(19) DANMARK

(10) DK/EP 3560953 T5

Patent- og Varemærkestyrelsen

(12) Rettet oversættelse af europæisk patentskrift

(51) Int.Cl.: *C 07 K 14/705 (2006.01)* *C 07 K 16/28 (2006.01)* *C 07 K 16/30 (2006.01)* *C 12 N 5/0783 (2010.01)*

(45) Oversættelsen bekendtgjort den: **2024-09-02**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2024-01-31**

(86) Europæisk ansøgning nr.: **19172397.2**

(86) Europæisk indleveringsdag: **2015-12-23**

(87) Den europæiske ansøgnings publiceringsdag: **2019-10-30**

(30) Prioritet: **2014-12-24 GB 201423172**

(62) Stamansøgningsnr: **15817520.8**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Autolus Limited, The MediaWorks , 191 Wood Lane, London W12 7FP, Storbritannien**

(72) Opfinder: **PULÉ, Martin, c/o Autolus Limited, The MediaWorks, 191 Wood Lane, London W12 7FP, Storbritannien**
CORDOBA, Shaun, c/o Autolus Limited, The MediaWorks, 191 Wood Lane, London W12 7FP, Storbritannien
ONUOHA, Shimobi, c/o Autolus Limited, The MediaWorks, 191 Wood Lane, London W12 7FP, Storbritannien
THOMAS, Simon, c/o Autolus Limited, The MediaWorks, 191 Wood Lane, London W12 7FP, Storbritannien

(74) Fuldmægtig i Danmark: **RWS Group, Europa House, Chiltern Park, Chiltern Hill, Chalfont St Peter, Bucks SL9 9FG, Storbritannien**

(54) Benævnelse: **CELLE**

(56) Fremdragne publikationer:
WO-A1-2014/055657
WO-A1-2015/075468
WO-A1-2016/210293
SHIVANI SRIVASTAVA ET AL: "Engineering CAR-T cells: Design concepts", TRENDS IN IMMUNOLOGY, vol. 36, no. 8, 1 August 2015 (2015-08-01) , pages 494-502, XP055251965, GB ISSN: 1471-4906, DOI: 10.1016/j.it.2015.06.004

DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates to a cell which comprises more than one chimeric antigen receptor (CAR).

BACKGROUND TO THE INVENTION

[0002] 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.

[0003] Typically these immunotherapeutic agents target a single antigen: for instance, Rituximab targets CD20; Myelotarg targets CD33; and Alemtuzumab targets CD52.

[0004] The human CD19 antigen is a 95 kd transmembrane glycoprotein belonging to the immunoglobulin superfamily. CD19 is expressed very early in B-cell differentiation and is only lost at terminal B-cell differentiation into plasma cells. Consequently, CD19 is expressed on all B-cell malignancies apart from multiple myeloma. Since loss of the normal B-cell compartment is an acceptable toxicity, CD19 is an attractive CAR target and clinical studies targeting CD19 with CARs have seen promising results.

[0005] A particular problem in the field of oncology is provided by the Goldie-Coldman hypothesis: which describes that the 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. This modulation of antigen expression may reduce the efficacy of known immunotherapeutics, including those which target CD19.

[0006] Thus a problem with immunotherapeutics targeted against CD19 is that a B-cell malignancy may mutate and become CD19-negative. This may result in relapse with CD19-negative cancers which are not responsive to CD19 targeted therapeutics. For example, in one paediatric study, Grupp *et al.* reported that half of all relapses following CD19-targeted chimeric antigen receptor therapy for B-acute Lymphoblastic leukaemia (B-ALL) were due to CD19-negative disease (56th American Society of Hematology Annual Meeting and Exposition).

[0007] There is thus a need for immunotherapeutic agents which are capable of targeting more than one cell surface structure to reflect the complex pattern of marker expression that is associated with many cancers, including CD19-positive cancers.

Chimeric Antigen Receptors (CARs)

[0008] Chimeric antigen receptors are proteins which graft the specificity of, for example, 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).

[0009] 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.

[0010] It has been observed that using a CAR approach for cancer treatment, tumour heterogeneity and immunoediting can cause escape from CAR treatment. For example, in the study described by Grupp et al (2013; New Eng. J. Med 368:1509-1518, paper No 380, ASH 2014) CAR-modified T cell approach was used for the treatment of acute B-lymphocytic leukemia. In that clinical trial it was found that 10 patients with a complete remission after one month did relapse and 5 of them relapsed with CD19-negative disease.

[0011] There is thus a need for alternative CAR treatment approaches which address the problems of cancer escape and tumour heterogeneity.

Expression of two CAR binding specificities

[0012] Bispecific CARs known as tandem CARs or TanCARs have been developed in an attempt to target multiple cancer specific markers simultaneously. In a TanCAR, the extracellular domain comprises two antigen binding specificities in tandem, joined by a linker. The two binding specificities (scFvs) are thus both linked to a single transmembrane portion: one scFv being juxtaposed to the membrane and the other being in a distal position.

[0013] Grada et al (2013, Mol Ther Nucleic Acids 2:e105) describes a TanCAR which includes a CD19-specific scFv, followed by a Gly-Ser linker and then a HER2-specific scFv. The HER2-scFv was in the juxta-membrane position, and the CD19-scFv in the distal position. The Tan CAR was shown to induce distinct T cell reactivity against each of the two tumour restricted antigens. This arrangement was chosen because the respective lengths of HER2 (632 aa/125Å) and CD19 (280aa, 65Å) lends itself to that particular spatial arrangement. It was also known that the HER2 scFv bound the distal-most 4 loops of HER2.

[0014] The problem with this approach is that the juxta-membrane scFv may be inaccessible due to the presence of the distal scFv, especially which it is bound to the antigen. In view of the need to choose the relative positions of the two scFvs in view of the spatial arrangement of the antigen on the target cell, it may not be possible to use this approach for all scFv binding pairs. Moreover, it is unlikely that the TanCar approach could be used for more than two scFvs, a TanCAR with three or more scFvs would be a very large molecule and the scFvs may well fold back on each other, obscuring the antigen-binding sites. It is also doubtful that antigen-binding by the most distal scFv, which is separated from the transmembrane domain by two or more further scFvs, would be capable of triggering T cell activation.

[0015] There is thus a need for an alternative approach to express two CAR binding specificities on the surface of a cell such as a T cell.

[0016] WO 2014/055657 discloses trans-signalling CAR T cells comprising a first CAR having a first signalling module and a second CAR having a distinct second signalling module.

SUMMARY OF THE INVENTION

[0017] The present invention provides a cell which co-expresses a first CAR and second CAR at the cell surface, each CAR comprising an intracellular signalling domain, wherein the intracellular signalling domain of the first CAR comprises a CD28 co-stimulatory domain and an ITAM-containing domain but does not comprise a TNF receptor family endodomain; and the intracellular signalling domain of the second CAR comprises a TNF receptor family endodomain selected from OX40 or 4-1BB endodomain and an ITAM-containing domain but does not comprise a co-stimulatory domain.

[0018] The antigen-binding domain of the first CAR may bind to CD19 and the antigen-binding domain of the second CAR may bind to CD22.

[0019] The fact the one CAR binds CD19 and the other CAR binds CD22 is advantageous because some lymphomas and leukaemias become CD19 negative after CD19 targeting, (or possibly CD22 negative after CD22 targeting), so it gives a "back-up" antigen, should this occur.

[0020] 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.

[0021] Each CAR may comprise:

1. (i) an antigen-binding domain;
2. (ii) a spacer; and
3. (iii) a trans-membrane domain.

[0022] The spacer of the first CAR may be different to the spacer of the second CAR, such the first and second CAR do not form heterodimers.

[0023] The spacer of the first CAR may have a different length and/or configuration from the spacer of the second CAR, such that each CAR is tailored for recognition of its respective target antigen.

[0024] The antigen-binding domain of the second CAR may bind to a membrane-distal epitope on CD22. The antigen-binding domain of the second CAR may bind to an epitope on Ig domain 1, 2, 3 or 4 of CD22, for example on Ig domain 3 of CD22.

[0025] The antigen-binding domain of the first CAR may bind to an epitope on CD19 which is encoded by exon 1, 3 or 4.

[0026] For example, one CAR (which may be CD19 or CD22-specific) may have the structure:

AgB1-spacer1-TM1- costim-ITAM

in which:

AgB1 is the antigen-binding domain;

spacer 1 is the spacer;

TM1 is the transmembrane domain;

costim is a CD28 co-stimulatory domain; and

ITAM is an ITAM-containing endodomain;

and the other CAR (which may be CD22 or CD19-specific) may have the structure:
AgB2-spacer2-TM2- TNF-ITAM

in which:

AgB2 is the antigen-binding domain;

spacer 2 is the spacer;

TM2 is the transmembrane domain;

TNF is a TNF receptor endodomain selected from OX40 or 4-1BB endodomain; and

ITAM is an ITAM-containing endodomain.

[0027] The present invention provides a nucleic acid sequence encoding both the first and second chimeric antigen receptors (CARs) as defined above.

[0028] The nucleic acid sequence may have the following structure:
AgB1-spacer1-TM1 - costim- ITAM1-coexpr-AbB2-spacer2-TM2-TNF-ITAM2

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; costim is a nucleic acid sequence encoding a CD28 co-stimulatory domain;

ITAM1 is a nucleic acid sequence encoding the ITAM-containing 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 nucleic acid sequence encoding the transmembrane domain of the second CAR;

TNF is a nucleic acid sequence encoding a TNF receptor endodomain selected from OX40 or 4-1BB endodomain;

ITAM2 is a nucleic acid sequence encoding the ITAM-containing endodomain of the second CAR;

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

[0029] 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 2^{nd} promoter or other such means whereby one skilled in the art can express two proteins from the same vector.

[0030] Alternative codons may be used in regions of sequence encoding the same or similar amino acid sequences, such as the transmembrane and/or intracellular T cell signalling domain (endodomain) in order to avoid homologous recombination. For example, alternative codons may be used in the portions of sequence encoding the spacer, the transmembrane domain and/or all or part of the endodomain, such that the two CARs have the same or similar amino acid sequences for this or these part(s) but are encoded by different nucleic acid sequences.

[0031] The present invention provides a kit which comprises

1. (i) a first nucleic acid sequence encoding the first chimeric antigen receptor (CAR), which nucleic acid sequence has the following structure:

   AgB1-spacer1-TM1- costim- ITAM1

   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;

   costim is a nucleic acid sequence encoding a CD28 co-stimulatory domain;

   ITAM1 is a nucleic acid sequence encoding the ITAM-containing endodomain of the first CAR; and

2. (ii) a second nucleic acid sequence encoding the second chimeric antigen receptor, which nucleic acid sequence has the following structure:

   AgB2-spacer2-TM2-TNF-ITAM2

   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 nucleic acid sequence encoding the transmembrane domain of the second CAR;

   TNF is a nucleic acid sequence encoding a TNF receptor endodomain selected from OX40 or 4-1 BB endodomain; and

   ITAM2 is a nucleic acid sequence encoding the ITAM-containing endodomain of the second CAR.

[0032] The present invention provides a vector comprising a nucleic acid sequence according to the invention. The vector may be a lentiviral vector.

[0033] The vector may be a plasmid vector, a retroviral vector or a transposon vector.

[0034] The present invention provides a method for making a cell according to 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 cell ex *vivo.*

[0035] The 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 cell line.

[0036] The present invention provides a pharmaceutical composition comprising a plurality of cells according to the invention.

[0037] Also described is a method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition according to the invention to a subject.

[0038] The method may comprise the following steps:

1. (i) isolation of a cell-containing sample from a subject;
2. (ii) transduction or transfection of the 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
3. (iii) administering the cells from (ii) to a the subject.

[0039] The disease may be cancer. The cancer may be a B cell malignancy.

[0040] The present invention provides a pharmaceutical composition according to the invention for use in treating and/or preventing a disease.

[0041] Also described is the use of a cell according to the invention in the manufacture of a medicament for treating and/or preventing a disease.

[0042] Described herein is a nucleic acid sequence which comprises:

1. a) a first nucleotide sequence encoding a first chimeric antigen receptor (CAR);
2. b) a second nucleotide sequence encoding a second CAR;
   wherein one CAR binds CD19 and the other CAR binds CD22; and
3. 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.

[0043] 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).

[0044] Also described herein is a vector and a cell comprising such a nucleic acid.

[0045] The present inventors have also developed new anti-CD19 and anti-CD22 CARs with improved properties.

[0046] Described herein is a chimeric antigen receptor (CAR) comprising a CD19-binding domain which comprises

1. a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

   CDR1 - SYWMN (SEQ ID No. 15);

   CDR2 - QIWPGDGDTNYNGKFK (SEQ ID No. 16)

   CDR3 - RETTTVGRYYYAMDY (SEQ ID No. 17); and

2. b) a light chain variable region (VL) having CDRs with the following sequences:

   CDR1 - KASQSVDYDGDSYLN (SEQ ID No. 18);

   CDR2 - DASNLVS (SEQ ID No. 19)

   CDR3 - QQSTEDPWT (SEQ ID No. 20).

[0047] The CD19 binding domain may comprise a VH domain having the sequence shown as SEQ ID No. 23, or SEQ ID NO 24; or a VL domain having the sequence shown as SEQ ID No 25, SEQ ID No. 26 or SEQ ID No. 40 or a variant thereof having at least 90% sequence identity which retains the capacity to bind CD19.

[0048] The CD19 binding domain may comprise the sequence shown as SEQ ID No 21, SEQ ID No. 22 or SEQ ID No. 39 or a variant thereof having at least 90% sequence identity which retains the capacity to bind CD19.

[0049] Described herein is a chimeric antigen receptor (CAR) comprising a CD22-binding domain which comprises

1. a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

   CDR1 - NYWIN (SEQ ID No. 27);

   CDR2 - NIYPSDSFTNYNQKFKD (SEQ ID No. 28)

   CDR3 - DTQERSWYFDV (SEQ ID No. 29); and

2. b) a light chain variable region (VL) having CDRs with the following sequences:

   CDR1 - RSSQSLVHSNGNTYLH (SEQ ID No. 30);

   CDR2 - KVSNRFS (SEQ ID No. 31)

CDR3 - SQSTHVPWT (SEQ ID No. 32).

[0050] The CD22 binding domain may comprise a VH domain having the sequence shown as SEQ ID No. 35, or SEQ ID NO 36; or a VL domain having the sequence shown as SEQ ID No 37, or SEQ ID No. 38 or a variant thereof having at least 90% sequence identity which retains the capacity to bind CD22.

[0051] The CD22 binding domain may comprise the sequence shown as SEQ ID No 33 or SEQ ID No. 34 or a variant thereof having at least 90% sequence identity which retains the capacity to bind CD22.

[0052] Described herein is a cell which expresses such a chimeric antigen receptor comprising a CD19 binding domain or such a chimeric antigen receptor comprising a CD22 binding domain at the cell surface.

[0053] Described herein is a nucleic acid sequence encoding such a chimeric antigen receptor comprising a CD19 binding domain or such a chimeric antigen receptor comprising a CD22 binding domain.

[0054] Described herein is a vector comprising a nucleic acid sequence as described above. The vector may be a lentiviral vector.

[0055] The vector may be a plasmid vector, a retroviral vector or a transposon vector.

[0056] Described herein is a method for making a cell as described above, which comprises the step of introducing one or more nucleic acid sequence(s); or one or more vector(s), as defined above, into a cell.

[0057] The cell may be a T-cell or a natural killer (NK) cell. The 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 cell line.

[0058] Described herein is a pharmaceutical composition comprising a plurality of cells as described above.

[0059] Described herein is a method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition as described above to a subject.

[0060] The method may comprise the following steps:

1. (i) isolation of a cell-containing sample from a subject;
2. (ii) transduction or transfection of the cells with a nucleic acid sequence encoding the

CAR or a vector comprising such a nucleic acid sequence; and

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

[0061] The disease may be cancer. The cancer may be a B cell malignancy.

[0062] Described herein is a pharmaceutical composition as described above for use in treating and/or preventing a disease.

[0063] Described herein is the use of a cell as described above in the manufacture of a medicament for treating and/or preventing a disease.

[0064] There is also provided a cell according to the invention, which comprises a first CAR comprising a CD19 binding domain as described above and a second CAR comprising a CD22 binding domain as described above.

[0065] There is also provided a nucleic acid sequence according to the invention, encoding a first CAR comprising a CD19 binding domain as described above and a second CAR comprising a CD22 binding domain as described above.

[0066] There is also provided a kit according to the invention, wherein the first nucleic acid sequence encodes a first CAR comprising a CD19 binding domain as described above and the second nucleic acid sequence encodes a second CAR comprising a CD22 binding domain as described above.

[0067] There is also provided a vector according to the invention, which comprises a nucleic acid sequence encoding a first CAR comprising a CD19 binding domain as described above and a second CAR comprising a CD22 binding domain as described above.

[0068] The present inventors have also found that, in an OR gate system, performance is improved if the co-stimulatory domain and domain producing survival signals are "split" between the two (or more) CARs.

[0069] By providing one CAR which targets CD19 and one CAR which targets CD22, it is possible to target each of these markers, thereby reducing the problem of cancer escape.

[0070] Because the CARs are expressed on the surface of the cell as separate molecules, this approach overcomes the spatial and accessibility issues associated with TanCARs. Cell activation efficiency is also improved. If each CAR has its own spacer, it is possible to tailor the spacer and therefore the distance that the binding domain projects from the cell surface and its flexibility etc. to the particular target antigen. This choice is unfettered by the design considerations associated with TanCARs, i.e. that one CAR needs to be juxtaposed to the T cell membrane and one CAR needs to be distal, positioned in tandem to the first CAR.

[0071] By providing a single nucleic acid which encodes the two CARs separated by a cleavage site, it is possible to engineer cells to co-express the two CARs using a simple single transduction procedure. A double transfection procedure could be used with CAR-encoding sequences in separate constructs, but this would be more complex and expensive and requires more integration sites for the nucleic acids. A double transfection procedure would also be associated with uncertainty as to whether both CAR-encoding nucleic acids had been transduced and expressed effectively.

[0072] The CARs will have portions of high homology, for example the transmembrane and/or intracellular signalling domains are likely to be highly homologous. If the same or similar linkers are used for the two CARs, then they will also be highly homologous. This would suggest that an approach where both CARs are provided on a single nucleic acid sequence would be inappropriate, because of the likelihood of homologous recombination between the sequences. However, the present inventors have found that by "codon wobbling" the portions of sequence encoding areas of high homology, it is possible to express two CARs from a single construct with high efficiency. Codon wobbling involves using alternative codons in regions of sequence encoding the same or similar amino acid sequences.

DESCRIPTION OF THE FIGURES

[0073]

Figure 1: a) Schematic diagram illustrating a classical CAR. (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 the same compound endodomain.

Figure 2: B-cell maturation pathway / B-cell ontogeny. DR=HLA-DR; cCD79 = cytoplasmic CD79; cCD22 = cytoplasmic CD22. Both CD19 and CD22 antigens are expressed during early stages in B-cell maturation. It is these cells that develop into B-cell acute leukaemias. Targeting both CD19 as well as CD22 simultaneously is most suited for targeting B-cell acute leukaemias.

Figure 3: Strategies for design of an anti-CD19 OR CD22 CAR cassette. Binders which recognize CD19 and binders which recognize CD22 are selected. An optimal spacer domain and signalling domain is selected for each CAR. (a) an OR gate cassette is constructed so that both CARs are co-expressed using a FMD-2A peptide. Any homologous sequences are codon-wobbled to avoid recombination. (c) The two CARs are co-expressed as separate proteins on the T-cell surface.

Figure 4: Example of codon-wobbling to allow co-expression in a retroviral vector of identical peptide sequences but avoiding homologous recombination. Here, wild-type HCH2CH3-CD28tmZeta is aligned with codon-wobbled HCH2CH3-CD28tmZeta.

Figure 5: Demonstrating functionality of anti-CD19 OR CD22 CAR gate. (a) Cartoon of construct: S1 - signal peptide 1; HA - haemagglutin tag; HCH2CH3 - hinge, CH2CH3 of IgG1 wild-type sequence; CD28tmZ - CD28 transmembrane domain and CD3 Zeta wobbled sequence; 2A - Foot and mouth disease 2A peptide; S2 - signal peptide 2; V5 - v5 epitope tag; aCD22 - anti-CD22 scFv; HCH2CH3' - hinge, CH2CH3 of IgG1 wobbled sequence; CD28tmZ - CD28 transmembrane domain and CD3 Zeta wobbled sequence.; (b) Co-expression of two receptors from a single vector. Peripheral blood T-cells were transduced with bicistronic vector after stimulation with OKT3 and anti-CD28. Cells were analysed five days after transduction by staining with anti-V5-FITC (invitrogen) and anti-HA-PE (abCam). The two CARs can be detected simultaneously on the T-cell surface. (c) Non-transduced T-cells, T-cells expressing just anti-CD19 CAR, T-cells expressing just anti-CD22 CAR and T-cells expressing the anti-CD19 OR CD22 CAR gate were challenged with target cells expressing neither CD19 or CD22, either CD19 or CD22 singly, or both antigen. T-cells expressing the anti-CD19 OR CD22 CAR gate could kill target cells even if one antigen was absent.

Figure 6: Biacore affinity determination for murine CD22ALAb scFv, humanised CD22ALAb scFv and M971 scFv

Figure 7: Biacore affinity determination for murine CD19ALAb scFv and humanised CD19ALAb

Figure 8: Comparison of the binding kinetics between soluble scFv-CD19 binding for CD19ALAb scFv and fmc63 scFv

Figure 9: Schematic diagram illustrating CD19ALAb CAR, fmc63 CAR, CD22ALAb CAR and M971 CAR used in the comparative studies

Figure 10: Killing assay of CD19 positive target cells comparing a CAR with a CD19ALAb antigen binding domain and an equivalent CAR with an fmc63 binding domain.

Figure 11: A) Killing assay of CD22 positive target cells comparing a CAR with a CD22ALAb antigen binding domain and an equivalent CAR with an M971 binding domain. B) Assay comparing IFNγ release following co-culture 1:1 with CD22 positive SupT1 cells

Figure 12: CD19 structure and exons

Figure 13: Schematic diagrams and construct maps illustrating the four constructs tested in Example 5. In the construct map, portions marked with ' are codon-wobbled.
A: CD19 and CD22 CAR both have 41BB-CD3zeta compound endodomains; B: CD19 and CD22 CAR both have OX40-CD3zeta compound endodomains; C: CD19 CAR has 41BB-CD3zeta compound endodomain and CD22 CAR has CD28-CD3zeta compound endodomain; and D: CD19 CAR has OX40-CD3zeta compound endodomain and CD22 CAR has CD28-CD3zeta compound endodomain

Figure 14: Target cell killing by cells expressing the constructs shown in Figure 13.

DETAILED DESCRIPTION

CHIMERIC ANTIGEN RECEPTORS (CARs)

[0074] 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.

[0075] 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.

[0076] 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.

[0077] Described herein is a cell which co-expresses a first CAR and a second CAR, wherein one CAR may bind CD19 and the other CAR may bind CD22, such that a T-cell can recognize a target cells expressing either of these markers.

[0078] Thus, as described herein, 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. The two CARs may comprise spacer domains which may be the same, or sufficiently different to prevent cross-pairing of the two different receptors. A cell can hence be engineered to activate upon recognition of either or both CD19 and CD22. 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.

[0079] It is important that the two CARs do not heterodimerize.

[0080] 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.

SIGNAL PEPTIDE

[0081] The CARs of the 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.

[0082] 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.

[0083] The signal peptide may be at the amino terminus of the molecule.

[0084] The signal peptide may comprise the SEQ ID No. 1, 2 or 3 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. 1: MGTSLLCWMALCLLGADHADG

[0085] The signal peptide of SEQ ID No. 1 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. 2: MSLPVTALLLPLALLLHAARP

[0086] The signal peptide of SEQ ID No. 2 is derived from IgG1.
SEQ ID No. 3: MAVPTQVLGLLLLWLTDARC

[0087] The signal peptide of SEQ ID No. 3 is derived from CD8.

[0088] The signal peptide for the first CAR may have a different sequence from the signal peptide of the second CAR.

CD19

[0089] The human CD19 antigen is a 95 kd transmembrane glycoprotein belonging to the immunoglobulin superfamily. CD19 is classified as a type I transmembrane protein, with a single transmembrane domain, a cytoplasmic C-terminus, and extracellular N-terminus. The general structure for CD19 is illustrated in Figure 12.

[0090] CD19 is a biomarker for normal and neoplastic B cells, as well as follicular dendritic cells. In fact, it is present on B cells from earliest recognizable B-lineage cells during development to B-cell blasts but is lost on maturation to plasma cells. It primarily acts as a B cell co-receptor in conjunction with CD21 and CD81. Upon activation, the cytoplasmic tail of CD19 becomes phosphorylated, which leads to binding by Src-family kinases and recruitment of PI-3 kinase. CD19 is expressed very early in B-cell differentiation and is only lost at terminal B-cell differentiation into plasma cells. Consequently, CD19 is expressed on all B-cell malignancies apart from multiple myeloma.

[0091] Different designs of CARs have been tested against CD19 in different centres, as outlined in the following Table:

Table 1

Centre	***Binder***	***Endodomain***	***Comment***
University College London	Fmc63	CD3-Zeta	Low-level brief persistence
Memorial Sloane Kettering	SJ25C1	CD28-Zeta	Short-term persistence
NCI/KITE	Fmc63	CD28-Zeta	Long-term low-level persistence
Baylor, Centre for Cell and Gene Therapy	Fmc63	CD3-Zeta/ CD28-Zeta	Short-term low-level persistence
UPENN/Novartis	Fmc63	41BB-Zeta	Long-term high-level persistence

[0092] As shown above, most of the studies conducted to date have used an scFv derived from the hybridoma fmc63 as part of the binding domain to recognize CD19.

[0093] As shown in Figure 12, the gene encoding CD19 comprises ten exons: exons 1 to 4 encode the extracellular domain; exon 5 encodes the transmembrane domain; and exons 6 to 10 encode the cytoplasmic domain,

[0094] In the CD19/CD22 OR gate, the antigen-binding domain of the anti-CD19 CAR may

bind an epitope of CD19 encoded by exon 1 of the CD19 gene.

[0095] In the CD19/CD22 OR gate, the antigen-binding domain of the anti-CD19 CAR may bind an epitope of CD19 encoded by exon 3 of the CD19 gene.

[0096] In the CD19/CD22 OR gate, the antigen-binding domain of the anti-CD19 CAR may bind an epitope of CD19 encoded by exon 4 of the CD19 gene.

CD19ALAb

[0097] The present inventors have developed a new anti-CD19 CAR which has improved properties compared to a known anti-CD19 CAR which comprises the binder fmc63 (see Examples 2 and 3). The antigen binding domain of the CAR may be based on the CD19 binder CD19ALAb, which has the CDRs and VH/VL regions identified below.

[0098] Described herein is a CAR which comprises a CD19-binding domain which comprises a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

CDR1 - SYWMN (SEQ ID No. 15);

CDR2 - QIWPGDGDTNYNGKFK (SEQ ID No. 16)

CDR3 - RETTTVGRYYYAMDY (SEQ ID No. 17); and

b) a light chain variable region (VL) having CDRs with the following sequences:

CDR1 - KASQSVDYDGDSYLN (SEQ ID No. 18);

CDR2 - DASNLVS (SEQ ID No. 19)

CDR3 - QQSTEDPWT (SEQ ID No. 20).

[0099] It may be possible to introduce one or more mutations (substitutions, additions or deletions) into the or each CDR without negatively affecting CD19-binding activity. Each CDR may, for example, have one, two or three amino acid mutations.

[0100] The CAR may comprise one of the following amino acid sequences:

SEQ ID No. 21 (Murine CD19ALAb scFv sequence)
QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDT
NYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQG
TTVTVSSDIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDA
SNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK

SEQ ID No. 22 (Humanised CD19ALAb scFv sequence - Heavy 19, Kappa 16)
QVQLVQSGAEVKKPGASVKLSCKASGYAFSSYWMNWVRQAPGQSLEWIGQIWPGDGDT
NYNGKFKGRATLTADESARTAYMELSSLRSGDTAVYFCARRETTTVGRYYYAMDYWGKG
TLVTVSSDIQLTQSPDSLAVSLGERATINCKASQSVDYDGDSYLNWYQQKPGQPPKLLIYDA
SNLVSGVPDRFSGSGSGTDFTLTISSLQAADVAVYHCQQSTEDPWTFGQGTKVEIKR

SEQ ID No. 39 (Humanised CD19ALAb scFv sequence - Heavy 19, Kappa 7)
QVQLVQSGAEVKKPGASVKLSCKASGYAFSSYWMNWVRQAPGQSLEWIGQIWPGDGDT
NYNGKFKGRATLTADESARTAYMELSSLRSGDTAVYFCARRETTTVGRYYYAMDYWGKG
TLVTVSSDIQLTQSPDSLAVSLGERATINCKASQSVDYDGDSYLNWYQQKPGQPPKVLIYD
ASNLVSGVPDRFSGSGSGTDFTLTISSLQAADVAVYYCQQSTEDPWTFGQGTKVEIKR

[0101] The scFv may be in a VH-VL orientation (as shown in SEQ ID No.s 21, 22 and 39) or a VL-VH orientation.

[0102] The CAR may comprise one of the following VH sequences:

SEQ ID No. 23 (Murine CD19ALAb VH sequence)
QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDT
NYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQG
TTVTVSS

SEQ ID No. 24 (Humanised CD19ALAb VH sequence)
QVQLVQSGAEVKKPGASVKLSCKASGYAFSSYWMNWVRQAPGQSLEWIGQIWPGDGDT
NYNGKFKGRATLTADESARTAYMELSSLRSGDTAVYFCARRETTTVGRYYYAMDYWGKG
TLVTVSS

[0103] The CAR may comprise one of the following VL sequences:

SEQ ID No. 25 (Murine CD19ALAb VL sequence)
DIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGI
PPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK

SEQ ID No. 26 (Humanised CD19ALAb VL sequence, Kappa 16)
DIQLTQSPDSLAVSLGERATINCKASQSVDYDGDSYLNWYQQKPGQPPKLLIYDASNLVSG
VPDRFSGSGSGTDFTLTISSLQAADVAVYHCQQSTEDPWTFGQGTKVEIKR

SEQ ID No. 40 (Humanised CD19ALAb VL sequence, Kappa 7)
DIQLTQSPDSLAVSLGERATINCKASQSVDYDGDSYLNWYQQKPGQPPKVLIYDASNLVSG
VPDRFSGSGSGTDFTLTISSLQAADVAVYYCQQSTEDPWTFGQGTKVEIKR

[0104] The CAR may comprise a variant of the sequence shown as SEQ ID No. 21, 22, 23, 24,

25, 26, 39 or 40 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retain the capacity to bind CD19 (when in conjunction with a complementary VL or VH domain, if appropriate).

[0105] The percentage identity between two polypeptide sequences may be readily determined by programs such as BLAST which is freely available at http://blast.ncbi.nlm.nih.gov.

CD22

[0106] The human CD22 antigen is a molecule belonging to the SIGLEC family of lectins. It is found on the surface of mature B cells and on some immature B cells. Generally speaking, CD22 is a regulatory molecule that prevents the overactivation of the immune system and the development of autoimmune diseases.

[0107] CD22 is a sugar binding transmembrane protein, which specifically binds sialic acid with an immunoglobulin (Ig) domain located at its N-terminus. The presence of Ig domains makes CD22 a member of the immunoglobulin superfamily. CD22 functions as an inhibitory receptor for B cell receptor (BCR) signaling.

[0108] CD22 is a molecule of the IgSF which may exist in two isoforms, one with seven domains and an intra-cytoplasmic tail comprising of three ITIMs (immune receptor tyrosine-based inhibitory motifs) and an ITAM; and a splicing variant which instead comprises of five extracellular domains and an intra-cytoplasmic tail carrying one ITIM. CD22 is thought to be an inhibitory receptor involved in the control of B-cell responses to antigen. Like CD19, CD22 is widely considered to be a pan-B antigen, although expression on some non-lymphoid tissue has been described. Targeting of CD22 with therapeutic monoclonal antibodies and immunoconjugates has entered clinical testing.

[0109] Examples of anti-CD22 CARs are described by Haso et al. (Blood; 2013; 121(7)). Specifically, anti-CD22 CARs with antigen-binding domains derived from m971, HA22 and BL22 scFvs are described.

[0110] The antigen-binding domain of the anti-CD22 CAR may bind CD22 with a K_D in the range 30-50nM, for example 30-40nM. The K_D may be about 32nM.

[0111] CD-22 has seven extracellular IgG-like domains, which are commonly identified as Ig domain 1 to Ig domain 7, with Ig domain 7 being most proximal to the B cell membrane and Ig domain 7 being the most distal from the Ig cell membrane (see Haso et al 2013 as above Figure 2B).

[0112] The positions of the Ig domains in terms of the amino acid sequence of CD22

(http://www.uniprot.org/uniprot/P20273) are summarised in the following table:

Ig domain	Amino acids
1	20-138
2	143-235
3	242-326
4	331-416
5	419-500
6	505-582
7	593-676

[0113] The antigen-binding domain of the second CAR may bind to a membrane-distal epitope on CD22. The antigen-binding domain of the second CAR may bind to an epitope on Ig domain 1, 2, 3 or 4 of CD22, for example on Ig domain 3 of CD22. The antigen-binding domain of the second CAR may bind to an epitope located between amino acids 20-416 of CD22, for example between amino acids 242-326 of CD22.

[0114] The anti-CD22 antibodies HA22 and BL22 (Haso et al 2013 as above) and CD22ALAb, described below, bind to an epitope on Ig domain 3 of CD22.

[0115] The antigen binding domain of the second CAR may not bind to a membrane-proximal epitope on CD22. The antigen-binding domain of the second CAR may not bind to an epitope on Ig domain 5, 6 or 7 of CD22. The antigen-binding domain of the second CAR may not bind to an epitope located between amino acids 419-676 of CD22, such as between 505-676 of CD22.

CD22ALAb

[0116] The present inventors have developed a new anti-CD22 CAR which has improved properties compared to a known anti-CD22 CAR which comprises the binder m971 (see Examples 2 and 3 and Haso et al (2013) as above). The antigen binding domain of the CAR is based on the CD22 binder CD22ALAb, which has the CDRs and VH/VL regions identified below.

[0117] Described herein is a CAR which comprises a CD22-binding domain which comprises

1. a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

   CDR1 - NYWIN (SEQ ID No. 27);

   CDR2 - NIYPSDSFTNYNQKFKD (SEQ ID No. 28)

CDR3 - DTQERSWYFDV (SEQ ID No. 29); and

2. b) a light chain variable region (VL) having CDRs with the following sequences:

CDR1 - RSSQSLVHSNGNTYLH (SEQ ID No. 30);

CDR2 - KVSNRFS (SEQ ID No. 31)

CDR3 - SQSTHVPWT (SEQ ID No. 32).

[0118] It may be possible to introduce one or more mutations (substitutions, additions or deletions) into the or each CDR without negatively affecting CD22-binding activity. Each CDR may, for example, have one, two or three amino acid mutations.

[0119] The CAR may comprise one of the following amino acid sequences:

SEQ ID No. 33 (Murine CD22ALAb scFv sequence)
QVQLQQPGAELVRPGASVKLSCKASGYTFTNYWINWVKQRPGQGLEWIGNIYPSDSFTNY
NQKFKDKATLTVDKSSSTAYMQLSSPTSEDSAVYYCTRDTQERSWYFDVWGAGTTVTVSS
DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFS
GVPDRFSGSGSGTDFTLKISRVEAEDLGLYFCSQSTHVPWTFGGGTKLEIK

SEQ ID No. 34 (Humanised CD22ALAb scFv sequence)
EVQLVESGAEVKKPGSSVKVSCKASGYTFTNYWINWVRQAPGQGLEWIGNIYPSDSFTNY
NQKFKDRATLTVDKSTSTAYLELRNLRSDDTAVYYCTRDTQERSWYFDVWGQGTLVTVSS
DIVMTQSPATLSVSPGERATLSCRSSQSLVHSNGNTYLHWYQQKPGQAPRLLIYKVSNRFS
GVPARFSGSGSGVEFTLTISSLQSEDFAVYYCSQSTHVPWTFGQGTRLEIK

[0120] The scFv may be in a VH-VL orientation (as shown in SEQ ID Nos 33 and 34) or a VL-VH orientation.

[0121] The CAR may comprise one of the following VH sequences:

SEQ ID No. 35 (Murine CD22ALAb VH sequence)
QVQLQQPGAELVRPGASVKLSCKASGYTFTNYWINWVKQRPGQGLEWIGNIYPSDSFTNY
NQKFKDKATLTVDKSSSTAYMQLSSPTSEDSAVYYCTRDTQERSWYFDVWGAGTTVTVSS

SEQ ID No. 36 (Humanised CD22ALAb VH sequence)
EVQLVESGAEVKKPGSSVKVSCKASGYTFTNYWINWVRQAPGQGLEWIGNIYPSDSFTNY
NQKFKDRATLTVDKSTSTAYLELRNLRSDDTAVYYCTRDTQERSWYFDVWGQGTLVTVSS

[0122] The CAR may comprise one of the following VL sequences:

SEQ ID No. 37 (Murine CD22ALAb VL sequence)
DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFS
GVPDRFSGSGSGTDFTLKISRVEAEDLGLYFCSQSTHVPWTFGGGTKLEIK

SEQ ID No. 38 (Humanised CD22ALAb VL sequence)
DIVMTQSPATLSVSPGERATLSCRSSQSLVHSNGNTYLHWYQQKPGQAPRLLIYKVSNRFS
GVPARFSGSGSGVEFTLTISSLQSEDFAVYYCSQSTHVPWTFGQGTRLEIK

[0123] The CAR may comprise a variant of the sequence shown as SEQ ID No. 33, 34, 35, 36, 37 or 38 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retain the capacity to bind CD22 (when in conjunction with a complementary VL or VH domain, if appropriate).

B-CELL ANTIGEN EXPRESSION DURING B-CELL ONTOGENY AND SUBSEQUENT TUMOURS

[0124] CD19 is widely considered a pan-B antigen, although very occasionally, it may display some lineage infidelity. The CD19 molecule comprises of two extracellular IgSF domains separated by a smaller domain and a long intracytoplasmic tail, nearly as big as the extracellular portion of the molecule, carrying one ITAM. CD19 is a key molecule in the development and activation of B-cells. CD22 is a molecule of the IgSF which may exist in two isoforms, one with seven domains and an intra-cytoplasmic tail comprising of three ITIMs (immune receptor tyrosine-based inhibitory motifs) and an ITAM; and a splicing variant which instead comprises of five extracellular domains and an intra-cytoplasmic tail carrying one ITIM. CD22 is thought to be an inhibitory receptor involved in the control of B-cell responses to antigen. Like CD19, CD22 is widely considered to be a pan-B antigen, although expression on some non-lymphoid tissue has been described (Wen et al. (2012) J. Immunol. Baltim. Md 1950 188, 1075-1082). Targeting of CD22 with therapeutic monoclonal antibodies and immunoconjugates has entered clinical testing. Generation of CD22 specific CARs have been described (Haso et al, 2013, Blood: Volume 121; 7: 1165-74, and James et al 2008, Journal of immunology, Volume 180; Issue 10; Pages 7028-38).

[0125] Detailed immunophentyping studies of B-cell leukaemias shows that while surface CD19 is always present, surface CD22 is almost always present. For instance, Raponi et al (2011, as above) studied the surface antigen phenotype of 427 cases of B-ALL and found CD22 present in 341 of cases studied.

[0126] The eventuality of CD19 down-regulation after CAR19 targeting described above may be explained by the Goldie-Coldman hypothesis. The Goldie-Coldman hypothesis predicts that tumor cells mutate to a resistant phenotype at a rate dependent on their intrinsic genetic instability and that the probability that a cancer would contain resistant clones depends on the

mutation rate and the size of the tumor. While it may be difficult for cancer cells to become intrinsically resistant to the direct killing of cytotoxic T-cells, antigen loss remains possible. Indeed this phenomenon has been reported before with targeting melanoma antigens and EBV-driven lymphomas. According to Goldie-Coldman hypothesis, the best chance of cure would be to simultaneously attack non-cross resistant targets. Given that CD22 is expressed on nearly all cases of B-ALL, simultaneous CAR targeting of CD19 along with CD22 may reduce the emergence of resistant CD19 negative clones.

ANTIGEN BINDING DOMAIN

[0127] 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.

[0128] The antigen binding domain of the CAR which binds to CD19 may be any domain which is capable of binding CD19. For example, the antigen binding domain may comprise a CD19 binder as described in Table 1.

[0129] The antigen binding domain of the CAR which binds to CD19 may comprise a sequence derived from one of the CD19 binders shown in Table 2.

Table 2

Binder	***References***
HD63	Pezzutto (Pezzutto, A. et al. J. Immunol. Baltim. Md 1950 138, 2793-2799 (1987)
4g7	Meeker et al (Meeker, T. C. et al. Hybridoma 3, 305-320 (1984)
Fmc63	Nicholson et al (Nicholson, I. C. et al. Mol. Immunol. 34, 1157-1165 (1997)
B43	Bejcek et al (Bejcek, B. E. et al. Cancer Res. 55, 2346-2351 (1995)
SJ25C1	Bejcek et al (1995, as above)
BLY3	Bejcek et al (1995, as above)
B4, or re-surfaced, or humanized B4	Roguska et al (Roguska, M. A. et al. Protein Eng. 9, 895-904 (1996)
HB12b, optimized and humanized	Kansas et al (Kansas, G. S. & Tedder, T. F. J. Immunol. Baltim. Md 1950 147, 4094-4102 (1991); Yazawa et al (Yazawa et al Proc. Natl. Acad. Sci. U. S. A. 102, 15178-15183 (2005); Herbst et al (Herbst, R. et al. J. Pharmacol. Exp. Ther. 335, 213-222

Binder	***References***
	(2010)

[0130] The antigen binding domain of the CAR which binds to CD22 may be any domain which is capable of binding CD22. For example, the antigen binding domain may comprise a CD22 binder as described in Table 3.

Table 3

Binder	***References***
M5/44 or humanized M5/44	John et al (J. Immunol. Baltim. Md 1950 170, 3534-3543 (2003); and DiJoseph et al (Cancer Immunol. Immunother. CII 54, 11-24 (2005)
M6/13	DiJoseph et al (as above)
HD39	Dorken et al (J. Immunol. Baltim. Md 1950 136, 4470-4479 (1986)
HD239	Dorken et al (as above)
HD6	Pezzutto et al (J. Immunol. Baltim. Md 1950 138, 98-103 (1987)
RFB-4, or humanized RFB-4, or affinity matured	Campana et al (J. Immunol. Baltim. Md 1950 134, 1524-1530 (1985); Krauss et al (Protein Eng. 16, 753-759 (2003), Kreitman et al (J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 30, 1822-1828 (2012))
Tol5	Mason et al (Blood 69, 836-840 (1987))
4KB128	Mason et al (as above)
S-HCL1	Schwarting et al (Blood 65, 974-983 (1985))
mLL2 (EPB-2), or humanized mLL2 - hLL2	Shih et al (Int. J. Cancer J. Int. Cancer 56, 538-545 (1994)), Leonard et al (J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 21, 3051-3059 (2003))
M971	Xiao et al (mAbs 1, 297-303 (2009))
BC-8	Engel et al (J. Exp. Med. 181, 1581-1586 (1995))
HB22-12	Engel et al (as above)

SPACER DOMAIN

[0131] 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.

[0132] In the cell of the present invention, the first and second CARs may 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.

[0133] The spacer for the anti-CD19 CAR may comprise a CD8 stalk spacer, or a spacer having a length equivalent to a CD8 stalk spacer. The spacer for the anti-CD19 CAR may have at least 30 amino acids or at least 40 amino acids. It may have between 35-55 amino acids, for example between 40-50 amino acids. It may have about 46 amino acids.

[0134] The spacer for the anti-CD22 CAR may comprise an IgG1 hinge spacer, or a spacer having a length equivalent to an IgG1 hinge spacer. The spacer for the anti-CD22 CAR may have fewer than 30 amino acids or fewer than 25 amino acids. It may have between 15-25 amino acids, for example between 18-22 amino acids. It may have about 20 amino acids.

[0135] Examples of amino acid sequences for these spacers are given below:

SEQ ID No. 4 (hinge-CH2CH3 of human IgG1)
AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL
DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD

SEQ ID No. 5 (human CD8 stalk):
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI

SEQ ID No. 6 (human IgG1 hinge):
AEPKSPDKTHTCPPCPKDPK

SEQ ID No. 7 (CD2 ectodomain)
KEITNALETWGALGQDINLDIPSFQMSDDIDDIKWEKTSDKKKIAQFRKEKETFKEKDTYKLF
KNGTLKIKHLKTDDQDIYKVSIYDTKGKNVLEKIFDLKIQERVSKPKISWTCINTTLTCEVMNG
TDPELNLYQDGKHLKLSQRVITHKWTTSLSAKFKCTAGNKVSKESSVEPVSCP
EKGLD

SEQ ID No. 8 (CD34 ectodomain)
SLDNNGTATPELPTQGTFSNVSTNVSYQETTTPSTLGSTSLHPVSQHGNEATTNITETTVKF
TSTSVITSVYGNTNSSVQSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLSTTSTSLATS
PTKPYTSSSPILSDIKAEIKCSGIREVKLTQGICLEQNKTSSCAEFKKDRGEGLARVLCGEEQ
ADADAGAQVCSLLLAQSEVRPQCLLLVLANRTEISSKLQLMKKHQSDLKKLGILDFTEQDVA
SHQSYSQKT

[0136] 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. The spacer of the first CAR may be 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.

TRANSMEMBRANE DOMAIN

[0137] The transmembrane domain is the sequence of the CAR that spans the membrane.

[0138] 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).

[0139] The transmembrane domain may be derived from CD28, which gives good receptor stability.

[0140] The transmembrane domain may be derived from human Tyrp-1. The tyrp-1 transmembrane sequence is shown as SEQ ID No. 45.
SEQ ID No. 45
IIAIAVVGALLLVALIFGTASYLI

ACTIVATING ENDODOMAIN

[0141] 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.

[0142] The cell of the present invention comprises two CARs, each with an intracellular signalling domain; the endodomain of the first CAR comprises a co-stimulatory domain, wherein the co-stimulatory domain is CD28 co-stimulatory domain, and an ITAM-containing domain but does not comprise a TNF receptor family endodomain; and the endodomain of the second CAR comprises a TNF receptor family endodomain, wherein the TNF receptor family endodomain is OX40 or 4-1BB endodomain, and an ITAM-containing domain but does not comprise a co-stimulatory domain.

[0143] As such, the co-stimulatory and survival signal-producing domains are "shared" between the two (or more) CARs in an OR gate. For example, where an OR gate has two CARs, CAR A and CAR B, CAR A comprises a CD28 co-stimulatory domain and CAR B comprises a TNF receptor family endodomain, selected from OX40 or 4-1BB endodomain.

[0144] An 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 2000/063372, which relates to synthetic signalling molecules).

[0145] 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. 9 or 10 or a variant thereof having at least 80% sequence identity.

SEQ ID No. 9 comprising CD28 transmembrane domain and CD3 Z endodomain
FWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEY
DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY
QGLSTATKDTYDALHMQALPPR

SEQ ID No. 10 comprising CD28 transmembrane domain and CD28 and CD3 Zeta endodomains
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPP
RDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
PR

SEQ ID No. 11 comprising CD28 transmembrane domain and CD28, OX40 and CD3 Zeta endodomains (for reference only).
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPP
RDFAAYRSRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQG
QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG

MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

[0146] A variant sequence may have at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity to SEQ ID No. 9 or 10, provided that the sequence provides an effective trans-membrane domain and an effective intracellular T cell signalling domain.

"SPLIT" OR GATE ENDODOMAINS

[0147] The present invention provides an OR gate in which the co-stimulatory/survival signal domains are "split" between the two CARs.

[0148] In this respect, 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 an intracellular signalling domain, wherein the intracellular signalling domain of the first CAR comprises a CD28 co-stimulatory domain and an ITAM-containing domain but does not comprise a TNF receptor family endodomain; and the intracellular signalling domain of the second CAR comprises a TNF receptor family endodomain selected from OX40 or 4-1BB endodomain, and an ITAM-containing domain but does not comprise a co-stimulatory domain.

[0149] The first and second CARs may bind to different antigens. For example, the first CAR may bind CD19 and the second CAR may bind CD22; alternatively the first CAR may bind CD22 and the second CAR may bind CD19.

[0150] The intracellular signalling domain of the first CAR comprises a CD28 co-stimulatory domain and does not comprise a domain which transmits survival signals (such as a TNF receptor family endodomain). The intracellular signalling domain of the second CAR comprises a TNF receptor family endodomain selected from OX40 or 4-1BB endodomain and does not comprise a co-stimulatory domain (such as CD28 endodomain).

[0151] The co-stimulatory domain is a CD28 co-stimulatory domain. The CD28 co-stimulatory domain may have the sequence shown as SEQ ID No. 41.
SEQ ID No. 41 (CD28 co-stimulatory endodomain)
SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS

[0152] The CAR of the invention may comprise a variant of the sequence shown as SEQ ID No. 41 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retains the capacity to co-stimulate T cells upon antigen recognition, i.e. provide signal 2 to T cells.

[0153] The TNF receptor family endodomain is an OX40 or 4-1BB endodomain. The OX40 endodomain may have the sequence shown as SEQ ID No. 42. The 4-1BB endodomain may

have the sequence shown as SEQ ID No. 43.

SEQ ID No. 42 (OX40 endodomain)
RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI

SEQ ID No. 43 (4-1BB endodomain)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[0154] The CAR may comprise a variant of the sequence shown as SEQ ID No. 42 or 43 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retains the capacity to transmit a survival signal to T cells upon antigen recognition..

[0155] The intracellular signalling domain of the first and the second CAR comprises an ITAM-containing domain, such as a CD3 zeta domain. The CD3 zeta domain may have the sequence shown as SEQ ID No. 44.
SEQ ID No. 44 (CD3zeta endodomain)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL
YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

[0156] The CAR may comprise a variant of the sequence shown as SEQ ID No. 44 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retains the capacity to induce T-cell signalling upon antigen recognition, i.e. provide signal 1 to T cells.

[0157] The first CAR may have the structure:
AgB1-spacer1-TM1- costim-ITAM
in which:

AgB1 is the antigen-binding domain of the first CAR;

spacer 1 is the spacer of the first CAR;

TM1 is the transmembrane domain of the first CAR;

costim is a co-stimulatory domain; and

ITAM is an ITAM-containing endodomain.

"Costim" is a CD28 co-stimulatory domain.

"ITAM" may be a CD3 zeta endodomain.

[0158] The second CAR may have the structure:
AgB2-spacer2-TM2- TNF-ITAM

in which:

AgB2 is the antigen-binding domain of the second CAR;

spacer 2 is the spacer of the second CAR;

TM2 is the transmembrane domain of the second CAR;

TNF is a TNF receptor endodomain; and

ITAM is an ITAM-containing endodomain.

"TNF" is a TNF receptor family endodomain OX40 or 4-1BB endodomain.

[0159] There is also provided a nucleic acid sequence encoding both the first and second chimeric antigen receptors (CARs) with "split" endodomains; and a kit comprising two nucleic acids one encoding a first CAR and one encoding a second CAR comprising split endodomains as defined above.

CO-EXPRESSION SITE

[0160] The invention relates to a nucleic acid which encodes the first and second CARs.

[0161] 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.

[0162] Various self-cleaving sites are known, including the Foot-and-Mouth disease virus (FMDV) 2A peptide and similar sequence (Donnelly et al, Journal of General Virology (2001), 82, 1027-1041), for instance like the 2A-like sequence from Thosea asigna virus which has the sequence shown as SEQ ID No. 12:
SEQ ID No. 12
RAEGRGSLLTCGDVEENPGP.

[0163] The co-expressing sequence may be an internal ribosome entry sequence (IRES). The co-expressing sequence may be an internal promoter

CELL

[0164] The cell of the invention may be any eukaryotic cell capable of expressing a CAR at the cell surface, such as an immunological cell.

[0165] In particular the cell may be an immune effector cell such as a T cell or a natural killer (NK) cell.

[0166] 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.

[0167] 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.

[0168] 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.

[0169] 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.

[0170] Two major classes of CD4+ Treg cells have been described - naturally occurring Treg cells and adaptive Treg cells.

[0171] 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.

[0172] Adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response.

[0173] The T cell of the invention may be any of the T cell types mentioned above, in particular a CTL.

[0174] 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

[0175] 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.

[0176] The CAR cells of the invention may be any of the cell types mentioned above.

[0177] 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 (1st party), or in the setting of a haematopoietic stem cell transplant from donor peripheral blood (2nd party), or peripheral blood from an unconnected donor (3rd party).

[0178] Described is 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 transducing a blood-sample *ex vivo* with a nucleic acid according to the present invention.

[0179] 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.

[0180] 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.

[0181] 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.

[0182] A CAR T cell of the invention may be made by:

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

and

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

[0183] 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

[0184] The invention relates to a nucleic acid sequence which codes for a first CAR and a second CAR as defined in the cell of the invention.

[0185] The nucleic acid sequence may be, for example, an RNA, a DNA or a cDNA sequence.

[0186] The nucleic acid sequence may encode one chimeric antigen receptor (CAR) which binds to CD19 and another CAR which binds to CD22.

[0187] The nucleic acid sequence may have the following structure:
AgB1-spacer1-TM1 -costim- ITAM1 -coexpr-AbB2-spacer2-TM2-TNF-ITAM2
in which

AgB1 is a nucleic acid sequence encoding the antigen-binding domain of a first CAR; spacer 1 is a nucleic acid sequence encoding the spacer of a first CAR;

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

costim is a nucleic acid sequence encoding a CD28 co-stimulatory domain;

ITAM1 is a nucleic acid sequence encoding the ITAM-containing 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 a second CAR;

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

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

TNF is a nucleic acid sequence encoding a TNF receptor endodomain selected from OX40 or 4-1BB endodomain;

ITAM2 is a nucleic acid sequence encoding the ITAM-containing endodomain of the second CAR;

which nucleic acid sequence, when expressed in a cell, encodes a polypeptide which is

cleaved at the cleavage site such that the first and second CARs are co-expressed at the cell surface.

[0188] The first CAR may bind CD19 and the second CAR may bind CD22. Alternatively the first CAR may bind CD22 and the second CAR may bind CD19.

[0189] Alternative codons may be used in regions of sequence encoding the same or similar amino acid sequences, in order to avoid homologous recombination.

[0190] Due to the degeneracy of the genetic code, it is possible to use alternative codons which encode the same amino acid sequence. For example, the codons "ccg" and "cca" both encode the amino acid proline, so using "ccg" may be exchanged for "cca" without affecting the amino acid in this position in the sequence of the translated protein.

[0191] The alternative RNA codons which may be used to encode each amino acid are summarised in Table 3.

Table 3

	U	C	A	G
U	UUU, UUC — Phe (F)	UCU, UCC, UCA, UCG — Ser (S)	UAU, UAC — Tyr (Y)	UGU, UGC — Cys (C)
	UUA, UUG — Leu (L)		UAA — Ocher; UAG — Amber	UGA — Opal; UGG — Trp(W)
C	CUU, CUC, CUA, CUG — Leu (L)	CCU, CCC, CCA, CCG — Pro (P)	CAU, CAC — His (H)	CGU, CGC, CGA, CGG — Arg (R)
			CAA, CAG — Gln (Q)	
A	AUU, AUC, AUA — Ile (I)	ACU, ACC, ACG, ACG — Thr (T)	AAU, AAC — Asn (N)	AGU, AGC — Ser (S)
			AAA, AAG — Lys (K)	AGA, AGG — Arg (R)

	U	C	A	G
	AUG Met(M)			
G	GUU, GUC, GUA, GUG — Val (V)	GCU, GCC, GCA, GCG — Ala (A)	GAU, GAU — Asp (D) GAA, GAG — Glu (E)	GGU, GGC, GGA, GGG — Gly (G)

[0192] Alternative codons may be used in the portions of nucleic acid sequence which encode the spacer of the first CAR and the spacer of the second CAR, especially if the same or similar spacers are used in the first and second CARs. Figure 4 shows two sequences encoding the spacer HCH2CH3 - hinge, in one of which alternative codons have been used.

[0193] Alternative codons may be used in the portions of nucleic acid sequence which encode the transmembrane domain of the first CAR and the transmembrane of the second CAR, especially if the same or similar transmembrane domains are used in the first and second CARs. Figure 4 shows two sequences encoding the CD28 transmembrane domain, in one of which alternative codons have been used.

[0194] Alternative codons may be used in the portions of nucleic acid sequence which encode all or part of the endodomain of the first CAR and all or part of the endodomain of the second CAR. Alternative codons may be used in the CD3 zeta endodomain. Figure 4 shows two sequences encoding the CD3 zeta endodomain, in one of which alternative codons have been used.

[0195] Alternative codons may be used in one or more co-stimulatory domains, such as the CD28 endodomain.

[0196] Alternative codons may be used in one or more domains which transmit survival signals, such as OX40 and 41BB endodomains.

[0197] Alternative codons may be used in the portions of nucleic acid sequence encoding a CD3zeta endodomain and/or the portions of nucleic acid sequence encoding one or more costimulatory domain(s) and/or the portions of nucleic acid sequence encoding one or more domain(s) which transmit survival signals.

VECTOR

[0198] The present invention also provides a vector, which comprises a CAR-encoding nucleic acid sequence. Such a vector may be used to introduce the nucleic acid sequence into a host cell so that it expresses the first and second CARs.

[0199] 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.

[0200] The vector may be capable of transfecting or transducing a T cell.

PHARMACEUTICAL COMPOSITION

[0201] 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 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

[0202] The cells of the present invention are capable of killing cancer cells, such as B-cell lymphoma cells. CAR- expressing cells, such as T cells, may either be created ex *vivo* either from a patient's own peripheral blood (1st party), or in the setting of a haematopoietic stem cell transplant from donor peripheral blood (2nd party), or peripheral blood from an unconnected donor (3rd party). Alternatively, CAR T-cells may be derived from *ex-vivo* differentiation of inducible progenitor cells or embryonic progenitor cells to T-cells. In these instances, CAR T-cells are generated by introducing DNA or RNA coding for the CAR by one of many means including transduction with a viral vector, transfection with DNA or RNA.

[0203] The cells of the present invention may be capable of killing target cells, such as cancer cells. The target cell is recognisable by expression of CD19 or CD22.

Table 4 - expression of lymphoid antigens on lymphoid leukaemias

	CD19	CD22	CD10	CD7	CD5	CD3	clg μ	slg μ
Early pre-B	100	>95	95	5	0	0	0	0
Pre-B	100	100	>95	0	0	0	100	0
Transitional pre-B	100	100	50	0	0	0	100	0
B	100	100	50	0	0	0	>95	>95
T	<5	0	0	100	95	100	0	0

[0204] Taken from Campana et al. (Immunophenotyping of leukemia. J. Immunol. Methods 243, 59-75 (2000)). clg μ - cytoplasic Immunoglobulin heavy chain; slg μ - surface Immunoglobulin heavy chain.

[0205] The expression of commonly studied lymphoid antigens on different types of B-cell leukaemias closely mirrors that of B-cell ontogeny (see Figure 2).

[0206] The cells of the present invention may be used to treat cancer, in particular B-cell malignancies.

[0207] Examples of cancers which express CD19 or CD22 are B-cell lymphomas, including Hodgkin's lymphoma and non-Hodgkins lymphoma; and B-cell leukaemias.

[0208] For example the B-cell lymphoma may be Diffuse large B cell lymphoma (DLBCL), Follicular lymphoma, Marginal zone lymphoma (MZL) or Mucosa-Associated Lymphatic Tissue lymphoma (MALT), Small cell lymphocytic lymphoma (overlaps with Chronic lymphocytic leukemia), Mantle cell lymphoma (MCL), Burkitt lymphoma, Primary mediastinal (thymic) large B-cell lymphoma, Lymphoplasmacytic lymphoma (may manifest as Waldenström macroglobulinemia), Nodal marginal zone B cell lymphoma (NMZL), Splenic marginal zone lymphoma (SMZL), Intravascular large B-cell lymphoma, Primary effusion lymphoma, Lymphomatoid granulomatosis, T cell/histiocyte-rich large B-cell lymphoma or Primary central nervous system lymphoma.

[0209] The B-cell leukaemia may be acute lymphoblastic leukaemia, B-cell chronic lymphocytic leukaemia, B-cell prolymphocytic leukaemia, precursor B lymphoblastic leukaemia or hairy cell leukaemia.

[0210] The B-cell leukaemia may be acute lymphoblastic leukaemia.

[0211] Treatment with the cells of the invention may help prevent the escape or release of tumour cells which often occurs with standard approaches.

[0212] 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 - Proof-of-concept of a CD19/CD22 Logical 'OR' gate (for reference)

[0213] A CD19 'OR' CD22 CAR gate was constructed by co-expression of a CD19 and a CD22

CAR in the same vector. The anti-CD19 binder was a scFv derived from the re-surfaced B4 antibody (Roguska et al. (1996) Protein Eng. 9, 895-904), and the anti-CD22 binder was a scFv derived from the humanized RFB4 antibody. A human IgG1 hinge-CH2-CH3 spacer was used for both CARs, the coding sequence of which was codon-wobbled to avoid homologous recombination by the integrating vector. The TM domain in both CARs was derived from that of CD28, and both CAR endodomains comprised of CD3-Zeta. Once again, these homologous sequences were codon-wobbled. Co-expression was achieved by cloning the two CARs in frame separated by a FMD-2A peptide. The nucleic acid and amino acid sequence of the CD19/CD22 'OR' gate construct are shown as SEQ ID NOs: 13 and 14; respectively.

SEQ ID NO: 13

ATGAGCCTGCCCGTGACCGCCCTGCTGCTGCCCCTGGCCCTGCTGCTGCACGCCGCCAGACCATACCCCTACGAC
GTGCCCGACTACGCCAGCCTGAGCGGAGGCGGCGGCAGCCAGGTGCAGCTGGTGCAGAGCGGAGCCGAGGTGAAG
AAGCCTGGCGCCAGCGTGAAGGTGTCCTGTAAGGCCAGCGGCTACACCTTCACCAGCAACTGGATGCACTGGGTG
AGGCAGGCCCCTGGACAGGGACTGGAGTGGATGGGCGAGATCGACCCCAGCGACAGCTACACCAACTACAACCAG
AAGTTCAAGGGCCGGGTGACCATCACCGTGGATAAGAGCGCCAGCACCGCCTACATGGAGCTGTCCAGCCTGAGA
AGCGAGGATACCGCCGTGTACTACTGTGCCAGAGGCAGCAACCCCTACTACTACGCTATGGACTACTGGGGCCAG
GGCACCCTGGTGACCGTGTCCAGCGGCGGAGGAGGAAGCGGAGGGGGCGGATCTGGCGGCGGAGGGAGCGAGATC
GTGCTGACCCAGAGCCCCGCCACCCTGAGCCTGAGCCCTGGCGAGAGAGCCACCCTGTCCTGTAGCGCCAGCAGC
GGCGTGAATTACATGCACTGGTATCAGCAGAAGCCCGGCCAGGCCCCCAGAAGATGGATCTACGACACCAGCAAG
CTGGCCAGCGGCGTGCCCGCCAGATTCAGCGGCAGCGGCTCCGGCACCAGCTACAGCCTGACCATCAGCAGCCTG
GAGCCTGAGGATTTCGCCGTGTATTATTGCCACCAGAGGGGCAGCTACACCTTTGGCGGCGGAACAAAGCTGGAG
ATCAAGCGCTCAGATCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGTCGCAGCCC
CTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGT
GATATCTTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATT
ATTTTCTGGGTGAGGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAACCAGCTC
TATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATG
GGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCC
TACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACA
GCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCTCCTCGCAGAGCCGAGGGCAGGGGAAGTCTT
CTAACATGCGGGGACGTGGAGGAAAATCCCGGGCCCATGGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATT
TTAAAAGGTGTCCAGTGCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCAGGGGGGTCCCTGCGC
CTCTCCTGTGCAGCCTCTGGATTCGCTTTCAGTATCTATGACATGTCTTGGGTCCGCCAGGTTCCGGGGAAGGGG
CTGGAGTGGGTCTCATATATTAGTAGTGGTGGTGGTACCACCTATTACCCGGACACTGTGAAGGGCCGCTTCACC
ATCTCCCGTGACAATTCCCGCAACACTCTGGATCTTCAAATGAACAGTCTGCGCGTCGAGGACACGGCTGTCTAT
TATTGTGCGCGTCATAGTGGCTACGGTAGTAGCTACGGGGTTTTGTTTGCTTACTGGGGCCAAGGAACCCTGGTC

ACCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCCAGATGACTCAG
TCTCCGTCCTCCCTGTCTGCATCTGTAGGAGACCGCGTCACCATCACCTGCCGTGCAAGTCAGGACATTAGCAAT
TATTTAAACTGGCTTCAACAGAAACCGGGGAAAGCCCCGAAGCTCCTGATTTACTACACATCAATCTTACACTCA
GGAGTCCCGTCACGCTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCGGAA
GATTTTGCAACTTATTACTGTCAACAGGGTAATACGCTTCCGTGGACGTTTGGCCAGGGGACCAAACTGGAAATC
AAACGTTCGGATCCAGCCGAACCAAAGAGCCCCGATAAGACCCACACCTGTCCCCCCTGCCCAGCCCCAGAGCTG
CTGGGAGGCCCCAGCGTGTTTCTGTTTCCACCCAAGCCAAAGGATACCCTGATGATTAGTAGAACACCCGAAGTG
ACCTGTGTGGTGGTGGATGTGTCTCACGAGGACCCCGAGGTGAAATTTAATTGGTATGTTGATGGTGTTGAAGTG
CACAACGCCAAAACCAAACCCAGAGAGGAGCAGTACAATTCTACCTATAGAGTCGTGTCTGTGCTGACAGTGCTG
CATCAGGATTGGCTGAACGGAAAAGAATACAAATGTAAAGTGAGCAATAAGGCCCTGCCCGCTCCAATTGAGAAG
ACAATTAGCAAGGCCAAGGGCCAGCCAAGGGAGCCCCAGGTGTATACACTGCCACCCAGTAGAGACGAACTGACA

AAGAATCAGGTGTCTCTGACATGTCTGGTGAAGGGATTTTACCCATCTGATATCGCCGTGGAATGGGAATCTAAC
GGCCAGCCCGAGAATAACTATAAGACAACCCCCACCAGTGCTGGATAGCGATGGCAGCTTTTTTCTGTATTCTAAG
CTGACAGTGGATAAGTCCCGGTGGCAGCAGGGAAATGTGTTTAGCTGTAGTGTCATGCATGAGGCCCTGCACAAT
CACTATACCCAGAAATCTCTGAGTCTGAGCCCAGGCAAGAAGGACCCCAAGTTCTGGGTCCTGGTGGTGGTGGGA
GGCGTGCTGGCCTGTTACTCTCTCCTGGTGACCGTGGCCTTCATCATCTTTTGGGTGCGCTCCCGGGTGAAGTTT
TCTCGCTCTGCCGATGCCCCAGCCTATCAGCAGGGCCAGAATCAGCTGTACAATGAACTGAACCTGGGCAGGCGG
GAGGAGTACGACGTGCTGGATAAGCGGAGAGGCAGAGACCCCGAGATGGGCGGCAAACCACGGCGCAAAAATCCC
CAGGAGGGACTCTATAACGAGCTGCAGAAGGACAAAATGGCCGAGGCCTATTCCGAGATCGGCATGAAGGGAGAG
AGAAGACGCGGAAAGGGCCACGACGGCCTGTATCAGGGATTGTCCACCGCTACAAAAGATACATATGATGCCCTG
CACATGCAGGCCCTGCCACCCAGATGA

SEQ ID NO: 14

MSLPVTALLLPLALLLHAARPYPYDVPDYASLSGGGGSQVQLVQSGAEVKKPGASVKVSCKASGYTFT
SNWMHWVRQAPGQGLEWMGEIDPSDSYTNYNQKFKGRVTITVDKSASTAYMELSSLRSEDTAVYYCAR
GSNPYYYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGERATLSCSASSGVNY
MHWYQQKPGQAPRRWIYDTSKLASGVPARFSGSGSGTSYSLTISSLEPEDFAVYYCHQRGSYTFGGGT
KLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLAC
YSLLVTVAFIIFWVRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN
PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRAEGRGSLL
TCGDVEENPGPMEFGLSWLFLVAILKGVQCEVQLVESGGGLVQPGGSLRLSCAASGFAFSIYDMSWVR
QVPGKGLEWVSYISSGGGTTYYPDTVKGRFTISRDNSRNTLDLQMNSLRVEDTAVYYCARHSGYGSSY
GVLFAYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWL
QQKPGKAPKLLIYYTSILHSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQGNTLPWTFGQGTKL
EIKRSDPAEPKSPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT
VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPKFWVLVVVGGVLACYSLLVTVAFIIFWVR
SRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM
AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

[0214] To demonstrate co-expression of both CARs, the scFv of each CAR was tagged with an epitope tag (HA or V5 respectively). This subsequent single open-reading frame was cloned into the SFG retroviral vector. T-cells were transduced with this vector and both CARs could be detected on the T-cells surface expressing the cassette by staining with anti-HA and anti-V5 and studying expression by flow cytometry.

[0215] Next, T-cells expressing the CD19 OR CD22 CAR gate were challenged with target cells, expressing neither, both or one antigen along with control T-cells which expressed no CARs, or just anti-CD19 CAR alone, or anti-CD22 CAR alone. We found that the OR-gated CAR T-cells could kill target cells expressing either one or both target antigens (Figure 5).

Example 2 - Identification and characterisation of CD19ALAb and CD22ALAb

[0216] A CD19-binder (CD19ALAb) was identified, humanised and the binding affinities of both murine and humanised IgGs and scFvs were identified and compared with the "gold-standard" anti-CD19 binder, fmc63. In parallel, and a CD22-binder (CD22ALAb) was identified,

humanised and the binding affinities of both murine and humanised IgGs and scFvs were identified and compared with the "gold-standard" anti-CD22 binder, M971.

[0217] Experiments were performed on a Biacore T200 instrument using HBS-P as running and dilution buffer. BIAevaluation software Version 2.0 was used for data processing. For binding kinetics, mouse anti-human IgG or goat anti-mouse IgG was covalently coupled to a CM5 Sensor Chip. IgG or scFv-Fc proteins were captured, and various concentrations of interaction partner protein injected over the flow cell at a flow rate of 30 µl/min. Kinetic rate constants were obtained by curve fitting according to a 1:1 Langmuir binding model. Bulk refractive index differences were subtracted using a blank control flow cell in which capture antibody had been immobilized to the same level as the active surface. A double reference subtraction was performed using buffer alone.

[0218] The results are shown in Figures 6 to 8.

[0219] The data show that humanised CD22ALAb has comparable binding affinity to CD22 to murine CD22ALAb (Figure 6) and similar binding kinetics. Both murine and humanised CD22ALAb in an scFv format have significantly higher binding affinity to CD22 than the gold-standard CD22-binding antibody, M971 (Figure 6).

[0220] Although the binding affinity of murine and humanised CD19ALAb in an IgG format was found to be similar (data not shown), surprisingly the binding affinity of humanised CD19ALAb was found to be higher than murine CD19ALAb in an scFv format (Figure 7). The binding affinity of CD19ALAb is comparable (possibly slightly better) than that of the gold-standard anti-CD19 Ab, fmc63 (Figure 8).

Example 3 - Comparative functional assays with CD19ALAb/fmc63 CARs and CD22ALAb/M971 CARs

[0221] The antigen binding domain of a CAR can affect its function. In this study, CARs were created comprising CD19ALAb and CD22ALAb and function was compared with an equivalent CAR having an antigen-binding domain based on fmc63 or M971.

[0222] CARs comprising scFvs based on fmc63 (anti-CD19) and M971 (anti-CD22) can be considered as the gold standard antibodies as both CARs are in clinical development.

[0223] CARs were constructed and expressed based on CD19ALAb, fmc63, CD22ALAb and M971. Their structure is shown in Figure 9. The CARs differed solely in their antigen binding domain. In all constructs, the binding domains were linked to the membrane with a CD8 stalk spacer and contained intracellular activatory motifs from 41BB and CD3-zeta.

[0224] Retroviruses were produced by transient transfection of 293T cells with plasmids encoding the CARs, gag/pol and the envelope protein RD114. After 3 days the supernatants

were harvested and used to transduce PHA/IL2-activated PBMCs with equal titres of retrovirus on retronectin-coated plates. Six days post-transduction CAR-expression was confirmed by flow cytometry and PBMCs were co-cultured in a 1:1 ratio with either CD19+ BFP SupT1 cells (fmc63 and CD19ALAb CARs) or CD22+ BFP SupT1 cells (M971 and CD22ALAb CARs). Target cell killing was assayed after one and three days. Also after one and three days, supernatants were removed and interferon-γ levels were assayed by ELISA.

[0225] The results are shown in Figures 10 and 11.

[0226] As shown in Figure 10, the CAR with a CD19ALAb antigen binding domain gave more killing of CD19+ve target cells (Figure 10) at both Day1 and Day 3, than the equivalent CAR with a fmc63 binding domain.

[0227] With regard to CD22, the CAR with a CD22ALAb antigen binding domain gave more killing of CD22+ve target cells (Figure 11a) after three days than the equivalent CAR with an M971 binding domain. IFNγ release was significantly higher with the CD22ALAb CAR than the M971 CAR after the same time frame.

[0228] CARs having an antigen-binding domain based on CD19ALAb and CD22ALAb therefore have improved properties in terms of target cell killing than equivalent CARs based on fmc63 and M971.

[0229] The CD22ALAb result is particularly surprising, given the findings reported in Haso et al (2013) as above. In that study, different anti-CD22 CARs were made and tested, with binding domains based on the anti-CD22 antibodies HA22, BL22 and m971. HA22 and BL22 scFvs bind to Ig domain 3 of CD22, whereas m971 binds within Ig domain 5-7 of CD22 (see Haso et al (2013) Figure 2B). It was reported that the m971-derived CAR showed superior target cell killing activity than HA22-derived CAR, which finding is attributed to the importance of the CD22 epitope targeted by the CAR (Haso et al (2013) page 1168, last full paragraph). It is concluded that targeting a membrane proximal domain of CD22 is "the key element" in developing a highly active anti-CD22 CAR (Discussion, last paragraph). Contrary to this finding, the data shown here in Figure 11 demonstrate that CD22ALAb, which targets an epitope in Ig domain 3 of CD22 - a "membrane distal" epitope compared to the Ig domain 5-7 epitope targeted by m971 - has superior target cell killing ability than an m971-based anti-CD22 CAR.

Example 4 - Investigating OR gate constructs with different endodomain combinations

[0230] Four OR gate constructs were developed as shown in Figure 13. They all encoded CD19/CD22 OR gates having identical antigen-binding domains, spacer domains and transmembrane domains: the only difference between the construct was in the endodomains, which were as shown in the following Table:

Construct	CD19 CAR endodomain	CD22 CAR endodomain
A	41BB-CD3ζ	41BB-CD3ζ
B	OX40-CD3ζ	OX40-CD3ζ
C	41BB-CD3ζ	CD28-CD3ζ
D	OX40-CD3ζ	CD28-CD3ζ

[0231] The capacity of cells expressing each CD19/CD22 OR gate to kill Raji cells in vitro was assayed as described above. Transduced PBMCs expressing the various OR gate combinations were co-cultured for 72 hours with CD19+/CD22+ Raji target cells at both a 1:1 and 1:10 effector:target cell ratio.

[0232] The results are shown in Figure 14. All four OR gates were found to kill target cells significantly better than the fmc63 and M971 CARs. With the 1:10 effector:target cell ratio, it was shown that the "split" endodomain OR gates, which have 4-1BBzeta/OX40zeta on one CAR and CD28zeta on the other CAR, had the best killing activity.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO2014055657A [0016]
- US7052906B1 [0138]
- WO2000063372A [0144]

Non-patent literature cited in the description

- **GRUPP et al.**New Eng. J. Med, 2013, vol. 368, 1509-1518 [0010]
- **GRADA et al.**Mol Ther Nucleic Acids, 2013, vol. 2, e105- [0013]
- **HASO et al.**Blood, 2013, vol. 121, 7- [0109]
- **WEN et al.**J. Immunol. Baltim. Md, 1950, vol. 188, 1075-1082 [0124]
- **HASO et al.**Blood, 2013, vol. 121, 71165-74 [0124]
- **JAMES et al.**Journal of immunology, 2008, vol. 180, 107028-38 [0124]
- **PEZZUTTO, A. et al.**J. Immunol. Baltim. Md, 1950, vol. 138, 2793-2799 [0129]
- **MEEKER, T. C. et al.**Hybridoma, 1984, vol. 3, 305-320 [0129]
- **NICHOLSON, I. C. et al.**Mol. Immunol., 1997, vol. 34, 1157-1165 [0129]
- **BEJCEK, B. E. et al.**Cancer Res., 1995, vol. 55, 2346-2351 [0129]
- **ROGUSKA, M. A. et al.**Protein Eng., 1996, vol. 9, 895-904 [0129]
- **KANSAS, G. S.TEDDER, T. F.**J. Immunol. Baltim. Md, 1950, vol. 147, 4094-4102 [0129]
- **YAZAWA et al.**Proc. Natl. Acad. Sci. U. S. A., 2005, vol. 102, 15178-15183 [0129]
- **HERBST, R. et al.**J. Pharmacol. Exp. Ther., 2010, vol. 335, 213-222 [0129]
- **JOHN et al.**J. Immunol. Baltim. Md, 1950, vol. 170, 3534-3543 [0130]
- **DIJOSEPH et al.**Cancer Immunol. Immunother. CII, 2005, vol. 54, 11-24 [0130]
- **DORKEN et al.**J. Immunol. Baltim. Md, 1950, vol. 136, 4470-4479 [0130]
- **PEZZUTTO et al.**J. Immunol. Baltim. Md, 1950, vol. 138, 98-103 [0130]
- **CAMPANA et al.**J. Immunol. Baltim. Md, 1950, vol. 134, 1524-1530 [0130]
- **KRAUSS et al.**Protein Eng., 2003, vol. 16, 753-759 [0130]
- **KREITMAN et al.**J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol., 2012, vol. 30, 1822-1828 [0130]
- **MASON et al.**Blood, 1987, vol. 69, 836-840 [0130]
- **SCHWARTING et al.**Blood, 1985, vol. 65, 974-983 [0130]
- **SHIH et al.**Int. J. Cancer J. Int. Cancer, 1994, vol. 56, 538-545 [0130]
- **LEONARD et al.**J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol., 2003, vol. 21, 3051-3059 [0130]
- **XIAO et al.**mAbs, 2009, vol. 1, 297-303 [0130]
- **ENGEL et al.**J. Exp. Med., 1995, vol. 181, 1581-1586 [0130]
- **DONNELLY et al.**Journal of General Virology, 2001, vol. 82, 1027-1041 [0162]
- **CAMPANA et al.**Immunophenotyping of leukemiaJ. Immunol. Methods, 2000, vol. 243, 59-75 [0204]
- **ROGUSKA et al.**Protein Eng., 1996, vol. 9, 895-904 [0213]

Patentkrav

1. Celle, som har coekspression af en første kimær antigenreceptor (CAR) og en anden CAR på celleoverfladen, idet hver CAR omfatter et intracellulært signaleringsdomæne, hvor det intracellulære signaleringsdomæne af den første CAR omfatter et CD28-costimulatorisk domæne og et ITAM-indeholdende domæne, men ikke omfatter et TNF-receptorfamilie-endodomæne; og det intracellulære signaleringsdomæne af den anden CAR omfatter et TNF-receptorfamilie-endodomæne valgt blandt OX40- eller 4-1BB-endodomæne og et ITAM-indeholdende domæne, men ikke omfatter et costimulatorisk domæne.

2. Celle ifølge krav 1, hvor den første CAR har strukturen:
AgB1-spacer1-TM1-costim-ITAM
hvor:
AgB1 er det antigenbindende domæne af den første CAR;
spacer 1 er spaceren i den første CAR;
TM1 er transmembrandomænet af den første CAR;
costim er et costimulatorisk domæne; og
ITAM er et ITAM-indeholdende endodomæne;
og den anden CAR har strukturen:
AgB2-spacer2-TM2-TNF-ITAM
hvor:
AgB2 er det antigenbindende domæne af den anden CAR;
spacer 2 er spaceren i den anden CAR;
TM2 er transmembrandomænet af den anden CAR;
TNF er et TNF-receptor-endodomæne; og
ITAM er et ITAM-indeholdende endodomæne.

3. Nukleinsyresekvens, som koder for både den første og anden kimære antigenreceptor (CAR) som defineret i et hvilket som helst af ovennævnte krav.

4. Nukleinsyresekvens ifølge krav 3, som har følgende struktur:
AgB1-spacer1-TM1-costim-ITAM1-coexpr-AbB2-spacer2-TM2-TNF-ITAM2

hvor
AgB1 er en nukleinsyresekvens, der koder for det antigenbindende domæne af den første CAR;
spacer 1 er en nukleinsyresekvens, der koder for spaceren i den første CAR;
TM1 er en nukleinsyresekvens, der koder for transmembrandomænet af den første CAR;
costim er en nukleinsyresekvens, der koder for et costimulatorisk domæne;
ITAM1 er en nukleinsyresekvens, der koder for det ITAM-indeholdende endodomæne af den første CAR;
coexpr er en nukleinsyresekvens, der muliggør coekspression af begge CAR'er;
AgB2 er en nukleinsyresekvens, der koder for det antigenbindende domæne af den anden CAR;
spacer 2 er en nukleinsyresekvens, der koder for spaceren i den anden CAR;
TM2 er en nukleinsyresekvens, der koder for transmembrandomænet af den anden CAR;
TNF er en nukleinsyresekvens, der koder for et TNF-receptor-endodomæne;
ITAM2 er en nukleinsyresekvens, der koder for det ITAM-indeholdende endodomæne af den anden CAR;
hvilken nukleinsyresekvens, når den udtrykkes i en celle, koder for et polypeptid, der spaltes i spaltningsstedet, hvorved der tilvejebringes coekspression af den første og anden CAR på celleoverfladen.

5. Kit, som omfatter
(i) en første nukleinsyresekvens, der koder for den første kimære antigenreceptor (CAR) som defineret i et hvilket som helst af kravene 1 til 2, hvilken nukleinsyresekvens har følgende struktur:
AgB1-spacer1-TM1-costim-ITAM1
hvor
AgB1 er en nukleinsyresekvens, der koder for det antigenbindende domæne af den første CAR;

spacer 1 er en nukleinsyresekvens, der koder for spaceren i den første CAR;
TM1 er en nukleinsyresekvens, der koder for transmembrandomænet af den første CAR;
costim er en nukleinsyresekvens, der koder for et costimulatorisk domæne;
ITAM1 er en nukleinsyresekvens, der koder for det ITAM-indeholdende endodomæne af den første CAR; og
(ii) en anden nukleinsyresekvens, der koder for den anden kimære antigenreceptor (CAR) som defineret i et hvilket som helst af kravene 1 til 2, hvilken nukleinsyresekvens har følgende struktur:
AbB2-spacer2-TM2-TNF-ITAM2
hvor
AgB2 er en nukleinsyresekvens, der koder for det antigenbindende domæne af den anden CAR;
spacer 2 er en nukleinsyresekvens, der koder for spaceren i den anden CAR;
TM2 er en nukleinsyresekvens, der koder for transmembrandomænet af den anden CAR;
TNF er en nukleinsyresekvens, der koder for et TNF-receptor-endodomæne; og
ITAM2 er en nukleinsyresekvens, der koder for det ITAM-indeholdende endodomæne af den anden CAR.

6. Vektor, som omfatter en nukleinsyresekvens ifølge krav 3 eller 4

7. Fremgangsmåde til frembringelse af en celle ifølge et hvilket som helst af kravene 1 til 2, som omfatter trinet indføring af: en nukleinsyresekvens ifølge krav 3 eller 4; en første nukleinsyresekvens og en anden nukleinsyresekvens som defineret i krav 5; eller en vektor ifølge krav 6 i en celle ex vivo.

8. Farmaceutisk sammensætning, som omfatter en flerhed af celler ifølge et hvilket som helst af kravene 1 til 2.

9. Farmaceutisk sammensætning ifølge krav 8 til anvendelse til behandling og/eller forebyggelse af en sygdom.

DRAWINGS

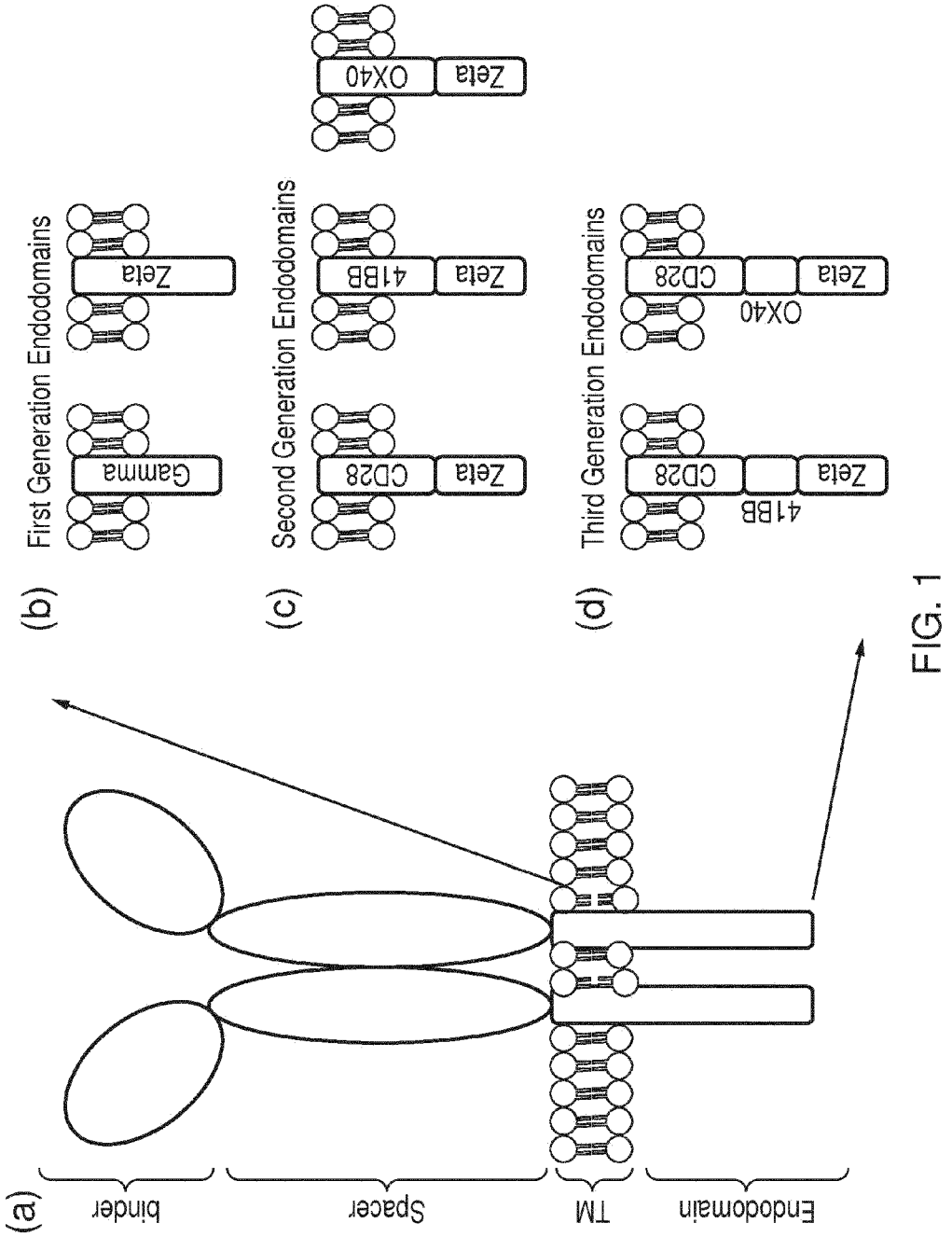


FIG. 1

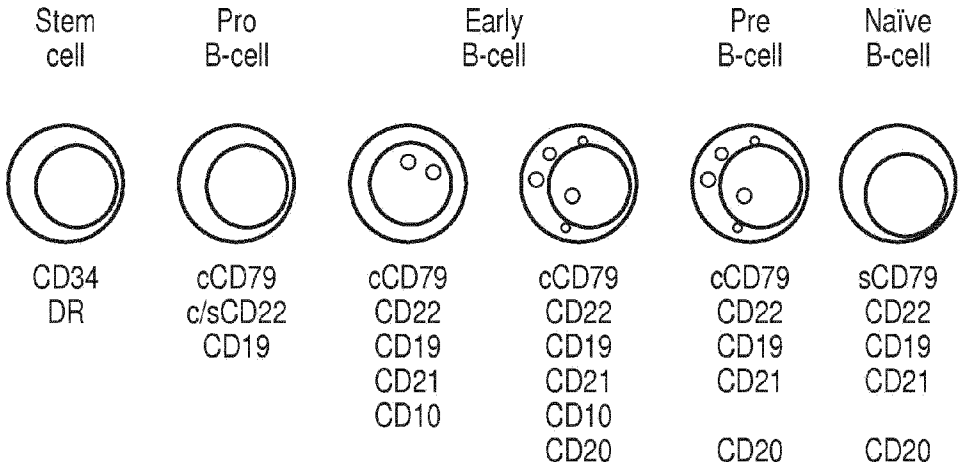

Stem cell
Pro B-cell
Early B-cell
Pre B-cell
Naïve B-cell
CD34 DR
cCD79 c/sCD22 CD19
cCD79 CD22 CD19 CD21 CD10
cCD79 CD22 CD19 CD21 CD10 CD20
cCD79 CD22 CD19 CD21 CD20
sCD79 CD22 CD19 CD21 CD20

FIG. 2

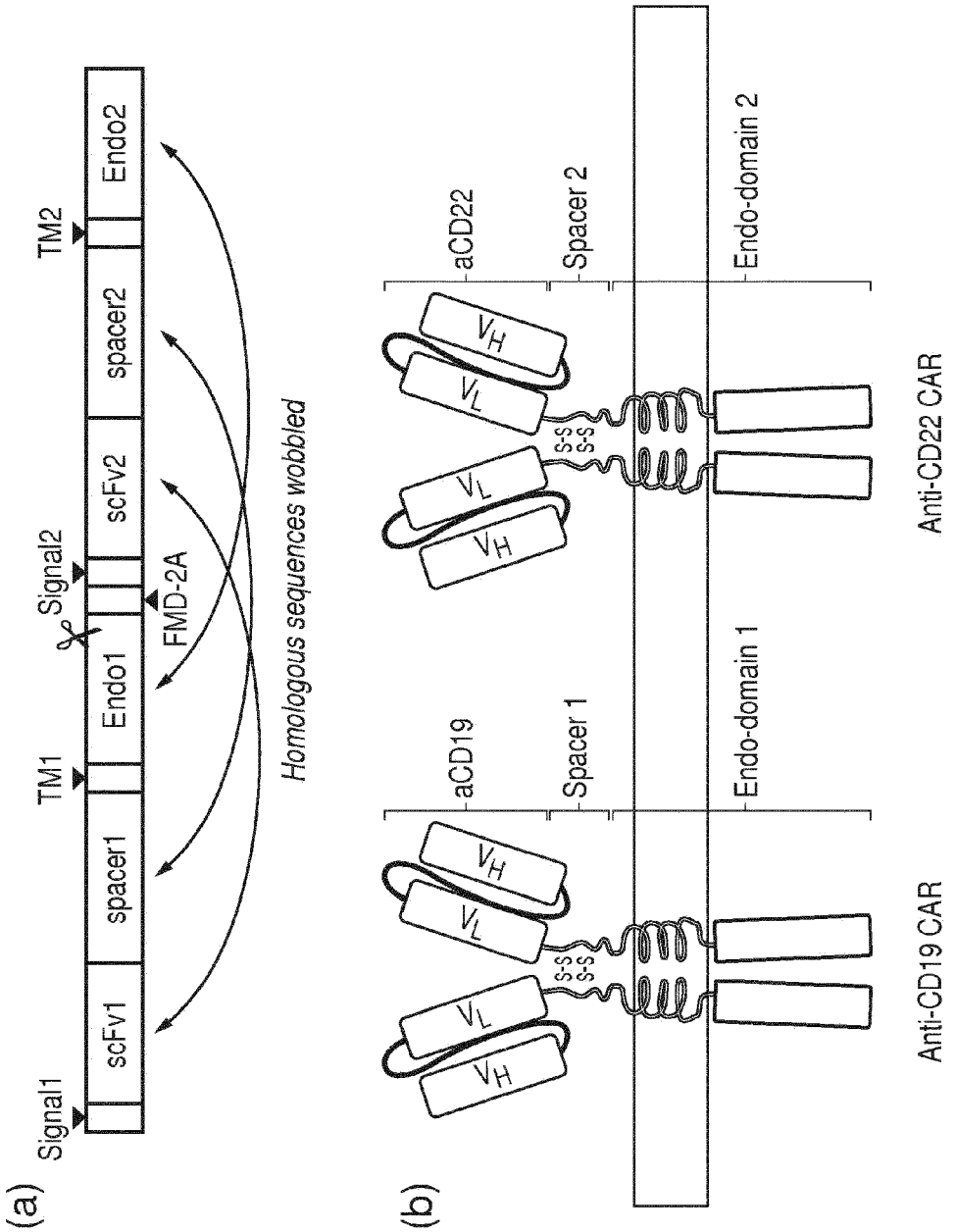

(a)
Signal1
scFv1
TM1
spacer1
Endo1
Signal2
FMD-2A
scFv2
TM2
spacer2
Endo2
Homologous sequences wobbled
(b)
aCD19
Spacer 1
Endo-domain 1
Anti-CD19 CAR
aCD22
Spacer 2
Endo-domain 2
Anti-CD22 CAR
VH
VL
S-S
S-S

FIG. 3

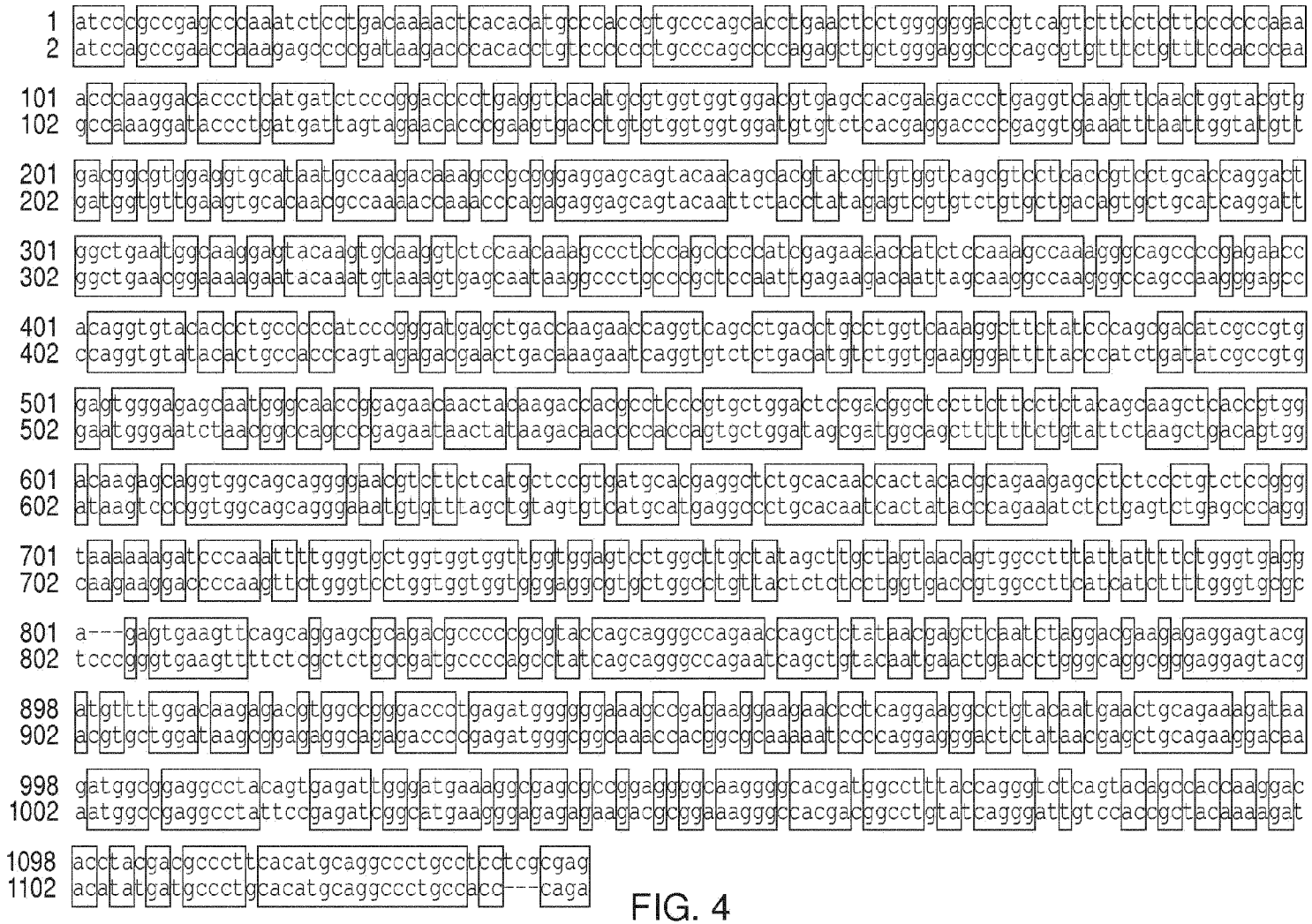


FIG. 4

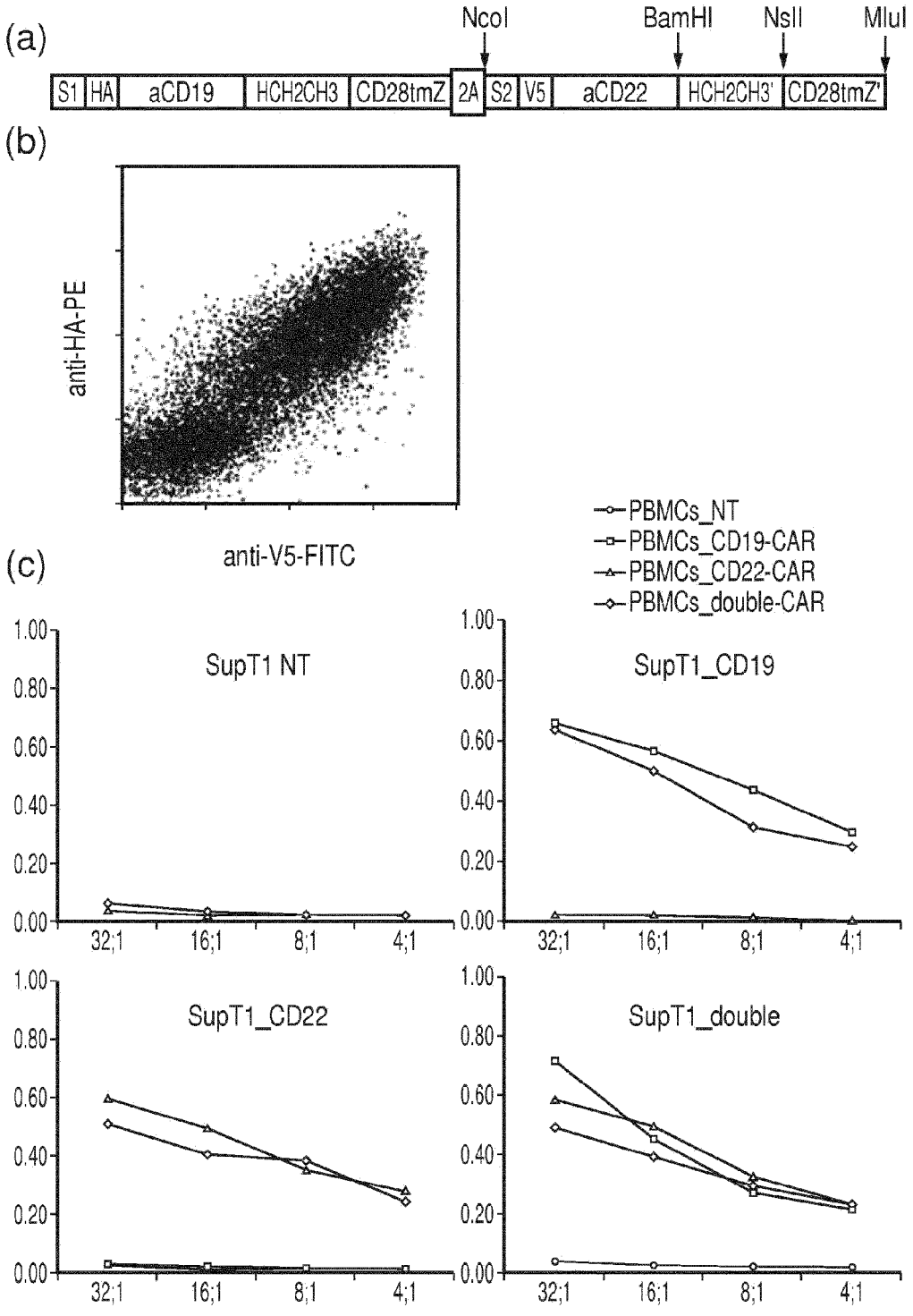
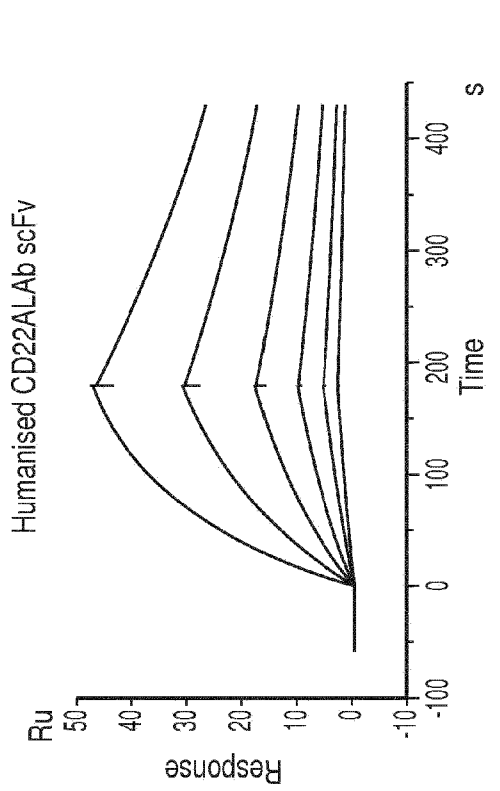
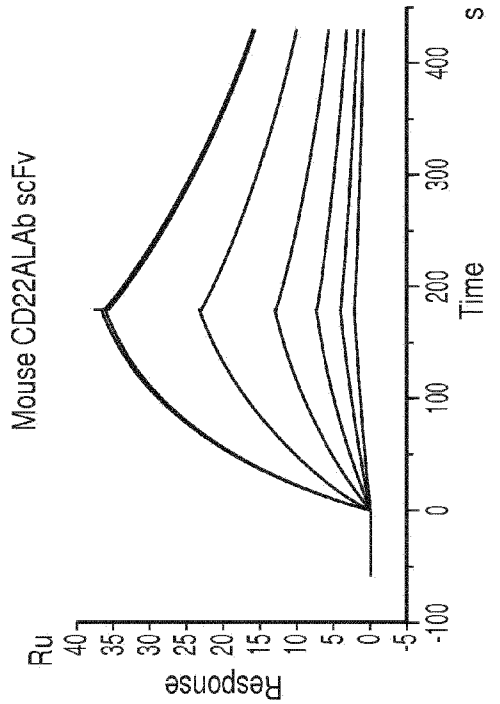

(a)
NcoI
BamHI
NsiI
MluI
S1
HA
aCD19
HCH2CH3
CD28tmZ
2A
S2
V5
aCD22
HCH2CH3'
CD28tmZ'
(b)
anti-HA-PE
anti-V5-FITC
(c)
PBMCs_NT
PBMCs_CD19-CAR
PBMCs_CD22-CAR
PBMCs_double-CAR
SupT1 NT
SupT1_CD19
SupT1_CD22
SupT1_double
1.00
0.80
0.60
0.40
0.20
0.00
32;1
16;1
8;1
4;1

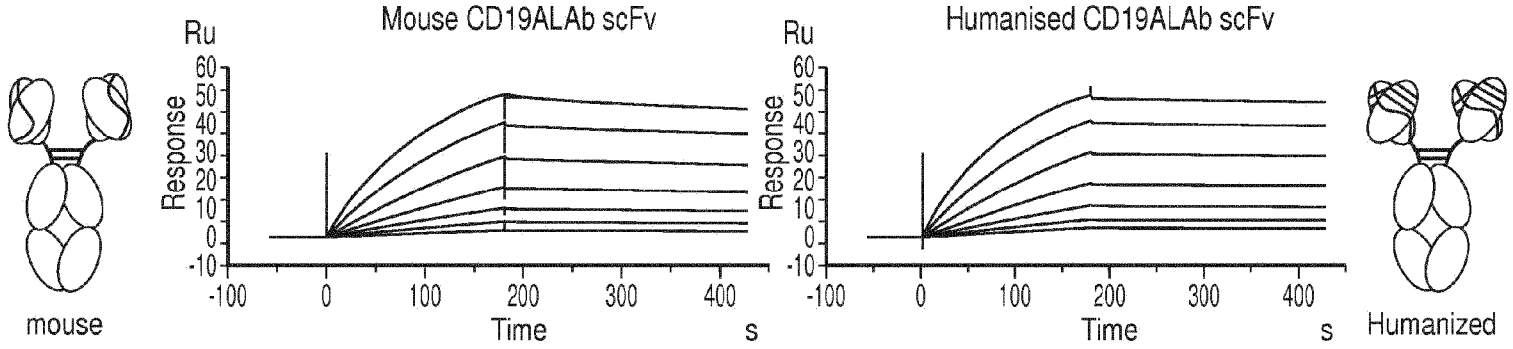
FIG. 5





Antibody	ka (1/Ms)	kd (1/s)	KD (nM)
Mouse CD22ALAb scFv	7.09 E4	2.8 e-3	38
Humanised CD22ALAb scFv	6.83 E4	2.26 e-3	32
M971 scFv	1.01 E5	6.19 e-3	62

FIG. 6



scFv	ka (1/Ms)	kd (1/s)	KD (nM)
mscFv CD19ALAb	1.78 E5	3.28 E-4	1.85
hscFv CD19ALAb	2.21 E5	2.11 E-4	1.09

FIG. 7

DK/EP 3560953 T5

scFv	ka (1/Ms)	kd (1/s)	KD (nM)	n
CD19ALAb	$1.65 \pm 0.143 \times 10^5$	$3.00 \pm 2.198 \times 10^{-4}$	1.1 ± 0.2	2
FMC63	$3.2 \pm 0.8 \times 10^5$	$3.9 \pm 1.2 \times 10^{-4}$	1.3 ± 0.7	2

FIG. 8

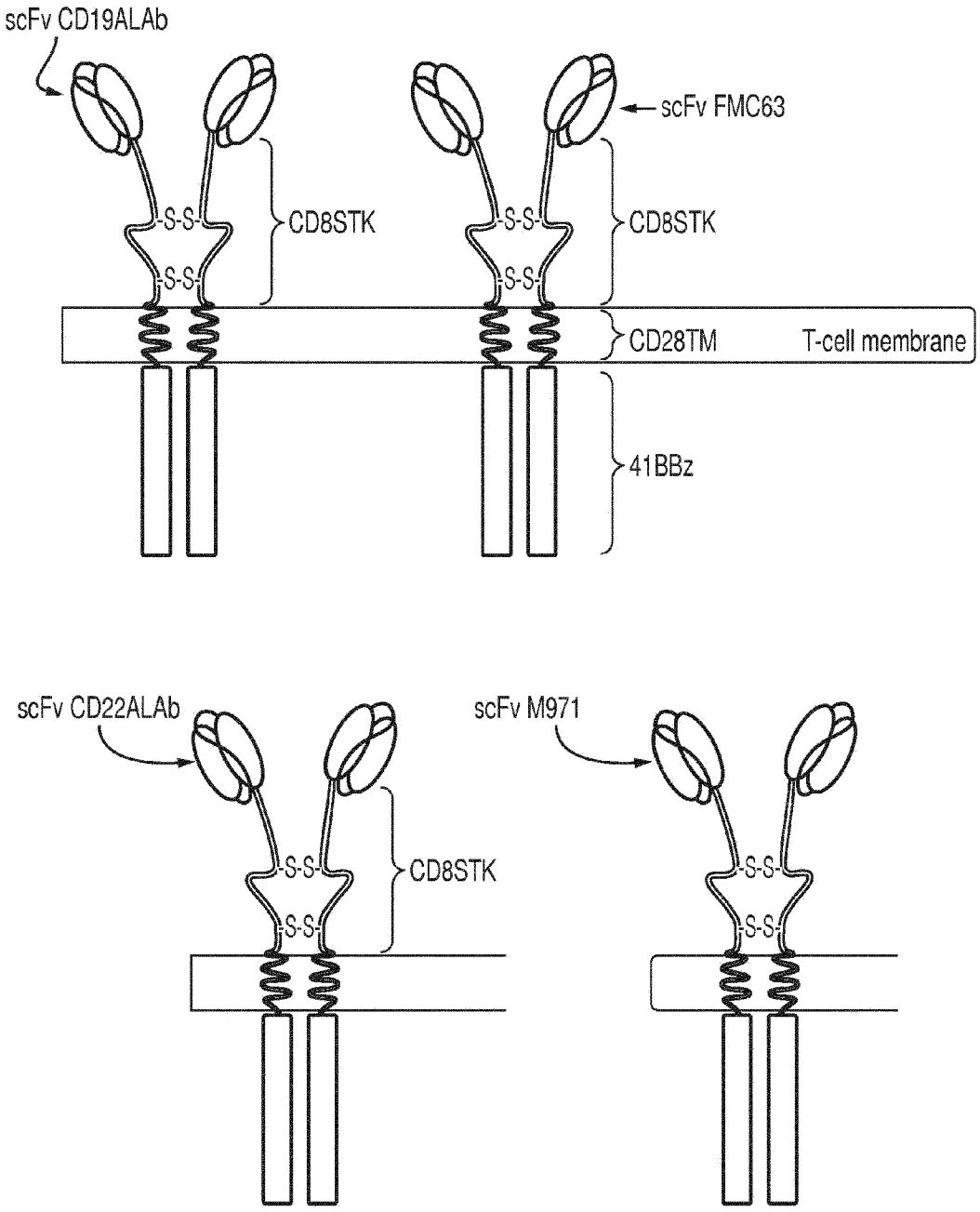

scFv CD19ALAb
scFv FMC63
S-S
S-S
CD8STK
CD8STK
CD28TM
T-cell membrane
41BBz
scFv CD22ALAb
scFv M971
CD8STK

FIG. 9

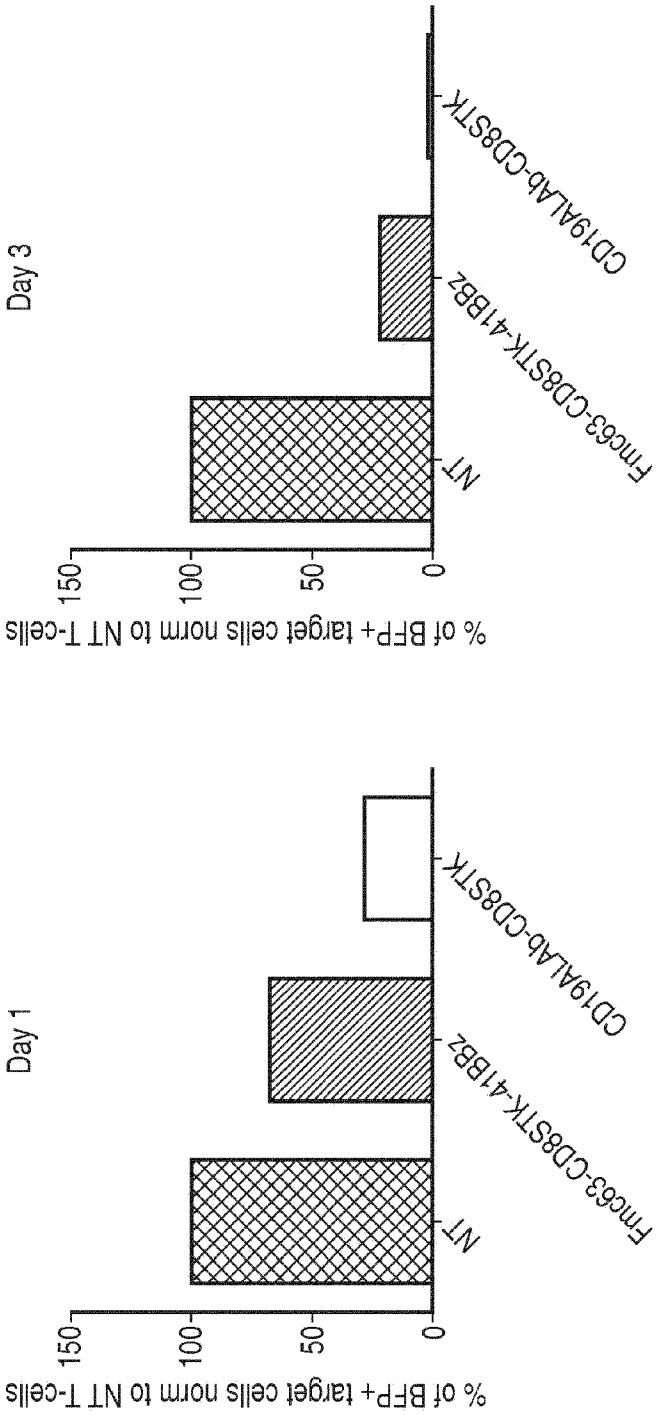

Day 3
% of BFP+ target cells norm to NT T-cells
150
100
50
0
NT
Fmc63-CD8STK-41BBz
CD19ALAb-CD8STK
Day 1
% of BFP+ target cells norm to NT T-cells
150
100
50
0
NT
Fmc63-CD8STK-41BBz
CD19ALAb-CD8STK

FIG. 10

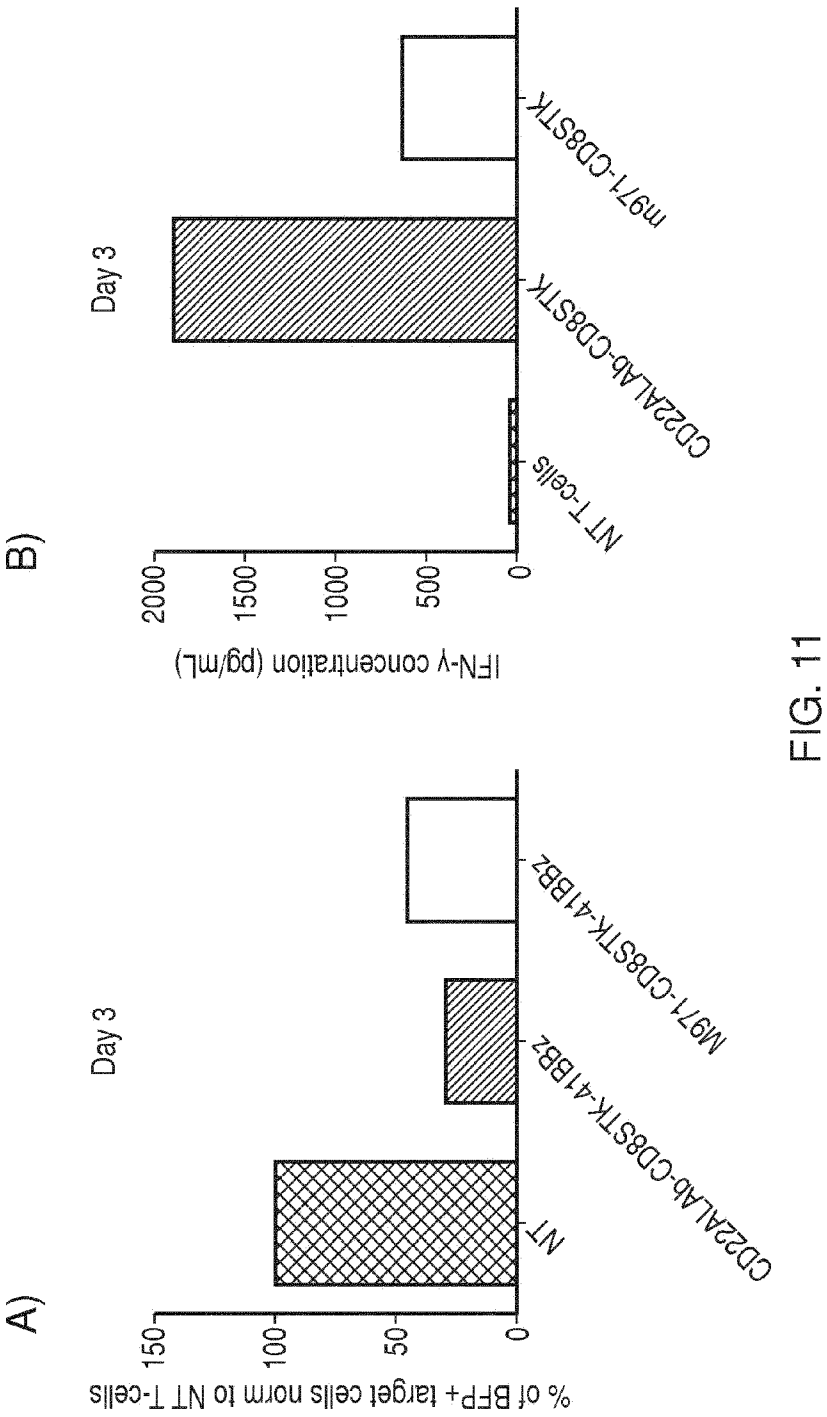

A)
% of BFP+ target cells norm to NT T-cells
150
100
50
0
Day 3
NT
CD22ALAb-CD8STK-41BBz
M971-CD8STK-41BBz
B)
IFN-γ concentration (pg/mL)
2000
1500
1000
500
0
Day 3
NT T-cells
CD22ALAb-CD8STK
m971-CD8STK

FIG. 11

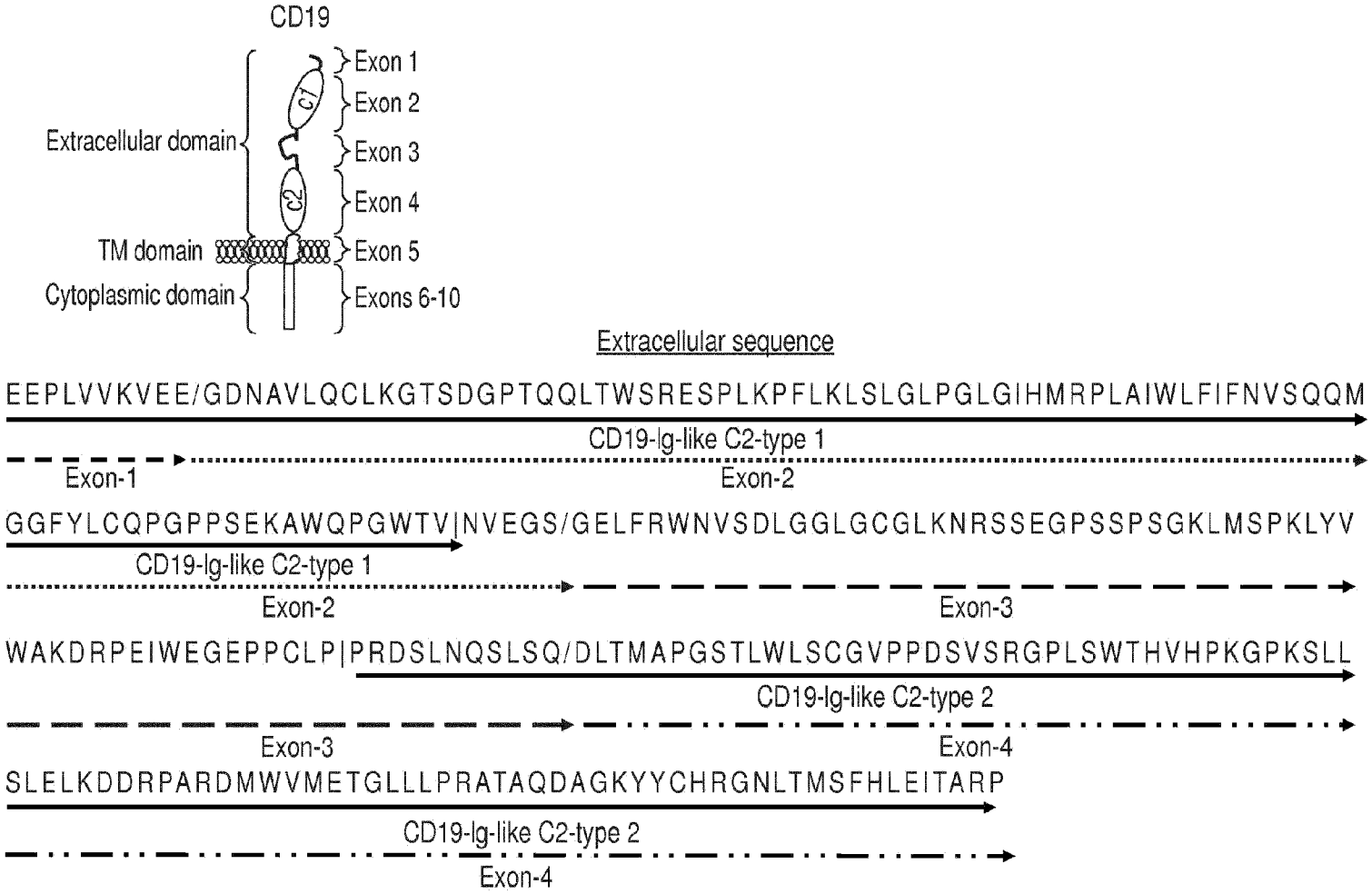


FIG. 12

DK/EP 3560953 T5

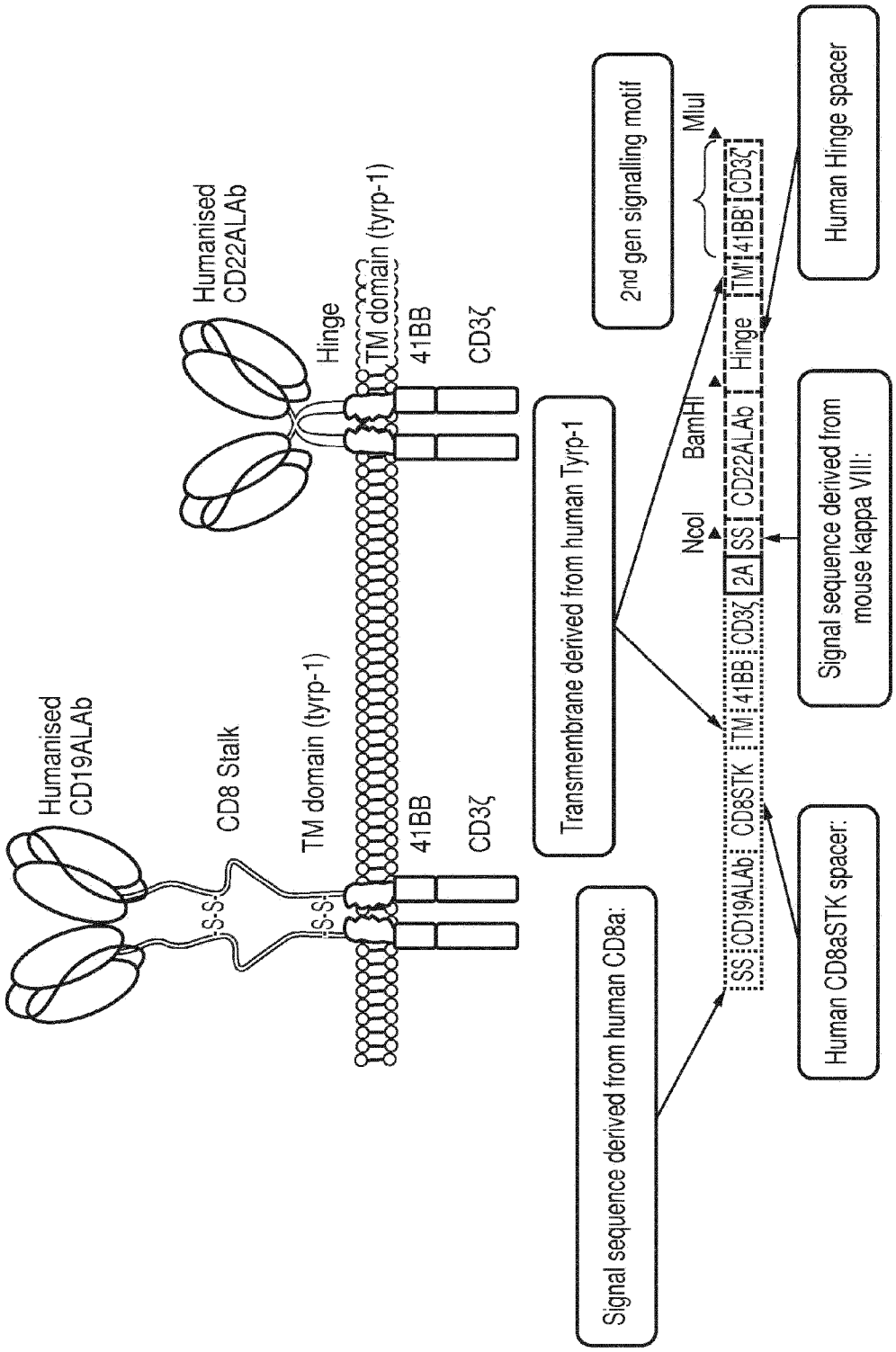

Humanised CD19ALAb
CD8 Stalk
TM domain (tyrp-1)
41BB
CD3ζ
S-S
S-S
Humanised CD22ALAb
Hinge
TM domain (tyrp-1)
41BB
CD3ζ
Transmembrane derived from human Tyrp-1
2nd gen signalling motif
MluI
NcoI
BamHI
SS
CD19ALAb
CD8STK
TM
41BB
CD3ζ
2A
SS
CD22ALAb
Hinge
TM
41BB
CD3ζ
Signal sequence derived from human CD8a:
Human CD8aSTK spacer:
Signal sequence derived from mouse kappa VIII:
Human Hinge spacer

FIG. 13A

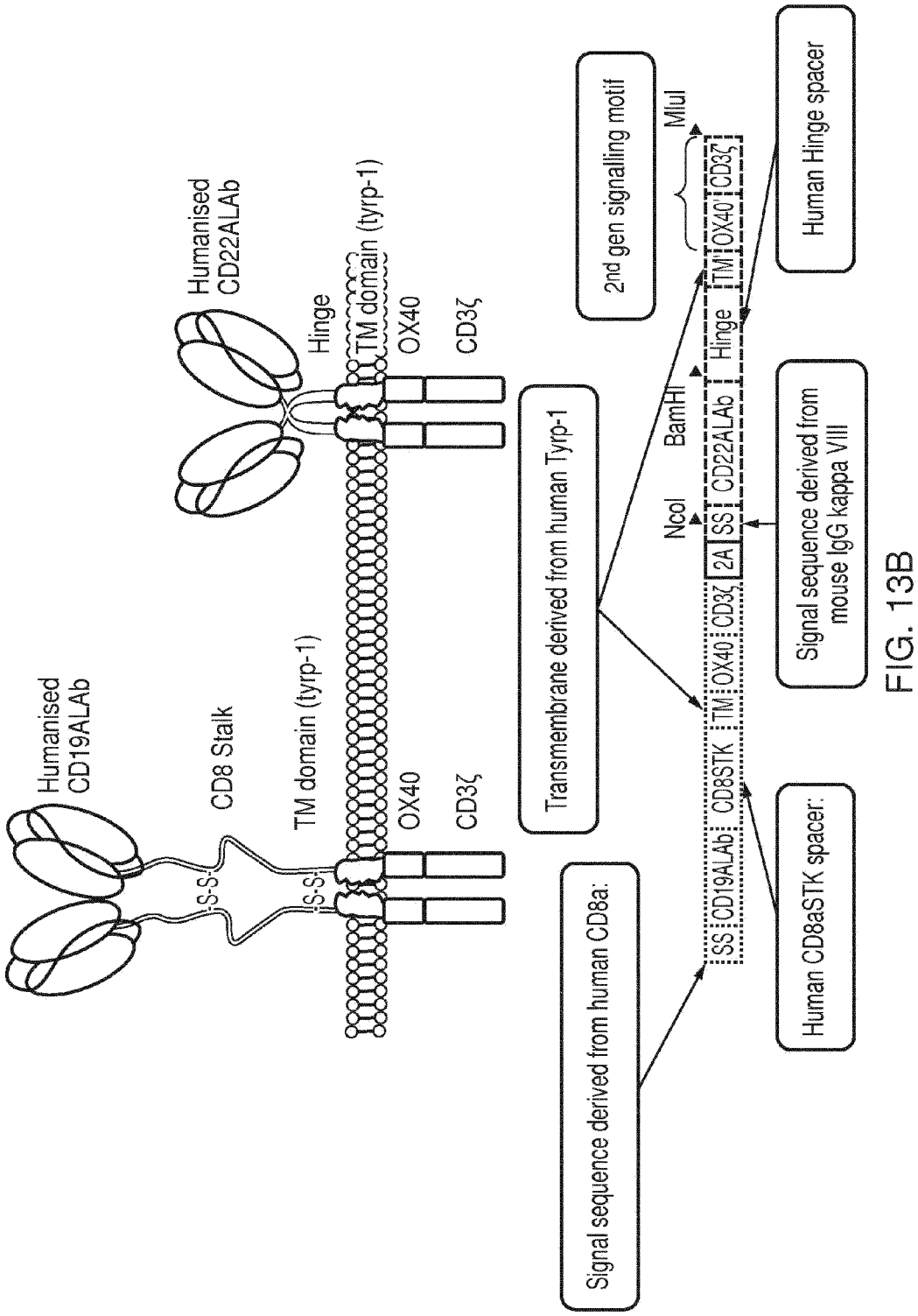

Humanised CD19ALAb
CD8 Stalk
S-S
S-S
TM domain (tyrp-1)
OX40
CD3ζ
Humanised CD22ALAb
Hinge
TM domain (tyrp-1)
OX40
CD3ζ
Transmembrane derived from human Tyrp-1
2nd gen signalling motif
MluI
NcoI
BamHI
SS
CD19ALAb
CD8STK
TM
OX40
CD3ζ
2A
SS
CD22ALAb
Hinge
TM
OX40
CD3ζ
Signal sequence derived from human CD8a:
Human CD8aSTK spacer:
Signal sequence derived from mouse IgG kappa VIII
Human Hinge spacer

FIG. 13B

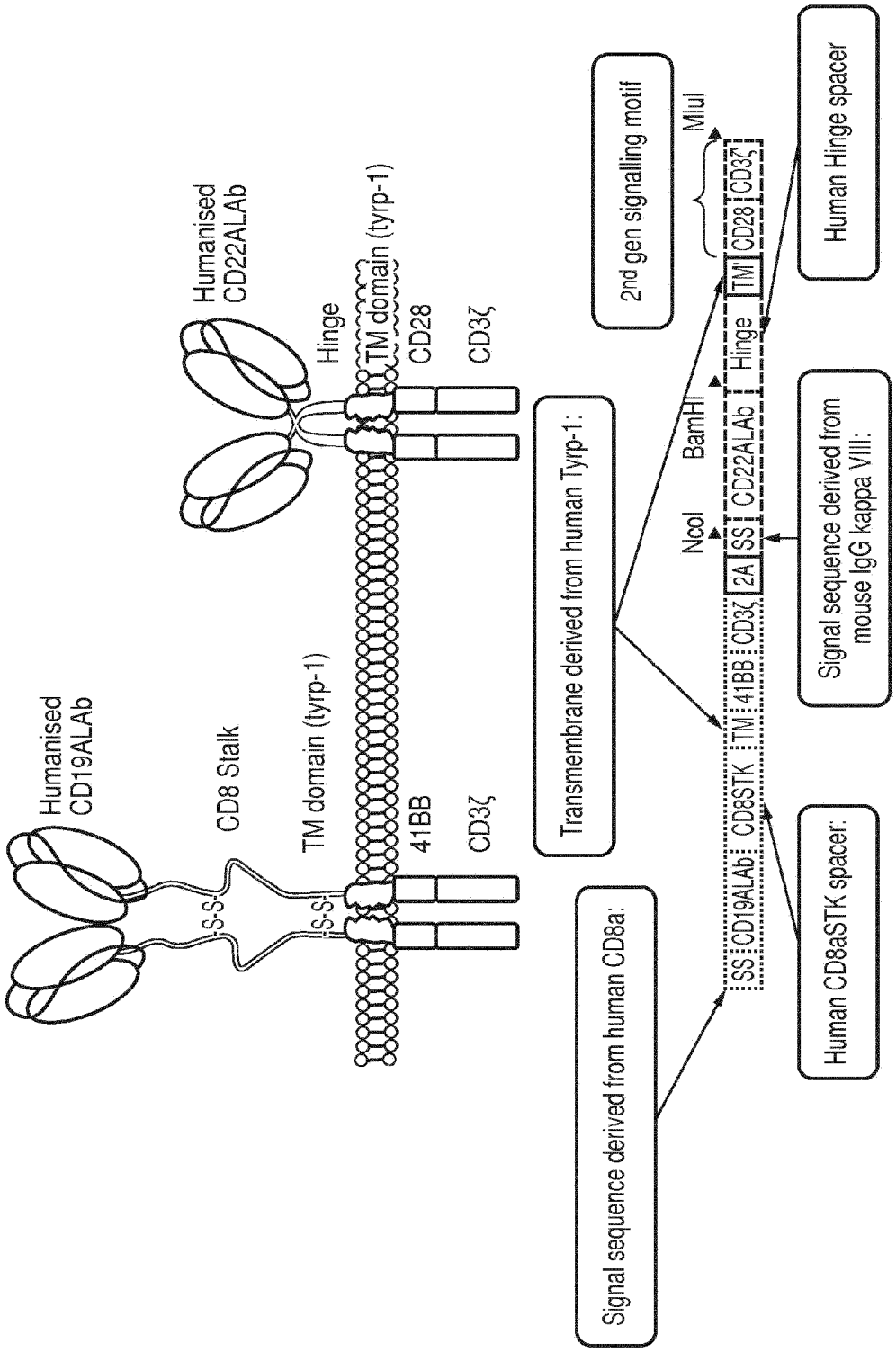

Humanised CD19ALAb
CD8 Stalk
TM domain (tyrp-1)
41BB
CD3ζ
S-S
S-S
Humanised CD22ALAb
Hinge
TM domain (tyrp-1)
CD28
CD3ζ
Transmembrane derived from human Tyrp-1:
Signal sequence derived from human CD8a:
2nd gen signalling motif
MluI
NcoI
BamHI
SS CD19ALAb CD8STK TM 41BB CD3ζ 2A SS CD22ALAb Hinge TM CD28 CD3ζ
Human CD8aSTK spacer:
Signal sequence derived from mouse IgG kappa VIII:
Human Hinge spacer

FIG. 13C

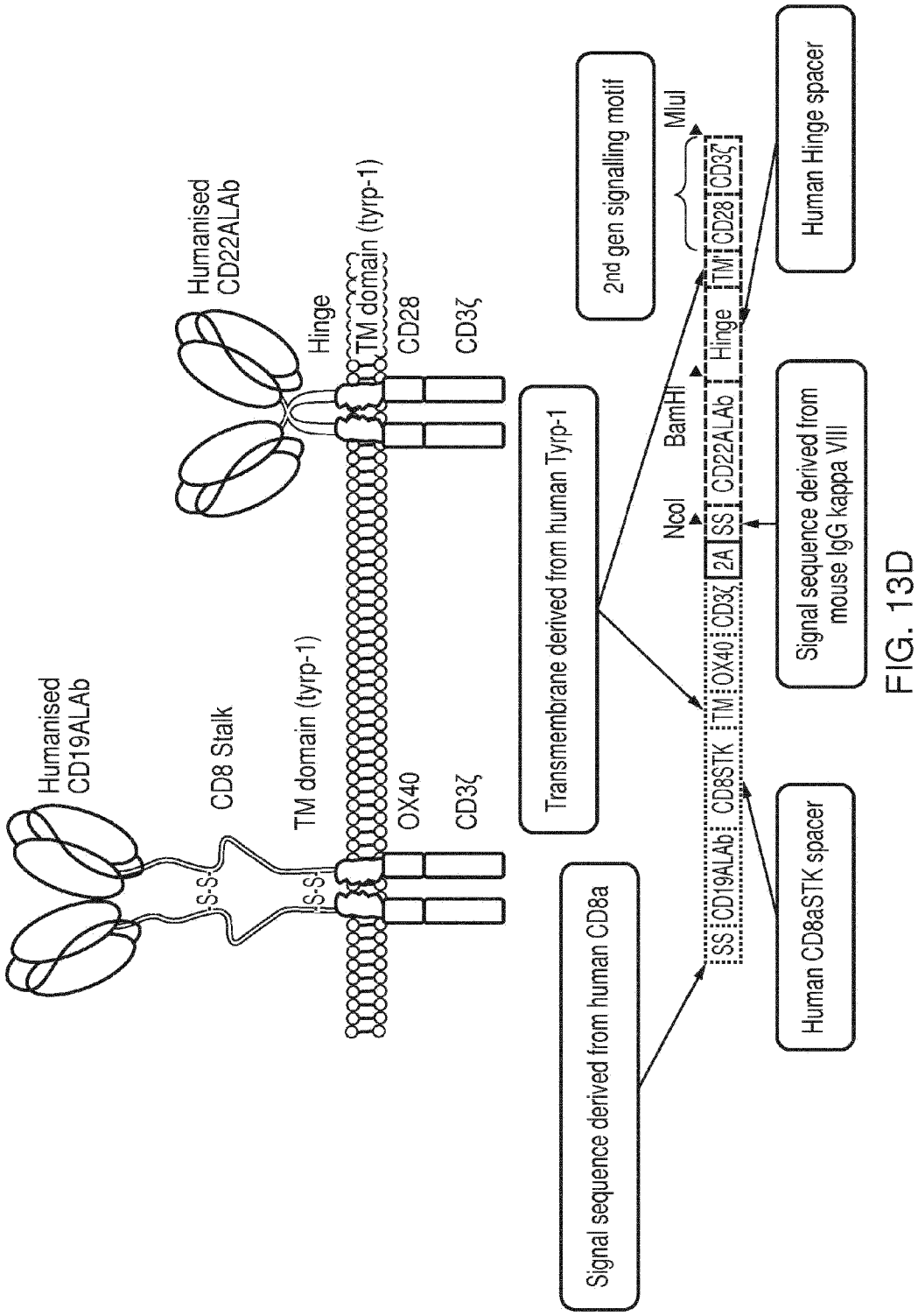

Humanised CD19ALAb
CD8 Stalk
TM domain (tyrp-1)
OX40
CD3ζ
S-S
S-S
Humanised CD22ALAb
Hinge
TM domain (tyrp-1)
CD28
CD3ζ
Transmembrane derived from human Tyrp-1
Signal sequence derived from human CD8a
2nd gen signalling motif
MluI
NcoI
BamHI
SS
CD19ALAb
CD8STK
TM
OX40
CD3ζ
2A
SS
CD22ALAb
Hinge
TM
CD28
CD3ζ
Human Hinge spacer
Signal sequence derived from mouse IgG kappa VIII
Human CD8aSTK spacer

FIG. 13D

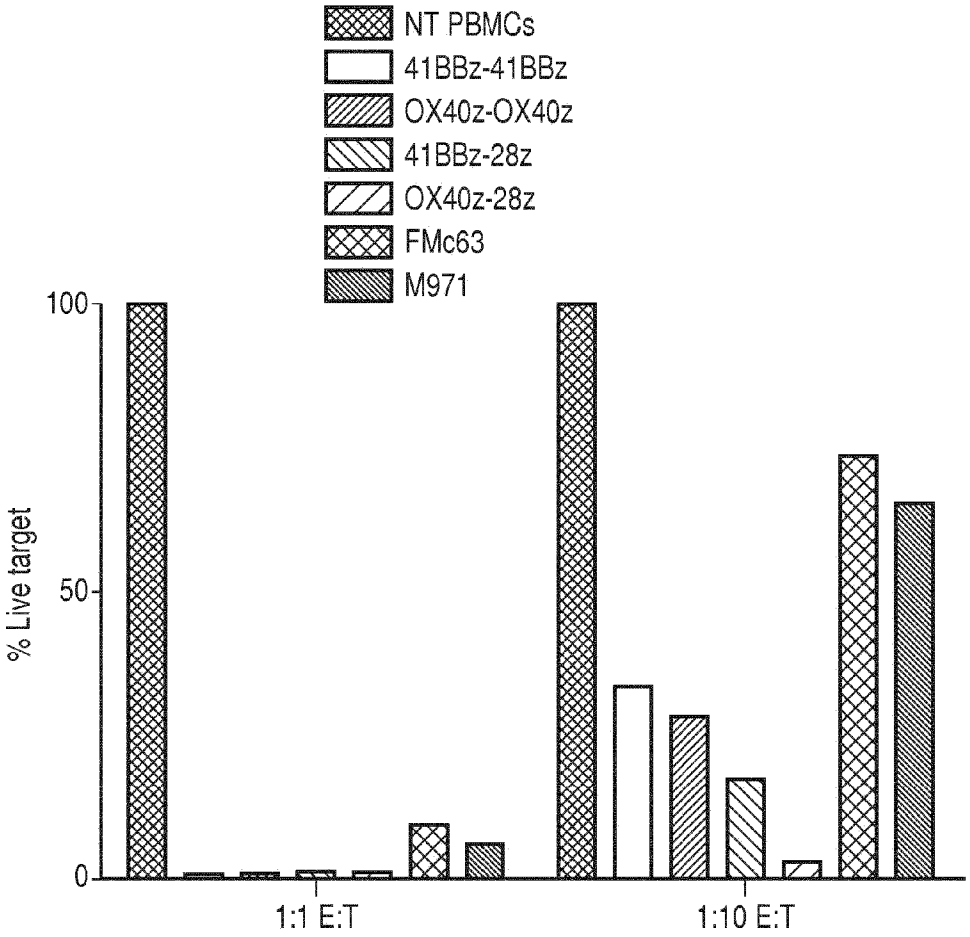

NT PBMCs
41BBz-41BBz
OX40z-OX40z
41BBz-28z
OX40z-28z
FMc63
M971
100
50
0
% Live target
1:1 E:T
1:10 E:T

FIG. 14

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.