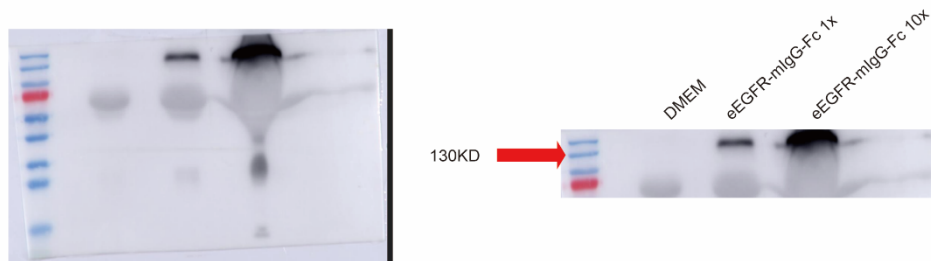


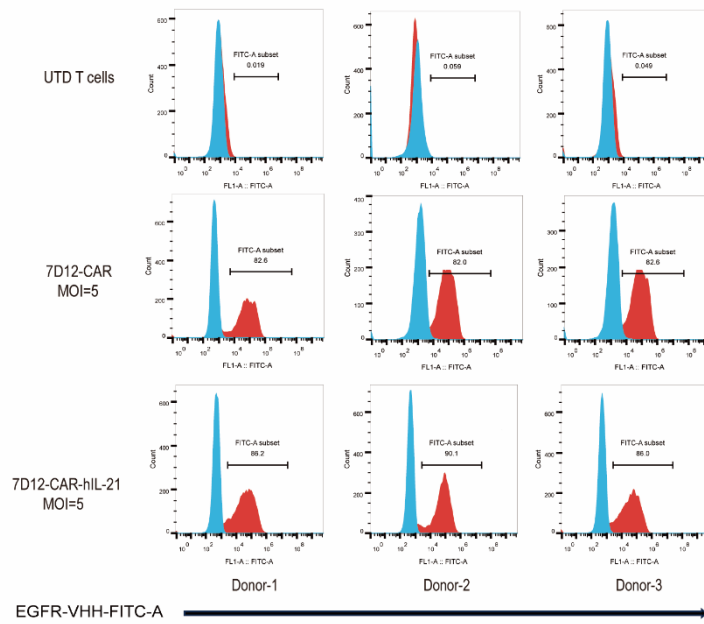
Supplement figure 1



Supplemental Figure 1: Western Blot Analysis of EGFR Met1-Ser645-mIgG-Fc Protein Concentration

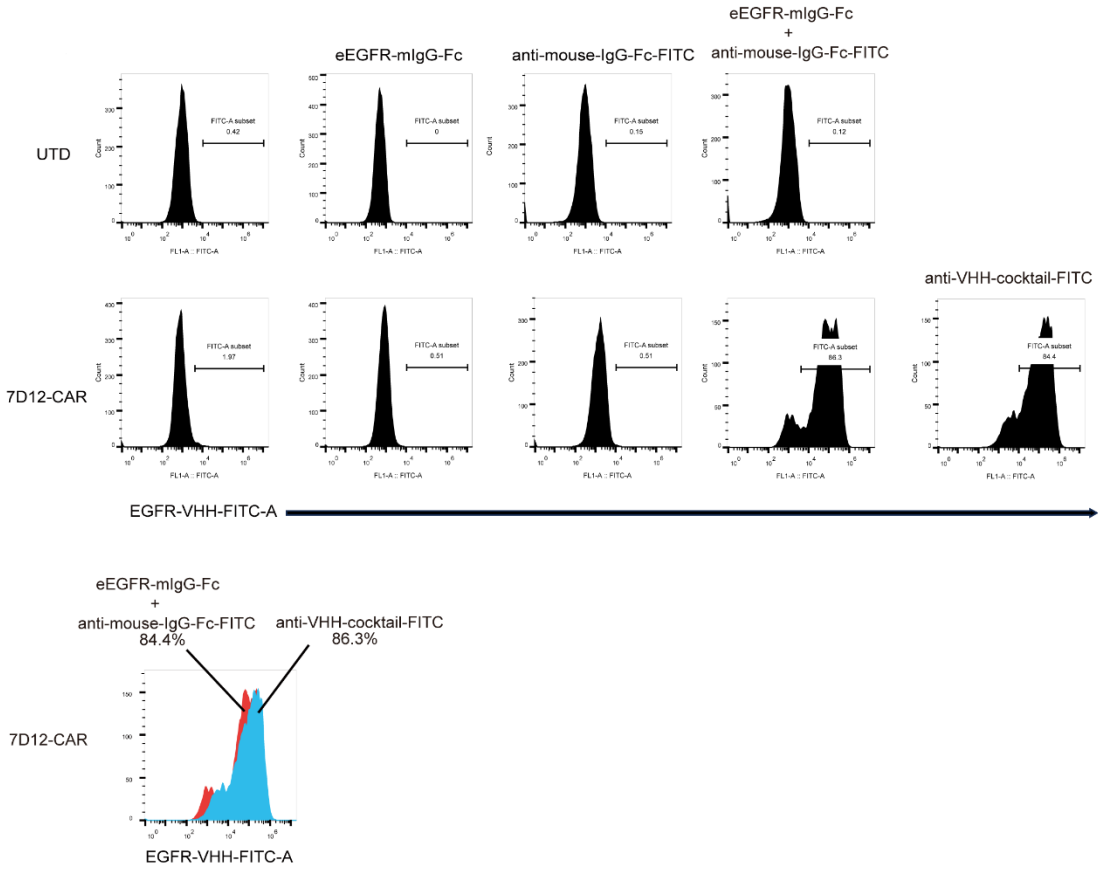
This figure presents a Western blot analysis aimed at quantifying the concentration of the EGFR Met1-Ser645-mIgG-Fc fusion protein in culture medium. The analysis compares samples from DMEM without added protein, with the standard concentration of EGFR Met1-Ser645-mIgG-Fc protein, and with a 10-fold concentrated EGFR Met1-Ser645-mIgG-Fc protein.

Supplement figure 2



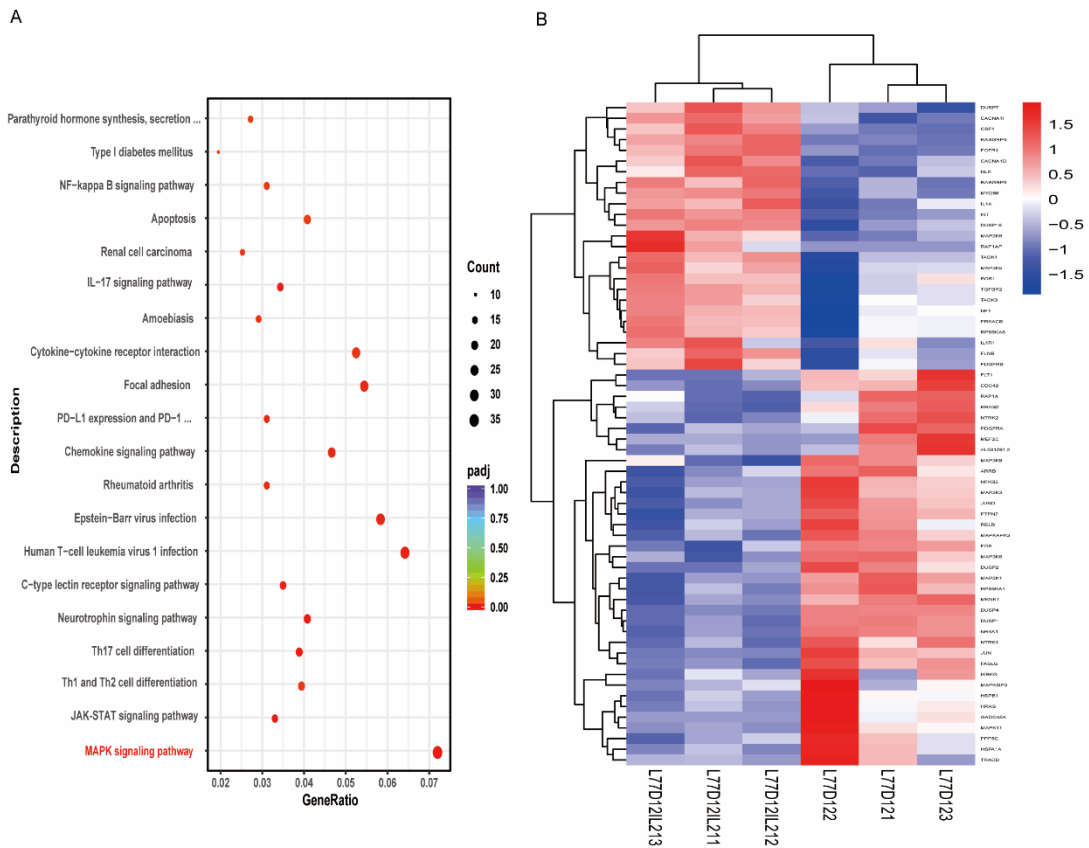
Supplemental Figure 2: The expression of EGFR-VHH-7D12 in UTD, EGFR-VHH-7D12-CAR and EGFR-VHH-7D12-CAR-hiL-21 T cells Flow cytometry analysis the expression of EGFR-VHH-7D12 in UTD, EGFR-VHH-7D12-CAR and EGFR-VHH-7D12-CAR-hiL-21 T cells at a multiplicity of infection (MOI) of 5. The top is UTD T cells, the middle is 7D12-VHH-CAR-T cells, and the bottom is 7D12-VHH-CAR-hiL-21-T cells.

Supplemental figure 3



Supplemental Figure 3: UTD T cells and EGFR-VHH-7D12-CAR-T cells that stained with eEGFR-mIgG-Fc, anti-mouse-IgG-Fc-FITC or dual-stained with eEGFR-mIgG-Fc and anti-mouse-IgG-Fc-FITC. EGFR-VHH-7D12-CAR-T cells stained with anti-VHH-cocktail-FITC.

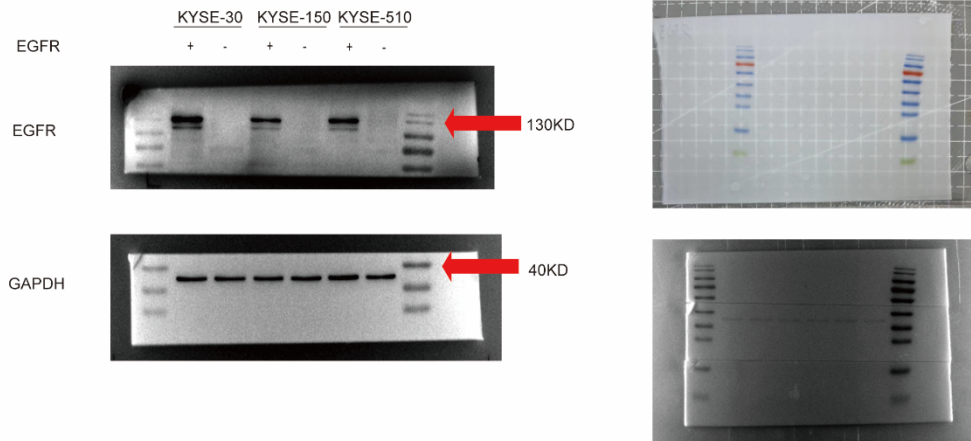
Supplemental figure 4



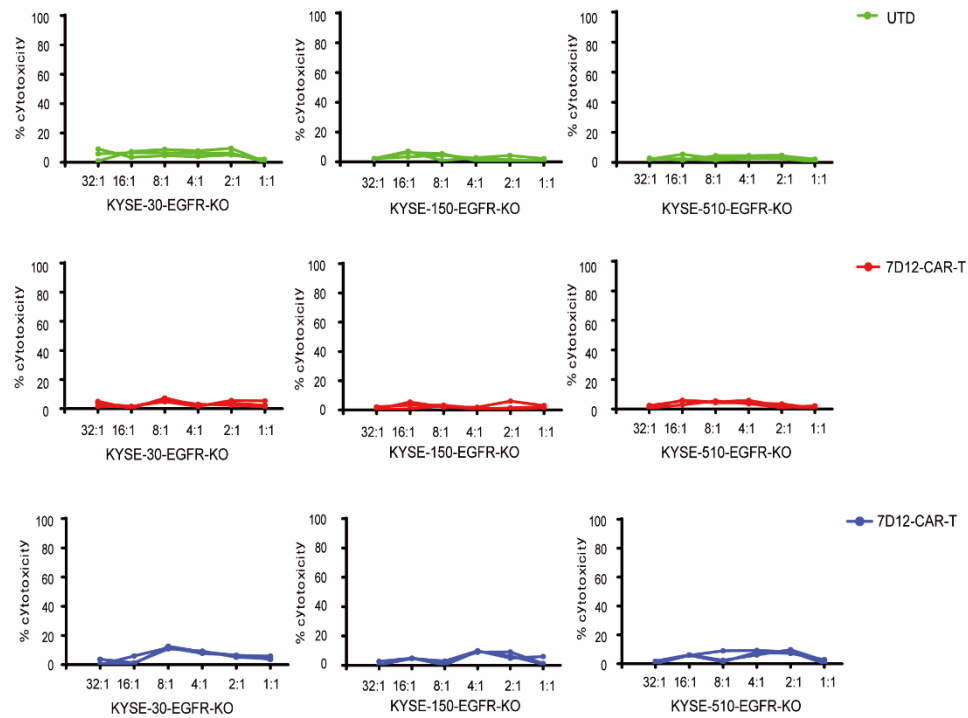
Supplemental Figure 4: RNA sequencing analysis of EGFR-VHH-7D12-CAR and EGFR-VHH-7D12-P2A-hiL-21-CAR T cells(A) KEGG enrichment analysis of theEGFR-VHH-7D12-CAR and EGFR-VHH-7D12-P2A-hiL-21-CAR T cells. (B) Heat map of the PMAK signaling pathway in EGFR-VHH-7D12-CAR and EGFR-VHH-7D12-P2A-hiL-21-CAR T cells. n=3 per group.

Supplemental figure 5

A



B



Supplemental Figure 5: Assessment of EGFR Knockout and Cytotoxicity of CAR-T Cells Against EGFR-KO ESCC Cell Lines(A) Western blot analysis for the confirmation of EGFR knockout in KYSE-30, KYSE-150, and KYSE-510 cell lines. (B) LDH release assay to evaluate the cytotoxicity of UTD T cells, EGFR-VHH-7D12-CAR T cells, and EGFR-VHH-7D12-P2A-hIL-21-CAR T cells when cocultured with EGFR-knockout (EGFR-KO) versions of the KYSE-30, KYSE-150, and KYSE-510 cell lines. The cocultures were maintained at various effector-to-target (E/T) ratios (32:1, 16:1, 8:1, 4:1, 2:1, and 1:1) at 37°C for 16 hours. This assay measures the release of LDH from lysed cells, serving as an indicator of CAR-T cell-mediated cytotoxicity against target cells lacking EGFR expression.