

## Supplementary Materials for

### **Focused ultrasound ablation of melanoma with boiling histotripsy yields abscopal tumor control and antigen-dependent dendritic cell activation**

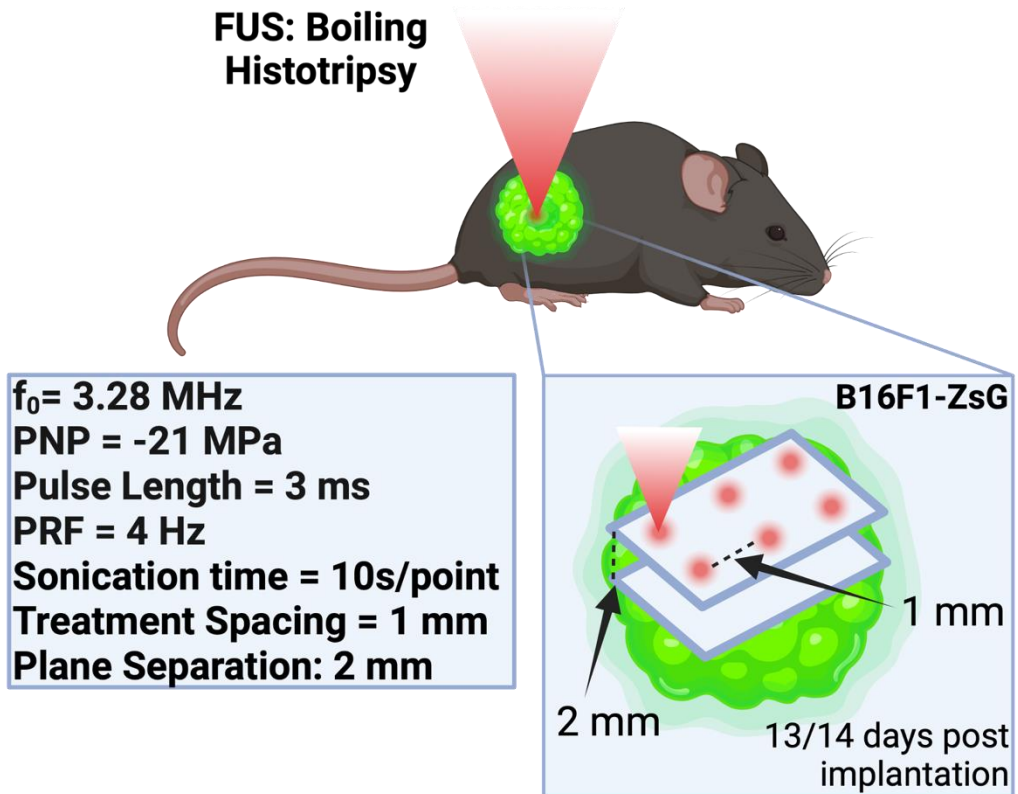
Eric A Thim *et al.*

\*Corresponding author: Richard J. Price, rprice@virginia.edu & Timothy N. J. Bullock, tb5v@virginia.edu

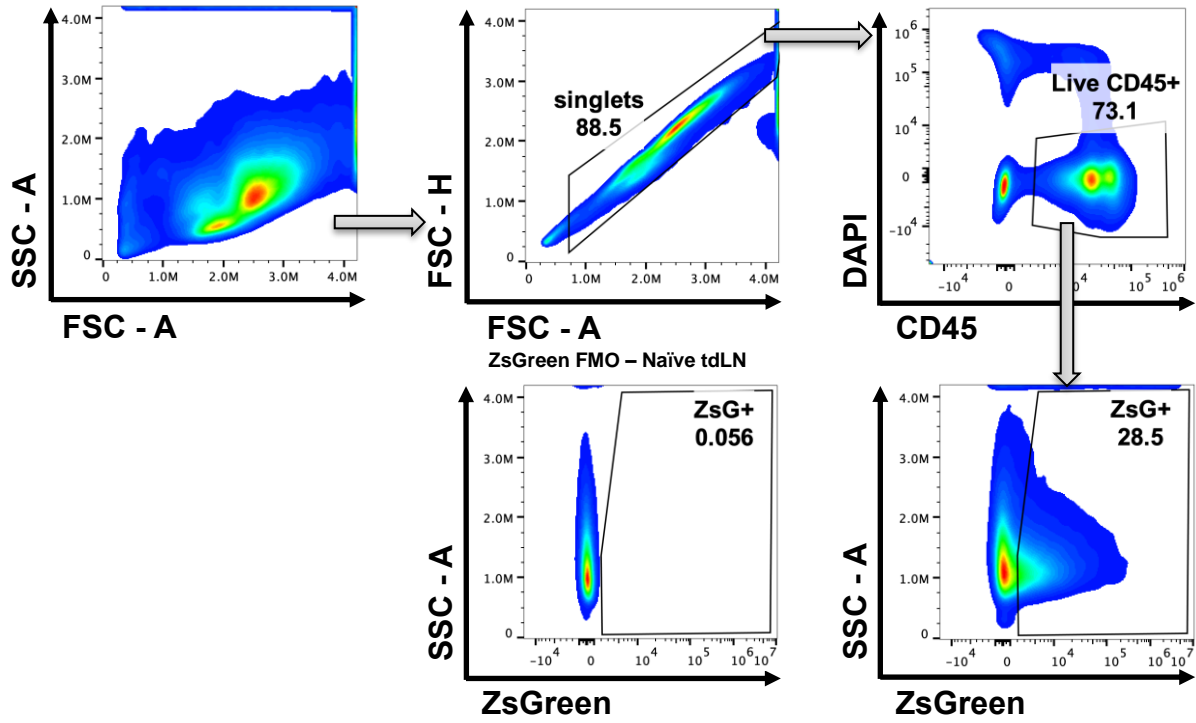
#### **This PDF file includes:**

Figs. S1 to S8

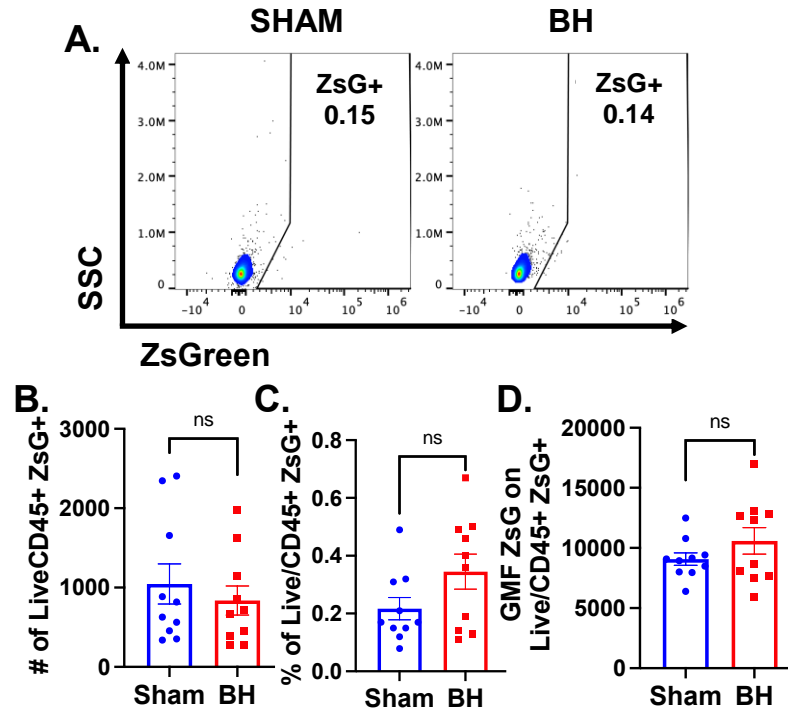
Tables S1 to S2



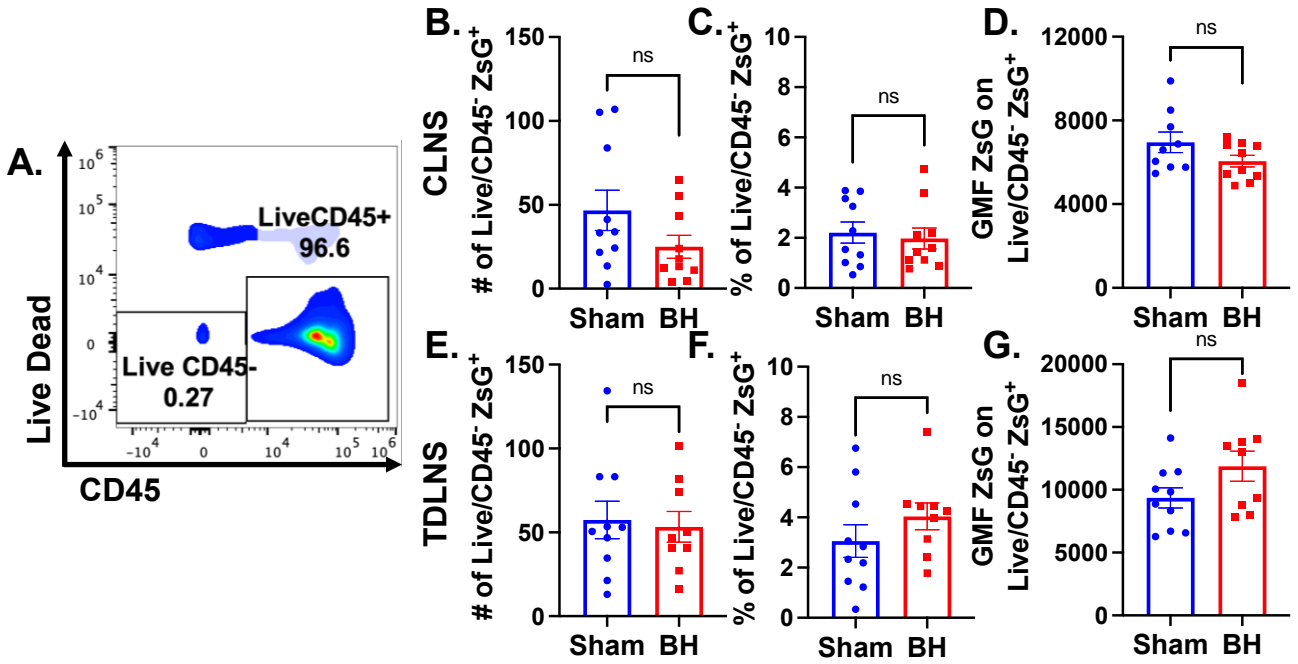
**Figure S1. Schematic for boiling histotripsy treatment.** Boiling histotripsy was applied with the following parameters: Operational frequency ( $f_0 = 3.28$  MHz); peak-negative pressure (PNP = -21 MPa); pulse length (3 ms); pulse repetition frequency (PRF = 4 Hz); sonication time = 10 s/point; treatment spacing = 1 mm; plane separation = 2 mm. With this treatment scheme, ~20% of the tumor volume is within the -6dB focus during BH application.



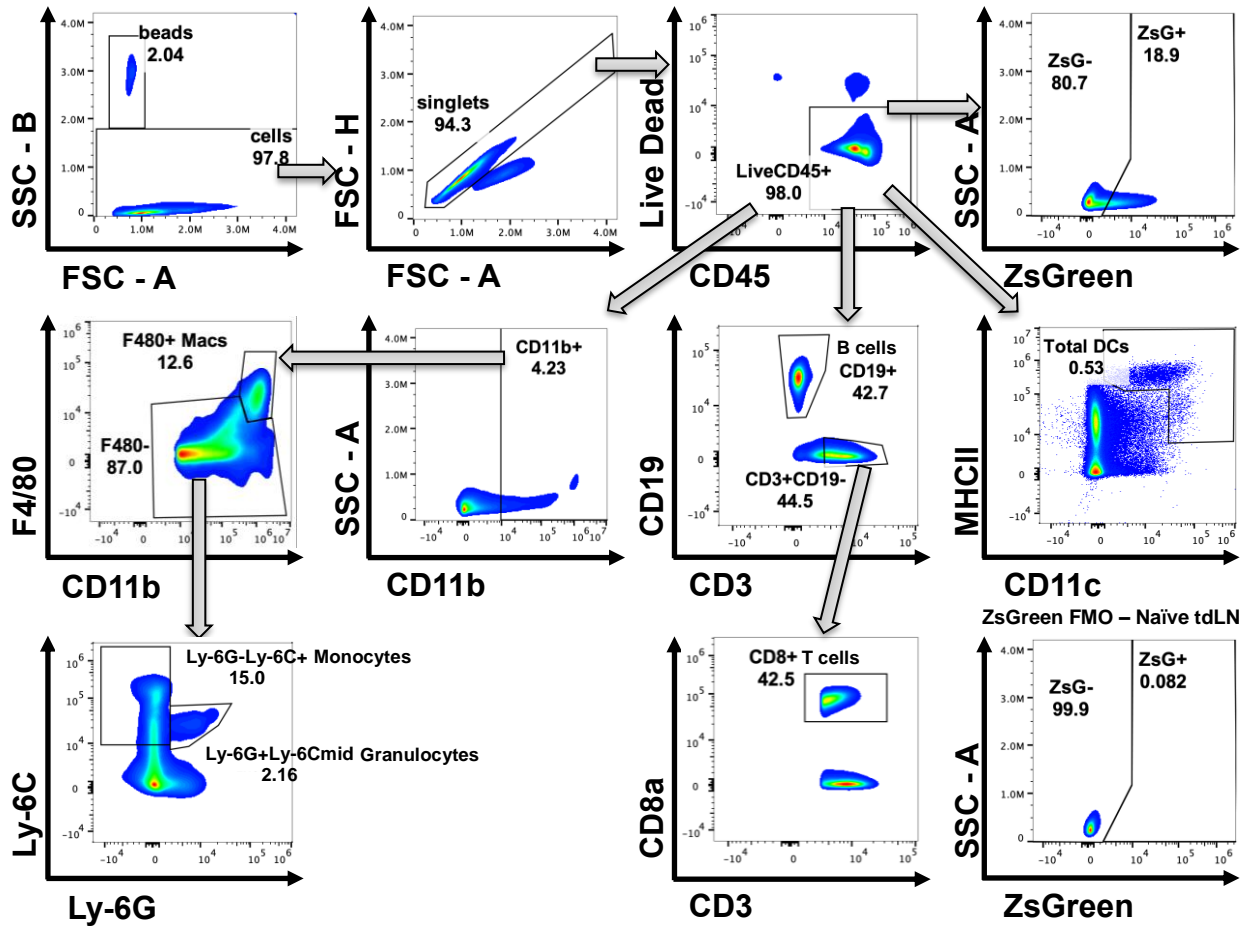
**Figure S2. Gating strategy for flow cytometry analysis of Figure 1.** ZsG-naïve mice were used as the gating control for ZsG positivity in LiveCD45<sup>+</sup> immune cells.



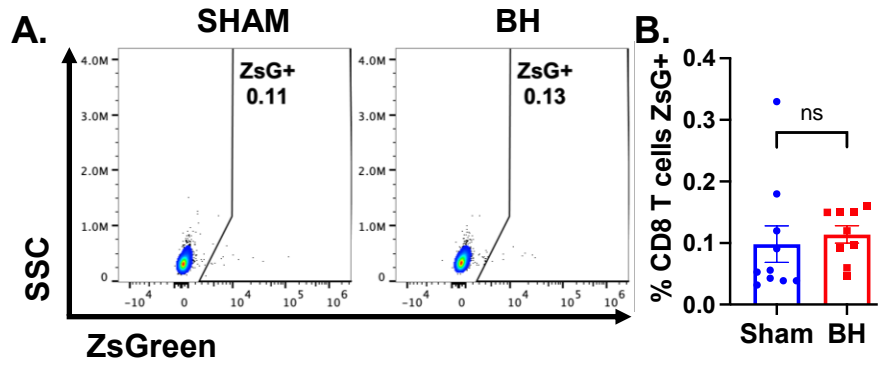
**Figure S3. BH does not promote ZsG tumor antigen acquisition in non-tumor draining lymph nodes 24 hrs post treatment. A.** Density scatter plots of side scatter vs ZsG indicating percent of Live/CD45<sup>+</sup> cells that are ZsG<sup>+</sup>. **B-D.** Summary of flow cytometry analysis. **B)** Number of LiveCD45<sup>+</sup>ZsG<sup>+</sup> cells. **C)** Percent of LiveCD45<sup>+</sup> cells that are ZsG<sup>+</sup>. **D)** Geometric mean fluorescent intensity (GMF) of ZsG on LiveCD45<sup>+</sup>ZsG<sup>+</sup> cells. n=10. Unpaired Welch's t-test: not significant. Means ± SEM.



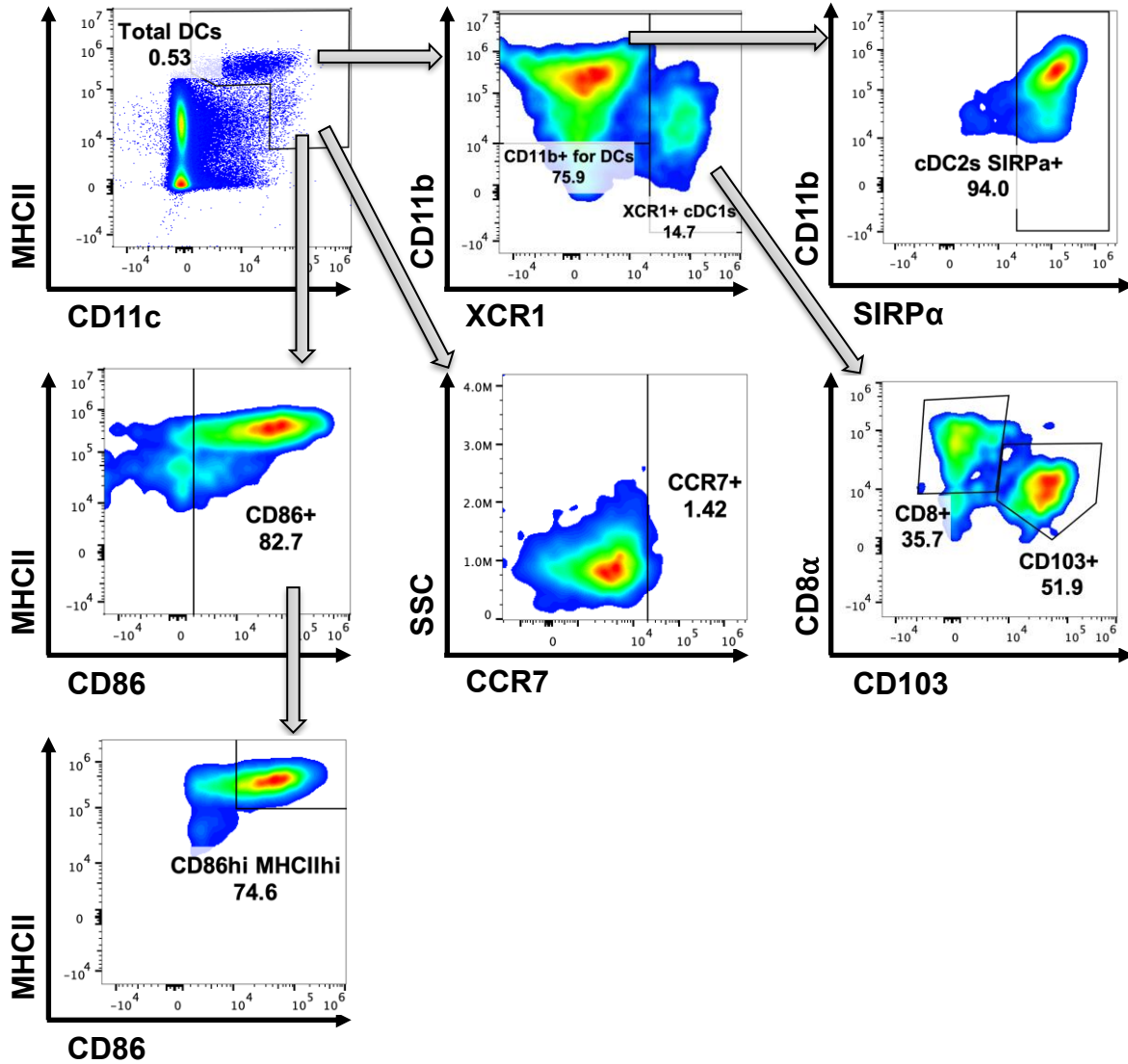
**Figure S4. BH does not promote metastatic spread of melanoma 24 hrs after treatment. A.** Density scatter plots of Live Dead vs CD45 indicating percent of singlet cells that are Live/CD45<sup>+</sup> and Live/CD45<sup>-</sup>. The subsequent summary plots are in reference to ZsG<sup>+</sup> portion of Live/CD45<sup>-</sup> cells. **B, E)** Number of Live/CD45<sup>-</sup>ZsG<sup>+</sup> cells in the contralateral and tumor draining lymph nodes. **C, F)** Percent of Live/CD45<sup>-</sup> cells that are ZsG<sup>+</sup> in CLNs and TDLN. **D, G)** The geometric mean fluorescent intensity (GMF) of ZsG on Live/CD45<sup>-</sup>ZsG<sup>+</sup> cells in CLNs and TDLN. n=10. Unpaired Welch's t-test: not significant. Means ± SEM.



**Figure S5. Gating strategy for flow cytometry analysis of Figure 2 and Figure S3.** Gating strategy presented for the following immune cell subsets after cells and singlets: Macrophages (LiveCD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>); Monocytes (LiveCD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>-</sup>Ly-6G<sup>+</sup>Ly-6C<sup>+</sup>); Granulocytes (LiveCD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>-</sup>Ly-6G<sup>+</sup>Ly-6C<sup>mid</sup>); B cells (LiveCD45<sup>+</sup>CD19<sup>+</sup>); CD8 T cells (LiveCD45<sup>+</sup>CD3<sup>+</sup>CD19<sup>-</sup>CD8<sup>+</sup>); Total DCs (LiveCD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>). Gating strategy for ZsGreen<sup>+</sup> (ZsG<sup>+</sup>) immune cells and the corresponding control from ZsG-naïve mice are outlined.

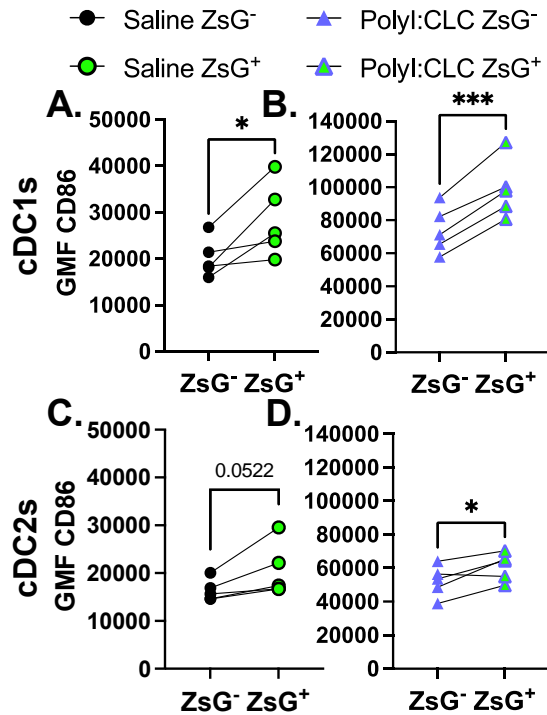


**Figure S6. Negative control for ZsG fluorescence showing antigen acquisition by non-APC and non-phagocytic cells ( $CD8^+$  T cells).** **A.** Density scatter plots of side scatter vs ZsG indicating percent of Live $CD45^+CD19^-CD3^+CD8^+$  T cells that are ZsG $^+$ . **B.** Summary of flow cytometry analysis in **A.**  $n=10$ . Unpaired Welch's t-test: not significant. Means  $\pm$  SEM.



**Figure S7. Gating strategy for flow cytometry analysis of cDCs and activated status.** Gating strategy for the following DC (LiveCD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>) subsets: cDC1s (XCR1<sup>+</sup>), migratory cDC1s (XCR1<sup>+</sup>CD103<sup>+</sup>), tissue-resident cDC1s (XCR1<sup>+</sup>CD8α<sup>+</sup>CD103<sup>-</sup>) and cDC2s (XCR1<sup>-</sup>CD11b<sup>+</sup>SIRPα<sup>+</sup>). Activation of DC subsets was defined as a subset of CD86<sup>+</sup>MHCII<sup>+</sup>: CD86<sup>hi</sup>MHCII<sup>hi</sup>. CD86<sup>+</sup> and CCR7<sup>+</sup> gating strategies are shown.





**Figure S8. CD86 expression on cDCs is augmented by toll-like receptor 3 agonist polyI:CLC and ZsG.** 400k B16F10-ZsG cells were inoculated in the right flanks of C57/B16 mice and tumors were treated with polyI:CLC (75 mg/0.1 mL saline I.P.) or saline (0.1 mL I.P.) 13 days post inoculation. Axial and brachial lymph nodes were harvested and pooled 24 h after saline or polyI:CLC injection. **A, B**) Geometric mean fluorescent (GMF) intensity of CD86 on cDC1s as a function of ZsG positivity. **C, D**) GMF intensity of CD86 on cDC2s as a function of ZsG. n = 10. Paired t-test: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. Means ± SEM.

**Table S1. Logistics model parameters.**

Tumor Side	<b>R<sup>2</sup></b>		<b>r</b>		<b>a</b>	
	Sham (n = 11)	BH (n = 8)	Sham (n = 11)	BH (n = 8)	Sham (n = 11)	BH (n = 8)
<b>Ipsilateral</b>	0.9678 ± 0.0077 (0.9086 to 0.9933)	0.7993 ± 0.1038 (0.0902 to 0.9956)	(4.66 ± 2.58)x10 <sup>4</sup> (897 to 2.93x10 <sup>5</sup> )	(3.34 ± 3.01)x10 <sup>6</sup> (0.0351 to 2.44x10 <sup>6</sup> )	0.4626 ± 0.0263 (0.2888 to 0.5908)	0.4013 ± 0.0431 (0.2465 to 0.5792)
<b>Contralateral</b>	0.9346 ± 0.0429 (0.5127 to 0.9975)	0.9393 ± 0.0298 (0.7489 to 0.9954)	(1.43 ± 0.51)x10 <sup>4</sup> (0.0094 to 4.77x10 <sup>4</sup> )	(3.31 ± 1.25)x10 <sup>4</sup> (53.55 to 9.67 x10 <sup>4</sup> )	0.3625 ± 0.0370 (0.0907 to 0.5028)	0.3018 ± 0.0497 (0.0250 to 0.4304)

**Values are Means ± SEM. Parentheses show range, from minimum to maximum. Sample sizes indicated below group labels.**

**Table S2. Key Resources Table.**

REAGENTS	SOURCE	IDENTIFIER
Anti-mouse CD45 clone 30-F11 BUV395	BD	Cat# 564279; RRID: AB_2651134
Anti-mouse CD3 clone 145-2C11 BUV563	BD	Cat# 749277; RRID: AB_287352
Anti-mouse CD19 clone 1D3 BUV737	BD	Cat# 612781; RRID: AB_2870111
Anti-mouse F4/80 clone BM8 BV421	BioLegend	Cat# 123137; RRID: AB_2563102
Anti-mouse CD11c clone N418 eF450	Invitrogen	Cat# 48-0114-82; RRID: AB_1548654
Anti-mouse Ly6G clone 1A8 BV605	BioLegend	Cat# 127639; RRID: AB_2565880
Anti-mouse/rat XCR1 clone ZET BV650	BioLegend	Cat# 148220; RRID: AB_2566410
Anti-mouse CD103 clone 2E7 BV785	BioLegend	Cat# 121439; RRID: AB_2800588
Anti-mouse Ly-6C clone HK1.4 PE/Dazzle 594	BioLegend	Cat# 128044; RRID: AB_2566577
Anti-mouse CD172a (SIRP $\alpha^+$ ) clone P84 PE	BioLegend	Cat# 144012; RRID: AB_2563550
Anti-mouse/human CD11b clone M1/70 PE/Cy5	BioLegend	Cat# 101209; RRID: AB_312792
Anti-mouse CD197 (CCR7) clone 4B12 PE/Cy7	Invitrogen	Cat# 25-1971-82; RRID: AB_469652
Anti-mouse CD86 (B7-2) clone GL1 APC	Invitrogen	Cat# 17-0862-82; RRID: AB_469419
Anti-mouse MHCII clone M5/114.15.2 AF700	Invitrogen	Cat# 56-5321-80; RRID: AB_494010
Anti-mouse CD8 $\alpha$ clone 53-6.7 APC/Fire 750	BioLegend	Cat# 100766; RRID: AB_2572113
Fixable Live/Dead Blue	Invitrogen	Cat# L23105; UV Excitation
DAPI	Sigma-Aldrich	Cat# D9542
Fc Block	Invitrogen	Cat# 14-0161-86; RRID: AB_467135
Brilliant Stain Buffer	BD	Cat# 563794
FACS Lysis	BD	Cat# 349202