Supplementary Materials for

Acoustogenetic Control of CAR T Cells via Focused Ultrasound

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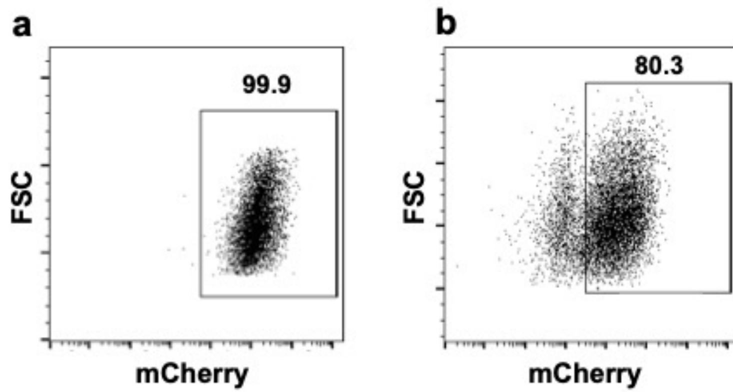

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9 **Fig. S1. Transduction efficiencies of the dual-promoter eGFP reporter.** Representative flow

10 cytometry data showing the lentiviral transduction efficiencies of the dual-promoter eGFP

11 reporter (Hsp-eGFP-PGK-mCherry) in **(a)** HEK293T cells (99.9%) and **(b)** primary human T

12 cells (80.3%). The mCherry+ gates are based on the profiles of the corresponding non-

13 engineered cells.

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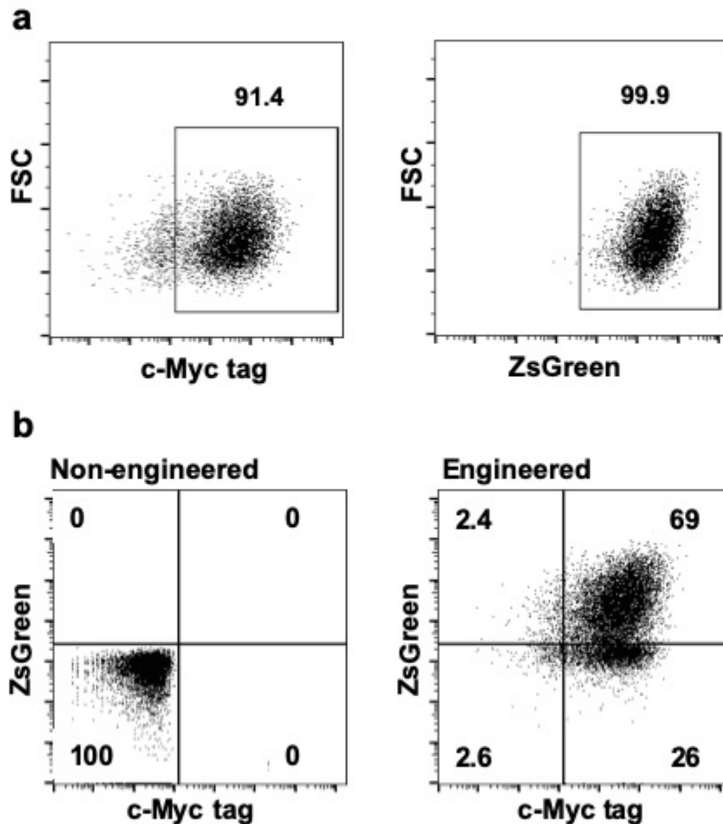

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16 **Fig. S2. Transduction efficiencies of the heat-inducible Cre-lox system in Jurkat and**

17 **primary human T cells. (a)** Jurkat cells were lentivirally co-transduced with inducible Cre

18 (Hsp-Cre-PGK-cMyc-LaG17) and lox-stop CAR reporter (PGK-LoxH-ZsGreen-stop-LoxP-

19 CAR). Shown are the transduction efficiencies of cells without HS. **(b)** Primary human T cells

20 were co-transduced with inducible Cre (Hsp-Cre-PGK-cMyc-LaG17) and lox-stop CAR reporter

21 (PGK-LoxH-ZsGreen-stop-LoxP-CAR) lentiviruses. The cells were sorted through MACS for c-

22 Myc+ cells. The representative transduction efficiency after MACS is 69% for the c-Myc and

23 ZsGreen double positive, 95% for the c-Myc+ and 71.4% for the ZsGreen+ cells. Gating is based

24 on the corresponding non-engineered cells with the same c-Myc antibody staining.

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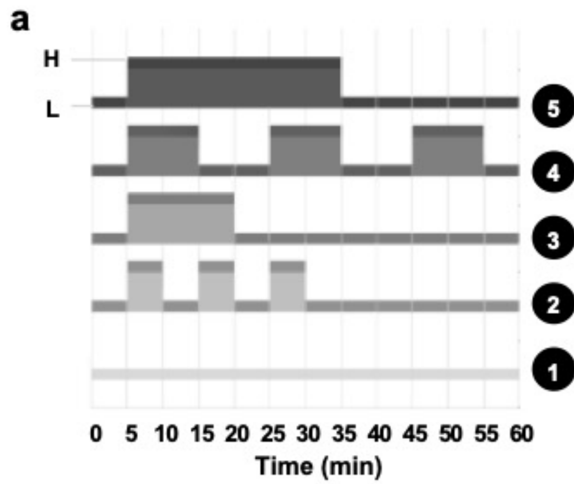

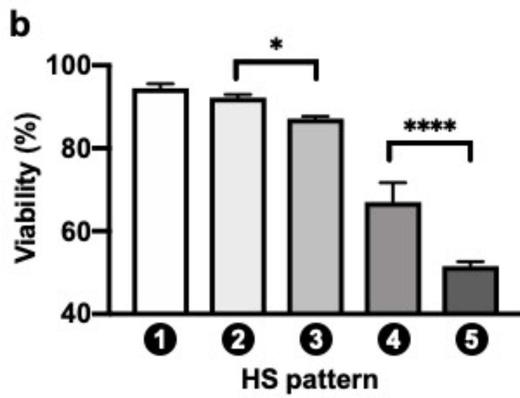

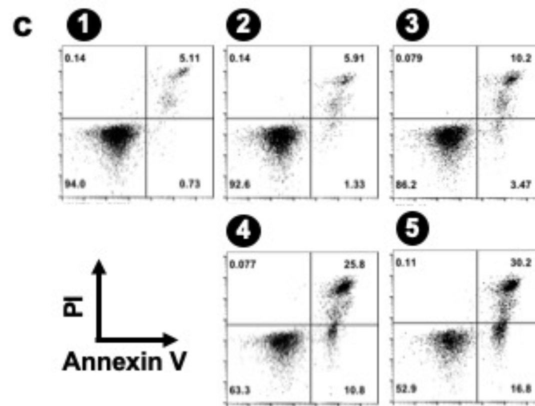

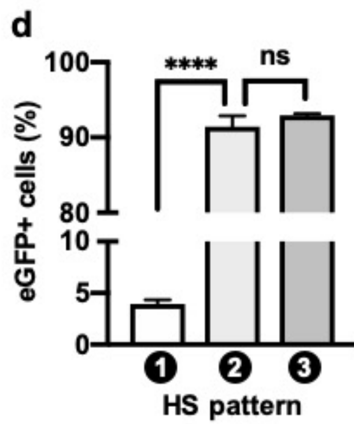

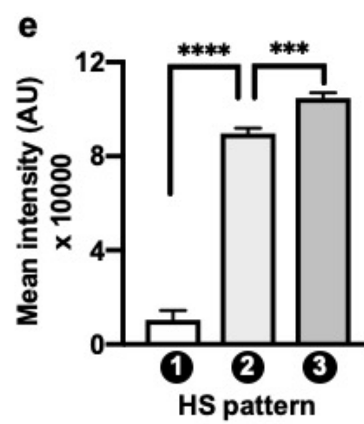

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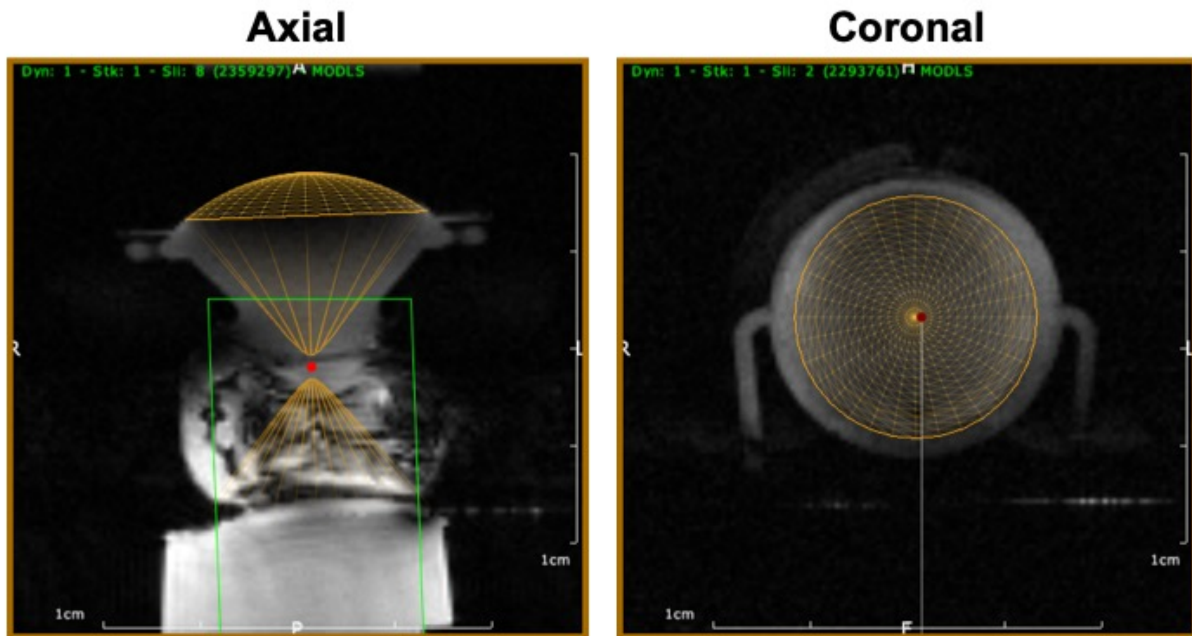
Fig. S3. Thermal tolerance of primary human T cells. (a) Different patterns of HS. H: heating at 43°C; L: resting at 37°C. **(b)** The viability of non-transduced primary human T cells 24 hr after different patterns of HS in (a) assayed by Annexin V and PI staining. **(c)** Representative flow cytometry data of (b). **(d-e) (d)** The percentage of eGFP+ cells and **(e)** their mean fluorescence intensity in primary human T cells engineered with the dual-promoter eGFP reporter in Fig. 1b under different HS patterns in (a). N = 3 repeats; error bar: SEM. *: $p < 0.05$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: no significant difference.



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36 **Fig. S4. MRI images of the *in vivo* FUS experimental setup.** An anesthetized mouse is laid on
37 its side on an MR bed containing an agarose gel pad. The ultrasound transducer is positioned
38 above the targeted region on the mouse's hindlimb. Three-dimensional representations of the
39 transducer generated by the ThermoGuide software are superimposed on the MRI images. Left:
40 an axial view of the experimental setup. The bright red dot indicates the theoretical ultrasound
41 focal point. Right: a coronal view showing the transducer.
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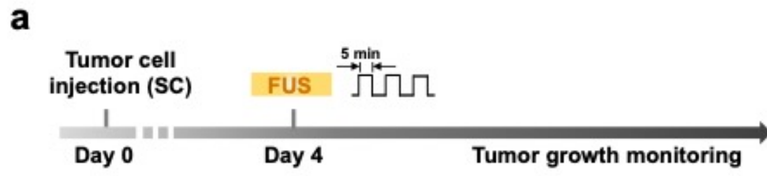
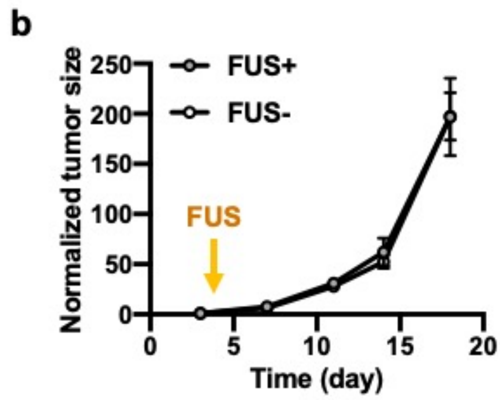
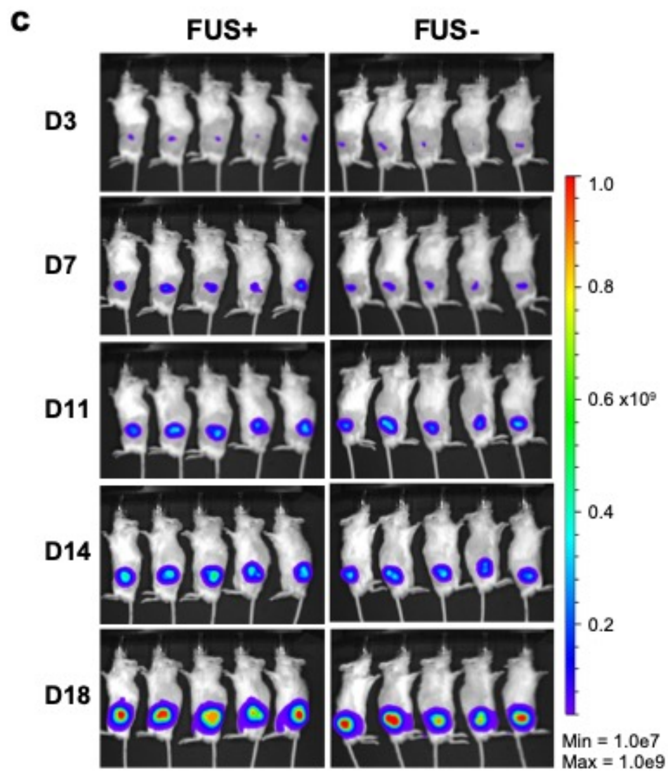
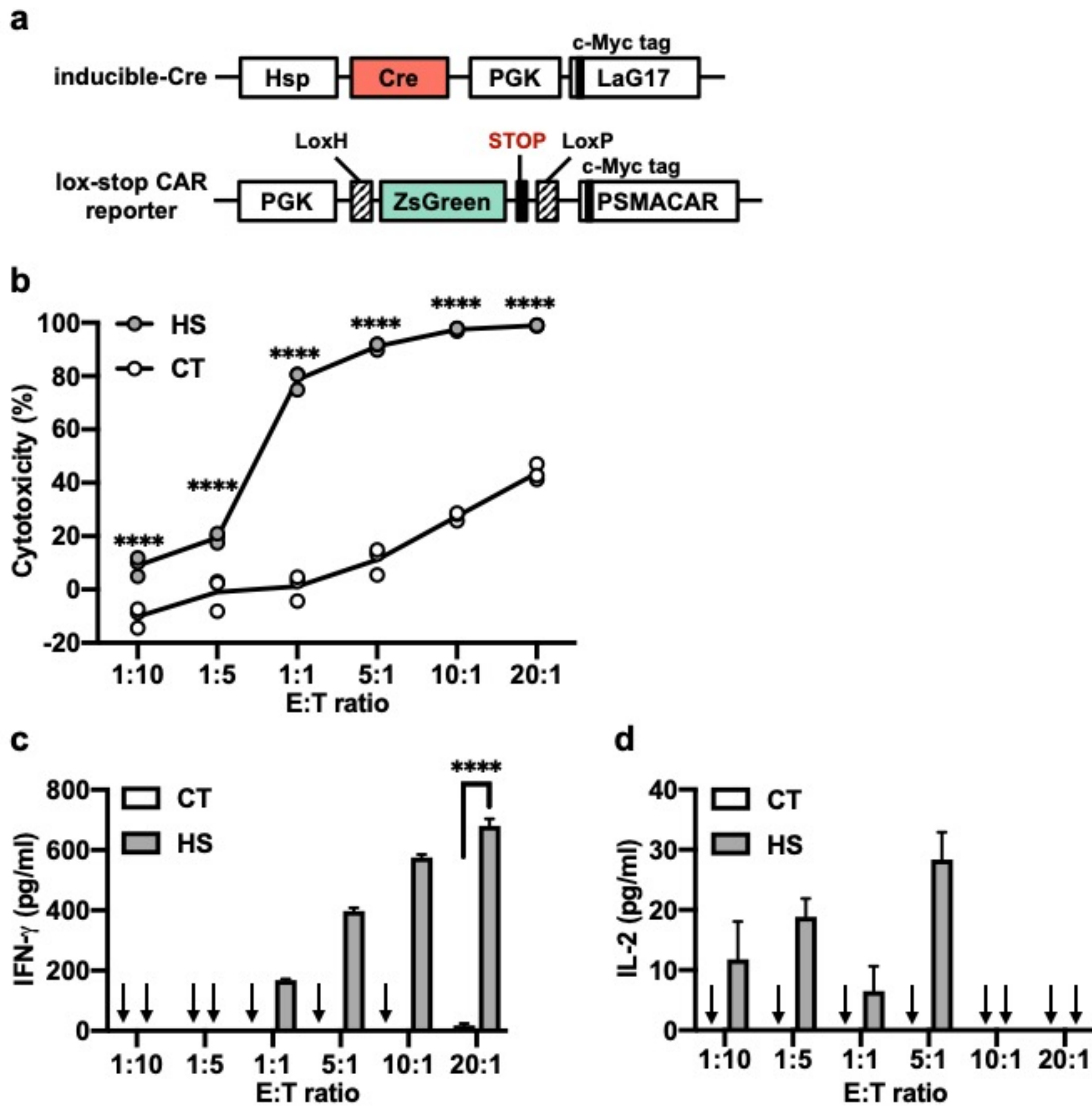

a
Tumor cell
injection (SC)
FUS
5 min
Day 0
Day 4
Tumor growth monitoring


b
Normalized tumor size
250
200
150
100
50
0
FUS+
FUS-
FUS
0
5
10
15
20
Time (day)


c
FUS+
FUS-
D3
D7
D11
D14
D18
1.0
0.8
0.6 x10⁹
0.4
0.2
Min = 1.0e7
Max = 1.0e9

46 **Fig. S5. FUS has no detectable effect on tumor growth. (a)** Timeline of *in vivo* experiment.
47 Fluc+ Nalm-6 cells are subcutaneously injected into NSG mice to generate matched bilateral
48 tumors. Four days later, three pulses of 5-min FUS stimulation targeted at 43°C was applied on
49 the tumor on one side (left), while the tumor on the other side (right) received no FUS
50 stimulation. Tumor growth after FUS stimulation was monitored by bioluminescence imaging.
51 **(b-c)** The **(b)** quantified tumor growth and **(c)** representative bioluminescence images of the
52 tumors with (FUS+) or without (FUS-) FUS stimulation. Tumor size is quantified using the
53 integrated luminescence intensity and normalized to that of the same tumor on the first
54 measurement. N = 5 mice. Error bar: SEM. No significant difference was detected.



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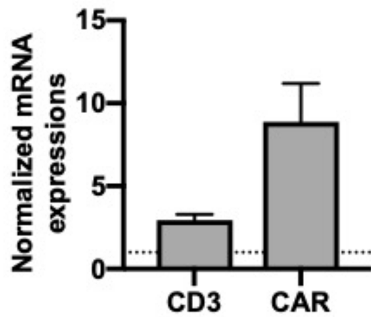
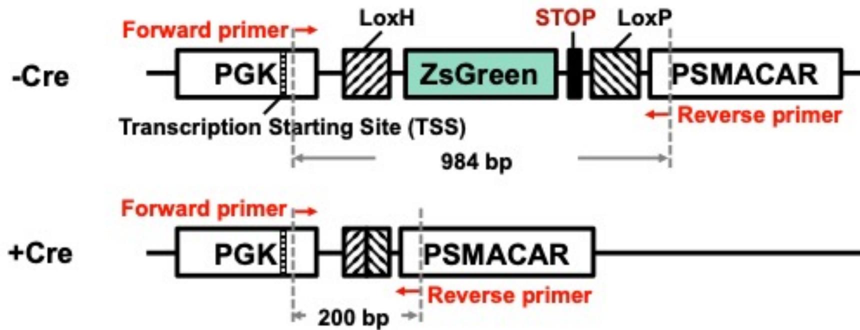
56 **Fig. S6. Functionality of the heat-inducible PSMACAR T cells *in vitro*. (a)** Schematics of the
57 transgenes used in this figure, including the heat-inducible Cre and the lox-stop PSMACAR
58 reporter. **(b)** Cytotoxicity of the T cells engineered with the transgenes in (a) against Fluc+
59 PSMA+ PC3 tumor cells at various E:T ratios. **(c-d)** Quantification of **(c)** IFN-γ and **(d)** IL-2
60 cytokine release associated with (b). Arrow: cytokine level not detectable. CT: without HS. HS:
61 with a continuous 15-min HS. N = 3; error bar: SEM. ****: $p < 0.0001$.

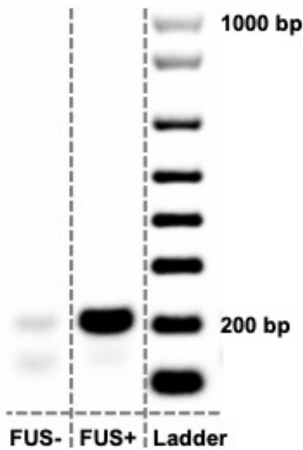
a



b



c



d

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Seq_1  1     -acgcttcaaaagcgcacgtctgccgcgctgttctcctcttcctcatctccgggcctttc  59
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     TACGCTTCAAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTTCCTCATCTCCGGGCCTTTC  60

Seq_1  60    gacctgcagcccaagcttaccGAGACCCAAGCTGGCTAGAATTACCTCATATAGCATACA  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    GACCTGCAGCCCAAGCTTACCGAGACCCAAGCTGGCTAGAATTACCTCATATAGCATACA  120

Seq_1  120   TTATACGAAGTTATGTTTcggAATTCGCGCGCAggaattgatccttcgaactagtGGATC  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   TTATACGAAGTTATGTTTCGGAATTCGCGCGCAGGAATTGATCCTTCGAACTAGTGGATC  180

Seq_1  180   CATGGCTCTCCCAGTGACTGC  200
             ||||||||||||||||||:::
Seq_2  181   CATGGCTCTCCCAGTGACNNN  201
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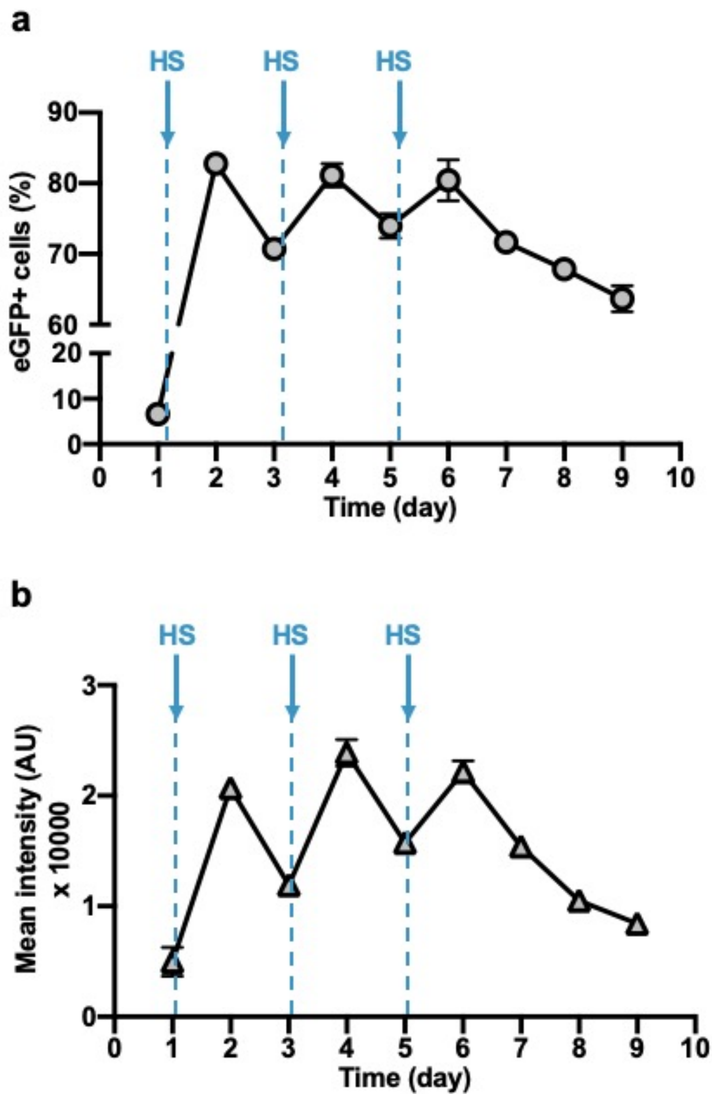
Seq_1: theoretical sequence after Cre-lox recombination
Seq_2: combined Sanger sequencing results
Pink: PGK promoter (partial)
Green: first half of LoxH
Blue: second half of LoxP
Red: PSMACAR (partial)

Fig. S7. Quantification of relative mRNA expression levels in tumors. (a) The expression levels of CD3 and CAR mRNA in tumors with FUS stimulation (Fig. 4E, FUS+), with their values normalized to the corresponding mRNA levels in tumors without FUS stimulation (Fig. 4e, FUS-). Dotted line: the corresponding mRNA levels in tumors without FUS stimulation are set to 1. **(b)** Schematics of the theoretical templates without (-Cre) or with (+Cre) Cre-lox recombination, and the expected products amplified by the designed primers. **(c)** Gel electrophoresis of the qPCR products of the tumors with FUS stimulation (FUS+) or without (FUS-). **(d)** Alignment of the combined Sanger sequencing results and the theoretical template with Cre-lox recombination.



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73 **Fig. S8. Gene activation with periodical HS. (a-b)** The dynamics of eGFP expression in terms

74 of **(a)** percentage of eGFP+ cells and **(b)** mean eGFP intensity in primary human T cells

75 transduced with the dual-promoter eGFP reporter and subjected to a 10-min HS every 48 hr on

76 Day 1, 3, and 5. N = 3; error bar: SEM.

Plasmid	Purpose	Used in	Source
pHR-Hsp-eGFP-PGK-mCherry	inducible GFP reporter	Figs. 1C-G, S1, S7	This work
pHR-PGK-LoxH-ZsGreen-STOP-LoxP-cMyc-CD19CAR	Lox recombination CD19CAR reporter	Figs. 2B, 2D-I, 4B-C, S2	This work
pHR-Hsp-Cre-PGK-cMyc-LaG17	inducible Cre	Figs. 2B, 2D-I, 4B-C, S2, S5, S6	This work
pHR-Hsp-Fluc-PGK-Rluc-P2A-mCherry	inducible dual luciferase reporter	Fig. 3D, 3G-H	This work
pHR-PGK-LoxH-ZsGreen-STOP-LoxP-cMyc-PSMACAR	Lox recombination PSMACAR reporter	Figs. 4D-E, S5, S6	This work
pHR-PGK-PSMA	PSMA antigen	Figs. 4D-E, S5	This work
pHIV-EF1a-Fluc-IRES-ZsGreen	firefly luciferase	Figs. 2G-I, 4B-E, S4, S5	Addgene #39196

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78

79 **Table S1. Plasmids used in this work.** Detailed information on the plasmids used in this work,

80 including the features of the constructs, their usage, and sources.

81

Pattern	Step	Temperature	Time
1		37°C	∞
2	Initial equilibrium	37°C	5 min
	3 Cycles	43°C	5 min
		37°C	5 min
3	Initial equilibrium	37°C	5 min
	1 Cycle	43°C	15 min
4	Initial equilibrium	37°C	5 min
	3 Cycles	43°C	10 min
		37°C	10 min
5	Initial equilibrium	37°C	5 min
	1 Cycle	43°C	30 min

82

83 **Table S2. Thermal cycler HS patterns used in this work.** The patterns of different HS

84 treatments performed in this work using a thermal cycler.

Cell type	Gene delivery method	Construct	Sorting	Efficiency	Application
HEK 293T	Lentivirus	Hsp-eGFP-PGK-mCherry	Not sorted	>99%	Gene activation in vitro by HS
Jurkat	Lentivirus	Hsp-eGFP-PGK-mCherry	Not sorted	>99%	Gene activation in vitro by HS
Jurkat	Lentivirus	Hsp-Cre-PGK-cMyc-LaG17	Not sorted	91.40%	Cre activation in vitro by HS, CD69 assay
	(co-transduction)	PGK-LoxH-ZsGreen-STOP-LoxP-CD19CAR	Not sorted	>99%	
Nalm-6	Lentivirus	Hsp-Fluc-PGK-Rluc-mCherry	FACS	>99%	Gene activation in vivo by FUS
Nalm-6	Gift from Michel Sadelain Lab	constitutive Fluc+	FACS	>99%	In vitro and in vivo cytotoxicity studies
PC3	Lentivirus	pHIV-EF1a-Fluc-IRES-ZsGreen (Addgene)	FACS	>99%	In vitro and in vivo cytotoxicity studies
	(co-transduction)	PGK-PSMA	FACS	>99%	

Table S3. Engineered cells used in this work. Detailed information on the engineered cells (excluding primary human T cells) used in this work, including the cell type, the transgene delivered and the associated delivery method and efficiency, sorting information and applications.

Movie S1. The dynamics of HS-activated eGFP expression. Real-time fluorescence imaging of two HEK 293T cells transduced with the dual-promoter eGFP reporter (Hsp-eGFP-PGK-mCherry). A 15-min HS at 43°C was applied from 30 to 45 min after the initiation of imaging using a heating stage integrated with the microscope. Imaging lasted for 12 hours. GFP: GFP channel showing the dynamic activation of eGFP; mCherry: mCherry channel showing the constitutive mCherry expression.

Movie S2. MRI-guided FUS stimulation on a targeted region on the hindlimb of an anesthetized mouse. MRI images are superimposed with color-coded temperature map of the region of interest. FUS stimulation was aimed at the hindlimb of an anesthetized mouse. The FUS stimulation is composed of a temperature rising phase (Dyn 1 - 10), a steady-state phase (Dyn 11 - 75), and a cool-down phase (Dyn 76-90). Dyn: dynamic; one dynamic of MRI scanning takes 4.6 sec.