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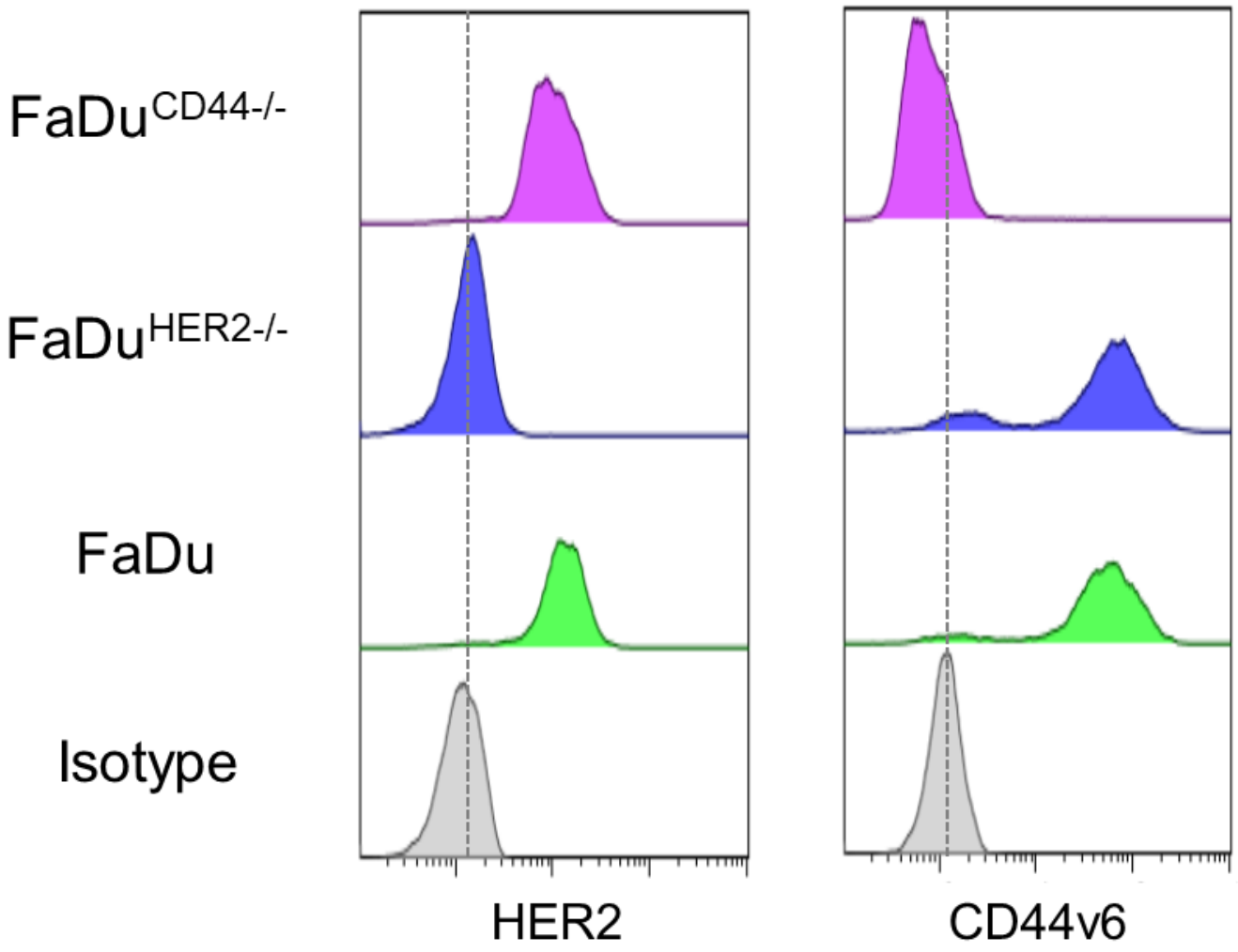
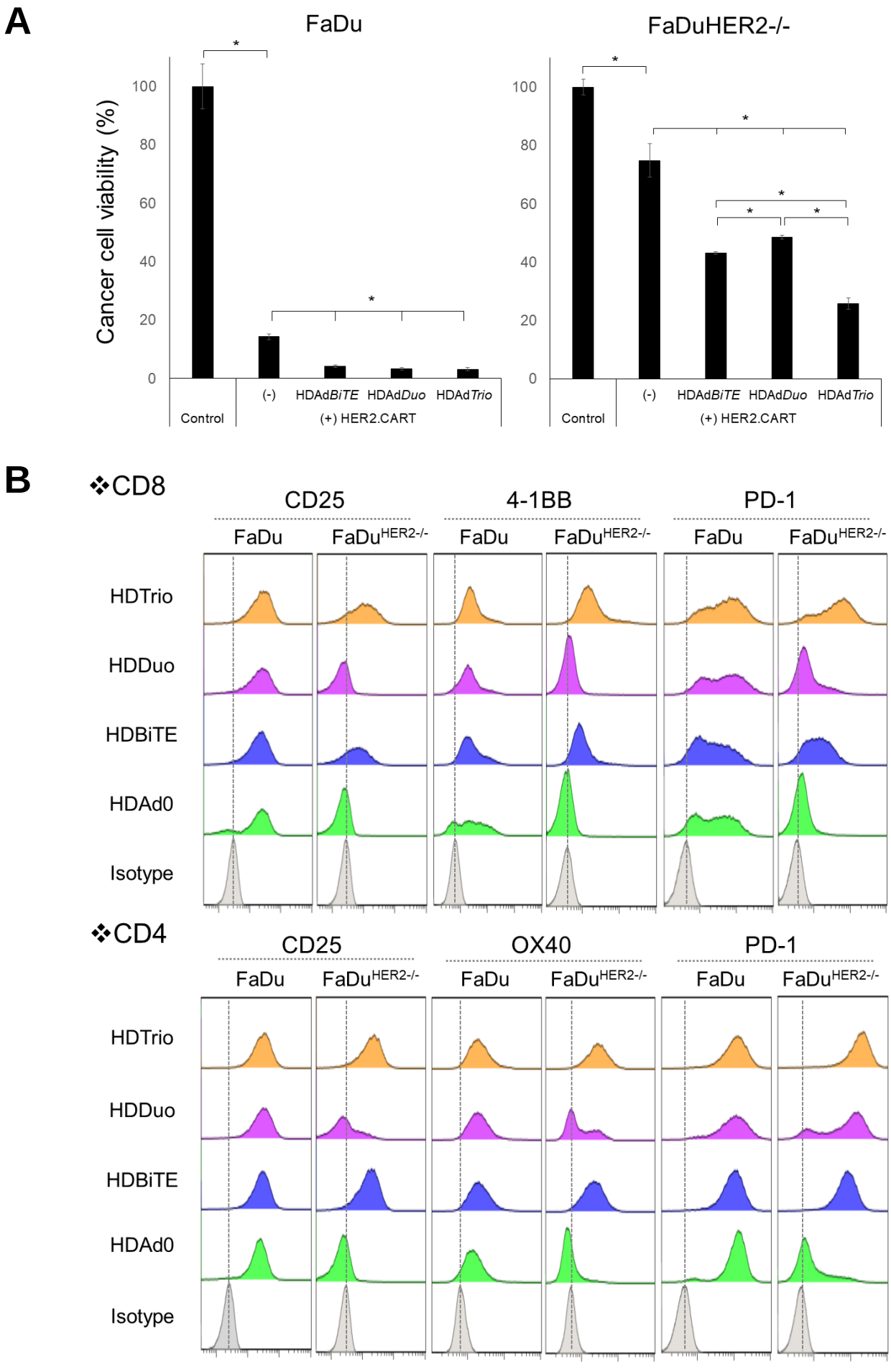
Supplemental Information

Oncolytic Adenovirus Armed with BiTE, Cytokine, and Checkpoint Inhibitor Enables CAR T Cells to Control the Growth of Heterogeneous Tumors

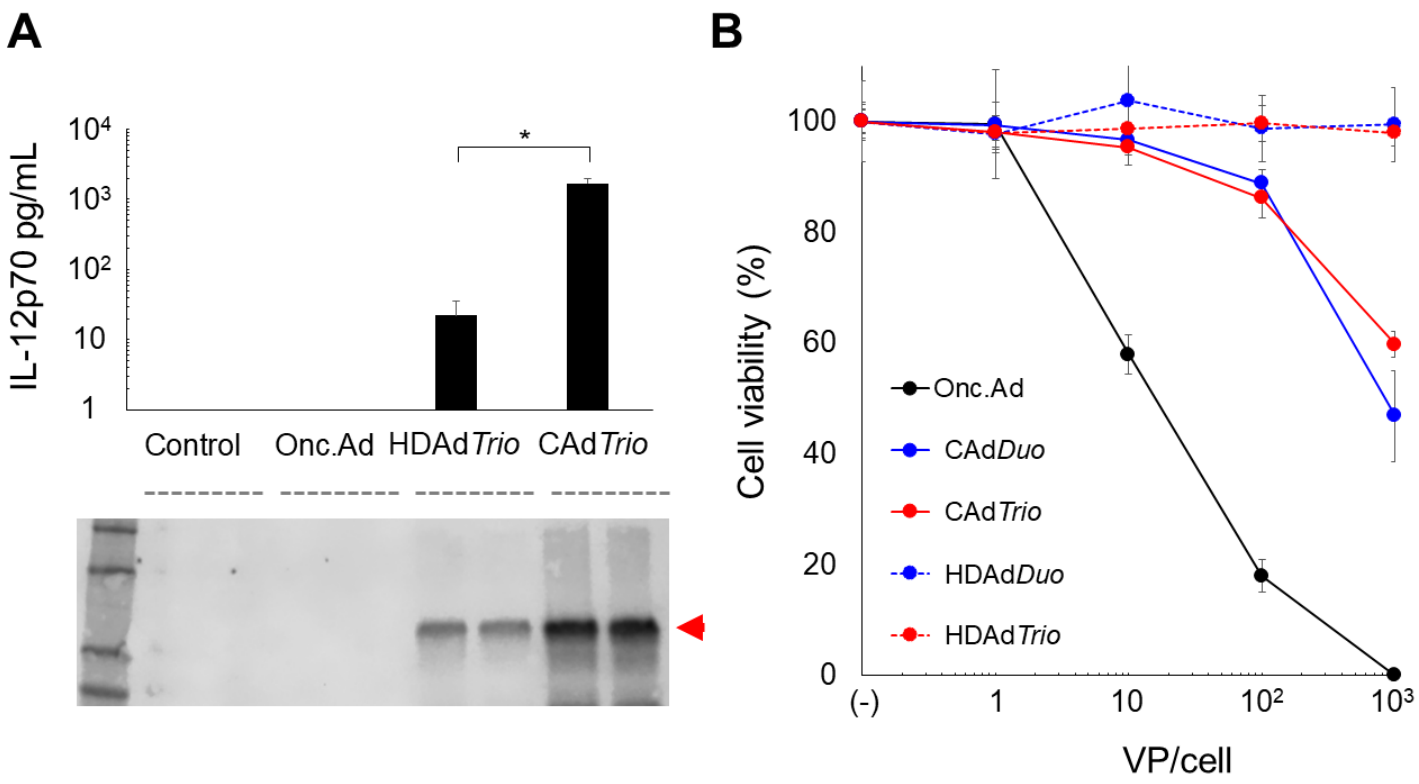
Caroline E. Porter, Amanda Rosewell Shaw, Youngrock Jung, Tiffany Yip, Patricia D. Castro, Vlad C. Sandulache, Andrew Sikora, Stephen Gottschalk, Michael M. Ittman, Malcolm K. Brenner, and Masataka Suzuki



Supplemental Fig. 1. *CRISPR/Cas9 system effectively knocks out target genes in FaDu cells*. We used the CRISPR/Cas9 system to knock either CD44 or HER2 out of FaDu cells. After sorting knockout lines, we confirmed CD44 or HER2 receptor expression on these cells.

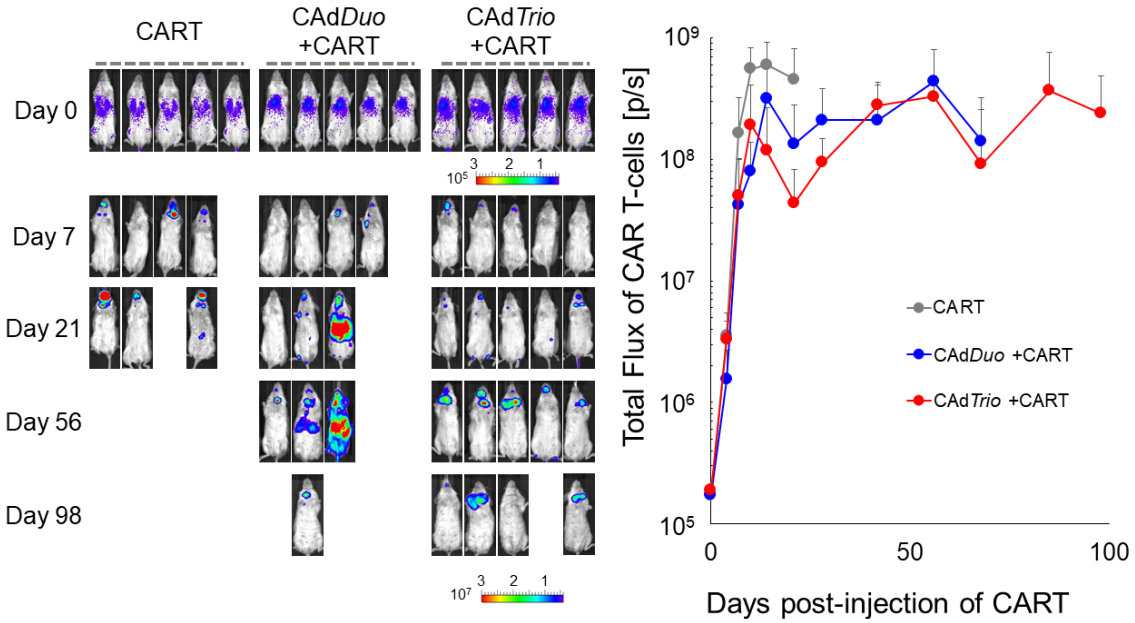

A
FaDu
FaDuHER2-/-
Cancer cell viability (%)
100
80
60
40
20
0
*
Control
(-)
HDAdBiTE
HDAdDuo
HDAdTrio
(+) HER2.CART
B
❖CD8
CD25
4-1BB
PD-1
FaDu
FaDuHER2-/-
HDTrio
HDDuo
HDBiTE
HDAd0
Isotype
❖CD4
CD25
OX40
PD-1

Supplemental Fig. 2. *HDAd-derived CD44v6.BiTE, IL-12p70 and PD-L1 blocking antibody increase the anti-tumor efficacy of HER2.CAR T-cells in vitro.* **(A)** FaDu and FaDuHER2-/- expressing *ffLuc* cells were infected with 100 vp/cell of HDAd0 (no transgene), HDAd*CD44v6.BiTE*, HDAd*Duo* or HDAd*Trio* (n=5 per group). HER2.CARTs were added at 24 hr post-infection (E:T=1:20). Cells were harvested 72 hours post-coculture, and viable cancer cells were analyzed by luciferase assay. Data are presented as means ± SD. **P* < 0.001. **(B)** HER2.CARTs were harvested 72 hours post-coculture, and CD25, PD-1, 4-1BB and OX40 expression were analyzed by flow cytometry.

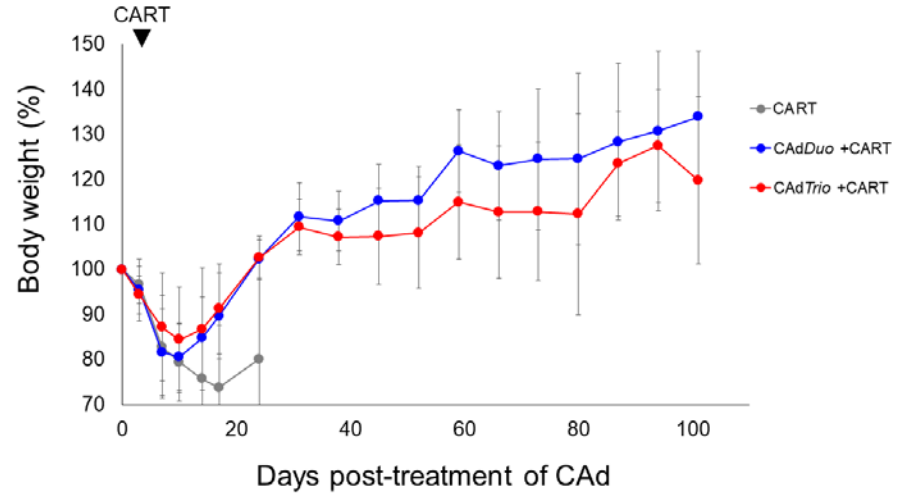


Supplemental Fig. 3. *Co-infection of Onc.Ad with HDAd expressing BiTE, IL-12p70 and PD-L1 mini-antibody amplifies IL-12p70 and PD-L1 blocking antibody expression with oncolysis in vitro.* **(A)** FaDu was infected with a total 20 Vp/cell of HDAd*Trio*, Onc.Ad or with an CAd*Trio* (Onc.Ad:HDAd=1:10) (n=4 per group). Medium samples were collected 48 hours post-infection. Levels of IL-12p70 and PD-L1 mini-antibody in media samples were quantified by IL-12p70 ELISA assay and western blotting for PD-L1 mini-antibody, respectively. Data are presented as means ± SD. *$P < 0.001$. **(B)** FaDu was infected with increasing doses of HDAds, Onc.Ad or with CAds (Onc.Ad: HDAd=1:10) (n=6 per group). Viable cells were analyzed at 96 hours by MTS assay. Data are presented as means ± SD.

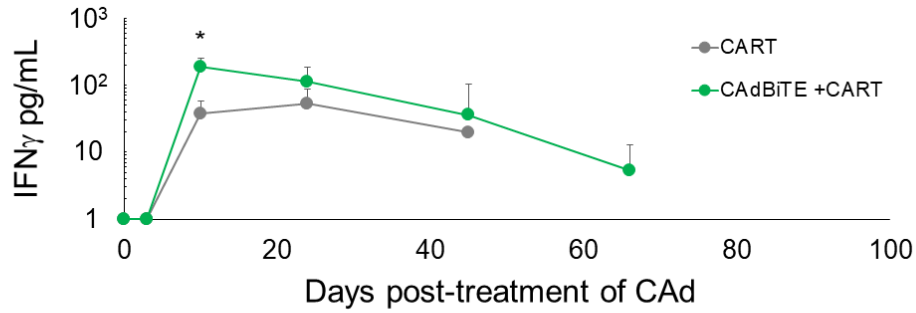
A

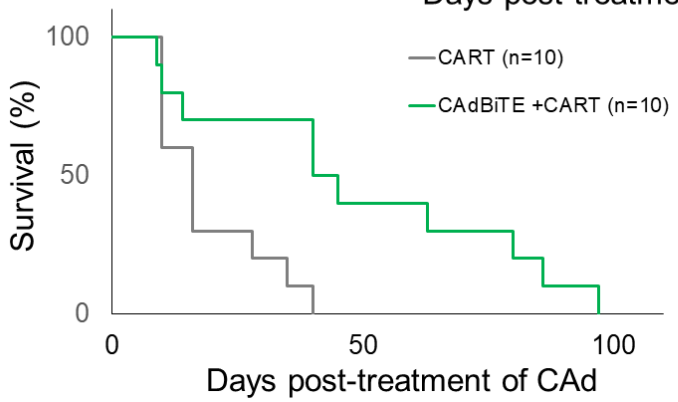


B



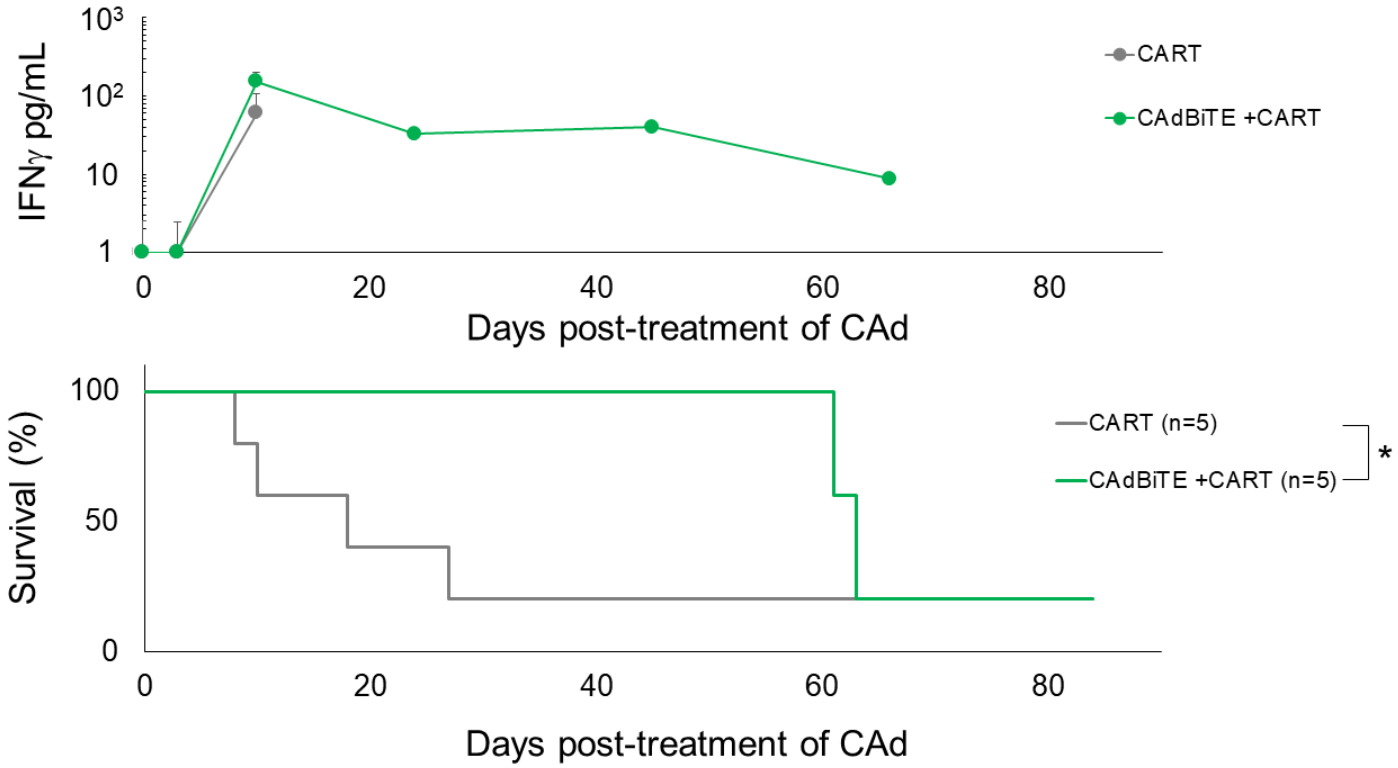
C





	Control	CART	CAd*BiTE* +CART
FaDu	14 day	18 day	45 day
FaDu$^{HER2-/-}$	16 day	18 day	63 day

Supplemental Fig. 4. *Combinatorial treatment can control both primary and metastasized tumors in an orthotopic HNSCC model*. **(A)** FaDu cells were transplanted into the tongues of NSG mice (n=5 per group). A total of $1x10^8$ vp of CAd*s* (Onc:HD=1:20) were injected into the tongue. A total of 0.2 $x10^6$ HER2.CARTs expressing *ffLuc* were systemically administered 3 days post-injection of CAd. Bioluminescence of HER2.CARTs at the tumor area was monitored at different time points. Data are presented as means ± SD. **(B)** Body weight was measured at different time points. Data are presented as means ± SD. **(C)** FaDu cells expressing *ffLuc* were transplanted into the tongues of NSG mice. $1x10^8$ vp of CAd*BiTE* (Onc:HD=1:20) were injected into the tongue. $0.2x10^6$ HER2.CARTs were systemically administered 3 days post-injection of CAds. IFNγ levels in were measured by ELISA from serum samples collected at 0, 3, 10, 24, 45, and 66 days post-injection of CAds (n=10). Data are presented as means ± SD. $*P < 0.001$. Kaplan-Meier survival curve after CAd*BiTE* administration in mice (n=10). Data are presented as means ± SD. **Table** shows median survival of animals treated with CART alone or CAd*BiTE* plus CART.



Supplemental Fig. 5. *CAd-derived CD44v6.BiTE improves HER2.CAR T-cell anti-tumor effects in an orthotopic HER2$^{-/-}$ HNSCC model*. FaDuHER2-/- cells expressing *ffLuc* were transplanted into the tongues of NSG mice. A total of 1x10^{8} vp of CAd*BiTE* (Onc:HD=1:20) were injected into the tongue. A total of 1x10^{6} HER2.CARTs were systemically administered 3 days post-injection of CAd*BiTE*. Bioluminescence of FaDuHER2-/- cells was monitored at different time points. Serum samples were collected at 0, 3, 10, 24, 45, 66 days post-injection of CAd*BiTE* (n=5), and IFNγ levels in serum were measured by ELISA. Data are presented as means ± SD. Kaplan-Meier survival curve after CAd*BiTE* administration (n=5). Data are presented as means ± SD. *$P < 0.02$.