

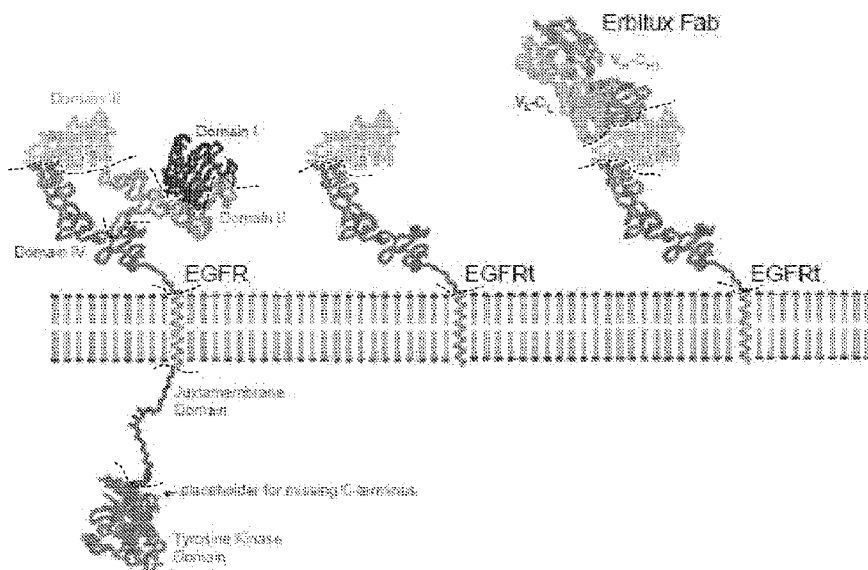


(86) **Date de dépôt PCT/PCT Filing Date:** 2010/11/03
(87) **Date publication PCT/PCT Publication Date:** 2011/05/12
(45) **Date de délivrance/Issue Date:** 2022/12/06
(85) **Entrée phase nationale/National Entry:** 2012/05/01
(86) **N° demande PCT/PCT Application No.:** US 2010/055329
(87) **N° publication PCT/PCT Publication No.:** 2011/056894
(30) **Priorité/Priority:** 2009/11/03 (US61/257,567)

(51) **Cl.Int./Int.Cl. C12N 15/12** (2006.01),
C07H 21/04 (2006.01), **C07K 14/705** (2006.01),
C07K 14/71 (2006.01), **C07K 16/28** (2006.01),
C12N 15/18 (2006.01), **C12N 5/0783** (2010.01),
G01N 33/567 (2006.01), **G01N 33/577** (2006.01)
(72) **Inventeur/Inventor:**
JENSEN, MICHAEL C., US
(73) **Propriétaire/Owner:**
CITY OF HOPE, US
(74) **Agent:** SMART & BIGGAR LP

(54) **Titre : RECEPTEUR DU FACTEUR DE CROISSANCE DE L'EPIDERME TRONQUE (EGFRT) POUR LA SELECTION DE LYMPHOCYTES T TRANSDUITS**

(54) **Title: TRUNCATED EPIDERMAL GROWTH FACTOR RECEPTOR (EGFRT) FOR TRANSDUCED T CELL SELECTION**



(57) **Abrégé/Abstract:**

A non-immunogenic selection epitope may be generated by removing certain amino acid sequences of the protein. For example, a gene encoding a truncated human epidermal growth factor receptor polypeptide (EGFRT) that lacks the membrane distal EGF-binding domain and the cytoplasmic signaling tail, but retains an extracellular epitope recognized by an anti-EGFR antibody is provided. Cells may be genetically modified to express EGFRT and then purified without the immunoactivity that would accompany the use of full-length EGFR immunoactivity. Through flow cytometric analysis, EGFRT was successfully utilized as an in vivo tracking marker for genetically modified human T cell engraftment in mice. Furthermore, EGFRT was demonstrated to have cellular depletion potential through cetuximab mediated antibody dependent cellular cytotoxicity (ADCC) pathways. Thus, EGFRT may be used as a non-immunogenic selection tool, tracking marker, a depletion tool or a suicide gene for genetically modified cells having therapeutic potential.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 May 2011 (12.05.2011)

(10) International Publication Number
WO 2011/056894 A3

PCT

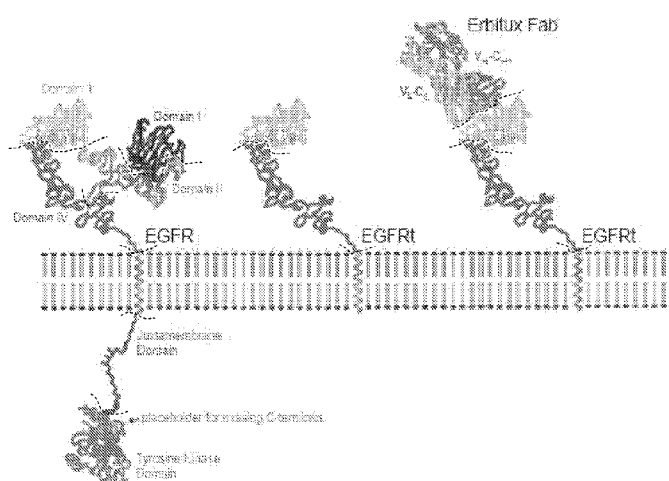
- (51) **International Patent Classification:**
C12N 15/00 (2006.01) *C07H 21/04* (2006.01)
- (21) **International Application Number:**
PCT/US2010/055329
- (22) **International Filing Date:**
3 November 2010 (03.11.2010)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
61/257,567 3 November 2009 (03.11.2009) US
- (72) **Inventor; and**
- (71) **Applicant :** JENSEN, Michael C. [US/US]; 1500 East Duarte Road, Duarte, California 91010 (US).
- (74) **Agent:** DUEPPEN, Lara J.; PERKINS COIE LLP, Patent - LA, P.O. Box 1208, Seattle, Washington 98111-1208 (US).
- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,

DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KI, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- *with international search report (Art. 21(3))*
 - *with sequence listing part of description (Rule 5.2(a))*
- (88) Date of publication of the international search report:**
- 24 November 2011

- (54) Title:** TRUNCATED EPIDERMAL GROWTH FACTOR RECEPTOR (EGFRt) FOR TRANSDUCED T CELL SELECTION

Figure 1



- (S7) Abstract:** A non-immunogenic selection epitope may be generated by removing certain amino acid sequences of the protein. For example, a gene encoding a truncated human epidermal growth factor receptor polypeptide (EGFRt) that lacks the membrane distal EGF-binding domain and the cytoplasmic signaling tail, but retains an extracellular epitope recognized by an anti-EGFR antibody is provided. Cells may be genetically modified to express EGFRt and then purified without the immunoactivity that would accompany the use of full-length EGFR immunoactivity. Through flow cytometric analysis, EGFRt was successfully utilized as an *in vivo* tracking marker for genetically modified human T cell engraftment in mice. Furthermore, EGFRt was demonstrated to have cellular depletion potential through cetuximab mediated antibody dependent cellular cytotoxicity (ADCC) pathways. Thus, EGFRt may be used as a non-immunogenic selection tool, tracking marker, a depletion tool or a suicide gene for genetically modified cells having therapeutic potential.

WO 2011/056894 A3

60412-4841

**TRUNCATED EPIDERMAL GROWTH FACTOR RECEPTOR (EGFRt) FOR
TRANSDUCED T CELL SELECTION**

PRIORITY CLAIM

[0001] This application claims priority from U.S. Patent Application No. 61/257,567, filed November 3, 2009.

TECHNICAL FIELD

[0002] The present products and methods relate to the fields of immunology and purification of genetically modified cells, specifically to a truncated or otherwise modified receptor paired with a corresponding antibody, such as a polypeptide derived from human epidermal growth factor receptor (EGFR) paired with cetuximab, for use in cancer immunotherapy.

BACKGROUND

[0003] Immune cell products with homogenous expression of tumor targeting chimeric antigen receptors (CARs) are desirable for clinical evaluation of adoptive therapy strategies to eliminate the product-to-product variability of transgene expression otherwise intrinsic to transduction and other genetic modification procedures without subsequent selection. Immunotherapy using genetically redirected immune cells is an attractive approach for treating minimal residual disease in a variety of cancer patients. However, immunologic rejection of cell products expressing antibiotic selection proteins as part of the transduction strategy has impeded this strategy. A novel selection marker that is not expressed on human lymphocytes, does not contain endogenous signaling or trafficking function, and is recognized by a known, preferably commercially available, pharmaceutical grade

antibody reagent that can be utilized for selection, *in vivo* tracking, and depletion of transduced cells would be a significant improvement in the art.

SUMMARY

[0004] Products and methods for purification, both *in vivo* and *ex vivo*, of genetically modified cells are provided herein. The genetically modified cells may be modified by transduction, or any other process that adds, deletes, alters, or disrupts an endogenous nucleotide sequence. The genetically modified cells may be transduced T cells with altered activity, including altered immunoactivity.

[0005] According to the embodiments described herein, a non-immunogenic selection epitope compatible with immunomagnetic selection facilitates immunotherapy in cancer patients without undesirable immunologic rejection of cell products (i.e. as seen when expressing antibiotic selection proteins) may be generated by removing certain amino acid sequences of the protein. In some embodiments, the non-immunogenic selection epitope is a gene encoding an endogenous cell-surface molecule that is modified or truncated to retain an extracellular epitope recognized by a known antibody or functional fragment thereof, and to remove any signaling or trafficking domains and/or any extracellular domains unrecognized by the known antibody. The removal of the signaling or trafficking domains and/or any extracellular domains unrecognized by the known antibody renders the endogenous cell-surface molecule inert, which is a desired property for the molecule. The non-immunogenic selection epitope may also be used for as a selection tool or tracking marker.

[0006] The modified endogenous cell-surface molecule may be, but is not limited to, any cell-surface related receptor, ligand, glycoprotein, cell adhesion molecule, antigen, integrin or cluster of differentiation (CD) that is modified as

described herein. In some embodiments, the modified endogenous cell-surface molecule is a truncated tyrosine kinase receptor. In one aspect, the truncated tyrosine kinase receptor is a member of the epidermal growth factor receptor family (e.g., ErbB1, ErbB2, ErbB3, ErbB4).

[0007] Epidermal growth factor receptor, also known as EGFR, ErbB1 and HER1, is a cell-surface receptor for members of the epidermal growth factor family of extracellular ligands. Alterations in EGFR activity have been implicated in certain cancers. In a first aspect, a gene encoding an EGFR polypeptide comprising human epidermal growth factor receptor (EGFR) that is constructed by removal of nucleic acid sequences that encode polypeptides including the membrane distal EGF-binding domain and the cytoplasmic signaling tail (a “truncated EGFR” or “EGFRt”), but retains the extracellular membrane proximal epitope recognized by an anti-EGFR antibody. Preferably, the antibody is a known, commercially available anti-EGFR monoclonal antibody, such as cetuximab, matuzumab, necitumumab or panitumumab.

[0008] Application of biotinylated-cetuximab to immunomagnetic selection in combination with anti-biotin microbeads successfully enriches T cells that have been lentivirally transduced with EGFRt-containing constructs from as low as 2% of the population to greater than 90% purity without observable toxicity to the cell preparation. Constitutive expression of this inert EGFRt molecule does not affect T cell phenotype or effector function as directed by the coordinately expressed chimeric antigen receptor (CAR), CD19R. Through flow cytometric analysis, EGFRt was successfully utilized as an *in vivo* tracking marker for T cell engraftment in mice. Furthermore, EGFRt was demonstrated to have suicide gene potential through Erbitux® mediated antibody dependent cellular cytotoxicity (ADCC) pathways. Thus,

EGFRt may be used as a non-immunogenic selection tool, tracking marker, and suicide gene for transduced T cells that have immunotherapeutic potential. The EGFRt nucleic acid may also be detected by means well known in the art.

[0009] In another embodiment, methods of discovering and designing modified, truncated or altered endogenous cell-surface molecules which bind to antibodies, preferably commercially available antibodies, as described herein are provided. The methods include modeling the protein of interest and truncating functional portions, while leaving the antibody-binding portions intact. The resulting modified receptor or ligand can be sorted using a labeled antibody and then enriched such that the concentration of the modified receptor or ligand is increased.

[0010] Yet another embodiment provides a method of selecting transduced T cells comprising transducing T cells with a modified, truncated or altered endogenous cell-surface molecule gene sequence (e.g., truncated EGFR) and then applying an antibody that binds the modified ligand or receptor sequence to the transduced T cells. If the modified receptor sequence is EGFRt, the antibody is preferably a biotinylated anti-EGFR monoclonal antibody. The T cells are then sorted by adding anti-biotin microbeads and selecting the T cells using immunomagnetic separation, adding fluorochrome-conjugated anti-biotin and selecting the T cells using Fluorescence Activated Cell Sorting, or any other reliable method of sorting the cells. The modified ligand or receptor sequences, such as the EGFRt sequence, may be contained in a suitable transfer vehicle such as a lentiviral vector.

[0010a] In one aspect, the invention provides a nucleic acid encoding a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

[0010b] In another aspect, the invention provides a modified Epidermal Growth Factor Receptor (EGFR) comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the truncated non-immunogenic endogenous cell surface molecule (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

[0010c] In another aspect, the invention provides a population of human T-cells expressing a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

[0010d] In another aspect, the invention provides a population of human T-cells comprising a nucleic acid encoding a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

[0011] These and other embodiments are further explained in the drawing and detailed description herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 is a molecular model of EGFR vs. EGFRt proteins based on the crystal structure files. The EGFR structure on the left shows a full-length EGFR with the structure of the four extracellular domains (Domains I-IV). The middle structure shows the truncated EGFR (EGFRt), which is missing Domain I, Domain II, the Juxatmembrane Domain, and the Tyrosine Kinase Domain as compared to an unmodified EGFR. The EGFRt on the right shows truncated structure bound to Eribitux® Fab, comprised of V_H-C_{H1} and V_L-C_L. The domains are separated with dotted lines.

[0013] Figure 2 illustrates the selection of EGFRt⁺ T cells using biotinylated cetuximab (referred to in the figure as Erbitux®). Figure 2a is a schematic of the cetuximab biotinylation and reformulation process. Figure 2b is a graph showing titration of biotinylated cetuximab. 10⁶ EGFR⁺ cells were stained with either 0μg (black), 1.45μg (red), 0.145μg (orange), 14.5ng (yellow), 1.45ng (green), 0.145ng (blue) or 14.5pg (purple) of biotinylated cetuximab followed by 0.5μg PE-conjugated streptavidin and analyzed by flow cytometry. 14.5ng or more of biotinylated cetuximab was deemed sufficient for future staining. Figure 2c depicts schematics of both the immunomagnetic (top) and the fluorescence activated cell sorting (bottom) EGFRt selection procedures.

[0014] Figure 2d shows immunomagnetic selection of various T cell lines lentivirally transduced with CAR and EGFRt containing constructs. Schematics of the CD19CAR-T2A-EGFRt (left) and CD19CAR-T2A-EGFRt-IMPDH2dm (right) constructs contained in lentiviral vectors are shown above the corresponding pre- and post-selection flow cytometric analyses for surface EGFRt expression. Codon optimized sequence portions of the CD19-specific, CD28 co-stimulatory CAR,

followed by the self-cleavable T2A, EGFRt and IMPDH2dm selection markers are indicated, along with the Elongation Factor 1 promoter sequences (EF-1p), and the GCSFR alpha chain signal sequences (GCSFRss, which directs surface expression). Flow cytometric analysis of lentivirally transduced T cell lines that had been stained with a biotinylated-cetuximab antibody and PE-conjugated anti-biotin antibody (black histograms) was performed on both the input T cells (PRE SLXN) and the positive fraction obtained from AutoMACS™ (POS FRXN). Open histograms represent staining with PE-conjugated anti-biotin antibody alone, and the percent positive cells are indicated in each histogram. Selection of CD19CAR⁺EGFRt⁺ Line A occurred 3 days after transduction of T cell blasts. Selection of CD19CAR⁺EGFRt⁺ Line B occurred after 3 REM stimulations of transduced CMVpp65-specific T_{CM}-derived cells. Selection of CD19CAR⁺EGFRt⁺ Line C occurred after 2 REM stimulations of transduced CD8⁺ T_{CM}-derived cells. Selection of CD19CAR⁺EGFRt⁺ Line D occurred after 1 REM stimulation of transduced T_{EM}-derived cells. Selection of CD19CAR⁺EGFRt⁺IMPDH2dm⁺ Line E occurred after 1 REM stimulation of transduced T_{CM}-derived cells.

[0015] Figure 3 shows that the EGFRt expressed on selected T cells is inert. In Figure 3a, EGFRt expressed on T cells is not phosphorylated upon co-incubation with EGF. Negative control T cells, CD19CAR⁺EGFRt⁺ Line A cells, or A431 cells were incubated for 5 minutes with or without either 100ng/mL EGF or cetuximab (referred to in the figure as Erbtx) and then lysed in the presence of phosphatase inhibitor. Lysates run on Western blots were then probed using antibodies specific for either β -actin, the cytoplasmic domain of EGFR, or the phosphorylated tyrosine at position 1068 of EGFR. Figure 3b shows that EGF does not bind to the surface of EGFRt expressing T cells. A431, Line A, and negative control T cells were stained

with PE-conjugated anti-EGFR, or either biotinylated cetuximab or biotinylated EGF followed by PE-conjugated streptavidin (black histogram) versus PE-conjugated isotype control Ab or streptavidin alone (open histogram) by flow cytometry. Percent positive staining is indicated in each histogram.

[0016] Figure 4 illustrates that selected EGFR^t CD19R⁺ T cells can be expanded with maintenance of effector phenotype. Figure 4a is a line graph showing expansion of EGFR^t-selected T cells, Lines A-E, over 12 or more days after rapid expansion medium (REM) stimulation was initiated on the day of AutoMACS™ selection (day 0). (MACS is magnetic activated cell sorting.) Expansion of T cells in rapid expansion medium (REM) involved the incubation of 10⁶ T cells with 30 ng/mL anti-CD3 ϵ (OKT3; Ortho Biotech, Raritan, NJ), 5 x 10⁷ γ -irradiated PBMCs (3500 cGy), and 10⁷ γ -irradiated LCLs (8000 cGy) in 50 mL CM; with addition of 50U/mL rhIL-2 and 10ng/ml rhIL-15 (CellGenix) every 48 hours, beginning on day 1. T cells were re-stimulated in this manner every 14 days. Figure 4b shows histograms representing EGFR^t-selected T cells (11 to 13 days after stimulation) that were phenotyped for surface EGFR (i.e., EGFR^t, with biotinylated cetuximab), Fc (i.e., CAR), and T cell markers CD4 or CD8, (black histogram) vs. isotype control Ab (open histogram) by flow cytometry. Percent positive staining is indicated in each histogram. "N.D." indicates no data. Figure 4C are five lines graphs, one for each of Lines A-E, of EGFR^t-selected T cells (within 11 to 15 days after REM stimulation) incubated for 4 hours with ⁵¹Cr-labeled NS0, U251T, CD19t-expressing NS0, CMV pp65-expressing U251T, CD19-expressing Daudi or SupB15, or OKT3-expressing LCL cells as targets at the indicated E:T ratios. Chromium release was measured to determine cytotoxic activity. Figure 4d is a graph showing MPA resistance of the CD19CAR⁺EGFR^tIMPDH2dm⁺ Line E. Control T cells that do not express

IMPDH2dm and EGFRt-selected IMPDH2dm-expressing Line E cells were cultured either with or without 1 μ M MPA and total cell numbers were monitored.

[0017] Figure 5 shows EGFRt expression can be used as a tracking marker for in vivo T cell engraftment. Day 36 bone marrow harvested from a control mouse or from a mouse that had received 10⁷ CD19CAR⁺EGFRt⁺ Line C at day 0 was stained using PerCP-conjugated anti-human CD45 and biotinylated cetuximab ("Bio-Erb") followed by PE-conjugated streptavidin. Quadrants were created based on isotype control staining, and percent positive staining in each quadrant is indicated in each histogram.

[0018] Figure 6 is a graph showing EGFRt expression targets T cells for cetuximab (referred to in the figure as Erbitux®) mediated ADCC. ⁵¹Cr-labeled Line A cells were pre-incubated either with or without up to 20 μ g/mL of cetuximab or the CD20-specific mAb Rituxan as a negative control prior to addition of human PBMC as effectors.

[0019] Figure 7 is the nucleotide (sense strand is SEQ ID NO: 1, antisense strand is SEQ ID NO: 2) and amino acid (SEQ ID NO: 3) sequences of GMCSFR alpha chain signal sequence linked to EGFRt. The GMCSFR alpha chain signal sequence, which directs surface expression, is encoded by nucleotides 1-66. EGFRt is encoded by nucleotides 67-1071.

[0020] Figure 8 is the nucleotide (sense strand is SEQ ID NO: 4, antisense strand is SEQ ID NO: 5) and amino acid (SEQ ID NO: 6) sequences of CD19R-CD28gg-Zeta(CO)-T2A-EGFRt. CD19R-CD28gg-Zeta(CO) is encoded by nucleotides 1-2040; T2A is encoded by nucleotides 2041-2112; GMCSFR is encoded by nucleotides 2113-2178; EGFRt is encoded by nucleotides 2179-3186.

[0021] Figure 9 is a graph showing CD19R-CD28gg-Zeta(CO)-T2A-EGFRt expression. Transduction of anti-CD3/anti-CD28 bead stimulated primary T cell blasts with the CD19R-CD28gg-Zeta(CO)-T2A-EGFRt_epHIV7 lentiviral vector (MOI = 3) results in surface detection of both the CAR (using a biotinylated anti-Fc Ab and streptavidin-PE) and the truncated EGFR molecule (using a biotinylated cetuximab Ab and streptavidin-PE) by flow cytometry on day 4. The white peak in each panel is non-transduced control T cell blasts.

[0022] Figure 10 is a schema showing a possible process flow for clinical trials for testing products of the present disclosure.

DETAILED DESCRIPTION

[0023] Certain embodiments of the invention are described in detail, using specific examples, sequences, and drawings. The enumerated embodiments are not intended to limit the invention to those embodiments, as the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention.

[0024] Erbitux® is a registered trademark for the anti-EGFR monoclonal antibody cetuximab and is intended to independently include the trade name product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product.

[0025] The term “genetic modification” means any process that adds, deletes, alters, or disrupts an endogenous nucleotide sequence and includes, but is not limited to viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction, e.g., viral mediated gene transfer such as the use of

vectors based on DNA viruses such as lentivirus, adenovirus, retroviruses, adeno-associated virus and herpes virus.

[0026] The term "antibody" includes monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies and antibody fragments that may be human, mouse, humanized, chimeric, or derived from another species. A "monoclonal antibody" is an antibody obtained from a population of substantially homogeneous antibodies that is being directed against a specific antigenic site.

[0027] "Variant" refers to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants will possess at least about 80% sequence identity, more preferably, at least about 90% homologous by sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the reference amino acid sequence.

[0028] "Percentage identity" or "percent identity" is defined as the percentage of residues in the amino acid sequence variant that are identical after best aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Methods and computer programs for the alignment are well known in the art. Such programs include GAP, BESTFIT, FASTA, BLAST or Align 2.

[0029] "Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors, such as natural killer cells, neutrophils, and macrophages, recognize bound antibody on a target cell and cause lysis of the target cell. ADCC activity may be assessed using methods, such as those described in U.S. Pat. No. 5,821,337.

[0030] "Effector cells" are leukocytes which express one or more constant region receptors and perform effector functions.

[0031] To "treat" a disease or a disorder, such as cancer, means to take either therapeutic measures or preventative measures to lessen or abate the disease or disorder. Such treatment includes prevention, alleviation of symptoms, diminishment or stabilization of scope, and/or remission.

[0032] The term "therapeutically effective amount" refers to an amount of a compound or molecule effective to treat a disease or disorder.

[0033] "Cancer" refers to cells undergoing uncontrolled cellular growth. Examples of cancer include colorectal cancer and head and neck cancer. A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer.

[0034] A "cytokine" is a protein released by one cell to act on another cell as an intercellular mediator.

[0035] "Non-immunogenic" refers to a material that does not initiate, provoke or enhance an immune response where the immune response includes the adaptive and/or innate immune responses.

[0036] The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons). Some genes may be developed which lack, in whole or in part, introns. Some leader sequences may enhance translation of the nucleic acid into polypeptides.

[0037] The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides

could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0038] As used herein, a "vector" may be any agent capable of delivering or maintaining nucleic acid in a host cell, and includes viral vectors (e.g. retroviral vectors, lentiviral vectors, adenoviral vectors, or adeno-associated viral vectors), plasmids, naked nucleic acids, nucleic acids complexed with polypeptide or other molecules and nucleic acids immobilized onto solid phase particles. The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art. Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0039] "Receptor" means a polypeptide that is capable of specific binding to a molecule. Whereas many receptors may typically operate on the surface of a cell, some receptors may bind ligands when located inside the cell (and prior to transport to the surface) or may reside predominantly intra-cellularly and bind ligand therein.

[0040] "Antibody or functional fragment thereof" means an immunoglobulin molecule that specifically binds to, or is immunologically reactive with a particular antigen or epitope, and includes both polyclonal and monoclonal antibodies. The

term antibody includes genetically engineered or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, and heteroconjugate antibodies (e.g., bispecific antibodies, diabodies, triabodies, and tetrabodies). The term functional antibody fragment includes antigen binding fragments of antibodies, including e.g., Fab', F(ab')₂, Fab, Fv, rIgG, and scFv fragments. The term scFv refers to a single chain Fv antibody in which the variable domains of the heavy chain and of the light chain of a traditional two chain antibody have been joined to form one chain.

[0041] In one embodiment, a gene encoding a modified endogenous cell-surface molecule that may be used as a non-immunogenic selection epitope compatible with immunomagnetic selection is provided. Such a non-immunogenic selection epitope may facilitate immunotherapy in cancer patients without undesirable immunologic rejection of cell products. The endogenous cell surface molecule may be modified or truncated to retain an extracellular epitope recognized by a known antibody or functional fragment thereof, and to remove any signaling or trafficking domains and/or any extracellular domains unrecognized by said known antibody. A modified endogenous cell surface molecule which lacks a signaling or trafficking domain and/or any extracellular domains unrecognized by said known antibody is rendered inert.

[0042] The modified endogenous cell-surface molecule may be, but is not limited to, any non-immunogenic cell-surface related receptor, glycoprotein, cell adhesion molecule, antigen, integrin or cluster of differentiation (CD) that is modified as described herein. Modification of such cell-surface molecules is accomplished by keeping an epitope that is recognized by a known antibody or functional fragment thereof; and removing any signaling or trafficking domains and/or any extracellular

domains unrecognized by a known antibody. Removal of the signaling or trafficking domains and/or any extracellular domains unrecognized by a known antibody renders the endogenous cell-surface molecule non-immunogenic and/or inert.

[0043] Examples of endogenous cell-surface molecules that may be modified or truncated according to the embodiments described herein include, but are not limited to EpCAM, VEGFR, integrins (e.g., integrins $\alpha v\beta 3$, $\alpha 4$, $\alpha IIb\beta 3$, $\alpha 4\beta 7$, $\alpha 5\beta 1$, $\alpha v\beta 3$, αv), TNF receptor superfamily (e.g., TRAIL-R1, TRAIL-R2), PDGF Receptor, interferon receptor, folate receptor, GPNMB, ICAM-1, HLA-DR, CEA, CA-125, MUC1, TAG-72, IL-6 receptor, 5T4, GD2, GD3, or clusters of differentiation (e.g., CD2, CD3, CD4, CD5, CD11, CD11a/LFA-1, CD15, CD18/ITGB2, CD19, CD20, CD22, CD23/IgE Receptor, CD25, CD28, CD30, CD33, CD38, CD40, CD41, CD44, CD51, CD52, CD62L, CD74, CD80, CD125, CD147/basigin, CD152/CTLA-4, CD154/CD40L, CD195/CCR5, CD319/SLAMF7).

[0044] Corresponding commercial antibodies that may be used to recognize a modified or truncated endogenous cell-surface molecule include, but are not limited to, 3F8, abagovomab, abciximab, adecatumumab, afutuzumab, alemtuzumab, altumomab pentetate, anatumomab mafenatox, apolizumab, arcitumomab, aselizumab, atlizumab (= tocilizumab), basiliximab, bectumomab, benralizumab, besilesomab, bivatumumab mertansine, blinatumomab, brentuximab vedotin, cantuzumab mertansine, capromab pendetide, catumaxomab, CC49, cedelizumab, celmoleukin, citatuzumab bogatox, clenoliximab, clivatuzumab tetraxetan, CNTO-95, conatumumab, dacetuzumab, daclizumab, daratumumab, detumomab, ecomeximab, edrecolomab, efalizumab, elotuzumab, enlimomab pegol, epitumomab cituxetan, epratuzumab, erlizumab, etaracizumab, fanolesomab, faralimomab, farletuzumab, galiximab, gavilimomab, gemtuzumab ozogamicin,

glembatumumab vedotin, gomiliximab, ibalizumab, ibritumomab tiuxetan, igovomab, intetumumab, iratumumab, inolimomab, inotuzumab ozogamicin, ipilimumab, keliximab, labetuzumab, lintuzumab, lexatumumab, lucatumumab, lumiliximab, mapatumumab, maslimomab, milatuzumab, minretumomab, mitumomab, muromonab-CD3, naptumomab estafenatox, natalizumab, ocrelizumab, odulimomab, ofatumumab, olaratumab, oportuzumab monatox, oregovomab, otelixizumab, pentumomab, priliximab, PRO 140, rituximab, rovelizumab, ruplizumab, satumomab pendetide, sipilizumab, sontuzumab, tadocizumab, taplitumomab paptox, teneliximab, teplizumab, TGN1412, ticilimumab (= tremelimumab), tigatuzumab, tocilizumab (= atlizumab), toralizumab, tositumomab, tremelimumab, tucotuzumab, vedolizumab, veltuzumab, visilizumab, vitaxin, volociximab, votumumab, zanolimumab, ziralimumab, zolimomab aritox.

[0045] In some embodiments, the modified endogenous cell-surface molecule is encoded by a modified or truncated tyrosine kinase receptor gene. Examples of tyrosine kinase receptors that may be modified or truncated according to the embodiments described herein include, but are not limited to, members of the endothelial growth factor receptor family (EGFR/ErbB1/HER1; ErbB2/HER2/neu; ErbB3/HER3; ErbB4/HER4), hepatocyte growth factor receptor (HGFR/c-MET) and insulin-like growth factor receptor-1 (IGF-1R). According to some embodiments, modified tyrosine kinase receptors retain an extracellular epitope recognized by a known antibody or functional fragment thereof, and lack at least a tyrosine kinase domain. A modified tyrosine kinase receptor which lacks at least a tyrosine kinase domain renders the receptor inert.

[0046] Commercial antibodies that may be used to recognize a modified tyrosine kinase receptor include, but are not limited to AMG-102, AMG-479,

BIIB022OA-5D5, CP-751,871, IMC-A12, R1507, cetuximab, cixutumumab, ertumaxomab, figitumumab, matuzumab, necitumumab, panitumumab, pertuzumab, nimotuzumab, robatumumab, trastuzumab, zalutumumab.

[0047] In one embodiment, the modified endogenous cell surface molecule is a truncated EGFR (tEGFR) that lacks the membrane distal EGF-binding domain and the cytoplasmic signaling tail, but retains the extracellular membrane proximal epitope recognized by a known antibody or functional fragment thereof (e.g., cetuximab, matuzumab, necitumumab or panitumumab). In another embodiment, the tEGFR is missing Domain I, Domain II, the Juxtamembrane Domain and the Tyrosine Kinase Domain as compared to an unmodified EGFR (Figure 1).

[0048] A gene encoding a modified endogenous cell surface molecule may be used as a cell selection or enrichment marker for a genetically modified population of immune cells (e.g., T cells). The gene encoding a modified endogenous cell surface molecule may be coupled to a gene encoding a tumor targeting chimeric antigen receptor (CAR). These genes may be inserted into a vector to transduce the population of T cells to be genetically modified. After transduction, the cells that are successfully transduced and express the CAR and modified endogenous cell-surface molecule are enriched by any suitable purification method, such as immunomagnetic purification with anti-biotin microbeads or fluorochrome-conjugated anti-biotin for fluorescence activated cell sorting, using a commercial antibody that recognizes the modified endogenous cell-surface molecule expressed by the transduced cell.

[0049] In another embodiment, a gene encoding a truncated human epidermal growth factor receptor (EGFR_t) that lacks the membrane distal EGF-binding domain and the cytoplasmic signaling tail, but retains the extracellular membrane proximal epitope recognized by the FDA-approved anti-EGFR monoclonal antibody (mAb)

cetuximab or another anti-EGFR antibody, is constructed and described herein. The EGFRt may be coupled with chimeric antigen receptors specific for a tumor associated antigen. The tumor associated antigen may be CD19, CD20, or CD22, or any other tumor associated antigen, but is preferably CD19 (CD19CAR). The tumor associated antigen is followed by a C-terminal 2A cleavable linker and the coding sequence for EGFRt. The biotinylated-cetuximab may be used in conjunction with commercially available anti-biotin microbeads for the purpose of immunomagnetic purification of the tumor associated antigen/CAR-expressing transductants. In the instance where the tumor associated antigen is CD19 the product is CD19CAR-expressing transductants. Alternatively, the biotinylated-cetuximab may be used in conjunction with Fluorochrome-conjugated anti-biotin for fluorescence activated cell sorting.

[0050] In another embodiment, a modified endogenous cell-surface molecule may be used as a marker for *in vivo* T cell engraftment. For example, when the modified endogenous cell-surface molecule is EGFRt, the EGFRt may be used to track the uptake of the T cells to which it is attached *in vivo* without affecting cellular function of the T cells or the cells to which the T cells are targeted, such as bone marrow cells in a transplant situation. The use of cetuximab conjugated to probes or reporter genes such as sr39TK may be used to improve the tracking potential of EGFRt-expressing cells to patients via PET imaging techniques.

[0051] In a separate embodiment, a modified endogenous cell-surface molecule may be used to induce cell suicide. For example, EGFRt may be used as a suicide gene via cetuximab mediated complement and/or antibody dependent cell mediated cytotoxicity (ADCC) pathways. The fact that cetuximab is a therapeutic

FDA-approved antibody further facilitates the suicide gene potential of EGFRt in the clinical setting.

[0052] In other embodiments, the truncated epidermal growth factor receptor (EGFRt) selection epitope or other modified cell-surface molecule is attached to other sequences. One exemplar sequence is the GMCSFR alpha chain signal sequence, which directs surface expression, attached to EGFRt. GMCSFR is encoded by nucleotides 1-66 and EGFRt is encoded by nucleotides 67-1071 of SEQ ID NO: 1. See Figure 7. Also in Figure 7 is the antisense strand (SEQ ID NO: 2) and amino acid (SEQ ID NO: 3) sequences of GMCSFR alpha chain signal sequence linked to EGFRt. Another such sequence is a codon-optimized cDNA sequence encoding an anti-CD19 costimulatory chimeric antigen receptor (CD19R-CD28gg-Zeta(CO)), and a cleavable T2A linker. Cytotoxic T lymphocytes (CTLs) modified to express a CD19-specific chimeric antigen receptor (CAR) that signals via a cytoplasmic costimulatory (CD28) domain fused to the cytoplasmic CD3- ζ domain exhibits superior anti-tumor potency that can be attributed to CD28-mediated survival and enhanced cytokine production. This construct may be further modified to incorporate a C-terminal 2A cleavable linker followed by the coding sequence for a truncated human EGFR (EGFRt) for the purpose of immunomagnetic purification of CAR-expressing transductants using cetuximab-biotin/anti-biotin microbeads. See the CD19R-CD28gg-Zeta(CO)-T2A-EGFRt sequence attached as Figure 8, SEQ ID NOS: 4 (nucleotide sense strand), 5 (nucleotide anti-sense strand), and 6 (protein). Lentivector transduction of primary human T cells with this codon-optimized cDNA directs the coordinated expression of the CAR and EGFRt (Fig. 9).

[0053] To eliminate variability between transgene expression products otherwise intrinsic to transduction procedures without subsequent selection, a non-

immunogenic selection epitope, EGFRt, compatible with immunomagnetic selection using the CliniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany) was developed. For example, EGFRt is a truncated human epidermal growth factor receptor that lacks the membrane distal EGF-binding domain and the ectoplasmic signaling tail, but retains the extracellular membrane proximal epitope recognized by the commercial anti-EGFR mAb cetuximab. See Figure 1. Biotinylated-cetuximab is applied to immunomagnetic selection in combination with anti-biotin microbeads (Miltenyi). Human OKT3 blasts that had been lentivirally transduced with CD19R-CD28gg-Zeta(CO)-T2A-EGFRt were subjected to immunomagnetic selection using the Miltenyi AutoMACS device, and the frequency of EGFRt+CAR+ T cells was enriched from 22% (pre-selection) to 99% (post-selection) without observable toxicity to the cell preparation (Fig. 3). It is also possible that, instead of or in addition to immunomagnetic sorting, the EGFRt can be purified using fluorescence-based cell sorting techniques.

[0054] Due to the absence of the EGF-binding domains and intracellular signaling domains, EGFRt is inactive when expressed by T cells. Importantly, the EGFRt-selected T cells maintain their desired effector phenotype – including anti-tumor cytotoxic activity mediated by the chimeric antigen receptor that is coordinately expressed with the EGFRt - and remain amenable to established expansion protocols.

[0055] Overall, this EGFRt has various advantages for immunotherapeutic cell products compared to other selection markers that have been previously reported. Specifically, unlike truncated CD4 and CD19, it is not endogenously expressed by subpopulations of lymphocytes. Furthermore, in contrast to truncated CD34 and low affinity nerve growth factor receptor, it does not have any activity that might

negatively affect the immune cell product (i.e., in terms of signaling or trafficking). Lastly, it alone can be bound/recognized by a known, preferably commercially available, pharmaceutical grade antibody reagent, i.e., cetuximab. Together, these attributes make EGFRt a superior selection marker for any transfection/transduction system that can be applied to the generation of cell products for adoptive immunotherapy. Thus, EGFRt is well suited to be used as a selection marker for lentivirally transduced T cells of immunotherapeutic relevance.

[0056] Also provided are methods for identifying new therapeutic cell products having the following criteria: a modified endogenous cell-surface molecule, ligand or receptor that is not, as modified, endogenously expressed in the subject in which it is intended to be therapeutically utilized, does not have any immunoactivity or other functional activity that would hinder the functioning of the product or the subject into which the product is administered, and that it can be recognized by a known antibody.

[0057] Having described the invention with reference to the embodiments and illustrative examples, those in the art may appreciate modifications to the invention as described and illustrated that do not depart from the spirit and scope of the invention as disclosed in the specification. The examples are set forth to aid in understanding the invention but are not intended to, and should not be construed to limit its scope in any way. The examples do not include detailed descriptions of conventional methods. Such methods are well known to those of ordinary skill in the art and are described in numerous publications.

**Example 1: Generation of EGFRt and Immunomagnetic selection of EGFRt
expressing T cells**

Materials & Methods

Antibodies and Flow Cytometry

[0058] FITC-, PE- and PerCP-conjugated isotype controls, PerCP-conjugated anti-CD8, FITC conjugated anti-CD4, PE-conjugated anti-IFN γ , PerCP-conjugated anti-CD45 and PE-conjugated streptavidin were obtained from BD Biosciences (San Jose, CA). Biotinylated anti-Fc was purchased from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, PA). PE-conjugated anti-Biotin was purchased from Miltenyi Biotec (Auburn, CA). Biotinylated EGF was purchased from Molecular Probes® Invitrogen (Carlsbad, CA). PE-conjugated anti-EGFR was purchased from Abcam Inc. (Cambridge, MA). All antibodies and biotin-EGF were used according to the manufacturer's instructions. Flow cytometric data acquisition was performed on a FACScalibur (BD Biosciences), and the percentage of cells in a region of analysis was calculated using FCS Express V3 (De Novo Software, Los Angeles, CA).

[0059] For generation of the biotinylated-cetuximab, 200mg of cetuximab (Erbix®) was buffer exchanged (19 hours) to PBS (D-PBS, pH 7.5 \pm 0.1) using a MidGee Hoop Cartridge (UFP-30-E-H42LA) with 527mL. The material at 2mg/mL was then modified at a 20:1 ratio using Sulfo-NHS-LC-Biotin in a reaction that was carried out for 1 hour at room temperature and then diafiltered to remove the excess biotin. The 200 mg of biotinylated cetuximab was then buffer exchanged (18 hours) to PBS (D-PBS, pH 7.5 \pm 0.1) using MidGee Hoop Cartridge (UFP-30-E-H42LA) with 533 mL. Glycerol was added to a final concentration of 20% and then the material was frozen in vials.

Cell lines

[0060] Unless otherwise indicated, all cell lines were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM L-glutamine (Irvine Scientific), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, Irvine Scientific), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Irvine Scientific), and 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT), hereafter referred to as culture media (CM).

[0061] To generate T cells, human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) from heparinized peripheral blood obtained from consented healthy donors participating on a City of Hope National Medical Center Internal Review Board-approved protocol. For generation of Line A, washed PBMC were stimulated with 25U/mL IL-2 and a 1:1 (cell:bead) ratio of Dynabeads® Human T expander CD3/CD28 (Invitrogen, Carlsbad, CA). For generation of the other lines, washed PBMC were first autoMACS™ depleted using anti-CD45RA beads (Miltenyi Biotec) per the manufacturer's protocol, and in some cases also depleted with PE-conjugated anti-CD4 (BD Biosciences) with anti-PE beads (Miltenyi Biotec). The resulting cells then underwent autoMACS™ positive selection using biotinylated DREG56 (anti-CD62L) and anti-biotin beads (Miltenyi Biotec) to produce purified CD62L⁺CD45RO⁺ T_{CM}. CD8⁺ cells were further selected in some cases using AutoMACS™ (Miltenyi Biotec) per the manufacturer's protocol. CMV-specific cells were generated by stimulating T cells with 5U/ml rhIL-2 (Chiron, Emeryville, CA) and autologous irradiated viral antigen presenting cells at a 4:1 (responder:stimulator) ratio once a week for three weeks, using 10% human serum instead of FCS to avoid non-specific stimulation. The viral antigen presenting cells

were derived from PBMC that had been genetically modified to express CMVpp65 antigen.

[0062] PBMC were resuspended in nucleofection solution using the Human T cell Nucleofector kit (Amaxa Inc., Gaithersburg, MD), and 5×10^7 cells were aliquoted into 0.2-cm cuvettes containing 10 μ g HygroR-pp65_pEK (or pmaxGFP from Amaxa Inc., as a transfection control) in a final volume of 100 μ L/cuvette, and electroporated using the Amaxa Nucleofector I (Amaxa Inc.), program U-14, after which cells were allowed to recover for 6 hours at 37°C prior to γ -irradiation (1200 cGy).

[0063] The CD19CAR-T2A-EGFRt_epHIV7 (pJ02104) and CD19CAR-T2A-EGFRt-T2A-IMPDH2dm_epHIV7 (pJ02111) lentiviral constructs contain a) the chimeric antigen receptor (CAR) sequences consisting of the V_H and V_L gene segments of the CD19-specific FmC63 mAb, an IgG1 hinge-C_{H2}-C_{H3}, the transmembrane and cytoplasmic signaling domains of the costimulatory molecule CD28, and the cytoplasmic domain of the CD3 ζ chain[10]; b) the self-cleaving T2A sequence[11]; c) the truncated EGFR sequence (See Fig. 1); and d) the IMPDH2 double mutant that confers MPA-resistance, as indicated. Lentiviral transduction was carried out on T cells that were stimulated with either 30 ng/mL anti-CD3 ϵ (OKT3; Ortho Biotech, Raritan, NJ) (i.e., for Line A) or human CD3/CD28Dyna beads at a 1:10 ratio (i.e., for Lines B, C, D and E) and 25U IL2/ml. Cells were cultured for up to 2 hours at 37°C on RetroNectin® (50ug/ml) coated plates prior to addition of the lentivirus at an MOI of 3 and 5 μ g/ml polybrene. After 4 hours, warm medium was added to triple to volume, and the cells were then washed and plated in fresh media after 48hours. AutoMACS™ sorting of EGFRt-expressing cells was carried out with biotinylated cetuximab and anti-biotin microbeads (Miltenyi Biotec)

as per the manufacturer's instructions. Expansion of T cells in rapid expansion medium (REM) involved the incubation of 10^6 T cells with 30 ng/mL anti-CD3 ϵ (OKT3; Ortho Biotech, Raritan, NJ), 5×10^7 γ -irradiated PBMCs (3500 cGy), and 10^7 γ -irradiated LCLs (8000 cGy) in 50 mL CM; with addition of 50U/mL rhIL-2 and 10ng/ml rhIL-15 (CellGenix) every 48 hours, beginning on day 1. T cells were re-stimulated in this manner every 14 days.

[0064] EBV-transformed lymphoblastoid cell lines (LCLs) were made from PBMC as previously described [13]. LCL-OKT3 cells were generated by resuspending LCL in nucleofection solution using the Amaxa Nucleofector kit T, adding OKT3-2A-Hygromycin_pEK (pJ01609) plasmid at $5\mu\text{g}/10^7$ cells, and electroporating cells using the Amaxa Nucleofector I, program T-20. The resulting LCL - OKT3-2A-Hygro_pEK (cJ03987) were grown in CM containing 0.4mg/ml hygromycin. The mouse myeloma line NS0 (gift from Andrew Raubitschek, City of Hope National Medical Center, Duarte, CA) was resuspended in nucleofection solution using the Nucleofector kit T (Amaxa Inc., Gaithersburg, MD), CD19t-DHFRdm-2A-IL12_pEK (pJ01607) or GFP-IMPDH2dm-2A-IL15_pcDNA3.1(+) (pJ01043) plasmid was added at $5\mu\text{g}/5 \times 10^6$ cells, and cells were electroporated using the Amaxa Nucleofector I, program T-27. The resulting NS0 - CD19t-DHFRdm-2A-IL12_pEK (cJ03935) and NS0 - GFP:IMPDH2-IL15(IL2ss)_pcDNA3.1(+) (cJ02096) were grown in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FCS, 25mM HEPES, and 2 mM L-glutamine in the presence of either 0.05uM methotrexate (MTX) or 6 μM mycophenolic acid (MPA). The tumorigenic strain of U251, termed U251T, was a kind gift of Dr. Waldemar Debinski (Wake Forest, NC). U251T-pp65 were generated by lentiviral transduction of U251T with pp65-2A-eGFP-ffluc_epHIV7 (pJ01928) at an

60412-4841

MOI of 1. The resulting U251T - pp65-2A-eGFP-fluc_epHIV7 were then FACS sorted for the GFP⁺ population (cJ05058). The Daudi lymphoma line was purchased from ATCC and grown in media consisting of RPMI 1640 (Irvine Scientific), 2 mM L-Glutamine (Irvine Scientific), 10% heat-inactivated FCS (Hyclone). SupB15 acute lymphoblastic leukemia cells and A431 epidermoid carcinoma cells were purchased from ATCC.

Protein analysis

[0065] Cells (up to 10^7) were lysed with 80 μ L of 1% Triton-XTM lysis buffer containing phosphatase inhibitor cocktail II (Sigma-Aldrich Corp., St. Louis, MO) (1:20 of inhibitor to buffer by volume). 50 μ g of protein was loaded in each lane, and Western blots were probed with antibodies from the Phospho-EGF receptor antibody sampler kit (Cell Signaling Technology, Inc., Danvers, MA) followed by IRDyeTM 680CW or 800CW conjugated goat anti-rabbit antibodies (LI-COR, Lincoln, NE), as well as the IRDyeTM 800 conjugated anti-beta-Actin antibody (LI-COR) as per the manufacturers' instructions. Blots were imaged on the Odyssey Infrared Imaging System (LI-COR).

Chromium-release assays

[0066] The cytolytic activity of T cells was determined by 4-hour chromium-release assay (CRA), where effector cells were seeded into triplicate wells of V-bottom 96-well micro-plates containing 5×10^3 ^{51}Cr -labeled target cells ($\text{Na}_2^{51}\text{CrO}_4$; (5mCi/mL); Amersham Pharmacia, Piscataway, NJ) at various E:T ratios in 200 μ L of CM and incubated for 4 hours at 5% CO_2 , 37°C. Plates were centrifuged, and 100 μ L of supernatant was removed from each well to assess chromium release using a γ -counter (Packard Cobra II, Downer's Grove, IL). The percent specific lysis was calculated as follows: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Maximum release was

determined by measuring the ^{51}Cr content of wells containing labeled targets lysed with 2% SDS.

[0067] Antibody dependent cell mediated cytotoxicity was determined by chromium release as above using 5×10^3 ^{51}Cr -labeled target cells that had been pre-incubated for 90 min with up to $10 \mu\text{g/mL}$ of either cetuximab or rituximab (a CD20-specific mAb), washed and then co-incubated with 5×10^5 freshly isolated PBMC.

T cell engraftment and cetuximab mediated suicide in vivo

[0068] For T cell engraftment, six- to ten-week old NOD/Scid IL-2R γ C^{null} mice are injected i.v. on day 0 with 10^7 T cells (Line C). 2×10^7 irradiated (8000 rads) NS0 - GFP:IMPDH2-IL15(IL2ss)_pcDNA3.1(+) (cJ02096) cells are administered i.p. 3 times a week starting on day 0 to provide a systemic supply of human IL-15 *in vivo*. Bone marrow was harvested from euthanized animals and analyzed by flow cytometry. Antibody dependent cell mediated cytotoxicity assays are performed to determine the activity of cetuximab against EGFRt⁺ T cells.

Results

Immunomagnetic selection of EGFRt expressing T cells

[0069] A truncated human EGFR (EGFRt), which contains only the transmembrane domain and extracellular domains III and IV of the full length EGFR, was generated as a non-immunogenic selection epitope compatible with immunomagnetic selection. As shown in the Figure 1 molecular model, the EGFRt retains the ability to be bound by cetuximab, but not have any signaling capacity due to the absence of the intracellular domains. Furthermore, it lacks the N-terminal domain required for EGF-binding.

[0070] To immunomagnetically select for EGFRt-expressing cells, biotinylated-cetuximab was generated (Fig. 2a, b) to be used in conjunction with

commercially available anti-biotin microbeads and an AutoMACS™ separator (Miltenyi Biotec) (Fig. 2c). Lentiviral transduction of various T cell lines with EGFRt-containing constructs, where the EGFRt gene was separated from other genes of interest on either one or both ends with the self-cleaving T2A sequence, consistently resulted in surface detection of the EGFRt molecule on less than 40% of the cells (Fig. 2d). Surface detection may also be accomplished with a EGFRt-sr39TK fusion. Immunomagnetic selection allowed for recovery of EGFRt⁺ T cell populations with greater than 90% purity. T cell populations that underwent this transduction and selection procedure included anti-CD3/anti-CD28 bead stimulated T cell blasts (for Line A), central memory (CD45RO⁺CD62L⁺ T_{CM}) derived T cells (for Lines B, C and E), which in some cases were also pre-selected for CMV specificity (via the endogenous TCR; for Line B) or CD8 expression (for Line C), as well as effector memory (CD62L⁻ CD45RO⁺ T_{EM}) derived T cells (for line D). These data show that EGFRt can successfully be used as a selection marker for various sources of T cell transductants, even when the original transduction efficiency was as low as 2%.

Inactivity of EGFRt on selected T cells

[0071] To confirm that the EGFRt is inactive, Western immunoblot analyses for EGFR phosphorylation were carried out on the EGFRt-selected T cells after culture with either EGF or cetuximab. As expected, cetuximab did not induce EGFR phosphorylation above background even in the EGFR⁺ cell line A431 (Fig 3a). Furthermore, in contrast to that seen with the A431 cells, no phosphorylation was seen in lysates of Line A after co-incubation with EGF. Indeed, using biotinylated EGF, flow cytometric analysis confirmed that EGF cannot bind the EGFRt-selected T cells (Fig. 3b), as expected due to the truncation in its N-terminus. These EGFRt⁺ T

cells were also not recognized by another anti-EGFR antibody distinct from cetuximab.

Maintenance of effector phenotype in expanded EGFRt⁺ CD19CAR⁺ T cells

[0072] Directly after AutoMACS™ separation, the selected T cells were expanded 30-fold or greater within 12 days after REM stimulation with OKT3, irradiated PBMC feeders and LCL, IL-2 and IL-15 (Fig. 4a). Flow cytometric analysis of the resulting expanded EGFRt⁺ T cells further confirmed that that they express the CD19CAR and T cell markers such as CD8, TCR, CD3, perforin, granzyme, etc. (Fig. 4b). Furthermore, CD19CAR-directed cytotoxic activity of these EGFRt-selected lines is evident in chromium release assays using CD19-expressing tumor targets (Fig. 4c). A direct comparison of the CD19-specific reactivity of Line E versus its non-selected or 'parental' counterpart shows that there is enhanced CD19CAR-mediated cytotoxicity upon EGFRt-selection. In addition, the CMV-specific T_{CM}-derived CD19CAR⁺EGFRt⁺ Line B cells also show cytotoxic activity through their endogenous T cell receptor against targets expressing CMV-pp65 antigen.

[0073] For the CD19CAR⁺EGFRt⁺IMPDH2dm⁺ Line E, the ability of the inosine monophosphate dehydrogenase 2 double mutant (IMPDH2dm) to confer resistance to the IMPDH2-inhibitor mycophenolic acid (MPA; a common immunosuppressant used to prevent rejection in organ transplantation) was also tested. Upon culture in 1uM MPA, the survival and/or proliferation of Line E cells is not inhibited (Fig. 4d). This is in contrast to the inhibition seen with a control T cell line that lacks expression of the IMPDH2dn gene. These data provide further evidence that EGFRt-mediated

selection results in the corresponding selection of the other genes present in the lentiviral construct used to transduce T cells.

Tracking of EGFRt⁺ T cells in vivo

[0074] To test the potential for detecting in vivo engrafted T cells, bone marrow cells collected from mice that had been engrafted with CD19CAR⁺EGFRt⁺ Line C was analyzed by flow cytometry using biotinylated cetuximab (Fig. 5). Control mice that did not receive T cells revealed that there was some cross-reaction of the cetuximab against murine EGFR. Thus, it was determined that successful detection of engrafted Line C cells required double staining for both human CD45 and EGFRt. Cells may also analyzed using immunohistochemistry to determine potential for screening biopsy material.

Cetuximab mediated cytotoxicity of EGFRt⁺ T cells

[0075] Because cetuximab is known to lyse EGFR-expressing cells via antibody dependent cell mediated cytotoxicity (ADCC), assays were performed to determine the ADCC activity of cetuximab against EGFRt⁺ T cells (Fig. 6). Using ⁵¹Cr-labeled Line A cells as targeted and freshly isolated human PBMC as effectors, cetuximab was found to significantly mediate chromium-release above that seen when using the CD20-specific humanized mAb Rituxan.

Example of therapeutic use of EGFRt⁺ T cells

[0076] Adult subjects with high-risk intermediate grade B-cell lymphomas who are candidates for an autologous myeloablative stem cell transplant procedure may receive post-transplant immunotherapy with adoptively transferred autologous Tcm-derived CD19R⁺ CD8⁺ EGFRt⁺ T cell grafts. A leukapheresis product collected from each patient undergoes selection of Tcm, transduction with clinical grade CD19CAR-

T2A-EGFRt_epHIV7, and then selection and expansion of the EGFRt⁺ cells in a closed system. After the resulting cell products have undergone quality control testing (including sterility and tumor specific cytotoxicity tests), they are cryopreserved. Meanwhile, following leukapheresis, study participants commence with standard salvage chemotherapy, with mobilization for auto HSC collection with cytoreductive chemotherapy and G-CSF. Since the EGFRt-selected, CD19-specific T cells will also target normal CD20⁺ (CD19⁺) B cells, the B cell numbers can first be lowered using Rituximab[™] to reduce the recipient's inflammatory response upon receiving the genetically modified CTL and also increase availability of infused T cells to immediately target lymphoma cells. Furthermore, Rituximab[™] may blunt a humoral immune response against the genetically modified T cells. If Rituximab[™] is not given as part of the Salvage/Priming chemotherapy regimen, research participants may receive a single intravenous infusion of Rituximab[™] (chimeric anti-CD20 antibody) at 375 mg/m² within 4-weeks of the planned auto-HSCT procedure. Rituximab[™] infusion would be carried out per standard practice including premedication with diphenhydramine and acetaminophen and hydrocortisone. On Day +2 or Day +3 after HSCT, the autologous cryopreserved CD19R⁺ CD8⁺ EGFRt⁺ T cell product will be transported, thawed and infused at the patient's bedside. Research participants can be pre-medicated at least 30 minutes prior to T cell infusion with 15mg/kg of acetaminophen P.O. (max. 650mg.) and diphenhydramine 0.5-1 mg/kg I.V. (max dose 50mg). Clinical and laboratory correlative follow-up studies can then be performed at the physician's discretion, and may include quantitative RT-PCR studies for the presence of CD19-expressing lymphoma cells and/or the adoptively transferred T cells; FDG-PET and/or CT scans;

bone marrow examination for disease specific pathologic evaluation; lymph node biopsy; and/or long-term follow up per the guidelines set forth by the FDA's Biologic Response Modifiers Advisory Committee that apply to gene transfer studies. Figure 10 provides a possible schematic for clinical testing of the present products and methods.

[0077] The present invention is not to be limited in scope by the specific embodiments disclosed in the examples which are intended as illustrations of a few aspects of the invention and any embodiments that are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims.

REFERENCES

1. Berger, C, Flowers, ME, Warren, EH, and Riddell, SR (2006). Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood* 107: 2294-302.
2. Tey, SK, Dotti, G, Rooney, CM, Heslop, HE, and Brenner, MK (2007). Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transplant* 13: 913-24.
3. Fehse, B, Richters, A, Putimtseva-Scharf, K, Klump, H, Li, Z, Ostertag, W, et al. (2000). CD34 splice variant: an attractive marker for selection of gene-modified cells. *Mol Ther* 1: 448-56.
4. Gaines, P, and Wojchowski, DM (1999). pIRES-CD4t, a dicistronic expression vector for MACS- or FACS-based selection of transfected cells. *Biotechniques* 26: 683-8.
5. Fehse, B, Uhde, A, Fehse, N, Eckert, HG, Clausen, J, Ruger, R, et al. (1997). Selective immunoaffinity-based enrichment of CD34+ cells transduced with retroviral vectors containing an intracytoplasmatically truncated version of the human low-affinity nerve growth factor receptor (deltaLNGFR) gene. *Hum Gene Ther* 8: 1815-24.
6. Lemoine, FM, Mesel-Lemoine, M, Cherai, M, Gallot, G, Vie, H, Leclercq, V, et al. (2004). Efficient transduction and selection of human T-lymphocytes with bicistronic Thy1/HSV1-TK retroviral vector produced by a human packaging cell line. *J Gene Med* 6: 374-86.

7. Li, S, Schmitz, KR, Jeffrey, PD, Wiltzius, JJ, Kussie, P, and Ferguson, KM (2005). Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 7: 301-11.
8. Dawson, JP, Berger, MB, Lin, CC, Schlessinger, J, Lemmon, MA, and Ferguson, KM (2005). Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. *Mol Cell Biol* 25: 7734-42.
9. Lange, C, Li, Z, Fang, L, Baum, C, and Fehse, B (2007). CD34 modulates the trafficking behavior of hematopoietic cells in vivo. *Stem Cells Dev* 16: 297-304.
10. Kowolik, CM, Topp, MS, Gonzalez, S, Pfeiffer, T, Olivares, S, Gonzalez, N, et al. (2006). CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* 66: 10995-1004.
11. Szymczak, AL, Workman, CJ, Wang, Y, Vignali, KM, Dilioglou, S, Vanin, EF, et al. (2004). Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol* 22: 589-94.
12. Yam, P, Jensen, M, Akkina, R, Anderson, J, Villacres, MC, Wu, J, et al. (2006). Ex vivo selection and expansion of cells based on expression of a mutated inosine monophosphate dehydrogenase 2 after HIV vector transduction: effects on lymphocytes, monocytes, and CD34+ stem cells. *Mol Ther* 14: 236-44.
13. Pelloquin, F, Lamelin, JP, and Lenoir, GM (1986). Human B lymphocytes immortalization by Epstein-Barr virus in the presence of cyclosporin A. *In Vitro Cell Dev Biol* 22: 689-94.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 51915-74 Seq 16-MAY-12 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. A nucleic acid encoding a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.
2. The nucleic acid of claim 1, wherein the nucleic acid comprises nucleotides 67-1071 of SEQ ID NO:1.
3. The nucleic acid of claim 1, wherein the nucleic acid comprises nucleotides 67-1071 of SEQ ID NO:2.
4. The nucleic acid of claim 1, wherein the nucleic acid encodes an amino acid sequence comprising residues 23-357 of SEQ ID NO:3.
5. The nucleic acid of claim 1, wherein the modified EGFR further comprises a GMCSFR alpha chain signal sequence.
6. The nucleic acid of claim 5, wherein the nucleic acid comprises SEQ ID NO:1.
7. The nucleic acid of claim 5, wherein the nucleic acid comprises SEQ ID NO:2.
8. The nucleic acid of claim 1, wherein the nucleic acid encodes an amino acid sequence at least 90% identical over its full length to SEQ ID NO:3.
9. The nucleic acid of claim 1, wherein the nucleic acid encodes an amino acid sequence comprising SEQ ID NO:3.

10. The nucleic acid of claim 1, wherein the nucleic acid is inserted into a vector.
11. The nucleic acid of claim 1, wherein the marker is used to enrich cells, to select cells, to deplete cells expressing the cell surface molecule, or to induce cell suicide in cells expressing the cell surface molecule.
12. The nucleic acid of claim 1, wherein the modified EGFR further comprises a C-terminal 2A cleavable linker and a chimeric antigen receptor specific for a tumor associated antigen selected from CD19, a codon-optimized anti-CD19 costimulatory chimeric antigen receptor (CD19CAR), CD20 or CD22.
13. The nucleic acid of claim 12, wherein the modified EGFR is coupled to a CD19CAR and a C-terminal 2A cleavable linker.
14. The nucleic acid of claim 12, wherein the modified EGFR comprises SEQ ID NO:6.
15. The nucleic acid of claim 1, wherein the marker is used to enrich cells, to select cells, to deplete cells expressing the cell surface molecule, or to induce cell suicide in cells expressing the cell surface molecule.
16. A modified Epidermal Growth Factor Receptor (EGFR) comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the truncated non-immunogenic endogenous cell surface molecule (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.
17. A population of human T-cells expressing a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine

Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

18. A population of human T-cells comprising a nucleic acid encoding a modified Epidermal Growth Factor Receptor (EGFR), said modified EGFR comprising an EGFR Domain III and an EGFR Domain IV; but lacking all of the domains consisting of an EGFR Domain I, an EGFR Domain II, an EGFR Juxtamembrane Domain, and an EGFR Tyrosine Kinase Domain; wherein the modified EGFR (i) does not have endogenous signaling or trafficking function; (ii) binds a therapeutic anti-EGFR antibody; (iii) does not bind an endogenous EGFR ligand; and (iv) acts as a marker.

19. The population of human T-cells of claim 18, wherein the nucleic acid comprises nucleotides 67-1071 of SEQ ID NO:1 or nucleotides 67-1071 of SEQ ID NO:2.

20. The population of human T-cells of claims 17 or 18, wherein the modified EGFR comprises an amino acid sequence comprising residues 23-357 of SEQ ID NO:3.

21. The population of human T-cells of claims 17 or 18, wherein the modified EGFR further comprises a GMCSFR alpha chain signal sequence.

22. The population of human T-cells of claim 18, wherein nucleic acid comprises SEQ ID NO:1 or SEQ ID NO:2.

23. The population of human T-cells of claim 19, wherein the nucleic acid further comprises a GMCSFR alpha chain signal sequence.

24. The population of human T-cells of claim 23, wherein nucleic acid comprises SEQ ID NO:2.

25. The population of human T-cells of claims 17 or 18, wherein the modified EGFR comprises an amino acid sequence at least 90% identical over its full length to SEQ ID NO:3.

26. The population of human T-cells of claims 17 or 18, wherein the modified EGFR comprises an amino acid sequence comprising SEQ ID NO:3.

27. The population of human T-cells of claims 17 or 18, wherein the marker is used to enrich cells, to select cells, to deplete cells expressing the cell surface molecule, or to induce cell suicide in cells expressing the cell surface molecule.

28. The population of human T-cells of claim 17 or 18, wherein the modified EGFR further comprises a C-terminal 2A cleavable linker and a chimeric antigen receptor specific for a tumor associated antigen selected from CD19, a codon-optimized anti-CD19 costimulatory chimeric antigen receptor (CD19CAR), CD20 or CD22.

29. The population of human T-cells of claim 17 or 18, wherein the modified EGFR is coupled to a CD19CAR and a C-terminal 2A cleavable linker.

30. The population of human T-cells of claim 17 or 18, wherein the modified EGFR comprises SEQ ID NO:6.

Figure 1

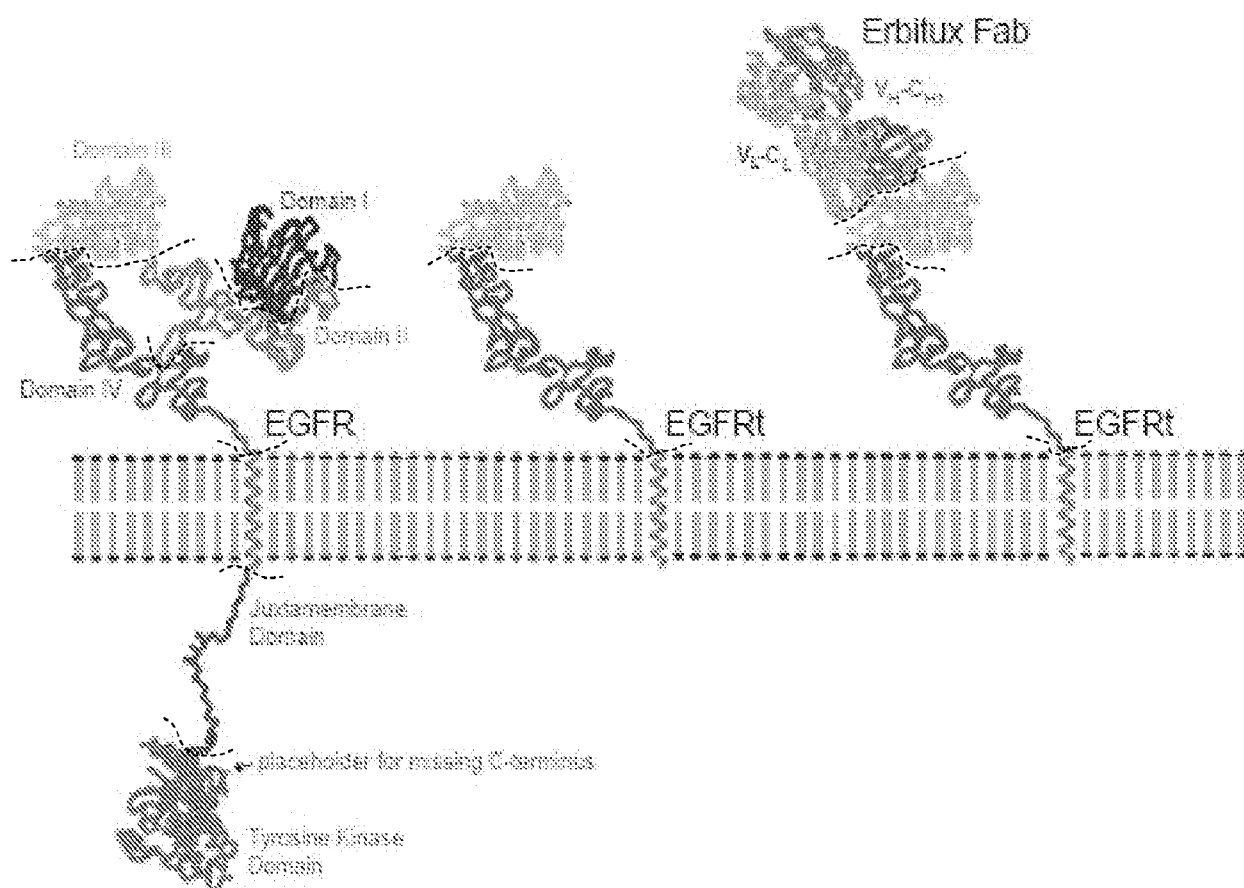


Figure 2A

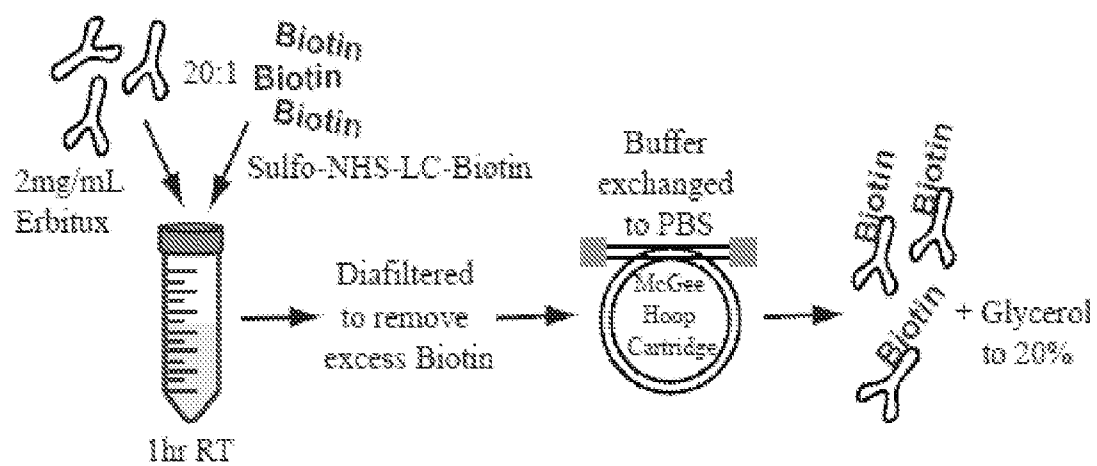


Figure 2B

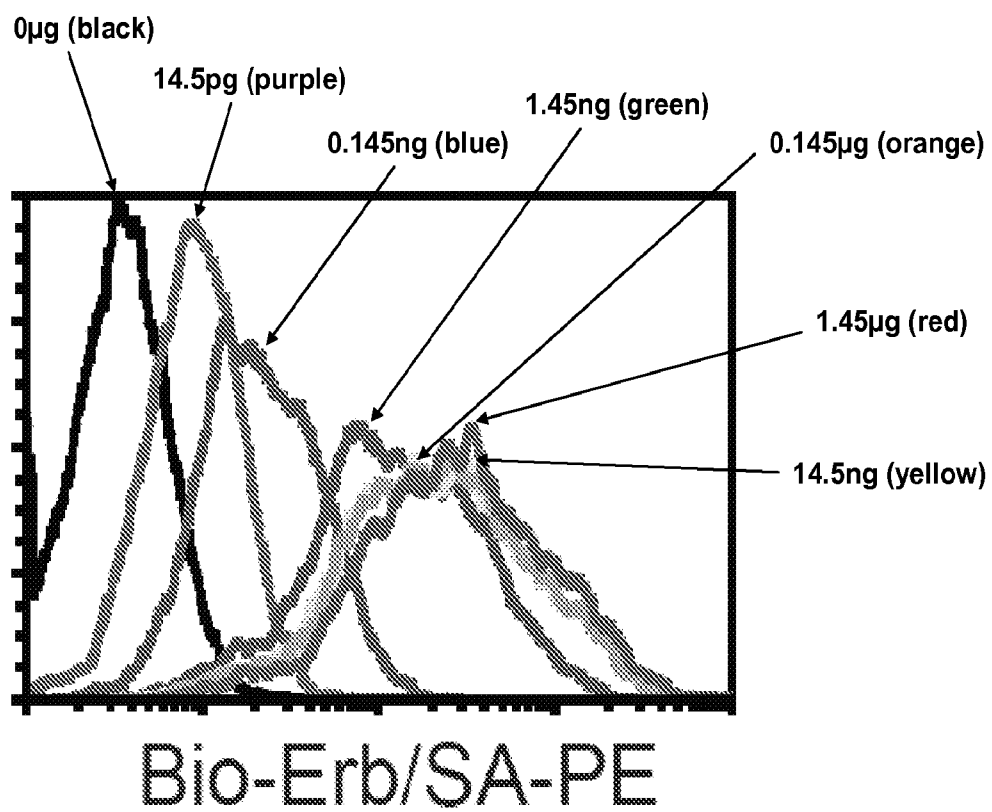


Figure 2C

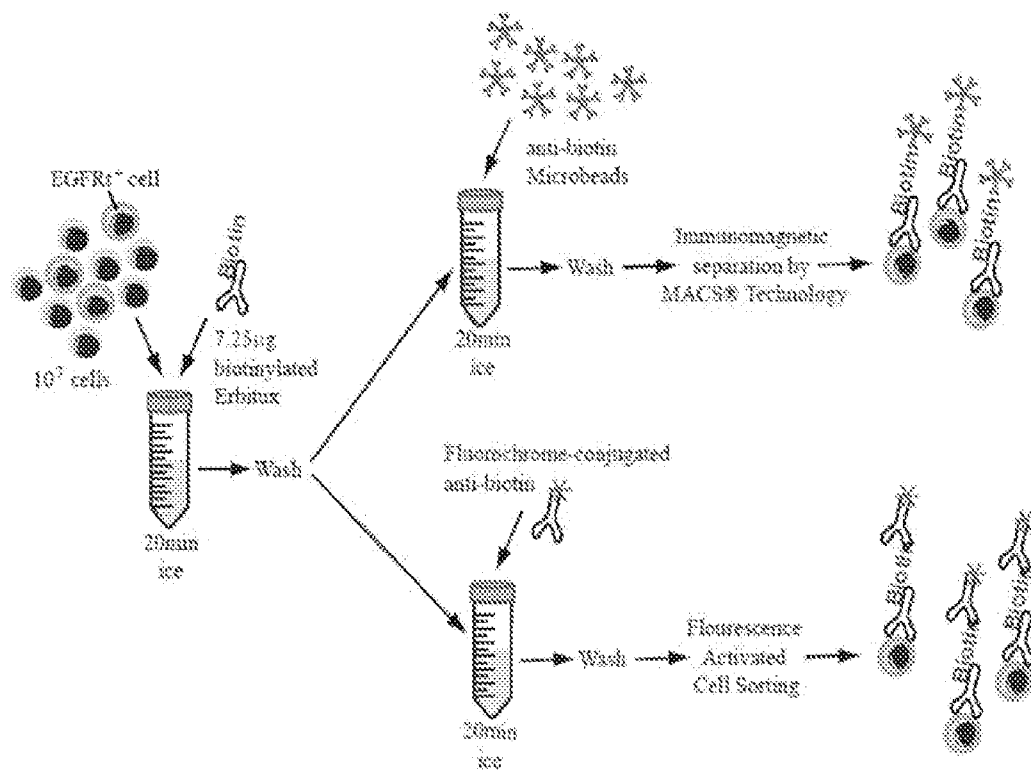


Figure 2D

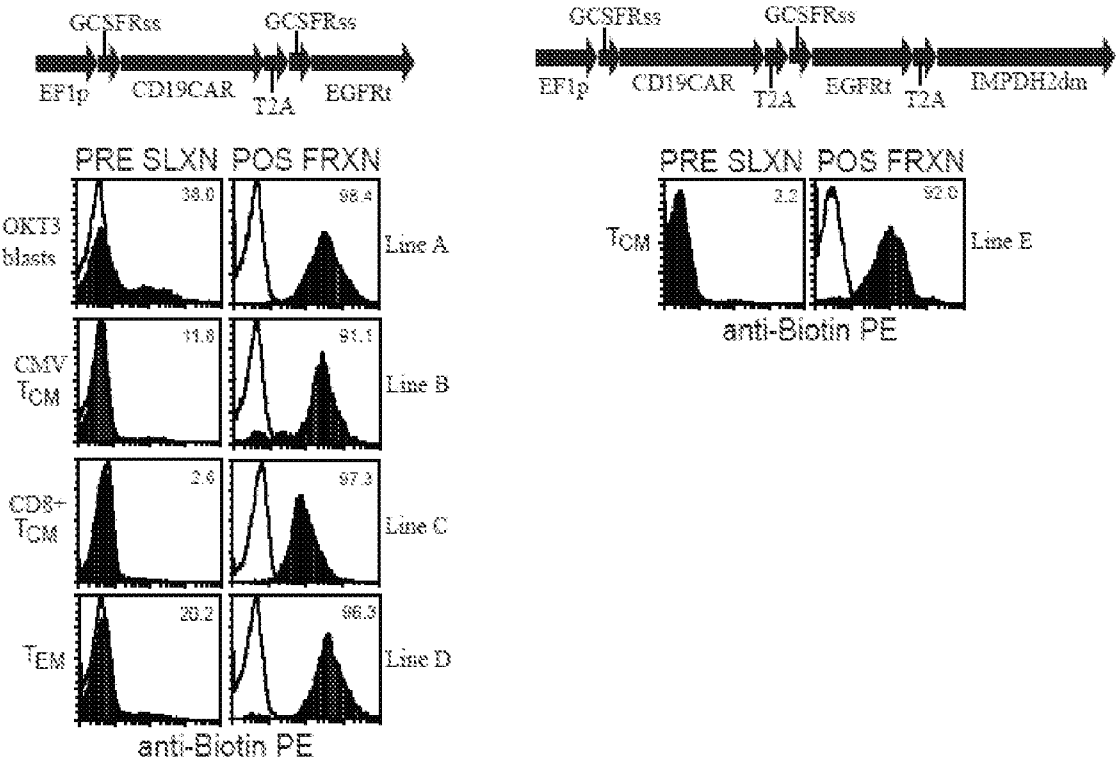


Figure 3A

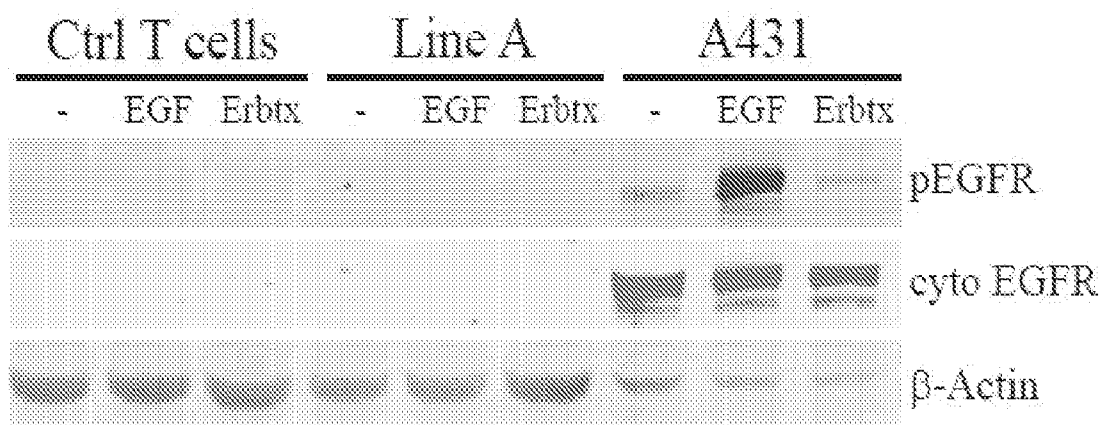


Figure 3B

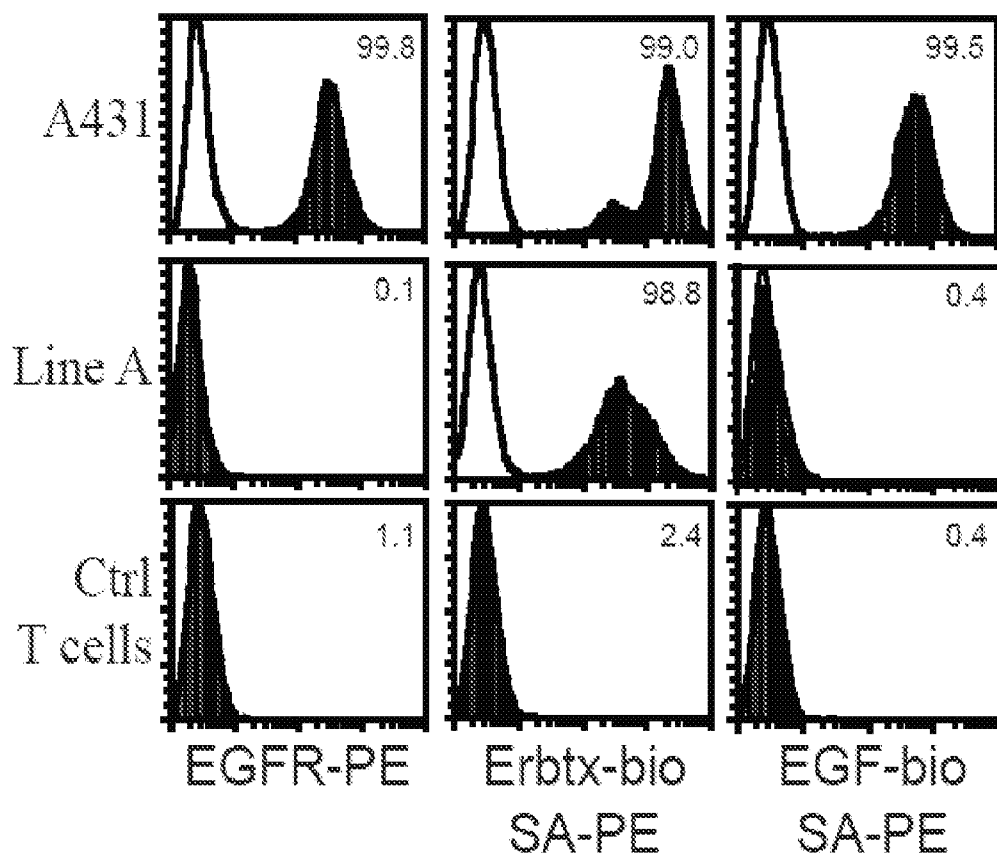


Figure 4A

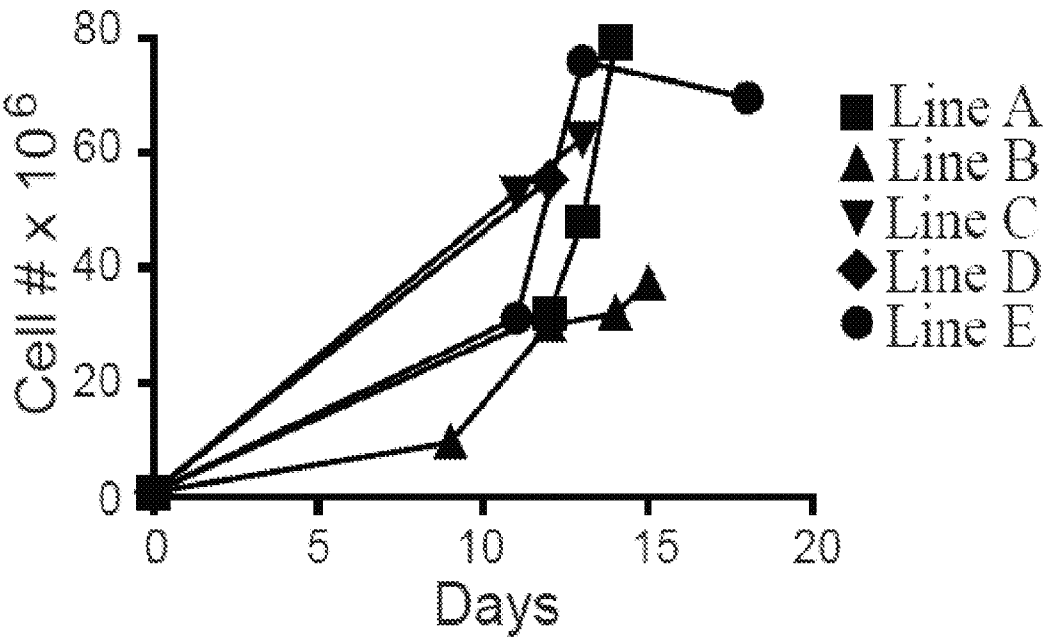


Figure 4B

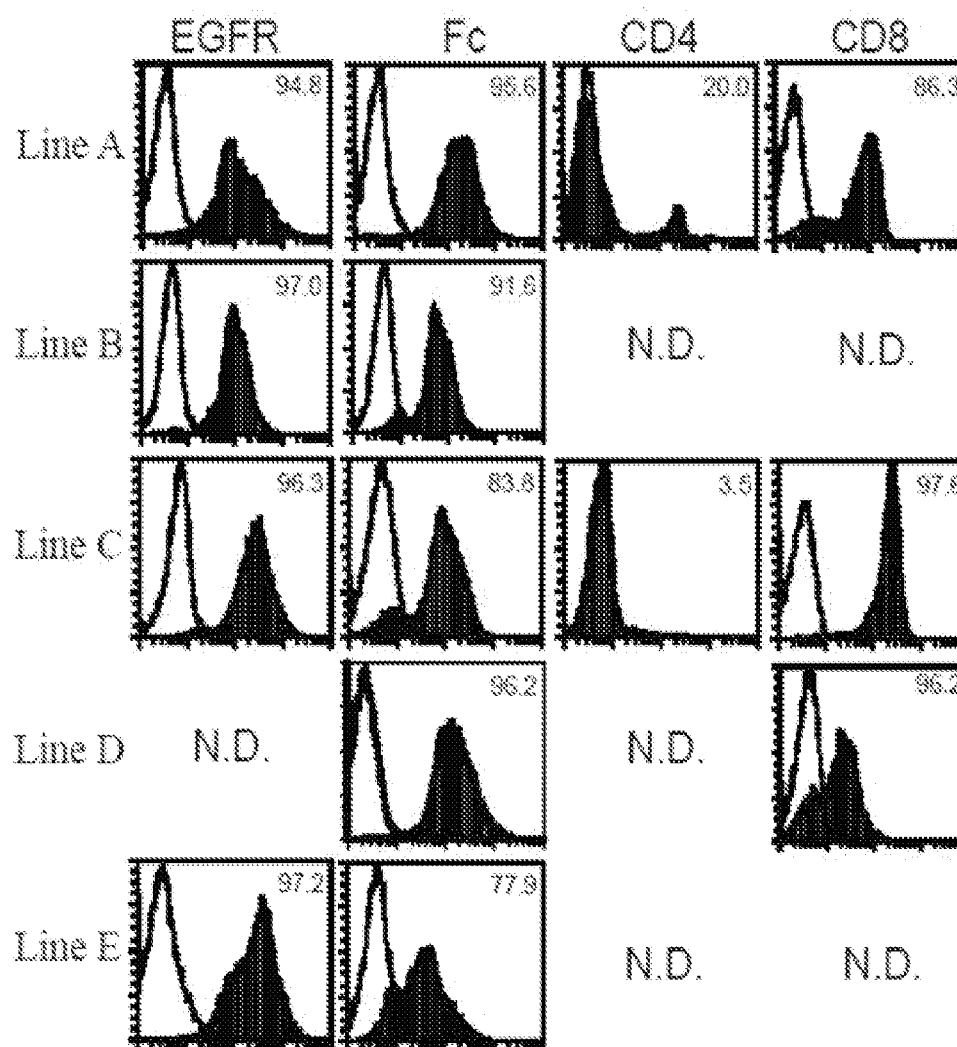


Figure 4C

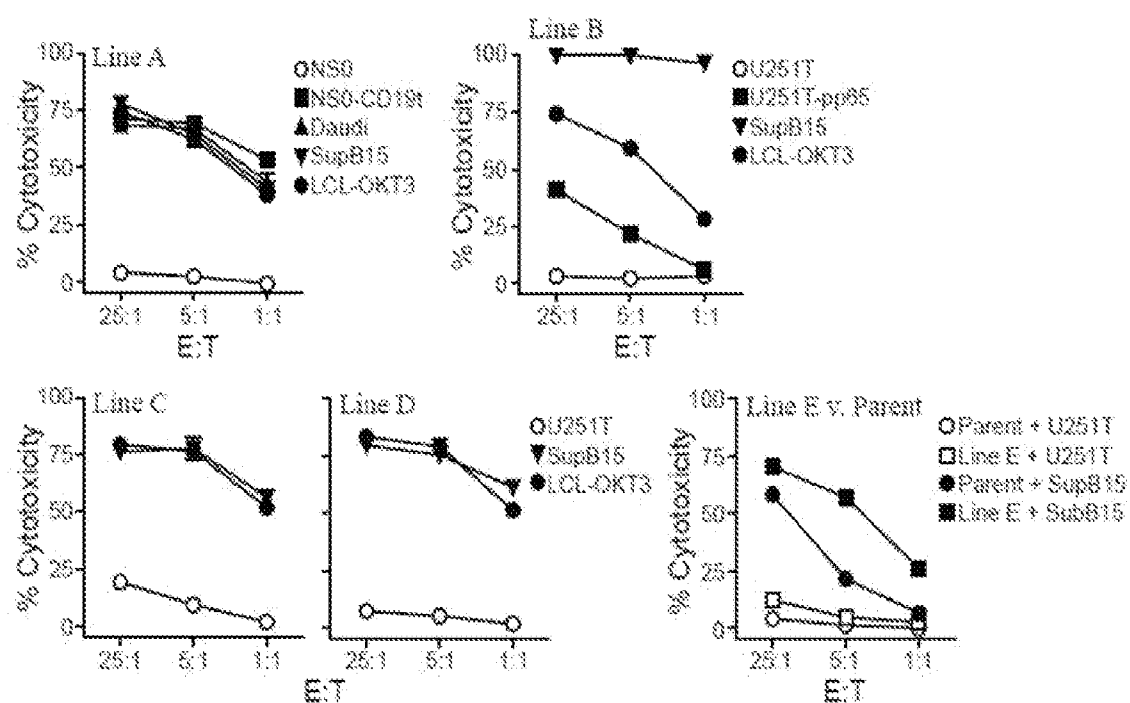


Figure 4D

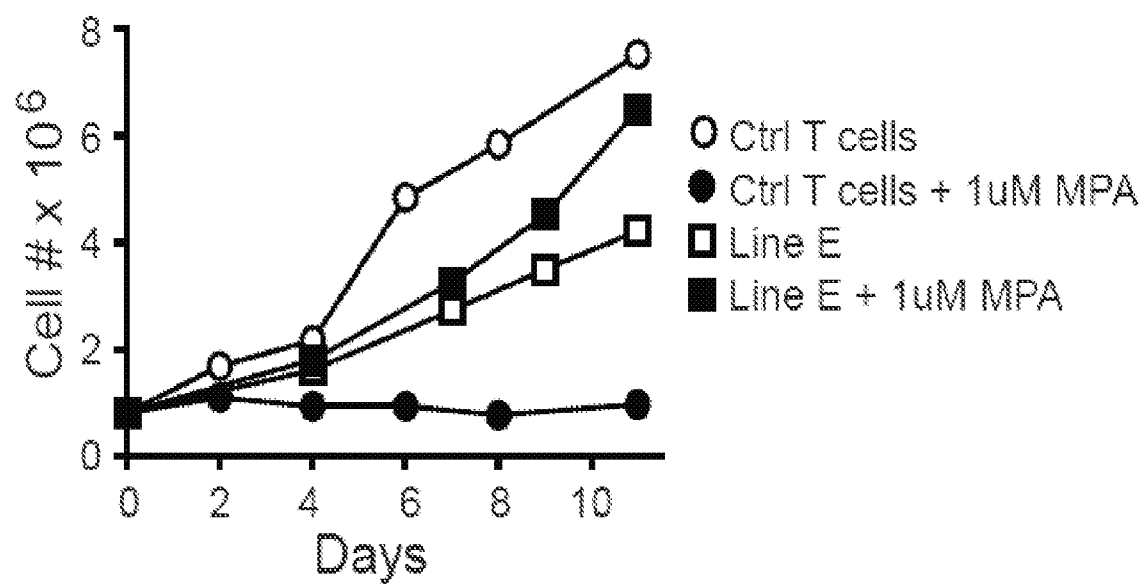


Figure 5

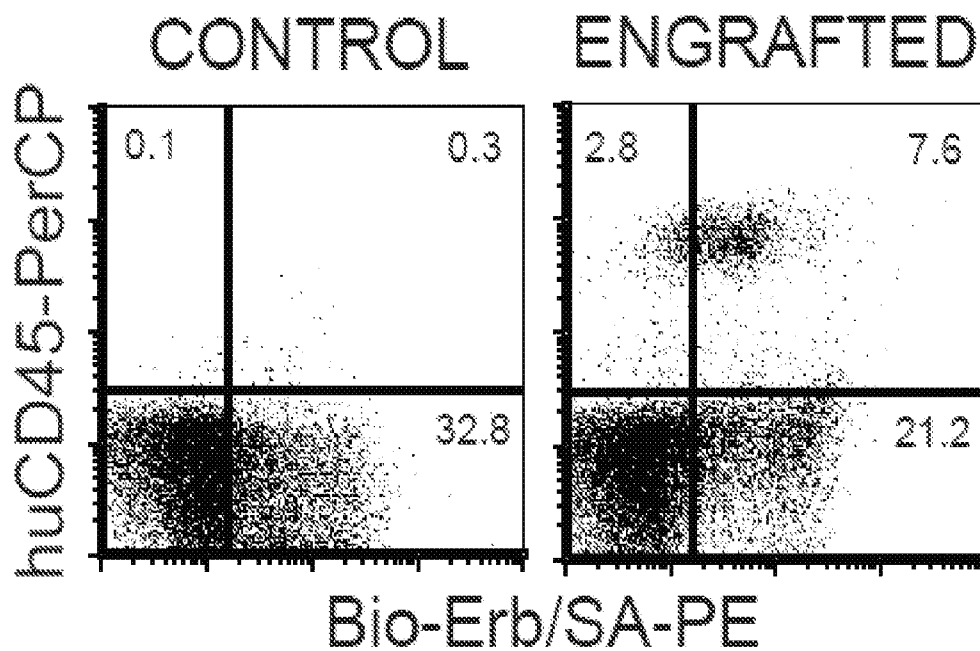


Figure 6

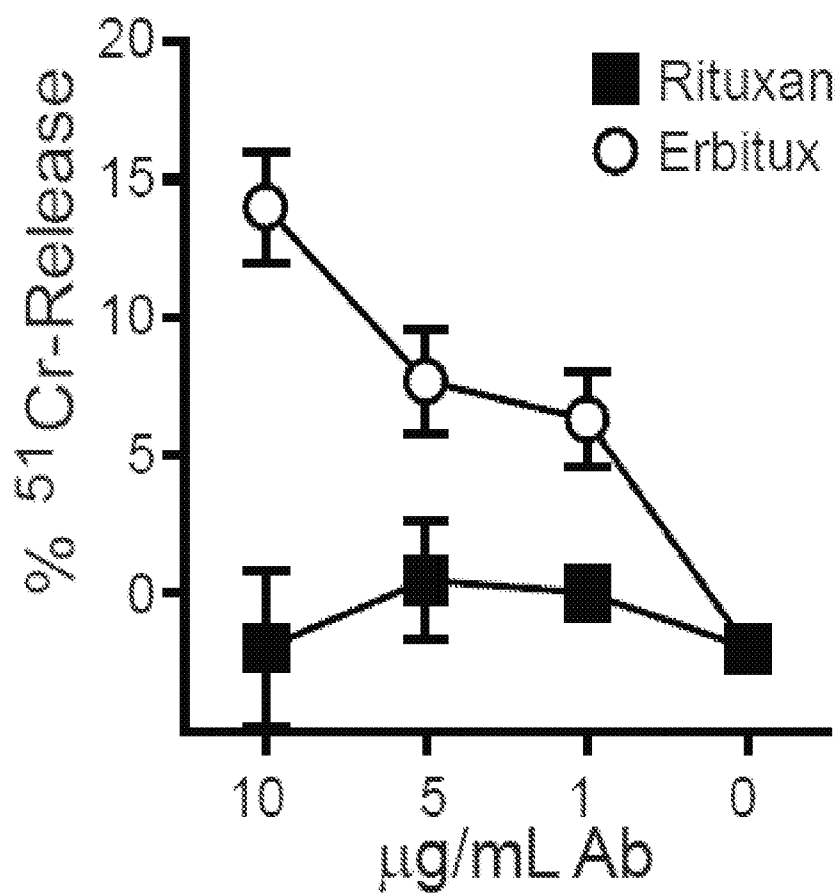


Figure 7

```

      M L L L V T S L L L C E L P H P A F L L
1  ATGCTTCTCC TGGTGACAAG CCTTCTGCTC TGTGAGTTAC CACACCCAGC ATTCTCCTG
   TACGAAGAGG ACCACTGTTC GGAAGACGAG ACACTCAATG GTGTGGGTCG TAAGGAGGAC
   I P R K V C N G I G I G E F K D S L S I
61 ATCCCACGCA AAGTGTGTAA CGGAATAGGT ATTGGTGAAT TTAAAGACTC ACTCTCCATA
   TAGGGTGCCT TTCACACATT GCCTTATCCA TAACCACTTA AATTTCTGAG TGAGAGGTAT
   N A T N I K H F K N C T S I S G D L H I
121 AATGCTACGA ATATTAAACA CTTCAAAAAC TGCACCTCCA TCAGTGGCGA TCTCCACATC
   TTACGATGCT TATAATTTGT GAAGTTTTTG ACGTGGAGGT AGTCACCGCT AGAGGTGTAG
   L P V A F R G D S F T H T P P L D P Q E
181 CTGCCGGTGG CATTTAGGGG TGACTCCTTC ACACATACTC CTCCTCTGGA TCCACAGGAA
   GACGGCCACC GTAAATCCCC ACTGAGGAAG TGTGTATGAG GAGGAGACCT AGGTGTCCTT
   L D I L K T V K E I T G F L L I P A W P
241 CTGGATATTC TGAAAACCGT AAAGGAAATC ACAGGGTTT TGCTGATTCA GGCTTGGCCT
   GACCTATAAG ACTTTTGGCA TTTCCTTTAG TGTCCCAAAA ACGACTAAGT CCGAACCGGA
   E N R T D L H A F E N L E I I R G R T K
301 GAAAACAGGA CGGACCTCCA TGCCTTTGAG AACCTAGAAA TCATACGCGG CAGGACCAAG
   CTTTGTGCTT GCCTGGAGGT ACGGAACTC TTGGATCTTT AGTATGCGCC GTCCTGGTTC
   Q H G Q F S L A V V S L N I T S L G L R
361 CAACATGGTC AGTTTCTCT TGCAGTCGTC AGCCTGAACA TAACATCCTT GGGATTACGC
   GTTGTACCAG TCAAAAGAGA ACGTCAGCAG TCGGACTTGT ATTGTAGGAA CCCTAATGCG
   S L K E I S D G D V I I S G N K N L C Y
421 TCCCTCAAGG AGATAAGTGA TGGAGATGTG ATAATTTTCTG GAAACAAAAA TTTGTGCTAT
   AGGGAGTTCC TCTATTCAC ACCTCTACAC TATTAAAGTC CTTTGTTTTT AAACACGATA
   A N T I N W K K L F G T S G Q K T K I I
481 GCAAATACAA TAACTGGAA AAACTGTTT GGGACCTCCG GTCAGAAAAC CAAAATTATA
   CGTTTATGTT ATTTGACCTT TTTTGACAAA CCCTGGAGGC CAGTCTTTTG GTTTTAATAT
   S N R G E N S C K A T G Q V C H A L C S
541 AGCAACAGAG GTGAAAACAG CTGCAAGGCC ACAGGCCAGG TCTGCCATGC CTTGTGCTCC
   TCGTTGTCTC CACTTTTGTG GACGTTCCGG TGTCCGGTCC AGACGGTACG GAACACGAGG
   P E G C W G P E P R D C V S C R N V S R
601 CCCGAGGGCT GCTGGGGCCC GGAGCCCAGG GACTGCGTCT CTTGCCGGAA TGTGAGCCGA
   GGGCTCCCGA CGACCCCGGG CCTCGGGTCC CTGACGCAGA GAACGGCCTT ACAGTCGGCT
   G R E C V D K C N L L E G E P R E F V E
661 GGCAGGGAAT GCGTGGACAA GTGCAACCTT CTGGAGGGTG AGCCAAGGGA GTTTGTGGAG
   CCGTCCCTTA CGCACCTGTT CACGTTGGAA GACCTCCAC TCGGTTCCCT CAAACACCTC
   N S E C I Q C H P E C L P Q A M N I T C
721 AACTCTGAGT GCATACAGTG CCACCCAGAG TGCCTGCCTC AGGCCATGAA CATCACCTGC
   TTGAGACTCA CGTATGTCAC GGTGGGTCTC ACGGACGGAG TCCGGTACTT GTAGTGGACG
   T G R G P D N C I Q C A H Y I D G P H C
781 ACAGGACGGG GACCAGACAA CTGTATCCAG TGTGCCCACT ACATTGACGG CCCCCACTGC
   TGTCTGCCC CTGGTCTGTT GACATAGGTC ACACGGGTGA TGTAAGTCC GGGGGTGACG
   V K T C P A G V M G E N N T L V W K Y A
841 GTCAAGACCT GCCCGGCAGG AGTCATGGGA GAAAACAACA CCCTGGTCTG GAAGTACGCA
   CAGTTCTGGA CGGGCCGTCC TCAGTACCCT CTTTGTGTGT GGGACCAGAC CTTTATGCGT
   D A G H V C H L C H P N C T Y G C T G P
901 GACGCCGGCC ATGTGTGCCA CCTGTGCCAT CCAAATGCA CCTACGGATG CACTGGGCCA
   CTGCGGCCGG TACACACGGT GGACACGGTA GGTGTGACGT GGATGCCTAC GTGACCCGGT
   G L E G C P T N G P K I P S I A T G M V
961 GGTCTTGAAG GCTGTCCAAC GAATGGGCCT AAGATCCCGT CCATCGCCAC TGGGATGGTG
   CCAGAACTTC CGACAGGTTG CTTACCCGGA TTCTAGGGCA GGTAGCGGTG ACCCTACCAC
   G A L L L L L V V A L G I G L F M
1021 GGGGCCCTCC TCTTGCTGCT GGTGGTGGCC CTGGGGATCG GCCTCTTCAT G
      CCCC GGAGG AGAACGACGA CCACCACCGG GACCCCTAGC CGGAGAAGTA C

```

Figure 8 CD19R-CD28gg-Zeta(CO)-T2A-EGFRt

```

      M L L L V T S L L L C E L P H P A F L L
1  ATGCTGCTGC TGGTGACCAG CCTGCTGCTG TGCAGAGCTGC CCCACCCCGC CTTTCTGCTG
   TACGACGACG ACCACTGGTC GGACGACGAC ACGCTCGACG GGGTGGGGCG GAAAGACGAC
   I P D I Q M T Q T T S S L S A S L G D R
61 ATCCCCGACA TCCAGATGAC CCAGACCACC TCCAGCCTGA GCGCCAGCCT GGGCGACCGG
   TAGGGGCTGT AGGTCTACTG GGTCTGGTGG AGGTGCGACT CGCGGTCGGA CCCGCTGGCC
   V T I S C R A S Q D I S K Y L N W Y Q Q
121 GTGACCATCA GCTGCCGGGC CAGCCAGGAC ATCAGCAAGT ACCTGAAGTGT GTATCAGCAG
   CACTGGTAGT CGACGGCCCG GTCGGTCCTG TAGTCGTTCA TGGACTTGAC CATAGTCGTC
   K P D G T V K L L I Y H T S R L H S G V
181 AAGCCCGACG GCACCGTCAA GCTGCTGATC TACCACACCA GCCGGCTGCA CAGCGGCGTG
   TTCGGGCTGC CGTGGCAGTT CGACGACTAG ATGGTGTGGT CGGCCGACGT GTCGCCGCAC
   P S R F S G S G S G T D Y S L T I S N L
241 CCCAGCCGGT TTAGCGGCAG CGGCTCCGGC ACCGACTACA GCCTGACCAT CTCCAACCTG
   GGGTCGGCCA AATCGCCGTC GCCGAGGCCG TGGCTGATGT CGGACTGGTA GAGGTGGAC
   E Q E D I A T Y F C Q Q G N T L P Y T F
301 GAACAGGAAG ATATCGCCAC CTACTTTTGC CAGCAGGGCA ACACACTGCC CTACACCTTT
   CTTGTCCTTC TATAGCGGTG GATGAAAACG GTCGTCCCGT TGTGTGACGG GATGTGGAAA
   G G G T K L E I T G S T S G S G K P G S
361 GCGCGGGAA CAAAGCTGGA AATCACCGGC AGCACCTCCG GCAGCGGCAA GCCTGGCAGC
   CCGCCGCCTT GTTTCGACCT TTAGTGGCCG TCGTGGAGGC CGTCGCCGTT CGGACCGTCG
   G E G S T K G E V K L Q E S G P G L V A
421 GCGGAGGGCA GCACCAAGGG CGAGGTGAAG CTGCAGGAAA GCGGCCCTGG CCTGGTGGCC
   CCGCTCCCGT CGTGGTTCCC GCTCCACTTC GACGTCCCTT CGCCGGGACC GGACCACCGG
   P S Q S L S V T C T V S G V S L P D Y G
481 CCCAGCCAGA GCCTGAGCGT GACCTGCACC GTGAGCGGCG TGAGCCTGCC CGACTACGGC
   GGGTCGGTCT CGGACTCGCA CTGGACGTGG CACTCGCCGC ACTCGGACGG GCTGATGCCG
   V S W I R Q P P R K G L E W L G V I W G
541 GTGAGCTGGA TCCGGCAGCC CCCCAGGAAG GGCCTGGAAT GGCTGGGCGT GATCTGGGGC
   CACTCGACCT AGGCCGTCCG GGGGTCTTTC CCGGACCTTA CCGACCCGCA CTAGACCCCG
   S E T T Y Y N S A L K S R L T I I K D N
601 AGCGAGACCA CCTACTACAA CAGCGCCCTG AAGAGCCGGC TGACCATCAT CAAGGACAAC
   TCGCTCTGGT GGATGATGTT GTCGCGGGAC TTCTCGGCCG ACTGGTAGTA GTTCTGTGTT
   S K S Q V F L K M N S L Q T D D T A I Y
661 AGCAAGAGCC AGGTGTTTCT GAAGATGAAC AGCCTGCAGA CCGACGACAC CGCCATCTAC
   TCGTTCTCGG TCCACAAGGA CTTCTACTTG TCGGACGTCT GGCTGCTGTG GCGGTAGATG
   Y C A K H Y Y Y G G S Y A M D Y W G G
721 TACTGCGCCA AGCACTACTA CTACGGCGGC AGCTACGCCA TGGACTACTG GGGCCAGGGC
   ATGACGCGGT TCGTGATGAT GATGCCGCCG TCGATGCGGT ACCTGATGAC CCCGGTCCCG
   T S V T V S S E S K Y G P P C P P C P A
781 ACCAGCGTGA CCGTGAGCAG CGAGAGCAAG TACGGCCCTC CCTGCCCCC TTGCCCTGCC
   TGGTCGCACT GGCACTCGTC GCTCTCGTTC ATGCCGGGAG GGACGGGGG AACGGGACGG
   P E F L G G P S V F L F P P K P K D T L
841 CCCGAGTTCC TGGGCGGACC CAGCGTGTTT CTGTTCCCCC CCAAGCCCAA GGACACCCTG
   GGGCTCAAGG ACCCGCCTGG GTCGCACAAG GACAAGGGGG GGTTCGGGTT CCTGTGGGAC
   M I S R T P E V T C V V V D V S Q E D P
901 ATGATCAGCC GGACCCCGA GGTGACCTGC GTGGTGGTGG ACGTGAGCCA GGAAGATCCC
   TACTAGTCGG CCTGGGGGCT CCACTGGACG CACCACCACC TGCACGCGT CTTCTAGGG
   E V Q F N W Y V D G V E V H N A K T K P
961 GAGGTCCAGT TCAATTGGTA CGTGACGGC GTGGAAGTGC ACAACGCCA GACCAAGCCC
   CTCCAGGTCA AGTTAACCAT GCACCTGCCG CACCTTCACG TGTTGCGGTT CTGGTTCCGG
      R E E Q F N S T Y R V V S V L T V L H Q
1021 AGAGAGGAAC AGTTCAACAG CACCTACCGG GTGGTGTCTG TGCTGACCGT GCTGCACCAG
   TCTCTCCTTG TCAAGTTGTC GTGGATGGCC CACCACAGAC ACGACTGGCA CGACGTGGTC
   D W L N G K E Y K C K V S N K G L P S S
1081 GACTGGCTGA ACGGCAAGA ATACAAGTGC AAGGTGTCCA ACAAGGGCCT GCCCAGCAGC
   CTGACCGACT TGCCGTTTCT TATGTTACG TTCCACAGGT TGTTCCCGGA CGGGTCGTCG

```



```

      I E K T I S K A K G Q P R E P Q V Y T L
1141 ATCGAAAAGA CCATCAGCAA GGCCAAAGGC CAGCCTCGCG AGCCCCAGGT GTACACCCTG
      TAGCTTTTCT GGTAGTCGTT CCGGTTCCCG GTCGGAGCGC TCGGGGTCCA CATGTGGGAC
      P P S Q E E M T K N Q V S L T C L V K G
1201 CCTCCCTCCC AGGAAGAGAT GACCAAGAAC CAGGTGTCCC TGACCTGCCT GGTGAAGGGC
      GGAGGGAGGG TCCTTCTCTA CTGGTTCTTG GTCCACAGGG ACTGGACGGA CCACTTCCCCG
      F Y P S D I A V E W E S N G Q P E N N Y
1261 TTCTACCCCA GCGACATCGC CGTGGAGTGG GAGAGCAACG GCCAGCCTGA GAACAACCTAC
      AAGATGGGGT CGCTGTAGCG GCACCTCACC CTCTCGTTGC CGGTCGGACT CTTGTTGATG
      K T T P P V L D S D G S F F L Y S R L T
1321 AAGACCACCC CTCCCGTGCT GGACAGCGAC GGCAGCTTCT TCCTGTACAG CCGGCTGACC
      TTCTGGTGGG GAGGGCACGA CCTGTCGCTG CCGTCGAAGA AGGACATGTC GGCCGACTGG
      V D G K S R W Q E G N V F S C S V M H E A
1381 GTGGACAAGA GCCGCTGGCA GGAAGGCAAC GTCTTTAGCT GCAGCGTGAT GCACGAGGCC
      CACCTGTTCT CGGCCACCGT CCTTCCGTTG CAGAAATCGA CGTCGCACTA CGTGCTCCGG
      L H N H Y T Q K S L S L S L G K M F W V
1441 CTGCACAACC ACTACACCCA GAAGAGCCTG AGCCTGTCCC TGGGCAAGAT GTTCTGGGTG
      GACGTGTTGG TGATGTGGGT CTTCTCGGAC TCGGACAGGG ACCCGTTCTA CAAGACCCAC
      L V V V G G V L A C Y S L L V T V A F I
1501 CTGGTGGTGG TGGGCGGGGT GCTGGCCTGC TACAGCCTGC TGGTGACAGT GGCCTTCATC
      GACCACCACC ACCCGCCCCA CGACCGGACG ATGTCGGACG ACCACTGTCA CCGGAAGTAG
      I F W V R S K R S R G G H S D Y M N M T
1561 ATCTTTTGGG TGCGGAGCAA GCGGAGCAGA GGCGGCCACA GCGACTACAT GAACATGACC
      TAGAAAACCC ACGCCTCGTT CGCCTCGTCT CCGCCGGTGT CGCTGATGTA CTTGTACTGG
      P R R P G P T R K H Y Q P Y A P P R D F
1621 CCCAGACGGC CTGGCCCCAC CCGGAAGCAC TACCAGCCCT ACGCCCCACC CAGGGACTTT
      GGGTCTGCGG GACCGGGGTG GGCTTCGTG ATGGTCGGGA TGCGGGGTGG GTCCCTGAAA
      A A Y R S G G G R V K F S R S A D A P A
1681 GCCCGCTACC GGTCCGGCGG AGGGCGGGT AAGTTCAGCA GAAGCGCCGA CGCCCCTGCC
      CGGCGGATGG CCAGGCCGCC TCCGCCCCAC TTCAAGTCGT CTTGCGGGCT CCGGGGACGG
      Y Q Q G Q N Q L Y N E L N L G R R E E Y
1741 TACCAGCAGG GCCAGAATCA GCTGTACAAC GAGCTGAACC TGGGCAGAAG GGAAGAGTAC
      ATGGTCGTCC CGGTCTTAGT CGACATGTTG CTCGACTTGG ACCCGTCTTC CTTTCTCATG
      D V L D K R R G R D P E M G G K P R R K
1801 GACGTCCTGG ATAAGCGGAG AGGCCGGGAC CCTGAGATGG GCGGCAAGCC TCGGCGGAAG
      CTGCAGGACC TATTCGCCTC TCCGGCCCTG GGACTCTACC CGCCGTTCCG AGCCGCCTTC
      N P Q E G L Y N E L Q K D K M A E A Y S
1861 AACCCCCAGG AAGGCCTGTA TAACGAACTG CAGAAAGACA AGATGGCCGA GGCCTACAGC
      TTGGGGGTCC TTCCGACAT ATTGCTTGAC GTCTTTCTGT TCTACCGGCT CCGGATGTGC
      E I G M K G E R R R G K G H D G L Y Q G
1921 GAGATCGGCA TGAAGGGCGA GCGGAGGCGG GGCAAGGGCC ACGACGGCCT GTATCAGGGC
      CTCTAGCCGT ACTTCCCGCT CGCCTCCGCC CCGTTCCCGG TGCTGCCGGA CATAGTCCCCG
      L S T A T K D T Y D A L H M Q A L P P R
1981 CTGTCCACCG CCACCAAGGA TACCTACGAC GCCCTGCACA TGCAGGCCCT GCCCCAAGG
      GACAGGTGGC GGTGGTTCCT ATGGATGCTG CGGGACGTGT ACGTCCGGGA CGGGGGTTCC
      L E G G G G G S L L T C G D V E E N
2041 CTCGAGGCG GCGGAGAGG CAGAGGAAGT CTTCTAACAT GCGGTGACV GGAGGAGAAT
      GAGCTCCCGC CGCCTCTCCC GTCTCCTTCA GAAGATTGTA CGCCACTGCA CCTCCTCTTA
      P G P R M L L L V T S L L L C E L P H P
2101 CCCGGCCCTA GGATGCTTCT CCTGGTGACA AGCCTTCTGC TCTGTGAGTT ACCACACCCA
      GGGCCGGGAT CCTACGAAGA GGACCACTGT TCGGAAGACG AGACACTCAA TGGTGTGGGT
      A F L L I P R K V C N G I G I G E F K D
2161 GCATTCTCTC TGATCCCACG CAAAGTGTGT AACGGAATAG GTATTGGTGA ATTTAAAGAC
      CGTAAGGAGG ACTAGGGTGC GTTTCACACA TTGCCTTATC CATAACCACT TAAATTTCTG
      S L S I N A T N I K H F K N C T S I S G
2221 TCACTCTCCA TAAATGCTAC GAATATTAAA CACTTCAAAA ACTGCACCTC CATCAGTGGC
      AGTGAGAGGT ATTTACGATG CTTATAATTT GTGAAGTTT TGACGTGGAG GTAGTCACCG
      D L H I L P V A F R G D S F T H T P P L
2281 GATCTCCACA TCCTGCCGGT GGCATTAGG GGTGACTCCT TCACACATAC TCCTCCTCTG
      CTAGAGGTGT AGGACGGCCA CCGTAAATCC CCACTGAGGA AGTGTGTATG AGGAGGAGAC
      D P Q E L D I L K T V K E I T G F L L I

```

2341 GATCCACAGG AACTGGATAT TCTGAAAACC GTAAAGGAAA TCACAGGGTT TTTGCTGATT
 CTAGGTGTCC TTGACCTATA AGACTTTTGG CATTTTCCTTT AGTGTCCCAA AAACGACTAA
 Q A W P E N R T D L H A F E N L E I I R
 2401 CAGGCTTGGC CTGAAAACAG GACGGACCTC CATGCCTTTG AGAACCTAGA AATCATACGC
 GTCCGAACCG GACTTTTGTC CTGCCTGGAG GTACGGAAAC TCTTGATCT TTAGTATGCG
 G R T K Q H G Q F S L A V V S L N I T S
 2461 GGCAGGACCA AGCAACATGG TCAGTTTCT CTTGCAGTCG TCAGCCTGAA CATAACATCC
 CCGTCTGGT TCGTTGTACC AGTCAAAAGA GAACGTCAGC AGTCGGACTT GTATTGTAGG
 L G L R S L K E I S D G D V I I S G N K
 2521 TTGGGATTAC GCTCCCTCAA GGAGATAAGT GATGGAGATG TGATAATTC AGGAAACAAA
 AACCTAATG CGAGGGAGTT CCTCTATTCA CTACCTCTAC ACTATTAAAG TCCTTTGTTT
 N L C Y A N T I N W K K L F G T S G Q K
 2581 AATTTGTGCT ATGCAAATAC AATAAACTGG AAAAAACTGT TTGGGACCTC CGGTCAGAAA
 TTAAACACGA TACGTTTATG TTATTTGACC TTTTTTGACA AACCTGGAG GCCAGTCTTT
 T K I I S N R G E N S C K A T G Q V C H
 2641 ACCAAAATTA TAAGCAACAG AGGTGAAAAC AGCTGCAAGG CCACAGGCCA GGTCTGCCAT
 TGGTTTTAAT ATTCGTTGTC TCCACTTTTG TCGACGTTCC GGTGTCCGGT CCAGACGGTA
 A L C S P E G C W G P E P R D C V S C R
 2701 GCCTTGTGCT CCCCCGAGGG CTGCTGGGGC CCGGAGCCCA GGGACTGCGT CTCTTGCCGG
 CGGAACACGA GGGGGCTCCC GACGACCCCG GGCCTCGGGT CCCTGACGCA GAGAACGGCC
 N V S R G R E C V D K C N L L E G E P R
 2761 AATGTCAGCC GAGGCAGGGA ATGCGTGGAC AAGTGCAACC TTCTGGAGGG TGAGCCAAGG
 TTACAGTCGG CTCCGTCCCT TACGCACCTG TTCACGTTGG AAGACCTCCC ACTCGGTTCC
 E F V E N S E C I Q C H P E C L P Q A M
 2821 GAGTTTGTGG AGAACTCTGA GTGCATACAG TGCCACCCAG AGTGCCTGCC TCAGGCCATG
 CTCAAACACC TCTTGAGACT CACGTATGTC ACGGTGGGTC TCACGGACGG AGTCCGGTAC
 N I T C T G R G P D N C I Q C A H Y I D
 2881 AACATCACCT GCACAGGACG GGGACCAGAC AACTGTATCC AGTGTGCCCA CTACATTGAC
 TTGTAGTGA CGTGTCTGTC CCCTGGTCTG TTGACATAGG TCACACGGGT GATGTAAGT
 G P H C V K T C P A G V M G E N N T L V
 2941 GGCCCCCACT GCGTCAAGAC CTGCCCCGCA GGAGTCATGG GAGAAAACAA CACCCTGGTC
 CCGGGGGTGA CGCAGTTCTG GACGGGCCGT CCTCAGTACC CTCTTTTGTT GTGGGACCAG
 W K Y A D A G H V C H L C H P N C T Y G
 3001 TGGAAGTACG CAGACGCCGG CCATGTGTGC CACCTGTGCC ATCCAAACTG CACCTACGGA
 ACCTTCATGC GTCTGCGGCC GGTACACACG GTGGACACGG TAGGTTTGAC GTGGATGCCT
 C T G P G L E G C P T N G P K I P S I A
 3061 TGCACTGGGC CAGGTCTTGA AGGCTGTCCA ACGAATGGGC CTAAGATCCC GTCCATCGCC
 ACGTGACCCG GTCCAGAACT TCCGACAGGT TGCTTACCCG GATTCTAGGG CAGGTAGCGG
 T G M V G A L L L L L V V A L G I G L F
 3121 ACTGGGATGG TGGGGGCCCT CCTCTTGCTG CTGGTGGTGG CCCTGGGGAT CGGCCTCTTC
 TGACCCTACC ACCCCCGGGA GGAGAACGAC GACCACCACC GGGACCCCTA GCCGGAGAAG
 M *
 3181 ATGTGA
 TACACT

Figure 9

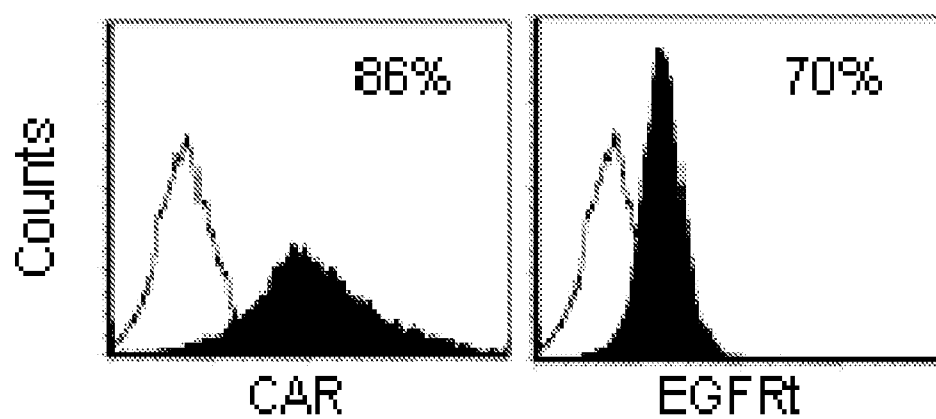


Figure 10

