Supplemental Methods and Materials

Plasma Stability

Plasma stability of EGFR Probody therapeutics was determined using a previously described method (44). Briefly, Oregon green-conjugated EGFR Probody therapeutics containing different cleavable linkers were incubated in plasma samples at 37°C for 24 hours. Plasma from a female cynomolgus monkey was incubated with 3 μM Probody therapeutic while 5 μM Probody therapeutic was used for pooled female nu/nu mouse plasma and human male donor plasma. Timepoints were taken at 0 and 24 hours, and Probody light chain cleavage was evaluated by capillary electrophoresis immunoassay with a Wes™ Simple Western instrument (ProteinSimple). Activated Probody therapeutic controls were generated by *in vitro* treatment with the protease MT-SP1.

k_{cat}/K_M Calculation

1 μM CI107 was incubated with a 1:3 serial dilution of MT-SP1 or MMP2 protease starting at 200 nM in TBST buffer for MT-SP1 and TCNB buffer for MMP2. At 0.5 hour, 1 hour, 2 hours, and 24 hours, samples were removed and light and heavy chain cleavage was evaluated by capillary electrophoresis peak area using a LabChip GXII Touch HT instrument (Perkin Elmer). The fixed ratio of k_{cat}/K_M was calculated using a method adapted from Stennicke and Salvesen (45).

EGFR Receptor Number Quantification

The EGFR receptor number was determined by flow cytometry using an indirect immunofluorescence assay (QIFI Kit, Dako) according to manufacturer's instructions. Detroit-562-Luc2, HCT116-Luc2, HT29-Luc2, and Lovo-Luc2 cells were stained with EGFR.1 mAb (BD Biosciences) at a saturating concentration of 10 μg/ml. Antibody binding capacity (ABC) was calculated after subtracting signal from the isotype control antibody.

Supplemental Figures/Tables

Supplemental Table 1: Protease cleavage kinetics of CI107

Probody	Target Arm	MT-SP1 ($M^{-1}s^{-1}$)	MMP2 ($M^{-1}s^{-1}$)
CI107	EGFR Linker 3	9.72×10^2	$< 2 \times 10^2$
	CD3 Linker 4	N/R	2.86×10^4

Cleavage kinetics of CI107 determined via measurement of K_{cat}/K_M using a serine protease and matrix metalloprotease. CI107 was incubated with MT-SP1 or MMP2 proteases, and light and heavy chain cleavage was evaluated by capillary electrophoresis peak area at 0.5, 1, 2, and 24 hours to calculate the fixed ratio of k_{cat}/K_M. N/R, not resolvable.

Supplemental Table 2: CI107 binding to EGFR and CD3 expressing cells is attenuated

Sample	HT29-Luc2 Kd (nM)	HCT116-Luc2 Kd (nM)	Jurkat Kd (nM)
CI107	91.28	98.19	ND
act-TCB	0.17	0.23	0.62
CI128	NA	NA	ND
act-CI128	NA	NA	0.57

Masking of EGFR and CD3 binding domains attenuates binding to EGFR and CD3 on the cell surface. HT29-luc2, HCT116-luc2, and Jurkat cells were incubated with CI107, act-TCB, CI128, or act-CI128 and binding was assessed by flow cytometry. Kd values represent the concentration achieving half maximal binding. NA, not applicable; ND, Kd could not be determined

Supplemental Table 3: Comparison of Cell Lines and T-cell donors on cytotoxic activity

	Activated CI107 EC_{50} (pM)	CI107 EC_{50} (pM)
HCT116 Donor 4	0.44	7297
HCT116 Donor A	0.50	4034
HT29 Donor 4	0.25	3678
HT29 Donor A	0.38	7193
Lovo Donor 4	0.05	1131
Lovo Donor 5	0.17	2013
Detroit 562 Donor 4	0.30	318.4
Detroit 562 Donor 5	1.29	976.7

Masking of EGFR and CD3 binding domains attenuates CI107-mediated cytotoxic effects of PBMCs across donors and cell lines. HCT116-Luc2, HT29-Luc2, Lovo-Luc2, and Detroit-562-Luc2 cells were co-cultured with human PBMCs from two separate donors and treated with increasing concentrations of CI107 or activated CI107. After 48 hours of culture, cell viability was measured by the ONE-Glo Luciferase Assay, and cytotoxicity was measured relative to untreated controls. EC_{50} values represent the concentration achieving half maximal cytotoxicity.

Supplemental Table 4: T cell activation assay from other donors

	Activated CI107 EC_{50} (pM)	CI107 EC_{50} (pM)
HCT116 Donor 4	7.65	14178
HCT116 Donor A	5.72	ND
HT29 Donor 4	8.75	65971
HT29 Donor A	1.75	ND

Masking of EGFR and CD3 binding domains attenuates CI107-mediated T cell activation in PBMCs from a separate donor co-cultured with human colorectal cancer cells. HCT116-Luc2 and HT29-Luc2 cells were co-cultured with human PBMCs from two separate donors and treated with increasing concentrations of CI107 or activated CI107. After 16 hours of culture, CD69 expression was measured by flow cytometry as a readout of T cell activation. EC_{50} values represent the concentration achieving half maximal CD69 expression. The EC_{50} values were ambiguous or could not be calculated due to poor curve fit in samples with PBMC Donor A treated with CI107. ND, not determined.

Supplemental Table 5: Cytokine release assay

Analyte	Donor 4 with HCT116 cells		Donor 4 with HT29 cells		Donor A with HCT116 cells		Donor A with HT29 cells	
	Act-CI107	CI107	Act-CI107	CI107	Act-CI107	CI107	Act-CI107	CI107
IFN-γ	7.49	17211	93.93	67545	6.72	84536	5.03	ND
IL-2	11.60	30511	24.77	54180	6.03	69715	5.07	78349
IL-6	3.99	22180	ND	ND	ND	ND	5.817	ND
MCP-1	0.98	8360	5.58	56778	2.65	ND	1.14	ND
TNF-α	9.36	25193	179.2	57414	5.84	74082	3.38	ND

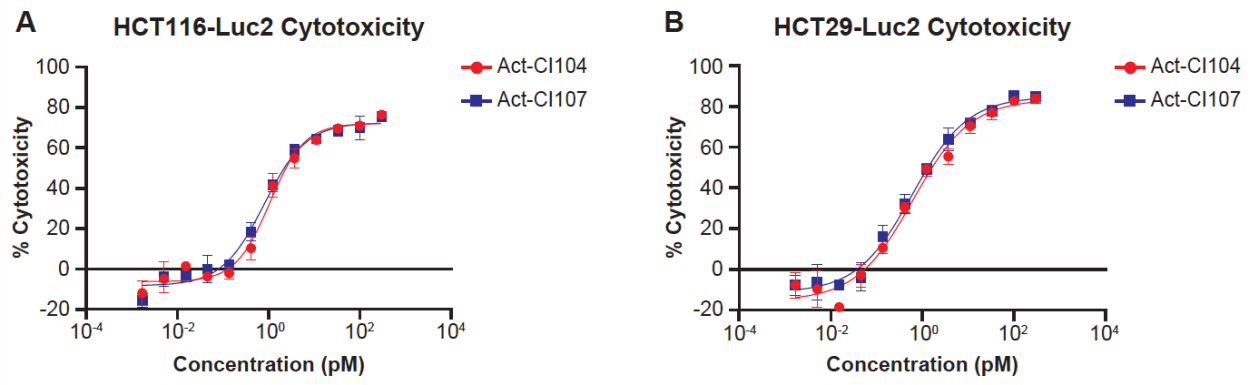
Masking of EGFR and CD3 binding domains attenuates CI107-mediated cytokine release from PBMCs from another donor co-cultured with human colorectal cancer cells. HCT116-Luc2 and HT29-Luc2 cells were co-cultured with human PBMCs from two separate donors and treated with increasing concentrations of CI107 or activated CI107 (Act-CI107). After 16 hours of culture, cytokine release was measured using a U-PLEX plate. EC_{50} values (pm/mL) represent the concentration achieving half maximal cytokine levels. ND indicates EC_{50} values that could not be calculated due to poor curve fit, insufficient data to fit a curve, or ambiguous EC_{50} values.

Supplemental Table 6: CI107 provides improved tolerability relative to Act-TCB in cynomolgus monkeys.

TCB	Dose (mg/kg)	Clinical Observations
Act-TCB	**0.06 *(MTD)***	Emesis, hunched posture
Act-TCB	0.18	Emesis, hunched posture, inappetence, pale and thin appearance
CI107	0.6	None
CI107	2.0	Emesis
CI107	**4.0 *(MTD)***	Emesis, inappetence
CI107	6.0	Not tolerated

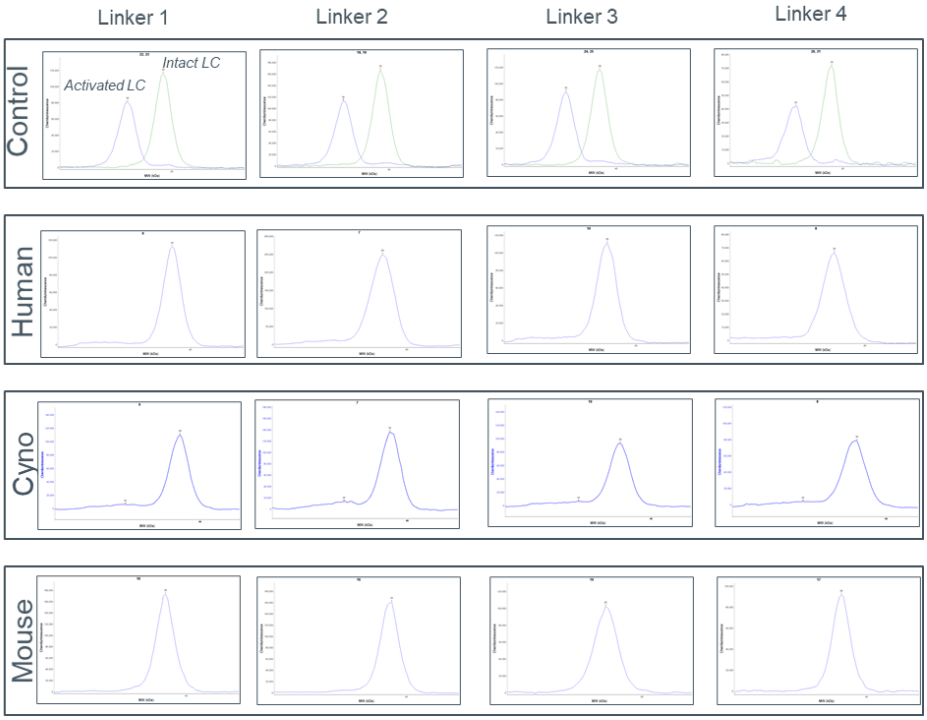
Severity of clinical signs observed after dosing were recorded following a single dose of 0.06 mg/kg or 0.18 mg/kg of activated TCB (Act-TCB) on Day 1 or 0.6 mg/kg, 2.0 mg/kg, 4.0 mg/kg, or 6.0 mg/kg CI107.

Supplemental Figure 1: Cytotoxicity of Act-CI107 is comparable to Act-CI104



Cytotoxicity of Act-CI107 is comparable to Act-CI104. HCT116-Luc2 and HT29-Luc2 cells were co-cultured with human PBMCs and treated with increasing concentrations of activated CI104 (Act-104) or activated CI107 (Act-CI107). After 48 hours of culture, cell viability of HCT116-Luc2 **(A)** or HT29-Luc2 **(B)** was measured by the ONE-Glo Luciferase Assay, and % cytotoxicity was calculated.

Supplemental Figure 2: Plasma stability of protease substrates used in Probody TCBs

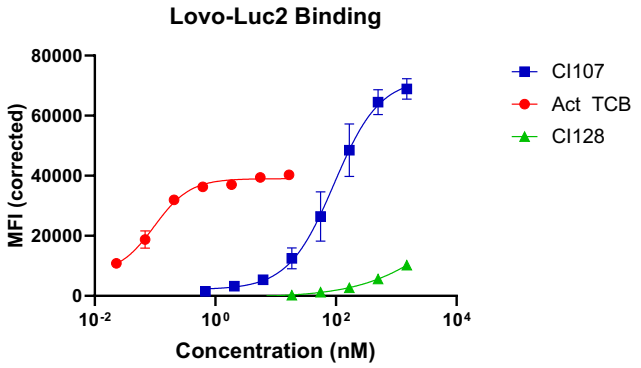


Substrate	% Cleavage		
	Mouse 24 h	Cyno 24 h	Human 24 h
Linker 1	0.0	0.0	0.0
Linker 2	0.0	0.0	0.0
Linker 3	0.0	0.0	0.0
Linker 4	0.0	0.0	0.0

EGFR Probody therapeutics have similar plasma stability. Plasma stability of EGFR Probody therapeutics containing cleavable linkers used in Probody TCB molecules CI011, CI040, and CI107 is comparable in human, cynomolgus monkey (cyno), and mouse plasma. No Probody activation was observed at 24 hours.

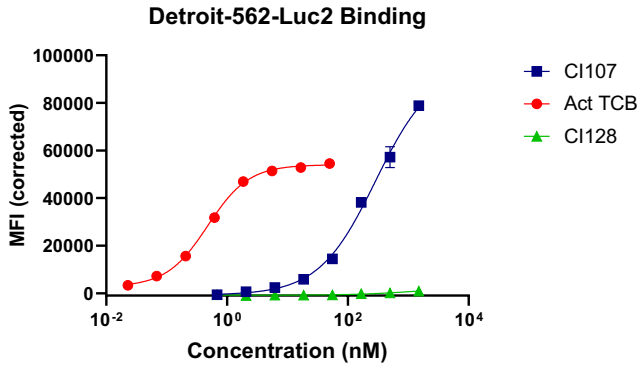
Supplemental Figure 3: CI107 binding is attenuated in additional EGFR-expressing cell lines

A



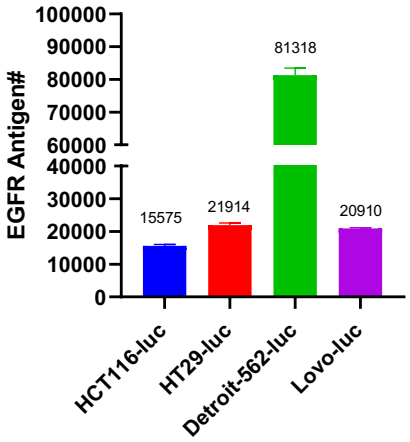
Sample	Kd (nM)
CI107	92.89
Act-TCB	0.10
CI128	NA

B



Sample	Kd (nM)
CI107	288.8
Act-TCB	0.48
CI128	NA

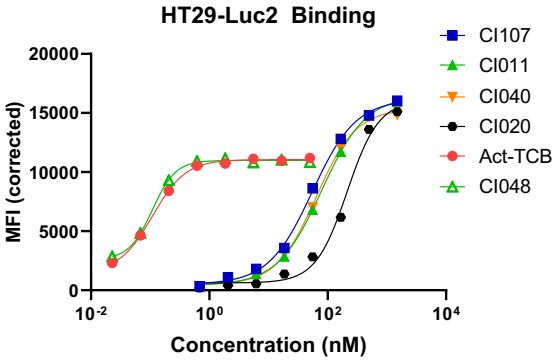
C



CI107 binding is attenuated across cell lines. Lovo **(A)** or Detroit-562 cells **(B)** were incubated with CI107 or protease-activated TCB (Act-TCB), and binding was assessed by flow cytometry. (C) EGFR expression levels for cell lines used in these experiments was quantified via QIFIKIT®. MFI, mean fluorescence intensity.

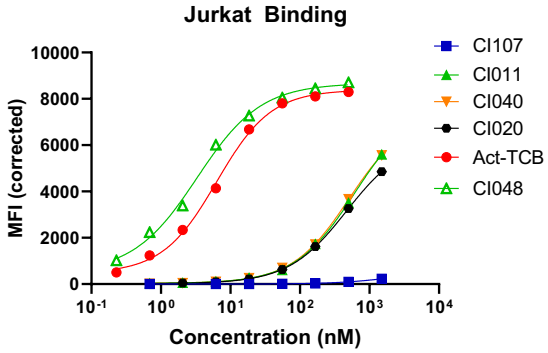
Supplemental Figure 4: EGFR and CD3 binding of additional Probody TCB molecules

A



Sample	Kd (nM)
CI107	54.79
CI011	79.06
CI040	64.17
CI020	213.2
Act-TCB	0.11
CI048	0.11

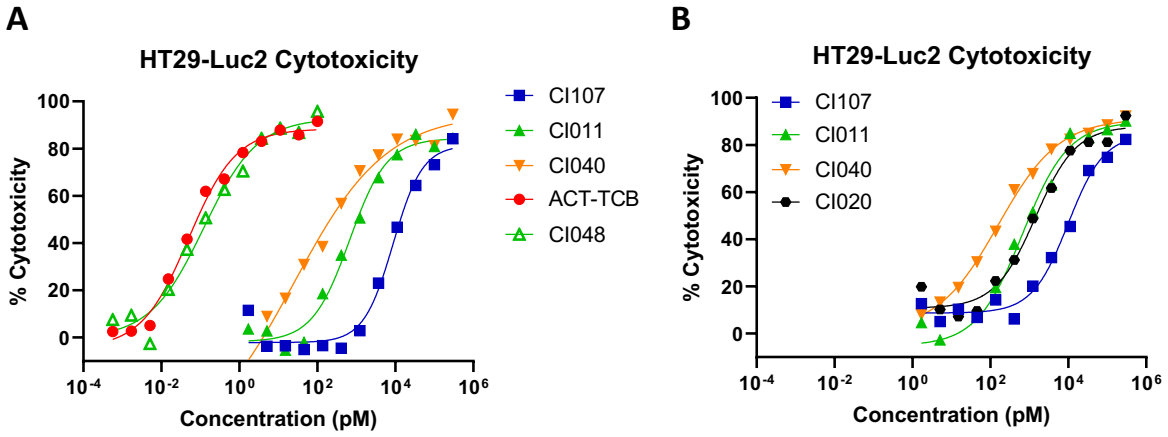
B



Sample	Kd (nM)
CI107	N/A
CI011	619.6
CI040	506.6
CI020	467.9
Act-TCB	6.36
CI048	3.30

Binding of CI011, CI020, and CI040 to EGFR and CD3 is attenuated relative to protease-activated TCB. HT29-Luc2 **(A)** or Jurkat **(B)** cells were incubated with Probody TCBs or protease-activated TCBs, and binding was assessed by flow cytometry. MFI, mean fluorescence intensity.

Supplemental Figure 5: Cytotoxic activity of additional Probody TCB molecules



Sample	Donor 2	Donor 4
CI107	5355	8760
CI011	397.5	673.5
CI040	105.7	25.48
Act-TCB	0.03	0.05
CI048	0.10	0.12

Sample	Donor 2	Donor 4
CI107	6240	9917
CI011	230.2	670.5
CI040	103.4	160
CI020	568.4	1441

Cytotoxic activity of CI011, CI020, and CI040 is attenuated relative to protease-activated TCB. HT29-Luc2 cells were co-cultured with human PBMCs from two donors and treated with increasing concentrations of Probody TCB (CI011, CI020, CI040, CI107) or protease-activated TCB (Act-TCB, CI048). After 48 hours of culture, HT29-Luc2 cell viability was measured by the ONE-Glo Luciferase Assay, and % cytotoxicity was calculated. Data from donor 4 are graphed. **(A)** Cytotoxic activity of protease cleavable Probody TCBs and protease-activated TCBs. **(B)** Cytotoxic activity of protease cleavable and non-cleavable Probody TCBs.