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Supplemental information

The IAP antagonist birinapant enhances chimeric antigen receptor T cell therapy for glioblastoma by overcoming antigen heterogeneity

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Supplemental Tables:

Table S1.

Concentration of human cytokines in mouse plasma on day 7 by Luminex from the experiment of Figure 6.

(Data uploaded separately in Excel spreadsheet.)

Table S2.

Histopathologic analysis of mouse major organs from the experiment of Figure 6. Heart, liver, kidney, and lung from each mouse were harvested at the endpoint of this experiment, followed by fixation in 10% formalin, processing, sectioning, H&E staining, and histopathologic assessment by a veterinary pathologist in a blinded fashion without prior knowledge of the experimental intervention and group distribution. Lesions were scored on a scale of 0 - 4 ranging from unremarkable (score of 0) to severe/marked change (score of 4).

(Data uploaded separately in Excel spreadsheet.)

Supplemental Figures:

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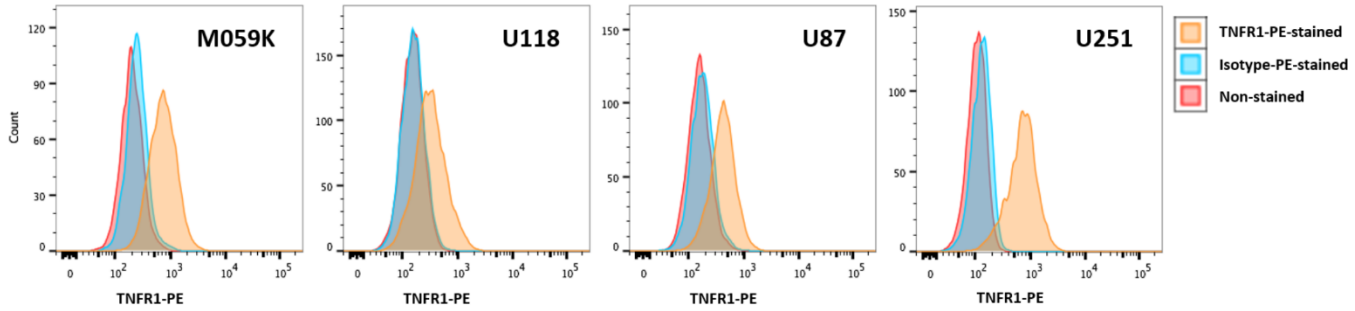

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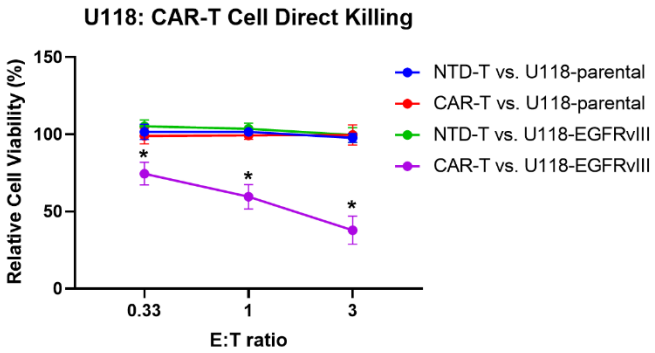

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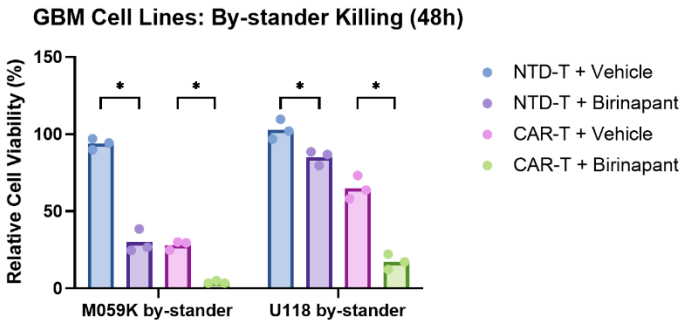

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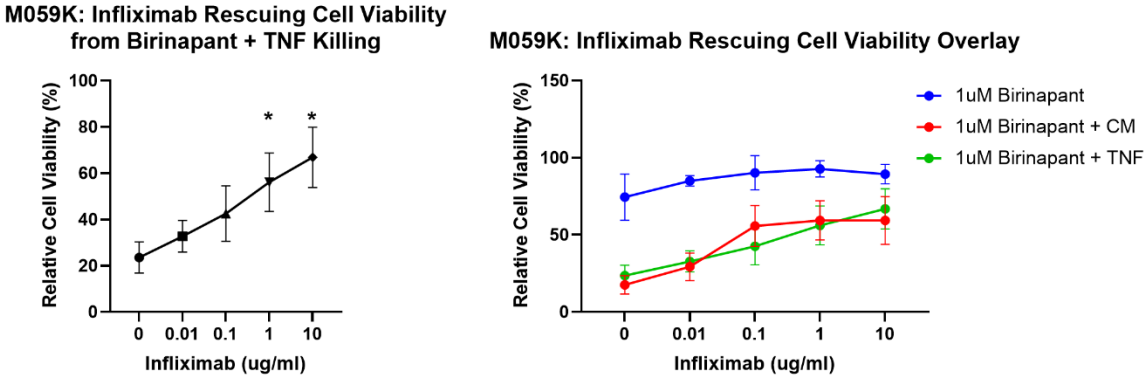

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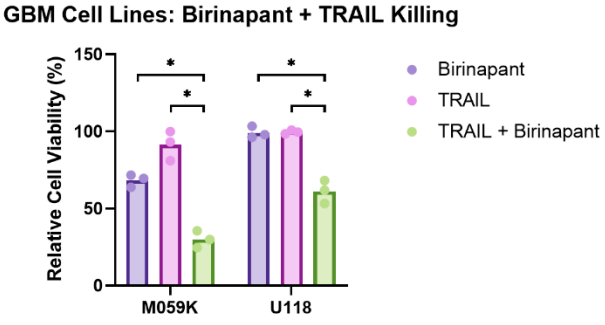

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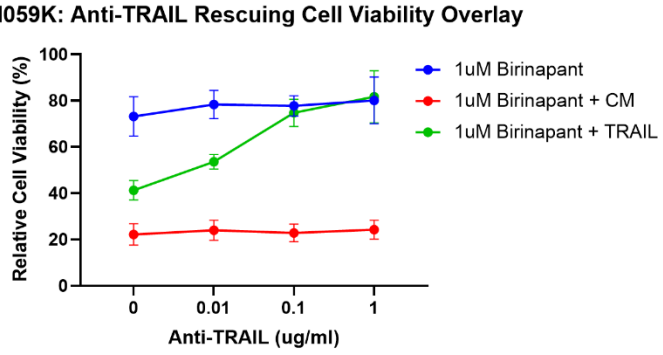

Figure S1.

A, TNF receptor 1 (TNFR1) expression on GBM cell lines by flow cytometry. **B,** Relative cell viability of U118-parental or U118-EGFRvIII cells after co-cultured with NTD T or CAR T cells at the indicated E:T ratios for 24 hours. **C,** Relative cell viability of the bystander GBM parental cells in a co-culture with EGFRvIII-expressing GBM cells and NTD T or CAR T cells (at E:T ratio of 1:10) in the presence of vehicle (0.1% DMSO) or 1 μM birinapant for 48 hours. **D,** Relative cell viability of M059K cells after treated with 1 μM birinapant with 10 pg/mL TNFα in the presence of the indicated concentrations of infliximab for 24 hours (left panel) and overlay of the relative M059K cell viability after treated with 1 μM birinapant, 1 μM birinapant with 1:100 diluted conditioned medium (CM), or 1 μM birinapant with 10 pg/mL TNFα in the presence of the indicated concentrations of infliximab for 24 hours (right panel). **E**, Relative cell viability of GBM cells after treated with vehicle (0.1% DMSO), 1 μM birinapant, 10 ng/mL TRAIL, or 10 ng/mL TRAIL with 1 μM birinapant for 24 hours. **F,** Overlay of the relative M059K cell viability after treated with 1 μM birinapant, 1 μM birinapant with 1:100 diluted conditioned medium (CM), or 1 μM birinapant with 10 ng/mL TRAIL in the presence of the indicated concentrations of anti-TRAIL antibody for 24 hours. Cell viability was normalized to the vehicle control without T cells in **B**, **C**, **D**, **E** and **F**. P values were determined by two-way ANOVA with Dunnett's multiple comparisons test (**B**), one-way ANOVA with Šídák's multiple comparisons test (**C**) or with Dunnett's multiple comparisons test (**D** and **E**) using data from three independent experiments. Significance was compared with CAR T vs. U118-parental at the indicated E:T ratio in **B**, and with 0 μg/mL infliximab in **D** (left). Results are presented as means ± SD in **B**, **D** and **F**. Significance is reported if $P < 0.05$ (*).

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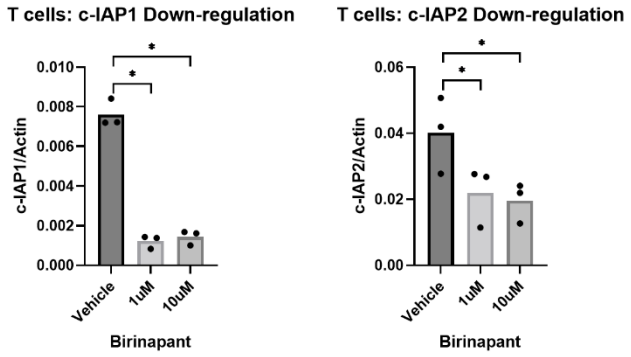

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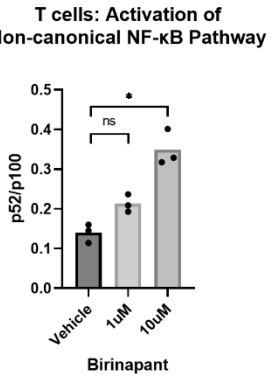

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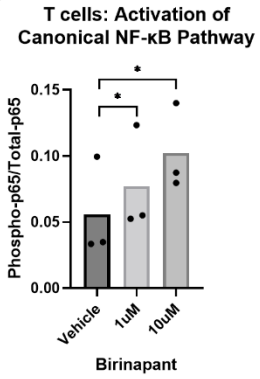

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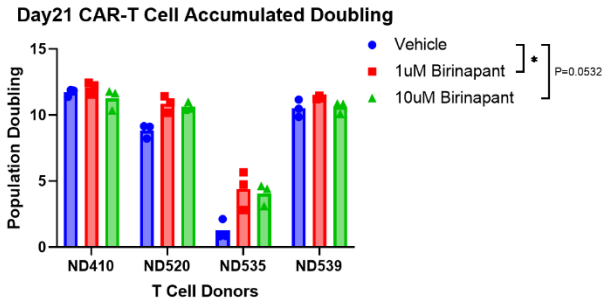

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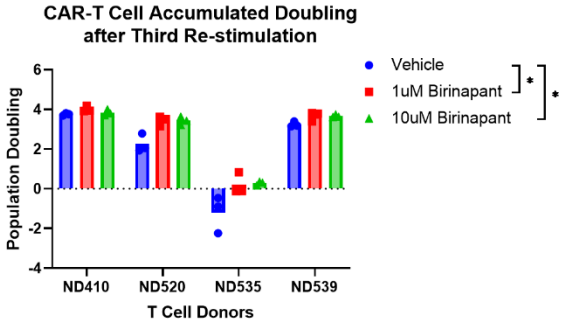

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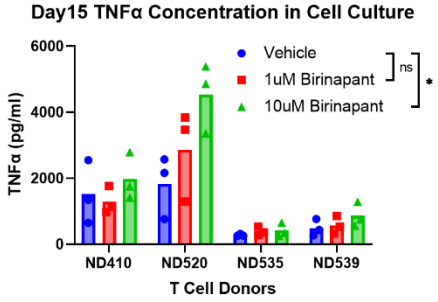

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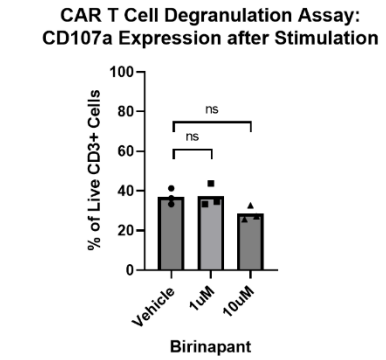

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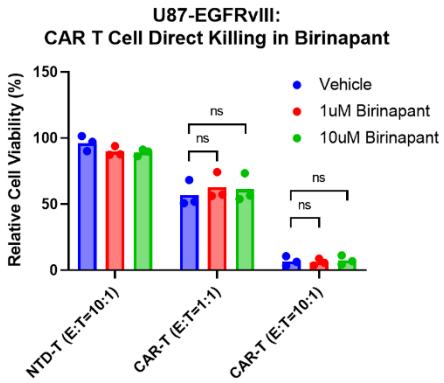

Figure S2.

A, Relative abundance of c-IAP1 (left panel) and c-IAP2 (right panel) protein normalized to β-actin loading control by western blotting assay from primary T cells treated with vehicle (0.1% DMSO), 1 μM birinapant or 10 μM birinapant for 6 hours. **B,** Relative abundance of NF-κB subunit p52 normalized to its precursor, p100, by western blotting assay from primary T cells treated with vehicle (0.1% DMSO), 1 μM birinapant, or 10 μM birinapant for 6 hours. **C,** Relative abundance of phosphorylated p65 normalized to total p65 by western blotting assay from samples as in **B**. **D,** Accumulated CAR T cell population doubling on day 21 of *in vitro* repeated stimulation assay. **E,** Accumulated CAR T cell population doubling after the third re-stimulation (day 14 to day 21). **F,** Concentration of TNFα in CAR T cell culture on day 15 by ELISA. **G**, Percentage of degranulated CAR T cells (CD107a+) among live CD3+ cells by flow cytometry after stimulated with U87-EGFRvIII cells at E:T ratio of 1:1 in the presence of vehicle (0.1% DMSO), 1 μM birinapant, or 10 μM birinapant for 5 hours. **H,** Relative cell viability of U87-EGFRvIII cells after co-cultured with NTD T or CAR T cells at the indicated E:T ratio in the presence of vehicle (0.1% DMSO), 1 μM birinapant, or 10 μM birinapant for 24 hours. P values were determined by randomized block (matching the data for each donor) one-way ANOVA with Dunnett's multiple comparisons test (**A**, **B**, **C**, **G**, **H**) using data from three independent experiments with different T cell donors, or randomized block (matching the data for each donor) two-way ANOVA with Dunnett's multiple comparisons test (**D**, **E**, **F**) using data from three independent experiments, each performed with all four donors side by side. Significance is reported if $P < 0.05$ (*).

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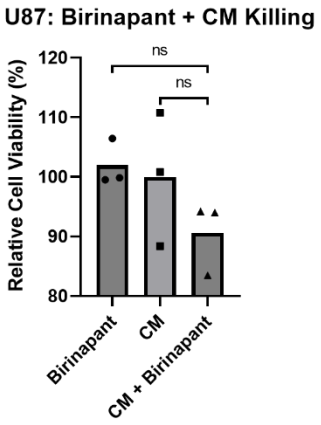

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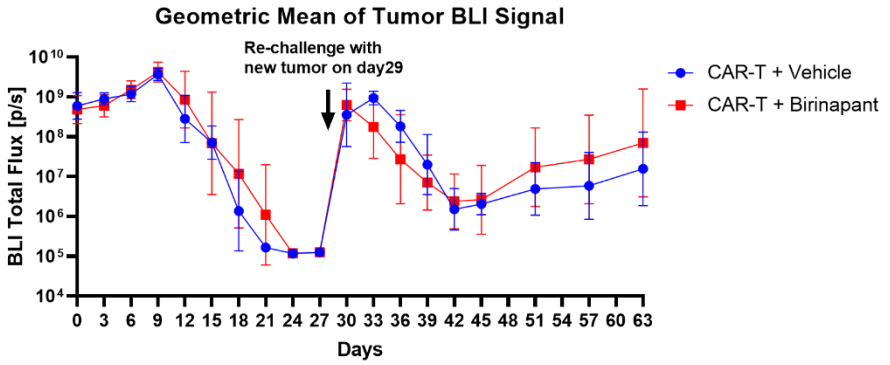

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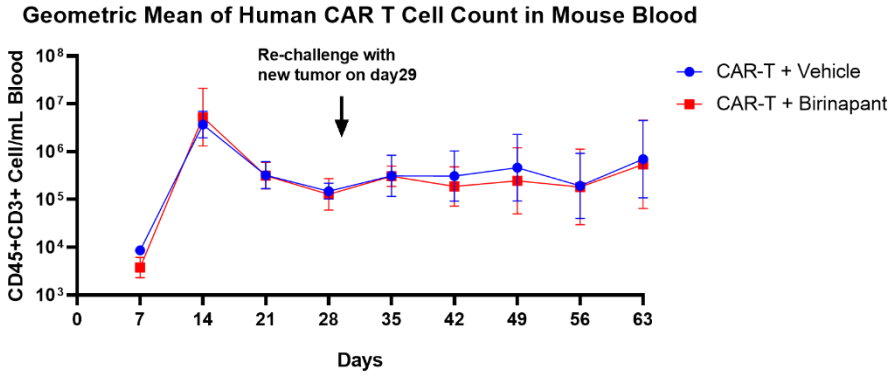

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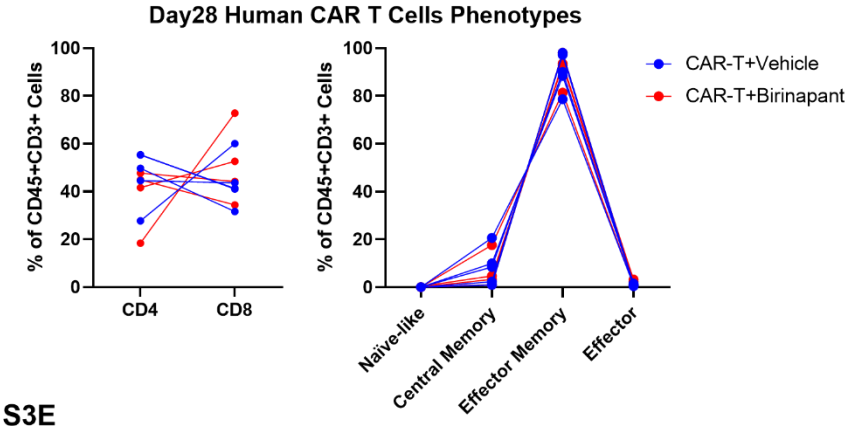

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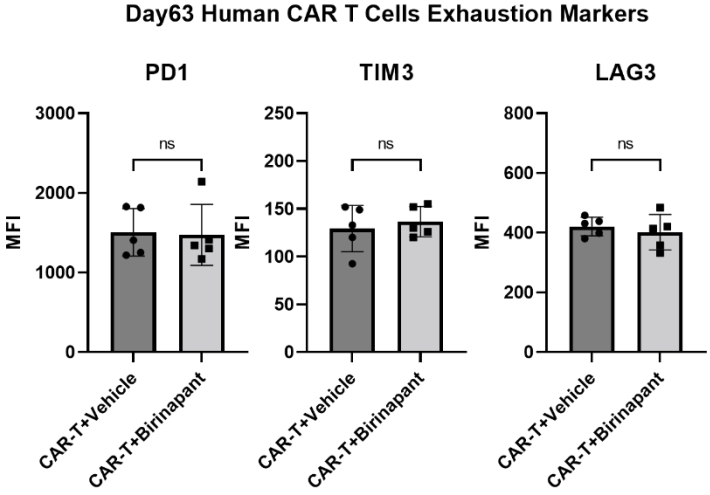

Figure S3.

A, Relative cell viability of U87 cells after treated with vehicle (0.1% DMSO), 1 μM birinapant, 1:100 diluted CAR T cell-derived conditioned medium (CM), or 1:100 diluted CM with 1 μM birinapant *in vitro* for 48 hours. **B-E**: NSG mice were subcutaneously implanted with 0.5 x 10^6 birinapant-resistant U87-EGFRvIII-CBG cells on the right flank. After 6 days (day 0), 0.5 x 10^6 2173-28ζ CAR T cells were intravenously injected. The vehicle (12.5% Captisol) or birinapant at 10 mg/kg were given by intraperitoneal injections every 3 days for 10 total doses since day 0. The mice were re-challenged with 3 x 10^6 U87-EGFRvIII-CBG cells subcutaneously implanted in the left flank on day 29. **B,** Geometric mean of bioluminescent total flux by imaging from U87-EGFRvIII-CBG tumors in each treatment group at the indicated time points. **C,** Geometric mean of human CAR T cell (CD45+/CD3+) count in mouse blood in each treatment group at the indicated time points. **D,** Percentage of CD4 (CD4+/CD8-), CD8 (CD4-/CD8+), naïve-like (CCR7+/CD45RO-), central memory (CCR7+/CD45RO+), effector memory (CCR7-/CD45RO+), and effector (CCR7-/CD45RO-) T cell subset among total human CAR T cells (CD45+/CD3+) in mouse blood on day 28 by flow cytometry. **E,** Median fluorescent intensity (MFI) of PD1, LAG3 and TIM3 on human CAR T cells (CD45+/CD3+) in mouse blood on day 63 by flow cytometry. Cell viability was normalized to the vehicle control in **A**. P values were determined by one-way ANOVA with Dunnett's multiple comparisons test (**A**) using data of three independent experiments, or unpaired Student's t test (**E**). Results are presented as geometric means ± geometric SD in **B** and **C**, and as means ± SD in **E**. Significance is reported if $P < 0.05$ (*). BLI, bioluminescent imaging.

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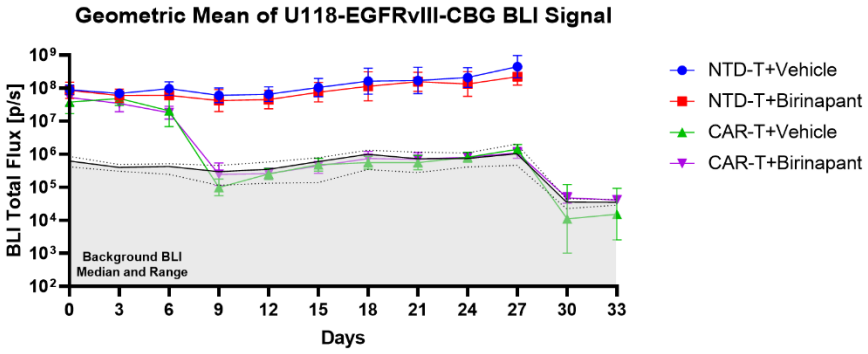

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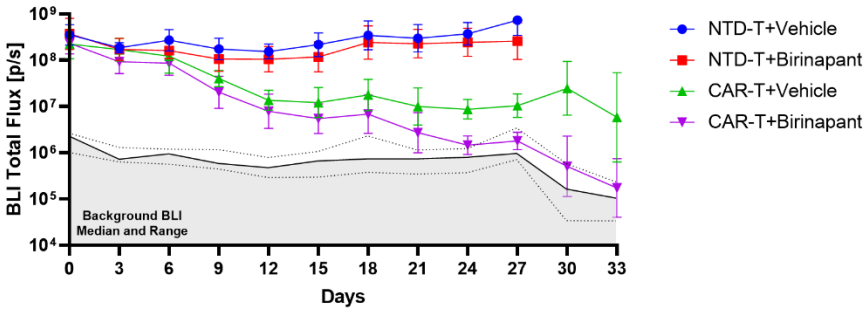

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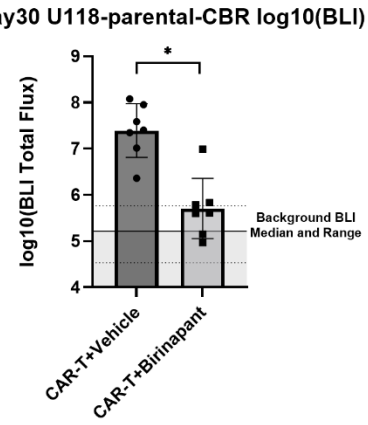

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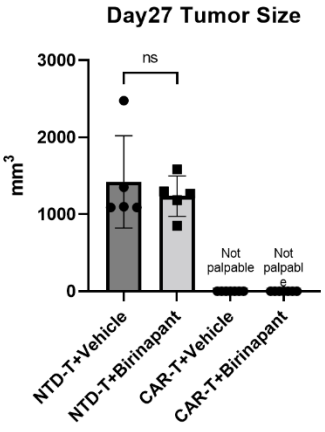

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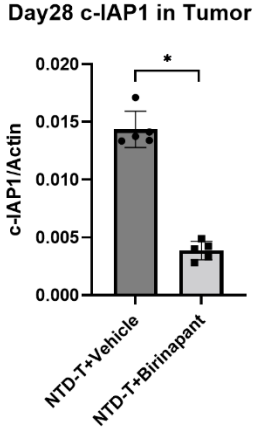

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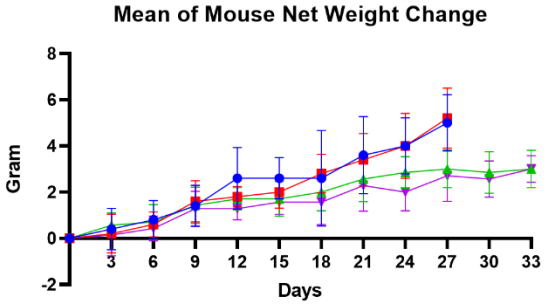

Figure S4.

NSG mice were subcutaneously implanted with a mixture of 4 x 10^6 U118-parental-CBR and 1 x 10^6 U118-EGFRvIII-CBG cells on the right flank. After 6 days (day 0), 0.5 x 10^6 2173-28ζ CAR T cells or the equivalent number of NTD T cells were intravenously injected. The vehicle (12.5% Captisol) or birinapant at 10 mg/kg were given by intraperitoneal injections every 3 days for 10 total doses since day 0. **A,** Geometric mean of spectrally unmixed bioluminescent total flux from U118-EGFRvIII-CBG cells in each treatment group at the indicated time points. **B,** Geometric mean of spectrally unmixed bioluminescent total flux from U118-parental-CBR cells in each treatment group at the indicated time points. **C,** Log_{10} of spectrally unmixed bioluminescent total flux from U118-parental-CBR cells on day 30. Solid and dotted black lines in **A**, **B** and **C** indicate median and range of background total flux from non-tumor area of each mouse at the indicated time points. **D,** Tumor size by caliper measurement on day 27. **E,** Relative abundance of c-IAP1 protein normalized to β-actin loading control by western blotting assay from tumor tissues harvested on day 28. **F**, Mean of mouse net weight change from day 0. P values were determined by unpaired t tests (**C**, **D**, and **E**). Results are presented as geometric means ± geometric SD in **A** and **B**, and as means ± SD in **C**, **D**, **E**, and **F**. Significance is reported if $P < 0.05$ (*). BLI, bioluminescent imaging.

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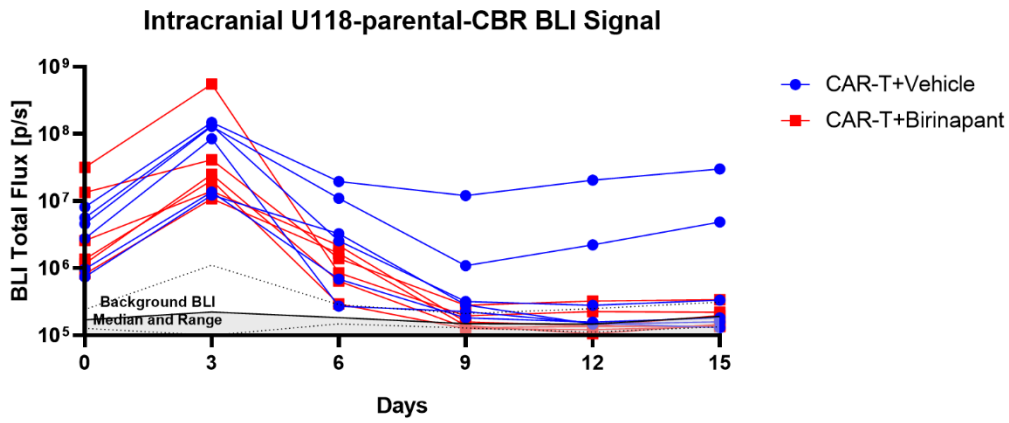

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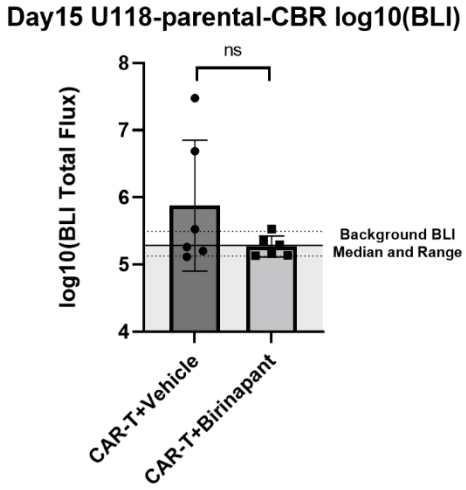

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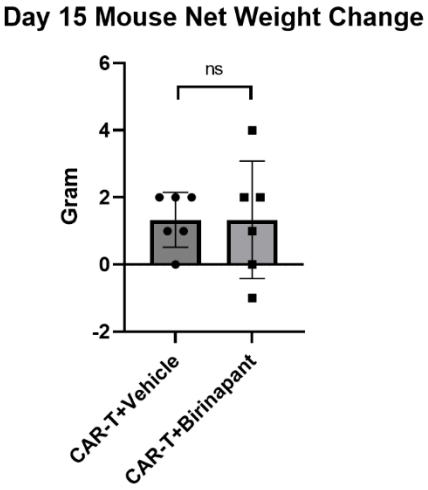

Figure S5.

NSG mice were intracranially implanted with a mixture of 200 x 10^3 U118-parental-CBR and 50 x 10^3 U118-EGFRvIII (without luciferase) cells. After 7 days (day 0), 1 x 10^6 2173-28ζ CAR T cells were intravenously injected. The vehicle (12.5% Captisol) or birinapant at 20 mg/kg were given by intraperitoneal injections every 3 days since day 0. **A,** Bioluminescent total flux from U118-parental-CBR cells in each mouse at the indicated time points. **B,** Log_{10} of bioluminescent total flux from U118-parental-CBR cells in each mouse on day 15. **C**, Mean of mouse net weight change from day 0 to day 15. Solid and dotted black lines in **A** and **B** indicate median and range of background total flux from non-tumor area of each mouse at the indicated time points. P values were determined by Mann-Whitney test (**B**) or unpaired t tests (**C**). Results are presented as means ± SD in **B** and **C**. Significance is reported if $P < 0.05$ (*). BLI, bioluminescent imaging.

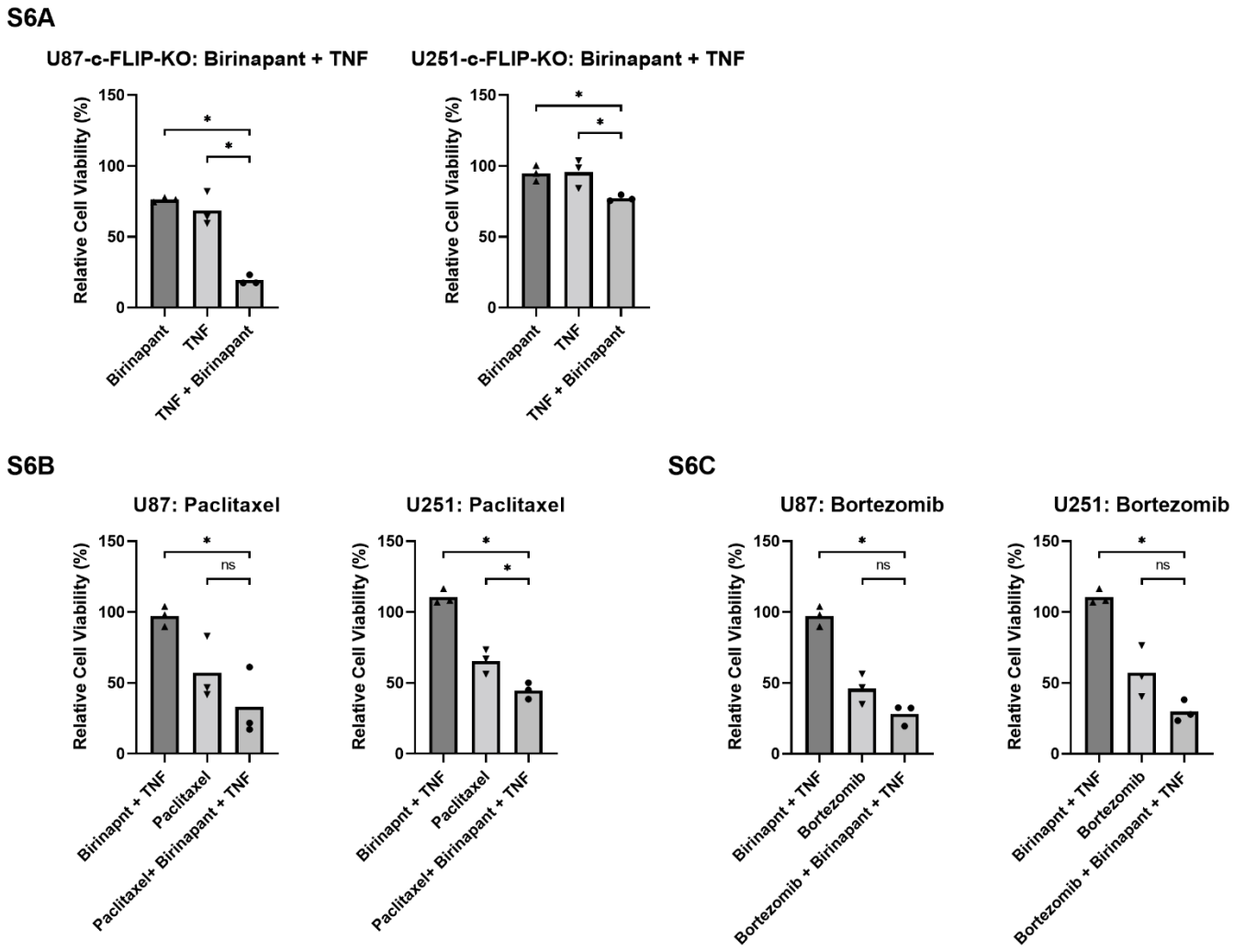

Figure S6.

A, Relative cell viability of GBM cell lines with c-FLIP knockout after treated with vehicle (0.1% DMSO), 1 μM birinapant, 100 pg/mL TNFα, or 100 pg/mL TNFα with 1 μM birinapant for 48 hours. **B,** Relative cell viability of GBM cell lines after treated with vehicle, 1 μM birinapant with 100 pg/mL TNFα, 5 nM paclitaxel, or 5 nM paclitaxel plus 1 μM birinapant and 100 pg/mL TNFα for 72 hours. **C,** Relative cell viability of GBM cell lines after treated with vehicle, 1 μM birinapant with 100 pg/mL TNFα, 5 nM bortezomib, or 5 nM bortezomib plus 1 μM birinapant and 100 pg/mL TNFα for 72 hours. Cell viability was normalized to the vehicle control. P values were determined by one-way ANOVA with Dunnett's multiple comparisons test using data from three independent experiments. Significance is reported if $P < 0.05$ (*).