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Supplemental Information

Ligand-Induced Degradation of a CAR Permits Reversible Remote Control of CAR T Cell Activity *In Vitro* and *In Vivo*

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Figure S1

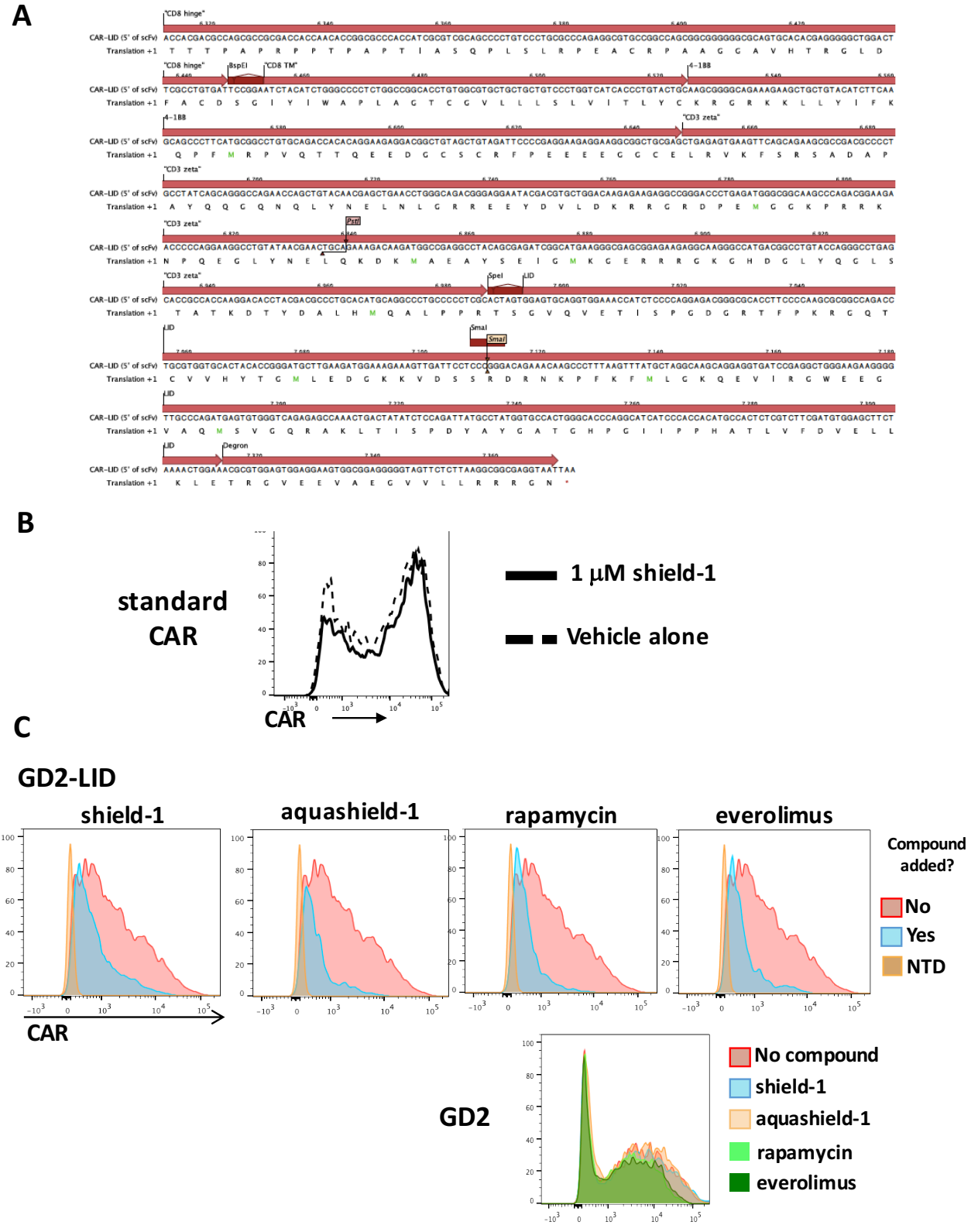

Figure S1. Specificity and versatility of LID/shield-1 system for CAR-LID downregulation
A. Annotated nucleotide and amino acid sequence of the CAR-LID construct (excluding the antigen binding portions). B. Standard GD2 CAR T cells were incubated for 24 hours with either 1 uM shield-1 or vehicle alone and subjected to CAR staining and flow cytometry. C. GD2-LID CAR T cells (top four panels) or standard GD2-CAR T cells (bottom panel) were incubated for 24 hours with 1 uM of either shield-1 or aquashield-1, 100 nM rapamycin, or 100 nM everolimus. T cells were then analyzed for surface CAR by flow cytometry.

Figure S2

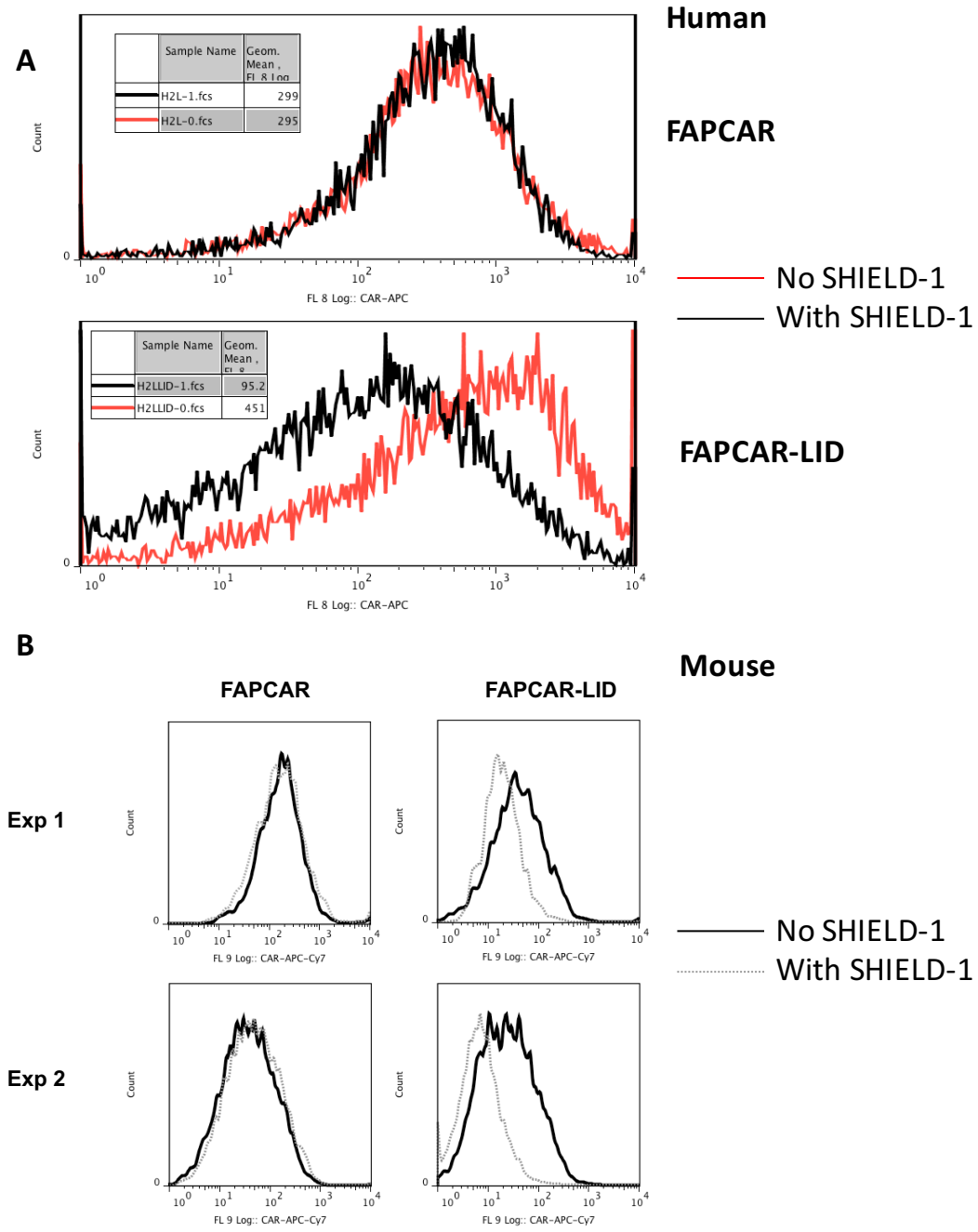

Figure S2. Shield-1-mediated downregulation of FAP-LID CAR T cells. A. Human FAPCAR (upper panel) or FAPCAR-LID (lower panel) was introduced into SupT1 cells, and CAR expression was detected by flow cytometry following a 24-hour incubation with shield-1 or vehicle only. B. Mouse T cells were transduced with mouse FAPCAR or FAPCAR-LID. CAR expression was detected by flow cytometry following a 24-hour incubation with shield-1 or vehicle only. Two independent experiments are shown.

Figure S3

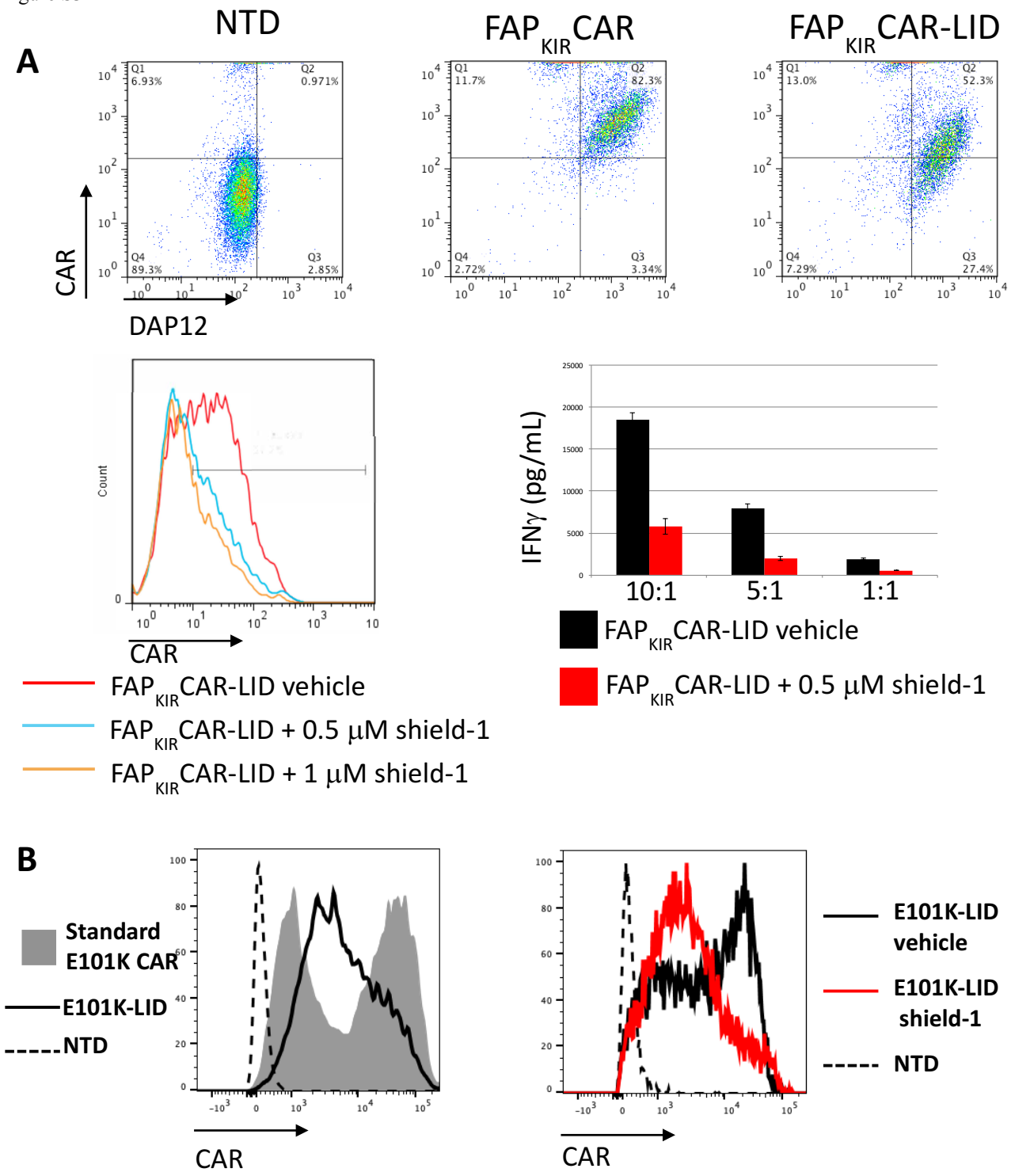

Figure S3. Additional in vitro characterization of CAR constructs used in in vivo studies. A. $FAP_{KIR}CAR$. Top row: Primary human T cells were co-transduced with either the $FAP_{KIR}CAR$ + DAP12 or $FAP_{KIR}CAR$-LID + DAP12. T cells were co-stained with anti-mouse (CAR stain) and anti-DAP12 antibody and analyzed by flow cytometry. Bottom row left: T cells expressing $FAP_{KIR}CAR$-LID were incubated with 0.5 µM shield-1, 1 µM shield-1, or vehicle alone and stained for CAR. Right: T cells expressing $FAP_{KIR}CAR$-LID were incubated with or without shield-1 for 24 hours prior to assay for IFNγ secretion. B. Left: Primary human T cells were transduced with either E101K or E101K-LID, stained for CAR expression, and analyzed by flow cytometry. Right: E101K-LID CAR T cells were incubated with either 1 µM shield-1 or vehicle alone for 24 hours and evaluated for CAR expression by flow cytometry.

Figure S4

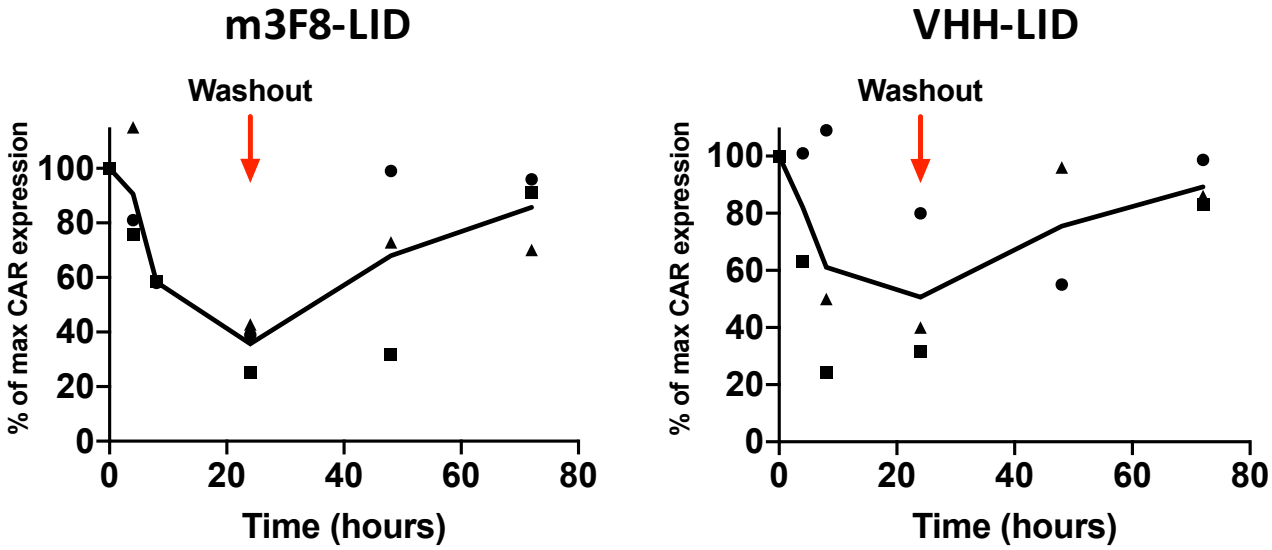

Figure S4. Kinetics of CAR downregulation and reversal in two additional CARs. CAR expression was monitored across time points for m3F8-LID and VHH-LID CAR T cells as described for Figure 2.

Figure S5

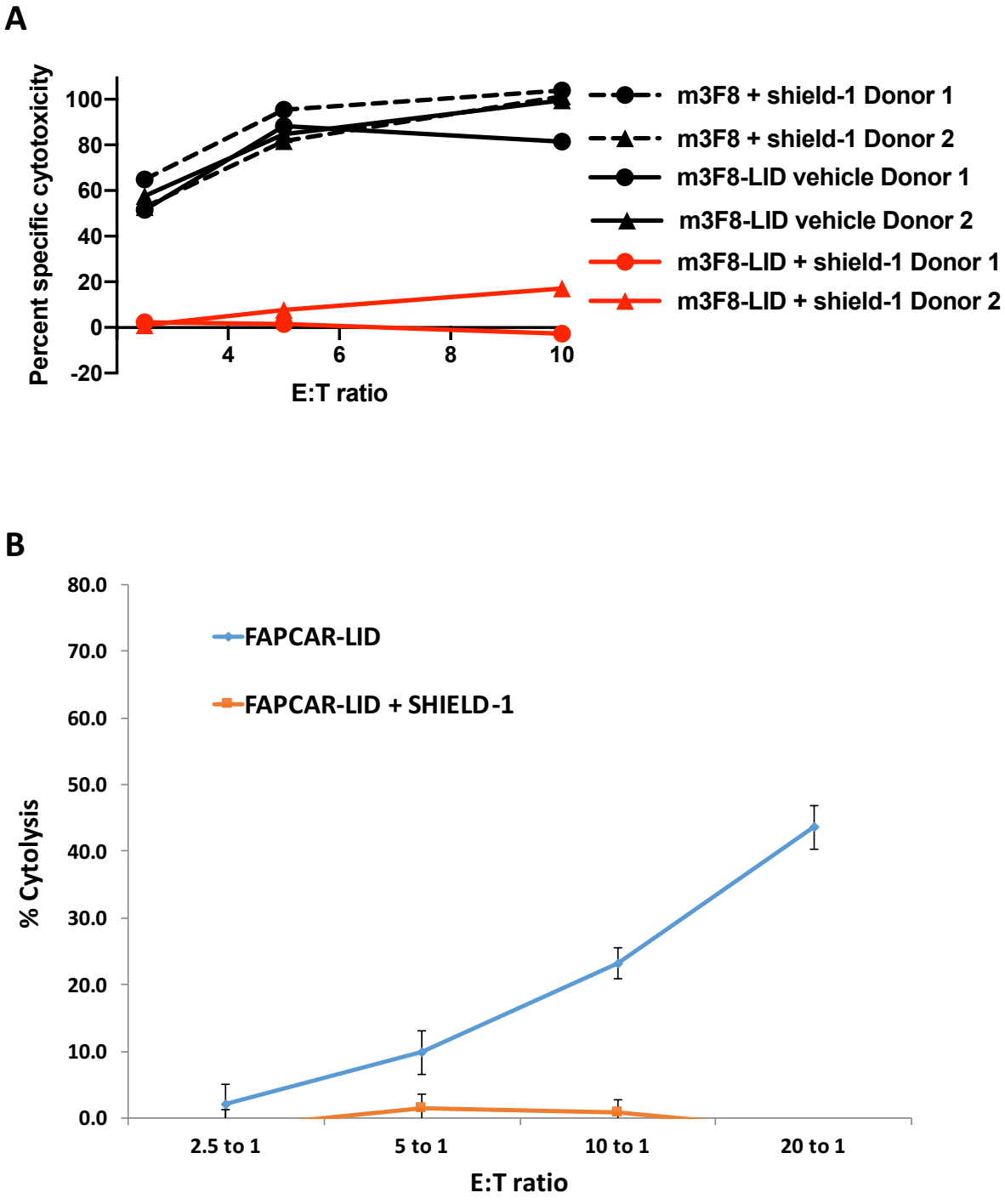

Figure S5. Shield-1-mediated CAR-LID downregulation inhibits T cell effector in vitro cytotoxicity in additional CARs. A. ^{51}Cr cytotoxicity assay performed using m3F8 or m3F8-LID CAR T cells as describe for Figure 3A. Curves are shown from 2 different T cell donors B. Murine T cells expressing FAPCAR-LID, incubated with Mouse 3T3Balb/C cells that had been transfected with both murine FAP and luciferase. Data points are mean +/- SEM of one donor performed in triplicate.

Figure S6

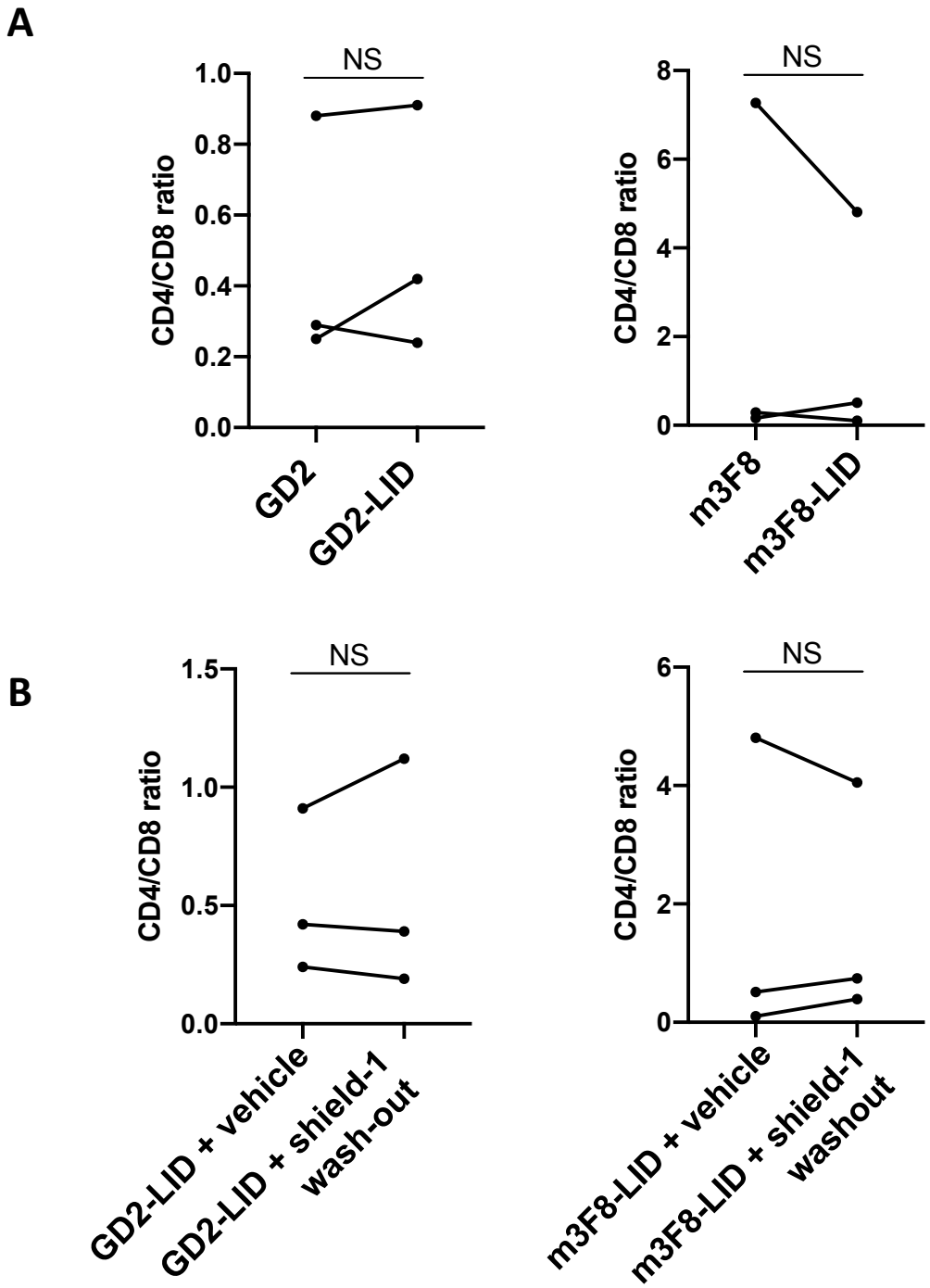

Figure S6. CD4/CD8 subset analysis. A. Standard CAR or CAR-LID T cells were co-incubated with irradiated SY5Y target cells and followed throughout expansion as described for Figure 3B. The ratios of CD4/CD8 T cells in the CAR and CAR-LID groups at the final time point (12 days) post antigen exposure are shown for the GD2 system (left panel) and m3F8 system (right panels) for each of 3 different T cell donors. B. The CD4/CD8 ratio of CAR-LID T cells exposed to vehicle alone or to shield-1 followed by washout (as for Fig 4) are shown at the final day of expansion. Lines connect data points from the same donor. Groups were compared using a two-sided paired t-test. NS = non-significant, n = 3 different donors.

Figure S7

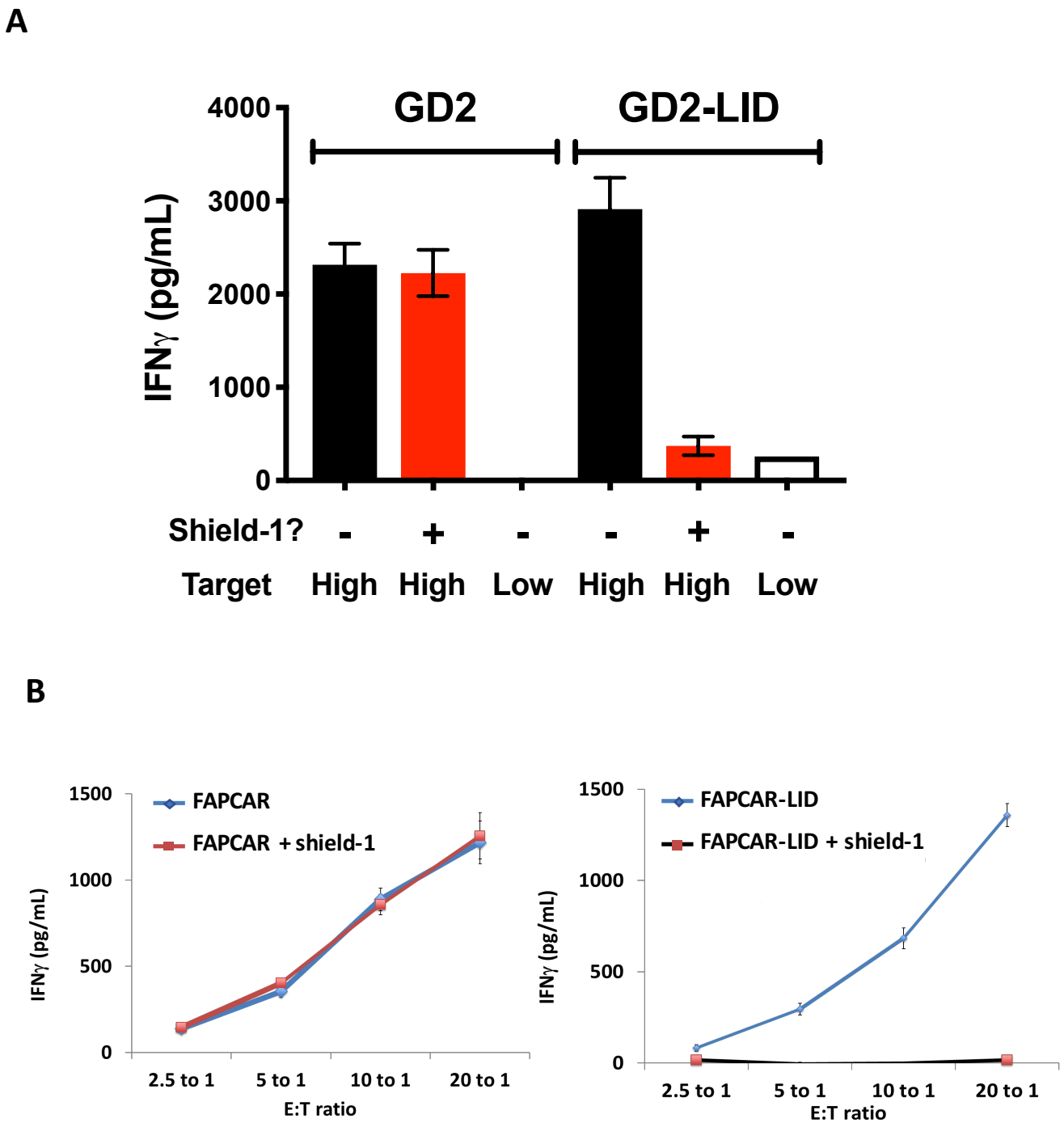

Figure S7. Shield-1-mediated CAR-LID downregulation inhibits T cell IFNγ release in vitro. A. GD2 or GD2-LID CAR T cells were pre-incubated for 24 hours with 1 uM shield-1 and then co-incubated overnight at an E:T ratio of 5:1 with either GD2-high SY5Y or GD2-low NB16 targets. The concentration of IFNγ in the supernatants was determined by ELISA. Results shown are from 1 T cell donor, performed in triplicate. Data points show the mean IFNγ concentration +/- SD. B. Murine T cells expressing mouse FAPCAR or FAPCAR-LID CAR were co-incubated with target cells at the indicated E:T ratios overnight. The concentration of IFNγ in the supernatants was determined by ELISA. Data points are the mean +/- SEM of one donor performed in triplicate.

Figure S8

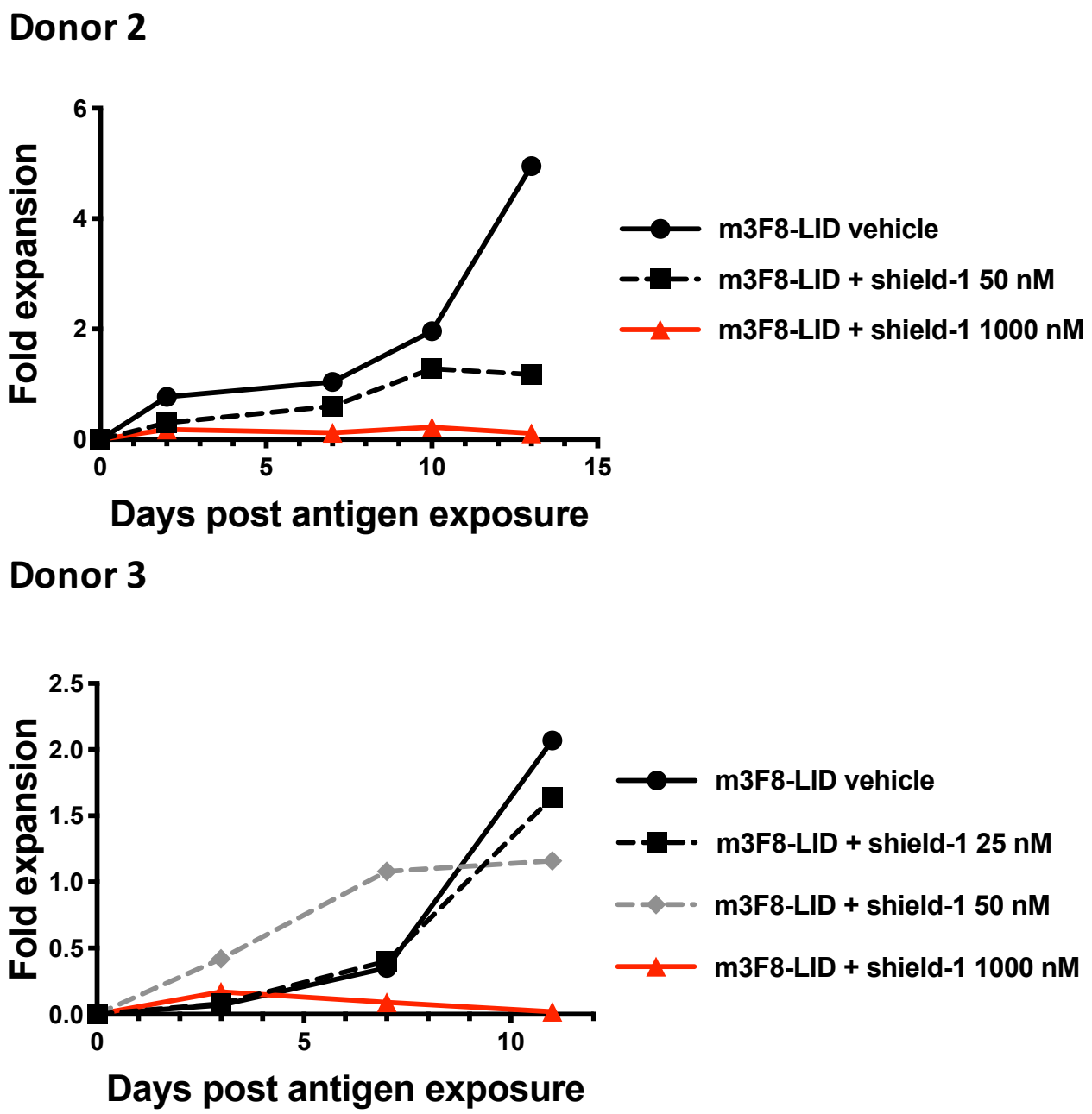

Figure S8. Antigen driven proliferation of m3F8-LID CAR T cells incubated with intermediate doses of shield-1, additional donors. m3F8-LID T cells were pre-incubated with 25-50 nM shield-1, 1000 nM, shield-1 or vehicle alone as described for Figure 5B.

Figure S9

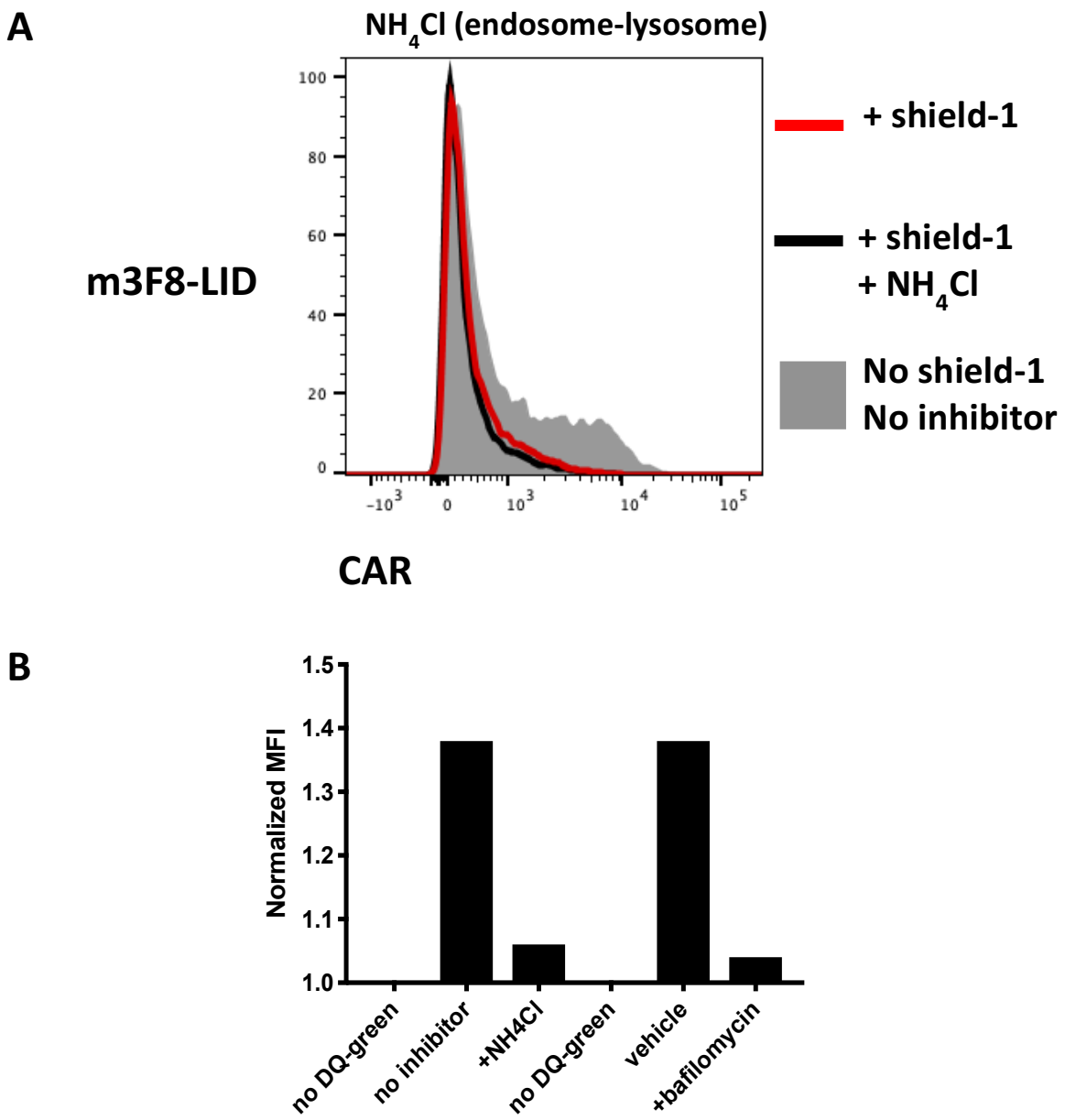

Figure S9. An additional endosome-lysosome inhibitor does not impact shield-1 mediated m3F8-LID downregulation. m3F8-LID CAR T cells were incubated with or without shield-1 for 24 hours. During the last 6 hours of shield-1 incubation, an aliquot was incubated with the endosome-lysosome inhibitor NH_4Cl. Cells were then stained for CAR and analyzed by flow cytometry. B. Positive control assay for endosome-lysosome inhibitors. Primary human T cells at day 6 post stimulation with anti-CD3/anti-CD28-coated beads were incubated with bafilomycin (100 nM), ammonium chloride (50 mM), or vehicle alone for 5.5 hours. Then 10 μg/mL DQ-green BSA was added to cells for 15 minutes. DQ-green BSA fluorescence is quenched at baseline and unquenched upon hydrolysis. Following incubation, cells were washed twice in PBS and returned to the incubator in fresh media for another 15 minutes. Viability dye was then added, and fluorescence was detected by flow cytometry. MFI from each group was normalized to MFI of cells treated in parallel that did not receive DQ-green BSA.

Figure S10

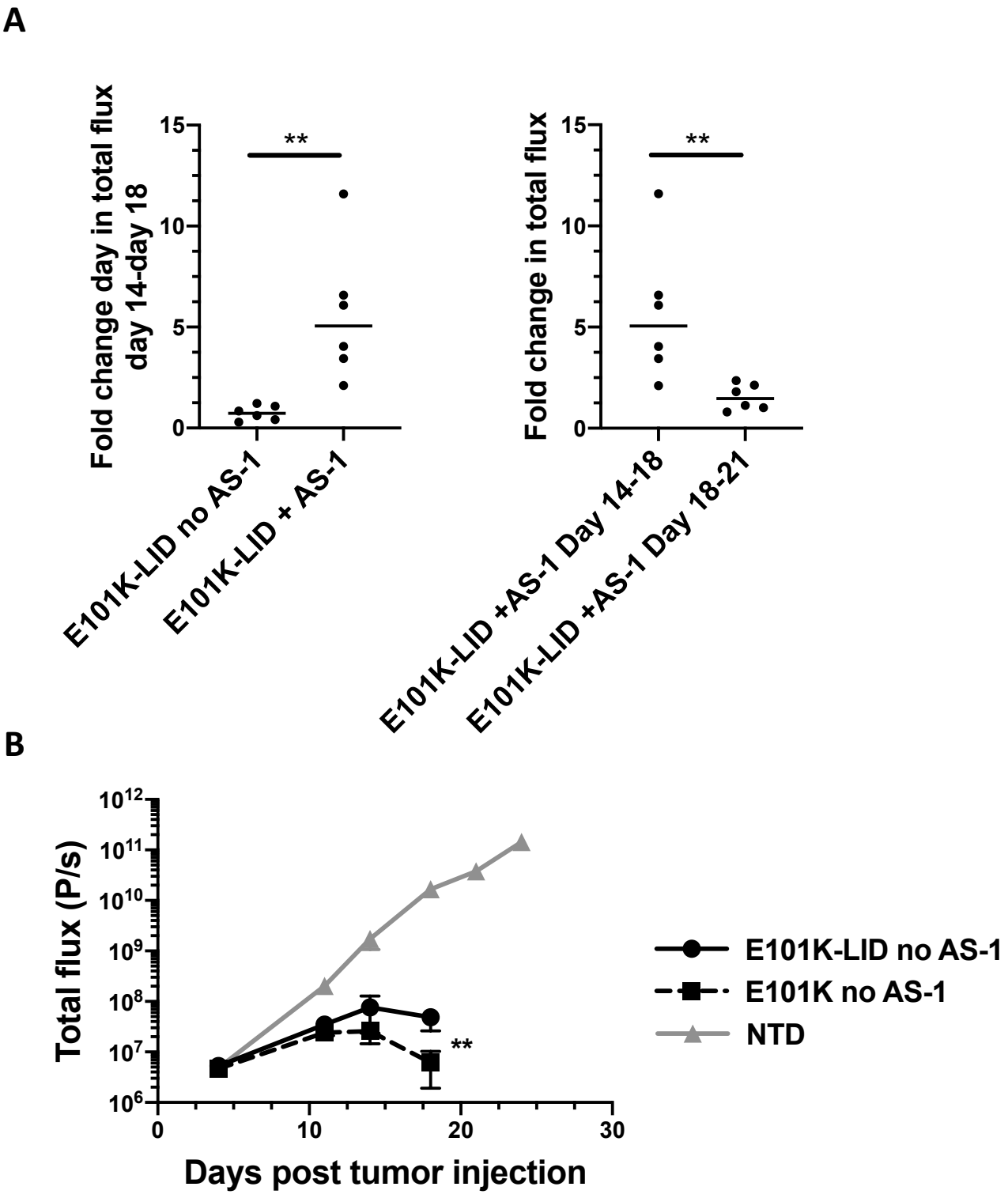

Figure S10. Analysis of impact of AS-1 and LID domain on tumor growth. A. Slope of total flux (tumor burden) compared between mice receiving AS-1 and those not receiving it between 2 days and 6 days following initiation of AS-1 delivery (left panel). Slope of total flux (tumor burden) of mice receiving AS-1 comparing the period during AS-1 delivery (day 14-day 18) to that following the end day of AS-1 delivery (day 18-day 21) (right panel). B. Tumor burden in mice receiving either standard E101K CAR, E101K-LID CAR, or control NTD T cells. Populations were compared using a two-tailed Mann-Whitney comparison. ** $p < 0.01$