SUPPLEMENTAL MATERIALS AND METHODS

In vitro oncolytic assay of TT1-GFP

2 x 10^5 TC-1 cells were plated in 6 well plates in RPMI-1640 complete medium composed of RPMI-1640 (Gibco; 52400-025), 10% FBS (Gibco; 011-90015M), 1% NEAA (Gibco; 11140-050), 1% Glutamax (Gibco; 35050-038), 1% Sodium Pyruvate (Gibco; 11360-070), 50 mg/ml Geneticine (Gibco; 10131-027) and 10.000 U/ml (Pen-Strep (Gibco; 15140-122). Cells were treated the day after with complete medium, 0.2 µM staurosporine (Sigma-Aldrich; S6942) or TT1-GFP at MOI 1 for 24, 48 or 72 hrs. To increase infection rates, treated cells were centrifuged at high speed (2671 rcf) for 2 hours at room temperature. Twenty-four, 48 or 72 hr after treatment, cells were incubated for 30 min at 4°C with LIVE/DEAD™ Fixable Aqua Dead (ThermoFisher; L34966) and fixed with fixation buffer (BD bioscience; 554655) before FACS analysis. Cell survival and TC-1 infection were quantified by measuring Aqua Dye negative cells and GFP^+ live cells respectively.

Antibodies used for flow-cytometry analysis of tumor-infiltrating immune cells

Antibody	Clone:	Company
APC Cy7 Anti-Mouse CD45	30-F11	BD Biosciences
BB515 Rat Anti-Mouse CD8+a	53-6.7	BD Biosciences
PE/Cy7 anti-mouse CD279 (PD-1) Antibody	RMP1-30	Biolegend
PE/Cy5 anti-mouse/human CD45R/B220 Antibody	RA3-6B2	Biolegend
BUV395 Hamster Anti-Mouse CD3e	145-2C11	BD
BV421 Rat Anti-Mouse CD4	GK1.5	BD
BUV737 Rat Anti-Mouse CD26	H194-112	BD Biosciences
BV421 Rat Anti-Mouse CD274 (PD-L1)	MIH5	BD Biosciences
Brilliant Violet 711™ anti-mouse CD64 (FcγRI) Antibody	X54-5/7.1	Biolegend
PE/Cy5 anti-mouse CD3ε Antibody	145-2C11	Biolegend
PE/Cy5 anti-mouse CD19 Antibody	6D5	Biolegend
PE/Cy7 anti-mouse CD11c Antibody	N418	Biolegend
APC anti-mouse F4/80 Antibody	BM8	Biolegend
Alexa Fluor® 700 anti-mouse CD45 Antibody Cat. N°: 103128	30-F11	Biolegend

APC/Cyanine7 anti-mouse I-A/I-E Antibody	M5/114.15.2	Biolegend
PE/Cy5 anti-mouse/human CD11b Antibody	M1/70	Biolegend
BV510 Rat Anti-Mouse CD335 (NKp46)	29A1.4	BD Biosciences
H-2 Db HPV16 E7-PE	Sequence RAHYNIVTF	Immudex
Fixable Viability Stain 700	-	BD Biosciences
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	-	ThermoFisher
Purified Rat Anti-Mouse CD16/CD32	2.4G2	BD Biosciences

SUPPLEMENTAL FIGURES

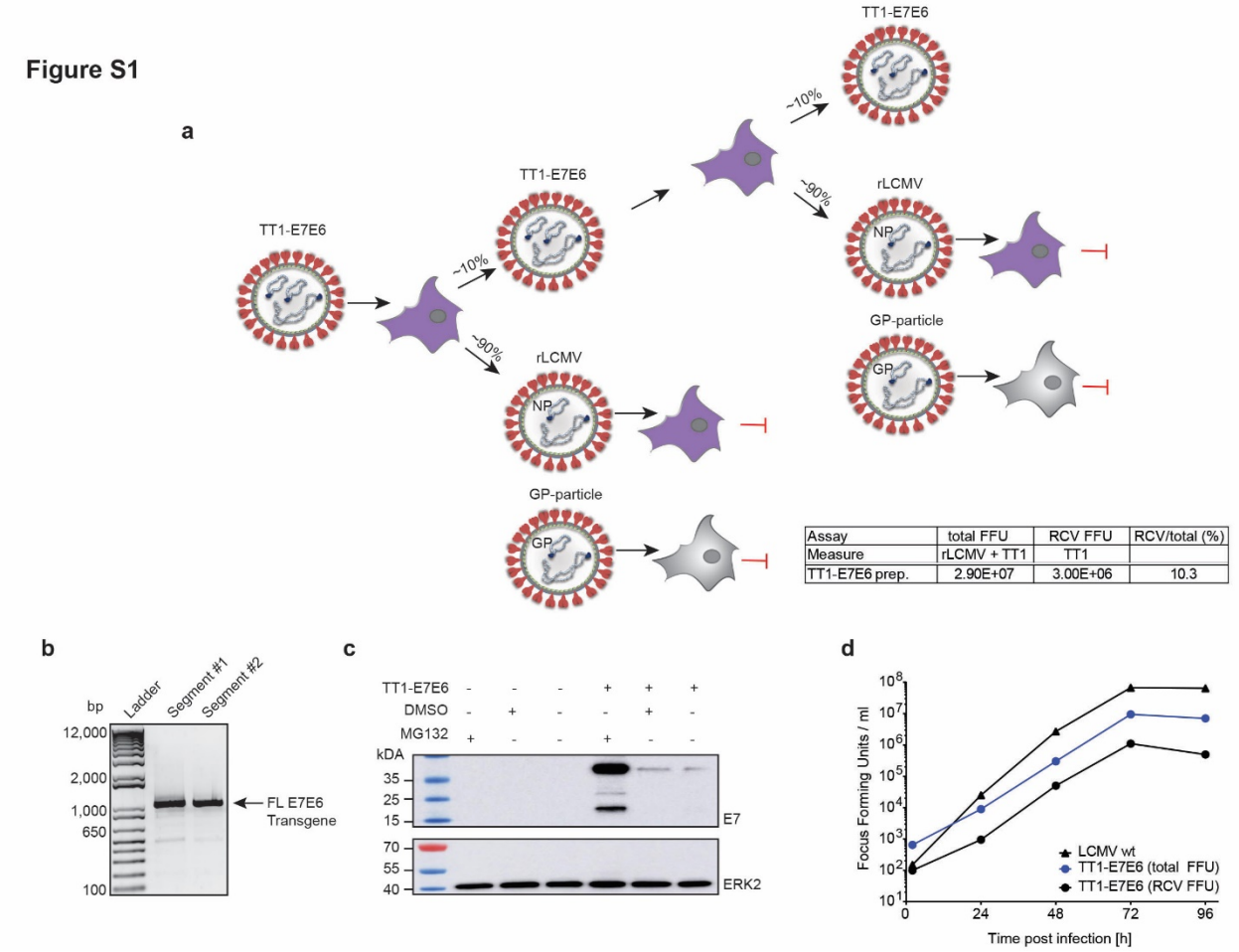
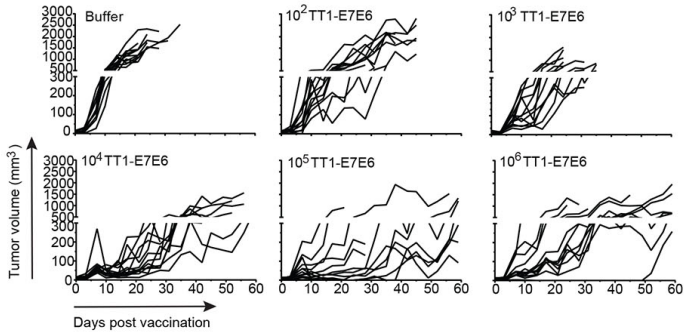


Figure S1. Characterization of TT1-E7E6 vectors. (a) Schematic illustration of tri-segmented TT1-E7E6 vector propagation. As wild-type arenaviruses have evolved to preferentially package two genome segments (one S-segment and one L-segment), co-packaging of the three genome segments is inefficient, resulting in a 10-fold lower abundance of three-segmented vectors compared to two-segmented vectors as shown by FFU analysis of culture supernantant from vector-infected cells in the lower right table. Two-segment vectors carry either S-Segment #1 (encoding the viral NP, rLCMV) or S-Segment #2 (encoding the viral GP). Both TT1-E7E6 and rLCMV are considered immunological active, as the vector facilitates at least one further round of replication/transgene expression (as indicated by purple color). (b-d) Characterization of TT1-E7E6 for transgene insertion, transgene expression and growth behavior. (b) Insertion of the transgene at the correct size on both S-Segments was verified by PCR. FL, full length, expected at 1050 bp. (c) Immunoblot analysis of E7 expression in HEK293 cells infected with TT1-E7E6. ERK2 expression was used as loading control. (d) Growth kinetics of TT1-E7E6 in GP complementing cells (Total) and in non-complementing HEK293 cells (RCV), as well as the parental virus LCMV wt in HEK293 cells. RCV, Replication Competent Virus.

Figure S2

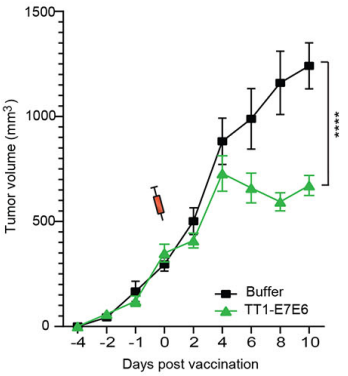
a



b

Group	MST in days (Median±SD)	*p* value vs TT1-E7E6 (10^2)	*p* value vs TT1-E7E6 (10^3)	*p* value vs TT1-E7E6 (10^4)	*p* value vs TT1-E7E6 (10^5)	*p* value vs TT1-E7E6 (10^6)
Buffer	24±0.5	0.0002 (***)	0.2326	0.0001(***)	0.00049(***)	0.0001(***)
TT1-E7E6 (10^2 RCV FFU)	40±5.2	N/A	--	--	--	--
TT1-E7E6 (10^3 RCV FFU)	28±N/A	0.002 (**)	N/A	--	--	--
TT1-E7E6 (10^4 RCV FFU)	51±7.5	0.079	0.001(**)	N/A	--	--
TT1-E7E6 (10^5 RCV FFU)	> 59	0.020 (*)	0.002(**)	0.099	N/A	--
TT1-E7E6 (10^6 RCV FFU)	> 59	0012 (*)	0.001(**)	0.111	0.915	N/A

c



d

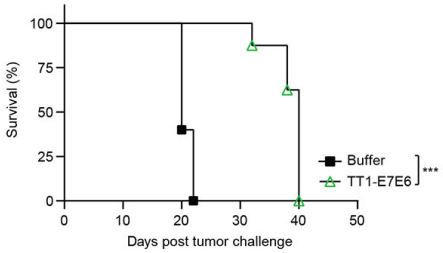


Figure S2. Therapeutic TT1-E7E6 vaccination promotes survival in a syngeneic TC-1 tumor model. (a, b) Mice were engrafted subcutaneously with TC-1 tumor cells (Day -5). Five days later (Day 0), upon appearance of palpable TC-1 tumors, mice were randomized and immunized i.v. with increasing doses of TT1-E7E6 (10^2, 10^3, 10^4, 10^5 and 10^6 RCV FFU) or buffer control. A second immunization with identical doses was administered two weeks post prime (Day 14). (a) Kinetics of tumor growth in individual animals that received indicated doses of TT1-E7E6 immunization. The mean tumor volumes of these animals shown here are provided in Figure 3d. (b) Median survival time (MST) and intergroup analysis of tumor-bearing mice with TT1-E7E6 treatment. Log-rank test.* $p<0.05$, ** $p<0.01$, *** $p<0.001$. (c) Efficacy in large tumors. Mice were inoculated subcutaneously with 10^5 TC-1 cells on Day -10. On Day-1 mice were allocated into groups based on tumor size. On Day 0 mice were immunized i.v. with 10^5 RCV FFU TT1-E7E6 or buffer followed by a boosting dose on Day 21. Means ± SEM. N=8 mice for TT1-E7E6 and n=5 mice for buffer control group. Two-way ANOVA, **** $p<0.0001$. (d) Kaplan-Meier survival curves of TC-1 large-tumor-bearing mice after TT1-E7E6 treatment. Mantel-Cox test. *** $p<0.001$.

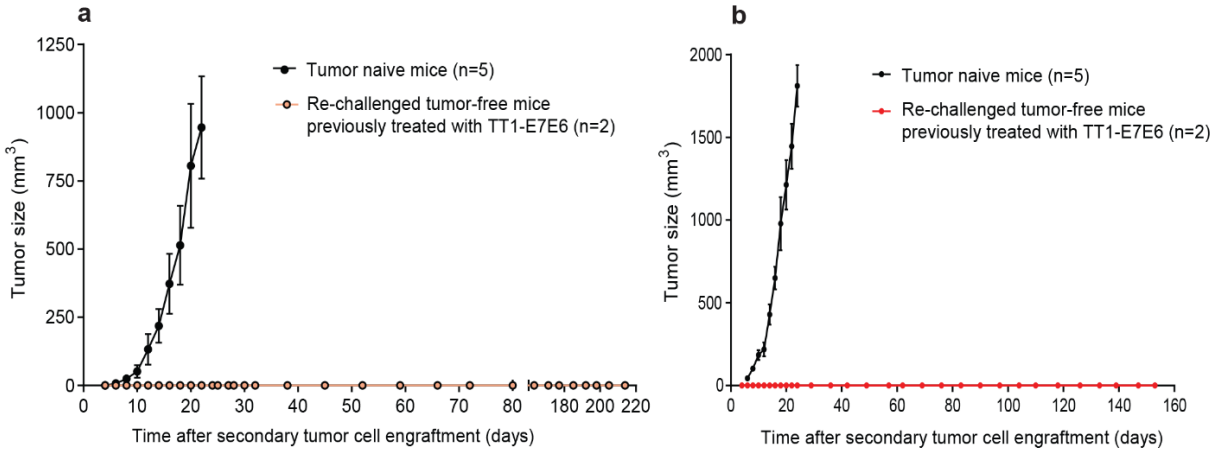


Figure S3. TC-1 tumor re-challenge in TT1-E7E6 cured mice. Mice inoculated with 10^5 TC-1 tumors on Day -5 were immunized with TT1-E7E6 (10^5 or 10^6 RCV FFU) or buffer i.v. on Day 0. Tumor growth was monitored. TT1-E7E6 cured, tumor-free mice were re-challenged with 10^5 TC-1 tumor cells On Day 143 (a) and 198 (b), respectively. Mice were followed up for tumor growth for 210 (a) or 150 (b) days. Means ± SEM. Two independent experiments, with N=2 mice in TT1-E7E6 treatment group and N=5 mice in buffer control group for both experiments.

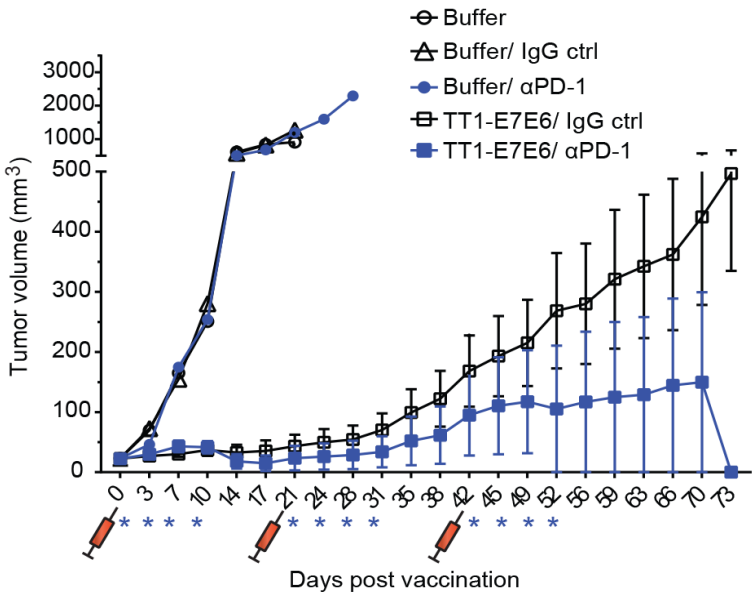


Figure S4. Superior tumor control by combination therapy with TT1-E7E6 and anti-PD-1 Ab. Mice were inoculated s.c. with 10^5 TC-1 tumor cells on Day -5. After tumors became measurable, mice were randomized and treated with 10^6 RCV FFU of TT1-E7E6 or buffer On Day 0, 21 and 42. Anti-PD-1 antibody (αPD1) or isoytpe control (IgG ctrl) was administered i.p at a dose of 200 µg/mouse on the same days of TT1-E7E6/buffer treatment, followed by 3 additional doses every 3 days. Tumor growth was monitored. The study was terminated 73 days after start of treatment. Kinetics of tumor growth until study termination was depicted. Syringe symbols above the graph indicate time of TT1-E7E6 or buffer treatment. Asterisks below the x-axis indicate the individual anti-PD1 treatment. Buffer/IgG control, n=6 mice; TT1-E7E6/IgG, n=10 mice; buffer/anti-PD-1, n=10 mice; TT1-E7E6/anti-PD-1, n=9 mice. Means ± SEM.

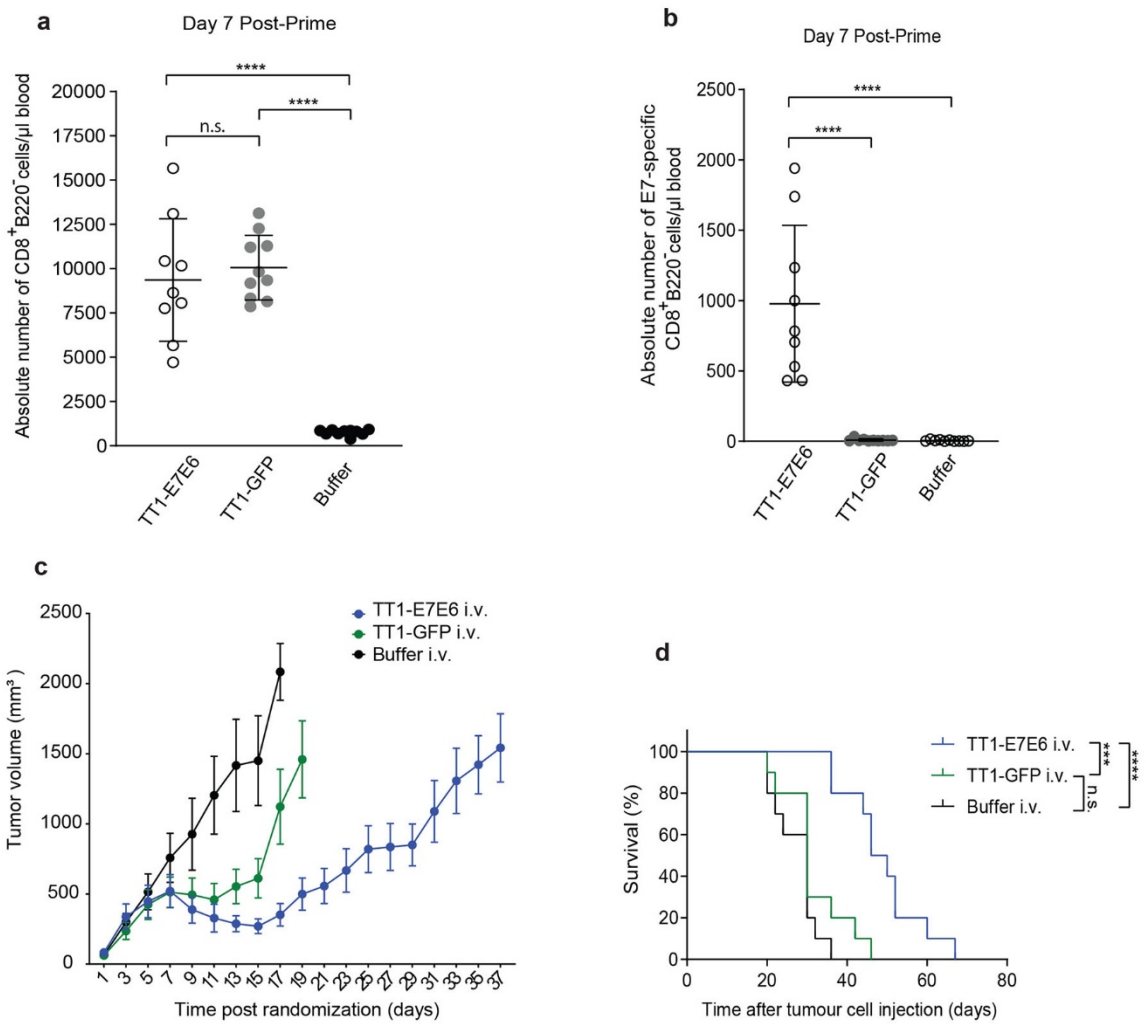


Figure S5. Evaluation of CD8$^+$ T cell response and tumor control efficacy by a control TT1-GFP vector. Mice inoculated with 10^5 TC-1 tumors on Day -5 were immunized with TT1-GFP, TT1-E7E6 (both at 10^5 RCV FFU) or buffer i.v. on Day 0. Total (a) and E7-specifc (b) CD8$^+$ T cells were measured on Day 7 after treatment. Kinetics of tumor growth (c) and survival (d) were monitored. N=9 mice for TT1-E7E6 group, N=10 mice per group for buffer and TT1-GFP treatment. (a, b) One-way ANOVA, (d) Mantel-Cox test. ns, not significant, *** p<0.001. **** p<0.0001.

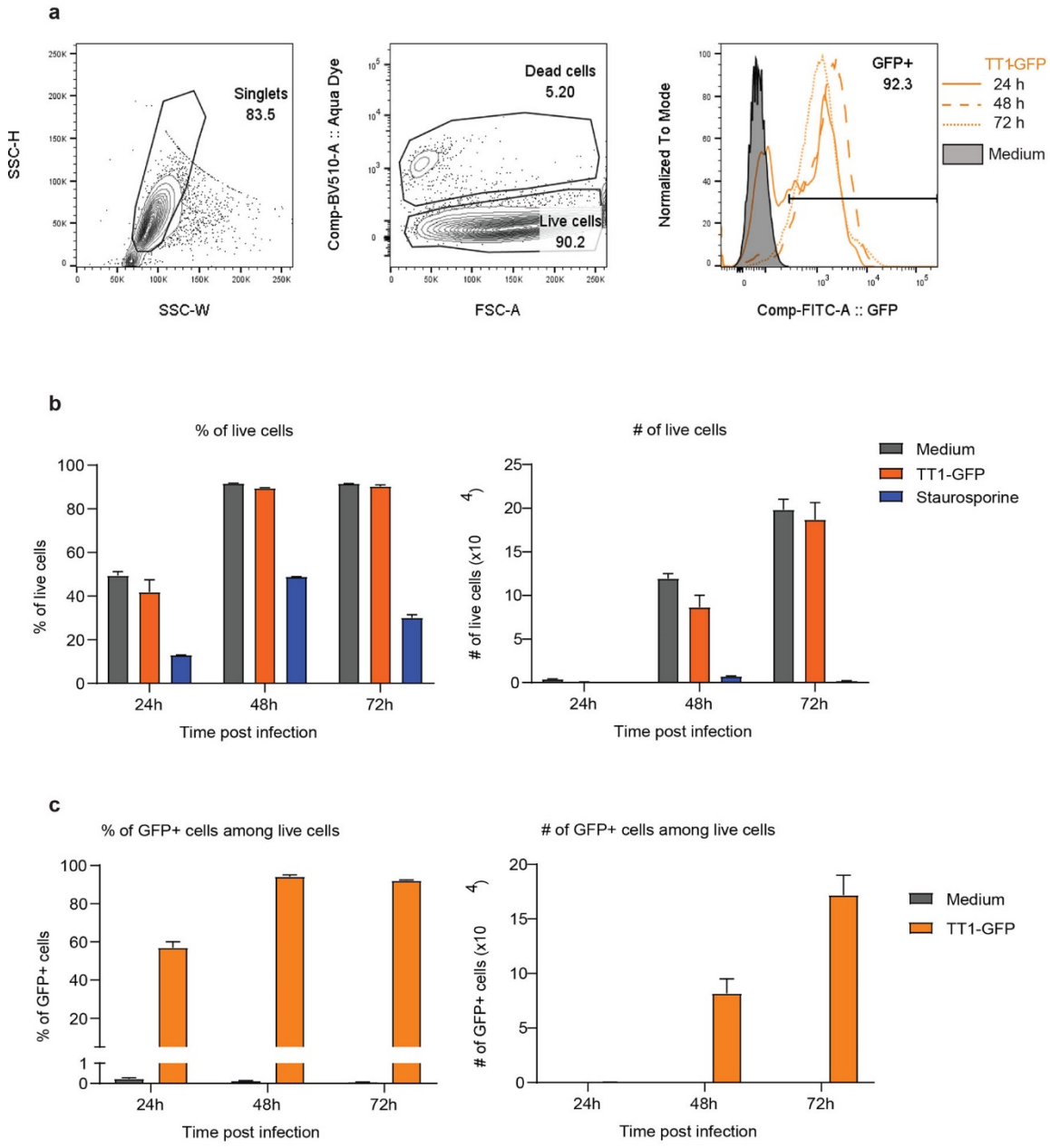


Figure S6. TT1-GFP does not induce cell death of TC-1 tumor cells in vitro. TC-1 tumor cells were treated in vitro with medium, TT1 vector expressing GFP (TT1-GFP) at MOI 1 or with the apoptosis inducer staurosporine for 24, 48 and 72 hrs. (a) FACS analysis gating strategy of TC-1 cells infected with TT1-GFP for different time points post infection. (b) Aqua Dye negative live cells were quantified to measure cell survival. Shown are percentage and cell number per well. (c) GFP^+ (infected) TC-1 cells are shown as percentage and total number per well. N=3. Bars show means ± SEM. Data are representative of two independent experiments.

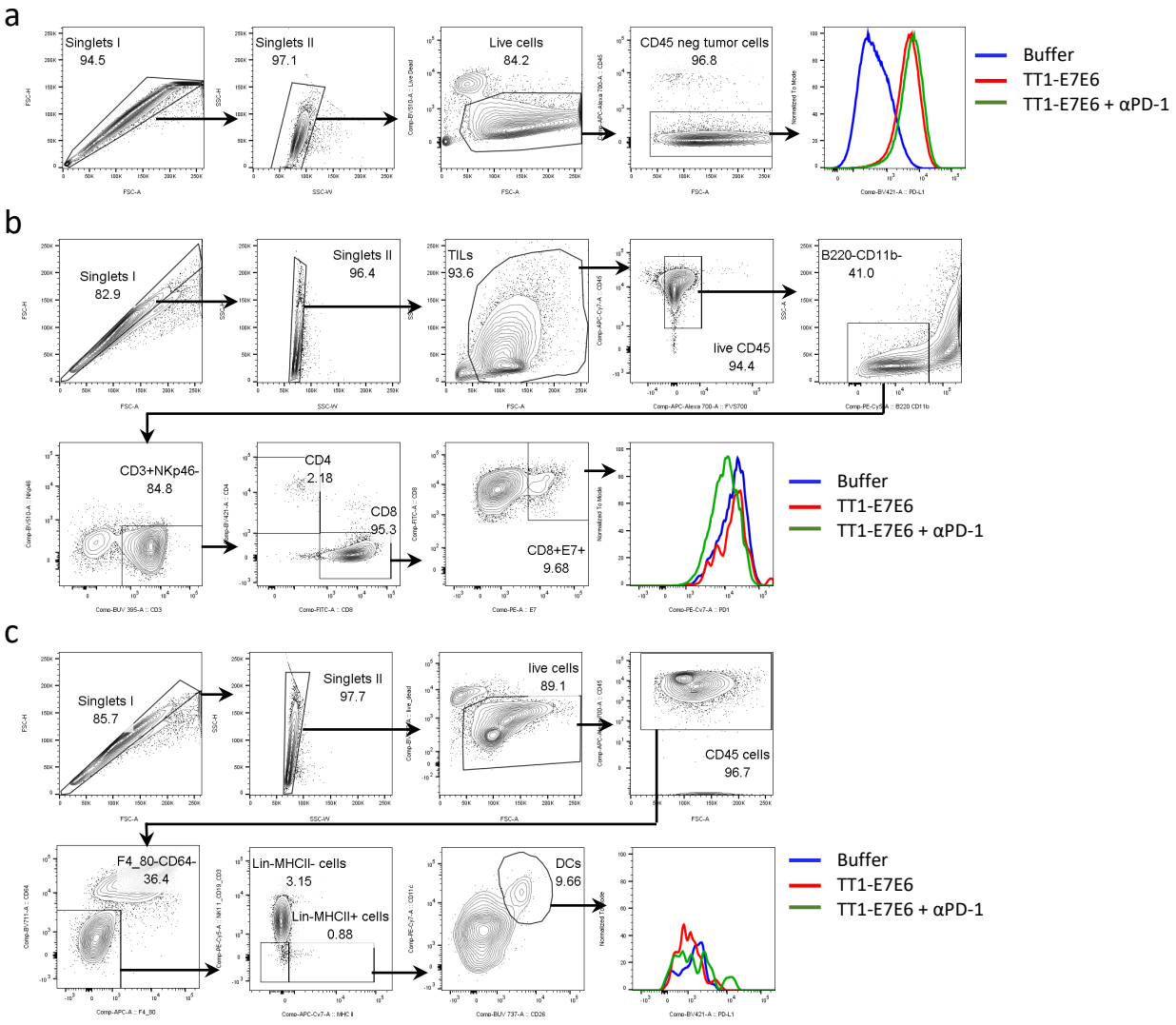


Figure S7. FACS gating strategy of the *ex vivo* TC-1 tumor tissue analysis. Single cells were selected by FSC-height vs. FSC area followed by SSC-width vs. SSC-height. Live cells were selected with LIVE/DEAD™ positivity. (a) tumor cells are defined as $CD45^-$ cells. (b) E7 specific-$CD8^+$ T cells are defined as $CD45^+B220^-NKp46^-CD11b^-CD3^+CD8^+E7^+$ cells. (c) Conventional dendritic cells (cDCs) are defined as $CD45^+CD64^-F4/80^-Lin^-I\text{-}A/I\text{-}E^+CD11c^+CD26^+$ cells. Expression of PD-1 was further analyzed on E7-specific $CD8^+$ T cells (b). Expression of PD-L1 was measured on tumor cells (a) and cDCs (c).