OMTO, Volume *28*

Supplemental information

Oncolytic adenovirus coding for bispecific T cell engager against human MUC-1 potentiates T cell response against solid tumors

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Table S1. List of antibodies used in this study.

Experiment	Antibodies	Manufacturer
Expression of cell surface antigens	APC anti- human MUC1 (clone 16A) FITC anti- human CD3 (clone SK7)	Biolegend, California, USA
Binding assay	Anti-human MUC1 (clone HMFG2)	Merck, New Jersey, USA
T cell activation and proliferation assay	PE-CF594 anti-human CD3 (clone UCHT1) PE anti-human CD4 (clone RPA-T4) FITC anti-human CD8 (clone RPA-T8) PE-Cy™7 anti-human CD69 (clone FN50) PE-Cy™5 anti-human CD25 (clone M-A251)	BioLegend, California, USA
Analysis of immune cells populations from *in vivo* tumor samples	FITC anti-human CD3 (clone, OKT3) AF700 anti-human CD4 (clone, A161A1) BV421 anti-human CD8 (clone, SK1) PE-Cy™7 anti-human CD69 (clone, 17A2) APC anti-human CD56 (clone, FN50) PE anti-human TIM3 (clone, F38-2E2) PE-CF594 anti-human PD-1 (clone, NAT105)	BioLegend, California, USA

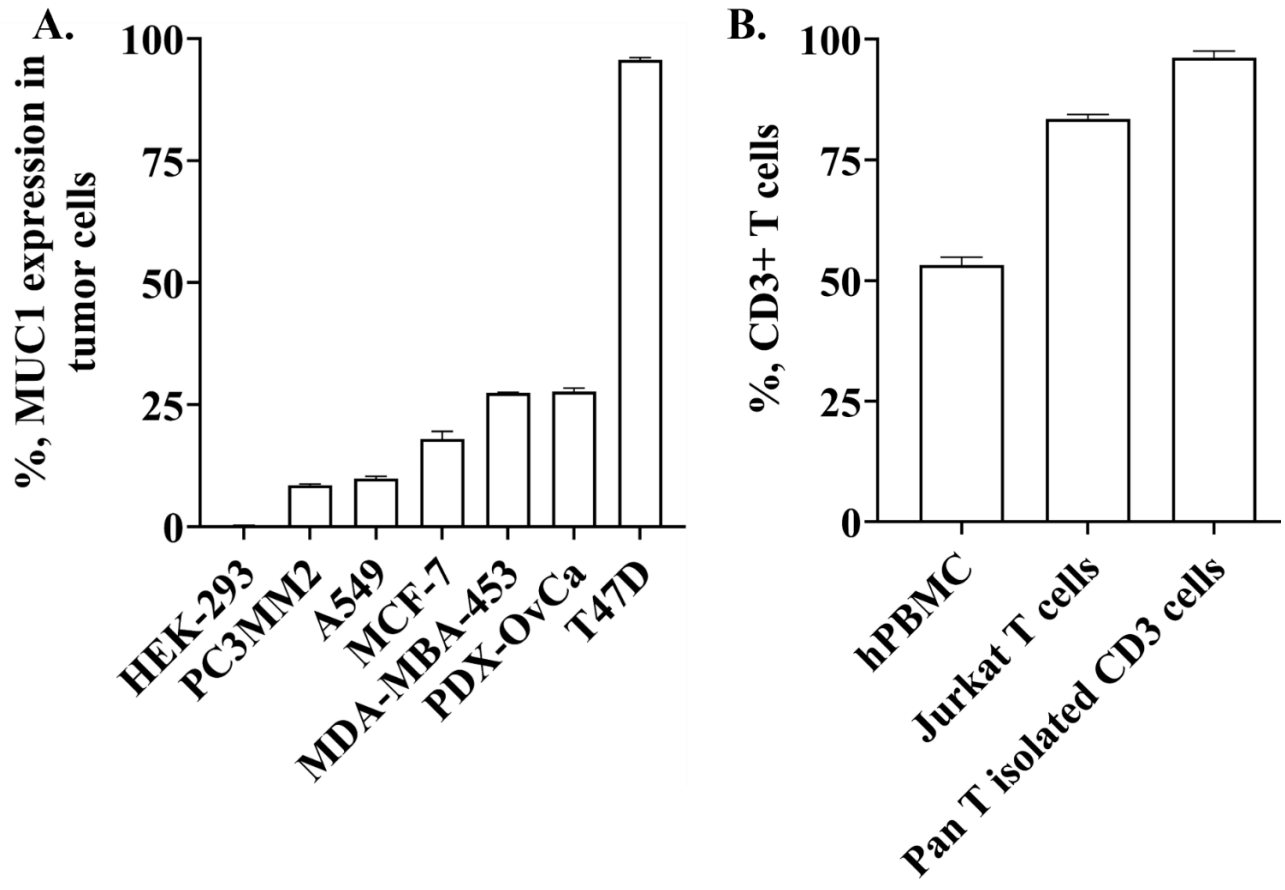

Figure S1. Expression of cell surface antigen from different cells. (A) MUC1 expression in a panel of different cell lines. **(B)** Expression of CD3 T cells in different cell lines used in this study.

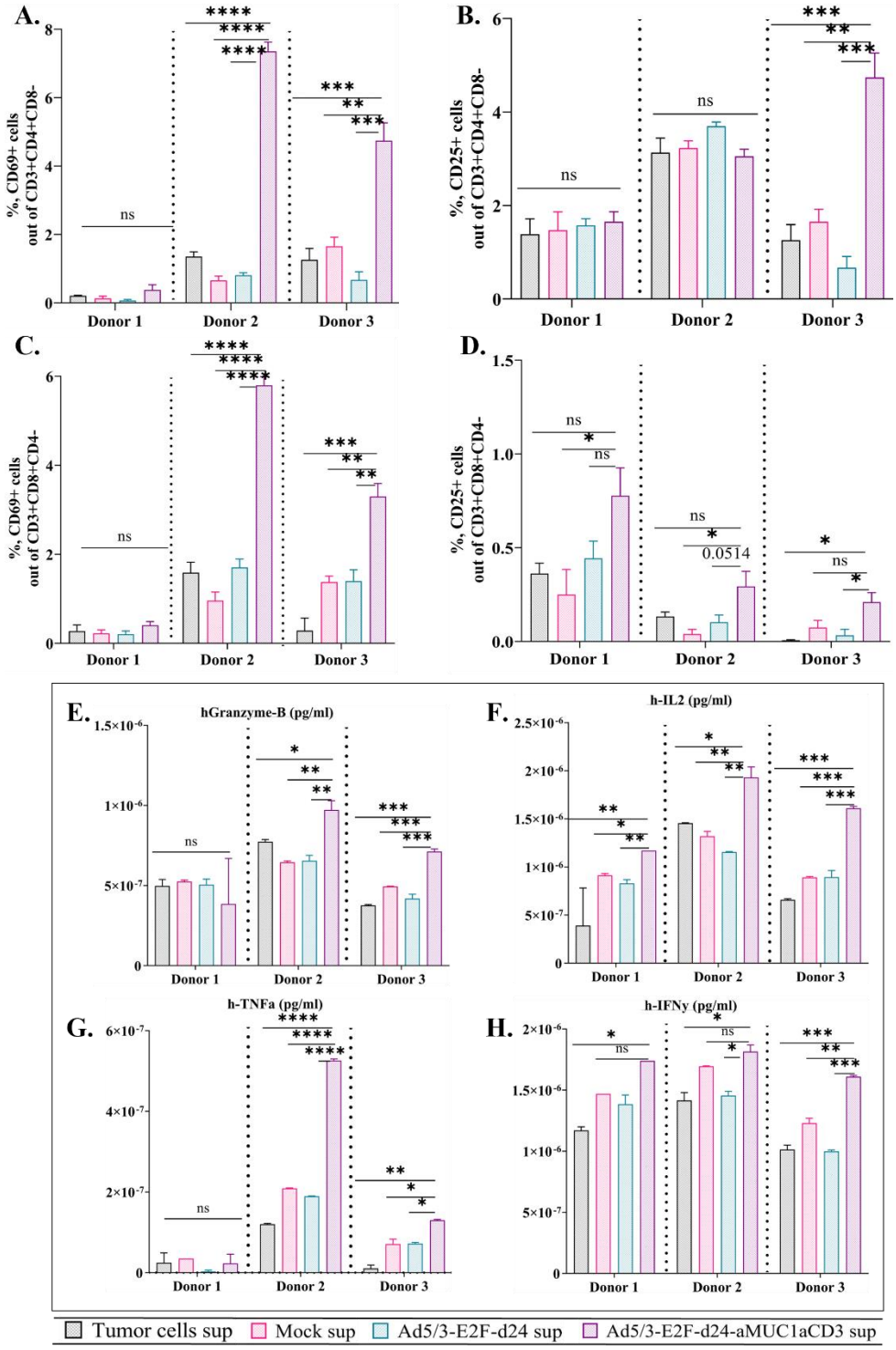

Figure S2. TILT-321 virus-derived aMUC1aCD3-BsTe functionality in T cells from three different healthy donors. (A-D) Isolated T cells were incubated with Ad5/3-E2F-d24 and Ad5/3-E2F-d24-aMUC1aCD3 supernatants. Uninfected (Mock) supernatants were used a negative control. 3 days after coculture, CD3+CD4+CD69+ or CD3+CD4+CD25+ and CD3+CD8+CD69+ or CD3+CD8+CD25+ T cells activation was assessed by flow cytometry. Cytokine concentrations in culture supernatants **(E)** GranzymeB, **(F)** IL2, **(G)** TNFa and **(H)** IFNy were quantified using a cytometric bead array. The mean

± SEM of quadruplets is shown. Statistical significance is represented as *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

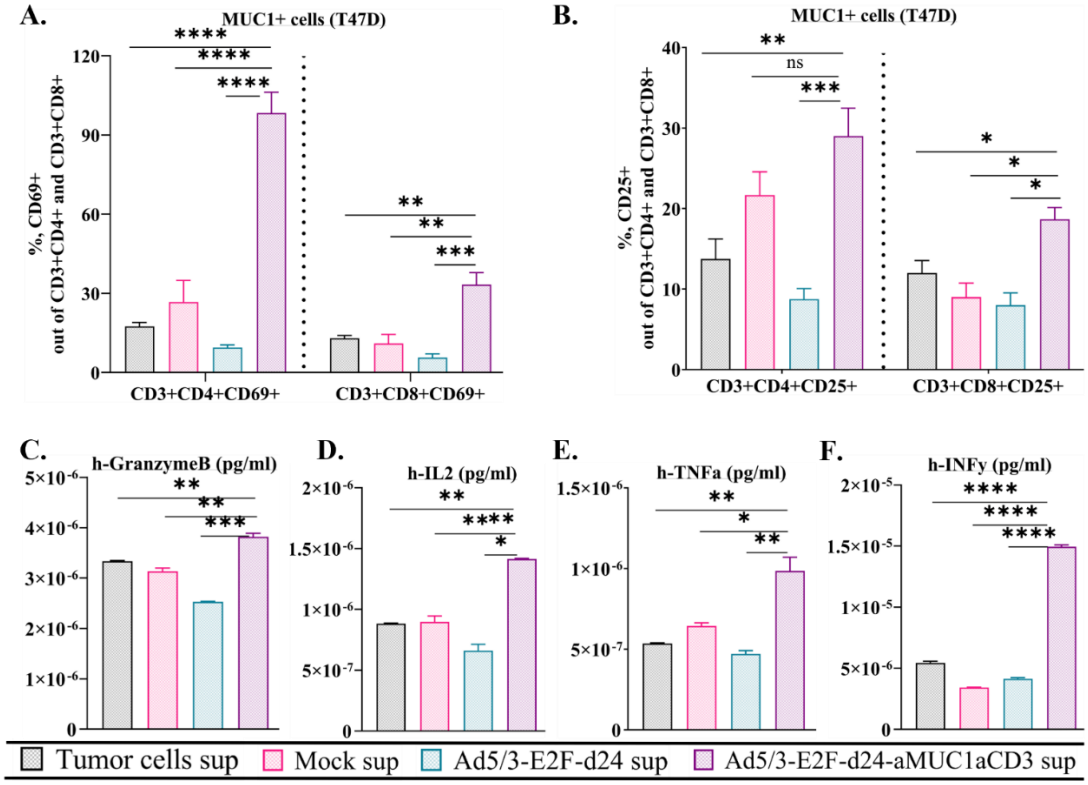

Figure S3. ***In vitro*** **characterization of virally released aMUC1aCD3-BsTe.** Ad5/3-E2F-d24-aMUC1aCD3-derived supernatant containing aMUC1aCD3-BsTe was added to cultures of MUC1 positive T47D cells monolayer in the presence of unstimulated T cell at ratio of T: E= 5. Uninfected supernatant was used as a Mock. Total percentage of activated T cells **(A)** CD3+CD4+CD69+ or CD3+CD4+CD25+ and, **(B)** CD3+CD8+CD69+ or CD3+CD8+CD25+ that are cytotoxic for MUC1+ tumor cells. The supernatants harvested after 72 hours of co-cultures were analyzed for **(C)** GranzymeB, **(D)** IL2, **(E)** TNFa and **(F)** IFNy using BD FACS Array bioanalyzer by Flow cytometry. The mean ± SEM of quadruplets is shown. Statistical significance is represented as *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

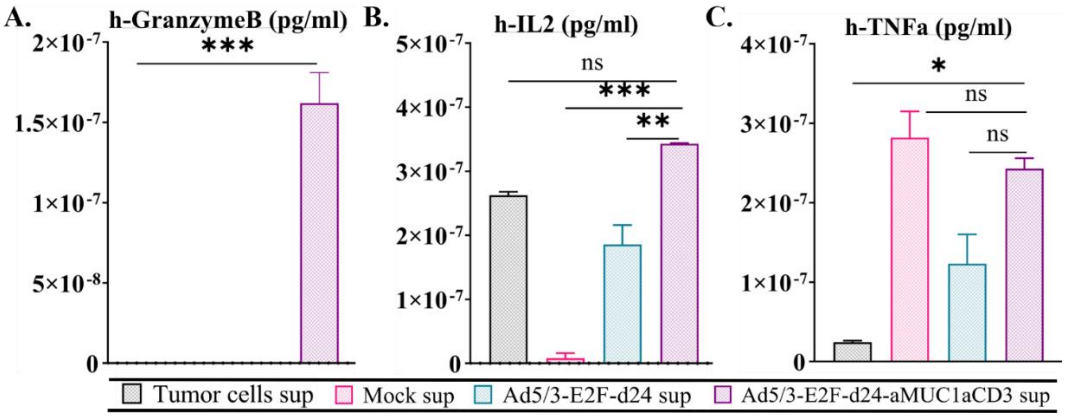

Figure S4. Evaluation of cytokine expression produced by proliferated T cells (CFSE stained). Supernatants were analyzed for **(A)** GranzymeB, **(B)** IL2 and, **(C)** TNFa by Flow cytometry.

The mean ± SEM of duplicates is shown. Statistical significance is represented as *p<0.05, **p<0.01, and ***p<0.001.

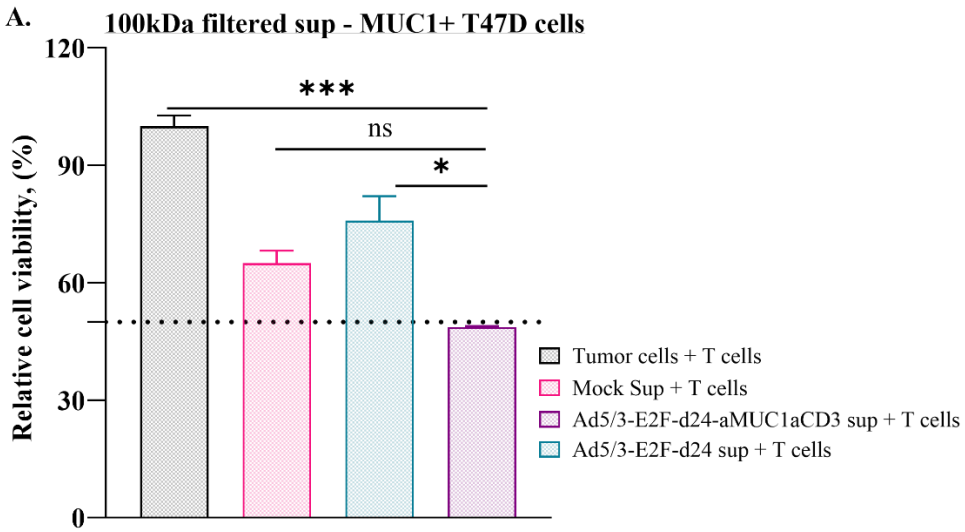

Figure S5. Cytotoxicity of virus-derived aMUC1aCD3 in co-cultures of T cells with MUC1+ tumor cells using 100 kDa filtered supernatant. (A) Ad5/3-E2F-d24-aMUC1aCD3-derived supernatant containing aMUC1aCD3-BsTe was added to cultures of MUC1 positive T47D cells monolayer in the presence of unstimulated T cell at T: E = 5. Uninfected supernatant is used as a Mock.

The mean ± SEM of triplicates is shown. Statistical significance is represented as *p<0.05 and ***p<0.001.

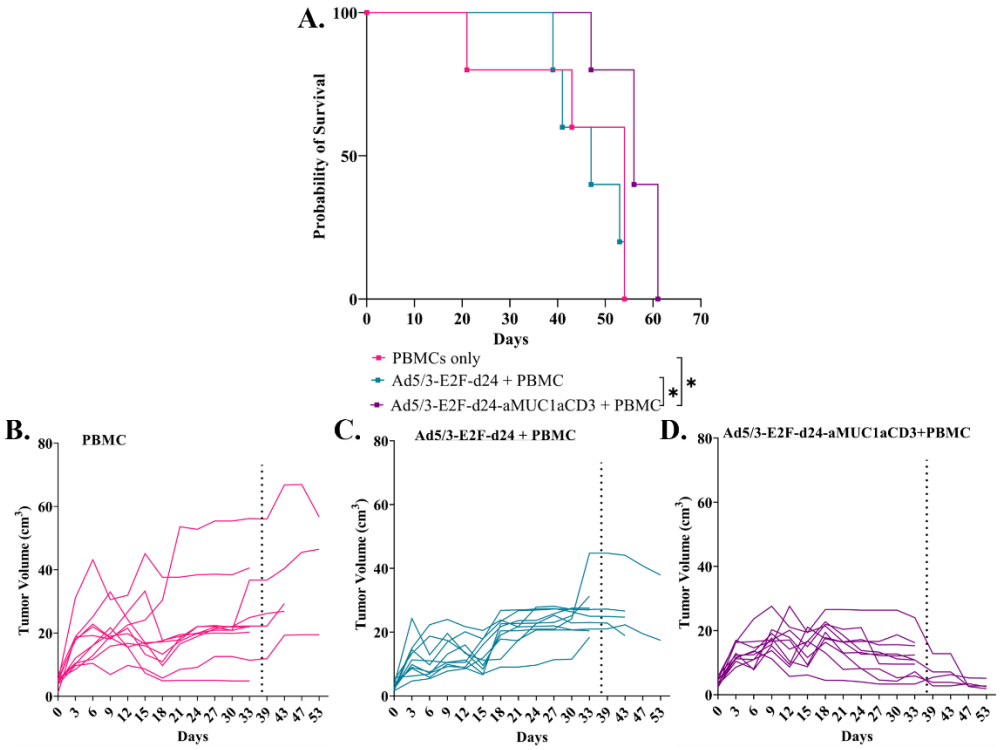

Figure S6. Probability of survival and Individual tumor growth curve of A549 humanized xenograft mice. (A) Ad5/3-E2F-d24-aMUC1aCD3+PBMC showed significantly better survival compared to Ad5/3-E2F-d24+PBMC and PBMC control group. Individual tumor growth of **(B)** PBMC **(C)** Ad5/3-E2F-d24+PBMC **(D)** Ad5/3-E2F-d24-aMUC1aCD3+PBMC treated mice. Dashed line is indicating the time point (day 35) for tumor sample collection from mice (n = 5).

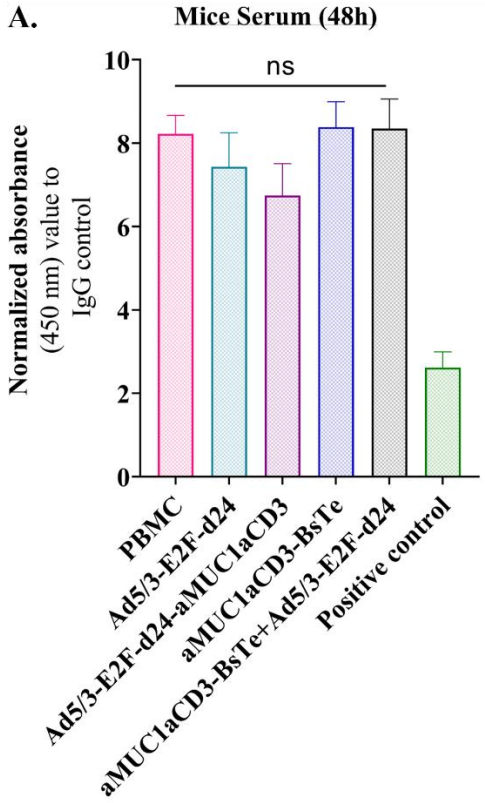

Figure S7. aMUC1aCD3-BsTe expression in serum sample collected from PDX-OvCa bearing mice. (A) Competitive binding In-cell ELISA was used to analyze the binding of aMUC1aCD3-BsTe to its target MUC1 antigen expressed by T47D cell monolayer. TILT-321 infected 30 kDa filtered cells supernatant was used as a positive control.