DESCRIPTION

TITLE OF THE INVENTION: COMPOSITION, IMMUNE CELLS COMPRISING SAME, AND USE OF SAID IMMUNE CELLS

[0001] The invention relates to a composition as well as to immune cells comprising said composition and to the use of cells comprising said composition.

[0002] T-lymphocytes are a category of white blood cells that participate in the immune defenses of the body by destroying pathogens and tumor cells. However, in the case of certain cancers, these tumor cells have the ability to inactivate the immune defenses of their host.

[0003] CAR-T or *Chimeric Antigenic Receptor-T* cells are a cellular immunotherapy approach aimed at combating certain cancers, especially B-cell leukemias and certain lymphomas, by boosting the patient's immune system.

[0004] This innovative approach consists in genetically modifying T-lymphocytes from the patient's own blood or from a compatible donor, so that they can effectively recognize and eliminate cancer cells. This *ex vivo* (outside the patient's body) modification enables better control of the genetic modification to be carried out, in this case the introduction of a gene enabling the production of surface receptors (CARs) that enables the T-cells to attack and destroy cancer cells. Once these cells have been produced in sufficient quantity to combat the disease, they are injected into the patient's bloodstream.

[0005] A plurality of genome-editing technologies have been adapted for the production of these CAR-T cells, such as CRISPR/CAS9, Zinc-finger and

TALENs. However, the latter are relatively ineffective at inserting the CAR gene into the T-cells.

[0006] Other systems are also used, such as viral vectors (AAV, Lentivirus) and the *sleeping beauty* transposon, but the pseudo-specific integration of the CAR gene with these approaches can lead to consequences downstream of their use (e.g. application WO2017180989A2).

[0007] Also, given the power of CAR technology, there is still a need for more efficient production of CAR-T modified T-cells.

[0008] The aim of the present invention is to overcome these drawbacks.

[0009] One aim of the invention is to propose a composition capable of enabling the simple and efficient production of CAR-T modified T-cells.

[0010] A further aim of the invention is to provide a method or a drug that is capable of treating diseases via said CAR-T modified T-cells.

[0011] The invention relates to a composition comprising

[0012] - a first single-stranded nucleic acid molecule comprising or consisting essentially of an A sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary sequence binding at 5′ to a first T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a second T-rich sequence of 40 to 60 nucleotides in length, said first and second T-rich sequences respectively comprising a first and a second domain of 6 to 12 G/C-rich nucleotides, the sequence of the first domain being complementary to the sequence of the second domain, said first and second domains being positioned 15 to 52 nucleotides from said A sequence, said first molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′

for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase,

[0013] - a second single-stranded nucleic acid molecule comprising or consisting essentially of a B sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary B sequence binding at 5′ to a third T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a fourth T-rich sequence of 40 to 60 nucleotides in length, said third and fourth T-rich sequences respectively comprising a third and a fourth domain of 6 to 12 G/C-rich nucleotides, the sequence of the third domain being complementary to the sequence of the fourth domain, said third and fourth domain being positioned 15 to 52 nucleotides from said B sequence, said second molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing said transposase and at its 3′ end the second sequence for recognizing said transposase,

[0014] said B sequence being a complementary sequence of said nucleic acid of interest, the A sequence being positioned at 5′ of a region of interest of said nucleic acid of interest and the B sequence positioned at 3′ of the region of interest of said nucleic acid of interest, and

[0015] - a third single-stranded molecule comprising

[0016] * in its 5′ part, at least one complementary sequence of said second sequence for recognizing said transposase of the first molecule,

[0017] * in its 3′ part, at least one complementary sequence of said first sequence for recognizing said transposase of the second molecule, and

[0018] * an intermediate region located between the complementary sequence of said second sequence for recognizing said transposase of the first molecule

and the complementary sequence of said first sequence for recognizing said transposase of the second molecule, said intermediate region comprising a sequence encoding a fusion protein, said fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the activation domain of the T-lymphocyte, i.e. the CD3ζ chain of the T-cell receptor or TCR,

[0019] the first and third single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase, and the second and third single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase.

[0020] The invention is based on the observation by the inventors that the use of a molecular complex as described hereinbefore, involving a bacterial transposase, enables targeted and efficient replacement of a genomic target, and the production of CAR-T fusions using a sequence of interest, enabling the custom production of T-cells targeting specific tumor cells.

[0021] This means that the invention relates to a molecular complex comprising a first single-stranded nucleic acid molecule, a second single-stranded nucleic acid molecule, and a third single-stranded nucleic acid molecule, said third single-stranded nucleic acid molecule comprising or consisting essentially at its 5′ end of at least one complementary sequence of said first sequence for recognizing said transposase, and said third single-stranded nucleic acid molecule comprising or consisting essentially at its 3′ end of at least one complementary sequence of said second sequence for recognizing said transposase,

[0022] said complex being such that the first, second, and third single-stranded nucleic acid molecules are paired according to the base complementarity

defined by Watson and Crick so as to define two double-stranded binding sites for said transposase in the 5′ end of the third molecule and two double-stranded binding sites for said transposase in the 3′ end of the third molecule.

[0023] The invention is based on the unexpected observation made by the inventor that the use of specific single-stranded guides capable of targeting a region of a nucleic acid of interest makes it possible to mobilize transposases in a controlled and “site-specific” manner, and thus to use the recombination properties of said transposases to replace sequences in molecules of interest.

[0024] The aforementioned molecular complex is in fact the basic unit of the technology defined in the invention. This basic unit is useful for guiding the recombinases to a specific site where the recombination, and therefore the sequence replacement, must take place. Unlike the CRISPR/Cas9 system, which requires the presence of PAM-type (NGG) sequences, the molecular tool defined herein may be used on any target sequence, regardless of its sequence.

[0025] The aforementioned molecular complex is therefore the basic unit to be completed by:

[0026] - a homology region of a 5′ region of the target sequence, said homology region being represented by the A sequence and

[0027] - a homology region of a 3′ region of the target sequence, said homology region being represented by the B sequence.

[0028] This is therefore an intermediate product of the tool as described hereinafter.

[0029] The molecular complex consists of three single-stranded nucleic acid molecules, which may be DNA molecules, RNA molecules or mixed RNA and DNA molecules.

[0030] These three molecules are partially complementary with each other two by two, according to the base complementarity of nucleic acids defined by Watson and Crick, that is to say that adenine pairs with thymidine or uracil, and cytosine pairs with guanine, and vice versa.

[0031] More particularly, the three molecules forming the aforementioned complex each comprise the sequence of one of the strands of a double-stranded molecule corresponding to the binding sequence of a transposase. Also, each single-stranded molecule therefore comprises a transposase binding “half sequence” and therefore cannot allow an interaction with said corresponding transposase. On the other hand, when the three molecules of the complex interact together, by base pairing as defined hereinbefore, a double-stranded molecule is thus formed, reconstituting a double-stranded binding site of said transposase, the latter thus being able to interact with the molecule formed.

[0032] The complex

[0033] In addition to the detailed definition of each of the first, second, and third molecules, each described hereinafter, the complex according to the invention is such that it comprises two pairs of binding sites to one or more transposases,

- a first pair of sites for binding to a first transposase as a result of pairing according to the nucleic acid base complementarity defined by Watson and Crick between the first single-stranded nucleic acid molecule and the third nucleic acid molecule, and
- a second pair of sites for binding to a second transposase as a result of pairing according to the nucleic acid base complementarity defined by Watson

and Crick between the second single-stranded nucleic acid molecule and the third nucleic acid molecule.

[0034] the first and second transposases may be the same or different.

[0035] Each site for binding to a transposase consists of two double-stranded motifs, a first double-stranded motif consisting of a first transposase binding half-site and a complementary sequence, according to the base complementarity defined by Watson and Crick, of said first transposase binding half-site, and a second double-stranded motif consisting of a second transposase binding half-site and a complementary sequence, according to the base complementarity defined by Watson and Crick, of said second transposase binding half-site.

[0036] In the invention, the complementary sequence of a half-sites for binding to a transposase is referred to as a complementary half-site of said site.

[0037] A site for binding to a transposase therefore consists of four half-sites:

[0038] - a first transposase binding half-site having a first sequence,

[0039] - a second transposase binding half-site having a second sequence,

[0040] - a third half-site which has a complementary sequence, according to the base complementarity defined by Watson and Crick, of the sequence of the first half-site, and

[0041] - a fourth half-site which has a complementary sequence, according to the base complementarity defined by Watson and Crick, of the sequence of the second half-site.

[0042] Also, in the complex there will thus be eight binding sites, four of them constituting the first pair of binding sites by pairing the first and third nucleic acid molecules, and the other four constituting the second pair of binding sites by pairing the second and third nucleic acid molecules.

[0043] It should be noted that, for the first pair of sites for binding to a transposase, the four half-binding sites are such that

- the first half-site and the second half-site are contained in the first nucleic acid sequence,
- the complementary site of the second half-site is included in the third nucleic acid sequence,

[0044] their positions in the complex are therefore defined, and

- the complementary site of the first half-site is either i) included in the first nucleic acid molecule, ii) or included in the third nucleic acid molecule

[0045] it is therefore not necessary to define its position in one (first) nucleic acid molecule or the other (third), the essential point being that when the first and third nucleic acid molecules are paired according to the base complementarity defined by Watson and Crick, the two half-sites are reconstituted, i.e. the first half-site is paired with the complementary half-site of the first half-site, and the second half-site is paired with the complementary half-site of the second half-site.

[0046] It should also be noted that for the second pair of sites for binding to a transposase, the four half-binding sites are such that

- the first half-site and the second half-site are contained in the second nucleic acid sequence,
- the complementary site of the first half-site is included in the third nucleic acid sequence,

[0047] their positions in the complex are therefore defined, and

- the complementary site of the second half-site is either i) included in the second nucleic acid molecule, or ii) included in the third nucleic acid molecule

[0048] it is therefore not necessary to define its position in one (second) nucleic acid molecule or the other (third), the essential point being that when the second and third nucleic acid molecules are paired according to the base complementarity defined by Watson and Crick, the two half-sites are reconstituted, i.e. the first half-site is paired with the complementary half-site of the first half-site, and the second half-site is paired with the complementary half-site of the second half-site.

[0049] The first molecule.

[0050] The first molecule of the complex is the molecule that comprises, once modified, a nucleic acid sequence that makes it possible to specifically target a region of interest of a nucleic acid molecule of interest. This sequence of interest is selected by the user of the system according to the selected target. This sequence of interest is inserted into the first molecule of said complex at the A region. This A region corresponds at least to two nucleic acids between which the sequence that makes it possible to target the target molecule is inserted. In view of the oriented structure of the nucleic acids (5′-to-3′ direction), it is important for the sequence that makes it possible to target the region of interest to be positioned in the correct direction, in order to allow pairing with the target sequence.

[0051] Also, advantageously, the A region comprises one or more sites recognizing restriction enzymes in order to promote an oriented insertion. One or more of the following sites may be present in the A region:

[0052] [Table 1]

		AA/CGTT	AclI

		A/AGCTT	HindIII
		AAT/ATT	SspI
		/AATT	MluCI
		A/CATGT	PciI
		A/CCGGT	AgeI
		ACCTGC(4/8)	BfuAI BspMI
		A/CCWGGT	SexAI
		A/CGCGT	MluI
		ACGGC(12/14)	BceAI
		A/CGT	HpyCH4IV
		ACN/GT	HpyCH4III
SEQ ID NO:	4	(10/15)ACNNNNGTAYC(12/7)	BaeI
SEQ ID NO:	5	(9/12)ACNNNNNCTCC(10/7)	BsaXI
		A/CRYGT	AflIII
		A/CTAGT	SpeI
		ACTGG(1/-1)	BsrI
		ACTGGG(5/4)	BmrI
		A/GATCT	BglII
		AGC/GCT	AfeI
		AG/CT	AluI
		AGG/CCT	StuI
		AGT/ACT	ScaI-
		AT/CGAT	ClaI BspDI
SEQ ID NO:	6	ATCTATGTCGGGTGCGGAGAAAGAGG TAAT(-15/-19)	PI-SceI
		ATGCA/T	NsiI
		AT/TAAT	AseI
		ATTT/AAAT	SwaI
SEQ ID NO:	7	(11/13)CAANNNNNGTGG(12/10)	CspCI
		C/AATTG	MfeI
		CACCTGC(4/8)	PaqCI
		CACGAG	Nb.BssSI
		CACGAG(-5/-1)	BssSI-v2
		CACGTC(-3/-3)	BmgBI
		CAC/GTG	PmlI
		CACNNN/GTG	DraIII
SEQ ID NO:	8	CACNN/NNGTG	AleI-v2
		CAGCAG(25/27)	EcoP15I
		CAG/CTG	PvuII
		CAGNNN/CTG	AlwNI
		CAGTG(2/0)	BtsIMutI
		CA/TATG	NdeI
		CATG/	NlaIII
		/CATG	FatI
		C/ATG	CviAII

SEQ ID NO:	9	CAYNN/NNRTG	MslI
		CC(12/16)	FspEI
SEQ ID NO:	10	CCANNNNN/NNNNTGG	XcmI
SEQ ID NO:	11	CCANNNNN/NTGG	BstXI
SEQ ID NO:	12	CCANNNN/NTGG	PflMI
		CCATC(4/5)	BccI
		C/CATGG	NcoI
		CCCAGC(-5/-1)	BseYI
		CCCGC(4/6)	FauI
		CCC/GGG	SmaI
		C/CCGGG(0/-1)CCD	TspMI XmaI Nt.CviPII
		CCDG(10/14)	LpnPI
		CCGC(-3/-1)	AciI
		CCGC/GG	SacII
		CCGCTC(-3/-3)	BsrBI
		C/CGG	MspI HpaII
		CC/NGG	ScrFI
		/CCNGG	StyD4I
		C/CNNGG	BsaJI
SEQ ID NO:	13	CCNNNNN/NNGG	BslI
		C/CRYGG	BtgI
		CC/SGG	NciI
		C/CTAGG	AvrII
		CCTC(7/6)	MnlI
		CCTCAGC	Nb.BbvCI
		CCTCAGC(-5/-7)	Nt.BbvCI
		CCTCAGC(-5/-2)	BbvCI
		CCTGCA/GG	SbfI
		CCTNAGC(-5/-2)	Bpu10I
		CC/TNAGG	Bsu36I
SEQ ID NO:	14	CCTNN/NNNAGG	EcoNI
		CCTTC(6/5)	HpyAV
		/CCWGG	PspGI
		CC/WGG	BstNI
		C/CWWGG	StyI
SEQ ID NO:	15	(10/12)CGANNNNNNTGC(12/10)	BcgI
		CGAT/CG	PvuI
		CG/CG	BstUI
		C/GGCCG	EagI
		CG/GWCCG	RsrII
		CGRY/CG	BsiEI
		C/GTACG	BsiWI
		CGTCTC	BsmBI-v2
		CGTCTC(1/5)	Esp3I

		CGWCG/	Hpy99I
		CMG/CKG	MspA1I
SEQ ID NO:	16	CNNNNNNNNNNN/NNNNNNNNNG	AbaSI
		CNNR(9/13)	MspJI
		CR/CCGGYG	SgrAI
		C/TAG	BfaI
		CTCAG(9/7)	BspCNI
		C/TCGAG	XhoI PaeR7I
		CTCTTC(1/4)	EarI
		CTGAAG(16/14)	AcuI
		CTGCA/G	PstI
		CTGGAG(16/14)	BpmI
		C/TNAG	DdeI
		C/TRYAG	SfcI
		C/TTAAG	AflII
		CTTGAG(16/14)	BpuEI
		C/TYRAG	SmlI
		C/YCGRG	BsoBI AvaI
		GAAGA(8/7)	MboII
		GAAGAC(2/6)	BbsI
SEQ ID NO:	17	GAANN/NNTTC	XmnI
		GAATGC(1/-1)	BsmI
		GAATGC	Nb.BsmI
		G/AATTC	EcoRI
		GACGC(5/10)	HgaI
		GACGT/C	AatII
		GAC/GTC	ZraI
		GACN/NNGTC	PflFI Tth111I
SEQ ID NO:	18	GACNN/NNGTC	PshAI
SEQ ID NO:	19	GACNNN/NNGTC	AhdI
SEQ ID NO:	20	GACNNNN/NNGTC	DrdI
		GAG/CTC	Eco53kI
		GAGCT/C	SacI
		GAGGAG(10/8)	BseRI
		GAGTC(4/-5)	Nt.BstNBI
		GAGTC(4/5)	PleI
		GAGTC(5/5)	MlyI
		G/ANTC	HinfI
		GAT/ATC	EcoRV
		GA/TC	DpnI
		/GATC	Sau3AI DpnII MboI
SEQ ID NO:	21	GATNN/NNATC	BsaBI
		G/AWTC	TfiI

		GCAATG	Nb.BsrDI
		GCAATG(2/0)	BsrDI
		GCAGC(8/12)	BbvI
		GCAGTG(2/0)	BtsI-v2
		GCAGTG	Nb.BtsI
SEQ ID NO:	22	GCANNNN/NTGC	BstAPI
		GCATC(5/9)	SfaNI
		GCATG/C	SphI
		GCCC/GGGC	SrfI
		GCCGAG(21/19)	NmeAIII
		G/CCGGC	NgoMIV
		GCC/GGC	NaeI
SEQ ID NO:	23	GCCNNNN/NGGC	BglI
		GCGAT/CGC	AsiSI
		GCGATG(10/14)	BtgZI
		GCG/C	HhaI
		G/CGC	HinP1I
		G/CGCGC	BssHII
		GC/GGCCGC	NotI
		GC/NGC	Fnu4HI
		GCN/NGC	Cac8I
SEQ ID NO:	24	GCNNNNN/NNGC	MwoI
		G/CTAGC	NheI
		GCTAG/C	BmtI
		GCTCTTC(1/-7)	Nt.BspQI
		GCTCTTC(1/4)	SapI BspQI
		GC/TNAGC	BlpI
		G/CWGC	ApeKI TseI
		GDGCH/C	Bsp1286I
		GGATC(4/5)	AlwI
		GGATC(4/-5)	Nt.AlwI
		G/GATCC	BamHI
		GGATG(9/13)	FokI
		GGATG(2/0)	BtsCI
		GG/CC	HaeIII
		GGCCGG/CC	FseI
SEQ ID NO:	25	GGCCNNNN/NGGCC	SfiI
		G/GCGCC	KasI
		GG/CGCC	NarI
		GGCGC/C	PluTI
		GGC/GCC	SfoI
		GG/CGCGCC	AscI
		GGCGGA(11/9)	EciI
		GGGAC(10/14)	BsmFI
		GGGCC/C	ApaI

		G/GGCCC	PspOMI
		G/GNCC	Sau96I
		GGN/NCC	NlaIV
		G/GTACC	Acc65I
		GGTAC/C	KpnI
		GGTCTC(1/5)	BsaI v2
		GGTGA(8/7)	HphI
		G/GTNACC	BstEII
		G/GWCC	AvaII
		G/GYRCC	BanI
		GKGCM/C	BaeGI
		GR/CGYC	BsaHI
		GRGCY/C	BanII
		GT/AC	RsaI
		G/TAC	CviQI
		GTATAC	BstZ17I
		GTATCC(6/5)	BciVI
		G/TCGAC	SalI
		GTCTC(1/5)	BsmAI BcoDI
		GTCTC(1/-5)	Nt.BsmAI
		G/TGCAC	ApaLI
		GTGCAG(16/14)	BsgI
		GT/MKAC	AccI
		GTN/NAC	Hpy166II
		/GTSAC	Tsp45I
		GTT/AAC	HpaI
		GTTT/AAAC	PmeI
		GTY/RAC	HincII
		GWGCW/C	BsiHKAI
		NNCASTGNN/	TspRI
		R/AATTY	ApoI
		RCATG/Y	NspI
		R/CCGGY	BsrFI-v2
		R/GATCY	BstYI
		RGCGC/Y	HaeII
		RG/CY	CviKI-1
		RG/GNCCY	EcoO109I
		RG/GWCCY	PpuMI
SEQ ID NO:	26	TAACTATAACGGTCCTAAGGTAGCGAA(-9/-13)	I-CeuI
		TAC/GTA	SnaBI
SEQ ID NO:	27	TAGGGATAACAGGGTAAT(-9/-13)	I-SceI
		T/CATGA	BspHI
		T/CCGGA	BspEI
		TCCRAC(20/18)	MmeI

		T/CGA	TaqI-v2
		TCG/CGA	NruI
		TCN/GA	Hpy188I
		TC/NNGA	Hpy188III
		T/CTAGA	XbaI
		T/GATCA	BclI
		TG/CA	HpyCH4V
		TGC/GCA	FspI
SEQ ID NO:	28	TGGCAAACAGCTATTATGGGTATTATGGGT(-13/-17)	PI-PspI
		TGG/CCA	MscI
		T/GTACA	BsrGI
		T/TAA	MseI
		TTAAT/TAA	PacI
		TTA/TAA	PsiI-v2
		TT/CGAA	BstBI
		TTT/AAA	DraI
		VC/TCGAGB	PspXI
		W/CCGGW	BsaWI
		YAC/GTR	BsaAI
		Y/GGCCR	EaeI

[0053] Obviously, in the context of a chemical synthesis of the first molecule, it is not necessary to have cloning (or insertion) sites of the sequence making it possible to target the target region, but rather to take good care to provide a correctly oriented sequence. This is of course however possible.

[0054] The first molecule further consists, on either side of the A region, of A/T-rich sequences, or in the case of RNA, of A/U-rich sequences, in order to allow a certain flexibility of the structure. A/T-rich or A/U rich are understood in the invention to mean a sequence that comprises more than 50 % of A or T, or U, preferably more than 50 % of T or U, with respect to the total number of nucleotides that constitute the sequence. These sequences on either side of the A region have a size in nucleotides ranging from 10 nucleotides to 60 nucleotides.

[0055] The flexibility of these sequences flanking the A region, due to the presence of numerous A, T or U bases, may have the effect of allowing a

recombination via recombinases that are not sufficiently controlled, maybe even when the complex still has not recognized the target molecule.

[0056] Also, in order to overcome this problem, G/C-rich sequences are introduced into each of the A/T-rich, especially T-rich, or A/U-rich, sequences bordering the A region. These G/C-rich regions consist of 6 to 12 nucleotides, in which the amount of C or G bases is greater than 50 % of the nucleotides contained in said G/C-rich sequence.

[0057] In order to stabilize the structure of the first molecule and, as described hereinbefore, prevent inadvertent recombination, the G/C-rich regions are positioned 15 to 52 nucleotides from the end of the A region.

[0058] For greater clarity, if the A region consists of three nucleotides, the central nucleotide corresponding to position 0, the A/T-rich or A/U-rich region begins on the left at position -2, and on the right at position +2. Therefore, on the left, the G/C-rich region is positioned from position -17 to position -54 and, on the right, from position +17 to position +54.

[0059] Another important element: the G/C-rich sequence to the right (or 5′) of the A region is necessarily complementary (according to the Watson and Crick pairing rule) to the G/C-rich region to the right (or 3′) of the A region. Also, the first single-stranded molecule pairs with itself at the G/C-rich regions, which prevents any recombination by the transposases, as long as there is no interaction with the complementary target sequence of the region that is inserted into the A region of the first molecule.

[0060] Finally, the first molecule comprises at its 5′ end a sequence corresponding to a first site for binding to a transposase, and at its 3′ end a second site for binding to said transposase.

[0061] The first binding site and the second binding site are advantageously the same, and above all both correspond to the same strand of the double-stranded binding site of said transposase. This means that the first transposase binding site present in the 5′ region of the first molecule can only be paired integrally, and therefore stably, with the transposase binding site present in the 3′ region.

[0062] The first binding site and the second binding site are advantageously the same, but each correspond to a different strand of the double-stranded transposase binding site. Also, for example, if the first transposase binding site corresponds to the sense strand, the second transposase binding site corresponds to the sequence of the complementary strand. It is then possible to have two configurations: either i) the second binding site which corresponds to the complementary strand is oriented in the 3′-to-5′ direction, in which case it is able to pair with the first transposase binding site and form the double-stranded site, or ii) the second binding site which corresponds to the complementary strand is oriented in the 5′-to-3′ direction, in which case it is not able to pair with the first transposase binding sequence, due to their orientation not being complementary. In the aforementioned case i), if the first single-stranded molecule pairs with itself at the first and second binding sites, it is not possible to form the aforementioned complex, since there are no more single-stranded complementary regions available to pair with the second molecule so as to form two double-stranded transposase binding sites.

[0063] Also, the first molecule, when it lacks a complementary sequence of the target region in the A part, or when it contains such a target sequence but the latter does not interact (does not pair) with said target sequence, forms a three-dimensional structure wherein the entire molecule is single-stranded with the exception of the region corresponding to the G/C-rich regions that pair with one another.

[0064] A schematic depiction of the matched form is depicted in **Fig. 1A to 1E**.

[0065] The second molecule.

[0066] The second molecule in the complex is structurally similar to the first molecule, so the aforementioned explanations apply *mutatis mutandis*. However, in the second molecule, the B region (or B sequence) and the transposase binding sites are organized differently.

[0067] First of all, the B region is different from the A region of the first molecule. Indeed, for the purposes of directed recombination, there can be no competition for the same target of interest between the first and second molecules.

[0068] It is therefore necessary for the B region to correspond to a second complementary sequence of the target sequence, this second complementary sequence of the target sequence being positioned at 3′ with respect to the first sequence recognized by the complementary sequence corresponding to the A sequence of the first molecule.

[0069] As a result, when the first molecule and the second molecule are paired with the target sequence, the target sequence will be bordered by these two molecules, with the first molecule located at 5′ and the second molecule positioned at 3′. The region of the target sequence located between the region of interaction with the first molecule and the region of interaction with the second molecule corresponds to the sequence that will be replaced with that of the third molecule.

[0070] The third molecule

[0071] The third molecule of the aforementioned complex is simpler than the first two (the first and second molecules). The third molecule comprises, in its 5′ part, a transposase binding site which is complementary to the binding site

for said transposase present in the 3′ part of the first molecule. Therefore, when the complex is formed, the (single-stranded) transposase binding half-site located at 3′ of the first molecule may pair with the (single-stranded) transposase binding half-site located at 5′ of the second molecule so as to form a double-stranded transposase binding site, a double-stranded site on which the transposase can bind.

[0072] The 3′ part of the third molecule comprises a transposase binding site which is complementary to the site for binding to said transposase present in the 5′ part of the second molecule. Therefore, when the complex is formed, the (single-stranded) transposase binding half-site located at 3′ of the third molecule may pair with the (single-stranded) transposase binding half-site located at 5′ of the second molecule so as to form a double-stranded transposase binding site, a double-stranded site on which the transposase can bind.

[0073] Between the 5′ part, which comprises a transposase binding half-site, and the 3′ part, which comprises a transposase binding half-site, the third molecule comprises a replacement sequence, i.e. the sequence that will eventually replace the target sequence. This replacement sequence is bordered at 5′ by at least one restriction site and at 3′ by at least one restriction site; these two restriction sites being different. Also, the third molecule comprises in the 5′ to 3′ direction: a transposase binding half-site which is complementary to the transposase binding site of the first molecule, followed by at least one restriction site, followed by the replacement sequence, followed by at least one restriction site different from the restriction site upstream of the replacement sequence, followed finally by a transposase binding half-site which is complementary to the transposase binding site of the second molecule.

[0074] The replacement sequence corresponds to the part of the encoding sequence of the CD247 gene encoding at least the intracellular domain of the CD3ζ (CD3 zeta) protein.

[0075] The ζ chain (or CD3ζ) is a transmembrane protein that is one of the components of the T-cell receptor (TCR) complex and is involved in signal transduction.

[0076] The ζ chain features a small extracellular N-terminal part (9 amino acids) that interacts with the CD3α and CD3β chains, a single transmembrane domain and a long cytoplasmic chain (120 amino acids). This long cytoplasmic chain contains three immunoreceptor tyrosine-based activation motifs (ITAMs), which are involved in its interaction with the T-cell cytoskeleton during T-cell activation.

[0077] The composition according to the invention makes it possible to obtain the aforementioned complex. [Fig. 2] schematically illustrates the complex formed between the first, second, and third molecules according to the invention.

[0078] The complex formed from the molecules of the composition of the invention, when the three molecules are correctly paired, comprises two pairs of transposase recognition double-stranded binding sites:

[0079] - the first pair being obtained by the hybridization of the first molecule with the third molecule, and

[0080] - the second pair being obtained by the hybridization of the second molecule with the third molecule.

[0081] The A sequence contained in the first molecule is complementary to the same strand of the nucleic acid which is complementary to the B sequence contained in the second molecule. In other words, the A sequence contained in the first molecule and the B sequence contained in the second molecule are capable of hybridizing with the same nucleic acid simultaneously, since the two sequences A and B do not recognize the same sequence.

[0082] In order to further clarify the remarks, the interest of the present invention is to propose a first molecule and a second molecule, both as defined hereinbefore, the respective sequences A and B being such that they are capable of recognizing, for one, a sequence located at 5′ of the target sequence of the nucleic acid of interest and, for the other, a sequence located at 3′ of the same target sequence of the nucleic acid of interest. The sequences A and B are therefore complementary to regions bordering the sequence of interest, which it is sought to replace, of the nucleic acid molecule of interest.

[0083] The first and second molecules of interest are therefore essential for targeting specifically the molecule of interest, in order to flank the sequence to be replaced.

[0084] The third molecule of the ensemble, in turn, is the one that provides the nucleic acid molecule that contains the replacement sequence., i.e. a sequence containing the intracellular part of the activation of the CD3ζ.

[0085] From a mechanistic point of view, the complex according to the invention is such that it consists of its three molecules, the first and second molecules being structurally organized in space so that their G/C-rich regions are paired.

[0086] On either side of the third molecule, that is to say at 5′ and at 3′, due to the hybridization with the first and second molecules, two pairs of sites for binding to a transposase allow, when transposase is present, transposase dimers to bind to the ensemble.

[0087] It will be noted that the transposase binding sites of the first molecule can be the same as those of the second molecule, or different. In the case where the binding sequences are the same, the transposases at 5′ of the third

molecule (by hybridization of the 5′ part of the third molecule with the first molecule), and at 3′ of the third molecule (by hybridization of the 3′ part of the third molecule with the second molecule) are the same. Also, by way of example, if the binding sites are all binding sites for the Tn5 transposase, the ensemble is associated with two Tn5 transposase dimers.

[0088] It is also possible for the binding sites of the first molecule and of the second molecule not to recognize the same transposase. In this case, and according to the definition given hereinbefore, the 5′ part of the third molecule forms, by hybridization with the first molecule, two double-stranded sites for binding to a first transposase, and the 3′ part of the third molecule forms, by hybridization with the second molecule, two double-stranded sites for binding to a second transposase.

[0089] If the aforementioned complex, linked to two transposase dimers, is next brought into the presence of a nucleic acid molecule of interest, the 5′ part of which is complementary to the A sequence of the first molecule of the ensemble and the 3′ part of which is complementary to the B sequence of the second molecule of the ensemble, then the nucleic acid molecule of interest pairs with the ensemble at the above-described regions A and B. This interaction has the consequence of breaking the interaction of the two G/C-rich sequences of each of the first and second molecules of the ensemble. Therefore, the third molecule and the nucleic acid molecule of interest are moved closer together in space and the transposases can exercise their tagmentation activity, the result of which is the replacement of the sequence of the molecule of interest, bordered by the complementary sequences of the A and B sequences, by the sequence of the third molecule of the ensemble, which is located between the half-sites for binding to the transposase(s) at 5′ and at 3′.

[0090] In the invention, the first molecules, second molecules and third molecule of the ensemble are advantageously molecules consisting of deoxyribonucleotides, in order to form single-stranded DNA molecules.

[0091] Even more advantageously, the first molecules, second molecules, and third molecule of the ensemble are hybrid DNA/RNA molecules, wherein the “backbone” of the molecules is DNA, and the recognition sequences A and B of the nucleic acid molecule of interest and the central region of the third molecule are RNA. This is particularly advantageous when the sequence replacement that enables the invention is to be carried out directly on an RNA molecule.

[0092] [Fig. 2] **-A** illustrates the interaction between the molecule of interest and the first/second/third molecule complex according to the invention.

[0093] Advantageously, the invention relates to the aforementioned complex, wherein said first molecule or said second molecule, or both molecules, are coupled with an enzyme, especially via a modified nucleotide. This enzyme is intended to promote the replacement of the molecule of interest. It can be

[0094] - a helicase, an enzyme capable of opening a double-stranded molecule that is supercoiled or even associated with proteins such as histones,

[0095] - a topoisomerase, an enzyme acting on the topological structure of the DNA by generating transient breaks,

[0096] - a ligase that makes it possible to form a phosphodiester linkage between a phosphate 5′ end of a nucleotide and the OH 3′ end of another nucleotide,

[0097] - a polymerase, which synthesizes a nucleic acid molecule from an initiation site, free 3′OH, in the 5′-to-3′ direction, especially by copying an antiparallel complementary strand according to the Watson and Crick model.

[0098] It is also possible to combine two or more of said enzymes in order to have all the enzymatic material necessary to enable the sequence replacement contemplated in the scope of the invention.

[0099] In one particular aspect, the first molecule of the ensemble can contain in its 5′ part, more precisely between the first transposase binding site and the A region, one or more modified nucleotides. In the same way, the third molecule of the ensemble can contain in its 3′ part, more precisely between the first transposase binding site and the 3 region, one or more modified nucleotides.

[0100] This modified nucleotide is especially modified by grafting a substituted carbon chain with a protein tag, or else a molecule enabling a specific interaction such as streptavidin or biotin.

[0101] Such modifications then make it possible to specifically bind, to the first molecule of the ensemble, enzymes which can be useful for promoting tagmentation, and sequence replacement. It is particularly advantageous to have, for example, streptavidin grafting, which makes it possible to graft a biotinylated helicase (or conversely a helicase grafted to the streptavidin and a biotinylated nucleotide) useful for opening a double-stranded molecule. It is also possible to contemplate grafting with a biotinylated ligase (or a ligase coupled with streptavidin), in order to connect the recombinant strand on the 3′ side.

[0102] It is also possible to associate with the first molecule or with the second molecule of the ensemble an oligonucleotide, so that said oligonucleotide pairs on a predetermined region of said first or second molecule. This oligonucleotide is then advantageously coupled with a grafting molecule as explained hereinbefore.

[0103] Advantageously, the invention relates to the aforementioned composition, wherein said fusion protein comprises the peptide capable of

binding to a membrane protein of a cell, fused to a transmembrane part of a membrane protein, the latter fused to the CD3ζ chain of the TCR.

[0104] In this advantageous embodiment, the third molecule comprises a sequence that encodes a fusion protein having the following organization in the N-terminal to C-terminal direction: a peptide capable of binding to a membrane protein of a cell, generally a tumor cell as will be developed hereinafter, this peptide being followed by a transmembrane domain, itself followed by the intracellular part of the CD3ζ chain of the TCR.

[0105] It is especially advantageous for the transmembrane part of the abovementioned fusion protein to be that of the CD3ζ chain of the TCR, but any other transmembrane domain can be used, provided that once synthesized in a cell the fusion protein is such that: the binding peptide is exposed outside the cell (extracellular), and the CD3ζ chain of the TCR is exposed inside the cell (intracellular) in order to enable signal transduction.

[0106] If no other domain is present in said fusion protein, it is referred to as a chimeric receptor or first-generation CAR.

[0107] Advantageously, the invention relates to the aforementioned composition, wherein said fusion protein comprises the peptide capable of binding to a membrane protein of a cell, fused to a transmembrane part of a membrane protein, the transmembrane domain of the membrane protein being fused to a co-activation domain, the latter being fused to the CD3ζ chain of the TCR.

[0108] Advantageously, the aforementioned fusion protein also comprises, between the transmembrane domain and the activation domain (i.e. the intracellular domain) of the CD3ζ chain of the TCR, a co-activation domain of a protein other than CD3ζ. This configuration is based on the normal TCR activation mechanism.

[0109] The intracellular double signal is the typical feature of T-cell activation. Three different types of receptors, including T-cell antigenic receptors, cytokine receptors and co-stimulatory receptors, are included in this progression. The first signal is the specific signal that is triggered by the TCR when it recognizes the antigenic peptide-Major Histocompatibility Complex (MHC) complex on the surface of antigen-presenting cells. The second signal is the co-stimulatory signal, produced by a co-stimulatory molecule such as CD28/B7, which promotes IL-2 synthesis to complete T-cell activation and avoid apoptosis.

[0110] Naïve T-cells cannot play their normal role if the co-stimulatory signal is absent, and the same is true if the T-cells are stimulated by the antigen.

[0111] The CARs that include only the CD3ζ sequence cannot activate the cells without an endogenous co-stimulatory signal to be recruited, which may delay the cellular response.

[0112] To avoid this drawback, it is preferable to group both the CD3ζ chain activation signal and the co-activation signal on the same fusion protein. When the TCR is activated after recognizing the antigen-presenting MHC, both signals are activated simultaneously. The proliferation, cytotoxicity, sustained response, and survival of cells expressing this fusion protein will therefore be enhanced.

[0113] It is also possible to include a third activation domain enabling the production of cytokines that stimulate IL-12 production. This third signal can be generated, for example, using the STAT3 factor-binding domain of the IL-2 receptor.

[0114] Other examples are conceivable, and a skilled person will be able to adapt the co-activation domains as required and as CAR cell technology evolves.

[0115] Even more advantageously, the invention relates to the aforementioned composition,

[0116] - wherein said A sequence comprises a complementary sequence of the sequence of a first region of the locus of the gene encoding the TRAC protein and

[0117] - wherein said B sequence comprises a complementary sequence of the sequence of a second region of the locus of the gene encoding the TRAC protein,

[0118] said A sequence and said B sequence being two different sequences, said first and second regions of the TRAC locus advantageously flanking a 5′ region of the locus of the gene encoding the TRAC protein.

[0119] As explained hereinbefore, in order to replace the target gene with the third molecule, it is advantageous for the sequences of part A of the first molecule and part B of the second molecule to be able to:

[0120] - recognize the same target molecule, and

[0121] - flank the target region to be replaced, meaning that the first molecule must, via its A region, recognize a sequence upstream of the sequence to be replaced of the target molecule, and the second molecule must, via its B region, recognize a sequence downstream of the sequence to be replaced of the target molecule, or vice versa, i.e. the first molecule must, via its A region, recognize a sequence downstream of the sequence to be replaced of the target molecule, and the second molecule must, via its B region, recognize a sequence downstream of the sequence to be replaced of the target molecule.

[0122] This means that any target sequence can be replaced with any replacement sequence, as long as the A and B sequences of the first and second molecules, respectively, recognize and flank the target region.

[0123] In the invention, it is particularly advantageous to insert the fusion encoded by the third molecule at a locus that allows its expression equivalently to the CD3ζ chain of the TCR, without replacing the CD3ζ chain of the endogenous TCR that is required for T-cell activity.

[0124] It is therefore possible to insert the fusion (i.e. the sequence of the third molecule encoding the fusion protein) anywhere in the target genome, as long as this insertion does not generate a mutation (interruption of a gene or regulatory element). In particular, it is interesting to propose a fusion insertion at the locus of the TRAC gene, a gene that encodes the constant part of the TCR α domain. Even more particularly, it is advantageous to perform an insertion at the level of the first exon of the TRAC locus, and even more advantageously, immediately at 5′ of the first exon.

[0125] Such an insertion at the TRAC locus not only results in basal expression similar to that of the TCR chains, but also offers enhanced anti-tumor efficacy.

[0126] In addition, such an insertion at the TRAC locus:

[0127] - reduces tonic activation signaling and the development of efficient internalization and balanced re-expression of CAR upon single or repeated exposure to an antigen.

[0128] - makes it possible to maintain a naïve/central memory phenotype, to delay the differentiation and exhaustion of effector T-cells, making them better effector cells.

[0129] - improves the safety profile. (Because the absence of TCR reduces the risk of TCR-induced alloreactivity and autoimmunity, and the precise insertion of the CAR encoding sequence reduces the risk of insertional oncogenesis).

[0130] Advantageously, the invention relates to the aforementioned composition, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence oriented 5′-to-3′ for recognizing said transposase and

[0131] wherein the second molecule comprises at its 5′ end a first complementary sequence of said first sequence for recognizing said transposase followed by a second complementary sequence of said second sequence for recognizing said transposase.

[0132] More advantageously, the invention relates to the composition as defined hereinbefore, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence oriented 5′-to-3′ for recognizing said transposase,

[0133] said second molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence oriented 5′-to-3′ for recognizing said transposase, and

[0134] wherein the third molecule comprises at its 5′ end a first complementary sequence of said first sequence for recognizing said transposase of the first molecule followed by a second complementary sequence of said second sequence for recognizing said transposase of the first molecule and at its 3′ end a first complementary sequence of said first sequence for recognizing said transposase of the second molecule followed by a second complementary sequence of said second sequence for recognizing said transposase of the second molecule.

[0135] A possibility for the format of the first and second molecules of the invention is defined herein. This configuration is schematically depicted in [Fig. 1B]. Of course, in the context of this example of the configuration of the first, second, and third molecules, the replacement sequence is located between the two transposase half-sites of the 5′ and 3′ parts of the third molecule, in this case, the sequence encoding the fusion protein containing the CD3ζ chain of the TCR.

[0136] Advantageously, the invention relates to the composition as defined hereinbefore, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase, followed by a first complementary sequence of said first sequence for recognizing said transposase and

[0137] wherein the second molecule comprises at its 5′ end a complementary sequence of said second sequence for recognizing said transposase.

[0138] Even more advantageously, the invention relates to the aforementioned composition, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase, followed by a first complementary sequence of said first sequence for recognizing said transposase of the first molecule,

[0139] the second molecule comprises at its 5′ end a first sequence for recognizing a transposase oriented 5′-3′ and at its 3′ end a second sequence for recognizing said transposase, said first sequence for recognizing said transposase of the 5′ end being preceded by a second complementary sequence of said second sequence for recognizing said transposase of the first molecule,

[0140] and

[0141] wherein the third molecule comprises at its 5′ end a first complementary sequence of said second sequence for recognizing said transposase of the first molecule and at its 3′ end a second complementary sequence of said first sequence for recognizing said transposase of the second molecule.

[0142] A further possibility for the format of the first and second molecules of the invention is defined herein. This configuration is schematically depicted in [Fig. 1C] and [Fig. 1D]. Of course, in the context of this example of the configuration of the first, second, and third molecules, the replacement sequence is located between the two transposase half-sites of the 5′ and 3′ parts of the third molecule, the sequence encoding the fusion protein containing the CD3ζ chain of the TCR.

[0143] Advantageously, the invention relates to the aforementioned composition, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a first complementary sequence of said first sequence for recognizing said transposase of the first molecule followed by a second sequence for recognizing said transposase, and

[0144] wherein the third molecule comprises at its 5′ end a complementary sequence of said second sequence for recognizing said transposase of the first molecule.

[0145] Even more advantageously, the invention relates to the aforementioned composition, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a first complementary sequence of said first sequence for recognizing said transposase of the first molecule followed by a second sequence for recognizing said transposase,

[0146] the second molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase of the first molecule, said first sequence being followed by a second complementary sequence of said second sequence for recognizing said transposase of the first molecule, and

[0147] wherein the third molecule comprises at its 5′ end a complementary sequence of said second sequence for recognizing said transposase of the first molecule and at its 3′ end a complementary sequence of the first sequence for recognizing the transposase of the second molecule.

[0148] This configuration is schematically depicted in [Fig. 1E]. Of course, in the context of this example of the configuration of the first, second, and third molecules, the replacement sequence is located between the two transposase half-sites of the 5′ and 3′ parts of the third molecule, the sequence encoding the fusion protein containing the CD3ζ chain of the TCR.

[0149] Advantageously, the invention relates to the aforementioned composition, wherein said transposase is a bacterial transposase, especially a transposase selected from Tn5, Tn9, Tn10 or Tc1/mariner.

[0150] Advantageously, the aforementioned transposase is a bacterial-type transposase selected from the transposase of transposon Tn5, the transposase of transposon Tn9, the transposase of transposon Tn10, Tn903, Tn602, or even the transposase of the transposon Tc1, or more generally of the mariner transposon superfamily.

[0151] Other examples of transposases that can be used in the context of the invention are: the Vibrio harveyi transposase (transposase characterized by Agilent and used in the product SureSelect QXT), the MutA transposase and a Mu transposase recognition site comprising the terminal sequences R1 and

R2, the transposase of Staphylococcus aureus transposon Tn552, the transposase of transposon Tn7, the Tn/O and IS10 transposase, the transposase of transposon Tn3.

[0152] The Tn5 transposase is the best known. It is coded by the Tnp gene of transposon Tn5. The transposase initiates the transposition by forming a transposase dimer which binds to its target sequences. In the context of this complex, the transposase then catalyzes four phosphoryl transfer reactions (DNA cleavage, DNA hairpin formation, hairpin resolution and strand transfer to the target DNA), resulting in the integration of the transposon into its new DNA site: this is what is known as “tagmentation”.

[0153] The invention is based on this tagmentation principle. By using the tagmentation properties of the transposases, it is possible to insert one sequence into another in a targeted manner, by virtue of the aforementioned complex.

[0154] Also in the context of the invention, when reference is made to a transposase, reference is being made to one of the aforementioned transposases, namely the transposases of transposons Tn5, Tn9, Tn10 or Tc1/mariner (or transposases mutated to increase their transposition or tagmentation activity).

[0155] In the invention, when several transposases are used simultaneously, one binding to the complex formed by the first molecule and the third molecule, and the other binding to the complex formed by the second molecule and the third molecule, the pairs of transposases resulting from transposons Tn5 and Tn10 are preferred.

[0156] Advantageously, the first and the second transposase recognition sequence is a sequence for recognizing the Tn5 transposase having one of the following sequences:

[0157] - CTGtCTCTTataCAcAtcT (SEQ ID NO: 29),

[0158] - CTGACTCTTataCACAagT (SEQ ID NO: 30), and

[0159] - CTGtCTCTTgatCAgATCT (SEQ ID NO: 31).

[0160] As a result, the corresponding complementary sequences are as follows:

[0161] - AgaTgTGtatAAGAGaCAG (SEQ ID NO: 32), complementary to the sequence SEQ ID NO: 29,

[0162] - ActTGTGtatAAGAGTCAG (SEQ ID NO: 33), complementary to the sequence SEQ ID NO: 30, and

[0163] - AGATcTGatcAAGAGaCAG (SEQ ID NO: 34), complementary to the sequence SEQ ID NO: 31.

[0164] Other transposase recognition sequences are as follows:

[0165] Tn5MErev,

[0166] 5′-[phos]CTGTCTCTTATACACATCT-3′ (SEQ ID NO: 35)

[0167] Tn5ME-A (Illumina FC-121-1030),

[0168] 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′; (SEQ ID NO: 36)

[0169] and Tn5ME-B (Illumina FC-121-1031),

[0170] 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ (SEQ ID NO: 37)

[0171] Other transposase recognition sequences are as follows

[0172] - sense sequence SEQ ID NO: i

[0173] - antisense sequence SEQ ID NO: i+1,

[0174] wherein i ranges from 38 to 192.

[0175] This means for example that the following respective sense and antisense sequence pairs are considered: SEQ ID NO: 38 and SEQ ID NO: 39, SEQ ID NO: 40 and SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43, SEQ ID NO: 44 and SEQ ID NO: 45, SEQ ID NO: 46 and SEQ ID NO: 47, SEQ ID NO: 48 and SEQ ID NO: 49, SEQ ID NO: 50 and SEQ ID NO: 51, SEQ ID NO: 52 and SEQ ID NO: 53, SEQ ID NO: 54 and SEQ ID NO: 55, SEQ ID NO: 56 and SEQ ID NO: 57, SEQ ID NO: 58 and SEQ ID NO: 59, SEQ ID NO: 60 and SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 63, SEQ ID NO: 64 and SEQ ID NO: 65, SEQ ID NO: 66 and SEQ ID NO: 67, SEQ ID NO: 68 and SEQ ID NO: 69, SEQ ID NO: 70 and SEQ ID NO: 71, SEQ ID NO: 72 and SEQ ID NO: 73, SEQ ID NO: 74 and SEQ ID NO: 75, SEQ ID NO: 76 and SEQ ID NO: 77, SEQ ID NO: 78 and SEQ ID NO: 79, SEQ ID NO: 80 and SEQ ID NO: 81, SEQ ID NO: 82 and SEQ ID NO: 83, SEQ ID NO: 84 and SEQ ID NO: 85, SEQ ID NO: 86 and SEQ ID NO: 87, SEQ ID NO: 88 and SEQ ID NO: 89, SEQ ID NO: 90 and SEQ ID NO: 91, SEQ ID NO: 92 and SEQ ID NO: 93, SEQ ID NO: 94 and SEQ ID NO: 95, SEQ ID NO: 96 and SEQ ID NO: 97, SEQ ID NO: 98 and SEQ ID NO: 99, SEQ ID NO: 100 and SEQ ID NO: 101, SEQ ID NO: 102 and SEQ ID NO: 103, SEQ ID NO: 104 and SEQ ID NO: 105, SEQ ID NO: 106 and SEQ ID NO: 107, SEQ ID NO: 108 and SEQ ID NO: 109, SEQ ID NO: 110 and SEQ ID NO: 111, SEQ ID NO: 112 and SEQ ID NO: 113, SEQ ID NO: 114 and SEQ ID NO: 115, SEQ ID NO: 116 and SEQ ID NO: 117, SEQ ID NO: 118

and SEQ ID NO: 119, SEQ ID NO: 120 and SEQ ID NO: 121, SEQ ID NO: 122 and SEQ ID NO: 123, SEQ ID NO: 124 and SEQ ID NO: 125, SEQ ID NO: 126 and SEQ ID NO: 127, SEQ ID NO: 128 and SEQ ID NO: 129, SEQ ID NO: 130 and SEQ ID NO: 131, SEQ ID NO: 132 and SEQ ID NO: 133, SEQ ID NO: 134 and SEQ ID NO: 135, SEQ ID NO: 136 and SEQ ID NO: 137, SEQ ID NO: 138 and SEQ ID NO: 139, SEQ ID NO: 140 and SEQ ID NO: 141, SEQ ID NO: 142 and SEQ ID NO: 143, SEQ ID NO: 144 and SEQ ID NO: 145, SEQ ID NO: 146 and SEQ ID NO: 147, SEQ ID NO: 148 and SEQ ID NO: 149, SEQ ID NO: 150 and SEQ ID NO: 151, SEQ ID NO: 152 and SEQ ID NO: 153, SEQ ID NO: 154 and SEQ ID NO: 155, SEQ ID NO: 156 and SEQ ID NO: 157, SEQ ID NO: 158 and SEQ ID NO: 159, SEQ ID NO: 160 and SEQ ID NO: 161, SEQ ID NO: 162 and SEQ ID NO: 163, SEQ ID NO: 164 and SEQ ID NO: 165, SEQ ID NO: 166 and SEQ ID NO: 167, SEQ ID NO: 168 and SEQ ID NO: 169, SEQ ID NO: 170 and SEQ ID NO: 171, SEQ ID NO: 172 and SEQ ID NO: 173, SEQ ID NO: 174 and SEQ ID NO: 175, SEQ ID NO: 176 and SEQ ID NO: 177, SEQ ID NO: 178 and SEQ ID NO: 179, SEQ ID NO: 180 and SEQ ID NO: 181, SEQ ID NO: 182 and SEQ ID NO: 183, SEQ ID NO: 184 and SEQ ID NO: 185, SEQ ID NO: 186 and SEQ ID NO: 187, SEQ ID NO: 188 and SEQ ID NO: 189, SEQ ID NO: 190 and SEQ ID NO: 191, and SEQ ID NO: 192 and SEQ ID NO: 193.

[0176] Advantageously, the first G/C-rich domain of the first molecule (or of the second molecule) corresponds to the following sequence GG CGATCGC (SEQ ID NO: 194) so that the second G/C-rich domain is the same. Indeed, due to the folding of the molecule onto itself, the second G/C-rich domain is in a complementary and antiparallel orientation with respect to the first G/C-rich domain, and the interaction takes place at the palindromic region (underlined in the sequence hereinbefore).

[0177] The first and second G/C-rich domains may also be the following sequence GCGGCGATCGGC (SEQ ID NO: 195). The explanations hereinbefore apply *mutatis mutandis*.

[0178] Other G/C-rich domain sequences of the first or second molecule may be as follows:

[0179] - first G/C-rich domain of sequence GGTCGC (SEQ ID NO: 196) and the second G/C-rich domain of sequence GCGACC (SEQ ID NO: 197).

[0180] These examples are given only by way of illustration and cannot limit the scope of the invention.

[0181] In one advantageous embodiment, the A/T-rich sequences of the first molecule of said complex consist essentially, or are made up of A or T.

[0182] Even more advantageously, the A/T-rich sequence of the first molecule of said complex consists of T.

[0183] Advantageously, the invention relates to the aforementioned composition, wherein the intermediate region of the third molecule comprises the sequence SEQ ID NO: 3 or the sequence SEQ ID NO: 1134.

[0184] As mentioned hereinbefore, the third molecule comprises a sequence encoding the intracellular part of the CD3ζ chain of the TCR. This sequence is preferentially oriented in the 5′-to-3′ direction.

[0185] The complete sequence of the CD3ζ protein of the TCR is represented by the sequence SEQ ID NO: 1, wherein

[0186] - the peptide part from 1 to 21 of said sequence SEQ ID NO: 1 corresponds to the propeptide, which is cleaved during CD3ζ maturation,

[0187] - the peptide part from 22 to 30 of said sequence SEQ ID NO: 1 corresponds to the extracellular domain of the CD3ζ,

[0188] - the peptide part from 31 to 51 of said sequence SEQ ID NO: 1 corresponds to the transmembrane domain of the CD3ζ, and

[0189] - the peptide part from 52 to 164 of said sequence SEQ ID NO: 1 corresponds to the intracellular domain of the CD3ζ, i.e. the signal transducing domain.

[0190] The sequence SEQ ID NO: 3 corresponds to the DNA encoding the intracellular part of sequence SEQ ID NO: 2.

[0191] The sequence SEQ ID NO: 1134 corresponds to the DNA encoding the transmembrane domain and the intracellular part of the CD3ζ.

[0192] Advantageously, the invention relates to the aforementioned composition, wherein the peptide capable of binding to a membrane protein is a single-chain variable fragment (scFv) recognizing a membrane protein, especially the extracellular part of a membrane protein.

[0193] scFv technology is particularly well-suited to fusion as described in the invention, as it enables the variable part of the antibodies to be reproduced by fusing the variable part of the light chain to the variable part of the heavy chain, the two variable parts being linked together by a linker. The protein thus generated retains the specificity of the original immunoglobulin, despite the elimination of the constant regions and the introduction of the linker. It is then possible to create a nucleic acid molecule that makes it possible to synthesize the corresponding peptide, thus obtaining a simplified "short antibody". The linker is generally 10 to 25 amino acids in size (or 30 to 75 nucleotides), and is generally rich in glycine for flexibility, and serine or threonine for solubility. The linker may connect the N-terminus of the variable part of the heavy chain (VH) to the C-terminus of the variable part of the light chain (VL), or vice versa.

[0194] The scFvs of interest in the context of the invention are especially the scFvs directed against membrane proteins widely expressed in tumors, such as CD19, BCma, CD123, CD20, CD22, CD38, LeY, ROR1, c-MET, CD133, CD171, CD70, CEA, EGFR-VIII, EpCAM, EphA2, FAP, c-KIT, Flt3, VEGFR2, PSMA, PSCA, MUC1, IL13Ra2, HER2, FAP, GD2, GPC3, mesothelin, EIIIB, gp100/HLA-A2, HLA-A2-WT1Db126, HA-1 H/HLA-A2, HLA-A*02:01, PD-1, PD-L1, CTLA-4 and TGFβ. This list is by no means exhaustive.

[0195] Advantageously, the invention relates to the aforementioned composition, wherein the peptide capable of binding to a membrane protein is a peptide fused to streptavidin. In this way, when the fusion peptide (extracellular streptavidin peptide and intracellular activation part comprising the CD3ζ chain of the TCR) is used, it is possible to couple specific antibodies in which the heavy chain is associated with biotin. Streptavidin and biotin will then interact specifically, creating a hybrid chimera that makes it possible to recognize the cellular target via the antibody and to activate the desired signal via the CD3ζ chain of the TCR.

[0196] The peptide capable of binding to a membrane protein may also be the ligand of a membrane receptor found on the target cell. This may be, for example, a cytokine, such as IL-13, which recognizes the IL13Ra receptor, but also the ligand of the receptors Flt3, c-Kit, c-Met, VEGFR2, PD1, HER2, EGFR, TNFRs, NKG2, CD116 (GM-CSF receptor). Again, this list is not exhaustive.

[0197] Advantageously, the invention relates to a composition as defined hereinbefore, wherein the co-activation domain corresponds to the co-activation domain of a protein selected from CD28, 4-1BB, OX40 or ICOS.

[0198] As used in the present description, the term "co-activation domain" "co-stimulatory signaling region" relates to the part of the chimeric T-cell receptor comprising the intracellular domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than the antigenic

receptors or their ligands, which are necessary for an effective response of the lymphocytes to the antigen. Examples of such molecules include CD28, 4-1BB, DAP-10 and ICOS. For example, chimeric TCRs containing the intracellular domain of

[0199] - CD28 (SEQ ID NO: 1135),

[0200] - 4-1BB (complete sequence given in SEQ ID NO: 1136),

[0201] - ICOS (complete sequence given in SEQ ID NO: 1137) and

[0202] - DAP-10 (SEQ ID NO: 1138) are used appropriately in the context of the present invention.

[0203] Even more advantageously, the invention relates to the composition as defined hereinbefore, wherein the first, second, and third molecules are chosen from the triplets as defined in Table 2 hereinafter.

[0204] Advantageously, the invention relates to a composition comprising one of the triplets of first, second, and third molecules described in the following Table 2:

[0205] [Table 2]

Triplet	**First molecule SEQ ID NO:**	**Second molecule SEQ ID NO:**	**Third molecule SEQ ID NO:**
#1	198	199	200
#2	201	202	203
#3	204	205	206
#4	207	208	209
#5	210	211	212
#6	213	214	215
#7	216	217	218
#8	219	220	221

#9	222	223	224
#10	225	226	227
#11	228	229	230
#12	231	232	233
#13	234	235	236
#14	237	238	239
#15	240	241	242
#16	243	244	245
#17	246	247	248
#18	249	250	251
#19	252	253	254
#20	255	256	257
#21	258	259	260
#22	261	262	263
#23	264	265	266
#24	267	268	269
#25	270	271	272
#26	273	274	275
#27	276	277	278
#28	279	280	281
#29	282	283	284
#30	285	286	287
#31	288	289	290
#32	291	292	293
#33	294	295	296
#34	297	298	299
#35	300	301	302
#36	303	304	305
#37	306	307	308
#38	309	310	311
#39	312	313	314
#40	315	316	317
#41	318	319	320
#42	321	322	323
#43	324	325	326
#44	327	328	329
#45	330	331	332
#46	333	334	335
#47	336	337	338
#48	339	340	341
#49	342	343	344
#50	345	346	347
#51	348	349	350
#52	351	352	353
#53	354	355	356

#54	357	358	359
#55	360	361	362
#56	363	364	365
#57	366	367	368
#58	369	370	371
#59	372	373	374
#60	375	376	377
#61	378	379	380
#62	381	382	383
#63	384	385	386
#64	387	388	389
#65	390	391	392
#66	393	394	395
#67	396	397	398
#68	399	400	401
#69	402	403	404
#70	405	406	407
#71	408	409	410
#72	411	412	413
#73	414	415	416
#74	417	418	419
#75	420	421	422
#76	423	424	425
#77	426	427	428
#78	429	430	431
#79	432	433	434
#80	435	436	437
#81	438	439	440
#82	441	442	443
#83	444	445	446
#84	447	448	449
#85	450	451	452
#86	453	454	455
#87	456	457	458
#88	459	460	461
#89	462	463	464
#90	465	466	467
#91	468	469	470
#92	471	472	473
#93	474	475	476
#94	477	478	479
#95	480	481	482
#96	483	484	485
#97	486	487	488
#98	489	490	491

#99	492	493	494
#100	495	496	497
#101	498	499	500
#102	501	502	503
#103	504	505	506
#104	507	508	509
#105	510	511	512
#106	513	514	515
#107	516	517	518
#108	519	520	521
#109	522	523	524
#110	525	526	527
#111	528	529	530
#112	531	532	533
#113	534	535	536
#114	537	538	539
#115	540	541	542
#116	543	544	545
#117	546	547	548
#118	549	550	551
#119	552	553	554
#120	555	556	557
#121	558	559	560
#122	561	562	563
#123	564	565	566
#124	567	568	569
#125	570	571	572
#126	573	574	575
#127	576	577	578
#128	579	580	581
#129	582	583	584
#130	585	586	587
#131	588	589	590
#132	591	592	593
#133	594	595	596
#134	597	598	599
#135	600	601	602
#136	603	604	605
#137	606	607	608
#138	609	610	611
#139	612	613	614
#140	615	616	617
#141	618	619	620
#142	621	622	623
#143	624	625	626

#144	627	628	629
#145	630	631	632
#146	633	634	635
#147	636	637	638
#148	639	640	641
#149	642	643	644
#150	645	646	647
#151	648	649	650
#152	651	652	653
#153	654	655	656
#154	657	658	659
#155	660	661	662
#156	663	664	665
#157	666	667	668
#158	669	670	671
#159	672	673	674
#160	675	676	677
#161	678	679	680
#162	681	682	683
#163	684	685	686
#164	687	688	689
#165	690	691	692
#166	693	694	695
#167	696	697	698
#168	699	700	701
#169	702	703	704
#170	705	706	707
#171	708	709	710
#172	711	712	713
#173	714	715	716
#174	717	718	719
#175	720	721	722
#176	723	724	725
#177	726	727	728
#178	729	730	731
#179	732	733	734
#180	735	736	737
#181	738	739	740
#182	741	742	743
#183	744	745	746
#184	747	748	749
#185	750	751	752
#186	753	754	755
#187	756	757	758
#188	759	760	761

#189	762	763	764
#190	765	766	767
#191	768	769	770
#192	771	772	773
#193	774	775	776
#194	777	778	779
#195	780	781	782
#196	783	784	785
#197	786	787	788
#198	789	790	791
#199	792	793	794
#200	795	796	797
#201	798	799	800
#202	801	802	803
#203	804	805	806
#204	807	808	809
#205	810	811	812
#206	813	814	815
#207	816	817	818
#208	819	820	821
#209	822	823	824
#210	825	826	827
#211	828	829	830
#212	831	832	833
#213	834	835	836
#214	837	838	839
#215	840	841	842
#216	843	844	845
#217	846	847	848
#218	849	850	851
#219	852	853	854
#220	855	856	857
#221	858	859	860
#222	861	862	863
#223	864	865	866
#224	867	868	869
#225	870	871	872
#226	873	874	875
#227	876	877	878
#228	879	880	881
#229	882	883	884
#230	885	886	887
#231	888	889	890
#232	891	892	893
#233	894	895	896

#234	897	898	899
#235	900	901	902
#236	903	904	905
#237	906	907	908
#238	909	910	911
#239	912	913	914
#240	915	916	917
#241	918	919	920
#242	921	922	923
#243	924	925	926
#244	927	928	929
#245	930	931	932
#246	933	934	935
#247	936	937	938
#248	939	940	941
#249	942	943	944
#250	945	946	947
#251	948	949	950
#252	951	952	953
#253	954	955	956
#254	957	958	959
#255	960	961	962
#256	963	964	965
#257	966	967	968
#258	969	970	971
#259	972	973	974
#260	975	976	977
#261	978	979	980
#262	981	982	983
#263	984	985	986
#264	987	988	989
#265	990	991	992
#266	993	994	995
#267	996	997	998
#268	999	1000	1001
#269	1002	1003	1004
#270	1005	1006	1007
#271	1008	1009	1010
#272	1011	1012	1013
#273	1014	1015	1016
#274	1017	1018	1019
#275	1020	1021	1022
#276	1023	1024	1025
#277	1026	1027	1028
#278	1029	1030	1031

#279	1032	1033	1034
#280	1035	1036	1037
#281	1038	1039	1040
#282	1041	1042	1043
#283	1044	1045	1046
#284	1047	1048	1049
#285	1050	1051	1052
#286	1053	1054	1055
#287	1056	1057	1058
#288	1059	1060	1061
#289	1062	1063	1064
#290	1065	1066	1067
#291	1068	1069	1070
#292	1071	1072	1073
#293	1074	1075	1076
#294	1077	1078	1079
#295	1080	1081	1082
#296	1083	1084	1085
#297	1086	1087	1088
#298	1089	1090	1091
#299	1092	1093	1094
#300	1095	1096	1097
#301	1098	1099	1100
#302	1101	1102	1103
#303	1104	1105	1106
#304	1107	1108	1109
#305	1110	1111	1112
#306	1113	1114	1115
#307	1116	1117	1118
#308	1119	1120	1121
#309	1122	1123	1124
#310	1125	1126	1127
#311	1128	1129	1130
#312	1131	1132	1133

[0206] Also, advantageously, the invention relates to the aforementioned composition, said composition comprising a triplet selected from the aforementioned triplets #1 to #312.

[0207] Advantageously, the invention relates to the composition as described hereinbefore, further comprising

[0208] ** a fourth single-stranded nucleic acid molecule comprising or consisting essentially of an A′ sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary sequence binding at 5′ to a fifth T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a sixth T-rich sequence of 40 to 60 nucleotides in length, said fifth and sixth T-rich sequences respectively comprising a fifth and a sixth domain of 6 to 12 G/C-rich nucleotides, the sequence of the fifth domain being complementary to the sequence of the sixth domain, said fifth and sixth domains being positioned 15 to 52 nucleotides from said A′ sequence, said fourth molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase,

[0209] ** a fifth single-stranded nucleic acid molecule comprising or consisting essentially of a B′ sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary B′ sequence binding at 5′ to a seventh T-rich sequence of 40 to 60 nucleotides in length and at 3′ to an eighth T-rich sequence of 40 to 60 nucleotides in length, said seventh and eighth T-rich sequence respectively comprising a seventh and an eighth domain of 6 to 12 G/C-rich nucleotides, the sequence of the seventh domain being complementary to the sequence of the eighth domain, said seventh and eighth domain being positioned 15 to 52 nucleotides from said B′ sequence, said fifth molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing said transposase and at its 3′ end the second sequence for recognizing said transposase,

[0210] said B′ sequence being a complementary sequence of said nucleic acid of interest, the A′ sequence being positioned at 5′ of a region of interest of said

nucleic acid of interest and the B′ sequence positioned at 3′ of the region of interest of said nucleic acid of interest, and

[0211] ** a sixth single-stranded molecule comprising

[0212] * in its 5′ part, at least one complementary sequence of said fifth sequence for recognizing said transposase of the first molecule,

[0213] * in its 3′ part, at least one complementary sequence of said fourth sequence for recognizing said transposase of the second molecule, and

[0214] * an intermediate region located between the complementary sequence of said fourth sequence for recognizing said transposase of the first molecule and the complementary sequence of said fifth sequence for recognizing said transposase of the second molecule, a complementary sequence of said sequence encoding said fusion protein contained in the third molecule, i.e. a complementary sequence of the sequence encoding the CD3ζ chain of the TCR,

[0215] the fourth and sixth single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase, and the fifth and sixth single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase.

[0216] Advantageously, the composition according to the invention comprises six molecules, i.e. two triplets enabling double-stranded replacement of a target molecule. This replacement can only take place if the A, B and A′ and B′ regions are correctly chosen, so that the region of interest to be replaced is correctly flanked.

[0217] Of course, all the definitions given for a composition comprising three molecules apply *mutatis mutandis* to a composition comprising six molecules.

[0218] Advantageously, the invention relates to the aforementioned composition, wherein

[0219] - said A sequence comprises a complementary sequence of the sequence of a first region of the locus of the gene encoding the TRAC protein,

[0220] - said B sequence comprises a complementary sequence of the sequence of a second region of the locus of the gene encoding the TRAC protein,

[0221] said A sequence and said B sequence being two different sequences, said first and second regions advantageously flanking a 5′ region of the locus of the gene encoding the TRAC protein,

[0222] and wherein

[0223] - said A′ sequence comprises a complementary sequence of the sequence of a third region of the locus of the gene encoding the TRAC protein,

[0224] - said B′ sequence comprises a complementary sequence of the sequence of a fourth region of the locus of the gene encoding the TRAC protein,

[0225] said A′ sequence and said B′ sequence being two different sequences, said third and fourth regions of the gene encoding the TRAC protein, advantageously flanking a complementary sequence of said 5′ region of the locus of the gene encoding the TRAC protein,

[0226] and wherein

[0227] the A sequence and the A′ sequence are at most partially complementary,

[0228] the A sequence and the B′ sequence are at most partially complementary,

[0229] the B sequence and the A′ sequence are at most partially complementary, and

[0230] the B sequence and the B′ sequence are at most partially complementary.

[0231] "At most partially complementary" is understood in the invention to mean that the sequences have sequences that are capable of pairing according to the base complementarity defined by Watson and Crick, but not over all the sequences. In other words, only part of the sequences is complementary. Advantageously, the A, A′, B and B′ sequences are partially complementary on less than 50% of the sequences, especially less than 30% of the sequences, in particular less than 10% of the sequences, especially are not at all complementary to each other.

[0232] In this way, it is possible to flank the 5′ part of the TRAC locus (just upstream of the first exon) in a specific and oriented manner, for both strands, in order to avoid disordered or inconsistent recombinations (i.e. tagmentations), so that the result would not be as expected.

[0233] It is also possible to create a molecule comprising the fusion sequence and exon 1 of the TRAC gene, so that insertion is carried out by replacing exon 1 with a sequence containing the encoding sequence of the fusion containing the CD3ζ of the TCR, followed by a termination sequence of the transcription,

itself followed by the sequence of TRAC exon 1. An example of recombination at the TRAC locus is depicted schematically in [Fig. 6].

[0234] Even more advantageously, the aforementioned composition comprises at least one of the sextuplets mentioned in the following Table 3:

[0235] [Table 3]

Sextuplet	First molecule SEQ ID NO:	Second molecule SEQ ID NO:	Third molecule SEQ ID NO:	Fifth molecule SEQ ID NO:	Fourth molecule SEQ ID NO:	Sixth molecule SEQ ID NO:
#1	198	199	200	201	202	203
#2	204	205	206	207	208	209
#3	210	211	212	213	214	215
#4	216	217	218	219	220	221
#5	222	223	224	225	226	227
#6	228	229	230	231	232	233
#7	234	235	236	237	238	239
#8	240	241	242	243	244	245
#9	246	247	248	249	250	251
#10	252	253	254	255	256	257
#11	258	259	260	261	262	263
#12	264	265	266	267	268	269
#13	270	271	272	273	274	275
#14	276	277	278	279	280	281
#15	282	283	284	285	286	287
#16	288	289	290	291	292	293
#17	294	295	296	297	298	299
#18	300	301	302	303	304	305
#19	306	307	308	309	310	311
#20	312	313	314	315	316	317
#21	318	319	320	321	322	323
#22	324	325	326	327	328	329
#23	330	331	332	333	334	335
#24	336	337	338	339	340	341
#25	342	343	344	345	346	347
#26	348	349	350	351	352	353
#27	354	355	356	357	358	359
#28	360	361	362	363	364	365
#29	366	367	368	369	370	371
#30	372	373	374	375	376	377

#31	378	379	380	381	382	383
#32	384	385	386	387	388	389
#33	390	391	392	393	394	395
#34	396	397	398	399	400	401
#35	402	403	404	405	406	407
#36	408	409	410	411	412	413
#37	414	415	416	417	418	419
#38	420	421	422	423	424	425
#39	426	427	428	429	430	431
#40	432	433	434	435	436	437
#41	438	439	440	441	442	443
#42	444	445	446	447	448	449
#43	450	451	452	453	454	455
#44	456	457	458	459	460	461
#45	462	463	464	465	466	467
#46	468	469	470	471	472	473
#47	474	475	476	477	478	479
#48	480	481	482	483	484	485
#49	486	487	488	489	490	491
#50	492	493	494	495	496	497
#51	498	499	500	501	502	503
#52	504	505	506	507	508	509
#53	510	511	512	513	514	515
#54	516	517	518	519	520	521
#55	522	523	524	525	526	527
#56	528	529	530	531	532	533
#57	534	535	536	537	538	539
#58	540	541	542	543	544	545
#59	546	547	548	549	550	551
#60	552	553	554	555	556	557
#61	558	559	560	561	562	563
#62	564	565	566	567	568	569
#63	570	571	572	573	574	575
#64	576	577	578	579	580	581
#65	582	583	584	585	586	587
#66	588	589	590	591	592	593
#67	594	595	596	597	598	599
#68	600	601	602	603	604	605
#69	606	607	608	609	610	611
#70	612	613	614	615	616	617
#71	618	619	620	621	622	623
#72	624	625	626	627	628	629
#73	630	631	632	633	634	635
#74	636	637	638	639	640	641
#75	642	643	644	645	646	647

#76	648	649	650	651	652	653
#77	654	655	656	657	658	659
#78	660	661	662	663	664	665
#79	666	667	668	669	670	671
#80	672	673	674	675	676	677
#81	678	679	680	681	682	683
#82	684	685	686	687	688	689
#83	690	691	692	693	694	695
#84	696	697	698	699	700	701
#85	702	703	704	705	706	707
#86	708	709	710	711	712	713
#87	714	715	716	717	718	719
#88	720	721	722	723	724	725
#89	726	727	728	729	730	731
#90	732	733	734	735	736	737
#91	738	739	740	741	742	743
#92	744	745	746	747	748	749
#93	750	751	752	753	754	755
#94	756	757	758	759	760	761
#95	762	763	764	765	766	767
#96	768	769	770	771	772	773
#97	774	775	776	777	778	779
#98	780	781	782	783	784	785
#99	786	787	788	789	790	791
#100	792	793	794	795	796	797
#101	798	799	800	801	802	803
#102	804	805	806	807	808	809
#103	810	811	812	813	814	815
#104	816	817	818	819	820	821
#105	822	823	824	825	826	827
#106	828	829	830	831	832	833
#107	834	835	836	837	838	839
#108	840	841	842	843	844	845
#109	846	847	848	849	850	851
#110	852	853	854	855	856	857
#111	858	859	860	861	862	863
#112	864	865	866	867	868	869
#113	870	871	872	873	874	875
#114	876	877	878	879	880	881
#115	882	883	884	885	886	887
#116	888	889	890	891	892	893
#117	894	895	896	897	898	899
#118	900	901	902	903	904	905
#119	906	907	908	909	910	911
#120	912	913	914	915	916	917

#121	918	919	920	921	922	923
#122	924	925	926	927	928	929
#123	930	931	932	933	934	935
#124	936	937	938	939	940	941
#125	942	943	944	945	946	947
#126	948	949	950	951	952	953
#127	954	955	956	957	958	959
#128	960	961	962	963	964	965
#129	966	967	968	969	970	971
#130	972	973	974	975	976	977
#131	978	979	980	981	982	983
#132	984	985	986	987	988	989
#133	990	991	992	993	994	995
#134	996	997	998	999	1000	1001
#135	1002	1003	1004	1005	1006	1007
#136	1008	1009	1010	1011	1012	1013
#137	1014	1015	1016	1017	1018	1019
#138	1020	1021	1022	1023	1024	1025
#139	1026	1027	1028	1029	1030	1031
#140	1032	1033	1034	1035	1036	1037
#141	1038	1039	1040	1041	1042	1043
#142	1044	1045	1046	1047	1048	1049
#143	1050	1051	1052	1053	1054	1055
#144	1056	1057	1058	1059	1060	1061
#145	1062	1063	1064	1065	1066	1067
#146	1068	1069	1070	1071	1072	1073
#147	1074	1075	1076	1077	1078	1079
#148	1080	1081	1082	1083	1084	1085
#149	1086	1087	1088	1089	1090	1091
#150	1092	1093	1094	1095	1096	1097
#151	1098	1099	1100	1101	1102	1103
#152	1104	1105	1106	1107	1108	1109
#153	1110	1111	1112	1113	1114	1115
#154	1116	1117	1118	1119	1120	1121
#155	1122	1123	1124	1125	1126	1127
#156	1128	1129	1130	1131	1132	1133

[0236] The composition according to the invention thus advantageously proposes 156 sextuplets of molecules enabling insertion at the locus of the TRAC gene of a hybrid sequence encoding a fusion protein comprising the CD3ζ chain of the TCR, and comprising at its N-terminus a protein sequence recognizing a peptide expressed on the surface of a target cell.

[0237] Depending on the target cell, this makes it possible to restore a function lost through mutation, and to achieve functional complementation.

[0238] In another aspect, the invention relates to an immune cell, especially a T-lymphocyte, comprising a composition as defined hereinbefore. The aforementioned immune cell is isolated and purified, outside its natural context.

[0239] The immune cell in question is in a state in which recombination, i.e. insertion, has not yet taken place. The cell contains in its cytoplasm or nucleus, or both, the molecular complex as formed by the triplets of the first, second, and third molecules, and optionally the sextuplets further comprising the fourth, fifth, and sixth molecules, mentioned hereinbefore.

[0240] Even more advantageously, the immune cell is a tumor-infiltrating immune cell, in particular an infiltrating T-cell.

[0241] If it is intended to perform a single-stranded insertion—for example in order to perform an insertion such that the resulting cell has two different alleles: one allele having an insertion, and one allele not having the insertion—it will be advantageous to use the composition comprising the first, second, and third molecules. The same applies if an RNA, and in particular messenger RNA, is replaced.

[0242] Conversely, if a double-stranded insertion is desired, it will be advantageous to use the first, second, third, fourth, fifth, and sixth molecules.

[0243] The composition according to the invention is introduced into the immune cell using transfection techniques well known to a skilled person, especially electroporation or calcium phosphate precipitation. A skilled person will know how to choose the best technique depending on the nature of the targeted immune cell.

[0244] It is also advantageous for the cell to comprise a composition comprising any one of the triplets in Table 2, or any one of the sextuplets in Table 3.

[0245] In another aspect, the invention relates to a pharmaceutical composition comprising the aforementioned composition, or an immune cell described hereinbefore, in association with a pharmaceutically acceptable carrier.

[0246] The aforementioned composition, in association with a pharmaceutically acceptable carrier, can be used in the treatment of diseases. The carrier is a commonly accepted carrier that is known to the skilled person, such as distilled water or a physiological buffer. This carrier must allow the formation of a complex as mentioned hereinbefore, but also be acceptable for a cell or a living being.

[0247] The same applies to the cell. The cell is placed in contact with a pharmaceutically acceptable carrier which has the property of keeping it alive (not affecting the membrane, and not inducing cell death phenomena) and being compatible with a living organism.

[0248] In another aspect, the invention relates to the aforementioned composition, or the aforementioned immune cell, as a drug.

[0249] It is furthermore advantageous for the composition comprising any one of the triplets in Table 2, or any one of the sextuplets in Table 3, to be used as a drug.

[0250] In yet another aspect, the invention relates to the aforementioned composition, or the aforementioned immune cell, for its use in the treatment of cancer.

[0251] Insofar as the aforementioned composition is intended to provide CAR cells, it is particularly relevant to use this composition to treat cancer.

[0252] The composition will be adapted to the target cancer cell, by selecting the extracellular part of the fusion protein to target a specific tumor cell.

[0253] Expression by the lymphocytes modified by the composition according to the invention thus ensures specificity towards the tumor antigen. It then suffices to inject the patient with a few tens or hundreds of millions of CAR-expressing lymphocytes. Finally, as they proliferate *in vivo*, the modified lymphocytes persist for weeks or even months, helping to reduce or even destroy tumors by killing cancer cells.

[0254] Advantageously, the invention relates to a method for treating cancer in a patient in need, the method comprising the administration of an effective dose of composition as described hereinbefore. In particular, the aforementioned method comprises administering an effective amount of immune cells comprising said composition as described hereinbefore.

[0255] Advantageously, the cancer can be of any type, including solid tumors and hematopoietic tumors of any type. The cancer can therefore especially be

[0256] - of hematopoietic type: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), multiple myeloma (MM), non-Hodgkin's lymphoma (NHL), acute multiple leukemia (AML), CD19-positive cancers, chronic myeloid leukemia (CML), acute myeloid leukemia (AML), small lymphocytic leukemia (SLL), and CD20-positive malignancies, or

[0257] - of solid type: neuroblastoma (NB), glioma, mesothelioma, lung cancer, ovarian cancer (OC), breast cancer (BC), sarcoma, osteosarcoma (OS), hepatocellular carcinoma (HCC), HER2-positive cancer, EGFRvIII-positive

cancer, glioblastoma (GBM), pancreatic cancer (PC), MUC1-positive cancer, prostate cancer (PCa), colorectal adenocarcinoma (COADREAD), colorectal cancer (CRC), melanoma (MEL), medulloblastoma (MB), gastric cancer (GC), liver cancer, neck cancer, head cancer, skin cancer, bone cancer, brain cancer, colorectal cancer, stomach cancer, associated bladder tumor, testicular cancer, thyroid cancer, endometrial cancer, cervical and endocervical cancer.

[0258] In another aspect, the invention relates to the use of an aforementioned composition, for inserting, into a T-lymphocyte cell, a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, provided that the use does not comprise a process for modifying the germline genetic identity of human beings and that said use is not a method for treating the human or animal body by surgery or therapy.

[0259] This insertion is preferentially located at the TRAC locus as described hereinbefore.

[0260] As explained hereinbefore, the composition makes it possible to target specifically a target region and to replace it with a sequence of interest, by using the transposase tagmentation properties.

[0261] The composition according to the invention is particularly advantageous for *in vitro* CAR cells, i.e. modified T-cells expressing, in addition to the T receptor (TCR), a chimeric T receptor that enables specific cell targeting of tumors.

[0262] The invention further relates to a method for insertion, especially *in vitro*, of a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

[0263] - placing a composition as defined hereinbefore in contact with a target nucleic acid comprising at least a 5′ region of the locus of the gene encoding the TRAC protein, in order to obtain a replacement complex,

[0264] said composition being such that

[0265] the A sequence of said first molecule comprises a complementary sequence of a first region of the locus of the gene encoding the TRAC protein,

[0266] the B sequence of said second molecule comprises a complementary sequence of a second region of the locus of the gene encoding the TRAC protein, and

[0267] the third molecule comprises the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR located between a complementary sequence of said second sequence for recognizing said transposase of the first molecule and the complementary sequence of said first sequence for recognizing said transposase of the second molecule,

[0268] - placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said composition, to obtain a recombination complex, and

[0269] - recombining the combination complex to obtain the sequence encoding the fusion protein inserted in the target nucleic acid between the first and second regions of the locus of the gene encoding the TRAC protein.

[0270] Advantageously, the invention relates to a method for *in vitro* insertion of a sequence encoding a fusion protein comprising, in the amino-terminal

part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

[0271] - placing a composition in contact with a target nucleic acid comprising at least one 5′ region of the locus of the gene encoding the TRAC protein, in order to obtain a replacement complex, said composition comprising a triplet as defined in Table 2 or a sextuplet as defined in Table 3

[0272] - placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said composition, to obtain a recombination complex, and

[0273] - recombining the combination complex to obtain the sequence encoding the fusion protein inserted in the target nucleic acid between the first and second regions of the locus of the gene encoding the TRAC protein.

[0274] In another aspect, the invention relates to a method for producing chimeric antigenic receptor-T cells or *CAR-T cells* comprising the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

[0275] - placing a composition as defined hereinbefore in contact with a purified T-cell, the genomic DNA of said purified T-cell comprising the locus of the gene encoding the TRAC protein,

[0276] said composition or said ensemble being such that

[0277] the A sequence of said first molecule comprises a complementary sequence of a first region of the locus of the gene encoding the TRAC protein,

[0278] the B sequence of said second molecule comprises a complementary sequence of a second region of the locus of the gene encoding the TRAC protein, and

[0279] the third molecule comprises the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR located between a complementary sequence of said second sequence for recognizing said transposase of the first molecule and the complementary sequence of said first sequence for recognizing said transposase of the second molecule, in order to obtain a replacement complex,

[0280] - placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said ensemble, to obtain a recombination complex,

[0281] - recombining the combination complex to obtain a *CAR-T* cell, the genomic DNA of said CAR-T cell comprising the sequence encoding said fusion protein at the locus of the gene encoding the TRAC protein, and

[0282] - purifying the *CAR-T* cell.

[0283] Advantageously, the invention relates to a method for producing chimeric antigenic receptor-T cells or *CAR-T cells* comprising the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

[0284] - placing a composition in contact with a purified T-cell, the genomic DNA of said purified T-cell comprising the locus of the gene encoding the TRAC protein, in order to obtain a replacement complex,

[0285] - placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said ensemble, to obtain a recombination complex,

[0286] - recombining the combination complex to obtain a *CAR-T* cell, the genomic DNA of said CAR-T cell comprising the sequence encoding said fusion protein at the locus of the gene encoding the TRAC protein, and

[0287] - purifying the *CAR-T* cell.

[0288] The invention also relates to a modified T lymphoid cell capable of being obtained by the aforementioned method, said modified T lymphoid cell comprising in its genome an insertion of a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said insertion being located at the locus of the gene encoding the TRAC protein.

[0289] Because of the “tagmentation” recombination carried out by the transposases, and the molecular signature that such recombination generates, i.e. a gene modification at the DNA cleavage zones by the transposases, these lymphocytic cells can only be defined more precisely by the method by which they are obtained. These cells can be recognized by the recognition zones of the A, B, and possibly A′ and B′ regions, which differ from the original sequences.

[0290] These cells are novel and differ from those that could be obtained, for example, with modern genome-editing technologies such as CRISPR/Cas9.

BRIEF DESCRIPTION OF THE FIGURES

[0291] The invention will be better understood from reading the examples hereinafter and the following figures:

[0292] [Fig. 1A] is a first schematic depiction of a first form of pairing the first, second, and third molecules of the composition according to the invention.

[0293] [Fig. 1B] is a second schematic depiction of a first form of pairing the first, second, and third molecules of the composition according to the invention.

[0294] [Fig. 1C] is a third schematic depiction of a first form of pairing the first, second, and third molecules of the composition according to the invention.

[0295] [Fig. 1D] is a fourth schematic depiction of a first form of pairing the first, second, and third molecules of the composition according to the invention.

[0296] [Fig. 1E] is a fifth schematic depiction of a first form of pairing the first, second, and third molecules of the composition according to the invention.

[0297] [Fig. 2] is a diagram showing the principle and the steps of single-strand replacement using the first, second, and third molecules of the composition according to the invention.

[0298] [Fig. 3] is a diagram showing the principle and the steps of double-strand replacement using the first, second, third, fourth, fifth, and sixth molecules of the composition according to the invention.

[0299] [Fig. 4] is a diagram showing the association of the molecules contained in tube 1 of example 1. The black ball depicts biotin.

[0300] [Fig. 5] is a diagram showing the association of the molecules contained in tube 2 of example 1. The black ball depicts biotin.

[0301] [Fig. 6] is a diagram showing the insertion of an ScFV-co-activation domain-CD3ζ fusion. A. illustrates the *TRAC* locus in the vicinity of the *TRAV* and *TRAJ* loci, and the recognition zone around exon 1 of the complex formed by the first, third (containing the TRAC fusion sequence and exon 1), and second molecules. B. depicts tagmentation in the presence of the transposases. C. depicts the result of insertion at the TRAC locus of the fusion sequence. D. schematically depicts the protein encoded by the fusion sequence inserted at the TRAC locus. * depicts the scFv exposed outside the T-cells, ** depicts the co-activation domain (e.g. CD28), and *** depicts the CD3ζ intracellular activation domain.

EXAMPLES

[0302] **Example 1 - Obtaining B-cells expressing a CAR fusion**

[0303] In order to treat patients suffering from cancer expressing the CD19 marker, it may be advantageous to propose a cell therapy aimed at grafting patients with CAR-T cells with CD19 scFv-CD3ζ+ that have been modified using a composition according to the invention, especially to insert the sequence encoding the fusion protein at the TRAC locus, into the T lymphocytes of the patients (in order to limit rejection).

A- Isolation of T-cells from blood

[0304] Blood samples from healthy donors are collected, either fresh or frozen.

[0305] The samples are centrifuged at 1000 g for 8 min on sterile density gradient centrifugation medium (e.g. Ficoll in the presence of EDTA) to isolate peripheral blood mononuclear cells (PBMC);

[0306] The white blood cell rings are collected and washed in PBS (phosphate buffered saline)

[0307] The white blood cells are cultured for 12 hours at 37°C and 5% CO_2 in a medium specific for hematopoietic cell culture, supplemented with IL-2 (20 ng/ml) and 5% human serum.

[0308] The T lymphocytes are then isolated in a cell sorter by positive selection using anti-CD3 antibody labelling or by negative selection using a cocktail of anti-CD2, anti-CD34, anti-CD14, anti-CD16, anti-CD19, anti-CD24, anti-CD56n, anti-CD66b and anti-CD61 antibodies;

[0309] The purified T-cells are then cultured at 37°C and 5% CO_2 for 72 h under a CD3/28 and IL-2 activation cocktail (40 ng/ml).

[0310] The cells are thus ready to be transfected with the composition according to the invention.

[0311] B- **Preparation of the molecules of the composition according to the invention**

[0312] To target the TRAC gene and verify insertion at the TRAC Chr14 locus: 22,547,500-22,552,358 HG38GR, a construct including GFP was prepared.

[0313] Two tubes were prepared as follows:

[0314] ** Tube 1 (10 µl):

[0315] - the oligo Great X1 (10 µM) corresponding to molecule 1 of the following sequence SEQ ID NO: 198

[0316] 5′-
AgaTgTGtatAAGAGaCAGGTAGTGTATTTTTTTTTTTTTTTTATCATCCTGtCT
CTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTTGGCGATCGCTTTTTTTTTTTT

TTTTGCAGACATCCAGCTGGATCCAAAACCAAATTTTTTTTTTTTTTTTGCG
ATCGCCTTTTTTTTTTTTTGATACATTTAgaTgTGtatAAGAGaCAGGATGAT-3′

[0317] - the oligo Great X3 (10 µM) corresponding to molecule 4 of the following sequence SEQ ID NO: 201

[0318] 5′-
CACGTGCTGtCTCTTataCAcAtcTTTTCTCGATCATTATTATTTTTTGGCGAT
CGCTTTTTTTTTTTTTTTTCATTGCCGAGGCCACCAGGGCTGGCTCAGCT
TTTTTTTTTTTTTTTGCGATCGCCTTTTTTTTTTTTTTTTTTTTAgaTgTGtat
AAGAGaCAGCACGTGTTTTTTTTTTTTTTTTTTTTCAGCTATACTGtCTCTTa
taCAcAtcT-3′

[0319] - the reverse oligo GREAT X1 Rev (10 µM) corresponding to the 5′ part of molecule 3 of the following sequence SEQ ID NO: 1139

[0320] 5′-
TACACTACCTGtCTCTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTTggttacGC
GGCCGCCGTACA-3′

[0321] - the reverse oligo GREAT X3 Rev (10 µM) corresponding to the 3′ part of molecule 6 of the following sequence SEQ ID NO: 1140

[0322] 5′-
TGTACGGCGGCCGCgtaaccTTTTTTTTTTTTTTTTTTTTTTTAgaTgTGtatAA
GAGaCAGTATAGCTG-3′

[0323] - the reverse oligo Helix Rev corresponding to an additional strand for binding a helicase via the biotinylated 3′ end, the oligo having the following sequence SEQ ID NO: 1141

[0324] 5′-
ATAATAATGATCGAGAACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT[Biot]-3′

[0325] ** Tube 2 (10 µl):

[0326] - the oligo Great X2 (10 µM) corresponding to molecule 2 of the following sequence SEQ ID NO: 199

[0327] 5′-
CACGTGCTGtCTCTTataCAcAtcTTTTCTCGATCATTATTATTTTTTGGCGAT
CGCTTTTTTTTTTTTTT

[0328] TTGTTGGAGCCACTGACCCTGCCAGAATATGGTTTTTTTTTTTTTT
TTGCGATCGCCTTTTTTTTTTTTTTTTTTTTAgaTgTGtatAAGAGaCAGCAC
GTGTTTTTTTTTTTTTTTTTTTGACGAATACTGtCTCTTataCAcAtcT-3′

[0329] - the oligo Great X4 (10 µM) corresponding to molecule 5 of the following sequence SEQ ID NO: 202:

[0330] 5′-
AgaTgTGtatAAGAGaCAGAGTATGAATTTTTTTTTTTTTTTTATCATCCTGtC
TCTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTGGCGATCGCTTTTTTTTTT
TTTTTTCTGCATGACTCACTAGCACTCTATCACGGCTTTTTTTTTTTTTTTT
GCGATCGCCTTTTTTTTTTTTTGATACATTTAgaTgTGtatAAGAGaCAGGAT
GAT-3′

[0331] - the reverse oligo GREAT X2 Rev (10 µM) corresponding to the 3′ part of molecule 3 of the following sequence SEQ ID NO: 1142

[0332] 5′-
CGATACGGTACCatgttcTTTTTTTTTTTTTTTTTTTTTTTAgaTgTGtatAAGAGa
CAGTATTCGTC-3′

[0333] - the reverse oligo GREAT X4 Rev (10 µM) corresponding to the 5′ part of molecule 6 of the following sequence SEQ ID NO: 1143

[0334] 5′-TTCATACTCTGtCTCTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTTTgaacatGGTACCGTATCG-3′

[0335] - the reverse oligo Helix Rev corresponding to an additional strand for binding a helicase via the biotinylated 3′ end, the oligo having the following sequence SEQ ID NO: 1142.

[0336] The two tubes are then heated to 95°C for 5 min and then left for 1 hour at room temperature, then each tube is digested with the corresponding restriction enzymes: Tube 1: NotI and Tube 2: KpnI then purified by PCR purification kit (elution 20 µl).

[0337] Figures 4 and 5 schematically depict the contents of tubes 1 and 2, respectively, before enzymatic digestion.

[0338] In parallel, the anti-CD19-CD3ζ-P2A-GFP sequence was obtained via direct amplification by DH5α bacteria of the pSLCAR-CD19-CD3ζ plasmid. This sequence is amplified (oligos with NotI and KpnI restriction site architecture digested after PCR) then digested with the corresponding restriction enzymes and purified by PCR purification kit eluted at a volume of 20 µl. The GFP-CD19-CD3ζ fusion is then provided with flanking ends (i.e. free NotI and KpnI half-sites) in tube 3.

[0339] The contents of the three tubes (tube 1, tube 2, and tube 3) are then mixed and T4 phage ligase is added to the mixture (4 µl, 100 U) in the presence of appropriate buffer (T4 buffer; 5 µl 10X) and 11 µl of distilled water. The mixture is left for 1 hour at room temperature. The mixture is then gel-

purified (30 µl elution) to isolate the largest GFP-CD19-CD3ζ fragment, larger than 1 Kb, and featuring the four sarcophages X1, X2, X3, and X4 at its 5′ and 3′ ends. The purified fragment is ready for use and includes the first, second, fourth, and fifth molecules at the ends, with the third and sixth molecules corresponding to the GFP-CD19-CD3ζ fusion (tube 4).

[0340] The GFP-CD19-CD3ζ replacement fragment with its four sarcophages positioned at the 5′ and 3′ ends is then ready to receive the transposase dimers.

[0341] At this stage, the complementary modified single-stranded oligonucleotide of part of the first and fourth molecules, with the 3′ part of the complementary modified single-stranded oligonucleotide coupled to biotin (biotinylated oligo), was added. This biotinylated molecule is not mandatory.

[0342] **C- Transfection of T lymphocytes with the composition according to the invention.**

[0343] The contents of tube 4, optionally containing the biotinylated oligo, are then used to transfect the isolated T lymphocyte cells by electroporation.

[0344] The cells are centrifuged at 300 g for 5 min and washed with PBS before being suspended in electrolyte buffer at a cell density of 75,000 to 100,000 cells per electroporation chamber. The contents of tube 4, together with a plasmid encoding the Tn5 protein, and optionally a plasmid encoding a helicase-streptavidin fusion (UVRD-mSA), are added to the electroporation chamber according to the "*Episomal iPSC Reprogramming Vectors*" protocol of Thermofisher's Neon® Transfection System.

[0345] The mixture is electroporated at 1650 V in three 10 ms sequences.

[0346] The cells are then immediately centrifuged at 300 g for 5 min and suspended in supplemented hematopoietic cell culture medium for 48 to 96 h at 37°C and 5% CO_2.

[0347] Tagmentation (i.e. gene replacement) is then possible. The cells are maintained in a suitable culture medium.

D- Transfection verification

[0348] The transfected cells with the molecule of interest inserted at the locus are selected using an antibiotic in which the resistance gene is contained in the replacement sequence (e.g. neomycin, bleomycin, blasticidin S, etc.).

[0349] A simple PCR using a 5′ sense oligonucleotide from the insertion and an 3′ antisense oligonucleotide from the insertion is performed *(Single-Tail Adapter/Tag (STAT)-PCR based method*). The PCR product is then sequenced using conventional techniques, in order to detect in the region of interest at least the presence of GFP, a sign of fusion insertion.

[0350] The transfected cells can also be detected by cytometry in order to detect GFP expression.

[0351] Reverse transcription can also be performed on isolated cells to obtain a cDNA library. The fusion sequence is then amplified by PCR using oligos for PCR and anti-CD19 sequencing: AGGAGTCCCATCAAGGTTCAGT (SEQ ID NO: 1144) and GFP Rev: CACGAACTCCAGCAGGACCATG (SEQ ID NO: 1145) and the amplified fragment is sequenced by Sanger sequencing and migrated onto agarose gel. As expected, after analysis (NCBI blast), very high similarity is found with the theoretical GFP-CD19-CD3ζ fragment at the TRAC locus.

[0352] **E- Functional analysis of T-cells modified by the composition according to the invention**

[0353] To verify that the cells have been effectively transfected and that the insertion is functional, a plurality of tests are performed:

1. CAR-T cell proliferation test

[0354] The T-cells produced are cultured in 24-well plates in medium unsupplemented with IL-2 and in the presence of irradiated Raji cells (1:1 ratio) at 37°C and 5% CO_2;

[0355] A fraction of the T-cells is harvested after 4, 7 and 10 days in order to count them by flow cytometry.

1. CAR-T cell cytotoxicity test

[0356] The T-cells produced are cultured in 12-well plates in medium unsupplemented with IL-2 and in the presence of Raji cells expressing a fluorochrome (3:1 ratio) at 37°C and 5% CO_2;

[0357] After incubating for 24 h, a fraction of the cells is harvested to quantify the presence of the fluorochrome by flow cytometry and the remaining cells are recultured with an additional ratio of Raji cells;

[0358] This step is repeated after incubating for 36 h and 48 h.

[0359] **Example 2 - Another CD3ζ fusion format**.

[0360] It is also possible to carry out another fusion by following the protocol described hereinbefore in example 1.

[0361] Tube 1 is prepared as described in the example suf for the following sequences

[0362] - the reverse oligo GREAT X1 Rev (10 µM) corresponding to the 5′ part of molecule 3 of the following sequence SEQ ID NO: 1146

[0363] 5′-TACACTACCTGtCTCTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTTTggttacACCGGTCGTACA-3′

[0364] - the reverse oligo GREAT X3 Rev (10 µM) corresponding to the 3′ part of molecule 6 of the following sequence SEQ ID NO: 1147

[0365] 5′-TGTACGACCGGTgtaaccTTTTTTTTTTTTTTTTTTTTTTTTAgaTgTGtatAAGAGaCAGTATAGCTG-3′

[0366] Tube 2 is prepared as follows

[0367] - the reverse oligo GREAT X2 Rev (10 µM) corresponding to the 3′ part of molecule 3 of the following sequence SEQ ID NO: 1148

[0368] 5′-CGATACCCGCGGatgttcTTTTTTTTTTTTTTTTTTTTTTTTAgaTgTGtatAAGAGaCAGTATTCGTC-3′

[0369] - the reverse oligo GREAT X4 Rev (10 µM) corresponding to the 5′ part of molecule 6 of the following sequence SEQ ID NO: 1149

[0370] 5′-TTCATACTCTGtCTCTTataCAcAtcTTTTTTTTTTTTTTTTTTTTTTTTgaacatCCGCGGGTATCG-3′

[0371] - the reverse oligo Helix Rev corresponding to an additional strand for binding a helicase via the biotinylated 3′ end, the oligo having the following sequence SEQ ID NO: 1142.

[0372] The two tubes are then heated to 95°C for 5 min and then left for 1 hour at room temperature, then each tube is digested with the corresponding restriction enzymes: Tube 1: AgeI and Tube 2: SacII then purified by PCR purification kit (elution 20 µl).

[0373] In parallel, the anti-CD19-CD3ζ-P2A-GFP sequence was obtained via direct amplification by DH5α bacteria of the pSLCAR-CD19-CD3ζ plasmid. This plasmid is then digested via the AgeI and SacII restriction sites and the CD19- CD3ζ-P2A-GFP sequence is then purified by gel purification kit at a volume of 20 µl. The CD19-CD3ζ-P2A-GFP fusion is then provided with flanking ends (i.e. free AgeI and SacII half-sites) in tube 3.

[0374] The contents of the three tubes (tube 1, tube 2, and tube 3) are then mixed and T4 phage ligase is added to the mixture (4 µl, 100 U) in the presence of appropriate buffer (T4 buffer; 5 µl 10X) and 11 µl of distilled water. The mixture is left for 1 hour at room temperature. The mixture is then gel-purified (30 µl elution) to isolate the largest CD19-CD3ζ-P2A-GFP fragment, larger than 1 Kb, and containing the four sarcophages X1, X2, X3, and X4 at its 5′ and 3′ ends. The purified fragment is ready for use and includes the first, second, fourth, and fifth molecules at the ends, with the third and sixth molecules corresponding to the CD19-CD3ζ-P2A-GFP fusion (tube 4).

[0375] The CD19-CD3ζ-P2A-GFP replacement fragment with its four sarcophages positioned at the 5′ and 3′ ends is then ready to receive the transposase dimers.

[0376] At this stage, the complementary modified single-stranded oligonucleotide of part of the first and fourth molecules, with the 3′ part of the

complementary modified single-stranded oligonucleotide coupled to biotin (biotinylated oligo), was added. This biotinylated molecule is not mandatory.

[0377] Transfection is carried out as described in example 1.

[0378] Transfection verification is carried out as described in example 1, except for the reverse transcriptase detection. The fusion sequence is then amplified by PCR using oligos for PCR and anti-CD19 sequencing: AGGAGTCCCATCAAGGTTCAGT (SEQ ID NO: 1144) and GFP Rev: CACGAACTCCAGCAGGACCATG (SEQ ID NO: 1145) and the amplified fragment is sequenced by Sanger sequencing and migrated onto agarose gel. As expected, after analysis (NCBI blast), very high similarity is found with the theoretical CD19-CD3ζ-P2A-GFP fragment at the TRAC locus.

[0379] **Example 3 - Obtaining T-cells expressing the CD3ζ-containing fusion from pluripotent stem cells.**

[0380] Since it can be difficult to obtain T-cells from the patient, it is possible to use induced pluripotent stem cell (iPSc) technology.

[0381] The iPSc cells can be obtained from cell banks, having an immunological profile that is compatible with the patients.

[0382] It is also possible to collect differentiated cells from the patient (e.g. fibroblasts or epithelial cells), and force dedifferentiation by expressing the Oct4 and Socs2 genes, as well as other genes such as Klf4, Dub3, c-Myc, Nanog. Such techniques are now well known to a skilled person, and they will be able to adapt the protocols described in the prior art according to the cell type they want to engage in a dedifferentiation model.

[0383] At this stage, the iPSc cells can be transfected with lipofectamine in the presence of tube 4 as described in example 1 or example 2.

[0384] The transfected cells are selected using the appropriate antibiotic, and the insertion is verified as described in example 1.

[0385] The iPSc cells are cultured on plates coated with extracellular matrix gel, co-cultured with inactivated murine embryonic fibroblasts for 24 h, in complete stem cell culture medium supplemented with bFGF and ROCK inhibitor.

[0386] The iPSc cells are then cultured for 7 days in 24 h culture media containing different differentiation cocktails each day:

[0387] o D0-1: medium supplemented with hBMP-4, hVEGF, hWnt3a, and KOSR;

[0388] o D2: medium supplemented with hBMP-4, hVEGF, and KOSR;

[0389] o D3: medium supplemented with hBMP-4, hVEGF, and bFGF;

[0390] o D4-5: medium supplemented with hVEGF and bFGF;

[0391] o D6: IMDM medium supplemented with F12, B27, N2, BSA, hVEGF, bFGF, human stem cell factor, and hFlt3 ligand;

[0392] o D7: IMDM medium supplemented with F12, B27, N2, BSA, hVEGF, bFGF, human stem cell factor, hFlt3 ligand, TPO, IL-6, hEPOgen, and FICZ (6-formylindolo [3,2-b]carbazole)

[0393] After seven days, the cells are then kept in culture for a further 3 to 7 days before continuing the experiments on the non-adherent cells harvested. These cells are tested to verify that they express the CD34+ marker by flow cytometry using an anti-CD34 antibody.

[0394] If the cells have not been transfected at the iPSc stage, they can be transfected at the CD34+ stage as described in example 1.

[0395] Their differentiation into T-cells is then induced as follows:

[0396] - Differentiation into progenitor cells of the lymphoid lineage:

[0397] The CD34+ cells obtained are cultured on plates coated with extracellular matrix gel specifically designed for the culture of lymphoid progenitors without feeder cells, in hematopoietic cell culture medium supplemented with recombinant human cytokines to specifically promote differentiation into CD7+CD5+ T progenitor cells (pro-T-cells).

[0398] The cells are kept in culture at 37°C 5% CO_2 for 14 days, refreshing half of the medium every 3-4 days.

[0399] - Maturation of progenitor cells into CD4+CD8+ T-cells:

[0400] At D14, the pro-T-cells are collected, transferred to new plates coated with extracellular matrix gel and cultured in a new hematopoietic cell culture medium supplemented with recombinant human cytokines to specifically promote pro-T-cell maturation into mature T-cells.

[0401] The cells are kept in culture at 37°C 5% CO_2 for 14 days, refreshing half of the medium every 3-4 days.

[0402] Maturation of T-cells into CD8+:

[0403] The cells obtained in the previous step are collected, transferred to new plates coated with extracellular matrix gel and cultured in the same culture

medium as before, supplemented with recombinant human IL-15 and CD3/28 activation cocktail.

[0404] The cells are kept in culture at 37°C 5% CO_2 for 7 days, refreshing half of the medium on D3-4.

Claims

[Claim 1] A composition comprising

- a first single-stranded nucleic acid molecule comprising or consisting essentially of an A sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary sequence binding at 5′ to a first T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a second T-rich sequence of 40 to 60 nucleotides in length, said first and second T-rich sequences respectively comprising a first and a second domain of 6 to 12 G/C-rich nucleotides, the sequence of the first domain being complementary to the sequence of the second domain, said first and second domains being positioned 15 to 52 nucleotides from said A sequence, said first molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase,

- a second single-stranded nucleic acid molecule comprising or consisting essentially of a B sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary B sequence binding at 5′ to a third T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a fourth T-rich sequence of 40 to 60 nucleotides in length, said third and fourth T-rich sequences respectively comprising a third and a fourth domain of 6 to 12 G/C-rich nucleotides, the sequence of the third domain being complementary to the sequence of the fourth domain, said third and fourth domains being positioned 15 to 52 nucleotides from said B sequence, said second molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing said transposase and at its 3′ end the second sequence for recognizing said transposase,

said B sequence being a complementary sequence of said nucleic acid of interest, the A sequence being positioned at 5′ of a region of interest of said

nucleic acid of interest and the B sequence positioned at 3′ of the region of interest of said nucleic acid of interest, and

- a third single-stranded molecule comprising

* in its 5′ part, at least one complementary sequence of said second sequence for recognizing said transposase of the first molecule,

* in its 3′ part, at least one complementary sequence of said first sequence for recognizing said transposase of the second molecule, and

* an intermediate region located between the complementary sequence of said second sequence for recognizing said transposase of the first molecule and the complementary sequence of said first sequence for recognizing said transposase of the second molecule, said intermediate region comprising a sequence encoding a fusion protein, said fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the activation domain of the T-lymphocyte, i.e. the CD3ζ chain of the T-cell receptor or TCR,

the first and third single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase, and the second and third single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase.

[Claim 2] The composition according to claim 1, wherein said fusion protein comprises the peptide capable of binding to a membrane protein of a cell, fused to a transmembrane part of a membrane protein, the latter fused to the CD3ζ chain of the TCR.

[Claim 3] The composition according to claim 1, wherein said fusion protein comprises the peptide capable of binding to a membrane protein of a cell, fused to a transmembrane part of a membrane protein, the transmembrane domain of the membrane protein being fused to a co-activation domain, the latter being fused to the CD3ζ chain of the TCR.

[Claim 4] The composition according to any one of claims 1 to 3,

- wherein said A sequence comprises a complementary sequence of the sequence of a first region of the locus of the gene encoding the TRAC protein and

- wherein said B sequence comprises a complementary sequence of the sequence of a second region of the locus of the gene encoding the TRAC protein,

said A sequence and said B sequence being two different sequences, said first and second regions of the TRAC locus advantageously flanking a 5′ region of the locus of the gene encoding the TRAC protein.

[Claim 5] The composition according to any one of claims 1 to 4, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence oriented 5′-to-3′ for recognizing said transposase and

wherein the second molecule comprises its 5′ end a first complementary sequence of said first sequence for recognizing said transposase followed by a second complementary sequence of said second sequence for recognizing said transposase.

[Claim 6] The composition according to any one of claims 1 to 5, wherein said first molecule comprises at its 5′ end a first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase, followed by a first complementary sequence of said first sequence for recognizing said transposase and

wherein the second molecule comprises at its 5′ end a complementary sequence of said second sequence for recognizing said transposase.

[Claim 7] The composition according to any one of claims 1 to 6, wherein said transposase is a bacterial transposase, especially a transposase selected from Tn5, Tn9, Tn10 or Tc1/mariner.

[Claim 8] The composition according to any one of claims 1 to 7, wherein the intermediate region of the third molecule comprises the sequence SEQ ID NO: 3 or the sequence SEQ ID NO: 1134.

[Claim 9] The composition according to any one of claims 1 to 8, wherein the first, second, and third molecules are selected from the triplets as defined in Table 2.

[Claim 10] The composition according to any one of claims 1 to 9, further comprising,

** a fourth single-stranded nucleic acid molecule comprising or consisting essentially of an A′ sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary sequence binding at 5′ to a fifth T-rich sequence of 40 to 60 nucleotides in length and at 3′ to a sixth T-rich sequence of 40 to 60 nucleotides in length, said fifth and sixth T-rich sequences respectively comprising a fifth and a sixth domain of 6 to 12 G/C-rich nucleotides, the sequence of the fifth domain being complementary to the sequence of the sixth domain, said fifth and sixth domains being positioned 15 to 52 nucleotides from said A′ sequence, said fourth molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing a transposase and at its 3′ end a second sequence for recognizing said transposase,

** a fifth single-stranded nucleic acid molecule comprising or consisting essentially of a B′ sequence allowing the insertion of a complementary sequence of a nucleic acid of interest, or comprising a complementary sequence of a nucleic acid of interest, said complementary B′ sequence binding at 5′ to a seventh T-rich sequence of 40 to 60 nucleotides in length and at 3′ to an eighth T-rich sequence of 40 to 60 nucleotides in length, said seventh and eighth T-rich sequence respectively comprising a seventh and an eighth domain of 6 to 12 G/C-rich nucleotides, the sequence of the seventh domain being complementary to the sequence of the eighth domain, said

seventh and eighth domains being positioned 15 to 52 nucleotides from said B′ sequence, said fifth molecule comprising at its 5′ end at least one first sequence oriented 5′-to-3′ for recognizing said transposase and at its 3′ end the second sequence for recognizing said transposase,

said B′ sequence being a complementary sequence of said nucleic acid of interest, the A′ sequence being positioned at 5′ of a region of interest of said nucleic acid of interest and the B′ sequence positioned at 3′ of the region of interest of said nucleic acid of interest, and

** a sixth single-stranded molecule comprising

* in its 5′ part, at least one complementary sequence of said fifth sequence for recognizing said transposase of the first molecule,

* in its 3′ part, at least one complementary sequence of said fourth sequence for recognizing said transposase of the second molecule, and

* an intermediate region located between the complementary sequence of said fourth sequence for recognizing said transposase of the first molecule and the complementary sequence of said fifth sequence for recognizing said transposase of the second molecule, a complementary sequence of said sequence encoding the fusion protein contained in the third molecule,

the fourth and sixth single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase, and the fifth and sixth single-stranded nucleic acid molecules being paired according to the base complementarity defined by Watson and Crick so as to define two double-stranded binding sites of said transposase.

[Claim 11] The composition according to claim 10, wherein

- said A sequence comprises a complementary sequence of the sequence of a first region of the locus of the gene encoding the TRAC protein,

- said B sequence comprises a complementary sequence of the sequence of a second region of the locus of the gene encoding the TRAC protein,

said A sequence and said B sequence being two different sequences, said first and second regions advantageously flanking a 5′ region of the locus of the gene encoding the TRAC protein,

and wherein

- said A′ sequence comprises a complementary sequence of the sequence of a third region of the locus of the gene encoding the TRAC protein,

- said B′ sequence comprises a complementary sequence of the sequence of a fourth region of the locus of the gene encoding the TRAC protein,

said A′ sequence and said B′ sequence being two different sequences, said third and fourth regions of the gene encoding the TRAC protein, advantageously flanking a complementary sequence of said 5′ region of the locus of the gene encoding the TRAC protein,

and wherein

the A sequence and the A′ sequence are at most partially complementary,

the A sequence and the B′ sequence are at most partially complementary,

the B sequence and the A′ sequence are at most partially complementary, and

the B sequence and the B′ sequence are at most partially complementary.

[Claim 12] An isolated and purified immune cell, especially a T-lymphocyte cell, comprising

a composition according to any one of claims 1 to 11.

[Claim 13] A pharmaceutical composition comprising:

- a composition according to any one of claims 1 to 11, or

- an immune cell according to claim 12,

in association with a pharmaceutically acceptable carrier.

[Claim 14] The composition according to any one of claims 1 to 11, for use as a drug.

[Claim 15] The composition according to any one of claims 1 to 11, for use in the treatment of cancer.

[Claim 16] A use of a composition according to any one of claims 1 to 11, for inserting, into a T-lymphocyte cell, a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, provided that the use does not comprise a process for modifying the germline genetic identity of human beings and that said use is not a method for treating the human or animal body by surgery or therapy.

[Claim 17] A method for *in vitro* insertion of a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

- placing a composition as defined in any one of claims 1 to 11 in contact with a target nucleic acid comprising at least a 5′ region of the locus of the gene encoding the TRAC protein, in order to obtain a replacement complex,

said composition being such that

the A sequence of said first molecule comprises a complementary sequence of a first region of the locus of the gene encoding the TRAC protein,

the B sequence of said second molecule comprises a complementary sequence of a second region of the locus of the gene encoding the TRAC protein, and

the third molecule comprises the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR located between a complementary sequence of said second sequence for recognizing said transposase of the first molecule and the

complementary sequence of said first sequence for recognizing said transposase of the second molecule,

- placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said composition, to obtain a recombination complex, and

- recombining the combination complex to obtain the sequence encoding the fusion protein inserted in the target nucleic acid between the first and second regions of the locus of the gene encoding the TRAC protein.

[Claim 18] A method for producing chimeric antigenic receptor-T cells or *CAR-T cells* comprising the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said method comprising:

- placing a composition as defined in any one of claims 1 to 11 in contact with a purified T-cell, the genomic DNA of said purified T-cell comprising the locus of the gene encoding the TRAC protein,

said composition or said ensemble being such that

the A sequence of said first molecule comprises a complementary sequence of a first region of the locus of the gene encoding the TRAC protein,

the B sequence of said second molecule comprises a complementary sequence of a second region of the locus of the gene encoding the TRAC protein, and

the third molecule comprises the sequence of a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR located between a complementary sequence of said second sequence for recognizing said transposase of the first molecule and the complementary sequence of said first sequence for recognizing said transposase of the second molecule, in order to obtain a replacement complex,

- placing the replacement complex in the presence of a transposase recognizing the double-stranded binding sites of said transposase contained in said ensemble, to obtain a recombination complex,

- recombining the combination complex to obtain a *CAR-T* cell, the genomic DNA of said CAR-T cell comprising the sequence encoding said fusion protein at the locus of the gene encoding the TRAC protein, and

- purifying the *CAR-T* cell.

[Claim 19] A modified T lymphoid cell capable of being obtained by the method according to claim 19, said modified T lymphoid cell comprising in its genome an insertion of a sequence encoding a fusion protein comprising, in the amino-terminal part, a peptide capable of binding to a membrane protein of a cell and, in the carboxy-terminal part, the CD3ζ chain of the TCR, said insertion being located at the locus of the gene encoding the TRAC protein.