Supplementary Material

**Generation of a symmetrical trispecific NK cell engager based on a two-in-one antibody**

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**Supplementary Figure 1.** Cellular binding on MRC-5 and NHEK cells. Cell titration of Cetuximab (black), HCP-LCE (pink), HC16-HCP (orange) and KiH variant (blue) on EGFR positive MRC-5 and NHEK cells. A variable slope three-parameter fit was utilized to for the resulting curves.

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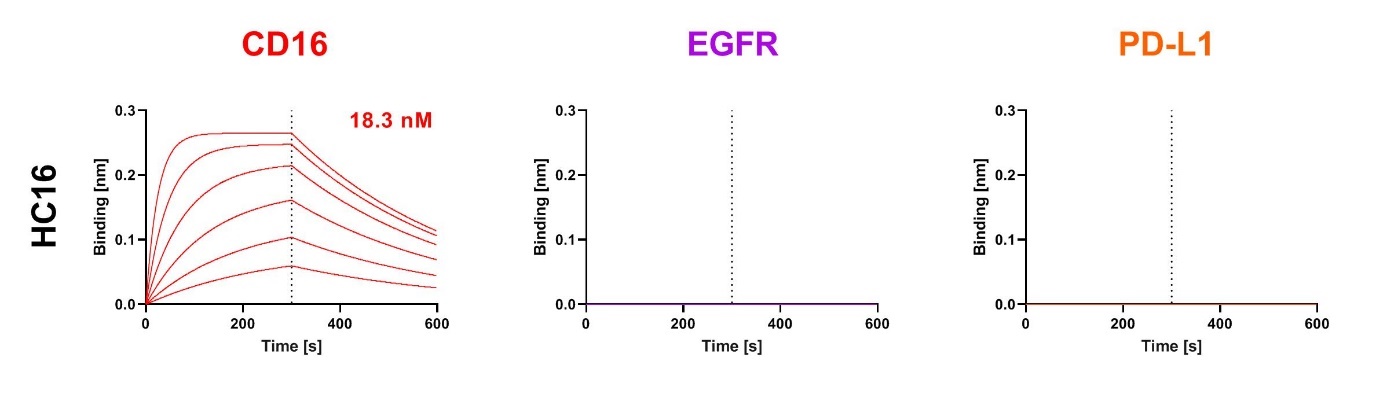
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Supplementary Figure 2. Isolation of HC16. A) Sorting of the diploid common light chain yeast library. Surface presentation is depicted on the y-axis utilizing the anti-human lambda chain antibody PE labelled, while CD16-His-Biotin or CD16-His binding is shown on the x-axis using Streptavidin APC conjugated or the anti-6xHis AF647 antibody. B) Flow cytometric analysis of the isolated single clone after four consecutive rounds of FACS screening. Surface presentation is depicted on the y-axis utilizing the anti-human lambda chain antibody PE labelled, while CD16-His (pink) and PD-L1-His (blue) binding is shown on the x-axis using the anti-6xHis AF647 antibody.

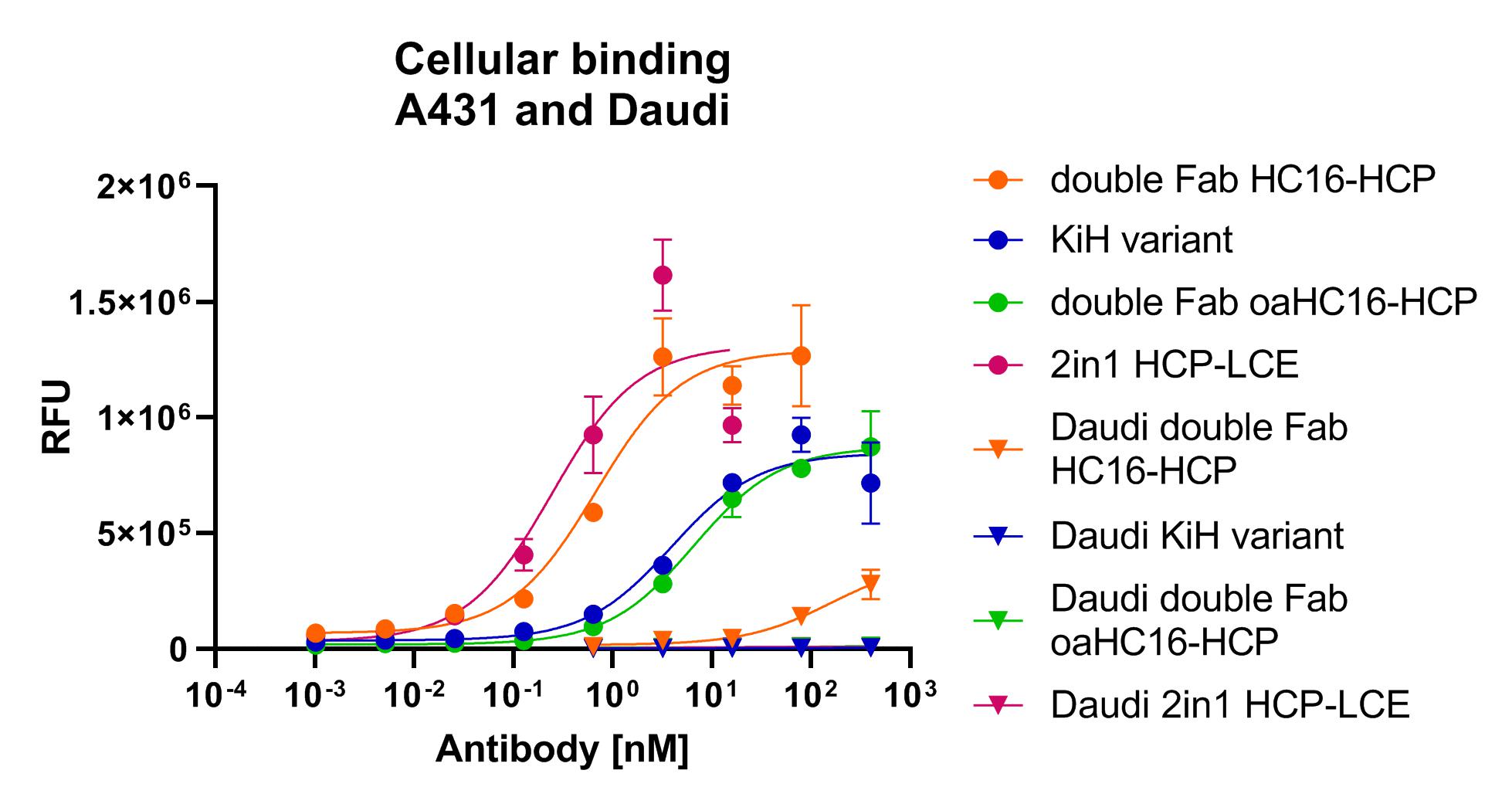
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Supplementary Figure 3. SDS-PAGE analysis and SEC profiles. A) SDS-PAGE analysis of HC16-HCP (orange), oaHC16-HCP (green), KiH variant (blue) and HCP-LCE (pink) under reducing conditions (left) and under non-reducing conditions (right) revealing high purity and the expected molecular weights. B) SEC profiles of HCP-LCE (pink) and HC16-HCP (orange) revealing high purity and the expected molecular weights.



Supplementary Figure 4. Binding kinetics of HC16 by BLI-assisted measurements. BLI-measurement of the anti-CD16 antibody HC16 against CD16a, EGFR and PD-L1. The antibody targets CD16a exclusively.



Supplementary Figure 5. Cellular binding on A431 cells and Daudi cells. Cell titration of HC16-HCP (orange), KiH variant (blue), oaHC16-HCP (green) and HCP-LCE (pink) on EGFR/PD-L1 double positive A431 cells (circles) and on EGFR/PD-L1 double negative Daudi cells (triangles). A variable slope three-parameter fit was utilized to fit the resulting curves. The assay was repeated twice, yielding comparable results.

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Supplementary Figure 6. ADCC cell-based reporter assay. The trispecific constructs HC16-HCP (orange) and KiH variant (blue) were tested in an ADCC reporter assay in the presence of EGFR/PD-L1 double-positive A549 cells (dots) and in the absence of A549 cells (triangles). Luciferase activity is plotted against the logarithmic antibody concentration. All measurements were performed in duplicates.