Supplementary Information

Harwood *et al.* ATTACK, a novel bispecific T cell-redirecting antibody with trivalent EGFR binding and monovalent CD3 binding for cancer immunotherapy

Supplementary Figures

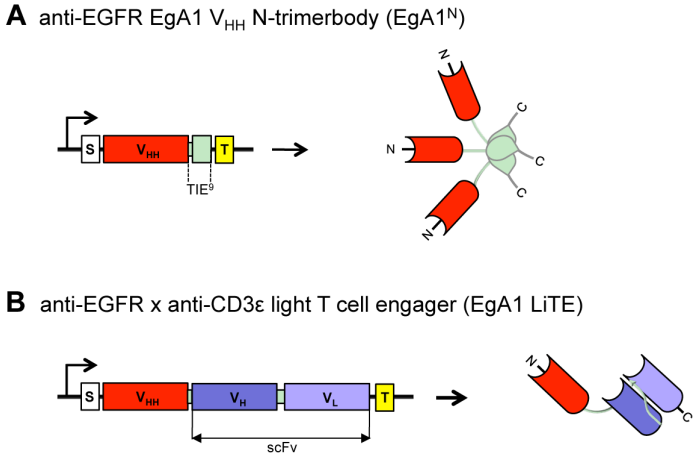

Figure S1. Schematic diagrams showing the genetic (left) and domain structure (right) of the anti-EGFR EgA1 multi-chain trimerbody (EgA1^N) (A) and the bispecific anti-EGFR x anti-CD3 *light T cell engager* (EgA1 LiTE) (B). The EgA1^N bears the signal peptide from the oncostatin M (white box), the anti-EGFR EgA1 V_{HH} gene (red box) and one TIE domain connected by 7-mer peptide linker (pale green box). The EgA1 LiTE bears the oncostatin M leader sequence and the EgA1 V_{HH} fused to the CD3-specific OKT3 scFv (V_H-V_L orientation, blue boxes) by 5-mer peptide linker. The myc/6xHis tags (yellow box) are appended for immunodetection and affinity purification, respectively. Arrow indicates the direction of transcription.

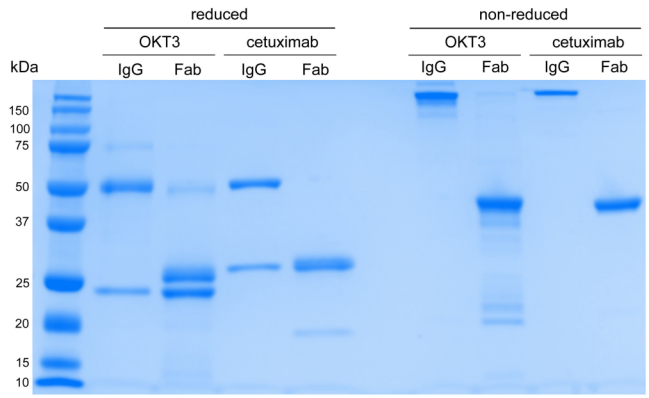

Figure S2. Coomassie-stained SDS-PAGE with OKT3 and cetuximab as full-length IgG and as F_{ab} (prepared by papain proteolysis and protein A clean-up of the IgG), in both reducing and non-reducing conditions.

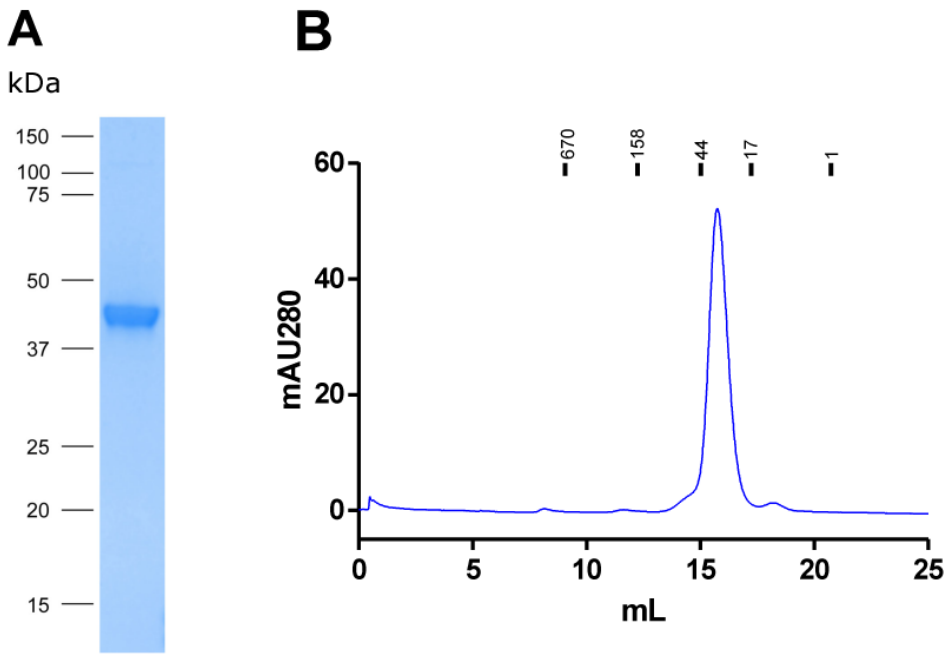

Figure S3. (A) Coomassie-stained SDS-PAGE of the purified EgA1 LiTE in reducing conditions. (B) Size Exclusion Chromatography profile of the EgA1 LiTE sample. The elution peak maxima for 5 standards proteins are marked above the profile with their MWs.

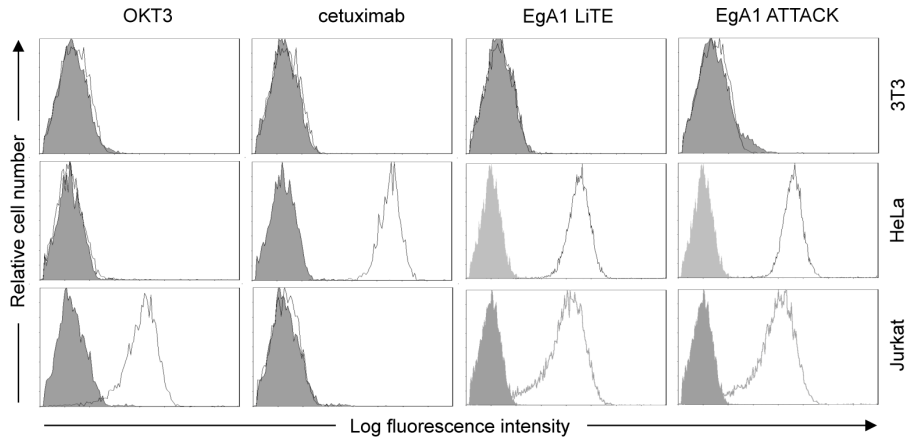

Figure S4. Functional characterization of the purified EgA1 LiTE and EgA1 ATTACK by flow cytometry. The anti-CD3 (OKT3) and anti-EGFR (cetuximab) mAbs were used as controls. The y-axis shows the number of cells and the x-axis represents the intensity of fluorescence, expressed on a logarithmic scale. One representative experiment out of three independent experiments is shown.

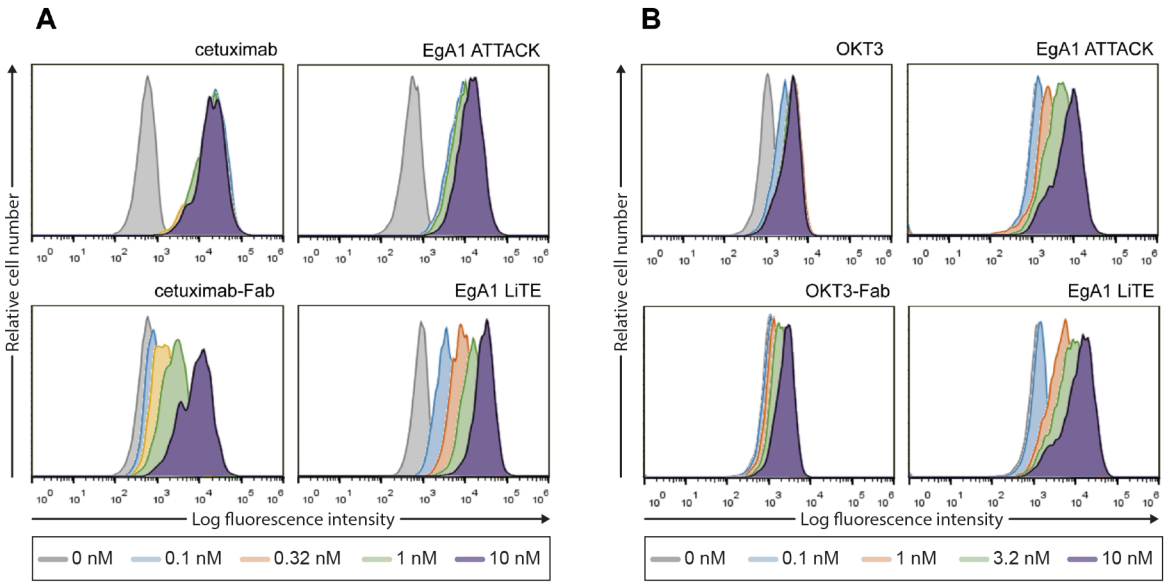

Figure S5. Fluorescence histograms obtained by flow cytometry for HeLa cells incubated with cetuximab, cetuximab-F_{ab}, EgA1 ATTACK, and EgA1 LiTE (A); and Jurkat cells incubated with OKT3, OKT3-F_{ab}, EgA1 ATTACK, and EgA1 LiTE (B). Each antibody was incubated at 4 different concentrations, represented with different colors. Fluorescence intensity (abscissa) is plotted against relative cell number (ordinate). All measurements were performed in triplicate; representative histograms are shown.

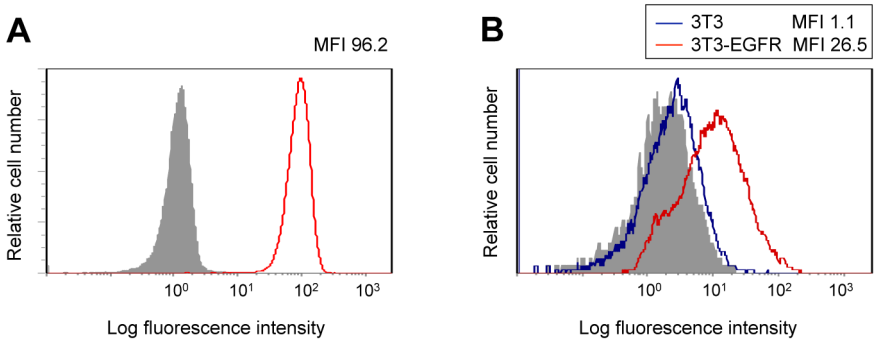

Figure S6. Analysis of EGFR expression by flow cytometry. A431 cells (A) and 3T3 or 3T3-EGFR cells (B) were incubated with anti-EGFR mAb (cetuximab) and PE-conjugated goat anti-human IgG. Fluorescence intensity (abscissa) is plotted against relative cell number (ordinate). The numbers above of each histogram indicate the mean fluorescence intensity (MFI).

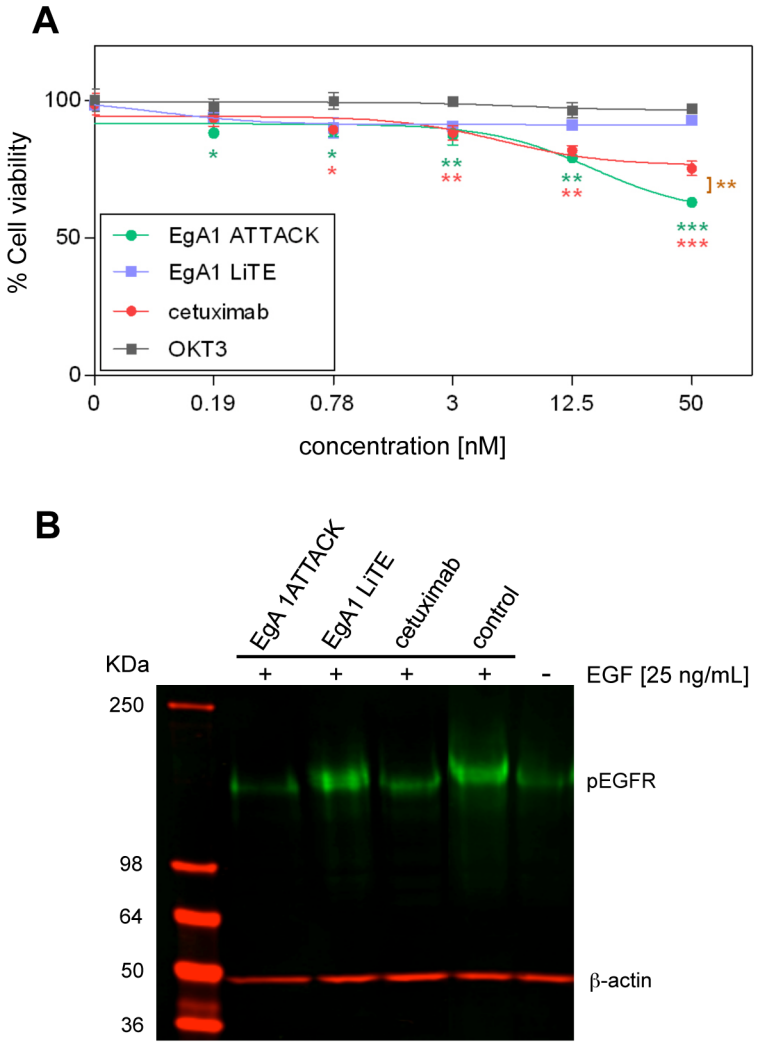

Figure S7. Effect of EgA1 ATTACK on EGFR-mediated signaling. (A) Inhibition of A431 cell proliferation**.** The cells were treated with the indicated doses of EgA1 ATTACK, EgA1 LiTE, cetuximab (positive control) or OKT3 (negative control). Viable cells were measured in triplicates after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls (* *p<0.05*, ** *p<0.01*, *** *p<0.001*; green asterisks, comparison of EgA1 ATTACK with OKT3; in red, comparison of cetuximab with OKT3; in brown, comparison of EgA1 ATTACK with cetuximab). (B) Inhibition of EGFR phosphorylation. Cells were pre-incubated with 50nM of each antibody 4 hours prior to stimulation for 5 minutes with EGF or vehicle. Phosphorylation status of EGFR was assessed by *Western* Blotting.

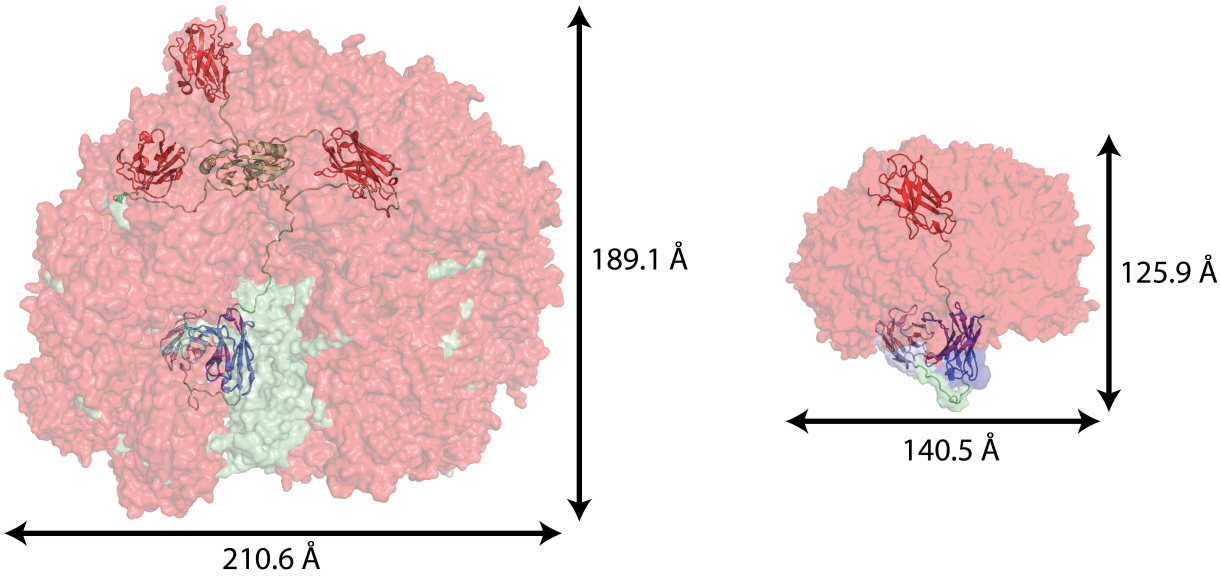

Figure S8. Comparative representation of the explored area by the anti-EFGR V_{HH} domain/s (red) when the anti-CD3ε scFv domain (dark blue/light blue) is fixed. ATTACK (A) and LiTE (B) are represented in scale to each other, with their area's axis labeled for each one. By structurally aligning all decoys of each molecule through the anti-CD3ε scFv domain, an ellipsoid-like shape representing the exploratory surface of the anti-EFGR V_{HH} domain/s was generated.

Supplementary Table 1

Kinetic rate constants and K_D values derived from fitting BLI data to a 1:1 binding model.

	K_D (nM)	**k_a ($M^{-1}s^{-1}$)**	**k_d (s^{-1})**
cetuximab-F_{ab}	0.603	$2.96*10^6$	$1.74*10^{-3}$
EgA1 V_{HH}	0.471	$3.25*10^6$	$1.53*10^{-3}$

The fitting curves are displayed in Figure 3B and C.

Supplementary Table 2

K_D values (i.e. EC50 values) obtained by fitting of flow cytometry binding data to a 1:1 binding model.

Antibody	**K_D (EGFR, HeLa)**	**K_D (CD3, Jurkat)**
cetuximab	3.6 pM	N/A
cetuximab-F_{ab}	5.42 nM	N/A
EgA1 LiTE	0.82 nM	2.96 nM
EgA1 ATTACK	31.8 pM*	8.01 nM
OKT3	N/A	0.10 nM
OKT3-F_{ab}	N/A	23.36 nM

*EgA1 ATTACK was not readily fit to a 1:1 model, and the K_D value given was obtained by omitting its 10 nM data point. N/A, not applicable.