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(72) Inventeurs/Inventors:
PULE, MARTIN, GB;
KONG, KHAI, GB;
CORDOBA, SHAUN, GB

(73) Propriétaire/Owner:
AUTOLUS LIMITED, GB

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

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(54) Title: A T CELL OR NATURAL KILLER (NK) CELL WHICH CO-EXPRESSES TWO CHIMERIC ANTIGEN RECEPTORS (CARS)

(57) Abrégé/Abstract:
The present invention provides a cell which co-expresses a first chimeric antigen receptor (CAR) and second CAR at the cell surface, each CAR comprising: (i) an antigen-binding domain; (ii) a spacer (iii) a trans-membrane domain; and (iv) an endodomain wherein the antigen binding domains of the first and second CARs bind to different antigens, wherein the spacer of the first CAR is different to the spacer of the second CAR and wherein one of the first or second CARs is an activating CAR comprising an activating endodomain and the other CAR is an inhibitory CAR comprising a ligation-off inhibitory endodomain.

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(71) Applicant: **UCL BUSINESS PLC** [GB/GB]; The Network Building, 97 Tottenham Court Road, London W1T 4TP (GB).

(72) Inventors: **PULÉ, Martin**; c/o UCL Business PLC, The Network Building, 97 Tottenham Court Road, London W1T 4TP (GB). **KONG, Khai**; c/o UCL Business PLC, The Network Building, 97 Tottenham Court Road, London W1T 4TP (GB). **CORDOBA, Shaun**; c/o UCL Business PLC, The Network Building, 97 Tottenham Court Road, London W1T 4TP (GB).

(74) Agent: **HOLLIDAY, Louise**; D Young & Co LLP, 120 Holborn, London, EC1N 2DY (GB).

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(54) Title: CELL

(57) Abstract: The present invention provides a cell which co-expresses a first chimeric antigen receptor (CAR) and second CAR at the cell surface, each CAR comprising: (i) an antigen-binding domain; (ii) a spacer (iii) a trans-membrane domain; and (iv) an endodomain wherein the antigen binding domains of the first and second CARs bind to different antigens, wherein the spacer of the first CAR is different to the spacer of the second CAR and wherein one of the first or second CARs is an activating CAR comprising an activating endodomain and the other CAR is an inhibitory CAR comprising a ligation-off inhibitory endodomain.

A T CELL OR NATURAL KILLER (NK) CELL WHICH CO-EXPRESSES TWO CHIMERIC ANTIGEN RECEPTORS (CARS)

FIELD OF THE INVENTION

The present invention relates to a cell which comprises more than one chimeric antigen receptor (CAR). The cell may be capable of specifically recognising a target cell, due to a differential pattern of expression (or non-expression) of two or more antigens by the target cell.

BACKGROUND TO THE INVENTION

A number of immunotherapeutic agents have been described for use in cancer treatment, including therapeutic monoclonal antibodies (mAbs), immunoconjugated mAbs, radioconjugated mAbs and bi-specific T-cell engagers.

Typically these immunotherapeutic agents target a single antigen: for instance, Rituximab targets CD20; Myelotarg targets CD33; and Alemtuzumab targets CD52.

However, it is relatively rare for the presence (or absence) of a single antigen effectively to describe a cancer, which can lead to a lack of specificity.

Most cancers cannot be differentiated from normal tissues on the basis of a single antigen. Hence, considerable "on-target off-tumour" toxicity occurs whereby normal tissues are damaged by the therapy. For instance, whilst targeting CD20 to treat B-cell lymphomas with Rituximab, the entire normal B-cell compartment is depleted, whilst targeting CD52 to treat chronic lymphocytic leukaemia, the entire lymphoid compartment is depleted, whilst targeting CD33 to treat acute myeloid leukaemia, the entire myeloid compartment is damaged etc.
The predicted problem of "on-target off-tumour" toxicity has been borne out by clinical trials. For example, an approach targeting ERBB2 caused death to a patient with colon cancer metastatic to the lungs and liver. ERBB2 is over-expressed in colon caner in some patients, but it is also expressed on several normal tissues, including heart and normal vasculature.

For some cancers, targeting the presence of two cancer antigens may be more selective and therefore effective than targeting one. For example, B-chronic lymphocytic leukaemia (B-CLL) is a common leukaemia which is currently treated by targeting CD19. This treats the lymphoma but also depletes the entire B-cell compartment such that the treatment has a considerable toxic effect. B-CLL has an unusual phenotype in that CD5 and CD19 are co-

expressed. By targeting only cells which express CD5 and CD19, it would be possible to considerably reduce on-target off-tumour toxicity.

There is thus a need for immunotherapeutic agents which are capable of more targeting to reflect the complex pattern of marker expression that is associated with many cancers.

Chimeric Antigen Receptors (CARs)

Chimeric antigen receptors are proteins which graft the specificity of a monoclonal antibody (mAb) to the effector function of a T-cell. Their usual form is that of a type I transmembrane domain protein with an antigen recognizing amino terminus, a spacer, a transmembrane domain all connected to a compound endodomain which transmits T-cell survival and activation signals (see Figure 1A).

The most common form of these molecules are fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies which recognize a target antigen, fused via a spacer and a trans-membrane domain to a signaling endodomain. Such molecules result in activation of the T-cell in response to recognition by the scFv of its target. When T cells express such a CAR, they recognize and kill target cells that express the target antigen. Several CARs have been developed against tumour associated antigens, and adoptive transfer approaches using such CAR-expressing T cells are currently in clinical trial for the treatment of various cancers.

However, the use of CAR-expressing T cells is also associated with on-target, off tumour toxicity. For example, a CAR-based approach targeting carboxy anyhydrase-IX (CAIX) to treat renal cell carcinoma resulted in liver toxicity which is thought to be caused by the specific attack on bile duct epithelial cells (Lamers et al (2013) Mol. Ther. 21:904-912).

Dual targeting CAR approaches

In order to address the problem of "on target, off tumour" toxicity, CAR T cells have been developed with dual antigen specificty. In the "dual targeting" approach, two complementary CARs are co-expressed in the same T-cell population, each directed to a distant tumour target and engineered to provide complementary signals.

Wlikie et al (2012 J Clin Immunol 32:1059-1070) describe a dual targeting approach in which ErbB2- and MUC1-specific CARs are co-expressed. The ErbB2-specific CAR provided the CD3ζ signal only and the MUC1-specific CAR provided the CD28 co-stimulatory signal only.

It was found that complementary signalling occurred in the presence of both antigens, leading to IL-2 production. However, IL-2 production was modest when compared to control CAR-engineered T cells in which signaling is delivered by a fused CD28+CD3ζ endodomain.

A similar approach was described by Kloss et al (2013 Nature Biotechnol. 31:71-75) in which a CD-19 specific CAR was used which provides a CD3ζ-mediated activation signal in combination with a chimeric co-stimulatory receptor specific for PSMA. With this 'co-CAR' design, the CAR T-cell receives an activation signal when it encounters a target cell with one antigen, and a co-stimulatory signal when it encounters a target cell with the other antigen, and only receives both activatory and co-stimulatory signals upon encountering target cells bearing both antigens.

This represents an early attempt at restricting CAR activity to only a target cell bearing two antigens. This approach however is limited: although CAR T-cell activity will be greatest against targets expressing both antigens, CAR T-cells will still kill targets expressing only antigen recognized by the activatory CAR; further, co-stimulation results in prolonged effects on T-cells which last long after release of target cell. Hence, activity against single-antigen positive T-cells equal to that against double-positives might be possible for example in a situation where single-positive tissues are adjacent to, or in a migratory path from double positive tumour.

There is thus a need for improved CAR-based therapeutic approaches with reduced on-target off-tumour toxicity where T-cell activation is wholly restricted to target cells which express both antigens.

DESCRIPTION OF THE FIGURES

Figure 1: (a) Generalized architecture of a CAR: A binding domain recognizes antigen; the spacer elevates the binding domain from the cell surface; the trans-membrane domain anchors the protein to the membrane and the endodomain transmits signals. (b) to (d): Different generations and permutations of CAR endodomains: (b) initial designs transmitted ITAM signals alone through FcεR1-γ or CD3ζ endodomain, while later designs transmitted additional (c) one or (d) two co-stimulatory signals in cis.

Figure 2: Schematic diagram illustrating the invention

The invention relates to engineering T-cells to respond to logical rules of target cell antigen expression. This is best illustrated with an imaginary FACS scatter-plot. Target cell

populations express both, either or neither of antigens "A" and "B". Different target populations (marked in red) are killed by T-cells transduced with a pair of CARs connected by different gates. With OR gated receptors, both single-positive and double-positive cells will be killed. With AND gated receptors, only double-positive target cells are killed. With AND NOT gating, double-positive targets are preserved while single-positive targets

Figure 3: Creation of target cell populations
SupT1 cells were used as target cells. These cells were transduced to express either CD19, CD33 or both CD19 and CD33. Target cells were stained with appropriate antibodies and analysed by flow cytometry.

Figure 4: Cassette design for an OR gate
A single open reading frame provides both CARs with an in-frame FMD-2A sequence resulting in two proteins. Signal1 is a signal peptide derived from IgG1 (but can be any effective signal peptide). scFv1 is the single-chain variable segment which recognizes CD19 (but can be a scFv or peptide loop or ligand or in fact any domain which recognizes any desired arbitrary target). STK is the CD8 stalk but may be any suitable extracellular domain. CD28tm is the CD28 trans-membrane domain but can by any stable type I protein transmembrane domain and CD3Z is the CD3 Zeta endodomain but can be any endodomain which contains ITAMs. Signal2 is a signal peptide derived from CD8 but can be any effective signal peptide which is different in DNA sequence from signal1. scFv recognizes CD33 but as for scFv1 is arbitrary. HC2CH3 is the hinge-CH2-CH3 of human IgG1 but can be any extracellular domain which does not cross-pair with the spacer used in the first CAR. CD28tm' and CD3Z' code for the same protein sequence as CD28tm and CD3Z but are codon-wobbled to prevent homologous recombination.

Figure 5: Schematic representation of the chimeric antigen receptors (CARs) for an OR gate
Stimulatory CARs were constructed consisting of either an N-terminal A) anti-CD19 scFv domain followed by the extracellular hinge region of human CD8 or B) anti-CD33 scFv domain followed by the extracellular hinge, CH2 and CH3 (containing a pvaa mutation to reduce FcR binding) region of human IgG1. Both receptors contain a human CD28 transmembrane domain and a human CD3 Zeta (CD247) intracellular domain. "S" depicts the presence of disulphide bonds.

Figure 6: Expression data showing co-expression of both CARs on the surface of one T-cell.

Figure 7: Functional analysis of the OR gate

Effector cells (5x10^4 cells) expressing the OR gate construct were co-incubated with a varying number of target cells and IL-2 was analysed after 16 hours by ELISA. The graph displays the average maximum IL-2 secretion from a chemical stimulation (PMA and Ionomycin) of the effector cells alone and the average background IL-2 from effector cells without any stimulus from three replicates.

Figure 8: Cartoon showing both versions of the cassette used to express both AND gates
Activating and inhibiting CARs were co-expressed once again using a FMD-2A sequence. Signal1 is a signal peptide derived from IgG1 (but can be any effective signal peptide). scFv1 is the single-chain variable segment which recognizes CD19 (but can be a scFv or peptide loop or ligand or in fact any domain which recognizes any desired arbitrary target). STK is the CD8 stalk but may be any non-bulky extracellular domain. CD28tm is the CD28 trans-membrane domain but can by any stable type I protein transmembrane domain and CD3Z is the CD3 Zeta endodomain but can be any endodomain which contains ITAMs. Signal2 is a signal peptide derived from CD8 but can be any effective signal peptide which is different in DNA sequence from signal1. scFv recognizes CD33 but as for scFv1 is arbitrary. HC2CH3 is the hinge-CH2-CH3 of human IgG1 but can be any bulky extracellular domain. CD45 and CD148 are the transmembrane and endodomains of CD45 and CD148 respectively but can be derived from any of this class of protein.

Figure 9: Schematic representation of the protein structure of chimeric antigen receptors (CARs) for the AND gates
The stimulatory CAR consisting of an N-terminal anti-CD19 scFv domain followed by the extracellular stalk region of human CD8, human CD28 transmembrane domain and human CD3 Zeta (CD247) intracellular domain. Two inhibitory CARs were tested. These consist of an N-terminal anti-CD33 scFv domain followed by the extracellular hinge, CH2 and CH3 (containing a pvaa mutation to reduce FcR binding) region of human IgG1 followed by the transmembrane and intracellular domain of either human CD148 or CD45. "S" depicts the presence of disulphide bonds.

Figure 10: Co-expression of activation and inhibitory CARs
BW5147 cells were used as effector cells and were transduced to express both the activation anti-CD19 CAR and one of the inhibitory anti-CD33 CARs. Effector cells were stained with CD19-mouse-Fc and CD33-rabbit-Fc and with appropriate secondary antibodies and analysed by flow cytometry.

Figure 11: Functional analysis of the AND gates

Effector cells (5x10^4 cells) expressing activation anti-CD19 CAR and the inhibitory anti-CD33 CAR with the A) CD148 or B) CXD45 intracellular domain were co- incubated with a varying number of target cells and IL-2 was analysed after 16hours by ELISA. The graph displays the maximum IL-2 secretion from a chemical stimulation (PMA and Ionomycin) of the effector cells alone and the background IL-2 from effector cells without any stimulus from three replicates.

Figure 12: Cartoon showing three versions of the cassette used to generate the AND NOT gate

Activating and inhibiting CARs were co-expressed once again using a FMD-2A sequence. Signal1 is a signal peptide derived from IgG1 (but can be any effective signal peptide). scFv1 is the single-chain variable segment which recognizes CD19 (but can be a scFv or peptide loop or ligand or in fact any domain which recognizes any desired arbitrary target). STK is the human CD8 stalk but may be any non-bulky extracellular domain. CD28tm is the CD28 trans-membrane domain but can by any stable type I protein transmembrane domain and CD3Z is the CD3 Zeta endodomain but can be any endodomain which contains ITAMs. Signal2 is a signal peptide derived from CD8 but can be any effective signal peptide which is different in DNA sequence from signal1. scFv recognizes CD33 but as for scFv1 is arbitrary. muSTK is the mouse CD8 stalk but can be any spacer which co-localises but does not cross-pair with that of the activating CAR. dPTPN6 is the phosphatase domain of PTPN6. LAIR1 is the transmembrane and endodomain of LAIR1. 2Aw is a codon-wobbled version of the FMD-2A sequence. SH2-CD148 is the SH2 domain of PTPN6 fused with the phosphatase domain of CD148.

Figure 13: Schematic representation of the chimeric antigen receptors (CARs) for the NOT AND gates

A) A stimulatory CAR consisting of an N-terminal anti-CD19 scFv domain followed by the stalk region of human CD8, human CD28 transmembrane domain and human CD247 intracellular domain. B) An inhibitory CAR consisting of an N-terminal anti-CD33 scFv domain followed by the stalk region of mouse CD8, transmembrane region of mouse CD8 and the phosphatase domain of PTPN6. C) an inhibitory CAR consisting of an N-terminal anti-CD33 scFv domain followed by the stalk region of mouse CD8 and the transmembrane and intracellular segments of LAIR1. D) An inhibitory CAR identical to previous CAR except it is co-expressed with a fusion protein of the PTPN6 SH2 domain and the CD148 phosphatase domain.

Figure 14: Functional analysis of the NOT AND gate

Effector cells (5x10^4 cells) expressing the A) full length SHP-1 or B) truncated form of SHP-1 were co- incubated with a varying number of target cells and IL-2 was analysed after 16 hours by ELISA. The graph displays the average maximum IL-2 secretion from a chemical stimulation (PMA and Ionomycin) of the effector cells alone and the average background IL-2 from effector cells without any stimulus from three replicates.

Figure 15: Amino acid sequence of an OR gate

Figure 16: Amino acid sequence of a CD148 and a CD145 based AND gate

Figure 17: Amino acid sequence of two AND NOT gates

Figure 18: Dissection of AND gate function
A. The prototype AND gate is illustrated on the right and its function in response to CD19, CD33 single and CD19, CD33 double positive targets is shown on the left. **B.** The scFvs are swapped so the activating endodomain is triggered by CD33 and the inhibitory endodomain is activated by CD19. This AND gate remains functional despite this scFv swap. **C.** The CD8 mouse stalk replaced Fc in the spacer of the inhibitory CAR. With this modification, the gate fails to respond to either CD19 single positive or CD19, CD33 double positive targets.

Figure 19: Expression of target antigens on artificial target cells
A. Shows flow cytometry scatter plots CD19 vs CD33 of the original set of artificial target cells derived from SupT1 cells. From left to right: double negative SupT1 cells, SupT1 cells positive for CD19, positive for CD33 and positive for both CD19 and CD33. **B**. Shows flow cytometry scatter plots CD19 vs GD2 of the artificial target cells generated to test the CD19 AND GD2 gate: From left to right: negative SupT1 cells, SupT1 cells expressing CD19, SupT1 cells transduced with GD2 and GM3 synthase vectors which become GD2 positive and SupT1 cells transduced with CD19 as well as GD2 and GM3 synthase which are positive for both GD2 and CD19. **C**. Shows flow cytometry scatter plots of CD19 vs EGFRvIII of the artificial targets generated to test the CD19 AND EGFRvIII gate. From left to right: negative SupT1 cells, SupT1 cells expressing CD19, SupT1 cells transduced with EGFRvIII and SupT1 cells transduced with both CD19 and EGFRvIII. **D**. Shows flow cytometry scatter plots of CD19 vs CD5 of the artificial targets generated to test the CD19 AND CD5 gate. From left to right: negative 293T cells, 293T cells transduced with CD19, 293T cells transduced with CD5, 293T cells transduced with both CD5 and CD19 vectors.

Figure 20: Generalizability of the AND gate

A. Cartoon of AND gate modified so the second CAR's specificity is changed from the original specificity of CD33, to generate 3 new CARs: CD19 AND GD2, CD19 AND EGFRvIII, CD19 AND CD5. **B.** CD19 AND GD2 AND gate: Left: expression of AND gate is shown recombinant CD19-Fc staining (x-axis) for the CD19 CAR, versus anti-human-Fc staining (Y-axis) for the GD2 CAR. Right: function in response to single positive and double positive targets. **C.** CD19 AND EGFRvIII AND gate: Left: expression of AND gate is shown recombinant CD19-Fc staining (x-axis) for the CD19 CAR, versus anti-human-Fc staining (Y-axis) for the EGFRvIII CAR. Right: function in response to single positive and double positive targets. **D.** CD19 AND CD5 AND gate: Left: expression of AND gate is shown recombinant CD19-Fc staining (x-axis) for the CD19 CAR, versus anti-human-Fc staining (Y-axis) for the CD5 CAR. Right: function in response to single positive and double positive targets.

Figure 21: Function of the AND NOT gates

Function of the three implementations of an AND NOT gate is shown. A cartoon of the gates tested is shown to the right, and function in response to single positive and double positive targets is shown to the left. **A.** PTPN6 based AND NOT gate whereby the first CAR recognizes CD19, has a human CD8 stalk spacer and an ITAM containing activating endodomain; is co-expressed with a second CAR that recognizes CD33, has a mouse CD8 stalk spacer and has an endodomain comprising of a PTPN6 phosphatase domain. **B.** ITIM based AND NOT gate is identical to the PTPN6 gate, except the endodomain is replaced by the endodomain from LAIR1. **C.** CD148 boosted AND NOT gate is identical to the ITIM based gate except an additional fusion between the PTPN6 SH2 and the endodomain of CD148 is expressed. All three gates work as expected with activation in response to CD19 but not in response to CD19 and CD33 together.

Figure 22: Dissection of PTPN6 based AND NOT gate function

The original PTPN6 based AND NOT gate is compared with several controls to demonstrate the model. A cartoon of the gates tested is shown to the right, and function in response to single positive and double positive targets is shown to the left. **A.** Original AND NOT gate whereby the first CAR recognizes CD19, has a human CD8 stalk spacer and an ITAM containing activating endodomain; is co-expressed with a second CAR recognizes CD33, has a mouse CD8 stalk spacer and has an endodomain comprising of a PTPN6 phosphatase domain. **B**. AND NOT gate modified so the mouse CD8 stalk spacer is replaced with an Fc spacer. **C** AND NOT gate modified so that the PTPN6 phosphatase domain is replaced with the endodomain from CD148. Original AND NOT gate (**A.**) functions as expected triggering in response to CD19, but not in response to both CD19 and CD33.

The gate in **B.** triggers both in response to CD19 along or CD19 and CD33 together. The gate in **C.** does not trigger in response to one or both targets.

Figure 23: Dissection of LAIR1 based AND NOT gate

Functional activity against CD19 positive, CD33 positive and CD19, CD33 double-positive targets is shown. **A**. Structure and activity of the original ITIM based AND NOT gate. This gate is composed of two CARs: the first recognizes CD19, has a human CD8 stalk spacer and an ITAM containing endodomain; the second CAR recognizes CD33, has a mouse CD8 stalk spacer and an ITIM containing endodomain. **B** Structure and activity of the control ITIM based gate where the mouse CD8 stalk spacer has been replaced by an Fc domain. This gate is composed of two CARs: the first recognizes CD19, has a human CD8 stalk spacer and an ITAM containing endodomain; the second CAR recognizes CD33, has an Fc spacer and an ITIM containing endodomain. Both gates respond to CD19 single positive targets, while only the original gate is inactive in response to CD19 and CD33 double positive targets.

Figure 24: Kinetic segregation model of CAR logic gates

Model of kinetic segregation and behaviour of AND gate, NOT AND gate and controls. CARs recognize either CD19 or CD33. The immunological synapse can be imagined between the blue line, which represents the target cell membrane and the red line, which represents the T-cell membrane. '45' is the native CD45 protein present on T-cells. 'H8' is a CAR ectodomain with human CD8 stalk as the spacer. 'Fc' is a CAR ectodomain with human HCH2CH3 as the spacer. 'M8' is a CAR ectodomain with murine CD8 stalk as the spacer. '19' represents CD19 on the target cell surface. '33' represents CD33 on the target cell surface. The symbol '⊕' represents an activating endodomain containing ITAMS. The symbol '⊖' represents a phosphatase with slow kinetics - a 'ligation on' endodomain such as one comprising of the catalytic domain of PTPN6 or an ITIM. The symbol '∅' represents a phosphatase with fast kinetics - a 'ligation off' endodomain such as the endodomain of CD45 or CD148. This symbol is enlarged in the figure to emphasize its potent activity.

(a) Shows the postulated behaviour of the functional AND gate which comprises of a pair of CARs whereby the first CAR recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; and the second CAR recognizes CD33, has an Fc spacer and a CD148 endodomain;

(b) Shows the postulated behaviour of the control AND gate. Here, the first CAR recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; and the second CAR recognizes CD33, but has a mouse CD8 stalk spacer and a CD148 endodomain;

(c) Shows the behaviour of a functional AND NOT gate which comprises of a pair of CARs whereby the first CAR recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; and the second CAR recognizes CD33, has a mouse CD8 stalk spacer and a PTPN6 endodomain;

(d) Shows the postulated behaviour of the control AND NOT gate which comprises of a pair of CARs whereby the first CAR recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; and the second CAR recognizes CD33, but has an Fc spacer and a PTPN6 endodomain;

In the first column, target cells are both CD19 and CD33 negative. In the second column, targets are CD19 negative and CD33 positive. In the third column, target cells are CD19 positive and CD33 negative. In the fourth column, target cells are positive for both CD19 and CD33.

Figure 25: Design of APRIL-based CARs.

The CAR design was modified so that the scFv was replaced with a modified form of A proliferation-inducing ligand (APRIL), which interacts with interacts with BCMA, TACI and proteoglycans, to act as an antigen binding domain: APRIL was truncated so that the proteoglycan binding amino-terminus is absent. A signal peptide was then attached to truncated APRIL amino-terminus to direct the protein to the cell surface. Three CARs were generated with this APRIL based binding domain: A. In the first CAR, the human CD8 stalk domain was used as a spacer domain. B. In the second CAR, the hinge from IgG1 was used as a spacer domain. C. In the third CAR, the hinge, CH2 and CH3 domains of human IgG1 modified with the pva/a mutations described by Hombach et al (2010 Gene Ther. 17:1206-1213) to reduce Fc Receptor binding was used as a spacer (henceforth referred as Fc-pvaa). In all CARs, these spacers were connected to the CD28 transmembrane domain and then to a tripartite endodomain containing a fusion of the CD28, OX40 and the CD3-Zeta endodomain (Pule et al, Molecular therapy, 2005: Volume 12; Issue 5; Pages 933-41).

Figure 26: Annotated Amino acid sequence of the above three APRIL-CARS

A: Shows the annotated amino acid sequence of the CD8 stalk APRIL CAR; B: Shows the annotated amino acid sequence of the APRIL IgG1 hinge based CAR; C: Shows the annotated amino acid sequence of the APRIL Fc-pvaa based CAR.

Figure 27: Expression and ligand binding of different APRIL based CARs

A. The receptors were co-expressed with a marker gene truncated CD34 in a retroviral gene vector. Expression of the marker gene on transduced cells allows confirmation of transduction. B. T-cells were transduced with APRIL based CARs with either the CD8 stalk

spacer, IgG1 hinge or Fc spacer. To test whether these receptors could be stably expressed on the cell surface, T-cells were then stained with anti-APRIL-biotin/Streptavidin APC and anti-CD34. Flow-cytometric analysis was performed. APRIL was equally detected on the cell surface in the three CARs suggesting they are equally stably expressed. C. Next, the capacity of the CARs to recognize TACI and BCMA was determined. The transduced T-cells were stained with either recombinant BCMA or TACI fused to mouse IgG2a Fc fusion along with an anti-mouse secondary and anti-CD34. All three receptor formats showed binding to both BCMA and TACI. A surprising finding was that binding to BCMA seemed greater than to TACI. A further surprising finding was that although all three CARs were equally expressed, the CD8 stalk and IgG1 hinge CARs appeared better at recognizing BCMA and TACI than that with the Fc spacer.

Figure 28: Function of the different CAR constructs.

Functional assays were performed with the three different APRIL based CARs. Normal donor peripheral blood T-cells either non-transduced (NT), or transduced to express the different CARs. Transduction was performed using equal titer supernatant. These T-cells were then CD56 depleted to remove non-specific NK activity and used as effectors. SupT1 cells either non-transduced (NT), or transduced to express BCMA or TACI were used as targets. Data shown is mean and standard deviation from 5 independent experiments. A. Specific killing of BCMA and TACI expressing T-cells was determined using Chromium release. B. Interferon-μ release was also determined. Targets and effectors were co-cultured at a ratio of 1:1. After 24 hours, Interferon-μ in the supernatant was assayed by ELISA. C. Proliferation / survival of CAR T-cells were also determined by counting number of CAR T-cells in the same co-culture incubated for a further 6 days. All 3 CARs direct responses against BCMA and TACI expressing targets. The responses to BCMA were greater than for TACI.

Figure 29: AND gate functionality in primary cells

PBMCs were isolated from blood and stimulated using PHA and IL-2. Two days later the cells were transduced on retronectin coated plates with retro virus containing the CD19:CD33 AND gate construct. On day 5 the expression level of the two CARs translated by the AND gate construct was evaluated via flow cytometry and the cells were depleted of CD56+ cells (predominantly NK cells). On day 6 the PBMCs were placed in a co-culture with target cells at a 1:2 effector to target cell ratio. On day 8 the supernatant was collected and analysed for IFN-gamma secretion via ELISA.

Figure 30: A selection / hierarchy of possible spacer domains of increasing size is shown. The ectodomain of CD3-Zeta is suggested as the shortest possible spacer, followed by the (b) the IgG1 hinge. (c) murine or human CD8 stalk and the CD28 ectodomains are considered intermediate in size and co-segregate. (d) The hinge, CH2 and CH3 domain of IgG1 is bigger and bulkier, and (e) the hinge, CH2, CH3 and CH4 domain of IgM is bigger still. Given the properties of the target molecules, and the epitope of the binding domains on said target molecules, it is possible to use this hierarchy of spacers to create a CAR signaling system which either co-segregates or segregates apart upon synapse formation.

Figure 31: Design rules for building logic gated CAR T-cells.
OR, AND NOT and AND gated CARs are shown in cartoon format with the target cell on top, and the T-cell at the bottom with the synapse in the middle. Target cells express arbitrary target antigens A, and B.
T-cells express two CARs which comprise of anti-A and anti-B recognition domains, spacers and endodomains. An OR gate requires (1) spacers simply which allow antigen recognition and CAR activation, and (2) both CARs to have activatory endodomains; An AND NOT gate requires (1) spacers which result in co-segregation of both CARs upon recognition of both antigens and (2) one CAR with an activatory endodomain, and the other whose endodomain comprises or recruits a weak phosphatase; An AND gate requires (1) spacers which result in segregation of both CARs into different parts of the immunological synapse upon recognition of both antigens and (2) one CAR with an activatory endodomain, and the other whose endodomain comprises of a potent phosphatase.

SUMMARY OF ASPECTS OF THE INVENTION

The present inventors have developed a panel of "logic-gated" chimeric antigen receptor pairs which, when expressed by a cell, such as a T cell, are capable of detecting a particular pattern of expression of at least two target antigens. If the at least two target antigens are arbitrarily denoted as antigen A and antigen B, the three possible options are as follows:

"OR GATE" – T cell triggers when either antigen A or antigen B is present on the target cell
"AND GATE" – T cell triggers only when both antigens A and B are present on the target cell
"AND NOT GATE" – T cell triggers if antigen A is present alone on the target cell, but not if both antigens A and B are present on the target cell

Engineered T cells expressing these CAR combinations can be tailored to be exquisitely specific for cancer cells, based on their particular expression (or lack of expression) of two or more markers.

Thus in a first aspect, the present invention provides a cell which co-expresses a first chimeric antigen receptor (CAR) and second CAR at the cell surface, each CAR comprising:

(i) an antigen-binding domain;

(ii) a spacer

(iii) a trans-membrane domain; and

(iv) an intracellular T cell signaling domain (endodomain)

wherein the antigen binding domains of the first and second CARs bind to different antigens, and wherein the spacer of the first CAR is different to the spacer of the second CAR, such that the first and second CARs do not form heterodimers, and wherein
one of the first or second CARs is an activating CAR comprising an activating intracellular T cell signaling domain and the other CAR is an inhibitory CAR comprising a "ligation-off" (as defined herein) inhibitory intracellular T cell signaling domain.

The cell may be an immune effector cell, such as a T-cell or natural killer (NK) cell. Features mentioned herein in connection with a T cell apply equally to other immune effector cells, such as NK cells.

The spacer of the first CAR may have a different length and/or charge and/or shape and/or configuration and/or glycosylation to the spacer of the second CAR, such that when the first CAR and the second CAR bind their respective target antigens, the first CAR and second CAR become spatially separated on the T cell. Ligation of the first and second CARs to their respective antigens causes them to be compartmentalized together or separately in the immunological synapse resulting in control of activation. This may be understood when one considers the kinetic separation model of T-cell activation (see below).

The first spacer or the second spacer may comprise a CD8 stalk and the other spacer may comprise the hinge, CH2 and CH3 domain of an IgG1.

In the present invention, which relates to the "AND" gate, one of the first or second CARs is an activating CAR comprising an activating endodomain, and the other CAR is a "ligation-off" inhibitory CAR comprising an inhibitory endodomain. The ligation-off inhibitory CAR inhibits T-cell activation by the activating CAR in the absence of inhibitory CAR ligation, but does not significantly inhibit T-cell activation by the activating CAR when the inhibitory CAR is ligated.

Since the spacer of the first CAR has a different length and/or charge and/or shape and/or configuration and/or glycosylation from the spacer of the second CAR, when both CARs are ligated they segregate. This causes the inhibitory CAR to be spatially separated from the activating CAR, so that T cell activation can occur. T cell activation therefore only occurs in response to a target cell bearing both cognate antigens.

The inhibitory endodomain may comprise all or part of the endodomain from a receptor-like tyrosine phosphatase, such as CD148 or CD45.

The antigen-binding domain of the first CAR may bind CD5 and the antigen-binding domain of the second CAR may bind CD19. This is of use in targeting chronic lymphocytic leukaemia (CLL). This disease can be treated by targeting CD19 alone, but at the cost of depleting the entire B-cell compartment. CLL cells are unusual in that they co-express CD5 and CD19. Targeting this pair of antigens with an AND gate will increase specificity and reduce toxicity.

In a second aspect, the present invention provides a nucleic acid sequence encoding both the first and second chimeric antigen receptors (CARs) as defined in the first aspect of the invention.

The nucleic acid sequence according may have the following structure:AgB1-spacer1-TM1-endo1-coexpr-AgB2-spacer2-TM2-endo2

in which
AgB1 is a nucleic acid sequence encoding the antigen-binding domain of the first CAR;
spacer 1 is a nucleic acid sequence encoding the spacer of the first CAR;
TM1 is a a nucleic acid sequence encoding the transmembrane domain of the first CAR;
endo 1 is a nucleic acid sequence encoding the endodomain of the first CAR;
coexpr is a nucleic acid sequence allowing co-expression of two CARs (e.g. a cleavage site);
AgB2 is a nucleic acid sequence encoding the antigen-binding domain of the second CAR;
spacer 2 is a nucleic acid sequence encoding the spacer of the second CAR;
TM2 is a a nucleic acid sequence encoding the transmembrane domain of the second CAR;
endo 2 is a nucleic acid sequence encoding the endodomain of the second CAR;
which nucleic acid sequence, when expressed in a T cell, encodes a polypeptide which is cleaved at the cleavage site such that the first and second CARs are co-expressed at the T cell surface.

The nucleic acid sequence allowing co-expression of two CARs may encode a self-cleaving peptide or a sequence which allows alternative means of co-expressing two CARs such as an internal ribosome entry sequence or a 2nd promoter or other such means whereby one skilled in the art can express two proteins from the same vector.

Alternative codons may be used in regions of sequence encoding the same or similar amino acid sequences, in order to avoid homologous recombination.

In a third aspect, the present invention provides a kit which comprises

(i) a first nucleic acid sequence encoding the first chimeric antigen receptor (CAR) as defined in the first aspect of the invention, which nucleic acid sequence has the following structure:

AgB1-spacer1-TM1-endo1

in which

AgB1 is a nucleic acid sequence encoding the antigen-binding domain of the first CAR;
spacer 1 is a nucleic acid sequence encoding the spacer of the first CAR;
TM1 is a a nucleic acid sequence encoding the transmembrane domain of the first CAR;
endo 1 is a nucleic acid sequence encoding the endodomain of the first CAR; and

(ii) a second nucleic acid sequence encoding the second chimeric antigen receptor (CAR) as defined in the first aspect of the invention, which nucleic acid sequence has the following structure:

AgB2-spacer2-TM2-endo2

AgB2 is a nucleic acid sequence encoding the antigen-binding domain of the second CAR;
spacer 2 is a nucleic acid sequence encoding the spacer of the second CAR;
TM2 is a a nucleic acid sequence encoding the transmembrane domain of the second CAR;
endo 2 is a nucleic acid sequence encoding the endodomain of the second CAR.

In a fourth aspect, the present invention provides a kit comprising: a first vector which comprises the first nucleic acid sequence as defined above; and a second vector which comprises the first nucleic acid sequence as defined above.

The vectors may be plasmid vectors, retroviral vectors or transposon vectors. The vectors may be lentiviral vectors.

In a fifth aspect, the present invention provides a vector comprising a nucleic acid sequence according to the second aspect of the invention. The vector may be a lentiviral vector.

The vector may be a plasmid vector, a retroviral vector or a transposon vector.

In a sixth aspect, the present invention involves co-expressing more than two CARs in such a fashion that a complex pattern of more than two antigens can be recognized on the target cell.

In a seventh aspect, the present invention provides a method for making a T cell according to the first aspect of the invention, which comprises the step of introducing one or more nucleic acid sequence (s) encoding the first and second CARs; or one or more vector(s) as defined above into a T cell.

The T cell may be from a sample isolated from a patient, a related or unrelated haematopoietic transplant donor, a completely unconnected donor, from cord blood, differentiated from an embryonic cell line, differentiated from an inducible progenitor cell line, or derived from a transformed T cell line.

In an eighth aspect, the present invention provides a pharmaceutical composition comprising a plurality of T cells according to the first aspect of the invention.

In a ninth aspect, the present invention provides a method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition according to the eighth aspect of the invention to a subject.

The method may comprise the following steps:

(i) isolation of a T cell as listed above.

(ii) transduction or transfection of the T cells with one or more nucleic acid sequence(s) encoding the first and second CAR or one or more vector(s) comprising such nucleic acid sequence(s); and

(iii) administering the T cells from (ii) to the subject.

The disease may be a cancer.

In a tenth aspect, the present invention provides a pharmaceutical composition according to the eighth aspect of the invention for use in treating and/or preventing a disease.

The disease may be a cancer.

In an eleventh aspect, the present invention provides use of a T cell according to the first aspect of the invention in the manufacture of a medicament for treating and/or preventing a disease.

The disease may be a cancer.

The present invention also provides a nucleic acid sequence which comprises:
a) a first nucleotide sequence encoding a first chimeric antigen receptor (CAR);
b) a second nucleotide sequence encoding a second CAR;
c) a sequence encoding a self-cleaving peptide positioned between the first and second nucleotide sequences, such that the two CARs are expressed as separate entities.

Alternative codons may be used in one or more portion(s) of the first and second nucleotide sequences in regions which encode the same or similar amino acid sequence(s).

The present invention also provides a vector and a cell comprising such a nucleic acid.

The kinetic-segregation based AND gate of the present invention offers a significant technical advantage to the previously described "co-CAR", i.e. the dual targeting approach in which two antigens are recognized by two CARs which supply either an activating or a co-stimulating signal to the T-cell.

With the co-CAR approach, although greatest activity might be expected against target cells bearing both antigens, considerable activity against tissues bearing only antigen recognized by the activating CAR can be expected. This activity can be expected to be at least that of a first-generation CAR. First generation CARs have resulted in considerable toxicity: for instance biliary toxicity was observed in clinical testing of a first generation CAR recognizing Carbonic anhydrase IX which was unexpectedly expressed on biliary epithelium (Rotterdam ref). Notably, terminally differentiated effectors do not require or respond to co-stimulatory signals, so any terminally differentiated CAR T-cells would act maximally despite the absence of a co-stimulatory CAR signal.

Further, co-stimulatory signals lead to long-lasting effects on the T-cell population. These effects long outlast the T-cell / target synapse interaction. Consequently, CAR T-cells which

become fully activated within the tumour and migrate could have maximally potent activity against single-antigen bearing normal tissues. This "spill-over" effect may be most pronounced in tissues within, near or which drain from the tumour. In fact, strategies based on the concept of the activity of a first generation CAR being enhanced by co-stimulatory signals engaged not CAR activation but through a distinct receptor, have been proposed and tested (Rossig, Blood. 2002 Mar 15;99(6):2009-16.).

The co-CAR approach hence can be expected to result at best to a reduction but not abolition of toxicity towards single antigen expressing normal tissue. The present invention uses kinetic segregation at the immunological synapse formed between the T-cell / target cell to regulate T-cell triggering itself. Consequently tight absolute control of triggering in the absence of the second antigen is achieved. Hence the totality of T-cell activation is restricted to target cells expressing both antigens, the AND gate should function irrespective of the effector cell type or differentiation state, and no "spill-over" effect AND gate T-cell activation is possible.

FURTHER ASPECTS OF THE INVENTION

The present invention also relates to the aspects listed in the following numbered paragraphs:

1. A T cell which co-expresses a first chimeric antigen receptor (CAR) and second CAR at the cell surface, each CAR comprising:

(i) an antigen-binding domain;

(ii) a spacer

(iii) a trans-membrane domain; and

(iv) an endodomain

wherein the antigen binding domains of the first and second CARs bind to different antigens, wherein the spacer of the first CAR is different to the spacer of the second CAR and wherein one of the first or second CARs is an activating CAR comprising an activating endodomain and the other CAR is either an activating CAR comprising an activating endodomain or an inhibitory CAR comprising a ligation-on or ligation-off inhibitory endodomain.

2. A T cell according to paragraph 1, wherein the spacer of the first CAR has a different length and/or charge and/or size and/or configuration and/or glycosylation of the spacer of the second CAR, such that when the first CAR and the second CAR bind their respective

target antigens, the first CAR and second CAR become spatially separated on the T cell membrane.

3. A T cell according to paragraph 2, wherein either the first spacer or the second spacer comprises a CD8 stalk and the other spacer comprises the hinge, CH2 and CH3 domain of IgG1.

4. A T cell according to paragraph 1, wherein both the first and second CARs are activating CARs.

5. A T cell according to paragraph 4, wherein one CAR binds CD19 and the other CAR binds CD20.

6. A T cell according to paragraph 2 or 3, wherein one of the first or second CARs is an activating CAR comprising an activating endodomain, and the other CAR is an inhibitory CAR comprising a ligation-off inhibitory endodomain, which inhibitory CAR inhibits T-cell activation by the activating CAR in the absence of inhibitory CAR ligation, but does not significantly inhibit T-cell activation by the activating CAR when the inhibitory CAR is ligated.

7. A T cell according to paragraph 6, wherein the inhibitory endodomain comprises all or part of the endodomain from CD148 or CD45.

8. A T cell according to paragraph 6 or 7, wherein the antigen-binding domain of the first CAR binds CD5 and the antigen-binding domain of the second CAR binds CD19.

9. A T cell according to paragraph 1 wherein the first and second spacers are sufficiently different so as to prevent cross-pairing of the first and second CARs but are sufficiently similar to result in co-localisation of the first and second CARs following ligation.

10. A T cell according to paragraph 9, wherein one of the first or second CARs in an activating CAR comprising an activating endodomain, and the other CAR is an inhibitory CAR comprising a ligation-on inhibitory endodomain, which inhibitory CAR does not significantly inhibit T-cell activation by the activating CAR in the absence of inhibitory CAR ligation, but inhibits T-cell activation by the activating CAR when the inhibitory CAR is ligated.

11. A T cell according to paragraph 10, wherein the ligation-on inhibitory endodomain comprises at least part of a phosphatase.

12. A T cell according to paragraph 11, wherein the ligation-on inhibitory endodomain comprises all or part of PTPN6.

13. A T cell according to paragraph 10, wherein the ligation-on inhibitory endodomain comprises at least one ITIM domain.

14. A T cell according to paragraph 13, wherein activity of the ligation-on inhibitory endodomain is enhanced by co-expression of a PTPN6-CD45 or –CD148 fusion protein.

15. A T cell according to any of paragraphs 10 to 14, wherein the CAR comprising the activating endodomain comprises an antigen-binding domain which binds CD33 and the CAR which comprises the ligation-on inhibitory endodomain comprises an antigen-binding domain which binds CD34.

16. A T cell which comprises more than two CARs as defined in the preceding paragraphs such that it is specifically stimulated by a cell, such as a T cell, bearing a distinct pattern of more than two antigens.

17. A nucleic acid sequence encoding both the first and second chimeric antigen receptors (CARs) as defined in any of paragraphs 1 to 16.

18. A nucleic acid sequence according to paragraph 17, which has the following structure:

AgB1-spacer1-TM1-endo1-coexpr-AbB2-spacer2-TM2-endo2

in which

AgB1 is a nucleic acid sequence encoding the antigen-binding domain of the first CAR;
spacer 1 is a nucleic acid sequence encoding the spacer of the first CAR;
TM1 is a a nucleic acid sequence encoding the transmembrane domain of the first CAR;
endo 1 is a nucleic acid sequence encoding the endodomain of the first CAR;
coexpr is a nucleic acid sequence enabling co-expression of both CARs
AgB2 is a nucleic acid sequence encoding the antigen-binding domain of the second CAR;
spacer 2 is a nucleic acid sequence encoding the spacer of the second CAR;

TM2 is a a nucleic acid sequence encoding the transmembrane domain of the second CAR;
endo 2 is a nucleic acid sequence encoding the endodomain of the second CAR;
which nucleic acid sequence, when expressed in a T cell, encodes a polypeptide which is cleaved at the cleavage site such that the first and second CARs are co-expressed at the T cell surface.

19. A nucleic acid sequence according to paragraph 18, wherein coexpr encodes a sequence comprising a self-cleaving peptide.

20. A nucleic acid sequence according to paragraph 18 or 19, wherein alternative codons are used in regions of sequence encoding the same or similar amino acid sequences, in order to avoid homologous recombination.

21. A kit which comprises

(i) a first nucleic acid sequence encoding the first chimeric antigen receptor (CAR) as defined in any of paragraphs 1 to 16, which nucleic acid sequence has the following structure:

AgB1-spacer1-TM1-endo1
in which
AgB1 is a nucleic acid sequence encoding the antigen-binding domain of the first CAR;
spacer 1 is a nucleic acid sequence encoding the spacer of the first CAR;
TM1 is a nucleic acid sequence encoding the transmembrane domain of the first CAR;
endo 1 is a nucleic acid sequence encoding the endodomain of the first CAR; and

(ii) a second nucleic acid sequence encoding the second chimeric antigen receptor (CAR) as defined in any of paragraphs 1 to 16, which nucleic acid sequence has the following structure:

AgB2-spacer2-TM2-endo2
AgB2 is a nucleic acid sequence encoding the antigen-binding domain of the second CAR;
spacer 2 is a nucleic acid sequence encoding the spacer of the second CAR;
TM2 is a a nucleic acid sequence encoding the transmembrane domain of the second CAR;
endo 2 is a nucleic acid sequence encoding the endodomain of the second CAR.

22. A kit comprising: a first vector which comprises the first nucleic acid sequence as defined in paragraph 21; and a second vector which comprises the first nucleic acid sequence as defined in paragraph 21.

23. A kit according to paragraph 22, wherein the vectors are integrating viral vectors or transposons.

24. A vector comprising a nucleic acid sequence according to any of paragraphs 17 to 20.

25. A retroviral vector or a lentiviral vector or a transposon according to paragraph 24.

26. A method for making a T cell according to any of paragraphs 1 to 16, which comprises the step of introducing: a nucleic acid sequence according to any of paragraphs 17 to 20; a first nucleic acid sequence and a second nucleic acid sequence as defined in paragraph 21; and/or a first vector and a second vector as defined in paragraph 22 or a vector according to paragraph 24 or 25, into a T cell.

27. A method according to paragraph 24, wherein the T cell is from a sample isolated from a subject.

28. A pharmaceutical composition comprising a plurality of T cells according to any of paragraphs 1 to 16.

29. A method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition according to paragraph 28 to a subject.

30. A method according to paragraph 29, which comprises the following steps:

(i) isolation of a T cell-containing sample from a subject;

(ii) transduction or transfection of the T cells with: a nucleic acid sequence according to any of paragraphs 17 to 20; a first nucleic acid sequence and a second nucleic acid sequence as defined in paragraph 21; a first vector and a second vector as defined in paragraph 22 or 23 or a vector according to paragraph 24 or 25; and

(iii) administering the T cells from (ii) to a the subject.

31. A method according to paragraph 29 or 30, wherein the disease is a cancer.

32. A pharmaceutical composition according to paragraph 28 for use in treating and/or preventing a disease.

33. The use of a T cell according to any of paragraphs 1 to 16 in the manufacture of a medicament for treating and/or preventing a disease.

DETAILED DESCRIPTION

CHIMERIC ANTIGEN RECEPTORS (CARs)

CARs, which are shown schematically in Figure 1, are chimeric type I trans-membrane proteins which connect an extracellular antigen-recognizing domain (binder) to an intracellular signalling domain (endodomain). The binder is typically a single-chain variable fragment (scFv) derived from a monoclonal antibody (mAb), but it can be based on other formats which comprise an antibody-like antigen binding site. A spacer domain is usually necessary to isolate the binder from the membrane and to allow it a suitable orientation. A common spacer domain used is the Fc of IgG1. More compact spacers can suffice e.g. the stalk from CD8α and even just the IgG1 hinge alone, depending on the antigen. A trans-membrane domain anchors the protein in the cell membrane and connects the spacer to the endodomain.

Early CAR designs had endodomains derived from the intracellular parts of either the γ chain of the FcεR1 or CD3ζ. Consequently, these first generation receptors transmitted immunological signal 1, which was sufficient to trigger T-cell killing of cognate target cells but failed to fully activate the T-cell to proliferate and survive. To overcome this limitation, compound endodomains have been constructed: fusion of the intracellular part of a T-cell co-stimulatory molecule to that of CD3ζ results in second generation receptors which can transmit an activating and co-stimulatory signal simultaneously after antigen recognition. The co-stimulatory domain most commonly used is that of CD28. This supplies the most potent co-stimulatory signal - namely immunological signal 2, which triggers T-cell proliferation. Some receptors have also been described which include TNF receptor family endodomains, such as the closely related OX40 and 41BB which transmit survival signals. Even more potent third generation CARs have now been described which have endodomains capable of transmitting activation, proliferation and survival signals.

CAR-encoding nucleic acids may be transferred to T cells using, for example, retroviral vectors. Lentiviral vectors may be employed. In this way, a large number of cancer-specific T cells can be generated for adoptive cell transfer. When the CAR binds the target-antigen, this results in the transmission of an activating signal to the T-cell it is expressed on. Thus

the CAR directs the specificity and cytotoxicity of the T cell towards tumour cells expressing the targeted antigen.

The first aspect of the invention relates to a T-cell which co-expresses a first CAR and a second CAR such that a T-cell can recognize a desired pattern of expression on target cells in the manner of a logic gate as detailed in the truth tables: table 1, 2 and 3.

Both the first and second (and optionally subsequent) CARs comprise:
(i) an antigen-binding domain;
(ii) a spacer;
(iii) a transmembrane domain; and
(iii) an intracellular domain.

Table 1: Truth Table for CAR OR GATE

Antigen A	*Antigen B*	*Response*
Absent	Absent	No activation
Absent	Present	Activation
Present	Absent	Activation
Present	Present	Activation

Table 2: Truth Table for CAR AND GATE

Antigen A	*Antigen B*	*Response*
Absent	Absent	No activation
Absent	Present	No Activation
Present	Absent	No Activation
Present	Present	Activation

Table 3: Truth Table for CAR AND NOT GATE

Antigen A	*Antigen B*	*Response*
Absent	Absent	No activation
Absent	Present	No Activation
Present	Absent	Activation
Present	Present	No Activation

The first and second CAR of the T cell of the present invention may be produced as a polypeptide comprising both CARs, together with a cleavage site.

SEQ ID No. 1 to 5 give examples of such polypeptides, which each comprise two CARs. The CAR may therefore comprise one or other part of the following amino acid sequences, which corresponds to a single CAR.

SEQ ID No 1 is a CAR OR gate which recognizes CD19 OR CD33
SEQ ID No 2 Is a CAR AND gate which recognizes CD19 AND CD33 using a CD148 phosphatase
SEQ ID No 3 Is an alternative implementation of the CAR AND GATE which recognizes CD19 AND CD33 which uses a CD45 phosphatase
SEQ ID No 4 Is a CAR AND NOT GATE which recognizes CD19 AND NOT CD33 based on PTPN6 phosphatase
SEQ ID No 5 Is an alternative implementation of the CAR AND NOT gate which recognizes CD19 AND NOT CD33 and is based on an ITIM containing endodomain from LAIR1
SEQ ID No 6. Is a further alternative implementation of the CAR AND NOT gate which recognizes CD19 AND NOT CD33 and recruits a PTPN6-CD148 fusion protein to an ITIM containing endodomain.

SEQ ID No. 1

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS
GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC
AAQDAYTGGYFDYWGQGTLVTVSSMDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPK
DTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK

GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGKKDPKFWVLVVVGGVLACYSLLVTVAFIIFWVRSRVKFSRSADAPA
YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA
YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 2

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS
GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC
AAQDAYTGGYFDYWGQGTLVTVSSMDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPK
DTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGKKDPKAVFGCIFGALVIVTVGGFIFWRKKRKDAKNNEVSFSQIKPK
KSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVLPY
DISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLT
KCVEQGRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHF
TSWPDHGVPDTTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENEN
TVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTTAMTIYENLAP
VTTFGKTNGYIA

SEQ ID No. 3

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL

YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS
GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC
AAQDAYTGGYFDYWGQGTLVTVSSMDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPK
DTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGKKDPKALIAFLAFLIIVTSIALLVVLYKIYDLHKKRSCNLDEQQELVER
DDEKQLMNVEPIHADILLETYKRKIADEGRLFLAEFQSIPRVFSKFPIKEARKPFNQNKNRYV
DILPYDYNRVELSEINGDAGSNYINASYIDGFKEPRKYIAAQGPRDETVDDFWRMIWEQKAT
VIVMVTRCEEGNRNKCAEYWPSMEEGTRAFGDVVVKINQHKRCPDYIIQKLNIVNKKEKAT
GREVTHIQFTSWPDHGVPEDPHLLLKLRRRVNAFSNFFSGPIVVHCSAGVGRTGTYIGIDA
MLEGLEAENKVDVYGYVVKLRRQRCLMVQVEAQYILIHQALVEYNQFGETEVNLSELHPYL
HNMKKRDPPSEPSPLEAEFQRLPSYRSWRTQHIGNQEENKSKNRNSNVIPYDYNRVPLKH
ELEMSKESEHDSDESSDDDSDSEEPSKYINASFIMSYWKPEVMIAAQGPLKETIGDFWQMI
FQRKVKVIVMLTELKHGDQEICAQYWGEGKQTYGDIEVDLKDTDKSSTYTLRVFELRHSKR
KDSRTVYQYQYTNWSVEQLPAEPKELISMIQVVKQKLPQKNSSEGNKHHKSTPLLIHCRDG
SQQTGIFCALLNLLESAETEEVVDIFQVVKALRKARPGMVSTFEQYQFLYDVIASTYPAQNG
QVKKNNHQEDKIEFDNEVDKVKQDANCVNPLGAPEKLPEAKEQAEGSEPTSGTEGPEHSV
NGPASPALNQGS

SEQ ID No. 4

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS
GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC

AAQDAYTGGYFDYWGQGTLVTVSSMDPATTTKPVLRTPSPVHPTGTSQPQRPEDCRPRG
SVKGTGLDFACDIYWAPLAGICVALLLSLIITLICYHRSRKRVCKSGGGSFWEEFESLQKQEV
KNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINANYIKNQLLGPDENA
KTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVPYWPEVGMQRAYGPY
SVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFLDQINQ
RQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTE
AQYKFIYVAIAQFIETTKKKL

SEQ ID No. 5

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS
GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC
AAQDAYTGGYFDYWGQGTLVTVSSMDPATTTKPVLRTPSPVHPTGTSQPQRPEDCRPRG
SVKGTGLDFACDILIGVSVVFLFCLLLLVLFCLHRQNQIKQGPPRSKDEEQKPQQRPDLAVD
VLERTADKATVNGLPEKDRETDTSALAAGSSQEVTYAQLDHWALTQRTARAVSPQSTKPM
AESITYAAVARH

SEQ ID No. 6

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPD
GTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTK
LEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYG
VSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC
AKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE
RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQ
VLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLI
YDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRS

GGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYC
AAQDAYTGGYFDYWGQGTLVTVSSMDPATTTKPVLRTPSPVHPTGTSQPQRPEDCRPRG
SVKGTGLDFACDILIGVSVVFLFCLLLLVLFCLHRQNQIKQGPPRSKDEEQKPQQRPDLAVD
VLERTADKATVNGLPEKDRETDTSALAAGSSQEVTYAQLDHWALTQRTARAVSPQSTKPM
AESITYAAVARHRAEGRGSLLTCGDVEENPGPWYHGHMSGGQAETLLQAKGEPWTFLVR
ESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKT
GIEEASGAFVYLRQPYSGGGGSFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAE
NRGKNRYNNVLPYDISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWR
MVWEKNVYAIIMLTKCVEQGRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNI
QTSESHPLRQFHFTSWPDHGVPDTTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTGT
FIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCVLDIVRSQKDSKVDLIY
QNTTAMTIYENLAPVTTFGKTNGYIASGS

The CAR may comprise a variant of the CAR-encoding part of the sequence shown as SEQ ID No. 1, 2, 3, 4, 5 or 6 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence is a CAR having the required properties.

Methods of sequence alignment are well known in the art and are accomplished using suitable alignment programs. The % sequence identity refers to the percentage of amino acid or nucleotide residues that are identical in the two sequences when they are optimally aligned. Nucleotide and protein sequence homology or identity may be determined using standard algorithms such as a BLAST program (Basic Local Alignment Search Tool at the National Center for Biotechnology Information) using default parameters. Other algorithms for determining sequence identity or homology include: LALIGN, AMAS (Analysis of Multiply Aligned Sequences), FASTA, Clustal Omega, SIM, and EMBOSS Needle.

CAR LOGICAL OR GATE

In this embodiment, the antigen binding domains of the first and second CARs of the present invention bind to different antigens and both CARs comprise an activating endodomain. Both CARs have different spacer domains to prevent cross-pairing of the two different receptors. A T cell can hence be engineered to activate upon recognition of either or both antigens.

This is useful in the field of oncology as indicated by the Goldie-Coldman hypothesis: sole targeting of a single antigen may result in tumour escape by modulation of said antigen due to the high mutation rate inherent in most cancers. By simultaneously targeting two antigens, the probably of such escape is exponentially reduced.

Various tumour associated antigens are known as shown in the following Table 4. For a given disease, the first CAR and second CAR may bind to two different TAAs associated with that disease. In this way, tumour escape by modulating a single antigen is prevented, since a second antigen is also targeted. For example, when targeting a B-cell malignancy, both CD19 and CD20 can be simultaneously targeted. In this embodiment, it is important that the two CARs do not heterodimerize.

TABLE 4

Cancer type	**TAA**
Diffuse Large B-cell Lymphoma	CD19, CD20
Breast cancer	ErbB2, MUC1
AML	CD13, CD33
Neuroblastoma	GD2, NCAM
B-CLL	CD19, CD52
Colorectal cancer	Folate binding protein, CA-125

KINETIC SEGREGATION MODEL

Subsequent pairing of CARs to generate the AND gate and the AND NOT gate are based on the kinetic segregation model (KS) of T-cell activation. This is a functional model, backed by experimental data, which explains how antigen recognition by a T-cell receptor is converted into down-stream activation signals. Briefly: at the ground state, the signalling components on the T-cell membrane are in dynamic homeostasis whereby dephosphorylated ITAMs are favoured over phosphorylated ITAMs. This is due to greater activity of the transmembrane CD45/CD148 phosphatases over membrane-tethered kinases such as lck. When a T-cell engages a target cell through a T-cell receptor (or CAR) recognition of cognate antigen, tight immunological synapses form. This close juxtapositioning of the T-cell and target membranes excludes CD45/CD148 due to their large ectodomains which cannot fit into the synapse. Segregation of a high concentration of T-cell receptor associated ITAMs and kinases in the synapse, in the absence of phosphatases, leads to a state whereby phosphorylated ITAMs are favoured. ZAP70 recognizes a threshold of phosphorylated ITAMs and propagates a T-cell activation signal. This advanced understanding of T-cell

activation is exploited by the present invention. In particular, the invention is based on this understanding of how ectodomains of different length and/or bulk and/or charge and/or configuration and/or glycosylation result in differential segregation upon synapse formation.

THE CAR LOGICAL AND GATE

In this embodiment, one CAR comprises an activating endodomain and one CAR comprises an inhibitory endodomain whereby the inhibitory CAR constitutively inhibits the first activating CAR, but upon recognition of its cognate antigen releases its inhibition of the activating CAR. In this manner, a T-cell can be engineered to trigger only if a target cell expresses both cognate antigens. This behaviour is achieved by the activating CAR comprising an activating endodomain containing ITAM domains for example the endodomain of CD3 Zeta, and the inhibitory CAR comprising the endodomain from a phosphatase able to dephosphorylate an ITAM (e.g. CD45 or CD148). Crucially, the spacer domains of both CARs are significantly different in size and/or shape and/or charge etc. When only the activating CAR is ligated, the inhibitory CAR is in solution on the T-cell surface and can diffuse in and out of the synapse inhibiting the activating CAR. When both CARs are ligated, due to differences in spacer properties, the activating and inhibiting CAR are differentially segregated allowing the activating CAR to trigger T-cell activation unhindered by the inhibiting CAR.

This is of considerable utility in the field of cancer therapy. Currently, immunotherapies typically target a single antigen. Most cancers cannot be differentiated from normal tissues on the basis of a single antigen. Hence, considerable "on-target off-tumour" toxicity occurs whereby normal tissues are damaged by the therapy. For instance, whilst targeting CD20 to treat B-cell lymphomas with Rituximab, the entire normal B-cell compartment is depleted. For instance, whilst targeting CD52 to treat chronic lymphocytic leukaemia, the entire lymphoid compartment is depleted. For instance, whilst targeting CD33 to treat acute myeloid leukaemia, the entire myeloid compartment is damaged etc. By restricting activity to a pair of antigens, much more refined targeting, and hence less toxic therapy can be developed. A practical example is targeting of CLL which expresses both CD5 and CD19. Only a small proportion of normal B-cells express both antigens, so the off-target toxicity of targeting both antigens with a logical AND gate is substantially less than targeting each antigen individually.

The design of the present invention is a considerable improvement on previous implementation as described by Wilkie *et al.* ((2012). *J. Clin. Immunol.* **32,** 1059–1070) and then tested *in vivo* (Kloss *et al* (2013) *Nat. Biotechnol.* **31,** 71–75). In this implementation, the first CAR comprises of an activating endodomain, and the second a co-stimulatory

domain. This way, a T-cell only receives an activating and co-stimulatory signal when both antigens are present. However, the T-cell still will activate in the sole presence of the first antigen resulting in the potential for off-target toxicity. Further, the implementation of the present invention allows for multiple compound linked gates whereby a cell can interpret a complex pattern of antigens.

TABLE 5

Cancer Type	*Antigens*
Chronic Lymphocytic Leukaemia	CD5, CD19
Neuroblastoma	ALK, GD2
Glioma	EGFR, Vimentin
Multiple myeloma	BCMA, CD138
Renal Cell Carcinoma	Carbonic anhydrase IX, G250
T-ALL	CD2, N-Cadherin
Prostate Cancer	PSMA, hepsin (or others)

THE CAR LOGICAL AND NOT GATE

In this embodiment, one CAR comprises an activating endodomain and one CAR comprises an inhibitory endodomain such that this inhibitory CAR is only active when it recognizes its cognate antigen. Hence a T-cell engineered in this manner is activated in response to the sole presence of the first antigen but is not activated when both antigens are present. This invention is implemented by inhibitory CARs with a spacer that co-localise with the first CAR but either the phosphatase activity of the inhibitory CAR should not be so potent that it inhibits in solution, or the inhibitory endodomain in fact recruits a phosphatase solely when the inhibitory CAR recognizes its cognate target. Such endodomains are termed "ligation-on" or semi-inhibitory herein.

This invention is of use in refining targeting when a tumour can be distinguished from normal tissue by the presence of tumour associated antigen and the loss of an antigen expressed on normal tissue. The AND NOT gate is of considerable utility in the field of oncology as it allows targeting of an antigen which is expressed by a normal cell, which normal cell also expresses the antigen recognised by the CAR comprising the activating endodomain. An example of such an antigen is CD33 which is expressed by normal stem cells and acute myeloid leukaemia (AML) cells. CD34 is expressed on stem cells but not typically expressed on AML cells. A T-cell recognizing CD33 AND NOT CD34 would result in destruction of leukaemia cells but sparing of normal stem cells.

Potential antigen pairs for use with AND NOT gates are shown in Table 6.

TABLE 6

Disease	**TAA**	**Normal cell which expresses TAA**	**Antigen expressed by normal cell but not cancer cell**
AML	CD33	stem cells	CD34
Myeloma	BCMA	Dendritic cells	CD1c
B-CLL	CD160	Natural Killer cells	CD56
Prostate cancer	PSMA	Neural Tissue	NCAM
Bowel cancer	A33	Normal bowel epithelium	HLA class I

COMPOUND GATES

The kinetic segregation model with the above components allows compound gates to be made e.g. a T-cell which triggers in response to patterns of more than two target antigens. For example, it is possible to make a T cell which only triggers when three antigens are present (A AND B AND C). Here, a cell expresses three CARs, each recognizing antigens A, B and C. One CAR is excitatory and two are inhibitory, which each CAR having spacer domains which result in differential segregation. Only when all three are ligated, will the T-cell activate. A further example: (A OR B) AND C: here, CARs recognizing antigens A and B are activating and have spacers which co-localise, while CAR recognizing antigen C is inhibitory and has a spacer which results in different co-segregation. A further example (A AND NOT B) AND C: Here CAR against antigen A has an activating endodomain and co-localises with CAR against antigen B which has a conditionally inhibiting endodomain. CAR against antigen C has a spacer who segregates differently from A or B and is inhibitory. In fact, ever more complex boolean logic can be programmed with these simple components of the invention with any number of CARs and spacers.

SIGNAL PEPTIDE

The CARs of the T cell of the present invention may comprise a signal peptide so that when the CAR is expressed inside a cell, such as a T-cell, the nascent protein is directed to the endoplasmic reticulum and subsequently to the cell surface, where it is expressed.

The core of the signal peptide may contain a long stretch of hydrophobic amino acids that has a tendency to form a single alpha-helix. The signal peptide may begin with a short positively charged stretch of amino acids, which helps to enforce proper topology of the polypeptide during translocation. At the end of the signal peptide there is typically a stretch of amino acids that is recognized and cleaved by signal peptidase. Signal peptidase may cleave either during or after completion of translocation to generate a free signal peptide and a mature protein. The free signal peptides are then digested by specific proteases.

The signal peptide may be at the amino terminus of the molecule.

The signal peptide may comprise the SEQ ID No. 7, 8 or 9 or a variant thereof having 5, 4, 3, 2 or 1 amino acid mutations (insertions, substitutions or additions) provided that the signal peptide still functions to cause cell surface expression of the CAR.

SEQ ID No. 7: MGTSLLCWMALCLLGADHADG

The signal peptide of SEQ ID No. 7 is compact and highly efficient. It is predicted to give about 95% cleavage after the terminal glycine, giving efficient removal by signal peptidase.

SEQ ID No. 8: MSLPVTALLLPLALLLHAARP

The signal peptide of SEQ ID No. 8 is derived from IgG1.

SEQ ID No. 9: MAVPTQVLGLLLLWLTDARC

The signal peptide of SEQ ID No. 9 is derived from CD8.

The signal peptide for the first CAR may have a different sequence from the signal peptide of the second CAR (and from the 3rd CAR and 4th CAR etc).

ANTIGEN BINDING DOMAIN

The antigen binding domain is the portion of the CAR which recognizes antigen. Numerous antigen-binding domains are known in the art, including those based on the antigen binding site of an antibody, antibody mimetics, and T-cell receptors. For example, the antigen-binding domain may comprise: a single-chain variable fragment (scFv) derived from a monoclonal antibody; a natural ligand of the target antigen; a peptide with sufficient affinity

for the target; a single domain antibody; an artificial single binder such as a Darpin (designed ankyrin repeat protein); or a single-chain derived from a T-cell receptor.

The antigen binding domain may comprise a domain which is not based on the antigen binding site of an antibody. For example the antigen binding domain may comprise a domain based on a protein/peptide which is a soluble ligand for a tumour cell surface receptor (e.g. a soluble peptide such as a cytokine or a chemokine); or an extracellular domain of a membrane anchored ligand or a receptor for which the binding pair counterpart is expressed on the tumour cell.

Examples 11 to 13 relate to a CAR which binds BCMA, in which the antigen binding doaimn comprises APRIL, a ligand for BCMA.

The antigen binding domain may be based on a natural ligand of the antigen.

The antigen binding domain may comprise an affinity peptide from a combinatorial library or a *de novo* designed affinity protein/peptide.

SPACER DOMAIN

CARs comprise a spacer sequence to connect the antigen-binding domain with the transmembrane domain and spatially separate the antigen-binding domain from the endodomain. A flexible spacer allows the antigen-binding domain to orient in different directions to facilitate binding.

In the T cell of the present invention, the first and second CARs comprise different spacer molecules. For example, the spacer sequence may, for example, comprise an IgG1 Fc region, an IgG1 hinge or a human CD8 stalk or the mouse CD8 stalk. The spacer may alternatively comprise an alternative linker sequence which has similar length and/or domain spacing properties as an IgG1 Fc region, an IgG1 hinge or a CD8 stalk. A human IgG1 spacer may be altered to remove Fc binding motifs.

Examples of amino acid sequences for these spacers are given below:

SEQ ID No. 10 (hinge-CH2CH3 of human IgG1)

AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS

KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL
DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD

SEQ ID No. 11 (human CD8 stalk):
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI

SEQ ID No. 12 (human IgG1 hinge):
AEPKSPDKTHTCPPCPKDPK

SEQ ID No. 13 (CD2 ectodomain)
KEITNALETWGALGQDINLDIPSFQMSDDIDDIKWEKTSDKKKIAQFRKEKETFKEKDTYKLF
KNGTLKIKHLKTDDQDIYKVSIYDTKGKNVLEKIFDLKIQERVSKPKISWTCINTTLTCEVMNG
TDPELNLYQDGKHLKLSQRVITHKWTTSLSAKFKCTAGNKVSKESSVEPVSCP
EKGLD

SEQ ID no. 14 (CD34 ectodomain)
SLDNNGTATPELPTQGTFSNVSTNVSYQETTTPSTLGSTSLHPVSQHGNEATTNITETTVKF
TSTSVITSVYGNTNSSVQSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLSTTSTSLATS
PTKPYTSSSPILSDIKAEIKCSGIREVKLTQGICLEQNKTSSCAEFKKDRGEGLARVLCGEEQ
ADADAGAQVCSLLLAQSEVRPQCLLLVLANRTEISSKLQLMKKHQSDLKKLGILDFTEQDVA
SHQSYSQKT

Since CARs are typically homodimers (see Figure 1a), cross-pairing may result in a heterodimeric chimeric antigen receptor. This is undesirable for various reasons, for example: (1) the epitope may not be at the same "level" on the target cell so that a cross-paired CAR may only be able to bind to one antigen; (2) the VH and VL from the two different scFv could swap over and either fail to recognize target or worse recognize an unexpected and unpredicted antigen. For the “OR” gate and the “AND NOT” gate, the spacer of the first CAR is sufficiently different from the spacer of the second CAR in order to avoid cross-pairing. The amino acid sequence of the first spacer may share less that 50%, 40%, 30% or 20% identity at the amino acid level with the second spacer.

In other aspects of the invention (for example the AND gate) it is important that the spacer of the first CAR has a different length, and/or charge and/or shape and/or configuration and/or glycosylation, such that when both first and second CARs bind their target antigen, the difference in spacer charge or dimensions results in spatial separation of the two types of CAR to different parts of the membrane to result in activation as predicted by the kinetic

separation model. In these aspects, the different length, shape and/or configuration of the spacers is carefully chosen bearing in mind the size and binding epitope on the target antigen to allow differential segregation upon cognate target recognition. For example the IgG1 Hinge, CD8 stalk, IgG1 Fc, ectodomain of CD34, ectodomain of CD45 are expected to differentially segregate.

Examples of spacer pairs which differentially segregate and are therefore suitable for use with the AND gate are shown in the following Table:

Stimulatory CAR spacer	Inhibitory CAR spacer
Human-CD8STK	Human-IgG-Hinge-CH2CH3
Human-CD3z ectodomain	Human-IgG-Hinge-CH2CH3
Human-IgG-Hinge	Human-IgG-Hinge-CH2CH3
Human-CD28STK	Human-IgG-Hinge-CH2CH3
Human-CD8STK	Human-IgM-Hinge-CH2CH3CD4
Human-CD3z ectodomain	Human-IgM-Hinge-CH2CH3CD4
Human-IgG-Hinge	Human-IgM-Hinge-CH2CH3CD4
Human-CD28STK	Human-IgM-Hinge-CH2CH3CD4

In other aspects of the invention (for example the AND NOT gate), it is important that the spacer be sufficiently different as to prevent cross-pairing, but to be sufficiently similar to co-localise. Pairs of orthologous spacer sequences may be employed. Examples are murine and human CD8 stalks, or alternatively spacer domains which are monomeric – for instance the ectodomain of CD2.

Examples of spacer pairs which co-localise and are therefore suitable for use with the AND NOT gate are shown in the following Table:

Stimulatory CAR spacer	Inhibitory CAR spacer
Human-CD8aSTK	Mouse CD8aSTK
Human-CD28STK	Mouse CD8aSTK
Human-IgG-Hinge	Human-CD3z ectodomain
Human-CD8aSTK	Mouse CD28STK
Human-CD28STK	Mouse CD28STK
Human-IgG-Hinge-CH2CH3	Human-IgM-Hinge-CH2CH3CD4

All the spacer domains mentioned above form homodimers. However the mechanism is not limited to using homodimeric receptors and should work with monomeric receptors as long as the spacer is sufficiently rigid. An example of such a spacer is CD2 or truncated CD22.

TRANSMEMBRANE DOMAIN

The transmembrane domain is the sequence of the CAR that spans the membrane.

A transmembrane domain may be any protein structure which is thermodynamically stable in a membrane. This is typically an alpha helix comprising of several hydrophobic residues. The transmembrane domain of any transmembrane protein can be used to supply the transmembrane portion of the invention. The presence and span of a transmembrane domain of a protein can be determined by those skilled in the art using the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Further, given that the transmembrane domain of a protein is a relatively simple structure, i.e a polypeptide sequence predicted to form a hydrophobic alpha helix of sufficient length to span the membrane, an artificially designed TM domain may also be used (US 7052906 B1 describes synthetic transmembrane components).

The transmembrane domain may be derived from CD28, which gives good receptor stability.

ACTIVATING ENDODOMAIN

The endodomain is the signal-transmission portion of the CAR. After antigen recognition, receptors cluster, native CD45 and CD148 are excluded from the synapse and a signal is transmitted to the cell. The most commonly used endodomain component is that of CD3-zeta which contains 3 ITAMs. This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully competent activation signal and additional co-stimulatory signaling may be needed. For example, chimeric CD28 and OX40 can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together.

Where the T cell of the present invention comprises a CAR with an activating endodomain, it may comprise the CD3-Zeta endodomain alone, the CD3-Zeta endodomain with that of either CD28 or OX40 or the CD28 endodomain and OX40 and CD3-Zeta endodomain.

Any endodomain which contains an ITAM motif can act as an activation endodomain in this invention. Several proteins are known to contain endodomains with one or more ITAM motifs. Examples of such proteins include the CD3 epsilon chain, the CD3 gamma chain and the CD3 delta chain to name a few. The ITAM motif can be easily recognized as a tyrosine separated from a leucine or isoleucine by any two other amino acids, giving the signature YxxL/I. Typically, but not always, two of these motifs are separated by between 6 and 8 amino acids in the tail of the molecule (YxxL/Ix(6-8)YxxL/I). Hence, one skilled in the art can

readily find existing proteins which contain one or more ITAM to transmit an activation signal. Further, given the motif is simple and a complex secondary structure is not required, one skilled in the art can design polypeptides containing artificial ITAMs to transmit an activation signal (see WO 2000063372, which relates to synthetic signalling molecules).

The transmembrane and intracellular T-cell signalling domain (endodomain) of a CAR with an activating endodomain may comprise the sequence shown as SEQ ID No. 15, 16 or 17 or a variant thereof having at least 80% sequence identity.

SEQ ID No. 15 comprising CD28 transmembrane domain and CD3 Z endodomain
FWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEY
DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY
QGLSTATKDTYDALHMQALPPR

SEQ ID No. 16 comprising CD28 transmembrane domain and CD28 and CD3 Zeta endodomains
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPP
RDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
PR

SEQ ID No. 17 comprising CD28 transmembrane domain and CD28, OX40 and CD3 Zeta endodomains.
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPP
RDFAAYRSRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQG
QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG
MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

A variant sequence may have at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity to SEQ ID No. 15, 16 or 17, provided that the sequence provides an effective trans-membrane domain and an effective intracellular T cell signaling domain.

"LIGATION-OFF" INHIBITORY ENDODOMAIN

In the embodiment referred above as the AND gate, one of the CARs comprises an inhibitory endodomain such that the inhibitory CAR inhibits T-cell activation by the activating CAR in the absence of inhibitory CAR ligation, but does not significantly inhibit T-cell

activation by the activating CAR when the inhibitory CAR is ligated. This is termed a "ligation-off" inhibitory endodomain.

In this case, the spacer of the inhibitory CAR is of a different length, charge, shape and/or configuration and/or glycosylation from the spacer of the activating CAR, such that when both receptors are ligated, the difference in spacer dimensions results in isolation of the activating CARs and the inhibitory CARs in different membrane compartments of the immunological synapse, so that the activating endodomain is released from inhibition by the inhibitory endodomain.

The inhibitory endodomains for use in a ligation-off inhibitory CAR may therefore comprise any sequence which inhibits T-cell signaling by the activating CAR when it is in the same membrane compartment (i.e. in the absence of the antigen for the inhibitory CAR) but which does not significantly inhibit T cell signaling when it is isolated in a separate part of the membrane from the inhibitory CAR.

The ligation-off inhibitory endodomain may be or comprise a tyrosine phosphatase, such as a receptor-like tyrosine phosphatase. An inhibitory endodomain may be or comprise any tyrosine phosphatase that is capable of inhibiting the TCR signalling when only the stimulatory receptor is ligated. An inhibitory endodomain may be or comprise any tyrosine phosphatase with a sufficiently fast catalytic rate for phosphorylated ITAMs that is capable of inhibiting the TCR signalling when only the stimulatory receptor is ligated.

For example, the inhibitory endodomain of an AND gate may comprise the endodomain of CD148 or CD45. CD148 and CD45 have been shown to act naturally on the phosphorylated tyrosines up-stream of TCR signalling.

CD148 is a receptor-like protein tyrosine phosphatase which negatively regulates TCR signaling by interfering with the phosphorylation and function of PLCγ1 and LAT.

CD45 present on all hematopoetic cells, is a protein tyrosine phosphatase which is capable of regulating signal transduction and functional responses, again by phosphorylating PLC γ1.

An inhibitory endodomain may comprise all of part of a receptor-like tyrosine phosphatase. The phospatase may interfere with the phosphorylation and/or function of elements involved in T-cell signalling, such as PLCγ1 and/or LAT.

The transmembrane and endodomain of CD45 and CD148 is shown as SEQ ID No. 18 and No.19 respectively.

SEQ ID 18 - CD45 trans-membrane and endodomain sequence
ALIAFLAFLIIVTSIALLVVLYKIYDLHKKRSCNLDEQQELVERDDEKQLMNVEPIHADILLETYK
RKIADEGRLFLAEFQSIPRVFSKFPIKEARKPFNQNKNRYVDILPYDYNRVELSEINGDAGSN
YINASYIDGFKEPRKYIAAQGPRDETVDDFWRMIWEQKATVIVMVTRCEEGNRNKCAEYWP
SMEEGTRAFGDVVVKINQHKRCPDYIIQKLNIVNKKEKATGREVTHIQFTSWPDHGVPEDPH
LLLKLRRRVNAFSNFFSGPIVVHCSAGVGRTGTYIGIDAMLEGLEAENKVDVYGYVVKLRRQ
RCLMVQVEAQYILIHQALVEYNQFGETEVNLSELHPYLHNMKKRDPPSEPSPLEAEFQRLP
SYRSWRTQHIGNQEENKSKNRNSNVIPYDYNRVPLKHELEMSKESEHDSDESSDDDSDSE
EPSKYINASFIMSYWKPEVMIAAQGPLKETIGDFWQMIFQRKVKVIVMLTELKHGDQEICAQ
YWGEGKQTYGDIEVDLKDTDKSSTYTLRVFELRHSKRKDSRTVYQYQYTNWSVEQLPAEP
KELISMIQVVKQKLPQKNSSEGNKHHKSTPLLIHCRDGSQQTGIFCALLNLLESAETEEVVDI
FQVVKALRKARPGMVSTFEQYQFLYDVIASTYPAQNGQVKKNNHQEDKIEFDNEVDKVKQ
DANCVNPLGAPEKLPEAKEQAEGSEPTSGTEGPEHSVNGPASPALNQGS

SEQ ID 19 - CD148 trans-membrane and endodomain sequence
AVFGCIFGALVIVTVGGFIFWRKKRKDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQADSN
CGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVLPYDISRVKLSVQTHSTDDYINANYM
PGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLTKCVEQGRTKCEEYWPSKQAQD
YGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPDTTDLLINFRYLVR
DYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTED
QYVFLNQCVLDIVRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGYIA

An inhibitory CAR may comprise all or part of SEQ ID No 18 or 19 (for example, it may comprise the phosphatase function of the endodomain). It may comprise a variant of the sequence or part thereof having at least 80% sequence identity, as long as the variant retains the capacity to basally inhibit T cell signalling by the activating CAR.

Other spacers and endodomains may be tested for example using the model system exemplified herein. Target cell populations can be created by transducing a suitable cell line such as a SupT1 cell line either singly or doubly to establish cells negative for both antigens (the wild-type), positive for either and positive for both (e.g. CD19-CD33-, CD19+CD33-, CD19-CD33+ and CD19+CD33+). T cells such as the mouse T cell line BW5147 which releases IL-2 upon activation may be transduced with pairs of CARs and their ability to function in a logic gate measured through measurement of IL-2 release (for example by

ELISA). For example, it is shown in Example 4 that both CD148 and CD45 endodomains can function as inhibitory CARs in combination with an activating CAR containing a CD3 Zeta endodomain. These CARs rely upon a short/non-bulky CD8 stalk spacer on one CAR and a bulky Fc spacer on the other CAR to achieve AND gating. When both receptors are ligated, the difference in spacer dimensions results in isolation of the different receptors in different membrane compartments, releasing the CD3 Zeta receptor from inhibition by the CD148 or CD45 endodomains. In this way, activation only occurs once both receptors are activated. It can be readily seen that this modular system can be used to test alternative spacer pairs and inhibitory endodomains. If the spacers do not achieve isolation following ligation of both receptors, the inhibition would not be released and so no activation would occur. If the inhibitory endodomain under test is ineffective, activation would be expected in the presence of ligation of the activating CAR irrespective of the ligation status of the inhibitory CAR.

"LIGATION-ON" ENDODOMAIN

In the embodiment referred above as the AND NOT gate, one of the CARs comprises a "ligation-on" inhibitory endodomain such that the inhibitory CAR does not significantly inhibit T-cell activation by the activating CAR in the absence of inhibitory CAR ligation, but inhibits T-cell activation by the activating CAR when the inhibitory CAR is ligated.

The "ligation-on" inhibitory endodomain may be or comprise a tyrosine phosphatase that is incapable of inhibiting the TCR signalling when only the stimulatory receptor is ligated.

The "ligation-on" inhibitory endodomain may be or comprise a tyrosine phosphatase with a sufficiently slow catalytic rate for phosphorylated ITAMs that is incapable of inhibiting the TCR signalling when only the stimulatory receptor is ligated but it is capable of inhibiting the TCR signalling response when concentrated at the synapse. Concentration at the synapse is achieved through inhibitory receptor ligation.

If a tyrosine phosphatase has a catalytic rate which is too fast for a "ligation-on" inhibitory endodomain, then it is possible to tune-down the catalytic rates of phosphatase through modification such as point mutations and short linkers (which cause steric hindrance) to make it suitable for a "ligation-on" inhibitory endodomain.

In this first embodiment the endodomain may be or comprise a phosphatase which is considerably less active than CD45 or CD148, such that significant dephosphorylation of ITAMS only occurs when activating and inhibitory endodomains are co-localised. Many

suitable sequences are known in the art. For example, the inhibitory endodomain of a NOT AND gate may comprise all or part of a protein-tyrosine phosphatase such as PTPN6.

Protein tyrosine phosphatases (PTPs) are signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. The N-terminal part of this PTP contains two tandem Src homolog (SH2) domains, which act as protein phospho-tyrosine binding domains, and mediate the interaction of this PTP with its substrates. This PTP is expressed primarily in hematopoietic cells, and functions as an important regulator of multiple signaling pathways in hematopoietic cells.

The inhibitor domain may comprise all of PTPN6 (SEQ ID No. 20) or just the phosphatase domain (SEQ ID No. 21).

SEQ ID 20 – sequence of PTPN6

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDF
YDLYGGEKFATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQA
ETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGG
LETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYYATRVNAADIENRVLELNKKQESEDTAKA
GFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYIN
ANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVP
YWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGV
PSEPGGVLSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKT
IQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKKKLEVLQSQKGQESEYGNITYPPAMKNAH
AKASRTSSKHKEDVYENLHTKNKREEKVKKQRSADKEKSKGSLKRK

SEQ ID 21 – sequence of phosphatase domain of PTPN6

FWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINA
NYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVPY
WPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVP
SEPGGVLSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTI
QMVRAQRSGMVQTEAQYKFIYVAIAQF

A second embodiment of a ligation-on inhibitory endodomain is an ITIM (Immunoreceptor Tyrosine-based Inhibition motif) containing endodomain such as that from CD22, LAIR-1, the Killer inhibitory receptor family (KIR), LILRB1, CTLA4, PD-1, BTLA etc. When phosphorylated, ITIMs recruits endogenous PTPN6 through its SH2 domain. If co-localised

with an ITAM containing endodomain, dephosphorylation occurs and the activating CAR is inhibited.

An ITIM is a conserved sequence of amino acids (S/I/V/LxYxxI/V/L) that is found in the cytoplasmic tails of many inhibitory receptors of the immune system. One skilled in the art can easily find protein domains containing an ITIM. A list of human candidate ITIM-containing proteins has been generated by proteome-wide scans (Staub, et al (2004) Cell. Signal. 16, 435–456). Further, since the consensus sequence is well known and little secondary structure appears to be required, one skilled in the art could generate an artificial ITIM.

ITIM endodomains from PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 and KIR3DL3 are shown in SEQ ID 22 to 31 respectively

SEQ ID 22 PDCD1 endodomain
CSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATI
VFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL

SEQ ID 23 BTLA4
KLQRRWKRTQSQQGLQENSSGQSFFVRNKKVRRAPLSEGPHSLGCYNPMMEDGISYTTL
RFPEMNIPRTGDAESSEMQRPPPDCDDTVTYSALHKRQVGDYENVIPDFPEDEGIHYSELI
QFGVGERPQAQENVDYVILKH

SEQ ID 24 LILRB1
LRHRRQGKHWTSTQRKADFQHPAGAVGPEPTDRGLQWRSSPAADAQEENLYAAVKHTQ
PEDGVEMDTRSPHDEDPQAVTYAEVKHSRPRREMASPPSPLSGEFLDTKDRQAEEDRQM
DTEAAASEAPQDVTYAQLHSLTLRREATEPPPSQEGPSPAVPSIYATLAIH

SEQ ID 25 LAIR1
HRQNQIKQGPPRSKDEEQKPQQRPDLAVDVLERTADKATVNGLPEKDRETDTSALAAGSS
QEVTYAQLDHWALTQRTARAVSPQSTKPMAESITYAAVARH

SEQ ID 26 CTLA4
FLLWILAAVSSGLFFYSFLLTAVSLSKMLKKRSPLTTGVYVKMPPTEPECEKQFQPYFIPIN

SEQ ID 27 KIR2DL1
GNSRHLHVLIGTSVVIIPFAILLFFLLHRWCANKKNAVVMDQEPAGNRTVNREDSDEQDP

QEVTYTQLNHCVFTQRKITRPSQRPKTPPTDIIVYTELPNAESRSKVVSCP

SEQ ID 28 KIR2DL4
GIARHLHAVIRYSVAIILFTILPFFLLHRWCSKKKENAAVMNQEPAGHRTVNREDSDEQDPQ
EVTYAQLDHCIFTQRKITGPSQRSKRPSTDTSVCIELPNAEPRALSPAHEHHSQALMGSSRE
TTALSQTQLASSNVPAAGI

SEQ ID 29 KIR2DL5
TGIRRHLHILIGTSVAIILFIILFFFLLHCCCSNKKNAAVMDQEPAGDRTVNREDSDDQDPQEV
TYAQLDHCVFTQTKITSPSQRPKTPPTDTTMYMELPNAKPRSLSPAHKHHSQALRGSSRET
TALSQNRVASSHVPAAGI

SEQ ID 30 KIR3DL1
KDPRHLHILIGTSVVIILFILLLFFLLHLWCSNKKNAAVMDQEPAGNRTANSEDSDEQDPEEV
TYAQLDHCVFTQRKITRPSQRPKTPPTDTILYTELPNAKPRSKVVSCP

SEQ ID 31 KIR3DL3
KDPGNSRHLHVLIGTSVVIIPFAILLFFLLHRWCANKKNAVVMDQEPAGNRTVNREDSDEQD
PQEVTYAQLNHCVFTQRKITRPSQRPKTPPTDTSV

A third embodiment of a ligation-on inhibitory endodomain is an ITIM containing endodomain co-expressed with a fusion protein. The fusion protein may comprise at least part of a protein-tyrosine phosphatase and at least part of a receptor-like tyrosine phosphatase. The fusion may comprise one or more SH2 domains from the protein-tyrosine phosphatase. For example, the fusion may be between a PTPN6 SH2 domain and CD45 endodomain or between a PTPN6 SH2 domain and CD148 endodomain. When phosphorylated, the ITIM domains recruit the fusion protein bring the highly potent CD45 or CD148 phosphatase to proximity to the activating endodomain blocking activation.

SEQUENCES of fusion proteins are listed 32 and 33

SEQ ID 32 PTPN6-CD45 fusion protein
WYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKV
MCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYKIYDLHKKRSCNLDEQQ
ELVERDDEKQLMNVEPIHADILLETYKRKIADEGRLFLAEFQSIPRVFSKFPIKEARKPFNQN
KNRYVDILPYDYNRVELSEINGDAGSNYINASYIDGFKEPRKYIAAQGPRDETVDDFWRMIW
EQKATVIVMVTRCEEGNRNKCAEYWPSMEEGTRAFGDVVVKINQHKRCPDYIIQKLNIVNK

KEKATGREVTHIQFTSWPDHGVPEDPHLLLKLRRRVNAFSNFFSGPIVVHCSAGVGRTGTY
IGIDAMLEGLEAENKVDVYGYVVKLRRQRCLMVQVEAQYILIHQALVEYNQFGETEVNLSEL
HPYLHNMKKRDPPSEPSPLEAEFQRLPSYRSWRTQHIGNQEENKSKNRNSNVIPYDYNRV
LKHELEMSKESEHDSDESSDDDSDSEEPSKYINASFIMSYWKPEVMIAAQGPLKETIGDFMI
QRKVKVIVMLTELKHGDQEICAQYWGEGKQTYGDIEVDLKDTDKSSTYTLRVFELRHSKRK
DSRTVYQYQYTNWSVEQLPAEPKELISMIQVVKQKLPQKNSSEGNKHHKSTPLLIHCRDGS
QQTGIFCALLNLLESAETEEVVDIFQVVKALRKARPGMVSTFEQYQFLYDVIASTYPAQNGQ
VKKNNHQEDKIEFDNEVDKVKQDANCVNPLGAPEKLPEAKEQAEGSEPTSGTEGPEHSVN
GPASPALNQGS

SEQ ID 33 PTPN6-CD148 fusion
ETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGG
LETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYRKKRKDAKNNEVSFSQIKPKKSKLIRVENF
EAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVLPYDISRVKLSVQ
THSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLTKCVEQGRTK
CEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVP
DTTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYD
LRMHRPLMVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGY
IA

A ligation-on inhibitory CAR may comprise all or part of SEQ ID No 20 or 21. It may comprise all or part of SEQ ID 22 to 31. It may comprise all or part of SEQ ID 22 to 31 co-expressed with either SEQ ID 32 or 33. It may comprise a variant of the sequence or part thereof having at least 80% sequence identity, as long as the variant retains the capacity to inhibit T cell signaling by the activating CAR upon ligation of the inhibitory CAR.

As above, alternative spacers and endodomains may be tested for example using the model system exemplified herein. It is shown in Example 5 that the PTPN6 endodomain can function as a semi-inhibitory CAR in combination with an activating CAR containing a CD3 Zeta endodomain. These CARs rely upon a human CD8 stalk spacer on one CAR and a mouse CD8 stalk spacer on the other CAR. The orthologous sequences prevent cross pairing. However, when both receptors are ligated, the similarity between the spacers results in co-segregation of the different receptors in the same membrane compartments. This results in inhibition of the CD3 Zeta receptor by the PTPN6 endodomain. If only the activating CAR is ligated the PTPN6 endodomain is not sufficiently active to prevent T cell activation. In this way, activation only occurs if the activating CAR is ligated and the inhibitory CAR is not ligated (AND NOT gating). It can be readily seen that this modular

system can be used to test alternative spacer pairs and inhibitory domains. If the spacers do not achieve co-segregation following ligation of both receptors, the inhibition would not be effective and so activation would occur. If the semi-inhibitory endodomain under test is ineffective, activation would be expected in the presence of ligation of the activating CAR irrespective of the ligation status of the semi-inhibitory CAR.

CO-EXPRESSION SITE

The second aspect of the invention relates to a nucleic acid which encodes the first and second CARs.

The nucleic acid may produce a polypeptide which comprises the two CAR molecules joined by a cleavage site. The cleavage site may be self-cleaving, such that when the polypeptide is produced, it is immediately cleaved into the first and second CARs without the need for any external cleavage activity.

Various self-cleaving sites are known, including the Foot-and-Mouth disease virus (FMDV) 2a self-cleaving peptide, which has the sequence shown as SEQ ID No. 34:

SEQ ID No. 34
RAEGRGSLLTCGDVEENPGP.

The co-expressing sequence may be an internal ribosome entry sequence (IRES). The co-expressing sequence may be an internal promoter.

CELL

The first aspect of the invention relates to a cell which co-expresses a first CAR and a second CAR at the cell surface.

The cell may be any eukaryotic cell capable of expressing a CAR at the cell surface, such as an immunological cell.

In particular the cell may be an immune effector cell such as a T cell or a natural killer (NK) cell

T cells or T lymphocytes are a type of lymphocyte that play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural

killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. There are various types of T cell, as summarised below.

Helper T helper cells (TH cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. TH cells express CD4 on their surface. TH cells become activated when they are presented with peptide antigens by MHC class II molecules on the surface of antigen presenting cells (APCs). These cells can differentiate into one of several subtypes, including TH1, TH2, TH3, TH17, Th9, or TFH, which secrete different cytokines to facilitate different types of immune responses.

Cytotoxic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. CTLs express the CD8 at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of all nucleated cells. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the CD8+ cells can be inactivated to an anergic state, which prevent autoimmune diseases such as experimental autoimmune encephalomyelitis.

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise three subtypes: central memory T cells (TCM cells) and two types of effector memory T cells (TEM cells and TEMRA cells). Memory cells may be either CD4+ or CD8+. Memory T cells typically express the cell surface protein CD45RO.

Regulatory T cells (Treg cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus.

Two major classes of CD4+ Treg cells have been described — naturally occurring Treg cells and adaptive Treg cells.

Naturally occurring Treg cells (also known as CD4+CD25+FoxP3+ Treg cells) arise in the thymus and have been linked to interactions between developing T cells with both myeloid (CD11c+) and plasmacytoid (CD123+) dendritic cells that have been activated with TSLP.

Naturally occurring Treg cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3. Mutations of the FOXP3 gene can prevent regulatory T cell development, causing the fatal autoimmune disease IPEX.

Adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response.

The T cell of the invention may be any of the T cell types mentioned above, in particular a CTL.

Natural killer (NK) cells are a type of cytolytic cell which forms part of the innate immune system. NK cells provide rapid responses to innate signals from virally infected cells in an MHC independent manner

NK cells (belonging to the group of innate lymphoid cells) are defined as large granular lymphocytes (LGL) and constitute the third kind of cells differentiated from the common lymphoid progenitor generating B and T lymphocytes. NK cells are known to differentiate and mature in the bone marrow, lymph node, spleen, tonsils and thymus where they then enter into the circulation.

The CAR cells of the invention may be any of the cell types mentioned above.

CAR- expressing cells , such as CAR-expressing T or NK cells, may either be created *ex vivo* either from a patient's own peripheral blood (1^{st} party), or in the setting of a haematopoietic stem cell transplant from donor peripheral blood (2^{nd} party), or peripheral blood from an unconnected donor (3^{rd} party).

The present invention also provide a cell composition comprising CAR expressing T cells and/or CAR expressing NK cells according to the present invention. The cell composition may be made by tranducing or transfecting a blood-sample ex vivo with a nucleic acid according to the present invention.

Alternatively, CAR-expressing cells may be derived from *ex vivo* differentiation of inducible progenitor cells or embryonic progenitor cells to the relevant cell type, such as T cells. Alternatively, an immortalized cell line such as a T-cell line which retains its lytic function and could act as a therapeutic may be used.

In all these embodiments, CAR cells are generated by introducing DNA or RNA coding for the CARs by one of many means including transduction with a viral vector, transfection with DNA or RNA.

A CAR T cell of the invention may be an *ex vivo* T cell from a subject. The T cell may be from a peripheral blood mononuclear cell (PBMC) sample. T cells may be activated and/or expanded prior to being transduced with CAR-encoding nucleic acid, for example by treatment with an anti-CD3 monoclonal antibody.

A CAR T cell of the invention may be made by:

(i) isolation of a T cell-containing sample from a subject or other sources listed above; and

(ii) transduction or transfection of the T cells with one or more nucleic acid sequence(s) encoding the first and second CAR.

The T cells may then by purified, for example, selected on the basis of co-expression of the first and second CAR.

NUCLEIC ACID SEQUENCES

The second aspect of the invention relates to one or more nucleic acid sequence(s) which codes for a first CAR and a second CAR as defined in the first aspect of the invention.

The nucleic acid sequence may comprise one of the following sequences, or a variant thereof

SEQ ID 35 OR gate
SEQ ID 36 AND gate using CD45
SEQ ID 37 AND gate using CD148
SEQ ID 38 AND NOT gate using PTPN6 as endodomain
SEQ ID 39 AND NOT gate using LAIR1 endodomain
SEQ ID 40 AND NOT gate using LAIR1 and PTPN6 SH2 fusion with CD148 phosphatase

SEQ ID No. 35:
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The nucleic acid sequence may encode the same amino acid sequence as that encoded by SEQ ID No. 35, 36, 37, 38, 39 or 40, but may have a different nucleic acid sequence, due to the degeneracy of the genetic code. The nucleic acid sequence may have at least 80, 85, 90, 95, 98 or 99% identity to the sequence shown as SEQ ID No. 35, 36, 37, 38, 39 or 40, provided that it encodes a first CAR and a second CAR as defined in the first aspect of the invention.

VECTOR

The present invention also provides a vector, or kit of vectors which comprises one or more CAR-encoding nucleic acid sequence(s). Such a vector may be used to introduce the nucleic acid sequence(s) into a host cell so that it expresses the first and second CARs.

The vector may, for example, be a plasmid or a viral vector, such as a retroviral vector or a lentiviral vector, or a transposon based vector or synthetic mRNA.

The vector may be capable of transfecting or transducing a T cell.

PHARMACEUTICAL COMPOSITION

The present invention also relates to a pharmaceutical composition containing a plurality of CAR-expressing cells, such as T cells or NK cells according to the first aspect of the invention. The pharmaceutical composition may additionally comprise a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical composition may optionally comprise one or more further pharmaceutically active polypeptides and/or compounds. Such a formulation may, for example, be in a form suitable for intravenous infusion.

METHOD OF TREATMENT

The T cells of the present invention may be capable of killing target cells, such as cancer cells. The target cell may be recognisable by a defined pattern of antigen expression, for example the expression of antigen A AND antigen B; the expression of antigen A OR antigen B; or the expression of antigen A AND NOT antigen B or complex iterations of these gates.

T cells of the present invention may be used for the treatment of an infection, such as a viral infection.

T cells of the invention may also be used for the control of pathogenic immune responses, for example in autoimmune diseases, allergies and graft-vs-host rejection.

T cells of the invention may be used for the treatment of a cancerous disease, such as bladder cancer, breast cancer, colon cancer, endometrial cancer, kidney cancer (renal cell), leukemia, lung cancer, melanoma, non-Hodgkin lymphoma, pancreatic cancer, prostate cancer and thyroid cancer.

It is particularly suited for treatment of solid tumours where the availability of good selective single targets is limited.

T cells of the invention may be used to treat: cancers of the oral cavity and pharynx which includes cancer of the tongue, mouth and pharynx; cancers of the digestive system which includes oesophageal, gastric and colorectal cancers; cancers of the liver and biliary tree which includes hepatocellular carcinomas and cholangiocarcinomas; cancers of the respiratory system which includes bronchogenic cancers and cancers of the larynx; cancers of bone and joints which includes osteosarcoma; cancers of the skin which includes melanoma; breast cancer; cancers of the genital tract which include uterine, ovarian and cervical cancer in women, prostate and testicular cancer in men; cancers of the renal tract which include renal cell carcinoma and transitional cell carcinomas of the utterers or bladder; brain cancers including gliomas, glioblastoma multiforme and medullobastomas; cancers of the endocrine system including thyroid cancer, adrenal carcinoma and cancers associated with multiple endocrine neoplasm syndromes; lymphomas including Hodgkin's lymphoma and non-Hodgkin lymphoma; Multiple Myeloma and plasmacytomas; leukaemias both acute and chronic, myeloid or lymphoid; and cancers of other and unspecified sites including neuroblastoma.

Treatment with the T cells of the invention may help prevent the escape or release of tumour cells which often occurs with standard approaches.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1 - Creation of target cell populations

For the purposes of proving the principle of the invention, receptors based on anti-CD19 and anti-CD33 were arbitrarily chosen. Using retroviral vectors, CD19 and CD33 were cloned. These proteins were truncated so that they do not signal and could be stably expressed for prolonged periods. Next, these vectors were used to transduce the SupT1 cell line either singly or doubly to establish cells negative for both antigen (the wild-type), positive for either and positive for both. The expression data are shown in Figure 3.

Example 2 - Design and function of the OR gate

To construct the OR gate, a pair of receptors recognizing CD19 and CD33 were co-expressed. Different spacers were used to prevent cross-pairing. Both receptors had a trans-membrane domain derived from CD28 to improve surface stability and an endodomain derived from that of CD3 Zeta to provide a simple activating signal. In this way, a pair of independent 1st generation CARs were co-expressed. The retroviral vector cassette used to co-express the sequences utilizes a foot-and-mouth 2A self-cleaving peptide to allow co-expression 1:1 of both receptors. The cassette design is shown in Figure 4, and the protein structures in Figure 5. The nucleotide sequence of homologous regions was codon-wobbled to prevent recombination during retroviral vector reverse transcription.

Example 3 - Testing the OR gate

Expression of both CARs was tested on the T-cell surface by staining with cognate antigen fused to Fc. By using different species of Fc domains (mouse for CD19 and rabbit for CD33), co-expression of both CARs was determined on the cell surface by staining with

different secondary antibodies conjugated with different fluorophores. This is shown in Figure 6.

Functional testing was then carried out using the mouse T-cell line BW5147. This cell line releases IL2 upon activation allowing a simple quantitative readout. These T-cells were co-cultured with increasing amounts of the artificial target cells described above. T-cells responded to target cells expressing either antigen, as shown by IL2 release measured by ELISA. Both CARs were shown to be expressed on the cell surfaces and the T-cells were shown to respond to either or both antigens. These data are show in Figure 7.

Example 4 - Design and function of the AND gate

The AND gate combines a simple activating receptor with a receptor which basally inhibits activity, but whose inhibition is turned off once the receptor is ligated. This was achieved by combining a standard 1st generation CAR with a short / non-bulky CD8 stalk spacer and a CD3 Zeta endodomain with a second receptor with a bulky Fc spacer whose endodomain contained either CD148 or CD45 endodomains. When both receptors are ligated, the difference in spacer dimensions results in isolation of the different receptors in different membrane compartments, releasing the CD3 Zeta receptor from inhibition by the CD148 or CD45 endodomains. In this way, activation only occurs once both receptors are activated. CD148 and CD45 were chosen for this as they function in this manner natively: for instance, the very bulky CD45 ectodomain excludes the entire receptor from the immunological synapse. The expression cassette is depicted in Figure 8 and the subsequent proteins in Figure 9.

Surface staining for the different specificity showed that both receptor pairs could be effectively expressed on the cell surface shown in Figure 10. Function in BW5147 shows that the T-cell is only activated in the presence of both antigens (Figure 11).

Example 5: Demonstration of Generalizability of the AND gate

To ensure that the observations were not a manifestation of some specific characteristic of CD19 / CD33 and their binders which had been used, the two targeting scFvs were swapped such that now, the activation (ITAM) signal was transmitted upon recognition of CD33, rather than CD19; and the inhibitory (CD148) signal was transmitted upon recognition of CD19, rather than of CD33. Since CD45 and CD148 endodomains are considered to be functionally similar, experimentation was restricted to AND gates with CD148 endodomain. This should

still result in a functional AND gate. T-cells expressing the new logic gate where challenged with targets bearing either CD19 or CD33 alone, or both. The T-cells responded to targets expressing both CD19 and CD33, but not to targets expressing only one or none of these antigens. This shows that the AND gate is still functional in this format (Figure 18B).

On the same lines, it was sought to establish how generalizable our AND gate is: the AND gate should be generalizable across different targets. While there may be lesser or greater fidelity of the gate given relative antigen density, cognate scFv binding kinetics and precise distance of the scFv binding epitope, one would expect to see some AND gate manifestations with a wide set of targets and binders. To test this, three additional AND gates were generated. Once again, experimentation was restricted to the CD148 version of the AND gate. The second scFv from the original CD148 AND gate was replaced with the anti-GD2 scFv huK666 (SEQ ID 41 and SEQ ID 42), or with the anti-CD5 scFv (SEQ ID 43 and SEQ ID 44), or the anti-EGFRvIII scFv MR1.1 (SEQ ID 45 AND SEQ ID 46) to generate the following CAR AND gates: CD19 AND GD2; CD19 AND CD5; CD19 AND EGFRvIII. The following artificial antigen expressing cell lines were also generated: by transducing SupT1, and our SupT1.CD19 with GM3 and GD2 synthases SupT1.GD2 and SupT1.CD19.GD2 were generated. By transducing SupT1 and SupT1.CD19 with a retroviral vector coding for EGFRvIII SupT1.EGFRvIII and SupT1.CD19.EGFRvIII were generated. Since CD5 is expressed on SupT1 cells, a different cell line was used to generate the target cells: 293T cells were generated which express CD19 alone, CD5 alone and both CD5 and CD19 together. Expression was confirmed by flow-cytometry (Figure 19). T-cells expressing the three new CAR AND gates were challenged with SupT1.CD19 and respective cognate double positive and single positive target cells. All three AND gates demonstrated reduced activation by the double positive cell lines in comparison with the single positive targets (Figure 20). This demonstrates generalizability of the AND gate design to arbitrary targets and cognate binders.

Example 6: Experimental proof of Kinetic segregation model of CAR AND gate

The aim was to prove the model that differential segregation caused by different spacers is the central mechanism behind the ability to generate these logic CAR gates. The model is that if only the activating CAR is ligated, the potent inhibiting 'ligation off' type CAR is in solution in the membrane and can inhibit the activating CAR. Once both CARs are ligated, if both CAR spacers are sufficiently different, they will segregate within the synapse and not co-localize. Hence, a key requirement is that the spacers are sufficiently different. If the model is correct, if both spacers are sufficiently similar so they co-localize when both

receptors are ligated, the gate will fail to function. To test this, the "bulky" Fc spacer in the original CAR we replaced with a murine CD8 spacer. It was predicted that this has the similar length, bulk and charge as human CD8 but so should not cross-pair with it. Hence, the new gate had a first CAR which recognizes CD19, a human CD8 stalk spacer and an activatory endodomain; while the second CAR recognizes CD33, has a mouse CD8 stalk spacer and a CD148 endodomain (Figure 18C). T-cells were transduced to express this new CAR gate. These T-cells were then challenged with SupT1 cells expressing CD19 alone, CD33 alone or CD19 and CD33 together. T-cells did not respond to SupT1 cells expressing either antigen alone as per the original AND gate. However, CAR T-cells failed to respond to SupT1 cells expressing both antigens, thereby confirming the model (Figure 18C). A functional AND gate requires both CARs to have spacers sufficiently different so that they do not co-localize within an immunological synapse (Figure 23A and B).

Example 7 - Design and function of an AND NOT gate

Phosphatases such as CD45 and CD148 are so potent that even a small amount entering an immunological synapse can inhibit ITAM activation. This is the basis of inhibition of the logical AND gate. Other classes of phosphatases are not as potent e.g. PTPN6 and related phosphatases. It was predicted that a small amount of PTPN6 entering a synapse by diffusion would not inhibit activation. In addition, it was predicted that if an inhibitory CAR had a sufficiently similar spacer to an activating CAR, it could co-localize within a synapse if both CARs were ligated. In this case, large amounts of the inhibitory endodomain would be sufficient to stop the ITAMS from activating when both antigens were present. In this way, an AND NOT gate could be created.

For the NOT AND gate, the second signal needs to "veto" activation. This is done by bringing an inhibitory signal into the immunological synapse, for example by bringing in the phosphatase of an enzyme such as PTPN6. We hence generated an initial AND NOT gate as follows: two CARs co-expressed whereby the first recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; co-expressed with an anti-CD33 CAR with a mouse CD8 stalk spacer and an endodomain comprising of the catalytic domain of PTPN6 (SEQ ID 38, Figure 13 A with B). A suitable cassette is shown in Figure 12 and preliminary functional data are shown in Figure 14.

In addition, an alternative strategy was developed for generating an AND NOT gate. Immune Tyrosinase Inhibitory Motifs (ITIMs) are activated in a similar manner to ITAMS, in that they become phosphorylated by lck upon clustering and exclusion of phosphatases.

Instead of triggering activation by binding ZAP70, phosphorylated ITIMs recruit phosphatases like PTPN6 through their cognate SH2 domains. An ITIM can function as an inhibitory endodomain, as long as the spacers on the activating and inhibiting CARs can co-localize. To generate this construct, an AND NOT gate was generated as follows: two CARs co-expressed - the first recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; co-expressed with an anti-CD33 CAR with a mouse CD8 stalk spacer and an ITIM containing endodomain derived from that of LAIR1 (SEQ ID 39, Figure 13 A with C).

A further, more complex AND NOT gate was also developed, whereby an ITIM is enhanced by the presence of an additional chimeric protein: an intracellular fusion of the SH2 domain of PTPN6 and the endodomain of CD148. In this design three proteins are expressed - the first recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; co-expressed with an anti-CD33 CAR with a mouse CD8 stalk spacer and an ITIM containing endodomain derived from that of LAIR1. A further 2A peptide, allows co-expression of the PTPN6-CD148 fusion (SEQ ID 40, Figure 13 A and D). It was predicted that these AND NOT gates would have a different range of inhibition: PTPN6-CD148 > PTPN6 > > ITIM.

T-cells were transduced with these gates and challenged with targets expressing either CD19 or CD33 alone, or both CD19 and CD33 together. All three gates responded to targets expressing only CD19, but not targets expressing both CD19 and CD33 together (Figure 21), confirming that all three of the AND NOT gates were functional.

Example 8: Experimental proof of Kinetic segregation model of PTPN6 based AND NOT gate.

The model of the AND NOT gate centres around the fact that the nature of the spacers used in both CARs is pivotal for the correct function of the gate. In the functional AND NOT gate with PTPN6, both CAR spacers are sufficiently similar that when both CARs are ligated, both co-localize within the synapse so the high concentration even the weak PTPN6 is sufficient to inhibit activation. If the spacers were different, segregation in the synapse will isolate the PTPN6 from the ITAM allowing activation disrupting the AND NOT gate. To test this, a control was generated replacing the murine CD8 stalk spacer with that of Fc. In this case, the test gate consisted of two CARs, the first recognizes CD19, has a human CD8 stalk spacer and an ITAM endodomain; while the second CAR recognizes CD33, has an Fc spacer and an endodomain comprising of the phosphatase from PTPN6. This gate activates in response to CD19, but also activates in response to CD19 and CD33 together (Figure 22B, where function of this gate is compared with that of the original AND NOT, and the

control AND gate variant described in Example 6). This experimental data proves the model that for a functional AND NOT gate with PTPN6, co-localizing spacers are needed.

Example 9: Experimental proof of kinetic segregation model of ITIM based AND NOT gate.

Similar to the PTPN6 based AND NOT gate, the ITIM based gate also requires co-localization in an immunological synapse to function as an AND NOT gate. To prove this hypothesis, a control ITIM based gate was generated as follows: two CARs co-expressed - the first recognizes CD19, has a human CD8 stalk spacer and an activating endodomain; co-expressed with an anti-CD33 CAR with an Fc spacer and an ITIM containing endodomain derived from that of LAIR1. The activity of this gate was compared with that of the original ITIM based AND NOT gate. In this case, the modified gate activated in response to targets expressing CD19, but also activated in response to cells expressing both CD19 and CD33. These data indicate that ITIM based AND NOT gates follow the kinetic segregation based model and a correct spacer must be selected to create a functional gate (Figure 23B).

Example 10: Summary of model of CAR logic gates generated by kinetic segregation

Based on current understanding of the kinetic-segregation model and the experimental data described herein, a summary of the model for a two-CAR gate is presented in Figure 24. The Figure shows a cell expressing two CARs, each recognizing a different antigen. When either or both CARs recognize a target antigen on a cell, a synapse forms and native CD45 and CD148 are excluded from the synapse due to the bulk of their ectodomain. This sets the stage for T-cell activation. In the case that the target cell bears only one cognate antigen, the cognate CAR is ligated and the cognate CAR segregates into the synapse. The unligated CAR remains in solution on the T-cell membrane and can diffuse in and out of the synapse so that an area of high local concentration of ligated CAR with low concentration of unligated CAR forms. In this case, if the ligated CAR has an ITAM and the non-ligated CAR has 'ligation off" type inhibitory endodomain such as that of CD148, the amount of non-ligated CAR is sufficient to inhibit activation and the gate is off. In contrast, in this case, if the ligated CAR has an ITAM and the non-ligated CAR has a 'ligation on' type inhibitory endodomain such as PTPN6, the amount of non-ligated CAR is insufficient to inhibit and the gate is on. When challenged by a target cell bearing both cognate antigens, both cognate CARs are ligated and form part of an immunological synapse. Importantly, if the CAR spacers are sufficiently similar, the CARs co-localize in the synapse but if the CAR spacers are sufficiently different the CARs segregate within the synapse. In this latter case, areas of membrane form whereby high concentrations of one CAR are present but the other CAR is

absent. In this case since segregation is complete, even if the inhibitory endodomain is a 'ligation off' type, the gate is on. In the former case, areas of membrane form with high concentrations of both CARs mixed together. In this case, since both endodomains are concentrated, even if the inhibitory endodomain is 'ligation on' type, the gate is off. By selecting the correct combination of spacer and endodomain logic can be programmed into a CAR T-cell.

Based on our work above, we have established a series of design rules to allow generation of logic-gated CARs (illustrated in figure 31). To generate an "antigen A OR antigen B" gated CAR T-cell, anti-A and anti-B CARs must be generated such that (1) each CAR has a spacer which simply allows antigen access and synapse formation such that the CAR functions, and (2) Each CAR has an activating endodomain; To generate an "antigen A AND NOT B" gated CAR T-cell, anti-A and anti-B CARs must be generated such that (1) both CARs have spacers which do not cross-pair, but which will allow the CARs to co-segregate upon recognition of both cognate antigens on the target cell, (2) and one CAR has an activating endodomain, while the other CAR has an endodomain which comprises or recruits a weak phosphatase (e.g. PTPN6); (3) To generate an "antigen A AND antigen B" gated CAR T-cell, anti-A and anti-B CARs must be generated such that (1) one CAR has a spacer sufficiently different from the other CAR such that both CARs will not co-segregate upon recognition of both cognate antigens on the target cell, (2) one CAR has an activating endodomain, while the other car has an endodomain which comprises of a potent phosphatase (e.g. that of CD45 or CD148). The correct spacers to achieve the desired effect can be selected from a set of spacers with known size/shape etc as well as taking into consideration size/shape etc of the target antigen (for instance see figure 30) and the location of the cognate epitope on the target antigen.

SEQ ID No 41: SFG.aCD19-CD8STK-CD28tmZ-2A-aGD2-HCH2CH3pvaa-dCD148

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLL
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GGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGS
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TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAF
IIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL
QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENP
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WLGVIWAGGSTNYNSALMSRLTISKDNSKNQVFLKMSSLTAADTAVYYCAKRSDDYSWFAYWGQGTLV
TVSSGGGGSGGGGSGGGGSENQMTQSPSSLSASVGDRVTMTCRASSSVSSSYLHWYQQKSGKAPKVWI
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KSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVLPYDISRVKL
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LVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQY
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SEQ ID No. 42: SFG.aCD19-CD8STK-CD28tmZ-2A-aGD2-HCH2CH3pvaa-dCD148

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CTACAGCCTGACCATCAGCAACCTGGAGCAGGAGGACATCGCCACCTACTTCTGCCAGCAGGGCAACA
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SEQ ID No. 43: SFG.aCD19-CD8STK-CD28tmZ-2A-aCD5-HCH2CH3pvaa-dCD148

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SEQ ID No. 44: SFG.aCD19-CD8STK-CD28tmZ-2A-aCD5-HCH2CH3pvaa-dCD148

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SEQ ID No. 45: SFG.aCD19-CD8STK-CD28tmZ-2A-aEGFRvIII-HCH2CH3pvaa-dCD148

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KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
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KKSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVLPYDISRVK
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YWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPDTTDLLINFR
YLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQ
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SEQ ID No. 46: SFG.aCD19-CD8STK-CD28tmZ-2A-aEGFRvIII-HCH2CH3pvaa-dCD148

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TGGATGTGTATGGGATTGTGTATGACCTTCGAATGCATAGGCCTTTAATGGTGCAGACAGAGGACCAG
TATGTTTTCCTCAATCAGTGTGTTTTGGATATTGTCAGATCCCAGAAAGACTCAAAAGTAGATCTTAT
CTACCAGAACACAACTGCAATGACAATCTATGAAAACCTTGCGCCCGTGACCACATTTGGAAAGACCA
ATGGTTACATCGCCTAA

Example 11: Design and construction of APRIL based CARs.

APRIL in its natural form is a secreted type II protein. The use of APRIL as a BCMA binding domain for a CAR requires conversion of this type II secreted protein to a type I membrane bound protein and for this protein to be stable and to retain binding to BCMA in this form. To generate candidate molecules, the extreme amino-terminus of APRIL was deleted to remove binding to proteoglycans. Next, a signal peptide was added to direct the nascent protein to the endoplasmic reticulum and hence the cell surface. Also, because the nature of spacer used can alter the function of a CAR, three different spacer domains were tested: an APRIL based CAR was generated comprising (i) a human IgG1 spacer altered to remove Fc binding motifs; (ii) a CD8 stalk; and (iii) the IgG1 hinge alone (cartoon in Figure 25 and amino acid sequences in Figure 26). These CARs were expressed in a bicistronic retroviral vector (Figure 27A) so that a marker protein – truncated CD34 could be co-expressed as a convenient marker gene.

Example 12: Expression and function of APRIL based CARs.

The aim of this study was to test whether the APRIL based CARs which had been constructed were expressed on the cell surface and whether APRIL had folded to form the native protein. T-cells were transduced with these different CAR constructs and stained using a commercially available anti-APRIL mAb, along with staining for the marker gene and analysed by flow-cytometry. The results of this experiment are shown in Figure 27B where APRIL binding is plotting against marker gene fluorescence. These data show that in this format, the APRIL based CARs are expressed on the cell surface and APRIL folds sufficiently to be recognized by an anti-APRIL mAb.

Next, it was determined whether APRIL in this format could recognize BCMA and TACI. Recombinant BCMA and TACI were generated as fusions with mouse IgG2a-Fc. These recombinant proteins were incubated with the transduced T-cells. After this, the cells were washed and stained with an anti-mouse fluorophore conjugated antibody and an antibody to detect the marker gene conjugated to a different fluorophore. The cells were analysed by flow cytometry and the results are presented in Figure 27C. The different CARs were able to bind both BCMA and TACI. Surprisingly, the CARs were better able to bind BCMA than TACI. Also, surprisingly CARs with a CD8 stalk or IgG1 hinge spacer were better able to bind BCMA and TACI than CAR with an Fc spacer.

Example 13: APRIL based chimeric antigen receptors are active against BCMA expressing cells

T-cells from normal donors were transduced with the different APRIL CARs and tested against SupT1 cells either wild-type, or engineered to express BCMA and TACI. Several different assays were used to determine function. A classical chromium release assay was performed. Here, the target cells (the SupT1 cells) were labelled with 51Cr and mixed with effectors (the transduced T-cells) at different ratio. Lysis of target cells was determined by counting 51Cr in the co-culture supernatant (Figure 28A shows the cumulative data).

In addition, supernatant from T-cells cultured 1:1 with SupT1 cells was assayed by ELISA for Interferon-gamma (Figure 28B shows cumulative data). Measurement of T-cell expansion after one week of co-culture with SupT1 cells was also performed (Figure 28C). T-cells were counted by flow-cytometry calibrated with counting beads. These experimental data show that APRIL based CARs can kill BCMA expressing targets. Further, these data show that CARs based on the CD8 stalk or IgG1 hinge performed better than the Fc-pvaa based CAR.

Example 14: Functional analysis of the AND gate in primary cells

PBMCs were isolated from blood and stimulated using PHA and IL-2. Two days later the cells were transduced on retronectin coated plates with retro virus containing the CD19:CD33 AND gate construct. On day 5 the expression level of the two CARs translated by the AND gate construct was evaluated via flow cytometry and the cells were depleted of CD56+ cells (predominantly NK cells). On day 6 the PBMCs were placed in a co-culture with target cells at a 1:2 effector to target cell ratio. On day 8 the supernatant was collected and analysed for IFN-gamma secretion via ELISA (Figure 29).

These data demonstrate that the AND gate functions in primary cells.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, cell biology or related fields are intended to be within the scope of the following claims.

Claims:

1. A T cell or natural killer (NK) cell which co-expresses a first chimeric antigen receptor (CAR) and second CAR at the cell surface, each CAR comprising:

(i) an antigen-binding domain;

(ii) a spacer

(iii) a trans-membrane domain; and

(iv) an endodomain

wherein the antigen binding domains of the first and second CARs bind to different antigens,

wherein the spacer of the first CAR is different from the spacer of the second CAR such that when both first and second CARs bind their target antigen, the difference in spacer dimensions results in spatial separation of the activating and inhibitory CARs to different parts of the membrane,

wherein one of the first or second CARs is an activating CAR comprising an activating endodomain and the other CAR is an inhibitory CAR comprising a ligation-off inhibitory endodomain, and

wherein the ligation-off inhibitory endodomain comprises a CD148 or CD45 tyrosine phosphatase domain.

2. The T or NK cell according to claim 1, wherein either the first spacer or the second spacer comprises a CD8 stalk and the other spacer comprises the hinge, CH2 and CH3 domain of IgG-I.

3. The T or NK cell according to claim 1, wherein the antigen-binding domain of the first CAR binds CD5 and the antigen-binding domain of the second CAR binds CD19.

4. A nucleic acid encoding both first and second chimeric antigen receptors (CARs) as defined in claim 1.

5. The nucleic acid according to claim 4, which has the following structure:
AgB1-spacer1-TM1-endo1-coexpr-AbB2-spacer2-TM2-endo2
in which

AgB1 is a nucleic acid encoding the antigen-binding domain of the first CAR;

spacer 1 is a nucleic acid encoding the spacer of the first CAR;

TM1 is a a nucleic acid encoding the transmembrane domain of the first CAR;

endo 1 is a nucleic acid encoding the endodomain of the first CAR;

coexpr is a nucleic acid enabling co-expression of both CARs

AgB2 is a nucleic acid encoding the antigen-binding domain of the second CAR;

spacer 2 is a nucleic acid encoding the spacer of the second CAR;

TM2 is a a nucleic acid encoding the transmembrane domain of the second CAR;

endo 2 is a nucleic acid encoding the endodomain of the second CAR;

which nucleic acid , when expressed in a T cell, encodes a polypeptide which is cleaved at the cleavage site such that the first and second CARs are co-expressed at the T cell surface.

6. The nucleic acid according to claim 5, wherein coexpr encodes a sequence comprising a self-cleaving peptide.

7. The nucleic acid according to claim 5, wherein alternative codons are used in regions of sequence encoding the same or similar amino acid sequences, in order to avoid homologous recombination.

8. A kit which comprises

(i) a first nucleic acid encoding the first chimeric antigen receptor (CAR) as defined in claim 1, which nucleic acid has the following structure:

AgB1-spacer1-TM1-endo1in which

AgB1 is a nucleic acid encoding the antigen-binding domain of the first CAR;

spacer 1 is a nucleic acid encoding the spacer of the first CAR;

TM1 is a nucleic acid encoding the transmembrane domain of the first CAR;

endo 1 is a nucleic acid encoding the endodomain of the first CAR; and

(ii) a second nucleic acid encoding the second chimeric antigen receptor (CAR) as defined in claim 1, which nucleic acid has the following structure:

AgB2-spacer2-TM2-endo2

AgB2 is a nucleic acid encoding the antigen-binding domain of the second CAR;

spacer 2 is a nucleic acid encoding the spacer of the second CAR;

TM2 is a nucleic acid encoding the transmembrane domain of the second CAR;

endo 2 is a nucleic acid encoding the endodomain of the second CAR.

9. The kit comprising: a first vector which comprises the first nucleic acid as defined in claim 8; and a second vector which comprises the second nucleic acid as defined in claim 8.

10. A vector comprising the nucleic acid according to claim 4.

11. The vector according to claim 10 which is a retroviral vector or a lentiviral vector or a transposon.

12. A method for making the T or NK cell according to claim 1, which comprises the step of introducing: the nucleic acid according to claim 4; the first nucleic acid and the second nucleic acid as defined in claim 8; and/or the first vector and the second vector as defined in claim 9, or the vector according to claim 10, into the T or NK cell.

13. The method according to claim 12, wherein the T or NK cell is from a sample isolated from a subject.

14. A pharmaceutical composition comprising a plurality of the T or NK cells according to any one of claims 1 to 3 and a carrier.

15. A use of a pharmaceutical composition according to claim 14 for treating a cancer.

16. The use according to claim 15, wherein the T cells are made according to claim 12 or 13.

Date Reçue/Date Received 2020-04-16

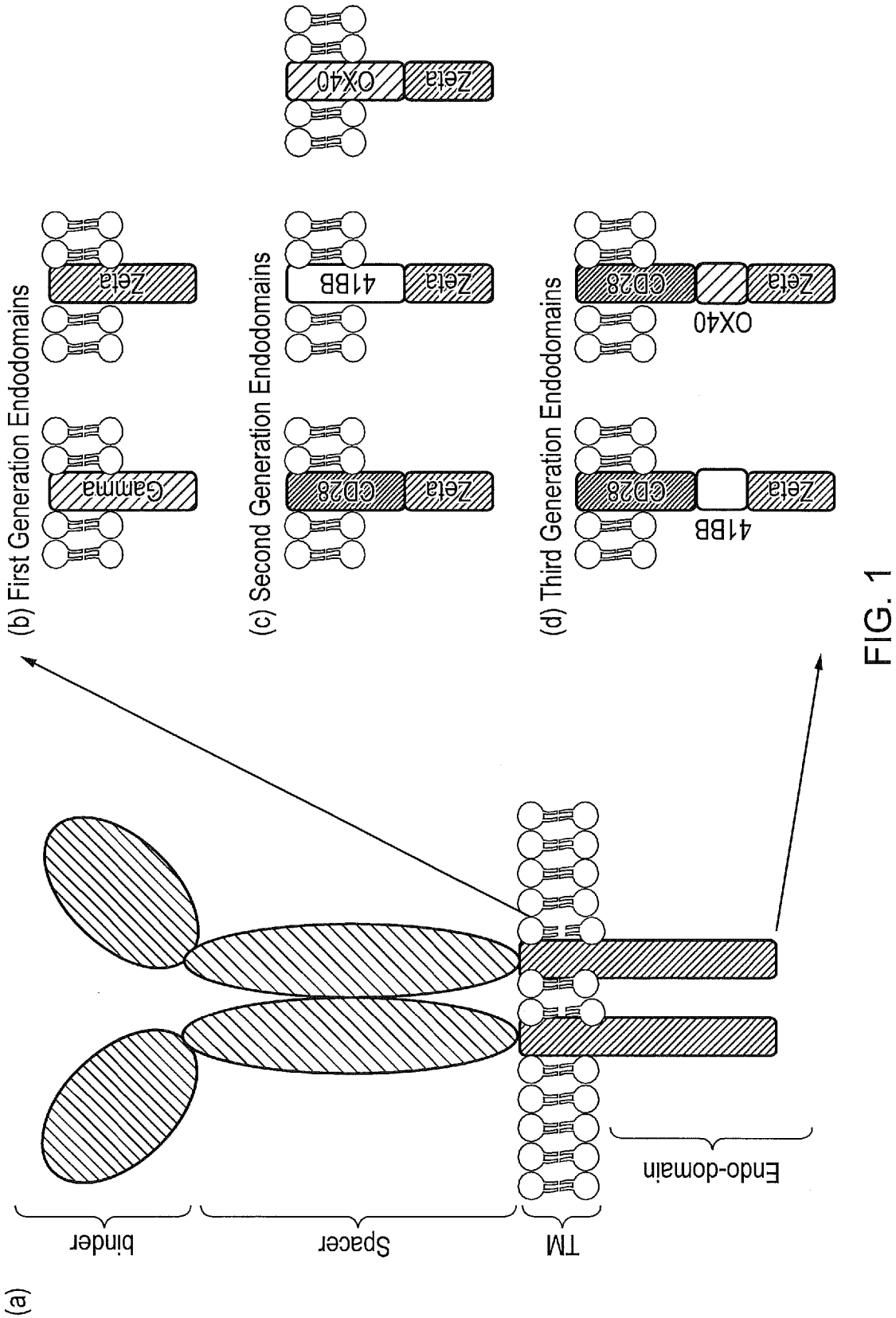
(a)
binder
Spacer
TM
Endo-domain
(b) First Generation Endodomains
Zeta
Gamma
(c) Second Generation Endodomains
Zeta
41BB
CD28
(d) Third Generation Endodomains
Zeta
CD28
OX40
41BB
OX40
Zeta

FIG. 1

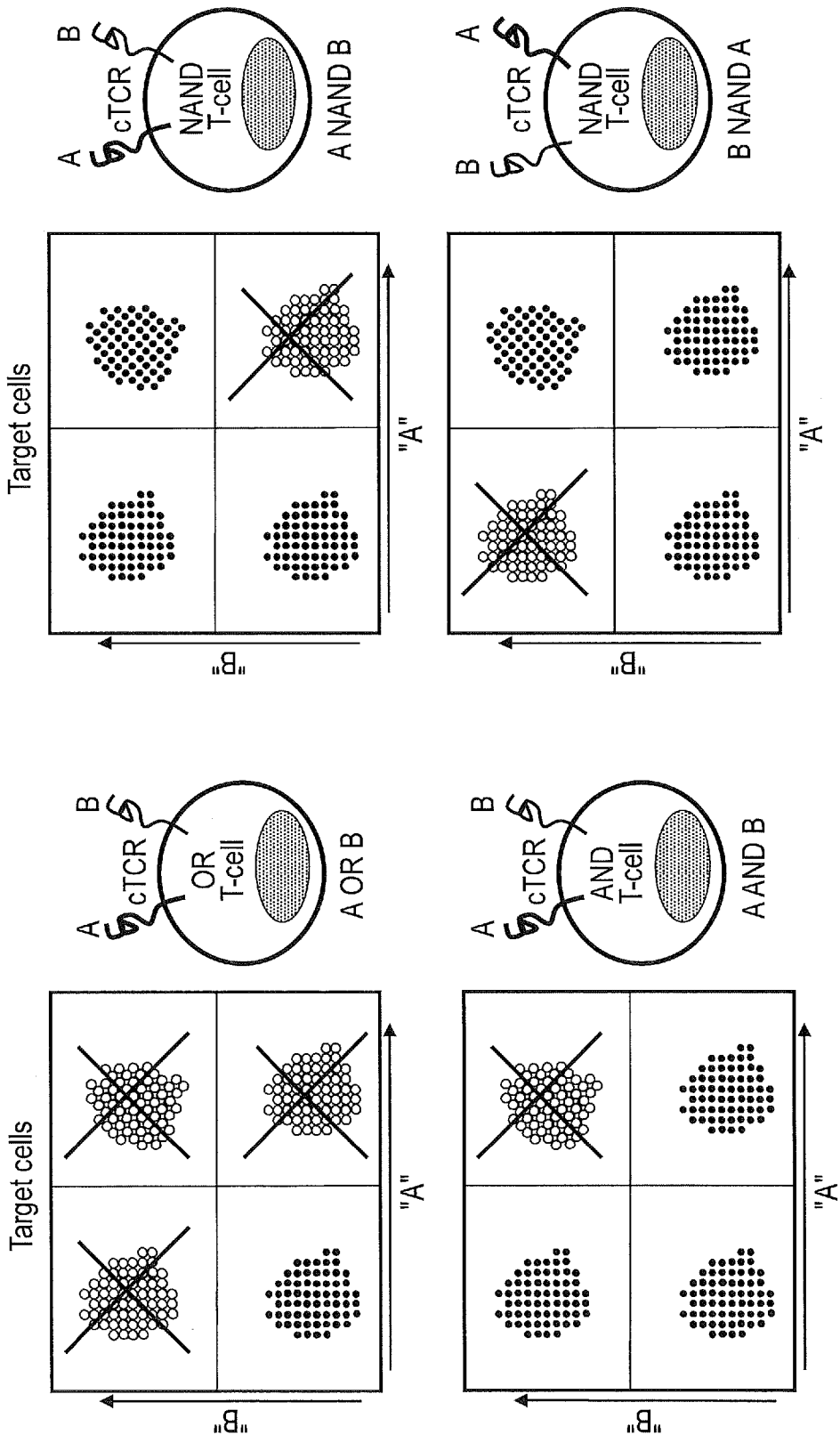
A
B
cTCR
NAND
T-cell
A NAND B
B NAND A
OR
T-cell
A OR B
AND
T-cell
A AND B
Target cells
"A"
"B"

FIG. 2

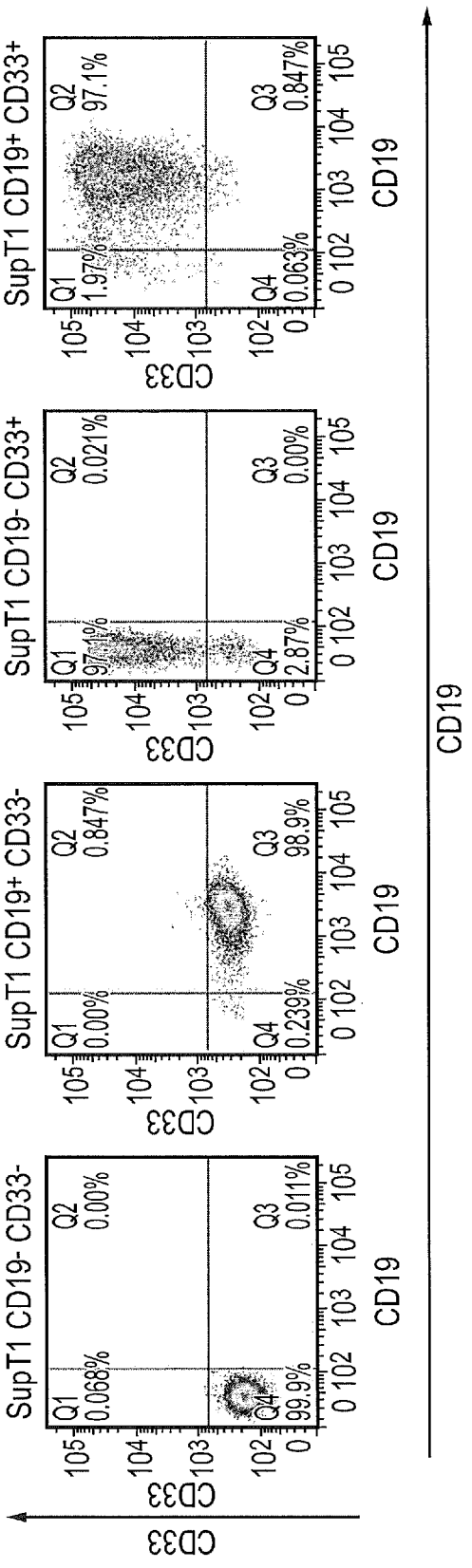
SupT1 CD19- CD33-
SupT1 CD19+ CD33-
SupT1 CD19- CD33+
SupT1 CD19+ CD33+
Q1 0.068%
Q2 0.00%
Q3 0.011%
Q4 99.9%
Q1 0.00%
Q2 0.847%
Q3 98.9%
Q4 0.239%
Q1 97.1%
Q2 0.021%
Q3 0.00%
Q4 2.87%
Q1 1.97%
Q2 97.1%
Q3 0.847%
Q4 0.063%
CD33
CD19
0 102 103 104 105

FIG. 3

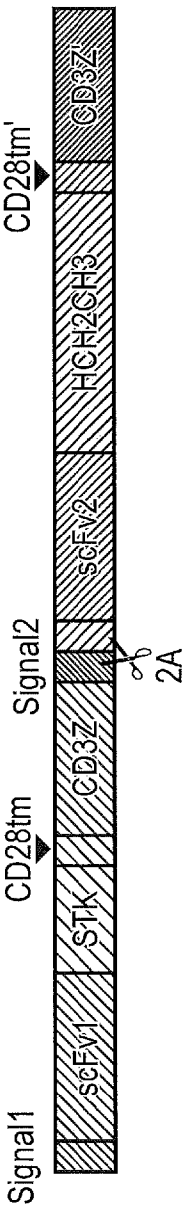
Signal1
scFv1
STk
CD28tm
CD3Z
Signal2
scFv2
2A
HCH2CH3
CD28tm'
CD3Z

FIG. 4

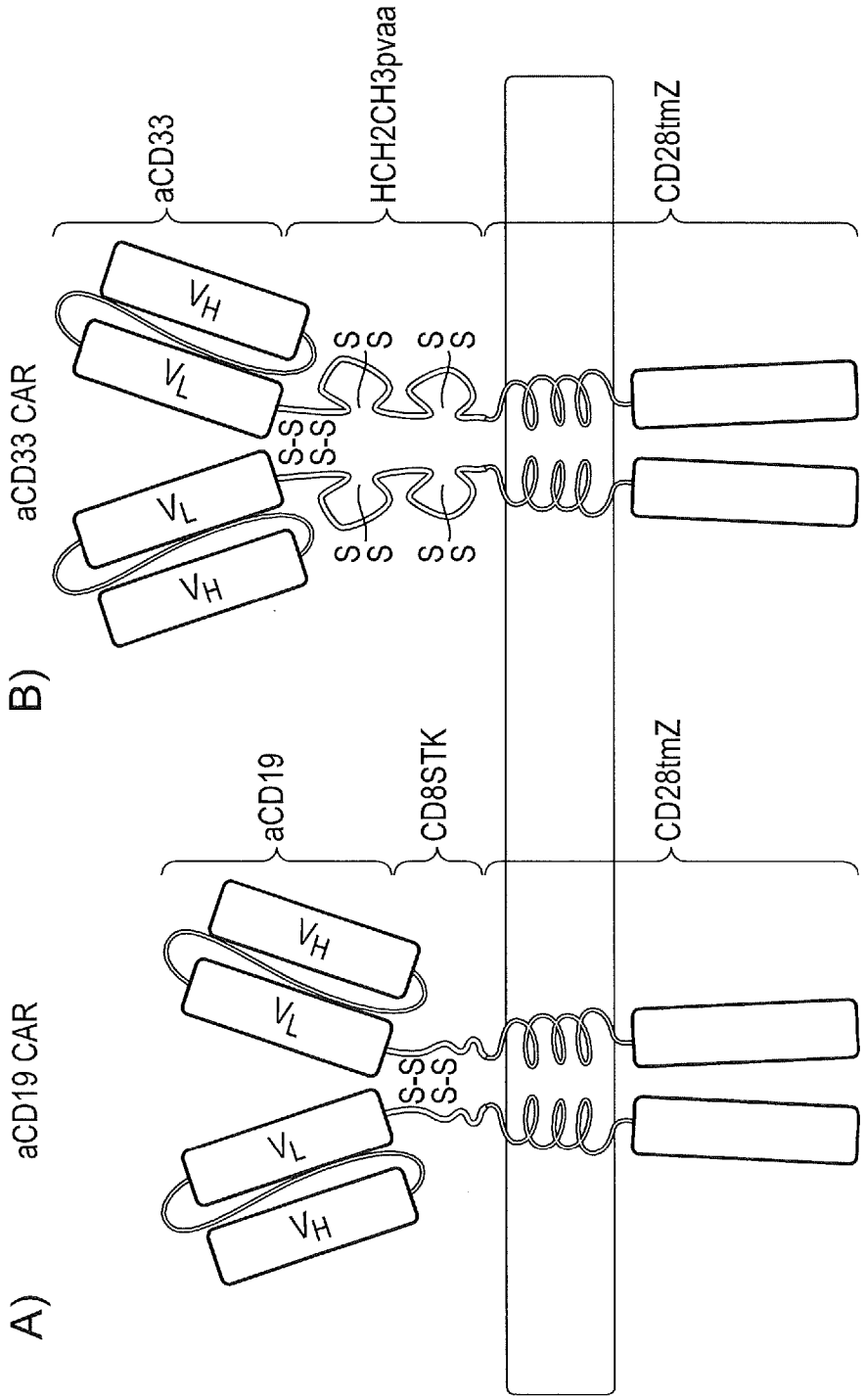
A)
aCD19 CAR
aCD19
CD8STK
CD28tmZ
VH
VL
S-S
B)
aCD33 CAR
aCD33
HCH2CH3pvaa
CD28tmZ
S

FIG. 5

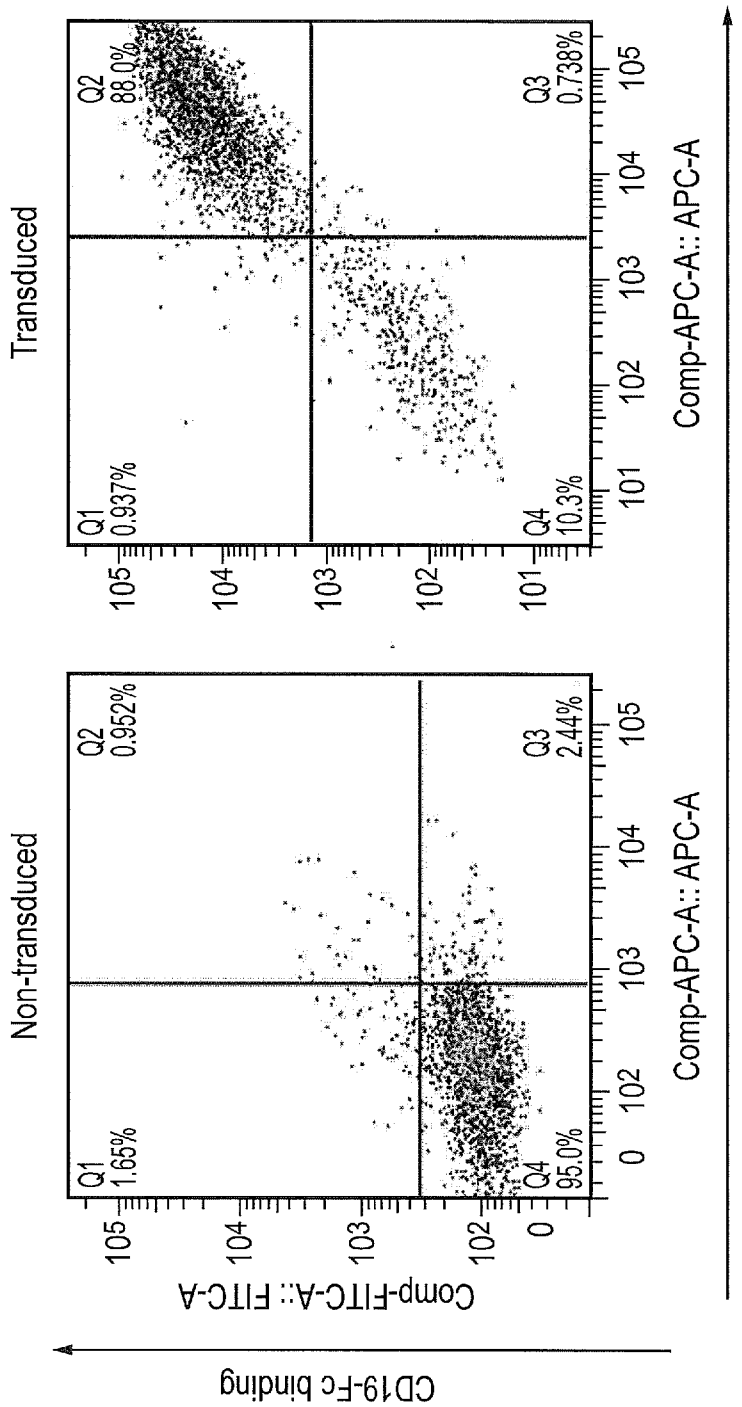
Non-transduced
Transduced
Q1
1.65%
Q2
0.952%
Q3
2.44%
Q4
95.0%
Q1
0.937%
Q2
88.0%
Q3
0.738%
Q4
10.3%
Comp-FITC-A:: FITC-A
CD19-Fc binding
Comp-APC-A:: APC-A
CD33-Fc binding
0
102
103
104
105
101

FIG. 6

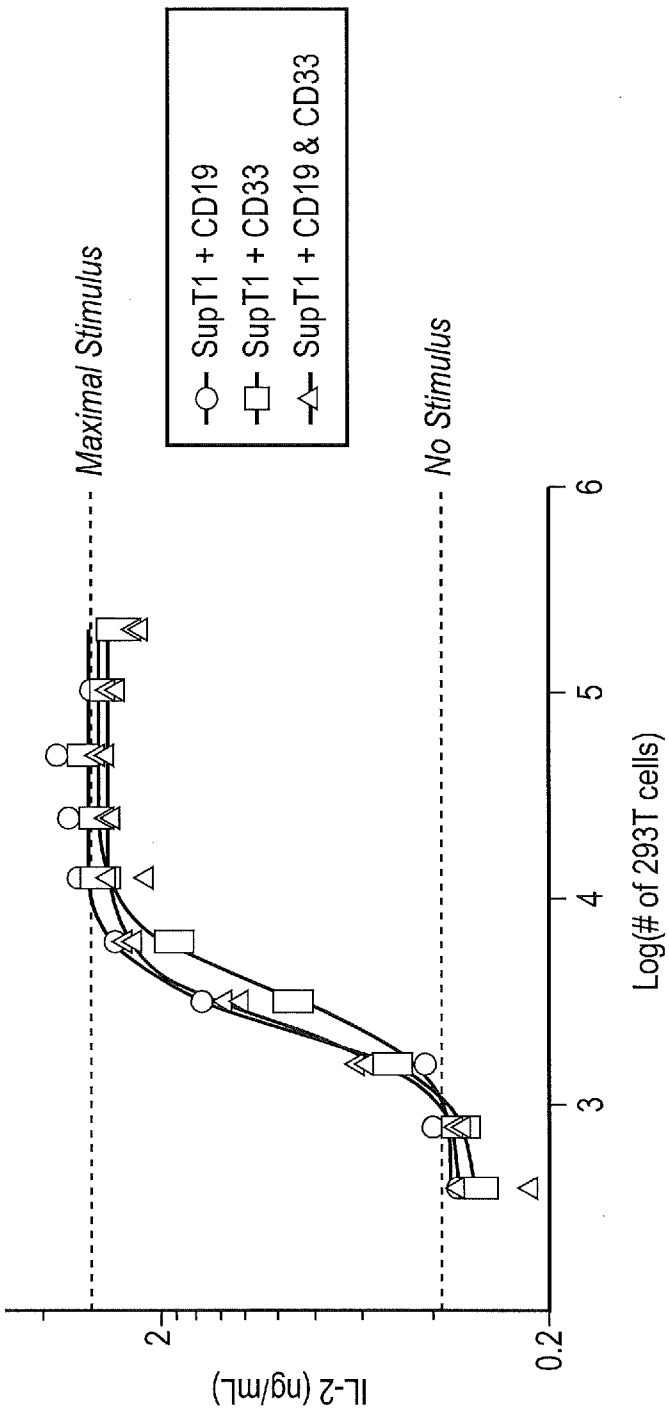
SupT1 + CD19
SupT1 + CD33
SupT1 + CD19 & CD33
Maximal Stimulus
No Stimulus
IL-2 (ng/mL)
2
0.2
3
4
5
6
Log(# of 293T cells)

FIG. 7

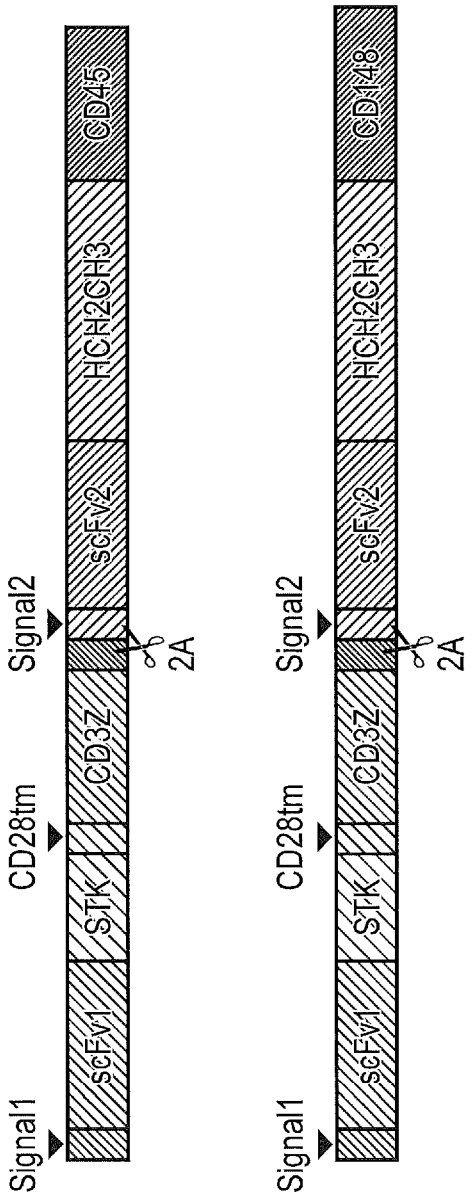
Signal1
scFv1
STK
CD28tm
CD3Z
Signal2
2A
scFv2
HCH2CH3
CD45
Signal1
scFv1
STK
CD28tm
CD3Z
Signal2
2A
scFv2
HCH2CH3
CD148

FIG. 8

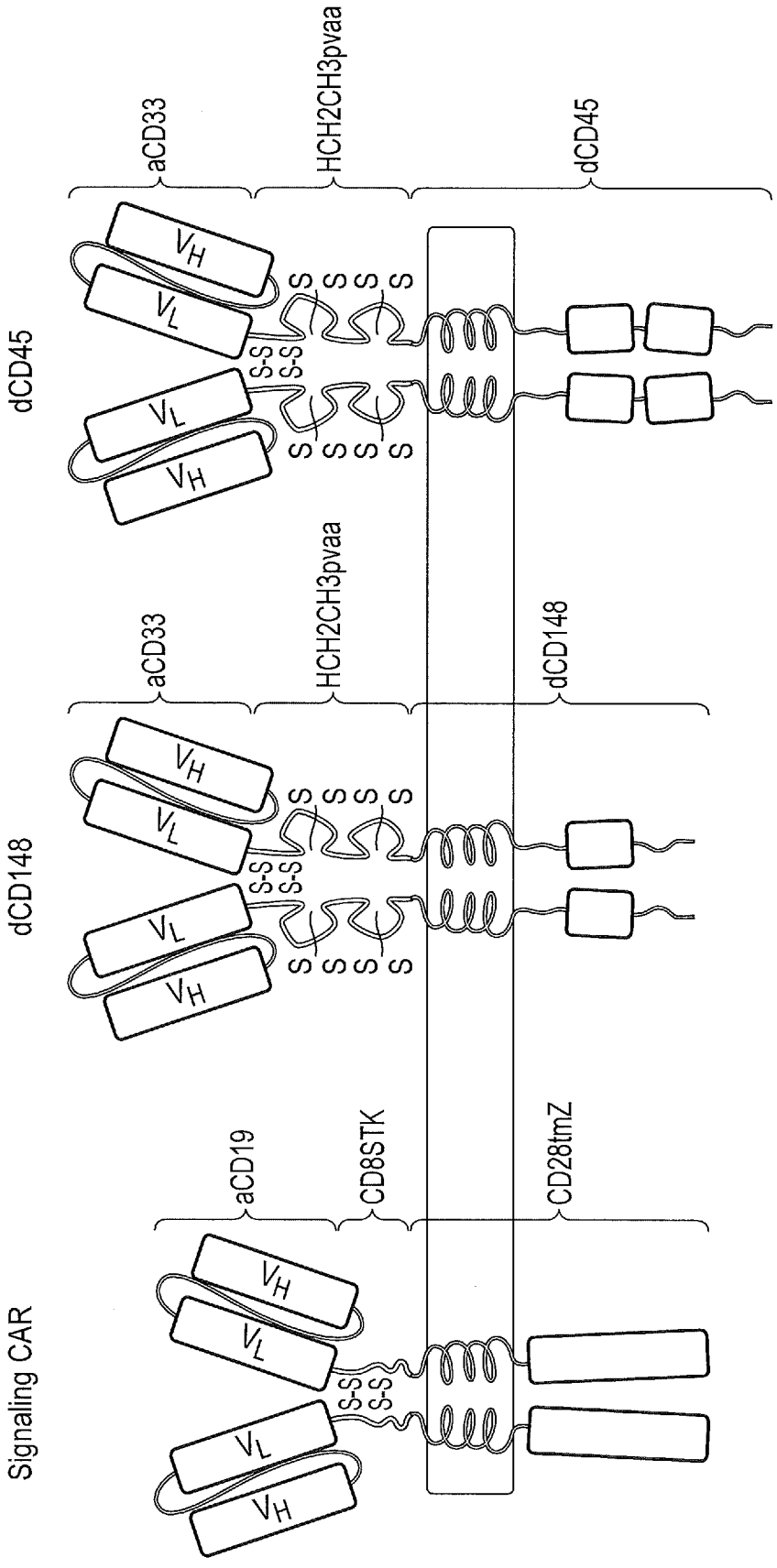
aCD33
HCH2CH3pvaa
dCD45
dCD45
VH
VL
VL
VH
S-S
S-S
aCD33
HCH2CH3pvaa
dCD148
dCD148
VH
VL
VL
VH
aCD19
CD8STK
CD28tmZ
Signaling CAR
VH
VL
VL
VH

FIG. 9

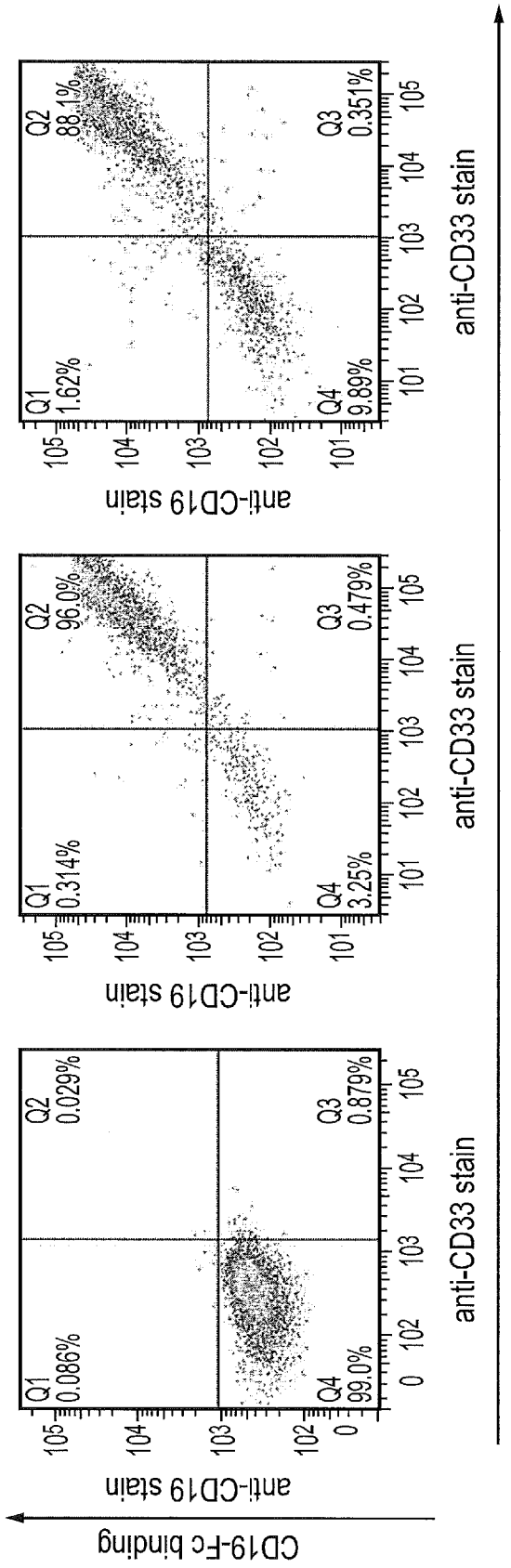
BW5147
BW5147 CD148+
BW5147 CD45+
Q1 0.086%
Q2 0.029%
Q3 0.879%
Q4 99.0%
Q1 0.314%
Q2 96.0%
Q3 0.479%
Q4 3.25%
Q1 1.62%
Q2 88.1%
Q3 0.351%
Q4 9.89%
anti-CD19 stain
anti-CD33 stain
CD19-Fc binding
CD33-Fc binding

FIG. 10

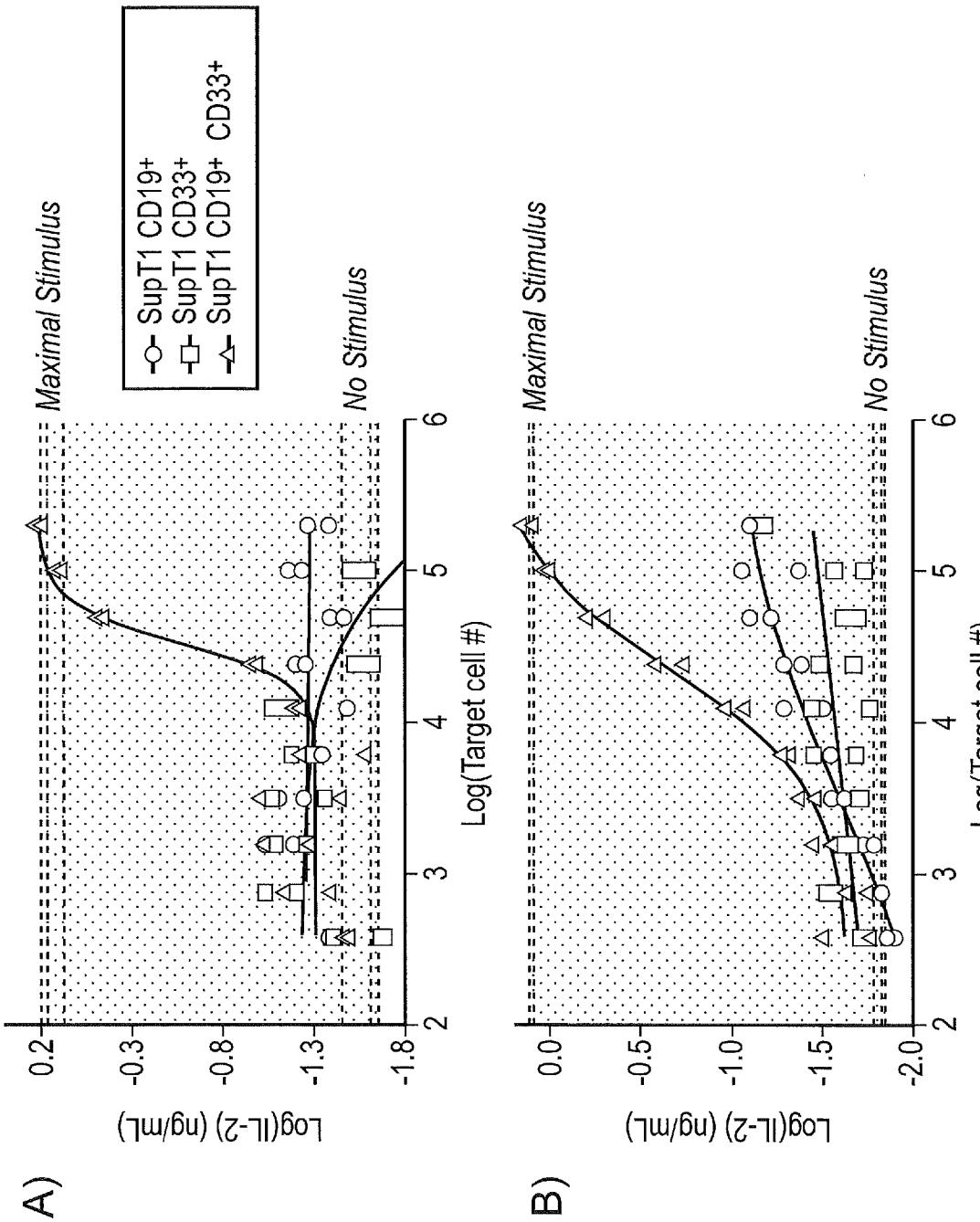
A)
B)
Maximal Stimulus
No Stimulus
SupT1 CD19+
SupT1 CD33+
SupT1 CD19+ CD33+
Log(IL-2) (ng/mL)
Log(Target cell #)
0.2
-0.3
-0.8
-1.3
-1.8
0.0
-0.5
-1.0
-1.5
-2.0
2
3
4
5
6

FIG. 11

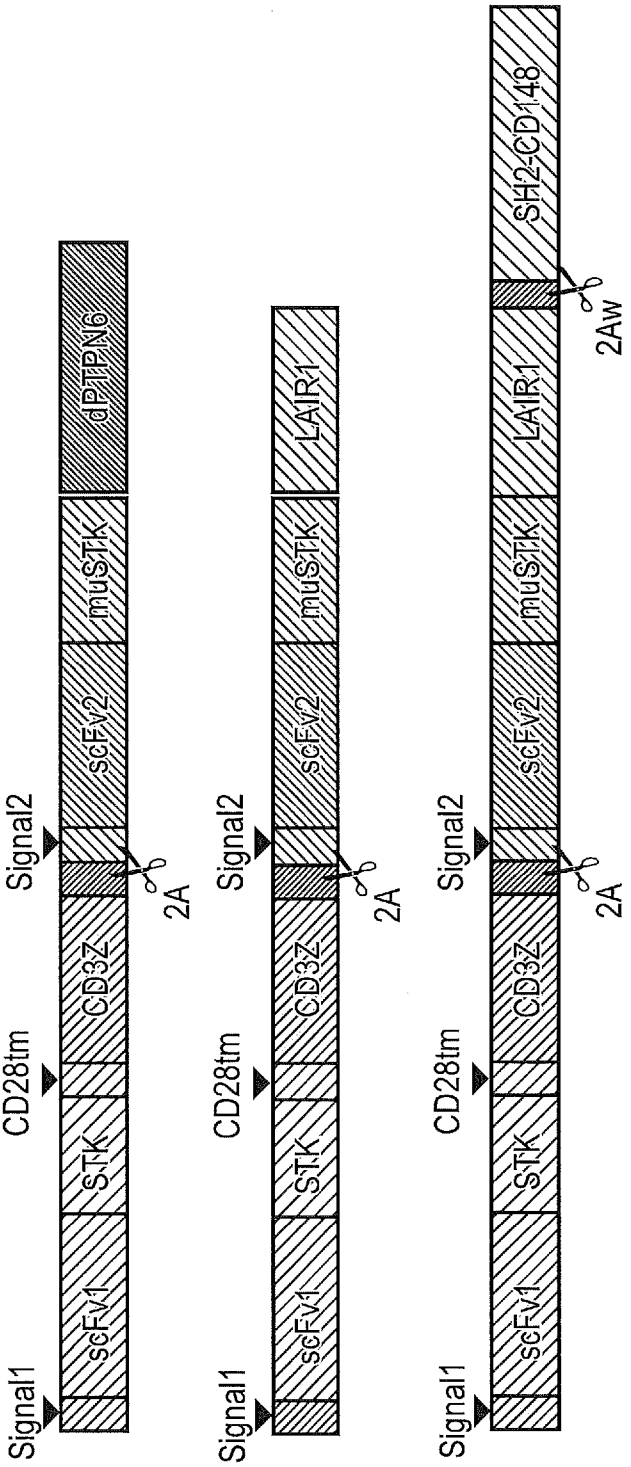
Signal1
scFv1
STK
CD28tm
CD3Z
2A
Signal2
scFv2
muSTK
dPTPN6
LAIR1
SH2-CD148
2Aw

FIG. 12

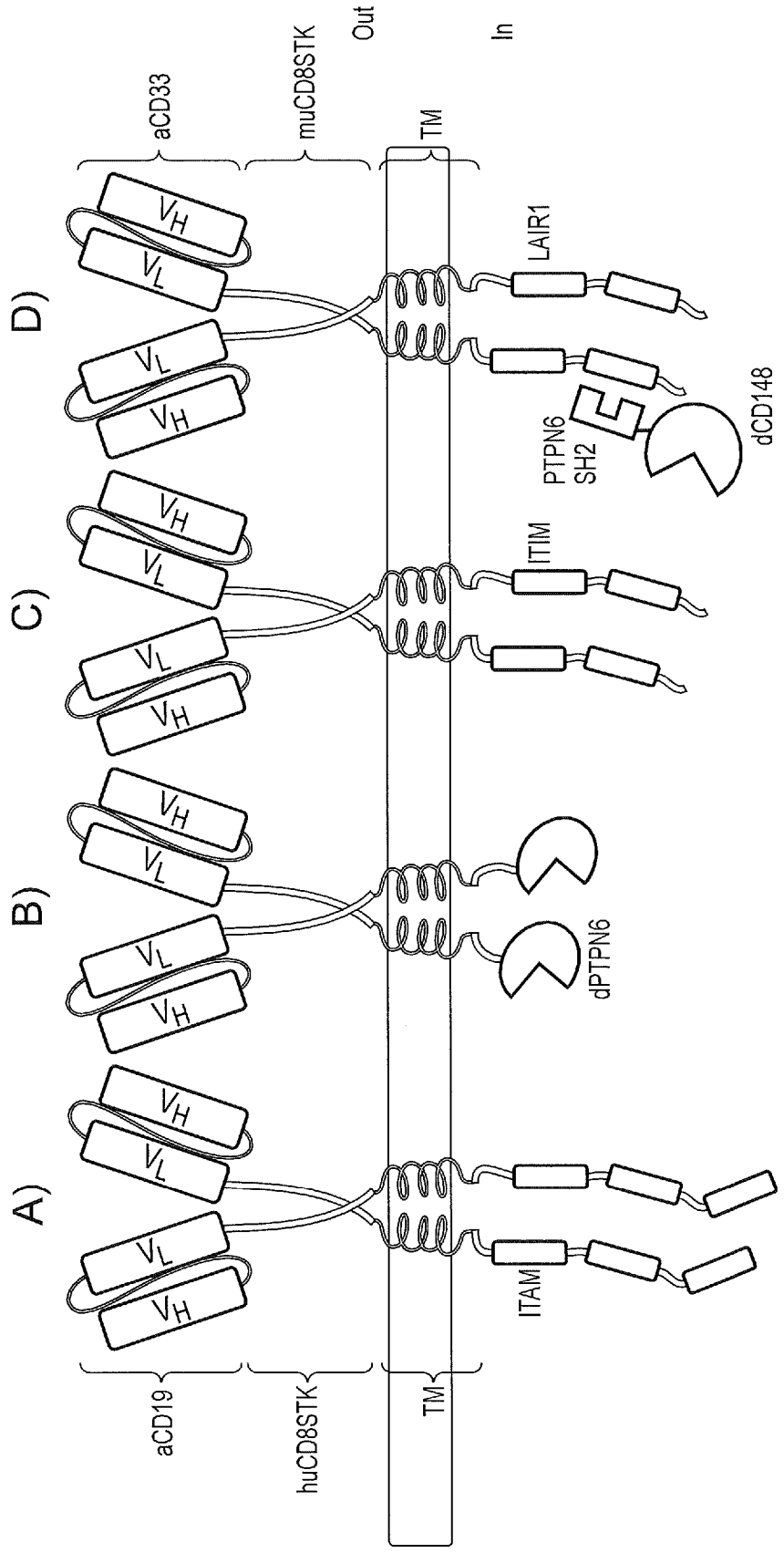
A)
B)
C)
D)
aCD19
huCD8STK
TM
aCD33
muCD8STK
TM
Out
In
ITAM
dPTPN6
ITIM
LAIR1
PTPN6
SH2
dCD148

FIG. 13

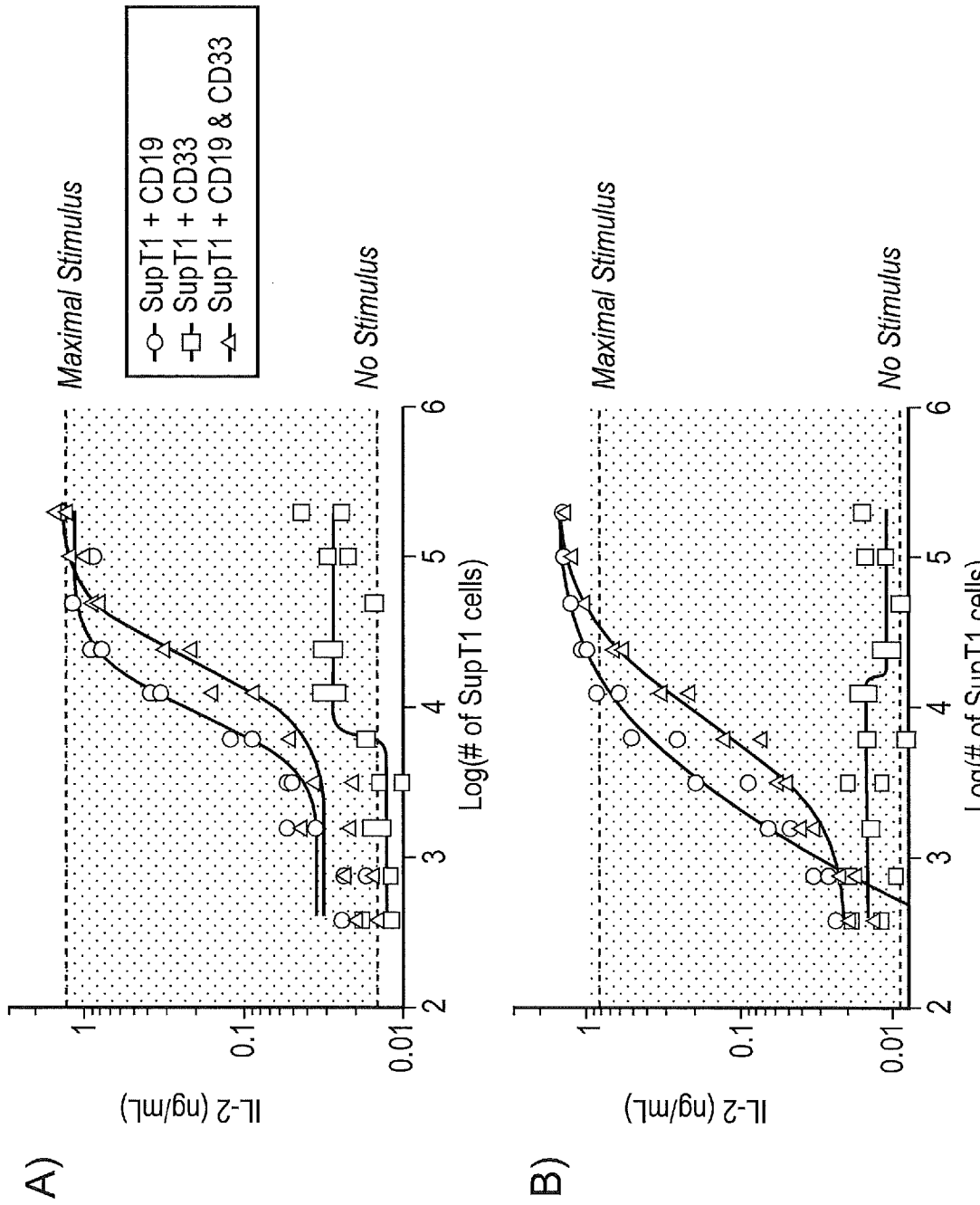
A)
B)
IL-2 (ng/mL)
Log(# of SupT1 cells)
Maximal Stimulus
No Stimulus
SupT1 + CD19
SupT1 + CD33
SupT1 + CD19 & CD33
1
0.1
0.01
2
3
4
5
6

FIG. 14

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>MP13974.SFG.aCD19fmc63_clean-CD8STK-CD28tmZ-2A-aCD33glx-HCH2CH3pvaa-CD28tmZw

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SLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
YYGGSYAMDYWGQGTSVTVS*SDP*TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACY
SLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE
AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQVLGLLLLWLTDARC
DIQMTQSPSSLSASVGDRVTITCRASEDIYENLVWYQQKPGKAPKLLIYDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDE
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WLRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTV
SS*MDP*AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE
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IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPKFWVLVVVG
GVLACYSLLVTVAFIIFWVRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL
QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

Region	*Description*
Signal1	Signal peptide 1
scFv1	scFv 1 – anti-CD19
SDP	Linker and chain break
STK	CD8alpha stalk
CD28tmZ	CD28 transmembrane domain and CD3 Zeta endodomain
FMD-2A	Foot-and-mouth disease 2A peptide
Signal2	Signal peptide 2
scFv2	scFv 2 – anti-CD33
MDP	Linker and chain break
HCH2CH3	Hinge, CH2 and CH3 of human IgG1
CD28tmZ'	CD28 transmembrane domain and CD3 Zeta endodomain

FIG. 15

16/32

>MP14801.SFG.aCD19fmc63_clean-CD8STK-CD28tmZ-2A-aCD33glx-HCH2CH3pvaa-dCD148

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRF
SGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQ
SLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
YYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACY
SLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE
AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQVLGLLLLWLTDARC
DIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLIYDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDF
ATYYCQHYKNYPLTFGQGTKLEIKRSGGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLSLNGGSTYYRDSVKGRFTLSRDNAKSTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTV
SSMDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE
EQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPKAVFGCIFG
ALVIVTVGGFIFWRKKRKDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENR
GKNRYNNVLPYDISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLTKCVEQGRTKC
EEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPDTTDLLINFRYLVRDYMKQSPPE
SPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTT
AMTIYENLAPVTTFGKTNGYIA

MP14802. SFG.aCD19fmc63_clean-CD8STK-CD28tmZ-2A-aCD33glx-HCH2CH3pvaa-dCD45

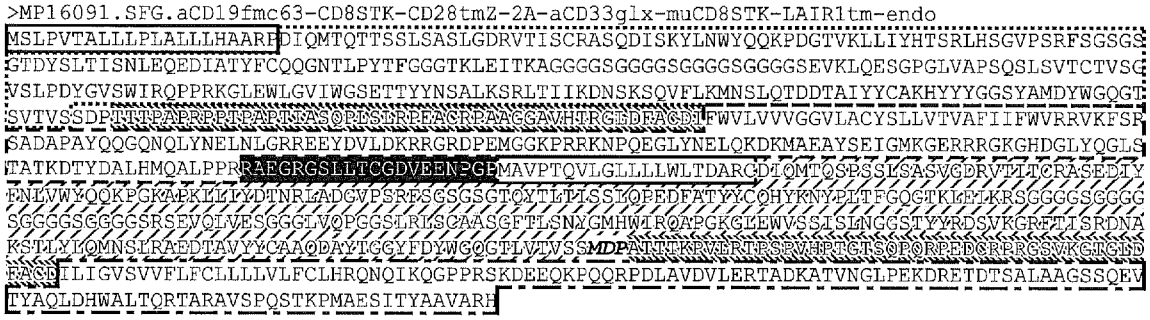
MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRF
SGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQ
SLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
YYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACY
SLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE
AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLLTCGDVEENPGPMAVPTQVLGLLLLWLTDARC
DIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLIYDTNRLADGVPSRFSGSGSGTQYTLTISSLQPEDF
ATYYCQHYKNYPLTFGQGTKLEIKRSGGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLSLNGGSTYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTV
SSMDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE
EQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPKALIAFLAF
LIIVTSIALLVVLYKIYDLHKKRSCNLDEQQELVERDDEKQLMNVEPIHADILLETYKRKIADEGRLFLAEFQSIPRVFSKFP
IKEARKPFNQNKNRYVDILPYDYNRVELSEINGDAGSNYINASYIDGFKEPRKYIAAQGPRDETVDDFWRMIWEQKATVIVMV
TRCEEGNRNKCAEYWPSMEEGTRAFGDVVVKINQHKRCPDYIIQKLNIVNKKEKATGREVTHIQFTSWPDHGVPEDPHLLLKL
RRRVNAFSNFFSGPIVVHCSAGVGRTGTYIGIDAMLEGLEAENKVDVYGYVVKLRRQRCLMVQVEAQYILIHQALVEYNQFGE
TEVNLSELHPYLHNMKKRDPPSEPSPLEAEFQRLPSYRSWRTQHIGNQEENKSKNRNSNVIPYDYNRVPLKHELEMSKESEHD
SDESSDDDSDSEEPSKYINASFIMSYWKPEVMIAAQGPLKETIGDFWQMIFQRKVKVIVMLTELKHGDQEICAQYWGEGKQTY
GDIEVDLKDTDKSSTYTLRVFELRHSKRKDSRTVYQYQYTNWSVEQLPAEPKELISMIQVVKQKLPQKNSSEGNKHHKSTPLL
IHCRDGSQQTGIFCALLNLLESAETEEVVDIFQVVKALRKARPGMVSTFEQYQFLYDVIASTYPAQNGQVKKNNHQEDKIEFD
NEVDKVKQDANCVNPLGAPEKLPEAKEQAEGSEPTSGTEGPEHSVNGPASPALNQGS

Region	*Description*
Signal1	Signal peptide 1
scFv1	scFv 1 – anti-CD19
SDP	Linker and chain break
STK	CD8alpha stalk
CD28tmZ	CD28 transmembrane domain and CD3 Zeta endodomain
FMD-2A	Foot-and-mouth disease 2A peptide
Signal2	Signal peptide 2
scFv2	scFv 2 – anti-CD33
MDP	Linker and chain break
HCH2CH3	Hinge, CH2 and CH3 of human IgG1
dCD148 / dCD45	Trans-membrane and endo-domains of CD148 and CD45

FIG. 16

>16076.SFG.aCD19fmc63-CD8STK-CD28tmZ-2A-aCD33glx-muCD8STK-tm-dPTPN6

>MP16091.SFG.aCD19fmc63-CD8STK-CD28tmZ-2A-aCD33glx-muCD8STK-LAIR1tm-endo



>MP16092.SFG.aCD19fmc63-CD8STK-CD28tmZ-2A-aCD33glx-muCD8STK-LAIR1tm-endo-2A-PTPN6_SH2-dCD148

Region	*Description*
Signal1	Signal peptide 1
scFv1	scFv 1 – anti-CD19
SDP	Linker and chain break
STK	Human CD8alpha stalk
CD28tmZ	CD28 transmembrane domain and CD3 Zeta endodomain
FMD-2A	Foot-and-mouth disease 2A peptide
Signal2	Signal peptide 2
scFv2	scFv 2 – anti-CD33
MDP	Linker and chain break
STK	Mouse CD8alpha stalk
dLAIR1	Hinge, CH2 and CH3 of human IgG1
dPTPN6	Phosphatase domain of PTPN6
FMD-2A'	Foot-and-mouth disease 2A peptide codon wobbled
PTPN6-SH2	SH2 domain of PTPN6
SGGGGS	Serine glycine linker and chain break
dCD148	Phosphatase domain of CD148

FIG. 17

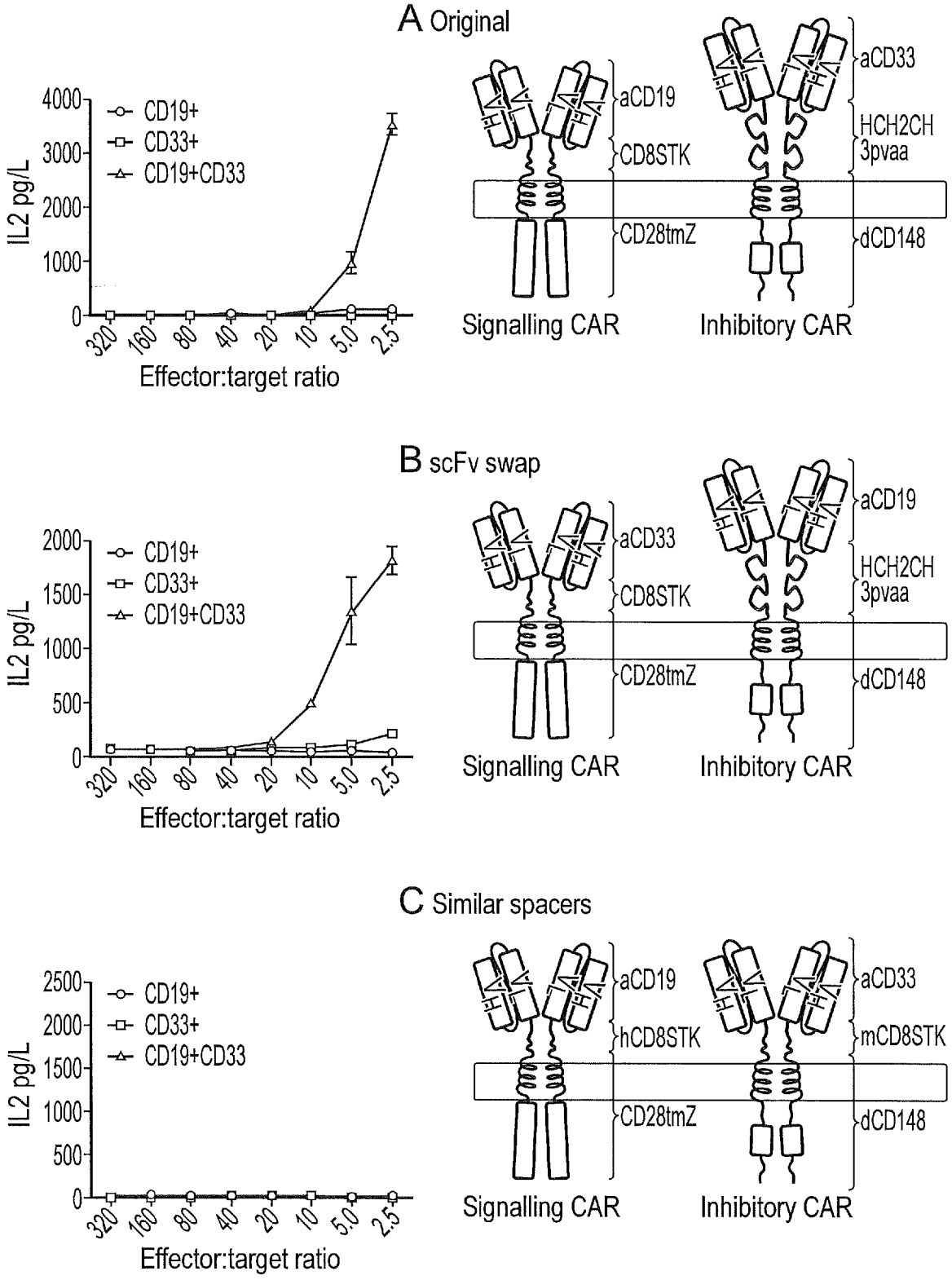

A Original
IL2 pg/L
4000
3000
2000
1000
0
CD19+
CD33+
CD19+CD33
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
CD8STK
CD28tmZ
aCD33
HCH2CH
3pvaa
dCD148
Signalling CAR
Inhibitory CAR
B scFv swap
IL2 pg/L
2000
1500
1000
500
0
CD19+
CD33+
CD19+CD33
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD33
CD8STK
CD28tmZ
aCD19
HCH2CH
3pvaa
dCD148
Signalling CAR
Inhibitory CAR
C Similar spacers
IL2 pg/L
2500
2000
1500
1000
500
0
CD19+
CD33+
CD19+CD33
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
hCD8STK
CD28tmZ
aCD33
mCD8STK
dCD148
Signalling CAR
Inhibitory CAR

FIG. 18

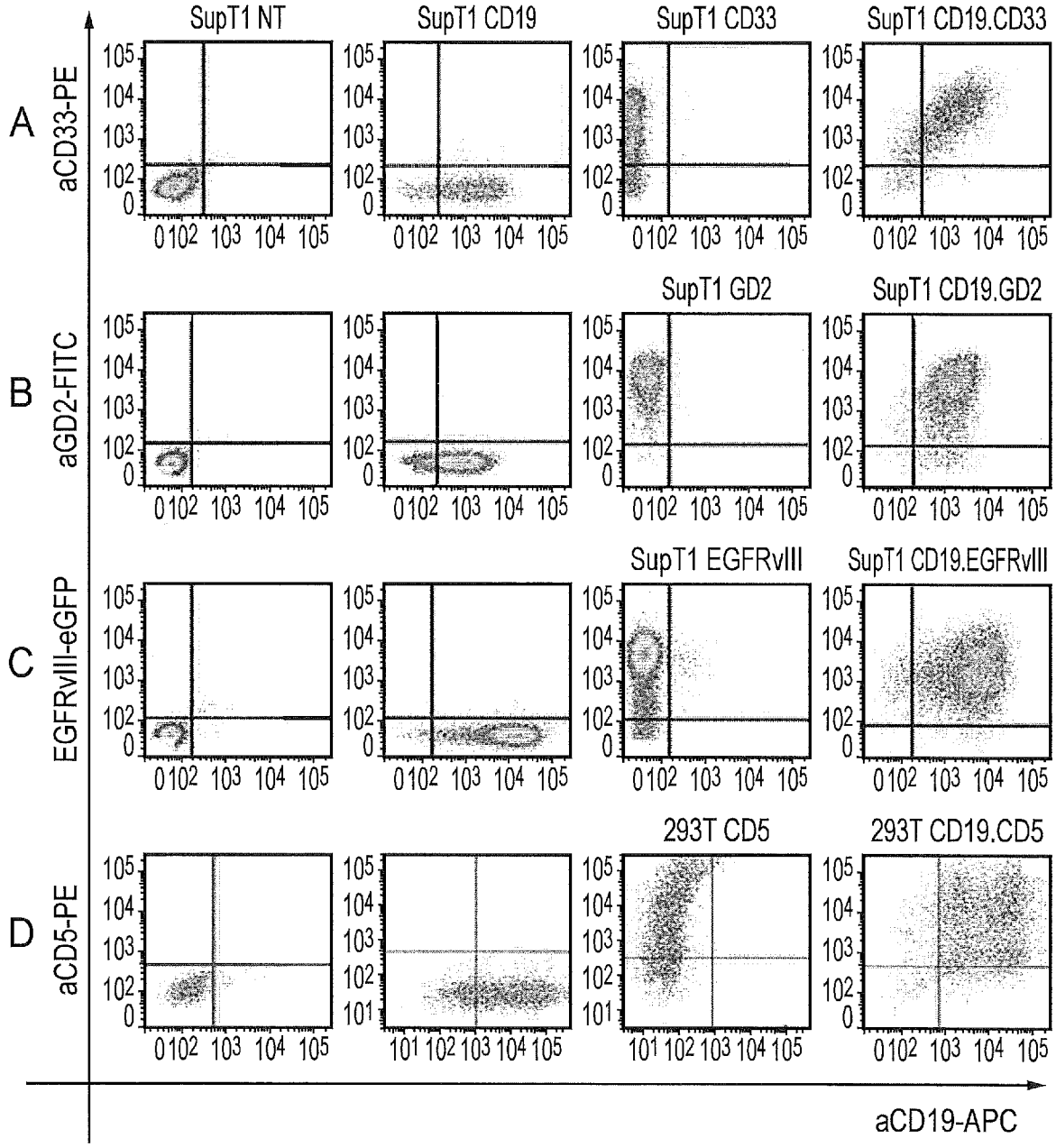

SupT1 NT
SupT1 CD19
SupT1 CD33
SupT1 CD19.CD33
A
aCD33-PE
SupT1 GD2
SupT1 CD19.GD2
B
aGD2-FITC
SupT1 EGFRvIII
SupT1 CD19.EGFRvIII
C
EGFRvIII-eGFP
293T CD5
293T CD19.CD5
D
aCD5-PE
aCD19-APC

FIG. 19

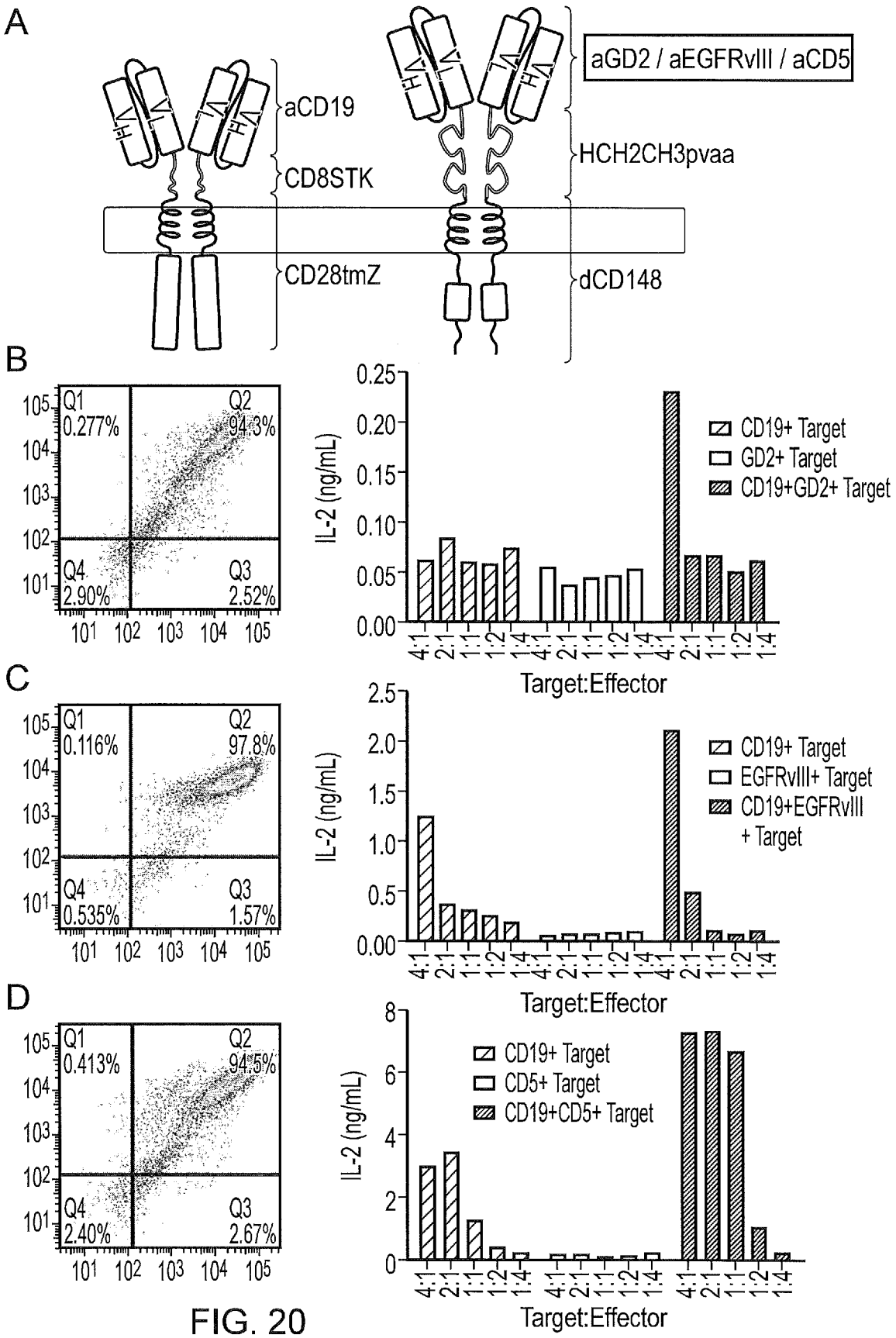

A
aCD19
CD8STK
CD28tmZ
aGD2 / aEGFRvIII / aCD5
HCH2CH3pvaa
dCD148
B
Q1 0.277%
Q2 94.3%
Q4 2.90%
Q3 2.52%
IL-2 (ng/mL)
CD19+ Target
GD2+ Target
CD19+GD2+ Target
Target:Effector
C
Q1 0.116%
Q2 97.8%
Q4 0.535%
Q3 1.57%
IL-2 (ng/mL)
CD19+ Target
EGFRvIII+ Target
CD19+EGFRvIII + Target
Target:Effector
D
Q1 0.413%
Q2 94.5%
Q4 2.40%
Q3 2.67%
IL-2 (ng/mL)
CD19+ Target
CD5+ Target
CD19+CD5+ Target
Target:Effector

FIG. 20

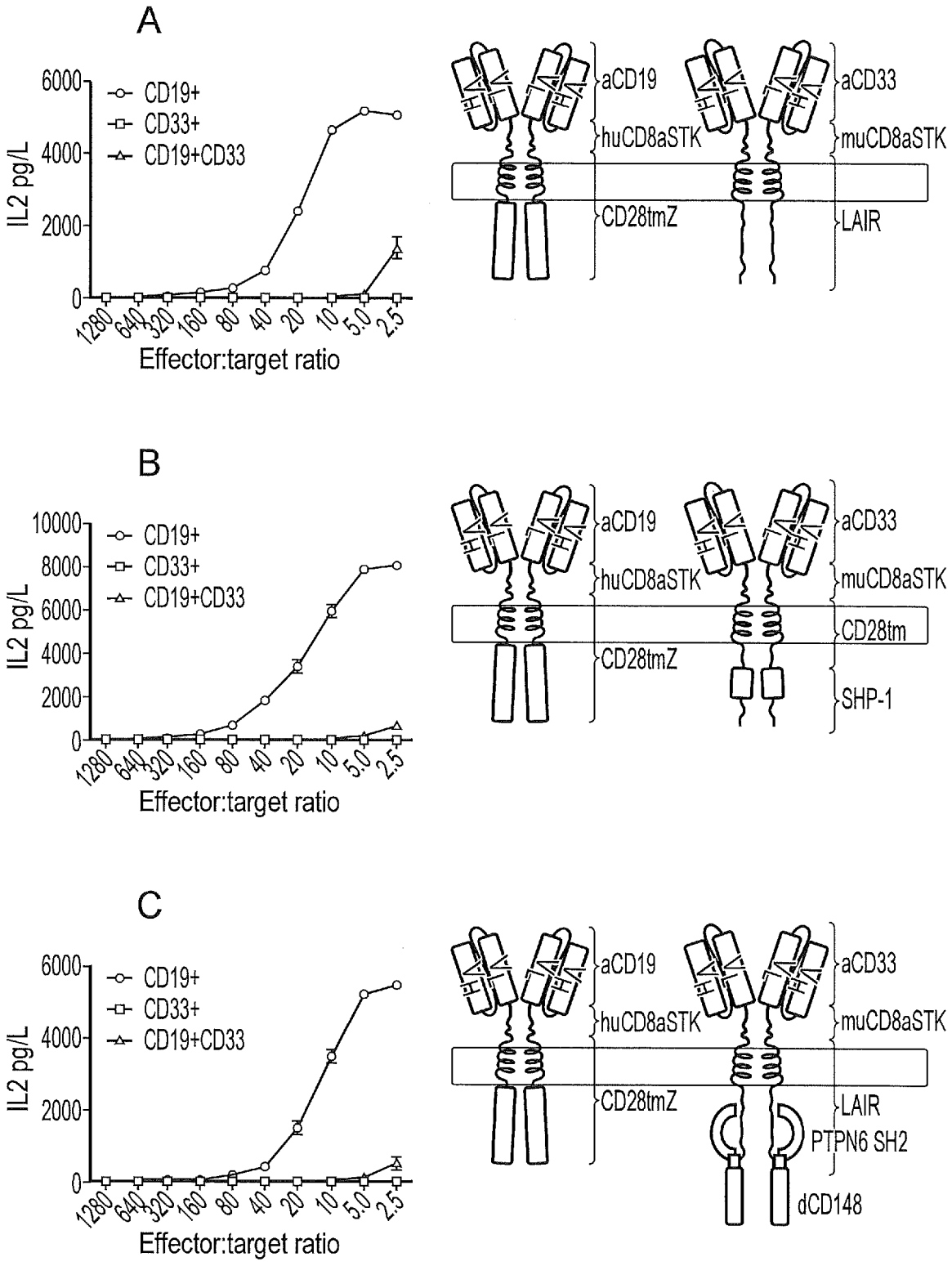

A
6000
4000
2000
0
IL2 pg/L
CD19+
CD33+
CD19+CD33
1280
640
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
muCD8aSTK
LAIR
B
10000
8000
6000
4000
2000
0
IL2 pg/L
CD19+
CD33+
CD19+CD33
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
muCD8aSTK
CD28tm
SHP-1
C
6000
4000
2000
0
IL2 pg/L
CD19+
CD33+
CD19+CD33
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
muCD8aSTK
LAIR
PTPN6 SH2
dCD148

FIG. 21

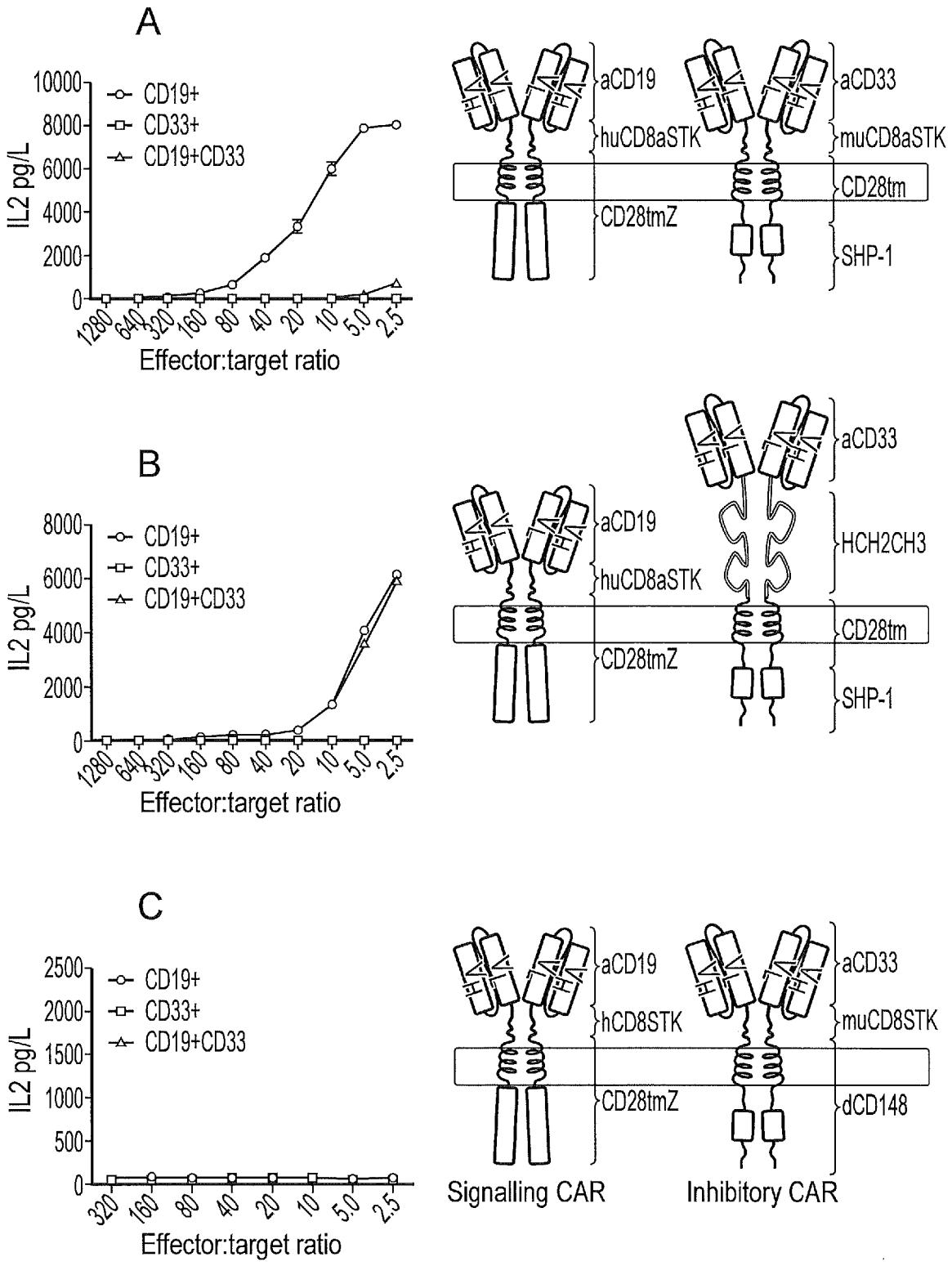

A
IL2 pg/L
10000
8000
6000
4000
2000
0
CD19+
CD33+
CD19+CD33
1280
640
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
muCD8aSTK
CD28tm
SHP-1
B
IL2 pg/L
8000
6000
4000
2000
0
CD19+
CD33+
CD19+CD33
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
HCH2CH3
CD28tm
SHP-1
C
IL2 pg/L
2500
2000
1500
1000
500
0
CD19+
CD33+
CD19+CD33
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
hCD8STK
CD28tmZ
aCD33
muCD8STK
dCD148
Signalling CAR
Inhibitory CAR

FIG. 22

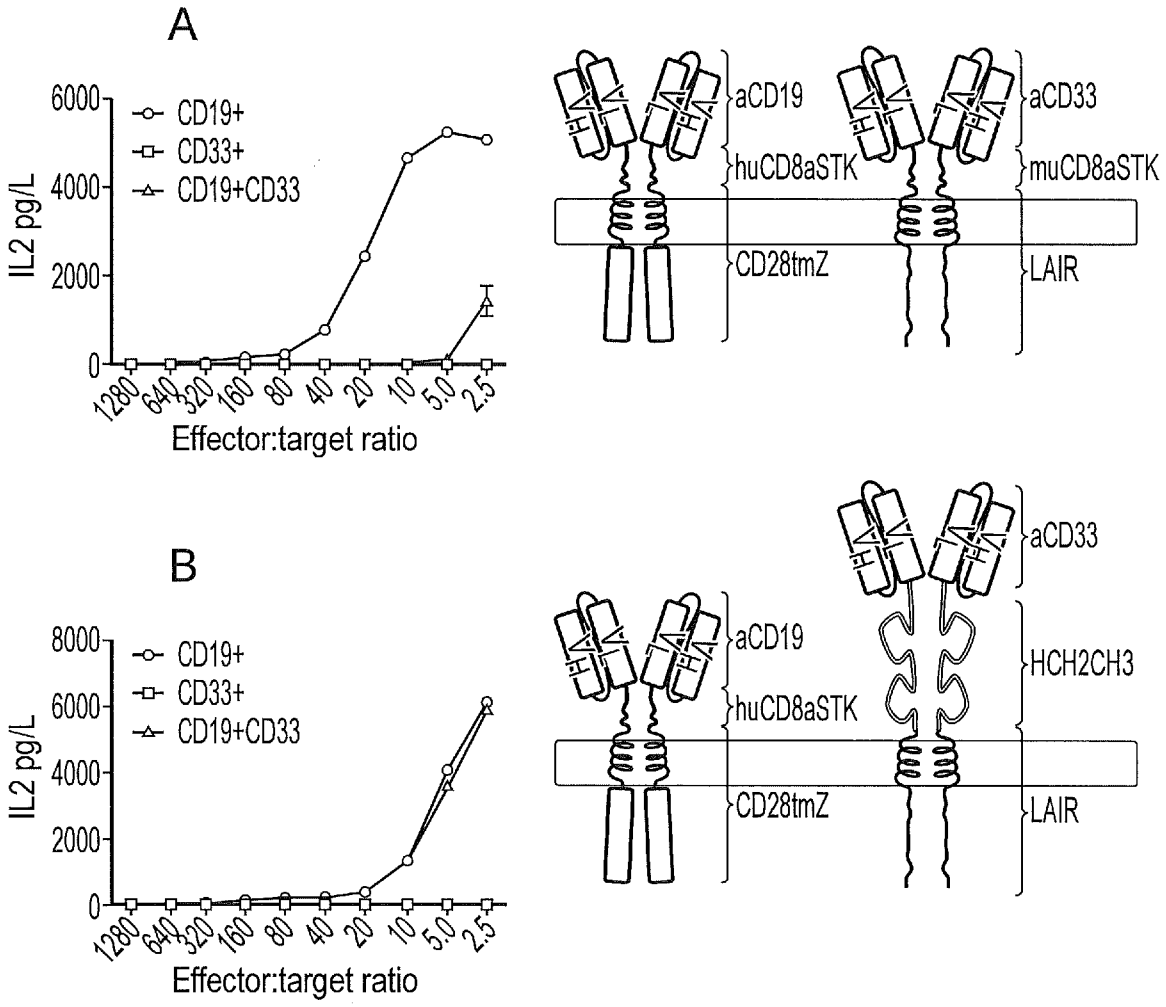

A
6000
4000
2000
0
IL2 pg/L
CD19+
CD33+
CD19+CD33
1280
640
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD19
huCD8aSTK
CD28tmZ
aCD33
muCD8aSTK
LAIR
B
8000
6000
4000
2000
0
IL2 pg/L
CD19+
CD33+
CD19+CD33
1280
640
320
160
80
40
20
10
5.0
2.5
Effector:target ratio
aCD33
aCD19
huCD8aSTK
CD28tmZ
HCH2CH3
LAIR

FIG. 23

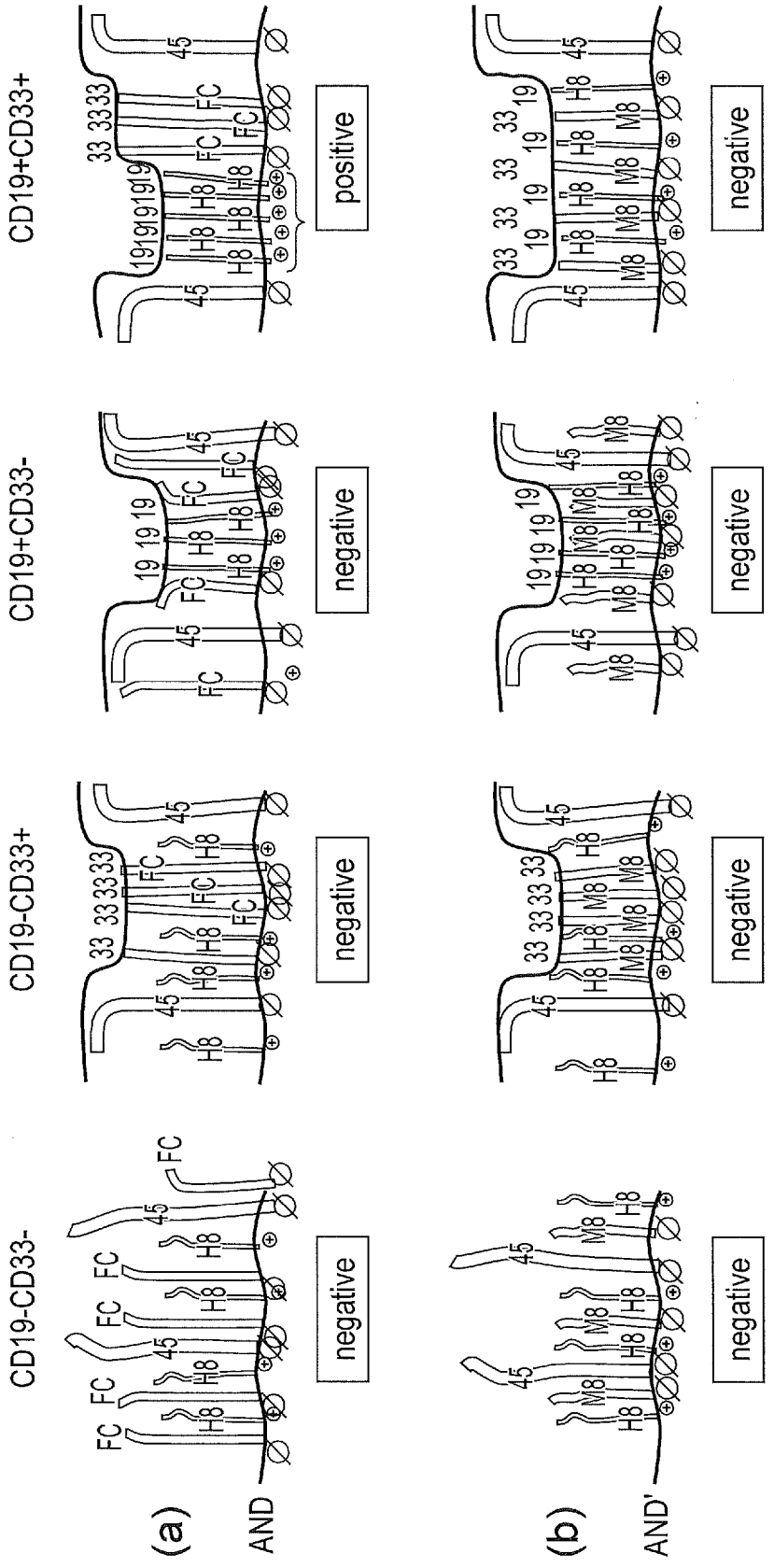

CD19-CD33-
CD19-CD33+
CD19+CD33-
CD19+CD33+
negative
negative
negative
positive
(a)
AND
(b)
AND'

FIG. 24

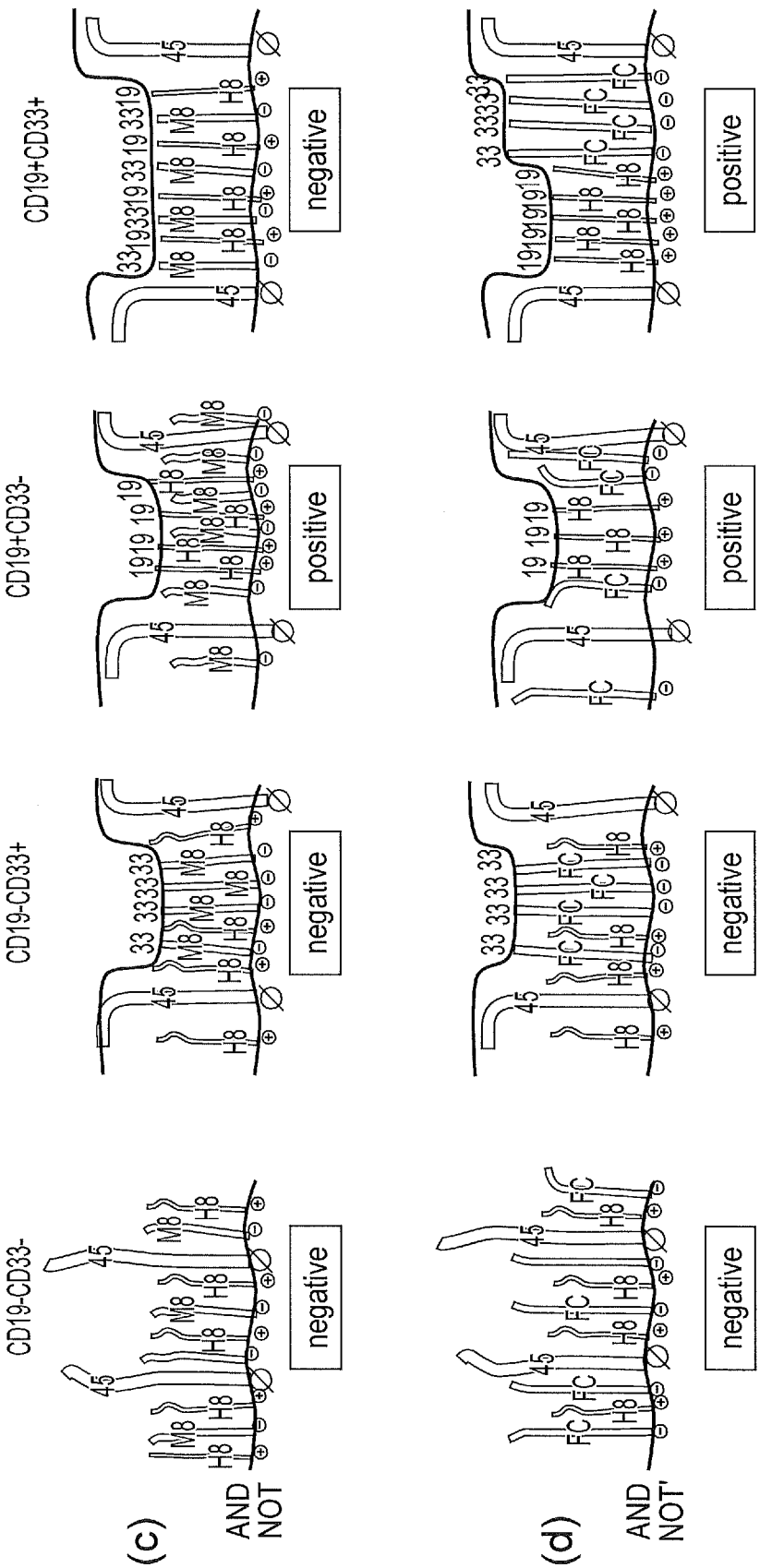


FIG. 24 (Continued)

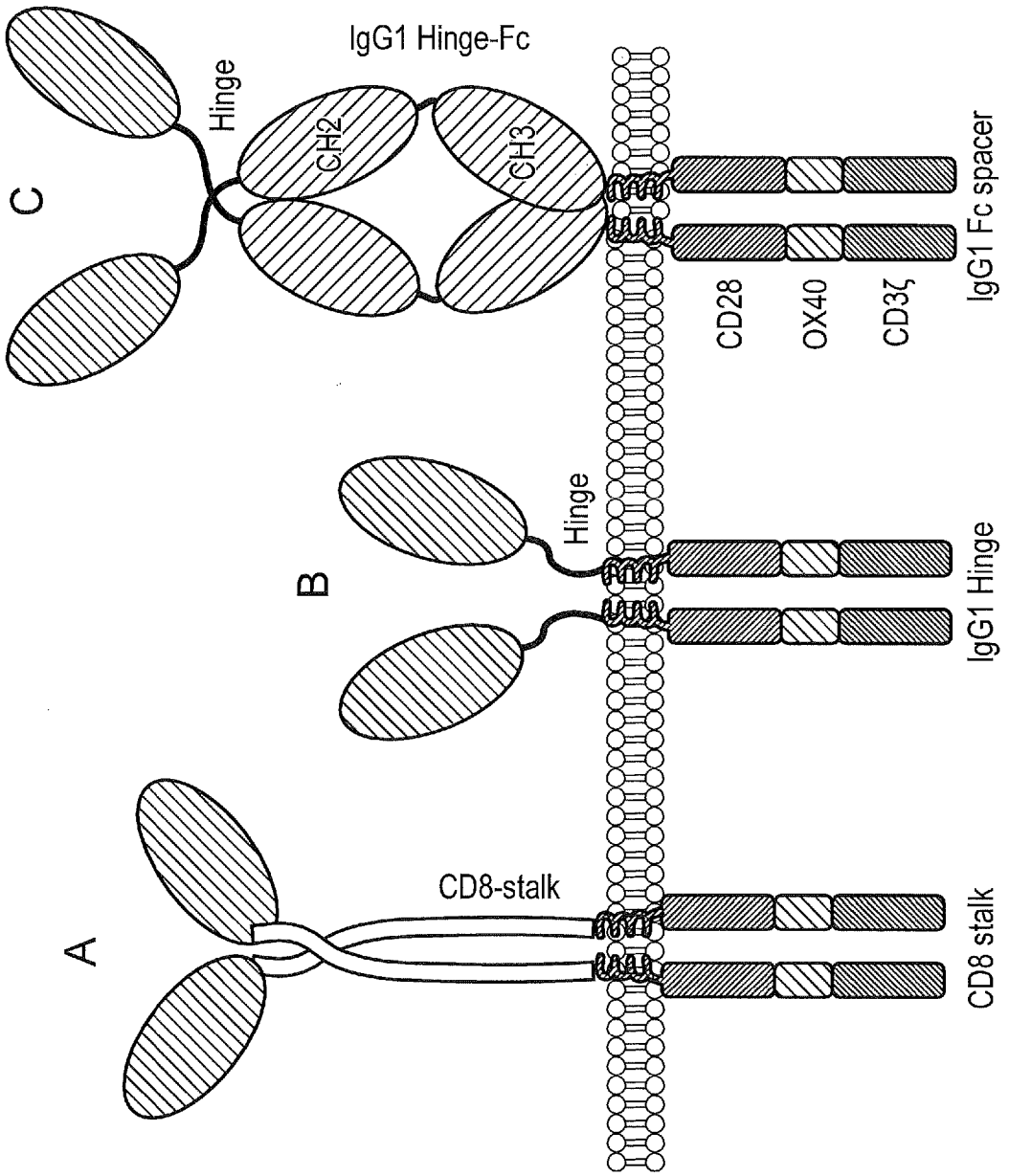
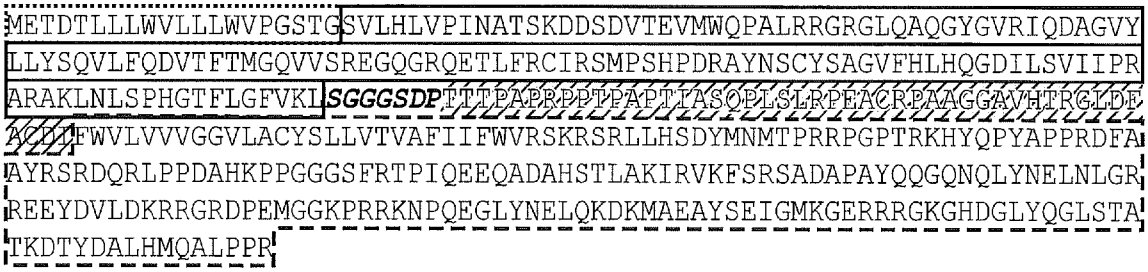
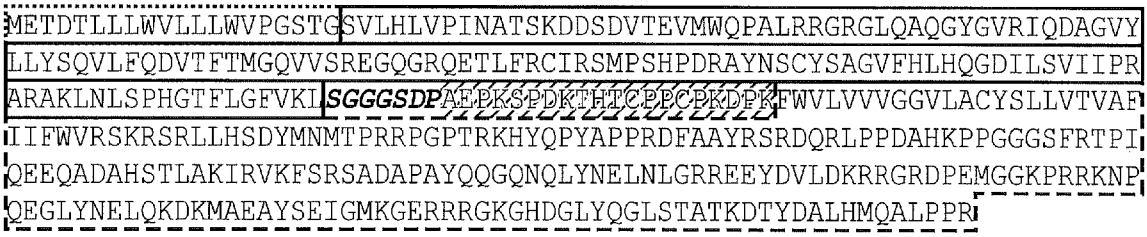

IgG1 Hinge-Fc
Hinge
CH2
CH3
C
CD28
OX40
CD3ζ
IgG1 Fc spacer
Hinge
B
IgG1 Hinge
CD8-stalk
A
CD8 stalk

FIG. 25

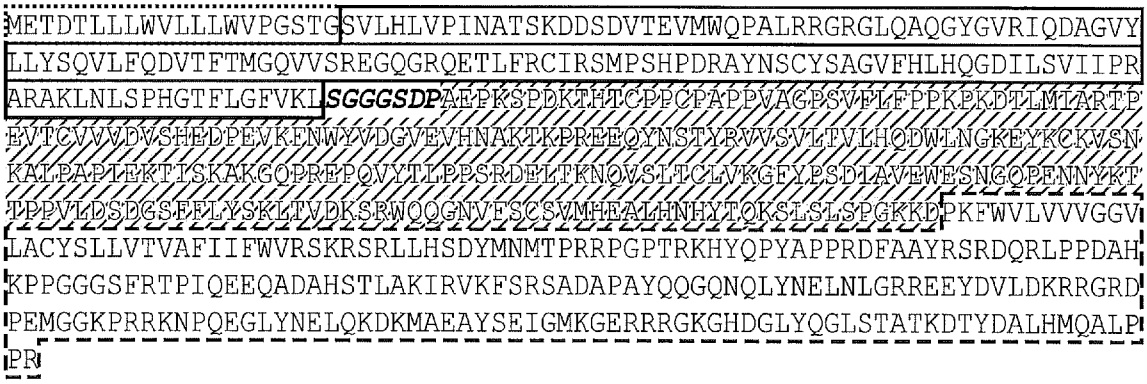
A



B



C



Signal Peptide	Efficient signal peptide
dAPRIL	Truncated APRIL
Spacer	Either hinge-CH2CH3 of human IgG1, human CD8 stalk and human IgG1 hinge
TM and endodomain	Compound endodomain comprising of the CD28TM domain, CD28 endodomain and OX40 and CD3-Zeta endodomains

FIG. 26

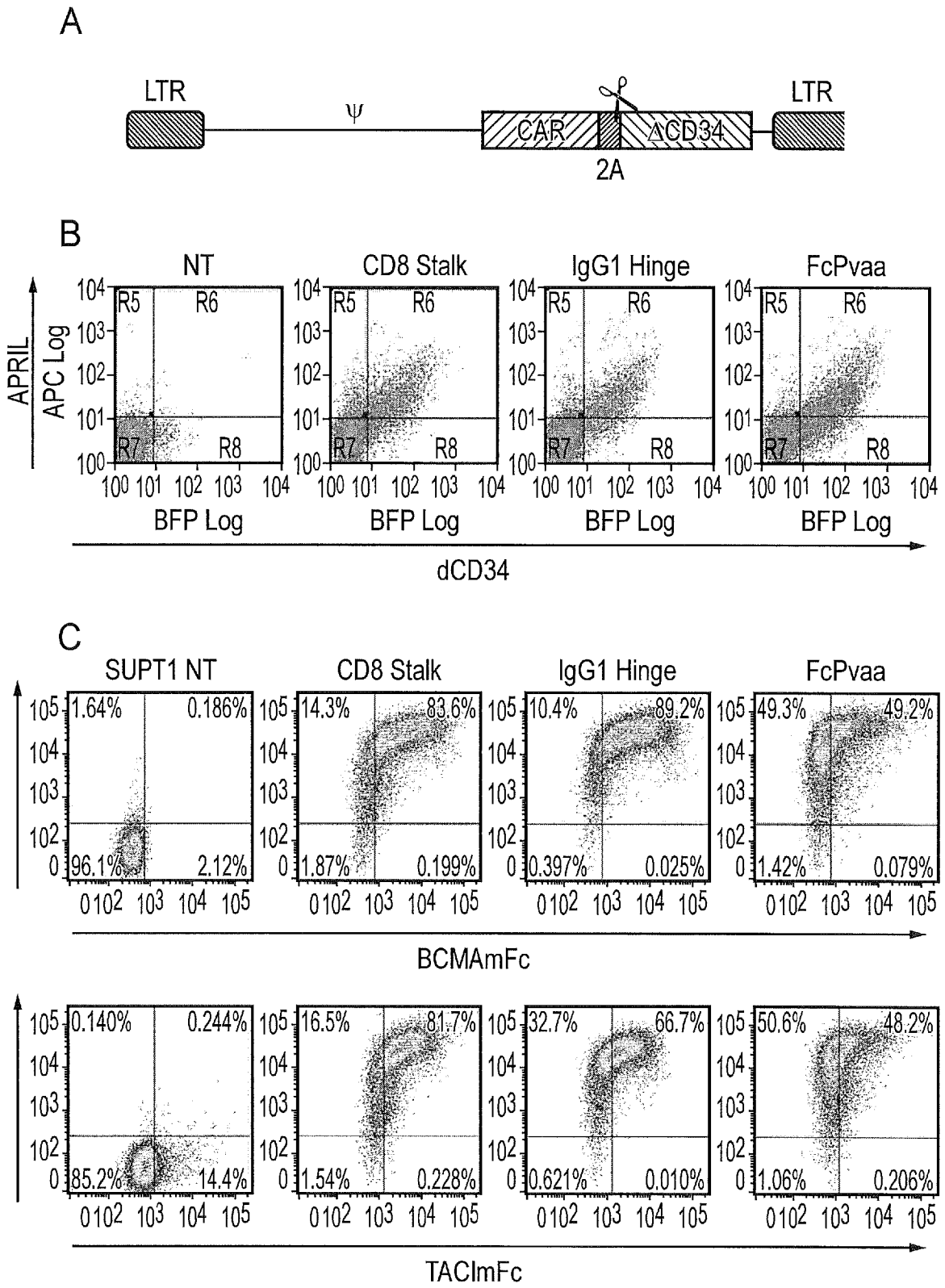

A
LTR
ψ
CAR
ΔCD34
LTR
2A
B
NT
CD8 Stalk
IgG1 Hinge
FcPvaa
APRIL
APC Log
R5
R6
R7
R8
BFP Log
dCD34
C
SUPT1 NT
CD8 Stalk
IgG1 Hinge
FcPvaa
1.64%
0.186%
96.1%
2.12%
14.3%
83.6%
1.87%
0.199%
10.4%
89.2%
0.397%
0.025%
49.3%
49.2%
1.42%
0.079%
BCMAmFc
0.140%
0.244%
85.2%
14.4%
16.5%
81.7%
1.54%
0.228%
32.7%
66.7%
0.621%
0.010%
50.6%
48.2%
1.06%
0.206%
TACImFc

FIG. 27

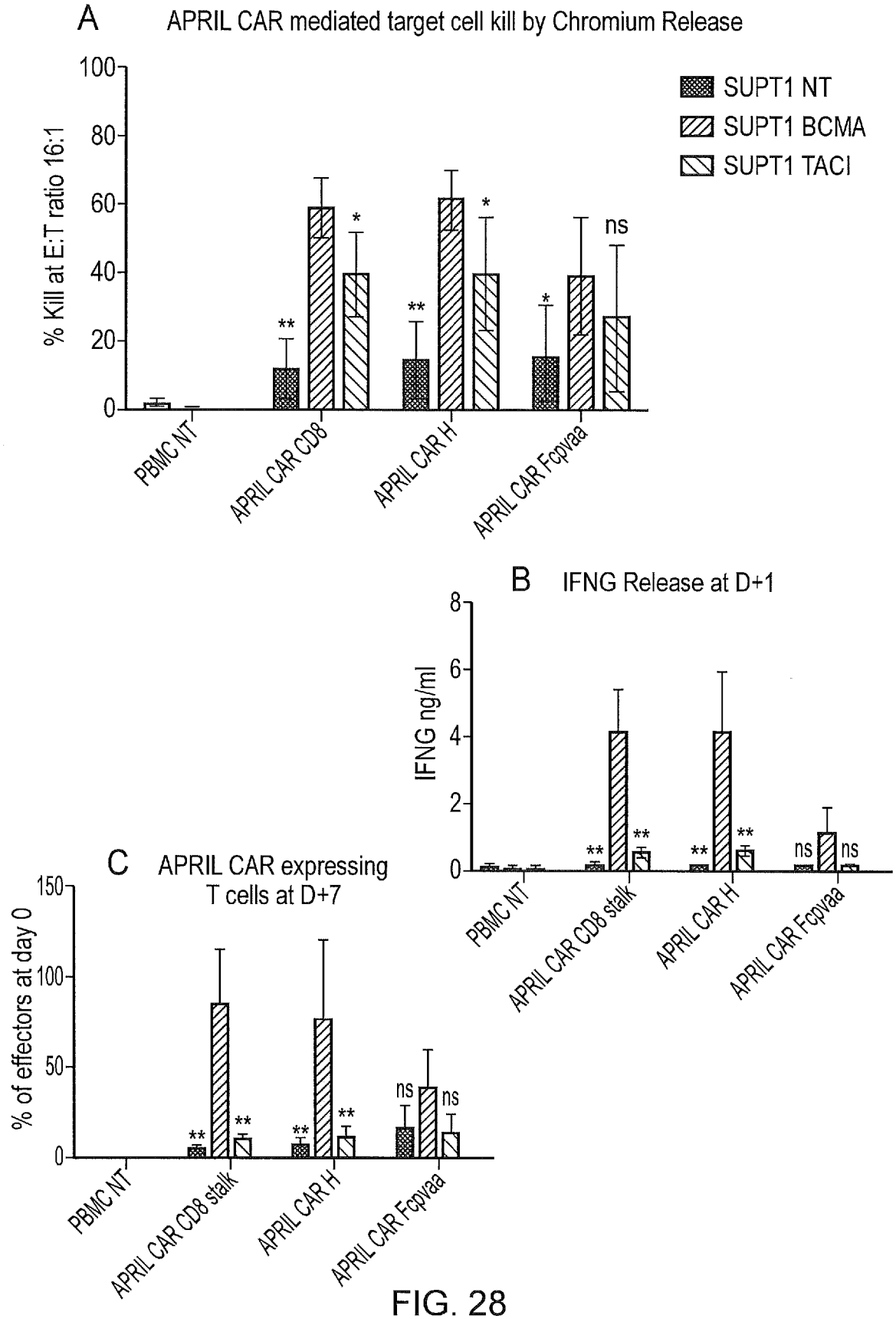

A
APRIL CAR mediated target cell kill by Chromium Release
% Kill at E:T ratio 16:1
100
80
60
40
20
0
SUPT1 NT
SUPT1 BCMA
SUPT1 TACI
**
*
**
*
*
ns
PBMC NT
APRIL CAR CD8
APRIL CAR H
APRIL CAR Fcpvaa
B
IFNG Release at D+1
IFNG ng/ml
8
6
4
2
0
**
**
**
**
ns
ns
PBMC NT
APRIL CAR CD8 stalk
APRIL CAR H
APRIL CAR Fcpvaa
C
APRIL CAR expressing
T cells at D+7
% of effectors at day 0
150
100
50
0
**
**
**
**
ns
ns
PBMC NT
APRIL CAR CD8 stalk
APRIL CAR H
APRIL CAR Fcpvaa

FIG. 28

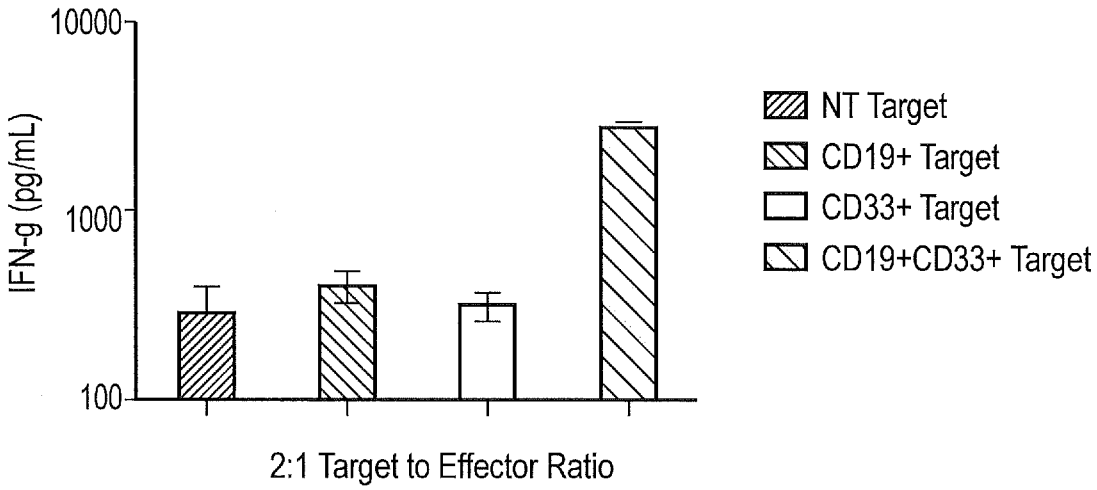

10000
1000
100
IFN-g (pg/mL)
2:1 Target to Effector Ratio
NT Target
CD19+ Target
CD33+ Target
CD19+CD33+ Target

FIG. 29

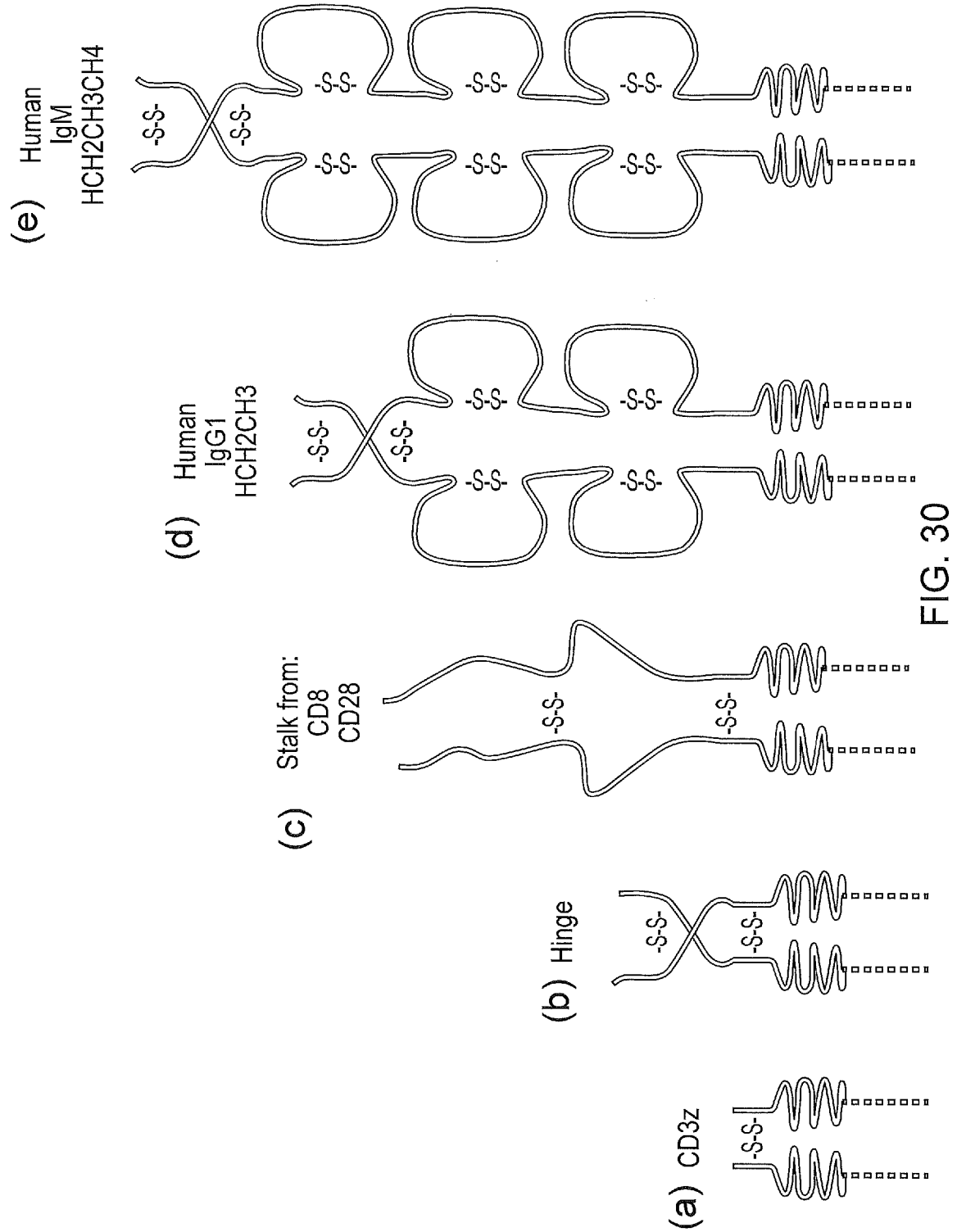

(e) Human IgM HCH2CH3CH4
-S-S-
(d) Human IgG1 HCH2CH3
(c) Stalk from: CD8 CD28
(b) Hinge
(a) CD3z

FIG. 30

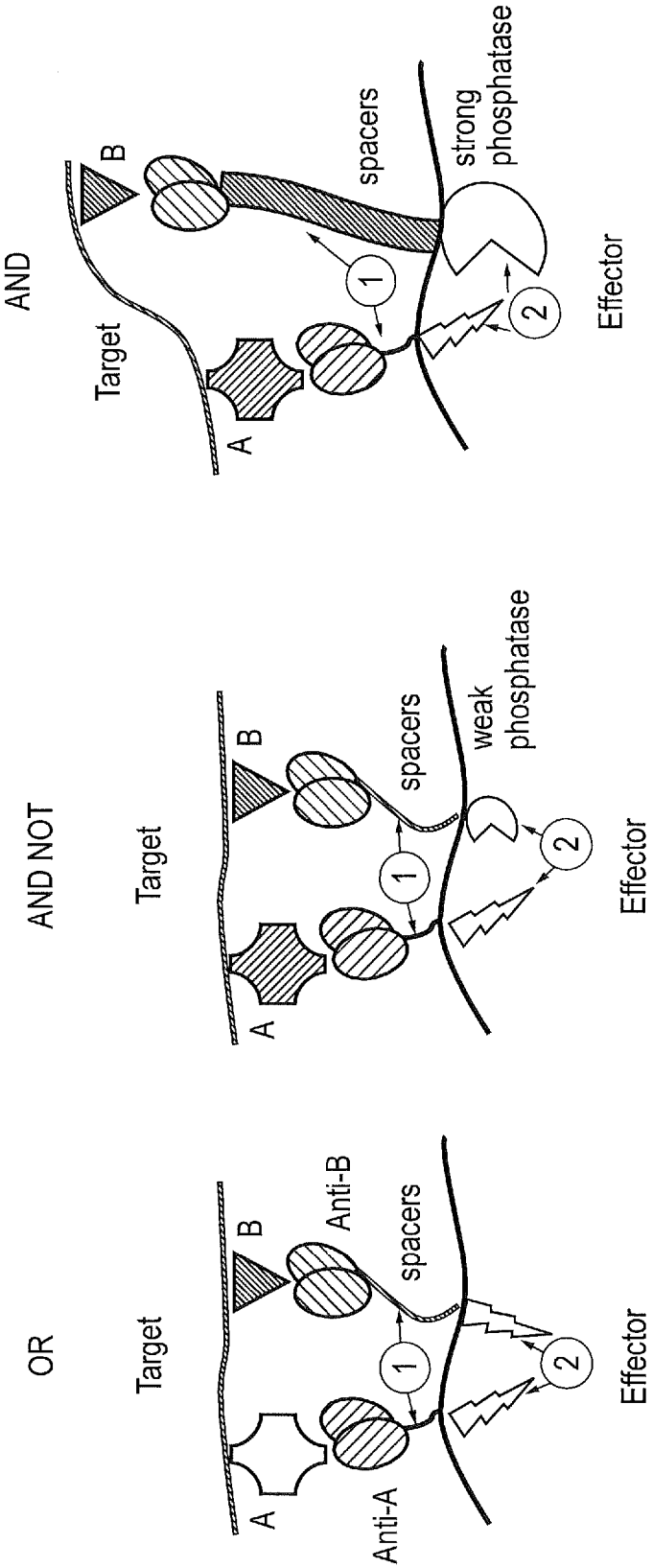

AND
Target
A
B
spacers
strong phosphatase
1
2
Effector
AND NOT
Target
A
B
spacers
weak phosphatase
1
2
Effector
OR
Target
A
Anti-A
B
Anti-B
spacers
1
2
Effector

FIG. 31