In vitro elimination of epidermal growth factor receptor-overexpressing cancer cells by CD32A-chimeric receptor T cells in combination with cetuximab or panitumumab

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Cetuximab and panitumumab bind the human epidermal growth factor receptor (EGFR). Although the chimeric cetuximab (IgG1) triggers antibody-dependent-cellular-cytotoxicity (ADCC) of EGFR positive target cells, panitumumab (a human IgG2) does not. The inability of panitumumab to trigger ADCC reflects the poor binding affinity of human IgG2 Fc for the FcγRII (CD16) on natural killer (NK) cells. However, both human IgG1 and IgG2 bind the FcγRII (CD32A) to a similar extent. Our study compares the ability of T cells, engineered with a novel low-affinity CD32A131R-chimeric receptor (CR), and those engineered with the low-affinity CD16158F.CR T cells, in eliminating EGFR positive epithelial cancer cells (ECCs) in combination with cetuximab or panitumumab. After T-cell transduction, the percentage of CD32A131R-CR T cells was 74 ± 10%, whereas the percentage of CD16158F.CR T cells was 46 ± 15%. Only CD32A131R-CR T cells bound panitumumab. CD32A131R-CR T cells combined with the mAb 8.26 (anti-CD32) and CD16158F.CR T cells combined with the mAb 3g8 (anti-CD16) eliminated colorectal carcinoma (CRC), HCT116(FcγR+) cells, in a reverse ADCC assay in vitro. Crosslinking of CD32A131R-CR on T cells by cetuximab or panitumumab and CD16158F.CR T cells by cetuximab induced elimination of triple negative breast cancer (TNBC) MDA-MB-468 cells, and the secretion of interferon gamma and tumor necrosis factor alpha. Neither cetuximab nor panitumumab induced Fcγ-CR T antitumor activity against Kirsten rat sarcoma (KRAS)-mutated HCT116, nonsmall-cell-lung-cancer, A549 and TNBC, MDA-MB-231 cells. The ADCC of Fcγ-CR T cells was associated with the overexpression of EGFR on ECCs. In conclusion, CD32A131R-CR T cells are efficiently redirected by cetuximab or panitumumab against breast cancer cells overexpressing EGFR.

Key words: CAR T cells, panitumumab, cetuximab, EGFR, breast cancer

Abbreviations: ADCC: antibody-dependent-cellular-cytotoxicity; APC: allogeneic osteosarcoma; BC: breast cancer; CM: complete medium; CR: chimeric receptor; CRC: colorectal carcinoma; DMEM: Dulbecco’s Modified Eagle’s Medium; DMSO: dimethyl sulfoxide; ECCs: EGFR positive epithelial cancer cells; EGFR: epidermal growth factor receptor; FBS: fetal bovine serum; FcR BR: Fc receptor blocking reagent; FITC: fluorescein isothiocyanate; IFN: interferon gamma; IL-15: interleukin-15; IL-7: interleukin-7; IMDM: Iscove’s Modified Dulbecco’s Medium; mAb: monoclonal antibody; MFI: mean fluorescence intensity; NSCLC: nonsmall cell lung cancer; OD: optical density; PBMCs: peripheral blood mononuclear cells; PE: phycoerythrin; PerCP-Cy5.5: peridinin-chlorophyll-protein complex cyanine5.5; PI: propidium iodide; RT-PCR: reverse-transcriptase polymerase chain reaction; TA: tumor antigen; TNBC: triple negative breast cancer; TNFα: tumor necrosis factor alpha

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Introduction

The role of antibody-dependent cellular cytotoxicity in the antitumor activity of tumor antigen-specific monoclonal antibodies has stimulated interest in genetically engineering T cells with the CD16 chimeric receptor (CD16-CR). Here, the authors expand the application of this methodology to triple negative breast cancer (TNBC) overexpressing EGFR by utilizing a novel CD32A\(^{131R}\)-CR in combination with anti-EGFR mAbs. The study supports the use of CD32A\(^{131R}\)-CR T cells combined with either panitumumab or cetuximab—an IgG2 and IgG1 monoclonal antibody, respectively—for targeting TNBC cells overexpressing EGFR. The results may be utilized as a platform for designing therapies targeting TNBC overexpressing EGFR.

Materials and Methods

Antibodies and reagents

Allophycocyanin (APC)-conjugated antihuman CD3 (cat. 555335), fluorescein isothiocyanate (FITC)-conjugated antihuman CD3 (cat. 555332), PerCP-Cy5.5-conjugated antihuman CD4 (cat. 332772), APC-conjugated antihuman CD8 (cat. 555369), FITC-conjugated antihuman CD107A (cat. 555800), FITC-conjugated antihuman CD279 (PD-1) clone MIH4 (cat. 557860), phycoerythrin (PE)-conjugated antihuman CD16 (cat. 555407), PE-conjugated antihuman CD8 (cat. 550586), FITC-conjugated Annexin V (cat. 556420), propidium iodide (PI) staining solution (cat. 5166211E), FITC-conjugated mouse antihuman IgG (cat. 555786), FITC-conjugated goat antimouse IgG (cat. 555988), mouse antihuman CD3 (cat. 555329) and antihuman CD28 (cat. 555725) were purchased from BD Bioscience (San Jose, CA). PE-conjugated goat antimouse IgG (1012-09) was purchased by Southern Biotech (Birmingham, AL). Mouse antihuman CD16 (clone 3g8), and PE-conjugated antihuman CD16 (cat. 555332), PerCP-Cy5.5-conjugated antihuman CD4 (cat. 555369), FITC-conjugated antihuman CD107A (cat. 555800), APC-conjugated antihuman CD3 (cat. 332772), APC-conjugated antihuman CD8 (cat. 555369), FITC-conjugated antihuman CD107A (cat. 555800), FITC-conjugated antihuman CD279 (PD-1) clone MIH4 (cat. 557860), phycoerythrin (PE)-conjugated antihuman CD16 (cat. 555407), PE-conjugated antihuman CD8 (cat. 550586), FITC-conjugated Annexin V (cat. 556420), propidium iodide (PI) staining solution (cat. 5166211E), FITC-conjugated mouse antihuman IgG (cat. 555786), FITC-conjugated goat antimouse IgG (cat. 555988), mouse antihuman CD3 (cat. 555329) and antihuman CD28 (cat. 555725) were purchased from BD Bioscience (San Jose, CA). PE-conjugated goat antimouse IgG (1012-09) was purchased by Southern Biotech (Birmingham, AL). Mouse antihuman CD16 (clone 3g8),
mouse antihuman CD247 (CD3ζ) mAb (clone 6B10.2) and mouse antihuman EGFR antibody (clone AY13) were purchased from BioLegend (San Diego, CA). Antihuman CD32 mAb (clone 8.26) was purchased from BD Bioscience (San Diego, CA). Antihuman B7-H3 (CD276) mAb 376.96 was developed and characterized as previously described.13 mAb 376.96 was purified from ascitic fluid by affinity chromatography on Protein A. The activity and purity of the mAb preparations was monitored using binding assays and SDS-PAGE.

2,5-diphenyltetrazolium bromide (MTT) was obtained from Aldrich (Saint Louis, MO). FcR blocking reagent (BR) was purchased from Miltenyi (Bergisch Gladbach, Germany). Geneljuice® Transfection Reagent (Novagen) was obtained from Millipore (Burlington, MA). Human recombinant interleukin-7 (IL-7) and interleukin-15 (IL-15) were obtained from Millipore (Burlington, MA). Human recombinant nontumor interleukin-7 (IL-7) and interleukin-15 (IL-15) were obtained from PeproTech (London, U.K.). Lipofectamine 2000 was purchased from Life Technologies (Carlsbad, CA) and Retronectin (Recombinant Human Fibronectin) was purchased from Takara Bio (Saint-Germain-en-Laye, France).

**Cell lines**

The 293T (RRID:CVCL_0063) packaging cell line was used to generate the helper-free retroviruses for T-cell transduction. 293T cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin, hereafter referred to as IMDM complete medium (CM). The Kirsten rat sarcoma (KRAS)-mutated A549 (RRID: CVCL_0023), HCT116 (RRID:CVCL_0291) and HCT116 cells stably transfected with CD32A131R-CR (HCT116CD32A131R), hereafter referred to as HCT116γC14FγC14R cells, were maintained in Roswell Park Memorial Institute (RPMI)-1640 CM. KRAS-mutated triple negative breast cancer (TNBC) cells, MDA-MB-231 (RRID:CVCL_0062) and KRAS wild-type TNBC cells, MDA-MB-468 (RRID:CVCL_0419), were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) CM (Thermo Fisher Scientific, Waltham, MA). The 293T cells were provided by Dr. Gianpietro Dotti, University of North Carolina, Chapel Hill, NC. The A549 cells were provided by Dr. Antonio Rossi, National Research Council, Italy. The MDA-MB-231 and MDA-MB-468 cells were provided by Dr. Maria Lucibello, National Research Council, Italy. The HCT116 cells were provided by Dr. Giulio Cesare Spagnoli, University of Basel, Switzerland. The mycoplasma-free cancer cell lines utilized in our study are part of our lab collection. An authentication test was successfully performed on November 21, 2018 using PCR-single-locus-technology (Eurofins, Ebersberg, Germany). The cell lines were passaged for four to eight times before use, or kept in culture for a maximum of 6 weeks.

**CD32A131R-CR construction**

The signal peptide (nucleotides 1-99) and the extracellular region (nucleotides 100-648) of the low-affinity variant CD32A131R were amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) from RNA extracted from freshly isolated peripheral blood mononuclear cells (PBMCs) using the following primers: forward 5’-GAGAATTCACCAT GACTATGGGAGACCCCAATG-3’ and reverse 5’-CGTAC GCCCATTTGGTGAAGAGCTGCC-3’ (Thermo Fisher Scientific, Waltham, MA). The PCR product was fused in tandem by restriction of the enzyme-compatible ends with the CD8α transmembrane domain and the CD28 and CD3ζ intracellular regions already available in the lab (CD32A131R.CR). The generation of CD16158F-CR has been previously described.10 The CD32A131R.CR and CD16158F.CR genes were subcloned into the Ncol and MluI sites of the SFG retroviral vector.

**Retrovirus production and T-cell transduction**

Retrovirus supernatants were obtained by transient transfection of the 293T packaging cells, using the Genejuice reagent, with the following vectors: the Peg-Pam vector, containing the Moloney murine leukemia virus gag and pol genes, the RDF vector, containing the RD114 envelope and the CD32A131R.CR or CD16158F.CR SFG retroviral vectors. Then, 48 hr and 72 hr posttransfection, the conditioned medium containing the retrovirus was harvested, filtered, snap frozen and stored at −80°C until use. To generate the Fcγ-γCR T cells, PBMCs (0.5 × 10⁶ PBMCs/ml) were cultured for 3 days in a nontissue culture treated in a 24-well plate precoated with 1 μg/ml of anti-CD3 and 1 μg/ml of anti-CD28 mAbs in the presence of 10 ng/ml of IL-7 and 5 ng/ml of IL-15. The viral supernatant was loaded on RetroNectin-coated nontissue culture treated 24 well plates, and spun for 1.5 hr at 2,000g. The activated T cells were seeded into the retrovirus loaded-plate, spun for 10min, and incubated for 72 hr at 37°C in 5% CO₂. After transduction, the T cells were expanded in RPMI-1640 CM supplemented with 10 ng/ml of IL-7 and 5 ng/ml of IL-15 for 12–13 days, and then analyzed.

**Western blot**

The CD32A131R.CR transduced and nontransduced T cells were lysed with Triton buffer composed of 1% (v/v) Triton X-100, 20 mM Tris–HCl pH 7.6, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM phenylmethylsulfonyl fluoride supplemented with phosphatase (Sigma–Aldrich, Saint Louis, MO), and protease (Roche, Basel, Switzerland) inhibitor cocktails. Thirty micrograms of protein lysates were resolved on Bolt 4–12% Bis–Tris plus gel (Invitrogen, Carlsbad, CA) under reducing conditions, and then transferred to a nitrocellulose filter. The filter was probed overnight at 4°C with a mouse antihuman CD3ζ or rabbit antiphospho-tyrosine CD3ζ.
(Y142) antibody. The former was detected using a horseradish peroxidase-conjugated donkey antimouse (Jackson Laboratory, Bar Harbor, ME) for 1 hr at room temperature. Antibody binding was visualized with an Amersham ECL Western blotting detection reagent (GE Healthcare, Little Chalfont, U.K.).

**Binding assay**

Direct immunofluorescence analysis was used to test the Fc antibody-binding ability of the FITC-conjugated anti-CD107A mAb to CD32A131R-CR and CD16158F-CR. The CD32A131R-CR and CD16158F-CR T cells were incubated with 5 μl of FITC-conjugated anti-CD107A, with or without the FcR BR, for 30 min at 4°C. Then, the cells were washed and analyzed using flow cytometry. Cetuximab or panitumumab Fc fragment binding to the CD32A131R-CR or CD16158F-CR T cells was evaluated by staining with an FITC-conjugated anti-human IgG.

**Flow cytometry**

Phenotypic analysis of transduced and nontransduced T cells was assessed by flow cytometry incubating the cells for 30 min at 4°C with various combinations of the following mAbs including FITC-conjugated anti-human CD3, PE-conjugated anti-human CD32, PE-conjugated anti-human CD16, APC-conjugated anti-human-CD8, PerCP-Cy5.5 anti-human CD4 and FITC-conjugated anti-human PD-1. The cells were then analyzed using a 2-laser BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The results were analyzed using Tree Star, Inc. FlowJo software.

**Cytokine release assay**

The CD32A131R-CR or CD16158F-CR transduced T cells (2 × 10^4/well) were added to 96-well plates, previously coated with 10 μg/ml of anti-CD3, 3g8, or 8.26 mAb. In the coculture experiments, the CD16158F-CR T cells or CD32A131R-CR T cells were plated in 96-well plates with target cell lines at a ratio of 5:1 E:T ratio in the presence or absence of 3 μg/ml of cetuximab or panitumumab or the anti-B7-H3 mAb, 376.96. Supernatants were collected 24 hr or 48 hr after culturing. The interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) levels were measured using ELISA (Thermo Fisher Scientific, Waltham, MA).

**In vitro tumor cell viability assay**

The tumor target cells (7 × 10^4/well) were seeded into 96-well plates and CD16158F-CR T cells or CD32A131R-CR T cells (35 × 10^4/well) were added in the presence or absence cetuximab or panitumumab or the anti-B7-H3 mAb 376.96 (3 μg/ml; see above). After 48 hr incubation at 37°C, the non-adherent T cells were removed. Then, a suspension of fresh medium (100 μl/well) supplemented with MTT (20 μg/ml mg/ml) was added to the adherent cells for 3 hr at 37°C. MTT was then removed, and 100 μl of dimethyl sulfoxide was added to each well. Absorbance (optical density) was measured at 570 nm.

In the cytotoxic assay, MDA-MB-468 cells were seeded in 24-well plates at a concentration of 0.2 × 10^4 cells/well. The following day, CD16158F-CR T cells or CD32A131R-CR T cells were added to the culture at E:T ratio of 1:1 for 4 hr at 37°C in the presence or absence of cetuximab, panitumumab or 376.96 mAb. The cells were then stained with PE-conjugated anti-human EGFR mAb, FITC-Annexin V and PI solution and analyzed by flow cytometry as previously described.14

**Statistical analysis**

The results were analyzed by using either the unpaired t-test or the Mann–Whitney test. The relationship between the two variables was measured using Spearman’s rank correlation coefficient. Differences with a p-value <0.05 were considered to be statistically significant.

**Data availability**

The data that support the findings of our study are available from the corresponding author upon reasonable request.

**Results**

**CD32A131R and CD16158F-CRs are differentially expressed on T cells**

The activated T cells were transduced in vitro with a gamma-retroviral vector encoding the CD32A131R-CR (Fig. 1a). Then, Western blot and flow cytometry analysis were used to test the cells for expression of CD32A131R-CR. For biochemical analysis, we used two mAbs specific for the nonphosphorylated and phosphorylated CD3ζ chain. Both mAbs detected two distinct bands. The molecular weight band slightly higher than 51 kDa matches the expected size of the CD32A131R-CR while the smaller 18 kDa band, detected in both the control and CD32A131R-CR-expressing T cells, corresponds to the endogenous CD3ζ chain (Fig. 1b). The flow cytometry results showed that CD32A131R-CR was clearly detectable on the cell surface of the engineered T cells (Fig. 1c, left panel). After a 3-day T-cell transduction, both CD32A131R-CR and CD16158F-CR T were found to be expressed on the surface of CD4 and CD8 T cells (Fig. 1c, middle panel). Interestingly, CD32A131R-CR T cells and to a lesser extent CD16158F-CR T cells expressed the checkpoint inhibitor molecule PD-1 (Fig. 1c, right panel).

**CD32A131R-CR specifically bound the Fc fragment of soluble immunoglobulins**

In the initial experiments, we compared the ability of the CD32A131R-CR and CD16158F-CR T cells to bind to the soluble IgG Fc fragment. As a model reagent, we chose the H4A3 mAb, a FITC-conjugated IgG1 specific for CD107A, an intracellular lysosomal-associated membrane protein (LAMP-1). First, we evaluated the binding of anti-CD107A mAb on the surface of the CD32A131R-CR T cells in comparison to the CD16158F-CR T cells. After a 30 min incubation, the CD32A131R-CR T cells effectively bound to the anti-CD107A mAb on their surfaces (Fig. 2a, left panel), whereas CD16158F-CR did not (Fig. 2a, right panel).
Binding was highly specific, because it was abrogated in the presence of FcR BR. To further evaluate whether the CD32A131R-CR T cells were capable of binding to the mAb Fc fragment in a more physiological condition, we tested whether the Fc fragment-binding capacity of CD32A131R-CR was preserved in the presence of human immunoglobulins. Therefore, we incubated anti-CD107A with the CD32A131R-CR T cells in a buffer containing 10% of human plasma (Fig. 2b). After a 30 min incubation, anti-CD107A mAb was still bound to the engineered T cells (Fig. 2b).

To assess the Fcγ-CR T-cell potential to target the EGFR+ ECCs upon incubation with the anti-EGFR mAbs, we tested their antibody-binding capacity by using cetuximab and panitumumab mAbs. Only the CD32A131R-CR T cells bound the Fc fragment of both of the soluble anti-EGFR mAbs (Fig. 2c); the binding capacity was higher for cetuximab than panitumumab (Fig. 2c, lower panels). It is important to note that the binding of both mAbs was prevented in the presence of FcR BR (Fig. 2c). Finally, we performed dose–response binding assays in which cetuximab was incubated at increasing concentrations with the Fcγ-CR T cells for 30 min at 4°C. As shown in Figure 2d, only the CD32A131R-CR T cells (solid lines) bound cetuximab. The maximum binding capacity of CD32A131R-CR, expressed as both percentages and mean fluorescence intensity...
CD16158F-CR T cells significantly reduced the number of viable HCT116FcγR+ cells, which is consistent with efficient Fc-mediated cytotoxic activity. Furthermore, crosslinking of either Fcγ-receptor with specific mAbs induced the release of comparable amounts of IFNγ and TNFα (Fig. 3b). These data indicate that both the CD32A131R-CR T cells and the CD16158F-CR T cells clearly mediate comparable levels of reverse ADCC when given in combination with the mAbs, 8.26 and 3g8, respectively.

CD32A131R-CR T cells and CD16158F-CR T cells differ in their ability to eliminate MDA-MB-468 cells in combination with cetuximab and panitumumab

The ability of CD32A131R-CR to specifically bind the cetuximab Fc fragment prompted us to investigate whether this binding triggers ADCC against EGFR+ ECC lines. The
CD32A131R-CR T cells and CD16158F-CR T cells were incubated with four ECC lines at an E:T ratio of 5:1. Tumor cell viability was assessed after 48 hr incubation at 37°C by a MTT assay. The CD32A131R-CR T cells significantly reduced the viability of the MDA-MB-468 cells in the presence of cetuximab or panitumumab, but the CD16158F-CR T cells were only effective in the presence of cetuximab (Fig. 4a). In contrast, anti-B7-H3, 376.96 mAb, which stained the MDA-MB-468 cells, did not cause detectable change in ECC viability. Neither cetuximab nor panitumumab had detrimental effects on the MDA-MB-468 cells in the absence of the Fcγ-CR T cells (Fig. 4a). However, the CD32A131R-CR T cells and CD16158F-CR T cells, in combination with cetuximab or panitumumab, failed to affect the viability of the EGFR+ MDA-MB-231, A549 and HCT116 cells (Fig. 4a).

To further demonstrate the cytotoxic effect of engineered T cells, both CD16158F-CR and CD32A131R-CR T cells were incubated, in the presence or absence of anti-EGFR mAbs, with or without MDA-MB-468 cells. After a 4 hr incubation at 37°C, in 5% CO₂, the cytotoxic activity of engineered T cells on MDA-MB-468 cells was assessed by a flow cytometry assay. Only CD16158F-CR T cells, in combination with cetuximab, induced apoptosis of TNBC cells while CD32A131R-CR T cells, in combination with either cetuximab or panitumumab, clearly induced MDA-MB-468 apoptosis. Interestingly, CD32A131R-CR, in combination with 376.96 mAb triggered detectable levels of MDA-MB-468 apoptosis (Fig. 4b). These data show that the engineered T cells exert cytotoxic activity on MDA-MB-468.

Crosslinking of CD16158F-CR on engineered T lymphocytes cultured with MDA-MB-468 BC cells promoted the release of IFNγ (Fig. 5a) and TNFα (Fig. 5b) in the presence of cetuximab but not panitumumab. In contrast, both anti-EGFR mAbs and 376.96 mAbs triggered the release of both cytokines by the CD32A131R-CR engineered T cells. Furthermore, although panitumumab and 376.96 mAb failed to mediate cell-dependent cytotoxicity against the A549 and HCT116 cell lines, they induced a significant release of IFNγ by the CD32A131R-CR T cells (Fig. 5a) incubated with the two cell lines.

**Correlation of EGFR expression level on targeted cancer cell lines with the cetuximab-dependent Fcγ-CR T-cell cytotoxicity**

Our results indicate that, among the evaluated EGFR+ ECC lines, only the MDA-MB-468 cells were efficiently killed by the CD16158F-CR T cells in combination with cetuximab and by the CD32A131R-CR T cells in combination with cetuximab or panitumumab (Fig. 4). Because MDA-MB-468 cells express high levels of EGFR, we hypothesized that the ability of cetuximab to mediate the ADCC activity of Fcγ-CR T cells against ECCs is associated with the EGFR expression level on the target cells. To test this hypothesis, we measured the EGFR expression level on the surface of the HCT116, A549, MDA-MB-231 and MDA-MB-468 cells, and we correlated it with the ability of cetuximab to mediate Fcγ-CR T-cell cytotoxicity with the target cells (Fig. 6). As expected, the MDA-MB-468 cells displayed the highest MFI upon indirect staining.
Figure 4. CD32A<sup>131R</sup>-chimeric receptor (CR) T cells eliminate MDA-MB-468 cells more efficiently than CD16<sup>158F</sup>-CR T cells. The antitumor activity of the CD32A<sup>131R</sup>-CR T cells and CD16<sup>158F</sup>-CR T cells was tested in combination with the indicated mAbs against the EGFR<sup>+</sup> and B7-H3<sup>+</sup> cancer cells. Triple negative breast cancer cells, MDA-MB-468 and KRAS-mutated MDA-MB-231, nonsmall cell lung cancer cells A549 and CRC cells HCT116, were chosen as the target cells. (a) MTT assays of tumor cell viability were performed after 48 hr incubation, at 37°C, with CD32A<sup>131R</sup>-CR or CD16<sup>158F</sup>-CR T cells, with or without mAbs. The figure shows the cumulative data, with mean ± SD values, of tumor cell viability obtained from three different donors at an E:T ratio of 5:1. Asterisks indicate: * = p < 0.05 and *** = p < 0.001. (b) Flow cytometry based-cytotoxic assay. CD16<sup>158F</sup>-CR T cells and CD32A<sup>131R</sup>-CR T cells were incubated at 37°C in 5% CO<sub>2</sub>, in the presence or absence of cetuximab (3 μg/ml) and panitumumab (3 μg/ml), with or without MDA-MB-468 at an E:T ratio of 1:1. After a 4 hr incubation, the cell mix was harvested and stained with PE-antihuman-EGFR mAb, FITC-Annexin V and PI. MDA-MB-468 cells were identified by posting an electronic gate of EGFR<sup>+</sup> cells. Numbers in the quadrants indicate percentages of cells after gating on MDA-MB-468 cells.
with an anti-EGFR mAb (Fig. 6a). Furthermore, we assessed the ability of both CD16158F-CR and CD32A131R-CR to reduce the viability of EGFR+ ECCs in combination with cetuximab. The CD16158F-CR and to a lesser extent CD32A131R-CR correlated with the MFI of the target marker by the cancer cell lines that were tested (Fig. 6b).

**Discussion**

Rituximab and trastuzumab have been used to redirect first and second generation CD16158V-CR T cells against CD20+ and HER2+ hematologic and solid malignancies, respectively.9,11,12 The results obtained in preclinical studies suggest that CD16158V-CR T cells may act as universal CR-effector cells capable of improving the therapeutic effectiveness of TA-specific mAbs by an ADCC mechanism. The rationale underlying the choice of the high-affinity extracellular CD16158V for manufacturing Fc chimeras is that CD16 triggers ADCC in NK cells.19 However, myeloid cells, such as monocyte/macrophages and granulocytes, can also mediate effector functions, such as proinflammatory cytokine production17,18 and cell-mediated cytotoxicity,19 including ADCC.20,21 Although CD16 is the major player in mediating ADCC, CD32A is also capable of promoting ADCC by myeloid cells.20 Similarly to CD16, CD32A is characterized by low (CD32A131R) and high affinity (CD32A131H) polymorphisms.3

To date, there is scant information about the antitumor activity of CD16158F-CR T cells, and the role of CD32A131R-CR T cells is completely unknown. Increasingly, experimental evidence suggests that CD16158F and CD32A131R polymorphisms show differential binding affinities for the mAb Fc portions of IgG1 and IgG2. Taking advantage of the availability of cetuximab (IgG1) and panitumumab (IgG2), we demonstrate, for the first time, that both CD32A131R-CR T cells and CD16158F-CR T cells trigger ADCC to the TNBC cells, MDA-MB-468. In a side-by-side comparison, we show the superiority of CD32A131R-CR over CD16158F-CR in redirecting engineered T cells against the MDA-MB-468 cells through anti-EGFR mAbs, at least in vitro. The affinity of CD16158F-CR and CD32A131R-CR for IgG1 and IgG2 mAbs was significantly different. The CD32A131R-CR bound Fc fragments of soluble IgG1 (anti-CD107A and cetuximab), and, to a lesser extent IgG2 (panitumumab), whereas CD16158F-CR T cells bound neither cetuximab nor panitumumab. The differential binding ability of cetuximab and panitumumab to CD32A131R-CR is not surprising, because the CD32A131R polymorphisms bind IgG1 with a significantly
Indeed, mAb differences and IgG1 functional Fc criteria: MB-HCT Mean fluorescence intensity (MFI) is indicated for each cell line. (a) Spearman’s correlation between EGFR expression levels (MFI) in the HCT116, A549, MDA-MB-231 and MDA-MB-468 cell lines and the ECC elimination by CD16\(^{158F}\)-CR T cells (left panel) and CD32A\(^{131R}\)-CR T cells (right panel) in combination with cetuximab. A regression line is shown in black. Spearman’s rank correlation coefficient (r) was 0.6316 for CD16\(^{158F}\)-CR and 0.533 for CD32A\(^{131R}\)-CR. This is a cumulative analysis of five experiments separately performed: 116 = HCT116, 231 = MDA-MB-231, 468 = MDA-MB-468 and 549 = A549 cells.

Figure 6. *In vitro* elimination of EGFR\(^{+}\) epithelial cancer cells (ECCs) by CD32A\(^{131R}\)-chimeric receptor (CR) and CD16\(^{158F}\)-CR T cells is associated with EGFR overexpression. (a) EGFR expression levels in the tumor cell lines as assessed by flow cytometry. The cells were incubated with 3 µg/ml of purified anti-EGFR antibody, washed and then stained with a phycoerythrin (PE)-conjugated antimouse IgG (gray filled histograms). The cells incubated with PE-conjugated antimouse IgG were used as a negative control (light gray filled histograms). Mean fluorescence intensity (MFI) is indicated for each cell line. (b) Spearman’s correlation between EGFR expression levels (MFI) in the HCT116, A549, MDA-MB-231 and MDA-MB-468 cell lines and the ECC elimination by CD16\(^{158F}\)-CR T cells (left panel) and CD32A\(^{131R}\)-CR T cells (right panel) in combination with cetuximab. A regression line is shown in black. Spearman’s rank correlation coefficient (r) was 0.6316 for CD16\(^{158F}\)-CR and 0.533 for CD32A\(^{131R}\)-CR. This is a cumulative analysis of five experiments separately performed: 116 = HCT116, 231 = MDA-MB-231, 468 = MDA-MB-468 and 549 = A549 cells.

higher affinity than IgG2. However, the failure of CD16\(^{158F}\)-CR to bind soluble cetuximab (IgG1) is surprising, because CD16\(^{158F}\) polymorphism has a slightly lower ability to bind IgG1 than CD32A\(^{131R}\). Our results are a bit different from those reported by Kudo *et al.*\(^9\), who showed weak, but detectable, binding of soluble IgG1 mAbs, such as rituximab and trastuzumab, to CD16\(^{158F}\)-CR. The differences in our results and the results reported by Kudo *et al.*\(^9\) may reflect structural differences between the CD16\(^{158F}\) and CD32A\(^{131R}\)-CR endodomains.

The induction of an effective ADCC of tumor cells by FcγR\(^^{+}\) cytotoxic T cells needs to meet at least three distinct criteria: (i) the presence of FcγR\(^{+}\) cytotoxic T cells with a functional lytic machinery; (ii) FcγR binding affinity for the tested mAb Fc fragment, sufficient to activate T cells; and (iii) surface expression level of the antigen targeted by the tested mAb, sufficient to activate the effector mechanisms in T cells. Indeed, both the CD16\(^{158F}\)-CR- and CD32A\(^{131R}\)-CR-engineered T cells fully satisfy the first condition, because anti-CD16 mAb and anti-CD32 mAb triggered a similar level of reverse ADCC when tested with KRAS-mutated, FcγR positive, HCT116 cells. However, the CD16\(^{158F}\)-CR T cells and the CD32A\(^{131R}\)-CR T cells, in combination with cetuximab, neither released IFNγ and TNFα nor eliminated the KRAS-mutated ECCs, including the A549, HCT116 and MDA-MB-231 cells,\(^{22,23}\) although they were fully activated by the EGFR overexpressing MDA-MB-468 cells.

The differential antitumor activity of both the Fcγ-CR T cells with wild-type and KRAS-mutated ECCs should be addressed. The inability of both Fcγ-CR T cells to eliminate the KRAS-mutated ECC cells does not reflect a mechanism of resistance of these target cells to the lytic activity of the two effector cells that were tested, because both of them can eliminate KRAS-mutated HCT116\(^{FcγR^{+}}\) cancer cells in a redirect ADCC assay. However, the higher sensitivity of the wild-type MDA-MB-468 in comparison to the tested KRAS-mutated ECCs is likely to reflect the differential levels of surface EGFR expression. Our data are also supported by Correale *et al.*, who showed that drug-mediated upregulation of EGFR enhanced the susceptibility of EGFR\(^{+}\) colorectal carcinoma (CRC) cells to cetuximab-based ADCC of LAK cells.\(^{24}\) Indeed, the KRAS wild-type, MDA-MB-468 cells overexpress EGFR\(^{25}\) at a level higher than the KRAS-mutated ECCs used in the
present study. Our hypothesis is supported by Derer et al.’s finding that a KRAS mutation impairs the sensitivity of CRC cells to anti-EGFR mAbs due to C/EBPβ-dependent down-regulation of EGFR expression.26

The restoration of the sensitivity of the KRAS-mutated ECCs to Fcγ-CR T-cell lytic activity may require the generation of CD32A-CR and CD16-CR with high affinity for the used mAb Fc fragments, such as CD32A131R-CR and CD16158V-CR. This strategy is supported by the ability of ECCs opsonized with anti-EGFR mAbs to induce a level of FcyR crosslinking insufficient to fully mediate ADCC, but sufficient to stimulate other Fcy-CR T-cell functions, such as cytokine production. In support of this, we show that HCT116 and A549 cells, opsonized with panitumumab or 376.96 mAb, promote IFNγ release from CD32A131R-CR T cells. In this context, we were surprised to see that CD32A131R-CR T cells combined with the IgG2, 376.96 mAb (anti-B7-H3) induced MDA-MB-468 apoptosis and cytokine release while no antitumor effects were detected by the MTT assay. These results may be explained by the possibility that the sensitivity of the two assays differ.

The failure to generate an effective ADCC could be related to inadequate expression of EGFR on the surface of ECCs. In support of this hypothesis, CD16158F-CR T and CD32A131R-CR T cells, in combination with cetuximab, damaged the wild-type MDA-MB-468 cells overexpressing EGFR. Indeed, the results shown in Fig. 6 indicate that the extent of ECC reduced viability induced by the combination of either the CD16158F-CR T cells or CD32A131R-CR T cells with cetuximab directly correlated with the EGFR expression levels on the targeted ECCs. EGFR crosslinking on MDA-MB-468 cells, with Fcγ-CR T cells and cetuximab, led to ADCC activation. The ability of the CD16158F-CR T cells to mediate ADCC in the presence of cetuximab was somewhat unexpected because no binding of soluble cetuximab to these cells could be detected. This finding may reflect the ability of CD16158F-CR T cells to bind cetuximab only after EGFR cancer cell opsonization, which stabilizes the ligand-receptor interactions.

Unlike cetuximab (IgG1), panitumumab (IgG2) did not induce significant ADCC by NK cells, thereby limiting its applications in cell-based cancer immunotherapy.27 However, panitumumab is still able to trigger ADCC by macrophages, which, with the exception of a small subset of cells,28 do not express CD16, but do express CD32 and CD64.29 As a logical consequence, engineering cytotoxic T cells with a CD32A131R-CR has allowed us to demonstrate that panitumumab can stimulate strong ADCC by CD32A131R-CR T cells against MDA-MB-468 cells overexpressing EGFR. These results provide new perspectives for the use of panitumumab in cell-based targeted immunotherapy of solid tumors.

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