Depletion of regulatory T cells in tumors with an anti-CD25 immunotoxin induces CD8 T cell-mediated systemic antitumor immunity

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SI APPENDIX

SUPPLEMENTAL FIGURES AND TABLES

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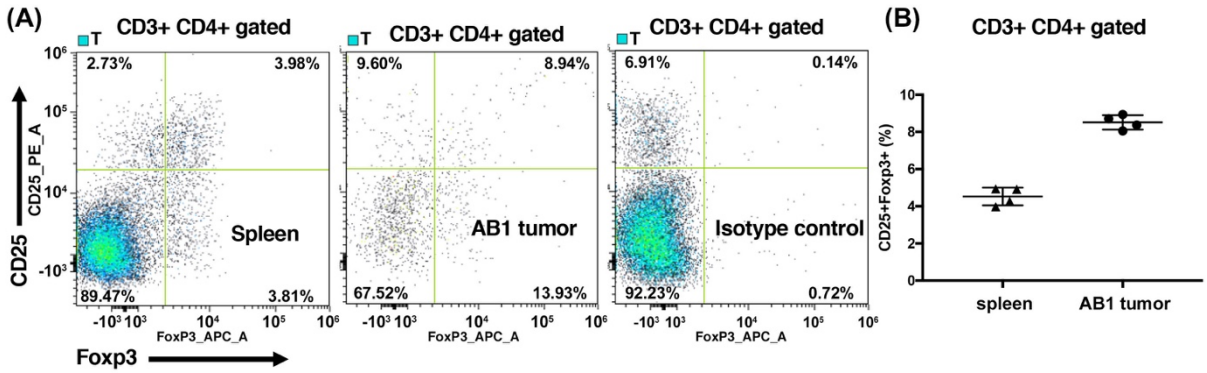

Fig. S1. Treg population in AB1 tumors compared to spleen. (**A**) Representative data of flow cytometry gated on $CD3^{+}CD4^{+}$ cells. (**B**) An increased $CD4^{+}CD25^{+}Foxp3^{+}$ Treg population among CD4 T cells was observed in AB1 tumors compared to that in the spleen (n = 4). Tregs are abundant in tumors.

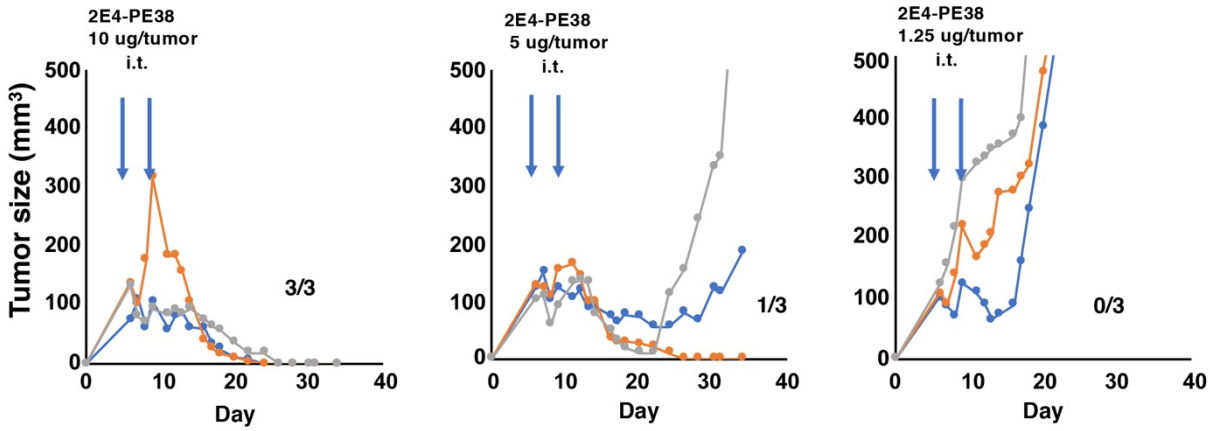

Fig. S2. Antitumor effect using various amounts of 2E4-PE38. BALB/c mice were implanted subcutaneously with AB1 tumors (5 x 10^6) on their dorsal side. Various amounts of 2E4-PE38 (left to right: 10, 5, and 1.25 μg) were injected into the tumor on days 5 and 9. Tumor growth was monitored with a caliper.

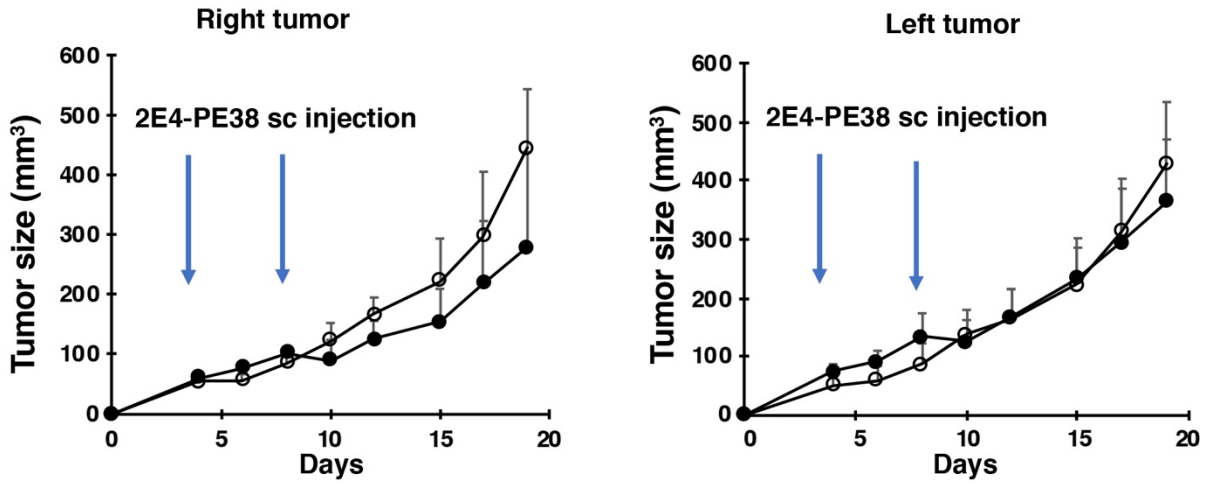

Fig. S3. No antitumor efficacy using subcutaneous injection of 2E4-PE38. BALB/c mice were implanted subcutaneously with 66c14 tumors (3 x 10^6) on both right and left dorsal side. On days 4 and 8, 2E4-PE38 (10 μg/100 μl) was injected subcutaneously (injection point was 1 cm distance from each tumor). Tumor growth was monitored with a caliper. The tumor growth shows no difference between s.c. treated and nontreated groups at each time point ($p > 0.5$). Mice bearing tumors did not have complete remission after s.c. treatment, indicating leaked 2E4-PE38 after intratumoral injection did not affect the shrinkage of untreated tumor, which was observed in Fig. 2.

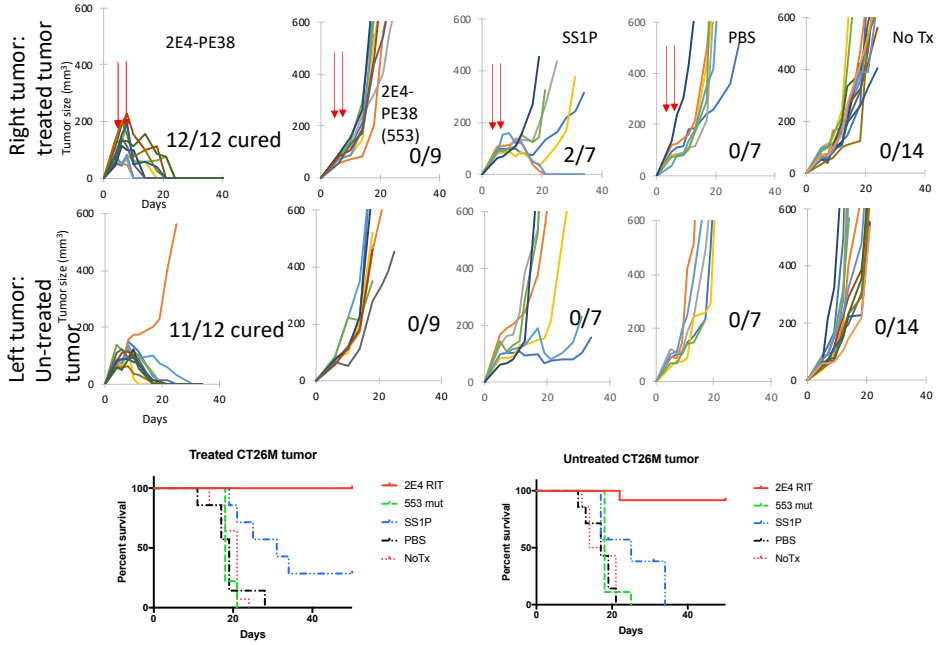

Fig. S4. Local 2E4-PE38 therapy is effective against colon cancer. BALB/c mice were implanted subcutaneously with CT26M (4 x 10^6) tumors on the back. Tumor growth was monitored with a digital caliper. 2E4-PE38 (10 µg), 2E4-PE38-E553D inactive mutant (10 µg), SS1P (10 µg) or PBS (100 µl) were injected into one right tumor site on days 5 and 9. Tumors were between 50-100 mm^3 on day 5. SS1P has some effect for shrinking right tumors because CT26M has some expression of human mesothelin. Mice survivals are shown in bottom graphs.

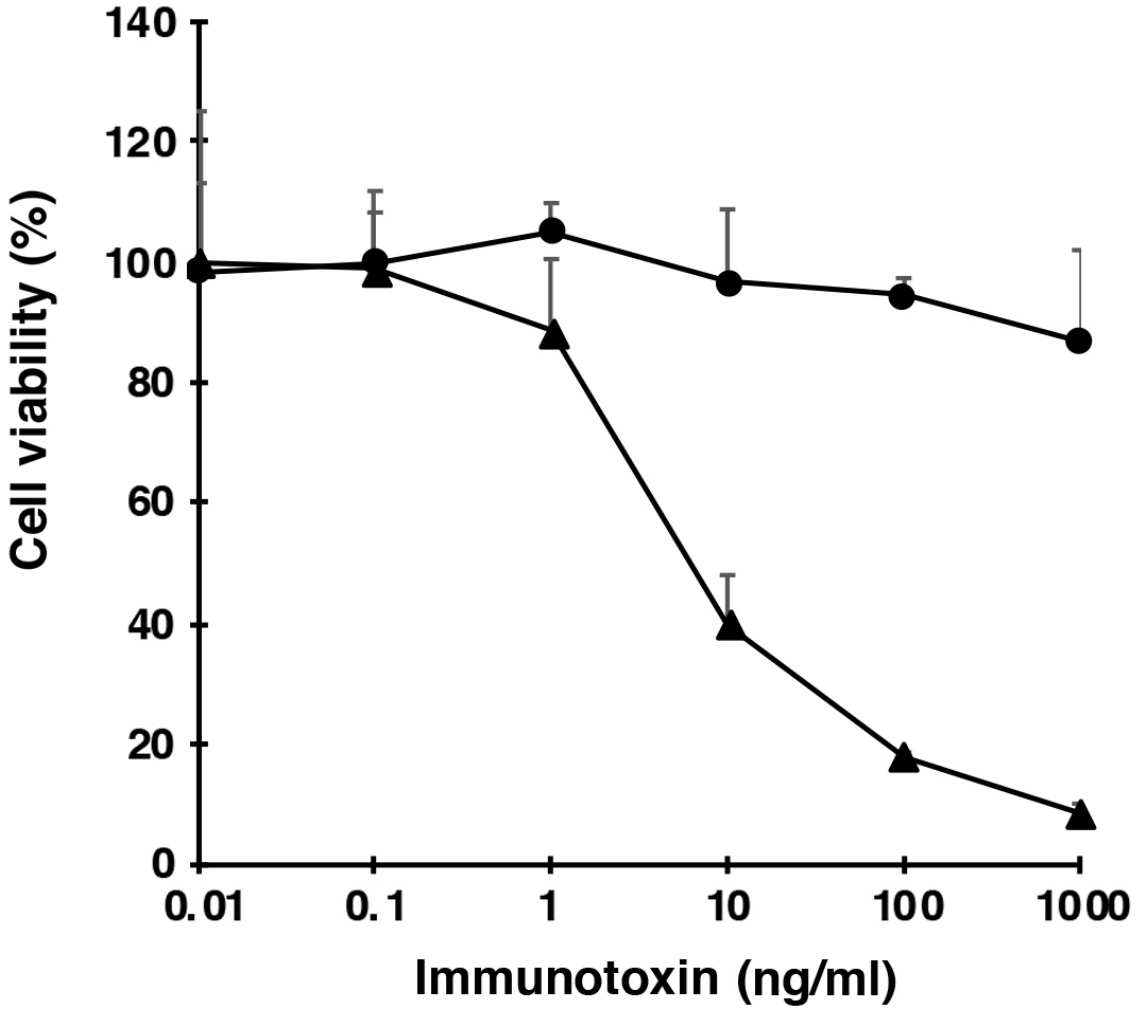

Fig. S5. Cytotoxic activity of SS1P using CT26M cells. Cell viability assay with CT26M cells shows that CT26M cells were sensitive to SS1P (IC_{50} = 3 ng/ml, ▲) and not sensitive for 2E4-PE38 (●).

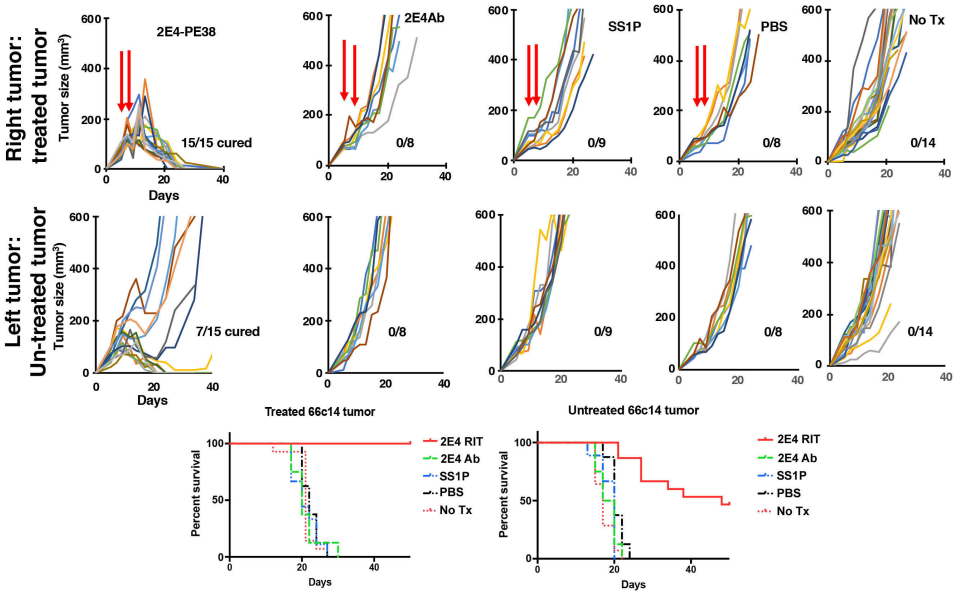

Fig. S6. Local 2E4-PE38 therapy is effective against breast cancer. BALB/c mice were implanted subcutaneously with 66c14 (3 x 10^6) tumors on both the right and left of their dorsal side. Tumor growth was monitored with a digital caliper. 2E4-PE38 (10 µg), 2E4 antibody (10 µg), SS1P (10 µg) or PBS (100 µl) were injected into one right tumor site on days 5 and 9. Tumors were between 50-100 mm^3 on day 5. Mice survivals are shown in bottom graphs. 2E4 Antibody is Rat IgG mAb, which may have a lower effector function in mice.

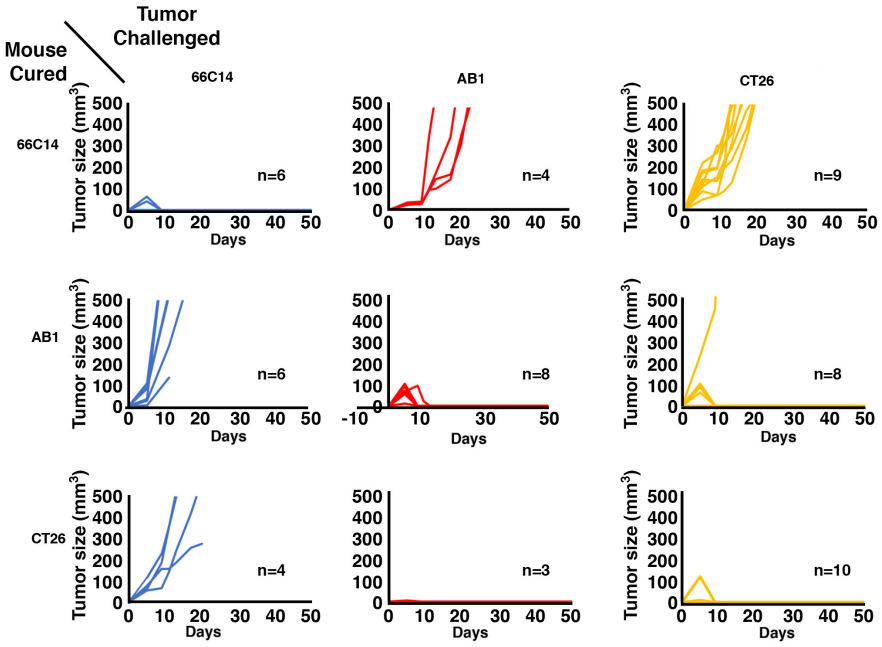

Fig. S7. Tumors undergoing complete regression are immune to re-challenge with other tumor cells. Mice with cured tumors (more than 50 days after complete remission of the tumor) were re-challenged with 66c14, AB1 and CT26M cells at three separate locations. Tumor size was measured by caliper. Upper column: cured mice with 66c14 tumors. Middle column: cured mice with AB1 tumors. Bottom column: cured mice with CT26M tumors. Survival plots are shown in Figure 3.

Table S1. 2E4-PE38 levels in serum after intratumoral injection (ng/ml)

Minutes	Mice bearing ABI tumors			
	#1	#2	#3	#4
3	-	571	777	-
10	-	465	543	-
30	-	311	422	-
50	-	-	252	-
80	-	-	-	--
120	-	-	-	

Mice bearing AB1 tumor (n=4) were injected with 10 μg of 2E4-PE38 intratumorally and serum 2E4-PE38 levels (ng/ml) were monitored over time using ELISA.
-, undetectable (less than 100 ng/ml)

Table S2. STR profiling of cells

Markers	**AB1**	**CT26M**
MCA-4-2	21.3, 22.3	21.3
MCA-5-5	14	14
MCA-6-4	18	18, 19
MCA-6-7	12	12
MCA-9-2	15	15
MCA-12-1	17, 18	16, 17
MCA-15-3	22.3, 23.3	21.3, 22.3
MCA-18-3	18, 19	19, 20
MCA-X-1	25	25, 26

Table S3. Antibodies for flow cytometry

Antibody (anti-mouse)	**Clone**	**Supplier**
anti-CD25-PE	PC61.5	eBioscience
anti-CD25-FITC	PC61.5.3	Invitrogen
anti-CD25-FITC	7D4	BD Pharmingen
Anti-Foxp3-APC	FJK-16s	eBioscience
Anti-CD4-FITC	RM4-4	BD Pharmingen
Anti-CD4-APC	RM4-5	eBioscience
anti-CD3-Brilliant Violet605	17A2	BioLegend
anti-CD8a-PE	53-6.7	SouthernBiotech
anti-CD69-PE-Cy7	H1.2F3	eBioscience
anti-IFNγ-PerCP-Cy5.5	XMG1.2	eBioscience
anti-CD11b-PE	M1/70	BD Pharmingen
anti-Ly6G-APC	RB6-BC5	eBioscience
anti-CD11c-FITC	N418	Invitrogen
anti-MCH-II-PE	NIMR-4	eBioscience
Arm Hamster IgG Iso Control-PE-Cy7	eBio299Arm	eBioscience
Rat IgG2b Iso Control-FITC	A95-1	BD Pharmingen
Rat IgG1 Iso Control-FITC	eBRG1	eBioscience
Rat IgG2a Iso Control-FITC	eBR2a	eBioscience
Rat IgG2a Iso Control-APC	eBR2a	eBioscience
Rat IgG1 Iso Control-PerCP-Cy5.5	eBRG1	eBioscience