Supplementary Materials for

A Novel Potent Anti-STEAP1 Bispecific antibody to Redirect T Cells for Cancer Immunotherapy

Running title: Anti-STEAP1 Bispecific Antibody for Ewing Sarcoma Family of Tumors

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Supplementary Table S1. Binding affinities of humanized STEAP1 IgG antibodies by cell-based fluorescent method.

	Chimeric X-120	VL1+VH1	VL1+VH2	VL1+VH5	VL2+VH2
Light chain humanness (%)	83	91	91	91	91
Heavy chain humanness (%)	75	87	87	87	87
Bmax	147 ± 20	150 ± 10	151 ± 5	115 ± 18	167 ± 9
KD (nM)	0.7 ± 0.6	1.5 ± 0.5	2.6 ± 0.3	39.7 ± 12.6	2.4 ± 0.6
R2	0.6	0.92	0.99	0.98	0.96

Supplementary Table S2. Purity and Stability of humanized STEAP1 IgG1 antibodies by HPLC.

Proteins	OD (280nm)	Conc. (mg/ml)	0 hr (%)	Freeze-thaw (%)	40℃ for 7 days (%)	40℃ for 14 days (%)	40℃ for 21 days (%)	40℃ for 28 days (%)
Chimeric X120	2.58	1.58	99.2	98	90.4	86	80.5	76.1
VL1+VH1	3.34	2.03	99.4	98.9	91.9	87.4	81.5	77.3
VL1+VH2	3	1.82	99.5	93.2	90.9	88.4	82.4	78.2
VL1+VH5	2.75	1.66	99.5	91	91.2	88.5	83.7	79.5
VL2+VH1	3.49	2.12	99.5	97.9	92.8	88.7	83.8	80.2
VL2+VH2	3.67	2.23	99.4	98	91.8	88.8	83.7	79.6
VL3+VH1	3.48	2.12	99.4	97.2	91.6	88.4	82.1	78
VL3+VH2	2.34	1.42	99.2	97.6	80.5	68.5	57.4	50.6
VL4+VH1	3.26	1.98	99.4	98.6	86.1	73.7	62	54.1
VL4+VH2	3.14	1.91	99.4	99.2	85.8	73.1	61.7	53.1

Supplementary Table S3. Amino acid sequences of VH and VL regions

Variable Region	Amino Acid sequence
VH1	QVQVQESGPGLVKPSQTLSLTCTVTGYSITSDYAWNWIRQPPGKGLEWMGYISNSGSTSYNPSLKSRITISRDTSKNQFSLKL SSVTAADTAVYYCARERNYDYDDYYYAMDYWGQGTTLTVSA
VH2	DVQVQESGPGLVKPSQTLSLTCTVTGYSITSDYAWNWIRQPPGKGLEWMGYISNSGSTSYNPSLKSRITISRDTSKNQFSLKL SSVTAADTAVYYCARERNYDYDDYYYAMDYWGQGTTLTVSA
VH5	DVQVQESGPGLVKPSQTLSLTCTVTGYSITSDYAWNWIRQPPGKGLEWMGYISNSGSTYYNPSLKSRITISRDTSKNQFSLKL SSVTAADTATYYCARERNYDYDDYYYAMDYWGQGTTLTVSA
VL1	DIVMTQSPDSLAVSLGERVTMNCKSSQSLLYRSNQKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFSGSGSGTDFTLTIS SVQAEDVAVYYCQQYYNYPRTFGGGTKVEIKR
VL2	DIVMTQSPDSLAVSVGERVTMNCKSSQSLLYRSNQKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTIS SVQAEDVAVYYCQQYYNYPRTFGGGTKVEIKR

Supplementary Table S4. Purity and Stability of anti-STEAP1 bispecific antibodies by HPLC.

BsAb	Conc. (mg/mL)	0 hour (%)	Freeze-thaw (%)	40℃ for 7 days (%)	40℃ for 14 days (%)	40℃ for 21 days (%)	40℃ for 28 days (%)
BC259	1.8	88.2	86.3	85.2	79.5	72.3	63.4
BC260	1.2	87.9	86.1	84.8	79.7	75.6	66.2
BC261	1.2	85.7	83.4	82.4	77.1	74.5	70.0
BC262	1.3	88.4	86.4	84.9	81.1	77.0	64.5

Supplementary Table S5. STEAP1-BsAb binding to varieties of tumor cell line.

Cancer	Cell line	MFI of unstained control	MFI of STEAP1 (BC261 binding)
Breast cancer	HTB 132	103	117
	MCF7	97	118
	HCC1954	108	130
Colorectal cancer	LS174T LUC	113	147
	SW1222	100	130
DSRCT	BER	102	122
	BZ	108	209
EFT	TC-32	94	554
	TC71	100	397
	SK-ES-1	101	292
	A673	105	341
	A4573	100	292
	SKELP	99	226
	TC71	103	271
	SKEAW	102	204
	SKERT	104	170
	SKNMC	150	161
Gastric cancer	AGS	107	116
	NCI-N87	105	127
Hepatocellular carcinoma	HEP G2	91	119
	SK-HEP-1	97	146
Melanoma	M14 Luc	109	135
	M14	103	100
Neuroblastoma	IMR32 LUC	103	120
	SK-N-FI	111	137
	BE(1)N	100	140
	IMR32	109	155
	SKNSH	89	112
Osteosarcoma	U2OS	116	175
	U2OS LUC	141	132
	SAOS2	107	165
	143B LUC	92	214
	CRL-1427	88	439
Canine osteosarcoma	D-17	106	227
	DAN	102	144
	DSN	102	219
	DSDH	105	146
Pancreatic cancer	SW1990 EGFR KO	100	135
	SW1990 Her2 KO	106	136

	KYSE-70	105	120
	MIA PaCa-2	104	123
Prostate cancer	DU145	94	158
	PC3 PSMA mTurqCBG	111	127
	PC3 tdrrsRLuc	98	118
	PC3 PSMA	104	118
	PC3	105	112
	LNCaP AR	104	297
	CWR22	116	252
	22Rv1	102	210
	VCaP	107	145
	DU145	98	210
Rhabdomyosarcoma	RH30	88	108
	RH41	105	116
	RH30	101	120
Small cell lung cancer	NCI-H524	87	133
	NCI-N417	104	147
Wilims tumor	Wilims1	108	361

Supplementary Table S6. STEAP1 expression (MFI, Mean Fluorescence Intensity) in multiple tumor cell lines and *in vitro* sensitivities (EC50, pM) to anti-STEAP1 BsAb (BC261). The MFI of unstained control was set to 5.

Cancer	Cell line	MFIs	EC_{50} (pM)
Ewing sarcoma family of tumor	TC-32	168	4.9
	TC71	128.8	1.1
	SK-ES-1	75.2	3
	A4573	74.9	5
	SKEAW	58.3	25.8
	SKELP	90	1.8
	SKERT	61.4	8.2
	SKNMC	12.8	10.9
Prostate cancer	LNCaP-AR	74	21.1
	CWR22	26	26.7
	VCaP	11	214.8
Canine osteosarcoma	D-17	121	24.8
	DSN	83	7.2
	DAN	26	29.5
	DSDh	23	16.2
Neuroblastoma	IMR32	5	>5000
Acute leukemia	HL-60	5	>5000

Supplementary Table S7. Purity, affinity, and endotoxin of STEAP1-BsAb formats.

Biclone	Antibody format	Purity (%), HPLC	Binding affinity KD (M)		Endotoxin (EU/mg)
			CD3	Target	
BC261	IgG-[L]-scFv	95	6.02E-09	ND	<2
BC328	Monomeric BiTE	90	4.39E-08	ND	<2
BC329	Dimeric BiTE	89	4.08E-09	ND	<2
BC330	BiTE-Fc	92	2.80E-09	ND	<2
BC365	IgG-[H]-scFv	95	9.46E-09	ND	<2
HD148	IgG-heterodimer	95	2.64E-08	ND	<2

Supplementary Table S8. Tumor infiltrating lymphocytes quantified by area under each BLI curves

Treatment	TIL quantified by BLI AUC			
	Mean	SEM	Relative to IgG[L]-scFv	*P*-value
No treatment	1.76E+04	8.78E+03	-	-
STEAP-1 IgG-[L]-scFv (BC261)	2.10E+07	1.05E+07	100%	-
STEAP-1 monomeric BiTE (BC327)	7.53E+05	3.77E+05	4%	0.01
STEAP-1 dimeric BiTE (BC329)	1.21E+06	6.06E+05	6%	0.01
STEAP-1 BiTE-Fc (BC330)	4.22E+05	2.11E+05	2%	0.01
STEAP-1 IgG-[H]-scFv (BC365)	6.56E+05	3.28E+05	3%	0.01
STEAP-1 IgG heterodimer (HD148)	1.02E+06	5.09E+05	5%	0.01

Supplementary Fig. S1. Characteristics of rehumanized anti-STEAP1 monoclonal antibodies and STEAP1 T cell-engaging bispecific antibodies

(A) Mean fluorescence intensities (MFIs) of STEAP1 IgG antibody binding to TC-32 EFT cell line, analyzed by flow cytometry. The MFI of unstained control was set to 5. (B) Binding avidity of humanized STEAP1 IgG clones following repeated cycles of washing using PBS/EDTA as dissociation buffer.

Supplementary Fig. S2. Properties of anti-STEAP1 T cell-engaging bispecific antibodies.

(A) Purity of STEAP1 BsAb BC261 by SEC-HPLC. (B) STEAP1 BsAb binding to STEAP1(+) tumor cell lines analyzed by flow cytometry. The MFI of unstained control was set to 5.

Supplementary Fig. S3. *In vitro* BC261 antibody-dependent T cell mediated cytotoxicity against STEAP1(+) canine osteosarcoma cell lines.

(A) Antibody-dependent T cell mediated cytotoxicity (ADTC) as a function of increasing doses of BC261 against canine osteosarcoma cell lines. Effector to target cell ratio (ET ratio) was 10:1.

Supplementary Fig. S4. *In vivo* TH1 cell cytokine release after STEAP1 BsAb armed T cells treatment.

(A) BC261 armed T cells (10μg of BC261/2x10^7 T cell) were administered in mice bearing EFT PDX (ES3a), and serum cytokine levels were measured at different time points post treatment. (B) *In vivo* cytokine release was compared among groups by analyzing each AUC.

Supplementary Fig. S5. In vivo anti-tumor efficacy: dose titration of BC261

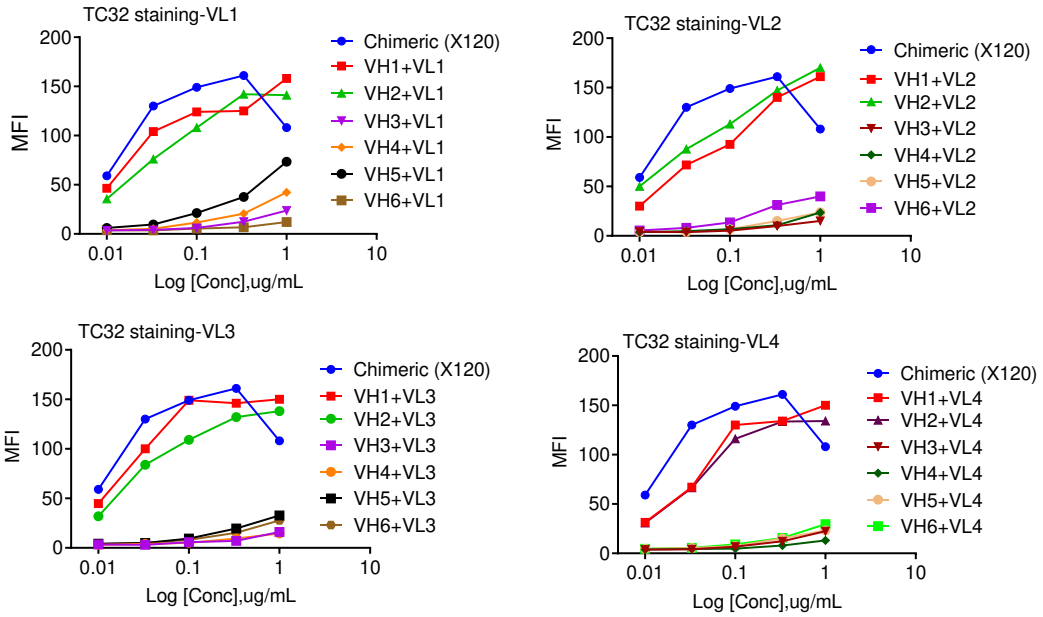
(A) Decreasing doses of BC261 were administered with $2x10^6$ T cells twice per week for 2-3 weeks. Subcutaneous IL-2 (1000 IU) was supplemented with each T cell injection. (B) *In vivo* anti-tumor effect of decreasing doses of STEAP1 BsAb BC261 against TC-32 cell line xenografts. (C) Monitoring of relative body weight after treatment. (D) Survival curves for the mice treated with decreasing doses of BC261.

Supplementary Fig. S6. *In vivo* anti-tumor effect of BC261 against STEAP1(+) EFT.

(A) 10μg of BC261 was administered with $2x10^7$ of T cells twice per week for 2-3 weeks to treat EFT PDXs. Subcutaneous IL-2 (1000 IU) was supplemented with each T cell injection. (B) *In vivo* anti-tumor effect of BC261 against EFT PDX ES03a. (C) *In vivo* anti-tumor effect of BC261 against EFT PDX ES15a. Tumor growth, relative body weight, and overall survival after treatment were plotted.

Supplementary Fig. S1

(A)



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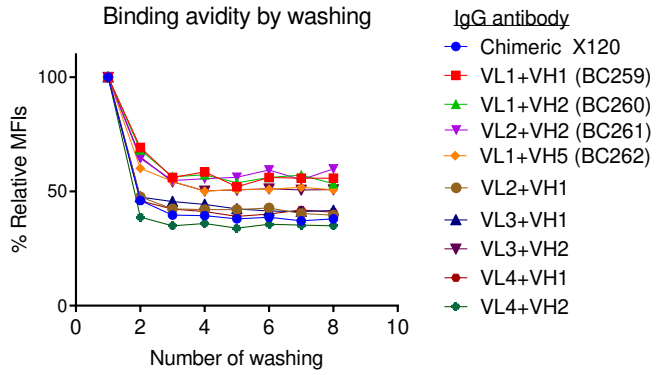
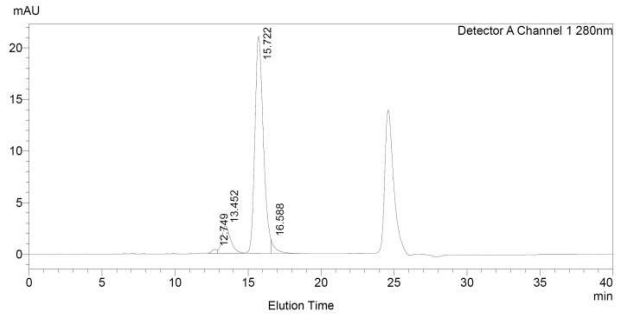


Supplementary Fig. S2

(A)



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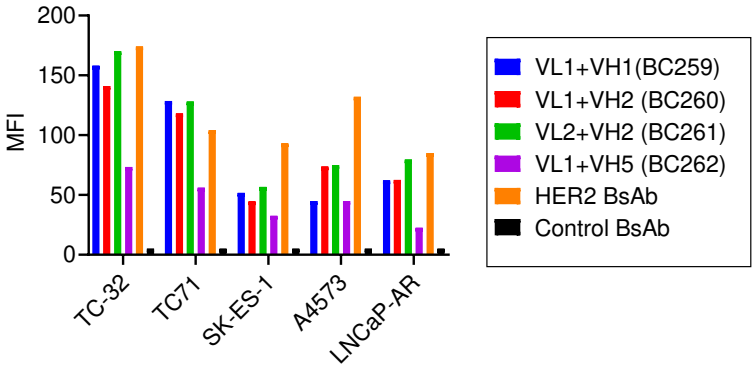

Supplementary Fig. S3

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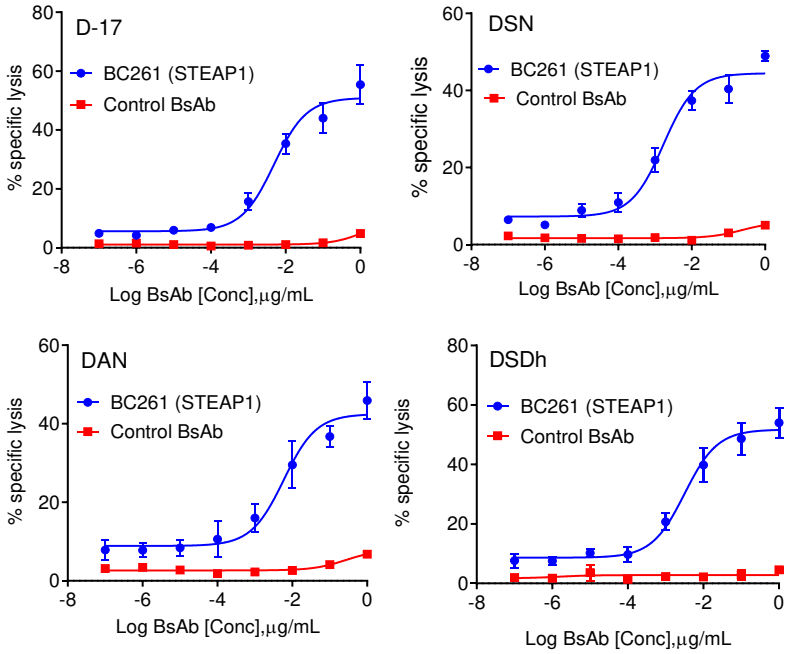
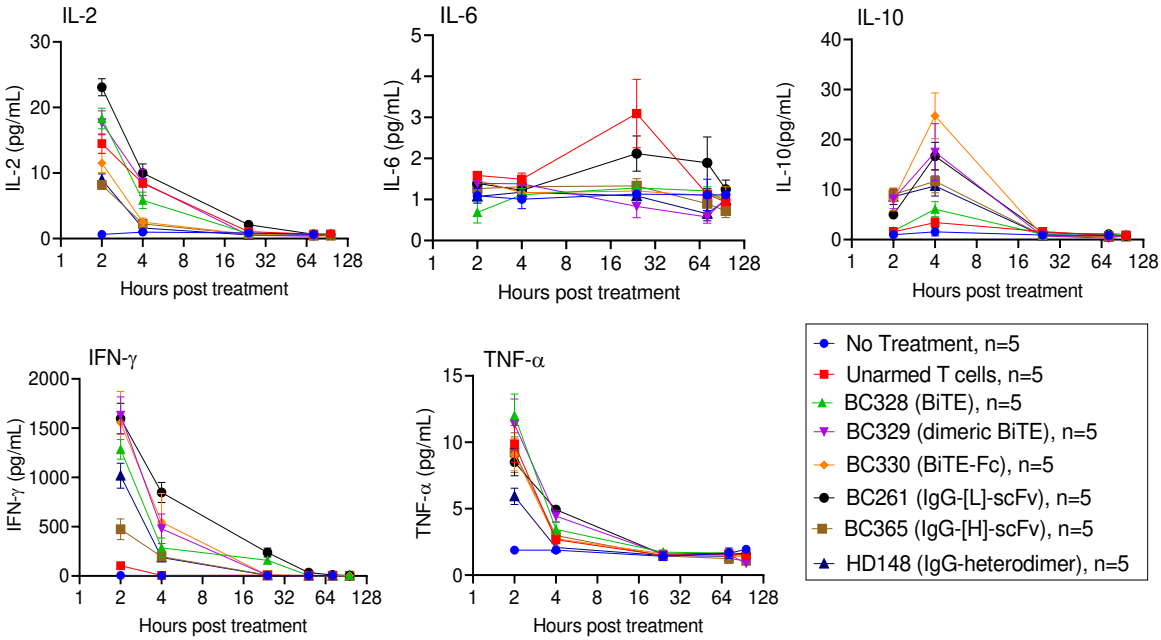


Supplementary Fig. S4

(A)



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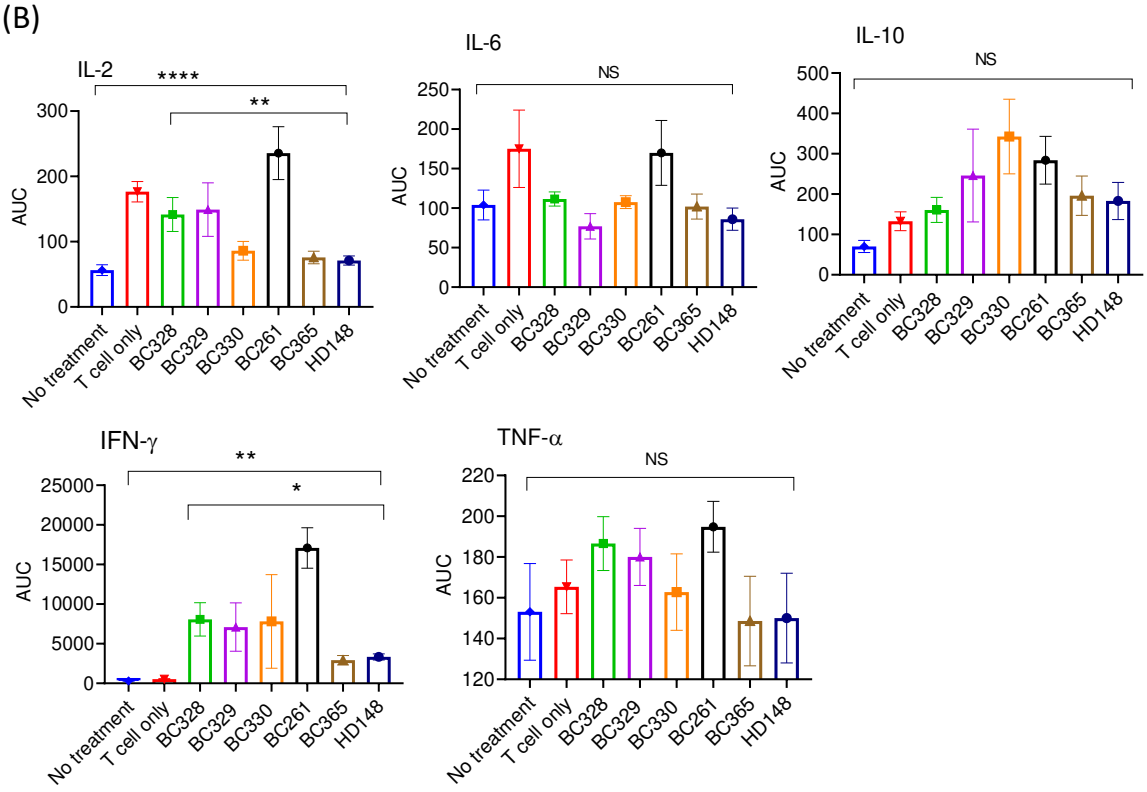
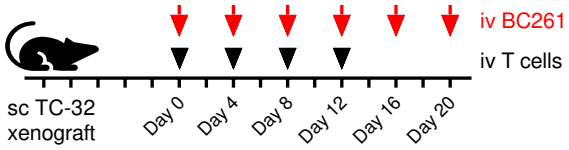


Supplementary Fig. S5

(A)



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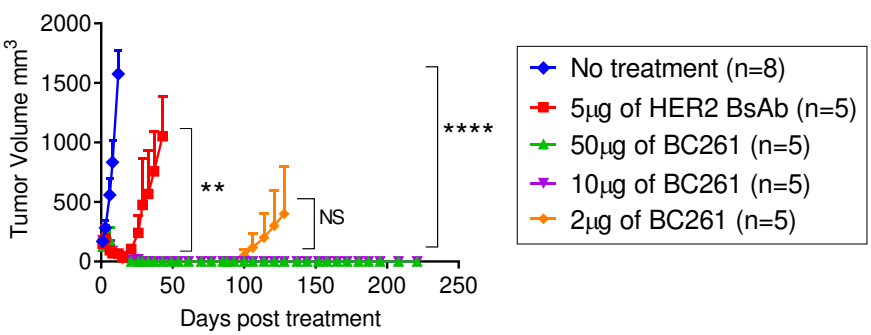

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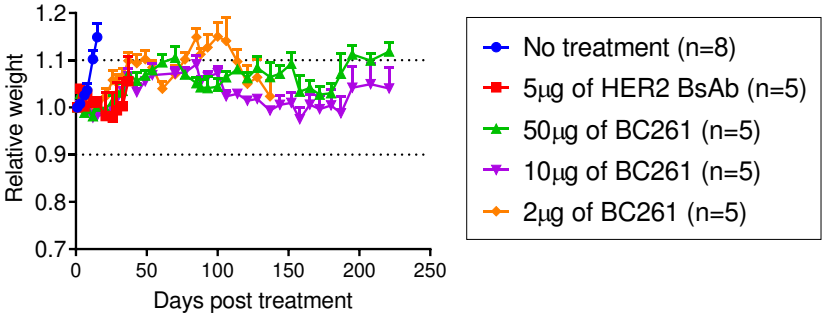

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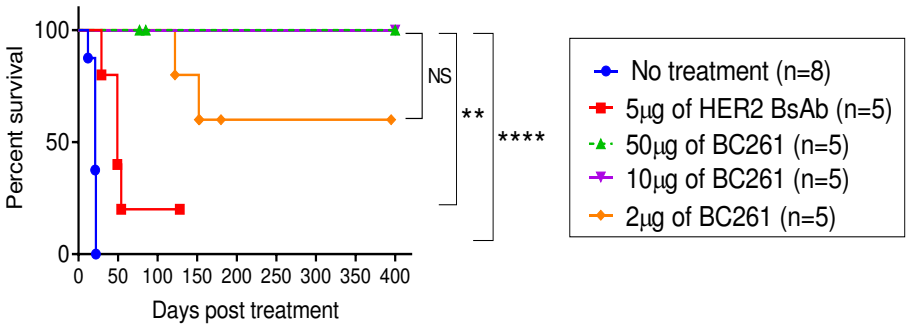

Supplementary Fig. S6