Supplementary Materials

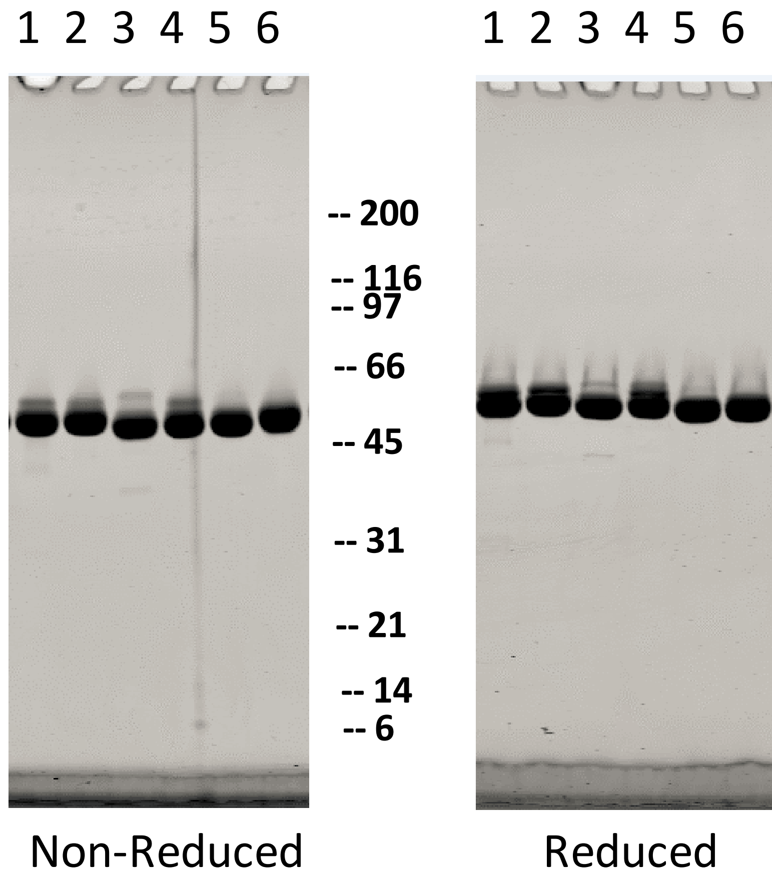

Fig. S1. Purity of EGFR-9G8 TriTACs as assessed by SDS-PAGE. SDS-PAGE of EGFR-9G8 TriTACs in six different configurations under non-reducing (left panel) or reducing (right panel) conditions. 2.5 ug of purified protein loaded in each lane visualized by Coomassie stain Lane 1: T:C:A configuration. Lane 2: T:A:C configuration. Lane 3: C:T:A configuration. Lane 4: A:T:C configuration. Lane 5: A:C:T configuration. Lane 6: C:A:T configuration. Similar results were obtained with the EGFR-7D12 and PSMA TriTACs (data not shown)

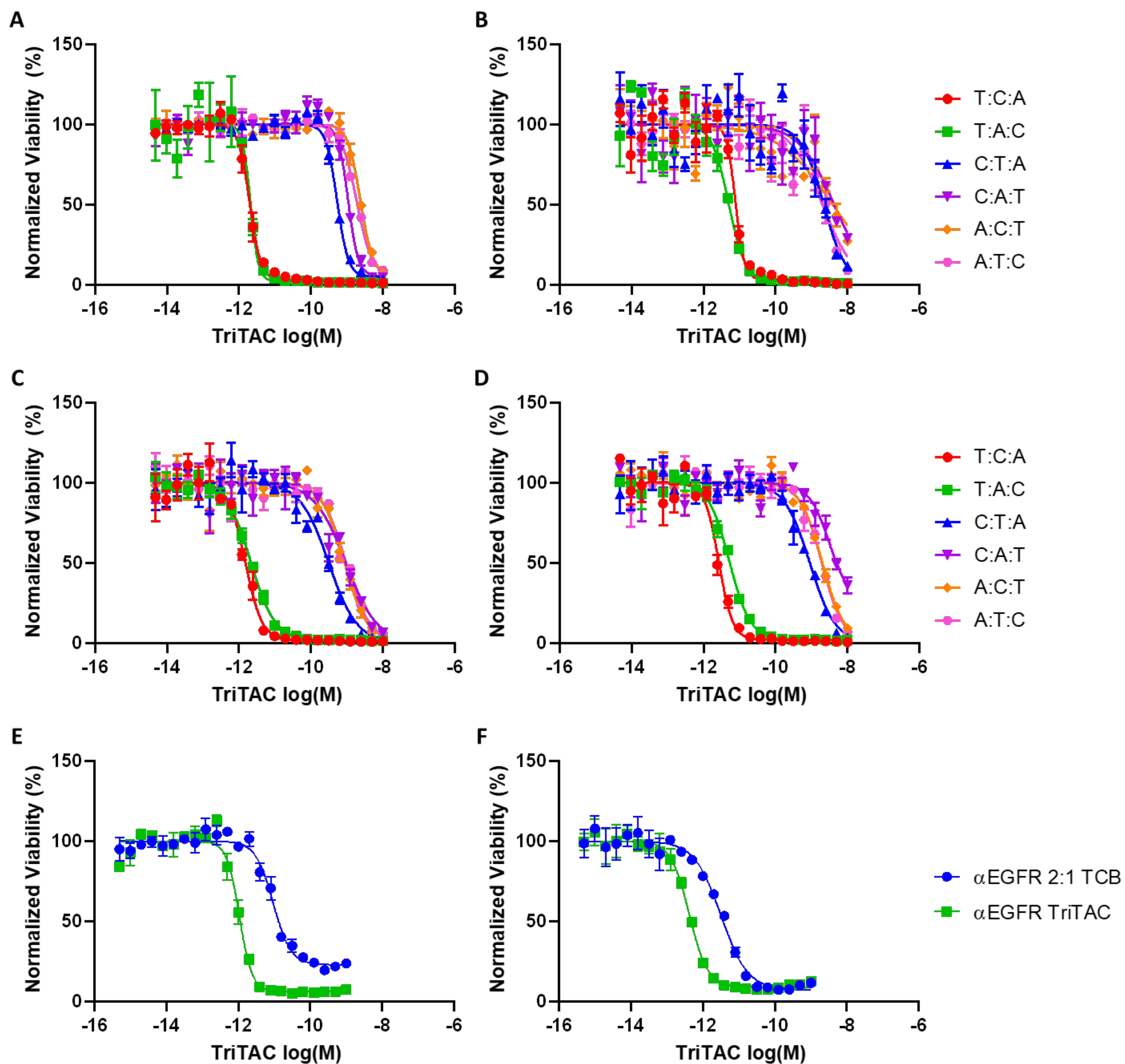

Fig. S2. Measuring activities of EGFR-7D12 TriTAC molecules produced in 6 different configurations. See Fig. 1A for a description of the six configurations. (**A** to **D**). NCI-H1563 cells (A and B) or CAPAN2 cells (C and D) and purified human T cells at a 1:10 ratio were incubated with a titration of EGFR-7D12 TriTAC molecules in 6 configurations for 48 hours, and then viability of the luciferase labelled tumor cell lines cells was measured. Human serum albumin (HSA) was present at 15 mg/ml in (B) and (D). (**E** to **F**) titrations of EGFR TriTAC in the T:A:C configuration or an EGFR T cell

engaging molecule in the 2:1 configuration (34) (2:1 TCB), engineered from the cetixumab sequence, were added to DMS-153 cells (E) or OVCAR8 (F) cells in the presence of purified human T cells. Forty-eight hours later, viability of the luciferase-labeled tumor cell lines was measured. EC_{50} values for cell killing of the DMS153 and OVCAR8 cells were, respectively, 1.0 and 0.4 pM for the TriTAC and 9.4 and 3.2 pM, respectively, for the 2:1 TCB. Plotted are mean values ± SEM, n=3.

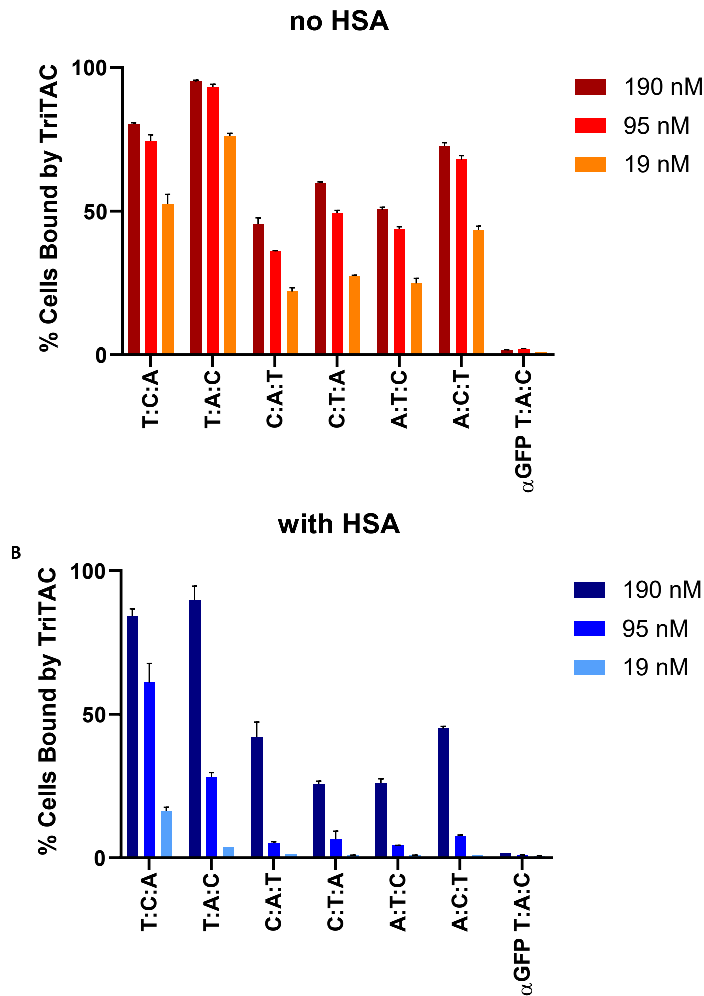

Fig. S3. Binding of PSMA TriTACs to PSMA-expressing MDAPCa2b cells. MDAPCa2b cells were incubated with PSMA TriTACs in six configurations at concentrations of 19, 95, or 190 nM (**A**) in the absence of HSA or (**B**) the presence of 15 mg/ml HSA. As a control, cells were incubated with an anti-GFP TriTAC in the T:A:C configuration. Bound TriTAC was detected with a fluorescently-labelled antibody recognized the anti-albumin domain in TriTAC molecules. Plotted in the graph are the mean percentage of cells ± SEM with detectable PSMA TriTAC binding relative to the control TriTAC as quantitated with FlowJo^{TM} software.

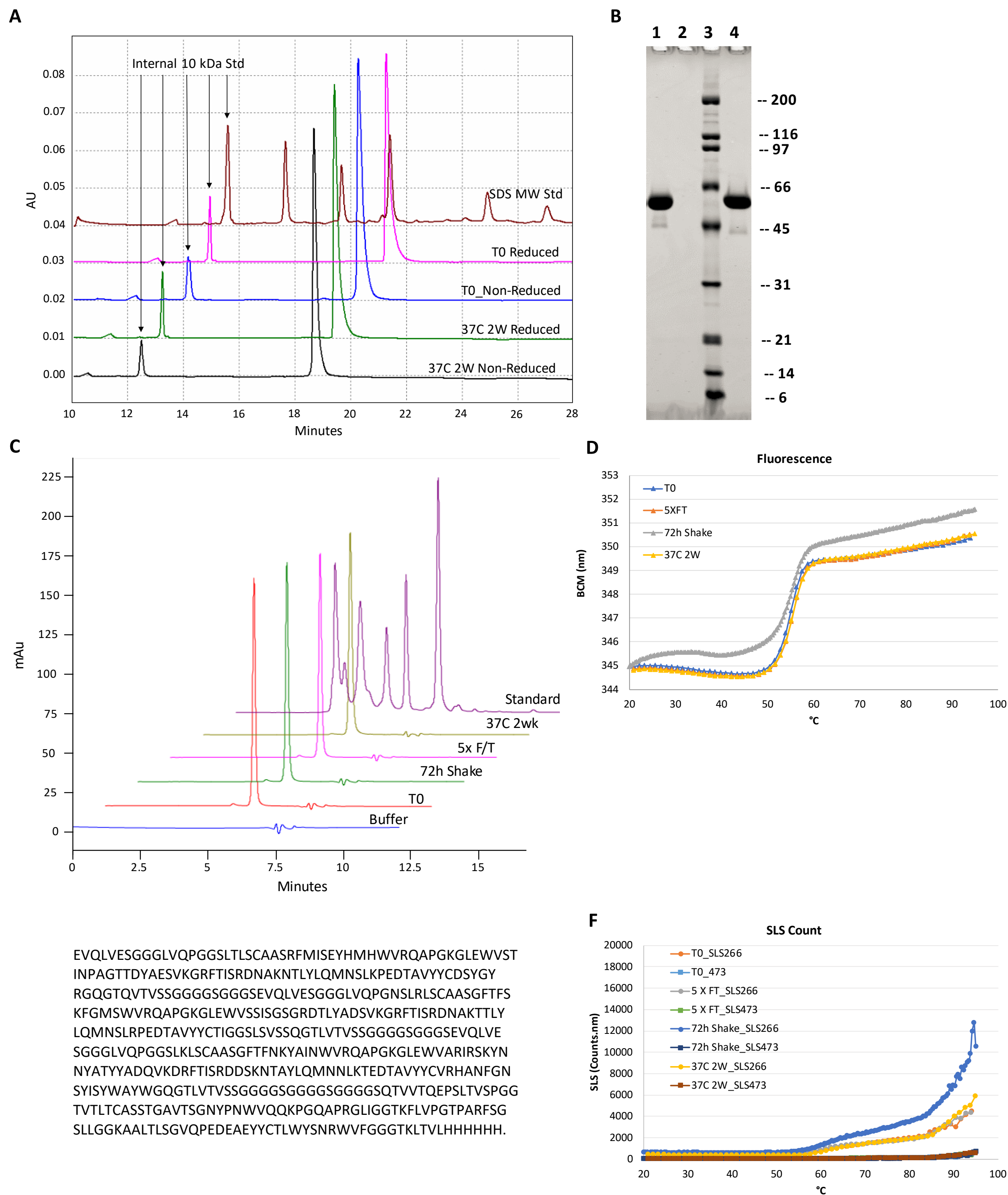

Fig. S4. Biophysical characterization of HPN424. (A) Overlay of electrophoretograms from SDS denatured capillary electrophoresis of HPN424 under reduced conditions (pink line), non-reduced conditions (blue line), reduced conditions after incubation at 37°C for two

weeks (green line), non-reduced conditions after incubation at 37°C for two weeks (black line). Molecular weight standards are graphed in brown. An internal 10 kDa standard is indicated by an arrow above each electrophoretogram. (**B**) SDS-PAGE of HPN424. Lane 1: HPN424 under non-reducing conditions. Lane 2: empty. Lane 3: molecular weight standard. Lane 4: HPN424 under reducing conditions. (**C**) Overlay of chromatograms of analytical size exclusion chromatography of HPN424 under stressed conditions. T_0: unstressed HPN424 (red line). 72 h Shake: Sample incubated with shaking at room temperature for 72 hours (green line), 5x F/T: sample frozen and thawed five times (pink line). 37°C 2 wk: HPN424 stored at 37°C for two weeks. Standard: molecular weight standards (purple line). Buffer (blue line). (**D**) Intrinsic fluorescence (barycentric mean plotted). T_0: unstressed HPN424 (blue triangles). 5x F/T: sample frozen and thawed five times (orange triangles). 72 h Shake: Sample incubated with shaking at room temperature for 72 hours (grey triangles). 37°C 2 wk: HPN424 stored at 37°C for two weeks (yellow triangles). (**E**) The primary structure of HPN424. (**F**) Static light scattering counts made at 266 nm and 473 nm during a 0.5ºC/min thermal ramp of HPN424 samples treated under stressed conditions T_0: unstressed HPN424 at 266 nM (orange circles) and 473 nM (light blue squares). 5x F/T: sample frozen and thawed five times at 266 nM (grey circles) and 473 nM (green squares). 72 h Shake: Sample incubated with shaking at room temperature for 72 hours at 266 nM (blue circles) and 473 nM (dark blue squares). 37°C 2 wk: HPN424 stored at 37°C for two weeks at 266 nM (yellow circles) and 473 nM (brown squares).

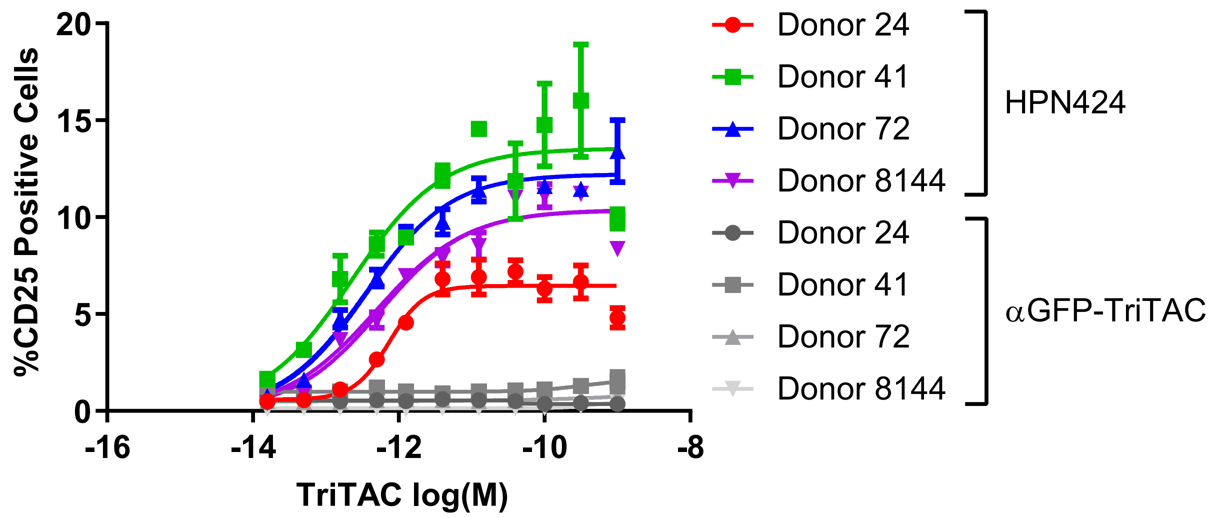

Fig. S5. HPN424-induced expression of CD25 on T cells. Co-cultures of LNCaP cells and purified T cells from four different healthy donors were treated with a titration of HPN424 or an anti-GFP TriTAC. After a 48 incubation, flow cytometry analyses were performed. Plotted is the expression of CD25 expression on $CD4^{+}$ and/or $CD8^{+}$ T cells (± SEM, n=2).

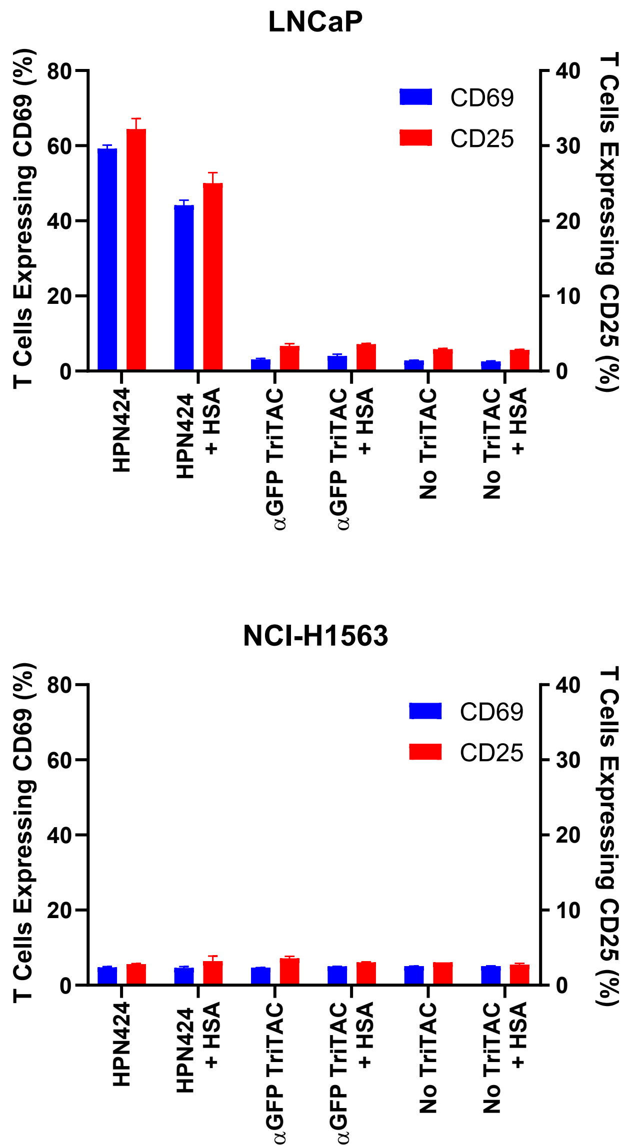

Fig. S6. Effect off HSA on PSMA-Dependent HPN424-induced expression of CD69 and CD25 on T cells. Co-cultures of purified T cells and (A) PSMA-expressing LNCaP cells or (B) PSMA-negative NCI-H1563 cells were treated with 1 nM HPN424 or anti-GFP TriTAC in the absence or presence of 15 mg/ml HSA. After a 48 incubation, flow cytometry analyses were performed. Plotted are the expression of CD69 (left y-axis) and CD25 expression (right y-axis) on $CD4^+$ and/or $CD8^+$ T cells (± SD, n=2).

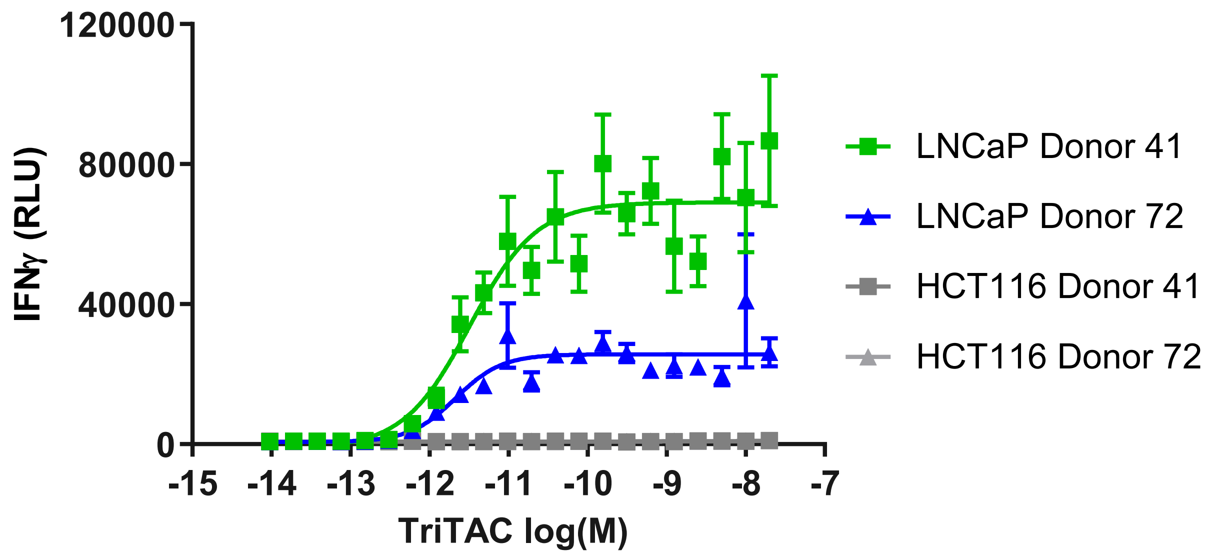

Fig. S7. HPN424-induced expression of IFNγ. Conditioned medium was collected from LNCaP or HCT116 cells co-cultured with purified human T cells from two different donors. Plotted are the mean relative IFNγ levels in the conditioned medium measured with an AlphaLISA assay (± SEM, n=3).

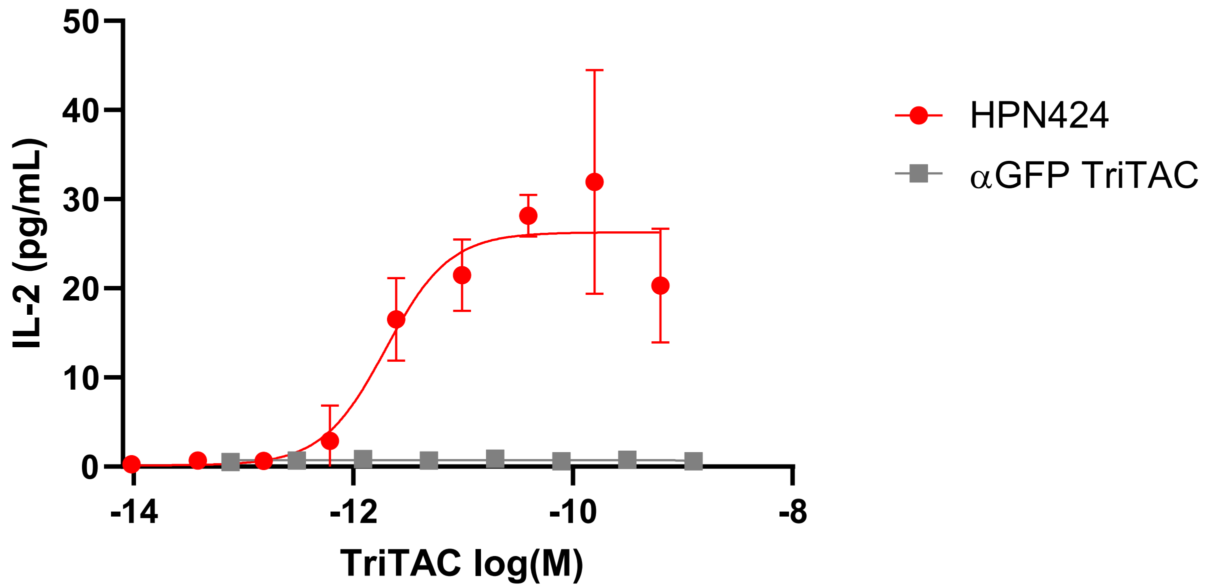

Fig. S8. HPN424-induced expression of IL-2. LNCaP cells were co-cultured with purified human T cells from Donor 02 treated with a titration of HPN424 (red circles) or anti-GFP TriTAC (αGFP TriTAC, grey squares). After a 48-hour incubation, conditioned media were collected, and IL-2 was measured using an electorchemilumiscent assay (Meso Scale Discovery) following the manufacturer's protocol. IL-2 concentrations were calculated from the raw data using a standard curve containing known IL-2 amounts. Plotted are the mean relative IL-2 concentrations (± SD, n=2).

Table S1. Purity and monomer content of EGFR-9G8 TriTACs. EGFR-9G8 TriTACs in six configurations were analyzed by size exclusion chromatography to determine high molecular weight, dimer, monomer, and low molecular weight content under native conditions. Similar results were obtained with the EGFR-7D12 and PSMA TriTACs (data not shown).

EGFR TriTAC Configuration	High Molecular Weight (%)	Dimer (%)	Monomer (%)	Low Molecular Weight (%)
T:C:A	0.0	0.5	98.0	1.5
T:A:C	0.0	0.3	98.4	1.3
C:T:A	0.0	0.0	98.5	1.5
C:A:T	0.0	0.3	98.1	1.6
A:T:C	0.0	0.3	98.6	1.1
A:C:T	0.0	0.3	98.5	1.2

Table S2. Pharmacokinetics of an EGFR TriTAC in mice. Pharmacokinetic values calculated from measured serum exposures of an EGFR-7D12 TriTAC in the T:A:C configuration (see Fig. 1A) after single 50 or 500 µg/kg i.v. bolus doses.

Dose Level	No. of points in lambda_z	Terminal t1/2 (h)	C_0 (ng/mL)	Cmax (ng/mL)	AUC, 0-last (h*ng/mL)	AUC, 0-inf (h*ng/mL)	Clearance (mL/h/kg)	Vss (mL/kg)
50 µg/kg	4	15.3	743	711	13400	13400	3.73	73.3
500 ug/kg	4	17.9	12400	11400	164000	164000	3.04	56.3

Table S3. Binding affinities of HPN424 to target proteins. Binding affinities of HPN424 for PSMA, CD3ε, and albumin (ALB) from different species were measured by biolayer interferometry.

Target	K_D (nM)	Replicates (n)
human PSMA	0.55 ± .20	11
cyno PSMA	indeterminate	2
human CD3ε	12 ± 1	7
cyno CD3ε	10 ± 1	4
human ALB	8.3 ± 1.0	10
cyno ALB	7.7 ± 1.4	5
mouse ALB	140 ± 1	3

Table S4. Capillary electrophoresis analysis of HPN424. HPN424 samples from an accelerated stability study were analyzed by capillary electrophoresis to measure monomer content under non-reduced and reduced denaturing conditions.

	Non-reduced			Reduced		
Condition	High Molecular Weight (%)	Monomer (%)	Low Molecular Weight (%)	High Molecular Weight (%)	Monomer (%)	Low Molecular Weight (%)
T0	0	99.7	0.3	0	99.4	0.6
5 X FT	0	98.6	1.4	0	98.2	1.8
72h Shake	0	98.2	1.8	0	98.3	1.7
37°C 2W	0	97.7	2.3	0	97.9	2.1

Table S5. Analytical size exclusion chromatography of HPN424. HPN424 samples from an accelerated stability study were analyzed by size exclusion chromatography to measure monomer content under non-denaturing conditions.

Condition	High Molecular Weight (%)	Monomer (%)	Low Molecular Weight (%)
Start (t=0)	1.9	98.1	0.0
72 h Shake	1.7	97.6	1.1
5x FT	1.6	97.6	0.8
37°C for 2 weeks	1.0	97.2	1.8

Table S6. Melting and aggregation temperatures of HPN424. HPN424 samples from an accelerated stability study were measured by full spectrum fluorescence and static light scattering at two wavelengths, 266 nm and 473 nm. Small aggregates initiated at the observed Tm of 55°C, but exponential particle growth occurred only above 83°C for all samples.

Condition	T_m (°C)	T_{agg} 266 (°C)*
T0	55.3	83.7
5 X FT	55.5	83.4
37°C 2W	55.5	84.3
72h Shake	55.2	83.3

Table S7. T cell directed killing of target cells by HPN424. EC50 values for HPN424-directed killing of four PSMA-expressing cell lines by T cells from four donors. No cell killing was observed in two cell lines lacking PSMA expression.

		TDCC EC_{50} (pM)			
Cell Line	**PSMA Expression**	**Donor 24**	**Donor 8144**	**Donor 72**	**Donor 41**
LNCaP	Positive	1.5	0.22	0.36	0.43
MDAPCa2b	Positive	4.8	0.41	0.49	0.65
VCaP	Positive	0.64	0.16	0.2	0.35
22Rv1	Positive	n/a	0.72	1.4	1.3
HCT116	Negative	>10,000	>10,000	>10,000	>10,000
NCI-1563	Negative	>10,000	>10,000	>10,000	>10,000

Table S8. Induction of CD69 expression on T cells by HPN424. EC50 values for HPN424-directed induction of CD69 expression on by T cells from four donors in the presence of PSMA-expressing cells. No induction of CD69 expression was observed in the presence of two cell lines lacking PSMA expression.

		CD69 EC_{50} (pM)			
Cell Line	**PSMA Expression**	**Donor 24**	**Donor 8144**	**Donor 72**	**Donor 41**
LNCaP	Positive	0.6	0.2	0.6	0.6
MDAPCa2b	Positive	0.3	0.1	0.2	0.2
VCaP	Positive	1.0	0.5	0.6	0.4
22Rv1	Positive	0.6	0.2	0.2	0.2
HCT116	Negative	>1000	>1000	>1000	>1000
NCI-1563	Negative	>1000	>1000	>1000	>1000

Table S9. **Induction of CD25 expression on T cells by HPN424.** EC50 values for HPN424-directed induction of CD25 expression on T cells from four donors in the presence of PSMA-expressing cells. No induction of CD25 expression was observed in the presence of two cell lines lacking PSMA expression.

		CD25 EC_{50} (pM)			
Cell Line	**PSMA Expression**	**Donor 24**	**Donor 8144**	**Donor 72**	**Donor 41**
LNCaP	Positive	0.7	0.2	0.4	0.6
MDAPCa2b	Positive	0.2	0.2	0.1	0.2
VCaP	Positive	1.5	1.2	0.9	0.8
22Rv1	Positive	0.9	0.3	0.5	0.6
HCT116	Negative	>1000	>1000	>1000	>1000
NCI-1563	Negative	>1000	>1000	>1000	>1000

Table S10. Induction of TNFα expression on T cells by HPN424. EC50 values for HPN424-directed induction of TNFα expression by T cells from four donors in the presence of PSMA-expressing cells. No induction of TNFα expression was observed in the presence of two cell lines lacking PSMA expression.

Cell Line	PSMA Expression	TNFα EC_{50} (pM) Donor 24	Donor 8144	Donor 72	Donor 41
LNCaP	Positive	4.9	2.8	4.0	3.2
MDAPCa2b	Positive	3.2	2.9	2.9	2.9
VCaP	Positive	21.0	4.0	5.5	3.6
22Rv1	Positive	8.9	2.5	4.0	3.3
HCT116	Negative	>10,000	>10,000	>10,000	>10,000
NCI-1563	Negative	>10,000	>10,000	>10,000	>10,000

Table S11. Induction of IFNγ expression on T cells by HPN424. EC50 values for HPN424-directed induction of IFNγ expression by T cells from four donors in the presence of PSMA-expressing cells. No induction of IFNγ expression was observed in the presence of two cell lines lacking PSMA expression.

		IFNγ EC_{50} (pM)			
Cell Line	**PSMA Expression**	**Donor 24**	**Donor 8144**	**Donor 72**	**Donor 41**
LNCaP	Positive	4.2	4.2	4.2	2.8
MDAPCa2b	Positive	5.1	15.0	3.4	4.9
VCaP	Positive	15.0	5.8	9.7	3.5
22Rv1	Positive	7.8	3.0	9.1	3.0
HCT116	Negative	>10,000	>10,000	>10,000	>10,000
NCI-1563	Negative	>10,000	>10,000	>10,000	>10,000

Table S12. Pharmacokinetics of an HPN424 TriTAC in Non-Human Primate.
Pharmacokinetic values calculated from measured serum exposures of HPN424 after single 0.03 or 0.1 mg/kg i.v. bolus doses.

Dose (mg/kg)	**Terminal $t_{1/2}$ (h)**	**C_{max} (nM)**	**AUC 0-168 h (h*nM)**	**AUC 0-inf (h*nM)**	**CL (mL/h/kg)**	**Vss (L/kg)**
0.1	79.9	65.5	3,530	3,570	0.53	0.051
0.03	80.9	25.4	1,260	1,270	0.45	0.047