YMTHE, Volume *25*

Supplemental Information

Adenovirotherapy Delivering Cytokine and Checkpoint Inhibitor Augments CAR T Cells against Metastatic Head and Neck Cancer

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Supplemental Table 1. *Primer sequences.*

Supplemental Fig. 1. *HNSCC lines heterogeneously express HER2*. HER2 expression was analyzed by flow cytometry on both HPV negative and HPV positive HNSCC lines. Triple negative breast cancer cell line MDA-MB-468, and MDA-expressing human HER2 (MDA*HER2*) were used as controls.

Supplemental Fig. 2. *HER2.CAR T-cells have minimal expansion in NSG mice without tumors*. A total of $1x10^6$ *ffLuc*-expressing HER2.CAR T-cells were systemically administered to NSG mice. Control mice received vehicle alone. Bioluminescence of HER2.CAR T-cells was monitored at different time points. Data are presented as means ± SD (n=5).

Supplemental Fig. 3. *Both HNSCC lines induce PD-L1 expression in the presence of IFNγ, and HDAdCyto and PD-L1 blocking antibody increase the anti-tumor efficacy of HER2.CAR T-cells in vitro*. **(A)** FaDu and SCC47 were cultured in the presence or absence of 10 ng/mL recombinant IFNγ. Cells were harvested at 24 hours, and the expression of PD-L1 was analyzed by flow cytometry. **(B)** SCC-47 or FaDu expressing *ffLuc* cells were co-infected with 100 vp/cell of HDAd*Cyto* and HDAd*PD-L1*. HER2.CAR T-cells were added at 24 hours post-infection (effector:target ratio of 1:40). Cells were harvested 120 hours post-co-culture, and viable cancer cells were analyzed by luciferase assay. Data are presented as means ± SD (n=4). $*P < 0.001$. The experiments were repeated with HER2.CAR T-cells derived from a second donor with similar results.

Supplemental Fig. 4. *Phenotype of HER2.CAR T-cells*. **(A)** Before infusion to mice, HER2.CAR expression, CD4 and CD8 ratio, memory phenotype and PD-1 expression of HER2.CAR T-cells were analyzed by flow cytometry. **(B)** T-cells were isolated from tumor sites at 22 days post-infusion, and CD4 and CD8 ratio, memory phenotype and PD-1, TIM3, LAG3 expression were analyzed by flow cytometry. The experiments were repeated with similar results.

Supplemental Fig. 5. *HNSCC lines resist Onc.Ad in vivo*. FaDu and SCC-47 cells were transplanted into the right flanks of NSG mice (pink: female, blue: male). A total of $1x10^8$ vp Onc.Ads were intra-tumorally injected. Tumor volumes were measured at different time points. Kaplan-Meier survival curve after injection of Onc.Ads. The end point was established as tumor volume of > 1,500 mm^3. Data are presented as means ± SD (n=8-9). *P=0.04.

Supplemental Fig. 6. *Tumor-infiltrating HER2. CAR T-cells were detected at tumor sites overtime*. A total of $1x10^8$ vp of Onc.Ad, CAd*PDL1*, CAd*IL-12* or CAd*12_PDL1* (Onc:HD=1:20) were injected intra-tumorally. A total of $1x10^6$ HER2.CAR T-cells expressing firefly luciferase (*ffLuc*) were systemically administered 3 days post-injection of Ads. Bioluminescence of HER2.CAR T-cells was monitored at different time points.

Supplemental Fig. 7. *Tumor-infiltrating HER2. CAR T-cells maintain HER2.CAR expression at 105 days post-injection*. T-cells were isolated from tumor sites at 105 days post-infusion, and HER2.CAR expression were analyzed by flow cytometry. The experiments were repeated with similar results.

Supplemental Fig. 8. *Combinatorial treatments did not cause weight loss in mice*. FaDu or SCC-47 cells were transplanted into the right flanks of NSG mice. A total of $1x10^8$ vp of Onc.Ad, CAd*PDL1*, CAd*IL-12* or CAd*12_PDL1* (Onc:HD=1:20) were injected intra-tumorally. A total of $1x10^6$ HER2.CAR T-cells were systemically administered 3 days post-injection of Ads. Body weights were measured at different time points. Data are presented as means ± SD (n=8-10).

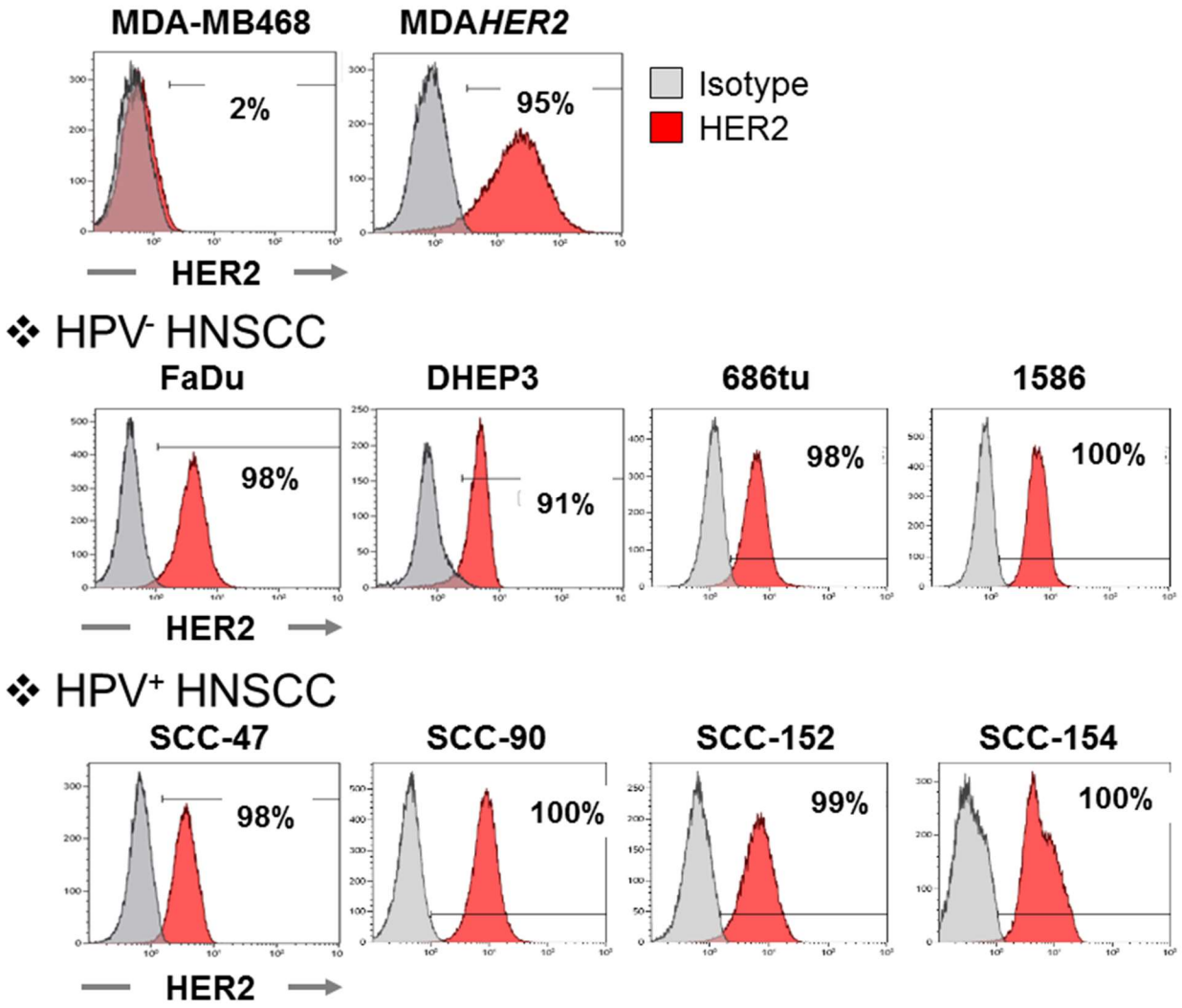
Supplemental Fig. 9. *HER2.CAR T-cells home both primary and metastasized tumors in FaDu orthotopic mice*. FaDu cells were transplanted into the tongues of NSG mice. A total of $1x10^8$ vp of CAd*12_PDL1* (Onc:HD=1:20) were injected into the tongue. A total of $1x10^6$ HER2.CAR T-cells were systemically administered 3 days post-injection of CAd. **(A)** Tongue and lymph node area were collected at 14 days post-injection of CAd and fixed. Paraffin sections were stained with H&E, anti-human CD3 and anti-human p53 antibodies. **(B)** Total DNA was extracted from tongue and lymph node area at 14 days post-injection of CAd, and HER2.CAR, Onc.Ad and HDAd copy

numbers in each tissue were quantified. After normalization of each copy number with mouse genomic GAPDH, copy numbers of tissue treated with CAd*12_PDL1* and HER2.CAR T-cells were normalized with that of control mice. Data are presented (n=2).

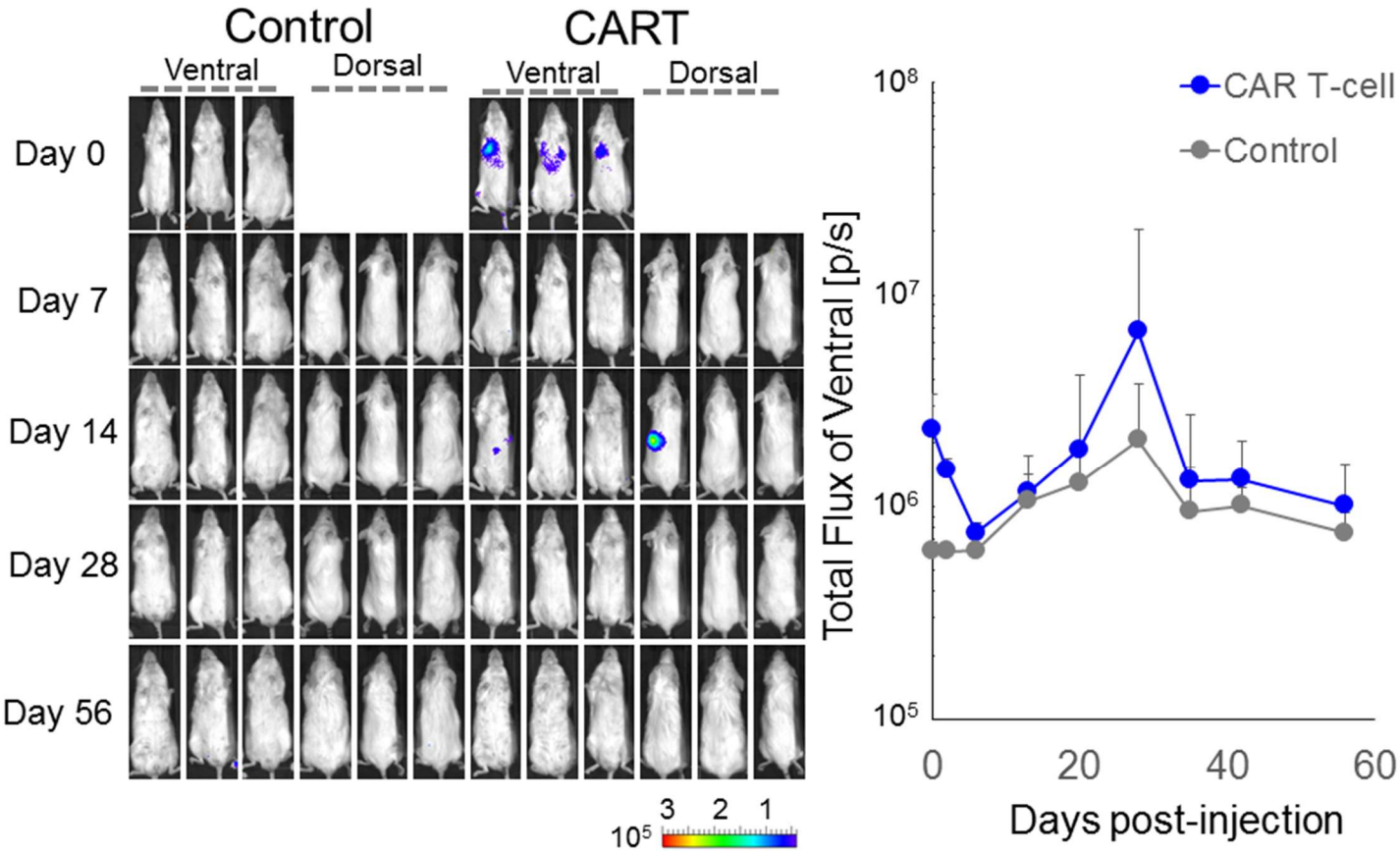
Supplemental Fig. 10. *Combinatorial treatments did not cause weight loss due to tumor growth in HNSCC orthotopic mice*. FaDu cells were transplanted into the tongues of NSG mice. A total of $1x10^8$ vp of CAd*12_PDL1* (Onc:HD=1:20) were injected into the tongue. A total of $1x10^6$ HER2.CAR T-cells expressing *ffLuc* were systemically administered 3 days post-injection of CAd. Body weights were measured at different time points. Data are presented as means ± SD (n=6).

Primer name	Sequence
E1A (Onc.Ad)	5’-TCCGGTTTCTATGCCAAACCT-3’ 5’-TCCTCCGGTGATAATGACAAGA-3’
Stuffer (HDAd)	5’-TCTGAATAATTTTGTGTTACTCATAGCGCG-3’ 5’-CCCATAAGCTCCTTTTAACTTGTTAAAGTC-3’
HER2.CAR (CAR T-cell)	5’-GAGGTACAACTGCAGCAGTCTGGA-3’ 5’-TTCCAAAGAGAAGTCAAACCGTCC-3’
Human genomic GAPDH	5’-CATGCCTTCTTGCCTCTTGTCTCTTAGAT-3’ 5’-CCATGGGTGGAATCATATTGGAACATGTAA-3’
Mouse genomic GAPDH	5’-TAGGCCAGGATGTAAAGGTCATTAAG-3’ 5’-CCAGAAAGGTCACACGGCTAAA-3’
Human β-actin	5’-GCCAACCGCGAGAAGATGACC-3’ 5’-CTCCTTAATGTCACGCACGATTTC-3’

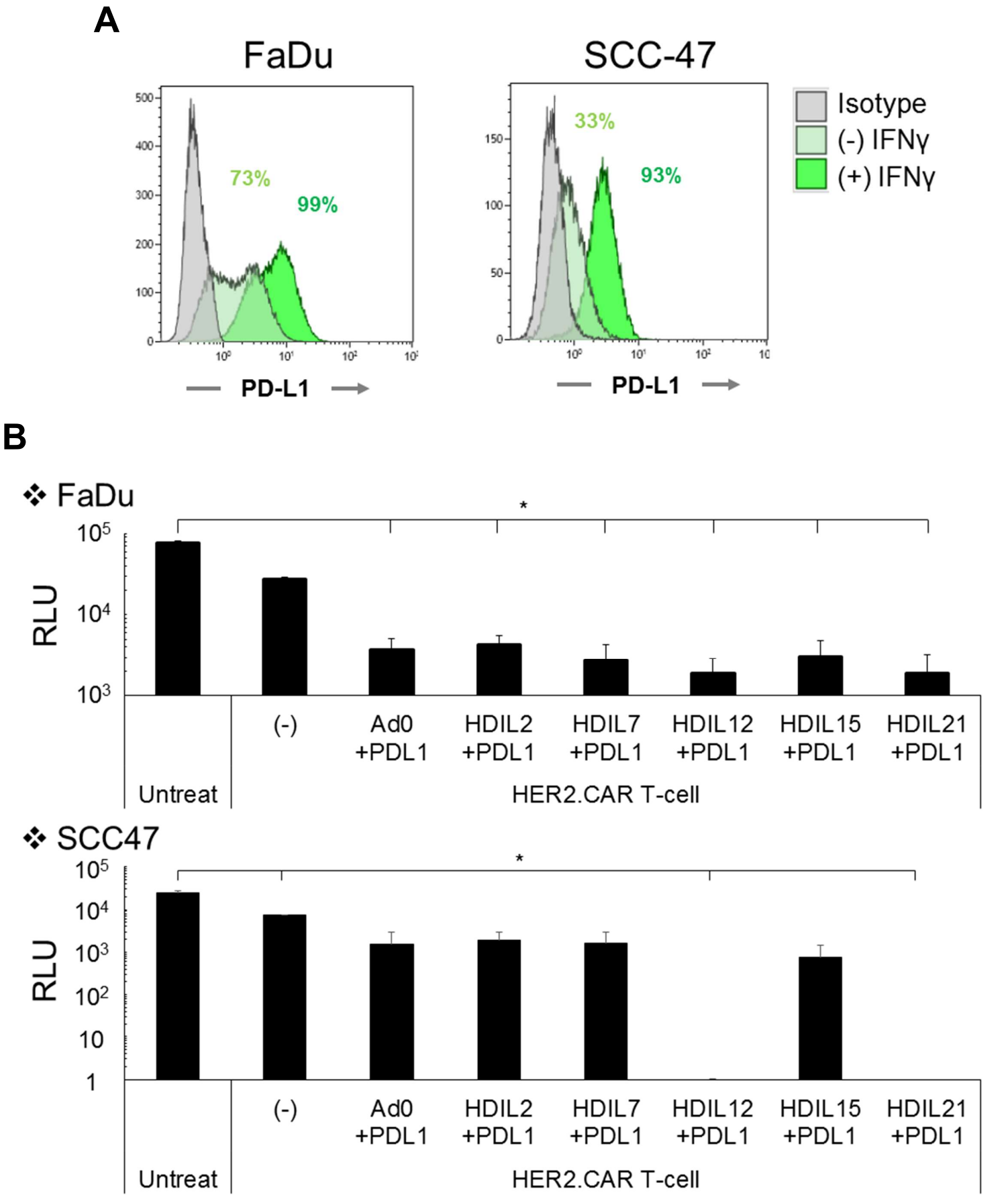
Supplemental Figure 1



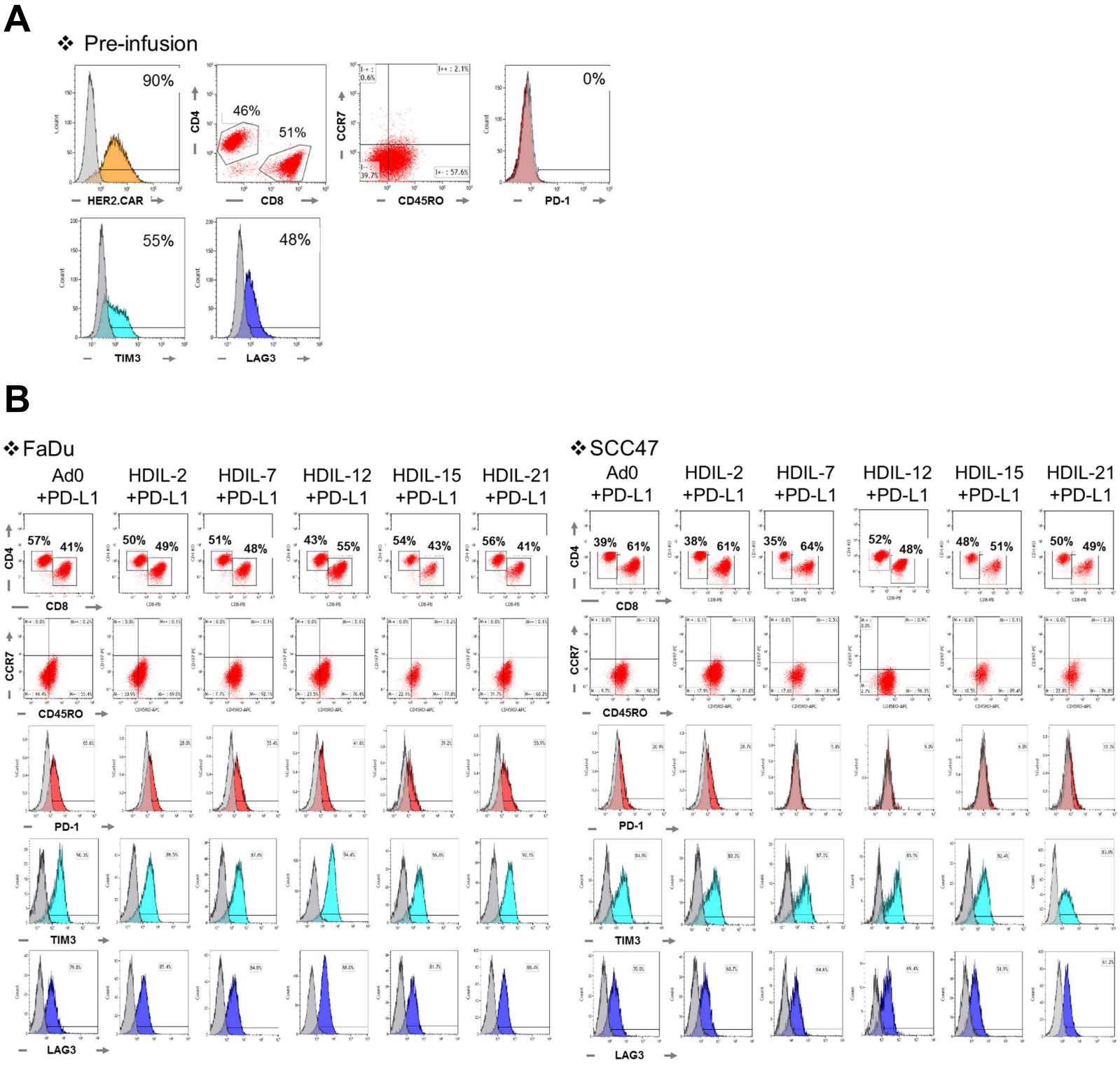
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



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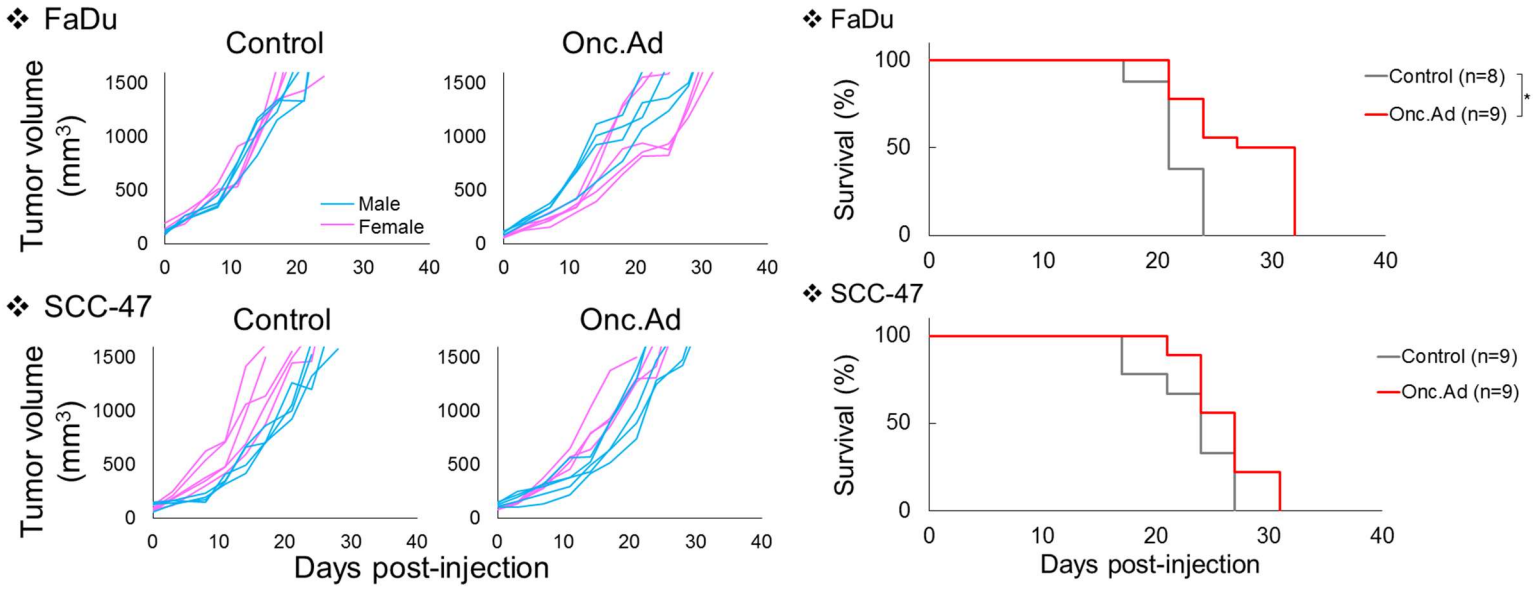
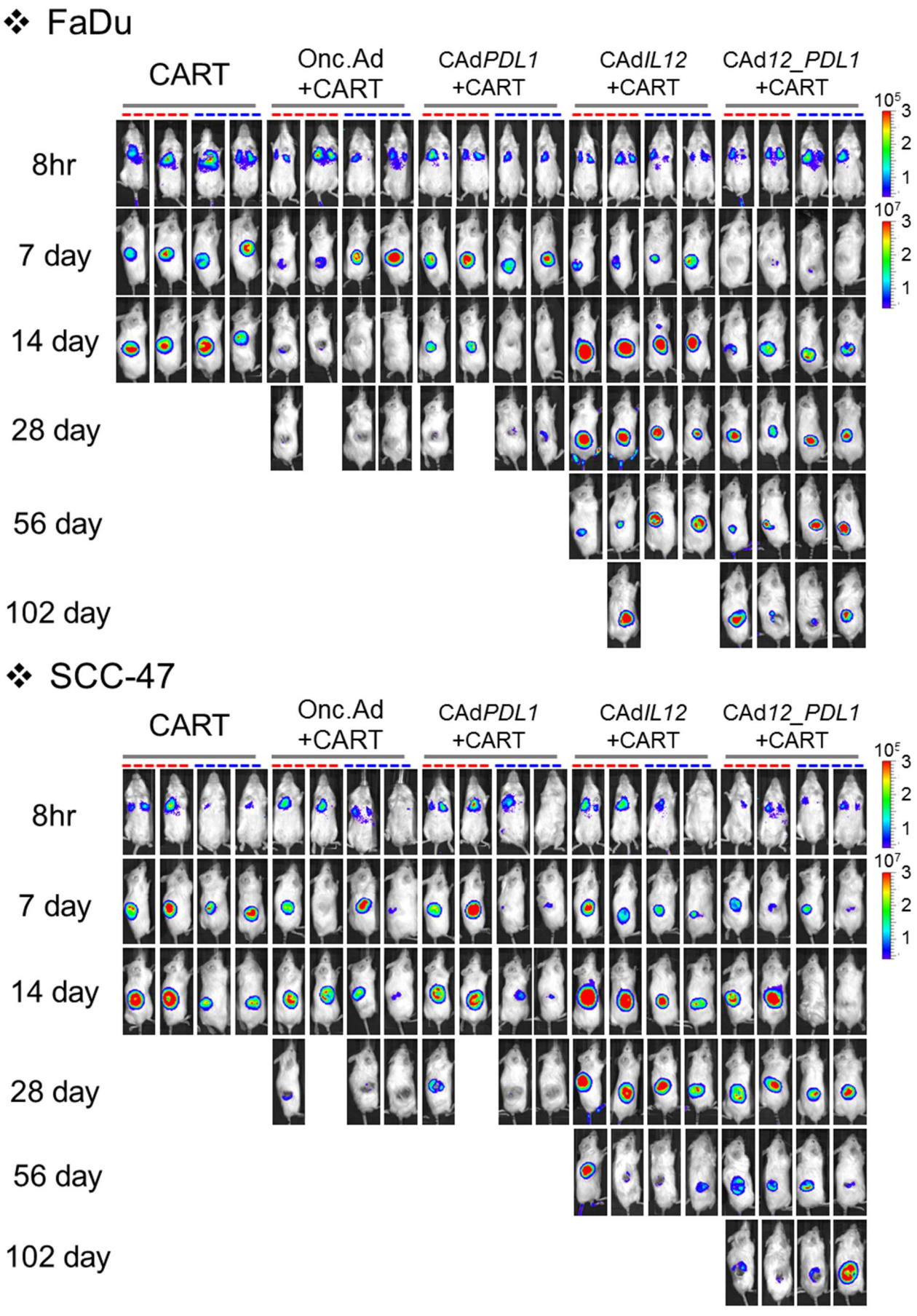
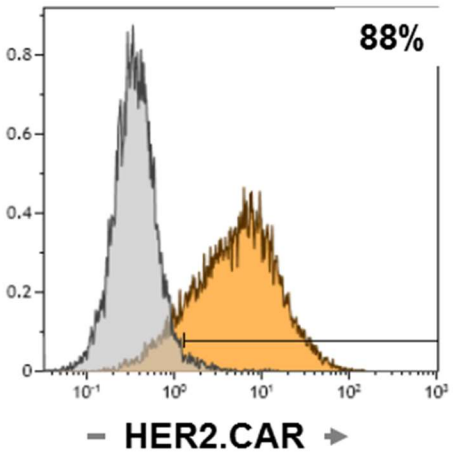


Supplemental Figure 6

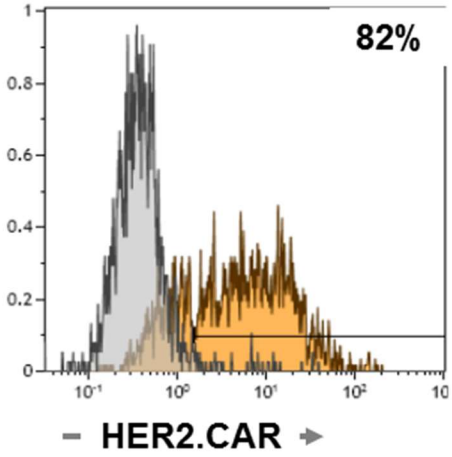


Supplemental Figure 7

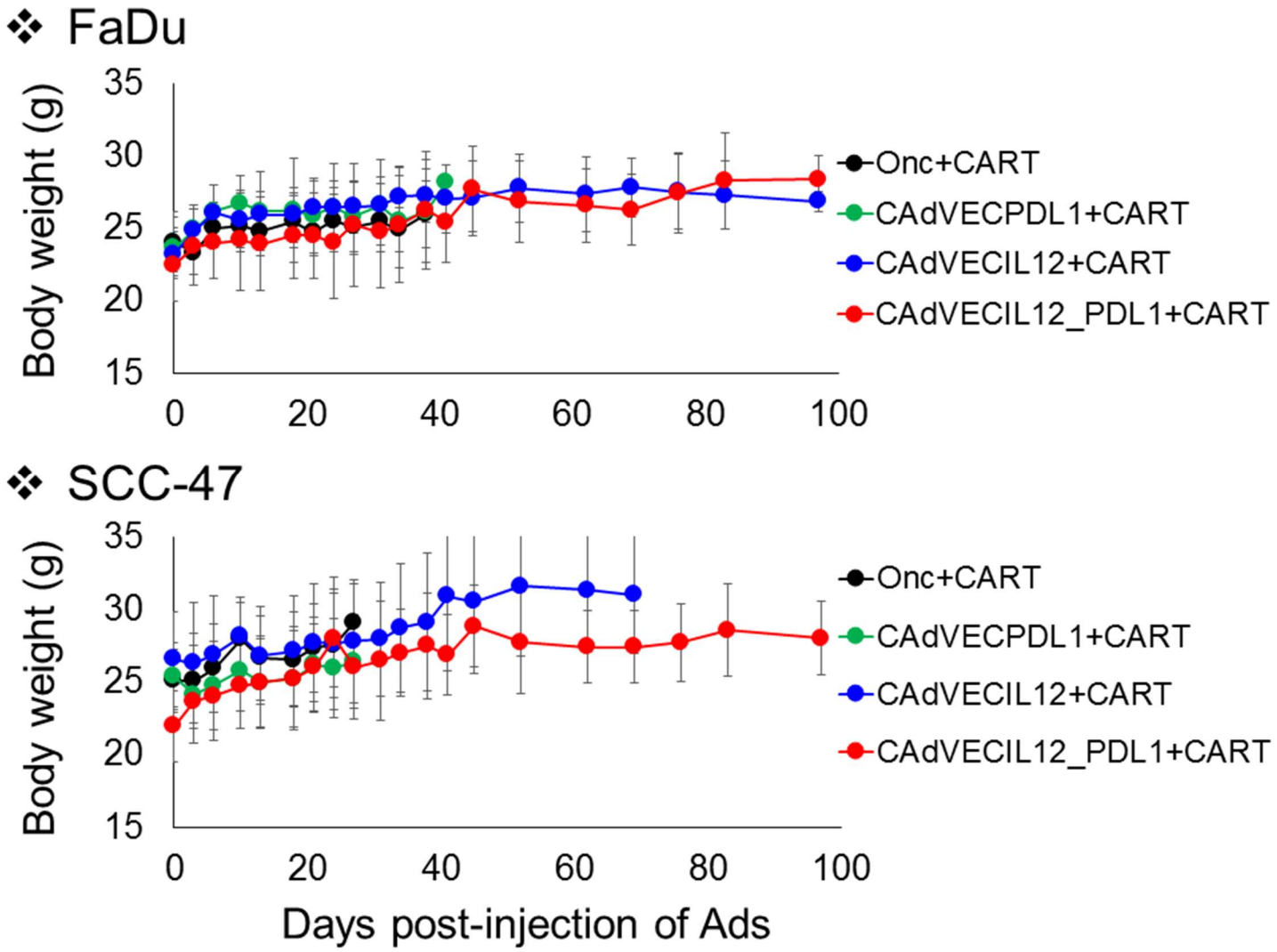
❖ FaDu TIL: 105 day



❖ SCC-47 TIL: 105 day



Supplemental Figure 8



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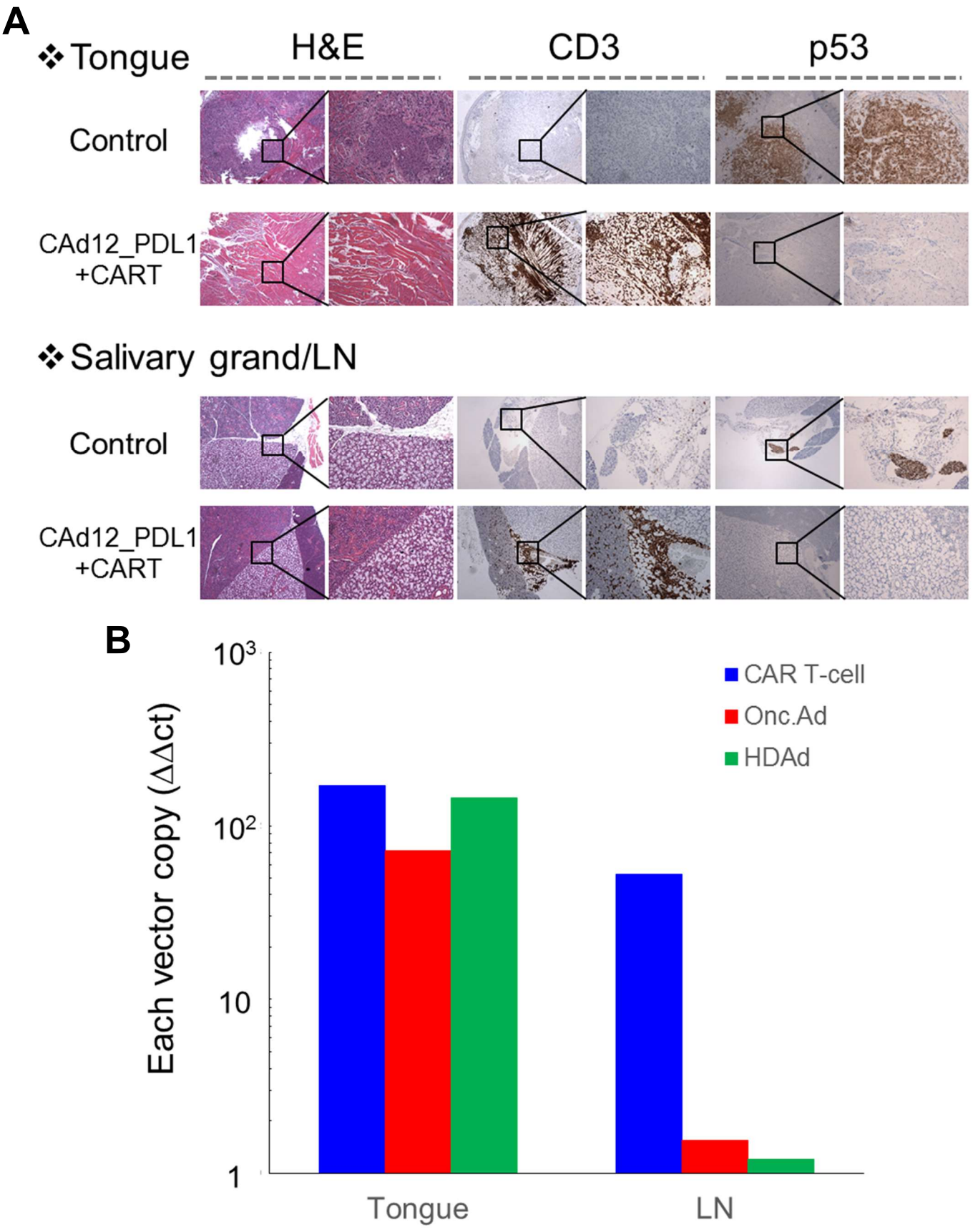

Supplemental Figure 10