Supplemental Figure 1. NCL-186 Demonstrates Tumor Growth Equivalent to Vehicle. HT29 (A) or LoVo (B) colorectal human tumor xenografts were dosed with vehicle, NCL-186, or TAK-186 every 3 days for a total of 7 doses. NCL-186 did not elicit anti-tumor response, with tumor growth equivalent to vehicle. Relative to both NCL-186 and vehicle, TAK-186 inhibited tumor growth by 100% in both studies (p <0.0001 HT29, p <0.0001 LoVo) Data representative of 2 or more independent experiments (n=6, error bars present SEM).

Supplemental Figure 2. pcTAK-186 Requires the Presence of EGFR-expressing Tumor Cells to Elicit T-cell Activation. Human PBMC were combined with NCL-186 or pcTAK-186 in the absence (A) or presence of HT29 tumor cells (B). T cell activation was assessed by staining for $CD3^+CD25^+$ or $CD3^+CD69^+$ populations by flow cytometry. Data is representative of 3 independent experiments.

Supplemental Figure 3. TAK-186 Tumor Biodistribution. TAK-186 was dosed at 3mg/kg in HT29 tumor-bearing mice. The concentration of cleaved and intact MVC-101 was assessed via Simple Western in tumor lysates concurrently via an anti-VHH antibody recognizing the sdAbs present in all forms of TAK-186 at 6h, 3 days and 7 days post dose. The % injected dose/g tumor of total TAK-186 peaked at 6h post dose (n=3, mean ± SD).

Supplemental Figure 4. Intra-tumoral protease-mediated TAK-186 cleavage: Intact and Cleaved TAK-186 are Differentiated by Size. TAK-186 was dosed at 3mg/kg in HT29 tumor-bearing mice. The concentration of cleaved and intact MVC-101 was assessed via Simple Western in tumor lysates concurrently via an anti-VHH antibody recognizing the sdAbs present in all forms of TAK-186, with cleaved TAK-186

differentiating from intact TAK-186 by size, as shown. The % cleaved relative to intact MVC-101 is increased at 3 days post dose (B) relative to 6h post dose (A). Chromatogram is representative of one tumor (n=3 per timepoint).

Supplemental Table 1. pcTAK-186 Mediates T-cell Mediated Killing and Cytokine Release Over Multiple Effector Cell Donors In Vitro. pcTAK-186 was tested in TDCC and Cytokine Release assays by co-incubating EGFR-expressing human tumor cell lines HT29 or LoVo in the presence of 5 healthy effector cell donors.

Supplemental Table 2. **TAK-186 Binding to Human, Cyno and Mouse Proteins.** Biosensor analysis was run on Octet to assess binding of adTAK-186, or TAK-186 to CD3ε, EGFR, EGFRviii and serum albumin from human, cynomolgus monkey or mouse recombinant protein. Binding to CD3ε is observed only in adTAK-186, and binding to serum albumin is present only in TAK-186 prodrug form, consistent with the COBRA design. Binding to EGFR in adTAK-186 is increased relative to TAK-186 as a result of the avidity provided by the 4 EGFR-binding sdAbs present in adTAK-186. TAK-186 binding to murine serum albumin is reduced relative to both human and cyno albumin. Data is the mean of 3 independent experiments.

Supplemental Table 3. pcTAK-186 Mediates T cell Activation in the Presence of Multiple Effector Cell Donors In Vitro. pcTAK-186 was tested in a T cell activation assay by co-incubating EGFR-expressing human tumor cell lines HT29 or LoVo in the presence of 5 healthy effector cell donors. Data was analyzed using GraphPad Prism (GraphPad software).

Supplemental Table 4. Pharmacokinetics of Cleaved and Intact TAK-186 in Tumor and Plasma of Tumor-bearing Mice. TAK-186 was dosed via i.v. bolus at 3 mg/kg in HT-29 tumor-bearing mice. The concentration of intact and cleaved TAK-186 were measured in tumor or plasma concurrently via Simple Western. PK parameters were calculated using WinNonlin (Certara). Data represents the mean (n=3).

Supplemental Methods

T-Cell Activation Assay

To assess T-cell activation, target cells were combined with human PBMC, NCL-186 and pcTAK-186 as above, and incubated at 37deg in AIM-V media (Thermo Fisher) for 24h. Lymphocytes were dislodged & collected, and washed prior to staining for CD3, CD69 and CD25 expression using fluorophore conjugated antibodes (Biolegend). Cells were stained on ice for 30 minutes. Data was collected on CytoFLEX LX, and analyzed using FlowJo (BD Biosciences) and GraphPad Prism (GraphPad Software).