressure of focused ultrasound (FUS) waves and the resultant cycles of mechanical
33 loading/unloading can lead to local heat generation in biological tissues. Aided by Magnetic
34 Resonance Imaging (MRI) thermometry, FUS has been widely applied to clinically ablate
35 tumors, and control drug delivery, vasodilation, neuromodulation (2), and transgene expression
36 (3-5). Transcription factors and genetic circuits have also been engineered to convert the FUS-
37 generated heat into genetic regulation to control microbial systems *in vivo* (6). However, there is
38 a lack of general methods using FUS to control mammalian cell functions *in vivo* for therapeutic
39 applications.

40

41 Chimeric antigen receptor (CAR) T cell therapy, where T cells are genetically programmed with
42 redirected specificity against malignant cells, is becoming a paradigm-shifting approach for
43 cancer treatment, especially for blood cancers (7). However, major challenges remain for solid
44 tumors before CAR-based immunotherapy can be widely adopted. For instance, the non-specific
45 targeting of the CAR T cells against normal tissues (on-target off-tumor toxicities) can be life-
46 threatening: off-tumor toxicities against the lung, the brain, and the heart have caused multiple
47 cases of deaths (7-10). Immunosuppressive corticosteroid therapy and suicide gene engineering
48 are relatively effective in suppressing off-tumor toxicities and related cytokine release syndrome
49 (CRS), but they fail to discriminate between beneficial T cell functions and toxic side effects

50 (11-13). Synthetic biology and genetic circuits have been used to enhance specificity and reduce
51 off-tumor toxicity by creating chemically inducible dimerization of split CARs, inhibitory CARs
52 (iCARs), and SynNotch to control CAR activation (8, 14-18). However, given the extensive
53 overlaps of antigens between solid tumors and normal tissues, especially those under conditions
54 of tissue injury/inflammation (19), it remains very difficult to identify ideal antigens and their
55 combinations to differentiate tumors from normal tissues. There is hence an urgent need for a
56 high-precision control of CAR-T cells to confine the activation at local sites of solid tumors.
57 Recently, we demonstrated that ultrasound signals can be amplified by microbubbles coupled to
58 cells engineered with the mechanosensor Piezo1 to precisely control CAR T cell activations (20).
59 However, the presence of microbubbles as cofactors limits the application of this system *in vivo*.
60 Here, we have engineered a new class of inducible CAR T cells that can be remotely and directly
61 controlled by FUS without any exogenous cofactor. We show that short-pulsed FUS stimulation
62 can activate the engineered T cells at the desired time and location to suppress tumor growth *in*
63 *vivo*.

64 **Results**

65 **Heat-induced reporter gene activation**

66 We propose to genetically engineer T cells with inducible CAR cassettes that can be remotely
67 and directly activated, without any exogenous cofactor, by MRI-guided FUS at local tumor sites
68 for recognizing and eradicating the tumor cells (Fig. 1a).

69

70 We first tested the inducible activation of a reporter eGFP under the control of the heat-shock-
71 protein promoter (Hsp). We assembled a dual-promoter reporter construct containing the Hsp-

72 driven eGFP and a constitutive PGK-driven mCherry (Fig. 1b). HEK 293T cells infected with
73 the reporter lentivirus (fig. S1a) were heated at 43°C for 15 min. Real-time fluorescence imaging
74 revealed that the heat-induced eGFP expression started as early as 2 hr after heat shock (HS) and
75 persisted throughout the course of observation (Fig. 1c and Movie S1). Quantitative tracking of
76 the dynamics of heat-induced eGFP expression by flow cytometry showed that 97% of the cells
77 expressed eGFP at 6 hr post HS, and the percentage increased to 99% at 12 hr and remained
78 stable for 2 days, while the mean fluorescence intensity peaked at 12 hr followed by a steady
79 decrease (Fig. 1d). We then investigated the inducible effect of HS in primary human T cells
80 hosting the dual-promoter eGFP reporter (fig. S1b). A 15-min HS induced a strong eGFP
81 expression in 92.9% of the engineered T cells, in contrast to a background of 3.9% in control
82 cells without HS (Fig. 1, e and f). The mean fluorescence intensity of the eGFP+ cells in the HS
83 group was 10-fold of that in the control group without HS (Fig. 1g).

84 **Heat-induced CAR expression and its functionality in Jurkat and primary human T cells**

85 In order to convert the transient heat stimulation to a sustained gene activation and cellular
86 functions for therapeutic actions, we integrated the Cre-lox gene switch into the inducible
87 system. The design is composed of two constructs, one containing the Hsp-driven Cre
88 recombinase and the PGK-driven membrane c-Myc tag for cell sorting (“inducible Cre”, Fig.
89 2a), and the other containing a lox-flanked “ZsGreen-STOP” sequence between a PGK promoter
90 and an anti-CD19 CAR (“lox-stop CAR reporter”, Fig. 2a). As such, the excision of the “STOP”
91 cassette mediated by the transient heat-induced Cre can cause a switch from ZsGreen to
92 sustained CD19CAR production.

93

94 We first tested this heat-inducible gene switch system in Jurkat T cell lines (fig. S2a). A 15-min
95 HS induced CAR expression in 76.6% of the cells when measured 24 hr after HS (Day 1), in
96 contrast to a basal value of 14.0% in control cells without HS and a minimal leakage of 0.6% in
97 cells infected with the lox-stop CAR reporter alone (Fig. 2b). The heat-induced CAR expression
98 remained stable when measured 6 days after HS (Day 6, Fig. 2b). We further examined the
99 functionality of the induced CD19CAR in engineered cells (Fig. 2c). Engineered Jurkat cells
100 with (HS) or without (CT, control) a 15-min HS were co-cultured with CD19-expressing Nalm-6
101 tumor cells for 24 hr. Quantification of the expression level of CD69 (an early T cell activation
102 marker) revealed a 73.4% CD69+ cell population in the engineered Jurkat cells in the HS group,
103 in contrast to a 11.9% in the control group (Fig. 2, d and e). These results indicate that the HS-
104 induced CD19CAR is efficient for functional changes in engineered Jurkat T cells.

105

106 We then examined our system in primary human T cells (Fig. 2, a and c; fig. S2b). CAR
107 antibody staining showed that a 15-min HS induced CAR expression in 29% of the T cells, in
108 contrast to 1.9% in control cells without HS (Fig. 2f). The heat-inducible CAR T cells were then
109 co-cultured with firefly luciferase (Fluc)-expressing Nalm-6 cells at different effector-to-target
110 (E:T) ratios for cytotoxicity assays. The luminescence of the remaining Nalm-6 cells was
111 quantified after 24-hr co-culture. The heat-stimulated T cells (HS) demonstrated increased
112 cytotoxicity with increased E:T ratio, with the largest difference in cytotoxicity between the HS
113 and control (CT) T cells observed at E:T = 1:5, eliminating 82.9% and 29.3% of the target tumor
114 cells, respectively (Fig. 2g). The heat-stimulated CAR T cells also released significantly higher
115 concentrations of cytokines (IFN- γ and IL-2) than the control cells when co-cultured with Nalm-

116 6 cells (Fig. 2, h and i), verifying the functional capability gained with the HS-induced CAR T
117 cells.

118

119 While the continuous 15 min HS could lead to strong gene inductions (Figs. 1 and 2), it may
120 cause toxicity to cells (21). We hence investigated the effect of different HS patterns in primary
121 human T cells (fig. S3). Our results showed that longer HS resulted in more cell death; however,
122 pulsed HS was able to alleviate this toxicity while achieving induction levels comparable to that
123 in response to continuous HS with the same total heating time (fig. S3). In particular, a pulsed
124 HS with 50% duty cycle and a total heating time of 15 min (fig. S3a, Pattern 2) caused a strong
125 induction of eGFP expression in 91.4% of the engineered T cells, with a minimal toxicity as
126 evidenced by the 92.2% cell viability measured 24 hr after HS (fig. S3, b to d). Therefore, we
127 applied this HS pattern (fig. S3a, Pattern 2) for *in vivo* therapeutic studies.

128 **MRI-guided FUS-induced gene activation in phantom and *in vivo***

129 MRI-guided FUS enables the delivery of thermal energy *in vivo* at confined local regions with
130 high spatiotemporal resolutions (3, 4). We integrated an MRI-guided FUS system (Image Guided
131 Therapy) with a 7T MRI as described in Methods. An annular array transducer is placed above
132 the target region of the object to be heated (phantoms or small animals) in the MRI bore. MR
133 images are acquired and transferred to Thermoguide software to calculate the temperature of the
134 target region in real-time, which is fed back to the PID controller to automatically regulate the
135 output power of the FUS generator, maintaining the temperature of the target region at the
136 desired level (Fig. 3a, fig. S4).

137

138 We transduced Nalm-6 cells with a lentiviral dual-luciferase reporter containing inducible Fluc
139 and constitutive Rluc (Hsp-Fluc-PGK-Rluc-mCherry; Rluc, *Renilla* luciferase; Fig. 3b) and
140 embedded them in a tofu phantom approximately 7 mm deep from the top surface (Fig. 3c and
141 Methods). We then focused the ultrasound on the embedded cells by changing the focal distance
142 in the z direction. Three pulses of 5-min FUS stimulations caused a significant induction of gene
143 expression as quantified by the Fluc/Rluc ratio of the cells assayed 8 hr later (Fig. 3d, Methods).
144 The induction level is comparable to that of the positive control using thermal cycler with the
145 same heating pattern (Fig. 3d), suggesting the acoustogenetic approach can remotely control
146 gene activation in engineered cells with high efficiency.

147

148 We then used MRI-guided FUS to control local temperature *in vivo* in mouse (Fig. 3, e and f and
149 Movie S2) and tested the FUS-induced gene activation using Nalm-6 cells with the dual-
150 luciferase reporter *in vivo*. Significant gene induction was observed in the implanted cells with
151 only two pulses of 5-min FUS stimulation (FUS+, after), in comparison to the basal level (FUS+,
152 before) and the control groups (FUS-, before and after) (Fig. 3, g and h).

153 **FUS-inducible tumor cytotoxicity of the engineered CAR T cells *in vivo***

154 We next tested the tumor cytotoxicity of the FUS-inducible CAR T cells *in vivo*. We
155 subcutaneously injected Nalm-6 cells (Fluc+) on both hindlimbs of NSG mice to generate
156 matched bilateral tumors (Fig. 4a). Four days later, engineered CD19CAR T cells were
157 subcutaneously injected at both tumor sites locally, followed by three pulses of 5-min FUS
158 stimulation at 43°C on the left but not on the right tumor (Fig. 4a). Bioluminescence imaging
159 revealed that FUS significantly suppressed tumor growth (Fig. 4, b and c). The results on the two
160 tumors (FUS+ and FUS-) on the same mouse indicate that the FUS-activated CAR T cells at the

161 local site had negligible off-site effects in attacking the distal tissues on the contralateral
162 hindlimb expressing the same antigens. We further performed a control experiment subjecting
163 mice carrying bilateral tumors to FUS stimulation on one side, with neither site subjected to the
164 injection of engineered CAR T cells (fig. S5a). The tumors with or without FUS stimulation
165 exhibited similar growth profiles, indicating that FUS itself (with the chosen pattern) had no
166 impact on tumor growth (fig. S5, b and c). Therefore, our results demonstrated that FUS can be
167 used to precisely control the cytotoxicity of the engineered CD19CAR T cells *in vivo* against
168 target tumor cells.

169

170 We further tested this acoustogenetic technology in controlling inducible CAR T cells against
171 other types of tumors, particularly solid tumors. We engineered solid tumor human prostate
172 cancer PC3 cells to express the prostate-specific membrane antigen (PSMA) and Fluc, and
173 engineered primary human T cells with the Cre-lox mediated heat-inducible anti-PSMA CAR
174 (PSMACAR; fig. S6a). We verified the functionality of the heat-inducible PSMACAR T cells
175 through *in vitro* co-culture cytotoxicity assays and the associated cytokine assays (fig. S6, b to
176 d). We then generated matched bilateral subcutaneous PC3 tumors (PSMA+, Fluc+) in NSG
177 mice; five days later we subcutaneously injected heat-inducible PSMACAR T cells next to the
178 tumor sites on both sides. The tumor regions on the left side were treated with three pulses of 5-
179 min FUS, while those on the right remained unstimulated. Consistently, the tumors with FUS
180 stimulation showed significantly inhibited growth as compared to the controls (Fig. 4, d and e).
181 We further harvested the tumor tissues and quantified the related mRNA amount. The CD3
182 mRNA in the FUS-treated tumors averaged 3-fold of that in the untreated ones, indicating more
183 T cell infiltration in the FUS-treated solid prostate tumors (fig. S7a). Moreover, the amount of

184 Cre-mediated recombined CAR mRNA in the FUS-treated tumors was 9-fold of that in the
185 untreated controls, verifying the FUS-induced DNA recombination and subsequent CAR
186 expression in the engineered T cells at the tumor sites (fig. S7, b and c, and Methods). These
187 results demonstrated the efficacy of FUS-based acoustogenetics in remote control of CAR T
188 cells for treating different types of tumors *in vivo*, including solid tumors of prostate cancer.

189 **Discussion**

190 We developed an FUS-based acoustogenetic approach to remotely control, without any
191 exogenous cofactor, the genetically engineered T cells capable of perceiving ultrasound signals
192 and transducing them into genetic and cellular activations for therapeutic applications *in vivo*.
193 This acoustogenetics technology enables the activation of CAR T cells at confined tissue regions,
194 thus allowing the targeting of the less ideal antigens without causing non-specific off-site
195 cytotoxicity. This is of critical importance given the extensive overlap of antigens between
196 tumors and normal cells, particularly those under conditions of tissue injury and inflammation.
197 The short-pulsed patterns of FUS stimulation should also minimize potential detrimental effects
198 of hyperthermia and induce transient expression of synthetic protein regulators to circumvent
199 severe immune responses. This acoustogenetic approach is highly modular, with the target CAR
200 genes switchable to aim at different cancer types.

201

202 We employ the Cre-mediated gene switch to convert transient FUS inputs into sustained outputs
203 of genetic and cellular activities for sufficient therapeutic efficiency. The nature of local
204 activation should limit the number of activated cells off the tumor site and the potential non-
205 specific cytotoxicity against normal tissues, as evidenced in our results (Fig. 4, b to e); if the Cre-
206 mediated permanent activation of CAR becomes an issue in the future, degradation domains such

207 as dihydrofolate reductase (DHFR) can be fused to CAR to control the protein lifetime with an
208 FDA-approved drug methotrexate (22). This “AND” gate with FUS and methotrexate should
209 enhance the precision of controllable CAR T immunotherapy.

210

211 We anticipate that comparable therapeutic outcomes can be achieved in a reversible heat-
212 inducible system without the Cre-lox gene switch, but this may require multiple rounds of FUS
213 stimulation. In such a system, Hsp directly drives the production of CAR (Hsp-CAR) under FUS
214 stimulation. Upon the withdrawal of FUS stimulation, HSFs gradually dissociate from Hsp,
215 returning Hsp and its downstream transcriptional activities to the resting state. This recovery
216 process is relatively fast, within 45 min after HS for *Drosophila* Hsp70 and approximately 60
217 min after HS for human Hsp70 (23, 24). The dynamics of this heat-induced CAR expression
218 hence largely depends on its protein lifetime, with the half-life of GFP-tagged CAR reported to
219 be around 8 hr (16). Therefore, repeated FUS stimulation can be applied to maintain the CAR
220 expression in the T cells (and hence their cytotoxicity) for a sustained period of time. We tested
221 this concept by applying a 10-min HS every 48 hr in T cells with Hsp-eGFP, and indeed
222 observed oscillatory patterns of the induced eGFP expression (fig. S8). We anticipate that T cells
223 with a simple Hsp-CAR can also be repeatedly activated by FUS to achieve sustained CAR
224 expression and cytotoxicity for a desired period of time or until tumor elimination. Such a
225 reversible FUS-inducible system can further prevent “on-target off-tumor” toxicity of canonical
226 CAR T therapy, as the T cells leaving the tumor site will no longer receive FUS stimulation and
227 gradually lose CAR molecules. The tunable FUS pulses should also allow the precise control of
228 the temporal activation patterns of CAR T cells for an optimized killing efficiency with
229 controllable exhaustion.

230

231 We chose local injection at the tumor site to deliver T cells *in vivo*. Local administration of CAR
232 T cells has been tested in animals and patients to overcome the obstacle of T cell homing
233 associated with intravenous delivery, and has achieved promising therapeutic effects (16, 25, 26).
234 For example, since the prostate is positioned near critical organ structures including urethra and
235 neurovascular bundles, surgery or radiation therapy targeting the whole prostate gland to treat the
236 prevalent locally-progressed prostate cancer (27) may cause adverse effects that would
237 significantly impact quality of life (28, 29). Local delivery and activation of inducible CAR T
238 cells using clinically available MRI-guided FUS systems should allow, without any exogenously
239 added nanoparticle or cofactor, a high degree of precision and safety in eradicating tumor cells in
240 these patients harboring locally progressed prostate cancer (29). In cases where intravenous
241 delivery is required, it is also possible to equip the FUS-inducible CAR T cells with additional
242 antigen binders and/or chemokine receptors to promote trafficking, infiltration, and the
243 enrichment of these engineered cells at the tumor site before FUS activation (30, 31).

244

245 The short-pulsed stimulation and the biocompatible Hsp capable of inducing transient
246 expressions of different synthetic protein regulators can potentially enhance the safety of gene
247 therapy, circumventing detrimental host immune response. For instance, CRISPR-Cas9 proteins
248 have been a powerful tool for research in genetic and epigenetic engineering, but can evoke
249 adaptive immune responses and tissue damage *in vivo*, and are therefore potentially pathogenic if
250 applied to correct inherited genetic defects to treat diseases (32). Protein engineering to remove
251 immunogenic epitopes and humanize these synthetic proteins to circumvent this issue can be
252 difficult owing to the high diversity of the human leukocyte antigen (HLA) loci (33). Using our

253 acoustogenetic approach, the transiently induced Hsp-driven synthetic regulators (e.g. Cas9) can
254 be cleared in a timely manner to mitigate or evade the adaptive immune response, hence offering
255 a new option for gene therapy.

256
257 Each component of this FUS-based acoustogenetics, i.e. ultrasound devices, molecular thermo-
258 sensors, and genetic/epigenetic transducing modules, is highly modular and will continue to evolve
259 for greater precision and reduced immunogenicity. In fact, stretchable electronic circuits are being
260 developed to fabricate wearable patches of ultrasound transducers (34). The leverage of
261 technological advancements of different fields into FUS-based acoustogenetics should be able to
262 drive the development of these fields to open up new frontiers. We envision that the current state
263 of acoustogenetics is analogous to optogenetics at its infancy. Before the functional demonstration
264 of channelrhodopsin in neuronal cells (35), it was challenging to manipulate molecular activities in
265 live cells at high spatiotemporal resolutions. With the technological integration and convergence of
266 optics, genetic circuits, and light-sensitive proteins, optogenetics is rapidly reaching its full potential.
267 Based on this analogy, acoustogenetics may undergo a similar trajectory to provide a broadly
268 applicable method and usher in an era of applying ultrasound for the direct, remote, and noninvasive
269 control of genetically engineered cells for therapeutics.

270 **Methods**

271 **Cloning**

272 Plasmids used in this paper are listed in Table S1. Cloning strategies include Gibson Assembly
273 (NEB, E2611L) and T4 ligation (NEB, M0202L). PCR was performed using synthesized primers
274 (Integrated DNA Technologies) and Q5 DNA polymerase (NEB, M0491). The sequences of the
275 constructed plasmids were verified by Sanger sequencing (Genewiz).

276

277 **General cell culture**

278 HEK 293T cells were cultured in DEME (Gibco, 11995115) with 10% FBS (Gibco, 10438026)
279 and 1% Penicillin-Streptomycin (Gibco, 15140122). Jurkat, Nalm-6, and PC3 cells were cultured
280 in RPMI 1640 (Gibco, 22400105) with 10% FBS and 1% P/S. Primary human T cells were
281 cultured in complete RPMI 1640 supplemented with 100 U/mL recombinant human IL-2
282 (PeproTech, 200-02). Cells were cultured at 37°C in a humidified 5% CO₂ incubator.

283

284 **Staining and flow cytometry**

285 Staining of cell surface markers (e.g., c-Myc, CD69, etc) for flow cytometry was performed
286 using fluorophore-conjugated antibodies according to manufacturers' protocols. In general, cells
287 were washed twice and resuspended in 100 µL wash buffer (PBS + 0.5% BSA) containing the
288 suggested amounts of antibodies, incubated in dark at room temperature for suggested durations,
289 and washed three times before being analyzed using a BD flow cytometer. Gating was based on
290 non-engineered cells with the same staining. Flow cytometry data were analyzed using FlowJo
291 software (Tree Star).

292

293 ***In vitro* heat shock**

294 For Fig. 1c and Movie S1, cells seeded in a glass bottom dish were heated at 43°C for 15 min
295 using a heating stage (Instec) integrated with a Nikon Eclipse Ti inverted microscope. Images
296 were acquired in real-time to obtain the kinetics of the induced fluorescent protein. For the
297 remainder of the *in vitro* heat shock (HS) experiment, unless otherwise specified, cells were
298 washed and resuspended in cell culture medium at a concentration of 2 x 10⁶ cells/mL, aliquoted

299 into 8-strip PCR tubes with 50 μ L/tube, and heat shocked at 43°C using a thermal cycler (Bio-
300 Rad, 1851148) with various patterns as indicated (Table S2). Cells were returned to standard
301 culture condition after HS. The gene induction levels were quantified by flow cytometry 12 hr
302 after HS in Fig. 1, f and g, and fig. S3, d and e.

303

304 **Engineered cells**

305 The engineered cells (excluding primary human T cells) used in this work are listed in Table S3.
306 Lentiviruses were used to deliver engineered genes into the cells. Fluorescence-activated cell
307 sorting (FACS), when needed, was performed at UCSD Human Embryonic Stem Cell Core
308 Facility by professional technicians following standard protocols.

309

310 **Quantification of CAR expression in Jurkat cells**

311 Jurkat cells were either transduced with a lentiviral cocktail (inducible Cre and lox-stop CAR
312 reporter, Fig. 2a) followed by indicated HS (Fig. 2b), or transduced with the lox-stop CAR
313 reporter lentivirus alone without HS. CAR expression was quantified by CAR antibody staining
314 (an anti-mouse IgG, F(ab')₂ fragment specific antibody; Jackson ImmunoResearch, 115-606-
315 072) and flow cytometry 24 hr after HS. Live single cells were gated for CAR expression
316 analysis. Non-engineered Jurkat cells were stained with the same antibody to generate the CAR+
317 gate.

318

319 **Quantification of CD69 expression in Jurkat cells**

320 Jurkat cells transduced with a lentiviral cocktail (inducible Cre and lox-stop CAR reporter, Fig.
321 2a) were treated with or without HS at 43°C for 15 min, and co-cultured with target tumor cells

322 for 24 hr. The cells were then stained by an APC anti-human CD69 antibody (BioLegend,
323 310910) and analyzed by flow cytometry. ZsGreen+ cells (representing the engineered Jurkat
324 cells) were gated for analysis of CD69 expression. Non-engineered Jurkat cells co-cultured with
325 target tumor cells were stained with the same antibody to generate the CD69+ (APC+) gate.

326

327 **Isolation, culture, transduction and MACS of primary human T cells**

328 Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (San Diego
329 Blood Bank) using Lymphocyte Separation Medium (Corning, 25-072-CV) following the
330 manufacturer's instructions. Primary human T cells were isolated from PBMCs using Pan T Cell
331 Isolation Kit (Miltenyi, 130-096-535) and activated with Dynabeads® Human T-Expander
332 CD3/CD28 (Gibco, 11141D). Three days later, lentivirus concentrated using PEG-it (SBI,
333 LV825A-1) was added to the T cells at MOI = 10, followed by spinoculation in a 96-well plate
334 coated with Retronectin (Takara, T100B). T cells were further expanded and dynabeads were
335 removed prior to downstream procedures (e.g., magnetic-activated cell sorting (MACS), *in vitro*
336 HS, *in vivo* injection, etc.).

337

338 For *in vitro* and *in vivo* cytotoxicity studies, T cells were transduced with a lentiviral cocktail of
339 inducible Cre and lox-stop CAR reporter (Fig. 2a). MACS was performed using Anti-c-Myc-
340 Biotin antibodies and Anti-Biotin microbeads (Miltenyi, 130-092-471 and 130-097-046)
341 following the manufacturer's instructions to enrich c-Myc+ cells. A representative double
342 positive efficiency after MACS is 69%, 95% for the c-Myc+ and 71.4% for the ZsGreen+ cells
343 (fig. S2b). CAR expression in the engineered inducible CAR T cells with or without HS was
344 quantified using the CAR antibody as described above.

345

346 **Luciferase-based cytotoxicity assay**

347 A constant number of 5×10^4 Fluc⁺ Nalm-6 cells were mixed with engineered primary human T
348 cells with or without HS (pre-washed and resuspended with complete RPMI without IL-2) at
349 effector-to-target (E:T) ratios of 1:50, 1:20, 1:10, 1:5, 1:1, 5:1 or no T cells (“target cell only”).
350 The mixtures were then cultured in round bottom 96 well plates for 24 hr, centrifuged to remove
351 the supernatant (which was harvested for quantification of cytokine production), and assayed
352 with the Bright-GloTM Luciferase Assay System (Promega, E2610) following the manufacturer's
353 instructions to quantify the luminescence of each sample. The cytotoxicity (%) of Sample X was
354 calculated as $(1 - \text{Luminescence of X} / \text{Luminescence of “target cell only”}) \times 100\%$.

355

356 For cytotoxicity assay using PC3 cells as the target, 1×10^4 PSMA⁺ Fluc⁺ PC3 cells were
357 seeded onto TC-treated flat bottom 96 well plates (Corning, 3603). Except for “target cell only”
358 wells, engineered primary human T cells with or without HS (washed and resuspended with
359 complete RPMI without IL-2) were added 6 hr later at E:T ratios of 1:10, 1:5, 1:1, 5:1, 10:1,
360 20:1. The luminescence was quantified 24 hr after co-culture as described above.

361

362 **Quantification of cytokine production**

363 The supernatant of effector-target cell co-culture was harvested. The concentrations of cytokines
364 IL-2 and IFN- γ were quantified using the corresponding ELISA kits (BD, 555190 and 555142).

365

366 **T cell viability assay**

367 Non-engineered primary human T cells were heat shocked as described above and kept under
368 normal culture condition for 24 hr. Cell viability was then assessed using the FITC Annexin V
369 Apoptosis Detection Kit I (BD, 556547) following the manufacturer's instructions. The cells
370 stained negative for both Annexin V and PI were counted as live cells.

371

372 **MRI-guided FUS**

373 The MRI-guided FUS system is composed of a 1.5 MHz 8-element annular array transducer, a
374 16-channel broadband RF generator, a piezo motor-based X-Y positioning stage, and a degassing
375 and water circulation system (Image Guided Therapy, France). MR images acquired using a
376 Bruker 7T MRI system were transferred to ThermoGuide software (Image Guided Therapy,
377 France) to generate phase images and real-time temperature maps. Using PID controller, the
378 software automatically regulates the output power of the generator to maintain the temperature at
379 the focal spot at a desired value as described elsewhere (6, 36).

380

381 Animal experiments were performed following Protocol S15285 approved by UCSD IACUC.
382 NSG mice (6-8 weeks old) were purchased from Jackson Laboratory (JAX) and shaved prior to
383 FUS stimulation. Anesthesia was induced using 2% isoflurane-oxygen mixture and maintained
384 with 1.5% isoflurane-oxygen mixture during FUS stimulation. The mouse was laid on its side on
385 an MR bed containing an agarose gel pad and a surface coil. A pressure pad was placed under
386 the mouse to monitor its respiration rate, and a rectal thermal probe was used to provide
387 feedback for the delivering of warm air into the bore to maintain the mouse's core temperature at
388 approximately 37°C. The ultrasound transducer was positioned right above the targeted region

389 on the mouse's hindlimb. Thin layers of SCAN ultrasound gel (Parker labs) were applied at the
390 skin-transducer and skin-bed interfaces.

391

392 The ThermoGuide software regulates the temperature in a 3 x 3 pixel square (3 - 4 mm²)
393 centered at the ultrasound focus (Fig. 3e). A PID controller is used to maintain the average
394 temperature of the target square at 6°C above reference by controlling the output power of the
395 FUS generator, with the reference temperature being 37°C as measured by the rectal thermal
396 probe. As such, the MRI-guided FUS enabled temperature elevation to 43°C locally at the focal
397 area in the hindlimb of an anesthetized mouse.

398

399 **FUS stimulation in tofu phantom**

400 For FUS stimulation on cells in the tofu phantom, Nalm-6 cells were lentivirally transduced with
401 the dual-luciferase reporter (Fig. 3b, Hsp-Fluc-PGK-Rluc-mCherry) and FACS-sorted. The cells
402 were resuspended in culture medium and mixed with matrigel (Corning, 354262) at 1:1 volume
403 ratio on ice. Extra-firm tofu was cut into a 15-mm thick pad, and an 8-mm deep hole of 8-mm
404 diameter was drilled from the top. A microcentrifuge tube of 7.5-mm diameter (Fisherbrand, 05-
405 408-120) was cut to 8-mm long by removing the lid and the conical bottom, and was inserted
406 into the hole in the tofu phantom. Cell-matrigel mixture of 150 µL was added into the hole (~3
407 mm thick) and allowed to gel at room temperature. The rest of the hole and the gap between the
408 tube and the tofu phantom were filled up with matrigel. After gelation, the assembly was
409 inverted and positioned onto the MR bed containing the surface coil. The ultrasound transducer
410 was positioned above the tofu phantom with its center aligned with that of the tube. Thin layers

411 of ultrasound gel were applied at the tofu-transducer and tofu-bed interfaces. A thermal probe
412 was inserted into the distal end of the tofu phantom to provide reference temperature readings.
413
414 MR images of the assembly were acquired and transferred to ThermoGuide to calculate the
415 theoretical ultrasound focal position. Test FUS shots were delivered to determine the actual focal
416 position. Steering was applied to focus the ultrasound at the region immediately above the cells.
417 Three pulses of 5-min FUS stimulations at 43°C were applied. The cell-matrigel mixture was
418 then recovered from the tube, placed in cell culture medium, and returned to a standard 37°C cell
419 culture incubator. After 6 hr, the culture was centrifuged to remove the supernatant, and the cell-
420 matrigel pellet was incubated in a Cell Recovery Solution (Corning, 354253) at 4°C for 1 hr to
421 retrieve the Nalm-6 cells from matrigel. The Fluc and Rluc luminescence of the cells was
422 quantified using the Dual-Luciferase® Reporter Assay System (Promega, E1910) following the
423 manufacturer's instructions.

424

425 ***In vivo* bioluminescence imaging**

426 *In vivo* bioluminescence imaging (BLI) was performed using an In vivo Imaging System (IVIS)
427 Lumina LT Series III (PerkinElmer). For Fluc imaging, 150 mg/kg D-Luciferin (GoldBio,
428 LUCK) was administered intraperitoneally (i.p.). BLI started 10 min after substrate injection
429 until peak signal was acquired. For Rluc imaging, 200 µL 0.295 mM ViviRen™ (Promega,
430 P1232) (37) was administered i.p. BLI started 15 min after substrate injection until peak signal
431 was acquired. BLI of Fluc and Rluc in the same mouse, when needed, was performed 4 hr apart.
432 Images were analyzed using Living Image software (PerkinElmer), and the integrated Fluc
433 luminescence intensities within regions of interest were quantified to represent tumor sizes.

434

435 **FUS-inducible gene activation *in vivo***

436 NSG mice (male, 6-8 weeks old) were subcutaneously injected with 2×10^6 dual-luciferase
437 reporter Nalm-6 cells at the hindlimb. One week later, the experimental mice received two pulses
438 of 5-min FUS stimulation at 43°C targeted at the implanted cells, while the control mice
439 remained unstimulated. The *in vivo* Fluc and Rluc luminescence was quantified 4 hr before and
440 12 hr after FUS stimulation, as described above.

441

442 ***In vivo* tumor cytotoxicity of FUS-inducible CAR T cells.**

443 NSG mice (male, 6-8 weeks old) were subcutaneously injected with 2×10^5 Fluc+ Nalm-6 cells
444 (or 2×10^5 PSMA+ Fluc+ PC3 cells, for PC3 tumors) on both hindlimbs to generate matched
445 bilateral tumors. Four days later (or five days later, for PC3 tumors), 1×10^6 inducible primary
446 human CAR T cells prepared as described above were injected subcutaneously and locally at
447 tumor regions. Within 4 - 8 hr after T cell injection, three pulses of 5-min FUS stimulation
448 targeted at 43°C were applied on the left tumor region as described above, while the tumor on the
449 right hindlimb received no FUS stimulation to serve as the control. Tumor aggressiveness was
450 monitored by BLI twice a week as described above until euthanasia criteria were met.

451

452 **Quantification of mRNA expression in tumor tissue.**

453 PC3 tumors (Fig. 4, d and e) were harvested 22 days after tumor implantation (17 days after T
454 cell injection and FUS stimulation). The tumors were disrupted and homogenized, and the same
455 amount of lysate from each tumor was used to extract total RNA with the RNeasy Mini Kit
456 (Qiagen, 74104) followed by reverse transcription using the same amount of template RNA.

457 Quantitative PCR (qPCR) was performed using iTaqTM Universal SYBR^{RTM} Green Supermix
458 (Bio-Rad, 1725121), the same amount of template cDNA, and the specific primers described
459 below. The mRNA levels were normalized to β -actin.

460

461 The first pair of specific primers were designed on human CD3 γ chain to detect the presence of
462 human T cells. The second pair of specific primers were designed based on the lox-stop
463 PSMACAR reporter sequence to reflect CAR expression after FUS-induced Cre recombination
464 (fig. S6, a and b). The forward primer anneals from -60 bp of the mouse PGK promoter,
465 downstream of the transcription starting site (TSS), and the reverse primer anneals from +20 bp
466 of the PSMACAR gene (38). With the presence of FUS-induced Cre recombinase, the sequence
467 from the second half of LoxH to the first half of LoxP will be excised, resulting in a 200 bp
468 qPCR product. Without Cre-mediated recombination, this pair of primers will theoretically
469 generate a 984-bp fragment. We adopted a two-step qPCR protocol with combined
470 annealing/extension at 60°C for only 15 sec to ensure the specific amplification of the 200-bp
471 fragment, but not the 984 bp fragment, as confirmed by gel electrophoresis and Sanger
472 sequencing of the qPCR product (fig. S6, c and d; sequence alignment performed in Serial
473 Cloner). Therefore, the second pair of specific primers can detect the successfully recombined
474 CAR mRNA amount.

475

476 **Statistics.**

477 One-way ANOVA followed by Tukey's multiple comparisons test is used for Figs. 1, f and g, and
478 fig. S3, b and d to e. Student's t-test is used for Fig. 2d. Two-way ANOVA followed by Sidak's
479 multiple comparisons test is used for Figs. 2, b, g to I, 3g, 4, b and d, fig. S4b, S5, b to d.

480 **Acknowledgments**

481 We thank Dr. Franck Couillaud (University of Bordeaux, France) for providing the Hsp template
482 and Dr. Michel Sadelain (Sloan Kettering Institute, USA) for the PSMA scFv and PSMA
483 constructs and the Nalm-6 cells. We also thank Dr. Erik Dumont and Ms. Stéphanie Hoarau-
484 Recco (Image Guided Therapy, France) for their most valuable help on the FUS system.

485 **Funding:** This work was supported in part by grants from NIH HL121365, GM125379,
486 GM126016, CA204704 and CA209629 (Y. Wang). **Author contributions:** Y.Wu, S.C. and
487 Y.Wang designed research; Y.Wu, Y.L, Z.H., X.W., Z.J., J.L., P.L., L.Z., M.A., Y.P., R.B., A.J.
488 performed research; Y.Wu and Y.L. analyzed data; Y.Wu, T. L., S.C. and Y.Wang wrote the
489 manuscript. All authors reviewed the manuscript and have given approval to the final version of
490 the manuscript. **Competing interests:** Y.Wang is a scientific co-founder of Cell E&G Inc and
491 Acoustic Cell Therapy Inc. These financial interests do not affect the design, conduct or
492 reporting of this research. **Data and materials availability:** All data is available in the main text
493 or the supplementary materials.

494 **Figure legends**

495 **Fig. 1. Heat-inducible gene activation. (a)** Design of the FUS-controllable CAR T therapy
496 technology. T cells engineered with the heat-inducible CAR and localized at the tumor region are
497 activated by MRI-guided FUS for recognizing and eradicating target tumor cells. **(b)** Schematics
498 of the dual-promoter eGFP reporter. **(c-d)** **(c)** Fluorescent images of inducible eGFP and
499 constitutive mCherry, and **(d)** the percentage of eGFP⁺ cells and their mean fluorescence
500 intensity after a 15-min HS at 43°C in HEK 293T cells containing the dual-promoter reporter. **(e-**
501 **g)** Gene induction in primary human T cells with the dual-promoter eGFP reporter. **(e)**

502 Representative flow cytometry profiles of eGFP expression. **(f)** The percentage of eGFP⁺ cells
503 and **(g)** their mean fluorescence intensity. In (e-g), CT: without HS; HS: with a continuous 15-
504 min HS. MCherry⁺ cells were gated for eGFP analysis. N = 3 repeats; error bar: SEM. ****: p <
505 0.0001.

506

507 **Fig. 2. Heat-inducible CD19CAR expression and functional outcomes in Jurkat and**
508 **primary T cells. (a)** Schematics of the transgenes: the inducible Cre and the lox-stop CAR
509 reporter. **(b)** Inducible CAR expression in Jurkat cells hosting the lox-stop CAR reporter alone
510 (lox), or both transgenes in (a) with (HS) or without HS (CT). **(c)** Schematics of assays accessing
511 the functionality of the heat-induced CAR T cells, including CD69 expression, cytotoxicity, and
512 cytokine release. **(d)** The percentage of CD69⁺ cells in Jurkat with both transgenes in (a). **(e)**
513 Representative flow cytometry data showing the histogram of CD69 expression in (d). **(f)**
514 Representative histograms showing the percentage of CAR⁺ cells in primary T cells with both
515 transgenes in (a). **(g)** The cytotoxicity of the T cells in (f) against Nalm-6 tumor cells at various
516 E:T ratios. **(h-i)** Quantification of **(h)** IFN- γ and **(i)** IL-2 cytokine release associated with (g).
517 Arrow: cytokine level not detectable. In (b) and (d) to (f), CT: without HS; HS: with a
518 continuous 15-min HS. N = 3; error bar: SEM. ***: p < 0.001; ****: p < 0.0001; ns: no
519 significant difference.

520

521 **Fig. 3. MRI-guided FUS-inducible gene activation in phantom and *in vivo*. (a)** Schematics of
522 the MRI-guided FUS system. **(b)** The dual-luciferase reporter containing the inducible Hsp-
523 driven Fluc and constitutive PGK-driven Rluc fused with mCherry. **(c)** The experimental setup
524 of FUS stimulation on cells in a tofu phantom. **(d)** Gene induction level in Nalm-6 cells

525 containing the dual-luciferase reporter with three pulses of 5-min heating by MRI-guided FUS in
526 tofu phantom (FUS) or by thermal cycler (HS). CT: without heating. Gene induction level is
527 quantified by the Fluc/Rluc ratio and normalized to CT. N = 3. **(e)** Left: color-coded temperature
528 map superimposed on MRI images at different time points during a 5-min FUS stimulation at
529 43°C on the hindlimb of an anesthetized mouse. Right: close-up of the red rectangle region on
530 the left. The dotted white square outlines the region of interest (ROI) for temperature regulation.
531 **(f)** The average temperature of the ROI during FUS stimulation in (e). The yellow shadow
532 represents the predefined target temperature (43°C) and duration (300 sec) of FUS stimulation.
533 **(g)** Gene induction *in vivo* by MRI-guided FUS. Nalm-6 cells containing the dual-luciferase
534 reporter were injected subcutaneously into NSG mice followed by FUS stimulation. FUS+ or
535 FUS-: with or without two pulses of 5-min FUS stimulation at 43°C. Gene induction was
536 quantified by the *in vivo* Fluc/Rluc ratio and normalized to the “FUS-, before” group, as
537 indicated by the dotted line. N = 4 mice. **(h)** Representative bioluminescence images of Fluc
538 expression before and after FUS stimulation in (g). Error bar: SEM. *: p < 0.05; **: p < 0.01; ns:
539 no significant difference.

540

541 **Fig. 4. FUS-controllable tumor suppression by the engineered CAR T cells *in vivo*. (a)**

542 Timeline of the *in vivo* experiment using NSG mouse bearing matched bilateral tumors as the
543 animal model. The tumor on the left flank received FUS stimulation (FUS+) and the one on the
544 right received no FUS (FUS-) following injection of engineered CAR T cells. **(b-e)** The
545 quantified tumor growth and representative bioluminescence images of **(b-c)** Nalm-6 tumors or
546 **(d-e)** PC3 tumors with (FUS+) or without (FUS-) FUS stimulation. Tumor size was quantified
547 using the integrated Fluc luminescence intensity of the tumor region and normalized to that of

548 the same tumor on the first measurement. N = 4 mice. Error bar: SEM. *: $p < 0.05$; **: $p < 0.01$;
549 ****: $p < 0.0001$.

550

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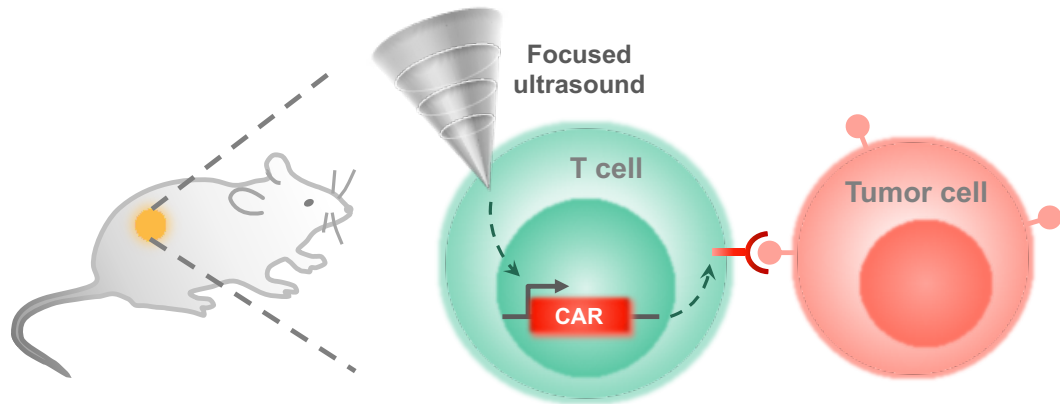
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643

Figure 1

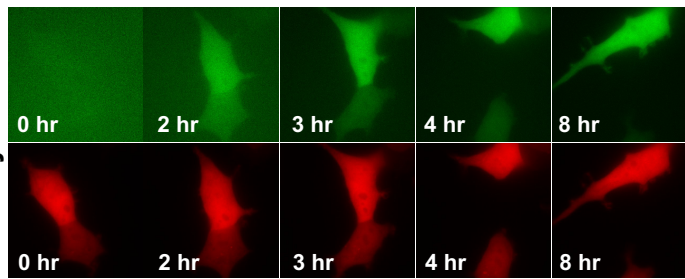
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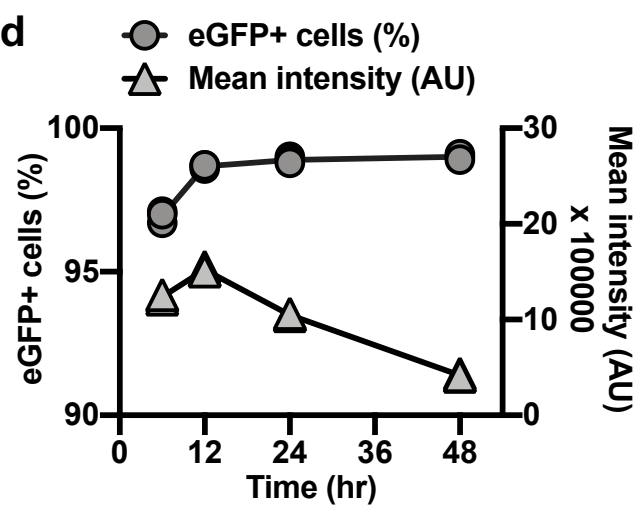
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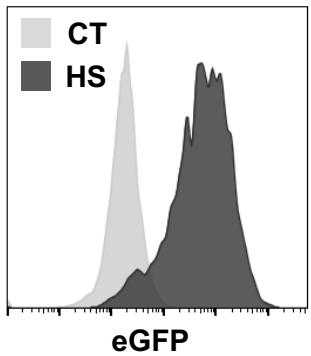
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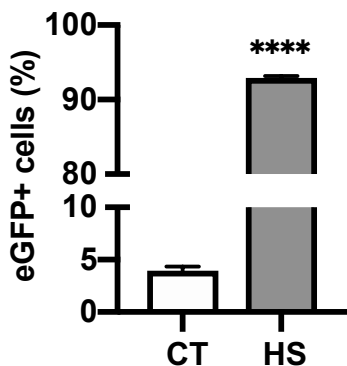
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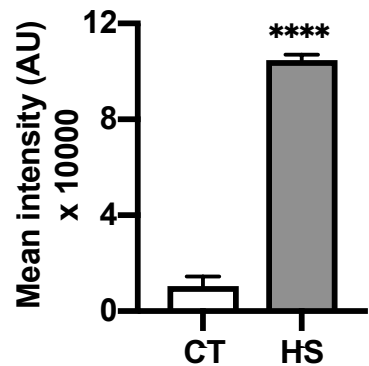


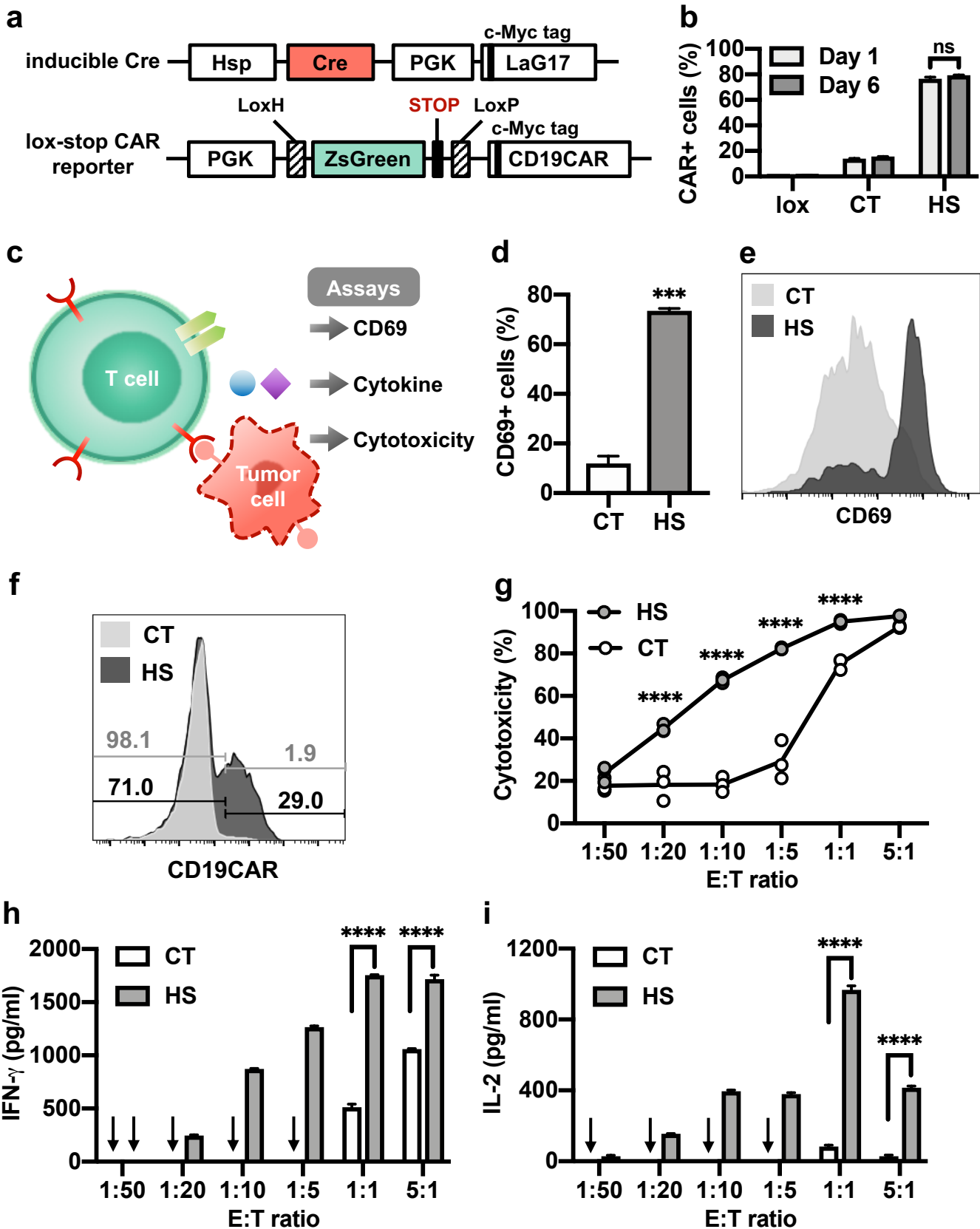
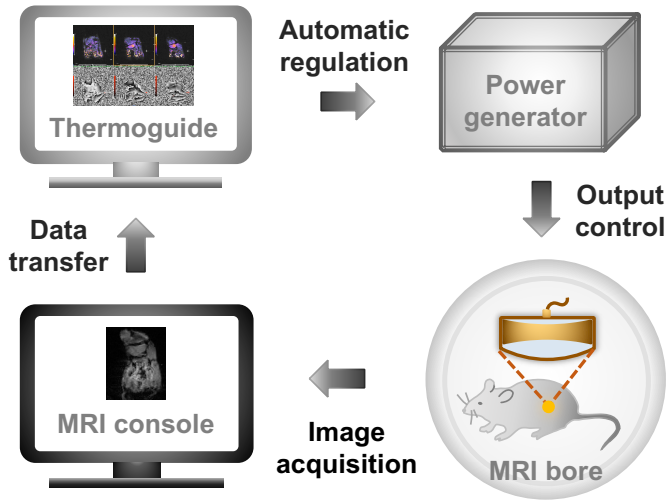
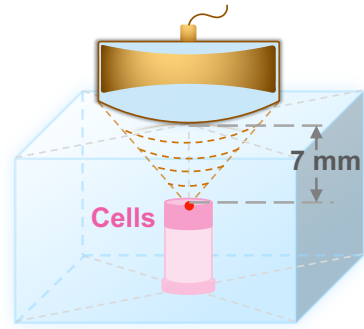
Figure 2

Figure 3

a



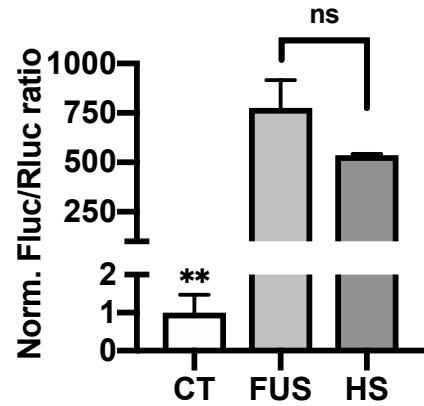
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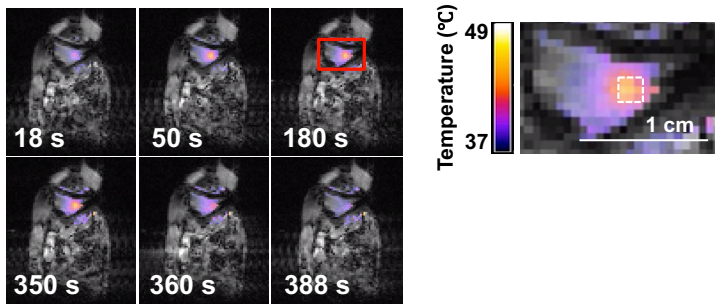
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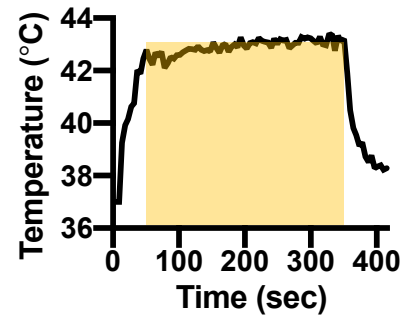
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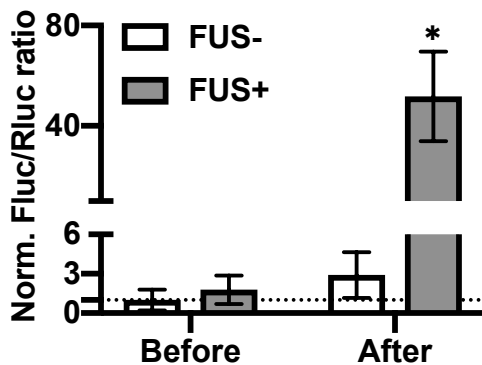
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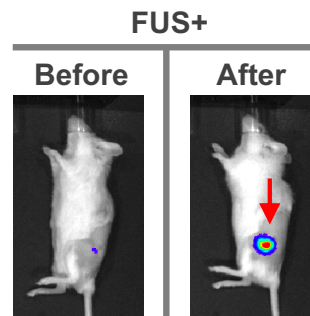


Figure 4