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Supplemental Information

Preclinical efficacy of a HER2 synNotch/CEA-CAR combinatorial immunotherapy against colorectal cancer with HER2 amplification

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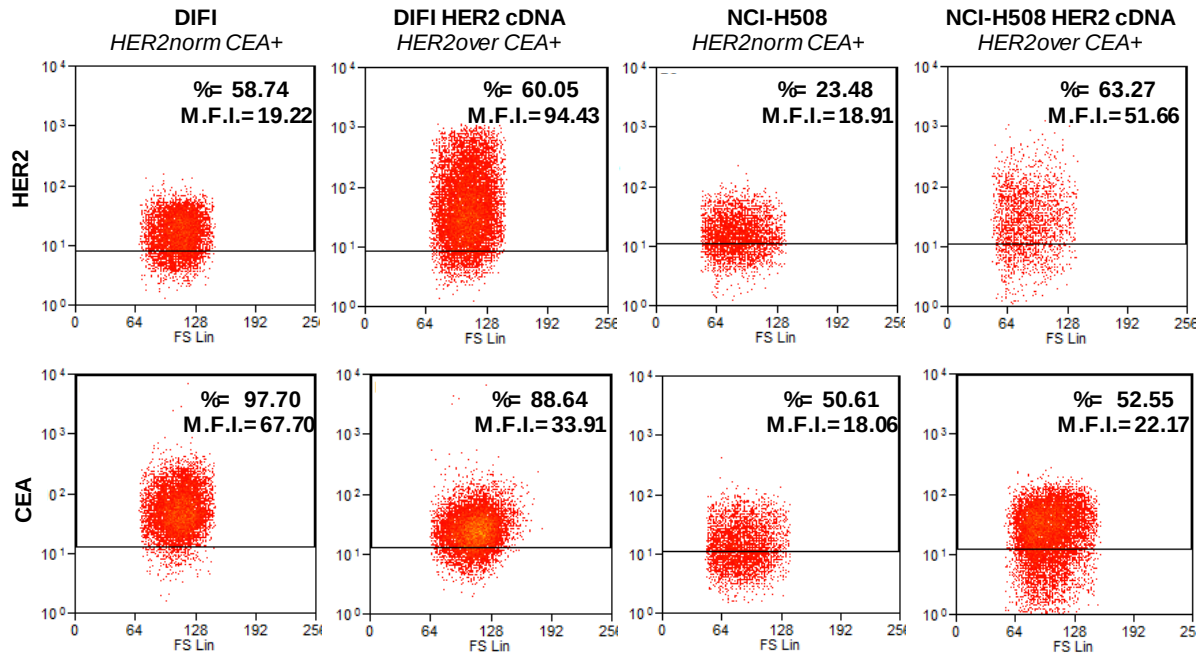

Figure S1. HER2 and CEA surface protein expression in engineered cell lines. Flow cytometry dot-plots reporting surface expression of HER2 (top panels) and CEA (bottom panels) in two CRC cell lines engineered or not with LV-expressing HER2 under the control of the CMV promoter; y-axis= fluorescence intensity, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescence Intensity (of all displayed cells).

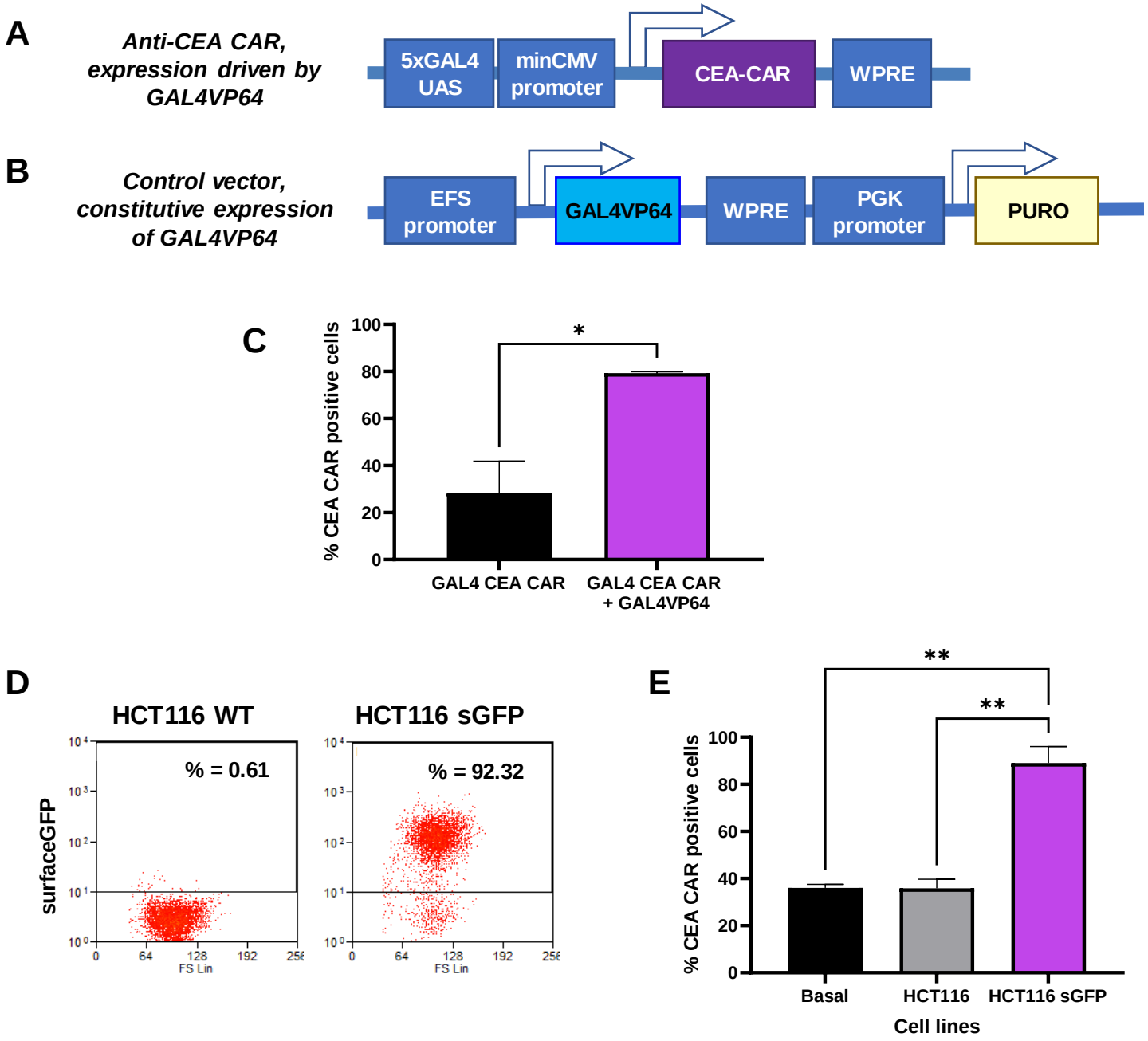

Figure S2. Technical validation of the GAL4-CEA-CAR vector in Jurkat cells. (**A-C**) Jurkat cells were transduced with GAL4 CEA-CAR with or without the additional GAL4VP64 vector, and checked for GAL4-induced CAR expression. The fraction of CEA-CAR-positive raised from 30% in the absence of GAL4VP64 to almost 80% in cells co-transduced with the constitutive GAL4VP64 vector, denoting marked induction. **A.** Schematic representation of the GAL4-CEA-CAR lentiviral vector (5xGAL4 UAS = 5x GAL4 Upstream Activation Sequence, minCMV= minimal cytomegalovirus promoter, WPRE= Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element) **B**. Schematic representation of the GAL4VP64 lentiviral vector (EFS= elongation factor 1α short, GAL4VP64 = transcription factor, PGK= Human phosphoglycerate kinase, PURO = puromycin resistance coding sequence). **C.** Bar graph showing the percentage of CEA-CAR-positive cells in the absence or presence of constitutively expressed GAL4VP64 (n. of independent experiments: 2).

(**D-E**) Validation of CEA-CAR induction by a synNotch against surface GFP (sGFP). Jurkat cells were transduced with the LAG16 anti-sGFP SynNotch, sorted for synNotch expression and then transduced with the GAL4 CEA-CAR. Transduced cells were then co-cultured with HCT116 CRC cells, either WT or transduced with sGFP, and tested for specific CEA-CAR indiction. Again the fraction of CEA-CAR-positive raised from 30% in the presence of WT HCT116 to almost 90% in the presence of sGFP-expressing HCT116 cells, denoting marked induction. **D.** Flow cytometry dot-plots showing the surface expression of sGFP in HCT116; y-axis= sGFP fluorescence intensity, x-axis= Forward Scatter (n. of independent experiments: 3). **E.** Bar graph showing the percentage of CEA-CAR positive cells induced by the LAG16 synNotch after co-culture with HCT116 transduced or not with sGFP (basal= without target cells; n. of independent experiments: 2).

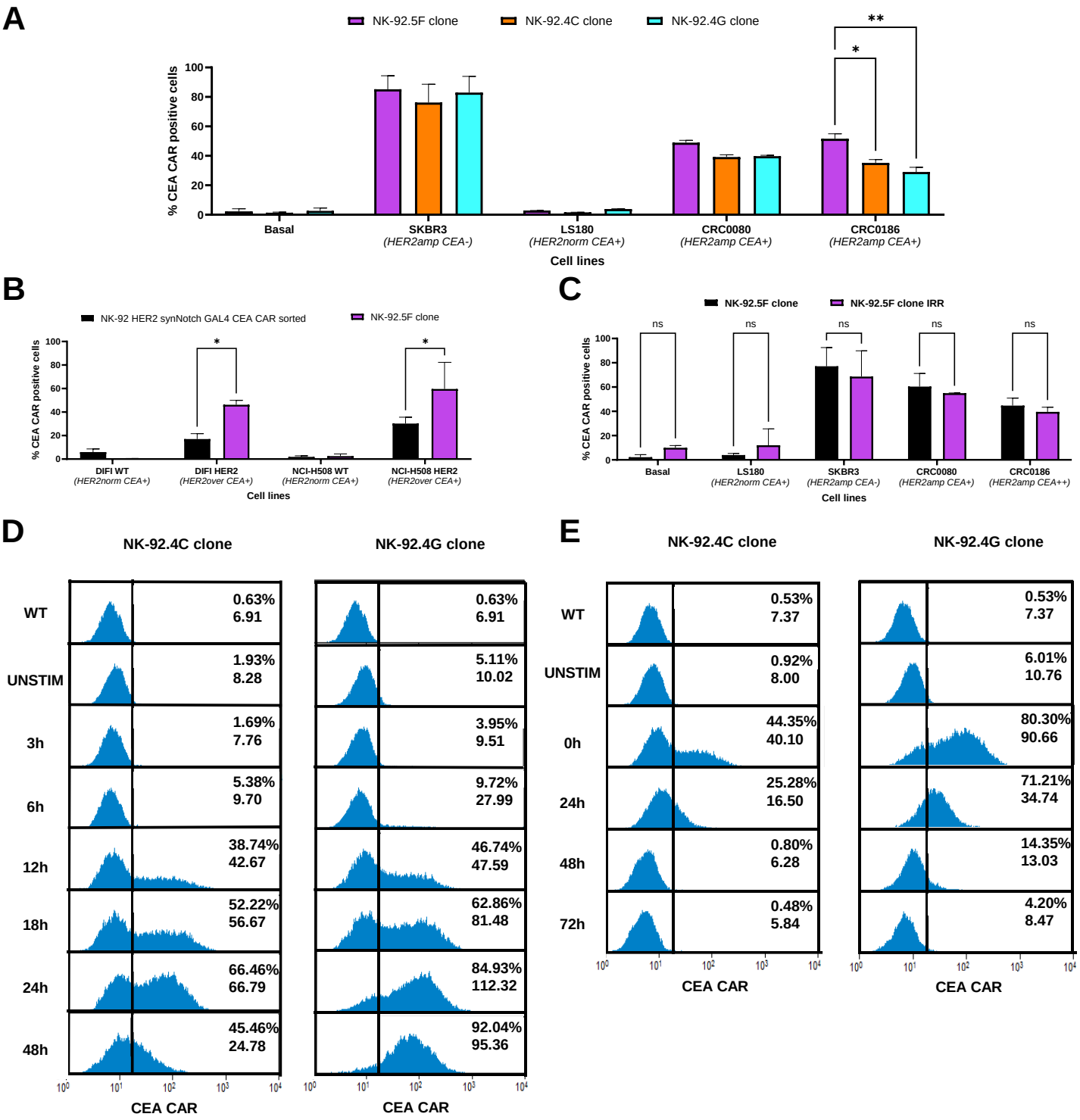

Figure S3. CEA CAR expression. A. Bar graph showing the fraction of CEA CAR-positive NK-92 cells after co-culture with HER2norm or HER2amp cells (Bars: Standard Deviations. Statistics: Two-way Anova. Stars = p-values: * ≤ 0.05, ** ≤ 0.01; n. of independent experiments: 2). **B.** Bar graph showing the fraction of CEA CAR-positive NK-92 cells after co-culture with HER2norm or HER2 artificially overexpressing cells (Bars: Standard Deviations. Statistics: Two-way Anova. Star = p-value ≤ 0.05; n. of independent experiments: 2). **C.** Bar graph showing the fraction of CEA CAR positive NK-92.5F cells irradiated or not, after co-culture with HER2norm or HER2amp cells. Basal= without target cells, Bars: Standard Deviations. Statistics: Two-way Anova. Stars indicate p-value ≤ 0.01 (n. of independent experiments: 3). **D.** Flow-cytometry histograms of CEA-CAR induction after co-culture for different times with HER2-amplified cells, of either the 4C clone (left panels) or the 4G clone (right panels; n. of independent experiments: 2). **E.** Flow-cytometry histograms of CEA-CAR induction and time-course suppression after removal of HER2amp target cells, of either the 4C clone (left panels) or the 4G clone clone (right panels). "%" = percent of cells above the depicted positivity threshold; M.F.I. = Mean Fluorescence Intensity of all cells (n. of independent experiments: 2).

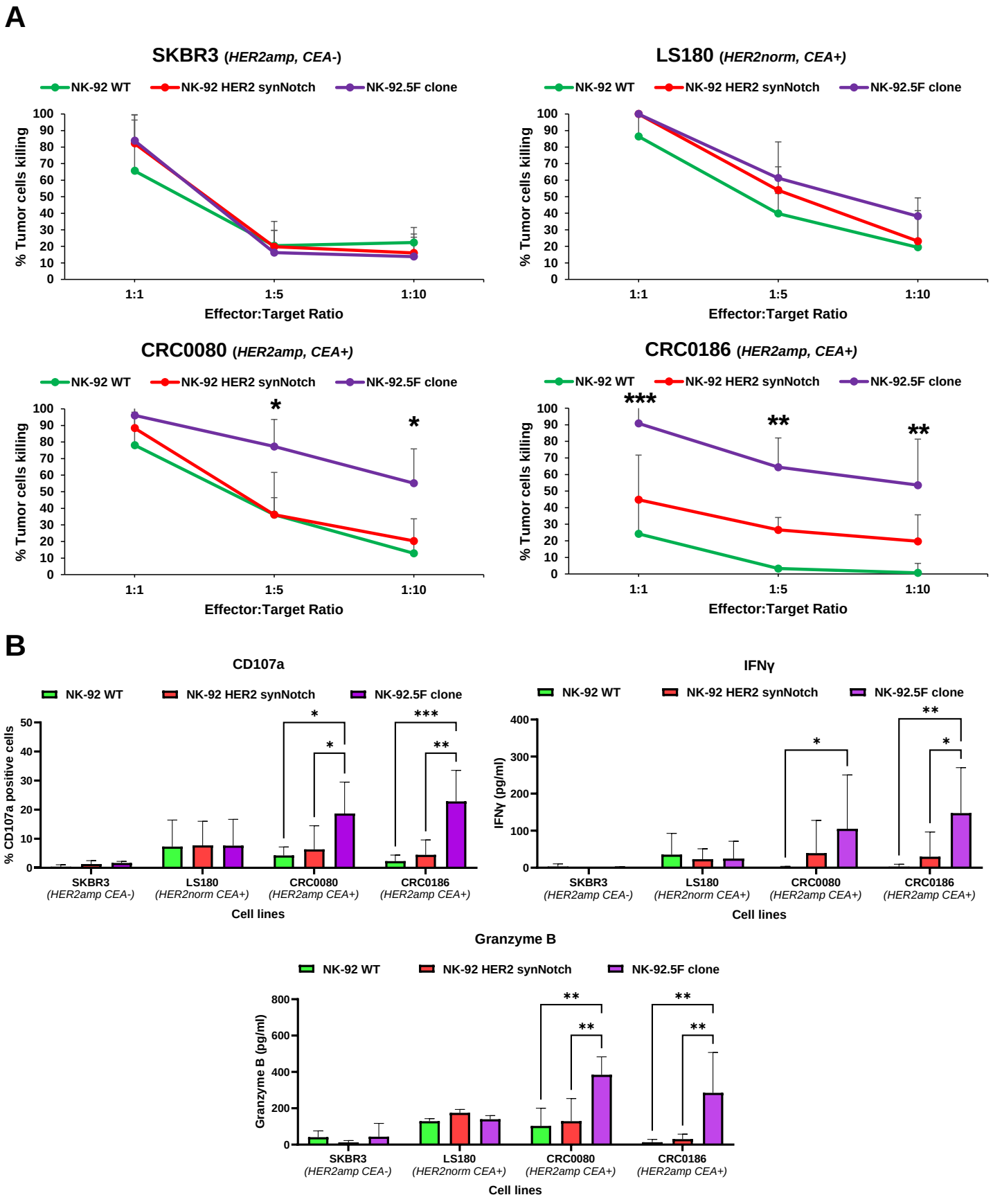

Figure S4. Cytotoxic activity of non-irradiated NK-92 effectors against human cancer cells. A. Specific killing activity of irradiated NK-92.5F or control cells against SKBR3, LS180, CRC0080 and CRC0186 cells after 48h of co-culture at different effector:target ratio. Bars: standard deviation. Statistics: Two-way Anova. Stars indicate p-values: * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; n. of independent experiments: 3). **B.** Degranulation and cytokine release upon co-culture with SKBR3, LS180, CRC0080 and CRC0186 with non-irradiated effectors. Bars: Standard Deviations. Statistics: Two-way Anova. Stars indicate p values: * ≤ 0.05; ** ≤ 0.01 (n. of independent experiments for CD107a: 4, for IFNg: 5, for Granzyme B: 3).

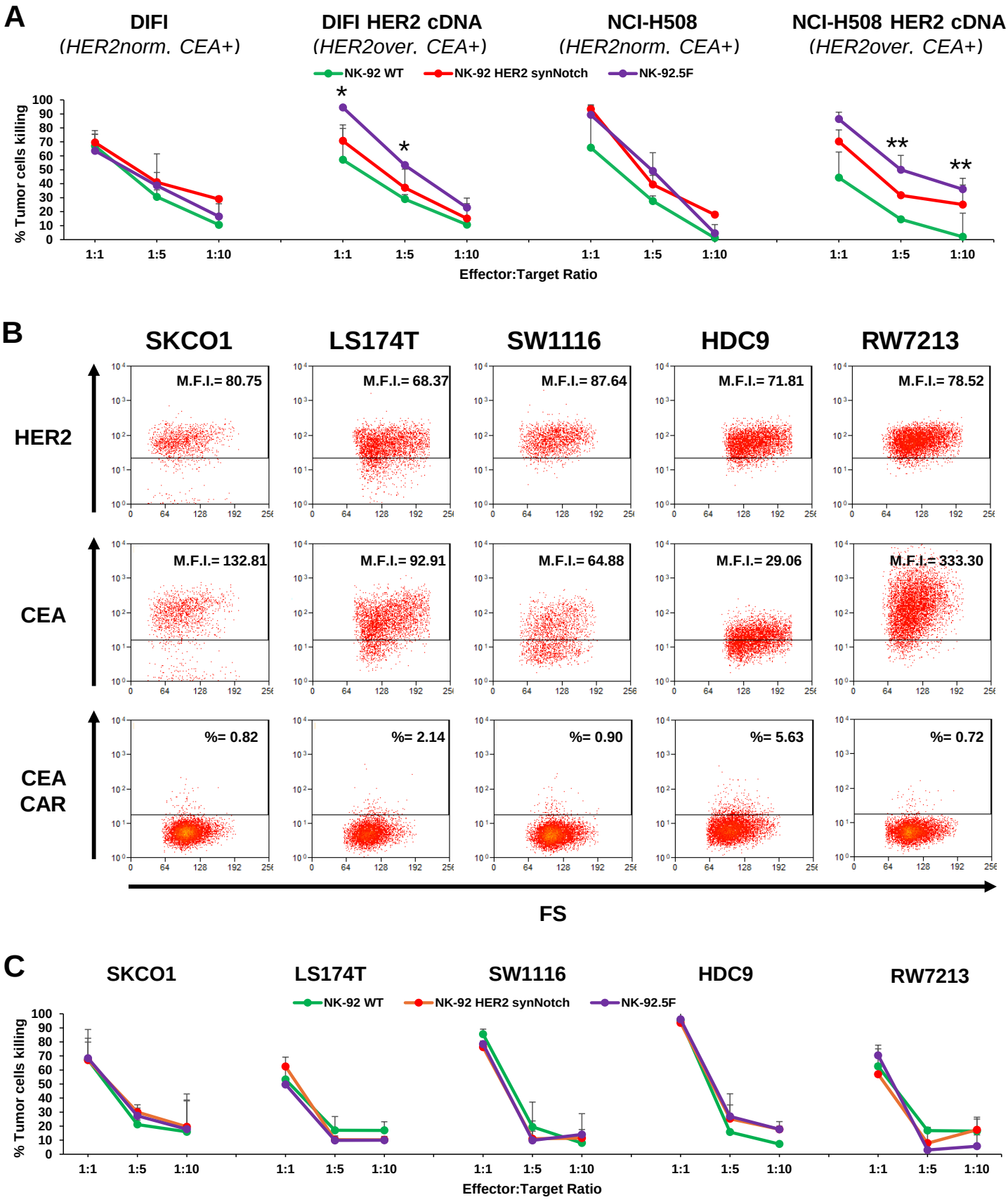

Figure S5. Cytotoxic activity of NK-92 effectors against additional human CRC cells. A. Specific killing activity of irradiated NK-92.5F or control cells against DIFI and H508, either normal or overexpressing HER2 (n. of independent experiments: 2). **B.** Flow cytometry dot-plots reporting surface expression of HER2 (top panels) and CEA (middle panels) in five additional CRC cell lines, plus the respective induction of CEA CAR in NK-92.5F cells (bottom panels); y-axis= fluorescence intensity, x-axis= Forward Scatter. Numbers in the squares indicate the fraction of CEA-CAR-positive cells. **C.** Specific killing activity of irradiated NK-92.5F or control cells against five additional CRC cell lines (n. of independent experiments: 2). Bars: Standard Deviations. Statistics: Two-way Anova. Stars indicate p-values: * ≤ 0.05; ** ≤ 0.01.

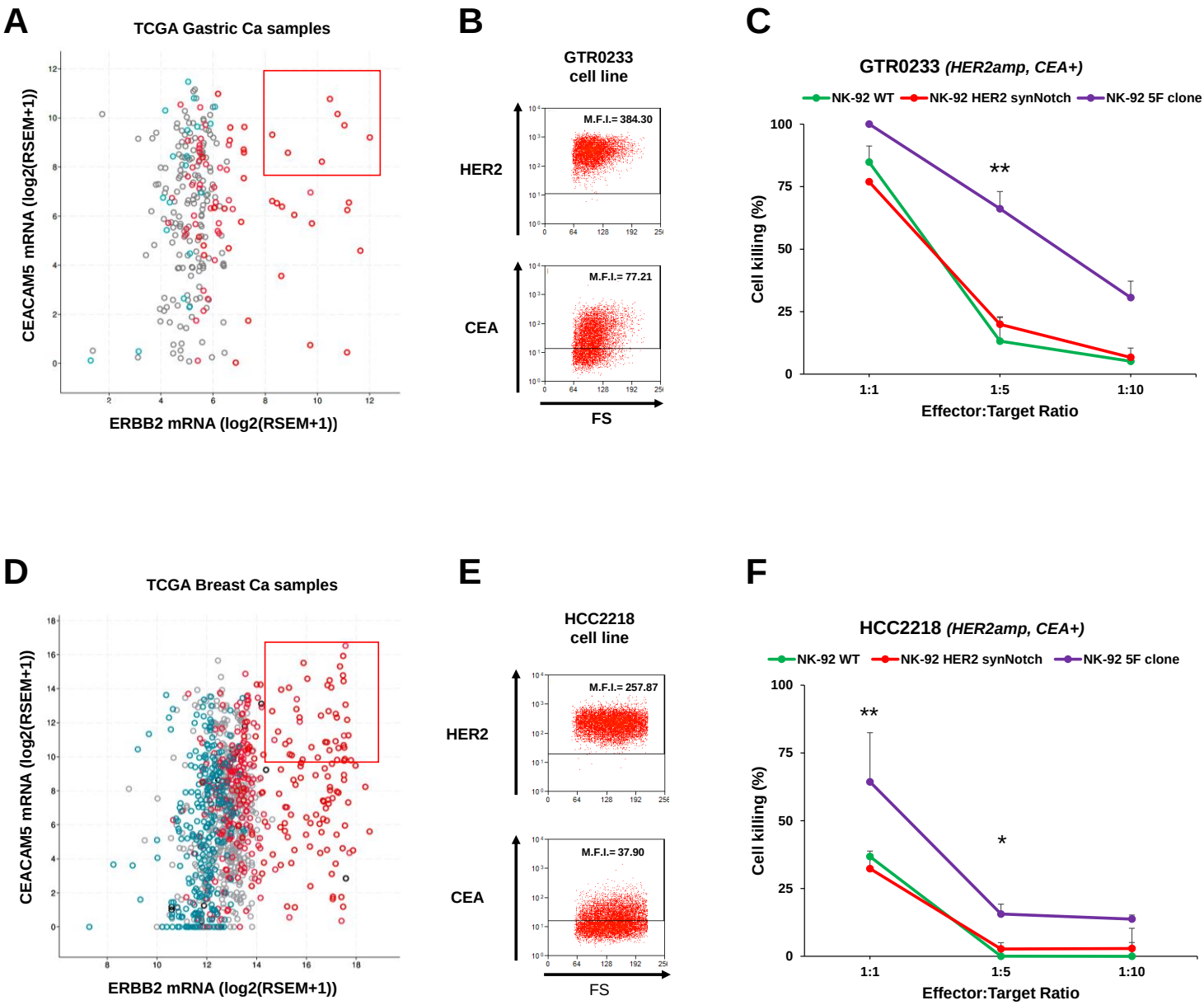

Figure S6. Efficacy of the NK-92.5F clone against gastric and breast cancer cells (A-C gastric cancer, D-E breast cancer). **A,D.** Dot plots displaying mRNA expression of ERBB2 (x-axis) vs CEACAM5 (y-axis) in gastric and breast cancer samples from TCGA. **B,E.** Flow cytometry dot-plots reporting surface expression of HER2 and CEA, as indicated; y-axis= fluorescence intensity, x-axis= Forward Scatter. **C,F.** *In vitro* killing activity of NK-92.5F against GTR0233 gastric cancer or HCC2218 breast cancer cells, as indicated, after 48h of co-culture at different effector:target ratio. Bars: standard deviation; n. of independent experiments: 2; statistics: Two-way Anova. Stars indicate p values: * ≤ 0.05; ** ≤ 0.01, *** ≤ 0.001.

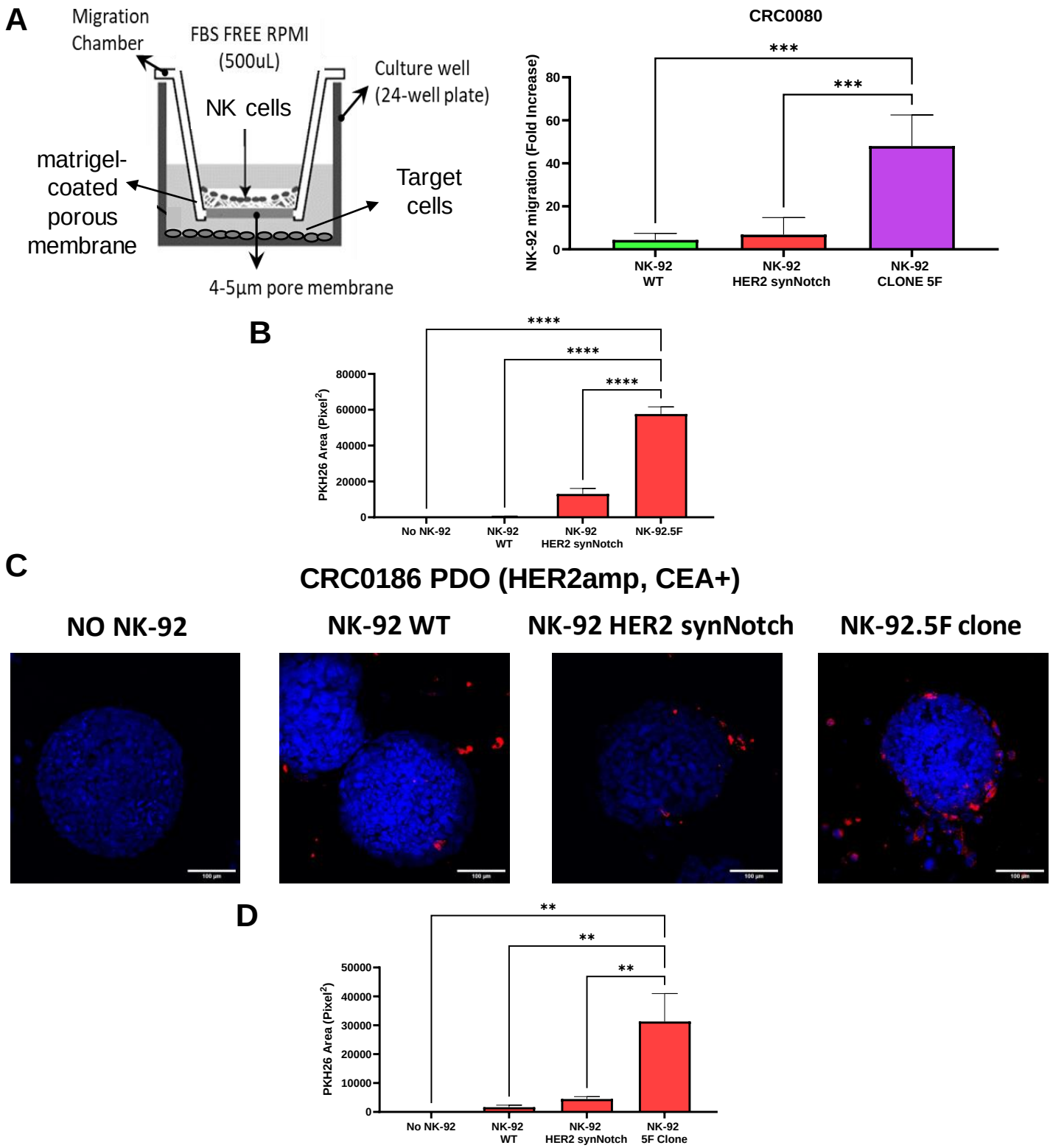

Figure S7. Homing and penetration assays. A. On the left, schematic representation of the transwell assays. On the right, bar graph representing the fold increase of effector cells, seeded in the upper migration chamber, migrated through a matrigel basal membrane in the presence of CRC0080 cells in the bottom chamber (n. of independent experiments: 4). **B.** Bar graph reporting the quantification of NK-92 effector cells infiltration into CRC0080 organoids by analyzing the red fluorescence area (square pixels). Data are from the experiment shown in Fig. 7C (n. of independent experiments: 2). **C.** Representative confocal microscopy images of CRC0186 organoids grown for 3 days in the presence of different NK-92 effectors, as indicated. Cancer cells were stained with NucBlue (blue), and NK-92 cells were stained with PHK26 (red). Magnification, 20x; scale bars, 100µm. **D.** Bar graphs reporting quantification of NK-92 effector cells infiltration into CRC0186 organoids by analyzing the red fluorescence area (square pixels). Data are from the experiment shown in C. Bars: Standard deviation. Statistics: Two-way Anova. Stars indicate p values: * ≤ 0.05; **** ≤ 0.0001 (n. of independent experiments: 2).

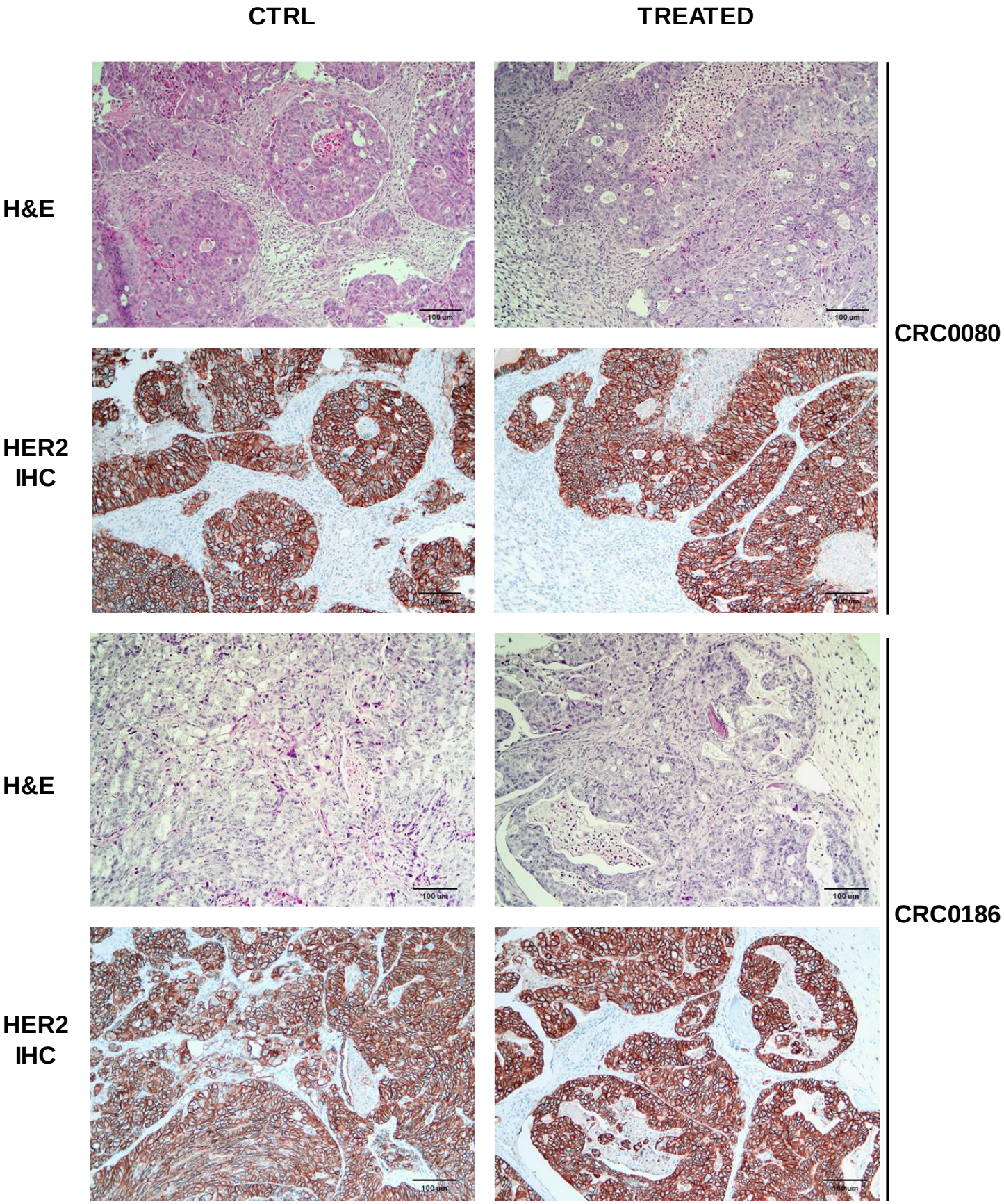

Figure S8. HER2 expression in post-treatment xenografts. Representative immunohistochemistry images of HER2 expression in CRC0080 and CRC0186 xenografts either untreated or after treatment with the 5F clone, as indicated; xenografts were explanted concomitantly with mouse sacrifice when reaching the ethical endpoint (IHC: immunohistochemistry; H&E: Hematossilin and Eosin). Magnification, 10x; scale bars: 100 µm.

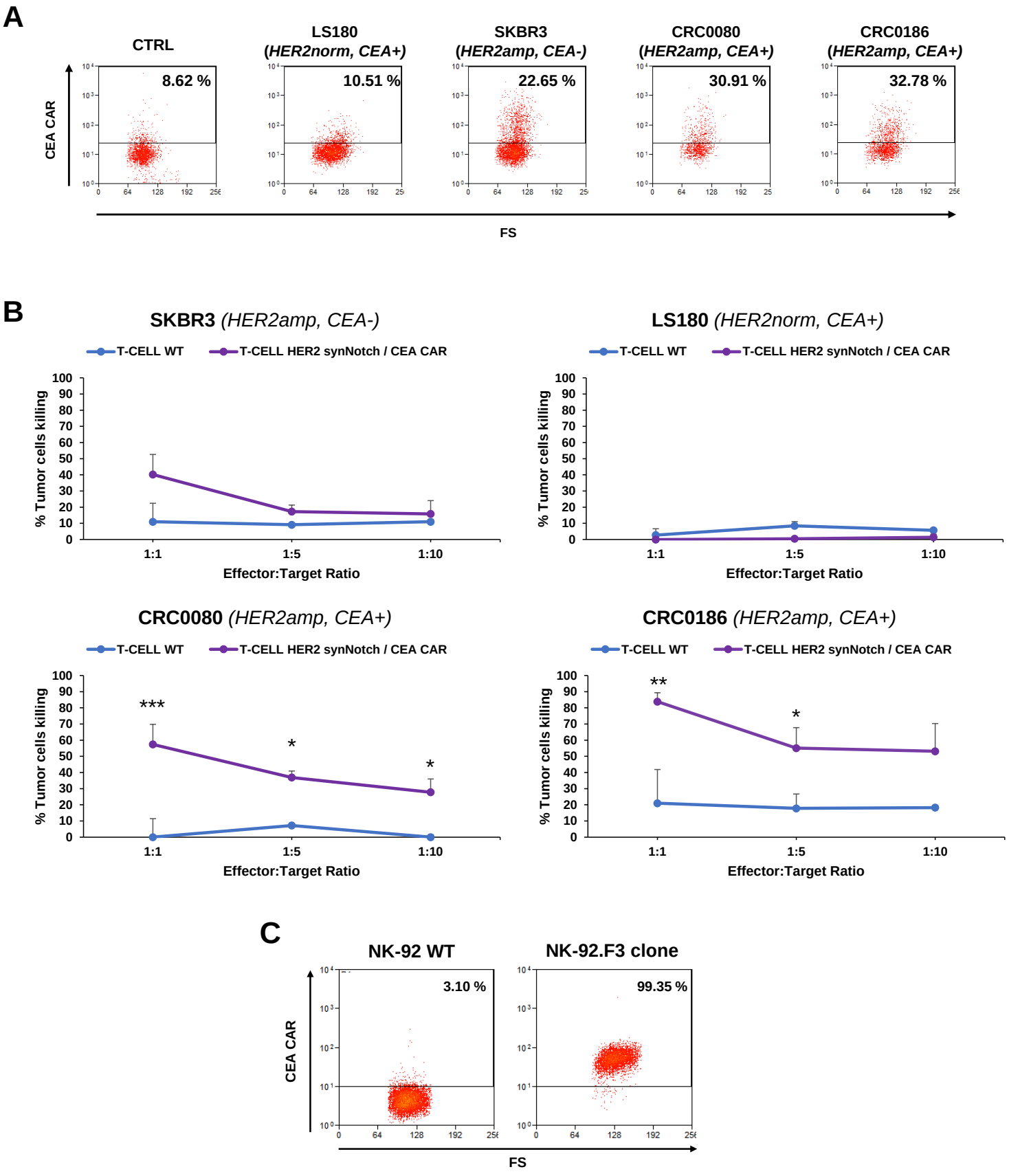

Figure S9. Additional efficacy studies. (**A-B**) Inducibility and efficacy of the HER2 synNotch/CEA-CAR system in T cells. **A.** Flow-cytometry plots displaying CEA-CAR induction by engaged synNotch in primary T cells after co-culture with target cells (numbers in the square indicate the fraction of CEA-CAR positive cells). **B.** Specific *in vitro* killing activity of HER2 synNotch/CEA-CAR T cells against SKBR3, LS180, CRC0080 and CRC0186 cells after 48h of co-culture at different effector:target ratio. Bars: standard deviation. (n. of independent experiments: 2). Statistics: Two-way Anova. Stars indicate p values: * ≤ 0.05; ** ≤ 0.01, *** ≤ 0.001. **C.** Flow-cytometry plots displaying constitutive CEA-CAR expression in the NK-92.F3 clone (numbers in the square indicate the fraction of CEA-CAR positive cells).