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(54) Title: ROR1 SPECIFIC MULTI-CHAIN CHIMERIC ANTIGEN RECEPTOR

(57) Abstract: The present invention relates to a new generation of chimeric antigen receptors (CAR) referred to as multi-chain CARs, which are made specific to the antigen ROR1. Such CARs aim to redirect immune cell specificity and reactivity toward malignant cells expressing the tumor antigen ROR1. The alpha, beta and gamma polypeptides composing these CARs are designed to assemble in juxtamembrane position, which forms flexible architecture closer to natural receptors, that confers optimal signal transduction. 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. The invention opens the way to efficient adoptive immunotherapy strategies for treating cancer, especially chronic lymphocytic leukemia or solid tumors.

ROR1 SPECIFIC MULTI-CHAIN CHIMERIC ANTIGEN RECEPTOR

Field of the invention

The present invention relates to a new generation of chimeric antigen receptors (CAR) referred to as multi-chain CARs, which are made specific to the antigen ROR1. Such CARs aim to redirect immune cell specificity and reactivity toward malignant cells expressing the tumor antigen ROR1. The alpha, beta and gamma polypeptides composing these CARs are designed to assemble in juxtamembrane position, which forms flexible architecture closer to natural receptors, that confers optimal signal transduction. 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. The invention opens the way to efficient adoptive immunotherapy strategies for treating cancer, especially chronic lymphocytic leukemia (CLL) or solid tumors such as breast, colon, lung, and kidney tumors.

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) Treating Cancer with Genetically Engineered T Cells. *Trends Biotechnol.* 29(11): 550–557) 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) Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood. 116(7): 1035–1044). CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains to form a single-chain fusion molecule. However, this approach has so far proven efficiency only with respect to patients with acute lymphoblastic leukemia (ALL) by targeting malignant B cells bearing the antigen CD19 (Porter, D.L. et al. (2011) Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia.

N. Engl. J. Med. 365:725-733). Chronic lymphocytic leukemia (CLL) is one of the most commonly diagnosed leukemias managed by practicing hematologists. For many years patients with CLL have been viewed as similar, with a long natural history and only marginally effective therapies that rarely yielded complete responses. Recently, several important observations related to the biologic significance of V_H mutational status and associated ZAP-70 overexpression, disrupted p53 function, and chromosomal aberrations have led to the ability to identify patients at high risk for early disease progression and inferior survival. Concurrent with these investigations, several treatments including the nucleoside analogues, monoclonal antibodies rituximab and alemtuzumab have been introduced. Combination of these therapies in clinical trials has led to high complete and overall response rates when applied as initial therapy for symptomatic CLL. Thus, the complexity of initial risk stratification of CLL and treatment has increased significantly. Furthermore, when these initial therapies do not work, approach of the CLL patient with fludarabine-refractory disease can be quite challenging (Byrd J.C et al, 2014).

One candidate antigen of immunotherapies for chronic lymphocytic leukemia (CLL) is Tyrosine-protein kinase transmembrane receptor ROR1 (also called NTRKR1; UniProtKB/TrEMBL) entries : Q01973). ROR1 (The receptor tyrosine kinase-like orphan receptor 1) is a 120-kDa glycoprotein containing an extracellular immunoglobulin (Ig)-like, Kringle, and Frizzled-like cysteine rich domain (Figure 1).The protein encoded by this gene is a receptor tyrosine kinase that modulates neurite growth in the central nervous system. It is a type I membrane protein and belongs to the ROR subfamily of cell surface receptors (Reddy et al, 1997). The Ror1 protein expression in patients with CLL but not in normal leukocytes merits further studies of its role in the pathobiology of CLL, which may provide a basis for development of Ror1 directed targeted therapy (Daneshmanesh et al; 2008). ROR1 is expressed on a variety of B-cell malignancies, and subsets of some solid tumors, including breast, colon, lung, and kidney tumors. ROR1 functions in oncogenic signaling to promote tumor cell survival in epithelial tumors. Importantly, ROR1 is not expressed on vital organs, except adipose and pancreatic tissue, which reduces potential toxicities from killing of normal cells (Hudecek et al, 2013). ROR1 is expressed during embryogenesis but absent from normal adult tissues, apart from a subset of immature B-cell precursors, and low-level expression on adipocytes (Hudecek et al., 2010; Matsuda et al., 2001). ROR1 was first shown to be expressed in B-cell chronic lymphocytic leukemia (B-CLL) by transcriptional profiling (Klein et al., 2001; Rosenwald et al., 2001) and was subsequently identified on the surface of many cancers

including mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL) with a t(1;19) chromosome translocation, and a subset of lung, breast, colon, pancreas, renal, and ovarian cancers (Baskar et al., 2008; Bicocca et al., 2012; Daneshmanesh et al., 2008; Dave et al., 2012; Fukuda et al., 2008; Yamaguchi et al., 2012; Zhang et al., 2012a, 2012b). In both lung adenocarcinoma and t(1;19) ALL, ROR1 cooperates in oncogenic signaling and knockdown of ROR1 with siRNA exposed a critical role for this molecule in maintaining tumor cell survival (Bicocca et al., 2012; Choudhury et al., 2010; Gentile et al., 2011; Yamaguchi et al., 2012). Thus, ROR1 loss may not be readily tolerated by tumors making it an attractive candidate for CAR directed T-cell therapy that could be broadly applied. It thus represents an appropriate target antigen for treating CLL or solid tumors, especially using CAR-expressing T cells.

The laboratories of Dr. Stanley Riddell and Dr. Laurence Cooper have previously engineered and validated anti-ROR1 scCARs containing the 4A5 and the 2A2 scFvs, respectively (Cooper et al 2010; Hudecek et al.,2013) . In particular, Hudecek et al discloses anti-ROR1 scCARs which contain an IgG4 hinge of diverse length and a CD28 transmembrane domain.

There is still the need for the improvement of CAR functionality by designing CAR architecture and using suitable components since these parameters play a role important and a fine tuning is necessary.

In the context of developing therapeutic grade engineered immune cells that can target malignant or infected cells, the inventors have sought for improved CAR architectures, which would be closer to natural ones and likely to behave accordingly using any extracellular mono or multi-specific ligand binding domains. In WO2014039523, they described a new generation of CARs involving separate polypeptide sub-units according to the present invention, referred to as “multi-chain CARs”. According to this architecture, the signaling domains and co-stimulatory domains are located on different polypeptide chains (Figure 2). Such multi-chain CARs can be derived from FcεRI, by replacing the high affinity IgE binding domain of FcεRI alpha chain by an extracellular ligand-binding domain such as scFv, whereas the N and/or C-termini tails of FcεRI beta and/or gamma chains are fused to signal transducing domains and co-stimulatory domains respectively. The extracellular ligand binding domain has the role of redirecting T-cell specificity towards cell targets, while the signal transducing domains activate the immune cell response. The fact that the different polypeptides derived from the alpha, beta and gamma polypeptides from FcεRI are transmembrane polypeptides sitting in juxtamembrane position, provides a more flexible architecture to CARs and reduces background activation of immune cells. However, this flexibility provides more variability from

one binding sequence to another, so that it is difficult to predict which binding domain and optimal architecture provide with an appropriate specificity towards ROR1.

It can be noted that single and multichain CAR architectures bearing the same scFvs may not perform the same way, depending of parameters which are not always controlled by the skilled man of the art. This remark may apply also to the type of expression used (transient or stable by using respectively, for instance, mRNA or lentivirus delivery).

Another aspect to be considered is the potential adverse effects linked to the infusion of engineered T cells to the patient, and in particular the cytokine-release syndrome (CRS). Thus, there is the need for designing the right CAR architecture and their specific components which can reduce the occurrence of such adverse events.

The invention provides with optimally designed multi-chain CAR bearing scFv extracellular domain, which are particularly suited to target malignant cells bearing ROR1 as a surface protein. It has been shown in the present invention that a particular architecture of multichain CAR with well-defined components can allow the engineered immune cells to be cytotoxic towards ROR1 antigen-bearing tumor cells. From those mcCARs, 2 of them csm13 and csm14 appear to be performant in terms of specific lysis while the immune cells keep their innate function.

This achievement opens the way to new immunotherapy treatments of malignant cells diagnosed to be ROR1 positive, such as those found in CLL and solid tumors in particular breast, colon, lung, and kidney tumors.

Summary of the invention

The inventors have generated ROR1 specific multichain CARs different scFV derived from ROR1 specific antibodies.

Following non-specific activation in vitro (e.g. with anti CD3/CD28 coated beads and recombinant IL2), T-cells from donors have been transformed with polynucleotides expressing these CARs using viral transduction. In certain instances, the T-cells may be further engineered to create non-alloreactive T-cells, more especially by disruption of a component of TCR (αβ – T-Cell receptors) to prevent Graft versus host reaction. The resulting engineered T-cells displayed reactivity in-vitro against ROR1 positive cells to various extend, showing that the CARs of the present invention contribute to antigen dependent activation, proliferation of the T-cells, and

also could be cytotoxic towards cells expressing ROR1, making them useful for immunotherapy.

The polypeptides and polynucleotide sequences encoding the CARs of the present invention are detailed in the present specification. Two anti-ROR1 multi-chain CARs (mcCARs) –csm13 and csm14- bearing scFvs from D10 and 2A2 monoclonal antibodies respectively, have shown remarkably their highly expression on the cell surface which could remain over a 2-weeks period. Moreover, csm13 and csm14 have shown their cytotoxic effect towards ROR1-expressing cells, while retaining their innate function.

The engineered immune cells of the present invention are particularly useful for treating haematological cancer conditions or for treating solid tumor.

Description of the Figures:

Figure 1: Structure of the ROR1 protein with its ecto- and endo-domain parts. Type 1 receptor tyrosine kinase evolutionarily conserved, co-receptor with Frizzled-2/4, with immunoglobulin (Ig) domain, cysteine-rich domain (CRD), and Kringle domain. The intracellular portion contains tyrosine kinase (TK) domain, proline-rich domain (PRD) flanked by Ser/Thr rich domains (S/TRD1 and 2.

Figure 2: Schematic representation of FcεRI from which derivate the multi-chain CAR architecture according to the invention. FcεRI is composed of 3 transmembrane chains α, β and γ.

Figure 3: General structure of the polycistronic construct encoding the ROR1 muti-chain CAR according to the invention. The one described in the examples of the present invention is based on a polycistronic lentiviral vector such as pSEW..

Figure 4: Different architectures of the ROR1 specific muti-chain CAR according to the invention. From left to right: polypeptide gamma (fused to ITAM of CD3zeta), polypeptide alpha (fused to ScFv), polypeptide beta (fused to co-stimulatory domain from 41BB in A and B, and CD28 in C and D). **A** and **B**: polypeptide beta is fused to co-stimulatory domain from 41BB, VL and VH fragments being in opposite orders.

Figure 5: FACS analysis showing cell surface expression of the multi-chain CARs mc13 and mc14 in transduced T cells. Data are presented as mean+/- SD of 3 independent experiments.

Figure 6: Degranulation assay performed on multi-chain CARs mc13 and mc14 in the presence of ROR1-positive cell line (Jeko-1), or ROR1-negative cell line (SupT1) or in absence of cell line (medium), or in PMA/ionomycin (positive control for T cell activation). A control was done for untransduced T cells (No LV). Data are presented as mean+/- SD of 3 independent experiments.)

Figure 7: Cytotoxicity assay performed for multi-chain CARs mc13 and mc14 in the presence of ROR1-positive cell line (Jeko-1). A control was done for untransduced T cells (No LV). Data are presented as mean+/- SD of 3 independent experiments.

Figure 8: INFγ secretion assay for multi-chain CARs mc13 and mc14 in the presence of ROR1-positive cell line (Jeko-1). A control was done for untransduced T cells (No LV). Data are presented as mean+/- SD of 3 independent experiments.

Figure 9: Schematic representation of the inactivation of TCR gene(s) in anti-ROR1 CAR T cells to render these allogeneic and therefore to minimize Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD).

The following Tables 1 to 6 show the components and their sequences which are used to assemble the multi-chain CARs of the present invention, as well as their architectures (assembly). Table 7 shows the polypeptide sequences of ROR1 multi-chain CARs.

Table 1: Exemplary sequences of the alpha polypeptide component of ROR1 muti-chain CAR

Functional domains	description	SEQ ID #	Raw amino acid sequence
FcεRI -SP	signal peptide	SEQ ID NO.1	MAPAMESPTLLCVALLFFAPDGV LA
CD8αhinge	hinge	SEQ ID NO.2	TTTPAPRPPTPAPTIASQPLSLRPE ACRPAAGGAVHTRGLDFACD
VH			See Table 5
G4SX3Linker	Linker VH-VL	SEQ ID NO.3	GGGGSGGGGSGGGGS
VL			See Table 5
FcεRI α-TM-IC	Fc Receptor for IgE, alpha chain, transmembrane and intracellular domain	SEQ ID NO.4	FFIPLLVVILFAVDTGLFISTQQQVT FLLKIKRTRKGFRLLNPHPKPNPKN N

Table 2: Exemplary sequences of the beta polypeptide component of ROR1 muti-chain CAR

Functional domains	description	SEQ ID #	Raw amino acid sequence
FcεR1β-ΔITAM	Fc Receptor for IgE, beta chain, without ITAM	SEQ ID NO.5	MDTESNRRANLALPQEPSSVPAF EVLEISPQEVSSGRLLKSASSPPLH TWLTVLKKEQEFLGVTQILTAMIC LCFGTVVCSVLDISHIEGDIFSSFKA GYPFWGAIFFSISGMLSIISERRNA TYLVRGSLGANTASSIAGGTGITILI INLKKSLAYIHIHSCQKFFETKCFM ASFSTEIVVMMLFLTILGLGSAVSL TICGAGEELKGNKVPE
41BB-IC	41BB co-stimulatory domain	SEQ ID NO.6	KRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCEL

Table 3: Exemplary sequences of the gamma polypeptide component of ROR1 muti-chain CAR

Functional domains	description	SEQ ID #	Raw amino acid sequence
FcεRI γ-SP	signal peptide	SEQ ID NO.7	MIPAVVLLLLLLVEQAAA
FcεRI γ -ΔITAM	Fc Receptor for IgE, gamma chain, without ITAM	SEQ ID NO.8	LGEPQLCYILDAILFLYGIVLTLLYCR LKIQVRKAAITSYEKS
CD3ζ-IC	CD3zeta intracellular domain comprising ITAM	SEQ ID NO.9	RVKFSRSADAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR

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Table 4: skip peptides linking the polypeptides forming the mutli-subunit CAR

Functional domains	description	SEQ ID #	Raw amino acid sequence
GSG-P2A	GSG-P2A ribosomal skip peptide	SEQ ID NO.10	GSGATNFSLLKQAGDVEENPGP
GSG-T2A	GSG-T2A ribosomal skip peptide	SEQ ID NO.11	GSGEGRGSLLTCGDVEENPGP

Table 5: Sequence of the 8 pairs of anti-ROR1 scFvs from murine origin, their CDRs of the scFv

ScFv sequences	SEQ ID #	Raw amino acid sequence
MURINE ORIGIN		
2A2 heavy chain variable region	SEQ ID NO.12	QVQLQQSGAELVRPGASVTLSCKASGYTFSDYEMHWVIQTPVHGLEWI GAIDPETGGTAYNQKFKGKAILTADKSSSTAYMELRSLTSEDSAVYYCTGY YDYDSFTYWGQGTLVTVSA
	SEQ ID NO.13	CDR1 : GYTFSDYE
	SEQ ID NO.14	CDR2 : IDPETGGT
	SEQ ID NO.15	CDR3 : TGYYDYDSFTY
2A2 light chain variable region	SEQ ID NO.16	DIVMTQSQKIMSTTVGDRVSITCKASQNVDAAVAWYQQKPGQSPKLLI YSASNRYTGVPDRFTGSGSGTDFTLTISNMQSEDLADYFCQQYDIYPYTF GGGTKLEIK
	SEQ ID NO.17	CDR1 : QNVDAA
	SEQ ID NO.18	CDR2 : SAS
	SEQ ID NO.19	CDR3 : QQYDIYPYT
4A5 heavy chain variable region	SEQ ID NO.20	EVKLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQIPEKRLEWVA SISRGGTTYYPDSVKGRFTISRDNVRNILYLQMSSLRSEDTAMYYCGRYD YDGYYAMDYWGQGTSVTVSS
	SEQ ID NO.21	CDR1 : GFTFSSYA
	SEQ ID NO.22	CDR2 : ISRGGTT
	SEQ ID NO.23	CDR3 : GRYDYDGYYAMDY
4A5 light chain variable region	SEQ ID NO.24	DIKMTQSPSSMYASLGERVTITCKASPDINSYLSWFQQKPGKSPKTLIYRA NRLVDGVPSRFSGGGSGQDYSLTINSLEYEDMGIYYCLQYDEFPYTFGGG TKLEMK
	SEQ ID NO.25	CDR1 : PDINSY
	SEQ ID NO.26	CDR2 : RAN
	SEQ ID NO.27	CDR3 : LQYDEFPYT
	SEQ ID NO.28	QVQLKESGPGLVAPSQTLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLG VIWAGGFTNYNSALKSRLSISKDNSKSQVLLKMTSLQTDDTAMYYCARR

D10 heavy chain variable region		GSSYSMDYWGQGTSVTVSS
	SEQ ID NO.29	CDR-H1: GFSLTSYG
	SEQ ID NO.30	CDR-H2: IWAGGFT
	SEQ ID NO.31	CDR-H3: ARRGSSYSMDY
D10 light chain variable region	SEQ ID NO.32	EIVLSQSPAITAASLGQKVTITCSASSNVSYIHWYQQRSGTSPRPWIYEISK LASGVPVRFSGSGSGTSYSLTISSMEAEDAAIYYCQQWNYPLITFGSGTKL EIQ
	SEQ ID NO.33	CDR-L1: SNVSY
	SEQ ID NO.34	CDR-L2: EIS
	SEQ ID NO.35	CDR-L3: QQWNYPLIT
G6 heavy chain variable region	SEQ ID NO.36	EVQLQQSGPELEKPGASVKISCKASGFAFTGYNMNWVKQTNGKSLEWI GSIDPYYGGSTYNQKFKDKATLTVDKSSSTAYMQLKSLTSDDSAVYYCAR SPGGDYAMDYWGQGTSVTVSS
	SEQ ID NO.37	CDR1 : GFAFTGYN
	SEQ ID NO.38	CDR2 : IDPYYGGS
	SEQ ID NO.39	CDR3 : ARSPGGDYAMDY
G6 light chain variable region	SEQ ID NO.40	DIKMTQSPSSMYASVGERVTITCKASQGINSYSGWFQQKPGKSPKTLIYR GNRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPYTFGG GTKLEIK
	SEQ ID NO.41	CDR1 : QGINSY
	SEQ ID NO.42	CDR2 : RGN
	SEQ ID NO.43	CDR3 : LQYDEFPYT
G3 heavy chain variable region	SEQ ID NO.44	QVQLQQPGAELVKPGTSVKLSCKASGYNFTNYWINWVKLRPGQGLEWI GEIYPGSGSTNYNEKFKSKATLTADTSSSTAYMQLSSLASEDSALYYCARD GNYYAMDYWGQGTSVTVSS
	SEQ ID NO.45	CDR1 : GYNFTNYW
	SEQ ID NO.46	CDR2 : IYPGSGST
	SEQ ID NO.47	CDR3 : ARDGNYYAMDY
	SEQ ID NO.48	DIQMTQTTSSLSASLGDRVTITCRASQDINNYLNWYQQKPDGTVKLLIYY

G3 light chain variable region		TSALHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPPYTFGG GTKLEIK
	SEQ ID NO.49	CDR1 : QDINNY
	SEQ ID NO.50	CDR2 : YTS
	SEQ ID NO.51	CDR3 : QQGNTLPPYT
H10 heavy chain variable region	SEQ ID NO.52	EVKLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVA SISTGASAYFPDSVKGRFTISRDNARNILYLQMSSLRSEDTAMYYCARITT STWYFDVWGAGTTVTVSS
	SEQ ID NO.53	CDR1-H1: GFTFSSYA
	SEQ ID NO.54	CDR-H2: ISTGASA
	SEQ ID NO.55	CDR-H3: ARITTSTWYFDV
H10 light chain variable region	SEQ ID NO.56	DIKMTQSPSSMYASLGERVTITCKASQDINSYLSWFQQKPGKSPKTLIYR ANRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPYTFGG GTKLEIK
	SEQ ID NO.57	CDR-L1: QDINSY
	SEQ ID NO.58	CDR-L2: RAN
	SEQ ID NO.59	CDR-L3: LQYDEFPYT
2A4 heavy chain variable region	SEQ ID NO.60	EVKLQQSGPELVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSLEWIG GINPNNGGTSYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCALQ GFAYWGQGTPLTVSS
	SEQ ID NO.61	CDR1 : GYTFTEYT
	SEQ ID NO.62	CDR2 : INPNNGGT
	SEQ ID NO.63	CDR3 : ALQGFAY
2A4 light chain variable region	SEQ ID NO.64	MEIEITQTPALMSASPGEKVTMTCSASSSVSYMYWYQQKPRSSPKPWIY LTSNLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPYTFG GGTRLELK
	SEQ ID NO.65	CDR1 : SSVSY
	SEQ ID NO.66	CDR2 : LTS
	SEQ ID NO.67	CDR3 : QQWSSNPYT

1C11 heavy chain variable region	SEQ ID NO.68	EVKLQESGAELARPGASVKMSCKASGYTFTSYTMHWVKQRPGQGLEWIGYINPSSGYTEYNQKFKDKTTLTADKSSSTAYMQLSSLTSGDSAVYYCARRVLWLRRGDYWGQGTILTVSA
	SEQ ID NO.69	CDR1 : GYTFTSYT
	SEQ ID NO.70	CDR2 : INPSSGYT
	SEQ ID NO.71	CDR3 : ARRVLWLRRGDY
1C11 light chain variable region	SEQ ID NO.72	MEVLITQTPSSLSASLGERVSLTCRASQDIGSSLNWLQQEPDGTIKRLIYATSSLDSGVPKRFSGSRSGSDYSLTISSLESEDFVDYYCLQYASSPYTFGGGTKLELK
	SEQ ID NO.73	CDR1 : QDIGSS
	SEQ ID NO.74	CDR2 : ATS
	SEQ ID NO.75	CDR3 : LQYASSP

Table 6: Exemplary Polypeptides forming anti-ROR1 multi-chain CAR

Multi chain CAR Designation	Precursor ROR1 muti-chain CAR polypeptide structure												
	Gamma polypeptide				Alpha polypeptide							Beta polypeptide	
	FcεRI γ-SP	FcεRI γ ΔTAM	CD3ζ-IC	P2A	FcεRI -SP	CD8α hinge	VH	G4SX3 Linker	VL	FcεRIα-TM-IC	T2A	FcεR1β- ΔTAM	Costimulation. domain
anti-ROR1 2A2 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.12	SEQ ID NO.3	SEQ ID NO.16	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 4A5 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.20	SEQ ID NO.3	SEQ ID NO.24	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 D10 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.28	SEQ ID NO.3	SEQ ID NO.32	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 G6 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.36	SEQ ID NO.3	SEQ ID NO.40	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 G3 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.44	SEQ ID NO.3	SEQ ID NO.48	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 H10 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.52	SEQ ID NO.3	SEQ ID NO.56	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 2A4 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.60	SEQ ID NO.3	SEQ ID NO.64	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6
anti- ROR1 1C11 (41BB)	SEQ ID NO.7	SEQ ID NO.8	SEQ ID NO.9	SEQ ID NO.10	SEQ ID NO.1	SEQ ID NO.2	SEQ ID NO.68	SEQ ID NO.3	SEQ ID NO.72	SEQ ID NO.4	SEQ ID NO.11	SEQ ID NO.5	SEQ ID NO.6

Table 7: Polypeptide sequences of exemplary anti-ROR1 multi-chain CARs

Name of mc CAR	SEQ ID NO.	Polypeptide sequence
anti-ROR1 2A2 mcCAR (4-1BB)	SEQ ID NO.76	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAQV QLQQSGAELVRPGASVTLSCKASGYTFSDYEMHWVIQTPVHGLEWIGAIDPETGGTAYNQKFKGKAILTADKSSSTAYMELRSLTSEDSAVYYCTGYYDYDSFTYWGQGT LVTVSAGGGGSGGGGSGGGGSDIVMTQSQKIMSTTVGDRVSITCKASQNVDAAVAWYQQKPGQSPKLLIYSASNRYTGVPDRFTGSGSGTDFTLTISNMQSEDLADYFC QQYDIYPYTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKN NGSGEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDI FSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKG NKVPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
anti- ROR1 4A5 mcCAR (4-1BB)	SEQ ID NO.77	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAEV KLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQIPEKRLEWVASISRGGTTYYPDSVKGRFTISRDNVRNILYLQMSSLRSEDTAMYYCGRYDYDGYYAMDYWGQG TSVTVSSGGGGSGGGGSGGGGSDIKMTQSPSSMYASLGERVTITCKASPDINSYLSWFQQKPGKSPKTLIYRANRLVDGVPSRFSGGGSGQDYSLTINSLEYEDMGIYYCL QYDEFPYTFGGGTKLEMKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKN NGSGEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDI FSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKG NKVPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
anti- ROR1 D10 (4-1BB)	SEQ ID NO.78	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAQV QLKESGPGLVAPSQTLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLGVIWAGGFTNYNSALKSRLSISKDNSKSQVLLKMTSLQTDDTAMYYCARRGSSYSMDYWGQGT SVTVSSGGGGSGGGGSGGGGSEIVLSQSPAITAASLGQKVTITCSASSNVSYIHWYQQRSGTSPRPWIYEISKLASGVPVRFSGSGSGTSYSLTISSMEAEDAAIYYCQQWN YPLITFGSGTKLEIQTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNGSGE GRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFSSFKA GYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPEK RGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

anti- ROR1 G6 mcCAR (4-1BB)	SEQ ID NO.79	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAEV QLQQSGPELEKPGASVKISCKASGFAFTGYNMNWVKQTNGKSLEWIGSIDPYYGGSTYNQKFKDKATLTVDKSSSTAYMQLKSLTSDDSAVYYCARSPGGDYAMDYWG QGTSVTVSSGGGGSGGGGSGGGGSDIKMTQSPSSMYASVGERVTITCKASQGINSYSGWFQQKPGKSPKTLIYRGNRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIY YCLQYDEFPYTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPK NNGSGEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEG DIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELK GNKVPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
anti- ROR1 G3 mcCAR (4-1BB)	SEQ ID NO.80	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAQV QLQQPGAELVKPGTSVKLSCKASGYNFTNYWINWVKLRPGQGLEWIGEIYPGSGSTNYNEKFKSKATLTADTSSSTAYMQLSSLASEDSALYYCARDGNYYAMDYWGQG TSVTVSSGGGGSGGGGSGGGGSDIQMTQTTSSLSASLGDRVTITCRASQDINNYLNWYQQKPDGTVKLLIYYTSALHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQ GNTLPPYTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN GSGEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFS SFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNK VPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
anti- ROR1 H10 mcCAR (4-1BB)	SEQ ID NO.81	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAEV KLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVASISTGASAYFPDSVKGRFTISRDNARNILYLQMSSLRSEDTAMYYCARITTSTWYFDVWGAGTT VTVSSGGGGSGGGGSGGGGSDIKMTQSPSSMYASLGERVTITCKASQDINSYLSWFQQKPGKSPKTLIYRANRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQY DEFPYTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNGS GEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFSSF KAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVP EKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

anti- ROR1 2A4 mcCAR (4-1BB)	SEQ ID NO.82	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAEV KLQQSGPELVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSLEWIGGINPNNGGTSYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCALQGFAYWGQGTPLTV SSGGGGSGGGGSGGGGSMEIEITQTPALMSASPGEKVTMTCSASSVSYMYWYQQKPRSSPKPWIYLTSNLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWS SNPYTFGGGTRLELKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNGSG EGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFSSFK AGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPE KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
anti- ROR1 1C11 mcCAR (4-1BB)	SEQ ID NO.83	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPGPMAPAMESPTLLCVALLFFAPDGVLAEV KLQESGAELARPGASVKMSCKASGYTFTSYTMHWVKQRPGQGLEWIGYINPSSGYTEYNQKFKDKTTLTADKSSSTAYMQLSSLTSGDSAVYYCARRVLWLRRGDYWG QGTILTVSAGGGGSGGGGSGGGGSMEVLITQTPSSLSASLGERVSLTCRASQDIGSSLNWLQQEPDGTIKRLIYATSSLDSGVPKRFSGSRSGSDYSLTISSLESEDFVDYYCL QYASSPYTFGGGTKLELKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN GSGEGRGSLLTCGDVEENPGPMDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFS SFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNK VPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

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.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will prevail. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

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).

Multi-chain Chimeric Antigen Receptor (CAR)

The present invention relates to a multi-chain chimeric antigen receptor (CAR) particularly adapted to immune cells used in immunotherapy.

The multi-chain CAR according to the invention generally comprises at least:

- one transmembrane polypeptide comprising at least one extracellular ligand-biding domain and;
- one transmembrane polypeptide comprising at least one signal-transducing domain;

such that said polypeptides assemble together to form a multi-chain Chimeric Antigen Receptor.

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.

In a preferred embodiment, said extracellular ligand-binding domain is a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody specific to ROR1 joined by a flexible linker. In a preferred embodiment, said scFv is an anti-ROR1 scFV, preferably provided in Table 5 as SEQ ID NO.12, 16, 20, 24, 28 and 32. Binding domain specific to ROR1 other than scFv can also be used for predefined targeting of lymphocytes, such as camelid or shark (VNAR) single-domain antibody fragments or receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13 mutein, antibody binding domains, antibody hypervariable loops or CDRs as non-limiting examples.

As other examples provided in Table 5 are the anti-ROR1 scFV of sequences SEQ ID NO.36, 40, 44, 48, 52, 56, 60, 64, 68 and 72.

The present invention relates more particularly to a ROR1 specific multi-chain Chimeric Antigen Receptor (mc CAR) comprising:

- a transmembrane polypeptide from the alpha chain of high-affinity IgE receptor (FcεRI) fused to an extracellular ROR1 ligand binding domain;
- a second transmembrane polypeptide from the gamma chain of FcεRI fused to a signal transducing domain;

- a third transmembrane polypeptide from the beta chain of FcεRI comprising a co-stimulatory domain.

wherein said ROR1 ligand binding domain fused to said alpha chain of FcεRI is a single-chain variable fragment (scFv) comprising heavy (V_H) and light (V_L) chains conferring specificity to ROR1,

wherein said V_H comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% identity to one selected from SEQ ID NO. 28 (D10), SEQ ID NO. 12 (2A2), SEQ ID NO. 20 (4A5), SEQ ID NO. 36 (G6), SEQ ID NO. 44 (G3), SEQ ID NO. 52 (H10), SEQ ID NO. 60 (2A4) and SEQ ID NO. 68 (1C11), and,

wherein said V_L comprises a polypeptide displaying at least 90 %, at least 95%, at least 98% or at least 99% sequence identity to one selected from SEQ ID NO. 32 (D10), SEQ ID NO. 16 (2A2), SEQ ID NO. 24 (4A5), SEQ ID NO. 40 (G6), SEQ ID NO. 48 (G3), SEQ ID NO. 56 (H10), SEQ ID NO. 64 (2A4) and SEQ ID NO. 72 (1C11). It is understood that the previously cited VH and VL chains function as pairs, i.e. for instance, the VH chain of F10 antibody is to be used in combination avec the VL chain of the same antibody (F10).

According to a more preferred embodiment, said V_H and VL comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% sequence identity respectively to SEQ ID NO. 28 and SEQ ID NO. 32 (D10), or respectively to SEQ ID.12 or SEQ ID NO. 16 (2A2).

According to another embodiment, wherein said extra cellular ligand binding-domain comprises:

- a VH chain comprising the CDRs from the mouse monoclonal antibody D10 of SEQ ID NO. 29 (CDR-H1), SEQ ID NO.30 (CDR-H2) and SEQ ID NO.31 (CDR-H3), and a VL chain comprising the CDRs from the mouse monoclonal antibody D10 of NO. 33 (CDR-L1), SEQ ID NO.34 (CDR-L2) and SEQ ID NO:35 (CDR-L3)

or ;

- a VH chain comprising the CDRs from the mouse monoclonal antibody 2A2 of SEQ ID NO. 13 (CDR-H1), SEQ ID NO.14 (CDR-H2) and SEQ ID NO.15 (CDR-H3) and a VL chain comprising the CDRs from the mouse monoclonal antibody 2A2 of SEQ ID NO. 17 (CDR-L1), SEQ ID NO:18 (CDR-L2) and SEQ ID NO:19 (CDR-L3).

In a preferred embodiment, said extracellular ligand-binding domain is a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody specific to ROR1 joined by a flexible linker.

In a preferred embodiment, said scFv is an anti-ROR1 scFV, or parts of them such as CDRs preferably provided in Table 5 as SEQ ID NO.12 to 75. From all scFvs cited in Table 5, the preferred pairs of scFvs correspond to the VH and VL chains of D10 (SEQ ID NO.28 and 32) and 2A2 (SEQ ID NO.12 and 16), as well as their respective CDRs (SEQ ID NO.29-31 and 33-35 corresponding respectively to VH and VL chains for D10 ; SEQ ID NO.13-15 and 17-19 corresponding respectively to VH and VL chains for 2A2).

Binding domain specific to ROR1 other than scFv can also be used for predefined targeting of lymphocytes, such as camelid or shark (VNAR) single-domain antibody fragments or receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13 mutein, antibody binding domains, antibody hypervariable loops or CDRs as non-limiting examples.

In a preferred embodiment said first transmembrane polypeptide further comprises a stalk region between said extracellular ligand-binding domain and said transmembrane domain. The term “stalk 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) (SEQ ID NO: 2).

Thus, the expression of multi-chain CAR in immune cells results in modified cells that selectively and eliminate defined targets, including but not limited to malignant cells carrying a respective tumor-associated surface antigen or virus infected cells carrying a virus-specific surface antigen, or target cells carrying a lineage-specific or tissue-specific surface antigen.

Downregulation or mutation of target antigens is commonly observed in cancer cells, creating antigen-loss escape variants. Thus, to offset tumor escape and render immune cell more specific to target, 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.

According to a preferred embodiment, the polypeptide encoding a ROR1 specific multi-chain Chimeric Antigen Receptor, comprises a polypeptide sequence displaying at least 80 %, at least 90 %, at least 95%, at least 98% or at least 99% identity to the full amino acid sequence of SEQ ID NO. 78 (anti-ROR1 mcCAR D10), SEQ ID NO.76 (anti-ROR1 mcCAR 2A2), SEQ ID NO.77 (anti-ROR1 mcCAR 4A5) , SEQ ID NO.79 (anti-ROR1 mcCAR G6), SEQ ID NO.80

(anti-ROR1 mcCAR G3), SEQ ID NO.81 (anti-ROR1 mcCAR H10), SEQ ID NO.82 (anti-ROR1 mcCAR 2A4) and SEQ ID NO.83 (anti-ROR1 mcCAR 1C11) as referred to in Table 7.

According to a more preferred embodiment, the polypeptide encoding a ROR1 specific multi-chain Chimeric Antigen Receptor comprises a polypeptide sequence displaying at least 80 %, at least 90 %, at least 95%, at least 98% or at least 99% identity to the full amino acid sequence of SEQ ID NO. 78 (anti-ROR1 mcCAR D10), SEQ ID NO.76 (anti-ROR1 mcCAR 2A2) as referred to in Table 7.

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. Thus, 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 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.

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 which is 4-1BB (GenBank: AAA53133.).

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.

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 a particular embodiment, the multi-chain CAR comprises a transmembrane polypeptide derived from a FcεRI chain. In more particular embodiment FcεRI chain is a FcεRI α chain, in which the extracellular domain is replaced by an extracellular ligand-binding domain, preferably by a scFV directed against ROR1.

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.

As non-limiting example, different versions (architectures) of multi-chain CAR are illustrated in Figure 4. In a more preferred embodiment, the multi-chain CARs of the present invention comprises a polypeptide comprising amino acid sequences as set forth in Table 6. In a preferred embodiment the multi-chain CAR comprise a polypeptide with amino acid sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with such amino amino acid sequences or with the polynucleotide sequence encoding one two or three of the polypeptides constitutive of the multi-chain polypeptide structure.

"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. 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. The polynucleotide sequences of similar polypeptides are deduced using the genetic code and may be obtained by conventional means, in particular by reverse translating its amino acid sequence using the genetic code.

Polynucleotides, vectors:

The present invention also relates to polynucleotides, vectors encoding the above described multi-chain CAR according to the invention. The present invention provides polynucleotides, including DNA and RNA molecules that encode the transmembrane polypeptides disclosed herein that can be included in the multi-chain CAR. In particular, the invention relates to a polynucleotide comprising a nucleic acid sequence encoding at least one transmembrane polypeptide composing the multi-chain CAR as described above. More particularly the invention relates to a polynucleotide comprising two or more nucleic acid sequences encoding transmembrane polypeptides composing the multi-chain CAR as described above.

The polynucleotide may consist in an expression cassette or expression vector (e.g. a plasmid for introduction into a bacterial host cell, or a viral vector such as a baculovirus vector for transfection of an insect host cell, or a plasmid or viral vector such as a lentivirus for transfection of a mammalian host cell).

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)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. 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.

Methods of engineering an immune cell:

In encompassed particular embodiment, the invention relates to a method of preparing immune cells for immunotherapy comprising introducing into said immune cells the polypeptides composing said multi-chain CAR and expanding said cells. In particular embodiment, the invention relates to a method of engineering an immune cell comprising providing a cell and expressing at the surface of said cell at least one multi-chain CAR as

described above. In particular embodiment, the method comprises transforming the cell with at least one polynucleotide encoding polypeptides composing at least one multi-chain CAR as described above, and expressing said polynucleotides into said cell.

In another embodiment, the present invention relates to a method of preparing cells for immunotherapy comprising introducing into said cells the different polypeptides composing said multi-chain CAR and expanding said cells. In a preferred embodiment, said polynucleotides are included in lentiviral vectors in view of being stably expressed in the cells.

Delivery methods

The different methods described above involve introducing multi-chain CAR, pTalpha or functional variants thereof, rare cutting endonuclease, TALE-nuclease, CAR optionally with DNA-end processing enzyme or exogenous nucleic acid into a cell.

As non-limiting example, said multi-chain CAR can be introduced as transgenes encoded by one or as different plasmidic vectors. Different transgenes can be included in one 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)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. 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 rare-cutting endonuclease and a DNA end-processing enzyme or the different polypeptides of the multi-chain CAR.

Said plasmid vector can also contain a selection marker which provides for identification and/or selection of cells which received said vector.

Polypeptides may be synthesized *in situ* in the cell as a result of the introduction of polynucleotides encoding said 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.

- *Electroporation*

In particular embodiment of the invention, polynucleotides encoding polypeptides according to the present invention can be mRNA which is introduced directly into the cells, for example by electroporation. The inventors determined the optimal condition for mRNA electroporation in T-cell.

The inventor used the cytoPulse technology which allows, by the use of pulsed electric fields, to transiently permeabilize living cells for delivery of material into the cells. The technology, based on the use of PulseAgile (Cellectis property) electroporation waveforms grants the precise control of pulse duration, intensity as well as the interval between pulses (U.S. patent 6,010,613 and International PCT application WO2004083379). All these parameters can be modified in order to reach the best conditions for high transfection efficiency with minimal mortality. Basically, the first high electric field pulses allow pore formation, while subsequent lower electric field pulses allow to move the polynucleotide into the cell. In one aspect of the present invention, the inventor describe the steps that led to achievement of >95% transfection efficiency of mRNA in T cells, and the use of the electroporation protocol to transiently express different kind of proteins in T cells. In particular the invention relates to a method of transforming T cell comprising contacting said T cell with RNA and applying to T cell an agile pulse sequence consisting of:

(a) one electrical pulse with a voltage range from 2250 to 3000 V per centimeter, a pulse width of 0.1 ms and a pulse interval of 0.2 to 10 ms between the electrical pulses of step (a) and (b);

(b) one electrical pulse with a voltage range from 2250 to 3000 V with a pulse width of 100 ms and a pulse interval of 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c) ; and

(c) 4 electrical pulses with a voltage of 325 V with a pulse width of 0.2 ms and a pulse interval of 2 ms between each of 4 electrical pulses.

In particular embodiment, the method of transforming T cell comprising contacting said T cell with RNA and applying to T cell an agile pulse sequence consisting of:

(a) one electrical pulse with a voltage of 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V per centimeter, a pulse width of 0.1 ms and a pulse interval of 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 ms between the electrical pulses of step (a) and (b);

(b) one electrical pulse with a voltage range from 2250, of 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V with a pulse width of 100 ms and a pulse interval of 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c); and

(c) 4 electrical pulses with a voltage of 325 V with a pulse width of 0.2 ms and a pulse interval of 2 ms between each of 4 electrical pulses.

Any values included in the value range described above are disclosed in the present application. Electroporation medium can be any suitable medium known in the art. Preferably, the electroporation medium has conductivity in a range spanning 0.01 to 1.0 milliSiemens.

In particular embodiments, as non-limiting examples, said RNA encodes a rare-cutting endonuclase, one monomer of the rare-cutting endonuclease such as Half-TALE-nuclease, a Chimeric Antigen Receptor, at least one component of the multi-chain chimeric antigen receptor, a pTalpha or functional variant thereof, an exogenous nucleic acid, one additional catalytic domain.

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.

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.

As mentioned previously, such cells can be also genetically engineered to inactivate one or several genes selected, for instance, 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 another 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 (Figure 9 for a schematic representation).

For instance, heterodimeric TALE-nuclease targeting two 17-bp long sequences (called half targets) separated by an 15-bp spacer within T-cell receptor alpha constant chain region (TRAC) gene were designed and produced. Each half target is recognized by repeats of the half TALE-nucleases listed in the following Table 8.

Table 8: TAL-nucleases targeting TCRalpha gene

Target	Target sequence	Repeat sequence	Half TALE-nuclease
TRAC_T01	TTGTCCCACAGATATCCAgaaccctgaccctg CCGTGTACCAGCTGAGA (SEQ ID NO: 84)	Repeat TRAC_T01-L (SEQ ID NO: 85)	TRAC_T01-L TALEN (SEQ ID NO: 87)
		Repeat TRAC_T01-R (SEQ ID NO: 86)	TRAC_T01-R TALEN (SEQ ID NO: 88)

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 another embodiment, said rare-cutting endonuclease can be a meganuclease, a Zinc finger nuclease or a TALE-nuclease. In a preferred embodiment, said rare-cutting endonuclease is a TALE-nuclease. Preferred methods and relevant TALE-nucleases have been described in WO2013176915

Anti-ROR1 T-cells made resistant to chemotherapy

According to a preferred embodiment of the invention, the T-cells endowed with anti ROR1 multi-chain CAR are engineered to be resistant to chemotherapy drugs, in particular to purine nucleotide analogues (PNAs), making them suitable for cancer treatments combining adoptive immunotherapy and chemotherapy.

Purine nucleotide analogues enter chemotherapy compositions for many cancer treatments and are used as a standard of care in CLL. The most widely used PNAs are clofarabine, fludarabine and cytarabine, alone or in combination.

PNAs are metabolized by deoxycytidine kinase (dCK) into mono, -di and tri-phosphate PNA. Their tri-phosphate forms and particularly clorofarabine triphosphate compete with ATP for DNA synthesis, acts as pro-apotptotic agent and are potent inhibitors of ribonucleotide reductase (RNR), which is involved in trinucleotide production.

The present inventors have successfully created anti-ROR1 T-cells resistant to purine nucleotide analogues, more particularly clorofarabine and fludarabine, by mediating the inactivation of dcK gene expression into said cells. Transfection of the T-cells using polynucleotides encoding specific TAL-nuclease directed against dck genes, preferably by using electroporation of mRNA, induced a significant resistance to the drugs, while maintaining T-cells cytotoxic activity towards ROR1 bearing cells.

The present application thus provides with anti-ROR1 T-cells, which expression of deoxycytidine kinase has been repressed or inactivated for the treatment of leukemia.

Activation and expansion of T cells

Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using 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. T cells can be expanded *in vitro* or *in vivo.*

Generally, the T cells of the invention 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, INFγ , 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

In another embodiment, isolated cell obtained by the different methods or cell line derived from said isolated cell as previously described can be used as a medicament. In another embodiment, said medicament can be used for treating cancer or infections in a patient diagnosed with a pathology linked to ROR1 positive cells. In another embodiment, said isolated cell according to the invention or cell line derived from said isolated cell can be used in the manufacture of a medicament for treatment of a cancer, especially CLL or solid tumors such as breast, colon, lung or kidney tumors

In another aspect, the present invention relies on methods for treating patients in need thereof, said method 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,

On one embodiment, said T cells of the invention can undergo robust *in vivo* T cell expansion and can persist 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.

Cells that can be used with the disclosed methods are described in the previous section. Said treatment can be used to treat patients diagnosed wherein a pre-malignant or malignant cancer condition characterized by ROR1-expressing cells, especially by an overabundance of ROR1-expressing cells. Such conditions are found in hematologic cancers, such as leukemia or malignant lymphoproliferative disorders.

Leukemia can be acute myelogenous leukemia, chronic myelogenous leukemia, melodysplastic syndrome, acute lymphoid leukemia, chronic lymphoid leukemia, and myelodysplastic syndrome.

Lymphoproliferative disorder can be lymphoma, in particular chronic lymphocytic leukemia, non-Hodgkin's lymphoma, Burkitt's lymphoma, and follicular lymphoma (small cell and large cell).

According to one preferred embodiment, said engineered T cells are provided for the treatment of the Chronic Lymphocytic Leukemia (CLL) or the Small Lymphocytic Lymphoma (SLL).

According to another preferred embodiment, said treatment of CLL or SLL is administered to patients who have been lympho-depleted before the ROR1-CAR-T cell infusion. Said lympho-depletion is performed usually by chemotherapy, and preferably by using drugs as fludarabine (F), cyclophosphamide (C), bendamustine (B) or rituximab (R) or a combination thereof. Typically, the combination of FCR or FBR can be used for lympho-depletion prior to CAR-T administration.

According to another preferred embodiment, said engineered T cells are provided for the treatment of Mantle Cell Lymphoma (MCL, Acute Lymphoblastic Leukemia (ALL) with a t(1;19) chromosome translocation.

Also, solid tumors such as breast, colon, lung, and kidney tumors can be treated by the CARs of the invention. Also, the engineered T cells of the invention can be used as a treatment of pancreas or ovarian cancers.

It can be a treatment 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. Indeed, the present invention preferably relies on cells or population of cells, which have been made resistant to at least one immunosuppressive agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. In this aspect, the immunosuppressive treatment should 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.

GENERAL METHODS

Primary cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation from buffy coats from healthy volunteer donors (Etablissement Français du Sang). T lymphocytes were then purified using the EasySep human T cell enrichment kit (Stemcell Technologies), and activated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in X-vivo 15 medium (Lonza) supplemented with 20 ng/ml IL-2 (Miltenyi) and 5% human AB serum (Seralab).

Cell lines

The Jeko-1 and SupT1 cell lines were obtained from the American Type Culture Collection. Jeko-1 cells were cultured in RPMI 1640 supplemented with 20% heat–inactivated FCS, 2mmol/L L-glutamine and 100 units/ml penicillin, and 100µg/mL streptomycin. SupT1 cells were cultured in RPMI 1640 supplemented with 10% heat–inactivated FCS, 2mmol/L L-glutamine and 100 units/ml penicillin, and 100µg/mL streptomycin.

Synthesis of DNA encoding mcCARs

The DNA encoding the mcCARs was synthesized by GenScript.

Construction of polycistronic lentiviral vectors

The DNA encoding the mcCARs was cloned in the pSEW lentiviral vector backbone between the SFFV promoter and the WPRE sequence.

Lentiviral vectors production

Concentrated lentiviral vectors were produced by Vectalys (Toulouse, France).

T cells transduction

After 3 days of activation, T cells were transduced on retronectin coated plates at an MOI of 5.

Detection of mcCAR

mcCARs detection at the surface of T cells was done using a recombinant protein consisting of the fusion of the extracellular domain of ROR1 protein together with a murine IgG1 Fc fragment (produced by LakePharma). Binding of this protein to the CAR molecule was detected with a PE-conjugated secondary antibody (Jackson Immunoresearch) targeting the mouse Fc portion of the protein, and analysed by flow cytoùmetry.

Degranulation assay

5×10^4 T cells were co-cultured with 5×10^4 ROR1-positive or ROR1-negative cells in 0.1 ml per well in a 96-well plate. APC-labeled anti-CD107a (BD Biosciences) was added at the beginning of the co-culture in addition to 1µg/ml of anti-CD49d (BD Biosciences), 1µg/ml of anti-CD28 (Miltenyi), and 1x Monensin solution (eBioscience). After a 6h incubation, the cells were stained with a fixable viability dye (eBioscience) and vioblue-labeled anti-CD8 (Miltenyi) and analyzed using the MACSQuant flow cytometer (Miltenyi). Of note: degranulating cytotoxic T cells correspond to CD8+CD107a+ cells.

Cytokine release assay

5 x 10^4 T cells were co-cultured with 5 x 10^4 ROR1-positive or ROR1-negative cells in 0.1 ml per well in a 96-well plate. After a 24 hours incubation, the culture supernatants were collected and analysed for INFγ□NFγ sed for e or ROR1-negative cells □

Cytotoxicity assay

ROR1-positive and ROR1-negative cells were respectively labeled with CellTrace CFSE and CellTrace Violet. A batch of 1 x 10^4 ROR1-positive cells were co-cultured with 1 x 10^4 ROR1negative cells with 1 x 10^5 T cells in 0.1ml per well in a 96-well plate. After a 4 hours incubation, the cells were harvested and stained with a fixable viability dye (eBioscience) and analyzed using the MACSQuant flow cytometer (Miltenyi).

The percentage of specific lysis was calculated using the following formula:

$$\% \text{ cell lysis} = 100\% - \frac{\dfrac{\% \text{ viable target cells upon coculture with CAR modified T cells}}{\% \text{ viable control cells upon coculture with CAR modified T cells}}}{\dfrac{\% \text{ viable target cells upon coculture with non modified T cells}}{\% \text{ viable control cells upon coculture with non modified T cells}}}$$

Example of ROR1 specific multi-chain CARs

A. Design of multi-chain CARs

Ten multi-chain CARs targeting the ROR1 antigen were designed based on the high affinity receptor for IgE (FcεRI). The FcεRI expressed on mast cells and basophiles triggers allergic reactions. It is a tetrameric complex composed of a single α subunit, a single β subunit and two disulfide-linked γ subunits. The α subunit contains the IgE-binding domain. The β and γ subunits contain ITAMs that mediate signal transduction. In every multi-chain CAR, the extracellular domain of the FcRα chain was deleted and replaced by the respective scFv referred to µIn Table 5 respectively and the CD8α hinge (SEQ ID NO: 2) and the ITAM of the FcRβ chain and/or the FcRγ chain was deleted. The resulting constructions had the structure detailed in table 6.

Architecture of ROR1-specific multi-chain CAR (csm13 and csm14)

The 2 mcCARs specific for ROR1 developed and tested in the present invention have a CAR architecture as depicted in Figure 4A and with components of α, β and γ chains as shown in Tables 1-3. These 2 receptors differ from each other only by their antigen-binding domain. The csm13 CAR contains the D10 scFv whereas csm14 CAR contains the 2A2 scFv as shown in Table 5. Both csm13 and csm14 contain the 41-BB costimulatory domain and the CD3zeta ITAMs as signaling domains.

The polycistronic expression cassettes in lentiviral vector encoding ROR1-specific mcCAR cms13 and cms14 are realized as in Figure 3.

The polypeptide sequence of cms13 and cms14 correspond to SEQ ID NO:78 and SEQ ID NO:76 as shown in Table 7.

B. Transiently expression in T cells

Multi-chain CARs are expressed in human T cells after electroporation of polycistronic mRNA. T cells were electroporated with capped and polyadenylated polycistronic mRNA that were produced using the mMESSAGE mMACHINE kit and linearized plasmids as template. The plasmids used as template contained the T7 RNA polymerase promoter followed by a polycistronic DNA sequence encoding the different CAR variants.

The electroporation of the polycistronic mRNAs into the human T cells was done using the CytoLVT-S device (Cellectis), according to the following protocol: $5X10^6$ T cells preactivated several days (3-5) with anti CD3/CD28 coated beads and IL2 were resuspended in cytoporation buffer T, and electroporated with 45µg of mRNA. Twenty-four hours after electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were labeled with a fixable viability dye eFluor-780 and a PE-conjugated goat anti mouse IgG F(ab')2 fragment specific, and analysed by flow cytometry.

The live T cells engineered using polycistronic mRNAs expressed the multi-chain CARs on their surface.

C. Lenviral expression in T cells

In vitro screening of ROR1-specific mcCAR

The polycistronic genes encoding csm13 and csm14 were vectorized in human T cells using lentiviral vectors as reported previously. Firstly the cell surface expression profile was assessed over time of csm13 and csm14 in transduced T cells. For that purpose, the ROR1/Fc fusion protein was used. As shown in Figure 5, it was observed that csm13 and csm14 were highly expressed on the cell surface 3 days post transduction and remained relatively highly expressed over a 2 weeks period. The capacity of csm13 and csm14 was then assessed to mediate antigen-dependent T cells activation. To address this issue, activity assays was performed using a ROR1-positive cell line (Jeko-1), and a ROR1-negative cell line (SupT1). It was observed that csm13 and csm14 were able to activate T cells in the presence of Jeko-1 but not in the presence of SupT1 as shown with the results of the degranulation assay, the cytotoxicity assay and the IFNγ secretion assay shown in Figure 6, 7 and 8 respectively.

D. The human T cells transiently expressing the multi-chain CARs degranulate following coculture with target cells

Twenty-four hours after electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 6 hours. The CD8+ T cells were then analyzed by flow cytometry to detect the expression of the degranulation marker CD107a at their surface. This experiment aims to check

that the human CD8+ T cells expressing the ROR1 multi-chain CARs degranulate in coculture with ROR1 expressing target cells but not in coculture with control cells.

E. The human T cells transiently expressing the multi-chain CARs secrete cytokines following coculture with target cells

Twenty-four hours after electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 24 hours. The supernatants were then harvested and analysed using the TH1/TH2 cytokine cytometric bead array kit to quantify the cytokines produced by the T cells. The assay aims to show that the human T cells expressing the multi-chain CARs produce IFNγ, IL8 and IL5 in coculture with ROR1 expressing target cells but not in coculture with control cells.

F. The human T cells transiently expressing the multi-chain CARs lyse target cells

Twenty-four hours after electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 4 hours. The target cells were then analysed by flow cytometry to analyse their viability. This assay aims to show that the different cells expressing the ROR1 multi-chain CARs lyse the ROR1 expressing target cells but not the control cells.

CLAIMS

1) A ROR1 specific multi-chain Chimeric Antigen Receptor (mc CAR) comprising:

- a transmembrane polypeptide from the alpha chain of high-affinity IgE receptor (FcεRI) fused to an extracellular ROR1 ligand binding domain;

- a second transmembrane polypeptide from the gamma chain of FcεRI fused to a signal transducing domain;

- a third transmembrane polypeptide from the beta chain of FcεRI comprising a co-stimulatory domain,

wherein said ROR1 ligand binding domain fused to said alpha chain of FcεRI is a single-chain variable fragment (scFv) comprising heavy (V_H) and light (V_L) chains conferring specificity to ROR1,

wherein said V_H comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% identity to one selected from SEQ ID NO. 28 (D10), SEQ ID NO. 12 (2A2), SEQ ID NO. 20 (4A5), SEQ ID NO. 36 (G6), SEQ ID NO. 44 (G3), SEQ ID NO. 52 (H10), SEQ ID NO. 60 (2A4) and SEQ ID NO. 68 (1C11), and,

wherein said V_L comprises a polypeptide displaying at least 90 %, at least 95%, at least 98% or at least 99% sequence identity to one selected from SEQ ID NO. 32 (D10), SEQ ID NO. 16 (2A2), SEQ ID NO. 24 (4A5), SEQ ID NO. 40 (G6), SEQ ID NO. 48 (G3), SEQ ID NO. 56 (H10), SEQ ID NO. 64 (2A4) and SEQ ID NO. 72 (1C11).

2) A ROR1 specific multi-chain Chimeric Antigen Receptor of claim 1, wherein said V_H and VL comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% sequence identity respectively to SEQ ID NO. 28 and SEQ ID NO. 32 (D10) and respectively to SEQ ID.12 or SEQ ID NO. 16 (2A2).

3) A ROR1 specific chimeric antigen receptor according to claim 1 or claim 2, wherein said extra cellular ligand binding-domain comprises:

- a VH chain comprising the CDRs from the mouse monoclonal antibody D10 of SEQ ID NO. 29 (CDR-H1), SEQ ID NO.30 (CDR-H2) and SEQ ID NO.31 (CDR-H3), and a VL chain

comprising the CDRs from the mouse monoclonal antibody D10 of NO. 33 (CDR-L1), SEQ ID NO.34 (CDR-L2) and SEQ ID NO:35 (CDR-L3)

or ;

- a VH chain comprising the CDRs from the mouse monoclonal antibody 2A2 of SEQ ID NO. 13 (CDR-H1), SEQ ID NO.14 (CDR-H2) and SEQ ID NO.15 (CDR-H3) and a VL chain comprising the CDRs from the mouse monoclonal antibody 2A2 of SEQ ID NO. 17 (CDR-L1), SEQ ID NO:18 (CDR-L2) and SEQ ID NO:19 (CDR-L3).

4) A ROR1 specific multi-chain Chimeric Antigen Receptor of anyone of claim 1-3, wherein said alpha chain of FcεRI is fused to said extracellular ligand-binding domain by a hinge from CD8α, IgG1 or FcγRIIIα proteins.

5) A ROR1 specific multi-chain Chimeric Antigen Receptor of claim 5, wherein said hinge comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% identity to SEQ ID NO.2.

6) A ROR1 specific multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 5, wherein said signal transducing domain fused to the gamma chain of FcεRI is from the TCR zeta chain, the FCεRβ chain, the FcεRIγ chain, or includes an immunoreceptor tyrosine-based activation motif (ITAM).

7) A ROR1 specific multi-chain Chimeric Antigen Receptor according to claim 6, wherein said signal transducing domain is from CD3zeta.

8) A ROR1 specific multi-chain Chimeric Antigen Receptor according to claim 7, wherein said signal transducing domain comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% identity to SEQ ID NO.9.

9) A ROR1 specific multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 8, wherein said second or third polypeptide comprises a co-stimulatory domain from the cytoplasmic domain of a costimulatory molecule selected from CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, CD8, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

10) A ROR1 specific multi-chain Chimeric Antigen Receptor according to claim 9, wherein said co-stimulatory domain is from 4-1BB and comprises a polypeptide sequence displaying at least 90 %, at least 95%, at least 98% or at least 99% identity to SEQ ID NO.6.

11) A polypeptide encoding a ROR1 specific multi-chain Chimeric Antigen Receptor according to anyone of claim 1 to 11, comprising a polypeptide sequence displaying at least 80 %, at least 90 %, at least 95%, at least 98% or at least 99% identity to the full amino acid sequence of SEQ ID NO. 78 (anti-ROR1 mcCAR D10), SEQ ID NO.76 (anti-ROR1 mcCAR 2A2), SEQ ID NO.77 (anti-ROR1 mcCAR 4A5) , SEQ ID NO.79 (anti-ROR1 mcCAR G6), SEQ ID NO.80 (anti-ROR1 mcCAR G3), SEQ ID NO.81 (anti-ROR1 mcCAR H10), SEQ ID NO.82 (anti-ROR1 mcCAR 2A4) and SEQ ID NO.83 (anti-ROR1 mcCAR 1C11).

12) A polypeptide encoding a ROR1 specific multi-chain Chimeric Antigen Receptor according to claim 11, comprising a polypeptide sequence displaying at least 80 %, at least 90 %, at least 95%, at least 98% or at least 99% identity to the full amino acid sequence of SEQ ID NO. 78 (anti-ROR1 mcCAR D10) and SEQ ID NO.76 (anti-ROR1 mcCAR 2A2).

13) A polynucleotide comprising a nucleic acid sequence encoding a ROR1 specific multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 12.

14) A vector comprising a polynucleotide of claim 13.

15) A method of engineering an immune cell comprising:

(a) Providing an immune cell;

(b) Expressing at the surface of said cells at least one multi-chain Chimeric Antigen Receptor according to any one of the claims 1 to 12.

16) The method of engineering an immune cell of claim 15 comprising:

(a) Providing an immune cell;

(b) Introducing into said cell at least one polynucleotide encoding polypeptides composing at least one multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 12;

(c) Expressing said polynucleotides into said cell.

17) The method of engineering an immune cell of anyone of claim 15 to 16 comprising:

(a) Providing an immune cell;

(b) Expressing at the surface of said cell a population of multi-chain Chimeric Antigen Receptors according to any one of the claims 1 to 12 each one comprising different extracellular ligand-binding domains.

18) The method of engineering an immune cell of anyone of claim 15 to 17 comprising:

(a) Providing an immune cell;

(b) Introducing into said cell at least one polynucleotide encoding polypeptides composing a population of multi-chain Chimeric Antigen Receptors according to any one of claims 1 to 12 each one comprising different extracellular ligand binding domains.

(c) Expressing said polynucleotides into said cell.

19) An isolated immune cell obtainable from the method according to any one of claims 15 to 18.

20) An isolated immune cell comprising at least one multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 12.

21) An isolated immune cell according to claim 19 or 20 for its use as a medicament.

22) An isolated immune cell according to claim 21 for use in human therapy.

23) An isolated immune cell according to anyone of claim 20 to 22 for use in therapy, wherein the condition is a pre-malignant or malignant cancer condition characterized by ROR1-expressing cells.

24) An isolated immune cell according to anyone of claim 20 to 23 for use in therapy, wherein the condition is a condition which is characterized by an overabundance of ROR1-expressing cells.

25) An isolated immune cell according to claim 24 for use in therapy, wherein the condition is a haematological cancer condition.

26) An isolated immune cell according to claim 25, for use in therapy, wherein the haematological cancer condition is leukemia.

27) An isolated immune cell according to claim 26 for use in therapy, wherein the haematological cancer condition is chronic lymphocytic leukemia (CLL) or the Small Lymphocytic Lymphoma (SLL).

28) An isolated immune cell according to claim 26 for use in therapy, wherein said leukemia is selected from the group consisting of acute myelogenous leukemia, chronic myelogenous leukemia and myelodysplastic syndrome.

29) An isolated immune cell according to claim 26 for use in therapy, wherein said leukemia is of Mantle Cell Lymphoma (MCL), Acute Lymphoblastic Leukemia (ALL) with a t(1;19) chromosome translocation.

30) An isolated immune cell according to anyone of claim 20 to 24 for use in therapy, wherein the condition is a solid tumor.

31) An isolated immune cell according to claim 30, wherein the tumor is a breast, colon, lung, or kidney tumor.

32) An isolated immune cell according to claim 30, wherein the tumor is a renal, pancreas or ovarian tumor.

33) An isolated cell according to any one of claims 20 to 32 derived from, NK cells, inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes.

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

a) Providing an immune cell obtainable by a method according to any one of the claims 16 to 18;

b) Administrating said T-cells to said patient.

35) The method for treating a patient of claim 34 wherein said immune cells are recovered from donors.

36) The method for treating a patient of claim 34 wherein said immune cells are recovered from patients.

5

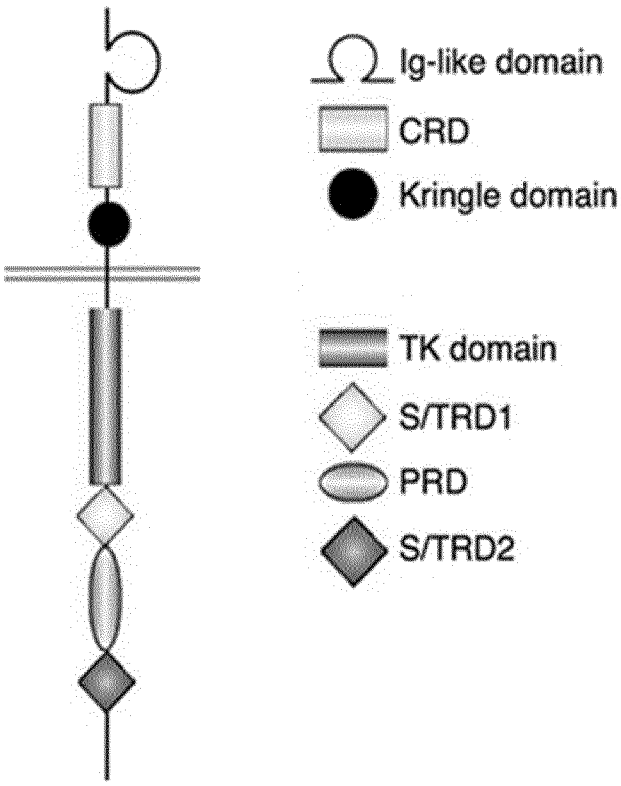
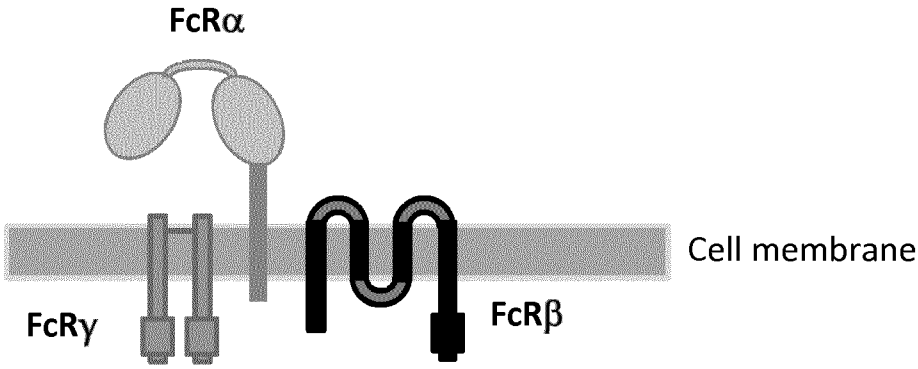


Figure 1

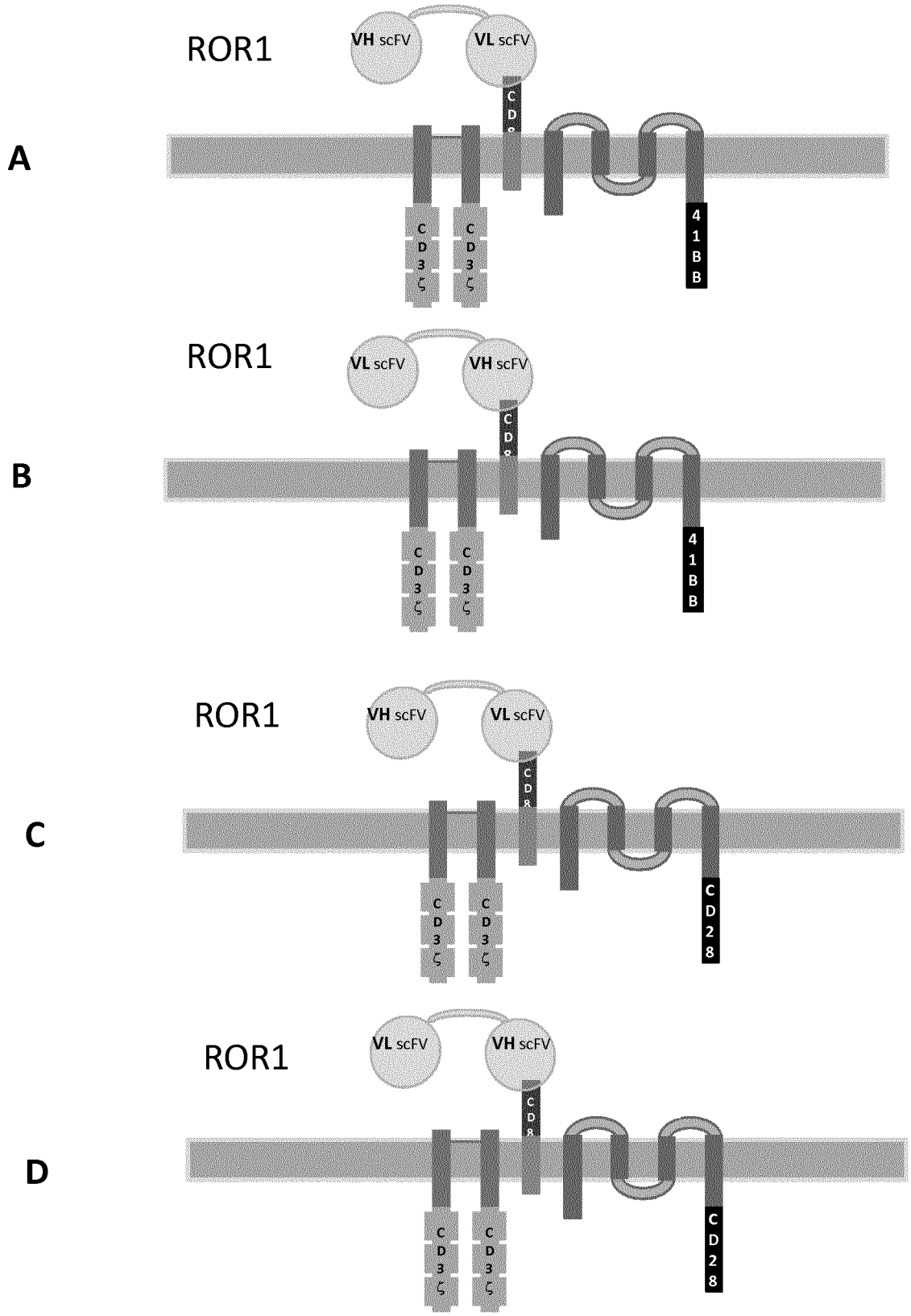


Native FcεRI

Figure 2

Structure of the polycistronic mcCAR construct

Figure 3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VL scFV
CD8
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41BB
B
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VH scFV
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CD3ζ
41BB
C
ROR1
VH scFV
VL scFV
CD8
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CD3ζ
CD28
D
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VL scFV
VH scFV
CD8
CD3ζ
CD3ζ
CD28

Figure 4

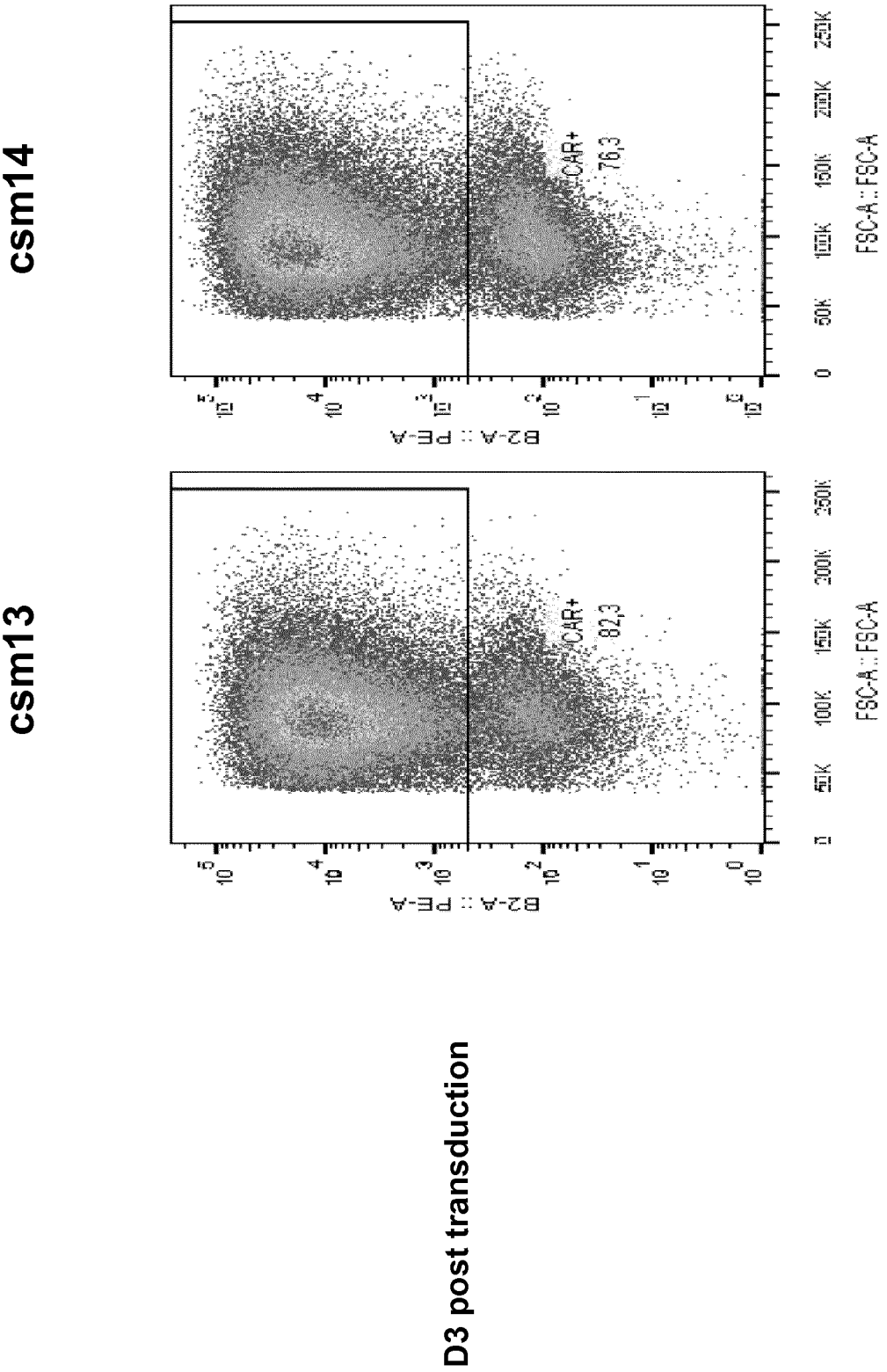
csm14
csm13
CAR+
76,3
CAR+
82,3
B2-A :: PE-A
FSC-A :: FSC-A
D3 post transduction

Figure 5

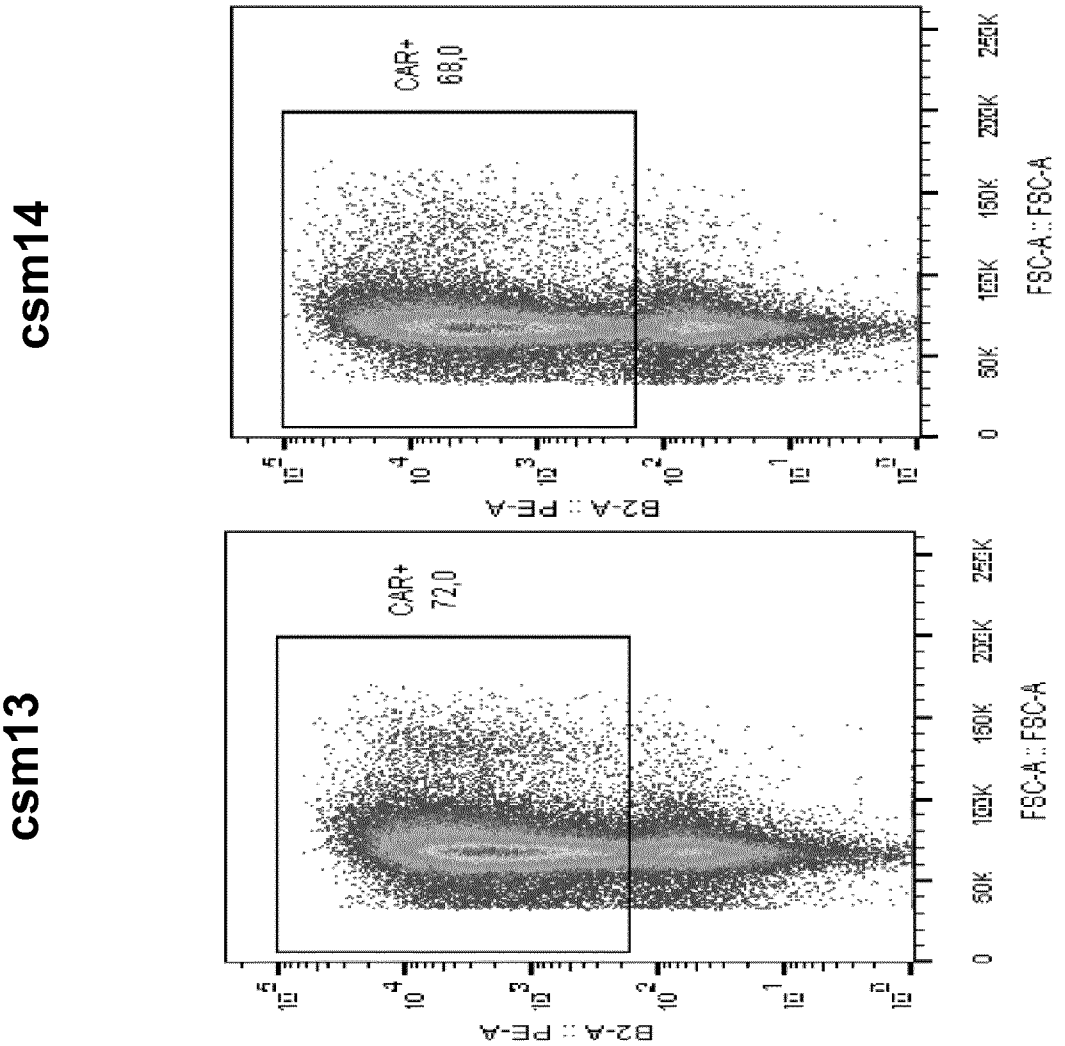

Figure 5 (cont.)

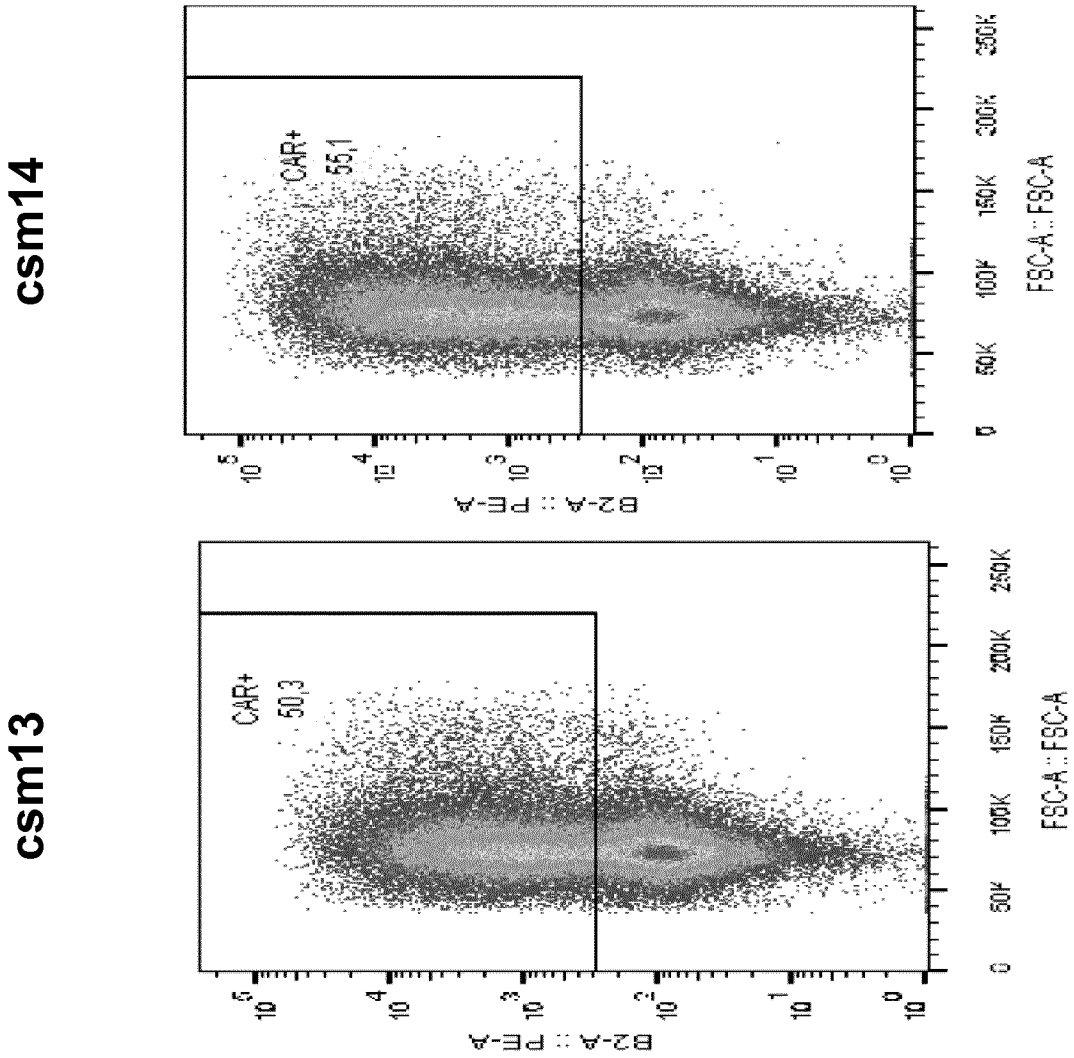

Figure 5 (cont.)

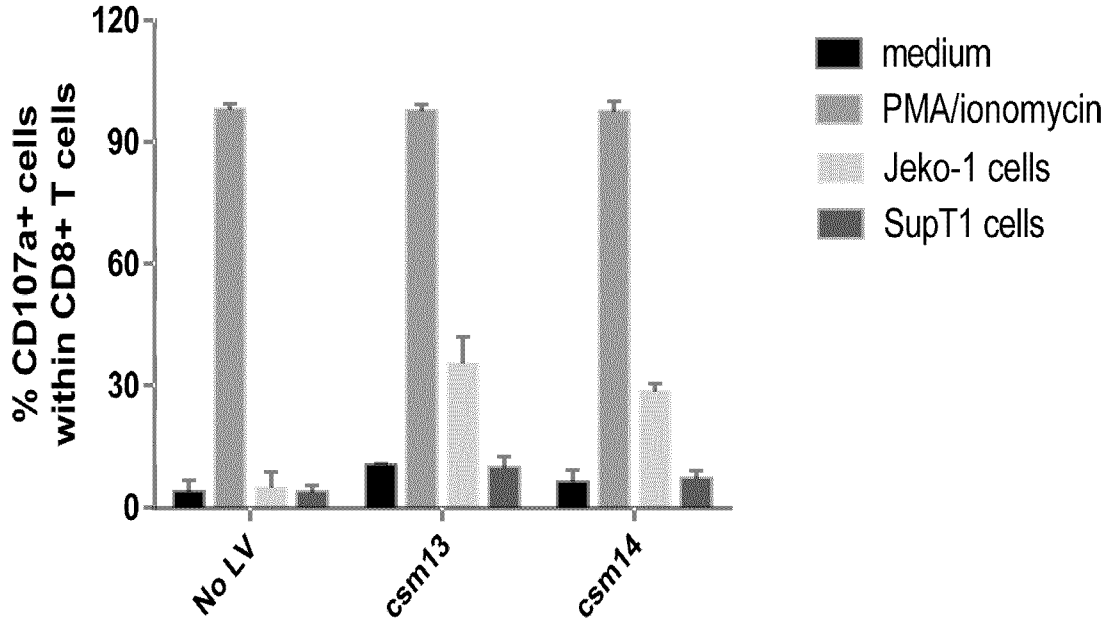

Figure 6

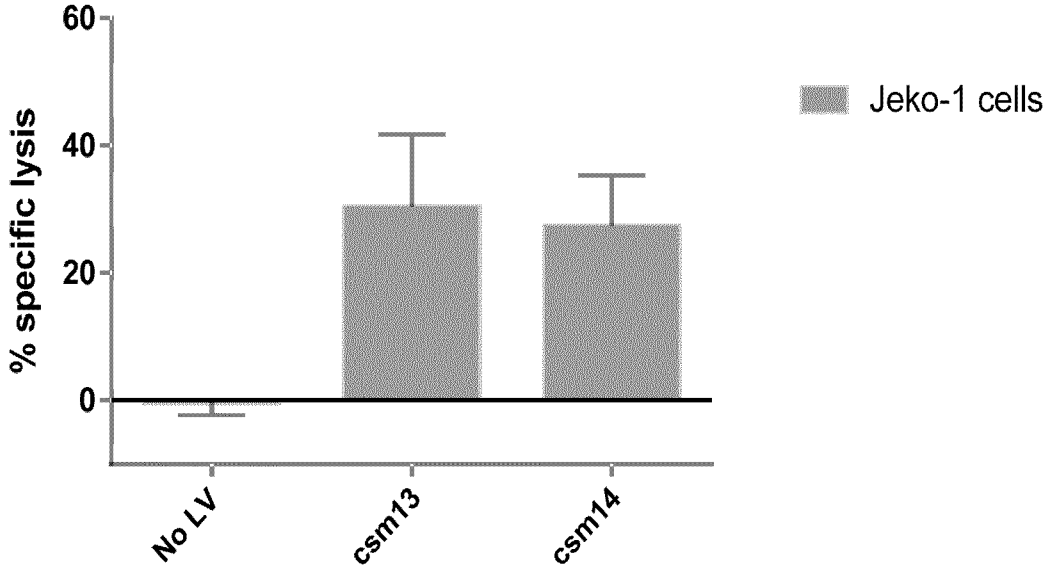

Figure 7

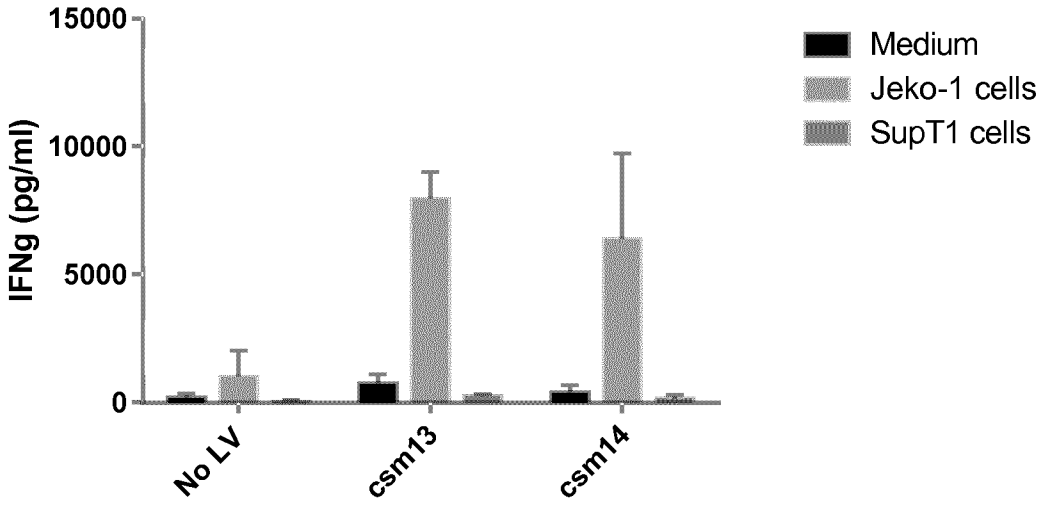
15000
10000
5000
0
IFNg (pg/ml)
No LV
csm13
csm14
Medium
Jeko-1 cells
SupT1 cells

Figure 8

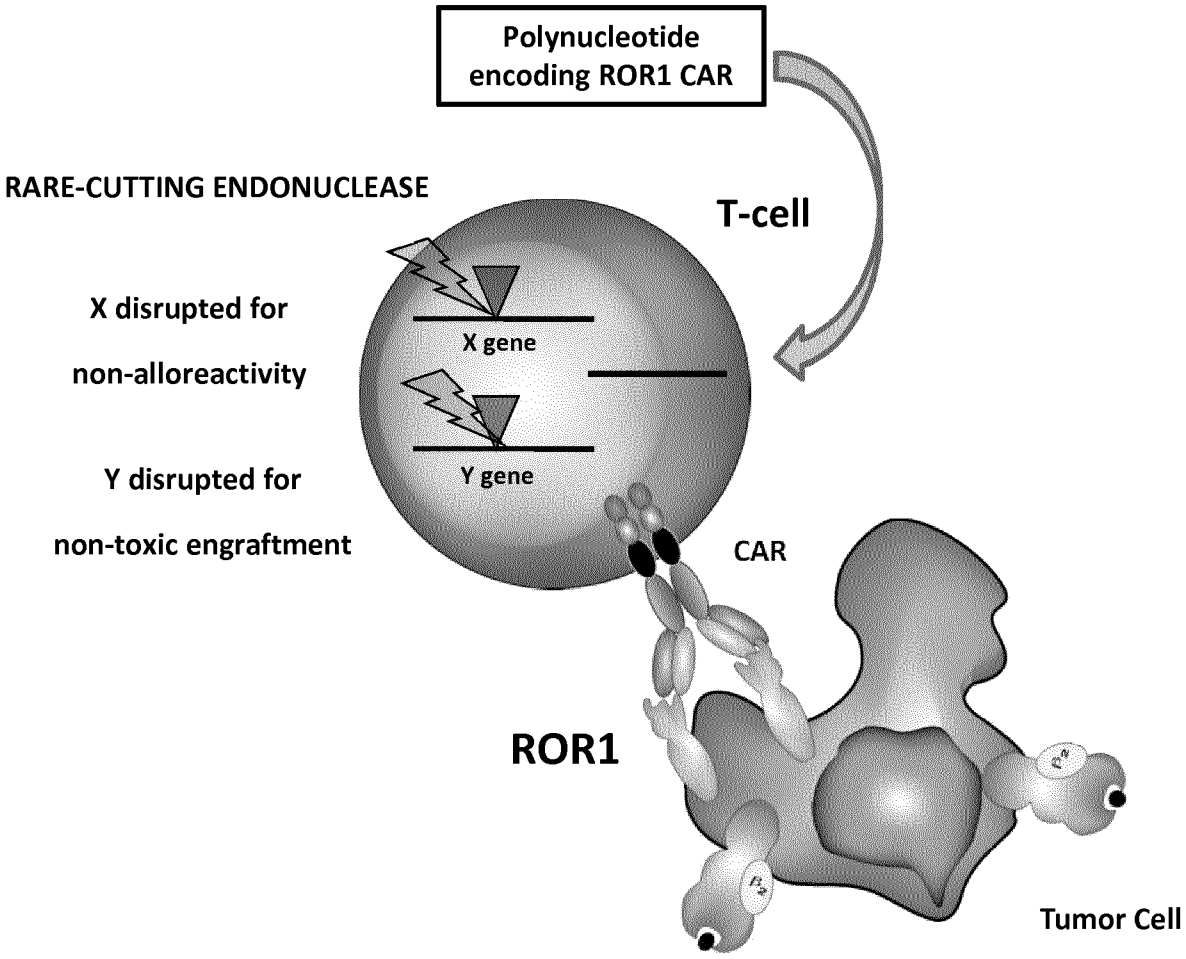
Polynucleotide encoding ROR1 CAR
RARE-CUTTING ENDONUCLEASE
T-cell
X disrupted for non-alloreactivity
X gene
Y disrupted for non-toxic engraftment
Y gene
CAR
ROR1
Tumor Cell

Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/067441

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 C07K16/28 C07K14/735 C07K16/2803
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)
C12N C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/039523 A1 (CELLECTIS [FR]) 13 March 2014 (2014-03-13) cited in the application page 15 - paragraph 1; claims	1-36
Y	WO 2013/123061 A1 (SEATTLE CHILDREN S HOSPITAL D B A SEATTLE CHILDREN S RES INST [US]) 22 August 2013 (2013-08-22) page 20 - paragraph 1; claims 63,98	1-36

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[X] Further documents are listed in the continuation of Box C.

[X] 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

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Date of the actual completion of the international search	Date of mailing of the international search report
26 October 2015	10/11/2015

Name and mailing address of the ISA/	Authorized officer
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