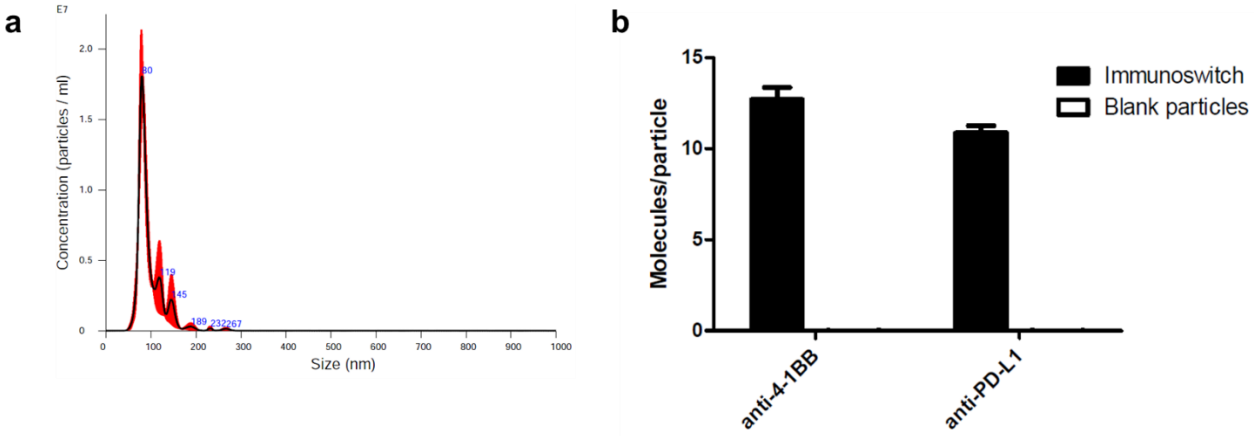
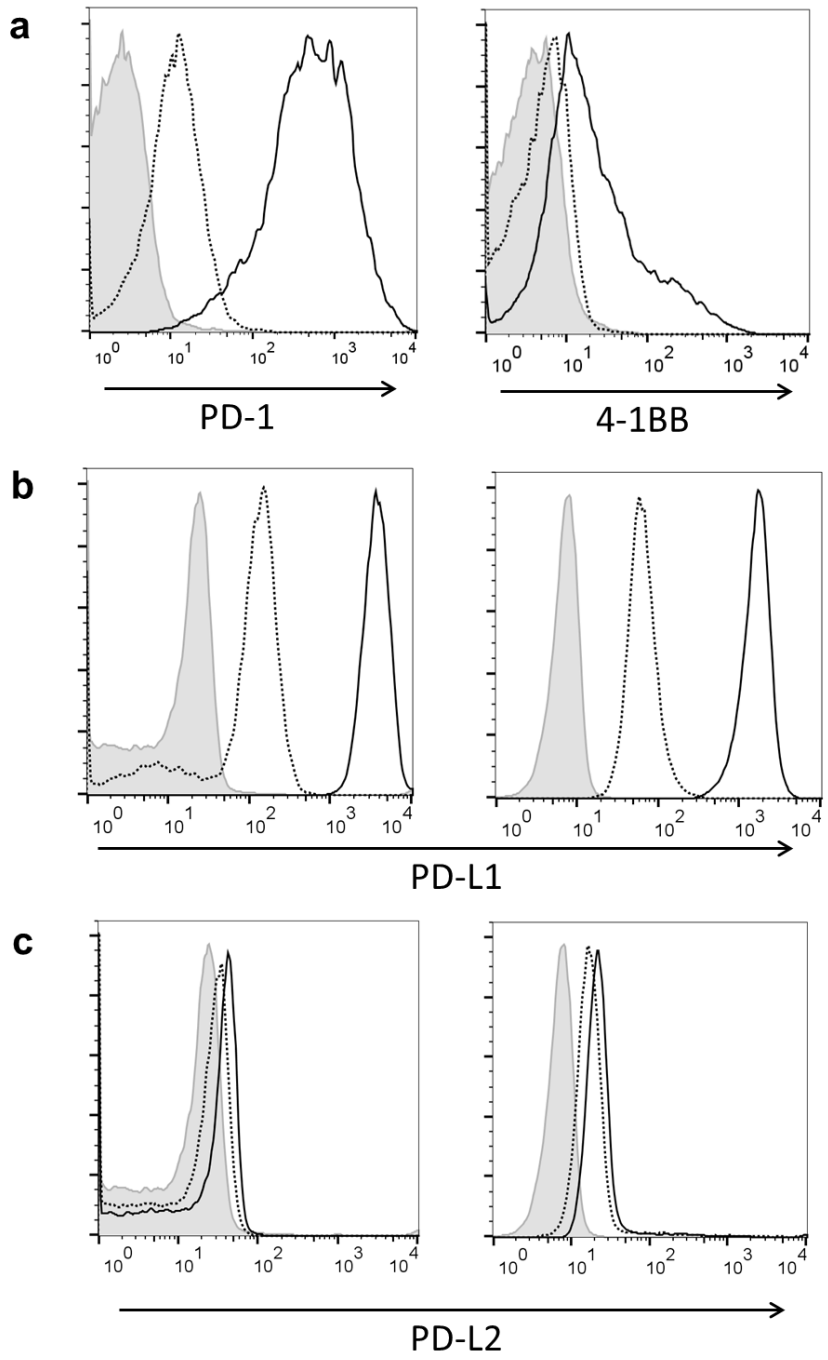
Dual Targeting Nanoparticle Stimulates the Immune System to Inhibit Tumor Growth

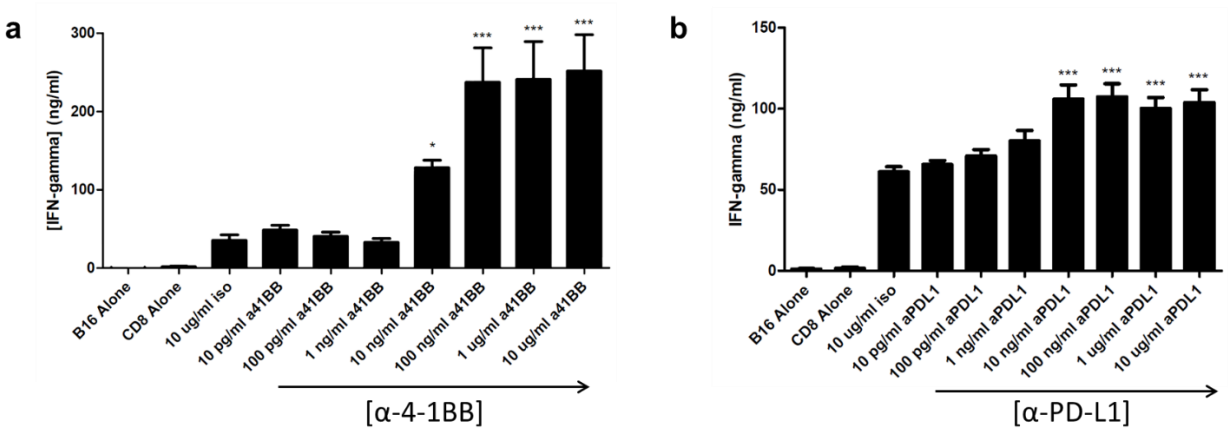
Alyssa K. Kosmides, John-William Sidhom, Andrew Fraser, Catherine A. Bessell, Jonathan P. Schneck[*]



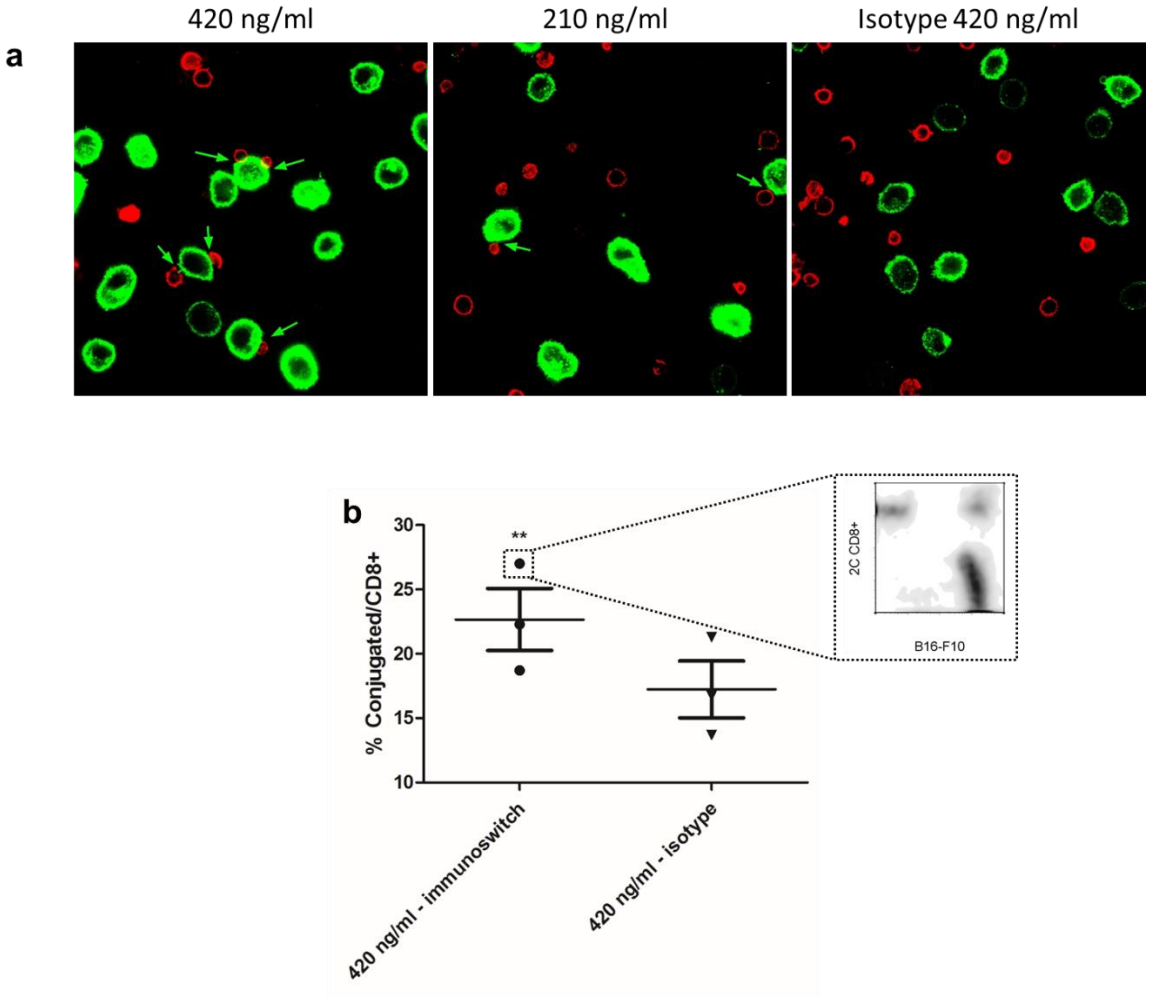
Supplemental Figure 1. Immunoswitch particles combine anti-PD-L1 mAb and anti-4-1BB mAb on a nanoparticle platform. (**a**) Iron-dextran nanoparticle platform is 80nm in diameter. Unconjugated iron-dextran nanoparticle size was measured by nanoparticle tracking analysis. Mean (black) and SEM (red) of 3 separate captures of a single sample is shown. The mode of the dataset is 81.1 +/- 1.6 nm and the mean of the dataset is 96.0 +/- 1.9 nm. (**b**) Number of anti-4-1BB and anti-PD-L1 molecules per particle, as measured by fluorescently labeled secondary antibody. Mean +/- SEM is shown, averaged across 3 independent experiments. No significant differences in antibody level per each particle type, as measured by two-way ANOVA with Bonferroni posttest.



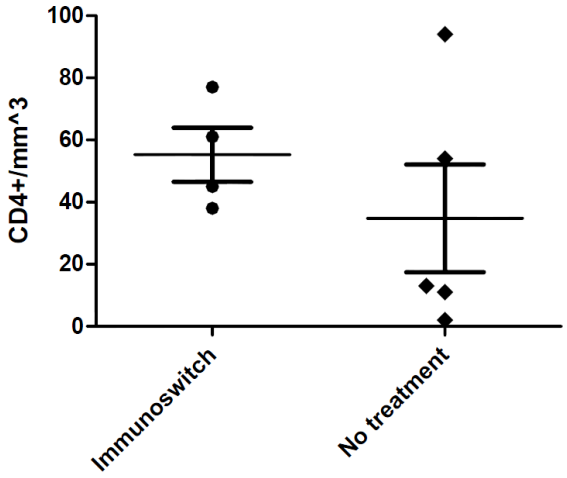
Supplemental Figure 2. *In vitro* model results in upregulated inhibitory molecules on CD8 and B16 cells. (**a**) Flow cytometry plots of PD-1 and 4-1BB expression on CD8 cells on day 0 (dotted) and day 8 after expander bead stimulation on days 0 and 4 (solid), as compared with isotype (grey, filled). B16 cells were cultured in media supplemented with 20 ng/ml IFN-γ for 48 hours, and PD-L1 and PD-L2 expression was assessed by flow cytometry. Flow cytometry plot of PD-L1 (**b**) and PD-L2 (**c**) expression by B16-SIY (left) and B16-F10 (right) cells on day 0 (dotted) and 48 hours after IFN-γ treatment (solid), as compared with isotype (grey). B16-SIY and B16-F10 upregulate PD-L1 but not PD-L2 in response to IFN-γ incubation.



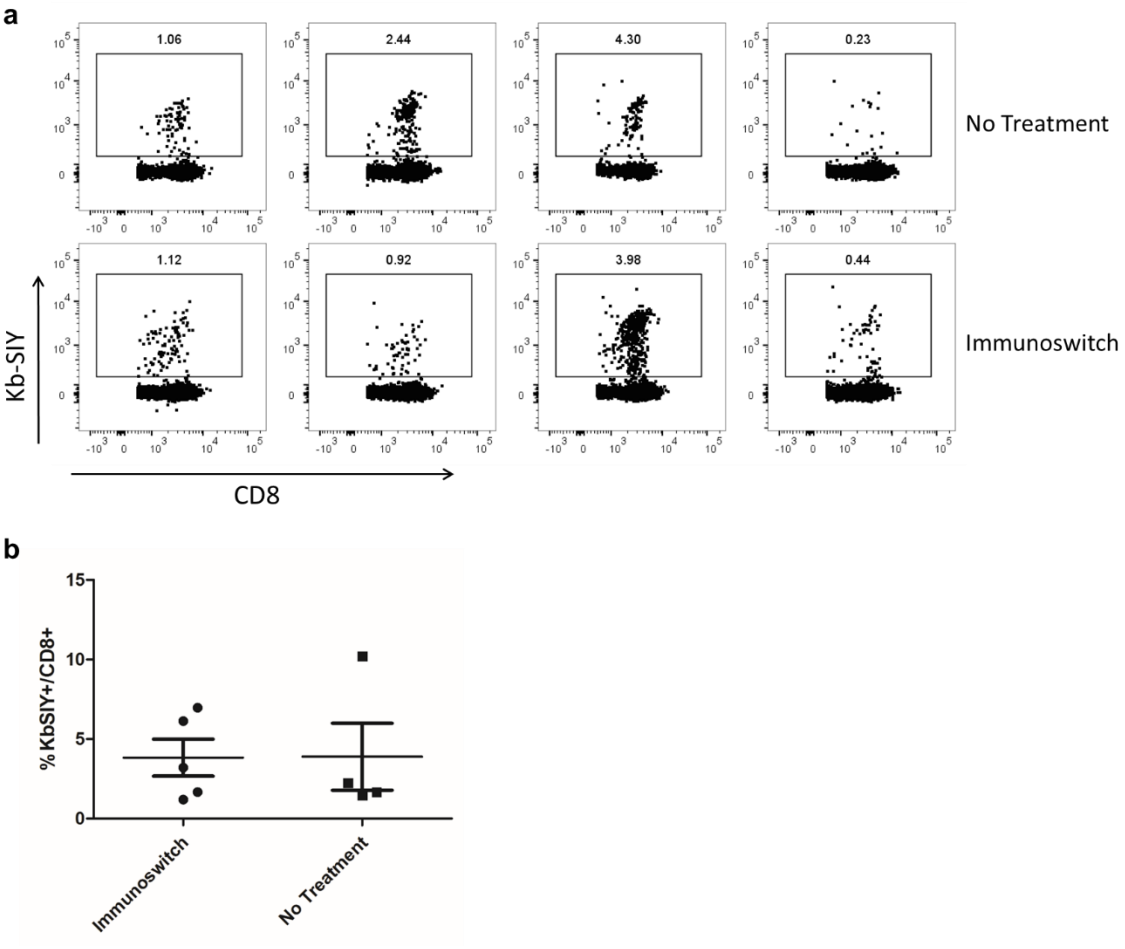
Supplemental Figure 3. Soluble anti-4-1BB and anti-PD-L1 mAb increase PD-1^{hi} CD8+ T cell activation when co-incubated with PD-L1^{hi} target cells. PD-1^{hi} 2C CD8 cells and PD-L1^{hi} cognate B16-SIY cells were co-incubated at a 1:1 ratio in the presence of titrating amounts of soluble anti-4-1BB (**a**) or anti-PD-L1 (**b**) antibody. IFN-γ secretion by CD8 cells was measured by ELISA after 18 hours. Both antibodies induced a significant increase in the level of IFN-γ secretion as compared to isotype at concentrations at and above 10 ng/ml. Mean +/- SEM are shown of three replicates. Significance measured by one-way ANOVA with Dunnett's posttest, comparing each condition to isotype. (*p<0.05, ***p<0.001)



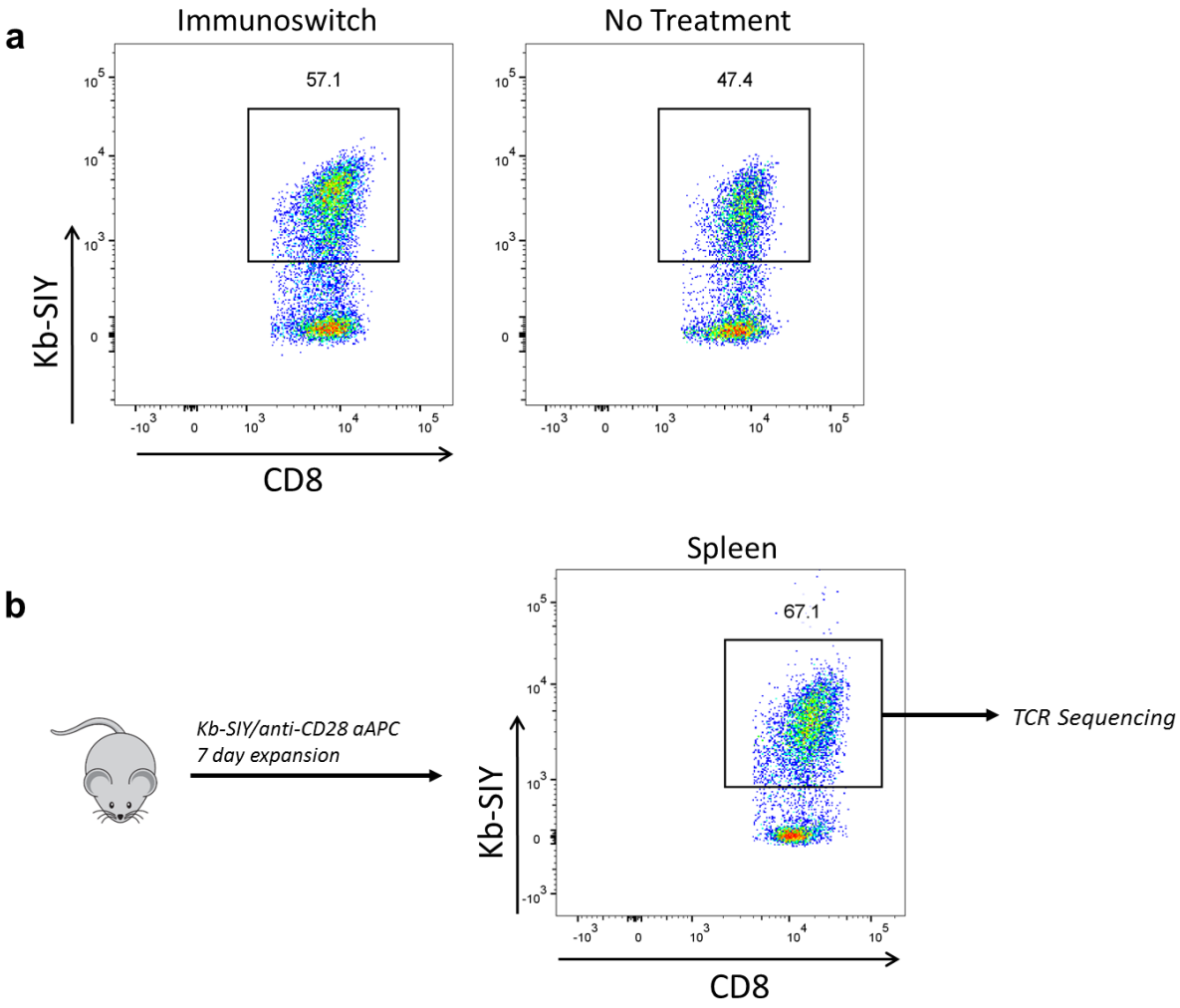
Supplemental Figure 4. Immunoswitch particles increase effector-target cell conjugation. (**a**) Representative confocal images of 2C CD8+ T cells (red) and B16-F10 cells (green) in the presence of immunoswitch or isotype particles. Arrows show conjugate formation. (**b**) Conjugate formation was measured by gating on double red and green positive cells by flow cytometry. Each data point represents an independent experiment. 420 ng/ml of immunoswitch particles significantly increased conjugation rate as measured by two-tailed paired t-test (p=0.0016).



Supplemental Figure 5. CD4+ T cell density within the tumor microenvironment is unchanged with immunoswitch treatment. C57BL/6 mice (n=5/group) were injected with B16-SIY cells SC on day 0, and half were treated with immunoswitch particles IT on days 8 and 11. On day 14 TILs were harvested and analyzed. CD4 density with the tumor was unchanged with treatment, measured by a two-tailed t-test.



Supplemental Figure 6. Tumor specific CD8+ T cells are present in the periphery at the same levels in treated and non-treated mice. (**a**) Flow plots of Kb-SIY staining on CD8+ T cells from the spleens of immunoswitch-treated and non-treated mice. (**b**) Percent of Kb-SIY+/CD8+ T cells in the peripheral blood of immunoswitch-treated and non-treated mice.



Supplemental Figure 7. A significant portion of the anti-tumor response in the presence and absence of immunoswitch treatment is Kb-SIY specific. (**a**) Representative flow plots of Kb-SIY staining on CD8+ T cells from the TILs of immunoswitch-treated and non-treated mice. (**b**) Expansion protocol used to sequence the Kb-SIY specific CD8+ T cell response. Splenocytes from non-tumor bearing C57BL/6 mice were expanded with Kb-SIY/anti-CD28 aAPC for 7 days. Kb-SIY+ cells were sorted and sequenced after 7 days.