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(54) Title: CHIMERIC ANTIGEN RECEPTOR USING ANTIGEN RECOGNITION DOMAINS DERIVED FROM CARTILAGINOUS FISH

(57) Abstract: The present invention relates to a new generation of chimeric antigen receptors (CAR), under single-chain or multi-chain forms, the specificity of which, to a desired antigen, is conferred by a VNAR polypeptide derived from monomeric antibodies from cartilaginous fish. Such CARs, which aim to redirect immune cell specificity toward selected undesired malignant cells, are compact and thus particularly adapted to target hollow antigens such as ions channels of efflux pumps present at the surface of drug-resistant cells. The invention encompasses the polynucleotides, vectors encoding said multi-chain CAR and the isolated cells expressing them at their surface, in particularly for their use in immunotherapy.

CHIMERIC ANTIGEN RECEPTOR USING ANTIGEN RECOGNITION DOMAINS DERIVED FROM CARTILAGINOUS FISH

Field of the invention

The present invention relates to the field of cell immunotherapy and more particularly to a new generation of chimeric antigen receptors (CAR), the specificity of which is conferred by VNAR polypeptides derived from monomeric antibodies of cartilaginous fish. The CAR of the invention can be expressed at the surface of immune cells to redirect their specificity toward specific antigens, in particular hollow antigens, such as components of ion channels and efflux pumps conferring drug resistance to malignant cells. The invention opens the way to efficient adoptive immunotherapy strategies, especially for the treatment of refractory cancer forms.

Background of the invention

Adoptive immunotherapy, which involves the transfer of autologous antigen-specific T cells generated *ex vivo*, is a promising strategy to treat viral infections and cancer. The T cells used for adoptive immunotherapy can be generated either by expansion of antigen-specific T cells or redirection of T cells through genetic engineering (Park, Rosenberg et al. 2011). Transfer of viral antigen specific T cells is a well-established procedure used for the treatment of transplant associated viral infections and rare viral-related malignancies. Similarly, isolation and transfer of tumor specific T cells has been shown to be successful in treating melanoma.

Novel specificities in T cells have been successfully generated through the genetic transfer of transgenic T cell receptors or chimeric antigen receptors (CARs) (Jena, Dotti et al. 2010). CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. In general, the binding moiety of a CAR consists of an antigen-binding domain of a single-chain antibody (scFv), comprising the light and heavy variable fragments of a monoclonal antibody joined by a flexible linker. Binding moieties based on receptor or ligand domains have also been used successfully. The signaling domains for first generation CARs are derived from the cytoplasmic region of the CD3zeta or the Fc receptor gamma chains. First generation CARs have been shown to successfully redirect T cell cytotoxicity, however, they failed to provide prolonged expansion and anti-tumor activity in vivo. Signaling domains from co-stimulatory molecules including CD28, OX-40 (CD134), ICOS and 4-1BB (CD137) have been added alone (second generation) or in combination (third generation) to enhance survival and increase proliferation of CAR modified T cells. CARs have successfully allowed T cells to be redirected against antigens expressed at the surface of tumor cells from various malignancies including lymphomas and solid tumors (Jena, Dotti et al. 2010). However, for example, some surface antigens will be difficult to target efficiently with classical antibodies as mAbs are not able to access epitopes embedded in the protein structures (e.g. numerous surface receptor may contain the ligand binding pocket). Moreover, single-chain antibody (scFv), CAR comprising the light and heavy variable fragments of a monoclonal antibody joined by a flexible linker have limitations due to their size and structural complexity that renders them problematic to manufacture and to predict their efficacy.

Here, the inventors have alleviated these limitations by creating new Chimeric Antigen Receptors in which antigen specificity is mediated through variable antigen receptors (VNAR) derived from cartilaginous fish.

Summary of the invention

Despite their success, IgG molecules have shown practical limitations as part of current CAR constructs. In particular they are large (~150 kDa) tetrameric structures prone to elicit immune reactions and expensive to develop.

VNAR (variable domain of the IgNAR, or Novel Antigen Receptor) forms a unique class of protein that have been identified in the serum of cartilaginous fish. The VNAR can be isolated as a monomeric binding domain of 12-15 kDa in size, i.e a much smaller size than IgG.

VNARs have been identified for several years as possible biotherapeutics based on their robustness and solubility, propensity to bind to antigen clefts and block active sites of enzymes, and high binding affinities for a range of antigens. However, they remain much less well understood structurally and biophysically than other types of antigen receptors. The VNAR domain shares structural features with the T-cell receptor Va and the IgG Vk-chain, but sequence homology with these domains is low (~35%). By contrast to scFv, VNAR polypeptides have the common feature of lacking CDR2 (CDR = Complementarity Determining Region). They usually contain a shorter CDR1 loop but a longer CDR3 loop, which create the main binding surface of the domain.

Given these features, it was not predictable that VNAR would be suitable for the construction of efficient chimeric receptors. Indeed, it had been so far considered that CAR architectures required rather extensive extracellular antigen recognition domains to reach antigens present at the surface of malignant or infected cells.

The invention relates to such new chimeric antigen receptor that includes VNAR polypeptides as antigen recognition domains.

The present invention also relates to the polypeptides encoding these new CARs referred to as “VNAR-CARs” and to methods of engineering immune cells, in particular T-cells, by expression of said cell polypeptides. The immune cells obtainable by these methods should be better tolerated by patient’s organism and more slowly destroyed by the immune system.

In more specific embodiments, different architectures are proposed for the VNAR-CARs of the invention depending on their single or multi-chain structure, allowing modulation of the interaction and/or activation of the immune cell upon antigen recognition. The VNAR may also be humanized in order contain less immunogenic sequences, such that T-cells expressing CAR would not trigger immune response from the receiver organism (e.g. human). The T-cells expressing the VNAR CARs can also be genetically engineered for allogeneic therapeutic use, for instance, by disruption of the genes encoding T-cell receptors (ΔTCR).

Brief description of the figures

Figure 1: General structure of VNAR polypeptides used as antigen recognition domains.

Figure 2: Sequence alignment of four representative exemplary VNAR Scaffolds from Shark corresponding to SEQ ID NO.1 (E06), SEQ ID NO.101 (5A7), SEQ ID NO.102 (7e80) and SEQ ID NO.115 (12A9).

Figure 3: schematic representation of an exemplary single-chain VNAR-CAR according to the invention comprising (1) an extracellular domain composed of a VNAR polypeptide comprising a CDR3 acting as the main antigen recognition domain and a hinge from CD8, (2) a transmembrane polypeptide comprising 4-1BB (co-stimulatory domain) and CD3zeta (signaling domain).

Figure 4: Schematic representation of an exemplary multi-chain VNAR-CARs according to the present invention based on the structure of the FcεRI receptor. The VNAR polypeptide is

fused to FcεRI alpha chain, whereas the co-stimulatory domain is fused to FcεRI gamma chain and the signaling domain to the FcεRI beta chain.

Figure 5 and 6: Schematic representations of different exemplary versions of the multi-chain CARs of the present invention (csm1 to csm10) comprising an extracellular VNAR polypeptide fused to a CD8 stalk/hinge region fused to the transmembrane domain of FcεRI alpha chain, whereas at least one co-strimulatory 41BB, CD28 and/or CD3 zeta domains are fused to either FcεRI alpha, beta and/or gamma chains.

Figure 7: schematic representation of the structure of the single-chain CAR according to the invention (SEQ ID NO.110) as described in example 1.

Figure 8: schematic representation of the structure of a multi-chain CAR according to the invention (SEQ ID NO.105) as described in example 1.

Detailed description of the invention

Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled artisan in the fields of gene therapy, biochemistry, genetics, and molecular biology.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Harries & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-

IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

The present invention primary focuses on a chimeric antigen receptor (CAR) characterized in that it comprises:

i) one extracellular antigen recognition domain comprising a VNAR polypeptide; and

ii) one transmembrane polypeptide comprising at least one signal-transducing domain;

VNAR polypeptides are distinct from typical Ig VH and VL domains, as well as from camelid VHH domains, in particular by sharing higher structural homology to immunoglobulin VL and T-cell receptor (TCR) V domains than with immunoglobulin VH.

The most unique feature of VNAR polypeptides is the absence of a CDR2 loop and of two β-strands, C′and C″, associated with it. Instead, a distinct “belt” is formed round the middle of the β-sandwich structure (Kovalenko et al., 2013). This region shows an elevated rate of somatic mutations and has thus been termed hypervariable region 2, HV2). Another region of increased mutation frequency is located between HV2 and CDR3, comprising a loop that links β-strands D and E similar to that in TCR V chains; thus, this region was termed HV4. Structurally, HV2 is most proximal to CDR3, whereas HV4 is in proximity to CDR1. Several structural types of IgNAR variable domains have been classified based on the number and position of extra cysteine residues in CDRs and frameworks (FW) in addition to the canonical cysteine pair (Cys-23/Cys-88 for VL, Kabat nomenclature) of the Ig fold. Type I V-NAR, found in nurse sharks, has 2 cysteines in CDR3 and 2 more in frameworks (FW2 and FW4). The more common type II has one extra cysteine pair that links CDR1 and CDR3. Type III, detected primarily in neonatal shark development, is similar to type II but has a conserved Trp residue in CDR1 and limited CDR3 diversity. Another structural type of V-NAR, which we have termed type IV, has 3 only two canonical cysteine residues. So far, this type has been found primarily in dogfish sharks, and was also isolated from semi-synthetic V-NAR libraries derived from wobbegong sharks. The single-domain nature and the lack of CDR2 in V-NARs heighten the requirement for CDR1 and CDR3 to provide specific and high-affinity binding to prospective antigens. CDR3, being more variable in terms of sequence, length and conformation, plays the key role in antigen recognition.

Also, the antigen recognition domain of the CAR according to the invention preferably comprises only two Complementary Determining Regions (CDRs) referred to as CDR1 and

CDR3, and more preferably, said antigen recognition domain has only one Complementary Determining Regions (CDR3).

In general, the specificity of recognition of the CAR for said antigen is determined by said CDR3. Most of the time, CDR3 accounts by more than 50 %, and more generally by more than 70 % in the T-cell activation (i.e. affinity is only reduced by 50 or 30 % when CDR1 is modified or removed). T-cell activation can be measured by different means, in particular by using the method described by Betts et al. (2003).

VNAR polypeptides having the advantage of being relatively small polypeptides (12-13kDa), they demonstrate the advantage of high biophysical stability, solubility and ability to bind to a variety of antigens, including epitopes located in clefts on protein surfaces (e.g. enzyme active sites) that are non-accessible by traditional antibody variable domains.

According to a preferred embodiment of the invention, the CDR3 region, which is often long between 10 to 25 residues, but preferably between 15 to 20, protrudes from the VNAR surface. This CD3 region preferably comprises at least two cysteine residues creating disulfide bounds with residues from the VNAR polypeptide to obtain a more protruding recognition surface.

The term “extracellular antigen recognition domain” as used herein is defined as an oligo- or polypeptide that is capable of binding a ligand, more specifically an antigen. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In particular, the extracellular ligand-binding domain can comprise an antigen binding domain derived from an antibody against an antigen of the target.

As non-limiting examples, the antigen of the target can be any cluster of differentiation molecules (e.g. CD16, CD64, CD78, CD96,CLL1, CD116, CD117, CD71, CD45, CD71, CD123 and CD138), a tumor-associated surface antigen, such as ErbB2 (HER2/neu), carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptor (EGFR), EGFR variant III (EGFRvIII), CD19, CD20, CD30, CD40, disialoganglioside GD2, ductal-epithelial mucine, gp36, TAG-72, glycosphingolipids, glioma-associated antigen, β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostase specific antigen (PSA), PAP, NY-ESO-1, LAGA-1a, p53, prostein, PSMA, surviving and telomerase, prostatecarcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrin B2, CD22, insulin growth factor (IGF1)-I, IGF-II, IGFI receptor, mesothelin, a major histocompatibility complex (MHC) molecule presenting a tumor-specific peptide epitope, 5T4, ROR1, Nkp30, NKG2D, tumor stromal antigens, the extra domain A (EDA) and extra domain B (EDB) of fibronectin and the A1 domain of tenascin-C (TnC A1) and fibroblast associated protein (fap); a lineage-specific or tissue specific antigen such as CD3, CD4, CD8, CD24, CD25, CD33, CD34, CD133, CD138, CTLA-4, B7-1 (CD80), B7-2 (CD86), GM-CSF, cytokine receptors, , endoglin, a major histocompatibility complex (MHC) molecule, BCMA (CD269, TNFRSF 17), or a virus-specific surface antigen such as an HIV-specific antigen (such as HIV gp120); an EBV-specific antigen, a CMV-specific antigen, a HPV-specific antigen, a Lasse Virus-specific antigen, an Influenza Virus-specific antigen as well as any derivate or variant of these surface markers. Antigens are not necessarily surface marker antigens but can be also endogenous small antigens presented by HLA class I at the surface of the cells.

The extracellular ligand-binding domain can also comprise a peptide binding an antigen of the target, a peptide or a protein binding an antibody that binds an antigen of the target, a peptide or a protein ligand such as a growth factor, a cytokine or a hormone as non-limiting examples binding a receptor on the target, or a domain derived from a receptor such as a growth factor receptor, a cytokine receptor or a hormone receptor as non-limiting examples, binding a peptide or a protein ligand on the target. Preferably the target is a cell, but can also be a virus or a microorganism. According to another aspect of the invention, the CARs according to the invention can be directed against antibodies or against other CARs comprising Fc immunoglobulin chains.

The chimeric antigen receptors according to the present invention display the advantage of having an extracellular domain smaller than other types of ligand binding domains. In general the VNAR polypeptide which forms this extracellular domain is shorter than 150 amino acids, preferably shorter than 140, more preferably shorter than 130, even more preferably shorter than 120 amino acids. In some instances, the VNAR polypeptide can be of less than 110 amino acids and sometimes less than 100 amino acids.

The inventors have established that the CARs of smaller extracellular domains according to the present invention could be particularly efficient to target antigens with a hollow structure present at the surface of cells, such as polypeptides involved into transport function. Indeed, Leukemias, as other cancers, bear several genetic alterations of tumor-related genes, such as point mutations, translocations, epigenetic modifications, often accompanied by gene amplification or inactivation. The identification of tumor-related genes provides considerable insight into the biology of leukemias and opens the way to more specific pharmacological treatments. These genes comprise several ion channels and pumps, as the transport mechanisms associated with volume control, proliferation and apoptosis are often altered in cancers. In leukemic cells, such changes are observed as early as the stem cell stage. Ion channels can regulate other malignant features, such as lack of differentiation, increased migratory and invasive phenotype and chemoresistance. Multidrug resistance (MDR), mediated by multiple drug efflux ATP-binding cassette (ABC) transporters, is a critical issue, particularly in the treatment of acute leukemia, with permeability (P)-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP, or ABCG2) consistently shown to be the key effectors of MDR in cell line studies. Studies have demonstrated that intrinsic MDR can arise due to specific gene expression profiles, and that drug-induced overexpression of P-gp and other MDR proteins can result in acquired resistance, with multiple ABC transporters having been shown to be overexpressed in cell lines selected for resistance to multiple drugs for acute leukemia. Other receptors such as sigma receptors (sigmaR)(S), namely sigmaR(1) and sigmaR(2), have been found to be overexpressed in breast cancer cells.

Thus because of their involvement in the genesis of cancer and their overexpression in this pathology, one aspect of the present invention would be to target such type of membrane pores or pumps using the CAR of the present invention for immunoadoptive therapy of cancer.

Table 1 below provides examples of ABC transporters, which could be targeted with the VNAR-CAR of the present invention for the treatment of malignant cells resistant to chemotherapy.

Table 1: ABC transporters involved into cell resistance to chemotherapy

ABC family	Chemotherapy substrates	Related cancer
ABCA		
ABCA2	Estramustine and mitoxantrone	Lung cancer cell lines and AML
ABCA3	Anthracyclines	Neuroblastoma
ABCB		
ABCB1	Colchicine, Anthracyclines, epipodophyllotoxins, vinca alkaloids, taxanes, camptothecins, bisantrene, imatinib, mitoxantrone, saquinivir, methotrexate and actinomycin D	AML and Lung cancer cell lines

ABCB4	Anthracyclines, vinca alkaloids, taxanes, mitoxantrone, epipodophyllotoxins	
ABCB5	Anthracyclines, camptothecins et thiopurines	Melanoma
ABCB11	Taxanes	
ABCC		
ABCC1	Anthracyclines, mitoxantrone, vinca alkaloids, imatinib, epipodophyllotoxins, camptothecins, mitoxantrone and saquinivir, Methotrexate	Squamous cell carcinoma lines, lung cancer lines, glioma and AML
ABCC2	Methotrexate, epipodophyllotoxins, vinca alkaloids, ciplatin, taxanes, anthracyclines, mitoxantrone, saquinivir, camptotechins	
ABCC3	Methotrexate, epipodophyllotoxins,	
ABCC4	Thiopurines, PMEA, methotrexate, AZT, camptotechins	
ABCC5	Thiopurines, PMEA, methotrexate, AZT, cisplatin	
ABCC6	anthracyclines, cisplatin, epipodophyllotoxins,	
ABCC10	Vinca alkaloids, ciplatin	
ABCC11	Thiopurines	
ABCG		
ABCG2	Mitoxantrone, camptotechins, anthracyclines, bisantrene imitaninib, methotrexate, flavopiridol, epipodophyllotoxins,	Lung cancer, AML, oesophageal carcinoma, glioma, neuroblastoma, squamous cell, carcinoma cell lines, melanoma, ovarian cancer and nasopharyngeal carcinoma cell lines

According to a particular embodiment of the invention, several VNAR polypeptides can be linked in tandem to provide multi-specificity, the increase size of the extracellular domain or in vivo half-life of molecule.

According to a further aspect of the invention, the VNAR polypeptide involved into the CAR construction can be humanized in order to reduce immunogenicity and/or improve thermodynamic stability, folding and expression properties. Considerable expertise has been accumulated in this subject area, particularly with rodent mAbs. Typically, CDRs of a murine antibody of interest are grafted onto an appropriate human germline framework (selected for sequence similarity, expression properties, or both) and then back-mutations are introduced at key positions responsible for particular CDR conformation and thus antigen binding. This approach has yielded many humanized Abs, with a number of them making it into the clinic. Although shark VNARs represent more challenge for humanization due to the structural differences (e.g., lack of CDR2) and low overall sequence identity (generally ~30%) to human VH/VL sequences, available crystal structures of VNAR domains demonstrate similar organization of key framework regions to human Ig variable domains, thus making an attempt at humanization possible (Kovelenko et al. 2013). Such humanization may lead to the replacement of up to 50 % of the initial overall amino acid sequence of the initial VNAR scaffold used as VNAR polypeptide. Accordingly, the present invention encompass the use of VNAR polypeptides having relatively low amino acid identity with any reported VNAR polypeptides originating from cartilaginous fish, although displaying preferably at least 50 %, more preferably at least 75 %, even more preferably at least 80%, most preferably at least 90 % amino acid sequence identity with the polypeptide sequences referred to as SEQ ID NO. 1 to 100 (Table 2). These sequences are provided as non-limiting examples of VNAR scaffold that can be used and humanized according to the invention.

The Chimeric Antigen Receptors according to the present invention generally further comprise a hinge (stalk) region between their transmembrane region and extracellular antigen recognition domain.

The term “hinge region” used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk region are used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A stalk region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4 or CD28, or from all or part of an antibody constant region. Alternatively the stalk region may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence. In a preferred embodiment said stalk region is a part of human CD8 alpha chain (e.g. NP_001139345.1).

Multi-chain VNAR-CARs

Example 1 and Figures 3 and 7 of the present specification illustrate Chimeric Antigen Receptors according to the invention based on a single-chain CAR, corresponding to the classical architecture of CARs, in which all relevant domains are contained within a single polypeptide as described in US 7,741,465.

However, the present invention encompasses also multi-chain architectures as shown in Example 2 and Figures 4, 5 and 8. According to such architectures, ligands binding domains and signaling domains are born on separate polypeptides. The different polypeptides are anchored into the membrane in a close proximity allowing interactions with each other. In such architectures, the signaling and co-stimulatory domains can be in juxtamembrane positions (i.e. adjacent to the cell membrane on the internal side of it), which is deemed to allow improved function of co-stimulatory domains. The multi-subunit architecture also offers more flexibility and possibilities of designing CARs with more control on T-cell activation. For instance, it is possible to include several extracellular antigen recognition domains having different specificity to obtain a multi-specific CAR architecture. It is also possible to control the relative ratio between the different subunits into the multi-chain CAR. This type of architecture has been recently described by the applicant in PCT/US2013/058005.

The assembly of the different chains as part of a single multi-chain CAR is made possible, for instance, by using the different alpha, beta and gamma chains of the high affinity receptor for IgE (FcεRI) (Metzger, Alcaraz et al. 1986) to which are fused the signaling and co-stimulatory domains. The gamma chain comprises a transmembrane region and cytoplasmic tail containing one immunoreceptor tyrosine-based activation motif (ITAM) (Cambier 1995).

The multi-chain CAR can comprise several extracellular ligand-binding domains, to simultaneously bind different elements in target thereby augmenting immune cell activation and function. In one embodiment, the extracellular ligand-binding domains can be placed in tandem on the same transmembrane polypeptide, and optionally can be separated by a linker. In another embodiment, said different extracellular ligand-binding domains can be placed on different transmembrane polypeptides composing the multi-chain CAR. In another embodiment, the present invention relates to a population of multi-chain CARs comprising each one different extracellular ligand binding domains. In a particular, the present invention relates to a method of engineering immune cells comprising providing an immune cell and expressing at the surface of said cell a population of multi-chain CAR each one comprising different extracellular ligand binding domains. In another particular embodiment, the present invention relates to a method of engineering an immune cell comprising providing an immune cell and introducing into said cell polynucleotides encoding polypeptides composing a population of multi-chain CAR each one comprising different extracellular ligand binding domains. In a particular embodiment the method of engineering an immune cell comprises expressing at the surface of the cell at least a part of FcεRI beta and/or gamma chain fused to a signal-transducing domain and several part of FcεRI alpha chains fused to different extracellular ligand binding domains. In a more particular embodiment, said method comprises introducing into said cell at least one polynucleotide which encodes a part of FcεRI beta and/or gamma chain fused to a signal-transducing domain and several FcεRI alpha chains fused to different extracellular ligand biniding domains. By population of multi-chain CARs, it is meant at least two, three, four, five, six or more multi-chain CARs each one comprising different extracellular ligand binding domains. The different extracellular ligand binding domains according to the present invention can preferably simultaneously bind different elements in target thereby augmenting immune cell activation and function.

The present invention also relates to an isolated immune cell which comprises a population of multi-chain CARs each one comprising different extracellular ligand binding domains.

The signal transducing domain or intracellular signaling domain of the multi-chain CAR of the invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response. In other words, the signal transducing domain is responsible for the activation of at least one of the normal effector functions of the immune cell in which the multi-chain 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.

In the present application, the term “signal transducing domain” refers to the portion of a protein which transduces the effector signal function signal and directs the cell to perform a specialized function.

Preferred examples of signal transducing domain for use in single or multi-chain CAR can be the cytoplasmic sequences of the Fc receptor or T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivate or variant of these sequences and any synthetic sequence that as the same functional capability. Signal transduction domain comprises two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. Primary cytoplasmic signaling sequence can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. ITAMs are well defined signaling motifs found in the intracytoplasmic tail of a variety of receptors that serve as binding sites for syk/zap70 class tyrosine kinases. Examples of ITAM used in the invention can include as non-limiting examples those derived from TCRzeta, FcRgamma, FcRbeta, FcRepsilon, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b and CD66d. In a preferred embodiment, the signaling transducing domain of the multi-chain CAR can comprise the CD3zeta signaling domain, or the intracytoplasmic domain of the FcεRI beta or gamma chains.

In particular embodiment the signal transduction domain of the multi-chain CAR of the present invention comprises a co-stimulatory signal molecule. A co-stimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient immune response.

"Co-stimulatory ligand" refers to a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T-cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation activation, differentiation and the like. A co-stimulatory ligand can include but is not limited to CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory igand (ICOS-L), intercellular adhesion molecule (ICAM, CD30L, CD40, CD70, CD83, HLA-G, MICA, M1CB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as but not limited to, CD27, CD28, 4-IBB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LTGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a T-cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

A “co-stimulatory signal” as used herein refers to a signal, which in combination with primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or downregulation of key molecules.

In another particular embodiment, said signal transducing domain is a TNFR-associated Factor 2 (TRAF2) binding motifs, intracytoplasmic tail of costimulatory TNFR member family. Cytoplasmic tail of costimulatory TNFR family member contains TRAF2 binding motifs consisting of the major conserved motif (P/S/A)X(Q/E)E) or the minor motif (PXQXXD), wherein X is any amino acid. TRAF proteins are recruited to the intracellular tails of many TNFRs in response to receptor trimerization. In a preferred embodiment, the signal transduction domain of the multi-chain CAR of the present invention comprises a part of co-stimulatory signal molecule selected from the group consisting of 4-1BB (GenBank: AAA53133.) and CD28 (NP_006130.1).

The distinguishing features of appropriate transmembrane polypeptides comprise the ability to be expressed at the surface of an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and to interact together for directing cellular response of immune cell against a predefined target cell. The different transmembrane polypeptides of the multi-chain CAR of the present invention comprising an extracellular ligand-biding domain and/or a signal transducing domain interact together to take part in signal transduction following the binding with a target ligand and induce an immune response. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non-limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ or □, polypeptide constituting CD3 complex, IL2 receptor p55 (α chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fcγ receptor III or CD proteins. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

In a preferred embodiment, the transmembrane polypeptide derived from the Fcε receptor chains or variant thereof, in particular comprises the FcεRI α, β and/or γ chains or a functional fragment or variant thereof. The term "derived from" means a polypeptide having an amino acid sequence which is equivalent to that an Fcε receptor which include one or more amino acid modification(s) of the sequence of the Fcε receptor. Such amino acid modification(s) may include amino acid substitution(s), deletion(s), addition(s) or a combination of any of those modifications, and may alter the biological activity of the Fc binding region relative to that of an Fc receptor. On the other hand, Fc binding regions derived from a particular Fc receptor may include one or more amino acid modification(s) which do not substantially alter the biological activity of the Fc binding region relative to that of an Fc receptor. Amino acid modification(s) of this kind will typically comprise conservative amino acid substitution(s).

In more particular embodiment, said multi-chain CAR can comprise a part of FcεRI alpha chain and a part of FcεRI beta chain or variant thereof such that said FcεRI chains spontaneously dimerize together to form a dimeric Chimeric Antigen Receptor. In another embodiment, the multi-chain Chimeric Antigen can comprise a part of FcεRI alpha chain and a part of a FcεRI gamma chain or variant thereof such that said FcεRI chains spontaneously trimerize together to form a trimeric Chimeric Antigen Receptor, and in another embodiment the multi-chain Chimeric Antigen Receptor can comprise a part of FcεRI alpha chain, a part of FcεRI beta chain and a part of FcεRI gamma chain or variants thereof such that said FcεRI chains spontaneously tetramerize together to form a tetrameric Chimeric Antigen Receptor.

In other words, the multi-chain CAR comprising at least two of the following components:

a) one polypeptide comprising a part of FcεRI alpha chain and an extracellular ligand-binding domain,

b) one polypeptide comprising a part of FcεRI beta chain and/or

c) one polypeptide comprising a part FcεRI gamma chain, whereby different polypeptides multimerize together spontaneously to form dimeric, trimeric or tetrameric CAR.

The term "functional fragment” used herein refers to any subset of a protein, retaining at least 50 % of the activity of the whole protein. Alternatively, the term “functional variants” refers to a polypeptide that can include, for example, deletions, or insertions or substitutions of amino acids with respect to an initial protein, while retaining at least 50 % of the activity of said initial protein. Such functional variants can be prepared by mutations in the DNA which encodes the polypeptide.

The functionality of the CARs of the invention with respect to a desired antigen can be assayed upon binding to Daudi cells expressing said antigen on their surface as described in the experimental part. Other assays known in the art are available involving measurement of the increase of calcium ion release, intracellular tyrosine phosphorylation, inositol phosphate turnover, or interleukin (IL) 2, interferon γ, GM-CSF, IL-3, IL-4 production by the targeted cells.

Polynucleotides, vectors:

In a particular embodiment, the different nucleic acid sequences can be included in one polynucleotide or vector which comprises a nucleic acid sequence encoding ribosomal skip sequence such as a sequence encoding a 2A peptide. 2A peptides, which were identified in the Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see Donnelly et al., J. of General Virology 82: 1013-1025 (2001); Donnelly et al., J. of Gen. Virology 78: 13-21 (1997); Doronina et al., Mol. And. Cell. Biology 28(13): 4227-4239 (2008); Atkins et al., RNA 13: 803-810 (2007)). Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an mRNA when the polypeptides are separated by a 2A oligopeptide sequence that is in frame. Such ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA. As non-limiting example, in the present invention, 2A peptides have been used to express into the cell the different polypeptides of the multi-chain CAR.

To direct, transmembrane polypeptide such as FcεR into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in polynucleotide sequence or vector sequence. The secretory signal sequence may be that of FcεR, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo.* The secretory signal sequence is operably linked to the transmembrane nucleic acid sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleic acid sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleic acid sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). In a preferred embodiment the signal peptide comprises the residues 1 to 25 of the FcεRI alpha chain (NP_001992.1).

Those skilled in the art will recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. Preferably, the nucleic acid sequences of the present invention are codon-optimized for expression in mammalian cells, preferably for expression in human cells. Codon-optimization refers to the exchange in a sequence of interest of codons that are generally rare in highly expressed genes of a given species by codons that are generally frequent in highly expressed genes of such species, such codons encoding the amino acids as the codons that are being exchanged.

Polypeptides may be synthesized *in situ* in the cell as a result of the introduction of polynucleotides encoding said polypeptides into the cell. Alternatively, said polypeptides could be produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into animal 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, 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), liposome and the like. For example, transient transformation methods include for example microinjection, electroporation or particle bombardment. Said polynucleotides may be included in vectors, more particularly plasmids or virus, in view of being expressed in cells.

Modified and engineered T-cells

The present invention also relates to isolated cells or cell lines susceptible to be obtained by said method to engineer cells. In particular said isolated cell comprises at least one multi-chain CAR as described above. In another embodiment, said isolated cell comprises a population of multi-chain CARs each one comprising different extracellular ligand binding domains. In particular, said isolated cell comprises exogenous polynucleotide sequences encoding polypeptides composing at least one multi-chain CAR. Said cells can also further comprise at least one inactivated gene selected from the group consisting of CD52, GR, TCR alpha, TCR beta, HLA gene, immune check point genes such as PD1 and CTLA-4, or can express a pTalpha transgene.

In the scope of the present invention is also encompassed an isolated immune cell, preferably a T-cell obtained according to any one of the methods previously described. Said immune cell refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptative immune response. Said immune cell according to the present invention can be derived from a stem cell. The stem cells can be adult stem cells, 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. Representative human cells are CD34+ cells. Said isolated cell can also be a dendritic cell, killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T-cell selected from the group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes. 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, from a patient diagnosed with cancer or from a patient diagnosed with an infection. In another embodiment, said cell is part of a mixed population of cells which present different phenotypic characteristics. In the scope of the present invention is also encompassed a cell line obtained from a transformed T- cell according to the method previously described. Modified cells resistant to an immunosuppressive treatment and susceptible to be obtained by the previous method are encompassed in the scope of the present invention.

In another embodiment, said isolated cell according to the present invention comprises one inactivated gene selected from the group consisting of CD52, GR, PD1, CTLA-4, LAG3, Tim3, BTLA, BY55, TIGIT, B7H5, LAIR1, SIGLEC10, 2B4, HLA, TCR alpha and TCR beta and/or expresses a CAR, a multi-chain CAR and/or a pTalpha transgene. In another particular embodiment, said isolated cell comprises polynucleotides encoding said polypeptides composing the CAR of the invention as previously described.

In another embodiment, said isolated cell according to the present invention comprises two inactivated genes selected from the group consisting of CD52 and GR, CD52 and TCR alpha, CDR52 and TCR beta, GR and TCR alpha, GR and TCR beta, TCR alpha and TCR beta, PD1 and TCR alpha, PD1 and TCR beta, CTLA-4 and TCR alpha, CTLA-4 and TCR beta, LAG3 and TCR alpha, LAG3 and TCR beta, Tim3 and TCR alpha, Tim3 and TCR beta, BTLA and TCR alpha, BTLA and TCR beta, BY55 and TCR alpha, BY55 and TCR beta, TIGIT and TCR alpha, TIGIT and TCR beta, B7H5 and TCR alpha, B7H5 and TCR beta, LAIR1 and TCR alpha, LAIR1 and TCR beta, SIGLEC10 and TCR alpha, SIGLEC10 and TCR beta, 2B4 and TCR alpha, 2B4 and TCR beta and/or expresses a CAR, a multi-chain CAR and/or a pTalpha transgene.

In a further embodiment, TCR is rendered not functional in the cells according to the invention by inactivating TCR alpha gene and/or TCR beta gene(s). The above strategies are used more particularly to avoid GvHD. In a particular aspect of the present invention is a method to obtain modified cells derived from an individual, wherein said cells can proliferate independently of the Major Histocompatibility Complex signaling pathway. Said method comprises the following steps:

(a) Recovering cells from said individual;

(b) Genetically modifying said cells ex-vivo by inactivating TCR alpha or TCR beta genes;

(c) Cultivating genetically modified T-cells in vitro in appropriate conditions to amplify said cells.

Modified cells, which can proliferate independently of the Major Histocompatibility Complex signaling pathway, susceptible to be obtained by this method are encompassed in the scope of the present invention. Said modified cells can be used in a particular aspect of the invention for treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD); therefore in the scope of the present invention is a method of treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD) comprising treating said patient by administering to said patient an effective amount of modified cells comprising inactivated TCR alpha and/or TCR beta genes.

- *Immunosuppressive resistant T cells:*

In a particular aspect, one of the steps of genetically modifying cells can be a method comprising :

(a) modifying T-cells by inactivating at least one gene expressing a target for an immunosuppressive agent, and

(b) Expanding said cells, optionally in presence of said immunosuppressive agent.

An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. In other words, an immunosuppressive agent is a role played by a compound which is exhibited by a capability to diminish the extent and/or voracity of an immune response. As non-limiting example, an immunosuppressive agent can be a calcineurin inhibitor, a target of rapamycin, an interleukin-2 α-chain blocker, an inhibitor of inosine monophosphate dehydrogenase, an inhibitor of dihydrofolic acid reductase, a corticosteroid or an immunosuppressive antimetabolite. Classical cytotoxic immunosuppressants act by inhibiting DNA synthesis. Others may act through activation of T-cells or by inhibiting the activation of helper cells. The method according to the invention allows conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for immunosuppressive agent can be a receptor for an immunosuppressive agent such as: CD52, glucocorticoid receptor (GR), a FKBP family gene member and a cyclophilin family gene member.

By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In particular embodiment, the genetic modification of the method relies on the expression, in provided cells to engineer, of one rare-cutting endonuclease such that said rare-cutting endonuclease specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. In a particular embodiment, said method to engineer cells comprises at least one of the following steps:

(a) Providing a T-cell, preferably from a cell culture or from a blood sample;

(b) Selecting a gene in said T-cell expressing a target for an immunosuppressive agent;

(c) Introducing into said T-cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break said gene encoding a target for said immunosuppressive agent, and

(d) Expanding said cells, optionally in presence of said immunosuppressive agent.

In a more preferred embodiment, said method comprises:

(a) Providing a T-cell, preferably from a cell culture or from a blood sample;

(b) Selecting a gene in said T-cell expressing a target for an immunosuppressive agent;

(c) Transforming said T cell with nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break said gene encoding a target for said immunosuppressive agent, and

(d) Expressing said rare-cutting endonucleases into said T-cells;

(e) Expanding said cells, optionally in presence of said immunosuppressive agent.

In particular embodiment, said rare-cutting endonuclease specifically targets one gene selected from the group consisting of CD52, GR. In another embodiment, said gene of step (b), specific for an immunosuppressive treatment, is CD52 and the immunosuppressive treatment of step (d) or (e) comprises a humanized antibody targeting CD52 antigen.

In another embodiment, said gene of step (b), specific for an immunosuppressive treatment, is a glucocorticoid receptor (GR) and the immunosuppressive treatment of step d) or (e) comprises a corticosteroid such as dexamethasone.

In another embodiment, said target gene of step (b), specific for an immunosuppressive treatment, is a FKBP family gene member or a variant thereof and the immunosuppressive treatment of step (d) or (e) comprises FK506 also known as Tacrolimus or fujimycin. In another embodiment, said FKBP family gene member is FKBP12 or a variant thereof.

In another embodiment, said gene of step (b), specific for an immunosuppressive treatment, is a cyclophilin family gene member or a variant thereof and the immunosuppressive treatment of step (d) or (e) comprises cyclosporine.

- *Highly active T cells for immunotherapy*

In a particular aspect, one particular step of genetically modifying cell can be a method comprising:

(a) modifying T-cells by inactivating at least one immune checkpoint gene; and

(b) expanding said cells.

T cell-mediated immunity includes multiple sequential steps involving the clonal selection of antigen specific cells, their activation and proliferation in secondary lymphoid tissue, their trafficking to sites of antigen and inflammation, the execution of direct effector function and the provision of help (through cytokines and membrane ligands) for a multitude of effector immune cells. Each of these steps is regulated by counterbalancing stimulatory and inhibitory signal that fine-tune the response. It will be understood by those of ordinary skill in the art, that the term "immune checkpoints" means a group of molecules expressed by T cells. These molecules effectively serve as "brakes" to down-modulate or inhibit an immune response. Immune checkpoint molecules include, but are not limited to Programmed Death 1 (PD-1, also known as PDCD1 or CD279, accession number: NM_005018), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4, also known as CD152, GenBank accession number AF414120.1), LAG3 (also known as CD223, accession number: NM_002286.5), Tim3 (also known as HAVCR2, GenBank accession number: JX049979.1), BTLA (also known as CD272, accession number: NM_181780.3), BY55 (also known as CD160, GenBank accession number: CR541888.1), TIGIT (also known as VSTM3, accession number: NM_173799), B7H5 (also known as C10orf54, homolog of mouse vista gene, accession number: NM_022153.1), LAIR1 (also known as CD305, GenBank accession number: CR542051.1), SIGLEC10 (GeneBank accession number: AY358337.1), 2B4 (also known as CD244, accession number: NM_001166664.1), which directly inhibit immune cells. For example, CTLA-4 is a cell-surface protein expressed on certain CD4 and CD8 T cells; when engaged by its ligands (B7-1 and B7-2) on antigen presenting cells, T-cell activation and effector function are inhibited. Thus the present invention relates to a method of engineering T-cells, especially for immunotherapy, comprising genetically modifying T-cells by inactivating at least one protein involved in the immune check-point, in particular PD1 and/or CTLA-4.

In a particular embodiment, said method to engineer cells comprises at least one of the following steps:

(a) providing a T-cell, preferably from a cell culture or from a blood sample;

(b) introducing into said T-cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break one gene encoding a immune checkpoint protein,

(c) expanding said cells.

In a more preferred embodiment, said method comprises:

(a) providing a T-cell, preferably from a cell culture or from a blood sample;

(b) transforming said T cell with nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break a gene encoding a immune checkpoint protein;

(c) expressing said rare-cutting endonucleases into said T-cells;

(d) expanding said cells.

In particular embodiment, said rare-cutting endonuclease specifically targets one gene selected from the group consisting of: PD1, CTLA-4, LAG3, Tim3, BTLA, BY55, TIGIT, B7H5, LAIR1, SIGLEC10, 2B4, TCR alpha and TCR beta. In another embodiment, said rare-cutting endonuclease can be a meganuclease, a Zinc finger nuclease, a TALE-nuclease or CAS9/CRISPR endonuclease complex. In a preferred embodiment, said rare-cutting endonuclease is a TALE-nuclease. By TALE-nuclease is intended a fusion protein consisting of a DNA-binding domain derived from a Transcription Activator Like Effector (TALE) and one nuclease catalytic domain to cleave a nucleic acid target sequence. (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009; Christian, Cermak et al. 2010; Cermak, Doyle et al. 2011; Geissler, Scholze et al. 2011; Huang, Xiao et al. 2011; Li, Huang et al. 2011; Mahfouz, Li et al. 2011; Miller, Tan et al. 2011; Morbitzer, Romer et al. 2011; Mussolino, Morbitzer et al. 2011; Sander, Cade et al. 2011; Tesson, Usal et al. 2011; Weber, Gruetzner et al. 2011; Zhang, Cong et al. 2011; Deng, Yan et al. 2012; Li, Piatek et al. 2012; Mahfouz, Li et al. 2012; Mak, Bradley et al. 2012).

- *Non alloreactive T cells:*

In another embodiment, the present invention can be particularly suitable for allogeneic immunotherapy. In this case, one of the steps of genetically modifying cells can be a method comprising :

(a) modifying T-cells by inactivating at least one gene encoding a component of the T-cell receptor (TCR)

(b) Expanding said cells.

In particular embodiment, the genetic modification of the method relies on the expression, in provided cells to engineer, of one rare-cutting endonuclease such that said rare-cutting endonuclease specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. In a particular embodiment, said method to engineer cells comprises at least one of the following steps:

(a) Providing a T-cell, preferably from a cell culture or from a blood sample;

Introducing into said T-cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break at least one gene encoding a component of the T-cell receptor (TCR).

(b) Expanding said cells.

In a more preferred embodiment, said method comprises:

(a) Providing a T-cell, preferably from a cell culture or from a blood sample;

(b) Transforming said T cell with nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break at least one gene encoding a component of the T-cell receptor (TCR);

(c) Expressing said rare-cutting endonucleases into said T-cells;

(d) Sorting the transformed T-cells, which do not express TCR on their cell surface;

(e) Expanding said cells.

In order to engineer genetically highly active modified immune cells, the invention also provides methods where immune checkpoints are blocked by lack of expression of genes such as PD1 and CTLA-4.

The present application further discloses engineered immune cells in particular T cells to be used as medicament, more particularly, for treating or preventing cancer by administrating such immune cells to a living organism.

The T cells used for adoptive immunotherapy according to the present invention can be generated either by expansion of antigen-specific T cells or redirection of T cells through genetic engineering (Park, Rosenberg et al. 2011). Transfer of viral antigen specific T cells is a well-established procedure used for the treatment of transplant associated viral infections and rare viral-related malignancies. Similarly, isolation and transfer of tumor specific T cells has been shown to be successful in treating melanoma.

Activation and expansion of T cells

T-cells can be activated prior to or after genetic modification and expanded *in vitro* or *in vivo* generally according to the methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005. In general, they are expanded by contact with an agent that stimulates a CD3 TCR complex and a co-stimulatory molecule on the surface of the T cells to create an activation signal for the T-cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T-cell. As non-limiting examples, T cell populations may be stimulated *in vitro* such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody. For example, the agents providing each signal may be in solution or coupled to a surface. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. In further embodiments of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 5, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-g , 1L-4, 1L-7, GM-CSF, -10, - 2, 1L-15, TGFp, and TNF- or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanoi. Media can include RPMI 1640, A1M-V, DMEM, MEM, a-MEM, F-12, X-Vivo 1, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% C02). T cells that have been exposed to varied stimulation times may exhibit different characteristics

In another particular embodiment, said cells can be expanded by co-culturing with tissue or cells. Said cells can also be expanded *in vivo*, for example in the subject's blood after administrating said cell into the subject.

Therapeutic applications

The engineered isolated immune cell as previously described can be used as a medicament, in particular for the treatment of cancers or infections in a patient in need thereof. The present invention more particularly to methods for treating patients comprising at least one of the following steps:

(a)providing an immune-cell obtainable by any one of the methods previously described;

(b)Administrating said transformed immune cells to said patient,

Prior to administrating the T cells of the invention, the cells can undergo robust *in vivo* T cell expansion to obtain persistance for an extended amount of time.

Said treatment can be ameliorating, curative or prophylactic. It may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment.

By autologous, it is meant that cells, cell line or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor. By allogeneic is meant that the cells or population of cells used for treating patients are not originating from said patient but from a donor.

The invention is particularly suited for allogenic immunotherapy, insofar as it enables the transformation of T-cells, typically obtained from donors, into non-alloreactive cells. This may be done under standard protocols and reproduced as many times as needed. The resulted modified T cells may be pooled and administrated to one or several patients, being made available as an “off the shelf” therapeutic product.

Cells that can be used with the disclosed methods are described in the previous section. Said treatment can be used to treat patients diagnosed with cancer, viral infection, autoimmune disorders or Graft versus Host Disease (GvHD). Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise nonsolid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the multi-chain CARs of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

The treatment may be administered to patients in combination with one or more therapies against cancer selected from the group of antibodies therapy, chemotherapy, cytokines therapy, dendritic cell therapy, gene therapy, hormone therapy, laser light therapy and radiation therapy.

According to a preferred embodiment of the invention, said treatment can be administrated into patients undergoing an immunosuppressive treatment or chemotherapy since the present invention preferably provides cells or population of cells, which have been made resistant to at least one immunosuppressive and/or chemotherapy agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent or making it resistant to the chemotherapy treatment. In this aspect, the immunosuppressive or chemotherapy treatment can help the selection and expansion of the T-cells according to the invention within the patient.

The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermaliy, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

The administration of the cells or population of cells can consist of the administration of 10^4-10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. The cells or population of cells can be administrated in one or more doses. In another embodiment, said effective amount of cells are administrated as a single dose. In another embodiment, said effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

In another embodiment, said effective amount of cells or composition comprising those cells are administrated parenterally. Said administration can be an intravenous administration. Said administration can be directly done by injection within a tumor.

In certain embodiments of the present invention, cells are administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or nataliziimab treatment for MS patients or efaliztimab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycoplienolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1 1; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Citrr. Opin. mm n. 5:763-773, 93). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH, In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery. Said modified cells obtained by any one of the methods described here can be used in a particular aspect of the invention for treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD); therefore in the scope of the present invention is a method of treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD) comprising treating said patient by administering to said patient an effective amount of modified cells comprising inactivated TCR alpha and/or TCR beta genes.

Other definitions

- Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Amino acid substitution means the replacement of one amino acid residue with another, for instance the replacement of an Arginine residue with a Glutamine residue in a peptide sequence is an amino acid substitution.

- Nucleotides are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- “As used herein, “nucleic acid” or “polynucleotides” refers to nucleotides and/or polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes.

Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Nucleic acids can be either single stranded or double stranded.

- By chimeric antigen receptor (CAR) is intended molecules that combine a binding domain against a component present on the target cell, for example an antibody-based specificity for a desired antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-target cellular immune activity. In the prior art, CAR consisted of single-chain polypeptides comprising an extracellular single chain antibody (scFvFc) fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain (scFvFc:ζ) and have the ability, when expressed in T cells, to redirect antigen recognition based on the monoclonal antibody's specificity. One example of CAR used in the prior art are CARs directed against CD19 antigen ( ). The CARs according to the present invention are present under single-chain or multi-chain architectures. The extracellular domain(s) thereof consist of single-chain antigen recognition domain comprising a VNAR polypeptide as previously defined. This extracellular domain is anchored to the cell membrane by fusion with a transmembrane domain. The CAR can adopt a single or multi-chain architecture. when the CAR is under a single-chain, said transmembrane domain is fused or includes the signaling domain to form a unique polypeptide. When the CAR is a multi-chain CAR, the signaling domain may be present on another polypeptide that will assemble with the fusion polypeptide comprising the VNAR polypeptide.

- By " delivery vector" or " delivery vectors" is intended any delivery vector which can be used in the present invention to put into cell contact (i.e "contacting") or deliver inside cells or subcellular compartments (i.e "introducing") agents/chemicals and molecules (proteins or nucleic acids) needed in the present invention. It includes, but is not limited to liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsions or other appropriate transfer vectors. These delivery vectors allow delivery of molecules, chemicals, macromolecules (genes, proteins), or other vectors such as plasmids, peptides developed by Diatos. In these cases, delivery vectors are molecule carriers. By "delivery vector" or "delivery vectors" is also intended delivery methods to perform transfection.

- The terms "vector" or "vectors" refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A "vector" in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

- By “lentiviral vector” is meant HIV-Based lentiviral vectors that are very promising for gene delivery because of their relatively large packaging capacity, reduced immunogenicity and their ability to stably transduce with high efficiency a large range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration in the DNA of infected cells. By “integrative lentiviral vectors (or LV)”, is meant such vectors as nonlimiting example, that are able to integrate the genome of a target cell. At the opposite by “non-integrative lentiviral vectors (or NILV)” is meant efficient gene delivery vectors that do not integrate the genome of a target cell through the action of the virus integrase.

- Delivery vectors and vectors can be associated or combined with any cellular permeabilization techniques such as sonoporation or electroporation or derivatives of these techniques.

- By cell or cells is intended any eukaryotic living cells, primary cells and cell lines derived from these organisms for *in vitro* cultures.

- By “primary cell” or “primary cells” are intended cells taken directly from living tissue (i.e. biopsy material) and established for growth in vitro, that have undergone very few population doublings and are therefore more representative of the main functional components and characteristics of tissues from which they are derived from, in comparison to continuous tumorigenic or artificially immortalized cell lines.

As non-limiting examples cell lines can be selected from the group consisting of CHO-K1 cells; HEK293 cells; Caco2 cells; U2-OS cells; NIH 3T3 cells; NSO cells; SP2 cells; CHO-S cells; DG44 cells; K-562 cells, U-937 cells; MRC5 cells; IMR90 cells; Jurkat cells; HepG2 cells; HeLa cells; HT-1080 cells; HCT-116 cells; Hu-h7 cells; Huvec cells; Molt 4 cells.

All these cell lines can be modified by the method of the present invention to provide cell line models to produce, express, quantify, detect, study a gene or a protein of interest; these models can also be used to screen biologically active molecules of interest in research and production and various fields such as chemical, biofuels, therapeutics and agronomy as non-limiting examples.

- by “mutation” is intended the substitution, deletion, insertion of up to one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, twenty, twenty five, thirty, fourty, fifty, or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. The mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

- by “variant(s)”, it is intended a repeat variant, a variant, a DNA binding variant, a TALE-nuclease variant, a polypeptide variant obtained by mutation or replacement of at least one residue in the amino acid sequence of the parent molecule.

- by “functional variant” is intended a catalytically active mutant of a protein or a protein domain; such mutant may have the same activity compared to its parent protein or protein domain or additional properties, or higher or lower activity.

- By “gene” is meant the basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome, which codes for a specific protein or segment of protein. A gene typically includes a promoter, a 5' untranslated region, one or more coding sequences (exons), optionally introns, a 3' untranslated region. The gene may further comprise a terminator, enhancers and/or silencers.

- By “fusion protein” is intended the result of a well-known process in the art consisting in the joining of two or more genes which originally encode for separate proteins or part of them, the translation of said “fusion gene” resulting in a single polypeptide with functional properties derived from each of the original proteins.

-"identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default setting. For example, polypeptides having at least 70%, 85%, 90%, 95%, 98% or 99% identity to specific polypeptides described herein and preferably exhibiting substantially the same functions, as well as polynucleotide encoding such polypeptides, are contemplated.

- “similarity” describes the relationship between the amino acid sequences of two or more polypeptides. BLASTP may also be used to identify an amino acid sequence having at least 70%, 75%, 80%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% sequence similarity to a reference amino acid sequence using a similarity matrix such as BLOSUM45, BLOSUM62 or BLOSUM80. Unless otherwise indicated a similarity score will be based on use of BLOSUM62. When BLASTP is used, the percent similarity is based on the BLASTP positives score and the percent sequence identity is based on the BLASTP identities score. BLASTP “Identities” shows the number and fraction of total residues in the high scoring sequence pairs which are identical; and BLASTP “Positives” shows the number and fraction of residues for which the alignment scores have positive values and which are similar to each other. Amino acid sequences having these degrees of identity or similarity or any intermediate degree of identity of similarity to the amino acid sequences disclosed herein are contemplated and encompassed by this disclosure.

- “signal-transducing domain” or “co-stimulatory ligand” refers to a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T-cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation activation, differentiation and the like. A co-stimulatory ligand can include but is not limited to CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory igand (ICOS-L), intercellular adhesion molecule (ICAM, CD30L, CD40, CD70, CD83, HLA-G, MICA, M1CB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as but not limited to, CD27, CD28, 4-IBB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LTGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

- “bispecific antibody” refers to an antibody that has binding sites for two different antigens within a single antibody molecule. It will be appreciated by those skilled in the art that other molecules in addition to the canonical antibody structure may be constructed with two binding specificities. It will further be appreciated that antigen binding by bispecific antibodies may be simultaneous or sequential. Bispecific antibodies can be produced by chemical techniques (see e.g., Kranz et al. (1981) Proc. Natl. Acad. Sci. USA 78, 5807), by “polydoma” techniques (See U.S. Pat. No. 4,474,893) or by recombinant DNA techniques, which all are known per se. As a non-limiting example, each binding domain comprises at least one variable region from an antibody heavy chain ("VH or H region"), wherein the VH region of the first binding domain specifically binds to the lymphocyte marker such as CD3, and the VH region of the second binding domain specifically binds to tumor antigen.

-The term “extracellular ligand-binding domain” as used herein is defined as an oligo- or polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells.

The term "subject" or “patient” as used herein includes all members of the animal kingdom including non-human primates and humans.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.

The following examples are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

Examples

Electroporation of T cells with mRNA encoding respectively for an anti-CD19 single-chain and multi-chain chimeric antigen receptor (CAR):

The same protocol was followed with the following transcripts respectively illustrated in Figure 6 and 7:

- Monocistronic transcript of SEQ ID NO.110 encoding a VNAR-CAR single chain polypeptide directed against CD19 antigen. This transcripts encodes a single chain polypeptide comprising a VNAR polypeptide anti-CD19 derived from the scaffold SEQ ID NO.1 fused to a transmembrane domain from CD8 alpha, itself fused to the co-stimulatory domain 4-1BB and the signaling domain CD3zeta comprising an ITAM.

- Polycistronic transcript of SEQ ID NO.105 encoding a multi subunit CAR directed against CD19 antigen.T2A and F2A sequences are introduced to split the translated sequences into the different chains. The first chain encode the external VNAR polypeptide anti-CD19 (the same as for the single chain CAR) linked to the transmembrane domain of the FcεRI alpha chain.

In both architectures, the hinge region of CD8 alpha chain was used because it is detectable through PE-conjugated goat antibody staining at the surface of the transformed T-cells.

The transcripts also contained a T cell specific Alpha Signal peptide sequence to enable an efficient addressing to the plasma membrane.

Humanization of the VNAR polypeptide use for targeting CD19 could be done by replacing different structural element of the VNAR primary structure (i.e mostly located outside of CDR3 and CDR1 regions) by amino acid sequence found in structurally similar human antibodies. As an example, such approach has been successfully used to humanize 5A7 VNAR using the human antibody DPK9, a member of variable kappa subgroup 1 (Vk1) as a framework

5X10^6 T cells preactivated several days (3-5) with anti-CD3/CD28 coated beads and IL2 were resuspended in cytoporation buffer T, and electroporated in 0.4cm cuvettes without mRNA or with 10µg of mRNA respectively encoding the single chain VNAR-CAR (SEQ ID NO: 110)and the multi-chain VNAR-CAR (SEQ ID NO.105).

24 hours post electroporation, cells were stained with a fixable viability dye eFluor-780 and a PE-conjugated goat anti-CD8 to assess the cell surface expression of the CAR on the live cells.

24 hours post electroporation, T cells were cocultured with Daudi ($CD19^+$) cells for 6 hours and analyzed by flow cytometry to detect the expression of the degranulation marker CD107a at their surface (Betts, Brenchley et al. 2003).

The results showed that the majority of the cells electroporated, either with the monocistronic mRNA or the polycistronic mRNA as described above degranulated in the presence of target cells expressing CD19. These results clearly demonstrate that the VNAR-CAR expressed at the surface of electroporated T cells were active under both single-chain and multi-chain architectures.

Table 2 - Sequences listed in the present specification

Sequence Description	**SEQ_ID_NO**
>gi|491668396|pdb|4HGK|D Chain D, Shark Ignar Variable Domain (E06)	SEQ_ID NO 1
>gi|491668397|pdb|4HGM|A Chain A, Shark Ignar Variable Domain	SEQ_ID NO 2
>gi|59892033|gb|AAX10148.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 3
>gi|59892031|gb|AAX10147.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 4
>gi|355525308|gb|AES92986.1| IgNAR immunoglobulin heavy chain secretory form, partial [Squalus acanthias]	SEQ_ID NO 5
>gi|355525312|gb|AES92988.1| IgNAR immunoglobulin heavy chain secretory form, partial [Squalus acanthias]	SEQ_ID NO 6
>gi|355525306|gb|AES92985.1| IgNAR immunoglobulin heavy chain secretory form, partial [Squalus acanthias]	SEQ_ID NO 7
>gi|59892021|gb|AAX10142.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 8
>gi|59892019|gb|AAX10141.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 9
>gi|59892017|gb|AAX10140.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 10
>gi|21539972|gb|AAM52970.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 11
>gi|355525310|gb|AES92987.1| IgNAR immunoglobulin heavy chain secretory form, partial [Squalus acanthias]	SEQ_ID NO 12
>gi|25987499|gb|AAN75876.1|AF447120_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 13
>gi|21805812|gb|AAM76812.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 14
>gi|25987497|gb|AAN75875.1|AF447119_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 15
>gi|307685087|dbj|BAJ20185.1| immunoglobulin NAR [Triakis scyllium]	SEQ_ID NO 16
>gi|59892015|gb|AAX10139.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 17
>gi|3982965|gb|AAC83733.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 18

>gi|21747962|gb|AAM76235.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 19
>gi|21898882|gb|AAM77162.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 20
>gi|21805800|gb|AAM76806.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 21
>gi|59892023|gb|AAX10143.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 22
>gi|21805822|gb|AAM76817.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 23
>gi|21898926|gb|AAM77183.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 24
>gi|21655108|gb|AAL58520.1| new antigen receptor variable domain [Orectolobus maculatus]	SEQ_ID NO 25
>gi|52696108|pdb|1VER|A Chain A, Structure Of New Antigen Receptor Variable Domain From Sharks >gi|32709090|gb|AAP86761.1| new antigen receptor variable domain [Orectolobus maculatus]	SEQ_ID NO 26
>gi|3986584|gb|AAC84086.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 27
>gi|3983003|gb|AAC83752.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 28
>gi|15420366|gb|AAK97360.1| new antigen receptor [Orectolobus maculatus]	SEQ_ID NO 29
>gi|59892029|gb|AAX10146.1| immunoglobulin NAR variable region [Heterodontus francisci]	SEQ_ID NO 30
>gi|59892025|gb|AAX10144.1| immunoglobulin NAR variable region, partial [Heterodontus francisci]	SEQ_ID NO 31
>gi|25987461|gb|AAN75857.1|AF447101_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 32
>gi|21898887|gb|AAM77164.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 33
>gi|21898924|gb|AAM77182.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 34
>gi|3983053|gb|AAC83777.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 35
>gi|21539902|gb|AAM52938.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 36
>gi|307685089|dbj|BAJ20186.1| immunoglobulin NAR [Triakis scyllium]	SEQ_ID NO 37
>gi|3986580|gb|AAC84084.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 38
>gi|126009471|gb|ABN64030.1| antigen receptor variable domain [Orectolobus maculatus]	SEQ_ID NO 39
>gi|25987459|gb|AAN75856.1|AF447100_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 40

>gi|307685093|dbj|BAJ20188.1| immunoglobulin NAR [Triakis scyllium]	SEQ_ID NO 41
>gi|21748031|gb|AAM76269.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 42
>gi|3986664|gb|AAC84126.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 43
>gi|3982949|gb|AAC83725.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 44
>gi|21885446|gb|AAM76964.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 45
>gi|21069163|gb|AAM33846.1|AF466396_1 new antigen receptor variable domain [Orectolobus maculatus]	SEQ_ID NO 46
>gi|21898928|gb|AAM77184.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 47
>gi|21885420|gb|AAM76954.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 48
>gi|21748025|gb|AAM76266.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 49
>gi|21748015|gb|AAM76261.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 50
>gi|21539976|gb|AAM52972.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 51
>gi|21747995|gb|AAM76251.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 52
>gi|21805816|gb|AAM76814.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 53
>gi|21747977|gb|AAM76242.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 54
>gi|21539983|gb|AAM52975.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 55
>gi|21885436|gb|AAM76960.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 56
>gi|25987495|gb|AAN75874.1|AF447118_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 57
>gi|21885442|gb|AAM76962.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 58
>gi|21885444|gb|AAM76963.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 59
>gi|21748009|gb|AAM76258.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 60
>gi|21539988|gb|AAM52977.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 61
>gi|21748029|gb|AAM76268.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 62
>gi|3986602|gb|AAC84095.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 63
>gi|699465|gb|AAB48206.1| novel antigen receptor, partial [Ginglymostoma cirratum]	SEQ_ID NO 64
>gi|21539974|gb|AAM52971.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 65

>gi|161172318|pdb|2Z8W|C Chain C, Structure Of An Ignar-Ama1 Complex >gi|161172319|pdb|2Z8W|D Chain D, Structure Of An Ignar-Ama1 Complex	SEQ_ID NO 66
>gi|21747979|gb|AAM76243.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 67
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>gi|52696109|pdb|1VES|A Chain A, Structure Of New Antigen Receptor Variable Domain From Sharks	SEQ_ID NO 71
>gi|21898858|gb|AAM77150.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 72
>gi|3986668|gb|AAC84128.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 73
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>gi|21747970|gb|AAM76239.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 75
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>gi|134104489|pdb|2I26|N Chain N, Crystal Structure Analysis Of The Nurse Shark New Antigen Receptor Ancestral Variable Domain In Complex With Lysozyme	SEQ_ID NO 77
>gi|3982937|gb|AAC83719.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 78
>gi|3982933|gb|AAC83717.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 79
>gi|3982955|gb|AAC83728.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 80
>gi|307685091|dbj|BAJ20187.1| immunoglobulin NAR [Triakis scyllium]	SEQ_ID NO 81
>gi|3982959|gb|AAC83730.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 82
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>gi|25987449|gb|AAN75851.1|AF447095_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 84
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>gi|25987493|gb|AAN75873.1|AF447117_1 novel antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 87
>gi|21885434|gb|AAM76959.1| antigen receptor [Ginglymostoma cirratum] >gi|21885454|gb|AAM76968.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 88

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>gi|3982975|gb|AAC83738.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 91
>gi|21885440|gb|AAM76961.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 92
>gi|3986588|gb|AAC84088.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 93
>gi|21885395|gb|AAM76942.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 94
>gi|21539954|gb|AAM52962.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 95
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>gi|699417|gb|AAB48359.1| novel antigen receptor, partial [Ginglymostoma cirratum]	SEQ_ID NO 97
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>gi|21805883|gb|AAM76843.1| antigen receptor [Ginglymostoma cirratum]	SEQ_ID NO 99
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New antigen receptor (Orectolobus) Q90XW8_9CHON amino acid sequence (Orectolobus maculatus clone 7E-80 new antigen receptor)	SEQ_ID NO 102
Alpha-Signal-peptide(from pCLS22370) amino acid sequence	SEQ_ID NO 103
Signal-peptide(from Q90XW8_9CHON) amino acid sequence	SEQ_ID NO 104
Chimeric VNAR-CAR2(multi-chain + endogeneous hinge domain)	SEQ_ID NO 105
Chimeric VNAR-CAR3 (multi-chain + IgG1 hinge domain)	SEQ_ID NO 106
Chimeric VNAR-CAR4 (multi-chain + CD8 hinge domain)	SEQ_ID NO 107
Chimeric VNAR-CAR5 (single chain + endogeneous hinge domain)	SEQ_ID NO 108
Chimeric VNAR-CAR6 (single chain + IgG1 hinge domain)	SEQ_ID NO 109
Chimeric VNAR-CAR7 (single chain + CD8 hinge domain)	SEQ_ID NO 110
IgG1 hinge CH2 CH3	SEQ_ID NO 111
CD8 alpha hinge	SEQ_ID NO 112
>sp|P02786|89-760 TFR1_HUMAN amino acid sequence of the extracellular region	SEQ_ID NO 113

>sp|Q9UP52|105-801 TFR2_HUMAN amino acid sequence of the extracellular region	SEQ_ID NO 114
12A9	SEQ_ID NO 115

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CLAIMS

1) A chimeric antigen receptor (CAR) characterized in that it comprises:

   i) one extracellular antigen recognition domain comprising a VNAR polypeptide; and

   ii) one transmembrane polypeptide comprising at least one signal-transducing domain;

2) A chimeric antigen receptor according to claim 1, wherein said antigen recognition domain comprises only two Complementary Determining Regions (CDRs) referred to as CDR1 and CDR3.

3) A chimeric antigen receptor according to claim 1, wherein said antigen recognition domain has only one Complementary Determining Regions (CDR3).

4) A chimeric antigen receptor according to any one of claims 1 to 3, wherein the specificity of recognition of the CAR for an antigen is determined by said CDR3.

5) A chimeric antigen receptor according to any one of claims 1 to 4, wherein said CDR3 comprises at least two cysteine residues creating disulfide bounds with residues from the VNAR polypeptide.

6) A Chimeric Antigen Receptor according to any one of claims 1 to 5, wherein said CAR further comprises a hinge region between its transmembrane region and its extracellular antigen recognition domain.

7) A chimeric antigen receptor according to any one of claims 1 to 6, wherein its entire extracellular domain is shorter than 150 amino acids.

8) A chimeric antigen receptor according to any one of claims 1 to 7, wherein said VNAR polypeptide has at least 50% sequence identity with any of SEQ ID NO.1 to 100.

9) A chimeric antigen receptor according to claim 8, wherein said VNAR polypeptide sequence is humanized.

10) A Chimeric Antigen Receptor according to any one of claims 1 to 9, wherein the transmembrane region of said CAR comprises a signal transducing domain selected from the group consisting of: TCR zeta chain, Fc receptor chain, immunoreceptor tyrosine-based activation motif (ITAM).

11) A Chimeric Antigen Receptor according to any one of claims 1 to 11, wherein it further comprises a co-stimulatory molecule selected from the group consisting of: CD28, OX40, ICOS, CD137 and CD8.

12) A Chimeric Antigen Receptor according to any one of claims 1 to 11, wherein it is under the form of a single-chain CAR.

13) A Chimeric Antigen Receptor according to any one of claims 1 to 12, wherein it is under the form of a multi-chain CAR.

14) The multi-chain Chimeric Antigen Receptor of claim 13, wherein the signal transducing domain and extracellular antigen recognition domain of said CAR are not born on the same chain, but on at least two different chains which interact to form a dimeric or a multimeric Chimeric Antigen Receptor.

15) The multi-chain Chimeric Antigen Receptor of claim 14, wherein said different chains comprise a portion of a FcεRI alpha chain, FcεRI beta chain and/or FcεRI gamma chain or a variant thereof, such that said FcεRI chains dimerize, trimerize or tetramerize together to form a multimeric CAR.

16) The multi-chain Chimeric Antigen Receptor of claim 14 or 15, wherein the different multimers assemble so that the co-stimulation or signalization domains are in a juxtamembrane position.

17) A polynucleotide comprising a nucleic acid sequence encoding a CAR according to any one of claims 1 to 13.

18) A polynucleotide comprising nucleic acid sequences encoding two or more transmembrane polypeptides composing the multi-chain CAR according to any one of claims 14 to 16.

19) A vector comprising a polynucleotide according to claim 17 or 18.

20) A method of engineering an immune cell comprising:

    (a) Providing an immune cell;

    (b) Expressing at the surface of said cells at least one Chimeric Antigen Receptor according to any one of claims 1 to 16.

21) The method of engineering an immune cell according to claim 20 comprising:

    (a) Providing an immune cell;

    (b) Introducing into said cell at least one polynucleotide according to claims 17 or 18;

(c) Expressing said polynucleotides into said cell.

22) An isolated immune cell obtainable from the method according to any one of claims 20 or 21.

23) An isolated immune cell comprising at least one Chimeric Antigen Receptor according to any one of claims 1 to 16.

24) An isolated cell according to any one of claims 22 or 23, wherein it is derived from inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes.

25) An isolated immune cell according to any one of claims 22 to 24 for its use as a medicament.

26) An isolated immune cell according to any one of claims 22 to 24 for its use as a medicament for treating cancer or a self-immune disease.

27) An isolated immune cell according to any one of claims 22 to 24 for its use as a medicament for treating liquid tumors.

28) An isolated immune cell according to any one of claims 22 to 24 for its use as a medicament for treating B cell malignancies.

29) An isolated immune cell according to any one of claims 22 to 24 for its use as a medicament for targeting drug resistant cells expressing ions channels of efflux pumps on their surface, alone or in combination with chemotherapy.

30) An isolated immune cell according to any one of claims 22 to 24 for its use in adoptive immunotherapy.

31) A method for treating a patient in need thereof comprising:

a) Providing immune cells comprising a Chimeric Antigen Receptor according to any one of claims 1 to 16;

b) Administrating said immune cells to said patient.

32) The method for treating a patient according to claim 31, wherein said immune cells under a) are recovered from donors (allogeneic mode).

33) The method for treating a patient according to claim 31, wherein said immune cells under a) are recovered from the patient in need thereof (autologous mode).

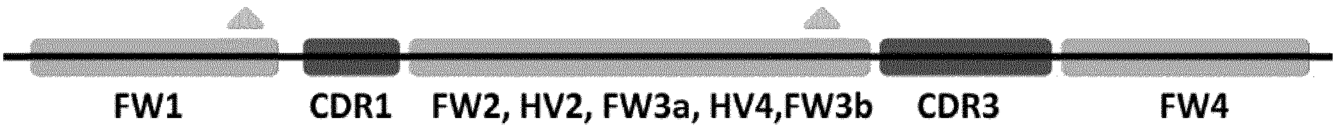

Fig. 1

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5A7xxx1          -------------------ARVDQTPRSVTKETGESLTINCVLRDASYALGSTCWYRKKS
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                 *. ::: ** .*** *:*:.*::* ** * ** * *..** *
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                          |         |         |         |
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12A9xx3          KE-GAGTVLTVK------------------------------------
5A7xxx1          AECGDGTAVTVN------------------------------------
E06xxx0          GD-GAGTVLTVNHHHHHH------------------------------
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7e80: SEQ ID NO. 102 (type IV)
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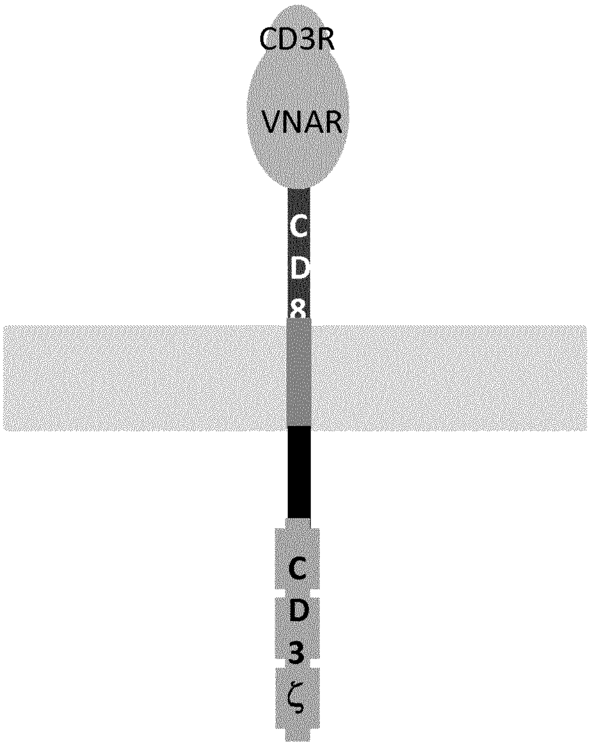
5A7: SEQ ID NO. 101 (type I)
E06: SEQ ID NO. 1

Fig. 2



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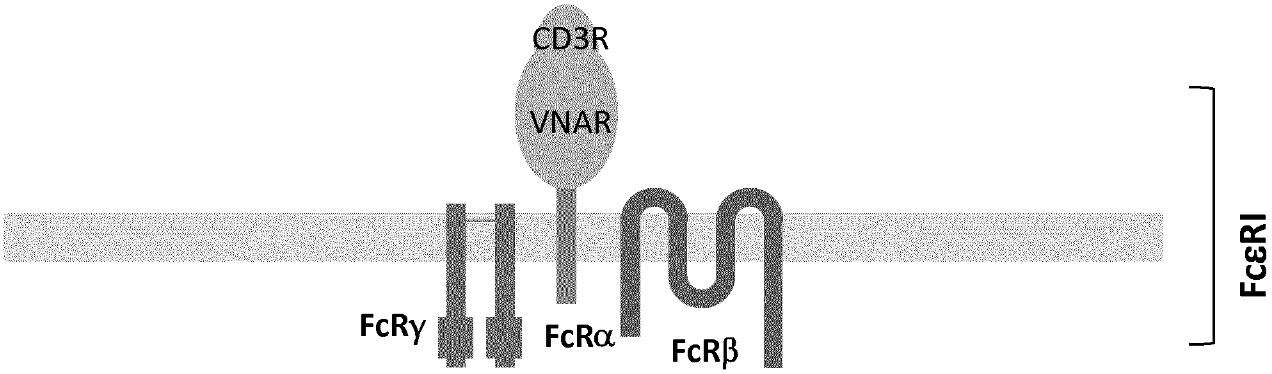

Fig. 4

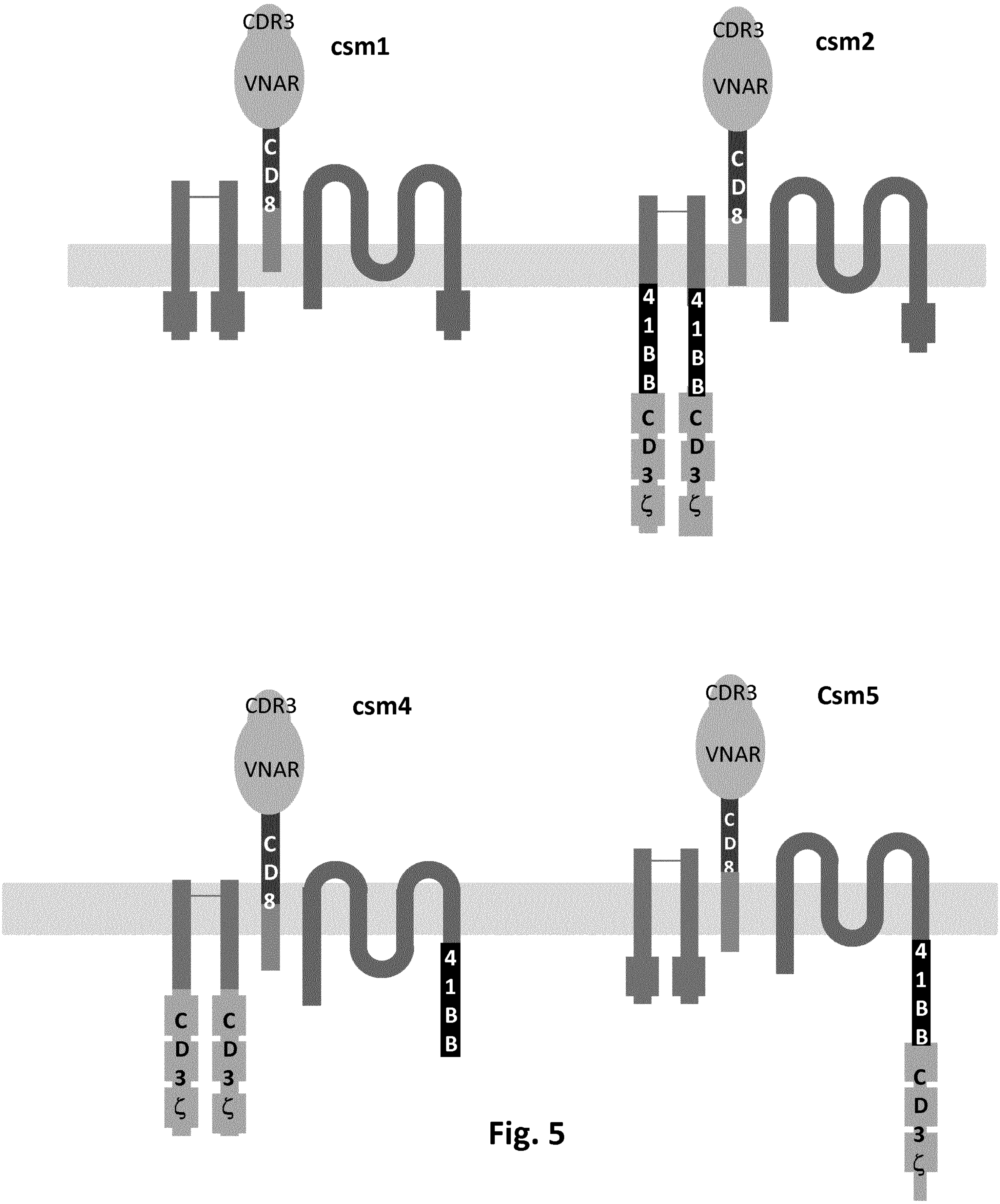
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Fig. 5

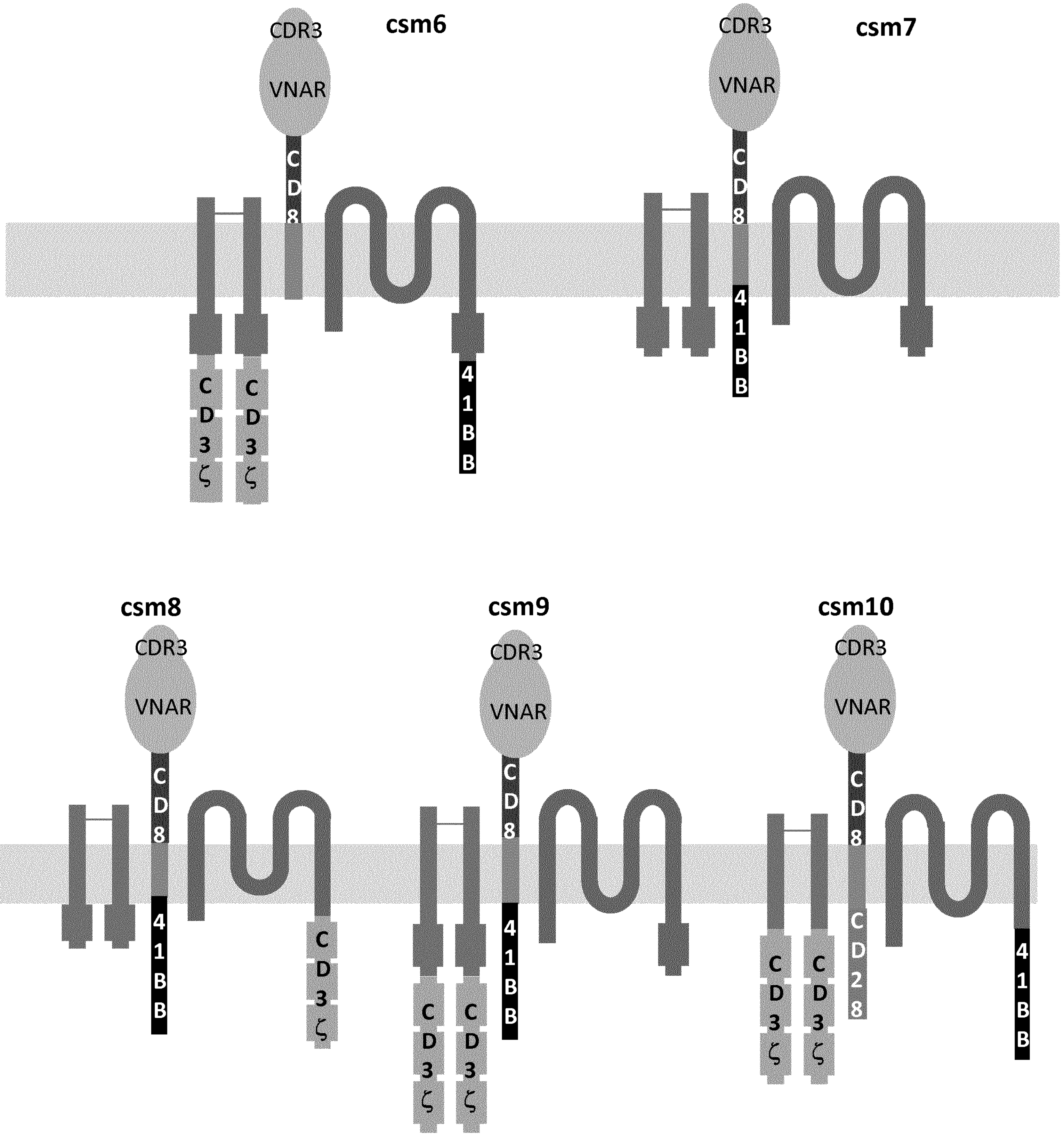
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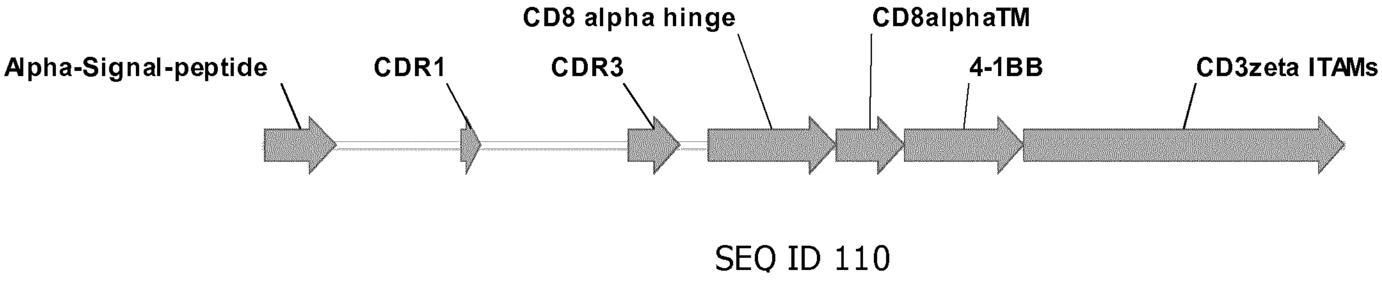
Fig. 6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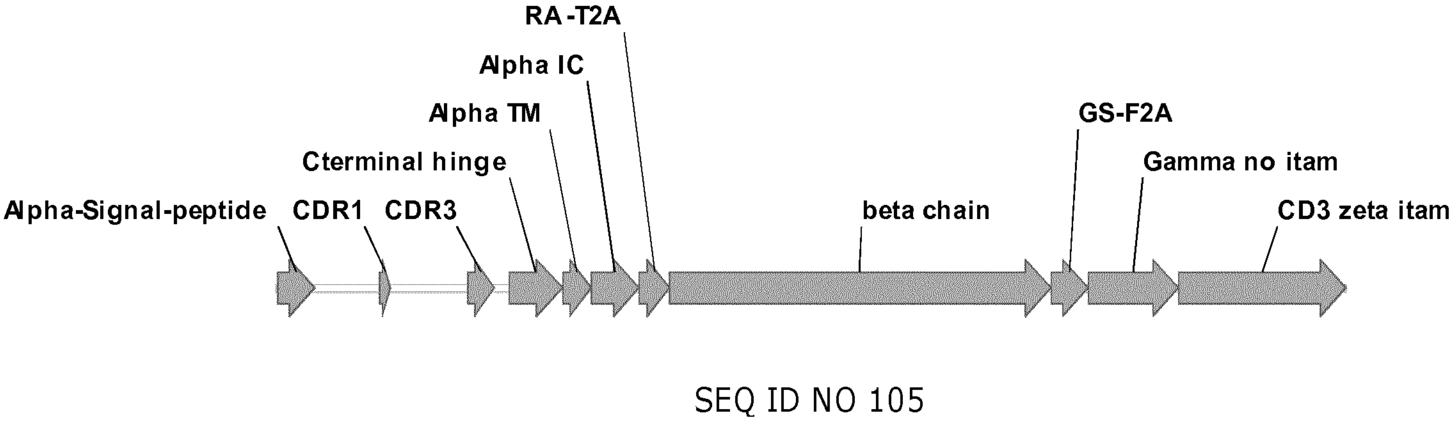
CD8alphaTM
Alpha-Signal-peptide
CDR1
CDR3
4-1BB
CD3zeta ITAMs
SEQ ID 110

Fig. 7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Alpha TM
GS-F2A
Cterminal hinge
Gamma no itam
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SEQ ID NO 105

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/050581

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 C07K14/725 C12N5/0783
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)
A61K C07K C12N

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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FATEMEH RAHIMI JAMNANI ET AL: "T cells expressing VHH-directed oligoclonal chimeric HER2 antigen receptors: Towards tumor-directed oligoclonal T cell therapy", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - GENERAL SUBJECTS, vol. 1840, no. 1, 27 September 2013 (2013-09-27), pages 378-386, XP055108962, ISSN: 0304-4165, DOI: 10.1016/j.bbagen.2013.09.029 the whole document ----- -/--	1-33

☒ 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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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 30 March 2015	Date of mailing of the international search report 23/04/2015
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 Bradbrook, Derek

1

International application No
PCT/EP2015/050581

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHARIFZADEH ZAHRA ET AL: "Genetically engineered T cells bearing chimeric nanoconstructed receptors harboring TAG-72-specific camelid single domain antibodies as targeting agents", CANCER LETTERS, vol. 334, no. 2, 1 July 2013 (2013-07-01), pages 237-244, XP028555132, ISSN: 0304-3835, DOI: 10.1016/J.CANLET.2012.08.010 the whole document -----	1-33
A	WO 2013/167883 A1 (UNIV ABERDEEN [GB]) 14 November 2013 (2013-11-14) the whole document -----	1-33
A	WO 2013/038203 A1 (HEALTH PROT AGENCY [GB]; SUTTON JOHN MARK [GB]; SKIPPER PHILIP JAMES A) 21 March 2013 (2013-03-21) the whole document -----	1-33
A	MARTIN F. FLAJNIK ET AL: "A Case Of Convergence: Why Did a Simple Alternative to Canonical Antibodies Arise in Sharks and Camels?", PLOS BIOLOGY, vol. 9, no. 8, 2 August 2011 (2011-08-02), page e1001120, XP055109496, DOI: 10.1371/journal.pbio.1001120 the whole document -----	1-33

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/050581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013167883 A1	14-11-2013	AU 2013257848 A1	27-11-2014
		EP 2847229 A1	18-03-2015
		US 2013302250 A1	14-11-2013
		WO 2013167883 A1	14-11-2013
WO 2013038203 A1	21-03-2013	AU 2012308132 A1	13-03-2014
		CA 2847607 A1	21-03-2013
		EP 2756081 A1	23-07-2014
		JP 2014531206 A	27-11-2014
		US 2014315215 A1	23-10-2014
		WO 2013038203 A1	21-03-2013

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/050581

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☒ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: