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- (71) **Applicant:** GLAXOSMITHKLINE INTELLECTUAL
PROPERTY DEVELOPMENT LIMITED [GB/GB];
980 Great West Road, Brentford Middlesex TW8 9GS
(GB).
- (72) **Inventor:** BATUWANGALA, Thil Dinuk; GlaxoSmithK-
line, Gunnels Wood Road, Stevenage Hertfordshire SG1
2NY (GB).
- (74) **Agent:** O'FARRELL, Damien John; GlaxoSmithKline,
Global Patents CN925.1, 980 Great West Road, Brentford
Middlesex TW8 9GS (GB).
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(54) **Title:** NOVEL CHIMERIC ANTIGEN RECEPTORS

(57) **Abstract:** The invention relates to chimeric antigen receptor (CAR) scaffolds comprising: a target binding domain; a spacer re-
gion; a transmembrane domain; and an intracellular effector domain, wherein the spacer region comprises at least one, or multiples
of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule. The invention also relates to polynucleotides and expression vec-
tors encoding said CAR scaffold and immunomodulatory cells comprising said CAR scaffold. The invention also relates to methods
of engineering an immunomodulatory cell to comprise said CAR scaffold.



NOVEL CHIMERIC ANTIGEN RECEPTORS

FIELD OF THE INVENTION

The invention relates to chimeric antigen receptors comprising a spacer region from a CD4 molecule. The present invention also relates to polynucleotides and vectors encoding said CAR and immunomodulatory cells expressing said CAR at their surface. The present invention also relates to methods for engineering immune cells expressing said CAR at their surface.

BACKGROUND TO THE INVENTION

T cells of the immune system recognize and interact with specific antigens through T cell receptors (TCRs) which, upon recognition or binding with such antigens, causes activation of the cell. TCRs are expressed on the T cell surface and comprise highly variable protein chains (such as alpha (α) and beta (β) chains) which are expressed as part of a complex with CD3 chain molecules. The CD3 chain molecules have an invariant structure and, in particular, the CD3zeta (CD3 ζ) chain is responsible for intracellular signalling upon TCR:antigen binding. The TCRs recognise antigenic peptides that are presented to it by the proteins of the major histocompatibility complex (MHC) which are expressed on the surface of antigen presenting cells and other T cell targets. In natural CD8⁺ T cell activation, antigenic peptides presented by MHC Class I on antigen presenting cells are recognised by the TCR and the TCR:peptide:MHC complex is formed. This forms intercellular membrane contact regions that are defined in width by the physical dimensions of the TCR:peptide:MHC complex. Inhibitory signalling receptors that are too large to fit in this space are excluded allowing triggering of the TCR/CD3 signals to activate cell killing (Choudhuri *et al.* (2005) *Nature* 436 (7050):578-582).

Chimeric antigen receptors (CARs) have been developed as artificial TCRs to generate novel specificities in T cells without the need to bind to MHC-antigenic peptide complexes. These synthetic receptors contain a target binding domain that is associated with one or more signalling domains via a flexible linker in a single fusion molecule. The target binding domain is used to target the T cell to specific targets on the surface of pathologic cells and the signalling domains contain molecular machinery for T cell activation and proliferation. The flexible linker which passes through the T cell membrane (*i.e.* forming a transmembrane domain) allows for cell membrane display of the target binding domain of the CAR. CARs have successfully allowed T cells to be redirected against antigens expressed at the surface of tumour cells from various malignancies including lymphomas and solid tumours (Jena *et al.* (2010) *Blood*, 116(7):1035-44).

The development of CARs has comprised three generations so far. The first generation CARs comprised target binding domains attached to a signalling domain derived from the cytoplasmic region of the CD3zeta or the Fc receptor gamma chains. First generation CARs were shown to successfully redirect T cells to the selected target, however, they failed to provide prolonged

expansion and antitumor activity *in vivo*. The second and third generation CARs have focussed on enhancing modified T cell survival and increasing proliferation by including co-stimulatory molecules, such as CD28, OX-40 (CD134) and 4-1BB (CD137).

T cells bearing CARs could be used to eliminate pathologic cells in a disease setting. One clinical aim would be to transform patient cells with recombinant DNA containing an expression construct for the CAR via a vector (e.g. a lentiviral vector) following aphaeresis and T cell isolation. Following expansion of the T cells they are re-introduced into the patient with the aim of targeting and killing the pathologic target cells.

However, there is still a need in the art to develop the construction of CARs to provide improved characteristics, such as enhanced binding properties. It is therefore an object of the present invention to provide CARs with improved characteristics.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a chimeric antigen receptor (CAR) comprising:

- a target binding domain;
- a spacer region;
- a transmembrane domain; and
- an intracellular effector domain,

wherein the spacer region comprises at least one, or multiples of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule .

According to a further aspect of the invention, there is provided a polynucleotide encoding the chimeric antigen receptor described herein.

According to a further aspect of the invention, there is provided an expression vector comprising the polynucleotide described herein.

According to a further aspect of the invention, there is provided an immunomodulatory cell comprising the chimeric antigen receptor described herein.

According to a further aspect of the invention, there is provided the immunomodulatory cell described herein for use in therapy.

According to a further aspect of the invention, there is provided a method of engineering an immunomodulatory cell, comprising:

- (a) providing an immunomodulatory cell;
- (b) introducing the expression vector described herein into said immunomodulatory cell; and
- (c) expressing said expression vector in the immunomodulatory cell.

According to a further aspect of the invention, there is provided an engineered immunomodulatory cell comprising a chimeric antigen receptor (CAR) which binds to a protein on a target cell, wherein said CAR comprises:

- a target binding domain,
- a spacer domain which comprises at least one, or multiples of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule,
- a transmembrane domain and
- an intracellular effector domain,

wherein the length of the spacer domain is such that the distance between the cell membranes of the target cell and engineered immunomodulatory cell creates an immune synapse.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Modelling of CAR with CD4 spacers. Structures are to scale and rendered as low-resolution globular surfaces. Spheres on scFV and CD4 domains show termini of protein chains where polypeptide chains could fuse. 14nm is the calculated distance between T-cell membrane (solid black line) and target cell membrane (top dotted grey line). In the case of BCMA, based on this modelling it is predicted that a type-2 CAR spacer gives optimal spacing for binding.

FIGURE 2: Cytotoxicity of transduced αBCMA CAR T-cells specific to target expressing cells. A) Gating strategy: gates were drawn around T-cells and target cells and counts used to determine ratios over control. B) Percentage (%) cytotoxicity of CAR T-cells as assessed by flow cytometry. Co-cultured transduced T-cells and target cells were incubated for 24 hours at an effector to target ratio of 1:1. A baseline % cytotoxicity is observed for negative target cells comparable to untransduced (UT) T-cells. All CD4 spacer variants show significant cytotoxic activity over background comparable to the CD8 comparator spacer. Nomenclature to **Figure 1**: 'No spacer' is type-0; 'short spacer' is type-1; 'intermediate spacer' is type-2; 'long spacer' is type-3.

FIGURE 3: Target specific cytokine expression after incubation with αBCMA CAR T-cells. A) Gating strategy to identify cytokine producing cells. B) IFN-γ and IL-2 specific staining, respectively, of target cells over control. Variable levels of cytokine production are possibly dependent on spacer length. Colour coding of bars and nomenclature of constructs as in **Figure 2**.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art (*e.g.*, in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and
10 Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference in their entirety) and chemical methods. All patents and publications referred to herein are incorporated by reference in their entirety.

The term "comprising" encompasses "including" or "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

15 The term "consisting essentially of" limits the scope of the feature to the specified materials or steps and those that do not materially affect the basic characteristic(s) of the claimed feature.

The term "consisting of" excludes the presence of any additional component(s).

The term "about" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, including $\pm 5\%$,
20 $\pm 1\%$, and $\pm 0.1\%$ from the specified value.

The term "chimeric antigen receptors" ("CARs") as used herein, refers to an engineered receptor which consists of an extracellular target binding domain (which is usually derived from a monoclonal antibody or fragment thereof), a spacer region, a transmembrane region, and one or more intracellular effector domains. CARs have also been referred to as chimeric T cell receptors or chimeric
25 immunoreceptors (CIRs). CARs are genetically introduced into hematopoietic cells, such as T cells, to redirect specificity for a desired cell-surface antigen.

The term "target binding domain" as used herein is defined as an oligo- or polypeptide that is capable of binding a specific target, such as an antigen or ligand. In particular, the target may be a cell surface molecule. For example, the target binding domain may be chosen to recognise a target
30 that acts as a cell surface marker on pathogenic cells, including pathogenic human cells, associated with a particular disease state.

The term "spacer region" as used herein, refers to an oligo- or polypeptide that functions to link the transmembrane domain to the target binding domain. This region may also be referred to as a "hinge region" or "stalk region". As explained in more detail herein, the size of the spacer can be
35 varied depending on the position of the target epitope in order to maintain a set distance (*e.g.* 14nm) upon CAR:target binding.

The term "domain" refers to a folded protein structure which retains its tertiary structure independent of the rest of the protein. Generally domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

5 The term "transmembrane domain" as used herein refers to the part of the CAR molecule which traverses the cell membrane.

 The term "intracellular effector domain" (also referred to as the "signalling domain") as used herein refers to the domain in the CAR which is responsible for intracellular signalling following the binding of the target binding domain to the target. The intracellular effector domain is responsible for
10 the activation of at least one of the normal effector functions of the immune cell in which the CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines.

 The term "antibody" is used herein in the broadest sense to refer to molecules with an immunoglobulin-like domain (for example IgG, IgM, IgA, IgD or IgE) and includes monoclonal,
15 recombinant, polyclonal, chimeric, human, humanised, multispecific antibodies, including bispecific antibodies, and heteroconjugate antibodies; a single variable domain (*e.g.*, VH, VHH, VL, domain antibody (dAbTM)), antigen binding antibody fragments, Fab, F(ab')₂, Fv, disulphide linked Fv, single chain Fv, disulphide-linked scFv, diabodies, TANDABSTM, etc. and modified versions of any of the foregoing.

20 The term "single variable domain" refers to a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains such as VH, VHH and VL and modified antibody variable domains, for example, in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as
25 well as folded fragments of variable domains which retain at least the binding activity and specificity of the full-length domain. A single variable domain is capable of binding an antigen or epitope independently of a different variable region or domain. A "domain antibody" or "dAbTM" may be considered the same as a "single variable domain". A single variable domain may be a human single variable domain, but also includes single variable domains from other species such as rodent (for
30 example, as disclosed in WO 00/29004), nurse shark and Camelid VHH dAbsTM. Camelid VHH are immunoglobulin single variable domain polypeptides that are derived from camelid species including bactrian and dromedary camels, llamas, vicuñas, alpacas, and guanacos, which produce heavy chain antibodies naturally devoid of light chains. Such VHH domains may be humanised according to standard techniques available in the art, and such domains are considered to be "single variable
35 domains". As used herein VH includes camelid VHH domains.

 "Affinity" is the strength of binding of one molecule, *e.g.* the target binding protein of the CAR molecule of the invention, to another, *e.g.* its target antigen, at a single binding site. The binding

affinity of an antigen binding protein to its target may be determined by equilibrium methods (e.g. enzyme-linked immunoabsorbent assay (ELISA) or radioimmunoassay (RIA)), or kinetics (e.g. BIACORE™ analysis).

The term "epitope" as used herein refers to that portion of the antigen that makes contact with a particular binding domain, e.g. the target binding domain of the CAR molecule. An epitope may be linear or conformational/discontinuous. A conformational or discontinuous epitope comprises amino acid residues that are separated by other sequences, i.e. not in a continuous sequence in the antigen's primary sequence. Although the residues may be from different regions of the peptide chain, they are in close proximity in the three dimensional structure of the antigen. In the case of multimeric antigens, a conformational or discontinuous epitope may include residues from different peptide chains. Particular residues comprised within an epitope can be determined through computer modelling programs or via three-dimensional structures obtained through methods known in the art, such as X-ray crystallography.

Sequence identity as used herein is the degree of relatedness between two or more amino acid sequences, or two or more nucleic acid sequences, as determined by comparing the sequences. The comparison of sequences and determination of sequence identity may be accomplished using a mathematical algorithm; those skilled in the art will be aware of computer programs available to align two sequences and determine the percent identity between them. The skilled person will appreciate that different algorithms may yield slightly different results.

Thus the "percent identity" between a query nucleic acid sequence and a subject nucleic acid sequence is the "Identities" value, expressed as a percentage, that is calculated by the BLASTN algorithm when a subject nucleic acid sequence has 100% query coverage with a query nucleic acid sequence after a pair-wise BLASTN alignment is performed. Such pair-wise BLASTN alignments between a query nucleic acid sequence and a subject nucleic acid sequence are performed by using the default settings of the BLASTN algorithm available on the National Center for Biotechnology Institute's website with the filter for low complexity regions turned off. Importantly, a query nucleic acid sequence may be described by a nucleic acid sequence identified in one or more claims herein.

Similarly, the "percent identity" between a query amino acid sequence and a subject amino acid sequence is the "Identities" value, expressed as a percentage, that is calculated by the BLASTP algorithm when a subject amino acid sequence has 100% query coverage with a query amino acid sequence after a pair-wise BLASTP alignment is performed. Such pair-wise BLASTP alignments between a query amino acid sequence and a subject amino acid sequence are performed by using the default settings of the BLASTP algorithm available on the National Center for Biotechnology Institute's website with the filter for low complexity regions turned off. Importantly, a query amino acid sequence may be described by an amino acid sequence identified in one or more claims herein.

The query sequence may be 100% identical to the subject sequence, or it may include up to a certain integer number of amino acid or nucleotide alterations as compared to the subject sequence

such that the % identity is less than 100%. For example, the query sequence is at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to the subject sequence. Such alterations include at least one amino acid deletion, substitution (including conservative and non-conservative substitution), or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the query sequence or anywhere between those terminal positions, interspersed either individually among the amino acids or nucleotides in the query sequence or in one or more contiguous groups within the query sequence.

The terms "individual", "subject" and "patient" are used herein interchangeably. In one embodiment, the subject is a mammal, such as a primate, for example a marmoset or monkey, or a human. In a further embodiment, the subject is a human.

The CAR described herein may also be used in methods of treatment of a subject in need thereof. Treatment can be therapeutic, prophylactic or preventative. Treatment encompasses alleviation, reduction, or prevention of at least one aspect or symptom of a disease and encompasses prevention or cure of the diseases described herein.

The CAR described herein is used in an effective amount for therapeutic, prophylactic or preventative treatment. A therapeutically effective amount of the antigen binding protein described herein is an amount effective to ameliorate or reduce one or more symptoms of, or to prevent or cure, the disease.

CHIMERIC ANTIGEN RECEPTORS

The present inventors have developed a CAR scaffold with improved binding properties by introducing a spacer region comprising the domains of a CD4 molecule. In natural CD8⁺ T cell activation, the TCR:peptide:MHC complex which is formed on antigen binding creates intercellular membrane contact regions that are defined in width by the physical dimensions of the TCR:peptide:MHC complex. Inhibitory signalling receptors that are too large to fit in this space are excluded allowing triggering of the TCR:CD3 signals to activate cell killing (see Choudhuri *et al.* (2005) *Nature* 436(7050):578-582). The present inventors have developed a method of designing CARs which takes into account this phenomenon of exclusion of inhibitory receptors by using spacer regions which are designed to mimic the dimensions of the TCR:peptide:MHC complex. For example, if the target epitope for the scFv is close to the target cell membrane then a larger spacer would be required for the scFv to reach it while maintaining the set distance between membranes. Dimensions of the TCR:peptide:MHC complex are such that the distance between membranes of opposing cells would be approximately 14 nm/14 Å (see Wild *et al.* (1999) *J. Exp. Med.*, 190(1):31-41, Garboczi *et al.* (1996) *Nature*, 384:134-141, Garcia *et al.* (1998) *Science*, 279:1166-1172)

Previously, spacers have been investigated using IgG Fc domains (e.g. see WO2014/031687 and Guest *et al.* (2005) *J. Immunother.*, 28(3):203-211, herein incorporated by reference). The problem with IgG Fc domains is that they naturally dimerise and form interactions with other

molecules. The present inventors recognized that a preferred CAR spacer domain would be as inert as possible so that it does not affect the binding ability of the target binding domain of the CAR scaffold. The present invention utilizes CD4 domains 2, 3 and/or 4 as a spacer, as these domains are inert and do not form dimerising interactions with other molecules.

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Therefore, according to a first aspect of the invention there is provided a chimeric antigen receptor (CAR) comprising:

a target binding domain;

a spacer region;

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a transmembrane domain; and

an intracellular effector domain,

wherein the spacer region comprises at least one, or multiples of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule.

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The epitope of an antigen may be positioned proximal (*i.e.* near) to the target cell membrane or distal (*i.e.* far) from the target cell membrane. Another factor which affects the size of spacer to be chosen is the size of the target molecule. As depicted in **Figure 1**, one example is the antigen BCMA (B-cell maturation antigen). This is a small antigen with the target epitope distal from the target cell membrane. Based on the modelling in **Figure 1**, the present inventors predicted that the most effective spacer to use in a BCMA-specific CAR is type-2 (*i.e.* a spacer comprising domains 3 and 4 of a CD4 molecule) because this results in a 14 nm distance between the target and T cell membranes upon CAR:antigen binding.

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Thus, according to the present invention, the size of the spacer is selected based upon the epitope position and/or size of the target antigen. For example, the CEA antigen is relatively large, but the epitope is positioned distal from the target cell membrane, therefore only a short spacer would be needed to improve CAR binding properties. In an alternative example, the NCAM (natural cell adhesion molecule) antigen is also relatively large, but the epitope is positioned proximal to the target cell membrane, therefore a large spacer would be needed to improve CAR binding properties. The size of the spacer selected for use in the CAR can therefore be decided when the target is selected based on the size of the target and position of the epitope. Methods of epitope mapping, in order to determine the position of a target epitope, are well known in the art, such as X-ray co-crystallography, array-based oligopeptide scanning (or pepscan analysis) and site directed mutagenesis.

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The term "CD4" as used herein, refers to a Cluster of Differentiation 4 molecule which is a member of the immunoglobulin superfamily. CD4 is a co-receptor that assists in the TCR:peptide:MHC Class II interaction and has a rigid rod-like structure. It has four immunoglobulin

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domains (domains 1 to 4) that are exposed on the extracellular surface of the cell. CD4 binds MHC Class II via domain 1, whilst domains 2-4 act as a scaffold (Yin *et al.* (2012) *PNAS*, 109(14):5405-5410). The amino acid sequence of CD4 is described in more detail on UniProt, ID number P01730. The term "domain" refers to a folded protein structure which retains its tertiary structure

independent of the rest of the protein. Generally domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

In one embodiment, the spacer region comprises or consists of domain 4 of a CD4 molecule. The spacer region may comprise at least one copy of domain 4 of a CD4 molecule. For example, in a further embodiment, the spacer region comprises or consists of multiple copies of domain 4 of a CD4 molecule (*e.g.* 2, 3 or 4 copies).

In one embodiment, the spacer region comprises or consists of domain 3 of a CD4 molecule. The spacer region may comprise at least one copy of domain 3 of a CD4 molecule. For example, in a further embodiment, the spacer region comprises or consists of multiple copies of domain 3 of a CD4 molecule (*e.g.* 2, 3 or 4 copies).

In one embodiment, the spacer region comprises or consists of domain 2 of a CD4 molecule. The spacer region may comprise at least one copy of domain 2 of a CD4 molecule. For example, in a further embodiment, the spacer region comprises or consists of multiple copies of domain 2 of a CD4 molecule (*e.g.* 2, 3 or 4 copies).

In one embodiment, the spacer region comprises or consists of domains 3 and 4 or combinations thereof of a CD4 molecule. For example, the spacer region may comprise or consist of one copy of each domain 3 and domain 4 of a CD4 molecule, or the spacer region may comprise or consist of one copy of domain 3 and two copies of domain 4 of a CD4 molecule, or vice versa.

In one embodiment, the spacer region comprises or consists of domains 2, 3 and 4 or combinations thereof of a CD4 molecule. In a further embodiment, the spacer region comprises or consists of domains 2 and 3 and two copies of domain 4 of a CD4 molecule.

In one embodiment, the spacer domain comprises or consists of an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with an amino acid sequence selected from the group consisting of: SEQ ID NOs 2, 3 and 4. In a further embodiment, the spacer region comprises or consists of an amino acid sequence selected from the group consisting of: SEQ ID NOs 2, 3 and 4.

The advantage of using CD4 domains is that they are easy to manipulate in order to create a spacer of the desired size depending on the epitope position and/or size of the target. Furthermore, domains 2, 3 and 4 of CD4 are relatively inert which makes them ideal for use as a spacer because they would not affect the target binding domain of the CAR molecule. The flexibility or rigidity of the spacer can also be tailored by using domains 2, 3 and 4 of CD4. Naturally, the connection between domains 2 and 3 of CD4 is more flexible than the connection between domains 3 and 4. Therefore,

if a more flexible CAR scaffold is required, then domains 2 and 3 of CD4 can be used, whereas if the CAR scaffold is required to be more rigid then domains 3 and 4 of CD4 can be used.

The boundaries of the CD4 domains are disclosed in more detail herein, however it will be understood by a person skilled in the art that the CD4 domains may be as defined by any domain databases, such as Uniprot or Interpro.

The CD4 molecule also contains domain 1 which binds MHC Class II, therefore it will be understood that this domain is not suitable for use as a spacer according to the present invention because the CAR molecule is not required to interact with an MHC molecule. Therefore, in one embodiment, the spacer region does not comprise domain 1 of a CD4 molecule. In one embodiment, domain 1 of a CD4 molecule starts at any one of amino acids 20 to 31 (*i.e.* amino acid 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31) of SEQ ID NO: 1 and ends at any one of amino acids 116 to 125 (*i.e.* amino acid 116, 117, 118, 119, 120, 121, 122, 123, 124 or 125) of SEQ ID NO: 1. In one embodiment, domain 1 of a CD4 molecule comprises amino acids 31 to 116 of SEQ ID NO: 1. In a further embodiment, domain 1 of a CD4 molecule comprises amino acids 26 to 125 of SEQ ID NO: 1. In an alternative embodiment, domain 1 of a CD4 molecule comprises amino acids 20 to 120 of SEQ ID NO: 1.

In one embodiment, domain 2 of a CD4 molecule starts at any one of amino acids 123 to 126 (*i.e.* amino acid 123, 124, 125 or 126) of SEQ ID NO: 1 and ends at any one of amino acids 197 to 203 (*i.e.* amino acid 197, 198, 199, 200, 201, 202 or 203) of SEQ ID NO: 1. In a further embodiment, domain 2 of a CD4 molecule comprises amino acids 126 to 197 of SEQ ID NO: 1. In a yet further embodiment, domain 2 of a CD4 molecule comprises amino acids 123 to 201 of SEQ ID NO: 1. In an alternative embodiment, domain 2 of a CD4 molecule comprises amino acids 126 to 203 of SEQ ID NO: 1. In another alternative embodiment, domain 2 of a CD4 molecule comprises amino acids 125 to 203 of SEQ ID NO: 1. In one embodiment, domain 3 of a CD4 molecule starts at any one of amino acids 202 to 208 (*i.e.* amino acids 202, 203, 204, 205, 206, 207 or 208) of SEQ ID NO: 1 and ends at amino acid 316 or 317 of SEQ ID NO: 1. In a further embodiment, domain 3 of a CD4 molecule comprises amino acids 208 to 316 of SEQ ID NO: 1. In a yet further embodiment, domain 3 of a CD4 molecule comprises amino acids 202 to 317 of SEQ ID NO: 1. In an alternative embodiment, domain 3 of a CD4 molecule comprises amino acids 204 to 316 of SEQ ID NO: 1.

In one embodiment, domain 4 of a CD4 molecule starts at any one of amino acids 315 to 318 (*i.e.* amino acids 315, 316, 317 or 318) of SEQ ID NO: 1 and ends at any one of amino acids 374 to 396 (*e.g.* 386 or 388) of SEQ ID NO: 1. In a further embodiment, domain 4 of a CD4 molecule comprises amino acids 318 to 374 of SEQ ID NO: 1. In a yet further embodiment, domain 4 of a CD4 molecule comprises amino acids 318 to 396 of SEQ ID NO: 1. In an alternative embodiment, domain 4 of the CD4 molecule comprises amino acids 318 to 388 of SEQ ID NO: 1. In another alternative embodiment, domain 4 of a CD4 molecule comprises amino acids 315 to 386 of SEQ ID NO: 1.

It will be understood that the sequences described herein may further comprise sequences to aid with cloning and expression of the CD4 domains. For example, amino acid sequences such as "FGL", "SVRS" or "LA" can be added to the synthesised domain to aid with cloning. Furthermore, the sequences of the domains as defined herein are based upon data available on the UniProt protein database, however it would be understood that the domain boundaries are not restricted to only those as defined on this database.

The target binding domain binds to a target, wherein the target is a tumour specific molecule, viral molecule, or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte. In one embodiment, the target binding domain comprises an antibody, an antigen binding fragment or a ligand. In one embodiment, the target binding domain comprises an antibody or fragment thereof. In one embodiment, the target binding domain is a ligand. In an alternative embodiment, the target binding domain is an antigen binding fragment. In a further embodiment, the antigen binding fragment is a single chain variable fragment (scFv) or a dAbTM. In a yet further embodiment, said scFv comprises the light (VL) and the heavy (VH) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker. In one embodiment, the target binding domain may bind to more than one target, for example two different targets. Such a target binding domain may be derived from a bispecific single chain antibody. For example, Blinatumomab (also known as AMG 103 or MT103) is a recombinant CD19 and CD3 bispecific scFv antibody consisting of four immunoglobulin variable domains assembled into a single polypeptide chain. Two of the variable domains form the binding site for CD19 which is a cell surface antigen expressed on most normal and malignant B cells. The other two variable domains form the binding site for CD3 which is part of the T cell-receptor complex on T cells. These variable domains may be arranged in the CAR in tandem, *i.e.* two single chain antibody variable fragments (scFv) tethered to a spacer, and transmembrane and signaling domains. The four variable domains can be arranged in any particular order within the CAR molecule (*e.g.* VL(first target)-VH(first target)- VH(second target)-VL(second target) or VL(second target)-VH(second target)- VH(first target)-VL(first target) etc.).

In one embodiment, the target binding domain and/or spacer domain may comprise a multimerization domain(s), for example as described in WO2015/017214. This enables the signal transduction of the CAR to be controlled through the addition of external agents, such as a chemical drug, which acts a bridging factor between the multimerization domains. Therefore, in one embodiment, the target binding domain and/or spacer domain comprises (a) a first multimerization domain; and (b) a second multimerization domain; wherein a first bridging factor promotes the formation of a polypeptide complex with the bridging factor associated with and disposed between the first and second multimerization domains.

The target binding domain may bind a variety of cell surface antigens, but in one embodiment, the target binding domain binds to a tumour associated antigen. In a further embodiment, the tumor associated antigen is selected from: BCMA, CD19, HER2, prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), carcinoembryonic antigen (CEA), cancer antigen-125, CA19-9, MUC-1, tyrosinase, CD34, CD45, CD117, protein melan-A, synaptophysin, CD22, CD27, CD30, CD70, ganglioside G2 (GD2), epidermal growth factor variant III (EGFRvIII), mesothelin, prostatic acid phosphatase (PAP), prostatein, TARP, Trp-p8 or six transmembrane epithelial antigen of the prostate I (STEAP1). In a yet further embodiment, the tumour associated antigen is BCMA.

In one embodiment, the target binding domain has a binding affinity of less than about 500 nanomolar (nM), such as less than about 400 nM, 350 nM, 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, 90 nM, 80 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 9 nM, 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.5 nM or 0.25 nM. In one embodiment, the target binding domain has a binding affinity of about 10 nM to about 0.25 nM. In a further embodiment, the target binding domain has a binding affinity of about 1 nM to about 0.5 nM (*i.e.* about 1000 pM to about 500 pM).

In one embodiment, the transmembrane domain can be derived either from a natural or from a synthetic source. In one embodiment, the transmembrane domain can be derived from any membrane-bound or transmembrane protein. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

For example, the transmembrane domain can be the transmembrane domain of CD proteins, such as CD4, CD8, CD3 or CD28, a subunit of the T cell receptor, such as α , β , γ or δ , a subunit of the IL-2 receptor (α chain), a subunit of the Low-Affinity Nerve Growth Factor Receptor (LNGFR or p75) (β chain or γ chain), or a subunit chain of Fc receptors. In one embodiment, the transmembrane domain comprises the transmembrane domain of CD4, CD8 or CD28. In a further embodiment, the transmembrane domain comprises the transmembrane domain of CD4 or CD8 (*e.g.* the CD8 alpha chain, as described in NCBI Reference Sequence: NP_001139345.1, incorporated herein by reference). In a yet further embodiment, the transmembrane domain comprises the transmembrane domain of CD4. The advantage of this embodiment is that the CD4 transmembrane domain is joined to the CD4 spacer domains, therefore this avoids using an unnatural junction and the CAR molecule is easier to construct. This is particularly advantageous over the prior art which describes using IgG domains as the spacer because these domains would not normally be linked to a transmembrane domain therefore they are forced into an unnatural junction which may affect the ability of the CAR scaffold to bind to a target.

In one embodiment, the transmembrane domain of the CD4 molecule comprises amino acids 397 to 418 of SEQ ID NO: 1. In a further embodiment, the transmembrane domain of the CD4 molecule comprises a sequence which starts at any one of amino acids 375 to 397 (*e.g.* 389) of SEQ ID NO: 1 and ends at amino acid 418 of SEQ ID NO: 1.

In one embodiment, the transmembrane domain comprises an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with an amino acid sequence of SEQ ID NO: 5. In a further embodiment, the transmembrane region comprises an amino acid sequence of SEQ ID NO: 5.

Preferred examples of the effector domain for use in a CAR scaffold can be the cytoplasmic sequences of the natural T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen binding, as well as any derivate or variant of these sequences and any synthetic sequence that has the same functional capability. Effector domains can be separated into two classes: those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or costimulatory signal. Primary activation effector domains can comprise signalling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs are well defined signalling motifs, commonly found in the intracytoplasmic tail of a variety of receptors, and serve as binding sites for syk/zap70 class tyrosine kinases. Examples of ITAMs used in the invention can include, as non limiting examples, those derived from CD3zeta, FcRgamma, FcRbeta, FcRepsilon, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b and CD66d. In one embodiment, the intracellular effector domain comprises a CD3zeta signalling domain (also known as CD247). Natural TCRs contain a CD3zeta signalling molecule, therefore the use of this effector domain is closest to the TCR construct which occurs in nature.

In one embodiment, the intracellular effector domain of the CAR comprises a CD3zeta signalling domain which has an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with SEQ ID NO: 7. In a further embodiment, the intracellular effector domain of the CAR comprises a CD3zeta signalling domain which comprises an amino acid sequence of SEQ ID NO: 7.

As described herein, effector domains may also provide a secondary or costimulatory signal. T cells additionally comprise costimulatory molecules which bind to cognate costimulatory ligands on antigen presenting cells in order to enhance the T cell response, for example by increasing proliferation activation, differentiation and the like. Therefore, in one embodiment, the intracellular effector domain additionally comprises a costimulatory domain. In a further embodiment, the costimulatory domain comprises the intracellular domain of a costimulatory molecule, selected from CD28, CD27, 4-1BB (CD137), OX40 (CD134), ICOS (CD278), CD30, CD40, PD-1 (CD279), CD2, CD7, NKG2C (CD94), B7-H3 (CD276) or any combination thereof. In a yet further embodiment, the costimulatory domain comprises the intracellular domain of a costimulatory molecule, selected from CD28, CD27, 4-1BB, OX40, ICOS or any combination thereof.

In one embodiment, the intracellular effector domain additionally comprises a CD28 intracellular domain which has an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with SEQ ID NO: 6. In a

further embodiment, the intracellular effector domain additionally comprises a CD28 intracellular domain which comprises an amino acid sequence of SEQ ID NO: 6.

5 POLYNUCLEOTIDES AND EXPRESSION VECTORS

According to a further aspect of the invention, there is provided a polynucleotide encoding the chimeric antigen receptor described herein.

The polynucleotide may be present in an expression cassette or expression vector (*e.g.* a plasmid for introduction into a bacterial host cell, or a viral vector such as a lentivirus for
10 transfection of a mammalian host cell). Therefore, according to a further aspect of the invention, there is provided an expression vector comprising the polynucleotide described herein.

The term "vector" refers to a vehicle which is able to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed. In one embodiment, the expression vector is a retroviral vector. In a further embodiment, the retroviral vector is derived
15 from, or selected from, a lentivirus, alpha-retrovirus, gamma-retrovirus or foamy-retrovirus, such as a lentivirus or gamma-retrovirus, in particular a lentivirus. In a further embodiment, the retroviral vector particle is a lentivirus selected from the group consisting of HIV-1, HIV-2, SIV, FIV, EIAV and Visna. Lentiviruses are able to infect non-dividing (*i.e.* quiescent) cells which makes them attractive vectors for gene therapy. In a yet further embodiment, the retroviral vector particle is HIV-1 or is
20 derived from HIV-1. The genomic structure of some retroviruses may be found in the art. For example, details on HIV-1 may be found from the NCBI Genbank (Genome Accession No. AF033819). HIV-1 is one of the best understood retroviruses and is therefore often used as a viral vector.

25 IMMUNOMODULATORY CELLS

According to a further aspect of the invention, there is provided an immunomodulatory cell comprising the chimeric antigen receptor described herein. In one embodiment, the immunomodulatory cell may be a human immunomodulatory cell.

The term "immunomodulatory cell" refers to a cell of hematopoietic origin functionally
30 involved in the modulation (*e.g.* the initiation and/or execution) of the innate and/or adaptive immune response. Said immunomodulatory cell according to the present invention can be derived from a stem cell. The stem cells can be adult stem cells, non-human embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow stem cells, induced pluripotent stem cells, totipotent stem cells or hematopoietic stem cells. Said
35 immunomodulatory cell can also be a dendritic cell, a killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T-cell. The T-cell may be selected from the group consisting of inflammatory T-

lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes, or a combination thereof. Therefore, in one embodiment, the immunomodulatory cell is derived from an inflammatory T-lymphocyte, cytotoxic T-lymphocyte, regulatory T-lymphocyte or helper T-lymphocyte. In another embodiment, said cell can be derived from the group consisting of CD4⁺ T-lymphocytes and CD8⁺ T-lymphocytes.

Prior to expansion and genetic modification of the cells of the invention, a source of cells can be obtained from a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments of the present invention, any number of T cell lines available and known to those skilled in the art, may be used. In another embodiment, said cell can be derived from a healthy donor or a diseased donor, such as a patient diagnosed with cancer or an infection. In another embodiment, said cell is part of a mixed population of cells which present different phenotypic characteristics.

It will be understood that the immunomodulatory cells may express the chimeric antigen receptor described herein transiently or stably/permanently (depending on the transfection method used and whether the polynucleotide encoding the chimeric antigen receptor has integrated into the immunomodulatory cell genome or not).

USES

According to a further aspect of the invention, there is provided the immunomodulatory cell described herein for use in therapy. In one embodiment, therapy comprises administration of the immunomodulatory cell to a human subject in need of such therapy.

In one embodiment, the therapy is adoptive cellular therapy. "Adoptive cellular therapy" (or "adoptive immunotherapy") refers to the adoptive transfer of human T lymphocytes that are engineered by gene transfer to express CARs (such as the CARs of the present invention) specific for surface molecules expressed on target cells. This can be used to treat a range of diseases depending upon the target chosen, *e.g.* tumour specific antigens to treat cancer. Adoptive cellular therapy involves removing a portion of the patient's white blood cells using a process called leukapheresis. The T cells may then be expanded and mixed with expression vectors comprising the CAR polynucleotide in order to permanently transfer the CAR scaffold to the T cells. The T cells are expanded again and at the end of the expansion, the T cells are washed, concentrated, and then frozen to allow time for testing, shipping and storage until the patient is ready to receive the infusion of engineered T cells.

METHODS

According to a further aspect of the invention, there is provided a method of engineering an immunomodulatory cell, comprising:

- (a) providing an immunomodulatory cell;
- (b) introducing the expression vector described herein into said immunomodulatory cell; and
- (c) expressing said expression vector in the immunomodulatory cell.

As a non-limiting example, the CAR can be introduced as transgenes encoded by an expression vector as described herein. The expression vector can also contain a selection marker which provides for identification and/or selection of cells which received said vector.

Polypeptides may be synthesized *in situ* in the cell as a result of the introduction of polynucleotides encoding said CAR into the cell. Alternatively, said polypeptides could be produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into cells are known in the art and including, as non limiting examples, stable transformation methods wherein the polynucleotide construct is integrated into the genome of the cell or transient transformation methods wherein the polynucleotide construct is not integrated into the genome of the cell and virus mediated methods. Said polynucleotides may be introduced into a cell by, for example, recombinant viral vectors (*e.g.* retroviruses, adenoviruses), liposomes and the like. For example, transient transformation methods include for example microinjection, electroporation or particle bombardment. The polynucleotides may be included in vectors, more particularly plasmids or viruses, in view of being expressed in cells.

The terms "transfection", "transformation" and "transduction" as used herein, may be used to describe the insertion of the expression vector into the target cell. Insertion of a vector is usually called transformation for bacterial cells and transfection for eukaryotic cells, although insertion of a viral vector may also be called transduction. The skilled person will also be aware of the different non-viral transfection methods commonly used, which include, but are not limited to, the use of physical methods (*e.g.* electroporation, cell squeezing, sonoporation, optical transfection, protoplast fusion, impalefection, magnetofection, gene gun or particle bombardment), chemical reagents (*e.g.* calcium phosphate, highly branched organic compounds or cationic polymers) or cationic lipids (*e.g.* lipofection). Many transfection methods require the contact of solutions of plasmid DNA to the cells, which are then grown and selected for a marker gene expression.

Once the CAR has been introduced into the immunomodulatory cell, said cell may be referred to as a "transformed immunomodulatory cell". Therefore, in the scope of the present invention is also encompassed a cell line obtained from a transformed immunomodulatory cell according to the method previously described.

According to a further aspect of the invention, there is provided an engineered immunomodulatory cell comprising a chimeric antigen receptor (CAR) which binds to a protein on a target cell, wherein said CAR comprises:

a target binding domain,

a spacer domain which comprises at least one, or multiples of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule,

a transmembrane domain and

an intracellular effector domain,

wherein the length of the spacer domain is such that the distance between the cell membranes of the target cell and engineered immunomodulatory cell creates an immune synapse.

The term "immune synapse" or "immunological synapse" refers to any stable, flattened interface between a lymphocyte or natural killer (NK) cell and a cell that they are in the process of recognising (as described in more detail in Huppa and Davis (2003) *Nat. Immunol.* 3, 973-983; Davis and van der Merwe (2006) *Nat. Immunol.* 7(8), 803-809; Rossy et al. (2012) *Front. Immun.* 3, 352, all of which are herein incorporated herein by reference in their entirety).

As explained herein, in natural CD8⁺ T cell and MHC Class I binding, an intercellular membrane contact region is formed which is defined in width by the physical dimensions of the TCR:antigen:MHC complex. Any inhibitory signals which are too large for this space are excluded which allows the TCR signals to activate cell killing. In one embodiment, the distance between the cells membranes is about 14 nm (or about 14 Å). This distance has been shown to be the dimensions of the natural TCR:peptide:MHC complex, therefore without being bound by theory, this is thought to be the optimum distance for creating an effective immune synapse.

EXAMPLES

EXAMPLE 1: Construction of CAR (chimeric antigen receptor) containing different extracellular linker length:

The generic CAR architecture investigated here comprises the target-specific scFv, variable length CD4 spacers (SEQ ID NOs: 2, 3 and 4), CD4 transmembrane domain (SEQ ID NO: 5), CD28 intracellular domain (SEQ ID NO: 6) and CD3zeta (CD3ζ) signaling domain (SEQ ID NO: 7).

The entire CAR construct is constructed allowing for the insertion of different CD4 spacer domains (SEQ ID NOs: 2, 3 and 4) as synthesised DNA-fragments by incorporating appropriate restriction sites in the CAR and DNA sequences. Standard molecular biology protocols are followed to PCR amplify, restriction enzyme digest, purify and ligate DNA fragments into expression vectors.

TABLE 1: Details of sequences used in CAR construct

SEQ ID NO.	Description	Sequence
2	CD4 Domain 4	RATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKREKAVWVLNPEAGMWQCCLSDSGQVLLESNIKVLP
3	CD4 Domain 3 and Domain 4	LAFQKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSKSWITFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSGNLTALAEAKTGKLGHEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKREKAVWVLNPEAGMWQCCLSDSGQVLLESNIKVLP
4	CD4 Domain 2, Domain 3, Domain 4	FGLTANS DTHLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVVLA FQKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKSWITFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSGNLTALAEAKTGKLGHEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKREKAVWVLNPEAGMWQCCLSDSGQVLLESNIKVLP
5	CD4 transmembrane domain	TWSTPVQPMALIVLGGVAGLLLFIGLGIFFSVRS
6	CD28 intracellular domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS
7	CD3 ζ signalling domain	RVKF SR SADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

EXAMPLE 2: Confirmation of antigen binding of scFv using SPR / BIAcore:

Soluble scFv fragments produced and purified from mammalian expression systems are subjected to *in vitro* affinity determination to their antigen. A dilution series of scFv protein in HBS-EP buffer is injected over a BIAcore T200 chip surface previously coated with the antigen at an appropriate 'Response Unit Density' and the sensogram recorded. Analysis of the binding kinetics is assisted by the proprietary software using an appropriate fitting model (mostly 1:1 binding). Affinity data can be used to confirm suitability of scFv fragments to be used in the CAR construct.

EXAMPLE 3: Expression of CAR on T-cells:

In brief, host T cells are transfected or transduced with the appropriate CAR construct using standard protocols known in the art. Mammalian expression vectors may be used for transient cell surface expression or retroviral vector transduction may be used for stably inserted CARs.

EXAMPLE 4: Determination of antigen binding of a CAR when expressed on a cell surface:

Affinity of scFvs in the context of CARs expressed on T-cells are determined by a receptor binding assay. Here, the fraction of soluble antigen bound to the CAR is determined over a range of increasing concentrations. The fraction bound is measured using flow cytometry and plotted against the concentration used providing the IC_{50%} (inhibitory concentration). The cytometer values are normalised for receptor numbers on T-cells by using Bangs Beads (Bangs Laboratories, Inc., Fishers, Indiana) coated with an anti-scFv detection mAb following standard protocols. The results from this assay are used to provide confidence that the signalling/T-cell stimulation originates from specific antigen binding.

EXAMPLE 5: Functional cell assay of target binding in the context of an 'Immune Synapse':

The ability of the different CAR-T constructs after transduction of T-cells is measured by using a commercially available reporter cell line (Promega Immunostimulatory Bioassay T-cell activation bioassay (IL-2; cat# CS1870002 or NFAT, cat#CS176404)). Binding of the cell-surface displayed CAR to its antigen on another cell type will activate signalling through the CD3 and CD28 signalling pathway, respectively. The reporter cell line (*i.e.* Jurkat cells) has been re-engineered in such a way that the T-cell activation will result in luciferase transcription/translation via an IL-2 promoter or NFAT-RE.

CAR constructs with different length CD4 spacers are compared using the data obtained from the assays used in Examples 4 and 5 to determine the optimum spacer length to be used, with a specific target antigen, for T-cell activation from immune synapse formation with target displaying cells.

EXAMPLE 6: Generation of target specific CARs:

Peripheral blood mononuclear cells (PBMCs) of healthy donors were obtained after centrifugation of fresh blood on a density gradient using Accuspin Sytem-Histopaque (Sigma, A7054) according to the manufacturer's instructions. Cells were then resuspended at 1×10^6 cells/ml and cultured in 24-well plates in TexMACs medium (Miltenyi Biotec; 130-097-196) containing 100IU/ml of IL-2 (Sigma; SRP3085) and beads coated with specific antibodies for CD3 and CD28 (TransAct beads, Miltenyi Biotec) to initiate outgrowth of T cells.

48 hours post activation, T-cells were infected with lentivirus encoding CARs targeted to BCMA (α BCMA CARs). A multiplicity of infection (MOI) of 5 was used. 5 days post transduction, expression of CARs on the T cell surface was assayed by flow cytometry using antigen-Fc AlexaFluor 647 (produced and conjugated in-house; ThermoFisher; A20006) in combination with ZsGreen expression.

Fresh medium and IL-2 were added 3 times per week during culture and cell concentration maintained at about 0.7×10^6 cells/mL. 12 days post transduction, CAR T-cells were harvested and effector function tested using assays described below.

5 Cytotoxicity Assay results

Cytotoxicity assay was evaluated by flow cytometry. The target negative and positive cell lines (in-house generated) were suspended in PBS at 1×10^6 cells/mL and stained with fluorescent Cell Trace Far Red (0.1 μ M, final concentration; ThermoFisher; C34564) and with Cell Trace Violet (0.1 μ M, final concentration; ThermoFisher; C34557) respectively. Cells were incubated at room temperature for 30 minutes, protected from light. The cells were then washed twice in medium containing 10% of serum and suspended in 4×10^5 cells/mL. Stained cell types were combined and 100 μ l of the obtained solution added to untransduced (UT) control or CAR-transduced T-cells at a 1:1 effector to target ratio.

The cultures were incubated for 24 hours at 37°C. Immediately after the incubation, a solution containing SytoxAADvanced (20 μ M, final concentration; ThermoFisher; S10349), EDTA (200 mM final concentration) and Dnase (10 mM final concentration) was added, incubated for 20 minutes and flow cytometry acquisition was performed. Samples were acquired using a MACSQuant flow cytometer (Miltyeni Biotec), and data analyzed using FlowJo. An example of the gating strategy is shown in **Figure 2A**.

The percentage of survival of target cells was calculated as follows:

$$100 - (\text{sample counts}/\text{maximum counts}) \times 100$$

where the maximum count is given by the number of target cells in the absence of any effector cells.

25 Intracellular cytokine staining assay (ICCS) results:

For intracellular cytokine staining, 2×10^5 T-cells were cultured alone or in the presence of 2×10^5 target cells (negative or positive target expressing cells as above). The samples were incubated at 37°C for 6 hours, in the presence of Brefeldin A (BD, 555029). The cells were surface stained with anti-CD3 (BioLegend, clone UCHT1), then permeabilized, and intracellular staining was conducted for IFN- γ (BioLegend, clone 5S.B3) and IL-2 (BioLegend, clone MQ1-17H12) by following the instructions of the Cytofix/Cytoperm kit (Caltagmedsystem, GAS-002). Samples were acquired using a MACSQuant flow cytometer (Miltyeni Biotec), and data analyzed using FlowJo. An example of the gating strategy is shown in the **Figure 3A**.

35 Table 2: Other relevant sequences

SEQ ID NO.	Description	Sequence

1	CD4 sequence (UniProt, ID number P01730)	MNRGVPFRHLLVLQLALLPAATQGKKVVLGKKGDTVELTCT ASQKKSIQFHWKNSNQIKILGNQGSFLT KGPSKLNDRADSR RSLWDQGNFPLIINKLKIEDSDTYICEVEDQKEEVQLLVFGLT ANSDTHLLQGQSLTLTLESPPGSSPSVQCRSPRGKNIQGGKT LSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVLAFQKASSI VYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKSWI TFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLQPALPQYAGS GNLTLALEAKTGKLGHEVNLVVMRATQLQKNLTCEVWGPTS PKLMLSLKENKEAKVSKREKAVWVLNPEAGMWQCLLSDSG QVLLESNIKVLPTWSTPVQPMALIVLGGVAGLLLFIGLGIFFCV RCRHRRRQAERMSQIKRLLSEKKTCCQPHRFQKTCSPI
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CLAIMS

1. A chimeric antigen receptor (CAR) comprising:
 - a target binding domain;
 - 5 a spacer region;
 - a transmembrane domain; and
 - an intracellular effector domain,
 wherein the spacer region comprises at least one, or multiples of, domains 2, 3 or 4 or a combination thereof of a CD4 molecule.
- 10 2. The chimeric antigen receptor of claim 1, wherein the spacer region comprises domain 4 of a CD4 molecule.
3. The chimeric antigen receptor of claim 1 or claim 2, wherein the spacer region comprises
 15 domains 3 and 4 of a CD4 molecule.
4. The chimeric antigen receptor of any one of claims 1 to 3, wherein the spacer region comprises domains 2, 3 and 4 of a CD4 molecule.
- 20 5. The chimeric antigen receptor of any one of claims 1 to 4, wherein the spacer region comprises domains 2 and 3 and two copies of domain 4 of a CD4 molecule.
6. The chimeric antigen receptor of claim 1, wherein domain 2 of a CD4 molecule comprises amino acids 126 to 203 of SEQ ID NO: 1.
 25
7. The chimeric antigen receptor of claim 1, wherein domain 3 of a CD4 molecule comprises amino acids 204 to 317 of SEQ ID NO: 1.
8. The chimeric antigen receptor of claim 1, wherein domain 4 of a CD4 molecule comprises
 30 amino acids 318 to 374 of SEQ ID NO: 1.
9. The chimeric antigen receptor of any one of claims 1 to 8, wherein the target binding domain comprises an antibody, an antigen binding fragment or a ligand.
- 35 10. The chimeric antigen receptor of any one of claims 1 to 9, wherein the target binding domain binds to a tumour associated antigen.

11. The chimeric antigen receptor of claim 10, wherein the tumour associated antigen is selected from: BCMA, CD19, HER2, prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), carcinoembryonic antigen (CEA), cancer antigen-125, CA19-9, MUC-1, tyrosinase, CD34, CD45, CD117, protein melan-A, synaptophysin, CD22, CD27, CD30, CD70, ganglioside G2 (GD2), epidermal growth factor variant III (EGFRvIII), mesothelin, prostatic acid phosphatase (PAP), prostatein, TARP, Trp-p8 or six transmembrane epithelial antigen of the prostate I (STEAP1).

12. The chimeric antigen receptor of any one of claims 1 to 11, wherein the target binding domain has a binding affinity of less than about 500 nanomolar (nM).

13. The chimeric antigen receptor of any one of claims 1 to 12, wherein the transmembrane domain comprises the transmembrane domain of CD4.

14. The chimeric antigen receptor of any one of claims 1 to 13, wherein the intracellular effector domain comprises a CD3zeta signalling domain.

15. The chimeric antigen receptor of any one of claims 1 to 14, wherein the intracellular effector domain additionally comprises a costimulatory domain.

16. The chimeric antigen receptor of claim 15, wherein the costimulatory domain comprises the intracellular domain of a costimulatory molecule, selected from CD28, CD27, 4-1BB, OX40, ICOS, CD30, CD40, PD-1, CD2, CD7, LIGHT, NKG2C, B7-H3 or any combination thereof.

17. A polynucleotide encoding the chimeric antigen receptor of any one of claims 1 to 16.

18. An expression vector comprising the polynucleotide of claim 17.

19. An immunomodulatory cell comprising the chimeric antigen receptor of any one of claims 1 to 16.

20. The immunomodulatory cell of claim 19, which is derived from an inflammatory T-lymphocyte, cytotoxic T-lymphocyte, regulatory T-lymphocyte or helper T-lymphocyte.

21. The immunomodulatory cell of claim 19 or claim 20 for use in therapy.

22. A method of engineering an immunomodulatory cell, comprising:
(a) providing an immunomodulatory cell;

- (b) introducing the expression vector of claim 18 into said immunomodulatory cell;
and
(c) expressing said expression vector in the immunomodulatory cell.

- 5 23. An engineered immunomodulatory cell comprising a chimeric antigen receptor (CAR) which binds to a protein on a target cell, wherein said CAR comprises:
- a target binding domain,
a spacer domain which comprises at least one, or multiples of, domains 2, 3 or 4 or
a combination thereof of a CD4 molecule,
10 a transmembrane domain and
an intracellular effector domain,
wherein the length of the spacer domain is such that the distance between the cell
membranes of the target cell and engineered immunomodulatory cell creates an immune synapse.
- 15 24. The immunomodulatory cell of claim 23, wherein the distance between the cells membranes is about 14 nm.

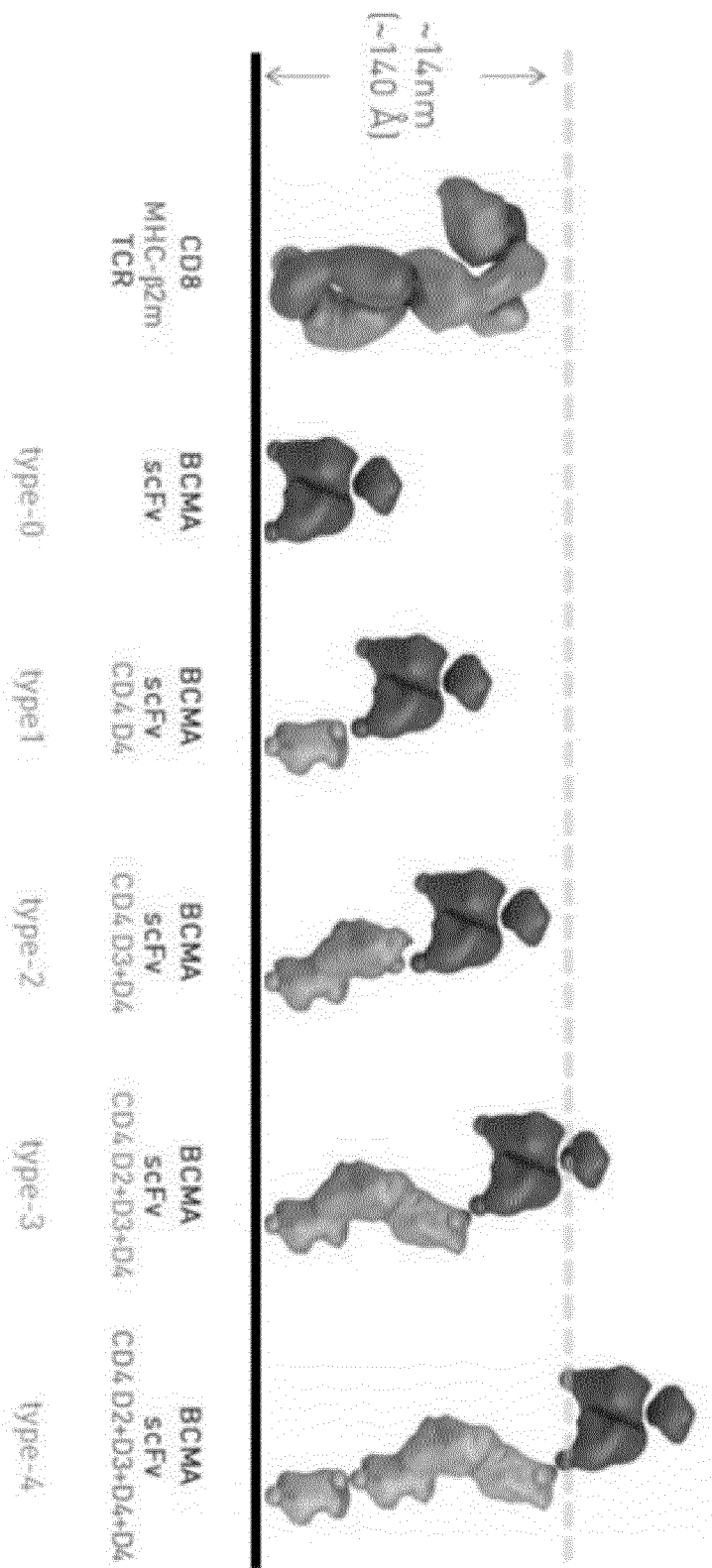
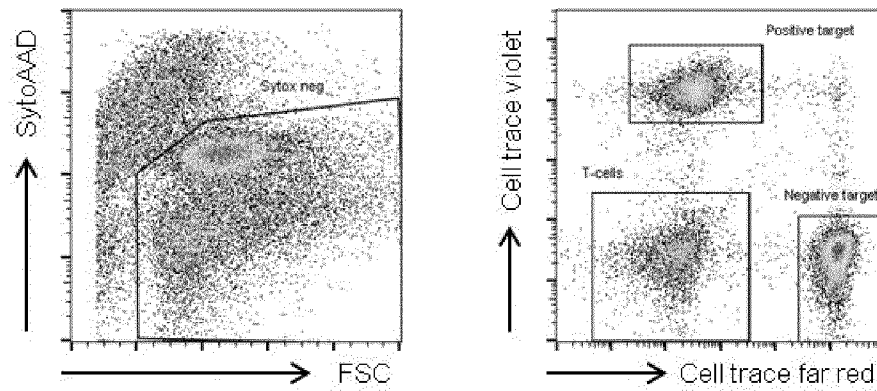


FIGURE 1

2a



2b

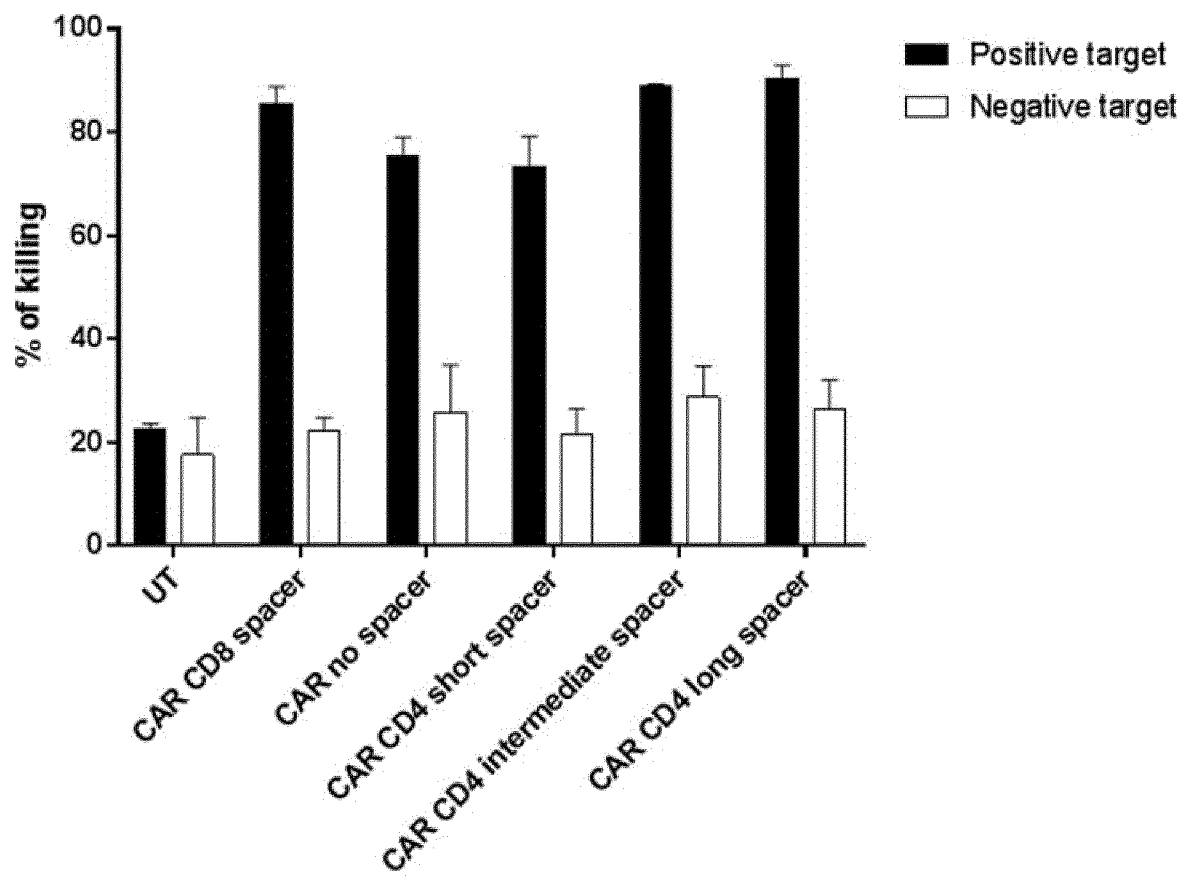


FIGURE 2

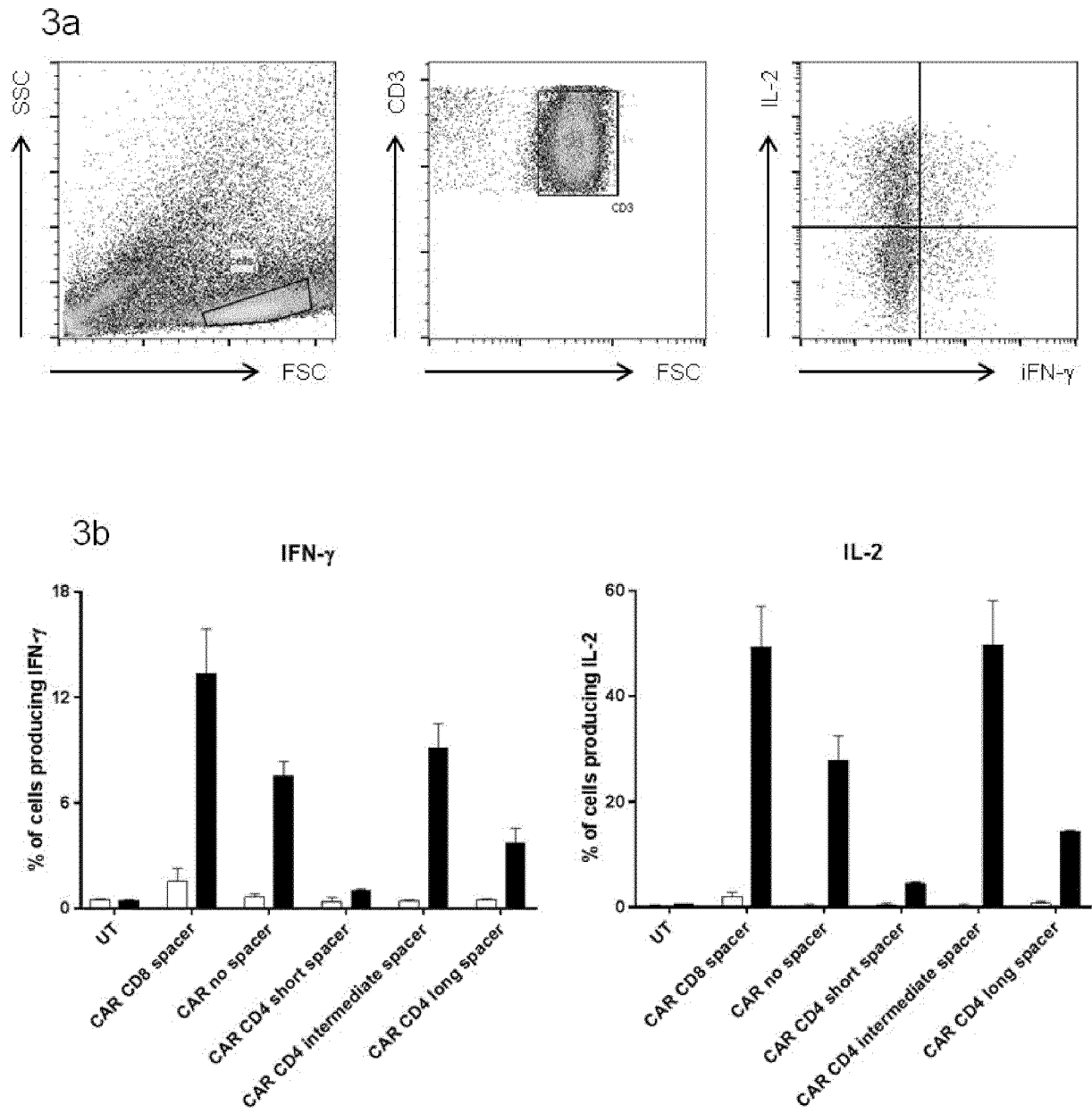


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/074382

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/725 C07K14/73
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATEL S D ET AL: "IMPACT OF CHIMERIC IMMUNE RECEPTOR EXTRACELLULAR PROTEIN DOMAINS ON T CELL FUNCTION", GENE THERAPY, NATURE PUBLISHING GROUP, GB, vol. 6, no. 6, 1 March 1999 (1999-03-01), pages 412-419, XP001095026, ISSN: 0969-7128, DOI: 10.1038/SJ.GT.3300831	1-5, 7-10, 12-14, 17-24
Y	page 417, left-hand column, last paragraph - right-hand column, paragraph 1; figure 1 ----- -/--	6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 December 2016

Date of mailing of the international search report

03/01/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Lanzrein, Markus

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/074382

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORITZ D ET AL: "A SPACER REGION BETWEEN THE SINGLE CHAIN ANTIBODY- AND THE CD3 ZETA-CHAIN DOMAIN OF CHIMERIC T CELL RECEPTOR COMPONENTS IS REQUIRED FOR EFFICIENT LIGAND BINDING AND SIGNALLING ACTIVITY", GENE THERAPY, NATURE PUBLISHING GROUP, GB, vol. 2, no. 8, 1 October 1995 (1995-10-01), pages 539-546, XP000646503, ISSN: 0969-7128	1-5,7, 10, 12-14, 17-24
Y	figure 1c	6
X	----- WO 2015/140268 A1 (CELLECTIS [FR]) 24 September 2015 (2015-09-24)	1,14-24
Y	figure 3 page 32, line 26 - line 29	6
A	----- M. HUDECEK ET AL: "Receptor Affinity and Extracellular Domain Modifications Affect Tumor Recognition by ROR1-Specific Chimeric Antigen Receptor T Cells", CLINICAL CANCER RESEARCH, vol. 19, no. 12, 25 April 2013 (2013-04-25), pages 3153-3164, XP055177780, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-13-0330 page 3154, left-hand column, last paragraph - right-hand column, paragraph 1 page 3155, right-hand column, paragraph 4	1-24
Y	----- WO 2012/031744 A1 (CHEMOTHERAPEUTISCHES FORSCHUNGSINSTITUT [DE]; SCHOENFELD KURT [DE]; KN) 15 March 2012 (2012-03-15) page 11, paragraph 1; figure 2 claim 1	6,11
Y	----- PULE M ET AL: "ARTIFICIAL T-CELL RECEPTORS", CYTOTHERAPY, ISIS MEDICAL MEDIA, OXFORD, GB, vol. 5, no. 3, 1 January 2003 (2003-01-01), pages 211-226, XP008028247, ISSN: 1465-3249, DOI: 10.1080/14653240310001488 table 1 -----	11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/074382

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			CA 2943008 A1 24-09-2015
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