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Supplemental information

Oncolytic herpes virus G47Δ works synergistically with CTLA-4 inhibition via dynamic intratumoral immune modulation

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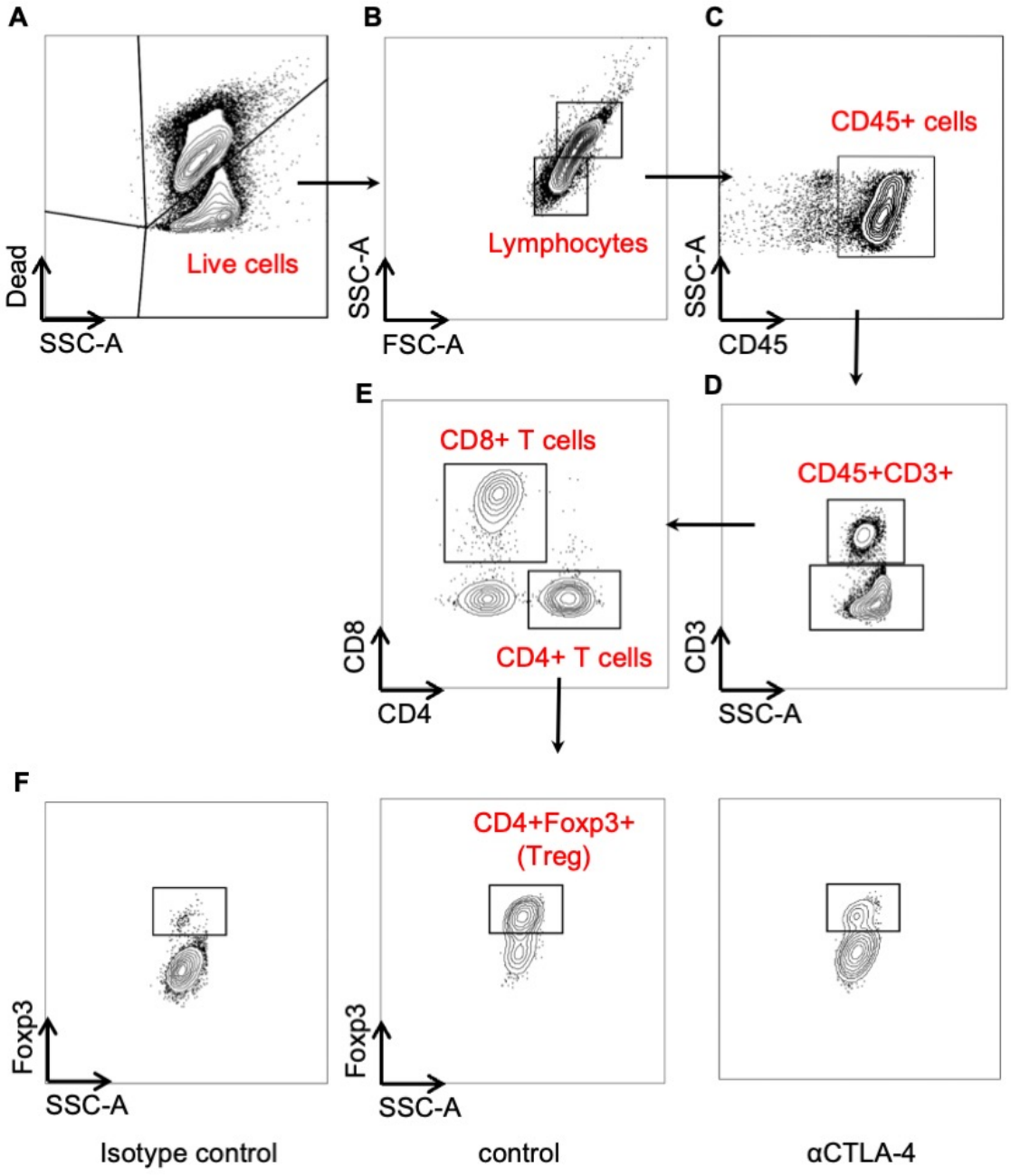
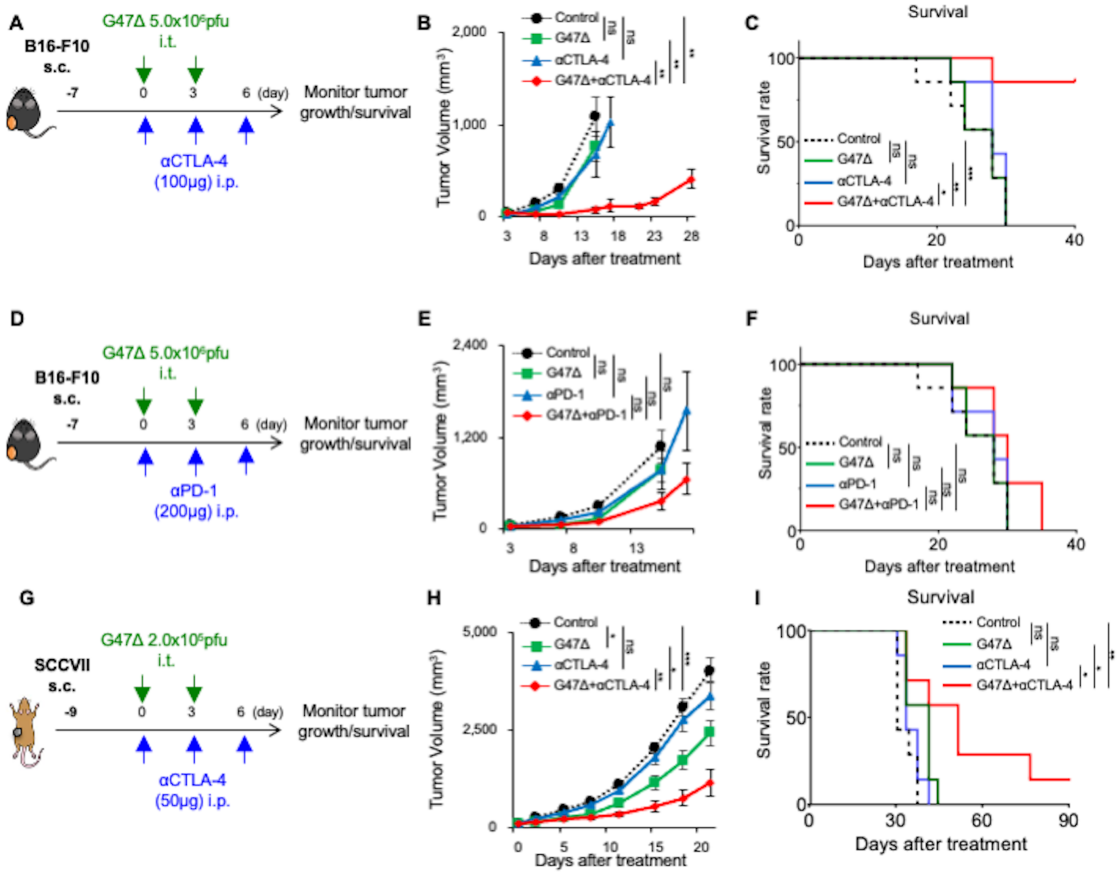
Supplementary Figures and Tables



Supplementary Figure 1. Gating strategy

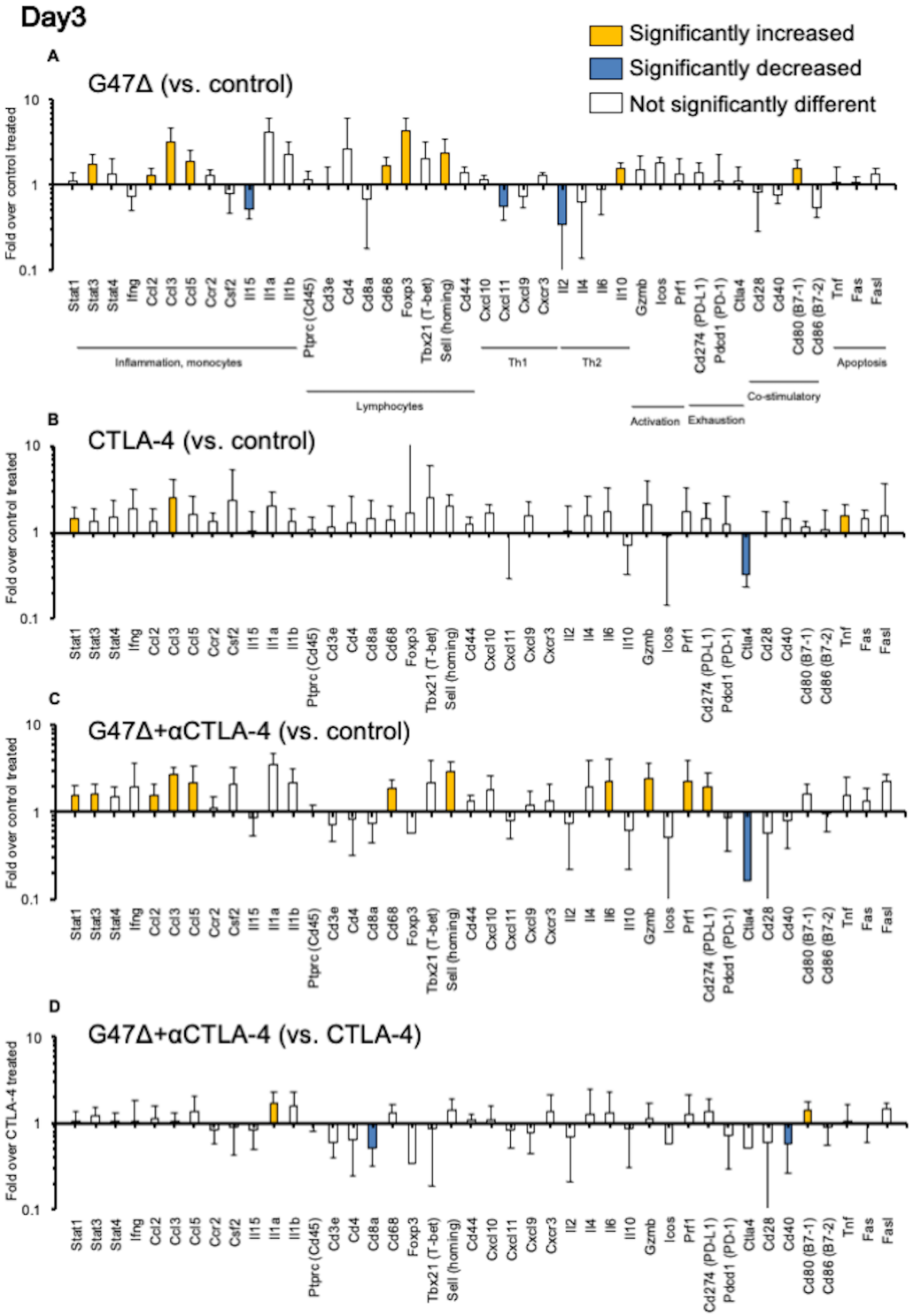
Representative flow cytometry plots and the gating strategy are shown. (A, B, C, D) T cells were assessed as CD45+CD3+ after dead cells and doublets had been removed. (E) CD8+ and CD4+ T cells and (F) Tregs (Foxp3+CD4+ T cells) were determined based on the isotype control. Left; isotype control, middle; control, right; anti-CTLA-4.



Supplementary Figure 2. Efficacy of the combination of G47Δ and ICIs in syngeneic B16-F10 and SCCVII subcutaneous tumors

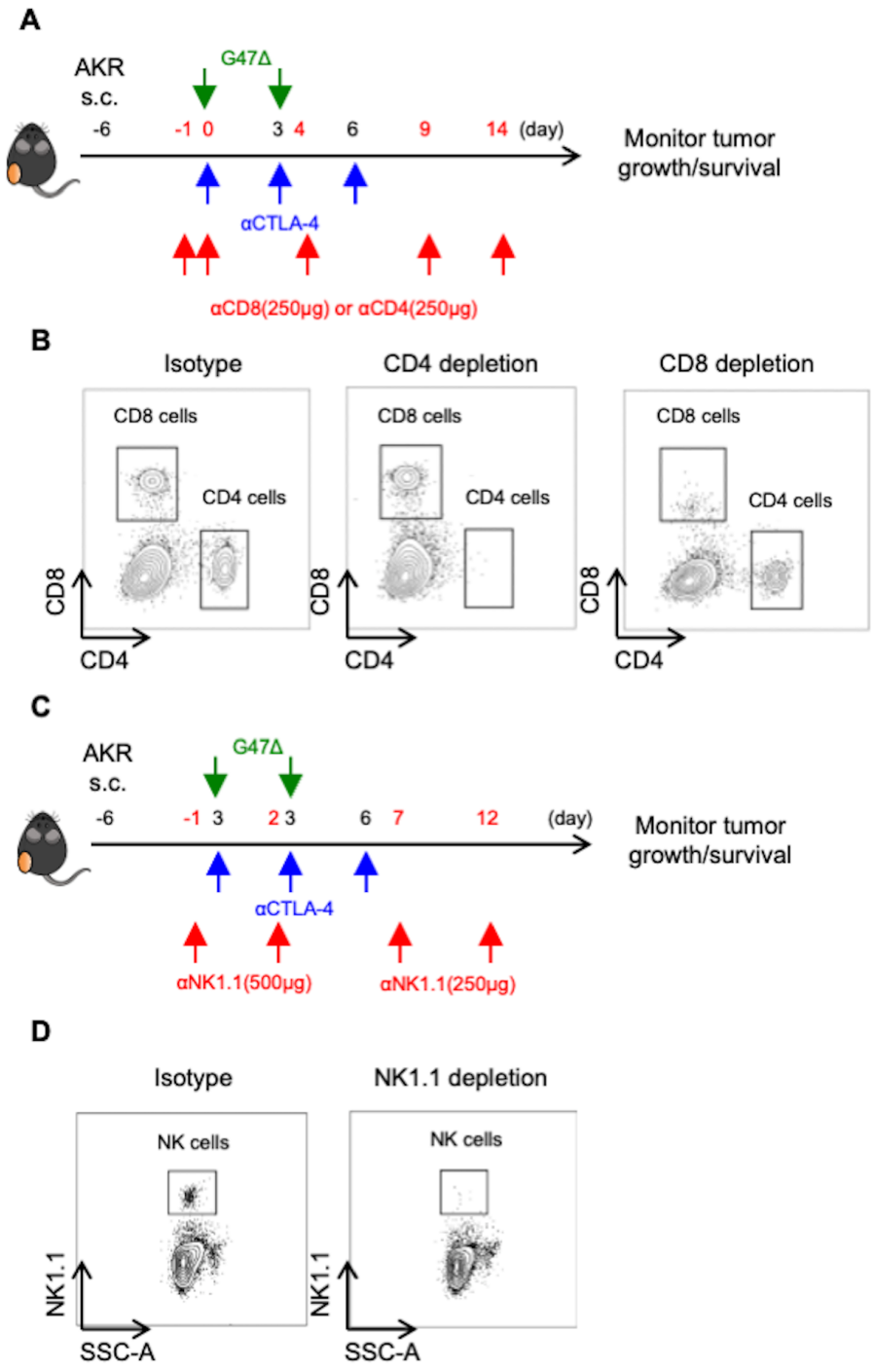
(A-C) Effects of G47Δ and CTLA-4 inhibition, either alone or in combination, on the growth of subcutaneous B16-F10 tumors in syngeneic C57BL/6 mice (n=7 per group). (A) Experimental design. Tumor growth (B) and Kaplan–Meier survival curves (C). The tumor growth was not suppressed by each monotherapy, but was significantly inhibited by the combination therapy (vs. control and monotherapies, $P < 0.01$). (D-F) Effects of G47Δ and PD-1 inhibition, either alone or in combination, on the growth of

subcutaneous B16-F10 tumor model (n=7 per group). (D) Experimental design. Tumor growth (E) and Kaplan–Meier survival curves (F). Neither tumor growth nor survival differed significantly among the four treatment groups. (G-I) Effects of G47Δ and CTLA-4 inhibition, either alone or in combination, on the growth of subcutaneous SCCVII tumors in syngeneic C3H mice (n=7 per group). (G) Experimental design. Tumor growth (H) and Kaplan–Meier survival curves (I). The tumor growth was inhibited by G47Δ alone (vs. control, $P < 0.05$) and the efficacy was enhanced by the combination therapy (vs. G47Δ; $P < 0.01$, vs. αCTLA-4; $P < 0.001$). One-way ANOVA followed by Dunnett's test was used for comparisons of tumor growth. For survival analysis, the log-rank test followed by Holm's sequential Bonferroni corrections was used to determine statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).



Supplementary Figure 3. Intratumoral immune-related gene expression changes in subcutaneous AKR tumors 3 days after the initial treatment

C57BL/6 mice bearing AKR tumors were treated according to the schedule shown in Figure 2A. Tumor tissues were harvested 3 days after the initial treatments, and gene expressions were analyzed using qPCR analysis. The fold change in expression of the indicated genes (A) with G47Δ treatment over control, (B) with CTLA-4 inhibition over control, (C) with the combination therapy over control and (D) the combination therapy over CTLA-4 inhibition. The bar represents mean fold change + SEM (n=6). The yellow bars represent mRNAs that were significantly upregulated ($P < 0.05$, fold change ≥ 2) as compared with the reference group. The blue bars show mRNAs that were significantly downregulated ($P < 0.05$, fold change < 0.5) as compared with the reference group. The expression data were normalized to the geometric mean of three housekeeping genes (*Actb*, *Gapdh,* and *Hprt1*). One-way ANOVA followed by Dunnett's test was used to determine statistical significance. All experiments were performed twice, with six samples for each group.



Supplementary Figure 4. Experimental design of depletion assays and validation of immune cell depletion

C57BL/6 mice were implanted with AKR cells in the left flank on day -6, intratumorally inoculated with G47Δ (5×10^6 pfu), or mock on days 0 and 3, and injected intraperitoneally with an anti-CTLA-4 antibody (25 μg) or Syrian hamster IgG on days 0, 3, and 6. Arrows indicate intratumoral injections with G47Δ (green), intraperitoneal injections with an anti-CTLA-4 antibody (blue) or administration of depletion antibodies (red). (A) Depletion antibodies against CD4 or CD8 (250 μg) were injected intraperitoneally on days -1, 0, 4, 9, and 14 as indicated. (B) On days 0 and 4, the mice were sacrificed, spleens collected, splenocytes isolated and stained with or without anti-mouse CD4 and CD8a antibodies, analyzed using flow cytometry, and adequate cell depletion of each cell subset was confirmed. (C) The mice were injected with 500 μg of the depletion antibody, NK1.1, on days -1 and 2, followed by injection of 250 μg every 5 days throughout the experiment as indicated. (D) On days 0 and 7, adequate NK cell depletion was confirmed.

Supplementary Table 1. Gene symbols of a custom panel

No.	Gene Symbols	Assay IDs	No.	Gene Symbols	Assay IDs
1	*Actb*	Mm00607939_s1	25	*Cd3e*	Mm00599683_m1
2	*Hprt*	Mm00446968_m1	26	*Cd4*	Mm00442754_m1
3	*Gapdh*	Mm99999915_g1	27	*Cd68*	Mm00839636_g1
4	*Stat1*	Mm00439518_m1	28	*Cd8a*	Mm01182106_g1
5	*Stat3*	Mm00456961_m1	29	*Ptprc*	Mm00448463_m1
6	*Stat4*	Mm00448890_m1	30	*Tbx21*	Mm00450960_m1
7	*Cd19*	Mm00515420_m1	31	*Foxp3*	Mm00475162_m1
8	*Ifng*	Mm00801778_m1	32	*Sell*	Mm00441291_m1
9	*Il1a*	Mm00439620_m1	33	*Cd44*	Mm01277161_m1
10	*Il1b*	Mm00434228_m1	34	*Gzmb*	Mm00442834_m1
11	*18S*	Hs99999901_s1	35	*Icos*	Mm00497600_m1
12	*Il15*	Mm00434210_m1	36	*Prf1*	Mm00812512_m1
13	*Ccl5*	Mm01302428_m1	37	*Ccr2*	Mm99999051_gH
14	*Cxcl10*	Mm00445235_m1	38	*Ccl2*	Mm00441242_m1
15	*Cxcl11*	Mm00444662_m1	39	*Ccl3*	Mm00441258_m1
16	*Cxcr3*	Mm00438259_m1	40	*Cd28*	Mm00483137_m1
17	*Cxcl9*	Mm00434946_m1	41	*Cd80*	Mm00711660_m1
18	*Ccr4*	Mm00438271_m1	42	*Cd86*	Mm00444543_m1
19	*Il10*	Mm00439616_m1	43	*Csf2*	Mm00438328_m1
20	*Il2*	Mm00434256_m1	44	*Cd40*	Mm00441895_m1
21	*Il4*	Mm00445259_m1	45	*Ctla4*	Mm00486849_m1
22	*Fas*	Mm00433237_m1	46	*Cd274*	Mm03048248_m1
23	*Fasl*	Mm00438864_m1	47	*Pdcd1*	Mm01285676_m1
24	*Tnf*	Mm00443258_m1	48	*Il6*	Mm00446190_m1

A custom panel includes one control gene (*18S*), three housekeeping genes (*Actb*, *Gapdh*, and *Hprt*) and 44 immune-related genes.

Supplementary Table 2. Combination index

Day [1]	FTV				CI [3]
	G47Δ	αCTLA-4	Combination		
			Expected [2]	Observed	
8	0.72	0.25	0.18	0.12	0.67
12	0.66	0.08	0.05	0.02	0.31

Abbreviations: FTV; fractional tumor volume, CI; combination index

[1] Day after the initial treatments
[2] (Mean FTV of G47Δ) × (mean FTV of αCTLA-4)
[3] Obtained by dividing the expected FTV by the observed FTV

FTV = (mean tumor volume experimental)/(mean tumor volume control)
CI < 1, CI = 1 and CI > 1 indicates a synergistic, an additive and antagonistic effect, respectively

The efficacy of treatments (G47Δ, αCTLA-4 and the combination) was assessed as the fractional tumor volume (FTV). FTV was calculated as described in the Materials and Methods, and the expected FTV and the combination index (CI) were estimated. CI < 1, CI = 1 and CI > 1 indicates a synergistic, an additive and antagonistic effect, respectively.