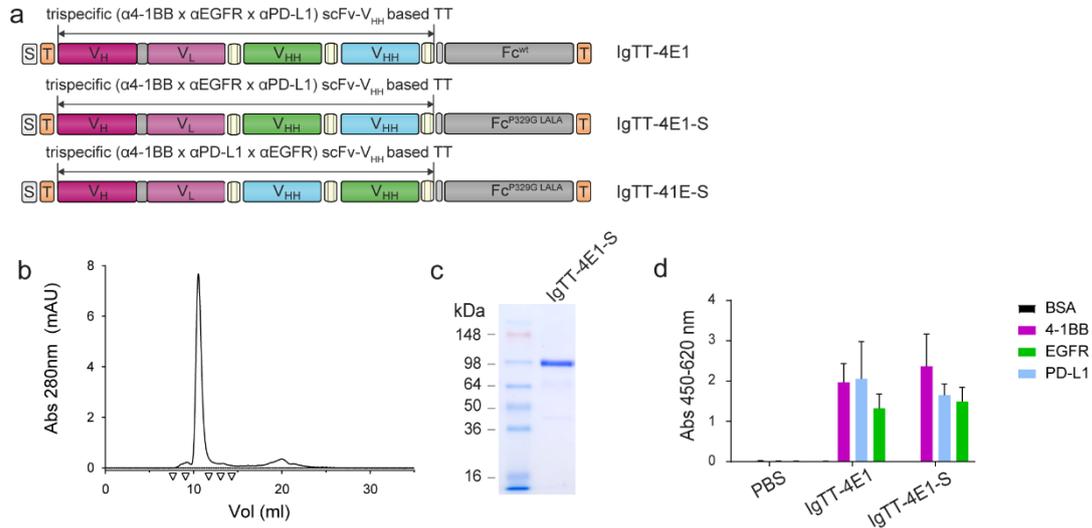
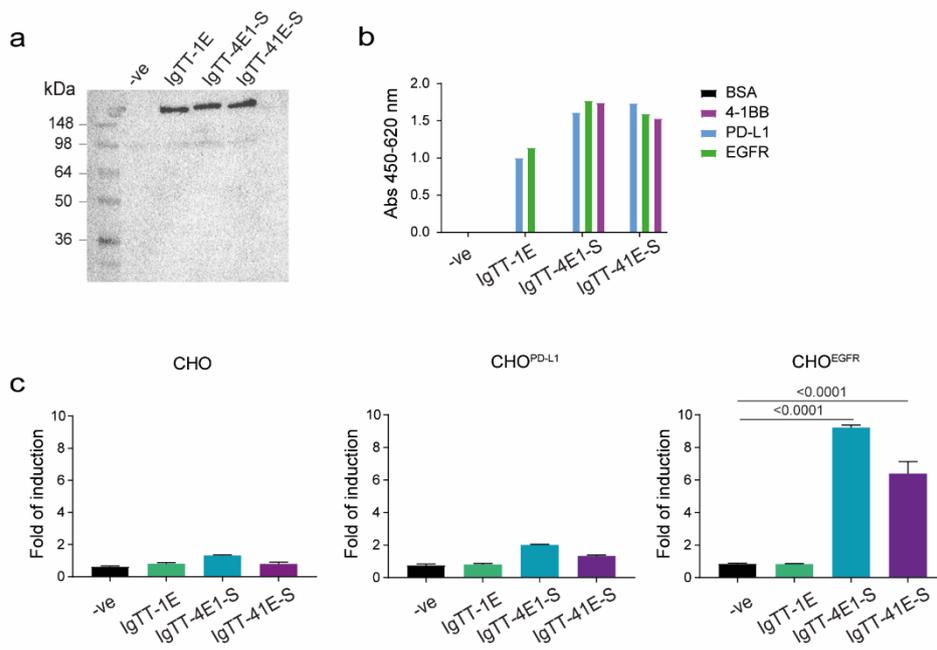


# Characterization of a Trispecific PD-L1 Blocking Antibody That Exhibits EGFR-Conditional 4-1BB Agonist Activity



**Figure S1. Gene constructs and structural characterization of IgTT-4E1-S antibody.** (a) Gene layout of the IgTT-4E1, IgTT-4E1-S and IgTT-41E-S bearing a signal peptide from oncostatin M (white box), one anti-4-1BB scFv (magenta boxes), one anti-EGFR  $V_{HH}$  (green box), one anti-PD-L1  $V_{HH}$  (blue box), three collagen-derived trimerization (TIE) domains (yellow boxes) flanked by peptide linkers and the Fc encoding element (gray boxes). N-terminal FLAG-Strep and C-terminal Myc-His tags (orange boxes) were appended for purification and immunodetection purposes. (b) SEC analysis of IgTT-4E1-S molecules in Superdex 200 Increase 10/300 GL column. Experiments were performed once after calibrating the column using a Gel Filtration HMW Calibration Kit from Cytiva.  $\nabla$  Corresponds to the elution volumes of the well-defined protein standards used for calibrating, from left to right: thyroglobulin (8.73ml), ferritin (9.89 ml), aldolase (12.11 ml), conalbumin (13.59 ml) and ovalbumin (14.60 ml). (c) Reducing SDS-PAGE analysis of the central fraction of the major peak from SEC experiment. (d) ELISA analysis where both purified protein IgTT-4E1 and IgTT-4E1-S specifically recognized human 4-1BB-Fc (4-1BB), human EGFR-Fc (EGFR) and human PD-L1-Fc (PD-L1) followed by anti-FLAG-HRP detection.



**Figure S2. Characterization of IgTT-41E-S antibody.** (a) Conditioned media (0.1% FBS) from negative control (vehicle), IgTT-1E, IgTT-4E1-S and IgTT-41E-S were analyzed by Western blot and probed with mouse anti-FLAG IgG1, followed by incubation with GAM-HRP. Three proteins were efficiently secreted by Expi293 cells and exhibited a migration pattern consistent with the molecular weight calculated from the amino acid sequences. (b) ELISA analysis where three purified proteins specifically recognized ELISA analysis where both purified protein IgTT-4E1 and IgTT-4E1-S specifically recognized human 4-1BB-Fc (4-1BB), human EGFR-Fc (EGFR) and human PD-L1- Fc (PD-L1) followed by anti-FLAG-HRP detection. (c) T cell co-stimulation of Jurkat<sup>NFκB-4-1BB</sup> cells co-cultured with CHO cells, CHO<sup>EGFR</sup> or CHO<sup>PD-L1</sup> cells in the presence of conditioned media of negative control (vehicle), IgTT-1E, IgTT-4E1-S and IgTT-41E-S, and after 6 hours at 37 °C luminescence determined. Data were presented as fold induction relative to the values obtained from unstimulated Jurkat<sup>NFκB-4-1BB</sup> cells. Results are expressed as mean ± SD (*n* = 3). Significance was measured by one-way ANOVA with Dunnett's multiple comparison test.

**Table S1. Oligonucleotides used in this study**

Name	Sequence (5'-3')
FwCMV	CGCAAATGGGCGGTAGGCGTG
RvBGH	TAGAAGGCACAGTCGAGG

Oligonucleotides were synthesized by Thermo Scientific.